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Original article (Orijinal araştırma)

Comparison of effectiveness of molecular markers linked to *Me1* and *N* genes in pepper (*Capsicum annuum* L.) (Solanales: Solanaceae)

Biber (*Capsicum annuum* L.) (Solanales: Solanaceae)' de *Me1* ve *N* genlerine bağlı moleküler markörlerin etkinliğinin karşılaştırılması

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Abstract

Pepper (*Capsicum annuum* L.) (Solanales: Solanaceae) is one of the most important agricultural products consumed in the world. Root-knot nematodes (RKNs (*Meloidogyne* spp.)) are major pests that occur dramatically damage on pepper. However, the management of RKNs has some difficulties and one of the most effective methods is using resistant cultivars in infested areas. In this study, the efficiency of molecular markers linked to *Me1* and *N* genes was investigated. The study was conducted in laboratory and under controlled conditions at Akdeniz University Faculty of Agriculture, Department of Plant Protection Nematology Laboratory in 2022. Pepper genotypes belonging to two main varieties (Charleston pepper and Bell pepper) were tested against S6 isolate of *Meloidogyne incognita* (Kofoid & White, 1919) Chitwood 1949 (Tylenchida: Heteroderidae), and screened with molecular markers. As a result, molecular markers linked to two genes gave compatible results with pathologic tests. These markers can be successfully used for marker assisted selection in pepper genotypes.

Keywords: *Meloidogyne incognita*, pathologic tests, PCR primers, resistance

Öz

Biber (*Capsicum annuum* L.) (Solanales: Solanaceae) dünyada tüketilen en önemli tarımsal ürünlerinden biridir. Kök-ur nematodları (*Meloidogyne* spp.) biberde ciddi hasara neden olan başlıca zararlılardır. Ancak Kök-ur nematodlarının mücadelesinde bazı zorluklar vardır ve bulaşık bölgelerde en etkili mücadele yöntemlerden biri dayanıklı çeşitlerin kullanılmasıdır. Bu çalışmada *Me1* ve *N* genlerine bağlı moleküler belirteçlerin etkinliği araştırılmıştır. Çalışma, 2022 yılında Akdeniz Üniversitesi Ziraat Fakültesi Bitki Koruma Bölümü Nematoloji laboratuvarında, laboratuvar ve kontrollü iklim odası koşullarında yürütülmüştür. İki ana çeşide (Charleston biberi ve Dolma biberi) ait biber genotipleri *Meloidogyne incognita* (Kofoid & White, 1919) Chitwood 1949 (Tylenchida: Heteroderidae) S6 izolatına karşı test edilmiş ve moleküler belirteçlerle taranmıştır. Sonuç olarak iki gene bağlı moleküler belirteçler patolojik testlerle uyumlu sonuçlar vermiştir. Bu belirteçler biber genotiplerinde markör destekli seleksiyonda başarılı bir şekilde kullanılabilir.

Anahtar sözcükler: *Meloidogyne incognita*, patolojik test, PCR primerler, dayanıklılık

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Introduction

Solanaceae family has significant importance in agricultural productivity due to economically produced crops. Pepper (*Capsicum annuum* L.) (Solanales: Solanaceae) belonging to Solanaceae family is one of the most important agricultural vegetable yields consumed in the world (Pinto et al., 2016; Barka & Lee, 2020). There are approximately 20-30 *Capsicum* species cultivated in the different parts of the agricultural areas. Among these species, there are five main cultivated species; *Capsicum annuum*, *Capsicum chinense* Jacq., *Capsicum frutescens* L. Kuntze., *Capsicum baccatum* L., and *Capsicum pubesces* Ruiz & Pav. (Solanales: Solanaceae) (Bosland & Votava, 2005).

Pepper cultivations have an important role in economy and pharmacy. Pepper is known as a high-value crop including carotenoid, provitamin A and vitamin C (Bosland et al., 2012). It is also preferred as spice, while has been consumed for nutraceutical and nutritional properties, and industrial use (Lu et al., 2020).

Many pathogens and pests can attack pepper during cultivation. Root-knot nematodes (RKNs) (*Meloidogyne* spp.) (Tylenchida: Heteroderidae) are major pests that cause dramatically damage on pepper (Lizardo et al., 2022). RKNs are well-adapted obligate endoparasites which have more than one hundred species all over the world (Rehak Biondić et al., 2023). Major RKN species are *Meloidogyne incognita* (Kofoid & White, 1919) Chitwood 1949*, Meloidogyne javanica* (Treub, 1885) Chitwood 1949*, Meloidogyne arenaria* (Neal, 1889) Chitwood 1949, and *Meloidogyne hapla* Chitwood 1949 (Tylenchida: Heteroderidae). It was reported that *M. incognita* is the most common and significant species of RKNs all around the world (Jones et al., 2013). They cause serious damage on plants due to forming galls which prevent the nutrient uptake and absorption of water from soil. It was reported that 52% yield losses were caused by RKNs in pepper-growing areas in the Mediterranean area (Talavera-Rubia et al., 2022).

In the management of RKNs, cultural practices including crop rotation, use of resistant plant etc., solarization, biological control agents, and chemical nematicides were employed (Liu et al., 2023). Using resistant cultivars is one of the most effective methods in infested areas (Devran & Söğüt, 2014; Devran et al., 2015; Bucki et al., 2017). Breeding of nematode-resistant cultivars offers an economically and environmentally friendly sustainable strategy to controlling RKNs (Devran et al., 2013). The resistance against RKNs in pepper confers with several genes which are *N, Me1, Me2, Me3, Me4, Me5, Me6, Me7, Mech1, Mech2* and *CaRKNR* (Wang & Bosland, 2006; Djian-Caporalino et al., 2007; Wang et al., 2009; Mao et al., 2015). Resistance response of the genes occurs as the hypersensitive response (HR), which is gene to gene reaction in the plant tissue (Wang et al., 2009, 2018; Özalp & Devran, 2018). *Me* and *N* resistance genes in pepper have been reported to have resistance against RKNs including *M. incognita, M. javanica*, and *M. arenaria* (Djian-Caporalino et al., 2007; Thies & Ariss, 2009; Barbary et al., 2014).

In the resistance screening programs mostly pathogenicity tests are used, and it depends on several factors such as seedling stage, temperature and nematode inoculation levels (Djian-Caporalino et al., 2001; Devran et al., 2013; Barbary et al., 2016). In addition, classical pathogenicity tests have some difficulties with respect to labor and time required and involve a long process as well as serving limited materials. To overcome these difficulties, molecular markers, which are a powerful tool in plant breeding, can be preferred for providing an accurate and fast screening of large populations (Francia et al., 2005; Özkaynak et al., 2014; Nadeem et al., 2018). Molecular markers are also more advantageous as they are cost effective, quick and help checking more plant material. Molecular markers linked to resistance genes against RKNs have been developed from pepper lines/cultivars (Djian-Caporalino et al., 2007; Wang et al., 2009; Fazari et al., 2012; Uncu et al., 2015; Çelik et al., 2016). However, there is limited information on the efficacy of markers in different pepper types carrying resistant genes. This study aimed to describe the efficiency of the previously published molecular markers closely linked to *N* and *Me1* genes in pepper genotypes.

Materials and Method

Nematode isolate

Meloidogyne incognita S6 isolate was used in this study. This isolate was identified in previous studies (Devran & Söğüt, 2009, 2011). The isolate has been cultured as a pure nematode population since 2008 in Devran's Laboratory (Devran et al., 2023). The study was conducted in laboratory and under controlled conditions at Akdeniz University, Faculty of Agriculture, Department of Plant Protection, Nematology Laboratory in 2022.

Nematode culturing

Reproduction of *M. incognita* S6 isolate was performed on susceptible pepper cultivar Safran F1 (Yüksel Seed, Türkiye). Pepper seedlings which had three or four true stages of leaves were planted into 250 cc plastic pot containing sterile soil. Approximately one week after the transplanting, egg masses of nematode were inoculated into plant roots 2-3 cm deep. Eight weeks after nematode inoculation, the plants were harvested, and the roots were washed carefully under tap water. Egg masses were counted under the light microscope and put into 1.5 ml centrifuge tubes to use inoculation.

Nematode inoculation

Egg masses of nematodes were put into petri and incubated at 27±2°C in an incubator to the hatching of second stage juveniles (J2s) (Hooper, 1986). Then, every day hatched J2s were taken to 15 ml tube and J2s were counted under the stereo microscope. Approximately one week after transplanting, plants were inoculated with 1000 J2s of *M. incognita* S6 isolate into the 2 cm depth (Devran & Söğüt, 2009; Mıstanoğlu, et al., 2016).

Plant material

Total 9 pepper genotypes belonging to Charleston and Bell pepper were used in this study. Both homozygous and heterozygous pepper cultivars for *Me1* and *N* genes and susceptible Safran F1 were used as control plants in the experiment (Table 1).

Table 1. Pepper plants used in this study

Plant Code	Pepper type/ cultivar
P1	Charleston pepper
P2	
P3	
P4	
P5	
P6	Bell pepper
P7	
P8	
P9	
P10	Resistant Control 1 (*N/n*)
P11	Resistant Control 2 (*Me1/me1)*
P12	Resistant Control 3 *(Me1/Me1)*
P13	Susceptible Control (Safran F1)

Safran F1: No *Me1* and *N* genes

Testing of pepper genotypes

The pepper seeds were germinated under 25±2°C controlled climate chamber in perlite and turf (1:1) mixture. Four true leaves seedlings were potted into 250 cc plastic pots filled with autoclaved (at 120°C, 2 h) sandy soil (75% sand, 15% silt, and 10% clay) after germination of seeds. When peppers have four true leaves, nematode inoculations were done. The study was conducted with 8 replicates in total plant (as 2 repeats with 4 replicates) according to the completely randomized design. Plants were grown in climate chamber at 25±1°C for 16:8 (light: dark) photoperiod with 60-65% relative humidity. Then plants were uprooted approximately sixty days after the inoculation, and plant roots were evaluated for the number of galls and egg masses and their indices (Hartman & Sasser, 1985).

Data analyses

Plant roots were evaluated according to parameters including the number of egg masses, number of galls, and root galling and egg masses index (Hartman & Sasser, 1985). Based on the index, it was represented as resistant between 0-2 while 3-5 as susceptible.

In order to clear observation of roots, each plant's roots were stained with 0.15 g/L Phloxine B solution (Hussey & Barker, 1973). Following this application, the egg masses were counted under the light microscope.

The statistical analysis of these parameters was done using the statistical package program SAS (v. 9.0). Significant differences in pepper genotypes were analyzed using ANOVA with multiple comparison test Tukey HSD at $p<0.05$.

DNA extraction

Total genomic DNA of pepper genotypes was isolated from young pepper leaves using Wizard Magnetic DNA Kit (Promega, Madison, WI, USA).

Molecular marker analyses

For *Me1* gene and *N* genes, molecular markers linked to these genes were used in this study (Table 2). PCR was performed according to references mentioned in Table 2 and previous study (Nas et al., 2023). PCR yields were run a agarose gel in buffer TAE, stained with Xpert Green DNA Stain (Grisp, Portugal), and visualized in a Gel iX Imager (Intas Science, Germany).

Table 2. Molecular markers linked to *Me1* and *N* genes used in this study

Gene	Marker name	Sequence (5′-3′)	Reference
Me1	SCAR_CD	GAAGCTTATGTGGTAMCC GCAAAGTAATTATATGCAAGAGT	Djian-Caporalino et al. (2007)
	SCAR_HM60	TATCCGTGGTCATCCTAGCC TGTGGTTCATCGGGACTGTA	Fazari et al. (2012)
	SCAR_PM54	CTGCAGGGTAGCAAAGTAATTATAT CCAAAATTAGTCATGTTCTTATGTTCTTAC	
	16880-1-V2	TGACCCCTCAGACTGAACAG CTCCTTCGCTGCTACCTTCT	Wang et al. (2018)
N	N-SCAR-315	AATTCAGAAAAAGACTTGGAAGG TAAAGGGATTCATTTTATGCATAC	Wang et al. (2009)
	CASSR37	ACATACCCAAAAACTCTCTCAC GATTGACCATGTTTCCGTAT	Çelik et al. (2016)

Results

Response of peppers to *Meloidogyne incognita*

In the pathogenicity test, two pepper types which are Charleston and Bell were used. Pathogenicity test data were evaluated based on quantified parameters, the number of gall and egg masses and gall and egg masses index in 1000 J2 inoculation, pepper genotypes showed different levels of resistance to *M. incognita* S6 isolate. As expected, resistant control genotypes which carried *Me1* and *N* genes showed the highest level of resistance to *M. incognita*. (Table 3).

All Charleston genotypes exhibited the highest resistance to *M. incognita* and were classified as resistant according to scale (Hartman & Sasser, 1985). The highest and lowest gall number of Charleston genotypes which are P5 and P1 was 3.87 and 1.75, respectively. The number of egg masses ranged between 0.75 and 2.50 on P1 and P4, respectively. Gall index values were found between 1.12 and 1.87 while egg masses index was 0.62 and 1.50 on P1 and P5, respectively (Table 3). The response of Charleston pepper genotypes to *M. incognita* S6 isolate resulted in significant differences in the variables; the number of galls, the number of egg masses, gall index and egg masses index ($p < 0.05$) (Table 3).

Table 3. The number of galls, egg masses and indices of Charleston and Bell pepper genotypes and their marker reactions

Plant Code	Gall	Egg masses	Gall index	Egg masses index	Pathological Reaction	Claimed phenotype[1]	SCAR_CD	SCAR_HM60	SCAR_PM54	166880-1-V2	NSCAR-315	CASSR37
P1	1.75±1.03 b*	0.75±1.03 b	1.12±0.64 bc	0.62±0.74 b	R	*Me1*	R	R	R	RR	S	S
P2	2.12±1.72 b	1.37±1.50 b	1.25±0.70 bc	0.87±0.99 b	R	*Me1*	R	R	R	RR	S	S
P3	2.00±1.06 b	1.75±1.28 b	1.25±0.46 bc	1.12±0.64 b	R	*Me1*	R	R	R	RR	S	S
P4	3.25±2.71 b	2.50±2.72 b	1.37±0.51 bc	1.12±0.83 b	R	*Me1*	R	R	R	RR	S	S
P5	3.87±1.45 b	2.50±1.30 b	1.87±0.35 b	1.50±0.53 b	R	*Me1*	R	R	R	RR	S	S
P6	3.37±3.11b	1.25±1.16 b	1.25±0.70 bc	0.75±0.70 b	R	*N*	S	S	S	S	RR	RR
P7	2.75±3.05 b	1.62±2.77 b	1.37±0.51 bc	0.75±0.88 b	R	*N*	S	S	S	S	RR	RR
P8	3.87±2.79 b	1.50±2.32 b	1.62±0.51 bc	0.75±0.70 b	R	*N*	S	S	S	S	Rr	Rr
P9	4.00±2.30 b	2.14±1.77 b	1.71±0.48 bc	1.28±0.75 b	R	*N*	S	S	S	S	RR	RR
P13	58.25± 3.15 a	61.75±3.10 a	4.00±0.0 a	4.00±0.0 a	S	*S*	S	S	S	S	S	S
P10	3.12±2.10 b	0.12±0.35 b	1.50±0.53 bc	0.12±0.35 a	R	*N*	S	S	S	S	Rr	Rr
P11	1.57±1.81 b	0.42±0.78 b	0.85±0.89 c	0.28±0.48 b	R	*Me1*	R	R	R	Rr	S	S
P12	1.87±1.55 b	1.25±1.66 b	1.12±0.64 bc	0.62±0.70 b	R	*Me1*	R	R	R	RR	S	S

*Means ± SD Different letters within a column show significant differences ($p<0.05$ by ANOVA) between genotypes analyzed by Tukey multiple comparison tests.

[1]Phenotype information was obtained from the company. R: Resistant, S: Susceptible, RR: Homozygote resistant, Rr: Heterozygote resistant.

Gall and Egg masses index were evaluated according to Hartman & Sasser (1985).

Bell pepper genotypes represented the highest resistance to *M. incognita* S6 isolate. All genotypes were found resistant according to gall and egg masses indices (0-5). The highest gall number was found on P9 with 4.0, while the lowest gall number was 2.75 on P7 bell pepper genotypes (Table 3). The number of egg masses ranged between 1.25 and 2.14 on P6 and P9, respectively. Gall index values were found between 1.25 and 1.71 while egg masses index was 0.75 and 1.28 on P6 and P9, respectively (Table 3). Bell pepper genotypes showed significant differences for *M. incognita* S6 isolate according to the number of galls, the number of egg masses, gall index and egg masses index ($p < 0.05$) (Table 3).

Molecular marker amplification

For *Me1* gene, SCAR_CD, SCAR_PM54, SCAR_HM60, and 16880-1-V2 molecular markers were used for screening of plants. Results showed that marker 16880-1-V2 was codominant, and the others were dominant. The presence of *Me1* gene in all Charleston pepper genotypes was determined successfully. Molecular markers were compatible with pathological tests (Table 3).

For *N* gene, NSCAR-315 and CASSR37FR markers which are codominant were used in bell pepper genotypes. Bell pepper genotypes were homozygous and heterozygous allele for *N* gene (Table 3). The presence of *N* gene in Bell pepper genotypes was determined successfully by molecular markers, NSCAR-315 and CASSR37FR. These markers were correctly determined resistant genotypes. Molecular markers were compatible with pathological tests of bell pepper genotypes (Table 3).

Discussion

Among the root-knot nematodes, *Meloidogyne incognita* is one of the most common species (Jones et al., 2013). Due to widespread geographical distribution of *M. incognita*, a significant reduction of yield and damage on pepper are observed in different locations of the world (Fullana et al., 2023). One of the main management strategies of RKN is validated as using resistant plant varieties especially in Solanaceous plants (Djian-Caporalino et al., 2007; Abdel-Mageed et al., 2023; Pradhan et al., 2023). Using resistance gene in plants helps growers to decrease economic losses in pepper production areas. In order to find resistant genes, plants need to screen both molecular and pathogenicity tests. In this study, the efficiency of the previously published molecular markers closely linked to *N* and *Me1* genes in pepper genotypes was described to test the response of peppers to *M. incognita*.

Charleston pepper genotypes bearing *Me1* gene were tested with *M. incognita*. Gall and egg masses indexes showed that all Charleston pepper genotypes used in this study were highly resistant. Similarly, pepper genotypes carrying *Me1* and *Me3* genes were tested with *M. incognita, M. javanica* and *M. arenaria* isolates and were found as resistant (Castagnone-Sereno et al., 1996, 2001). In another study conducted by Bucki et al. (2017), pathological response of accessions of pepper carrying the *Me1, Me3*, and *N* genes was found highly variable against *M. incognita* populations. Göze Özdemir & Uysal (2018) reported that the number of egg mass and gall reduced in pepper cultivars carrying *Me1* and *N* genes tested with *M. incognita*. Gürkan et al. (2018) reported the reactions of *M. incognita* race 1 against some pepper lines and varieties and all lines and varieties of pepper were found as susceptible. In this study, molecular markers SCAR_CD, SCAR_PM54, SCAR_HM60, and 16880-1-V2 were used for screening of *Me1* gene in Charleston peppers and the results of them were in accordance with pathology tests. SCAR_CD and SCAR_PM54 markers are dominant and SCAR_HM60 and 16880-1-V2 markers are codominant (Djian-Caporalino et al., 2007; Fazari et al., 2012; Wang et al., 2018). These markers help to find homozygous and heterozygous positions of gene in pepper genotypes. Pinar et al. (2016) used some molecular markers which are SCAR_CD and SCAR_PM54 to determine nematode resistance in diverse peppers genotypes and SCAR_PM54 were found fully successful in confirming both pathogenicity test and resistant genotypes. In the study dominant CAPS markers were used to screen the pepper to predict resistant and susceptible genotypes.

Bell pepper genotypes carrying *N* gene were tested with *M. incognita*. Gall and egg masses indexes showed that all Bell pepper genotypes were highly resistance. Thies et al. (1997) reported that pepper genotype of Carolina Ceyenne bearing *N* gene was resistance. In the other study De Souza- Sobrinho et al. (2002), it was determined the high resistance to *M. incognita* in backcrossed as seed parents of hot pepper cv. Carolina Cayenne and the sweet pepper cv. Agronômico-8. Wang et al. (2009) developed the molecular marker called as N-SCAR-315 linked *N* gene in pepper genotype using 320 F2 individuals obtained crossing of sweet pepper line (Carolina Wonder, *N* gene), and an inbred line '20080-5-29' (*C. annuum*). In this study, N-SCAR-315 marker was successful in the detection of *N* gene. In another study, the *N* gene linked to marker CASSR37FR were developed from F2 populations of crossed with resistant Carolina Wonder and susceptible pepper cultivar (Çelik et al., 2016). In the present study, molecular markers NSCAR-315 and CASSR37FR were in accordance with pathology tests in Bell peppers. These markers successfully predicted the homozygous and heterozygous in bell pepper genotypes.

In conclusion, pepper genetical variation was crucial in pepper breeding to nematode resistance. Our study emphasizes the molecular markers of *Me1* and *N* genes successfully determined the resistance of Charleston and Bell pepper genotypes. During molecular screening of pepper cultivars, we found all *Me1* gene markers produced positive results on all Charleston pepper genotypes, and *N* gene markers successfully gave products as expected size on Bell pepper genotypes. Both results pathogenicity and molecular markers were well matched. These marker sets can be used to determine the *Me1* and *N* genes in pepper breeding programs. However, further research is needed to better determine the efficacy of these markers. The markers should be screened in more pepper genotypes with different genetic backgrounds and types.

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