EPIDEMIOLOGY AND DISTRIBUTIONAL PATTERNS OF *MELOIDOGYNE* (NEMATODA: MELOIDOGYNIDAE) IN A MONOCROPPING SYSTEM

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Summary. Epidemiological incidence of root-knot nematodes (*Meloidogyne* spp.) is not well known in the Iberian Peninsula. The apomictic *Meloiodogyne arenaria*, together with *M. javanica* and *M. incognita*, are responsible for major crops losses in this region. In the area of La Vera (Extremadura), where agricultural systems are more or less limited to monocropping with tobacco, *M. arenaria* is predominant (> 95% of samples). As an epidemiological evaluation, three fields naturally infested with *M. arenaria* populations were used to assess resistant and susceptible tobacco genotypes under natural conditions. Also, a distributional pattern approach of different *M. arenaria* multilocus enzyme electrophoresis (MLEE) variants was carried out in this area. Results showed a highly significant differential interaction of locality × variety × time ($P < 0.000$), as well as revealing considerable inter-population genetic variability of these pathogens. Distributional pattern analysis of 18 different MLEE variants revealed that geographic structure is lacking in the area of study. Consequently, the potential of management strategies based on the employment of resistant varieties is considered highly limited. An epidemiological zoning based on similarity groups is proposed as a starting point for the development of control programmes for these pathogens in the high-risk infested areas.

Key words: Aggressiveness, multilocus enzyme electrophoresis, root-knot nematodes, spatial analysis, tobacco, virulence.

Plant nematodes respond to a diversity of environmental factors at both the population and species level, but the majority of related research focuses on host variability rather than variability of the pathogen. Nevertheless, the occurrence of resistance/virulence interactions regarding plant parasitic nematodes, has been described on different hosts (Kort *et al*., 1977; Cook and Evans, 1987; Müller, 1992; Roberts, 1995; Lasserre *et al*., 1996).

Host plant resistance has proven to be effective for controlling *Meloidogyne* spp. (root-knot nematodes) as an alternative to chemical nematicides, which are environmentally detrimental and often not justified economically (Janssen *et al*., 1996; Ijani *et al*., 2000). Most *Meloidogyne* populations of the same race vary in their aggressiveness (parasitic potential with respect to susceptible host) or in their virulence (response to host genetic resistance) (Swanson and Van Gundy, 1984). This condition can be genetically stable, showing up years later after an initial selection (Netscher, 1976; Dalmasso *et al*., 1991; Jarquin-Barberena *et al*., 1991). Inter- and intra-specific *Meloidogyne* variability is masked by the current race classification, so is often not considered in control programmes. However, knowledge of this variability is essential in the screening for genetic resistance because undetected populations may adapt to the host genotype, and result in a decrease in cultivar efficiency (Cook and Evans, 1987; Navas *et al*., 2001).

In La Vera (Extremadura Region), where the agriculture is limited to a tobacco monocropping system, *M. arenaria* (Neal) Chitw. predominates, being found in more than 95% of the samples (Navas *et al*., 2001; Flores-Romero and Navas, 2005). Resistance to *M. arenaria* is lacking in traditionally used cultivars in this area. In recent years several hybrid tobacco cultivars resistant to *M. incognita* (Kofoid *et* White) Chitw. have been identified (Young, 1992). To validate whether such resistance is effective against *M. arenaria*, it is necessary to evaluate the level of susceptibility or resistance in the field on growing plants using naturally infested soils with prolonged exposure of plants to multiple generations during a growing season.

Thus, the detection of genetic divergence and geographic variation patterns among *Meloidogyne* populations and their relationship with the characteristics defined in terms of host-pathogens interaction of these populations can be of great value in the design and management of successful control programmes. Also, the application of such programmes requires accurate knowledge of population densities, ecology and biology, especially in the case of monocropping systems (Navas *et al*., 1992). Spatial pattern can be considered one of the most characteristic ecological properties of any species, reflecting environmental and genetic heterogeneity and acting on several biological processes, such as reproduction, dispersal and mortality (Ristaino and Gumpertz, 2000). Such knowledge is important for improved crop disease management (Jeger, 1999). Patterns of disease development in natural plant populations, whether homogeneous or heterogeneous, also provide insight into

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plant-pathogen population interactions (Jeger, 2000).

The aims of this study were: (i) an evaluation of *M. arenaria* populations, genetically classified as MLEE (multilocus enzyme electrophoresis) variants, for their aggressiveness and virulence under natural conditions (against susceptible and resistant tobacco genotypes); and (ii) to estimate spatial distributional patterns of the 18 *M. arenaria* MLEE variants previously detected (Navas *et al*., 2001; Flores-Romero and Navas, 2005).

MATERIALS AND METHODS

Host-Nematode interaction

Field experiments design. Experiments were carried out in three localities, La Cañalera (Locality 1), Rosalejo (Locality 2) and Cuaternos (Locality 3), in the area of La Vera (Extremadura, Spain). Two resistant varieties of tobacco, hybrid genotypes H1 and H2, selected for their resistance to *M. incognita*, and one susceptible cultivar, Virginia K326 genotype (planted in roughly 90% of the cultivated area of the study zone since 1982), were used.

In each of the three localities, three randomly positioned blocks of 50.4 m^2 (7.2 m × 7 m) were marked out. Three rows of tobacco were planted in the centre of each block. Rows were 1.2 m apart and each contained 14 plants spaced 0.5 m. Six samples (individual root systems and the associated soil) were collected randomly from each block at each of the three sampling times. Samplings were made, approximately every four weeks, between 12 July and the end of the crop season (23 September). A total of 162 samples (3 blocks × 6 samples × 3 samplings × 3 localities), 54 from each locality, were analysed.

The nematode populations were characterized by allozyme electrophoresis, and classified into MLEE variants using the same enzyme systems and the alloenzymatic methodology described in Navas *et al*. (2001). Phenological assessments of the crops and the development of the soil and root nematode populations were carried out. Their aggressiveness/virulence was estimated through assays of adult females in roots and in soil, for each of the three sampling dates.

Nematode extraction from soil and roots. The J_2 were extracted from soil by a sugar centrifugation method (Barker, 1985). Extraction of adult females was carried out after a 12-hour digestion of infected roots (washed and free of soil) in a solution of pectinase (15%) plus cellulase (30%), prepared from commercial Pectinex and Celluclast (Novonordisk®). Roots then were mechanically ground and the larger debris removed by sieving with a colander. All the nematode stages were then sieved again (150 µm mesh) to retain mature females and small organic debris. Retained adult females were separated from small pieces of debris by sugar centrifugation with kaolin added to the suspension. Finally, clean adult females were obtained by washing on a 150 µm sieve. On occasion, adult females were collected by manual dissection of the roots with a lancet under a 40-power magnifying glass.

Root systems of plants younger than two months (first sampling) were processed as a whole, while only sub-samples of older root systems were processed. At least 20 g of root were analyzed and final results were normalized to a standard weight of 20 g (number of females found x 20/weight of root examined).

Counts of nematode stages (J_2 in soil and adult females) were carried out under stereoscopic microscope in saline (physiological) solution.

Data analysis. Statistical evaluation was performed on the density of juveniles in 100 cm^3 of soil, and the density of adult females in 20 g of root, as affected by tobacco variety, locality, and sample date. Data were normalized by means of base N logarithm [Log (x+1)] transformation and subjected to Analysis of Variance for detection of factorial effects using STATISTICA v6.0 software programme (StatSoft Inc., 2006).

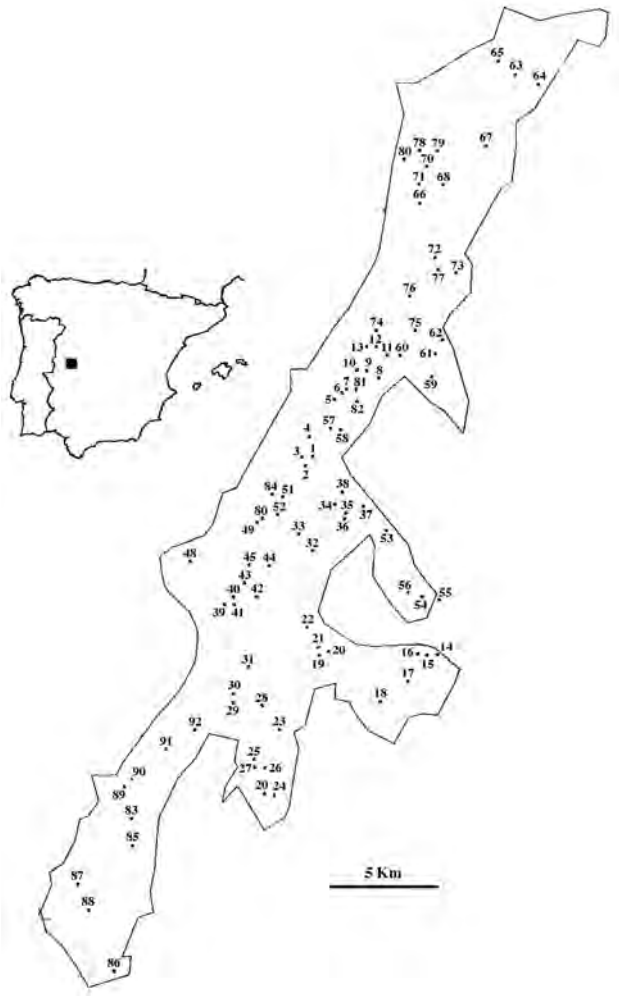

Fig. 1. Sampling points corresponding to the samples used in the geographic distributional patterns assessment in the area of study (La Vera, Extremadura).

Geographic distributional patterns

A total of 92 samples were used from the area of La Vera (Extremadura-Spain) (Fig. 1). The contents of the samples corresponded to those found in samples from previous work and classified into 18 MLEE variants (Flores-Romero and Navas, 2005). All variants were identified as *M. arenaria*, according to the method of Esbenshade and Triantaphyllou (1990) except for the VER18 variant, which was *M. javanica* (Treub) Chitw. (Table I).

Sample processing. Each sample consisted of a plant root system and the associated rhizosphere soil (totalling 1 kg of soil). To define nematode population densities, J_2 in the soil and adult females in the roots were extracted and counted following the methodology described previously. Sand, silt and clay content and pH were estimated for each soil sample.

Data analysis. The total weight of roots from which adult females were extracted, was used to calculate adult females per 20 g of root. This data, as well as that of J_2 per 100 cm^3 of soil, was normalized by N logarithm [Log (x+1)] transformation. Correlations between edaphic and nematode density parameters (adult females and J_2 in soil) of the MLEE variants were assessed by means of correlation and correspondence analysis followed by calculation of the Euclidean distances between samples as a function of epidemiological similarity. Thus, groups defined as of equal similitude were then compared by Discriminant Analysis. The STATISTICA v6.0 software programme (StatSoft Inc., 2006) was used for the Correlation and Correspondence Analysis, the Ward Classification and the Discriminant Analysis.

Finally, values obtained from the similarity index were mapped using SURFER v6.01 (Surfer (win32), 1995), with extrapolation between adjacent points to locate areas of potential nematode infestation.

Table I. MLEE variants of *Meloidogyne spp.* detected in 92 samples analysed based on the phenotypes obtained; distribution or sample points as in Fig. 1 and the corresponding MLEE variant defined by Navas *et al.* (2001). The corresponding species* are determined according to Esbenshade and Triantaphyllou (1990).

MLEE Variant	Distribution (sample points in Fig. 1)	Navas *et al.*, 2001	Species*
VER1	2, 12, 38, 48, 62, 70, 71, 73, 79	A1	*M. arenaria*
VER2	61, 75, 90	B1	*M. arenaria*
VER3	8, 58	G	*M. arenaria*
VER4	60, 67	A3	*M. arenaria*
VER5	47	47b	*M. arenaria*
VER6	41	41b	*M. arenaria*
VER7	59	59b	*M. arenaria*
VER8	3, 5, 6, 21, 22, 24, 35, 82, 84	D2	*M. arenaria*
VER9	52, 55	D3	*M. arenaria*
VER10	4, 11, 26, 29, 30, 31, 33, 34, 36, 51, 53, 87, 88	D1	*M. arenaria*
VER11	46, 63, 64, 86, 89, 91	E1	*M. arenaria*
VER12	16, 32, 69, 78, 80	A2	*M. arenaria*
VER13	54	54b	*M. arenaria*
VER14	23, 37, 57, 65, 68, 74, 76, 83, 85, 92	E2	*M. arenaria*
VER15	9, 13, 14, 15, 17, 18, 19, 20, 25, 28, 42, 43, 44, 72, 77	F	*M. arenaria*
VER16	66, 81	B2	*M. arenaria*
VER17	7, 10, 27, 35, 39, 40, 45, 50	C	*M. arenaria*
VER18	49, 56	J	*M. javanica*

RESULTS

Host-Nematode interaction

The *Meloidogyne* populations found in the three localities sampled belonged to genetically different MLEE groups or variants, based on the allozymephenotypes from each of the enzyme systems analysed (Table II).

Meloidogyne stages were tested as indicators of the effect or relation between sites (localities or populations), date (sampling time) and varieties by means of a traditional analysis of their interaction (analysis of variance). The dynamics of nematode females and J_2, as affected by tobacco cultivar, locality and time, is shown in Fig. 2. Both females in root (Fig. 2A) and J_2 in soil (Fig. 2B) expressed a strong 3-way interaction (locality × variety × time; $F = 5.33$, $P < 0.000$).

The locality was an important factor in the interaction, mainly for J_2 in soil (locality 1 has significant lower values than the others) but also associated with varieties K326 and H1. Regarding females in the roots, significant difference was detected only for the variety H1. In the three localities, time and variety discriminated for the average density of the nematode stages separately. Some differences with respect to variety were detected when they were compared using all of the samples for each variety ($F = 2.24$, $P < 0.06571$). Thus, a hypothetical range of resistance could be established for each, where the H1 hybrid was the most resistant, the H2 hybrid less so, and K326 the most susceptible.

Ecological Structure and Geographic Distributional Patterns

Although the sample areas had been treated previously with nematicides (1,3-dichloropropene), all the samples were positive (J_2 in soil). The overall average densities were 97 females/20 g of roots and 491 J_2/100 cm^3 of soil.

Correspondence analysis was carried out on the MLEE variants studied (considering the 92 samples of Fig. 1 and Table I), together with the normalized classes

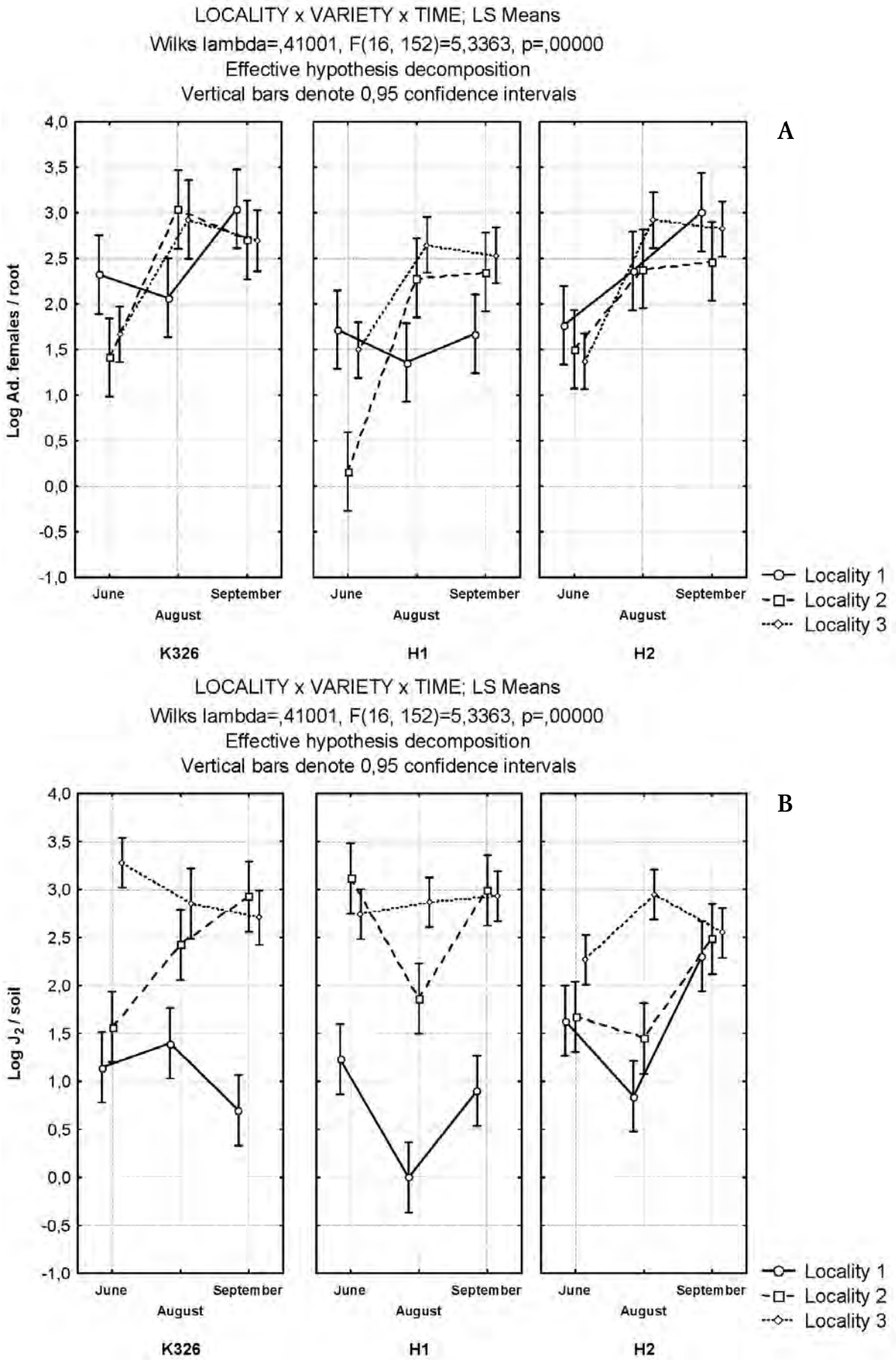

Fig. 2. Analysis of Variance showing significant differential interaction (F = 5.33; P< 0.000) among Localities, Time and Varieties. Nematological parameters considered are: A, adult females (Log Ad. females) in roots and B, juveniles (Log J_2) in soil. Values are average of transformed N base logarithm (Log (x+1)) nematological parameters.

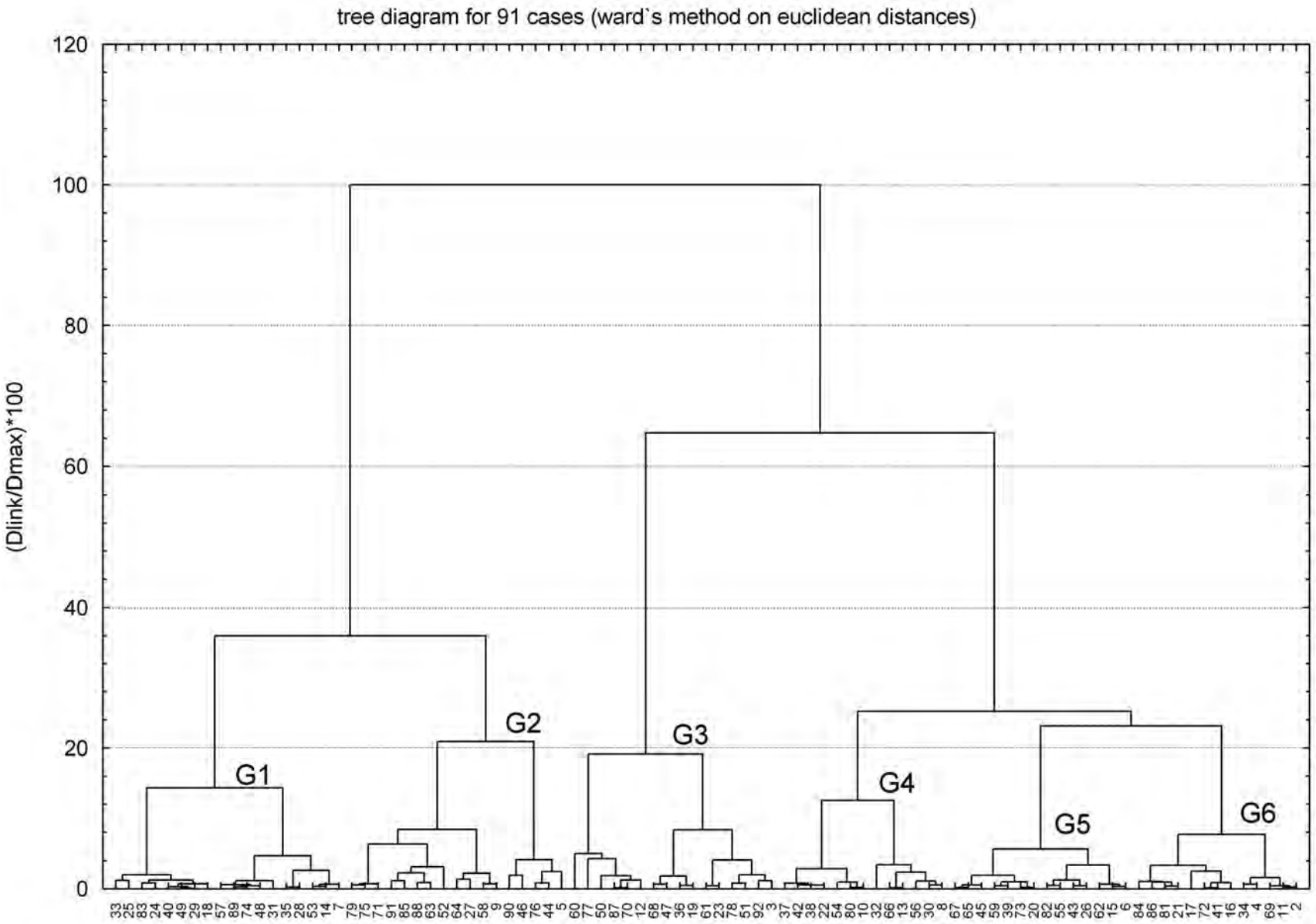

Fig. 3. Ward classification obtained on the basis of similarity index values of juveniles/kg of soil and adult females/20 g of root. The six groups found to be significantly different (P<0.000) are indicated in the figure (G1-G6) and their epidemiological characteristics are in Table III.

Table II. Classification of *M. arenaria* populations collected at each of the three localities where experiments on host-nematode interaction were conducted. Populations were characterized according to the phenotypes obtained in the seven enzyme systems analysed. Combinations of phenotype patterns defined the MLEE variants according to Navas *et al.* (2001).

MLEE variants		Localities		
		L1	L2	L3
Locus	Allele	VER11	VER1	VER3
EST-1	A	0.0	0.0	0.0
	B	0.0	0.0	0.0
	Null	1.0	1.0	1.0
EST-2	A	0.5	0.5	0.5
	B	0.5	0.5	0.5
	Null	0.0	0.0	0.0
MDH	A	1.0	1.0	1.0
CAT	A	0.0	0.0	0.0
	B	0.0	1.0	0.0
	C	1.0	0.0	1.0
SOD-1	A	1.0	1.0	1.0
	Null	0.0	0.0	0.0
DIA-1	A	0.0	0.5	0.0
	B	1.0	0.5	1.0
PGI-1	A	0.5	0.5	0.0
	B	0.5	0.5	1.0
PGI-2	A	0.5	0.5	0.5
	B	0.5	0.5	0.5
PGI-3	A	0.0	0.0	0.0
	B	0.0	0.0	0.0
	Null	1.0	1.0	1.0
GOT	A	1.0	1.0	1.0

*Enzyme systems. a-EST: a-esterase (E.C.: 3.1.1.1), MDH: malate dehydrogenase (E.C.: 1.1.1.37), CAT: catalase (E.C.: 1.11.1.6), SOD: superoxide dismutase (E.C.: 1.15.1.1), DIA: diaphorase (E.C.: 1.6.2.2), PGI: glucose phosphate isomerase (E.C.: 5.3.1.9), and GOT: glutamate oxalacetate transaminase (E.C.: 2.6.1.1).

of edaphic parameters and the nematode densities (adult females and J_2 in soil) as the best parameters representing the host-nematode interaction. The inertia percentages of the first three axis were: 7.55% (axis I), 5.49% (axis II) and 4.47% (axis III). The contribution of the continuous parameters (sand, silt, clay, soil pH and density of nematodes) (not shown) to the detected variation was small (18% of total explained). Consequently, no association was found between the MLEE variants, the edaphic parameters and nematode densities. Similarly, there was no independent correlation between adult females and J_2 densities of any of the MLEE variants or the edaphic parameters. Densities of adult females and J_2 in soil were more closely related with sand content ($r = 0.8$) and soil pH ($r = 0.68$) than with *Meloidogyne* population diversity.

Stable global epidemiological relationships can be seen from the similarity index, which defines areas or groups of equal similitude. Logically, the areas most affected by these pathogens matched with those of highest adult female and J_2 densities, while those less affected coincided with the lowest densities of both parameters. Between these extremes, classification was achieved by means of Ward's method (Fig. 3). In this way, similarity between sample points or areas based solely on similitude of adult female and J_2 densities led to the definition of six groups (Fig. 3) (the degree of significance corresponded to one standard deviation in the Euclidean distance). These six groups were different from each other ($P < 0.000$) according to Discriminant Analysis. For each, the classification percentage was very high. The highest values were from groups G3 and

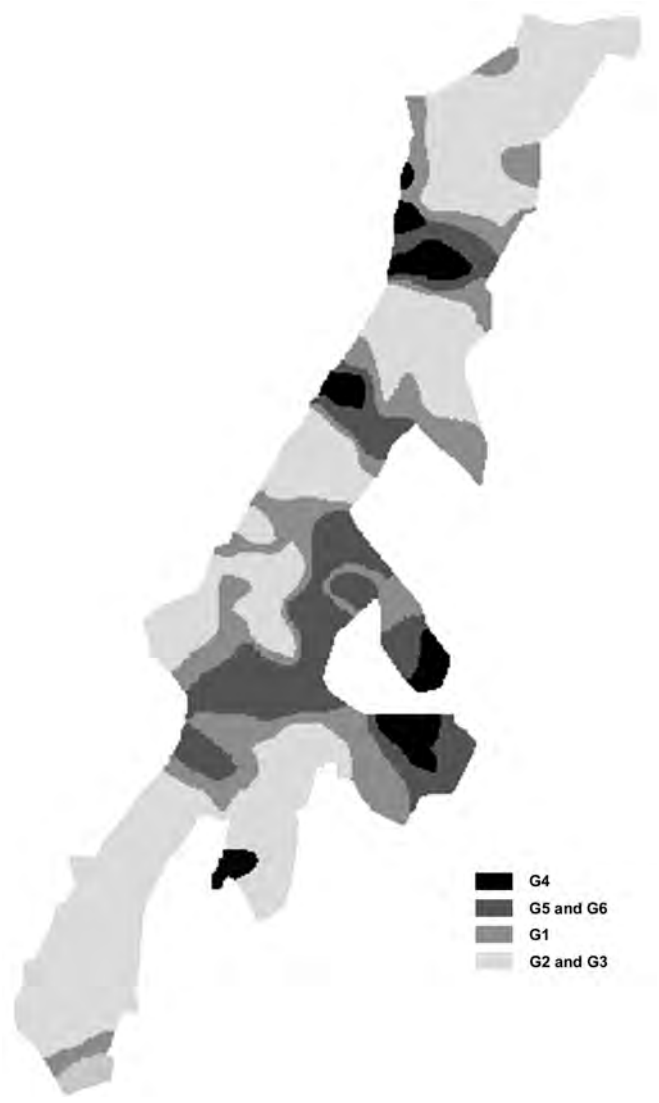

Fig. 4. Geographic plotting of the equal similarity groups as infestation areas, taking into account the range of variation of the similarity index values for each group. Isolines join points with equivalent infestation values.

Table III. Classification percentage, similarity index and epidemiological characteristics of the six groups obtained using Ward´s method of clustering. Values in brackets are the averages of females/20 g of roots or juveniles/kg of soil.

Groups	Classification %	Similarity Index	Epidemiological Characteristics (abundance of nematodes)	
			(females in root)	(juveniles in soil)
G1	100%	6.4-6.9	medium (53)	high (8103)
G2	94.11%	4.4-6.2	low (16)	medium (3294)
G3	81.25%	4.8-6.4	high (121)	low (854)
G4	92.30%	7.6-8.2	very high (601)	high (7331)
G5	92.85%	6.6-7.2	high (181)	medium (4447)
G6	92.30%	6.8-7.6	high (134)	very high (16317)

G1, 81.2% and 100%, respectively (Table III), indicating the existence of non-random value epidemiological zoning and the definition of four density-classes (epidemiological characteristics in Table III: low, medium, high and very high), which are characteristic of each of the groups, or zones. The highest densities were found in groups G4 (adult females: very high/J_2 in soil: high) and G6 (adult females: high/J_2 in soil: very high). Densities in the group G2, the area least affected, were less (adult females: low/J_2 in soil: medium). Geographic plotting of the variant groups is shown as areas of infestation in Fig. 4. Although the geographic representation is relatively complex, the range of variation of the similarity index for each group from Table III facilitated an interpretation. Isolines join points of equal similarity and by extrapolation define probability zones. The overlap of range of variation of G2 on G3 and G5 on G6 allowed us to group them together in a clearer graphic representation.

DISCUSSION

A combined population genetics and ecology approach is important in designing efficient control programmes for crop diseases (Jeger, 2000). Therefore, correlation between genetic and epidemiological characterization can be highly informative regarding such programmes, based on evaluation of pathology-process components and the prediction of their role in disease development. An epidemiological approach was carried out on genetically characterized *M. arenaria* MLEE variants. According to our alloenzymatic analyses, *Meloidogyne* populations at the three experimental localities belonged to different MLEE variants, VER11, VER1 and VER3 at L1, L2 and L3, respectively, which had been detected in the study area during previous studies (Navas *et al.*, 2001; Flores-Romero and Navas, 2005). Experimental analysis of the differential interaction corroborated the existence of a highly significant influence of combined effects of time, variety and locality (population). Our results indicated that all the parameters implicated in the host-interaction must be taken into account. Different MLEE variants behaved in different ways on both the same and different hosts according to seasonal fluctuation, suggesting that detection of *Meloidogyne* MLEE variants should be included in future epidemiologic studies in order to improve the effectiveness of control programmes.

The definition of pathosystem (Robinson, 1980), in which pathotypes are included, involves two types of relations, horizontal and vertical. The former involves polygenic host resistance in the absence of differential interaction, whereas the latter involves gene-to-gene response, the existence of races or pathotypes, and consequently, differential interaction, as suggested in the current study. The relation between population diversity and plant resistance has been demonstrated in many pathosystems (Fritz and Simms, 1992). Host diversity affects pathogen distribution, abundance and stability. Evolution is then driven by genetic adaptation to, or preferences for, varying level of host resistance, especially in areas of intensive agriculture. In this study, resistance level differences of host varieties allowed us to establish a range of resistance rankings where the hybrid H1 was the most resistant, H2 less so, and K326 was the most susceptible. When levels of resistance vary, as in this study, selection favours pathogen populations that can overcome such resistance (Dalmasso *et al.*, 1991; Semblat *et al.*, 2000). This could be a possible explanation for the predominance of *M. arenaria* populations in the area of study (Navas *et al.*, 2001; Flores-Romero and Navas, 2005). These populations could have been selected by widespread and constant employment of the Virginia K326 cultivar before using varieties with resistance to *M. incognita* races 1 and 3, but with some resistance against *M. arenaria*. Currently, the incidence of *M. incognita* in the study area is less than 6% (Navas *et al.*, 2001). Accordingly, the employment of resistant cultivars should be re-considered after careful evaluation of the resistance in order to minimize problems related with resistance losses. It cannot be assumed that resis-

tance of one host to a given nematode will be effective for the same nematode in another host. In addition, nematode species genetically variable at the inter- and intra-individual levels, as reported for *Meloidogyne* species (Esbenshade and Triantaphyllou, 1990; Hyman and Powers, 1991; Castagnone-Sereno *et al.*, 1994; Hugall *et al.*, 1999; Navas *et al.*, 2002; Molinari *et al.*, 2005; Flores-Romero and Navas, 2005), may behave differently on the same host genotype.

The employment of resistant varieties would require accurate knowledge of the geographic patterns of these pathogens in the area under consideration (Gould, 1983). Several studies carried out on plant nematode species have recognized that distribution is important for the evaluation of host-parasite response (Davis, 1984; Noe and Campbell, 1985; Duncan, 1986) and that ecological studies are valuable (Bello *et al.*, 1986). However, according to our results, the MLEE variants analysed together with the nematode densities of the nematode (adults females and J_2) were unrelated to the edaphic variables (soil pH, soil sand, clay and silt content) in our study, indicating that there was no geographic and ecologic structure based on the parameters analysed. Therefore, we can conclude that the La Vera study area represents an ecologically optimum niche for the *M. arenaria* MLEE variants detected. Thus, the potential of strategies based on the employment of resistant varieties is highly limited because of absence of geographic structure. Founding events and agricultural practices are recognized as important factors determining genetic structure in plant parasitic nematodes (Folkertsma *et al.*, 2001) in addition to the influence of host selection (Semblat and Castagnone-Sereno, 2001). Nevertheless, the proposed zoning based on similarity groups obtained (Fig. 4) could provide a starting point to implement control of these pathogens in the high-risk areas. Spatial analysis is an emerging area in models of ecological processes whose main objective is the quantification of spatial patterns of distribution of organisms (Liebhold and Gurevitch, 2002). Methods have been developed to estimate values at unsampled locations via interpolation for mapping spatial data (Dale *et al.*, 2002). Application of this approach would ease the control of plant parasitic nematodes by crop rotation after a monocropping in the studied area and, given the relation between diversity and the biological cycles of these nematodes, the proposed zoning could also facilitate the efficient use of nematicides through their application to the appropriate areas at the appropriate time. The procedure used in this study will produce effective zones of infection potential when the entire geographic range of the pathogen is taken into account. Although this study has been carried out in a monocropping area, we assume that these results would correspond well not only to other crops with similar characteristics (i.e., monocropping areas), but also to commercial greenhouse crops, as has been reported by Janssen *et al.* (1996) for these nematodes.

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