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Project leader:	Dr Steve Ellis, ADAS
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The results and conclusions in this report are based on an investigation conducted over a one-year period. The conditions under which the experiments were carried out and the results have been reported in detail and with accuracy. However, because of the biological nature of the work it must be borne in mind that different circumstances and conditions could produce different results. Therefore, care must be taken with interpretation of the results, especially if they are used as the basis for commercial product recommendations.

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AUTHENTICATION

We declare that this work was done under our supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

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- The Seinhorst two-flask technique was most effective at extracting stem nematode from soil and on average extracted almost three times as many stem nematodes as the Flegg modified Cobb technique and about twice as many nematodes as the Whitehead tray and centrifugation method.
- Intensive soil sampling using differential GPS sample location showed that there are distinct patches and gaps in the nematode distribution across a field and that it is not just random. This will have to be taken into account when developing sampling protocols.
- Sampling at an intensity of *c.* 100 sample points per 4 ha gives an acceptable measure of the average infestation in that area, but does not adequately distinguish patches and gaps in nematode distribution.

Background and expected deliverables

Stem nematode (*Ditylenchus dipsaci*) is a very destructive pest of bulb onions. Assured Produce protocols strongly recommend the use of representative soil sampling as part of risk assessment for the pest. This ignores the inherent problems of detecting it in soil. The pest is primarily an endoparasite (spending most of its life in the plant) so numbers in soil are usually small. This project aims to develop optimum methods of soil sampling and analysis for detecting the pest at typical population densities encountered in commercial practice. This will involve the use of soil sampling linked to differential GPS (DGPS) sample location. In particular, the project will investigate within field distribution of the pest and develop sampling protocols to give the maximum chance of detection. As not all soil extraction methods are equally effective at recovering stem nematode, different techniques will be compared to determine best practice.

The expected deliverables from this work include:

- Clear guidelines on which extraction method to request from analytical laboratories, to give the best chance of recovering stem nematode from soil.
- Graphical representation of the distribution of stem nematodes within fields at high, medium and low risk from the pest to aid generation of sampling plans.

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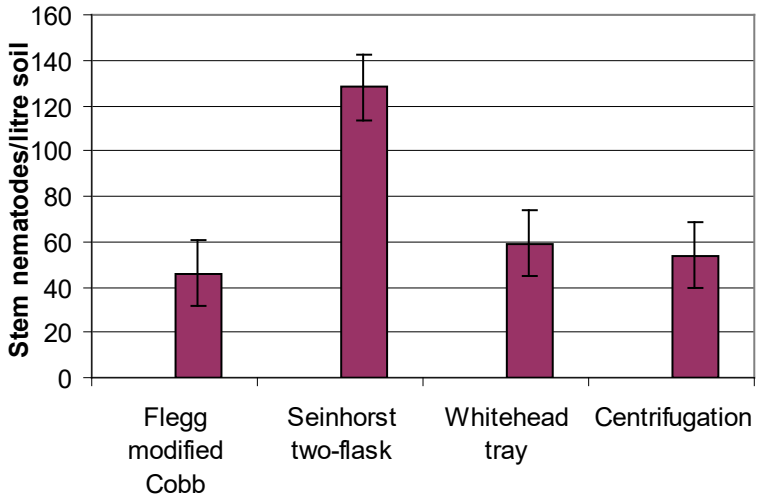
- Sampling protocols that optimise the chance of detecting stem nematode at typical levels of infestation
- Guidelines to give improved confidence in risk assessment for stem nematode.

Summary of the project and main conclusions

Comparing nematode extraction methods

Soil samples from fields known to be heavily infested with stem nematode were mixed thoroughly and a sub-sample extracted using the Whitehead tray, Seinhorst two-flask, Flegg modified Cobb and centrifugation techniques. A total of five replicate samples were extracted using each technique. There were clear differences between the methods in terms of their efficiency of extraction of stem nematode. Overall the Seinhorst two-flask technique was most effective and on average extracted almost three times as many stem nematodes as the Flegg modified Cobb technique and just over twice as many nematodes as the Whitehead tray and centrifugation techniques (Figure 1). Interestingly, the Seinhorst two-flask technique was also most effective at extracting all other nematode groups recovered from the samples.

Figure 1. Numbers of stem nematodes (± standard errors) recovered from soil using different extraction methods



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Mapping the in-field distribution of stem nematode

This part of the project is taking advantage of automated soil sampling equipment linked to differential GPS (DGPS) provided by Fresh Produce Consultancy. Intensive soil sampling concentrated on three fields known to be at high, medium and low risk from stem nematode. GPS-generated sampling grids were prepared for a four hectare block in each field to provide 400 10 x 10m sampling plots. A total of five soil cores were taken from within each plot to provide a bulked sample of about 50 g of soil. Coordinates for the location of each sample were obtained using DGPS. Individual soil samples were extracted using the Seinhorst two-flask technique and the number of stem nematodes counted.

The nematode data was linked to sampling coordinates and used to map the characteristics of the pest's distribution within each field. As expected the distribution of nematodes varied depending on whether the field was perceived to be at low, medium or high risk from stem nematode. Detailed analysis of these distributions is underway and so far results suggest that there are distinct patches and gaps in the nematode distribution across a field and that it is not just random. This will have to be taken into account when developing sampling protocols. Using the distribution maps as a tool some preliminary work has been done to determine how reducing the number of sampling points affects the mean nematode count. This will help to indicate how few sampling points are needed to produce an accurate assessment of pest numbers and risk of crop damage. For example, by systematically omitting every eighth, fourth or second count it is possible to reduce the number of sampling points to 350, 300 and 200 respectively. Results to date suggest that this has little effect on the apparent mean nematode population in the field. More work needs to be done on how reducing the number of sample points affects the maps of pest distribution. As the pest shows a patchy distribution, a systematic sampling plan is probably the best option as it covers the sample area more uniformly and increases the chances of locating a patch of stem nematode. If you simply sampled randomly, the chances of missing the patch could be higher.

Financial benefits

Estimates from historical records of soil sampling suggest that about 2.5% of land sampled for stem nematode is infested. Therefore for every 1,000 ha of onions grown there may be 25 ha lost due to stem nematode. With a total UK crop area of onions of about 8,000 ha the total annual area lost is about 200 ha with a value of around £1,000,000. Greater confidence in the results of soil sampling to predict stem nematode risk would significantly

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reduce this loss. Improved risk assessment would also help to minimise unnecessary use of nematicides as insurance treatments and allow growers to move towards nematicide-free production as requested by many supermarkets

Action points for growers

- Request that labs offering analysis of soil samples for detection of stem nematode are extracted using the Seinhorst two-flask technique to give the best chance of recovering the pest.
- When sampling fields for stem nematodes, individual soil samples (which can be bulked) should be taken from at least 100 uniformly distributed points per 4 ha to ensure that an acceptable measure of the average numbers of nematodes present is obtained.

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Science Section

Introduction

Stem nematode (*Ditylenchus dipsaci* (Kuhn) Filipjev) is a destructive pest of many plants. Onions may be attacked at any time after germination and may be killed outright before they reach the seedling stage even by apparently low levels of nematodes in soil. Following the withdrawal from use of Temik 10G (aldicarb) on 31 December 2007 only Vydate (oxamyl) remains with a specific off label (SOLA) for stem nematode control on bulb onions (SOLA 2006/1890). As nematode numbers in soil are usually small and show an aggregated distribution, work is needed to define best practice for soil sampling to provide the maximum chance of detecting the pest. This project will address this need by investigating in-field distribution of stem nematode, optimum sampling patterns for pest detection and comparing soil extraction methods to determine the most effective at recovering nematodes.

The risk from stem nematode damage is currently assessed by considering field history, previous cropping and by representative soil sampling. Soil sampling is recommended by Assured Produce protocols, but in practice current soil sampling strategies ignore the inherent problems of detecting low numbers of stem nematode in soil. With the loss of Temik 10G and pressure from retailers to reduce nematicide use, reliable soil sampling will become increasingly important for identifying fields at risk. Alternative detection strategies, such as sampling weed species for stem nematode, are not likely to be effective as weed distribution across fields is never uniform.

There is little information available on soil sampling for stem nematode in both the UK and Europe. Some workers have commented on the number of samples and depth of sampling that should be used. Caubel (1975) suggest that 50 x 20g samples to a depth of 15 to 20 cm are required where the nematode is concentrated. However, there is no mention of the maximum area to be sampled and the technique may not be effective in fields where there is little information on the existing nematode population, for example fields potentially available for rent. One third hectare areas were sampled 60 times to a depth of 20 cm by Kleijburg (1960) to provide 1 kg of soil for extraction whereas currently in the UK fields may be hand sampled by walking a W shaped path and taking 25 cores or when using mechanised sampling up to 100 cores may be taken from a 4 ha area (David Norman, pers comm). There is clearly no agreement over the number of cores to be taken over a particular area and this is something that the proposed project will address.

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Muller *et al*. (1993) divided each of 20 replicate 2 kg soil samples into 16 x 125 g sub-samples. This provided 320 samples in which numbers of stem nematodes were assessed. These data were used to calculate the nematode numbers in a range of sample sizes and predict the number of samples required to detect the pest. It was concluded that two 125 g sub-samples needed to be examined to detect stem nematode. It is not clear whether the field was considered at high risk of damage but the principle of intensively sampling onion fields and using these data to predict the accuracy of different sampling patterns to detect the pest will be adopted in this study.

Automated soil sampling equipment linked to differential GPS (DGPS) sample location offers the opportunity to take large numbers of soil cores at known locations. This will provide data on stem nematode distribution and allow better guidelines for the correct approach to soil sampling for the pest to be developed. This in turn should increase the chances of detecting low levels of stem nematode.

Experience has shown that not all laboratory extraction procedures are equally effective at recovering stem nematode from soil; extraction procedures need to be optimised to ensure that extraction of low numbers of the pest can be reliably achieved. There is no published literature comparing extraction methods for stem nematode. Therefore this study will compare the efficiency at which the Whitehead tray, the Flegg modified Cobb technique, centrifugation and the Seinhorst two-flask technique are able to recover stem nematode from soil.

Overall, improved soil sampling, pest extraction and results interpretation will increase growers' confidence in making valid risk assessments, leading to reduced/targeted use of nematicides and allowing cost savings for growers.

Materials and methods

Comparison of nematode extraction methods

A total of four soil samples, each weighing approximately 10 kg, were taken in September 2007 from a field in Cambridgeshire known to be heavily infested with stem nematode. The samples were bulked together and thoroughly mixed. This sample was then sub-sampled to create 20 samples each of approximately 1 kg. Five replicate samples were then extracted to determine the level of stem nematode infestation using each of the following extraction methods.

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1. Whitehead Tray (Whitehead and Hemming, 1965)
2. Flegg modified Cobb technique (Flegg, 1967)
3. Seinhorst two-flask technique (Seinhorst, 1955)
4. Centrifugation (Jenkins, 1964)

Nematode suspensions were cleaned to aid microscopic examination. This was done using a small sieve consisting of a plastic ring (8 cm diameter) cut from vinyl drain-pipe to which a circle of plastic mesh (48 µ mesh) was glued at one end. This sieve was placed inside a Petri dish and the nematode suspension plus debris poured onto it. More water was added carefully down the inside of the Petri dish until the debris in the small sieve was immersed. The Petri dish was then covered and allowed to stand undisturbed for 48 hours. During this period the nematodes wriggled through the mesh and into the Petri dish where they could be collected. The numbers of nematodes recovered were counted and expressed as numbers/L of soil. These data were subjected to the analysis of variance. The time taken to undertake each stage of the extraction was also noted.

Mapping stem nematode distribution within fields

Intensive soil sampling was done in 4 ha blocks in each of three fields in Cambridgeshire, known to be at high (Block A, Denton Lodge Farm, Holme, Peterborough, Cambs. Grid ref TL 183884), medium (Block B, Carolls Farm, Manea, Cambs. Grid ref TL 494904) and low (Block C, Carolls Farm, Manea, Cambs. Grid ref TL 495902) risk respectively from stem nematodes. Soil samples were taken using automated soil sampling equipment linked to differential GPS (DGPS). GPS generated sampling grids were prepared for a four hectare block in each field to provide 400 10 x 10 m sampling plots. A total of five soil cores were taken from within each plot to provide a bulked sample of about 50 g of soil. Co-ordinates for the location of each sample were taken using the DGPS. Individual soil samples were extracted using the Seinhorst two-flask technique and the number of stem nematodes counted. Numbers were converted to number/litre soil. The nematode data was linked to sampling co-ordinates and used to map the characteristics of the pest's distribution within each field.

Impact of reducing sample size on sampling accuracy

Analyses were done to assess the impact of reducing sample size on the accuracy of both the overall mean infestation level identified by the sampling, and the apparent spatial distribution of nematodes in the sample blocks. To generate appropriate reduced data sets, two approaches were taken:

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a) *Systematic reduction*: all sample points from each block were ranked in the order in which they were taken during the sampling process. Data sets reduced by 50, 100, 200, 300, 350 and 375 points for each block were obtained by removing the 8th data point in every series of 8 (50 points removed), every 4th data point in every series of 4 (100 points removed), every other data point (200 data points removed). Data sets reduced by 300, 350 and 375 points were obtained by retaining only every 6th, 10th and 18th data point respectively.

b) *Random reduction:* each sample point in each block was assigned a random number (using the random number function in Microsoft Excel). To obtain a sample reduced by 50 points, all sample points were ranked in ascending order by the random number and the first 50 points removed. The process was repeated to remove the number of data points required to generate data sets reduced by 100, 200, 300, 350 and 375 points for each block, using a new randomisation for each selection.

Mean and 95% confidence limits were calculated for each sample size for each block.

To assess the impact on the apparent spatial distribution of the nematodes in each block of reducing sample size, the systematically reduced data sets (nematode counts and their associated x, y coordinates within the sampling block) were used to calculate an index of aggregation (I_a) using SADIE (Spatial Analysis by DistancE Indices (Perry, 1995)). Index values significantly greater than 1 indicate that counts exhibit a degree of aggregation, and an associated probability value indicates whether the degree of aggregation is significantly different from what would be obtained from random permutations of the counts. In essence therefore, I_a indicates whether or not the counts are randomly distributed.

In addition, SADIE 'red-blue' plots (Perry *et al.*, 1999) were generated for the full data sets and for data sets systematically reduced to 100 points. In simple terms, this analysis calculates an index of clustering for each data point. The analysis assesses whether a high count at a particular location is associated with other high counts at neighbouring data points = a 'patch', and similarly whether low/zero counts are associated with low values at neighbouring points = a 'gap'). These values can then be interpolated to produce a contour map with patches (red) where the cluster index is >1.5 and gaps (blue) where the index is <1.5.

These analyses could only be done on data from blocks A and B as the data set from block C contained too many zeroes to give reliable results.

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Results

Comparison of nematode extraction methods

Time to perform nematode extractions

The total time taken to perform each of the nematode extraction methods is shown in Table 1. Centrifugation was the least time consuming extraction method. This is mainly because the other methods require samples to be left for 24-48 hours as part of the extraction or to clear the nematode suspension. During this waiting time no staff input is required so other tasks can be undertaken.

Table 1. Time (minutes) taken to extract one soil sample for stem nematodes using the Whitehead tray, Flegg modified Cobb technique, Seinhorst two flask technique and centrifugation

	Time (minutes) for different stages of extraction			
Extraction method	**Soil preparation**	**Extraction**	**Clearing suspension**	**Total**
Whitehead tray	5	1440	3	1448
Flegg modified Cobb technique	5	6	2880	2891
Seinhorst two-flask technique	5	15	2880	2900
Centrifugation	5	36	0	41

Table 2. Mean numbers of a range of nematodes recovered from replicate soil samples using four extraction methods.

	Nematode group (number/l soil)			
Extraction method	**Stem nematode**	**Stunt/spiral nematodes**	**Root lesion nematodes**	**Cyst juveniles**
Whitehead tray	59	225	205	80

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Flegg modified Cobb technique	46	95	0	45
Seinhorst two-flask technique	128	920	250	110
Centrifugation	54	45	5	10
SED (16 DF)	9.7	37.6	37.4	20.2

Extraction efficiency

A total of four nematode groups were recovered in sufficient numbers using each extraction method to subject to statistical analysis. These were stunt/spiral nematodes (*Tylenchorynchus* spp), cyst juveniles (larvae of *Globodera* spp or *Heterodera* spp), root lesion nematodes (*Pratylenchus* spp) and stem nematode (*Ditylenchus dipsaci*). Numbers of all nematodes recovered differed significantly between extraction methods. (P<0.001 for each nematode group, Table 2.) The Seinhorst two-flask technique was the most effective extraction method for all four nematode groups studied. It was significantly better (P<0.05) than all other extraction methods for stem nematode and stunt/spiral nematodes and statistically better (P<0.05) than centrifugation and the Flegg modified Cobb technique for cyst juveniles and root lesion nematodes. The Whitehead tray was significantly better (P<0.05) than centrifugation and the Flegg modified Cobb technique for stunt/spiral nematodes and root lesion nematodes and significantly better than centrifugation for cyst juveniles. Overall centrifugation was probably the least effective extraction method.

Mapping stem nematode distribution within field

Numbers of stem nematode recovered from fields at high medium and low risk from stem nematode were linked to sampling co-ordinates and used to map the characteristics of the pest's distribution within each of the high, medium and low risk field areas (Figures1 and 2) using punctual kriging as the interpolation routine. These maps tend to suggest the presence of a patchy distribution, particularly for stem nematode in the high risk (Block A) field (Figure 1). Medium infestations (Block B) suggested are more random distribution (Figure 2), or at least a distribution with fewer and less well-defined patches. In the low risk block (Block C), distinct but small patches were evident.

Figure 1. Punctual kriging contour map (courtesy of Fresh Produce Consultancy) to show distribution of stem nematode in a 4ha block known to be at high (Block A) risk from stem nematode

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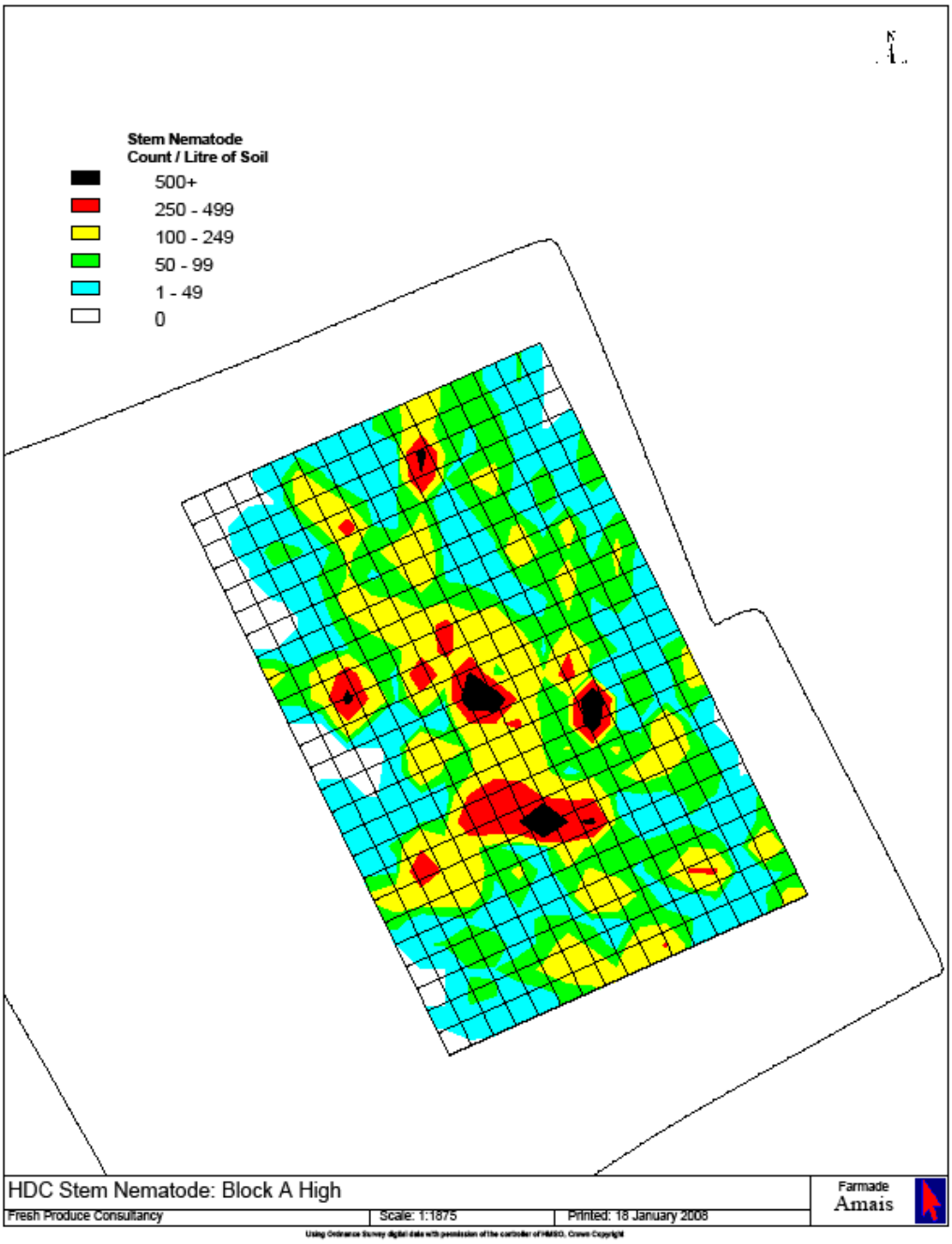

Figure 2. Punctual Kriging contour map (courtesy of Fresh Produce Consultancy) to show distribution of stem nematode in a 4ha block known to be at medium (Block B) and low (Block C) risk from stem nematode

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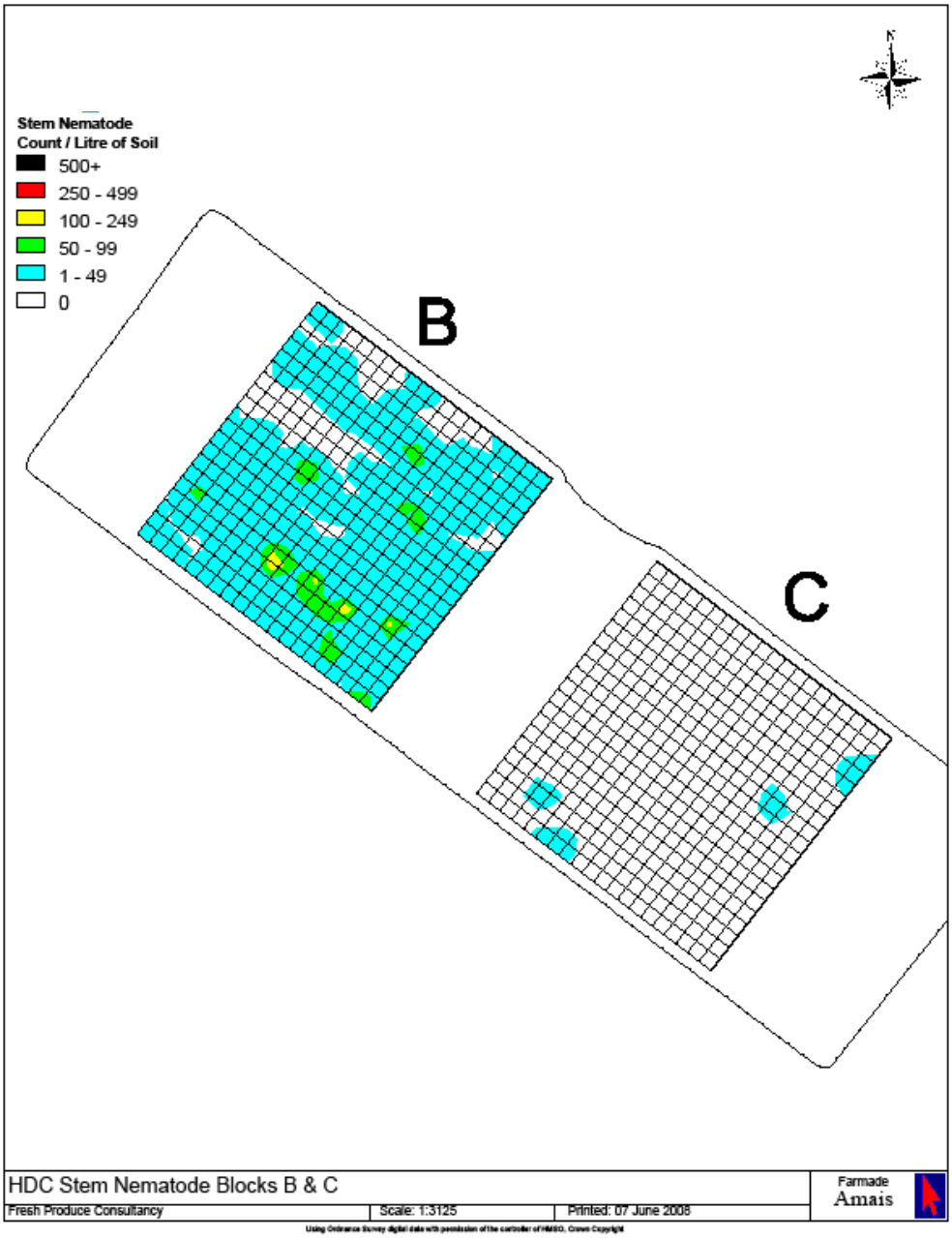

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Impact of reducing sample size on sampling accuracy

The effect of reducing data sets for each block by systematic and random reduction are shown in Figures 3, 4 and 5 for blocks A, B and C respectively. The overall mean populations levels for the three blocks for the full data sets (400 points) were block A: 95.8 nematodes/litre of soil; block B: 16.3 nematodes/litre of soil; and block C: 0.14 nematodes/litre of soil (approximately one order of magnitude between each block). Reducing sample sizes from 400 to any value down to 100 had very little effect of the overall mean in any of the blocks, whether sample points were randomly or systematically removed. Sample sizes of <100 tended to have higher errors associated with them. Plots of log mean against log variance for each data set for each block (data not shown) generally showed that outlying points were associated with sample sizes of <100, suggesting that mean/variance relationships were more stable for sample sizes >100. Even where infestation levels were very low (Block C) nematodes were found at all sample sizes except 25 samples (Figure 3).

Figure 3. The effect on the mean nematode infestation level (± 95% confidence limits) in Block A (high risk) of reducing the sample point number randomly and systematically

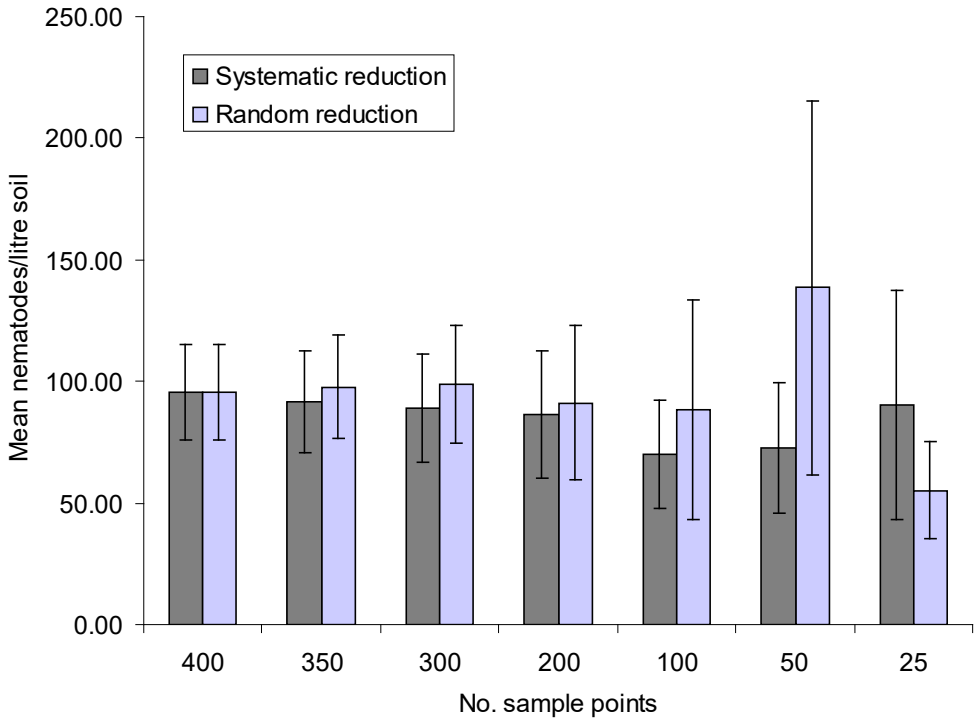

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Figure 4. The effect on the mean nematode infestation level (± 95% confidence limits) in Block B (medium risk) of reducing the sample point number randomly and systematically

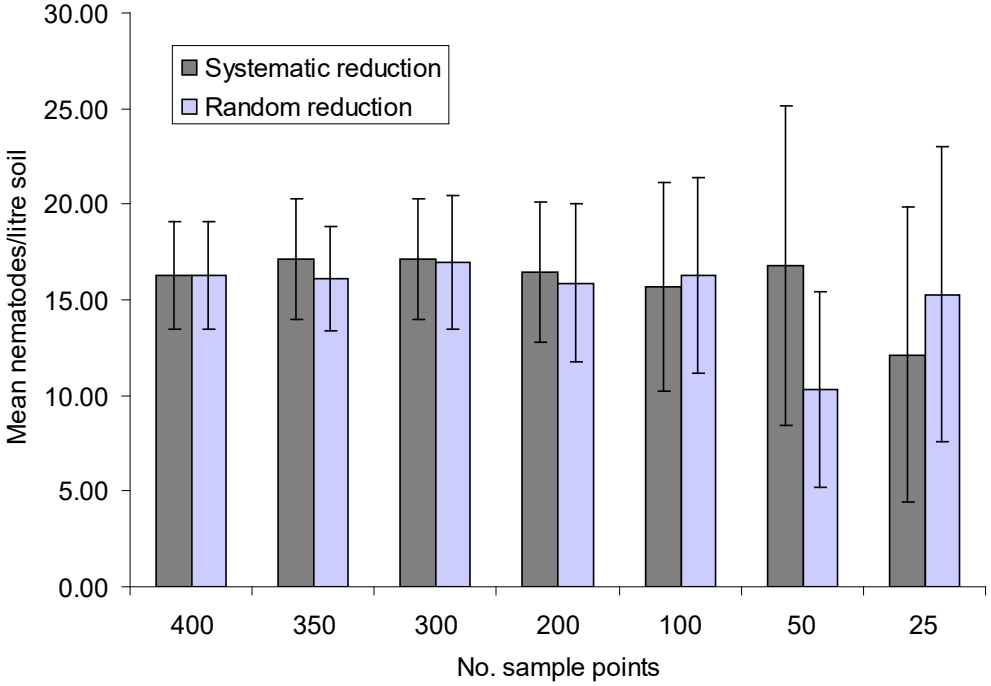
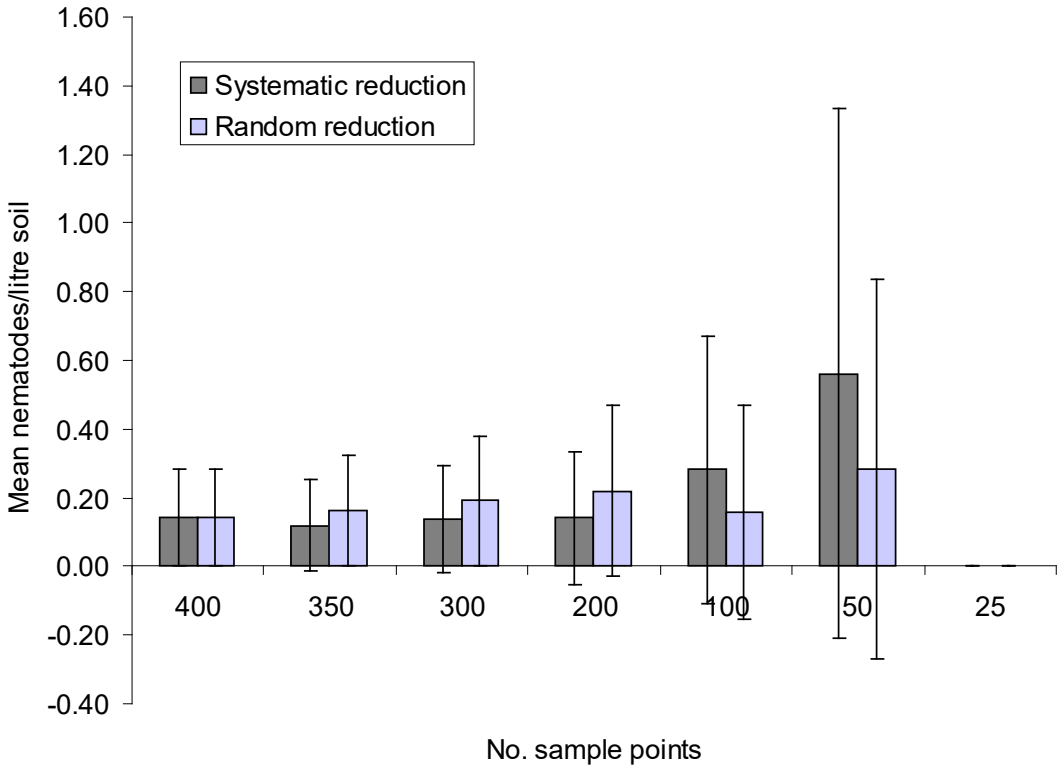


Figure 5. The effect on the mean nematode infestation level (± 95% confidence limits) in Block C (low risk) of reducing the sample point number randomly and systematically



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Spatial analyses

The values of I_a obtained from the full and systematically reduced data sets from Blocks A and B are given in Table 3.

Table 3. Values of I_a and associated probabilities (*P*) for full and systematically reduced data sets in Blocks A and B. Values of *P* in bold are significant

	Block A		Block B	
Sample size	**I_a**	***P***	**I_a**	***P***
50	1.012	0.396	1.006	0.400
100	1.139	0.208	1.198	0.118
200	1.184	0.148	1.741	**0.005**
300	1.271	0.094	2.021	**0.005**
350	1.427	**0.031**	2.095	**0.005**
400	1.486	**0.039**	2.220	**0.013**

In both blocks, values of I_a declined as sample size decreased, indicating that the observed nematode distribution tended to become more random as the sample size decreased. For block A (high infestation), values of I_a were significant at sample sizes of 400 and 350, but not at smaller sample sizes, suggesting that at samples sizes <350, the nematode distribution was random rather than patchy. For block B, evidence of aggregation in the counts was observed down to a sample size of 200.

The SADIE 'red-blue' plots (Figures 6 and 7) show that significant patches and gaps can be identified in the distribution of nematode in both block A and block B when the full data sets (400 data points are used). When the same analysis is done using at much reduced data sets (100 points), patches and gaps are still present to a lesser extent, but do not correlate particularly well with those indicated on the maps for the full data sets.

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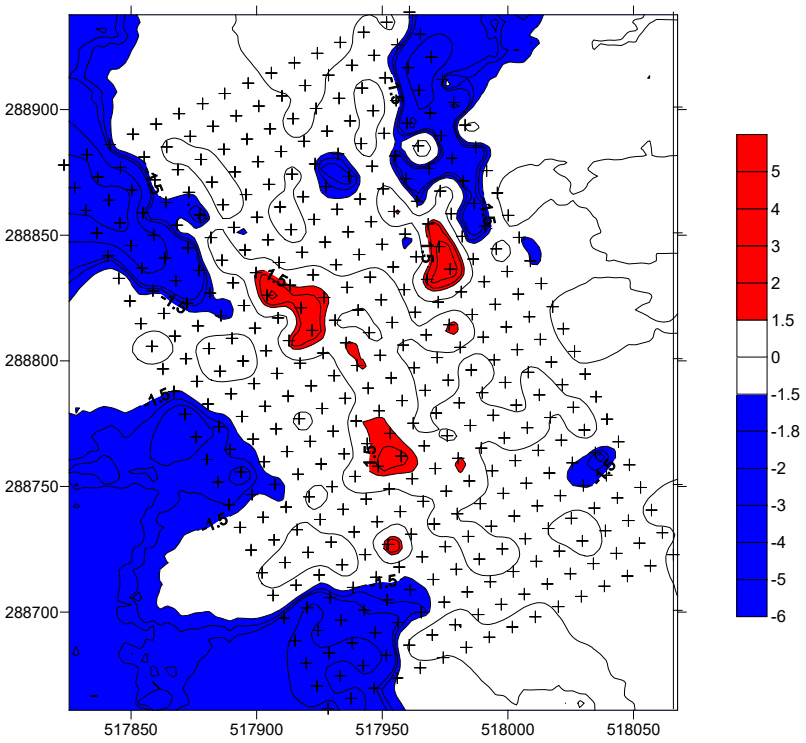
Figure 6. SADIE 'red-blue' plots for block A for full (400 data points) and reduced (100 data points) data sets. Crosses indicate the location of the sample points (data are interpolated beyond the physical limits of the sampling block). Red areas = patches, blue areas = gaps

a) 400 sample points



b) 100 sample points

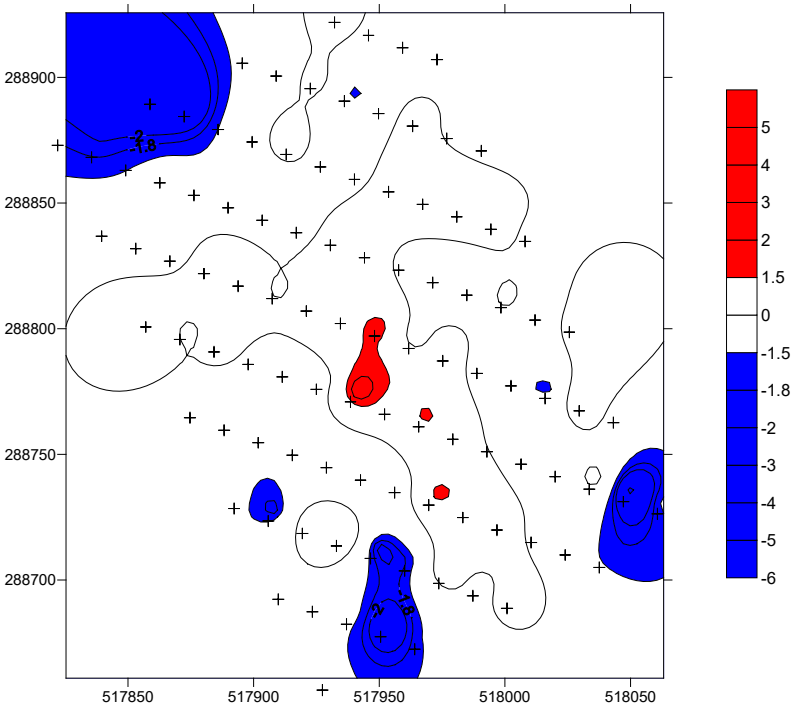


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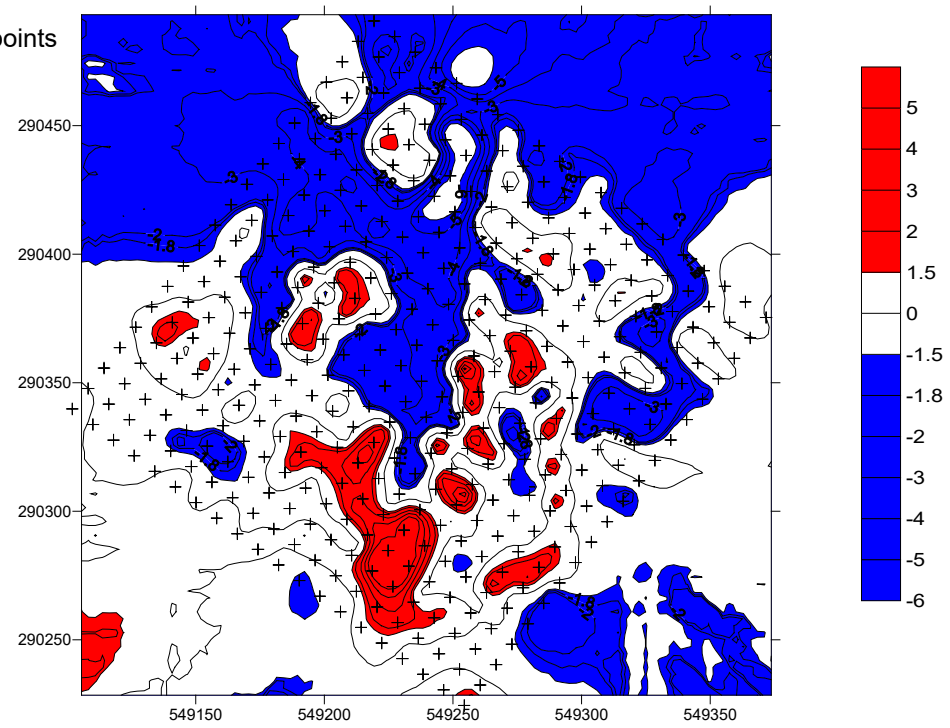
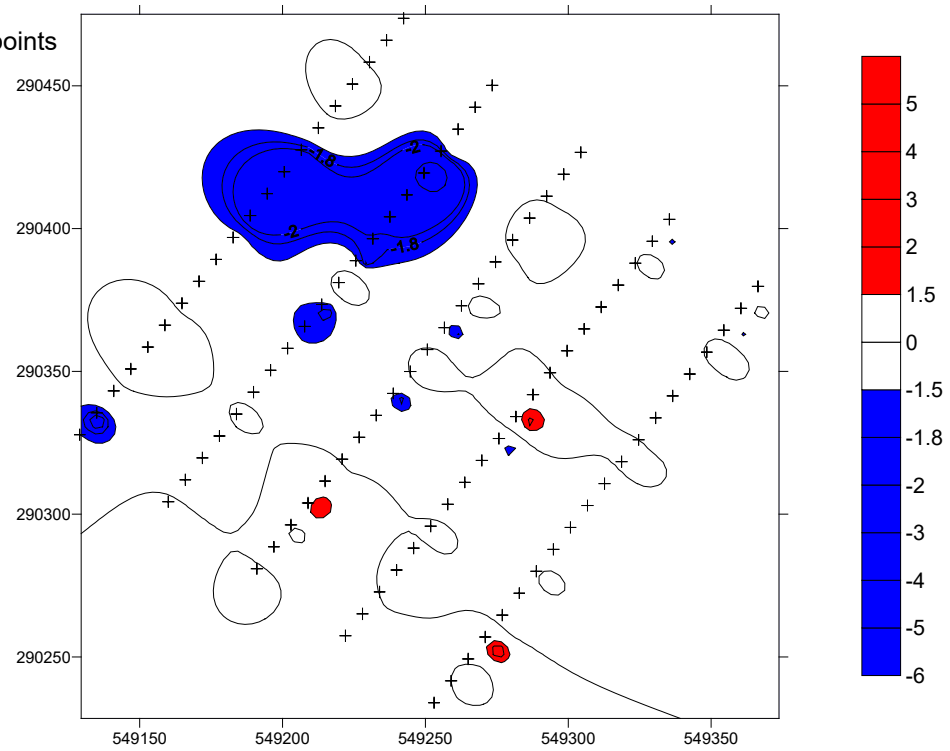
Figure 7. SADIE 'red-blue' plots for block B for full (400 data points) and reduced (100 data points) data sets. Crosses indicate the location of the sample points (data are interpolated beyond the physical limits of the sampling block). Red areas = patches, blue areas = gaps

a) 400 sample points



b) 100 sample points



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Discussion

Stem nematodes were recovered from all replicate samples used to compare the efficacy of extraction methods, confirming that the sampled field was at high risk from the pest. Numbers recovered using all four extraction methods were sufficiently high to suggest that the field should not be cropped with onions.

The Seinhorst two-flask technique was the most effective extraction method for all nematode groups recovered from the samples. This was most obvious with stem nematode and stunt/spiral nematodes. The Seinhorst two-flask technique recovered at least twice as many stem nematodes and at least four times as many stunt/spiral nematodes as any other extraction methods. The Whitehead tray was generally the second most effective extraction method.

The Flegg modified Cobb technique and centrifugation were least effective of all the extraction methods compared. The Flegg modified Cobb technique is principally intended for extraction of larger nematode species such as needle (*Longidorus* spp) and dagger nematodes (*Xiphinema* spp). Therefore, it is not surprising that it was ineffective for lesion nematodes, cyst juveniles and stem nematode which are all relatively small species.

Although centrifugation was the least time consuming extraction method, the extracted nematodes were less easy to examine than those from any of the other methods tested. This is because the nematodes became distorted following extraction in sucrose solution and so were less easy to identify than those extracted in water. The effect of the sucrose solution was more marked with stunt/spiral nematodes, root lesion nematodes and cyst juveniles than with stem nematodes. A further disadvantage of centrifugation is that some organic debris remained in the sample which made microscopic examination more difficult than with the other extraction methods. Although centrifugation has the advantage over other extraction methods in that it will extract dead nematodes, it was the least user friendly method of all those tested.

The analysis of the sampling data clearly indicated that if all that was required from the sampling data was a reasonable estimate of the mean nematode infestation present, then the number of sampling points per 4 ha could be systematically reduced from 400 to 100 without significantly altering the mean. However, the SADIE analysis (Table 3) clearly shows that as sampling intensity is reduced, the nematode aggregation (patchiness) evident when 400 sampling points are used is lost (i.e. the distribution appears more random).

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Conclusions

- The Seinhorst two-flask technique was the most effective extraction method for stem nematode and most other nematode groups.

- Growers should request analytical laboratories use the Seinhorst two-flask extraction to optimise chances of recovering stem nematode from soil samples.

- Centrifugation was the least user friendly extraction method of those tested.

- It is likely that a systematic sampling grid of 100 points covering a 4 ha area will be the best compromise between sampling accuracy and economy of sampling effort. A standard 25 core sample along a W pattern across the same area may not be sufficient to identify low populations.

Technology transfer

The project has featured in all of the following
HDC News May 2008, Plant, feed, spray and weed with accuracy
HDC News July 2008, How to find a needle in a haystack
Precision Farming Event, Springfields Spalding, March 26 2008
Article in Vegetable Farmer, October 2007
BOPA R&D group meeting at PG Rix, Stourgarden, 29 January 2008
BCGA Carrot meeting PGRO 21 February 2008
Elsoms Onion Conference, November 2008

Glossary

Cluster index – within SADIE (see below), a method for describing a region of either relatively large counts close to one another in two-dimensional space (i.e. a patch), or of relatively small counts (i.e. a gap).

Differential GPS – a means of accurately locating sampling points using the Global Positioning System satellite network linked to known reference points on the ground to obtain very high (sub-metre) accuracy.

Endoparasite – a parasite which spends most of its time within its host

Index of aggregation (I_a) – the degree to which a set of counts is identified as being aggregated spatially as defined using SADIE (see below).

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Punctual krigging – a statistical method for interpolating point data sets into a contour map format.

SADIE – Spatial Analysis by Distance IndicEs. A statistical technique for assessing the degree of aggregation in a spatially-referenced set of counts.

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