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Parasitism and pathogenicity of curly-leaf parsley with the root-knot nematode *Meloidogyne javanica* in Southern Italy

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Summary

Severe infections of parsley plants and soil infestations with *Meloidogyne javanica* during an autumn surveys for the pathogenic root-knot nematode infestations were found in Monopoli at Bari province in Southern Italy. This unusual severe infection of parsley, considered a winter crop, was possibly instigated by a very warm autumn from the previous year. Nematodes were extracted from soil samples according to the Coolen's method. Morphological analysis (based on stylet length, tail length and shape, adult females perineal pattern, excretory pore position and Ep/stylet ratio) and molecular studies were used for the nematode characterization and identification. In the soil of infested area a severely deformed root systems were observed, showing a galling rate = 2.5 - 4 (scale 0-5) and a soil nematode population densities ranging from 350 to 2,730 eggs and J2 per 5 g of fresh root. *M. javanica* attack on parsley roots is a limiting factor for plant growth. Considering that curly-leaf parsley varieties resistant to the nematodes are not yet available control strategies must be focused on reduction of soil infestation level below tolerance limit of the target nematode species. Due to the higher cost and reduced availability of fumigant and non-fumigant nematicides, soil solarization, organic amendments or biological control approaches should be preferably used as alternatives.
Keywords: giant cells; histopathology; nematode reproduction; *Petroselinum crispum;* root-knot nematode population density

Introduction

Parsley, *Petroselinum crispum* (Mill.) Nyman ex A.W. Hill, a member of the *Apiaceae* family, is one of the world's most popular herbs. The edible foliage is grown as an annual plant and used as a garnish and food ingredient. Both curly-leaf (*P. crispum* var. *crispum*) and flat-leaf or Italian parsley (*P. crispum* var. *neapolitanum*) are of agricultural importance, and largely cultivated in the Adriatic coastal areas of Apulia region in Southern Italy. Parsley has higher vitamin and nutrient content (i.e., β-carotene, thiamin, riboflavin and vitamins C and E, calcium, iron and folate) than most of the other vegetables (Benamotz & Fishler, 1998; Athar *et al*., 1999).

Root-knot nematodes (*Meloidogyne* spp.) are major group of plant-parasitic nematodes that have a severe effect on yield and quality in a wide range of economically important crops. Plant growth impairment caused by *Meloidogyne* spp. to vegetable crops is influenced by nematode species and/or physiological race as well as the initial nematode population density in soil at transplant or sowing (Sasanelli, 1994; Vovlas *et al*., 2008). Since, the host-plant resistance could be used to reduce the initial nematode population density below the tolerance threshold levels of vegetable crops (Sasser & Carter, 1985) an appropriate nematode identification is especially important in the root-knot nematodes. It is well established that a minimum nematode population density (*T,* tolerance limit) is required before measurable yield loss occurs (Seinhorst, 1965; 1979). Parsley has been reported as a suitable host for several root-knot nematodes what including *M. arenaria*, *M. enterolobii* (= *M. mayaguensis*), *M. floridensis*, *M. incognita*, *M. hapla, M. hispanica* and *M. javanica* (Doucet & Pinochet 1992; Sikora & Fernández, 2005; Mennan *et al*., 2011; Quénéhervé *et al*., 2011; Maleita *et al*., 2012). Root-knot nematode-infected parsley plants show symptoms of decline, stunting and leaf yellowing

(Sikora & Fernández, 2005; Mennan *et al*., 2011). Because parsley leaves are marketed this damage is economically very important for the farmers. In fact, Aguirre *et al*. (2003) in greenhouse test with *M. incognita* found tolerance limits (*T*) as low a 0.17, 0.025 and 0.02 eggs and second-stage juveniles (J_2)/cm^3 soil for top fresh and dry weight and total fresh weight of parsley plant cv. Double Curledas, respectively. Maximum nematode reproduction rate was 37 fold at *Pi* = 0.25 eggs and J_2/cm^3, confirming that parsley is a good host for this nematode (Aguirre *et al*., 2003). However, no information is available regarding parsley growth suppression by other *Meloidogyne* spp. in infested soil in open field.

During the last growth season periods 2012 and 2013 a severe feeder root infections of curly-leaf parsley cv. Robustus and soil heavily infested (350 – 2,730 units of eggs and J_2/ cm^3 soil) by *Meloidogyne* sp. were found on commercial fields at Monopoli in Bari province in Southern Italy. Unhealthy plants showed damage that occurred in patches (20 – 25 m diameter) within the field with symptoms including severe stunting and heavily affected root systems. The abundance of root-knot nematode affected roots suggested a highly specialized nematode-plant interaction. Since the *Meloidogyne* spp. have probably a widest host range among the plant parasitic nematodes an accurate identification and precise estimation of their population density in the soil is crucial for designing of effective control measures within the context of sustainability and integrated pest management practices. In fact, it is well established that the extent of crop growth suppression is influenced by the nematode species. Therefore, the objectives of this study were: (i) to identify the root-knot nematode species attacking parsley in Southern Italy, (ii) to provide morpho-biological information on the host-parasite relationships of this nematode species in parsley-nematode-feeding sites, and (iii) to assess the effects of natural soil infestations by the root-knot nematode on the growth of curly-leaf parsley plants.

Materials and Methods

Nematode identification

Samples of root-knot nematodes infected curly-leaf parsley cv. Robustus roots together with rhizosphere and bulk soil were taken from a commercial field at Monopoli (Bari province), in Southern Italy. Composite soil samples of 40 cores were collected with a soil probe 1.5 cm diameter wide and 30 cm long. The root-knot nematode was identified by microscopic examinations, analysis of isozyme esterases and by species-specific molecular markers amplified using polymerase chain reaction (PCR) (Zijlstra *et al*., 2000). Second-stage juveniles and males were extracted from soil (Coolen, 1979) and females were recovered from naturally-infected root tissues and mounted in glycerine. Glycerine infiltrated specimens were examined by light microscopy. Twenty adult females were identified according to the perineal pattern morphology and excretory pore position. Perineal patterns and anterior body portions were prepared as described in Hartman & Sasser (1985) and examined under a light microscope.

To obtain females for isozyme pattern identification, the field population and a reference population of *M. javanica* from Córdoba, Southern Spain (Nico *et al*., 2002) both derived from a single egg mass, were raised on tomato (*Solanum lycopersicum* L. cv. Rutgers) in a glasshouse at 25 ± 2 ºC. Forty days after inoculation, tomato plants were uprooted. Their roots gently washed out of adhering soil and the root tissues teased apart with forceps to remove adult females.

Isozyme analysis

Esterase (Est) and malate dehydrogenase (Mdh) phenotypes of *Meloidogyne* from the Monopoli population were compared with the reference *M. javanica* population from Córdoba, Spain. One to three young egg-laying females of each nematode isolate were macerated in microtubes containing 5 µL of 20 % (w:v) sucrose, 1 % (v:v) Triton X-100 and 0.01 % (w:v) bromophenol blue. Electrophoresis was performed with Tris-glycine buffer on 7 × 8-cm stacking (pH 6.8) and separating (pH 8.8) homogeneous gels containing 4 % or 7 % polyacrylamide (Mini Protean II electrophoresis unit, BioRad, Madrid, Spain). 0.75-mm thick gels were stained after running for about 120 min at 150 V with following substrates: α-naphthyl acetate for Est (Sigma-Aldrich, Madrid, Spain) and Fast Blue RR (Sigma-Aldrich, Madrid, Spain) for Mdh (Esbenshade & Triantaphyllou, 1985).

DNA extraction and PCR assays

For molecular analyses two young females were temporarily mounted in a drop of 1M NaCl containing glass beads. After taking the measurements and photomicrographs for nematode analysis the slides were dismantled and DNA extracted. Nematode DNA was extracted from a single female nematode and PCR assays were conducted as described by Castillo *et al*. (2003). PCR assays were carried out with the species-specific SCAR primer pairs Far/Rar (*M. arenaria*), Finc/Rinc (*M. incognita*) and Fjav/Rjav (*M. javanica*). The reaction conditions used were according to Zijlstra *et al*. (2000). Amplifications reactions were performed on BioRad C1000 thermal cycler (BioRad, Madrid, Spain). Amplification products were analyzed by electrophoresis on 1.5 % agarose gels in 1× TAE buffer ran for 2 – 3 h at 100 V. Gels were stained with ethidium bromide and visualized under UV light. The 0.1-kb DNA ladder XIV size marker (Roche Diagnostics, Mannheim, Germany) was used during electrophoresis. Reactions were repeated at least twice and negative controls (no DNA) and positive controls DNA from *M. arenaria, M. incognita* and *M. javanica* adult females from olive nurseries (Nico *et al*., 2002) were always included.

Histopathology

For histopathological observations naturally infected roots of curly-leaf parsley cv. Robustus were gently washed out of adhering soil and debris. Infected and healthy root pieces were fixed in FAA (formaldehyde-acetic acid- alcohol) for a minimum of 48 h, then dehydrated in tertiary butyl alcohol series (70-85-90-100 %) and embedded in Histosec® embedding paraffin (Merck, Darmstadt, Germany). Embedded tissues were sectioned transversely and longitudinally at 10 – 12 µm with a rotary microtome (Historange 2218, LKB Bromma) and mounted on glass slides stained with safranin and fast green (Johansen, 1940). Finally slides were mounted permanently in 40 % xylene solution of a polymethacrylic ester (Synocril 9122X, Cray Valley Products, NJ) and examined under

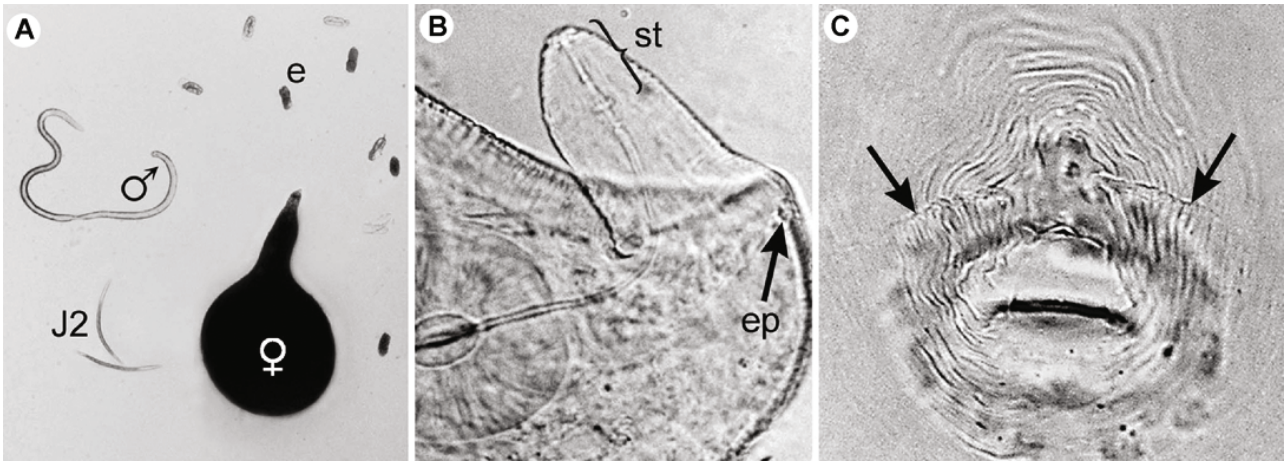

Fig. 1. Microphotographs of nematode morphology with taxonomic value for *Meloidogyne javanica*. A: Life stages of the nematode (e = eggs; J_{2s} = infective second stage juvenile; ♂ and ♀ = adult male and female). B: Female anterior body portion, showing the stylet (st) and the position of excretory pore (ep). C: Perineal pattern of adult female of *M. javanica*. Note the distinct lateral lines (arrowed) characteristic structure for this species

optical microscope (Reichert-Jung at 1,000 x). Images were taken with a Leica DFC 425 system.

Effect of the root-knot nematode soil infestation on growth of curly-leaf parsley plants

A stunted area was selected, in a curly-leaf parsley root-knot nematode-infested field near Monopoli (Bari Province) in Southern Italy where the cv. Robustus had been grown for 72 days and sampled in order to assess the effects of the nematode on the growth of this crop. A total of 24 curly-leaf parsley plants, six from each of the four concentric sectors considered in the patched area, were randomly dug up with a shovel. The sampling points in the non-stunted area were at least ten meters from plants in the stunted area. The plants sampled from the two areas were put in two separate large plastic bags to avoid drying. Within an hour after sampling the heights of the plants were measured and degrees of root infection rated according to a scale from 0 (no galls) to 5 (root system completely deformed by the presence of numerous and large galls) (Lamberti, 1971). Soil nematode population density in each sector of the stunted area was determined by processing 250 cm^3 of soil samples close to the uprooted parsley plants according to the Coolen's method (Coolen, 1979).

Results

Nematode identification and field symptoms

Morphological observations based on J_2, male stylet knobs, features of the female perineal pattern and excretory pore position/stylet length ratio (EP/st = 3.4 – 3.6) agree with typical traits of *M. javanica* (Fig. 1). The isozyme electrophoretic analyses of the sampled young egg-laying females revealed a single Est band typical of J_3 phenotypes (Fig. 2A), which characterize *M. javanica*. Similarly, PCR assays using the SCAR primer pairs specific for *Meloidogyne* species amplified the predicted products from DNA of the reference isolates (a band of 720 bp for *M. javanica*, primer pair Fjav/Rjav) (Zijlstra *et al*., 2000) (Fig. 2B).

Curly-leaf parsley plants cv. Robustus in commercial fields at Monopoli infected by the nematode showed severe decline with stunting, a patched distribution of field, and heavily infected roots with severe galled roots. Roots showed numerous large galls that usually contained one or more *Meloidogyne* females, males and eggs. Population density ranged from 420 to 53,400 eggs and J_2/g of galled fresh roots. Population density in naturally infested soil ranged from 0.12 to 14.25 eggs and J_2/cm^3 of soil.

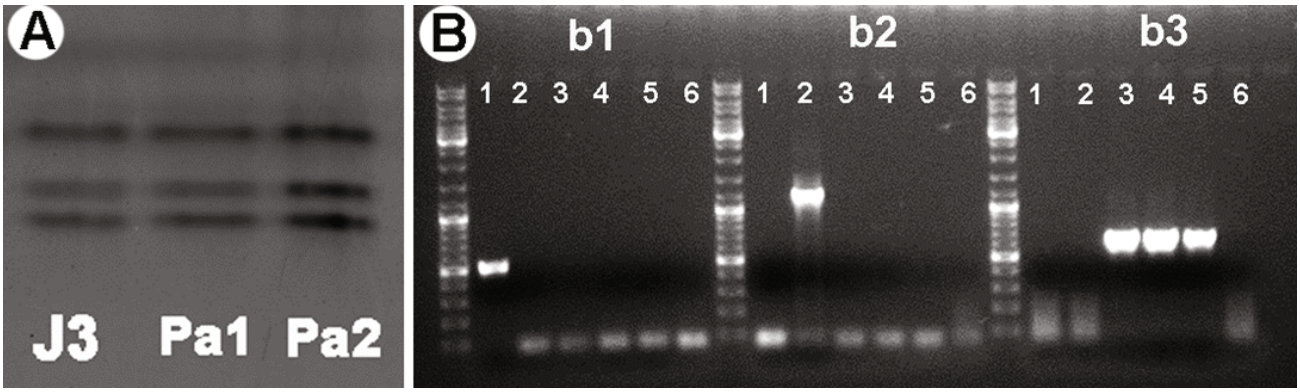

Fig. 2. A: Esterase electrophoresis pattern of protein homogenates from one to three egg-laying females (Pa1, Pa2, respectively) of *Meloidogyne javanica* infecting curly-leaf parsley cv. Robustus in southern Italy and single young egg-laying female (J3) of a reference isolate of *M. javanica*. B: Typical amplification products of PCR using primers (b1) *Far/Rar*, (b2) *Fin/Rin* or (b3) *Fjav/Rjav* using 5 ng of template DNA of b1: *M. arenaria*, b2 *M. incognita*, b3 *M. javanica,* references isolates*, M. javanica* from curly-leaf parsley cv. Robustus from southern Italy, lanes 4-5 and lane 6 control (no template DNA)

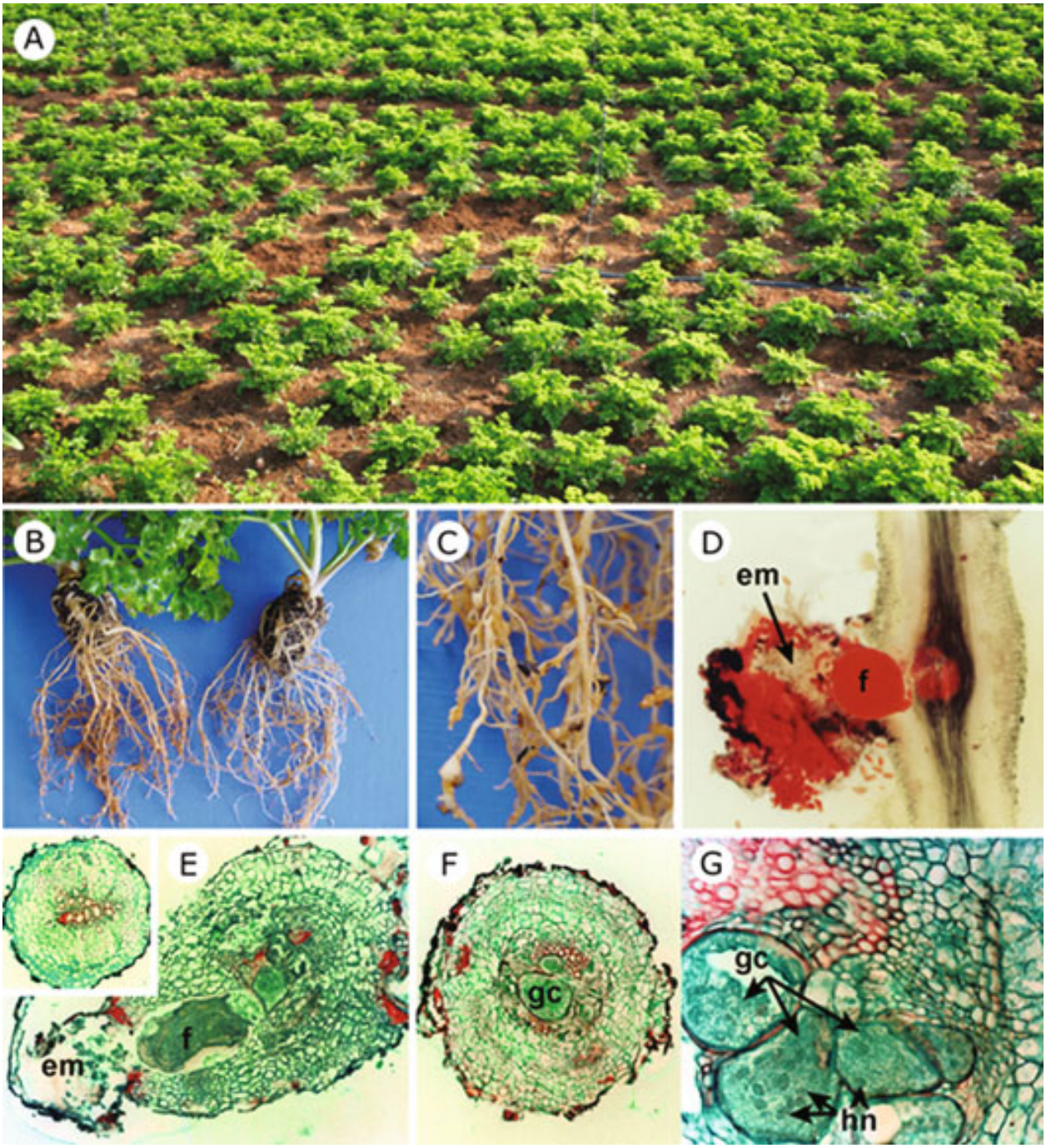

Fig. 3. A: Stunting and yellowing of curly-leaf parsley cv. Robustus plants, severely affected by *M. javanica*, collected in the field at Monopoli (Bari province). B and C: Galled parsley roots in plants from the depressed area (A) of selected for the field observations. D: Feeding site, note slight swelling of the root and eggs production by a single female. E, F and G: cross histological sections at galled feeder roots infected by *M. javanica*, showing the specialized host relationship at the feeding point sites. Insert on Fig. E: cross sections of an uninfected root portion. Abbreviations: gc = multinucleate giant cell; em = egg mass; f = female; hn = hypertrophied nuclei

Histopathology

Root galls induced by *M. javanica* on roots of curly-leaf parsley cv. Robustus varied in size and location (Fig. 3, B and C). Generally, large, spherical regular galls were present on root tips, and several were also present along the root axes. Galls occurred either individually or in clusters which could encircle the entire root. In this latter case, the root diameter was from two to four times greater than that of uninfected roots. The majority of individual galls randomly selected for inspection contained an egg mass and, in many cases, up to three or four mature globose females were found associated with the largest galls. Occasionally, an egg mass was found inside the cortical root tissues, but the majority of egg masses were observed protruding from the root surface.

Comparative histological observation of healthy (Fig. 3, E) and *M. javanica*-infected parsley roots (Fig. 3, F and G) showed cellular alterations in tissues of the cortex, endodermis and pericycle.

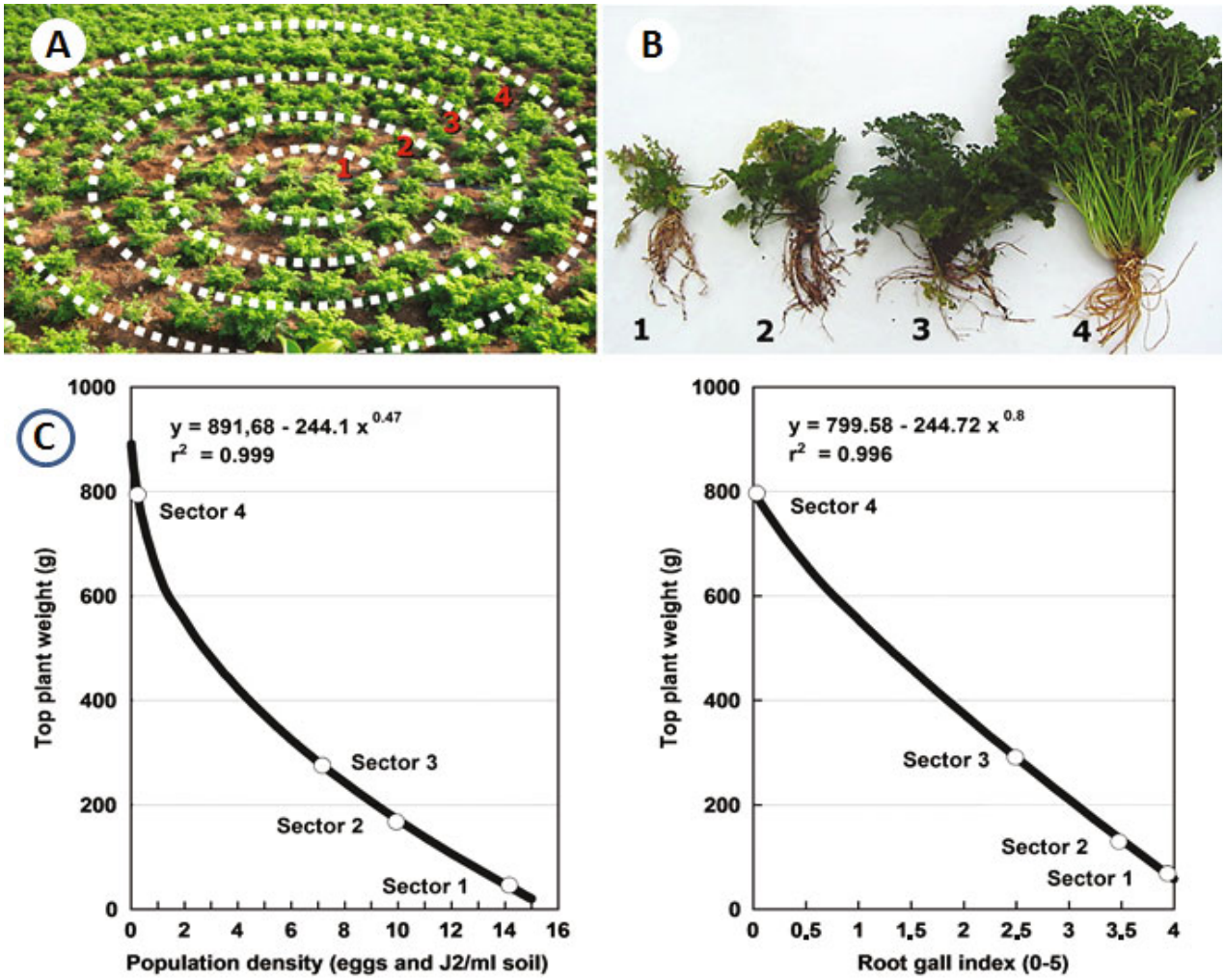

Fig. 4. A: Sectors selected in the *M. javanica*-infested field, to assess the effect of different infection levels on growth of parsley. B: Plant growth in each sector. C: relationship between top plant weight and soil nematode population density and root gall index (0 – 5 scale)

The greater damage was caused in the vascular system where the nematode induced cellular expansion of nurse cells and also by the expanding bodies of the nematode females. In the permanent feeding sites, nematode-induced formation of large multinucleate giant cells (three to six giant cells) adjacent to the vascular tissues and it was observed in all infected parsley galls. Giant cells consisted of dense cytoplasm and variable numbers of hypertrophied nuclei and nucleoli. Feeding site formation led to tissues distortion and crushing (Fig. 3). Hyperplasia of tissues adjacent to giant cells contributed to the formation of root galls.

Effect of the nematode on growth of parsley plants

The effect of the nematode on the growth of this crop was assessed by sampling of four concentric sectors in the infested area. A strict correlation was observed between top plant weight and soil nematode population density. In the different sectors of the stunted area the average of total root-weight of curly-leaf parsley cv. Robustus per plant were 18, 26, 32 and 46 g with an average of 30.5 g, about 34 % less in comparison to those observed in the non-stunted area (46 g). The average of aerial part weights of plants in the sectors 1, 2, 3 and 4 of the stunted area (Fig. 4) were 40, 160, 280 and 800 g, respectively. The average of these values was 320 g corresponding to 40 % than that recorded in the non-stunted area (800 g). In the fourth sector, nematode population density was inconsiderable and close to 0 (Fig. 4). For this sector, no significant differences were observed between root and aerial part weights of plants in comparison to those in non-stunted areas. Chlorosis, distinct mainly at the basal leaves was observed on the parsley plants in the stunted area especially in the sectors 1 and 2. A strict correlation was observed between top plant weight and soil nematode population density, expressed as eggs and J_2/cm^3 soil and root gall index (Fig. 4). Also a high correlation was found between root weight and the previous mentioned nematological parameters. Root weight/soil nematode population density and root weight/root gall index fitted the power equations $y = 891.68 - 244.1x^{0.47}$, $r^2=0.999$ and $y = 799.58 - 244.72x^{0.8}$, $r^2=0.996$, respectively, using the Table Curve Windows v. 1.0 Program (Fig. 4).

Discussion

The primary goal of this study was to determine the extent of soil infestation and curly-leaf parsley cv. Robustus infection by root-knot

nematodes in commercial fields of Southern Italy. The results provide an unequivocal diagnosis and bionomics of *M. javanica* attacking curly-leaf parsley cv. Robustus in Southern Italy. Morphology and morphometrics of perineal patterns, second-stage juveniles and males fit closely with those of the original description of the javanese root-knot nematode, *M. javanica* (Vovlas *et al*., 2005).

This new record of *M. javanica* on curly-leaf parsley cv. Robustus in Southern Italy and the severe plant growth impairment by this nematode reported in this work advice on the potential spread risk of this pathogen from early season plants to the other vegetable growing areas.

The histopathological changes induced by *M. javanica* on curly-leaf parsley cv. Robustus are quite similar to those induced by the other root-knot nematodes affecting vegetables, (Sikora & Fernández, 2005). The development and parasitic habit of *M. javanica* on curly-leaf parsley cv. Robustus confirmed a typical susceptible reaction of *M. javanica* hosts (Sikora & Fernández, 2005). Feeding sites are metabolic sinks sequestering nutrients from the host plants and limiting water and nutrient translocation from infected roots to above ground plant tissues (Hussey & Williamson, 1997). Results also suggest that these infections support successful root-knot nematode reproduction that increases the soil inoculum for successive growing seasons. The large distribution of *M. javanica* in Southern Italy (Candido *et al*., 2004) resulting in severe growth reduction can be a potentially severe threat to parsley crop in the warm autumn climate conditions.

In summary, the present study enlarges the knowledge about the pathogenicity of the javanese root-knot nematode *M. javanica* at field conditions. Since production of curly-leaf parsley will continue to expand in the southern regions of Italy, often in rotation with other vegetable crops that may be susceptible to root-knot nematodes, adequate management procedures need to be implemented in order to prevent losses during field production. Because curly-leaf parsley varieties resistant to the plant nematodes are not yet available and the intensive nature of the crop make crop rotation nearly impossible control strategies should be focused on reduction of infestation levels below to the tolerance limit of the target nematode species (Sasanelli, 1994; Vovlas *et al*., 2008). Due to the higher cost and reduced availability of fumigant and non-fumigant nematicides (Sasanelli, 2000; Sasanelli *et al*., 2008; Renčo *et al*., 2009; Maistrello *et al*., 2010; Abdel-Dayem *et al*., 2012; Daragò *et al*., 2013; Zouhar *et al*., 2013; Jahanshahi Afshar *et al*., 2014) soil solarization, organic amendments or biological control are suggested to use. Soil sampling to assess the risk of nematode damage is necessary for decision making on cropping sequences and possible control strategies.

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