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Effect of *Meloidogyne ethiopica* parasitism on water management and physiological stress in tomato

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Abstract Root-knot nematodes (RKN) are obligate endoparasites that severely damage the host root system. Nutrient and water uptake are substantially reduced in infested plants, resulting into altered physiological processes and reduced plant growth. The effect of nematode infestation on the morphological changes of roots and subsequent physiological plant responses of infested tomatoes with the RKN *Meloidogyne ethiopica* was studied in a pot experiment. Plants were infested with two inoculum densities (10 or 50 eggs per cm^3 substrate) and its effect was evaluated 74 and 102 days post inoculation (DPI). Morphological changes and root growth was determined by analysing scanned images of the whole root system. Nematode infestation reduced the portion of fine roots and increased that of coarse roots due to gall formation. Fine roots of non-infested control plants represented around 51% of the area of the whole root system at 74 and 102 DPI. In comparison to controls, plants inoculated with low and high nematode density had 2.1 and 3.2-times lower surface area of fine roots at 102 DPI. Root analyses revealed that plants had a very limited ability to mitigate the effects of the root-knot nematodes infestation by altering root growth. Root galls had a major influence on the hydraulic conductivity of the root system, which was significantly reduced. The low leaf water potential of infested plants coincided with decreased stomatal conductivity, transpiration and photosynthesis. The latter two were reduced by 60–70% when compared to non-infested control plants.

Keywords Leaf water potential · Root hydraulic conductivity · Root morphology · Root-knot nematodes · Photosynthesis

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Introduction

Plant parasitic nematodes are major agricultural pests worldwide responsible for global agricultural losses. Among them, root-knot nematodes (RKN) of the genus *Meloidogyne* Göldi represent the most important polyphagous group, including highly adapted obligate plant pathogens in temperate and tropical regions (Karssen and Moens 2006). Anatomical changes in roots, such as giant-cell development and gall formation, are the primary symptoms of RKN

infestation on susceptible plants. RKN invade the roots in the zone of elongation and then migrate intercellularly to the vascular cylinder, where they establish feeding sites and disrupt the vascular tissue (Abad et al. 2003). As a consequence, the water supply to the shoot is impaired. Dorhout et al. (1991) reported that infestation with *M. incognita* increased axial resistance to water flow and reduced total water uptake in tomato plants. Similar results were obtained by Rahi et al. (1988) on tobacco plants infested with *M. incognita* or *M. javanica* where plants extracted less water from the soil in comparison to non-inoculated plants, even when soil contained water at or near field capacity. Disturbance of water transport lead to water stress that is manifested in above-ground symptoms, such as stunting, wilting and chlorosis. Furthermore, a reduction in the water supply has been found to deleteriously influence physiological and biochemical processes in plants such as photosynthesis, respiration, translocation, ion uptake, nutrient levels, pigments composition, carbohydrate levels, growth promoters and metabolism (Jaleel et al. 2008, 2009). One of the theories for reduction of physiological processes in plants is also that the RKN interferes with the synthesis and translocation of growth hormones produced in the roots (Bird 1974). Several authors have reported a decrease in water potential, a reduction of the stomatal conductivity and a decrease in the photosynthetic rate when plants were infested with RKN (Rahi et al. 1988; Loveys and Bird 1973; Wallace 1974; Meon et al. 1978; Poskuta et al. 1986; Melakeberhan et al. 1987; Kirkpatrick et al. 1991; Ramakrishnan and Rajendran 1998). However, Melakeberhan et al. (1990) did not detect any reduction of stomatal conductance and transpiration rate on *Vitis vinifera* infested with *M. incognita* 120 days after inoculation, but they commented that an effect might have been shown only when infested plants are exposed to nematodes for longer period and therefore facing a greater stress. The infestation with RKN can also influence nutrient element changes and related physiological effects in plants. In beans infested with *M. incognita*, the total content of several elements decreased with the increased duration of the infestation and with an increasing number of nematodes. It was established that the lower potassium concentration in the shoots and leaves of infested beans usually corresponded to increases in root gall formation, giant cell size and metabolic activity (Melakeberhan et al. 1987).

The effects of RKN have been widely studied, mainly focusing on particular processes but rarely combine the root morphology with the host plant physiological processes. The present study was designed to evaluate the effect of morphological changes of roots on physiological response of tomato plants infested with *M. ethiopica*. Some parameters of the physiology of the infested tomato plants, such as leaf water potential, hydraulic conductivity, photosynthesis, stomatal conductance and transpiration were evaluated. The response of the photosynthetic metabolism to infestation was analysed by measuring leaf gas exchange and the content of pigments of xanthophylls cycle. Formation of zeaxanthin and antheraxanthin from violaxanthin by the enzyme violaxanthin de-epoxidase is a known energy dissipation mechanism by which plants avoid photoinhibitory damage during water deficit (Demmig et al. 1987; Demmig-Adams and Adams 1992; Niyogi 1999).

In our study, *Meloidogyne ethiopica* was used because it is amongst the most important emerging RKN species (Hunt and Handoo 2009) and has a wide host range including monocotyledonous and dicotyledonous plants (Strajnar et al. 2009, 2011). In addition, this species can represent a serious risk for agricultural production, especially in Southern Europe, because it was shown to be able to survive through the winter period in field, in sub-Mediterranean and continental climate conditions (Strajnar et al. 2011).

Materials and methods

Nematode inoculum

The *M. ethiopica* isolate from Slovenia (Širca et al. 2004) was maintained on tomato, *Lycopersicum esculentum Mill*. cv. Cuor di bue in a greenhouse pot culture. Hussey and Barker's (1973) method was used to prepare the inoculum from infested roots. The obtained suspensions of eggs were further purified following McClure et al. (1973) with slight modifications. Nematode eggs on the 32 μm sieve were rinsed from the sieve in 40 ml of water into 50 ml polycarbonate centrifuge tubes and centrifuged at 952×*g* for 5 min. The pellets were re-suspended in 40 ml of sucrose solution (454 g sucrose per 1 l of tap

water) and centrifuged at 635×*g* for 1 min. The supernatants with eggs were poured through a 32 μm banked sieve and washed with tap water. Finally, the eggs were rinsed from the sieve and counted.

Pot experiment

The experiment was conducted outdoors, from May to August, 2009, at the Agricultural Institute of Slovenia (Ljubljana, Slovenia), under the plastic roof to protect direct precipitation, on plants. The tomato cv. Cuor di bue, which is an excellent host for *M. ethiopica* was also used in the experiment (Strajnar et al. 2011). During the experimental period, the mean air temperature was 20.6°C and the mean minimum and maximum temperatures ranged from 12.9°C to 25.8°C in May, 13.4°C to 24.3°C in June, 15.6°C to 27.2°C in July and 17.1°C to 29.1°C in August. Temperature data were obtained from the Environmental Agency of Slovenia. Forty-five-day-old plants were transferred to 25 cm diameters pots (volume 5 l) filled with a mixture of sterilised sand (0.25–1.0 mm) and hydroculture granules (size 1–4 mm) in a 3:1 volume ratio. Plants were watered daily with nutrient solutions for hydroponic growth (General Hydroponics Europe). The nutrient concentration depended on the stage of plant development. This solution was prepared by mixing the commercial solutions Flora Micro 5-0-1, Flora Bloom 0-5-4 and Flora Gro 3-1-7 accordingly to the manufacturer's instructions. Two-month-old tomato plants were inoculated with ten eggs per cm^3 substrate or 50,000 eggs pot^{-1} (low inoculum density) and 50 eggs per cm^3 substrate or 250,000 eggs pot^{-1} (high inoculum density) with ten replicates per treatment and control.

Data records

The root morphology, hydraulic conductivity and leaf water potential were evaluated at 74 days post inoculation (DPI) and at 102 DPI when the second generation juveniles were expected to enter the roots and cause additional damage. The gas exchange parameters (net photosynthesis, stomatal conductance and transpiration rate) and the leaf pigments were also recorded at 102 DPI. All the evaluations were made on a clear sunny day and 1 day after the pots were watered to saturation to achieve equal moisture in all pots.

Root morphology

The root length and root surface area (RSA) of three plants per treatment were measured at 74 and 102 DPI. The root systems were washed, dissected, immersed in water and scanned with the optical scanner Epson Perfection V700 photo. Three diameter classes (DC) were defined: up to 0.5 mm for fine roots; more than 0.5 and less than 1.5 mm for middle roots; and more than 1.5 mm for coarse roots. Images were analysed using the software WinRHIZO (v2002c, Régent Instruments Inc.).

Hydraulic conductivity

The hydraulic conductivity of the root systems of three plants per treatment was estimated. Tomato plants were removed from the pots and the root systems were cleaned by gentle shaking. The stem was cut off, approximately 10 cm above the growth medium level, and the conductivity was determined after pressurising the root system at 0.2 MPa in a pressure chamber. Xylem sap was collected, with a pipette, during 5 min. The hydraulic conductivity was expressed as weight of extracted xylem sap per minute (Scholander et al. 1964).

Leaf water potential

Leaves of five young tomato plants per treatment were analysed 74 DPI, at midday (12:00–13:00) and 102 DPI every 2 h from 6:00 to 19:00. The measurements were performed on leaves exposed to the sun using the pressure chamber 3005–1223 from Soil Moisture Equipment Corp (Scholander et al. 1964).

Gas exchange parameters

All the parameters (net photosynthesis, stomatal conductance and transpiration rate) were measured seven times at 102 DPI, between 9:40 and 18:40, in one sun-exposed leaf per plant (five plants per treatment) with the LI- 6400 (LI-COR Biosciences). The measurements were performed at a controlled reference CO_2 concentration (380 μmol mol^{-1}), ambient temperature varying from 22.3 to 37.7°C during the day, air humidity (RH) ranging from 55.8 to 25% and light intensity (photon flux density, PFD) from 90 to 2,300 μmol photons m^{-2} s^{-1}. The ambient PFD

was measured by the external light sensor of the LI-6400 at the beginning of each measurement and kept stable by using an internal light source to prevent fluctuations during the measurements.

Leaf pigments

Three sun-exposed leaves per treatment were collected at midday at 102 DPI, immediately frozen in liquid nitrogen and stored at −80°C until analysis. Chloroplast pigment concentrations were determined using the method described by Šircelj and Batič (2007). Pigments were extracted from the dry leaf powder with ice-cold acetone on an ice bath, using a T-25 Ultra-Turrax (Ika-Labortechnik) homogeniser for 25 s. All the extraction procedures were performed in dim light. The acetone extracts were filtered through a 0.2 μm Minisart SRP 15 filter (Sartorius Stedim Biotech GmbH) and subjected to HPLC gradient analysis (a Spherisorb S5 ODS-2 250×4.6 mm column with an S5 ODS-2 50×4.6 mm precolumn, Alltech Associates, Inc.), using two solvents: solvent A- acetonitrile/methanol/water (100/10/5, *v*/*v*/*v*); and solvent B- acetone/ethyl acetate (2/1, *v*/*v*), at a flow rate of 1 ml min^{-1}. A linear gradient from 10% solvent B to 70% solvent B in 18 min was used, with a run time 30 min, and photometric detection was performed at 440 nm. HPLC analyses were performed using a Spectra-Physics HPLC system with a Spectra Focus UV–VIS detector. Identification of the compounds was achieved by comparing the retention times and spectra and by the addition of standards. The concentrations of pigments were calculated by comparing to corresponding external standards. The following high purity standards (DHI LAB products) were used for the determination of photosynthetic pigments: β-carotene, neoxanthin, violaxanthin, antheraxanthin, zeaxanthin, lutein and chlorophyll. Acetone, ethyl acetate, methanol and acetonitrile were HPLC graded from Merck.

Statistical analysis

The data were analysed by ANOVA for statistical comparison of hydraulic conductivity, leaf water potential, photosynthesis, chloroplast pigments and root parameters presented as mean ± standard error (SE); Duncan's multiple range test at $P=0.05$ was used to separate means. Tests were performed with the Statgraphics XVI software package (StatPoint Technologies Inc., USA).

Results

Root morphology

The nematode infestation had a significant effect on the percentage of fine and coarse roots at 74 DPI. The total RSA for control plants, plants with low and high inoculum density were not significantly different ($P=0.646$). The RSA of the fine roots of nematode infested plants was significantly reduced over control while in the case of coarse roots was significantly increased (Fig. 1; Table 1).

The total length of the control plant roots was 11323.4±126.0 cm and in infested plants with low and high nematode densities was 8974.6±1133.8 and 8967.8±1237.0 cm respectively and did not differ significantly.

Non-inoculated control plants retained the same portion of RSA for fine roots when compared 74 and 102 DPI evaluation, representing about 51% of the total root system (Table 1). In plants with low and high inoculum density, this portion was reduced significantly to 24.9 and 22.8% at 102 DPI, respectively. The percentage of middle roots at 102 DPI was significantly lower in nematode infested plants over control while that of coarse roots of infested plants represented 41% of the RSA when compared to 5% of non-inoculated plants (Table 1). The total root length after nematode infestation was also significantly reduced. The total length of the control plants roots was 17755.9±3581.0 cm and in infested plants with low and high nematode densities was 10614.4±306.7 and 6997.7±1596.0 cm respectively, being the root length of the infested plants with the high nematode density 2.5-times reduced ($P=0.0394$).

Hydraulic conductivity

The hydraulic conductivity of the root systems was significantly different between the treatments at 74 DPI. It was 1.4–4.3 fold reduced in plants with low and high inoculum densities (Table 2) when compared to non-inoculated plants. At 102 DPI, the xylem sap was collected only from non-

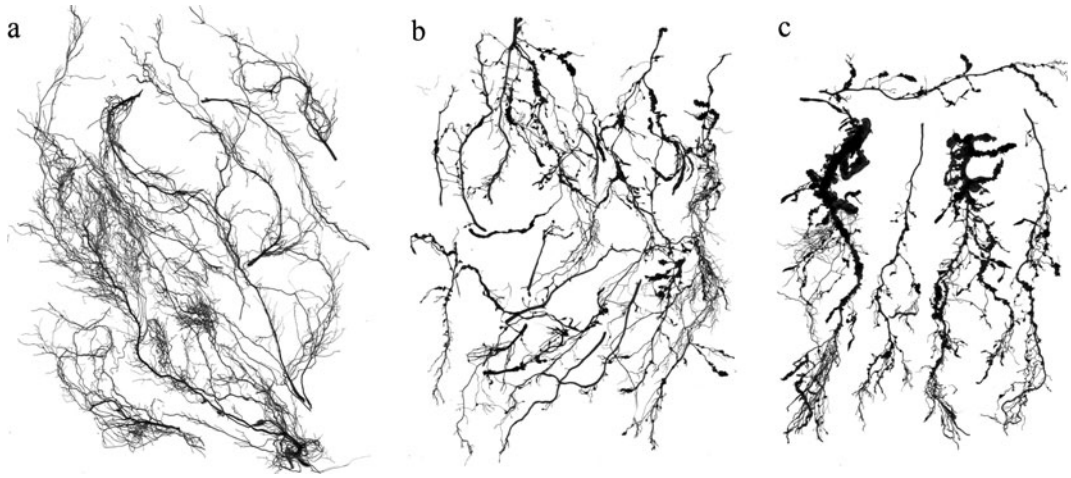

Fig. 1 Effect of *Meloidogyne ethiopica* on the growth of tomato root systems, 74 days post inoculation. Non-inoculated tomato plants (**a**); tomato plants inoculated with low inoculum density (**b**); tomato plants inoculated with high inoculum density (**c**)

inoculated plants to which the hydraulic conductivity was 0.26±0.01 gmin^{-1}.

Leaf water potential

At 74 DPI, the nematode infestation reduced the leaf water potential approximately 1.2-times and significant differences were found only between inoculated and non-inoculated plants (Table 2). At 102 DPI, the midday leaf water potential of non-inoculated plants was −1.01±0.04 MPa and significantly lower (P=0.0040) in plants with low nematode density, -1.17±0.03 MPa, or −1.24±0.05 MPa in plants with high inoculum density (Fig. 2). However, no effect of inoculum density on leaf water potential was measured at 102 DPI.

Gas exchange parameters

The nematode infestation significantly reduced the photosynthetic rate, the stomatal conductance and the transpiration rate over the entire day (Fig. 3). The average daily net photosynthesis in non-inoculated plants was approximately 2-fold higher in comparison to infested plants. Statistically significant differences were found at midday (P=0.0000 for all parameters). The midday net photosynthesis of non-inoculated plants was 14.3±1.4 μmol CO_2 m^{-2} s^{-1}, whereas in plants

Table 1 Effect of inoculum density of *Meloidogyne ethiopica* on tomato root surface area (RSA), 74 and 102 days post inoculation (DPI)

DC [mm]		RSA [%]		
Treatment (Total RSA cm^2)		0.0<DC≤0.5 (fine roots)	0.5<DC≤1.5 (middle roots)	DC>1.5 (coarse roots)
74 DPI	Non inoculated (Control) (1260.3±26.7)	51.4±0.6 a	44.1±0.7 ab	4.5±0.4 a
	50,000 eggs pot^{-1} (1193.6±146.3)	39.2±1.7 b	47.7±0.2 a	13.1±1.6 b
	250,000 eggs pot^{-1} (1345.0±121.2)	33.1±2.6 c	42.5±0.2 b	24.4±2.6 c
102 DPI	Non inoculated (Control) (1941.0±454.8)	51.7±2.8 a	43.6±1.5 ab	4.7±1.3 a
	50,000 eggs pot^{-1} (1915.2±47.3)	24.9±1.7 d	34.8±1.8 c	40.3±2.5 d
	250,000 eggs pot^{-1} (1367.0±319.2)	22.8±0.7 d	35.7±2.1 c	41.5±2.7 d
ANOVA P		0.0000	0.0001	0.0000

DC-Diameter classes (mm); Mean ± SE are presented, n=3. Different letters that follow the means in a column indicate a significant difference at P<0.05

Table 2 Effect of inoculum densities of *Meloidogyne ethiopica* on hydraulic conductivity and leaf water potential of tomato, 74 days post inoculation

Treatment	Hydraulic conductivity [g min^{-1}]	Leaf water potential [MPa]
Control	0.26±0.02 a	−0.84±0.03 a
50,000 eggs pot^{-1}	0.18±0.02 b	−0.98±0.04 b
250,000 eggs pot^{-1}	0.06±0.03 c	−1.07±0.04 b
ANOVA *P*	0.0015	0.0038

Means ± SE are presented, $n=3$ (hydraulic conductivity) and $n=5$ (leaf water potential). Means followed by different letters are significantly different at $P<0.05$

inoculated with low and high nematode densities was 4.9±1.1 and 5.6±1.3 CO_2 m^{-2} s^{-1} respectively. A similar pattern was also observed for the stomatal conductance and transpiration rate where the average daily values in infested plants were approximately 3.3-fold and 2.6-fold lower, respectively, in comparison to non-inoculated plants. At midday, the stomatal conductance in control plants was 0.30±0.03 mol H_2O m^{-2} s^{-1} while, at low and high inoculum density, the plants exhibited stomatal conductances of 0.05±0.01 mol H_2O m^{-2} s^{-1} and 0.07±0.02 mol H_2O m^{-2} s^{-1}, respectively. The transpiration rates at midday were 2.05±0.33 mmol H_2O m^{-2} s^{-1} and 2.44±0.37 mmol H_2O m^{-2} s^{-1} in plants with low and high nematode densities, respectively, while a higher rate was found for non-inoculated plants (7.28±0.70 mmol H_2O m^{-2} s^{-1}) (Fig. 3).

Leaf pigments

The photosynthetic pigments analysis in the tomato leaves showed no clear response to the nematode infestation, which can be partly attributed to the high variability of the data (Table 3). The only significant difference was observed for the content of violaxanthin, which was approximately 1.4-times lower in plants with high inoculum densities in comparison to non-inoculated plants. Despite its decrease we were not able to prove any differences in the ratio of violaxanthin to other pigments of the xanthophyll cycle.

Discussion

Root knot nematodes induce severe changes on the root system of their hosts, which are most commonly estimated by a root gall index that evaluates the frequency of root galls (Taylor and Sasser 1978). However, the root gall index does not consider the impact of nematode infestation on the fine roots, which are crucial for water absorption and necessary for normal physiological and biochemical processes in plants. The morphological analyses of the root

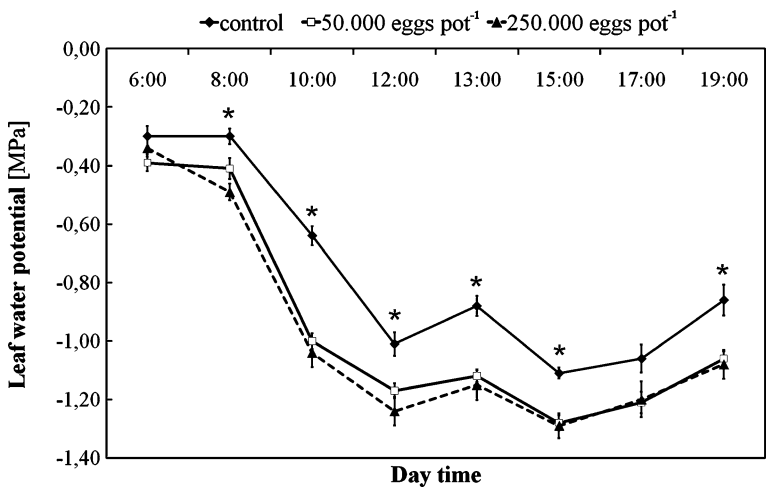

Fig. 2 Fluctuations of the leaf water potential of tomato plants inoculated with *Meloidogyne ethiopica*, 102 days post inoculation. Means ± SE of five replicates are shown; * indicates significant differences between infested and non-infested plants ($P<0.05$)

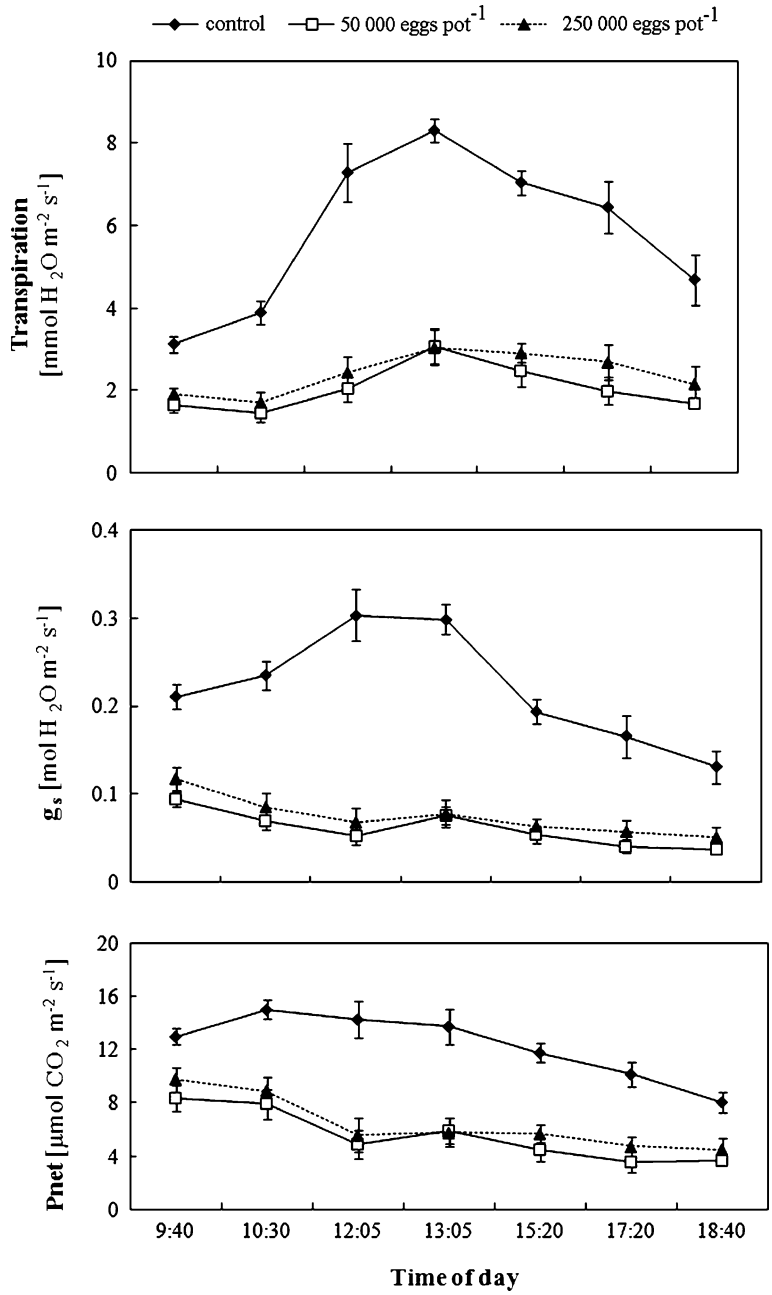

Fig. 3 Fluctuations of the transpiration rate, stomatal conductance (g_s) and net photosynthesis rate (Pnet) of tomato plants inoculated with *Meloidogyne ethiopica*, 102 days post inoculation. Means ± SE of five replicates are shown. Measurements of non-inoculated plants were in all cases significantly higher ($P<0.05$)

system indicate the effect of the nematode inoculum density on some plant physiological processes. Previous experiments showed that *M. ethiopica* needs 48 days to complete a reproduction cycle at 22.7°C. On the basis of the mathematical equation for the reproduction cycle (Strajnar et al. 2011), we calculated that the second generation juveniles of *M. ethiopica* had infested the roots and caused additional damage at 102 DPI. Therefore, the most significant effect of nematode infestation was observed at 102 DPI when the total length of the root system as well as the RSA of fine roots was significantly reduced. The RSA of fine roots was 2.1 and 3.2-times lower in plants inoculated with low and high nematode density, respectively and the total length of root was lower at 1.7 and 2.5-fold, respectively. On the contrary, the RSA of the coarse roots significantly increased in infested plants because of the root galls development.

Table 3 Photosynthetic pigments in tomato inoculated with different *Meloidogyne ethiopica* inoculum densities, 102 days post inoculation

Photosynthetic pigments	Non-inoculated (control)	50,000 eggs pot^{-1}	250,000 eggs pot^{-1}	ANOVA *P*
Chlorophyll *a* [mg gDW^{-1}]	7805.7±928.9	6894.3±1411.1	7681.2±780.5	0.8151
Chlorophyll *b* [mg gDW^{-1}]	3115.9±431.8	2448.8±383.8	3075.4±286.7	0.4191
Chl *a*/Chl *b*	2.52±0.06	2.78±0.16	2.49±0.05	0.1707
β-carotene [μg gDW^{-1}]	197.9±37.9	231.9±92.9	517.6±400.9	0.6078
Violaxanthin [μg gDW^{-1}]	225.8±9.8a	184.6±15.7ab	165.4±12.1b	0.0391
Antheraxanthin [μg gDW^{-1}]	58.6±7.0	46.3±9.9	78.3±13.2	0.1698
Zeaxanthin [μg gDW^{-1}]	8.7±4.2	55.6±46.2	9.8±2.2	0.4223
VAZ	293.1±3.1	286.5±66.4	253.5±24.2	0.7724
V/VAZ	0.77±0.03	0.69±0.10	0.66±0.03	0.4948
Neoxanthin [μg gDW^{-1}]	320.8±39.4	271.1±40.3	318.2±25.9	0.5747
Lutein [μg gDW^{-1}]	1129.1±100.6	889.0±117.8	1193.8±137.8	0.2443

Means ± SE are presented, n=3. Different letters that follow the means in a row indicate a significant difference at $P<0.05$

The duration of the nematode infestation intensified the root galling which was evident by the increased portion of the coarse roots in plant with low inoculum from 13.1 to 40.3% and in plants with high inoculum from 24.4 to 41.5% of the total RSA between 74 and 102 DPI. In non-inoculated control plants, the RSA of coarse roots at 74 and 102 DPI was the same, representing about 4.6% of the total RSA. The damage effect of nematode inoculum density was clearly demonstrated at 74 DPI but it was not detected at 102 DPI. Plants with both inoculum densities had approximately equal extent of nematode infestation at 102 DPI represented by 41% coarse roots of their total RSA. This observation can be explained by the lower nematode reproduction rates when the initial nematode populations are high, which was already observed previously with *M. incognita* on common beans (Di Vito et al. 2004) and *M. ethiopica* on grapevine (Di Vito et al. 2009). The changes in the root morphology such as the development of root galls had a decisive role in the efficiency of root water transport in infested plants. The amount of extracted xylem sap per time was lower in more damaged root systems but in non-inoculated plants was equal at both evaluations since the portion of coarse roots remained the same. The differences found in coarse roots of the plants with low and high inoculum density reflected the differences in the amount of xylem sap at 74 DPI. However, no xylem sap was collected in plants of both inoculum treatments at 102 DPI when the coarse roots reached the level of approximately 41% of the total RSA. The impairment of xylem-conducting efficiency is a consequence of the intrusion of giant cells into the vascular system, which breaks larger vessels continuity and the development of abnormal xylem-like elements (Meon et al. 1978). The nematode densities had no effect on the leaf water potential, but the differences between inoculated and non-inoculated plants were substantial and corresponded with the results of previous reports where RKN caused a decrease of plant water availability (Meon et al. 1978; Rahi et al. 1988; Kirkpatrick et al. 1991). The highest differences in leaf water potential were detected at 102 DPI between inoculated and non-inoculated plants at midday that was correlated with the stomatal conductivity and transpiration decrease. Furthermore, a clear stomatal inhibition of photosynthesis was observed. Analyses of the photosynthetic pigments showed that photosynthesis could be affected not only by limited stomatal conductivity but also by less efficient thylakoid processes as indicated by differences found for violaxanthin levels. Since we were not able to unambiguously prove xanthophyll cycle related energy dissipation, by changed ratios of violaxanthin to other pigments of the xanthophyll cycle, this presumption should be confirmed by further experiments.

It is concluded that *M. ethiopica* can develop a significant infestation on tomato plants with root damage increasing with the development of a second nematode generation. Furthermore, root gall formation disturbed seriously water transportation in infested

plants, which led to water stress that substantially hindered photosynthetic carbon assimilation.

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