

PT346
Identification of potato cyst nematode
pathotypes

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Agriculture Victoria



Know-how for Horticulture™

PT346

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INDUSTRY SUMMARY

This report details research undertaken at Plant Sciences & Biotechnology, La Trobe University, Bundoora and RMIT, Melbourne on developing methods to aid in the differentiation of biological variants or pathotypes within the potato cyst nematode (PCN) species complex. This work is of direct relevance to the potato industry in Australia as PCN is a quarantine soil borne pest of potatoes which has now been detected in two Australian states, including Victoria, the largest potato producing state.

The best strategy for control of PCN is to plant resistant potato cultivars, combined with long crop rotation schedules. At present there is good resistance to some pathotypes but the majority of pathotypes cannot be controlled adequately by potato resistant genes. If accurate advice can be given to farmers and quarantine officers on the pathotype present then resistant cultivars can be planted with confidence.

Nematode identification is difficult and traditionally based on taxonomy. However, many important biological differences relating to host range and pathogenicity cannot be determined by morphology. Biochemical analyses of nematode populations of potato cyst nematode were used to distinguish differences that may be useful for characterising nematode populations. Electrophoretic techniques such as polyacrylamide gel electrophoresis, isoelectric focusing, isozyme analysis and high performance capillary electrophoresis showed reproducible differences that aid the identification of nematode genera, species and subspecific variants.

Methods based on protein analysis of nematodes are outlined that were used to determine the species of potato cyst nematode present in Victorian infestations. Cysts from Victorian properties were tested at Rothamsted, UK on a standard set of differential cultivars containing different resistance genes. A new method relying on the microanalytical preparative technique of high performance capillary electrophoresis was developed to aid the differentiation of pathotypes of PCN. After analysis of 1,000's of cysts the Victorian PCN samples were identified as *Globodera rostochiensis* Ro1 pathotype. This pathotype is controlled by the H1 gene in the resistant potato cultivar Atlantic.

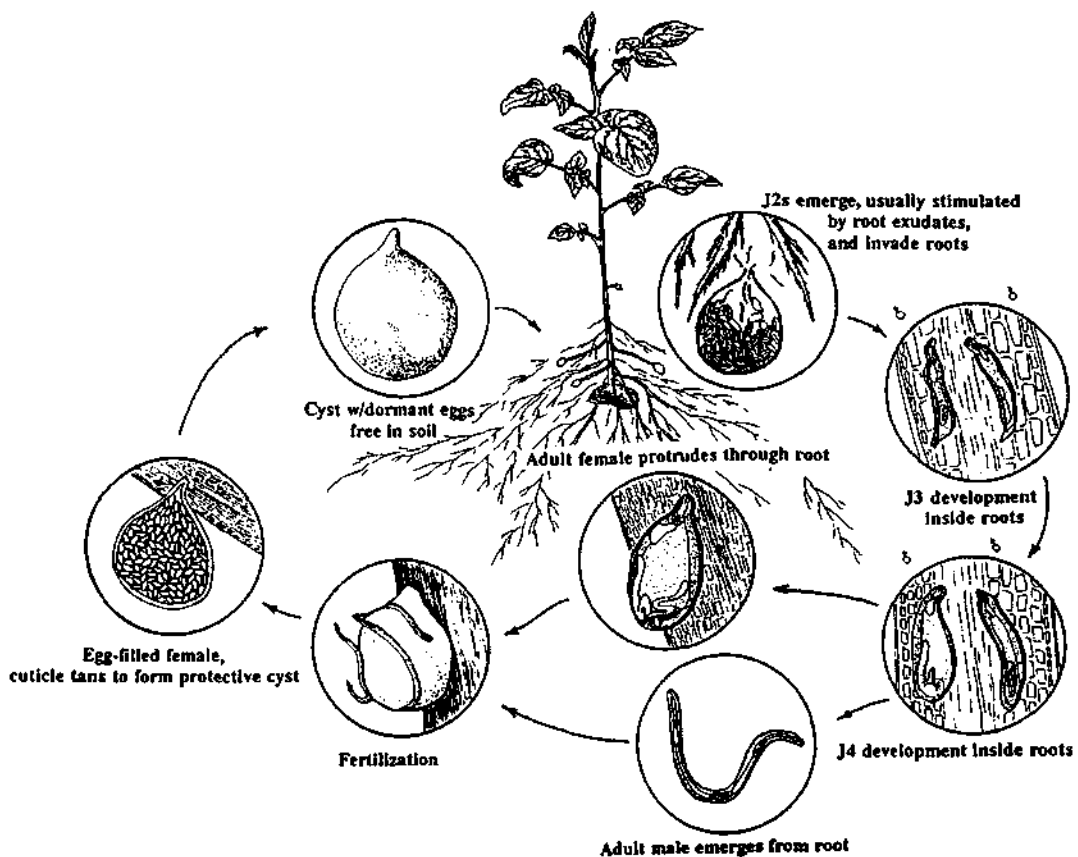
This finding is significant for the potato industry as a reliable control option is thus available for potato cyst nematode in Victoria. These findings confirm the breeding strategy for the National Potato Breeding Program to continue to develop agronomically suitable lines incorporating the H1 gene.

The work described in the following pages is written in a condensed form and aimed at a general audience. In depth methodology and precise technical interpretations of the analyses is provided in manuscript form attached for the specialist reader.

Jill Hinch, December, 1995

INTRODUCTION:

Nematodes, or eelworms, are microscopic soil borne pests (approx. 1 mm long) that invade and parasitise roots causing cell death, and damage to the root system resulting in reduced yield. There are many different types of nematodes that attack a wide range of pasture and crop plants causing extensive economic damage. Cyst nematodes are highly specialised sedentary endoparasitic nematodes with limited host ranges (potato, tomato, egg plant and some solanaceous weeds). The potato cyst nematode (PCN) has a complex life cycle (see below).



Life cycle of potato cyst nematode.

The free living juvenile larvae (J2) hatch from nematode eggs in the presence of potato roots, then move through the soil to a potato root, penetrate then align themselves along the vascular system (J3), feeding from potato root cells. Eventually they induce multinucleate feeding cells and as the female nematodes (J4) grow they are fertilized by males. Females retain many of their eggs inside their body walls which form the protective cyst. At or before potato harvest the cyst drops off the root into the soil ready to provide a new source of eggs that contain unhatched larvae (J1, J2).

Management options and advice given to Australian farmers to control economically important nematodes such as the potato cyst nematodes, *Globodera rostochiensis* and *G. pallida*, depends on accurate identification and knowledge of pathogenicity. These two species are morphologically similar, each contains several subspecific biological variants or pathotypes that are also morphologically identical. Potato cultivars with good levels of resistance to *G. rostochiensis* (pathotypes Ro1 and Ro4) are available, but potatoes resistant to *G. pallida* are not available in commercial quantities.

Since January 1991 and the present, some 14 properties in Victoria have been classified as infested with the potato cyst nematode (Figure 1A). These properties have been quarantined and restrictions placed on crops grown on the infested paddocks and the use and movement of machinery. Because of these restrictions many farmers have had not only their incomes reduced but have faced operational difficulties. The present quarantine program involves soil fumigation which is environmentally undesirable. Research work undertaken by the USDA over many years has determined that an effective method for reducing PCN cysts and eggs in the soil is to plant 6 consecutive years of a resistant cultivar providing only *Globodera rostochiensis* pathotype Ro1 is present, and only in low initial population densities (Evans and Brodie, 1980).

Our aim in this study was to determine accurately the species and pathotype present in Victorian populations of PCN in order to advise farmers and quarantine officers appropriately on the best strategies to effectively reduce PCN levels on potato properties. This work was funded for a period of 18 months.

The approach of protein analysis was selected for this study because experience shows that peptide differences may be a more effective diagnostic tool than the molecular approach which has so far failed to differentiate between Ro1 and the other *rostochiensis* pathotypes (Dr Shields UK pers comm., Dr Marshall pers. comm.). Analysis of proteins by two dimensional gel electrophoresis has shown differences between the pathotypes (Janssen et al., 1990). The new technique of high performance capillary electrophoresis was chosen to analyse potato cyst nematode pathotypes. This is a microanalytical technique that has high efficiency and sensitivity, short analysis times, nanolitre injection volumes and highly reproducible analysis of samples.

METHODS:

1. Identification of cyst nematode genera and species

In order to confirm that cysts isolated from potato properties were indeed potato cyst nematode and not some other species of cyst nematode samples were subjected to gel electrophoresis as described in the following method.

Cysts (Figures 1C, 1D) from defined European pathotypes of *G. rostochiensis* and *G. pallida*, and from Australian populations of potato cyst nematode, beet cyst nematode (*Heterodera schachtii*), clover cyst nematode (*H. trifolii*) and cereal cyst nematode (*H. avenae*) were homogenized on ice in extraction buffer (20 mM 3 (-N-morpholino) propane-sulphonic acid (MOPS) buffer pH 7.0, 5 mM EDTA and 10 mM β -mercaptoethanol prior to gel electrophoresis.

Isoelectric focusing (IEF) was performed in order to determine the genera of cyst nematode present (i.e. *Globodera* or the closely related *Heterodera*) and which species of *Globodera* present. Nematode extracts were applied to the cathodal end of PhastGels pH 3-9 or pH 5-8 range and analysed using the PhastSystem (Pharmacia, LKB Biotechnology). Gels were silver stained.

2. Generation of pathotype protein profiles

In order to develop a reliable, reproducible method for analysis of potato cyst nematode pathotype identification samples were subjected to capillary electrophoresis for micro level protein and peptide separation as described in the following method.

Capillary electrophoresis was performed on pathotypes of *G. rostochiensis* and *G. pallida* (Table 1). For high performance capillary electrophoresis of PCN samples the addition of proteinase inhibitors 5mM phenylmethanesulfonyl fluoride (PMSF) and 5 mM pepstatin A was included prior to homogenisation in the extraction buffer. The soluble nematode protein extracts (20 μ l) were mixed with eCAP Sample Buffer 60/200 (20 μ l, 120 mM Tris / HCl, pH 6.6, 1% SDS) with the addition of 5% β mercaptoethanol and Orange G as an internal marker dye, boiled for 5 mins prior to pressure loading for 35 secs on a pre conditioned eCAP SDS Coated Capillary in a Beckman P/ACE 5510 Capillary Electrophoresis System (16 kV-57 μ A, reverse polarity). Run times were typically 30 mins and peaks were monitored at 214 nm. Post run analysis of data was performed with the System Gold Chromatography Software Data System (Beckman Instruments) (Figure 4).

Table 1: Defined European populations of potato cyst nematode, *Globodera rostochiensis* (Ro) and *G. pallida* (Pa).

Designated pathotype	Population
Ro1	Ecosse Hannover Woburn
Ro2	Grabau Obersteinbach
Ro3	Wageningen
Ro4	Dutch F
Ro5	Harmerz G1524
Pa1	Port Glenone
Pa2/3	Avereest

3. Cultivar assessments of nematodes

In order to characterise the pathotype of potato cyst nematode present by conventional methods, cysts were inoculated onto potato plants containing different resistant genes effective against different pathotypes as described in the following method.

Cysts of potato cyst nematode isolated from Victorian infestations were tested at IACR, Rothamsted for reproductive ability on standard cultivars. Cysts (25 per replicate, three replicates) were placed into an inoculation bag (polyester voile) and placed into a 10 cm pot beneath a single sprout potato tuber piece planted in sterile loam in a screen house. Any new cysts which formed (Figure 1B) were kept separate from the original inoculum. Each population was exposed to a fully susceptible control cultivar (*Désirée*), a cultivar containing the resistance gene H1 (*Maris Piper*) or an experimental line containing the resistance H2 gene (P55/7).

RESULTS:

1. Identification of cyst nematode genera and species

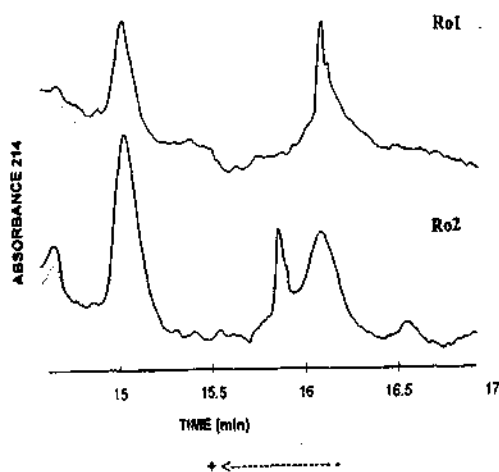
Homogenised cyst extracts of Australian isolates of potato cyst nematode (*Globodera rostochiensis*), beet cyst nematode (*Heterodera schachtii*), clover cyst nematode (*H. trifolii*) and cereal cyst nematode (*H. avenae*) subjected to isoelectric focusing pH gradient 5-8 were readily distinguished from each other by differences in the pI values of major proteins (Figure 2).

Cyst extracts of the Victorian isolates of potato cyst nematode from all 14 infested properties displayed the characteristic proteins of isoelectric point pI 5.9 indicative of *Globodera rostochiensis* pathotypes when subjected to isoelectric focusing on PhastGels (Figure 3). Defined populations from Europe and United Kingdom also displayed the species specific proteins appropriate for their classification i.e. populations characterised as *Globodera rostochiensis* at pI 5.9 and *G. pallida* displayed major proteins at pI 5.7. Species specific proteins could be detected with as little as one cyst per lane with the sensitive silver staining Pharmacia protocol. When extracts consisted of two cysts, one cyst from *G. rostochiensis* and one from *G. pallida*, both proteins (pI 5.9 and pI 5.7) were observed.

2. Pathotype protein profiles

Different pathotypes within *G. rostochiensis* and *G. pallida* can be distinguished by detection of protein peaks within the 29-66 kD molecular weight range by capillary electrophoresis (Figure 5). Different combinations of peaks were present for the different pathotypes (Table 2). However different populations of the same pathotype gave similar patterns with good reproducibility.

Figure 5: Peptide profiles of pathotypes Ro1 and Ro2 with distinguishing differences seen at 16 mins



SDS-Capillary electrophoresis was performed on nematode protein extracts with a SDS Coated Capillary in a Beckman P/ACE 5510 Capillary Electrophoresis System (16 kV-57 μ A, reverse polarity). Post run analysis of data was performed with the System Gold Chromatography Software Data System (Beckman Instruments).

Patterns generated by the Victorian isolates of PCN were all similar to each other and to that generated by material of Ro1 type which had undergone freeze drying. The Victorian PCN had low levels of protein present which also impeded analyses. These factors indicate that field collected material is in poor condition in comparison to that obtained from glasshouse grown nematodes from Europe.

Table 2: Relative migration rates of protein peaks detected by SDS-capillary electrophoresis with different pathotypes of potato cyst nematode.

PATHOTYPE	RELATIVE MIGRATION RATES*					
	1.37	1.41	1.43	1.44	1.47	1.52
Ro1	+	-	-	-	+	-
Ro2	+	-	-	+	+	-
Ro3	-	-	-	-	-	+
						doublet
Ro4	-	+	-	-	-	-
Ro5	+	+	-	-	+	-
Pa1	-	+	+	-	-	+
Pa2/3	+	+	-	-	-	-

In order to aid comparisons relative migration rates* were determined for each peak as the ratio of its absolute migration time relative to that of Orange G (time protein / time Orange G).

3. Cultivar assessments of nematodes

When cysts of Victorian isolates of PCN were inoculated onto potato lines containing different resistant genes, multiplication rates observed indicated that all isolates were *Globodera rostochiensis* pathotype Ro1 (or Ro4) i.e. controlled by the H1 gene (Table 3). The mean multiplication rates of the populations are expressed as P_f / P_i (where P_i = the initial cyst population and P_f = the final cyst population). If P_f / P_i is < 1.0 then nematode reproduction is poor and the cultivar is resistant, if P_f / P_i is 1-2, the cultivar is partially resistant and if $P_f / P_i > 2.0$ then nematode reproduction is good and the cultivar is susceptible (Turner, 1989). There were some differences between reproductive ability of some of the isolates, with an isolate from Gembrook (2) giving the highest reproductive ratio on the susceptible cv. Désirée. This is a result of differences in egg numbers per cyst as well as reproductive potential and vitality (i.e. factors other than virulence).

If *Globodera pallida* was present then high multiplication rates (i.e. $P_f / P_i > 2$) would be seen on Maris Piper. When *Globodera rostochiensis* Ro1 (or the rarer Ro4) is present there is always some low level multiplication on resistant hosts such as Maris Piper because the H1 gene does not inhibit nematode hatching but acts on inhibiting feeding site establishment. However, some Ro1 nematodes can still penetrate, form feeding sites and develop into females on resistant H1 plants. Good nematode reproduction is seen on the cultivar containing the H2 gene as this gene is not effective against the Ro1 pathotype. If other pathotypes of Ro were present then high multiplication ratios ($P_f / P_i > 2.0$) would be recorded as the H1 gene does not control the Ro2 / Ro3 or Ro5 pathotypes.

Table 3: Mean multiplication rate P_f/P_i * of Victorian populations of PCN on potato cultivars and lines with varying resistance.

PCN POPULATIONS	POTATO HOST		
	Desiree (Suscept)	Maris Piper (H1)	P55/7 (H2)
Wandin East	32.85* (3.225)**	0.23 (0.071)	21.15 (0.889)
Silvan South	47.24 (0.677)	0.52 (0.189)	42.49 (3.434)
Gembrook 1	12.07 (4.170)	0.32 (0.122)	n.d.
Gembrook 2	87.91 (3.280)	0.07 (0.035)	n.d.
Gembrook 3	28.49 (10.495)	0.12 (0.040)	n.d.

*Where P_i = initial cyst population (25 cysts), P_f = final cyst population. ** standard error of mean
 If $P_f/P_i < 1.0$ the plant is PCN resistant,
 If $P_f/P_i > 2.0$ the plant is PCN susceptible.
 n.d. not determined

DISCUSSION:

Biochemical analyses of nematode extracts reveal reproducible differences in major and minor protein components of different nematode species and pathotypes within individual species. Using the technique of high performance capillary electrophoresis, isoelectric focusing of potato cyst nematode isolates and cultivar assessment from thousands of cysts from Victorian populations, the Victorian populations were typed as *Globodera rostochiensis* Ro1. These techniques, including the technique developed in this project of capillary electrophoresis pathotype analysis, were also able to correlate protein profiles with defined European populations.

Data collected by the technique of capillary electrophoresis is stored in a computer data base so that if new infestations occur the new protein profiles can be compared with those obtained in this study.

Difficulties were encountered with the low amounts of protein present in the Victorian material which hindered comparisons of protein profiles with the European material. This is a common condition with field collected cysts in Europe. Hence in order to optimise pathotype identification in future fresh cysts (high egg numbers, high viability) need to be multiplied up. An additional quarantine permit should be available to multiply up fresh cysts, under high containment conditions,.

Management options and strategies for controlling potato cyst nematode populations in Victoria can now be developed with confidence as extensive testing has revealed that all populations are *Globodera rostochiensis* Ro1. An integrated management program based on resistant cultivars and crop rotations in addition to chemical controls will be the best long term option for the potato industry. Control options will need to proceed cautiously as other pathotypes may build up in time if resistant cultivars are used extensively.

ACKNOWLEDGMENTS:

This work was funded by the Horticultural Research & Development Corporation. Thanks to Dr Rumpfenhorst for providing defined populations, and my collaborators, Dr Ken Evans, Dr James Woodward, Dr Stuart Smith and Fred Alberdi. The excellent technical assistance of L. Nambiar and L. McLeish is gratefully acknowledged.

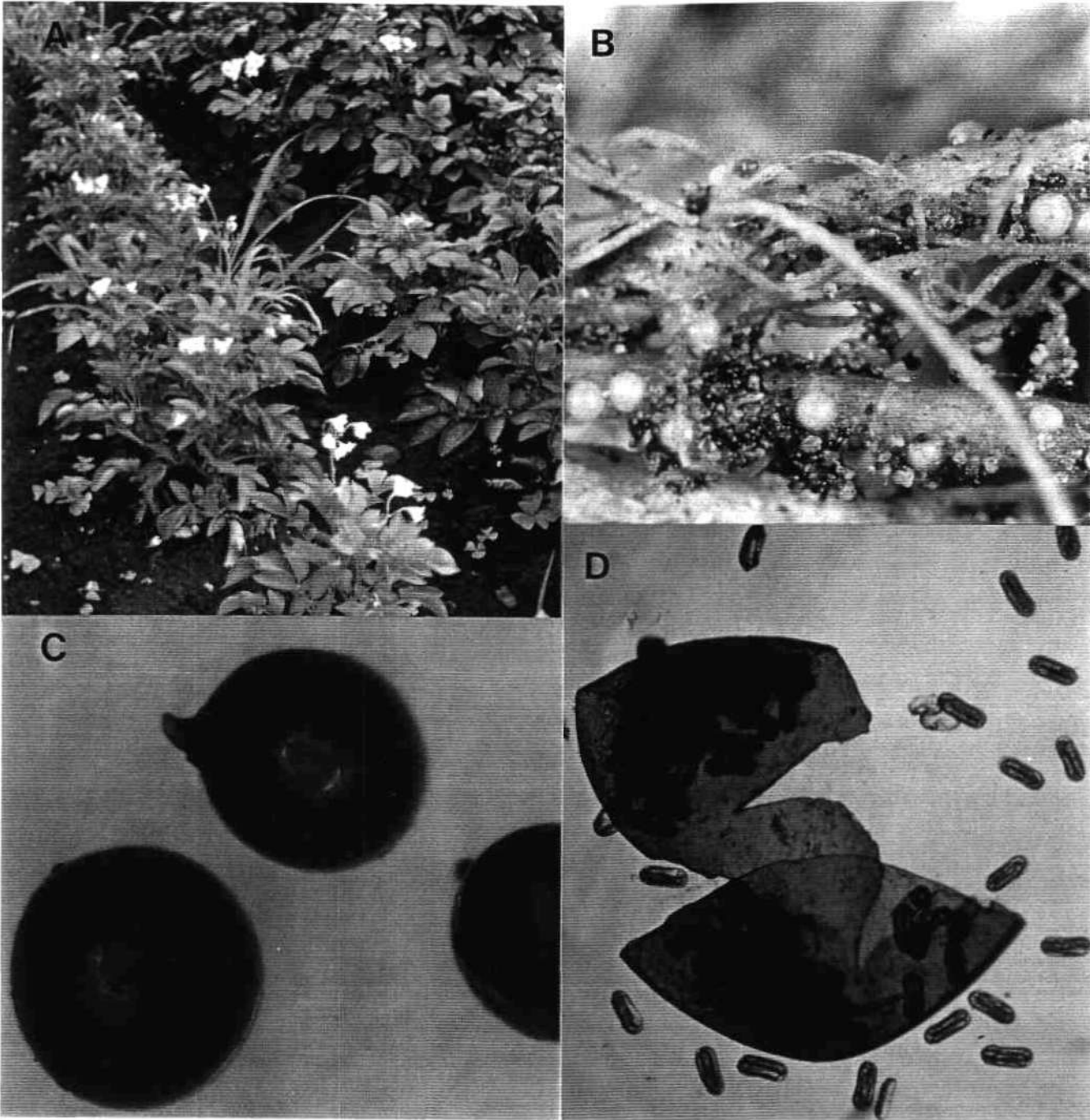
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Janssen, R., Bakker, J., & Gommers, F.J., (1990). Assessing intra-specific variations in virulence in *Globodera rostochiensis* and *G. pallida*. *Revue Nematol.*, **13**: 11-15.

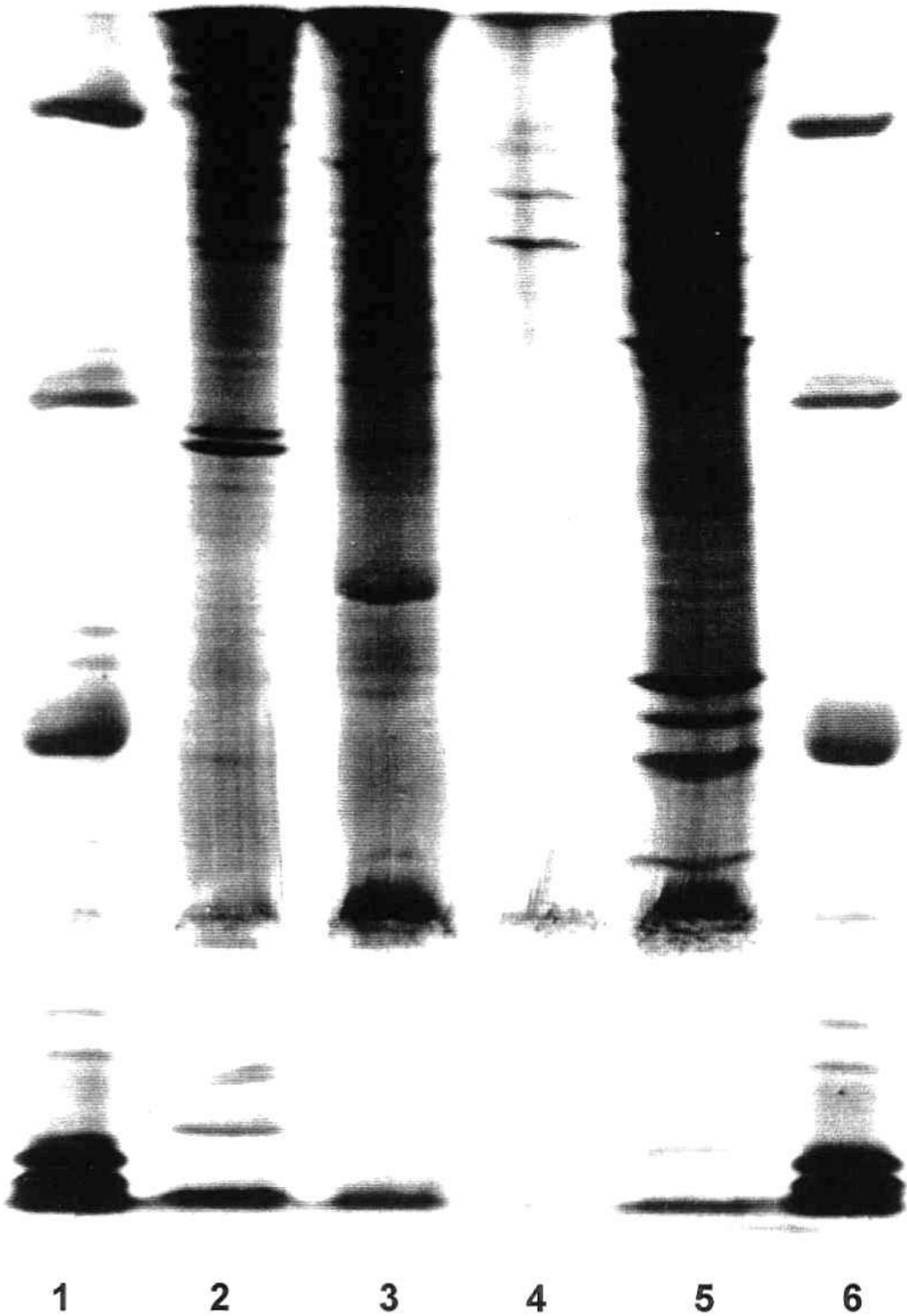
Turner, S., (1989) New sources of resistance to potato cyst nematodes in the Commonwealth Potato Collection. *Euphytica* **42**: 145-153.

Figure 1:



(A) Victorian property with potato plants, on the left hand side, stunted due to a heavy infestation of potato cyst nematode. (B) Potato roots with cysts (tanned remains of female nematodes) attached. The cysts are the size of a pin head. Young females are pale in colour but darken as they age. (C) Mature cysts as they appear after soil extraction. (D) Cyst crushed open to reveal hundreds of nematode eggs that were used for protein extraction.

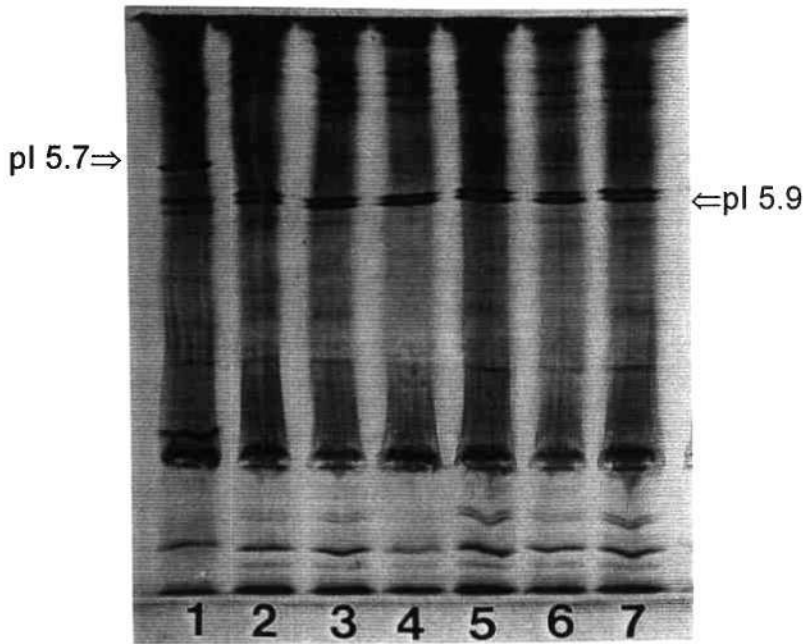
Figure 2:



Genera of cyst nematodes can be identified by major proteins on silver stained IEF gels pH 5-8. Lanes 1 and 6 standards, Lane 2 potato cyst nematode, Lane 3 beet cyst nematode, Lane 4 clover cyst nematode, Lane 5 cereal cyst nematode.

13

Figure 3



Potato cyst nematode species can be distinguished by major proteins at pI 5.7 (*G. pallida*, English population Lane 1) and pI 5.9 (*G. rostochiensis*, Victorian populations Lanes 2-7) on IEF gels pH 5-8.

Figure 4



The Beckman P/ACE 5510 Capillary Electrophoresis System and System Gold Chromatography Software Data System.

APPENDIX

Discrimination of European and Australian *Globodera rostochiensis* and *G. pallida* pathotypes by high performance capillary electrophoresis.

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Key words: capillary electrophoresis, potato cyst nematode, *Globodera rostochiensis*, *Globodera pallida*, pathotype, isoelectric focusing, polypeptide profiles, microanalytical, PhastSystem®.

Summary

High performance capillary electrophoresis (CE) is a family of related microanalytical preparative techniques that permits the rapid separation of proteins, peptides oligonucleotides and oligosaccharides. This paper describes the use of CE as a tool to differentiate between populations representative of the pathotypes of the potato cyst nematode species *Globodera rostochiensis* and *G. pallida*. Reproducible polypeptide profiles obtained by Sodium dodecyl sulphate (SDS)-capillary gel electrophoresis displayed peptides which were characteristic of *G. rostochiensis* and some which were characteristic of *G. pallida*. Polypeptide profiles of each of the pathotypes of *G. rostochiensis* Ro1, Ro2, Ro3, Ro4, Ro5 and *G. pallida* Pa1, Pa2/Pa3 were obtained. Profiles of Australian isolates from Victoria were of the Ro1 type and this pathotype classification was also determined by standard differential cultivar tests. The CE technique outlined is a reliable, reproducible technique which could aid the differentiation of these pathotypes with minimum sample preparation.

Introduction

Potato cyst nematodes (PCN) are soil borne pests of quarantine status in many countries. There are two species of PCN, namely *Globodera rostochiensis* (Woll.) Skarbilovich and *G. pallida* Stone. These two species are morphologically similar and each contains several subspecific biological variants or pathotypes that are morphologically identical. Differentiation of the pathotypes is traditionally by nematode reproductive ability on a standard set of non-isogenic potato lines that contain different resistance genes to PCN (Kort *et al.*, 1977) or by using the revised method of Nijboer & Parlevliet (1990). These methods are time consuming and responses can be influenced by the environment (Phillips, 1985). Attempts to develop molecular probes to distinguish between the pathotypes so far are only able to distinguish between species or between one or two pathotypes (Burrows & Perry, 1988; Schnick, Rumpfenhorst & Burgermeister, 1990; Stratford, Shields, Goldsbrough & Fleming, 1992; Roosien *et al.*, 1993; Ferris, Miller, Faghihi & Ferris, 1995). Analysis of nematode proteins by isoelectric focusing discriminates between the species (Fleming & Marks, 1983; Fox & Atkinson, 1984) and isozyme gel analysis has shown differences between some of the pathotypes, as has analysis by high resolution two-dimensional gel electrophoresis (Janssen, Bakker & Gommers, 1990). However, because of the difficulty of obtaining reproducible two dimensional gels, this method is not one that lends itself to routine identification. Also slab gel electrophoresis generally suffers from long analysis times and difficulties in detection and separation.

In this study, the microanalytical technique of high performance capillary electrophoresis was assessed for its potential to discriminate between the pathotypes within the two species. Capillary electrophoresis is a technique for the separation of micro-amounts of proteins and peptides. The technology consists of a family of related techniques that employ narrow bore (25-200 μm internal diameter) fused silica capillaries to perform high efficiency separations of both large and small molecules (Landers *et al.*, 1993). Various column matrices can be employed, and high voltages and electric fields are applied across the capillaries. While capillary electrophoresis is still a developing technology, the method has several advantages over traditional approaches, namely: high efficiency and sensitivity e.g. picomole-femtomole (10^{-12} - 10^{-15} mol) detection, short analysis times, nanolitre injection volumes and on-column, highly reproducible analysis of samples.

Materials and methods

EXTRACTION OF NEMATODE PROTEINS

Cysts of defined pathotypes of *Globodera rostochiensis* and *G. pallida* were gifts from the collection of Dr H. Rumpfenhorst (Münster, FRG) and from the collection of one of the authors (K. E.), whilst Australian cysts of *Globodera rostochiensis* were collected from infested paddocks in Victoria (J.M.H.). Cysts (over 200 in total) of each pathotype were homogenised in extraction buffer in a 1.5 ml microtube with a disposable PELLET PESTLE® mixer (Kontes, Vineland, New Jersey, USA) fitted to

1 a cordless drill (for 60 sec, low speed on ice). The extraction buffer for PhastSystem®
2 isoelectric focusing consisted of 20 mM 3 (-N- morpholino) propane-sulphonic acid
3 (MOPS) (BDH Ltd., Poole, England) buffer (pH 7.0), 5 mM EDTA (ethylenediamine
4 tetra-acetic acid disodium salt, Sigma Chemical Co., St.Louis, MO, USA) and 10 mM
5 β -mercaptoethanol (BDH Ltd., Poole, England) with 5 cysts homogenised in 10 μ l of
6 buffer. The extraction buffer for high performance capillary electrophoresis contained
7 20 mM MOPS buffer (pH 7.0), 5 mM EDTA, 10 mM β -mercaptoethanol, with the
8 addition of proteinase inhibitors, 5mM PMSF (phenylmethanesulfonyl fluoride, Sigma
9 Chemical Co., St Louis, MO, U.S.A.), 5mM Pepstatin A (Calbiochem, La Jolla, CA,
10 USA.) and a few grains of Polyclar AT (Polyvinylpyrrolidone, BDH Ltd., Poole,
11 England) prior to extraction. Fifty cysts were homogenised in 30 μ l of extraction
12 buffer. Samples were centrifuged at 13,000 rpm for 5 mins at 5°C.
13

14 ISOELECTRIC FOCUSING

15 Isoelectric focusing (IEF) was performed in order to confirm the species of *Globodera*
16 present. Nematode extracts (1 μ l of sample per lane) were applied to the cathodic end
17 of PhastGels pH 3-9 or 5-8 range and analysed using the PhastSystem® (Pharmacia,
18 LKB Biotechnology AB, Uppsala, Sweden). Gels were stained with a sensitive silver
19 staining protocol optimised for PhastGel IEF media (Pharmacia, LKB Biotechnology,
20 Development Technique File No 210). Species-specific proteins, differing in their
21 isoelectric points (as described by Fleming & Marks, 1983) were detected by this
22 method.
23

24 CAPILLARY ELECTROPHORESIS

25 For capillary electrophoresis the supernatant from the extracts were snap frozen in
26 liquid nitrogen and stored at -70°C until analysed. The nematode extracts (20 μ l) were
27 mixed with eCAP Sample Buffer 60/200 (20 μ l, 120 mM Tris / HCl, pH 6.6, 1% SDS)
28 and Orange G (5 μ l, 0.1%) as an internal marker dye, with the addition of 5% β -
29 mercaptoethanol, then boiled for 5 mins prior to pressure loading for 35 secs on a pre
30 conditioned eCAP SDS Coated Capillary (65 cm length, 100 μ m internal diameter,
31 Beckman Instruments Inc., Fullerton, CA, USA.) in a Beckman P/ACE 5510 Capillary
32 Electrophoresis System. Prior to sample application by pressure injection the
33 polyacrylamide coated column was rinsed for 2 mins with 1.0 N HCl, then the column
34 was filled with an SDS non-acrylamide low viscosity gel of proprietary formulation
35 (Beckman Gel Buffer 200) for 4 minutes. Electrophoresis was performed at 16kV-
36 57 μ A, using reverse polarity (inlet -, outlet +) at 22°C. Run times were typically less
37 than 30 mins and peaks were monitored at 200 nm, 214 nm real time spectral data
38 (wavelength of 190-300nm with a 2nm bandwidth). Peaks were detected by UV
39 absorbance through a transparent window in the capillary. Molecular weights were
40 determined from a calibration curve generated with a mixture of six proteins ranging
41 from 14.4 to 94 kDa (α -lactalbumin, 14.4 kDa; trypsin inhibitor, 20.1 kDa; carbonic
42 anhydrase, 30 kDa; ovalbumin, 43 kDa; albumin, 67 kDa; phosphorylase b, 94 kDa).
43 In order to aid comparisons, a relative migration time was determined for each detected
44 peak as the ratio of its absolute migration time to that of Orange G (time_{protein} / time_{Orange G}).
45 Orange G migration real time was 11.00 min. Post run analysis of data was
46 performed with the System Gold Chromatography Software Data System (Beckman

1 Instruments).

2 ASSESSMENT OF NEMATODES ON STANDARD POTATO CULTIVARS

3 Australian cysts of potato cyst nematode isolated from Victorian infestations at Wandin
4 East, Silvan South and Gembrook were tested at IACR-Rothamsted, for reproductive
5 ability on standard cultivars. Cysts (25 per replicate, three replicates), were enclosed
6 in an inoculation bag (polyester voile) and placed in a 10cm pot beneath a single sprout
7 potato tuber piece, planted in sterile loam. Any new cysts which formed were thereby
8 kept separate from the original inoculum. Each population was exposed to a fully
9 susceptible control cultivar (Désirée), a cultivar containing the resistance gene H₁
10 (Maris Piper) or a potato line containing the resistance gene H₂ (P55/7). The pots were
11 kept in a glasshouse for 12 weeks, after which the numbers of new cysts were counted.
12
13

14 Results

15 ISOELECTRIC FOCUSING

16 Cyst extracts of defined populations from Europe and the United Kingdom displayed
17 the species specific proteins appropriate for their classification. Populations
18 characterised as *G. rostochiensis* displayed major proteins at isoelectric point pI 5.9
19 (Figure 1A) and *G. pallida* at pI 5.7 (Figure 1B). Australian isolates of potato cyst
20 nematode from the State of Victoria displayed the protein of pI 5.9 characteristic of *G.*
21 *rostochiensis* when subjected to isoelectric focusing on PhastGels® (Figures 1C and
22 1D). Species specific proteins could be detected with as little as one cyst per lane with
23 the sensitive silver staining Pharmacia protocol. When extracts consisted of two cysts,
24 one cyst from *G. rostochiensis* and one from *G. pallida*, both proteins (pI 5.9 and pI
25 5.7) were observed.
26
27

28 CAPILLARY ELECTROPHORESIS

29 The method of choice for analysing nematode protein extracts with high performance
30 capillary electrophoresis was SDS-capillary gel electrophoresis (SDS-CE). Protein
31 extracts were initially run under Free-Zone Capillary Electrophoresis (FZCE) conditions
32 and differences between samples could be detected (data not shown). However, as the
33 samples consisted of total nematode protein extracts, choosing a single buffer which
34 was the best for all proteins in the crude mixture was difficult. When capillary gel
35 electrophoresis was performed on nematode protein extracts under reducing conditions
36 (with protease inhibitors) on a coated capillary column containing sodium dodecyl
37 sulphate (Beckman eCAP SDS gel capillary electrophoresis), many protein /
38 polypeptide peaks were detected. Nanolitre injection volumes could be applied in this
39 technique and fast analysis times were achieved.
40

41 Automated loading of samples in the SDS-P/ACE system combined with pressure
42 rinsing of the gel matrix with eCAP Gel Buffer 200 between runs achieved excellent
43 reproducibility of sample runs i.e. of both molecular weight standards and nematode
44 extracts. A single nematode extract was run nine times in one day and variability of
45 peak relative migration times was only 1%. Excellent reproducibility was also obtained
46

1 when separate replicates of the same nematode extracts were run over different days.
2 Peak area measurements and retention times recorded by the Gold System Software
3 indicated the relative amounts of reduced, denatured proteins/peptides present and
4 estimates of molecular weights were made on the basis of peak migration times relative
5 to molecular weight standards.
6

7 Analysis of crude nematode extracts detected more than 20 different major peaks when
8 subjected to SDS-capillary electrophoresis. Different peaks could be detected which
9 were indicative of *G. rostochiensis* and *G. pallida*. Analysis of *G. rostochiensis* and *G.*
10 *pallida* samples (except Ro3) displayed a major, dominant doublet peak cluster (peaks
11 1,2) at relative migration times of 1.10,1.11, respectively. In some samples peak one
12 was not always clearly resolved from peak two and so was seen as a single broad peak
13 of MW<14.4 kDa. Similarly another broad but major peak cluster was resolved into
14 a triplet (peaks 4,5,6) at relative migration times of 1.20,1.21,1.22 also less than 14.4
15 kDa. Another major peak occurred at a relative migration time of 1.24. Major
16 differences occurred between all pathotypes examined in the 14.4 kDa to 43 kDa range
17 (relative migration times of 1.37-1.52, see Table 1). Isolates of *G. rostochiensis*
18 pathotype Ro1 (including Australian populations from Wandin East, Silvan South and
19 Gembrook in Victoria) had major peaks at relative migration rates of 1.37 (15.1 min
20 real time) and 1.47, Ro2 at 1.37, 1.44 and 1.47 (Figure 2), Ro3 a doublet at 1.52, Ro4
21 with a peak at 1.41 but no peak at 1.37, whilst Ro5 had peaks at 1.37, 1.41 and 1.47. *G.*
22 *pallida* pathotype Pa1 displayed major peaks at 1.41, 1.43 and 1.52 whilst Pa2/3
23 displayed peaks at 1.37 and 1.41 (Table 1 and Figure 4). Quantitative differences
24 occurred in the peak cluster 4,5,6 for some isolates of Ro and in Pa pathotypes.
25

26 Good peak reproducibility was observed in isolates of the same pathotype designation
27 from different localities e.g. *G. rostochiensis* Ro₂ from Obersteinbach and Grabau, and
28 *G. rostochiensis* Ro5 from Harmerz and isolate G1524 (Figure 3 and Table 1). There
29 were minor differences detected between some of the *G. rostochiensis* Ro1 populations
30 (Ecosse and Hannover) suggesting greater heterogeneity in the Ro1 group than with the
31 other *G. rostochiensis* pathotype groups (Table 1). In *G. pallida* (both Pa1 and Pa2/3)
32 isolates examined, the dominant peak cluster (1.24) was not present or present in a
33 reduced form (data not shown).
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ASSESSMENT OF NEMATODES ON STANDARD POTATO CULTIVARS

When cysts of Victorian isolates of PCN were inoculated onto potato lines containing different resistance genes, multiplication rates observed indicated that all isolates were *Globodera rostochiensis* pathotype Ro1 (or Ro4) (Table 2). The mean multiplication rates of the populations are expressed as P_f / P_i (where P_i = the initial cyst population and P_f = the final cyst population). If P_f / P_i is < 1.0 then nematode reproduction is poor and the cultivar is resistant, if P_f / P_i is 1-2, the cultivar is partially resistant and if $P_f / P_i > 2.0$ then nematode reproduction is good and the cultivar is susceptible (Turner, 1989). There were some differences between reproductive ability of some of the isolates, with an isolate from Gembrook giving the highest reproductive ratio on the susceptible cv. Désirée. This is a result of differences in egg numbers per cyst as well as reproductive potential and vitality (i.e. factors other than virulence).

If *Globodera pallida* was present, then high multiplication rates (i.e. $P_f / P_i > 2$) would be seen on Maris Piper. When *G. rostochiensis* Ro₁ (or the rarer Ro₄) is present there is always some low level multiplication on resistant hosts such as Maris Piper because the H₁ gene does not inhibit nematode hatching but acts by slowing nematode development at all stages as well as inhibiting feeding site establishment. A small proportion of Ro₁ nematodes can still penetrate and form feeding sites on resistant H₁ plants. Good nematode reproduction was seen on the cultivar containing the H₂ gene as this gene is not effective against the Ro₁ pathotype. If other pathotypes of Ro were present then high multiplication ratios ($P_f / P_i > 2.0$) would be recorded on cv. Maris Piper as the H₁ gene does not control the Ro₂, Ro₃ or Ro₅ pathotypes.

Discussion

In this paper we report an evaluation of sodium dodecyl sulphate - capillary gel electrophoresis (SDS - CE) as a technique for discriminating between populations of biological variants of potato cyst nematodes. On the basis of the results presented it is possible to detect differences in protein profiles between pathotypes which differ genetically (based on the traditional cultivar testing schemes). Such differences are not unexpected as the technique of two dimensional gel electrophoresis has shown differences between some of the pathotypes (Janssen *et al.*, 1990). It is not clear how or if these differences relate to virulence.

Fully reduced protein monomers are detected by SDS-CE so the 34 kDa dominant protein (Robinson *et al.*, 1993) seen on non-denaturing and IEF gels is not seen under reducing conditions but rather its subunit components. The subunits for these species specific proteins (of differing isoelectric point) would be less than 29 kDa. We chose the range greater than 14.4 kDa to examine in detail for pathotype specific variability and several consistent differences were observed in this range. The capillary electrophoresis protein profiles of *G. rostochiensis* populations were similar in appearance and displayed common peaks which clearly set the *G. rostochiensis* group apart from the peak patterns displayed by the *G. pallida* populations i.e. the *G. rostochiensis* group (except for Ro3) had similar overall patterns with minor differences

1 between them. The differences were detected in the 14.4 - 43 kDa range. Profiles of
2 *G. pallida* populations on the other hand exhibited more differences between the
3 pathotypes. Indeed on the basis of these profiles the *G. pallida* group appears to be
4 quite diverse as opposed to the uniformity seen in the *G. rostochiensis* groups examined
5 in this study. The observed heterogeneity in the *G. rostochiensis* Ro1 populations, as
6 opposed to the reproducible patterns seen for two populations of each of Ro2 and Ro5,
7 may be indicative of the wide and early distribution of the Ro1 pathotype. Analysis of
8 more Ro1 populations would be necessary to confirm this heterogeneity.
9

10 There were several restrictions in this study. One of the restrictions was the need to
11 obtain very concentrated protein solutions so that when nanogram samples were
12 injected or run, enough protein in individual peaks could be detected without extending
13 the injection time to 60 seconds, which could have resulted in overlap of one peak on
14 another. Problems can be encountered in SDS CE if sample proteins are overloaded,
15 such as a skewed peak shape. However, if protein loading is too low then peak area
16 varies considerably (Tsuji, 1993). A second problem consistently confronted in this
17 work was the low level of protein present in field collected cysts of Australian isolates
18 in contrast to European glasshouse derived cyst samples. However, this study provided
19 evidence that SDS CE is a useful technique for determining very small protein
20 differences which relate to pathotype or population differences. Analyses of a larger
21 collection of populations will clarify this potential.
22

23 The use of capillaries filled with viscous buffers combined with linear polymers and
24 SDS has enabled the separation of proteins by size in gel capillary electrophoresis.
25 Capillary electrophoresis is still a developing technology and whilst isoelectric focusing
26 is possible in CE this is not yet a perfected technique. Capillary electrophoresis is a
27 simpler and more reproducible technique than two dimensional gel electrophoresis and
28 will enable the automated analysis of large numbers of samples. More importantly,
29 SDS - CE provides a computer access and retrieval system which can act as a central
30 diagnostic facility and as new outbreaks or new pathotypes emerge the information can
31 be added to the existing data bank. This technique will also allow the on-line
32 identification of polypeptides of interest and the isolation of these polypeptides for
33 future sequencing and molecular cloning. SDS - CE should thus lead more quickly to
34 developing probes to species specific or pathotype specific components than traditional
35 molecular approaches.
36

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43 United Kingdom.
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Table 1: Relative migration rates of protein peaks detected by SDS-capillary electrophoresis with different pathotype populations of potato cyst nematodes in the molecular weight range 14.4 - 43 kDa.

RELATIVE MIGRATION RATES*

PATHOTYPE	POPULATION	1.37	1.41	1.43	1.44	1.47	1.52
Ro1	Ecosse	+	-	-	-	+	-
	Australia*	+	-	-	-	+	-
	Hannover	-	-	-	+	+	-
Ro2	Grabau	+	-	-	+	+	-
	Obersteinbach	+	-	-	+	+	-
Ro3	Wageningen	-	-	-	-	-	++
Ro4	Dutch F	-	+	-	-	-	-
Ro5	Harmerz	+	+	-	-	+	-
	G1524	+	+	-	-	+	-
Pa1	Port Glenone	-	+	+	-	-	+
Pa2/3	Avereest	+	+	-	-	-	-

In order to aid comparisons relative migration rates were determined for each peak as the ratio of its absolute migration time relative to that of Orange G ($\text{time}_{\text{protein}} / \text{time}_{\text{Orange G}}$). Ro= *Globodera rostochiensis*, Pa= *G. pallida*. Peak present (+), peak absent (-), peak doublet (++) . *= Australian populations from Wandin East, Silvan South, Gembrook, Victoria.

Table 2: Mean Multiplication Rate P_f / P_i * of Australian populations of PCN on potato cultivars and lines with varying resistance.

PCN POPULATION	POTATO HOST		
	cv Désirée (Susceptible)	cv Maris Piper (H ₁ resistance gene)	line P55/7 (H ₂ resistance gene)
Wandin East	32.85 (3.225)	0.23 (0.071)	21.15 (0.889)
Silvan South	47.24 (0.677)**	0.52 (0.189)	42.49 (3.434)
Gembrook 1	12.07 (4.170)	0.32 (0.122)	nd
Gembrook 2	87.91 (3.280)	0.07 (0.035)	nd
Gembrook 3	28.49 (10.495)	0.12 (0.040)	nd

*Where P_i = initial cyst population (25 cysts), P_f = final cyst population. ** standard error of mean

If $P_f / P_i < 1.0$ the plant is PCN resistant,

If $P_f / P_i = 1-2$ the plant is partially resistant

If $P_f / P_i > 2.0$ the plant is PCN susceptible.

n.d. not determined

Figures**Figure 1.**

Polyacrylamide gel electrophoresis (Phastgels, IEF) of protein extracts from PCN cysts of European and Australian isolates.

1A.

Phastgel (IEF 3-9, 5 cysts in 10 μ L of buffer, 1 μ L); Lanes 1-6 are extracts of European *Globodera rostochiensis* isolates - Ro1 = Hannover (Lane 1), Ro2 = Grabau (Lane 2) and Obersteinbach (Lane 3), Ro3 = Wageningen (Lane 4), Ro4 = Dutch F (Lane 5), Ro5 = Harmerz (Lane 6).

1B.

Phastgel (IEF 3-9, 5 cysts in 10 μ L of buffer, 1 μ L); Lanes 7-13 are extracts of European *Globodera pallida* isolates- Pa1 = Port Glenone (Lane 7), Pa2/3 = Chavornay (Lane 8), Wegberg (Lane 9), Delmsen (Lane 10), Kalle (Lane 11), Avereest (Lane 12), Frenswegen (Lane 13).

1C and 1D.

Composite photograph from several phastgels (2 cysts per lane in 2 μ L of buffer, 1 μ L loaded, Figure 1C=IEF 5-8, 1D=IEF 3-9); Lane 1 extract from English *G. pallida* isolate arrow indicates pathotype Pa2 dominant proteins including pI 5.7 protein, Lanes 2-9 are extracts of Australian *Globodera rostochiensis* isolates with Ro dominant proteins at pI 5.9, Ro1= Silvan South (Lane 2), Wandin East (Lane 3), Gembrook 1 (Lanes 4 and 5), Gembrook 2 (Lanes 6 and 7), Gembrook 3 (Lanes 8 and 9).

Figure 2.

Two examples of SDS-capillary electrophoretic separations of the potato cyst nematode crude extracts, molecular weight range 29 - 43 kDa of *Globodera rostochiensis* pathotypes Ro1(Ecosse) and Ro2 (Grabau) as monitored at 214 nm.

Figure 3

SDS-capillary electrophoretic separations of the potato cyst nematode crude extracts, molecular weight range 29 - 43 kDa. In order to aid comparisons and highlight pathotype consistencies of peaks for Ro5 populations, electropherograms of populations with the same pathotype designation were super imposed, isolate G1524(_____) and isolate Harmerz (_ _ _ _ _).

Figure 4

SDS-capillary electrophoretic separations of the potato cyst nematode crude extracts, molecular weight range 29 - 43 kDa of *Globodera pallida* pathotype populations Pa1 from Port Glenone and Pa2/3 from Avereest.

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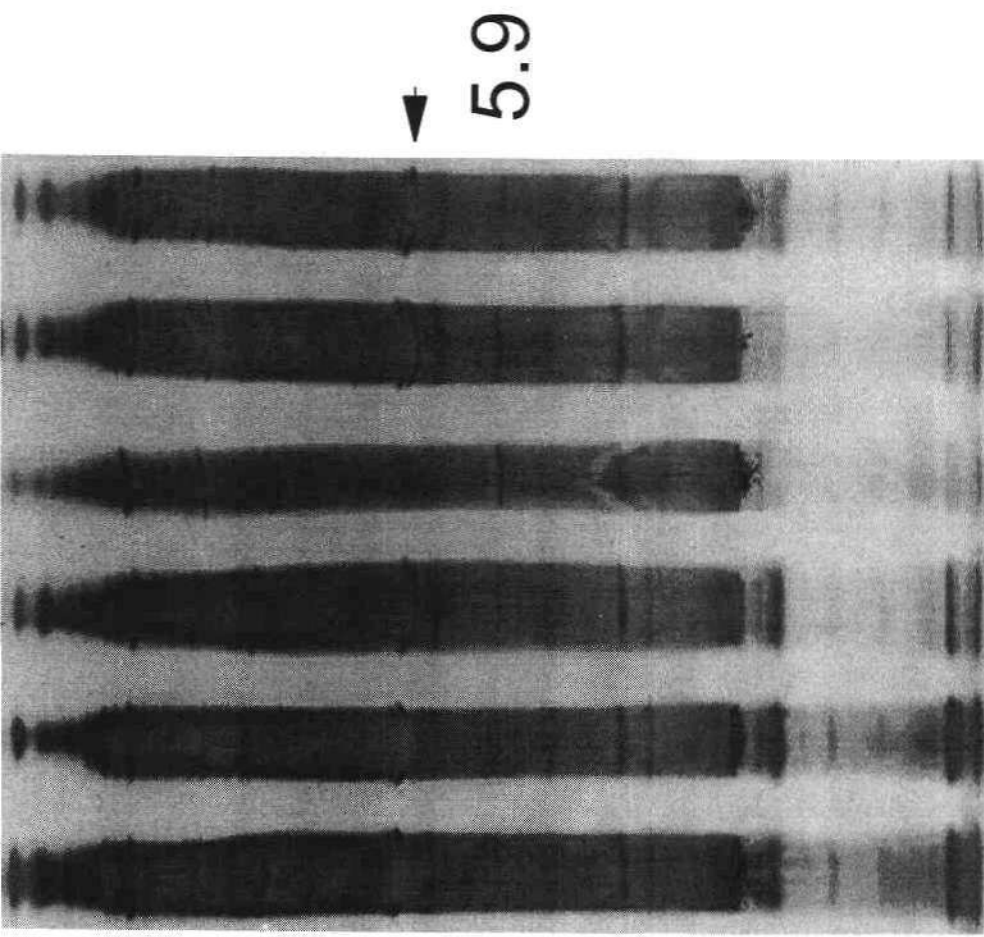
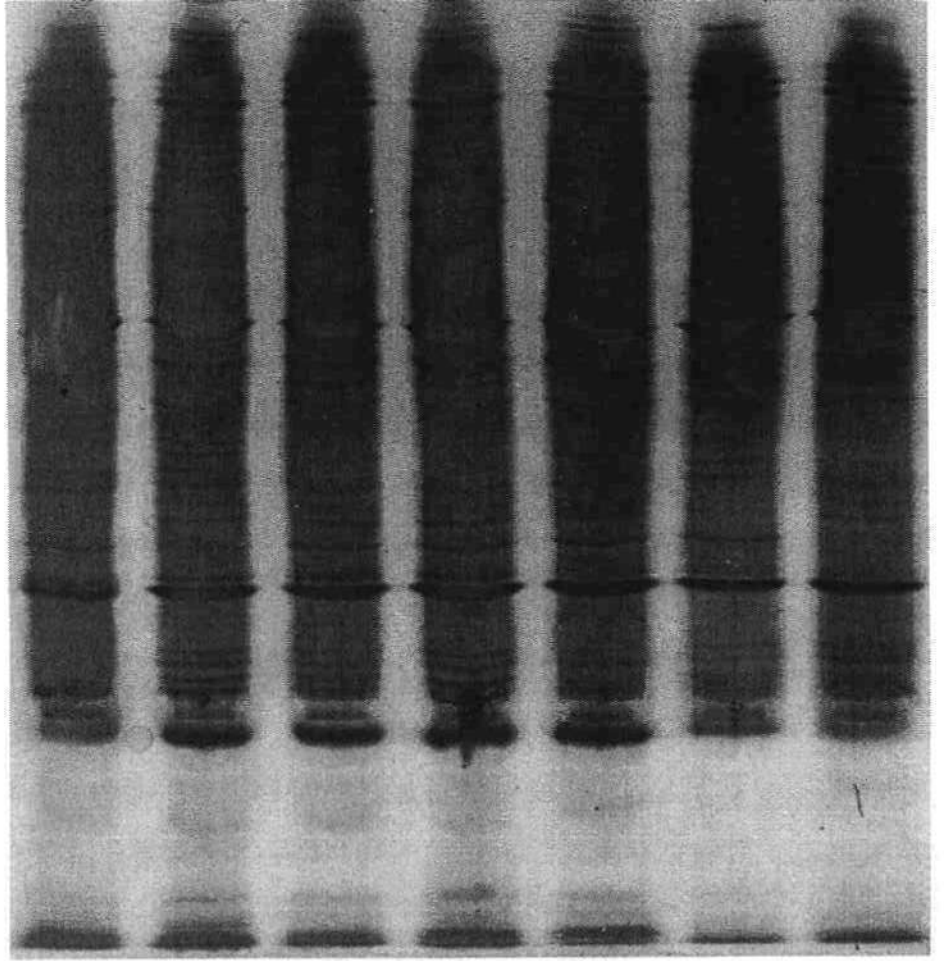


Figure 1A

1000

5.7
▶



7 8 9 10 11 12 13

Figure 1B

Hinch et al, 1996

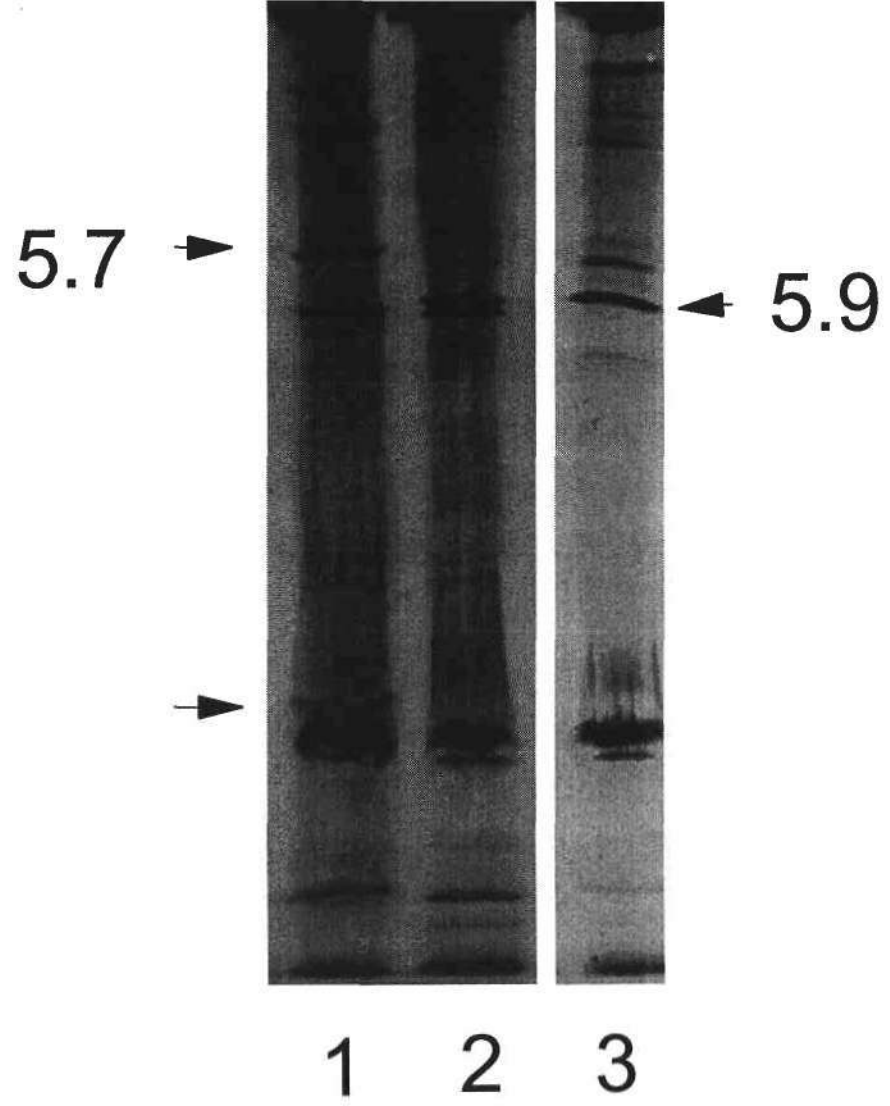
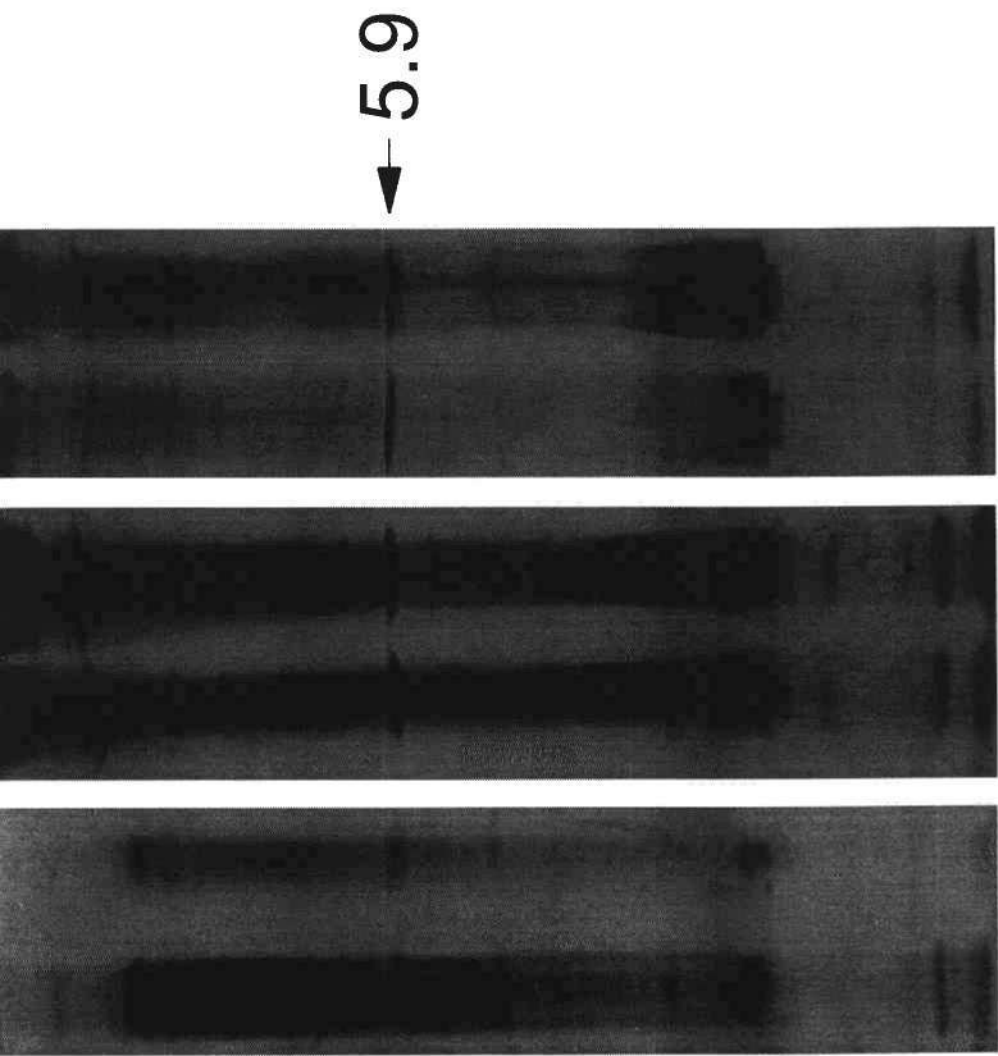


Figure 1C



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Figure 1D

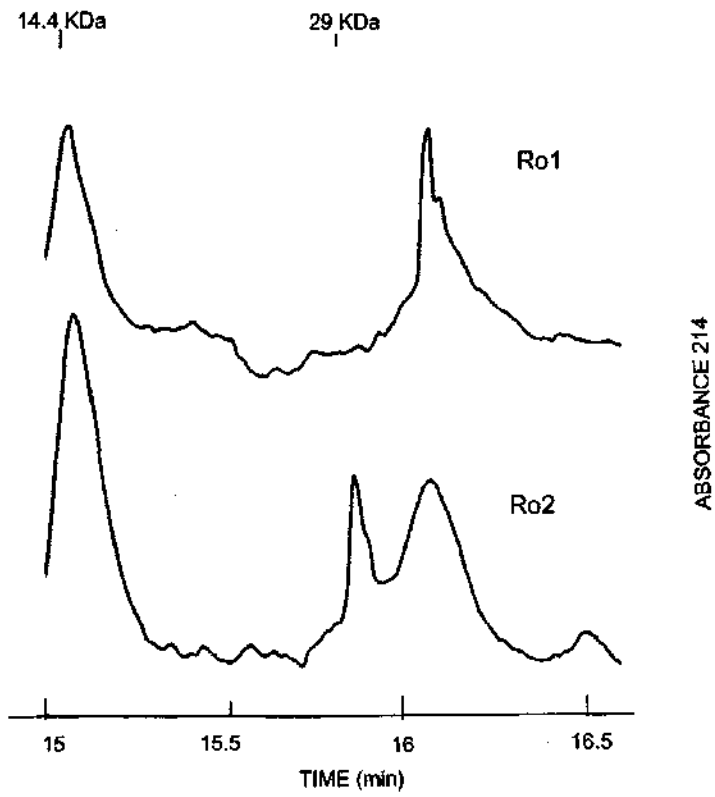


Figure 2, *Hinch et al., 1996*

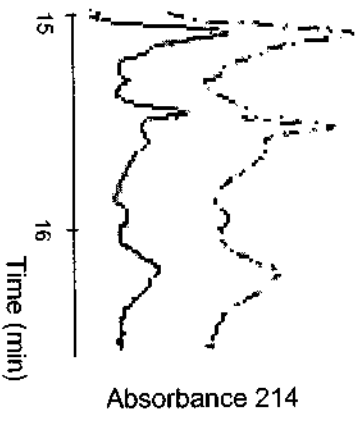


Figure 3,

Hirach et al, 1996

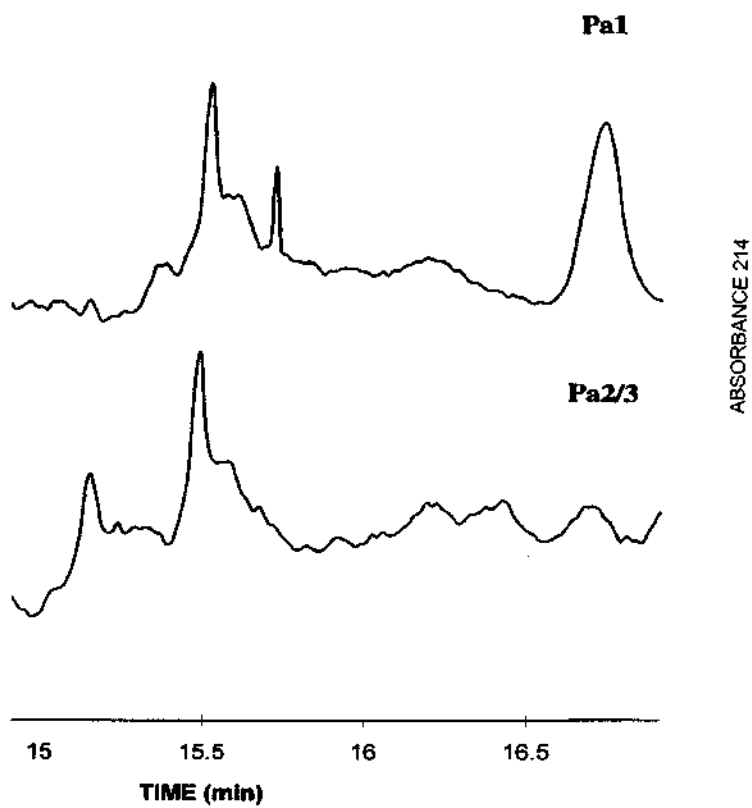


Figure 4, *Hinch et al., 1996*