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

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Summary – High performance capillary electrophoresis (CE) is a family of related microanalytical preparative techniques for the rapid separation of proteins, peptides oligonucleotides, and oligosaccharides. This article describes the use of CE as a tool to differentiate between representative populations of 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 some peptides characteristic of *G. rostochiensis* and others 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 with the differentiation of these pathotypes with minimum sample preparation. © Elsevier - ORSTOM

Résumé – ***Séparation des pathotypes européens et australiens de* Globodera rostochiensis *et* G. pallida *par électrophorèse capillaire à haute performance*** - L'électrophorèse capillaire (CE) à haute performance relève d'un groupe de techniques microanalytiques voisines permettant la séparation rapide de protéines et de peptides tels les oligonucléotides et les oligosaccharides. Le présent article décrit l'utilisation de la CE comme outil pour différencier des populations appartenant aux pathotypes des espèces de nématodes à kyste de la pomme de terre *Globodera rostochiensis* et *G. pallida*. Des profils polypeptidiques reproductibles obtenus par CE sur gel à l'aide du dodecyle sulfate révèlent des peptides caractéristiques de *G. rostochiensis* et d'autres de *G. pallida*. Ont été ainsi obtenus les profils polypeptidiques de chacun des pathotypes de *G. rostochiensis* (Ro1, Ro2, Ro3, Ro4, Ro5) et de *G. pallida* (Pa1, Pa2/Pa3). Les profils des isolats australiens provenant de l'État de Victoria appartiennent au type Ro1. Cette classification des pathotypes a été également précisée par des tests sur des cultivars différentiels standards. La technique CE utilisée, fiable et reproductible, peut faciliter la différenciation de ces pathotypes avec une préparation minimale des échantillons. © Elsevier - ORSTOM

Keywords: capillary electrophoresis, *Globodera rostochiensis*, *Globodera pallida*, isoelectric focusing, microanalytical, pathotype, PhastSystem™, polypeptide profiles, potato cyst nematode

Potato cyst nematodes (PCN) are soil borne pests of quarantine status in many countries. The two species of PCN, *Globodera rostochiensis* (Woll.) Behrens and *G. pallida* Stone, are morphologically similar and each includes several subspecific biological variants or pathotypes that are morphologically identical. Nematode pathotypes are traditionally differentiated by their 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 and Parlevliet (1990). These methods are time consuming and responses can be influenced by the environment (Phillips, 1985). The molecular probes developed so far are able to distinguish only between species or between one or two pathotypes (Burrows & Perry, 1988; Schnick *et al.*, 1990; Stratford *et al.*, 1992; Roosien *et al.*, 1993; Ferris *et al.*, 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 *et al.*, 1990). However, because of the difficulty of obtaining reproducible two-dimensional gels, this last method does not lend itself to routine identification. Furthermore 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

Cysts of defined pathotypes of *Globodera rostochiensis* and *G. pallida* were obtained from the collection of Dr H. Rumpenhorst (Münster, Germany) and Australian cysts of *Globodera rostochiensis* were collected from infested paddocks in Victoria.

Extraction of nematode proteins

Cysts of each pathotype were homogenized in extraction buffer in a 1.5 ml microtube with a disposable Pellet Pestle™ mixer (Kontes, Vineland, NJ, USA) fitted to a cordless drill for 60 s, low speed (approximately 150 rpm) on ice. The extraction buffer for PhastSystem™ isoelectric focusing consisted of 20 mM 3 (-N- morpholino) propane-sulphonic acid (MOPS) (BDH Ltd., Poole, UK) buffer (pH 7.0), 5 mM EDTA (ethylenediamine tetra-acetic acid disodium salt; Sigma Chemical Co., St Louis, MO, USA) and 10 mM β-mercaptoethanol (BDH Ltd., Poole, UK) with five cysts homogenized in 10 µl of buffer. The extraction buffer for high performance capillary electrophoresis contained 20 mM MOPS buffer (pH 7.0), 5 mM EDTA, 10 mM β-mercaptoethanol, with the addition of proteinase inhibitors, 5 mM PMSF (phenylmethanesulphonyl fluoride; Sigma Chemical Co., St Louis, MO, USA), 5 mM Pepstatin A (Calbiochem, La Jolla, CA, USA) and a few grains of Polyclar AT (Polyvinylpyrrolidone, BDH Ltd., Poole, UK) prior to extraction. For SDS-CE four replicates, each containing 50 cysts homogenised in 30 µl of extraction buffer, (200 cysts in total) were extracted for each population. Samples were centrifuged at 13000 rpm for 5 min at 5°C.

Isoelectric focusing

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

Capillary electrophoresis

The method of choice for analysing nematode protein extracts with high performance capillary electrophoresis was sodium dodecyl sulphate-capillary gel electrophoresis (SDS-CE). Protein extracts were initially run under Free-Zone Capillary Eletrophoresis (FZCE) conditions and differences between samples could be detected (data not shown).

For SDS-capillary electrophoresis, the supernatants from the extracts were snap frozen in liquid nitrogen and stored at -70°C until analysed. The nematode extracts (20 µl) were mixed with eCAP Sample Buffer 60/200 (20 µl, 120 mM Tris/HCl, pH 6.6, 1% SDS) and Orange G (5 µl, 0.1%; Beckman) as an internal marker dye, with the addition of 5% β-mercaptoethanol, then boiled for 5 min prior to pressure loading for 35 s on a pre conditioned eCAP SDS Coated Capillary (65 cm length, 100 µm internal diameter; Beckman Instruments Inc., Fullerton, CA, USA) in a Beckman P/ACE 5510 Capillary Electrophoresis System. Prior to sample application by pressure injection the polyacrylamide coated column was rinsed for 2 min with 1.0 N HCl, then the column was filled with an SDS non-acrylamide low viscosity gel of proprietary formulation (Beckman Gel Buffer 200) for 4 min. Electrophoresis was performed at 16 kV 57 µA, using reverse polarity (inlet –, outlet +) at 22°C. Run times were typically less than 30 min with a sample loading of 3 ηg of protein, and peaks were monitored at 200 ηm, 214 ηm real time spectral data (wavelength of 190-300 ηm with a 2 ηm bandwidth). Each replicate was run at least twice. Peaks were detected by UV absorbance through a transparent window in the capillary. Molecular weights were determined from a calibration curve generated with a mixture of six proteins ranging from 14.4 to 94 kDa (α-lactalbumin, 14.4 kDa; trypsin inhibitor, 20.1 kDa; carbonic anhydrase, 30 kDa; ovalbumin, 43 kDa; albumin, 67 kDa; phosphorylase b, 94 kDa). For comparison purposes, a relative migration time was determined for each detected peak as the ratio of its absolute migration time to that of Orange G ($\text{time}_{\text{protein}} / \text{time}_{\text{Orange G}}$). Orange G migration real time was 11.00 min. A single nematode extract was run nine times in one day and variability

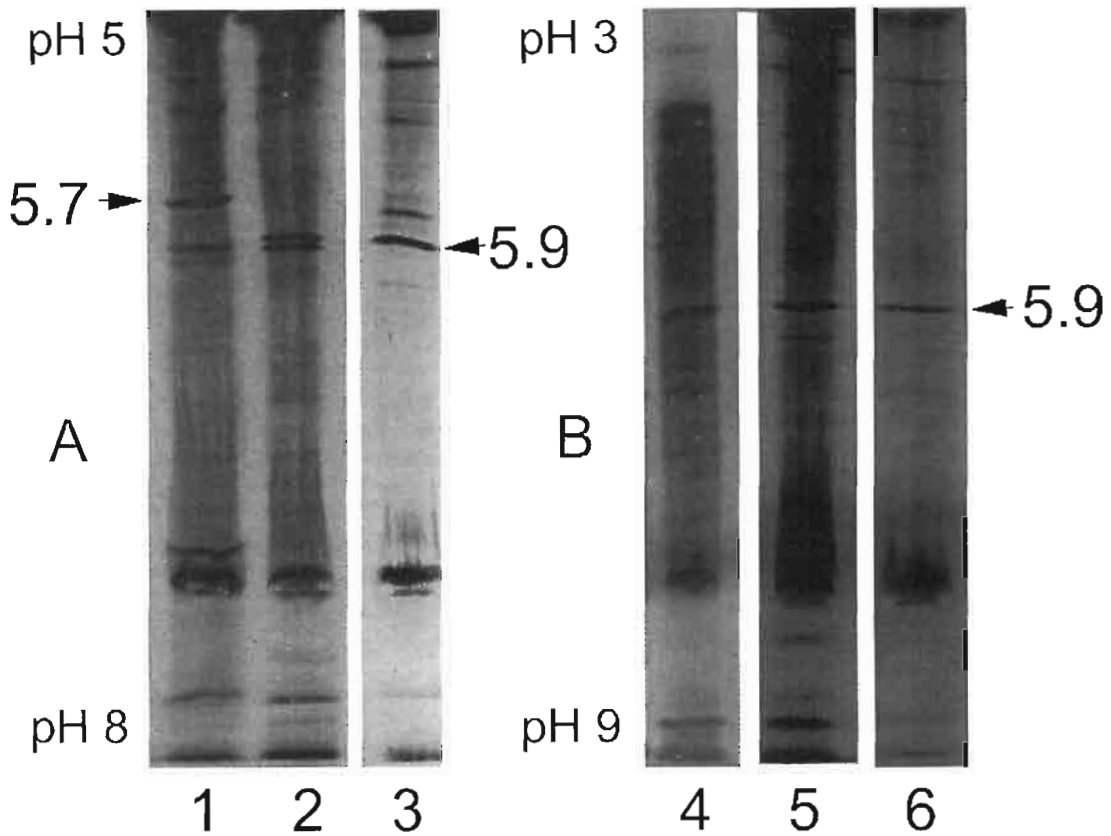

Fig. 1. *Composite photograph from several phastgels (two cysts per lane in 2 µl of buffer, 1 µl loaded, A: Lanes 1-3=IEF 5-8; B: Lanes 4-6=IEF 3-9); Lane 1 extract from English* Globodera pallida *isolate arrow indicates pathotype Pa2 dominant proteins including pI 5.7 protein, Lanes 2-6 are extracts of Australian* G. rostochiensis *isolates with species-specific protein at pI 5.9, Silvan South (Lane 2), Wandin East (Lane 3), Gembrook 1 (Lane 4), Gembrook 2 (Lane 5), Gembrook 3 (Lane 6).*

of peak relative migration times was only 1%. Post run analysis of data was performed with the System Gold Chromatography Software Data System (Beckman Instruments). Peak area measurements and retention times recorded by the Gold System Software indicated the relative amounts of reduced, denatured proteins/peptides present and estimates of molecular weights were made based on peak migration times relative to molecular weight standards.

Assessment of Australian populations on standard potato cultivars

Australian PCN cysts isolated from Victorian infestations (approximately 50 km east of Melbourne, all within a 10 km radius) at Wandin East, Silvan South, and Gembrook were tested at IACR-Rothamsted for reproductive ability on standard cultivars. Cysts (25 per replicate, three replicates), were enclosed in an inoculation bag (polyester voile) and placed in a 10 cm pot beneath a single sprout potato tuber piece, planted in sterile loam. Any new cysts formed were thereby 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 a potato line containing the resistance gene H2 (P55/7). The pots were kept in a glasshouse for 12 weeks, after which the numbers of new cysts were counted.

Results

Isoelectric focusing

European populations characterised as *G. rostochiensis* displayed major proteins at isoelectric point pI 5.9 and *G. pallida* at pI 5.7 . Australian isolates of potato cyst nematode from the State of Victoria (Wandin East, Silvan South, and Gembrook) displayed the protein of pI 5.9 characteristic of *G. rostochiensis* (Fig. 1).

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 14.4-30 kDa.*

Pathotype	Population	Relative migration rates*					
		1.37	1.41	1.43	1.44	1.47	1.52
Ro1	Ecosse	+	-	-	-	+	-
	Wandin East**	+	-	-	-	+	-
	Silvan South**	+	-	-	-	+	-
	Gembrook**	+	-	-	-	+	-
	Hannover	-	-	-	+	+	-
Ro2	Grabau	+	-	-	+	+	-
	Obersteinbach	+	-	-	+	+	-
Ro3	Wageningen	-	-	-	-	-	++
Ro4	Dutch F	-	+	-	-	-	-
Ro5	Harmerz	+	+	-	-	+	-
	G1524	+	+	-	-	+	-
Pa1	Port Glenone	-	+	+	-	-	+
Pa2/3	Avereest	+	+	-	-	-	-

*For comparative purposes, 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 $_{Orange\ G}$). Ro= *Globodera rostochiensis*, Pa= *G. pallida*. Peak present (+), peak absent (-), peak doublet (++). **= Australian populations.

Table 2. *Mean multiplication rate P_f / P_i * of Australian populations of potato cyst nematodes on potato cultivars and lines with major resistance genes.*

PCN population	Potato host		
	cv. Désirée (susceptible)	cv. Maris Piper (H1 resistance gene)	line P55/7 (H2 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)	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; n.d.=not determined.

Capillary electrophoresis

When capillary gel electrophoresis was performed on nematode protein extracts under reducing conditions (with protease inhibitors) on a coated capillary column containing sodium dodecyl sulphate (Beckman eCAP SDS gel capillary electrophoresis), many protein/polypeptide peaks were detected. Nanolitre injection volumes can be used with this technique and fast analysis times were achieved.

Automated loading of samples in the SDS-P/ACE system combined with pressure rinsing of the gel matrix with eCAP Gel Buffer 200 between runs achieved excellent reproducibility of sample runs, *i.e.*, of both molecular weight standards and nematode extracts. Excellent reproducibility was also obtained when separate replicates of the same nematode extracts were run over different days.

Over twenty different major peaks indicative of *G. rostochiensis* and *G. pallida* were detected. *G. rostochiensis* and *G. pallida* samples (except Ro3) displayed a major, doublet peak (peaks 1 and 2) at relative migration times of 1.10 and 1.11, respectively. In some samples, peak 1 was not always clearly resolved from peak 2 and so was seen as a single broad peak of MW<14.4 kDa. Similarly, another broad but major peak cluster was resolved into a triplet (peaks 4, 5, and 6) at relative migration times of 1.20, 1.21, and 1.22, of MW also less than 14.4 kDa. Another major peak occurred at a relative migration time of 1.24. Major differences occurred between all pathotypes examined in the 14.4 kDa to 30 kDa range (relative migration times of 1.27-1.56, see Table 1). Isolates of *G. rostochiensis* pathotype Ro1 (including Australian populations from Wandin East, Silvan South and Gembrook in Victoria) had major peaks at relative migration rates of 1.37 (15.1 min real time, 17.9 kDa) and 1.47 (23 kDa), Ro2 at 1.37, 1.44 (20.1 kDa), and

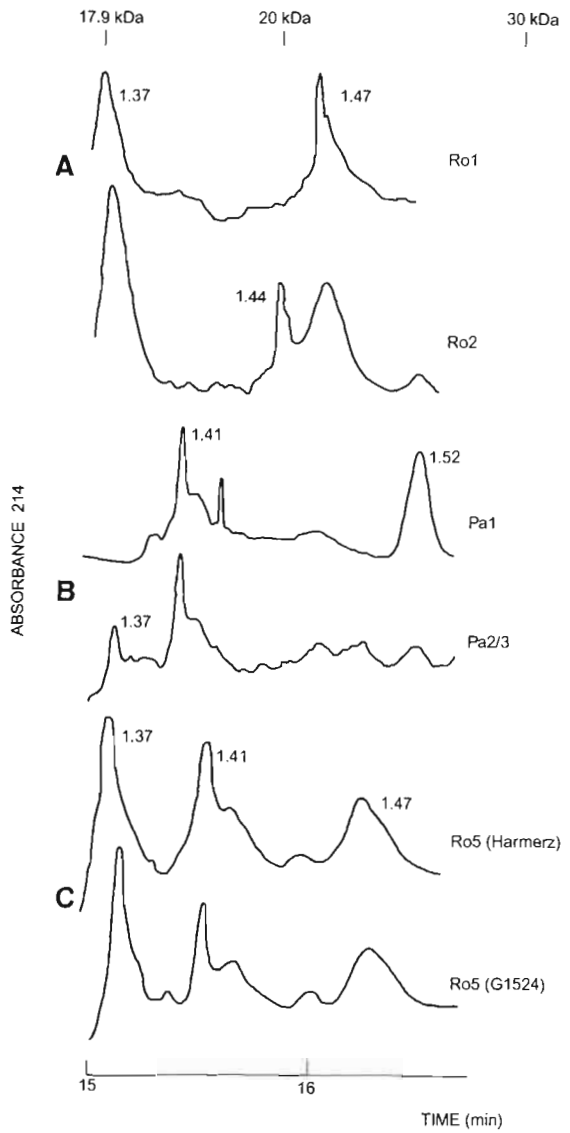

Fig. 2. *A: Two examples of SDS-capillary electrophoretic separations of the potato cyst nematode crude extracts, molecular weight range 17.9-30 kDa, of* Globodera rostochiensis *pathotypes Ro1 (Ecosse) and Ro2 (Grabau) as monitored at 214 nm. Relative migration times are indicated beside each peak; B: SDS-capillary electrophoretic separations of crude extracts, molecular weight range 17.9-30 kDa, of* G. pallida *pathotype Pa1 from Port Glenone and Pa2/3 from Avereest. Relative migration times are indicated beside each peak; C: SDS-capillary electrophoretic separations of crude extracts, molecular weight range 17.9-30 kDa, of* G. rostochiensis *Ro5 populations. In order to aid comparisons and highlight pathotype consistencies of peaks for Ro5 populations, electropherograms of populations with the same pathotype designation (isolate G1524 and isolate Harmerz) have been super imposed. Relative migration times are indicated beside each peak.*

1.47 (Fig. 2A), Ro3 a doublet at 1.52 (28 kDa), Ro4 with a peak at 1.41 (19 kDa) but no peak at 1.37, whilst Ro5 had peaks at 1.37, 1.41, and 1.47. *G. pallida* pathotype Pa1 displayed major peaks at 1.41, 1.43 (19.5 kDa), and 1.52 whilst Pa2/3 displayed peaks at 1.37 and 1.41 (Table 1; Fig. 2B). Quantitative differences occurred in the peak cluster 4, 5, and 6 for some isolates of Ro and in Pa pathotypes.

Good peak reproduciblity was observed in isolates of the same pathotype designation from different localities, *e.g.*, *G. rostochiensis* Ro2 from Obersteinbach and Grabau, and *G. rostochiensis* Ro5 from Harmerz and isolate G1524 (Fig. 2C; Table 1). Minor differences were detected between some of the *G. rostochiensis* Ro1 populations (Ecosse and Hannover) suggesting greater heterogeneity in the Ro1 group than in the other *G. rostochiensis* pathotype groups (Table 1). In the *G. pallida* (both Pa1 and Pa2/3) isolates examined, a peak at 1.24 (relative migration rate) was not present or present in a reduced form (data not shown).

Assessment of nematodes on standard potato cultivars

The mean multiplication rates of the populations are expressed as P_f / P_i (where P_i = initial cyst population and P_f = final cyst population). When cysts of Victorian PCN isolates were inoculated onto potato lines containing different resistance genes, none was able to multiply on cv. Maris Piper with the H1 gene, which indicates that all isolates were *G. rostochiensis* pathotype Ro1 (or Ro4) (Table 2) and that *G. pallida* was not present. There were some differences in reproductive ability between 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).

Discussion

This article presents 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. It was possible to detect differences in protein profiles between genetically different pathotypes (according to traditional cultivar testing schemes). Such differences were not unexpected as the technique of two-dimensional gel electrophoresis has already 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 kD 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 them apart from *G. pallida.* Minor differences between *G. rostochiensis* populations were detected in the 14.4-30 kDa range. On the other hand, profiles of *G. pallida* populations exhibited more differences between the pathotypes. Indeed, based on these profiles, the *G. pallida* group appears to be quite diverse as opposed to the uniformity seen in the *G. rostochiensis* groups examined in this study.

One problem encountered was the need to obtain very concentrated protein solutions so that when nanogram samples were injected or run, enough protein in individual peaks could be detected without extending the injection time to 60 s, which could have resulted in overlap of one peak on another. However, if protein loading is too low then peak area varies considerably (Tsuji, 1993). A second problem was the consistently low level of protein present in field collected cysts of Australian isolates in contrast to European glasshouse derived cyst samples. However, this study did provide evidence that SDS-CE could be a useful technique for determining very small protein differences which relate to pathotype or population differences. Analyses of a larger collection of populations will clarify this potential.

Capillary electrophoresis is a simpler and more reproducible technique than two-dimensional gel electrophoresis and will enable the automated analysis of large numbers of samples. More importantly, SDS-CE provides a computer access and retrieval system which can act as a central diagnostic facility, and as new outbreaks or new pathotypes emerge the information can be added to the existing data bank. This technique will also allow the on-line identification of polypeptides of interest and the isolation of these polypeptides for future sequencing and molecular cloning. SDS-CE should thus lead more quickly to developing probes to species-specific or pathotype-specific components than traditional molecular approaches.

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