Journal of Nematology 37(3):328–335. 2005.

Characterization of *Heterodera zeae* Populations from Portugal

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Abstract: Three populations of the corn cyst nematode *Heterodera zeae,* one found in the rhizosphere of a fig tree and two infecting corn, were studied using the morphology and morphometry of cysts and second-stage juveniles, and compared with other populations. The intrapopulation and intraspecific variability are discussed. A simple and improved technique to prepare vulval cones for SEM is described. The non-specific esterase patterns of females, isolated from infected corn, were analyzed by electrophoresis in polyacrylamide gels. Two bands of esterase activity were detected. The occurrence of *H. zeae* is reported for the first time in Portugal and Europe.

Key words: corn cyst nematode, electrophoresis, esterases, Europe, geographical distribution, *Heterodera zeae,* identification, Portugal, taxonomy.

The corn cyst nematode (CCN), *Heterodera zeae* Koshy, Swarup and Sethi, 1971, was originally found parasitizing corn, *Zea mays,* in India (Koshy et al., 1971). Subsequently, CCN was found to be widely distributed in India (Koshy and Swarup, 1971; Makadia et al., 1989; Sakhuja et al., 1987; Srivastava and Kaushal, 1991; Swarup and Sosa-Moss, 1990) and in Egypt (Aboul-Eid and Ghorab, 1983), Pakistan (Ahmed and Qasim, 1990; Maqbool, 1981; Maqbool and Hashmi, 1984; Shahina and Maqbool, 1990), the United States (Eisenback et al., 1993; Krusberg, 1988; Sardanelli et al., 1981), and Thailand (Chinnasri et al., 1995).

During a survey in Portugal of *Heterodera* spp., cysts and second-stage juveniles (J2) of *H. zeae* were recovered from soil samples collected near a fig tree (*Ficus carica*) and from two corn fields.

In the present study, the morphological and morphometric characters of the three Portuguese populations of *H. zeae* are compared with other populations. The non-specific esterase patterns of the three populations are also characterized.

Additionally, a simple and improved technique for preparing vulval cones for scanning electron microscopy (SEM) is described.

Materials and Methods

Soil and root samples were collected from a fig tree and two corn fields in Pego, Abrantes, and S. Facundo and Granja, Coimbra, respectively (Centre of Portugal). Cysts were extracted from soil samples using a Fenwick can. They were separated from the extracted material in a Fenwick counting tray using fine forceps (Oostenbrink, 1950; Shepherd, 1986) and a stereomicroscope, and stored at ± 4 °C.

Light microscopy (LM) studies: Vulval cones were prepared from cysts using the glycerine-agar technique (Correia and Abrantes, 1997) and J2, released from cysts by crushing the cysts, were fixed in 4% formaldehyde. The specimens were mounted on glass slides for observation and measurements with a compound microscope. In some cases, differential interference contrast (DIC) was also used. All measurements were in micrometers (µm) and presented as mean ± standard deviation with the range in parentheses and the coefficient of variability.

Scanning electron microscopy (SEM) studies: Cyst terminal areas were prepared using a simple and improved technique developed for improved observation of the external and internal morphology of the vulval cones and avoidance of the interaction between the processing chemicals and the cone wall. The terminal areas/vulval cones were selected individually (some of them previously used in LM studies), transferred to a 0.5-ml Eppendorf tube containing 45% lactic acid, and sonicated using a 115-V sonicator for 20 sec. This technique was modified from Mota and Eisenback (1993), where whole cysts were used. Cyst terminal areas were transferred to 4% formaldehyde and sonicated a second time. Cyst terminal areas/vulval cones were mounted onto a SEM stub with double-sided adhesive tape, allowed to dry in a desiccator at room temperature for at least 24 hrs, and kept in the desiccator until being coated with gold for SEM observations.

Excised stylets of J2 were prepared for SEM observations. Whole specimens were placed in a chamber with a drop of 22.5% lactic acid and the stylets were excised and cleaned. A drop of 2% formaldehyde was added to the chamber to wash off the first solution and the chamber was mounted onto a SEM stub with double-sided adhesive tape (Abrantes and Santos, 1989; Eisenback and Hirschmann, 1982).

Cyst structures and J2 stylets were coated with 200 Å gold and examined using a JEOL-35C SEM.

Biochemical studies: Mature females of the Granja population were hand-picked from infected corn roots and carefully cleaned. Protein extracts were prepared from 1, 5, or 10 females placed in different microhematocrit tubes that were sealed at one end and contained 5 µl (1 female) or 10 µl (5 and 10 females) of extraction buffer (20% sucrose, 10 mM Tris-HCl, 1 mM

Received for publication 3 August 2004.

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Supported in part by Fundação para a Ciência e a Tecnologia through a PhD grant (BD/2846/93) of PRAXIS XXI Program to the first author. Scientific illustrations and photographs authorship: Fernando J. S. Correia (2003).

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This paper was edited by R. T. Robbins.

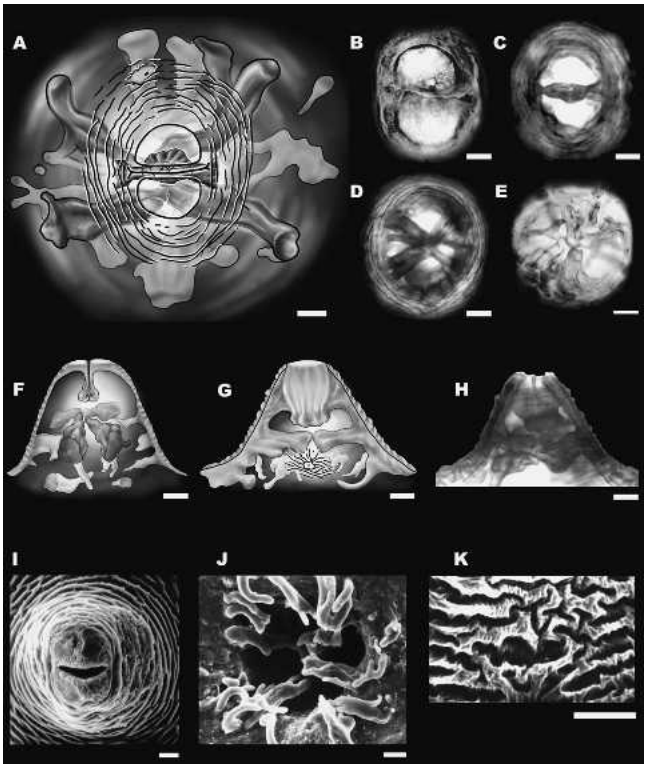

FIG. 1. *Heterodera zeae* cyst vulval cones. A–E) Top views. A) Drawing of external and internal morphology. B–E) LM photographs, respectively, of fenestrae and vulval bridge, vagina, bullae. F–H) Vagina and bullae, sagittal view. F) Lateral (drawing). G,H) Ventral (drawing and LM photograph). I–K) SEM photographs of top view, bullae level view, and perineal fenestration pattern. Scale bars = 10 μm.

EDTA, 1% Triton X-100, pH 8.0). The females were macerated with a metal pestle and used immediately or frozen at −80 °C. Shortly before electrophoresis the homogenates were centrifuged at 10,000*g* for 15 min at −5 °C. The supernatants plus a drop of bromophenol blue marker dye (1 mg/ml) were transferred to the stacking gel. The stacking and separating gels were homogenous 3% (2.92% total monomer concentration [T]; 2.66% crosslinking monomer concentration [C]) and 7% (6.99% T; 2.66% C) polyacrylamide, respectively (Laemmli, 1970). Soluble proteins were separated by native polyacrylamide gel electrophoresis (PAGE) in a Mini-PROTEAN II (Bio-Rad Laboratories, Hercules, CA) apparatus, using vertical thin-slabs immersed in 25 mM Tris-Base, 192 mM glycine bridge buffer (pH 8.2). Electrophoresis was carried out at 6 mA/gel for 15 min and at 20 mA/gel until the marker dye reached the base of the separation gel (ca 45 min). Gels were stained for non-specific esterase activity at 37 °C for 45 min (Abrantes et al., 1992), fixed in 20% ethanol-water solution, and air-dried between two cellophane sheets (48 hr). *Meloidogyne javanica* soluble protein extract aliquots (5-μl) were included in one lane on each gel as a reference (Pais and Abrantes, 1989). Relative electrophoretic mobility (Rm) for each band was calculated as the ratio of its movement toward the marker dye. Phenotypes were named as described by Esbenshade and Triantaphyllou (1985).

RESULTS

The morphological (Figs. 1,2) and morphometric studies of cysts and J2 of the three Portuguese populations were compared with those of other CCN populations previously published (Tables 1,2).

Description

Cysts: Morphometric data of eight populations are listed in Table 1. Cysts were light brown, lemon-shaped with terminal area protruding; vulval cone ambifenestrate and semifenestrate separated by a rather wide vulva bridge (Fig. 1A,B,I); vulval slit quite long, crenate (Fig. 1A,B,I); anus not conspicuous (Fig. 1G,K); underbridge very short and thin, not forked at ends (Fig. 1A,C,F,G,H); bullae prominent in specific arrangements on two different levels: (i) four finger-like projections, in ''x''-shaped set, located immediately below

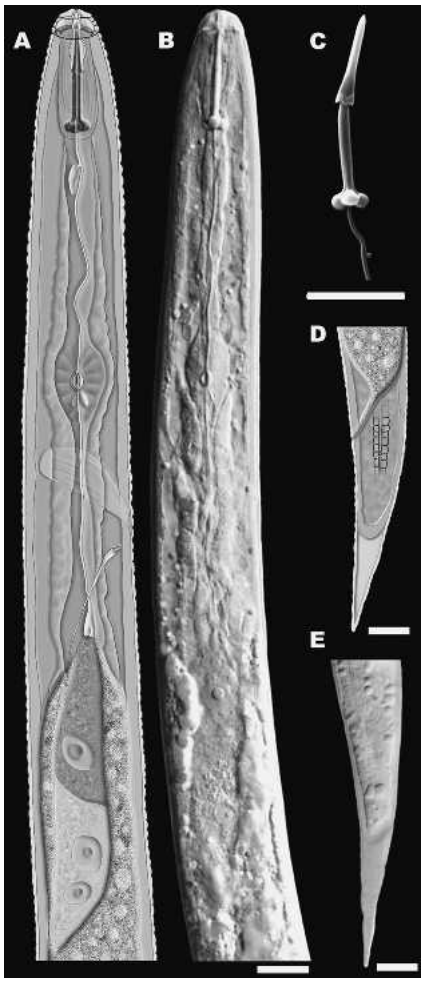

FIG. 2. *Heterodera zeae* second-stage juveniles. A) Drawing and B) DIC photograph of anterior region. C) SEM photograph of excised stylet. D) Drawing and E) DIC photograph of tail. Scale bars = 10 μm.

TABLE 1. Morphometric data of cysts of *Heterodera zeae* populations (mean ± standard deviation, range in parentheses and coefficient of variability [%]).

Character	Portugal Pego	Portugal S. Facundo	Portugal Granja	India (Koshy et al., 1971)	India (?) (Mulvey, 1972)	India (Golden and Mulvey, 1983)	USA (Golden and Mulvey, 1983)	Thailand (Chinnasri et al., 1994)
N	6	20	20	80	10	38	40	25
Linear (µm)								
Body length (L)	668.8 ± 79.1 (505–758) 11.8%	636.3 ± 73.7 (520–805) 11.6%	606.5 ± 83.9 (480–740) 13.8%	501 ± 10 (342–684)	—[a]	565 ± 92 (428–785)	588 ± 88 (454–785)	585.2 ± 71.2 (470.4–688.3)
Body length without neck	620.7 ± 63.3 (505–695) 10.2%	554.5 ± 76.5 (385–730) 13.8%	541.5 ± 87.9 (400–685) 16.2%	—[a]	—[a]	—[a]	—[a]	—[a]
Body width at midbody (W)	456.7 ± 69.1 (340–551) 15.1%	426.3 ± 54.9 (340–530) 12.9%	390.5 ± 63.9 (300–530) 16.4%	396 ± 8.8 (260–537)	—[a]	380 ± 74 (245–525)	347 ± 63 (255–551)	454.9 ± 31.2 (392–528)
Vulval cone height (VCH)	63 ± 7.2 (55–75) 11.4%	79 ± 8.2 (65–95) 10.3%	75.3 ± 10.4 (50–95) 13.9%	—[a]	—[a]	—[a]	—[a]	—[a]
Vulval cone width (VCW)	95.8 ± 12.2 (79–111) 12.8%	109.5 ± 13.4 (90–135) 12.2%	107.3 ± 9.6 (95–125) 8.9%	—[a]	—[a]	—[a]	—[a]	—[a]
Neck length	96.3[b] ± 6.3 (90–105) 6.6%	77.8 ± 27.9 (35–150) 35.9%	66.5 ± 16.7 (45–110) 25.1%	—[a]	—[a]	—[a]	—[a]	—[a]
Neck width	53.3[b] ± 9.4 (40–60) 17.7%	56.3 ± 14.1 (30–85) 25.1%	61.5 ± 13.2 (40–85) 21.5%	—[a]	—[a]	—[a]	—[a]	—[a]
Vulval slit length (VSL)	32 ± 2.5 (30–37) 7.9%	37.7 ± 3.4 (31–45) 8.9%	35.1 ± 2.5 (31–39.5) 7.1%	44.4 ± 1 (36–58)	—[a] (38–46)	40.4 ± 3.5 (38–45)	36.5 ± 3.7 (29–42)	44.2 ± 1.6 (41.4–47.2)
Vulval bridge length (VBL)	26.5 ± 1.7 (25–30) 6.4%	28 ± 4.1 (21–30) 14.5%	26.6 ± 2.2 (21–30) 8.3%	27.8[c] ± 0.8 (20–32)	—[a] (22–25)	27.4 ± 5.3 (19–38)	26.3 ± 3.9 (16–34)	32.9 ± 2.4 (28.8 ± 38.4)
Vulval bridge width	6 ± 1.4 (5–8) 23.6%	7.1 ± 1.8 (5–10) 24.9%	6.4 ± 1.1 (5–8) 16.6%	6.7 ± 0.2 (4.5–9)	—[a]	—[a]	—[a]	—[a]
Mean semifenestral width (MSW)	17.7 ± 2 (15–21) 11.2%	18.7 ± 2.9 (15–26) 15.3%	18.2 ± 0.9 (16.5–20) 5%	18.7 ± 0.4 (16–21)	—[a]	—[a]	—[a]	—[a]
Fenestrae length	41.3 ± 3.3 (37–47) 8%	44.5 ± 4.8 (37–58) 10.9%	42.8 ± 2.4 (38–48) 5.5%	44 ± 0.8 (37–53)	—[a] (42–45)	46 ± 4.5 (39–57)	40.4 ± 3.3 (35–45)	48.5 ± 2.1 (44.3–53.1)
Underbridge length (UL)	49.2 ± 5.8 (40–58) 11.9%	48.4 ± 5.1 (40–59) 10.6%	43.7 ± 4 (40–51) 9.1%	—[a]	—[a] (40–50)	38.7 ± 3.5 (34–51)	36.8 ± 3 (30–41)	33.9 ± 1.5 (30.6–36.4)
Mean underbridge width	7.2 ± 2 (5–11) 13.4%	5 ± 0.9 (3.5–6.5) 17.9%	5 ± 0.6 (4–6) 12.7%	—[a]	—[a] (10–12)	9.1 ± 1.9 (7.6–10.2)	10.3 ± 1.4 (7.6–12)	9.2 ± 0.9 (7.9–10.3)
Ratios								
L/W	1.5 ± 0.3 (1.1–1.9) 19.4%	1.5 ± 0.2 (1.2–1.9) 11.6%	1.6 ± 0.2 (1.3–1.9) 10.1%	1.3 ± 0 (1–1.5)	—[a]	1.4 ± 0.3 (1.2–1.8)	1.7 ± 3.3 (1.4–2.2)	1.3 ± 0.1 (1.0–1.5)
VCH/VCW	0.7 ± 0.1 (0.6–0.7) 8.2%	0.7 ± 0.1 (0.6–1) 13.4%	0.7 ± 0.1 (0.5–1) 14.1%	—[a]	—[a]	—[a]	—[a]	—[a]
VBL/MSW	1.5 ± 0.1 (1.4–1.7) 7.3%	1.5 ± 0.1 (1.1–1.9) 13.3%	1.5 ± 0.1 (1.2–1.6)	—[a]	—[a]	—[a]	—[a]	—[a]
Percentages								
(VSL/UL) × 100	66.3 ± 12.2 (51.7–82.5) 19.5%	78.3 ± 6.9 (64–90.4) 8.7%	80.8 ± 8.0 (62.8–94.1) 9.9%	—[a]	—[a]	—[a]	—[a]	—[a]
(VBL/VSL) × 100	82.9 ± 2.1 (81.1–86.7) 2.5%	74.1 ± 8.1 (63.3–89.2) 10.9%	75.9 ± 6.3 (63.6–85.7) 8.3%	—[a]	—[a]	—[a]	—[a]	—[a]

[a] Character not observed or available.
[b] Value obtained from three specimens.
[c] Vulval bridge length considered as synonym to fenestra width.

TABLE 2. Morphometric data of second-stage juveniles of *Heterodera zeae* populations (mean ± standard deviation, range in parentheses and coefficient of variability [%]).

Character	Portugal Pego	Portugal S. Facundo	Portugal Granja	India (Koshy et al., 1971)	India (Golden and Mulvey, 1983)	USA (Golden and Mulvey, 1983)	Pakistan (Maqbool and Hashmi, 1984)	Thailand (Chinnasri et al., 1994)
N	30	30	30	25	30	50	—[a]	25
Linear (µm)								
Body length (L)	437.9 ± 16.7 (402–484) 3.8%	381.8 ± 17.9 (350–430) 4.7%	447.7 ± 22.9 (400–500) 5.1%	410 ± 40 (360–430)	423 ± 15 (392–451)	431 ± 14 (399–460)	(340–450)	451.7 ± 18.8 (416.3–483.5)
Body width at midbody	—[a]	22.8 ± 0.7 (21–24) 3.3%	21 ± 0.9 (19–22.5) 4.4%	—[a]	19.7 ± 0.1 (19.6–20.2)	19.6 ± 0.7 (18.5–20.7)	—[a]	19.8 ± 0.1 (18.3–21.5)
Body width at stylet knobs	—[a]	15.5 ± 0.9 (14–17.5) 5.6%	16.0 ± 1.2 (15–20.5) 7.4%	—[a]	—[a]	—[a]	—[a]	—[a]
Lip region height (LRH)	3.3 ± 0.4 (3–4) 10.3%	3.4 ± 0.9 (3–4) 10.4%	3.3 ± 0.4 (3–4) 11.1%	—[a]	4.3 ± 0.3 (3.9–4.5)	3.9 ± 0.1 (3.4–4.5)	—[a]	—[a]
Lip region width (LRW)	9.9 ± 0.3 (8.5–10.5) 3.4%	9.2 ± 0.3 (8.5–9.5) 2.9%	9.2 ± 0.5 (8.5–10) 5.0%	—[a]	9.1 ± 0.2 (9–9.5)	9.1 ± 0.2 (8.4–9.5)	—[a]	—[a]
Stylet length (SL)	19.5 ± 0.7 (18–20.7) 3.4%	19.4 ± 0.9 (17–21) 4.7%	20.5 ± 0.5 (19.5–21.5) 2.6%	23 ± 0.26 (20–25.0)	19.9 ± 0.3 (19.6–20.2)	19.9 ± 0.4 (19–20.7)	(19.4–24)	21.9 ± 0.8 (20.7–23.2)
Stylet shaft and knobs length (SSKL)	10.8 ± 10.4 (10–11.7) 3.5%	11.3 ± 0.5 (10.1–12.5) 4.6%	11.1 ± 0.3 (10.5–11.5) 2.5%	—[a]	—[a]	—[a]	—[a]	—[a]
Stylet knobs height (SKH)	1.8 ± 0.2 (1.4–2.2) 11.2%	1.7 ± 0.2 (1.5–2) 10.9%	2 ± 0.2 (1.5–2.5) 8.8%	—[a]	—[a]	—[a]	—[a]	—[a]
Stylet knobs width (SKW)	4.7 ± 0.3 (4.3–5.3) 5.3%	4.5 ± 0.3 (4–5.2) 5.8%	4.6 ± 0.3 (4.5–5) 6.8%	—[a]	—[a]	—[a]	—[a]	—[a]
Stylet base to anterior end	22.1 ± 1.0 (19.8–24.5) 4.6%	22.2 ± 0.8 (21–24) 3.5%	23.2 ± 0.5 (22.5–24.5) 2.3%	—[a]	—[a]	—[a]	—[a]	—[a]
Dorsal oesophageal gland opening to stylet base	5.8 ± 0.5 (4.8–7.1) 8.6%	4.4 ± 0.4 (3.8–5.2) 9.4%	4.9 ± 0.6 (3.5–6) 11.7%	(4–5)	4.3 ± 0.4 (3.9–5.6)	4.3 ± 0.4 (3.9–5.6)	—[a]	3.8 ± 0.4 (3.2–4.8)
Metacorpus valve length (MVL)	2.7 ± 0.6 (1.5–3.3) 21.4%	2.7 ± 0.3 (2.2–3.1) 10%	2.9 ± 0.2 (2.5–3.5) 7.8%	—[a]	—[a]	—[a]	—[a]	—[a]
Metacorpus valve width (MBW)	2.2 ± 0.4 (1.8–3) 19.8%	1.7 ± 0.2 (1.5–2.2) 14.3%	1.9 ± 0.2 (1.5–2.2) 10%	—[a]	—[a]	—[a]	—[a]	—[a]
Metacorpus valve to anterior end (MVAE)	68.7 ± 2.5 (64–73.8) 3.6%	58.3 ± 3.4 (51–65.5) 5.9%	73 ± 3.4 (65.5–79) 4.7%	—[a]	69.5 ± 3.1 (63–77)	65.3 ± 2.7 (61–67)	—[a]	63.2 ± 1.8 (60–66.4)
Body width at excretory pore level	—[a]	20.3 ± 1.3 (18.0–22) 6.2%	20.2 ± 1.1 (18.1–22.5) 5.2%	—[a]	—[a]	—[a]	—[a]	—[a]
Excretory pore to anterior end (EPAE)	97.6 ± 0.7 (89.3–106.0) 3.9%	86.4 ± 4 (75–94) 4.6%	100.8 ± 3.3 (94–106.5) 3.3%	—[a]	—[a]	—[a]	—[a]	—[a]
Tail length (TL)	43.6 ± 3.3 (34.5–50) 7.6%	40.9 ± 2 (36.5–45) 4.9%	47.4 ± 2.5 (42.5–51.5) 5.2%	41 ± 1.1 (32–50)	45.9 ± 2.2 (40–51)	44.2 ± 2.4 (40–49)	—[a]	41.4 ± 3.5 (36.5–48.8)
Body width at anus (BWA)	—[a]	12 ± 1.1 (10–14) 8.8%	12.9 ± 0.9 (11.5–15) 7.0%	—[a]	—[a]	—[a]	—[a]	—[a]
Hyaline tail terminus length (HTL)	23.2 ± 1.6 (18.9–27) 6.9%	20.3 ± 1.8 (16.5–23) 8.9%	23 ± 1.9 (19.5–26) 8.3	24 ± 0.67 (16–30)	22.7 ± 2.3 (17.9–26.3)	21.9 ± 1.7 (16.8–25.2)	—[a]	24 ± 1.5 (20.8–26.4)
Hyaline tail terminus width at beginning (HTW)	—[a]	5.4 ± 0.5 (4.7–6.5) 9.2%	6.2 ± 0.4 (5.5–6.8) 5.8%	—[a]	—[a]	—[a]	—[a]	—[a]
Phasmids to tail tip (PTT)	36.7 ± 3.1 (30–45) 8.5%	27.2 ± 3.3 (21–31.5) 12.3%	—[a]	—[a]	—[a]	—[a]	—[a]	—[a]

TABLE 2. *Continued*

Character	Portugal Pego	Portugal S. Facundo	Portugal Granja	India (Koshy et al., 1971)	India (Golden and Mulvey, 1983)	USA (Golden and Mulvey, 1983)	Pakistan (Maqbool and Hashmi, 1984)	Thailand (Chinnasri et al., 1994)
Ratios								
a	—[a]	16.7 ± 0.9 (14.6–18.7) 5.6%	21.4 ± 1 (19.5–23.4) 4.8%	—[a]	21.6 ± 1.2 (20–23.3)	22.1 ± 0.9 (20.4–23.4)	(20–24.6)	22.8 ± 0.9 (20.6–24.1)
L/MVAE	6.4 ± 0.2 (5.9–6.9) 3.8%	6.6 ± 0.5 (5.6–7.7) 7.5%	6.1 ± 0.3 (5.4–6.9) 5.6%	—[a]	—[a]	—[a]	—[a]	—[a]
c	10.1 ± 0.2 (9–12.2) 7.9%	9.4 ± 0.6 (8.2–10.4) 6.6%	9.5 ± 0.5 (8.6–10.9) 5.6%	8.8 ± 0.2 (8–13)	9.2 ± 0.3 (8.8–9.8)	9.9 ± 0.3 (9.3–10.5)	(8.5–13.4)	11.9 ± 1.1 (9.0–12.5)
c′	—[a]	3.4 ± 0.3 (2.8–4.4) 9%	3.7 ± 0.3 (3–4.4) 8.1%	—[a]	—[a]	—[a]	—[a]	—[a]
HTL/HTW	—[a]	3.8 ± 0.4 (2.8–4.4) 10.7%	3.7 ± 0.3 (3.2–4.4) 8.5%	—[a]	—[a]	—[a]	—[a]	—[a]
BWA/HTW	—[a]	2.2 ± 0.2 (1.7–2.8) 11.1%	2.1 ± 0.2 (1.9–2.5) 8.9%	—[a]	—[a]	—[a]	—[a]	—[a]
LRW/LRH	3 ± 0.3 (2.4–3.5) 10.3%	2.7 ± 0.3 (2.3–3.2) 10.4%	2.8 ± 0.3 (2.3–3.3) 11.4%	—[a]	2.1 ± 0.1 (2–2.4)	2.3 ± 0.1 (2.3–2.7)	—[a]	—[a]
SKW/SKH	2.6 ± 0.4 (2.1–3.3) 13.6%	2.7 ± 0.3 (2–3.3) 11.3%	2.4 ± 0.2 (1.8–2.7) 8.6%	—[a]	—[a]	—[a]	—[a]	—[a]
MVL/MVW	1.6 ± 0.2 (1.2–2) 10.3%	1.6 ± 0.2 (1.3–2) 15.9%	1.6 ± 0.2 (1.3–2) 13.9%	—[a]	—[a]	—[a]	—[a]	—[a]
HTL/SL	1.2 ± 0.1 (1–1.4) 7.4%	1 ± 0.1 (0.8–1.3) 10.3%	1.1 ± 0.1 (0.9–1.3) 7.7%	—[a]	—[a]	—[a]	—[a]	1.1 ± 0.1 (1.0–1.3)
Percentages								
(EPAE/L) × 100	22.3 ± 0.6 (21.1–24.1) 2.7%	22.7 ± 1.4 (19.0–24.4) 6.1%	22.6 ± 0.9 (20.3–24) 3.9%	—[a]	—[a]	—[a]	—[a]	—[a]
(HTL/TL) × 100	53.6 ± 5.3 (43.8–63.1) 9.9%	49.6 ± 3.8 (41.8–57.2) 7.6%	48.6 ± 3.3 (43–59.1) 6.7%	—[a]	—[a]	—[a]	—[a]	—[a]
(PTT/TL) × 100	84.3 ± 4.2 (74.5–90) 5%	66.7 ± 7.6 (52.2–78.2) 11.5%	—[a]	—[a]	—[a]	—[a]	—[a]	—[a]
(SSKL/SL) × 100	55.5 ± 1.6 (52.6–60) 2.8%	58.1 ± 3.2 (50–64.7) 5.6%	54.3 ± 1.4 (51.2–57.5) 2.6%	—[a]	—[a]	—[a]	—[a]	—[a]

[a] Character not observed or available.

underbridge (Fig. 1A,D,F,G,H,J) and (ii) scattered, random, and irregular projections immediately below the first set of bullae (Fig. 1A,E,F,G,H,J).

Second-stage juveniles: Morphometric data of J2 of eight populations are listed in Table 2. Body tapering more posteriorly than anteriorly, short; cuticular body annulation distinct, lateral field with four lines, the two outer bands areolated (Fig. 2D); cephalic region with 3–4 annules (Fig. 2A,B); stylet slender, with round knobs linked to shaft by a slender bridge (Fig. 2A–C); excretory pore (EP) distinct, hemizonid two annules anterior to EP, three body annules long (Fig. 2A,B); phasmids conspicuous, usually midway between anus and anterior end of hyaline tail terminus (Fig. 2D); tail short, with a conical terminus; hyaline tail terminus length about one-half of tail length (Fig. 2D,E).

Biochemical analysis: A characteristic non-polymorphic phenotype was shown with two electrophoretic bands (Rm 0.27 and 0.31) for the non-specific esterases in the Granja population (Fig. 3). Native-PAGE gave reproducible esterase banding profiles, and the esterase activity was detected from homogenates prepared from 1, 5, or 10 females. The intensity of the bands was related with the number of females and therefore with the final soluble protein concentration of each sample.

Discussion

The morphological and morphometric characteristics of the cysts and J2 of the three Portuguese populations were similar to each other and to those reported in the original description or from other CCN populations (Tables 1,2).

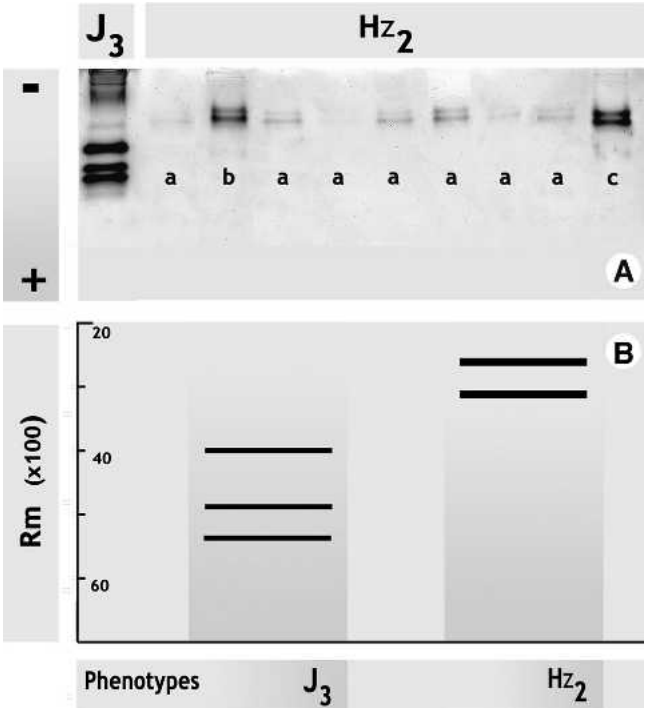

Fig. 3. Non-specific esterase phenotypes of Granja *Heterodera zeae* population. A) Native-PAGE electrophoretogram. Lane a—1 female; Lane b—5 females; Lane c—10 females. B) Zymogram. Phenotypes are designated with a letter or letters and a number indicating the number of bands. J_3 = *Meloidogyne javanica;* Hz_2 = *H. zeae.* Rm = relative mobility.

Heterodera zeae from Portugal presented the two main diagnostic characters: (i) the unique two levels of bullae with characteristic arrangement and orientation and (ii) a stylet mean length usually smaller than 20 µm. These characters are also found in other CCN populations (Golden and Mulvey, 1983; Stone, 1986).

The three populations were morphologically identical with similar mean values for cyst L/W ratio, and underbridge and stylet lengths. However, the S. Facundo population showed slight differences in some cyst characters, such as the bigger fenestrae fenestrae and greater vulva slit lengths, and in generally smaller J2 measurements.

According to a previous description by Golden and Mulvey (1983), the anterior face views of the J2 stylet knobs were described as shallowly concave using LM. This configuration was not detected in SEM observations, in which stylet knobs appeared to be round. Because the anterior slender bridges binding shaft and knobs were not seen in LM, it may be highly translucent and, therefore, without any optical interference in LM observations.

Intrapopulation variability was found in most of the cyst characters except for vulva, semifenestrae, and underbridge lengths (Table 1). The major J2 variability was found in the cephalic region and stylet knob heights, and in metacorpus valve dimensions and related ratios (Table 2).

Intraspecific variability was observed in some *H. zeae* population characteristics such as vulva length, cyst length, and width (Table 1). The fenestrae dimensions had a very low variability. However, the measurements found for Portuguese populations are very similar to those described by Golden and Mulvey (1983). The majority of the diagnostic characters referred to J2 (Table 2) show a very low intraspecific variability, especially the cephalic region width, the distance of dorsal oesophageal gland orifice to the stylet base, and the stylet, tail, and hyaline tail terminus lengths.

The technique described for preparing vulval cones for SEM observations (Fig. 1I–K) is easy to use and the vulval cones can be used for morphological studies.

A distinct and reproducible phenotype was detected in the non-specific esterase system for *H. zeae.* No faint bands due to an "aging" effect, commonly encountered in allozymes (Richardson, 1986), was detected in any of the homogenates, frozen at −80 °C or not. The multiple forms of esterase appear to be due to two different and active monomorphic loci rather than the expression of some allelic variation. This unique phenotype was the same as reported for other geographical isolates (Meher et al., 1998), suggesting that this enzyme pattern is species-specific, showing not only intrapopulation homozygosity but homozygosity for the spe-

cies itself. This procedure can detect and differentiate iso-esterase bands from a single female, providing a good tool not only to assess isozyme polymorphism but also to provide a fast and reliable method for the identification of *H. zeae* populations. This study corroborates previous reports of the usefulness of esterase markers for plant-parasitic nematode identification (Ibrahim et al., 1995; Mokabli et al., 2001; Nobbs et al., 1992; Pais and Abrantes, 1989; Powers and Fleming, 1998; Romero et al., 1996).

It has been reported that damage resulting from *H. zeae* could be limited by high-temperature requirements (Baldwin and Mundo-Ocampo, 1991; Whitehead, 1998). The occurrence of *H. zeae* in Portugal suggests that CCN is also adapted to temperate climates. *Heterodera zeae* is reported for the first time in Portugal and Europe.

The economic damage due to this plant-parasitic nematode in corn, wheat, and barley has been considered significant in some countries but not others (Maqbool, pers. comm.; Sharma et al., 1997; Swarup and Sosa-Moss, 1990). Because corn production has increased greatly in recent years in Europe (Sharma et al., 1997), further studies are required to find the real distribution and host status, and to determine whether this nematode species damages cereal crops in Portugal.

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