Pathogenicity of the root-knot nematode *Meloidogyne javanica* on potato

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Host–parasite relationships and pathogenicity of *Meloidogyne javanica* on potatoes (newly recorded from Malta) were studied under glasshouse and natural conditions. Potato cvs Cara and Spunta showed a typical susceptible reaction to *M. javanica* under natural and artificial infections, respectively. In potato tubers, *M. javanica* induced feeding sites that consisted of three to four hypertrophied giant cells per adult female. Infection of feeder roots by the nematode resulted in mature large galls which usually contained at least one mature female and egg mass. In both tubers and roots, feeding sites were characterized by giant cells containing granular cytoplasm and many hypertrophied nuclei. Cytoplasm in giant cells was aggregated alongside the thickened cell walls. Stelar tissues within galls appeared disorganized. The relationship between initial nematode population density (P) [0–64 eggs + second-stage juveniles (J2s) per cm^3 soil] and growth of cv. Spunta potato seedlings was tested under glasshouse conditions. A Seinhorst model [$y = m + (1 − m)z^{(P−T)}$] was fitted to fresh shoot weight and shoot height data of nematode-inoculated and control plants. Tolerance limits (T) for fresh shoot weight and shoot height of cv. Spunta plants infected with *M. javanica* were 0·50 and 0·64 eggs + J2s per cm^3 soil, respectively. The m parameter in that model (i.e. the minimum possible y-values) for fresh shoot weight and shoot height were 0·60 and 0·20, respectively, at $P = 64$ eggs + J2s per cm^3 soil. Root galling was proportional to the initial nematode population density. Maximum nematode reproduction rate was 51·2 at a moderate initial population density ($P = 4$ eggs + J2s per cm^3 soil).

Keywords: histopathology, host–parasite relationships, nematode reproduction, *Solanum tuberosum*, threshold level

Introduction

Root-knot nematodes (*Meloidogyne* spp.) can severely damage and cause significant losses in potato (*Solanum tuberosum*), in both warm and cool climates, depending upon the nematode species (Brodie *et al.*, 1993). Besides direct yield losses, root-knot nematodes may cause indirect damage in the form of blisters on tubers, as well as brown spots in tuber flesh where the maturing egg mass is located directly under the epidermis. These deformations and blemishes make tubers unmarketable and, more importantly, facilitate dissemination of the pathogen in infected seed tubers to new areas. Although many species of *Meloidogyne* are known to infect potato, only six are considered to be of global importance: *Meloidogyne chitwoodi*, *M. fallax*, *M. hapla*, *M. arenaria*, *M. incognita*

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and *M. javanica* (Netscher, 1970; Jatala & Bridge, 1990; Brodie *et al.*, 1993; Molendijk & Mulder, 1996). The first three of those six species are found in cool temperate regions, whereas the others are more important in warm temperate, tropical and subtropical regions of the world (Brodie *et al.*, 1993).

Infection of potato tubers by *Meloidogyne* spp. has been reported previously in Argentina (Chaves & Torres, 2001), Brazil (Charchar, 1997), Florida (Chitwood, 1949), Japan (Nakasono *et al.*, 1990), Libya (Dabaj & Khan, 1981), Rhodesia (now Zimbabwe; Mitchell *et al.*, 1971), Saudi Arabia (Al-Hazmi *et al.*, 1993) and Turkey (Cinarli & Eterkin, 1996). The present study was carried out on samples of soil heavily infested with *M. javanica* [36 eggs + second-stage juveniles (J2s) cm^{-3}] at Zabbar in Malta to determine: (i) the histopathology of nematode feeding sites in potato tubers and feeder roots infected by *M. javanica*; and (ii) the relationship between the initial population density of the nematode and growth of potato seedlings under glasshouse conditions. This is a new occurrence and first record of the nematode in Europe.

Materials and methods

Nematode diagnosis

The root-knot nematode infecting potato tubers and feeder roots of potato cv. Cara was identified by means of microscopic examinations, as well as by analysis of isozyme esterases and by species-specific molecular markers amplified in polymerase chain reaction (PCR) based on sequence-characterized amplified regions (SCARs; Zijlstra *et al.*, 2000). Samples of potato tubers together with rhizosphere and bulk soil were taken with a shovel from the upper 30 cm of soil from a commercial field at Zabbar, Malta. Second-stage juveniles (J2s) and males were extracted from roots and soil (Coolen, 1979), and females were recovered from infected root tissues and mounted in glycerin. Glycerin-infiltrated specimens were examined by light microscopy for nematode diagnosis. Thirty adult females from this population were analysed by perineal pattern morphology and excretory pore position. Perineal patterns and anterior body portions were prepared as described by Hartman & Sasser (1985) and examined under a light microscope.

To obtain females for electrophoretic and molecular analyses, the nematode population under study and a reference *M. javanica* population from peach at San Ferdinando in the province of Foggia, Italy, were increased on tomato (*Lycopersicon esculentum* cv. Rutgers) in a glasshouse at 25 ± 3°C. Forty days after inoculation, tomato plants were uprooted, their roots gently washed free of soil, and the root tissues teased apart with forceps and transfer needles to remove adult females.

Esterase isozyme analysis

One, three or five young egg-laying females of the *M. javanica* population from Zabbar, Malta, and five young egg-laying females of the *M. javanica* reference population were macerated in microtubes containing 5 μL of 20% sucrose and 1% Triton X-100. Electrophoresis was carried out in 7 × 8-cm homogeneous polyacrylamide gels, 0·75 mm thick, in a Mini Protean II electrophoresis unit (BIORAD, Madrid, Spain). Gels were stained for esterases with the substrate α-naphthyl acetate (Pais *et al.*, 1986).

DNA extraction and PCR assays

Total genomic DNA was extracted from single adult females according to Castillo *et al.* (2003). PCR assays were carried out using the SCAR primers and reaction conditions described by Zijlstra *et al.* (2000). Amplifications were performed with a PTC 100 thermocycler (MJ Research). Amplification products were separated by electrophoresis in 1·5% agarose gels in 1 × TAE buffer for 2–3 h at 100 V, stained with ethidium bromide and visualized under UV light. The 0·1-kb DNA ladder XIV size marker (Roche Diagnostics, Germany) was used for electrophoresis. Reactions were repeated at least twice and always included negative controls (no DNA) and positive control

DNA from *M. arenaria* and *M. incognita* adult females from olive nurseries (Nico *et al.*, 2002).

Histopathology

Plants of potato cv. Spunta were artificially inoculated with the sampled *M. javanica* population and incubated in a glasshouse at 25 ± 3°C for 60 days. Tubers of potato cv. Cara naturally infected by *M. javanica* at Zabbar (Malta), together with feeder roots from artificially infected cv. Spunta potatoes, were selected for histopathological studies. Small portions (5 × 5 × 5 cm) of the outermost layer (5–8 mm depth) of naturally infected potato tubers, including the vascular ring, as well as artificially infected feeder roots (4–5 mm long), were gently washed free of soil and debris and the galled tissues, together with portions of healthy tuber and feeder roots, were selected for histological examination. Tissues were fixed in formaldehyde chromo-acetic solution for 48 h, dehydrated in a tertiary butyl alcohol series (40-70-85-90-100%), embedded in paraffin with a melting point of 58°C and sectioned with a rotary microtome. Sections 10–12 μm thick were placed on glass slides, stained with safranin and fast-green, mounted permanently in a 40% xylene solution of a polymethacrylic ester (Synocril 9122X), examined microscopically and photographed (Johansen, 1940).

Relationship between inoculum density and plant growth

Inoculum of *M. javanica* from Zabbar was increased on cv. Rutgers tomato plants inoculated as described above and maintained in a glasshouse adjusted to 25 ± 3°C. Inoculum was obtained by extracting eggs + J2s using 1% sodium hypochlorite solution (Hussey & Barker, 1973). Two months after inoculation, when egg masses were well formed in the tomato roots, the inoculated plants were uprooted and their roots gently washed free of soil and finely chopped. To estimate the numbers of eggs + J2s in the chopped tissue, six 5 g aliquots of infected chopped roots were suspended in a 1% aqueous solution of sodium hypochlorite in 100 mL jars for 4 min (Hussey & Barker, 1973) and all the eggs and J2s in the suspension were counted using a Peter's 1 mL counting slide. For inoculation, chopped infected roots were thoroughly mixed with 2 kg of steam-sterilized sandy soil and this mixture was combined in different proportions with a potting medium of steam-sterilized sandy soil (87·5% sand, 5% silt, 5% clay and 2·5% organic matter) to obtain a range of increasing population densities: 0, 0·125, 0·25, 0·5, 1, 4, 16 and 64 eggs + J2s per cm^3 soil (Di Vito *et al.*, 1986).

Single-bud pregerminated seed tubers of potato cv. Spunta with good root primordials were planted singly in 500 mL clay pots filled with 500 g of infested soil mixture. Plants were incubated in a glasshouse at 25 ± 3°C. There were six replicated pots for each level of inoculum density, arranged in a randomized complete block design. Plants in pots were watered as needed and fertilized with 100 mL of a 0·1%, 20-5-32 + micronutrients hydro-sol

fertilizer (Haifa Chemicals Ltd, Israel) solution every week. Data on the appearance of symptoms of nematode attack (stunting and yellowing) were recorded during the experiment. Sixty days after planting, fresh shoot weight and total plant shoot height were measured. Plants were uprooted and the roots were washed free of soil and weighed. Root infection by the nematode was assessed by estimating root gall severity (RGS) on a 0–5 scale: 0, no galls; 1, one to five galls; 2, six to 20 small galls; 3, more than 20 galls homogeneously distributed in the root system; 4, reduced and deformed root system with some large galls; and 5, completely deformed root system with few but large galls (Di Vito *et al*., 1979). Eggs and J2s in the egg masses in roots were extracted by the sodium hypochlorite method (Hussey & Barker, 1973) and counted. Nematodes in soil were extracted by a modification of Coolen's method (Coolen, 1979). The final nematode population density (P_f) was calculated as the total number of nematodes extracted from roots and soil.

Statistical analysis

The relationship between the initial nematode population density (P) and plant growth (indicated by fresh shoot weight and total plant height) was determined by fitting the data to the Seinhorst model: $y = m + (1 - m)z^{(P-T)}$ when $P \geq T$, and $y = 1$ when $P < T$ (Seinhorst, 1965, 1979). In this model, y is the relative value of the plant growth parameter; m is the minimum value of y (at a very large initial nematode population density); P is the initial nematode population density; T is the tolerance limit (initial population at which plant growth is not impaired); and z is a constant < 1 reflecting nematode damage, with $z^{-T} = 1{\cdot}05$ (Seinhorst, 1965, 1979). The Seinhorst equation was fitted using the SEINFIT program (Viaene *et al*., 1997). The coefficient of determination (R^2) and the residual sum of squares were used to indicate the goodness-of-fit of data to the model.

The experiment was performed twice. Similarity among experiments was tested by preliminary analyses of variance using experimental runs as factors, which allowed the experiment × treatment interaction to be determined. This interaction was not significant ($P = 0{\cdot}05$) and allowed data to be combined for analyses of variance and fitting to the Seinhorst model. Data on root gall severity (RGS), final nematode population (P_f) and reproduction rate (R_f = final population/initial population) were normalized before analysis by transforming them into $\log_{10}(X + 1)$ (Gomez & Gomez, 1984). Analyses of variance were carried out using STATISTIX 8·0 (NH Analytical Software, USA). Means of RGS, P_f and R_f values were compared using Fisher's protected least-significant-difference test (LSD) at $P = 0{\cdot}05$.

Results

Symptoms and nematode diagnosis

Potato cv. Cara plants in commercial fields at Zabbar (Malta) showed severe yellowing, decline (Fig. 1a) and

heavily damaged tubers. Infected tubers had numerous blisters on the tuber surface (Fig. 1b and c) that usually contained one or more *Meloidogyne* females, males and egg masses with eggs (Fig. 1d and e). A mean population density of 8889 eggs + J2s/g of infected tuber tissue (based on the total weight of small-sized tubers) was estimated.

Morphological observations based on J2s, male stylet knobs, features of the female perineal pattern and excretory pore position/stylet length ratio matched typical traits of *M. javanica*. Isozyme electrophoretic analyses of the sampled young egg-laying females revealed three esterase bands identical to the esterase phenotype of the reference *M. javanica* (Fig. 2a). Similarly, PCR assays (with primer set Fj/Rj) using DNA extracted from potato nematodes in Malta and from the *M. javanica* reference isolate from peach gave rise to the *M. javanica* marker amplicon of 670 bp (Zijlstra *et al*., 2000) (Fig. 2b, lanes 3–5 and 1–2, respectively). No amplification occurred with *M. arenaria* or *M. incognita* DNA, or with the control (Fig. 2b, lanes 6–7, 8–9 and 10, respectively).

Histopathology

The irregular swellings induced by *M. javanica* on potato tubers (cv. Cara) and the galls on feeder roots (cv. Spunta) varied in size and location. Usually, irregular blisters covering 50–60% of the tuber surface were present on some 80% of sampled tubers, giving them a warty appearance (Fig. 1b–d). Most of the infected tissues randomly selected for examination contained an egg mass (Fig. 1g); however, adult males and up to four mature globose females were found associated with the same feeding site in many cases. Additionally, similar infections by adult females of *M. javanica* were observed in several potato tubers without external symptoms of nematode attack. Histopathological observations on infected cv. Cara tubers revealed well established feeding sites located in the outermost layer of the tuber that included the vascular ring (Fig. 1d). Each adult female was surrounded by three to six large giant cells (originating from protoxylematic cells) showing thickened cell walls, granulated cytoplasm and numerous hypertrophied nuclei and nucleoli (Fig. 1f). Nematode bodies and egg masses containing 0–255 eggs were observed in the internal tuber tissues.

Galls on artificially infected feeder roots of cv. Spunta occurred either singly or in clusters, which encircled the entire root. Occasionally, an egg mass was found inside the cortical root tissues (Fig. 1g), but the majority of egg masses were observed at the root surface. Comparative histological observations of healthy and *M. javanica*-infected feeder roots of cv. Spunta showed cellular alterations in tissues of the cortex, endodermis, pericycle and stele induced by the nematode. In the permanent feeding sites, each adult female was surrounded by three to four large giant cells showing granulated cytoplasm and numerous hypertrophied nuclei and nucleoli. Nematode-induced formation of large multinucleate giant cells adjacent to the stele tissues was observed in all infected potato feeder roots. This formation modified the internal
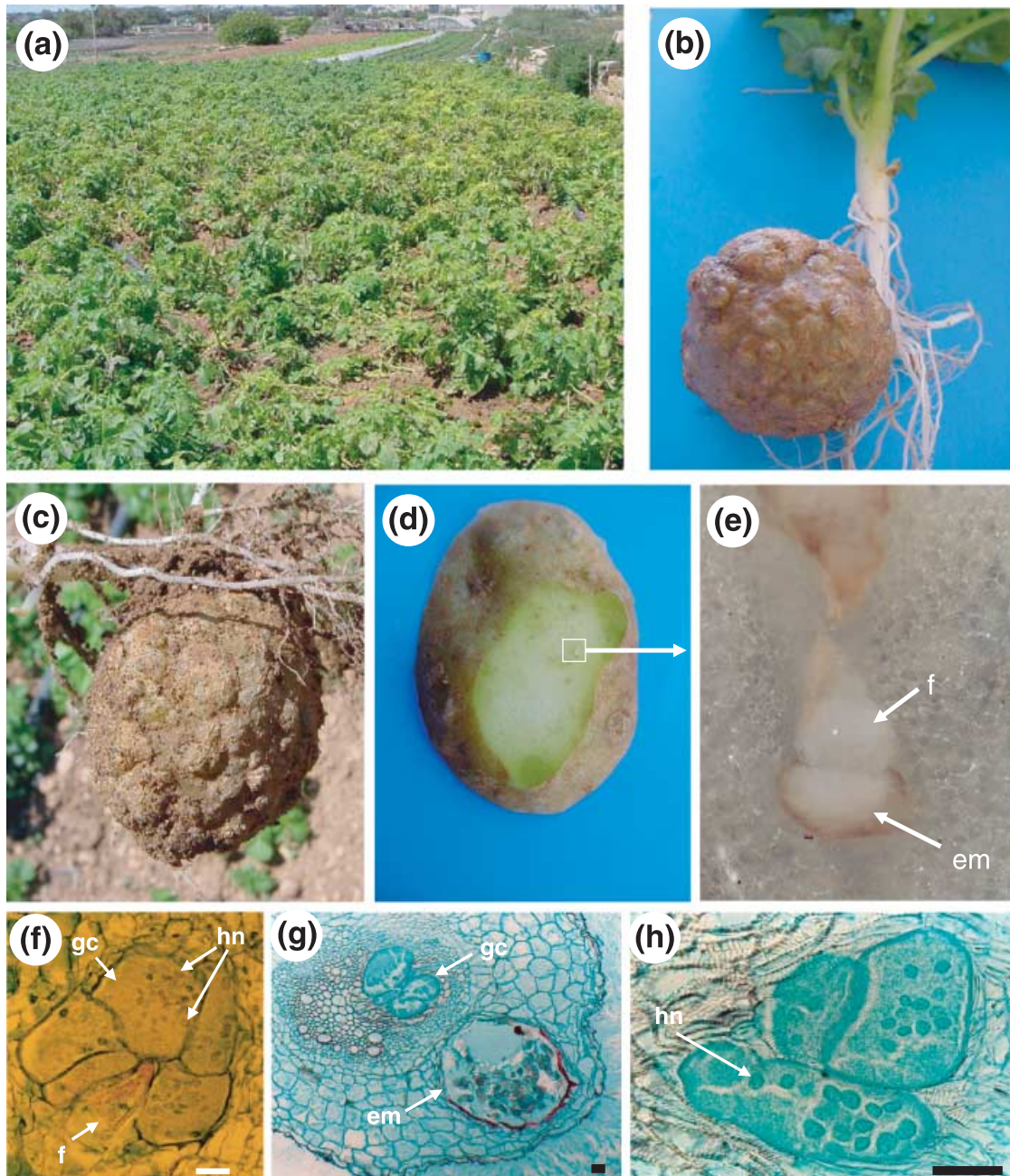
Figure 1 (a) Stunting and yellowing of potato cv. Cara plants severely affected by *Meloidogyne javanica* in a commercial field at Zabbar, Malta. (b, c) Diseased potato tubers naturally infected by *M. javanica* showing strong deformation of the outermost layer of the tuber. (d) Section of potato tuber showing numerous adult females with mature egg masses. (e) Higher magnification of (d). (f) Feeding site on tuber induced by nematode female. (g, h) Feeder roots of potato cv. Spunta infected by *M. javanica*. em, egg-mass; f, adult female; gc, giant cells; hn, hypertrophied nuclei. Scale bars: (f) 200 μm; (g, h) 50 μm.

structure of the vascular cylinder, causing extensive distortion, as well as disorganization of the root cortex (Fig. 1g). Giant cells showed dense cytoplasm and variable numbers of hypertrophied nuclei (15–25) and nucleoli (Fig. 1h). Hyperplasia of tissues adjacent to giant cells contributed to formation of root galls.

Relationship between inoculum density and plant growth

Inoculation of potato cv. Spunta with *M. javanica* impaired plant growth at all nematode inoculum densities in the study (Fig. 3). The relationship between fresh shoot weight and
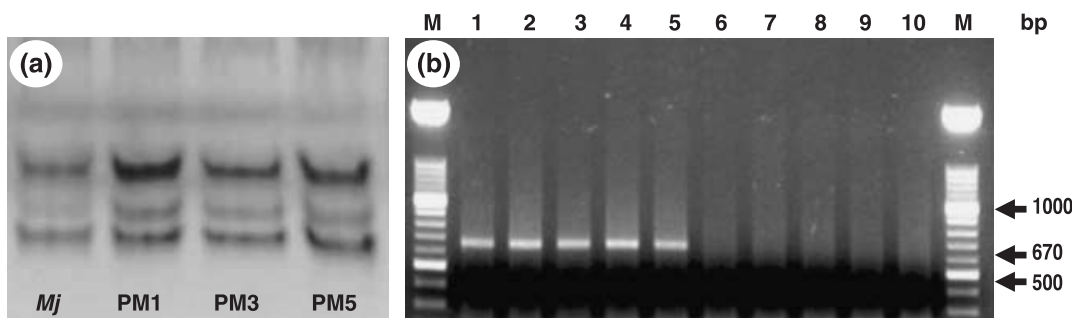
Figure 2 (a) Esterase electrophoresis pattern of protein homogenates from one, three and five egg-laying females (PM1, PM3 and PM5, respectively) of *Meloidogyne javanica* infecting potato cv. Cara in Malta, and a single young egg-laying female (*Mj*) of a reference isolate of *M. javanica*. (b) PCR amplification product using primers Fj and Rj and 1–10 ng of template DNA of *Meloidogyne* isolates: lanes 1–2, *M. javanica* reference isolate from peach; lanes 3–5, *M. javanica* from potato in Zabbar, Malta; lanes 6–7, *M. arenaria* from olive nurseries in Spain; lanes 8–9, *M. incognita* from olive nurseries in Spain; lane 10, control (no template DNA).

total shoot height of plants and the initial nematode population density in soil was adequately described by the Seinhorst equation, with R^2 values ranging from 0·672 to 0·747 (Fig. 3a and b). Plant stunting and yellowing caused by *M. javanica*, and reduction of plant shoot growth, were obvious by 30 days after inoculation with 16 eggs + J2s per cm^3 soil. Forty-five days after inoculation, all plants inoculated with more than 4 eggs + J2s per cm^3 soil showed stunting and yellowing. The tolerance limits (*T*) of potato to *M. javanica* were 0·50 and 0·64 eggs + J2s per cm^3 soil for fresh shoot and total shoot weight, respectively (Fig. 3a and b). The minimum possible relative values (*m*) for fresh shoot and total shoot weight were 0·60 and 0·20, respectively, at *P* = 64 eggs + J2s per cm^3 soil (Fig. 3a and b). The maximum nematode reproduction rate (R_f) was 51·2 at a moderate initial population density of 4 eggs + J2s per cm^3 soil. In general, the reproduction rate increased as the initial nematode population increased up to 1 egg + J2 per cm^3 soil, and decreased at the highest initial nematode population (Table 1). The highest final population density was found in plants infected with the highest initial population density (Table 1). Similarly, the severity of root galling of potato feeder roots increased with the increase in initial nematode population, with the highest value at 64 eggs + J2s per cm^3 soil (Table 1).

Discussion

Accurate identification of *Meloidogyne* spp. infecting potato tubers in potato-growing areas is a prerequisite for quarantine measures, the efficient use of host plant resistance and effective management of these nematodes on potato. Similarly, information on plant growth impairment and yield losses caused by root-knot nematodes is of use in implementing effective integrated management programmes, as well as a prerequisite for farmers to be aware of the importance of managing the diseases caused by these nematodes. Therefore, the severe infections of potato tubers by *M. javanica* found at Zabbar (Malta) and the severe potato growth impairment caused by this nematode reported in this work highlight the potential risk of spread

Table 1 Relationship between initial population density of *Meloidogyne javanica* and root galling, final population density and reproduction rate on seed potato cv. Spunta, 50 days after inoculation

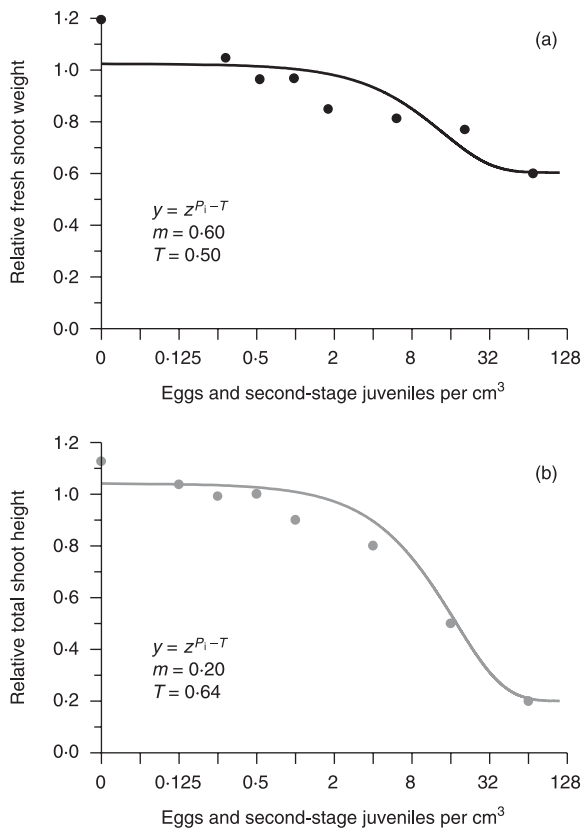| Initial population density (*P*)[a]	Population density (*P*) per pot	RGS[b]	P_f	R_f[c]
0·125	62·5	0·75 d	310 d	4·9 c
0·25	125	1·5 c	591 d	4·7 c
0·5	250	3·0 b	3 825 c	15·3 b
1	500	3·0 b	6 680 c	13·4 b
4	2 000	4·5 a	102 353 b	51·2 a
16	8 000	4·75 a	403 085 a	50·4 a
64	32 000	5·0 a	619 213 a	19·4 b

[a]Pregerminated potato cv. Spunta seed tubers were planted (one per pot) in a potting mixture infested with the appropriate *P* (numbers of eggs + second-stage juveniles per cm^3 soil). Plants were grown in a glasshouse adjusted to 25 ± 3°C for 60 days. Data are the average of two trials each with six replicated plants per treatment combination. Means followed by the same letter do not differ significantly (*P* > 0·05) according to Fisher's protected LSD test.
[b]Severity of root galling (RGS) was rated on a 0–5 scale: 0, no galls; 1, one to five galls; 2, six to 20 galls; 3, more than 20 galls homogeneously distributed in the root system; 4, reduced and deformed root system with some large galls; and 5, completely deformed root system with a few large galls.
[c]R_f (nematode reproduction rate) = P_f (final nematode population per plant)/*P*.

of this pathogen from infected early season potatoes to the potato-growing areas of the European Union.

The results of the histopathological studies indicate a typical susceptible reaction of potato cvs Cara and Spunta to infection by *M. javanica*. The extensive galling on potato tubers naturally infected by the nematode suggests that environmental conditions in the Mediterranean Basin are highly conducive for infection of potato tubers, thus making it possible for the pathogen to be disseminated in them to new areas (Nakasono *et al*., 1990). In particular, special care must be taken with seed-potato production in *Meloidogyne*-infested fields, since seed tubers without

Figure 3 Relationship between initial population densities (P_i) of *Meloidogyne javanica* and relative fresh shoot weight (a) and shoot height (b) of potato cv. Spunta plants grown in pots at 25 ± 3°C in the glasshouse for 60 days. Each point is the mean of two trials with six replicated plants each. Lines represent the predicted function calculated by fitting the Seinhorst model to data of fresh shoot weight and shoot height. For the fitted model of fresh shoot weight (a), $R^2 = 0.6718$ and sum of squares = 0.7495; for shoot height (b), $R^2 = 0.7469$ and sum of squares = 0.1771.

surface galling at harvest or initial storage may still be potential carriers of *Meloidogyne* egg-laying females, as demonstrated by Jatala *et al.* (1982). The observations of the present study indicate that symptoms may develop internally, resulting in external symptoms being visible only after several months of storage (Jatala *et al.*, 1982). Therefore, visual examination of tubers is not always accurate enough for diagnosis of infection, while extraction of nematodes from tubers undoubtedly improves detection and confirms infection (Viaene *et al.*, 2004).

Giant cells and tissue modifications induced by nematode infections were found to sequester nutrients from the host plant and limit water and nutrient translocation from infected roots to above-ground plant tissues (Hussey & Williamson, 1997) with subsequent plant growth impairment. The development and parasitic habit of *M. javanica* observed in potato tubers and feeder roots in the present study were similar to those reported for *M. incognita* (Vovlas *et al.*, 1994), suggesting that infection by *M. javanica* has the potential to severely damage potato. The severity of

root galling of cv. Spunta feeder roots, as well as the severe impairment of plant growth with increase in initial inoculum densities in soil, confirmed that *M. javanica* has great damage potential and reproductive capacity on potato, and agreed with data reported by Di Vito *et al.* (2003) for several susceptible *Solanum* spp.

The Seinhorst function adequately described the pathogenic relationship of *M. javanica* inoculum on potato. Using this model, the estimated tolerance limit of this plant to the nematode was as low as 0·50 and 0·64 eggs + J2s per cm³ soil (equivalent to 125 and 160 eggs + J2s per 250 cm³ soil) for fresh shoot weight and total shoot height, respectively. An initial population density of this pathogen exceeding 64 eggs + J2s per cm³ soil may decrease shoot height by 80% compared with noninfected control plants. Preplant inoculum thresholds for root-knot nematodes in potato were estimated at 1 egg per 250 cm³ soil for *M. chitwoodi* (Santo *et al.*, 1981) and 50 eggs per 250 cm³ soil for *M. hapla* (Brodie *et al.*, 1993). Thus, the tolerance limits in the present study of 125 and 160 eggs + J2s per 250 cm³ soil for fresh shoot weight and shoot height of potato cv. Spunta, respectively, indicate that under conditions conducive to infection, *M. javanica* is less aggressive on potato than *M. chitwoodi* and *M. hapla*. Nevertheless, damage by *M. hapla* on potato has been reported in the northeastern and north-central USA, eastern and central Canada, and in the Netherlands. It has also recently been found to increase as a result of reduction in the use of soil pesticides and cereal crop rotation (Brodie *et al.*, 1993; Wishart *et al.*, 2002).

The reduction of nematode reproduction rate with increasing initial nematode inoculum density has been reported for infections of several crops by *Meloidogyne* spp. (Di Vito *et al.*, 1986, 2004). Reproduction rates of *M. javanica* on potato at an initial inoculum density of 1 egg + J2 per cm³ soil were similar to those observed for *M. chitwoodi*, and higher than those for *M. fallax* or *M. hapla*, on several potato cultivars (Van der Beek *et al.*, 1997).

In view of the aggressiveness of *M. javanica* to potato demonstrated in the present study, management of this nematode species should be considered when implementing integrated pest management programmes in potato-growing areas where environmental conditions and potato-culture practices may favour the development of *M. javanica*. The results also highlight the need for additional studies on the resistance of potato to *M. javanica*. Although some attention was given to screening and breeding for resistance to *Meloidogyne* spp. in potato (Anter, 1989; Phillis, 1994; Grammatikaki *et al.*, 1999; Berthou *et al.*, 2003; Di Vito *et al.*, 2003), no potato cultivars resistant to these nematodes appear to be available in Europe. This is probably a consequence of the highly variable genetic make-up of *Meloidogyne* species.

This study shows that *M. javanica* is less aggressive on potato than *M. chitwoodi* and *M. hapla*, but nevertheless can severely impair potato growth. Thus, correct diagnosis and estimation of soil population levels of *M. javanica*, as well as of other root-knot nematodes, should be carried

out before planting to facilitate the effective integrated management of these nematodes on potato.

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