The development of an immunoassay to identify and quantify the two species of the potato Cyst Nematode, G.rostochiensis & G.pallida.

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The Development of an Immunoassay to Identify and Quantify the Two Species of the Potato Cyst Nematode, *G.rostochiensis & G.pallida.*

MSc Thesis 1997

by Jacqueline A. Dunn

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ABSTRACT

The potato cyst nematodes (PCN) *G.rostochiensis* (Woll.) and *G.pallida* (stone) are sedentary endoparasites and serious economic pests of potato crops throughout the temperate world. Therefore there is an agricultural need for a quick, cheap and user-friendly method to assess the specific population density in the field. This thesis describes the development of just such a diagnostic immunoassay procedure.

Firstly two monoclonal antibodies (Mabs), MR8/4 and MR8/5 were selected from a screen of hybridoma lines for their specificity to *G.pallida* and *G.rostochiensis* respectively.

The specific nature of the antigen and antibody binding was then determined before a procedure was standardized to identify and quantify PCN in both pure and mixed populations. Further tests proved that the Mabs only recognized live eggs.

A procedure was then developed to extract the nematode antigen direct from soil samples. The robustness of the extraction procedure and immunoassay was confirmed by comparison with the lengthier traditional flotation and counting by microscope method.

The traditional counting procedure does not take into account the viability of the cysts therefore the new extraction procedure and immunoassay was used to determine the efficiency of nematicide treatment from a field fumigation trial. This confirmed the influence of soil type and conditions at time of application on fumigation efficiency.

Finally a methodology was evolved to convert the assay procedures into a marketable commercial technique.

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ABBREVIATIONS

PCN	Potato Cyst Nematode
Pabs	Polyclonal Antibodies
Mabs	Monoclonal Antibodies
DAS ELISA	Double-Antibody Sandwich, Enzyme Linked Immunoassay
TAS ELISA	Triple-Antibody Sandwich, Enzyme Linked Immunoassay
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline plus 0.1% Tween 20
TMB	3,3'5,5'-Tetramethyl-Benzidine
OM	Organic Matter
ADAS	British Agricultural Development & Advisory Service
w/v	weight by volume
v/v	volume by volume
M	Molar concentration
rpm	revolutions per minute
GPS	Global positioning Systems
ICM	Integrated Crop Management
IgG	Immunoglobulin G
IEF	Isoelectric focusing
RO	*G. rostochiensis*
PA	*G. pallida*

1. INTRODUCTION

Nematodes occur in all natural environments and their numbers most probably exceed the total of all other animals living on Earth. Their success in colonisation is due largely to their relatively simple and adaptable body plan which allows for a range of sizes - from the 0.3 millimetres of an adult *Pratylenchus* to the 8 metres of *Placentonema gigantissima*. Most nematodes are microscopic and free-living but some parasitise animals and others plants. Of the plant parasites, cyst-nematodes are the most highly specialized and among the most successful. Their outstanding features are the swelling of the female into spherical or lemon-shaped, protective cysts.

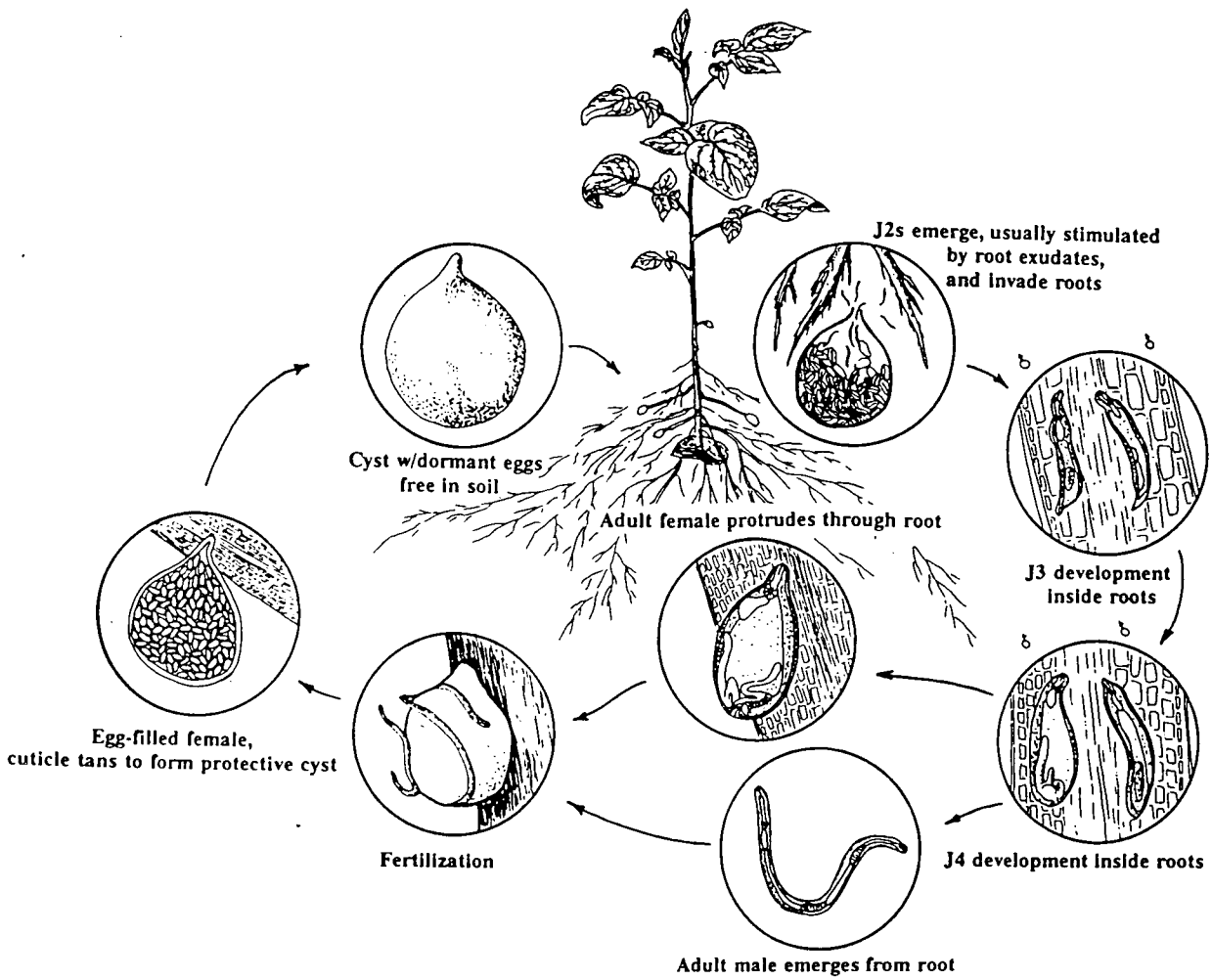
The potato cyst nematodes (PCN), *Globodera rostochiensis* (Woll.) and *G.pallida* Stone are sedentary endoparasites and serious pests of potato crops. They are therefore of economic importance throughout temperate regions of the world. The seriousness or potential seriousness of their threat to potato production is recognized in all of the 58 countries in which they are known to occur (EPPO, 1992), and also in the many additional countries that impose quarantine or regulatory action against them in the hope that such action will continue to exclude these pests.

Originating in the South American Andes, PCN co-evolved with their preferred host, the potato (*Solanum* spp.) (Evans, Franco and de Scurrah, 1975). It was from this mountainous region that PCN spread to many other countries throughout the world (Cotten and van Riel, 1993). For many years there was only one known species of PCN, *Heterodera rostochiensis* Woll., or the 'golden nematode', so named due to the golden-yellow phase of the maturing female. But in 1973 Stone described a second species - *H. pallida*. The two species were distinguished apart by the relatively larger body length (484 μm compared to 468 μm), stylet and tail length of the *G. pallida* larvae, and that the maturing female of *G. pallida* was pallid or cream white in colour. The sub-genus *Globodera* was elevated to generic rank by Behrens (1975) and all round cyst nematode species, including PCN, were assigned to this genus with proper authorities (Loof and Bakker, 1992).

FIGURE 1. Adult females (pale) and cysts (dark) of *Globodera rostochiensis* on potato roots (courtesy of C. C. Doncaster, Rothamsted)

FIGURE 2. Life cycle of potato cyst nematodes

(after Brodie, 1984, revised by M. Brucato)



As with other cyst nematode species, PCN show marked sexual dimorphism. The males retain a typical vermiform shape and are motile but the females enlarge as they mature, eventually becoming spherical and remaining sedentary throughout. The eggs that form after fertilisation are retained inside the female body, which tans to form a protective cyst enclosing up to 400 or more eggs. Exudates from the roots of a host plant species then stimulates hatching of 60 - 80% of these eggs (Rawsthorne and Brodie, 1986). In the absence of a host, few eggs hatch. This dependence on a hatching stimulus from the host allows the eggs to remain dormant for long periods of a time, further specialized as a diapause (Hominick, Forrest and Evans, 1995), and serves to synchronise the life cycle of the nematode with that of its host.

The very survival strategies of PCN also serve to aid their dissemination. In soil, the eggs can survive within cysts for long periods in desiccating conditions, so are well able to withstand the long periods in transport which took them from their origins in the Andes throughout the world, probably in the soil adhering to potato root tubers (Evans and Brodie, 1980). It is likely that Europe became a secondary distribution centre, from where PCN spread with the seed tubers of improved cultivars that were traded around the world (Evans and Trudgill, 1978). Local dissemination within countries is largely by farming activities, which inevitably move soil (and any cysts it may contain) around.

Wherever they are able to establish, which is virtually wherever potatoes are grown, PCN quickly multiply to population densities at which they begin to cause noticeable crop damage, although this is dependent on the frequency with which potatoes are grown. The first sign of PCN presence is the poor growth of plants in small patches which enlarge with frequent growing of potatoes. Affected plants exhibit symptoms of water stress and/or mineral deficiency (Jones, 1970). The yield of affected plants can be much reduced and cysts may also form on the surface of tubers (Inagaki,1974) and disfigure them, causing a loss of quality as well as quantity. Perhaps most importantly, each potato crop leaves a legacy of newly-formed eggs.

Main crop potato production allows only one generation of PCN to develop each season, and it is possible to predict the amount of reproduction that will occur from a given starting population density (Jones & Perry, 1978). In the same way, it is also possible to predict the amount of damage and yield loss that will be caused by a given initial population density, although the several mathematical models that have been produced for this relationship are not entirely satisfactory (Trudgill, 1986). Nevertheless, the estimation of pre-planting population densities of PCN form the basis of our management strategies for these pests. Such estimates indicate whether specific control measures are required for the crop about to be grown and show the overall effectiveness (or otherwise) of management programmes.

The traditional method of controlling the damage caused by PCN is that of rotating potatoes (the only field host for PCN) with non-host crops, a practice that has its roots in the 7-course rotation enforced by the Incas (Anon, 1993). This allows the population density of PCN to decline naturally to non-damaging levels. The use of rotation for control was supplemented by chemical control after the Second World War, first by fumigants and then by granular formulations of organophosphates and carbamate nematicides (Hooper and Evans,1993). In the 1960's, resistant cultivars of potato for PCN control also became available. Discovery of populations of PCN able to break the resistance of these early resistant cultivars led to the recognition and description of *G.pallida.* However, repeated growing of cultivars resistant only to *G.rostochiensis* has allowed the selective reproduction of *G.pallida* so that this latter species has increased in relative abundance (Evans, 1993) and so it is very important to diagnose which of these two species are present in infested fields.

It is possible to trace the separate introductions of the two species of PCN that have been made to Europe and to surmise that the original introductions probably comprised only a few cysts, so that their virulence against different resistance genes was restricted and their overall heterogeneity reduced (Trudgill, *et al.*, 1995). Despite this, complete resistance to *G.pallida* has not been found and cultivars bred for their resistance to this species rely upon polygenic sources, which not only confer incomplete resistance but also are susceptible to eventual failure due to selection for

virulence in the nematode. Further concern over the possible long-term control of *G.pallida* was expressed when it was discovered that granular nematicides were less effective against this species than against *G.rostochiensis*, and that decline rates in the absence of a host crop might be as low as 15% per annum for *G.pallida* compared with c. 33% per annum for *G.rostochiensis* (Evans, 1993).

These differences between the two species of PCN, and the implications that they have for their management, mean that it is very important to be able to recognise and quantify the two species accurately. Standard procedures for the extraction of PCN cysts from soil samples and quantification of their egg contents, using an elutriation and sieving apparatus known as the Fenwick Can, were defined many years before the existence of two species was suspected (Fenwick, 1940). However, the description of a second species and the recognition that pathotypes which vary in their reaction to host resistance genes exist within both species, meant that better diagnostic systems were required for PCN. The first problem of rapid identification of the two species and the quantification of their proportions in mixed populations was solved by the use of isoelectric focusing to separate two species-specific proteins, which occur in equal concentrations in the two species (Fleming and Marks, 1983). When combined with the traditional Fenwick procedure for egg counting, this system gave a reliable estimate of the population densities of the two species in field soil samples.

Since the late 1970's antibody based, or serological methods, have made a big impact in many areas of crop protection. Diagnostic antibodies can be derived from three basic routes, two of which (the production of polyclonal antisera and monoclonal antibodies) have been reviewed by Harlow and Lane (1988). Polyclonal antisera are produced by immunising antibodies, typically rabbits (as with those described in this paper), with selected immunogens and subsequently collecting blood samples after suitable time periods. Other animals such as sheep and goats have also been used, particularly if large volumes of antisera are required. Blood samples are allowed to clot and serum can be simply decanted off and may be used whole or can be purified to yield antibody containing fractions, depending on the required use.

A desire to produce more defined reagents led to the development of methods for the production of monoclonal antibodies opening up many new possibilities in immunodiagnostics. The technique involves fusing antibody secreting lymphocytes, obtained from the spleens of immunised mice or rats, with cultured myeloma cells. The resultant hybrid cells (hybridomas) can be selected for and share both the antibody secreting properties of their parent cell-lines. Individual hybridoma cell lines, derived from a single cell, by dilution plating, which have the ability to be grown in cell-culture can then be established. Those clonal cell-lines that retain the ability to secrete antibody will produce only a single (monoclonal) antibody which can be harvested from the culture medium. A number of schemes have been devised to produce and then purify monoclonal antibodies in large amounts and include both culture and *in vivo* (ascites) methods.

New opportunities for improvement of diagnostic procedures are continually becoming available (Curran and Robinson, 1993) and the use of monoclonal antibodies (Mabs) for both diagnosis and quantification of the two species of PCN is becoming an increasingly attractive proposition. The potential benefits of such a system can be most easily judged by reference to Table 1, which lists the steps required for diagnosis and quantification of the two species using the best currently available, traditionally-based procedures and the steps that would be required in an immunoassay based on two Mabs able to recognise the two species both specifically and quantitatively.

TABLE 1. Steps required for the quantification of the two species of PCN using either current methodology or an immunoassay.

CURRENT	IMMUNOASSAY
1) Dry soil	1) Flotation
2) Sieve soil	2) Sample preparation
3) Fenwick Can	3) ELISA
4) Clean extract	N.B. The above 3 steps are capable
5) Count cysts	of automation.
6) Count eggs	
7) IEF	

Clearly, many fewer steps are involved in the immunoassay procedure and all are capable of being automated. This opens the way for much more rapid, cheaper and possibly more reliable assays of PCN population densities. Such possibilities have already been referred to for PCN by Schots *et al.* (1990) and for root knot nematode by Robinson (1989) and Davies and Lander (1992). In fact, a quantitative procedure based on ELISA for the detection of PCN in soil samples was described by Schots *et al.* (1992), but this was based on three antibodies and has proved difficult to operate. If individual Mab's which specifically recognise the two species of PCN, as described by Robinson *et al.* (1993) and Evans *et al.* (1995), could be selected, then the basis of a suitable immunoassay for PCN would be established.

It may even be possible to use Mab's for sub-specific recognition and quantification of PCN populations. It is important to be able to recognise pathotypes within species of PCN but the classification system for PCN pathotypes proposed by Kort *et al.* (1977), is no longer thought to be valid. True pathotypes can only be recognised by single major resistance genes and, since the scheme of Kort *et al.* uses only two differential host clones with single major resistance genes, only four true pathotypes can be recognised, *i.e.* two in each of the two species. Although PCN populations differ in

their ability to reproduce on potato clones with polygenic resistance, they are best not thought of as different pathotypes, but simply as having different complements of virulence genes. The eventual challenge will be to diagnose the degree of virulence in field populations. This is unlikely to be possible using a serologically based procedure but may well be possible using DNA-based technology, as referred to by Curran and Robinson (1993).

Numerous variations of the enzyme-linked immunosorbent assay (ELISA) have been developed but in these tests only the four most standard types were applied. All use a colour reaction to quantify an antigen of interest and the intensity of the colour reaction depends upon the amount of specific enzyme present, the colour reaction occurring when a specific substrate is added. The amount of enzyme present is determined by the amount of antigen present because the enzyme is attached as a label either to the antibody which recognises the antigen or to second antibody which recognises that first antibody. Thus, the four commonest types of ELISA are:

1) DIRECT	Antigen bound directly to ELISA plate, amount present determined by antibody with enzyme label attached.
2) INDIRECT	Antigen bound directly to ELISA plate, amount present determined by first antibody. Amount of first antibody present determined by second antibody with enzyme label attached.
3) DOUBLE-ANTIBODY SANDWICH (DAS)	Antibody which recognises antigen bound directly to ELISA plate and used to 'trap' antigen from solution. Amount of antigen trapped determined by second antibody with enzyme label.
4) TRIPLE ANTIBODY SANDWICH (TAS)	Antibody which recognises antigen bound directly to ELISA plate and used to 'trap' antigen from solution. Amount of antigen trapped determined by second antibody. Amount of second antibody present determined by third antibody with enzyme label attached.

2. AIM

This MSc project seeks to improve the diagnostic capabilities for PCN by the use of immunoassays. The aims are addressed in the following objectives:

1. To provide an immunoassay to quantify *Globodera pallida* and *Globodera rostochiensis* in mixed or pure populations.

2. To investigate the possibility of direct use of an immunoassay on soil samples.

3. To test the robustness of any resulting immunoassay with a view to producing marketable diagnostic tests.

3. MATERIALS AND METHODS

3.1. Antibody & Antigen Screening

3.1.1 Antigen preparation

Antigens for screening were produced by homogenization in 0.1 M Phosphate Buffered Saline (PBS). Much of the screening was against antigenic preparations from *G. rostochiensis* and *G. pallida* but, for the purposes of checking cross-reactivity, preparations were also made from *G. tabacum, G. t. solanacearum, Heterodera avenae, H. cajani, H. carotae, H. cruciferae, H. glycines, H. goettingiana, H. mani, H. sorghi, H. schachtii. H. trifolii, H. urticae, Anguina tritica, Aphelenchoides besseyi, Ditylenchus myceliophagus, Meloidogyne arenaria, Panagrellus redivivus, Pratylenchus neglectus, Rotylenchulus reniformis* and *Rhabditis oxycerca.* Extracts from cyst nematodes were adjusted to a protein concentration of 2 μg/ml and those from free-living species to a concentration of 12 μg/ml. (Nematodes supplied by Rothamsted).

3.1.2 Initial Antibody Screening

For the first batch of antibodies, two female DA rats were immunised intramuscularly with 100 μg of antigen preparation plus Freund's complete adjuvant, followed on day 21 with a subcutaneos boost of 100 μg of antigen in Freund's incomplete adjuvant. On day 31, bleeds were taken and screened by ELISA. Both animals had antiserum titres greater than 1/5000. A further 100 μg of antigen was administered intravenously on day 97 and fusion was carried out 3 days later. Mabs were prepared using standard protocols (Galfre & Milstein, 1981). Spleen cells were fused with rat cell line Y3Ag1.2.3 in a ratio of 2 spleen:1 myeloma cell in polyethylene glycol. After fusion, the cells were diluted to 100 000 cells per microtitre late well and grown in the presence of 1% of 50X HAZA (hypoxanthine-azaserine, Sigma).

All other Mab production was by intraperitoneal immunisation of Balb/c mice. Three immunisations of 100 μg of nematode homogenate, emulsified in Freund's complete

adjuvant, were made, with a further intraperitoneal booster of 100 μg given 3 days before fusion. Standard protocols were used and 5×10^7 spleen cells were fused with 5×10^6 SP2/0-Ag14 myeloma cells.

Two of the fusions that were made used spleens from mice that had been immunised following protocols designed to improve Mab specificity, namely immunosupression using cyclophosphamide and anti-idiotype immunisation.

For initial screening of supernatants from fusions, 96-well microtitre plates (Dynatech, Immulon 1) were coated overnight with 50 μl per well of 2 μg/ml antigen in coating buffer. Supernatants were screened by Indirect ELISA, using goat anti-rat or goat anti-mouse IgG labelled with horse radish peroxidase (Sigma) as the second antibody. An optical density ratio of at least 3:1 in the recognition of the two species was chosen as a discriminatory threshold for potentially diagnostic antibodies. Cell lines of interest were cloned at least twice by limiting dilution.

3.1.3 Enzyme-Linked Immunosorbent Assay (ELISA)

Further investigations of Mabs and antisera were also made by ELISA. For this, 96-well microtitre plates (Nunc Immuno Plates, Maxi-Sorp) were coated at 4^0C with 50 μl per well of extracts prepared as described above. After incubation, excess antigen was removed from the wells and the plates were rinsed in three changes of 0.1M PBST (PBS plus 0.05% v/v Tween 20, Sigma), for 3 minutes each time. Plates were incubated for 30 minutes on a shaking platform at room temperature with 50 μl per well of PBST plus 5% milk powder (Marvel). Plates were rinsed as before and incubated with tissue culture supernatants of Mabs or 1:1000 dilutions in PBST of polyclonal antisera for 1 hour on a shaking platform at room temperature. After three more rinses, 50 μl of a second antibody (horse radish peroxidase conjugated goat anti-rat, anti-mouse or anti-rabbit IgG, Sigma) at 1:1000 dilution in PBST was added to the wells and the plates were incubated for 1 hour on a shaking platform at room temperature. Finally, after three rinses with PBST, 100 μl of 1 mg/ml of TMB substrate (3,3',5,5'-tetramethyl-benzidine from Sigma) 0.1% v/v hydrogen peroxidase

and 10% w/v sodium acetate, pH 5.8 in distilled water were added to each well and incubated at room temperature for 20 minutes. The reaction was stopped by adding 3 M sulphuric acid (25 µl per well). The intensity of the colour reaction was measured photometrically at 450nm and 690nm in a microtitre plate reader (Multiscan MCC/340, Titertek).

To determine the cross-reactivity of potential diagnostic antibodies with species of soil-dwelling nematodes other than PCN, microtitre plates were coated with twofold serial dilutions of homogenates of these other species and probed with the antibodies and antisera using the standard protocol.

3.1.4 Separation of cyst components

In order to separate cyst components, so that their antigenicity could be tested separately, crushed cysts of *G. pallida* were added to a pre-formed stepped sucrose gradient (15-65%) and centrifuged at 25 000 rpm in a Beckman L8M centrifuge. This permitted live eggs, dead eggs, and empty eggshells to be collected from separate fractions. Cyst wall fragments were collected by washing the crushed suspension through a 100-mesh sieve (aperture size 150 µm). The antigenicity of each of these components was then checked in ELISA.

When whole eggs were required for quantification studies, cysts were cut open individually, using a scalpel, in 0.1M PBS, pH 7.2, and the eggs concentrated by centrifugation before homogenization in a Biomedix homogenizer.

3.1.5 Antigen source

For studies on the source of antigens recognised by species-specific Mabs, some eggs were deliberately ruptured in PBS, so that the antigenicity of the vitelline fluid to these Mabs, and to polyclonal antiserum raised to juvenile whole body homogenates, could be assessed by testing the antigenicity of the resulting supernatant. This was compared to the antigenicity of homogenates of free juveniles, a mixture of eggs and juveniles, and whole cysts.

3.1.6 Antigen extraction

The effectiveness of different extraction agents, including water, buffer solutions, detergents and organic solvents, in the extraction of antigens recognized by two Mabs, chosen for their differential recognition of the two species of PCN, was compared. Homogenates of *G. pallida* and *G. rostochiensis* were prepared in the following solutions:

- 0.1M PBS, pH 7.2
- 1% Triton X-100 in 0.05 M Tris/HCl, pH 7.5
- 2% sodium deoxycholate (NaDOC) in Tris/HCl, pH 7.5
- 0.5% hexadecyltrimethylammonium bromide (CTAB) in Tris/HCl, pH 7.5
- 2.5% sodium dodecyl sulphate (SDS)
- 0.01% Tween 20 in 0.1M PBS, pH 7.2 (PBST)
- 0.01% Tween 20 in 0.1M PBS, pH 7.2 and 5% skimmed milk powder (PBSTM)
- 5% skimmed milk powder (Marvel)
- 8.3% polyvinylpyrrolidone (PVP)
- acetone
- butanol
- ethanol
- 1% urea

Five cysts were homogenised in 50 µl of solution/reagent on ice using a Biomedix plastic homogenizer. The supernatants were recovered after centrifugation for 1 minute in a microcentrifuge, diluted to 1 ml with 0.1M PBS pH 7.2 and used immediately to coat microtitre plates for assay by indirect ELISA. Homogenates prepared in PBS were also boiled for 10 minutes before centrifugation and testing as above.

3.1.7 Methods of antigen extraction

This section of the work was done with *G. pallida* only. Several methods of antigen release were tried. Suspensions of whole cysts or eggs in 0.5 ml of 0.1 M PBS, pH 7.2 were disruptured by adding 1g of Ballotini beads (0.09-0.13 mm) to the suspension before agitation for 2 minutes on a vortex shaker at maximum speed (Davies and Carter, 1995). Alternatively, cysts were sonicated (Vibra-Cell; Sonics & Materials, Danbury, Conneticut, USA. Output control No. 8 & % duty cycle at 90) in 0.5ml of 0.1 M PBS pH 7.2 held on ice, using a microtip; sonication was repeated for 2-3 seconds at a time until no intact nematode bodies remained (10 times, three cycles each time). This was confirmed by the use of a light microscope. The antigens extracted from the equivalent of five cysts after the milling and sonication procedures were centrifuged for 5 minutes in a microcentrifuge before dilution to 1ml with PBS, pH 7.2.

Chemical antigen release was attempted by incubating cysts in 1 ml of sodium hypochlorite (NaOCl, available chlorine 8% minimum) for 20 minutes. The reaction was stopped by neutralization of the NaOCl with 0.8ml of 30% hydrogen peroxide (H_2O_2).

The efficiency of antigen extraction by these techniques was compared with antigen extraction by homogenization of whole cysts in 0.1M PBS. All antigen extracts were then diluted in PBS to equivalent concentrations and used to coat microtitre plates for assay by indirect ELISA.

3.1.8 Screening for Globodera *specificity*

End-users of PCN immunoassays might, for certain purposes, require kits which are very sensitive and *Globodera* specific i.e. which could be used for the detection of presence (or absence in terms of not exceeding a threshold) of cysts of any species of *Globodera*. This would, of course, usually be PCN and such a test would most probably be used by quarantine authorities.

For this reason, the screening of Mabs produced on the project included a screen for Mabs which recognised the two species of PCN but no other plant parasitic species of nematodes.

3.1.9 Screening for PCN pathotype specificity

Although it seemed unlikely that pathotypes within the species of PCN could be differentiated by Mabs, eight populations of PCN that were representative of the PCN pathotype classification scheme of Kort *et al.* (1977), were used to screen Mabs which had shown differential recognition of the two species of PCN.

3.2. Assaying PCN population densities from soil

3.2.1 Quantification by ELISA

From the screen of Mabs described previously, two Mabs were chosen for their excellent differential recognition of the two species of PCN, one recognising *G. pallida* predominantly and the other *G. rostochiensis*, and given the code numbers MR8/4 and MR8/5 respectively. To analyse this reactivity more specifically, twofold serially diluted protein preparations made by homogenization in PBS were used to coat microtitre plates and these were probed with tissue culture supernatant of the two Mabs. An indirect ELISA protocol was followed (See appendix).

Once the basic protocol for PCN quantification was defined, a procedure to confirm quantification from soil was followed. Known numbers of *G. pallida* cysts were added to 200g batches of soil (4:1 mixture, Kettering loam:coarse sand) and then re-extracted by Fenwick Can (Fenwick, 1940). The dried extract was placed on a polyester sieve (aperture size 100μm) in a Petri dish in 1.5 ml 0.1 M PBS, pH 7.2 and the cysts broken open by crushing with a glass rod. The filtrate was collected and sonicated as above (10 times, three cycles each time). The extract was then centrifuged for 10 minutes in a microcentrifuge before testing by both indirect ELISA and Double-Antibody-Sandwich (DAS)-ELISA. Solution volumes were 100 μl in both cases, and soil samples with no cysts added were also processed in order to provide controls. For the DAS-ELISA, microtitre plates were pre-coated with polyclonal antiserum diluted 1:24 000 with 0.1M PBS pH 7.2, and MR8/4 was used as the detecting antibody.

3.2.2 PCN extraction from soil using magnesium sulphate solution

To maximise the advantages of speed, convenience, accuracy and cheapness that an immunoassay potentially offers for PCN quantification, a rapid, cheap and efficient method for the recovery of cysts from soil that is also capable of being automated is required. In this part of the work, an extraction procedure based on flotation with $MgSO_4$ solution was developed. Even wet cysts will float in a 30% w/v

solution of $MgSO_4$ in water. In this method, a 100g sample of soil is placed in a plastic bag (30cm × 12cm, 500 gauge) and 200ml of 30% $MgSO_4$ solution was added. The bag is heat-sealed across the top and the contents mixed and shaken thoroughly for 30 seconds. The bag is then allowed to stand upright for 60 seconds before folding it over at the mid-point to separate the floating material (which includes the cysts)from the inorganic residue. The bag is heat-sealed across the middle to ensure separation and the lower portion discarded. The floating material is then passed through a pair of filters - a course, bronze filter of 840 μm aperture which allows the cysts to pass through and a fine, polyester sieve of 100 μm aperture which retains the cysts. Much of the rest of the extract either passes through both sieves or is retained by the top sieve. The above process is illustrated diagrammatically in Fig. 3.

The efficiency of the process utilising $MgSO_4$ flotation was compared with flotation in water alone, Fenwick Can extraction, and the Schuiling Centrifuge which was expensive and complicated and therefore not considered further.

FIGURE 3. Rapid extraction of PCN cysts from soil samples using magnesium sulphate flotation

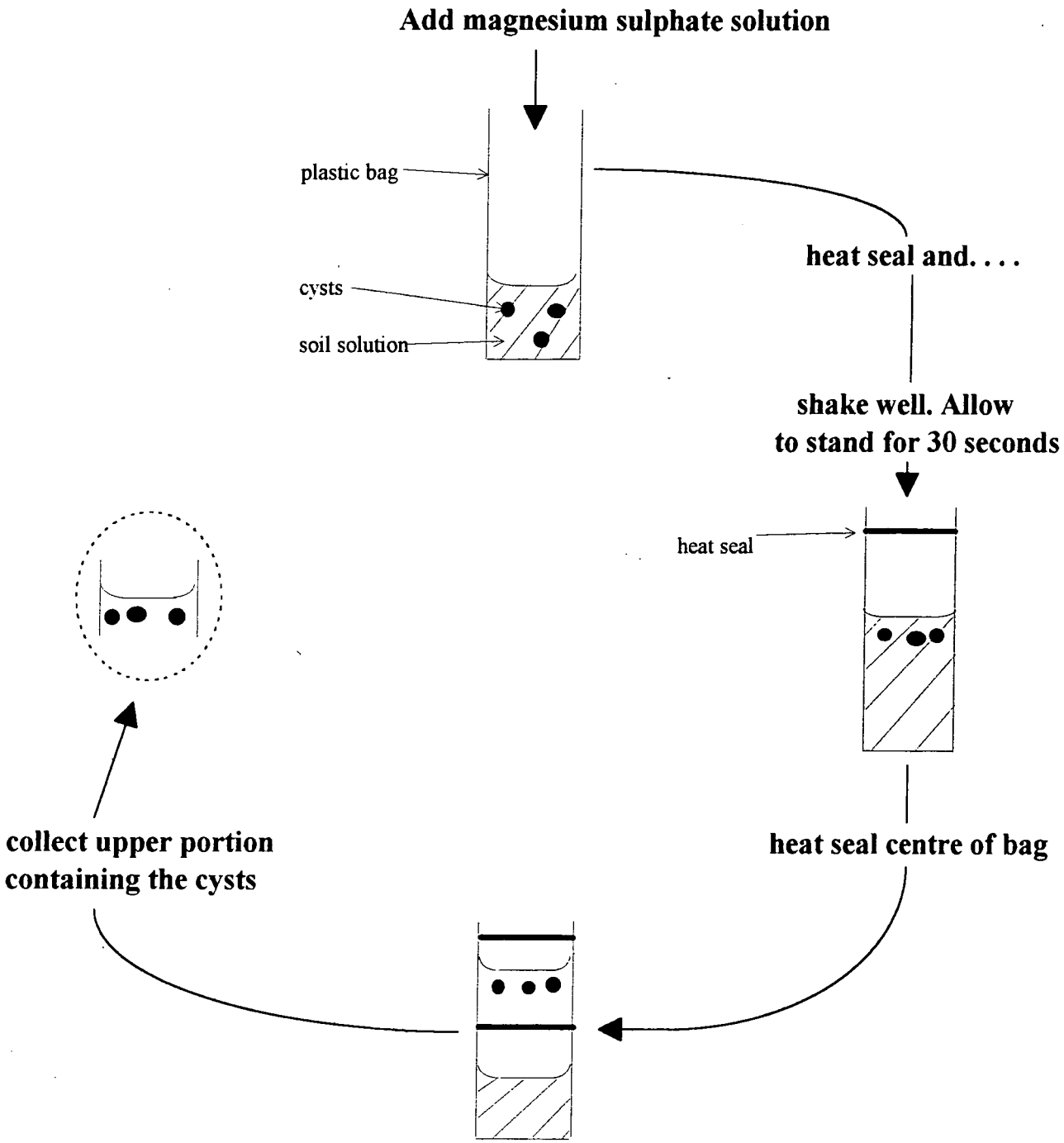


FIGURE 4 The Fenwick Can .

3.2.3 Physical antigen extraction

For rapid and convenient extraction of antigens from cyst samples, the filter plus cysts from the $MgSO_4$ process was sealed in a plastic bag (16cm × 12cm, 500 gauge) along with 2 ml of extraction buffer. The standard extraction buffer was 0.1M PBS, pH 7.2. To improve antigen extraction, various additions to this standard were made, including protease inhibitor (phenylmethylsulphonyl fluoride - PMSF, 0.05%) to prevent antigen breakdown by proteases released during extraction, detergent (Tween 20, 0.05%) to aid solubilisation of membrane-bound proteins, and skimmed milk powder (Marvel, 10%) to prevent non-specific binding of released antigen to organic matter.

Antigen release was achieved by passing the sample sealed in the plastic bag through the stainless steal rollers of a domestic pasta (lasagne) making machine. The rollers were set to their closest position. To prevent the sealed bag from rupturing as the contents were squeezed under pressure, a spiral groove was cut around one of the rollers. Finally the bag contents were centrifuged to remove particulate matter and the supernatant was retained for assay.

As an alternative, a simpler extraction procedure, samples were crushed in their plastic bags using a domestic rolling pin to roll the bag on a hard surface (laminated laboratory bench).

After a review of all the methods tested a decision to use a mechanical method of antigen release was taken after trying three chemical, four mechanical, two freeze-thawing and one ultrasonication extraction procedures. The procedures were: soaking in PBS or solutions of sodium deoxycholate or sodium hypochlorite; the pasta machine or a Homex 5, Tecan or hand-held homogenisers; freeze-thawing on ice or at -20^0C; ultrasonication for 30 minutes.

3.2.4 Assay of mixed species populations of PCN by ELISA

To test the robustness of Mab specificity in likely field situations, the two Mabs referred to above (MR8/4 and MR8/5) were used in an indirect ELISA to assay

double-diluted extracts of each of the PCN species against a constant background of the other. The dilution series was made up such that a volume of 50 µl added to each well of the microtitre plate represented 0.025 to 50 eggs per well. Extract equivalent to a constant 25 eggs per well of the other species was added to each well.

In addition, standard curves in indirect ELISA were produced for the two Mabs above that were chosen for further assay development. In arriving at the standard assay for this further development, various options had been tested, including the direct conjugation referred to above, indirect and double and triple antibody sandwich formats (using various antibody combinations), and amplification of the reactions using biotin-avidin complexes.

3.2.5 Assay to determine whether Mabs can distinguish between live and dead eggs

In any work with PCN, the estimations that are made of population densities for management purposes must be able to discriminate between live and dead eggs as it is only the live eggs that will yield live juveniles capable of invading plant roots and causing damage.

The preliminary investigations on antigenicity of cyst components suggested that only live eggs and whole cysts were antigenic. Further proof was required that only live eggs are antigenic towards the Mabs chosen for development of the diagnostic assay. For this investigation, samples were taken from Rothamsted's archived PCN collection, taking cysts from as early as 1935. These were extracted by homogenization in 25 µl of PBS and processed by Indirect ELISA.

Since our Mabs appeared to be reacting to live eggs only, the next step deliberately killed eggs by various methods, including treatment with formal-acetic-alcohol (FAA):-

50 cysts were soaked overnight in FAA, 25 cysts were homogenized in 25 µl of PBS and the volume increased to 5mls, the other cysts were removed to root diffusate to confirm that the cysts were dead;

parasitic fungi (*Cylindrocarpon destructans*):-

C. destructans was cultured on agar plates with PCN cysts. Ten infected cysts were then homogenized in 25 µl of PBS and the volume increased to 2mls; and heat (30^0 C, 40^0 C, 50^0 C & 60^0 C)

Fifty cysts were incubated at the above temperatures for three hours, they were then homogenized in 25 µl PBS;

The antigen from each assay was then tested for its reactivity with the Mabs in an Indirect ELISA.

3.2.6 The effect of soil type and organic matter content on PCN estimation

For maximum advantage to be gained from the potential offered by the use of immunoassay in conjunction with the $MgSO_4$ flotation system, it is important that the efficiency of recovery of cysts by the new methodology compared to the standard Fenwick Can be known. This requires comparison to be made over a comprehensive range of soil types. At the same time, the effects of the different soil characteristics on immunoassay results must be determined. For these reasons, a comprehensive experiment was designed to include PCN estimations by standard methodology and by immunoassay following cyst extraction either by Fenwick Can or $MgSO_4$ flotation. The experiment used soil types typical of an area of northern Portugal where PCN is common. In this area, soil parent materials are either schist or granite, and organic matter (OM) contents vary between less than 1% and in excess of 11%.

The experiment used artificially infested soils in order that a range of precise PCN population densities could be established. A range of OM contents was also created artificially by taking a large bulk of soil of each parent material which was of either low or high OM content. These were mixed in different proportions to give five different levels of OM for each parent material. Full chemical and mechanical analyses were made on each of these mixtures in order to characterize them fully. The soils were then infested with PCN by adding known numbers of cysts to each parent material x OM combination such as to give infestation levels corresponding to median values in each of the categories of infestation levels used by the British Agricultural Development and

Advisory Service (ADAS). Two 100g replicate sub-samples of each type of soil created by this procedure were then processed by both Fenwick Can and by flotation. Cysts and eggs were then counted by standard methodology and a note was made of the time taken for each step in each method. A duplicate series of soil samples was processed for assay by immunoassay, in order that the full comparison of all procedures could be completed.

3.3. Direct use of the assay in a field fumigation trial

At the southern end of a 15 hectare field in Nocton Rise, Lincolnshire, two sites, A & B were marked at approximately 100 metres (from each other) apart. Each area was approximately 30m × 30m and 11 strips in A and 13 in B were fumigated with 1,3-dichloropropene (1,3-D). The strips were the width of the application machinery (3m) and were alternated with untreated strips. Forty soil cores were then taken per strip with a hand auger on days 2, 4, 12 & 26, after fumigation, from site A and days 0, 2, 3, 10 & 24 after fumigation, from site B. The forty cores were bulked into single samples on each sampling occasion .

Cyst Extraction:

From each sample on each date, four replicate sub-samples of soil (200g each) were taken. All four were processed by a Fenwick Can (as was any remaining soil so as to retrieve cysts for a root invasion test). Two of the Fenwick floats were analyzed by traditional counting methods and the remaining two by ELISA.

The Can was filled with water and the collecting sieves (840μm over a 250 μm) were wetted. A 200g soil sample was then placed in the 2mm-aperture sieve in the funnel and washed through into the can with a strong jet of water from above. (The Fenwick Can used was modified slightly from the original design (Fenwick, 1940) by a water inlet at the side of the can which provides a current of water to prevent cysts becoming trapped by falling sediment).

The 'float' was washed on the 840μm sieve in order to carry any cysts through to the 250μm sieve. Further separation of cysts was achieved by flotation in a glass conical flask filled with water. The float was then air dried.

The cysts were separated from the organic material by flotation in acetone, in a conical flask.

Cyst Assessment:

1) Indirect ELISA (See appendix)

2) Traditional:

The number of cysts in samples were counted under a stereo-microscope and their contents assessed by counting the eggs in a sub-sample from a suspension of crushed cysts.

Root Invasion Test:

1. Single sprout potato plants were grown inside a greenhouse at Rothamsted for 27 days in soil in 10cm pots, to which 50 cysts from each treated and untreated field sample had been added.

2. After 27 days the potato plants were washed free of soil and their roots, shoots and stolons weighed.

3. The root systems were cut into 1 cm lengths, thoroughly mixed, and a 2g sub-sample preserved in FAA.

4. The nematodes in the roots were stained by boiling for 3mins in 0.05% acid fuchsin in equal volumes of glycerol, lactic acid and distilled water (Bridge, et al., 1981).

5. The roots were cleared in a 50:50 solution of glycerol and distilled water and then chopped for 30 seconds in an "Atomix" blender.

6. The samples were diluted to 200ml with distilled water, agitated and two 2ml sub-samples removed.

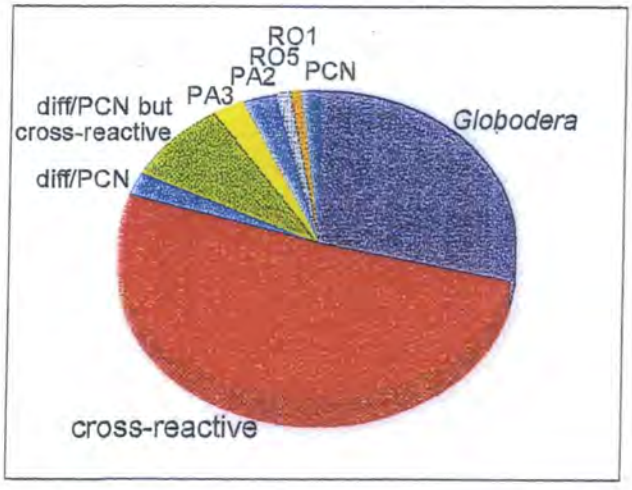
7. The numbers of nematodes in the sub-samples were counted by microscope and their developmental stages noted.

4. RESULTS

4.1. Antibody & Antigen Screening

Of the 1339 hybridoma lines, approximately 200 reacted positively in ELISA to PCN, 30 recognised *G. pallida* and *G. rostochiensis* differentially (12 of these without cross-reacting with other soil nematodes), and 72 were able to recognise the genus *Globodera* specifically. When screened against populations representative of pathotypes within the international pathotype scheme of Kort *et al.* (1977), some were able to recognise individual pathotypes. By way of example, the recognition characteristics of antibodies produced by the hybridoma lines resulting from one fusion (using low molecular weight proteins from *G. rostochiensis* JJ2 as immunogens) are shown in Fig. 5. Mabs able to recognise six of the eight international pathotypes were identified and the only two pathotypes not recognised were *G. rostochiensis* Ro3 and *G. pallida* Pa1.

FIGURE 5 The recognition characteristics of *c.* 200 cell lines produced by immunising mice with low molecular weight protein preparations from *G. rostochiensis.*



For diagnostic use, the antibodies finally selected must, in addition to their differential recognition of the two species of PCN, have minimal or zero cross-reaction with other species of plant-parasitic or soil nematodes. The cross-reactivities of perhaps the best two Mabs (coded MR8/4 - *G.pallida* specific, and MR8/5 - *G.rostochiensis* specific) are compared with those of PC 266 and PC 267 in Table 2.

MR8/4 and MR8/5 showed essentially no cross-reaction with other species of plant parasitic and soil nematodes, the only strongly cross-reaction being with the two sub-species within *G. tabacum*. Since these occur only in the New World and then only sporadically, the cross-reaction is not a problem in most locations. A very low level of cross-reaction occurred with *Heterodera goettingiana* and *Rhabditis oxycerca* but the method of extraction of PCN from soils and the type of soil in which they occur mean that this also is not a serious problem. The Pab's, PC 266 and PC 267, on the other hand, cross-reacted with all nematode species tested, presumably because they recognise many epitopes on many proteins shared by the different species. This means that Pabs will never have the level of discrimination required for the recognition and quantification of individual nematode species but, because of their potentially great sensitivity, they may still have a role to play in the development of immunoassays.

Although MR8/4 and MR8/5 were some of the best Mabs produced in terms of ability to differentiate the species of PCN, it proved impossible to produce large amounts of MR8/4 because the cell line seemed to be unstable. However, repeated testing finally showed that it was infected by a mycoplasma. Intensive drug treatment and repeated sub-culturing finally yielded a mycoplasma-free cell line and allowed both Mabs to be produced successfully.

TABLE 2. Cross-reactivities of MR8/4, MR8/5, PC 266 and PC 267 with nematode species.

	MR8/4	MR8/5	PC266	PC267
G.rostochiensis	-	++++	++++	++++
G.pallida	++++	-	++++	++++
G.tabacum tabacum	++++	-	++++	++++
G.tabacum solanacearum	-	++++	++++	++++
H.avenae	-	-	+++	+++
H.cajani	-	-	+++	+++
H.carotae	-	-	+++	+++
H.cruciferae	-	-	+++	+++
H.glycines	-	-	+++	+++
H.goettingiana	+	-	+++	+++
H.mani	-	-	+++	+++
H.schachtii	-	-	+++	+++
H.sorghi	-	-	+++	+++
H.trifolii	-	-	+++	+++
H.urticae	-	-	+++	+++
A.tritici	-	-	++++	+++
A.besseyi	-	-	+++	+++
D.myceliophagus	-	-	+++	+++
M.arenaria	-	-	+++	+++
P.redivivus	-	-	+++	+++
P.neglectus	-	-	+++	+++
R.reniformis	-	-	+++	+++
R.oxycerca	+	-	++++	+++

In Table 2, reaction was considered positive when the absorbance value in ELISA was at least three times the value of the negative controls, which had a mean optical density value of 0.054. Positive values were graded as follows:

++++ Optical density values above 1.000
+++ Optical density values above 0.500
++ Optical density values above 0.350
+ Optical density values above 0.150

4.1.1 Testing antibodies and antisera in ELISA

The simplest type of ELISA and, therefore, also the most rapid to perform is the direct ELISA, where the diagnostic antibody is labelled directly with an enzyme. Once diagnostic Mabs had been chosen, the next step was to attempt direct conjugation of these Mabs with appropriate enzymes (either alkaline phosphatase or horseradish peroxidase). Several conjugations were made using commercially available kits and several more were performed by specialist companies. All conjugations, including those made with biotin rather than an enzyme in an attempt to amplify the signal obtained, resulted in impaired differentiation of the two species of PCN. Only a slight improvement in differentiation was obtained when the Mab was purified prior to conjugation.

Indirect ELISA conjugation procedures gave better species differentiation with both of the diagnostic Mabs. Trapping ELISA's using a Mab as the trapping antibody and a Pab as the detecting antibody gave poor species differentiation but good differentiation was obtained when a Pab was used as the trapping and a Mab as the detecting antibodies, especially when the final detection was indirect (i.e. a Triple Antibody Sandwich). The usefulness of the various ELISA protocols for immunoassay of PCN using the chosen diagnostic antibodies, in terms of quantification, differentiation and sensitivity, are summarized in Table 3, information based on a series of tests.

TABLE 3 Quantification, differentiation and sensitivity of different ELISA types for PCN species.

ELISA type	Quantification		Differentiation		Sensitivity
	G.p.	G.r.	G.p.	G.r.	(eggs/sample)
Direct	+++	+++	-	+	30
Indirect	+++	+++	++	+	30
DAS	+++	+++	++	-	3
TAS	+++	++	+++	+	30

- = poor + = good

The final choice of ELISA type will depend on the results of optimisation studies.

4.1.2. Standardization of antigen extraction and ELISA protocol

In order to standardise the identification and quantification procedure, a standard antigen extraction system was defined. In this, PCN cysts were first recovered from soil by Fenwick Can and the resulting float placed on a nylon sieve inside a Petri dish, where the cysts were crushed in 1.5 ml of PBS. The extract, containing both eggs and juveniles, passed through the sieve and extraction of antigen completed by a combination of homogenization and sonication. The extract was centrifuged before taking the supernatant for assay. Figs. 6 and 7 show the assay of *G.pallida* with MR8/4 in indirect (Fig. 6) and triple antibody sandwich (Fig. 7) ELISA.

FIGURE 6. Assay of *G.pallida* in Indirect ELISA with MR8/4

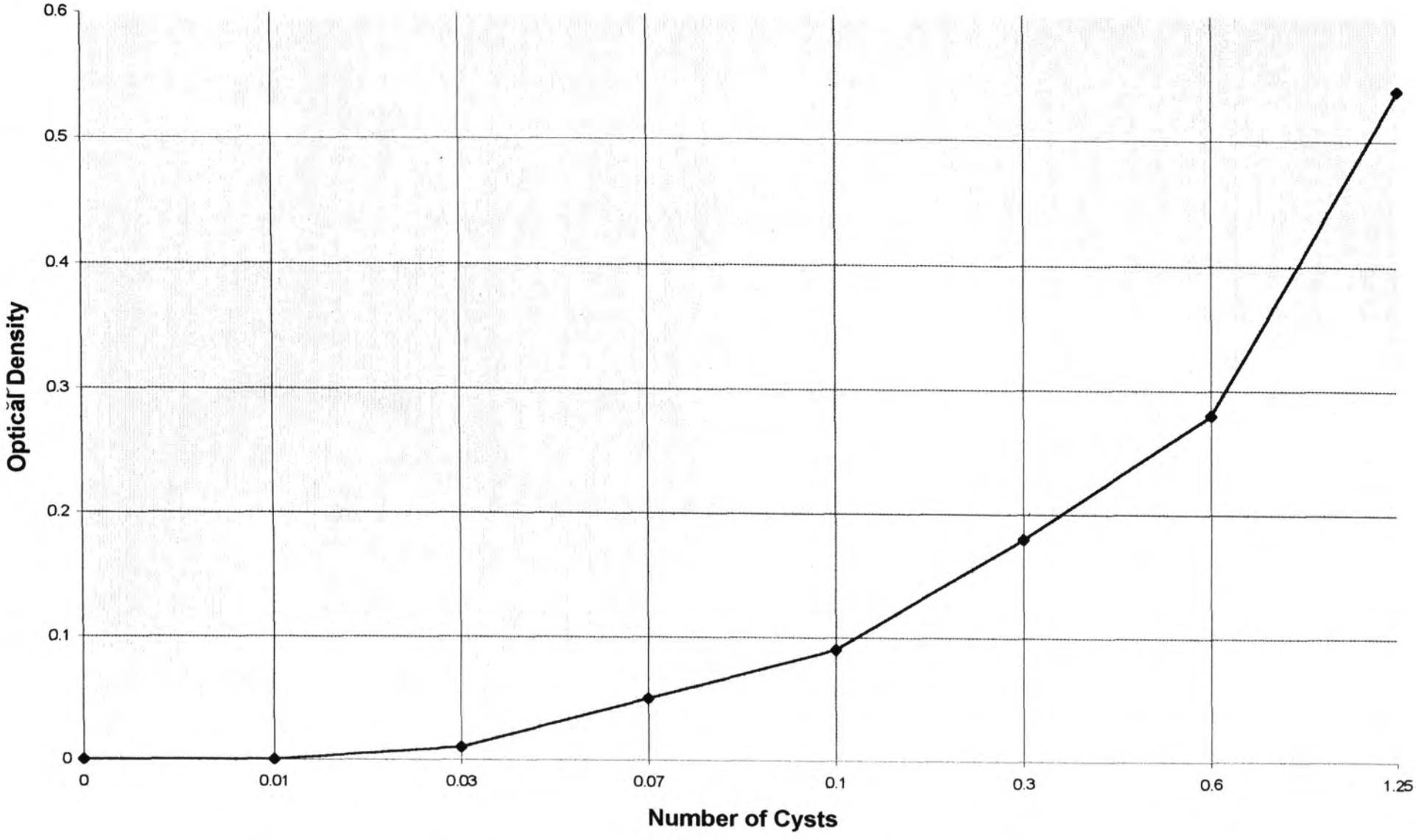
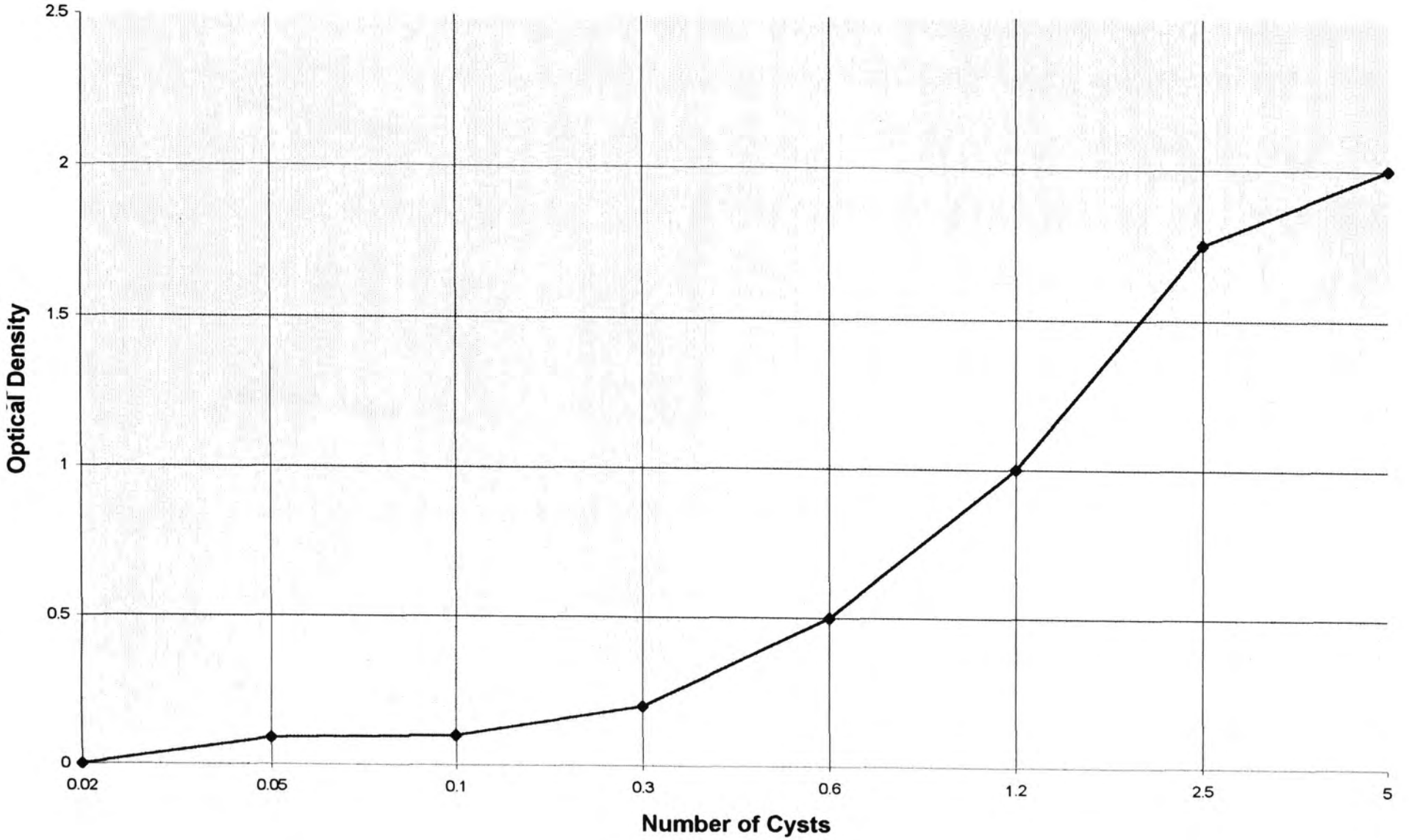


FIGURE 7. Assay of *G.pallida* in TAS-ELISA with MR8/4 and using PC 267 as the trapping antibody

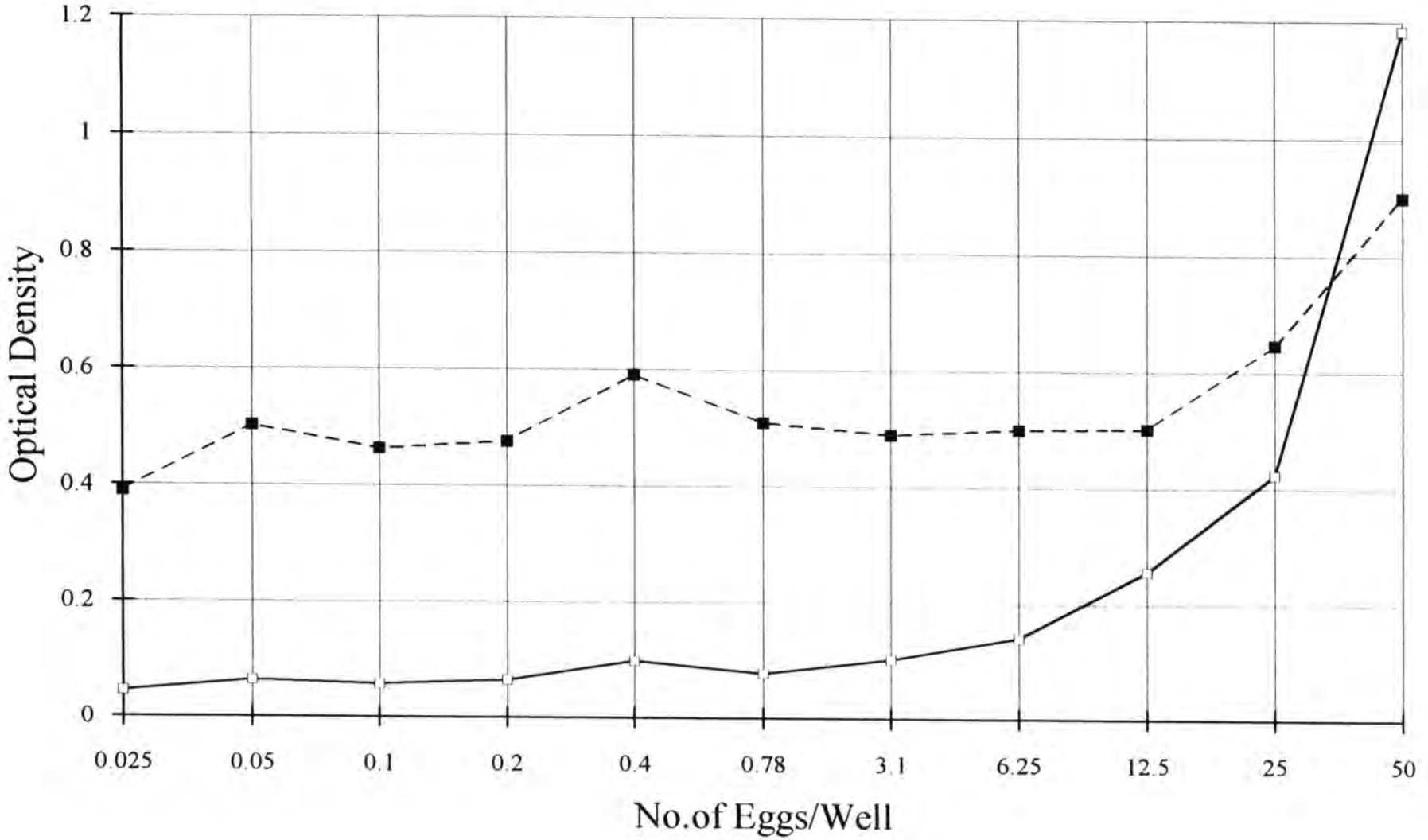


The potentially greater sensitivity of TAS-ELISA can be seen here but the assay procedure must be optimised for its full potential to be exploited.

4.1.3. ELISA with mixed species populations of PCN

In assays of populations consisting of mixtures of the two species of PCN, there was very little interference with the specific recognition of either antibody. In Fig. 8, ELISA's with double dilutions of extracts of the two species are shown in which a fixed number of eggs of the other species was present in each well. The exercise was performed with both diagnostic Mabs and confirmed the low level of interference with specific recognition.

FIGURE 8. ELISA with *G.rostochensis* specific Mab of -■- double dilution of *G.pallida* with fixed number (25) of *G.rostochiensis* eggs per well, and -□- double dilution of *G.rostochiensis* with fixed number (25) of *G.pallida* eggs per well.



4.1.4. Antigenicity of components of cysts

Because the infective stage of PCN (the second stage juvenile) occurs inside the eggs which are enclosed within a protective cyst, and because it is the whole cyst that is extracted from soil samples, it is important to know the relative antigenicity of the different components of the cysts and to ensure that it is impossible for empty cysts or cysts full of dead eggs to give high readings in immunoassay. For this reason, crushed cysts were sieved to remove the cyst wall fragments and their contents separated by sucrose gradient centrifugation. The separation into free ('hatched') juveniles, live eggs, dead eggs and empty eggshells that was obtained is illustrated in Fig. 9.

The antigenicities of the separated cyst components, including the cyst wall fragments removed by sieving and whole cysts, were compared using PC 267. Extracts were made and their antigenicities compared in indirect ELISA. The results are in Fig. 10.

Only whole cysts and live eggs showed strong antigenicities. In addition, the antigenicity of these two components was very similar. This is a very important result as it means that the response in immunoassay is confined to live, and therefore infective, eggs. The low antigenicity of hatched juveniles was surprising but was explained by subsequent tests.

FIGURE 9. Separation of cyst contents using stepped sucrose gradient centrifugation.

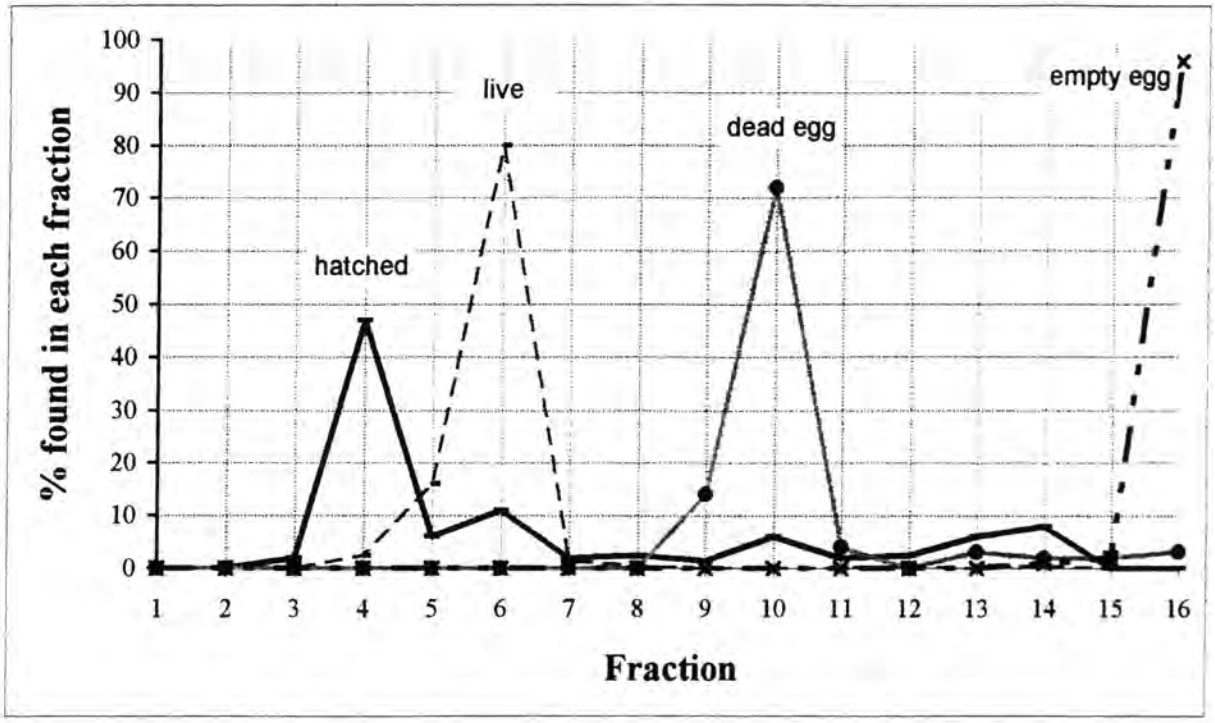
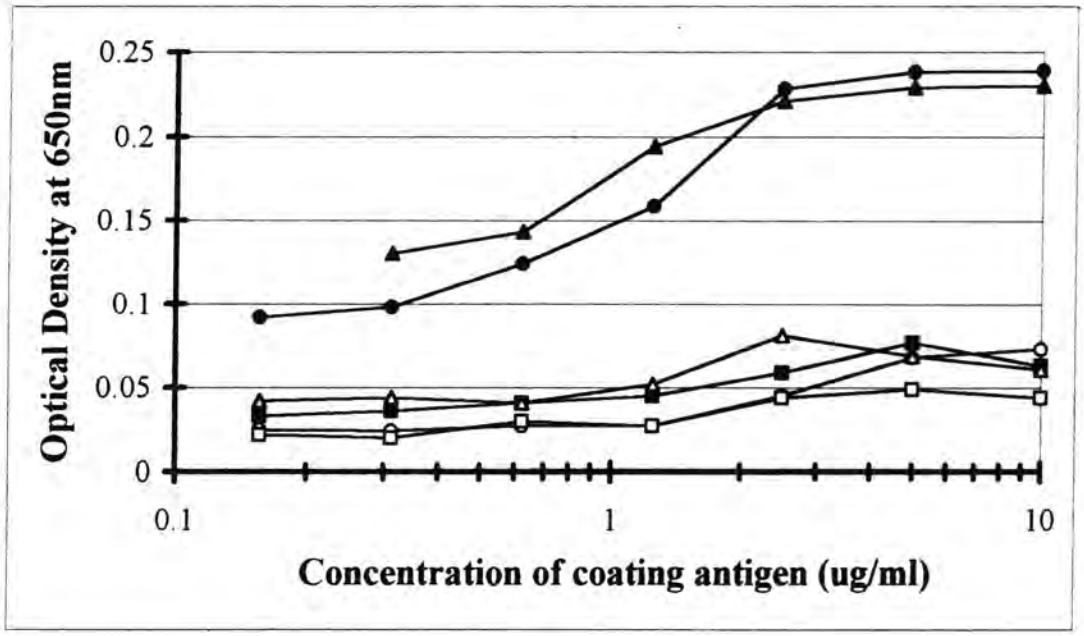


FIGURE 10. Antigenicity of components of *G.rostochiensis* cysts, compared in indirect ELISA using PC 267 -○- free ('hatched') juveniles, -●- live eggs, -□- dead eggs, -■- empty eggs, -Δ- cyst wall fragments, -▲- whole cysts.



4.1.5. Antigenicity of cysts with time

Further indirect ELISA tests on cysts from Rothamsted's PCN archive, showed that cysts lost their antigenicity with age, and that there was barely any reaction in cysts produced earlier than 1970. This suggests that the contents of cysts die completely after about 25 years, a period of time noted (more as hearsay than fact) in the literature but which perhaps now has some scientific backing (fig. 11)

Since the Mabs appeared to be reacting to live eggs only, the next study deliberately killed eggs by various methods, including treatment with formal-acetic alcohol, parasitic fungi and heat (fig.12)

All killing treatments resulted in total loss of antigenicity of the cysts. Standard counting procedures for PCN take no account of whether or not eggs are alive. This shortcoming may not be important some of the time because dead eggs eventually decay and are not then confused with live eggs. However, it is particularly important after treatment with nematicides, when it is necessary to be able to assess the degree of kill. If the nematicide is a fumigant it can actually have a "preservative" effect on the nematodes so that they appear live for many weeks after treatment. This immunoassay has the potential to overcome this problem and give a direct estimate of the surviving population density after nematicide treatment.

FIGURE 11. Optical Density in Indirect ELISA to MR8/4 of extracts made from PCN cysts of various ages.

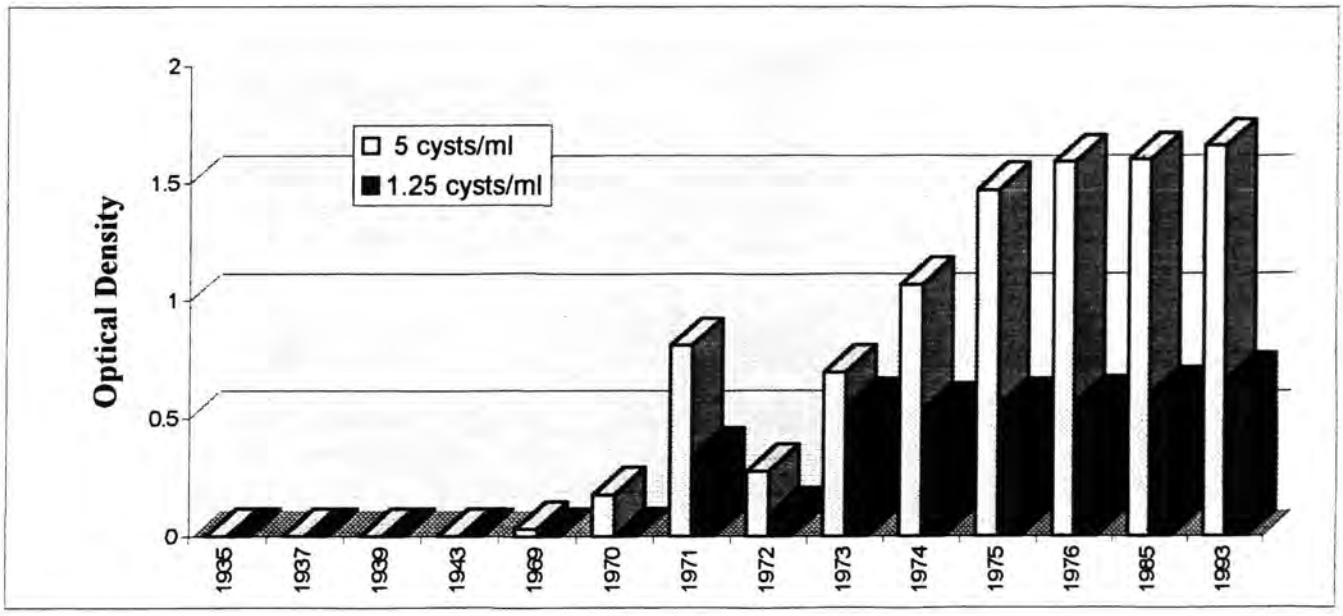
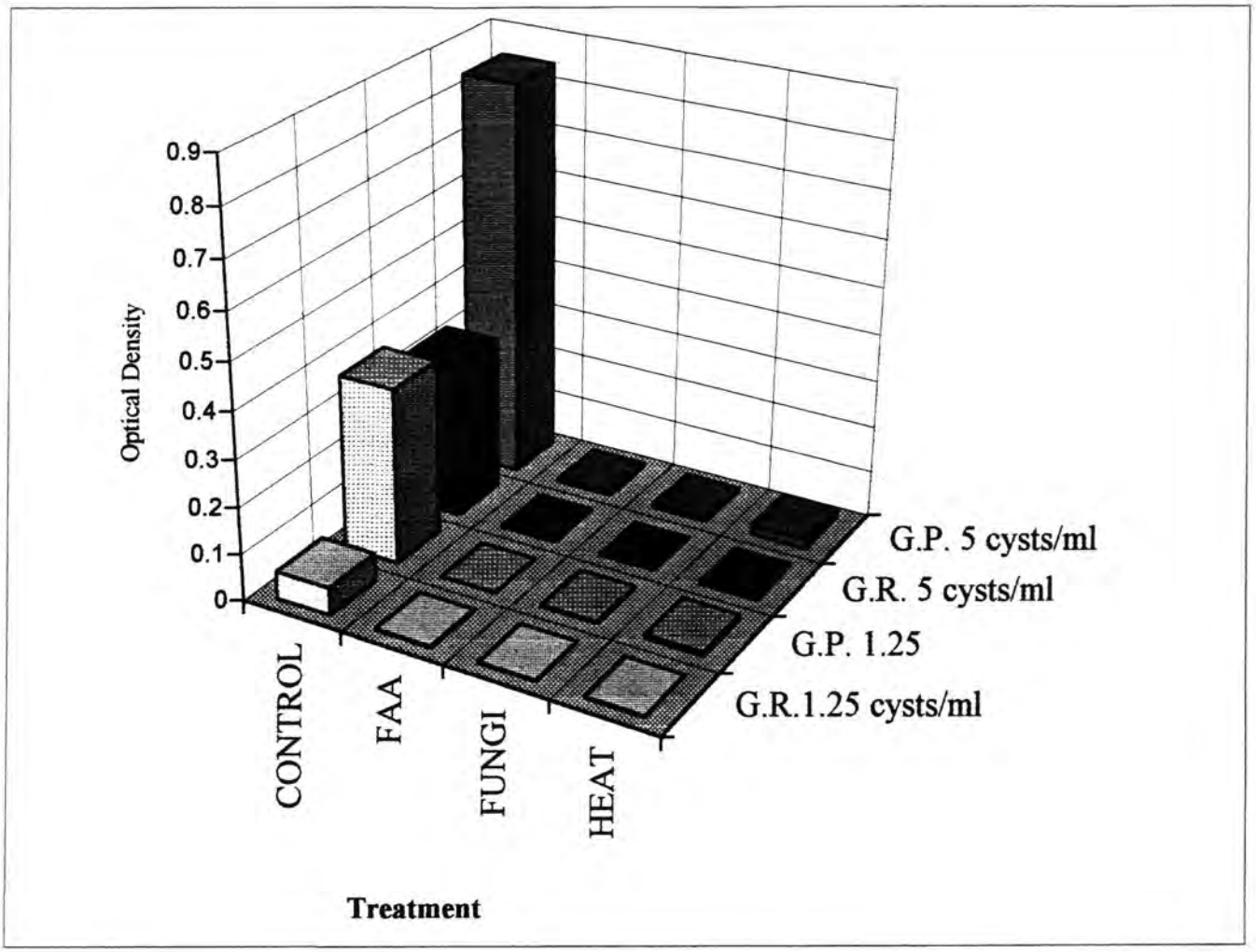


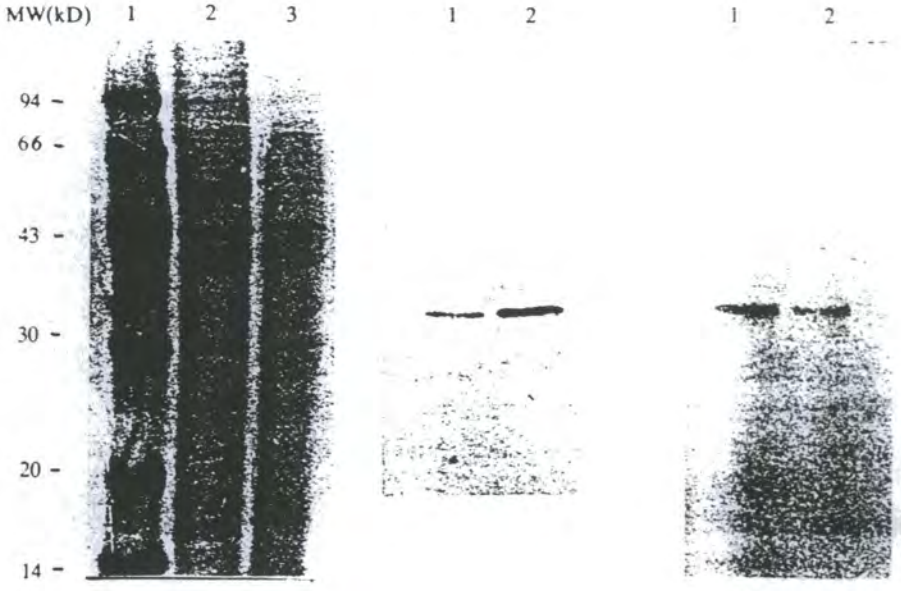
FIGURE 12. Optical densities recorded in Indirect ELISA with PCN cysts either untreated or killed in various ways.



4.1.6 Characterization of antigens recognised by chosen diagnostic Mabs

The molecular weights of the antigens recognised by MR8/4 and MR8/5 were determined by Western blotting of *G.rostochiensis* and *G.pallida* homogenates followed by probing with the two Mabs; both recognised a 34kD antigen in both species, though somewhat differentially (Fig. 13).

FIGURE 13. Coomassie blue staining of SDS-PAGE of molecular weight markers (lane 1) followed by *G.rostochiensis* (2) and *G.pallida* (3) homogenates. b) Western blots of (1) *G.rostochiensis* and (2) *G.pallida* cyst homogenates probed with the Mabs MR8/4 and MR8/5.



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Both MR8/4 and MR8/5 reacted with antigens present in both species of PCN but MR8/4 produced a more intense band in *G.pallida*, whilst MR8/5 produced a more intense band in *G.rostochiensis*. Immunoblotting of IEF gels of homogenates of the two nematode species with MR8/4 and MR8/5 demonstrated that the Mabs recognised antigens with isoelectric points at pH values of 5.7 in *G.pallida* and 5.9 in *G.rostochiensis*. The results for MR8/5 are in Fig. 14(b). These are the diagnostic proteins described by Fleming and Marks (1983) and can be used to identify the species of PCN in Coomassie Brilliant Blue stained IEF gels as in Fig.14(a).

The bands corresponding to the *G.pallida* and *G.rostochiensis* diagnostic proteins were excised from IEF gels and shown by SDS-PAGE to have the same molecular weight (34kD) as the proteins recognised by the Mabs (Fig.15), and Western blotting of the diagnostic proteins followed by probing with the Mabs confirmed their recognition by MR8/5 (Fig. 16). The same result was obtained with MR8/4 (not shown).

4.1.7 Immunolocalization of antigens

In addition to biochemical characterization of the antigens recognised by the diagnostic Mabs, it was felt necessary to determine the location of the antigens as a prelude to examination of extracts and procedures which would maximise recovery of the antigens in a quantitative manner. The first stage was to examine the relative antigenicity of the extraction buffer in which eggs were deliberately ruptured to allow the determination of the antigenicity of the vitelline fluid contained within the egg.

FIGURE 14. (a) Coomassie Brilliant blue staining gel of (1) *G.rostochiensis* and (2) *G.pallida* homogenates. b) Immunoblot of IEF gel of (1) *G.rostochiensis* and (2) *G.pallida* homogenates probed with MR8/5.

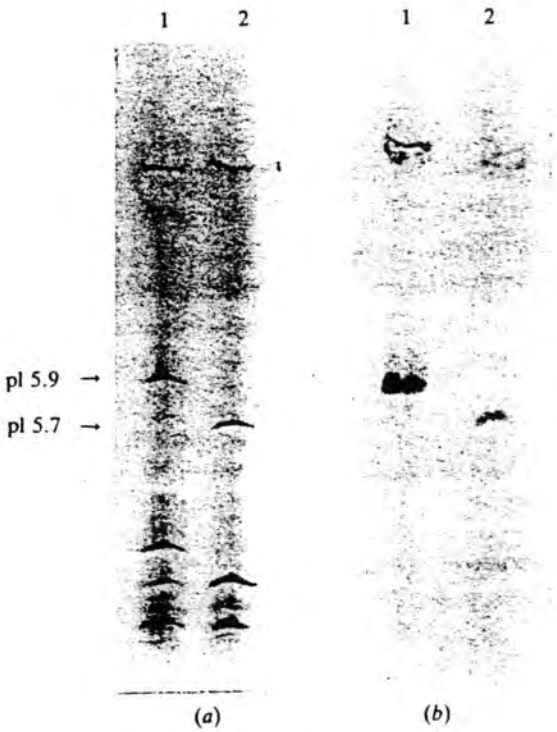


FIGURE 15. Coomassie Brilliant blue staining of SDS-PAGE of (1) *G.rostochiensis* homogenate, (2) *G.rostochiensis* diagnostic band, (3) molecular weight markers, (4) *G.pallida* diagnostic band, and (5) *G.pallida* homogenate.

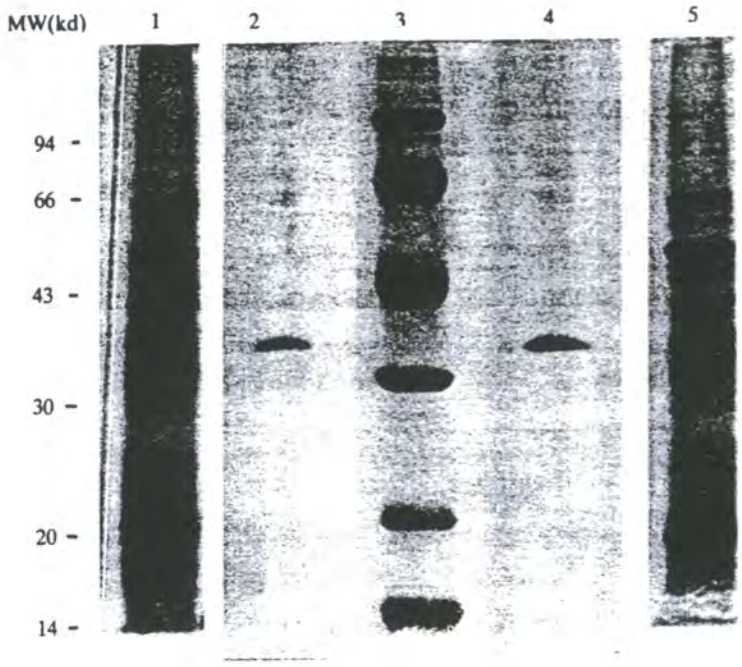
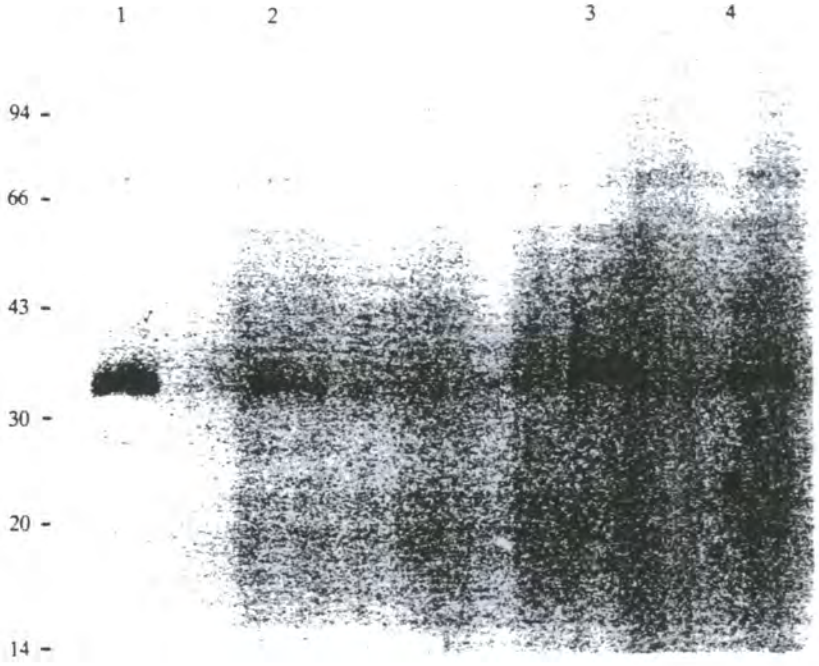


FIGURE 16. Western blots of (1) *G.rostochiensis* diagnostic band, (2) *G.pallida* diagnostic band, (3) *G.rostochiensis* homogenate and (4) *G.pallida* homogenate, all probed with MR8/5.



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Vitelline fluid antigenicity to the diagnostic Mabs and to PC 266 was compared with the antigenicities of homogenates of free juveniles, a mixture of eggs and juveniles, and whole cysts (Fig. 17). Whilst PC 266 recognised all four samples more or less equally (as would be expected from recognition of a wide range of juvenile proteins) with the lowest optical density for the buffer supernatant containing mainly vitelline fluid, MR8/4 failed to recognise juveniles strongly but, at the same time, recognised whole eggs and buffer supernatant equally strongly. This result implied either that the antigen recognised resides in the vitelline fluid of the egg and is released as soon as the eggshell is ruptured, or that the act of rupturing the eggshell causes the juvenile to release this antigen suddenly, or both of these things. Whatever the explanation, it means that the antigen is very easily released from eggs, a valuable characteristic when rapid antigen release procedures are being sought.

Immunolocalisation studies on cryostat sections of J2 of both species of PCN showed that both diagnostic Mabs recognised an antigen within the amphids of the juveniles (Fig. 18).

FIGURE 17. Antigen detection by MR8/4 and PC 266 in homogenates of JJ2, mixtures of eggs and JJ2, whole cysts, and the supernatant of buffer in which eggs have been ruptured.

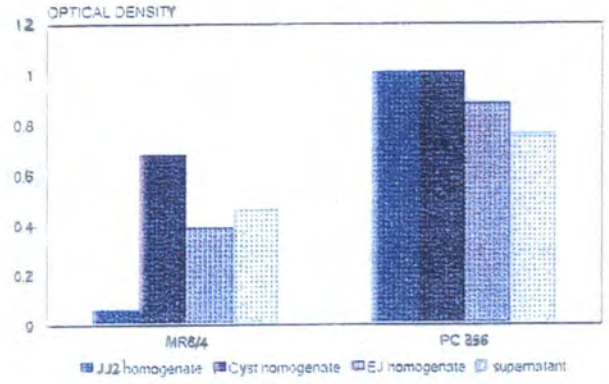
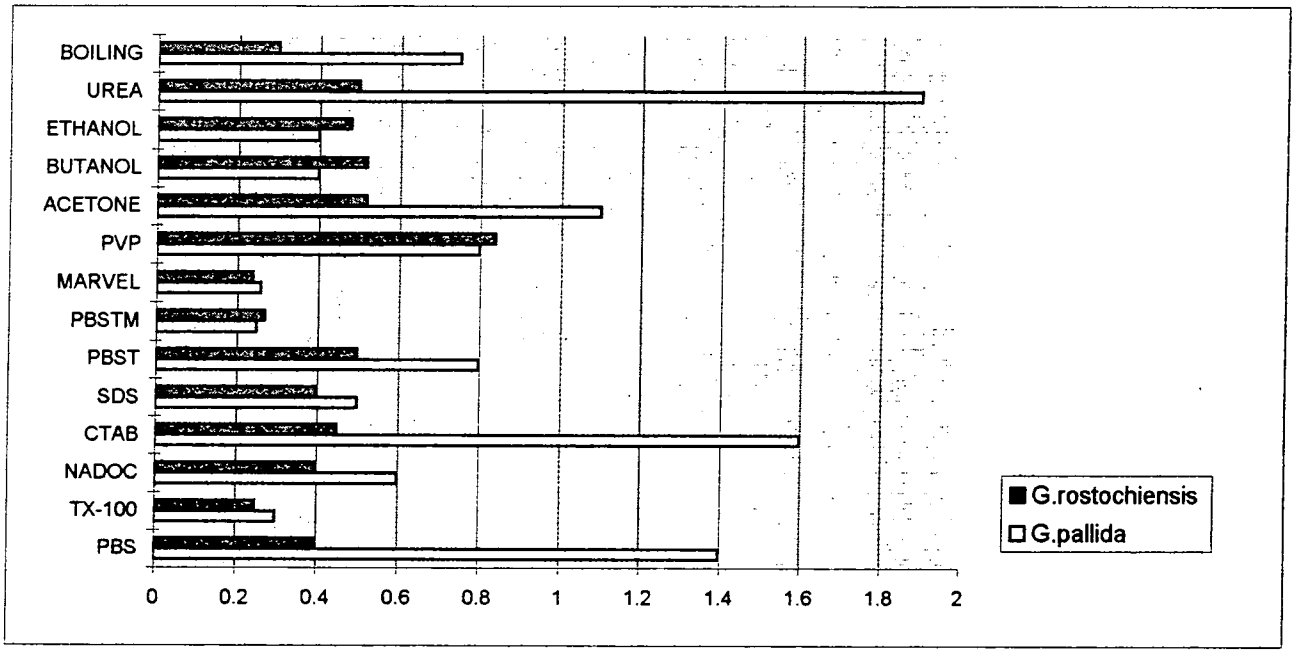


FIGURE 18. Cryostat section of anterior end of J2 of *G. pallida* probed with MR8/4.

4.2. Antigen extraction reagents and procedures with soil

Whilst it is desirable, in the long term, to develop methods of extracting antigen of interest directly from soil containing nematodes, a first stage is to extract antigen with maximum efficiency, in terms of both recovery and effort, from clean cysts. The standard extraction procedure, adopted early on in the work, was to homogenise cysts in PBS using a hand-held plastic homogenizer (Biomedix). Once the diagnostic Mabs had been chosen, it was important to establish whether other extraction reagents were more appropriate. A variety of reagents was tested with the Biomedix homogeniser and compared with PBS. The results are given in Fig. 19.

FIGURE 19. Antigenicity in indirect ELISA to MR8/4 of extracts made by homogenising cysts of *G.pallida* and *G.rostochiensis* in a range of extraction solutions and reagents. Also shown are the results obtained after boiling extracts made by homogenising cysts in PBS.



In general, more antigen was detected from cysts of *G.pallida* than from those of *G.rostochiensis*, reflecting the greater specificity of the detecting antibody (MR8/4) for the former. Most extractants yielded less detectable antigen than the standard, PBS, but both CTAB and especially 1% urea extracted more antigen than PBS. Boiling for 10 minutes after extraction in PBS decreased the optical density readings obtained but differentiation of the two species was preserved. Few other treatments gave good antigen yields and many caused a loss of differentiation between the species.

Homogenization by hand gave excellent levels of antigen recovery but was slow and physically tiring. For these reasons, various physical and chemical procedures were tried as alternatives to hand homogenization. In Table 4, the effects of sonication and agitation with Ballotini beads are compared with homogenization and with extraction by sodium hypochlorite, reported as a possible extraction procedure by Haydock (1990).

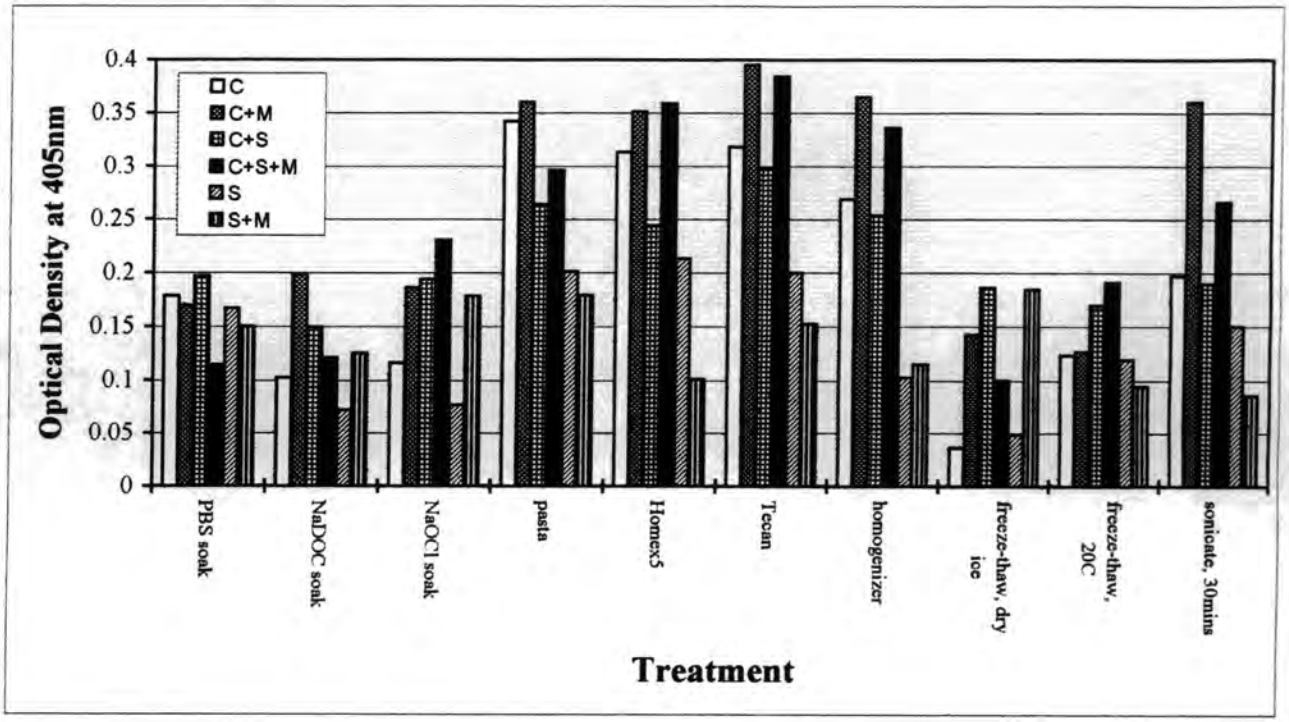
TABLE 4. Optical density values in indirect ELISA, using MR8/4, of a homogenate of whole cysts of *G.pallida,* an extract made by sonication of whole cysts, extracts made by disruption of cysts or eggs with Ballotini beads agitated in a vortex shaker, and an extract made by incubating cysts in sodium hypochlorite solution for 20 minutes.

	Optical Density
Homogenised cysts	0.830 +/- 0.040
Sonicated cysts	1.017 +/- 0.020
Cysts disruptured by beads	0.000
Eggs disruptured by beads	0.317 +/- 0.004
Cysts in sodium hypochlorite	0.201 +/- 0.060

The sonication procedure gave better antigen extraction from cysts than homogenisation, but extraction with sodium hypochlorite and disruption of either cysts or eggs with Ballotini beads gave poor yields of antigen, especially when beads were used on cysts.

Since practical use of immunoassays for PCN may require the operation to be carried out in the presence of contaminating "float" material (i.e. soil organic matter etc. which is extracted along with the cysts), further tests of physical antigen extraction procedures were made in the presence of soil extracts and Marvel ™ skimmed milk protein as a potential blocking agent for non-specific antibody binding. The results obtained are shown in Fig. 20.

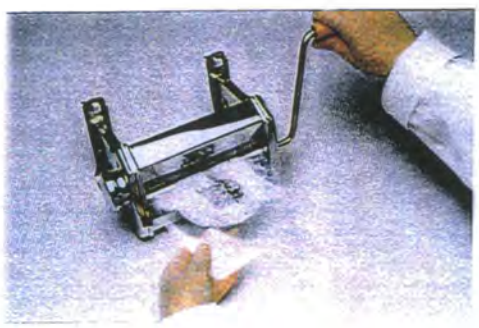
FIGURE 20. Antigenicity in Indirect ELISA, using MR8/4, of extracts of *G.pallida* cysts and soil "float" extract, made with a variety of extraction procedures in the presence or absence of Marvel. C = cysts; S = soil extract; M = Marvel.



When cysts alone were treated, a modified pasta rolling machine was the most effective extraction system. Other physical methods of antigen release, including hand homogenization and two powered blending machines, were also very effective. Freeze-thawing was poor and simply soaking the cysts in PBS, strong detergent (sodium deoxycholate), or sodium hypochlorite solution gave only slightly better recovery of antigen. Addition of soil extract generally decreased the optical density readings, presumably due to the antigen of interest becoming bound to the material in the soil extract and therefore unavailable for recognition by the Mab. Inclusion of Marvel restored the readings, presumably because it was able to block the binding sites on the soil extract. Most extraction procedures gave significant optical density readings from soil alone but, at least in the more efficient physical extraction processes, these readings were less when Marvel was included with the soil.

In the quest for simplicity of procedure and the possibility of eventual automation, it was felt that a process akin to the pasta machine offered the most potential. To crush samples with this machine, they were enclosed within a sealed plastic bag. A problem which arose was the tendency of the bag to split under pressure and this was overcome by cutting a spiral groove in the surface of one of the rollers. The machine is shown in operation in Fig. 21. Several passes through the machine proved to be necessary to achieve maximum antigen release and the alternative of crushing the sample contained within the plastic bag with a domestic rolling pin was eventually used extensively.

FIGURE 21. Pasta rolling machine adapted for crushing cyst samples or soil samples in a plastic bag.



4.2.1. Extraction of cysts from soil

Since the first problem to be addressed in recovering PCN antigen from soil is that of efficient cyst recovery, attempts were made to simplify and improve the current commonly used methods based on the Fenwick Can and, latterly, the Schuiling Centrifuge. Both of these are fairly labour-intensive to operate and would be difficult to automate. In a search for a simple, automatable alternative to these procedures, the magnesium sulphate solution flotation technique described in the methods was devised. The operation of this system is shown in Fig. 22. Normal use of the Fenwick Can and Schuiling Centrifuge requires that soil samples, and any cysts they contain, are dried before extraction. This is because they rely upon the cysts floating to achieve good extraction. Choice of an appropriate concentration of magnesium sulphate solution should allow cysts to be floated out of soil samples without prior drying. To check this possibility, dry or wet cysts were prepared and processed by the magnesium sulphate method, Fenwick Can or Schuiling centrifuge and the extraction efficiencies with flotation in plain water.

The results for air dried samples are in Fig. 23(a) and show that flotation in either medium and the Fenwick Can gave consistently close to 100% extraction whilst the Schuiling Centrifuge was less effective. When recovery of wet cysts from un-dried soil was attempted (Fig.23(b)), only flotation in magnesium sulphate solution retained close to 100% efficiency; flotation in water failed to recover any cysts and the two standard methods were hardly any better.

On the basis of the above results, magnesium sulphate flotation was used further in the assay validation tests reported later.

FIGURE 22. Operation of the magnesium sulphate flotation system for extraction of PCN cysts with minimum pre-treatment of soil samples and maximum efficiency of recovery. 1) Plastic bag containing soil sample and 30% magnesium sulphate solution, 2) division of sample into sealed upper section of bag in order to separate "float" from unwanted mineral matter, 3) top of bag flipped over to complete the separation of cysts from mineral matter, 4) appearance of sample after heat-sealing across centre of bag.

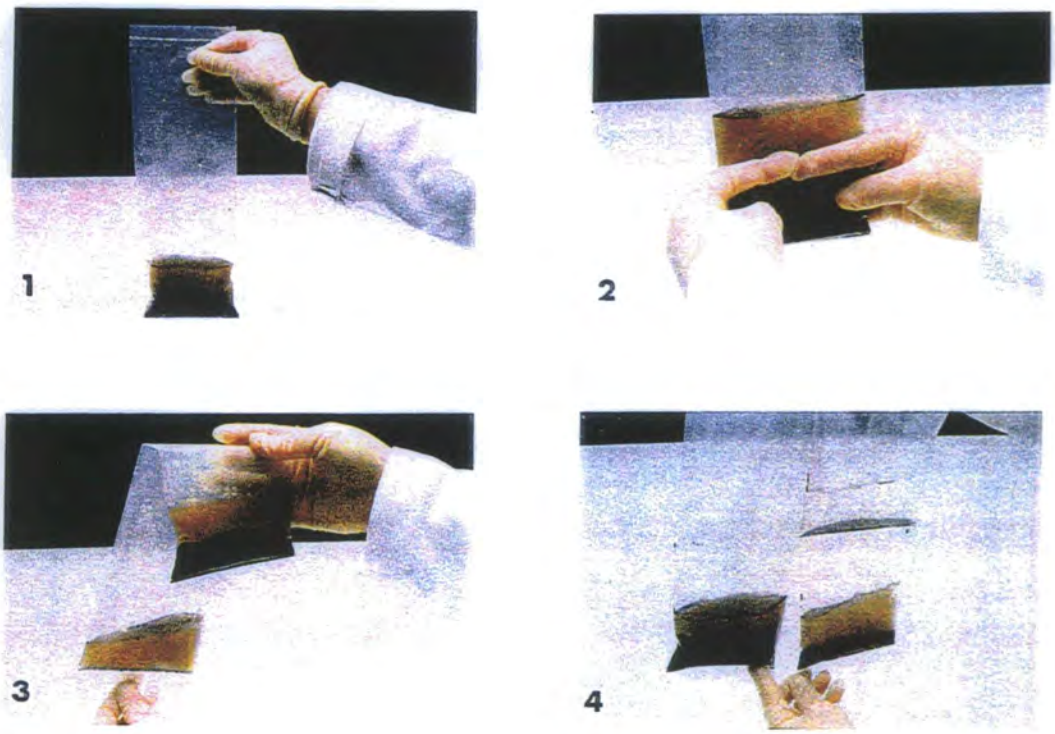
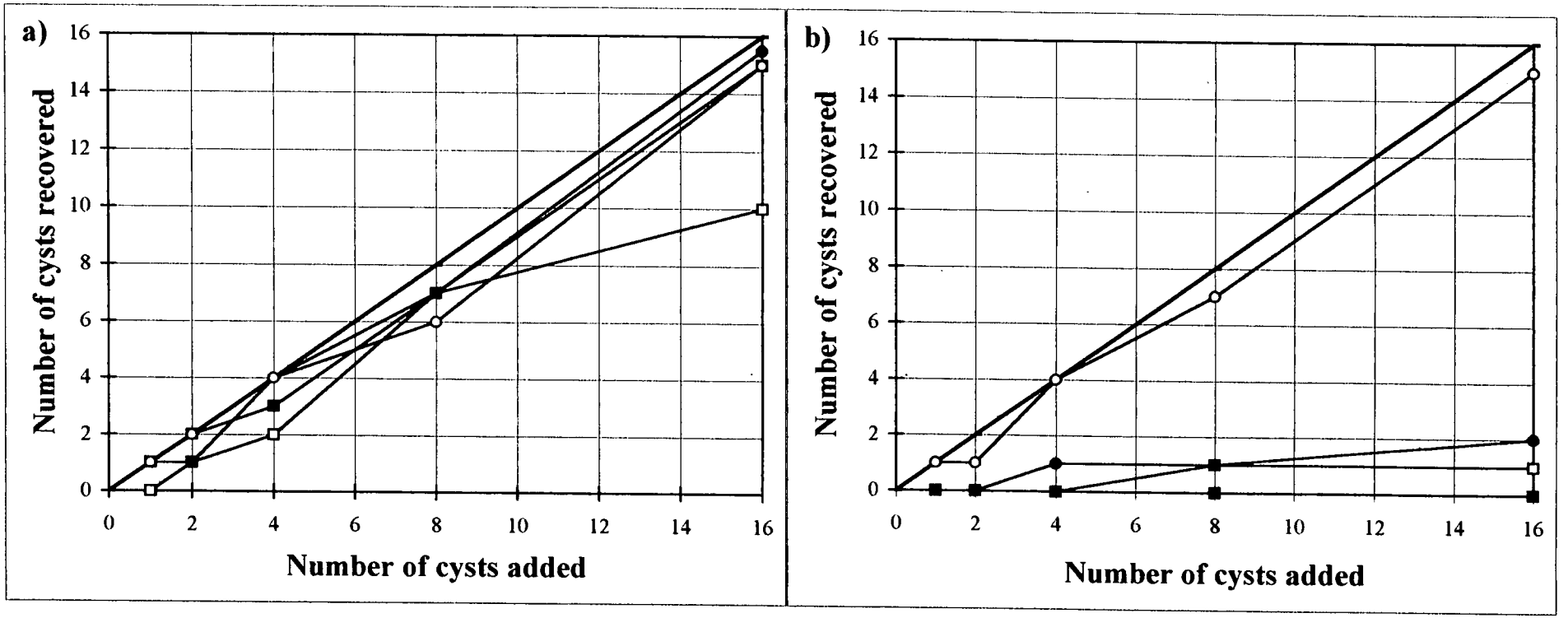


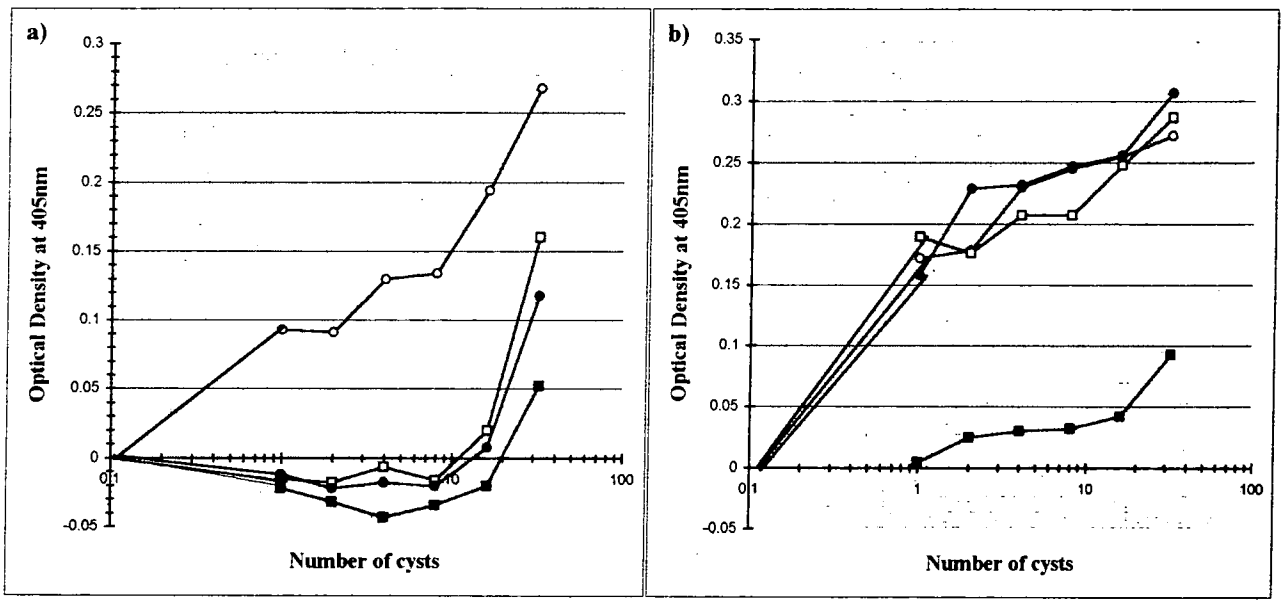
FIGURE 23. Recovery of a) air-dried PCN cysts and b) wet cysts from soil samles using -○- magnesium sulphate flotation, -●- Fenwick Can, -□- Schuiling Centrifuge, -■- flotation in water.



4.2.2 *Blocking of non-specific binding*

In order to investigate further the potential of skimmed milk powder (Marvel) to block non-specific binding sites on material contained in soil extracts, and therefore to maximise recovery of nematode antigen for subsequent ELISA, a series of soils of low medium and high organic matter content with a range of infestation levels of *G.pallida* were extracted by magnesium sulphate flotation. Antigen was released by passing the floats obtained through the pasta machine, sealed in a plastic bag in extraction buffer in the presence or absence of 10% Marvel. The results are in Fig.24.

FIGURE 24. Optical densities obtained in DAS-ELISA, using MR8/4, with extracts from -○- *G.pallida* cysts and extracts of floats obtained from soils of -●- low, -□- medium, and -■- high organic matter contents, infested with varying numbers of cysts of *G.pallida*. a) No Marvel added to extraction buffer, b) 10% Marvel added to extraction buffer.

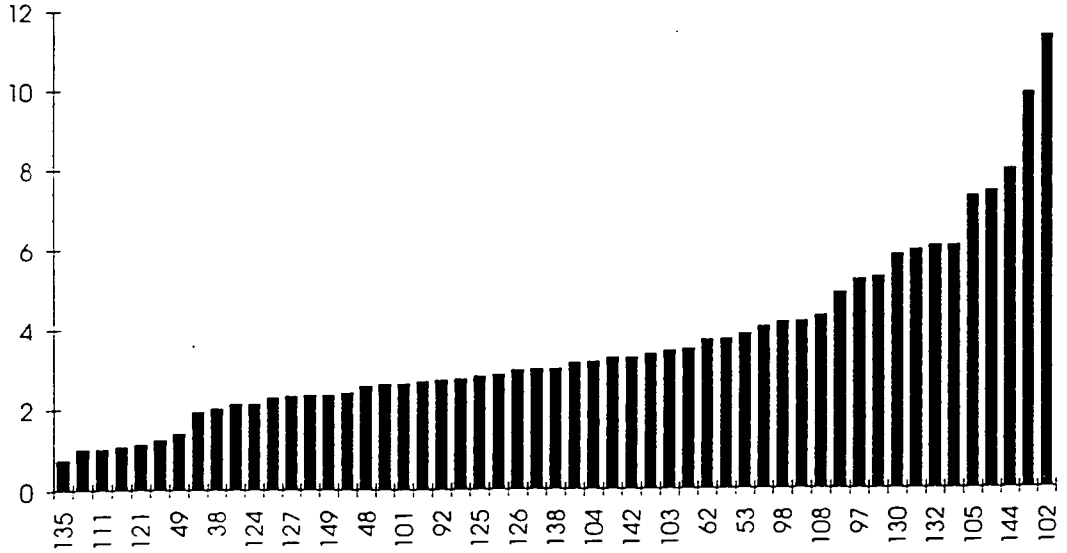


When no Marvel was added to the extraction buffer, a reading in the ELISA was only obtained at the highest population density of *G.pallida*, whatever the organic matter content of the soil. In the presence of Marvel, the ELISA gave similar readings to the clean cyst sample in all soils except that with the highest organic matter content and, even in this soil, higher ELISA readings than from the unblocked samples were obtained. Since the high organic matter soil was a 1:1 mixture of John Innes compost with white sand, the use of Marvel to block non-specific binding sites on soil extracts holds great promise, although it was not used routinely in subsequent tests.

4.2.3. Soil type range in proving tests

It was felt that the single most important characteristic of field soils in determining the success of an immunoassay for PCN would be the organic matter content. Since it was planned to conduct a large scale proving test on PCN-infested soils of northern Portugal, a survey was made of organic matter content variation across the region. From Fig. 25 it can be seen that organic matter content varied between less than 1% to about 12%.

FIGURE 25. Organic matter content of 27 soils taken across a PCN-infested region of northern Portugal.



4.2.4. The effects of soil type and organic matter content on PCN estimations

An extensive experiment was done to compare the efficiencies of recovery from soils composed of different parent rock materials and of different organic matter (OM) contents, using the magnesium sulphate flotation method or a Fenwick Can. The soil extracts so obtained were either used for immunoassay or the cysts simply picked out for routine egg counting.

From the survey of soil types noted above, it was also clear that the region concerned had soils which originated from two main parent rock types, namely schist and granite. In order to obtain soils of different parent rock type and a controlled range of OM contents it was decided to take natural soils of high and low contents within each parent rock type and mix them in a range of fixed proportions. The proportions used are listed in Table 5 and the mechanical analyses of the resulting mixtures are given in Table 6. OM content ranged from 0.83% to 6.67% in the soils of granite origin and from 0.26% to 3.62% in the soils of schist origin. Other important differences in particle size distribution are also shown in these tables.

Having created a range of soil types, a range of levels of infestation with PCN was created by adding 3, 9, or 18 cysts of *G.pallida* to 100g samples of each type of soil. This gave infestation levels corresponding to 15, 35 or 81 eggs per g of soil. Eight replicate samples of each soil type × infestation level combination were prepared, so that four samples could be processed by each extraction method. Of these four, two were used for standard cyst and egg counting and two were used for immunoassay.

TABLE 5. Proportions of different soils mixed to give a range of soil types.

Mixture	Parent rock	Low OM (%)	High OM (%)	OM level
G1	Granite	100	0	1
G2	Granite	75	25	2
G3	Granite	50	50	3
G4	Granite	25	75	4
G5	Granite	0	100	5
S1	Schist	100	0	1
S2	Schist	75	25	2
S3	Schist	50	50	3
S4	Schist	25	75	4
S5	Schist	0	100	5

TABLE 6. Mechanical analysis of soil mixtures (figures are percentages).

Mixture	C.sand	F.sand	Silt	Clay	OM (%)
G1	57.4	25.3	10.0	7.3	0.83
G2	55.2	27.0	10.7	7.2	2.29
G3	52.9	28.6	11.4	7.1	3.75
G4	50.7	30.3	12.1	7.0	5.21
G5	48.4	31.9	12.8	6.9	6.67
S1	8.1	44.4	38.5	9.0	0.26
S2	17.9	42.5	30.6	9.0	1.10
S3	27.7	40.6	22.7	9.1	1.94
S4	37.5	38.7	14.7	9.1	2.10
S5	47.3	36.8	6.8	9.1	3.62

Great effort was made to pick out all the cysts from the "floats" generated by the two extraction methods and the results are in Table 7.

TABLE 7. Percentage recovery of cysts by two extraction methods from ten soil types.

Mixture	Fenwick Can	Flotation	Mean
G1	100	93.5	96.8
G2	92.7	96.3	94.5
G3	100	96.3	98.2
G4	100	90.8	95.4
G5	100	91.7	95.8
S1	99.0	95.3	97.2
S2	100	96.2	98.1
S3	98.0	90.7	94.3
S4	97.2	91.7	94.4
S5	99.0	87.2	93.1
Mean	98.6	93.0	95.8

The ease of recovery of cysts was greatly influenced by the amount of material in each float and the average weights of the air-dried floats are given in Table 8, along with the average time taken to search the floats from each soil type for cysts.

TABLE 8. Mean weights (g) of soil extracts from the two extraction methods and the average time taken to search for cysts.

Mixture	Fenwick Can float weight	Flotation float wt.	Mean float weight	Average time (minutes)
G1	0.567	0.267	0.417	16.4
G2	0.567	0.350	0.458	24.2
G3	1.133	0.533	0.833	24.2
G4	1.267	0.700	0.983	19.0
G5	1.233	0.967	1.100	24.3
S1	0.267	0.250	0.258	10.7
S2	1.750	1.300	1.525	21.4
S3	2.933	2.317	2.625	31.3
S4	4.950	3.317	4.133	40.6
S5	7.750	4.583	6.167	50.8

Floats were heavier from soils of higher OM content and heavier from schist than from granite based soils. These trends in float weight and bulk were mirrored in the trend time taken to pick out the cysts. The increase in time to pick out the cysts was not only due to the amount of material in the float but also to the colour of that material - if it was in contrast to the colour of the cysts then the cysts were easier to see, and *vice versa*.

Both extraction methods were quite efficient, with better than 90% recovery. The Fenwick Can was slightly more efficient than flotation but tended to produce heavier floats, with consequent increase in cyst recovery time. The numbers of cysts recovered were not affected by the soil parent rock type but fewer were recovered the greater the soil OM content (not significantly, however). As expected, the level of PCN infestation had no effect on cyst recovery. Cyst recovery, expressed as an index (10 = 100%), is summarized for soil type, OM level and level of PCN infestation in Table 9.

TABLE 9. Index of cyst recovery (10 = 100%) from two parent soil types with five levels of organic matter and three different levels of PCN infestation.

	Granite				*Schist*			
OM level	3	9	18	Mean	3	9	18	Mean
1	10.0	9.5	9.6	9.7	10.0	10.0	9.3	9.8
2	9.2	9.5	9.7	9.5	10.0	10.0	9.4	9.8
3	10.0	9.7	9.7	9.8	9.2	9.7	9.4	9.4
4	9.2	10.0	9.5	9.6	9.2	9.5	9.7	9.5
5	9.2	9.7	9.9	9.6	9.2	9.7	9.0	9.3
Mean	9.5	9.7	9.7	9.6	9.5	9.8	9.4	9.6
Grand Mean								9.6

Although there were effects of treatments on cysts recovery, there seemed to be no consistent effects of treatment on the numbers of eggs found in the cysts that were recovered.

For the immunoassay of the duplicate series of samples, the floats were sealed in 500 gauge plastic bags and soaked overnight in 2 ml of PBS, pH 7.4. Antigen was extracted not with the pasta machine but by crushing the float in the bag with a domestic rolling pin. The samples were then centrifuged at 300 rpm for 5 minutes in a Beckman centrifuge equipped with a blood bag adapter. The amount of *G.pallida* antigen in each sample was quantified by ELISA using MR8/4. Some samples failed to give readings. For this reason, the results are summarized in the form of a series of correlation coefficients, wherein the correlations between immunoassay results and standard counts within soil types and extraction procedures are calculated separately (see Table 10).

The best correlation was obtained with the soil samples of granite rock type, lowest OM content and after extraction by the flotation technique (correlation coefficient = 0.99206 in Table 10). Within samples G1 to G5 extracted by flotation, as OM content increased so the correlation between the immunoassay results and standard egg count decreased. This effect is presumably due to the non-specific binding of PCN antigen by the organic matter in the float material so that, in the samples derived from soils of high OM content, most of the antigen is bound as soon as it is released and is therefore unavailable for recognition by the Mab in the ELISA. The estimate of PCN population density is therefore low, and the correlation of the results obtained by the two different methods is poor. The poor (i.e. totally non-significant and apparently random) correlations between counts from the two methods for samples G1 to G5 extracted by Fenwick Can is presumably due to the greater amount of material in the floats from the Fenwick Can method (see Table 8) and the consequently greater opportunities for non-specific binding. Samples S1 to S5, from the soils of schist parent rock, yielded even greater amounts of float material after both extraction methods, despite having a lower range of OM contents (see Table 6), and this may have been due to carryover of clay and silt particles with the float material. The schist soils had greater clay and silt contents than the granite soils (Table 6) and these particles have greater binding capacities than the sand particles dominant in the granite soils. The result of this is that an even greater proportion of nematode antigen was lost immediately after release due to non-specific binding to material in the floats. The effect is reflected in the total absence of readings in the ELISA for a number of the schist samples (Table 10).

TABLE 10. Correlation coefficient and levels of probability for comparisons between immunoassay estimates and standard counts of *G.pallida* population densities in different soil mixtures subjected to Fenwick Can or magnesium sulphate flotation extraction procedures.

Mixture	Fenwick Can		Flotation	
	Correlation	*P* level	Correlation	*P* level
G1	-0.02315	> 0.1	0.99206	0.001
G2	-0.15286	>0.1	0.84181	>0.05
G3	0.68330	>0.1	0.90725	>0.02
G4	0.03097	>0.1	0.42386	>0.1
G5	-0.04921	>0.1	0.39078	>0.1
S1	0.55097	>0.1	0.13325	>0.1
S2	-0.56577	>0.1	-0.32949	>0.1
S3	-	-	-0.03307	>0.1
S4	-	-	-	-
S5	-	-	-	-

4.3. Field Fumigation Trial

Globodera pallida was the species of PCN detected as only MR8/4 produced positive optical density values. Figure 26 depicts the mean number of eggs/g of soil from the pairs of sub-samples processed by ELISA or the traditional method. The number of eggs/g of varied between samples (and in some cases, sub-samples) and sites. Site B had lower egg counts than those from site A - a mean of 2.2 eggs/g in site B samples whereas those from site A had approximately nine times as many: a mean number of 19 eggs/g of soil (Traditional results).

In general, the values from the Traditional and ELISA techniques showed some similarity - with slightly fewer eggs detected by ELISA. Some samples, however, (e.g. A8) showed an increasing disparity between the two methods over the 26 day period.

The first nine graphs in Figures 26 & 27 are a comparison of a) the two techniques and b) the population density between the two sites. To assess the effect of the fumigant over time the ELISA results were examined in isolation as the Mabs only detect live eggs.

Figures 28 & 29 show the percent viable eggs (ELISA count ÷ traditional count) on each sampling occasion during the sampling period. On average 80% of the untreated eggs from site A were viable but samples taken from fumigated strips on A showed a 15% decrease in egg viability over the 26 days from initial treatment. Site B results showed a less obvious relationship. Cysts from untreated samples contained eggs that varied in viability from 60 to 82 %. Treated samples had a minimum of 72% and a maximum of 99% viable eggs, with no observable trend over the sampling period.

The root invasion test results (Figures 30a & 30b), carried out to confirm the egg viability results, showed that juveniles from cysts taken from each field sample were capable of hatching, invading and developing inside potato roots. The actual number of juveniles (J) that invaded, though, varied in both untreated and treated samples. Untreated A samples decreased from 100 to 50 J/g of root and untreated B samples

from 30 to 10 J/g of root. Those from treated samples also decreased; from 99 to 10 J/g of root in site A and 38 to 9 J/g of root in site B. It is interesting to note that there was less invading nematodes in B than in A untreated samples (which relates well to the egg counts, Fig.'s 26 & 27) but the samples taken later from treated strips in A had cysts that contained less or almost the same number of invading juveniles to treated B samples - a trend also seen in Figure 29 which depicts egg viability over the sampling period.

Figures 31a & 31b show the correlation between traditional and ELISA counts and the results of linear regression analysis. As the Mabs used in the ELISA detect only viable eggs, whereas the traditional method is non-discriminatory, it should be possible to determine the effect of the fumigant over time (as in percent viable eggs, Figures 28 & 29). If the fumigant killed the eggs, there should be a change in the regressions between the two methods over the sampling period. This does appear to happen on both sites but, as with the bar charts (Figures 26 & 27), the number of egg/g was highly variable.

1,3-dichloropropene (trade name Telone II) is a volatile halogenated aliphatic hydrocarbon which belongs to the 'True Nematicide' type of fumigant, as it is not a general biocide at the rates used. As with any other nematicide, 1,3-D needs to persist in the soil to be effective, so, as it is a gas, the soil surface must be sealed. The soil in this field was a clay/loam with greater clay content in the area of site B. Poor conditions at the time of treatment left the soil with numerous clods such that the surface could be only poorly sealed, especially noted at site B. The clayey nature also meant that diffusion of the gas would be blocked as clays are characteristically impervious, often with a high moisture content. The many small particles with large surface areas and small pore spaces result in sorption of the fumigant onto the soil, and any remaining gas molecules that do dissolve in the soil water remain relatively static.

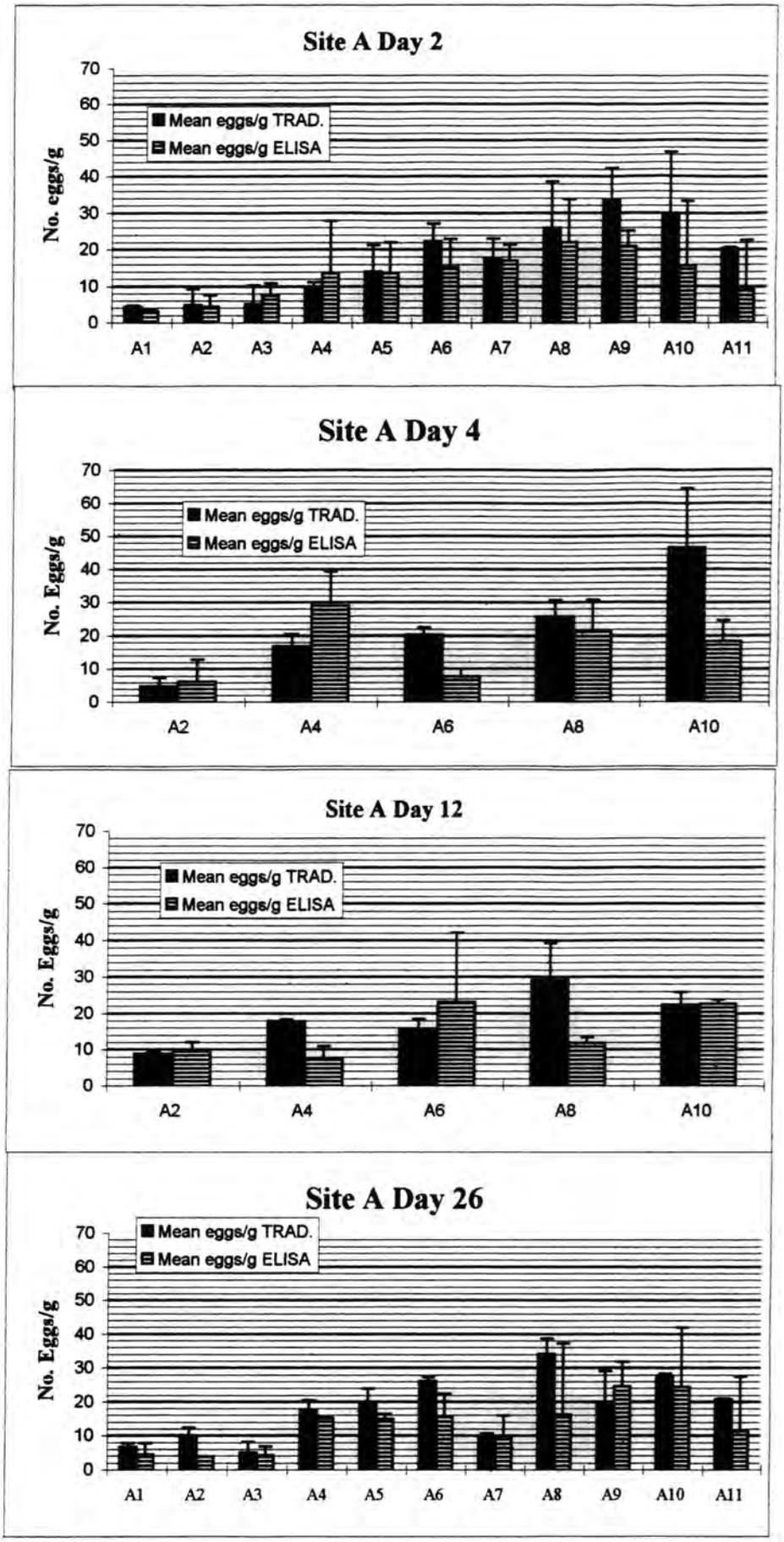
Thus, inefficient control of PCN at Nocton Rise was the result of inadequate exposure to the chemical at a high enough concentration for a sufficient length of time.

Vaporization to the atmosphere and adsorption by the clay particles were probably the main factors contributing to this effect.

The results also show that the distribution of this pest in this field trial was patchy both between and within plots in the same field. This is a characteristic of this species as regular or uniform distribution is rare (Boag and Neilson, 1994). Many physical, biological and agronomic factors contribute to this uneven horizontal distribution, e.g. soil type, cropping history (potatoes were last grown in this field five years ago), egg deposition, relative pathogenicity, root distribution, response to microclimates, interaction with natural enemies, agricultural practices, etc.(McSorley, 1987). But at the same time it appears that an immunoassay (ELISA) could be the ideal diagnostic tool, for not only has it assessed the efficiency of the fumigant 1,3-D but has analysed the nematode distribution in the field on only four, 96-well, microtitre plates in (theoretically) less than five hours.

FIGURE 26.

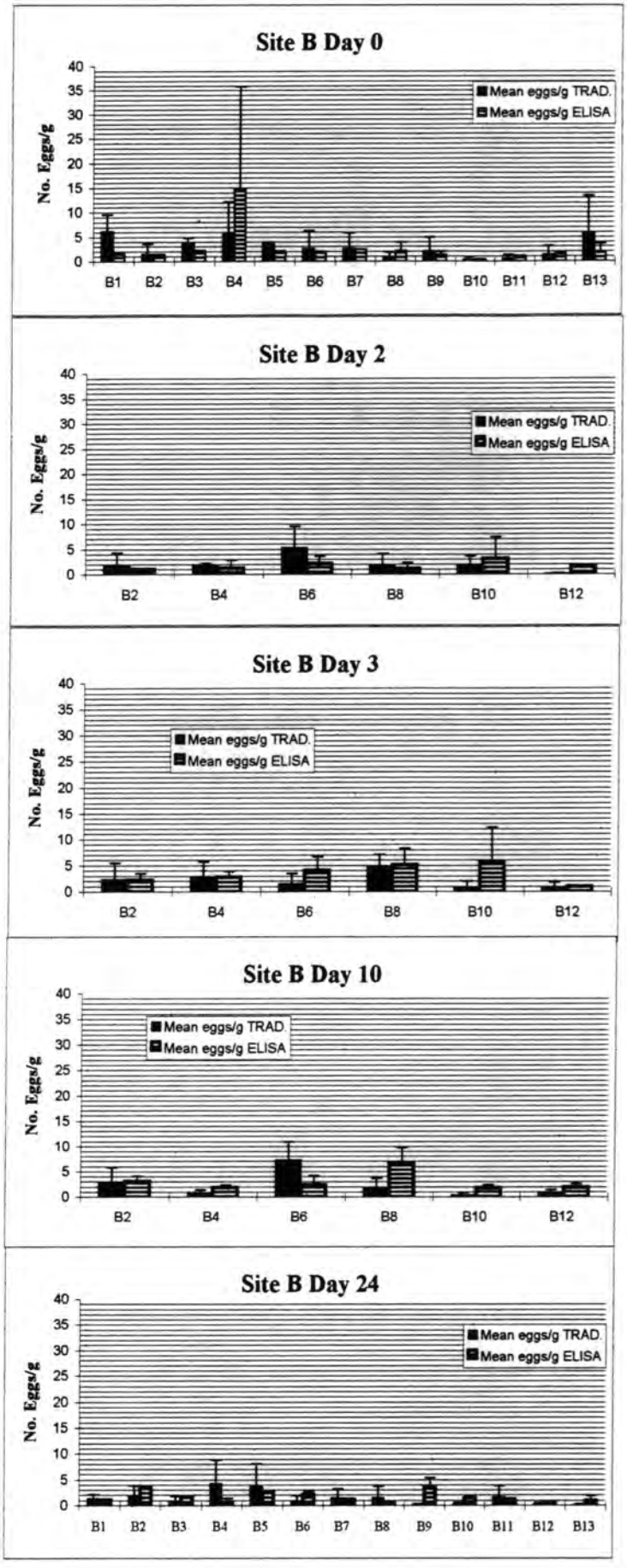
Comparison Between the Traditional (TRAD) and ELISA results on Site A



Even numbered samples are fumigated
Odd numbered samples are unfumigated

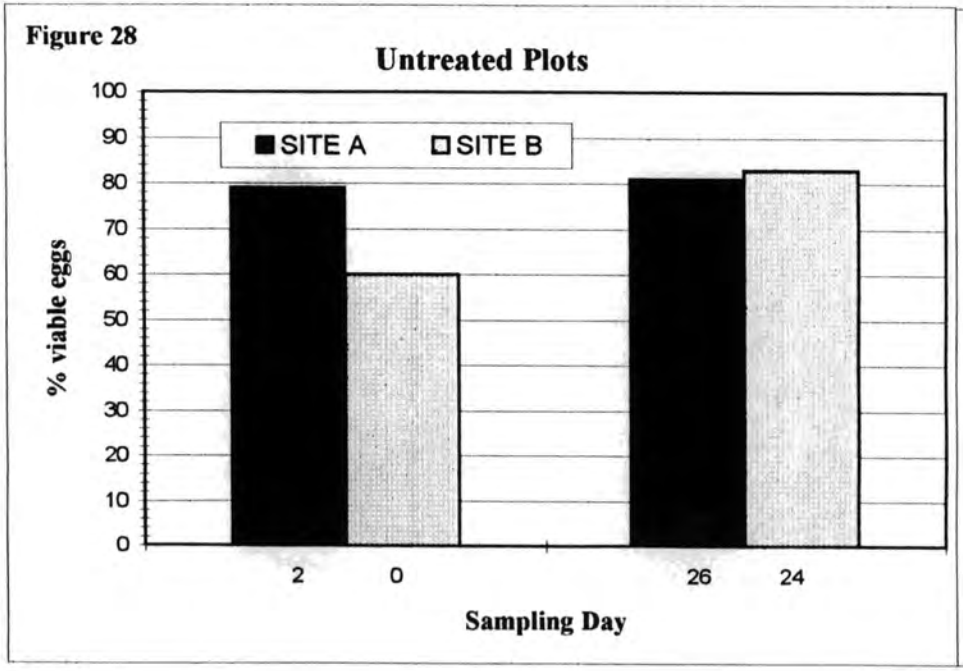
FIGURE 27.

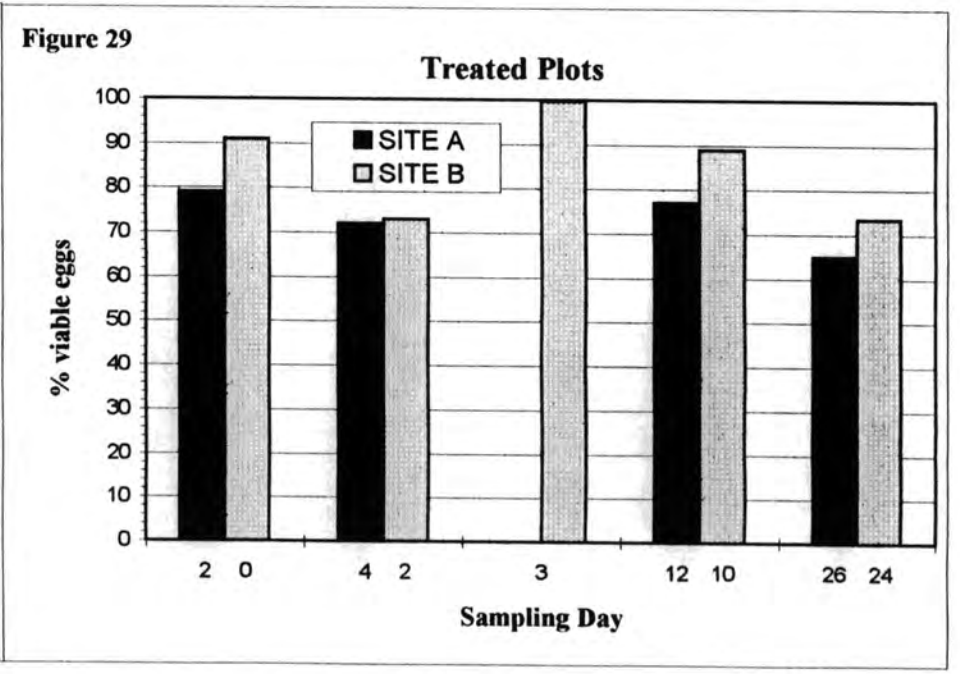
Comparison Between the Traditional (TRAD) and ELISA results on Site B



Even numbered samples are fumigated
Odd numbered samples are unfumigated

Percent viable PCN eggs on Sites A and B over time





The average number of nematodes that invaded roots of greenhouse-grown potato plants from each sample

Figure 30a

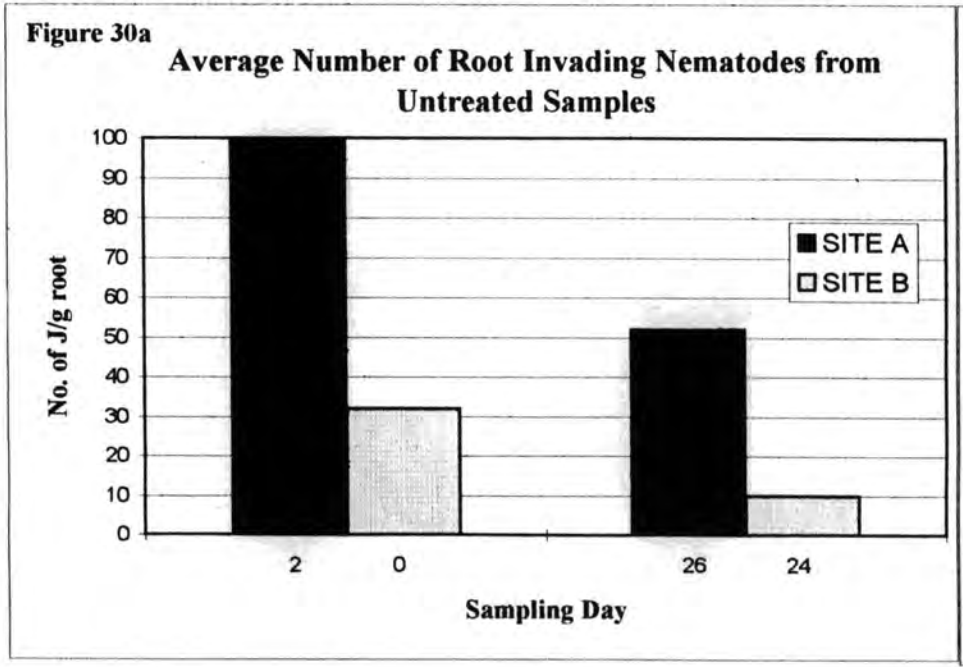
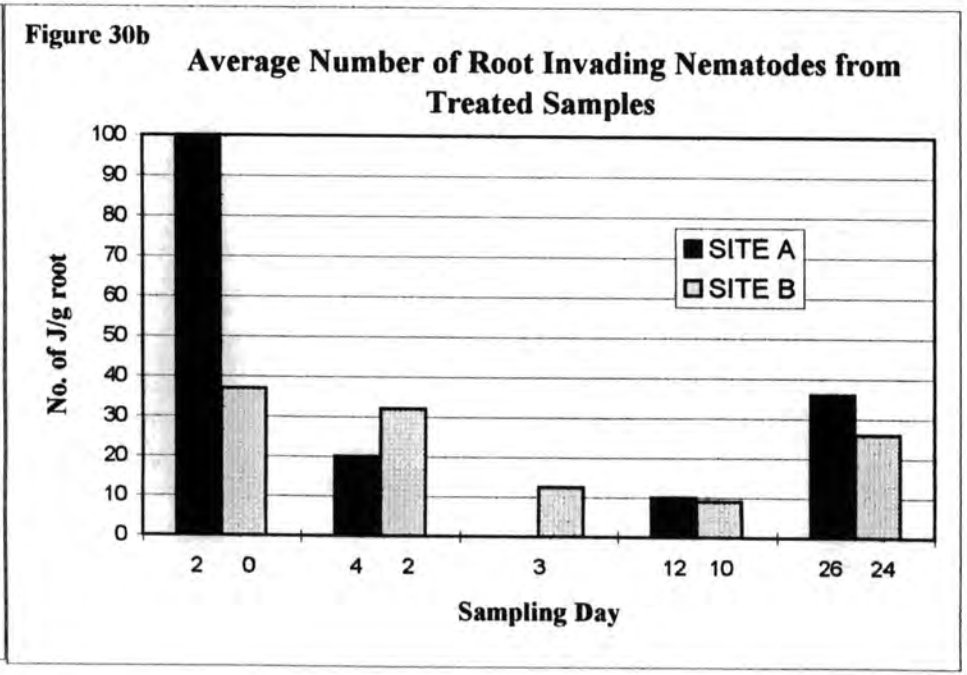


Figure 30b

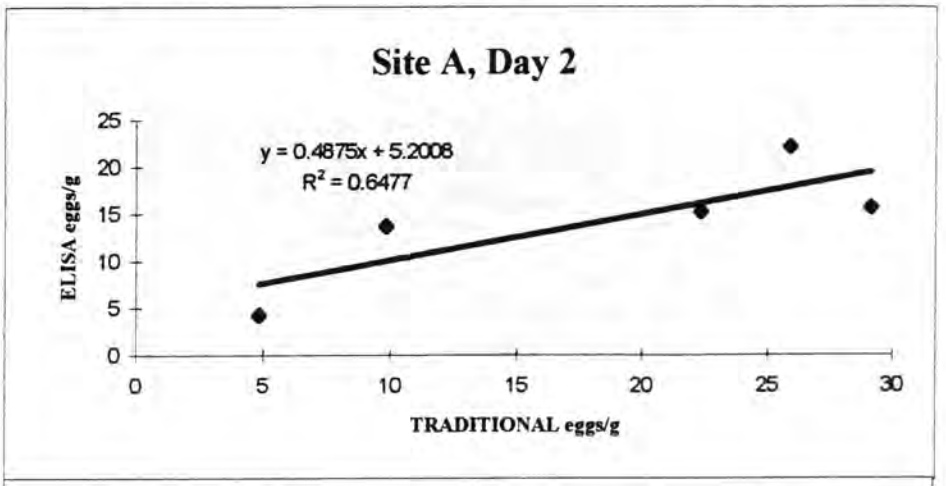


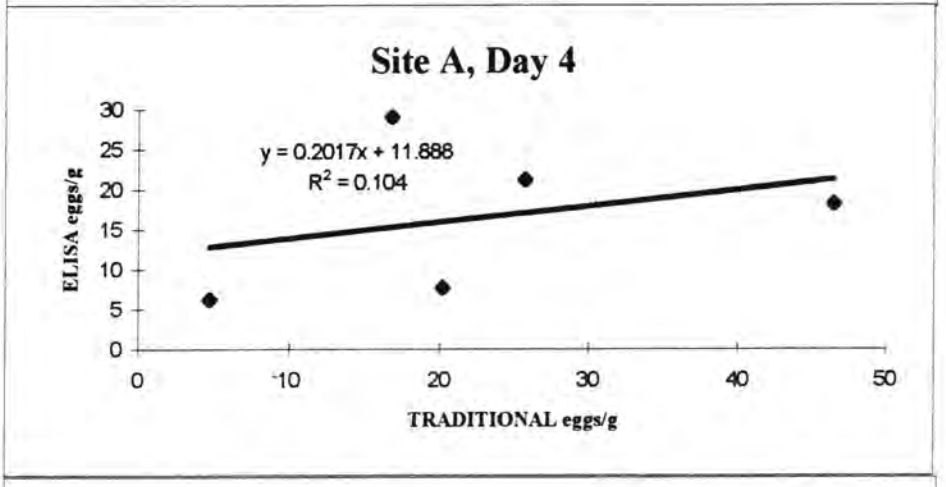
KEY:

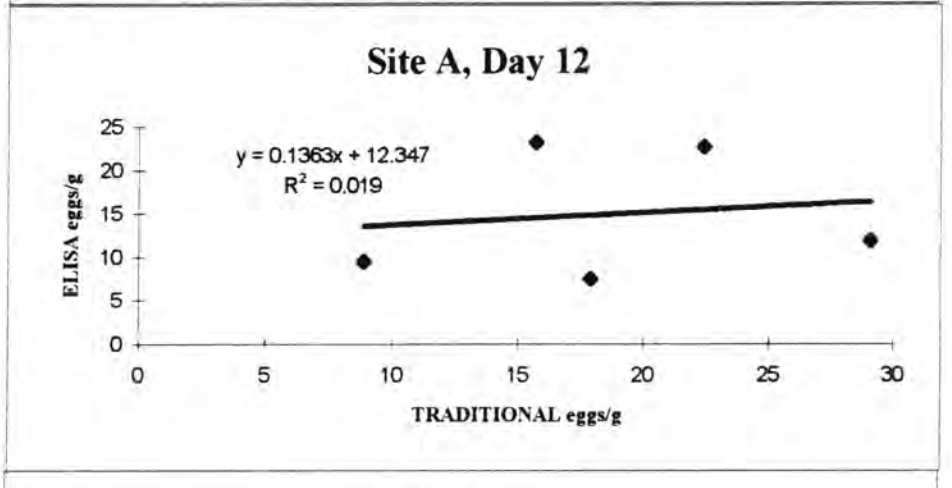
J/g of root :- number of juveniles per gram of root

FIGURE 31a.

LINEAR REGRESSIONS BETWEEN THE TRADITIONAL AND ELISA ESTIMATED MEAN NO. OF EGGS/G ON THE FUMIGATED SAMPLES, SITE A







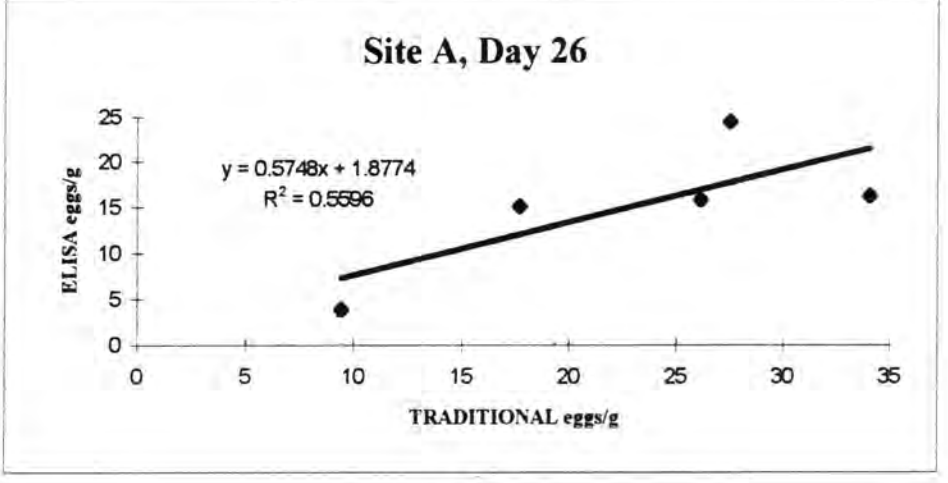
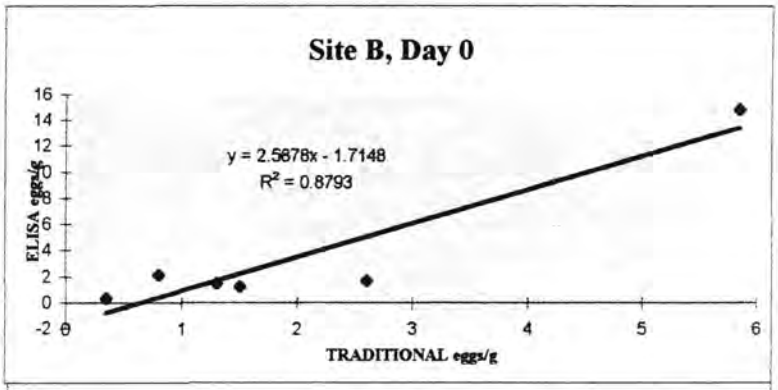
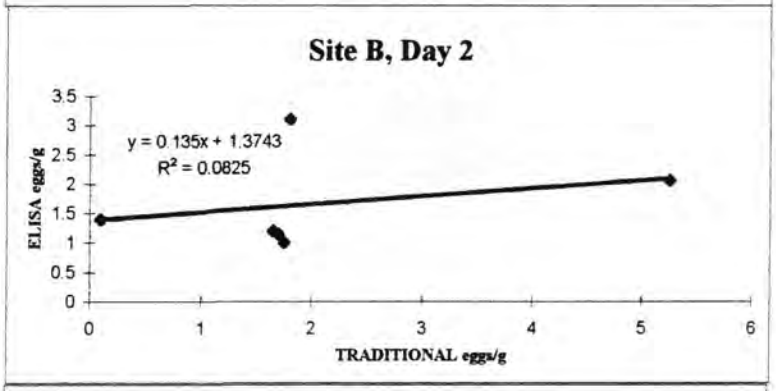
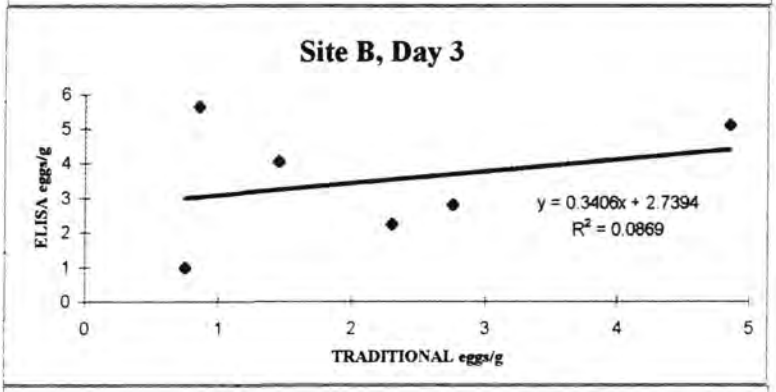


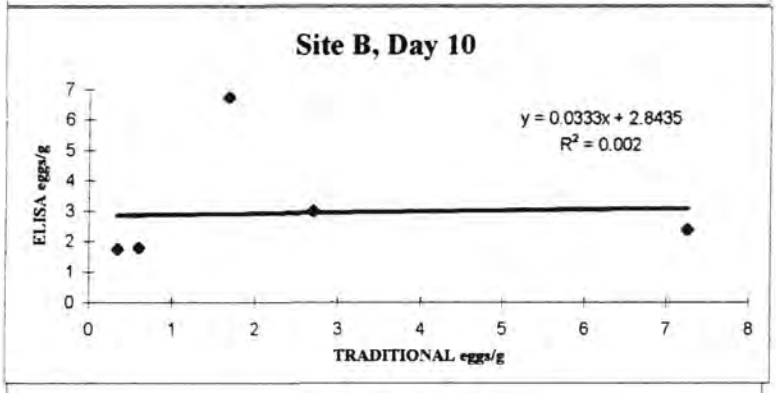
FIGURE 31b.

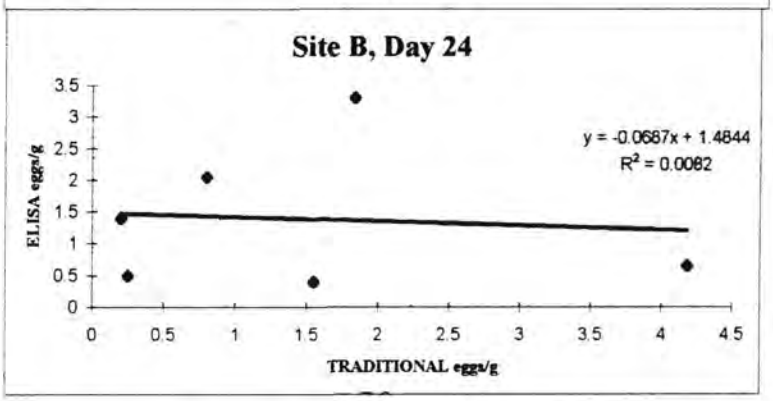
LINEAR REGRESSIONS BETWEEN THE TRADITIONAL AND ELISA ESTIMATED MEAN NO. OF EGGS/G ON THE FUMIGATED SAMPLES, SITE B







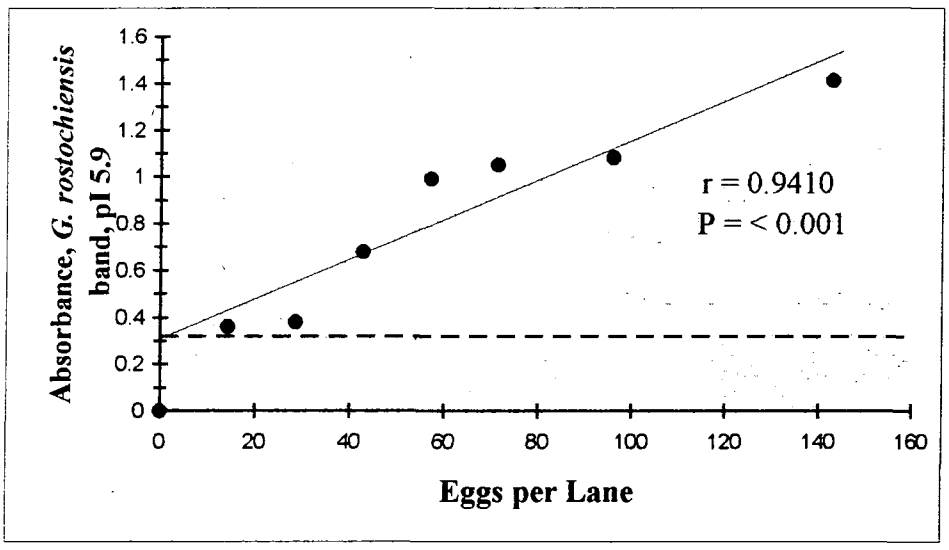




5. DISCUSSION

The currently used procedure for identification and quantification of the two species of PCN for advisory and management purposes depends ultimately on the determination of the relative proportions of the two species in a mixed field population by use of isolectric focusing. Combined with cyst and egg counts made on samples processed by the Fenwick Can, IEF gives an acceptably accurate estimate of the actual population densities of the two species. Use of an optical densitometer to measure the density of critical species specific IEF bands would perhaps be a way forward to semi-automate and, therefore, speed up the counting process but such a system appeared not to have the required sensitivity even when used with the most sensitive protein staining procedures in an automated IEF system (LKB Pharmacia Phast-system). A good correlation between optical density of species-specific bands and population density was obtained (see Fig. 32) but the required sensitivity was lacking.

FIGURE 32. Correlation between optical density of silver stained species specific IEF band (pI 5.9) of *G.rostochiensis* and number of eggs per lane added to gel.



When the species-specific band density was twice the background optical density of the gel, there were 37 eggs per lane. With the particular procedure used, this translates to a detection limit of 15.2 eggs per g of soil. This is too high to be useful, so automated processing of PCN samples will require a different approach. The approach described, of using an immunoassay, will give that sensitivity and this has been calculated at considerably less than 1 egg per g of soil, a population density range where sampling technology becomes more important than the accuracy of the count performed.

The use of the species-specific proteins of pI's 5.7 and 5.9 for recognition by the Mabs chosen for the immunoassay was fortuitous rather than deliberate but has the added value of providing continuity with earlier and existing procedures. Choice of good differential Mabs was naturally followed by some characterization of the antigens that they recognise. It was quickly shown that both proteins had the same molecular weight of 34 kD (Robinson *et al.*,1993) and it was possible to surmise that the proteins were probably very similar in the two species, with similar functions, occurring in similar concentrations and therefore being equally easy to release from the two species. All of these characters are ideal characters for antigens which are to be used for differential recognition and quantification of two closely-related species.

Immunofluorescence studies showed that the antigens recognised by the chosen differential Mabs resided in the amphids of the second stage juveniles and also that a considerable proportion of the total amount found in an egg was likely to be in the vitelline fluid. This confirmed just how easily antigen could be released from whole eggs. Further, antigenicity studies had shown that only live, and not dead, eggs were antigenic. This is a particularly important quality for an immunoassay aimed at assessing the risk of crop damage from soil populations of PCN. Further studies on the antigenicity of eggs killed by different agents would be particularly relevant.

Having identified such important differential proteins, preparations were made of them and used for the production of monospecific polyclonal antisera. These proved to have no differential recognition characteristics but along with other Pabs produced to whole

body homogenates of juveniles, were very useful in the development of sandwich ELISA's with the correct combination of sensitivity and differentiation. The attempts to simplify ELISA procedure by direct conjugation of the Mabs to enzymes were disappointing in that they caused loss of differentiation and sensitivity, but alternative indirect ELISA procedures proved quite adequate.

The potential specificity of Mabs for pathotypes of PCN was explored, and it proved possible to recognise six of the eight pathotypes listed in the so-called "international" pathotype scheme. As yet, due to shortage of time, this has not been followed up further. Other Mabs had the potential to recognise the genus *Globodera* specifically and yet others appeared to recognise PCN specifically. Both types would have potential uses in quarantine situations. An alternative to selecting a PCN specific antibody would be, of course, to mix the chosen species-specific Mabs MR8/4 and MR8/5.

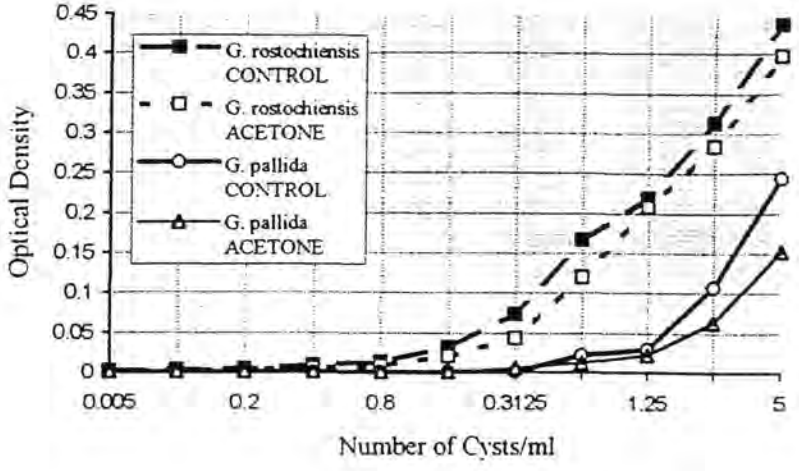
It was fortunate that there were no cross-reaction problems between the diagnostic Mabs and species of soil nematodes other than PCN. There was significant cross-reaction with *Globodera tabacum* but, since the sub-species occur only in the New World, this is not a problem for the practical operation of an immunoassay.

Although Western blots indicated some cross-reactivity between the relevant antigens when the two species were probed with both diagnostic Mabs, this proved not to be important when assaying mixtures of species in verification tests.

The extraction of cysts from soil samples by flotation in magnesium sulphate solution in plastic bags was probably the most innovative development made in the project and is a procedure which should be readily automatable. Also, it provided the cleanest and most usable extracts from the field samples in the Portuguese verification trial. However, the prototype immunoassay procedure only worked well with soils of quite low organic matter content. This means that an extra step will be required in the procedure. This could take the form of appropriate blocking, for which it has already been shown that skimmed milk powder has some potential and which might be

increased further if protease inhibitors were also included in the extraction buffer. Alternatively, the offending material (i.e. organic matter and fine soil particles) could be taken out of the samples by the extra procedural step of flotation in acetone. In preliminary tests, such a step had only a small effect on the sensitivity of recognition of the diagnostic antigens, a disadvantage which would be greatly outweighed by elimination of antigen loss by a non-specific binding to soil organic matter or clay mineral particles (see Fig. 33).

FIGURE 33. Recognition of *G.rostochiensis* and *G.pallida* by species-specific Mabs before and after treatment with acetone.

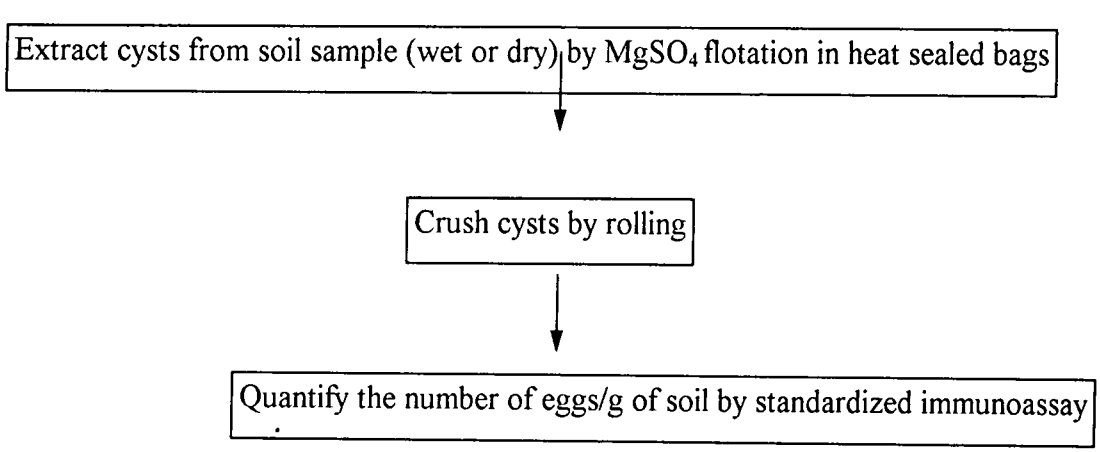


The initial promise of the pasta machine for antigen extraction was not fulfilled despite modification of the rollers to prevent sample bag splitting. This was because the machine could not be adjusted to give a small enough clearance between the rollers to ensure complete crushing of samples. Sufficient crushing could undoubtedly be achieved with purpose made rollers but, for the low volume of samples processed in this initial proving test, a domestic rolling pin gave perfectly satisfactory sample crushing and antigen release.

Direct extraction from soil of the antigens required for PCN quantification is obviously the most attractive eventual target but, to release this objective, the use of blocking agents, particularly on organic soils, requires fuller investigation. It is possible that an acetone flotation procedure could be made to solve all the problems. Further tests have shown that the addition of this step reduces OM from a number of different field soil types to a similar amount, thus making quantification reproducibility, by the use of known standards, a much more amenable task.

One possible agent for direct extraction of PCN antigens from soil is sodium hypochlorite, as it has already been shown that this will break down PCN cysts and yield antigenic products capable of recognition by polyclonal antisera raised against PCN (Haydock, 1990). It may be possible to base an assay on this extraction procedure and to make suitable Mabs by raising them to sodium hypochlorite extracts of PCN.

Potato growers today require a single diagnostic system that positively identifies only live PCN. This would obviate the use of prophylactic agrochemical treatments and improve the targeting and efficiency of those that were needed, following positive diagnosis. For routine use, such a diagnostic system needs to be cheap, easy to use and to provide clear, uncomplicated, totally reliable results. An immunoassay could be the reality.



Precision agriculture is likely to have a significant impact on crop protection practices over the next 10 years, especially now with the availability of global positioning systems (GPS). This advent means that the farmer can pinpoint accurately his position in the field; and with field data collected not only by conventional methods but be remote sensing by satellite, the data could be transformed by the farm's computer, digatilized by the farm equipment and then the appropriate application rates applied using the latest variable application technology. Precision agriculture will allow farmers to use their inputs in the most cost effective way, and will be an important component of practical Integrated Crop Management (ICM).

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APPENDIX

DETAILS OF INDIRECT ELISA (USING ALKALINE PHOSPHATASE)

1. **Sample Preparation:** Extract nematode antigen as required. Dispense 50µl of antigen per well.

2. **Incubation:** Leave plates covered overnight at 4^0C.

3. **Washing**: Samples and solutions should be washed from the wells by first flicking the samples from the wells while the plate is held upside down over a sink. Flood the wells with PBST. Repeat this twice more allowing the plates to stand for three minutes between each wash, at room temperature. Pat the plates dry with paper towelling.

4. **Block:** Excess binding sites on the plate to which the sample components are bound should be blocked by a 5% (w/v) solution of Marvel dried milk powder in PBST, using 100 µl/well.

5. **Incubation:** Leave plates to shake at room temperature, on the electronic plate shaker, for 30 minutes at 300/min.

6. **Washing:** Repeat procedure 2.

7. **Antibody preparation:** 50 µl of MR8/4.15.1 (17/1/94) [1:3200] - *G. pallida* specific MAB; 50 µl of MR8/5 (21/10/94) [1:2000] - *G. rostochiensis* specific MAB; diluted in PBST.

8. **Incubation:** Leave plates to shake on the electronic plate shaker at 33^0C for 2 hours at 300/min.

9. **Washing:** Repeat procedure 2.

10. **Enzyme Conjugate:** Dispense 50 µl/well of SIGMA Goat α-Rat IgG (whole) AP [1:1000] (diluted in PBST).

11. **Incubation:** Leave plates to shake on the electronic plate shaker at 33^0C for 50 minutes at 300/min.

12. **Washing:** Repeat procedure 2.

13. **Substrate preparation for alkaline phosphatase:** 1ml of diethanolamine buffer (5×) was mixed with 4ml of distilled water. Add one tablet of *p*-NPP and mix thoroughly to dissolve tablet. Warm solution to room temperature before dispensing 100 µl/well.

14. **Test result evaluation:** Leave plates in the dark at room temperature for 60 minutes then read on a plate reader at 405nm.

APPENDIX 2

NEMATODE CULTURE

Routine

For routine maintenance of cyst nematode populations, multiplication was carried out in 10 cm plastic pots filled with a steam-sterilized loam soil. The Desiree (for *G. rostochiensis*) and Cara (for *G. pallida*) were grown in the pots form single sprouts, cut from seed tubers kept at 10^0C in diffuse light. Nematode inoculum was in the form of cysts (30 per pot) added at the time of planting. Pots were kept in a glasshouse with natural lighting, a daytime maximum temperature of 25^0C and a night-time maximum temperature of 15^0C. The host plants were grown for a period of 12 weeks before watering was withheld. When the soil was dry, it was sieved and the new cysts extracted by Fenwick Can. Cyst stocks were held in a refrigerator at 4^0C.

Hatching of PCN juveniles

For certain purposes, a nematode inoculum of freshly-hatched juveniles was required. To obtain these juveniles, cysts were placed on a 10 cm diameter sieve made of polyester voile (aperture sieve *c.* 100 μm) stretched across a perspex ring. The sieve was placed in a shallow dish with the cysts just submerged in potato root diffusate. This diffusate was collected by growing Desiree potato plants in pots for *c.* 4 weeks before leaching the pot with water and collecting the leachate. Each 10 cm pot was leached with some 50 ml of water. The diffusate was filtered before storing it at 4^0C. For hatching, the diffusate was diluted 1:4 with water.