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Corresponding address:
Turkish Journal of Entomology
Ege Üniversitesi Kampüsü PTT Şubesi, P. O. Box: 10, 35100 Bornova, İzmir, Turkey
e-mail: dergi@entomoloji.org.tr
web : http://www.entomoloji.org.tr

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Original article (Orijinal araştırma)

Chemical components and insecticidal effects of essential oils from three lavender cultivars against adult *Sitophilus granarius* (L., 1758) (Coleoptera: Curculionidae)[1]

Farklı lavanta çeşitlerinin kimyasal bileşenleri ve *Sitophilus granarius* (L., 1758) (Coleoptera: Curculionidae) erginlerine karşı insektisidal etkisi

Mustafa ALKAN[2*] Merve GÜZEL[3] Hüseyin AKŞİT[4]
Reyhan BAHTİYARCA BAĞDAT[5] Fatma Ruveyda ALKAN[5] Emre EVLİCE[2]

Abstract

The study was conducted to determine the insecticidal and behavioral effect of essential oils (EOs) extracted from three lavender cultivars, Hemus, Raya and Yubileina, against adult stage of *Sitophilus granarius* (L., 1758) (Coleoptera: Curculionidae) under laboratory conditions. The contact, fumigant and repellent effects of plant EOs were investigated and possible germination inhibitory effects of the EOs on wheat germination were evaluated. EOs were extracted from the plant samples using a Neo-Clevenger type apparatus where the plant material is subjected to hydro distillation. The chemical constituents of EOs were detected by gas chromatography-mass spectrometry. The experiments were conducted under laboratory conditions in 2019 and 2020. As a result of the study, it was determined that the fumigant activity changed according to the cultivars. While the LC_{90} value in the fumigant activity of the EOs of the Hemus was determined as 0.157 µl/ml air, this value was recorded as 0.139 µl/ml air and 0.118 µl/ml air in Raya and Yubileina, respectively. Six h after treatment, the highest repellent activity was 86% at 0.05 µl/cm^2 with Yubileina. Main EOs components of each cultivar were: Yubileina, linalool (36.0%), linalyl acetate (24.2%) and lavandulyl acetate (5.86%); Hemus, linalool (28.5%), linalyl acetate (23.1%) and lavandulyl acetate (6.59%); and Raya, linalool (42.5%), linalyl acetate (30.0%) and α-terpineol (5.45%). There was no negative effect on the germination of wheat with any of essential oils. These results show that lavender EOs could be useful for the control of *S. granarius*.

Keywords: Biplot analyses, fumigant activity, GC-MS, granary weevil, *Lavandula angustifolia*, repellent activity

Öz

Bu çalışma, ticari amaçlı üretimi yapılan üç farklı lavanta çeşidi Hemus, Raya ve Yubileina'dan izole edilen uçucu yağların *Sitophilus granarius* (L., 1758) (Coleoptera: Curculionidae)'un ergin dönemlerine karşı insektisidal ve davranışsal etkisini laboratuvar koşullarında belirlemek amacıyla yapılmıştır. Bitki uçucu yağlarının kontakt, fumigant ve repellent etkileri araştırılmış ve bu yağların buğdayın çimlenme gücü üzerine olası etkileri değerlendirilmiştir. Uçucu yağlar, bitki materyallerinden Neo-Clevenger tipi aparat kullanılarak hidrodistilasyon yöntemine göre elde edilmiştir. Uçucu yağların kimyasal bileşenleri gaz kromatografisi-kütle spektrometrisi ile tespit edilmiştir. Denemeler 2019 ve 2020 yıllarında laboratuvar koşullarında yürütülmüştür. Çalışma sonucunda bitki çeşitlerine göre fumigant aktivitesinin değiştiği tespit edilmiştir. Hemus uçucu yağının fumigant aktivitesindeki LC_{90} değeri 0.157 µl/ml hava olarak belirlenirken, bu değer Raya ve Yubileina'da sırasıyla 0.139 µl/ml hava ve 0.118 µl/ml hava olarak belirlenmiştir. Uygulamadan altı saat sonra en yüksek repellent etki, Yubileina'nın 0.05 µl/cm^2 uygulama dozunda %86 repellent etki ile gözlenmiştir. Yubileina'nın ana uçucu yağ bileşenleri, linalool (%36.0), linalil asetat (%24.2) ve lavandulil asetat (%5.86) olarak belirlenirken Hemus'un temel bileşenleri linalool (%28.5), linalil asetat (%23.1) ve lavandulil asetat (%6.59), Raya'nın ana bileşenleri ise linalool (%42.5), linalil asetat (%30.0) ve α-terpineol (%5.45) olarak belirlenmiştir. Her üç uçucu yağ için de buğdayın çimlenme gücü üzerinde olumsuz bir etki belirlenmemiştir. Bu sonuçlar, lavanta uçucu yağlarının *S. granarius*'un mücadelesinde önemli bir potansiyele sahip olabileceğini göstermektedir.

Anahtar sözcükler: Biplot analizi, fumigant aktivite, GC-MS, buğday biti, *Lavandula angustifolia*, repellent aktivite

[1] Part of this study was presented as an oral presentation at the International Erciyes Agriculture, Animal & Food Sciences Conference.

[2] Directorate of Plant Protection Central Research Institute, 06172, Ankara, Turkey
[3] Directorate of Trakya Agricultural Research Institute, 22100, Edirne, Turkey
[4] Erzincan Binali Yıldırım University, Faculty of Pharmacy, 24002, Erzincan, Turkey
[5] Fields Crops Central Research Institute, 06172, Ankara, Turkey
* Corresponding author (Sorumlu yazar) e-mail: alkan0101@gmail.com
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Introduction

The world population is increasing rapidly and as a result the growing demand for safe food demand is a major problem for agricultural systems with limited resources. Therefore, the current global agricultural production has to be protected from biotic and abiotic factors from harvest to the table. The largest insect order, Coleoptera, includes the major and significant stored product pests. These pests can live under in a wide range of environment conditions. The feeding behavior and levels of storage pests vary, so some are considered primary pests whereas others are classified as secondary pests. *Sitophilus granarius* (L., 1758) (Coleoptera: Curculionidae) is considered one of the primary pests. Qualitative and quantitative losses occur in the stored products because of the detrimental effects of this pest. Various cultural, physical and chemical control methods are used to protect the stored products. Stored product pests are potentially present in all warehouses and cause 10-30% damage to annual global cereal production (Singh et al., 2009). The most widely used approach to control stored product pests globally is pesticides and in particular fumigation (Mutungi et al., 2014). However, both the direct consumption of stored products and the increase in concerns about pesticide use in recent years have led to the development of more environmentally-friendly approaches.

Lavender spp. which is a member of the Lamiaceae are known to have a Mediterranean origin (Aslancan & Sarıbaş, 2011). The lavender is a semi-bushy, perennial herb that can grow up to 1 m. There are more than 30 species of lavender, but only a few are used commercially for essential oil (EOs). The essential oil obtained from the flowers and flower stalks is one of the 15 most traded EOs in the world (Aslancan & Sarıbaş, 2011). The essential oil of the lavender is extracted using water vapor distillation apparatus. Lavender essential oil is still in high demand around the world. In near future, over 200,000 ha are expected to be planted with lavender in Europe and the quality of the essential oil produced is crucial for medical, pharmaceutical and aromatherapy applications (Hassiotis et al., 2010).

Plants consist of very different chemical components that cause insecticidal effects. These secondary metabolites are complex mixtures and can be classified as alkaloids, glycosides, phenols, terpenoids, tannins and saponins (Shanker & Solanki, 2000). Lis-Balchin & Hart (1999) found that lavender oil from *Lavandula angustifolia* Mill. (Lamiaceae) flowers contains linalyl acetate, linalool, lavandulol, 1,8-cineole, lavandulyl acetate, camphor and borneol. The antimicrobial activity of EOs, along with their sweetener/aromatic properties, has been widely used in the pharmaceutical, cosmetic and food industries (Prashar et al., 2004). Lavender essential oil is used in the culinary industry to flavor drinks, ice cream, confectionery, baked goods and chewing gum (Kim & Lee, 2002). Recently, aromatherapy has become increasingly popular and lavender has been used as a sedative (Lis-Balchin & Hart, 1999). Lavender species is also used as pain relievers, antifungal and antibacterial agents in the treatment of burns and insect bites (Cavanagh & Wilkinson, 2002).

Many studies have been conducted in terms of insecticidal and some other biological activities of lavender species. Different activities of *Lavandula* spp. such as antioxidant (Yakoubi et al., 2021), cytotoxic (Siddiqui et al., 2020), antimicrobial (Leong et al., 2021), anti-acetylcholinesterase (Vairinhos & Miguel, 2020), antibacterial (Sayout et al., 2020), repellent (Huang et al., 2020), antifungal (Domingues et al., 2021), allelopathic (Nazemi et al., 2016) have been recorded recently.

In this study, the insecticidal and behavioral effects of EOs of lavender cvs Hemus, Raya and Yubileina were evaluated under laboratory conditions against *S. granarius* and also possible germination inhibitory effects of the EOs on wheat germination. In addition, essential oil components were determined by GC-MS.

Material and Methods

Plant material and essential oil extraction

Plant materials, *L. angustifolia* (cvs Hemus, Raya and Yubileina) cultivars were provided from the cultivation areas of Trakya Agricultural Research Institute (Edirne, Turkey). The four-year-old plants were harvested for each cultivar at the flowering time in cloudless sunny weather at midday in June 2019. EOs were extracted from 100 g dry flower samples of each cultivars using Neo-Clevenger apparatus by hydrodistillation for 4 h. Oils were kept in amber vials at -20°C until identified.

Gas chromatography-mass spectrometry analysis

Gas chromatography-mass spectrometry (GC-MS) analyses were performed using a Thermo Scientific Trace 1310 GC-MS system, equipped with HP-5MS capillary column (30 m x 0.25 mm and 0.25 m ID). 20 mg of EOs were solved in 1 ml of acetone and directly injected to GC-MS in 1 µl volumes. Helium (constant flow, 1.2 ml/min) was used as a carrier gas in split mode by 50:1. The injection site and mass transfer line temperature were set at 280°C. The column oven temperature was programmed accordingly: the initial column oven temperature was 60°C, held for 3 min then ramped to 200°C at a rate of 3°C/min and then immediately ramped to 240°C at a rate of 5°C/min and held for 5 min. The mass spectrometer conditions were: the ion source temperature was 280°C and the ionization energy was 70 eV in EI mode. The retention indexes were calculated for all the components using the Van den Dool & Kratz equation (Dool & Kratz, 1963) based on homolog n-alkane series retention times. Wiley and NIST2004 MS libraries were used to confirm the identities of the compounds. The relative peak area percentages of each compound were calculated based on the MS chromatograms. Relative peak area percentages were calculated by multiplying the division of the relevant peak area by the total peak area by 100.

Insect collection and rearing

The population of *S. granarius* population in the Plant Protection Central Research Institute (Ankara, Turkey) stock culture was used in the study. Soft wheat kernels which were stored in a freezer at -18°C for 72 h to eliminate the risk of possible contamination were used for rearing *S. granarius*. Mixed-sex adults were placed in 1 L glass jars and the monitoring of emerged adults was recorded daily. The adults that emerged between 7 and 28 days after first emergence were used in the study.

Fumigant toxicity bioassays

Glass tubes (10 ml) with airtight caps were used in the single concentration for fumigant activity assays. Discs of 10 mm in diameter were cut from Whatman No. 1 filter paper and attached to the glass tube caps with needle. EOs were diluted using acetone 0.1 (v/v) and 10 µl applied to each filter disc with a micropipette. The same amount of acetone was applied to the filter paper in the control groups. The tubes were placed in a fume hood for 5 min to evaporate the acetone, the tubes were closed using a motor creeper with a silicone septic cap afterwards. The tubes were placed in an incubator at 25±2°C and dead insects were recorded after 24 h (Polatoğlu et al., 2013). The insects were considered dead when they did not move when probing with a sable brush. The experiment was set up with a completely randomized design with 18 replicates and five adult insects were used in each replicate. Since 70-100% mortality was detected in the applications of Hemus, Raya and Yubileina EOs, all three EOs were included in the dose-response assays. In order to calculate the LC_{50} and LC_{90} values, bioassays were set up at doses ranging from 0.05 to 0.15 (v/v).

Contact activity bioassays

EOs which were diluted with acetone at a concentration of 0.15 (v/v) applied to the second segment of the dorsal surface of thorax of the insects (1 µl/insect) with a micro applicator (Hamilton Company, PB-

600, Reno, NV, USA) in single-dose contact activity assays. In the control group, the insects were treated with the same amount of acetone. Adult individuals were placed into Petri dishes (6 cm diameter) containing food and were maintained in the dark at 25±2°C and 65% RH in the incubator. After 24 and 48 h, the dead adults were counted. The insects were considered dead when they did not move when probing with a sable brush (Hossain et al., 2019). The experiment was conducted according to completely randomized design with 10 replicates. In each replicate, 20 mixed sexed adult insects were used and the whole experiment was repeated two times.

Repellent activity assays

The method developed by McDonald et al. (1970) was used in order to evaluate the repellent effect of lavender cultivars EOs. For this purpose, 9 cm discs were cut from Whatman No. 1 filter paper. Acetone was applied to half of the filter paper as a control and EOs at 0.05 and 0.25 µl/cm^2 concentrations were separately applied to the other half. The filter papers, which were kept under a fume hood for 5 min to evaporate the acetone, were then fixed to the bottom of Petri dishes and 20 adult individuals were placed in the middle of the filter. The plates were covered with Parafilm to prevent evaporation and were kept at 25±2°C and 65% RH in the incubator. The area where the insects were present was recorded after 2, 4 and 6 h. The experiment was conducted according to completely randomized design with three replications. In each replicate 20 mixed sexed adult insects were used and the whole experiment was repeated two times. The following formula (McDonald et al., 1970) was used to compute the percent repellent efficacy:

$$\text{Repellent activity (\%)} = (Nc - Nt) / (Nc + Nt) \times 100$$

where, Nc is the number of insects in control and Nt is number of insects in respective EOs treatment.

After the calculation of percentage repellent effectivity, it was scored on 5-point scale used by Juliana & Su (1983): 0, 0.1% repellent activity; 1, 0.1-20.0%; 2, 20.1-40.0%; 3, 40.1-60.0%; 4, 60.1-80.0%; and 5, 80.1-100%.

Grain germination assays

Wheat seeds [*Triticum aestivum* L. cv. Eser (Poaceae)] have >95% germination rate obtained from the Field Crops Central Research Institute (Ankara, Turkey) were used in the study. Wheat seeds were surface-sterilized by NaOCl (5%) and ethanol (96%) for 5 and 30 min respectively and rinsed with distilled water. EOs concentrations tested in wheat germination were determined according to LC_{50} values obtained as a result of fumigant activity tests. Accordingly, 0.4l, 0.8l, 1.6l and 3.2 µl/ml doses for Hemus EOs, 0.35, 0.7, 1.4 and, 2.8 µl/ml doses for Raya EOs, and 0.3, 0.6, 1.2 and, 2.4 µl/ml doses for Yubileina EOs were used. Twenty seeds were treated with EOs concentrations in closed 10 ml airtight glass bottles and the lids open after 2 days were kept under a fume hood for 1 day. Seeds placed in Petri dishes containing filter paper moistened with purified water were incubated at 15-20°C at room temperature. After 7 days, the number of germinated seeds was determined. Seeds were considered as germinated when the shoot and root development reached the half-size and the same size of the seed, respectively. The experiment was laid out in a completely randomized design with five replicates, and repeated two times.

Statistical analysis

The mortality data recorded in single-dose assays were converted to percent mortality and then transformed by arcsine transformation technique. One-way analysis of variance was used to test the significance and treatment means were separated by Tukey's multiple comparison test. The statistical analyses were performed using the MINITAB 18 computer program (Minitab Inc., PA, USA). The data from dose-response tests were analyzed using the Polo-PC probit package program and the LC_{50} and LC_{90} values, as well as confidence intervals, were calculated. The GenStat computer program (VSN International, Hemel Hempstead, UK) was used to perform principal component analysis.

Results

GC-MS analysis revealed 25, 20 and 24 compounds in Yubileina, Raya and Hemus EOs, respectively, and these represented 97.8, 97.1 and 97.2% of total EOs, respectively (Table 1).

Table 1. Essential oil components extracted from three lavender cultivars

RT[a]	RI[b]	RI Lit[c]	Essential oils component	Yubileina (% area)	Raya (% area)	Hemus (% area)	RI Lit.	IM[d]
4.98	956	954	camphene	-	0.3	0.3	Asuming et al., 2005	MS[e], RI[f]
5.46	980	981	1-octen-3-ol	0.4	0.4	1.9	Oliveira et al., 2006	MS, RI
5.62	987	988	3-octanone	3.4	2.1	-	Zhao et al., 2006	MS, RI
5.72	992	986	*α*-myrcene	1.0	1.2	1.1	Shang et al., 2002	MS, RI
5.80	996	994	3-Octanol	0.4	-	0.4	Juliani et al., 2004	MS, RI
6.60	1034	1032	limonen	0.6	-	0.8	Mevy et al., 2006	MS, RI
6.68	1037	1038	1,8-Cineole	1.4	1.1	0.5	Jalali-Heravi et al., 2006	MS, RI
6.75	1040	1040	*cis*-ocimene	1.6	1.4	2.1	Oliveira et al., 2006	MS, RI
7.00	1051	1050	*β*-ocimene	1.1	1.4	1.4	Karioti et al., 2003	MS, RI
7.64	1077	1078	linalool oxide	0.7	-	1.8	Liu et al., 2006	MS, RI
8.02	1092	1092	terpinolene	0.7	0.7	1.6	Novak et al., 2001	MS, RI
8.33	1105	1103	linalool	36.0	42.5	28.5	Bouzouita et al., 2003	MS, RI
8.54	1114	1112	1-octen-3-yl acetate	2.0	2.1	2.1	Benzo et al., 2007	MS, RI
9.48	1152	1153	camphor	0.4	-	0.6	Radulescu et al., 2004	MS, RI
10.01	1172	1171	borneol	2.4	-	2.7	Zeng et al., 2007	MS, RI
10.32	1183	1182	4-terpineol	-	-	7.7	Avato et al., 2004	MS, RI
10.54	1191	1192	cryptone	0.7	1.0	0.3	Lazarević et al., 2010	MS, RI
10.65	1195	1196	α-terpineol	5.4	5.5	6,0	Nickavar et al., 2002	MS, RI
11.58	1230	1229	nerol	1.1	0.6	1.3	Mevy et al., 2006	MS, RI
12.32	1255	1257	linalyl acetate	24.2	30,0	23.1	Quijano et al., 2007	MS, RI
13.19	1291	1293	lavandulyl acetate	5.9	3.3	6.6	Saroglou et al., 2006	MS, RI
15.07	1368	1364	neryl acetate	1.7	-	1.7	Saroglou et al., 2006	MS, RI
15.56	1379	1380	geranyl acetate	2.8	1.7	2.4	Bonaïti et al., 2005	MS, RI
16.59	1415	1419	caryophyllene	1.1	1.1	1.1	Benkaci-Ali et al., 2007	MS, RI
17.38	1461	1459	*β*-farnesene	1.2	0.6	-	Kundakovic et al., 2007	MS, RI
18.08	1478	1477	germacrene-D	0.4	0.4	-	Kundakovic et al., 2007	MS, RI
20.51	1590	1592	caryophyllene oxide	1.3	-	1.8	Kundakovic et al., 2007	MS, RI
21.78	1620	1623	*α*-cadinol	-	0.3	-	Pavlović et al., 2006	MS, RI
Monoterpene hydrocarbons				4.9	4.9	7.2		
Oxygenated monoterpenes				88.3	89.0	87.2		
Sesquiterpene hydrocarbons				2.7	2.10	1.1		
Oxygenated sesquiterpenes				1.3	0.3	1.8		
Total				97.8	97.12	97.2		

[a]RT, retention time (min); [b]RI, retention index; [c]RI Lit., RI of the compound at same GC column and similar GC-MS condition; [d]IM, Identification method; [e]MS; mass spectrometry match in database; and [f]RI, comparison of retention index from the literature.

It was concluded that the EOs of Hemus, Raya and Yubileina contain high amounts of linalool and linalyl acetate. It was found that Raya EOs contained 42.5% linalool whereas Yubileina and Hemus EOs contained 36.0 and 28.5% linalool, respectively. Similarly, the highest linalyl acetate content was determined in Raya EOs with 30.0%, followed by Yubileina and Hemus EOs with 24.2 and 23.1% contents, respectively. In addition, lavandulyl acetate content was determined in all three EOs, ranging from 3.33 to 6.59%.

Raya and Yubileina EOs generated a similar composition as pointed in Figure 1. Raya and Yubileina EOs have been closer to the center at the end of the biplot analysis.

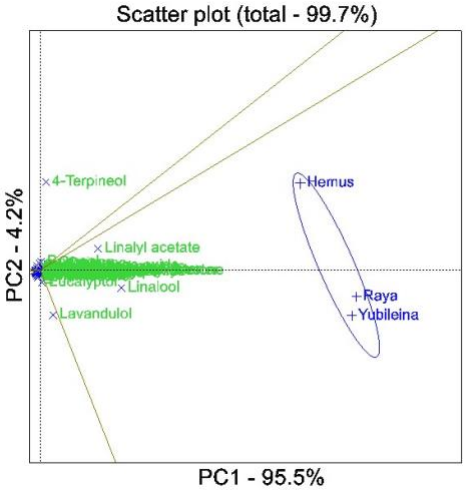

Figure 1. Principal component (PC) analysis of essential oil composition of lavender cvs Hemus, Raya, Yubileina.

As a result of the germination tests, no negative effects of EOs on wheat germination were observed. After 7 days, 100% germination was observed in all treatments.

Although there were significant differences between the EOs and their doses ($P < 0.05$) in terms of contact activity of the EOs evaluated in this study against *S. granarius* adults, the high-level effect was not observed (Table 2). The highest contact activity was determined at the 10% (v/v) application dose of Yubileina EOs with 27.9% ($F = 27.3$; $df = 6,28$; $P < 0.05$) and 62.2% ($F = 51.2$; $df = 6,28$; $P < 0.05$), respectively, after 24 and 48 h, while other EOs and doses did not show useful activity.

Table 2. Contact activities on *Sitophilus granarius* of essential oil obtain from lavender cvs Hemus, Raya, Yubileina

Treatment	Concentration (% v/v)	Mortality (%)±SEM	
		24 HAT	48 HAT
Control		0.0±0.0 c	0.0±0.0 c
Hemus	5	0.0±0.0 c	0.2±0.5 bc
	10	4.3±1.6 b	4.3±1.6 b
Raya	5	0.0±0.0 c	0.0±0.0 c
	10	1.8±0.7 bc	4.7±0.8 b
Yubileina	5	0.0±0.0 c	0.0±0.0 c
	10	27.9±0.1 a	62.2±0.4 a

Means followed by the same letter within a column are not statistically different (ANOVA $P < 0.05$ Tukey's test); HAT, hours after treatment; and SEM, standard error of the mean.

The fumigant effects of EOs isolated from three lavender cultivars showed similar fumigant effect on *S. granarius*, while the highest effect was shown in Yubileina EOs (Table 3). LC_{50} and LC_{90} values were 0.079 and 0.118 µl/ml air with Yubileina EOs and 0.094 and, 0.157 µl/ml air with Hemus EOs, 0.09, 0.139 µl/ml Raya EOs.

Table 3. The results of dose-response assays used to evaluate the fumigant activity of lavender cvs Hemus, Raya and Yubileina essential oils 24 h after treatment against *Sitophilus granarius*

Cutlivar	Slope±SE	LC_{50} (µl/ml air)	95% CI	LC_{90} (µl/ml air)	95% CI	χ2	Heterogeneity
Hemus	5.83±0.56	0.094	0.088-0.101	0.157	0.143-0.176	27.9	0.93
Raya	6.92±0.66	0.091	0.085-0.097	0.139	0.130-0.153	16.4	0.55
Yubileina	7.22±0.63	0.079	0.073-0.084	0.118	0.110-0.129	26.9	0.90

HAT, hours after treatment; SE, standard error; LC_{50}, 50% lethal concentration; LC_{90}, 90% lethal concentration; and CI, confidence interval.

Hemus, Raya and Yubileina EOs were in the same repellent group representing both doses, but the effects varied depending on the application time and dose. Similar to the fumigant effect, the highest average repellent activity was obtained with Yubileina EOs at 70 and 75% at both doses. In Raya and Hemus, 64 and 70% effect were at 0.25 µl/cm^2 dose, respectively, while 66 and 64% at 0.05 µl/cm^2 dose.

Table 4. Repellent effects of essential oil obtain from three lavender cultivars on *Sitophilus granarius*

Cultivar	Dose (µl/cm^2)	RA (%)±SE (95 % CI)			
		2 HAT	4 HAT	6 HAT	Mean (repellency score)
Raya	0.05	74.0±5.1 (52.3-95.7)	56.0±10.3 (34.9-77.1)	62.0±11.6 (44.2-79.8)	64.0 (4)
	0.25	64.0±16.9 (42.3-85.7)	64.0±8.7 (42.9-85.2)	70.0±7.7 (52.2-87.8)	66.0 (4)
Hemus	0.05	74.0±5.1 (52.3-95.7)	64.0±8.1 (42.9-85.2)	72.0±8.0 (54.2-89.8)	70.0 (4)
	0.25	64.0±11.7 (42.3-85.7)	72.0±12.4 (50.9-93.1)	56.0±6.8 (38.2-73.8)	64.0 (4)
Yubileina	0.05	78.0±10.2 (56.3-99.7)	62.0±8.6 (40.9-83.2)	86.0±6.0 (68.2-104)	75.3 (4)
	0.25	74.0±9.3(52.3-95.7)	72.0±12.4 (50.9-93.1)	64.0±10.3 (46.2-81.8)	70.0 (4)

HAT, hours after treatment; RA, repellent activity; and CI, Confidence interval.

Discussion

The main EOs components of lavender were 28.5-42.5% linalool and 23.1-30.0% linalyl acetate, which is consistent with earlier studies (Smigielski et al., 2018; Najibullah et al., 2021). Linalool, linalyl acetate, 1,8-cineole and borneol have already been identified as important components of EOs of various lavender cultivars, although in varying proportions (Cosimi et al., 2009; Fouad et al., 2021). It has been reported that there can be high variability in the component contents of lavender EOs and that some compounds such as linalyl acetate can be detected at highly variable concentrations depending on the production area and cultivar (Dušková et al., 2016). It is also known that biotic and abiotic factors affect the chemical composition of the plant species (Fernández-Sestelo & Carrillo, 2020) and the chemical composition can vary according to the part of the plant used (Smigielski et al., 2018). The other factors affecting chemical composition are the distillation time and method.

There are many studies on the potential use of plant EOs on controlling them against pests (Koul et al., 2008; Lopez et al., 2008). In this study, the possibilities of using lavender cultivars in the control of *S. granarius* were investigated. The biological activities of EOs of lavender species, as well as some of its main components, against stored product pests have been evaluated by different researchers (Al-Ansari et al., 2021; Al-Harbi et al., 2021; Fouad et al., 2021). Considering the results of contact activity, Yubileina EOs, whose main components were determined as linalool and linalyl acetate, showed the highest contact activity at the end of 48 h. However, no significant effect was detected with Hemus and Raya EOs, which have the same main components. This suggests that the activity detected in Yubileina EOs may be due to other compounds. Similar results were obtained with other insect species and EOs. Many researchers found that the same plant EOs or extract from the same genus, as well as various types of insects, react differently to these varying quantities (Guo et al., 2017, Liang et al., 2017).

Lavender cultivars used in this study showed fumigant activity against *S. granarius*, in parallel with the results of other studies against various stored product pests (Al-Ansari et al., 2021; Al-Harbi et al., 2021). Kheloul et al. (2020) reported that *L. angustifolia* EOs showed significant fumigant activity against *S. granarius* and the LC_{50} value was 1.57 mg/l. In addition, it has been reported that the main components of the EOs of *L. angustifolia* are linalool (23.8%), 1,8-cineole (12.0%) and borneol (10.7%). Similarly, the amount of linalool (28.5-42.5%) detected as the main component with a higher area, but 1,8-cineole was not detected in our study. Between the *L. angustifolia* cvs, borneol in Hemus (2.72%) and Yubileina (2.41%) EOs was lower, but not in Raya. Previous studies indicated that linalool or linalool-rich EOs have fumigant activity against stored product insects (Yang et al., 2014; Zhou et al., 2012). Our findings have confirmed these results.

Lavender EOs showed significant repellent activity against *S. granarius* depending on the application dose and time. There are many studies in parallel with this study, in which the effect of repellent activities of EOs obtained from plants in the Lamiaceae with storage pests were investigated (Mishra et al., 2012; Moazeni et al., 2014). The repellent activity of 10 plant EOs, including *L. angustifolia* Mill., was evaluated against *Sitophilus oryzae* (L., 1763) (Coleoptera: Curculionidae) and the EOs of *L. angustifolia* was the oil with the third highest repellent activity (Jayakumar et al., 2017). Similarly, Sabbour & El-Aziz (2020) were evaluated the repellent activity of lavender, *Nepeta* sp., *Geranium* sp. (Geraniaceae) and *Chamaemelum* sp. (Asteraceae) EOs against *Tribolium confusum* Jacquelin du Val, 1863 and *Tribolium castaneum* (Herbst, 1797) (Coleoptera: Tenebrionidae). They showed that the highest repellent activity in geranium EOs and reported that the repellent activity of this oil was followed by the activity of lavender EOs. However, there are conflicting results about which component provides the repellent activity. Fouad et al. (2021) reported that linalool, which was the most abundant component in our study, did not have a significant repellent activity, but eα-pinene, which was not detected in our study, showed significant repellent activity. Linalyl acetate was the second most abundant component detected in this study. Cosimi et al. (2009) was reported that *Citrus bergamia* Risso (Rutaceae) and *Lavandula* hybrid EOs rich in linalool and linalyl acetate showed significant repellent activity against *Sitophilus zeamais* (Motschulsky), 1855 (Coleoptera: Curculionidae). Repellent activity of linalyl acetate was also determined for *Rhodnius prolixus* (Sfara et al., 2009). Germinara et al. (2017) was also reported that the EOs of *L. angustifolia*, in which 23.8% linalool, 6.90% linalyl acetate, 12.0% 1,8-cineole and 10.7% borneol were detected, showed significant repellent activity against *S. granarius*. In our study, the components of the EOs we used were similar in terms of linalool content, while the linalyl acetate content was found at a high level, the borneol content at a very low level and 1,8-cineole was not detected. Obtaining similar results at different component densities suggests that the repellent activity is not caused by a single chemical component in plant EOs, but rather multiple EOs components act together on the repellant activity.

Studies on the use of EOs in controlling agricultural pests have recently gained momentum. In parallel with the high level of toxicity of pesticides used in pest control and increasing consumer awareness, interest in the use of agents derived from natural or natural products is increasing. One of the limiting factors for the use of EOs for pest control is their low stability in open areas and field conditions. The decrease in fumigant activities due to low vapor pressures is another factor limiting the use of EOs. However, this can be eliminated with new formulation studies such as slow-release mechanisms and propellant applications. Studies with EOs are mostly aimed at determining the biological activity of the total EOs composition and these studies will gain more meaning with studies on the determination of the biological activities of the main components and their purification. It is extremely important to increase the number of these studies in order for the biomolecules of EOs, whose effectiveness has been demonstrated, to be an alternative to synthetic fumigants. In this study, the insecticidal and behavioral of EOs obtained from lavender cultivars that can be produced commercially were tested against *S. granarius* under laboratory conditions. The results of the experiment show that these oils have a considerable potential for pest control. However, in order for this potential to be realized, application method and formulation need to be optimized.

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Original article (Orijinal araştırma)

Bioactivity of a betabaculovirus, *Hyphantria cunea* granulovirus, in six lepidopteran insects as potential hosts[1]

Betabaculovirüs, *Hyphantria cunea* granulovirüs'ün potansiyel konukçu olarak altı lepidopter böcekteki biyoaktivitesi

Zeynep BAYRAMOĞLU[2] **Dönüş GENÇER[3]** **İsmail DEMİR[4*]**

Abstract

The aim of this study, conducted in 2018 and 2020, was to investigate the bioactivity of a local baculovirus isolate, *Hyphantria cunea* granulovirus (HycuGV), in seven lepidopteran pests. Based on data collected 10 days after exposure, HycuGV was found to infect *Malacosoma neustria* (L., 1758) (Lepidoptera: Lasiocampidae), *Lymantria dispar* (L., 1758) (Lepidoptera: Erebidae), *Helicoverpa armigera* (Hübner, 1805) (Lepidoptera: Noctuidae) and *Spodoptera exigua* (Hübner, 1808) (Lepidoptera: Noctuidae) larvae as well as its host *Hyphantria cunea* (Drury, 1773) (Lepidoptera: Erebidae). However, it did not infect *Spodoptera littoralis* (Boisduval, 1883) (Lepidoptera: Noctuidae) and *Cydia pomonella* (L., 1758) (Lepidoptera: Tortricidae). A HycuGV dose rate experiment indicated LC_{50} of $4.7x10^5$ occlusion bodies (OBs)/ml in *H. cunea*, $5.6x10^6$ OBs/ml in *L. dispar*, $7x10^7$ OBs/ml in *S. exigua*, $1.5x10^9$ OBs/ml in *M. neustria* and $7.7x10^9$ OBs/ml in *H. armigera*. HycuGV was infectious to *S. exigua* and *L. dispar*, but only provided effective control in *M. neustria* and *H. armigera* at high dose rates. These findings demonstrate that HycuGV can be highly effective for control of *S. exigua*, *L. dispar* and *H. cunea*.

Keywords: Baculovirus, bioactivity, biological control, host range, *Hyphantria cunea* granulovirus

Öz

2018 ve 2020 yıllarında yürütülen bu çalışmanın amacı, yerel bir bakülovirüs izolatı *Hyphantria cunea* granulovirus (HycuGV)'ün yedi lepidopter zararlısı üzerindeki biyoaktivitesinin araştırılmasıdır. Denemeden sonraki 10. günde elde edilen verilere göre HycuGV'nin kendi konukçusu olan *Hyphantria cunea* (Drury, 1773) (Lepidoptera: Erebidae)'nın yanı sıra *Malacosoma neustria* (L., 1758) (Lepidoptera: Lasiocampidae), *Lymantria dispar* (L., 1758) (Lepidoptera: Erebidae), *Helicoverpa armigera* (Hübner, 1805) (Lepidoptera: Noctuidae) ve *Spodoptera exigua* (Hübner, 1808) (Lepidoptera: Noctuidae) larvaları üzerinde de enfeksiyon oluşturma kabiliyetine sahip olduğu belirlenirken, *Spodoptera littoralis* (Boisduval, 1883) (Lepidoptera: Noctuidae) ve *Cydia pomonella* (L., 1758) (Lepidoptera: Tortricidae) larvalarında enfeksiyon oluşturmadığı tespit edildi. HycuGV'nin doz denemelerinde LC_{50} değeri, *H. cunea*'da $4.7x10^5$ OBs/ml, *L. dispar*'da $5.6x10^6$ OBs/ml, *S. exigua*'da $7x10^7$ OBs/ml, *M. neustria*'da $1.5x10^9$ OBs/ml ve *H. armigera*'da $7.7x10^9$ OBs/ml olarak hesaplandı. Bu sonuçlar HycuGV'nin *S. exigua* ve *L. dispar* için bulaşıcı olduğunu, ancak *M. neustria* ve *H. armigera*'da ise yüksek doz oranlarında etkili olduğunu gösterdi. Bu bulgular, HycuGV'nin *S. exigua*, *L. dispar* ve *H. cunea*'nın mücadelesi için oldukça etkili olabileceğini göstermektedir.

Anahtar sözcükler: Bakülovirüs, biyoaktivite, biyolojik mücadele, konukçu aralığı, *Hyphantria cunea* granulovirus

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[2] Recep Tayyip Erdoğan University, Pazar Vocational School, Department of Plant and Animal Production, 53300, Pazar, Rize, Turkey
[3] Trabzon University, Şalpazarı Vocational School, Department of Property Protection and Security, 61670, Şalpazarı Trabzon, Turkey
[4] Karadeniz Technical University, Faculty of Science, Department of Biology, 61080, Trabzon, Turkey
* Corresponding author (Sorumlu yazar) e-mail: idemir@ktu.edu.tr
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Introduction

Baculoviruses are generally characterized by a narrow host range, which can be important when compared to synthetic chemical insecticides or other microbial control agents. However, there are significant differences between Baculoviridae host ranges. Due to limited knowledge of their host ranges, it is difficult to predict the infectiveness of a particular isolate under laboratory conditions, let alone in the field (Cory & Entwistle, 1990; Cory et al., 1997).

Baculoviruses have been isolated from more than 700 insect species in the Lepidoptera, Hymenoptera and Diptera (Moscardi, 1999; Herniou & Jehle, 2007). They are often preferred as agents for integrated pest management due to their high specificity (Ahmad et al., 2011). Baculoviruses are categorized into two groups based on morphology: nucleopolyhedrovirus (NPV) and granulovirus (GV). The Baculoviridae family is divided into four genera: *Alphabaculovirus* (nucleopolyhedroviruses isolated from Lepidoptera), *Betabaculovirus* (nucleopolyhedroviruses isolated from Lepidoptera), *Gammabaculovirus* (nucleopolyhedroviruses isolated from Hymenoptera) and *Deltabaculovirus* (nucleopolyhedroviruses isolated from Diptera) (Jehle et al., 2006). More than 150 insect species (Lepidoptera) are known to be susceptible to granuloviruses (Rohrmann, 2013).

Host range information is important for determining the properties of a microbial biocontrol agents. The host ranges of NPVs were reported to be broader than GVs (Ignoffo, 1968); however, one recent study did not support this view (Hamm, 1982). Although cross-infection tests are commonly used in the study of baculoviruses, comparative test data are rarely reported for different hosts. Nevertheless, knowing the effect of baculoviruses on different hosts is important for recommending the deployment of microbial biocontrol agents. Standard bioassays are needed to determine the host range and specificity of NPVs (Cory et al., 1997). NPVs are prevalent among 400 arthropod species in seven insect genera (Murphy et al., 1995). Mostly, the host range of NPVs are limited to one or more genera, or the host species from which it was isolated (Moscardi, 1999). Host range and cross infectivity of baculoviruses have been reviewed by Gröner (1986). The infectivity of NPV and GV in alternate hosts was typically determined on virus infection and mortality of the test larvae after oral virus application. However, these examinations are biased towards Lepidopteran species and other economically important insects. To date, no standardized bioassays have been developed to determine the host range and specificity of baculoviruses (Cory, 2003).

Baculoviruses are often reported to have various host ranges. Certain baculoviruses such as *Autographa californica multicapsid nucleopolyhedrovirus* and *Mamestra brassicae multicapsid nucleopolyhedrovirus* have broad host ranges and are infectious in certain insect species in different families. Also, the host range of certain baculoviruses, such as *Spodoptera exigua multiple nucleopolyhedrovirus*, are limited to a single (or a few) insect species (Federici, 1997; Goulson, 2003). High host specificity provides advantages for biocontrol; however, it limits the commercialization of baculoviruses as bioinsecticides. The production of a baculovirus with a broad host range is more economically attractive compared to producing many host-specific baculovirus that control only one (or a few) target species (Haase et al., 2015).

Host range is important in the determination of the persistence of the baculovirus isolates in an ecosystem based on the presence of primary and alternative hosts. Similarly, a broad host range is significant for the development of effective commercial biocontrol agents (Brodeur, 2012).

Hyphantria cunea granulovirus (HycuGV) has been isolated from *Hyphantria cunea* (Drury, 1773) (Lepidoptera: Erebidae) larvae and characterized by Bayramoglu et al. (2018) and Gencer et al. (2020). The aim of present study is to investigate the bioactivity and the host ranges of this virus in a range of pest species: *Malacosoma neustria* (L., 1758) (Lepidoptera: Lasiocampidae), *Lymantria dispar* (L., 1758) (Lepidoptera: Erebidae), *Helicoverpa armigera* (Hübner, 1805) (Lepidoptera: Noctuidae), *Spodoptera littoralis* (Boisduval, 1883) (Lepidoptera: Noctuidae), *Spodoptera exigua* (Hübner, 1808) (Lepidoptera: Noctuidae) and *Cydia pomonella* (L., 1758) (Lepidoptera: Tortricidae).

Materials and Methods

Virus preparation

A single isolate of Betabaculovirus (HycuGV) was obtained from *H. cunea* larvae and identification, phylogeny, biological activity and whole-genome sequence determined in previous studies (Bayramoglu et al., 2018; Gencer et al., 2020). The present study was conducted at Karadeniz Technical University, Department of Biology between 2018 and 2020. For propagation of the virus, *H. cunea* larvae collected from the field in Trabzon and Rize Provinces, Turkey and then cultured in the laboratory for use in bioassays. Fresh mulberry leaves (*Morus* sp., Rosales: Moraceae) were surface sterilized initially with 70% ethanol then 2% sodium hypochlorite before rinsing with sterile dH_2O. A suspension of $1x10^7$ occlusion bodies (OBs)/ml HycuGV was applied to the leaves along with 100 *H. cunea* third-stage larvae. The larvae infected with HycuGV were collected and the viruses were reisolated by the method of Opoku-Debrah et al. (2013). Individual infected larvae were homogenized in 1 ml 0.1% sodium dodecyl sulfate (SDS) and filtered through cheesecloth. An equal volume of 0.1% SDS was added and the process was repeated. The resulting filtrate was centrifuged at 7,840 × g for 30 min at 4°C. The supernatant was discarded and the pellet suspended in dH_2O. A sucrose gradient (30-80% w/v) was prepared in Beckman Avanti centrifuge tubes (Beckman Coulter Inc., CA, USA) and kept overnight at 4°C. Test virus suspensions was loaded on top of a sucrose gradient and centrifuged for 30 min at 29,774 × g at 4°C in a Beckman Avanti (J-30I) ultracentrifuge. The visible OB band in the middle of the tube was extracted using a pipette, placed into a new sterile tube, filled to the brim with dH_2O and centrifuged again for 30 min at 29,774 × g at 4°C. The final pellet was resuspended with dH_2O and the OBs were quantified visually with a Neubauer hemocytometer under a phase-contrast microscope (Nikon Eclipse LH-M100C1, Tokyo, Japan) at 400X. The purified HycuGV stock was stored at -20°C until the bioassays were conducted.

Insects

The third larval stages of *H. cunea*, *M. neustria* (L., 1758) (Lepidoptera: Lasiocampidae), *S. exigua* (Hübner, 1808), *S. littoralis* (Boisduval, 1883) (Lepidoptera: Noctuidae), *C. pomonella* (L., 1758) (Lepidoptera: Tortricidae), *H. armigera* (Hübner, 1805) (Lepidoptera: Noctuidae) and *L. dispar* (L., 1758) (Lepidoptera: Erebidae) larvae were tested in bioassays. *Spodoptera exigua*, *S. littoralis* and *H. armigera* were cultured on an artificial diet under laboratory conditions at 60-70% RH and 16:8 h L:D photoperiod at 26±1°C. The other (*M. neustria*, *L. dispar* and *C. pomonella*) larvae were collected in Gümüşhane, Bingöl and Trabzon, and fed with fresh rose leaves (*Rosa canina* L., Rosales: Rosaceae), oak leaves (*Quercus petraea* L., Fagales: Fagaceae) and apples (*Malus domestica* Borkh, Rosales: Rosaceae), respectively. Only apparently healthy larvae were selected for inclusion in the bioassays.

Insecticidal activity trials on the hosts

The pathogenicity of HycuGV was tested at five concentrations (10^{5-9} OBs/ml) on the seven-lepidopteran species (*H. cunea*, *M. neustria*, *S. exigua*, *S. littoralis*, *C. pomonella*, *H. armigera* and *L. dispar*). Thirty third-stage larvae were used in tests and all tests were repeated three times at different times. For bioassays, five concentrations ($1x10^5$, 10^6, 10^7, 10^8 and 10^9 OBs/ml) were applied on 3 x 3 cm leaf disks and artificial diet disks. Larvae incubated in a 16:8 h L:D photoperiod at 26±1°C and those that consumed the entire disks were supplied with fresh food as needed. The control groups for each pest species, only water was added to each leave and placed into box. Mortality was recorded daily for 10 days. Dead larvae were examined for symptoms of viral infection and then the presence of viral structures under a phase-contrast microscope.

Confirmation of HycuGV infection with polymerase chain reaction

HycuGV infection in dead larvae (*M. neustria*, *L. dispar*, *H. armigera* and *S. exigua*) was confirmed using a PCR kit (Phire Animal Tissue Direct PCR Kit, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. The presence of granulovirus was confirmed by polymerase chain reaction (PCR) using granulin primers (forward, ATG GGA TAY AAC ARA KCW YTR MGK TAY AGY MRH CAC, and reverse, TTA RTA VGC BGG DCC DGT RWA YAR WGG YAC RTC). The PCR products were analyzed on 1% agarose gel electrophoresis. The bands detected after PCR were considered positive for insect infection.

Statistical analysis

Mortality data were corrected by Abbott's formula (Abbott, 1925). Lethal concentrations (LC_{50}) for the virus against third-stage larvae of hosts were calculated by probit analysis using MS Excel (Finney, 1952).

Results

The mortalities of all insects infected with HycuGV are shown in Figure 1. The highest mortalities with HycuGV treatment were 90, 80 and 70% for *H. cunea, L. dispar* and *S. exigua*, respectively, at the 10^9 OBs/ml. Although, *L. dispar* larvae had a mortality, they do not have the soft body tissues like the other host larvae (Figure 2). The mortalities were similar for *M. neustria* (46%) and *H. armigera* (33%) larvae but low than the *L. dispar* and *S. exigua*. Based on the results, it was determined that HycuGV can infect and kill *H. cunea, M. neustria*, *L. dispar*, *H. armigera* and *S. exigua* larvae to different degrees. However, it was not lethal to *S. littoralis* and *C. pomonella* hosts. The LC_{50} values calculated in the experiments are presented in Table 1. The LC_{50} calculated by probit analysis were lower at $5.6x10^6$ OBs/ml for L. dispar and $4.7x10^5$ OBs/ml for *H. cunea* host, but higher in *S. exigua*, *M. neustria* and *H. armigera* at 7.0, 1.5 and $17.7x10^9$ OBs/ml, respectively.

During the bioassays, viral disease symptoms such as the soft body tissues were observed in dead larvae (Figure 2). Dead tissue samples were examined under a phase-contrast microscopy observing GV structures and PCR confirmed granulin gene region amplification bands (~800 bp) (Figure 3). Control groups of each insect species remained in healthy throughout the bioassay period.

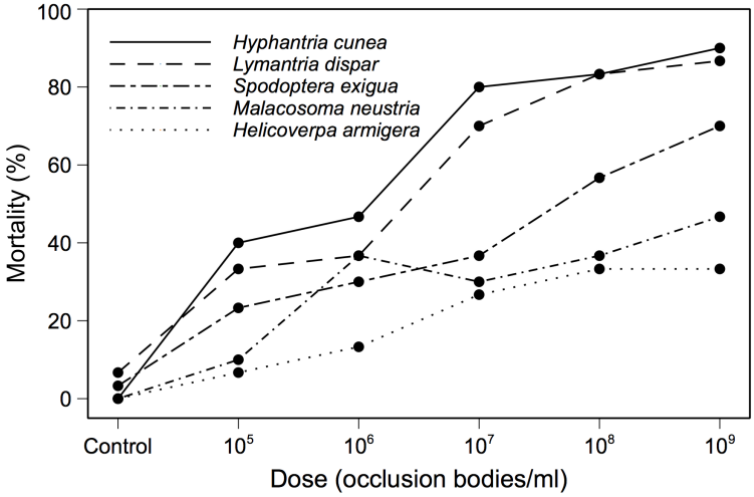

Figure 1. Mortality of insect larvae resulting from HycuGV application.

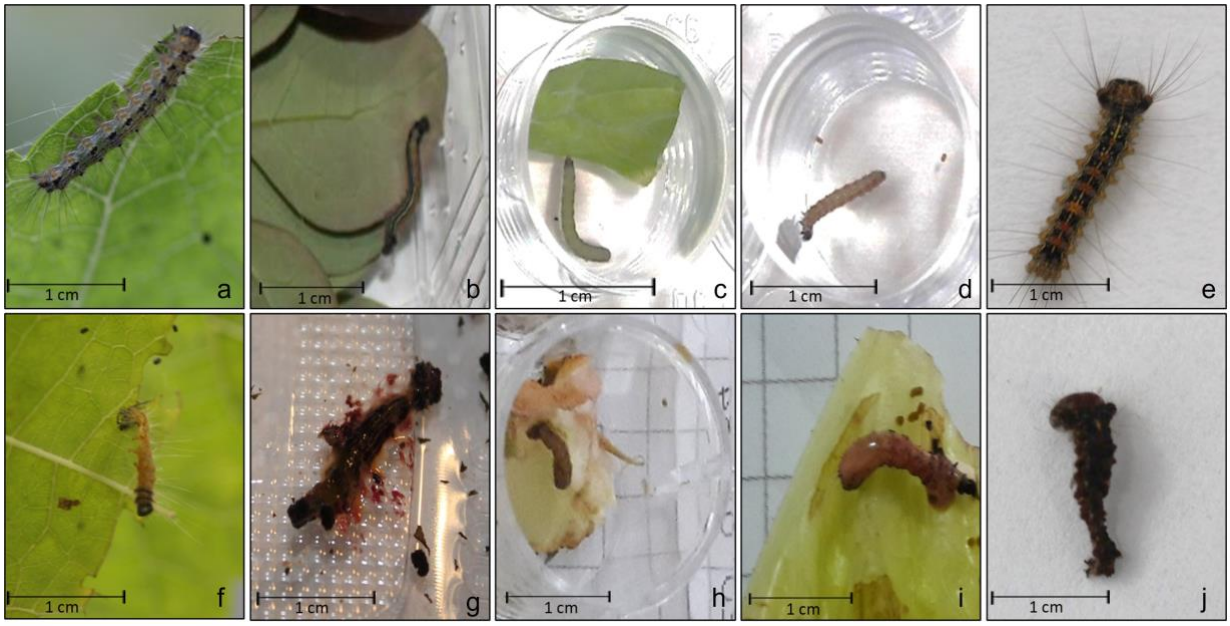

Figure 2. Pre- and post-*Hyphantria cunea* granulovirus treated larvae: a) *Hyphantria cunea*, b) *Malacosoma neustria*, c) *Spodoptera exigua*, d) *Helicoverpa armigera*, and e) *Lymantria dispar* healthy larvae; f) *Hyphantria cunea*, g) *Malacosoma neustria*, h) *Spodoptera exigua*, i) *Helicoverpa armigera*, and j) *Lymantria dispar* infected larvae.

Table 1. Median lethal concentrations (LC_{50}) of HycuGV on seven lepidopteran pests

Hosts	LC_{50} (OBs/ml)	Slope±*SE*	LC_{95} (OBs/ml)	*df*	X^2
Helicoverpa armigera	$7.7x10^9$ ($2.9x10^8$ - $2x10^{11}$)	0.324±0.521	$5.3x10^{15}$	3	0.847
Malacosoma neustria	$1.5x10^9$ ($4.3x10^7$ - $5.4x10^{10}$)	0.237±0.764	$2.9x10^{16}$	3	0.446
Lymantria dispar	$5.6x10^6$ ($5.6x10^5$ - $3.3x10^7$)	0.471±0.452	$4.0x10^{10}$	3	0.893
Spodoptera exigua	$7x10^7$ ($5.2x10^6$ - $9.3x10^8$)	0.345±0.578	$8.3x10^{12}$	3	0.970
Hyphantria cunea	$4.7x10^5$ ($5.5x10^4$ - $4x10^6$)	0.427±0.480	$4.7x10^9$	3	0.961

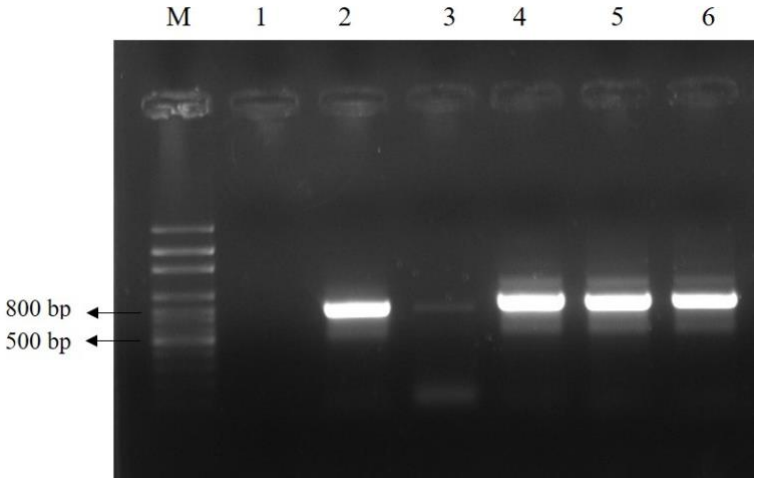

Figure 3. Agarose gel image of the granulin gene region (~800 bp) obtained from dead larvae by PCR. (M: 1 kb; 1: negative control; 2: positive control (from *Hyphantria cunea granulovirus* DNA sample); 3: from *Malacosoma neustria* cadaver; 4: from *Lymantria dispar* cadaver; 5: from *Spodoptera exigua* cadaver; 6: from *Helicoverpa armigera* cadaver).

Discussion

We conducted bioassays with *H. cunea*, *M. neustria*, *S. exigua*, *S. littoralis*, *C. pomonella*, *H. armigera* and *L. dispar* larvae to obtain a better insight into the of host range of HycuGV. Different rates of mortality were determined for *L. dispar*, *S. exigua*, *M. neustria* and *H. armigera*, but *S. littoralis* and *C. pomonella* were not susceptible to this virus. The mortality of *H. cunea* was higher than the others. Other studies have investigated the effects of HycuGV on some other hosts (Vasiljevic, 1968; Ignoffo, 1968; Hukuhara et al., 1969; Vaughn, 1974; Tomita & Ebihara, 1982). Vasiljevic (1968) examined the effect of GV from *H. cunea* on *Bombyx mori* (L., 1758) (Lepidoptera: Bombycidae) and *Pieris rapae* (L., 1758) (Lepidoptera: Pieridae) with HycuGV lethal to only *P. rapae* larvae. Vasiljevic (1968) also reported that HycuGV was infective *P. rapae* larvae but not to domestic *B. mori* larvae. Tomita & Ebihara (1982) tested HycuGV on eight species, *Euproctis pseudoconspersa* (Strand, 1923) (Lepidoptera: Erebidae), *Euproctis similis* (Füssli, 1775) (Lepidoptera: Erebidae), *Numenes disparilis* (Staudinger, 1887) (Lepidoptera: Erebidae), *Clostera anastomosis tristis* (L., 1758) (Lepidoptera: Notodontidae), *Diaphania pyloalis* (Hampson, 1859) (Lepidoptera: Crambidae), *B. mori*, *Spilarctia subcarnea* (Walker, 1855), *Lemyra imparilis* (Butler, 1877) (Lepidoptera: Erebidae) and *H. cunea*. The lepidopteran species, *S. subcarnea* and *L. imparilis*, in the same family (Erebidae) as *H. cunea*, were found to be susceptible to HycuGV. However, HycuGV was not lethal to *E. pseudoconspersa*, *N. disparilis albofascia*, *E. similis*, *C. anastomosis tristis*, *D. pyloalis* and *B. mori* (Tomita & Ebihara, 1982). In the present study, HycuGV provided useful control of *L. dispar* larvae, also in the Erebidae. Similarly, Hukuhara et al. (1969) reported that HycuGV was not lethal to *B. mori*. Ignoffo (1968) and Vaughn (1974) also reported that HycuGV is highly host specific.

Given these results, it is clear that the host range of HycuGV is not limited to a single species and this should be further investigated. In the present study, it was determined that the impact of HycuGV on *L. dispar* and *S. exigua* was higher than on *M. neustria* and *H. armigera*. Therefore, HycuGV could potentially be used as a biocontrol agent for *L. dispar* and *S. exigua*. HycuGV had some impact on *M. neustria* and *H. armigera,* however, but a higher dose was required for 50% mortality. Thus, the effect of HycuGV on these two insects was limited. No effect was observed on *S. littoralis* and *C. pomonella*. The two *Spodoptera* species used in this study had quite different mortality when exposed to HycuGV. HycuGV gave more than 80% mortality of *S. exigua* but did not affect *S. littoralis*. This may be because *S. littoralis* is a more highly resistant species. Some studies have shown that *S. littoralis* larvae are resistant to baculovirus infection (Riwkin et al., 2006; Ghulam et al., 2017).

GV infections have been recorded in more than 100 insects, but only in Lepidoptera (Murphy et al., 1995). Unlike NPVs, the host range of the GVs is narrower and mostly limited to a single species. In several studies, baculoviruses were tested in various species without any pathogenicity observed (Del Rincon-Castro & Ibarra, 1997). In the present study, it was determined that HycuGV was a weaker pathogen of *M. neustria*, *S. littoralis*, *C. pomonella* and *H. armigera* larvae than the species from which it was originally isolated. In contrast, however, *L. dispar* and *S. exigua* larvae were susceptible to HycuGV infection.

In conclusion, it was demonstrated that HycuGV was infectious and lethal to various lepidopteran species in addition to its source host. These results show that HycuGV has a wider host range confirming some earlier studies.

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Original article (Orijinal araştırma)

Diversity and plant interactions of aphids (Hemiptera: Aphidomorpha) adjacent to Çardak Lagoon with new aphid and host records for Turkey

Türkiye için yeni afit ve konukçu kayıtları ile birlikte Çardak Lagün alanındaki afitlerin (Hemiptera: Aphidomorpha) çeşitliliği ve bitki etkileşimleri

Şahin KÖK[1*]

Abstract

This study aimed to reveal the diversity of aphid species and aphid-plant interactions adjacent to Çardak Lagoon, which is close to agricultural areas in Çanakkale Province of Turkey. Twenty-seven aphid species belonging to 17 genera in three subfamilies from the Aphididae (Hemiptera) were identified. Of these, *Staticobium latifoliae* (Bozhko, 1950) is new record from the genus *Staticobium* Mordvilko, 1914 and *Aphis symphyti* Schrank, 1801 are new records for the aphid fauna of Turkey. With these new records, the number of the aphid fauna of Turkey has increased to 596 species from 149 genera in the infraorder Aphidomorpha (Hemiptera). Of the hosts, *Geranium pusillum* L., *Geranium columbinum* L. (Geraniaceae), *Anagallis arvensis* L. (Primulaceae), *Polygonum maritimum* L. (Polygonaceae), *Myosotis* sp. (Boraginaceae), *Anthriscus caucalis* M. Bieb. (Apiaceae), *Raphanus raphanistrum* L. (Brassicaceae), *Anthemis* sp. (Asteraceae) and *Silene* sp. (Caryophyllaceae) are new hosts for the aphids in Turkey, respectively. Results of this detailed study conducted adjacent to the lagoon which has a diversity of hosts native to saline and sandy soils for the first time in Turkey provides important data on the diversity of aphids and the interactions with their hosts, and that this data will contribute a better understanding of aphid-plant interactions in agricultural and non-agricultural habitats as well as the biology and control strategies of host-alternate aphid pests.

Keywords: Aphid, Çanakkale, aphid-plant interactions, lagoon, Turkey

Öz

Bu çalışma 2020 ve 2021 yıllarında Nisan-Eylül arasında Türkiye'nin Çanakkale İli'nde tarım alanlarına yakın bir bölgede yer alan Çardak Lagününde bulunan afit türlerinin çeşitliliği ve afit-bitki etkileşimlerini ortaya çıkarmayı amaçlamaktadır. Aphididae (Hemiptera) familyasından üç altfamilya içerisinde 17 cinse ait 27 afit tespit edilmiştir. Bu türlerden, *Staticobium* Mordvilko, 1914 cinsinden yeni kayıt olan *Staticobium latifoliae* (Bozhko, 1950) ve *Aphis symphyti* Schrank, 1801 Türkiye afit faunası için yeni kayıtlardır. Bu yeni kayıtlar ile birlikte Türkiye afit faunası Aphidomorpha (Hemiptera) alttakımı içerisinde 149 cinse ait 596 türe yükselmiştir. Konukçulardan, *Geranium pusillum* L., *Geranium columbinum* L. (Geraniaceae), *Anagallis arvensis* L. (Primulaceae), *Polygonum maritimum* L. (Polygonaceae), *Myosotis* sp. (Boraginaceae), *Anthriscus caucalis* M. Bieb. (Apiaceae), *Raphanus raphanistrum* L. (Brassicaceae), *Anthemis* sp. (Asteraceae) ve *Silene* sp. (Caryophyllaceae) Türkiye'de afitler için yeni konukçu kayıtları olarak tespit edilmiştir. Türkiye'de ilk kez tuzlu ve kumlu topraklara özgü konukçu çeşitliliğine sahip bir lagünde yürütülen bu detaylı çalışmanın sonuçları afitlerin çeşitliliği ve onların konukçuları ile etkileşimleri üzerine önemli veriler sağlamaktadır. Bu veriler tarım ve tarımdışı habitatlardaki afit-bitki etkileşimlerinin daha iyi anlaşılmasının yanı sıra konukçu değişimi gösteren zararlı afitlerin biyoloji ve kontrol stratejilerine katkı sağlayacaktır.

Anahtar sözcükler: Afit, Çanakkale, afit-bitki etkileşimleri, lagün, Türkiye

[1] Çanakkale Onsekiz Mart University, Lapseki Vocational School, Department of Plant and Animal Production, 17800, Çanakkale, Turkey
* Corresponding author (Sorumlu yazar) e-mail: sahinkok@gmail.com
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Introduction

Aphids (Hemiptera: Aphidomorpha) are one of the most important pest groups that can cause serious economic losses on agricultural crops throughout the world. Aphids can damage crops by sucking plant sap, causing gall and deformities from toxins in their saliva, causing sooty mold due to secreted honeydew and by transmitting more than 270 plant viruses that cause serious economic damage to agricultural crops (Katis et al., 2007). All crops are known to be a host for at least one pest aphid (Peters et al., 1991). About 40% of aphid species live on trees, the other 55% prefer to feed on flowering herbaceous plants and shrubs (the host selection of the remaining 5% is unknown). Consequently, it is known that aphids feed on hosts in 300 families (Blackman & Eastop, 2021). Several studies, including Toros et al. (2002), Özdemir et al. (2005), Görür et al. (2012), Kanturski et al. (2014, 2018), Şenol et al. (2015), Kök et al. (2016), Kök & Kasap (2019), Özdemir (2020), have identified more species, hereby increasing the number of aphid species in Turkey to a total of 596 aphid species, 26 of which are subspecies, belonging to 149 genera (Kök & Özdemir, 2021; Patlar et al., 2021).

Aphids are an insect group commonly known to specialize on a particular host even if they have a range of possible hosts. Aphids can show different responses to different habitats such as agricultural, non-agricultural, urban and landscape areas in terms of their diversity, species richness, abundance, plant interactions and diversity of natural enemies (Janković et al., 2017; Kök et al., 2020; Barczak et al., 2021). Also, non-crop contexts such as stubble and pasture areas, roadsides, urban and landscape areas are important as refuge habitats for aphids with a migratory life cycle (Wilkaniec et al., 2015). Among these areas, lagoons are defined as semi-connected coastal ponds located on the edge of seas with a large coastal zone; they are also considered as geographical heritage due to their rare geomorphological formations (Kocataş, 1999; Doğaner, 2003). Lagoons are biological and economic rich at points where aquatic and terrestrial ecosystems converge (Viaroli et al., 2007). Çardak Lagoon in Çanakkale Province, Turkey, particularly, is of great importance as it is close to agricultural areas producing fruits, vegetables and cereal crops, and the plant diversity found there as potential secondary hosts to migrating aphids. Considering this situation, aphid diversity and aphid-plant interactions not only in agricultural areas, but also in non-agricultural areas should be examined in detail.

Aphids have a rich diversity and distribution in both agricultural and non-agricultural areas due to their host and non-host-alternating life cycles. Today, about 15% of aphids, most of which are polyphagous pest species, have a host-alternating life cycle (Blackman & Eastop, 2007). Detailed examination of host-alternating aphid species and their plant interactions within agricultural and nearby non-agricultural areas is important in terms of understanding the biology, damage and population densities of such aphids as well as their control strategies on agricultural crops. In this study, it is hypothesized that the vegetation adjacent to a coastal lagoon, consisting mostly halophytic and dune plants, and close to agricultural areas can contribute to the local diversity and number of aphid fauna of Turkey and it can contain secondary hosts for migrating aphid pests. In this context, this study aimed to reveal the diversity of aphid species and aphid-plant interactions adjacent to Çardak Lagoon, which is close to agricultural areas producing fruit, vegetables and cereal crops in Çanakkale Province, Turkey.

Materials and Methods

Sampling site

Çardak Lagoon is located on the northeastern shore of the Dardanelles Strait, which separates the European and Asian continents. Coordinates of this lagoon are 40°22'36"-40°23'36" N and 26°42'45"-26°44'18" E. The sampling area generally consists of a spit and a lagoon lake between it and the mainland. The soil structure of the sampling area consists of alluviums, brown forest soils and coastal sand soils. Also, the sampling area has a transitional character between Mediterranean and Black Sea climate types.

The lagoon and its surroundings, including the sampling area, are windy throughout the year. Considering the vegetation of the sampling area, there are reeds along the mainland shore of the lagoon. Also, there are more trees to the east of the lagoon and dune plants are distributed on the surface of the spit (Çalışkan & Tosunoğlu, 2010).

Figure 1. Map showing the sampling area adjacent to Çardak Lagoon in the Çanakkale Province of northwest Turkey (Anonymous, 2021).

Collection, preparation and identification of aphids

Aphid sampling was conducted from their hosts including flower herbaceous plants, shrubs and trees found adjacent to Çardak Lagoon in Çanakkale Province of northwest Turkey between April-September from 2020 to 2021. The apterous and alate aphids collected from hosts were transferred with a soft brush (#00) into the Eppendorf tubes containing 70% ethanol and brought to the laboratory for the identification. The preparation of the aphid specimens followed the method of Hille Ris Lambers (1950). The identification of aphids was done by the author using a LEICA DM 2500 microscope with a mounted HD camera and LAS 4.1 version software according to Blackman & Eastop (2006; 2021). Current taxonomic status and names of the identified aphid species were stated as in Favret (2021). For new aphid species to the fauna of Turkey, detailed measurements of morphological characters, ratios of different body parts and chaetotaxy were also examined. Abbreviations of morphological characters for apterous and alatae aphids used in this study are: BL, body length; HW, head width; ANT, whole antenna length; ANT I, ANT II, ANT III, ANT IV, ANT V and ANT VI, antennal segments lengths; ANT III BD, antennal segment III basal diameter; LsH on ANT III, antennal segment III longest hair length; ANT VI base, antennal segment VI base length; ANT VI PT, processus terminalis of antennal segment VI; Urs (R IV+V), ultimate rostral segment length; HFem, hind femur length; HTib, hind tibia length; Ht I, hind tibia first segment length; Ht II, hind tibia second segment length; Siph, siphinculi; hairs on ABD Tergite III, abdominal tergite segment III hair length; LFh, length of frontal hairs; and DFf, deeper frontal furrow. The slides of the identified aphid specimens were deposited in the Systematic Laboratory in the Department of Plant Protection of Çanakkale Onsekiz Mart University.

Aphid-plant interactions

The graphs of bipartite network interactions were constructed based on the data of aphid and host relative abundances for aphid-plant interactions for all years using functions of bipartite in R version 3.6.1 (R Core Team, 2021).

Results and Discussion

Diversity of aphids adjacent to Çardak Lagoon and new records for the aphid fauna of Turkey

Twenty-seven aphid species belonging to 17 genera in three subfamilies from family Aphididae (Hemiptera: Aphidomorpha) were identified from the study area. The identified aphid species and their hosts according to current taxonomic status follow. Also, for new aphids of the aphid fauna of Turkey, detailed description, measurements of morphological characters, number of setae on different body parts and chaetotaxy, ratio of different body parts, preparation figures, distribution and short biology are provided.

Order Hemiptera

Infraorder Aphidomorpha

Family Aphididae

Subfamily Aphidinae

***Acyrthosiphon malvae* (Mosley, 1841)**

Material examined. Çanakkale, Çardak, 19.V.2020, apt. 7♀♀, alt. 2♀♀, *Geranium pusillum* L. (Geraniaceae); 27.V.2021, apt. 4♀♀, alt. ♀, *Geranium columbinum* L. (Geraniaceae).

***Aphis fabae* Scopoli, 1763**

Material examined. Çanakkale, Çardak, 11.VI.2020, apt. 5♀♀, alt. 3♀♀, *Chenopodium* sp. (Amaranthaceae); 16.V.2021, apt. 4♀♀, alt. ♀, *Galium aparine* L. (Rubiaceae); 10.VII.2020, apt. 5♀♀, alt. 3♀♀, *Rumex* sp. (Polygonaceae).

***Aphis nasturtii* Kaltenbach, 1843**

Material examined. Çanakkale, Çardak, 10.V.2020, apt. 6♀♀, alt. 2♀♀, *Anagallis arvensis* L. (Primulaceae).

***Aphis polygonata* (Nevsky, 1929)**

Material examined. Çanakkale, Çardak, 19.VI.2020, apt. 6♀♀, alt. 2♀♀, *Polygonum maritimum* L. (Polygonaceae).

***Aphis ruborum* (Börner, 1931)**

Material examined. Çanakkale, Çardak, 21.VI.2021, apt. 4♀♀, alt. 2♀♀, *Rubus sanctus* Schreb. (Rosaceae).

***Aphis solanella* Theobald, 1914**

Material examined. Çanakkale, Çardak, 24.V.2020, apt. 4♀♀, *Papaver rhoeas* L. (Papaveraceae); 14.IV.2021, apt. 6♀♀, *Capsella rubella* Reut. (Brassicaceae).

***Aphis symphyti* Schrank, 1801**

A. symphyti is a new species record for the aphid fauna of Turkey.

Material examined. Çanakkale, Çardak, 11.V.2020, apt. 4♀♀, alt. 2♀♀, *Anchusa hybrida* Ten. (Boraginaceae).

Description. Color of apterous viviparous female specimens on slide; ANT I dark brown, ANT II paler brown, ANT III and IV pale, ANT V pale with brown apices, ANT VI wholly brown or dark brown; head dark; coxa dark or dusky, trochanter dusky, femur and tibia dusky with dark brown apices, hind tibia I and II dark brown, rostrum pale in base, III and URS brown or dark brown; siph wholly dark, and cauda usually paler

than siph. Body of apterous viviparous female more rounded or elliptical (Figure 2b). HW about 0.24 x BL. ANT PT 2.08-2.58 x ANT VI base (Figure 2e). Antennal tubercle weakly developed (Figure 2c). ANT III of alate female with 8-9, ANT IV 5 and ANT V 2-3 secondary rhinaria. The number of hairs of antennal segments of apterous females: ANT I 5-6, ANT II 3-5 and ANT III 10-11. Longest hairs on ANT III about 0.01-0.02 mm, and 0.45-0.67 x ANT BD III. Rostrum reach to the hind coxa and URS 1.24-1.36 x HT II, and generally two hairs present on URS (Figure 2f). Dorsal abdomen without dark markings and dorsal hairs on ABD tergites about 0.02-0.03 mm. Siph wholly dark and without reticulated zone (Figure 2h). Siph 1.59-1.79x cauda, and 0.16-0.21 x body length. Tongue-shaped cauda paler than siph, and bearing 6-7 hairs (Figure 2i), and its length 1.56-1.71 x width (Table 1). *A. symphyti* is difficult to distinguish morphologically from *Aphis* (*Aphis*) *gossypii* Glover, especially on the hosts belonging to the Boraginaceae. Considering the host difference, it is known that *A. symphyti* living under leaves, stems and inflorescence of *Symphytum officinale* L. (Boraginaceae) (Stroyan, 1984). Also, *A. symphyti* has a monoecious holocyclic life cycle, which is only known to be completed on *S. officinale* (Blackman & Eastop, 2021).

Key for identification of *Aphis symphyti* of apterous females on the hosts, *Anchusa* sp. (Boraginaceae) and *Symphytum* sp. (Boraginaceae) (Blackman & Eastop, 2021)

1. ANT VI PT/Base less than 1. Siph absent…….....*Geoica* sp.

- ANT VI PT/Base more than 1. Siph present……......2

2. ANT tubercles undeveloped or weakly developed.....3

- ANT tubercles well developed.....*Macrosiphum* spp., *Ovatomyzus* spp., other polyphagous aphids

3. Cauda helmet shaped.....*Brachycaudus* spp.

- Cauda tongue shaped.....4

4. Cauda and siph black, and bearing 11-24 hairs.....*Aphis fabae*

- Cauda usually paler than siph, and bearing 4-8 hairs.....5

5. R IV+V 0.120-0.160 mm (more than 0.135 mm) and siph 0.330-0.380 mm.....*Aphis symphyti*

- R IV+V 0.075-0.135 mm and siph 0.330-0.440 mm.....*Aphis gossypii*

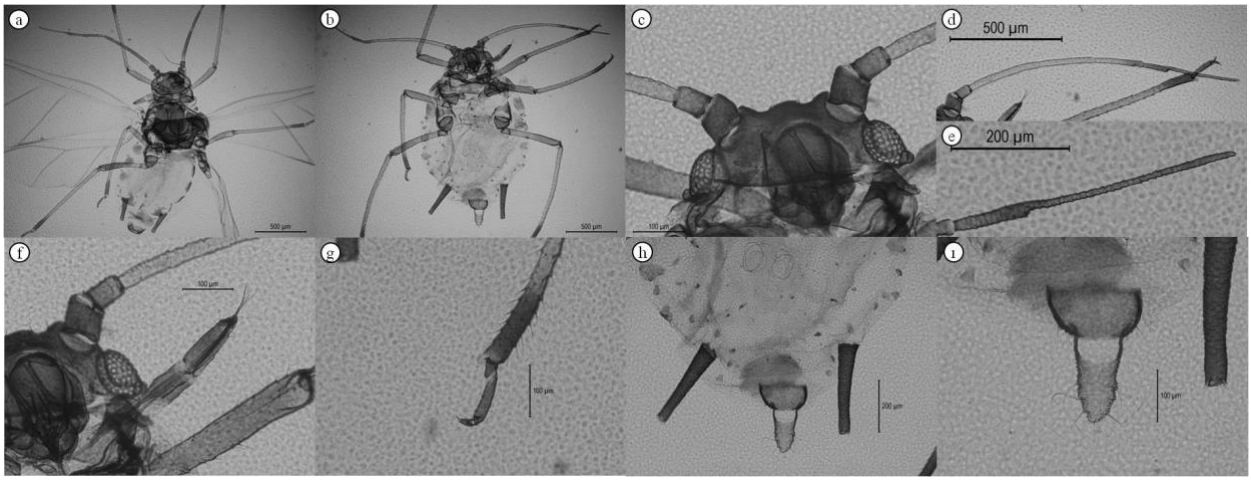

Figure 2. *Aphis symphyti*: a) body of alatae viviparous female; b) body of apterous viviparous female; c) antennal tubercle of apterous female; d) wholly antenna of apterous female; e) ANT VI PT of apterous female; f) URS (R IV+V) of apterous female; g) Ht I and Ht II of apterous female; h) siph of apterous female; and i) cauda of apterous female.

Table 1. Morphometric data (mm) for *Aphis symphyti* from Çanakkale

	Morphometric characters (mm)	*Aphis symphyti*	
		Apterous female (n=4)	Alate female (n=2)
Length of body parts	BL	1.711 (1.578-2.001)	1.649 (1.674-1.625)
	HW	0.410 (0.393-0.436)	0.388 (0.396-0.380)
	ANT	1.379 (1.358-1.436)	1.334 (1.328-1.340)
	ANT I	0.074 (0.067-0.084)	0.065 (0.061-0.070)
	ANT II	0.069 (0.066-0.071)	0.067 (0.070-0.065)
	ANT III	0.329 (0.307-0.345)	0.323 (0.325-0.320)
	ANT III BD	0.028 (0.027-0.029)	0.026 (0.022-0.030)
	LsH on ANT III	0.016 (0.013-0.018)	0.013 (0.011-0.014)
	ANT IV	0.238 (0.235-0.243)	0.215 (0.210-0.220)
	ANT V	0.223 (0.217-0.231)	0.208 (0.211-0.205)
	ANT VI	0.444 (0.425-0.481)	0.456 (0.451-0.460)
	ANT VI base	0.132 (0.122-0.156)	0.123 (0.126-0.120)
	ANT VI PT	0.312 (0.301-0.325)	0.333 (0.325-0.340)
	URS (R IV+V)	0.145 (0.139-0.153)	0.142 (0.139-0.145)
	HFem	0.513 (0.501-0.534)	0.415 (0.410-0.420)
	HTib	0.933 (0.892-0.979)	0.891 (0.883-0.900)
	Ht I	0.038 (0.036-0.041)	0.037 (0.034-0.040)
	Ht II	0.112 (0.107-0.116)	0.108 (0.105-0.110)
	Siph	0.365 (0.318-0.340)	0.223 (0.216-0.230)
	Cauda length	0.193 (0.182-0.203)	0.145 (0.142-0.148)
	Cauda width	0.118 (0.108-0.126)	0.109 (0.105-0.112)
	Hairs on ABD tergite III	0.030 (0.024-0.031)	0.016 (0.014-0.018)
Number of setae on different body parts	ANT I	6 (5-6)	7 (6-8)
	ANT II	4 (3-5)	5 (5-5)
	ANT III	10 (10-11)	9 (8-9)
	URS (R IV+V)	2 (2-2)	2 (2-2)
	Cauda	7 (6-7)	6 (5-7)
Number of secondary rhinaria on antennal segments	ANT III	0	9 (8-9)
	ANT IV	0	5 (5-5)
	ANT V	0	3 (2-3)
Ratio of different body parts	Whole antenna/body	0.811 (0.717-0.836)	0.808 (0.793-0.824)
	PT/base	2.392 (2.083-2.581)	2.817 (2.801-2.833)
	PT/ANT III	0.949 (0.891-1.000)	1.031 (1.000-1.062)
	URS/Ht II	1.297 (1.241-1.353)	1.320 (1.323-1.318)
	Siph/ANT III	0.987 (0.936-1.052)	0.691 (0.664-0.718)
	Siph/body length	0.190 (0.163-0.208)	0.135 (0.129-0.141)
	Siph/cauda	1.686 (1.591-1.791)	1.537 (1.520-1.556)
	Siph/hind femur	0.633 (0.604-0.664)	0.536 (0.526-0.547)
	Cauda length/cauda width	1.639 (1.563-1.705)	1.336 (1.352-1.321)
	LsH on ANT III/BD III	0.561 (0.448-0.666)	0.483 (0.500-0.466)

Distribution. *Aphis symphyti* is distributed throughout the European continent except for Scandinavia and Iberian Peninsula (Blackman & Eastop, 2021).

Biology. *Aphis symphyti* lives generally under leaves, stems and inflorescences of *Symphytum officinale* L. (Boraginaceae) and on other hosts in the Boraginaceae. This species has a monoecious

holocyclic life cycle with alate males and it completes its life cycle only on *S. officinale* (Stroyan, 1984; Blackman & Eastop, 2021).

***Brachycaudus amygdalinus* (Schouteden, 1905)**

Material examined. Çanakkale, Çardak, 19.V.2020, apt. 8♀♀, alt. 3♀♀, *Prunus dulcis* (Mill.) D.A. Webb (Rosaceae).

***Brachycaudus cardui* (Linnaeus, 1758)**

Material examined. Çanakkale, Çardak, 19.VIII.2021, apt. 7♀♀, Unknown plant species (Asteraceae).

***Brachycaudus helichrysi* (Kaltenbach, 1843)**

Material examined. Çanakkale, Çardak, 11.V.2020, apt. 4♀♀, alt. 3♀♀, *Anthemis* sp. (Asteraceae); 14.VI.2021, apt. 4♀♀, alt. ♀, *Artemisia santolina* Schrenk (Asteraceae); 14.VI.2020, apt. 5♀♀, alt. 2♀♀, *Myosotis* sp. (Boraginaceae); 16.IV.2021, apt. 5♀♀, alt. 2♀♀, *Senecio vulgaris* L. (Asteraceae); 12.VI.2021, apt. 4♀♀, alt. ♀, *Silybum marianum* (L.) Gaertn. (Asteraceae).

***Brevicoryne brassicae* (Linnaeus, 1758)**

Material examined. Çanakkale, Çardak, 16.VI.2020, apt. 6♀♀, alt. ♀, *Brassica nigra* (L.) K. Koch (Brassicaceae).

***Capitophorus similis* van der Goot, 1915**

Material examined. Çanakkale, Çardak, 11.VI.2021, apt. 7♀♀, *Elaeagnus angustifolia* L. (Elaeagnaceae).

***Hyadaphis foeniculi* (Passerini, 1860)**

Material examined. Çanakkale, Çardak, 23.IV.2020, apt. 4♀♀, *Anthriscus caucalis* M. Bieb. (Apiaceae)

***Hyalopterus pruni* (Geoffroy, 1762)**

Material examined. Çanakkale, Çardak, 13.VI.2021, apt. 3♀♀, alt. ♀, *Phragmites australis* (Cav.) Trin. Steud. (Poaceae).

***Hyperomyzus lactucae* (Linnaeus, 1758)**

Material examined. Çanakkale, Çardak, 14.V.2020, apt. 4♀♀, alt. 3♀♀, *Crepis* sp. (Asteraceae), 25.VI.2020; apt. 4♀♀, alt. 2♀♀, *Sonchus oleraceus* (L.) L. (Asteraceae), 15.VII.2021; apt. 5♀♀, alt. 2♀♀, *Sonchus* sp. (Asteraceae).

***Lipaphis pseudobrassicae* (Davis, 1914)**

Material examined. Çanakkale, Çardak, 11.V.2020, apt. 4♀♀, *Raphanus raphanistrum* L. (Brassicaceae).

***Macrosiphoniella tapuskae* (Hottes & Frison, 1931)**

Material examined. Çanakkale, Çardak, 19.V.2021, apt. 6♀♀, *Anthemis* sp. (Asteraceae).

***Macrosiphoniella pulvera* (Walker, 1848)**

Material examined. Çanakkale, Çardak, 15.V.2020, apt. 5♀♀, *Artemisia* sp. (Asteraceae).

***Myzus* sp.**

Material examined. Çanakkale, Çardak, 10.IX.2021, apt. 4♀♀, alt. 2♀♀, *Geranium molle* L. (Geraniaceae).

***Rhopalosiphum padi* (Linnaeus, 1758)**

Material examined. Çanakkale, Çardak, 24.V.2020, apt. 4♀♀, *Hordeum murinum* L. (Poaceae).

***Staticobium latifoliae* (Bozhko, 1950)**

S. latifoliae is a new species record for the aphid fauna of Turkey.

Material examined. Çanakkale, Çardak, 11.V.2020, apt. 4♀♀, alt. ♀, *Limonium narbonense* Mill. (Plumbaginaceae); 14.VI.2021, apt. 2♀♀, alt. ♀, *Limonium* sp. (Plumbaginaceae).

The genus *Staticobium* Mordvilko, 1914 contains 12 aphid species associated with the hosts in the Plumbaginaceae in salt-marsh and coastal habitats. The aphids in this genus are morphologically close to the genus *Macrosiphoniella* Del Guercio (Blackman & Eastop, 2021).

Description. Color of apterous viviparous female specimens on slide; ANT I and II dark brown or dark, ANT III and IV dusky with dark apices, ANT V wholly dark brown, base of ANT VI dark brown and PT of ANT VI dusky with paler apices; head dusky brown; coxa dark, trochanter dusky or pale, femur and tibia mostly pale with dark brown apices, hind tibia I and II dusky, rostrum generally pale and URS paler brown; siph dark with paler base, and cauda wholly pale. Body of apterous viviparous female mainly elliptical (Figure 3c); HW about 0.25 x BL. ANT PT 3.57-4.05 x ANT VI base (Figure 3f). Antennal tubercle well developed with diverging apices (Figure 3d). Length of frontal hairs 0.04-0.06 mm (Figure 3d). ANT III of apterous female with 0-3 secondary rhinaria mainly in base, ANT IV and V without secondary rhinaria while ANT III of alate female with 4-5 secondary rhinaria, ANT IV and V without secondary rhinaria (Figure 3h,i). The number of hairs of antennal segments of apterous females: ANT I 6-8, ANT II 4-6 and ANT III 11-14. Longest hairs on ANT III about 0.02-0.04 mm, and 0.68-0.94 x ANT BD III. Rostrum exceeds the middle coxa and URS 0.97-1.13 x HT II, and 4-6 hairs present on URS (Figure 3g). ABD tergites I-VI with dorsal hairs mostly arising from dark scleroites, and about 0.02-0.05 mm (Figure 3j). Siph dark with paler base, and reticulated zone of it (%) 37.24-51.36 (Figure 3k). Siph 1.30-2.30 x cauda, and 0.20-0.27 x body length. Cauda with 7-10 hairs (Figure 3l), and its length 1.45-2.29 x width (Table 2). On *S. latifoliae*, depth of frontal grove 0.15-0.28 (on average 0.21) x distance between apices of antennal tubercle, on *Staticobium smailovae* Kadyrbekov, 2004 which is morphologically a taxon between *S. latifoliae* and *Staticobium staticis* (Theobald, 1923) depth of frontal grove 0.14-0.18 x distance between apices of antennal tubercle. The apterous antennae of *S. latifoliae* are 1.0-1.3 times the body length and the apterous antennae of *S. staticis* are 0.6-0.9 times the body length.

Key for identification of *Staticobium latifoliae* of apterous females on the host, *Limonium* sp. (Plumbaginaceae) (Blackman & Eastop, 2021)

1. Siph dark in distally and pale in basal, polygonal reticulation of siph extending over distal 0.300-0.550 of length. Hairs on dorsal arising from small dark scleroites.....2

- Siph pale or dark, polygonal reticulation of siph extends only 0.130-0.200 of length. Hairs on dorsal not arising from small dark scleroites.....*Aphis* sp. and other polyphagous aphids

2. Hairs on dorsal very small, longest hairs on ANT III 0.200-0.300 × BD III.....*Staticobium gmelini*

- Hairs on dorsal evident, longest hairs on ANT III 0.500-1.500 × BD III.....3

3. Cauda bearing 4 (sometimes 5) hairs.....*Staticobium loochooense*

- Cauda bearings 6-13 hairs.....4

4. Cauda 1.0-1.5 × its basal width and rounded at apex.....*Staticobium longisetosum*/*caucasicum*

- Cauda 1.4-2.4 × its basal width and pointed at apex.....5

5. Hairs on dorsal not arising from scleroites, R IV+V 0.730-0.900 × HT II…*Staticobium otolepidis*/*suffruticosum*

- Hairs on dorsal arising from dark scleroites, R IV+V 0.900-1.300 × HT II.....6

6. ANT VI PT/Base 3.100-3.700 mm. R IV+V 1.000-1.100 × HT II.....*Staticobium* sp.

- ANT VI PT/Base 3.500-5.500 mm. R IV+V 0.900-1.300 × HT II.....7

7. Siph 0.290-0.370 × BL, siph 1.000-1.200 × ANT III; reticulation over 0.350-0.420 of length….*Staticobium limonii*

- Siph 0.140-0.300 × BL, siph 0.580-1.150 × ANT III; reticulation over distal 0.350-0.550 of length.....8

8. Siph 0.140-0.230 × BL, siph 0.900-1.500 × cauda, which bearing 6-11 (usually 7-8) hairs....*Staticobium staticis*

- Siph 0.230-0.300 × BL, siph 1.250-2.00 × cauda, which bearing 7-14 (usually 8-11) hairs.....*Staticobium latifoliae*

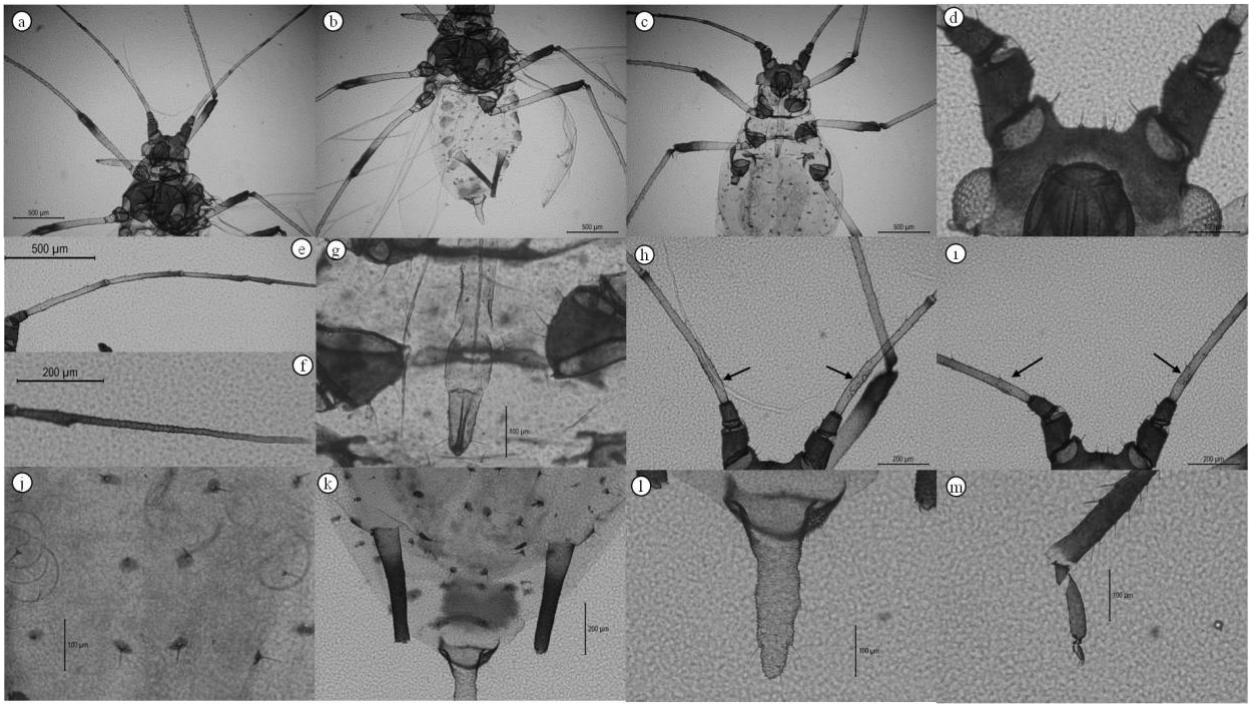

Figure 3. *Staticobium latifoliae*: a,b) body of alatae viviparous female; c) body of apterous viviparous female; d) antennal tubercle and frontal hairs of apterous female; e) wholly antenna of apterous female; f) ANT VI PT of apterous female; g) URS (R IV+V) of apterous female; h) secondary rhinaria on antennal segments of alatae female; i) secondary rhinaria on antennal segments of apterous female; j) dorsal hairs on ABD tergites of apterous female; k) siph of apterous female; l) cauda of apterous female; and m) Ht I and Ht II of apterous female.

Distribution. *S. latifoliae* is distributed in Bulgaria, Greece, Hungary, Iran, Italy, Kazakhstan, Lebanon, Pakistan, Russia, Romania, Tajikistan and Ukraine (Blackman & Eastop, 2021).

Biology. *S. latifoliae* feed on undersides of leaves and flower stalks of *Limonium* spp. (Blackman & Eastop 2021).

Table 2. Morphometric data (mm) for *Staticobium latifoliae* from Çanakkale

	Morphometric characters (mm)	*Staticobium latifoliae*	
		Apterous female (n=6)	Alate female (n=2)
Length of body parts	BL	2.082 (1.938-2.335)	1.965 (1.806-2.124)
	HW	0.523 (0.450-0.557)	0.481 (0.475-0.488)
	ANT	2.222 (1.972-2.510)	2.537 (2.438-2.637)
	ANT I	0.144 (0.140-0.150)	0.126 (0.116-0.136)
	ANT II	0.096 (0.088-0.101)	0.102 (0.100-0.103)
	ANT III	0.519 (0.459-0.562)	0.566 (0.530-0.603)
	ANT III BD	0.035 (0.030-0.040)	0.036 (0.035-0.037)
	LsH on ANT III	0.029 (0.024-0.036)	0.029 (0.028-0.030)
	ANT IV	0.425 (0.368-0.487)	0.490 (0.475-0.506)
	ANT V	0.345 (0.299-0.378)	0.421 (0.410-0.432)
	ANT VI	0.690 (0.594-0.844)	0.837 (0.817-0.857)
	ANT VI base	0.145 (0.130-0.168)	0.141 (0.136-0.146)
	ANT VI PT	0.545 (0.464-0.676)	0.696 (0.681-0.711)
	URS (R IV+V)	0.148 (0.142-0.157)	0.148 (0.147-0.148)
	HFem	0.806 (0.733-0.867)	0.797 (0.722-0.872)
	HTib	1.443 (1.270-1.568)	1.498 (1.352-1.645)
	Ht I	0.046 (0.041-0.050)	0.043 (0.041-0.045)
	Ht II	0.141 (0.127-0.155)	0.135 (0.135-0.135)
	Siph	0.486 (0.440-0.607)	0.457 (0.418-0.488)
	Cauda length	0.287 (0.243-0.350)	0.255 (0,222-0.289)
	Cauda width	0.152 (0.130-0.175)	0.119 (0.100-0.138)
	Hairs on ABD tergite III	0.036 (0.022-0.050)	0.037 (0.030-0.048)
	LFh	0.050 (0.039-0.061)	0.030 (0.027-0.035)
	DFf	0.053 (0.042-0.065)	0.054 (0.052-0.059)
Number of setae on different body parts	ANT I	7 (6-8)	6 (5-6)
	ANT II	5 (4-6)	4 (3-4)
	ANT III	13 (11-14)	14 (12-15)
	URS (R IV+V)	5 (4-6)	7 (6-8)
	Cauda	9 (7-10)	8 (8-8)
Number of secondary rhinaria on antennal segments	ANT III	2 (0-3)	5 (4-5)
	ANT IV	0	0
	ANT V	0	0
Ratio of different body parts	Whole antenna/body	1.060 (0.960-1.286)	1.295 (1.241-1.349)
	PT/Base	3.755 (3.569-4.050)	4.934 (4.869-5,000)
	PT/ANT III	1.047 (0.965-1.202)	1.231 (1.179-1.284)
	URS/Ht II	1.050 (0.974-1.125)	1.092 (1.088-1.096)
	Siph/ANT III	0.939 (0.782-1.089)	0.807 (0.784-0,847)
	Siph/body length	0.233 (0.197-0.265)	0.232 (0.222-0.248)
	Siph/cauda	1.734 (1.302-2.290)	1.807 (1.636-2.022)
	Siph/hind femur	0.602 (0.534-0.700)	0.575 (0.542-0.621)
	Cauda length/cauda width	1.890 (1.446-2.291)	4.314 (2.094-2.220)
	LsH on Ant III/BD III	0.825 (0.675-0.939)	0.805 (0.800-0.810)
	Depth of frontal grove / distance between apices of antennal tubercle	0.204 (0.150-0.275)	0.235 (0.227-0.253)
	Reticulated zone of siph (%)	42.71 (37.24-51.36)	45.042 (41.86-47.60)

***Uroleucon aeneum* (Hille Ris Lambers, 1939)**

Material examined. Çanakkale, Çardak, 11.VI.2020, apt. 4♀♀, *Carduus pycnocephalus* L. (Asteraceae), 12.VI.2020; apt. 4♀♀; 15.V.2021, apt. 5♀♀, *Carduus* sp. (Asteraceae).

***Uroleucon jaceae* (Linnaeus, 1758)**

Material examined. Çanakkale, Çardak, 23.IV.2020, apt. 6♀♀, *Centaurea spinosa* L. (Asteraceae).

***Uroleucon sonchi* (Linnaeus, 1767)**

Material examined. Çanakkale, Çardak, 11.VII.2020, apt. 4♀♀, alt. 2♀♀, *Sonchus oleraceus* (L.) L. (Asteraceae); 14.VI.2021, apt. 5♀♀, alt. ♀, *Sonchus* sp. (Asteraceae).

***Volutaphis schusteri* (Börner, 1939)**

Material examined. Çanakkale, Çardak, 19.IV.2020, apt. 6♀♀, *Silene* sp. (Caryophyllaceae).

Subfamily Chaitophorinae

Chaitophorus salicti (Schrank, 1801)

Material examined. Çanakkale, Çardak, 27.V.2021, apt. 7♀♀, *Salix alba* L. (Salicaceae).

Subfamily Eriosomatinae

***Pemphigus immunis* Buckton, 1896**

Material examined. Çanakkale, Çardak, 14.VI.2020, alt. 7♀, *Populus canadensis* Moench (Salicaceae).

Considering the taxonomic diversity of the aphids in this study, 25 of the identified species are in the subfamily Aphidinae. The subfamily Aphidinae, with 361 species belonging to 74 genera, is the largest subfamily of the Aphididae in Turkey and constitute about 61% of all aphid species (Kök & Özdemir, 2021). About 93% of the identified species are in the Aphidinae subfamily. Also, of the 27 identified aphids in this study, six species (about 22% of species) belong to the genus *Aphis* which is one of the largest genera with 90 species among 148 genera from the Aphididae in Turkey.

Of the identified aphids, *A. fabae*, *B. helichrysi* and *H. lactucae* were as most common aphid species in the study area. *Aphis fabae*, black bean aphid, is one of the most commonly distributed aphid species; it has been reported from almost 50 provinces in Turkey. Similarly, *B. helichrysi*, known as the leaf-curling plum aphid, was reported in more than 30 provinces and *H. lactucae* in more than 20 provinces in Turkey (Kök & Özdemir, 2021). Some aphid species identified from this study area are rarely recorded in Turkey. For example, *L. pseudobrassicae*, an important worldwide pest of brassica crops, and *V. schusteri*, widely distributed in continental Europe, were previously only recorded from Samsun Province in Turkey (Remaudière et al., 2006). Similarly, *M. pulvera* was previously only recorded from Ankara, Eskişehir and Erzurum Provinces of Turkey (Tuatay, 1990). In terms of aphid diversity, the results of this study significantly contribute to the regional diversity of aphids in Turkey. *Aphis symphyti* on *A. hybrida* and *S. latifoliae* on *Limonium* sp. and *L. narbonense* are reported for the first time for the aphid fauna of Turkey. Also, *S. latifoliae* is the first aphid species of the genus *Staticobium* to be identified in Turkey.

Aphid-plant interactions adjacent to Çardak Lagoon and new host records for aphids in Turkey

In this study, 37 host species belonging to 16 families, viz., Apiaceae, Amaranthaceae, Asteraceae, Brassicaceae, Boraginaceae, Caryophyllaceae, Elaeagnaceae, Geraniaceae, Papaveraceae, Plumbaginaceae, Poaceae, Polygonaceae, Primulaceae, Rubiaceae, Rosaceae and Salicaceae, were revealed as hosts of aphids adjacent to Çardak Lagoon of Çanakkale. From these plant families, Asteraceae, with 15 species

in the sampling area, provided the highest number of host species for the aphids. In contrast, the Amaranthaceae, Apiaceae, Caryophyllaceae, Elaeagnaceae, Rubiaceae and Primulaceae had only one host species each. In the case of the aphids, *B. helichrysi* feeding on five host species was the aphid species that had the highest number of host species. Similarly, it was determined that *A. fabae* and *H. lactucae* feed on two host species (Figure 4).

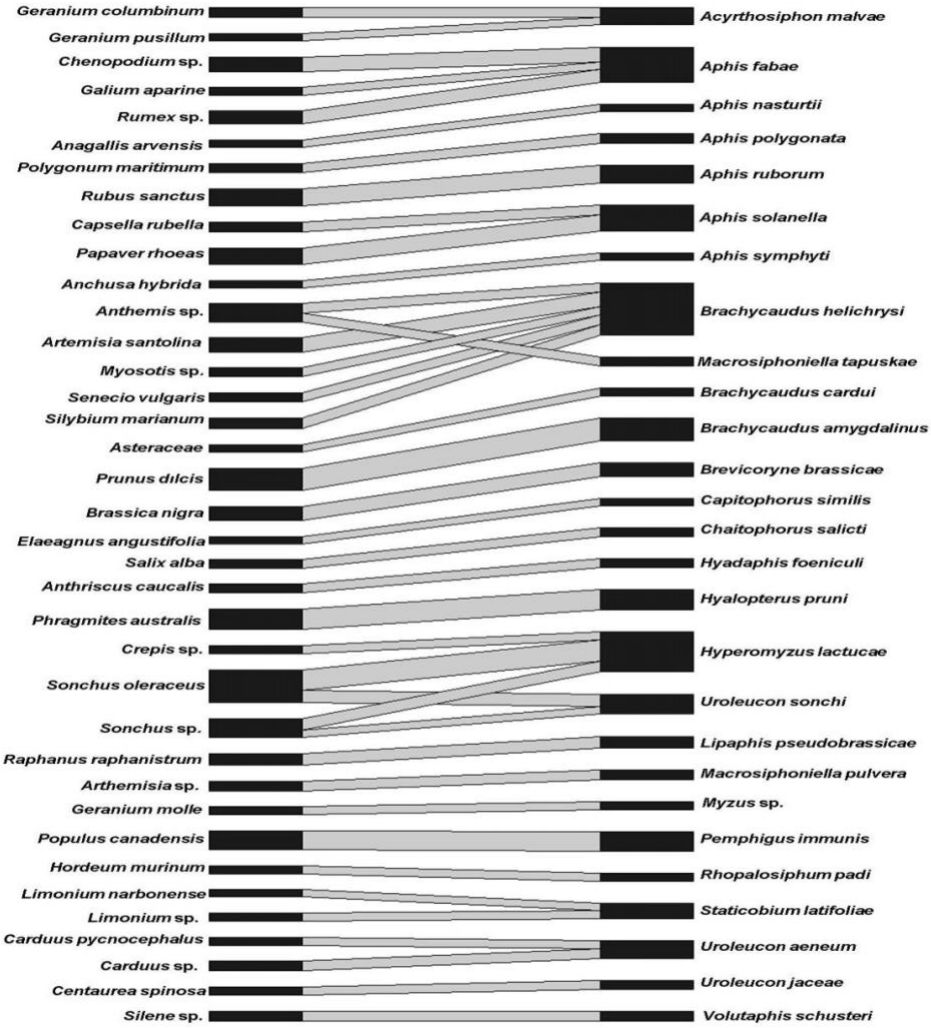

Figure 4. Bipartite network interaction between aphids (right) and hosts (left) species adjacent to Çardak Lagoon in Çanakkale Province, Turkey. Black bars represent the abundance of the species and gray bars represent interactions.

Also, a large number of new host records for aphids in Turkey were determined from the sampling area. Of these, *G. pusillum* and *G. columbinum* for *A. malvae*, *A. arvensis* for *A. nasturtii*, *P. maritimum* for *A. polygonata*, *Myosotis* sp. for *B. helichrysi*, *A. caucalis* for *H. foeniculi*, *R. raphanistrum* for *L. pseudobrassicae*, *Anthemis* sp. for *M. tapuskae* and *Silene* sp. for *V. schusteri* were revealed for the first time as new hosts of aphids in Turkey.

With this study, the important results were obtained confirming the hypothesis presented. With this study, *A. symphyti* distributed throughout European and *S. latifoliae* distributed in salt-marsh and coastal habitats of European, Middle and North Asia were introduced in to the aphid fauna of Turkey. With these new records, the number of the aphid fauna of Turkey has reached to 596 species from 149 genera in the infraorder Aphidomorpha (Hemiptera). Also, some aphid species, which are rarely reported in Turkey such

as *L. pseudobrassicae*, *M. pulvera* and *V. schusteri*, were identified with this study and these results contribute significantly to the local diversity of the aphid fauna of Turkey. The new hosts reported for some aphid species such as *A. malvae*, *A. nasturtii*, *A. polygonata*, *B. helichrysi*, *H. foeniculi*, *L. pseudobrassicae*, *M. tapuskae* and *V. schusteri* provide significant contributions to the emergence of new data on the biology and control of aphid pests, which are economically harmful in agricultural areas.

A remarkable finding was that *B. helichrysi*, a cosmopolitan pest on *Prunus* spp. (Rosaceae) (especially *Prunus domestica* L. and *Prunus persica* (L.) Batsch) worldwide and in Turkey, was the most common aphid species in the sampling area. So far, *B. helichrysi* has been reported on *Achillea* sp. (Asteraceae), *A. millefolium* L. (Asteraceae), *A. nobilis* L. (Asteraceae), *Anchusa* sp. (Boraginaceae), *A. leptophylla* Roem. & Schult. (Boraginaceae), *A. pusilla* Gusul. (Boraginaceae), *Anthemis* sp. (Asteraceae), *Calendula* spp. (Asteraceae), *C. arvensis* M. Bieb. (Asteraceae), *C. officinalis* L. (Asteraceae), *Caltha* sp. (Ranunculaceae), *Campsis* sp. (Bignoniaceae), *Carduus* sp. (Asteraceae), *C. pycnocephalus*, *Carlina* sp. (Asteraceae), *Carthamus dentatus* Wahl (Asteraceae), *C. tinctorius* L. (Asteraceae), *Centaurea* sp. (Asteraceae), *C. solstitialis* L. (Asteraceae), *Cerasus* sp. (Rosaceae), *Chrysanthemum* sp. (Asteraceae), *C. frutescens* L. (Asteraceae), *C. nivellei* Braun-Blanq. & Maire (Asteraceae), *Cineraria* sp. (Asteraceae), *Cirsium arvense* (L.) Scop. (Asteraceae), *C. cephalotes* Boiss. (Asteraceae), *Cucurbita pepo* L. (Cucurbitaceae), *Cydonia* sp. (Rosaceae), *Cynoglossum* sp. (Boraginaceae), *Eryngium* sp. (Apiaceae), *Euphorbia* spp. (Euphorbiaceae), *Gazania* sp. (Asteraceae), *Helianthus* sp. (Asteraceae), *H. annuus* L. (Asteraceae), *Hyacinthus* sp. (Asparagaceae), *Leucanthemum vulgare* (Vaill.) Lam. (Asteraceae), *Lycopersicum esculentum* L. (Solanaceae), *Matricaria* sp. (Asteraceae), *M. chamomilla* L. (Asteraceae), *Onopordum* sp. (Asteraceae), *Pulicaria dysenterica* (L.) Gaertn. (Asteraceae), *Pyrus* sp. (Rosaceae), *Rubus* sp. (Rosaceae), *Rumex crispus* L. (Polygonaceae), *Sambucus nigra* L. (Adoxaceae), *Senecio* sp. (Asteraceae), *S. vernalis* Waldst. & Kit. (Asteraceae), *Silene* sp. (Caryophyllaceae), *Sorbus* sp. (Rosaceae), *Spirea* spp. (Rosaceae), *Symphytum asperum* Lepech. (Boraginaceae), *Taraxacum officinale* L. (Asteraceae), *Tripleurospermum inodorum* (L.) Sch.Bip. (Asteraceae), *Urtica dioica* L. (Urticaceae), *Verbascum* sp. (Scrophulariaceae), *Veronica anagallis-aquatica* L. (Plantaginaceae) and *Vinca minor* L. (Apocynaceae) in Turkey (Toros et al., 2002; Özdemir, 2004; Aslan & Uygun, 2005; Görür et al., 2009; Görür, 2014; Kök et al., 2016; Öztürk & Muştu, 2017; Kök & Kasap, 2019; Akyıldırım Beğen & Görür, 2021; Başer & Tozlu, 2020). *Brachycaudus helichrysi* has a heteroecious holocyclic life cycle with sexual phase on *Prunus* spp. (especially *P. domestica*) in colder climates. This aphid, which uses *Prunus* species as the primary host on which sexual reproduction occurs during the autumn, migrates to secondary host on which parthenogenetic reproduction occurs throughout the spring and the summer period (Blackman & Eastop, 2021). *Brachycaudus helichrysi* is highly polyphagous on its secondary hosts and it was reported linked with more than 300 herbaceous plant genera in the Palearctic region (Holman, 2009). In this respect, the secondary hosts are vital for this aphid pest to complete its life cycle. This study clearly revealed that the secondary hosts of this aphid pest is important in terms of the better understanding of the biology and control strategies of the aphid in the Çanakkale Province, where plums and peaches are produced in high quantities.

Plant interactions are a major driver of reproductive isolation in aphids in different habitats (Peccoud et al*.,* 2010). In this regard, it is important that faunal and biological studies are conducted on hosts in different habitats to contribute to aphid diversity in the world and in our country. This phenomenon appears in the results of this study which was conducted in a small area with different habitats and is close to agricultural areas. Therefore, it is believed that a detailed examination of aphid-plant interactions not only in agricultural areas but also non-agricultural areas with different habitats as in this study will enhance understanding of the diversity of aphids and their hosts in Turkey. Also, uncovering the secondary hosts of migrating aphids, which have continually increasing distributions due to global warming and climate change, and their interactions with these plants will provide important data for the cultural and biological control of these aphid pests in crops.

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Original article (Orijinal araştırma)

Optimization of *in vitro* solid culture of *Heterorhabditis bacteriophora* Poinar, 1976 (Rhabditida: Heterorhabditidae) HBH hybrid strain[1]

Heterorhabditis bacteriophora Poinar, 1976 (Rhabditida: Heterorhabditidae) HBH hibrit ırkının *in vitro* katı kültürde üretiminin optimizasyonu

Tufan Can ULU[2] **İ. Alper SUSURLUK[3*]**

Abstract

Entomopathogenic nematodes are soil-dwelling biocontrol agents. EPNs need an insect host to complete their life cycle, and they kill their host during its development. The major disadvantage of EPNs is the high cost of commercial products. Thus, there are many studies focused on reducing production costs by optimization of mass production. In a previous project, *Heterorhabditis bacteriophora* Poinar, 1976 (Rhabditida: Heterorhabditidae) HBH hybrid strain was developed from local isolates. This hybrid strain was patented due to superior bioecological characteristics. This study aimed to optimize *in vitro* solid mass production of hybrid strain. All laboratory trials were performed between 2017 and 2018, in Nematology Laboratory of Bursa Uludağ University, Faculty of Agriculture, Department of Plant Protection. For optimization, additional supplementary ingredients (soy lecithin and egg yolk), temperature (24, 28 and 32°C) and medium pH (5, 7 and 9) were selected as production parameters. Optimization was evaluated based on hermaphrodite egg numbers, total infective juveniles (IJs), IJ body length and IJ virulence. Based on results, best production combination was found as agar containing soy lecithin, 28°C and pH 7. Also, agar media with pH 9 markedly reduced production yield. Consequently, optimum values for some important *in vitro* solid production parameters of HBH hybrid strain were determined.

Keywords: *Heterorhabditis bacteriophora*, mass production, monoxenic culture, optimization

Öz

Entomopatojen nematodlar (EPN), toprakta yaşayan biyolojik mücadele ajanlarıdır. EPN'ler yaşam döngüleri boyunca bir konukçu böceğe ihtiyaç duyarlar ve gelişimleri sırasında konukçusunu öldürürler. EPN'lerin en büyük dezavantajı, ticari ürünlerin yüksek maliyetidir. Bu nedenle kitle üretimin optimizasyonu ile üretim maliyetlerinin düşürülmesine odaklanan birçok çalışma bulunmaktadır. Daha önceki bir proje kapsamında iki yerel izolattan *Heterorhabditis bacteriophora* Poinar, 1976 (Rhabditida: Heterorhabditidae) HBH hibrit ırkı geliştirilmiştir. Bu hibrit ırk, üstün biyo-ekolojik özelliklerinden dolayı patentlidir. Bu çalışmada, hibrit HBH ırkının *in vitro* katı kültürde kitle üretiminin optimize edilmesi amaçlanmıştır. Tüm laboratuvar denemeleri 2017-2018 yılları arasında Bursa Uludağ Üniversitesi, Ziraat Fakültesi, Bitki Koruma Bölümü, Nematoloji Laboratuvarı'nda gerçekleştirilmiştir. Optimizasyonda standart ortama ek katkı maddeleri (soya lesitini ve yumurta sarısı), sıcaklık (24, 28 ve 32°C) ve ortam pH'ı (5, 7 ve 9) üretim parametreleri olarak seçilmiştir. Optimizasyon, hermafrodit yumurta sayıları, toplam infektif jüvenil (IJ) sayısı, IJ vücut uzunluğu ve IJ etkinliği ile değerlendirilmiştir. Sonuçlara göre, en iyi üretim kombinasyonu, soya lesitini içeren agar, 28°C ve pH 7 olarak bulunmuştur. Ayrıca pH 9'lu agarlar, üretim verimini önemli ölçüde azaltmıştır. Sonuç olarak, HBH hibrit ırkının bazı önemli *in vitro* katı üretim parametreleri için optimum değerler belirlenmiştir.

Anahtar sözcükler: *Heterorhabditis bacteriophora*, kitle üretimi, monoksenik kültür, optimizasyon

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[2] Bilecik Seyh Edebali Univ., Faculty of Agriculture and Natural Sciences, Department of Plant Protection, 11230, Bilecik, Turkey
[3] Bursa Uludağ University, Faculty of Agriculture, Department of Plant Protection, 16059, Bursa, Turkey
* Corresponding author (Sorumlu yazar) e-mail: susurluk@uludag.edu.tr
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Introduction

Today, the most widely used method against pathogens, pests and weeds in agriculture is chemical control (Sarwar, 2015). Until now, the most successful and applicable alternative method to chemical control has been biological control. Although the market share for biocontrol agents seems quite low, it is thought that the biological products will exceed chemical products in the 2050s (Glare et al., 2012; Olson, 2015). Chemical pesticides are preferred due to their high efficacy, quick mode of action and low price. For this reason, alternative methods to chemicals should be able to compete with pesticides. There are some major problems restricting the adoption of biological control agents such as high production costs, short shelf life, and slow effectiveness. For such reasons, many studies are conducted to overcome these disadvantages. A large part of this work is directed towards increasing the effectiveness of biological control agents, reducing production and storage costs, increasing production efficiency, and developing new formulations that improve ease of use (Grewal, 2002; Schumann et al., 2014).

Entomopathogenic nematodes (EPN) are obligate endoparasites that spend more than 90% of their life cycle in the soil and need an insect host to continue their development. EPNs can be found in many regions and climates around the world (Griffin et al., 1990; Hominick, 2002), they have a wide host range (Peters, 1996), no negative effects on other non-targeted organisms (Lacey et al., 2015), they have active host search capabilities (Lewis et al., 1992), they can be easily applicable with many agricultural equipment (Georgis, 1990; Wright et al., 2005) and they are compatible with pesticides (García del Pino & Jové, 2005; Atwa et al., 2013).

EPNs are suitable organisms for mass production, and improvements in production techniques have been going on for many years (Ehlers, 2001; Shapiro-Ilan et al., 2002). The most widely used *in vitro* production method of EPNs, which have been produced by a range of methods, was developed by Lunau et al. (1993). EPNs are mass produced *in vivo* or *in vitro*, depending on their intended use (Ehlers, 2001). While *in vivo* methods are preferred for laboratory studies, greenhouse and small-scale field trials, *in vitro* methods are preferred for commercial production and large-scale applications (Gaugler et al., 2002). *In vitro* methods are basically divided into two groups using solid and liquid media. Solid media production is generally conducted in Petri dishes of certain sizes. In some conditions, solid media are preferred to liquid media due to being more convenient and risk-free.

Although mass production of a biological control agent is an important feature, expensive commercial products are less likely to become popular in the market. There have been many developments in the production of EPNs in artificial environment over the years (Chavarría-Hernández et al., 2011; Testa & Shields, 2017). Most of these developments have been realized on increasing production efficiency, reducing production costs, or increasing the quality of the product. Some of these studies examined the effects of mass production in artificial environment and environmental factors during the production process (Hirao & Ehlers, 2009; Leite et al., 2017).

There are many factors that affect the mass production of EPNs using solid and liquid media. Some of these factors can be summarized as temperature, humidity, pH, viscosity, salinity, electrical conductivity, agitation speed, pressure, media content, type, or race of EPN used (Shapiro-Ilan et al., 2012). All these factors directly affect the yield during production and the quality of the product after production. With the optimization of mass production of EPNs, commercial biological products have gained ground to compete with pesticides and increase their market share.

This study aimed to optimize the *in vitro* solid mass production of *Heterorhabditis bacteriophora* Poinar, 1976 (Rhabditida: Heterorhabditidae) HBH hybrid strain. The HBH hybrid strain was patented in 2018 due to its superior bioecological features (Patent No: TR 2013 06141 B). Egg yolk and soy lecithin were added as protein and fat sources into the standard solid medium used for optimization. In addition, the effects of temperature and pH on production efficiency were also investigated. The effects of these

variables were evaluated using parameters such as the number of eggs of hermaphrodites, the body length of infective juveniles (IJs), the total number of IJs produced and the effectiveness of IJs. By this study, it was also aimed to reveal some optimum production parameters of our local EPN strain and speed up liquid production processes in future.

Materials and Methods

Heterorhabditis bacteriophora HBH hybrid strain

Heterorhabditis bacteriophora is one the most abundant EPN species in Turkey. Two local Turkish isolates of *H. bacteriophora* were hybridized and HBH strain obtained with superior bioecological traits. HBH hybrid strain has high virulence, reproduction capacity and longevity compared to commercial EPN strains. *H. bacteriophora* HBH strain was *in vivo* reared on last instar of *Galleria mellonella (*L., 1758) (Lepidoptera: Pyralidae) larvae (Kaya & Stock, 1997).

Egg isolation and bacteria cultures

Egg and bacteria isolation are the first steps of monoxenic cultures. Eggs are usually extracted from the first-generation hermaphrodites (*Heterorhabditis*) or gravid females (*Steinernema*). For egg isolation, each *G. mellonella* larva was inoculated with 100 IJs of the hybrid strain. Four days after inoculation, dead cadavers were dissected in a Petri dish and fertilized hermaphrodites were collected in a tube. Hermaphrodites were rinsed with Ringer's solution several times and they were cut into little pieces with razor blades using a vortex. Dispersed eggs were filtered through a 50-µm sieve and washed with distilled water to eliminate hermaphrodite particles. Finally, surface of the eggs was sterilized with sterilization solution, and they were incubated in sterile YS medium until they hatch contamination-free (Lunau et al., 1993).

Freshly hatched eggs need to feed immediately. So, frozen bacterial cultures were thawed, and YS medium inoculated with symbiotic bacteria. To establish food source for nematodes, modified agar media inoculated with precultured bacteria and incubated for 2 days before nematode transfer (Hirao & Ehlers, 2009).

Optimization parameters

Production media has significant impact on yield (Ramakuwela et al., 2016; Alsaidi et al., 2018). There are many different solid media for EPN production. Wouts agar is mostly used for small-scale production (Wouts, 1981; El-Sadawy, 2011). Thus, we modified Wouts agar to improve yield and quality of IJs (Yoo et al., 2000). In addition to the ingredients of Wouts agar, we used egg yolk for additional protein and soy lecithin for fat source. List of the modified agar media is given in Table 1. Standard Wouts agar was used as control.

Table 1. Modified Wouts agar media for *in vitro* solid culture (100 ml)

	W	WL	WE	WLE
Nutrient broth	1.6	1.6	1.6	1.6
Agar (g)	1.2	1.2	1.2	1.2
Sunflower oil	0.5	0.5	0.5	0.5
Egg yolk (g)	-	-	0.5	0.5
Soy lecithin (g)	-	0.5	-	0.5

W, Wouts (control); WL, Wouts + lecithin; WE, Wouts + egg yolk; and WLE, Wouts + lecithin + egg yolk.

Production temperature is another important parameter (Serwe-Rodriguez et al., 2004; Anbesse et al., 2013). Since different EPN species have different temperature adaptations, *in vitro* productions were conducted at 24, 28 and 32°C to find the optimum temperature. Incubators were preheated, and agar media were incubated at each temperature during production.

Lastly, even slight changes of pH may have detrimental effect on bacteria and nematodes (Yoo et al., 2000; Hirao et al., 2010). Though pH is more important for liquid cultures, it is also an important parameter for solid production optimization. It is known that bacteria secrete different metabolites under different pH values, which will eventually change production yield. We prepared agar media with 3 pH values (5, 7 and 9). The pH of the agar media was adjusted with ascorbic acid and sodium bicarbonate during preparation.

Evaluation of the optimization

First-generation hermaphrodites of *Heterorhabditis* genus highly correlate with the yield of reproduction (Zioni et al., 1992; Strauch & Ehlers, 2000; Ferreira et al., 2014). Thus, one criterion of the evaluation was the eggs of the hermaphrodites. Five first-generation hermaphrodites were collected from surface of each agar and dissected separately in Petri dishes. Dispersed eggs were counted under microscope and average egg number was calculated.

Each EPN production aims to achieve maximum IJ yield. Although the number of hermaphrodite eggs is closely related with final yield, a linear relationship cannot be established between the total number of IJs and the number of hermaphrodite eggs due to many reasons during production. After 14 days of incubation, the Petri dishes were washed with distilled water and all produced IJs were transferred to a tube. Six 10 µl samples were taken from the tubes and counted under the microscope. The average number of IJ obtained from the samples was scaled to the total suspension in the tube and the total number of IJ obtained from the Petri dish was calculated.

Another part of the evaluation is to measure the length of IJs (Hirao et al., 2010; Ferreira et al., 2014). IJs are the only free-living, non-feeding and infective stage of EPNs. IJs consume their fat reserves to stay alive and higher body fat ratio provides longer life. Body length of ten in vitro produced IJs from each Petri were measured under an invert microscope using Leica Application Suite v3.2.

Virulence of the IJs is an important indicator for the quality of production. We tested the virulence of *in vitro* produced IJs on the larvae of *G. mellonella*. Each larva inoculated with 50 IJs and incubated at room temperature. After incubated, dead cadavers were dissected, and mortality rates was calculated. All experiments were repeated three times.

Statistical analyses

Results were subjected to one-way and full factorial ANOVA. Means of the treatments were compared using least significant difference at $\alpha = 0.05$ level. All analyses were done using JMP v11.0 software.

Results

Hermaphrodite eggs

WL agar containing lecithin had a statistically significant effect compared to the W (control) agar as well as the other agar media. However, WE agar was not statistically different from the control group W medium in which you were evaluated in terms of the number of hermaphrodite eggs ($F = 144$; $df = 3, 104$; $p < 0.0001$). When the effect of temperature on the number of hermaphrodite eggs was examined, 28°C had positive effect compared to other temperatures and was statistically different. The most detrimental temperature was 32°C ($F = 39.2$; $df = 2, 105$; $p < 0.0001$). There were significant differences between the effect of pH values on the number of hermaphrodite eggs ($F = 5780$; $df = 2, 105$; $p < 0.0001$). In the pH 9 agar media, a statistically significant decrease occurred, and the number of hermaphrodite eggs was approximately three times lower than the control, pH 7 (Figure 1).

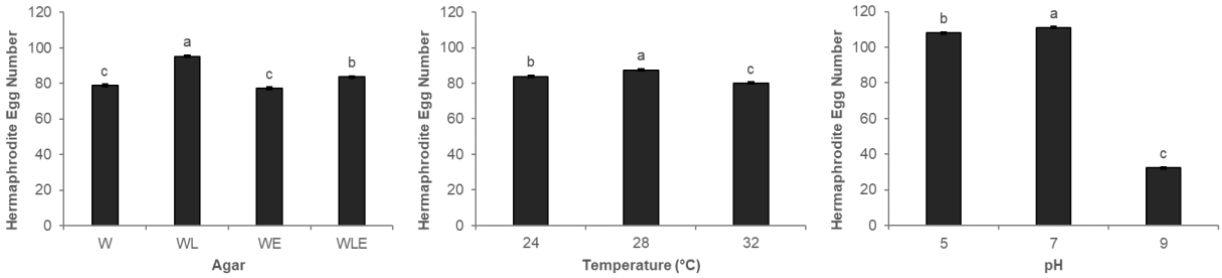

Figure 1. Effect of optimization parameters on hermaphrodite egg numbers.

Total IJs

Considering the total number of IJs, the agar ingredients had significant effect on IJ yield. Like the number of hermaphrodite eggs, it was determined that the WL agar containing lecithin gave better results than the other media in total IJ number and provided greater production efficiency ($F = 146$; $df = 3, 104$; $p < 0.0001$). When the effect of temperature on the total IJ number was examined, it was determined that the yield was statistically higher at 28°C than the other two temperatures. No statistical difference was found between the other two temperatures, 24 and 32°C ($F = 7.97$; $df = 2, 105$; $p = 0.0007$). Similar to the results of hermaphrodite egg count, the result with the lowest total IJs was at pH 9 ($F = 2840$; $df = 2, 105$; $p < 0.0001$). While no statistical difference was found between the other two pH values, the pH 9 value resulted in lower production yields (Figure 2).

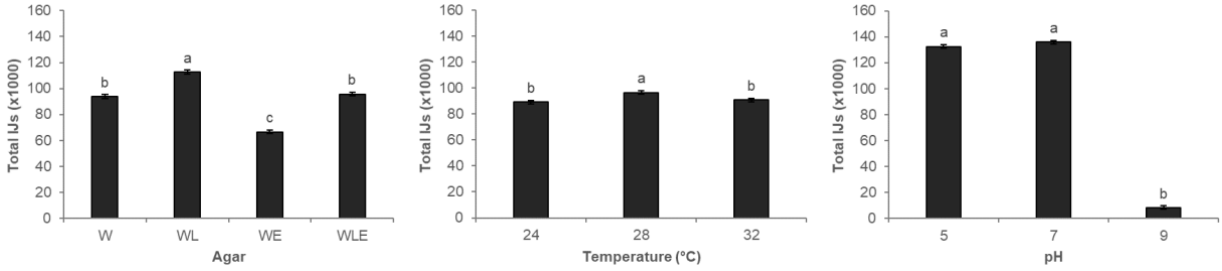

Figure 2. Effect of optimization parameters on total infective juveniles.

Body length of IJs

When the measurements were evaluated, it was determined that the media content ($F = 0.090$; $df = 3, 104$; $p = 0.97$) and temperature ($F = 0.460$; $df = 2, 105$; $p = 0.63$) did not have a statistically significant effect on the length of the produced IJs. Considering that IJs feed on bacteria growing on solid media, this result shows that media content and temperature do not have a positive or negative effect on the chemical compounds secreted by bacteria (Cabral et al., 2004). When the effect of pH value on IJ length was examined, it was determined that a negative effect occurred especially at pH 9 ($F = 7.73$; $df = 2, 105$; $p = 0.0009$). Although the optimum pH demand of symbiotic bacteria varies according to the species, it varies between 6 and 7 (Yoo et al., 2001). The chemical compounds secreted by the bacteria during development cause the pH value to change and have a negative effect in the long term. For this reason, pH is constantly monitored in liquid culture production and the production process is directed accordingly. Due to the high pH value of the agar in this study, it is thought that the length of the IJs was decreased (588.17 µ for pH 9 and a mean of 592.42 µ for the other treatments), especially due to the negative effects during the development of symbiotic bacteria.

Virulence of IJs

When the results were examined, it was determined that the agar content did not have any effect on the virulence of IJs on the *G. mellonella* larvae ($F = 1.00$; $df = 3, 104$; $p = 0.398$). Similar results were obtained in the temperature experiment ($F = 1.00$; $df = 2, 105$; $p = 0.398$). When the pH test results were examined, no statistically significant effect was observed on the efficacy, similar to the results in the other two criteria ($F = 1.00$; $df = 2, 105$; $p = 0.373$). All larvae were dead and virulence of all treatments was 100%.

Discussion

One of the obstacles to the widespread use of biological control is that biological products are expensive and unattractive to the producers. EPNs cannot compete with chemical products, especially due to the high production, formulation, and storage costs. By improving virulence, storage life or reproduction capacity, these costs can be also reduced indirectly (Shapiro-Ilan et al., 2012; Blackburn et al., 2016). In this study, it was aimed to optimize the *in vitro* solid mass production of the *H. bacteriophora* HBH hybrid strain. Effect of modified agar media, temperature and pH was tested on hermaphrodite egg number, total IJ number, IJ body length and virulence of IJs.

Optimization parameters have statistically significant effect on the number of hermaphrodite eggs. Hermaphrodite egg number is a key indicator in *in vitro* solid and liquid production (Zioni et al., 1992; Ciche et al., 2008; Clarke, 2008). The source of IJs is the eggs in hermaphrodites (Ehlers, 2001). Even though there are many other factors, a good hermaphrodite development has the potential to increase production efficiency.

The total number of IJs is generally accepted as one of the most important production criteria of *in vitro* production (Hirao & Ehlers, 2010; Addis et al., 2016; Leite et al., 2017). Although the number of hermaphrodite eggs and the total number of IJs can be seen as two directly related criteria, sometimes a parallel relationship cannot be established between these two criteria due to various reasons related to symbiotic bacteria (Johnigk & Ehlers, 1999; Ehlers et al., 2000). During the *in vitro* solid and liquid production process, the metabolites secreted by the bacteria change due to the changes in the content of the medium, and this result directly affect the production efficiency. Actually, when the criteria for the number of hermaphrodite eggs and the total number of IJs are examined, the results vary. It is thought that adverse conditions for the development of IJs occur in the later stages of production because of differences in media content and the effects of pH on symbiotic bacteria. However, when the yield results in the WL medium containing lecithin, which is the most efficient production medium, are examined, approximately 10,000 IJs are produced per 1 g medium. These results are comparable with the yields obtained in a previous study (El-Sadawy, 2011). In some studies, more than 100,000 IJ per 1 g medium was produced in solid culture (Tabassum & Shahina, 2004). The main reason is that the solid medium used in their work is sponge instead of agar. Sponge media is both a lower density than agar and an environment that allows three-dimensional production. In the agar, growth mostly occurs on the surface.

IJs are the only stage that can survive under the soil for a long time without feeding and are essential for EPNs to colonize insects. It is known that IJs use the lipid reserves in their bodies during survival without nutrition (Smart, 1995; Qiu & Bedding, 2002). In addition, EPNs accumulate lipids from the environment where they are fed (Blackburn et al., 2016). For this reason, providing more nutrients can increase the production quality (Yoo et al., 2000; Singh & Upadhyay, 2018). In the current study, lecithin was used as a fat source. It has been shown that symbiotic bacteria break down lecithin and reveal fatty acids due to secretion of lecithinase enzymes (Boemare et al., 1996). In the data obtained in the present study, the positive effect of the agar media containing lecithin was consistent with previous studies.

When the production yield was examined, there was very little production in some experiments. Despite low production, high virulence ratios can cause confusion. The main reason for this discrepancy is the use of 50 IJs per larva for all media, regardless of production yield. This result can be evaluated from different perspectives. One of the important features of the final product of *in vivo* or *in vitro* production is virulence. The data obtained from the virulence trials show that the efficacy of IJs produced under control combination (W-24-7) is not statistically different when compared with all other interactions. High virulence can be considered as a positive trait. Although the agar content was not suitable for the growth of IJs, ingredients of the agar did not adversely affect the symbiotic bacteria. However, since the production yield is very low, the importance of efficiency falls into the background. This has also been emphasized in some studies (Susurluk et al., 2013; Ulu & Susurluk, 2014). Based on the results of the current study, the best combination was WL-28-7 for the hybrid strain.

This study is one of the first studies in Turkey on the optimization of mass production of EPNs. Although studies have been conducted for many years around the world, it is thought that the study is important due to the use of a patented strain. There are many parameters used in *in vitro* production, however, the most important parameters for solid and liquid culture have been used to find optimized conditions for hybrid strain. More work is needed to improve the production efficiency and the quality of the IJs for efficient field application.

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Investigations on soil nematode diversity in three contrasting habitat types in Bolu, Turkey

Bolu İli'nde üç farklı habitat tipinde karasal nematod topluluklarındaki çeşitliliğinin incelenmesi

Şenol YILDIZ[1*] Emine GÖK[2] Göksel ÖZER[3] Mustafa İMREN[3]

Abstract

The study investigated nematode diversity in three contrasting habitat types around the Yeniçağa Lake, Bolu Province, Turkey, in 2019 and 2020. The Shannon-Wiener index was used to evaluate nematode diversity in different taxonomic categories (order, family and genus level), trophic group and colonizer-persister (c-p) group in grassland, cropland and peatland in two locations, Hamzabey and Adaköy, in the Yeniçağa Lake Reserve. The results revealed that there was statistically significant variation in the composition of nematode fauna between the study sites by the assessments of the higher taxa whereas genus level and family level lower taxa did not differentiate such variation in Hamzabey and Adaköy. In addition, the variation in nematode diversity in relation to soil types were better reflected by assessing the trophic group structures rather than the c-p groups. The findings indicated that the diversity at higher taxa might serve as a better indicator than the diversity of lower taxa (family and genera) variation among the habitat types of the study areas.

Keywords: C-p groups, diversity, indicator, terrestrial nematodes

Öz

Çalışma, 2019 ve 2020 yıllarında Bolu İli'ndeki Yeniçağa Gölü çevresinde üç farklı habitat tipi altında nematod çeşitliliğini incelemiştir. Shannon-Wiener indeksi, Yeniçağa Gölü rezerv alanındaki Hamzabey ve Adaköy lokasyonlarında otlak, tarım arazisi ve turbalık alanlardan üç arazi tipinde farklı taksonomik kategorileri (takım, familya ve cins seviyesi), trofik grup ve kolonizör-persister (c-p) grupları ve nematod çeşitliliğini değerlendirmek için kullanılmıştır. Sonuçlara göre, çalışma sahaları arasında nematod fauna kompozisyonunda, takım seviyesindeki taksonlarda istatistiksel olarak önemli farklılıklar olduğunu, cins seviyesi ve familya seviyesindeki düşük taksonlarda ise Hamzabey ve Adaköy'deki varyasyonları ayırt etmede daha zayıf kaldığı görülmüştür. Ayrıca, toprak tiplerine göre nematod çeşitliliğindeki varyasyonlar, c-p grupları yerine trofik grup yapıları değerlendirildiğinde daha iyi sonuçlar alınmıştır. Bulgular, yüksek taksonlardaki çeşitliliğin daha düşük taksonlara (familya ve cins) göre, çalışma alanındaki habitatlar arası farkın ayırımında daha iyi bir gösterge olabileceğini ortaya koymuştur.

Anahtar sözcükler: C-p grupları, çeşitlilik, indikatör, karasal nematodlar

[1] Kyrgyz-Turkish Manas University, Faculty of Agriculture, Department of Plant Protection, 720038, Bishkek, Kyrgyzstan
[2] Bolu Abant İzzet Baysal University, Faculty of Agriculture, Department of Wildlife Ecology, 14030, Bolu, Turkey
[3] Bolu Abant İzzet Baysal University, Faculty of Agriculture, Department of Plant Protection, 14030, Bolu, Turkey
* Corresponding author (Sorumlu yazar) e-mail: s7yildiz@gmail.com
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Introduction

Nematodes are one of the most important organisms in terrestrial habitats from which to draw information about the state of the ecosystem in which they live by analyzing their diversity. Due to their commonality and prevalence, which can exceed 10^6 individuals/m^2, they are a useful tool for studying soil biodiversity (Bongers & Bongers, 1998). Nematode diversity can vary widely depending on the type of ecosystem (Boag & Yeates, 1998; Nielsen et al., 2014; Song et al., 2017; Melakeberhan et al., 2021), and assessing nematode diversity in terrestrial habitats has been widely adopted as a research method.

Community structure of nematodes can reflect the state of soil biodiversity, soil conditions, including a source of information as the indicator of nutrient availability in ecosystems, and the current state of soil biota (Neher & Barbercheck, 1998; Ferris et al., 2001, 2012; Ferris & Tuomisto, 2015). Thus, due to their diversity and adaptability, they become the most functional secondary consumers of the soil fauna (Ferris et al., 2001; Mulder et al., 2005). As a reliable soil ecological indicator, they are expected to possess the potential reliable ecological soil indicator that can accurately determine the effects of soil variables on the soil biota. Any relationship with ecosystem services and fauna composition of nematodes still has some uncertainty, and there are no set of rules and protocols to follow, therefore; most assemblages of nematodes have shortcomings to rely on a single evaluation tool (Neher & Darby, 2009).

Nematodes have a number of biological characteristics that give them priority as the indicator organisms over other organisms in environmental monitoring programs since nematodes are easy to isolate from soil, they are relatively easier to examine under a light microscope and no deep expertise is needed to count and allocate to trophic groups (Freckman, 1988). These communities in various terrestrial habitats and in almost all parts of the world have been extensively studied. Taxon diversity is one of the most common methods of assessing nematode diversity whereas some researchers prefer to use indices of nematode fauna (Bhusal et al., 2014). Like all other organisms, nematodes are classified into taxonomic categories and assigned to certain groups depending on their ecological role in the soil environment, and it is also possible to assign nematodes to functional groups, e.g., trophic adaptations and c-p groups (colonizer-persister), or combining both groups (Bongers & Bongers, 1998; Ferris et al., 2001; Yeates, 2003). Nematodes at the genera level can react differently to environmental change (Fiscus & Neher, 2002; Zhao & Neher, 2013). The Shannon-Wiener index (H') has been a widely used tool to define the diversity of the terrestrial nematode fauna (Yeates & Bongers, 1999; Neher & Darby, 2009). H' gives the best results for rare specimens (Neher & Darby, 2009; Zhao et al., 2014). A c-p scaling has been formulated including determining the ordination of nematode life-history strategy based on the ability of taxa to colonize and persist in habitats (Bongers, 1990).

A nematode community is usually arranged into feeding groups, i.e., bacterivores, fungivores, herbivores, omnivores and predators. Also, each nematode family can be allocated into a c-p scale (1-5) based on the idea that closely related species have similarities in terms of life-history traits based on an affinity in phylogenetic origin, morphology, anatomy, physiology and genetics (Bongers, 1990).

Although, there are numerous studies around the world on the influence of habitat and environmental variables on nematode community assemblages, large scale studies on the diversity of the nematode communities are not always applicable to every geographical region due to the existing variation from region to region, habitat to habitat. Therefore, local studies on nematode assemblages and some taxa associated with certain disturbances are considered more reliable and informative for land use in local areas. As such, this study collected data for the study area based on the local nematode communities affected by the Yeniçağa Lake surrounding habitat types which mainly differ in soil composition and land use regimes.

In the present study, H' was used to assess the taxonomic diversity of nematodes and c-p groups in grassland, cropland and peatland in the Lake Yeniçağa Reserve (Adaköy and Hamzabey) in Bolu Province of Turkey. The aim of this study was to determine (1) if the analysis of nematode diversity requires fine-level arrangements to monitor environmental differences between habitats, and (2) how the taxonomic diversity, trophic groups and c-p groups reflect environmental differences.

Materials and Methods

Study site

Lake Yeniçağa is located in Bolu, in the northwestern Black Sea Region, Turkey. The climate of the area is influenced by a warm temperate climate regime in the summer season and a cool and rainy in the winter season. Lake Yeniçağa lies between the 40º46'12"-40º47'24" N and 32º00'36"-32º02'24" E and extends over an area of about 2.78 km^2 with surroundings. The lake is located at an altitude of 990 m above sea level with the following characteristics: shallow, non-stratified, with a surface area of about 1.8 km^2, and an average depth of 4 m. The water level usually fluctuates depending on the season and extends to the surrounding reedy and peat zones.

Soil samples were collected from the Lake Yeniçağa study areas (Adaköy and Hamzabey) by selecting three types of habitat in each site: peatland, grassland and cropland. The peatland was the closest to the lake with minor disturbance; grassland was second after peatland with a typical transition zone with a high organic matter than arable land used as pasture for domestic animals and arable land with a slightly sloppy mineral soil structure that was cultivated for wheat and barley and/or maize.

Sampling

Soil samples were collected in May, July, and September 2018 and 2019 from three types of habitat in two locations. Thirty-two sampling sites were marked from each land cluster and a total of 96 samples were collected. The sampling points were in an S-shape for analyzing soil nematode communities across the entire area of each site. Each soil sample was taken from five sub-sampling points, which were then mixed thoroughly to produce a composite sample. All soil samples were placed in sterile plastic bags, in a box to keep cool and protect from sunlight before being transported to the laboratory.

Soil properties of the study site were as follows. Peat soils were dark in color due to the high content of the organic matter (≥50%). Arable land was loamy with 1-2% organic matter content, neutral to alkaline mineral soils. The grassland soil was between the peat and arable land soil having both types of properties especially high content of organic matter (≥15-20%).

Extraction and identification of nematodes

To extract nematodes from the samples, 100 g of soil was processed by using the Cobb sieving method (van Bezooijen, 2006). Each soil sample was immersed in 1 L of water for 60 min to allow soil clumps to dissolve and allow nematodes to move into the water. The suspension was then gently poured onto a 1-mm sieve to remove the debris and allowed to settle for 2 min in a 10 L bucket. The sample suspension was then poured onto a fine sieve (50 µm) to remove fine particles from the suspension containing nematodes. The resultant suspension was then poured onto cotton filter discs placed on Baermann funnels, and the nematodes were left to migrate to the bottom of the funnels over 24 h. Finally, the accumulated nematodes were collected from the tips of the funnels.

The nematodes were immediately counted to determine the total number using a stereomicroscope at 40× magnification and preserved in 4% hot formaldehyde. Temporary mounts of the nematode suspension were made, and nematodes were identified using a compound microscope at 400× magnification to the genus level, with the exception of individuals belonging to the Rhabditidae according to Bongers (1994).

Nematode trophy groups were defined as described in Yeates et al. (1993) and Okada et al. (2005); and the c-p g scale (1-5) were classified according to descriptions of Bongers & Bongers (1998).

Data analysis

In order to study the diversity of the nematodes, they were allocated to genera, families and orders to test the impact of the taxonomic composition. Although the division of nematodes into trophic groups and c-p groups in which nematodes are grouped into a 1-5 scale based on their sensitivities to the environmental differences besides their r-K life strategist features, is usually also based on the properties of the genera and family features (Yeates et al., 1993; Bongers & Bongers, 1998), and the feeding types (trophic groups) were identified based on the food preference of nematodes based on the structure of mouthparts described by Yeates et al. (1993) and Okada et al. (2005).

H' enables the weighting of rare specimens with the formula: $H' = -\Sigma P_i (\ln P_i)$, where P is the proportion of in the i-th taxon for the entire community (McSorley & Frederick, 1996; Pen-Mouratov et al., 2003; Pen-Mouratov & Steinberger, 2005). H' is frequently used to obtain information from various classification levels and varying mixed groups. However, the data require logarithmic transformation (x + 1) to adjust variance for normality and uniformity. The occurrence of nematodes was calculated as sites with nematodes/total sites × 100. To assess variation in the nematode compositions, a one-way ANOVA was calculated for habitat types. In case, the ANOVA result was significantly different ($P < 0.05$), the LSD test was run to evaluate the variation between the habitat types. In addition, Tamhane's (T2) was employed when the transformed data variances were not at the desired level to assess the effect of habitat type variation on nematode composition. One-way ANOVA calculations were performed using the SPSS24 (IBM, Armonk, NY, USA).

Results

Forty taxa of nematodes were recovered from the soil samples: Fifteen taxa of plant parasites, thirteen bacterivores, four fungivores, two predators and six omnivores. The abundance of nematodes ranged from 2 to 420 nematodes/100 g of soil and total numbers of 482 to 2,485 nematodes/100 g of soil in the samples. The abundance and frequencies of recovered nematodes are given in Table 1.

Diversity of nematode community

Diversity patterns of the order, family and genus of nematode generated by H' had significant differences ($p < 0.05$) among the three types of habitat (grassland, cropland, and peatland) in the two locations: Adaköy and Hamzabey in the Lake Yeniçağa lake reserve areas (Figure 1).

In Hamzabey, H' of cropland was significantly higher at the order level when compared to grassland and peatland. Significant differences ($p < 0.05$) were also found between the grassland and peatland at the order level (Figure 1a). In the family, H' of grassland and cropland differed significantly ($p < 0.05$) from peatland but not significantly between grassland and cropland (Figure 1c). At the genus level, H' of cropland was significantly ($p < 0.05$) higher than that of grassland and peatland, but significantly different between grassland and cropland (Figure 1c).

In Adaköy, H' for the grassland and cropland was significantly ($p < 0.05$) higher than that of peatland at the order level whereas there was no significant difference between the cropland and grassland at the order level (Figure 1d). H' of grassland was significantly ($p < 0.05$) different from that of cropland and peatland in the family level, but significantly different between cropland and peatland (Figure 1e). At the genus level, H' of grassland had a significantly ($p < 0.05$) higher value than that of cropland and peatland but was significantly different between cropland and peatland (Figure 1f).

Table 1. The incidence values of nematode communities in grassland, cropland, and peatland areas (mean±SEM)

Nematode	Cropland		Grassland		Peatland	
	Abundance	Occurrence (%)	Abundance	Occurrence (%)	Abundance	Occurrence (%)
Plant Parasitic						
Helicotylenchus Steiner, 1945	7.6±2.2	38.6	6.4±1.8	22.5	4.5±1.6	13.5
Rotylenchus Linford & Oliveira, 1940	1.4±0.2	8.2	2.2±0.6	12.8	0.5±0.2	3.8
Heterodera Schmidt, 1871	1.2±0.1	8.3	0±0.0	0.0	0±0.0	0.0
Meloidogyne Göldi, 1889	1.4 ±0.1	4.3	0±0.0	0.0	0±0.0	0.0
Merlinius Siddiqi, 1970	42.4 ±4.2	72.5	48.6 ±5.1	76.4	31.5 ±5.1	36.7
Tylenchorhynchus Cobb, 1913	51.4 ±4.9	75.8	46.8 ±5.1	69.7	36.4 ±5.1	43.8
Paratylenchus Micoletzky, 1922	12.8 ±2.1	36.8	20.4 ±3.2	43.4	10.2 ±2.1	23.1
Pratylenchoides Winslow, 1958	21.2±2.2	43.7	28.6±2.2	51.8	16.2±2.8	26.5
Pratylenchus Filipjev, 1936	22.4 ±3.9	56.4	34.5 ±3.2	73.8	6.6 ±2.8	36.6
Trophurus Loof, 1956	0.6±0.2	4.7	0.4±0.2	3.2	0±0.0	0.0
Paratrophurus Arias, 1970	0.8±0.3	6.8	0±0.0	0.0	0.6±0.4	4.1
Filenchus Andrassy, 1954	70.6±7.4	64.4	0±0.0	0.0	0±0.0	0.0
Tylenchus Bastian, 1865	26.8 ±4.2	28.6	36.8 ±5.3	64.4	18.4 ±3.4	22.4
Psilenchus De Man, 1921	0.8 ±0.2	4.2	1.5 ±0.4	8.9	0.6 ±0.8	2.1
Criconema Hofmanner & Menzel, 1914	46.5±8.6	35.4	58.4±9.2	52.8	32.4±3.5	26.3
Bacterivores						
Rhabditis Dujardin, 1845	26.4±5.7	65.8	38.2±9.2	70.4	15.3±1.4	34.1
Monhysteridae De Man, 1876	74.4±5.4	88.7	80.6±6.5	92.4	55.4±1.6	62.4
Cephalobus Bastian, 1865	123.2±11.6	100.0	132.8±6.5	100	84.6±1.6	92.5
Eucephalobus Steiner, 1936	102.6±8.8	100.0	118±5.5	100	84.6±1.6	92.5
Acrobeloides Cobb, 1924	108.6±9.8	100.0	55.4±4.8	84.5	38.4±3.4	45.4
Acrobeles Von Linstow, 1877	88.6±5.5	96.4	64.8±6.3	76.3	42.4±4.6	49.8
Achramodora	5.9±0.8	18.8	8.7±0.6	24.4	0±0.0	0.0
Cervidellus Thorne, 1937	4.4±0.6	21.4	0.±0.0	0.0	6.7±0.7	24.4
Alaimidae Thorne 1934	2.2±0.2	11.2	0±0.0	0.0	0±0.0	0.0
Alaimus De Man, 1880	6.2±5.5	14.3	8.3±0.6	26.4	2.9±0.6	6.4
Wilsonema Cobb, 1913	4.8±0.6	11.4	6.3±0.8	17.6	3.2±0.2	4.2
Plectus Bastian, 1865	88.4±4.6	92.8	103.4±5.5	100	52.4±4.8	70.4
Panagrolaimus	1.1±0.1	4.5	0±0.0	0.0	0±0.0	0.0
Fungivores						
Aphelenchoides Fischer, 1894	82.8±5.4	100.0	114.8±8.4	100	66.8±5.9	86.8
Aphelenchus Bastian, 1865	66.6±7.9	98.8	94.6±4.5	96.5	64.6±6.5	72.8
Ditylenchus Filipjev, 1936	48.8±5.4	79.6	86.6±6.4	88.4	58.6±4.9	68.8
Tylencholaimus De Man, 1876	0±0.0	0.0	0±0.0	0.0	4.6±0.4	8.2
Predators						
Mononchus Bastian, 1865	1.9±0.4	22.2	1.4±0.5	65.6	0.6±6.5	12.4
Seinura Fuchs, 1931	0.6±0.1	18.2	1.1±0.2	36.4	0±0.0	0.0
Omnivores						
Dorylaimidae De Man, 1876	2.9±0.5	6.6	3.4±0.8	8.8	1.6±0.9	8.8
Dorylaimus Dujardin, 1845	0±0.0	0.0	1.4±0.6-2	5.4	0±0.0	0.0
Mesodorylaimus Andrassy 1959	0.9±0.2	5.4	0.8±0.8	4.2	0.4±0.1	1.2
Prodorylaimus	10.2±0.7	11.4	12.4±0.6	14.6	6.8±0.8	7.2
Aporcelaimus Thorne & Swanger, 1936	5.9±1.1	8.4	0±0.0	0.0	5.5±0.7	6.9
Aporcelaimellus Heyns, 1965	44.2±3.4	74.6	48.6±1.4	85.4	78.4±4.6	54.4

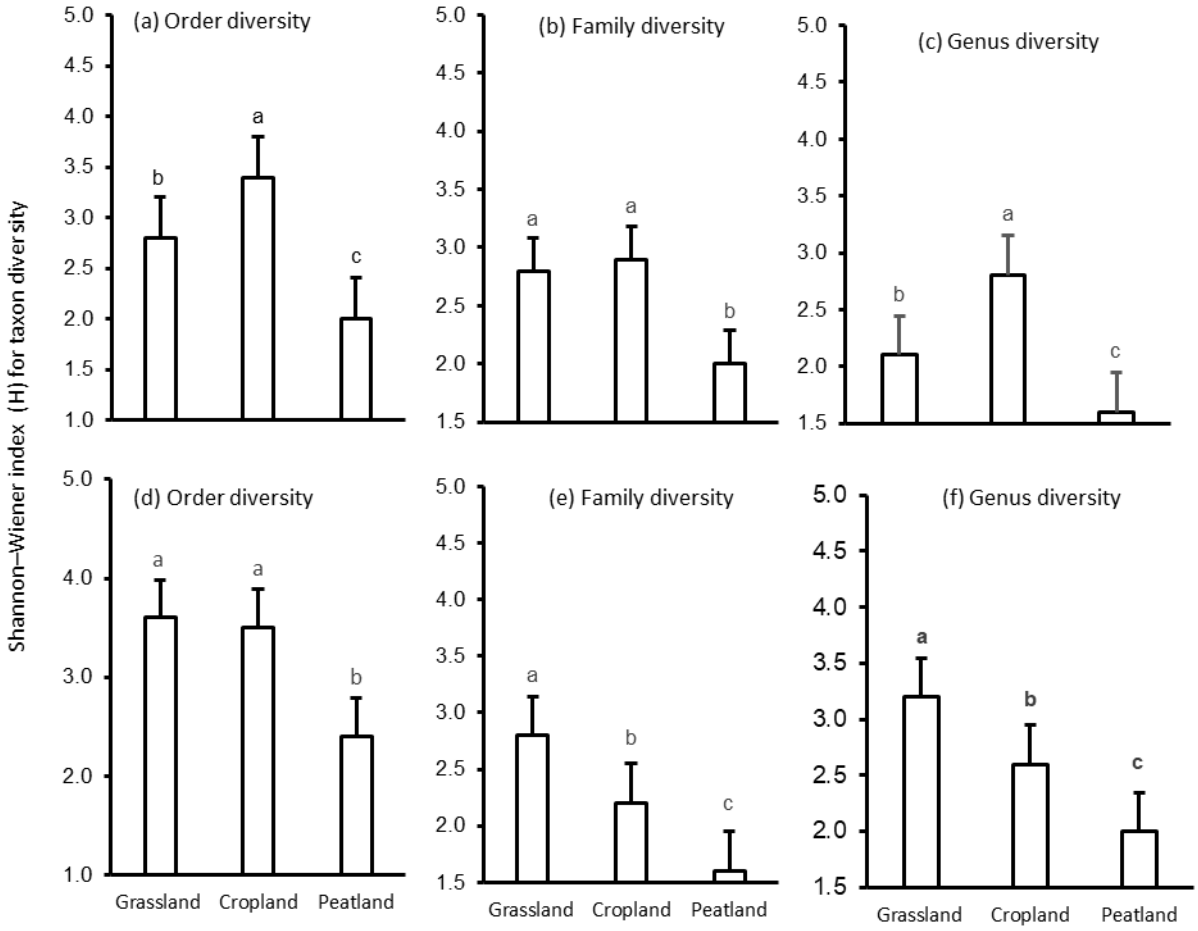

Figure 1. Distribution of Shannon-Wiener indices indicating the taxonomic structure of study sites (Hamzabey (a-c) and Adaköy (d-f) and significance levels ($P < 0.05$) and SEM for each study plot.

Diversity indices for trophic structure

The trophic structure of the nematode community and H' had significant differences ($p < 0.05$) among the three types of habitat in the two reserve areas (Figure 2). In Hamzabey, H' of bacterivores, and fungivores for the peatland was significantly lower ($p < 0.05$) than that of grassland and cropland. It was also significantly lower for the grassland than for the cropland (Figure 2a, b). There were no significant differences in the value of H' of herbivores nematodes among the three habitats (Figure 2c). H' of defining the omnivores was significantly higher in the grassland when compared to the cropland and peatland. Also, it had a significantly lower value in the cropland than in the peatland (Figure 2d). H' of the predator nematodes had no significant difference between grassland, cropland and peatland (Figure 2e).

In Adaköy, the cropland and grassland had higher index values that might be associated with H' of cropland and peatland for bacterivores, and fungivores. There were also significant differences ($p < 0.05$) between cropland and grassland (Figure 2g, f). For the herbivores and omnivores, H' was significantly higher value for the grassland than for peatland and cropland. There was a significant difference ($p < 0.05$) between the cropland and peatland (Figure 2h, i). Also, the H' was significantly higher for the grassland than the cropland and peatland for predators (Figure 2j).

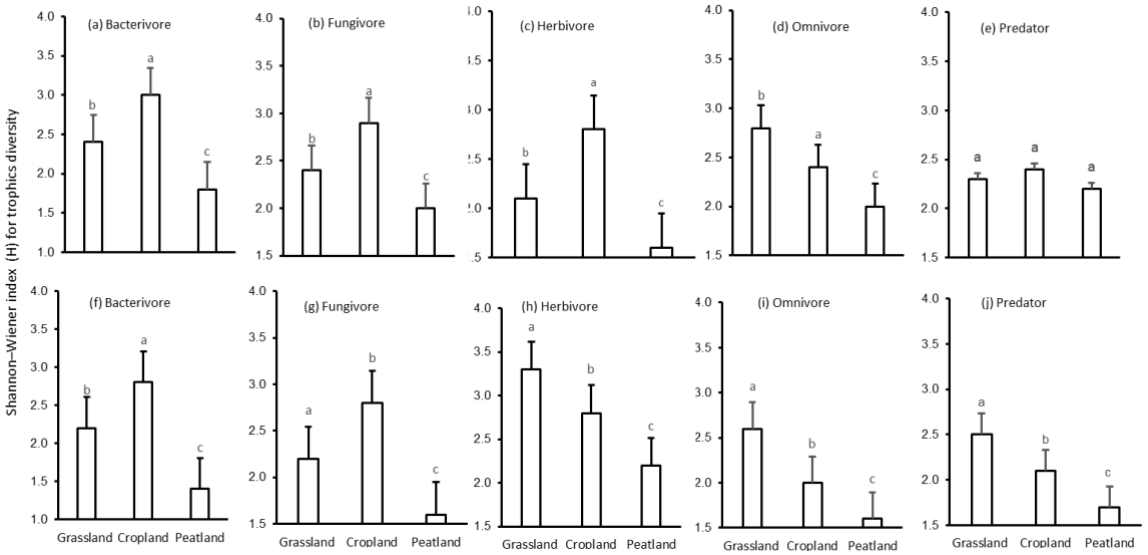

Figure 2. Distribution of Shannon-Wiener indices indicating the trophic structure of study sites (Hamzabey (a-e) and Adaköy (f-j) and significance levels ($P < 0.05$) and SEM for each study plot.

Diversity indices for c-p groups

For the diversity of groups cp1, c-p2, c-p3, c-p4, and c-p5, H' had significant differences ($p < 0.05$) among the three habitats in the two study sites (Figure 3). In Hamzabey, H' of cp1 group, c-p4 group, and c-p5 group for cropland and peatland were significantly higher for the grassland than (Figure 3a, d, e). There was also a significant difference between the cropland and peatland. However, the c-p2 and the c-p3 groups had high index values in cropland when compared to that of grassland and peatland. There was also a slight difference between grassland and peatland in Hamzabey (Figure 3b, c).

In Adaköy, H' of the cp2 group and the cp3 group for the cropland and grassland were significantly higher than for peatland, with no significant differences between cropland and grassland (Figure 3g, h). The cp1 group, the cp4 group, and the cp5 group also had a low H' and did not show significant differences among the three habitats (Figure 3f-j).

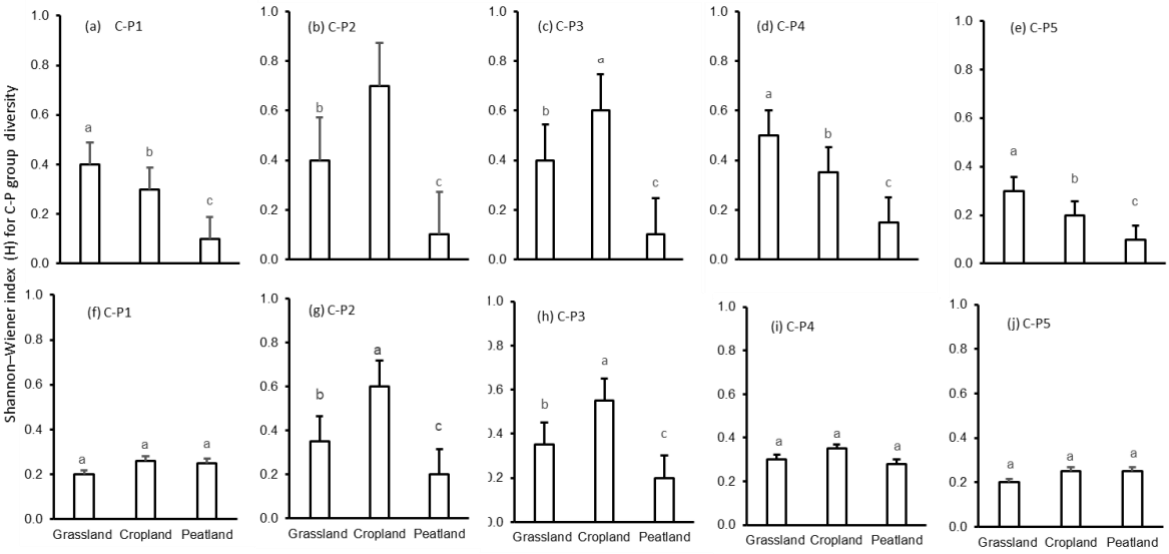

Figure 3. Distribution of Shannon-Wiener indices indicating the colonizer-persister groups of study sites (Hamzabey (a-e) and Adaköy (f-j) and significance levels ($P < 0.05$) and SEM for each study plot.

Discussion

The study revealed that the diversity of nematode fauna in the upper taxa, particularly at the order level, provides more reliable information on habitat type than the lower taxa (at family or genus level), so assessments based on the lower taxonomic categories are not always more sensitive than higher taxa that have a larger number of members to reflect habitat conditions. However, large scale studies on the diversity of the entire terrestrial nematode communities is practically impossible due to the variation from region to region, instead, local studies on nematode assemblages and some taxa associated with certain disturbances are considered more reliable.

The study findings revealed that difference in the nematode genera and family structures had similar patterns as in previous studies (Bhusal et al., 2014; Li et al., 2020). The inconsistency in the differences of the orders, families and genera compositions associated with the habitat types may be attributed to variation in the number of taxa recovered. It is well-documented that soil nematodes are strongly influenced by their microenvironment, which can also reflect the health and function of soil (Mulder et al., 2005). Agricultural management measures (e.g., tillage, irrigation and fertilization) cause disturbances in the soil ecosystem (Bongers et al., 1997) and influence communities of the soil nematode (Yardirn & Edwards, 1998). The abundance of nematodes reflects the ability of the soil to perform basic ecological functions (e.g., nutrient cycling) (Overstreet et al., 2010). Differences in the numbers of nematodes, trophic groups and c-p groups were observed among the three habitats: in grassland, cropland and peatland, indicating that reclamation had a significant impact on the nematode communities. The abundance and diversity of nematodes are closely related to the organic matter in the soil (Wall et al., 2002). The increase in organic matter provides an abundant source of food, especially for the free-living nematodes. In the process of reclamation, the application of organic or mineral fertilizers, and long-term agricultural cultivation resulted in the residue of plant roots into the soil, which increased the organic matter content in the grassland and cropland soil. However, due to their compact structure and low oxygen content, peatlands were not conducive to the survival of nematodes, which limited their abundance to relatively low levels.

The diversity pattern, as well as the structures of trophic groups and the compositions of c-p groups of the nematode communities in the study area, had some differences. In particular, the trophic groups differed more strongly between habitat type than the c-p groups. The findings of the study were consistent with those of Bushal et al. (2014), who suggested that trophic groups might be a better tool as an indicator of ecological aspects of the nematode fauna. In addition, the structure of trophic groups, described through H', were significantly different between the habitats in the current study. As a final statement, the results indicated that there were similar patterns but higher taxonomic grades might be preferable to the diversity of trophic groups to reflect different characteristics of the heterogeneous ecosystems or habitat types. Nematodes occupy several different trophic levels of soil biota and have adapted to take several different food sources (Bongers, 1990; Yeates et al., 1993; Zhao & Neher, 2013). Thus, environmental conditions especially food sources have different availabilities among the ecosystems; therefore, trophic groups of nematodes may better reflect differences in the variation of resource types and as well as environmental change (Ferris & Tuomisto, 2015).

In this study, the dominant trophic groups were bacterivores and fungivores. They contained many individuals that constitute the bulk of nematode communities in the cropland and grassland. This result differs from the study of Ou et al. (2005), who found herbivores to be the dominant trophic group in maize fields with yellow brown soil. This may be due to differences in climate and soil type (Yeates & Bongers, 1999). Bacterivores are usually abundant in planting soils (Wardle et al., 1995). Our result contradicted his view. The high abundance of bacterivores and fungivores in the cropland and grassland means that they are important in the nutrient cycling (Mikola & Sulkava, 2001). After bacterivores and fungivores, herbivores dominated nematode communities in cropland and grassland. The reason why the high abundance of

bacterivores indicates that they feed on bacteria and the intake of organic matter nourishes many of the bacteria in the farmland (Bulluck III et al., 2002). Van der Putten & van der Stoel (1998) noted that the feeding of herbivores accelerated the transportation of nutrients from plants to the soil ecosystem and promoted the accumulation of soil organic matter. The next trophic level organism (i.e., omnivores-predators) is slow to respond to prey, and soil pores can be effective in reducing the efficiency of predator access to prey (Mikola & Setälä, 1998). These results indicate that the flow of matter and energy in the soil food web run in the bottom-up direction. The frequency of nematodes feeding on bacteria and fungi is much higher than that of herbivores, omnivores and predators. A study by Sánchez-Moreno et al. (2006) indicated that agricultural management measures had a significant impact on growth than that of the reproduction of K-strategist nematodes. In grassland and cropland, the mineralization of nitrogen is accelerated when the microbivores (bacterivore and fungivore) nematodes dominate, increasing the availability of nitrogen to plants (Ferris et al., 2004).

The patterns of the c-p groups were different depending on the type of habitat, only the nematodes of the c-p2 group and the c-p3 group were significantly different between habitat types. The nematodes of the c-p2 and c-p3 groups were found to be relatively sensitive to environmental change (Bongers & Bongers, 1998). Thus, the variables of soil environment are the driving forces of these influences affecting differences in nematode fauna in ecosystems. As an example, mineral fertilization can reduce the incidence of nematodes in the omnivorous group (Neher & Barbercheck, 1998). Studies have shown that grassland with minor human intervention has had the potential to produce a substantial range of plant diversity over the last 25 years, which could ultimately become a rich resource of food for the soil fauna (Zhao et al., 2015). It can be concluded that abundant food resources, in addition to an undisturbed environment, may be more favorable for omnivore nematodes as well as the c-p3 nematode group. As a result, the taxonomic diversity of nematodes can also indicate a rich soil biota, the availability of nutritional resources, as well as disturbances in the ecosystem.

As mentioned above, when assessing diversity of taxonomic categories, the nematodes of trophic groups and c-p groups revealed significant effects of habitat types based on nematode community structures and can serve as acceptable indicators. In conclusion, analysis of nematode diversity at the higher level of classification (Ordo level) was more reliable for tracking variation in habitat types compared to the lower level of classification. In addition, the nematodes in trophic groups and c-p groups had significantly different patterns among habitat types. In the light of the study, the main order of the nematode diversity to express the responses of nematode fauna in these three types of habitat was peatland < cropland ≤ grassland, which is consistent with that from by Zhao et al. (2015) and Li et al. (2020). Conversely, environmental disturbance had a suppressive effect on the diversity of nematode fauna. The findings revealed that the diversity of nematodes in environmental monitoring might give beneficial information on soil biota via (1) the taxonomic structure or richness of the nematode fauna, (2) the structure of trophic groups and c-p groups of the given nematode community, and (3) taxon richness of each nematode trophic group.

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Original article (Orijinal araştırma)

Determination of the wing morphology differentiation of old and recent honey bee samples from western Turkey using geometric morphometrics[1]

Batı Anadolu eski ve yeni bal arısı örneklerinin kanat morfolojisi farklılaşmasının geometrik morfometri kullanılarak belirlenmesi

Mustafa KÖSOĞLU[2*] **Rahşan İVGİN TUNCA[3]** **Neslihan ÖZSOY[2]**
Yahya Tuncay TUNA[4] **Erkan TOPAL[2]** **Belgin GÜNBEY[5]**

Abstract

In this study, old and recent honey bee, *Apis mellifera* L., 1758 (Hymenoptera: Apidae) specimens were compared using geometric morphometrics. The old honey bee samples were collected from different apiaries in Edirne, Balıkesir, Çanakkale, Denizli and Muğla Provinces, and Gökçeada (an Aegean island) in Turkey in 1987-1988 under a project of the Aegean Agricultural Research Institute and the recent samples were collected in the same locations in 2017. The mean values determined for each region were grouped using Mahalanobis distances, and the results were summarized on dendrogram. While the old samples constituted one group, the recent samples constituted another one. When the results of the discriminant function analysis were compared, it was observed that overall old and recent samples were statistically different from each other ($P < 0.0001$). The evaluation of both groups has revealed that the recent population of Gökçeada was different from morphologically the other mainland populations in the current situation. However, the Thrace (Edirne) honey bee specimens were different from the Anatolian (Çanakkale, Denizli, Balıkesir, Muğla) and island (Gökçeada) specimens in the past according to dendrogram relationships.

Keywords: *Apis mellifera*, geometric morphometrics, Gökçeada, honey bee, Thrace, Turkey

Öz

Bu çalışmada eski ve yeni bal arısı, *Apis mellifera* L., 1758 (Hymenoptera: Apidae) örnekleri geometrik morfometrik yöntemlerle morfolojik olarak karşılaştırılmıştır. Eski bal arısı örnekleri, Ege Tarımsal Araştırma Enstitüsü tarafından yürütülen bir proje kapsamında, 1987-1988 yıllarında Edirne, Balıkesir, Çanakkale, Denizli, Muğla illeri ve Gökçeada Adası'ndaki farklı arılıklardan toplanmıştır ve 2017 yılında aynı lokasyonlardan alınan güncel bal arısı örnekleri ile çalışılmıştır. Her bölge için belirlenen ortalama değerler Mahalanobis mesafesi kullanılarak gruplandırılmış ve sonuçlar dendrogram üzerinde özetlenmiştir. Eski örnekler bir grubu oluştururken, güncel örnekler bir diğerini oluşturmuştur. Eski ve güncel örnekler için diskriminant fonksiyon analizi sonuçları karşılaştırıldığında, geçmiş ve şimdiki genel örneklerin istatistiksel olarak birbirinden farklı olduğu gözlenmiştir ($P < 0.0001$). Her iki grubun değerlendirmesi, Ada'nın (Gökçeada) mevcut popülasyonunun, mevcut durumda diğer anakara popülasyonlarından morfolojik olarak farklı olduğunu ortaya koymuştur. Öte yandan Trakya (Edirne) bal arısı popülasyonunun, dendrograma göre geçmişte Anadolu (Çanakkale, Denizli, Balıkesir, Muğla) ve Ada (Gökçeada) bal arısı örneklerinden farklı olduğu belirlenmiştir.

Anahtar sözcükler: *Apis mellifera*, geometrik morfometrik, Gökçeada, bal arısı, Trakya, Türkiye

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[2] Apiculture Research Center, Aegean Agricultural Research Institute, 35661, Menemen, Izmir, Turkey
[3] Muğla Sıtkı Koçman University, Ula Ali Koçman Vocational School, Department of Plant and Animal Breeding, Apiculture Program, 48640, Ula, Muğla, Turkey
[4] Tekirdağ Namık Kemal University, Agriculture Faculty, Department of Animal Science, 59030, Tekirdağ, Turkey
[5] Republic of Turkey Ministry of Agriculture and Forestry, General Directorate of Agricultural Research and Policies (TAGEM), 06800, Ankara, Turkey
* Corresponding author (Sorumlu yazar) e-mail: mustafakosoglu@gmail.com
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Introduction

The previous studies conducted in Turkey revealed that there are different honey bee subspecies and ecotypes in Turkey (Kandemir et al., 2006; Bodur et al., 2007; Tunca, 2009; Tunca & Kence, 2011). Bodenheimer (1941) defined the honey bees, *Apis mellifera* L., 1758 (Hymenoptera: Apidae) morphologically in Anatolia and reported that there were different honey bee subspecies from western to eastern of Turkey. Despite the low level of economic efficiency of local bee races and subspecies in Turkey, they adapt to the specific conditions of their geographical regions, resistant to various diseases, and are able to produce in extreme environmental conditions and maintain the reproductive ability. For the existence of these genetic resources, it is necessary to reveal their superior characteristics and to benefit from these qualities both today and for future. This situation can be revealed with current methods (Ertuğrul et al., 2000; Kence, 2006).

The studies on genetic variation in different populations have been conducted for a long time (Smith, 2002). It was reported that there was a risk of losing the genetic differences of the bees in Turkey before fully characterizing them. The debates on the subject about the threat of the genetic diversity of the honey bees have recently gained importance in Turkey. Consequently, morphological and genetic studies have been conducted to examine the effects of migratory beekeeping (Kükrer, 2013; Kambur et al., 2018). In order to morphologically distinguish honey bee samples, there has been a transition from the standard to geometric morphometrics methods (Tofilski, 2008; Turan, 2011; Koca, 2012; Koca & Kandemir, 2013). The honey bees identified by Ruttner (1988) in the Middle East have been now analyzed by way of geometric morphometrics, which is more reliable to distinguish the honey bee subspecies (Koca & Kandemir, 2013).

The geometric morphometric method can help to distinguish shape differences more clearly despite the body size being more readily affected by the environmental conditions, as differences in the, shape of the body mostly originate from genetic differences (Kence, 2006). As a result, the data obtained from geometric morphometry gives more reliable classification of honey bees than standard morphometrics (Kence, 2006; Koca & Kandemir, 2013; Kambur et al., 2018). Nowadays, both geometric morphometric and DNA-based studies are used to determination of evolutionary lineages or subspecies and in evaluating of genetic structure within honey bee subspecies (Barour & Baylac, 2016; Zammit-Mangion et al., 2017; Alattal et al., 2019; Henriques et al., 2020).

In this study, honey bee samples collected from western Turkey 30 years ago were compared with recent bee samples collected in 2017 from local beekeepers from the same regions. The aim of the study is to reveal possible changes in populations with the possible effects of climate change, production activities and migratory beekeeping over this period. In a previous project conducted by the Aegean Agricultural Research Institute in 1987-1988, the number of colonies was around 2.8 million in Turkey. Now there are approximately 8 million colonies in Turkey based on the 2018 data FAO (FAO, 2018). This study was conducted to determine if there is any change in the wing morphology between the old and recent honeybee samples.

Materials and Methods

Honey bee samples examined were composed of two groups; old and recent samples (Table 1). All of the old and recent samples were obtained from the same apiaries located in Edirne, Balıkesir, Çanakkale, Denizli, Muğla and Gökçeada. The old samples had been collected in 1986 (Öztürk et al., 1992) and deposited as a collection at the Aegean Agricultural Research Institute. The recent samples represented were collected from the same apiaries and regions as for the old ones. For each location, 50 wing samples were used (300 old and 300 recent samples; 600 as total).

Table 1. Areas (number of sites and apiaries) where measured samples (50 bees each, 600 total) were collected

Old samples (1987-1988)			Recent samples (2017)		
Area	Sites	Apiaries	Area	Sites	Apiaries
Balıkesir	2	6	Balıkesir	4	7
Çanakkale	7	33	Çanakkale	6	6
Denizli	6	13	Denizli	3	6
Edirne	3	8	Edirne	4	10
Muğla	9	74	Gökçeada	1	6
Gökçeada	1	11	Muğla	7	19

All samples were collected from local beekeepers that were not in contact with migratory beekeepers. Six hundred honey bee samples were measured in this study. At least 20 samples from each hive were collected from local beekeepers. The forewings of worker bees were dissected and prepared on slides and their high-resolution photos were taken under microscope (BAB-STR 45) for geometric morphometric analysis. Twenty landmarks on the right-side forewings were digitized according to Bookstein's landmark definition (Bookstein, 1990) (Figure 1). Data files (tps) were prepared using tpsUtil 1.40 and landmarks were digitized on the images using tpsDig 2.11 (Rohlf, 2008a; Rohlf, 2008b). In order to assess the variation among honey bee samples, procrustes ANOVA test, canonical variate analysis (CVA), and discriminant function analysis (DFA) were performed with MorphoJ version 1.06d program (Klingenberg, 2011). A UPGMA cluster analysis was performed on Mahalanobis distances of data to show the clustering among honey bee populations using NTSYS-PC (2.2) (Rohlf, 2000).

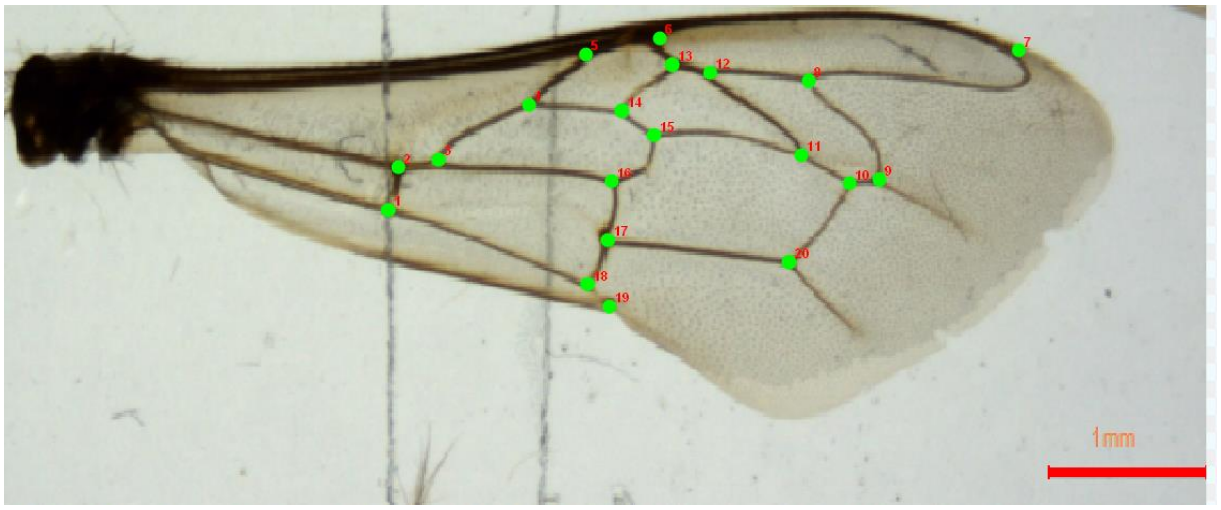

Figure 1. Location of landmarks on *A. mellifera* worker the fore wing.

Results

Old (1987-1988) honey bee samples - 300 wings were analyzed from the old samples. Procrustes ANOVA test applied to assess the population differences showed significant shape differences between locations ($P < 0.0001$) but not significant in terms of centroid size (Table 2).

Table 2. Procrustes ANOVA for old honey bee samples

Centroid size							
Effect		SD		MS	df	F	P (param.)
Individual		12373		2475	5	1.77	0.118
Residual		410518		1396	294		
Shape, procrustes ANOVA							
Effect	SD	MS	df	F	P (param.)	Pillai trace	P (param.)
Individual	0.0205	0.000114	180	5.34	< 0.0001	1.55	< 0.0001
Residual	0.225	0.0000213	10584				

CVA indicated that the total shape variation was explained by five axes as 41.8, 25.6, 13.8, 11.3, and 7.47%, respectively. The first three axes explained 81.3% (cumulative) of the total variation among the honey bee groups. Edirne (Thrace), Gökçeada (Aegean island) and Balıkesir (Anatolia) each of them formed a separate group, while other populations including Muğla, Denizli and Çanakkale populations formed one group according (Figure 2).

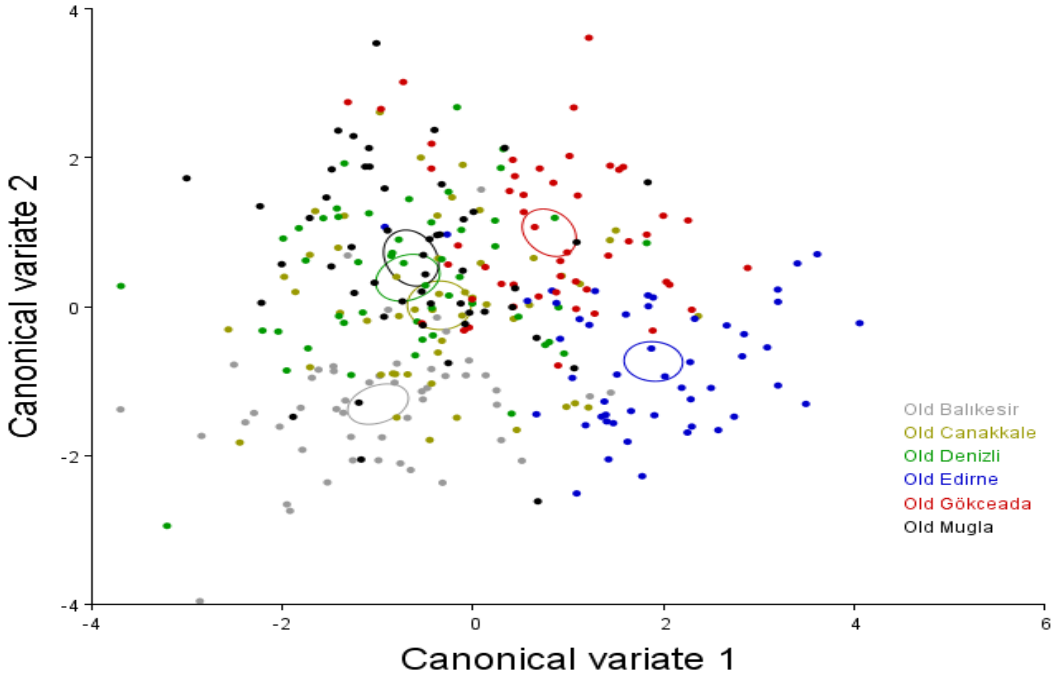

Figure 2. Distribution of the form differences generated by 20 landmarks in the old worker bee wing samples on the first two axes (canonical variate analysis).

DFA showed that differences both means of procrustes and Mahalanobis distances and also true allocation of the population comparisons between old Çanakkale-old Denizli, old Çanakkale-old Muğla, old Denizli-old Muğla were not statistically significant (P > 0.0001). Comparisons old Balıkesir-old Çanakkale, old Balıkesir-old Denizli pairs were also not significant (P > 0.0001) according to the t-square calculated from Mahalanobis distances (Table 5).

Recent (2017) honey bee samples - 300 worker bee wing samples were analyzed and compared by locations. In the procrustes ANOVA test, the shape and centroid were estimated from total variation based on size (Table 3).

Table 3. Procrustes ANOVA for recent honey bee samples

Centroid size							
Effect	SS	MS	df	F	P (param.)		
Individual	25855	5171	5	5.34	0.0001		
Residual	284472	968	294				
Shape, procrustes ANOVA							
Effect	SS	MS	df	F	P (param.)	Pillai trace	P (param.)
Individual	0.0125	0.0000694	180	3.8	< 0.0001	1.52	< 0.0001
Residual	0.190	0.0000180	10584				

Procrustes ANOVA test showed that there were statistically significant differences between locations in terms of shape (P < 0.0001). Three main clusters were observed on CVA distribution diagram. Gökçeada and Edirne populations were different from other populations collected from the west part of Anatolian populations (Figure 3).

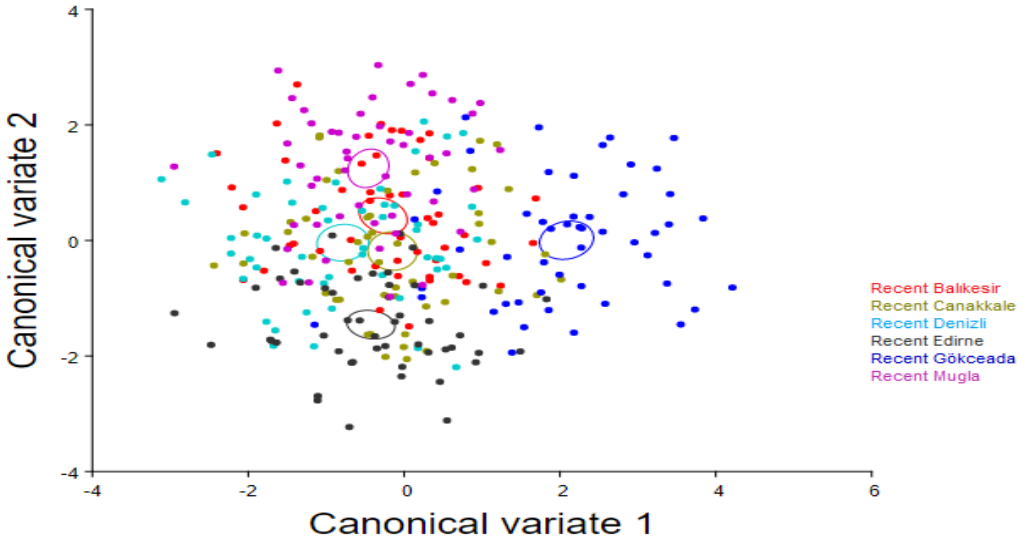

Figure 3. Distribution of form differences generated by 20 landmarks in recent worker bee wing samples on first two axes (canonical variate analysis).

With CVA of the six populations, the total shape variation was explained by five axes as 38.0, 27.1, 18.2, 11.4 and 5.34%, respectively. The first three axes explained 83.3% (cumulative) of the total variation. DFA showed that differences both means of procrustes and Mahalanobis distances and also true allocation of the population comparisons between the recent populations of Balıkesir-Çanakkale and Çanakkale-Denizli were not statistically significant (P > 0.0001) (Table 5).

Old (1987-1988) and recent (2017) honey bee samples - procrustes ANOVA test revealed that there are significant differences between the locations of the old and recent samples in terms of both centroid size and shape (P < 0.0001) (Table 4).

Table 4. Procrustes ANOVA for old versus recent honey bee samples

Centroid size							
Effect	SS	MS		df	F	P (param.)	
Individual	1301016	118274		11	100	< 0.0001	
Residual	694990	1181		588			
Shape, procrustes ANOVA							
Effect	SS	MS	df	F	P (param.)	Pillai	P (param.)
Individual	0.0556	0.000140	396	8.36	< 0.0001	2.21	< 0.0001
Residual	0.415	0.000019	21168				

The total shape variation was explained by eleven axes. The first three axes explained 70.5% (cumulative) of the morphological variation of populations (first three axes: 39.6,19.2 and 11.8%). The CVA from populations also showed that old and recent populations were clustered distinctly from each other. While old and recent populations were in two groups on the plot, it was also observed the differences the populations represented within their groups (old or recent) (Figure 4). According to DFA, there were significant differences between population both in procrustes and Mahalanobis distances. (Table 5).

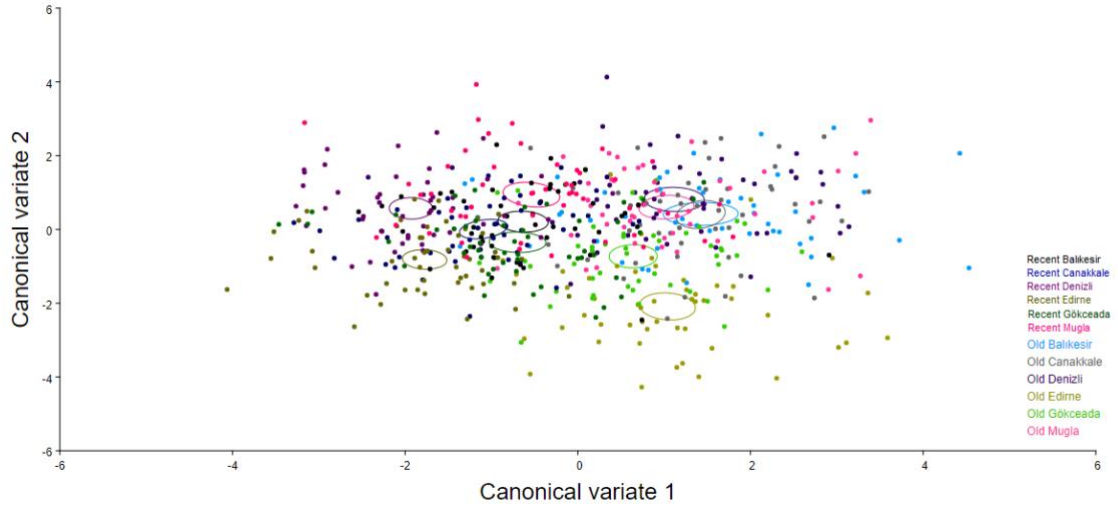

Figure 4. Distribution of the form differences generated by 20 landmarks in the old and recent worker bee wing samples on the first two axes (canonical variate analysis).

Comparisons recent Balıkesir-recent Muğla and recent Denizli-recent Edirne were not significant for procrustes distances (P > 0.0001). Comparisons old Çanakkale-old Denizli, Old Çanakkale-old Muğla, Old Denizli-old Muğla, recent Balıkesir-recent Çanakkale, recent Çanakkale-recent Denizli, recent Çanakkale-recent Edirne were not significant in terms of discriminant function and cross-validation (P > 0.0001). In the evaluation of the results regarding the old and recent bee samples simultaneously, the old and recent populations were grouped into two main clusters on the dendrogram (Figure 5). All old populations were placed on the one main branch. The important point is that old Edirne population was located from different branch from other old populations. However, the recent honey bee populations from Western Anatolia and Thrace specimens were clustered together, and Gökçeada population was separated from them. Evaluation of both groups shows that there are wing morphological differences in populations from past to present. According to analysis, Gökçeada population is separated from recent populations but in the past, it was clustered with Western Anatolia (Figure 5).

While the statistical differences among the old honey bee populations for Balıkesir-Denizli were not significant in the past, recent populations had significant differences for both locations according to Mahalanobis distances. However, the differences between the old Balıkesir and Muğla were significant in the past but this difference was not observed between both populations according to procrustes distances (Table 5).

Table 5. Discriminant function analysis for old, recent and combined honey bee populations

Comparison	Procrustes distance	Procrustes distance (P)	T-square (P)	Mahalanobis distance	T-square (value)	T-square (P)
Old Balıkesir-old Mugla	1.39 X10^{-2}	<0.0001	<0.0001	2.67	178	<0.0001
Old Balıkesir-old Canakkale	1.14X10^{-2}	<0.0001	<0.0001	2.35	138	0.0009
Old Balıkesir-old Denizli	0.98 X10^{-2}	<0.0001	<0.0001	2.45	150	0.0003
Old Balıkesir-old Edirne	1.69 X10^{-2}	<0.0001	<0.0001	3.36	282	<0.0001
Old Balıkesir-old Gökceada	1.84 X10^{-2}	<0.0001	<0.0001	3.52	309	<0.0001
Old Canakkale-old Denizli	0.15 X10^{-2}	0.017	0.324	1.59	63.3	0.329
Old Canakkale-old Edirne	1.29 X10^{-2}	<0.0001	<0.0001	2.83	200	<0.0001
Old Canakkale-old Gökceada	1.26 X10^{-2}	<0.0001	<0.0001	2.63	173	<0.0001
Old Canakkale-old Mugla	0.72 X10^{-2}	0.043	0.012	2.03	103	0.0172
Old Denizli-old Edirne	1.44 X10^{-2}	<0.0001	<0.0001	3.46	299	<0.0001
Old Denizli-old Gökceada	1.26 X10^{-2}	<0.0001	<0.0001	2.94	215	<0.0001
Old Denizli-old Mugla	0.92 X10^{-2}	0.002	0.002	2.28	130	0.0017
Old Edirne-old Gökceada	1.22 X10^{-2}	<0.0001	<0.0001	2.95	217	<0.0001
Old Edirne-old Mugla	1.43 X10^{-2}	<0.0001	<0.0001	3.58	321	<0.0001
Old Gökceada-old Mugla	1.27 X10^{-2}	<0.0001	<0.0001	2.90	210	<0.0001
Recent Balıkesir-recent Canakkale	0.70 X10^{-2}	0.009	0.15	1.73	74.8	0.155
Recent Balıkesir-recent Denizli	0.95 X10^{-2}	<0.0001	<0.0001	2.70	182	<0.0001
Recent Balıkesir-recent Edirne	1.16 X10^{-2}	<0.0001	<0.0001	3.14	246	<0.0001
Recent Balıkesir-recent Gökceada	0.97 X10^{-2}	<0.0001	<0.0001	2.99	224	<0.0001
Recent Balıkesir-recent Mugla	0.60 X10^{-2}	0.117	<0.0001	2.30	133	0.0013
Recent Canakkale-recent Denizli	0.63 X10^{-2}	0.037	0.002	2.25	126	0.0024
Recent Canakkale-recent Edirne	0.78 X10^{-2}	<0.0001	0.005	2.23	125	0.0027
Recent Canakkale-RecentGökceada	1.11 X10^{-2}	<0.0001	<0.0001	2.90	210	<0.0001
Recent Canakkale-recent Mugla	0.81 X10^{-2}	<0.0001	0.001	2.41	146	0.0004
Recent Denizli-recent Edirne	0.80 X10^{-2}	0.001	<0.0001	2.79	195	<0.0001
Recent Denizli-recent Gökceada	1.39 X10^{-2}	<0.0001	<0.0001	3.21	257	<0.0001
Recent Denizli-recent Mugla	0.86 X10^{-2}	<0.0001	0.004	2.32	134	0.0011
Recent Edirne-recent Gökceada	1.40 X10^{-2}	<0.0001	<0.0001	4.18	436	<0.0001
Recent Edirne-recent Mugla	1.18 X10^{-2}	<0.0001	<0.0001	3.52	310	<0.0001
Recent Gökceada-recent Mugla	1.16 X10^{-2}	<0.0001	<0.0001	4.04	408	<0.0001

Table 5. Continued

Comparison	Procrustes distance	Procrustes distance (P)	T-square (P)	Mahalanobis distance	T-square (value)	T-square (P)
Recent Balıkesir-old Balıkesir	1.53 X10^{-2}	<0.0001	<0.0001	2.97	220	<0.0001
Recent Balıkesir-old Canakkale	1.40 X10^{-2}	<0.0001	<0.0001	3.41	290	<0.0001
Recent Balıkesir-old Denizli	1.18 X10^{-2}	<0.0001	<0.0001	3.02	228	<0.0001
Recent Balıkesir-old Edirne	1.62 X10^{-2}	<0.0001	<0.0001	3.53	311	<0.0001
Recent Balıkesir-old Gökceada	1.28 X10^{-2}	<0.0001	<0.0001	3.10	240	<0.0001
Recent Balıkesir-old Mugla	1.60 X10^{-2}	<0.0001	<0.0001	3.33	278	<0.0001
Recent Canakkale-old Balıkesir	1.57 X10^{-2}	<0.0001	<0.0001	3.36	282	<0.0001
Recent Canakkale-old Canakkale	1.53 X10^{-2}	<0.0001	<0.0001	3.86	373	<0.0001
Recent Canakkale-old Denizli	1.39 X10^{-2}	<0.0001	<0.0001	3.87	373	<0.0001
Recent Canakkale-old Edirne	1.66 X10^{-2}	<0.0001	<0.0001	4.11	423	<0.0001
Recent Canakkale-old Gökceada	1.52 X10^{-2}	<0.0001	<0.0001	3.75	352	<0.0001
Recent Canakkale-old Mugla	1.80 X10^{-2}	<0.0001	<0.0001	3.91	382	<0.0001
Recent Denizli-old Balıkesir	1.18 X10^{-2}	<0.0001	<0.0001	3.95	390	<0.0001
Recent Denizli-old Canakkale	1.84 X10^{-2}	<0.0001	<0.0001	4.65	540	<0.0001
Recent Denizli-old Denizli	1.65 X10^{-2}	<0.0001	<0.0001	4.14	429	<0.0001
Recent Denizli-old Edirne	1.93 X10^{-2}	<0.0001	<0.0001	4.83	584	<0.0001
Recent Denizli-old Gökceada	1.75 X10^{-2}	<0.0001	<0.0001	4.47	500	<0.0001
Recent Denizli-old Mugla	2.06 X10^{-2}	<0.0001	<0.0001	4.45	496	<0.0001
Recent Edirne-old Balıkesir	1.91 X10^{-2}	<0.0001	<0.0001	3.93	386	<0.0001
Recent Edirne-old Canakkale	1.92 X10^{-2}	<0.0001	<0.0001	4.86	592	<0.0001
Recent Edirne-old Denizli	1.79 X10^{-2}	<0.0001	<0.0001	4.97	616	<0.0001
Recent Edirne-old Edirne	1.57 X10^{-2}	<0.0001	<0.0001	4.40	485	<0.0001
Recent Edirne-old Gökceada	1.71 X10^{-2}	<0.0001	<0.0001	4.25	452	<0.0001
Recent Edirne-old Mugla	2.13 X10^{-2}	<0.0001	<0.0001	4.24	449	<0.0001
Recent Gökceada-old Balıkesir	1.67 X10^{-2}	<0.0001	<0.0001	3.43	293	<0.0001
Recent Gökceada-old Canakkale	1.51 X10^{-2}	<0.0001	<0.0001	3.61	326	<0.0001
Recent Gökceada-old Denizli	1.37 X10^{-2}	<0.0001	<0.0001	3.76	354	<0.0001
Recent Gökceada-old Edirne	1.60 X10^{-2}	<0.0001	<0.0001	4.31	465	<0.0001
Recent Gökceada-old Gökceada	1.21 X10^{-2}	<0.0001	<0.0001	3.13	245	<0.0001
Recent Gökceada-old Mugla	1.65 X10^{-2}	<0.0001	<0.0001	3.66	334	<0.0001
Recent Mugla-old Balıkesir	1.46 X10^{-2}	<0.0001	<0.0001	3.40	288	<0.0001
Recent Mugla-old Canakkale	1.26 X10^{-2}	<0.0001	<0.0001	2.94	217	<0.0001
Recent Mugla-old Denizli	0.98 X10^{-2}	<0.0001	<0.0001	2.18	119	0.0045
Recent Mugla-old Edirne	1.60 X10^{-2}	<0.0001	<0.0001	3.56	317	<0.0001
Recent Mugla-old Gökceada	1.30 X10^{-2}	<0.0001	<0.0001	3.02	228	<0.0001
Recent Mugla-old Mugla	1.43 X10^{-2}	<0.0001	<0.0001	2.59	167	<0.0001

Permutation tests using the T-square statistic is equivalent to tests using Mahalanobis distances.

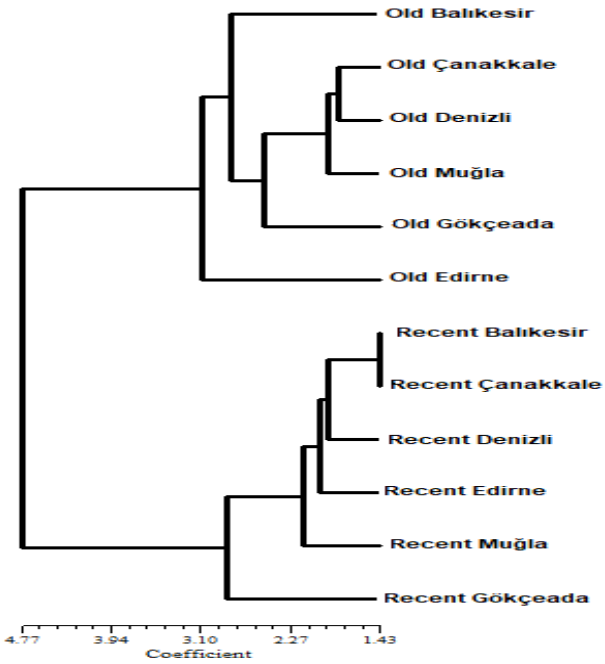

Figure 5. Dendrogram of old and recent honey bee populations.

Discussion

Morphological characters have been used to determine the insect populations with both traditional and geometric morphometrics (Zhou et al., 2018; Power et al., 2019). For more than 10 years, geometric morphometrics have been widely used in determine the variation of honey bee populations (Francoy et al., 2008; Tofilski, 2008; Francoy et al., 2009; Kandemir et al., 2009; Özkan & Kandemir, 2010; Santoso et al., 2018). The geometric morphometrics has enabled the construction of more meaningful clusters for the old and recent honey bees' specimens. The populations represented in the same locations from the past to the present have also revealed the differences from each other in this study.

The results of this study showed that there has been change in honey bee populations since 1987. Results for past and recent populations are consistent with previous studies (Kandemir et al., 2000; Palmer et al., 2000; Koca & Kandemir, 2013). In the recent honey bee populations, the significant differences were not observed pairs of Western Anatolia populations especially Balıkesir, Çanakkale, Muğla, Denizli and Edirne. The possible explanation of this is that these populations could be influenced by each other through the selling the queen bees and colonies during migratory beekeeping activities.

While there was a difference between old Balıkesir samples and old Muğla samples, no difference was observed between Balıkesir and Muğla recent samples. A reason for this, could be that pine honey production areas have moved northward due to climate change over recent years. Especially in Balıkesir, pine honey production areas have been expanding recently. This may be the reason that the producer of the region obtains queen bees from the colonies that are successful in pine honey production and chooses the colonies that are similar to Muğla bees in creating new colonies. While there is no difference between the old Çanakkale-old Denizli and recent Çanakkale-recent Denizli samples, the difference in comparing the old and recent samples explains that the change is holistic. This is also evident in the analysis results. Overall, there is a difference when all old and recent sampling locations are compared, and this includes the island population from Gökçeada.

Gökçeada population is different from other Anatolian and Thrace populations in present. It should be mentioned that Gökçeada bee registered officially by Republic of Turkey Ministry of Agriculture and Forestry in 2018 (Official Gazette 16/05/2019/30776). It showed that the island honey bee population can be maintained as long as there is no new introduction to the gene pool of the island from mainland. However, that the population may contract in the long term due to the inbreeding in island populations. The government agencies should development an alternative plan for this situation.

Overall data revealed that the bees from Thrace, Western Anatolia and Gökçeada are different from the past to the present and this change is significant (based on DFA). Even if there are some changes in the genetic material due to the intense migratory beekeeping and particularly due to the preference of queen bees of different genotypes from outside the region, so it is possible that the changes occurred gradually. Hence, both government institutions and beekeeper associations have to implement their strategic action plans for conservation of local bee populations as soon as possible. The observed changes in Turkish bee populations were reported in a previous study (Kambur & Kekeçoğlu, 2018).

As a result, although honey bees, whose lives are completely dependent on nature, can survive for thousands of years, it is known that beekeepers turn to new sources and want to increase production using different genotypes that are not suited to their regions in the long term. The effects of these choices made by beekeepers and their relationship with climate change have emerged as issues that should be studied and researched further. According to the results of this analysis, although the reason for the changes in the populations from the past to the present is possibly queen bee changes or migratory beekeeping, these two factors are actually a consequence of other factors. Yield or colony losses due to poor colony management led to the need for queen bee changes and migratory beekeeping. Also, uncontrolled sales of queens and colonies belonging to different subspecies and beekeeping activities conducted in different regions, especially during queen rearing seasons, can contribute to changes in the gene pools of populations. All problems on this subject can be solved with the joint decisions of producers, beekeeper associations and government institutions.

It is hoped that bees, whose lives are completely dependent on nature and have lived for thousands of years, will always continue their lives as an inseparable part of nature.

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Original article (Orijinal araştırma)

Determination of lambda-cyhalothrin and imidacloprid resistance and synergistic activity of diatomaceous earth in *Leptinotarsa decemlineata* Say, 1824 (Coleoptera: Chrysomelidae) populations[1]

Leptinotarsa decemlineata Say, 1824 (Coleoptera: Chrysomelidae) popülasyonlarında lambda-cyhalothrin ve imidacloprid direncinin ve diatom toprağının sinerjistik etkinliğinin belirlenmesi

Osman ÇAĞIRGAN[2*] **Hüseyin ÇETİN[3]**

Abstract

In this study conducted in 2020, the resistance of *Leptinotarsa decemlineata* Say, 1824 (Coleoptera: Chrysomelidae) populations collected from Karapınar, Çumra, Seydişehir, Güneysınır and Doğanhisar Districts of Konya Province, Turkey to lambda-cyhalothrin and imidacloprid was investigated. Insecticides were applied topically to fourth instar larvae. In addition, the synergistic efficiency of diatomaceous earth (DE) was investigated by applying imidacloprid plus DE (2% w/v) with the same methodology in the Karapınar population. As a result of the research, the resistance rate of all populations was found to be higher in imidacloprid than in lambda-cyhalothrin. The highest LD_{50} was found in the Çumra (0.212 µg/larvae) and Karapınar (0.456 µg/larvae) population for lambda-cyhalothrin and imidacloprid respectively. The highest synergism ratio was determined as 1.93 after 48 hours in imidacloprid plus DE application. As a result of the research, it is considered that the use of DE will make a great contribution to the control and resistance management of the imidacloprid resistant potato beetle.

Keywords: Diatomaceous earth, insecticide, *Leptinotarsa decemlineata*, resistance, synergism

Öz

Bu çalışma 2020 yılında yürütülmüş olup, Konya'nın Karapınar, Çumra, Seydişehir, Güneysınır ve Doğanhisar ilçelerinden toplanan patates böceği, *Leptinotarsa decemlineata* Say, 1824 (Coleoptera: Chrysomelidae) popülasyonların lambda-cyhalothrin ve imidacloprid etken maddelerine direnci araştırılmıştır. İnsektisit uygulamaları 4. dönem larvalara topikal olarak gerçekleştirilmiştir. Ayrıca Karapınar popülasyonunda aynı metodoloji ile imidacloprid ve diatom (%2 w/v) uygulanarak, diatom toprağının sinerjistik etkinliği araştırılmıştır. Araştırma sonucunda tüm popülasyonların direnç oranı imidacloprid aktif maddesinde, lambda-cyhalothrine nazaran daha yüksek bulunmuştur. En yüksek LD_{50} değeri lambda-cyhalothrin ve imidacloprid için sırasıyla Çumra (0.212 µg larva^{-1}) ve Karapınar (0.456 µg larva^{-1}) popülasyonunda tespit edilmiştir. İmidacloprid ve diatom uygulamasında en yüksek sinerji oranı uygulamadan 48 saat sonra 1.93 olarak belirlenmiştir. Araştırma sonucunda diatom toprağı kullanımının imidacloprid etken maddesine direnç geliştirmiş olan patates böceğinin mücadelesinde ve direnç yönetiminde büyük katkı sağlayacağı değerlendirilmektedir.

Anahtar sözcükler: Diatom toprağı, insektisit, *Leptinotarsa decemlineata*, direnç, sinerjizm

[1] This study was a part of PhD thesis of the first author in Selçuk University, Institute of Science, Department of Plant Protection.

[2] Directorate of Soil Water and Deserting Control Research Institute, 42010, Konya
[3] Selçuk University, Faculty of Agriculture, Department of Plant Protection,42250, Konya
* Corresponding author (Sorumlu yazar) e-mail: osman.cagirgan@tarimorman.gov.tr
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Introduction

Colorado potato beetle, *Leptinotarsa decemlineata* Say, 1824 (Coleoptera: Chrysomelidae), the main pest of potato crops, feeds voraciously in the larval and adult stages and causes a 70-80% decrease in yield depending on population density (Oerke et al., 1994). In addition to the direct damage from its herbivory, it is a vector for pathogens such as brown rot bacterium, and ringspot and spindle tuber viruses (Yüceer, 2011). Currently, control methods other than chemical control and rotation are not used by growers as they are not easily applied nor effective. Presently, 262 plant protection products with 20 different active substances are registered for Colorado potato beetle in Turkey (Anonymous, 2021a). It is one of the species that develop dangerous resistance as a result of intense insecticide pressure, its high reproduction rate (Bishop & Grafius, 1996), and the development of physiological capacity for detoxification (Ferro,1993) as a result of the glycoalkaloids found in the solanaceous plants, which are its normal hosts (Weisz et al., 1994). The first resistance was observed in 1952 to DDT (Quinton, 1955), resistance was detected to permethrin after 2 years and imidacloprid after 5 years from their first registration (Huseth et al., 2014). Ioannidis et al. (1991) report that resistance is 1000-fold to azinphos-methyl and 2000-fold to carbofuran. It has been reported that resistance develops 26 and 130-fold, respectively, for thiamethoxam and imidacloprid, which are neonicotinoid group insecticides that have been used extensively in the recent past (Szendrei et al., 2011), and resistance can reach up to 300-fold to imidacloprid (Mota-Sanchez et al., 2006). Since the pest develops cross resistance to insecticides, rotation among insecticides with the same mode of action is ineffective in delaying resistance.

In Colorado potato beetle, although it varies in different populations, reducing insecticide penetration, increasing secretions enhance detoxification (Clark et al., 2001), desensitization of target tissue and conversion of active substance to low-toxicity metabolites (Mota-Sanchez, 2003) by increasing esterase, carboxylesterase, monooxygenase enzyme secretions are known to be significant resistance mechanisms. Recently, studies on the use of some compounds with known synergistic effects with insecticides in resistance management have attracted attention. For example, it has been reported that the susceptibility to permethrin (Silcox et al., 1985), azinphos-methyl (Ahammad-Sahib et al., 1994), abamectin (Yoon et al., 2002) of insect pests increases with the use of piperonyl butoxide (PBO), an oxygenase enzyme inhibitor. Zamojska et al. (2011) reported that DEF (S-S-S-tributyl phosphorotrithioate), DEM (diethyl malonate) inhibitors have synergism rates of 15.3 and 3.63, respectively, when used with deltamethrin in resistant populations.

Another substance with the potential for synergistic benefit is diatomaceous earth (DE), which is a siliceous sedimentary material consisting of fossilized diatoms that is applied to many stored-product insect pests (Başkaya, 2020). DE can act by adsorption or abrasion. In adsorption, the powders can adhere to the cuticle and lead to the disruption of the lipid layer. This disruption causes the loss of water by dehydration and, death (Golob,1997). Başkaya (2020) reported that DE is also effective in different pests such as *Callosobruchus maculatus* Fabricius, 1775 (Coleoptera: Bruchidae) and *Tetranychus urticae* Koch, 1836 (Acari: Tetranychidae).

In this study, the resistance to imidacloprid and lambda-cyhalothrin in fourth instar larvae of Colorado potato beetle collected from different districts of Konya was investigated. In addition, the synergistic effect of DE was studied.

Materials and Methods

Insect populations

In preliminary experiments conducted in 2018, it was observed that the susceptibility to imidacloprid has decreased in Colorado potato beetle populations from Karapınar District and LD_{50} were determined. In 2020, the fourth instar larvae of Colorado potato beetle were collected from potato production areas in Çumra, Doğanhisar, Güneysınır, Karapınar, Meram and Seydişehir Districts of Konya Province, Turkey,

placed in plastic containers and kept in refrigerated boxes until transferred to the laboratory. In the laboratory, they were fed on insecticide-free potato leaves for 1 day at 25 ± 1°C with 16:8 h L:D photoperiod and 60 ± 10% RH, thus, it was ensured that the impacts of collection and transportation were eliminated (Huseth & Groves, 2013). The population with the lowest LD_{50} came from an organic farm that has been operating for 6 years in Meram District was considered as a susceptible population. The food of larvae (insecticide-free potato leaves) was changed daily.

Insecticides application

In the study, imidacloprid (Confidor SC 350 Bayer Crop Science) and lambda-cyhalothrin (Petra 5 EC, Agrobest), which are commonly used insecticides for Colorado potato beetle control, were used. In the preliminary experiments, a dose range for 10-90% mortality was determined for imidacloprid and lambda-cyhalothrin using 8 and 7 dose range series and control, respectively. The dose range series was created by diluting 50% at each step. The insecticides were applied with 3 replicates for each dose to 20 larvae, and as a control, distilled water was applied. Two µl of insecticide solution was dripped to the dorsal surface of larvae with a hand microapplicator using a microsyringe (Burkard Scientific, Uxbridge, UK). The larvae were then placed in 90-mm plastic Petri dishes with sufficient potato leaves in the incubator (Nüve EN500). After 24 h, the larvae that did not react when touched their body gently with a soft brush were considered to be dead (Alkan et al., 2017). Resistance ratio (RR) was classified according to Lee et al., (1999): RR < 2, no resistance or very low resistance; RR = 2-5, low level resistance; RR=5-10, moderate level resistance; and RR > 10, high level resistance.

Diatomaceous earth and its synergistic activity

A 10 µm diameter powder of Turkish DE product (Turco 010) was obtained from Beg Tuğ Mineral Company (Istanbul, Turkey) was used. Turco 010 has a pH of 5.1 and contains 83.4% SiO_2 and 1.3% CaO (Anonymous, 2021b). The synergistic effect of DE was investigated in the Karapınar population, with the highest LD_{50}, by using it together with imidacloprid. For this purpose, separate treatment for each day were created and imidacloprid and imidacloprid plus 2% (w/v) DE mixture were applied by the same method as described above. Distilled water plus 2% (w/v) DE was used as a control. Mortality and LD_{50} for imidacloprid alone and its mixture with DE were determined after 24, 48, 72, and 96 h of exposure. Corrected mortality data were calculated by using Abbott's formula (Abbott, 1925). The synergy rate (SR) was calculated with the following formula (Zamojska et al., 2011): SR = LD of active substance alone/LD of active substance with a synergist, SR < 1, antagonist; SR = 1, the lack of synergism or antagonism; and SR > 1, synergism.

Data analysis

The probit analysis for the data of mortality vs dose was conducted using the POLO computer package program (LeOra, 2002) to estimate LD_{50} and LD_{90}. Susceptibility ratios were determined by dividing the LD_{50} of tested field population by the LD_{50} of the susceptible population. Percentage mortality in imidacloprid alone and imidacloprid plus DE 2% (w/v) treatments were evaluated variance with LSD in the JUMP statistical program.

Result and Discussion

Resistance to lambda-cyhalothrin

The highest LD_{50} of 0.212 (0.164-0.263) µg ai $larvae^{-1}$ for lambda-cyhalothrin was found in Çumra population (Table 1). The resistance rate based on LD_{50} was determined as 2.98, 2.63 and 2.40 in the Çumra, Karapınar and Seydişehir populations, respectively. It is considered that the relatively low resistance rate in the Güneysınır and Doğanhisar populations is most probably because that the crop production in these areas is in the form of small family businesses and less insecticide is used in the control of Colorado potato beetles.

Table 1. Contact toxicity of lambda-cyhalothrin to fourth instar larvae of *Leptinotarsa decemlineata* after 24 h

Population	n	LD_{50} (µg ai larvae^{-1}) (95% CL)	LD_{90} (µg ai larvae^{-1}) (95% CL)	Slope ± SEM	λ^2 (df)	H	RR
Susceptible	420	0.071 (0.052-0.090)	0.317 (0.232-0.518)	1.97 ± 0.278	3.47 (4)	0.867	-
Çumra	420	0.212 (0.164-0.263)	0.892 (0.636-1.571)	2.05 ± 0.305	2.75 (4)	0.687	2.98
Karapınar	420	0.187 (0.147-0.234)	0.843 (0.603-1.419)	1.96 ± 0.258	2.30 (4)	0.574	2.63
Seydişehir	420	0.171 (0.127-0.214)	0.672 (0.497-1.103)	2.16 ± 0.327	2.54 (4)	0.635	2.40
Güneysınır	420	0.117 (0.086-0.162)	0.577 (0.364-1.248)	1.85 ± 0.172	5.25 (4)	1.312	1.64
Doğanhisar	420	0.092 (0.071-0.116)	0.407 (0.307-0.602)	1.99 ± 0.226	2.95 (4)	0.737	1.29

n, number of larvae tested; SEM, standard error of the mean; λ^2, chi-square; H, heterogeneity; RR, resistance ratio (resistant population LD_{50}/susceptible population LD_{50}).

It has been reported in similar studies that the Colorado potato beetle develops resistance against lambda-cyhalothrin and other pyrethroids (Cutler et al., 2005; Zamojska et al. 2011). For example, Keskin & Yorulmaz Salman (2020) found that different populations collected from Afyonkarahisar developed resistance to deltamethrin in the range of 9.41-77.2 times on third instar larvae. Slađan et al. (2012) observed that adults developed up to 60 times resistance to cypermethrin in Romania. In another study using adults, resistance rates for lambda-cyhalothrin in different populations were found to be in the range of 14.3 to 617 times, and it was determined that the resistance rates for cypermethrin and deltamethrin increased up to 41 and 2,325 times, respectively (Jiang et al., 2010). This difference in resistance may be due to differences in life stages and insecticide pressure.

Resistance to imidacloprid

Contact toxicity of imidacloprid in different populations of *L. decemlineata* is given in Table 2. Although, the LD_{50} of the susceptible population was 0.065 (0.048-0.087) µg ai larvae^{-1}, the highest LD_{50} was 0.456 (0.337-0.599) µg ai larvae^{-1} in the Karapınar population. The lowest LD_{50} was found in the Doğanhisar population at 0.159 (0.101-0.220) µg ai larvae^{-1}. Resistance rates were found in the range of 2.44 to 7.01 times. Imidacloprid resistance level was found to be moderate in the Karapınar population and low in the populations from other districts. Similar studies demonstrate that Colorado potato beetle develops resistance to imidacloprid at varying rates. Olson et al. (2000) found that the resistance rate in larvae was 30 times, Baker et al. (2014) stated that it varies between 7.6 to 71 times in different populations. However, Slađan et al. (2012) showed the resistance rate in adults 1.56 to 82.9 times, Mota-Sanchez et al. (2006) determined it 310 times. Keskin & Yorulmaz Salman (2020) determined that the populations of Afyonkarahisar districts gained resistance to imidacloprid in the range of 3.96 to 27.3 times.

Crassley et al. (2018) found that the LD_{50} of different populations were in the range of 0.0005-0.16 µg larvae^{-1} for second instar larvae and 0.051-2.401 µg adults^{-1}. According to the results of the same study, it is determined that resistance develops up to 320 times in larvae and up to 47 times in adults.

In the preliminary study conducted in 2018, it was determined that the LD_{50} of the population collected from the same field in Karapınar District was 0.342 µg larvae^{-1} but had increased to 0.456 µg larvae^{-1} by 2020 (Table 3). In a similar study, it was observed that LD_{50} of four populations increased 1.8-3.75 times in the third year in Poland (Wegorek, 2005). This situation is considered to be a result of intensive production in the region and spraying tubers and foliage with imidacloprid and other neonicotinoid group insecticides. Jeschke & Nauen (2008) reported that cross resistance developed even when imidacloprid was not used. Resistance development is detected as a decrease in insecticide effectiveness and a shortening of the control period under field conditions (Stewart et al., 1997).

Table 2. Contact toxicity of imidacloprid to fourth instar larvae of *Leptinotarsa decemlineata* after 24-h treatment

Population	n	LD_{50} (µg ai $larvae^{-1}$) (95% CL)	LD_{90} (µg ai $larvae^{-1}$) (95% CL)	Slope ± SEM	λ^2(df)	H	RR
Susceptible	540	0.065 (0.048-0.087)	0.67 (0.45-1.15)	1.27 ± 0.123	2.65 (5)	0.441	-
Karapınar	480	0.456 (0.337-0.599)	3.68 (2.47-6.46)	1.41 ± 0.152	2.59 (5)	0.518	7.01
Çumra	300	0.301 (0.198-0.416)	2.22 (1.44-4.41)	1.48 ± 0.216	2.60 (4)	0.651	4.63
Seydişehir	540	0.271 (0.185-0.364)	1.75 (1.26-2.73)	1.58 ± 0.184	4.48 (5)	0.746	4.16
Güneysınır	540	0.185 (0.132-0.245)	1.22 (0.89-1.84)	1.57 ± 0.163	5.37 (5)	0.895	2.84
Doğanhisar	540	0.159 (0.101-0.220)	0.85 (0.63-1.26)	1.76 ± 0.231	5.30 (5)	0.883	2.44

n, number of larvae tested; SEM, standard error of the mean; λ^2, chi-square; H, heterogeneity; RR, resistance ratio (resistant population LD_{50}/susceptible population LD_{50}).

Table 3. Contact toxicity of imidacloprid to fourth instar larvae of *Leptinotarsa decemlineata* in different years

Population	n	LD_{50} (µg ai $larvae^{-1}$) (95% CL)	LD_{90} (µg ai $larvae^{-1}$) (95% CL)	Slope ± SEM	λ^2(df)	H
Karapınar (2018)	480	0.342 (0.242-0.457)	2.77 (1.91-4.66)	1.41 ± 0.256	2.58 (6)	0.441
Karapınar (2020)	480	0.456 (0.337-0.599)	3.68 (2.47-6.46)	1.41 ± 0.152	2.59 (5)	0.518

n, number of larvae tested; SEM, standard error of the mean; λ^2, chi-square; H, heterogeneity.

Synergistic effect of diatomaceous earth application

For imidacloprid, the LD_{50} was in the range of 0.387-0.456 µg $larvae^{-1}$ according to exposure times (Table 4). The LD_{50} was 0.354, 0.231, 0.223 and 0.214 µg $larvae^{-1}$ after 24, 48, 72 and 96 h exposure respectively, when imidacloprid plus DE mixtures were applied. According to the exposure times, the maximum synergy rate was 1.93 for 48 h exposure.

Mortality rates in imidacloprid alone and imidacloprid plus DE mixtures treatments were close to each other for 24 h exposure and were found to be 48.5 and 53.9%, respectively (Table 5, 6). For the increase of exposure time from 24 to 48 h of exposure, the mean mortality rate was 48.5% in imidacloprid alone treatment whereas it was 59.0% with a 10% increase in imdacloprid plus DE treatment.

In the studies using synergism in the control of Colorado potato beetle, inhibitors of enzymes that are involved in resistance mechanisms were tested. For example, Sharif et al. (2007), found synergism rates of 3.5 and 2.3, respectively, for DEF (S-S-S tributyl phosphorotrithioate) and PBO (Piperonyl butoxide) with endosulfan in fourth instar larvae. In another study, it was determined that the synergism rates increased up to 48.2, 15.2 and 3.6 levels as a result of the use of deltamethrin and PBO, DEF and DEM (diethyl malonate), respectively (Zamojska et al., 2011). However, Jiang et al. (2010) reported that when carbofuran and PBO, DEM, triphenyl phosphate (TPP) were used in adults from different populations, the maximum synergism rates were 5.7, 2.9, and 2.6, respectively. Zhao et al. (2000), found that when they used DEF together with imidacloprid, there was no DEF synergistic or antagonistic effect in the susceptible larvae, but antagonistic effect in susceptible adults. In the same study, synergism was observed with the use of imidacloprid plus PBO at a rate of 1.1 and 1.3 in susceptible adults and larvae, respectively.

Table 4. Contact toxicity of imidacloprid and imidacloprid plus diatomaceous earth (DE) 2% (w/v) against fourth instar larvae of *Leptinotarsa decemlineata* in the Karapınar population

Application	Exposure (h)	n	LD_{50} (µg ai larvae^{-1}) (95% CL)	LD_{90} (µg ai larvae^{-1}) (95% CL)	Slope ± SEM	λ^2 (df)	H	SR
Imidacloprid	24	480	0.456 (0.337-0.599)	3.68 (2.47-6.46)	1.41 ± 0.152	2.59 (5)	0.518	-
	48	480	0.447 (0.321-0.596)	3.62 (2.42-6.42)	1.41 ± 0.159	2.45 (5)	0.490	-
	72	480	0.401 (0.284-0.540)	3.50 (2.32-6.31)	1.36 ± 0.155	3.64 (5)	0.728	-
	96	480	0.387 (0.263-0.550)	4.15 (2.34-10.70)	1.24 ± 0.179	0.39 (5)	0.162	-
Imidacloprid + DE	24	480	0.354 (0.260-0.464)	2.65 (1.84-4.38)	1.47 ± 0.155	1.04 (5)	0.208	1.28
	48	480	0.231 (0.151-0.322)	1.85 (1.27-3.12)	1.42 ± 0.166	3.37 (5)	0.673	1.93
	72	480	0.223 (0.139-0.318)	1.65 (1.13-2.76)	1.48 ± 0.184	1.48 (5)	0.296	1.79
	96	480	0.214 (0.123-0.317)	1.49 (1.01-2.52)	1.52 ± 0.209	3.14 (5)	0.628	1.80

n, number of larvae tested; SEM, standard error of the mean; λ^2, chi-square; H, heterogeneity; SR, synergy ratio (LD_{50} of imidacloprid alone/LD_{50} of imidacloprid plus diatomaceaus earth).

Table 5. Mortality (%) with imidacloprid application in fourth instar larvae of *Leptinotarsa decemlineata* at different doses and times

Dose (µg imidacloprid ai larvae^{-1})	Hours after treatment									
	24[1]		48		72		96		Mean[1]	
0.052	6.9 ± 1.66	e	7.1 ± 1.66	f	10.6 ± 2.88	g	12.8 ± 1.85	e	9.36 ± 1.66	G
0.105	18.9 ± 7.31	e	19.7 ± 2.18	e	21.4 ± 3.21	f	23.7 ± 1.85	de	20.9 ± 1.88	F
0.210	35.2 ± 2.40	d	37.3 ± 5.78	d	37.4 ± 5.03	e	36.4 ± 4.91	d	36.6 ± 2.02	E
0.420	50.7 ± 3.28	c	46.2 ± 6.96	d	53.5 ± 2.08	d	56.2 ± 6.02	c	51.7 ± 2.41	D
0.840	61.6 ± 6.64	bc	62.4 ± 3.78	c	64.2 ± 2.08	c	63.6 ± 4.91	c	63.0 ± 2.01	C
1.680	73.6 ± 5.45	b	75.0 ± 1.85	b	73.1 ± 3.48	b	78.4 ± 5.78	b	75.0 ± 1.98	B
3.360	92.9 ± 2.18	a	92.8 ± 3.84	a	94.5 ± 3.46	a	100.0 ± 0.00	a	95.1 ± 1.53	A
Mean[2]	48.5 ± 6.45	B	48.5 ± 6.41	B	50.7 ± 6.36	AB	53.0 ± 6.55	A		

[1] Means followed by same letters within columns are not statistical different at P<0.05; [2] Means same letters within the row and not statistical different at P<0.05.

In previous studies, it was shown that the synergistic activity of DE can be used for storage pests. For example, Başkaya (2020), when using DE (0.4% w/v) with cypermethrin for *Callosobruchus maculatus* (Coleoptera: Bruchidae) found synergism of 2.6, 4.0, 3.3 after 48, 72 and 96 h of the treatment, respectively. The effectiveness of DE varies according to the physical and chemical properties of the material, dose, type of insect, life stage, application method and temperature (Losic & Korunic, 2018). However, it is also known that DE has a repellent effect as well as an insecticidal effect (Bayram, 2018).

Table 6. Mortality (%) with imidacloprid plus diatomaceous earth (2% w/v) application in fourth instar larvae of *Leptinotarsa decemlineata* at different doses and exposure times

Dose (µg imidacloprid ai larvae^{-1})	Hours after treatment									
	24[1]		48		72		96		Mean[1]	
0.052	12.2 ± 1.52	g	14.7 ± 7.62	e	14.5 ± 4.58	e	15.3 ± 2.51	e	14.16 ± 2.02	G
0.105	22.7 ± 3.17	f	32.8 ± 2.40	de	35.4 ± 9.16	d	39.2 ± 7.83	d	32.52 ± 3.26	F
0.210	36.8 ± 2.72	e	47.7 ± 6.48	cd	49.4 ± 7.37	d	49.9 ± 3.33	d	45.95 ± 2.77	E
0.420	59.6 ± 3.84	d	66.6 ± 6.42	bc	67.3 ± 5.20	c	71.9 ± 5.23	c	66.36 ± 2.60	D
0.840	70.4 ± 3.60	c	69.6 ± 10.10	b	77.9 ± 4.66	bc	78.2 ± 2.51	bc	74.03 ± 2.83	C
1.680	80.9 ± 4.33	b	83.0 ± 6.11	ab	87.5 ± 4.05	ab	89.3 ± 4.00	ab	85.15 ± 2.26	B
3.360	94.6 ± 3.17	a	98.2 ± 2.00	a	97.8 ± 2.33	a	100.0 ± 0.00	a	97.66 ± 1.12	A
Mean[2]	53.9 ± 6.44	B	59.0 ± 6.35	A	61.4 ± 6.45	A	63.4 ± 6.37	A		

[1] Means followed by same letters within columns are not statistical different at P<0.05; [2] Means same letters within the row and not statistical different at P<0.05.

In the current study, it was determined that the pest has developed resistance to both imidacloprid and lambda-cyhalothrin in different populations. It has been determined that relatively high rates of resistance had developed in the Karapınar and Çumra populations from districts where crop production is intensive and the both active substances are intensively applied. It is considered that the higher rate of imidacloprid resistance compared to lambda-cyhalothrin is due to imidacloprid being used in both tuber and spray applications. It is observed that other neonicotinoid class insecticides, such as acetamiprid, are also used in spray applications, and it is thought that this may increase imidacloprid resistance due to the development of cross resistance. Although lambda-cyhalothrin is older than imidacloprid in Colorado potato beetle control, there has been an increase in spray applications of lambda-cyhalothrin, due to the decrease in imidacloprid efficacy. Since the use of insecticides is the most common and effective method in the control of Colorado potato beetle, delaying and management of resistance against insecticides gains great importance. In the present study, it was shown that DE can be used as an effective synergist for insecticide treatments against Colorado potato beetle larvae. In future studies, the synergistic effect of DE against adults and its effectiveness in field conditions should be investigated.

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Original article (Orijinal araştırma)

Toxic effects of some acaricides on *Aceria oleae* (Nalepa, 1900) (Acari: Eriophyidae) and its predator *Neoseiulus californicus* (McGregor, 1954) (Acari: Phytoseiidae) under laboratory conditions[1]

Laboratuvar koşullarında *Aceria oleae* (Nalepa, 1900) (Acari: Eriophyidae) ve avcısı *Neoseiulus californicus* (McGregor, 1954) (Acari: Phytoseiidae)'a bazı akarisitlerin toksik etkileri

Nabi Alper KUMRAL*[2] **Sultan ÇOBANOĞLU[3]**

Pınar HEPHIZLI GÖKSEL[4],[5] **Anıl AKSOY[5]**

Abstract

Olive bud mite, *Aceria oleae* (Nalepa, 1900) (Acari: Eriophyidae) is one of the key pests that regularly needs control with acaricides in olive orchards of Bursa Province, Turkey. For the chemical control of *A. oleae*, it is critical the use of acaricides does not reduce the survival and fecundity of its natural enemies. The toxic effects of three concentrations of seven acaricides were assessed against both *A. oleae* and its predator *Neoseiulus californicus* (McGregor, 1954) (Acari: Phytoseiidae) using a residual method under controlled conditions at Bursa Uludağ University during 2020-2021. The highest recommended concentrations of acequinocyl, azadirachtin, fenbutatin oxide, milbemectin, pyridaben, spirodiclofen and sulfur killed *A. oleae* adults with rates varying from 80 to 100%. Two concentrations of milbemectin, pyridaben and sulfur showed high mortality rates. Nevertheless, highest recommended concentrations of acequinocyl, pyridaben, spirodiclofen and sulfur were found to be highly toxic to *N. californicus* adults with rates varied from 82 to 100%. The high mortalities for mobile immature stages and reducing in the fecundity of *N. californicus* occurred by highest recommended concentrations of all tested acaricides. Based on the scale recommended by the International Organization for Biological Control, some sublethal concentrations of fenbutatin oxide, spirodiclofen and sulfur were found to be slightly harmful to both mature and immature of *N. californicus.*

Keywords: Acaricide, olive bud mite, phytoseiids, side effect, toxicology

Öz

Zeytin tomurcuk akarı, *Aceria oleae* (Nalepa, 1900) (Acari: Eriophyidae) Bursa İli (Türkiye) zeytin bahçelerinde sürekli mücadelesinin yapılması gereken ana zararlılar arasında yer almaktadır. Zararlı akarın kimyasal mücadelesinde kullanılan akarisitlerin, doğal düşmanlarının canlılığını ve üremesini düşürmemesi çok kritiktir. Bu nedenle Bursa Uludağ Üniversitesi'nde 2019-2020 yıllarında 7 akarisitin 3 farklı konsantrasyonunun hem *A. oleae* hem de avcısı *Neoseiulus californicus* (McGregor, 1954) (Acari: Phytoseiidae) üzerindeki toksik etkileri kontrollü koşullarda bir kalıntı metodu kullanılarak belirlenmiştir. Azadirachtin, acequinocyl, fenbutatin oxide, milbemectin, pyridaben, spirodiclofen ve kükürtün Türkiye'de diğer akarlara önerilen en yüksek konsantrasyonları *A. oleae*'nın erginlerini %80 ile 100 arasında öldürmüştür. Milbemectin, pyridaben ve kükürtün iki alt konsantrasyonunun *A. oleae* erginleri üzerindeki öldürücü etkisi çok yüksek bulunmuştur. Buna karşılık, acequinocyl, pyridaben, spirodiclofen ve kükürtün en yüksek konsantrasyonları *N. californicus*'un erginlerinin, %82 ile 100 oranında zehirli bulunmuştur. Tüm akarisitlerin en yüksek konsantrasyonları *N. californicus*'un hareketli ergin öncesi dönemlerinde yüksek ölüm oluşturmuş ve erginlerin yumurta bırakma miktarını çok azaltmıştır. Uluslararası Biyolojik Mücadele Organizasyonu'nun skalasına göre fenbutatin oxide, spirodiclofen ve kükürtün bazı düşük konsantrasyonları *N. californicus*'un hem ergin hem de ergin öncesi dönemleri için hafif zararlı bulunmuştur.

Anahtar sözcükler: Akarisit, zeytin tomurcuk akarı, phytoseiidler, yan etki, toksikoloji

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[2] Bursa Uludağ University, Agricultural Faculty, Department of Plant Protection, 16059, Bursa, Turkey
[3] Ankara University, Agricultural Faculty, Department of Plant Protection, Ankara, Turkey
[4] Ataturk Horticultural Research Institute, 77102, Yalova
[5] Bursa Uludağ University, Institute of Natural and Applied Science, Department of Entomology, 16059, Bursa, Turkey
* Corresponding author (Sorumlu yazar) e-mail: akumral@uludag.edu.tr
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Introduction

Olive bud mite, *Aceria oleae* (Nalepa, 1900) (Acari: Eriophyidae) damages both flowers and new formed fruits of olive trees, and significantly decreases olive yield. The mite attacks cause flower dropping and the formation of small/premature fruit in olives (Hatzinikolis, 1986; Tzanakakis, 2003). With 26-82% infestation rate of trees and 8-59% injury rate of fruit, this pest is a serious olive pest in some locations with moist conditions in the Mediterranean Basin (Avidov & Harpaz, 1969; Hatzinikolis, 1986; Abou-Awad et al., 2000; Tzanakakis, 2003; Shahini et al., 2009; Chatti et al., 2017). Previous studies reported three eriophyid mite species from olive trees of Turkey: *A. oleae, Aculus olearius* Castagnoli, 1977 and *Tegolophus hassani* (Keifer, 1959) (Kumral & Kovancı, 2004; Çetin & Alaoğlu, 2006; Kaçar et al., 2010; Çetin et al., 2012; Denizhan et al., 2015; Ersin et al., 2020; Kaya, 2020). Among these mites, *A. oleae* was reported as the predominant species in Marmara, Eagan and Mediterranean Regions (Alkan, 1952; Kumral & Kovancı, 2004; Kaçar et al., 2010; Kaya, 2020).

In recent years, because the pressure of natural enemies has disappeared due to the intensive use of synthetic chemicals in the olive orchards of Bursa Province, *A. oleae* has become one of the key pests that must be regularly controlled with synthetic acaricides (Kumral et al., 2020). Considering the size of the olive groves, the sole method is the chemical control of *A. oleae*. However, the toxic effects of many acaricides, including botanical and mineral preparations, on *A. oleae* are not known. In a previous study, the effectiveness of a translaminar acaricide, abamectin, against *A. oleae* was determined in an olive grove of Bursa (Kılınç & Kumral, 2016). According to results of that study, three different doses (2.25, 4.5 and 9.0 g ai/100 L water) reduced *A. oleae* density by 87, 93 and 94%, respectively, 7 days after spraying. In addition, the study showed that the dose (4.5 g ai/100 L water) of abamectin reduced density of its predator mites, phytoseiids (mixed population contain 2-3 different species) by 67% under field conditions. The authors suggested that abamectin cannot be used in integrated mite control strategies due to its moderately harmful effect to phytoseiids and should be investigated new environmentally-friendly alternative solutions in the future. According to the European Union Directive 2009/128/EC, member and associated countries should prefer to environmentally-friendly pesticides that are safe for non-target organisms such as biological control agents. Among these agents, phytoseiids can control eriophyid mites successfully (Gerson et al. 2003). Also, several phytoseiid mite species related to eriophyid mite populations in olive trees, *Typhlodromus (Typhlodromus) athiasae* Porath & Swirski, 1965, *Typhlodromus (Typhlodromus) rarus* Wainstein, 1961, *Typhlodromus (Anthoseius) recki* Wainstein, 1958, *Typhlodromus (Anthoseius) psyllakisi* Swirski & Ragusa, 1976, *Typhlodromus (Anthoseius) involutus* Livshitz & Kuznetsov, 1972, *Typhlodromus (Anthoseius) athenas* Swirski & Ragusa, 1976, *Typhlodromus (Anthoseius) rapidus* Wainstein & Arutunjan, 1968, *Neoseiulus californicus* (McGregor, 1954), *Neoseiulus barkeri* Hughes, 1948, *Amblyseius andersoni* (Chant, 1957), *Euseius stipulatus* (Athias-Henriot, 1960) and *Paraseiulus talbii* (Athias-Henriot, 1960), were reported in different locations by some researchers (El-Laithy, 1999; Kumral & Kovanci, 2004; Kumral et al., 2010; Chatti et al., 2017; Ersin et al., 2020; Kaya, 2020; Elhalawany et al., 2021). Also, Abou-Awad et al. (2000) reported that phytoseiids are capable of preventing the outbreak of *A. oleae* in olive groves. *Neoseiulus californicus* is used commercially around the world to control eriophyids and other economically important spider mite species on several crop species (Castagnoli & Simoni, 2003). The predator is widely distributed in the Mediterranean Region of Turkey (Cakmak & Cobanoğlu, 2006; Yorulmaz & Ay, 2012). The phytoseiid is the predominant species in Bursa Province probably due to its adaptation to cold winter conditions and resistance development against some insecticides and acaricides in conventional production areas (Kumral & Kovancı, 2007; Yorulmaz & Ay, 2012). The presence of *N. californicus* in olive orchards was previously reported by Chatti et al. (2017) and Kumral et al. (2021). Additionally, the predation capacity of *N. californicus* fed on *A. oleae* has been shown under laboratory conditions by Kumral et al. (2021), recently.

It is critical that the acaricides used in the chemical control of *A. oleae* do not reduce the survival and fecundity of its natural enemies. If, it is chosen to use any acaricide, their side effects on phytoseiids like *N. californicus*, must be taken into consideration. A sustainable chemical control can be provided, only in cases when biological control agents are not affected negatively (Overmeer & van Zon, 1982; Blümel et al., 2000; Norris et al., 2003). Therefore, laboratory-based side effect studies on female predators should evaluate, not only the toxic effects, but also the negative effects on fecundity (Overmeer & van Zon, 1982). Additionally, the juvenile stages (e.g., larvae and nymphs) of phytoseiid mites are in general more susceptible to pesticides compared with adults and the survival of juveniles is greatly important for maintaining the population of the predator (van Zon & Wysoki, 1978). For that reason, during this study, firstly the effects of different concentrations of seven acaricides having different modes of action (acequinocyl, azadirachtin, fenbutatin oxide, milbemectin, pyridaben, spirodiclofen and sulfur) for *A. oleae* were established. Subsequently, the side effects of these concentrations were shown for *N. californicus* in laboratory conditions based on the scale of International Organization for Biological Control (IOBC).

Materials and Methods

Mite cultures

A field colony of *A. oleae* was used in this experiment. The *A. oleae* colony was collected from Mirzaova Village, Mudanya District, Bursa Province, Turkey. The Turkish *N. californicus* strain was obtained from Gorukle Campus of Bursa Uludağ University (Bursa, Turkey). The identification of both mite species was made by senior author based on following literature: Keifer (1975) and Hatzinikolis (1986) for *A. oleae;* Schuster & Pritchard (1963) and Okassa et al. (2011) for *N. californicus*. Predator mites were mass reared in glass Petri dishes at 27 ± 1ºC, 65% RH and 16:8 h L:D photoperiod in a controlled climate chamber. For mass rearing, the predator mites were fed on individuals of *Tetranychus urticae* Koch, 1836, (Acari: Tetranychidae) and pollen of *Typha latifolia* L., 1753, (Poales: Thyphaceae) (Overmeer, 1985).

Bioassays on *Aceria oleae*

The following commercial acaricides belonging to seven chemical groups and modes of action were used in bioassays: acequinocyl, azadirachtin, fenbutatin oxide, milbemectin, pyridaben, spirodiclofen and sulfur (IRAC, 2021). Details about acaricides are given in Table 1. The toxic effect of acaricide on *A. oleae* adults was evaluated using a modified residual method under laboratory conditions (Monteiro et al., 2012; Simon, 2014). As a test tool, Plexiglas Munger cells (8 x 10 x 1 cm) with a circular hole (5 cm diameter) in the center were used (Overmeer, 1985). Moistened cotton wool and then black leather (8 x 10 cm) were placed between Plexiglass with or without a circular hole. Stalks of olive flower bud cluster were insert into the layers (Figure 1). At least five concentrations of acaricides were used and randomly applied to *A. oleae*, at concentrations between 50 and 100% that caused death. The highest recommended concentrations (HRC) and two sublethal concentrations resulted in >50% mortality was prepared with distilled water and used for the bioassays. For each bioassay, three replicates were assessed for both treatments and controls (distilled water). Two mL of different acaricide concentrations were sprayed to olive bud clusters for 3 s with spray tower resulting in a deposition of 1.5 mg/cm^2 (Potter precision, Burkard Manufacturing Co. Ltd., Rickmansworth, UK). The plant parts were sprayed with acaricides once during all bioassays. All sprayed olive bud clusters were then dried at 25ºC (room temperature) for 10-15 min (Potter, 1952). Fifty *A. oleae* adults were put onto the olive bud clusters by a paintbrush. To prevent the escape of the mites, the upper Plexiglas lid was closed. Plexiglas plate and whole fragments of Munger cell which were hold together with the aid of four clips. Then, the Munger cells were placed into a controlled climate chamber in Petri dishes at 27 ± 1ºC, 65% RH and 16:8 h L:D photoperiod. The survival of mites was checked daily under a stereomicroscope after the acaricide application for 3 days. It was decided the test duration (3 days) according to mite viability in the control treatments. Mites unable to move when touched with a hairbrush were considered dead. The rates of mortality in control trials did not exceed 10%.

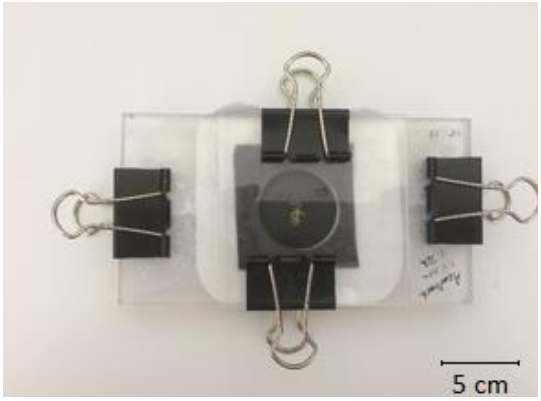

Figure 1. Munger cells used in bioassays.

Table 1. The information about tested acaricides

Active substance	Chemical group[1]	Mode of Action[1]	Mode of action classes[1]	Commercial name	Company	Formulation type[2]	Rate of active substance (g/L)[2]	HRC (mg/L)[2]
Acequinocyl	Unclassified	Mitochondrial complex III electron transport inhibitors	20A	Kanemite	SumiAgro	SC	156	195
Azadirachtin	Botanical acaricides	Compounds of unknown or multible MoA	UN	Nimbecidine	Agrobest	SC	0.3	1.5
Fenbutatine oxide	Organometal	Inhibitors of mitochondrial ATP synthase	12B	Quiz	Hektaş	SC	550	330
Milbemectin	Avermectins	Glutamate-gated chloride channel (GluCl) allosteric modulators	6	Milbeknock	SumiAgro	EC	9.3	9.3
Pyridaben	Pyridazinone	Mitochondrial complex I electron transport inhibitors	21A	Sanmite	SumiAgro	WP	20%	150
Spirodiclofen	Tetronic& Tetramic acid derivates	Inhibitors of acetyl CoA carboxylase	23	Smach	Hektaş	SC	240	60
Sulfur	Minerals	Compounds of unknown or multible MoA	UN	Power sulfur'H	Safa Tarım	WP	80%	3200

[1] The data were obtained from mode of action database of Insecticide Resistance Action Committee (IRAC, 2021);

[2] HRC, highest recommended concentration for other mite pests in Turkey. The data were provided from Turkish Agricultural Ministry Pesticide Registration Database (BKU, 2021).

Toxicity tests on *Neoseiulus californicus*

The toxic effect of the acaricides on both juveniles and adults of *N. californicus* was determined using a modification of the method described by Overmeer & van Zon (1982) in laboratory conditions. Two mL of the three concentrations were sprayed on the undersurface of olive leaves with the spray tower adjusted for the same conditions. Then, the leaves were allowed to air dry for 15 min under room conditions. As a control, the leaves treated with distilled water were used (Potter, 1952). Same Plexiglas Munger cells and

procedures were used for bioassays. An equal amount of eriophyid individuals as prey was put into a cell, followed by the same age females of *N. californicus* (~3 days old) obtained from synchronized population. For juvenile test, freshly deposited eggs (1 day old) were used. The eggs were transferred by paint brush onto sprayed leaves. The trials for immature stages began with egg spraying and continued until the individuals reached adult stage. A total of five female mites and eggs were used for each test. For each acaricide, five replicates were assessed. The cells were put in a climate room at the same conditions. The number of dead female and juvenile mites were recorded daily under a stereomicroscope. Females were considered as dead at the end of 3 days if no movement was observed after a gentle touch by a camel hair brush. Juveniles were recorded as dead when the individuals did not mature or survive during 10 days.

Fecundity inhibition tests on *Neoseiulus californicus*

The inhibition effects of acaricides on the fecundity of *N. californicus* were assessed using the method described by Overmeer & van Zon (1982). The three concentrations were applied to olive leaves based on the above tests. Following this, a mated *N. californicus* female (each newly emerged female was paired with a male adult for 12 h) were put into a Munger cell. An equal amount of prey was put into a cell. The leaves treated with distilled water only served as the control. Five females were treated in each replicate and each treatment was applied to five replicates. The mortality and the number of eggs deposited by females were determined daily and removed until all of the females died naturally.

Data Analysis

Mortality percentages for *A. oleae* and *N. californicus* were corrected using control percentages with Abbott's formula (Abbott, 1925):

$$CM = \frac{CA - TA}{CA} x100$$

where, *CM* is the corrected mortality, *CA* is the proportion alive in control and *TA* is the proportion alive in the treatment (Simon, 2014). A one-way ANOVA was conducted to analyze variation in the corrected mortality data of *N. californicus* juvenile stages and females treated with different acaricides (SAS Institute, 2015). Before the analyses, corrected mortality data were transformed to arcsin. Means obtained by ANOVA were separated using Tukey's HSD post-hoc test. Also, the combined total side effect (E) of the acaricides on *N. californicus* was calculated using the following formula (Overmeer & van Zon, 1982):

$$E = 100 - ((100 - M) \times R)$$

where, *E* is the coefficient of toxicity, *M* is the corrected (Abbott, 1925) mortality effects of acaricides on both juvenile stages or females of *N. californicus* and *R* is the ratio between the mean number of eggs deposited by *N. californicus* females treated acaricides and the mean number of eggs producing by the females exposed to distilled water (control group). The concentrations of each acaricides were classified using these *E* results according to the following IOBC toxicity categories: class I (<30%, harmless), class II (30-79%, slightly harmful), class III (80-99%, moderately harmful), class IV (>99%, harmful) (Sterk et al., 1999). The influence of acaricide exposure on phytoseiid females was analyzed using Kaplan-Meier survival and log-rank test with SPSS 23 version.

Results

Acute toxicity effects on *Aceria oleae*

Table 1 shows the toxicity results for 3 days after application of the highest recommended concentrations (HRC, for other mite pests in Turkey) of seven acaricides to *A. oleae* adults. HRCs of acequinocyl, azadirachtin, fenbutatin oxide, milbemectin, pyridaben, spirodiclofen and sulfur killed *A. oleae* with rates of 97, 80, 91, 100, 100, 80 and 100%, respectively (Table 2). HRCs of acequinocyl, milbemectin,

pyridaben and sulfur were found to be significantly toxic against *A. oleae* adults ($F_{6,20}$ = 93.1; P < 0.01). In addition, *A. oleae* adult mortality rates of their two sublethal concentrations were found to be 54 and 74%, 57 and 66%, 65 and 75%, 93 and 98%, 86 and 98%, 48 and 69%, and 90 and 94%, respectively. Some concentrations of milbemectin (0.29 and 1.26 ai mg/L.), pyridaben (0.75 and 1.5 ai mg/L) and sulfur (3.2 and 12.8 ai mg/L) were significantly more toxic for *A. oleae* adults compared with other acaricides (low conc. $F_{6,20}$ = 69.2; HRC conc. P < 0.01; $F_{6,20}$ = 38.6; P < 0.01). Reducing the concentrations of all tested acaricides was significantly decreased mortality rates of *A. oleae* adults (acequinocyl $F_{2,8}$ = 55.0; P < 0.01; azadirachtin $F_{2,8}$ = 21.6; P < 0.01; fenbutatin oxide $F_{2,8}$= 50.8; P < 0.01; milbemectin $F_{2,8}$= 10.4; P = 0.011; pyridaben $F_{2,8}$ = 15.3; P < 0.01; spirodiclofen $F_{2,8}$= 9.10; P = 0.015; sulfur $F_{2,8}$ = 528; P < 0.01).

Table 2. The bioassay results for *Aceria oleae* adults

Active substance	Number of individuals[a]	Tested conc.	Mean corrected death rates (%)[b]	The tested conc.	Mean corrected death rates (%)[b]	The tested conc.	Mean corrected death rates (%)[b]
Acequinocyl	120	24.4	54.2 bc[d]C[e]	97.5	73.9 bB	195[c]	97.2 aA
Azadirachtin	120	0.75	57.0 bcB	1.5	66.4 bAB	3[c]	80.4 cA
Fenbutatin oxide	120	20.6	65.4 bB	82.5	74.8 bB	330[c]	90.7 bA
Milbemectin	120	0.29	92.5 aB	1.16	98.2 aA	9.8[c]	100 aA
Pyridaben	120	0.75	86.0 aB	1.5	98.1 aAB	150[c]	100 aA
Spirodiclofen	120	7.50	47.8 cC	30	69.4 bB	60[c]	80.4 cA
Sulfur	120	3.20	89.7 aB	12.8	94.4 aAB	3200[c]	100 aA

[a] 120 adult mites were used each concentration; [b] corrected mortality rates by Abbott formula; [c] high recommended concentration for other mite pests in Turkey; [d] means followed by the same lowercase letters within columns are not significantly different (P < 0.01); [e] means followed by the same uppercase letters within rows are not significantly different (P < 0.01).

Side effects on *Neoseiulus californicus*

The side effects of HRC and two sublethal concentrations of seven acaricides on juveniles and females of *N. californicus* are summarized in Table 3. The corrected mortality for juveniles (JM) varied significantly with exposure to different acaricides ($F_{20,96}$ = 8.76; P < 0.01). JM were 96, 95, 96, 94, 100, 100 and 82% with exposure to HRCs of acequinocyl, azadirachtin, fenbutatin oxide, milbemectin, pyridaben, spirodiclofen and sulfur, respectively. Also, JM of their two concentrations were 76 and 95%, 67 and 71%, 42 and 94%, 31 and 75%, 92 and 94%, 44 and 100% and 22 and 35%, respectively. Significantly low JM values were determined when the juveniles were treated with some concentrations of milbemectin (0.29 ai mg/L) and sulfur (3.2 and 12.8 ai mg/L). The corrected mortality for females (FM) differed significantly with exposure to different acaricides ($F_{20,90}$ = 10.4; P < 0.01). FM were 100, 67, 42, 62, 81, 100 and 92% with exposure to HRCs of acequinocyl, azadirachtin, fenbutatin oxide, milbemectin, pyridaben, spirodiclofen and sulfur, respectively. In addition, FM values of their two sublethal concentrations were found to be 13 and 76%, 13 and 16%, 7 and 21%, 60 and 62%, 44 and 81%, 58 and 96%, and 13 and 83%, respectively. Significantly low FM were detected in treated with some concentrations of acequinocyl (24.4 ai mg/L), azadirachtin (0.75 and 1.5 ai mg/L) and sulfur (3.2 ai mg/L) (Table 3).

Table 3 also shows fecundity reduction (R) of *N. californicus* exposed to the HRCs and two sublethal concentrations of the seven acaricides. The HRCs of the acaricides significantly decreased the fecundity of *N. californicus* females compared untreated females ($F_{20,96}$ = 12.8; P < 0.01). R was considerably less in females exposed to the HRCs of all tested acaricides (from 0 to 0.3). Moderate R (0.4 to 0.6) were observed in some concentrations of azadirachtin (0.75 ai mg/L), fenbutatin oxide (82.5 ai mg/L), milbemectin (1.16 ai mg/L), sulfur (3.2 ai mg/L) and spirodiclofen (7.5 ai mg/L), low to high, respectively.

According to the side effect scale suggested by IOBC, HRCs of acequinocyl, pyridaben, spirodiclofen and sulfur were harmful and others were moderately harmful to females (Table 3). However, HRCs of all tested acaricides were found to be harmful to juveniles except sulfur. The lowest concentrations of acequinocyl, azadirachtin, spirodiclofen and sulfur found to be slightly harmful to females, a few concentrations of fenbutatin oxide (20.6 ai mg/L), spirodiclofen (7.5 ai mg/L) and sulfur (3.2 ai mg/L) were found to be slightly harmful to both juveniles and females of *N. californicus.* Fenbutatin oxide (20.6 ai mg/L) was found to be harmless to females of *N. californicus.*

Table 3. The side effects on *Neoseiulus californicus* of seven acaricides in laboratory

Active substance	Applied concentration (ai mg/L)	Number of individuals	JM[1] (%)		FM[2] (%)		R[3]	E[4] (%)	Juvenile toxicity[6]	E[5] (%)	Female toxicity[6]
Acequinocyl	24.4	50	76.0	a-e[7]	13.3	e	0.3	92.8	III	73.4	II
	97.5	50	95.1	a	76.0	a-c	0.0	100	IV	100	IV
	195.0	50	95.9	a	100	a	0.0	100	IV	100	IV
Azadirachtin	0.75	50	67.2	a-e	12.7	e	0.4	87.9	III	67.7	II
	1.5	50	70.6	a-e	16.2	e	0.2	95.3	III	86.6	III
	3.0	50	95.1	a	66.7	a-e	0.1	99.7	IV	97.7	III
Fenbutatin oxide	20.6	50	42.1	c-e	6.7	e	1.2	42.14	II	6.67	I
	82.5	50	94.1	ab	20.8	de	0.4	97.9	III	72.3	II
	330	50	95.8	a	41.7	a-e	0.2	98.9	IV	86.0	III
Milbemectin	0.29	50	31.3	de	60.1	a-e	0.3	83.5	III	90.5	III
	1.16	50	75.0	a-e	61.9	a-e	0.4	90.5	III	85.5	III
	9.8	50	94.1	ab	61.9	a-e	0.3	98.5	IV	90.5	III
Pyridaben	0.75	50	91.6	a-c	44.4	a-e	0.3	97.7	III	84.4	III
	1.5	50	94.1	ab	71.4	a-d	0.2	99.1	IV	95.7	III
	150	50	100	a	80.9	ab	0.0	100	IV	100	IV
Spirodiclofen	7.5	50	43.9	b-e	58.3	a-e	0.6	65.8	II	74.6	II
	30	50	100	a	96.0	a	0.0	100	IV	100	IV
	60	50	100	a	100	a	0.0	100	IV	100	IV
Sulfur	3.2	50	21.6	e	13.2	e	0.5	59.2	II	54.8	II
	12.8	50	35.3	de	83.3	a	0.2	85.8	III	96.3	III
	3200	50	82.4	a-d	91.7	a	0.1	98.1	III	99.1	IV

[1] Corrected death rates of juveniles (survival rate from egg to adult); [2] corrected death rates of females; [3] reproduction reduction rate of treated females compared with untreated ones; [4] total side effect according to juvenile deaths= 100 -((100-JM) x R); [5] total side effect according to female deaths= 100-((100-FM) x R); [6] side effect scale, I = harmless (<%30), II = slightly harmful (30-79%), III = moderately harmful (80-99%), IV = harmful (>99); [7] means followed by the same letter with columns are not significantly different ($P < 0.05$).

Figures 2 and 3 show that the fecundity and lifespan of *N. californicus* females exposed to HRCs and two sublethal concentrations of seven acaricides. Based on Kaplan-Meier survival analysis, HRCs of the acaricides significantly reduced the lifespan of *N. californicus* females ($\chi 2 = 60.2$; $df = 2$, $P < 0.01$). Besides increased concentration of the acaricides, active substance differences resulted in a significant decrease in survivals of *N. californicus* females ($\chi 2 = 187$; $df = 6$, $P < 0.01$).

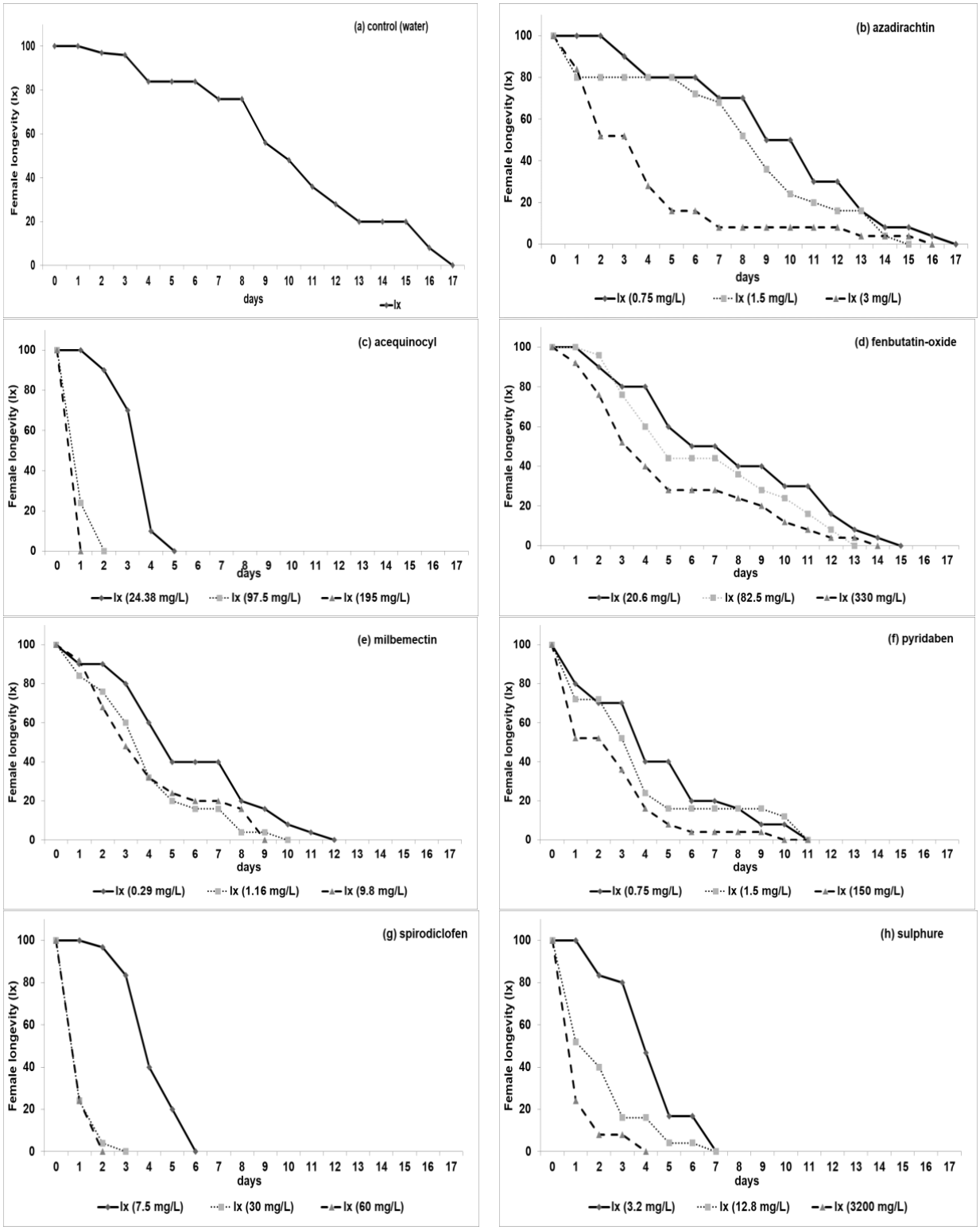

Figure 2. The longevity of a) untreated *Neoseiulus californicus* females versus females exposed to HRCs and two sublethal concentrations of b) azadirachtin, c) acequinocyl and d) fenbutatin oxide, e) milbemectin, f) pyridaben, g) spirodiclofen, and h) sulfur.

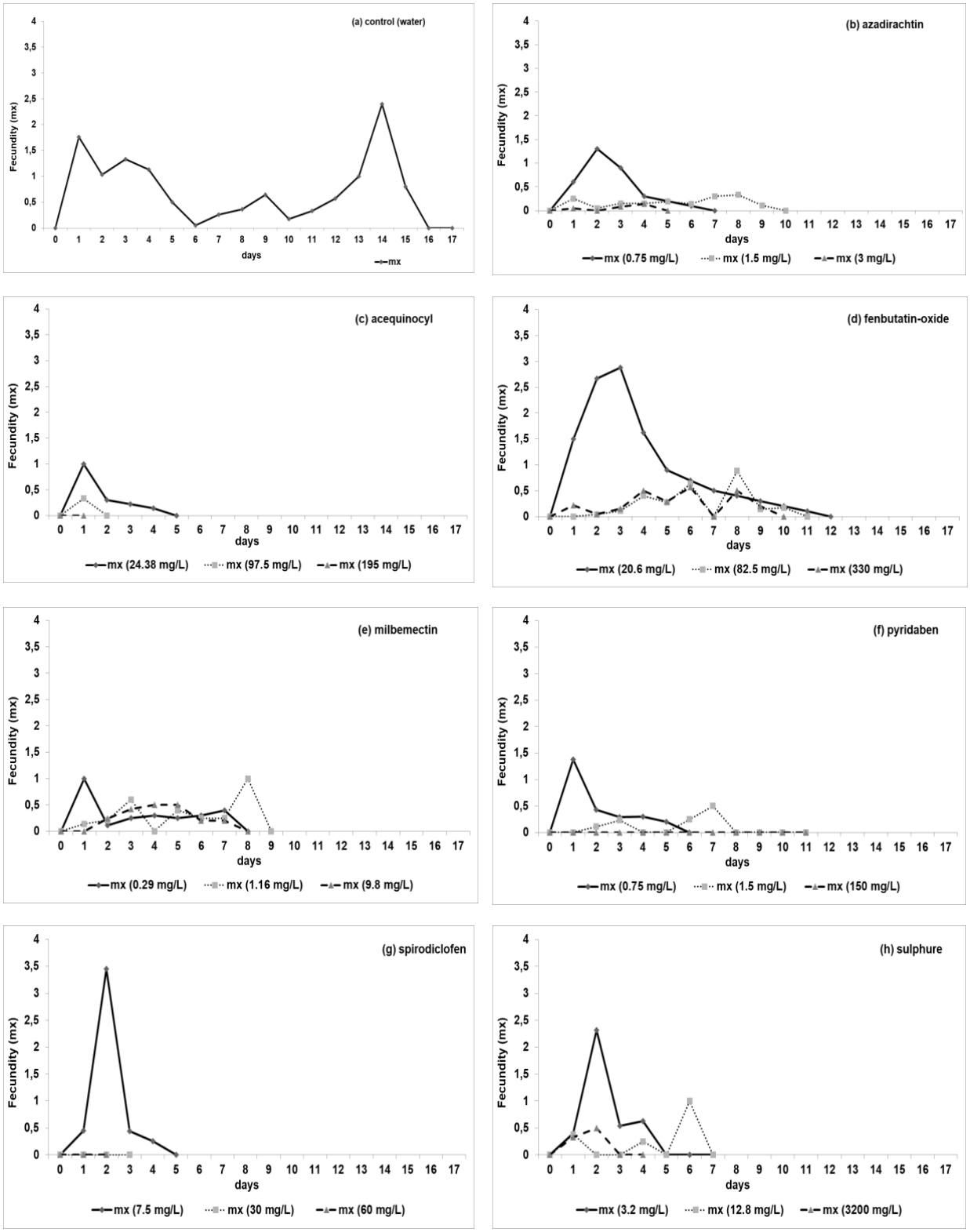

Figure 3. The fecundity of a) untreated *Neoseiulus californicus* females versus females exposed to HRCs and two sublethal concentrations of b) azadirachtin, c) acequinocyl and d) fenbutatin oxide, e) milbemectin, f) pyridaben, g) spirodiclofen and h) sulfur.

The lifespans of females were 1, 2, 4, 9, 10, 14 and 16 days for acequinocyl, spirodiclofen, sulfur, milbemectin, pyridaben, fenbutatin oxide and azadirachtin, respectively. Females exposed to the lowest concentrations of fenbutatin oxide (20.6 ai mg/L) and azadirachtin (0.75 ai mg/L) survived at a significantly higher rate than those exposed to other acaricides (Figure 2). HRCs of the acaricides significantly reduced oviposition of *N. californicus* females compared untreated females ($F_{20,96}$ = 11.6; P < 0.01). The fecundity of females was 0, 0, 0, 0.3, 0.8, 2.1 and 2.5 eggs/days for acequinocyl, pyridaben, spirodiclofen, azadirachtin, sulfur, milbemectin and fenbutatin oxide, respectively. The lowest concentrations of fenbutatin oxide (20.6 ai mg/L) did not affect the fecundity of females compared with untreated females (Figure 3).

Discussion

The current study showed that different concentrations of acequinocyl, azadirachtin, fenbutatin oxide, milbemectin, pyridaben, spirodiclofen and sulfur were toxic to *A. oleae* adults. Although the toxicity effect of acaricides on some other species of the family Eriophyidae has been widely reported in several crops, there is no record about their biological effects on *A. oleae* (Ky & Shepherd, 1988; Silva et al., 1988; van Leeuwen et al., 2010; Fischer & Klötzli, 2015; Kashyap et al., 2015; Kolcu & Kumral, 2018). In our study, HRCs of acequinocyl, milbemectin, pyridaben and sulfur were found to be significantly more toxic to *A. oleae* compared with other tested acaricides. When the concentrations of milbemectin, pyridaben and sulfur were reduced, these low concentrations were still highly toxic with mortality of more than 90%. Similarly, some researchers reported that different concentrations of milbemectin, pyridaben and sulfur are highly toxic to eriophyid species such as *Aculops lycopersici* (Massee, 1937) (Acari: Eriophyidae) (Royalty & Perring, 1987; Silva et al., 1988; van Leeuwen et al., 2010; Fischer & Klötzli, 2015; Kolcu & Kumral, 2018).

This study also provided laboratory findings about the side effects of acaricides at three concentrations to *N. californicus*. HRCs of all tested acaricides were harmful to phytoseiid juveniles. In spite of the fact that azadirachtin, fenbutatin oxide and milbemectin were found to be moderately harmful to adults, their high toxicity to juveniles showed that the acaricides are unfavorable for *N. californicus*. The current study also showed that some low concentrations of fenbutatin oxide, milbemectin, sulfur and spirodiclofen were slightly toxic to both females and juveniles of *N. californicus*. Based on R, low concentrations of fenbutatin oxide, spirodiclofen and sulfur slightly reduced the fecundity of females. In addition, their low concentrations were highly toxic to *A. oleae*. Therefore, acequinocyl and pyridaben are not a favorable acaricide for *N. californicus* and they cannot be recommended to use with this predatory mite in integrated management (Ghadim Mollaloo et al., 2018). Similar to our results, some researchers reported that minor side effects on phytoseiids were observed from evaporation and dusts arising from application of sulfur (Pijnakker & Ramakers, 2009; Gazquez et al., 2011). Also, previous studies also showed the compatibility of sulfur with phytoseiids in different agricultural ecosystems (Pijnakker & Ramakers, 2009; Gazquez et al., 2011; Fiedler & Sosnowska, 2012). In contrast, some researchers who reported that HRC of spirodiclofen was harmful to some phytoseiids due to considerably negative effects on their fecundity and lifespan (Maroufpoor et al., 2016; Evans et al., 2018), while only sublethal doses were slightly harmful (Kaplan et al., 2012; Audenaert et al., 2014; Alinejad et al., 2016; Döker & Kazak, 2019). Our results indicated that low concentrations of fenbutatin oxide have no negative effect on *N. californicus*, which is consistent with the results of other studies on the effects of the acaricide on *Phytoseiulus persimilis* (Athias-Henriot, 1957) and *N. californicus* (Kim & Yoo, 2002; Evans et al., 2018).

In the present study, the lowest concentration of azadirachtin was slightly harmful to *N. californicus* females whereas it was moderately harmful to its juveniles. Similar with the findings of Kurubal & Ay (2015), the lifespan of females was not affected when applied the concentration of azadirachtin. Our findings on adult toxicity of azadirachtin are similar with the results of some studies demonstrated that, the botanical insecticide/acaricide did not cause high mortality of phytoseiid mites due to quick degradation of the botanical acaricide under field conditions (Castagnoli et al., 2005; Audenaert et al., 2014). When making

comparisons with other studies, this variation in mortality of phytoseiid juveniles might be a result of different formulations and concentration as well as test conditions (Castagnoli et al., 2005; Audenaert et al., 2014). Our results are similar to those of some authors who showed azadirachtin properties stem from strong antifeedant activity against many arthropod species including mites, which is supplemented also by marked insect growth regulatory and sterility effects (Mordue & Blackwell, 1993; Sundaram & Sloane, 1995).

In conclusion, in order to conserve biological control agents, the most important factor for controlling the key pests is to reduce the concentrations applied by using sublethal doses, which do not have biological, behavioral and/or demographic effects on natural enemies (Roush, 1989; Dent, 2000; Desneux et al., 2007). Consequently, the results of our study show that the low concentrations of tested acaricides may conserve populations of *N. californicus*. The findings are consistent with the findings of Dent (2000) who reported that acaricides at reduced rates might be used in combination with biological control agents for IPM, reducing the selection pressure and development of acaricide resistance. Among tested acaricides, the low concentration of sulfur was highly toxic to *A. oleae*, but this concentration was not harmful to females and juveniles of *N. californicus.* For *A. oleae* control, a low concentration of sulfur is compatible with *N. californicus.* Alternatively, when *A. oleae* population reaches high population densities in olive orchards, low concentrations fenbutation oxide and spirodiclofen, which have moderately toxic effects, can be applied, and so the phytoseiid population could be conserved due to only slightly harmful effects to them. Azadirachtin could be used due to its quick degradation potential under field conditions. However, this hypothesis should be tested under field conditions. This integrated strategy could both reduce the chemical residue in olives while also conserving the population of the predatory mite. The tested acaricides and their determined concentrations have potential for the control of *A. oleae* in olive orchards, although more studies are needed for verification of the results under field conditions.

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Original article (Orijinal araştırma)

Size and shape variations in wing morphology of *Anopheles maculipennis* s.s. Meigen, 1818 (Diptera: Culicidae) from northeastern Turkey

Türkiye'nin Kuzeydoğu Anadolu Bölgesi'ndeki *Anopheles maculipennis* s.s. Meigen, 1818 (Diptera: Culicidae) türünün kanat morfolojisindeki büyüklük ve şekil farklılıkları

Berna DEMİRCİ[1*]

Abstract

Anopheles maculipennis Meigen, 1818 (Diptera: Culicidae) complex was discovered as the first sibling species complex among mosquito species and identified as a highly important malaria vector in the Middle East and Europe. *Anopheles maculipennis* s.s. is the nominotypical member species of the complex, and widely spread across the whole of Europe. Body size and shape are the most important characters of organisms and is related to numerous variables. Biological size and shape may be affected by altitude and altitudinal differences. In this study, the variation in wing size and shape of *An. maculipennis* s.s. populations collected from four sampling stations in Iğdır Province (Mürşitali, Sürmeli, Yukarıçıyrıklı and Zülfikarköy) and two sampling stations in Kars Province (Kötek and Kozlu) at different altitudes and in different habitats from northeastern Turkey in 2019 were investigated. It was assumed that altitude and the environmental differences related with altitude may affect the wing (body) size or shape of *An. maculipennis* s.s. This is the first comparative geometric morphometric study of *An. maculipennis* s.s. populations in Turkey and the results indicate size and shape differences among some populations. While centroid size did not show a linear association with altitude, samples from the highest altitude population had larger wings than the other populations.

Keywords: *Anopheles maculipennis* complex, geometric morphometric, malaria, plasticity

Öz

Anopheles maculipennis kompleksi sivrisinek türleri arasında ilk ikiz tür kompleksi olarak keşfedilmiş ve Orta Doğu ve Avrupa'da çok önemli sıtma vektörü olarak tanımlanmıştır. *Anopheles maculipennis* s.s. Meigen, 1818 (Diptera: Culicidae) kompleksin nominotipik üye türü olarak bilinmektedir ve tüm Avrupa'da yaygın bir şekilde bulunmaktadır. Vücut büyüklüğü ve şekli organizmaların en önemli özelliklerindendir ve çok sayıda değişkenle ilişkilidirler. Vücut büyüklüğü ve şekli yükseklikten ve yüksekliğe bağlı farklılıklardan etkilenebilir. Bu çalışmada Türkiye'nin Kuzeydoğu Anadolu Bölgesi'nde farklı yükseklik ve habitatlarda bulunan ve 4 tanesi Iğdır İli (Mürşitali, Zülfikarköy, Sürmeli, Yukarıçıyrıklı) ve 2 tanesi ise Kars iline (Kötek ve Kozlu) ait farklı örnekleme istasyonlarından 2019 yılında toplanan *An. maculipennis* s.s. popülasyonlarının kanat büyüklük ve şekil varyasyonları araştırılmıştır. Yükseklik ve yüksekliğe bağlı çevresel farklılıkların *An. maculipennis* s.s.'in kanat (vücut) büyüklüğü veya şeklini etkileyebileceği varsayılmıştır. Bu çalışma ile Türkiye'de bulunan *An. maculipennis* s.s. türleri geometrik morfometri yöntemi ile ilk kez değerlendirilmiştir ve sonuçlar bazı popülasyonlar arasında bazı büyüklük ve şekil farklılıklarına işaret etmektedir. Geometrik merkez her ne kadar yükseklikle doğrusal bir ilişki göstermese de en yüksek rakımdan toplanan popülasyon diğer popülasyonlara göre göreceli olarak daha büyük kanatlara sahiptir.

Anahtar sözcükler: *Anopheles maculipennis* kompleks, geometrik morfometri, malarya, plastisite

[1] Kafkas University, Faculty of Science and Literature, Department of Molecular Biology and Genetics, 36100, Kars, Turkey
* Corresponding author (Sorumlu yazar) e-mail: demirciberna80@gmail.com
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Introduction

Malaria is still a particularly serious health problem in the modern world, and one of the major reasons of death of all infectious diseases. It has been endemic in Turkey for years. In recent years, while endemic malaria cases were observed in the southeastern part of Turkey, only imported cases have been reported since 2012. The case counts remained under 150 per year prior to 2012, when a peak was observed with 376 cases associated with the Syrian refugee influx; imported cases have since remained over 200 per year between 2012-2017 (WHO, 2018; Ergönül et al., 2020). Given the persistence of malaria vectors in south and southeastern part of Turkey, there will be always a threat for malaria for Turkey.

Among mosquito species, *An. maculipennis* complex was discovered as the first sibling species complex and was previously identified as a highly important malaria vector in the Middle East and Europe (Falleroni, 1926; van Thiel, 1927). *Anopheles maculipennis* complex has eleven species in the Palearctic region. Of these eleven members, four species have been recorded in Turkey: *An. maculipennis* s.s. Meigen, 1818 (Diptera: Culicidae), *Anopheles messeae* Falleroni, 1926 (Diptera: Culicidae), *Anopheles melanoon* Hackett, 1934 (Diptera: Culicidae) and *Anopheles sacharovi* Favre, 1903 (Diptera: Culicidae), (Parrish, 1959; Postiglione et al., 1973; Kasap & Kasap, 1983; Ramsdale et al., 2001; Simsek et al., 2011). *Anopheles maculipennis* s.s. is the nominotypical member species of the complex, and widely spread across the whole of Europe, except the most northerly regions (Ramsdale & Snow, 2000). However, a recent study showed the expansion of this species to northeastern Europe and northwestern Asia (Novikov & Vaulin, 2014). This species is thought to be the only species of the complex occurring at altitudes of 1000 m and above, and often has been sampled in small open water bodies in cultivated land (Becker et al., 2010). Even if plasmodial sporozoites have been found in the salivary glands of this species (Barber & Rice, 1935), the feeding preference on domestic animals over humans make this species a moderate malaria vector (Jetten & Takken, 1994).

Variations between size and shape are particularly useful for understanding of ecological relationships because of the correlation with many biological features of different species. Insect wings show ecological responses to environmental changes such as climate and altitude (Morales et al., 2010; Stephens & Juliano, 2012; Gómez et al., 2014; Phanitchat et al., 2019; Prudhomme et al., 2019). Dispersal capacity for blood seeking, maneuverability, and transmission of *Plasmodium* sporozoites into the host are particularly important features for malaria for infected anophelines and directly associated with flight performance (Dudley, 2000). Some studies have shown a positive correlation between size and vector competence with the support of much more parasites in larger mosquitoes (Pulkkinen & Ebert, 2004; Moller-Jacobs et al., 2014). Previous studies indicated wing size and wing shape differences among *Aedes vexans* (Meigen, 1830) (Diptera: Culicidae), (Kuclu et al., 2011) and *Culex theileri* Theobald, 1903 (Diptera: Culicidae) (Demirci et al., 2012) populations.

Geometric morphometry is a powerful tool for assessing shape and size variation and the correlations between them (Dujardin, 2011). Wing size is often used as an alternative index of body size (Cowley & Atchley, 1990), and their flattened two-dimensional structure is particularly useful for landmark-based shape/size studies (Grodnitsky, 1999; Zelditch et al., 2004).

This study investigated the variation in wing size and shape of *An. maculipennis* s.s. populations in different altitudes and habitats from northeastern Turkey. The hypothesis was that altitude and the environmental differences related with altitude may have influence on the wing (body) size or shape of *An. maculipennis* s.s. More detailed information on morphological changes can improve understanding of the epidemiological patterns of medically important mosquito vectors (Dujardin, 2011).

Materials and Methods

Study sites and mosquito collection

Field studies were performed from June to September 2019 in northeastern Turkey, along habitat-climate-elevation gradients ranging from plain habitats to mid-range montane areas (848-1780 m) (Figure 1). Collection sites and coordinates information were given in Table 1.

Figure 1. Mosquito sampling sites (mapped using www.earth.google.com).

Table 1. *Anopheles maculipennis* s.s. collection site and number of specimens examined

Locality	Abbreviation	Habitat type	Altitude (m)	Latitude (N)	Longitude (E)	Specimens
Mürşitali	MA	Plain	848	40°01’15"	44°08’10"	35
Zülfikarköy	ZÜ	Plain	860	39°59’24"	44°08’44"	37
Sürmeli	SÜ	Plain-Low montane	944	40°03’56"	43°47’11"	19
Yukarıçıyrıklı	YÇ	Low montane	1,031	40°06’46"	43°34’57"	33
Kötek	KÖ	Low montane	1,350	40°13’06"	43°01’06"	23
Kozlu	KO	Mid-range montane	1,780	40°11’11"	42°56’59"	18

Active adults were collected with New Jersey light traps run between 1700 and 0700 hours at each collection site for one night per month. Additionally, two experimental collectors were collected indoor resting mosquitoes. Samples were stored at −20°C until analyzed.

Molecular identification of *An. maculipennis* s.s.

Schaffner et al. (2001) descriptions and keys were used first to identify *An*. *maculipennis* complex by morphology. Multiplex polymerase chain reaction (PCR) was then used for identification of complex members (Proft et al., 1999). Sequences from the isolates belong to some *An*. *maculipennis* s.s. species were deposited in the NCBI GenBank database under accession numbers MZ666139-48.

Wing preparation and data acquisition

The wings of specimens were separated from the thorax and fixed on labeled slides with Entellan (Merck KgaA, Darmstadt, Germany). To avoid errors in the analyses of within-individual correlation, only the left side was used. A stereoscopic zoom dissection microscope was used to photograph and digitize the wings. A single experimenter scored all the wings to avoid inter-rater bias, and to evaluate repeatability of the measurements. Procrustes ANOVA was performed with MorphoJ software (Klingenberg, 2011) on individuals for 19 landmarks, which were scored two times for each specimen.

The tpsUtil (Rohlf, 2019a) was used to build a data file (tps) from wing images. Using TpsDig (Rohlf, 2018), 19 landmarks were digitized and analyzed (Figure 2). The position of these landmarks was chosen based on the clarity of the intersection of the wing lines to prevent visual error. The generalized least squares Procrustes superimposition method was used to scaling, translation and rotation of the landmark configurations against the consensus configuration (Bookstein, 2007). Tps-Relw (Rohlf, 2019b) was used to analyze the coordinates and compute the eigenvalues for each principal warp. The consensus configurations per wing for each female mosquito were subjected to relative warps analysis by defining the variability in the shape space using the scores acquired for each individual landmark; this is technically a principal component analysis (PCA). The relative warps correspond to the principal components and define a shape space in which individual landmarks are replaced.

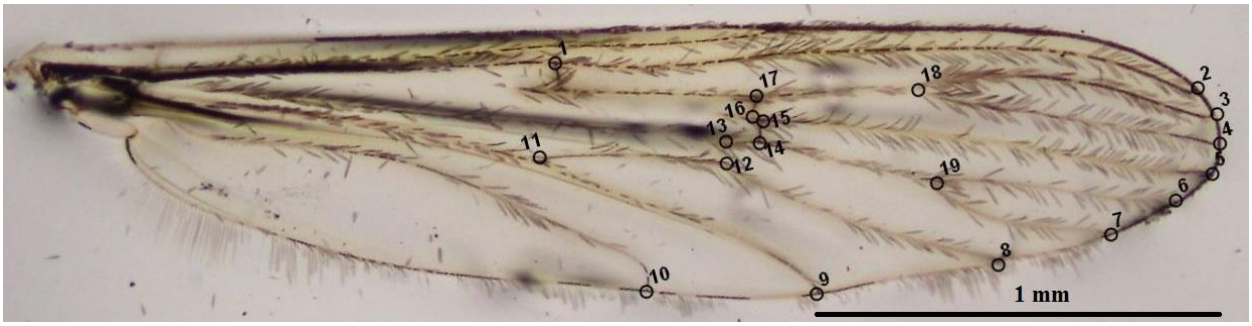

Figure 2. Positions of landmarks on the wing of *Anopheles maculipennis* s.s.

MorphoJ was used for canonical variates analysis to compare the different altitude populations using group membership information via landmark data. Squared Mahalanobis distances were provided from the computed CVA to measure the intraspecific phenetic correlations. Pair-Mahalanobis distances between populations were used for a non-parametric permutation (10,000 runs) after a Bonferroni correction test to analyze the statistical significance of wing shape differences. The matrix was used to produce a dendrogram using the unweighted pair-group method with arithmetic average (UPGMA).

The centroid sizes of the wings were used as an estimator of the body size of *An*. *maculipennis* s.s via a Mann-Whitney U-test, performed in Past 3.10. Centroid size was defined as the square root of the sum of the squared distances between the center of the configuration of all landmarks and each individual landmark (Bookstein, 2007). Differences in average wing CS between each location were analyzed with a

non-parametric permutation (10,000 runs) and a Bonferroni correction test. A linear regression between size and altitude were tested for each altitude. Additionally, the contribution of size to wing shape (residual allometry) was estimated by multivariate regression of the shape and log-transformed centroid size variation based on the Procrustes coordinates with 10,000 permutations in MorphoJ.

Results

For the geometric morphometrics analyses, 165 female mosquito specimens were measured (Table 1). The PCR results belonging to the *An*. *maculipennis* s.s. are given in Figure 3. The results of the Procrustes ANOVA testing individual variation relative to measurement error were summarized in Table 2. Individual differences for size and shape calculated for significantly more variance than error (P < 0.0001).

Table 2. Procrustes ANOVA for *Anopheles maculipennis* s.s. used to calculate the measurement errors

Data	Effect	SS	MS	df	F	P
Centroid size	Individual	934	934	1	20.9	<0.0001
	Residual	14656	44.7	328		
Shape	Individual	0.0152	0.000449	34	15.8	<0.0001
	Residual	0.317	0.0000284	11252		

SS, sum of squares; MS, mean square; df, degrees of freedom; F, F statistic; P, probability.

Size variation

The size differences were significant between some populations (Table 3). While centroid size did not show a linear association with altitude, samples from Kozlu, located at the highest altitude, had larger wings than other populations (Figure 3). The relationship of overall shape variation with size was 1.04% and not significant (P = 0.78).

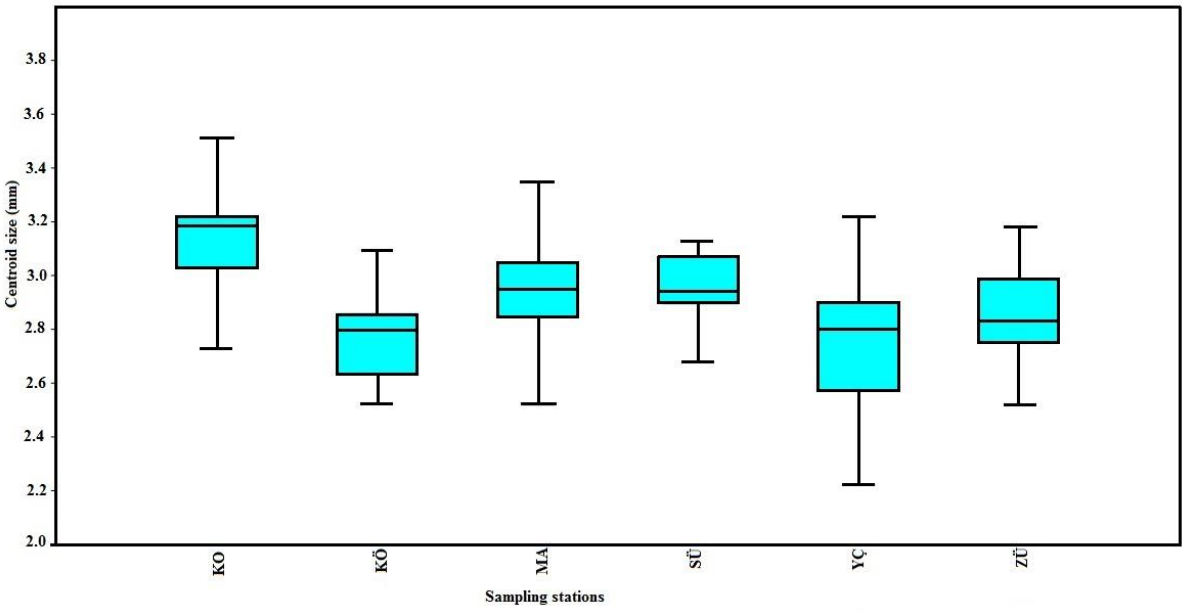

Figure 3. Centroid sizes of each *Anopheles maculipennis* s.s. population from different altitudes: Zülfikarköy (ZÜ) 848 m, Mürşitali (MA) 860 m, Sürmeli (SRM) 944 m, Yukarıçıyrıklı (YÇ) 1,031 m, Kötek (KÖ) 1,350 m and Kozlu (KO) 1,780 m.

Table 3. Statistical significance (P < 0.05) for size (upper half) and shape (lower half). Results in bold denote statistical significance after a Bonferroni correction test

Population	KO	KÖ	MA	SÜ	YÇ	ZÜ
KO	-	**<0.001**	**0.105**	**0.013**	**<0.001**	**<0.001**
KÖ	0.591	-	**0.001**	**0.036**	1.000	1.000
MA	0.360	**0.045**	-	1.000	**0.005**	0.212
SÜ	**0.001**	**0.036**	0.777	-	**0.033**	0.671
YÇ	**0.001**	1.000	0.364	1.000	-	1.000
ZÜ	**0.001**	**0.006**	0.415	**0.001**	**0.001**	-

Shape variation

When the PCA was performed on the 19 landmarks, the first three discriminant factors explained 20.2, 14.4, and 10.1% of the total variance. Canonical variables 1 and 2, which explained 32.3 and 30.9%, respectively, indicating the distinction between populations. The distributions of the individuals along the first two canonical variables are shown in Figure 4. The wing shape differences were supported by permutation tests (10,000 rounds of permutation) of Mahalobonis distances for most of the populations (Table 3). When the shape differences between populations were analyzed by UPGM provided from Mahalobonis distances: Sürmeli, Yukarıçıyrıklı and Zülfikarköy populations comprised the first group and Kötek, Mürşitali, and Kozlu populations the second group (Figure 5).

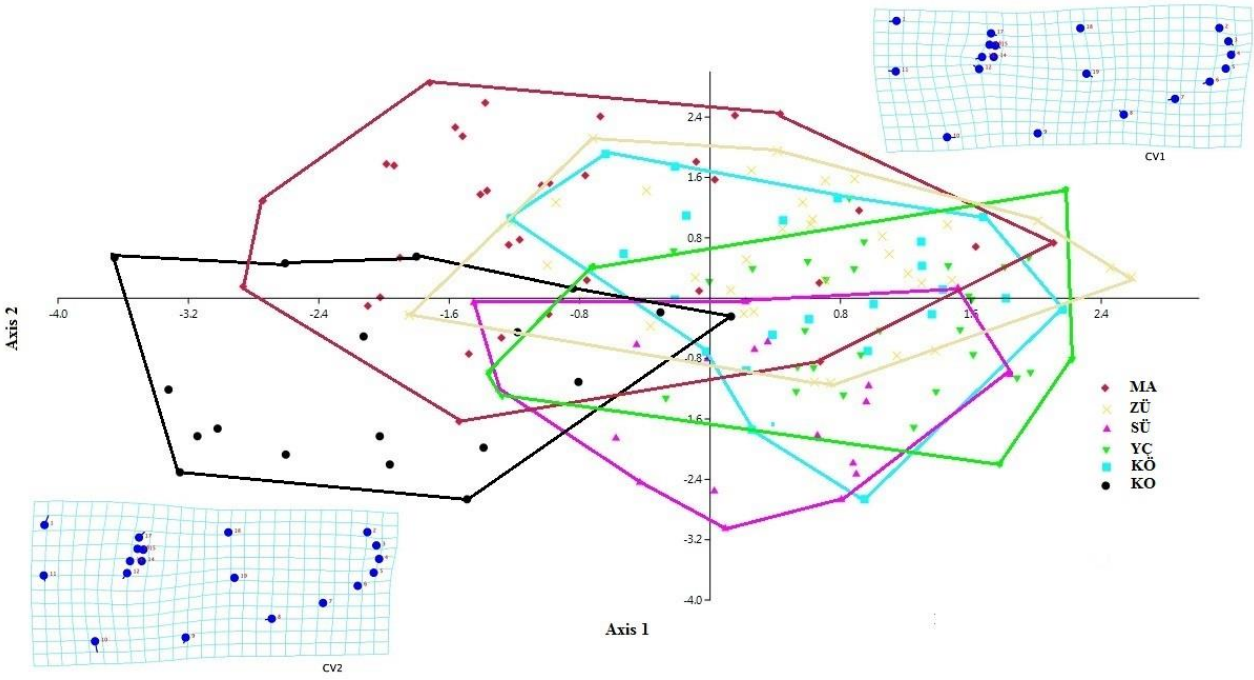

Figure 4. Distribution of the female *Anopheles maculipennis* s.s. along the first two canonical variables.

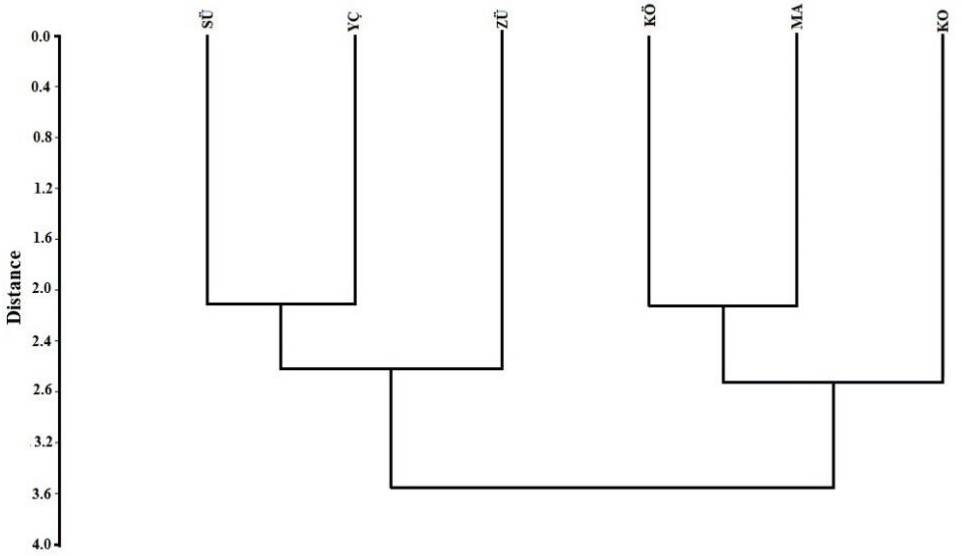

Figure 5. UPGMA conducted by the data derived from Mahalanobis distances.

Discussion

This is the first comparative geometric morphometric study of *An*. *maculipennis* s.s. populations in Turkey or elsewhere. Although the results indicate different levels of wing size/shape differences between populations, it may not be possible to draw any conclusions concerning the effect of month on mosquito phenotypes. Environmental and climatic features could change depending on the month. Also, sampling size may be limiting for discriminant analyses. Additional studies should be considered with larger sample sizes according to month and altitude.

Although the results indicate different levels of wing size/shape differences between populations, there was not an obvious difference between populations due to altitude. Similar to the present results, *Ae. vexans* populations from northeastern Anatolia (Kuclu et al., 2011), *An*. *sacharovi* populations from southeast Anatolia (Yurttas & Alten, 2006), and *Phlebotomus papatasi* (Scopoli 1786) (Diptera: Psychodidae), populations from Şanlıurfa Province of Turkey (Belen et al., 2004) did not show a clear difference related to altitude. Nevertheless, *C. theileri* populations collected from nine altitudes from 808 to 2,130 m in northeastern Anatolia appeared to have a clear phenotypic difference related to altitude (Demirci et al., 2012). However, while centroid size did not show a linear association with altitude in the present study, samples from Kozlu, located at the highest altitude, had larger wings than other populations.

Several species show geographical clines in body size with larger individuals presenting in higher latitudes/altitudes and this adaptation pattern was first explained by Bergmann (1847), with the explanation that small surface area to volume ratios may be more suitable for not losing heat in cold areas (Blanckenhorn & Demont, 2004). Previous studies conducted with *Anopheles gambiae* Giles 1902 (Diptera: Culicidae) revealed a positive correlation between mosquito body size and malaria transmission due to the fact that larger females have a longer lifespan, feed more often and use the blood meal more effectively compared to smaller individuals (Takken et al., 1998; Manoukis et al., 2006; Aboagye-Antwi & Tripet, 2010; Christiansen-Jucht et al., 2014). It might be expected that the larger mosquitoes from Kozlu (1,620 m) populations might be more effective malaria vectors than the other populations. Nevertheless, there are numerous other regional factors known to affect the vector and pathogen transmission capacity of mosquito populations (Cohuet et al., 2010).

However, other studies conducted with *Aedes albopictus* (Skuse 1895) (Diptera: Culicidae), and *Aedes aegypti* (Linnaeus 1762) (Diptera: Culicidae), (Noden et al., 2016; Reiskind & Lounibos, 2009) did not show a correlation between body size and longevity, and studies conducted with *Anopheles coluzzii* Coetzee & Wilkerson, 2013 (Diptera: Culicidae), (Vantaux et al., 2016) and *Ae*. *aegypti* (Zeller & Koella, 2016) showed a negative correlation between body size and longevity.

The size of adult mosquitoes is primarily associated with temperature (Bar-Zeev, 1958; Lyimo et al., 1992; Zeller & Koella, 2016) and larval diet, which are associated with various factors such as food availability, larval habitat quality, larval density and competition (Nasci & Mitchell, 1994; Renshaw et al., 1994; Gimnig et al., 2002; Strickman & Kittayapong, 2003; Schneider et al., 2007; Moller-Jacobs et al., 2014; Shapiro et al., 2016; Vantaux et al., 2016). Larval development conditions might thus be an explanation for larger size. Unfortunately, there is no data on larval habitats, so the relationship between larval habitats and size changes is unknown.

In this study, although mean wing shape comparisons revealed significant differences between populations such as the Sürmeli, Yukarıçıyırıklı, and Zülfikarköy populations comprised the first group, Kötek, Mürşitali, and Kozlu populations comprised the second group, a clear-site/altitude specific population differentiation is not evident in this species. Various factors such as genetic, biological, ecological, environmental and physiological influences may be associated with shape differences between populations, similar to size differences. Although geographical clines associated with latitude or temperature were described in many animal species for size-related features, the relations between shape and environment are still not particularly clear (James et al., 1997; Huey et al., 2000). There is conditional confirmation suggesting that developmental and evolutionary temperature-connected cell size variation have opposite effects on wing shape in *Drosophila subobscura* Collin, 1936 (Diptera: Drosophilidae) (Calboli et al., 2003). Variations in wing shape could have relation with flight performance and be an indicator for an adaptation related to flight dynamics. A previous study conducted between migrant and non-migrant populations in dragonflies showed important wing shape modifications (Johansson et al., 2009). A previous morphometrics study revealed that thinner wings could be more adaptive for *Aedes albifasciatus* (Macquart, 1838) (Diptera: Culicidae) populations to avoid disturbance in a windy environment (Garzón & Schweigmann, 2018). Other studies conducted with *Ae*. *aegypti* populations in multiple geographical locations (coastal, residential and cultivated areas) of Samut Songkhram, Thailand (Chaiphongpachara & Laojun, 2020) and with *Anopheles darlingi* Root, 1926 (Diptera: Culicidae) in five major Brazilian ecoregions (Motoki et al., 2012) revealed differences in wing shapes between all geographical areas and ecoregions due to environmental factors such as wind current and weather. A previous study conducted with *Ae*. *albopictus* populations from Black Sea and Aegean coastal populations in Turkey indicated some shape and size differences between some populations and also a high-level significant difference was found between Aegean and Black Sea coastal populations in wing shapes (Demirci et al., 2021). A study conducted on sandflies comparing island and mainland locations in Thailand showed differences between populations with greater morphological variations in island populations (Sumruayphol et al., 2017). Contrasting ecosystems results variation in mosquito populations (Vicente et al., 2011; Motoki et al., 2012). Studies have also indicated that vector competence differences, such as transmission and infection rates, may exist in different geographical locations (Bennett et al., 2002; Goddard et al., 2002).

It is known that the wing traits are not only affected by environmental conditions but also genetics and changes at the molecular level (Fusco & Minelli, 2010). Ayala et al. (2011) showed the effect of environmental conditions and chromosome polymorphisms on phenotypic variation of a very important African malaria vector, *Anopheles funestus* Giles, 1900 (Diptera: Culicidae) in different eco-climatic regions of Cameroon.

In conclusion, these results highlight the adaptability and plasticity of this mosquito species even over short distances, however, the reasons for observed shape and size variations in wing morphology need more exploration. The investigation of phenotypic and genetic differences in *An*. *maculipennis* s.s. populations could shed light on malaria transmission dynamics. It is necessary to conduct further studies to determine the drivers of these variations and if they have a genetic basis or a phenotypic reflection in different environments.

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Original article (Orijinal araştırma)

Translocation of clothianidin to guttation fluid and its potential impact on honey bee, *Apis mellifera anatoliaca* Maa, 1953 (Hymenoptera: Apidae)[1]

Clothianidinin gutasyon sıvısında taşınımı ve bal arısı, *Apis mellifera anatoliaca* Maa, 1953 (Hymenoptera: Apidae) üzerindeki etkisinin belirlenmesi

Melis YALÇIN[2*] İlknur KAPIZ[2] Mustafa KÖSOĞLU[3] Yakup Onur KOCA[4] Nalan TURGUT[2] Erkan TOPAL[3] Perihan Binnur KURT KARAKUŞ[5] Levent ATATANIR[6] Serhan MERMER[7] Merve ÖZKALELİ AKÇETİN[5] Jens PISTORIUS[8] Cafer TURGUT[2]

Abstract

Honey bees, *Apis mellifera anatoliaca* Maa, 1953 (Hymenoptera: Apidae) forage water from guttation fluid so transported neonicotinoid insecticides in guttation fluid poses a risk to the bees. The first aim of this study was to determine the toxicity and risk of clothianidin to honey bees. In addition, the changes of clothianidin residue in the guttation fluid of maize plants in Turkey were determined in 2018 and 2019. Also, the toxicity of guttation fluid collected from the maize plants to bees was determined in ecotoxicological tests. The acute oral LD_{50} of clothianidin to honey bees in the first 24 h was 1.80 ng bee^{-1} and residue analysis demonstrated that honey bees were exposed to clothianidin concentration in guttation fluid ranging from 0.02 to 6.0 mg L^{-1} with mortality ranging between 80 and 100%. As the measured concentration of clothianidin in guttation fluid can lead to the mortality of honey bees, present study indicates that clothianidin, and possibly related pesticides in treated maize seed poses a risk to honey bees. Future studies are needed to determine the scale and distribution of this risk in Turkey.

Keywords: Clothianidin, exposure risk, guttation, honey bees, residue

Öz

Bal arılarının, *Apis mellifera anatoliaca* Maa, 1953 (Hymenoptera: Apidae) gutasyon sıvısından su ihtiyacını karşılaması sırasında gutasyon sıvısında tohum ilacı olarak kullanılan neonicotinoid insektisitlerin taşınması arılarda risk yaratmaktadır. Bu çalışmanın ilk amacı clothianidinin arılara toksisitesi ve riskinin belirlenmesidir. Bunun yanında 2018 ve 2019 yıllarında Türkiye'de mısır bitkisindeki gutasyon sıvısında clothianidin kalıntısının değişimleri belirlenmiştir. Ayrıca mısır bitkisinden toplanan gutasyon sıvılarının arılara toksisitesinde yapılan ekotoksikolojik testlerde tespit edilmiştir. Clothianidinin ilk 24 saatteki bal arılarına olan toksisitesi incelendiğinde ortalama akut oral LD_{50} değeri 1.80 ng arı$^{-1}$ olarak saptanmıştır. Bununla birlikte kalıntı analizleri sonucunda bal arılarının gutasyon sıvısında 0.02 mg L^{-1} ile 6 mg L^{-1} arasında değişen clothianidin konsantrasyonuna maruz kaldığı tespit edilmiş olup bal arılarının ölüm oranının %80 ile %100 arasında değiştiği gözlenmiştir. Gutasyon sıvısında ölçülen clothianidin konsantrasyonu bal arılarının ölümüne yol açabileceğinden, bu çalışma, mısır tohumlarına uygulanan clothianidin ve benzer grupta olan pestisitlerin, bal arıları için risk oluşturduğunu göstermektedir. Bu riskin Türkiye'deki miktarını ve dağılımını belirlemek için gelecekte yapılacak çalışmalara ihtiyaç vardır.

Anahtar sözcükler: Clothianidin, maruziyet riski, gutasyon, bal arıları, kalıntı

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[2] Aydın Adnan Menderes University, Faculty of Agriculture, Department of Plant Protection, 09100 Aydın, Turkey
[3] Aegean Agricultural Research Institute, Apiculture R&D Innovation and Breeding Queen Bee Production Section, İzmir, Turkey
[4] Aydın Adnan Menderes University, Faculty of Agriculture, Department of Field Crops, 09100 Aydın, Turkey
[5] Bursa Technical University, Architecture and Engineering, Faculty of Natural Sciences, Department of Environmental Engineering, 16310 Bursa, Turkey
[6] Aydın Adnan Menderes University, Faculty of Agriculture, Department of Soil Science and Plant Nutrition, 09100 Aydın, Turkey
[7] Oregon State University, Department of Horticulture, Corvallis, Oregon 09331, USA
[8] Julius Kuehn-Institut (JKI), Federal Research Centre for Cultivated Plants, Institute for Bee Protection, Messeweg11/12, Braunschweig, Germany
* Corresponding author (Sorumlu yazar) e-mail: melisusluy@adu.edu.tr
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Introduction

Seed coating with pesticides is widely used in agriculture to control and prevent pests and diseases. Pesticides used for seed coating also provide protection after emergence of the crop (Mancini & Romanazzi, 2014). Systemic insecticides are the most prevalent as a seed coating for agricultural crops. They are taken up from roots and transported to different parts of plants (e.g., stalk and leaves) (Karmakar et al., 2006; Huff Hartz et al., 2017). The high-water solubility, low use rates, broad spectrum activity and systemic movement in plants are desired properties for insecticides in pest management; however, their transportation leads to contaminated nectar, pollen and guttation fluid which can be a route of exposure for honey bees (Schmuck et al., 2001; Krupke et al., 2012; Cutler et al., 2014; Rundlöf et al., 2015; Alkassab & Kirchner, 2016; Schenke et al., 2018; Mörtl et al., 2020). Especially the neonicotinoids are the most significant systemic insecticides of the past few decades (Tomizawa & Casida, 2003). Their introduction was well-received by farmers, pesticide regulators and toxicologists due to the high efficacy against pests, selectivity and new modes of action (Matsuda et al., 2001; Jeschke & Nauen, 2008; Cutler et al., 2014). Due to the systemic properties, neonicotinoid insecticides have been widely applied to soil via irrigation, seed coatings, as well as foliar application. Neonicotinoids act as a competitive modulator of nicotinic acetylcholine receptor that causes immobility, abnormal behavior, excitation, and death of target and non-target insects (Jeschke & Nauen, 2008).

Several adverse effects of neonicotinoids are reported in honey bees including mortality, reduction in the hive fitness, increased susceptibility to pests and pathogens, long term colony viability, impaired learning, homing behavior, memory function, colony strength, reproductivity, flight dynamics, foraging behavior, cognitive functions, no production of new queens, and fewer total brood cells, and all these abnormalities can lead to colony disruption (Schneider et al., 2012; Di Prisco et al., 2013; Palmer et al., 2013; Sandrock et al., 2014; Scholer & Krischik, 2014; Williamson et al., 2014; Karahan et al., 2015; Larson et al., 2015; Alkassab & Kirchner, 2016; Brandt et al., 2016; Solomon & Stephenson, 2017; Woodcock et al., 2017; Çakmak, 2018; Abdelkader et al., 2021). There are lots of exposure routes of neonicotinoids to honey bees such as nectar of crops, accumulation of neonicotinoids on the plant parts such as flowers, transportation by the root system and contact exposure (soil and planter dust) (Krupke et al., 2012). In addition, guttation fluid that exudes from xylem pores on leaf margins can be an additional route of exposure for honey bees (Girolami et al., 2009; Thompson, 2010; Pistorius et al., 2012). Guttation is an active physiological process that occurs when leaves cannot transpire water because of the unfavorable conditions. In the humid and windless times, root pressure is so strong that plants exude xylem fluid (Shawki et al., 2006). In that situation, guttation fluid will be exude from xylem pores known as hydathodes. In general water demand of the beehive is high during spring and summer seasons, and honey bees use water to regulate the temperature and humidity of the beehive, to dilute thick honey and to feed the brood. Plants offering pollen and nectar will attract honey bees from long distances whereas water is gathered close to the colony (Pistorius et al., 2012).

Clothianidin, which breaks down slowly in the environment (US-EPA, 2005), is a neonicotinoid insecticide registered for seed coating (Tomlin, 2004) in various countries including Turkey. It is used for the management of stink bugs, planthoppers, whiteflies, aphids in vegetables, fruits and citrus. It is effective on various pests in the insect orders Coleoptera, Diptera, Hemiptera, Isoptera, Lepidoptera, Orthoptera and Thysanoptera. The properties of clothianidin consists of broad insecticidal spectrum, low use rate, systemic action, high efficacy of control effect and variety of application method (Uneme, 2011). However, clothianidin belongs to nitro substitution group in neonicotinoids which is one of the most toxic insecticide to bees (Iwasa et al., 2004). The maximum residue limit of clothianidin in maize is 0.02 mg kg^{-1} (Anonymous, 2021). Clothianidin is used worldwide and there are many studies on its residue in pollen, wax syrup and honey (Tapparo et al., 2012; Scholer & Krischik, 2014; Codling et al., 2016; Sanchez-Hernandez et al., 2016; Çil et al., 2020), in guttation (Reetz et al., 2016; Marzaro et al., 2011; Tapparo et

al., 2011; Scmolke et al., 2018), in dust (Girolami et al., 2012; Krupke et al., 2012), and in bee bread and adult bees (Frommberger et al., 2011; Flores et al., 2021). In addition, many researchers have reported evidence of the toxic impacts of clothianidin on honey bees, bumble bees and other bees (Schneider et al., 2012; Palmer et al., 2013; Sandrock et al., 2014; Williamson et al., 2014; Scholer & Krischik, 2014; Larson et al., 2015; Çakmak, 2018; Abdelkader et al., 2021).

Honey bee colonies use water close to the hive and long-distant flight is avoided to save energy, therefore, the location of the bee hive and proximity to water sources such as guttation fluid determines the risk of insecticide exposure. Laboratory chronic exposure and long-term exposure tests have been used to clarify possible effects of clothianidin on bees (Schmuck & Keppler, 2003; Cutler & Scott-Dupree, 2007; Alkassab & Kirchner, 2016). Although the effects of clothianidin residues on honey bees have been studied under various meteorological conditions, there is insufficient information on the relationship between clothianidin residues in guttation fluid from seed treated maize and honey bee mortality in Turkey. Specifically, there is no information on the effects of clothianidin on *Apis mellifera anatoliaca* Maa, 1953 (Hymenoptera: Apidae), which is the most common as well as economically and ecologically important honey bee race in Turkey (Akyol & Kaftanoğlu, 2001). Therefore, the main aim of this study was to determine whether under Turkish conditions, clothianidin used in seed coating is transformed into guttation fluid of maize and if this causes toxicity to *A. mellifera anatolica*. In addition, the toxicity of clothianidin in guttation fluid to *A. mellifera anatolica* was assessed over a range of concentrations in the laboratory test.

Materials and Methods

Bees

Worker honey bees, *A. mellifera anatoliaca* were collected from a single colony (disease free and queen-right) at the Aegean Agricultural Research Institute, Izmir, Turkey in 2018 and 2019. Honey bees were collected and transformed in stainless-steel test cages (8 × 6 × 4 cm) with aeration holes. Ten individuals (5-10 days old) were place in each cage and maintained in darkness at 25°C and under a controlled humidity of 50-70%. Sucrose solution and water were given together by syringe through the hole on the cages before starting the experiment and then replaced by guttation fluid collected with syringes from the field mixed with sucrose solution (1:1).

Field sites and planting

Maize fields of the Faculty of Agriculture Research Farm (37°45’32" N 27°45’35" E), Aydin Adnan Menderes University, Aydin, Turkey were uses in the experiments. Treatment plots consist of three field replicates (with clothianidin and control) and each experimental plot had an area of 250 m^2. The maize row and intra-row spacing was 70 and 18 cm, respectively.

Three plots were selected based on the randomized block design for the treatments. The maize sown was the hybrid Pioneer P2088 (Corteva Agriscience, Wilmington, DE, USA) with clothianidin applied to the seed as Poncho FS formulation (BASF, Ludwigshafen, Germany) provided by SeedEFE Ltd. (Aydın, Turkey) at 84 ml of product per 50,000 seed (Anonymous, 2021).

Collection of guttation fluid

Guttation fluid was collected from maize seedlings when the plants were at the 2-3 leaf-stage. Sampling was conducted throughout the growing period and the minimum time between any two sampling was 24 h. Sampling was done by using a Pasteur pipette from May until mid-June in 2018 and 2019. Approximately 10 ml of guttation fluid was collected from each row and field collections were conducted early in the morning (from 06:00 to 08:00 h). Samples were stored at -20ºC until the analyzed. Half of each sample was used for the residue analysis and the other half was used for the exposure experiment.

Chemicals

Acetonitrile (99.9% HPLC grade), acetic acid 96% and sodium acetate anhydrous for analysis were obtained from Merck (Darmstadt, Germany). $MgSO_4$ anhydrous for analysis was obtained from Sigma Aldrich (Merck). PSA (primary and secondary amines) bonded silica bulk was obtained from Supelco (Bellefonte, PA, USA). Hydrophilic PTFE, 0.20 µm pore size, 25 mm diameter syringe filters were obtained from Merck. Clothianidin (purity ≥98%) was obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany).

LC-MS/MS analysis

Confirmation of the clothianidin results were provided by European Commission Guidelines (Pihlström, 2011). Experiments were performed with a Shimadzu 8030 (Tokyo, Japan) liquid chromatography (LC) tandem mass spectrometry (MS/MS) apparatus on a C18 Column (GL Services Luertsil OD54 - 3 µm 4.1 × 50 mm (UP), Cat No: 5020-04012 Merck. Mobile phase solvents were 0.1% formic acid and acetonitrile. The mobile phases were milli deionized water with of 0.03% of 5 mM ammonium formate (solvent A) and methanol with 0.03% ammonium formate (solvent B). The gradient elution program was: 0.0-6.5 min 95% B, 6.5-7.5 min 95% B, 7.5-8 min 5% B held for 12 min. The injection volume was 20 µL with a flow rate of 0.4 mL min^{-1}. Other operating conditions were: nebulizer gas flow 3 L min^{-1}, drying gas (N_2) flow rate 15 mL min^{-1} and drying gas temperature 400°C. The calibration curve was linear ($r^2 > 0.99$) in the range of 5-200 µg L^{-1}. Precursor ion (m/z) was 250 while product ions (m/z) were 169 and 132 for multiple reaction monitoring and related voltage was 12 and 16 V. Retention time was 3.7 min.

Toxicity tests

Dose response assessments was performed according to the OECD Guideline No 213/214 having a control and seven concentrations of clothianidin (OECD, 1998). Three replicate tests were performed for each test dose and totally 27 cages were used in the experiment. Each replicate group had 10 bees and dosed with each test concentration ranging from 0.04 to 2.7 ng mL^{-1}. During the experiment, honey bees were fed with 50% sucrose solution and incubated at 25°C (except during observation) under a controlled humidity of 50-70%. Before the test, honey bees were kept in stainless-steel test cages for at most 2 h without sucrose solution. Then 50% sucrose solution and different clothianidin doses (details given below) were added to each cage from weighed syringe feeders. For a calculation of exact dose uptake of the solution, the amount of consumed clothianidin with sucrose solution was determined from the weight difference of the syringes. Total of seven clothianidin doses according to the already established acute LD_{50} value (0.00379 µg bee^{-1}) (EFSA, 2013) of honey bees were used in the experiment. Mortality was assessed at 4 and 24 h intervals for up to 48 and 72 h after dosing. Honey bees were considered dead or moribund if they stopped moving. Mortality values were compared with values from positive control. The median lethal dose (LD_{50}) of clothianidin was established by using probit analysis using the POLO Plus program (LeOra Software, Berkeley, CA, USA).

Acute oral toxicity assessments were performed to determine the risks of guttation fluid to honey bees according to the draft OECD Guideline 213 (OECD, 1998). Honey bees were transferred to stainless-steel test cages in randomized groups of 10 individuals. The test conditions were same as the dose response assessments. The test solutions consisted of guttation fluid from 27 collection days with the addition of 50% w/v sucrose solution. The consumed quantity per cage was recorded after 4, 24 and 48 h by weighing the syringes at each time and also dead bees were recorded to determine mortality. Three replicate assessments were performed for each guttation fluid sample.

Clothianidin content in guttation fluid

Residues of clothianidin in guttation fluid were determined by the modified QuEChERS (quick, easy, cheap, effective, rugged and safe) method (Anastassiades et al., 2003). Ten ml acetonitrile acetic acid mixture (100:1 v/v) was added into 5 ml of guttation fluid. Sample was mixed for 15 s on a vortex mixer. $MgSO_4$ (4 g) and CH_3COONa (1 g) was then added to this mixture and mixed on a vortex mixer immediately for 1 min and centrifuged for 5 min at 4000 rpm. Two ml aliquot of supernatant was transferred into 15-ml centrifuge tubes by micropipette. $MgSO_4$ (0.3 g) and PSA (0.1 g) were added to the aliquot and mixed for 10 min, and then centrifuged for 2 min at 4000 rpm. The supernatant was transferred to glass centrifuge tubes and the volume of the extract was reduced to 0.25 ml by vacuum centrifugal evaporation filtered through into PTFE filters (25 mm x 0.22 µm) and transferred to the auto sampler vials for LC-MS/MS analysis. The extracts were stored at -20°C until analyzed.

Results

The toxicity (LD_{50}) of clothianidin to *A. mellifera anatoliaca* was 1.80 ng bee^{-1} (slope = 1.55; $\chi 2$ = 11.6) and the LD_{90} was 12.0 ng bee^{-1} (slope= 1.55; $\chi 2$ = 11.6).

The concentration of clothianidin in guttation fluid was slightly higher in 2018 than in 2019 with both residue levels comparably high. The highest concentrations of clothianidin in guttation fluid in 2018 and 2019 were 5.7 and 4.9 mg L^{-1}, respectively. In both years, the residues of the clothianidin were detected from the onset of guttation but declined by time. The residual toxicity of clothianidin in the guttation fluid collected in 2018 declined on day 12, and concentrations ranged between 0.5 and 5.7 mg L^{-1} (Figure 1). In 2019, clothianidin concentrations ranged from 0.02 to 4.9 mg L^{-1} (Figure 2) with the maximum concentration recorded on day 12 and the clothianidin residue followed a similar trend as in the first-year experiment (Figure 2). For guttation fluid the limit of detection for clothianidin was 0.001 mg L^{-1}, and the limit of quantitation was 0.005 mg L^{-1}.

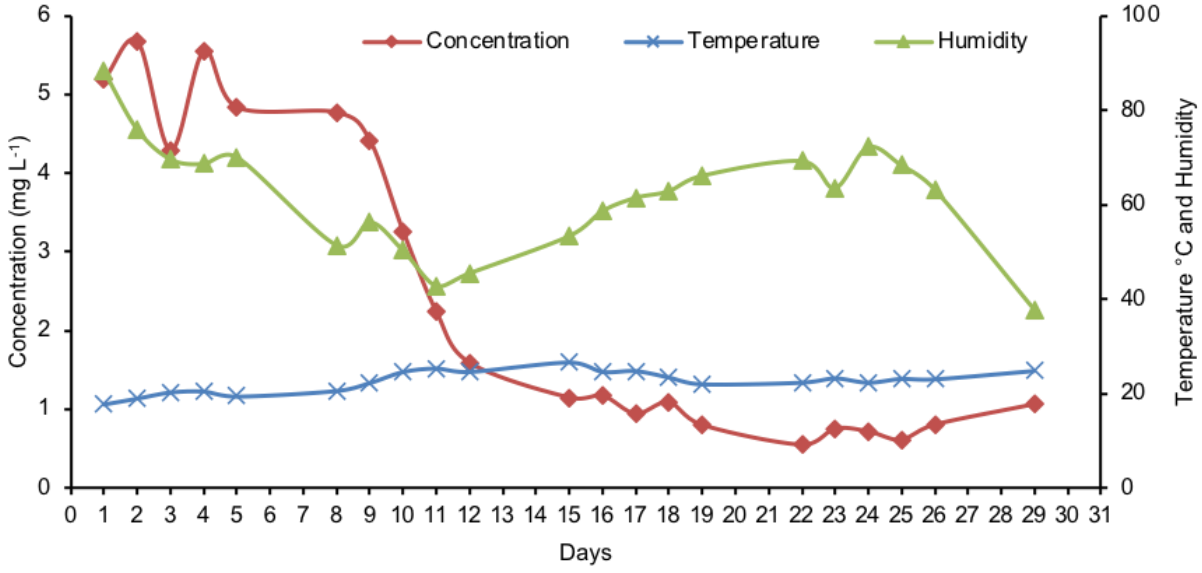

Figure 1. Concentration of clothianidin in sampled guttation fluid in maize by days in 2018.

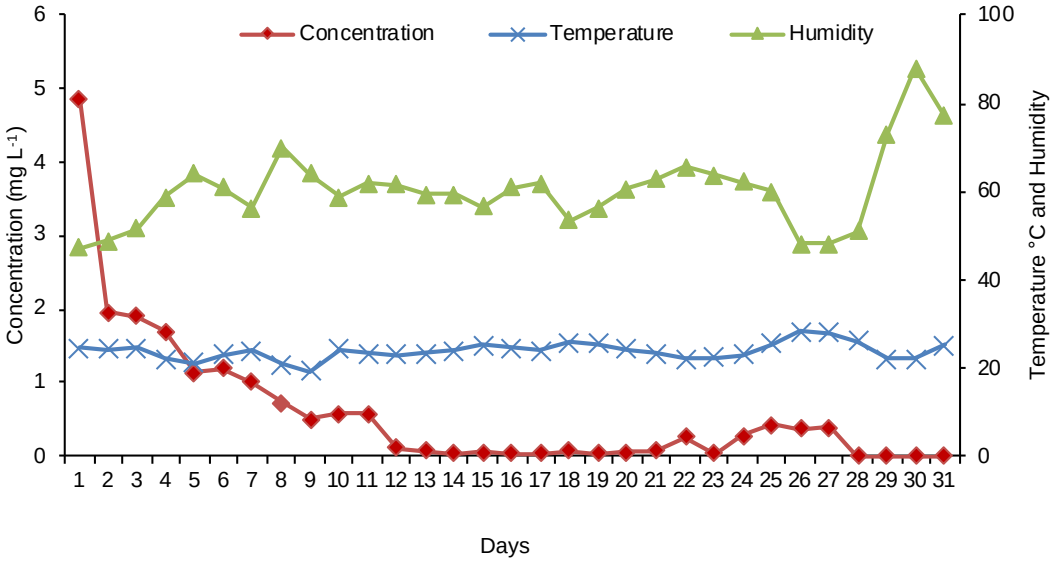

Figure 2. Concentration of clothianidin in sampled guttation fluid in maize by days in 2019.

In 2018 (Figure 3), mortality decreased irregularly, especially in the 4 h observations, and mortality in the 24 and 48 h observations were significantly higher than after 4 h. Mortality varied between 70 and 100% after 4 h due to the high concentration of clothianidin in guttation fluid until day 12. From day 12, mortality decreased unevenly. After 24 h, 100% mortality was observed for the first 15 days then it decreased in parallel with diminished clothianidin concentrations. After 48 h, 100% mortality continued for 22 days and decreased to 37% by day 29. In 2019 (Figure 4), up to day 13, mortality ranged from 50 to 100% after 4 h and 100% after 24 and 48 h. After day 13, mortality decreased irregularly after 4, 24 and 48 h. The concentration of clothianidin in guttation fluid in 2019 decreased to day 12 (Figure 2), so the decreased mortality observed was consistent with this.

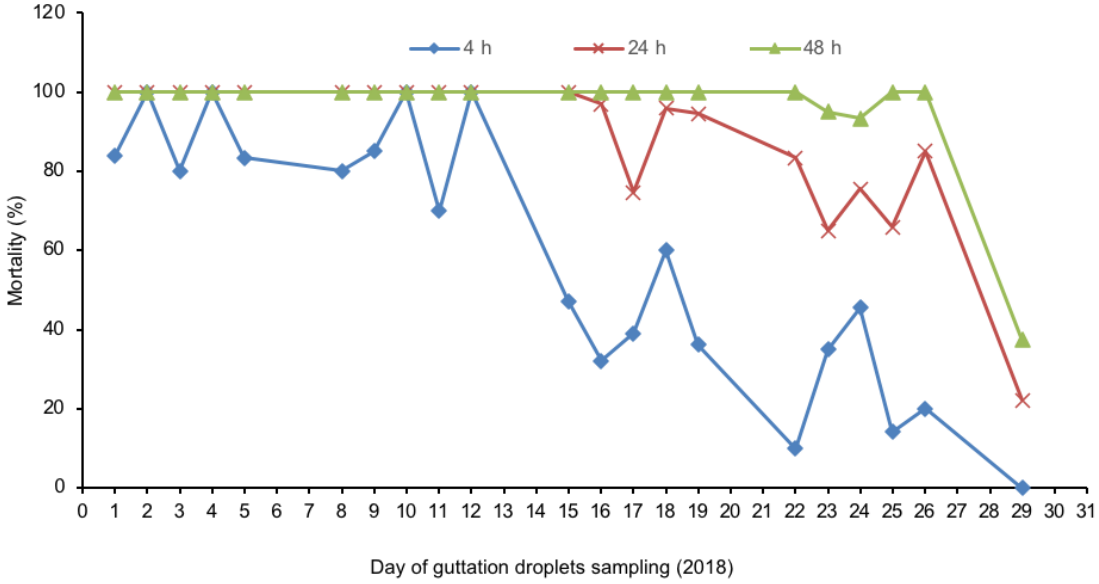

Figure 3. Bee mortality observed at 4, 24, 48 h after oral application of guttation fluid collected from different days from maize plants in year 2018.

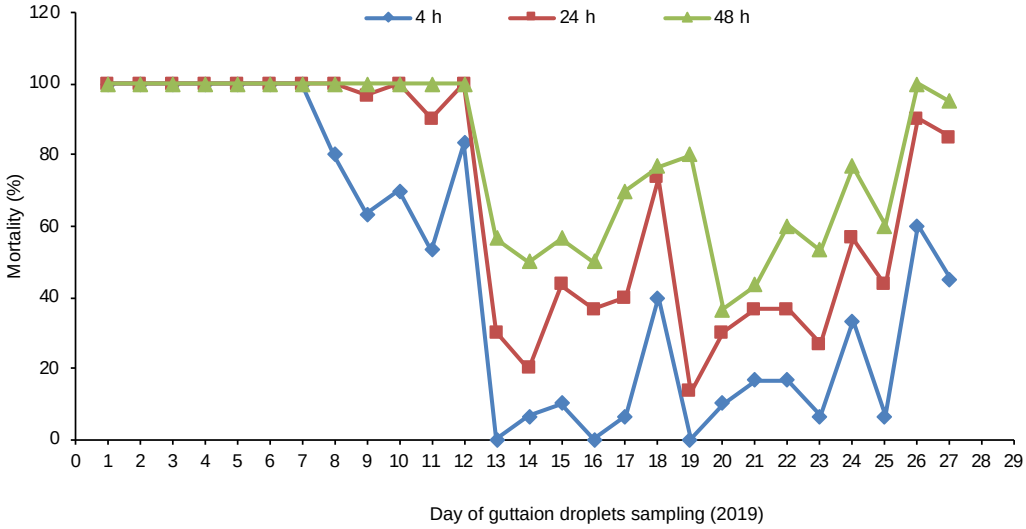

Figure 4. Bee mortality observed at 4, 24, 48 h after oral application of guttation fluid collected from different days from maize plants in year 2019.

Discussion

This study showed that clothianidin was significantly toxic to honey bees with LD_{50} of 1.80 ng bee^{-1} at 24 h. According to the Insecticide Classification of WSDA (2010) pesticides, LD_{50} <2 µg bee^{-1} is classified as significantly toxic to honey bees. Also, Laurino et al. (2013) reported that mean acute oral toxicity LD_{50} of clothianidin at 24 h was 3.53 ng bee^{-1} and at 48 and 72 h was 3.35 and 3.28 ng bee^{-1}, respectively. Pistorius et al. (2012) reported that after consuming 3.7 µL water containing 1 ng µL^{-1} clothianidin, the LD_{50} value of clothianidin in honey bee was 3.7 ng bee^{-1} would be reached. European Commission also reports that for the *A. mellifera* acute oral LD_{50} for clothianidin is 3.79 ng bee^{-1}. When published LD_{50} values are compared to the LD_{50} value detected in the present study, European Commission's value and Pistorius et al. (2012) results are less toxic. Suchail et al. (2001) reported that imidacloprid, which is in the same group with clothianidin (Group 4 in IRAC modes of action), has an LD_{50} of 60 ng bee^{-1} at 48 h and 40 ng bee^{-1} at 72 and 96 h, whereas Laurino et al. (2013) reported an LD_{50} of 90.1 ng bee^{-1} at 48 h and 69.7 ng bee^{-1} at 72 h. These contrasting results of the present study would likely be due to the particular features of pesticide toxicology or variation in the amounts ingested (Nauen et al., 2001; Suchail et al., 2001) or the differences could also be due to the different bee genotypes. For example, the acute oral LD_{50} of clothianidin in *Apis mellifera ligustica* Spinola, 1806 (Hymenoptera: Apidae) was 2.0 ng bee^{-1} whereas in *Apis cerana cerana* Fabricus, 1793 (Hymenoptera: Apidae) it was 0.5 ng bee^{-1} (Li et al., 2017), with the latter much lower.

In this study, the clothianidin residues in guttation fluid of maize was found to be between 0.02 to 6 mg L^{-1}. Similar clothianidin residue concentrations (up to 8 µg mL^{-1}) were reported in Germany for treated maize guttation fluid and this remained detectable over several weeks (Reetz et al., 2011). Nikolakis et al. (2014) who reported that guttation fluid collected in the early stages of the crop were more harmful due to the peak residue levels of neonicotinoids. Peak residue levels which were 8.5 mg L^{-1} for clothianidin and 6.7 mg L^{-1} for imidacloprid in winter barley (Nikolakis et al., 2014). The concentrations of three different neonicotinoids in guttation fluid of maize were: imidacloprid from 8.2 to 346 mg L^{-1}, clothianidin from 7.3 mg L^{-1} to 102 mg L^{-1} and thiamethoxam from 2.9 to 40.8 mg L^{-1} (Tapparo et al., 2011). The residue of clothianidin in guttation fluid of sugar beet was reported as 9.04 mg L^{-1} (Wirtz et al., 2018). In combination, these results suggest that neonicotinoid concentrations in guttation fluid are highly variable. Residues of clothianidin used as seed coating have been reported in guttation fluid at lower concentrations in oilseed rape than in maize (Nikolakis et al., 2014). Similar results were found in Germany where honey bees exposed to up to 130 µg L^{-1} clothianidin in guttation fluid of oilseed rape. It is reported that rapid growth of the plants parallels

a rapid decrease in pesticide residues in the oilseed rape guttation fluid (Reetz et al., 2016). Girolami et al. (2009) found that the high concentrations of clothianidin (23.3 ± 4.2 mg L^{-1}) occurred in guttation fluid collected from potted plants in the laboratory. Similar clothianidin concentrations were measured in some studies (Reetz et al., 2011; Nikolakis et al., 2014) and higher in others (Girolami, 2009; Tapparo, 2011; Schenke, 2018; Mörtl et al, 2020). The higher concentrations of clothianidin can be more variable due to various factors including amount of water evaporation, the rate of seedling emergence and time of the day of sample collection (Girolami et al., 2009; Sing et al., 2013; Mörtl et al., 2020). In the present study, sampling time and the time interval for collecting guttation fluid were constant and water evaporation was assessed for each replicate. Also, humidity and temperature can affect the guttation fluid quantity and residue concentrations. Marzaro et al. (2011) reported that highly concentrated insecticide sufficient to kill bees was related to variation in humidity in the field. Also, Wirtz et al. (2018) reported that increased humidity caused the highest occurrence of guttation and Nikolakis et al. (2014) found seasonal difference in residue levels in guttation water. These findings are consistent with the present study. High mortality was observed especially at the beginning of the present experiment, which could be linked to the humidity. At the commitment of the study in 2018, the humidity was about 88% and at about 17°C (Figure 1) whereas in 2019, it was 47% and 24°C, respectively (Figure 2). This indicates that humidity and other environmental factors can influence the pesticide concentration in guttation fluid.

The mortality of honey bees paralleled the residues of clothianidin in guttation fluid of treated maize in the field. Laurino et al. (2013) reported that according to the acute oral toxicity of clothianidin to honey bees, 24 and 48 h results were found similar because most mortality was observed in the first 24 h, as observed in the present study. In contrast, in a field experiment on colony health instead of individual mortality, it was reported that number of adults per colony did not change when they were exposed to clothianidin seed treated canola and exposure time did not affect the honey bee mortality. However, it was observed that queens ceased depositing egg and overwintering was reduced (Cutler et al., 2014). Similarly, in a semi-artificial study, homing flights of honey bees decreased with the exposure to one tenth of median lethal dose of clothianidin (0.002 µg bee^{-1}) (Matsumoto, 2013). Also, honey bees exposed to 10 µg L^{-1} clothianidin had increase hemocyte density than bees exposed to 50 µg L^{-1} (Brandt et al., 2017). Although, in some cases, clothianidin residues in guttation fluid decreased over time, the amount of pesticides required to cause mortality in honey bees decreases and mortality increases (Suchail et al., 2001; Moncharmont et al., 2003; Alkassab & Kirchner, 2016). According to Pistorius et al. (2012), honey bee mortality and colony health were normal but although dead bees were observed, but there was no increase in the mortality and results indicated that single bees came in contact with clothianidin but this did not lead to increase of mortality.

In two experiments, with treated seed and untreated maize crops under semi field conditions, the effects of clothianidin to *A. mellifera* were examined. In the first experiment two treatment variants were used. In the first one, none of the colonies had additional water source while in the second one, all colonies had, uncontaminated water source as an artificial water source. As a result, in the variant with no additional water source high adult bee mortality was observed and the mortality increased in time while no increase in mortality was observed in colonies with alternative water source. Thus, in the extreme semi field scenario it could be demonstrated that bees may use guttation water as a water source; in this scenario no additional water was available and high mortality of foragers and also effects on the colony level were observed in contrast to semi field tunnels in which an uncontaminated water source or uncontaminated guttation fluid were available (Frommberger et al., 2011). This highlights the need to investigate if, and to what extent, honey bees may use guttation fluid in different climate zones and environmental conditions, to exclude that such scenarios as found by Frommberger et al. (2011) might take place in a field-realistic scenario. In the field experiment conducted by Nikolakis et al. (2014) maize seeds treated with clothianidin at a rate of 0.5 mg $seed^{-1}$. The mean numbers of dead bees were 12.7, 46.3 and 38.4 after exposure of 48, 43 and 32

days, respectively. Based on the results observed for control group, mortality cases were uncommon, however, it was concluded that clothianidin did not affect the bee health, overwintering success and honey production. In contrast one of the surveys which compared mortality rate of honey bees located in maize-dominated and maize-free contexts showed that colonies located in maize-dominated areas had 3.51 times higher daily mortality counts compared to those found in maize-free areas (Samson-Robert et al., 2017).

In conclusion, this study demonstrated that clothianidin was transported from seed coated maize and exuded via guttation under Turkish environmental conditions. The concentration of clothianidin in guttation fluid declined over time to a low level for both years. The LD_{50} for clothianidin was lower than reported to published literature; therefore, clothianidin can be classified as one of the most potent insecticide to honey bees *A. mellifera anatoliaca*. In the laboratory experiments, honey bees exposed to clothianidin from guttation fluid from treated maize crop had a wide range of acute oral toxicity and peak residue concentrations coincided with high honey bee mortality indicating the relation between residue concentration and mortality. Also, in the laboratory test clothianidin toxicity increased with exposure time, varying about twofold from 4 to 48 h in both years. Thus, the present study documented clothianidin residues in guttation fluid collected from maize in the field and their effects on the mortality of honey bees in the laboratory. However, the effects of field conditions need to be investigated in future work since the effects of environmental stress factors and realistic water consumption behavior are not addressed in the laboratory tests. Also, winter survival of colonies near clothianidin treated field should be investigated for since bees will consume clothianidin deposited pollen and honey in the hive. In addition, future investigations should consider the effects of other insecticides applied to seed both individually and in a combination.

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Türkiye Entomoloji Dergisi Yayın İlkeleri

Derginin yayın ilkeleri aşağıda özet olarak sunulmuştur. Ayrıntılar için web adresine (www.entomoloji.org.tr) bakınız.

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Türkiye Entomoloji Dergisi
Ege Üniversitesi Kampüsü PTT Şubesi, PK. 10, 35100 Bornova, İzmir
e-posta : dergi@entomoloji.org.tr
web : http://www.entomoloji.org.tr