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Original article (Orijinal araştırma)

First record of three plant parasitic nematode species from Mount Ararat (Ağrı) in Turkey[1]

Türkiye bitki paraziti nematod faunası için Ağrı Dağı (Ağrı)'ndan 3 yeni kayıt

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Abstract

In this study, plant parasitic nematode fauna from Mount Ararat was determined according to altitude. A total of 30 soil samples were taken in 2013 during summer. Nematodes were extracted by a modified Baermann funnel technique. Nematodes were identified by morphology, morphometric and phylogenetic analysis based on sequences of the D2-D3 and ITS1-rRNA gene sequences. Permanent slides of individuals were made and species-specific characters were screened by scanning electron microscopy (SEM). SEM studies were processed at the Department of Animal Biology, Vegetal Biology and Ecology, SEM laboratory University of Jaén in Spain during 2014. A total of 19 plant parasitic nematodes were identified from Mount Ararat. Three plant parasitic nematodes *Rotylenchus conicaudatus* Atighi et al., 2011 (Nematoda: Hoplolaimidae), *Heterodera trifolii* Goffart, 1932 (Nematoda: Heteroderidae) and *Tylenchorhynchus mangiferae* (Luqman & Khan, 1986) (Nematoda: Belonolaimidae) from this study are new records for plant parasitic nematode fauna of the Turkish.

Keywords: Morphometrics, nematode fauna, new record, phylogeny, Turkey

Öz

Bu çalışmada Ağrı Dağı bitki paraziti nematod faunası yükseklikliere bağlı olarak belirlenmiştir. Toplam 30 toprak örneği 2013 yılında yaz döneminde alınmıştır. Nematodlar alınan toprak örneklerinden geliştirilmiş Baermann huni yöntemi ile elde edilmiştir. Nematodların tür teşhisleri morfolojik karakterler, morfometrik ölçümler ve moleküler olarak rDNA'nın D2-D3 ve ITS1 bölgelerinin sekans analizleri yapılarak belirlenmiştir. Elde edilen bireylerin daimî preparatları yapılarak türe özgü karakterler taramalı elektron mikroskobu (SEM) ile görüntülenmiştir. SEM çalışmaları İspanya'da Jaen Üniversitesi, Hayvan ve Bitki Biyolojisi ve Ekolojisi Bölümü, SEM laboratuvarında 2014 yılında yapılmıştır. Ağrı Dağı'ndan toplam 19 bitki paraziti nematod türü belirlenmiştir. Bu çalışmada elde edilen bitki paraziti nematodlardan *Rotylenchus conicaudatus* Atighi et al., 2011 (Nematoda: Hoplolaimidae), *Heterodera trifolii* Goffart, 1932 (Nematoda: Heteroderidae) ve *Tylenchorhynchus mangiferae* (Luqman & Khan, 1986) (Nematoda: Belonolaimidae) türleri Türkiye bitki paraziti nematod faunası için ilk kayıt niteliğindedir.

Anahtar sözcükler: Morfometrik, nematod faunası, yeni kayıt, filogeni, Türkiye

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Introduction

Nematodes are highly diverse, complex and biologically specialized metazoans (De Ley et al., 1999). There are estimates of between 40.000 and 10.000.000 species in the phylum Nematoda (Blaxter et al., 1998). One challenging estimate speculates that there might be as many as 100.000.000 species, even before considering the cryptic diversity among morphologically indistinguishable taxa (Lambshead, 1993). The key roles of nematodes in agricultural and natural ecosystems, as well as their usefulness for indicator and molecular studies, make them an important focus for taxonomic, ecological, physiological and molecular research.

Turkey is situated between latitudes 35º and 43º N, and longitudes 25º and 45º E within subtropical climate zone. Turkey is geographically consisting of seven regions and each region has different climatic conditions. As a result of this, in recent decades, plant parasitic nematode fauna of the Turkish has been studied by several authors and important nematode species were reported (Ökten, 1982; Elekçioğlu et al., 1994; Elekçioğlu, 1996; Kepenekçi & Ökten, 2000a, b, c; Kepenekçi & Öztürk, 2000; Ökten et al., 2000; Erdal et al., 2001; Gözel & Yıldız, 2015; Mıstanoğlu et al., 2015; Muşdağı & Gözel, 2015; Aydınlı, 2018). Also, Kepenekçi (2014) published a detailed list of 240 nematode species of plant parasitic nematodes belonging to 56 genera of Tylenchida from Turkey.

Various biogeographic reasons explain the unique diversity met in Turkey´s nature. Due to its "crossroad" location and the variations of its geographic features and climatic conditions, Turkey has a rich biodiversity. Another factor, that has shaped Turkey´s biodiversity is the ice ages ranging from 1.8 million to ten thousand years ago. The global map of biodiversity hotspots clearly reflects this richness. Three out of 34 biodiversity hotspots meet in Turkey and one of them is the Irano-Anatolian spot.

The Mount Ararat (Ağrı) (5137 m) is the highest mountain in Turkey. As a result of its geographical conditions, the nematode fauna has not been studied to date. In the present study, a total of 2.560 of nematodes were identified. Three new reports for Turkey, *Rotylenchus conicaudatus* Atighi et al., 2011 (Nematoda: Hoplolaimidae), *Heterodera trifolii* Goffart, 1932 (Nematoda: Heteroderidae) and *Tylenchorhynchus mangiferae* (Luqman & Khan, 1986) (Nematoda: Belonolaimidae) are described from the Mount Ararat. Detailed morphological and morphometric characterization of the taxa are discussed and molecular comparison with closely related *Rotylenchus* and *Heterodera* species using the ITS1 of rRNA and D2-D3 of 28S gene sequences is provided.

Material and Methods

Sampling

Nematodes were collected from the root zone of wildflower meadows, mountain grasslands, riverbeds, chalk grasslands, igneous soil and marshland from 1523 to 4957 m a.s.l. on Mount Ararat (5.137 m), northern Turkey (39º41'3.91" N, 44º16'49.80" E) in July 2013. Samples were taken from 15 x 15 cm quadrats, placed into ziplock sampling bags and stored in portable cooler during the transport to the laboratory for extraction.

Extraction

Nematodes were extracted by using a modified Baermann (1917) funnel technique. After separating rocks and large organic particles, further processing was done based on improving the uniformity of samples of equal volume in 200 ml beakers. Samples were then placed on plastic trays lined with paper towels and incubated on the laboratory. Nematodes were collected after 48 h by pouring the extraction tray over a 500 mesh sieve (25 µm opening) and put into DESS solution according to Yoder et al. (2006). Each extract was then labeled with corresponding sample number and transported in plastic tubes to the University of Jaén, Spain.

Preparation of nematodes for light microscope

After picking out individuals, the nematodes were rinsed with purified water to remove the debris. Glass staining blocks with extracted nematodes were then placed in an airtight jar with 1.25 cm deep volume of 96% ethanol, a few drops of glycerol-formalin (4%) (1:99) was added and the specimens left overnight at room temperature. Next morning, the glass blocks were removed from the jars and a few drops of five parts glycerol and 95 parts 96% ethanol solution added. Two-thirds of cavity in the glass blocks covered with a glass square and it was placed in an incubator at 40ºC. For gradual transition of glycerin, a few drops of glycerol-ethanol (5:95) solution was added every 2 h. The next day, individual nematodes were permanently mounted on glass slides (Yoder et al., 2006).

Scanning Electron Microscopy

Fixed individual specimens were rinsed into deionized water to remove all traces of fixatives. After 2 h for dehydration of specimens, nematodes were passage into 100% ethanol by passing them through series of 25 (overnight), 30, 50, 70, 80, 90, 95, 100% (each for 2 h). The next day the specimens were put in acetone for 1 h before critical point dried with CO_2, coated with gold (Abolafia & Peña-Santiago, 2005) and observed with a Jeol JSM-5800 microscope. SEM studies were done at Department of Animal Biology, Vegetal Biology and Ecology, SEM Laboratory University of Jaén during 2014.

Measurements

Measurements and drawings were made using a drawing tube attached to an Olympus microscope (Olympus Optical, Tokyo, Japan). Light microscope pictures were taken using a Nikon Eclipse 80i microscope equipped with a Nikon Digital Sight DS-5M digital video camera. Illustrations were prepared based on light microscope drawings using Illustrator® CS3 Extended software, version 10.0 (Adobe Systems, CA, USA).

Molecular characterization

DNA extraction, PCR and sequencing

DNA was extracted from single individuals. After initial selection, nematodes were individually picked from the DESS solution, rinsed with deionized water, put in a drop of worm lyses buffer (50 mM KCl, 10 mM Tris-Cl pH 8.3, 2.5 mM $MgCl_2$, 0.45% NP 40 and 0.45% Tween 20), cut into small pieces and after adding 60 µg ml^{-1} Proteinase K, nematodes were placed in a microcentrifuge tube and transferred to −80ºC for 10 min. DNA suspension was added to PCR mixture after preparation the tubes by incubating for 1 h at 65ºC followed by enzyme deactivation for 10 min at 90ºC and centrifugation for 1 min at 16,000g. PCR was performed with a final volume of 25 µL suspension, containing 2.5 µL genomic DNA template, 2.5 µL of 10× reaction buffer with $MgCl_2$, dNTP-mix at 0.2 mM each, 0.5 m of each primers; D2Ab (5´-ACAAGTACCGTGAGGGAAAGTTG-3´), D3B (5´-TCGGAAGGAACCAGCTACTA-3´) for the D2-D3 domain of the large subunit rDNA gene (De Ley et al., 1999) and Vrain2(f) (5´-CTTTGTACACACCGCCCGTCGCT-3´), Vrain2(r) (5´-TTTCACTCGCCGTTACTAAGGGAATC-3´) for the ITS1-rRNA (Vrain et al., 1992). The PCR amplification was performed with initial denaturation at 94ºC for 5 min, followed by 35 cycles of 94ºC for 1 min, 54ºC for 90 s and 72ºC for 2 min, and a final extension at 72ºC for 10 min. PCR products were sized with 1 kb DNA ladder (Promega, Madison, WI, USA) on a 1% agarose gel stained with 0.0003% ethidium bromide. In order to obtain sequences of the forward and reverse DNA strand, PCR products were sent to Macrogen Inc., Amsterdam, the Netherlands and sequencing was done in both directions with the appropriate primers. The sequences were then edited by performing BioEdit 7.0.4.1 (Hall, 1999). These sequences were then aligned with available sequences from Genbank (NCBI).

Phylogenetic analysis

D2-D3 domains of 28S and ITS1-rRNA were used for phylogenetic analyses. Previously published sequences were obtained from GenBank to perform phylogenetic reconstruction (Tables 1&2). Alignment of sequences were checked by using program CLUSTAL W (Thompson et al., 1994). Bayesian phylogenetic inference was performed with MrBayes v3.1.2 (Ronquist & Huelsenbeck, 2003). The best fitting model with rate variation across sites and a proportion of invariable sites (GTR+I+G) as estimated by Mega 6.0 program model testing 1.0b was performed for entire alignment. Two independent runs were performed for each 3 million generations and the trees were obtained by using the last 1 million generations with well beyond the burn-in value. Final statistics to check two different runs as average standard deviation of split frequencies between the two analyses were convincing as the value approached zero (<0.0012). Geneious (Basic 5.6.7) program was used to visualize the trees.

Table 1. *Rotylenchus* species and populations used in this study

Species	Locality	Accession Number		Reference
		D2/D3	ITS-rRNA	
R. buxophilus	California, USA	JX015421	JX015432	Cantalapiedra-Navarrete et al. (2013)
R. conicaudatus	Mazandaran, Iran	HQ700698	HQ700700	Atighi et al. (2011)
R. eximius	Brindisi, Italy	EU280794	EU373663	Cantalapiedra-Navarrete et al. (2013)
R. goodeyi	Vejer, Spain	DQ328756	-	Subbotin et al. (2007)
R. incultus	Niebla, Spain	EU280796	EU373673	Cantalapiedra-Navarrete et al. (2013)
R. iranicus	Mazandaran, Iran	HQ700697	HQ700699	Atighi et al. (2011)
R. jaeni	Santa Elena, Spain	EU280791	EU280791	Cantalapiedra-Navarrete et al. (2013)
R. laurentinus	Torre Canne, Italy	DQ328757	-	Cantalapiedra-Navarrete et al. (2013)
R. magnus	Arévalo, Spain	EU280789	EU373660	Cantalapiedra-Navarrete et al. (2013)
R. magnus	Arévalo, Spain	-	EU373676	Cantalapiedra-Navarrete et al. (2013)
R. montanus	Trentino, Italy	-	EU280801	Cantalapiedra-Navarrete et al. (2013)
R. montanus	Trentino, Italy	DQ328743	EU280800	Vovlas et al. (2008)
R. paravitis	Jerez, Spain	JX015422	JX015434	Cantalapiedra-Navarrete et al. (2013)
R. pumilus	California, USA	JX015423	JX015435	Cantalapiedra-Navarrete et al. (2013)
R. robustus	Lucena del Puerto, Spain	JX015424	JX015437	Cantalapiedra-Navarrete et al. (2013)
R. uniformis	Bruges, Belgium	DQ328735	-	Vovlas et al. (2008)
R. unisexus	Seville, Spain	-	EU373675	Vovlas et al. (2008)
R. unisexus	Seville, Spain	EU280799	EU373674	Vovlas et al. (2008)
R. vitis	Montemayor, Spain	JN032581	JN032582	Cantalapiedra-Navarrete et al. (2013)
Rotylenchus sp.	Russia	-	EU280802	Vovlas et al. (2008)

The phylogenetic relationships of the *R. conicaudatus* isolate from Mount Ararat were inferred from large subunit rDNA sequences (D2/D3 domains of the 28S gene) and ITS-rRNA available from GenBank.

Table 2. *Heterodera* species and populations used in this study

Species	Locality	Accession Number	Reference
H. trifolii	Iran, Khozestan	GU475089	Heydari et al. (2010)
H. schachtii	Iran, Khorasan	GU475088	Heydari et al. (2010)
H. schachtii	USA, North Dakota	JQ040526	Nelson et al. (2012)
H. schachtii	USA, North Dakota	JQ040527	Nelson et al. (2012)
H. glycines	Iran, Mazandaran	GU475087	Heydari et al. (2010)
H. glycines	China, Gansu province	GU595446	Peng and Ye (2011)
H. glycines	China, Gansu province	GU595450	Peng & Ye (2011)
H. glycines	China, Gansu province	GU595452	Peng & Ye (2011)
H. glycines	China, Gansu province	GU595447	Peng & Ye (2011)
H. glycines	China, Gansu province	GU595448	Peng & Ye (2011)
H. glycines	China, Gansu province	GU595445	Peng & Ye (2011)
H. glycines	USA	DQ328692	Subbotin et al. (2007)
H. glycines	Canada, Quebec	KF453623	Mimee et al. (2014)
H. glycines	China	JQ067683	Peng & Xu (2013)
H. glycines	China, Zhongwei province	HM560850	Ye et al. (2010)
H. glycines	China, Gansu province	JN684907	Wei et al. (2013)
H. glycines	China, Gansu province	JN684906	Wei et al. (2013)
H. avenae	China, Anhui province	HM560801	Ye et al. (2010)
H. avenae	China, Anhui province	GU595436	Ye et al. (2010)
H. avenae	China, Anhui province	GU595437	Ye et al. (2010)
H. avenae	China, Anhui province	GU595438	Ye et al. (2010)
H. cruciferae	Italy, Castellanetta	JX402414	Sasanelli et al. (2013)

Results and Discussion

Hoplolaimidae

Rotylenchus conicaudatus Atighi et al. 2011

Material examined. Mount Ararat, Turkey, 23.VII.2013, 30 ♀♀ and 32 ♂♂, altitude 3757, 3754, 3563, 2554 and 2337m a.s.l., in mountain grassland.

Description (Table 3).

Table 3. Main morphometric data of *Rotylenchus conicaudatus* from Mount Ararat (measurements in µm)

Characters	♀	♀♀	♂♂
n	1	30	32
L (µm)	735.0	780.0±38.0 (724-882)	722.0±27.0 (671-772)
a	26.9	28.1±0.9 (27.3-30.4)	32.1±1.6 (29.1-33.8)
b	6.6	6.47±0.6 (5.1-8.1)	5.96±0.6 (5.2-8.6)
b´	5.8	5.6±0.5 (4.6-6.8)	5.5±0.5 (4.8-7.7)
c	44.4	46.4±3.1 (41-55)	33.7±2.5 (30.5-38.1)
c'	1.1	1.0±0.1 (0.8-1.2)	1.6±0.1 (1.5-1.8)
V (%) or T %	60.0	60.0±0.02 (54-64)	33.3±1.7 (30-38)
G1 (%)	38.3	31.2±5.3.0 (21.2-41.6)	-
G2 (%)	35.8	29.3±3.0 (21-36)	-
N. lip annuli	6.0	(5-6)	(5-6)
Lip width (µm)	7.7	7.5±0.2 (7.3-8)	7.2±0.3 (6.5-7.8)
Lip height (µm)	3.9	4.1±0.3 (3.6-5.3)	4.0±0.3 (3.5-4.6)
Stylet length	25.2	25.6±0.5 (24.7-26.4)	22.7±0.8 (21.4-24)
Conus	10.5	10.6±0.4 (10.2-11.5)	9.8±1.0 (7.4-11.5)
D.G.O.	4.2	4.31±0.7 (2.8-5.4)	4.5±0.7 (3.3-5.8)
O (%)	16.7	16.8±2.9 (10.6-21.3)	5.1±0.8 (4.1-6.9)
Anterior end to centre of median bulb	86.0	90.3±5.1 (82-99)	78.6±7.2 (65-95)
Anterior end to excretory pore	124.0	118.4±9.8 (103-141)	111±8.9 (91-127)
Nerve ring	116.0	107±10.5 (97-121)	103.6±8.1 (88-119)
Pharynx length (µm)	130.0	140.4±11.4 (119-159)	131.0±13.3 (96-156)
Pharyngeal overlap (µm)	24.0	29.4±5.2 (21-40)	15.0±1.9 (11.8-19)
Max. body diam. (µm)	28.0	27.8±1.0 (26-29.5)	22.5±0.8 (20-24)
Anal/cloacal body diameter (µm)	16.0	16.2±1.0 (14.2-18)	12.7±0.8 (11.2-14.5)
Tail length (µm)	17.0	16.8±1.1 (15-20)	21.6±1.3 (19.4-24.8)
Tail annuli	11.0	10.0±0.8 (9-12)	-
Phasmid to terminus (µm)	25.0	25±2 (22-31.5)	24.2±1.0 (22.4-26.6)
Spicules (µm)	-	-	27.9±1.2 (26-31)
Gubernaculum (µm)	-	-	11.6±0.9 (10-13)
Testis length (µm)	-	-	323.0±33.3 (230-387)

Female, habitus ventrally curved to C-shaped. Body 0.7-0.8 mm long. Lip region rounded to hemispherical, offset with five to six annules and without longitudinal lines. Labial disc not distinct. Cephalic framework strongly sclerotized. Lateral field areolated from middle of stylet to pharyngeal region only. Stylet 24-26 µm, conus slightly shorter than shaft (10 µm) and basal knobs rounded. Dorsal pharyngeal gland orifice 3-5.5 µm posterior to stylet base. Excretory pore 70-118 µm from anterior end. Median bulb oval to rounded, isthmus short, encircled with nerve ring at anterior level. Basal glandular lobe of pharynx fairly variable in size from 21 to 40 µm long. Excretory pore 95-141 µm from anterior level. Genital system didelphic with paired, equally developed ovaries, oocytes arranged in a single row. Spermatheca rounded filled with sperms. Vulva a depressed transverse slit and epiptygma not seen. Phasmid position at level of anus varying from two to five annuli anterior to anus. Tail rounded, slightly curved ventrally 15-20 µm or 10-12 annuli long.

Male, common, as abundant as female. Habitus ventrally curved. Body length slightly shorter than females, 0.6-0.7 µm long. Labial region identical to female. Stylet slightly shorter and weaker than that of female, 21-24 µm long; basal knobs rounded. Dorsal pharyngeal gland orifice 3.3-5-8 µm long, posterior to stylet knobs. Excretory pore 100-127 µm from anterior end. Testis outstretched. Spicule simple, 26-31 µm long and resting on 10-13 µm long gubernaculum. Bursa varying on length 36-62 µm and enveloping tail. Phasmids position at level of anus. Tail conoid, 19-25 µm long (Figures 1&2).

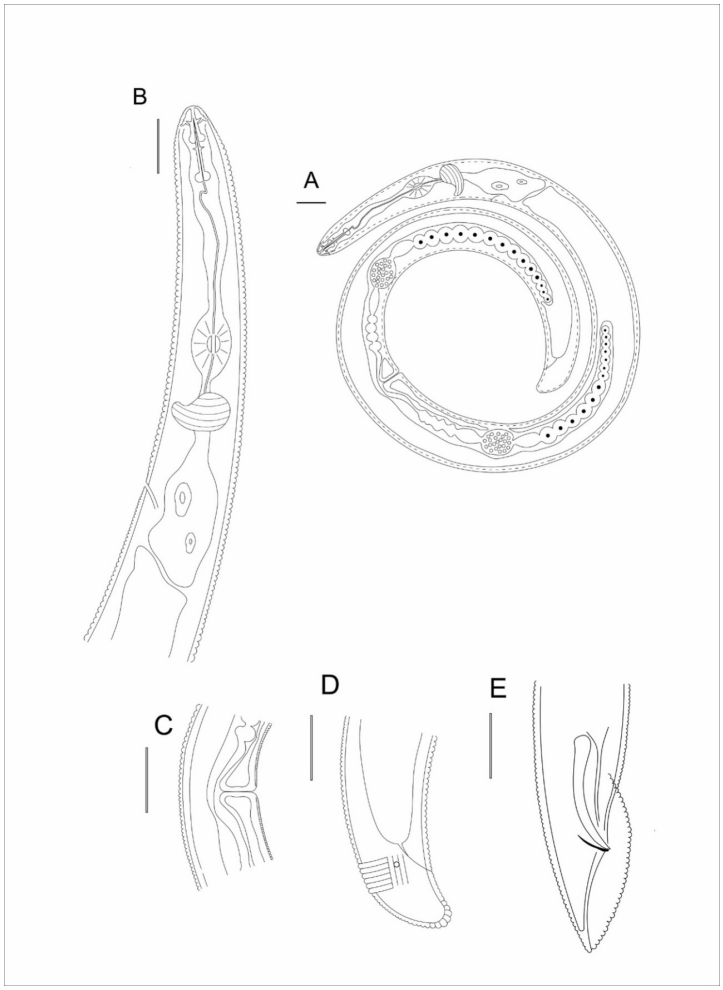

Figure 1. *Rotylenchus conicaudatus*, A) entire female, B) female labial region, C) vulva region, D) female tail, and E) mail tail (scale bars: 20 µm).

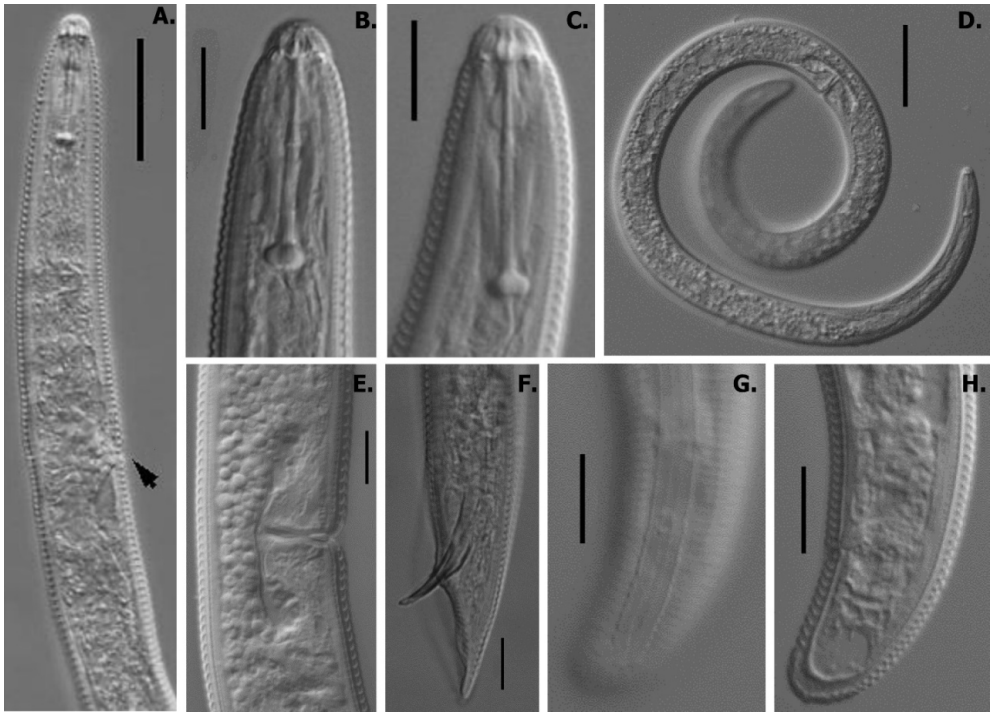

Figure 2. Photomicrographs of *Rotylenchus conicaudatus*: A) female pharyngeal region, B) female lip region, C) male lip region, D) whole body, E) vulval region, F) male tail, G) female tail (showing phasmid and lateral fields), and H) female tail (Scale bars: A: 30 µm; D: 60 µm; and B, C, E, F, G and H: 10 µm).

Remarks. Morphology of *R. conicaudatus* from Mount Ararat showed slight differences in morphometrics from *R. conicaudatus* from Iran in having (i) an offset and hemispherical head region vs conoid-rounded lip region (Figures 3&4); (ii) shorter body (724-882 µm vs 758-1049 µm), (iii) shorter stylet (25-26 µm vs 27-32 µm) and (iv) shorter stylet cone (10 µm vs 15 µm) with less developed and rounded knobs vs wider knobs on *R. conicaudatus* (4.0-4.5 µm vs 5.0-6.5 µm) (Atighi et al. 2011).

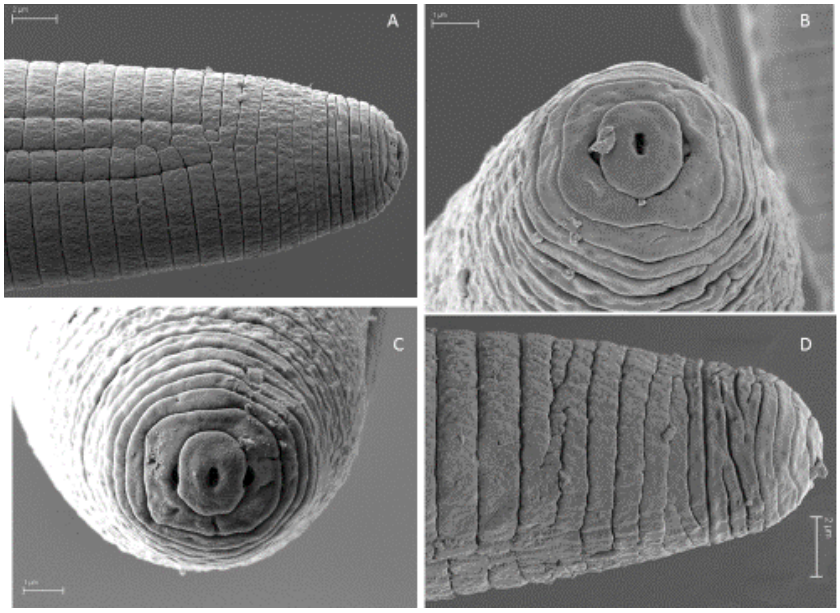

Figure 3. SEM pictures of *Rotylenchus conicaudatus*: A) anterior region in lateral view, B and C) lip region in frontal view, and D) anterior region in ventral view.

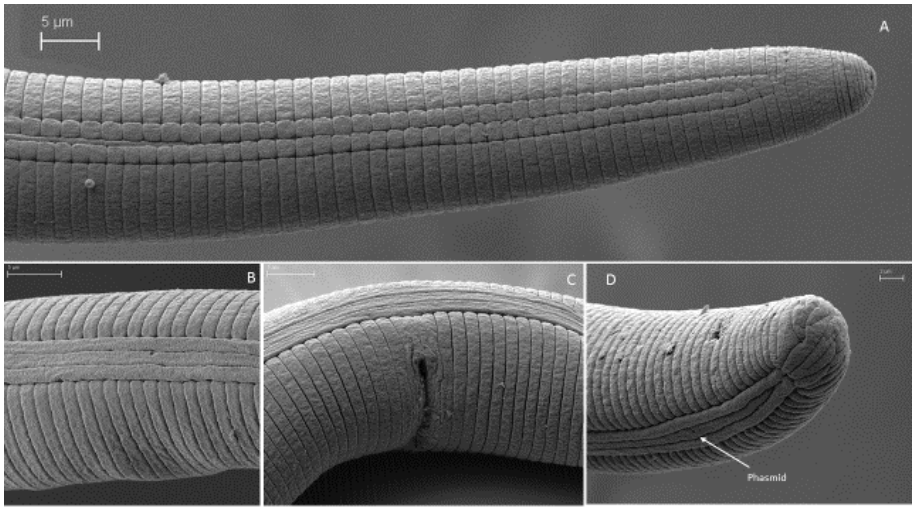

Figure 4. SEM pictures of *Rotylenchus conicaudatus*: A) lateral field at neck region, B) lateral lines, C) vulva region, and D) female tail.

The phylogenetic analysis based on the ITS-rRNA gene confirmed the identification of *R. conicaudatus*. Figures 5 and 6 presents the 50% majority rule consensus phylogenetic tree generated with 17 aligned sequences of *Rotylenchus* spp, from the ITS1 rRNA alignment by Bayesian analysis. The tree confirmed and supported that specimens from Mount Ararat were closely related with *R. conicaudatus* even though there are some morphological difference with the Iranian population. It is clear that there must be further studies on these two population with more different gene regions (Atighi et al., 2011).

Figure 5. Bayesian inference 50% majority rule consensus phylogeny, generated from the D2-D3 of 28S rRNA gene dataset of *Rotylenchus conicaudatus* and other closely related species sequences from GenBank. *Basiria gracilis* (Thorne, 1949) was selected as outgroup.

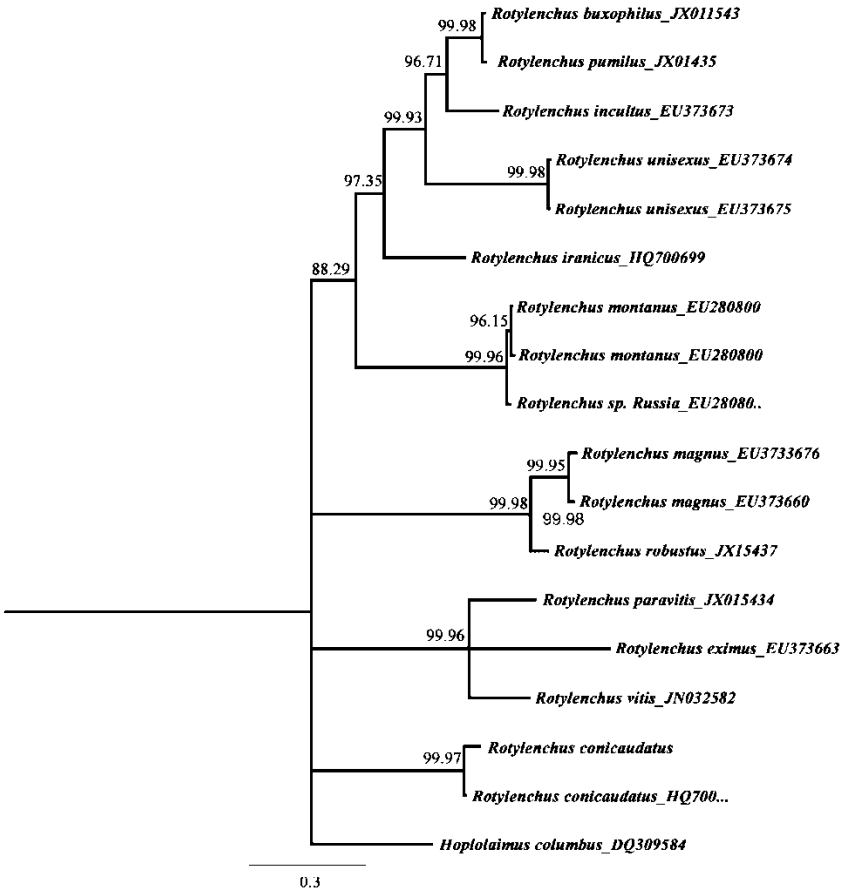

Figure 6. Bayesian inference 50% majority rule consensus phylogeny, generated from the ITS-rRNA gene dataset of *Rotylenchus conicaudatus* and other closely related species sequences from GenBank. *Hoplolaimus columbus* Sher, 1963 was selected as outgroup.

Heteroderidae

***Heterodera trifolii* Goffart, 1932**

Material examined. Mount Ararat, Turkey, 23.VII.2013, three second stage juveniles, altitude 2952 m a.s.l., in riverbed.

Description (Table 4).

Second stage juvenile, body straight, 0.43-0.5 µm. Head offset, cephalic framework heavily sclerotized. Lip region with four annules. Stylet robust (≥28 µm), anchor shape, anteriorly concave. Dorsal gland orifice short, 3.3 µm posterior to spear knobs. Subventral pharyngeal glands extending ventrally to intestine junction. Conoid tail uniformly tapering to a finely rounded terminus. Hyaline portion occupies 55% of total tail length. Phasmids located at the middle level of tail.

Remarks. *Heterodera trifolii* has a wide host range (Siddiqi, 2000) including plants in the Caryophyllaceae, Leguminosae and Polygonaceae. However, we collected *H. trifolii* on a species in the Poaceae.

Phylogenetic analysis. The phylogenetic relationships of the *H. trifolii* isolate from Mount Ararat were inferred from large subunit rDNA sequences (D2/D3 domains of the 28S gene) including 24 selected species of *Heterodera* (Figure 7). The *H. trifolii* isolate from Iran (Heydari et al., 2010) was identical to *H. trifolii* from Mount Ararat without any single nucleotide difference.

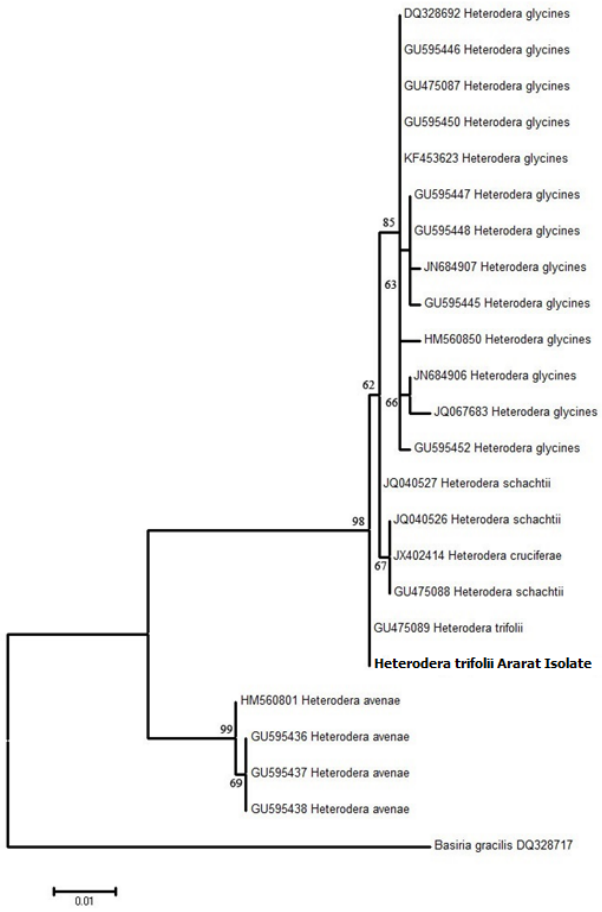

Figure 7. Bayesian inference 50% majority rule consensus phylogeny, generated from the D2-D3 of 28S rRNA gene data set of *Heterodera* species sequences from GenBank. *Basiria gracilis* was designated as outgroup.

Pratylenchidae

***Pratylenchus neglectus* (Reusch, 1924) Filipjev and Schuurmans Stekhoven, 1941**

Material examined. Mount Ararat, Turkey, 23.VII.2013, 1 ♀, altitude 3372 m a.s.l., in wildflower meadow.

Description (Table 4).

Female, body small. Labial region bluntly rounded, the dorsal and ventral submedian lips are fused, labia with two annules, second annulus wider than first. Lateral fields with four lines. Stylet knobs 4-6 µm across, typically indented on anterior surfaces. Pharyngeal gland overlapping intestine ventrally or ventrolaterally, subventral gland nuclei at end of lobes, not in tandem. Excretory pore 75 µm from anterior end. Hemizonid immediately anterior to excretory pore. Female reproductive system monodelphic, prodelphic, ovary outstretched with oocytes in tandem, reaching to base of pharynx. Postvulval uterine sac less than corresponding body diameter. Tail conoid with little curvature of ventral surface and with 15 annules. Tail terminus without annulation, rounded. Phasmids in posterior half of tail.

Male, not found.

Table 4. Morphometrics of plant parasitic species from Mount Ararat (measurements in µm)

Characters	*Heterodera trifolii*	*Pratylenchus neglectus*	*Pratylenchus thornei*	*Malenchus* sp.	
	3 J2	♀	♀	2♀♀	♂
n	3	1	1	2	1
L (mm)	0.43-0.5	0.4	0.5	0.32-0.33	0.36
a	21.0-24.0	24.3	29.9	26.5-27.8	25.2
b	3.1-3.8	6.8	7.4	3.8	3.9
b´	-	5.8	5.6	-	-
c	8.6-11.6	20.9	18.6	3.3	3.6
c'	3.1-4.1	1.9	2.1	10.5	9.2
V (%) or T %	57	79	75	82	?
N. lip annuli	-	2	3	-	-
Lip width (µm)	10.0	7.1	6.8	4.7-4.9	4.5
Lip height (µm)	4.3-4.5	2.4	3.1	3.0-3.1	2.8
Stylet (µm)	28.3	15.7	16.3	11.5-11.7	10.8
D.G.O.	3.3	3.0	2.4	-	-
Ex. Pore	102-109	75	79	55-58	59
Nerve ring	83-86	49	60	47-49	51
Pharynx length (µm)	114-162	58	72	81-87	85
Pharyngeal overlap (µm)	-	20	27	-	-
Max. body diam. (µm)	20.5	16.1	18.0	12.0	11.5
ABD (µm)	14.0	9.8	13.0	9.1-9.5	9.0
Tail length (µm)	58.0	18.8	27.8	98-101	91.5
Spicules (µm)	-	-	-	-	14
Gubernaculum (µm)	-	-	-	-	3

Remarks. Identification of genus *Pratylenchus* is quite complex. Although it is difficult to justify the identification of *P. neglectus* based on only one individual specimen, the specimen examined closely matched to the published descriptions.

***Pratylenchus thornei* Sher & Allen**

Material examined. Mount Ararat, Turkey, 23.VII.2013, 1 ♀, altitude 2552 and 2554 m a.s.l., in chalk grassland and mountain grassland.

Description (Table 4).

Female, body small (0.5 mm), slender, slightly ventrally curved after fixation. Lateral fields with four lines, outer ones straight or weakly crenate. Labial region with three annules, not offset from body. Stylet relatively short (16 µm) with rounded knobs. Stylet guiding apparatus extending posteriorly from basal

plate. Dorsal gland orifice (DGO) 3 µm posterior to stylet base. Nerve ring immediately posterior to pharyngeal bulb, hemizonid located one annulus anterior to excretory pore. Ovary not extending up to pharynx. Oocytes in single row, oviduct indistinct, uterus short. Spermatheca obscure, postvulval uterine sac slightly more than 1.5 vulval body diam. long. Phasmids slightly posterior to midtail region, all four incisures extending posterior to phasmids. Tail dorsally convex-conoid with truncate terminus.

Male, not found.

Remarks. The material examined closely matched the published descriptions. A further phylogenetic analysis was made for a precise identification.

Telotylenchidae

***Nagelus camelliae* (Kheiri, 1972) Siddiqi, 1979**

Material examined. Mount Ararat, Turkey, 23.VII.2013, 4 ♀♀, altitude 4184, 3972, and 3757 m a.s.l., in wildflower meadow and mountain grassland.

Description (Table 5).

Female, body 0.8-0.9 mm, slightly ventrally curved after fixation. Lateral field with six incisures. Labial region slightly offset, with five annules. Cephalic framework weakly sclerotized. Stylet slender, 25-27 µm long with large knobs, sloping posteriorly. DGO 4.5-5.5 µm long. Pharynx 120-132 µm, median bulb oval. Nerve ring position slightly posterior to excretory pore. Body diameter 23-25 µm at midbody. Vulva equatorial (50%), vagina with thin walls. Spermatheca dorsally slightly offset. Tail conical, 72-78 µm long.

Male, not found.

Remarks. According to our observations, *N. camelliae* species from Mount Ararat have longer body (0.8-0.9 mm vs 0.59-0.73 mm) and longer tail (72-78 µm vs 39-51 µm) comparing with the original description (Geraert, 2019).

***Nagelus hexagrammus* (Sturhan, 1966) Siddiqi, 1970**

Material examined. Mount Ararat, Turkey, 23.VII.2013, 1 ♀ and 4 ♂♂, altitude 2337 and 1900 m a.s.l., in wildflower meadow and mountain grassland.

Description (Table 5).

Female, body 1.3 mm, slightly ventrally curved after fixation. Lateral field with six lines, occupying one-third of corresponding body diameter. Deirids finely visible on fourth line. Body slightly narrowing anteriorly, labial region with six annules, and cephalic framework prominent and strong. Stylet robust, 30 µm long, knobs somehow laterally elongated. DGO 6.7 µm. Pharynx long, 185 µm with oval median bulb. Nerve ring at 80% of pharynx length. Excretory pore anterior to basal bulb and more or less at the same level of nerve ring. Cardia hemispherical. Max width 30 µm at midbody. Genital branches short. Tail conical, 74 µm.

Male, general morphology of males are same as female. Body 1.0-1.3 mm. Stylet length 30-33 µm. DGO slightly longer than female, 7.5-8.5 µm. Tail longer than female, 95-104 µm. Spicules 32-36 µm, gubernaculum 11-13 µm. Anal body diameter 11-13 µm, bursa narrow, enveloping the terminus.

Remarks. This species was described from Germany and reported from Turkey (Elekçioğlu, 1996). *Nagelus hexagrammus* population from Mount Ararat closely matches the original description.

Table 5. Morphometrics of plant parasitic species from Mount Ararat (all measurements in µm)

Characters	*Geocenamus koreanus*		*Nagelus camelliae*	*Nagelus hexagrammus*		*Tylenchorhynchus mangiferae*		*Tylenchorhynchus maximus*
	♀♀	♂♂	♀♀	♀	♂♂	♀♀	♂♂	♀
n	5	5	4	1	4	3	2	1
L (mm)	0.69-0.76	0.67-0.72	0.8-0.9	1.1-1.3	1.0-1.3	0.61-0.65	0.54-0.58	1.1
a	28.0-29.0	26.0-28.0	31.0-32.0	37.3	36.0-38.1	31.6-31.8	32.5-33.1	44.7
b	6.4-8.6	6.5-8.4	6.4-7.2	6.0	6.1	5.1-5.4	5.8-6.0	6.9
b´	-	-	-	-	-	-	-	-
c	12.5-13.0	11.0-13.5	10.0-11.5	15.1	11.5	12.0-12.5	8.1	19.1
c'	3.1-3.3	2.9-3.2	4.2-5.1	2.9	4.7	3.5.3.6	3.0-3.3	3.3
V (%) or T %	54-56	38-40	50	54	33-35	54-56	42	50
N. lip annuli	6	6	5	6	6	5	5	5
Lip width (µm)	8.1-9.5	8.2-9.0	7.3-7.7	8.2	8.6-8.9	7.3-7.5	7.1-7.3	8.0
Lip height (µm)	3.2-5.5	3.3-5	4.2-4.5	5.0	5.4-5.8	3.5-3.7	3.2-3.4	3.7
Stylet (µm)	20.0-22.0	19.0-21.0	25.0-27.0	30.0	30.0-33.0	18.2-18.8	18.0-18.5	23.7
D.G.O.	2.2-3.4	2.0-3.2	4.3-5.6	6.7	7.5.8.5	2.0-3.0	2.5-3.0	4.0
Ex. Pore	85-97	79-86	102-110	150	154-156	111-114	110-113	154
Nerve ring	68-84	65-72	98-102	142.0	140-147	63-74	66-72	69
Pharynx length (µm)	109-119	105-112	120-132	185	180-193	120-136	118-120	162
Max. body diam. (µm)	22.3-24.0	22.0	23.4-25.0	30.0	31.0	19.5-20.0	18.0	25.0
ABD (µm)	17.0-19.0	18.0	17.0-18.0	26.0	19.0-22.0	14.5-15.0	14.0	18.0
Tail length (µm)	49.0-52.0	55.0-72.0	72.0-78.0	74.0	95.0-104.0	51.0-58.5	64.0-66.0	58.5
Spicules (µm)	-	25.0-27.0	-	-	32.0-36.0	-	26.5	-
Gubernaculum (µm)	-	6.1-7.2	-	-	11.2-13.0	-	7.7	-

Belonolaimidae

***Tylenchorhynchus mangiferae* (Luqman & Khan, 1986)**

Material examined. Mount Ararat, Turkey, 23.VII.2013, 3 ♀♀ and 2 ♀♀, altitude 3372, 3140, and 1900 m a.s.l., in wildflower meadow and mountain grassland.

Description (Table 5).

Female, habitus slightly arcuate ventrally. Head slightly off set from body with five annules, rounded shape, lateral fields with four lines, occupying one-third of corresponding body width; Stylet not strongly developed with rounded knobs. DGO 2-3 µm. Pharynx with oval median bulb and pyriform basal bulb, slightly distinct from intestine. Cardia hemispherical. Genital system amphidelphic with rounded spermathecae, vulva depressed, vagina appears with thin-walled structure. Tail bluntly rounded with fine annulations at terminus; pore-like phasmid at post anal region.

Male, crenate bursa surrounding tail. 22-23 µm long spicules, slightly ventrally curved, gubernaculum 8-10 µm long.

Remarks. Described from Uttar Pradesh, India. *Tylenchorhynchus mangiferae* population from Mount Ararat has a longer tail (51-58 µm versus 39 µm) than the type specimen.

***Tylenchorhynchus maximus* (Allen, 1955)**

Material examined. Mount Ararat, Turkey, 23.VII.2013, 1 ♀, altitude 3563, 3140, and 3053 m a.s.l., in chalk grassland and mountain grassland.

Description (Table 5).

Female, habitus strongly arcuate. Body widely annulated, annuli width 1.3-1.6 µm on pharyngeal region, narrowing on mid body. Lateral field with 4 lines occupying 8-10 µm of corresponding body diameter. Head rounded and slightly set off from body, 8 µm wide and 4 µm high. Five to seven distinct annules present in head construction. Delicate stylet around 22-23 µm long with small knobs posteriorly slightly oblique. DGO 3 µm. Median bulb rounded, basal bulb pyriform off set from intestine. Cardia conoid. Vulva without epiptygma structure. Tail subcylindrical to cylindrical with 30-32 annules to broad terminus. Phasmids posterior to anus.

Male, not found.

Remarks. *Tylenchorhynchus maximus* is widely distributed in different continents, and was first described from New York, USA, and has since been reported from North America, Europe, Pakistan (Maqbool & Shahina, 1987) and Trinidad (Gómez-Barcina et al., 1992). In our study morphometric measurements showed mostly the same numbers as it is described in Siddiqi, 2000.

Dolichoridae

***Geocenamus koreanus* (Choi & Geraert, 1971) Brzeski, 1991**

Material examined. Mount Ararat, Turkey, 23.VII.2013, 5 ♀♀ and 5 ♂♂, altitude range 2337-3144 m a.s.l., in mountain grassland.

Description (Table 5).

Female, habitus, body slightly curved ventrally. Longitudinal striations are prominently formed, resembling divided blocks with the transverse striations, each of them with 1.5 µm width. Lateral field occupying one fourth of the body width. Head slightly off set with six annules, and cephalic framework with six lips and slightly sclerotized. Conus of stylet very fine and delicate, measuring about 60% of the

spear length, and surrounded by a guiding apparatus consisting of a tubular and oval part, and stylet knobs well developed and flattened anteriorly. DGO about 2 µm. Nerve ring positioned in 60% of pharynx length. Median bulb oval. Deirids obscure. Excretory pore near to posterior end of isthmus. Vulva not more than 4 µm wide and provided with anterior and posterior epiptygma. Subcylindrical tail slightly tapering with widely rounded annulation at tip. Phasmids at posterior to anus.

Male, clearly developed hypoptygma observed in cloacal region. Conical and rounded tail. Bursa variable in size. Spicules 25-27 µm long, gubernaculum slightly curved and 6-7 µm long.

Remarks. Described from Korea and reported from Pakistan (Choi & Geraert, 1972) *Geocenamus koreanus* population from Mount Ararat differs from type population with slightly shorter body and stylet length.

Tylenchomorpha

***Malenchus* sp. Andrássy, 1968**

Material examined. Mount Ararat, Turkey, 23.VII.2013, 2 ♀♀ and 1 ♂, altitude 1523-3754 m a.s.l., in mountain grassland, chalk grassland, wildflower meadow and marshland.

Description (Table 4).

Female, body slender, ventrally arcuate. Cuticle 1 µm thick with fine transverse striae. Lateral field with crenate margins starting from 18 µm from anterior end and occupying about one-third of body diameter. Head elevated and slightly offset from body by small depression. Stylet delicate, 10 µm long with distinct knobs. Median bulb oval, basal bulb pyriform. Excretory pore 55-58 µm from anterior end. Female genital system prodelphic, ovary consisting single row of cells. Vulva transverse slit with vulval flaps at 82% of body length. Postvulval uterine sac short about 5 µm. Tail 98-101 µm, tapering gradually and pointed at tip.

Male, general appearance of both sexes similar. Spicules 14 µm, gubernaculum 3 µm in length. Bursa about 32 µm long.

Remarks. This species, showed closest relation to *Malenchus kausari* (Khan & Ahmad, 1991) in morphology. However, *Malenchus* sp. from Mount Ararat differs in having abundance of males in population and in several morphometric characters such as the pharynx is longer longer (114-162 µm vs 71-97 µm) and the vulva position is more posterior (82% vs 59-64% in *M. kausari*). The latter is exceptional for the genus.

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Influence of different grain storage types on Khapra beetle, *Trogoderma granarium* Everts, 1898 (Coleoptera: Dermestidae), infestation in southeastern Anatolia (Turkey) and its resistance to malathion and deltamethrin[1]

Güneydoğu Anadolu Bölgesi (Türkiye)'nde farklı depo tiplerinin Khapra böceğinin, *Trogoderma granarium* Everts, 1898 (Coleoptera: Dermestidae), bulaşıklığına olan etkisi ve zararlının malathion ve deltametrine olan direnci

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Abstract

This study was conducted to determine the effect of different storage types on the infestation of Khapra beetle, *Trogoderma granarium* Everts, 1898 (Coleoptera: Dermestidae), and its resistance to malathion and deltamethrin in southeastern Anatolia, Turkey. A total 355 various grain storage facilities (metal silo, concrete wall and basic plasterless) were surveyed in five provinces, (Diyarbakır, Mardin, Şanlıurfa, Adıyaman and Batman) during April-December in 2014-2016 and wheat grain samples were collected. Also, 24 populations of Khapra beetle were collected for bioassay studies. The provinces and storage types significantly influenced the infestation rate of Khapra beetle. The highest infestation of the beetle was recorded in Mardin (77.5%), followed by Şanlıurfa (67.5%). Whereas the lowest infestation observed was in Diyarbakır (43.4%) and Adıyaman (44.1%). For storage types, the highest infestation was observed in basic plasterless storage type (80.0%), while the lowest (27.1%) was noted for storage type of metal silos. Bioassay studies indicated that Khapra beetle has evolved low resistance to deltamethrin, whereas it was tolerant to malathion. Resistance ratios of the populations exposed to deltamethrin were 4-10.7 times, while the ratios for malathion were 1.32-1.92 times. It is concluded that the higher resistance ratios for deltamethrin were linked to its frequent use compared to malathion.

Keywords: Infestation, insecticide resistance, storage types, survey, *Trogoderma granarium*

Öz

Bu çalışma Güneydoğu Anadolu Bölgesi'nde farklı depo tiplerinin Khapra böceğinin, *Trogoderma granarium* Everts, 1898 (Coleoptera: Dermestidae), bulaşıklığına olan etkisi ve zararlının malathion ve deltametrine olan direncini belirlemek amacıyla yapılmıştır. Bölgedeki beş ilden (Diyarbakır, Mardin, Şanlıurfa, Adıyaman ve Batman) farklı tahıl depo tiplerinden (metal silo, betonarme ve basit sıvasız) 2014-2016 yıllarında nisan-aralık ayları arasında sürveyler yapılmış ve toplam 355 depodan buğday örnekleri alınmıştır. Ayrıca, direnç çalışmaları için farklı depolardan 24 Khapra böceği popülasyonu toplanmıştır. Çalışma sonucunda, iller ve depo tipleri Khapra böceği bulaşıklığına önemli derecede etki etmiştir. En yüksek bulaşıklık Mardin ilinde (%77.5), bunu takiben Şanlıurfa ilinde (%67.5) kayıt edilmiştir. Buna karşın en düşük Khapra böceği bulaşıklığı Diyarbakır (%43.4) ve Adıyaman (%44.1) ilinde kayıt edilmiştir. Depo tiplerine göre en yüksek bulaşıklık basit sıvasız depolarda (%80) belirlenmiş iken, en düşük bulaşıklık metal silolarda (%27.1) bulunmuştur. Direnç çalışmaları, Kapra böceğinin deltametrine karşı düşük düzeyde direnç geliştirdiği, buna karşın malathiona toleranslı olduğu göstermiştir. Deltametrin uygulanmış popülasyonların 4-10.7 kat direnç geliştirmiş olduğu, ancak malathion için bu rakamın 1.32-1.92 kat olduğu kayıt edilmiştir. Deltametrin için belirlenen yüksek direnç oranları, malathiona kıyasla bu insektisitin daha sık kullanımını ile ilgili olduğu düşünülmektedir.

Anahtar sözcükler: Bulaşıklık, insektisit direnci, depo tipleri, sürvey, *Trogoderma granarium*

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Introduction

Southeastern Anatolia is one of the important production areas for cereals in Turkey. The region produces ~15% of the national wheat production of the country (TUIK, 2017). Since cereals are harvested at a certain time and used throughout the year for human and animal feed, their safe storage is an important issue. Stored-grain pests, such as, *Sitophilus* spp. Schoenherr, 1838 (Coleoptera: Curculionidae) *Tribolium* spp. MacLeay, 1825 (Coleoptera: Tenebrionidae), *Rhyzopertha dominica* (Fabricius, 1792) (Coleoptera: Bostrychidae), *Trogoderma granarium* Everts, 1898 (Coleoptera: Dermestidae) and *Oryzaephilus surinamensis* (Linnaeus, 1758) (Coleoptera: Silvanidae), are responsible for weight, germination and quality losses during storage of wheat in Turkey (Ergül et al., 1972; Erakay, 1974; Özar & Yücel, 1982, 1988; Özer et al., 1989; Işıkber et al., 2005; Anonymous, 2008).

Khapra beetle (*T. granarium*) is one of the most important pests of stored wheat, and is subject to quarantine restrictions (Banks, 1977; USDA, 1983; Lowe et al., 2004; EPPO, 2005, 2007; French & Venette, 2005; Hasan et al., 2006; CABI, 2018). The beetle is included in the list of 100 worst invasive species worldwide (Lowe et al., 2004). Khapra beetle is very common in granaries, bins, silos as well as farmhouses in southeastern Anatolia due to suitable climatic and storage conditions. Wheat produced in the region is used commercially (i.e., stored and then processed); therefore, Khapra beetle should be carefully monitored in stored grains to avoid economic losses. Stored-grain pests are known to cause ~10% losses during storage of grain in Turkey (Emekçi & Ferizli, 2000).

The use of synthetic insecticides is the most common method of controlling agricultural insect pests around the world (Matthews, 1993; Sathyan et al., 2016; Wojciechowska et al., 2016); however, their excessive and unconscious use leads to the evolution of insecticide resistance (Fragoso et al., 2003; Ribeiro et al., 2003; Hasan et al., 2006; Wojciechowska et al., 2016). Aluminum phosphide fumigation is the most prevalent method used to control stored-grain pests in Turkey. However, stored-grain pests have developed resistance to phosphine used for the fumigation of grain storage facilities (Zettler & Keever, 1994; Benhalima et al., 2004; Pimentel et al., 2010). In addition, malathion has been used as a protective insecticide against stored-grain pests for a long time in Turkey. A synthetic pyrethroid (deltamethrin) was used excessively in grain storage facilities before the use of fumigation to control Khapra beetle and other stored-grain pests in the world as well as Turkey. Consequently, numerous researchers have reported that Khapra beetle has developed resistance against deltamethrin (Irshad & Iqbal, 1994; Tarakanov et al., 1994; Saxena & Sinha, 1995; Kumar et al., 2010; Hafiz et al., 2018). Also, malathion is more widely used in empty storage facilities than other registered insecticides in the country. During 1998, 297 t of pesticide were used against stored-product pests in Turkey (Emekçi & Ferizli, 2000). The use of same insecticide, for example malathion, for extended periods leads to the evolution of insecticide resistance in stored-grain pests (Champ & Dyte, 1976; Navarro et al., 1986). The frequent use of malathion has led to the evolution of resistance in *Tribolium castaneum* (Herbst) and *Sitophilus* spp. (Dyte & Blackman, 1970).

Khapra beetle usually has four to five generations per year and it can have 12 generations under suitable conditions. The female can lay 50-100 eggs, which are loosely scattered on host material (Harris, 2006; Szito, 2007). Larvae are able to hide in cracks and crevices of shipping containers, bulk cargo holds and packing material. Khapra beetle can stay in diapause for up to 6 years, until the onset of suitable conditions for development (Burges, 1962; Pasek, 1998; Stibick, 2007). Khapra beetle damages stored wheat by reducing weight and grade of the grain. The damage caused by Khapra beetle to stored wheat grain may reach to 73% (Rahman et al., 1945; Kalkan, 1963; Prasad et al., 1977). The control and eradication of Khapra beetle is difficult, which may reduce its susceptibility to some control methods (Ahmad, 1994). Phosphine gas fumigation, deltamethrin and malathion are widely used to control Khapra beetle in Turkey.

There are no studies reporting the distribution, infestation and resistance of Khapra beetle to certain insecticides in southeastern Anatolia region of Turkey. The only study related to the distribution and infestation of Khapra beetle in southeastern Anatolia dates back to 1982 (Özar & Yücel, 1982). A recent study in 2005 investigated the stored-grain pest species in Kahramanmaraş and Adıyaman Provinces, and found a lower infestation of Khapra beetle in Adıyaman (Işıkber et al., 2005). The precautionary measures based on the knowledge on the distribution of pest, resistance level to insecticides used could effectively control Khapra beetle. However, no such data are available for southeastern Anatolia. This study was

therefore conducted to determine the distribution, infestation and resistance level of Khapra beetle to malathion and deltamethrin insecticides in southeastern Anatolia. The results of the study will help to devise effective precautionary measures for reducing Khapra beetle infestation and subsequently damage to stored wheat grain in the region.

Material and Method

Field study

Infestation level of the Khapra beetle

Different storage types in five provinces (Adıyaman, Batman, Diyarbakır, Mardin and Şanlıurfa) in southeastern Anatolia were surveyed to investigate the infestation of Khapra beetle in the region. Various kinds of storage are used to store wheat grain in Turkey; however, the most prevalent storage types in southeastern Anatolia are metal silos, basic plasterless and reinforced concrete. Grain samples were collected from 355 different storage types (metal silos, basic plasterless and reinforced concrete) through April to December following Işıkber et al. (2005). Five different grain samples (~800 g each) were collected from five different points and depths of stored-grain using a 2-m long probe. The collected samples were pooled, which made a composite sample of 4 kg from each storage facility. The stored grain close to the walls and corners were also inspected for Khapra infestation. Samples were placed in plastic containers and brought to the laboratory. The presence of Khapra beetle (either larvae or adults) in the samples was visually determined in the laboratory. The samples were regarded as infested when presence of the beetle was confirmed. Whereas, where beetle presence was not observed in the samples, were regarded as uninfested. The percentage infestation was calculated as follows:

$$\text{Infestation (\%)} = 100 \times n / N$$

where, n is the number of infested samples and N is total number of samples.

Laboratory study

Collection of test populations

Test populations for resistance studies were collected from different storage types where malathion and deltamethrin have been extensively used. The grain samples were collected from the same five provinces of southeastern Anatolia where field study was conducted. A total of 24 putative resistant populations were collected for laboratory study. Also, a susceptible population was obtained from a on-farm storage where insecticides have never been used.

Insect cultures

Khapra beetle larvae were obtained from the collected grain samples from different types of storage. The insects were reared in glass jars (3 L) covered with muslin cloth. The jars were incubated in continuous darkness at 32±1°C and 60% RH (Hasan et al., 2006). The larvae were fed with bread wheat cv. Pehlivan. The cultured insects were reared for two generations until adequate number of insects was obtained for experiments. The reared insects were kept at 10±1°C and 60% RH until used in the experiments.

Selection of Khapra beetle populations for bioassay studies

The recommended dose (discriminate dose) of both insecticides (malathion and deltamethrin) was applied to the collected populations to select the populations for bioassay studies. However, 100% mortality was noted in all populations 24 h after the application of the insecticides, indicating absence of resistance in these populations. Several pretests were conducted on the available susceptible population, which indicated a higher sensitivity of the susceptible populations compared to the other susceptible populations used in the literature (Singh & Yadav, 1994). LC_{50} (lethal concentration$_{50}$) value of the available susceptible population was determined and two times of the LC_{50} was considered as discriminatory dose for the selection of populations for bioassay studies. Six populations were selected for use in bioassay studies. Adult individuals from the selected six populations were kept on sterilized grain of wheat cv. Pehlivan to oviposit for 2 d, after which the adults were removed. In this way, larvae with the same age (fourth instar) were obtained and used in the bioassay studies.

Preparation of pesticide solutions

The pure active ingredients of malathion and deltamethrin, produced by Sigma-Aldrich under the trademark Pestanal®, were used for the preparation of solutions in desired concentration. The active substances were first dissolved in pure acetone (Merck) and 100% stock solution was prepared. The target concentrations were then obtained by making dilutions of this solution with distilled water containing 0.02% Triton X-100 (Immaraju et al., 1989).

Bioassay studies

Film residue method

The method devised by Busvine (1971) was followed to test larval mortality in the film residue studies. Five different doses of malathion and deltamethrin were included in bioassay studies. Deltamethrin doses were 0.1, 0.2, 0.4, 0.8 and 1.6 ppm, while 10, 15, 20, 30 and 40 ppm doses were used for malathion. Bioassay experiments were conducted in Petri dishes (10 cm diameter). One ml solution of each dose was dropped by automatic pipette in each Petri dish. The Petri dishes were carefully shaken for the homogenous distribution of the solution. After drying at room temperature for up to 2 h, 25 larvae were released in each Petri dish. For control treatment, 0.02% Triton X-100 was used instead of insecticide. Larval mortality was recorded 72 h after treatment. The experiments were conducted in completely randomized design with four replicates of each dose of each insecticide and there was one Petri dish in each replicate.

Topical application method

Five different doses of both insecticides were used in the topical application experiment. Deltamethrin doses were 0.5, 1, 1.5, 2 and 2.5 ppm, whereas 50, 60, 70, 80 and 90 ppm doses were used for malathion. The fourth instar larvae were released in Petri dishes (25 larvae per dish) and 0.1 µl of the prepared solutions were applied to each larva by using Eppendorf micropipette following IRAC method 029 (www.irac-online.org/methods/euschistus-heros-adults-2). For control treatment, 0.1 µl of 0.02% Triton X-100 was applied to each larva instead of insecticides. The experiment was conducted in completely randomized design with four replicates. The Petri dishes were placed in an electronically controlled climate chamber at 25±1°C and 60% RH. Mortality was observed 72 h after the application of insecticides.

Statistical analyses

Analysis of variance (ANOVA) was used to test the differences among surveyed provinces and storage types using infestation percentage data. The normality in the data was tested prior to ANOVA by Shapiro-Wilk test, which indicated non-normal distribution. Therefor data was normalized by arcsine transformation method and two-way ANOVA was conducted on transformed data. Least significant difference test at 5% probability was applied where ANOVA indicated significant differences. The mortality percentage data of the bioassay studies was corrected using Abbott's formula (Abbott, 1925). The corrected mortality data was subjected to probit analysis (Busvine, 1971; Finney, 1971) and LC_{50}/LD_{50} values were calculated using the POLO Plus-PC software (LeOra, 1987). Resistance ratio was calculated by dividing the LC_{50}/LD_{50} of each population by the LC_{50} value of susceptible population. Confidence intervals were generated by POLO Plus-PC software as described by Robertson et al. (2007).

Results

Field study

Infestation level of the Khapra beetle

A total of 355 grain samples were collected from three different storage types in five provinces. Different provinces and storage types significantly influenced Khapra beetle infestation rate, whereas their interaction was non-significant (Table 1).

Table 1. Analysis pf variance of different provinces, storage types and their interactions on infestation rate of Khapra beetle in southeastern Anatolia, Turkey

Source of Variation	DF	Sum of squares	Mean squares	F Value	P Value
Province (P)	4	5531.88	1382.97	9.64	0.0001*
Storage Type (S)	2	17088.45	8544.23	59.53	0.0001*
P × S	8	1491.48	186.43	1.30	0.2968^{NS}

DF = Degree of freedom, * = significant at P ≤ 0.05, NS = non-significant.

Khapra infestation rate varied from 43.5 to 77.5% in the surveyed provinces (Table 2). Similarly, high variation was noted for storage types surveyed within different provinces. The number of metal silos in the surveyed provinces ranged from eight to 48, whereas the infestation rate varied from 15.6 to 50%. The number of concrete wall storage types varied from three to 57, whereas the infestation rate was between 44.4 and 84.6%.

Table 2. Infestation of Khapra beetle in wheat grain storage facilities in different provinces of southeastern Anatolia, Turkey

Sampled provinces	No. of facilities sampled	No. of infested facilities	Infestation (%)	No. of sampled populations
Adıyaman	34	15	44.1	2
Batman	25	13	52.0	2
Diyarbakır	145	63	43.5	9
Mardin	40	31	77.5	3
Şanlıurfa	111	75	67.6	8
Total	355	197	Average 56.92	Total 24

Similarly, the number of basic plasterless storage ranged from seven to 43 with an infestation range of 71.4-90.9% (Table 3). Overall, the highest infestation of Khapra beetle was noted for Mardin Province, followed by Şanlıurfa Province (Figure 1). Similarly, the lowest infestation of the beetle was noted for Adıyaman and Diyarbakır Provinces (Figure 1).

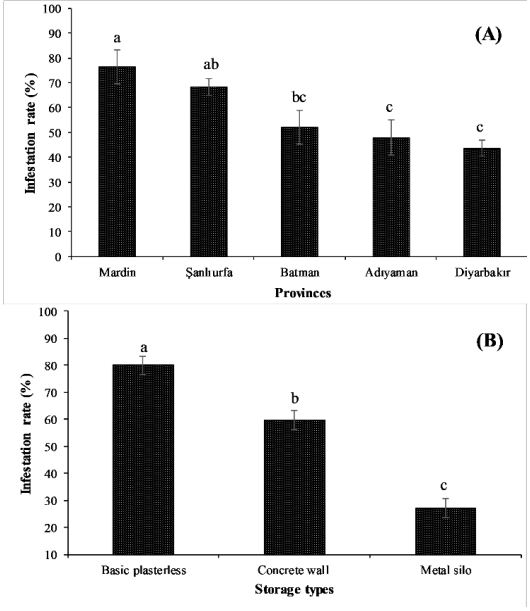

Figure 1. The influence of different provinces (A) and storage types (B) on Khapra beetle infestation in southeastern Anatolia region, Turkey.

For storage type, the highest Khapra infestation was recorded for basic plasterless storage, whereas the lowest infestation was noted for metal silos (Figure 1). The province by storage type interaction was not significant for Khapra infestation.

Table 3. Infestation level of Khapra beetle in different storage types of southeastern Anatolia, Turkey

Metal Silos			
Provinces	Collected Samples	Infested Samples	Infestation (%)
Adıyaman	10	2	20.0
Batman	8	2	25.0
Diyarbakır	45	7	15.6
Mardin	12	6	50.0
Şanlıurfa	48	19	39.6
Total	123	36	Mean 30.0
Concrete Wall			
Adıyaman	13	6	46.2
Batman	10	6	60.0
Diyarbakır	57	23	40.4
Mardin	17	15	88.2
Şanlıurfa	39	33	84.6
Total	136	83	Mean 63.9
Basic Plasterless			
Adıyaman	9	7	77.8
Batman	7	5	71.4
Diyarbakır	43	33	76.7
Mardin	11	10	90.9
Şanlıurfa	26	23	88.4
Total	96	78	Mean 81.0

Bioassay studies

Film residue method

A high variation was observed in LC_{50} values and resistance ratio of susceptible control population and the test populations for deltamethrin, whereas little variation was recorded for malathion (Table 4). The highest LC_{50} values were observed for Mardin, Şanlıurfa and Batman populations exposed to deltamethrin, while the lowest LC_{50} value was detected in susceptible populations. The resistance ratios of Mardin, Şanlıurfa and Batman populations were 10.7, 8.09 and 7.43 times, respectively (Table 4). The resistance ratio of the test populations exposed to malathion ranged from 1.47 to 1.92 times with the highest resistance ratio in Şanlıurfa population. Overall the resistance ratios of the populations exposed to deltamethrin were higher than malathion exposure (Table 4).

Table 4. LC_{50} values of different Khapra beetle populations treated with different doses of malathion and delthamethrin in film residue method

	Deltamethrin				Malathion			
Population	LC_{50}	CI 95%	Slope	RR	LC_{50}	CI 95%	Slope	RR
Susceptible	0.03	0.02-0.04	0.89±0.13	Ref.	19.45	14.1-25.7	3.44±0.31	Ref.
Batman	0.26	0.15-0.82	0.66±0.14	7.43	28.63	21.6-51.8	2.41±0.29	1.47
Diyarbakır-1	0.13	0.08-0.25	0.67±0.13	3.71	31.36	23.3-67.8	2.46±0.30	1.61
Diyarbakır-2	0.14	0.09-0.31	0.64±0.13	4.11	32.79	24.8-64.5	2.35±0.30	1.69
Diyarbakır-3	0.12	0.08-0.32	0.66±0.13	3.69	35.37	30.7-43.4	2.33±0.30	1.82
Mardin	0.37	0.20-1.54	0.64±0.14	10.7	33.74	24.9-81.8	2.44±0.30	1.73
Şanlıurfa	0.28	0.15-1.22	0.57±0.13	8.09	37.26	27.6-91.8	2.50±0.32	1.92

CI = confidence interval; RR = resistance ratio.

Topical application method

Slight variation was noted in LD_{50} values and resistance ratio of susceptible and test populations exposed to deltamethrin and malathion (Table 5). Overall the LD_{50} values and resistance ratios were higher for the populations exposed to deltamethrin than those for malathion (Table 5). The highest LD_{50} values were observed for Mardin, Şanlıurfa and Batman populations exposed to deltamethrin as noted in film application method. The resistance ratios of Mardin, Batman and Şanlıurfa populations were 4.00, 3.27 and 3.22 times, respectively (Table 5). The resistance ratio of the test populations exposed to malathion ranged from 1.18 to 1.29 times with the highest resistance ratio in Diyarbakır-3 population (Table 5).

Table 5. LD_{50} values of different Khapra beetle populations treated with different doses of malathion and delthamethrin in topical application method

	Deltamethrin				Malathion			
Populations	LD_{50}	CI 95%	Slope	RR	LD_{50}	CI 95%	Slope	RR
Susceptible	0.102	0.05-0.14	1.44±0.23	Ref.	68.78	64.3-73.4	8.14±0.73	Ref.
Batman	0.334	0.23-0.72	1.04±0.24	3.27	80.85	74.2-93.5	5.89±0.72	1.18
Diyarbakır-1	0.222	0.17-0.34	1.19±0.24	2.18	84.01	75.6-106.2	5.81±0.73	1.22
Diyarbakır-2	0.246	0.18-0.42	1.09±0.24	2.41	86.15	81.2-93.9	5.54±0.73	1.25
Diyarbakır-3	0.282	0.21-0.49	1.20±0.24	2.76	88.63	83.3-97.3	5.63±0.75	1.29
Mardin	0.408	0.27-1.01	1.13±0.25	4.00	86.33	77.8-109.2	6.00±0.76	1.26
Şanlıurfa	0.328	0.22-0.80	0.99±0.24	3.22	90.66	82.04-112.27	5.99±0.73	1.32

CI = confidence interval; RR = resistance ratio.

Discussion

The field study indicated that different provinces and storage types significantly influenced the Khapra beetle infestation in southeastern Anatolia. The most infested provinces were Mardin and Şanlıurfa, and the lowest were Diyarbakır and Batman Provinces. Although the highest number of storage facilities was surveyed in Diyarbakır Province, the beetle infestation was the lowest in that province. The interprovincial differences may arise due to management options used to control Khapra beetle, storage types and conditions, product circulation, storage sanitation and farmer awareness. A study conducted in 1982 in southeastern Anatolia revealed no significant effect of storage types on the infestation rate of

Khapra beetle (Özar & Yücel, 1982). However, the current study indicated significant effect of the provinces and storage types. These differences could be explained by the improvements in storage types over the last two decades in the region. Metal silos, which were not prevalent in 1982, have significantly increased with rising awareness of the private companies storing wheat grain.

Moreover, wheat-dependent industrial zones of Mardin and Şanlıurfa Provinces have large number of flour, bulgur and feed factories, and cereals are purchased from different provinces in southeastern Anatolia. The grain is stored to ensure the continuous availability for processing. The main storage type in the region is reinforced concrete, which is owned by most of the wheat grain traders. Metal silos are not widely used because of their high investment cost. Therefore, surveyed storage types in the study were mostly reinforced concrete. Whereas, the storage owned by wheat farmers are generally simple plasterless type with briquette. Cracks and crevices were frequently observed in reinforced concrete and basic plasterless storage during the survey.

Khapra beetle has a refuge seeking behavior (Bell & Wilson, 1995; EPPO, 2005; French & Venette, 2005; Harris, 2006; Anonymous, 2008); therefore, cracks and crevices found in reinforced concrete storage provide shelter to the beetle. Grain in factory storage is used in the manufacturing process within a short time, and the grain is continually stored without frequent use of insecticides to control Khapra beetle and other pests. For this reason, a significant increase in infestation level and distribution of various pests could easily occur in these storage situations. The highest infestation level was noted for basic plasterless storage, which was followed by reinforced concrete storage. The main reason of high infestation rate in the region is unsatisfactory control of Khapra beetle due to unsuitable storage conditions and incorrect application of insecticides (unpublished field observations).

The cracks and crevices formed in the walls made of bricks, mud, concrete or stone provide highly suitable shelter for Khapra beetle, making control of the beetle almost impossible. Furthermore, the leftover grain (when the storage facilities are emptied) remains permanently in the cracks and crevices of these facilities, providing a long-term food source for the beetle (EPPO, 2005; French & Venette, 2005; Harris, 2006; Anonymous, 2008). Since Khapra beetle individuals settled in the crevices and cracks do not come in contact with the insecticides applied, sustainable control and eradication becomes impractical and expensive (Lindgren & Vincent, 1959; Ahmad, 1994; Harris, 2006; Saidana et al., 2010; Singh et al., 2017). Moreover, ensuring sufficient gas tightness in reinforced concrete and basic plasterless storage facilities is difficult; the desired level of fumigation success cannot be achieved in such storage types. However, the beetle infestation was the lowest in the metal siloes. The gas tightness, adjustable temperature and relative humidity, controlled environmental conditions (Fidan & Satuk, 2011; Pekmez, 2016), relatively less crevices and cracks, easy cleaning and subsequent high sanitation, high awareness and trained workers in these storage types are the reasons linked with the low infestation of Khapra beetle. Low temperature forces the pest to enter diapause and metal silos are usually equipped with aeration system that reduces the temperature of the grain bulk. Therefore, lower temperature of the metal silos is another reason of reduced infestation of the pest. Under the subtropical climate of Israel Khapra beetle was suppressed in the grain bulks after the introduction of aeration system (Navarro et al., 1969).

The management of stored-grain pests is as important field as grain production (Ahmad, 1994). The insecticides are generally applied in March-April in empty storage facilities for controlling Khapra beetle in the southeastern Anatolia. The insecticide application time coincides the inactive period of the beetle in concrete wall and basic plasterless storage types; thus, leading to poor control. Whereas, the metal silos heat up earlier than the other storage types and insecticide application timing coincides with the active period of the beetle, giving better control. The reduced infestation in metal silos compared to other storage types could also be explained by better control of the beetle compared to the other storage types. Khapra larvae enter diapause when temperatures fall below 25°C or when populations are very dense (Anonymous, 1978). Therefore, insecticides should be applied in the storage facility when temperature is >25°C for effective control of Khapra beetle.

Complete mortality was recorded in all populations in preliminary tests to select the populations for bioassay studies. These results indicated that the selected populations have not become resistance to deltamethrin and malathion insecticides. Similar to the results of current study, Dörtbudak et al., (1987) also reported that Khapra beetle has not developed resistance to malathion. However, several researchers have indicated that excessive use of deltamethrin and malathion led to resistance development in Khapra beetle and other stored-grain pests (Dyte & Blackman, 1970; Champ & Dyte, 1976; Irshad & Iqbal, 1994; Tarakanov et al., 1994; Saxena & Sinha, 1995; Kumar et al., 2010; Hafiz et al., 2017, 2018). The absence of insecticide resistance in our studies could be explained by the lower use of these pesticides compared to other countries where resistance has been confirmed. Unfortunately, no data on the use of deltamethrin and malathion insecticides is available for southeastern Anatolia to strengthen our argument. Therefore, a detailed survey study is needed to obtain the data on insecticide use and link it with the absence of resistance in the region.

Although preliminary studies conducted with recommended dose indicated absence of resistance, using double the LC_{50}/LD_{50} of susceptible population suggested some resistance in six populations. The bioassay studies indicated that the highest resistance ratio for the populations exposed to deltamethrin was 10.6 for the Mardin population (Table 4), while the highest resistance ratio for the populations exposed to malathion was recorded for Şanlıurfa (Table 4). Testing resistance with two different methods yielded almost similar results for resistance ratios and LC_{50}/LD_{50} values (Tables 4 & 5). Nonetheless, deltamethrin is preferred over malathion due to its weaker smell in empty storage facilities and easier availability in the market (unpublished field observation). Therefore, deltamethrin is more frequently used than malathion, which resulted in higher resistance ratio for deltamethrin compared to malathion. The results of Singh & Yadav (1994) support our finding that Khapra beetle was more resistant to deltamethrin than malathion. Overall, the results of the current study indicate that the beetle has not developed resistance to the frequently used pesticide; however, slight resistance was observed compared with the susceptible populations. These results suggest that intensive use without rotating the insecticides with different mode of action/active ingredients could lead to the development insecticide resistance in future. Therefore, the use of these insecticides should be carefully monitored and farmers should be warned of the possible negative outcomes of over using these insecticides.

The successful management of stored-grain pests requires sound knowledge of their distribution, infestation ratio, biology of pests and storage conditions (such as, sanitation, temperature, relative humidity, prevention of new infestations, and avoiding use of old and new products in the same storage). The current study has provided useful information on the infestation and storage conditions, which provide valuable insights for effective control of Khapra beetle in the region. In conclusion, use of metal silos should be encouraged in the region and use of insecticides and other management options should be improved based on the biology of insect, product circulation and environmental conditions.

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Original article (Orijinal araştırma)

Investigation of the damage of Miridae species on cotton in Çukurova Region of Turkey

Çukurova Bölgesi (Türkiye)'nde Miridae türlerinin pamukta meydana getirdikleri zararın araştırılması

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Abstract

Damage of the Miridae species on cotton was determined in cotton fields in two locations of Adana Province (Çukurova Region), Turkey during the years, 2013-2014. Experiments were conducted over 2 years with sprayed and unsprayed plots. *Creontiades pallidus* (Rambur, 1839) and *Lygus italicus* Wagner, 1950 were detected at both experimental areas. Also, the correlations between the mirid population and stained bolls have been investigated. The damage caused by the mirids on squares and bolls was measured in caged branch experiments. Mirid population increased from mid-July reaching the highest population density in late July or early August. Where mirid numbers were high, stained bolls and shed bolls were also high. As the number of *C. pallidus* nymphs released into the cages increased, the damage rates increased on the squares after 7 and 14 d. In cages with zero, one, two and four individuals released, damage rates in squares were 0, 0.9, 5.9 and 33.6%, respectively, after 7 d, and 0, 22.2, 41.4 and 58.0%, respectively, after 14 d. In addition, all bolls with a diameter of 0.5-0.9 cm after 7 d in cages with nymphs released were damaged.

Keywords: Boll, cotton, damage, Miridae, square

Öz

Adana İli (Çukurova Bölgesi), Türkiye'de pamuk tarlalarında 2013 ve 2014 yıllarında iki farklı bölgede yürütülen çalışmada Miridae türlerinin pamukta meydana getirdikleri zararlar tespit edilmiştir. Denemeler iki farklı lokasyonda ilaçlı ve ilaçsız parseller şeklinde 2 yıl üst üste kurulmuştur. Deneme yapılan 2 tarlada hem *Creontiades pallidus* (Rambur, 1839) hem de *Lygus italicus* Wagner, 1950 türü bulunmuştur. Ayrıca, mirid popülasyonu ile lekeli kozalar arasındaki ilişki incelenmiştir. Miridlerin tarak ve kozalarda oluşturduğu zarar dal-kafes denemeleriyle ortaya konulmuştur. Mirid popülasyonu temmuz ayı ortasından sonra artmış, temmuz sonu veya ağustos başında en yüksek noktaya ulaşmıştır. Mirid popülasyonu yüksek olduğunda, lekeli koza sayısı ve yere dökülen kozaların sayısı da yüksektir. *C. pallidus*'un kafeslere salınan nimf sayıları arttıkça 7 ve 14 gün sonunda taraklarda verdikleri zarar oranlarının da arttığı görülmüştür. Sıfır, bir, iki ve dört birey salınan kafeslerde taraklarda zarar oranları 7 gün sonra sırasıyla %0, 0.9, 5.9 ve 33,6 bulunmuşken, 14 gün sonra %0, 22,2, 41,4 ve 58,0 bulunmuştur. Ayrıca kozalardaki zarar oranı, 0.5-0.9 cm çaplı kozalarda 7 gün sonra kafeslerde tüm bireyler için %100 bulunmuştur.

Anahtar sözcükler: Koza, pamuk, zarar, Miridae, tarak

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Introduction

The Miridae family is the largest family of insects (Hemiptera: Heteroptera) with about 1400 genera containing over 10,000 described species (Schuh, 1995). Miridae species are both pests and predators of some important pests depending on environmental conditions (zoophytophagous species). Miridae species globally cause significant damage in a wide array of hosts such as cotton, clover, soybean, mung bean, strawberry, sorghum, cocoa, apple and tea (Wheeler, 2001). In some parts of the world where transgenic cotton cultivars are grown, the mirids were dominant species due to a reduced number of insecticide sprays against *Helicoverpa armigera* Hübner, 1827 and the increased use of targeted selective insecticides in recent years. Most chemical control is against cotton pests including the mirids in China and the USA. (Wu et al., 2002; Musser et al., 2009). Mirids become important especially for the damage which they give to generative organs in cotton. They suck leaves, shoots, squares (floral buds), flowers and young bolls and cause damage of drying and shedding. *Lygus hesperus* Knight, 1917 (Hemiptera: Miridae) adults and nymphs feeding on small and medium sized squares cause shedding after 3-4 d (Hake et al., 1996). Some studies conducted in the USA found that *L. hesperus* negatively affects yield mostly by feeding on fingers (Jubb & Carruth, 1971; Mauney & Henneberry, 1979; Bailey, 1982; Leight et al., 1988; Zink & Rosenheim, 2005). Karman & Akşit (1961) identified as *Lygus pratensis* Leston, 1957, *Creontiades* spp. and *Creontiades pallidus* (Rambur, 1839) (Hemiptera: Miridae) in cotton fields in Aydın (Turkey). In addition, Efil & İlkan (2003) and Efil & Bayram (2009) reported that *C. pallidus* causes shedding of young bolls of the cotton in the southeastern Anatolia (Turkey). While there are many reasons for shedding of squares and bolls, this shedding can reach significant levels at times when the mirids are high density. Efil & İlkan (2003) monitored the population development of *C. pallidus* in cotton fields on the Harran Plain (Şanlıurfa, Turkey). During a 3-year study, the pest population reached the peak density during the last 3 weeks of August and the first week of September.

Çukurova is one of the important production areas for cotton. In the region, farmer concerns have increased in recent years due to damage caused by mirids, as well as the main pests thrips, cotton aphids, leafhoppers and whiteflies. Despite concerns about the increase of mirid damage in the cultivated area of cotton in Turkey, studies on this pest have been quite limited. This study aimed to reveal some important features of pest control. For this purpose, experiments were conducted in Çukurova in order to determine the population fluctuations of pest mirids in cotton fields in two locations. In addition to the development of mirid populations, stains on bolls and mirid damage in generative organs were investigated.

Material and Methods

Sampling of harmful mirids

In order to investigate the damage status of the mirids on cotton, sprayed plots (20 rows x 10 m, 142.5 m^2) which were kept at the lowest level of the pest and a unsprayed plot (20 rows x 10 m, 142.5 m^2) which allow a certain population of mirids were laid out with four replicates. Plots were sprayed at intervals of 2 weeks during the season to keep the mirid population at the lowest level (Table 1). Experiments were established in Çukurova University Research and Implementation Area Balcalı, and Research Area of the Eastern Mediterranean Agricultural Research Institute, Hacıali, Adana, Turkey in 2013 and 2014.

Two sampling methods were used to determine the chemical control of the mirids according to the Integrated Management Technical Instructions; (i) by direct counting of generative organs (economic threshold: four mirids per 100 generative organs after square formation, 20 mirids after 80% of bolls begin to mature), and (ii) by sweep net (economic threshold: seven mirids per 50 sweep net after square formation, 30 mirids after 80% of bolls beginning to be mature) (TOB, 2017). The population fluctuations of harmful mirids were recorded weekly in the mornings (8-10 am) by both methods from the 2-3 leaf stages until harvest (in Hacıali from 2 July to 17 September 2013 and 18 June to 10 September 2014, and in Balcalı from 3 July to 18 September 2013 and 12 to 4 September 2014). For sweep netting, the net was swept 25 times in four subplots to a total of 100 times for each insecticide-sprayed and unsprayed plot. Every 25 sweep samples were transferred to fabric bags (30 x 40 cm) and labeled.

Table 1. Insecticides which were applied for the mirids in experiment areas

Hacıali		Balcalı	
2013	2014	2013	2014
Dimethoate 1 l/ha (9 July)	Thiacloprid & Deltamethrin 0.8 l/ha(3 July)	Dimethoate 1 l/ha (4 July)	Thiacloprid & Deltamethrin 0.5 l/ha (19 June)
Dimethoate 1 l/ha (23 July)	Thiacloprid & Deltamethrin 0.8 l/ha (18 July)	Dimethoate 1 l/ha (19 July)	Thiacloprid & Deltamethrin 0.8 l/ha (5 July)
Thiacloprid & Deltamethrin 0.5 l/ha (6 August)	Thiacloprid & Deltamethrin (0.8 l/ha) Cypermethrin (0.3 l/ha)	Thiacloprid & Deltamethrin 0.5 l/ha (5 August)	Thiacloprid & Deltamethrin (0.8 l/ha) Cypermethrin (0.6 l/ha)
Thiacloprid & Deltamethrin 0.5 l/ha (21 August)	Thiacloprid & Deltamethrin (0.8 l/ha) Cypermethrin (0.3 l/ha)	Thiacloprid & Deltamethrin 0.5 l/ha (16 August)	Dimethoate 1 l/ha (22 July)
Thiacloprid & Deltamethrin 0.5 l/ha (12 September)	Thiacloprid & Deltamethrin 0.8 l/ha (21 August)	Thiacloprid & Deltamethrin 0.5 l/ha (6 September)	Thiacloprid & Deltamethrin (0.8 l/ha) Cypermethrin (0.3 l/ha)
			Thiacloprid & Deltamethrin (0.8 l/ha) Cypermethrin (0.3 l/ha)

For direct counting, 25 squares, 25 flowers (10 flowers during the periods when the numbers are decreasing; in Hacıali from 23 July to 10 September 2013 and 31 July to 20 August 2014, and in Balcalı from 24 July to 11 September 2013 and 24 July to 14 August 2014), 25 bolls (mostly young green bolls) and 25 opened bolls were randomly sampled in each insecticide-sprayed and unsprayed plot. Nymphs and adults of the two Miridae species were easily distinguished. Nymphs and adults of *Lygus italicus* Wagner, 1950 have dark spots on the pronotum and scutellum.

Detection of stains on bolls

At the beginning of boll formation, 25 bolls from four subplots of each insecticide-sprayed and unsprayed plot were examined at weekly intervals. The stains caused by the mirid feeding were counted and recorded.

Determination of boll shedding

In each unsprayed subplot, 3 rows of cotton 3-m long were selected for sampling and marked. On each sampling date, shed bolls were collected from the ground below the plants. Shed bolls were cut to record color changes in anthers, damage to anther sac, color changes on external surfaces of squares, color changes in fruit tissue, and stain density on the boll surface. If there was no insect damage evident, the shedding of these bolls was assumed to have been physiological.

Determination of *Creontiades pallidus* damage in caged branches

Rearing of insects

Creontiades pallidus was collected by sweep net in a clover field (cultivar Elçi) in Balcalı (37º02'16.7" N, 35º22'12.5" E). *Creontiades pallidus* was the common species and regularly found in alfalfa fields. Under laboratory conditions (climatic chamber at 25±1ºC, 60% RH, 16:8 h L:D photoperiod), they were fed on green fresh beans and laid eggs on these beans. Hatched nymphs were fed on the fresh green beans obtained from the local market.

Determination of the damage on squares

Two cage experiments were conducted in 2014 in Balcalı. In the first experiment, two healthy squares selected from the upper half of the plant, were placed in a cage with dimensions of 47 x 27.5 cm (49 holes/cm^2, i.e., 10 mesh). The branches were cleared of other insects before Miridae insects were added to cages. The plant and the cages were labeled. Squares larger than 0.3 cm in diameter were selected for these cages. Fruiting branches were selected on the same position of the plants. Then, fourth stage nymphs of *C. pallidus* were released as one, two and four individuals into each cage on 25 June 2014. Also, the control cages were established with no nymphs released. Seven d after the releasing of the nymphs, fruit branches were cut and taken to laboratory. Squares were examined under the stereomicroscope with X45 magnifications (1 July 2014). The experiment was replicated 11 times.

A second experiment was established on 26 June 2014 as above but with 10 replicates. Fourteen d after nymph release, fruit branches were taken to laboratory and examined (10 July 2014). Assessments were made as in the first experiment; the damage symptom was investigated. The squares were examined individually. Damage indications in squares such as discoloration at the anther, softening and deformation in the tissues, shrinkage, dryness or shedding were examined and evaluated as observable percentage loss.

Determination of the damage on bolls

Damage indications in the squares and the bolls such as discoloration at the anther, softening and deformation in the tissues, shrinkage, dryness or shedding were examined and evaluated as observable percentage loss (Figure 1).

Figure 1. Some damage views caused by the Miridae species in bolls and squares: a) stains on the boll, b) rots in the boll tissue, c) desiccation of the boll, d) rots in the square tissue, and e) desiccation of the square.

Cage experiments for boll damage were conducted on two dates in Balcalı and Hacıali locations (Adana Province) in 2014. First experiment was established in Balcalı on 14 July 2014. One healthy boll larger than 1 cm in diameter on the upper half of the plant, were placed into cages, and the experiment conducted as for the caged squares as described above. Seven d after releasing the nymphs, fruit branches were taken to laboratory and they were examined (21 June 2014) for Miridae damage. Diameters of the bolls were measured by caliper. The experiment had 20 replicates. A second experiment was established in Hacıali on 17 July, healthy bolls larger than 0.5 cm in diameter caged and again treated as above. Seven d after releasing the nymphs, fruit branches were taken to laboratory and examined (24 June 2014). The second experiment had five replicates. The bolls were examined individually under the stereomicroscope with X45 magnifications and damage assessed as described above.

Statistical analysis

Relationships between the Miridae population, and stained and shed bolls for both sampling methods were analyzed. The average number of stains on bolls in unsprayed and sprayed plots were compared by simple t-test. Differences in damage rates of *C. pallidus* on the squares and bolls in the cage experiments were evaluated by analysis of variance (one-way ANOVA). Differences between means were grouped by Duncan's test ($P < 0.05$).

Results and Discussion

Relationship between mirid populations and number of stains on bolls

Both *C. pallidus* and *L. italicus* species were present in the experimental fields. The apparent damage of the Miridae species on the bolls was often dark round, slightly precipitated stains, which resulted from sucking.

In 2013 in Hacıali, Miridae populations reached the highest number on generative organs on 6 August (14.5 mirids/100 generative organs) in unsprayed plots. When mirid population peaked, the number of stains due to mirid feeding increased. There was a significant and positive relationship between Miridae population density and number of stains on bolls ($r^2 = 0.72$, $y = 0.522x + 1.32$) (Figure 2).

On the generative organs in sprayed plots, the mirid population reached the highest density on 23 July (8.17 mirids/100 generative organs). However, the number of stains was highest on 6 August. So, there was no close relationship between the mirid population and stain density on the bolls ($r^2 = 0.27$, $y = 0.350x + 0.863$) (Figure 2).

In unsprayed plots, mirid population reached the highest number on 30 July (15.5 mirids/sweep net) as determined by sweep net sampling (Figure 2). The number of stains on the bolls also increased following the pest population density. The mirid population generally decreased after 6 August, but had a small increase on 2 September. Due to the short-term increase in the mirid population in September, the number of stains increased a little. However, there was no relationship between mirid population densities and stain densities on the bolls ($r^2 = 0.086$, $y = 0.18x + 2.23$).

In the sprayed plots, mirid populations were low during 16 to 30 July, but then reached the highest level with a mean of 17 mirids/sweep net on 6 August (Figure 2). The number of stains on the bolls increased following the increase of the pest population and decreased following the decrease of the pest population. As a result, there was a significant positive relationship ($r^2 = 0.51$, $y = 0.290x + 0.650$). The densities of stains on bolls in the sprayed plot were lower than found in unsprayed plots.

The mirid population exceeded the economic threshold for the two sampling methods in the early period for unsprayed and sprayed plots.

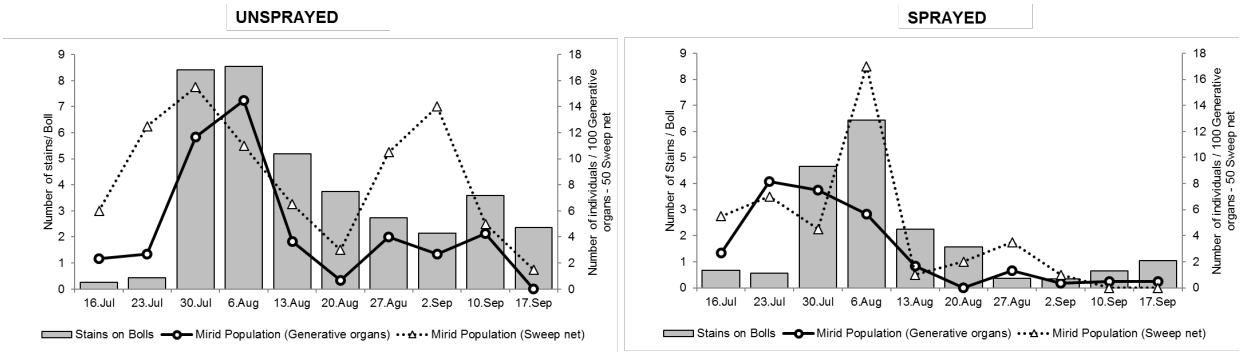

Figure 2. The relationship between numbers of mirids (counted directly and by sweep net) and densities of stains on the bolls in unsprayed and sprayed plots in Hacıali in 2013.

In 2014 in Hacıali, stained bolls appeared after the decrease in the mirid population in unsprayed plots (Figure 3). On 27 August, the number of stains on the bolls increased relatively on the other sampling dates. In other words, the numbers of mirids on generative organs were lower on sampling dates when the dense stains were observed on the bolls. There was no correlation between the number of stains and the number of pests on the bolls ($r^2 = 0.04$, $y = -0.156x + 0.791$) (Figure 3).

In the generative organs of sprayed plots, following a slightly increase in the mirid population, number of stained bolls increased on that date. Although the mirid population decreased after 13 August, there was a slight increase in the number of stains on the bolls. No correlation was detected ($r^2 = 0.1$, $y = 0.149x + 0.281$) (Figure 3).

In the unsprayed plots, the number of stains increased in early August and early September following mirid feeding (Figure 3). There was no relationship between the number of stains and the number of individuals ($r^2 = 0.02$, $y = -0.056x + 0.792$).

Mirid population determined by sweep net was low throughout the season in the sprayed pots. The number of stains on bolls had increased by a low rate by 13 August and the following dates. The numbers of mirids counted by both methods were slightly lower than those found in unsprayed plots. The densities of stains were also found to be lower than that of unsprayed plots (Figure 3).

Economic threshold was not exceeded in two sampling methods during the season for unsprayed and sprayed plots.

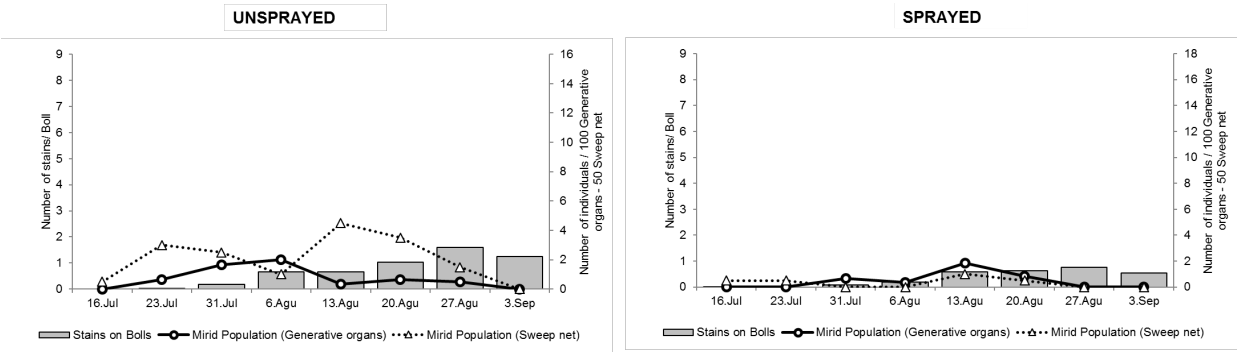

Figure 3. The relationship between numbers of mirids (counted directly and by sweep net) and densities of stains on the bolls in unsprayed and sprayed plots in Hacıali in 2014.

In 2013 in Balcalı, in the unsprayed plots, the number of stains on bolls increased from 31 July to 21 August. During this period, the population density of mirids on the generative organs increased for a short time, but then remained stable. After that date, although the numbers of the pest increased for a short time, the density of stains on bolls decreased. There was no relationship between the number of stains and the number of mirids ($r^2 = 0.13$, $y = -0.354x + 2.34$) (Figure 4).

In sprayed plots, the density of the mirid population on the generative organs remained low throughout the cotton growing season (Figure 4). No correlation was found between the number of mirids and number of stains on bolls during the season ($r^2 = 0.002$, $y = -0.0472x + 1.03$).

For sweep net sampling, the number of stains increased together with the mirid population, which had stable densities throughout the season in the unsprayed plots until 21 August. Although numbers of stains apparently increased according to population increase of pest mirids, no significant relationship was found between the number of stains and the number of pests ($r^2 = 0.16$, $y = 0.262x + 0.913$).

Population density of mirids reached 4 individuals/50 sweep net on 31 July in sprayed plots. Although number of mirids decreased after this date, the number of stains did not decrease (Figure 4). There was no correlation between them ($r^2 = 0.07$, $y = 0.108 + 0.848$) (Figure 4). During the season, relatively higher mirid populations in unsprayed plots than sprayed plots caused higher stain density.

Mirid population did not exceed the economic damage threshold in both sampling methods during the season for unsprayed and sprayed plots.

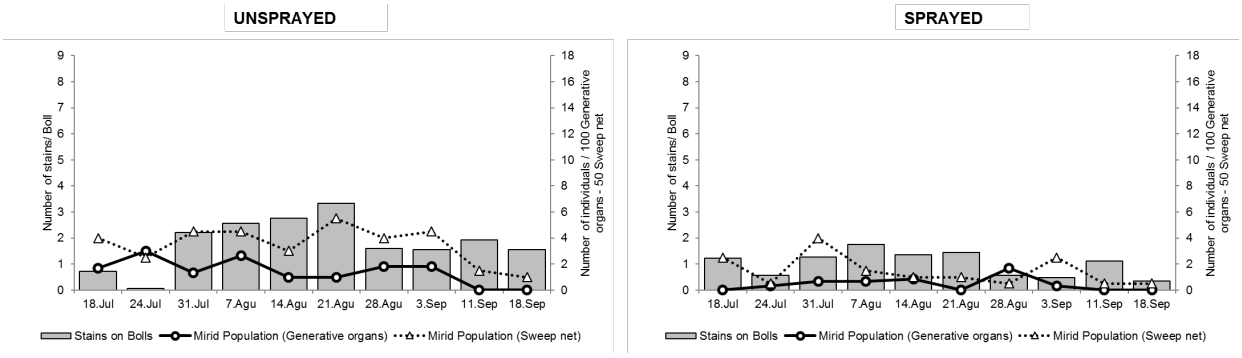

Figure 4. The relationship between numbers of mirids (counted directly and by sweep net) and densities of stains on the bolls in unsprayed and sprayed plots in Balcalı in 2013.

In 2014 in Balcalı, mirid populations on the generative organs in unsprayed plots increased in the last week of July and the first week of August. When the mirid population reached the highest level, on 31 July, the density of stains on the bolls was found to be relatively low. There was no relationship between number of individuals and number of stains ($r^2 = 0.04$, y = -0.0686x + 1.68) (Figure 5).

The mirid density on generative organs in sprayed plots peaked on 31July. Similar to the other sampling, numbers of the stains increased after the high mirid population occurred (Figure 5). However, there was no relationship between the number of stains and the number of pest ($r^2 = 0.17$, y = -0.0672x + 0.782).

In unsprayed plots, population densities of mirids were similar for direct and sweep net counting as well as by stain sampling of bolls. The pest population increased on 31 July, while the density of stains increased towards the end of August (Figure 5). There is no correlation between number of individuals and number of stains as a result of the analysis ($r^2 = 0.02$, y = -0.124x + 1.77).

In the sweep net sampling in sprayed plots, the mirid population remained low throughout the sampling period. There was no relationship between pest and stain density ($r^2 = 0.11$, y = -0.175x + 0.814) (Figure 5).

Mirid population reached the economic threshold 31 July on generative organs for the unsprayed plots.

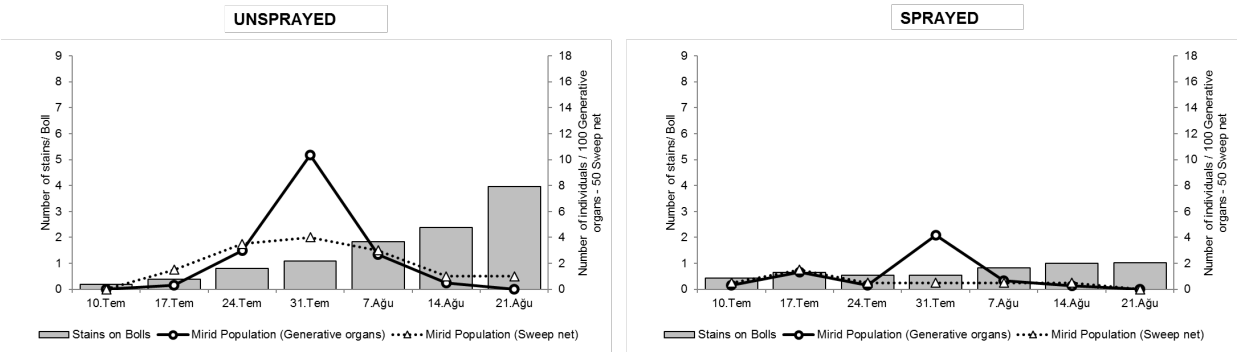

Figure 5. The relationship between numbers of mirids (counted directly and by sweep net) and densities of stains on the bolls in unsprayed and sprayed plots in Balcalı in 2014.

Numbers of stains in unsprayed and sprayed plots

The number of stains on bolls in unsprayed and sprayed plots are given Figure 6. The difference between the number of stains in sprayed and unsprayed plots was statistically significant on 30 July (t = -3.61, P = 0.01), 6 August (t = -3.22, P = 0.02), 13 August (t = -3.25, P = 0.02), 20 August (t = -7.59, P = 0.0002),

27 August (t = -5.66, P = 0.001), 10 September (t = -4.53, P = 0.004) and 17 September (t = -2.81, P = 0.03). The number of stains was higher in the unsprayed plots (Figure 6).

In unsprayed plots in Hacıali (Figure 6), no stained bolls were found between 16 and 23 July 2014. The differences in the number of stains in unsprayed and sprayed plots were found to be significant on 6 August (t = -4.73, P = 0.003), 27 August (t = -3.24, P = 0.02) and 3 September (t = -3.21, P = 0.02). Both number of stains on bolls and mirid population were lower in 2014 than in 2013.

In Balcalı in 2013, the stain densities on the bolls in unsprayed plots remained quite low level on 18 to 24 July (Figure 6). The numbers of stains in sprayed plots were statistically significant on 14 August (t = -3.61, P = 0.01), 21 August (t = -4.03, P = 0.007), 28 August ($F_{1.6}$ = 12.675, t = -3.56, P = 0.01), 3 September (t = -3.20, P = 0.02), 18 September (t = -4.35, P = 0.01) than unsprayed plots.

In Balcalı in 2014, the stain density on the bolls in unsprayed plots remained quite low level on 10 July. The number of stains increased towards the end of the season and reached the highest level on 21 August. Differences in stain numbers were found to be higher and statistically significant on 31 July (t = -3.01, P = 0.02), 14 August (t =-2.49, P = 0.05) and 21 August (t = -5.30, P = 0.002) for the unsprayed plot. The number of stains on boll in sprayed plot was significancy higher than that of unsprayed plot on 17 July (t = 4.42, P = 0.004).

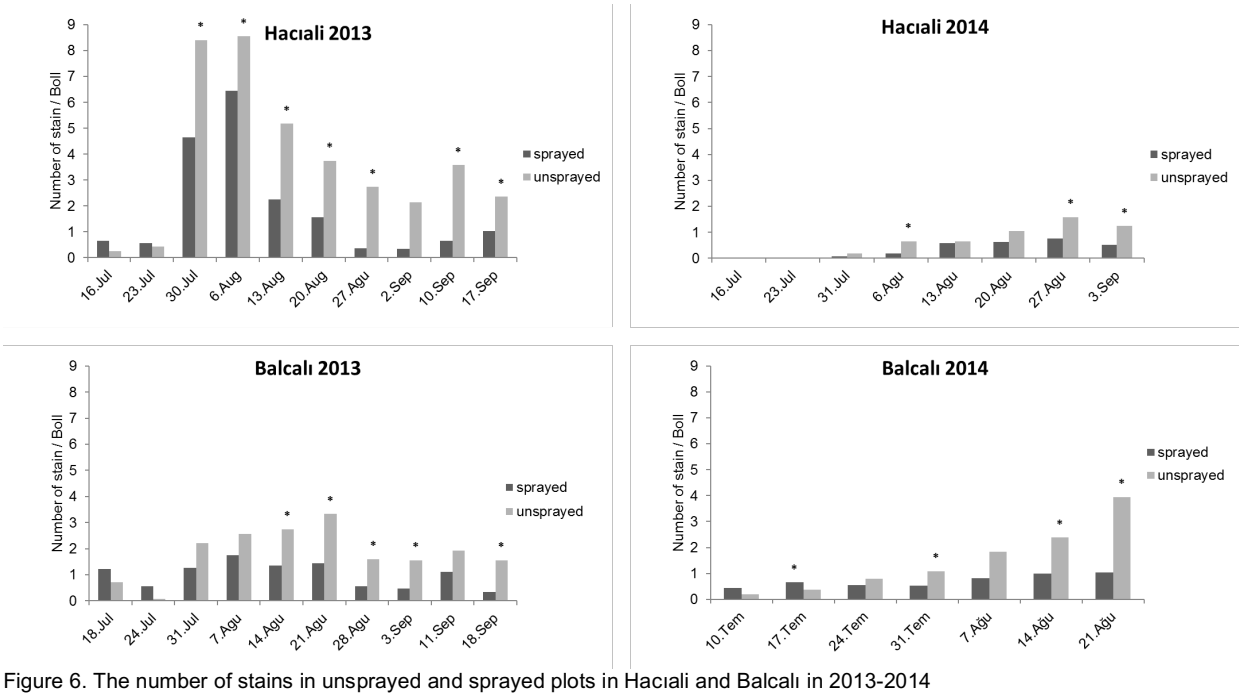

Figure 6. The number of stains in unsprayed and sprayed plots in Hacıali and Balcalı in 2013-2014

*Means on the bars are significant according to t-test (P < 0.05).

Findings in both Hacıali and Balcalı in 2013 and 2014 indicated that harmful mirid populations reached the highest density at the end of July or at the beginning of August. Stains on the bolls were detected after occurrence of mirid populations on plants in both sampling locations. Nakash et al. (1990) reported that puncture of *C. pallidus* is easily detected as a small black shiny spot on small bolls. Efil & İlkan (2003) revealed that population of *C. pallidus* in cotton fields reached to peak point in last week of August and first week of September in Şanlıurfa, Turkey. Some researchers reported that the population of mirids increased in the cotton fields after July in the USA. (Gore et al., 2012; Asiimwe et al., 2014). Khan et al. (2006) stated that during December-January, many of the cotton fields in Australia were in the early

boll period and that in this period *Creontiades dilutus* (Stål, 1859) (Hemiptera: Miridae) could lead to significant damage. He reported that feeding generally causes shedding of squares and yield losses.

On a weekly basis, there was no significant relationship between the dynamics of the number of mirids and the stains on the bolls. This may be related to the physiology of the bolls, stage of the nymphs and adults of mirids, the time required for stain formation, the feeding behavior on bolls and the boll preference. However, when the number of mirids and stains were taken into consideration, the number of stains were higher in the years, locations and the plots (sprayed and unsprayed) where the number of mirids was higher. In both locations and years, spraying kept the number of pest low, but it was not able to prevent the occurrence of the mirid damage (stained bolls). Stained bolls were also seen at low levels of the mirid densities.

Relationship between boll shedding and mirid population

Mirid damage was examined in bolls collected from the soil and the bolls were cut to determine the mirid sucking damage. Also, stains on the bolls caused by mirids were noted. In Hacıali, in 2013, the number of the bolls shed on soil was quite low at the beginning of sampling (in July). While number of mirids was the highest on generative organs and by sweep net sampling (30 July and 6 August), number of the bolls shed on soil was the highest. At the end of August and at the beginning of September, the numbers of shed bolls did not increase, although the population of the mirid increased for a short time. The reason for this could be that mature bolls have hardened tissues which may not be suitable for mirid feeding. Although there was a significant relationship between the number of mirids on generative organs and the numbers of shed bolls ($r^2 = 0.84$, $y = 0.294x - 0.352$), the relationship was not high with sweep net sampling ($r^2 = 0.28$, $y = 0.155x - 0.344$) (Figure 7).

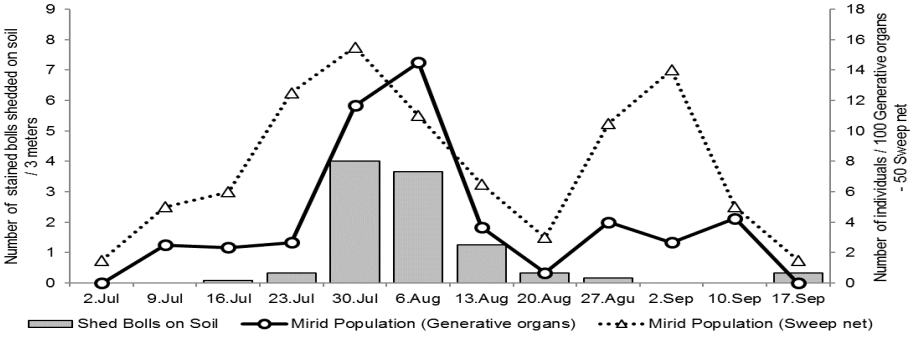

Figure 7. The relationship between the number of mirid (counted directly and by sweep net) and shed bolls on soil in unsprayed plots in Hacıali in 2013.

In 2014, there was an increase at the mirid population on generative organs and sweep net at the end of July and the beginning of August in unsprayed plots in Hacıali (Figure 8). After mirid population densities on bolls slightly increased, shed boll numbers also increased, Similar case was seen at the sweep net sampling. However, there was no relationship to between the number of shed bolls and the number of pests on generative organs ($r^2 = 0.35$, $y = 0.627x + 0.161$) and by sweep net sampling ($r^2 = 0.19$, $y = 0.212x + 0.125$) (Figure 8).

The numbers of stained and shed bolls in 2014 was lower than in 2013. This may be related to the fact that mirid numbers in 2014 were lower than in 2013.

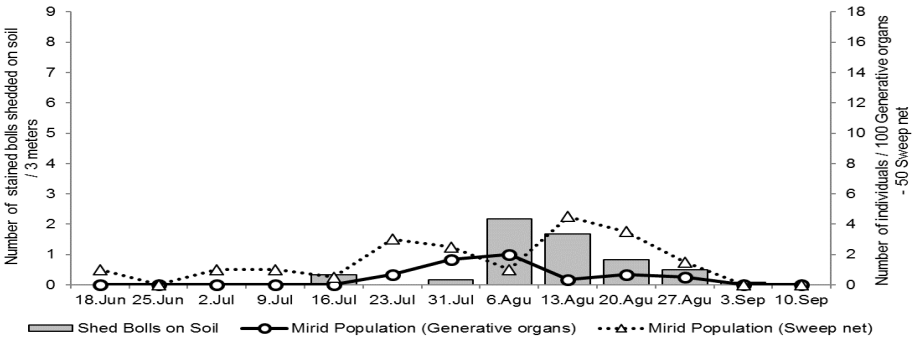

Figure 8. The relationship between the number of mirid (counted directly and by sweep net) and shed bolls on soil in unsprayed plots in Hacıali in 2014.

In Balcalı at the beginning of sampling in 2013, the number of shed bolls was quite low in the unsprayed plots (Figure 9). According to direct and sweep net counting, the mirid population generally showed similar and low population densities on most sampling dates. The number of shed bolls had increased by 31 July and 7 August. At the end of the season, the number of mirids increased for a short time, but no shed bolls that were found. There was no significant relationship between the number of shed bolls and the number of mirids on generative organs ($r^2 = 0.13$, $y = 0.348x + 0.116$) and by sweep net sampling ($r^2 = 0.12$, $y = 0.215x - 0.092$) (Figure 9).

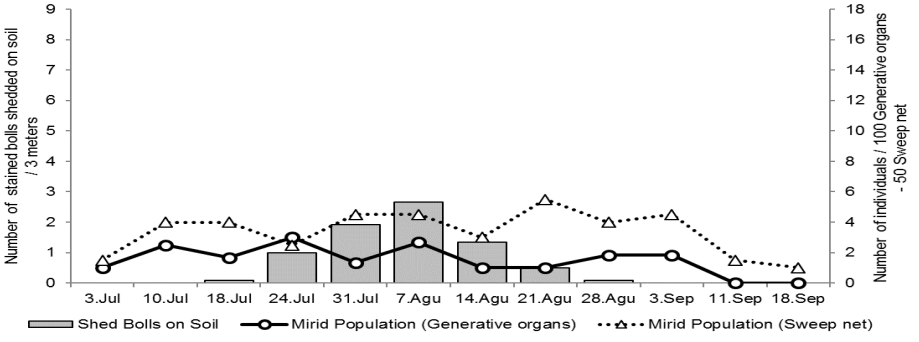

Figure 9. The relationship between the number of mirid (counted directly and by sweep net) and shed bolls on soil in unsprayed plots in Balcalı in 2013.

In Balcalı, in 2014, the mirid population on generative organs and sweep net in unsprayed plots peaked on 31 July (Figure 10). For sweep net sampling, the mirid population was detected in the middle of June and early July, in this period no shed bolls were recorded. The number of bolls was higher in the period when the mirid population started to increase (24 July). After 31 July, the mirid population declined rapidly, and numbers of shed bolls was low during that period (14 August). There was no significant relationship between number of shed bolls and the number of pests on the generative organs ($r^2 = 0.24$, $y = 0.061x + 0.172$) and by sweep net sampling ($r^2 = 0.22$, $y = 0.117x + 0.079$) (Figure 10).

In both locations, mainly young bolls were shed due to mirid feeding. Shedding of stained bolls were detected mostly in mid-July and mid-August. In this period, the number of young bolls on plants were relatively higher and the number of mirids were also higher. After early or mid-August, even though mirids were present, ratios of shed bolls were low. In other words, mature bolls were not shed even if they were exposed to mirid feeding.

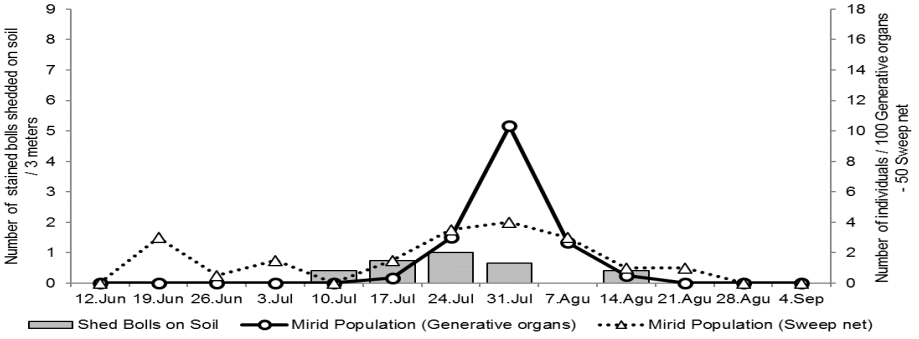

Figure 10. The relationship between the number of mirid (counted directly and by sweep net) and shed bolls on soil in unsprayed plots in Balcalı in 2014.

Pack & Tugwell (1976) reported that *L. lineolaris* does not always cause shedding of older squares, but leads to damage in developing flower anthers. Researchers noted that feeding damage, which did not lead to shedding of larger squares, usually resulted in abnormal flower formation. If the damage to the anthers is severe, pollination does not occur and this leads to the development of small abnormal bolls. These bolls are usually shed a few days after pollination. Nakash et al. (1990) reported that the puncturing of squares by *C. pallidus* caused a rapid deterioration of the flowers and caused them to shed later. They also reported that small bolls were sensitive to shedding as quickly as flowers. Mature and larger bolls were not shed due to *C. pallidus* infestations. Layton (2000) noted that feeding on the young bolls generally causes shedding and yield loss. Rosenheim et al. (2006) noted that *Lygus* feeds on squares and bolls. Mirids damage anthers and seeds then causes shedding of squares and bolls. Researchers noted that with *Lygus* feeding, the damage that occurs in cotton can vary considerably. Square and boll losses are often inconsistent with *Lygus* population estimates. Armstrong et al. (2010) noted that smaller bolls are more susceptible to feeding of *Creontiades signatus* (Distant, 1884) but damaged more than larger bolls. According to researchers, *C. signatus* seeks developing embryos and feeds actively on them. In the present study, nymphs and adults were recorded on the squares but few squares were found to have shed. It was thought that squares could have been shed for physiological reasons given that no significant feeding damage was observed on the squares. The reason for this may be the increase in the number of bolls during the periods of high mirid population and the fact that these mirid species prefer fresh bolls rather than squares. There was no mirid damage to flowers on which most adults were encountered. No linear relationships were recorded between weekly dynamics of the mirid population and the number of shed bolls during the season. However, it is clear that the number of shed bolls were higher in the years, locations and the plots (sprayed and unsprayed) where the number of mirids was higher.

Damage to squares

Seven d after the release of *C. pallidus* nymphs into the cages, less feeding damage was seen in cage with one individual (0.9%) and two individuals (5.9%) than in cage with four individuals (33.6%) on squares. There was no statistically significant difference between cages with no nymphs (control), and one and two individuals in the occurrence of damage. Whereas, feeding damage is greater in cages with four individuals and damage rate was statistically significant ($F_{3.84} = 9.53$, $P = 0.0001$). There was no damage to squares in cages with no nymphs released. Figure 11 shows the damage to squares 14 d after release of *C. pallidus* nymphs into the cages. Accordingly, no damage was observed in the cages with no nymphs released. When the number of individuals that released into the cages increased, damage ratios of squares also increased. The highest losses (58.0%) were seen in cage with four individuals ($F_{3.72} = 7.66$, $P = 0.0001$). As the number of nymphs which released into the cages increased, the damage rates also increased after 7 and 14 d.

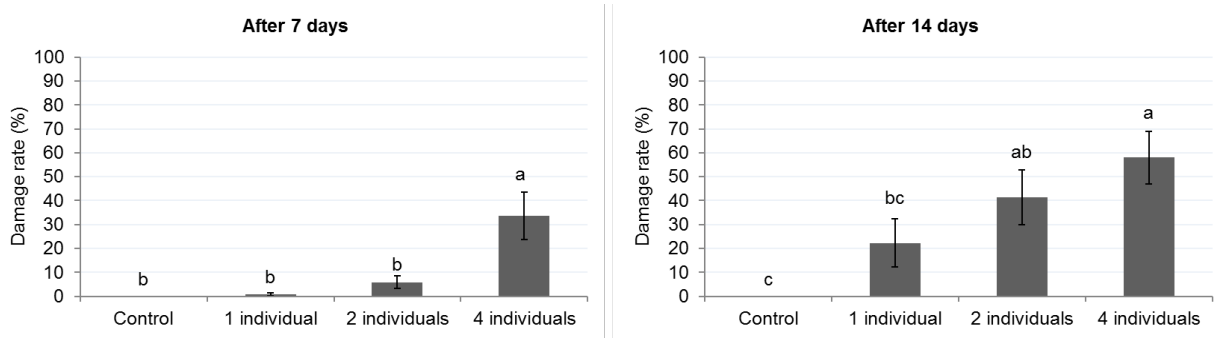

Figure 11. Damage (%) caused by *Creontiades pallidus* nymphs in squares (0.3-0.5 cm in diameter) in branch cages after 7 and 14 d in Balcalı in 20141

*The mean numbers with same letter on bars are not significant according to the Duncan multiple range test (P > 0.05).

Stam (1987) noted, the developing anthers in the squares were dark brown and narrowed in Syrian cotton fields. He indicated that damaged squares and bolls were shed and there was a significant relationship between the *C. pallidus* population and the damaged squares. Leight et al. (1988) reported a positive relationship between the density of *L. hesperus* and the number of shed squares in their study in San Joaquin Valley, CA, USA.

Damage to bolls

Seven d after the release of the *C. pallidus* nymphs to the bolls with 0.5-0.9 cm in diameter, no damage was seen in the control cages. However, all of the bolls in the cages with one, two, and four individuals all bolls were damaged. Seven d after the release of the *C. pallidus* nymphs to the bolls with a 1-1.5 cm in diameter, damage rates in the cages with two individuals (68.4%) and four individuals (70.0%) were similar but more than the bolls infested with 0 (5.26%) and one individual (36.8%). The difference for boll damage among the treatments was statistically significant ($F_{3.71} = 9.83$, $P = 0.0001$) (Figure 12).

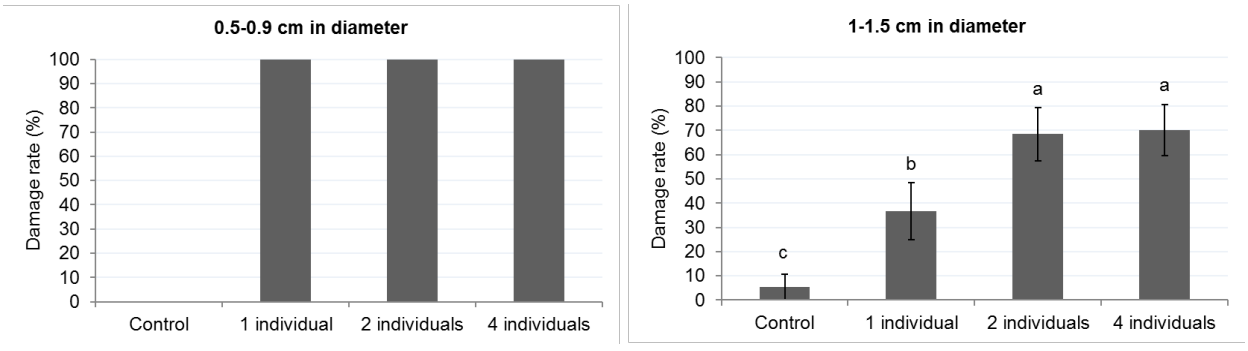

Figure 12. Damage (%) caused by *Creontiades pallidus* nymphs in bolls (0.5-0.9 cm) in Hacıali and (1-1.5 cm) in Balcalı in branch cages after 7 d in 2014.

*The mean numbers with same letter on bars are not significant according to the Duncan multiple range test (P > 0.05).

Creontiades pallidus nymphs negatively affected boll development, with 7-day feeding. The smaller bolls were more damaged in comparison to the matured bolls 7 d after releasing nymphs to the bolls with a diameter of 0.5-0.9 cm. No damage was seen in control cages but with one, two and four individuals release all bolls were damaged. The damage rate on bolls with 1-1.5 cm in diameter increased with increased density of nymphs. Damage rates of the bolls were 70% in the cages with two and four individuals. The differences between the damage rates of bolls were also found to be statistically significant (Figure 12). Stam (1987) reported that *C. pallidus* was found frequently on squares and bolls in cotton, and that small bolls had more black stains caused by mirids sucking. Small bolls are very sensitive to mirid

feeding compared to the larger bolls. In other words, small green boll stage is highly susceptible to mirid damage. Greene et al. (1999) reported that *Lygus lineolaris* (Palisot de Beauvois, 1818) caused significant damage to small bolls (8 d from white flowers) in the cage experiments. Layton (2000) reported that *L. lineolaris* usually leads to the loss of small bolls and thus to yield losses. Rosenheim et al. (2006) reported that *Lygus* fed on squares and bolls in California. The pest caused shedding of squares and bolls due to feeding damage to anthers and seeds. Armstrong et al. (2010), investigated *C. signatus* over a 10-year period in Texas. They found that younger bolls were more susceptible to feeding of mirids and thus, they were more damaged. Some of the digestive enzymes are released through the insertion of the stigmata of the mirid and slowing the growth of the bolls by preventing accumulation of assimilates. The developing fiber and seed are damaged, or boll shedding occurs.

Conclusions

The main population development of the pest mirids in Çukurova starts in July and continues until the beginning of August. This period is a critical for development of cotton fruiting bodies as the young square and boll ratio is high on the plants. This period would be suitable for control mirid pests. Young bolls should be taken into consideration when considering insecticide applications to prevent mirid damage to cotton in the region. Cultivation of early cotton cultivars in the region may be as important as a cultural practice. Furthermore, early planting cotton may be also be important in other cotton growing areas where the mirids are regarded as a problem. There was significant relationship between mirid densities and stain numbers on bolls sampled in some experimental years and locations. It was clear that low or high populations of mirids caused significant damage. In fact, stains occur several days after mirid feeding on young and mature bolls. The stage of the pest, the age of bolls and the amount of secreted digestive enzyme are the important factors that affect the occurrence of stains on bolls. It is therefore difficult to correlate a weekly relationship between the number of mirids and the number of stains. However, it is clear that the total number of stains was higher in the year and plots when the total number of mirids was high. It was also found that mirid populations in Adana can exceed the economic threshold. The mirids should be sampled using the recommended methods and chemical control should be applied if needed.

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Original article (Orijinal araştırma)

Integrated management of *Mi-1* virulent *Meloidogyne incognita* (Kofoid & White, 1919) Chitwood, 1949 (Tylenchida: Meloidogynidae) in greenhouse tomatoes[1]

Örtüaltı domates yetiştiriciliğinde *Mi-1* virülent *Meloidogyne incognita* (Kofoid & White, 1919) Chitwood, 1949 (Tylenchida: Meloidogynidae)'ya karşı entegre mücadele

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Abstract

The *Mi-1* gene in tomato provides a safe and economical strategy for managing root-knot nematodes (RKN). However, the resistance conferred by the *Mi-1* gene is not effective against virulent populations of RKNs. In this study, the efficacy of combining the dose effect of the *Mi-1* gene with *Bacillus firmus* Bredemann & Wermer, 1933 (Bacillales: Bacillaceae) + fluopyram against *Mi-1*-virulent *Meloidogyne incognita* (Kofoid & White, 1919) Chitwood, 1949 (Tylenchida: Meloidogynidae) after soil solarization was assessed for tomatoes grown in a greenhouse. The study was conducted in a greenhouse in Kepez, Antalya between 2015 and 2016. The second-stage juveniles *M. incognita* were not detected in the soil for 2 months after solarization completed. The reactions of homozygous or heterozygous resistant tomato plants against *Mi-1*-virulent populations were not statistically different under greenhouse conditions. Furthermore, *B. firmus* + fluopyram controlled the RKN population when applied to the soil both during the planting period and when J2 count in the soil started to increase. This study suggests that the soil solarization should be combined with other management methods, and the dose effect of the *Mi-1* gene is not important against virulent RKN populations. Additionally, combined *B. firmus* + fluopyram have the potential to be used as a suitable management tool for RKN control in tomato production. These findings will help improve integrated management practices for controlling *Mi-1*-virulent RKN populations.

Keywords: Management, *Mi-1* gene, root-knot nematode, solarization, tomato, virulent

Öz

Domatesteki *Mi-1* geni, kök-ur nematodları kontrol için güvenli ve ekonomik mücadele sağlamaktadır. Bununla birlikte, *Mi-1* geninin sağladığı dayanıklılık virülent kök-ur nematodlarına karşı etkili değildir. Bu çalışmada, solarizasyon uygulamasından sonra serada yetiştirilen domatesler için *Mi-1* virülent *Meloidogyne incognita* (Kofoid & White, 1919) Chitwood, 1949 (Tylenchida: Meloidogynidae) popülasyonuna karşı *Bacillus firmus* Bredemann & Wermer, 1933 (Bacillales: Bacillaceae) + fluopyram kombinasyonları ile *Mi-1* geninin doz etkisinin kombinasyonunun etkinliği değerlendirilmiştir. Çalışma 2015-2016 yılları arasında Antalya, Kepez bölgesindeki serada yürütülmüştür. Solarizasyon uygulamasından sonra toprakta iki ay boyunca *M. incognita* ikinci dönem larva tespit edilmemiştir. Sera koşullarında *Mi-1* virülent popülasyona karşı, homozigot-heterezigot dayanıklı domates bitkilerinin reaksiyonlarında istatistiksel farklılık olmamıştır. Ayrıca, *B. firmus* + fluopyram kombinasyonu, hem bitki dikim döneminde uygulandığında hem de toprakta J2 sayısı artmaya başladığı anda uygulandığında kök-ur nematod popülasyonunu kontrol altına almıştır. Bu çalışma, toprak solarizasyonunun diğer mücadele yöntemleriyle kombine edilebilir ve *Mi-1* geninin doz etkisinin virülent kök-ur nematodlarına karşı önemli olmadığını göstermiştir. Ayrıca, *B. firmus* + fluopyram kombinasyonu, domates üretimlerinde kök-ur nematolarının kontrolünde uygun bir mücadele aracı olarak kullanılabilme potansiyeline sahiptir. Bu bulgular, *Mi-1* virülent kök-ur nematod popülasyonlarının kontrolü için entegre mücadele yöntemlerinin gelişmesine yardım edebilecektir.

Anahtar sözcükler: Mücadele, *Mi-1* geni, kök-ur nematodları, solarizasyon, domates, virülent

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Introduction

Tomato is one of the most important vegetables in Turkey, grown over about 170 kha with an annual yield of 12 Mt in 2015 (TUİK, 2018). Tomatoes are produced in many parts of Turkey and the western Mediterranean Region of Turkey is the most significant production area.

Root-knot nematodes (RKNs) are widespread in many parts of the world. So far, 98 RKN species have been described worldwide (Jones et al., 2013). In previous studies, the presence of *Meloidogyne incognita* (Kofoid & White, 1919) Chitwood, 1949 (Tylenchida: Meloidogynidae), *Meloidogyne javanica* (Treub, 1885) Chitwood, 1949 and *Meloidogyne arenaria* (Neal, 1889) Chitwood, 1949 was determined in greenhouses of the western Mediterranean Region of Turkey (Devran & Söğüt, 2009; Devran et al., 2017).

RKNs feed on plant roots, resulting in the root gall formation that causes poor uptake of water and nutrients (Abad et al., 2003). In addition, infested plants can be more susceptible to soilborne plant pathogens (Karssen & Moens, 2006). Therefore, RKNs cause yield losses in conjunction with other factors in crops (Schomaker & Been, 2006). Several management tactics such as soil solarization, organic amendments, biological agents, chemicals and resistant cultivars are used to control RKNs (Collange et al., 2011). Soil solarization is also commonly used alone or in combination with other methods to manage soilborne pathogens (Katan, 1996). Biological control has no residual effects and is eco-friendly. Fungi, such as *Purpureocillium lilacinum* (Thom) Luangsa-ard, Houbraken, Hywel-Jones & Samson, 2011 (Hypocreales: Ophiocordycipitaceae) and *Hirsutella rhossiliensis* Minter & Brady 1980 (Hypocreales: Ophiocordycipitaceae), and bacteria, such as *Pasteuria penetrans* Thorne, 1940 (Bacillales: Pasteuriaceae) and *Bacillus* spp., are parasites of nematodes (Keren-Zur et al., 2000; Walia et al., 2000; Walia & Vats, 2000; Anastasiadis et al., 2008). Moreover, biological agents, including *P. lilacinus, P. penetrans* and *Bacillus firmus* Bredemann & Wermer, 1933 (Bacillales: Bacillaceae), are commercially available (Hallman et al., 2009). However, the efficacy of these agents can decrease under field conditions. Nematicides are commonly used to control RKN, although the use of many nematicide are banned or restricted because of health concerns (Devran & Söğüt, 2010; Wesemael et al., 2011). Recently, fluopyram was developed initially as a fungicide by Bayer Crop Science. It is a succinate dehydrogenase inhibitor (SDHI) in the phenyl-benzamide chemical group that is used to manage fungal diseases in plants. Additionally, fluopyram has been used against plant-parasitic nematodes including RKNs (Faske & Hurd, 2015).

Plant resistance is considered an alternative, economical and environment-friendly method to manage RKNs (Devran & Söğüt, 2010). In tomatoes, resistance to RKN is controlled by the *Mi-1* gene. The *Mi-1* gene was introgressed into cultivated tomato from *Solanum peruvianum* L. (Solanaceae) in the 1940s (Smith, 1944). This gene confers resistance against *M. incognita*, *M. javanica* and *M. arenaria* (Roberts & Thomason, 1986). The *Mi-1* gene has been successfully incorporated into many commercially-available tomato cultivars. However, virulent RKN populations overcome the *Mi-1* gene in tomato (Kaloshian et al., 1996; Ornat et al., 2001; Tzortzakakis et al., 2005). In nature, virulent populations occur spontaneously without selection (Castagnone-Sereno et al., 1994). Besides, Jarquin-Barberena et al. (1991) reported that populations of *M. incognita* virulent to *Mi-1* have developed with repeated exposure to this resistance gene under laboratory and field conditions.

The existence of virulent populations has also been documented in many countries (Roberts, 1995; Ornat et al., 2001; Tzortzakakis et al., 2005; Devran & Söğüt 2010; Iberkleid et al., 2014). Therefore, the development of new management methods is needed in the presence *Mi-1* virulent population of RKNs. The objectives of this study were a) to determine the performance of homozygous and heterozygous resistant tomato cultivars grown in a greenhouse infested with *Mi-1*-virulent *M. incognita,* b) to identify the population density of second-stage juveniles (J2s) in the soil planted tomato plants, during the growing season after soil solarization, c) to determine the effect of two different applications of commercial formulations including the combination of chemical and biological components on *Mi-1*-virulent *M. incognita* population and tomato yields in the greenhouse.

Material and Methods

Plant materials

Susceptible tomato cv. Tueza F_1 (*mimi*), heterozygous resistant cv. Seval F_1 (*Mimi*) and homozygous resistant cv. Browny F_1 (*MiMi*) were used in this experiment. All tomato seedlings were provided by Multi Tohum Tar. San. Tic. A.Ş. (Antalya, Turkey).

Commercial formulations

Velum Prime SC 400 and Flocter WP 5, registered by Bayer CropScience LP (Monheim, Germany) were used to control the RKNs. Velum Prime SC 400 is a nematicide containing the active ingredients 400 g/l fluopyram, which inhibits mitochondrial respiration by blocking the electron transport in the respiratory chain of succinate dehydrogenase (Garris, 2017; Heiken, 2017). Flocter WP 5 includes the active biological agent 50 g/kg *B. firmus* I-1582, a gram-positive bacterium. The studies reported that *B. firmus* paralyzes *M. incognita* J2s and inhibits hatching of eggs (Giannakouet et al., 2004; Mendoza et al., 2008; Terefe et al., 2009).

Glasshouse location

This study was conducted in a glasshouse in Kepez District, Antalya, Turkey (36°54'46.83" N, 30°45'13.11" E). The soil was 55% sand, 30% silt, 15% clay, and heavily infested with *M. incognita.* Initial nematode population density in the greenhouse was 3,328 J2s/100 g of soil.

Nematode culture

Soil samples were collected from different parts of the greenhouse used for the experiment in a zigzag pattern. Initially, seven populations of *Meloidogyne* spp. were collected from the root systems of a commercial candidate tomato cultivar with *Mi-1* gene grown in a previous season in the glasshouse. Afterwards, pure cultures were multiplied on susceptible tomato according to Mıstanoğlu et al. (2016).

DNA isolation

DNA was isolated from J2s with the DNAeasy Tissue and Blood Kit (Qiagen, Hilden, Germany), based on the manufacturer's protocol.

Molecular identification

The RKN populations were identified according to previous studies using species-specific primers Inc14F/Inc14R (Randig et al., 2002), Fjav/Rjav (Zijlstra et al., 2000) and Far/Rar (Zijlstra et al., 2000).

Virulence test

Seedlings of susceptible tomato cv. Tueza F_1 and resistant cv. Seval F_1 were planted singly in 250ml plastic pots containing steam-sterilized sandy soil. The establishment of the experiment and nematode inoculation were conducted as previously described (Mıstanoğlu et al., 2016).

Egg masses on roots of plants were examined under a stereomicroscope with. the number of egg masses on each plant root scored on a 0-5 scale according to Hartman & Sasser (1985) as follows: 0, no galls (resistant); 1, 1-2 galls or egg masses (resistant); 2, 3-10 galls or egg masses (resistant); 3, 11-30 galls or egg masses (susceptible); 4, 31-100 galls or egg masses (susceptible); and 5, more than 100 galls or egg masses per root system.

The J2s from 100 g of soil from each pot were extracted by modified Baermann funnel (Hooper, 1986). Reproduction factor (RF; i.e., final J2 population density/initial nematode population, 1000 J2s) was calculated (Ferris & Noling, 1987).

Soil preparation

The tomato plants from the previous season were removed from the glasshouse in 1 June 2015. About 15-20 cm depth of soil was processed with rotavators. The soil was then arranged using a shovel and 12 bands (double rows) were formed for the planting of seedlings. Drip irrigation pipes were placed in the soil.

Soil solarization

Soil solarization in the greenhouse was performed from 11 July to 22 August 2015 (6 weeks). No chemical was applied to the soil during the solarization period. The soil was covered with plastic of 20µ thickness and watered in the early morning at 5-d intervals for heat transmission to the deep layers of the soil. To detect the effectiveness of solarization, soil samples were taken from the greenhouse using a soil drill after soil solarization was completed. Three samples were taken from each subsection and then combined for analysis. The J2s were extracted from the soil (100 g) by modified Baermann funnel and counted under a microscope.

Planting of tomato seedlings

The glasshouse was divided into three sections, each formed from four bands. In addition, each band was split into three subsections (Figure 1). Seedlings of tomato cvs Tueza F_1, Seval F_1 and Browny F_1 were arranged in a randomized block design, with four replicates in each section (Figure 1). Forty tomato seedlings were planted for each replicate. The plants were planted 20 cm apart from each other in glasshouse. In total, 1440 seedlings were planted on 4 September 2015. Fertilization, irrigation and pests and pathogen management were done as needed.

Bands	Sub-Sections			Sections
Band 1	Seval F1	Tueza F1	Browny F1	**Section 1**
Band 2	Tueza F1	Browny F1	Seval F1	
Band 3	Browny F1	Seval F1	Tueza F1	
Band 4	Seval F1	Tueza F1	Browny F1	
Band 5	Tueza F1	Browny F1	Seval F1	**Section 2**
Band 6	Browny F1	Seval F1	Tueza F1	
Band 7	Seval F1	Tueza F1	Browny F1	
Band 8	Tueza F1	Browny F1	Seval F1	
Band 9	Seval F1	Tueza F1	Browny F1	**Section 3**
Band 10	Tueza F1	Browny F1	Seval F1	
Band 11	Browny F1	Seval F1	Tueza F1	
Band 12	Seval F1	Tueza F1	Browny F1	

Figure 1. Experimental layout.

Application of commercial formulations

As mentioned above, the glasshouse was divided into three sections corresponding to the three treatments (Figure 1). Commercial formulations were applied in Sections 1 and 2 via drip tubes into the soil according to the recommended doses (40 kg/ha for Flocter WP 5 and 600 ml/ha for Velum Prime SC 400). Treatments of the sections were:

Section 1: Flocter WP 5 was applied 1 week before (28.08.2015) planting and 1 week after (11.09.2015) planting. Velum Prime SC 400 was applied 1 d (05.09.2015) after planting and 15 d after the first application (20.09.2015) (Figure 1).

Section 2: Flocter WP 5 and Velum Prime SC 400 were applied twice at 15 d apart (01.12.2015 and 16.12.2015) because the number of J2s in the soil increased (Figure 1).

Section 3: Control, no commercial formulations.

Monitoring of J2s

The soil samples were collected 27 times at 10-d intervals, after the soil solarization. Three samples were obtained from each subsection and were combined to detect the number of J2s in the soil by modified Baermann funnel. The number of J2s was monitored during the growing season.

Evaluation of treatments

Plants were harvested at two different periods, 16 April and 31 May 2016. Ten tomato plants were harvested from each subsection. A total of 720 plants were harvested and evaluated at the end of the experiment. The gall indices of the roots were rated on a scale of 0 to 10 (Zeck, 1971).

Applications effects on yield

Tomato fruit of 20 plants from each subsection were harvested from 15 November 2015 to 20 March 2016 and the cumulative yield calculated.

Data analysis

Raw data were used in all analyses. ANOVA was used to compare triplet groups, and the Sidak test for binary comparisons. Two-way repeated measures ANOVA was used for the number of J2 and yield. One-way ANOVA was used for egg masses. Statistical analyses were performed using the SPSS 22.0 software (SPSS Inc., Chicago, IL, USA) at $p < 0.05$.

Results and Discussion

Molecular confirmation of *M. incognita*

Pure cultures were confirmed by PCR, using species-specific primers. Only PCR primers Inc14F/Inc14R, which are specific primers for *M. incognita*, produced an ~400 bp amplicon in all nematode samples, while the other primers did not yield any PCR products (data not shown). The results indicated that all populations were *M. incognita* and concur with previous studies (Randig et al., 2002; Devran & Söğüt, 2009).

Virulence test

Meloidogyne incognita pure cultures were tested for virulence. All *M. incognita* populations multiplied well in both susceptible cv. Tueza F_1 and resistant cv. Seval F_1. The RF values of populations were >1 in both susceptible and resistant cultivars. Similarly, the number of egg masses per root system was more than 100 (Table 1). All *M. incognita* populations were virulent for the *Mi-1* gene. The *Mi-1* gene confers resistance against three RKNs including *M. incognita*, *M. javanica* and *M. arenaria* (Roberts & Thomason, 1986). However, the *Mi-1* gene has been overcome by virulent populations that occur naturally or by a selection pressure in field and laboratory experiments (Jarquin-Barberena et al., 1991; Devran & Söğüt, 2010; Verdejo-Lucas et al., 2012). Results indicated that all *M. incognita* populations collected from the glasshouse were *Mi-1* virulent.

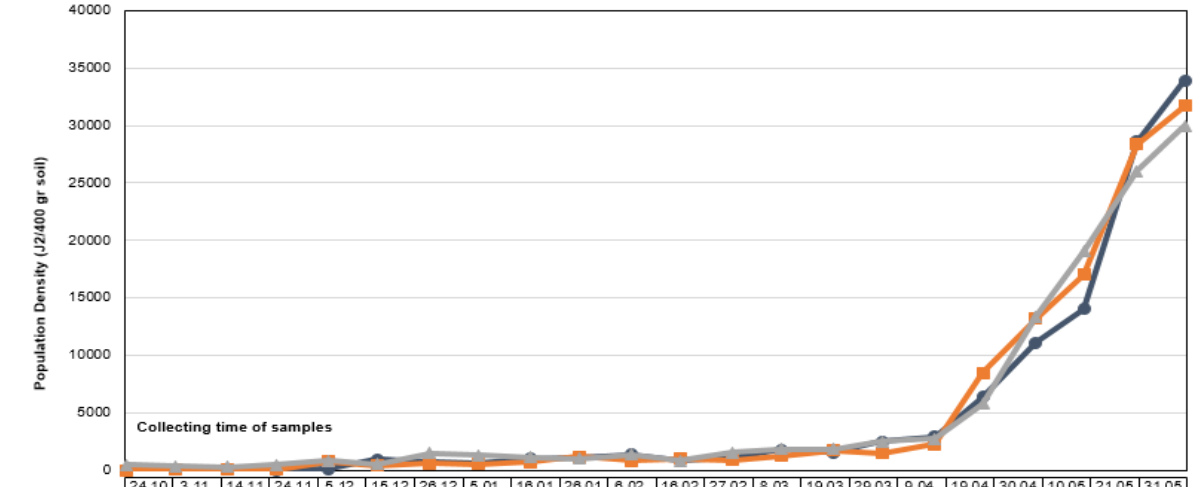
Table 1. The determination of the virulence of *Meloidogyne incognita* populations under laboratory conditions

Sample No	Egg mass index*		Reproduction factor	
	Tueza F_1	Seval F_1	Tueza F_1	Seval F_1
1	5	5	26.5	27.5
2	5	5	53.1	46.5
3	5	5	9.6	8.0
4	5	5	11.8	13.3
5	5	5	27.6	28.0
6	5	5	31.7	25.3
7	5	5	31.1	34.7

* Hartman and Sasser (1985) scale. Tueza F1, susceptible (*mimi*); and Seval F1, heterozygous resistant (*Mimi*).

Effect of soil solarization on J2s

J2s were first detected in the soil 2 months after the soil solarization completed and were also found in the soil for 51 d after planting of seedlings (Figure 2). Greco (1999) demonstrated that soil solarization for 45-60 d during July-August can eradicate nematodes from top soil, even in heavily infested plastic houses. However, the effectiveness of solarization depends on some parameters, such as the soil color, structure and moisture, the air temperature, length of day and sunlight intensity (Souza, 1994).



	24.10.2015	3.11.2015	14.11.2015	24.11.2015	5.12.2015	15.12.2015	26.12.2015	5.01.2016	16.01.2016	26.01.2016	6.02.2016	16.02.2016	27.02.2016	8.03.2016	19.03.2016	29.03.2016	9.04.2016	19.04.2016	30.04.2016	10.05.2016	21.05.2016	31.05.2016
Tueza F1	0	80	60	20	140	920	700	660	980	1060	1340	900	1070	1780	1540	2500	2930	6400	11120	14050	28570	33920
Seval F1	0	100	40	60	760	420	580	520	720	1200	840	1020	880	1260	1700	1510	2240	8500	13160	17000	28350	31720
Browny F1	470	360	280	460	840	540	1460	1300	1100	1000	1370	850	1560	1860	1830	2520	2720	5780	13380	19040	26020	29960

Figure. 2. Population density curves of *Mi*-1 virulent *Meloidogyne incognita* J2s in Section 3.

Effect of commercial formulations and cultivars on J2 numbers

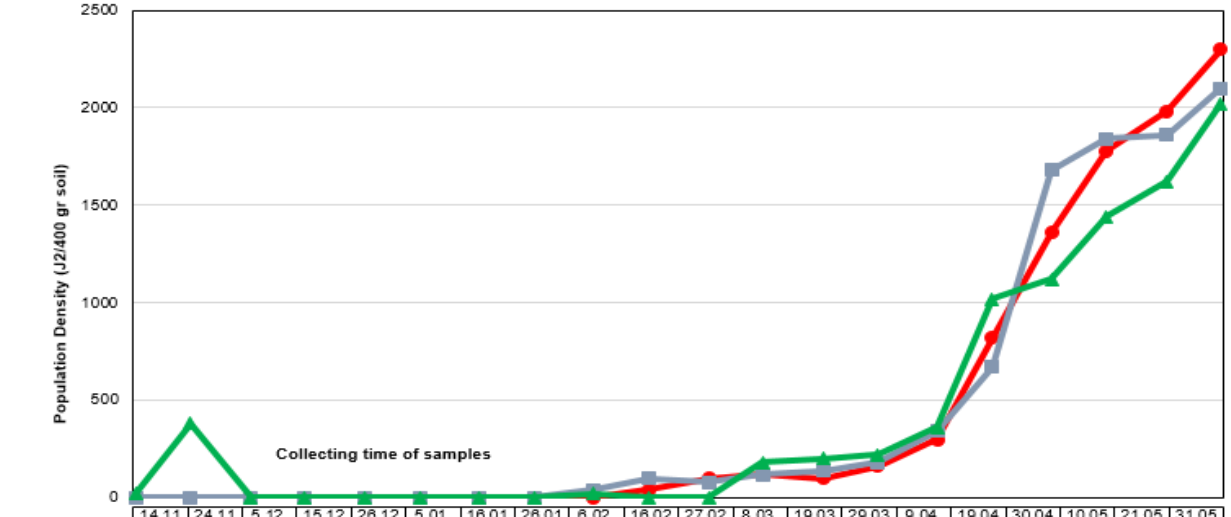
The number of J2s in the soil of the three tomato cultivars within each section were not statistically different ($F = 1.06$, $p > 0.05$) (Table 2). Nonetheless, when comparing the sections, the number of J2, in Section 3 was significantly high than 1 and 2 sections ($F = 4.56$, $F = 4.55$, $F = 5.49$, $p < 0.05$) (Table 2). Curves of J2s in the soil shown in Figures 2-4. The lowest J2 population density curve was detected in

Section 2, followed by Section 1, and the highest J2 density curve corresponded to Section 3, during the growing season (Figures 2-4). In Section 2, the combination of Flocter WP 5 + Velum SC 400 was applied to the soil when the soil population of J2 started to increase. No J2s were detected in the samples colleced between 5 December 2015 and 6 February 2016 (Figure 3).

Table 2. Analysis of mean number of J2 between treatments within tomato cultivars and between cultivars (n = 108)

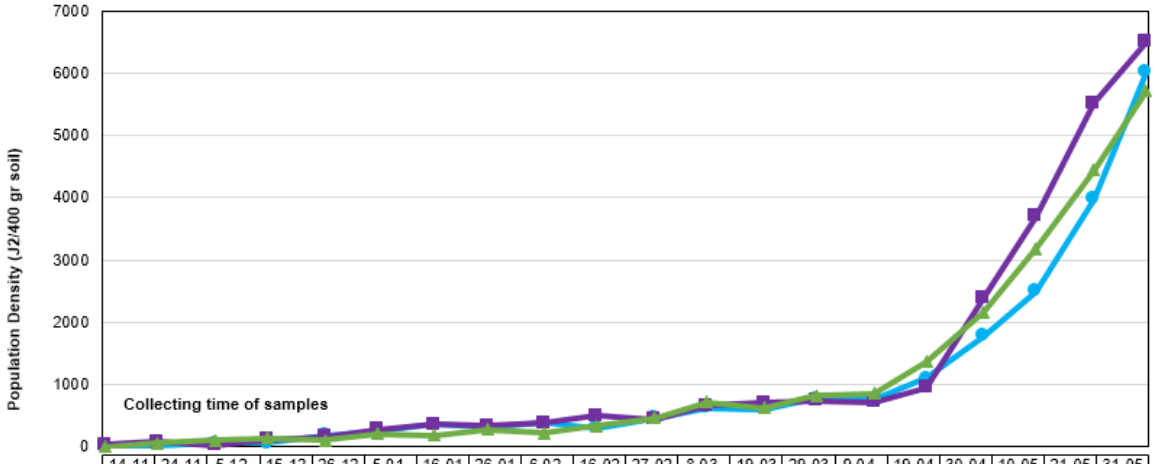
Tomato cultivar	Treatment	Mean J2 number	Standard deviation	Between treatments	Pairwise comparison*	Between cultivars
Tueza F1	Application 1	761	1384	F = 4.56 p = 0.01	3 > 1 and 2	
	Application 2	336	680			
	Control	4101	8554			
Seval F1	Application 1	911	1682	F = 4.55 p = 0.01	3 > 1 and 2	F = 1.06 p = 0.22
	Application 2	339	669			
	Control	4170	8532			
Browny F1	Application 1	814	1442	F = 5.49 p = 0.01	3 > 1 and 2	
	Application 2	319	579			
	Control	4248	8076			

*3 = Control.
Tueza F1: Susceptible (*mimi*), Seval F1: Heterozygous resistant (*Mimi*), Browny F1: Homozygous resistant (*MiMi*)
Application 1 (Section 1): Flocter WP 5 + Velum Prime SC 400 (applied in the soil when plants were planted)
Application 2 (Section 2): Flocter WP 5 + Velum Prime SC 400 (applied in the the soil when the J2 population started to increase)
Control (Section 3): No application.



	14.11.2015	24.11.2015	5.12.2015	15.12.2015	26.12.2015	5.01.2016	16.01.2016	26.01.2016	6.02.2016	16.02.2016	27.02.2016	8.03.2016	19.03.2016	29.03.2016	9.04.2016	19.04.2016	30.04.2016	10.05.2016	21.05.2016	31.05.2016
Tueza F1	0	0	0	0	0	0	0	0	0	40	100	120	100	160	300	820	1360	1780	1980	2300
Seval F1	0	0	0	0	0	0	0	0	40	100	80	120	140	180	340	670	1680	1840	1860	2100
Browny F1	20	380	0	0	0	0	0	0	20	0	0	180	200	220	360	1020	1120	1440	1620	2020

Figure 3. Population density curves of *Mi*-1 virulent *M. incognita* J2s in Section 2.



	14.11.2015	24.11.2015	5.12.2015	15.12.2015	26.12.2015	5.01.2016	16.01.2016	26.01.2016	6.02.2016	16.02.2016	27.02.2016	8.03.2016	19.03.2016	29.03.2016	9.04.2016	19.04.2016	30.04.2016	10.05.2016	21.05.2016	31.05.2016
Tueza F1	20	20	60	60	180	260	360	320	380	300	460	620	600	780	760	1100	1780	2500	3980	6020
Seval F1	40	80	20	120	160	280	360	340	380	500	440	660	700	740	720	950	2400	3700	5520	6500
Browny F1	0	60	100	140	100	210	180	280	220	340	460	720	620	820	860	1360	2160	3180	4440	5720

Figure 4. Population density curves of *Mi*-1 virulent *M. incognita* J2s in Section 1.

The data established that this application was effective against *M. incognita* during this period. In addition, the J2 population densities in the soil fluctuated though winter because of the low soil temperature. However, J2 population densities steadily increased with every sampling from March to June. These findings show that the combination of *B. firmus* + fluopyram was effective against RKNs and the individual components were not antagonist against each other. Faske & Hurd (2015) revealed that fluopyram could be used successfully to control *M. incognita* and *Rotylenchulus reniformis* Linford & Oliveira, 1940 (Tylenchida: Hoplolaimidae). Giannakou et al. (2004) investigated the efficacy of bionematicide including *B. firmus* in a field naturally infected with *Meloidogyne* spp. and under laboratory conditions. The treatments were compared with fumigant nematicides and the biocontrol agent *P. penetrans*. Under field conditions, 1,3-dichloropropene and dazomet + sodium tetrathiocarbonate were typically superior to bionematicide application. However, the recommended dose of the bionematicide significantly suppressed the numbers of J2s at the end of the cropping season in comparison with 1,3-dichloropropene. In addition, in pot experiments, findings indicated that the bio-nematicide was typically more efficient in controlling RKNs than the biocontrol agent *P. penetrans*. In another study, Giannakou et al. (2007) evaluated the effectiveness of a formulated bionematicide product containing lyophilized *B. firmus* spores against RKNs under greenhouse and field conditions. In the laboratory, a decrease in J2s hatching was recorded with bionematicide at a dose of 0.9 g/kg of soil, while a further decline was noted by doubling the dose. In a field experiment, the combination of soil solarization with bionematicide improved nematode control and gave results similar to the chemical treatment.

Dose effect of *Mi-1* gene

Gall indices did not differ between susceptible, and homozygous resistant and heterozygous resistant tomatoes in two separate harvest periods, against *Mi-1*-virulent *M. incognita* populations ($F = 0.09$, $F = 0.05$, $p > 0.05$) (Table 3). Therefore, no significant difference was evident in the reactions of homozygous and heterozygous resistant tomato plants against *Mi-1*-virulent *M. incognita* population, under greenhouse conditions. Tzortzakakis et al. (1998) stated that virulent isolate 1 was unaffected by the *Mi* gene copy number. In another study, Iberkleid et al. (2014) found that the virulent nematodes reproduced well on all susceptible and resistant tomatoes and showed both high reproduction on the susceptible, and

heterozygous and homozygous *Mi-1* genotypes. These findings indicate that reproduction of the *Mi-1*-virulent population was not affected by the allelic variation. Our findings agreed with these studies. In contrast, for an avirulent RKN population, the dosage of the *Mi-1* gene negatively impacted the reproduction of RKNs. Tzortzakakis et al. (1998) observed that expression of the *Mi-1* gene could be affected by gene zygosity, depending on whether the *Mi-1* gene is homozygous or heterozygous, and temperature. Many factors, including the background genotype, the structure of the nematode population and the use of resistant genotypes, can affect virulence (Castagnone-Sereno et al., 1994; Jacquet et al., 2005; Verdejo-Lucas et al., 2009).

Table 3. Comparison of gall index ratings in tomato cultivars (n = 120)

Harvest	Tomato cultivar	Average gall index*	Standard deviation	ANOVA F value	Probability (p)
Harvest 1	Tueza F1	1.25	1.93	0.09	0.91
	Seval F1	1.15	1.89		
	Browny F1	1.18	1.61		
Harvest 2	Tueza F1	1.88	2.39	0.05	0.95
	Seval F1	1.86	2.43		
	Browny F1	1.95	2.46		

* Samples were evaluated according to Zeck (1971). Harvest 1, 16 April 2016; and Harvest 2, 31 May 2016.
Tueza F1, susceptible (*mimi*); Seval F1, heterozygous resistant (*Mimi*); and Browny F1, homozygous resistant (*MiMi*).

Effect of applications on gall formation

Gall indices of the roots of susceptible, and heterozygous and homozygous resistant tomato cultivars were evaluated in two separate harvest periods. Gall indices of the roots did not differ between susceptible, and homozygous and heterozygous resistant tomatoes planted in all sections (Table 3). Nevertheless, Flocter WP 5 + Velum SC 400, which were applied in different periods, decreased galls on roots compared to plants in the control section. At the first harvest, there was no difference between Sections 1 and 2. Whereas, at the second harvest, galls numbers on the root of plants in Section 2 were lower than in Section 1 ($F = 88.36$, $F = 82.17$, $p < 0.05$) (Table 4). As mentioned above, fluopyram is an SDHI fungicide that is being evaluated for management of soilborne fungi and plant-parasitic nematodes in agronomic crops. The effect of fluopyramy on various fungi has been assessed, including its ability to control *Neocosmospora virguliformis* (O'Donnell & T. Aoki) L. Lombard & Crous, 2015 (Hypocreales: Nectriaceae) and many isolates of *F. virguliforme* showed sensitivity to fluopyram (Wang et al., 2017). Recently, a formulation that consists of fluopyram + imidacloprid has been considered as an in-furrow treatment for suppression of *M. incognita* and *R. reniformis* in cotton. In these field trials, fluopyram was found to suppress nematode densities at levels that were numerically more effective than those achieved by thiodicarb applied as a seed treatment (Lawrence et al., 2015). Faske & Hurd (2015) reported that in tomatoes nematode infestation of roots was reduced, and root galling lowered by 31 to 84% at concentrations of 1.3 to 5.3 mg/ml fluopyram. In another study, Giannakou et al. (2004) showed that band application of the bionematicide at all dose levels failed to decrease the midseason nematode population in soil compared to the control. However, there were significantly fewer nematodes at the end of the season in plots treated with the bionematicide at all dose levels than in the control plots. Terefe et al. (2009) examined the influence of BioNem on *M. incognita* in the greenhouse and field. They reported that BioNem applied at 8 g/pot planted with tomato seedlings reduced gall formation by 91%, final nematode populations by 76% and the number of eggs by 45% in the greenhouse trials. In addition, in the field trails, BioNem applied at 200 and 400 kg/ha was effective in reducing the number of galls (75-84%), and increased shoot height (29-31%) and weight (20-24%) over the untreated control, at 45 d after treatment.

Table 4. Effect of applications on gall index ratings (n = 120)

Harvest	Application	Average gall index*	Standard deviation	ANOVA F value	Possibility (p)	Pair-wise comparison
Harvest 1	Application 1	0.66	1.04	88.4	0.01	3 > 1 and 2
	Application 2	0.28	0.73			
	Control	2.65	2.24			
Harvest 2	Application 1	1.33	1.87	82.2	0.01	3 > 1 > 2
	Application 2	0.59	1.02			
	Control	3.77	2.75			

* Samples were evaluated according to Zeck (1971). Harvest 1: 16 April 2016, Harvest 2: 31 May 2016.
Tueza F1: Susceptible (*mimi*), Seval F1: Heterozygous resistant (*Mimi*), Browny F1: Homozygous resistant (*MiMi*)
Application 1 (Section 1): Flocter WP 5 + Velum Prime SC 400 (applied in the soil when plants were planted)
Application 2 (Section 2): Flocter WP 5 + Velum Prime SC 400 (applied in the the soil when the J2 population started to increase)
Control (Section 3): No application.

Effect of applications on yield

Given the differences in the genetic backgrounds of the tomato cultivars used in the experiment, the yields were separately evaluated for each cultivar. The yields of tomato cultivars planted in the Sections 1 and 2 were higher than Section 3. Also, yields of susceptible and heterozygous resistant tomato cultivars planted in Section 2 were higher relative to Section 1 ($F = 4.12$, $F = 3.86$, $F = 5.22$, $p < 0.05$) (Table 5). Whereas, the yields of homozygous resistant cultivars grown in Sections 1 and 2 were not statistically different from each other ($F = 4.12$, $F = 3.86$, $F = 5.22$, $p < 0.05$) (Table 3). The results showed that Flocter WP 5 + Velum Prime SC 400 decreased yield losses caused by *M. incognita*. Moreover, application with increasing of *M. incognita* J2 population the soil was more successful than application before planting. Terefe et al. (2012) investigated the effect of BioNem on nematode infestation and plant growth and yield of tomato, and compared two methods of BioNem application for the control of root-knot under field conditions. A significant increase in crop growth and yield relative to the untreated control was reported.

Table 5. Effect of different applications on yield (n = 10)

Tomato cultivar	Application	Mean yield (kg)	Standard deviation	ANOVA F value	Probability (p)	Pair-wise comparison
Tueza F1	Application 1	25.28	11.5	4.12	0.01	2 > 1 > 3
	Application 2	27.65	11.7			
	Control	21.85	10.4			
Seval F1	Application 1	24.73	12.3	3.86	0.01	2 > 1 > 3
	Application 2	26.53	11.2			
	Control	21.44	12.4			
Browny F1	Application 1	15.92	10.1	5.22	0.01	1 and 2 > 3
	Application 2	17.52	11.2			
	Control	13.58	10.2			

Tueza F1, susceptible (*mimi*); Seval F1, heterozygous resistant (*Mimi*); and Browny F1, Homozygous resistant (*MiMi*).
Application 1 (Section 1), Flocter WP 5 + Velum Prime SC 400 (applied in the soil when plants were planted).
Application 2 (Section 2), Flocter WP 5 + Velum Prime SC 400 (applied in the the soil when the J2 population started to increase).
Control (Section 3), No application.

Conclusion

Importantly, no J2s were detected in the soil for 2 months after solarization completed. Furthermore, to control RKNs, soil solarization should be performed in the summer, providing a great opportunity to transplant seedlings into the soil confidently. The solarization effect can be further improved when combined with other management practices. Under these greenhouse conditions, the homozygous or heterozygous resistant tomato plants were not effective against virulent RKN. However, *B. firmus* + fluopyram applied both at planting and when the J2 population in the soil was increasing provided some control of *M. incognita*, with the latter more effective at reducing the *M. incognita* population at the end of the experiment. In future research on integrated management practices, there should be a focus on controlling *Mi-1*-virulent RKNs.

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Original article (Orijinal araştırma)

Reaction of peach and nectarine rootstocks to different populations of root-knot nematode species, *Meloidogyne incognita* (Kofoid & White, 1919) and *Meloidogyne javanica* (Treub, 1885)[1]

Şeftali ve nektarin anaçlarının, Kök ur nematodu türleri; *Meloidogyne incognita* (Kofoid & White, 1919) ve *Meloidogyne javanica* (Treub, 1885)'nın farklı popülasyonlarına karşı reaksiyonu

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Abstract

The reaction of peach and nectarine rootstocks, Garnem, Cadaman, GF 677, Barrier, Nemaguard and M-29, used in Turkey was investigated to five populations of root knot nematode species, *Meloidogyne incognita* (Kofoid & White, 1919) and *Meloidogyne javanica* (Treub, 1885), under controlled conditions. The study was conducted at the Plant Protection Central Research Institute of the Laboratory of Nematology (Republic of Turkey Ministry of Agriculture and Forestry) in 2013-2016. Three *M. javanica* (TR16-2, TR12-1 and S5-1) and two *M. incognita* (TR10-3, S4-1) populations were obtained from infested peach orchards and established pure culture. All rootstocks were inoculated with 3000 second stage juveniles (J2s) from each *a* population. Each combination was replicated five times. One hundred and twenty d after inoculation, the ratio of galling on the roots and the number of nematode J2s in the soil were assessed and thus the response of rootstocks was determined. M-29, Cadaman and Garnem rootstocks were resistant to all populations, whereas GF 677 was susceptible to all populations. Nemaguard was resistant to TR16-2 and TR12-1 populations, but this rootstock was susceptible to S5-1, TR10-3 and S4-1 populations. Barrier rootstock was moderately resistant to TR16-2 and TR12 populations but susceptible to S5-1 and S4-1 populations. The findings could be used for control root-knot nematodes as well in breeding programs.

Keywords: *Meloidogyne incognita, Meloidogyne javanica*, nectarine, peach, resistance, rootstocks

Öz

Türkiye'de kullanılan Garnem, Cadaman, GF 677, Barrier, Nemaguard ve M-29 olarak adlandırılan şeftali ve nektarin anaçlarının kontrollü koşullar altında Kök ur nematodu türleri *Meloidogyne incognita* (Kofoid & White, 1919) ve *Meloidogyne javanica* (Treub, 1885)'nın beş popülasyonuna karşı reaksiyonu incelenmiştir. Çalışma, 2013-2016 yılları arasında gerçekleştirilmiştir. Çalışma, Bitki Koruma Merkezi Araştırma Enstitüsü Nematoloji Laboratuvarı (T.C. Tarım ve Orman Bakanlığı)'nda 2013-2016 yılında gerçekleştirilmiştir. Bulaşık şeftali bahçelerinden elde edilen üç *M. javanica* (TR16-2, TR12-1 ve S5-1) ve iki *M. incognita* (TR10-3, S4-1) popülasyonu ile çalışılmış olup, söz konusu popülasyonların saf kültürleri oluşturulmuştur. Bütün anaçlara her popülasyondan 3000 ikinci dönem larva (J2s) inokulasyonu yapılmıştır. Deneme, her bir anaç için beş tekerrürlü olarak kurulmuştur. Bulaştırmadan yüz yirmi gün sonra, köklerdeki ur oluşum oranı ve topraktaki ikinci dönem larva (J2s) sayısı analiz edilmiş ve böylece anaçların direnci belirlenmiştir. GF 677 tüm popülasyonlara karşı hassas iken, M-29, Cadaman ve Garnem anaçlarının tüm popülasyonlara karşı dayanıklı bulunmuştur. Nemaguard, TR16-2 ve TR12-1 popülasyonlarına dayanıklı iken, S5-1, TR10-3 ve S4-1 popülasyonlarına hassas olarak saptanmıştır. Barrier anacı TR16-2 ve TR12 popülasyonlarına karşı orta derecede dirençli iken S5-1 ve S4-1 popülasyonlarına karşı hassas olarak kaydedilmiştir. Elde edilen bulgular, kök-ur nematodlarının kontrolünde ve ıslah programlarında kullanılabilir.

Anahtar sözcükler: *Meloidogyne incognita, Meloidogyne javanica*, nektarin, şeftali, dayanıklılık, anaç

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Introduction

Turkey is among the main countries, producing peaches and nectarines in the world; China produces the most, and Turkey is the fourth largest producer (USDA, 2018). Peach-nectarine cultivation has spread to many parts of the world with mild climates. The Mediterranean basin is one of the important cultivation centers. These fruits are rich in nutrients and malic acid constitutes 80-90% of the acid found in peach. Sugars constitutes 60-65% of water-soluble dry matter. This concentration is higher in clingstone peach. One hundred g peach contains 7-12 g sugar, 2-20 mg vitamin C, nitrogen, and vitamins A and B at different ratios (Anonymous, 2008). Fruit is consumed fresh as well as jam, compote and fruit juice (Anonymous, 2004).

Today, various pest and disease problems are increasing and cause peach crop losses. Root-knot nematodes are one of the most important. Since root-knot nematodes have a very wide host range, it is difficult control. As a result of nematode feeding, large galls form throughout the root system of infected plants. Plants infested with nematodes have symptoms on the aboveground parts, including foliage yellowing and smaller leaves, because of their reduced ability to absorb and transport nutrients from the soil.

The damage varies depending on the nematode density and the sensitivity of the plant. Most plant parasitic nematodes live underground and are thus difficult to control. Therefore, plant resistance is an important management strategy. One of the most effective, environmentally friendly control measures in plants is the use of genetic host resistance. Using resistant cultivars can prevent the reproduction of the nematodes and it does not require any special application techniques or equipment, so It has a lower cost compared with other control methods (Lopez-Perez et al., 2006). The detection of the resistance of rootstock against the nematodes is very important to control the nematode and selection for establish new orchards.

There are four main root-knot nematode species, *Meloidogyne arenaria* (Neal, 1889), *Meloidogyne hapla* Chitwood, 1949, *Meloidogyne incognita* (Kofoid & White, 1919) and *Meloidogyne javanica* (Treub, 1885), that can cause damage to *Prunus* spp., but *M. incognita* and *M. javanica* are the predominant species in peach and plum (Ye et al., 2009). Root-knot nematodes cause serious problems in warm, sandy and well-drained soils (Pinochet et al., 1999). In South Carolina, *M. incognita* and *M. javanica* were found in 95 and 5% of peach orchards, respectively (Nyczepir et al., 1997). The most common symptom of root-knot nematode problems in peach is stunted growth of young trees. Thus, quantity and quality of fruit can be reduced in peach growing areas infested with RKNs. Maquilan et al. (2018) reported that RKNs caused disease complexes with fungi and bacteria in peach orchards.

Root-knot nematodes has been found on different cultivated plants in Turkey (Elekçioğlu et al., 1994; Kaşkavalcı & Öncüer, 1999; Devran & Söğüt 2009; İmren et al., 2014; Aydınlı, 2018). In Turkey, the limited studies have been conducted on peach and nectarine rootstocks. The aim of this study was to determine the reaction of the rootstocks widely used peach and nectarine production in Turkey to populations of *M. incognita* and *M. javanica*.

Material and Methods

Nematode material

Meloidogyne incognita and *M. javanica* populations were originally isolated from a peach and nectarine orchards in the Aegean Region. Soil and root samples were taken from infected peach and nectarine orchards in 2012-2013 (Yağcı et al., 2018). Three *M. javanica (*TR16-2, TR12-1 and S5-1) and two *M. incognita* (TR10-3 and S4-1) populations were obtained from these orchards (Figure 1).

Figure 1. Peach and nectarine orchards in Aegean Region.

Plant material

GF677, Garnem, M-29, Barrier, Cadaman (the clones of *Prunus cerasifera* Ehrh.) and Nemaguard (open pollinated peach seedling rootstock), which are commonly grown for peach production in Turkey, were used in this study. Tomato cv. SC 2121 was used for mass rearing of root-knot nematodes and was planted into 500 ml pots containing soil mixture. Peach materials were transplanted into 3 L pots containing sand-soil mixture previously sterilized at 120ºC (Robbins & Barker, 1974; Chen et al., 1995).

Pure culture of populations

Egg masses were collected with the help of forceps under a stereo-binocular microscope and second stage juveniles (J2s) were obtained from infested roots and the soil. They were inoculated to susceptible tomato cv. SC 2121 at the four leaf stage. After 8 weeks egg masses were collected with forceps under the stereo-binocular microscope. Pure culture of nematode populations was identified morphologically (Yağcı, 2017).

Mass rearing of nematode populations

Two *M. incognita* and three *M. javanica* populations were reared for use in this study. Experiments were conducted in a temperature-controlled glasshouse at 25-30ºC. Tomatoes were harvested and egg masses collected from roots 3 months after inoculation. The J2s were extracted from the eggs using a Baermann funnel (Hooper, 1986). About 3,000 J2s were collected under a light microscope (Leica DM 300, Wetzlar, Germany) from each population for inoculation.

Inoculation of root-knot nematode populations

Plants of uniform height of 15-20 cm and 10-20 leaves were inoculated through three 3-cm deep holes with a suspension of 3000 J2s per plant containing an equal proportion of populations (*M. incognita* and *M. javanica*) (Fernandez et al., 1995). The study was conducted at the Plant Protection Central Research Institute of the Laboratory of Nematology (Republic of Turkey Ministry of Agriculture and Forestry) in 2013-2016. Each experiment was laid out in a completely randomized design with five replicates. Plants were watered daily or as needed during the study. Experiments were conducted in a climate chamber at 25±2ºC and 65% RH with a 14:8 h L:D photoperiod. Plants were harvested 120 d after inoculation.

Data analysis

The number of galls and egg masses per root system were recorded using the 0-5 gall index scale of Hartman & Sasser (1985). Plants in scale 0 to 2 were rated as resistant, and 3 to 5 as susceptible. One-hundred-g soil samples were taken from each pot and collected to determine of the J2 density in the soil. J2s were extracted from the soil with a Baermann funnel. SPSS statistical program was used in the analysis and averages compared according to the Duncan test at $P \leq 0.01$ level.

Results and Discussion

Meloidogyne incognita and *M. javanica* were examined 120 d after inoculation and the ratio of the population densities were determined.

The reaction of rootstocks to *M. javanica* TR 16-2 was calculated according to gall index, gall number and final nematode population (Pf). M-29, Cadaman, Garnem, Nemaguard and Barrier were resistant to TR 16-2 while GF 677 was susceptible with the highest final population (Table 1).

Table 1. Number of galls, gall index and final population for *Meloidogyne javanica* population TR 16-2 on different rootstocks [(mean±SD) (min, max)], (n = 5)

Rootstock	Gall number		Gall index		Final population	
M-29	0.00±0.00 (0.00-0.00)	a*	0.00±0.00 (0.00-0.00)	a	0.00±0.00 (0.00-0.00)	a
Cadaman	0.20±0.20 (0.00-1.00)	a	0.20±0.20 (0.00-1.00)	a	0.00±0.00 (0.00- 0.00)	a
Garnem	0.00±0.00 (0.00-0.00)	a	0.00±0.00 (0.00-0.00)	a	0.00±0.00 (0.00-0.00)	a
Nemaguard	0.00±0.00 (0.00-0.00)	a	0.00±0.00 (0.00-0.00)	a	0.00±0.00 (0.00-0.00)	a
Barrier	1.40±1.16 0.00-6.00	a	0.60±0.40 (0.00-2,00)	a	32.00±20.20 (0.00-96.00)	a
GF677	28.20±6.77 12.00-48.00	b	3.60±0.24 (3.00-4.00)	b	282.00±22.00 (216.00-340.00)	b

*Means followed by the same letter are not statistically different according to the Duncan test (P ≤ 0.01).

Rootstocks M-29, Cadaman, Garnem and Nemaguard were resistant to *M. javanica* population TR 12-1. Barrier with gall index of 2.20 in the second group was susceptible. GF 677 was susceptible with the highest number of final population and with gall index of 3.60 (Table 2).

Table 2. Number of galls, gall index and final population for *Meloidogyne javanica* population TR 12-1 on different rootstocks [(mean±SD) (min, max)], (n = 5)

Rootstock	Gall number		Gall index		Final population	
M-29	0.00±0.00 (0.00-0.00)	a*	0.00±0.00 (0.00-0.00)	a	0.00±0.00 (0.00-0.00)	a
Cadaman	0.00±0.00 (0.00-0.00)	a	0.00±0.00 (0.00- 0.00)	a	0.00±0.00 (0.00- 0.00)	a
Garnem	0.80±0.49 (0.00-2.00)	a	0.40±0.24 (0.00-1.00)	a	0.00±0.00 (0.00-0.00)	a
Nemaguard	0.00±0.00 (0.00-0.00)	a	0.00±0.00 (0.00-0.00)	a	0.00±0.00 (0.00-0.00)	a
Barrier	6.80±1,35 (3.00-11.00)	a	2.20±0.20 (2.00-3.00)	b	51.80±10.90 (30.00-90.00)	b
GF677	35.80±7.34 (9.00-52.00)	b	3.60±0.40 (2.00-4.00)	c	199.00±31.00 (81.00-256.00)	c

*Means followed by the same letter are not statistically different according to the Duncan test (P ≤ 0.01).

Rootstocks M-29, Cadaman, Garnem and Nemaguard were resistant to population TR S5-1. Barrier and GF677 rootstocks were susceptible with gall indices of 2.60 and 2.80, respectively. The highest number of J2s was found in GF677 (Table 3).

Table 3. Number of galls, gall index and final population for *Meloidogyne javanica* population S5-1 on different rootstocks [(mean±SD) (min, max)], (n = 5)

Rootstock	Gall number		Gall index		Final population	
M-29	0.00±0.00 (0.00-0.00)	a*	0.00±0.00 (0.00-0.00)	a	0.00±0.00 (0.00-0.00)	a
Cadaman	0.00±0.00 (0.00-0.00)	a	0.00±0.00 (0.00-0.00)	a	0.00±0.00 (0.00-0.00)	a
Garnem	0.00±0.00 (0.00-0.00)	a	0.00±0.00 (0.00-0.00)	a	0.00±0.00 (0.00-0.00)	a
Nemaguard	3.60±1.83 (0.00-9.00)	a	1.20±0.37 (0.00-2.00)	ab	35.60±20.00 (0.00-108.00)	a
Barrier	26.80±12.00 (2.00-72.00)	ab	2.60±0.49 (1.00-4.00)	c	182.00±47.50 (30.00-314.00)	c
GF677	53.00±22.30 (0.00-107.00)	b	2.80±1.07 (0.00-5.00)	b	223.00±93.70 (0.00-441.00)	c

*Means followed by the same letter are not statistically different according to the Duncan test (P ≤ 0.01).

Rootstocks M-29, Cadaman and Garnem were resistant to *M. incognita* population S4-1. Nemaguard, Barrier and GF677 were susceptible with gall indices of 4.00, 3.80 and 4.80. The highest final population was found in GF677, no J2s were found in M-29 and Garnem (Table 4).

Table 4. Number of galls, gall index and final population for *Meloidogyne incognita* population S4-1 on different rootstocks [(mean±SD) (min, max)], (n = 5)

Rootstock	Gall number		Gall index		Final population	
M-29	0.00±0.00 (0.00-0.00)	a*	0.00±0.00 (0.00-0.00)	a	0.00±0.00 (0.00-0.00)	a
Cadaman	1.60±1.60 (0.00-8.00)	a	0.40±0.40 (0.00-2.00)	a	11.80±11.80 (0.00-59.0)	a
Garnem	0.00±0.00 (0.00-0.00)	a	0.00±0.00 (0.00-0.00)	a	0.00±0.00 (0.00-0.00)	a
Nemaguard	71.80±24.80 (4.00-131)	b	4.00±0.54 (2.00-5.00)	bc	182.00±30.20 (95.00-282.00)	b
Barrier	52.80±9.10 (18.00-68.00)	b	3.80±0.20 (3.00-4.00)	b	287.00±28.30 (204.00-370.00)	c
GF677	115.00±17.60 (70.00-178.00)	c	4.80±0.20 (4.00-5.00)	c	459.00±52.30 (269.00-583.00)	d

*Means followed by the same letter are not statistically different according to the Duncan test (P ≤ 0.01).

Rootstocks Cadaman and Garnem were resistant to *M. incognita* population TR10-3. Nemaguard and GF677 were susceptible with gall indices of 3.00 and 5.00, respectively. Final population score of Cadaman and Garnem were 0, whereas GF677 was 554 (Table 5).

Table 5. Number of galls, gall index and final population for *Meloidogyne incognita* population TR10-3 on different rootstocks [(mean±SD) (min, max)], (n = 5)

Rootstock	Gall number		Gall index		Final population	
Cadaman	0.00±0.00 (0.00-0.00)	a*	0.00±0.00 (0.00-0.00)	a	0.00±0.00 (0.00-0.00)	a
Nemaguard	21.70±2.96 (16.00-26.00)	a	3.00± 0.00 (3.00-3.00)	a	110.00±18.70 (89.00-147.00)	a
Garnem	0.00±0.00 (0.00-0.00)	a	0.00±0.00 (0.00-0.00)	a	0.00±0.00 (0.00-0.00)	a
GF677	151.00±21.90 (113.00-189.00)	b	5.00±0.00 (5.00-5.00)	b	554.00±66.20 (433.00-661.00)	b

*Means followed by the same letter are not statistically different according to the Duncan test (P ≤ 0.01).

Rootstocks M-29, Cadaman, Garnem, Nemaguard and Barrier were resistant to TR 16-2 while GF 677 was susceptible with the highest final population. GF 677 and Barrier were susceptible to *M. javanica* population TR 12-1 and M-29, Cadaman, Garnem and Nemaguard resistant. Whereas, M-29, Cadaman, Garnem and Nemaguard were resistant to TR S5-1 and Barrier and GF677 were were susceptible. M-29, Cadaman and Garnem were resistant to *M. incognita* population S4-1 and Nemaguard, Barrier and GF677 were susceptible. Cadaman and Garnem were resistant to *M. incognita* population TR10-3 and Nemaguard and GF677 were susceptible (Figure 2). Yoshikawa et al. (1989) reported that Nemaguard, Nemared and Lovell rootstocks were widely used in California, and Nemaguard and Nemared rootstocks were resistant to root-knot nematodes.

Rootstocks M-29, Cadaman and Garnem were resistant to all nematode populations. However, Pinochet et al. (1999) found that Garnem and Cadaman were resistant to *M. javanica*. Likewise, Özbek et al. (2014) reported that Garnem, Cadaman and Myrobalan 29-C when inoculated J2s of *M. incognita* race 2 and *M. javanica* all rootstocks were resistant. Esmenjaud et al. (1997) reported that M-29 was resistant to populations of *M. arenaria, M. incognita* and *M. javanica*. In another study, Özarslandan & Tanrıver (2018) showed that Myrobalan 29-C, Garnem, Patrones Arda, Cadaman, Patrones Toro, Mariana GF 8-1 rootstocks were resistant to *M. incognita* while Myrobalan B and GF677 were susceptible.

Figure 2. Galls on the peach and nectarine roots caused by *M. incognita*.

Nemaguard and Barrier are reported to be resistant to root-knot nematodes (Sherman & Lyrene, 1983; Huettel & Hammerschlag, 1993; Pinochet et al., 1996, 1999; Layne & Bassi, 2008). However, in the present study these rootstocks were susceptible to some of nematode populations. Similarly, Esmenjaud et al. (1997) reported that Nemaguard was resistant to populations of *M. arenaria, M. incogn*ita and *M. javanica* but it was susceptible to Florida isolates (*M. incognita* race 3). Meza et al. (2016) showed that Nemaguard had variable resistance to each of the most aggressive isolates.

GF 677 was susceptible to all root-knot nematode populations. In previous studies, Pinochet al. (1996) showed that GF677 was susceptible to *M. incognita* (5 populations), *M. javanica* (5 populations), *M. arenaria* (5 populations), *M. hapla* (1 population), *Meloidogyne hispanica* Hirschmann, 1986 (1 population) and Barrier was moderately resistant. Cadaman and Nemaguard rootstocks were also resistant to all populations. In another study, Fernandez (1995) reported that GF677 was susceptible while Barrier was moderately resistant to *M. incognita.* Nyczepir & Wood (2012) reported that Nemaguard was highly resistant to *Meloidogyne partityla* Kleynhans, (1986) and no egg masses were present on the roots. Additionally, Marull et al. (1991) reported that GF-677 was susceptible to *M. arenaria.*

Several studies have been conducted on *Prunus*. Esmenjaud et al. (1995) found that in their study 15-month-old hardwood cuttings of Myrobalan (*P. cerasifera*) were resistant to *M. arenaria* populations. In another study, Ye et al. (2009) showed that root-knot nematodes caused serious damage to the *Prunus* rootstocks in China and some cultivars such as Tsukuba-4 and Tsukuba-5 were immune to *M. incognita*.

In conclusion, Barrier, Nemaguard and GF 677 rootstocks should not be selected to establish new orchards in the Aegean Region of Turkey. Garnem, M-29 and Cadaman rootstocks are resistant to all root-knot nematode populations in our study. Thus, they should be used in the peach and nectarine cultivation. These resistant rootstocks can be used to control *M. incognita* and *M. javanica* in IPM programs. The results could provide important knowledge for plant breeders, and peach and nectarine growers.

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Original article (Orijinal araştırma)

Fumigant toxicity of mustard essential oil and its main compound alone and combinations with modified atmosphere treatments against *Tribolium confusum* du Val., 1863 (Coleoptera: Tenebrionidae)[1]

Hardal uçucu yağı ve ana bileşiğinin tek başına ve değiştirilmiş atmosfer uygulamaları ile kombinasyonun *Tribolium confusum* du Val., 1863 (Coleoptera: Tenebrionidae)'a karşı fümigant etkisi

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Abstract

This study was carried out in 2017 in Entomology Laboratory of Kahramanmaraş Sütçü İmam University to determine fumigant toxicity of mustard essential oil and its main compound (allyl isothiocyanate) alone and in combination with high concentration (92%) of CO_2 or N_2 to all life stages of *Tribolium confusum* du Val., 1863 (Coleoptera: Tenebrionidae) was determined. Preliminary bioassay tests indicated that 10 µl/l of mustard essential oil and allyl isothiocyanate alone resulted in 100% mortality for all life stages of *T. confusum* without any necessity of CO_2 and N_2 combinations. Lethal concentration tests indicated that combinations of mustard essential oil or allyl isothiocyanate with 92% CO_2 produced 1.8 to 7.3 times reductions in LC_{90} values for larvae, pupae and adults of *T. confusum*. Generally, the combinations of mustard essential oil or allyl isothiocyanate with 92% CO_2 were more toxic to larvae, pupae and adults of *T. confusum* than those in combinations with 92% N_2 as evidenced by significant decrements in their LC_{50} and LC_{90} values. It appears that high concentration of CO_2 or N_2 might have a synergistic effect on larvae, pupae and adults of *T. confusum* when exposed together with mustard essential oil or allyl isothiocyanate. In conclusion, this study indicates that combinations of mustard essential oil or its main compound, allyl isothiocyanate with modified atmospheres can be a potential alternative to the most commonly used commercial fumigants, methyl bromide and phosphine.

Keywords: Allyl isothiocyanate, essential oil, modified atmosphere, mustard, *Tribolium confusum*

Öz

Bu çalışma 2017 yılında Kahramanmaraş Sütçü İmam Üniversitesi'nin Entomoloji Laboratuvarı'nda hardal uçucu yağı ve bunun ana bileşiği olan allyl isothiocyanate'ın yüksek CO_2 veya N_2 ile kombinasyonunun *Tribolium confusum* du Val., 1863 (Coleoptera: Tenebrionidae)'un tüm biyolojik dönemlerine karşı fümigant etkinlikleri belirlemek amacıyla yürütülmüştür. Ön biyolojik etkinlik test sonuçları, hardal uçucu yağı veya allyl isothiocyanate'ın tek başına 10 µl/l konsantrasyonda *T. confusum* 'un tüm biyolojik dönemlerinde %100 ölümüne neden olduğunu göstermiştir. Hardal uçucu yağı veya allyl isothiocyanate'ın %92 oranındaki CO_2 veya N_2 ile birlikte uygulanması *T. confusum*'un larva, pupa ve erginlerine ait LC_{90} değerlerinde 1.8-7.3 arasında değişen oranlarda azalmalara neden olmuştur. Genel olarak hardal uçucu yağı veya allyl isothiocyanate'in %92 CO_2 ile kombinasyonun *T. confusum*'un larva, pupa ve erginlerine karşı bunların %92 N_2 ile kombinasyonuna kıyasla daha toksik olduğu belirlenmiştir. Biyolojik etkinlik testleri sonunda hardal uçucu yağı ve bunun ana bileşiği olan allyl isothiocyanate'ın yüksek konsantrasyonda CO_2 veya N_2 gazıyla birlikte uygulanmasının *T. confusum*'un larva, pupa ve erginlerine ait toksisite değerlerinde önemli azalmalara neden olduğu ve dolayısıyla CO_2 veya N_2 kullanımının sinerjik etki gösterebileceği görülmüştür. Sonuç olarak, hardal uçucu yağı ve ana bileşiği olan allyl isothiocyanate'ın değiştirilmiş atmosfer ile kombinasyon halinde uygulanmasının depolanmış ürün zararlılarının mücadelesinde konvansiyonel fümigantlara potansiyel alternatif olabileceği değerlendirilmiştir.

Anahtar sözcükler: Allyl isothiocyanate, uçucu yağ, değiştirilmiş atmosfer, hardal, *Tribolium confusum*

[1] This study was presented as an oral presentation at the VII Turkish Plant Protection Congress with International Participation (Muğla, Turkey, 14-17 November 2018) and published as an abstract in the abstract book.

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Introduction

Plant essential oils in general have been recognized as an important natural resource and their major components, often various monoterpenoids, are among the best-known substances to have attracted attention in recent years as potential pest control agents due to their insecticidal, repellent and/or antifeedant properties (Tunç et al., 2000; Papachristos & Stamopoulos, 2002a,b; Lee et al., 2003; Trypathy, 2004; Ketoh et al., 2005; Isman, 2006). Most of these substances are volatile and can act as fumigants, thus offering the prospect of use against stored-product insects. Several studies have shown fumigant action of various essential oils and their compounds against some stored-product insects (Shaaya et al., 1997; Huang et al., 2000; Tunç et al., 2000). A number of studies showed that mustard essential oil and its major component, allyl isothiocyanate, have high toxicity against both *Sitophilus zeamais* (Motschulsky, 1855), *Tribolium confusum* du Val., 1863 (Coleoptera: Tenebrionidae) and *Rhyzopertha dominica* (Fabricius, 1792) (Worfel et al., 1997; Tsao et al., 2002; Paes et al., 2011), indicating their potential use in protecting grain and other stored products. Paes et al. (2011) reported that the vapor of mustard essential oil resulted in high mortality of all life stages of *S. zeamais*. However, there were significant differences in toxicity of mustard essential oil to its life stages. The egg was the most tolerant developmental stage, followed by pupae and larvae, respectively. This study indicates that mustard essential oil is a possible candidate for development of an environmentally-friendly fumigant for control of grain insect pests. However, the successful implementation of essential oils as fumigants is hampered by the relatively high concentrations needed for effective protection of stored grain against insect pests, the great difference in the sensitivity of various insect species, poor penetration into grain bulk current market prices and the effects on stored products being fumigated (Korunic & Rozman, 2008; Rajendran & Sriranjini, 2008; Rozman et al., 2008).

The use of CO_2 together with various fumigants has also been studied. Carbon dioxide, a respiratory stimulant, is known as an adjuvant for fumigants, such as methyl bromide. There are some advantages of using CO_2 in the mixture; higher toxicity of the fumigant, better gas distribution, limitation of harmful residue levels on the treated commodity, and also elimination of flammable hazard of some fumigants. Several investigations of fumigant and CO_2 mixtures have been done in the past (Cotton & Young, 1929; Jones, 1938), and these were followed by investigations which indicated that methyl bromide and CO_2 mixture caused an increase in the susceptibilities of some stored-product insects (Calderon & Leesch, 1983; Williams, 1985). Laboratory tests with essential oils have shown a similar joint action with CO_2 atmospheres. Shaaya et al. (1999) reported increased toxicity of essential oil, SEM76 (extracted from a species in the family Lamiaceae), in the presence CO_2 to *Tribolium castaneum* (Herbst, 1797) (adults, pupae and larvae), *Plodia interpunctella* (Hübner, [1813]) (pupae and larvae), *R. dominica*), *Sitophilus oryzae* (Linnaeus, 1763) and *Oryzaephilus surinamensis* (Linnaeus, 1763) (adults). However, toxicity studies on essential oils and CO_2 mixtures against stored-product insects to exhibit additive, synergistic or antagonistic effects are limited.

Several studies revealed that mustard essential oil and its major component, allyl isothiocyanate, had high fumigant toxicity against stored grain insects (Worfel et al., 1997; Tsao et al., 2002; Paes et al., 2011) and potential use for controlling stored grain insects. However, the problems regarding to decrease in the efficacy of mustard essential oil and its major component due to requiring their high toxicity values and exposure times to obtain the complete mortality of stored-product insects and a possible weak penetration power in bulk commodity limited to be potential alternative to current fumigants (especially phosphine) for controlling stored-product insects. The use of mustard essential oil and its compound, allyl isothiocyanate, with high concentrations of inert gases, such as N_2 and CO_2, may contribute to increase their toxicity against stored-product insects and penetration into bulk commodity. In this context, the present study was conducted to determine fumigant toxicity of mustard essential oil and its main compound (allyl isothiocyanate) in combination with high concentration (92%) of CO_2 and N_2 to all life stages of *T. confusum*.

Material and Methods

Test insects

Biological tests were conducted on all life stages (egg, larva, pupa and adult) of *T. confusum*. *Tribolium confusum* were obtained from standard cultures reared in 1-L glass jars at 25±1ºC and 65±5% RH on a standard diet with wheat flour mixture with dry brewer's yeast (17:1 by weight). Eggs were obtained by daily separation from oviposition jars by sieving (60 mesh, 250 µm sieve; Retsch, Haan, Germany). Eggs for exposure to treatments were transferred into the glass vials (5 cm long × 2.5 cm diameter). The 10 mL glass tube (18 cm high × 18 cm wide × 12 cm diameter), each containing 50 eggs 1 to 2-d old, was exposed to each treatment. Larvae were removed from insect culture jars 25-30 d after oviposition and exposed to the treatments. Two-d-old pupae for exposure to treatments were separated from insect culture jars and kept in wheat flour for 24-h before the treatment. Newly emerged adults were kept in pre-exposure vials containing wheat flour, and were exposed to treatment 7-10 d after emergence of the adults.

Fumigation chambers

Fumigation chambers consisted of 3-L glass jar, each capped with a ground-glass stopper equipped with entry and exit tubing. Two pieces of rubber tubing (5 cm long × 6.2 mm ID) were attached to the tubing and sealed with pinch-clamps.

Mustard essential oil and its main compound

Essential oil from mustard (*Sinapis nigra* L.) and its main compound, allyl isothiocyanate were tested against all life stages of *T. confusum*. Mustard essential oil extracted by stem distillation method was provided commercially from ATL Canada Company. Allyl isothiocyanate (Merck, 800260, 95% purity) was provided commercially by Sigma-Aldrich. After purchase, mustard essential oil and allyl isothiocyanate were collected in sealed glass containers and refrigerated in the dark at 4ºC until their use.

Carbon dioxide and nitrogen gas

Compressed CO_2 and N_2 were supplied by Linde Gas (Ankara, Turkey) and were >99.9% pure.

Bioassay and experimental procedures

Mustard oil and its main compound, allyl isothiocyanate were introduced as a liquid into the fumigation chamber using 10 or 50 µl gastight syringes (Hamilton Company, Switzerland). CO_2 and N_2 were transferred from the supply cylinder through a pipe equipped with a regulator valve. Concentrations of CO_2 and N_2 inside the glass jars were checked by hand-operated O_2/CO_2 analyzer (PBI Dansensor) and portable O_2/N_2 gas analyzer (Brotie, Beijing, China) respectively. Relative humidity during fumigations was also measured by placing small hygrometers within the fumigation chamber. Prior to each test, 20 larvae, pupae, adults and 50 eggs of *T. confusum* were confined, separately, inside 2.5 cm diameter by 5 cm long glass vials.

Firstly, preliminary bioassay tests on fumigation activity of mustard essential oil and allyl isothiocyanate alone; mustard essential oil and allyl isothiocyanate combination with 92% CO_2 or 92% N_2 were conducted to determine the effective concentrations of each treatment against all life stages of *T. confusum*. For mustard essential oil and allyl isothiocyanate alone treatment, all life stages of *T. confusum* were exposed to a concentration of 10 µl/l of mustard essential oil and allyl isothiocyanate for 24-h. Mustard essential oil and allyl isothiocyanate were applied on filter paper (2 x 8 cm) attached to lower side of the lids of fumigation chamber by using 50 µl syringe. After all life stages of *T. confusum* held in the glass vials, insects were transferred separately into fumigation chamber, fumigation chambers were closed by screwed

lids, which were made airtight. Each treatment and control were replicated three times. For the treatments with mustard essential oil and allyl isothiocyanate in a CO_2 and N_2 atmosphere, test insects were first placed in the fumigation chambers. Then, prior to the introduction of 10 µl/l of mustard essential oil or allyl isothiocyanate concentration, the fumigation chambers were briefly evacuated to 60.8 or 295 mm Hg followed by flushing with CO_2 or N_2 respectively until restoration of atmospheric pressure so as to achieve a uniform concentration of 92±3% CO_2 or 92±3% N_2. The 24-h exposure was used throughout all the experiments. In addition to these treatments, separate exposure to 92% CO_2 or 92% N_2 alone was made and untreated control insects were exposed to atmospheric conditions. For all fumigations, relative humidity and temperature were maintained at 65±5% at atmospheric pressure and 25±1°C, respectively. The relative humidity level was maintained in the fumigation chamber by using saturated solutions of sodium nitrite (Greenspan, 1977).

Concentration-mortality tests were conducted to determine LC_{50} and LC_{90} values of mustard essential oil and allyl isothiocyanate alone and in their combination with 92% CO_2 or 92% N_2 for all life stages of *T. confusum*. Each stage of *T. confusum* was exposed to four to five different concentrations of mustard essential oil or allyl isothiocyanate for 24-h. With mustard essential oil and allyl isothiocyanate alone a range of five concentrations from 0.25 to 5 µl/l and from 0.25 to 2 µl/l for all life stages of *T. confusum* was used, respectively. With mustard essential oil and allyl isothiocyanate in combination with 92% CO_2 ranges consisted of five concentrations from 0.25 to 1.5 µl/l and from 0.25 to 2 µl/l for all life stages of *T. confusum,* respectively. With mustard essential oil and allyl isothiocyanate in combination with 92% N_2 ranges consisted of five concentrations from 0.25 to 5 µl/l and from 0.25 to 2.6 µl/l for all life stages of *T. confusum,* respectively. Concentrations were selected for all life stages of *T. confusum* on basis of preliminary bioassay tests. Each concentration and control treatment were replicated three times. Fumigation procedures were the same as described above.

Data processing and analysis

After each treatment, larvae, pupae, and adults were transferred to clean 200-ml jars containing wheat flour and were kept at 25±1ºC and 65±5% RH until mortality checking. The eggs were held in their Perspex slides under the same conditions and examined for egg hatch after 7 d. Mortality data obtained from preliminary tests were normalized using arcsine transformation and then were analyzed using one-way ANOVA. The means were separated using the LSD test at 5% significance level (SAS Institute, 1985). Data obtained from each zero dose control and concentration-response mortality were subjected to probit analysis PoloPlus (LeOra Software, Petaluma, CA) (LeOra Software, 2005) to determine LC_{50} (Lethal Concentration$_{50}$) and LC_{90} (Lethal Concentration$_{90}$) values for each treatment and life stage of *T. confusum* and their 95% confidence intervals.

Results and Discussion

Preliminary bioassay tests indicated that all treatments (mustard essential oil and allyl isothiocyanate alone, mustard essential oil and allyl isothiocyanate+92% N_2 or 92% CO_2) except 92% CO_2 and N_2 alone and control resulted in 100% mortality for all life stages of *T. confusum* (Table 1). However, exposure to 92% CO_2 and 92% N_2 alone produced very low mortality of adults, pupae and larvae of *T. confusum*, but they resulted in relatively high mortality of *T. confusum* eggs (54-78%). Preliminary bioassay tests indicated that 10 µl/l concentration of mustard essential oil and allyl isothiocyanate alone resulted in 100% mortality for all life stages of *T. confusum* without any necessity of CO_2 and N_2 combinations (Table 1).

Table 1. Percentage mortalities (%) of all life stages of *T. confusum* exposed to 10 µl/l concentration of mustard essential oil and allyl isothiocyanate alone, 10 µl/l of mustard essential oil in combination with 92% CO_2 or 92% N_2, and 92% CO_2 and 92% N_2 alone for 24-h exposure time.

Treatments	Mean mortality(%)±Standard error			
	Egg	Larva	Pupa	Adult
Mustard oil	100.00±0.00 A*	100.00±0.00 A	100.00±0.00 A	100.00±0.00 A
Mustard oil+92% CO_2	100.00±0.00 A	100.00±0.00 A	100.00±0.00 A	100.00±0.00 A
Mustard oil+92% N_2	100.00±0.00 A	100.00±0.00 A	100.00±0.00 A	100.00±0.00 A
Allyl isothiocyanate	100.00±0.00 A	100.00±0.00 A	100.00±0.00 A	100.00±0.00 A
Allyl isothiocyanate+92% CO_2	100.00±0.00 A	100.00±0.00 A	100.00±0.00 A	100.00±0.00 A
Allyl isothiocyanate+92% N_2	100.00±0.00 A	100.00±0.00 A	100.00±0.00 A	100.00±0.00 A
92% CO_2	78.00±1.16 B	8.33±3.33 B	28.33±1.67 B	15.00±0.00 B
92% N_2	54.00±1.15 C	8.33±1.67 B	15.00±2.89 C	10.00±2.89 C
Control	10.67±0.67 D	1.67±1.67 C	8.33±3.33 C	0.00±0.00 D
F and P value	$F_{8,18}$ = 3548.9 P < 0.0001	$F_{8,18}$ = 253.3 P < 0.0001	$F_{8,18}$ = 242.7 P < 0.0001	$F_{8,18}$ = 1331.6 P < 0.0001
LSD value	2.338	8.708	7.114	3.591

* Means within a column with the same letter are not significantly different (LSD at 5% level). One-way ANOVA was applied to the data.

Probit analysis data of mustard essential oil alone and mustard essential oil in combination with 92% CO_2 or 92% N_2 for larval to adult stages of *T. confusum* resulting from 24-h laboratory fumigations are given Table 2. Since the lowest concentration of mustard essential oil alone (0.25 µl/l of air) resulted 100% mortality of *T. confusum* eggs, the lethal concentration values were not able to be estimated. Mustard essential oil in combination with 92% CO_2 or 92% N_2 reduced LC_{50} and LC_{90} values for adults, larvae and pupae of *T. confusum*. Mustard essential oil in combination with 92% CO_2 had 7.3, 1.8 and 4.6 times reduction in LC_{90} values for larvae, pupae and adults of *T. confusum,* respectively, compared with mustard essential oil alone (Table 2). Mustard essential oil in combination with 92% N_2 had 1.2, 1.1 and 3.1 times reduction in LC_{90} values for larvae, pupae and adults of *T. confusum,* respectively, compared with mustard essential oil alone. Generally, the combinations of mustard essential oil with 92% CO_2 were more toxic to larvae, pupae and adults of *T. confusum* than those in combinations with 92% N_2 as evidenced by significant decrements in their LC_{90} values.

Toxicity data of allyl isothiocyanate alone and allyl isothiocyanate in combination with 92% CO_2 or 92% N_2 for all life stages of *T. confusum* resulting from 24-h laboratory fumigations are given Table 3. Allyl isothiocyanate in combination with 92% CO_2 and N_2 reduced LC_{50} and LC_{90} values of all life stages of *T. confusum*. Allyl isothiocyanate in combination with 92% CO_2 had 4.1, 1.9 and 4.1 times reduction in LC_{90} values for larvae, pupae and adults of *T. confusum,* respectively, compared with allyl isothiocyanate alone (Table 3). Allyl isothiocyanate in combination with 92% N_2 had 2.3, 1.2 and 2.1 time reduction in LC_{90} values for larvae, pupae and adults of *T. confusum,* respectively, compared with allyl isothiocyanate alone. Generally, the combinations of allyl isothiocyanate with 92% CO_2 were more toxic to larvae, pupae and adults of *T. confusum* than those in combinations with 92% N_2 as evidenced by significant decrements in their LC_{90} values.

Table 2. Probit analysis data of mustard essential oil alone and mustard essential oil in combination with 92% CO_2 or 92% N_2 for larval to adult stages of *Tribolium confusum* resulting from 24-h laboratory fumigations

Life stage[f]	Treatments	N[a]	Slope[b]±S.E.	LC_{50} (µl/l) (Fiducial limit)[c]	LC_{90} (µl/l) (Fiducial limit)[c]	χ^2 [d] H[e]
Larva	Mustard oil alone	420	4.59±0.52	1.76 (1.54-1.97)	3.35 (2.94-3.98)	6.19 0.39
	Mustard oil+ 92% CO_2	300	8.33±1.31	0.32 (0.29-0.34)	0.46 (0.42-0.52)	5.22 0.58
	Mustard oil+ 92% N_2	420	6.05±0.70	1.73 (1.57-1.87)	2.82 (2.58-3.19)	8.55 0.43
Pupa	Mustard oil alone	360	4.37±0.69	0.83 (0.67-0.95)	1.75 (1.55-2.06)	7.93 0.61
	Mustard oil+ 92% CO_2	300	3.41±0.52	0.41 (0.33-0.48)	0.98 (0.82-1.31)	5.81 0.58
	Mustard oil+ 92% N_2	420	4.76±0.73	0.92 (0.76- 1.07)	1.62 (1.39-2.03)	10.71 0.67
Adult	Mustard oil alone	360	6.02±0.55	2.25 (2.08-2.43)	3.67 (3.32-4.19)	9.87 0.76
	Mustard oil+ 92% CO_2	360	4.00±0.41	0.42 (0.37-0.47)	0.88 (0.77-1.05)	5.39 0.41
	Mustard oil+ 92% N_2	420	4.71±0.42	0.65 (0.59-0.71)	1.21 (1.09-1.41)	9.85 0.62

[a]Number treated, excluding controls, [b]Slopes are non-parallel and unequal where noted, [c]Value in parentheses refers to the 95% confidence range, [d]Chi-square value, [e]Heterogeneity value, [f]The lethal concentration values were not able to be estimated since the lowest concentration of mustard essential oil alone (0.25 µl/l of air) resulted 100% mortality of *Tribolium confusum* eggs.

The results of probit analyses indicate that the use of 92% CO_2 or 92% N_2 with mustard essential oil and allyl isothiocyanate clearly resulted in significant reductions of LC_{50} and LC_{90} values for larvae, pupae and adults of *T. confusum*. This was particularly effective for the most tolerant stages, larvae and adults, where combining mustard essential oil and allyl isothiocyanate with 92% CO_2 decreased the LC_{90} value from 3.35 to 0.46 µl/l and 2.65 to 0.65 µl/l; from 3.67 to 0.88 µl/l and 1.98 to 0.49 µl/l, respectively. Similarly, mustard essential oil and allyl isothiocyanate with 92% N_2 also decreased the LC_{90} value of larval and adults' stage from 3.35 to 2.82 µl/l and 2.65 to 1.16 µl/l; from 3.67 to 1.21 µl/l and 1.98 to 0.93 µl/l, respectively. It might be argued that low O_2 concentrations could influence the potentiating effect of CO_2 and N_2 for the most tolerant stages of *T. confusum* (larvae and adults). However, data without mustard essential oil and allyl isothiocyanate indicated that exposure of 92% CO_2 alone for 24 h resulted in only limited mortality of the larvae and adults. Therefore, the results suggest that CO_2 or N_2 might have a synergistic effect on target insects when exposed together with mustard essential oil and allyl isothiocyanate. The addition of CO_2 has long been known to increase the toxicity of fumigant gases for some storage insect pests (Bond & Buckland, 1978; Navarro et al., 2004) and has been recommended as a possible method of lowering the amount of methyl bromide required (Kawakami et al., 1996). Several modes of action have been proposed for the toxic action of elevated CO_2 levels (Friedlander, 1983), which include a reduction in various detoxification pathways from the mixed function oxidizes to the regeneration of acetylcholine. At CO_2 levels as low as 1% in air, the insects increase their spiracle opening, allowing the diffusion of fumigant gases into the tracheae to increase (Wigglesworth, 1972). Depending on insect species, insect spiracles remain open in the CO_2 concentration ranging from 2% to 5%, (Wigglesworth, 1972), which facilities the entrance of the toxic gases into the insect body. In the present study, CO_2 might have had a synergistic effect on target insects when exposed together with mustard essential oil and allyl isothiocyanate, which can be explained by mode of action of CO_2 as described above.

Other studies have indicated that the addition of CO_2 can increase the toxicity of methyl bromide (Dumas et al., 1969; Calderon & Leesch, 1983; Williams, 1985; Donahaye & Navarro, 1989). All these studies reported that the susceptibilities of target insects to fumigants mixture with CO_2 were increased by a factor of one to three. A similar joint action with CO_2 atmospheres has been recorded for some essential oils. The peel oils of *Citrus* spp. and *Eucalyptus citriodora* Hook at 10 and 20 µl/l concentrations in presence of two different modified atmospheres (15% CO_2+1% O_2+84% N_2 and 12% CO_2+5% O_2+83% N_2) were more toxic to the psocid, *Liposcelis bostrychophila* Badonnel, 1931 (Wang et al., 2001). However, toxicity results from present study show that reductions in LC_{50} and LC_{90} caused by mustard essential oil and allyl isothiocyanate in combination CO_2 and N_2 are higher than those reported by Wang et al. (2001).

Table 3. Probit analysis data of allyl isothiocyanate alone and allyl isothiocyanate in combination with 92% CO_2 or 92% N_2 for larval to adult stages of *Tribolium confusum* resulting from 24-h laboratory fumigations

Life stage[f]	Treatments	N[a]	Slope[b]±S.E.	LC_{50} (µl/l) (Fiducial limit)[c]	LC_{90} (µl/l) (Fiducial limit)[c]	χ^{2} [d] H[d]
Larva	Allyl isothiocyanate alone	360	7.52±1.11	1.79 (1.61-1.93)	2.65 (2.45-2.99)	4.96 0.31
	Allyl isothiocyanate+92% CO_2	300	4.35±0.59	0.33 (0.27-0.38)	0.65 (0.56-0.79)	6.16 0.62
	Allyl isothiocyanate+92% N_2	360	4.09±0.59	0.57 (0.46- 0.65)	1.16 (1.02-1.41)	5.81 0.36
Pupa	Allyl isothiocyanate alone	360	4.14±0.56	0.53 (0.43-0.62)	1.08 (0.93-1.32)	4.09 0.32
	Allyl isothiocyanate+92% CO_2	300	3.74±0.59	0.26 (0.20-0.31)	0.58 (0.49-0.73)	7.06 0.71
	Allyl isothiocyanate+92% N_2	420	4.25±0.55	0.45 (0.37-0.51)	0.89 (0.79-1.07)	6.13 0.38
Adult	Allyl isothiocyanate alone	360	5.69±0.55	1.18 (1.08-1.79)	1.98 (1.79-2.25)	11.39 0.88
	Allyl isothiocyanate+92% CO_2	300	3.21±0.58	0.19 (0.13-0.25)	0.49 (0.41-0.62)	4.78 0.48
	Allyl isothiocyanate+92% N_2	360	3.90±0.40	0.44 (0.38-0.49)	0.93 (0.81-1.11)	11.50 0.88

[a]Number treated, excluding controls, [b]Slopes are non-parallel and unequal where noted, [c]Value in parentheses refers to the 95% confidence range, [d]Chi-square, [e]Heterogeneity value, [f]The lethal concentration values were not able to be estimated since the lowest concentration of mustard essential oil alone (0.25 µl/l of air) resulted 100% mortality of *Tribolium confusum* eggs.

The combinations of mustard essential oil and allyl isothiocyanate with 92% CO_2 were more toxic to larvae, pupae and adults of *T. confusum* than those in combinations with 92% N_2 as evidenced by significant decrements in their LC_{50} and LC_{90} values. N_2 is not directly toxic to the insects, but is only lethal to the insects by producing a progressive hypoxia or anoxia (decreasing O_2 availability) only when used alone at a high concentration, producing low O_2 atmospheres. However, a higher CO_2 concentration, accompanied by a reduction of O_2, leads to hypercarbia, which directly affects the nervous, endocrine, respiratory and circulatory systems, as well as general metabolism (Wong-Corral et al., 2013). Insects are generally killed more rapidly by CO_2 than by lack of O_2 (high level of N_2 atmosphere) and therefore, CO_2 is more effective method than use of N_2 because CO_2 stimulates insect respiration by displacing O_2 (Bell et al., 1980; Jayas & Jeyamkondan, 2002). The higher toxicity of combination of mustard essential oil and allyl isothiocyanate with CO_2 can be attributed to these insecticidal properties of CO_2 as described above.

In conclusion, the use of high concentration of CO_2 or N_2 with mustard essential oil and allyl isothiocyanate might have a possible synergistic effect on larvae, pupae and adults of *T. confusum* as evidenced by significant reductions in their LC_{50} and LC_{90} values. These results indicate that combination of mustard essential oil and allyl isothiocyanate with CO_2 or N_2 can be potential alternative to the most commonly used commercial fumigants, methyl bromide and phosphine.

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Original article (Orijinal araştırma)

A study of the Zygaenidae (Lepidoptera) fauna of Central Anatolia, Turkey[1]

Orta Anadolu Bölgesi (Türkiye) Zygaenidae (Lepidoptera) faunası üzerine bir araştırma

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Abstract

The Zygaenidae fauna in some provinces (Aksaray, Karaman, Kayseri, Konya, Nevşehir and Niğde) of Central Anatolia was studied using attractant traps as well as by netting specimens in biotopes in 2017. The sex attractants for the Procridinae had been produced in the Crimean Federal University and were esters of 2-dodecenoic acid and isomers of 2-butanol: EFETOV-2 [racemic mixture of *R*- and *S*-enantiomers], EFETOV-S-2 [R-enantiomer] and EFETOV-S-S-2 [*S*-enantiomer]. *Zygaena* attractants were made at Canterbury Christ Church University using a range of acetate compounds with known attraction to various genera as originally identified by Priesner et al. (1984). Fourteen Zygaenidae species from four genera belonging to Procridinae and Zygaeninae subfamilies were found: *Rhagades* Wallengren, 1863 (1 species), *Adscita* Retzius, 1783 (1 species), *Jordanita* Verity, 1946 (4 species), and *Zygaena* Fabricius, 1775 (8 species).

Keywords: Central Anatolia, Procridinae, sex attractants, Turkey, Zygaenidae, Zygaeninae

Öz

Orta Anadolu'nun bazı illerinde (Aksaray, Karaman, Kayseri, Konya, Nevşehir ve Niğde) Zygaenidae faunasını belirlemek için 2017 yılında, türlerin yaşam alanlarında, çekici tuzaklar ve atrap kullanılarak çalışılmıştır. Procridinae alt familyası için cinsel çekiciler 2-asit esterleri ve 2-butanol isomerleri: EFETOV-2 [*R*- ve *S*-enantiyomerlerinin rasemik karışımı], EFETOV-S-2 [*R*-enantiyomer] ve EFETOV-S-S-2 [*S*-enantiyomer] kullanılarak, Crimean Federal Üniversitesinde üretilmiştir. *Zygaena* türleri için çekiciler, Priesner et al. (1984) tarafından ilk defa tanımlanan ve birçok cins için çekici olduğu bilinen asetat bileşimlerinin farklı oranları kullanılarak Canterbury Christ Church Üniversitesinde hazırlanmıştır. Procridinae ve Zygaeninae alt familyalarına ait, 4 cinse giren, on dört Zygaenidae türü bulunmuştur: *Rhagades* Wallengren, 1863 (1 tür), *Adscita* Retzius, 1783 (1 tür), *Jordanita* Verity, 1946 (4 tür) ve *Zygaena* Fabricius, 1775 (8 tür).

Anahtar sözcükler: Orta Anadolu Bölgesi, Procridinae, cinsel çekiciler, Türkiye, Zygaenidae, Zygaeninae

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Introduction

Asian Turkey (Anatolia) is a biologically diverse region since the variable topography and climate provide many different macro or microhabitats; the region is a bridge between Asia and Europe and in the south to the Ethiopian region via the Arabian Peninsula. It thus provides a natural pathway for the spread of species both north-south and east-west. Its tectonic evolution has continuously changed through Tertiary and Quaternary periods, providing an important refuge during the Quaternary ice ages receiving populations via the Balkans and/or the Caucasus (Cıplak, 2003). Consequently, Anatolia has a distinctive zoogeography and biodiversity.

According to the contemporary classification the family Zygaenidae (Lepidoptera) is divided into five subfamilies: Inouelinae Efetov & Tarmann, 2017; Procridinae Boisduval, 1828; Chalcosiinae Walker, 1865; Callizygaeninae Alberti, 1954; and Zygaeninae Latreille, 1809 (Efetov et al., 2014a, b; Efetov & Tarmann, 2017). The family includes more than 1000 species (Efetov, 1996, 1997a, b, 1998, 1999, 2006, 2010; Efetov et al., 2004; Efetov & Hayashi, 2008; Efetov & Tarmann, 2013a, b, 2014a, b, 2016; Mutanen et al., 2016; Hofmann & Tremewan, 2017).

Fifty-four Zygaenidae species are known from Turkey (Mollet, 1995; Naumann et al., 1999; Efetov, 2001, 2005; Koçak & Kemal, 2007; Kemal & Koçak, 2010; Efetov & Tarmann, 2012). Recently, in the Thrace Region (the European part of Turkey) two Procridinae species new for Turkey were found, viz., *Rhagades* (*Rhagades*) *pruni* (Denis & Schiffermüller, 1775) and *Jordanita* (*Jordanita*) *globulariae* (Hübner, 1793), which were discovered with the help of sex attractants of the 'EFETOV-2' series (Can Cengiz et al., 2018). Now, the Zygaenidae fauna of Turkey includes 23 Procridinae species and 31 Zygaeninae species. Five of them are endemics of Turkey: *Jordanita* (*Jordanita*) *chloronota* (Staudinger, 1871), *Zygaena* (*Mesembrynus*) *lydia* Staudinger, 1887, *Zygaena* (*Agrumenia*) *formosa* (Herrich-Schäffer, 1852), *Zygaena* (*Agrumenia*) *peschmerga* Eckweiler & Görgner, 1981, and *Zygaena* (*Zygaena*) *problematica* Naumann, 1966. Identification of endemic and other zygaenid species, determination of their biological characteristics and definition of their distribution sites are crucial for the understanding, protection and control of the Turkish fauna. The family Zygaenidae also comprises many pest species (Tarmann, 2003). In addition, zygaenid moths colonize a great diversity of natural and occasionally secondary habitats, from coastal dunes and cliffs and dry Mediterranean landscapes to various arboreal habitats and even high alpine and extreme boreal regions.

Zygaenidae from Anatolia have been studied for many years (Staudinger, 1887; Alberti, 1954, 1958; Mollet, 1995; Efetov et al., 2010b). Sex pheromones and sex attractants are fast becoming key instruments in ecology-faunistic investigations to monitor distribution, seasonal flight and population density of Lepidoptera species as well as in agriculture and horticulture for pest control (Can et al., 2010; Efetov et al., 2010a, 2011, 2014a, 2015; Subchev et al., 2010, 2012, 2013, 2016; Witzgall et al., 2010; Oleander et al., 2015; Thackery & Burman, 2016; Razov et al., 2017). The aim of this research was to study the occurrence and distribution of Zygaenidae in Central Anatolia because the fauna of this family has not been sufficiently studied in this region of Turkey.

Material and Methods

The Zygaenidae fauna of Central Anatolia was studied using attractant traps and by netting specimens in different biotopes in 2017. The Procridinae sex attractants were synthesized from chiral alcohol *sec*-butanol and lauric acid in Simferopol (Crimea) as described in Efetov et al. (2014c). Three different attractants were used and are designated as EFETOV-2 [a racemic mixture of the two esters (2*R*)-butyl 2-dodecenoate and (2*S*)-butyl 2-dodecenoate], EFETOV-S-2 [*R*-enantiomer] and EFETOV-S-S-2 [*S*-enantiomer]. Previously, the biological activity of these attractants for some species of Procridinae had been confirmed in field tests conducted in the Crimea and Japan (Efetov et al., 2016a, 2018).

Zygaeninae attractants were made at Canterbury Christ Church University using a range of acetate compounds with known attraction to various genera as originally identified by Priesner et al. (1984), as well as modified combinations of (*Z*)-9-tetradecenyl acetate, (*Z*)-11-hexadecenyl acetate and (*Z*)-11-tetradecenyl acetate designated ZtA, ZtB and ZtC (Table 1).

Table 1. Blends of acetate attractants formulated as lures for the attraction of various *Zygaena* spp. numbers in table show the quantity of each component added to a single rubber lure

Attractant*	Attractant dose (µg/lure)						
	Z7-12:Ac	Z9-14:Ac	Z5-12:Ac	Z7-14:Ac	Z11-16:Ac	Z11-14:Ac	Z9-12:Ac
Za						100	
Zc	100	30			3		
Zf	100	10	3	3			
Zl	3		100				
Zp	100	100		10			
ZtA		100			10	10	
ZtB		10			100	10	
ZtC		10			10	100	
Zv	100	10	10		5		1

* For taxa: *Zygaena* (*Zygaena*) *angelicae* Ochsenheimer, 1808, *Zygaena* (*Agrumenia*) *carniolica* (Scopoli, 1763), *Zygaena* (*Zygaena*) *filipendulae* (Linnaeus, 1758), *Zygaena* (*Zygaena*) *lonicerae* (Scheven, 1777), *Zygaena* (*Mesembrynus*) *purpuralis* (Brünnich, 1763), *Zygaena* (*Mesembrynus*) *tamara* Christoph, 1889, and *Zygaena* (*Agrumenia*) *viciae* (Denis & Schiffermuller, 1775).

Rubber caps with different attractants were fixed in cardboard rectangles with corresponding labels. Prepared lures were placed in transparent Delta traps with removable sticky sheets. The traps were attached to branches of bushes or trees at a height of 1.0-1.5 m above the ground. Control traps without attractant baits were also present in all studied localities. The distance between the traps was more than 10 m.

Traps with attractants EFETOV-2, EFETOV-S-2, EFETOV-S-S-2 and the nine acetate blends as detailed in Table 1, and controls with no attractant were set up in six localities (detailed below). The baits were deployed from 13-18 May 2017 and inspected from 29 May-4 June 2017, 17-23 June 2017 and 3-9 July 2017.

Fieldwork was undertaken in 42 localities in six provinces of Central Anatolia: Aksaray, Karaman, Kayseri, Konya, Nevşehir and Niğde (Table 2). Specimens were dissected in the laboratory, with the genitalia embedded on slides in either in Entellan or in Euparal following standard procedures. The material collected was deposited in the collection of Hatay Mustafa Kemal University (Hatay, Turkey).

Localities of traps

The location of the traps were: Aksaray-Saratlı, Bilişim Valley, 38º28’04” N, 34º13’10” E, 1184 m; Karaman-Seyithasan, 37º03’37” N, 33º13’08” E, 1202 m; Kayseri-Yeşilhisar, 38º17’00” N, 35º06’19” E, 1128 m; Konya-Selçuk University Campus, 38º01’55” N, 32º30’25” E, 1166 m; Nevşehir-Hacıbektaş, Kızılağıl, 39º01’05” N, 34º47’12” E, 1203 m; and Niğde-Çamardı, Çukurbağ, 37º49’55” N, 35º02’06” E, 1460 m.

Table 2. Localities studied in Central Anatolia, Turkey

Location no.	Province and town	Latitude, longitude and elevation
1	Niğde-Çamardı, Yelatantown	37º40’31” N, 35º00’27” E, 1250 m
2	Niğde-University Campus	37º56’36” N, 34º37’43” E, 1206 m
3	Kayseri-Yeşilhisar	38º28’28” N, 35º09’26” E, 1088 m
4	Nevşehir-Ürgüp, Başderetown	38º34’30” N, 35º03’52” E, 1372 m
5	Nevşehir-Ürgüp, Akköy1	38º34’54” N, 35º03’06” E, 1323 m
6	Nevşehir-Ürgüp, Akköy 2	38º35’31” N, 35º01’47” E, 1240 m
7	Nevşehir-Ürgüp, Boyalıköy	38º35’35” N, 35º00’09” E, 1213 m
8	Nevşehir-Göreme	38º39’43” N, 34º49’55” E, 1060 m
9	Nevşehir-Göreme, Çavuşin Köyü	38º43’54” N, 34º52’47” E, 944 m
10	Nevşehir-Gülşehir	38º43’46” N, 34º40’43” E, 972 m
11	Aksaray	38º24’23” N, 34º02’16” E, 1113 m
12	Karaman-Seyithasan 1	37º04’18” N, 33º13’26” E, 1158 m
13	Karaman-Seyithasan 2	37º05’20” N, 33º14’20” E, 1218 m
14	Karaman-Organize Sanayi	37º13’09” N, 33º18’00” E, 1027 m
15	Niğde-Darboğaz I	37º29’17” N, 34º33’57” E, 1364 m
16	Niğde-Alpu	37º28’06” N, 34º52’14” E, 859 m
17	Niğde-Emli	37º47’02” N, 35º03’55” E, 1631 m
18	Kayseri-Niğde border	38º12’36” N, 35º13’54” E, 1356 m
19	Kayseri-Develi	38º26’31” N, 35º13’40” E, 1077 m
20	Kayseri-Develi, Hüseyin Şahin Vocational School	38º22’48” N, 35º27’13” E, 1201 m
21	Kayseri-Soysallı	38º23’31” N, 35º21’44” E, 1080 m
22	Nevşehir	38º30’18” N, 35º07’49” E, 1269 m
23	Nevşehir-Karacaören	38º37’00” N, 34º58’16” E, 1168 m
24	Nevşehir-Ürgüp	38º39’34” N, 34º53’59” E, 1066 m
25	Nevşehir-Avanos	38º40’58” N, 34º34’03” E, 1030 m
26	Nevşehir-Acıgöl	38º33’09” N, 35º34’03” E, 1327 m
27	Aksaray-Alayhan	38º31’15” N, 34º21’55” E, 1293 m
28	Konya-Karatay, Yenice	38º10’36” N, 33º17’47” E, 990 m
29	Konya-Karatay	38º03’22” N, 32º59’24” E, 1120 m
30	Konya-Karatay, Ortakonak	37º59’16” N, 32º44’10” E, 1022 m
31	Konya-Selçuklu, Silile	37º55’07” N, 32º26’27” E, 1112 m
32	Konya-Selçuklu, Tatköy	37º56’33” N, 32º22’39” E, 1353 m
33	Karaman-Kazımkarabekir, Kızılkuyu	37º19’55” N, 32º49’50” E, 1056 m
34	Konya-Adabağ, Ereğli	37º27’46” N, 33º54’24” E, 1018 m
35	Niğde-Darboğaz II	37º30’16” N, 34º34’14” E, 1270 m

Table 2. (Continued)

36	Kayseri-Sarız, Yeşilkent	38º16’25” N, 36º26’15” E, 1545 m
37	Kayseri-Sarız, İncemağara	38º22’47” N, 36º26’37” E, 1498 m
38	Kayseri-Pınarbaşı	38º47’15” N, 36º27’01” E, 1538 m
39	Kayseri-Büyüktuzhisar	38º57’06” N, 35º52’26” E, 1217 m
40	Kayseri-Sarımsaklı	38º53’23” N, 35º42’45” E, 1169 m
41	Kayseri-Melikgazi, Hisarcık	38º37’22” N, 35º30’56” E, 1600 m
42	Niğde-Çamardı, Bademdere	37º56’07” N, 35º04’52” E, 1609 m

Results and Discussion

The attractants used attracted males of four Procridinae species: *Rhagades* (*Wiegelia*) *amasina* (Herrich-Schäffer, 1851), *Adscita* (*Adscita*) *obscura* (Zeller, 1847), *Jordanita* (*Praviela*) *anatolica* (Naufock, 1929) and *Jordanita* (*Solaniterna*) *subsolana* (Staudinger, 1862) (Figure 1, Table 3).

Figure 1. Sticky trap baited with EFETOV-2 with 49 males of *Jordanita* (*Praviela*) *anatolica*, Karaman-Seyithasan, 17-23 June 2017.

Jordanita (*P.*) *anatolica* was mainly found on sticky traps baited with EFETOV-2, whereas, *Rh.* (*W.*) *amasina* was attracted to EFETOV-S-S-2. *Adscita* (*A.*) *obscura* was mainly attracted to EFETOV-2 and EFETOV-S-2. *Jordanita* (*S.*) *subsolana* was attracted to EFETOV-S-2. This was as expected and confirmed data obtained in the Crimea, Austria, Italy, and Greece (Efetov et al., 2016b, 2017). No specimens were found in the control traps during the study.

Zygaena (*A.*) *carniolica*, *Zygaena* (*Zygaena*) *ephialtes* (Linnaeus, 1767) and *Z.* (*Z.*) *filipendulae* were attracted to the acetates used. *Zygaena filipendulae* was attracted to lures containing (*Z*)-7-dodecenyl acetate, (*Z*)-9-tetradecenyl acetate and (*Z*)-5-dodecenyl acetate, and this blend also attracted some *Z. carniolica*, possibly because both blends contained a similar ratio of (*Z*)-7-dodecenyl acetate to (*Z*)-9-tetradecenyl acetate. *Zygaena ephialtes* was also attracted to a lure containing (*Z*)-11-tetradecenyl acetate as its primary component (Table 4).

Table 3. Number of males of Procridinae species attracted the three attractants during three trapping periods

Species trapped	29 May-4 June 2017			17-23 June 2017			3-9 July 2017		
	EFETOV -2	EFETOV -S-2	EFETOV -S-S-2	EFETOV -2	EFETOV -S-2	EFETOV -S-S-2	EFETOV -2	EFETOV -S-2	EFETOV -S-S-2
Adscita obscura	1♂ Aksaray	-	-	3♂♂ Aksaray	3♂♂ Nevşehir 1♂ Karaman	1♂ Karaman	-	-	-
Jordanita anatolica	-	-	-	49♂♂ Karaman	14♂♂ Karaman	-	2♂♂ Konya 11♂♂ Karaman	1♂ Konya 5♂♂ Karaman	-
Jordanita subsolana	-	-	-	-	-	-	-	5♂♂ Niğde	-
Rhagades amasina	-	-	-	-	-	-	1♂ Konya	-	11♂♂ Konya 20♂♂ Karaman

Table 4. Number of males of three *Zygaena* spp. attracted to nine attractants in two tapping periods

Attractant	17-23 June 2017			3-9 July 2017		
	Z. carniolica	*Z. ephialtes*	*Z. filipendulae*	*Z. carniolica*	*Z. ephialtes*	*Z. filipendulae*
Za	-	-	-	-	-	-
Zc	-	-	-	3♂♂ Karaman 1♂ Konya	-	-
Zf	-	-	2♂♂ Karaman 1♂ Nevşehir	7♂♂ Karaman 1♂ Konya	-	1♂ Karaman
Zl	-	-	-	-	-	-
Zp	-	-	-	5♂♂ Karaman	-	-
ZtA	-	-	-	-	-	-
ZtB	-	-	-	-	-	-
ZtC	-	-	-	-	1♂ Karaman	-
Zv	-	-	-	2♂♂ Karaman	-	1♂ Niğde

Also, nine Zygaenidae species were collected by netting, viz., *Adscita* (*Adscita*) *obscura* (Zeller, 1847), *Jordanita* (*Jordanita*) *graeca* (Jordan, 1907), *Jordanita* (*Jordanita*) *chloros* (Hübner, 1813), *Jordanita* (*Praviela*) *anatolica* (Naufock, 1929) (Procridinae), *Zygaena* (*Mesembrynus*) *brizae* (Esper, 1800) (Figure 2), *Zygaena* (*Mesembrynus*) *diaphana* Staudinger, 1887, *Zygaena* (*Mesembrynus*) *purpuralis* (Brünnich, 1763), *Zygaena* (*Mesembrynus*) *laeta* (Hübner, 1790) and *Zygaena* (*Agrumenia*) *loti* (Denis & Schiffermüller, 1775) (Zygaeninae) (Table 5).

Table 5. Zygaenidae species caught by netting in Central Anatolia in 2017

Species netted	Localities										
	2	4	5	11	17	25	26	29	31	38	41
Adscita obscura	-	-	1♂	-	-	-	-	-	-	-	-
Jordanita anatolica	1♀	-	-	-	-	1♂	-	-	-	-	-
Jordanita chloros	-	-	-	2♂♂ 1♀	-	-	-	-	1♂	-	-
Jordanita graeca	-	-	-	-	-	2♂♂	1♂	-	-	5♂♂	-
Zygaena brizae	-	-	-	-	2♂♂	-	-	2♂♂	-	-	-
Zygaena diaphana	-	4♂♂ 3♀♀	1♂ 1♀	-	-	-	-	-	-	-	-
Zygaena laeta	-	-	-	-	-	-	-	-	-	-	1♂ 1♀
Zygaena loti	-	5♂♂ 2♀♀	1♂	-	-	-	-	-	-	-	-
Zygaena purpuralis	-	1♂	-	-	-	-	-	-	-	-	-

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Rhagades (*Wiegelia*) *amasina* (Herrich-Schäffer, 1851): 1♂ to EFETOV-2 (Konya); 31♂♂ to EFETOV-S-S-2 (Konya and Karaman).

Adscita (*Adscita*) *obscura* (Zeller, 1847): 4♂♂ to EFETOV-2 (Aksaray); 1♂ to EFETOV-S-S-2 (Karaman); 4♂♂ to EFETOV-S-2 (Nevşehir and Karaman); 1♂ by net (Nevşehir).

Jordanita (*Jordanita*) *graeca* (Jordan, 1907): 10♂♂ and 1♀ by net (Aksaray, Kayseri and Nevşehir).

Jordanita (*Jordanita*) *chloros* (Hübner, 1813): 1♂ by net (Konya).

Jordanita (*Praviela*) *anatolica* (Naufock, 1929): 62♂♂ to EFETOV-2 (Karaman and Konya); 20♂♂ to EFETOV-S-2 (Konya and Karaman); 1♂ and 1♀ by net (Niğde and Nevşehir).

Jordanita (*Solaniterna*) *subsolana* (Staudinger, 1862): 5♂♂ to EFETOV-S-2 (Niğde).

Zygaena (*Mesembrynus*) *brizae* (Esper, 1800): 4♂♂ by net (Konya and Niğde).

Zygaena (*Mesembrynus*) *diaphana* Staudinger, 1887: 6♂♂ and 3♀♀ by net (Nevşehir).

Zygaena (*Mesembrynus*) *purpuralis* (Brünnich, 1763): 1♂ by net (Nevşehir).

Zygaena (*Mesembrynus*) *laeta* (Hübner, 1790): 1♂ and 1♀ by net (Kayseri).

Zygaena (*Agrumenia*) *carniolica* (Scopoli, 1763): 4♂♂ to Zc trap (Konya and Karaman); 8♂♂ to Zf trap (Konya and Karaman); 2♂♂ to Zv trap (Karaman); 5♂♂ to Zp trap (Karaman).

Zygaena (*Agrumenia*) *loti* ([Denis & Schiffermüller], 1775): 6♂♂ and 2♀♀ by net (Nevşehir).

Zygaena (*Zygaena*) *ephialtes* (Linnaeus, 1767): 1♂ to ZtC trap (Karaman).

Zygaena (*Zygaena*) *filipendulae* (Linnaeus, 1758): 4♂♂ to Zf trap (Karaman and Nevşehir); 1♂ to Zv trap (Niğde).

Figure 2. *Zygaena* (*Mesembrynus*) *brizae,* Niğde-Emli, 21 June 2017.

Conclusions

It was found that EFETOV-2, EFETOV-S-2 and EFETOV-S-S-2 attracted males of four Procridinae species, *Rh.* (*W.*) *amasina*, *A.* (*A.*) *obscura*, *J.* (*P.*) *anatolica*, and *J.* (*S.*) *subsolana*. Four species of Procridinae were also collected by netting, *A.* (*A.*) *obscura*, *J.* (*J.*) *graeca*, *J.* (*J.*) *chloros*, and *J.* (*P.*) *anatolica*.

The lures containing the *Zygaena* acetates were attractive for three Zygaeninae species, viz., *Z.* (*A.*) *carniolica*, *Z.* (*Z.*) *ephialtes*, and *Z.* (*Z.*) *filipendulae*. Five Zygaeninae spp. were collected also by netting, viz*., Z.* (*M.*) *brizae*, *Z.* (*M.*) *diaphana*, *Z.* (*M.*) *purpuralis*, *Z.* (*M.*) *laeta*, and *Z.* (*A.*) *loti*.

Jordanita (*P.*) *anatolica* was the most numerous species caught in this study. The attractants applied in this work helped to discover the biotopes with *Rh.* (*W.*) *amasina*, a potential pest species for apple, pear, plum and cherry orchards in Central Anatolia.

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Original article (Orijinal araştırma)

Comparative toxic potential of some plant extracts and spinetoram against *Tribolium castaneum* (Herbst, 1797) (Coleoptera: Tenebrionidae)

Bazı bitkisel özütlerin ve spinetoram ile kombinasyonlarının *Tribolium castaneum* (Herbst, 1997) (Coleoptera: Tenebrionidae)'a karşılaştırmalı olarak zehirlilik potansiyeli

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Abstract

The comparative toxicity of lemon grass, *Cymbopogon citratus* (Stapf) (Poales: Poaceae); neem, *Azadirachta indica* (A. Juss.) (Sapindales: Meliaceae); castor oil plant, *Ricinus communis* (L.) (Malpighiales: Euphorbiaceae); and yellow oleander, *Thevetia peruviana* (L.) Lippold (Gentianales: Apocynaceae) and spinetoram alone and in combinations were evaluated against *Tribolium castaneum* (Herbst, 1797) in the Entomology Laboratory of Punjab Bioenergy Institute, University of Agriculture, Faisalabad, Punjab, Pakistan from May to October, 2018. Different concentrations of plant extracts (5, 10 and 15%) and spinetoram (0.01, 0.02 and 0.03%) were applied in filter paper toxicity bioassay. The results of single applications of all treatments showed that spinetoram was more effective with the highest mortality of 79.8% by followed by 57.9% for *A. indica*, 49.5% for *C. citratus*, 40.1% for *T. peruviana* and 28.9% for *R. communis* extracts. In combinations, *A. indica*+spinetoram gave the highest mortality of 84.9% with a lower mortality of 25.2% observed for *R. communis*+spinetoram. LC_{50} values of spinetoram were comparatively low indicating that it is the more toxic than the plant extracts. Overall the results indicated that these plant extracts and spinetoram can be used as combined form in IPM for the efficient management of insect pests of stored grains.

Keywords: Concentrations, LC_{50}, management, mortality, synthetic insecticides, toxic

Öz

Limon otu (*Cymbopogon citratus* (Stapf) (Poales: Poaceae), neem (*Azadirachta indica*) (A. Juss.) (Sapindales: Meliaceae), hint otu (*Ricinus communis*) (L.) (Malpighiales: Euphorbiaceae) ve meksika zakkumu (*Thevetia peruviana*) (L.) Lippold (Gentianales: Apocynaceae) 'nun tek başına ve spinetoram ile kombinasyonlarının *Tribolium castaneum* (Herbst, 1797)'a karşı toksikolojik etkileri Mayıs-Ekim 2018 tarihlerinde Tarım Üniversitesi, Punjab Bioenergy Enstitüsü (Faisalabad, Punjab, Pakistan), Entomoloji laboratuvarında değerlendirilmiştir. Filtre kâğıdı yöntemi ile yapılan biyolojik testlerde farklı bitki konsantrasyonları (%5, 10 ve 15) ve spinetoramın (%0.01, 0.02 ve 0.03) farklı dozları uygulanmıştır. Tüm muameleler tek başına uygulandığında, spinetoram %79.8 oranla en yüksek öldürücü etkiye sahip olurken, bunu sırasıyla *A. indica* (%57.9), *C. citratus* (%49.5) ve *T. peruviana* (%40.1) izlemiştir. En düşük etki %28.9 oranla *R. communis* özütünde kaydedilmiştir. Özütler spinetoram ile birlikte uygulandığında, *A. indica* kombinasyonu %84.9'lik oranla en yüksek öldürücülüğe sahip olurken, nispeten düşük ölüm oranı (%25.2) spinetoramın *R. communis* ile kombinasyonunda gözlenmiştir. Spinetoramın LC_{50} değerleri denemede kullanılan bitki özütleriyle karşılaştırıldığında en yüksek değeri veren özüte göre düşük bulunmuştur. Sonuç olarak, bu çalışma bu bitki özütlerinin spinetoram ile kombinasyonun depolanmış tahıllardaki zararlı böcekler ile etkili mücadele için IPM'de kullanılabileceğini göstermiştir.

Anahtar sözcükler: Konsantrasyonlar, LC_{50}, mücadele, öldürücü etki, sentetik insektisitler, zehirlilik

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Introduction

Insect pest infestations in stored commodities are increasingly important worldwide. Many different insect pests can infest the stored grain (Phillips & Throne, 2010). The red flour beetle, *Tribolium castaneum* (Herbst, 1797) (Coleoptera: Tenebrionidae) is a cosmopolitan and the most widespread insect pest of wheat flour and cereals in Pakistan (Lee et al., 2002; Shafique et al., 2006; Nadeem et al., 2013) and worldwide (Ogendo et al., 2008). Quality of infested commodities is seriously impacted, for example., baking ability, nutritional value, decreased germination and increase proportion of damaged (Mondal, 1994). Both larvae and adults of *T. castaneum* feed on wheat flour and broken grain (Dars et al., 2001; Karunakaran et al., 2004), secrete quinines and other chemicals (Ho et al., 2008).

Synthetic chemicals (fumigants and other insecticides) have been the main means of control different insect pests in stored grains. With fumigants, such as methyl bromide and phosphine, have been mostly used in the management of different insect pests of stored grain. However, frequent and inappropriate use of these pesticides has resulted in adverse effects to human beings, and natural flora and fauna. Furthermore, some insect populations have developed resistance against these chemicals (Bell & Wilson, 1995; Benhalima et al., 2004). Many stored grain insect pests have also developed resistance against organophosphates (Hussain et al., 2005). Moreover, the residual effects synthetic insecticides in grains have been reported, leaving the food commodities unfit for human consumption (Benhalima et al., 2004; Phillips & Throne, 2010). Therefore, there is an imperative to develop and deploy bioinsecticides from plant materials and the bio-derived spinetoram, which are eco-friendly, safe to humans and can effectively manage the insect pests of stored commodities in storage facilities.

Plant extracts are potential substitute to synthetic insecticides (Copping & Menn, 2000; Mondal et al., 2006; Koul et al., 2008; Nenaah, 2011; Usha et al., 2011), having different effects, such as, fumigant action (Rajendran & Sriranjini, 2008), antifeedant action (Kamruzaman et al., 2005), toxicity (Islam et al., 2010), repellence (Dwivedi & Shekhawat, 2004; Susana et al., 2013; Hassan et al., 2017) and growth inhibition (Tatun et al., 2014). Being natural products and less persistent in nature, they are eco-friendly to surrounding flora and fauna (Isman, 2006; Sanna et al., 2004; Tapondjou et al., 2005; Saroukolai et al., 2010; Regnault-Roger et al., 2012). Many researchers have evaluated plant extracts for the management of different insect pests, for example, *Mentha royleana* Benth. (Lamiales: Lamiaceae) and *Artemisia absinthium* (L.) (Asterales: Asteraceae) extracts against *T. castaneum* (Zuhra et al., 2018), datura leaf extract against *Trogoderma granarium* (Everts, 1898) (Coleoptera: Dermestidae) (Ali et al., 2012), *Elletaria cardamomum* (L.) (Zingiberales: Zingiberaceae) against *Sitophilus zeamais* (Motschulsky), 1855 (Coleoptera: Curculionidae) and *T. castaneum* (Huang et al., 2000), *Abroma augusta* (L.) (Malvales: Malvaceae) and garlic extracts against *T. castaneum* (Mondal et al., 2006; Yang et al., 2010), *Azadirachta indica* (A. Juss.) (Sapindales: Meliaceae) extract against *T. granarium* (Odeyemi & Ashamo, 2005), *Datura alba* and some other plan extracts against *S. zeamais* and *Oryzaephilus surinamensis* (L., 1758) (Coleoptera: Silvanidae) (Rehman et al., 2018). Spinetoram is regarded as new member of spinosyn family and has been introduced commercially for the control of different insect pests (Sparks et al., 2008; Jones et al., 2010; Sial et al., 2011; Ali et al., 2017). Spinetoram is a product of secondary metabolites spinosyn J and L (Herbert, 2010). Another new chemistry insecticide, spinosad has also been used against *T. castaneum*. Spinetoram has been used by many researchers and proved comparatively more effective for the control of stored grain insect pests than spinosad (Sparks et al., 2008; Jones et al., 2010; Dripps et al., 2011; Yee & Alston, 2012; Saglam et al., 2016). To address the situation described above, experiments were conducted in the Entomology Laboratory of Punjab Bioenergy Institute, University of Agriculture, Faisalabad, Punjab, Pakistan from May to October, 2018 to evaluate toxic effects of some plant extracts and spinetoram on *T. castaneum*.

Material and Methods

Collection and rearing of test insects

Tribolium castaneum adults were collected from flour mills and grain markets of Kasur and Faisalabad in Punjab, Pakistan. The insects were cultured in the Entomology Laboratory of Punjab Bioenergy Institute, University of Agriculture, Faisalabad, Punjab, Pakistan from May to October 2018 on sterilized wheat flour in small plastic jars at 30±2ºC and 65±5% RH. Fifty pairs of *T. castaneum* were released into jars containing 2 kg of wheat flour. The jars were covered with muslin, secured with a rubber band to prevent the insects escape. The insects were kept in the jars for abut 3 to 4 d to lay eggs. After oviposition, the insects were transferred to new jars of wheat flour using a camel hair brush to lay more eggs and then the flour combined with that in the original jar. Complete emergence of adult beetles was achieved after 30-35 d and these adults were used in toxicity bioassays.

Preparation of plants extracts

Leaves of four plant species (Table 1) were collected from different sites of the University of Agriculture Faisalabad.

Table 1. Description and altitude of collection of plant materials collected from May to October 2018

Common name	Scientific name	Description	Altitude (m)
Lemon grass	*Cymbopogon citratus* (Stapf) (Poales: Poaceae)	lofty perennial grass**,** silky heads, citronella fever grass	184
Neem	*Azadirachta indica* (A. Juss.) (Sapindales: Meliaceae)	pinnate leaves, purple-red when young, developing to a medium green color when mature, produces small, fragrant white flowers and olive-like fruits	168
Castor oil plant	*Ricinus communis* (L.) (Malpighiales: Euphorbiaceae)	perennial flowering plant, reproducing with a mixed pollination system which favors selfing by geitonogamy	184
Yellow oleander	*Thevetia peruviana* (L.) Lippold (Gentianales: Apocynaceae)	iornamental, medicinal shrub and is an evergreen shrub often planted close to stables and paddocks, highly toxic, a potent cardiotoxic plant	189

Plant materials were washed with distilled water, dried in the shade and ground into a powder using an electric mill. The plant powders were sieved using a suitable mesh sieve to get a fine powder. Plant materials were extracted with acetone as by mixing 50 g of plant powder with 100 ml of solvent as described by Valladares et al. (1997) and Ahmad et al. (2006). To minimize solvent evaporation during extraction process, conical flasks were plugged with cotton plugs covered aluminum foil. The flasks were then placed on rotary shaker at 220 rpm for 24 h. Filtration was performed with using the Whatman filter papers.

Bioassay of plant extracts and spinetoram against *Tribolium castaneum*

Completely randomized design (CRD) used for the bioassay. Dilutions (5, 10 and 15%) of the stock solution of each plant extract were prepared in acetone. Each dilution was applied on 40 g of wheat grain in jars using a glass pipette. Controls were treated with acetone only. The grain was shaken for 2 min to ensure uniform distribution of the applied treatments. After evaporation of the solvent for few minutes, twenty unsexed adults of *T. castaneum* were added to each jar, and the jar covered with muslin secured with a rubber to prevent insects escape. The jars kept in incubator under optimum conditions. Insect mortality was recorded at regular intervals from 24 to 72 h. Also, three dilutions (0.01, 0.02 and 0.03%) of a stock solution of spinetoram were prepared in acetone for a separate bioassay using the method given above. All experiments were performed with three replicates.

Percentage corrected mortality was calculated using Abbott's formula (Abbott, 1925) prior to statistical analysis as:

$$\% \text{ Corrected Mortality} = \frac{\text{Mo (\%)} - \text{Mc (\%)}}{100 - \text{Mc (\%)}} \text{ X } 100$$

where, Mo is the observed mortality and Mc is the mortality in control unit.

Bioassay of plant extract and spinetoram combinations

Combined effects were evaluated using the most effective concentrations of plant extracts and spinetoram against *T. castaneum* as determined in the bioassays dcribed above. Treated filter papers (with treatment combinations) were placed in separate Petri dishes along with broken grains and 20 adult beetles (10 pairs). Mortality was recorded at regular intervals from 24 to 72 h.

Statistical analysis

The recorded data was subjected to Abbott's formula and then analyzed by using STATISTICA and means of the treatments were compared by Tukey's HSD at α of 5%.

Results and Discussion

Figures 1, 2 and 3 shows the treatment and concentration effects, which were significant after 24, 48 and 72 h. of treatment application. The combined effect of time ($F_{2,24}$ = 206; $p < 0.05$) and concentration ($F_{2,24}$ = 20.8; $p < 0.05$) were also significant. The highest mortality of 79.9% was observed for 0.03% spinetoram and for the plant extracts, 57.9% for *A. indica* followed by 49.5% for *C. citratus* and 40.1% for *T. peruviana* after 72 h. Th lowest mortality 28.9% was for *R. communis* at 15% after 72 h. Exposure of 24 and 48 h was less effective with mortality at 24 h of 21.3% for spinetoram, 20.0% for *A. indica*, 18.3% for *C. citratus*, 11.2% for *R. communis*, 8.33% for *T. peruviana* and at 48 h of 45.0, 33.7, 29. 3, 17.7 and 15.3%, respectively.

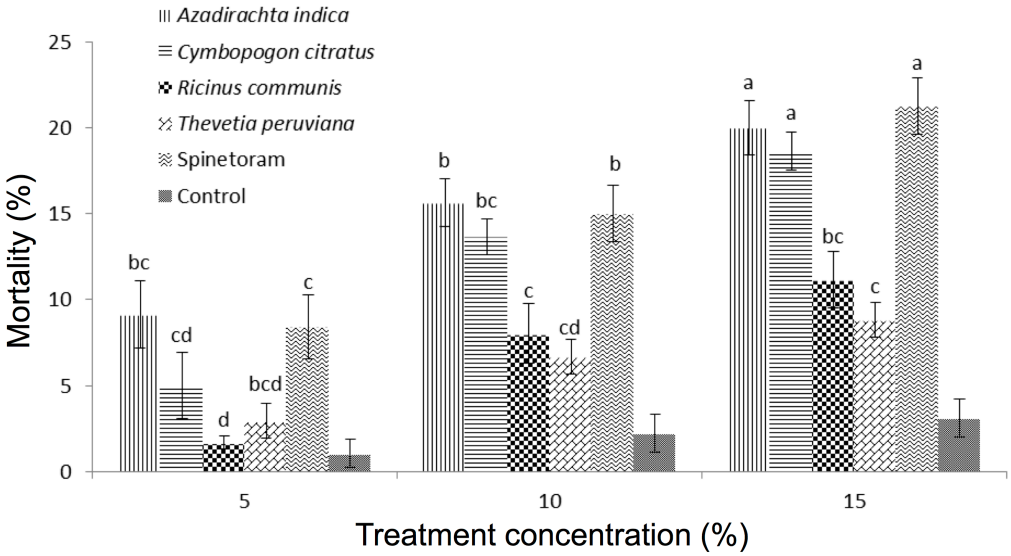

Figure 1. Efficacy of extracts of *Azadirachta indica*, *Cymbopogon citratus*, *Ricinus communis*, and *Thevetia peruviana,* and spinetoram against *Tribolium castaneum* adults after exposure of 24 h. Bars represent treatment means and error bars are 95% CI. The lowercase superscript letters above each bar represent post hoc pairwise comparisons between treatments. Treatment $F_{3,24}$ = 4.66, $p < 0.05$ and concentration: $F_{2,24}$ = 2.14, $p < 0.05$.

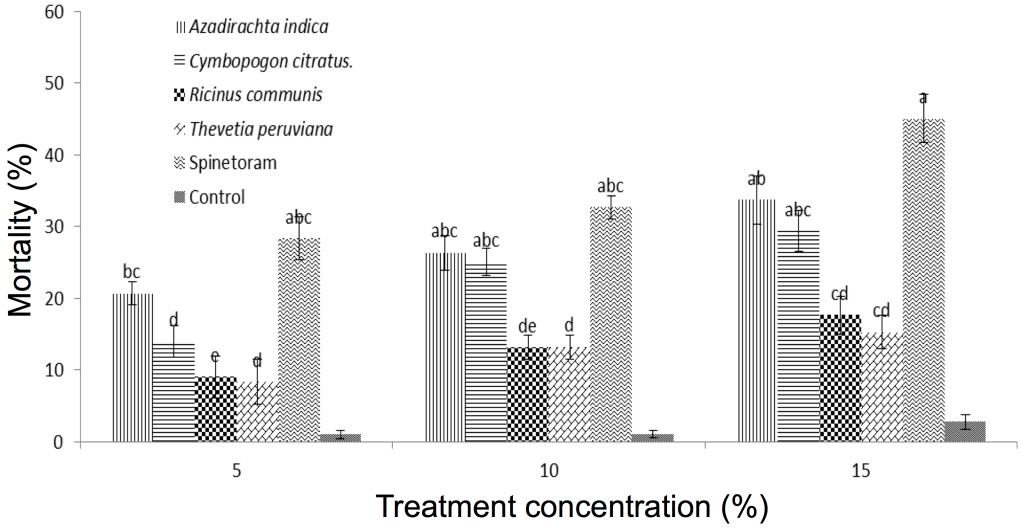

Figure 2. Efficacy of extracts of *Azadirachta indica*, *Cymbopogon citratus*, *Ricinus communis*, and *Thevetia peruviana,* and spinetoram against *Tribolium castaneum* adults after exposure of 48 h. Bars represent treatment means and error bars are 95% CI. The lowercase superscript letters above each bar represent post hoc pairwise comparisons between treatments. Treatment, $F_{3,24} = 12.3$, $p < 0.05$ and concentration $F_{2,24} = 11.5$, $p < 0.05$.

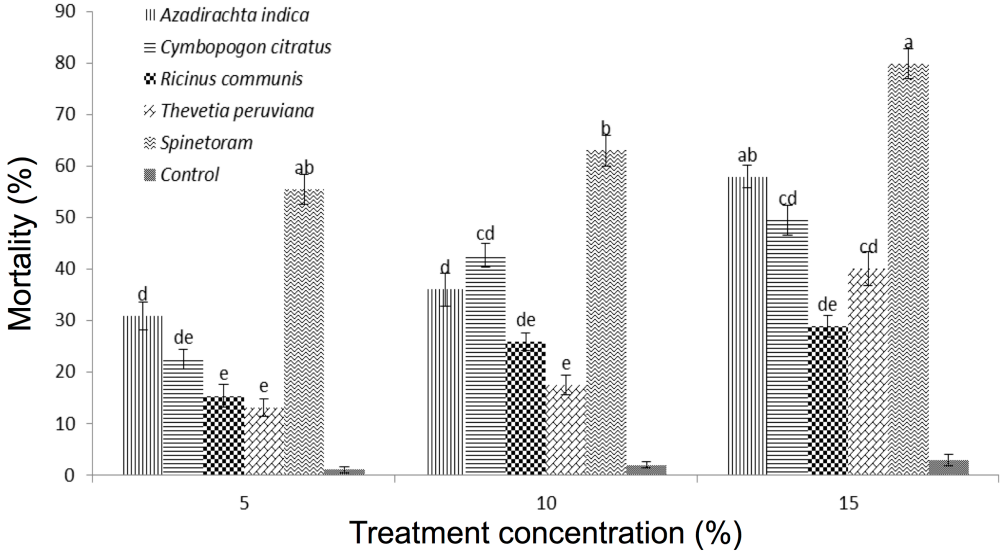

Figure 3. Efficacy of extracts of *Azadirachta indica*, *Cymbopogon citratus*, *Ricinus communis*, and *Thevetia peruviana,* and spinetoram against *Tribolium castaneum* adults after exposure of 72 h. Bars represent treatment means and error bars are 95% CI. The lowercase superscript letters above each bar represent post hoc pairwise comparisons between treatments. Treatment $F_{3,24} = 4.72$, $p < 0.05$ and concentration $F_{2,24} = 11.1$, $p < 0.05$.

For the combined treatments, the effect of time and concentration were also significant (Figure 4). The results showed that the combined action was more effective with the highest mortality of 84.9% for *A. indica*+spinetoram followed by 61.1% for *C. citratus*+spinetoram and 52.7% for *T. peruviana*+spinetoram). The lowest mortality was 39.4% for *R. communis*+spinetoram.

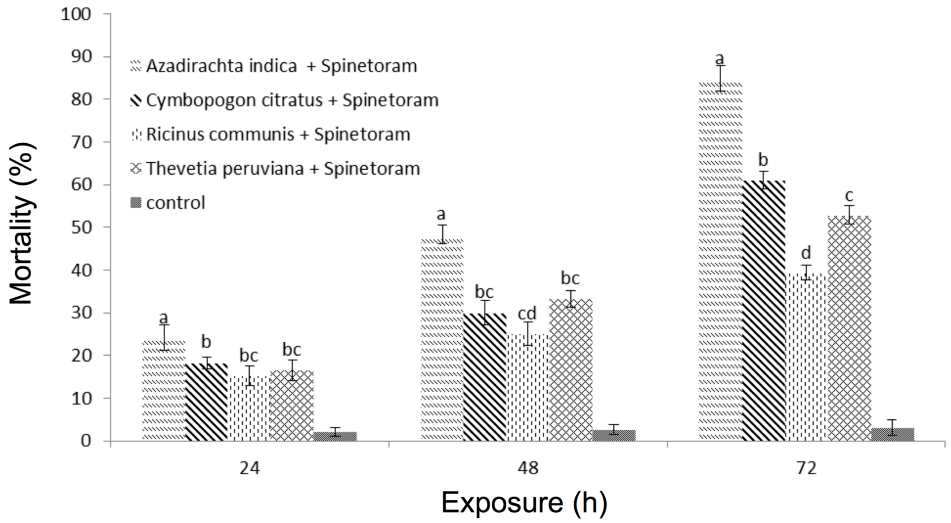

Figure 4. Combined action of optimal concentration of extracts of *Azadirachta indica*, *Cymbopogon citratus*, *Ricinus communis* and *Thevetia peruviana*, and spinetoram against *Tribolium castaneum* adults after different exposure times. Bars represent treatment means and error bars are 95% CI. The lowercase superscript letters above each bar represent post hoc pairwise comparisons between treatments. Time $F_{2,24} = 206$, $p < 0.05$ and concentration $F_{2,24} = 20.8$, $p < 0.05$.

LC_{50}

Probit analysis gave approximate LC_{50} values for plant extracts of 13.3% for *A. indica*, 14.7% *C. citratus*, 20.9% for *T. peruviana* and 39.7% for *R. communis* after 72 h. LC_{50} values after 48 and 24 h were higher, with the highest being 63.7% for for *A. indica* at 24 h (Table 2). The lower the value the greater the toxicity. The results indicated that extract of *A. indica* was more toxic than other extracts with extended exposure.

Table 2. LC_{50} of plant extracts and spinetoram against *Tribolium castaneum* after three exposure periods (24, 48 and 72 h)

Treatments	Exposure (h)	Slope	Z-value	P-value	R^2-value	LC_{50} (%)	Confidence limits (95%)	
Azadirachta indica	24	5.5	2.15	0.032	0.997	63.70	25.7	88.4
	48	6.5	2.03	0.042	0.998	44.00	19.9	76.3
	72	13.5	3.61	0.000	0.906	13.30	10.6	21.1
Cymbopogon citratus	24	6.5	2.74	0.006	0.982	41.40	23.0	82.4
	48	7.5	2.55	0.011	0.932	36.80	20.1	78.1
	72	13.5	3.78	0.000	0.906	14.70	11.7	24.0
Ricinus communis	24	4.5	2.40	0.016	0.964	48.30	24.5	84.6
	48	4.0	1.48	0.139	0.984	44.20	7.8	60.7
	72	6.5	2.38	0.015	0.911	39.70	20.4	79.8
Thevetia peruviana	24	3.0	1.50	0.133	0.988	70.10	4.1	82.7
	48	3.5	1.54	0.123	0.942	67.30	4.0	76.8
	72	18.5	4.05	0.000	0.985	20.90	16.1	39.9
Spinetoram	24	6.5	2.52	0.012	0.998	9.37	4.7	16.1
	48	8.5	2.38	0.017	0.914	4.42	2.8	8.2
	72	14.5	4.11	0.000	0.918	2.10	0.5	4.3

Management of *T. castaneum* has been mainly achieved with fumigants such as methyl bromide and phosphine, and some dusts chemicals such as permethrin (Price & Mills, 1988). However, frequent and inappropriate use of such insecticides has resulted in problems such as environmental pollution, residues in food and hazardous effects on non-target organisms (Benhalima et al., 2004). These problems have motivated researchers to search for alternative management options such as the use of plant extracts and bio-derived insecticides such as spinetoram.

The research reported here, was were to evaluate the toxic effects of four plant extracts and spinetoram against *T. castaneum*. Concentrations as well as exposure period effects were found to be significant in bioassays. After 72 h at the highest concentration, the highest toxicity was 79.8% for spinetoram and the lowest 28.9% was for *R. communis*. These findings are close to Khoshnoud et al. (2008) who used plant extracts against two stored grain insect pests and recorded increased mortality at highest concentration and exposure period similar to the present study. Likewise, the findings are consistent with Mamun et al. (2009) and Ahmed et al. (2018), who recorded increased mortality at increased plant extract concentrations confirming the toxicity. The highest mortality (79.8%) was close to Mostafa et al. (2012) who used *Cucumus sativus* and recorded 80% mortality of *T. castaneum.* In present study, the extract of *A. indica* gave mortality up to 57% at highest concentration after exposure of 72 h is similar to Padín et al. (2013) who examined the toxicity of some plant extracts against *T. castaneum* and recorded mortality up to 57% with extract of *Matricaria chamomilla* L. LC_{50} values in the present study are close to Mamun et al. (2009) who evaluated neem oil and some other plant oil against the same target insect. Slight difference may be due to different concentrations of plant extracts. The LC_{50} are similar to the findings of Reddy et al. (1999) who recorded similar but somewhat different values due to difference in insect species in both studies. Findings of the present study are close to Huang et al. (2000) who recorded values up to 51 and 52%, close to the 48.3% for *R. communis*. Slight difference may be due to difference in plant extracts and concentrations, used. The toxicity (70%) in the bioassays with spinetoram was also close to Vassilakos et al. (2014) who recorded 72.4% against *Rhyzopertha dominica* (Fabricius, 1792) (Coleoptera: Bostrichidae). A slight difference may be due to different species. Findings of the present study are close to Hameed et al. (2012) who evaluated the insecticidal activity of a bio-base insecticide spinosad and two extracts, neem (*A. indica*) and *Nerium oleander* L., against *T. castaneum*. Mortality values of *T. castaneum* were up to 50%. Slight differences may be due to difference in new chemistry insecticide than in the present study (spinetoram). Mortality finding of the present study were close Vassilakos et al. (2012) who found mortality similar to the present study with application of spinetoram against the adults of *Sitophilus oryzae* (L., 1763) (Coleoptera: Curculionidae), *R dominica*, *Prostephanus truncatus* (Horn, 1878) (Coleoptera: Bostrichidae), *Tribolium confusum* Jacquelin du Val, 1863 (Coleoptera: Tenebrionidae), *Sitophilus granarius* (L., 1758) Coleoptera: Curculionidae) and *O. surinamensis.* The mortality trend was similar to the present study, increased mortality was found at increased concentrations.

It is concluded that spinetoram and the tested plant extracts can provide effective control of *T. castaneum*. However, the efficacy of spinetoram was enhanced when combined with plant extracts, especially in case of *A. indica.* Therefore, it is suggested that the combine application of plant extracts and spinetoram at higher concentration along with longer exposure periods can be an effective alternative to synthetic insecticides for eco-friendly management of stored commodity insect pests.

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Original article (Orijinal araştırma)

Notes on the genus *Xantholinus* Dejean, 1821 (Coleoptera: Staphylinidae, Staphylininae, Xantholinini) from the western Palearctic Region

Batı Palearktik Bölgedeki *Xantholinus* Dejean, 1821 (Coleoptera: Staphylinidae, Staphylininae, Xantholinini) cinsi üzerine notlar

Sinan ANLAŞ[1*]

Abstract

The genus *Xantholinus* Dejean, 1821 (Coleoptera: Staphylinidae, Staphylininae, Xantholinini) contains 105 species in the western Palearctic region. In the present study, new and additional distribution data for 22 species of the genus *Xantholinus* are reported from various countries of the western Palearctic Region. The material examined was collected between 1898 and 2017; and contained types and additional specimens mostly in European museums. Among them, five species are the first country records: Azerbaijan (1), Armenia (1), Bosnia Herzegovina (1), Iraq (2), Jordan (1) and Macedonia (2). The original specimens of *Xantholinus araxis* Reitter, 1898 have been studied, the lectotype designated, the species redescribed and illustrated. Besides, the type specimens of *Xantholinus phenicius* Coiffait, 1971 (syn of *Xantholinus rufipennis* Erichson, 1839), *Xantholinus kirschenblati* Bordoni, 1975, *Xantholinus fageli* Coiffait, 1971 and *Xantholinus gridellii carius* Coiffait, 1972 (syn of *Xantholinus varnensis* Coiffait, 1972) are illustrated.

Keywords: Fauna, lectotype, new records, Staphylinidae, *Xantholinus,* western Palearctic Region

Öz

Xantholinus Dejean, 1821 (Coleoptera: Staphylinidae, Staphylininae, Xantholinini) Batı Palearktik Bölgede 105 türle temsil edilen bir cinstir. Bu çalışmada, Batı Palearktik Bölgenin farklı ülkelerdeki *Xantholinus* cinsine dâhil 22 türe ait yeni ve ek yayılışsal kayıtlar rapor edilmiştir. İncelenen materyal, 1889-2017 yılları arasında toplanmış olup, çoğunlukla Avrupa müzeleri'nde bulunan tip ve diğer örnekleri içermektedir. Belirtilen türlerden, beş tanesi ilk ülke kaydı niteliğindedir: Azerbaycan (1), Ermenistan (1), Bosna Hersek (1), Irak (2), Ürdün (1) ve Makedonya (2). *Xantholinus araxis* Reitter, 1898 türünün tip örnekleri incelenmiş, lektotipi seçilmiş ve bu tür yeniden tanımlanarak şekillendirilmiştir. Ayrıca, *Xantholinus phenicius* Coiffait, 1971 (*Xantholinus rufipennis* Erichson, 1839 türünün sinonimi), *Xantholinus kirschenblati* Bordoni, 1975, *Xantholinus fageli* Coiffait, 1971 ve *Xantholinus gridellii carius* Coiffait, 1972 (*Xantholinus varnensis* Coiffait, 1972 türünün sinonimi) türlerinin tip örnekleri şekillerle gösterilmiştir.

Anahtar sözcükler: Fauna, lektotip, yeni kayıtlar, Staphylinidae, *Xantholinus*, Batı Palaearktik Bölge

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Introduction

The genus *Xantholinus* Dejean, 1821 (Coleoptera, Staphylinidae, Staphylininae, Xantholinini) is divided into 14 subgenera (Schülke & Smetana, 2015). *Xantholinus* contains 105 species, with three species as incertae sedis and one species as nomen dubium in the western Palearctic region (Schülke & Smetana, 2015; Anlaş, 2017; Assing, 2017). The main center of diversity of the genus is in the Mediterranean countries and adjacent regions, especially in Anatolia with 38 species, Italy with 26 species, Caucasus with 20 species, Spain with 15 species and Greece with 12 species.

Members of the genus can be seen in many habitats, but most often in plant matter, under stones or bark, in grasslands, on flowers and in leaf litter. They are free-living and most probably predators of small insects and mites.

The present study is the result of the examination of the types of the *Xantholinus* species in the Hungarian Natural History Museum and the Institut Royal des Sciences Naturelles de Belgique. In this context, the syntypes of *Xantholinus araxis* Reitter, 1898 have been examined, the lectotype designated, the species redescribed and illustrated. Additionally, the type specimens of *Xantholinus fageli* Coiffait, 1971, *Xantholinus phenicius* Coiffait, 1971, *Xantholinus gridellii carius* Coiffait, 1972 and *Xantholinus kirschenblati* Bordoni, 1975 were studied for the first time after their description and internal structures of the aedeagus and habitus of these species are illustrated. Consequently, it was confirmed that *Xantholinus phenicius* is a synonym of *Xantholinus rufipennis* Erichson, 1839 and *Xantholinus gridellii carius* Coiffait, 1972 is a synonym of *Xantholinus varnensis* Coiffait, 1972. Also, numerous records from Palearctic Region of zoogeographic interest for *Xantholinus* species are given.

Material and Methods

Examination of the material was collected between 1898 and 2017, and included types and additional specimens in museums. Specimens were examined using a Stemi 2000-C microscope (Zeiss, Oberkochen, Germany). Photographs of the habitus, forebody and aedeagus were taken with a digital camera (Zeiss Axiocam ERC5s). All photographs were edited with Helicon Focus V6 (Helicon Soft Ltd., Kharkov, Ukraine) and CorelDRAW Graphics Suite X5 (Corel Corporation, Ottawa, Canada). Nomenclature of the terminalia and abbreviations of other morphological measurements (in mm) follow Assing (2007): HL, head length from anterior margin of clypeus to posterior margin of head; HW, head width (including eyes); AL, length of antenna; PL, length of pronotum along median line; PW, maximal width of pronotum; EL, length of elytra from apex of scutellum to posterior margin; EW, combined width of elytra; AW, maximal width of abdomen; TaL, length of metatarsus; TiL, length of metatibia; ML, length of aedeagus from apex of ventral process to base; and TL, total body length.

The material referred to in this study is deposited in the following collections: AZMM, Alaşehir Zoological Museum, Manisa, Turkey (S. Anlaş); HNHM, Hungarian Natural History Museum, Budapest, Hungary (G. Makranczy, O. Merkl); IRSNB, Institut Royal des Sciences Naturelles de Belgique, Bruxelles, Belgium (W. Dekoninck); MHNG, Muséum d'Histoire Naturelle, Genève, Switzerland (G. Cuccodoro); NMPC, National Museum, Praha, Czech Republic (M. Fikáček, Jiří Hájek); and NMNHS, National Museum of Natural History, Sofia, Bulgaria (R. Bekchiev).

Results

Genus *Xantholinus* Dejean, 1821

Subgenus *Calolinus* Coiffait, 1956

***Xantholinus rufipennis* Erichson, 1839** (Figure 1)

Syn: *Xantholinus phenicius* Coiffait, 1971 (Figure 1a-e)

Material examined: Cyprus: 1♂, 12.III.2011, Lefkoşa, Değirmenlik, Yaylatepe 2 km S, environs Alevkayası, 35°17'28" N, 33°33'03" E, 820 m, leg. Anlaş (AZMM). Greece: 1♂, 1♀, 11.IV.1977, Rhodes Profitis, Ilias, 650 m, leg. Besuchet (MHNG). 1♀, 15.IV.1977, Rhodes, Ebouas, leg. Besuchet & Löbl (MHNG). 2♂♂, 25-26.IV.1935, Chios Island, Ayio Georgios, leg. Fodor (HNHM). Macedonia: 1♂, 16.VII.1937, Han Mavrova, leg. Fodor (HNHM). Turkey: 1♂, 2♀♀, 29.IV.2006, Kilis Province, Küplüce, leg. Yağmur (AZMM); 1♂, 16.III.2008, Ömerli 1 km SE, 1200 m, 36°52'01" N, 37°12'02" E, leg. Yağmur (AZMM). 2♂♂, 5♀♀, 03.IV.2017, Adıyaman Province, 37°50'04" N, 38°18'56" E, 980 m. leg. Yağmur & Örgel (AZMM). 2♂♂, 4♀♀, 23.VIII.2017, Gaziantep Province, Oğuzeli, Çaybaşı 2 km S, 36°59'58" N, 37°30'48" E, 720 m, pitfall traps, leg. Yağmur.

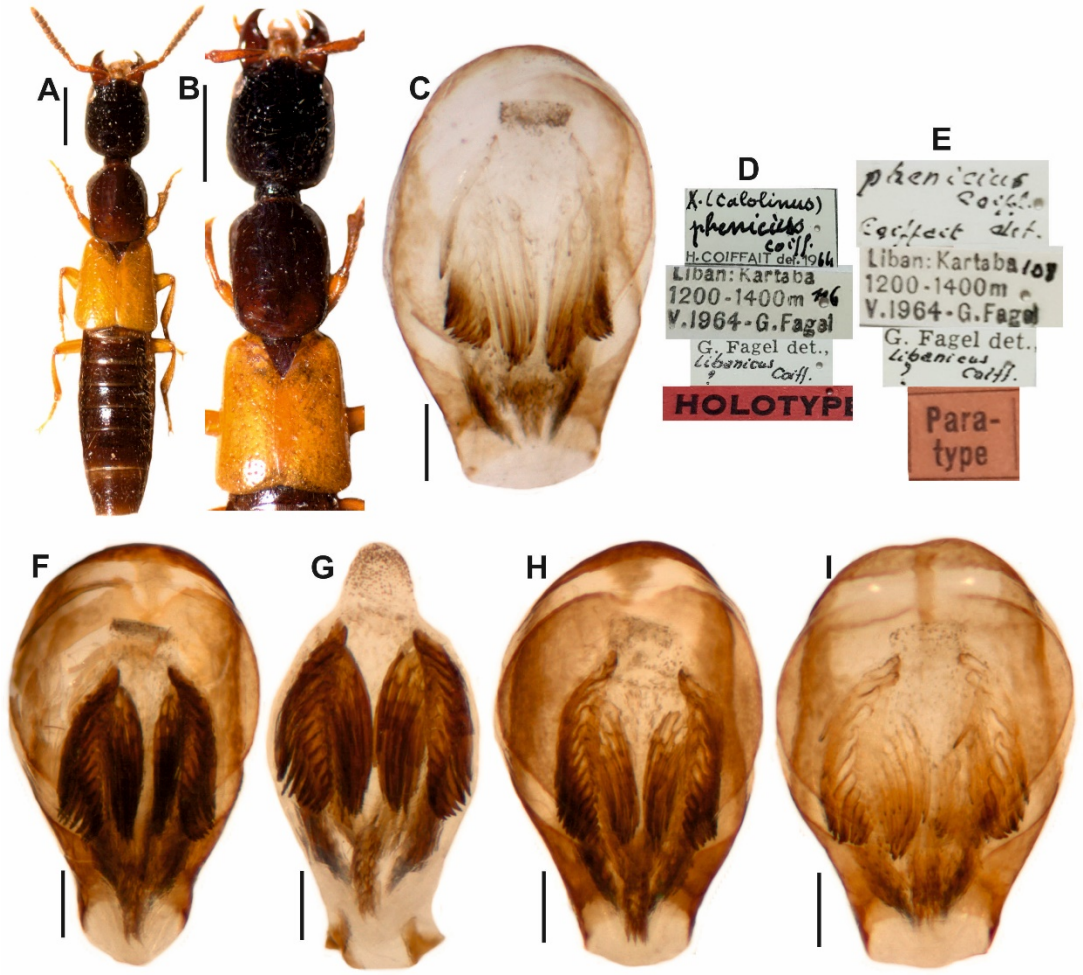

Figure 1. *Xantholinus rufipennis*: A-C) types of *X. phenicius*: A) habitus (Holotype); B) forebody (Paratype); C) aedeagus (Holotype); D-E) type labels of *X. phenicius*; F-I) aedeagus (F-G: Adıyaman, Turkey; H-I: Gaziantep, Turkey). (Scale bars: A-B: 1 mm; C, F-I: 0.2 mm).

Type examined: *Xantholinus phenicius* Coiffait: Holotype: 1♂: "Liban: Kartaba, 1200-1400 m, 116, V.1964-G. Fagel / G. Fagel det., *libanicus* Coiff. ? / *X.* (*Calolinus*) *phenicus* [sic] Coiff., H. Coiffait det. 1964 / Holotype" (IRSNB). Paratype: 1♀ "Liban: Kartaba, 1200-1400 m, 108, V.1964-G. Fagel / G. Fagel det., *libanicus* Coiff. ? / *X.* (*Calolinus*) *phenicus* [sic] Coiff., H. Coiffait det. 1964 / Paratype" (IRSNB).

Remarks: Coiffait (1971) based the original description of *Xantholinus phenicus* (Coiffait, 1971) on a male holotype and a female paratype from "Kartaba, Liban." The types are deposited in the Fagel collection in Brussels (IRSNB). This species has been proposed as a synonym of *X. rufipennis* by Assing (2007) without seeing the male holotype.The type specimens of *X. phenicus* was found at IRSNB during a visit in 2016. An examination of the aedeagus of the male holotype of *X. phenicius* revealed that it is identical to *X. rufipennis*. Consequently, it was confirmed that *X. phenicius* is a synonym of *X. rufipennis*. For illustrations of the type specimens of *X. phenicus* see Figures 1a-e.

Distribution: This species widespread in the Eastern Mediterranean region (Assing, 2007; Schülke & Smetana, 2015; Anlaş, 2017). But, it had not been recorded from Macedonia.

Subgenus *Helicophallus* Coiffait, 1956

***Xantholinus araxis* Reitter, 1898** (Figure 2)

Type examined: Lectotype (here designated): 1♂: "Caucasus, Araxesthal. Leder. Reitter. / *Xantholinus* (*Helicophallus*) *araxis* Reitter sensu / det. A. Bordoni 19….. / aedeotypus / *Xantholinus* (*Helicophallus*) *araxis* Reitter sensu Bordoni, 1972 / Paratypus, 1898, *Xantholinus araxis* Reitter" with red printed label reading "Lectotype", *Xantholinus araxis* Reitter, 1898; des. S. Anlaş 2017. (HNHM). Paralectotypes (here designated): 1♀: "Caucasus, Araxesthal. Leder. Reitter. / coll. Reitter / *Xantholinus araxis* m. / Holotypus, 1898, *Xantholinus araxis* Reitter" with red printed label reading "Paralectotype", *Xantholinus araxis* Reitter, 1898; des. S. Anlaş 2017. (HNHM). 2♀♀: "Caucasus, Araxesthal. Leder. Reitter. / coll. Reitter / Paratypus, 1898, *Xantholinus araxis* Reitter / with red printed label reading "Paralectotype", *Xantholinus araxis* Reitter, 1898; des. S. Anlaş 2017. (HNHM).

Redescription: Measurements (in mm) and ratios (range, n = 4): AL: 1.75-1.88, 182; HL: 1.23-1.33, 128; HW: 0.95-1.06, 1.01; PL: 1.25-1.34, 1.30; PW: 0.82-0.90, 0.86; EL: 0.90-0.99, 0.95; EW: 1.12-1.22, 1.17; AW: 1.14-1.26, 1.20; ML: 1.38 (n=1); TiL: 0.78-0.82, 0.80; TaL: 0.63-0.69, 0.66; TL: 9.1-9.4, 9.3; HL/HW 1.26-1.29, 1.27; PW/HW 0.85-0.86, 0.85; PW/PL 0.66-0.67, 0.66; EL/PL 0.72-0.74, 0.73; EW/PW 1.36-1.37, 1.36; EL/EW 0.80-0.81, 0.80; AW/EW 1.01-1.03, 1.02; TiL/TaL: 1.19-1.24, 1.21.

Habitus as in Figure 2a. Coloration: head blackish, pronotum dark brown to black, elytra yellowish red to bright reddish; abdomen dark brown to black; legs yellowish red; antennae reddish to brown.

Head strongly oblong, average 1.25-1.30 times as long as wide (see ratio HL/HW and Figure 2a), and weakly dilated posteriorly; eyes small, not distinctly projecting from lateral outline of head, about a fifth the length of postocular region in dorsal view; dorsal surface with sparse, not well-defined, and relatively coarse punctation and with a few interspersed micropunctures, central dorsal region without punctures, microsculpture absent; antenna not slender, with eight to 10 antennomeres weakly transverse (Figure 2a). Pronotum narrower than head (see ratio PW/HW and Figure 2a), and strongly oblong (see ratio PW/PL and Figure 2a); distinctly tapering posteriad; lateral margins almost straight in dorsal view; dorsal series composed of nine to 11 punctures; microsculpture absent. Elytra distinctly wider than pronotum and, at suture distinctly shorter than pronotum (see ratio EL/PL, EW/PW and Figure 2a), puncturation well-defined. Hind wings at least in some specimens of reduced length. Legs relatively long (see measurements TiL and TaL). Abdomen about as wide as elytra (see ratio AW/EW and Figure 2a); punctation fine and moderately dense; all tergites with distinct transverse microsculpture; pubescense blackish; posterior margin of tergite VII with narrow palisade fringe.

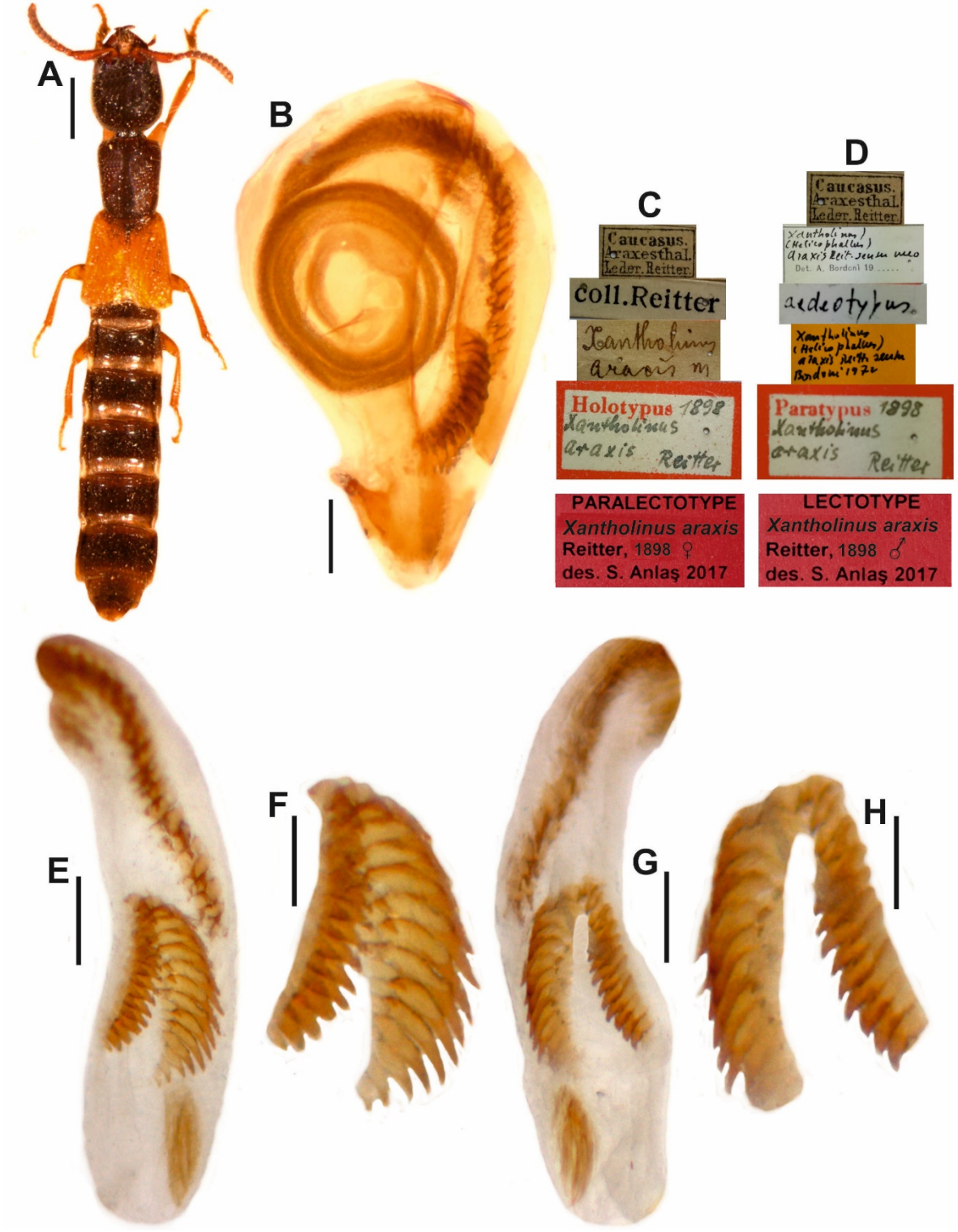

Figure 2. *Xantholinus araxis*: A) habitus; B) aedeagus in dorsal view; C) paralectotype labels; D) lectotype labels; E-H) internal structures of aedeagus in squeeze preparation. (Scale bars: A: 1 mm; B, E-H: 0.2 mm).

♂: Posterior margins of tergite and sternite VIII weakly convex and narrowly semitransparent; aedeagus with internal structures composed of a proximal series of about 20 relatively short and sclerotized spines, a distal series of about 15 long and distinctly sclerotized spines, a second distal series of about 15 moderately long and distinctly sclerotized spines, an intermediate distal series composed of eight short and weakly sclerotized spines of gradually decreasing length, and a distal brush-like cluster of long and weakly sclerotized spines (Figure 2b and e-h).

Comparative notes: *Xantholinus araxis* is distinguished from all its congeners by the internal structures of the aedeagus. The similarly derived morphology of the aedeagal characters suggests that this new species is most closely related to *Xantholinus luteipennis* Coiffait, 1970, which is distributed in Anatolia. *X. luteipennis* is readily separated from *X. araxis* by the presence of more distinct sclerotized spines in intermediate distal series and by the less oblong pronotum.

Remarks: Although the types of *X. araxis* have labels “Holotypus” and “Paratypus”, Reitter (1898), in the original descriptions of species, mentioned a series of specimens without selecting a holotype. The curators in HNHM most probably labeled the types as holotypus (a female) and paratypes (a male and two females). Therefore, the types of *X. araxis* actually were syntypes. Reitter (1898) mentioned specimens from only one location, “Araxesthal, near Ordubad” (now in Nakhchivan, Azerbaijan), without specifying the numbers of specimens. A dissected male, is designated here as a lectotype, in order to fix the identity of the species for future studies. The species is illustrated in Figure 2.

Distribution: This species is known from Armenia, Azerbaijan and Iran (Bordoni, 1975; Schülke & Smetana, 2015; Assing, 2017).

***Xantholinus bulgaricus* Coiffait, 1972**

Material examined: Bulgaria: 1♂, 1936, Čamkorija Mussalla, leg. Purkyně (NMPC).

Distribution: This species is only known from Bulgaria (Schülke & Smetana, 2015).

***Xantholinus distans* Mulsant & Rey, 1853** (Figure 3a-d)

Material examined: Belgium: 1♂, 1♀, 25.IV.1951, Dinant route de Neufchâteau, leg. and det. Fagel. (IRSNB). 1♂, 2♀♀, 18.IV.1949, Ben-Ahin, ruisseau de soliére, leg. Fagel. (IRSNB). 1♂, 16.VI.1943, Andenne (Ankan), leg. and det. Fagel. (IRSNB). Germany: 1♂, 2♀♀, Stuttgart 26 km SW, leg. Anlaş (AZMM). Italy: 1♂, 14.VI.1994, Calabria, Castlglione Cos. (CS) torr. Padula, leg. Angelini (AZMM).

Distribution: This species is widespread in Europe (Schülke & Smetana, 2015). The species is illustrated in Figure 3a-d.

***Xantholinus kirschenblati* Bordoni, 1975** (Figure 3e-g)

Material examined: Azerbaijan: 2♂♂, 26.VI.2003, Ismalinsky nat. reserved, environs Valyasin village, leg. Nabozhenko (AZMM).

Type examined: *Xantholinus kirschenblati* Bordoni: Paratype: 1♂ "Kaukas, Leder / *Xanth. variabilis* Hochh. Coll. Reitter / *Xantholinus kirschenblati* n. sp. det. A. Bordoni 1972, paratypus / *Xantholinus kirschenblati* Paratypus, Bordoni 1972" (HNHM).

Remarks: Bordoni (1975) based the original description of *X. kirschenblati* on a male holotype from “Achti, distr. Novo, Bajazet (=Armenia, Yerevan, Sevan lake)” and a male paratype from “Kaukas”, without specification of the locality. The paratype is deposited in the Reitter collection in Budapest (HNHM). The paratype of this species in the collections of the HNHM were studied during a visit in 2015. The species is illustrated in Figure 3e-f.

Distribution: This species is known from Armenia (Bordoni, 1975; Schülke & Smetana, 2015; Assing, 2017). The specimens from Azerbaijan detailed here represent the first record for that country.

Figure 3. A-D) *Xantholinus distans*, E-G) *X. kirschenblati*: A) habitus; B) forebody; C-F) aedeagus in dorsal view; G) paratype labels. (Scale bars: A: 1 mm; B: 0.5 mm; C-F: 0.2 mm).

***Xantholinus maykopensis* Coiffait, 1966** (Figure 4)

Material examined: Russia: 2♂♂, 2♀♀, V.2017, Adygeya, env. Maykop, by pitfall traps (AZMM).

Type examined: 1♂, Caucas. occ. Circassien, Leder Reitter, *Xantholinus forcepunctatus* coll. Reitter (HNHM). 1♂, Caucas. occ. Circassien, Leder Reitter, G. Fagel det. *X. forcepunctatus* Mots. (IRSNB).

Remarks: Coiffait (1966) based the original description of *X. maykopensis* on a male holotype and six female paratypes from the surroundings "Maykop" in the Northwestern Caucasus region. According to Bordoni (2011), the type specimens of *X. maykopensis* is deposited in the Muséum national d'Histoire Naturelle of Paris and the holotype was without genital segment and aedeagus. For that reason, A. Bordoni designated a neotypus for this species (Bordoni, 2011) from "Krasnaya Polyana", preserved in the Zoological Museum of Copenhagen. The aedeagus illustrations of this species provided by Coiffait (1966, 1972) and Bordoni (1975). But later, the illustrations of aedeagus were drawn differently by Bordoni (2011). The species is illustrated in Figure 4.

Distribution: The species was known from Northwestern Caucasus region and Georgia (Coiffait, 1966, 1972; Bordoni, 1975, 2011; Assing, 2007).

Subgenus *Heterolius* Coiffait, 1983

***Xantholinus fortepunctatus* Motschulsky, 1860**

Material examined: Georgia: 1♂, 07-09.VII.1987, Cauc. C., Bubya Fl. 1850 m, leg. Odvarka, coll. Dvořák (NMPC). 1♂, 05.VII.1989, Caucasus, Dagestan, Samursky forest, *Quercus robur-Carpinus caucasicus* sifted from litter, Leg. Z. Korsos (HNHM). Russia: 1♂, 28.VII.2000, Rostov region, Sholovsky district, Kalininsky village, leg. Khachikov (AZMM). Uzbekistan: 1♂, 08-25.VI.1989, Uzbecká SSR, Samarkand, leg. K. Hůrka (NMPC); 1♂, 11.VI.1989, Uzbec, SSR, Turk. chr, Džum-džum-saj, sníh, 2000-2400 m, alp, pásma, leg. Hůrka (NMPC).

Distribution: This species occurs in Caucasus, Moldavia, Poland, Ukrania, the southern European territory of Russia, Middle Asia, Iran and Turkey (Anlaş, 2014; Schülke & Smetana, 2015).

***Xantholinus khnzoriani* Coiffait, 1966**

Syn: *Xantholinus caucasicus* Bordoni, 1975

Material examined: Azerbaijan: 3♂♂, 4♀♀, 08.V.2004, Leriksy District, Ukhary Village, Gan lake, leg. Kasatkin (AZMM). Georgia: 2♂♂, 24.V.2004, Abkhazia, Avadchara, leg. Kasatkin (AZMM). Russia: 2♂♂, 1♀, 05.VIII.2006, Kabardino Balkariya, Lesistyi range, env. Belaya rechka vill., Beshenka River, leg. Nabozhenko (AZMM). 1♂, 2♀♀, Caucasus, Teberda, VI.1902, coll. Rambausek (NMPC).

Distribution: *Xantholinus khnzoriani* is known from Caucasus and Turkey (Anlaş, 2017; Assing, 2017). Recently, *X. caucasicus* has been proposed as a synonym of *X. khnzoriani* by Assing (2017).

Subgenus Idiolinus Casey, 1906

***Xantholinus crassicornis* Hochhuth, 1851**

Syn: *Xantholinus lederi* Coiffait, 1966

Material examined: 2♂♂, Caucasus, Leder Reitter, 494, coll. Roelfs (misidentification as *X. variabilis* Hochhuth, 1851 det. Fagel and as *X. lederi* Coiffait, det. A. Bordoni) (IRSNB). Georgia: 1♂, Caucasus (=Ossetia), Meskisches Geb. Leder (Reitter coll.) (NMPC). 1♂, 1♀, Caucasus, Meskisches Geb., coll. Leder (Reitter) (HNHM).

Distribution: This species is known from Azerbaijan, Georgia, southern European Russia and Turkey (Schülke & Smetana, 2015; Anlaş, 2017; Assing, 2017).

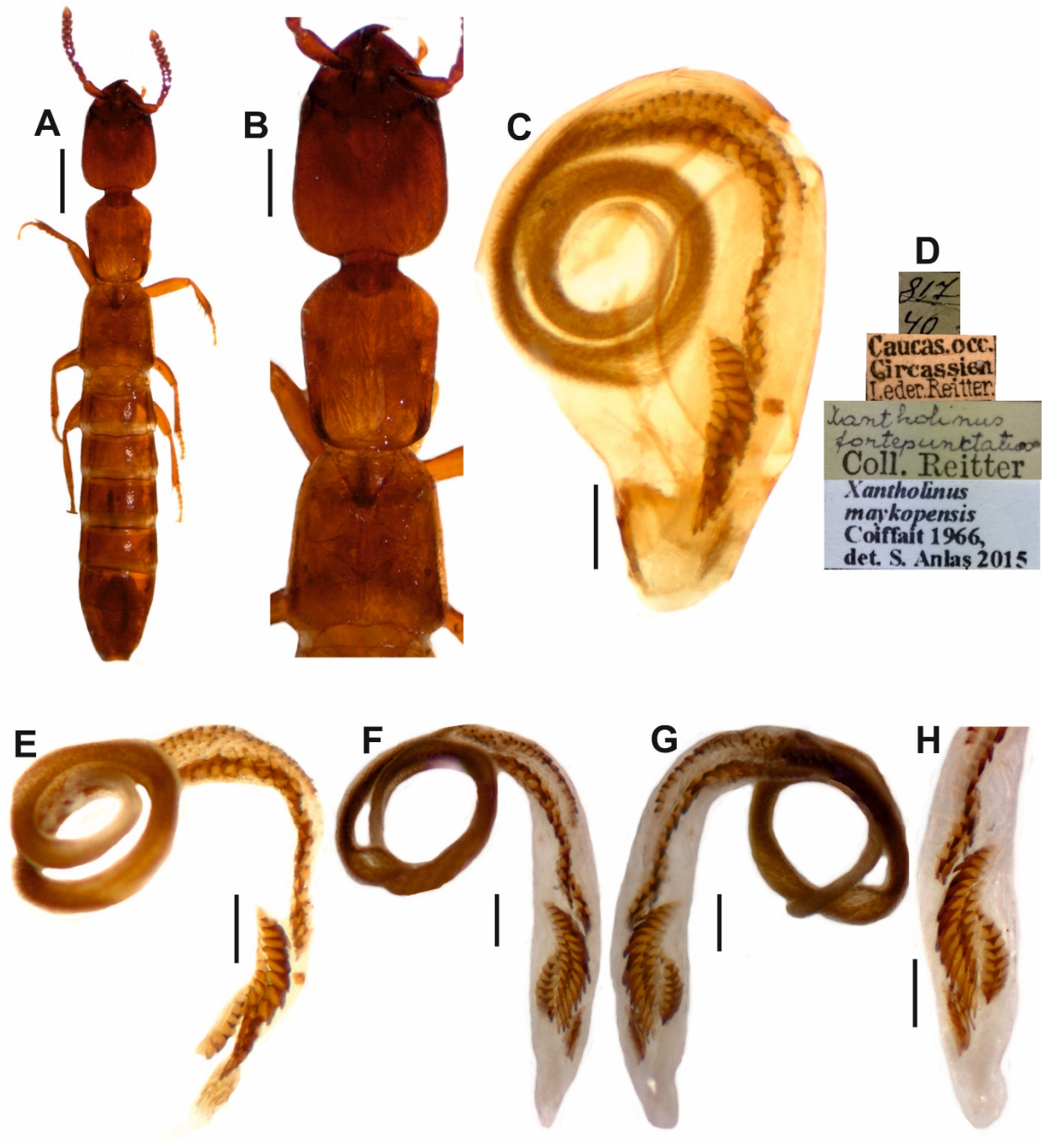

Figure 4. *Xantholinus maykopensis*: A) habitus; B) forebody; C) aedeagus in dorsal view; D) labels; E, H) internal structures of aedeagus in squeeze preparation. (Scale bars: A: 1 mm; B: 0.5 mm; C, E-H: 0.2 mm).

Subgenus *Paracyclinus* Bordoni, 1975

***Xantholinus procerus* (Erichson, 1839)**

Material examined: Armenia: 1♂, 14-19.VI.1999, Razdansky distr., Tsakhkunyats Range, high Arzakan vill., leg. Nabozhenko (AZMM). Bosnia Herzegovina: 1♂, Bosnie (NMPC).

Distribution: *Xantholinus procerus* is distributed in Azerbaijan, Albania, Bosnia Herzegovine, Georgia, Romania, Ukrania and Turkey (Schülke & Smetana, 2015). The record from Armenia is reported here for the first time.

Subgenus *Polydontophallus* Bordoni, 1972

***Xantholinus elegans* (Olivier, 1795)**

Material examined: Italy: 1♂, 28.V.1969, Passo la Futa, leg. Mlynář (NMPC).

Distribution: This species occurs in Europe and also Nearctic Region (Schülke & Smetana, 2015).

Xantholinus fageli Coiffait, 1971 (Figure 5a-f)

Type examined: *Xantholinus fageli* Coiffait: Holotype: 1♂: "Liban: Kartaba, 1200-1400 m, 114, V.1964-G. Fagel / G. Fagel det. sp. apud *semirufus* Steel. / X. (*Purrolinus*) *fageli* [sic] Coiff., H. Coiffait det. 1964 / Holotype" (IRSNB). Paratypes: 4♀♀ "Liban: Kartaba, 1200-1400 m, 116, V.1964-G. Fagel / G. Fagel det. sp. apud *semirufus* Steel. / *fageli* Coiffait, Coiffait det. / Paratype" (IRSNB). Allotype: 1♀ "Liban: Kartaba, 1200-1400 m, 116, V.1964-G. Fagel / G. Fagel det. sp. apud *semirufus* Steel. / *fageli* Coiffait, Coiffait det. / Paratype" (IRSNB).

Remarks: Coiffait (1971) based the original description of *X. fageli* on a male holotype and a male paratype from “Kartaba, Liban”. The types are deposited in the Fagel collection in Brussels (IRSNB). The type specimens of *X. fageli* in the collections of the IRSNB were studied during a visit in 2016. The male paratype was not found, but there were five females (four paratypes and one allotype). Although the female types of *X. fageli* have labels “Allotype” and “Paratype”, the author did not select any female types while describing the new species. G. Fagel most probably labeled yourself the types as allotype and paratypes. Coiffait (1971) illustrated the aedeagus of *X. fageli,* but this illustration is imprecise. For this reason, the species is illustrated in Figures 5.

Distribution: This species is known only from the type locality to Kartaba (=Qartaba, Jbel District) in Lebanon (Coiffait, 1971).

Subgenus *Purrolinus* Coiffait, 1956

***Xantholinus tricolor* (Fabricius, 1787)**

Material examined: Bulgaria: 1♂, VIII.1908, Rila, Čamkorija, coll. Rambousek (NMPC). Georgia: 1♂, 24.VI.2004, Abkhazia, Turetskaya Shapka Mountains, leg. Kolbachev (AZMM). Hungary: 1♂, 25.V.2005, Hungary, pest m. Pilisszentlászló Kopaniyoe, Cseres-tölgyes, Fühálózás egyelés, leg. Merkl (NMHM). Slovakia: 1♂, 19.V.2002, Herlany env., 500 m, leg. Skuhrovec (NMPC).

Distribution: *Xantholinus tricolor* is known from Europe, including the southern European territory of Russia, Turkey, Tajikistan, and eastern and western Siberia (Anlaş, 2014; Schülke & Smetana, 2015).

Subgenus *Typhlolinus* Reitter, 1908

***Xantholinus graecus* Kraatz, 1858**

Material examined: Bulgaria: 1♂, 28.V.2010, Strandzha Mts., Brashlian vill., 41°46'14" N, 23°01'35" E, leg. Bekchiev (NMNHS). Cyprus: 1♂, 2♀♀, 12.III.2011, Girne, Beşparmak, 530 m, 35°16'59" N, 33°28'21" E, leg. Anlaş (AZMM). 2♂♂, 1♀, Mt. Arménien (NMPC). Greece: 1♂, 11.IV.1977, Rhodes Profitis, Ilias, 650 m, leg. Besuchet (MHNG). Italy: 1♂, 11-12.V.1980, Appennino, Lombardo, Staffora-Tal, leg. Zwick (MHNG). Turkey: 1♂, 2♀♀, 04.IV.2014, Uşak Province, Banaz, Susuz, 1087 m, 38°37'41" N, 29°44'10" E, leg.

Anlaş & Yağmur (AZMM). 1♂, 3♀♀, 14.III.2014, Manisa Province, Selendi, Omurlar Village, 974 m, 38°52'15" N, 28°49'11" E, leg. Anlaş & Yağmur (AZMM).

Distribution: This species occurs in Albania, Macedonia, Greece, Italy and the southern European territory of Russia from Europe, and Lebanon, Israel, Cyprus and Turkey (Schülke & Smetana, 2015; Anlaş, 2017).

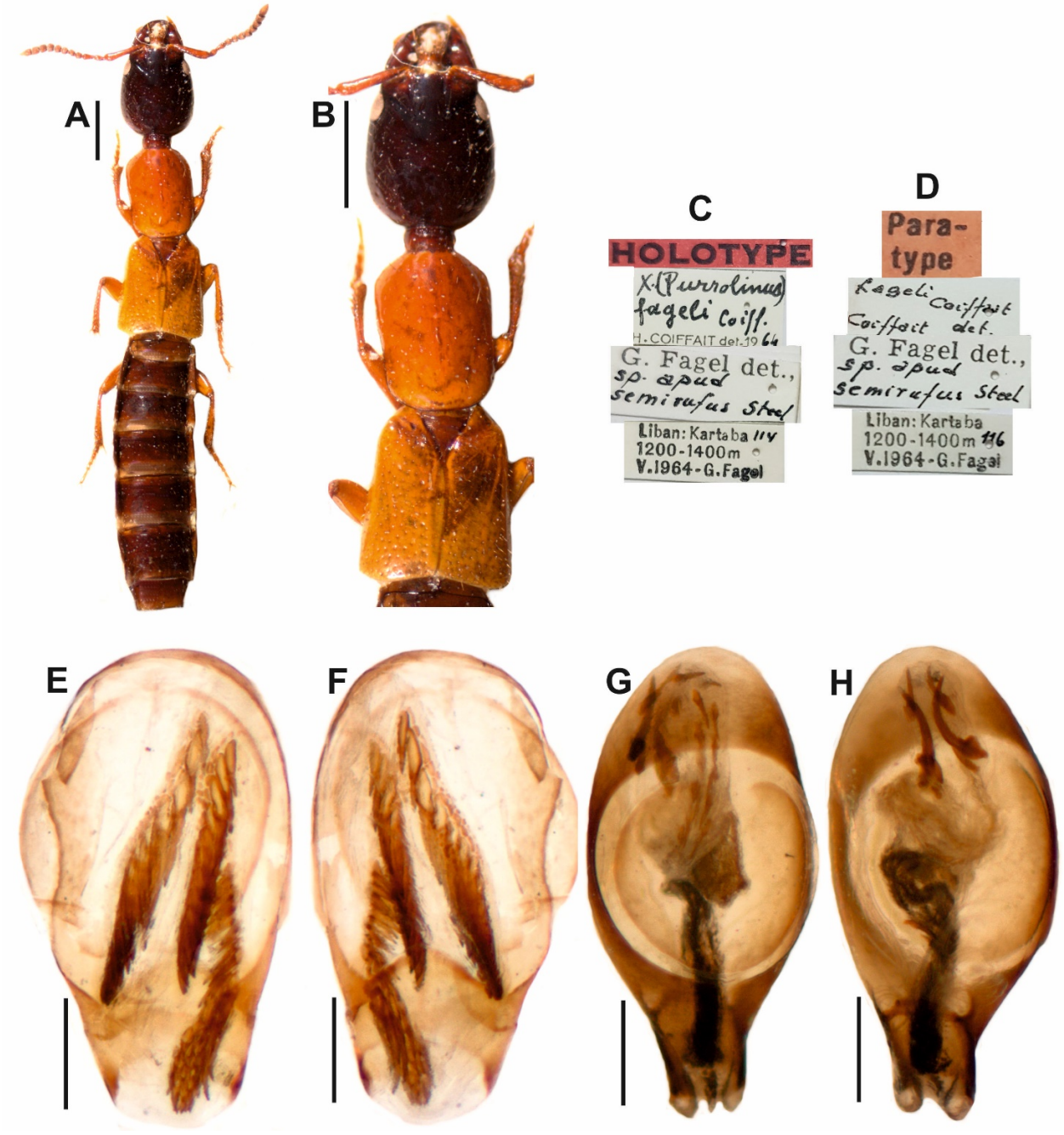

Figure 5. A-F) *Xantholinus fageli*, G-H) *X. dvoraki*: A) habitus (Holotype); B) forebody (Holotype); C) holotype labels; D) paratype labels; E, F) aedeagus in dorsal and ventral view (Paratype); G, H) aedeagus in dorsal and ventral view. (Scale bars: A-B: 1 mm; E-H: 0.2 mm).

***Xantholinus varnensis* Coiffait, 1972** (Figure 6a-f)

Syn: *Xantholinus gridellii carius* Coiffait, 1972 (Figure 6a-f)

Material examined: Turkey: 10♂♂, 7♀♀, 04.V.2013, Aydın, Bozdoğan, Koyuncular, 2 km N, 257 m, 37°39'27" N, 28°25'34" E leg. Anlaş & Yağmur (AZMM). 3♂♂, 4♀♀, 16.VI.2017, Bursa, Karacabey, Longoz Forest, leg. Yağmur (AZMM).

Type examined: *Xantholinus gridellii carius* Coiffait: Holotype: 1♂: "Anatolia merid., Marmaris, V.1969, G. Fagel / Type [sic] / *X.* (*Acanthophallus*) *gridellii* ssp. *carius* H. Coiffait 1971" (IRSNB). Paratypes: 2♂♂, 2♀♀: "Anatolia merid., Marmaris, V.1969, G. Fagel / Paratype [sic] / *X.* (*Acanthophallus*) *gridellii* ssp. *carius* H. Coiffait 1971" (IRSNB).

Remarks: Coiffait (1972) based the original description of *X. gridellii carius* on a "type" from "Anatolia merid., Marmaris". Assing (2016) remarked that the "…but the Coiffait collection contains only a male labelled as paratype (not dissected by Coiffait). There are two possible explanations: either this type is the holotype, but was mislabeled or the holotype was returned to Fagel and Coiffait (1972) failed to mention the paratype in the original description." During a visit to the Fagel collection in Brussels (IRSNB) in 2016, A "type", and two males and two females "paratype" were found. Although the specimens of *X. gridellii carius* have labels "Paratype", the author did not select any paratypes while describing the new species. It is considered that the second suggestion of Assing (2006) is more plausible.

This subspecies has been proposed as a synonym of *X. graecus* by Assing (2006). In the same paper, the type material of *X. varnensis* was studied and referred to *X. graecus*. However, *X. varnensis* was revalidated by Assing (2008) and *X. gridellii carius* has been proposed as a synonym of *X. varnensis*. The type specimen of *X. gridellii carius* in the collections of the IRSNB were studied. An examination of the aedeagus of the male type of *X. gridellii carius* revealed that it is identical to *X. varnensis.* Consequently, it was confirmed that *X. gridellii carius* is a synonym of *X. varnensis*. For illustrations of the type specimens of *X. gridellii carius* see Figure 6a-f.

Distribution: This species is known from Bulgaria, Greece and Turkey (Schülke & Smetana, 2015; Anlaş, 2017).

***Xantholinus gridellii* Coiffait, 1956**

Material examined: Cyprus: 1♂, 1♀, 24.IV.2015, Lefkoşa, Değirmenlik, 650 m, leg. Yağmur (AZMM). Iraq: 2♂♂, 17-20.V.2008, northern Iraq, ca 10 km NW Suleimaniyah Province, leg. Sevinç (AZMM). Jordan: 1♂, Jordan (NMPC).

Distribution: This species is known from Israel, Lebanon, Syria, Cyprus and Turkey (Anlaş, 2017; Assing, 2017). The records from Iraq and Jordan are reported here for the first time.

***Xantholinus laevigatus* Jacobsen, 1849** (Figure 6g)

Material examined: Austria: 1♂, Kärnten, R. I. Sc. N. B. 17.479. (IRSNB). Belgium: 1♂, 27.VII.1948, Mirwart, le Parfondry, leg. Fagel (coll. Fagel). (IRSNB). Bosnia Herzegovina: 1♂, Mac., Jablanica Plan., VII.1930, leg. Rambousek (NMPC). Bulgaria: 1♂, 1♀, 11-14.VII.1927, Rila, Rila Kloster, leg. Fodor (HNHM). Czech Republic: 1♂, Hluboká, 31VIII.1907 (NMPC). Macedonia: 1♂, Maced. Perister SV. Petka, VII.1914, leg. Rambousek (NMPC). Italy: 1♂, 1♀, 1905, Calabria, Sta. E, d'Aspromonte, coll. Fodor (HNHM). Locality Not Found: 1♂, 22.VIII.1911, Bricnot, leg. Vrecerick (coll. Fagel). (IRSNB).

Distribution: This species is widespread in Europe and Turkey (Schülke & Smetana, 2015; Anlaş, 2017). However, it had not been recorded from Bosnia Herzegovina and Macedonia.

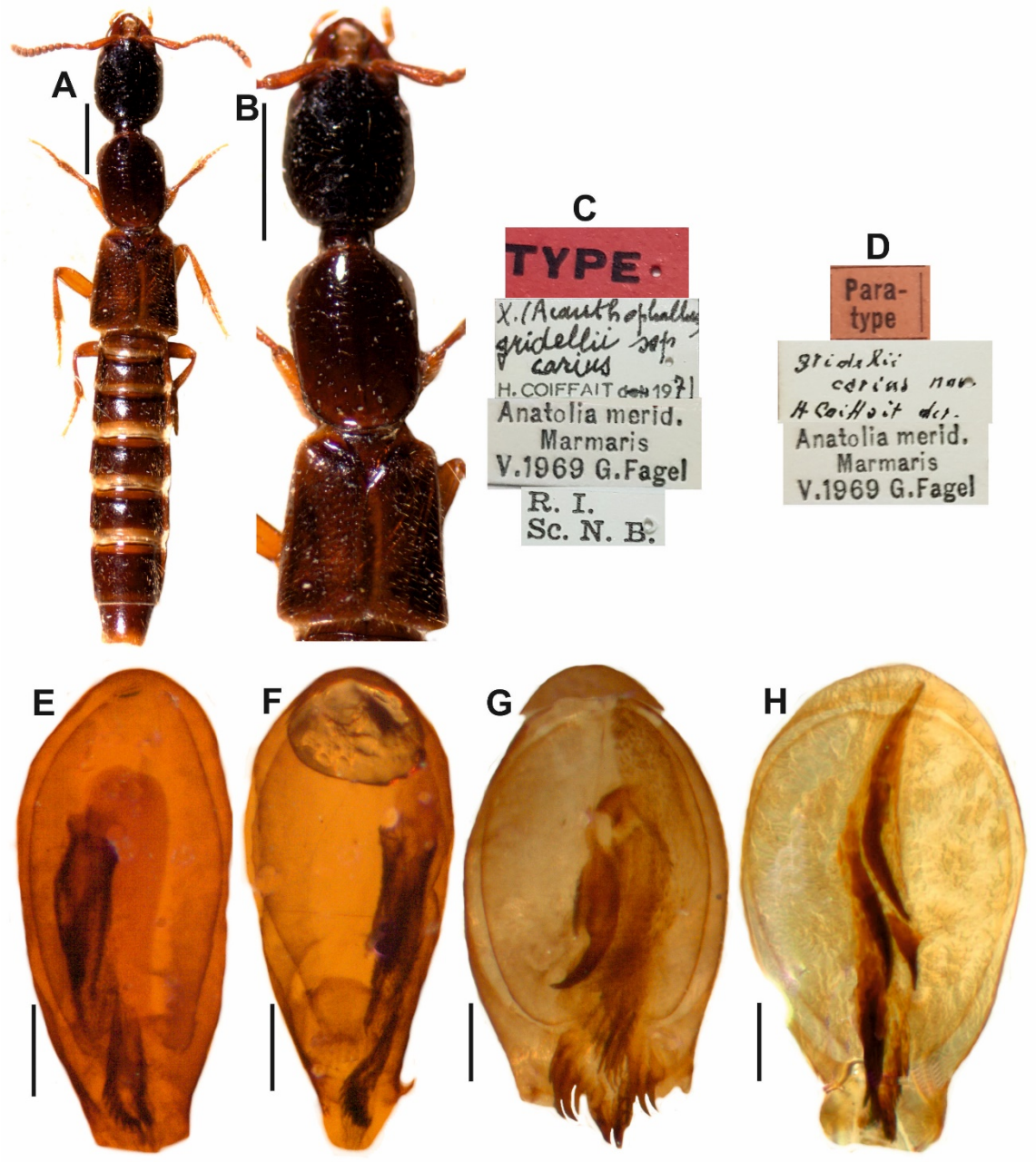

Figure 6. A-F) *Xantholinus gridellii carius* (syn of *X. varnensis*), G) *X. laevigatus*, H) *X. linearis*: A) habitus (Holotype); B) forebody (Holotype); C) type labels; D) paratype labels; E, F) aedeagus in dorsal and ventral view (E-Paratype; F-Holotype); G) aedeagus in dorsal view (G-Brussels, Belgium; H-Algeria). (Scale bars: A-B: 1 mm; E-H: 0.2 mm).

Subgenus *Xantholinus* Dejean, 1821

***Xantholinus coiffaiti* Franz, 1966**

Material examined: Bulgaria: 1♂, V.1919, Sozopel, leg. Hlisnikowski (NMPC).

Distribution: According to Schülke & Smetana (2015), this species distributed in Austria, Bulgaria, Czech Republic, Germany, Greece, Hungary, Italy, Slovakia, Slovenia and Turkey.

***Xantholinus dvoraki* Coiffait, 1956** (Figure 5g-h)

Material examined: Iraq: 1♂, 17-20.V.2008, northern Iraq, ca 10 km NW Suleimaniyah Province, leg. Sevinc (AZMM). Czech Republic: 1♂, 28.II.1937, Bohemia, Celakovice, leg. Kodym (NMPC). 1♂, Neratovice, Heyrovsky (NMPC).

Distribution: *Xantholinus dvoraki* occurs in Europe, Caucasus, Central Asia and Turkey (Bordoni, 2011, Schülke & Smetana, 2015; Anlaş, 2017). The above specimen represents the first records from Iraq.

***Xantholinus linearis* (Olivier, 1795)** (Figure 6h)

Material examined: Algeria: 1♂, 12.V.1988, Gde Kabylie Yakouren, 730 m, leg. Besuchet, Löbl & Burckhardt (MHNG). Belgium: 1♂, Calmpthout (=*Kalmthout)*, coll. et det. A. Fauvel. R. I. Sc. N. B. 17.479. (IRSNB). Greece: 1♂, Corfu, leg. J. Sahlberg, R. I. Sc. N. B. 17.479. (IRSNB). Czech Republic: 1♂, 2♀♀, 09.XI.2002, Bohemia centr., leg. Danék (NMPC). 1♂, Bezkydy, leg. F. Kouřil (NMPC). 1♂, Volšany, leg. Křiženeck, 1934 (NMPC). 1♂, Bohemie, Zlonice, 25.III.1914, leg. A. Procházka (NMPC).

Distribution: According to Schülke & Smetana (2015), this species is widespread in the western Palearctic Region and the Nearctic.

***Xantholinus longiventris* Heer, 1839**

Material examined: Czech Republic: 1♂, 10.V.1914, Bohemia, Klobuky, coll. A. Procházka (NMPC).

Distribution: According to Schülke & Smetana (2015), this species is widespread in the western Palearctic Region and the Nearctic.

***Xantholinus morandi* Coiffait, 1958**

Material examined: Portugal: 1♂, 2♀♀, 08.IX.1969, Setubal, Marateca/Setubal, 730 m, leg. Senglet (MHNG).

Distribution: This species is known from Italy, Portugal, Spain, Algeria and Morocco (Schülke & Smetana, 2015).

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Original article (Orijinal araştırma)

Phylogenetics of *Buchnera aphidicola* Munson et al., 1991 (Enterobacteriales: Enterobacteriaceae) based on 16S rRNA amplified from seven aphid species[1]

Farklı yaprak biti türlerinden izole edilen *Buchnera aphidicola* Munson et al., 1991 (Enterobacteriales: Enterobacteriaceae)'nın 16S rRNA'ya göre filogenetiği

Gül SATAR[2*]

Abstract

The obligate symbiont, *Buchnera aphidicola* Munson et al., 1991 (Enterobacteriales: Enterobacteriaceae) is important for the physiological processes of aphids. *Buchnera aphidicola* genes detected in seven aphid species, collected in 2017 from different plants and altitudes in Adana Province, Turkey were analyzed to reveal phylogenetic interactions between *Buchnera* and aphids. The 16S rRNA gene was amplified and sequenced for this purpose and a phylogenetic tree built up by the neighbor-joining method. A significant correlation between *B. aphidicola* genes and the aphid species was revealed by this phylogenetic tree and the haplotype network. Specimens collected in Feke from *Solanum melongena* L. was distinguished from the other *B. aphidicola* genes on *Aphis gossypii* Glover, 1877 (Hemiptera: Aphididae) with a high bootstrap value of 99. *Buchnera aphidicola* in *Myzus* spp. was differentiated from others, and the difference between *Myzus cerasi* (Fabricius, 1775) and *Myzus persicae* (Sulzer, 1776) was clear. Although, *B. aphidicola* is specific to its host aphid, certain nucleotide differences obtained within the species could enable specification to geographic region or host plant in the future.

Keywords: Aphid, genetic similarity, phylogenetics, symbiotic bacterium

Öz

Obligat simbiyont, *Buchnera aphidicola* Munson et al., 1991 (Enterobacteriales: Enterobacteriaceae), yaprak bitlerinin fizyolojik olaylarının sürdürülmesinde önemli bir rol oynar. Adana (Türkiye)' dan 2017 yılında farklı bitki ve yüksekliklerden toplanan yedi yaprak biti türünde saptanan *B. aphidicola* genleri ile yaprakbiti türleri arasındaki filogenetik etkileşimi ortaya çıkarmak için analiz edilmiştir. Bu amaçla, 16S rRNA'nın gen bölgeleri kullanılmış ve filogenetik ağaç, neighbor-joining ile oluşturulmuştur. *Buchrena aphidicola* genleri ve aphid türleri arasında filogenetik ağaç ve haploid networke göre anlamlı bir korelasyon tespit edilmiştir. *Solanum melongena* L. toplanan Feke örneği, diğer *Aphis gossypii* Glover, 1877 (Hemiptera: Aphididae)'lerdeki *B. aphidicola* genlerinden çok yüksek bir boostrap değeri (99) ile ayrılmıştır. *Myzus* cinsindeki *B. aphidicola* genleri diğer cinslerden ayrı dallanmış ve *Myzus cerasi* (Fabricus, 1775) ve *Myzus persicae* Sulzer, 1776 arasındaki ayrım belirgindir. *Buchera aphidicola* konukçu yaprakbiti türüne özelleşmiş olsa da tür içinde elde edilen bazı nükleotid farklılıkları ilerde coğrafik bölgeye ya da bitkiye de özelleşmeye neden olabilir.

Anahtar sözcükler: Yaprak biti, genetik benzerlik, filogenetik, simbiyotik bakteri

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Introduction

Aphids (Hemiptera: Aphididae) are a group of diverse insect species highly adapted to feed on plants by sucking sap from phloem (Blackman & Eastop, 2000). Turkey has more than 500 aphid species across its different geographic and climatic regions, with more than 80 aphid species have been recorded in Adana Province (Uygun et al., 2001; Çalışkan et al., 2012; Görür et al., 2017). Worldwide there are about 5000 aphid species, and most are considered have an intracellular bacterial symbiont (Fukatsu, 2001). These symbionts can be obligating like *Buchnera aphidicola* Munson et al., 1991 (Enterobacteriales: Enterobacteriaceae) or secondary symbionts such as *Rickettsia* spp. da Rocha-Lima, 1916*, Wolbachia* spp. Hertig & Burt 1924 (Rickettsiales: Rickettsiaceae), *Hamiltonella defensa* Moran et al., 2005*, Regiella insecticola* Moran et al., 2005, and *Serratia symbiotica* Moran et al., 2005 (Enterobacteriales: Enterobacteriaceae) (Peccoud et al., 2014). Endosymbionts have active roles in nutritional support (Akman Gündüz & Douglas, 2009; Chen et al., 2009), insecticide resistance (Martine et al., 2013; Pan et al., 2013), reproductive behavior (Baldo et al., 2006; Simon et al., 2011), predator-prey and parasitoid-prey interactions (Tsuchida et al., 2010; Vorburger et al., 2010; Telesnicki et al., 2012; Martine et al., 2013), adaptation of the insects to external conditions (Russell & Moran, 2006), and the physiology of host-associated populations of polyphagous insects (Brady & White, 2013). Physiological processes, such as development and reproduction in aphids, depend on the existence of the obligate symbiont *B. aphidicola* (Baumann et al., 1995; Douglas, 2003; Peccoud et al., 2014). Aphids need *B. aphidicola* for synthesizing essential amino acids and riboflavin, nutrients that are usually not present in phloem sap (Akman Gündüz & Douglas, 2009; Chen et al., 2009; Liu et al., 2013). Without *B. aphidicola*, aphids produce dwarfed offspring, or have lower or no capacity to reproduce. The benefit for *B. aphidicola* is that aphids create a safe and stable environment for the bacterium in specialized bacteriocytes (Chen et al., 2009). Therefore, the relationship between aphids and *B. aphidicola* is mutualistic (Baumann et al., 1995).

For the evolutionary role of such mutualisms, the term symbiogenesis was first used by Mereschkowsky (1909) and then in the context of the work of Buchner (1912) on hemipteran symbionts. In 1924, Kozo-Polyansky (see Kozo-Polyansky & Fet, 2010) proposed a role for symbiont organisms in the evolutionary process of the all living organisms. Within this context, *B. aphidicola* is a highly fascinating symbiont. It is located in maternal bacteriocytes and transmits through eggs or embryos (Liu et al., 2013). Improved understanding the relationship between aphids and their symbionts, a relationship that evolved 150-200 million years ago (Jousselin et al., 2009), will undoubtedly provide deeper insights into the biology of both organisms. There have been a range of studies on this coevolution published (e.g., Fukatsu, 2001; Liu et al., 2013, 2014). In order to reveal the coevolutionary process between the aphid species and *B. aphidicola*, molecular examination of the 16S rRNA gene has been used (Munson et al., 1991; Baumann et al., 1997; Liu et al., 2014). These studies suggest that *B. aphidicola* has lost some genes and/or aphids acquired some highly transcribed genes including LD-carboxypeptidases (LdcA1, LdcA2 and yLdcA), lipoprotein As (RlpA1-5), DNA polymerase III alpha chain (yDnaE) and ATP synthase delta chain (yAtpH) from *B. aphidicola* (Nikoh et al., 2010; Liu et al., 2013; Lagos et al., 2014; Güz et al., 2015). Given their close evolutionary relationship, phylogenetics of the host and its symbiont mirror each other in deeper evolutionary divergences. Therefore, data obtained from the symbionts can be used to reconstruct the evolutionary process of hosts (Nováková et al., 2013).

Satar et al. (2013) studied *Aphis gossypii* Glover, 1877 on different host plant species in the eastern Mediterranean Region, and discovered that *A. gossypii* on cucurbits had distinctly different biology. However, it is known if *B. aphidicola* contributes to this observed biological diversity. Also, it is not known whether different aphid taxa on the same host plant possess similar or diverse *B. aphidicola* genotypes. Both are possible because host-plant relationships in polyphagous insects are important in the evolution of the insect. In addition, relationships between aphids and *B. aphidicola* have generally been studied on

a wide geographic scale, with geography potentially acting as a major driver for differentiation of *B. aphidicola*. Therefore, research focusing on a smaller geographic scale may provide valuable information by eliminating the wider geographic effects on climate, host plants and natural enemies.

Therefore, the aim of this study was to examine the genetic interaction between *B. aphidicola* and seven different aphid species collected from different plants at different altitudes in Adana Province, Turkey, which represents the first genetic investigation of this symbiotic system in Turkey. Additionally, this study aimed to increase the genetic database on *B. aphidicola*, and provide deeper insights into the coevolutionary process between this obligate bacterium and its aphid hosts.

Material and Methods

Sampling of aphids

Aphid populations were collected in 2017 from a range of plants (trees, weeds and vegetables) and altitudes in Adana Province, Turkey (Table 1, Figure 1). Aphids were removed from infested plants with a fine brush and transferred to Eppendorf tubes containing 96% alcohol. Collection date, geographic location and host plant species were recorded, and samples stored at -80ºC until DNA extraction. Aphid species were determined morphologically by Dr. Işıl Özdemir (Plant Protection Central Research Institute, Ankara, Turkey) according to Blackman & Eastop (2019). Seven aphid species were determined in three genera of the Aphidinae subfamily, and used for constructing molecular phylogeny of *B. aphidicola* (Table 1). According to morphological identification, the specimens were *Aphis craccivora* Koch, *Aphis fabae* Scopoli, 1763, *A. gossypii*, *Aphis pomi* De Geer, 1773, and *Rhopalosiphum maidis* (Fitch, 1856) from the Aphidini tribe, and *Myzus cerasi* (Fabricius, 1775) and *Myzus persicae* Sulzer, 1776 from the Macrosiphini tribe.

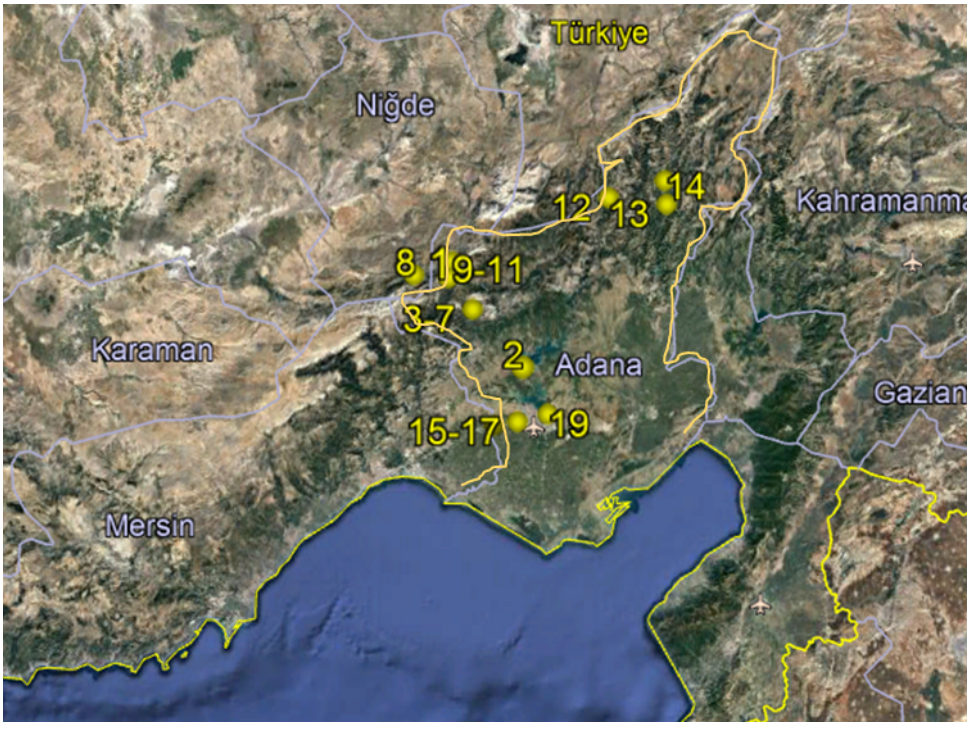

Figure 1. Collection locations of aphid samples from Adana Province, Turkey (satellite image from Anonymous, 2019a).

Table 1. Sample numbers, sampling date, location, altitude, host plants, and host aphid species for detection of genetic diversity of *Buchnera aphidicola*

Sample number	Date	Location	Altitude (m)	Host plant	Aphid species
1	27.07.2017	Fındıklı/Pozantı	1139	*Cucumis sativus* L.	*Aphis gossypii*
2	17.08.2017	Sağdıkali/Karaisalı	141	*Abelmoschus esculentus* L.	*A. gossypii*
3	17.08.2017	Kızıldağ	1666	*Prunus avium* L.	*Myzus cerasi*
4	17.08.2017	Kızıldağ	1666	*Zea mays* L.	*Rhopalosiphum maidis*
5	17.08.2017	Kızıldağ	1650	*A. esculentus*	*A. gossypii*
6	17.08.2017	Kızıldağ	1650	*Malus communis* L.	*Aphis pomi*
7	17.08.2017	Kızıldağ	1650	*Phaseolus vulgaris* L.	*Aphis fabae*
8	07.09.2017	Çiftehan	950	*P. avium*	*M. cerasi*
9	07.09.2017	Kamışlı Pozantı	1220	*Robinia pseudoacacia* L.	*Aphis craccivora*
10	07.09.2017	Kamışlı Pozantı	1220	*M. communis*	*A. pomi*
11	07.09.2017	Kamışlı Pozantı	1220	*Capsicum annuum* L.	*Myzus persicae*
12	20.09.2018	Görbeyaz-Feke	1050	*C. annuum*	*M. persicae*
13	20.09.2017	Feke-Akkaya	772	*Z. mays*	*R. maidis*
14	20.09.2017	Düşmüş-Feke	620	*Solanum melongena* L.	*A. gossypii*
15	03.11.2017	Seyhan	27	*C. annuum*	*A. gossypii* *A. craccivora*
16	01.11.2017	Seyhan	23	*Vigna unguiculata* (L.)	*A. craccivora*
17	01.11.2017	Seyhan	23	*Cucumis melo* L.	*A. gossypii*
18	24.02.2018	Zeytinli	24	*Capsella bursa-pastoris* (L.)	*A. craccivora*
19	27.02.2018	Balcalı	137	*C. bursa-pastoris*	*M. persicae*

DNA extraction and amplification

Genomic DNA for 37 specimens, representing all 19 populations (Table 1), were extracted from parasitoid free single aphids with a DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions in order to analyze *B. aphidicola* 16S rRNA gene (Table 2). The primer pairs, 16sF (5'-AGAGTTTGATCATGGCTCAGATTG-3') and 16sR (5'-TACCTTGTTACGACTTCACCCCAG-3') belonging to 16S rRNA gene region were used for PCR (Liu et al., 2013). The reaction mixture was prepared to achieve a final volume of 25 µl with inclusion of Taq buffer (10X), 2.5 mM $MgCl_2$, 250 µM dNTPs, 1 µM primer, 0.5 U Taq and 10 µM DNA template. The thermocycler conditions were: 5 min at

94ºC for predenaturation, followed by 35 cycles at 94ºC for 1 min, 50ºC for 1 min, at 72ºC for 1 min, and a final period at 72ºC for 7 min. The PCR products were run on 1% agarose gel, stained with ethidium bromide and viewed with a gel imaging system before two-way sequencing by a commercial company (Molgentek, Adana, Turkey).

Table 2. Country of origin, aphid species, and sample number (this study) or GenBank accession number used for examine of 16S rRNA gene of *Buchnera aphidicola*

Country	Aphid species	n	Sample number
Turkey (this study)	*Aphis craccivora*	7	9-1, 9-2, 15-1 15-2, 15-10, 16-1, 16-2
	Aphis fabae	2	7-1 7-2
	Aphis gossypii	12	1-1, 1-2, 2-1, 2-2, 5-1, 5-2, 14-1*, 14-2*, 15-20, 17-1, 17-2, 18-1
	Aphis pomi	3	3-2, 8-1, 8-2
	Myzus cerasi	3	10-2, 122-1,122-2
	Myzus persicae	6	11-1 11-2 12-1 12-2 19-1 19-2
	Rhopalosiphum maidis	4	4-1 4-2, 13-1 13-2
India	*A. fabae*	7	KT175935.1, KT175936.1, KT175937.1, KT175938.1, KT175939.1, KT175941.1, KT175940.1
	A. gossypii	18	KT175910.1, KT175911.1, KT175912.1, KT175913.1, KT175914.1, KT175915.1, KT175916.1, KT175917.1, KT175918.1, KT175919.1, KT175920.1, KT175921.1, KT175922.1, KT175923.1, KT175924.1, KT175925.1, KT175926.1, KT175927.1
USA	*A. fabae*	1	AY518294.1
	M. persicae	1	M63249.1
Taiwan	*A. craccivora*	1	EF614236.1
China	*R. maidis*	1	JX998123.1

* Different haplotype of *B. aphidicola* in *A gossypii* from Adana, Turkey.

Data analysis

The multiple alignments were made using ClustalW, and MEGA6 software (Tamura et al., 2013) was used to make necessary comparisons. Two-way sequences were controlled for each different base, edited on the Finch TV (FinchTV, 2019) and combined with MEGA6 software (Tamura et al., 2013). The reference sequences for the 16S rRNA gene of *B. aphidicola* from different countries obtained from GenBank were compared with these new results. DnaSP 5 software was used to detect haplotypes (Librado & Rozas, 2009.). Along with the reference sequences, 9, 4, 2, 1, 1, 2, and 2 haplotypes were used for construction of a phylogenetic tree for *A. gossypii*, *A. fabae, A. craccivora*, *A. pomi*, *M. cerasi*, *M. persicae*, and *R. maidis,* respectively (Table 2). The seven haplotypes were added to GenBank with accession number MK676083-90.

The neighbor-joining method based on Kimura 2-parameter (K2P) and Gamma distributed (5 categories, +G, parameter = 0.4153), and the best-fit model for the sequences in MEGA6, was used to reconstruct phylogenetic tree (Tamura et al., 2013). The bootstrap consensus tree inferred from 500 replicates (Nei & Kumar, 2000) was taken to represent the evolutionary history of the taxa analyzed. *Escherichia coli* T. Escherich, 1885 (Enterobacteriales: Enterobacteriaceae) (Migula, 1895) was selected

as an out-group. Nucleotide distance matrix based on the K2P calculated according to Kimura (1980) and Tamura et al. (2013) in MEGA6 software. All genes from this study and corresponding reference genes were used to establishment the 16S rRNA gene-specific haplotype network using PopArt (Anonymous, 2019b) software by the median-joining method (Bandelt et al., 1999). Nucleotide distance matrix based on the K2P was calculated according to Kimura (1980) and Tamura et al. (2013) in MEGA6 software.

Results and Discussion

Amplification of the 16S rRNA gene of *B. aphidicola* from the seven aphid species yielded 1500-bp products for 37 specimens on the agarose gel. After cleaning and editing of the specimen sequences, 66 sequences (1357-bp) were included from this study and reference sequences were analyzed to construct the phylogenetic tree (Table 2, Figure 2). Specimens belonging to the same haplotypes are shown in the brackets at the same tip on the phylogenetic tree.

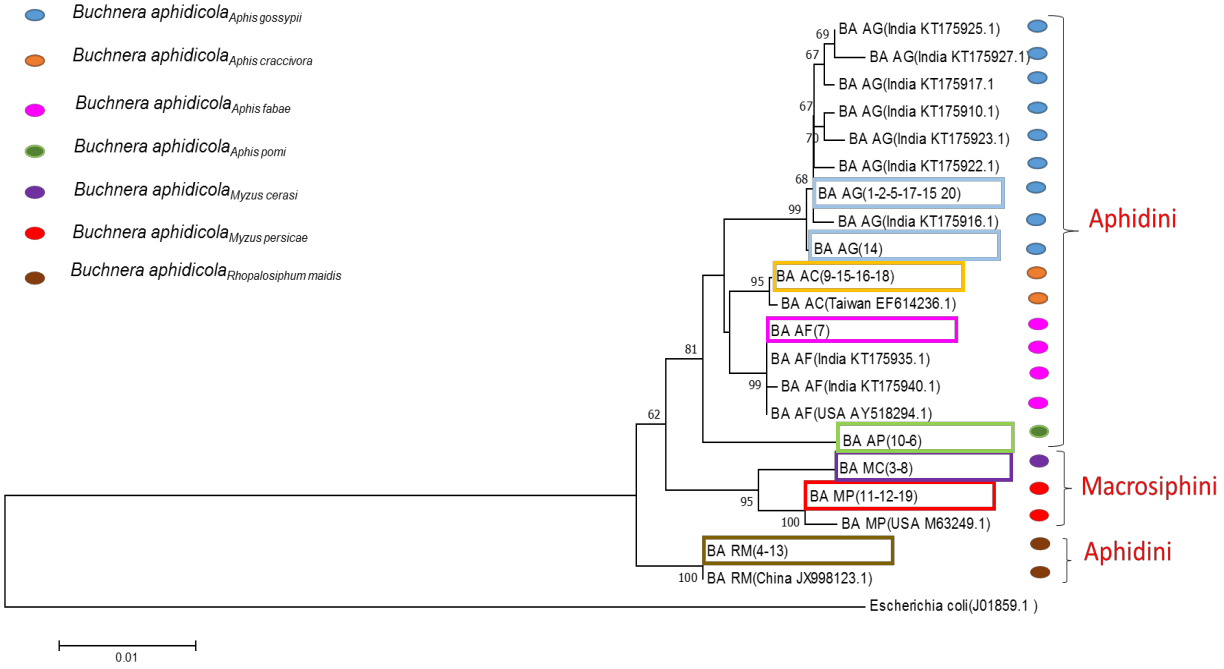

Figure 2. Phylogenetic tree of *Buchnera aphidicola* (BA) from seven aphid species based on neighbor-joining method (bootstrap 500). The genes used in this study, highlighted with different colors, came from to *B. aphidicola* from seven aphid species: AC, *Aphis craccivora;* AF, *Aphis fabae;* AG, *Aphis gossypii;* AP, *Aphis pomi*; MC, *Myzus cerasi*; MP, *Myzus persicae*; and RM, *Rhopalosiphum maidis*.

The *E. coli* sequence, used as the out-group, was well separated from the *B. aphidicola* 16S rRNA sequences. *Buchnera aphidicola* is a close relative of *E. coli*, but its genome size is only one seventh of *E. coli* (Shigenobu et al., 2000). Differentiation of Aphidini and Macrosiphini tribes is evident in the tree (Figure 2). However, *R. maidis* belonging to Aphidini tribe was separated from the *Aphis* spp. in the same tribe. This might relate to the feeding of these two genera on different host plants. *Rhopalosiphum maidis* feeds on monocotyledonous plants, whereas, *Aphis* spp. feed exclusively on dicotyledonous plants (Holman, 2009; Betsiashvili et al., 2014). *Buchnera aphidicola* provides essential amino acids to aphids, and this might create genetic distance between monocotyledonous and dicotyledonous plants because of their different composition of metabolites (Schobert et al., 1998; Qi et al., 2018). The range of the amino acids provided and the metabolic versatility of *B. aphidicola* may vary between higher aphid taxa or between aphid species that have narrow and broad host plant ranges (Douglas, 1998).

Buchnera aphidicola genes are distinguished according to aphid genus in the phylogenetic tree (Figure 2). *Myzus* spp. are differentiated from *Aphis* spp. with bootstrap value of 62. Two *Myzus* spp. are also distinguished from each other with a high bootstrap value. Species within *Aphis* was distinguished with low bootstrap value except for *A. pomi*. When *A. gossypii* was evaluated intraspecifically, *A. gossypii* samples collected from Adana Province were clustered in a group with homologous sequences from specimens from India. However, the *B. aphidicola* sample collected from *Solanum melongena* L. (14) in Feke District was distinguished from others with a bootstrap value of 99. Although one nucleotide difference was determined among the specimens from Turkey, higher numbers of differences were found in Indian samples (Figures 2 and 3). Aphids are holocyclic in regions with warmer climates including the coast areas of the East Mediterranean Region of Turkey. However, they are heterocyclic in colder areas like plateaus of the same region. Holocyclic aphid populations consist of females only, and the offspring are genetically identical to their parent. However, heterocyclic aphids that overwinter under harsher climatic conditions as eggs have both females and males. Therefore, genetic differentiation can occur more readily in colder regions. Samples collected in Feke District, a transition zone between upland and coastal areas, have higher variation than the *B. aphidicola* in *A. gossypii* specimens, which is probably a resulted of to the mating individuals from the two different areas. Chong & Moran (2016) showed that the same *B. aphidicola* haplotype in different aphid genotypes differently affected the fitness cost of aphid clones. The intraspecific genotypic variation of aphids could be important for the potential long-term evolution of *B. aphidicola* and aphids. In this respect, molecular studies alone are insufficient for explaining this interaction.

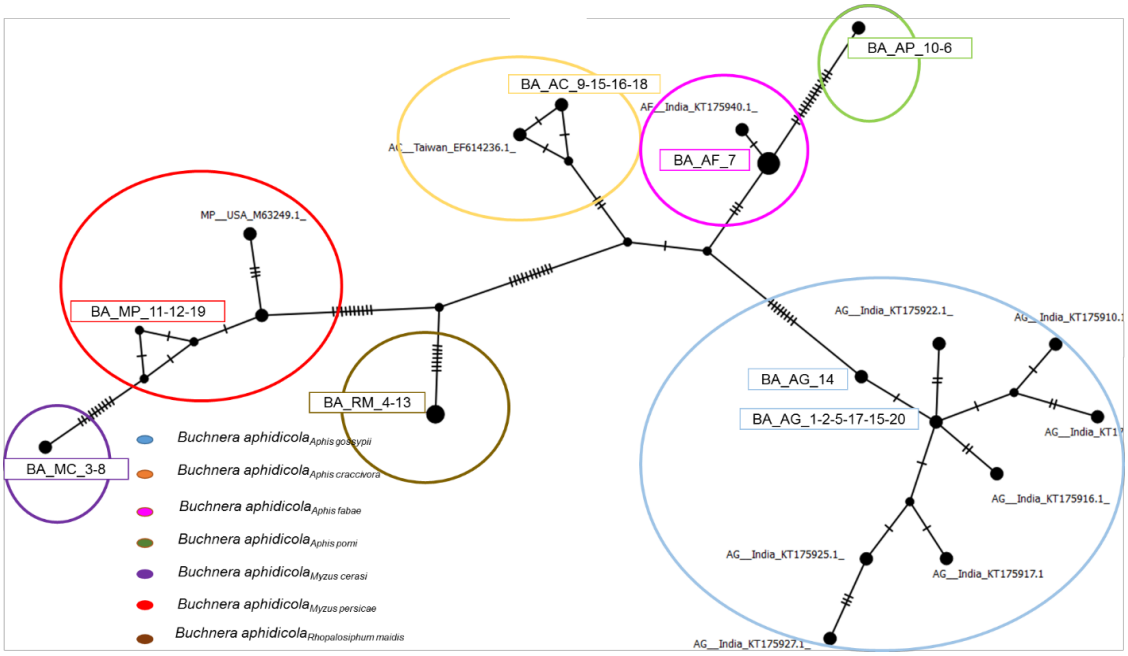

Figure 3. Haplotype network of *Buchnera aphidicola* (BA) 16S rRNA gene from seven host aphid species: AC, *Aphis craccivora*; AF, *Aphis fabae*; AG, *Aphis gossypii*; AP, *Aphis pomi*; MC, *Myzus cerasi*; MP, *Myzus persicae*; and RM, *Rhopalosiphum maidis*.

Buchnera aphidicola 16S rRNA genes from seven aphid species were grouped separately on the haplotype network in parallel with the phylogenetic tree. While *B. aphidicola* in *A. gossypii* and *M. cerasi* are the furthest species with more than 28 single nucleotide shifts, *A. fabae* was closest to *A. craccivora* with only eight to nine single nucleotide shifts (Figure 3). The pairwise K2P nucleotide distance analyses showed that interspecific and intraspecific mean distance values were 1-2% and 0%, respectively. General pairwise distance is ranged up to 3% (Table 3). In general, low genetic distance was observed between *B. aphidicola* in the seven-aphid species.

Although *A. gossypii* and *A. craccivora* species were collected from same *Capsicum annuum* L. plant (15), their *B. aphidicola* appears to be specific to the aphid species (Table 1). Also, no specific differences were observed in relation to altitude or plant species. For example, the samples on *C. annuum* collected from the coastline (15) and upland area (11 and 12) were found to be identical in terms of genetic structure. Satar et al. (2013) studied *A. gossypii* on different host plant species and found that *A. gossypii* has distinctly different biology on cucurbits compared to other host plants. However, it was found that *B. aphidicola* from *A. gossypii* on cucurbits (1 and 17) did not have any nucleotide differences from other *A. gossypii* specimens in the region, except for the *S. melongena* specimens. Thus, it appears that host plant species is not a driver of *B. aphidicola* diversity.

As Turkey is at the intersection between Europe and Asia, it is rich in terms of aphid biodiversity compared to the neighboring countries (Kök et al., 2016). It has diverse geographical features and climate types that have led to many endemic aphid species and genetic variation within these species. Over 540 aphid species have been recorded in Turkey, as significant proportion of the 5000 aphidofauna and 13 subspecies belong to 141 genera recorded worldwide (Görür et al., 2017). The present study was by intent restricted to a narrow geographic area and limited number of aphid species. However, it is the first study to demonstrate the relationship between aphid species and *B. aphidicola* in Turkey. Future studies of a wider range of aphids from different ecosystems could potentially provide reasons for the genetic variation such as seen in the Feke specimen.

Table 3. Nucleotide distance matrix based on Kimura 2-parameter model of *Buchnera aphidicola* (BA) 16S rRNA gene on seven host aphid species: AC, *Aphis craccivora*; AF, *Aphis fabae*; AG, *Aphis gossypii*; AP, *Aphis pomi*; MC, *Myzus cerasi*; MP, *Myzus persicae*; and RM, *Rhopalosiphum maidis*

No	Specimen Name	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1	BA_AG (1, 2, 5, 15_20, 17)																					
2	BA_AG (14)	0.00																				
3	BA_AG (India_KT175910.1)	0.00	0.00																			
4	BA_AG (India_KT175916.1)	0.00	0.00	0.00																		
5	BA_AG (India_KT175917.1)	0.00	0.00	0.00	0.00																	
6	BA_AG (India_KT175922.1)	0.00	0.00	0.00	0.00	0.00																
7	BA_AG (India_KT175923.1)	0.00	0.00	0.00	0.00	0.00	0.00															
8	BA_AG (India_KT175925.1)	0.00	0.00	0.00	0.00	0.00	0.00	0.00														
9	BA_AG (India_KT175927.1)	0.00	0.00	0.01	0.01	0.00	0.01	0.01	0.00													
10	BA_AC (9, 15, 16, 18)	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01												
11	BA_AC (Taiwan_EF614236.1)	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.00											
12	BA_MC (3, 8)	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.03	0.02	0.02										
13	BA_AP (6, 10)	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02									
14	BA_RM (4, 13)	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02								
15	BA_RM (China_JX998123.1)	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.00							
16	BA_MP (11, 12, 19)	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.03	0.02	0.02	0.01	0.02	0.01	0.01						
17	BA_MP (USA_M63249.1)	0.02	0.02	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.02	0.02	0.01	0.02	0.02	0.02	0.00					
18	BA_AF (7)	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.01	0.02	0.02	0.02	0.02				
19	BA_AF (India_KT175935.1)	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.01	0.02	0.02	0.02	0.02	0.00			
20	BA_AF (India_KT175940.1)	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.01	0.02	0.02	0.02	0.02	0.00	0.00		
21	BA_AF (USA_AY518294.1)	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.01	0.02	0.02	0.02	0.02	0.00	0.00	0.00	
22	*Escherichia coli* (J01859.1)	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.13	0.12	0.11	0.13	0.12	0.11	0.11	0.12	0.13	0.12	0.12	0.12	0.12

Theories on the coevolution and coexistence of aphid species and *B. aphidicola* have been offered by a number of researchers (Munson et al., 1991; Funk et al., 2000; Liu et al., 2013), although there is no universal agreement on these ideas (van Ham et al., 1997). Aphids and *B. aphidicola*, which coevolved 150-200 million years ago (Jousselin et al., 2009), may have been affected by many factors, such as host plant, environment and geographic region. However, detailed investigations of aphid fitness cost, effects of environmental factors, geographic differences are needed to fully understand this symbiotic relationship. Although, *B. aphidicola* is specific to host aphid, some intraspecific nucleotide differences may be found to be related to geographic region or host plant in future studies. In conclusion, sampling from different geographic regions and different plants at different intervals would be helpful to further examine the coevolutionary processes.

Acknowledgments

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