In vitro testing for nonfumigant-nematicide resistance in *Heterodera schachtii*

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SUMMARY

The *in vitro* response to touch stimulus tests the immediate capacity of a nematode to cope with immersion in nonfumigant nematicide solutions. Behavioral characteristics exhibited in this immediate reaction may be distinguished from those of longer-term trials involving growth, development and reproduction. Larvae of unstressed *Heterodera schachtii* populations and others stressed with carbofuran, oxamyl, phenamiphos or aldicarb were immersed in nematicide solutions. The oxamyl-stressed population was the only one indicating a significant loss of responsiveness. Protection was generally observed in stressed populations with few exceptions. Correlation of *in vitro* tests with greenhouse trial results was poor. A high proportion of *H. schachtii* retained the ability to respond to touch whether wild or stressed, after exposure to nonfumigant nematicide concentrations approaching 0.1 % for 24 hours. The existence of cross-tolerance or cross-resistance is strong presumptive evidence for a common biological site of action for these nematicides; however, the high proportion of *H. schachtii* larvae exhibiting moderate activity in the touch test suggests that the four nonfumigant nematicides employed in these experiments are inefficient nematode acetylcholinesterase inhibitors.

RÉSUMÉ

Test in vitro *de la résistance d'*Heterodera schachtii *aux nématicides non-fumigants*

La réaction, *in vitro*, aux stimuli de contact indique si un nématode est immédiatement capable de surmonter une immersion dans des solutions de nématicides non fumigants. Les caractéristiques comportementales liées à cette réaction doivent être distinguées de celles caractérisant les essais à long terme, ceux-ci impliquant croissance, développement et reproduction. Des larves provenant de populations non-sensibilisées d'*Heterodera schachtii* et de populations sensibilisées au carbofuran, à l'oxamyle, au phenamiphos ou à l'aldicarbe sont immergées dans des solutions de nématicides. A de rares exceptions, une protection est observée chez les populations sensibilisées. La corrélation entre les résultats des essais *in vitro* et ceux des expériences en serre s'est révélée faible. Une forte proportion de *H. schachtii*, provenant indifféremment de populations sauvages ou sensibilisées, conserve la possibilité de répondre aux stimuli de contact après exposition à des concentrations de nématicides non-fumigants voisines de 0,1 % pendant 24 heures. L'existence d'une tolérance croisée ou d'une résistance croisée suggère fortement un site biologique commun aux différents nématicides; cependant, la forte proportion de larves d'*H. schachtii* montrant une faible réaction aux tests par contact suggère que les quatre nématicides non fumigants utilisés dans les expériences sont inefficaces en tant qu'inhibiteurs de l'acétylcholinestérase.

Populations of *Heterodera schachtii* stressed over a year with subnematicidal doses of carbofuran, oxamyl, phenamiphos and aldicarb were observed to have altered reproductive responses to nematicide applications (Viglierchio, Brown & Kuo, 1989). The altered responses indicated various changes in population characteristics, including various forms of resistance, cross-resistance and apparent chemical habituation to subnematicidal doses. These greenhouse studies used concentrations of nonfumigant nematicides (NFN) approximating those recommended for field applications.

Conventional wisdom indicates that most NFN, particularly the organophosphates and carbamates, interfere with the function of the acetylcholinesterase in related nerve transmission centers of nematodes (Corbett, 1974). Recent evidence suggests that NFN action impairs or modifies nematode behavior (Marban-Mendoza & Viglierchio, 1980*a, b, c;* Yamashita & Viglierchio, 1986*a, b;* Yamashita, Viglierchio & Schmitt, 1986). The complexity of the nematode-host-NFN interaction has forstalled understanding the precise mechanisms of NFN activity. Greenhouse trials may indicate long-term behavioral modifications, *i.e*, changes in reproductive potential, and changes in susceptibility, resistance, cross-resistance and chemical habituation perhaps mediated by selection processes. *In vitro* trials on the other

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hand may reveal short-term characteristics that may help explain population trends observed in greenhouse studies. The two approaches are complementary and may contribute to an improved understanding of the effects of NFN on different species. This study was conducted using a motility *in vitro* assay to measure immediate responses that characterize *H. schachtii*-NFN behavior modification.

Materials and methods

Populations of *H. schachtii* used in these tests included : a wild population (W-P; with no previous history of nematicide treatments) and populations stressed more than twelve months with monthly subnematicidal doses of carbofuran (C-S-P), oxamyl (Ox-S-P), phenamiphos (P-S-P) and aldicarb (A-S-P). All populations were cultured on sugar beet plants in 4-liter pots containing a sterilized mixture of equal parts river sand and white quartz sand, and watered with half-strength Hoagland's nutrient solution. The NFN stressing regime was described elsewhere (Viglierchio, Brown & Kuo, 1989). Soil and root cores were removed from stock pots and the cysts were collected in a 246-µm sieve by wet-sieving. The cysts were further purified by sugar flotation then crushed to release eggs. The resulting mixture was placed on a modified Baermann funnel in a mister. At 24-hour intervals thereafter, the freshly-hatched larvae were collected and stored at 10°. An excess of 75 aerated nematodes were transferred to 60 mm glass Petri dishes to which was added an equal volume of either carbofuran, oxamyl, phenamiphos or aldicarb of sufficient concentration to provide the following concentrations (total volume 5 ml) of each nematicide : carbofuran-0.50 mM, 1.00 mM, 2.0 mM; oxamyl-0.25 mM, 0.50 mM, 1.00 mM; phenamiphos-0.20 mM, 0.40 mM, 0.80 mM; and aldicarb-0.05 mM, 0.25 mM, 1.25 mM. The control consisted of substituting an equal volume of tap water for the nematicide; each treatment was replicated three times. The covered Petri dishes were placed on wet cheesecloth in aluminium-covered plastic containers to retard evaporation and eliminate light. After 24 hours at 25°, the Petri dishes were removed, and the nematodes were assessed as follows while bathed in the nematicide solution. A fine pick was rolled across the middle of each of 75 nematodes two times; those responding with muscular movements were recorded as active.

In preliminary trials, nematodes of a W-P exposed to increasing concentrations of nematicides exhibited a decreasing activity S-shaped curve as a function of activity *vs* concentration. The range of concentrations of each nematicide tested, fell within the region of inflection of each curve. The data were evaluated following an arcsine transformation using Duncan's multiple range test for all values with an upper significance level of 5 %.

Results

Bioassay results revealed that activity (activity signifies those nematodes responding to touch stimulus by muscular contraction) in stressed control populations, *i.e.,* nematodes placed in water for 24 hours and bioassayed, depends upon the stressing NFN. Although activity in C-S-P, P-S-P and A-S-P was equivalent to W-P, activity of Ox-S-P was reduced, while that of A-S-P was greater than that of the other three stressed populations (Tab. 1).

Table 1

In vitro bioassays with carbofuran : percent activity of various populations of *Heterodera schachtii* at three concentrations of carbofuran.

H. schachtii *population*	*Control*	*Carbofuran treatments*		
		0.50 mM	*1.00 mM*	*2.00 mM*
W-P	70 *fg* (100 h)	41 *bc*	35 *b*	26 *a*
C-S-P	61 *ef* (100 h)	58 *def*	63 *ef*	78 *g*
Ox-S-P	45 *bcd* (100 h)	79 *g*	73 *fg*	63 *ef*
P-S-P	61 *ef* (100 h)	51 *cde*	49 *bcde*	42 *bc*
A-S-P	79 *g* (100 h)	63 *ef*	59 *def*	61 *ef*

W-P = Wild population, C-S-P = carbofuran-stressed population, likewise for Ox = oxamyl, P = phenamiphos and A = aldicarb. Control column values not in parentheses represent actual survival percentages. All other values have been adjusted relative to 100 % for each population control. Numbers not followed by a common letter are different at a significance level of 5 % or less.

Bioassays with carbofuran

Except for P-S-P (0.50 mM, 1.0 mM), percent activity in all cases was significantly greater than W-P at the corresponding concentrations; in terms of numbers of nematodes, activity in all stressed populations was greater than W-P at all concentrations (Tab. 1). Percent activity appeared to be concentration-dependent for W-P and Ox-S-P, but not for C-S-P (except 2.00 mM), P-S-P or A-S-P. Furthermore, the data indicate that in all treatments of W-P, A-S-P and P-S-P (2.00 mM) percent activity was significantly below, while that of all treatments of Ox-S-P and C-S-P (2.00 mM) percent activity was significantly above the corresponding population controls.

Bioassays with oxamyl

Except for C-S-P at all oxamyl concentrations and P-S-P at 0.25 mM, percent activity was significantly

Table 2

In vitro bioassays with oxamyl : percent activity of various populations of *Heterodera schachtii* at three concentrations of oxamyl.

H. schachtii *population*	*Control*	*Oxamyl treatments*		
		0.25 mM	*0.50 mM*	*1.00 mM*
W-P	70 *def* (100 g)	43 *ab*	41 *a*	40 *a*
C-S-P	61 *bcde* (100 g)	58 *abcd*	50 *abc*	57 *abcd*
Ox-S-P	45 *ab* (100 g)	83 *fg*	72 *def*	75 *def*
P-S-P	61 *bcde* (100 g)	58 *abcd*	70 *def*	71 *def*
A-S-P	79 *ef* (100 g)	64 *cdef*	64 *cdef*	65 *cde*

W-P = wild population, C-S-P = carbofuran-stressed population, likewise for Ox = oxamyl, P = phenamiphos and A = aldicarb. Control column values not in parentheses represent actual survival percentages. All other values have been adjusted relative to 100 % for each population control. Numbers not followed by a common letter are different at a significance level of 5 % or less.

greater than the corresponding treatments of W-P (Tab. 2). There was no evidence of oxamyl concentration dependence in percent activity for any of the five populations tested. The percent activity for all oxamyl concentrations applied to W-P was significantly below the W-P control but significantly greater than the Ox-S-P control for all oxamyl concentrations applied to Ox-S-P. For all other populations, controls and treatments were the same.

Bioassays with phenamiphos

The percent activity of all phenamiphos treatments for all stressed populations except A-S-P (0.80 mM) was significantly greater than the corresponding treatments of W-P (Tab. 3). A concentration-dependence trend was observed for Ox-S-P and at the higher concentrations for P-S-P. The significant difference in Ox-S-P between 0.40 mM and 0.8 mM may be an anomaly in the trend. The results indicate that for all phenamiphos concentrations, the percent activity was less than that of the respective controls of W-P and A-S-P, unchanged for C-S-P and greater than controls for Ox-S-P and P-S-P. It is striking to note that the 0.20 mM phenamiphos treatment of Ox-S-P and the 0.20 mM and 0.40 mM phenamiphos treatments of P-S-P demonstrated percent activity values equivalent to the respective control.

Bioassays with aldicarb

The general pattern of percent activity with stressed populations relative to W-P was somewhat different

Table 3

In vitro bioassays with phenamiphos : percent activity of various populations of *Heterodera schachtii* at three concentrations of phenamiphos.

H. schachtii *population*	*Control*	*Phenamiphos treatments*		
		0.20 mM	*0.40 mM*	*0.80 mM*
W-P	70 *cd* (100 f)	41 *a*	43 *a*	46 *ab*
C-S-P	61 *c* (100 f)	69 *cd*	66 *cd*	70 *cd*
Ox-S-P	45 *ab* (100 f)	90 *ef*	61 *c*	77 *d*
P-S-P	61 *c* (100 f)	90 *ef*	90 *ef*	77 *d*
A-S-P	79 *de* (100 f)	61 *c*	58 *bc*	59 *bc*

W-P = wild population, C-S-P = carbofuran-stressed population, likewise for Ox = oxamyl, P = phenamiphos and A = aldicarb. Control column values not in parentheses represent actual survival percentages. All other values have been adjusted relative to 100 % for each population control. Numbers not followed by a common letter are different at a significance level of 5 % or less.

with aldicarb treatments (Tab. 4). The percent activity of P-S-P and A-S-P was significantly less, at 0.05 mM aldicarb, but significantly greater for Ox-S-P at 0.25 mM aldicarb and significantly less for P-S-P, but significantly greater for Ox-S-P at 1.25 mM concentration of aldicarb than the respective treatments of W-P.

Table 4

In vitro bioassays with aldicarb : percent activity of various populations of *Heterodera schachtii* at three concentrations of aldicarb.

H. schachtii *population*	*Control*	*Aldicarb treatments*		
		0.05 mM	*0.25 mM*	*1.25 mM*
W-P	70 *defg* (100 i)	85 *ghi*	62 *cd*	57 *bcd*
C-S-P	61 *cd* (100 i)	83 *fgh*	60 *cd*	69 *def*
Ox-S-P	45 *ab* (100 i)	79 *efgh*	90 *hi*	85 *ghi*
P-S-P	61 *cd* (100 i)	69 *def*	49 *abc*	38 *a*
A-S-P	79 *efgh* (100 i)	65 *cde*	65 *cde*	66 *cde*

W-P = wild population, C-S-P = carbofuran-stressed population, likewise for Ox = oxamyl, P = phenamiphos and A = aldicarb. Control column values not in parentheses represent actuel survival percentages. All other values have been adjusted relative to 100 % for each population control. Numbers not followed by a common letter are different at a significance level of 5 % or less.

A negative concentration-dependence (a decrease in activity with an increase in concentration) occurred between the two lower concentration treatments of W-P, C-S-P and P-S-P; no differences existed between the higher aldicarb concentration treatments of any population. Of the aldicarb treatments of the five populations, only the 0.05 mM treatment of W-P and probably all treatments of Ox-S-P were no different than the respective water control; all other treatments were significantly different from the water control.

Discussion

The percent activity values in the control column less than that of W-P indicate a potential residual loss of muscular activity in response to the stressing nematicide. The Ox-S-P of *H. schachtii* constitutes the only stressed population with a significant loss of response capability. In no case did any treatment increase percent activity over the respective population control; however, seven events were observed (Ox-S-P, 0.25 mM oxamyl; Ox-S-P, 0.20 mM and P-S-P 0.20 mM and 0.40 mM phenamiphos; W-P, 0.05 mM and Ox-S-P, 0.25 mM and 1.25 mM aldicarb) to exhibit percent activity equivalent to the respective population control.

The percent activity values in the rows (stressed populations treated with NFN) greater than the respective treatment of W-P signify a measure of protection (resistance-tolerance), but if less, an increased susceptibility. The general pattern for carbofuran, oxamyl, and phenamiphos treatments was protection with several exceptions (for carbofuran, P-S-P, 0.50 mM and 1.00 mM; for oxamyl, C-S-P, all concentrations and P-S-P, 0.25 mM; for phenamiphos, A-S-P, 0.80 mM). In contrast, aldicarb treatments generated a different pattern of responses : two events demonstrated protection (Ox-S-P, 0.25 mM and 1.25 mM), three events demonstrated increased susceptibility (P-S-P, 0.05 mM and 1.25 mM and A-S-P, 0.05 mM), while the remaining observations were equivalent to the respective treatments of W-P.

The strong concentration dependence observed with *in vitro* bioassays of *Meloidogyne incognita* and *Pratylenchus vulnus* (Yamashita & Viglierchio, 1986*b*) and *Xiphinema index* (Yamashita & Viglierchio, 1987) was not evident with *Heterodera schachtii.* The results of the *in vitro* bioassays did not coincide with the observations in the greenhouse assays. Protection for stressed populations was evident in both assays in a substantial number of treatments. Though the Ox-S-P *in vitro* control was significantly reduced as was that of the greenhouse test, the C-S-P *in vitro* control value was not. In fact, the C-S-P and the P-S-P *in vitro* controls were comparable but inconsistent with the greenhouse trial control values. Those *in vitro* situations where increasing NFN concentration resulted in increased activity for touch stimulus (C-S-P, C-treated; Ox-S-P, P-treated) may be explained in part by competitive inhibition via a two-point site enzyme attachment. It remains striking to note that a high proportion of nematodes (approximately a quarter) retained the ability to respond to touch stimulus, whether wild or stressed, to NFN concentrations approaching 0.1 % for 24 hours.

In a simplified scheme of nerve conduction a depolarization pulse travels down the axon and upon arriving in the region of the pre-synaptic plate initiates a physical discharge of chemical transmitter into the synaptic gap. The transmitter moves towards the receptors of the post-synaptic plate to initiate a depolarization pulse in the ongoing conductor. The activation potential of the pulse appears to be generated primarily by two mechanisms, a membrane flow of sodium ion (sodium channel) or calcium ion (calcium channel). More frequently, the synaptic gap chemical transmitter is acetylcholine but may be an acylcholine, noradrenaline, adrenaline or other agent depending upon the neurofunction. To prepare the system for the next message, the ready-state membrane potential must be restored by reversing the sodium ion/calcium ion flow and the synapse chemical transmitter degraded; if acetylcholine, it must be hydrolyzed to acetic acid and choline by acetylcholinesterase. It is at this point that NFN activity is believed to intervene by binding with the acetylcholinesterase, thereby preventing the hydrolysis of the acetylcholine and subsequent release of components from the receptor site of the post-synaptic membrane. It is known that an inhibitor enzyme complex of this nature " ages " *i.e.,* initially the complex is reversible, but gradually transforms to one that is irreversible. In the avian brain, the half-life of a similar complex has been reported as approximately two hours (Witter & Gaines, 1963), whereas with plant-parasitic nematodes the half-life appeared to be on the order of days (Marban-Mendoza & Viglierchio, 1980*a*). Since the *in vitro* tests were completed within 24 hours, aging was considered to be a minor factor. If the NFN were an ideal acetylcholinesterase inhibitor, acetylcholine could not be hydrolyzed, and the post-synaptic membrane receptor sites would remain blocked, message transmission could not occur and the animal could not move. This is not the case since a high proportion of plant-parasitic nematodes bathed in high concentrations of NFN are able to respond to touch stimulus. It may be useful to consider some events or factors which can moderate or influence the system function.

The jellyfish, *Aglantha digitale* (order Hydromedusae) is capable of two distinct kinds of locomotion; " slow " swimming effected by a weak contraction of the bell-shaped body wall and " fast " swimming effected by a violent contraction of the bell-shaped body wall. In both cases, swimming is accomplished by expulsion of a jet of water from the opening at the base of the animal. Giant motor axons mediate both kinds of activity by

conducting different sorts of impulses. For fast swimming, a rapidly conducted sodium ion-dependent action potential and for slow swimming, a low amplitude calcium ion action potential (Mackey & Meech, 1985). Although this was the first observation where both mechanisms operate through the same nervous transmission system, both mechanisms were found in a variety of tissues in different animals (Hagiwara, Ozawa & Sand, 1975).

The second component of the nerve transmission system involves the synapse in which chemical transmitters convey a message across the synaptic gap. There appear to be a wealth of chemical transmitters depending upon nervous system function and location, e.g., in the brain the number of transmitters is upwards of two dozen and growing; in other tissues besides acetylcholine; acylcholines and other transmitters, there are the transmitter deactivating systems consisting of a wide array of isozymes and allozymes (Jacobs, 1987; Fest & Schmidt, 1975; National Research Council, 1986). Organophosphates for example, not only inhibit acetylcholinesterase-type enzymes via anionic and esteric binding, but also receptor sites of the post-synaptic plate to modify resultant message transmission. Moreover, they can evoke the liberation of indigenous substances which stimulate electrical activity and which effect an excess of acetylcholine to modify message transmission. Furthermore, the protein molecules which constitute receptor sites have slightly different conformations for each transmitter subtype and which can be inhibited in varying degrees depending upon the applied agent. The observation of cross-tolerance or resistance provides presumptive evidence for common biological site of action of major importance; however, the high proportion of nematodes exhibiting moderate activity in response to touch stimulus implicate, as a substantial component, a host of factors as outlined that in part may explain the *in vitro* observations for different nematodes treated with different NFN.

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