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Original article (Orijinal araştırma)

Response of tomato seedlings with different number of true leaves to *Meloidogyne incognita* (Kofoid & White, 1919) Chitwood, 1949

Farklı sayıda gerçek yapraklı döneme sahip domates fidelerinin *Meloidogyne incognita* (Kofoid & White, 1919) Chitwood, 1949'ya tepkileri

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Summary

Tomato is one of the most important vegetables grown throughout the world. Root-knot nematodes cause significant economic yield losses in tomato. Development of tomato cultivars which are resistant to root-knot nematodes is the most effective management method. In tomato, resistance to root-knot nematodes is determined by bioassay and molecular markers. Molecular markers are commonly used to screen for resistance genes in breeding programs. However, bioassays are also required for identification of the performance of resistance genes. Different parameters such as stage of seedling, soil temperature, nematode quantity and nematode virulence effect performance of bioassays. In the present study, the response of tomato seedlings with different numbers of true leaves to *Meloidogyne incognita* isolate S6 was compared under controlled conditions. Seedlings showed different reactions to *M. incognita* inoculation. The results indicated that stage of tomato seedlings can be important for bioassay and that tomato seedlings with four true leaves are best for nematode testing. These results will help in the optimization of root-knot nematode tests used in tomato breeding.

Keywords: Bioassay, *Meloidogyne*, tomato seedling, reaction

Özet

Domates dünyada yetiştiriciliği yapılan en önemli sebzelerden birisidir. Kök-ur nematodları domateste önemli düzeyde ekonomik kayıplara neden olmaktadır. Kök-ur nematodlarına dayanıklı domates çeşitlerinin geliştirilmesi en önemli mücadele yöntemidir. Domateste kök-ur nematodlarına dayanıklılık biyolojik testler ve moleküler markörler tarafından belirlenebilmektedir. Moleküler markörler ıslah programlarında dayanıklılık geninin tespitinde yaygın şekilde kullanılmaktadır. Bununla birlikte dayanıklılık geninin performansının belirlenmesi için biyolojik testler gereklidir. Nematod virülensliği, nematod sayısı, toprak sıcaklığı ve fidenin dönemi gibi farklı parametreler biyolojik testin performansını etkilemektedir. Bu çalışmada, farklı sayıda gerçek yaprak dönemine sahip domates fidelerinin *M. incognita*' nın S6 izolatına tepkisi kontrollü koşullar altında karşılaştırılmıştır. Fideler *M. incognita* inokulasyonuna farklı farklı tepkiler göstermiştir. Sonuçlar, biyolojik test için domates fidesinin döneminin önemli olduğunu ve nematod testi için dördüncü gerçek yapraklı döneme sahip fidelerin en uygun olduğunu göstermiştir. Bu sonuçlar, domates ıslahında kullanılacak olan kök-ur nematodlar testlerinin optimizasyonuna yardım edecektir.

Anahtar sözcükler: Biyolojik test, *Meloidogyne*, domates fidesi, reaksiyon

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Introduction

Root-knot nematodes are one of the most important pathogens attacking cultivated plants. The second-stage juveniles of the root-knot nematodes move intercellularly after penetrating the root (Williamson, 1998). They secrete substances through their stylet. These secretions emanate from two subventral and one dorsal esophageal gland cells, and are crucial for infection and for the formation of host feeding cells (Bird, 1967; Hussey & Mims, 1990). They establish a permanent feeding site in the differentiation zone of the root. Root-knot nematodes cause nuclear division without cytokinesis in host cells because of feeding. This process cause large multinucleate cells, known as giant cells (Huang, 1985). Galled roots impair the ability of the plants to take up water and nutrients, resulting in reduced translocation of minerals and photosynthesis (Abad et al., 2003). Affected plants often show symptoms of stunting, wilting or chlorosis (Karssen & Moens, 2006; Schomaker & Been, 2006). In addition, root-knot nematodes interact with soil-borne plant pathogens, resulting in increased damage from other diseases (Williamson, 1998; Karssen & Moens, 2006). Therefore, root-knot nematodes cause significant economic yield losses alone or in combination with other biotic and abiotic factors in crop fields (Schomaker & Been, 2006).

Tomato is one of the most important vegetables grown around the world. Root-knot nematodes are considered to be a major pest of tomato. Managing nematode problems can be difficult in tomato growing fields. Chemical treatments have been used for controlling root-knot nematodes. However, environmental effects and legal regulations have limited their use (Wesemael et al., 2011). Therefore, alternative management methods are required. Resistance breeding is obviously the most effective method to control of root-knot nematode. Genetic resistance to root-knot nematodes has been shown to reduce nematode populations, and thereby decrease the need for pesticides (Williamson, 1999). Therefore, development of the tomato cultivars resistant to root-knot nematodes is one of the most important strategies for controlling root-knot nematodes (Devran et al., 2013). Resistance to root-knot nematodes in tomato plants is determined by bioassays and molecular methods. Bioassays are required for determining the performance of resistant genes in plant-nematode interactions. Bioassays also give reliable and logical information about the resistance of plants. These assays are carried out under controlled conditions in a growth chamber and different parameters, such as stage of seedling, soil temperature and nematode quantity, are important (Devran et al., 2013). Also, knowing the virulence of the nematode species or race is essential. In tomato, resistance assays for root-knot nematodes are actively carried out by researcher focusing nematode-host interactions (Ramsay et al., 2004; Melillo et al., 2006). However, there is no detailed information about the response of tomato seedlings with different number of true leaves to nematode infection. Also, there has been no assessment of the effect of tomato seedling stage on bioassays performance. Therefore, in this study, response of tomato seedlings with different number of true leaf stages to *Meloidogyne incognita* (Kofoid & White, 1919) Chitwood, 1949 was evaluated under controlled conditions.

Material and Methods

Plant material

The susceptible tomato cv. Tueza F_1 seeds were provided by Multi Seed (Antalya, Turkey). The seeds were sown in seedling trays in facilities of Multi Seed. Seedlings were transferred singly to 250-ml plastic pots containing steam-sterilized sandy soil (75% sand, 15% silt, and 10% clay) five weeks after sowing.

Nematode isolate

Meloidogyne incognita race 2 isolate S6 was used in this study. The isolate was identified using the molecular methods and host reaction tests described in previous studies (Devran & Söğüt, 2009; Devran & Söğüt, 2011).

Nematode culture

Egg masses of *M. incognita* were collected from roots of infected tomato plants using a needle and incubated in a petri dish at room temperate. Second-stage juveniles that hatched from the egg masses were collected, placed in a refrigerator at 4^{o}C and used within 1 day. Number of juveniles were counted under microscope.

Nematode inoculation

Tomato seedlings with two, three, four, five, six, seven and eight true leaves were inoculated with 1000 second-stage juveniles each. The juveniles were injected into a 2-cm deep hole close to the stem of the plants. Five replicates for each true leave stage seedlings were laid out in a randomized block design in a growth chamber (16-h photoperiod, 25±0.5°C and 65% RH). The plants were harvested 8 weeks after inoculation. They were gently uprooted and roots of plants were washed under tap water before scoring of egg masses and galls.

DNA isolation

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

PCR amplification

Meloidogyne incognita was checked using the species-specific primers MincF/MincR (unpublished data). The other nematode species, *M. javanica*, *M. arenaria* and *M. ethiopica* were also used as negative control. The absence of the *Mi-1* gene in Tueza F1 was checked using the Mi23 marker (Seah et al., 2007). Browny F_1 and Seval F_1 were used as homozygous resistant and heterozygous resistant for *Mi-1* gene, respectively. The PCR reaction was performed in a total volume of 25 µL with 2.5 µL of 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 (Fermentas, Vilnius, Lithuania). Amplification was performed in a thermal cycler (Veriti 96-Well, Applied Biosystems, Foster City, CA, USA) using the following conditions: 3 min at 94^{o}C, 35 cycles at 94^{o}C for 30 s, 56^{o}C for 30 s and 72^{o}C for 1 min with a final extension at 72^{o}C for 7 min. Amplified products were analyzed on a 2% agarose gel in 1X TAE buffer and visualized by ethidium bromide staining.

Data collection and analysis

Egg masses and galls on roots of tomato seedlings were counted. Second-stage juveniles from 100 g soil per pot were extracted using a modified Baermann funnel technique (Hooper, 1986) and counted under microscope. These data were analyzed by ANOVA using the statistical package SAS (v. 9.0 for Windows; SAS Institute Inc., Cary, NC, USA). Significant differences within treatments were tested using least significant difference (LSD).

Results and Discussion

Molecular identification

Meloidogyne incognita was confirmed using species-specific primers MincF/MincR. The primer pairs produced an approximately 150 bp amplicon in *M. incognita* samples, and did not yield any PCR product in another nematode species, *Meloidogyne javanica*, *Meloidogyne arenaria* and *Meloidogyne ethiopica* as expected (Figure 1). Our findings were in accordance with an earlier study (unpublished data). These results indicated that the *M. incognita* isolate S6 was a pure culture.

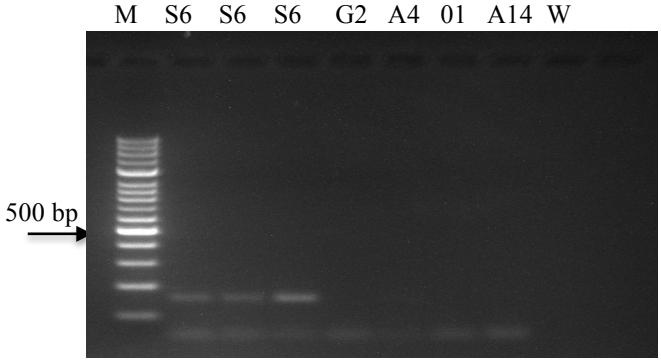

Figure 1. PCR products of MincF and MincR primer sets. M: Molecular marker (100 bp DNA ladder, GeneAll), S6: *Meloidogyne incognita*, G2 and A4: *Meloidogyne javanica*, O1: *Meloidogyne arenaria*, A14: *Meloidogyne ethiopica*: W: Water.

The absence of the *Mi-1* gene in the tomato seedling was verified with molecular marker Mi23. Mi23 primer pairs yielded 380 bp and 430 bp fragments in homozygous resistant and susceptible plants, respectively. Heterozygous plants produced 380 and 430 bp fragments (Figure 2). Marker analysis showed that Tueza F_1 was susceptible as expected. Our results were consistent with earlier studies (Devran et al., 2013; Devran & Söğüt, 2014).

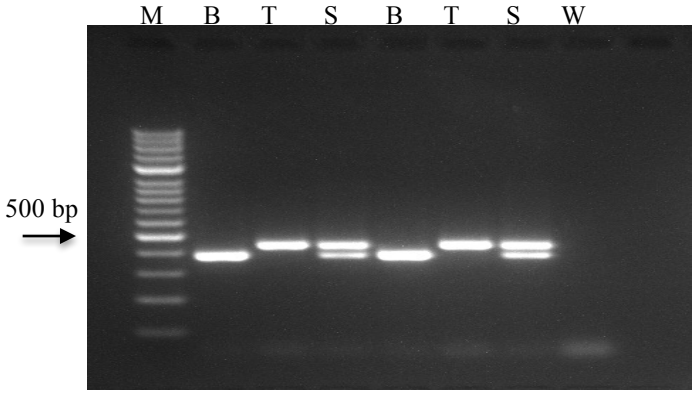

Figure 2. PCR products of Mi23F and Mi23R primer sets. M: Molecular marker (100 bp DNA ladder, GeneAll), B: Browny F_1 (homozygous resistant), T: Tueza F_1 (susceptible), S: Seval F_1 (heterozygous resistant), W: water.

Bioassay of seedlings

Meloidogyne incognita isolate S6 showed different reactions according to the stage of tomato seedlings inoculated. There were significant differences in the number of egg masses produced (Table 1). *Meloidogyne incognita* isolate S6 produced the highest number of egg masses on seedlings with four, five and six true leaves and the lowest number on seedlings the two true leaves. There were no statistically significant differences among seedlings with four, five and six true leaves, between those with seven and eight true leaves.

Table 1. Number of egg masses on the roots of tomato seedlings with different numbers of true leaves

Number of true leaves	Number of egg masses on roots
2	91 ± 68 c
3	151 ± 64 bc
4	314 ± 74 a
5	294 ± 31 a
6	298 ± 77 a
7	250 ± 88 ab
8	233 ± 33 ab

Means in columns followed by the same letter are not significantly different (P ≤ 0.05) according to LSD test.

Tomato seedlings with four true leaves stages had the highest number of gall on their roots (Table 2). Seedlings with two and three true leaves had the lowest number of gall. The number of galls was not statistically significant among seedlings with two and three true leaves, those with five and six true leaves or those with seven and eight true leaves.

Table 2. Number of galls on roots of tomato seedlings with different numbers of true leaves

Number of true leaves	The number of gall on roots
2	100 ± 40 c
3	131 ± 43 c
4	330 ± 33 a
5	280 ± 41 ab
6	276 ± 30 ab
7	261 ± 42 b
8	257 ± 40 b

Means in columns followed by the same letter are not significantly different (P ≤ 0.05) according to to LSD test.

The number of second-stage juveniles was not statistically significant in seedlings with two, three and six true leaves (Table 3). Tomato seedlings with five true leaves had the highest number of second-stage juveniles. Seedlings with two and tree true leaves had the lowest number of second-stage juveniles. There was no correlation number of second-stage juveniles according to stages of true leaves. Therefore, number of second-stage juveniles is not useful unless supported by assessment of number of egg masses and galls.

Table 3. Number of second-stage juveniles in soil from the pots with tomato seedlings with different number of true leaves

Number of true leaves	Number of second-stage juveniles into pots (100 g soil from per pot)
2	15220 ± 13890 c
3	11652 ± 11007 c
4	41470 ± 13498 bc
5	86436 ± 21809 a
6	37920 ± 32282 c
7	83124 ± 37629 ab
8	43050 ± 27940 bc

Means in columns followed by the same letter are not significantly different (P≤0.05) according to to LSD test.

Our findings showed that the stage of tomato seedling is important for nematode inoculation. Therefore, tomato seedlings with same number of true leaves should be inoculated with root-knot nematodes for consistent results in bioassay tests. The seedlings with four, five and six true leaves had higher number of egg masses and galls on their roots than seedling at other stages. Our results indicated that tomato seedlings with four leaves are the best for root-knot nematode testing according to the number of egg masses and galls on root. In the previous studies (Khan et al., 2000; Wasemael et al., 2006), the number of egg masses on roots of young plants was higher than the number of egg masses on roots of old plants. However, our results showed that the number of egg masses and galls on roots of seedlings with two true leaves was lower than other stages. In this study, *M. incognita* isolate S6 affected to tomato seedlings with two and three true leaves more than old plants. Therefore, roots and shoots of these did not grow effectively. Also, the number of egg masses and galls on the seedlings with two and three true leaves were the lowest. This may be because of weak development of root system or stunting of roots. Accordingly, Shane & Barker (1986) reported that plant development can be adversely affected when young seedlings are inoculated with nematode. Consequently, results can be used for optimizing root-knot nematode testing in tomato breeding programs.

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