Fundam. appl. Nematol., 1992, **15** (6), 495-501

Protein polymorphisms within *Globodera pallida* assessed with mini two dimensional gel electrophoresis of single females

Jan M. DE BOER, Hein OVERMARS, Liesbeth BOUWMAN-SMITS, Marjan DE BOEVERE, Fred J. GOMMERS and Jaap BAKKER

Department of Nematology, Agricultural University, P.O. Box 8123, Binnenhaven 10, 6709 PD Wageningen, The Netherlands.

Accepted for publication 26 december 1991.

Summary — Single females of *Globodera pallida* were analysed with mini two dimensional gel electrophoresis (2-DGE) followed by a sensitive silver stain. Comparison of the protein patterns of 35 individuals from one population revealed 62 invariant protein spots and sixteen variant proteins having the typical characteristics expected from mutations that change the isoelectric point (IP-variants). The sixteen IP-variants are assumed to be encoded by sixteen alleles at eight putative loci. The frequencies of the presumed genotypes showed no significant deviations from Hardy-Weinberg equilibrium. The average heterozygosity per locus was 5 %. A detailed procedure for analysing minute amounts of protein with mini 2-DGE is described.

Résumé — ***Établissement du polymorphisme protéique sur des femelles isolées de* Globodera pallida *par micro-électrophorèse sur gel en deux dimensions*** — Des femelles isolées de *Globodera pallida* sont analysées par micro-électrophorèse sur gel en deux dimensions (2-DGE), suivie d'une imprégnation argentique. La comparaison de la répartition des protéines chez 35 individus d'une même population révèle 62 spots protéiques constants et 16 spots protéiques variables ayant typiquement les caractéristiques attendues de mutations modifiant le point isoélectrique (IP-variants). Les seize IP-variants sont supposés être codés par seize allèles sur huit loci présumés. La fréquence des génotypes supposés ne montre pas de déviation significative par rapport à l'équilibre de Hardy-Weinberg. L'hétérozygotie par locus est de 5 %. Une procédure pour l'analyse de faibles quantités de protéines à l'aide de micro- 2-DGE est décrite de façon détaillée.

Key-words : Nematodes, potato, *Globodera*, pathotypes, virulence, allele frequencies, heterozygosity, electrophoresis.

In plant nematology the use of electrophoretic techniques for the discrimination of nematode populations has been largely confined to the analysis of mixtures of individuals. Standard electrophoretic techniques are too insensitive for analysing proteins from single nematodes of microscopic size. A number of investigators have developed micromethods of which micro disc electrophoresis (Dalmasso & Bergé, 1978; Bergé *et al.*, 1981) and more recently thin slab gel electrophoresis (Esbenshade & Triantaphyllou, 1985) have proved especially useful in studying the genetic variation between individuals. Various enzyme loci of *Meloidogyne* species have been screened for polymorphisms with these micromethods.

Another powerful technique for studying genetic variation is two dimensional gel electrophoresis (2-DGE), according to O'Farrell (1975), which enables the examination of several hundred polypeptides in a single sample. In a parallel paper, we reported on the genetic variation between populations of potato cyst nematodes revealed by 2-DGE of mixtures of 100 individuals (Bakker *et al.*, 1992). The objective of the present study is to extend 2-DGE to the protein analysis of single females of potato cyst nematodes and to examine the protein polymorphisms within a population of *Globodera pallida*. In addition we evaluated the accuracy of allele frequency data derived from mixtures of individuals (Bakker *et al.*, 1992) by comparing them with data obtained by electrophoresis of single individuals.

Materials and methods

SAMPLE PREPARATION

G. pallida population ROOK, classified as pathotype Pa_3, was obtained from the collection of the CPRO, Wageningen, the Netherlands. The population was originally collected at Valthe, the Netherlands. Adult, white females were reared on the susceptible cv. " Eigenheimer " at 18 °C and 16 h daylength. Samples containing a mixture of individuals were prepared as described by Bakker and Bouwman-Smits (1988). Samples for electrophoresing single individuals were prepared by transferring one female into an homogenization tube (length 2 cm, inner diameter 1.7 mm). The tube was made from a glass capillary, which was heat sealed at one end. Single females were homogenized in 10 µl buffer with a small glass pestle, which was made by heating the tip of a capillary (outer diameter 0.9 mm). The size of the small ball formed at the end of the

capillary was checked under the stereoscope to ensure a perfect fit inside the homogenization tube. The homogenization buffer was prepared by mixing 64.7 mg urea, 57 µl 10 mM Tris-HCl, pH 7.4 and 13.3 µl of a solution containing 25 % 2-mercaptoethanol, 8 % (w/v) ampholytes pH 5-7 and 2 % (w/v) ampholytes pH 3-10. After homogenization the extract was frozen for 30 min at — 80 °C and after thawing immediately used for electrophoresis. Protein determinations were made according to Bradford (1976).

MINI TWO-DIMENSIONAL ELECTROPHORESIS

Chemicals for electrophoresis and Coomassie Brilliant Blue G-250 were from Bio-Rad Laboratories (Richmond, CA). Silver nitrate and citric acid were from BDH Chemicals Ltd. (Poole, England.) All other chemicals were from Merck (Darmstadt, FRG). Distilled, deionized water was used for preparing the solutions and for the washing steps of the silver stain.

Mini two-dimensional electrophoresis was performed with the Mini Protean II 2-D Cell system of Bio-Rad Laboratories. Isoelectric focusing was performed in capillary tubes of 77 mm length and 1.1 mm inner diameter. The capillary tubes were cleaned by immersion in chromic acid and ethanol, and washed with distilled deionized water. The isoelectric focusing gels contained 3.78 % (w/v) acrylamide, 0.22 % (w/v) Bis, 9.15 M urea, 1.6 % (w/v) ampholytes pH 5-7, 0.4 % (w/v) ampholytes pH 3-10, 0.013 % (w/v) ammonium persulfate, and 0.10 % (v/v) TEMED. The tubes were filled by capillary action with gel solution up to 10 mm below the top. They were pressed in a cushion of plasticine covered with parafilm, and allowed to polymerize for 1 h. Samples were applied on top of the gel with a glass capillary pipette. The remaining space in the focusing tubes was filled with sample overlay solution containing 5.2 mM Tris HCl pH 7.4, 8.41 M urea, 0.84 % (w/v) ampholytes pH 5-7, and 0.21 % (w/v) ampholytes pH 3-10. The cathode buffer was at the top of the focusing gels and contained 20 mM NaOH. The anode buffer was 10 mM phosphoric acid. Focusing was performed without prerun and was accomplished with the following voltage schedule : 16.5 h 10 V, 90 min 180 V, 30 min 270 V and 80 min 578 V.

After focusing, the tube-gels were extruded in equilibration buffer containing 62.7 mM Tris HCl pH 6.8, 2.3 % (w/v) SDS and 10 % (v/v) glycerol. The separation gels were 84 × 59 × 0.75 mm (width × height × thickness). They contained 12.65 % (w/v) acrylamide, 0.35 % (w/v) Bis, 375 mM Tris HCl pH 8.8, 0.1 % (w/v) SDS, and were polymerized with 0.05 % (w/v) ammonium persulfate, and 0.05 % (v/v) TEMED. The stacking gel (height 13 mm) was poured up to 1 mm below the top of the inner glas plate, and contained 3.894 % (w/v) acrylamide, 0.106 % (w/v) Bis, 125 mM Tris HCl pH 6.8, 0.1 % (w/v) SDS, 0.05 % (w/v) ammonium persulfate, and 0.10 % (v/v) TEMED. The focusing gels were positioned on top of the stacking gel by pushing them in the slit between the two glass plates. At both ends of the tube gel a few drops of SDS-sample buffer (86 mM Tris HCl pH 6.8, 2 % (w/v) SDS, 12.5 % (v/v) glycerol, 0.004 % (w/v) Bromophenol Blue) were added. Both the upper and the lower buffer reservoirs were filled with a solution of 24.7 mM Tris, 193 mM glycine, and 0.1 % (w/v) SDS. Second dimension electrophoresis was performed with 10 mA per gel for 20 min, followed by 20 mA per gel for approximately 55 min, until the dye front had reached the bottom of the gel.

The gels were stained separately in plastic Petri dishes (diameter 13.5 cm) with 50 ml of solution for each step. First, the gels were transferred to 50 % (v/v) methanol, 10 % (v/v) acetic acid for 30 min. This was followed by incubation in 5 % (v/v) methanol, 7 % (v/v) acetic acid either for 30 min or overnight, and a fixation in 7 % (v/v) glutaraldehyde for 1 h. After four washings steps of 15 min in water, the gels were impregnated with ammoniacal silver solution (11.4 mM $AgNO_3$, 4.7 mM NaOH, 134 mM NH_3) for 20-25 min. After one washing step of 15 s and two 5 min washings in water, the gels were stained in 0.00925 % (w/v) paraformaldehyde, 0.025 % (w/v) citric acid solution for 20-25 min. The staining reaction was stopped with water. Protein patterns were evaluated visually by superimposing the original gels on a bench viewer. Variant protein spots (loci C, E, F, H, I, J) and a number of other conspicuous proteins which were also resolved with macro 2-DGE (Bakker *et al.*, 1992) were used as internal standards to estimate the pH gradient and molecular weights.

Results

To optimize the mini 2-DGE system, various conditions were tested by electrophoresing protein samples containing a mixture of individuals. A low initial voltage for isoelectric focusing proved to be essential for good quality protein patterns. The high initial voltages used in other studies (Poehling & Neuhoff, 1980; Neukirchen *et al.*, 1982; Sanderink *et al.*, 1988; Tanaka & Kawamura, 1988) resulted in streaking of the proteins, especially basic proteins with a high molecular weight. Application of a range of protein amounts demonstrated that 5 µg was the optimal concentration for mini 2-DGE. Approximately 350 protein spots were routinely visible with this amount (data not shown). Higher sample loads introduced streaking of the neutral and basic proteins and led to a loss of resolution in this area. Attempts to increase the sensitivity of the protein stain failed. Our staining procedure, which is essentially an adaptation of the ammoniacal silver stain of Oakley *et al.* (1980), was more sensitive than other staining methods (Oshawa & Ebata, 1983; De Moreno *et al.*, 1985; Chaudhuri & Green, 1987; Neuhoff *et al.*, 1988). Only the procedure of Poehling and Neuhoff (1981) was equally sensitive. The colour of the proteins visualized with our stain ranged

from red, reddish brown, blackish brown, brownish grey to grey or any shade in between.

Thirty five individuals of *G. pallida* population ROOK were analysed with mini 2-DGE. Examples of the protein patterns of single females are shown in Figure 1. The protein amount per female averaged 2.5 µg. The number of protein spots, including minor and faint spots, resolved per individual ranged from 233 to 439 with a mean of 360. Comparison of the protein patterns of 35 individuals revealed three types of protein spots : *i)* 62 invariant protein spots, which were consistently present in all 35 individuals, *ii)* 16 isoelectric point variants (IP-variants), which had the typical characteristics expected from mutations that change the isoelectric point (e.g. Fig. 2) and *iii)* a remaining group of more than 350 variant protein spots, which did not have the characteristics of the IP-variants. The latter group may also include genetically determined variant protein spots, but the majority of the variations is probably due to artefacts, such as, slight differences in the electrophoretic procedure, sample preparation and physiological state of the females.

The 16 IP-variants are assigned with a capital letter referring to the putative locus and a number referring to the allele. Corresponding IP-variants have moderately different isoelectric points, but have similar molecular weight, colour and quantity. As illustrated in Figure 2, corresponding IP-variants are encoded by codominant alleles of which the products are synthesized in similar amounts. The genetic interpretation was confirmed by a chi-square test showing that the observed number of genotypes did not deviate significantly from the number expected under Hardy-Weinberg equilibrium conditions (Table 1).

The average heterozygosity per locus was 5 % and was calculated according to Leigh Brown and Langley (1979) by assuming that the 62 invariant spots and 16 IP-variants represent the protein products of 70 loci. Obviously this is coarse estimate. As has been established in other studies (Racine & Langley, 1980) some alleles will display more than one protein spot; we also noticed this phenomenon. Each allele of locus H displays probably two protein spots (Fig. 1). The presence (or absence) of allele H_1 was accompanied in each individual by the presence (or absence) of protein spot no. 1 (Fig. 1). Similar results were obtained for allele H_3 and protein spot no. 2 (Fig. 1). The protein spots no. 1 and no. 2 were therefore recorded as separate IP-variants.

The IP-variant loci C, E, F, H, I, J (Fig. 1) were also recorded as polymorphic for population ROOK in a previous study with macro 2-DGE (Bakker *et al.*, 1992). As shown in Table 2 the allele frequency data obtained by mini 2-DGE of individuals are within the same range as the data obtained by macro 2-DGE of a mixture of 100 individuals. In the latter case the relative protein quantities of the variant protein spots were used as a measure for the allele frequencies (Bakker *et al.*, 1992). The largest differences between the two methods were observed for locus E and I. The macro 2-DGE data for these two loci fall just outside the 95 % confidence interval of the mini 2-DGE data (Table 2).

Differences in experimental conditions and data collection mean that not all the protein polymorphisms detected with macro 2-DGE are revealed with mini 2-DGE and *vice versa*. Allele F_1 having a frequency of 0.03 (Bakker *et al.*, 1991) was probably not traced in this study because of the smaller number of individuals sampled (35 *vs* 100). The IP-variant loci A, D and K (Bakker *et al.*, 1992) were not detected with mini 2-DGE and the IP-variant loci L and M (Fig. 1) were not resolved with macro 2-DGE.

Discussion

Since its introduction 2-DGE has become a widespread technique in various disciplines. As an alternative to radioactive labelling methods and Coomassie Blue staining, ultra sensitive silver stains were developed. With the introduction of micro 2-DGE (Poehling & Neuhoff, 1980) the sensitivity of protein detection was further increased. However, the use of micro 2-DGE is still limited and applications deal mainly with the analysis of a low number of polypeptides (Poehling *et al.*, 1980; Ohms & Heinicke, 1985; Manabe *et al.*, 1987; Sanderink *et al.*, 1988). A new development in 2-DGE is the use of mini gels (Sakurai *et al.*, 1986; Tanaka & Kawamura, 1988); which have a size intermediate between conventional and micro gels. As shown in this report mini 2-DGE allows the analysis of complex protein samples containing only a few µg of total protein. The high resolution of conventional 2-DGE is retained and the sensitivity of detection is increased by about a factor ten. In addition the system is easier to handle, faster, and very economical in the use of chemicals.

Analysing and evaluating 100 or more proteins on a single 2-DGE pattern is evidently more difficult and less straightforward than interpreting isozyme patterns. In this report we used stringent criteria to collect data from 2-DGE patterns. Only protein variations having the typical characteristics of corresponding allele products differing in one or a few net charge-changing amino acids were included. The assumption that such variations are indeed encoded by alleles at a single locus is supported by the observation that the relative frequencies of the homozygous and heterozygous genotypes do not deviate from Hardy-Weinberg expectations. Other types of variation are difficult to assess by electrophoresis of single individuals. During the course of this study we observed numerous qualitative and quantitative differences for which no homologues could be detected. These variations are difficult to evaluate, because genetically determined variants cannot be distinguished

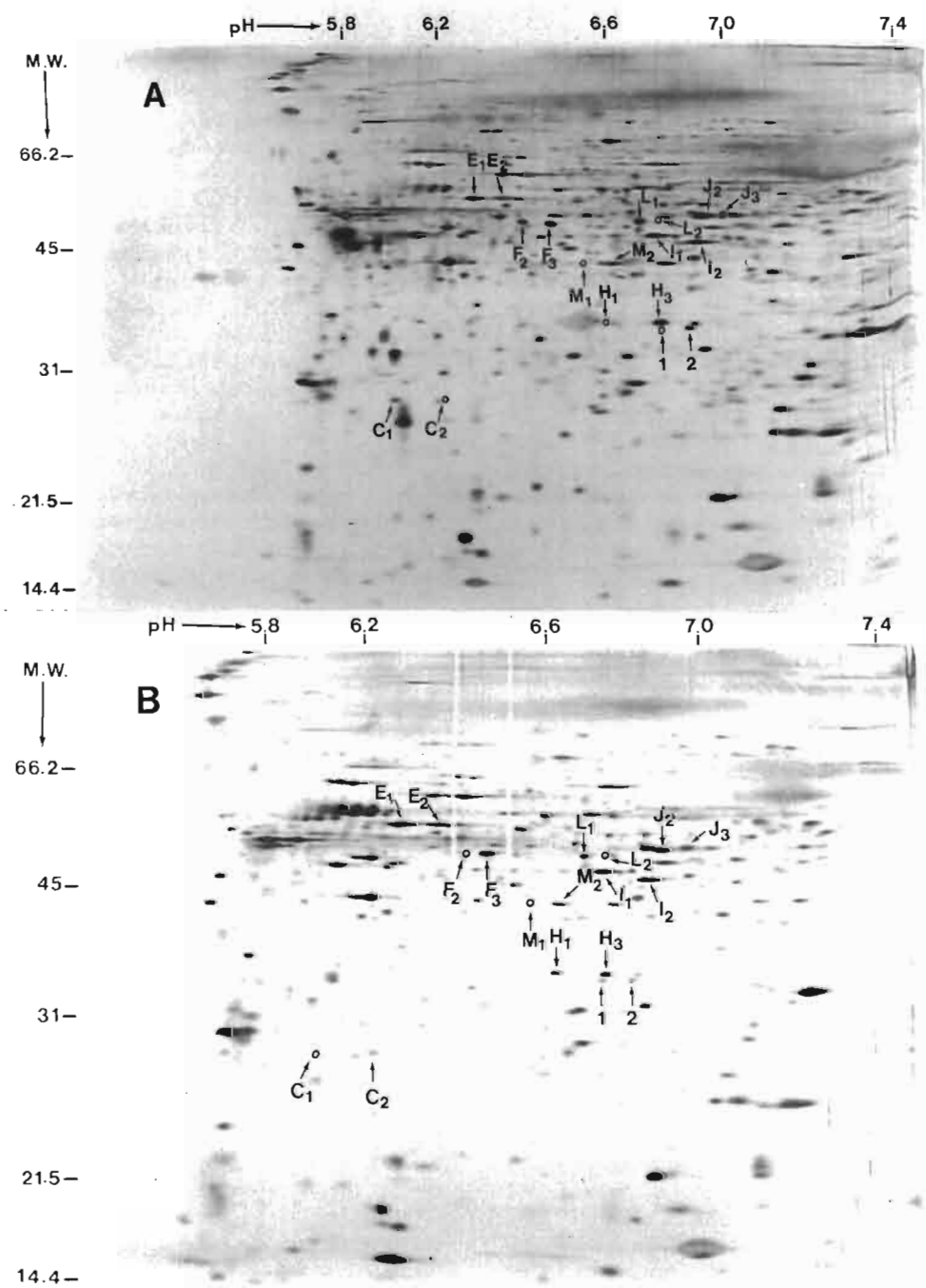

Fig. 1. Protein patterns of single individuals from *Globodera pallida* population ROOK. The positions of the isoelectric point variants (IP-variants) detected by the comparison of 35 individuals are shown. The capitals and numbers refer to the putative loci and alleles, respectively. The presumed genotypes for the individual shown in A are; C_1C_1, E_1E_2, F_2F_3, H_3H_3, I_1I_2, J_2J_2, L_1L_1, M_2M_2, and for the individual shown in B; C_2C_2, E_1E_2, F_3F_3, H_1H_3, I_1I_2, J_2J_3, L_1L_1, M_2M_2. The proteins indicated with arabic numbers (no. 1 and no. 2) are referred to in the text. Molecular masses are given in kilodaltons.

Table 1. Hardy Weinberg Equilibrium Test on presumed genotypes (1) detected in *G. pallida* population ROOK after mini 2-DGE of 35 indiduals.

	C_1C_1	C_1C_2	C_2C_2	E_1E_1	E_1E_2	E_2E_2	F_2F_2	F_2F_3	F_3F_3	H_1H_1	H_1H_3	H_3H_3	I_1I_1	I_1I_2	I_2I_2	J_2J_2	J_2J_3	J_3J_3	L_1L_1	L_1L_1	L_2L_2	M_1M_1	M_1M_2	M_2M_2
Observed	8	14	13	4	17	14	6	15	14	5	18	12	6	18	11	14	17	4	23	12	0	4	12	19
Expected	6.4	17.1	11.4	4.5	16.0	14.4	5.2	16.6	13.2	5.6	16.8	12.6	6.4	17.1	11.4	14.4	16.0	4.5	24	9.9	1	2.8	14.3	17.8
Total χ^2 (2)	1.17			0.014			0.325			0.177			0.087			0.116			0.53			0.388		

(1) The presumptive loci and alleles of the isoelectric point variants (IP-variants) are designated with capitals and arabic numbers, respectively.
(2) One degree of freedom. $\chi^2 = 3.84$ corresponds with the 5 % level of significance. Yate's correction was applied in case the expected number of one genotype was smaller than five (Ayala & Kriger, 1980).

Table 2. Allele frequencies of isoelectric point variants (IP-variants) (1) estimated by mini 2-DGE of single individuals and macro 2-DGE of a mixture of individuals (2).

	C_1	C_2	E_1	E_2	F_1	F_2	F_3	H_1	H_3	I_1	I_2	J_2	J_3	L_1	L_2	M_1	M_2
mini 2-DGE	0.43	0.57	0.36	0.64	0	0.38	0.62	0.40	0.60	0.43	0.57	0.64	0.36	0.83	0.17	0.29	0.71
macro 2-DGE (2)	0.35	0.65	0.23	0.77	0.03	0.29	0.68	0.42	0.58	0.30	0.70	0.59	0.41	n.d.	n.d.	n.d.	n.d.
95 % interval (3)	0.31-0.55		0.25-0.47			0.26-0.50		0.28-0.52		0.31-0.55		0.53-0.75		0.74-0.92		0.18-0.40	
pI (4)	5.96	6.20	6.23	6.29	6.32	6.42	6.48	6.62	6.78	6.71	6.84	6.90	6.97	6.69	6.77	6.56	6.63
MW (5)	28		60		54			36		50	49	56		53		42	
color (6)	r.		r.b.		r.b.			b.b.		r.b.		r.b.		r.b.		r.b.	

(1) The presumptive loci and alleles of the IP-variants are designated with capitals and arabic numbers, respectively.
(2) Data from Bakker *et al.*, 1992.
(3) Confidence intervals of the allele frequencies estimated by electrophoresing single individuals. Only the range of the first allele is given.
(4) Isoelectric points.
(5) Molecular masses in kilodaltons.
(6) Red (r.), reddish brown (r.b.) and blackish brown (b.b.).

Fig. 2. Details of protein patterns made by electrophoresing single individuals of *Globodera pallida* population ROOK. The positions of the isoelectric point variants (IP-variants) are designated with capitals and numbers referring to the putative loci and alleles, respectively. The presumed genotypes of the three individuals are; E_1E_2 (left), E_1E_1 (middle), E_2E_2 (right).

from artefacts due to, for example, experimental variations or differences in physiological or developmental stage.

Genetic variation in abundant proteins revealed by 2-DGE has been studied in organisms such as *Drosophila melanogaster,* (Leigh Brown & Langley, 1979), mice (Racine & Langley, 1980) and man (McConkey *et al.*, 1979; Rosenblum *et al.*, 1983). In these studies the average heterozygosity per locus based on variations in net charge ranged from 1 to 6 %. The variability of *G. pallida* population ROOK is not exceptional (5 %). The actual heterozygosity is evidently much higher. Only about 30 % of the amino acid substitutions result in a displacement in the isoelectric focusing dimension and other types of mutations resulting in qualitative or quantitative variations are not recorded. Quantitative and qualitative variations, for example due to mutations in regulatory genes, seem a prominent group among the variants resolved by 2-DGE. Klose (1982) studied inbred strains of mice and demonstrated that only 13 % of the protein variants resulted from changes in net charge and that the vast majority (87 %) of the variants were expressed as quantitative or qualitative differences.

In a parallel report we estimated the genetic relationships between potato cyst nematode populations by electrophoresing mixtures of individuals and using the ratio between the protein quantities of corresponding IP-variants as a measure for the allele frequencies (Bakker *et al.*, 1992). In this report we showed that this method is reasonably accurate. The genetic interpretation of the IP-variants was confirmed by electrophoresing single individuals (Table 1) and the two sets of allele frequency data were within a narrow range (Table 2). Evidently electrophoresing single individuals is more precise but is too laborious as an adjunctive technique for breeding for resistance (Bakker *et al.*, 1992)

Modern plant nematology is confronted with the need to analyse gene products from minute amounts of biological material. As shown here the mini 2-DGE system extends the possibilities of biochemical research in plant nematology. Mini 2-DGE allows the analysis of single individuals, but obviously can also be applied to other problems in plant nematology where a high sensitivity is required.

References

AYALA, F. J. & KIGER, J. A. (1980). *Modern genetics.* Benjamin/Cummings Publ. Co. 844 p.

BAKKER, J. & BOUWMAN-SMITS, L. (1988). Genetic variation in polypeptide maps of two *Globodera rostochiensis* pathotypes. *Phytopathology,* 78 : 894-900.

BAKKER, J., BOUWMAN-SMITS, L. & GOMMERS, F. J. (1992). Genetic relationships between *Globodera pallida* pathotypes in Europe assessed by using two dimensional gel electrophoresis of proteins. *Fund. appl. Nematol.,* 15 : 481-490.

BERGÉ, J. B., DALMASSO, A., PERSON, F., RIVOAL, R. & THOMAS, D. (1981). Isoesterases chez le nématode *Heterodera avenae.* 1. Polymorphisme chez différentes races françaises. *Revue Nématol.,* 4 : 99-105.

BRADFORD M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.,* 72 : 248-254.

CHAUDHURI, T. R. & GREEN, T. J. (1987). A sensitive urea-silver stain method for detecting trace quantities of separated proteins in polyacrylamide gels. *Preparative Biochem.,* 17 : 93-99.

DALMASSO, A. & BERGE, J. B. (1978). Molecular polymorphism and phylogenetic relationship in some *Meloidogyne* sp. : Application to the taxonomy of *Meloidogyne. J. Nematol.,* 10 : 323-332.

DE MORENO, M. R., SMITH, J. F. & SMITH, R. V. (1985). Silver staining of proteins in polyacrylamide gels : increased sensitivity through a combined coomassie blue-silver stain procedure. *Anal. Biochem.,* 151 : 466-470.

ESBENSHADE, P. R. & TRIANTAPHYLLOU, A. C. (1985). Use of enzyme phenotypes for identification of *Meloidogyne* species. *J. Nematol.,* 17 : 6-20.

KLOSE, J. (1982). Genetic variability of soluble proteins studied by two-dimensional electrophoresis on different inbred

mouse strains and on different mouse organs. *J. molec. Evolution,* 18 : 315-328.

LEIGH BROWN, A. J. & LANGLEY, C. H. (1979). Reevaluation of level of genetic heterozygosity in natural population of *Drosophila melanogaster* by two-dimensional electrophoresis. *Proc. natn. Acad. Sci. USA,* 76 : 2381-2384.

MANABE, T., VISVIKIS, S., STEINMETZ, J., GALTEAU, M. M., OKUYAMA, T. & SIEST, G. (1987). Systematic analysis of serum lipoproteins and apolipoproteins by a combined technique of micro two-dimensional electrophoresis. *Electrophoresis,* 8 : 325-330.

MCCONKEY, E. H., TAYLOR, B. J. & PHAN, D. (1979). Human heterozygosity : A new estimate. *Proc. natn. Acad. Sci. USA,* 76 : 6500-6504.

NEUHOFF, V., AROLD, N., TAUBE, D. & EHRHARDT, W. (1988). Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. *Electrophoresis,* 9 : 255-262.

NEUKIRCHEN, R. O., SCHLOSSHAUER, B., BAARS, S., JACKLE, H. & SCHWARZ, U. (1982). Two-dimensional protein analysis at high resolution on a microscale. *J. biol. Chem.,* 257 : 15229-15234.

OAKLEY, B. R., KIRSCH, D. R. & MORRIS, N. R. (1980). A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. *Anal. Biochem.,* 105 : 361-363.

O'FARRELL, P. H. (1975). High resolution two-dimensional electrophoresis of proteins. *J. biol. Chem.,* 250 : 4007-4021.

OHMS, J. P. & HEINICKE, D. H. K. (1985). Pathotypes of the potato cyst nematode. II. Identification of the pathotypes of *Globodera rostochiensis* by micro-2D-electrophoresis of single cysts. *J. Pl. Dis. & Protect.,* 92 : 225-232.

OHSAWA, K. & EBATA, N. (1983). Silver stain for detecting 10-femtogram quantities of protein after polyacrylamide gel electrophoresis. *Anal. Biochem.,* 135 : 409-415.

POEHLING, H. M. & NEUHOFF, V. (1980). One- and two-dimensional electrophoresis in micro-slab gels. *Electrophoresis,* 1 : 90-102.

POEHLING, H. M. & NEUHOFF, V. (1981). Visualization of proteins with a sliver " stain " : A critical analysis. *Electrophoresis,* 2 : 141-147.

POEHLING, H. M., WYSS, U. & NEUHOFF, V. (1980). Two-dimensional micro-electrophoresis of proteins from plant-parasitic nematodes : increased sensitivity of protein detection by silver staining or *in vivo* labelling. *Electrophoresis,* 1 : 198-200.

RACINE, R. R. & LANGLEY, C. H. (1980). Genetic heterozygosity in a natural population of *Mus musculus* assessed using two-dimensional electrophoresis. *Nature,* 283 : 855-857.

ROSENBLUM, B. B., NEEL, J. V., & HANASH, S. M. (1983). Two-dimensional electrophoresis of plasma polypeptides reveals " high " heterozygosity indices. *Proc. natn. Acad. Sci. USA,* 80 : 5002-5006.

SAKURAI, H., TSUTSUI, K., KOBAYASHI, K., & SHOHMORI, T. (1986). Microscale two-dimensional electrophoresis of human cerebrospinal fluid proteins. *Neurosciences,* 12 : 158-159.

SANDERINK, G. J., ARTUR, Y., PAILLE, F., GALTEAU, M. M., & SIEST, G. (1988). Micro-scale two-dimensional electrophoresis of alkaline phosphatase from serum. *Clin. Chem.,* 34 : 730-735.

TANAKA, T. & KAWAMURA, K. (1988). Cardiac muscle protein analysis by high-resolution and microscale two-dimensional gel electrophoresis. *Electrophoresis,* 9 : 94-100.