nematicide stressed *H. schachtii* stock cultures were employed to ensure direct relevance to the non-fumigant nematicide control of this nematode.

Materials and methods

ISOLATION OF SOIL MICROORGANISMS FROM NEMATICIDE TREATED AND UNTREATED STOCK GREENHOUSE NEMATODE CULTURES

Soil samples were collected from long-term low dosdosage nematicide stressed greenhouse cultures of *H. schachtii* (Viglierchio *et al.*, 1989). At the time of sampling, the sub-lethal concentrations used to stress the nematode populations were 0.008 mM carbofuran and oxamyl and 0.0012 mM fenamiphos, and aldicarb; a wild population of nematode stock cultures served as control samples. Three cores of soil were collected from each pot and mixed together thoroughly to comprise one replicate composite sample. Each treatment was replicated three times.

The isolation of microorganisms from composite soil samples was initiated by taking 10 g of soil from each composite and transferring it to an Erlenmeyer flask for suspension in 90 ml of sterilized deionized water by shaking on a mechanical shaker for 30 min. A serial dilution was made for each soil suspension and each dilution was plated out on Petri dishes containing selective media (one set with and one without added nematicides) to isolate different groups of microorganisms. There were three replicates for each dilution. Total aerobic bacteria except *Pseudomonas* species were estimated by the dilution plate method using Thornton's standardized medium plus Nystatin (mycostatin) (Thornton, 1922). Peptone dextrose agar plus rose bengal (1:30 000) and Streptomycin (30 µg/ml, Martin, 1950) or Aureomycin (2 µg/ml, Johnson, 1957) was used to isolate fungi. *Pseudomonas* isolation agar (Difco) was used for isolating *Pseudomonas* species. A similar set of dilution plates but with the addition of each of the following filter sterilized nematicide concentrations : 0.008 mM carbofuran or oxamyl, and 0.0012 mM fenamiphos or aldicarb served for comparison. The plate cultures were placed in the 30 °C incubator for five days after which bacterial colonies including *Pseudomonas* were sub-cultured on nutrient agar (Difco) slant tubes and fungal colonies were sub-cultured on slants of Czapek-Dox agar (Thom & Raper, 1945) at ambient temperature.

TRANSMITTANCE AND ESTIMATION OF BACTERIAL COLONY FORMING UNITS (CFU)

Pseudomonas species from slant cultures were transferred into AC broth (Difco) and shaken on a mechanical shaker at 30 °C for 24 h. During the incubation period, at times 0, 1, 2, 3, 4, 5, 6, 7 and 24 h, 3.5 ml of liquid Mother culture from each flask of isolate was transferred to colorimeter tubes to estimate transmittance optically at 465 nm with a Spectronic 20 (Bausch & Lomb colorimeter). Each liquid bacterial culture isolate was treated in the same fashion. Simultaneously, a known volume from each mother culture cell suspension was plated out as a dilution series onto Petri dishes. These plates were incubated at 30 °C for 24 h after which the number of CFU per plate was determined to prepare a standard curve for transmittance as a function of number of *Pseudomonas* CFU.

THE INHIBITIVE EFFECT OF NEMATICIDE TO ACETYLCHOLINESTERASE ACTIVITY

Acetylcholinesterase catalyses a reaction producing thiols from a precursor substrate, acetylthiocholine iodide. These thiols can then react with dithiobisnitrobenzoic acid (DTNB) to yield a yellow chromophore (Ellman *et al.*, 1961) that can be used to measure the inhibitive effect of nematicides on cholinesterase activity. Three replicate tubes were used for all the following test conditions.

Test of optimum incubation period for the enzyme-substrate reaction : The assay-reaction mixture consisted of the following components : 9 ml of culture supernatant (cell free prepared from liquid culture aerobic bacteria), enzyme-substrate mixture as follows-9 ml of 0.1 M phosphate buffer, pH 8.0; 75 µl bovine erythrocyte cholinesterase (Sigma, 5 units/ml water); 30 µl 0.075 M substrate (acetylthiocholine iodide, Sigma); 150 µl of 0.01 M DTNB (39.6 mg dissolved in 10 ml of 0.1 M phosphate buffer, pH 7.0 to which 15 mg sodium bicarbonate was added). A control blank was prepared with all the components described above but omitting bovine erythrocyte cholinesterase. The reaction mixtures were placed in a 30 °C shaker water bath. Three ml of the reaction mixture from the same flask were transferred to cuvettes after 20 min, 2 h and 3 h of reaction time to measure transmittance with a Spectronic 20 colorimeter at 412 nm (the wave length of chromophore absorption). The 2 h reaction period was selected as the most favorable for the following experiments.

Selection of nematicide : Nematicides were added to the cholinesterase enzyme-substrate reaction mixture at the following rates : 0.005, 0.001, 0.002, 0.004 and 0.008 mM for carbofuran or oxamyl; and 0.0006, 0.0012, 0.0024, 0.0048, 0.0096 and 0.012 mM for fenamiphos or aldicarb. The enzyme-substrate reaction mixture with water substituted for nematicides served as a control. Each of four replicates was incubated at 30 °C in a water bath shaker for two hours before transmittance was measured as an indication of effectiveness as an inhibitor. The optimum reaction was obtained with 0.008 mM carbofuran which was therefore selected for further testing.

Inactivation effects of microbial metabolites on carbofuran activity :

i) A standard transmittance curve was prepared for the range of substrate concentration anticipated in the enzymatic reaction.

A series of concentrations of acetylcholine iodide (0.024, 0.049, 0.097, 0.15, 0.19 and 0.24 mM) were added in the enzyme reaction mixture so that it could be used to estimate millimoles of substrate in the reaction mixture. There were three replicates for each testing condition and this experiment was repeated four times.

ii) Effect of culture cell free supernatant from *Pseudomonas* species, aerobic bacteria and *Trichoderma* species on carbofuran activity. Three replicates of each of four different kinds of treatments which each microbial isolate were prepared as follows :

a - 1 ml cell free supernatant plus enzyme-substrate reaction mixture composed of the following : 3 ml of 0.1 M phosphate buffer, pH 8.0; 25 µl bovine erythrocyte cholinesterase (Sigma, 5 units/ml water); 10 µl of 0.075 M substrate-acetylthiocholine iodide (Sigma); 50 µl 0.01 M - DTNB (39.6 mg dissolved in 10 ml of 0.1 M phosphate buffer, pH 7.0 to which 15 mg of sodium bicarbonate was added).

b - 1 ml cell free supernatant plus the enzyme-substrate reaction mixture but substituting 25 µl of water for the enzyme solutions;

c - 1 ml cell free supernatant, enzyme-substrate reaction mixture as mentioned (*a*) and carbofuran solution to result in a final concentration 0.008 mM;

d - 1 ml cell free supernatant, prepared from liquid microbial culture with 0.008 mM carbofuran in the medium during culture incubation, enzyme-substrate reaction mixture as described (*a*).

Results

The numbers of CFU of groups of microorganisms isolated from the soil solution dilutions of composite samples by plating out on different isolation media with and without added nematicide are indicated in Table 1. Aerobic bacteria and *Pseudomonas* species were isolated from the soil samples whether or not the isolation medium contained nematicide. *Trichoderma* was the overwhelmingly dominant fungus isolate regardless of isolation process except that perhaps more colony forming units (CFU) were obtained from fenamiphos and aldicarb treated soils (Table 1). There were no significant differences in the number of CFU between the isolation media with or without nematicide from nematicide-treated soils; however, there were greater numbers of *Trichoderma* CFU isolated from nematicide stressed culture soil samples than from the soil of wild population nematode cultures. Substantially more CFU of aerobic bacteria and *Pseudomonas* species were isolated from the carbofuran treated soil than from all other nematicide stressed or unstressed nematode stock culture soils.

A standard curve was prepared as a basis to estimate the CFU of microbial cultures producing supernatants used in cholinesterase reactions in order to compare results of different isolates on the basis of CFU (Fig. 1).

Under our laboratory conditions, the two hour incubation was selected for the enzyme-substrate reaction

Table 1. Micro-organism colony forming units (CFU) isolated from nematicide treated and untreated soil from *H. schachtii* stock cultures in the presence or absences of nematicide in the isolation media. Nematicide concentrations in isolation media or soil treatments were : C = 0.008 mM Carbofuran; O = 0.008 mM Oxamyl; F = 0.0012 mM Fenamiphos; A = 0.0012 mM Aldicarb; CTL = Control, untreated. (Mean and standard deviation of three replicates.)

		Number of CFU/g of composite soil samples					
		Aerobic bacteria (10^6)		*Pseudomonas* (10^3)		*Trichoderma* (10^3)	
Nematicide		Present (Media)	Absent (Media)	Present (Media)	Absent (Media)	Present (Media)	Absent (Media)
Soil	Media						
C	C	4.6 ± 0.1	4.8 ± 0.3	10.7 ± 2.0	10.4 ± 2.0	560 ± 36	490 ± 86
O	O	3.4 ± 0.1	2.7 ± 0.2	1.7 ± 0.4	1.4 ± 0.5	520 ± 80	630 ± 7
F	F	1.8 ± 0.2	2.8 ± 0.1	0.4 ± 0.4	0	610 ± 96	740 ± 104
A	A	2.6 ± 0.07	2.9 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	680 ± 45	710 ± 72
CTL	C	2.3 ± 0.4	3.1 ± 0.27	0.6 ± 0.1	0.6 ± 0.1	150 ± 10	150 ± 32
CTL	O	2.4 ± 0.05	3.1 ± 0.27	1.1 ± 0.2	0.6 ± 0.1	110 ± 20	150 ± 32
CTL	F	1.5 ± 0.2	3.1 ± 0.27	1.2 ± 0.2	0.6 ± 0.1	110 ± 21	150 ± 32
CTL	A	2.0 ± 0.3	3.1 ± 0.27	1.5 ± 0.5	0.6 ± 0.1	100 ± 25	150 ± 32

Table 2. The interaction of *Pseudomonas* isolate culture supernatant on the carbofuran inhibited and uninhibited cholinesterase reaction. A = Normal cholinesterase reaction mixture incorporating supernatant (material and methods); B = Normal cholinesterase reaction mixture incorporating supernatant but substituting an equivalent volume of water for enzyme solution - non specific hydrolysis (material and methods; C = Normal cholinesterase reaction mixture incorporating supernatant and 0.008 mM carbofuran as reaction inhibitor (material and methods; D = Normal cholinesterase reaction mixture incorporating supernatant from a culture grown in a medium containing 0.008 mM carbofuran. No supplemental carbofuran was added to the reaction mixture which therefore contained 0.002 mM carbofuran.

	Substrate hydrolyzed							
	A		B		C		D	
Isolate	μM	nM/10^7 CFU	μM	nM/10^7 CFU	μM	nM/10^7 CFU	μM	nM/10^7 CFU
1	0.44	9700	0.06	1400	0.08	1800	0.07	1500
2	0.38	5	0.06	1	0.07	1	0.08	0.3
3	0.35	14	0.06	2	0.08	3	0.08	9
4	0.43	21	0.06	3	0.08	4	0.08	2
5	0.87	6	0.06	1	0.08	1	0.08	1
6	0.53	110	0.06	12	0.07	15	0.07	31
7	0.54	113	0.06	13	0.08	16	0.08	27
8	0.50	2	0.06	0.2	0.07	0.2	0.07	15
9	0.45	15	0.06	2	0.07	3	0.07	104
10	0.40	45	0.06	7	0.07	8	0.07	36
11	0.39	39	0.05	6	0.06	6	0.07	82
12	0.45	630	0.06	8.7	0.07	103	0.08	94
13	0.42	525	0.06	77	0.07	90	0.08	99
14	0.43	24	0.06	3	0.08	4	0.07	1
15	0.43	478	0.06	72	0.07	93	0.08	74
16	0.41	1	0.06	0.1	0.07	0.1	0.08	0.1
17	0.26	78	0.05	15	0.05	16	0.05	5
18	0.23	38	0.05	8	0.05	9	0.06	3
19	0.39	91	0.05	13	0.06	13	0.06	1
20	0.48	19	0.05	2	0.06	2	0.06	9
21	0.41	123	0.05	16	0.06	17	0.06	14
22	0.52	11 400	0.05	1200	0.06	1300	0.06	1300
23	0.58	107	0.05	10	0.06	11	0.06	16

seventeen randomly selected aerobic bacteria isolates (Table 3) also indicate that about half the substrate present was hydrolyzed with a range among isolates of between 40 and 75 %. Whereas the range was perhaps somewhat narrower, than for *Pseudomonas* the range on a 10^7 colony forming unit basis was greater, 17 to 55 000 %. In the absence of added enzyme the nonspecific hydrolysis of substrate was similar to that of *Pseudomonas*, on the order of 9 % of the initial substrate. In the enzyme reaction mixture the presence of carbofuran inhibited the cholinesterase activity of the added enzyme.

Cholinesterase reaction studies incorporating supernatants from randomly selected *Trichoderma* isolates (Table 4) indicated that approximately 20 % of the substrate was hydrolyzed and the variability among isolates was much reduced from that of bacterial observations. In terms of substrate hydrolyzed per gram of mycelium in the culture, there appeared to be a correlation with fungal growth, i.e., more mycelium, more substrate hydrolyzed. In the absence of added enzyme to the reaction mixture, the nonspecific hydrolysis was on the order of 2 % of the initial substrate added. As with bacterial extracts, the addition of carbofuran to the enzyme reaction mixture effectively inhibited cholinesterase activity.

Discussion

The presence of nematicide in the medium appeared to make little difference to the number of colony forming units isolated from nematicide treated soils of aerobic bacteria except perhaps from fenamiphos treated soils. The number of CFU from control soils appeared to be somewhat lower with nematicides in the medium for aerobic bacteria and perhaps *Trichoderma*

Table 3. The interaction of aerobic bacteria isolate culture supernatant on carbofuran inhibited and uninhibited cholinesterase reaction. A = Normal cholinesterase reaction mixture incorporating supernatant (material and methods); B = Normal cholinesterase reaction mixture incorporating supernatant but substituting an equivalent volume of water for enzyme solution - non specific hydrolysis (material and methods). C = Normal cholinesterase reaction mixture incorporating supernatant and 0.008 mM carbofuran as reaction inhibitor (material and methods). D = Normal cholinesterase reaction mixture incorporating supernatant from a culture grown in a medium containing 0.008 mM carbofuran. No supplemental carbofuran was added to the reaction mixture which therefore contained 0.002 mM carbofuran.

	Substrate hydrolyzed							
	A		B		C		D	
Isolate	μM	nM/10^7 CFU	μM	nM/10^7 CFU	μM	nM/10^7 CFU	μM	nM/10^7 CFU
1	0.55	0.4	0.07	0.1	0.09	0.1	0.08	0.2
2	0.58	18	0.08	2	0.1	3	0.08	0.1
3	0.45	346	0.07	52	0.09	66	0.09	11
4	0.54	43	0.08	6	0.10	8	0.10	56
5	0.53	410	0.07	56	0.10	74	0.09	188
6	0.45	2	0.07	0.3	0.10	0.4	0.10	0.9
7	0.40	1	0.07	0.1	0.10	0.2	0.10	0.3
8	0.37	0.4	0.07	0.1	0.09	0.1	0.10	0.2
9	0.47	2	0.06	0.3	0.08	0.4	0.08	2
10	0.44	210	0.07	3.2	0.08	40	0.09	0.2
11	0.47	252	0.07	86	0.09	46	0.09	0.1
12	0.44	24	0.06	4	0.08	4	0.09	20
13	0.38	0.5	0.07	0.1	0.09	0.1	0.10	0.2
14	0.32	46	0.07	10	0.09	12	0.09	4
15	0.31	0.1	0.06	0.03	0.08	0.03	0.09	0.2
16	0.44	15	0.06	2	0.08	3	0.09	1
17	0.50	51	0.06	7	0.08	8	0.10	0.3

Table 4. The interaction of *Trichoderma* isolate culture supernatant on carbofuran inhibited and uninhibited cholinesterase activity. * = nanomoles/g of dry mycelium from the culture. A = Normal cholinesterase reaction mixture incorporating supernatant (material and methods). B = Normal cholinesterase reaction mixture incorporating supernatant but substituting an equivalent volume of water for enzyme solution - non specific hydrolysis (material and methods. C = Normal cholinesterase reaction mixture incorporating supernatant and 0.008 mM carbofuran as the reaction inhibitor (material and methods). D = Normal cholinesterase reaction mixture incorporating supernatant from a culture grown in a medium containing 0.008 mM carbofuran. No additional carbofuran was added to the reaction mixture which therefore contained 0.002 mM carbofuran.

	Substrate hydrolyzed							
	A		B		C		D	
Isolate	μM	nM/g *	μM	nM/g *	μM	nM/g *	μM	nM/g *
1	0.13	19	0.003	0.44	0.005	0.74	0.009	1.3
2	0.18	34	0.003	0.58	0.005	0.97	0.007	1.1
3	0.15	20	0.003	0.42	0.005	0.70	0.006	0.8
4	0.18	24	0.003	0.41	0.005	0.68	0.009	1.2
5	0.17	22	0.003	0.40	0.005	0.67	0.009	1.3

or oxamyl, fenamiphos, and aldicarb whereas those of *Pseudomonas* were somewhat higher for oxamyl, fenamiphos and aldicarb. The number of colony forming units of *Trichoderma* from treated soils were consistently greater than from untreated ones. The number of colony forming units of aerobic bacteria and *Pseudomonas* species was substantially greater from carbofuran treated soils than those noted from untreated soils or soils treated with oxamyl, fenamiphos or aldicarb.

The standard curve relating transmittance to number of colony forming units was based on the log phase growth of *Pseudomonas* species (Fig. 1). This was believed to provide a more reliable indicator of colony forming units than a standard curve obtained by diluting a mature population.

The capacity of nematicides to inhibit the bovine erythrocyte cholinesterase reaction (Fig. 2) revealed that carbofuran was substantially more effective than the other nematicides under the conditions of these experiments. Inasmuch as it was more active in the range of concentrations utilized in the stock culture stressing regime, 0.008 mM carbofuran was selected as a candidate nematicide for the cholinesterase reaction experiments. During the course of these experiments randomly selected isolates were spot checked by repeating the entire reaction series; reproducibility of the results were consistently within 5 % of the mean of the initial trial.

Bacterial supernatant (aerobic bacteria and *Pseudomonas*) reduced the substrate hydrolysis by half from that occurring in the presence of microorganism free medium; products of microbial growth inhibited the cholinesterase reaction. In cholinesterase free systems nonspecific hydrolysis by microbe by-products was of the order of 8 to 9 % of the initial substrate and not affected by the presence of carbofuran. The substrate hydrolysis was not correlated with microbe growth as indicated by CFU counts; i.e., although the number of CFU may have been hundreds or thousands of times greater in some isolates than others, this was not reflected in the substrate hydrolysis. A 0.008 mM concentration of carbofuran added to the enzyme reaction mixture completely inhibited the activity of the cholinesterase enzyme thereby indicating that the bacterial culture supernatant caused no reduction in carbofuran activity either by degrading or complexing it. The observations were confirmed by the results from the enzyme reaction mixture incorporating supernatant from microbial cultures growing in the presence of 0.008 mM carbofuran. These enzyme reaction mixtures contained 0.002 mM carbofuran, a concentration on the shoulder of the steep slope of the inhibitory curve (Fig. 2). At this concentration any reduction in carbofuran activity would have been easily noted; no reduction was observed.

The results with *Trichoderma* were somewhat similar (Table 4). In the normal reaction mixture with the supernatant, approximately 80 % of the substrate remained unhydrolyzed with the amount hydrolyzed correlated with mycelial growth. Nonspecific hydrolysis was extremely low and unlikely to be of much impact on the system. As was observed in the bacterial systems, the *Trichoderma* reaction mixture in the presence of carbofuran was totally inhibited. None of the isolate supernatants from aerobic bacteria, *Pseudomonas* or *Trichoderma* appeared to have any affect on carbofuran activity whether by degradation or complexing. The supernatants did however, reduce the Bovine erythrocyte cholinesterase reaction by approximately half for aerobic bacteria and *Pseudomonas* and by 80 % for *Trichoderma*. Whether microbial growth products in nature have any inhibitory effect on the cholinesterase reaction within nematodes remains to be established. In the greenhouse stock nematode cultures, *Trichoderma* was the overwhelmingly dominant fungal group in contrast to field soils where the fungal population is varied. The *Trichoderma* dominance is a well-known observation for greenhouse stock cultures in which both soil and pots are heat sterilized; it reinvades quickly, grows rapidly and out competes other fungal contaminants. Although in these experiments the goal was to establish the parameters of the nematicide stressed nematode culture system it would be useful to understand how other fungal populations in field soils affect the nematode cholinesterase reactions or nematicide activity.

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