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A PATHOGENIC AND BIOCHEMICAL COMPARISON OF TWO SPANISH POPULATIONS OF THE CEREAL CYST NEMATODE

by
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Summary. A series of pathogenicity tests, biochemical (2-D PAGE protein patterns, isozymes) and molecular (-RAPD/PCR) techniques, were used to compare two Spanish populations of the cereal cyst nematode from Santa Olalla (P1) and from Torralba de Calatrava (P2). Previous morphometric studies defined P1 as a member of the strict *Heterodera avenae* group and P2 as a member of the "Gotland strain" or "British pathotype 3" of *H. avenae.* The two populations were found to belong to the resistance groups Ha1 and Ha2 respectively. The 2-D protein patterns of the two populations differed by at least 12 polypeptides. Of the eight isozyme systems analyzed, six differed in number, concentration and relative mobility of bands. Catalase isozymes are suggested as an enzymatic system for routine use in the biochemical characterization of nematodes. Of 43 markers obtained by the RAPD technique, 31 showed differences between the two populations. These results, along with the differences described for these populations in a previous morphometric study, support the idea that P1 and P2 belong to specific groups. They also help to observe genetic dissimilarity between some members of the *H. avenae* complex.

The *Heterodera avenae* complex is formed by a group of species that attack cereals and grasses. The most representative species is *H. avenae* Woll., because of its wide distribution and economic importance. Some of the species can easily be distinguished by their morphology, whilst some show few morphological differences. Further, the "Gotland strain" (Andersson, 1973) or the "British pathotype 3" (Cook, 1975) of *H. avenae* differ from strict *H. avenae* in morphology and host range and could be considered as different species (Romero, 1982, Stone and Hill, 1982).

A morphometric study by Valdeolivas and Romero (1990) on eight Spanish, three British, and two Swedish populations, allowed their classification into two groups: the strict *H. avenae* and the "Gotland strain" of *H. avenae.* The Spanish populations from Santa Olalla (P1) and Torralba de Calatrava (P2) were selected for the present study as they show the greatest degree of difference and are representative of these two groups.

In both groups, the strict *H. avenae* and the "Gotland strain", pathotypes have been found, differing in host ranges among cereal species and their varieties. Pathotypes are defined by virulence on three known resistance genes in barley (*Hordeum vulgare* L.) (Nielsen, 1971; Andersen and Andersen 1982), and evaluated by the International Test Assortment of cereal cultivars as resistant, when less than 5% of white females are present on the roots compared to susceptible controls (Lücke, 1976). More recently, plants with intermediate resistance-susceptibility have been found and, in the last few

years, many pathotypes have been recognized that do not correspond to those already defined. This makes it difficult to know the correct identity and relationships of pathotypes, essential for the most efficient use of the plant breeder's resources. There are a few sources of genetic resistance to cereal cyst nematode in hexaploid wheat, *Triticum aestivum*. Until now, four different resistance genes (*Cre1* to *Cre4*) have been described (McIntosh *et al.*, 1995). The wheat line Aus 10894/Loros carrying the gene *Cr1* (Slootmaker *et al.*, 1974; O'Brien *et al.*, 1980) is resistant to P1 population (Sanchez and Zancada, 1987). The H93-8 wheat/*Ae. ventricosa* introgression line carrying the gene *Cre2* is resistant to P1 and P2 populations, as well as to most European pathotypes (Delibes *et al.*, 1993).

Comparison of protein patterns obtained by two dimensional polyacrylamide gel electrophoresis of different isolates of cereal cyst nematodes (Ferris *et al.*, 1989; Bossis and Rivoal, 1996) and of ribosomal DNA (Ferris *et al.*, 1994), revealed differences between the strict *H. avenae* and the "Gotland strain" groups of *H. avenae*. Enzymatic analysis by electrophoresis using the thin-slab technique has been reported to be an effective method for identifying nematode genera and species. Esterase isozymes were found to be useful in differentiating *Meloidogyne* or *Heterodera* spp. (Rumpenhorst, 1985; Esbenshade and Triantaphyllou, 1985b, 1990), populations of *H. sacchari* (Nobbs *et al.*, 1992), pathotypes of *H. avenae* (Bergé *et al.*, 1981) and in population genetics of field subpopulations (Bossis and Rivoal, 1989). Moreover, RAPD analysis has been used to identify populations of four major species of *Meloidogyne* (Cenis, 1993), to differentiate *H. schachtii* from *H. cruciferae* and to assess intraspecific variation in both species (Caswell-Chen *et al.*, 1992).

In this investigation, biochemical and pathogenicity studies were performed on two Spanish populations of *H. avenae*, previously differentiated on the basis of their morphometric characteristics (Valdeolivas and Romero, 1990), with the aim of providing new information that might clarify their status within the "*H. avenae* complex".

Material and methods

Experiments for pathotype identification of a population from Torralba de Calatrava (P2) were undertaken during 1991-94. Plastic cylinders were filled with sterilized soil and were kept outdoors. Twenty five cysts (40 for oat cultivars), contained in a nylon bag, were placed in the soil at a depth of 5-10 cm. In each of five replicate cylinders, a pre-germinated seed from an International Tester Set (Andersen and Andersen, 1982) was planted at the beginning of December 1991. At the beginning of May 1992 the roots were rinsed free of soil and the females counted.

Wheat plants (*Triticum aestivum* L.) cv. Capa, susceptible to all pathotypes were grown outdoors and infected with Santa Olalla (P1) and Torralba de Calatrava (P2) cysts. Mature white females were hand-picked from the roots for biochemical analysis.

For each population, a soluble nematode protein extract was prepared by grinding about 100 females with 10 µl of 10 mM Tris HCl pH = 7.4 and 30 µl of a homogenizing solution (9M urea, 5% mercaptoethanol and 2% ampholytes pH = 3-10, 6-8 and 5-7) (Bossis and Mugniery, 1993). The homogenate was centrifuged at 10,000 g for 10 minutes, and the supernatant frozen at -70 °C. Just before use, samples were thawed and protein concentration was quantified by the bicinchoninic acid assay (Smith *et al.*, 1985).

Electrophoresis was carried out essentially as described by O' Farrell (1975) with some modifications, in a mini protean II 2D electrophoresis cell (Bio-Rad). IEF was performed using a wide range of ampholytes (pH = 3-10, 6-8 and 5-7). 8-16 µg protein/cylindrical gel was loaded.

Focusing was carried out without pre-run and was accomplished with the following voltage schedule: 10 minutes, 500 V and 3 h, 1,500 V. The gels were not equilibrated and were placed (without agarose) on top of a 10% SDS (W/V) polyacrylamide slab gel, 1 mm thick. Electrophoresis was then performed at a constant, 2,000 V for two hours and at 2-4 °C. Proteins were stained using a Bio-Rad silver staining kit according to the manufacturer's instructions. At least five repetitions were performed for each population. Proteins from both populations were run in both dimensions in the same electrophoresis cell.

The following enzymatic activities were studied by electrophoresis: malate dehydrogenase (MDH) E.C.1.1.1.37, catalase (CAT) E.C.1.11.1.6., superoxide dismutase (SOD) E.C.1.15.1.1., lactate dehydrogenase (LDH) E.C.1.1.1.27, glyceraldehyde-3-P-dehydrogenase (G-3-PDH) E.C.1.2.1.12, esterase (EST) E.C.3.1.1.2, phosphoglucomutase (PGM) E.C.2.7.5.1, NADH dehydrogenase (NADH) E.C.1.6.99.3, alkaline phosphatase (AlkPH) E.C.3.1.3.1, and glucose-6-phosphate dehydrogenase (G-6-PDH) E.C.1.1.1.49.

Enzyme extraction was performed as described by Esbenshade and Triantaphyllou (1985 a, b) and the same homogenates were used for all enzymatic assays, except for MDH. Six hundred white females from each population were homogenized by a small plastic pestle with 600 µl of extraction media (20% sucrose, 2% triton X-100) and then centrifuged at 13,000 g for 10 minutes at 4 °C. A 30 µl aliquot of the supernatant was immediately introduced into an electrophoresis cell for each isozyme analysis and the remaining supernatant frozen for use in other enzyme essays. Nematodes to be assayed for malate dehydrogenase activity were extracted in a similar manner using Tris buffer (Esbenshade and Triantaphyllou, 1985a).

The isozymes present in the nematode samples were fractionated by native polyacrylamide gel electrophoresis in a mini slab apparatus (Bio-Rad) with buffer, as described by Esbenshade and Triantaphyllou (1985a). A 4% acrylamide stacking gel and 7.5% separating gel were used for PGM, G-3-PDH and EST; a 4-20% acrylamide gradient gel for CAT, NADH, AlkPH and G-6-PDH, and a three layer gel (4%, 6% and 10% of acrylamide) for LDH, SOD and MDH. Electrophoresis was performed at a constant voltage (200 V) at 2-4 °C, until the marker dye (bromophenol blue) reached the base of the separating gel. The gels were stained for specific enzymes (Brewer and Sing, 1970) except for CAT and G-3-PDH (Dickson *et al.*, 1971). After staining, gels were fixed in ethanol-water (1:1).

DNA extraction from ten brown cysts (previously stored at 4 °C in 0.8% NaCl for seven days) was done by the method of Caswell-Chen *et al.* (1992). Nine different random decamer primers (primer kit E, Operon Technologies, Alameda, CA) were used according to manufacturer's instructions. RAPD amplifications were made as described by Devos and Gale (1992) with minor modifications. A reaction mix of 30 µl containing 1ng of template DNA, 0.8 units of Taq polymerase (Amersham), 0.1 mM dNTPs, 6 pmoles of primer, 1.5 mM Mg_2Cl and reaction buffer, supplied by the enzyme manufacturer, were amplified in a Perkin-Elmer Cetus thermal cycler for: 1 cycle of 4 minutes at 96 °C; 5 cycles of 1 minute at 94 °C, 1 minute at 38 °C, 2 minutes at 72 °C; 55 cycles of 1 minute at 90 °C, 1 minute at 38 °C, 2 minutes at 72 °C and one cycle of 5 minutes at 72 °C. The number of cycles, annealment and denaturation temperatures and the enzyme concentration, were optimized to minimize the DNA needed for amplifications. Accordingly, the number of cysts required for DNA extraction was reduced to ten. Amplification products were resolved by electrophoresis in a 1.7% agarose gel in TAE buffer (pH = 8.0) at 60 V for approximately 5 hours, visualized by ethidium bromide staining (0.5 µg/ml) and examined on a UV transilluminator. Bacteriofage Lambda DNA cut with Eco RI/Hind III was used to pro-

vide molecular size markers. All amplifications were repeated at least three times, including a negative control without DNA.

Results

The results of the quantitative test for pathotype identification of the Torralba de Calatrava population (P2), expressed as ♀/plant and ♀/g root, are summarized in Table I.

Table II shows the scheme for the identification of pathotypes (Andersen and Andersen, 1982), as modified by Rivoal and Cook (1993). Two previously identified Spanish pathotypes – Ha71 (Population P1) and Ha22 (Sanchez and Zancada, 1987) – are included, as well as the new pathotype corresponding to the P2 population described in this report.

Host reactions to P2 were similar in all the cvs tested over the three years of the investigation. However, as the highest multiplication rate was obtained in 1993-94, these results were chosen for reporting in Table I. Barley (*Hordeum vulgare* L.) cvs Siri, KVL 191, Martin 403-2 and Bajo Aragón, all with resistance gene Rha2, gave a resistant response to infection by the P2 nematode population, as did Morocco (Rha3) and Harlan 43. In contrast, barley cv. Dalmatische could be considered as moderately resistant. The other barley cvs tested, including Ortolan with the Rha1 gene, were susceptible. All the oat (*Avena sativa* L.) cultivars (Sun II, Nidar, Pusa Hybrid B51, Silva, *Avena sterilis* L. and IGVH 72.646) showed high resistance but all the wheat cvs (Capa, Loros, Iskamish K-2 light, Psathias and AUS 10894) proved to be susceptible.

Fig. 1 shows the two dimensional protein patterns, with molecular weight and pH scale, obtained from P1 and P2. Patterns in both cases were highly reproducible (at least five times). In general, the smaller amount of protein loaded on the gel, the better the resolution. Large quantities of protein caused a distortion of spot shape. The optimum amount of protein/gel proved to be 8 μg. A pattern showing all major spots was obtained and zones with poorly separated proteins were discarded. The number, staining intensity, and electrophoretic mobilities of spots were sufficiently different to distinguish the two populations. The major discriminating spots of proteins were localized in a molecular weight range between 67,000 and 45,000 at pH 5.0 for the P1 population (Fig. 1A), and at a molecular weight of 45,000 and a pH range of 3.5-4.0 for P2 (Fig. 1B). Of the main spots ob-

TABLE I - *Female rating and host reaction of the International Test Assortment for resistance to Spanish population P2 of* Heterodera avenae.

	♀/plant[a]	♀/g root[a]	Reaction[b]
Barley			
Emir	193	109	S
Ortolan (Hal)	112	54	S
Siri (Ha2)	4	1	R
Morocco (Ha3)	5	4	R
Varde	189	145	S
KVL 191 (Ha2)	5	2	R
Bajo Aragon (Ha2)	3	1	R
Herta	156	111	S
Martín 403-2 (Ha2)	1	1	R
La Estanzuela	117	38	S
Harlan 43	0	0	R
Dalmatische	8	4	R
Oats			
Sun II	0	0	R
Nidar	0	0	R
Pusa Hybrid B-51	0	0	R
Silva	0	0	R
Avena sterilis	0	0	R
IGVH 72-646	0	0	R
Wheat			
Capa	75	43	S
Loros (Crel)	314	92	S
Iskamish K-2 light	57	15	S-(S)
Psathias	51	27	S
AUS 10894 (Crel)	136	43	S

[a]Mean number of white females in five replicates. [b]R = Resistant; S = Susceptible; ( ) = Moderatedly.

tained by two-dimensional electrophoresis for P1, and 24 for P2, only 12 were common to both populations.

The banding pattern of isozymes obtained by native gel electrophoresis (Fig. 2) was highly reproducible (at least three repetitions) for eight of ten enzymes (MDH, CAT, SOD, LDH, G-3-PDH, EST, PGM and NADH) analyzed. AlkPH and G-6-PDH were discarded because only a single diffuse band was detected with the former enzyme, and no activity at all was detected for the second one. Banding patterns of all enzymes analyzed were simple (no more than four bands per sample). No detectable differences in LDH and SOD banding patterns were found for the P1 and P2 populations (Fig. 2C).

TABLE II - *Pathotypes of* H. avenae *defined by the International Test Assortment of cereal cultivars*[a].

Pathotype	*H. avenae* group Ha1 pathotypes								Ha2 pathotypes			Ha3 pathotypes		
	Ha11 Fr3	Ha21	Ha31	Ha41 Fr1	Ha51	Ha61	Ha71	Ha71[d] P1	Ha12 Fr2-4	Ha22[d]	P2[e]	Ha13	Ha23	Ha33
Barley														
Emir (Rha?[c])	S[b]	S	–	S	–	S	S	S	S	S	S	S	S	S
Ortolan (Rha1)	R	R	R	R	R	R	R	R	S	S	S	S	S	S
Siri (Ra2)	R	R	R	S	S	S	R	R	R	R	R	S	S	S
Morocco (Rha3)	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Varde	S	–	–	S	–	S	S	S	S	S	S	S	S	S
KVL191 (Rha2)	R	R	R	–	S	S	S	–	R	–	R	–	–	–
Bajo Aragon (Rha2)	R	–	–	R	–	R	R	S	R	R	R	S	S	R
Herta	S	S	R	–	R	–	R	–	S	–	S	S	–	–
Martín 403-2 (Rha2)	R	–	–	R	–	R	R	S	R	S	R	R	S	S
La Estanzuela	–	–	–	–	–	–	S	–	–	–	S	–	(R)	–
Harlan 43 (Rha?)	R	–	–	–	–	–	R	R	R	–	R	–	R	S
Dalmastiche	(R)	–	–	S	–	R	(S)	S	S	S	R	S	(R)	S
Oats														
Sun II	S	R	R	R	R	S	R	R	S	R	R	S	S	S
Nidar	S	–	–	S	–	S	R	R	S	R	R	S	S	S
Pusa Hybrid B51	R	R	–	R	R	R	R	R	R	–	R	S	R	S
Silva	(R)	–	–	R	–	(R)	R	R	(R)	R	R	(R)	(R)	S
Avena sterilis	R	R	–	R	R	R	R	R	R	R	R	R	R	R
IGVH 72.646	R	–	–	R	–	R	R	R	R	R	R	S	S	S
Wheat														
Capa	S	S	–	S	–	S	S	S	S	S	S	S	S	S
Loros (Crel)	R	R	–	R	–	(R)	R	R	R	R	S	(R)	S	S
Iskamish K-2 light	S	–	–	R	–	(R)	–	(R)	S	S	S-(S)	S	S	S
Psathias	–	–	–	S	–	–	–	R	S	R	S	S	S	S
AUS 10894 (Crel)	R	–	–	R	–	R	S	R	R	S	S	(R)	S	S

[a] From Rivoal and Cook, 1993. [d] Sanchez and Zancada, 1987. [e] Pathotype identified in this paper.
[b] S = Susceptible; R = Resistant; ( ) = Moderatedly; - No observation.
[c] Resistance genes 1 to 3 in barley defining 3 pathotype groups.

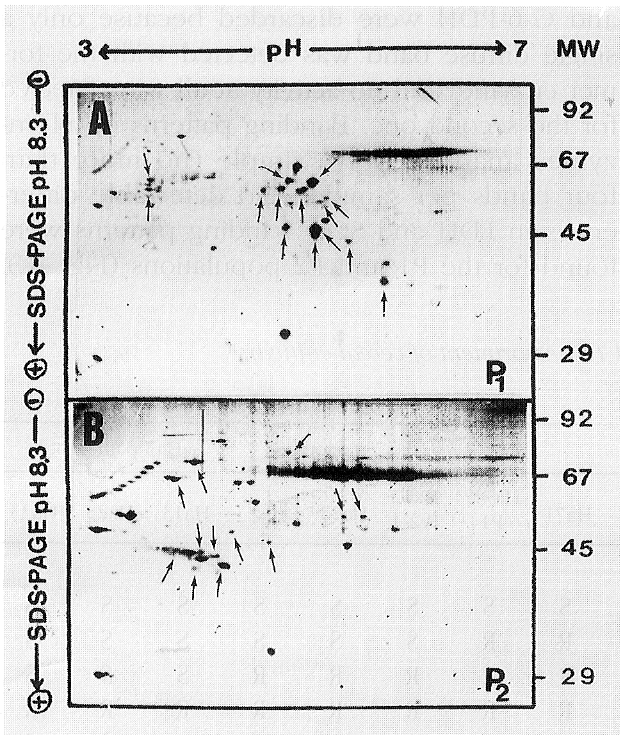

Fig. 1 - Comparison of protein patterns of populations Sta Olalla (P1) and Torralba de Calatrava (P2) of *H. avenae* complex analyzed by high resolution two-dimenisonal polyacrilamide gel electrophoresis. Relevant spots present in one nematode population and absent in the other are indicated by arrowheads. Molecular weights are given x 1000.

LDH was slow in showing on the gel (1h 30 minutes) and was weakly stained as a thin dark band. A broad, clear band with the same rate of migration (Rm) for SOD also appeared on the gel (Fig. 2C). G-3-PDH, PGM and NADH also showed low activity, but with characteristic enzyme profiles for each population (Fig. 2D, 2F and 2G). MDH, CAT and EST stained rapidly but only CAT and EST showed a clear enzyme profile (Fig. 2A, 2B and 2E).

Fragments from 0.2 to 2.1 Kbp were obtained with each RAPD primer and the number of fragments varied from two to seven. All random primers tested gave different pattern for P1 and P2, yielding complex patterns with several bands common to both populations. In contrast, primer OPE-18 yielded a simple pattern, with two main fragments of approximately 1.2 and 1 Kbp which were the same in both populations. The most complex pattern, two bands for P1 and five for P2, with no shared bands, was obtained with primer OPE-19 (Fig. 3). RAPD analysis detected 24 and 31 bands in P1 and P2 populations respectively.

Discussion

The pathogenicity tests performed on Torralba de Calatrava population (P2) show that it does not belong to the same pathotype grouping as Santa Olalla population (P1). P2 belongs to a pathotype included within the Ha2 resistance group characterized by a resistant response of the barley cvs Siri, KVL 191, Martín 403-2 and Bajo Aragón. Nevertheless, according to the virulence to Cre1 gene present in wheat cvs Loros (Slootmaker *et al.*, 1974) and Australian 10894 (O'Brien *et al.*, 1980), P2 population show similar behaviour to that of pathotypes included in Ha3 group, which are also similar in morphology (Gotland type), although until now correlation between morphology and pathogenicity has not been found.

A comparison of the P2 population with the other two pathotypes described in the Ha2 group (Ha12 and Ha22) reveals several differences: Sun II and Nidar oats are both susceptible to Ha12 but are resistant to P2; Loros and AUS. 10894 are both resistant to Ha12 and susceptible to P2; barley cvs Martín 403-2 and Dalmatische are both susceptible to Ha22 but are both resistant to P2; and the wheats Loros and Psathias are both resistant to Ha22 but susceptible to P2. All the oat cultivars in the International Test Assortment were resistant to P2, as are all pathotypes previously identified in Spain by Sanchez and Zancada (1987). This same characteristic has also been described for populations of *H. avenae* in Israel (Mor *et al.*, 1992).

In 2-D PAGE the number of spots considered

valid were fewer than those reported in previous studies comparing strict *H. avenae* and "Gotland strain" (Ferris *et al.*, 1989, 1994; Bossis and Rivoal, 1996). This difference could be due to the fact that the techniques used to develop the gels are different. Also, in the 67,000D molecular weight zone, some of the most basic proteins were eliminated because they were not well resolved. In addition, faint spots were not evaluated. The analysis of the main spots obtained from the electrophoresis patterns of the two populations compared, showed 12 common spots (representing 31.5%), 14 spots were only present in P1 and 12 spots in P2 populations. Similarly, in another investigation where Swedish populations belonging to these two groups were studied, Ferris *et al.*, 1989 found that strict *H. avenae* and "Gotland strain" shared only about 25% of the proteins that they scored.

Enzyme analysis could be of great taxonomic value for identification of cyst nematode species. It has been demonstrated that for *Meloidogyne* species, enzyme patterns were useful for taxonomic purposes (Dickson *et al.*, 1971; Dalmasso and Bergé, 1978, 1983; Esbenshade and Triantaphyllou, 1985b, 1990). In the present investigation, LDH showed patterns similar to those in *Meloidogyne* spp. *Ditylenchus triformis* and *Aphelenchus avenae*, whereas AlkPH and G-6-PDH expressed no enzymatic acitvity reported by Dickson *et al.*, (1971). Moreover G-3-PDH, PGM and NADH, which have not previously been used for enzymatic analysis of *Heterodera* spp., showed faint banding patterns nevertheless useful to differentiate both populations.

This study confirms the usefulness of esterase marker for nematode identification (Dickson *et al.*, 1971; Bergé *et al.*, 1981; Esbenshade and Trianthaphyllou, 1990; Nobbs *et al.*, 1992). In addition, catalase (CAT) appeared to be a new potential biochemical character to distinguish populations of nematodes, as demonstrated by different isozyme phenotypes exhibited by Santa Olalla and Torralba de Calatrava populations.

P1 and P2 populations showed few similarities in the enzymes studied (29.1% of the band

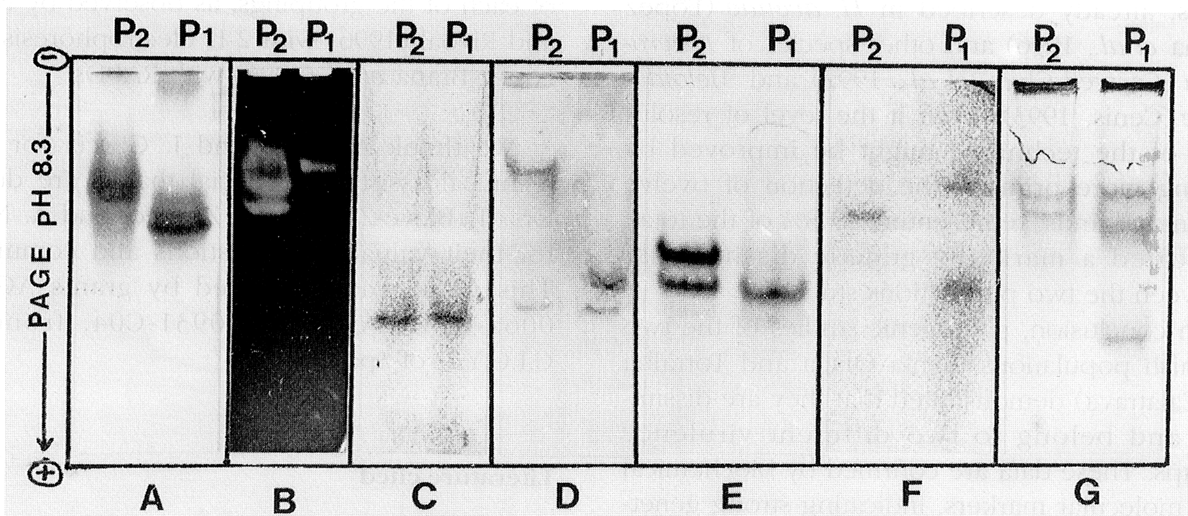

Fig. 2 - Zymogram phenotypes of malate dehydrogenase (A); catalase (B); superoxide dismutase white band, and lactate dehydrogenase black band (C); glyceraldehyde-3-P-dehydrogenase (D); esterase (E); phosphoglucomutase (F) and NADH dehydrogenase (G) produced by populations of *H. avenae* complex (Sta Olalla P1 and Torralba de Calatrava P2).

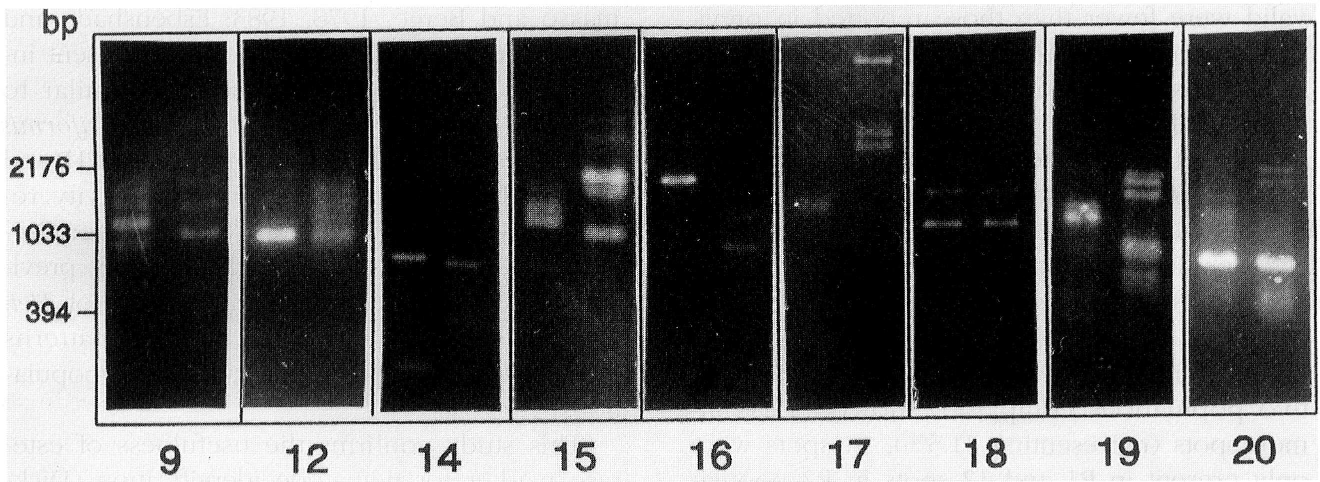

Fig. 3 - Polymorphic bands of amplified DNA from populations P1 (Sta Olalla) (left) and P2 (Torralba de Calatrava) (right) produced using nine random decamer primers of Kit E (9, 12, 14, 15, 16, 17, 18, 19, and 20) of Operon Technologies. Molecular weights standards are given in base pairs.

scored) and certain bands appear only in one population (eight in P1 and nine in P2). Such markedly different profiles support evidence of interspecific variation as previously reported (Dickson *et al.*, 1971; Hussey and Krusberg, 1971; Nobbs *et al.*, 1992).

The variability in RAPD markers indicates that this technique may be useful in the establishment of inter and intra-specific relationships, already described in *H. avenae* (López Braña *et al.*, 1996) and other species of *Heterodera* (Caswell-Chen *et al.*, 1992) and *Meloidogyne* (Cenis, 1993). Even if the level of resolution of the technique might be improved by testing more primers, the detection of twelve common bands, representing 27.8% of the total, indicated a markedly genetic dissimilarity between the two populations studied.

In conclusion, pathogenic studies of the two Spanish populations (Santa Olalla and Torralba de Calatrava) demonstrated that they are dissimilar and belong to two different virulence groups. These data are cofirmed by biochemical and molecular markers, indicating strong genetic differences between the populations compared, and possibly a specific variation as reported by Ferris *et al.* (1994). Some of these techniques have been used for the first time in the study of genetic variability in *H. avenae*. All these data are in agreement with the results of an earleir morphometric study (Valdeolivas and Romero, 1990) which classified the P1 and P2 populations as belonging to the strict *H. avenae* and "Gotland strain", respectively. It would be interesting to see whether the biochemical and molecular patterns occur in other populations of each of the groupings, as observed by Bossis and Rivoal (1996) with 2-D electrophoresis, and López-Braña *et al.* (1996) with RAPDs.

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