

# *In vitro* testing for nonfumigant nematicide resistance in *Meloidogyne incognita* and *Pratylenchus vulnus*

Tom T. YAMASHITA and David R. VIGLIERCHIO

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

## SUMMARY

Populations of *Meloidogyne incognita* and *Pratylenchus vulnus* were stressed with subnematicidal levels of carbofuran, oxamyl and phenamiphos for over five years (stressed populations). After the third year of stressing, however, one half of the stressed cultures were released from monthly stress treatments (unstressed populations). These various stressed and unstressed populations were tested for resistance to the three nonfumigant nematicides (NFN). The *in vitro* bioassay employed high NFN concentrations. In most cases the NFN concentrations were more than ten times the estimated field dose concentrations. Following 24 hours of exposure, the nematodes were assessed as living or dead. Using this *in vitro* bioassay, it was possible to detect resistance in stressed and unstressed populations. The method also helped to express population differences that went undetected in previous greenhouse tests. Expression of these differences was most evident at the highest NFN concentrations.

## RÉSUMÉ

*Essais in vitro de résistance aux nématicides non fumigants chez Meloidogyne incognita et Pratylenchus vulnus*

Des populations de *Meloidogyne incognita* et de *Pratylenchus vulnus* ont été sensibilisées par application mensuelle, pendant cinq ans, de doses subnématicides de carbofuran, oxamyl et phenamiphos (populations sensibilisées). Cependant, après trois années de sensibilisation, la moitié de ces populations n'a plus été soumise au traitement (populations non sensibilisées). Ces deux lots de populations ont été ensuite testés pour leur résistance à trois nématicides non fumigants (NNF). Lors des bio-essais *in vitro* les doses de NNF employées étaient très élevées; dans certains cas ces doses atteignaient plus de dix fois les concentrations estimées au champ. Après 24 heures de traitement, les nématodes morts et vivants étaient dénombrés. Un tel bio-essai a permis de détecter l'existence d'une résistance chez des populations sensibilisées et non sensibilisées. Cette méthode est également utile pour mettre en évidence entre populations des différences qui n'avaient pas été perçues dans les essais antérieurs en serre. L'expression de ces différences était la plus évidente aux fortes concentrations de NNF.

Greenhouse trials have been conducted with populations of *Xiphinema index*, *Meloidogyne incognita* and *Pratylenchus vulnus* stressed with nonfumigant subnematicidal doses and released from stress for extended periods. Diverse altered behavior patterns were observed (Yamashita & Viglierchio, 1986a, 1986b; Yamashita, Viglierchio & Schmitt, 1986).

In the range of recommended field doses nonfumigant nematicides (NFN) can effect behavioral changes including motility, dispersion, orientation, attraction to hosts or reproductive potentials (Marban-Mendoza & Viglierchio, 1980a, 1980b, 1980c). Previous tests with stressed and unstressed nematodes had indicated that populations differing from the wild population did not always share the same characteristic differences (Yamashita & Viglierchio, 1986a, 1986b; Yamashita, Viglierchio & Schmitt, 1986). A rapid and convenient *in vitro* bioassay, designed to detect NFN resistance or increased susceptibility and able to

integrate a broader spectrum of nematode characteristics would have merit.

Because field doses tended to test a particular behavioral change, they were not considered in the design of an *in vitro* bioassay. The authors felt that the use of higher concentrations would be more appropriate in these *in vitro* tests. The nematodes subjected to an *in vitro* bioassay would be placed under sufficient stress to manifest a broader spectrum of physiological processes contributing to its survival. An *in vitro* bioassay employing this concept proved to be a satisfactory means of detecting NFN resistance and increased susceptibility in various populations of *X. index* (Yamashita & Viglierchio, 1987). Furthermore, the method was able to express differences which went undetected in greenhouse tests.

The concept of differences between species is well-understood. These experiments were conducted to test the feasibility of a previously utilized *in vitro*

bioassay (with *X. index*) for detecting NFN resistance and increased susceptibility in *M. incognita* and *P. vulnus*.

## Materials and methods

The seven populations of *M. incognita* 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 stress (Ox-U-P) and a third from phenamiphos stress (Ph-U-P).

For the first three years, the wild and stressed populations had been cultured on French Colombard grapevines in four liter pots. The soil consisted of a sterilized mixture of equal parts white sand and river sand. Inspection of the stock cultures at the end of the third year indicated that the population levels had dropped dramatically low. All cultures were contaminated with an organism resembling *Pasteuria penetrans*. All cultures were salvaged by sterilizing the *M. incognita* egg masses. The emerging J2 larvae were inoculated onto three week old Murietta tomato plants. One half of the stressed populations were allowed to rebuild for two months before monthly subnematicidal treatments were resumed. The other half of the stressed cultures were released completely from monthly NFN stress (unstressed populations). All stock cultures were maintained in a greenhouse and watered daily with half strength Hoagland's nutrient solution. Temperatures were maintained at about 25°.

Low population levels were also found in all *P. vulnus* stock cultures. Stock populations on Thompson Seedless grapevines were extracted through sieving and misting techniques. Salvaged nematodes were inoculated onto two week old Kentucky Wonder bean plants to rebuild the population levels. The populations that were available for immediate *in vitro* testing included the following :

1. Wild population (W-P).
2. A population stressed for five years with phenamiphos (Ph-S-P).
3. Two populations with a three year history of NFN stressing followed by two years in the absence of subnematicidal stress. One had been released from carbofuran stress (C-U-P) and the other from oxamyl stress (Ox-U-P).

For both *M. incognita* and *P. vulnus* extractions, portions of the host roots were removed from stock culture plants. These were cut into 1 cm pieces and placed into mist chambers for two days. The collected nematodes were caught on a 45 µm sieve. They were collected in flasks and aerated in tap water for five minutes. Following aeration, approximately 50 nematodes were aliquanted into 100 ml glass beakers (2.5 mls suspension). An equal volume (2.5 ml) of either carbofuran, oxamyl or phenamiphos was then added to bring the total volume of nematicide and nematode suspension up to 5 ml. Three concentrations of each nematicide were used : carbofuran : 0.20 mM, 0.40 mM, 0.60 mM; oxamyl : 0.04 mM, 0.20 mM, 0.40 mM; phenamiphos : 0.08 mM, 0.20 mM, 0.40 mM. The nematicide solutions were made up fresh before each experiment. The control treatment consisted of adding an equal volume (2.5 ml) of tap water. Each treatment was replicated five times. The beakers were then placed into a plastic container 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 24 hours incubation, the nematodes were assessed as living or dead. A fishing line pick was rolled across the middle of the nematode. Those responding with movement were recorded as living. All assessments of living and dead nematodes were conducted within the tested NFN solution. The experiment was repeated three times. Each time an experiment was repeated, the tested population was extracted from a different stock culture pot. This was done to account for any natural variations between stock culture pots within a population.

Due to slight variations in the numbers of nematodes aliquanted into beakers, the data was evaluated following a logit transformation [ $\ln(\text{number of living} + 0.5) / \ln(\text{number of dead} + 0.5)$ ]. Mean comparisons were conducted using Duncan's Multiple Range Test. The means represent the averages of fifteen replications. An upper significance level of 5 % was used in these analyses.

## Results

### MELOIDOGYNE INCOGNITA

#### *In vitro* bioassays with carbofuran

Differences in the ability of certain populations to survive the 24 hours carbofuran exposures were best expressed at the higher concentrations (Tab. 1). Significantly lower survival percentages of the W-P at 0.40 mM (78 %) and 0.60 mM (22 %) suggested a degree of resistance to carbofuran in all stressed and unstressed

Table 1

*In vitro* bioassays with carbofuran : percent survival of various populations of *Meloidogyne incognita* at three concentrations of carbofuran

M. incognita population	Carbofuran Treatment			
	Control	0.20 mM	0.40 mM	0.60 mM
Wild	92 abcde	88 cdef	78 gh	22 i
C-S-P	95 abc	93 abcd	88 cdef	82 fgh
Ox-S-P	100 a	94 abc	99 a	91 bcdef
Ph-S-P	100 a	98 a	99 a	83 fg
C-U-P	92 abcde	92 abcde	84 ef	76 h
Ox-U-P	95 abc	97 ab	96 ab	92 abcde
Ph-U-P	95 abc	93 abcd	93 abcd	86 def

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

populations. Releasing the stressed populations from monthly subnematicidal treatments (unstressing) appeared to have had little effect on the unstressed populations inasmuch as the percent survival in stressed populations closely matched the percent survival in their respective unstressed populations (for example, C-S-P vs C-U-P). Aside from the W-P, the C-S-P and C-U-P survival percentages tended to be lower than others. This is especially evident at the 0.40 mM carbofuran exposure (C-S-P at 88 % and C-U-P at 84 %). In an earlier greenhouse test the C-S-P also appeared to be more susceptible to carbofuran than the Ox-S-P and Ph-S-P (Yamashita, Viglierchio & Schmitt, 1986). Because a larger percentage of nematodes survived the 0.60 mM exposures, the Ox-S-P (91 %) and Ox-U-P (92 %) appeared to have the greater degree of resistance to carbofuran.

#### *In vitro* bioassays with oxamyl

The low survival percentages from the 0.40 mM oxamyl exposures suggested that *M. incognita* may be more sensitive to this nematicide than to carbofuran or phenamiphos (Tab. 2). Also, the interactions between *M. incognita* and oxamyl appeared to be more complex than the interactions with carbofuran (Tab. 1) or phenamiphos (Tab. 3). This interpretation was suggested because, the comparative percentages of survival tended to vary more with changes in the oxamyl concentrations. For example, all but the C-U-P (89 %) showed a resistance to oxamyl at the 0.04 mM exposures (W-P at 82 %). At 0.20 mM, however, only the Ox-U-P (87 %) and the Ph-U-P (95 %) had significantly higher survival percentages over the W-P (68 %). When the

Table 2

*In vitro* bioassays with oxamyl : percent survival of various populations of *Meloidogyne incognita* at three concentrations of oxamyl

M. incognita population	Oxamyl Treatment			
	Control	0.04 mM	0.20 mM	0.40 mM
Wild	92 bc	82 ef	68 g	1 i
C-S-P	95 abc	90 bcd	75 fg	2 i
Ox-S-P	100 a	99 ab	70 g	16 h
Ph-S-P	100 a	97 abc	73 g	2 i
C-U-P	92 bc	89 cde	69 g	10 h
Ox-U-P	95 abc	93 abc	87 de	10 h
Ph-U-P	95 abc	94 abc	95 abc	71 g

Numbers represent the means of fifteen replications. Nematodes were exposed to oxamyl for 24 hours and then evaluated for live vs dead using a touch-response method. Means not followed by a common letter are significantly different at an  $\alpha$  level of 5 % or less.

Table 3

*In vitro* bioassays with phenamiphos : percent survival of various populations of *Meloidogyne incognita* at three concentrations of phenamiphos

M. incognita population	Phenamiphos Treatment			
	Control	0.08 mM	0.20 mM	0.40 mM
Wild	92 bcd	79 gh	69 jk	22 l
C-S-P	95 abc	77 hi	72 ijk	66 k
Ox-S-P	100 a	92 bcd	82 fgh	81 fgh
Ph-S-P	100 a	97 ab	84 efg	89 cde
C-U-P	92 bcd	76 hij	82 fgh	72 ijk
Ox-U-P	95 abc	89 cde	89 cde	86 def
Ph-U-P	95 abc	94 abc	92 bcd	84 efg

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

nematodes were tested at 0.40 mM, the Ox-S-P (16 %), C-U-P (10 %), Ox-U-P (10 %) and Ph-U-P (71 %) expressed resistance to oxamyl (W-P at 1 %). The expression of resistance to oxamyl in all stressed populations agreed with findings of an earlier greenhouse test (Yamashita, Viglierchio & Schmitt,

1986). In general, unstressed populations appeared to be able to withstand the oxamyl exposures better than their respective stressed populations. This was most evident with the Ph-U-P at the 0.20 mM (Ph-S-P at 73 % vs Ph-U-P at 95 %) and 0.40 mM (Ph-S-P at 2 % vs Ph-U-P at 71 %) oxamyl exposures.

*In vitro bioassays with phenamiphos*

The concentration-dependent changes in survival percentages resembled those seen in the carbofuran bioassays (Tab. 3). All stressed and unstressed populations demonstrated a degree of resistance to phenamiphos at 0.40 mM. In earlier greenhouse experiments, all stressed *M. incognita* populations had demonstrated a degree of resistance to phenamiphos (Yamashita, Viglierchio & Schmitt, 1986). This expression of resistance was not as obvious at the two lower concentrations. As found in the carbofuran bioassays, the C-S-P and C-U-P tended to show a higher degree of sensitivity to phenamiphos than did the other stressed and unstressed populations. This higher sensitivity was best expressed at 0.40 mM (C-S-P at 66 %; C-U-P at 72 %). Except for a minor difference between the C-S-P and C-U-P at 0.20 mM (C-S-P at 72 % vs C-U-P at 82 %), there were no apparent differences between the stressed and respective unstressed populations. That is, unstresssing did not appear to affect the degree of phenamiphos resistance seen in the stressed populations.

*PRATYLENCHUS VULNUS*

*In vitro bioassays with carbofuran*

Resistance to carbofuran was expressed at the 0.40 mM and 0.60 mM exposures (Tab. 4). The Ph-S-P, C-U-P and Ox-U-P were all capable of surviving both

Table 4

*In vitro* bioassays with carbofuran : percent survival of various populations of *Pratylenchus vulnus* at three concentrations of carbofuran

P. vulnus population	Carbofuran Treatment			
	Control	0.20 mM	0.40 mM	0.60 mM
Wild	92 ab	94 ab	84 c	63 d
Ph-S-P	100 a	100 a	100 a	100 a
C-U-P	100 a	100 a	100 a	94 ab
Ox-U-P	100 a	100 a	100 a	97 ab

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

concentrations of carbofuran better than the W-P. This was best demonstrated at the highest concentration of carbofuran. Earlier, all stressed populations of *P. vulnus* had shown resistance to carbofuran (Yamashita, Viglierchio & Schmitt, 1986). There were no apparent differences observed between the Ph-S-P, C-U-P and Ox-U-P.

*In vitro bioassays with oxamyl*

As with the carbofuran bioassay with *P. vulnus*, there appeared to be a concentration-dependent expression of resistance (Tab. 5). Again, the indications of resistance were most evident at the highest concentration of oxamyl. The C-U-P (at 100 %) demonstrated a higher degree of resistance than the Ph-S-P (93 %) and Ox-U-P (95 %) at the 0.20 mM oxamyl exposures. However, this difference was not seen at 0.40 mM oxamyl.

Table 5

*In vitro* bioassays with oxamyl : percent survival of various populations of *Pratylenchus vulnus* at three concentrations of oxamyl

P. vulnus population	Oxamyl Treatment			
	Control	0.04 mM	0.20 mM	0.40 mM
Wild	92 d	90 de	81 ef	67 f
Ph-S-P	100 a	99 ab	93 d	94 cd
C-U-P	100 a	100 a	100 a	93 d
Ox-U-P	100 a	98 abc	95 bcd	93 d

Numbers represent the means of fifteen replications. Nematodes were exposed to oxamyl for 24 hours and then evaluated for live vs dead using a touch-response method. Means not followed by a common letter are significantly different at an  $\alpha$  level of 5 % or less.

*In vitro bioassays with phenamiphos*

*Pratylenchus vulnus* appeared to be more sensitive to phenamiphos than to carbofuran or oxamyl (Tab. 6). Resistance was expressed even at the lowest concentration (0.08 mM). Furthermore, as the concentrations of phenamiphos were increased, there was relatively little change from the results observed at 0.08 mM. While the C-U-P of *P. vulnus* had demonstrated resistance to carbofuran and oxamyl, it did not show signs of resistance to phenamiphos. Resistance to phenamiphos was demonstrated in only the Ph-S-P and Ox-U-P.

**Discussion**

In almost all of these *in vitro* bioassays there is a distinct concentration-dependent expression of

Table 6

*In vitro* bioassays with phenamiphos : percent survival of various populations of *Pratylenchus vulnus* at three concentrations of phenamiphos

P. vulnus population	Phenamiphos Treatment			
	Control	0.08 mM	0.20 mM	0.40 mM
Wild	92 b	56 e	49 ef	46 f
Ph-S-P	100 a	100 a	91 bc	84 cd
C-U-P	100 a	50 ef	43 f	44 f
Ox-U-P	100 a	91 bc	92 b	83 d

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

population differences. Population differences are best expressed at the highest concentrations of the NFN. Resistance was seen in unstressed, as well as stressed populations. This suggested that the NFN resistance, which had developed in stressed populations, persisted even after two years in the absence of selective pressures. Persistence of various altered behaviors was also seen with *X. index* (Yamashita & Viglierchio, 1986a, 1987) and this phenomenon has been observed in insects (Brown, 1971). The ability of the Ox-U-P and Ph-U-P of *M. incognita* to withstand oxamyl exposures better than the Ox-S-P and Ph-S-P is difficult to explain at this time and requires further research. The concept of "vigor tolerance" has helped to explain an apparent resistance in insects (Hoskins & Gordon, 1956). With vigor tolerance, certain strains of insects are able to withstand higher concentrations of insecticides. This is made possible by virtue of a superior range of secondary physiological mechanisms which are capable of supporting the primary mechanism injured by the toxic agent. In previous *in vitro* tests unstressed populations of *X. index* were observed to survive NFN exposures better than their respective stressed populations (Yamashita & Viglierchio, 1987). In the current tests the unstressed populations were found to be more resistant than the stressed populations to 0.096 mM phenamiphos.

The relative toxicities of carbofuran, oxamyl and phenamiphos have been studied (Bunt, 1975; Marban-Mendoza & Viglierchio, 1980a, 1980b, 1980c).

current *in vitro* bioassays and past greenhouse and

laboratory tests indicate that the effectiveness of any one NFN tends to vary with the specific nematode species treated (Yamashita & Viglierchio, 1986a, 1986b, 1987; Yamashita, Viglierchio & Schmitt, 1986). For example, the current tests with *M. incognita* and *P. vulnus* indicated that *M. incognita* was more sensitive to oxamyl than to carbofuran or phenamiphos (Tab. 2). *Pratylenchus vulnus* was found to be more sensitive to phenamiphos than to carbofuran or oxamyl (Tab. 6). A similar *in vitro* bioassay with *X. index* revealed that this species was more sensitive to phenamiphos than to carbofuran or oxamyl (Yamashita & Viglierchio, 1987). With these types of results, it may appear as though carbofuran may be of minor economic importance. However, in both the carbofuran and phenamiphos bioassays with *M. incognita* (Tab. 1 & 3) and the phenamiphos bioassays with *P. vulnus* (Tab. 6), the carbofuran-stressed and unstressed populations demonstrated lower percentages of survival than did other populations (besides the W-P). Secondly, with *X. index*, carbofuran stressing appeared to have reduced reproductive potentials significantly lower than was obtained with either oxamyl or phenamiphos stressing (Yamashita, Viglierchio & Schmitt, 1986).

### Conclusive statements

Testing nematode populations with field doses of NFN may surface various behavioral differences. For example, under normal conditions, two nematode populations with subtle physiological differences may appear identical in behavior. Their otherwise unnoticeable differences may be expressed during exposures to field concentrations. However, the characterizing of a population in one or more aspects of behavior does not necessarily constitute a correlation with the population levels that develop. These concepts were covered in other papers (Yamashita & Viglierchio, 1986a, 1986b, 1987; Yamashita, Viglierchio & Schmitt, 1986). These previously cited studies had also indicated that stressed populations with resistance to NFN generally had lower reproductive potentials. However, when these NFN-resistant populations were released from selective pressures, their reproductive potentials were generally improved. More importantly, in most cases they were shown to retain the NFN resistance character. With these thoughts in mind, then, a primary concern for practical considerations should focus on detecting the characters of resistance and/or increased susceptibility.

The use of a living vs dead type of *in vitro* bioassay necessitated the use of high NFN concentrations. In the estimated field doses. These *in vitro* bioassays may tend to test a broad spectrum of physiological processes

contributing to the life of the nematode. Because of this, the results of *in vitro* tests should not be totally equated with field and greenhouse data, which measure behavioral changes. However, the results from these *in vitro* bioassays indicated that the method can be a viable means of detecting resistance in various populations of *M. incognita* and *P. vulnus*.

#### ACKNOWLEDGEMENTS

The authors would like to thank the American Vineyard Foundation (San Francisco, California) and the Western Pesticide Impact Assessment Program (WPIAP) for their partial support of this project. We would also like to thank Kathleen Yamashita for her invaluable help in this project.

This research constitutes partial fulfillment of advanced degree requirements.

#### REFERENCES

- BROWN, A. W. A. (1971). Pest resistance to pesticides. In : White-Stevens, R. (Ed.) *Pesticides in the environment*, New York, Marcel Dekker, I (II) : 457-552.
- BUNT, J. A. (1975). Effect and mode of action of some systemic nematicides. *Meded. Landbhogesch. Wageningen*, 75, 127 p.
- HOSKINS, W. M. & GORDON, H. T. (1956). Arthropod resistance to chemicals *Ann. Rev. Entomol.*, 1 : 89-122.
- MARBAN-MENDOZA, N. & VIGLIERCHIO, D. R. (1980a). Behavioral effects of carbofuran and phenamiphos on *Pratylenchus vulnus*. I. Motility and dispersion. *J. Nematol.*, 12 : 102-114.
- MARBAN-MENDOZA, N. & VIGLIERCHIO, D. R. (1980b). Behavioral effects of carbofuran and phenamiphos on *Pratylenchus vulnus*. II. Attraction to bean roots. *J. Nematol.*, 12 : 114-118.
- MARBAN-MENDOZA, N. & VIGLIERCHIO, D. R. (1980c). Behavioral effects of carbofuran and phenamiphos on *Pratylenchus vulnus*. III. Penetration and development. *J. Nematol.*, 12 : 119-129.
- YAMASHITA, T. T. & VIGLIERCHIO, D. R. (1986a). The behavior of nonfumigant nematicide-stressed, unstressed and wild populations of *Pratylenchus vulnus* cultured on grapevines and bean plants. *Revue Nématol.*, 9 : 267-276.
- YAMASHITA, T. T. & VIGLIERCHIO, D. R. (1986b). Variations in the stability of behavioral changes in nonfumigant nematicide-stressed populations of *Xiphinema index* following release from subnematicidal stress. *Revue Nématol.*, 9 : 377-383.
- YAMASHITA, T. T. & VIGLIERCHIO, D. R. (1987). In vitro testing for nonfumigant nematicide resistance in *Xiphinema index*. *Revue Nématol.*, 10 (in press).
- YAMASHITA, T. T., VIGLIERCHIO, D. R. & SCHMITT, R. V. (1986). Responses of nematodes to nematicidal applications following extended exposures to subnematicidal stress. *Revue Nématol.*, 9 : 49-60.

Accepté pour publication le 15 octobre 1985.