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ORIGINAL ARTICLE

The reaction of tomato plants carrying *Mi-1* gene to different inoculation densities of *Meloidogyne incognita* (Kofoid and White, 1919) Chitwood, 1949

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Abstract

The response of the *Mi-1* gene to different densities of *Meloidogyne incognita* race 2 was investigated under controlled conditions. Susceptible and resistant tomato seedlings were inoculated with 25, 50, 100, 200, 400, 1000, 2000, 5000 and 10 000 second-stage juveniles of *M. incognita*. Plants were uprooted 8 weeks after inoculation and the numbers of egg masses and galls on the roots, and second-stage juveniles in 100 g soil per pot were counted. In susceptible plants, there was a correlation between the number of egg masses on roots until 2000 J2 inoculum densities. In resistant plants, when inoculum densities increased, the number of egg masses and galls also increased. The reproduction factor ratio was >1 in the susceptible plant and <1 in the resistant plant. The data showed that the 5000 J2 inoculum was a critical limit, and 10 000 J2s were above threshold for resistant plants. The data indicate that densities of *M. incognita* can seriously affect the performance of the *Mi-1* gene.

Key words: gene *Mi-1*, *Meloidogyne incognita*, root-knot nematodes, tomato

Introduction

Tomato is one of the most important vegetables worldwide. However, this plant can be severely damaged by various pests and diseases. Root-knot nematodes (*Meloidogyne* spp.) are considered to be major parasites of tomato. These worms feed on plant roots, inducing the formation of galls and impairing uptake of water and nutrients. Infected plants show stunting, wilting, chlorosis and a poor yield (Williamson and Hussey 1996; Karssen and Moens 2006; Schomaker and Been 2006; Moens *et al.* 2009). Moreover, infected plant roots may rot because of secondary soil-borne fungi, and synergistically interact with other soil-borne pathogens, thereby greatly increasing crop damage (Williamson 1999; Karssen and Moens 2006; Moens *et al.* 2009).

Different management tactics, including soil fumigants, non-fumigant nematicides, resistant varieties and rootstocks, are commonly used to control root-knot nematodes (Devran *et al.* 2010). Chemical treatments have been extensively employed to manage root-knot nematodes worldwide. However, increasing environmental and health concerns have restricted their use (Nyczepir and Thomas 2009; Wesemael *et al.* 2011). Resistant cultivars are an environmentally-safe method for controlling root-knot nematodes in infested soil. Resistant cultivars show good yield performance, and suppress nematode population increases (Cook and Starr 2006). Furthermore, they do not require significant changes in farming operations or market supply (Ornat *et al.* 2001).

Resistance to root-knot nematodes in tomato is controlled by a single dominant gene *Mi-1* (Smith 1944; Milligan *et al.* 1998). This gene confers resistance

to the three root-knot nematode species: *M. incognita*, *M. javanica* and *M. arenaria* (Roberts and Thomason 1986). The *Mi-1* gene has been successfully incorporated into many commercially available tomato varieties and is currently the only source of root-knot nematode resistance in all commercial tomatoes (Devran *et al.* 2010; Seid *et al.* 2015). In addition, the gene also confers resistance to the aphid *Macrosiphum euphorbiae*, as well as *Bemisia tabaci* biotypes Q and B (Rossi *et al.* 1998; Nombela *et al.* 2003). Nonetheless, the performance and efficiency of the *Mi-1* gene are affected by many factors, such as high-soil temperature (Dropkin 1969; Araujo *et al.* 1982a; Haroon *et al.* 1993; Abdul-Baki *et al.* 1996), *Mi-1* virulent populations (Castagnone-Sereno 1994; Kaloshian *et al.* 1996; Ornat *et al.* 2001; Devran and Söğüt 2010) and population densities (Araujo *et al.* 1982b; Maleita *et al.* 2012).

To the best of our knowledge, there are limited studies on the efficiency of the *Mi-1* gene against high population densities of root-knot nematodes. Maleita *et al.* (2012) reported that increasing inoculation densities of *M. javanica* and *M. hispanica* increased the number of galls in resistant plants. Another study indicated that the intensity of root-knot nematode infection negatively affected the growth of plant and biomass (Sharma and Sharma 2015). Here, we report the response of susceptible and resistant tomato plants carrying the *Mi-1* gene to different inoculum densities of *M. incognita* race 2 under controlled conditions.

Materials and Methods

Plant material

Root-knot nematode-susceptible Tueza F1 and root-knot nematode-resistant Seval F1 tomato varieties were used in the bioassay. Tomato seeds were sown in seedling trays. After 4 weeks, tomato seedlings were transferred singly to 250-ml plastic pots containing steam-sterilised sandy soil.

Nematode isolate

The G2 isolate of *M. incognita* race 2 was used in this study. This isolate had been identified by a molecular method and host reaction test in previous studies (Devran and Söğüt 2009, 2011).

DNA isolation

Nematode DNA was also isolated from ten second-stage juveniles (J2s) with the DNAeasy Tissue and Blood Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Plant genomic DNA was extracted from young leaf tissue using the Wizard Magnetic Kit (Promega, Madison, WI, USA), based on the manufacturer's protocol.

PCR amplification

The G2 isolates of *M. incognita* race 2 were confirmed using the species-specific primers Inc14F/Inc14R (Randig *et al.* 2002). The absence of the *Mi-1* gene in tomato plants was checked using the Mi23 marker (Seah *et al.* 2007). The PCR reactions were performed in a total volume of 25 µl with 2.5 µl DNA, 2 mM $MgCl_2$, 200 µM dNTPs, 0.4 µM of each primer, 2.5 µl 10X PCR buffer and 1 U Taq DNA polymerase. Amplification was performed in a DNA Engine PTC-200 thermal cycler (Bio-Rad, Hercules, CA, USA) using the following conditions: 94°C for 3 min, 35 cycles at 94°C for 30 s, 60°C for 30 s and 72°C for 1 min, with a final extension at 72°C for 7 min. Amplified products were analysed on a 2% agarose gel in 1X TAE buffer and visualised by ethidium bromide staining.

Nematode culture

In order to obtain the nematode culture, susceptible tomato seedlings at the two true-leaf stage were transferred singly to 250-ml plastic pots containing steam-sterilised sandy soil. The plants at the four true-leaf stage were inoculated with 1000 J2s. The plants were grown in a growth chamber (16-h photoperiod, 25 ± 0.5°C, 65% relative humidity) for 8 weeks after nematode inoculation. The plants were then uprooted, and their roots were gently washed under running tap-water. Then, egg masses were detached from plant roots using a needle and incubated in a Petri dish at room temperature for 1 day. The J2s which emerged from the egg masses were collected and counted under a light microscope (Mıstanoğlu *et al.* 2016).

Nematode inoculation

The seedlings of Tueza F1 and Seval F1 were tested with the verified G2 isolates of *M. incognita* race 2. The susceptible and resistant tomato seedlings at the four true-leaf stage were inoculated with 25, 50, 100, 200, 400, 1000, 2000, 5000 and 10 000 freshly emerged (< 24 h) J2s, respectively. The J2s were poured into a hole, 2 cm deep, close to the stem of the plants. Quintuplicates for each inoculum level were taken for the screening test. The plants were then kept in a growth chamber (16-h photoperiod, 25 ± 0.5°C, 65% relative humidity) for 8 weeks after nematode inoculations. Inoculated plants were uprooted, and the roots were washed under running tap water.

Data analysis

Egg masses and galls on roots were counted under a stereomicroscope. The J2s from the soil of each pot (100 g soil/pot) were extracted using a modified Baermann funnel technique (Hooper 1986) and counted under a light microscope. The reproduction factor (Rf) was calculated by the formula, Rf = Pf/Pi, where Pf = final *M. incognita* population and Pi = initial *M. incognita* population (Ferris and Noling 1987).

All data were analysed using analysis of variance (ANOVA) procedures. Differences among treatments were compared according to the least significant difference (LSD) test. All data analyses were performed using SAS (v. 9.0 for Windows; SAS Institute Inc., Cary, NC, USA).

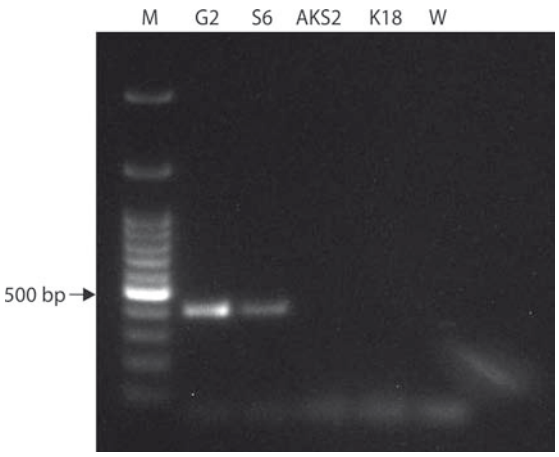

Fig. 1. PCR products obtained with Inck14F/Ink14R primers. M – marker (100 bp PlusOpti DNA, abm, Canada), G2 and S6 – *Meloidogyne incognita*, AKS2 – *M. javanica*, K18 – *M. arenaria*, W – water

Results and Discussion

The authenticated G2 isolate of *M. incognita* race 2 produced a DNA fragment about 400 bp in length (Fig. 1), which agrees with previous studies (Randing *et al.* 2002; Devran and Söğüt 2009, 2014). Verification of the *Mi-1* gene in tomato varieties based on the presence of the Mi23 molecular marker (Devran *et al.* 2013; Mıstanoğlu *et al.* 2016) confirmed that of the two F1 varieties, one was root-knot nematode-susceptible and the other was root-knot nematode-resistant.

In susceptible plants, the highest number of egg masses per root system occurred in pots inoculated with 2000 J2s and the least at 25 and 50 J2s inoculum levels. The number of egg masses was not statistically different in susceptible plants inoculated with 400 and 1000 J2s; 25 and 50 J2s, but in the pots inoculated with 2000 J2s, it was significantly greater than all the other pots (Table 1). Khan *et al.* (2000) noted a correlation between egg masses on roots and inoculum densities (2000, 4000 and 6000 J2s) in susceptible tomato. Our data showed a correlation between the number of egg masses on roots until 2000 J2s inoculum densities but then decreased in susceptible plants. Given that the growth of plant roots was affected by a heavy nematode density, the number of egg masses on roots could decrease. Sharma and Sharma (2015) observed that increasing the intensity of root-knot nematode infection (500, 750, 1000, 1250 and 1500 J2s) negatively affected the growth of the plant and biomass. The largest number of galls per root system was recorded at 10 000 J2s inoculation levels and the least at 200 J2s inoculation. The number of galls was not statistically different in susceptible plants inoculated with 25, 50, 100 and 400; and 2000, 5000 and 10 000 J2s (Table 1). Our findings concur with Kankam and Adomako (2014), who inoculated plants with 0, 500, 1000 and 2000 J2s *Meloidogyne* spp. In this study, the J2s was obtained from each pot using Baermann funnel. The highest number of juveniles was observed in the pots inoculated with 2000 J2s, and the least at 25 J2s. The number of juveniles increased until 2000 J2s and then decreased. The number of juveniles in the soil of pots showed differences according to J2 inoculation densities. In all inoculation densities, the Rf ratio was >1. The highest Rf ratio was 400 J2s inoculation, and the lowest was 10 000 J2s inoculation (Table 1). Di Vito and Ekanayake (1983) inoculated susceptible tomatoes with *M. incognita* race 1 using increasing inoculum densities according to a geometrical series. They found that the highest Rf ratio was 1 (eggs + J2/ml soil), and the lowest was 512 (eggs + J2/ml soil). Khan *et al.* (2000) inoculated susceptible tomato plants using three different inoculation densities, including 2000, 4000, and 6000 J2s. The highest J2 number was 6000 J2s inoculation. These differences can be associated with the stage of the seedlings, virulence of the nematode and differences in inoculation methods.

In resistant plants, the highest numbers of egg masses per root system were in the pots inoculated at 10 000 J2s and, the least at 50 J2s. The number of egg masses was not statistically different in resistant plants inoculated with 25, 100 and 400 J2s (Table 2). The number of galls was not statistically different in resistant plants inoculated with 25 and 50 J2s; 100 and 400 J2s (Table 2). However, increases in gall formation occurred at comparatively larger inoculum densities, with the greatest number observed at the 10 000 J2s inoculum level. Maleita *et al.* (2012) found that increasing the inoculation densities (2500, 5000

Table 1. Number of egg masses, galls per root and J2s in pots planted with susceptible plants

Number of J2s inoculum	Number of egg masses	Number of galls	Number of J2	Rf ratio
0	0 e	0 d	0 c	0 f
25	14.8 e	192.2 bc	492 c	19.68 def
50	19.4 e	187.8 bc	1564 c	31.28 cde
100	40.4 de	156.0 bc	3516 c	35.16 cd
200	80.4 cd	140.0 c	10 338 bc	51.69 abc
400	164.8 b	160.4 bc	26 120 b	65.30 a
1000	168.4 b	221.8 b	58 680 a	58.68 ab
2000	279.6 a	347.0 a	78 946 a	39.473 bcd
5000	134.6 bc	367.0 a	61 204 a	12.24 ef
10 000	85.4 cd	402.0 a	12 268 bc	1.22 f

Means in columns followed by the same letter are not significantly different ($p \leq 0.05$) according to LSD test

Table 2. Number of egg masses, galls per root and J2s in the soil of pots planted with resistant plants

Number of J2s inoculum	Number of egg masses	Number of galls	Number of J2 per pot	Rf ratio
0	0 d	0 e	0 c	0 a
25	1.0 cd	0.4 e	0 c	0 a
50	0.2 d	0.4 e	16 c	0.32 a
100	1.0 cd	3.0 de	50 c	0.5 a
200	1.6 bcd	6.2 cde	108 bc	0.54 a
400	1.0 cd	2.6 de	46 c	0.115 a
1000	3.8 ab	9.8 cd	324 a	0.324 a
2000	3.0 abc	12.4 bc	256 ab	0.128 a
5000	3.8 ab	17.6 b	164 abc	0.0328 a
10 000	4.6 a	25.2 a	130 abc	0.013 a

Means in columns followed by the same letter are not significantly different ($p \leq 0.05$) according to LSD test

and 10 000 eggs + J2s) of *M. javanica* and *M. hispanica* increased the number of galls in resistant plants. In this study, the highest number of J2 was in pots inoculated with 1000 J2s, and the least was at 25 J2s. There was also no significant difference at this inoculation range. At all inoculation densities, the Pf/Pi ratio was <1 (Table 2). Maleita *et al.* (2012) noticed that the number of eggs + J2s did not consistently increase when inoculation levels of *M. javanica* and *M. hispanica* were increased to 2500, 5000 or 10 000 J2s. Our findings corroborate these results. Our data showed that an inoculation density of 5000 J2s of *M. incognita* was the critical level and, hence, 10 000 J2s were above the threshold for appropriate testing of resistant plants. Also, the results indicated that inoculation densities ≥1000 J2s were more convenient for bioassay.

In conclusion, the resistance provided by the *Mi-1* gene has some critical limitations, such as high-soil temperature and *Mi-1* virulent populations (Dropkin 1969; Castagnone-Sereno 1994). Here, we also report that population densities of root-knot nematodes are very important in plants carrying the *Mi-1* gene. Our findings showed the inoculum level around 25 J2s, may cause problems in the screening of plants and miss testing of plant resistance. Five thousand J2s were the critical level for resistance plants. Root-knot nematode populations may sometimes have high densities in vegetable-growing areas. Our data showed that inoculum density adversely affected the performance of the *Mi-1* gene. Similarly, it was previously stated that increasing root-knot nematode densities affected the growth of the plant and biomass (Sharma and Sharma 2015). We assessed three parameters, including the number of egg masses, galls and J2 in pot soil, in the bioassay. Three parameters should be evaluated when plant comparisons are intended.

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