RESEARCH ARTICLE

Penetration and post-infection development of root-knot nematodes in watermelon

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Abstract

Meloidogyne javanica has showed less reproductive success than *M. incognita* in watermelon genotypes. This study was conducted to elucidate the low reproduction of *M. javanica* in watermelon. The post-infection development of *M. javanica* in watermelon 'Sugar Baby' was determined at progressively higher initial population (Pi) levels at two time points during the life cycle. Plants were inoculated with 0, 25, 50, 100, 200, and 300 second-stage juveniles (J2)/plant. The increase in Pi was correlated with the penetration rates ($R^2 = 0.603$, $p<0.001$) and total numbers of nematodes in the root ($R^2 = 0.963$, $p< 0.001$) but there was no correlation between the Pi and the reproduction factor (eggs/plant/Pi). The population in the roots at 26 days post-inoculation (dpi) consisted primarily of third-stage juveniles (J3) with a small presence of J2 and fourth stages, and egg-laying females. The dominance of the J3, when egg-laying females are expected, point to the malfunction of the feeding sites that failed to support nematode development beyond the J3 stage. The similarities in egg-laying females at 26 and 60 dpi imply the disruption of the life cycle. Watermelon compensated for *M. javanica* parasitism by increasing vine length (19% to 33%) and dry top weight (40%) in comparison with the non-inoculated plants. The area under the vine length progress curve was significantly larger as the Pi progressively increased ($R^2 = 0.417$, $p<0.001$). Physiological variation was detected between the *M. incognita* populations. *M. arenaria* had less ability to invade watermelon roots than did *M. incognita* and *M. javanica*.

Additional keywords: *Citrullus lanatus*; growth stimulation; *Meloidogyne arenaria*; *Meloidogyne incognita*; *Meloidogyne javanica*; parasitic variation; reproduction factor.

Abbreviations used: AUC (area under the curve); Dpi (days post-inoculation); EM (egg masses); F (females); HSD (honestly significant difference); J2, J3, J4 (second-, third- and fourth-stage juveniles); LCC (leaf-chlorophyll content); Pi (initial population density); Pf (final population density); Rf (reproduction factor; Rf =Pf /Pi); RKN (root-knot nematodes).

Introduction

Root-knot nematodes (RKN) of the genus *Meloidogyne* are polyphagous obligate sedentary endoparasites that depend on the induction of feeding sites in the roots to complete their life cycle. Second-stage juveniles (J2) penetrate the root; migrate through the intercellular space to enter the vascular cylinder in order to induce the formation of a feeding site. Afterwards, the nematode has three consecutive moults to third (J3) and fourth (J4)-stage juveniles and to adult females. Mature females lay eggs into a gelatinous matrix attached to the posterior end of the female (Escobar *et al.*, 2015).

Watermelon, *Citrullus lanatus* (Thunb) Matsum & Nakai, is susceptible to the most common RKN species, *M. arenaria* (Neal) Chitwood, *M. incognita* (Kofoid & White) Chitwood and *M. javanica* (Treub) Chitwood (Thies & Levi, 2003; Anwar & McKenry, 2010; Thies *et al.*, 2010). However, physiological variability among RKN species, in terms of penetration rates, root galling severity, and final population densities (Pf) were also found among isolates of these RKN species in watermelon (Winstead & Riggs, 1959; Edelstein *et al.*, 2010; Cohen *et al.*, 2014; López-Gómez & Verdejo-Lucas, 2014). *Meloidogyne javanica* showed less ability than *M. incognita* to form galls, produce egg masses (EM), and lay eggs

in watermelon genotypes (Cohen *et al.*, 2014; López-Gómez *et al.*, 2016).

The reproductive success of the nematode depends on the status of the host plant, population size, and soil temperatures, which in turn affect the length of the life cycle, which is slower in poor and resistant hosts than in good hosts (Trudgill, 1995). The reproduction factor (Rf =Pf/Pi-initial population density), is used in Nematology as an indicator of the suitability of a host plant for the nematode. Thus, susceptible plants show a Pf/Pi >1 whereas resistant or non-hosts, a Pf/Pi <1 (Seinhorst, 1967). Watermelon germplasm and commercial cultivars vary in host suitability levels as demonstrated by the Rf values in different genotypes. The *M. incognita* Rf ranged from 2.9 in 'Sugar Baby' to 9.6 in 'Charleston 76' (Montalvo & Esnard, 1994). The Rf for *M. arenaria* was 2.8, 1.8, and 5.4 on watermelon 'Crimson Sweet', 'Dixie Lee', and 'Charleston Gray', respectively (Thies & Levi, 2003). The Rf for *M. incognita* on Watermelon 'Congo' and 'Charleston Gray' was 7.5 and 7.0, respectively (Pofu *et al.*, 2011). Davis (2007) reported Rf = 6.6 for *M. incognita* on watermelon 'Cooperstown' whereas in 'Royal Sweet' the Rf was 1.2 for *M. incognita* (Xing & Westphal, 2012). However, cultivation of susceptible watermelon in RKN-infested fields decreased the Pf despite the presence of root galling (Vawdrey & Stirling, 1996; Davis, 2007; Anwar & McKenry, 2010; Xing & Westphal, 2012; López-Gómez *et al.*, 2014). Reduced Pf could be due to root damage directly related to the Pi. As the Pi increases so does damage and the competition for available feeding sites which can impair growth of the nematode as well as of the host plant. In fact, reduction in plant-growth parameters is the most frequently reported outcome of the host-parasite interaction.

The leaf chlorophyll content (LCC) has been used as an indicator for evaluation of nematode damage (Loveys & Bird, 1973; Melakeberhan *et al.*, 1985; Giné *et al.*, 2014; López-Gómez *et al.*, 2015) due to the strong correlation between LCC and the nitrogen content of the plant (Gholizadeh *et al.*, 2009; Pôrto *et al.*, 2011). Changes in LCC may be detected before the appearance of disease symptoms (Wagner *et al.*, 2006).

This research was conducted to elucidate the low reproduction of *M. javanica* in watermelon. The post-infection development of the nematode at progressively higher initial inoculum levels was determined by quantifying nematode traits at two time points during the life cycle. The physiological variability among populations of *Meloidogyne* spp. in watermelon was also determined.

Material and methods

Post-infection development

Watermelon 'Sugar Baby' (Intersemillas, S. A., Loriguilla, Valencia, Spain) seeds were soaked overnight and germinated in vermiculite trays. Seedlings were transplanted at the first true leaf stage to Styrofoam cups filled with 500 cm³ of sterilized river sand. Plants were maintained in a greenhouse for one week before inoculation with *M. javanica* (code Mj05). Nematode eggs from infected tomato 'Roma' roots were extracted by blender maceration in a 0.5% NaOCl solution for 5 min (Hussey & Barker, 1973). The egg suspension was passed through a 74-µm aperture sieve to remove root debris, and the dispersed eggs were collected onto a 25-µm sieve. The J2 were obtained by incubating the egg suspension in Baermann trays at 26°C, and those emerging within 72 h were used as the inoculum.

The experimental design was a completely randomized block with six treatments (Pi), each with seven replicates. Treatments consisted of six inoculum levels, 0, 25, 50, 100, 200, and 300 J2/plant. The experiment was repeated once. Plants were maintained in a greenhouse, watered daily as needed, and fertilized with a slow-release fertilizer Osmocote® Scotts Company, Netherlands (15% N +10% P$_2$O$_5$ +12% K$_2$O + 2% MgO$_2$ + microelements) at the beginning of the experiments.

The length (cm) of the watermelon vines and LCC were determined weekly for 8 weeks from the time of nematode inoculation. The vine length was measured using a measuring tape from the base to the tip of each stem. Then, the measured lengths of all stems were added together as the total vine length. The LCC was measured with a portable chlorophyll meter SPAD 502® (Minolta, Osaka, Japan). Three SPAD readings were taken in the largest fully expanded leaf/plant at two-thirds the distance from the leaf tip to the stem.

The post-infection development of *M. javanica* was assessed at 26 days post-inoculation (dpi). At this time the presence of egg-laying females was expected according to the thermal time requirement of *M. javanica* in watermelon (López-Gómez *et al.*, 2014). An egg-laying female was recognized by the presence of an egg mass (EM) attached to the posterior end of its body. Roots were washed free of soil and stained with acid fuchsin 0.05% (Bridge & Page, 1982). The number of nematodes inside the roots was quantified and categorized as J2, J3, J4, and egg-laying females (F).

Seven additional plants/treatment were included in each experiment to determine the number of females, eggs, and Rf. Plants were harvested at 60 dpi, when

completion of the life cycle from J2 to J2 was expected (López-Gómez *et al.*, 2014). Tops were cut at ground level and their dry weight determined after desiccation in an oven at 60ºC for 72 h. Roots were washed free of soil, weighed and then stained in a 0.1 g/L erioglaucine solution (Aldrich Chemical Company, Milwaukee, WI, USA) for 2 h (Omwega *et al.*, 1988) to facilitate the EM counting. To determine egg production, all EM/root system were handpicked, placed into an Eppendorf tube and macerated in a 0.5% NaOCl solution for 10 min (Hussey & Barker, 1973), as described for the inoculum preparation. The dispersed eggs were counted, expressed as eggs/plant and the Rf of the nematode calculated.

Physiological variability

Two populations each of *M. arenaria* (MaAL30, MaAL47), *M. incognita* (MiAL10, MiAL48) and *M. javanica* (MjAL01, MjAL39) were used to determine the physiological variability among *Meloidogyne* spp. populations in watermelon 'Sugar Baby'. These populations came originally from tomato cultivated plants in plastic greenhouse (Verdejo-Lucas *et al.*, 2012) and had been maintained in pot cultures in a greenhouse.

Watermelon seedlings were transplanted at the cotyledon stage to clay pots filled with 20 cm³ of sterilized river sand and allowed to grow for two weeks before inoculation with 200 J2 of the respective populations. The treatments were replicated seven times. The nematode inoculum was obtained following a procedure similar to that described for the initial inoculum experiments. The experiment was repeated once. Plants were maintained in a growth chamber at 26ºC with a 16-h light photoperiod, watered daily as needed and fertilized with Osmocote® at the beginning of the experiment. Plants were harvested seven days post-inoculation. The roots were washed free of soil, stained with 0.05% acid fuchsine (Bridge & Page, 1982) and the numbers of infection sites, nematodes inside the roots, and their developmental stages were determined.

Data analyses

The SAS system V8 (SAS Institute Inc., Cary, NC, USA) was used for statistical analyses. Prior to the analyses, when needed, nematode data were log transformed [log10 (x+1)] to homogenize the variances. Data from the repeated experiments were combined because there was no significant difference between the individual experiments. The relationship between Pi and the area under the vine-length curve

(AUC) was estimated using linear regression. The AUC over time was determined using the trapezoidal method as implemented in Matlab R2102a (MathWorks Inc., Natick, MA, USA). The relationship between Pi and penetration rates and nematodes in the roots was tested by fitting an asymptotic regression approach to the data. The relationship between Pi and top dry weight and root fresh weight were also explored using an asymptotic regression curve fitting approach. Data on the number of females and on the physiological variability among RKN populations were analysed using analysis of variance (ANOVA). When the analyses showed statistical differences ($p<0.05$), the means were separated according to Tukey HSD (Honestly Significant Difference) Test.

Results

Post-infection development

The percentage of J2 penetrating watermelon roots ranged from 28 to 67% (Fig. 1). The increase in Pi was correlated with the penetration rates ($R^2 = 0.603$, $p< 0.001$). There was a correlation between the progressive increase in Pi and nematodes in the roots ($R^2 = 0.963$, $p<0.001$). (Fig. 2a). The highest number (165 J2/ plant) was observed at the highest Pi. The nematode completed its life cycle and produced offspring in watermelon, and the Rf was highest at the lowest Pi (25 J2/plant) (Fig. 2b). There was no good correlation between the Pi and the Rf. At 26 dpi, the *M. javanica* population in the watermelon roots consisted primarily of J3, with a small percentage of J2 and J4 plus a few egg-laying females, this situation being consistent for all Pi levels (Fig. 3). At the second harvest (60 dpi), the numbers of
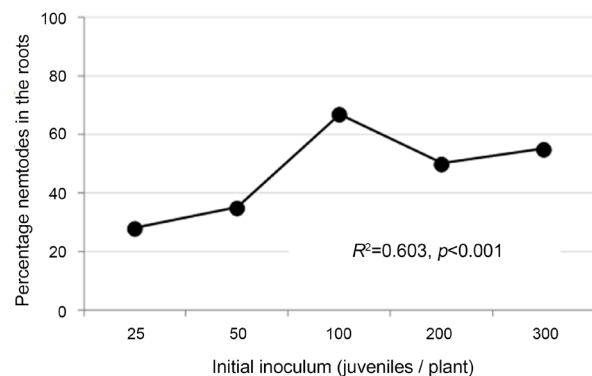

Figure 1. Percentage of *Meloidogyne javanica* invading watermelon roots 'Sugar Baby' at progressively higher second-stage juveniles (J2) inoculum levels 26 days post-inoculation in greenhouse pot experiments. Values are means of 14 replicates (seven replicates/experiment × two experiments).
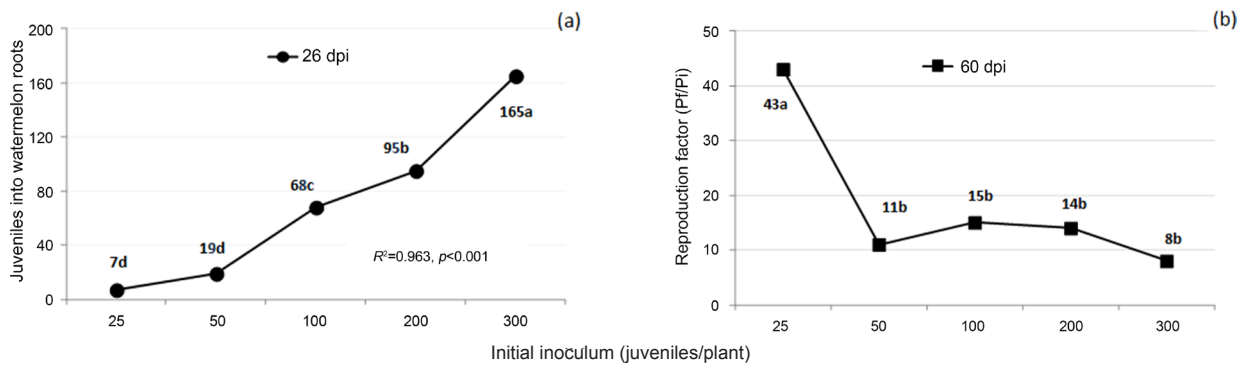
Figure 2. Numbers of *Meloidogyne javanica* inside the roots of watermelon 'Sugar Baby' at progressively higher second-stage juveniles (J2) inoculum levels 26 days post-inoculation (dpi) (a), and nematode reproduction factor (eggs/plant/initial inoculum) 60 days post-inoculation (b) in greenhouse pot experiments. Values are means of 14 replicates (seven replicates/experiment × two experiments).

egg-laying females were similar to those observed at 26 dpi (Fig. 4).

All plants increased their vine length linearly along the experimental period independently of the Pi. Regression analysis indicated that the vine length curve AUC was significantly larger as the Pi progressively increased ($R^2 = 0.417$, $p<0.001$) (Fig. 5a). The progressive increase in Pi was correlated with top dry weight ($R^2 = 0.431$, $p< 0.01$), and root fresh weight ($R^2 = 0.149$, $p< 0.022$) (Fig. 5 b and 5c, respectively). The Pi level did not affect the watermelon LCC (data not shown).

Physiological variability

The nematodes inside the watermelon roots at 7dpi were J2 and J3, the number of infection sites ranged from 13 (MaAL30) to 48 sites (MiAL10)/ root system, and only MiAL10 showed a higher ($p<0.05$) number of infection sites than the remaining populations (Table 1). Nematodes in the roots varied greatly among
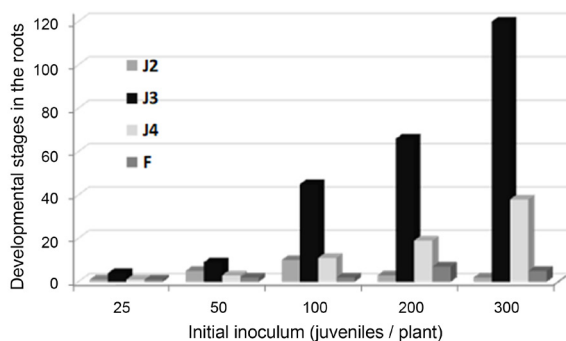
populations and ranged from 47 to 112 individuals. The *M. incognita* populations differed in their ability to penetrate roots and MiAL10 did at higher ($p<0.05$) rates than MiAL48. By contrast, both populations of *M. javanica* and *M. arenaria* invaded roots in a similar way, although fewer ($p<0.05$) individuals of *M. arenaria* did so in comparison with *M. javanica*.

Discussion

The penetration of *M. javanica* in watermelon roots followed a similar pattern to that previously described (López-Gómez & Verdejo-Lucas, 2014). The high correlation between Pi and total number of nematodes in the roots suggests that the range of Pi values did not surpass the capacity of the root system to host the nematode. The increase in the *M. javanica*

Figure 3. Post-infection developmental stages of *Meloidogyne javanica* in watermelon 'Sugar Baby' at progressively higher initial inoculum levels 26 days post-inoculation in pot experiments conducted in a greenhouse. Values are means of 14 replicates (seven replicates/experiment × two experiments). J2, J3, J4: second-, third- and fourth-stage juveniles, respectively; F: egg-laying females.

Figure 4. Number of *Meloidogyne javanica* females in watermelon 'Sugar Baby' at progressively higher second-stage juveniles (J2) inoculum levels 26 and 60 days post-inoculation (dpi) in pot experiments conducted in a greenhouse. Values are means of 14 replicates (seven replicates/ experiment × two experiments). Different letters within harvest point (26 dpi and 60 dpi) indicate statistical differences according to Tukey's HSD (honestly significant difference) test ($p<0.05$).

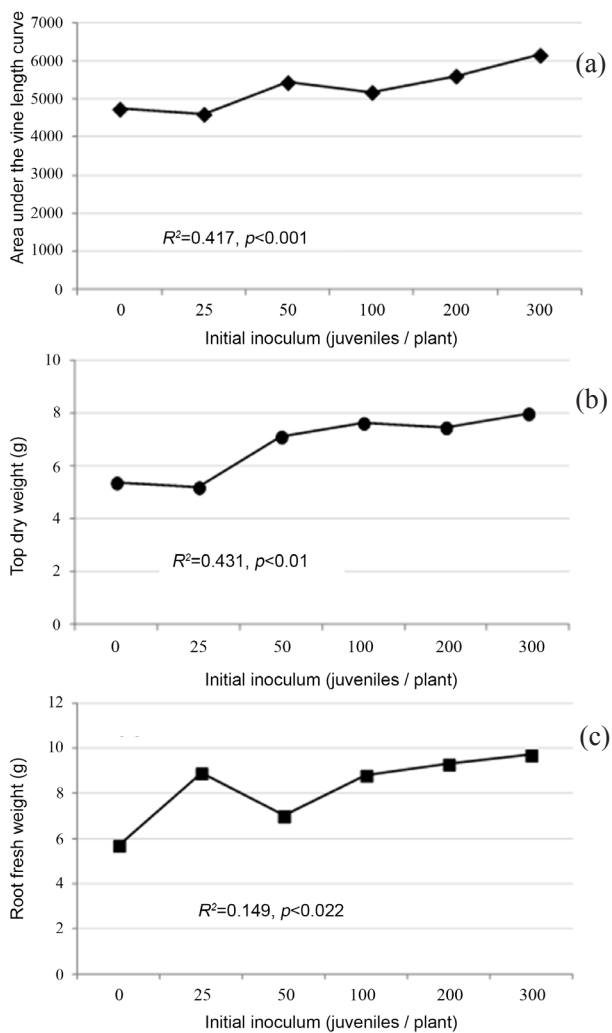**Figure 5.** Plant growth parameters of watermelon 'Sugar Baby' inoculated with second-stage juveniles (J2) at progressively higher inoculum levels of *Meloidogyne javanica* in greenhouse pot experiments. Area under the vine length curve (AUC) over time measured at weekly intervals from 0 to 56 days-post-inoculation (a), top dry weight (b), and root fresh weight (c) 60 days post-inoculation. Values are mean of 14 replicates/treatment (seven replicates/experiment × two experiments).

population densities (Rf >1) in watermelon confirmed its suitability as a host for the nematode. However, an inverse relationship was not found between Pi and Rf typically occurring in good host plants (Seinhorst, 1967). The population dynamics of *M. javanica* in watermelon revealed low magnitudes for the maximum reproduction rate and equilibrium density (López-Gómez *et al.*, 2014), which is in agreement with the poor-host status definition given by Seinhorst (1967). Any trait preventing pathogen establishment or limiting their proliferation can be considered a source of resistance. In fact, watermelon shows low to moderate RKN population increases and decreased susceptibility in comparison with other susceptible crops. The Rf for *M. incognita* in watermelon 'Sugar Baby' was 2.9 and 9.6 in 'Charleston 76` compared with 24.8 in tomato 'Rutgers' (Montalvo & Esnard, 1994). Anwar & McKenry (2010) reported Rf= 2.5 for *M. incognita* in watermelon in contrast to Rf= 125 in tomato. Reproduction of *M. incognita* and *M. javanica* in watermelon genotypes was lower than in cucumber and melon (Cohen *et al.*, 2014), as well as in hybrids of *Cucurbita maxima* × *C. moschata* used for grafting watermelon (Thies *et al.*, 2015; López-Gómez *et al.*, 2016).

The low Rf of *M. javanica* in watermelon was formerly explained by a reduction in J2 penetration and a delay in nematode development in the roots during the first 11dpi in comparison with other cucurbits (López-Gómez & Verdejo-Lucas, 2014). *Meloidogyne javanica* induced the formation of feeding sites as the presence of large numbers J3 in the roots indicated. This observation concurs with their successful establishment in the roots because the moult from J2 to J3 occurs only after the feeding site is established (Escobar *et al.*, 2015). The establishment of feeding sites is required to satisfy the nematode nutritional demands for development and thus those sites need to be

Table 1. Number of infection sites, nematodes and percentage of root penetration of *Meloidogyne arenaria*, *M. incognita* and *M. javanica* into roots of watermelon 'Sugar Baby' inoculated with 200 second-stage juveniles/plant seven days post-inoculation in pot experiments conducted in a growth chamber.

Meloidogyne species	Population code	Infection sites	Nematodes	Percentage root penetration (%)
M. incognita	MiAL10	48 ± 0.5 a	112 ± 2 a	56
	MiAL48	26 ± 0.4 b	66 ± 1 bc	33
M. javanica	MjAL01	26 ± 0.5 b	91 ± 1 b	41
	MjAL39	18 ± 0.2 b	96 ± 2 b	48
M. arenaria	MaAL30	13 ± 0.3 b	47 ± 1 c	24
	MaAL47	25 ± 0.6 b	47 ± 1 c	24

Values are mean ± standard error of 14 replicates (seven replicates/ experiment × two experiments). Different letters within a column indicate statistical differences according to Tukey's HSD (honestly significant difference) test (*p*<0.05).

maintained throughout the life cycle until reproduction is completed (Abad *et al.*, 2009). The dominance of J3 at 26 dpi, when egg-laying adult females are expected to have developed (López-Gómez *et al.*, 2014) suggests the failure of the feeding sites to support nematode development beyond the J3 stage. The disruption of the life cycle at the J3 stage is reflected by the similarities in the number of egg-laying adult females at 26 and 60 dpi. Furthermore, these similarities exclude the hypothesis of delayed development as previous observations have implied (López-Gómez & Verdejo-Lucas, 2014; this study). Nematodes penetrating roots but stopping their development could degrade and eventually die, leaving the galls empty (Stephan & Trudgill, 1982; Faske, 2013; López-Gómez *et al.*, 2015) and their vestiges would not be recognized when dissecting the galls. Because the total numbers of nematodes inside the root rose with the Pi level, the low reproduction of *M. javanica* was not explained by crowding of the invading J2. Therefore, the key event in the host-parasite interaction was the development from J3 to J4, which was interrupted. Consequently, only a small number of the invading J2 reached the egg-laying female stage and produced offspring. These results agree with field observations on low Pf densities after watermelon cultivation (Davis, 2007; Xing & Westphal, 2012; López-Gómez *et al.*, 2015). On the other hand, the display of profuse root galling (Thies & Levi, 2003; Davis, 2007) but suppressed nematode reproduction suggests the hypersensitivity of watermelon to RKN, as previously noted (Anwar & McKenry, 2010).

Meloidogyne javanica did not cause disease in watermelon in this study as the plant growth parameters increased instead of decreasing with the progressive increase in Pi. Indeed, watermelon compensated for the *M. javanica* parasitism by increasing the length of the vines (19 to 33%) and the dry top weight (40%) in comparison with the non-inoculated plants. Plants inoculated with the highest inoculums level (300J2/plant) grew faster than the non-inoculated plants. Plant growth is influenced by the nematode population density, and stimulation of top weight has been detected at low Pi values (Wallace, 1971). The increased root weight, from 23 to 70% with respect to non-inoculated plants could be expected as it is characteristic of *Meloidogyne* infection caused by root-tissue hyperplasia and hypertrophy. Root weight of 'Royal Sweet' increased with the increase in Pi of *M. incognita* (Xing & Westphal, 2012). However, in certain host-parasite combinations, the large size of the root system offsets nematode damage, as in the cucurbit hybrid rootstocks used for grafting watermelon (Edelstein *et al.*, 2010; Thies *et al.*, 2010). The lack of differences in LCC suggests that the range of Pi tested (0.05 to 0.06 J2/cm^3 soil) were below those that may

cause RKN damage and associated nutrient deficiencies in watermelon. Declines in LCC have been recorded in zucchini at higher Pi values but not lower than 1.81 *M. javanica* J2/cm^3 soil (López-Gómez *et al.*, 2015).

The physiological variability typically associated with the genus *Meloidogyne* was noted both between populations and species of the nematode. Thus, the *M. incognita* populations differed in invasion rates but not those of *M. arenaria* or *M. javanica*. *Meloidogyne arenaria* had lesser ability than *M. incognita* and *M. javanica* to invade watermelon roots, as also shown in tomato, zucchini and cucumber (Verdejo-Lucas *et al.*, 2012; López-Gómez *et al.*, 2015). The extent of nematode damage and the severity of the disease are affected by the physiological and genetic characteristics of the species or population infesting a given site, and thus, plant damage would change from site to site. A 5-fold increase in *M. javanica* Pi did not increase the Rf in 'Sugar Baby' and had no effect on dry top weight (López-Gómez *et al.*, 2016). By contrast, a 10-fold increase in *M. incognita* Pi resulted in Rf <1 and reduced dry top weight in 'Royal Sweet' (Xing & Westphal, 2012). The estimated tolerance limit (the nematode density below which there is no yield loss) for *M. incognita* infecting watermelon was 4 J2/100 cm^3 soil (Xing & Westphal, 2012) whereas for *M. javanica* the value was 20 J2/100 cm^3 soil (López-Gómez *et al.*, 2014). Watermelon genotypes showed less root galling when infected by *M. javanica* than *M. incognita* (Cohen *et al.*, 2014). These results suggest that watermelon has higher tolerance to *M. javanica* than to *M. incognita*, and therefore would tolerate higher Pi levels before showing symptoms of damage and yield losses. Additional research would be needed to establish how the physiological variability in RKN can affect the host-parasite relationship in watermelon.

In summary, this study demonstrates that *M. javanica* development in watermelon roots was disrupted at the J3 stage. Further development of the nematode leading to completion of the life cycle was attained only by a low proportion on the invading nematodes. Consequently, *M. javanica* exhibited low to moderate reproductive success in watermelon. The range of Pi tested had a positive stimulating effect on plant growth parameters. These findings help elucidate the host-parasite interactions in a crop where currently no commercial watermelon cultivars are resistant to *Meloidogyne*.

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