In vitro testing for nonfumigant nematicide resistance in *Xiphinema index*

Tom T. YAMASHITA and David R. VIGLIERCHIO

Division of Nematology, University of California, Davis, Ca 95616 USA.

SUMMARY

An *in vitro* method was used for detecting nonfumigant nematicide resistance in *Xiphinema index*. This consisted of a 24 hour exposure of tested populations to three concentrations each of carbofuran, oxamyl and phenamiphos. Nematodes were evaluated active or inactive by using a touch-response technique. The results tended to vary with the concentration of nematicide. In most cases the highest nematicide concentration best expressed differences between stressed, unstressed and wild populations. Detection of resistance and increased susceptibility coincided with results from previous greenhouse testing. The *in vitro* method also detected differences between populations that went unnoticed in greenhouse tests. Results from the *in vitro* experiments with carbofuran, oxamyl and especially phenamiphos suggested that these nematicides react in an intricate and complex manner with this nematode species.

RÉSUMÉ

Test in vitro *de la résistance de* Xiphinema index *aux nématicides non fumigants*

Une technique *in vitro* a été utilisée pour détecter la résistance de *Xiphinema index* aux nématicides non fumigants. Elle a consisté à exposer pendant 24 h les populations à tester à trois concentrations de chacun des nématicides carbofuran, oxamyl et phenamiphos. Les nématodes actifs ou inactifs ont été différenciés par réaction au contact. Les résultats varient avec la concentration du nématicide. Dans la plupart des cas les plus fortes concentrations donnent les meilleures différences entre populations sensibilisées, désensibilisées et naturelles. La détection de la résistance et l'accroissement de la sensibilité correspondent aux résultats antérieurement acquis lors d'essais en serre. Cette technique *in vitro* permet de détecter entre populations des différences qui n'avaient pas été mises en évidence lors des essais en serre. A partir de ces tests *in vitro* les résultats obtenus avec le carbofuran, l'oxamyl, et plus particulièrement le phenamiphos, suggèrent que ces nématicides agissent sur le nématode d'une manière complexe.

Populations of *Xiphinema index* stressed for over three years with subnematicidal doses of carbofuran, oxamyl and phenamiphos were observed to have altered responses to nematicidal-level applications (Yamashita, Viglierchio & Schmitt, 1986). These altered responses gave indications of various changes in population characters. Some of these altered characteristics included reduced reproductive potentials, increased susceptibility to nonfumigant nematicides (NFN), resistance to NFN and an apparent habituation to subnematicidal doses. The populations were released from monthly stressing for about two years and tested for retention of their altered characters (Yamashita & Viglierchio, 1986). In many cases the characters of resistance and increased susceptibility were retained, but this varied with the specific nematode population-NFN interaction. Lowered reproductive potentials observed in stressed populations were restored to wild-type behavior. However, in a population released from phenamiphos stressing, there were strong indications of increased reproductive potentials.

The design of these preliminary studies attempted to simulate field conditions, while minimizing the variable field factors. As a result, these experiments were conducted under controlled greenhouse conditions. The concentrations of NFN used approximated those applied under actual field conditions.

At recommended field doses, the great majority of these NFN appear to act by impairing nematode behavior (Marban-Mendoza & Viglierchio, 1983*a*, 1983*b*, 1983*c*). Characterizing and screening of NFN have largely relied upon bioassays which isolate one or more effects on behavioral changes (Bunt, 1975; Marban-Mendoza & Viglierchio, 1983*a*, 1983*b*, 1983*c*). An investigation into possible resistance of animal parasites to anthelmintics, as an example, had relied upon an egg hatch *in vitro* assay (Le Jambre, 1976).

Acetylcholinesterase and biogenic amines have been implicated as responsible nerve transmission factors in nematodes (Hogger & Estey, 1978; Kisiel, Deubert & Zuckerman, 1976; Sulston, Dew & Brenner, 1975; Wright & Awan, 1976). In addition, the overall

matabolism of nematodes appears to parallel that of higher metazoans. This includes demonstrated TCA, glycolytic, cytochrome and electron transport systems, glyoxalate, beta oxidation and phosphogluconate and gluconeogenesis pathways, to mention a few (Bolla, 1980). A majority of the NFN (in particular the organophosphates and carbamates) are believed to act on the acetylcholinesterase and related nerve transmission centers of nematodes (Corbett, 1974). However, the exact nature of how NFN affect nematodes is unknown. Furthermore, the exact nature of resistance to NFN is unknown at this time. *In vitro* bioassays, which test a particular isolated nematode behavior, such as motility, dispersion or attraction to hosts, may help characterize resistance or increased susceptibility. These types of *in vitro* bioassays may not, however, lend themselves to a quick and thorough detection of such characteristics as resistance or increased susceptibility. *In vitro* bioassays which test the death or survival (as is oftentimes used in testing insecticides) of a nematode species may test a broader spectrum of the total physiological processes contributing to resistance or increased susceptibility. This study was conducted to determine wether a death-survival type of *in vitro* bioassay could detect characteristics such as NFN resistance and increased susceptibility in *X. index.*

Materials and methods

The seven populations of *X. index* tested in these experiments included the following :

1. Wild population (W-P; with no previous history of nematicide treatments).
2. Nematicide-stressed populations with a five year history of continuous monthly subnematicidal stressing. One population was stressed with carbofuran (C-S-P), another with oxamyl (Ox-S-P) and a third with phenamiphos (Ph-S-P).
3. Nematicide-unstressed populations with a three year history of continuous subnematicidal stressing followed by two years in the absence of stress treatments. One population was released from carbofuran stress (C-U-P), another from oxamyl (Ox-U-P) and a third from phenamiphos stress (Ph-U-P).

All populations were cultured on Carignane grapevines in four liter pots. The soil used consisted of a sterilized mixture of equal parts loam and river sand. Stock cultures were maintained in a greenhouse and watered daily with half strength Hoagland's nutrient solution. Temperatures were maintained at about 25°.

Soil and root cores were removed from stock pots and the nematodes extracted using 833 µm and 147 µm sieves. The suspension of nematodes caught on the 147 µm sieve was further freed from debris by using a Baermann Funnel with three layers of cheesecloth. After two hours, nematodes were collected from the Baermann Funnel and washed in a 38 µm sieve with a gentle stream of tap water. The cleaned suspension of nematodes was then aerated in tap water for five minutes. Following aeration, approximately 50 nematodes were aliquanted into 100 ml glass beakers (2.5 mls of suspension). An equal volume (2.5 mls) of either carbofuran, oxamyl or phenamiphos was then added bringing the total volume of nematicide and nematode suspension up to 5 mls. The resultant concentrations of each nematicide were used : carbofuran — 0.02 mM, 0.20 mM, 0.60 mM; oxamyl — 0.06 mM, 0.30 mM, 0.60 mM; phenamiphos — 0.032 mM, 0.096 mM, 0.160 mM. The control treatment consisted of adding an equal volume (2.5 mls) of tap water. Each treatment was replicated five times. The beakers were then placed into plastic containers covered with aluminum foil (to eliminate light and reduce evaporation). A six-layered piece of cheesecloth (10 cm × 5 cm) was placed into the container and saturated with tap water to maintain humidity. The container with beakers was left at 25° for 24 hours. Following a 24 hour incubation, the nematodes were assessed as active or inactive while bathed in nematicide solution. A fishing line pick was rolled across the middle of the nematode. Those responding with movement were recorded as active. Other detection methods were tested, such as the use of dyes. While the use of a touch-response was more tedious, it proved to be more accurate, since the results with dyes tended to be variable.

As preliminary testing revealed a tendency for slight variations between different stock culture pots within one population, the experiment was repeated three times. Each time an experiment was repeated, the nematodes were extracted from a different stock culture pot (from the population evaluated).

Due to slight variations in the numbers of nematodes aliquanted, the data was evaluated following a logit transformation [ln (number of active + 0.5 divided by number of inactive + 0.5)]. Mean comparisons were conducted using Duncan's Multiple Range Test. The means represent the average of fifteen replications. An upper significance level of 5 % was used in these analyses.

Results

The results will be divided into three sections :

1. *In vitro* bioassays with carbofuran.
2. *In vitro* bioassays with oxamyl.
3. *In vitro* bioassays with phenamiphos.

In vitro bioassays with carbofuran

Results from these tests are summarized in Table 1. The effects of different concentrations are best viewed

Table 1

In vitro bioassays with carbofuran : percent survival of various populations of *Xiphinema index* at three concentrations of carbofuran

X. index *population*	*Carbofuran Treatment*			
	Control	0.02 *mM*	0.20 *mM*	0.60 *mM*
Wild	93 *abc*	87 *bcde*	83 *def*	74 *g*
C-S-P	96 *ab*	94 *abc*	94 *abc*	94 *abc*
Ox-S-P	94 *abc*	93 *abc*	88 *bcde*	82 *efg*
Ph-S-P	93 *abc*	89 *bcde*	88 *bcde*	91 *bcd*
C-U-P	95 *ab*	98 *a*	99 *a*	93 *abc*
Ox-U-P	96 *ab*	95 *ab*	95 *ab*	93 *abc*
Ph-U-P	96 *ab*	96 *ab*	95 *ab*	84 *def*

Numbers represent the means of fifteen replications. Nematodes were exposed to carbofuran for 24 hours and then evaluated for active *vs* inactive using a touch-response method. Means not followed by a common letter are significantly different at an α level of 5 % or less.

by moving across a row. Comparisons between populations are made by moving down a column. Differences between populations were best expressed at the highest concentration of carbofuran (far right column with 0.60 mM carbofuran). The most evident feature was that the percent of survival in all stressed and unstressed populations were significantly higher than the W-P (74 %). This agrees with the previous greenhouse tests, since all stressed and unstressed populations displayed a resistance to carbofuran (Yamashita, Viglierchio & Schmitt, 1986; Yamashita & Viglierchio, 1986).

The expression of population differences appeared to be dependent on the concentration of carbofuran. In the absence of chemical (control) there were no differences expressed. At 0.02 mM carbofuran, only the C-U-P (at 98 %) showed a significantly higher survival than the W-P (at 87 %). At the 0.20 mM concentration exposures, four populations had higher survival percentages than the W-P (C-S-P at 94 %, C-U-P at 99 %, Ox-U-P at 95 % and Ph-U-P at 95 %; W-P = 83 %). At the highest carbofuran exposure of 0.60 mM, all the stressed and unstressed populations were found to have higher survival percentages than the W-P.

IN VITRO BIOASSAYS WITH OXAMYL

Unlike carbofuran, differences between populations are best expressed at the intermediate concentration of oxamyl (0.30 mM; Tab. 2). The survival in all stressed and unstressed populations exceeded that of the W-P (78 %). In earlier greenhouse tests (Yamashita, Viglierchio & Schmitt, 1986) all stressed populations displayed resistance to oxamyl. In these previous tests resistance was hinted at because the C-S-P, Ox-S-P and Ph-S-P appeared to be indifferent to oxamyl applications. When tests were conducted with the unstressed populations, however, this resistance appeared to have been lost (Yamashita & Viglierchio, 1986). Contrary to these latter greenhouse findings, however, the *in vitro* tests indicated that all unstressed, as well as stressed, populations had resistance to oxamyl.

Again, the expression of population differences appeared to be dependent on the oxamyl concentration. There were no differences between controls, but the exposure to 0.06 mM oxamyl indicated higher percentages of survival in the C-S-P (96 %), C-U-P (96 %), Ox-U-P (96 %) and Ph-U-P (98 %) over that of the W-P (85 %). Differences were expressed most clearly at 0.30 mM oxamyl. At the highest concentration of 0.60 mM, the Ph-S-P (67 %) did not exhibit the higher percent survival (over the W-P at 62 %) as it did at the intermediate concentration of 0.30 mM oxamyl.

Table 2

In vitro bioassays with oxamyl : percent survival of various populations of *Xiphinema index* at three concentrations of oxamyl

X. index *population*	*Oxamyl Treatment*			
	Control	0.06 *mM*	0.30 *mM*	0.60 *mM*
Wild	93 *abc*	85 *def*	78 *fgh*	62 *j*
C-S-P	96 *ab*	96 *ab*	96 *ab*	72 *hi*
Ox-S-P	94 *abc*	89 *bcde*	91 *abcde*	76 *ghi*
Ph-S-P	93 *abc*	92 *abcd*	87 *cde*	67 *ij*
C-U-P	95 *ab*	96 *ab*	95 *abc*	87 *cde*
Ox-U-P	96 *ab*	96 *ab*	96 *ab*	83 *efg*
Ph-U-P	96 *ab*	98 *a*	91 *abcde*	76 *ghi*

Numbers represent the mean of fifteen replications. Nematodes were exposed to oxamyl for 24 hours and then evaluated for active *vs* inactive using a touch-response method. Means not followed by a common letter are significantly different at an α level of 5 % of less.

IN VITRO BIOASSAYS WITH PHENAMIPHOS

There were no differences between control treatments (Tab. 3). However, at the 0.032 mM phenamiphos

Table 3

In vitro bioassays with phenamiphos : percent survival of various populations of *Xiphinema index* at three concentrations of phenamiphos

X. index *population*	*Phenamiphos Treatment*			
	Control	0.032 *mM*	0.96 *mM*	0.16 *mM*
Wild	93 *a*	77 *b*	48 *ef*	15 *hi*
C-S-P	96 *a*	93 *a*	50 *e*	6 *j*
Ox-S-P	94 *a*	91 *a*	39 *fg*	5 *j*
Ph-S-P	93 *a*	92 *a*	56 *de*	2 *j*
C-U-P	95 *a*	93 *a*	61 *cd*	9 *ij*
Ox-U-P	96 *a*	95 *a*	76 *b*	19 *h*
Ph-U-P	96 *a*	96 *a*	73 *bc*	29 *g*

Numbers represent the means of fifteen replications. Nematodes were exposed to phenamiphos for 24 hours and then evaluated for active *vs* inactive using a touch-response method. Means not followed by a common letter are significantly different at an α level of 5 % or less.

exposures, the percent survival in the W-P (77 %) was significantly below that of all stressed and unstressed populations. This pattern of differences was seen to change at the higher concentration exposures. At 0.096 mM, all unstressed populations remained significantly higher in survival over the W-P. The stressed populations, however, did not display significant differences in survival from the W-P. What is also expressed at the 0.096 mM exposures are differences between the stressed and unstressed populations. The percent survival in all stressed populations (C-S-P at 50 %; Ox-S-P at 39 %; Ph-S-P at 56 %) were significantly lower than each of their respective unstressed populations (C-U-P at 61 %; Ox-U-P at 76 %; Ph-U-P at 73 %).

At the 0.160 mM phenamiphos exposures, the percent survival in the Ox-S-P and Ph-S-P are again significantly lower than their respective unstressed populations (Ox-S-P at 5 % *vs* Ox-U-P at 19 %; Ph-S-P at 2 % *vs* Ph-U-P at 29 %). However, the differences between the C-S-P and C-U-P that were expressed at 0.096 mM are not expressed at the 0.160 mM phenamiphos exposures. Only one population (Ph-U-P at 29 %) expressed signs of resistance. All stressed populations, however, expressed signs of increased susceptibility to 0.160 mM phenamiphos. This increased susceptibility to phenamiphos was observed with the C-S-P and Ox-S-P in earlier greenhouse tests (Yamashita, Viglierchio & Schmitt, 1985).

Discussion

What is very evident in these tests is that the expression of population differences appears to be concentration-dependent. In all of the chemical exposures and at one or more of the concentrations tested, there was an indication of resistance. However at the 0.160 mM phenamiphos exposures, the C-S-P, Ox-S-P and Ph-S-P gave signs of increased susceptibility. These findings support earlier observations in greenhouse experiments (Yamashita, Viglierchio & Schmitt, 1986; Yamashita & Viglierchio, 1986).

The indications of resistance to carbofuran by all stressed and unstressed populations coincide with results found in earlier greenhouse studies. However, the *in vitro* bioassay results with oxamyl and phenamiphos do not always agree so closely with earlier greenhouse test results. For example, at the 0.30 mM exposures to oxamyl, all stressed and unstressed populations displayed signs of resistance. The earlier greenhouse tests results, however, indicated that only the stressed populations were resistant to oxamyl. As another example, all stressed and unstressed populations showed resistance to phenamiphos at the 0.032 mM exposures. However, in neither of the greenhouse tests were there signs of resistance to phenamiphos.

Conclusive statements

Greenhouse tests, using actual field doses of NFN, help to express behavioral differences in various populations of *X. index*. Tests with *Pratylenchus vulnus* had indicated that NFN acted by impairing such behavioral characteristics as motility, dispersion, orientation and attraction to hosts (Marban-Mendoza & Viglierchio, 1983*a*, 1983*b*, 1983*c*). The methods used in these *in vitro* bioassays, however, place the nematodes under extremely stressful conditions. The entire spectrum of physiological processes, contributing to the immediate survival of the organism, are put to the test. This type of bioassay approximates that commonly used with insecticides to test LD_{50} ranges of selected insects and selected insecticides (Tahori, 1971).

Findings from these bioassays do not always coincide with previous greenhouse test results. However, they appear to express differences that might otherwise go undetected in tests which utilize only field concentrations. The concentration-dependent expressions of various differences attests to the complexities of nematode-nematicide type interactions. These interactions appear to be most intricate with phenamiphos, since each concentration helps to express a slightly different response.

With further research to characterize correlations between greenhouse and *in vitro* bioassay results, it may

be possible to utilize this method for detecting differences in field populations. As the use of NFN increases, farm advisors may find an *in vitro* inspection for resistance and increased susceptibility a necessary part of their programs.

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