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Potato cyst nematode sampling and identification

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Contents

1 Executive summary 1

2 Objective 2

3 Method 3

3.1 Plot trial 3

3.2 Development of duplex primers for quantifying PCR 4

4 Results and discussion 5

4.1 Main trial 5

4.2 Cultivar trial 7

4.3 Mapping of PCN infestations within a paddock using GPS technology 8

4.4 Development of duplex primers for quantifying PCN 9

5 Summary 10

6 References 11

7 Acknowledgements 11

Appendix I 12

1 Executive summary

Potato cyst nematode (PCN) is known to affect potato yields. The presence of PCN within harvested crops may also compromise existing and new export markets due to quarantine issues. The development of integrated control practices such as crop rotation, effective soil sampling techniques, and the use of resistant cultivars will be necessary to ensure the infestation is maintained at as low a level as possible. A potato plot trial was established within a naturally infested paddock near Pukekawa to determine the effect of different initial PCN (Pi) populations on potato (Ilam Hardy) yield and multiplication rates of PCN. Potato yields were significantly lower in plots where Pi was high. Yields were reduced to as little as 30% of crops grown in a nematode-free environment, suggesting high marginal costs to the grower. With the resistant potato cultivars (Nadine, Karaka, Moonlight and Summit), Nadine showed partial resistance, while the remaining three cultivars maintained a high level of resistance to PCN multiplication.

Effective control of PCN relies on knowledge of the species and pathotype present in the soil. Fluorescence detection of PCR products using the Perkin-Elmer ABI® 7700 Sequence Detector (TaqMan™), allows for rapid detection and quantification of DNA sequences. By using fluorescence detection to quantify DNA a relationship between the number of cysts extracted and the Ct value from the TaqMan™ was estimated. This enabled a minimum detection of 1 cyst (260 eggs/cyst) per 100 ml of soil sample.

Direct estimation of Ct values relating egg content in cysts (derived from field sites) was attempted. A poor relationship was found, which resulted from the high variability of eggs per cyst values within replicates. This research is ongoing to further understand the relationship between Ct values and egg content in soil.

The outcome of this research will be an improved detection monitoring system that will provide enhanced levels of assurance of phytosanitary regulations, thus allowing access to new export markets for New Zealand potatoes. This work will be integrated into collaborative research being undertaken between Australian and New Zealand researchers with continuing financial support from Vegfed and matching funds provided by HAL Australia. This research will be undertaken over the next three years.

2 Objective

Potato cyst nematode (PCN) is known to affect potato yields. The presence of PCN within harvested crops may also compromise existing and new export markets due to quarantine issues. A recent survey of paddocks in the Franklin District has shown that PCN is present on a number of properties. The development of integrated control practices such as crop rotation, effective soil sampling techniques, and the use of resistant cultivars will be necessary to ensure the infestation is maintained at as low a level as possible. This will assist with quality assurance of potato exports, as it will be necessary that export orders can be guaranteed to be free of PCN. If PCN levels are not well managed, it is possible potato production in New Zealand may follow the lead of England and other parts of Europe where PCN is a major factor risking sustainable production.

Effective control of PCN also relies on knowledge of the species and pathotype present in the soil. Physical detection methods are slow and labour intensive as well as sometimes being ineffective and so in recent years molecular biology tools such as Polymerase Chain Reaction (PCR) have been used to examine the PCN species and pathotypes. PCR has been used to distinguish the two main species of PCN in New Zealand. However, this gives us information on the species present, not the quantity of each species. Fluorescence detection of PCR products using the Perkin-Elmer ABI® 7700 Sequence Detector (TaqMan™), allows for rapid detection and quantification of DNA sequences. This method uses a fluorescent oligonucleotide probe with a 5-prime (5') reporter dye and a downstream 3-prime (3') quencher dye. During PCR, the reporter dye is released and the resultant fluorescence is detected. Relative normalised fluorescence (Rn, (emission intensity of reporter) over (emission intensity of reference)) v. time (PCR cycle number) is plotted to allow real time assessment of the PCR. Delta-Rn is the Rn value at any given cycle number after subtracting the baseline Rn setting. The average background fluorescence emission is calculated and the standard deviation derived. A threshold fluorescence intensity is established at 10 times this standard deviation. Any sample that reaches a fluorescence value exceeding the fluorescence threshold is considered positive and the cycle at which this first occurs is defined as the threshold cycle (Ct). The Ct values are obtained for known quantities of DNA sequence and linear regression was used to estimate a relationship between Ct value and DNA amount. This allows us to then estimate a quantity for the Ct value of the unknown sample.

The objective of this project was to:

- establish a plot trial within a naturally infested paddock to determine the effect of different initial PCN (*Globodera pallida*) population levels on potato yield and multiplication rates of PCN. The effect of resistant potato cultivars on multiplication rates of PCN was also assessed.
- develop duplex primers for the quantitative PCR for the potato cyst nematodes *G. pallida* and *G. rostochiensis*.

3 Method

3.1 Plot trial

To ensure a range of initial PCN population levels were included within the main potato plot trial, it was necessary to initially map the existing infestation. This was conducted in June 2000 by intensively sampling an area of 1.2 hectares within the paddock using a sampling grid of 4 x 5 metres, where approximately 1 kg of soil was collected from each position. Soil samples were brought back to Crop & Food, Lincoln, for assessment of cyst numbers using standard procedures. This involves the soil samples being elutriated to recover cysts which are then counted under a stereoscopic microscope. From this initial mapping of the infestation, it was possible to establish plots across the full range of initial PCN levels to assess the effect of differing initial population levels on potato yield and PCN multiplication rates (= Pf/Pi, where Pf is the final cyst population, determined at crop harvest). Multiplication rates provide a measure of the PCN population dynamics during the potato crop's growing season. These rates are influenced by a number of factors, including the potato cultivar's resistance to PCN infestation. This part of the project is known as the "main trial".

Thirty two potato plots were established in September 2000, where each plot consisted of 7 tubers by 5 rows. Soil samples were collected from each plot at the time of planting and analysed in the laboratory to determine the initial (Pi) populations at planting, which ranged from nil to 66 eggs/ml soil. This confirmed that the positioning of the plots for the main trial encompassed a range of initial PCN levels. The cultivar selected for this trial was Ilam Hardy as this particular cultivar is susceptible to PCN. To determine the number of eggs in a sample, the first step involves removing and counting cyst numbers, as described above. The eggs are then released from the cysts by staining with 0.1% w/v new blue^{R} overnight and egg numbers determined in a Doncaster counting cell under a stereoscopic microscope at 30 x magnification.

An additional trial, termed a "cultivar trial", was established adjacent to some of the main trial's plots to assess the effect of resistant cultivars on multiplication rates of PCN. The additional cultivars planted were Nadine, Karaka, Moonlight and Summit. A brief description of each cultivar used is given below.

Ilam Hardy is the most widely grown early cultivar in the area and is the susceptible control. **Nadine** is a Scottish bred second early to main crop fresh market potato, which has claims for partial resistance to *G. pallida*. **Karaka** is a Crop & Food Research fresh market and French fry second early to early main crop cultivar, which has shown high resistance to a range of PCN populations in testing both in New Zealand and in Northern Ireland, and overall has shown a higher level of PCN resistance to a range of populations than any other released potato cultivar. **Moonlight**, a newly released Crop & Food Research fresh market and French fry second early to main crop potato cultivar, shows moderately high resistance to the Pukekohe Pa2 PCN population and to a range of populations tested, both in New Zealand and Northern Ireland. Lastly, **Summit**, a newly released Crop & Food Research

early fresh market potato, has shown high resistance to the Pukekohe Pa2 PCN population, and is similar to Moonlight with regard to the range of resistance to PCN populations.

For the cultivar trial, five replicates of the five cultivars were planted in a randomised block layout. Soil samples were collected from each plot to determine the initial (Pi) populations following the same procedure used for the main trial.

The entire trial site was managed using standard commercial fertiliser, herbicide, and fungicide programmes.

On 7 February 2001 the plots were harvested and soil samples collected. To exclude "edge effects", yield assessments for the main trial were based on the middle 5 plants by 3 rows; equivalent to an effective sampling area of 3.4 m2 per plot. Tubers were graded into table and seed, and the number and weights of each grade were recorded. Unfortunately, machinery damage to some of the plots meant that the number of plots used for yield assessments was reduced to 26. Soil samples were collected from all 32 plots and assessed for cyst and egg counts to determine final (Pf) PCN populations.

In the cultivar trial, it was originally intended that the effect of resistant cultivars on yields and multiplication rates of PCN would be determined. Unfortunately, damage from machinery compaction meant that no reliable yield data from the cultivar plots could be obtained. The machinery damage also affected the number of plots available for soil sampling. Consequently, soil sampling was restricted to 4 replicate plots of each cultivar, apart from Karaka, where only 3 replicates could be sampled. Soil samples were assessed for cyst and egg counts to determine final (Pf) PCN populations. This data was used to determine the effect of cultivar on multiplication rates of PCN.

In addition, map coordinates of each of the 32 potato plots in the main trial were captured using GPS (Global Positioning System) technology to ensure the sites could be accurately found in future. This is further discussed in section 4.3.

3.2 *Development of duplex primers for quantifying PCR*

Nematode cysts were ground in eppendorf tubes, using plastic micro-pestles with 500ul of solution containing 5M guanidine isothiocyanate, 10mM EDTA, 50mM Tris-HCl (pH7.5) and 8% mercaptoethanol. After room temperature incubation for up to 1 hour, the DNA-containing solution was extracted once with equal volumes of phenol and chloroform-isoamyl alcohol (24:1) and once with chloroform-isoamyl alcohol, then precipitated with 0.3M sodium acetate and two volumes of isopropanol. DNA was resuspended in 100ul of H_2O.

Quantitative PCR was performed on the Perkin-Elmer ABI® 7700 Sequence Detector (TaqMan™). The two primers used were:

PCNTQPr1f $^{5'}$-CACATGCCTCCGTTTGTTGT-$^{3'}$

PCNTQPr1r $^{5'}$-CGCTCAACGACGCACAGA-$^{3'}$

The primers were designed to amplify the DNA from both *Globodera pallida* and *G. rostochiensis* equally well. The fluorescent probes were designed to be species specific and had the following sequences:

Pallida $^{5'}$Fam-ACAGCAATCGTCGAGTCACCCATTG-Tamra$^{3'}$

Rostochiensis $^{5'}$Viv-CAGCAATCGTCGGCTCACCCATA-Tamra$^{3'}$

All reaction components for quantitative PCR were purchased from Applied Biosystems. For the template 5µl of DNA was used in a final PCR reaction volume of 25µl. PCR reagents were 1x TaqMan™ universal PCR master mix, 300nM of each primer, 200nM of pallida probe and 100nM of rostochiensis probe. The thermal cycling conditions for the reactions were: a hold step for AmpErase activation at 50°C for 2 min; a second hold step for AmpliTaq Gold activation at 95°C for 10 min; then 40 cycles of a denaturation at 95°C for 15 seconds and an annealing phase at 60°C for 1 minute.

4 Results and discussion

4.1 Main trial

The multiplication rates (= Pf/Pi) were approximately x 8 for the range of Pi levels found within the potato plots (Figure 1). To assist with the interpretation of the data, Figure 1 also shows the theoretical x 1 and x 10 multiplication rates. At high Pi levels, a theoretical maximum final population is reached, beyond which a decrease in the population is known to occur, largely because of increasing competition between individuals and decreasing food supplies (Southey 1978; Been et al. 1995). This would be illustrated in Figure 1 as a drop in the multiplication rate below the x 1 line for high Pi levels. Clearly, such conditions did not occur in this plot trial, and, instead, at all Pi levels there was a similar increase in the PCN population.

Potato yields were directly influenced by the PCN infestation. The infestation had a greater impact on the more marketable table grade, with a reduction in tuber numbers and weight being observed. Figure 2 illustrates the substantial decline in table grade yield with increasing Pi. This decline is apparent from Pi levels of 5 eggs/ml soil and greater. It is unlikely that an equilibrium density has been reached, as there appears to be no change in the rate of yield decline. The equilibrium density can be defined as the population at which no further plant response is observed. The highest levels of Pi is 66 eggs/ml, at which point yields are approximately 30% of that obtained from the nematode-free plots. Clearly, this is a marked reduction in yield which is likely to affect the marginal profit for a grower.

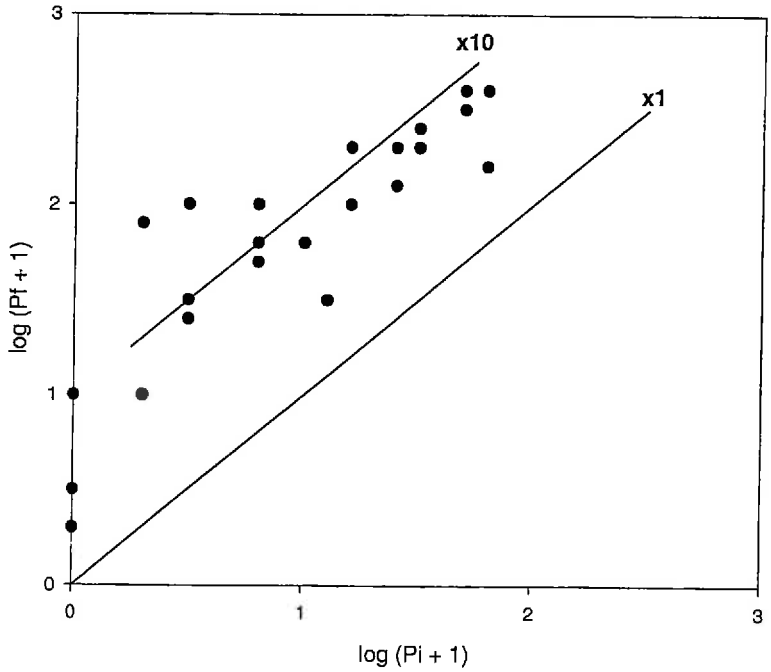

Figure 1. Relationship between the initial PCN population (Pi) and post-harvest populations (Pf), with theoretical x 1 and x 10 multiplication rates also shown.

Results from the main trial clearly illustrate the effect of initial PCN populations on both the final populations and potato yields. To ensure PCN levels remain manageable, it is recommended that crop rotations and effective soil sampling techniques are employed as part of integrated control practices. By taking lightly infested paddocks with no groundkeepers out of potato production for at least 4 years, PCN populations will decline to negligible levels. However, heavily infested paddocks, with groundkeepers present, even at low levels, will require a considerably longer period of time out of potato production before negligible levels of PCN are achieved. Consequently, if potato production is to continue on infested land, it will be necessary to grow more resistant cultivars (see Section 4.2 below).

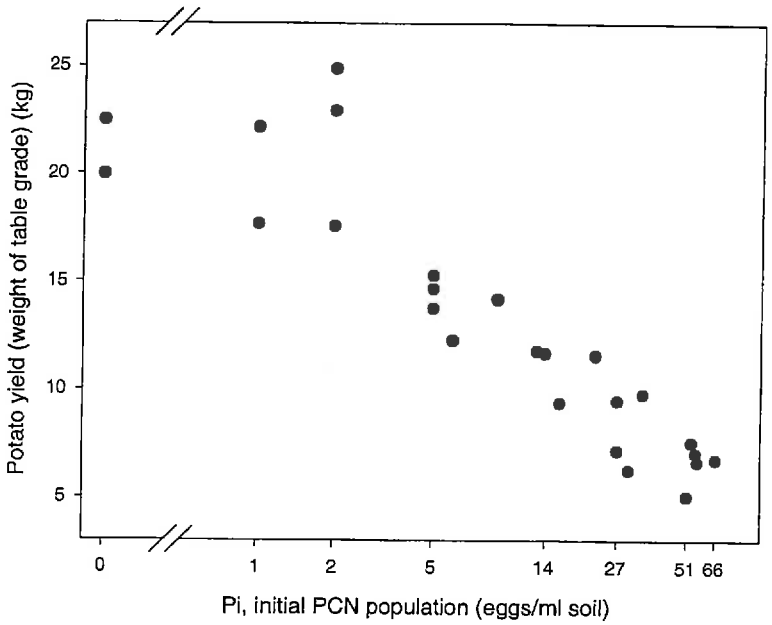

Figure 2. Relationship between yield of Ilam Hardy (weight of table grade) and population density of PCN at planting time.

Soil sampling of paddocks needs to be conducted at such an intensity that the probability of detection is high. Recent Dutch research into soil sampling strategies for PCN detection show that a recommended sampling grid of 5 x 6 m provided a detection probability of 90% (Been & Schomaker 2000). An intensive sampling protocol may be necessary for New Zealand conditions to ensure quality assurance process satisfy existing and potential export markets. Such sampling procedures in practice may prove to be expensive, therefore the development of simpler sampling strategies and mechanisation of soil sampling techniques would be more cost-effective.

4.2 Cultivar trial

Potato cultivar clearly affects PCN multiplication rates (Table 1). Soil sampled from non-resistant Ilam Hardy plots exhibited much higher final PCN populations (Pf) and multiplication rates, far greater than the other cultivars. Therefore, for the purpose of statistical analysis, data for this cultivar was not included. Nadine clearly showed a degree of partial resistance with a much reduced multiplication rate compared with the Ilam Hardy control. The other three resistant cultivars (Karaka, Moonlight and Summit) all had similar effects on PCN development. There were no significant differences ($P<0.05$) between these three cultivars in final population or multiplication rate. Although not a significant difference, it is interesting that Karaka, which is generally considered as the most resistant line of these three, had the lowest population reduction of the three lines.

Table 1: Influence of resistant and non-resistant potato cultivars on multiplication rates of PCN.

	Pi egg/g	Pf egg/g	Multiplication rate[a]
ILAM HARDY	7.9	88.2	28.2
NADINE	9.7	18.1	2.45
KARAKA	12.9	8.0	0.69
MOONLIGHT	9.4	4.0	0.37
SUMMIT	12.9	4.1	0.47
LSD(0.05)[b]	6.8	6.3	1.16
CV%[b]	41.5	45.1	71.6

[a] Means of multiplication rates from individual plot replicates are presented.
[b] For statistical analysis, Ilam Hardy data is excluded.

These results clearly show that cultivar resistance can greatly influence the population level of PCN. This resistance is potentially a very useful part of an integrated PCN control programme but it must be used appropriately to avoid breakdown of the resistance. For instance, resistant varieties should not be planted into soils containing high PCN populations but should be used where PCN levels are low or undetectable, as part of a sensible crop rotation. Use of resistant varieties as part of an integrated approach will ensure effective PCN control in the long term.

4.3 *Mapping of PCN infestations within a paddock using GPS technology*

At the time of potato harvest, the central position of each of the 26 plots used in the main trial was "captured" using a GPS unit. GPS relies on a constellation of 24 satellites that provides worldwide accurate position coordinates. The level of accuracy associated with the GPS coordinates is approximately 1.0 metre. Appendix I contains three maps illustrating the location of the trial site (red markers on Mile Bush Rd, Pukekawa) and the central position of each plot (blue markers). Each potato plot had an effective soil sampling area of approximately 3.4 m^2 (3 potato rows x 5 potato plants). The GPS unit will allow staff to return to within the effective sampling area of each plot. This will allow the study of PCN infestations within paddocks to be conducted over time with the knowledge that the quality of the data is not being compromised by potential errors in accurately locating field sampling positions.

4.4 *Development of duplex primers for quantifying PCN*

Various levels of *G. pallida* cysts (100, 75, 50, 25, 10 5 and 1 cyst) were extracted to obtain the DNA. The resultant DNA was then analysed on the TaqMan™ in triplicate and an amplification plot of the DNA was generated (Figure 3; mean data for each level is presented).

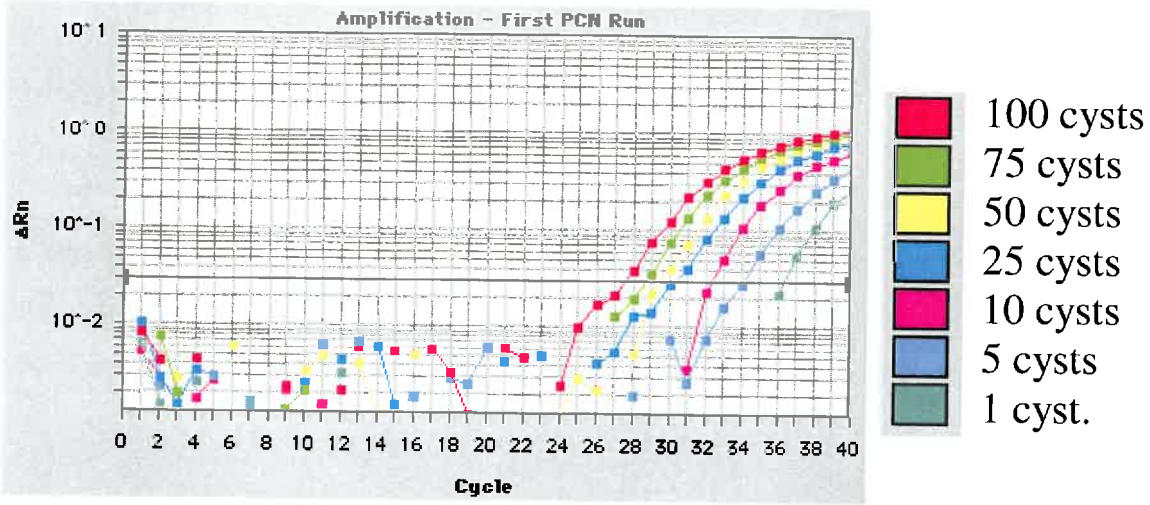

Figure 3: Plot of the fluorescence signal detected as an indication of the amplification of DNA. A low cycle number indicates high concentrations of DNA.

The TaqMan™ Ct value was then plotted against the log number of cysts extracted to allow a regression line to be fitted. The mean data of one run conducted in triplicate is presented in Figure 4. This produced an equation to estimate the relationship between the Ct value and the amount of cysts. This relationship can then be used to estimate the number of cysts contained in an unknown sample. The Ct values of egg content in cysts relating to field populations were determined. This involved counting all the cysts in a given soil sample, taking a portion of the cysts and counting the eggs contained, extracting DNA from remaining cysts and using the eggs contained in a portion of the cysts to obtain the number of eggs contained in extracted cysts. The DNA was put through the TaqMan™ as before and Ct values were subsequently related to the egg counts obtained. A weak relationship was identified between egg content, cyst numbers and Ct values.

The variability in the relationship between Ct values and egg count was caused by large uncontrolled variability in the number of eggs per cyst between samples. Resolution of this problem is ongoing and forms one of the primary objectives in future funded studies.

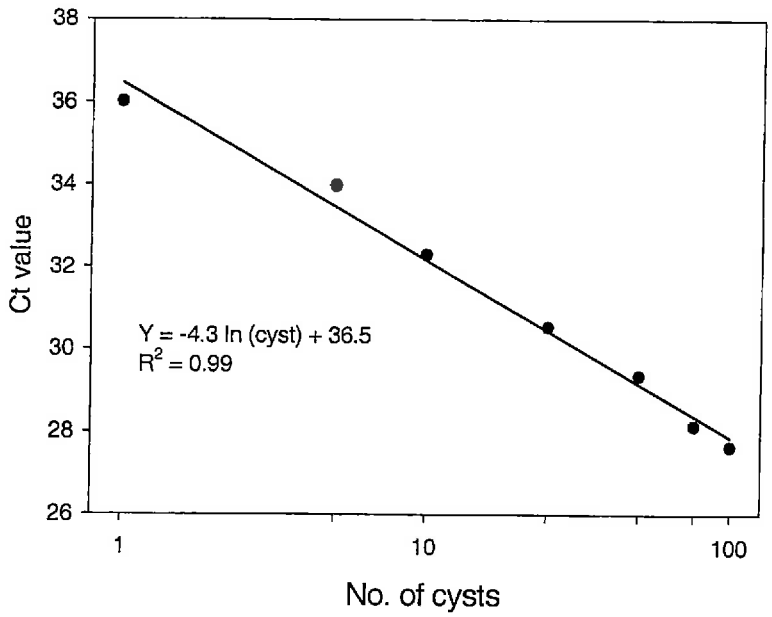

Figure 4. Relationship between Ct value from TaqMan™ and cyst numbers (mean of one run in triplicate). Fitted line with regression equation is shown.

5 Summary

A potato plot trial was established within a naturally infested paddock near Pukekawa to determine the effect of different initial PCN (Pi) populations on potato (Ilam Hardy) yield and PCN multiplication rates.

Multiplication rates were approximately 8 in plots where Pi ranged from 0 to 66 eggs/ml soil, suggesting that the PCN population would increase rapidly if control measures were not taken. Potato yields were significantly lower in plots where Pi was high, compared with yields obtained from plots with low Pi. Yields were reduced to as little as 30% of crops grown in a nematode-free environment, suggesting high marginal costs to the grower.

The effect of resistant potato cultivars (Nadine, Karaka, Moonlight and Summit) on multiplication rates of PCN was also determined. Of these, Nadine showed partial resistance, while the remaining 3 cultivars maintained a high level of resistance to PCN multiplication.

To ensure PCN levels are controlled in the long term, crop rotations, effective soil sampling techniques and the use of resistant cultivars all play important roles in integrated PCN control programmes.

By using fluorescence detection for quantifying DNA, a relationship for the number of cysts extracted compared to the Ct value from the TaqMan™ was estimated. This allowed us to calibrate the Ct value for unknown DNA quantities, but repeat sampling will be undertaken to enable this relationship to be confirmed. This work is ongoing.

This method of detection has been successful for the individual species of *G. pallida* and *G. rostochiensis,* with a corresponding minimum detection level of 1 cyst per 100 ml of soil sample. The next step in the project is to duplex both probes together, enabling quantification of mixed species to be identified.

Resolution of the variability in egg content versus cyst numbers in soil will also be carried out.

6 References

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7 Acknowledgements

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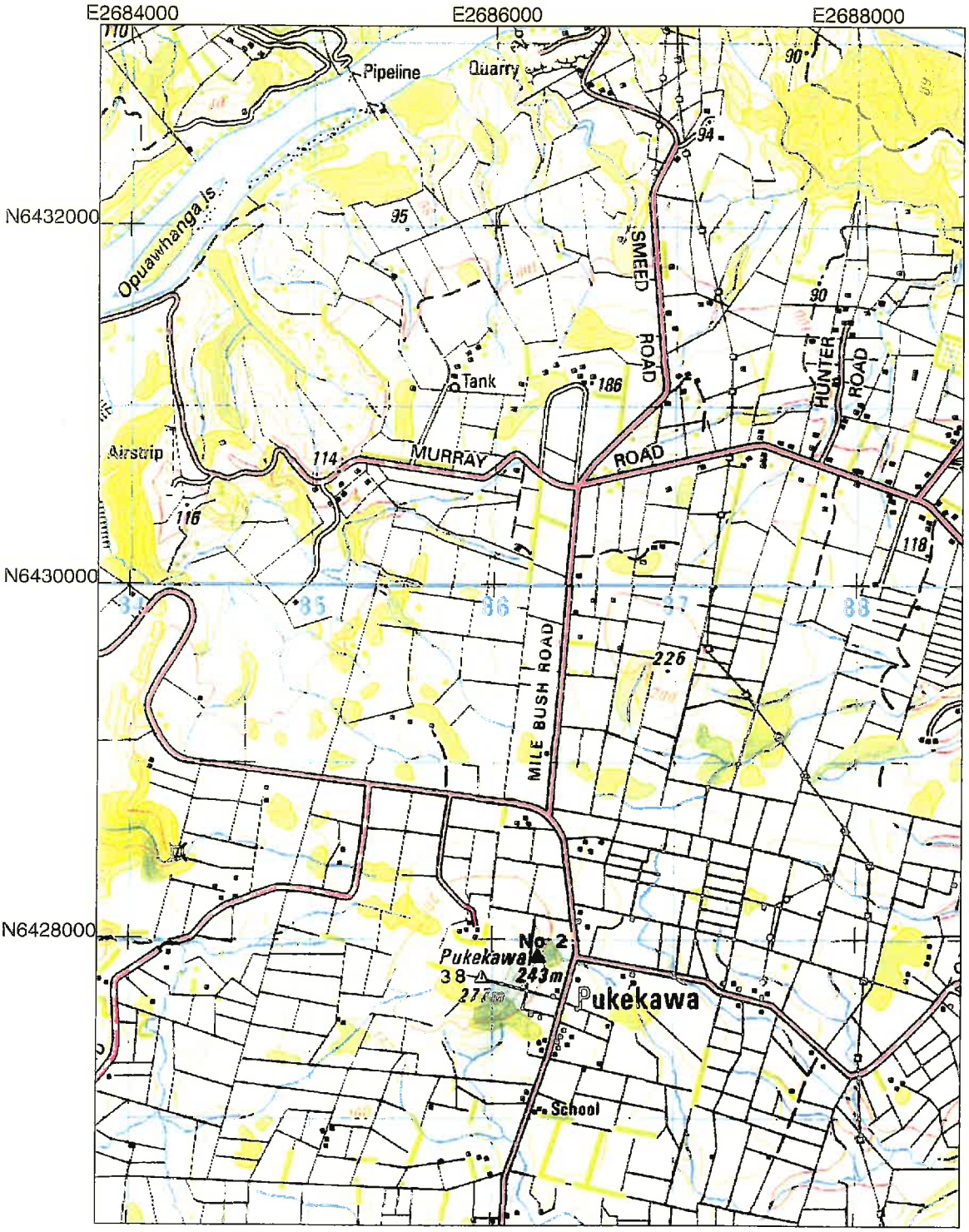
Appendix I

Three maps are shown to illustrate the location of the trial site (red markers on Mile Bush Rd, Pukekawa) and the central position of each of the 32 potato plots, forming the main trial (blue markers).



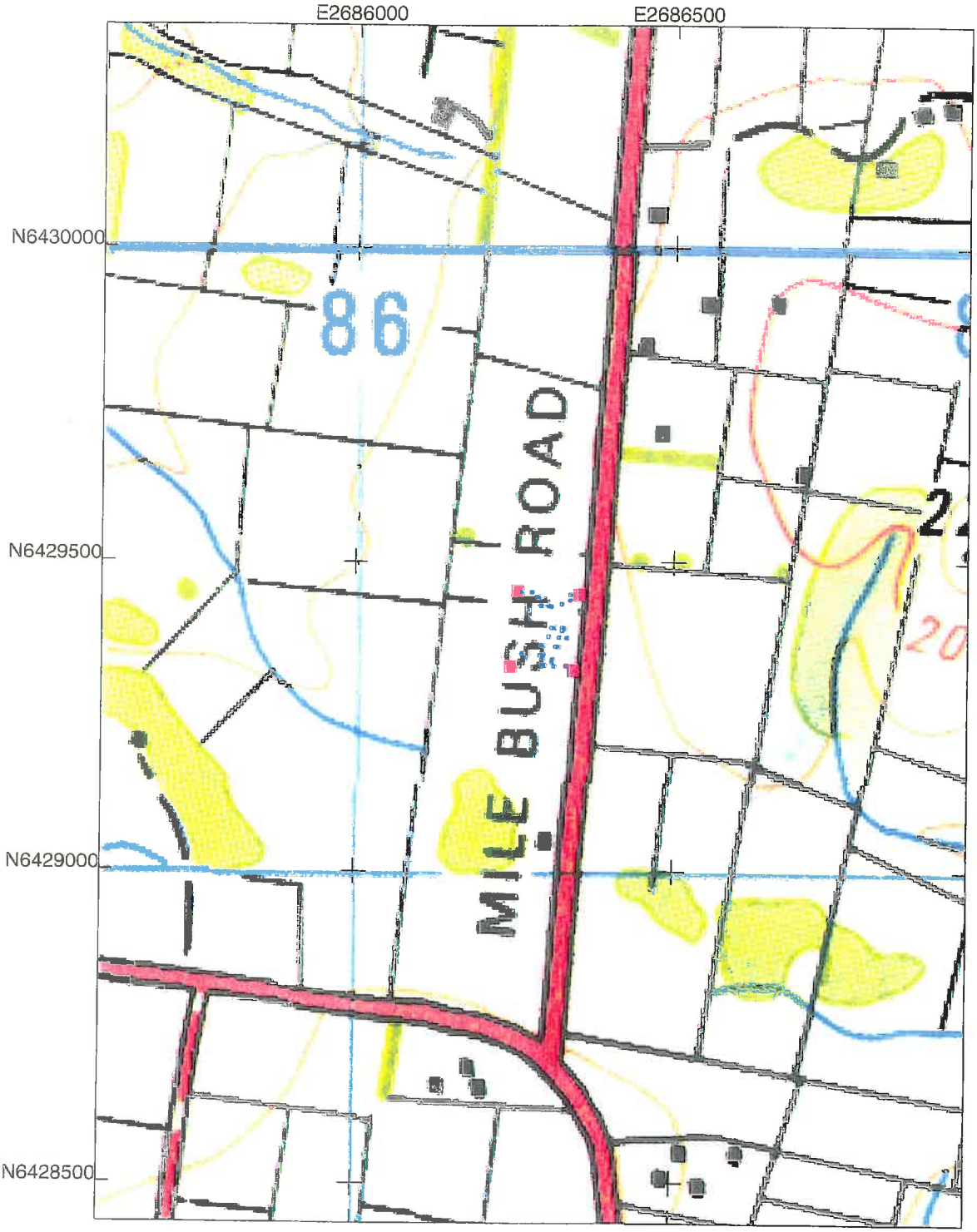
Region of trial site: Pukekawa (south of Pukekohe)

New Zealand Map Grid
New Zealand
New Zealand Geodetic 1949

N

Scale 1:35,000
0 1.250
Kilometers

2/19/01
GPS Pathfinder
Trimble



Trial site on Mile Bush Road, Pukekawa

New Zealand Map Grid
New Zealand
New Zealand Geodetic 1949

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Scale 1:10,000
0 0.300
Kilometers

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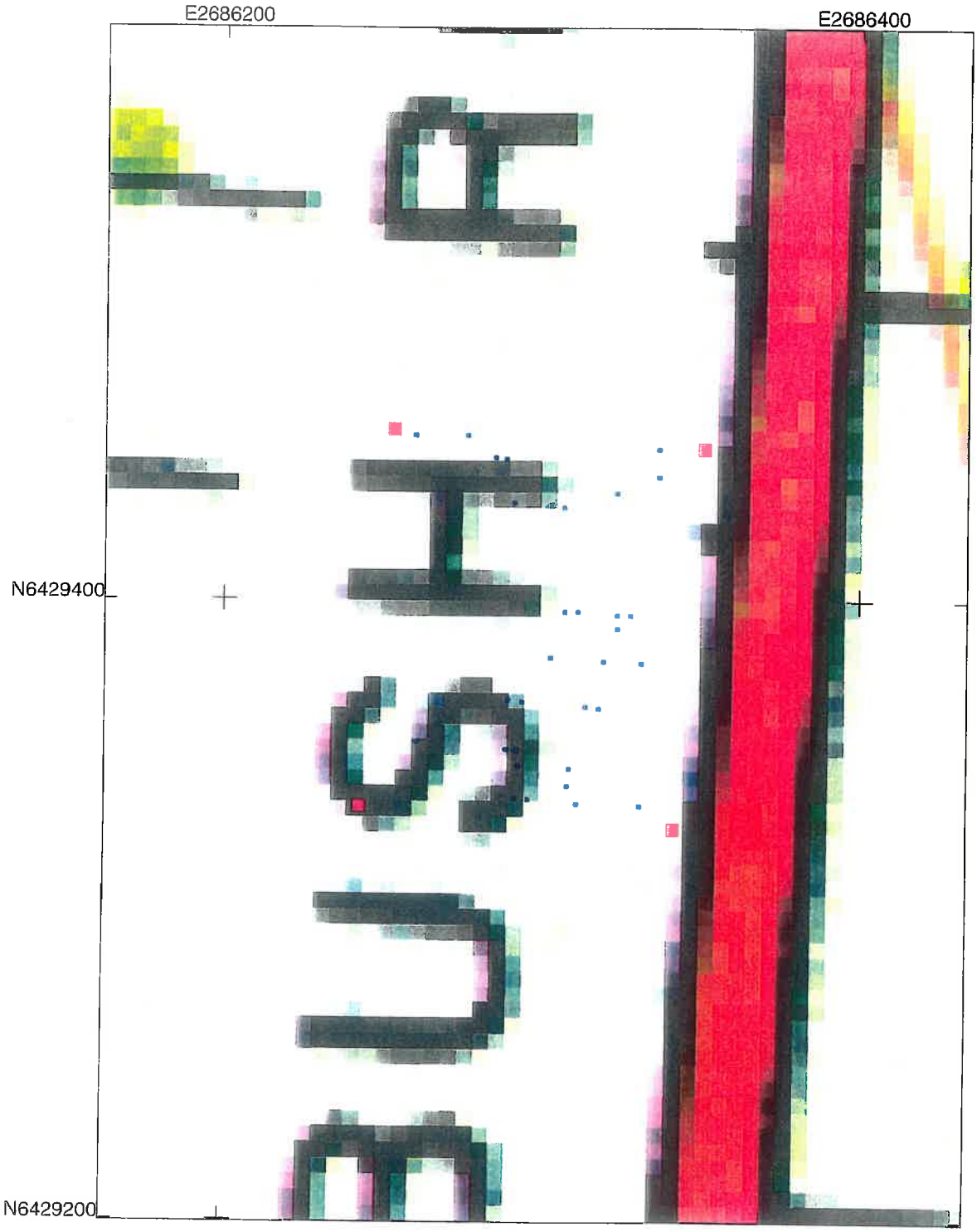
GPS Pathfinder
Trimble



Position of Plots (blue) within Trial site (Red showing corners)

New Zealand Map Grid
New Zealand
New Zealand Geodetic 1949

N

Scale 1:2,000
0 0.060
Kilometers

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