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Control and identification of root knot nematode

A report prepared for
Vegfed

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CONTENTS

1 EXECUTIVE SUMMARY

This report consists of two parts. Part One describes a field trial to evaluate the effect on yield of applying a nematocide to nematode-infested ground before planting a carrot crop. Part Two discusses the successful use of PCR primers to identify the root knot nematode, *Meloidogyne hapla*, and plans to develop a suite of PCR primers to identify other nematode species in New Zealand.

1.1 Nematocide trial

A field trial was undertaken to determine the effect of Nemacur (20 ℓ/ha) on the marketable yield of carrots grown in nematode-infested ground. Three methods of incorporating Nemacur were examined:

- Nemacur was sprayed on to the bed and incorporated; the bed was seeded immediately.
- Nemacur was sprayed on to the bed and incorporated; the bed was seeded 10 days later.
- Nemacur was sprayed onto the surface of the bed immediately after seeding, but not incorporated.

The growing season was difficult because of the hot dry conditions that prevailed, and only 30% of potential yield was achieved throughout the trial. Yields were variable and final analysis was confined to the percentage marketable yield of carrots.

The results showed that Nemacur applied at 20 ℓ/ha (product) had a significant effect and improved the yield of marketable grade carrots by approximately 10% over yields from untreated plots. The various methods of applying and incorporating Nemacur did not result in significant differences in marketable yields from different treatments.

1.2 Rapid identification of root knot nematode and its species with PCR detection methods

A number of PCR primers have been developed overseas for selected root knot nematode species. The results of a preliminary study show that some of these primers can detect *Meloidogyne hapla* and some other species, but not all species.

We will use the published primers as a basis for developing additional primers to determine the distribution and species composition of all *Meloidogyne* species within New Zealand.

Part One

Evaluation of liquid nematocides for the control of root knot nematode and other plant parasitic nematodes on yield and quality of fresh carrots

2 INTRODUCTION

Carrots are grown throughout New Zealand and are marketed as fresh or processed produce. The fresh produce is sold locally or exported. The value of export carrots has continued to rise from $1 million in 1993 to $5 million in 1996. Because some importing countries (e.g. Japan) do not tolerate any root knot nematode (*Meloidogyne*) on carrots there is a demand for cost effective control of nematodes in carrot crops destined for the export market. In addition the local market is also demanding higher quality. The important standards required are:

- freedom from obvious disease
- uniform appearance
- consistent size

The results of disease are obvious but many other factors affect the appearance of carrots including soil type, growing conditions, damage by mechanical equipment, and nematodes. The effects of nematode damage on the appearance of carrots are illustrated in Plate 1. When carrots are repeatedly grown in nematode-infested ground the level of damage can make the crop uneconomic to harvest. This seldom happens, however, as nematodes are usually distributed unevenly across a field and damage occurs in patches.

Control of nematodes is difficult and expensive. There are only two registered control agents--Nemacur and Vydate. The most frequently used product is Nemacur (fenamiphos (Bayer)) but within the industry there are a number of application practices. Crop & Food Research therefore carried out a trial (Plate 2) to:

- test different methods of applying and incorporating Nemacur in crops;
- determine the optimum method of application;
- identify the effect of Nemacur on the marketable yield of fresh carrots.

Plate 1: Carrots with low levels of nematode damage; note the contrasting appearance of carrots grown in untreated control plots (right) and carrots grown in nematocide-treated plots (left).

Plate 2: Trial site showing field layout.

3 EXPERIMENTAL METHODS AND MATERIALS

3.1 Trial site

A trial site in the Cambridge district was selected as it had a history of carrot production and nematode damage had been reported. Root knot nematode (*Meloidogyne*) and other plant parasitic nematodes (*Pratylenchus, Paratylenchus, Heliocotylenchus* and *Heterodera*) were identified from field samples of the area. The fact that *Meloidogyne* levels were low was seen as an advantage as it gave an opportunity to examine the collective effects of a range of plant parasitic nematodes without the obvious effects of root knot masking other nematode damage. (As a result of recent field observations and literature searches we now know that nematodes other than root knot nematode can affect carrot quality).

3.2 Site preparation

In the preceding autumn a green crop of barley was grown and incorporated over the winter. This procedure reduced root knot nematode levels but a significant nematode population still remained in the soil.

The trial site was fertilised according to the farmer's usual practice for carrots, and seed beds were preformed with a rotary hoe. Before planting, soil samples of all the plots were collected to identify the distribution of plant parasitic nematodes in the paddock.

3.3 Plot size

Each plot was one bed wide, 1500 mm between wheel centres, and 2000 mm long. The total area of the trial was 18 m x 18 m.

3.4 Soil treatments

The following treatments were applied to the plots:

1. Control. No Nemacur was applied.
2. Nemacur was applied and rotary hoed into the plot to a depth of 200 mm. The plot was seeded immediately.

3. Nemacur was applied to the plot and rotary hoed to a depth of 200 mm. The crop was not seeded for 10 days.
4. Nemacur was applied to the plots immediately after the beds had been seeded.

3.5 Materials

Nemacur (400 g/litre fenamiphos) was obtained from the farmer's own new stocks and applied with a watering can to the preformed beds to give an equivalent rate of 20 ℓ/ha.

New, certified seed of the carrot cultivar, Explorer, was purchased and coated with Apron and Promet to industry standards. In addition to sowing Promet-coated seed, a line of seed without Promet in its coating was sown to allow for analysis of the component effects of Promet and Nemacur (see Section 3.6).

3.6 Experimental design

The trial was designed as a meshing of two components: one for soil treatments 1, 2 and 4 in four field rows, and one for treatments 1 and 3 in two field rows randomly placed among the first four field rows.

A second trial was established as a result of the farmer's enthusiasm and interest in seeing whether Promet-treated seed had an effect on seedling establishment, or offered early protection from nematode damage. This trial was laid in six rows alongside the first trial. It was planned to be identical to the first trial except that no Promet was included in the seed coating. Because of an accidental mis-sowing of the ten day delayed seeding of Promet and non-Promet treated seed, it was not possible to compare soil treatments directly between the two component field trials. To get maximum precision from the results of the work and to test for differences between the two seed treatments it was necessary to use both the farmer's additional trial and the original one, and to do separate analyses of the two component trials, i.e. treatments 1, 2 and 4 (trial A), and 1 and 3 (trial B).

Trial A was a non-standard balanced block design with two seed treatments (with Promet, without Promet) applied to four field rows, and three soil treatments randomised to plots within field rows. An additional soil treatment not part of this trial was also included in both Trial A and Trial B, described below. Field rows contained nine plots placed end-to-end within the row, so there were 72 plots in the trial. The difference between seed treatments was confounded with differences between halves of the trial; each half had the same treatment layout. The trial was seeded and soil treatments were applied on 22 October 1997.

Trial B was similar, with two field rows of each seed treatment, and three plots of each soil treatment per field row. The seed treatments were confounded with the two halves of the trial, as they were for trial A. There were 36 plots for trial B. The Nemacur soil treatment was applied and incorporated on 22 October 1997 and all plots were seeded on 1 November 1997.

Thus, in each trial, the soil treatments were compared as subplot treatments within each field row (99% and 100% of the information on soil treatment differences was at this level in trials A and B respectively).

3.7 Growing season

The pre-emergence herbicide spray, Linuron (2.5 ℓ/ha), was applied seven days after planting. The crop was not irrigated although the 1997 season was a difficult one and there were periods of severe drought stress during the growing season.

The trial was harvested on 12 February 1998. All carrots were hand pulled and topped, and the number and weight of marketable and unmarketable carrots were recorded in the field.

4 RESULTS

4.1 Nematode numbers and distribution

Immediately before laying down the trial a 500 g soil sample was taken from 45% of all plots in the six adjacent field rows that comprised the original trial. The sample was examined for nematode numbers and species composition. Nematodes were unevenly distributed across the trial area (an average of 50/100 ml of soil). The species composition was typical of intensively cropped soil: the plant parasitic genera, *Pratylenchus, Meloidogyne, Paratylenchus, Heterodera* and *Helicotylenchus,* were all represented (Appendix I). The same method was used to take postharvest nematode counts on the same plots. The results are presented in Appendix II.

Nemacur-treated plots showed no clear pattern of reduced nematode numbers. It was obvious, however, that in all plots and treatments total nematode numbers had decreased (mean 18/100 ml of soil), and often only *Pratylenchus* was present in the nematode counts. It is possible that the effect of the nematocide on nematode numbers was masked by the subsequent recolonisation of the soil by surviving nematodes after the compound had degraded. We expected to find higher numbers of nematodes in the control plots but this was not the case, probably because the hot dry season restricted the rate of nematode multiplication in all plots.

4.2 Quality and yield

All yield and grading data were analysed with the GENSTAT statistical package.

The hot, dry season was a very difficult one. Overall yields from the trial were only 30% of yields in an average season and there was a large variation in yields between rows. This substantial variation in row yields was accompanied by sizeable variation among the plots within rows. (Coefficients of variation in total weight were 22% for trial A and 17% for trial B.) Effectiveness of the soil treatments was most precisely evaluated by the analysis of the proportion of the carrots which were of "marketable grade". The precision achieved in the experiments was sufficient to detect differences in marketable yields due to soil treatments greater than 9% for trial A and 12% for trial B (testing significance at the 5% level and using angular transformed data).

4.3 Marketable yield

Because many of the plots had responses over 80% the angular transformation was required to stabilise variances; and because there was substantial variation in average numbers of carrots between plots a weighted analysis of variance on the angular scale was used. Statistical tests were made on transformed data but bias-adjusted back-transformed means are given also as estimates of "% marketable carrots" for each trial. There were no significant differences between the two halves (one with and one without Promet in the seed coating) of either trial in the percentage of carrots that were marketable. Results for the soil treatments are summarised in Table 1.

Table 1: Percentage of marketable carrots from two adjacent trials of the nematocide, Nemacur.

	Trial A		Trial B	
Treatments	Angle	% marketable carrots	Angle	% marketable carrots
(1) control	58.4	71.7a[1]	62.6	77.4a
(2) Nem. incorporated	69.3	86.0b	-	-
(4) Nem. surface	69.6	86.4b	-	-
(3) Nem. incorporated. Planting delayed 10 days	-	-	70.4	86.8b
LSD (5%)	5.6	-	8	-

[1] Treatments with the same letter are not significantly different at the 5% level, using Fisher's protected LSD test.

4.4 Summary of treatment results

1. Nemacur used at 20 ℓ/ha resulted in a 12% higher marketable yield of fresh carrots than yields from other treatments.

2. The three different methods of applying and incorporating Nemacur did not result in any significant differences in marketable yields.

3. Plant parasitic nematodes other than root knot nematodes can affect carrot quality, but a damage threshold could not be established from the results of these trials.

5 DISCUSSION

The results of these trials indicate that the use of nematocides has the potential to improve the percentage yield of marketable carrots. It was not possible to calculate the costs and benefits of applying a nematocide as overall crop yields were too low.

It should also be noted that despite the relatively low incidence of root knot nematode, symptoms that are often associated with root knot nematode damage were present. These data indicate that a range of nematode species can affect carrot quality, and in this trial, at least 10% of marketable yield losses could be attributed to nematode damage.

Significant losses in marketable yield still occurred in the Nemacur-treated plots—14% in trial A and 13% in trial B. These losses could be attributed to a range of factors such as soil texture, wind damage to the developing roots, fungal disease, and damage from mechanical equipment to plants in rows close to the wheel rows.

Part two

Rapid identification of the root knot nematode, *Meloidogyne hapla*, using PCR primers to amplify its ribosomal intergenic spacer

6 INTRODUCTION

6.1 Identifying nematodes

Meloidogyne species are important pathogens of potatoes and other crops worldwide. Because many countries including New Zealand are implementing quarantine restrictions for the species, simple, rapid, and reliable means of identification are essential. Nematode identification usually requires morphometric measurements of juveniles and mature females, as well as differential host range studies. Although reliable, these methods remain specialised and time-consuming. However, recently developed molecular techniques are simplifying the process of identifying the species *Meloidogyne*.

6.2 Developing molecular techniques

Castagnone-Sereno et al. (1993) separated *Meloidogyne arenaria, M. incognita, M. javanica,* and *M. hapla,* using highly repetitive sequences such as satellite DNA. Other advances rely on PCR (polymerase chain reaction) amplification of a specific sequence, and restriction fragment length polymorphisms (RFLPs) in that sequence of interest. Powers and Harris (1993) were able to identify *M. incognita, M. javanica, M. arenaria, M. hapla,* and *M. chitwoodi,* from RFLP analysis of a mitochondrial intergenic region, while Ziljstra et al. (1995) separated *M. chitwoodi* from *M. hapla, M. fallax, M. incognita,* and *M. javanica,* using RFLPs in the ITS of ribosomal DNA (rDNA) repeats. Still more advanced are the methods developed by Ziljstra (1997) which eliminate the need for restriction enzyme digestion by directly separating nematode species by the size of the PCR amplified fragment. The ITS regions of *M. incognita, M. fallax, M. hapla,* and *M. chitwoodi* were cloned and sequenced and PCR primers were designed to amplify the variable regions to separate these four species. The amplification product from *M. chitwoodi* was easily separated from *M. hapla* and *M. incognita,* but the size of the PCR product of *M. fallax* differed from *M. chitwoodi* by only eight base pairs making it very difficult to distinguish by standard agarose gel electrophoresis. Peterson and Vrain hypothesised that the variable length of rDNA IGS sequences could be used to separate species of root knot nematodes. Primers used to amplify the ITS were reorientated divergently and used to amplify the IGS region of *M. chitwoodi* via PCR. Additional primers that could amplify the IGS with only minimal flanking of the 28S and 18S rDNA were constructed. Agarose gel electrophoresis of amplified fragments obtained from PCR amplification of DNA from *M. chitwoodi , M. hapla,* and *M. fallax* demonstrated size polymorphisms.

In summary, a combination of the protocols and primers developed by Ziljstra (1997) and Peterson & Vrain (1997) makes it possible to identify and differentiate between *Meloidogyne hapla, M. incognita, M. fallax* and *M. chitwoodi.* The objective of this project was to obtain existing primers known to specifically amplify genomic DNA of *M. hapla,* and trial these to see if they are also specific to reference New Zealand isolates.

7 OBJECTIVE AND EXPERIMENTAL METHOD

7.1 Objective

Our objective was to test existing primers known to specifically amplify genomic DNA of *Meloidogyne hapla* to see if they are also specific to reference New Zealand isolates. The primers tested are listed in Table 1.

Table 1: Primer sets 1 and 2.

Primer	Sequence	Product size (bp)	Origin
H-18S	5'-CTT GGA GAC TGT TGA TC-3'	660	Ziljstra (1997)
HCFI-28S	5'-TTC CTC CGC TTA CTG ATA TG-3'		Ferris (1993)

7.2 Preparation of genomic DNA

Preparation of template DNA: Frozen stocks of nematode larvae (bulk) were defrosted and immediately suspended in 500μl of solution (containing 5*M* guanidine isothiocyanate, 10 m*M* EDTA, 50 m*M* Tris-HCL (pH 7.5), and 8% mercaptoethanol) and ground as finely as possible in Eppendorf tubes using plastic micro-pestles (Treff). After incubation for 1 h at 65°C, the solution was extracted once with equal volumes of phenol and chloroform-isoamyl alcohol (24:1) and once with chloroform- isoamyl alcohol. The DNA was precipitated with 1/10th volume of 0.3 *M* sodium acetate and two volumes of 100% ethanol. Following a 30 minute incubation at -20°C, the DNA was pelleted by centrifugation, washed in 70% ethanol and dissolved in 50 μl dH_2O.

7.3 The PCR reaction

One μl of DNA was used as the template in the final PCR reaction volume of 25 μl. PCR conditions were 20 m*M* Tris-HCl (pH 8.3), 50 m*M* KCl, 2.0 m*M* $MgCl_2$, 160 μl each dNTP, 250 μl each primer, and 0.6 U Taq DNA polymerase (Boehringer Mannheim). A touch down cycling profile was used: denaturation at 94°C for 4 min, followed by five cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 1 min with a decrease of 1°C per cycle for the annealing temperature followed by 25 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min.

All PCR reactions included a no DNA control. Reactions showing amplification in the negative control were discarded. Amplification products were analysed by electrophoresis in 1% agarose gels.

8 RESULTS AND DISCUSSION

8.1 Results

The combination of primers H-18S and HCF1-28S resulted in an amplified fragment of 660 bp when *Meloidogyne hapla* DNA was used as template DNA (Figure 1).

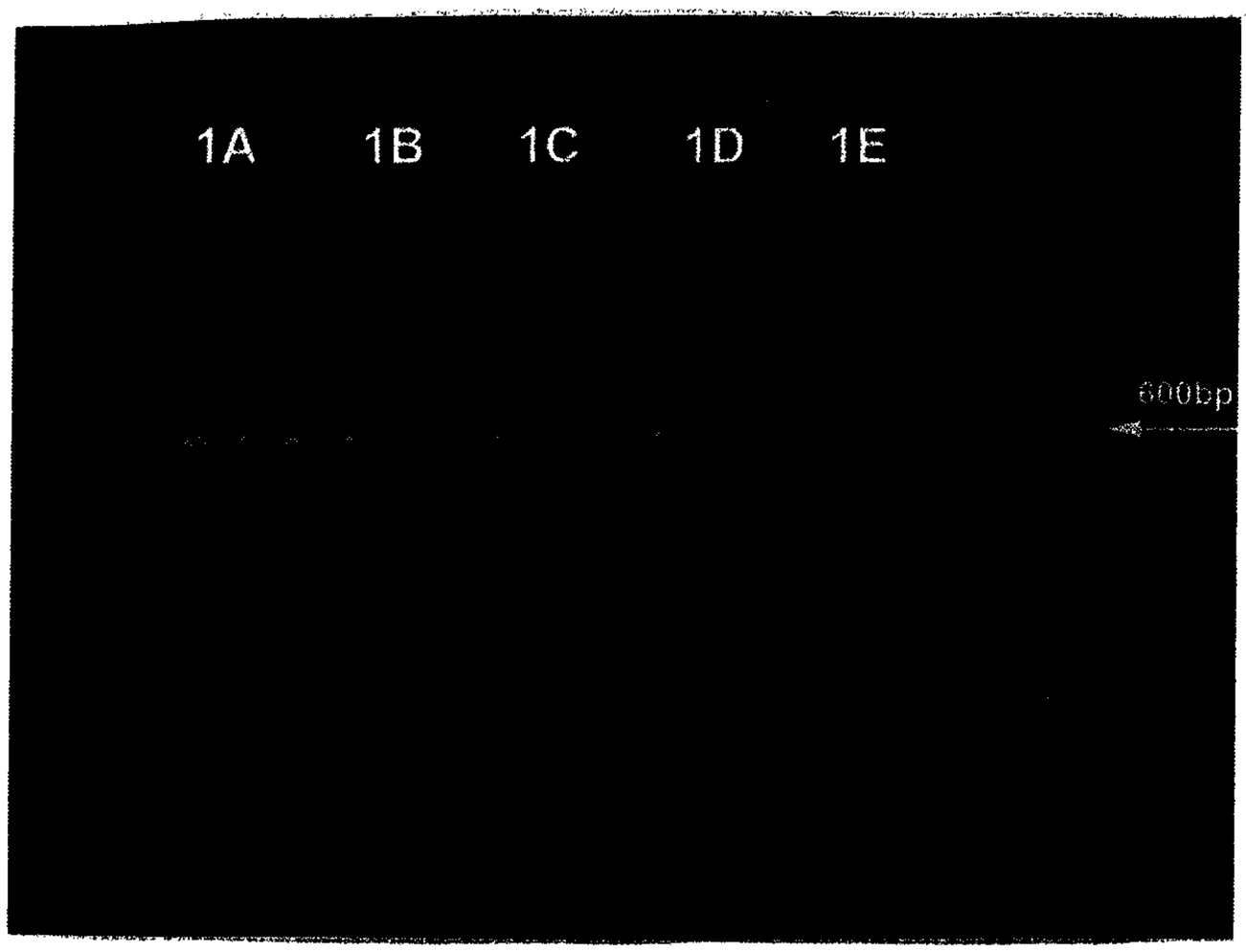

Figure 1: Amplification of a 660 bp band from a New Zealand isolate of *Meloidogyne hapla*. 1A, 1B, 1C, and 1D are local isolates of *M. hapla*. 1E is the negative control.

8.2 Future work

This limited study indicates that it is possible to apply PCR techniques to New Zealand populations to resolve questions about which species are present in New Zealand, and their distribution.

As a result of a successful 1998 bid to FfRST (Foundation for Research, Science and Technology), this work will be continued as a PGSF (Public Good Science Fund) programme. Over the next three to four years we will develop a comprehensive suite of PCR primers capable of identifying root knot nematode in New Zealand. The results of the work will have a direct application for assessing export material that requires an endorsement for freedom from root knot nematode. In addition we will be able to use the technology in longer term studies to develop new methods to control nematodes in the absence of traditional chemical control methods.

9 REFERENCES

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10 APPENDICES

Appendix I

Table 1A: Numbers and species of nematode detected in field plots, pre-planting

Plot number	*Pratylenchus*	*Meloidogyne*	*Paratylenchus*	*Heterodera*	*Helicotylenchus*
35796	40	-	-	-	-
35800	50	-	-	-	-
35801	120	20	10	-	10
35802	90	-			
35826	100	-	-	-	10
35827	30	-	-	10	-
35829	20	-	-	20	-
35832	10	-	20	-	-
35856	20	20	-	10	-
35858	20	-	-	10	-
35860	20	-	10	-	-
35862	40	-	10	-	-
35885	20	-	-	-	-
35886	30	-	-	-	-
35889	60	-	20	-	-
35890	40	-	-	-	-
35893	20	-	-	-	-
35916	10	10	-	-	-
35918	10	-	-	-	-
35919	30	-	-	-	-
35922	80	10	20	-	-
35923	70				
35947	-	-	-	40	-
35950	30	-	10	-	-
35952	20	-	-	-	-

Appendix II

Table 2A: Nematode numbers in all plots, postharvest.

Plot number	*Pratylenchus*	*Meloidogyne*	*Paratylenchus*	*Heterodera*	*Helicotylenchus*
35796	30	-			
35800	20	-			
35801	-	-			
35802	20	-	20	10	
35826	40	-			
35827	20	-			
35829	20	-			10
35832	-	-			
35856	30	-		40	
35858	-	-			
35860	-	-		10	
35862	10	-			
35885	20	-			
35886	10	-			
35889	30	-	20		
35890	20	-			
35893	10	-			
35916	10	-	20		
35918	-	-			
35919	-	-			
35922	10	-			
35923	10	-			
35947	10	-			
35950	-	-			
35952	-	-			