Protein variability in cereal cyst nematodes from different geographic regions assessed by two-dimensional gel electrophoresis

Michel Bossis and Roger Rivoal

Laboratoire de Recherches de la Chaire de Zoologie, INRA,
Centre de Recherches de Rennes, Domaine de la Motte-au-Vicomte,
B.P. 29, 35650 Le Rheu, France.

Summary – The protein variability of twelve populations of cereal cyst nematodes, originating from seven countries (four continents), was studied on three samples of white females per population, reared in controlled conditions on the same wheat, *Triticum aestivum* cv. Arminda. The pathogenic characteristics of each isolate were obtained from the literature and/or complementary host tests. Three electrophoreses were run for each protein sample with light modifications in migration (O' Farrell, 1975) and staining (Oakley *et al.,* 1980) techniques. 320 polypeptide spots were identified on all the populations tested. The protein patterns were compared by an image computing analysis with a Vax station (4000.60), a scanner (Eikonix, Kodak), and a Kepler software combination. Different levels of protein acceptance were applied, based on their volume and/or amplitude range. The similarity indices (F) and genetic distances (D =1-F) were calculated from identical spots. Dendrograms were built following the UPGMA method. Although some variability was observed between populations, clear genetic dissimilarities separate *H. avenae sensu stricto* from Gotland strain isolates. In the *H. avenae sensu stricto* group, two French populations and one isolate from South Australia were very similar. Specific proteins common to both French Ha12 and Ha12/Fr2 pathotypes were characterized. The use of image computing analysis to compare protein patterns is discussed.

Résumé – *Variabilité protéique chez les nématodes à kyste des céréales de différentes régions géographiques étudiées par l'électrophorèse en deux dimensions* – La variabilité protéique de douze populations de nématodes à kyste des céréales provenant de sept pays (quatre continents) a été étudiée à partir de trois échantillons par population, chacun constitué de 40 femelles blanches âgées de 50 jours, produites sur le même blé, *Triticum aestivum* cv. Arminda, en conditions contrôlées. La pathogénie de chaque isolat est précisée par référence à la littérature ou/et à des tests d'hôtes complémentaires. Trois électrophorèses sont réalisées par échantillon protéique avec de légères modifications apportées aux techniques de migration (O' Farrell, 1975) et de coloration (Oakley *et al.,* 1980). 320 polypeptides sont détectés sur l'ensemble des populations. Les profils protéiques sont comparés par analyse d'images informatisée à l'aide d'une station Vax (4000.60), d'un scanner (Eikonix, Kodak) et du logiciel Kepler (L.S.B. Corporation). Différents degrés de sévérité dans l'acceptation de la détection des spots ont été établis selon leur volume et/ou une gamme d'amplitudes. Les indices de similarité (F) et les distances génétiques (D = 1-F) sont calculés à partir des spots homologues. Les dendrogrammes correspondants sont construits selon la méthode UPGMA. Les résultats montrent une grande variabilité protéique entre populations et une séparation nette entre le groupe *Heterodera avenae sensu stricto* et le groupe Gotland. Dans le groupe *H. avenae sensu stricto*, les deux populations françaises et un isolat d'Australie du sud sont fortement apparentés. Des protéines spécifiques des deux pathotypes français Ha12 et Ha12/Fr2 sont caractérisées. La comparaison des profils protéiques par analyse d'images informatisée est discutée.

Key-words : Cereal cyst nematodes, *Heterodera avenae*, pathotypes, two-dimensional electrophoresis, protein patterns, image computing analysis, genetic distances, dendrograms.

A complex of cyst nematode species, more or less easy to identify, is found on cereal crops (Cook, 1982). *Heterodera avenae* Woll. is the dominant species, widespread in the world. Populations can be differentiated by differences in hatching cycles (ecotypes) and in virulence to the hosts (pathotypes) (Rivoal & Cook, 1993). From reactions to a panel of test cultivars of barley, oats and wheat, Andersen and Andersen (1982) proposed a new terminology for pathotypes instead of the letters, arabic or roman numerals used previously. This terminology took into account both the (a)virulence status in relation to three dominant genes found in different cultivars of barley (*Ha1* = cvs Drost and Ortolan, *Ha2* = cvs KVL 191 and Siri, *Ha3* = cv. Morocco) and, within each virulence group, the reactions of other differential hosts. Rivoal (1977) and later Sanchez and Zancada (1987) observed that reactions of oat cultivars could differentiate pathotypes of *H. avenae*. In the Andersen and Andersen (1982) classification, the French pathotypes Ha12 and Fr2 are identical in spite of differences be-

tween northern (Ha12) and southern (Fr2) populations in their ability to develop on several cultivars of spring oats (Rivoal, 1977). Several populations called the Gotland strain in Sweden and pathotype 3 in Great Britain, overcome the dominant resistant gene *Cre1* in the wheat Loros; they exhibit also some morphological differences with *H. avenae sensu stricto* (Cook, 1982).

The use of biochemical and molecular techniques is developing to characterize inter and intraspecific variability of plant parasitic nematodes. The electrophoresis of native proteins from cysts or second stage juveniles by isoelectric focussing (IEF) or of denatured proteins (SDS PAGE) permitted differentiation between species in the Heteroderidae family (Rumpenhorst, 1985; Bossis, 1991). Two-dimensional gel electrophoresis (2-DGE) was concurrently evaluated for distinguishing races and pathotypes in the potato cyst nematodes *Globodera* (Bakker & Gommers, 1982; Bakker, 1987) and in the soybean cyst nematode *H. glycines* Ichinohe (Ferris *et al.*, 1986). Protein patterns from populations of cereal cyst nematodes attributed to *H. avenae s. str.* and to the Gotland strain revealed greater differences than previously described from the virulence and morphological data (Ferris *et al.*, 1989). Nevertheless, the use of proteins for diagnosis of pathotypes in each group of populations was as inconsistent as was for *H. glycines* and *Globodera pallida* (Ferris *et al.*, 1986; Bakker & Bouwman-Smits, 1988 *a*).

Comparisons of protein patterns obtained by 2-DGE by a computing analysis procedure allowed us to study the genetic variability in populations of cereal cyst nematodes with the aim of characterizing specific proteins for virulence types. The present publication gives the result from a set of isolates from distinct geographic origins (Asia, Australia, North Africa, Europe). They were compared to three populations from Sweden belonging to *H. avenae s. str.* and to the Gotland strain previously studied by Ferris *et al.* (1989).

Material and methods

Populations

We compared twelve populations originating from seven countries : Algeria, Australia, Bulgaria, France, India, Spain and Sweden (Table 1). They were obtained from the Institut National Agronomique, El Harrach, Algeria; Department of Crop Protection, The University of Adelaïde, Glen Osmond, Australia; Plant Protection Institute, Kostinbrod, Bulgaria; C.S.I.C., Centro de Ciencias Medioambientales, Madrid, Spain; Institut National de la Recherche Agronomique, Le Rheu, France; Division of Nematology, IARI, New Delhi, India; Departement of Plant Protection Sciences, Swedish Univesity of Agricultural Sciences, Alnarp, Sweden.

For each isolate, a sample of 50 to 100 cysts was kept at 8 °C to obtain hatched second stage juveniles. The

Table 1. *Origin of the cereal cyst populations used.*

Location	Country (region)	Pathotype*
Sidi Hosni	Algeria (Tiaret)	Ha41
South Australia	Australia	Ha13
Karnobat	Bulgaria (South)	Gotland strain
Santa Olalla	Spain (Toledo)	Ha71
Torralba de Calatrava	Spain (Ciudad Real)	Gotland strain
St-Christophe-le-Jajolet	France (Normandie)	Ha12
St-Georges-du-Bois	France (Charentes-Poitou)	Ha12/Fr2
Chezelles	France (Centre)	Ha??
Najafgarh	India (Delhi)	Ha?1
Ask	Sweden (Skåne)	Ha11
Våxtorp	Sweden (Halland)	Ha?3
Etelhem	Sweden (Gotland)	Gotland strain

* Nomenclature of *Heterodera avenae* pathotypes according to Andersen and Andersen (1982).

susceptible wheat cultivar Arminda (*Triticum aestivum* L.), cultivated on 2 % agar in Petri dishes (Mugniéry & Person, 1976), was inoculated with the juvenile nematodes of each isolate. The inoculum per plant varied from 24 to 48 juveniles according to the demonstrated or presumed ability to produce females (Rivoal & Person-Dedryver, 1982). The seedlings were grown at 15 ± 1 or $17 °C \pm 1 °C$, depending on the geographical origin of the isolates (Table 2). The numbers of inoculated plants ranged from 16 to 30 per nematode isolate. The white females were counted and harvested 50 days after inoculation. Some populations whose virulence was unknown were tested using a reduced range of differential hosts in the same conditions. White females from these pathotype identifications were counted 60 days after inoculation, but not used for protein extraction.

Preparation of protein samples

White females were handpicked with a needle from roots under the dissecting microscope, and placed on wet filter paper in a Petri dish. Plant fragments and secretions attached near the head were discarded with forceps and a needle. For each population, three samples of 40 white females were collected at random. Females were washed three times in 100 µl of 10 mM Tris HCl, pH 7.4 buffer and crushed in a mixture of 10 µl of

Table 2. *Reactions of differential hosts to various populations of cereal cyst nematodes, established from the literature or complementary tests.*

Differential hosts	H. avenae sensu stricto pathotypes									Gotland strain	
	Ha11 Ask	Ha41 Sidi Hosni	Ha71 Santa Olalla	Ha12 St-Christophe-le-Jajolet	Ha12/Fr2 St-Georges-du-Bois	Ha13 South Australia	Ha?3 Vâxtorp	Ha?1 Najafgarh	Ha?? Chezelles	Etelhem	Karnobat
	Ferris et al., 1989		Sanchez & Zancada, 1987			Andersen & Andersen, 1982	Ireholm 1994	Swarup et al., 1979		Ferris et al., 1989 Ireholm, 1994	
Barley											
Ingrid/Varde/ Emir	S*	4.1**	S	5.0	2.5	0.2 S	S	S***	1.3	S	8.2
Drost/Ortolan *(Ha1)*	R	0.1	R	2.7	2.6	0.7 S	S	R	3.6	S	4.0
KVL 191/Siri *(Ha2)*	R	1.1	R	0.1	0.8	0.2 S	S	R	1.7	S	1.6
Morocco C.I. 3902 *(Ha3)*	R	.0	R	ND	ND	ND R	R	R	ND	(R)	0.8
Oats											
– Winter type Peniarth/Prieuré	ND	1.8	ND	6.2	3.7	1.5 ND	ND	ND	0	ND	2.8
– Spring type Noire de Moyencourt/ Manoire Nidar	S	0	R	7.8	0.7	0.9 S	S	ND	2.0	S	5.8
Wheat											
Capa/Prins	S	4.1	S	2.0	6.4	1.3 S	S	S	5.7	S	8.6
Loros *(Cre1)*	R	0.1	R	0	0	0.3 [R]	R	ND	0	S	6.4

* S : susceptible; (R) : moderately resistant; R : resistant; ND : not determined (from the literature).

** Higher average number of white females per cultivar and plant, cultivated in Petri dish; eight to ten replicates (from complementary tests).

*** Host reactions to this isolate are referred Pathotype No. 1 in India (Kaushal, pers. comm.).

the same buffer plus 20 µl of the homogenizing solution with urea, ß mercaptoethanol and ampholytes (Bossis & Mugniéry, 1993). The concentration of the protein sample obtained by centrifugation was estimated by the Bradford's technique (1976). 2-DGE were run according to O'Farrell (1975) and gels were stained according to Oakley *et al.* (1980) with modifications (Bossis & Mugniéry, 1993). Three electrophoreses were run for each sample (nine for each population). Molecular weights were estimated with a calibration kit (Pharmacia) containing : phosphorylase b (94.0 kD), bovin serum albumin (67.0 kD), ovalbumin (43.0 kD), carbonic anhydrase (30.0 kD), soybean trypsin inhibitor (20.1 kD) and α-lactalbumin (14.4 kD). Gels were dried in pairs between two sheets of cellophane, washed in 20 % ethanol water solution and stretched with clips on a 20 × 25 cm frame.

ANALYSIS OF PROTEIN PATTERNS

Gels were digitized (2048 × 2048 elements) by means of a scanner (Eikonix EC78/99 Kodak). The focus was adjusted so that the resolution in the object plane was 100 µm per pixel. Images were compared on a Vax station 4000.60 computer (Digital Equipment Corporation) with the Kepler software (Large Scale Biology Corporation, Rockville, USA). For each image, the zone of interest was defined by eliminating of the left part (acid part without spot) and the top part of the right zone in which overlapping spots made modelling impossible. The first step of this process produces a digitized

image (4 Mb) stored in an image file. Image files were subjected to a sequence of image processing procedures that removed the background, streaks and sharp noise. Then, for each image, a spot file was generated (20 Kb) by optimizing the parameters of a set of 2D Gaussian distributions so that they approximate as closely as possible the peaks detected in the processed image. The following parameters were used : *x, y, sx, sy* and *amp* (*x* and *y* = the *x* and *y* position of the spot in the image, *sx* = the *x* width, *sy* = the *y* width, *amp* = the amplitude or density of the spot). The fitting process optimized the parameters by minimizing the sum of squares differences between the model and the processed image. The amplitude depends on the optical density (O.D.) of each element of the spot and was translated on a scale from 0 (O.D. = 0.0) to 255 (O.D. = 1.5). The volume of each spot was calculated according to the equation : $vol. = 2 \times \pi \times amp.sx \times sy$. Spot files were used in a setting depending on the experimental plan with a master (image of reference). This allowed numerous comparisons at different levels : inside populations and between populations. The three gels having the best quality of image were selected for each population. Five levels of protein acceptance were applied :

– All spots detected.
– Spots with the amplitude > 5.
– Spots with the amplitude > 7.5.
– Spots with the amplitude > 10.
– Spots with the amplitude > 5 and volume > 500.

Moreover, at the level where a spot was accepted as being specific to one population, two degrees of severity were tested : present in the three gels or only in two gels.

The number of spots shared between two populations was taken into account in the estimation of the similarity index *F*, according to $F = 2Nxy/(nx+ny)$ in which *nx* and *ny* were the total number of protein spots scored for population *x* and *y;* and *Nxy* the number of spots shared by *x* and *y*. The genetic distance was $D = 1-F$ (Aquadro & Avise, 1981). Dendrograms were constructed with a UPGMA algorithm (Unweighted Pair-Group Method using Arithmetic averages), calculated with SAS software (SAS Institut INC, Cary, North Carolina, USA).

Results

Depending on the presence or absence of an underbridge in the cyst vulval cone and reactions of differential hosts observed in our tests or as reported in the literature, populations were divided into two groups : 1) *H. avenae s. str.* with different pathotypes and 2) Gotland strain (Table 2). Virulence features were unknown for the Spanish Torralba de Calatrava which is assumed to belong to the Gotland strain group based on morphometric and therefore is not given in Table 2 (Valdeolivas & Romero, 1990). All populations tested showed a real virulence to wheat cv. Arminda with numbers of white
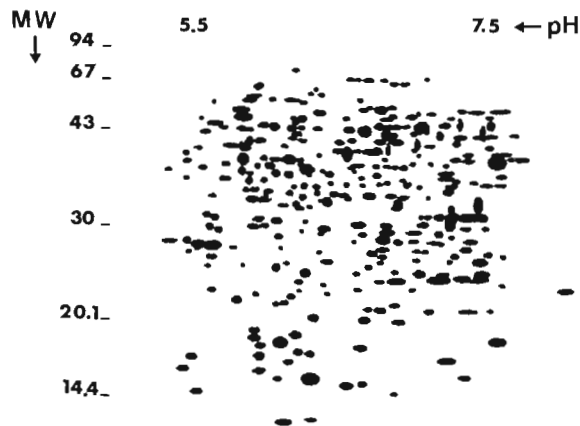

Fig. 1. *Synthetic image of the master with all spots identified in the twelve populations of cereal cyst nematodes.*

females per plant ranging from 2.7 ± 2.41 (Torralba de Calatrava) to 8.4 ± 3.96 (Etelhem).

For all populations, 320 protein spots were identified on the synthetic pattern based on the selected spots (Fig. 1). Table 3 gives the number of spots selected at different levels of filtering when they were present in two of three gels. Discarding spots of amplitude < 5 obviously increased the average number of matched spots. It suppressed also several instances of ambiguous matching between spots. The selection of the zone of interest on gels for the computing analysis and the level of filtering severity on homologous spots amplify the relative genetic distances. Table 4 gives the number of major spots selected for each population, the number of shared spots (above the diagonal) used to estimate the similarity coefficients *(F)* and the genetic distances *(D)* (below the diagonal).

The smaller genetic distances (*D* = 0.120 to 0.183) concern the populations St-Christophe-le-Jajolet, St-Georges-du-Bois, South Australia, Santa Olalla, Ask, Chezelles and Sidi Hosni placed in *H. avenae s. str.* Greater genetic distances are found in comparisons of different pathotypes of *H. avenae* and the Gotland strain populations (Etelhem, Karnobat). The largest distance (*D* = 0.258 to 0.276) are between the Indian population Najafgarh and different pathotypes of *H. avenae* (Sidi Hosni, Ask) and the Gotland strain isolates (Torralba de Calatrava, Etelhem), as well as the Gotland strain populations (Etelhem, Karnobat) and *H. avenae* (Ask). These distance comparisons are reflected in the similarity dendrograms constructed following the UPGMA analysis of protein spots > 5 amplitude. The values of the normalized root-mean-square distances are given under the branch lengths. All populations belonging to *H. avenae s. str.* are grouped together, despite substantial genetic differences, in comparison between the most of isolates that were reflected in clusters 4, 5, 7, 9, 10 (Fig. 2). The two French populations St-Christophe-le-

Table 3. *Number of matched proteins extracted from white females in different populations of cereal cyst nematodes, according to different levels of filtering.*

Populations	Without elimination	amp > 5	amp > 7,5	amp > 10	amp > 5 vol > 500
St-Georges-du-Bois	250	281	242	228	245
St-Christophe-le-Jajolet	300	294	279	260	265
Sidi Hosni	216	250	193	160	192
Etelhem	205	231	168	157	170
Våxtorp	207	225	181	151	172
Ask	211	233	164	154	176
Chezelles	201	237	182	158	188
Torralba de Calatrava	204	226	168	151	171
Santa Olalla	229	250	201	179	200
Najafgarh	200	214	156	135	164
South Australia	229	251	190	166	197
Karnobat	208	225	169	158	178
Means	222	243	191	171	193
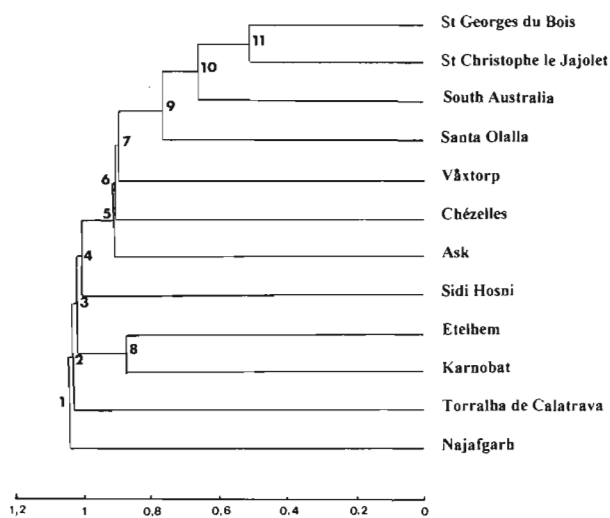
Fig. 2. *Similarity dendrogram of twelve cereal cyst nematodes populations constructed from the genetic distances following the UP-GMA method without spots of amplitude < 5. The cluster numbers are given with arabic numerals.*

Jajolet and St-Georges-du-Bois cluster together (cluster 11) with the South Australian isolate nearby (cluster 10). The Algerian Sidi Hosni is genetically the farthest isolate in this grouping (cluster 4). The Gotland strain populations, Swedish Etelhem and Bulgarian Karnobat are clustered together (cluster 8); the Spanish isolate Torralba de Calatrava is quite distant (cluster 2). The Indian population Najafgarh shows the highest normalized root-mean-square distance (cluster 1).

Examples of typical protein patterns obtained from six different populations of cereal cyst nematodes are shown in Figure 3. Protein spots which might be specific to populations or pathotypes are indicated in lower case letters. For example, the two French populations are easily distinguished by a vertical row of three protein spots : " a ", " b ", " c ", very conspicuous in pathotype Ha12/Fr2 (St-Georges-du-Bois) with mean volume : 7514, 7081, 3896 respectively, and absent or very faint in pathotype Ha12 (St-Christophe-Le-Jajolet); the spots " d ", " e ", " f ", " g " and " h " are specific to the former pathotype and the spots " i ", " j ", " k " to the latter (Fig. 3 A and 3 B). The spot " l " (Fig. 3 C, D) seems to be an isoelectric point variant (I.P. variant) specific to both Santa Olalla and South Australia but " m ", " n " and " o " are observed only in the South Australia isolate. Arrows (Fig. 3 D, F) indicate that very conspicuous proteins are lacking in South Australia and Etelhem populations. Compared to other protein patterns, the spot " r " (Fig. 3 F) seems to be also an I.P. variant, with the spots " s " and " t " present only in the Etelhem isolate. Though they are also present in other isolates, " p " and " q " spots are expressed in the Santa Olalla isolate in greater volume and could therefore be diagnostic (Fig. 3 C).

Table 4. *Number of proteins shared (above diagonal) and genetic distance (below diagonal) between populations of cereal cyst nematodes.*

Populations	Number of spots	St-Georges-du-Bois	St-Christophe-le-Jajolet	Sidi Hosni	Etelhem	Våxtorp	Ask	Chezelles	Torralba de Calatrava	Santa Olalla	Najafgarh	South Australia	Karnobat
St-Georges-du-Bois	281		253	212	200	199	201	203	195	220	189	223	197
St-Christophe-le-Jajolet	294	0,120		223	210	210	217	217	208	232	201	235	210
Sidi Hosni	250	0,202	0,180		180	177	182	189	181	189	168	193	178
Etelhem	231	0,219	0,200	0,252		173	172	178	173	182	165	191	183
Våxtorp	225	0,213	0,191	0,255	0,241		177	180	169	186	165	186	173
Ask	233	0,218	0,176	0,246	0,259	0,227		184	174	183	164	191	168
Chezelles	237	0,216	0,183	0,224	0,239	0,221	0,217		175	190	171	190	173
Torralba de Calatrava	226	0,231	0,200	0,239	0,243	0,251	0,242	0,244		177	163	180	169
Santa Olalla	250	0,171	0,147	0,244	0,243	0,217	0,242	0,220	0,256		174	205	187
Najafgarh	214	0,236	0,209	0,276	0,258	0,248	0,266	0,242	0,259	0,250		179	166
South Australia	251	0,162	0,138	0,230	0,207	0,218	0,211	0,221	0,245	0,182	0,230		185
Karnobat	225	0,221	0,191	0,251	0,197	0,231	0,266	0,251	0,251	0,213	0,244	0,223	

Discussion

TECHNIQUE

The very low variability between gels for a geographic isolate of nematodes indicates an adequate sampling of adult white females (40 per isolate) for the extraction of proteins. This sampling process reduced the great variation observed between single females of *G. pallida* (de Boer *et al.*, 1992 *b*). The production of females from each population on the same host, their harvest and treatment, systematically 50 days after inoculation, also certainly minimized variations in quality of proteins which could change according to the host plant (Greet & Firth, 1977) and with the developmental stage of the nematodes (de Boer *et al.*, 1992 *a*). It should be noted that Bakker and Bouwman-Smits (1998 *a*) did not observe an influence of host on protein patterns of *G. rostochiensis* pathotypes.

Ferris *et al.* (1989) gave the first results on protein variability in cereal cyst nematodes comparing 2-DGE patterns with a maximum of 130 consistent protein spots per isolate. Our data revealed in the same group of nematodes a higher number of polypeptides, 320. They were certainly the direct result of modifications to the O'Farrel's electrophoresis method, mainly bisacryla

mide replaced by piperazine di-acrylamide in the preparation of gels for the first and the second dimension and the use of sodium thiosulfate in gels of second dimension as well as modifications in the staining method (Bossis & Mugniéry, 1993).

The use of the LSB's Kepler software enhanced the discriminating analyses of the protein profiles. The synthetic images established from all the populations tested allowed simultaneous comparisons of a higher number of gels than *de visu*. On each image all spots matched to their presumed homologues in the reference pattern were numbered and could be easily referenced. These synthetic images provided a data base for a further comparison of protein variability inside this group of nematodes and with other species or genera. In addition, a distance matrix derived by UPGMA was used to construct similarity dendrograms (Felsenstein, 1988).

PROTEIN VARIABILITY

From their data on *H. glycines,* Ferris *et al.* (1986) suggested that the 2 DGE protein analysis was a better method than one-dimensional isozyme tests to visualize and measure relative evolutionary divergence among isolates across a broad spectrum of proteins, although one-dimensional isozyme test might be preferable for
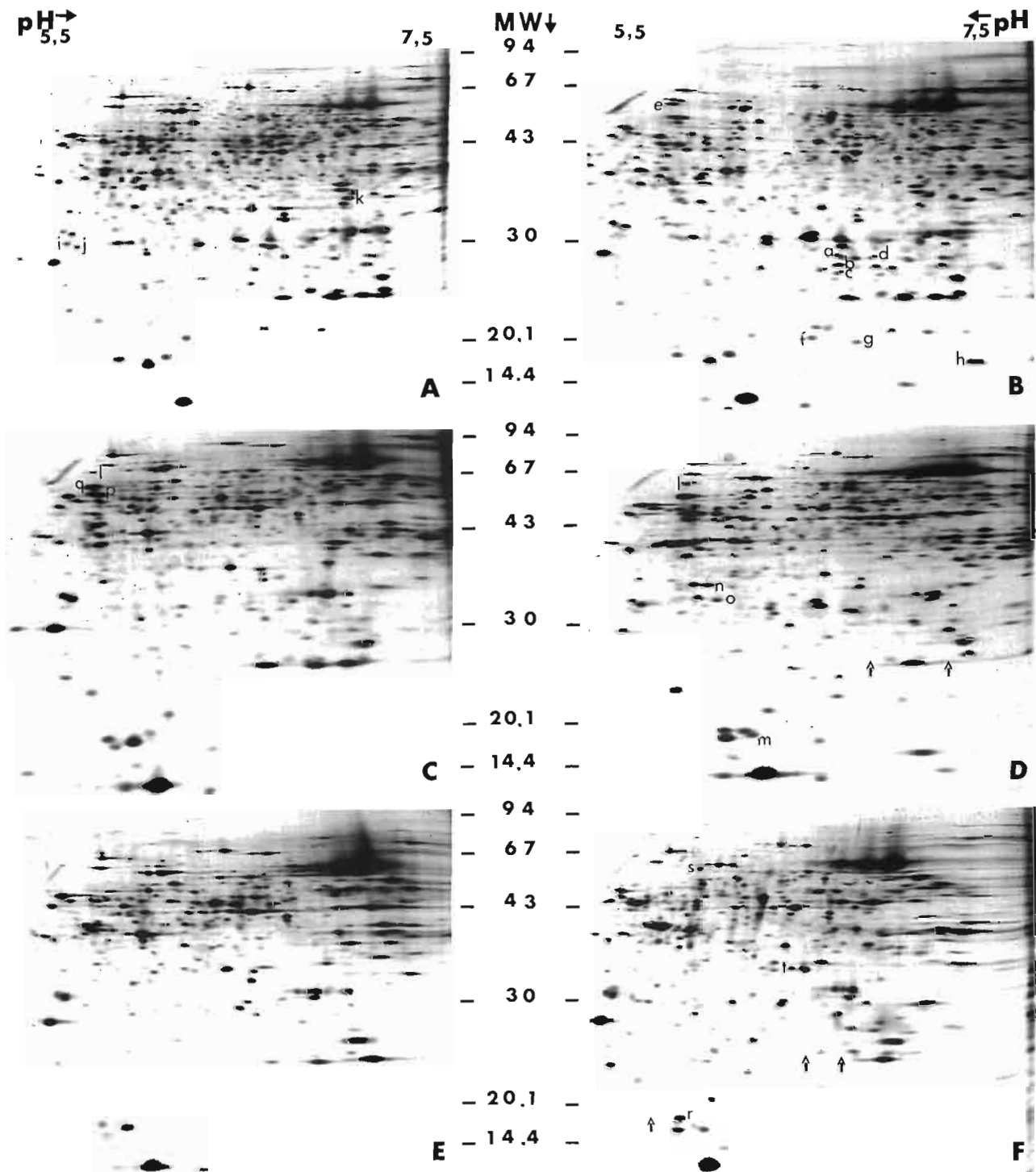
Fig. 3. *2-DGE protein patterns of white females of cereal cyst nematodes : A and B : French populations, St-Christophe-le-Jajolet (Ha12) and St-Georges-du-Bois (Ha12/Fr2) respectively; C : Spanish population, Santa Olalla (Ha71); D : South Australian population (Ha13); E, F : Swedish populations, Våxtorp (Ha?3) and Etelhem (Gotland strain), respectively. Lower case letters and arrows indicate main differences referred in the text.*

routine identification of populations. Later, Ferris *et al.* (1989) were unable to find consistent protein pattern differences diagnostic for pathotypes for the cereal cyst nematode isolates they examined. Nevertheless, the use of micro 2-DGE had proved useful to study the protein polymorphism in single females of *Globodera pallida* (de Boer *et al.*, 1992 *b*).

In our data the grouping of *H. avenae s. str.* isolates was convincing in spite of degrees of genetic dissimilarity which may reflect adaptative and selective features in response to their respective ecological niches. Even though the isolates originated from distinct geographic areas, the protein pattern data were in agreement with their morphological and virulence characteristics. In addition, these data confirmed that the Etelhem isolate of the Gotland strain differs from strict *H. avenae* (Ferris *et al.*, 1989). The Bulgarian population Karnobat was similar to the Swedish population in proteins and had the same morphological particularities including cyst color and the presence of a distinct underbridge in the cyst vulval cone. Neither isolate hybridized with several French pathotypes of strict *H. avenae* (Person-Dedryver, pers. comm.). This comparison might establish a more convincing example of synchrony between small morphological changes, protein similarities, and genetic drift than in solanaceous *Globodera* species (Bakker & Bouwman-Smits, 1988 *b*). Even though they developed well on wheat, the Gotland strain from Spain (Torralba de Calatrava) and the Indian isolate Najafgarh appeared to be genetically different from the rest. A detailed morphological and biometrical study indicated similarity of the Spanish population to the Gotland strain group (Valdeolivas & Romero, 1990). But in contrast, based on reactions of differential hosts, Andersen and Andersen (1982) still considered there was little doubt that the Indian populations really differ from European pathotypes of *H. avenae*.

Previous successful hybridizations between French Ha41, Ha12/Fr2 and Australian Ha13 pathotypes demonstrated that they belong to the strict *H. avenae* species (Person-Dedryver, pers. com.). Andersen and Andersen (1982) considered the Australian pathotype quite different from European *H. avenae* populations, partly overcoming the gene *Cre1* in wheat Loros (20 % of cysts that occur on the susceptible cultivars), exactly as in our host tests (Table 2). So, it was surprising to find the Australian isolate so similar to the French populations. A direct analysis of autoradiographic protein patterns had not shown such a closed relation between two Australian isolates and strict *H. avenae* from Sweden and USA isolates (Ferris *et al.*, 1994). The similarity between the Australian and French populations of *H. avenae* could be explained by the hypothesis that *H. avenae* was imported to the south of the Australian continent in soil originating from Northern Europe (Fisher, pers. comm.). In contrast, the greatest genetic

distance found for the Algerian isolate (Sidi Hosni), with a virulence typical of strict *H. avenae,* raised questions concerning the species identity in the cyst samples analyzed. Where cereal crops are not completely controlled for weeds, a community of cultivated and wild gramineous plants could establish a complex of cereals cyst nematode species. The grass cyst nematode *Heterodera mani* is morphologically indistinguishable from *H. avenae* upon cyst characteristics but does not develop on wheat, barley and oats (Mathews, 1971). But Cook (1982) observed in different populations of this species a variation of host reactions in barley cultivars.

The speculative prospects in using 2-DGE to separate pathotypes based on the biochemical composition of females were tempered when inconsistent relations between protein variants and (a)virulence genes were found in *G. rostochiensis* and *G. pallida* populations (Bakker & Bouwman-Smits, 1988 *a*; Bakker *et al.*, 1992). Furthermore the genetic distances within pathotypes were not necessarily smaller than between pathotypes. The main explanations for this situation were given by several authors : 1) the doubt about the translation of (a)virulence genes in reliable protein, 2) the analysis of a limited portion of proteins expressed from the genome, 3) the genes for virulence may not be expressed at the female development stage. Nevertheless, Dalmasso *et al.* (1991) observed that a pair of near-isogenic lines of *Meloidogyne incognita,* virulent and avirulent to gene Mi in tomato could be differentiated by one peptide related to the avirulence feature. In addition, based on protein patterns, the two French populations St-Christophe-le-Jajolet and St-Georges-du-Bois seemed to differ by the presence or absence of several spots, in agreement with their virulence reactions to spring oat cultivars.

Previous results with 2-DGE in plant parasitic nematodes were based mainly on a qualitative evaluation of divergence between populations. A higher number of protein spots produced with a more accurate electrophoretic technique on larger polyacrylamide gels combined with the application of the computing image analysis, might lead to a better prospect for finding diagnostic protein markers in the future. As this combined procedure could allow more populations to be tested, protein differences between pathotypes might be sought in nematode lines previously selected for their ability to overcome different genes of resistance (Bakker *et al.*, 1992). Future investigations will concern protein variability between and within pathotypes of *H. avenae* occuring in France.

References

ANDERSEN, S. & ANDERSEN, K. (1982). Suggestions for determination and terminology of pathotypes and genes for resistance in cyst-forming nematodes, especially *Heterodera avenae*. *EPPO Bull.*, 12 : 379-386.

AQUADRO, C. F. & AVISE, J. C. (1981). Genetic divergence between rodent species assessed by using two-dimensional electrophoresis. *Proc. natn. Acad. Sci. USA*, 78 : 3784-3788.

BAKKER, J. (1987). *Protein variation in cyst nematodes.* Thesis, Landbouwuniversiteit, Wageningen, Nederlands, 159 p.

BAKKER, J. & BOUWMAN-SMITS, L. (1988 *a*). Genetic variation in polypeptide maps on two *Globodera rostochiensis* pathotypes. *Phytopathology*, 78 : 894-899.

BAKKER, J. & BOUWMAN-SMITS, L. (1988 *b*). Contrasting rates of protein and morphological evolution in cyst nematode species. *Phytopathology*, 78 : 900-904.

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. *Fundam. appl. Nematol.*, 15 : 481-490.

BAKKER, J. & GOMMERS, F. J. (1982). Differentiation of the potato cyst nematodes *Globodera rostochiensis* and *G. pallida* and of two *G. rostochiensis* pathotypes by means of two-dimensional electrophoresis. *Proc. K. nederl. Akad. Wetensch., Ser. C*, 85 : 309-314.

DE BOER, J. M., OVERMARS, H. A., BAKKER, J. & GOMMERS, F. J. (1992 *a*). Analysis of two-dimensional protein patterns from developmental stages of the potato cyst-nematode, *Globodera rostochiensis. Parasitology*, 105 : 461-474.

DE BOER, J. M., OVERMARS, H. A., BOUWMAN-SMITS, L., DE BOEVERE, M., GOMMERS, F. J. & BAKKER, J. (1992 *b*). Protein polymorphisms within *Globodera pallida* assessed with mini two-dimensional gel electrophoresis of single females. *Fundam. appl. Nematol.*, 15 : 495-501.

BOSSIS, M. (1991). Discrimination entre *Heterodera carotae* et *H. cruciferae* par l'électrophorèse (SDS PAGE) de protéines solubles extraites à partir de kystes individualisés. *Revue Nématol.*, 14 : 325-327.

BOSSIS, M. & MUGNIÉRY, D. (1993). Specific status of six *Globodera* parasites of solanaceous plants studied by means of two-dimensional gel electrophoresis with a comparison of gel patterns by a computed system. *Fundam. appl. Nematol.*, 16 : 47-56.

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

COOK, R. (1982). Cereal and grass hosts of some gramineous cyst nematodes. *EPPO Bull.*, 12 : 399-411.

DALMASSO, A., CASTAGNONE-SERENO, P., BONGIOVANNI, M. & DE JONG, A. (1991). Acquired virulence in the plant parasitic nematode *Meloidogyne incognita*. 2. Two-dimensional analysis of isogenic isolates. *Fundam. appl. Nematol.*, 14 : 305-308.

FELSENSTEIN, J. (1988). Phylogenies from molecular sequences : inference and reliability. *A. Rev. Genetics*, 22 : 521-565.

FERRIS, V. R., FERRIS, J. M., MURDOCK, L. L. & FAGHIHI, J. (1986). *Heterodera glycines* in Indiana : III. 2-D protein patterns of geographical isolates. *J. Nematol.*, 18 : 177-182.

FERRIS, V. R., FAGHIHI, J., IREHOLM, A. & FERRIS, J. M. (1989). Two-dimensional protein patterns of cereal cyst nematodes. *Phytopathology*, 79 : 927-933.

FERRIS, V. R., FERRIS, J. M., FAGHIHI, J. & IREHOLM, A. (1994). Comparisons of isolates of *Heterodera avenae* using 2-D PAGE protein patterns and ribosomal DNA. *J. Nematol.*, 26 : 144-151.

GREET, D. N. & FIRTH, J. (1977). Influence of host plant on electrophoretic protein patterns of some round-cyst nematode females and use of larvae to obtain less ambiguous results. *Nematologica*, 23 : 411-415.

IREHOLM, A. (1994). Characterization of pathotypes of cereal cyst nematodes, *Heterodera* spp., in Sweden. *Nematologica*, 40 : 399-411.

MATHEWS, H. J.P. (1971). Two new species of cyst nematode, *Heterodera mani* n. sp. and *H. iri* n. sp., from northern Ireland. *Nematologica*, 17 : 553-565.

MUGNIÉRY, D. & PERSON, F. (1976). Méthode d'élevage de quelques nématodes à kystes du genre *Heterodera. Sci. agron., Rennes*, 217-220.

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

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

RIVOAL, R. (1977). Identification des races biologiques du nématode à kystes des céréales *Heterodera avenae* Woll., en France. *Annls. Zool., Ecol. anim.*, 9 : 261-272.

RIVOAL, R. & COOK, R. (1993). Nematode pests of cereals. *In :* Evans, K., Trudgill, D. L. & Webster, J. M. (Eds). *Plant parasitic nematodes in temperate agriculture.*, Wallingford, UK, CAB International : 259-303.

RIVOAL, R. & PERSON-DDRYVER, F. (1982). Caractérisation des pathotypes d'*Heterodera avenae* en France : influence de la période de culture sur le pouvoir discriminant de cultivars d'*Avena sativa* et différences dans la capacité à former des femelles. *EPPO Bull.*, 12 : 387-391.

RUMPENHORST, H. J. (1985). Vergleichende elektrophoretische Untersuchungen von Proteinen einiger Zystennematoden von Getreide und Gräsern. *Beitr. Nematodensforsch. Inst. Nematol. 25 Jahre Münster-Gievenbeck :* 64-74.

SANCHEZ, A. & ZANCADA, M. C. (1987). Characterization of *Heterodera avenae* pathotypes from Spain. *Nematologica*, 33 : 55-60.

SWARUP, G., SETHI, C. L., SESHADRI, A. R. & KAUSHAL, K. K. (1979). On the biotypes of *Heterodera avenae,* the causal organism of " Molya " disease of wheat and barley in India. *Indian J. Nematol.,* 9 : 164-168.

VALDEOLIVAS, A. & ROMERO, M. D. (1990). Morphometric relationships of some members of the *Heterodera avenae* complex (Nematoda : Heteroderidae). *Nematologica,* 36 : 292-303.