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Original article

Screening of snap and dry bean (*Phaseolus vulgaris* L.) genotypes for resistance to Bean common mosaic virus and Bean common mosaic necrosis virus

Taze ve kuru fasulye (*Phaseolus vulgaris* L.) genotiplerinin Bean common mosaic virus ve Bean common mosaic necrosis virus'a dayanıklılık durumlarının araştırılması

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ABSTRACT

The most effective control of Bean common mosaic virus (BCMV) and Bean common mosaic necrosis virus (BCMNV) is achieved by using the seeds of resistant cultivars. During conventional breeding, resistance against BCMV and BCMNV in common bean can be developed by pyramiding the strain-nonspecific dominant *I* gene with strain-specific recessive (*bc*-) resistance genes for long-term virus control. In this study, a total of 58 bean genotypes involving registered green and dry bean cultivars, local genotypes, and breeding lines were tested for the presence of known resistance genes. First of all, each genotype was inoculated with the NL-3 strain of BCMNV and the NL-4 strain of BCMV separately, and the plants were evaluated for the symptom appearance and tested by DAS-ELISA to confirm the presence or absence of the virus after three weeks of sap-inoculation. In the last part of the study, the resistance genes in bean genotypes were investigated by SCAR markers of SW-13 linked with the *I* gene and SBD-5 linked to *bc-1*^{2}. According to the phenotypic and molecular tests, out of 58 common bean genotypes tested, 37 involved the *I* gene, and seven and three genotypes contained *bc-2*^{2} and *bc-1*^{2} genes, respectively.

INTRODUCTION

Common bean (*Phaseolus vulgaris* L.) is an important leguminous crop in human consumption worldwide. Common bean crops can be affected by several viral agents, and Bean common mosaic virus (BCMV) and Bean common mosaic necrosis virus (BCMNV) are among the most important viral pathogens limiting common bean production. Both viruses belong to the *Potyvirus* genus in the *Potyviridae* family, which is the largest plant virus family (Kyle and Provvidenti 1993). BCMV and BCMNV are transmitted by aphids in a non-persistent manner, seed and pollen (Galvez and Morales 1989, Silbernagel et al. 2001). In susceptible cultivars, the seed transmission ratio may reach up to 39.7-54.4% (Morales and Castano 1987). Combining the use of healthy seeds and resistant cultivars

is the most effective management method for these viruses (Drijfhout 1978, Worrall et al. 2015). BCMV and BCMNV strains are classified into eight pathogroups (PG) based on interactions of resistance genes in differential bean cultivars with pathogenicity genes (Drijfhout et al. 1978, Feng et al. 2015).

Resistance to BCMV and BCMNV in common bean are conferred by the *I* gene (Ali 1950) and six recessive alleles (*bc-1, bc-1^2, bc-2, bc-2^2, bc-3*, and *bc-u*) distributed across four loci (Drijfhout 1978). Dominant *I* gene is widely used in breeding new bean cultivars, and it is associated with either immunity or systemic vascular necrosis in the infected common bean plants (Kelly 1997). Vascular necrosis occurs as a result of hypersensitive reaction of the *I* gene-bearing plant, which is also termed "black root syndrome" or "top necrosis," which may subsequently be followed by plant death, especially in situations without protection by recessive genes (Kelly 1997, Silbernagel et al. 2001). BCMV strains are classified as necrotic and non-necrotic strains according to reactions of the *I* gene-carrying bean cultivars. The necrotic strains usually produce vascular necrosis at higher temperatures (>30 °C) in the dominant *I* gene-carrying cultivars, and this condition is called temperature-dependent necrosis (TDN). However, recently, a strain that induces top necrosis in common bean cv. Jubila carrying *I+bc-1* (Arli-Sokmen et al. 2016, Feng et al. 2014) and cvs. Amanda and Isabella having *I+bc-1^2* (Arli-Sokmen et al. 2016) at lower temperatures were identified. The *I* gene-carrying plants normally show extreme resistance against non-necrotic strains of BCMV. These plants do not exhibit any symptoms, and no virus is recoverable from inoculated leaves at typical growing temperatures and higher temperatures (McKern et al. 1992). On the other hand, when the *I* gene-carrying plants are challenged with BCMNV, vascular top necrosis occurs regardless of temperature, called temperature-independent necrosis (TIN) (Kelly 1997, Worrall et al. 2015). The dominant *I* gene provides broad-spectrum resistance; namely, it gives resistance to BCMV and some other BCMV-related potyviruses such as Watermelon mosaic virus, Cowpea aphid borne mosaic virus, Passionfruit woodiness virus-K (Fisher and Kyle 1994). Apart from the *I* gene, several recessive *bc* genes (*bc-1, bc-1^2, bc-2, bc-2^2, bc-3*, and *bc-u*) have been shown to protect bean plants against both BCMV and BCMNV strains. The *bc-u* is the strain-nonspecific helper gene and is necessary if the *I* gene is absent and for the other recessive *bc* genes to express; the rest are strain-specific genes (Drijfhout 1978, Kelly 1997). The *bc-3* gene has been shown to be translation initiation factors, *eIF4E* and *eIF*(*iso*)*4E* (Naderpour et al. 2010).

The dominant *I* gene and recessive *bc*-genes have been used to obtain virus-resistant common bean cultivars in breeding programs. Although gene pyramiding studies mostly require intensive and challenging long-term efforts, common bean genotypes with *I+bc-3* or *I+bc-2^2* gene combinations, which are known to confer resistance to most of BCMV and BCMNV strains, have been used in breeding new common bean cultivars (Drijfhout 1994, Kelly 1997). For instance, common bean genotypes carrying the *I+bc-2^2* genes respond to BCMNV and necrotic strains of BCMV by giving local necrotic lesions or limited vein necrosis (Deligoz and Sokmen 2013, Kelly 1997).

Combining the dominant *I* gene with recessive genes offers long-lasting resistance since the two types of genes have different mechanisms (Tryphone et al. 2013). Molecular markers have been used in breeding studies to incorporate monogenic resistance genes during gene pyramiding strategy for more durable resistance. Virus resistance studies based on screening by a combination of phenotypic evaluations after biological test and marker-assisted selection (MAS) have been used in plant breeding to improve new common bean cultivars (Basavaraja et al. 2020, Kelly et al. 2003, Miklas et al. 2000, Mondo et al. 2019, Ruhimbana and Mutlu 2019). Molecular markers such as Sequence Characterized Amplified Region (SCAR), Simple Sequence Repeats (SSR), Random Amplified Polymorphic DNA (RAPD), and Amplified Fragment Length Polymorphism (AFLP) increase the efficiency of breeding programs. Gene-specific markers have been developed and used for the *I* gene on chromosome Pv02 (Bello et al. 2014, Haley et al. 1994, Melotto et al. 1996), *bc-3* on chromosome Pv06 (Johnson and Gepts 1994, Johnson et al. 1997, Miklas et al. 1996, Mukeshimana et al. 2005, Naderpour et al. 2010) and *bc-1^2* on chromosome Pv03 (Miklas et al. 2000, Myers et al. 1996).

BCMV was detected in common bean crops in Türkiye more than thirty-five years ago (Acikgoz 1984), and it is more prevalent than BCMNV (Arli Sokmen et al. 2016, Kilic et al. 2020). There are limited studies on screening bean genotypes for resistance genes to BCMV and BCMNV in Türkiye (Cetin et al. 2021, Deligoz et al. 2013, Deligoz et al. 2021, Palacioglu et al. 2020, Yeken et al. 2018). The majority of bean genotypes grown in Türkiye have been poorly characterized for the presence of virus resistance genes.

In this study, 58 bean genotypes including dry and snap bean cultivars, local populations, and breeding lines were tested to evaluate their responses to the NL-4 strain of BCMV and the NL-3 strain of BCMNV under controlled room conditions by using a mechanical sap-inoculation method and molecular markers linked to the resistance genes, *I* and *bc-1^2*.

MATERIALS AND METHODS

Bean seed materials

In the present study, 58 bean genotypes consisting of registered bean cultivars, local populations, and breeding lines were tested. The seeds of registered snap and dry bean cultivars were supplied from private companies and research institutes of the Ministry of Agriculture and Forestry of Türkiye. The seeds of local bean populations and breeding lines were obtained from the Black Sea Agricultural Research Institute, Samsun, Türkiye. Differential varieties Sutter Pink, Redlands Greenleaf B (*bc-1*^{2}), Monroe (*bc-2*^{2}), IVT-7214 (*bc-3*), Widusa (*I*), Amanda (*I, bc-1*^{2}), IVT-7233 (*I, bc-2*^{2}), BRB-195 (*I, bc-3*) were included in the study as a control. The seeds of resistant and susceptible bean controls were supplied by USDA-ARS (United States Department of Agriculture- Agricultural Research Service).

Virus inoculum

Seeds of common bean plants infected with the NL-4 strain [Pathogroup (PG)-VII] of BCMV or the NL-3 strain (PG-VI) of BCMNV, which has been maintained since our previous study (Deligoz and Arli Sokmen 2013) were used as an inoculum source. The seeds were germinated in a plastic tray with an organic substrate and transferred into plastic pots of 7 cm in diameter. Symptomatic seedlings were tested to confirm the presence of both viruses by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA), and the infected seedlings were used to inoculate ten days-old plants of susceptible bean cv. Sutter Pink for virus propagation and maintenance.

Phenotypic evaluations and serological testing

The seeds of each bean genotype to be tested were planted into plastic pot trays containing sterile compost and placed in a controlled growth room. The inoculums was prepared by grinding 1 g of leaf tissue from systemically infected plants in 10 ml phosphate buffer (1% K_2HPO_4, 0.1% Na_2SO_3, pH: 7.5) and used to inoculate Carborundum 400 mesh-dusted primary leaves of bean seedlings. At least five seedlings of each bean genotype were individually inoculated with NL-3 and NL-4 strains on different occasions to prevent strain contamination. One seedling of each genotype was also mock-inoculated as a control. Inoculated plants were kept at 25 °C (light) and 20 °C (dark) for 14 hr photoperiod. All bean genotypes tested were assessed periodically for virus symptoms and vigour up to 28 days after inoculation. Non-inoculated leaves of the plants were tested by DAS-ELISA to evaluate the presence of BCMV or BCMNV using a commercial kit (Bioreba, Switzerland) according to Clark and Adams (1977) and manufacturer's instructions. Samples were considered positive if absorbance readings (405 nm) were greater than two times those of healthy control plants using an ELISA microplate reader (Tecan Spectra II, Austria).

According to symptomatic reactions of plants challenged by NL-3 (PG VI) and NL-4 (PG VII) strains (Drijfhout et al. 1978, Kelly 1997), the presence of the dominant *I* gene and bc genes, except the *bc-3* gene, were predicted (Table 1). The plants with the obvious systemic symptom or no symptom but reacting positively in ELISA four weeks after inoculation were evaluated as susceptible, otherwise resistant.

Molecular evaluations

The presence of dominant *I* and recessive *bc-1*^{2} genes in common bean genotypes was screened by multiplex polymerase chain reaction (PCR) using SCAR markers SW-13 (Melotto et al. 1996) and SBD-5 (Miklas et al. 2000), respectively (Table 2).

Table 1. Evaluation of different common bean genotypes regarding to resistance genes after inoculation with NL-4 (BCMV) and NL-3 (BCMNV) strains (Drijfhout et al. 1978, Kelly 1997)

Genotype*	BCMNV/ NL-3	BCMV/NL-4
i	Susceptible – mosaic	Susceptible - mosaic
I	Susceptible – systemic necrosis	Resistant - no reaction
i+bc-1^2*+bc-u*	Susceptible – mild mosaic	Susceptible - mosaic
I+bc-1^2	Resistant- vein necrosis	Resistant - no reaction
i+bc-2^2*+ bc-u*	Resistant - no reaction	Susceptible – mosaic
I+ bc-2^2*+ bc-u*	Resistant- necrotic local lesion	Resistant - no reaction
i+bc-3+ bc-u	Resistant- no reaction	Resistant - no reaction
I+ bc-3	Resistant – no reaction	Resistant – no reaction

Table 2. SCAR markers used in this study

Marker	Gene	Primer Sequence (5'…3')	Size (bp)
SW-13	*I*	Forward: CACAGCGACATTAATTTTCCTTTC Reverse: CACAGCGACGAGGAGCTTATTA	690
SBD-5	*bc-1*^{2}	Forward: GTGCGGGAGAGGCCATCCATTGGTG Reverse: GTGCGGAGAGTTTCAGTGTTGACA	1300

Total genomic DNAs were extracted from bean leaves according to the protocol of DNeasy Plant Mini Kit (Qiagen, USA). The constituents of PCR reagents included 5 µl 5xPCR buffer, 2 µl 10 mM dNTPs, 0.25 µl (25 µM) each primer, 0.12 µl Taq DNA polymerase (5 u/µl), 5 µl 25 mM MgCl2 and 0.5 µl DNA (50 ng). The total reaction volume was completed to 25 µl with nuclease-free sterile water. Amplification conditions for SBD-5 and SW-13 primers were 2 min at 94 °C, 34 cycles of 10 s at 94 °C, 40 s at 66 °C, 2 min at 72 °C, and the reaction was completed with one cycle of 5 min at 72 °C (Strausbaugh et al. 2003). PCR products were visualized and photographed under the GelDoc XR system (Biorad) after running in 1% agarose gel prepared with TBE buffer at 80 mA for 90 min.

RESULTS AND DISCUSSION

Reactions of bean genotypes

Seventeen dry bean cultivars, 12 dry bean local genotypes, 16 dry bean breeding lines, and 13 green (snap) bean cultivars were analyzed for resistance to BCMV and BCMNV (Table 3). Reactions of different bean genotypes against to the NL-4 strain of BCMV and the NL-3 strain of BCMNV are recorded periodically after inoculation until the fourth week. Some bean genotypes started to show symptoms in 3-4 days on inoculated leaves and 12-16 days on non-inoculated trifoliate leaves after inoculation, depending on virus strain and bean genotypes. Systemic mosaic was the only symptom in susceptible plants after inoculation with the NL-4 strain (Figure 1). In contrast, two types of symptoms occurred in susceptible plants inoculated with NL-3; one is necrotic lesions and vein necrosis which is spread out to other parts of the plant resulting in premature death within 3-5 days (Figure 2), and the other is systemic mosaic (Figure 3). These reactions had consistency with the results of our previous studies (Deligoz and Sokmen 2013, Deligoz et al. 2021).

Figure 1. Mosaic symptom on trifoliate leaf of dry bean breeding line "TB 773" inoculated with Bean common mosaic virus NL-4 strain

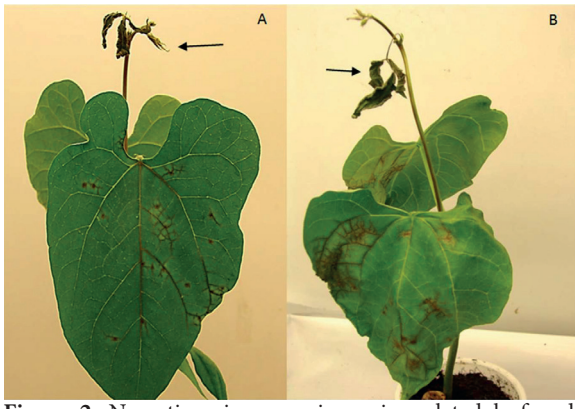

Figure 2. Necrotic vein necrosis on inoculated leaf and systemic vascular necrosis in dry bean cv."Yunus-90" (A) and breeding line "TB- 543" (B) challenged with NL-3 strain of Bean common mosaic necrosis virus. Arrow shows top necrosis

Figure 3. Mosaic symptom on trifoliate leaf of dry bean local genotype "Beyaz Bodur 25" inoculated with Bean common mosaic necrosis virus NL-3 strain

Out of the 58 dry bean cultivars tested, 36 of them showed systemic necrosis to the NL-3 strain of BCMNV, and they were found to be susceptible (Table 3). Most of the genotypes died within a couple of days after inoculation with the NL-3 strain of BCMNV due to systemic top-necrosis. However, top-necrosis progressed slowly in some bean genotypes like Noyanbey, Aras-98, Önceler-98, and Yakutiye, especially in some replicates of these cultivars that relatively stayed alive for a longer time. These genotypes did not show any visible symptoms either on inoculated or non-inoculated leaves after inoculation with NL-4 and were found to be resistant to NL-4 strain. The resistance studies showed that a total of 36 bean genotypes carried the *I* gene. Similarly, NL-3 strain caused systemic necrosis in genotypes with the unprotected *I* gene, while NL-4 strain did not infect these genotypes as obtained similarly in the studies by Kelly (1997) and Strausbaugh et al. (2003).

Table 3. Resistance genes in dry bean genotypes determined by a combination of phenotypic scoring, ELISA and molecular markers

	Phenotypic evaluation						Molecular evaluation		Proposed
	NL-3			NL-4					
	Symptom	ELISA	Reaction	Symptom	ELISA	Reaction	*I*	*bc-1²*	Genotype
Dry Bean Cultivars									
Akman 98	SN	*	S	AS	N	R	+	-	*I*
Aras 98	SN	P	S	AS	N	R	+	+	*I*
Elkoca-05	M	P	S	M	P	S	-	+	*i*
Eskişehir 855	M	P	S	M	P	S	-	+	*i*
Göynük	SN	*	S	AS	N	R	+	+	*I*
Güngör	AS	N	R	M	P	S	-	+	*i+bc-2²*
Kantar-05	AS	P	S	MM	P	S	-	+	*i+bc-1²*
Karacaşehir 90	SN	*	S	AS	N	R	-	-	*I*
Nihatbey	M	P	S	M	P	S	-	+	*i*
Noyanbey 98	SN	P	S	AS	N	R*	+	+	*I*
Önceler 98	SN	P	S	AS	N	R	+	+	*I*
Şahin 90	M	P	S	M	P	S	-	+	*i*
Şehirali 90	SN	*	S	AS	N	R	-	-	*I*
Şeker	M	P	S	M	P	S	-	+	*i*
Terzibaba	AS	P	S	MM	P	S	-	+	*i+bc-1²*
Yakutiye 98	SN	P	S	AS	N	R	+	+	*I*
Yunus 90	SN	*	S	AS	N	R	+	+	*I*
Dry Bean Local Genotypes									
Arda Şeker	SN	P	S	AS	N	R	+	+	*I*
İspir Şeker	R	N	R	M	P	S	-	+	*i+bc-2²*
Kelkit Şeker	SN	*	S	AS	N	R	+	+	*I*
Kara Yaprak	SN	P	S	AS	N	R	+	+	*I*
Ladik Şekeri	M	P	S	M	P	S	-	+	*i*
Beyaz Bodur 20	AS	N	R	M	P	S	-	+	*i+bc-2²*
Beyaz Bodur 24	M	P	S	M		S	-	+	*i*
Beyaz Bodur 25	AS	N	R	M	P	S	-	+	*i+bc-2²*
Beyaz Bodur 28	SN	N	S	AS	N	R	+	+	*I*
Beyaz Bodur 78	NL,VN,SN	P	S	AS, M	P/N	V	+	+	*I*
Beyaz Bodur 82	SN	N	S	AS	N	R	+	-	*I*
Beyaz Bodur 84	M	P	S	M	P	S	-	+	*i*
Dry Bean Breeding Lines									
TB 543	SN	*	S	AS	N	R	+	+	*I*
TB 773	AS	N	R	M	P	S	-	+	*i+bc-2²*
TB 542	SN	*	S	AS	N	R	+	+	*I*
TB 112	SN	*	S	AS	N	R	+	+	*I*
TB 198	SN	*	S	AS	N	R	+	+	*I*
TB 138	SN	*	S	AS	N	R	+	+	*I*
TB 146	SN	*	S	AS	N	R	+	+	*I*
TB 277	SN	*	S	AS	N	R	+	+	*I*
TB 280	SN	*	S	AS	N	R	+	+	*I*
TB 125	SN	*	S	AS	N	R	+	+	*I*
TB 174	SN	*	S	AS	N	R	+	+	*I*
Cranberry	SN	*	S	AS	N	R	-	+	*I*
Arjantin Şeker	SN	*	S	AS	N	R	+	+	*I*
Pinto 1	SN	*	S	AS	N	R	+	+	*I*
Pinto 2	SN	*	S	AS	N	R	+	+	*I*
CB686	SN	*	S	AS	N	R	+	+	*I*
Snap Bean Cultivars									
Nadide	SN	*	S	AS	N	R	+	+	*I*
Sofia	SN	*	S	AS	N	R	-	+	*I*
Gina	SN	P	S	AS	N	R	+	+	*I*
Öz Ayşe	M	P	S	M	P	S	-	+	*i*
Limka	SN	*	S	AS	N	R	-	+	*I*
Ferasetsiz	AS	P	S	AS	P	S	-	+	*i+bc-1²*
Helda	SN	*	S	AS	N	R	-	+	*I*
Volare	SN	*	S	AS	N	R	+	+	*I*
Yalova 5	AS	N	R	M	P	S	-	+	*i+bc-2²*
Yalova 17	AS	N	R	M	P	S	-	+	*i+bc-2²*
Kara Ayşe	M	P	S	M	P	S	-	+	*i*
Y-39 Şekerpare	M	P	S	M	P	S	-	+	*i*
Magnum	SN	*	S	AS	N	R	+	+	*I*
Control Varieties									
Sutter Pink	M	P	S	M	P	S	*	*	*i*
RGB	MM	P	S	MM	P	S	*	*	*i+bc-1²*
Monroe	AS	N	R	M	P	S	*	*	*i+bc-2²*
IVT 7214	AS	N	R	AS	N	R	*	*	*bc-2+bc-3*
Widusa	SN	*	S	AS	N	R	*	*	*I*
Amanda	VN	N	R	AS	N	R	+	+	*I+bc-1²*
IVT 7233	NL	N	R	AS	N	R	*	*	*I+bc1²+bc-2²*
BRB-195	AS	N	R	AS	N	R	*	*	*I+bc-3*

E: ELISA, R: Resistant, S: Susceptible, P: positive, N: negative, AS: Asymptomatic, M: Mosaic, MM: Mild mosaic, SN: systemic necrosis, VN: Vein necrosis, Mo: Mosaic, V: variable (reactions ranged from susceptible to resistant), +: presence of the marker, -: absence of marker, *: No data

Interestingly, the other genotype Beyaz Bodur 78 appeared to have phenotypically two different situations. Out of five plants, two died due to top necrosis; the rest showed necrotic lesions and limited vein necrosis when tested with the NL-3 strain of BCMNV. On the other hand, when Beyaz Bodur 78 was tested with NL-4 strain, two plants occurred to have mosaic and positivity in ELISA for BCMV. The rest was negative and did not show any symptoms. These results indicated that Beyaz Bodur 78 seeds had genetic heterogeneity and some plants of this genotype have the dominant *I* gene (Table 3). On the other hand, when five dry bean genotypes (Güngör, İspir Şekeri, Beyaz Bodur 20, Beyaz Bodur 25, TB-773) and two snap bean genotypes (Yalova-5 and Yalova-17) were challenged with NL-3 strain, none of them showed any symptom, and they gave negative result against BCMNV antiserum in DAS-ELISA (Table 3). These genotypes showed systemic mosaic symptoms after inoculation with NL-4 and positive result in ELISA for BCMV. These seven genotypes were evaluated to be resistant to NL-3 strain but susceptible to NL-4, similar to the control variety Monroe (Table 3). These results indicated that seven common bean genotypes are more likely to carry *bc-2²*, as shown by the studies of Drijfhout et al. (1978) and Kelly (1997) (Table 1). In our previous studies, one dry bean cultivar (Deligoz et al. 2013) and five breeding lines (Deligoz et al. 2021) were also found to be resistant to NL-3 but susceptible to NL-4.

Two dry bean cultivars (Kantar-05 and Terzibaba) did not show any visible symptoms either on inoculated or non-inoculated leaves after inoculation with NL-3, but their ELISA results were positive. Mild mosaic symptom after inoculation with NL-4 and positive ELISA results indicated that these cultivars might contain the *bc-1²* gene (Table 3). Interestingly, snap bean cultivar "Ferasetsiz" did not show any symptoms after verified by inoculation with both virus strains, but ELISA showed the presence of BCMV and BCMNV in non-inoculated leaves. These results showed that Ferasetsiz also might carry the *bc-1²* gene (Table 3). Kelly (1997) reported that varieties possessing the *bc-1²* gene may exhibit mild mosaic symptoms to NL-3 and NL4 and show delayed development of the NL-3 strain of BCMNV, with mild mosaic symptoms appearing within four to six weeks after inoculation. The absence of virus symptoms in these cultivars after inoculation with the NL-3 strain could be related to the early symptom assessment time of the fourth week after inoculation in the current study. Eleven common bean genotypes reacted positively to NL-3 and NL-4 strains in phenotypic and serological tests. They are verified as susceptible to both BCMV and BCMNV (Table 3).

According to the result of phenotypic tests, out of 58 bean genotypes tested, 37 involved the *I* gene, and seven and three genotypes contain *bc-2²* and *bc-1²* genes, respectively (Table 3). However, none of the tested bean genotypes was found to carry the *bc-3* gene. In the phenotypic evaluations completed in Türkiye so far, any common bean genotype identified to be resistant against both NL-3 and NL-4 strains are not present. However, Palacioglu et al. (2020) recently identified *bc-3* gene in three snap bean cultivars (4F-89 Fransız, 40 Günlük ve Karabacak) by using ROC-11 and eIFE4 markers.

Analysis with SCAR markers

Screening of 58 common bean genotypes for resistance to BCMV and BCMNV was conducted using SCAR markers tightly linked to the genes of resistance to these viruses. The dominant *I* gene and *bc-1²* genes were analyzed with SCAR markers, SBD-5, and SW-13, respectively. Total DNAs were extracted from all bean genotypes tested, and the presence of the resistance gene was investigated by multiplex RT-PCR. Amanda (*I+bc-1²*) was used as a control. Out of the 58 bean genotypes tested, 31 gave the expected product of 690 bp with SW-13 marker, which is linked to the *I* gene, while 25 genotypes gave the only a 1300 bp product specific for SBD-5 marker known to be linked to the *bc-1²* (Tablo 3, Figure 4). On the other hand, both the dominant *I* gene and *bc-1²*-specific products were determined in 29 genotypes, as similar to Amanda control (Figure 4) whereas none of the gene-specific products was obtained in two common bean genotypes (Şehirali 90 and Karacaşehir 90).

The results of phenotypic evaluation indicated that 37 common

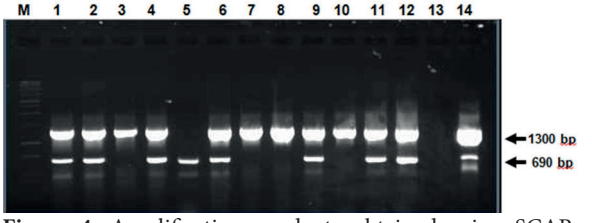

Figure 4. Amplification products obtained using SCAR markers of SW-13 and SBD-5 linked with the dominant *I* and *bc-1²* genes, respectively. M: 1 kb Ladder (Promega), 1: Göynük, 2: Aras 98, 3: Erzurum Şeker, 4: Arda Şeker, 5: Akman, 6: Önceler 98, 7: Terzibaba, 8: Şahin 98, 9: Volare, 10: Erkoca, 11: Yakutiye, 12: Amerikan Kara Yaprak, 13: Negative control, 14: Positive control (Amanda: *I+ bc-1²*)

bean genotypes had the dominant *I* gene, whereas molecular marker-based tests revealed that 31 out 37 genotypes had (84%). Deligoz et al. (2021) had similar observation with the SW-13 marker, and the success rate in identifying the *I* gene was 87% (133 out of 153) when compared to the phenotypic test (153). Palacioglu et al. (2020) investigated resistance genes in 39 common bean cultivars using DNA markers (SW-13, SBD-5, ROC11, eIFE4) and identified the *I* and *bc-1²*-related sequences in most of the cultivars, and the *b-3* gene in three cultivars. Similar to our findings, they revealed

that cvs Gina, Magnum, Önceler, Karacaşehir 90, Göynük 98, Yakutiye 98, and Aras 98 involved the *I* gene and *bc-1²* genes. However, the same SCAR marker (SW-13) failed to identify the *I* gene in common bean cv. Sofia in their study, while both phenotypic test and SW-13 marker detected the *I* gene in the present study. On the other hand, when Yeken et al. (2018) evaluated common bean cvs. Gina, Göynük, Aras 98, Akman 98, Önceler 98, and Yakutiye 98 with SW-13 marker to determine the effectiveness of it in these cultivars, they found that SW-13 marker worked well as similar to the results of the current study. However, in cvs. Güngör, Elkoca 05, Kantar 05, and Karacaşehir 90, the *I* gene was not molecularly detected by SW-13 marker in both studies. In the present study, phenotypic tests based on symptoms indicated that Karacasehir 90 might have the *I* gene (Table 3), as it was molecularly detected in this dry bean cultivar by Palacioglu et al. (2020). Other controversial results belong to snap bean cv. Helda and dry bean cv. Terzibaba. In the current study, screening resistance genes with phenotypic observations and molecular marker (SW-13) suggested that the *I* gene was not present in cv. Terzibaba, but present in cv. Helda. When Yeken et al. (2018) tested these cvs. with the same marker, they identified the *I* gene in Terzibaba, but did not in Helda. These conflicting results could be due to the low efficiency of the SW-13 marker in detecting the *I* gene indicating limitations of the use of marker. Alternatively, an SNP marker used for MAS of the *I* gene (Bello et al. 2014) could be tested for these genotypes in the future.

Phenotypic test results revealed that only three genotypes involved the *bc-1²* gene, whereas the SBD-5 marker resulted in *bc-1²* in 44 genotypes in the present study (Table 3). Yeken et al. (2018) and Palacioglu et al. (2020) reported that the SBD-5 marker gave high positive results through a polymerase chain reaction. Previous studies also reported that the results of SBD-5 marker deviated from phenotypic observations significantly (Deligoz et al. 2021, Miklas et al. 2000, Pasev et al. 2014, Strausbaugh et al. 2003). This could be attributed to the factors involving the genetic background of genotype. Therefore, phenotypic tests are necessary to confirm the results of molecular analysis.

Their rapid dispersal by aphid species and a high percentage of seed transmission make the control of BCMV and BCMNV difficult. The use of resistant plants is known to be the most economical and efficient way of virus control. In this study, 58 common bean genotypes were screened by phenotypic evaluations and molecular markers. More than half the genotypes (37) tested were found to carry the dominant I gene, while seven and three genotypes were likely to have *bc-2²* and *bc-1²*, respectively. Resistant cultivars are recommended to be grown in common bean areas where BCMV and BCMNV are problematic. Also, these resistant genotypes could be used in plant breeding as a parental source. The SBD-5 marker gave inconsistent results in some common bean genotypes to determine *bc-1²* in the present study. The use of this marker in selecting resistant plants during breeding studies seems to be not suitable. Although the SW-13 marker was found to be reasonably accurate in identifying the *I* gene, testing plants for observable traits by biological methods is recommended.

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ÖZET

Fasulyede Bean common mosaic virus (BCMV) ve Bean common mosaic necrosis virus (BCMNV)'a karşı mücadelede en etkili yol dayanıklı çeşit kullanmaktır. Fasulye ıslah çalışmalarında, dominant *I* geni ve ırka-spesifik resesif genlerin (*bc*-) kombine edilmesi ile fasulyede BCMV ve BCMNV'ye karşı uzun süreli dayanıklılık sağlanabilmektedir. Bu çalışmada kuru ve taze fasulye çeşitlerini, yerel genotipleri ve ıslah hatlarını içeren toplam 58 fasulye genotipi, BCMV ve BCMNV'ye karşı test edilmiş ve genotiplerin sahip oldukları dayanıklılık genleri araştırılmıştır. Öncelikle her bir genotip, BCMV'nin NL-4 ve BCMNV'nin NL-3 ırkı ile ayrı ayrı inokule edilmiş; inokulasyondan üç hafta sonra genotipler, ortaya çıkan simptomlara ve DAS-ELISA sonuçlarına göre değerlendirilmiştir. Çalışmanın son bölümünde; fasulye genotiplerinin içerdiği dayanıklılık genleri, *I* genine spesifik SCAR markör SW-13 ve *bc-1²* geni ile ilişkili SCAR markör SBD-5 kullanılarak araştırılmıştır. Fenotipik ve moleküler test sonuçlarına göre test edilen 58 genotipin 37'sinin *I* geni, yedi tanesinin *bc-2²* geni ve üç tanesinin ise bc-12 genine sahip olduğu ortaya konulmuştur.

Anahtar kelimeler: ELISA, mekanik inokulasyon, moleküler markör, *I* geni, *bc* genleri

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Original article

Research on the efficacy of three application techniques of entomopathogenic nematodes against the Colorado potato beetle [*Leptinotarsa decemlineata* (Say), (Coleoptera: Chrysomelidae)] under greenhouse conditions

Entomopatojen nematodların üç uygulama tekniği ile patates böceği [*Leptinotarsa decemlineata* (Say), (Coleoptera: Chrysomelidae)] üzerinde etkilerinin sera koşullarında araştırılması

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ABSTRACT

Colorado Potato Beetle [*Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae)] (CPB) is one of the most destructive pests of potatoes. CPB is a polyphagous pest that damages every stage of the potato. Although entomopathogenic nematodes (EPNs) in the world have been demonstrated by many laboratories and field/garden studies activity against many harmful groups, very few studies have been conducted on CPB in our country (Türkiye). In the scope of work intended to be used EPNs against CPB. The main objective of the study is to reveal greenhouse-pot applications (soil, green limbs, and cadaver applications) of EPN [*Steinernema feltiae* (isolate 09-31) and *Heterorhabditis bacteriophora* (isolate 09-43)], which are present in our laboratories in our stocks. For the first time in Türkiye, greenhouse-pot applications studies have been carried out against this harmful group. There is also a lot of work in the world about the use of aqueous concentrations of EPNs against harmful effects. In recent years, efforts have been made to use EPNs in cadavers instead of aqueous concentrates. This application (cadaver applications) against CPB is the first working. The results revealed that EPNs performed better in soil applications, and the highest mortality rate was obtained from *S. feltiae* (65.23±4.45 and 77.33±2.59). Other applications (green limbs and cadaver applications) are seen to have a low level of efficacy. In trials, the mortality rate in cadaver applications did not exceed 40%, and the lowest mortality rate in *H.bacteriophora* was 37,40±8,88%. In the case of green devices, the mortality rate did not exceed 30%, and the highest mortality rate was 29.14±6.09 in *H.bacteriophora*. According to EPN greenhouse-pot experiments results, soil applications of *S. feltiae* (isolate 09-31) should be included within the scope of field trials.

INTRODUCTION

The potato (*Solanum tuberosum* L.) is the most important field crop both in Türkiye and the world. However, under current field conditions, potatoes are under attack by a large number of insect pests including aphids, beetles, leafhoppers, and lepidopterous pests. One such pest that negatively impacts potato production is the Colorado Potato Beetle (CPB) [*Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae)]. CPB is a polyphagous pest especially of potatoes that can damage all stages of potato development.

Pests such as the CPB are responsible for the important losses observed during potato production. *L. decemlineata* is a polyphagous pest that damages all above-ground organs of every stage of potato plants. The first and second larval stages consume the leaf epidermis of the plant whereas the third and fourth larval stages feed on the leaf. Importantly, the last instar transitions to a pupa libera after passing into the soil, where it overwinters as an adult.

Adult and all larval stages of CPB feed on the green portions of plants. Especially, last instar larvae cause lots of damage in the field. In the absence of pest control, up to 100% of the crop can be damaged. Additionally, CPB acts as a vector for bacterial ring rot, which can cause significant loss depending on climate conditions (Christie et al. 1991).

Chemical control of the CPB has led to high levels of resistance to insecticides (Forgash 1985, Mota-Sanchez et al. 2000, Stewart et al. 1997) and the contamination of groundwater with aldicarb, carbofuran, and oxamyl (Leach et al. 1986). Moreover, each year the control of CPB becomes more difficult because of the pest's cross-persistence against insecticides (Whalon et al. 2011). For that reason, alternative methods for the chemical control of CPB are required, and a number of investigations are being conducted worldwide. These investigations focused on plant extracts (Hough-Goldstein 1990, Scott et al. 2003, 2004) and biological control agents such as natural enemies including parasitoids, predators, and entomopathogens (Cantwell et al. 1985, Gelernter 1990, Grissel 1981, Lashomb et al. 1987, Obrycki et al. 1987, Özdemir et al. 2021, Toba et al. 1983, Welch 1958, Welch and Briand 1961, Zehnder and Gelernter 1989).

Entomopathogenic nematodes (EPNs) offer a good alternative to chemical insecticides for CPB control (Toba et al. 1983, Welch 1958, Welch and Briand 1961). EPNs, *Heterorhabditis* spp. and *Steinernema* spp., are soil-inhabiting parasites of insects. These nematodes carry symbiotic bacteria in the genera *Photorhabdus* and *Xenorhabdus* spp. (Fam: Morganellaceae) in their intestine; these bacteria help nematodes kill their host within 48 h by producing a copious set of virulence factors in host hemocoel. For years, these organisms have been mass-produced and used effectively as biological control agents against harmful pests, especially against larval stages, in pest management programs (Koppenhöfer et al. 2020, Shapiro-Ilan et al. 2020). This study determined the effects of EPNs [*Steinernema feltiae* (isolate 09-31) and *Heterorhabditis bacteriophora* (isolate 09-43)], which showed promising results in previous laboratory studies (Kepenekci et al. 2013a, Kepenekci et al. 2016), against different larval stages of the CPB (against 3rd larval stage in above-ground applications and last stage larvae in soil applications) in the greenhouse (pot) trials and soil, cadaver and above-ground applications. Many studies have previously assessed the effects of aqueous suspensions of EPNs against pests in the world (Yüksel et al. 2022). Recently, some studies have also applied EPNs in cadavers instead of aqueous concentrations (Shapiro-Ilan et al. 2003). Results from this study as well as information available in the literature will promote the use of EPNs against CPB, and reduce the use of chemical pesticides with negative effects on the environment. EPNs will serve as a sustainable and reliable method of *L. decemlineata* control.

MATERIALS AND METHODS

Nematode and insect sources

Native Turkish EPNs, *Steinernema feltiae* (isolate 09-31) and *Heterorhabditis bacteriophora* (isolate 09-43) were isolated from vegetable gardens and peach orchards in Aydın, Türkiye. Both nematodes were supplied by Prof. Dr. Selçuk Hazır (Adnan Menderes University, Aydın, Türkiye). The nematodes were cultured on the last instar larvae of the wax moth, *Galleria mellonella* (Linnaeus) (Lepidoptera: Pyralidae) at room temperature (23-24 °C) using methods described by Kaya and Stock (1997). The harvested infective juveniles (IJs) were used within two weeks after emergence in the experiments. The Colorado potato beetle (CPB) was also reared on potato plants. Because studies using populations obtained from the field (natural conditions) provide more practical results as compared to studies using laboratory-reared populations which are more sensitive. In this study, CPB populations were collected from the infested fields in potato cultivated areas and transferred to potato plants in a 1.5 decares area for field studies. CPB populations collected from the field were kept at 10±1 °C until used in greenhouse-pot studies.

Greenhouse-pot activity studies

Pot trial studies were carried out in the Tokat Gaziosmanpaşa University greenhouse. The plants used were all of the similar sizes in all applications. EPN was applied to the soil and the soil surface area (cm^2) was measured. Soils used in the pot experiment were collected from the potato field, sterilized, and then moistened with water. One potato tuber was planted in each before to use to eliminate dead or live larvae, pupae, and adults. EPNs were applied at a concentration of 25 infective juveniles (IJs) cm^{-2} to the soil. Tap water without nematodes was applied to the plants in the control group. After the applications, the pots were covered with gauze to prevent insects from escaping. Each treatment had 10 pots with one potato plant and the experiment was conducted twice. The 3rd larval stage was used in above-ground applications and the last stage larvae in soil applications. The third larval stage is the most destructive. The fourth stage only stays in the green parts for 2-3 days depending on the climate and nutrition and then descends to the ground.

Above-ground application

EPNs have sprayed at 25 IJs cm^{-2} concentration on the above-ground organs of the plants including the 3rd larval stage (10 insects in each pot). Before this, we made sure that nematodes could be passed through the sieve in the application tools and equipment by conducting a preliminary study. For this purpose, nematode suspensions were sprayed into a Petri dish and the contents were examined under a stereomicroscope. Only water was applied in the controls.

Soil applications

EPNs have applied at 25 IJs cm^{-2} concentration to the soil as in the greenhouse pot experiments. EPN applications were applied on the soil surface as the last stage larvae of the CPB migrate to the soil to pupate and may encounter with IJs in the treated soil. To obtain a concentration of 25 IJs cm^{-2} in pots with a ground surface radius of 8.5 cm, 5.670 IJs in 20 ml of water were applied to each pot (plant or soil surface applications). Only water was applied in the controls.

Cadaver applications

First *G. mellonella* last instar larvae were infected with 500 IJs in 9 cm Petri dishes (5 larvae per Petri dish) and incubated for 6 days for *Steinernema* spp., and 10 days for *Heterorhabditis* spp. Two of these *G. mellonella* larvae infected with EPNs were placed at a depth of 2-3 cm near each potato plant to each pot. Only water was applied in the controls (no cadaver).

Statistical analysis

In the laboratory, greenhouse, and field experiments data were calculated by Abbott formula (Abbott 1925). SPSS Packet Programme was used for data analysis with ANOVA and Duncan Multiple Comparison was used to determine differences in the means among the treatments.

RESULTS AND DISCUSSION

In the first repeat, the highest mortality rate was observed in the treatments with *S. feltiae* and *H. bacteriophora* (65.23 ±4.45% and 41.01±8.05%, respectively) for the soil application. This was followed by cadaver applications with *H. bacteriophora*. The effect of *S. feltiae* cadaver and above-ground application was found to be low [F (2.17) 20.18 P<0.05] (Table 1). In the 2nd repetition of the greenhouse trials, the highest mortality rate was obtained in *S. feltiae* soil application (77.33±2.59%). [F (2.17) 29.52 P<0.05]. The mortality rate in cadaver applications did not exceed 40% with the highest mortality rate of 37.40±8.88% exhibited by *H. bacteriophora*. In the above-ground applications, the mortality rate did not exceed 30% and the highest mortality rate was recorded as 29.14±6.09% in *H. bacteriophora* (Table 1).

Table 1. Mortality of *Leptinotarsa decemlineata* of soil, cadaver, and above-ground applications of *Steinernema feltiae* (isolate 09-31) and *Heterorhabditis bacteriophora* (isolate 09-43) isolates and controls over 10 days from treatment

Application	Mortality±SEM*(%)	
	First repeat	Second repeat
Steinernema feltiae Soil application	65.23 ±4.45 a**	77.33±2.59 a
Heterorhabditis bacteriophora Soil application	40.01±8.53 ab	37.81±5.47 b
S. feltiae Cadaver application	10.78±4.00 c	7.22±3.45 c
H. bacteriophora Cadaver application	37.40±8.88 b	28.14±4.24 b
Control (just water in soil no nematodes and no cadaver)	0.00±0.00 d	0.00±0.00 d
S. feltiae Above-ground application	2.11±1.41 d	6.43±3.15 c
H. bacteriophora Above-ground application	21.84±5.09 b	29.14±6.09 b
Control (just water above-ground application and no nematodes)	0.00±0.00 d	0.00±0.00 d

* SEM: Standard error of the mean;

** Means in a line followed by the same letter are not statistically significantly different (ANOVA P<0.05, Tukey's test).

This study assessed the performance of EPNs [*Steinernema feltiae* (isolate 09-31) and *Heterorhabditis bacteriophora* (isolate 09-43)] which were previously reported as effective in the laboratory studies (Kepenekci et al. 2013a, Kepenekci et al. 2016) against different stages of the CPB (*L. decemlineata*) (3rd larval stage in above-ground applications and last stage larvae in soil applications) in greenhouse conditions. Many studies have applied aqueous suspensions of EPNs against pests in the world. Recently some studies utilized EPN-infected cadavers instead of aqueous suspensions (Shapiro-Ilan et al. 2003). This is also the first report of the use of this approach against CPB under greenhouse (pots) conditions in the Türkiye (soil, cadaver, and above-ground applications).

Until mid-2011, no studies have been conducted on the use of EPNs in the control of CPB in Türkiye (Kepenekci 2012, Kepenekci 2014). Kepenekci et al. (2013a) conducted a preliminary study investigating the potential use EPNs, *Steinernema feltiae*, *S. carpocapsae*, and *Heterorhabditis bacteriophora*, detected in Türkiye against the last-stage larvae of CPB. Trials were conducted under laboratory conditions using two different EPN concentrations (1000 and 2000 IJs insect-1) at 25 °C. The authors observed that *H. bacteriophora* (97.63±6.99) showed the highest effect at 2000 IJs concentration, followed by *S. feltiae* (86.05±11.72%). *S. carpocapsae* was the least effective with 53.34±1.34%. Results obtained indicated that more detailed laboratory studies should be conducted especially under natural conditions. A detailed laboratory study carried out by Kepenekci et al. (2016) with *S. carpocapsae*, *H. bacteriophora*, and *S. feltiae* revealed that these nematodes were 52, 83, and 99% effective, respectively. In the same order, these nematodes applied as infected cadavers were 72, 80, and 99% effective. In another study, Kepenekci et al. (2018) investigate the efficacy of two isolates of *S. carpocapsae* isolates (GOP72 and GOP81) against CPB using infected cadavers trial, different substrates (filter paper and soil) with a single dose and different doses. Experiments were set up at different temperatures, concentrations, and time intervals, to determine the most suitable temperature, density, and time interval in the fight against CPB. In cadaver application, both isolates exhibited more than 60% mortality at 25 °C which was the most suitable temperature. In dose-response trials, the most effective dose for *S. carpocapsae* GOP81 was 500 IJs application, and for *S. carpocapsae* GOP72 1000 IJs application. After the 7th day in a single-dose experiment with filter paper, the highest mortality rate was caused by *S. carpocapsae* GOP72 isolate. At the end of the 10th day, 100% mortality was detected in both isolates. In the filter paper trials, at the end of the 7th day, insect mortality was 100% at the doses. The effects of *S. carpocapsae* GOP81 isolate in the dose measurement with soil was dose-dependent increasing until the end of the 10th day. The authors stated that it was necessary to switch to greenhouse pot trials and then investigate the effectiveness of these EPNs under natural conditions.

The efficacy of the nematode species [*S. carpocapsae* (Black Sea isolate), *S. feltiae* (Aydın isolate- isolate 09-31), and *H. bacteriophora* (Aydın isolate- isolate 09-43)] used in the study have been evaluated against other insect groups in Türkiye. In a study conducted against the forest pest, *Dendroctonus micans* (Kugelann), (Coleoptera: Scolytidae), *S. feltiae* (Aydin isolate- isolate 09-31), and *H. bacteriophora* (Aydin isolate- isolate 09-43) at 1000 IJ ml^{-1} concentration were 98.04% and 94.04% effective at 25 °C, respectively. In the same study, *S. carpocapsae* (Black Sea isolate), however, was found to be ineffective as mortality caused did not exceed 40% (Kepenekci and Atay 2014). Atay et al. (2015) demonstrated that *S. carpocapsae* was more effective (89%) than *S. feltiae* and *H. bacteriophora* at different temperatures against *Acanthoscelides obtectus* (Say.) (Coleoptera: Bruchidae), which is an important storage pest. In this case, *S. feltiae* was considered to be ineffective with mortality not exceeding 60%. Likewise, in another study using these nematodes at 1000 IJ concentration against *Phthorimaea operculella* (Zeller) (Lepidoptera: Gelechiidae), *S. carpocapsae* caused 96% whereas *H. bacteriophora* presented with 80% mortality at 25 °C; *S. feltiae* was ineffective with mortality less than 40% (Kepenekci et al. 2013b). The study by Tülek et al. (2015) determined that *S. carpocapsae* caused the highest mortality rate (54%) of *Rhyzopertha dominica* (F.) (Coleoptera: Bostrichidae). They reported that *S. feltiae* and *H. bacteriophora* caused 28% and 12% larval mortality, respectively. Atay and Kepenekci (2015) demonstrated the effectiveness of *S. feltiae*, *S. carpocapsae*, and *H. bacteriophora* against *Holotrichapion pullum* (Gyllenhal) (Coleoptera, Apionidae), an important clover pest, under laboratory conditions. EPNs were applied at 3 concentrations (500, 1000, and 5000 IJs ml-1) and the setup was incubated at two different temperatures (15, 20 °C). *S. carpocapsae* had the highest mortality rates (80%, 83%, 82%) at all concentrations at 20 °C, followed by *S. feltiae* (30%, 41%, 35%), and *H. bacteriophora* (24%, 27%, 30%).

Armer et al (2004) reported that *H. marelata* was an effective biological control agent against CPB under laboratory and field experiments but stated that it was not commercially viable due to costs. Trdan et al. (2009) tested *S. feltiae*, *S. carpocapsae*, *H. bacteriophora*, and *H. megidis* against CPB larvae and adults at 15, 20, and 25 °C and at doses of 200, 1000, and 2000 IJs under laboratory conditions. All nematodes were found effective on CPB at 20, and 25 °C. Adel and Hussein (2010) assessed the effects of *S. feltiae*

and *H. bacteriophora* against CPB and found that *H. bacteriophora* was more effective against the 4th stage larvae compared to *S. feltiae*. Kary et al. (2010), evaluated 4 different geographic isolates of *H. bacteriophora* and 3 different species of *Steinernema* (*S. bicornutum*, *S. carpocapsae* and *S. feltiae*) against CPB using 5 different concentrations and 3 different methods. The highest mortality was caused by *H. bacteriophora*. Laznik et al. (2010) tested a Slovenian strain of *S. feltiae*, commercial product Entonem (*S. feltiae*), and insecticide thiamethoxam against CPB under field conditions. At the end of the experiment both EPNs were found to be effective biological agents, especially in the larval stage of CPB. In our study, *S. feltiae* was more effective against CPB than *H. bacteriophora*.

In our study, the nematodes had little effect on CPB when applied to aboveground parts of plants. *S. feltiae* was 2.11-6.63% effective, whereas was *H. bacteriophora* 21.84%-29.14% effective. The main reasons for the low success of EPNs in such applications are that nematode IJs are sensitive to drying (Lello et al. 1996), high temperatures (Grewal et al. 1994), and ultraviolet radiation (UV) (Gaugler et al. 1992, Gaugler and Boush 1978). Most EPNs are ineffective at temperatures exceeding 32 °C. Tolerance to drying and UV also differs from different EPN species. According to Lello et al. (1996), the effect of sunlight can be minimized by applying nematodes at dusk. Although adjuvants generally increase nematode activity (Bauer et al. 1997, Broadbent and Olthof 1995, Eidt 1991, Glazer et al. 1992, MacVean et al. 1982), this level of increase is generally considered insufficient to be recommended green component applications (Bauer et al. 1997, Mason et al. 1998). However, other avenues to optimize the use of EPNs for more service in the biocontrol of such insect pests (Abd-Elgawad 2019) and exploit available opportunities for their effective applications (Askary and Abd-Elgawad 2021) should be sought.

According to the results, the highest mortality rate was obtained in *S. feltiae* (65.23 ±4.45% and 77.33±2.59%) when EPNs were applied to the soil. It was seen that the effect was lower than in other applications (above-ground and cadaver applications). According to the results of the EPN greenhouse-pot test, it would be appropriate to conduct field trials with *S. feltiae* (isolate 09-31).

Many studies have used aqueous concentrations of EPNs against harmful insects in the world. In recent years, efforts to use EPN-infected cadavers instead of aqueous concentrate are increased. This study was also used against CPB in the greenhouse (pots) studies different three applications (soil, cadaver, and above-ground applications) using EPNs [*Steinernema feltiae* (isolate 09-31) and *Heterorhabditis bacteriophora* (isolate 09-43)] for the first time in the world. According to EPN greenhouse-pot experiments results, field trials should be conducted with *S. feltiae*. EPNs can serve as a sustainable and reliable method of *L. decemlineata* control.

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ÖZET

Patates üretimini azaltan zararlı organizmalardan biri de patates böceği [*Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae)] (PB)'dir. Patates bitkisinin her dönemine zarar veren polifag bir zararlıdır. Dünya'da entomopatojen nematodların (EPN) birçok zararlı grubuna karşı etkinliği laboratuvar ve tarla/bahçe çalışmaları ile ortaya konulmuş olmasına karşın ülkemizde PB ile ilgili çok az çalışma yapılmıştır. Yapılan bu çalışma kapsamında PB ile mücadelede EPN'lerin kullanılması amaçlanmıştır. Çalışmanın ana hedefi, laboratuvar stoklarımızda mevcut olan EPN [*Steinernema feltiae* (izolat 09-31) and *Heterorhabditis bacteriophora* (izolat 09-43)]'lerin toprak, yeşil aksam ve kadavra uygulamaları şeklinde sera-saksı denemeleri oluşturmuştur. Türkiye'de ilk defa bu zararlı grubuna karşı sera-saksı çalışmaları yürütülmüştür. Ayrıca dünyada EPN'lerin sulu konsantrasyonlarının zararlılara karşı kullanımı ile ilgili çok çalışma bulunmaktadır. Son yıllarda sulu konsantrasyonların yerine kadavra içinde EPN'lerin kullanılması çalışmaları başlamıştır. PB'ye karşı bu uygulama (kadavra uygulamaları) ilk çalışma niteliğindedir. Araştırma sonucu elde edilen sonuçlara göre EPN'lerin toprağa yapılan uygulamalarda en yüksek ölüm oranı *S. feltiae* (65.23±4.45 ve 77.33±2.59)'da elde edilmiştir. Diğer uygulamalar (yeşil aksam ve kadavra uygulamaları)'da etkisinin düşük düzeyde olduğu görülmektedir. Deneme sonuçlarında, kadavra uygulamalarında ölüm oranı %40'ı geçmemiş ve en yüksek ölüm oranı *H. bacteriophora*'da %37.40±8,88 olarak tespit edilmiştir. Yeşil aksam uygulamalarında ise ölüm oranı %30'u geçmemiş ve en yük ölüm oranı *H. bacteriophora*'da 29.14±6.09 olarak tespit edilmiştir. EPN sera-saksı deneme sonuçlarına göre *S. feltiae* (izolat 09-31)'nın toprak uygulamalarının arazi denemeleri kapsamına alınması uygun olacaktır.

Anahtar kelimeler: entomopatojen nematodlar, patates böceği, *Leptinotarsa decemlineata*, biyolojik mücadele.

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Original article

Investigation of aedeagus and spermatheca ultrastructure of *Cryptocephalus turcicus* Suffrian, 1847 (Coleoptera: Chrysomelidae: Cryptocephalinae) from Türkiye by using SEM

Türkiye'den *Cryptocephalus turcicus* Suffrian, 1847'nin (Coleoptera: Chrysomelidae: Cryptocephalinae) aedeagus ve spermatheca ince yapısının SEM kullanılarak araştırılması

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ABSTRACT

This paper presents ultrastructures of aedeagus and spermatheca observed for the first time by SEM of a plant pest (Coleoptera: Chrysomelidae: Cryptocephalinae) *Cryptocephalus turcicus* Suffrian, 1847 from Türkiye. In the present study, the morphologies of male and female genitalia of this species belonging to the nominative subgenus *Cryptocephalus* (s.str.) collected from Ankara, Kastamonu and Samsun provinces are examined using Scanning Electron Microscopy (SEM). To date, there has not been a study in Türkiye in this context in which male genitalia (aedeagus) and female genitalia (spermatheca) morphologies of the *Cryptocephalus turcicus* Suffrian, 1847 have been studied together. The genus *Cryptocephalus* is represented by 73 species in Türkiye. Detailed investigations of aedeagus "and spermatheca are very important to obtain new diagnostic characters in the genus *Cryptocephalus*. For this reason, ultrastructural and detailed investigations of aedeagus and spermatheca of *Cryptocephalus turcicus* Suffrian, 1847 from Türkiye were firstly studied with SEM and stereo microscope to obtain new diagnostic characters in the genus *Cryptocephalus*. The new diagnostic characters obtained by examining the ultrastructure of aedeagus and spermatheca belonging to the species will be used in the distinction of species that are especially similar to each other, by determining the differences and important characters among the species in the genus *Cryptocephalus*. Photos in SEM, as well as photos in the stereo microscope, are also given in the text.

INTRODUCTION

Chrysomelids constitute an important group of herbivores in the order Coleoptera (Jolivet 1988). Many species are monophagic and oligophagic with respect to host plants (Strauss 1988). Adults feed on flowers and leaves, while larvae feed on roots and leaves (Matsuda 1988). Adults and larvae of some species are therefore important pests of trees and shrubs (Mirzoeva 2001).

One of the biggest and most numerous subfamilies of the Chrysomelidae is the Cryptocephalinae. One of the most

common tribes of the Cryptocephalini, Cryptocephalus Geoffroy, 1762, has a variety of species throughout the Palearctic. One of the most widespread members of the genus is Cryptocephalus (Schöller 2008). Fauna Cryptocephalinae Türkiye is represented by 107 taxa of the species group (92 species and 15 subspecies) (Lopatin and Dovgailo 2002, Özdikmen and Cihan 2014, Warchalowski 2003). Leaf beetles are nearly all phytophagous, and their success in ecosystems is determined by their ability to occupy many different feeding niches (Jolivet 1988), and by their host specificity to almost all groups of plants (Riley et al. 2002). Most adults feed on living plant parts, such as leaves, polen, flowers, young stems or fruit (Flowers 1996, Jolivet and Verma 2008, Riley et al. 2002, Staines 2002, White 1968, Wilcox 1972). Larvae are found on the surface of leaves or as leaf diggers; others feed on droppings, roots or submerged parts of plants (Jolivet and Verma 2008, Riley et al. 2002, Staines 2002, White 1968). Some larvae feed on eggs and ant waste in their nests (Chamorro Lacayo 2014). Thus, beetles are an important group in the ecosystem as primary consumers, in direct competition with other herbivores (González-Megías and Gómez 2003), and an important in the food web (Basset and Samuelson 1996). *Cryptocephalus turcicus* is generally harmful to *Crataegus monogyna*, *Quercus petraea*, *Quercus rolar* and *Quercus ilex* plants (Gök and Çilbirlioğlu 2005, Selmi 1982).

Cryptocephalus turcicus Suffrian, 1847 is a member of the subgenus *Cryptocephalus* (s.str.) Geoffroy, 1762. This subgenus is well distinguished from other subgenera of *Cryptocephalus* Geoffroy, 1762 by the combination of following characters: Epipleuron of elytra inclined, in side view visible in whole length. Anterior tibiae not explosively broadened and smoothed, elytra without long, erect hairs, occasionally shortly pubescent (Warchałowski 2003). *Cryptocephalus turcicus Suffrian*, 1847 is in the *Cryptocephalus flavipes* species-group.

MATERIALS AND METHODS

The available specimens (a total of 15 specimens) for the present work were collected from Ankara, Kastamonu and Samsun provinces in Türkiye in 1991, 1997 and 2004. The specimens were deposited at Gazi University (Türkiye, Ankara). The aedeagus and spermatheca were dissected from the abdomen, and the remaining tissue was removed with fine tweezers. After cleaning, the samples were placed in 70% ethanol for microscopic examination and examined using an Olympus SZX7 stereo microscope and a Leica Z-16 APO stereo microscope.

For Scanning Electron Microscopy (SEM), the cleaned samples were dehydrated using an increasing series of ethanol (70%-100%) and then dried in air. Afterwards, the samples were mounted on SEM stubs with double-sided adhesive tape, gold-plated using Polaron SC 502 Sputter Coater in Gazi University Prof. Dr. Zekiye Suludere Electron Microscope Center. It was investigated by JEOL JSM 6060 SEM at 10 kV.

RESULTS AND DISCUSSION

Cryptocephalus turcicus Suffrian 1847

Cryptocephalus turcicus Suffrian, 1847 is a Turano-Mediterranean (Turano-Apenninian) species (Figure 1). It is distributed in Europe (Albania, Bosnia and Herzegovina, Bulgaria, Croatia, France, Germany, Italy, Macedonia, Romania, Slovenia, Türkiye, Serbia and Montenegro and Asia (Iranian, Jordan, Syria, Türkiye) (Borowiec and Sekerka 2010, Warchalowski 2010).

Figure 1. The species is widely distributed in Türkiye. It has been recorded from 17 provinces in all Turkish regions. It is reported from Ankara, Antalya, Balıkesir, Bilecik, Bursa, Çankırı, Düzce, Eskişehir, Isparta, İstanbul, İzmir, Kastamonu, Karabük, Samsun, Trabzon, Yalova and Zonguldak provinces in Türkiye (Ekiz et al. 2013, Özdikmen and Kaya 2014).

Materyal examined: Ankara Prov: Kızılcahamam, Soğuksu National Park, 1500m, 21.VI.1991,1 ex; Kızılcahamam, Soğuksu National Park, 21.VI.1991, 1700m, 1 ex; Kızılcahamam, Yukarıçanlı,14.VI.1997, 1540m, 1 ex. Kızılcahamam, Güvem, 26.VI.1997, 1000m, 1 ex; Kastamonu Prov: İnebolu road, 17.V.2004, 3 exs; Doğanyurt 18.VI.2004, 950m, 2 exs; Dadaş driveway, 19.VI.2004,1010m, 1 ex; Samsun Prov: Alaçam, Kapaklık village, 16.IV.2004, 620 m,4 exs. Alaçam, Dürtmen hill skirts, 16.VI.2004, 1460m, 1ex.

Aedeagus and spermatheca of *Cryptocephalus turcicus* were studied with SEM and stereo microscope. Obtaining observations on ultrastructural and detailed morphologies of them are presented as follows:

Aedeagus

In stereo microscope (Figures 1A-1B):

The median lobe is brown.

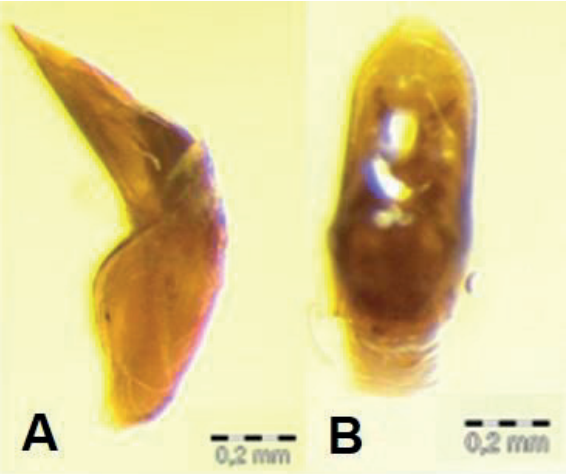

Figure 1. Aedeagus of *Cryptocephalus turcicus* Suffrian, 1847, A. Lateral view, B. Dorsal view

In lateral view, the median lobe gradually narrows slightly from the base to the apical portion. The median lobe is curved at an angle of 120 degrees in the 1/3 part starting from the base. The apex of the median lobe is flat and pointed.

In dorsal view, the median lobe moves parallel to the apex in the 2/3 part while the apex gradually narrows in the 1/3 part. The median lobe is quite narrow and elongated at the apex. The apex part is round in appearance. The median lobe in lateral parts and in the fore part thickened. Thickening in lateral part is distinctly smaller than in the fore parts.

In ventral view, the median lobe moves parallel to the apex in the 2/3 part while the apex gradually narrows in the 1/3 part. The median lobe is quite narrow and elongated at the apex. The apex part is round in appearance.

In SEM (Scanning Electron Microscope) (Figures. 2A-2C, 3A-3C):

SEM images are similar to images from a stereo microscope in general. Only the different characters in the SEM image are shown below. Median lobe especially in the anterior half with scattered, irregular and sparsely ultrastructural pits and sensillae. There are quite a lot of pits and sensillae, especially in the lateral parts and ventral part in the terminal area of the median lobe. Operculum of median lobe without ultrastructural pits and sensillae in dorsal view. The apex of the median lobe gradually narrowed, additionally prolonged.

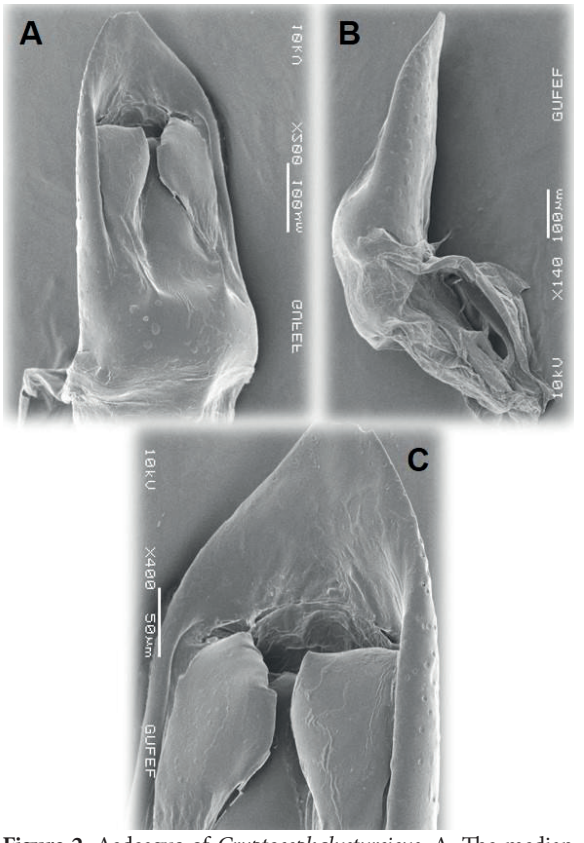

Figure 2. Aedeagus of *Cryptocephalusturcicus*, A. The median tube in dorso-lateral view (SEM), B. The median tube in lateral view (SEM), C. Apical part of the median tube dorsal view (SEM)

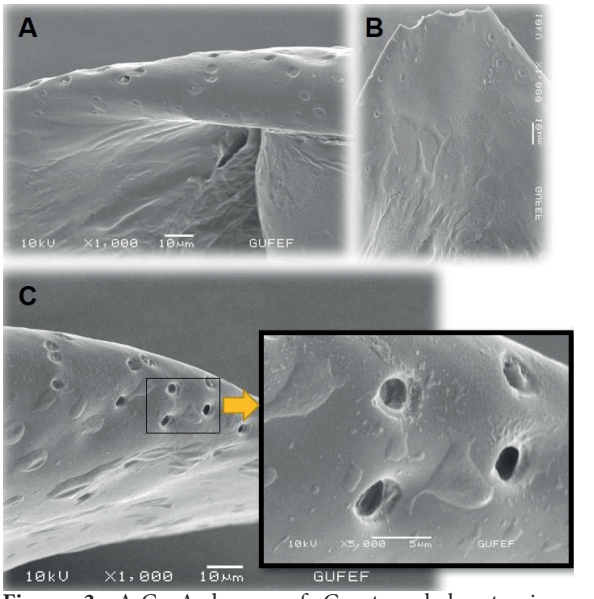

Figure 3. A-C. Aedeagus of Cryptocephalus turcicus, Sensilla on median part of median tube in lateral view (SEM)

Spermatheca

In stereo microscope (Figure 4):

Spermatheca are generally yellow. The general appearance of spermatheca is similar to neutrophil from white blood cells in the blood. The vasculum consists of gnarled partitions. Cornu Ç-shaped. Cornu gradually narrowed towards to apex and the apex of cornu round. Nodulus and cornu are circular in shape and nodulus has a larger structure than cornu. The nodulus is more bulging than the Cornu, and there is a narrowing towards the apex. Ampulla (Collum + Ramus) is not clearly visible. Therefore, spermathecal duct and spermathecal gland outlets cannot be clearly seen in the stereo microscope image.

Figure 4. Spermatheca of *Cryptocephalus turcicus* Suffrian, 1847 in lateral view

In SEM (Scanning Electron Microscope) (Figures 5A-5C, 6A-6D)

SEM images are similar to images from a stereo microscope in general. Only the different characters in the SEM image are shown below. The exit points of the spermathecal gland and spermathecal duct, which cannot be distinguished in stereo microscope pictures, are quite evident in the SEM microscope. The thick one is the spermathecal duct, which is directly connected to the bottom, and the thinner spermathecal gland structure is connected from the side. Nodulus, cornu, proximal tube of ductus spermatheca with scattered, irregular and sparsely ultrastructural pits. The number of pits increases from the cornu to the nodulus.

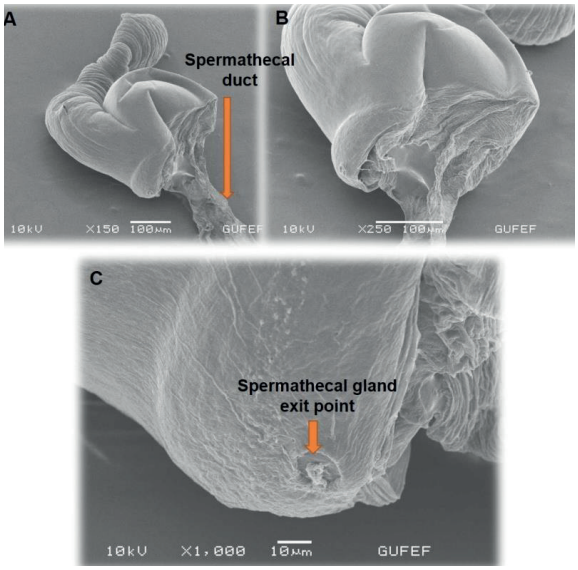

Figure 5. *Cryptocephalus turcicus* Suffrian, 1847, A. General view of spermatheca (SEM), B. Nodulus part (Basal part of spermatheca) of spermatheca (SEM), C. Spermathecal gland exit point of Spermatheca (SEM)

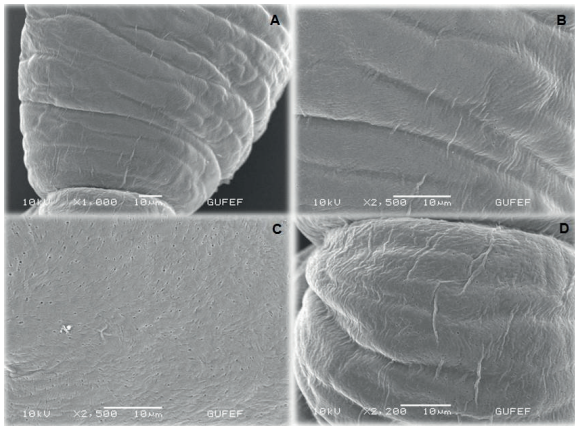

Figure 6. Spermatheca of *Cryptocephalus turcicus* Suffrian, 1847, A- B. Pits on Cornuin lateral view (SEM), C-D. Pits on nodulus in lateral view (SEM)

External morphological features are not sufficient in determining the species diversity in Türkiye and separating the problematic species groups. As a result of the study, the male and female genitals of *Cryptocephalus turcicus* species were identified and sampled using both stereo and Scanning electron microscopy and included in the study. By looking at the genital pictures, it is seen that the species has a very different structure from the other species in the *Cryptocephalus* nominative genus in which it is included. This suggests to us that a taxonomic category arrangement can be made again within this group. The importance of male and female genitalia in this distinction is great. Examining the ultrastructural structure of male and female genitalia will facilitate the identification of the species, the possibility of comparing the male and female genital structures of the species and the taxonomic categories of the species.

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ÖZET

Makale, Türkiye'den bir bitki zararlısının (Coleoptera: Chrysomelidae: Cryptocephalinae) *Cryptocephalus turcicus* Suffrian, 1847'de aedeagus ve spermatecanın SEM tarafından ilk kez gözlemlenen ultrastrüktürlerini sunmaktadır. Bu çalışmada, *Cryptocephalus* (s.str.) nominatif alt cinsine ait bu türün Ankara, Kastamonu ve Samsun illerinden toplanan türün erkek ve dişi üreme organlarının morfolojileri Taramalı Elektron Mikroskobu (Scanning Electron Microscopy-SEM) kullanılarak incelenmiştir. Bugüne kadar Türkiye'de *Cryptocephalus turcicus* Suffrian, 1847'nin erkek genital (aedeagus) ve dişi genital (spermatheca) morfolojilerinin birlikte çalışıldığı bu kapsamda bir çalışmaya rastlanmamıştır. *Cryptocephalus* cinsi Türkiye'de 73 tür ile temsil edilmektedir. Aedeagus ve spermatecanın detaylı araştırılması *Cryptocephalus* cinsinde yeni tanısal karakterler elde etmek için çok önemlidir. Bu nedenle, *Cryptocephalus turcicus* Suffrian, 1847'nin Türkiye'den aedeagus ve spermatecalarının ultrastrüktürel ve detaylı incelemeleri ilk olarak *Cryptocephalus* cinsinde yeni tanısal karakterler elde etmek için SEM ve stereo mikroskop ile incelenmiştir. Türlere ait aedeagus ve spermatecanın ultrastrüktürünün incelenmesiyle elde edilen yeni tanısal karakterler, *Cryptocephalus* cinsindeki türler arasındaki farklar ve önemli karakterler belirlenerek özellikle birbirine benzeyen türlerin ayrımında kullanılacaktır. SEM'deki fotoğraflar ve stereo mikroskoptaki fotoğraflar da metinde verilmiştir.

Anahtar kelimeler: *Cryptocephalus turcicus*, aedeagus, spermatheca, SEM, Türkiye

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Original article

Management of disease complex of *Meloidogyne incognita* and *Fusarium oxysporum* f.sp. *radicis lycopersici* on tomato using some essential oils

Domateste *Meloidogyne incognita* ve *Fusarium oxysporum* f.sp. *radicis lycopersici* hastalık kompleksinin bazı esansiyel yağlar kullanılarak yönetimi

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ABSTRACT

The effects of commercial thyme (*Origanum vulgare* L.), sage (*Salvia officinalis* L.), garlic (*Allium sativum* L.), sesame (*Sesame indicum* L.), rosemary (*Rosmarinus officinalis* L., syn. *Salvia rosmarinus* Spenn.), lemon (*Citrus limon* (L.) Osbeck) and mustard (*Brassica nigra* L.) essential oils (Botalife Natural and Aromatic Products Inc., Türkiye) on disease severity were investigated in simultaneous inoculation of *Meloidogyne incognita* (Kofoid and White, 1919) Chitwood, 1949 and *Fusarium oxysporum* f.sp. *radicis lycopersici* (Jarvis & Shoemaker) (FORL) on tomato. Nematicide (Velum®, Fluopyram, Bayer Crop Production Inc., Türkiye) and fungicide (Cebir®, Fludioxonil + Metalaxyl, Hektaş Crop Production Inc., Türkiye) were used as positive controls. The negative control was only plants with nematode and fungus inoculation. The study was set up in a randomized plot design with 5 replications for each essential oil. In simultaneous inoculations, 1000 *M. incognita* second juvenile larvae/1ml and $3x10^6$ spore/ml FORL were used for each seedling. The essential oil applications were applied to the soil at a dose of 1000 ppm for each pot, one day after the nematode and fungus inoculation. The study was terminated after 60 days, and the evaluation was based on gall, egg mass, and disease severity. Fungal growth and nematode development on roots were found lower in all tested oils applications than in negative control but fungicidal and nematicidal activity varied. Thyme and garlic essential oils had the highest control effect on nematode and fungus with 55.20% in simultaneous inoculation and this effect was higher than only nematicide (38.84%) and only fungicide (33.20%) applications. Sage (38.84%), rosemary (33.28%), and mustard (38.92%) essential oils were found to suppress disease severity higher than sesame (22.16%) and lemon (22.16%). It has been determined that thyme and garlic essential oils are good alternatives to manage root-knot nematode and FORL disease complexes.

INTRODUCTION

Fusarium oxysporum f.sp. *radicis lycopersici* (Jarvis & Shoemaker) (FORL), which causes tomato root rot, is an important pathogen species that causes more than 60% yield loss in the open field and greenhouse tomato production

(Arıcı et al. 2013, Hibar et al. 2007, Manzo et al. 2016, Ozbay et al. 2004). In Turkey, this pathogen was first detected by Can et al. (2004) and it causes significant yield losses in tomato-growing regions (Çolak and Biçici 2013). While it causes stunting and yellowing in tomato seedlings, root rot, wilting and death occur in plants in later periods. Rot in the root area and necrosis in the stem vascular bundles is around 15-30 cm from the soil surface at most (Singh et al. 2022). Although 105 species of root knot nematodes have been reported so far, the most common species in vegetable growing areas in the world and Turkey are *Meloidogyne incognita* (Kofoid and White, 1919) Chitwood, 1949 (Tylenchida: Meloidogynidae) *Meloidogyne javanica* (Treub, 1885) Chitwood, 1949, *Meloidogyne arenaria* (Neal, 1889) Chitwood, 1949 and *Meloidogyne hapla* Chitwood, 1949 (Adam et al. 2007, Ghaderi and Karssen 2020, Gürkan et al. 2019, Maleita et al. 2021, Uysal et al. 2017). Root-knot nematodes feed on roots and vascular tissues, cause cancer formation, disrupt water and nutrient flow, and show symptoms such as slow growth, yellowing of leaves, wilting, and early plant death in infected plants (Asaturova et al. 2022). *Meloidogyne incognita* and Fusarium disease complexes cause serious losses in horticultural and vegetable crops worldwide (Abuzar and Haseeb 2006, Patil et al. 2021).

In many studies of nematode-fungal disease interactions, pathogens are observed earlier in plants in the presence of nematodes, disease severity increases, and the plant dies completely (Göze Özdemir et al. 2022a, Lobna et al. 2017). Wilt and root rot diseases caused by Fusarium species mostly use resistant cultivars as well as cultivation methods and chemical control programs (Aydın 2019, Bilici et al. 2021). However, cultural methods are insufficient due to the saprophytic viability and the limited development of resistant varieties (Çolak and Biçici 2011, Jiménez-Díaz et al. 2015). In some studies, root-knot nematodes were found to break the plant's resistance even in resistant cultivars developed against Fusarium wilt (Colak-Ates et al. 2018, Göze Özdemir et al. 2022a, Lobna et al. 2016). The general approach to the control of disease complexes formed by root-knot nematodes and fungi is the use of nematicides. However, nematicides are not very effective against both pathogens (Giacometti et al. 2010, Nicolopoulou-Stamati et al. 2016). In addition, the high cost of nematicides, resistance development, health, and environmental hazards, residue, negative effects on soil fauna and beneficial microflora, and phytotoxic effects on plants are the factors limiting their use (Haydock et al. 2013, Silva et al. 2019). A possible alternative to nematicides in the control of nematode-fungal disease complexes is the use of biological agents and plant essential oils (Dutta and Thakur 2017, Türktaş and Koral 2018). Some researchers report that plant essential oils have a broad spectrum activity in controlling bacterial and fungal diseases in many vegetables (Arici et al. 2011, 2013, Bajpai et al. 2009, Shuping and Eloff 2017, Sivakumar and Bautista-Banos 2014). Fungal growth disorders are caused by changes in the structure of fungi associated with the interaction of essential oils on the enzymes responsible for cell wall synthesis (Krzyśko-Łupicka et al. 2020). It has been demonstrated by some studies that isothiocyanates, glucosides, alkaloids, ketones, aldehydes, phenolics, and fatty acids in plants show nematicidal activity (Chitwood 2002, Göze Özdemir et al. 2021, 2022b, Kabera et al. 2014, Stavropoulou et al. 2021). These essential oil components can affect the nematode nervous system as well as disrupt the cell membrane of the nematode and change its permeability (Echeverrigaray et al. 2010, Oka et al. 2000).

The management of disease complexes is more difficult than you suppose. The most effective management is to control one of the interacting organisms and apply appropriate methods to prevent the formation of the disease complex (Abuzar 2012). The effectiveness of essential oils on fungi and nematodes was evaluated separately by researchers and it was found that many of them could be used as an alternative to chemicals. Therefore, it is important to study the effect of essential oils in controlling *M. incognita* and FORL disease complex. This study aims to investigate the effect of thyme (*Origanum vulgare* L.), sage (*Salvia officinalis* L.), garlic (*Allium sativum* L.), sesame (*Sesame indicum* L.) , rosemary (*Rosmarinus officinalis* L., syn. *Salvia rosmarinus* Spenn.), lemon (*Citrus limon* (L.) Osbeck) and mustard (*Brassica nigra* L.) essential oils in the treatment of *M. incognita* and FORL disease complex in tomato.

MATERIALS AND METHODS

Material

The FORL isolate used in this study was isolated from a tomato plant in Antalya / Serik district, and its diagnosis was made according to Gerlach and Nirenberg (1982) and Davis and Raid (2002) (Göze Özdemir et al. 2022a). *Meloidogyne incognita* isolate DR17 was used (Uysal et al. 2017) and whose mass production continues under climate room conditions (24 ± 1 °C, 60% ± 5% humidity). The study was carried out on a tomato cultivar Gulizar F1 which is known to be susceptible to root knot nematode and FORL (Göze Özdemir et al. 2022). Essential oils are commercially available from Botalife Natural and Aromatic Products Inc. (Isparta, Türkiye). Nematicide (Velum®, Fluopyram) and fungicide (Cebir®, Fludioxonil + Metalaxyl) used as positive control were produced by Bayer and Hektaş Crop Protection Inc., respectively.

Method

Preparation of fungal inoculum

FORL isolate was incubated at 25 °C for 7 days in sterile petri dishes (9 cm) containing potato dextrose agar medium (PDA). Then, 5 fungal disc pieces (1 cm^2) were cultured in autoclaved 250 ml flasks containing 50 ml of PDB (potato dextrose brooth agar) and incubated at 25 °C in the dark for 7 days. Handshaking was performed daily during the incubation period. After seven days, the culture filtrate was first filtered through two layers of filter paper (Whatman No. 1) and then refiltered through a 0.45 lm pore-size filter to remove fungal spores and mycelium. The filtrate was kept at +4 °C until the experiment was established (Lobna et al. 2016).

Preparation of nematode inoculum

A thousand second juvenile larvae (J2) of *M. incognita* were used as nematode inoculum. After mass-produced tomato roots of Tueza F1, tomato variety were washed in tap water, egg masses were removed from the roots under a stereo microscope and incubated in water at 25±2 °C for three days in a petri dish containing a sterile sieve of 3 cm diameter. After three days, the J2s hatched from the eggs were counted under a light microscope and placed in 1 ml tubes, adjusted to the number to be used in the experiment (Lobna et al. 2017).

Effect of some essential oils on Meloidogyne incognita and FORL disease severity on tomato

In this study, the effects of thyme (*O. vulgare*), sage (*S. officinalis*), garlic (*A. sativum*), sesame (*S. indicum*) , rosemary (*R. officinalis*, syn. *S. rosmarinus*), lemon (*C. limon*) and mustard (*B. nigra* L.) essential oils and positive controls of nematicide and fungicide on nematode and fungus in the simultaneous inoculation of *M. incognita* and FORL in Gülizar F1 tomato cultivar were investigated between January and April 2022 in Isparta, Türkiye. Only plants with simultaneous application of *M. incognita* and FORL were evaluated as negative control. The study was set up in a climate room under controlled conditions (24 ± 1 °C, 60% ± 5% humidity) in plastic pots and in a randomized plot design for each essential oil with 5 replications. Three-week-old tomato seedlings were transplanted into plastic pots with a diameter of 14 cm containing approximately 1500 g of sterile, soil (68% sand, 21% silt and 11% clay). In the initial inoculum density per pot, 1000 *M. incognita* J2/1ml and $3x10^6$/10 ml FORL were used and simultaneous inoculation was performed. The nematode inoculum was evenly dispersed with a pipette into three 2-3 cm deep holes drilled around the seedling stem and deep enough to contact the roots in the soil. Fungi inoculation was poured into these holes opened on the soil surface of the pots with the help of a measuring tape (Lobna et al. 2016, 2017). One day after nematode and fungus inoculation, 1000 ppm/pot for each essential oil was applied to the soil (Simsek 2020). The maximum field recommendation doses of Velum and Cebir were used 0.16 ml/l and 0.25 ml/l, respectively.

The study was terminated 60 days after the essential oil applications. Tomato plants related to each application were carefully removed from the soil and their soils were washed with tap water. Evaluation is 1-9 root gall scale for nematodes (1= no gall, 2= 5% root gall, 3= 6-10% root gall, 4= 11-18% root gall, 5= 19-25% root gall, 6= 26-50% root gall, 7= 51-65% root gall, 8= 66-75% root gall, 9= 76-100% root gall) and egg mass production rate scale (1= no egg mass, 2 = 1 or 2 egg masses, 3= 3-6 egg masses, 4= 7-10 egg masses, 5= 11-20 egg masses, 6= 21-30 egg masses, 7= 31-60 egg masses, 8= 61 -100 egg masses, 9= more than 100 egg masses) (Göze Özdemir and Karaman 2020, Mullin et al. 1991). The severity of disease caused by FORL was evaluated according to the 0-4 scale of Chandler and Santelman (1968) (0: No damage to the seedling, 1: Discoloration and small lesions at the junction of the seedling with the soil surface, 2: Larger lesions turned stem, 3: Large lesions surrounding the stem, resulting in a concave appearance, 4: Dead plant due to fungal damage) (Erol and Tunalı 2007). The percentages of suppressing gall, egg mass and disease severity were calculated with the formula % = (nematode alone – treatment/Nematode alone) X100 (Xiang et al. 2020). The mean of the data obtained was compared according to the LSD test (P≤0.05) using the SAS (version 17.00) program.

RESULTS AND DISCUSSION

In this study, the gall and egg mass index were significantly lower in all essential oil, nematicide and fungicide applications than the negative control application (P≤0.05). Gall and egg mass index of essential oils were determined to be higher when compared to nematicide while lower than fungicide application. Among the essential oils, the highest gall index was found in sage application with 5.4 and the lowest in thyme application with 3.2 (P≤0.05). The gall index of garlic (3.8) and mustard (3.6) essential oil applications were lower than sesame (4.6), rosemary (4.2) and lemon (4.2) applications. In garlic, mustard, rosemary and lemon essential oil applications, the suppressive effect on gall was found to be over 50%. The highest suppressive effect on galling was found in thyme essential oil application with 62.4% compared to the negative control (P≤0.05). Sesame essential oil suppressed galling by 46.4% and its effect was higher than sage. It was observed that the egg mass index were higher than gall index in essential oil applications. It was found that the egg mass index varied between 3.6 and

5.8 in essential oil applications. The suppressive effect on the egg mass was determined above 50% only in mustard (56.78%) and thyme (59.06%) essential oil applications. The suppressive effect on the egg mass of other essential oils used in the study varied between 34-55% (Table 1).

In simultaneous application of *M. incognita* and FORL, the disease severity scale was found to be 3.6. The nematicide (2.2) and fungicide (2.4) applications to the simultaneous *M. incognita* and FORL inoculation significantly reduced the disease scale compared to negative control (P≤0.05) and suppressed by 33%. In sesame, rosemary, lemon, mustard and sage essential oil applications, the disease scale was in the same statistical group as the fungicide application (P≥0.05). The disease severity scale was determined as 1.6 in thyme and garlic oil applications. It was found that only two of the seven essential oils (thyme and garlic) suppressed the disease severity by more than 50%. The sage (38.84%), rosemary (33.28%) and mustard (38.92%) essential oils applications were found to suppress disease severity higher than sesame (22.16%) and lemon (22.16%). Except for sesame and lemon oil, the percentage of suppressing disease severity of the other essential oils used in the study was higher than the fungicide application (Table 1).

The suppressive effect of the essential oils used in the study on nematode and fungus in tomato was found to be significantly higher when compared to the negative control. It was determined that the application of fungicide in simultaneous inoculations suppressed the FORL and *M. incognita* by 30% compared to the negative control. However, disease severity was suppressed by 40% when nematicide is applied. Since essential oils of sage, sesame, rosemary, lemon and mustard are effective on both organisms in the nematode-disease complex, their suppressive effect on disease severity was included in the same statistical group as fungicide application. Although higher gall and egg mass index were determined in thyme and garlic essential oil applications than nematicide application, these 2 essential oils showed a higher suppressive effect on disease severity than nematicide and fungicide applications. These results show that applications that are effective on both organisms are more successful in the control against *M. incognita*-FORL disease complex. In additionit, it represents that nematode management is important while controlling the disease complex, and it also reveals that it should be a priority. This is due to plant parasitic that nematodes can cause various lesions in the roots of the host plant that facilitate infection of fungal hyphae, or cause physiological

Table 1. Effect of some essential oils on *Meloidogyne incognita* and *Fusarium oxysporum* f.sp. *radicis lycopersici* (FORL) disease severity on tomato

		LSD test					
	Applications of essential oil	Gall index (1-9)*	Percent suppressive effect on gall (%)	Egg mass index (1-9)**	Percent suppressive effect on egg mass (%)	Disease severity scale***	Percent suppressive effect on disease (%)
1	N+FORL+thyme	3.2 f****	62.74 b	3.6 e	59.06 b	1.6 c	55.52 a
2	N+FORL+sage	5.4 bc	37.16 ef	5.8 b	34.06 e	2.4 b	33.32 b
3	N+FORL+garlic	3.8 def	55.76 bcd	4.4 cd	49.94 cd	1.6 c	55.52 a
4	N+FORL+sesame	4.6 cd	46.44 de	4.4 cd	49.94 cd	2.8 b	22.16 b
5	N+FORL+rosemary	4.2 de	51.10 cd	4.8 c	45.38 d	2.4 b	33.28 b
6	N+FORL+lemon	4.2 de	51.08 cd	4.6 c	47.66 d	2.8 b	22.16 b
7	N+FORL+mustard	3.6 ef	58.08 bc	3.8 de	56.78 bc	2.2 bc	38.92 ab
8	N+FORL+Nematicide (Positive control)	1.4 g	83.66 a	1.4 f	84.04 a	2.2 bc	38.84 ab
9	N+FORL+Fungicide (Positive control)	6.0 b	32.52 f	6.2 b	29.52 e	2.4 b	33.20 b
10	N+FORL (Negative control)	8.6 a	0 g	8.8 a	0 f	3.6 a	0 c
	LSD (5%)	0.83	9.63	0.70	7.70	0.71	18.82
	CV(%)	14.57	15.75	11.45	13.20	23.19	44.24

*Scale of 1-9 root galling index (Muller et al. 1991); 1= no gall, 2= 5% root gall, 3= 6-10% root gall, 4= 11-18% root gall, 5= 19-25% root gall, 6= 26-50% root gall, 7= 51-65% root gall, 8= 66-75% root gall, 9= 76-100% root gall)

**Scale of 1-9 Egg mass index (Muller et al. 1991);1= no egg mass, 2= 1 or 2 egg masses, 3= 3-6 egg masses, 4= 7-10 egg masses, 5= 11-20 egg masses, 6= 21-30 egg masses, 7= 31-60 egg masses, 8= 61-100 egg masses, 9= more than 100 egg masses

*** Scale of 0-4 disease severity (Chandler and Santelman 1968); 0=No damage to the seedling, 1=Discoloration and small lesions at the junction of the seedling with the soil surface, 2= Larger lesions turned stem, 3= Large lesions surrounding the stem, resulting in a concave appearance, 4= Dead plant due to fungal damage).

****Means statistically compared with LSD test at P≤ 0.05 significance level.

changes lead to increased root secretions (LaMondia 2003). However, increased number of lateral roots and changes in the chemical composition of root exudates that may attract fungi may increase susceptibility to fungal infection (Back et al. 2002). Fungal infection may result in impaired or reduced host resistance, which can lead to larger nematode populations (Viketoft et al. 2020). According to Göze Özdemir et al. (2022a) determined that disease severity caused by FORL on tomatoes increased in the presence of *M. incognita* and determined that nematode infection was important in the durability of FORL resistance on tomatoes. It has been reported in different studies that simultaneous infection by root-knot nematode and FORL causes more severe damage to the host plant than infection by each pathogen alone (Hajji et al. 2016, McGawely 2001).

The most effectıve essential oils on the nematode and fungus disease complex were found thyme and garlic. It was determined that these two essential oils suppressed the *M. incognita* and FORL by more than 55%. The thyme and garlic essential oils have good performance against both pathogens in their simultaneous disease-complex agents. This fungicidal and nematicidal effect may be due to the secondary metabolites of essential oils. Thymol, carvacrol, linalool, p-cimen, geraniol, borneol monoterpenes are found in the essential oil components of most of the thyme species (Paşa 2019). Thymol is a nematicidal compound that can be found in many plants such as thyme and is highly toxic to root-knot nematodes (Deng et al. 2022). Carvacrol and thymol may act on TA receptors and interrupt a signal cascade of receptors in nematode cells (Lei et al. 2010). Garlic has high antimicrobial and antibacterial activity due to the allicin compound it contains (Khashan et al. 2014, Rahman et al. 2022, Tijjani et al. 2017, Wolde et al. 2018). Diallyl disulfide iswhich are the primary sulfur compounds, can effectively control the occurrence of bean root rot, tomato root-knot nematode, and pepper blight (Avato et al. 2000, Ma et al. 2009). Khairan et al. (2021) found that all garlic extracts in their study contained flavonoids, alkaloids and saponin secondary metabolites and showed that ethyl acetate garlic extracts had the highest nematicidal activity against root-knot nematodes. Galisteo et al. (2022) identified the nematicidal components of garlic oil as diallyl disulfide and diallyl trisulfidine and cited it as an important resource for the development of new nematode control products.

The antifungal effect of thyme essential oil on FORL has been demonstrated by previous studies (Arıcı et al. 2013, Bilici et al. 2021). It has also been reported that thyme essential oil inhibits the development of many pathogens (Aksit et al. 2022, Arıcı et al. 2011, Arıcı and Koç 2021, Koçak and Boyraz 2006). Garlic extracts were inhibit F. *Fusarium oxysporum*, *Botrytis cinerea*, *Phytophthora capsici*, *P. nicotianae* and *Verticillium dahliae* (Hayat et al. 2016, Wang et al. 2019). It was determined that the mortality effect of thyme essential oil on *M. incognita* J2 was higher (El-Gindi et al. 2005). Özdemir and Gözel (2018) reported that 3% and 5% doses of thyme (*Thymus serpyllum*) essential oil used in their study suppressed the *M. incognita* gall and egg mass on the roots. Cetintaş and Yarba (2010) reported that essential oils of rosemary, thyme and peppermint suppress root-knot nematode and that the effect of thyme is higher. Root gall index was found to be low in the applications of thyme essential oil with planting (Göze et al. 2014). It has been determined that the most effective application against *M. incognita* race 2 in sugar beet is 2000 ppm dose application of *Thymus vulgaris* L. essential oil to the soil (Tosun et al. 2018). Garlic methanol extract is found effective against *Aphelenchoides sacchari* in mushrooms and *Aphelenchoides grayi* in carrots (Block 2010). Nematicidal activity of garlic essential oil against *M. incognita* on tomatoes and cucumbers and pine tree nematode *Bursaphelenchus xylophilus* has been reported (Al-Shalaby 2009, El-Saedy et al. 2014, Jardim et al. 2020, Park et al. 2005).

In the present study, it was found that nematode control in nematode and fungal disease complexes is very important and should be a priority. When controlling the nematode disease complex, it was found that the effect of nematicide application on disease severity was higher than fungicide application. Furthermore, mustard essential oil and nematicide had similar effects in controlling disease severity in the nematode and fungal disease complex. The main finding of the study is that thyme and garlic essential oil applications are more effective than chemical nematicide and fungicide applications and other essential oils in the control of nematode and fungus disease complex.

Consequently, thyme (*O. vulgare*) and garlic (*A. sativum*) essential oils were determined as the best alternatives to chemicals for managing root-knot nematode and FORL disease complexes. However, mustard essential oil also looks promising. The application of essential oils to the soil will be an effective alternative control method to reduce pesticide use and increase yield in control of root knot nematode and FORL. It can be seen that thyme and garlic essential oils may be presented as a potential for nematode and disease control in organic farming where fewer control options are available. In addition, it is belived that thyme and garlic essential oils may be effective in nematode interactions with other soil-borne pathogens. However, due to the evaporation nature of essential oils, detailed studies should be conducted for their application under field conditions.

ÖZET

Domateste, ticari kekik (*Origanum vulgare* L.), adaçayı (*Salvia officinalis* L.), sarımsak (*Allium sativum* L.), susam (*Sesame indicum* L.), biberiye (*Rosmarinus officinalis* L., syn. *Salvia rosmarinus* Spenn.), limon (*Citrus limon* (L.) Osbeck) ve hardal (*Brassica nigra* L.) esansiyel yağlarının (Botalife Natural and Aromatic Products Inc., Türkiye) eş zamanlı *Meloidogyne incognita* (Kofoid and White, 1919) Chitwood, 1949 ve *Fusarium oxysporum* f.sp. *radicis lycopersici* (Jarvis & Shoemaker) (FORL) inokulasyonunda hastalık şiddeti üzerine etkileri araştırılmıştır. Pozitif kontrol olarak nematisit (Velum®, Fluopyram, Bayer Crop Production Inc., Türkiye) ve fungisit (Cebir®, Fludioxonil + Metalaxyl, Hektaş Crop Production Inc., Türkiye) kullanılmıştır. Negatif kontrol sadece nematod ve fungus inokulasyonu yapılan bitkilerden oluşmuştur. Çalışma, her bir uçucu yağ için 5 tekerrürlü tesadüf parselleri deneme deseninde kurulmuştur. Eş zamanlı inokulasyonlarda her bir fide için 1000 *M. incognita* ikinci dönem larva/1ml ve 3x10^6 spor/ml FORL kullanılmıştır. Uçucu yağ uygulamaları, nematod ve fungus inokulasyonundan bir gün sonra her saksı için 1000 ppm dozunda toprağa uygulanmıştır. Çalışma 60 gün sonra sonlandırılmış ve değerlendirme gal, yumurta paketi ve hastalık şiddetine göre yapılmıştır. Köklerde fungus büyümesi ve nematod gelişimi, test edilen tüm yağ uygulamalarında negatif kontrole göre daha düşük bulunmuştur, ancak fungus gelişimi ve nematod aktivitesi değişiklik göstermiştir. Kekik ve sarımsak uçucu yağları eş zamanlı inokulasyonda nematod ve fungus üzerinde %55.20 ile en yüksek kontrol etkisine sahip olmuş ve bu etki sadece nematod (%38.84) ve sadece fungisit (%33.20) uygulamalarından daha yüksek bulunmuştur. Adaçayı (%38.84), biberiye (%33.28) ve hardal (%38.92) esansiyel yağlarının, susam (%22.16) ve limona (%22.16) göre hastalık şiddetini daha fazla baskıladığı bulunmuştur. Kekik ve sarımsak esansiyel yağlarının kök ur nematodu ve FORL hastalık komplekslerini yönetmek için iyi birer alternatif olduğu belirlenmiştir.

Anahtar kelimeler: hastalık kompleksi, uçucu yağ, sarımsak, kök ur nematodu, kekik

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