E-ISSN 2536-491X

Türkiye Entomoloji Dergisi

Turkish Journal of Entomology

Cilt (Vol.): 46 Sayı (No.): 1 2022

Türkiye Entomoloji Dergisi
(Turkish Journal of Entomology)

Cilt (Vol.) **46** **Sayı** (No.) **1** **Mart** (March) **2022**

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Turkish Journal of Entomology

The Turkish Journal of Entomology is a quarterly journal which has been published by the Entomological Society of Turkey. İt accepts original research articles in the fields of entomology and agricultural zoology in Turkish or English.

Abstracted/Indexed in Biological Abstracts, BIOSIS Previews, CABAbstracts, FAO AGRIS, Elsevier Scopus, Global Health, Information Reference Library, Review of Agricultural Entomology, SCI-E, TÜBİTAK/ULAKBİM, VINITI, Zoological Record.

Annual subscription price:€75 Price of asingle issue: €20

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Türk. entomol. derg., 2022, 46 (1): 03-11
DOI: http://dx.doi.org/10.16970/entoted.992984

ISSN 1010-6960
E-ISSN 2536-491X

Original article (Orijinal araştırma)

Quantitation of neuroxin-1, ataxin-3 and atlastin genes related to grooming behavior in five races of honey bee, *Apis mellifera* L., 1758 (Hymenoptera: Apidae), in Turkey[1]

Türkiye'deki beş bal arısı, *Apis mellifera* L., 1758 (Hymenoptera: Apidae) ırkında tımar davranışı ile ilgili neuroxin-1, ataxin-3 ve atlastin genlerinin kantitasyonu

Berkant İsmail YILDIZ[2] **Kemal KARABAĞ[3*]**

Abstract

Although many methods have been used to control *Varroa destructor* Anderson & Trueman, 2000 (Acari: Varroidae), the satisfactory results have not yet been achieved. However, research has shown that some colonies of honey bee, *Apis mellifera* L., 1758 (Hymenoptera: Apidae), exhibit higher resistance or sensitivity to Varroa mites than others. One of the resistance mechanisms based on genetics is grooming behavior and it has been promising for beekeeping. The fact that there are many unanswered questions about grooming behavior led to the idea of this study. Worker bees from five honey bee races in Turkey were individually tested for their grooming behavior in response to *V. destructor* mite infestation. The quantitation of the expression levels of three candidate genes (neurexin-1, ataxin-3 and atlastin) in each honey bee race with and without grooming behavior was evaluated by quantitative polymerase chain reaction. Although expression levels of neurexin-1, ataxin-3 and atlastin genes showed significant differences among individuals, grooming levels of individuals were not related to the expression levels of these genes except in Syrian honeybees. Also, phenotypically no statistical differences were found among the honey bee races in terms of grooming behavior. The results show that grooming behavior may not be associated with neural gene expression alone. However, it is seen that more molecular studies related to grooming behavior are needed.

Keywords: *Apis mellifera,* candidate gene, grooming behavior, quantitation, *Varroa destructor*

Öz

Varroa destructor Anderson & Trueman, 2000 (Acari: Varroidae) kontrolünde birçok yöntem kullanılmış olmasına rağmen, tatmin edici sonuçlar henüz elde edilememiştir. Araştırmalar, bazı bal arısı, *Apis mellifera* L., 1758 (Hymenoptera: Apidae) kolonilerinin Varroa akarlarına diğerlerinden daha yüksek direnç veya duyarlılık sergilediğini göstermiştir. Genetik temele dayanan direnç mekanizmalarından birisi de tımar davranışıdır ve arıcılık için umut vericidir. Tımar davranışı ile ilgili cevaplanmamış birçok sorunun olması bu çalışmanın ortaya çıkmasına neden olmuştur. Türkiye'deki beş bal arısı ırkından işçi arıların *V. destructor* istilasına tepki olarak tımar davranışları test edilmiştir. Tımar davranışı gösteren ve göstermeyen her bir bal arısı ırkında üç aday genin (neuroxin-1, ataxin-3 ve atlastin) ekspresyon seviyelerinin kantitasyonu kantitatif polimeraz zincir reaksiyonu yöntemi ile değerlendirilmiştir. Neuroxin-1, ataxin-3 ve atlastin genlerinin ekspresyon seviyeleri önemli farklılıklar gösterse de Suriye ırkı haricinde tımar davranışı bu genlerin ekspresyon seviyeleri ile ilişkili bulunmamıştır. Ayrıca fenotipik olarak bal arısı ırkları arasında tımar davranışı açısından istatistiksel farklılık bulunmamıştır. Elde edilen sonuçlar, tımar davranışının, tek başına nöral gen ifadeleriyle ilişkili olmayabileceğini göstermektedir. Bununla birlikte tımar davranışı ile ilgili daha fazla moleküler çalışmaya ihtiyaç duyulduğu görülmektedir.

Anahtar sözcükler: *Apis mellifera*, aday gen, tımar davranışı, kantitasyon, *Varroa destructor*

[1] This study is a part of FYL-2019-4245 grant no project supported by University of Akdeniz, Scientific Research Project Unit; an also, was a part of Master thesis of the first author in Akdeniz University, Institute of Natural and Applied Sciences.
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Received (Alınış): 08.09.2021 Accepted (Kabul ediliş): 31.01.2022 Published Online (Çevrimiçi Yayın Tarihi): 16.02.2022

Introduction

The European honey bee, *Apis mellifera* L., 1758 (Hymenoptera: Apidae) has an important role in honey production and pollination of plants. Close to 84% of agricultural crop species depend on pollinators, especially bees (Gallai et al., 2009). However, colony losses in honey bees have been a major problem since the beginning of the 1800s, when modern beekeeping began. Although a number of factors have been shown to cause colony losses, research indicates that the main cause of the colony losses is *Varroa destructor* Anderson & Trueman, 2000 (Acari: Varroidae) (Eliash et al., 2017). *Varroa destructor* is an obligate ectoparasite primarily feeding on honey bee fat body tissue but also the hemolymph (Oldroyd, 1999; Ramsey et al., 2019). If effective control is not used, a Varroa-infested colony collapses within 2-3 years (Boecking & Genersch, 2008; Rosenkranz et al., 2010).

Honey bee colonies have natural resistance mechanisms for various diseases and parasites, and some colonies may show higher resistance or sensitivity than others. Resistance mechanisms work through physical, behavioral and immune systems due to genetic diversity of honey bee races. Some European honey bee populations have been reported to exhibit one or more behaviors for counteracting diseases and parasites through some physiological properties they have gained through natural selection, and survive without any problem (Fries et al., 2006; Le Conte et al., 2007; Seeley, 2007). For these reasons, in Varroa mite control focused on the determination of resistant races and genotypes by seeking alternative methods such as bee breeding in terms of behavioral resistance (Rosenkranz et al., 2010). One of the most important defensive behaviors against ectoparasites in honey bees is grooming behavior (Aumeier, 2001). This behavior in bees has been developed to protect the health of individual workers and the entire colony (De Figueiró Santos et al., 2016). Grooming behavior in adult bees involves detecting and eliminating mites from their own bodies (autogrooming) or from other bees (allogrooming) (De Figueiró Santos et al., 2016). Removal of mites from other bees is also known as social grooming behavior (Peng, 1988). When bees self-clean quickly with their legs, their body waggles and bends. So, this behavior is also called a grooming dance (Milum, 1947). This stimulates social grooming behavior in temporarily specialized groomer bees, and often clean several other bees in a row (Kolmes, 1989).

Various studies have been conducted on several candidates including genes coding for atlastin, ataxin-3, neurexin-1 (AmNrx1), poly U binding factor kd 68, vitellogenin, autophagy linked FYVE protein, blue cheese (BlCh) and immune-related hymenoptaecin, which have potential neurodevelopmental and behavioral effects, and thought to be related to the grooming behavior of honey bees. Generally, expression levels of genes have been investigated in Varroa mite-treated bees. Navajas et al. (2008), investigated the susceptibility of *A. mellifera* to Varroa mite parasitism and whether mite invasion caused changes in gene expression. As a result of such studies, it was found that most of the genes expressed differently between tolerant and sensitive bees are important in the development of the nervous system. Arechavaleta-Velasco et al. (2012) identified a region on chromosome 5 for honey bee grooming behavior using a QTL mapping approach. This region contained 27 genes, including neuroxin-1, ataxin-3 and atlastin which have potential neurodevelopmental and behavioral effects. Hamiduzzaman et al. (2017) investigated associations between grooming behavior and the expressions of immune, neural, detoxification, developmental and health-related genes. AmNrx-1 expression was found to be significantly higher in bees showing intense grooming behavior. As a result, neurexin-1 has been reported to be useful as a biomarker for behavioral characteristics in bees. Morfin et al. (2020), reported that the rate of mites damaged by mite-biter bees, the severity of the mutilation and winter colony survival was higher in selected Indiana mite-biter honey bees colonies than the unselected Italian honey bees colonies. In addition, the expression of the AmNrx-1 gene associated with grooming behavior was significantly higher in Indiana mite-biter bees. Although the mechanism of grooming behavior has not been fully resolved, it is clear that grooming behavior is negatively correlated with the number of mites in the colonies. It is also known that this behavior varies among honey bee species

and races (Delfinado-Baker et al., 1992; Boecking & Ritter, 1993; Büchler, 1993; Arechavaleta-Velasco & Guzman-Novoa, 2001; Mondragon et al., 2005; Andino & Hunt, 2011; Guzman-Novoa et al., 2012).

This study investigated whether changes in the expression of three candidate genes (neuroxin-1, ataxin-3 and atlastin) were associated with grooming behavior according to grooming in five honey bee races (*Apis mellifera anatoliaca* Maa, 1953, *Apis mellifera caucasica* Gorbachev, 1916, *Apis mellifera carnica* Pollmann, 1879, *Apis mellifera ligustica* Spinola, 1806 and *Apis mellifera syriaca* Skorikov, 1929) in Turkey.

Materials and Methods

Collection of honey bee specimens

This study was conducted in the Akdeniz University Animal Biotechnology Laboratory in 2019. A total of 100 similar physiological age worker bees from the five honey bee races including Anatolian Bee (*A. mellifera anatoliaca*) from Muğla Province, Caucasian Bee (*A. mellifera caucasica*) from Artvin Province, Syrian Bee (*A. mellifera syriaca*) from Hatay Province, Carniolan Bee (*A. mellifera carnica*) from Kırklareli Province and Italian Bee (*A. mellifera ligustica*) from Hatay Province used in Turkey were purchased from beekeepers. Commercially obtained bees were transferred on the same day in five feeding boxes to a laboratory at 30°C. In the boxes, sugar syrup containing 10 ml of 50% sucrose was present. Bees that spent one night in this manner were allowed to de-stress until the next day's grooming test. Considering the phenotypic images and beekeeper's advice, care was taken to ensure that the selected bee samples represent their races in the best possible way.

Collection of Varroa mite samples

Varroa mite samples were obtained from honey bee colonies in Antalya Province. The honeycombs taken from these colonies were brought to the laboratory and mites were removed from the capped drone cells with the help of sterile tweezers. Mites were fed with white-eye phase drone pupae in Petri dishes until used in grooming tests. The mites were stored in Petri dishes at 28°C and 75% RH in an incubator (Huang et al., 2017).

Testing and analysis of grooming behavior

Grooming behavior tests were performed in individual 6-cm Petri dishes of 20 bees for each race. Petri dishes were humidified with moist pieces of paper, and the temperature was maintained at 30°C. Given that the honey bees had been removed from their hives, the tests were performed with minimal delay. In order to prevent stress-induced problems in bees, these tests were completed on the same day for all bees. In order to achieve this, two video camera systems were set above the Petri dishes used in the tests and grooming behavior of bees was recorded. Individually grooming behavior test were a modified version of the method described by Aumeier (2000). When testing grooming behavior, one worker bee from each race was placed in the Petri dish at a time. Then one Varroa mite was put onto its thorax using insect brush. The bees with Varroa mite were monitored for 3 min then each bee immediately stored at -80°C by in sterile 1.5 ml tubes containing RNAlater (Thermo Fisher Scientific, Waltham, MA, USA) solution. A total of 100 worker bees, 20 from each race, were tested for their grooming behavior. After completion of the grooming tests, the video recordings were transferred to the computer and examined to determine whether each bee showed grooming behavior. Also, grooming time for each bee was recorded and individuals who attempted rid themselves of the Varroa mite during the 3 min period were considered as grooming otherwise they were coded as no-grooming.

Total RNA extraction and cDNA synthesis

Thorax of each worker bee tested for grooming behavior was crushed with liquid nitrogen and RNAs were extracted using the Norgen Total RNA Extraction Kit (Norgen Biotek Corp., Thorold, ON, Canada). The quality of the extracted RNAs were determined by Biodrop device. The RNA samples were stored at −80°C. Five µg of extracted total RNAs were converted to cDNA by reverse transcription with EvoScript cDNA Synthesis Kit (Roche, Basel, Switzerland) in Thermal Cycler for use in qPCR.

Primers

A primer sets was used to amplify each candidate genes and β-actin used as a housekeeping gene are given in Table 1. Sequences of the genes required for the primary design were obtained from the National Biotechnology Information Center.

Table 1. The primer sets used to amplified candidate genes to grooming behavior in honey bees and β-actin

Gene Name	Forward Primer (5'-3')	Reverse Primer (5'-3')	NCBI ID
Neurexin-1	tctgcacataaagcctgttc	actccattttcacccctc	LOC724217
Ataxin-3	tgcacttttacaaggtccg	ccccaaacttttaatgcactac	LOC410162
Atlastin	ggcatacattagatacagcgg	gggacacaaagggaatgaac	LOC550886
β-actin	gacgaagcccaatcaaagag	ggcgacatacatagcaggag	AB023025

qPCR amplifications

qPCR amplifications were performed using the LightCycler 96 Real-Time PCR instrument with SYBR Green fluorescent dye method. Each qPCR reaction in a 96-well plate contained 1.5 µL H_2O (PCR-grade), 5 µL of SYBR Green Master (Roche LightCycler 480 SYBR Green-I Master), 0.5 µL (for forward and reverse total 1 µL) of each gene-specific primer, and 2.5 µL of DNA in a final volume of 10 µL. Gene-specific primers (Table 1) were designed using Primer3Plus and Primer-BLAST programs.

Statistical analysis

The expression level of candidate genes was calculated using $2^{-\Delta\Delta}$ method (Livak & Schmittgen, 2001). A chi-square test was used to determine whether the number of individuals grooming behavior varied depending between races and whether there was a relationship between races in terms of $2^{-\Delta\Delta}$ values. Kolmogorov-Smirnov normal distribution test was performed on all data before calculating gene expression. Log10 transformation was applied for non-normal data. Box plots was used to visually summarize gene expression data. Differences of the gene expression between the races were compared with Kruskal-Wallis test. The differences of expression levels between individuals with and without grooming behavior were analyzed with the Mann-Whitney U test.

Results

The division of the honey bees into grooming and no-grooming behavior is given in Table 2. The number of individuals grooming was the highest in the Italian race and the least in the Caucasian race. The total number of individuals grooming was slightly less than those not grooming. The movement of mites placed in the thorax of bees towards other body regions also affected the grooming behavior of the bees. The presence of mite in the head region of the bee triggered the grooming behavior more whereas the grooming behavior in the abdomen was less. Grooming behavior was not observed when mites were moved to the propodium. The chi-square test indicated that the was no significant differences between the races in grooming behavior ($p = 0.07$).

Table 2. Classification of grooming behavior success of five honey bee races from Turkey

	Honey bee Races					
	A. mellifera anatoliaca	*A. mellifera caucasica*	*A. mellifera carnica*	*A. mellifera ligustica*	*A. mellifera syriaca*	Total
NG	9	15	13	7	9	53
G	11	5	7	13	11	47
Total	20	20	20	20	20	100

Expressions of neurexin-1, ataxin-3 and atlastin genes in the honey bee races are shown as box plots in Figure 1. The figure is analyzed it is understood that the expression levels of genes showed almost the same pattern for all honey bee races. Interestingly, Carniolan honey bees had the highest values in terms of expression levels for all genes. However, Caucasian bees had the lowest expression of the neurexin-1 gene, Anatolian and Italian bees had the lowest expression for the ataxin-3 gene, and Anatolian bees had the lowest expression of the atlastin gene.

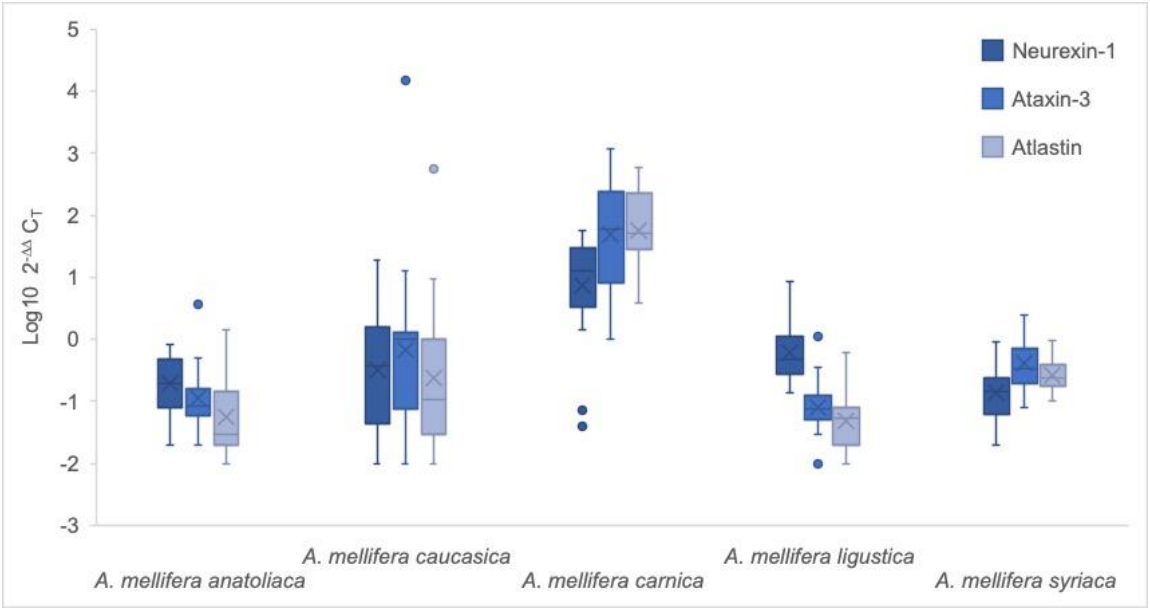

Figure 1. Box plots of Log10 transformed mRNA expression rates of neurexin, ataxin, atlastin in honey bee races.

The expression of neurexin-1, ataxin-3 and atlastin genes had significant differences between bee races ($p < 0.01$). There were also differences in the expression of these genes between individuals within a race. However, based on the Mann-Whitney U test, there were no differences between the individuals with and without grooming behavior in of expression of the genes except for the Syrian race ($p < 0.05$ and $p < 0.01$, respectively).

Discussion

Varroa mites continue to directly and indirectly threaten the health of honey bees. These harmful effects on the health of worker bees and all colony have led to the development of behavioral resistance, including grooming behavior. Although differences in grooming behavior among honey bee genotypes have been demonstrated (Moretto et al., 1993; Guzman-Novoa et al., 2012; Rinderer et al., 2013), the molecular mechanisms underlying grooming behavior are still not fully understood. However, the results obtained in gene expression studies related to neural genes are quite remarkable. On this basis, the aim of this study was to determine whether the expression levels of three candidate genes (neurexin-1, ataxin-3 and

atlastin), which are thought to be related to grooming behavior, change according to five bee races (*A. mellifera anatoliaca, A. mellifera caucasica, A. mellifera carnica, A. mellifera ligustica* and *A. mellifera syriaca*) and grooming behavior.

The individual grooming test described by Aumeier (2001) has been demonstrated to be effective in determining of grooming behavior in different honey bee genotypes. Aumeier (2001) reported that 66% of artificially infested Carniolan bees react to the presence of mites on their bodies within the first 30 s. However, in our study, this was observed in the Carniolan race. Compared to the other bee races, the Carniolan race was the third race in grooming response. In another study, mites that had fallen onto the bottom-board traps from naturally infested colonies were collected, to quantify the active grooming reactions by the Syrian honey bees towards the mite under natural conditions. A total of 22.8% of all dropping mites had body injuries and reported that workers of *A. mellifera syriaca* have an extraordinary potential to actively react to ectoparasitic Varroa mites (Zaitoun et al., 2001). Likewise, our grooming test results showed that the Syrian honey bees were had a 55% grooming rate. The Syrian race, which was noted for its aggressiveness during the tests, is the second most responsive race in terms of grooming behavior and there is little difference between first race, Italian honey bees. Bak & Wilde (2015) reported that *A. mellifera caucasica*, among the five artificially infested honey bee groups (*A. mellifera caucasica* Woźnica line, *A. mellifera mellifera* Augustowska line, *A. mellifera carnica* represented by two lines: Kortówka and Dobra bees, crossbreed of two subspecies: *A. mellifera capensis* × *A. mellifera carnica*) was the second most responsive race in grooming behavior. Also, they reported 86% of Caucasian bees tried to get rid of mites. In contrast to these results, the Caucasian bee known for its calmness was the the least responsive race in our study, and only 25% of the bees showed grooming behavior. In this regard, even in a small area of Turkey genetically diverse honey bee races can be found (Kandemir et al., 2000; Bodur et al., 2007; Solorzano et al., 2009; Kence et al., 2013). Consistently, the high variation in gene expression of Caucasian bees is an indicator of this diversity (Figure 1). Grooming behavior in response to Varroa mites is also associated with injured mites falling from bees in colonies (Arechavaleta-Velasco & Guzman-Novoa, 2001) and the rate of damaged mites per hive varies according to the honey bee race. Van Alphen & Fernhout (2020) reported Italian race had an average of about 6% mite damage. In contrast, Rosenkranz et al. (1997) recorded an average of 45% mite damage in Italian and Carniolan bees, while Africanized *A. mellifera* damaged 39%. In our grooming behavior test, the Italian bees were the most responsive with 65% grooming behavior. When looking at grooming behavior responses, it is not possible to drawn strong conclusions about the races differences. The fact that the results obtained in the tests are variable suggests that this behavior may be affected by the testing method and the parameters of the test conditions. Bak & Wilde (2015) reported that mites were successfully removed when located in the head, legs and distal region of abdomen. They reported none of the bees removed mites when these were on the propodium. Vandame et al. (2002) reported similar results in their studies. Therefore, grooming behavior is also influenced by the location of mites on the bee.

It should be noted that it is difficult to assess the intensity and effectiveness of grooming behavior against Varroa mites (Aumeier, 2001). The preparation of studies in full-size colonies or observation hives (Peng et al., 1987; Moretto et al., 1993, 1997; Bozic & Valentincic, 1995; Fries et al., 1996) is time-consuming, and even then, the continuous recording of the behavior of a particular bee is not guaranteed (Peng et al., 1987; Büchler et al., 1992; Thakur et al., 1997).

Genes expression was the highest in the Carniolan race (Figure 1), however that were relatively low proportion of grooming behavior (Table 2). Caucasian bees failed in terms of both grooming behavior and gene expressions compared to other races. Italian bees, the most responsive race in grooming behavior, did not show the same response in terms of gene expression. The expression profiles of the Syrian and Anatolian bee, which had the same proportion of grooming behavior, has similar expression of the three genes. When the bees with and without grooming behavior were compare in for neurexin-1, ataxin-3 and

atlastin, the expression of these genes was higher in grooming individuals only in the Syrian race. Although there are not many studies, intensive grooming behavior in general has been associated with high neural gene expression. Hamiduzzaman et al. (2017) determined that honey bees that had intense grooming behavior had greater neurexin-1 gene expression. They also reported that intense physical activity during grooming may be related to the nervous system induced byproducts of some neural genes and may cause these genes to suppress some other genes. Morfin et al. (2020) reported in their study that the expression of neurexin-1 was positively correlated the rate of mites injured in bred bees exhibiting of grooming behavior, but did not correlate with the growth of the mite population. Varroa mite-tolerant bees are mainly characterized by differences in the expression of genes regulating neural development, neural sensitivity and olfactory perception, although Navajas et al. (2008) reported that the expression of BlCh (an autophagy-dependent gene) was higher in Varroa mite-tolerated bees, and that the expression of the Dlic2 and Atg18 genes affecting neural reactions decreased. In contrast, the Strn-Mlck neural gene expression decreased in both Varroa mite-tolerant and intolerant bees. According to these studies and our findings, it seems that neural gene expressions may not always be related to grooming behavior. Also, genes selected as candidate genes may not always give consistent results (Navajas et al., 2008).

The present study is one of a few studies using phenotypic methods to address this issue. Also, there appears to have been no earlier studies of the grooming behavior of honey bee races and the comparison of candidate genes that may be responsible for this behavior. However, there is a need for further studies on neurexin-1, ataxin-3 and atlastin genes, which are reported to be candidate genes for grooming behavior in honey bees, and their relationship to grooming behavior. In addition to these candidate genes, other genes that may be involved should be investigated.

Acknowledgements

This study was financially supported by the Scientific Research Projects Unit of Akdeniz University under the project number of FYL-2019-4245.

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Türk. entomol. derg., 2022, 46 (1): 13-23
DOI: http://dx.doi.org/10.16970/entoted.1018061

ISSN 1010-6960
E-ISSN 2536-491X

Original article (Orijinal araştırma)

Characterization and taxonomic utility of ITS2 in *Dolerus* Panzer, 1801 (Hymenoptera: Tenthredinidae)[1]

Dolerus Panzer, 1801 (Hymenoptera: Tenthredinidae) cinsinde ITS2'nin karakterizasyonu ve taksonomik kullanımı

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Abstract

The widespread use of ITS2 as a potential marker in insects has accelerated species-level phylogenetic studies. Reliable and quality data can be obtained thanks to the features such as rapid evolution and secondary structure of this marker. This paper presents the phylogenetic relationship among 36 individuals of *Dolerus* Panzer, 1801 (Hymenoptera: Tenthredinidae) collected between 2002 and 2018 to obtain the first data on ITS2 secondary structure. Aligned of ITS2 data were analyzed by application of maximum likelihood method to reveal phylogenetic relationship among the specimens. Also, the structural properties, length variation and presence of compensatory base changes make the ITS2 useful marker in determination of species boundaries of closely related species. Four species (*Dolerus triplicatus* (Klug, 1818)*, Dolerus germanicus* (Fabricius, 1775)*, Dolerus puncticollis* Thomson, 1871 and *Dolerus nigratus* (Müller, 1776) and two putative species (*Dolerus* spp. 1 and 2) were determined from 36 individuals belonging to *Dolerus*.

Keywords: Compensatory base changes, *Dolerus*, Hymenoptera, ITS2, molecular marker

Öz

Böceklerde ITS2'nin moleküler belirteç olarak kullanılmasının yaygınlaşması, tür düzeyindeki filogeni çalışmalarına hız kazandırmıştır. Bu belirtecin hem hızlı evrime sahip olması hem de ikincil yapı oluşturması gibi özellikler sayesinde güvenilir ve kaliteli veriler elde edilebilir. Bu makalede, *Dolerus* Panzer, 1801 cinsine ait 2002-2018 yılları arasında toplanmış olan 36 bireyin (Hymenoptera: Tenthredinidae) aralarındaki filogenetik ilişkiyi ve ITS2 ikincil yapı özelliklerine ilişkin ilk verileri sunuyoruz. ITS2'nin hizalanmış verileri, örnekler arasındaki ilişkiyi görmek için maksimum likelihood yöntemi uygulanarak analiz edildi. Aynı zamanda ITS2'nin yapısal özellikleri, uzunluk polimorfizmi ve CBC'lerin olması, yakından ilişkili türlerin tür sınırlarının belirlenmesinde faydalıdır, *Dolerus* cinsine ait 36 bireyden, 4 tür (*Dolerus triplicatus* (Klug, 1818)*, Dolerus germanicus* (Fabricus, 1775)*, Dolerus puncticollis* Thomson, 1871 *ve Dolerus nigratus* (Müller, 1776) ve 2 olası tür (*Dolerus* spp. 1 ve 2) belirlenmiştir.

Anahtar kelimeler: CBC, *Dolerus*, Hymenoptera, ITS2, moleküler belirteç

[1] This study was supported by TÜBITAK (The Scientific and Technological Research Council of Turkey) Turkey, Grant Project No: 113Z753.
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Received (Alınış): 02.11.2021 Accepted (Kabul ediliş): 31.01.2022 Published Online (Çevrimiçi Yayın Tarihi): 16.02.2022

Introduction

The order Hymenoptera is one of the large insect orders comprising 153 thousand species described with very diverse life-histories (Niu et al., 2019; Çalmaşur, 2020; Taeger et al., 2021). Symphyta (Gerstacker, 1867) (sawflies) which is economically important insect linage that include major forest and horticultural pests (Heidemaa, 2004; Niu et al., 2021), is a small suborder of Hymenoptera largely distributed in the Palearctic region with 4,396 species (Taeger et al., 2021). Most of these species are members of Tenthredinoidea (Latreille, 1803), the most diverse lineage of non-apocritan Hymenoptera (Çalmaşur & Özbek, 2004a; Katılmış & Kıyak, 2015; Vilhelmsen, 2015). The Tenthredinoidea, known as typical sawflies, is the most well-known superfamily of Symphyta (Taeger et al., 2021). Tenthredinidae, the largest family of Symphyta, is one of seven families in Tenthredinoidea (Çalmaşur & Özbek, 2004b). This family comprises more than half of symphytan species mostly distributed in the Palearctic (Taeger et al., 2021). *Dolerus* Panzer, 1801 (Hymenoptera: Symphyta: Tenthredinidae) with 259 species, is a widespread genus in the Palearctic and Nearctic regions (Barker, 1998; Taeger et al., 2021).

Hymenoptera have diverse life-histories that range from feeding on or inside plants to highly variable forms of parasitism, social life and predation (Malm & Nyman, 2015; Niu et al., 2021). Adult sawflies feed on sap, such as maples, apples, pears, and nectar, such as willows, cherries and plums (Çalmaşur & Özbek, 2004b). Also, some larvae feed on plant in different ways such as holes or notches in the leaves, tunneling through the plant stem and making galls on the foliage (Goulet, 1986; Çalmaşur & Özbek, 2004b).

The two most common species of sawflies in rural habitats are graminivore *Dolerus* and *Pachynematus* (Barker et al., 1999). In recent studies, it has been reported that the genus *Dolerus*, known as wheat-sawflies, is not only found in wheat, but also in seed-grass plants and has become a more important pest, especially *Dolerus nigratus* (Müller, 1776) and *Dolerus puncticollis* Thomson, 1871. The larvae of *Dolerus* generally feed on plants in the Cyperaceae, Equisetaceae, Juncaceae, Poaceae (Haris, 1995). There are numerous reports of *Dolerus* larvae feeding on grain or grass crops highlighting their economic importance as pests (Haris, 1995). In addition, *Dolerus* larvae are known as an important food source for juvenile birds in rural habitats (Barker et al., 1999).

There are only a few phylogenetic studies of *Dolerus*. Vilhelmsen (2015), based on a morphological study on phylogeny of the Tenthredinidae, suggested that *Dolerus* is not monophyletic (Vilhelmsen, 2015). Also, Malm & Nyman (2015) using DNA data concluded that Selandriinae and Doleriini were clearly separate groups. However, more studies are required to determine the phylogeny of this group.

There are numerous phylogeny studies using both morphological characters and molecular markers on the family of Tenthredinidae. Cytochrome oxidase subunit I (COI) and internal transcribed spacers 2 (ITS2) genes have been preferred as molecular markers in many studies. Also, ITS2 has conserved primer sequences across many different taxa (Schulmeister, 2003; Prous et al., 2011; Kearse et al., 2012; Leppänen et al., 2012; Budak et al., 2016).

Analyzing DNA (or RNA) sequences is an important method for phylogenetic and taxonomic studies of protistan, plant and animal species (Young & Coleman, 2004; Salvi & Mariottini, 2012; Hong et al., 2019). Given its rapid evolution, ITS2 region is used to phylogenetic analyses of closely related species (Zhao et al., 2018; Verma & Mishra, 2020). Also, it can be used as a molecular marker for species-level phylogeny and molecular clock (Uluar & Çıplak, 2020) like COI (Wagener et al., 2006; Schwarzfeld & Sperling, 2015). ITS2 is located between 5.8S and 28S ribosomal genes and has a function in the regulation and maturation of rRNA genes (Caisová et al., 2013; Jørgensen et al., 2013; Poczai et al., 2015; Fagan-Jeffries et al., 2019). ITS2 commonly has a conserved secondary structure with four helices around a loop. This structure has regions both conserved (Helix II and III) and quite variable (Helix I and Helix IV) (Coleman, 2009; Salvi & Mariottini, 2012; Poczai, et al., 2015; Fagan-Jeffries et al., 2019).

Given secondary structure of ITS2, compensatory base changes (CBCs) are formed on the helices (Torres-Suárez, 2014). CBCs are defined as mutations in both nucleotides of a paired position in a double-stranded structure of the transcribed RNA (Ponce-Gordo et al., 2011; Salvi & Mariottini, 2012), for example, when G-C mutates to A-U (Gutell et al., 1994; Coleman, 2003; Schill et al., 2010). Hemi-CBCs consist of a change of one of the nucleotides in the pair. If a CBC occurs between the two organisms, there is a 93% probability that these organisms are different species (Müller et al., 2007; Ruhl et al., 2010; Pawłowska et al., 2013; Torres-Suárez, 2014). Thus, the use of the secondary structure of ITS2 is an important method in phylogeny and species delimitation (Vandivier et al., 2016; Verma & Mishra, 2020). Helix I is variable in sequence unlike Helix II which has more conserved structure and almost represents at least one pyrimidine-pyrimidine mismatch (UxU, UxC and CxC). Helix III is usually much longer and branched with conserved region in its apical region and includes a conserved four nucleotide motif (YGGY). Helix IV is a short structure that highly variable and may not be present in all species (Caisová et al., 2011; Coleman, 2015).

In this study, the secondary structure of ITS2 was investigated in *Dolerus* for the first time. This region was sequenced and then characterized the predicted secondary structure of ITS2 for 36 specimens of *Dolerus* representing four morphotypes identified using existing morphological keys. Then, phylogenetic analyses were applied based on the structural alignment and CBCs were defined between all samples.

Materials and Methods

Sample preparation

The specimens, collected with sweeping netting between 2002 and 2018 years from different locations in Turkey (Table 1), were provided from the Entomological Collection of Cumhuriyet University, Sivas which was protected in 99% ethanol at -20ºC. A total of 36 *Dolerus* specimens were identified to species using identification keys of Haris, (2000).

Molecular analysis

Molecular analyses of all specimens were performed during 2019. Whole genomic DNA was extracted from the hind legs of the specimens by the salting-out method of Aljanabi & Martinez (1997). The primers used for amplification of ITS2 region were CAS5p8sFc (5'-ATG AAC ATC GAC ATT TCG AAC GCA CAT-3') and CAS28sB1d (5'-TTC TTT TCC TCC GCT TAG TAA TAT GCT TAA-3') (Ji et al., 2003). Amplifications were performed in 50-ml volumes containing 0.5 U of Taq polymerase, 5 ml of 10x reaction buffer (100 mM Tris-HCl, pH 8.8, 500 mM KCl and 0.8% Nonidet P-40), 10 pmol of each of the primers, 0.2 mM of each of the four dNTPs, 1.5 mM $MgCl_2$ and 1 ml of DNA template (50-100 ng). PCR cycle conditions were: 94ºC for 5 min; 35 cycles of 94ºC for 30 s, 46ºC for 30 s and 72ºC for 60 s., and finally 72ºC for 5 min. The resulting PCR products were electrophoresed on 1% agarose gel and viewed. The purification and sequencing of amplification products were performed using a commercial sequencing service (Macrogen Ltd., Seoul, Korea.). Sequencing was performed in both directions using the same primers as in PCR reactions. The forward and reverse nucleotide sequences were assembled, edited and aligned by eye using Genious R9 (Kearse et al., 2012) and Mega7 (Kumar et al., 2016). The sequences were deposited to GenBank under the accession numbers OK642104-OK642139.

Inference of secondary structure

ITS2 sequences were identified using the ITS2 database V (Ankenbrand et al., 2015). E-value of <0.01 and metazoan HMMs with ITS2 minimum size of >150 nt were used to describe the borders of ITS2. Then defined sequences were progressed in the RNA Mfold Server folding program (Zuker, 2003). As parameters, linear sequence, RNA version 2.3 energy rules, 25ºC were applied. The structures and sequences were synchronously aligned by 4SALE (Seibel et al., 2006; Wolf et al., 2014) in locally implemented Clustal W (Larkin et al., 2007). The secondary structures were redrawn and annotated to improve visualization using VARNA 3.9 (Darty et al., 2009).

Phylogenetic analyses and mapping of synapomorphic compensatory base changes

ITS2 aligned sequences were used to show phylogenetic relationship of 36 *Dolerus* specimens. *Tenthredopsis tessellata* (Klug, 1817), is most closely related to *Dolerus* in database, was used as an outgroup because ITS2 data of Selandriinae was not available in the database. As a model, p-distance parameter was used to determine intra- and interspecific distance in Mega7 (Kumar et al., 2016). Phylogenetic tree was built by application of maximum likelihood (ML) using phangorn (Schliep, 2011) as performed in the statistical framework R (R core team, 2014). The R script used in the construction of ML structure tree was reached from the 4SALE website (4sale.bioapps.biozentrum.uni-wuerzburg.de) and for further details see Wolf et al. (2014). The robustness of the ML trees was tested by 1,000 bootstrap replicates.

Results and Discussion

The thirty-six specimens identified according to morphological characters are given in Table 1. The identified species were *Dolerus triplicatus* (Klug, 1818) (spcmn1-6), *Dolerus germanicus* (Fabricus, 1775) (spcmn7-16), *D. puncticollis* (spcmn17-26) and *D. nigratus* (spcmn27-36).

Table 1. Locality information and ITS2 secondary structure characteristic features of the specimens

Specimens	*Dolerus* morphospecies	Collection localities	Length (nt)	% GC content	ΔG value kcal/mol	Length (nt)			
						Helix I	Helix II	Helix III	Helix IV
spcmn1-3	*Dolerus triplicatus*	Erzurum-Tortum	647	57,0	-318,96	10	108	498	-
spcmn4-6	*Dolerus triplicatus*	Erzincan-Refahiye	647	57,0	-318,96	10	108	498	-
spcmn7	*Dolerus germanicus*	Kütahya-Altıntaş	640	55,9	-316.14	84	60	481	-
spcmn8	*Dolerus germanicus*	Kütahya-Altıntaş	640	55,6	-314.31	84	60	481	-
spcmn9	*Dolerus germanicus*	Uşak-Banaz	640	55,9	-313.77	84	60	481	-
spcmn10	*Dolerus germanicus*	Ankara-Bala	640	55,8	-317.95	84	60	481	-
spcmn 11-12-14-15-16	Dolerus *germanicus*	Erzincan-Refahiye	640	55,6	-313.15	84	60	481	-
spcmn13	*Dolerus* germanicus	Erzincan-Refahiye	640	55,8	-313.20	84	60	481	-
spcmn17	*Dolerus puncticollis*	Erzurum-Tortum	622	55,5	-321.39	48	63	475	15
spcmn18	*Dolerus puncticollis*	Erzurum-Tortum	619	55,6	-321.33	47	63	472	9
spcmn19	*Dolerus puncticollis*	Nevşehir-Ürgüp	619	55,6	-322.51	47	63	472	9
spcmn20	*Dolerus puncticollis*	Nevşehir-Ürgüp	621	54,6	-293.92	46	68	473	14
spcmn21	*Dolerus puncticollis*	Nevşehir-Ürgüp	621	55,4	-307.90	47	68	474	9
spcmn22	*Dolerus puncticollis*	Ankara-Beyşehir	620	55,5	-319.87	47	63	473	9
spcmn23	*Dolerus puncticollis**	Sivas-Gürün	559	55,5	-282.82	47	70	380	18
spcmn24	*Dolerus puncticollis*	Ankara-Beyşehir	622	55,5	-320.21	47	63	475	13
spcmn25	*Dolerus puncticollis*	Niğde-Çamardı	619	55,6	-322.51	47	63	472	9
spcmn26	*Dolerus puncticollis*	Niğde-Çamardı	624	57,2	-308,47	47	68	476	15
spcmn27	*Dolerus nigratus*	Kastamonu-Tosya	605	56,7	-280.35	90	80	394	27
spcmn28	*Dolerus nigratus*	Kastamonu-Tosya	605	56,9	-279.62	91	80	394	27
spcmn29	*Dolerus nigratus*	Kastamonu-Tosya	605	56,7	-282.61	88	74	394	27
spcmn30	*Dolerus nigratus*	Erzincan-Refahiye	605	56,5	-282.53	88	74	395	27
spcmn31	*Dolerus nigratus*	Erzurum-Oltu	607	55,7	-275.23	94	80	404	27
spcmn32	*Dolerus nigratus*	Erzincan-Refahiye	605	56,9	-279.62	91	80	395	27
spcmn33	*Dolerus nigratus*	Erzurum-Oltu	605	56,7	-282.61	88	74	395	27
spcmn34	*Dolerus nigratus***	Kütahya-Altıntaş	621	55,7	-321.15	9	152	432	9
spcmn35	*Dolerus nigratus***	Kütahya-Altıntaş	621	55,6	-321.06	9	149	432	9
spcmn36	*Dolerus nigratus***	Kütahya-Altıntaş	624	55,1	-316.27	9	152	432	9

*,** From the molecular analysis, these species was defined as *Dolerus* sp. 1 and 2, respectively.

The average GC content of the ITS2 region of 36 samples in the study was 56.0%. The GC contents of the examined sequences were ranged between 54.6% (spcmn20) and 57.2%. (spcmn26). These values were consistent with previous studies on eukaryotes (Mullineux & Hausner, 2009). The ITS2 sequence lengths were ranged between 559 and 647 bp (Table 1). Length polymorphism was not observed in *D. triplicatus* (647 bp) and *D. germanicus* (640 bp). The intra-specific length variation of ITS2 was found mostly in *D. puncticollis* (619-624 bp). The length variation of ITS2 in all studied animals was ranged between 100 and 2052 bp (Budak et al., 2016).

Inter-specific genetic distance was determined as a maximum of 28.8% (*D. nigratus* vs *D. triplicatus)*, and a minimum of 14.8% (*D. germanicus* vs *D. triplicatus*). Also, interspecific standard error was at most maximum 2% (*D. triplicatus* vs *D. nigratus)* and down to 1.4% (*D. triplicatus* vs *D. germanicus*) (Table 2). Intraspecific genetic distance was determined as a maximum of 3% (*D. puncticollis*) and a minimum of 0% (*D. triplicatus*) (Table 3). The results show that despite the high genetic distances between species, the absence of nucleotide variation within the species such as *D. triplicatus* strongly suggests that ITS2 evolved through concerted evolution (Dover, 1982). Concerted evolution is defined as a process in which related genes within a species experience genetic exchange, leading to their nucleotide evolution to be concerted over some period of time (Liao, 1999).

Table 2. Interspecific genetic distance

	Standard error			
Genetic	*D. triplicatus*	*D. germanicus*	*D. puncticollis*	*D. nigratus*
Dolerus triplicatus	-	0.0147	0.0173	0.0202
Dolerus germanicus	0.1480	-	0.0164	0.0187
Dolerus puncticollis	0.2352	0.2148	-	0.0177
Dolerus nigratus	0.2883	0.2629	0.2793	-

Table 3. Intraspecific genetic distance

Species	n	d	SE
Dolerus triplicatus	*6*	0.00000	0.00000
Dolerus germanicus	*10*	0.00531	0.00170
Dolerus puncticollis	*10*	0.03012	0.00397
Dolerus nigratus	*10*	0.00500	0.00157

The ITS2 secondary structures were predicted by the approach of energy minimization (Mathews et al., 1999) for all sequences. Predicted thermodynamic energy values of the putative secondary structures varied from -275.23 to -322.51 kcal/mol (spcmn31 vs spcmn19 and -25). The ITS2 structures in the database for eukaryotes display a common core structure (Ankenbrand et al., 2015). However, in the previous study of the same family (Budak et al., 2016), the ITS2 structures showed numerous branching. Despite diverged nucleotide sequences of ITS2 for studied taxon, secondary structures displayed a similar pattern. *Dolerus triplicatus* and *D. germanicus* have three helices (Helix I, II and III) and, *D. puncticollis* and *D. nigratus* have four helices (Helix I, II, III and IV) in their ITS2 secondary structures. Similar secondary structures were found in *Tenthredopsis* by Budak et al. (2016).

The length of Helix I ranged from 9 to 94 bp (*D. nigratus*). Helix I forms a non-dichotomous structure as seen in eukaryotes (Caisová & Melkonian, 2014). The length of Helix II changed between 60 bp (*D. germanicus*) and 152 bp (*D. nigratus*). Although these helix lengths show similarities with the study by

Budak et al., (2016), they are longer than helix length of many eukaryotes (Coleman, 2007). Helix III was the longest, as in most eukaryotes. The length of Helix III varied between specimens (380 bp in *D. puncticollis* and 498 in *D. triplicatus*). Despite the variation in length between species, Helix III had similar branching and folding structures. Also, Helix III had the conserved motifs, the sequences of these motifs were 5 'AUCGUCCGCGG (11 bp) and GUCGUUCCGUGAAU (15 bp). These conserved sequences have been suggested both as a protein binding site and a cleavage site because of their locations, lengths as well as conserved nucleotides (Coleman, 2007). The previously reported YGGY motif as a likely division site on the 5' side and near the apex (Coleman, 2007; Caisová et al., 2013) was also found in *Dolerus* spp. Helix IV structure of *D. nigratus* and *D. puncticollis* was similar to the genus *Tenthredopsis* (Tenthredinidae) (Budak et al., 2016). However, this helix was not found in *D. triplicatus* and *D. germanicus*. The length of Helix IV ranged from 9 to 27 bp and had no branching in its secondary structure (Figure 1).

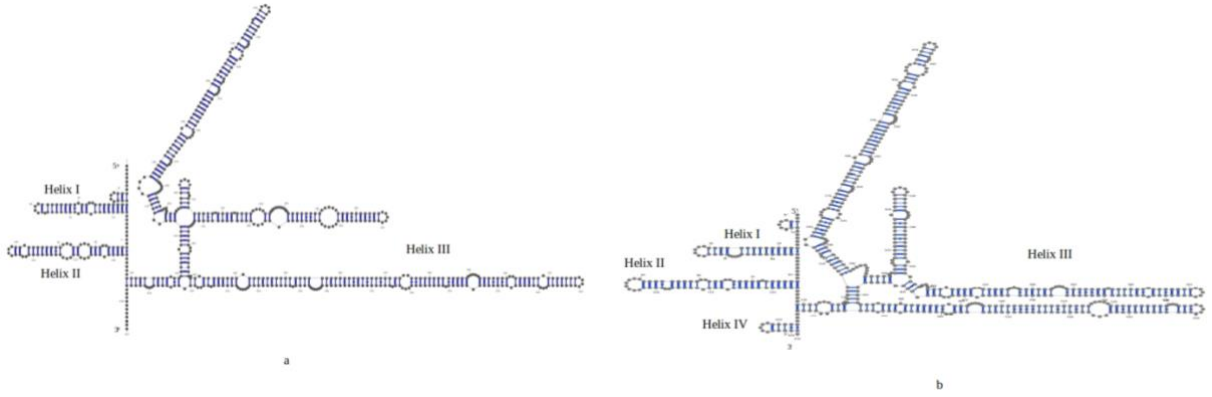

Figure 1. Secondary structures of ITS2 in *Dolerus:* a) the three-helix structure, and b) the four-helix structure.

The presence of a CBC between two specimens indicates that they belonging to different species with a probability of 93% (Coleman, 2007). The CBC formation was observed on Helix II and III of ITS2, but for the exceptional cases, the CBCs were equally distributed over all four helices (Müller et al., 2007). Also, the length differences of ITS2 are important in distinguishing species (Tang et al., 1996; Kitthawee, 2003; Nelson et al., 2008). Morphology, CBCs and length polymorphism data were used to define species boundaries.

Prior to phylogenetic analyses, the samples were designated numerically with the prefix *spcmn* (Table 1). The ML tree was built using the 36 ITS2 secondary structures (Figure 2). Only apomorphic CBCs were shown on the ITS2 tree (Figure 2). In total, 24 CBCs were identified among the six morphologically distinct taxa belonging to four identified species: three in Helix I, six in Helix II and fifteen in Helix III as given Table 4. In the ML tree, 36 *Dolerus* samples were divided into two main clades. The split of these two clades was supported with five CBCs (H1CBC3, H3CBC12, H3CBC13, H3CBC14 and H3CBC15) (Figure 2). The basal covered the specimen of *D. nigratus* without any CBCs. The second clade contained other taxa. This clade was divided into two subclades and each subclade was supported by the intra and interspecific CBCs (H2CBC4, H2CBC5, H2CBC6, H2CBC7, H3CBC6, H3CBC7, H3CBC8, H3CBC9, H3CBC10 and H3CBC11). Although the first subclade contained *D. germanicus* and *D. triplicatus*, *D. puncticollis* and two morphotypes, *Dolerus* sp. 1 and 2, were in the second subclade (Figure 2).

Table 4. Position of compensatory base changes in ITS2 secondary structure

CBC Name	Base Change	Position
H1CBC1	G:C-A:U	24-47
H1CBC2	A:U-G:C	26-45
H1CBC3	U:A-G:C	49-58
H2CBC1	A:U-G:C	122-155
H2CBC2	A:U-G:C	36-46
H2CBC3	C:G-G:U	38-43
H2CBC4	G:C-A:U	110-151
H2CBC5	C:G-A:U	125-138
H2CBC6	C:G-U:A	117-146
H3CBC1	G:C-C:G	185-267
H3CBC2	G:C-A:U	463-472
H3CBC3	U:A-G:C	406-444
H3CBC4	U:A-C:G	316-441
H3CBC5	U:A-C:G	315-338
H3CBC6	G:C-C:G	200-316
H3CBC7	G:C-A:U	434-466
H3CBC8	U:A-A:U	298-311
H3CBC9	A:U-G:C	320-406
H3CBC10	U:A-C:G	477-599
H3CBC11	C:G-A:U	335-340
H3CBC12	C:G-G:C	214-301
H3CBC13	U:A-C:G	225-289
H3CBC14	G:C-U:A	529-571
H3CBC15	G:C-A:U	436-464

Dolerus triplicatus and *D. germanicus* had similar ITS2 secondary structures, length variation and nucleotide similarity data, as reflected in the topology of the ML tree (Figure 2). However, the other two species (*D. puncticollis* and *D. nigratus*) were not monophyletic because of length variation and secondary structure differences of ITS2. It is suggested that these morphotypes (as *Dolerus* spp. 1 and 2) which are thought to have morphological similarities, may be distinct species. Only the spcmn31 in *D. nigratus* clade showed length variation. This length difference can be considered as intraspecific variation. spcmn34, spcmn35 and spcmn36 were morphologically identified as *D. nigratus* according to the morphological keys. However, phylogenetic analyses imply that these specimens could not be placed in the *D. nigratus* clade (Figure 2). Length differences of ITS2 gene and the presence of CBCs support this finding. In the light of these results, these specimens were temporarily designated as *Dolerus* sp. 2. with spcmn23 as species 1 being similar to *D. puncticollis* in terms of morphological characters. However, the high length variation (Table 1) and genetic distance of ITS2 (Table 2) separate *Dolerus* sp. 1 from *D. puncticollis*. The presence of CBCs (H1CBC1 and H2CBC3) between these specimens strongly support. *Dolerus* sp. 1 being considered as a distinct species. Although the presence of CBCs (H1CBC2, H3CBC4 and H3CBC5) between *D. puncticollis* samples, they were not considered as distinct species. Both the phylogenetic analyses and morphological similarities supported the monophyly of *D. puncticollis*. As a result, these CBCs can be considered as intraspecific variations. The sequence data of *D. triplicatus* samples were identical for all specimens. This is compatible with concerted evolution thus it can be said that ITS2 undergo genetic exchange between populations of the species (Table 3). All the samples of *D. triplicatus* had monophyletic relationship as expected. The presence of three CBCs (H2CBC2, H2CBC3 and H3CBC1) among the sister clade can be seen as the main feature that distinguishes this species from *D. germanicus*.

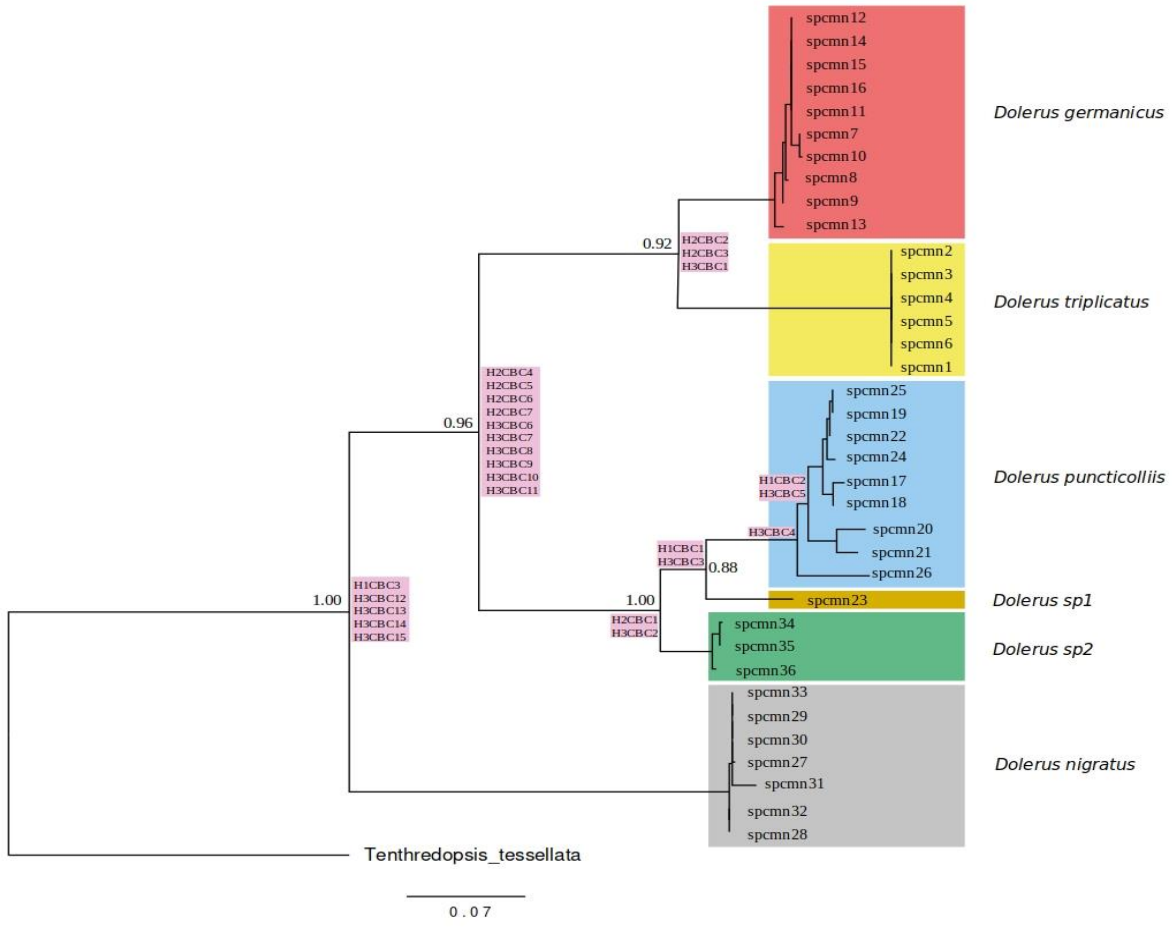

Figure 2. ML tree based on the structural alignment data of ITS2 in *Dolerus*.

Acknowledgment

This study was financially supported by the TÜBITAK (The Scientific and Technological Research Council of Turkey) via a research project with grant number 113Z753. The members of Cumhuriyet University Evolutionary Bioinformatics Research Group (EBRG) are thanked for their contributions in bioinformatic work. Mehmet Gülmez is supported by the Council of Higher Education (CoHE) of Turkey with 100/2000 PhD Scholarship.

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Türk. entomol. derg., 2022, 46 (1): 25-36
DOI: http://dx.doi.org/10.16970/entoted.1019437

ISSN 1010-6960
E-ISSN 2536-491X

Original article (Orijinal araştırma)

Spider mite predator *Feltiella acarisuga* (Vallot, 1827) (Diptera: Cecidomyiidae) in greenhouse strawberry cultivation in Antalya Province: recognition, population dynamics and parasitization by *Aphanogmus* sp.

Antalya İli örtüaltı çilek üretiminde kırmızı örümcek predatörü *Feltiella acarisuga* (Vallot, 1827) (Diptera: Cecidomyiidae): tanınması, popülasyon dinamikleri ve *Aphanogmus* sp. tarafından parazitlenmesi

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Abstract

Feltiella acarisuga (Vallot, 1827) (Diptera: Cecidomyiidae) is an effective and common beneficial species that feeds on many species of Tetranychid mites. Although it has been detected in Turkey, there are few studies on this predatory species. In this study, weekly leaf samples were taken to represent the growing area in the soilless strawberry greenhouse of Akdeniz University in 2020 and 2021. In leaf examinations, it was determined that *F. acarisuga* had an average egg size of 0.21 mm, young and mature larva of 0.26-1.51 mm, and a cocoon size of 2.01 mm. The average body length of male and female individuals was measured as 0.89 mm and 1.11 mm, respectively. It was determined that *F. acarisuga* can feed on all biological stages of *Tetranychus urticae* Koch, 1836 (Acari: Tetranychidae), as eggs, larvae, nymphs and adults were seen in the greenhouse in all seasons. *Feltiella acarisuga* is the most common natural enemy of *T. urticae*, with a maximum average of 0.38 larvae + cocoons/leaf in the greenhouse in the first year and 0.62 larvae + cocoons/leaf in the second year. In this study, a new species was detected, *Aphanogmus* sp., which was determined to be the parasitoid of *F. acarisuga* and different from its known congeners. The parasitization rate of *F. acarisuga* was found to be between 0 and 51.5%.

Keywords: *Feltiella acarisuga,* parasitoid, population dynamics, strawberry

Öz

Feltiella acarisuga (Vallot, 1827) (Diptera: Cecidomyiidae), Tetranychid akarların birçok türü ile beslenen etkili ve yaygın bir doğal düşman türüdür. Türkiye'de varlığı tespit edilmiş olmakla birlikte bu predatör tür ile ilgili olarak son derece kısıtlı çalışmalar bulunmaktadır. Bu çalışmada 2020 ve 2021 yıllarında, Akdeniz Üniversitesine ait topraksız çilek serasında üretim alanını temsilen haftalık yaprak örnekleri alınmıştır. Yaprak incelemelerinde, *F. acarisuga*'nın ortalama 0,21 mm yumurta, 0,26-1,51 mm genç ve olgun larva, 2,01 mm kokon büyüklüğüne sahip olduğu belirlenmiştir. Erkek ve dişi bireylerin vücut uzunluğu ise sırasıyla ortalama 0,89 mm ve 1,11 mm olarak ölçülmüştür. *Feltiella acarisuga*'nın, *Tetranychus urticae* Koch, 1836 (Acari: Tetranychidae)'nin yumurta, larva, nimf ve ergin olmak üzere tüm biyolojik evrelerinde beslenebildiği ve tüm sezon boyunca serada görülebildiği tespit edilmiştir. En yoğun olarak, ilk yıl serada ortalama 0.38 adet larva + kokon/yaprak, ikinci yıl ise 0,62 adet larva + kokon/yaprak tespit edilmiş olan *F. acarisuga*'nın, *T. urticae*'nin en yaygın doğal düşmanı olduğu belirlenmiştir. *Feltiella acarisuga*'nın parazitoiti olarak belirlenen ve bilinen türdeşlerinden farklı yeni bir tür olan *Aphanogmus* sp'nin tespit edildiği bu çalışmada, *F. acarisuga*'nın parazitlenme oranı %0-51,5 arasında bulunmuştur.

Anahtar sözcükler: *Feltiella acarisuga*, parazitoit, popülasyon dalgalanması, çilek

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Received (Alınış): 05.11.2021 Accepted (Kabul ediliş): 31.01.2022 Published Online (Çevrimiçi Yayın Tarihi): 16.02.2022

Introduction

Cecidomyiidae is among the families with the richest species in Diptera (Gagne, 2004). Gagne & Jaschhof (2014) listed 6203 species belonging to 736 genera worldwide. The Cecidomyiidae family, known as gall midges, also includes mycophagous, saprophagous and zoophagous species as well as phytophagous species that are plant pests (Skuhrava et al., 2010, 2014).

It has been reported that all known species of the genus *Feltiella* in the Cecidomyiidae family feed on tetranychid mites (Gagne, 2010). *Feltiella acarisuga* (Vallot, 1827) (Diptera: Cecidomyiidae) (syn. *Therodiplosis persicae* Kieffer, 1912) is a common species that can feed on many species of spider mites that cause economic damage to many host plants (Mo & Liu, 2007). It is an important species due to its efficiency and cosmopolitan distribution (Choi et al., 2021), and it is the most common predator of spider mites (Gillespie et al.,1997). It has been found that ~20°C and 90% RH are optimum conditions for *F. acarisuga*, which develop and reproduce at 15-27°C and 60-95% RH in greenhouses (Gillespie et al., 1998, 2000). The life history and life table parameters of *F. acarisuga* were determined using *Tetranychus cinnabarinus* (Boisduval, 1867) eggs under laboratory conditions (Mo & Liu, 2006). Xiao et al. (2011) reported that *F. acarisuga* is an excellent predator of both *Tetranychus urticae* Koch, 1836 (Acari: Tetranychidae) and *Oligonychus pratensis* (Banks, 1912) (Acari: Tetranychidae). Xiao et al. (2013) showed that *F. acarisuga* was highly effective on *T. urticae* eggs under laboratory conditions, while *Neoseiulus californicus* (McGregor, 1954) (Acari: Phytoseiidae) and *Amblyseius swirskii* (Anthias-Henriot, 1962) were moderately effective.

There are only a few studies on the Cecidomyiidae family or on *F. acarisuga* as a predator of spider mites in Turkey. In some of these studies, it is reported that a species of the Cecidomyiidae family is a predator of spider mites. For example, in the Antalya Province, a Cecidomyiid predator species, which could not be identified, was found in eggplant in addition to many natural enemies of spider mites (Soysal & Yayla, 1988). Çakmak (2002) detected an unidentified species from the Cecidomyiidae family in greenhouse strawberry fields in the Aydın Province and determined that the larvae of this species feed on *Tetranychus* species and locally put pressure on the leaves where the pest is dense. One of the few studies in which *F. acarisuga* was found in production areas at the species level was conducted in the Erzincan Province. In this study, besides many natural enemies of *T. urticae* in beans, *T. persicae* from Cecidomyiidae was identified as a specific mite predator, a new record for the fauna of Turkey (Aydemir & Toros, 1990). Bulut & Göçmen (2000) investigated pests and their natural enemies in greenhouse vegetable cultivation in the Antalya Province and identified *F. acarisuga* as a predatory species.

Although there are some studies on the natural enemies of spider mites, which are important pests in open and greenhouse cultivation, there is a lack of information on the predatory insect *F. acarisuga* in Turkey. Although the existence of this species has been reported in Turkey, detailed information has not been reported. Accordingly, the present study sought to determine the population dynamics, distinctive morphological features, parasitoid and parasitization rates of *F. acarisuga* on strawberry plants. The results of this study will provide useful information for researchers and biological control studies.

Materials and Methods

Study area and studies on *Feltiella acarisuga*

This study was conducted in the soilless research greenhouse of the Faculty of Agriculture of Akdeniz University in 2020 and 2021. A total of 110 cultivation pots (70 x 25 cm) were used, each containing cocopeat, and seven strawberry plants (cv. Festival) were planted in each pot in a growing area of 150 m^2. The ionic composition of the nutrient solution was as follows: 11.5 mM $NO3^-$, 1.5 mM $H2PO4^-$, 1.5 mM $SO4^{-2}$, 0.5 mM $NH4^+$, 3.5 mM K^+, 4.5 mM Ca^{+2}, and 1.5 mM Mg^{+2}. The EC and pH values of the nutrient solution were 1.5 dS/m and 6.0, respectively. The greenhouse was 5.5 m high, with a semi-circular roof

(without a heating system). Greenhouse controls were made on a weekly basis starting in November following planting, and the study continued until June. At least 50 leaf samples were taken to represent the greenhouse. The leaves were first placed in a paper bag and then in a polyethylene bag and brought to the laboratory in an icebox. Eggs, larvae and pupae of *F. acarisuga* as well as the feeding behavior of larvae on the pest were examined and viewed under a microscope. Adults emerged from larvae and pupae, which were placed into glass or plastic pots covered with gauze under laboratory conditions for examination. *Feltiella acarisuga* adults were preserved in 70% ethanol for species identification and labeled. In addition, due to their sensitive structure, female and male *F. acarisuga* were directly mounted in Hoyer's medium. Antenna, tarsus and genital parts of the samples were examined under a binocular microscope and photographed in their original form. The measurement values of at least three samples of the biological stages of *F. acarisuga* were obtained and used as an average. The morphological description of *F. acarisuga* was made according to Gagne (2018) and Abe et al. (2011), and the definitive species diagnosis was made by Dr. Marcela Skuhrava (CSc. Bítovská 1227/9 CZ-140 00 Praha 4 Czech Republic).

Population dynamics of *Feltiella acarisuga* and other natural enemies

The leaf samples were brought to the laboratory and kept in the refrigerator until they were examined. The larvae and cocoons of *F. acarisuga* on the leaves were examined under a stereo-microscope and counted. *Tetranychus urticae* and other natural enemies were counted. The larvae and cocoons of *F. acarisuga* and the adults, larvae and nymphs of other natural enemies were counted together and evaluated. To identify the spider mite species, the samples preserved in 70% ethanol were cleaned in lactophenol solution and prepared using Hoyer's medium (Henderson, 2001). The definitive diagnosis of spider mites was made by Edward A. Ueckermann (School of Environmental Sciences and Development, North-West University, South Africa). Despite the desire not to use pesticides in the growing area, spraying (240 g/l spiromesifen) was done twice in the first year against pests that exceeded the economic threshold (15 active individual/leaf) several times.

Parasitoid of *Feltiella acarisuga* and parasitization %

During the examination of leaf samples in the first year, parasitoid exit holes were found in the cocoons of *F. acarisuga*, and leaves with the larvae and cocoons were cultured in laboratory conditions. The adult parasitoids obtained were preserved in 70% ethanol and identified at the genus level by Dr. Kazunori Matsuo (Biosystematics Laboratory, Faculty of Social and Cultural Studies, Kyushu University, Motooka, Nishi-ku, Fukuoka 819-0395, Japan). When it was assumed that *F. acarisuga* was parasitized, leaf samples were taken in the second year of the study to determine the parasitization rate. Between April and June 2021, at least 20 leaves with *F. acarisuga* cocoons were collected weekly and cultured in laboratory conditions. Adults of *F. acarisuga* and parasitoid species emerging from the cultured leaves were counted, and the parasitization rate was calculated using the following formula:

Parasitization (%) = Number of adult parasitoids / (number of adult *F. acarisuga* + number of adult parasitoids) x 100

Results

Determination of *Feltiella acarisuga*

Feltiella acarisuga, which is seen in egg, larva and pupa stages on strawberry leaves, has also been found in adults flying around the plant from time to time. It has been observed that *F. acarisuga* lays its eggs on leaves with *T. urticae* eggs, especially on leaves with a high population of spider mites. Similarly, the egg size was found to be 0.21 mm on average. *Feltiella acarisuga* eggs can be easily distinguished from those of *T. urticae* under a microscope due to their thin and elongated structure (Figure 1a). At first, the newly hatched larvae were almost the same color as the eggs (whitish-yellow), and their color mostly darkened (yellow-pink-red) depending on feeding and in the following stages (Figure 1b-c).

Figure 1. a) Eggs of *Feltiella acarisuga* with *Tetranychus urticae* eggs; b-c) Larva of *Feltiella acarisuga*.

Both young and mature larvae of *F. acarisuga* feed on all biological stages of *T. urticae* indiscriminately (Figure 2a-b). In this study, young and mature larvae had a body length of 0.26-1.51 mm.

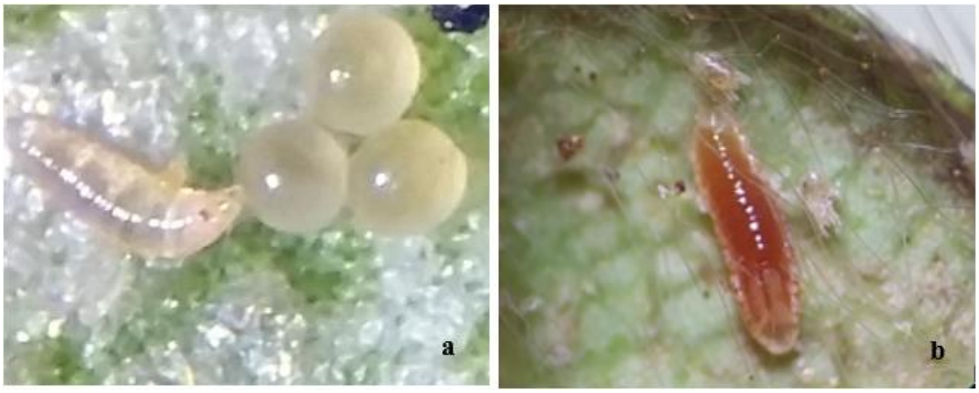

Figure 2. a) First instar larvae of *Feltiella acarisuga* feeding on *Tetranychus urticae* egg; b) mature larvae of *Feltiella acarisuga* feeding on *Tetranychus urticae*.

Feltiella acarisuga larvae, which feed on *T. urticae* eggs, larvae, nymphs and adults, did not differentiate between male and female.

Feltiella acarisuga forms cocoons and pupae mostly near the leaf veins (Figure 3a-b). Although rare, it is possible to encounter cocoons on any part of the leaf other than near the vein. In the study, the average cocoon length was 2.01 mm, while pupae size was found to be 1.35 mm on average. In the visual controls on strawberry leaves, the cocoons stood out as white small bumps and suggested the population density (Figure 3c). Cocoons and larvae could be seen widely among the *T. urticae* colony.

Figure 3. Pupa and cocoon of *Feltiella acarisuga* at the leaf vein margin. a-c) cocoon; b) pupa.

Adults of *F. acarisuga* with pinkish-brownish color have long legs. Males are distinguished from females by their thinner body structures and differences in antenna structures (Figure 4a-b). The antennae of males are longer than those of females, and the flagellomeres are completely different structurally, as shown in Figure 5 (a-b). In the study, the average body length of males and females were 0.89 and 1.11 mm, respectively.

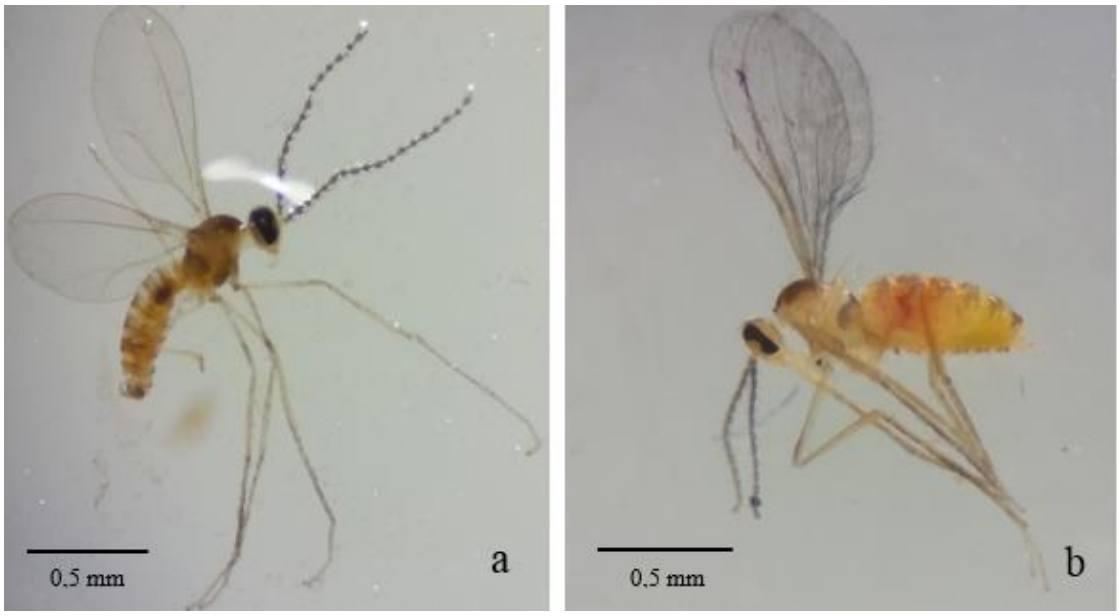

Figure 4. Adult of *Feltiella acarisuga* a) male; b) female.

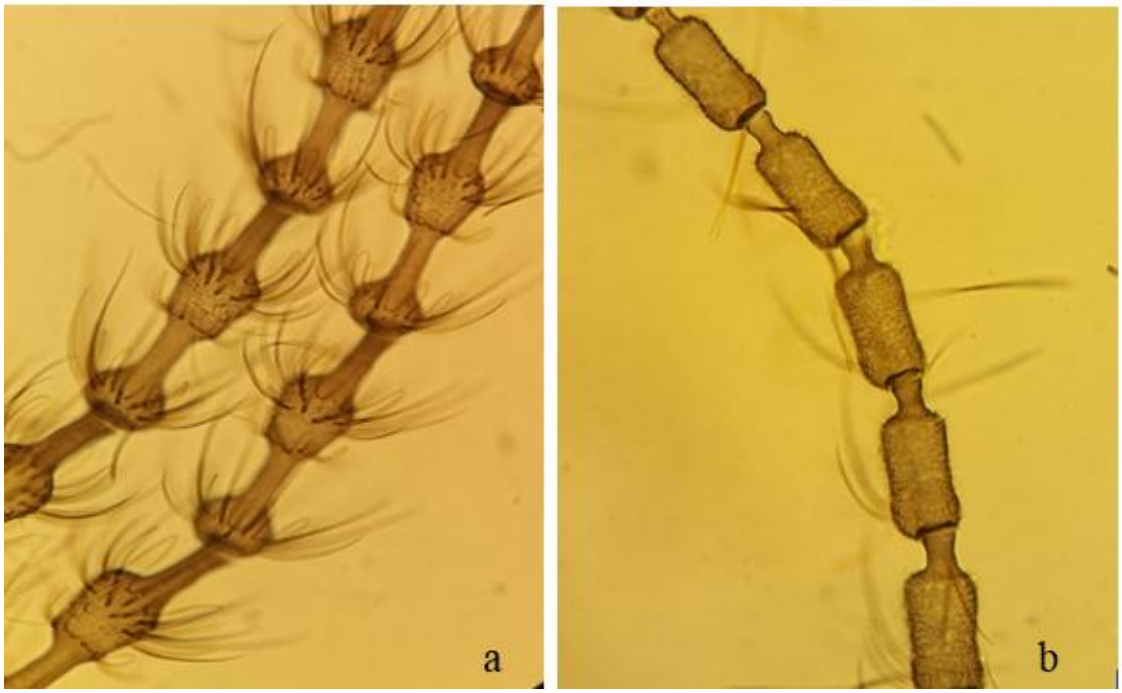

Figure 5. Antenna structure of *Feltiella acarisuga*: a) male flagellomeres; b) female flagellomeres.

The aedeagus was longer than the cerci and hypoproct in male specimens (Figure 6a), and the tarsal claws, which were simple in the middle and hind legs, were toothed in the forelegs (Figure 6b).

Figure 6. a) Male genitalia of *Feltiella acarisuga*: A: Aedegus, H: hypoproct, and C: cercus; and b) toothed tarsal claws on forelegs.

Population dynamics of *Feltiella acarisuga*

Feltiella acarisuga started to appear shortly after the appearance of *T. urticae* in the greenhouse and were found throughout the whole season. *Feltiella acarisuga* was present on the leaves as eggs, larvae and cocoons as long as the spider mites were present on the plant, and adults were also encountered occasionally during the sampling. In addition to *F. acarisuga*, which is the most common natural enemy of spider mites, *Stethorus gilvifrons (*Mulsant, 1850) (Coleoptera: Coccinellidae), *Scolothrips longicornis* Priesner, 1926 (Thysanoptera: Thripidae), *Orius* sp. (Hemiptera: Anthocoridae) and phytoseiids were determined as other natural enemies, respectively, according to their densities. The spider mites in the greenhouse were identified as *T. urticae*. In a study in 2018 in the same greenhouse, *Tetranychus solanacearum* Çobanoğlu & Ueckermann, 2015 (Acari: Tetranychidae) were detected (Topakci et al., 2021). This suggests the possibility of coexistence of both species or that *T. urticae*, which develops resistance to pesticides and has a wide host range, may be in a more dominant position than *T. solanacearum.*

In 2020, acaricide was applied twice when the *T. urticae* density averaged 25.2 and 29.4 per leaf (February and April). Despite this, the pest density was often above the economic threshold. *Feltiella acarisuga* started to appear from the third week after the start of greenhouse monitoring, and it was found almost throughout the entire season depending on the *T. urticae* population density. However, it was not effective in suppressing the pest. *Feltiella acarisuga* was most numerous (0.38 larvae + cocoons/leaf) on 18.05.2020 when the density of *T. urticae* was 18.2 per leaf (Figure 7). Twenty-nine *F. acarisuga* larvae and 164 cocoons were detected on the leaves, the highest number of natural enemies of *T. urticae*, and for the longest time during the season. Other natural enemies that were detected were *S. gilvifrons*, *S. longicornis* and phytoseiid species, with *S. longicornis* the second most common species. The highest number of *S. gilvifrons* was found on 2.06.2020, with 0.38 individuals/leaf, and the highest number of *S. longicornis* was 0.70 individuals/leaf on 08.06.2020. On 08.06.2020, the number of phytoseiid species was 0.02 individuals/leaf, but *Orius* was not found.

In 2021, *T. urticae* did not reach a population density as high as in 2020, and leaf drying was sometimes observed due to fungal infections. The pest population exceeded the economic threshold many times, and the density of the pest increased toward the end of the season. *Feltiella acarisuga* started to appear from the fifth week after the start of greenhouse monitoring and was detected throughout the entire season. It was observed that the beneficial species were more common in the greenhouse in 2021 compared to 2020 and reached greater numbers per leaf. The highest density of *F. acarisuga* was found on 20.05.2021, with 0.62 larvae + cocoons/leaf, and the density of *T. urticae* was 15.0 per leaf on that date (Figure 8). One

hundred and forty-eight larvae and 182 cocoons were found on the leaves sampled in the second year. Other natural enemies detected in the greenhouse were *S. gilvifrons*, *S. longicornis*, *Orius* and phytoseiid species, and these species were seen after 15.04.2021. After *F. acarisuga*, the second most common natural enemy species was *S. gilvifrons*, with the highest average number of 0.10 individuals/leaf on 27.05.2021. On 20.05.2021, there were 0.02 *Orius* individuals/leaf on average; on 2.06.2021 there were 0.08 *S. longicornis* individuals/leaf on average; and on 22.04.2021 there were 0.02 phytoseiid species individuals/leaf on average. *Orius* species, not found in 2020, were found in 2021.

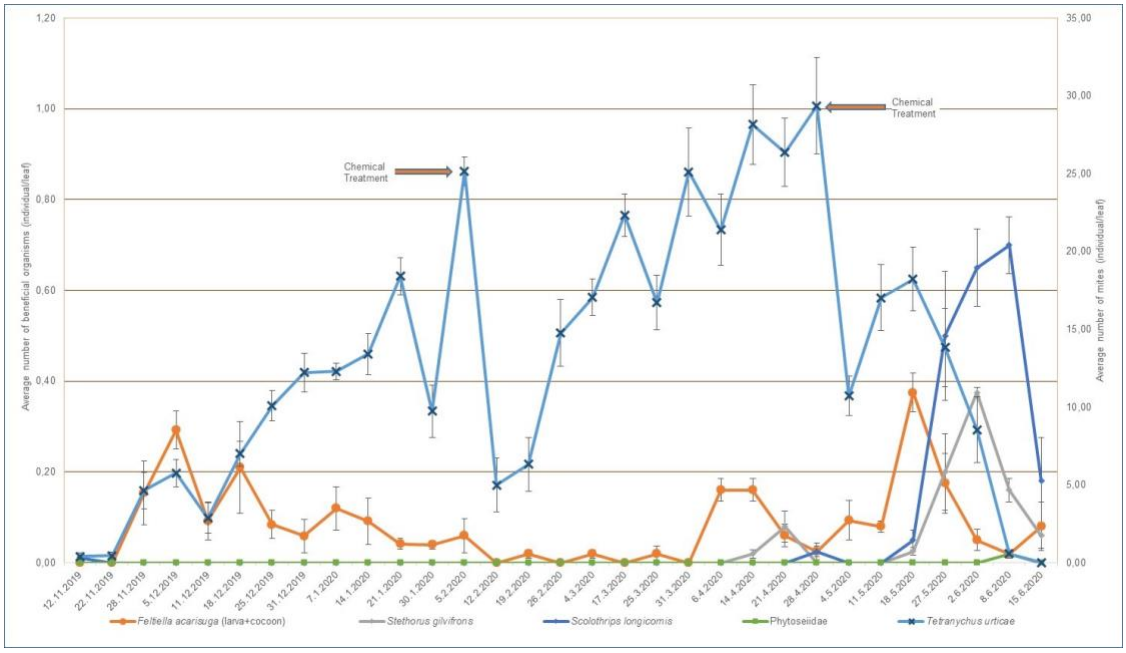

Figure 7. Population densities of *Tetranychus urticae* and beneficial organisms in 2020.

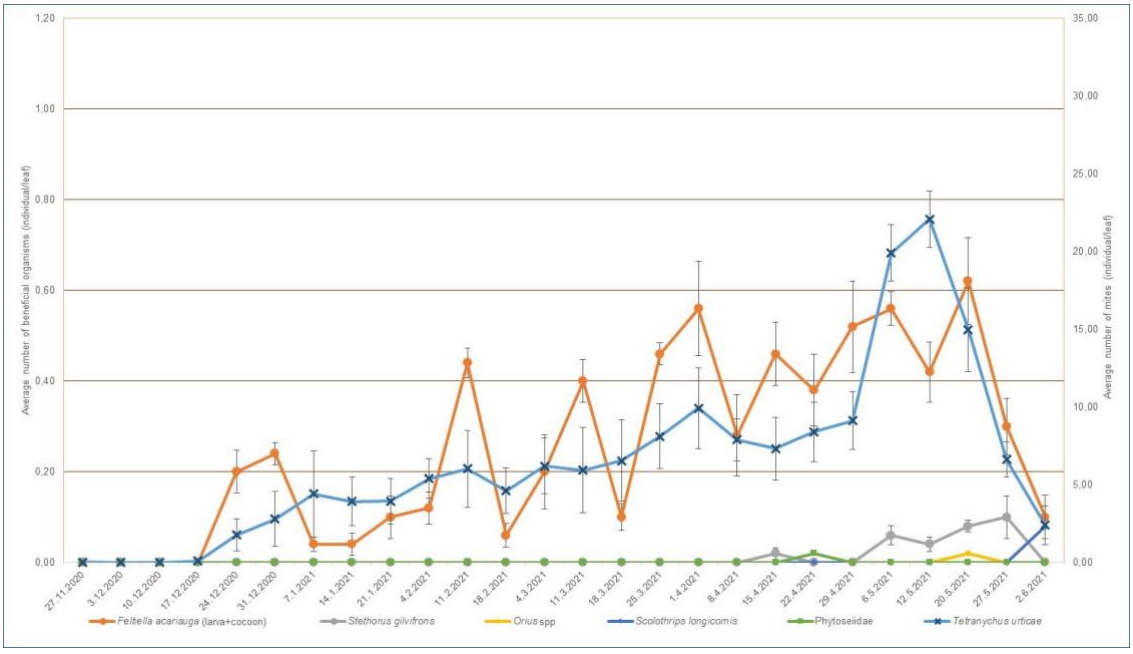

Figure 8. Population densities of *Tetranychus urticae* and beneficial organisms in 2021.

Parasitoid of *Feltiella acarisuga* and parasitization percentage

Characteristic parasitoid exit holes were observed in the cocoons examined under the microscope. The samples were cultured and it was determined that the holes were caused by a parasitoid exit. This species, a parasitoid of *F. acarisuga*, was thought to attack the host in the larval stage. Parasitized cocoons were partially distinguishable from non-parasitized cocoons due to their dark coloration (Figure 9a). The specimens that completed the prepupa (Figure 9b) and pupal stages (Figure 9c) in the cocoon and reached the adult stage (Figure 9d) were identified as belonging to the genus *Aphanogmus* (Hymenoptera: Ceraphronidae). The *Aphanogmus* sp. has different morphological characteristics from known congeners and has not yet been named (K. Matsuo, personal communication).

Figure 9. a) Parasitized *Feltiella acarisuga* cocoon; b) prepupa of *Aphanogmus* sp. with meconium; c) pupa of *Aphanogmus* sp. with meconium; d) adult of *Aphanogmus* sp.

Aphanogmus sp. was identified as the parasitoid of *F. acarisuga* for the first time in Turkey. The leaves with cocoons in the sampling between April and June 2021 were cultured in order to follow the emergence of adult parasitoids. *Aphanogmus* adults obtained from parasitized *F. acarisuga* cocoons were recorded, and data on the parasitization rate were obtained (Table 1). It was determined that the parasitization was higher in May, and the parasitization rate of *F. acarisuga* by *Aphanogmus* was found to be in the range of 0-51.5%.

Table 1. Parasitization rate of *Feltiella acarisuga* by *Aphanogmus* sp.

Date	*Feltiella acarisuga* adult	*Aphanogmus* adult	Parasitization rate (%)
01.04.2021	25	0	0
08.04.2021	14	0	0
15.04.2021	3	0	0
22.04.2021	15	1	6.25
29.04.2021	20	0	0
06.05.2021	27	2	6.89
12.05.2021	27	7	20.58
20.05.2021	16	17	51.51
27.05.2021	7	4	36.36
02.06.2021	5	5	50.00

Discussion

Spider mites are one of the most important pests affecting quality and yields in greenhouse strawberry cultivation. They increase their population in a very short time and cause severe damage to strawberries. *Tetranychus cinnabarinus* (Boisduval, 1867) (Acari: Tetranychidae) *T. urticae* and *Tetranychus turkestani* Ugarov & Nikolskii, 1937 (Acari: Tetranychidae) have been detected in greenhouse strawberry fields in Turkey, of which *T. cinnabarinus* is the most common (Çakmak, 2002). In another study conducted on the strawberry plant in 2018, *T. solanacearum* was identified (Topakcı et al., 2021).

In the current study, *F. acarisuga*, was present as long as *T. urticae* were present on the strawberry plant. It was found that *F. acarisuga*, whose egg, larva and pupa stages were examined on the leaves, could feed on all biological stages of *T. urticae*. It has been determined that the predator is also effective on adult spider mites, both females and males. Similarly, it has been reported that *F. acarisuga* larvae feed on all stages of spider mites (Zhang, 2003). It has been shown that *F. acarisuga* has no preference for male or female mites, killing females as well as males when both preys are present in equal numbers (Opit et al., 1997). It has been observed that *F. acarisuga*, which occasionally flies around strawberry plants, lays its eggs on leaves where the *T. urticae* population is dense. Xiao et al. (2011) reported that *F. acarisuga* flew 4.0-7.0 m in search of new prey in the greenhouse. Gillespie et al. (1998) noted that females tend to lay more eggs on leaves with more mites. Larvae can survive for several days in the absence of prey (Gillespie et al., 1998). Kawano (1969) reported that mature larvae have a body color ranging from pale orange to dark red depending on the prey species. Lee et al. (2004) reported that the larvae were cream-yellow-brown in color, 0.29 mm in size for the first instar larvae and 1.14 mm for the fourth instar. In the present study, the average body length of young and mature larvae was found to be 0.26-1.51 mm, which is consistent with these prior studies, and the colors of the larvae differed depending on feeding, but they were mostly a dark pinkish-reddish color.

Pupae in white cocoons and close to the leaf veins were observed commonly on the leaves. There were differences in the size of the cocoons. Zhang (2003) reported that this predator develops best in nutrient abundance, but in the case of food shortage the pupae can be smaller in size. Pupae are 1.1-1.47 mm in size and can be found in a white cocoon (Lee et al., 2004).

Fedotova & Kozlova (2019) found the body length of *F. acarisuga* to be 0.99-1.38 mm in females and 1.16-1.20 mm in males, and the antennae lengths were 0.61-0.77 and 1.69 mm, respectively. Lee et al. (2004) reported that adult males have a length of 1.11-1.33 mm and females 1.27-1.58 mm. In this study, the body measurements of male and female individuals were close to the ranges specified in the literature. Gagne (2018) highlighted the morphological characteristics of *F. acarisuga* in a diagnostic key. Abe et al. (2011) reported that *Feltiella* females could not be identified at the species level by morphological characteristics. In the present study, the morphological characteristics of *F. acarisuga* individuals were examined, and it was determined that the tarsal claws in males were toothed on the forelegs, simple in the middle and the hind legs and the aedeagus were much longer than the cerci and hypoproct. These results are consistent with the literature.

In this study, which looked for all the natural enemies of *T. urticae* in the strawberry greenhouse, the most prominent beneficial species was *F. acarisuga*. *F. acarisuga* started to appear 3-5 weeks after the start of the greenhouse controls and was found on the plants throughout the whole season. The number of *F. acarisuga* cocoons on the leaves was higher than the number of larvae, and 177 larvae and 346 cocoons were recorded over the two years. Other natural enemies of spider mites detected alongside *F. acarisuga* included *S. gilvifrons*, *S. longicornis*, *Orius* sp. and the unidentified phytoseiid species. It has been reported that *S. gilvifrons*, *S. longicornis* and cecidomyiid species, which are the natural enemies of spider mites on greenhouse strawberry plants in the Aydın Province, are unable to reach the population density needed to put pressure on spider mites with intensive spraying (Çakmak, 2002). Similarly, the most common beneficial species, *F. acarisuga*, was insufficient to completely suppress spider mites, despite the careful

application of pesticides. However, the number of mites *F. acarisuga* kills is high on leaves where pest density is high, indicating that *F. acarisuga* is successful when evaluated on a leaf basis. Similarly, Çakmak (2002) found that in greenhouse strawberry fields the larvae of an unidentified species of the Cecidomyiidae fed on *Tetranychus* species and exerted local pressure on the leaves where the pest was dense.

In this study, the number of *F. acarisuga* cocoons was higher than the number of larvae on the sampled leaves in both years. Although non-cocoon-forming pupae were detected at times, this situation was found to be negligible. The density of *F. acarisuga* was determined to be in the range of 0.01-0.62 larvae + cocoons/leaf. Çakmak (2002) reported a population density of cecidomyiids in the greenhouse as 0.01-0.27 larvae/leaflet. The higher number of predators cecidomyiids in the present study could be attributed to the combination of larvae and cocoon numbers. In addition, the cultivation conditions may have also been more effective, as the strawberry fields in which Çakmak (2002) conducted his study are covered after February.

Aphanogmus species are usually parasitoids of cecidomyiid flies (Evans et al., 2005). Gillespie et al. (1998) reported that the parasitoid *Aphanogmus floridanus* Ashmead, 1893 (Hymenoptera: Ceraphronidae) attacks the larvae of *F. acarisuga* and emerges from the cocoon. Oatman (1985) determined that *A. floridanus* on strawberries significantly affected the population of *Feltiella acarivora* (Felt, 1907) (Diptera: Cecidomyiidae), a predator of *T. urticae*, and parasitism ranged from 0 to 89.7%. Although there is a possibility that the parasitoid found in this study may be a different species, the evaluation of the parasitoid rate appears to provide sufficient information about the parasitization of *F. acarisuga*. Here, the parasitization ranged from 0% to over 50%, similar to the study of Oatman (1985). Gillespie et al. (1998) observed that parasitoids are most numerous in the summer months, and the decrease in parasitism in some periods may be due to diapause. Some *Aphanogmus* species have been reported to emerge from the pupa of *F. acarisuga* in other parts of the world, but not in Japan. It has been suggeest that the reason for this may be that another unidentified species, *F. acarisuga* and *F. acarivora*, also attacked *F. acarivora* when coexisting (Ganaha-Kikumura et al., 2012).

The *Aphanogmus* species, which was determined as a parasitoid of *F. acarisuga* in the present study, may be a factor affecting the successful control of spider mites. In addition, even individuals of *F. acarisuga* from natural populations that occasionally enter greenhouses can effectively reduce the pest density on cultivated plants (Sharaf, 1984). Commercially available as a biocontrol agent against tetranychid mites, *F. acarisuga* has a superior prey consumption rate to *Phytoseiulus persimilis* Athias-Henriot, 1957 (Acari: Phytoseiidae), an important and common spider mite predator (Opit et al., 1997). *Feltiella acarisuga* can naturally invade greenhouses, and its larvae can consume several times as many spider mites as *P. persimilis* per day. However, augmentative releases are required for spider mite control at high population densities (Gillespie et al., 1998). It has been found that a larva feeds on an average of 32.3 adult *Tetranychus* during the entire larval stage (Kawano, 1969). Larvae feed for 4-6 days depending on temperature and prey status, and they consume more than 150 eggs during their lifespan (Gillespie et al., 1998, Mo & Liu, 2006). Xiao e al. (2011) determined that the predation of *F. acarisuga* against *T. urticae* and *O. pratensis* ranged from 43.7 to 67.9% and 59.2 to 90.3%, respectively, under laboratory conditions. Xiao et al. (2013) showed that *F. acarisuga* is the most effective predator of *T. urticae* among three predator species, *N. californicus*, *A. swirskii* and *F. acarisuga*, and the daily predation by a larval *F. acarisuga* was 50 eggs per day. *Phytoseiulus persimilis* females were found to consume an average of 16.7 *T. cinnabarinus* eggs during their development at different temperatures (Kazak, 2006), while adult females consumed a maximum of 20.6 larvae and adult males a maximum of 3.25 larvae at different temperatures (Kazak, 2008).

Although the performance of *F. acarisuga* varies with temperature, all developmental stages can develop at 11.5-31.9°C (Choi et al., 2021). In the present study, the maximum daily temperatures in the greenhouse ranged from 18.5 to 32.9°C and the minimum daily temperature ranged from 7.2 to 26.6°C. It was found that greenhouses provide a suitable environment for *F. acarisuga.* In addition, it is thought that being careful about the use of pesticides in the greenhouse can facilitate the establishment of predator

species compared to commercial strawberry greenhouses where pesticides are commonly used. However, it is clear that the parasitism status of *F. acarisuga* should be considered. Matsuo et al. (2016) advised that the identification of *Aphanogmus* species is essential to evaluate their possible negative effects on the activity of *Feltiella* species. There is no commercial use of *F. acarisuga* for the biological control of spider mites in Turkey. However, as determined in this study, *F. acarisuga* can be found in strawberry greenhouses during the growing season.

Acknowledgments

I am grateful to Dr. Marcela Skuhravá (CSc.Bítovská 1227/9 CZ-140 00 Praha 4 Czech Republic), Assist. Prof. Kazunori Matsuo (Biosystematics Laboratory, Faculty of Social and Cultural Studies, Kyushu University, Motooka, Nishi-ku, Fukuoka 819-0395, Japan) and Prof. Edward A. Ueckermann (School of Environmental Sciences and Development, North-West University, South Africa), Prof. Dr. Ekrem Atakan (Çukurova University, Faculty of Agriculture, Department of Plant Protection, Adana) and Prof. Dr. Nedim Uygun (retired Professor, Adana) for their contributions and support in the diagnosis of specimens. I also thank Assoc. Prof. Dr. Nafiye Adak (Akdeniz University, Faculty of Agriculture, Department of Horticulture, Antalya) for her assistance in the greenhouse study.

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Türk. entomol. derg., 2022, 46 (1): 37-49
DOI: http://dx.doi.org/10.16970/entoted.1000271

ISSN 1010-6960
E-ISSN 2536-491X

Original article (Orijinal araştırma)

Effect of plant phenolic compounds on the hemocyte concentration and antioxidant enzyme activity in *Hyphantria cunea* (Drury, 1773) (Lepidoptera: Arctiidae) larvae infected by *Hyphantria cunea* granulovirus[1]

Hyphantria cunea granulovirus tarafından enfekte edilen *Hyphantria cunea* (Drury,1773) (Lepidoptera: Arctiidae) larvalarının hemosit konsantrasyonu ve antioksidan enzim aktivitelerine bitki fenolik bileşiklerinin etkisi

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Abstract

The aim of this study was to determine the effects of phenolic substances of four plants (apple, mulberry, plum and walnut) on hemocyte concentrations and antioxidant enzyme activity of *Hyphantria cunea* (Drury, 1773) (Lepidoptera: Arctiidae) larvae infected with *Hyphantria cunea* granulovirus and uninfected. The plants used in this study were collected in Bafra, Samsun, Turkey in 2019. The phenolic concentrations of the leaves of these plants were determined. Then, the effect of these phenolic on hemocyte concentrations and antioxidant enzyme activity of infected and uninfected larvae were determined. The hemocyte concentrations of all groups increased with virus infection. The concentration of malondialdehyde decreased in all groups as a result of viral infection. The highest superoxide dismutase and catalase activities among both infected and uninfected larvae were in the plum groups with the highest concentration of chlorogenic acid, the lowest glutathione peroxidase activity was also in these groups. All this showed that different phenolic concentrations of host plants affected the hemocyte concentrations and antioxidant enzyme activity of *H. cunea* larvae.

Keywords: Antioxidant activity, granulovirus, hemocyte, *Hyphantria cunea*, phenolic compounds

Öz

Bu çalışmanın amacı, dört bitkide (elma, dut, erik ve ceviz) bulunan fenolik maddelerin *Hyphantria cunea* granulovirus ile enfekte ve enfekte olmamış *Hyphantria cunea* (Drury, 1773) (Lepidoptera: Arctiidae) larvalarının hemosit konsantrasyonları ve antioksidan enzim aktivitesine olan etkilerini belirlemekti. Bu çalışmada kullanılan bitkiler Bafra, Samsun, Türkiye'de 2019 yılında toplandı. Bu bitkilerin yapraklarının fenolik konsantrasyonları belirlendi. Daha sonra, bu fenoliğin enfekte ve enfekte olmayan larvaların hemosit konsantrasyonlarına ve antioksidan enzim aktivitesine olan etkisi belirlendi. Tüm grupların hemosit konsantrasyonlarının virüs enfeksiyonu ile arttığı belirlendi. Viral enfeksiyon sonucu tüm gruplarda malondialdehit konsantrasyonu azaldı. Hem enfekte hem de enfekte olmayan larvalar arasında en yüksek süperoksit dismutaz ve katalaz aktiviteleri klorojenik asit konsantrasyonunun en yüksek olduğu erik gruplarında iken, en düşük glutatyon peroksidaz aktivitesi de bu gruplardaydı. Bütün bunlar, konukçu bitkilerin farklı fenolik konsantrasyonlarının *H. cunea* larvalarının hemosit konsantrasyonlarını ve antioksidan enzim aktivitesini etkilediğini göstermiştir.

Anahtar sözcükler: Antioksidan aktivite, granulovirüs, hemosit, *Hyphantria cunea*, fenolik bileşikler

[1] This study was supported by Ondokuz Mayıs University Research Foundation Turkey, Grant Project No: PYO.FEN. 1904.18.002.
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Received (Alınış): 24.09.2021 Accepted (Kabul ediliş): 07.02.2022 Published Online (Çevrimiçi Yayın Tarihi): 16.02.2022

Introduction

Lepidopteran larvae consume a range of plants with varying nutritional content and allelochemical defenses. Plants contain metabolites known as plant secondary metabolites (PSMs), and insects take these metabolites into their bodies while feeding. PSMs are important in plant defense as they can disrupt the metabolic, biochemical, physiological functions and metabolic pathways of herbivorous insects (Alon et al., 2012). Phenolic compounds and flavonoids are PSMs that are widely present in different plant species. There are numerous studies on the effects of phenolic compounds and flavonoids on insects (Hafeez et al., 2019; Simmonds et al., 2019; Huang et al., 2020). Antioxidant activity is one of the most important effects of these metabolites in insects. Various studies have shown that tannic acid (Türkan et al., 2019), rutin and catechin (Iacopini et al., 2008), chlorogenic acid (Naveed et al., 2018), benzoic acid (Giannenas et al., 2014), protocatechuic acid (Girsang et al., 2020), and rosmarinic acid (Adomako-Bonsu et al., 2017) have antioxidant properties.

Plants not only saturate herbivorous insects, but also affect the immunological resistance of these insects to viruses that an important group of the entomopathogens. In this tritrophic interaction network, since different host plants contain different PSMs, their effects on the susceptibility of herbivorous insects to insect viruses can differ. For example, the cornworm caterpillars were found to be more susceptible to these viruses when fed corn rather than cotton (Farrar & Ridgway, 2000). *Spodoptera exigua* (Hübner, 1808) (Lepidoptera: Noctuidae) larvae fed on soybeans were found to be more susceptible to a virus than when fed on kale or water convolvulus (Wan et al., 2018). The phytochemicals of the host plants influence the susceptibility of herbivorous insects to insect viruses, and such differences in susceptibility are closely related to insect immunity (Wan et al., 2018). The immune response is a physiological process that protects the organism from natural enemies, and the strength of this response is affected by the PSMs of the plant consumed by herbivores (Gowler et al., 2015; Trowbridge et al., 2016). Feeding on various host plants has been suggest to enhance the immune response of herbivores, and in this case, herbivores may be better protected from pathogen infection, thereby increasing their probability of survival (Muller et al., 2015; Barthel et al., 2016).

Reactive oxygen species (ROS) have beneficial effects on immune function at low or moderate concentrations (Janssen-Heininger et al., 2008), while they cause oxidative stress and damage cell structures, such as proteins and lipids, and DNA at high concentrations (Kobayashi et al., 2019). Insects are constantly exposed to numerous environmental stressors such as ultraviolet radiation, bacteria and viruses, and agrochemicals. All of these can lead to ROS production, so insects are constantly exposed to ROS-induced oxidative stress. Insects, like other organisms, are equipped with antioxidant defense systems to reduce the harmful effects of free radicals. An effective antioxidant system promotes free radical scavenging activity and repairs damage to biomolecules required for life, making it necessary for organisms. Superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) are antioxidant enzymes involved in antioxidant defense. SOD degrades the superoxide radical (O_2^-) to hydrogen peroxide (H_2O_2), and then the resulting H_2O_2 is degraded to water by CAT or GSH-Px (Kamalakkannan & Prince, 2006). Scavenging free radicals and increasing antioxidant enzyme activity can directly suppress oxidative damage. In addition to antioxidant defense, hemocytes involved in phagocytosis, nodulation and encapsulation are important in cellular immunity. Also, the enzyme phenoloxidase (PO), a critical component of the immune system, is involved in infection defense.

Hyphantria cunea (Drury, 1773) (Lepidoptera: Arctiidae), originally from North America, is a common pest that has spread worldwide including Turkey. The larval stage of *H. cunea* can effectively defoliate a wide variety of crops and many plant species. Although various methods are used to control *H. cunea*, it continues to spread and cause damage around the world (Gencer et al., 2020). In the fight against *H. cunea*, it is more reliable to use eco-friendly and cost-effective entomopathogens rather than chemical insecticides.

Baculoviruses are thought to be one of the biocontrol methods for lepidopteran species including *H. cunea* (Gencer et al., 2018, 2020; Sayed et al., 2020). Various studies have shown that baculoviruses are effective against a wide variety of insects (Woestmann et al., 2018; Gencer et al., 2019; Sun et al., 2020).

In nature, herbivorous insects feed on a range of plants with different PSMs. These qualitative differences among plants may affect various mechanisms of insects, including immunological resistance (Smilanich et al., 2018). Also, baculovirus infection affects host immune responses (Ikeda et al., 2013), and viruses are also one of the sources of oxidative stress. Antioxidant enzyme activity, hemocyte concentrations, phenoloxidase activity and malondialdehyde (MDA) concentrations in insects change in response to oxidative stress. Determining these changes is critical in determining how insects respond to stress. The effects of PSMs on these parameters in *H. cunea* were investigated using apple, mulberry, plum and walnut plants, which are commonly eaten by *H. cunea* larvae and have economic value. In addition, the impact of *Hyphantria cunea* granulovirus (HycuGV) infection on these parameters was evaluated.

Materials and Methods

Source of insect larvae and plants

Hyphantria cunea larvae were collected during field surveys in the Bafra District of Samsun, Turkey, in 2019 (41°30' N, 36°05' E). The larvae were kept in the laboratory at 25 ± 2°C and 60% RH under a 16:8 h L:D photoperiod. They were divided into four groups and fed on *Malus pumila* (Borkh, 1803) (Rosales: Rosaceae) (apple), *Morus alba* (L., 1753) (Rosales: Moraceae) (mulberry), *Prunus domestica* (L.) (Rosales: Rosaceae) (plum) and *Juglans regia* (L.) (Fagales: Juglandaceae) (walnut) until they reached the pupal stage. Adults emerged from the pupae were mated and females deposited eggs. The newly hatched larvae were divided into four groups to be fed on the four plant species. The leaves used in the study were collected daily, and the leaves was sterilized with 50% ethyl alcohol before being fed to the larvae.

Phenolic and gallotannin analysis of the leaves

The determination of phenolic compound was made with HPLC (Thermo-Finnigan Surveyor, Thermo Finnigan, San Jose, CA, USA). HPLC-UV analyses were performed on a reverse phase C18 column (150 mm × 4.6 mm id, 5 µm particle; Fortis, France) using a UV detector which is simultaneously operating dual-UV wavelength. Gradient elution was used for HPLC analyses. The mobile phase was 2% acetic acid in water (A) and 70:30 acetonitrile:water (B). The following gradient was used; 0-3 min 5% B; 3-8 min 5-15% B; 8-10 min 15-20% B; 10-12 min 20-25% B; 12-20 min 25-40% B; 20-30 min 40-80% B. The injection volume was 25 µl, the column temperature was 30°C and the flow rate was 1.2 ml/min.

The method used to determine the gallotannin contents of the leaf samples was described by Bate-Smith (1977). For gallotannin analysis, leaf samples from each plant species were taken daily and dried in an oven to constant weight and then were ground. Leaf samples (0.5 mg) were placed in 10-ml tubes with 1 ml of 5% KIO_3 solution added to three of the tubes and 1 ml of distilled water to the fourth tube (as a control). The tubs were kept on ice for 1 h before measuring their absorbance in a spectrophotometer at a wavelength of 550 nm. A standard curve was prepared with tannic acid solutions (0.1-0.7 mg/ml) to estimate the gallotannin concentrations of the samples.

Virus propagation

HycuGV-Hc1 was obtained from the entomopathogenic virus culture collection of the microbiology laboratory at Karadeniz Technical University (Bayramoglu et al., 2018). Propagation of virus was performed in healthy *H. cunea* larvae collected from the field. Mulberry leaves surface contaminated with 10 µl of viral stock culture were fed to *H. cunea* larvae. The infected larvae were homogenized in sterile distilled water and filtered through double-layer cheesecloth 7 days after treatment to remove larval debris. The filtrate was centrifuged at 5,000 g for 30 min. Pellets were suspended in 1 ml dH_2O, loaded onto 3 ml of 30%

sucrose and centrifuged at 5,000 g for 30 min (Bayramoglu et al., 2018). The resulting pellets were then washed with dH_2O and resuspended in 1 ml of dH_2O. The concentration of the virus was adjusted to 10^5 OB/ml by using a Neubauer hemocytometer.

Feeding experiments

Feeding experiments were conducted in two stages: uninfected and infected larvae. One hundred fifth instar larvae were used in both groups: 50 each for determination of enzyme activity and hemocyte concentration. The larvae in the uninfected groups were fed on uncontaminated leaves for 7 days. Five days after the start of the experiment, the larvae in the infected groups were infected by feeding leaves treated with 1 ml of HycuGV suspension for two more days. After 7 days for each group, hemolymph of the larvae in the infected groups was taken by cutting the third legs of the larvae.

Examination and counting of hemocytes by Giemsa staining

Hemolymph was placed in Eppendorf tubes and 10 µl spread on a glass slide and air-dried for 20-30 min to allow hemocytes to adhere to the glass. The cells were fixed in methanol: acetic acid solution (3:1) for 10 min. The slides were stained for 10 min with Giemsa (Merck, Darmstadt, Germany) and then washed with distilled water. After air-drying, the slides were treated with xylene and then mounted in Entellan. A Zeiss Primo Star microscope was used to count the hemocytes. The hemocytes were counted in twenty randomly selected areas on each slide. The hemocyte concentrations were calculated (number per 10 µl) by multiplying the mean cell concentrations by the microscope factor determined from the microscope sight field (Fitts & Laird, 2004).

Enzyme analysis

Hemolymph samples collected from the larvae were homogenized with an ultrasonic processor (VCX 130 Sonics, Newtown, CT, USA). The homogenates (20 ml), were transferred to Eppendorf tubes and centrifuged at +4°C for 20 min at 15,000 rpm in a refrigerated centrifuge (model 3500, Kubota, Tokyo, Japan). After centrifugation, the supernatant was kept at -80°C until total protein determination and enzyme activity analyses. Protein determination was made according to the method of Lowry et al. (1951). Solution A containing 2% Na_2CO_3, 1% $CuSO_4$, and 2% Na-K-tartrate mixture and solution B containing a 1:1 diluted Folin-Ciocalteu phenol reagent were prepared for protein determination. A 10-µl aliquot was then added to 2.5 ml of solution A and vortexed. After that, 250 µl of solution B was added and vortexed again. The mixture was incubated in the dark for 30-60 min. Spectrophotometric measurement was performed at 595 nm. Standards were prepared with bovine serum albumin and plotted. The absorbance values obtained from the samples were calculated by proportioning by the standard. The activity of superoxide dismutase was determined using the method of Flohé & Ötting (1984) and the spectrophotometric method of McCord & Fridovich (1969). A 0.76 mg (5 µl) xanthine solution in 10 ml 0.001 N NaOH was mixed with a 24.8 mg (2 µmol) cytochrome c solution in 100 ml 50 Mm pH 7.8 phosphate buffer containing 0.1 M EDTA. A freshly prepared 0.2 U/ml xanthine oxidase solution in 0.1 mM EDTA was used in this experiment. The reduction of cytochrome c by the xanthine/xanthine oxidase system was spectrophotometrically measured at 550 nm to determine SOD activity. Catalase activity was determined by the Luck (1963) method. $Na_2HPO_4.H_2O$-KH_2PO_4 buffer (67 mM) was prepared at pH 7 to determine CAT activity. For each activity measurement, 160 µl of H_2O_2 was added to 100 ml of Na-K buffer. When the samples were added to the mixture, the CAT activity was determined spectrophotometrically with the decrease in absorbance at 240 nm due to H_2O_2 degradation. Glutathione peroxidase activity was determined by the method of Lawrence & Burk (1976). GSH-Px catalyzes the oxidation of glutathione by Cumene hydroperoxide. $NADP^+$ oxidation occurs in the reaction medium during the conversion of oxidized glutathione to reduced glutathione with the cofactors glutathione reductase and NADPH. Potassium phosphate buffer (50 mM) was prepared at pH 7 to measure GSH-Px activity. The decreases in absorbance were measured using a spectrophotometer at 340 nm.

Activity of PO were determined according to Ashida & Söderhäll (1984). MDA concentrations were determined according to Draper & Hadley (1990). The activity was measured in terms of µmol of oxidized NADPH per min. T70 UV/VIS spectrophotometer was used to determine the enzyme activity.

Statistical analysis

In this study, SPSS 21.0 software was used for statistical analysis. The effects of phenolic compounds in plants on hemocyte concentrations and antioxidant enzyme activity of *H. cunea* larvae were determined using the ANOVA and Tukey's test. Two independent samples t-test was used to determine the relationship between these parameters based on PSMs. All analyzes were performed in three replicates.

Results

Plant phenolic compounds

Phenolic compounds in the plants are given in Table 1. Plum leaves contained the most chlorogenic acid and mulberry the least. Benzoic acid was only found in walnut leaves, and catechin and rutin were only found in mulberry leaves. Rosmarinic acid and protocatechuic acid were only found in apple and plum leaves with apple leaves having the higher concentration. For gallotannin (Table 1), the highest concentration of was found in walnut leaves and the least in the mulberry leaves.

Table 1. Phenolic compounds and gallotannin (µg/mg) in the plant leaves used in this study

Plant	Benzoic acid	Catechin	Chlorogenic acid	Gallotannin	Protocatechuic acid	Rosmarinic acid	Rutin
Apple	0	0	3.3	58	1.5	200	0
Mulberry	0	16.7	1.3	16	0	0	1.7
Plum	0	0	18.4	84	1.1	7	0
Walnut	12.3	0	4.1	116	0	0	0

Hemocyte concentrations

Hemocyte concentrations are given in Table 2. In the uninfected groups, larvae fed on apple leaves had the lowest hemocyte concentration and those fed on walnut leaves had the highest. The mean hemocyte concentration of all virus-infected groups increased compared to the uninfected groups. In the infected groups, larvae fed on mulberry leaves had the lowest hemocyte concentration and those fed on walnut larvae had the highest.

Table 2. Hemocyte concentrations (no./µl; mean ± SE) of *Hyphantria cunea* larvae in the uninfected and infected groups

Plant	Uninfected	Infected	t	P	Uninfected SD (Tukey's test)*	Infected SD (Tukey's test)*
Apple	275 ± 0.25	450 ± 0.51	-59.2	<0.001	4.48 a	4.14 c
Mulberry	284 ± 0.30	310 ± 0.40	-12.4	<0.001	2.23 b	4.00 a
Plum	281 ± 0.33	363 ± 0.30	-22.4	<0.001	5. 25 b	5.41 b
Walnut	359 ± 0.59	528 ± 0.17	-56.8	<0.001	2.48 c	4.33 d

* Values within the column followed by the same letter are not significantly different (P < 0.001).

Phenoloxidase activity

PO activity is given in Table 3. In the uninfected groups, larvae fed on apple leaves had the lowest PO activity and those fed on walnut leaves had the highest activity. In the infected groups, larvae fed on mulberry leaves had the lowest PO activity and those fed on plum leaves had the highest activity.

Table 3. Phenoloxidase activity (IU; mean ± SE) of *Hyphantria cunea* larvae in the uninfected and infected groups

Plant	Uninfected	Infected	t	P	Uninfected SD (Tukey's test)*	Infected SD (Tukey's test)*
Apple	26 ± 0.6	35 ± 0.7	-11.6	<0.001	1.24 a	1.17 b
Mulberry	33 ± 0.9	15 ± 0.5	23.3	<0.001	1.41 b	0.89 a
Plum	33 ± 0.9	36 ± 0.9	-3.5	<0.001	1.21 b	1.28 b
Walnut	40 ± 0.1	36 ± 0.1	4.5	<0.001	1.40 c	1.43 b

* Values within the column followed by the same letter are not significantly different (P < 0.001).

Malondialdehyde concentrations

MDA concentrations are given in Table 4. In the uninfected groups larvae fed on mulberry leaves had the lowest MDA concentration and those fed on apple leaves had the highest. The MDA concentrations of all groups decreased in virus infection compared to the uninfected groups. In the infected groups, larvae fed on apple leaves had the highest MDA concentration and those fed on mulberry leaves had the least.

Table 4. Malondialdehyde concentrations (IU; mean ± SE) of *Hyphantria cunea* larvae in the uninfected and infected groups

Plant	Uninfected	Infected	t	P	Uninfected SD (Tukey's test)*	Infected SD (Tukey's test)*
Apple	418 ± 0.1	354 ± 0.3	12	<0.001	5.80 c	9.83 c
Mulberry	236 ± 0.7	210 ± 0.2	8.6	<0.001	4.51 a	4.49 a
Plum	365 ± 0.3	335 ± 0.3	4.8	<0.001	11.16 b	6.43 b
Walnut	371 ± 0.6	353 ± 0.9	3.1	<0.001	9.40 b	9.01 c

* Values within the column followed by the same letter are not significantly different (P < 0.001).

Superoxide dismutase activity

SOD activity is given in Table 5 with activity with larvae fed on mulberry leaves having highest and those fed on plum leaves the lowest. In the infected groups, larvae fed on walnut had the lowest SOD activity and those fed on plum leaves-the highest.

Table 5. Superoxide dismutase activity (IU; mean ± SE) of *Hyphantria cunea* larvae in the uninfected and infected groups

Plant	Uninfected	Infected	t	P	Uninfected SD (Tukey's test)*	Infected SD (Tukey's test)*
Apple	237 ± 0.4	186 ± 0.1	10.2	<0.001	7.56 c	7.32 c
Mulberry	198 ± 0.5	149 ± 0.6	10	<0.001	7.03 b	6.98 b
Plum	791 ± 0.3	470 ± 0.9	51.7	<0.001	7.56 c	8.55 d
Walnut	170 ± 0.8	137 ± 0.4	7.8	<0.001	9.95 d	6.03 a

* Values within the column followed by the same letter are not significantly different (P < 0.001).

Catalase activity

CAT activity is shown in Table 6 with the uninfected groups ranked from the highest to the lowest as plum > mulberry > walnut > apple. In the infected groups, larvae fed on plum had the highest CAT activity and those fed on apple leaves had lowest.

Table 6. Catalase activity (IU; mean ± SE) of *Hyphantria cunea* larvae in the uninfected and infected groups

Plant	Uninfected	Infected	t	P	Uninfected SD (Tukey's test)*	Infected SD (Tukey's test)*
Apple	130 ± 0.8	202 ± 0.8	-15.6	<0.001	6.64 a	6.94 a
Mulberry	606 ± 0.3	580 ± 0.3	4.6	<0.001	9.23 c	8.20 c
Plum	1112 ± 0.8	804 ± 0.2	27.5	<0.001	17.05 d	9.75 d
Walnut	329 ± 0.1	244 ± 0.2	15.4	<0.001	7.78 b	8.73 b

* Values within the column followed by the same letter are not significantly different (P < 0.001).

Glutathione peroxidase activity

GSH-Px activity is shown in Table 7 with uninfected groups ranked from highest to lowest as walnut > mulberry > apple > plum. For the infected groups, the ranking was walnut > apple > mulberry > plum.

Table 7. Glutathione peroxidase activity (IU; mean ± SE) of *Hyphantria cunea* larvae in the uninfected and infected groups

Plant	Uninfected	Infected	t	P	Uninfected SD (Tukey's test)*	Infected SD (Tukey's test)*
Apple	229 ± 0.8	235 ± 0.3	-1.6	<0.001	7.48 b	6.82 c
Mulberry	299 ± 0.9	129 ± 0.3	35	<0.001	8.13 c	6.24 b
Plum	55 ± 0.7	91 ± 0.9	-13.6	<0.001	2.82 a	4.57 a
Walnut	418 ± 0.3	287 ± 0.2	26.8	<0.001	7.60 d	7.02 d

* Values within the column followed by the same letter are not significantly different (P < 0.001).

Discussion

The hemocyte concentrations of insects is related to the effectiveness of the immune system (Ghosh et al., 2018). Larvae fed on apple leaves had the lowest mean hemocyte concentration in the uninfected groups. The highest concentration of rosmarinic acid was present in the apple leaves, and in this case, a high concentration of rosmarinic acid can be assumed to reduce the hemocyte concentrations. Also, we found that larvae fed on walnut, the species containing the highest gallotannin concentration, had the highest hemocyte concentration. Both the findings of this study and the results of other studies (Pandey et al., 2012; Smilanich et al., 2018) show that feeding on different plants can affect the hemocyte concentrations of insects due to differences in plant constituents. The hemocyte concentration is an important indicator that reflects an insect's ability to resist entomopathogens. Viral infections induce cellular defense reactions (Millanta et al., 2019). It was found that the hemocyte concentrations of *S. exigua* larvae infected with the virus varied with the plant species consumed (Wang et al., 2021). Povey et al. (2013) found that the virus-treated *Spodoptera exempta* (Walker, 1856) (Lepidoptera: Noctuidae) larvae had higher hemocyte concentrations than control larvae. In this study, it was determined that the mean hemocyte concentrations of all virus-infected groups increased compared to the uninfected groups. It was found that the highest mean hemocyte concentration in the infected groups was in larvae fed on walnut leaves, which was the only plant with benzoic acid among the plants. Additionally, the concentration of gallotannin was the greatest in the walnut leaves. Hemocytes have an active role in clearing viruses from the hemolymph (Mahmoud & Soliman, 2015), and they do so by increasing their numbers, usually in response to infection. A higher hemocyte concentration means stronger resistance to insect virus infection as well as immunity. In this case, the presence of benzoic acid and high gallotannin may give the larvae an advantage in cellular defense.

Phenoloxidase is an enzyme that plays a critical role in the immune response (Wang et al., 2020) and is one of the main factors in insect immunity due to its role in the melanization process (Dudzic et al., 2015). Larvae fed on apple leaves had the lowest PO activity in the uninfected groups. In this case, the high concentration of rosmarinic acid found in the apple leaves may be effective. Also, the maximum PO activity was in larvae fed on walnut leaves containing a high concentration of gallotannin. Our findings show that different PSMs at high concentrations have different effects on the PO activity of *H. cunea* larvae. In addition, the fact that the larvae feeding with various plants have different PO activity (Ebrahimi & Ajamhassani, 2020) supports our findings. Since PO is the main component of hemocytes (Akita & Hoshi, 1995), the correlation between hemocyte concentration and PO activity was also confirmed by our findings in uninfected groups. The PO activity in the groups with the highest and lowest hemocyte concentrations (walnut and apple uninfected groups, respectively) also showed parallel results. The activation of the PO system in the hemolymph is one of the first responses of the insect immune system to infection. Phenoloxidase is present in the hemolymph as prophenoloxidase (proPO) and is activated by a serine proteinase (Nakhleh et al., 2017). The proPO activation cascade may facilitate in the protection of host larvae from baculovirus infection (Jiang et al., 2010). Studies have reported that hemolymph PO activity of lepidopteran larvae are affected by viral infection. For example, Millanta et al. (2019) found that virus-treated honeybees had higher PO activity than the control. Li et al. (2021) reported that the PO activity of *S. exigua* larvae infected with the virus was significantly increased. In this study, the PO activity of larvae fed on apple and plum leaves increased with viral infection compared to the uninfected larvae, which is consistent with the findings of these studies. Rosmarinic acid and protocatechuic acid, which were only found in the apple leaves and the plum leaves, could have been effective. In contrast, the PO activity of larvae fed on mulberry and walnut leaves were found to decrease relative to the uninfected larvae. The decrease in PO activity of the virus-infected larvae was somewhat unexpected, but it is consistent with the findings of previous studies (Rao et al., 2010; Yuan et al., 2017; Wang et al., 2020).

Malondialdehyde is an indicator of free radical-induced lipid peroxidation and acts as a marker of oxidative stress (Schuessel et al., 2006). The MDA concentration was lowest in larvae fed on mulberry leaves in both the uninfected and infected groups. Flavonoids prevent the formation of free radicals increased by the oxidation of saturated lipids (Oboh et al., 2015). Rutin can remove lipid peroxidation products (Zhu et al., 2019). In the study of Iacopini et al. (2008), rutin and catechin were shown to have antiradical and antioxidant properties. Our findings are consistent with these studies, and we conclude that catechin and rutin flavonoids, which are only found in the mulberry leaves, protect larvae from lipid peroxidation. Lipid peroxidation as a result of oxidative stress during the viral infection has been established in insect cell lines (Wang et al., 2001); however, we found that the concentrations of MDA with viral infection were lower than those of the uninfected groups. This situation can be explained by PSMs having antioxidant properties (Zhu et al., 2019) that protect larvae from infection against lipid peroxidation.

The activity of antioxidant enzymes in insect tissues is related to the host plant spectrum (Dampc et al., 2020). According to a study (Durak et al., 2018), *Aphis pomi* (de Geer, 1773) (Hemiptera: Aphididae), an oligophagous species, had higher SOD activity than *Cinara tujafilina* (Del Guercio, 1909) (Hemiptera: Aphidoidea), which has a broad host plant spectrum. Lukasik (2007) found that the SOD activity of *Rhopalosiphum padi* (L., 1758) (Hemiptera: Aphididae) and *Sitobion avenae* (Fabricius, 1775) (Hemiptera: Aphididae) were influenced by their host plants, which is consistent with our findings. These differences in SOD activity are most probably due to the different PSMs of the host plants. Larvae fed on walnut leaves had the lowest SOD activity in both the uninfected and infected groups. In this case, the benzoic acid found in the walnut leaves may have been effective. Velika & Kron (2012) found that benzoic acid has antioxidant properties against superoxide radicals, which is in contrast to our findings. In addition, it was determined that the highest SOD activity in both the uninfected and infected groups were in larvae fed on plum leaves, which contained the highest concentration of chlorogenic acid. Given that chlorogenic acid is a potent

antioxidant and has free radical scavenging properties (Kim et al., 2018), it is not surprising that this phenolic compound causes SOD activity to increase to eliminate ROS. SOD activity decreased in all groups with viral infection compared to the uninfected groups. It is counterintuitive because, in the face of a stress factor such as infection, the larvae are expected to upregulate their SOD activity to eliminate ROS.

Perić-Mataruga et al. (2014) reported that *Lymantria dispar* (L., 1758) (Lepidoptera: Lymantriidae) larvae exhibited different CAT activity when fed on various plants. It has been determined that *Myzus persicae* (Sulzer, 1776) (Hemiptera: Aphididae) fed on various host plants had varying CAT activity (Abdelsalam et al., 2016). In this study, we recorded that *H. cunea* larvae fed on various plants had different CAT activity. The lowest CAT activity in both the uninfected and infected groups were in larvae fed on apple leaves. Possibly the maximum concentration of rosmarinic acid present in the apple leaves may have caused CAT activity to be minimal. Larvae fed on plum leaves had the highest CAT activity in both the uninfected and infected groups. In this case, the highest concentration of chlorogenic acid found in plum leaves may have been effective. Also, the maximum SOD activity were in larvae fed on plum leaves. Since the H_2O_2 formed as a result of SOD activity is degraded by CAT (Kaushal et al., 2018), this explains the maximum CAT activity of the larvae fed on plum leaves having the highest SOD activity. Catalase activity is affected by virus application. For example, Wang et al. (2001) determined that virus application changed the CAT activity of larvae over time. It was found that CAT activity in all groups (except the apple group) with the viral infection were lower than those of the uninfected groups in this study.

The highest GSH-Px activity in both the uninfected and infected groups were in larvae fed on walnut leaves. Since tannins have powerful antioxidant properties (Payab et al., 2019), the highest concentration of gallotannin in walnut leaves may have given the larvae an advantage in GSH-Px activity. Hydrogen peroxide is detoxified by either CAT or GSH-Px enzymes converting it to water. When there is a high H_2O_2 concentration, CAT acts, whereas GSH-Px acts when there is a low H_2O_2 concentration (Baud et al., 2004). The fact that the larvae fed on plum leaves (both the uninfected and infected) had the highest CAT activity and the lowest GSH-Px activity confirms that these two enzymes work in a coordinated manner. GSH-Px plays a role in defense against pathogenic organisms (Brigelius-Flohé & Maiorino, 2013). The enzyme activity of larvae fed on apple and plum leaves increased due to viral infection. In this case, the rosmarinic acid and protocatechuic acid found in the apple leaves and the plum leaves may have given the larvae an advantage in overcoming the cytotoxic effect of H_2O_2.

In this study, in which we correlated the phenolic content of host plants with the concentrations of hemocytes and antioxidant enzyme activity, it was observed that the responses of *H. cunea* larvae to the various parameters measured differed depending on the phenolic compounds present. In addition, our findings determined that when *H. cunea* larvae infected with *Hyphantria cunea* granulovirus, they gave priority to cellular defense with increased hemocyte concentration rather than antioxidant activity. Plant-herbivore-entomopathogen interactions are quite complex. Identifying any immune changes should be considered as a significant issue in insecticide application in agroecosystems. Understanding which factors affect the insect immune system will be critical in combating harmful insects (especially microbial control).

Acknowledgments

We thank Dr. Ardahan Eski from Bilecik Seyh Edebali University for his contributions. This study was supported by the Ondokuz Mayıs University Research Foundation (PYO.FEN. 1904.18.002).

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Türk. entomol. derg., 2022, 46 (1): 51-62
DOI: http://dx.doi.org/10.16970/entoted.1048846

ISSN 1010-6960
E-ISSN 2536-491X

Original article (Orijinal araştırma)

Interactions of aphids (Hemiptera: Aphididae) with their primary and secondary host plants in orchards in Çanakkale Province, Turkey[1]

Türkiye'nin Çanakkale İli'ndeki bahçelerde afitler (Hemiptera: Aphididae) ile ana ve ara konukçu bitkilerinin etkileşimleri

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Abstract

This study aimed to reveal the interactions of aphids with their primary hosts (pome and stone fruit trees) and secondary hosts (herbaceous plants) in and around orchards in the Çanakkale Province in northwestern Turkey between April-September from 2020 to 2021. Thirty-eight aphid species belonging to 18 genera from two subfamilies, Aphidinae and Calaphidinae, of Aphididae in the order Hemiptera were identified on 53 host plants. Also, 92 aphid-primary-secondary host plant interactions, including several new host plant records for some aphid species in Turkey were revealed in the orchards. Of these hosts, *Ballota* sp. (Lamiaceae), *Carduus pycnocephalus* L. (Asteraceae), *Conyza* sp. (Asteraceae), *Diplotaxis* sp. (Brassicaceae), *Echium plantagineum* L. (Boraginaceae), *Galium* sp. (Rubiaceae), *Geranium columbinum* L. (Geraniaceae), *Papaver rhoeas* L. (Papaveraceae), *Ranunculus muricatus* L. (Ranunculaceae) and *Scandix pecten-veneris* L. (Apiaceae) are determined as new host plants for some aphids in Turkey. It is believed that the new data on the interactions of aphids with their primary and secondary hosts in orchards will support both a better understanding of the biology of fruit pest aphids and the development of new strategies for the control of aphid pests.

Keywords: Aphid, fruit tree, herbaceous plant, interaction, Turkey

Öz

Bu çalışma 2020 ve 2021 yıllarında Nisan ve Eylül ayları arasında Türkiye'nin kuzeybatısında yer alan Çanakkale İli'ndeki meyve bahçelerinde afitler ile ana konukçuları olan yumuşak ve sert çekirdekli meyve ağaçları ve bahçelerin içinde ve etrafında bulunan ara konukçuları olan yabancı otlar arasındaki etkileşimlerin ortaya çıkarılmasını amaçlamaktadır. Teşhislerin sonucunda, 53 konukçu üzerinde Hemiptera takımı Aphididae familyasından Aphidinae ve Calaphidinae altfamilyalarına ait 18 cins içerisinde toplam 38 afit türü belirlenmiştir. Ayrıca, meyve bahçelerinde Türkiye'de bazı afit türleri için çok sayıda yeni konukçu bitki kaydını içeren 92 afit-ana-ara konukçu bitki etkileşimi ortaya çıkarılmıştır. Bu konukçulardan, *Ballota* sp. (Lamiaceae), *Carduus pycnocephalus* L. (Asteraceae), *Conyza* sp. (Asteraceae), *Diplotaxis* sp. (Brassicaceae), *Echium plantagineum* L. (Boraginaceae), *Galium* sp. (Rubiaceae), *Geranium columbinum* L. (Geraniaceae), *Papaver rhoeas* L. (Papaveraceae), *Ranunculus muricatus* L. (Ranunculaceae) ve *Scandix pecten-veneris* L. (Apiaceae) Türkiye'de afitler için yeni konukçu bitki kayıtları olarak belirlenmiştir. Sonuç olarak, meyve bahçelerindeki afitler ile ana ve ara konukçu bitkilerinin etkileşimleri üzerine sunulan bu yeni verilerin hem meyve zararlısı afitlerin biyolojilerinin daha iyi anlaşılmasını hem de zararlı afitler için yeni mücadele stratejilerinin geliştirilmesini destekleyeceğine inanılmaktadır.

Anahtar sözcükler: Afit, meyve ağacı, yabancı ot, etkileşim, Türkiye

[1] This study was supported by Çanakkale Onsekiz Mart University, Scientific Research Coordination Unit, Çanakkale, Turkey, Grant Project No: FBA-2019-3082.
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Received (Alınış): 27.12.2021 Accepted (Kabul ediliş): 07.02.2022 Published Online (Çevrimiçi Yayın Tarihi): 16.02.2022

Introduction

Agricultural areas in Turkey have many fruit species due to the range of geographical and climatic characteristics of these areas. More than half of the 138 fruit species cultivated in the world can be grown economically in Turkey (Ağaoğlu et al., 2015). In 2020, about 8.7 Mt of pome and stone fruits was produced on 540 kha in Turkey. Çanakkale Province, where this study was conducted, is one of the important agricultural regions located in northwestern Turkey produces about 360 kt of pome and stone fruit on 15 kha (Anonymous, 2020).

Many diseases and pest species affect fruit production around the world and cause economically significant losses in yield. Of these pests, aphids (Hemiptera: Aphididae) are one of the most important agricultural pests that damage a large number of crop and forest plants as a result of the suck of plant sap and the secreting of honeydew. They are also vector more than 270 phytopathogenic viruses that cause serious economic damage to agricultural crops (Katis et al., 2007).

Some aphid species have more complex life cycles with host alternation, known as heteroecy. The haploid and fertilized eggs of these aphids occur on tree and shrub plants which serve as primary hosts, and later regularly migrate to other unrelated plants including flowering herbaceous plants which serve as secondary hosts (Blackman & Eastop, 2021). About 10% of aphids, mostly from the subfamily Aphidinae, are heteroecious species that spend seasons like winter, spring and autumn on their primary host plants including trees or shrubs and then switch to unrelated secondary hosts including herbaceous host plants during summer. It is an important that the majority of heteroecious aphids are highly specialized on both primary and secondary host plants (Dixon, 1987). Also, certain heteroecious aphids such as the green peach aphid, *Myzus* (*Nectarosiphon*) *persicae* (Sulzer, 1776) (Hemiptera: Aphididae) transmit plum pox virus (Potyviridae), which is one of the most significant and devastating pathogens of plants, among primary host plants [*Prunus persica* (L.) Batsch (Rosaceae)] and secondary host plants [herbaceous plants including *Centaurea* sp. (Asteraceae), *Matricaria* sp. (Asteraceae), *Papaver* sp. (Papaveraceae) and *Trifolium* sp. (Leguminosae)] (Manachini et al., 2007).

Many uncultivated herbaceous flowering plants occur in and around orchards and are neighbors of fruit trees in these orchards, the interactions of fruit pest aphids with their primary and secondary host plants need to be investigated in more detail. This study aimed to provide basic data on the interactions of aphids with their primary and secondary host plants in pome and stone fruit trees and herbaceous host plants in and around orchards in the Çanakkale Province in northwestern Turkey.

Materials and Methods

Collection, preparation and identification of aphids and their host plants

To reveal the interactions of aphids and primary and secondary hosts, aphid sampling was conducted from pome and stone fruit trees such as quince, apple, plum, cherry, and peach, and herbaceous host plants in and around orchards in the Çanakkale Province between April-September from 2020 to 2021 (Figure 1). Different plant parts, including as trunk, branches, shoots and leaves, of all fruit trees were visually checked and aphid sampling was done from infested fruit trees. To reveal secondary herbaceous hosts of aphids in these orchards, the flowering herbaceous plants in and around the orchards were also examined and aphids collected.

To identify the aphids, apterous and alate individuals from the colonies on infested fruit trees and herbaceous hosts were put into with a brush (#00) into the Eppendorf tubes containing 70% ethyl alcohol and then brought to the laboratory for preparation and identification. The preparation of collected aphids were conducted according to the method of Hille Ris Lambers (1950). The aphids were identified by the corresponding author according to the identification keys of Blackman & Eastop (2006; 2021) using a light microscope (Leica DM 2500) with HD camera and LAS software (version 4.1). The current taxonomic status and names of identified aphid are given as in Favret (2021). The specimens of identified aphids were deposited in the Department of Plant Protection, Faculty of Agriculture, Çanakkale Onsekiz Mart University, Turkey.

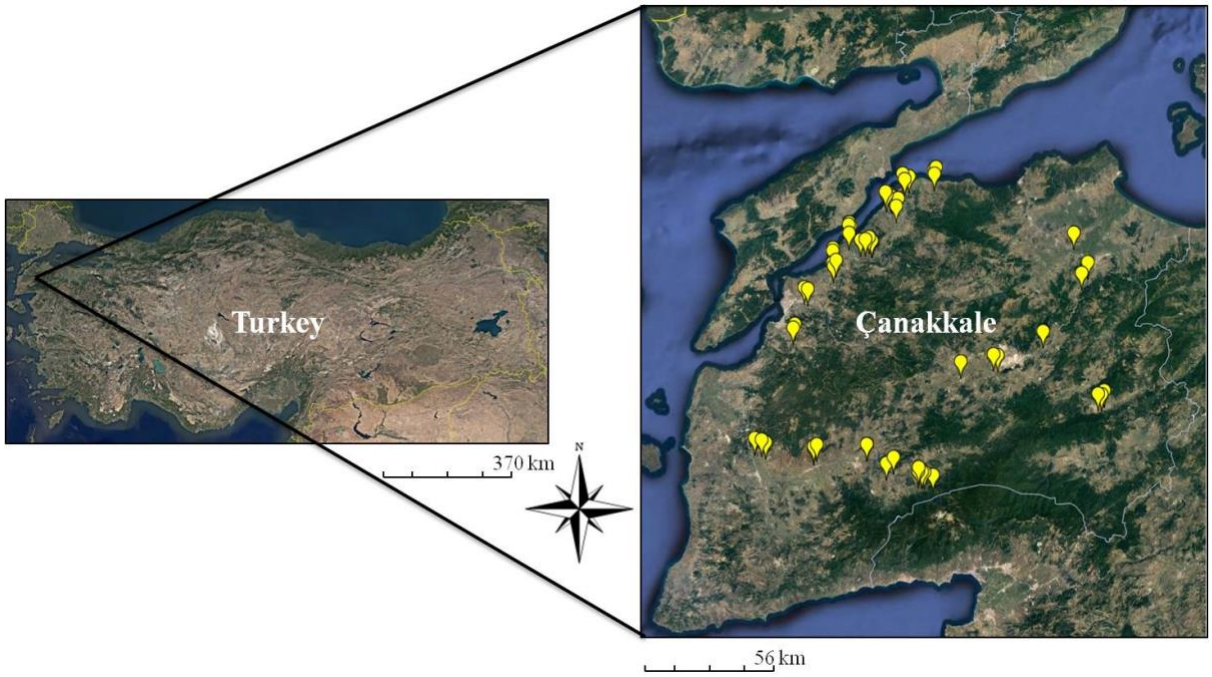

Figure 1. Satellite image showing the sampling area including the orchards in Çanakkale Province, Turkey (Google, 2022).

Plant samples determined to be hosts of aphids were prepared as herbarium specimens for identification. The host plants associated with aphids were identified by Assoc. Prof. Dr. Ersin Karabacak (Department of Biology, Faculty of Arts and Sciences, Çanakkale Onsekiz Mart University, Turkey).

Aphid - Primary and Secondary Host Plant Interactions

To visualize the structural patterns of the aphid - primary and secondary host plant interaction networks in orchards in Çanakkale Province, graphs of bipartite interactions were constructed on the basis of data on aphid, primary and secondary host plant relative abundances using the *plotweb* function in R package "bipartite" under R version 2.04 (Dormann et al., 2014; R Core Team, 2021).

Results and Discussion

Thirty-eight aphid species belonging to 18 genera from two subfamilies, Aphidinae and Calaphidinae, of Aphididae in the order Hemiptera were identified on 53 host plants. Of these, 15 aphids belonging to seven genera were determined on the fruit trees and 29 aphids belonging to 17 genera on the herbaceous hosts in the orchards. Taxonomic status, locality, collection date, the number of apterous and alatae individuals, and host plants of aphids on the fruit trees and herbaceous host plants in the orchards are presented below:

Order Hemiptera

Infraorder Aphidomorpha

Family Aphididae Latreille, 1802

Subfamily Aphidinae Latreille, 1802

***Acyrthosiphon* (*Acyrthosiphon*) *ilka* Mordvilko, 1914**

Material examined. Çanakkale, Lâpseki, Subaşı Village, 17.V.2020, apt. 5♀♀, *Papaver rhoeas* L. (Papaveraceae).

Comments. *Papaver rhoeas* is recorded for the first time as a new host for *A.* (*A.*) *ilka* in Turkey.

Hosts in Turkey. *Glaucium corniculatum* (L.) Rudolph (Papaveraceae), *Lactuca sativa* L. (Asteraceae), *Papaver somniferum* L. (Papaveraceae) (Tuatay & Remaudière, 1964; Tuatay et al., 1972; Tuatay, 1988; Aslan & Uygun, 2005; Islamoğlu & Tarla, 2018; Alaserhat et al., 2020).

***Acyrthosiphon* (*Acyrthosiphon*) *pisum* (Harris, 1776)**

Material examined. Çanakkale, Bayramiç, 15.VII.2020, apt. 4♀♀, alt. 2♀♀, *Trifolium spumosum* L. (Fabaceae); Lâpseki, Çardak, 23.IV.2021, apt. 5♀♀, alt. 2♀♀, *Vicia* sp. (Leguminosae).

***Aphis* (*Aphis*) *acetosae* L., 1767**

Material examined. Çanakkale, Bayramiç, 10.V.2020, apt. 5♀♀, *Rumex* sp. L. (Polygonaceae).

***Aphis* (*Aphis*) *ballotae* Passerini, 1860**

Material examined. Çanakkale, Lâpseki, Subaşı Village, 10.IV.2020, apt. 4♀♀, alt. 2♀♀, *Ballota* sp. (Lamiaceae).

Comments. *Ballota* sp. is recorded for the first time as a new host for *A.* (*A.*) *ballotae* in Turkey.

Hosts in Turkey. *Marrubium* sp. (Lamiaceae) (Tuatay & Remaudière, 1964).

***Aphis* (*Aphis*) *craccae* L., 1758**

Material examined. Çanakkale, Lâpseki, Çardak, 23.IV.2021, apt. 6♀♀, *Vicia* sp.

***Aphis* (*Aphis*) *craccivora* Koch, 1854**

Material examined. Çanakkale, Ezine, Akköy Village, 6.V.2020, apt. 3♀♀, alt. 2♀♀, *Anthemis* sp. (Asteraceae); Bayramiç, 19.V.2020, apt. 4♀♀, *Vicia* sp.; 21.V.2020, apt. 5♀♀, alt. 2♀♀, *Diplotaxis* sp. (Brassicaceae); 20.VI.2020, apt. 3♀♀, alt. 2♀♀, *Vicia* sp.; 15.VII.2020, apt. 5♀♀, alt. 3♀♀, *T. spumosum*; Umurbey, 2.IX.2020, apt. 5♀♀, alt. 2♀♀, *Melilotus* sp. (Leguminosae); 10.X.2020, apt. 3♀♀, alt. 2♀♀, *Malus* sp. (Rosaceae); Lâpseki, 25.V.2021, apt. 4♀♀, *Trifolium purpureum* Loisel. (Leguminosae); Musaköy Village, 25.VI.2021, apt. 5♀♀, alt. 2♀♀, *Trifolium* sp.; Lâpseki, Çardak, 10.VII.2021, apt. 4♀♀, alt. 3♀♀, *T. purpureum*.

Comments. *Diplotaxis* sp. is recorded for the first time as a new host for *A.* (*A.*) *craccivora* in Turkey.

Hosts in Turkey. *Aphis* (*Aphis*) *craccivora* is a polyphagous aphid species. In Turkey, it has been recorded on numerous host plants in many plant families, especially Leguminosae and Fabaceae (Düzgüneş & Tuatay, 1956; Bodenheimer & Swirski, 1957; Tuatay & Remaudière, 1964; Çanakçıoğlu, 1966, 1975; Tuatay, 1993; Toros et al., 1996, 2002; Ölmez Bayhan et al., 2003; Görür, 2004; Aslan & Uygun, 2005; Görür et al., 2009; Güçlü et al., 2015; Öztürk & Muştu, 2017; Kök & Kasap, 2019; Başer & Tozlu, 2020).

***Aphis* (*Aphis*) *fabae* Scopoli, 1763**

Material examined. Çanakkale, Lâpseki, Subaşı Village, 2.IV.2020, apt. 4♀♀, *Galium aparine* L. (Rubiaceae); Bayramiç, Evciler Village, 10.IV.2021, apt. 5♀♀, *Chenopodium album* L. (Amaranthaceae); 17.IV.2021, apt. 5♀♀, alt. 2♀♀, *Rumex* sp.; 25.IV.2021, apt. 4♀♀, alt. 2♀♀, *Rumex* sp.; Lâpseki, 19.V.2021, apt. 3♀♀, alt. 2♀♀, *Galium* sp. (Rubiaceae); 2.VI.2021, apt. 5♀♀, alt. 2♀♀, *C. album*; 5.VI.2021, apt. 4♀♀, *Rumex* sp.; 15.VI.2021, apt. 3♀♀, alt. 2♀♀, *Rumex* sp.; 21.VI.2021, apt. 5♀♀, alt. 2♀♀, *Chenopodium* sp. (Amaranthaceae); Lâpseki, Çardak, 15.VII.2021, apt. 3♀♀, *C. album*.

***Aphis* (*Aphis*) *frangulae* Kaltenbach, 1845**

Material examined. Çanakkale, Ezine, Akköy Village, 25.IV.2020, apt. 4♀♀, *Lamium amplexicaule* L. (Lamiaceae).

***Aphis* (*Aphis*) *gossypii* Glover, 1877**

Material examined. Çanakkale, Ezine, Akköy Village, 18.IV.2020, apt. 5♀♀, *Capsella rubella* Reut. (Brassicaceae); 21.IV.2020, apt. 7♀♀, *Carduus pycnocephalus* L. (Asteraceae); 21.IV.2020, apt. 5♀♀, alt. 2♀♀, *Scandix pecten-veneris* L. (Apiaceae); 21.IV.2020, apt. 6♀♀, *Galium* sp.; 29.IV.2020, apt. 6♀♀, *C. rubella*; Central, Saraycık Village, 21.V.2020, apt. 7♀♀, *Geranium columbinum* L. (Geraniaceae); 2.VI.2020, apt. 3♀♀, alt. 2♀♀, *Malus* sp.; Bayramiç, Evciler Village, 10.VI.2020, apt. 4♀♀, alt. 2♀♀, *Malus domestica* Borkh. (Rosaceae); Umurbey, 10.X.2020, apt. 4♀♀, alt. 2♀♀, *Malus* sp.; Umurbey, 16.VI.2020, apt. 5♀♀, *Punica*

granatum L. (Lythraceae); Yapıldak Village, 5.V.2021, apt. 3♀♀, alt. 2♀♀, *Prunus armeniaca* L. (Rosaceae); Musaköy Village, 16.V.2021, apt. 7♀♀, alt. 2♀♀, *M. domestica*.

Comments. *Carduus pycnocephalus* and *S. pecten-veneris* are recorded for the first time as new hosts for *A.* (*A.*) *gossypii* in Turkey.

Hosts in Turkey. *Aphis* (*Aphis*) *gossypii* is a highly polyphagous aphid species on a very wide range of host plants. In Turkey, it has been recorded on numerous host plants (Iyriboz, 1937; Çanakçıoğlu, 1967, 1975; Tuatay, 1993; Toros et al., 1996, 2002; Ölmez Bayhan et al., 2003; Görür, 2004; Aslan & Uygun, 2005; Ayyıldız & Atlıhan, 2006; Özdemir et al., 2006; Alaserhat & Kaplan, 2017; Kuloğlu & Özder, 2017; Öztürk & Muştu, 2017; Bayındır Erol et al., 2018).

***Aphis* (*Aphis*) *lamiorum* (Börner, 1950)**

Material examined. Çanakkale, Lâpseki, Subaşı Village, 16.IV.2020, apt. 8♀♀, *Lamium purpureum* L. (Lamiaceae).

***Aphis* (*Aphis*) *pomi* De Geer, 1773**

Material examined. Çanakkale, Bayramiç, Evciler Village, 6.IX.2020, apt. 5♀♀, *M. domestica*.

***Aphis* (*Aphis*) *rumicis* L., 1758**

Material examined. Çanakkale, Lâpseki, Subaşı Village, 1.IV.2020, apt. 5♀♀, *Rumex* sp.; Kepez, 10.IV.2020, apt. 5♀♀, *Rumex* sp.; 12.IV.2020, apt. 3♀♀, *Rumex* sp.; Biga, Aziziye Village, 13.V.2020, apt. 5♀♀, alt. 2♀♀, *Rumex* sp.; Çan, 20.V.2020, apt. 4♀♀, alt. 2♀♀, *Rumex* sp.; Central, Saraycık Village, 27.V.2020, apt. 5♀♀, *Rumex* sp.; 29.V.2020, apt. 3♀♀, alt. 2♀♀, *Rumex* sp.; Bayramiç, 19.VI.2020, apt. 3♀♀, alt. 2♀♀, *Rumex* sp.; 3.VII.2020, apt. 5♀♀, *Rumex* sp.

***Aphis* (*Aphis*) *solanella* Theobald, 1914**

Material examined. Çanakkale, Bayramiç, 5.VI.2020, apt. 5♀♀, *C. pycnocephalus*; Lâpseki, 10.VI.2021, apt. 4♀♀, *Ranunculus muricatus* L. (Ranunculaceae).

Comments. *Carduus pycnocephalus* and *R. muricatus* are recorded for the first time as new hosts for *A.* (*A.*) *solanella* in Turkey.

Hosts in Turkey. *Arctium minus* (Hill) Bernh. (Asteraceae), *C. album*, *C. rubella*, *Centaurea iberica* Trevir. ex Spreng. (Asteraceae), *Cirsium* sp. (Asteraceae), *Matricaria* sp., *P. rhoeas*, *Persicaria hydropiper* (L.) Spach (Polygonaceae), *Prunus cerasifera* Ehrh. (Rosaceae), *Rumex* sp., *Solanum americanum* Mill. (Solanaceae), *Solanum dulcamara* L. (Solanaceae), *Spirea* spp. (Rosaceae), *Urtica urens* L. (Urticaceae), *Vitis vinifera* L. (Vitaceae) and *Yucca glauca* Nutt. (Asparagaceae) (Giray, 1974; Çanakçıoğlu, 1975; Düzgüneş et al., 1982; Görür et al., 2009; Güleç, 2011; Kök & Kasap, 2019; Kök, 2021).

***Aphis* (*Aphis*) *spiraecola* Patch, 1914**

Material examined. Çanakkale, Ezine, Akköy Village, 29.IV.2020, apt. 7♀♀, *Cydonia oblonga* Mill. (Rosaceae); 15.V.2020, apt. 5♀♀, alt. 2♀♀, *C. oblonga*.

***Aulacorthum* (*Aulacorthum*) *solani* (Kaltenbach, 1843)**

Material examined. Çanakkale, Ezine, Akköy Village, 29.IV.2020, apt. 4♀♀, *C. oblonga*; 6.V.2020, apt. 5♀♀, *Galium* sp.; 8.V.2020, apt. 5♀♀, *Anthemis* sp.; 21.IV.2020, apt. 4♀♀, *S. pecten-veneris*; Çan, 13.V.2020, apt. 5♀♀, *Senecio* sp. (Asteraceae); Central, Saraycık Village, 25.V.2020, apt. 5♀♀, *G. columbinum*; 29.V.2020, apt. 3♀♀, *G. columbinum*.

Comments. *Galium* sp., *S. pecten-veneris* and *G. columbinum* are recorded for the first time as new host plants for *A.* (*A.*) *solani* in Turkey.

Hosts in Turkey. *Acer* sp. (Sapindaceae), *Achillea* sp. (Asteraceae), *Ajuga orientalis* L. (Lamiaceae), *Antirrhinum* sp. (Plantaginaceae), *Ballota* sp., *Begonia cucullata* Willd. (Begoniaceae), *Tecoma capensis* (Thunb.)

Lindl. (Bignoniaceae), *Calendula* sp. (Asteraceae), *Canna indica* L. (Cannaceae), *Convolvulus arvensis* L. (Convolvulaceae), *C. oblonga*, *Dianthus anatolicus* Boiss. (Caryophyllaceae), *Dianthus barbatus* L. (Caryophyllaceae), *Digitalis* sp. (Plantaginaceae), *Helianthus annuus* L. (Asteraceae), *Hibiscus rosa-sinensis* L. (Malvaceae), *Hydrangea macrophylla* (Thunb.) Ser. (Hydrangeaceae), *Hyoscyamus* sp. (Solanaceae), *L. sativa*, *Ligustrum vulgare* L. (Oleaceae), *Ocimum* sp. (Lamiaceae), *Populus nigra* L. (Salicaceae), *Prunus laurocerasus* L. (Rosaceae), *Pyrus communis* L. (Rosaceae), *Quercus* sp. (Fagaceae), *Robinia pseudoacacia* L. (Leguminosae), *Rubus* sp. (Rosaceae), *Russelia equisetiformis* Schltdl. & Cham. (Plantaginaceae), *Solanum lycopersicum* L. (Solanaceae), *Solanum tuberosum* L. (Solanaceae), *Taraxacum scaturiginosum* G.E. Haglund (Asteraceae), *Trifolium* sp., *Tulipa gesneriana* L. (Liliaceae), *Veronica anagalloides* Guss. (Plantaginaceae), *Viburnum opulus* L. (Adoxaceae), *Yucca filamentosa* L. (Asparagaceae) (Bodenheimer & Swirski, 1957; Tuatay, 1988; Ölmez Bayhan et al., 2003; Görür, 2004, 2014; Görür et al., 2009; Güleç, 2011; Kök et al., 2016; Kuloğlu & Özder, 2017; Öztürk & Muştu, 2017; Kök & Kasap, 2019; Oner et al., 2021; Patlar et al., 2021).

***Brachycaudus* (*Appelia*) *tragopogonis* (Kaltenbach, 1843)**

Material examined. Çanakkale, Bayramiç, 6.VI.2020, apt. 5♀♀, *Tragopogon dubius* Scop. (Asteraceae); Lâpseki, 10.VI.2021, apt. 5♀♀, *Tragopogon* sp. (Asteraceae); 14.VI.2021, apt. 6♀♀, *Tragopogon* sp.

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

Material examined. Çanakkale, Lâpseki, Subaşı Village, 4.IV.2020, apt. 5♀♀, *Prunus domestica* L. (Rosaceae); 7.IV.2020, apt. 7♀♀, *P. domestica*; Lâpseki, 11.IV.2020, apt. 6♀♀, *P. domestica*; Umurbey, 15.IV.2020, apt. 4♀♀, *P. domestica*; Ezine, Akköy Village, 18.IV.2020, apt. 5♀♀, *Cynoglossum creticum* Mill. (Boraginaceae); 21.IV.2020, apt. 7♀♀, alt. 2♀♀, *C. pycnocephalus*; 8.V.2020, apt. 5♀♀, *Anthemis* sp.; 11.V.2020, apt. 6♀♀, alt. 2♀♀, *C. creticum*; 19.V.2020, apt. 5♀♀, *P. domestica*; Biga, Aziziye Village, 22.V.2020, apt. 7♀♀, alt. 2♀♀, *Senecio vulgaris* L. (Asteraceae); 24.V.2020, apt. 5♀♀, alt. 2♀♀, *Matricaria* sp.; Biga, 24.V.2020, apt. 5♀♀, alt. 2♀♀, *P. domestica*; 28.V.2020, apt. 5♀♀, alt. 2♀♀, *P. domestica*; Central, Saraycık Village, 29.V.2020, apt. 6♀♀, alt. 2♀♀, *Conyza* sp. (Asteraceae); 1.VI.2020, apt. 3♀♀, alt. 2♀♀, *Anthemis* sp.; Bayramiç, 10.VI.2020, apt. 6♀♀, alt. 2♀♀, *Echium plantagineum* L. (Boraginaceae); 18.VI.2020, apt. 4♀♀, alt. 3♀♀, *C. creticum*; 2.VII.2020, apt. 3♀♀, alt. 2♀♀, *Anthemis* sp.; 11.VII.2020, apt. 3♀♀, alt. 2♀♀, *Anthemis* sp.

Comments. *Conyza* sp. and *E. plantagineum* are recorded for the first time as new hosts for *B.* (*B.*) *helichrysi* in Turkey.

Hosts in Turkey. *Brachycaudus* (*Brachycaudus*) *helichrysi* is a polyphagous aphid species. In Turkey, it has been recorded on numerous host plants in many plant families, especially Asteraceae and Boraginaceae (Tuatay & Remaudière, 1964; Giray, 1974; Tuatay, 1988; Toros et al., 2002; Ölmez Bayhan et al., 2003; Görür, 2004, 2014; Aslan & Uygun, 2005; Görür et al., 2009; Güleç, 2011; Öztürk & Muştu, 2017; Kök & Kasap, 2019).

***Brachycaudus* (*Prunaphis*) *cardui* (L., 1758)**

Material examined. Çanakkale, Lâpseki, Subaşı Village, 11.IV.2020, apt. 5♀♀, *C. pycnocephalus*; Lâpseki, 14.V.2021, apt. 4♀♀, *C. pycnocephalus*; Central, Musaköy Village, 21.V.2021, apt. 5♀♀, *C. pycnocephalus*.

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

Material examined. Çanakkale, Bayramiç, Ahmetçeli Village, 28.V.2020, apt. 7♀♀, alt. 2♀♀, *Prunus dulcis* (Mill.) D. A. Webb (Rosaceae).

***Brevicoryne brassicae* (L., 1758)**

Material examined. Çanakkale, Biga, Aziziye Village, 10.V.2020, apt. 5♀♀, *Raphanus raphanistrum* L. (Brassicaceae).

***Corylobium avellanae* (Schrank, 1801)**

Material examined. Çanakkale, Bayramiç Evciler Village, 27.V.2021, apt. 5♀♀, alt. 2♀♀, *Corylus* sp. (Betulaceae).

***Dysaphis* (*Pomaphis*) *plantaginea* (Passerini, 1860)**

Material examined. Çanakkale, Çan, 15.IV.2020, apt. 6♀♀, *M. domestica*; Saraycık Village, 24.IV.2020, apt. 5♀♀, *M. domestica*; Bayramiç, Ahmetçeli Village, 18.IV.2020, apt. 4♀♀, *M. domestica*; Bayramiç, Evciler Village, 29.V.2020, apt. 5♀♀, *M. domestica*; Central, Musaköy Village, 16.V.2021, apt. 6♀♀, *M. domestica*; Bayramiç, 26.V.2021, apt. 5♀♀, alt. 2♀♀, *M. domestica*; Evciler Village, 2.IX.2021, apt. 5♀♀, *M. domestica*; Ayvacık, Kösedere Village, 5.IX.2021, apt. 6♀♀, *M. domestica*.

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

Material examined. Çanakkale, Umurbey, 25.VI.2020, apt. 4♀♀, alt. 5♀♀, *Phragmites australis* (Cav.) Trin. ex Steud. (Poaceae); 10.VII.2021, apt. 4♀♀, alt. 2♀♀, *P. australis*.

***Hyperomyzus* (*Hyperomyzus*) *lactucae* (L., 1758)**

Material examined. Çanakkale, Lâpseki, Subaşı Village, 4.IV.2020, apt. 5♀♀, *Sonchus oleraceus* (L.) L. (Asteraceae); Çan, 11.IV.2020, apt. 4♀♀, *Sonchus* sp. (Asteraceae); Lâpseki, Alpagut Village, 18.IV.2020, apt. 4♀♀, *Sonchus* sp.; 24.IV.2020, apt. 5♀♀, alt. 2♀♀, *Sonchus* sp.; Central, Saraycık Village, 27.IV.2020, apt. 4♀♀, alt. 2♀♀, *Sonchus* sp.; 4.V.2020, apt. 4♀♀, alt. 2♀♀, *Sonchus* sp.; 17.V.2020, apt. 4♀♀, alt. 2♀♀, *Sonchus* sp.; 28.V.2020, apt. 5♀♀, alt. 3♀♀, *Sonchus* sp.; 29.V.2020, apt. 5♀♀, *G. columbinum*; Bayramiç, 3.VI.2020, apt. 5♀♀, alt. 2♀♀, *Sonchus* sp.; Central, Musaköy Village, 11.VI.2021, apt. 5♀♀, alt. 2♀♀, *Sonchus* sp.; Lâpseki, Çardak, 16.VI.2021, apt. 4♀♀, *Sonchus* sp.

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

Material examined. Çanakkale, Lâpseki, Subaşı Village, 11.IV.2020, apt. 4♀♀, *Moricandia arvensis* (L.) DC. (Brassicaceae); Ezine, Akköy Village, 23.IV.2020, apt. 7♀♀, alt. 2♀♀, *C. rubella*; 29.IV.2020, apt. 5♀♀, *C. rubella*; 30.IV.2020, apt. 5♀♀, alt. 3♀♀, *Raphanus* sp. (Brassicaceae); 3.V.2020, apt. 4♀♀, *Brassica* sp. (Brassicaceae); Biga, Aziziye Village, 15.V.2020, apt. 6♀♀, alt. 3♀♀, *C. rubella*; Bayramiç, 29.V.2020, apt. 7♀♀, alt. 2♀♀, *Bunias erucago* L. (Brassicaceae).

***Macrosiphum* (*Macrosiphum*) *euphorbiae* (Thomas, 1878)**

Material examined. Çanakkale, Biga, 20.V.2020, apt. 5♀♀, *P. communis*; Biga, Aziziye Village, 15.V.2020, apt. 5♀♀, alt. 2♀♀, *C. rubella*; 22.V.2020, apt. 5♀♀, alt. 2♀♀, *S. vulgaris*; Bayramiç, 10.VI.2020, apt. 6♀♀, *E. plantagineum*.

***Myzus* (*Myzus*) *cerasi* (Fabricius, 1775)**

Material examined. Çanakkale, Biga, 18.V.2020, apt. 7♀♀, *Prunus avium* (L.) L. (Rosaceae); Bayramiç, Evciler Village, 6.VI.2020, apt. 5♀♀, alt. 3♀♀, *P. avium*; Umurbey, 25.IV.2021, apt. 4♀♀, *P. avium*; Central, Musaköy Village, 29.IV.2021, apt. 5♀♀, *P. avium*; Bayramiç, Evciler Village, 20.V.2021, apt. 6♀♀, *P. avium*; 2.VI.2021, apt. 4♀♀, alt. 3♀♀, *P. avium*; 8.VI.2021, apt. 3♀♀, alt. 3♀♀, *P. avium*.

***Myzus* (*Myzus*) *lythri* (Schrank, 1801)**

Material examined. Çanakkale, Ezine, Akköy Village, 10.V.2020, apt. 5♀♀, alt. 2♀♀, *P. armeniaca*.

***Myzus* (*Myzus*) *varians* Davidson, 1912**

Material examined. Çanakkale, Çan, 27.V.2020, apt. 4♀♀, alt. 3♀♀, *P. persica*.

***Myzus* (*Nectarosiphon*) *persicae* (Sulzer, 1776)**

Material examined. Çanakkale, Lâpseki, Subaşı Village, 14.IV.2020, apt. 7♀♀, *Euphorbia helioscopia* L. (Euphorbiaceae); Ezine, Akköy Village, 21.IV.2020, apt. 6♀♀, alt. 2♀♀, *S. pecten-veneris*; Saraycık Village, 14.V.2020, apt. 5♀♀, *P. persica*; 16.V.2020, apt. 3♀♀, *P. persica*; Bayramiç, 17.V.2020, apt. 5♀♀, alt. 2♀♀, *P. persica*; 21.V.2020, apt. 4♀♀, alt. 2♀♀, *Diplotaxis* sp.; 24.V.2020, apt. 4♀♀, alt. 2♀♀, *P. persica*; Bayramiç, Evciler Village, 27.V.2020, apt. 5♀♀, alt. 2♀♀, *P. persica*; Bayramiç, 29.V.2020, apt. 5♀♀, alt. 3♀♀, *B. erucago*; 10.VI.2020, apt. 5♀♀, alt. 2♀♀, *E. plantagineum*; 19.VI.2020, apt. 4♀♀, alt. 3♀♀, *Rumex* sp.; 2.VII.2020, apt.

5♀♀, alt. 2♀♀, *Anthemis* sp.; Umurbey, 2.V.2021, apt. 5♀♀, alt. 2♀♀, *P. persica*; Central, Yapıldak Village, 5.V.2021, apt. 3♀♀, alt. 2♀♀, *P. armeniaca*; Lâpseki, 26.V.2021, apt. 6♀♀, alt. 3♀♀, *Prunus avium*.

***Myzus* sp.**

Material examined. Çanakkale, Lâpseki, Subaşı Village, 15.IV.2020, alt. 6♀♀, Poaceae.

***Ovatus* (*Ovatus*) *insitus* (Walker, 1849)**

Material examined. Çanakkale, Lâpseki, 21.IV.2020, apt. 5♀♀, *C. oblonga*; Umurbey, 25.IV.2020, apt. 6♀♀, *C. oblonga*; Ezine, Akköy Village, 29.IV.2020, apt. 4♀♀, *C. oblonga*; Çanakkale, Umurbey, 10.X.2020, apt. 5♀♀, *Malus* sp.

***Phorodon* (*Phorodon*) *humuli* (Schrank, 1801)**

Material examined. Çanakkale, Ezine, Akköy Village, 19.V.2020, apt. 5♀♀, *P. domestica*; Çan, 3.VI.2021, apt. 6♀♀, *Prunus* sp. (Rosaceae).

***Semiaphis dauci* (Fabricius, 1775)**

Material examined. Çanakkale, Musaköy Village, 11.VI.2021, apt. 4♀♀, alt. 3♀♀, *Daucus* sp. (Apiaceae).

***Sitobion* (*Sitobion*) *avenae* (Fabricius, 1775)**

Material examined. Çanakkale, Musaköy Village, 14.VI.2021, apt. 5♀♀, Poaceae.

***Uroleucon* (*Uroleucon*) *sonchi* (L., 1767)**

Material examined. Çanakkale, Lâpseki, Alpagut Village, 18.IV.2020, apt. 5♀♀, *Sonchus* sp.; Central, Saraycık Village, 27.IV.2020, apt. 4♀♀, *Sonchus* sp.; Central, Saraycık Village, 4.V.2020, apt. 5♀♀, *Sonchus* sp.; 12.V.2020, apt. 5♀♀, *Sonchus* sp.; Bayramiç, 3.VI.2020, apt. 3♀♀, alt. 2♀♀, *Sonchus* sp.; Lâpseki, Çardak, 16.VI.2021, apt. 5♀♀, alt. 2♀♀, *Sonchus* sp.; 19.VI.2021, apt. 6♀♀, alt. 3♀♀, *Sonchus* sp.; 24.VI.2021, apt. 5♀♀, alt. 2♀♀, *Sonchus* sp.

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

Material examined. Çanakkale, Bayramiç, 23.V.2020, apt. 4♀♀, alt. 2♀♀, *C. pycnocephalus*; Lâpseki, 4.VI.2021, apt. 6♀♀, alt. 2♀♀, *Carduus* sp. (Asteraceae).

Subfamily Calaphidinae Oestlund, 1919

***Therioaphis* (*Pterocallidium*) *trifolii* (Monell, 1882)**

Material examined. Çanakkale, Bayramiç, 15.VII.2020, apt. 6♀♀, *T. spumosum*.

Ninety-two aphid-host plant interactions, including many new host plant records for some aphid species in Turkey, were determined from the orchards. Of these hosts, *P. rhoeas* for *A.* (*A.*) *ilka*, *Ballota* sp. for *A.* (*A.*) *ballotae*, *Diplotaxis* sp. for *A.* (*A.*) *craccivora*, *C. pycnocephalus* and *S. pecten-veneris* for *A.* (*A.*) *gossypii*, *C. pycnocephalus* and *R. muricatus* for *A.* (*A.*) *solanella*, *Galium* sp., *S. pecten-veneris* and *G. columbinum* for *A.* (*A.*) *solani*, *Conyza* sp. and *E. plantagineum* for *B.* (*B.*) *helichrysi* were recorded for the first time as new hosts of aphids in Turkey.

It was revealed that the identified aphid species have varying degrees of diversity on both the fruit trees that are primary hosts and the herbaceous plants that are secondary hosts. Fifteen aphid species were identified on fruit trees of which, *A.* (*A.*) *gossypi* with four fruit trees hosts and *M.* (*N.*) *persicae* with three fruit trees hosts were recorded as the most common aphids in the orchards. However, *B.* (*B.*) *helichrysi*, known as the leaf-curling plum aphid, and *D.* (*P.*) *plantaginea*, forming yellowish crumpled-leaf galls on apple, were determined only on *P. domestica* and *M. domestica*, respectively (Figure 2).

As for herbaceous plants that are secondary hosts, 29 aphid species were identified on these herbaceous plants in and around the orchards. Similarly, *A.* (*A.*) *craccivora*, *M.* (*N.*) *persicae* and *B.* (*B.*) *helichrysi* was found on seven herbaceous host species and *A.* (*A.*) *fabae* and *A.* (*A.*) *gossypii* on five herbaceous host species (Figure 3).

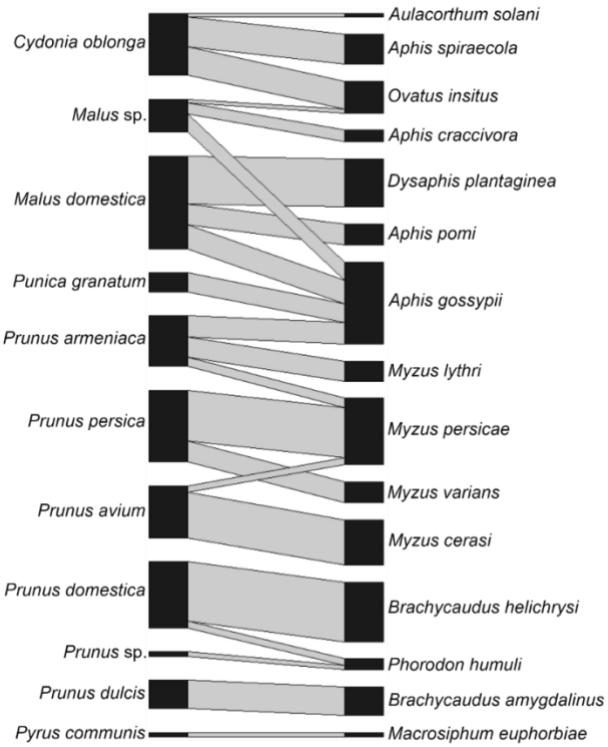

Figure 2. Quantitative bipartite network of interactions between host trees (left) and aphids (right) in orchards in Çanakkale Province, Turkey. Black bars show abundance of the plants and aphids, and gray bars shows their interactions (width of the bars indicates the intensity of interactions).

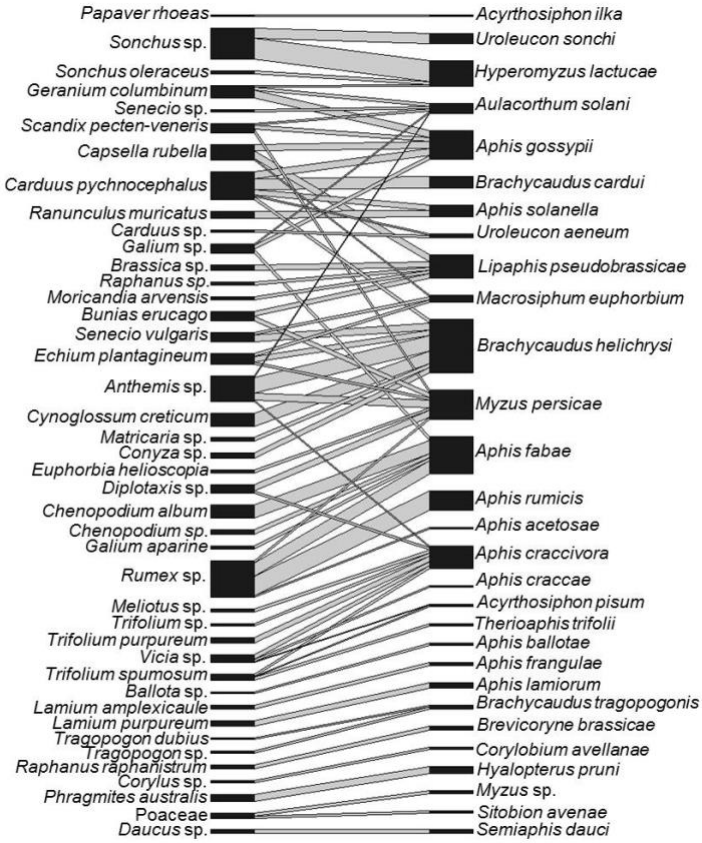

Figure 3. Quantitative bipartite network of interactions between host herbaceous plants (left) and aphids (right) in orchards in Çanakkale Province, Turkey. Black bars show abundance of the plants and aphids, and gray bars shows their interactions (width of the bars indicates the intensity of interactions).

Studies conducted in fruit fields in different regions of Turkey revealed that aphid pests have a significant diversity and density on both fruit trees and herbaceous host plants. Of these studies, Aslan & Karaca (2005) showed that *A.* (*A.*) *pomi*, *Dysaphis* (*Dysaphis*) *devecta* (Walker, 1849) (Hemiptera: Aphididae) and *D.* (*P.*) *plantaginea* are the most common aphids on fruit trees in the Isparta region because of the high amount of apple production. Aslan (2015) reported that *Hyalopterus amygdali* (Blanchard, 1840) (Hemiptera: Aphididae) and *M.* (*N.*) *persicae* are the most common species among the twelve identified species on fruit trees in Burdur. Also, other similar studies showed that many aphid species such as *A.* (*A.*) *craccivora*, *A.* (*A.*) *pomi*, *D.* (*D.*) *devecta*, *D.* (*P.*) *plantaginea*, *H. pruni* and *Rhopalosiphum oxyacanthae* (Schrank, 1801) (Hemiptera: Aphididae) are mostly distributed on trees in orchards in Antalya, Erzurum, Iğdır and Niğde Provinces of Turkey (Görür, 2004; Daşcı & Güçlü, 2008; Narmanlıoğlu & Güçlü, 2008; Saraç et al., 2015).

Determination of the primary and secondary hosts of aphids with heteroecious life cycles will make significant contributions to a clearer understanding of the biology and control strategies of these aphids. Unlike the studies mentioned above, we have revealed important data on herbaceous plants that are secondary hosts as well as fruit tree that are primary hosts of aphids in orchards. A study similar to ours conducted in orchards in Spain showed that 181 herbaceous host plants can serve as reservoirs for 27 aphid pests on fruit trees. In this study, the most common aphids, *M.* (*N.*) *persicae* and *B*. (*B.*) *helichrysi*, were reported on 30 and 13 secondary herbaceous host plants, respectively, in orchards (Tizado & Nunez Perez, 1998). Similarly, in our study, *M.* (*N.*) *persicae* and *B.* (*B.*) *helichrysi*, pests of fruit species, were found on seven herbaceous host species as secondary hosts the most in orchards (Figure 3). Ahmed et al. (2007) reported that *A.* (*A.*) *gossypii* is the most common aphid species on both fruit trees and herbaceous host plants in the areas of economic crops in Egypt. In contrast, the results of our study showed that *B.* (*B.*) *helichrysi* and *M.* (*N.*) *persicae* are common on secondary host plants, while *A.* (*A.*) *gossypii*, a polyphagous species, is one of the most common aphids on both fruit trees and secondary hosts in orchards.

As is clear from our study, and other studies conducted in orchards, aphid diversity is remarkable rich on fruit trees that are the primary hosts, as well as herbaceous plants that are the secondary hosts in orchards. Especially, considering that fruit pest aphids migrate to secondary hosts in summer, it is clear that revealing both primary-secondary hosts of pest aphids and their migration season in more detail will make significant contributions to the integrated pest management of aphids. In the context of these results, it is believed that the data presented in this study on the primary and secondary hosts and their interactions with aphids in orchards in northwestern Turkey will support both a better understanding of the biology of fruit aphids and the development of new strategies for the control of aphid pests.

Acknowledgments

This study was supported by Çanakkale Onsekiz Mart University, the Scientific Research Coordination Unit (Project number: FBA-2019-3082).

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Türk. entomol. derg., 2022, 46 (1): 63-73
DOI: http: //dx.doi.org/10.16970/entoted.1027969

ISSN 1010-6960
E-ISSN 2536-491X

Original article (Orijinal araştırma)

Interaction of *Meloidogyne incognita* (Kofoid & White, 1919) (Nemata: Meloidogynidae) and *Fusarium oxysporum* f. sp. *radicis-lycopersici* Jarvis & Shoemaker in tomato F1 hybrids with differing levels of resistance to these pathogens

Meloidogyne incognita (Kofoid & White, 1919) (Nemata: Meloidogynidae) ve *Fusarium oxysporum* f. sp. *radicis-lycopersici* Jarvis & Shoemaker'ya karşı farklı seviyelerde dayanıklılık sağlayan domates hibritlerinde bu patojenlerin etkileşimi

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Abstract

The interaction of *Meloidogyne incognita* (Kofoid & White, 1919) (Rhabditida: Meloidogynidae) and *Fusarium oxysporum* f. sp. *radicis-lycopersici* Jarvis & Shoemaker (FORL) on nematode reproduction and wilt severity was investigated in tomato hybrids in this study. The study included with five combinations of individual, simultaneous and sequential inoculations of *M. incognita* and FORL to tomato F1 hybrids Adel, Alberty, Armstrong, Body, Gülizar and Kaplan in January-May 2021 under controlled conditions. The experiment was completed after 60 days. Simultaneous inoculation increased *M. incognita* galls and egg masses in Adel, Armstrong, Body, and Gülizar. The highest gall and egg mass numbers occurred with FORL inoculation 10 days after *M. incognita* inoculation (N+10 FORL) in Alberty and Kaplan. The highest disease incidence occurred in all tomato hybrids at the application of N+10 FORL and was followed by Gülizar, Kaplan, Body, Alberty, Armstrong and Adel. *Meloidogyne incognita* showed high reproductive rates in Alberty and Body, and FORL resistance was overcome with treatment N+10 FORL. *Meloidogyne incognita* was unable to reproduce in Adel and Armstrong and thus no disease was seen. The results indicated that the development of root-knot nematodes is a significant factor affecting the durability of FORL resistance.

Keywords: FORL, interaction, resistance, root-knot nematode, tomato

Öz

Bu çalışmada, *Meloidogyne incognita* (Kofoid & White, 1919) (Rhabditida: Meloidogynidae) ve *Fusarium oxysporum* f. sp. *radicis-lycopersici* Jarvis & Shoemaker (FORL) etkileşiminin, nematod üremesi ve solgunluk şiddeti üzerine etkisi hibrit domates çeşitlerinde araştırılmıştır. Çalışma, kontrollü koşullar altında 2021 yılı Ocak-Mayıs ayları arasında Adel, Alberty, Armstrong, Body, Gülizar ve Kaplan F1 domates hibrit çeşitlerinde, *M. incognita* ve FORL'nin bireysel, eş zamanlı ve sıralı inokulasyonlarından oluşan beş kombinasyonu içermektedir. Deneme 60 gün sonra sonlandırılmıştır. Eş zamanlı inokulasyon Adel, Armstrong, Body ve Gülizar domates çeşitlerinde *M. incognita* gal ve yumurta paketi sayısını artırmıştır. Alberty ve Kaplan domates çeşitlerinde ise en yüksek gal ve yumurta paketi sayısı *M. incognita* inokulasyonundan 10 gün sonra FORL inokulasyonunda (N+10FORL) tespit edilmiştir. En yüksek hastalık şiddeti tüm domates hibritlerinde N+10FORL uygulamasında meydana gelmiş ve Gülizar, Kaplan, Body, Alberty, Armstrong ve Adel şeklinde izlemiştir. *Meloidogyne incognita* Alberty ve Body çeşitlerinde yüksek üreme göstermiş ve N+10FORL uygulaması ile FORL dayanımı kırılmıştır. *Meloidogyne incognita* Adel ve Armstrong domates hibritlerinde üreyememiş ve bu nedenle hastalık görülmemiştir. Sonuçlar, kök-ur nematodlarının gelişiminin FORL dayanımının sürekliliğini etkileyen önemli bir faktör olduğunu göstermiştir.

Anahtar sözcükler: FORL, interaksiyon, dayanıklılık, kök ur nematodu, domates

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Received (Alınış): 25.11.2021 Accepted (Kabul ediliş): 22.02.2022 Published Online (Çevrimiçi Yayın Tarihi): 02.03.2022

Introduction

Root-knot nematodes (RKNs), one of the most important plant parasitic nematode groups, cause heavy economic losses worldwide (Bird et al., 2009). The galls formed by RKNs limit the intake of water and nutrients from the soil. In high population densities, it causes the plants to dry out completely. Also, wounds inflicted on the roots by RKNs facilitate the entry of soilborne fungal and bacterial pathogens (Back et al., 2002). *Meloidogyne arenaria* (Neal, 1889), *Meloidogyne incognita* (Kofoid & White, 1919), and *Meloidogyne javanica* (Treub, 1885) (Rhabditida: Meloidogynidae) have been identified as most common and economically important RKN species in vegetable growing areas of Turkey (Elekcioglu et al., 1994; Kaşkavalcı & Öncüer, 1999; Devran & Söğüt, 2009; Özarslandan & Elekcioğlu, 2010; Cetintaş & Cakmak, 2016; Özarslandan, 2016; Uysal et al., 2017; Gürkan et al., 2019). *Meloidogyne incognita* is the most common RKN species and can infest almost all plants and causes significant economic damage (Sasser & Freckman, 1987). The most common method used for controlling RKNs is genetic resistance (Gilbert & McGuire, 1956; Jacquet et al., 2005; Lobna et al., 2016). *Mi* gene provides high resistance to *M. arenaria, M. incognita* and *M. javanica* (Roberts & Thomason, 1986; Verdejo-Lucas et al., 2009; Devran & Söğüt, 2011). However, *Mi* virulent *M. arenaria*, *M. incognita* and *M. javanica* populations that overcome this resistance have also been reported in many countries (Ornat et al., 2001; Tzortzakakis et al., 2005; Devran & Söğüt, 2010; Aydınlı & Mennan, 2019).

Fusarium oxysporum Schltdl. is one of the most widespread soilborne pathogens of tomato plants and has two forms: *Fusarium oxysporum* f. sp. *lycopersici* W.C. Snyder & H.N. Hansen (FOL) and *F. oxysporum* f. sp. *radicis-lycopersici* Jarvis & Shoemaker (FORL). While FOL causes Fusarium wilt, FORL causes Fusarium root and root rot (Attitalla et al., 2004). FORL, which causes tomato root rot, is an important pathogen species and causes more than 60% yield loss in open field and greenhouse tomato cultivation (Ozbay & Newman, 2004; Hibar et al., 2007; Manzo et al., 2016). FORL was first identified by Can et al. (2004) in 2004 in Turkey and since then it has started to spread in tomato growing areas (Erol & Tunalı, 2007; Yücel et al., 2008). Although various methods have been used to control this pathogen, the use of resistant hybrids is the most preferred and economic control method (Szczechura et al., 2013). The single genetic locus *Frl*, the gene expressing resistance to FORL in the tomato plant, was integrated into *Solanum lycopersicum* L. (Solanaecae) cultured from the wild *Solanum peruvianum* L. (Laterrot & Moretti, 1991; Fazio et al., 1999) and has been used in commercial production (Devran et al., 2018).

In previous studies on nematode and fungal pathogen interactions, early infestion of RKN was associated with increased severity of the disease observed (Lobna et al., 2016, 2017). The interaction between the RKN and *F. oxysporum* has been observed in plants such as bananas, beans, cotton, gerbera and grapes (Harris & Ferris, 1991; France & Abawi, 1994; Jonathan & Gajendran, 1998; Jeffers & Roberts, 2003; Meena et al., 2015). Simultaneous infection of both RKN and FORL causes greater and enhanced damage to the host plant compared to the pathogens alone (El-Sherif & Elwakil, 1991; McGawely, 2001; Hajji-Hedfi et al., 2018). It has been reported that the *M. javanica* and FORL interaction in FORL sensitive and resistant species can affect tomato growth and disease severity in different ways (Hajji-Hedfi et al., 2018). Since RKN can form disease complexes with *Fusarium* spp., the use of resistant hybrids is not suitable for the Fusarium wilt disease control. In general, *Meloidogyne* spp. overcome wilt resistance in the host plant (Morrell & Bloom, 1981; Fattah & Webster, 1983; Lobna et al., 2016). Çolak-Ates et al. (2018) found that AL-4, AL-9 and AL-21 tomato genotypes with FORL resistance, lost their resistance to FORL in simultaneous and sequential inoculations with *M. incognita*.

There are only a few studies on the interaction of RKNs and FORL in tomato plants and most of these studies have been conducted with *M. javanica*. A few studies have investigated the *M. incognita* interaction, which is the most widespread species in the world and in Turkey. In this study, the aim was to evaluate the interaction of *M. incognita* and FORL on reproduction of *M. incognita* and severity of Fusarium wilt diseases using resistant and susceptible tomato hybrids.

Materials and Method

Materials

The FORL isolate used in this study was obtained from a tomato plant in the Serik District of Antalya Province, and its morphologically identification was made in accordance with Gerlach et al. (1982) and Davis & Raid (2002). *Meloidogyne incognita* DR17 isolate, obtained in a previous study (N: 37°47'44" N, 30°30'47" E), was mass produced on tomato F1 hybrid Tueza under controlled climate conditions (24 ± 1°C, 60 ± 5% RH) (Uysal et al., 2017). In the study, six tomato F1 hybrids (Adel, Alberty, Armstrong, Body, Gülizar and Kaplan) that have resistance to RKNs and FORL were used (Table 1).

Table 1. Resistance of tomato hybrids to *Meloidogyne incognita* and *Fusarium oxysporum* f. sp. *radicis-lycopersici*

Tomato F1 hybrids	Nematode	Fungal resistance*
Adel	HR	HR
Alberty	X	HR
Armstrong	IR	HR
Body	IR	IR
Gülizar	X	X
Kaplan	IR	X

*HR, high/standard resistance; IR, moderate/intermediate resistance; and X, unknown

Method

Preparation of fungal inoculum

FORL isolate was incubated in sterile Petri dishes at 25°C for 7 days on potato dextrose agar medium. Then, 1 cm^2 pieces of the fungus colonies were taken and five pieces were added to 250 ml sterile flasks with 50 ml potato dextrose broth. The flasks were incubated in the dark at 25°C for 7 days in the laboratory and shaken manually on a daily basis. Seven days later, the culture filtrate was passed through two layers of filter paper (Whatman No. 1) to remove fungal spores and mycelium. Using a hemacytometer in the light microscope, each plant was adjusted to 3×10^6 ml suspension and kept at 4°C until the experiment was set up (Lobna et al., 2016).

Preparation of nematode inoculum

Mass production of *M. incognita* was done on Tueza with 20 replicates under climate room conditions (24 ± 1°C, 60 ± 5% RH). Tomato seedlings were transplanted into pots containing sterilized soil (68% sand, 21% silt and 11% clay) and about 1000 second-stage juveniles (J2s) inoculated into the soil. The tomato roots were removed 8 weeks after inoculation and were washed in tap water. Then the egg masses were removed from the roots under a stereomicroscope. Collected egg masses were incubated in water at 25 ± 2ºC for 3 days in a Petri dish containing a sterile sieve (3 cm diameter). The J2s hatched after 3 days were counted under the light microscope and placed in 1 ml tubes, adjusted to 1000 J2s to be used in the experiment (Lobna et al., 2017).

Fusarium oxysporum f. sp. *radicis-lycopersici* and *Meloidogyne incognita* interaction in tomato hybrids with different levels of resistance to these pathogens

The study was conducted between January and May 2021. There were five treatment combinations consisting of individual, simultaneous and sequential inoculations of *M. incognita* and FORL on tomato hybrids with different resistance levels to RKNs and FORL. Treatments were consisted of (1) *M. incognita* only (N); (2) FORL only; (3) Simultaneous inoculation of *M. incognita* and FORL (N+FORL); (4) first inoculation of FORL and 10 days later inoculation of *M. incognita* (FORL+10N); and (5) first inoculation of *M. incognita* and 10 days later FORL (N+10FORL). The study was conducted in a climate room under controlled conditions (24 ± 1°C, 60 ± 5% RH) in plastic pots and in a randomized block design with five

replicates. Three-week-old tomato seedlings were transplanted into 14-cm plastic pots containing approximately 1500 g of sterile soil (68% sand, 21% silt and 11% clay). As initial inoculum density, 1000 J2s/1 ml *M. incognita* and 3×10^6/10 ml FORL/seedling were used. Inoculations were made according to the treatment priority. The nematode inoculum was evenly distributed by a pipette into three small 2-3-cm holes drilled in the soil around the seedling stem and deep enough to contact the roots. Fungi inoculation was poured into these holes opened on the soil surface of the pots using a graduated cylinder (Lobna et al., 2016, 2017).

The study was completed 60 days after the fungi and nematode inoculation of the plants. At the end of the treatment, the tomato roots were washed carefully under tap water and then exposed to 0.25% triptan blue for 3 min (Sharma & Ashokkumar, 1991). Then, the gall formations and egg masses were counted under a stereomicroscope. The J2 density in soil (using a 100 g sample) was calculated using the Baermann funnel technique (Hooper, 1986). The severity of disease caused by FORL was scored on the 0 to 4 scale of Chandler & Santelman (1968): 0, no damage to the seedling (resistant); 1, discoloration and small lesions at the junction of the seedling with the soil surface; 2, larger lesions bending the stem (sensitive); 3, large lesions surrounding the stem, resulting in a concave appearance (vulnerable); and 4, dead plant due to fungal damage (very vulnerable) (Erol & Tunalı, 2007).

Statistical analysis

SPSS (version 20.0) program was used for statistical analysis of the data and one-way analysis of variance was performed to test the differences between the means. Tukey's test was used to determine the means of different groups when variances were homogeneous ($P \leq 0.05$).

Results and Discussion

The results showed that differences in treatment time with *M. incognita* and FORL in tomato hybrids were affected the number of galls and egg masses. The lowest gall formation was detected in the Armstrong with treatment FORL+10N (5 per pot) and 22 per pot with treatment N+10 FORL. Although the number of galls was higher with treatment N+FORL in Armstrong than with treatment N, there was no significant difference between groups (Table 2). There was no significant difference between treatments N (117 per pot), N+FORL (126 per pot) and N+10FORL (143 per pot) in terms of gall number in Alberty. The lowest gall number in Alberty was found with treatment FORL+10N (69 per pot). Although the number of galls with treatment N+FORL (42 per pot) in Body, which is resistant to RKNs and FORL, was higher than with treatment N (36 per pot), there was no significant difference. However, gall number with treatments FORL+10N (18 per pot) and N+10FORL (21 per pot) in Body were lower than these treatments. Although the number of galls with treatment N+10FORL (41 per pot) in Kaplan, which is tolerant to RKN, was higher with treatment N (34 per pot), there was no significant difference between them. The lowest gall number was in Kaplan with treatment FORL+10N (18 per pot). Adel, which is resistant to RKNs and FORL, had the highest gall with treatments N (26 per pot) and N+FORL (32 per pot) and the lowest with treatments FORL+10N (7 per pot) and N+10FORL (10 per pot). The highest gall number in the Gülizar, which is susceptible to RKNs and FORL, was found with treatment N+FORL (166 per pot) and the lowest with treatment FORL+10N (86 per pot). Also, the lowest gall number was determined with treatment FORL+10N in all tomato hybrids. With *M. incognita* infection most gall formation occurred in susceptible Gülizar and Alberty. The gall numbers of *M. incognita* were similar in Adel, Armstrong, Body and Kaplan with RKN resistance.

Table 2. Effect of sequentially and concomitantly inoculation of *Meloidogyne incognita* (N) and *Fusarium oxysporum* f. sp. *radicis-lycopersici* (FORL) on number of galls per pot (mean ± SE[1]) of resistant and susceptible tomato hybrids

Treatment[2]	Adel		Alberty		Armstrong		Body		Gülizar		Kaplan	
N	26 ± 1.4	a B	117 ± 10.5	a A	29 ± 3.0	ab B	36 ± 5.1	ab B	137 ± 4.9	b A	34 ± 2.6	ab B
N+FORL	32 ± 2.4	a C	126 ± 6.7	a B	35 ± 2.8	a C	42 ± 3.1	a C	166 ± 4.5	a A	27 ± 3.5	b C
FORL+10N	7 ± 1.2	b C	68 ± 7.7	b A	5 ± 1.2	c BC	18 ± 3.2	c BC	86 ± 3.7	c A	18± 3.2	c B
N+10FORL	10 ± 1.0	b C	143 ± 13.0	a A	22 ± 4.6	b BC	21 ± 4.6	bc BC	140 ± 6.0	b A	41 ± 4.1	a B

[1] Means followed by the same lowercase letter within columns or uppercase letter within rows are not significantly different ($p \leq 0.05$).
[2] Treatments: N, *M. incognita* only; N+FORL, simultaneous inoculation of *M. incognita* and FORL; FORL+10N, first inoculation with FORL and 10 days later inoculation with *M. incognita;* and N+10FORL, first inoculation with *M. incognita* and 10 days later with FORL.

The lowest number of egg masses in Armstrong was with treatment FORL+10N (6 per pot) and the highest with treatments N+FORL (40 per pot) and N (32 per pot) (Table 3). The number of egg masses with treatment FORL+10N (76 per pot) in Alberty was lower than with treatments N (129 per pot), N+FORL (136 per pot) and N+10FORL (152 per pot) (Table 3). There was no significant difference between treatments N (38 per pot), FORL+10N (21 per pot) and N+10FORL (25 per pot) in egg mass numbers in Body. In Kaplan, significant difference was found only between treatments N+10FORL (43 per pot) and FORL+10N (28 per pot) in egg mass numbers. The highest number of egg masses in Adel was with treatments N (30 per pot) and N+FORL (34 per pot) and the lowest with treatments FORL+10N (8 per pot) and N+10FORL (12 per pot). The highest number of egg mass in Gülizar was with treatment N+FORL (182 per pot) and the lowest with treatment FORL+10N (91 per pot). The number of egg masses parallel to the gall numbers in Gülizar and Alberty, which were most susceptible to RKN. The number of egg masses in Adel, Armstrong, Body and Kaplan, which have resistance and tolerance to RKN, were similar with treatment N.

Table 3. Effect of sequentially and concomitantly inoculation by *Meloidogyne incognita* (N) and *Fusarium oxysporum* f. sp. *radicis-lycopersici* (FORL) on number of egg masses per pot (mean ± SE[1]) of resistant and susceptible tomato hybrids

Treatment[2]	Adel		Alberty		Armstrong		Body		Gülizar		Kaplan	
N	30 ± 1.6	a B	129 ± 10.6	a A	32 ± 2.8	ab B	38 ± 5.7	ab B	150 ± 4.5	b A	37 ± 2.8	ab B
N+FORL	34 ± 3.0	a C	136 ± 6.4	a B	40 ± 2.2	a C	49 ± 4.5	a C	182 ± 4.8	a A	36 ± 3.5	ab C
FORL+10N	8 ± 1.4	b C	76 ± 8.4	b A	6 ± 1.6	c C	21 ± 3.1	b BC	91 ± 3.3	c A	28 ± 2.3	b B
N+10FORL	12 ± 1.8	b C	152 ± 11.7	a A	25 ± 5.7	b BC	25 ± 5.7	b BC	144 ± 5.9	b A	43 ± 3.5	a B

[1] Means followed by the same lowercase letter within columns or uppercase letter within rows are not significantly different ($p \leq 0.05$).
[2] Treatments: N, *M. incognita* only; N+FORL, simultaneous inoculation of *M. incognita* and FORL; FORL+10N, first inoculation with FORL and 10 days later inoculation with *M. incognita;* and N+10FORL, first inoculation with *M. incognita* and 10 days later with FORL.

The lowest J2 density with Armstrong was with treatment FORL+10N (31 per kg soil) and the highest with treatment N+FORL (95 per kg soil) (Table 4). The highest J2 density with Alberty was with treatment N+10FORL (210 per kg soil) and the lowest with treatment FORL+10N (130 per kg soil). The J2 density with Body was 23 per kg soil with treatment FORL+10N, lower than with treatment N+10FORL (48 per kg soil). There was no significant difference in J2 density treatments N (70 per kg soil) and N+FORL (71 per kg soil) with Body. The J2 density was highest with treatments N+10FORL (116 per kg soil) and N (100 per kg soil), and lowest with treatment FORL+10N (51 per kg soil) in the Kaplan. The J2 density with Adel with treatment FORL+10N was 20 per kg soil, lower than with treatment N+10FORL (50 per kg soil). There was no significant difference between treatments N (68 per kg soil) and N+FORL (76 per kg soil) in J2 density in Adel. The highest J2 density in the soil was with Gülizar, which is the most susceptible to RKN, followed by the RKN susceptible Alberty. In Gülizar, there was no significant difference between J2 densities with

treatments N (368 per kg soil), N+FORL (383 per kg soil) and N+10FORL (354 per kg soil) but these treatments gave densities than treatment FORL+10N (304 per kg soil). In the Adel, Armstrong, Body and Kaplan, with RKN resistance, the J2 densities were similar with treatment N.

Table 4. Effect of sequentially and concomitantly inoculation of *Meloidogyne incognita* (N) and *Fusarium oxysporum* f. sp. *radicis-lycopersici* (FORL) on second-stage juvenile density in soil (per kg, mean ± SE[1]) of resistant and susceptible tomato hybrids

Treatment[2]	Adel		Alberty		Armstrong		Body		Gülizar		Kaplan	
N	68 ± 6.6	a C	162 ± 9.0	bc B	74 ± 7.5	ab C	70 ± 6.9	a C	368 ± 5.9	ab A	100 ± 6.9	ab C
N+FORL	76 ± 4.3	a C	180 ± 7.2	ab B	95 ± 3.8	a C	71 ± 5.5	a C	383 ± 10.9	a A	81 ± 6.2	bc C
FORL+10N	20 ± 3.8	c D	130 ± 9.7	c B	31 ± 5.4	c CD	23 ± 4.2	c C	304 ± 22.9	b A	51 ± 9.0	c BC
N+10FORL	50 ± 3.7	b C	210 ± 13.9	a B	52 ± 6.1	bc C	48 ± 4.9	b C	354 ± 20.4	ab A	116 ± 12.0	a BC

[1] Means followed by the same lowercase letter within columns or uppercase letter within rows are not significantly different ($p \leq 0.05$).
[2] Treatments: N, *M. incognita* only; N+FORL, simultaneous inoculation of *M. incognita* and FORL; FORL+10N, first inoculation with FORL and 10 days later inoculation with *M. incognita;* and N+10FORL, first inoculation with *M. incognita* and 10 days later with FORL.

The disease severity in Armstrong, which is resistant to RKNs and FORL, was scored a 1.0 with treatments N+FORL and N+10FORL, which was higher than with treatments FORL (score 0.4) and FORL+10N (score 0.2) (Table 5). This shows that *M. incognita* contributed to the increase in disease severity in the Armstrong, however, the FORL resistance was not overcome. With Alberty which is only resistant to FORL only, Body, which is resistant to RKN and FORL, the disease severity was lower with treatments FORL and FORL+10N than with treatments N+FORL and N+10FORL. As disease severity was high with treatment FORL+10N, this shows that *M. incognita* enhances the fungal disease severity. In these two hybrids, especially with treatment N+10FORL, it was observed that the plant was not resistant to FORL and becomes susceptible. In Kaplan, which is susceptible to FORL but resistant to RKN, the lowest disease severity was with treatment FORL+10N (score 1.8) and the highest with treatment N+10FORL (score 4.0). The disease severity in the Adel, with RKN and FORL resistance, was low, and no significant difference was found between these treatments. It was found that *M. incognita* did not enhance the disease severity and FORL resistance was not overcome in Adel. There was no significant difference between disease severity in Gülizar, which is susceptible to RKNs and FORL. With treatment FORL, the highest disease severity among tomato hybrids was in Gülizar (score 3.4) without FORL resistance, followed by FORL-resistant Body (score 1.2) and FORL-susceptible Kaplan (score 2.0). With treatment N+10FORL, the disease severity was ranked highest to lowest as Gülizar > Kaplan > Body > Alberty > Armstrong > Adel.

Table 5. Effect of sequentially and concomitantly inoculation by *Meloidogyne incognita* (N) and *F*usarium *oxysporum* f. sp. *radicis-lycopersici* (FORL) on disease severity scores (mean ± SE[1]) of resistant and susceptible tomato hybrids

Treatment[2]	Adel		Alberty		Armstrong		Body		Gülizar		Kaplan	
FORL	0.4 ± 0.2	a C	0.6 ± 0.2	b C	0.4 ± 0.2	b C	1.2 ± 0.2	b BC	3.4 ± 0.2	a A	2.0 ± 0.3	b B
N+FORL	0.6 ± 0.2	a D	1.4 ± 0.2	ab CD	1.0 ± 0.0	a CD	1.6 ± 0.2	ab BC	3.6 ± 0.2	a A	2.4 ± 0.2	b B
FORL+10N	0.4 ± 0.2	a C	0.8 ± 0.2	b BC	0.2 ± 0.2	b C	1.2 ± 0.2	b BC	3.6 ± 0.2	a A	1.8 ± 0.3	c B
N+10FORL	0.6 ± 0.2	a C	2.2 ± 0.2	a B	1.0 ± 0.0	a C	2.4 ± 0.2	a B	4.0 ± 0.0	a A	4.0 ± 0.0	a A

[1] Means followed by the same lowercase letter within columns or uppercase letter within rows are not significantly different ($p \leq 0.05$).
[2] Treatments: N, *M. incognita* only; N+FORL, simultaneous inoculation of *M. incognita* and FORL; FORL+10N, first inoculation with FORL and 10 days later inoculation with *M. incognita;* and N+10FORL, first inoculation with *M. incognita* and 10 days later with FORL.

In the present study, tomato hybrids were found to be affected by a *M. incognita* and FORL interaction. Although the nematode density changed with treatment in Adel, Armstrong, Body, and Kaplan, resistance to RKN remained evident. Treatment time differences for *M. incognita* affected the response of

the tomato hybrids in the number of galls, egg masses and J2 density. Simultaneous inoculation enhanced the number of galls and egg masses in Adel, Armstrong, Body and Gülizar. The highest number of galls and egg masses in Alberty and Kaplan were with treatment N+10FORL. Treatments N+FORL and N+10FORL increased the number of galls, egg masses and J2 density. These data show that FORL has a positive influence on nematode reproduction. In some studies, it has been reported that nematode penetration was enhanced by formation of fungal pathogen enzymes in the roots (Edmunds & Mai, 1966a, 1966b, 1967; Nordmeyer & Sikora, 1983). Although root lesion nematodes have a different trophic behavior to RKNs, there are studies that show interaction between these nematodes and plant pathogenic fungi increases the nematode density (Vrain, 1987; Hasan, 1988). Also, the lowest number of galls and egg masses in all tomato hybrids in the present study was with treatment FORL+10N because of the root rot caused by FORL affected the nematode feeding process in the root tissues and then negatively affected nematode reproduction. Either the existence of a fungal hyphae prevents nematode penetration or invasion sites the nematode chooses to feed may cause a decrease in nematode density (Davide & Triantaphyllou, 1967; Mokbel et al., 2007). Additionally, *Fusarium* species secrete toxic compounds against plant parasitic nematodes and these compounds affect hatching, viability and juvenile movement (Nitao et al., 1999; 2001). In order to develop and deposit eggs of RKNs, they must provide their nutritional needs from giant cells. If *Fusarium spp*. colonizes these feeding cells and depletes their nutrient content, the female nematode can die without depositing eggs (Nordmeyer & Sikora, 1983). It has been reported that the reproduction of *M. incognita* and galling in the roots of blackgram plants (*Vigna mungo* L.) are significantly reduced in the presence of *F. oxysporum* (Mahapatra & Swain, 2001). Akram & Khan (2006) found that gall formation, egg mass production and soil population of *M. incognita* were negatively affected by FOL in greenhouse tomato plants. In this study, the change in the interaction of nematode and fungus according to the treatment time shows that the pathogen enters the plant first is important in the development of other pathogens. Lobna et al. (2016), in their study with *M. javanica* and FOL in tomato, found that disease severity depends on nematode population and inoculation time. Ramalingam (2019) found that when *M. incognita* is applied to tomato plants before FOL inoculation, wilt intensity is highest, followed by simultaneous inoculation, and the lowest wilt was with FOL application before nematode inoculation. Göze Özdemir (2020), in her research on *Pratylenchus thornei* Sher & Allen, 1953, *Pratylenchus neglectus* (Rensch, 1924) Filipjev & Schuurmans-Stekhoven, 1941 and *Pratylenchus penetrans* (Cobb, 1917) Filipjev & Schuurmans-Stekhoven, 1941, and with *F. culmorum* (WG Smith) Sacc. under controlled conditions, determined that the inoculation time of *F. culmorum* is important and that the pathogen that enters the wheat first negatively affects the development of the other pathogen. These results also confirm the findings of the present study.

Nematode development affecting FORL resistance of tomatoes is one of the important findings of the present study. Although there were differences between the treatments on Adel and Armstrong with RKN and FORL resistance, J2 density, galls and egg masses, and disease severity were found to be low. The nematode in Alberty caused increased gall formation, egg masses and J2 density, and the disease severity increased. With treatment N+10FORL, the highest number of galls and egg masses, and J2 density was with Alberty. In parallel, the highest disease severity was with treatment N+10FORL and FORL resistance of the plant was overcome. Although the RKN and FORL-tolerant Body developed less *M. incognita* than Alberty, disease severity was found to be similar. Although *M. incognita* developed well in Body, the highest disease severity with treatment N+10FORL and FORL resistance was found to be overcome. In Kaplan, which is sensitive to FORL but tolerant to RKN, disease severity was higher than with treatmentsFORL in N+FORL and N+10FORL. In Kaplan, nematode development was higher than with other treatments, especially with N+10FORL, and the disease severity was the highest with this treatment. Compared to Gülizar, Kaplan appeared to remain nematode resistant, and this resulted in lower disease severity in other treatments, except for N+10FORL. Gülizar, which is sensitive to RKN and FORL, had the highest number of galls and egg massed, and J2 density, and disease severity among all tomato hybrids. Also, the disease severity was found to be similarly high in all treatments in Gülizar. Co-infection of the two

pathogens was determined to increase the severity of Fusarium wilt susceptibly. RKN resistance was found to be important for ensuring the durability of FORL resistance. Resistance of the fungus was overcome with treatment N+10FORL, with the highest disease. This may be due to the wounds caused by RKNs in the roots, or by physiological and biochemical changes in the host cells (Moussa & Hague, 1988; Khan & Hosseini-Nejad, 1991; Marley & Hillocks, 1996). Porter & Powell (1967), in their study with RKNs in tobacco, found severe wilting in plants that were treated with nematodes before the fungus. Bowman & Bloom (1996) reported that the resistance to Fusarium wilt in tomato plants was overcome in the treatments with *M. incognita* inoculation before fungal inoculation. Vargas et al. (1996) found that *Phytophthora capsici* Leon. resistance was overcome when *Nacobbus aberrans* (Thorne, 1935) Thorne & Allen, 1944 (Rhabditida: Pratylenchidae) was applied to chili pepper. Colak-Ates et al. (2018) found that AL-4, AL-9 and AL-21 tomato genotypes with FORL resistance lost their resistance to FORL disease in simultaneous and sequential inoculations with *M. incognita*. In the present study, it was determined that although nematode resistance was not lost in tomato hybrids with RKN resistance, there was a change in application-based nematode development and this change was due to the synergistic or antagonistic interaction between *M. incognita* and FORL. It was found that these interactions are dependent on the pathogen that was first inoculated onto the plant.

When FORL was first inoculated on tomato plants under controlled conditions, gall formation, egg mass production and soil population of *M. incognita* were found to be negatively affected. It was determined that tomato FORL resistance was overcome with increasing nematode density with treatment N+10FORL in some tomato hybrids. This result shows that the density of *M. incognita* is important in the durability of FORL resistance, and as the population density increases, there may be a risk in the durability of resistance. It was found that the maintenance of FORL resistance is possible with RKN resistance. The disease was not observed in hybrids resistant to both pathogens. Farmers can prevent disease development by choosing tomato hybrids that are resistant to both RKNs and FORL.

It was concluded that RKN or fungal resistance can contribute to the prevention of yield losses caused by co-infection of *M. incognita* and FORL. When cost of resistant hybrids is taken into consideration, it is essential that resistance is not lost under field conditions. Nematode control should be successfully applied whenever FORL resistant hybrids are used. Integrated control methods should be considered to manage these disease complexes.

Acknowledgments

Olympos Seedling Production Facility and Agricultural Engineer Tuğçe Okumuş Erol are thanked for providing tomato seedlings.

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Türk. entomol. derg., 2022, 46 (1): 75-88
DOI: http://dx.doi.org/10.16970/entoted.1032262

ISSN 1010-6960
E-ISSN 2536-491X

Original article (Orijinal araştırma)

Insecticidal and repellency effects of a Turkish diatomaceous earth formulation (Detech) on adults of three important pests of stored grain

Yeni bir Türk diatom toprağı formülasyonunun (Detech) üç ana depolanmış tahıl zararlıların erginlerine karşı insektisidal ve kaçırıcı etkisi

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Abstract

In this study, laboratory experiments were conducted in order to assess the insecticidal and repellency effects of a novel Turkish diatomaceous earth (DE) formulation (Detech) on adults of the rice weevil, *Sitophilus oryzae* (L., 1763) (Coleoptera: Curculionidae), the confused flour beetle, *Tribolium confusum* du Val., 1863 (Coleoptera: Tenebrionidae), and the lesser grain borer, *Rhyzopertha dominica* (F., 1792) (Coleoptera: Bostrichidae). For the insecticidal activity, bioassays were conducted with soft wheat treated with 0, 600 and 900 ppm of Detech at 25 ± 1°C and 65 ± 5% RH and progeny production was assessed after 45 days. For the repellency tests, two-choice tests on mono-layer wheat were conducted at 1,000 ppm of Detech at 25 ± 1°C and 65 ± 5% RH. Overall, Detech was effective against *T. confusum* and *S. oryzae* adults in wheat, causing 82% to 100% mortality at 600 and 900 ppm after 7 and 14 days exposure. The complete, or almost complete, progeny inhibition of *S. oryzae* were found at both concentrations, whereas the highest reduction in *R. dominica* progeny (84%) was obtained at 900 ppm. Detech was highly and moderately repellent to *T. confusum* and *S. oryzae* adults, respectively, whereas it had no or low repellency effect on *R. dominica* adults. In conclusion, Detech, which consists of a mixture of three DE deposits with different diatom frustules has potential for use against stored-grain insect pests as a promising grain protectant. Experiments were conducted in 2020-2021 in Entomology Laboratory of Tekirdağ Namık Kemal University and Kahramanmaraş Sütçü İmam University.

Keywords: Natural insecticides, repellency, *Rhyzopertha dominica, Sitophilus oryzae, Tribolium confusum,* wheat

Öz

Bu çalışmada, yeni bir Türk diyatom toprağı formülasyonunun (Detech) pirinç biti *Sitophilus oryzae* (L., 1763) (Coleoptera: Curculionidae), kırma biti, *Tribolium confusum* du Val., 1863 (Coleoptera: Tenebrionidae) ve ekin kambur biti, *Rhyzopertha dominica* (F., 1792) erginlerine karşı insektisidal ve kaçırıcı etkisini değerlendirmek için laboratuvar denemeleri yürütülmüştür. İnsektisidal aktivite için, Detech'in 0, 600 ve 900 ppm konsantasyonu uygulanan ekmeklik yumuşak buğday üzerinde 25 ± 1°C sıcaklık ve %65 ± 5 nispi nemde biyolojik testler yürütülmüş ve 45 gün sonra yeni nesil ergin sayıları değerlendirilmiştir. Kaçırıcı etki için, 25 ± 1°C sıcaklık ve %65 ± 5 nispi nemde ve Detech'nin 1000 ppm konsantrasyonu uygulanan tek katmanlı buğday üzerinde iki seçenekli testler gerçekleştirilmiştir. Genel olarak, Detech buğday üzerinde *T. confusum* ve *S. oryzae* erginlerine karşı çok etkili olurken 7 ve 14 gün maruz bırakma süresi sonunda 600 ve 900 ppm'de *T. confusum* ve *S. oryzae* erginlerin %82-100 ölüme neden olmuştur. Her iki konsantrasyonda da *S. oryzae*'nın yeni nesil ergin çıkışı tamamen veya tamama yayın engellenirken, *R. dominica* da ise yeni nesil ergin çıkışındaki en yüksek azalma (%84) 900 ppm'de elde edilmiştir. Detech, *T. confusum* ve *S. oryzae* erginlerinde karşı oldukça yüksek düzeyde, *R. dominica* erginlerinde düşük kaçırıcı etki göstermiştir. Sonuç olarak, Detech'in, tahıllarda umut verici bir koruyucu insektisit olarak depolanmış tahıl zararlılarına karşı etkin bir şekilde kullanım potansiyeline sahip olduğunu göstermektedir. Denemeler 2020-2021 yılında Tekirdağ Namık Kemal ve Kahramanmaraş Sütçü İmam Üniversitesi Entomoloji laboratuvarlarında yürütülmüştür.

Anahtar sözcükler: Doğal insektisitler, kaçırıcı etki, *Rhyzopertha dominica*, *Sitophilus oryzae, Tribolium confusum,* buğday

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Received (Alınış): 04.12.2021 Accepted (Kabul ediliş): 02.03.2022 Published Online (Çevrimiçi Yayın Tarihi): 13.03.2022

Introduction

One of the most promising alternatives to the use of insecticides, including synthetic, petroleum-based contact organophosphate and pyrethroid compounds, in durable stored products is the use of diatomaceous earths (DEs). DEs are composed by the fossil skeletons of phytoplankton, also known as diatoms, which occur in fresh and salt water (Quarles, 1992; Losic & Korunic, 2018). Differences in physical properties of the diatoms include shape, bulk density, surface area, oil and water absorption capabilities, average pore size and pore size distribution (Losic & Korunic, 2018). Therefore, the DEs currently mined vary markably in their insecticidal activity, depending upon species composition, geological and geographical origin as well as certain chemical characteristics, such as SiO_2 content, pH and tapped density (Korunic, 1997). DEs are probably the most efficacious natural resource-based dry materials that can be used as insecticides (Korunic, 1998). The mode of action of DEs has been described indicatively in many studies (Ebeling, 1971; La Hue, 1978; Golob, 1997; Korunic, 1998; Korunic & Fields, 2016). In general, DEs abrade the insect cuticle, absorb lipids from the epicuticle, and cause death by desiccation and water loss (Ebeling, 1971; Golob, 1997; Korunic, 1998; Subramanyam & Roesli, 2000). DEs are also repellent to insects and this repellence depends on the dose (Quarles, 1992; White et al., 1966; Rigaux et al., 2001; Mohan & Fields, 2002). Since DEs are inert (silicaceous) materials, no interaction with the environment occurs. Thus, DEs persist in the treated substrate, providing a long-term protection against insect pests.

There are several (>30) DE formulations, based on natural deposits, which are now commercially available throughout the world (Subramanyam & Roesli, 2000; Fields & Korunic, 2000; Kavallieratos et al., 2005; Losic & Korunic, 2018). Many studies have shown that they can be quite effective against a wide range of stored-grain insect species (Subramanyam et al., 1994; Korunic, 1998; Subramanyam & Roesli, 2000; Wakil et al., 2006; Athanassiou et al., 2007, 2011; Kostyukovsky et al., 2010). However, the use of diatomaceous earth was limited because the required dose rates of 1.0 to 3.5 g per kg of grain (1,000 to 3,500 ppm) for most DE products significantly reduced the grain bulk density and flowability, and left visible dust residues, which are not widely accepted by quality standards in many countries (Subramanyam et al., 1994; Golob, 1997). Considerable research has been conducted to address these problems and develop new DE formulations with enhanced performance and reduced DE dosage that have no adverse effect on grain quality (Arnaud, 2005; Nikpay, 2006; Athanassiou & Korunic, 2007; Korunic, 2013).

Based on initial evidence and preliminary samples, it seems that Turkey is a rich source of natural DE deposits, and there is clear evidence for the existence of large DE deposits in the Central and West Anatolia Regions of Turkey (Özbey & Atamer, 1987; Mete, 1988; Sıvacı & Dere, 2006; Çetin & Taş, 2012). Diatomite reserves of Turkey are around 125 Mt. However, there are few published data on the potential use of some local DEs against stored-grain insect pests. Doğanay (2013), Işıkber et al. (2016) and Akçalı et al. (2018) evaluated some local DEs collected from different regions of Turkey against stored-grain insects with promising results. Akçalı et al. (2018) investigated efficacy of nine local DEs collected from different regions of Turkey against stored-grain insects, the rice weevil, *Sitophilus oryzae* (L., 1763) (Coleoptera: Curculionidae), the confused flour beetle, *Tribolium confusum* du Val., 1863 (Coleoptera: Tenebrionidae), and the lesser grain borer, *Rhyzopertha dominica* (F., 1792) (Coleoptera: Bostrichidae) and reported that CB2N-1 and BGN-1 local DEs had high efficacy against these stored-grain insects and thus, could be potential to be successfully used for controlling stored-grain insect pests as a grain protectant.

The search for newer, naturally occurring DEs that are more effective for insect control is still in progress, especially in areas rich in silicaceous rocks. Thus, as result of extensive screening tests of DEs from several regions of Turkey, a novel Turkish DE deposit, Detech, which consists of a mixture of three freshwater DEs collected from different DE reserves located in different regions of Turkey, has been commercially formulated by Entoteam R&D Food Agriculture Co. This novel DE formulation, Detech, is consisted of two different diatom frustules, which are completely different from the other commercial DE

formulations with single diatom frustules. However, there is inadequate or limited information about the effectiveness of Detech against stored-grain insects. The lack of information on insecticidal and repellency effects of Detech on stored-grain insects justifies the need of the present study. The objective of our study was to evaluate the insecticidal and repellency effects of novel Turkish diatomaceous earth formulation, Detech against adults of *S. oryzae*, *T. confusum*, and *R. dominica* on wheat.

Materials and Methods

Experiments were conducted in 2020-2021 at Entomology Laboratory of Tekirdağ Namık Kemal University and Kahramanmaraş Sütçü İmam University.

Test insects

For both the insecticidal and repellency tests, *S. oryzae, T. confusum* and *R. dominica* adults were reared in the Entomology Laboratory of Plant Protection Department, Tekirdağ Namık Kemal University and Kahramanmaraş Sütçü İmam University, Turkey. *Sitophilus oryzae* and *R. dominica* individuals were reared on whole soft wheat with 11% moisture content at 26 ± 1ºC, 65 ± 5% RH and 30 ± 1ºC, 65 ± 5% RH, respectively, whereas *T. confusum* was reared at 26 ± 1°C and 65 ± 5% RH in mixture of wheat flour with yeast (17:1, w/w). Seven- to 10-day-old adults of all the above species were used for all bioassays.

DE formulation

A Turkish diatomaceous earth formulation (Detech) was used in the bioassays and repellency tests. Detech is a mixture of freshwater diatomaceous earth deposits collected from there different DE reserves located in Central Anatolia of Turkey. It is consisted of naturally occurring amorphous silica and has white-gray color and is formulated by Entoteam R&D Food Agriculture Co. to use for insect pests control as an insecticide (pest control grade). According to total quantitative chemical analysis of Detech by atomic absorption spectroscopy, it contains 80.6% SiO_2, 4.75% CaO, 4.7% Al_2O_3, 1.5% Fe_2O_3, 0.85% MgO, 0.5% K_2O, 0.4% Na_2O, and less than 0.01% TiO_2. Some physical properties of Detech used in bioassays are given in Table 1. Scanning electron microscopy images of diatom frustules present in Turkish diatomaceous earth formulation (Detech) is presented in Figure 1. Strong uptake and attachment were observed when analyzing the images of Detech, it consists of a large number of cylindrical- and rod-shape diatom skeletons.

Table 1. Some physical properties of a proprietary Turkish diatomaceous earth (DE) formulation, Detech

DE formulation	Diatom type	Median particle diameter (µm)[1]	Adherence of DE to wheat kernels (%) mean ± SE (n = 3)[2]	Tapped bulk density (g/L) mean ± SE (n = 3)[3]	pH, mean ± SE (n = 3)	Color
Detech	Freshwater	14.1	84.0 ± 1.7	300.0 ± 2.9	8.25 ± 0.01	Yellowish-white

[1] The median particle diameter value that corresponds to 50% of the total particle volume in the volumetric cumulative particle size distribution. Particle size analysis was conducted using laser light diffraction technique by Accredited Mineralogy and Petrography Laboratory of General Directorate of Mineral, Research and Exploration, Turkey.

[2] Tests for adherence rate of DE on wheat kernels were performed using method described by Korunic (1997) in Stored Product Insects Laboratory of Kahramanmaraş Sütçü Imam University.

[3] Tests for bulk density of DE on wheat kernels were performed using method described by Korunic (1997) in Stored Product Insects Laboratory of Kahramanmaraş Sütçü Imam University.

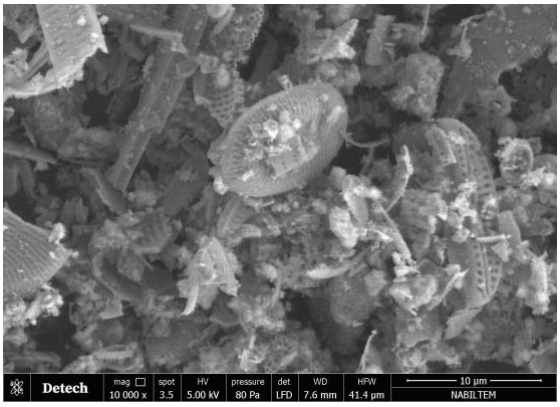

Figure 1. Scanning electron microscopy image of diatom frustules present in Turkish DE formulation (Detech) (10,000x magnification), (NABILTEM, Tekirdağ Namık Kemal University).

Commodity

Untreated, clean, with low dockage (0.8%) and infestation-free soft wheat (*Triticum aestivum* L., cv. Elbistan Yazlığı) was used for the bioassays. The moisture content of wheat cultivar, as determined by a Dickey-John moisture meter (Dickey-John Multigrain CAC II, DICKEY-John Co., Lawrence, KS), ranged between 11.0 and 11.4%.

Experimental arena used for the repellency tests

For two-choice test on mono-layer wheat, experimental test arena consisted plastic rectangular containers (25 × 13 × 6 cm LWH; Akyüz Plastic Co., Istanbul, Turkey). Dry concrete mix (Daracon C30/37, Draco Co., Istanbul, Turkey) was mixed with three parts water and poured into plastic rectangular container to a depth of 2 cm. After the concrete surface was completely dry, it was divided by 2 mm metal sieve into three different areas given below: 1. The area containing DE-treated wheat (10 × 13 cm), 2. Empty area between DE-treated and non-treated wheat (transition area) (5 × 13 cm), 3. The area containing non-DE-treated wheat (10 × 13 cm).

Insecticidal bioassays

Bioassays were conducted according to randomized block design with 3 x 2 factorial layout and with three replicates for all tested insect species and two concentrations 600 and 900 ppm (mg DE/kg wheat), which were selected according to according preliminary tests. Bioassays were conducted in the lockable 80 l (26 cm × 36.5 cm × 15 cm) plastic container; Akyüz Plastic Co., Istanbul, Turkey) in the incubator (IPP55 Plus, Memmert, Germany) at 25 ± 1°C temperature, 65 ± 5% RH and continuous darkness. The desired RH was maintained by using saturated salt solution of magnesium nitrate as recommend by Greenspan (1977). One kg lots of grain were prepared for each replicate and each was treated with 600 and 900 ppm of Detech. Additional 1 kg lots of wheat grain were left untreated and served as control. The treated wheat lots were shaken manually for approximately 15 min to achieve equal distribution of the DE particles inside the grain mass (Subramanyam & Roesli, 2000). For each trial (insect species-DE concentration combination), three sub-samples of 50 g wheat were taken from Detech-treated lots, and placed in 50 ml small cylindrical self-standing centrifuge tube (Isolab, Labor Teknik Co., Istanbul, Turkey) that was closed, apart from a hole 1.5 cm in diameter (at the top of the tube), and that was covered with muslin cloth for sufficient ventilation. Three additional tubes containing untreated wheat served as control in each treatment. Then, 30 one-week-old adults of each species were introduced into each tube. All tubes were placed in the lockable plastic container which contained saturated salt solution of magnesium nitrate under the plastic and set at the desired temperature level. Three replicates were used for each trial (tested insect species-DE concentration combination). Adult mortality was counted after 7 and 14 days exposure.

After 14 days individuals of *S. oryzae* and *R. dominica* were removed from the vials and the grain were left for an additional period of 45 days. Then, the vials were opened and the number of total progenies per treated commodity was counted. Since *T. confusum* is secondary pest that is not able to feed on the whole wheat grain, its progeny production was not assessed in present study. Temperature and RH during all the bioassays were monitored by using HOBO digital recorders (HOBO H8, Onset Computers, Bourne, MA, USA).

Repellency bioassays

Repellency bioassays were conducted according to randomized parcel design with five replicates at 1,000 ppm concentration of DE (mg DE/kg wheat) using the two-way choice method to assess the likelihood of the insects avoiding contact with DE-treated grain. Three kg lots of grain were prepared and each of them was treated with 1,000 ppm of Detech. Furthermore, an additional 1 kg lot of grain was left untreated and served as control. The treated wheat lots were shaken manually for approximately 15 min to achieve equal distribution of the DE particles inside the grain mass (Subramanyam & Roesli, 2000). For each treatment, 25 g of Detech-treated and untreated (control) wheat grain were placed in a single layer on each area of plastic rectangular respectively. Fluon (polytetrafluoroethylene, Sigma Aldrich, St. Louis, MO, USA) was applied to the edges of the containers to prevent insects from climbing and escaping from the experimental arena. Thirty adults of mixed sex and >14 days old were placed in empty area without wheat grain and then experimental arena was closed with a plexiglass with small holes. The number of insects found within DE-treated or untreated wheat was counted after 0.5, 1, 3, 5 and 7 days. No dead insects were observed for each observation time. The repellency tests were conducted under laboratory conditions at 25 ± 1ºC, 60 ± 5% RH and complete darkness.

Data analysis

Mortality counts were corrected by Abbott's formula (Abbott, 1925). All mortality data after 7 and 14 days for each species were normalized using arcsine transformation and then subjected to two-way ANOVA with main factors, insect species and DE concentrations by using the GLM Procedure of SAS/STAT 12.1 (SAS, 2012). Mean mortality percentages for each species and DE concentrations were separated by using the Tukey's HSD (honestly significant difference) test and a two-sided *t*-test (comparing treatment groups) at 5% significant level respectively. The differences in number of adult emergences of the new generation from the control were tested using Dunnett's test (P = 0.005). Adult progeny in each insect species-DE concentration combination was compared with adult progeny in the control vials using Dunnett's test at 5% significant level. The formula was used to determine the percentage reduction in the number of adults in new generation: ((Nc-Nt) / Nc) x 100, where, Nc was the number of adult progenies in the control and Nt the number of adult progenies in DE treatments. The means of percentage reduction in the number of adults of new generation for each insect species were separated by using a two-sided *t*-test (comparing treatment groups) at 5% significant level.

For the repellency tests, adults of *S. oryzae*, *T. confusum* and *R. dominica* adults, the proportion of the insects present in arenas with Detech-treated and untreated wheat for each observation time was analyzed using a two-sample Chi-square test for equality of proportions with continuity correction to see whether mean proportions of the insects in arenas with DE-treated and untreated wheat differ significantly from an equal distribution of 50%. The percentage repellence (PR) of DE treatment for each insect species was also calculated after each observation time using the formula: ((Nc-Nt) / (Nc+Nt)) × 100, where, Nc is the number of insects present in untreated wheat arena and Nt is the number of insects present in DE-treated wheat arena. The averages were then assigned to different classes (0 to V) using the scale given in Table 2 (Juliana & Su, 1983).

Table 2. Repellency classes assigned according to the percentage repellency ranges

Class	Repellency (%)	Class	Repellency (%)*
0	0.01-0.10	III	40.1-60.0
I	0.11-20.0	IV	60.1-80.0
II	20.1-40.0	V	80.1-100

***** All percentage repellency data for each species were normalized using arcsine transformation and then subjected to two-way ANOVA with main factors, insect species and observation intervals by using the GLM Procedure of SAS/STAT 12.1 (SAS, 2012). Mean percentage repellency for each species and observation interval was separated by using the Tukey's HSD (honestly significant difference) test at 5% significant level.

Results

Mortality

The two-way ANOVA analysis for insect mortality indicated significant differences for the main effects, tested insect species, DE concentration, and tested insect species x DE concentration interaction after 7 and 14 days exposure (Table 3). After 7 and 14 days exposure, no significant differences were obtained between mortality of *S. oryzae* and *T. confusum* at both concentrations, whereas the mortality of both *S. oryzae* and *T. confusum* were significantly higher than those of *R. dominica* (Table 4). After 7 days exposure, the highest mortality (93-97%) was observed in *T. confusum* at both concentrations, followed by *S. oryzae* (82-92%) whereas the lowest mortality (24-61%) was recorded in *R. dominica* adults. Total mortality was observed for *T. confusum* after 14 days at both concentrations, followed by *S. oryzae* (97-100%) whereas the lowest mortality (28-67%) was recorded in *R. dominica*. No complete mortality (100%) was observed for *R. dominica* after 7 and 14 days exposure whereas mortality ranged between 82-100% for *S. oryzae* and *T. confusum*.

Table 3. ANOVA parameters for main effects and associated interactions for mortality of *Sitophilus oryzae, Tribolium confusum* and *Rhyzopertha dominica* after two exposure intervals (total df = 12)

Exposure interval	Source	df	F	P
7 days	Insect species	2	101.0	<0.0001
	DE concentration	1	27.0	<0.0001
	Insect species*DE concentration	2	4.01	<0.046
14 days	Insect species	2	296.0	<0.0001
	DE concentration	1	36.9	<0.0001
	Insect species*DE concentration	2	15.0	<0.0001

DE concentration effects on adult mortality of the tested insect species are shown in Table 4. Increasing the concentration from 600 to 900 ppm resulted in significant increase of mortality of *R. dominica* after 7 and 14 days exposure whereas increasing the concentration from 600 to 900 ppm did not result in a significant increase of mortality of *S. oryzae* and *T. confusum* after 7 and 14 days exposure. Mortality of *R. dominica* was significantly higher at 900 ppm than that at 600 ppm after 7 and 14 days exposure, whereas there were not significant differences in the mortality of *S. oryzae* and *T. confusum* at 600 and 900 ppm for both exposure time.

Table 4. Mean mortality (mean ± SE) of *Sitophilus oryzae, Tribolium confusum* and *Rhyzopertha dominica* adults exposed for 7 and 14 days on wheat treated with at 600 and 900 ppm concentration of Detech in wheat

Exposure time	Concentration (ppm)	Insect species						F and P* value
		S. oryzae		*T. confusum*		*R. dominica*		
7 days	600	82.1 ± 4.0	A	93.3 ± 1.9	A	23.6 ± 4.0	Bb	$F_{2,6}$ = 82.3 P < 0.0001*
	900	92.2 ± 4.4	A	96.6 ± 0	A	60.7 ± 2.9	Ba	$F_{2,6}$ = 29.4 P = 0.0008*
	Control	0 ± 0		0 ± 0		1.1 ± 1.1		
	t and P value	*t* (3) = 1.77 P = 0.220		*t* (3) = 1.80 P = 0.214		*t* (3) = 7.49 P = 0.017**		
14 days	600	96.6 ± 1.9	A	100 ± 0	A	28.4 ± 3.4	Bb	$F_{2,6}$ = 82.25 P < 0.0001*
	900	100 ± 0	A	100 ± 0	A	67.1 ± 2.3	Ba	$F_{2,6}$ = 29.42 P = 0.0008*
	Control	1.1 ± 1.1		1.1 ± 1.1		2.2 ± 1.1		
	t and P* value	*t* (3) = 1.91 P = 0.196		- -		*t* (3) = 7.29 P = 0.018**		

* One-way ANOVA was applied to the data for insect species in the row for each exposure time. Means within rows followed by the same uppercase letter are not significantly different (Tukey's HSD test at 5% level).

** Student's t-test was applied to the data for DE concentration in the column for each exposure time and means within columns followed by the same lowercase letter are not significantly different (at 5% level).

Progeny production

Dunnett's test showed that progeny production of *S. oryzae*, *T. confusum* and *R. dominica* in control groups was significantly higher than that recorded in Detech-treated wheat (Table 5). All main effects were significant for progeny production in Detech-treated wheat. High progeny suppression was recorded for *S. oryzae* and *T. confusum* individuals (94-100%) whereas lower suppression was recorded for *R. dominica* (60-85%). When Detech at both concentrations were applied, significantly more progeny was suppressed for *S. oryzae* and *T. confusum* than for *R. dominica*. DE concentration also had a significant effect on the suppression of progeny production. Detech treatment at 900 ppm caused significantly more progeny suppression of *S. oryzae* and *R. dominica* than that at 600 ppm. However, in the case of *T. confusum* DE concentration was not significantly associated with the progeny suppression.

Table 5. Number of adult emergence (mean ± SE) and suppression rate of progeny production for *Sitophilus oryzae* and *Rhyzopertha dominica* adults exposed to 600 and 900 ppm Detech 45 d after the removal of their parental adults from the treated wheat

Insect species	Concentration (ppm)	Adult emergences (no.), mean ± SE	P value***	Reduction in progeny production (%), mean ± SE
Sitophilus oryzae	600	6.3 ± 1.4 (114 ± 3.6)**	0.016	94.0 ± 0.7 B
	900	1.6 ± 0.6 (114 ± 3.6)	0.013	98.3 ± 0.5 A
	t and P value*	-	-	*t* (3) = 4.25 P = 0.042*
Rhyzopertha dominica	600	13.3 ± 2.9 (33 ± 2)**	0.001	60.3 ± 6.4 B
	900	4.6 ± 1.4 (33 ± 2)	0.000	85.2 ± 5.0 A
	t and P value*	-	-	*t* (3) = 4.38 P = 0.039*

* Means within columns followed by the same letter are not significantly different (Student's t test at 5% level).

** Values in brackets are the average number of new generation adults obtained from the control treatment.

*** Dunnett's test was used to compare the number of new generation adults obtained from DE treatments with that in control treatment.

Repellency

Mean proportions of *S. oryzae, T. confusum* and *R. dominica* adults present in arenas with Detech-treated and untreated mono-layer wheat at each observation for the repellency tests in plastic container are given Figure 2.

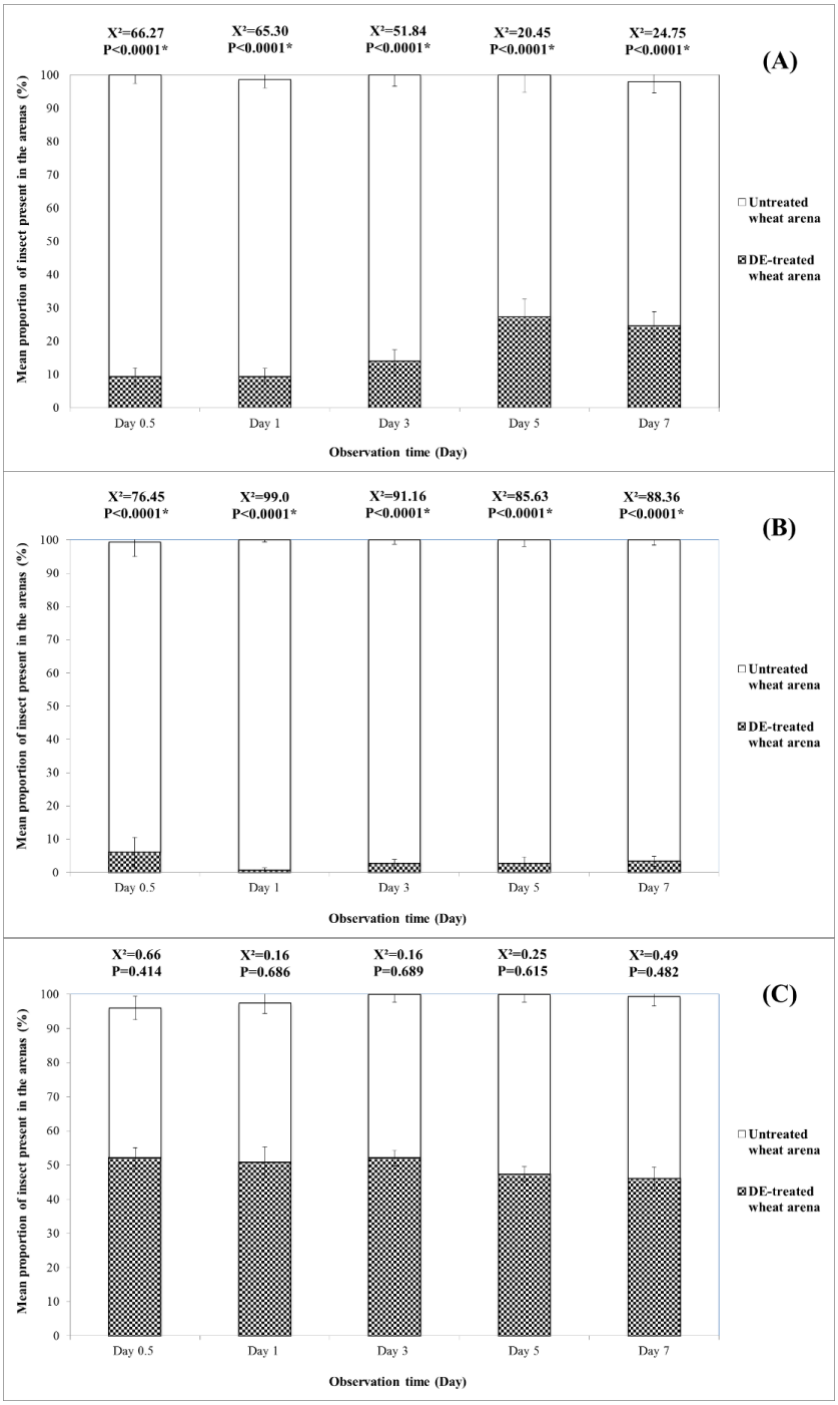

Figure 2. Mean proportion of *Sitophilus oryzae* (A), *Tribolium confusum* (B) and *Rhyzopertha dominica* (C) adults present in arenas with Detech-treated (1,000 ppm) and untreated mono-layer wheat at each observation for the repellency tests.

In the repellency tests conducted on single-layer wheat, there were significant differences between mean proportions of *S. oryzae* and *T. confusum* adults present in arenas with 1,000 ppm Detech-treated and untreated mono-layer wheat at all observation intervals (*S. oryzae* for 0.5, 1, 3, 5 and 7 days respectively; $\chi^2 = 66.3$, $\chi^2 = 65.3$, $\chi^2 = 51.8$, $\chi^2 = 20.5$ and $\chi^2 = 24.8$ $P < 0.001$; *T. confusum* for 0.5, 1, 3, 5 and 7 days, respectively; $\chi^2 = 76.5$, $\chi^2 = 99.0$, $\chi^2 = 91.2$, $\chi^2 = 85.6$ and $\chi^2 = 88.4$ $P < 0.001$). However, there were not significant differences between mean proportions of *S. oryzae* and *T. confusum* adults present in arenas with 1,000 ppm Detech-treated and untreated mono-layer wheat at all observation intervals (chi-square test; 0.5, 1, 3, 5, and 7 day respectively; $\chi^2 = 0.66$ P = 0.414, $\chi^2 = 0.16$ $P = 0.686$, $\chi^2 = 0.16$ $P = 0.686$, $\chi^2 = 0.25$ $P = 0.615$ and $\chi^2 = 0.49$ $P = 0.482$). In the repellency test, the highest proportions of *S. oryzae* and *T. confusum* adults were found in arena with untreated wheat at each observation time. However, the numbers of the insects for *R. dominica* were about the same in arena with Detech-treated and untreated wheat, and therefore, there was no preference for arena with Detech-treated and untreated wheat.

All main effects were significant for the percentage repellency in Detech-treated wheat (for tested insect species, $F_{2,60} = 167$, $P < 0.0001$; for observation intervals, $F_{4,60} = 2.52$, $P = 0.05$), whereas their interaction was not significant (for insect species x observation *intervals;* $F_{8,60} = 1.74$, $P = 0.107$). Repellency of *S. oryzae, T. confusum* and *R. dominica* in arenas with Detech-treated and untreated mono-layer wheat for each observation for the repellency tests are given Table 6. For all observation intervals, the percentages of repellency for *T. confusum* were significantly higher than those for *S. oryzae* and *R. dominica* with exception of the day 0.5). The highest percentage of repellency (88.0-98.8%) was observed in *T. confusum* at all observation intervals, followed by *S. oryzae* adults (45.3-81.3%) whereas the lowest percentage of repellency (1.3-8.9%) was recorded in *R. dominica*. The repellency tests indicated that Detech at 1,000 ppm was highly repellency to *T. confusum* with repellency class V and moderately repellency to *S. oryzae* with repellency class III-V whereas it has no or low repellency to *R. dominica*.

Table 6. Repellency percentage (mean ± SE) of *Sitophilus oryzae, Tribolium confusum* and *Rhyzopertha dominica* adults in arenas with Detech-treated and untreated mono-layer wheat at each observation for the repellency tests

Observation interval (day)	Repellency (%)						F and P value
	S. oryzae		*T. confusum*		*R. dominica*		
Day 0.5	81.3 ± 5.3 (V)**	Aa	88.0 ± 9.1 (V)**	A	2.1 ± 1.3 (I)**	B	$F_{2,12} = 61.3$ P < 0.0001*
Day 1	81.1 ± 4.9 (V)	Ba	98.7 ± 1.3 (V)	A	3.6 ± 2.3 (I)	C	$F_{2,12} = 243$ P < 0.0001*
Day 3	72.0 ± 6.8 (IV)	Bab	94.7 ± 2.5 (V)	A	1.3 ± 1.3 (I)	C	$F_{2,12} = 131$ P < 0.0001*
Day 5	45.3 ± 10.6 (III)	Bb	94.7 ± 3.9 (V)	A	6.7 ± 3.7 (I)	C	$F_{2,12} = 41.3$ P < 0.0001*
Day 7	49.9 ± 8.1 (III)	Bb	93.3 ± 3.0 (V)	A	8.9 ± 5.7 (I)	C	$F_{2,12} = 50.3$ P < 0.0001*
F and P value	$F_{4,20} = 5.35$ P = 0.004*		$F_{4,20} = 0.65$ P = 0.635		$F_{4,20} = 0.93$ P = 0.467		

* Means within rows followed by the same uppercase letter or in columns followed by the same lowercase letter are not significantly different (Tukey's HSD test at 5% level).

** The number in the brackets refers to repellency classes.

Considering observation interval effect on the repellency to tested insect species, increasing observation period did not result in significant increase in repellency to *T. confusum* and *R. dominica* (Table 6). However, increasing observation period resulted in significant reduction in repellency to *S. oryza*. In early observation period (0.5 and 1 day), repellency to *S. oryzae* was significantly higher than those in longer observation period (5 and 7 days).

Discussion

Commercially-available DEs (Insecto, USA; Dryacide, Australia; Protect-It, Canada) have been tested against a wide range of stored-product insect species (Korunic, 1998; Subramanyam et al., 1994; Subramanyam & Roesli, 2000; Wakil et al., 2006; Athanassiou et al., 2007, 2011; Kostyukovsky et al., 2010). They vary markably in their efficacy on different stored-product insect species (Fields & Korunic, 2000; Subramanyam & Roesli, 2000; Fields et al., 2003; Athanassiou et al., 2004). Generally, the recommended dose rate which is effective against stored-product insects (under certain temperature and moisture conditions) is ≥1,000 ppm. Many researchers underline the need for using new DEs, which are effective at low dose rates (≤1,000 ppm) (Arthur & Throne, 2003; Athanassiou et al., 2006, 2007). The search for new, naturally occurring DEs that are more effective for insect control is still in progress, especially in areas rich in silicaceous rocks. In present study, novel Turkish DE deposit, Detech, which consists of a mixture of three freshwater DEs collected from different DE reserves located in different regions of Turkey was tested for its efficacy against and repellency to *S. oryzae*, *T. confusum* and *R. dominica* adults.

Our bioassays indicated that Detech is highly effective against *T. confusum* on soft wheat, causing 93% and 97% mortality rate at 600 and 900 ppm, respectively, after 7 days exposure. Also, it is confirmed that *T. confusum* is one of the most tolerant stored-grain insects to DE (Korunic, 1998; Arthur, 2000). For example, Kavallieratos et al. (2007) evaluated the efficacy of three commercially available modified DE formulations (PyriSec, Insecto and Protect-It) against adults of *T. confusum* originating from different European geographical locations in laboratory tests. In their study, PyriSec, Insecto and Protect-It at 500 ppm with 7 days exposure resulted in the mortality rates ranging from 13-36%, 5-25% and 18-39%, respectively, depending on the tested *T. confusum* population. According to these results, Detech appears more effective against *T. confusum* adults than PyriSec, Insecto and Protect-It. In the case of *S. oryzae*, Detech was also highly effective against *S. oryzae* adults in wheat, causing 82 and 92% mortality rates at 600 and 900 ppm, respectively, after 7 days exposure. Athanassiou et al. (2007) reported that PyriSec, Insecto and Protect-It on wheat at 500 ppm caused 99.9, 96.5 and 99.7% mortality, respectively, after 7 days exposure. Based on these mortality results it appears that Detech is slightly less effective against *S. oryzae* than PyriSec, Insecto and Protect-It. Also, Detech was less effective against *R. dominica* on soft wheat, causing 24 and 61% mortality at 600 and 900 ppm, respectively, after 7 days exposure.

The type of the commodity affected the efficacy of DEs (Korunic & Mackay, 2000; Athanassiou et al. 2003, 2007; Kavallieratos et al., 2005). For example, Athanassiou et al. (2007) tested PyriSec, Insecto and Protect-It treatments on hard wheat at 500 ppm with 7 days exposure causing 97.4, 92.0 and 98.5% mortality, against *R. dominica*. In contrast, in the current study, Detech was less effective against *R. dominica* adults than PyriSec, Insecto and Protect-It. The large difference between mortality results of *R. dominica* can be attributed to the wheat cultivar (hard or soft) used in the bioassays. Also, Ferizli & Beris (2005) found that Protect-It gave a low mortality rate (20%) of *R. dominica* on a soft wheat cultivar at 500 ppm after 14 days exposure. These findings suggested that DEs are more effective on hard wheat than on soft wheat. We assume that this is likely to be due to the fact that *R. dominica* adults rapidly penetrate soft wheat kernels even with DE treatment and therefore these insects that stay inside wheat kernels have less exposure to DE dust particles from the surface of grain than other more active insect species which feed predominantly outside the kernel. Similar observations were also reported by Ferizli & Beris (2005).

DEs vary markably in their insecticidal effect on different stored-grain insects (Desmarchelier & Dines, 1987; White & Loschiavo, 1989; Korunic, 1998; Arthur, 2000; Fields & Korunic, 2000; Athanassiou et al., 2005; Kavallieratos et al., 2005). Though there are different rankings among these studies, overall the rankings (most to least susceptible) are: *Cryptolestes* spp., *Oryzaephilus* spp., *Sitophilus* spp., *R. dominica* and *Tribolium* spp. Of the species tested in our study, *T. confusum* is considered the most DE-susceptible, followed by *S. oryzae* and *R. dominica*. This is interesting finding because the most of previous studies

reported that *T. confusum* was considered the most DE-tolerant. This difference in DE-susceptibility of *T. confusum* can be attributed to the physical and chemical properties of Detech, which consists of a mixture of three freshwater DEs collected from different DE reserves located in different regions of Turkey. Arnaud et al. (2005) proposed as a possible solution the use of a DE mixture, in which more than one DE is present. In the same study, the mixture of the tested DEs was more effective than the application of each DE alone. Generally, a mixture can combine all the positive characteristics of different DEs, such as the use of low insecticidal rates and the presence of food additives. Athanassiou et al. (2007) found that a mixture of Protect-It, PyriSec and Insecto was more effective than a single DE application on both wheat and maize, against adults of *T. confusum*. There are also only a few studies that have examined the reasons for different susceptibilities of pest species. Nair (1957) using magnesite dust and White and Loschiavo (1989) using silica aerogel found that susceptible insects had more dust adhering to the cuticle. Fields & Korunic (2000) reported that general resistance to desiccation, either through better water retention, better water acquisition, or greater tolerance of desiccation could also be responsible for these differences in susceptibility.

Subramanyam & Roesli (2000) noted that it is often more important in practical conditions of cereal storage to prevent progeny formation than to concentrate on obtaining direct lethal effects of DE against parent insects. In the present study, *S. oryzae* showed complete, or almost complete, progeny inhibition, 94 and 98%, at 600 and 900 ppm, whereas the highest progeny reduction (84%) was observed in *R. dominica* at 900 ppm. Ferizli & Beris (2005) found that Protect-It resulted in reduced F_1 adult progeny of *R. dominica* according to application dosage and almost complete suppression (99%) of progeny production was achieved at 1,000 ppm on soft wheat. Desmarchelier & Dines (1987) also indicated that at least 1,000 ppm Dryacide applied to wheat with 28 days exposure was necessary to achieve 100% mortality and progeny control of *R. dominica*. These findings are almost in accordance with the present study. In the case of *S. oryzae,* similar results for suppression of progeny production are reported for several commercially available DE formulations, such as SilicoSec, Insecto and Protect-It (Subramanyam & Roesli, 2000; Athanassiou et al., 2003, 2005, 2008).

The repellence phenomena of inert dust to stored-grain insects has been described by several authors (White et al., 1966; Quarles, 1992; Rigaux et al., 2001). Our experiments were conducted to determine whether there was any evidence for avoidance of DE deposit by the insects when given a choice between DE-treated and untreated wheat surfaces. In our study, the repellency tests on mono-layer wheat indicated that there were significant differences in repellency of DE to tested insect species. *Sitophilus oryzae* and *T. confusum* adults preferred untreated wheat. Detech at 1,000 ppm was highly repellent to *T. confusum* adults with repellency class V and moderately repellent to *S. oryzae* adults with repellency class III-V. Similar high repellency observations have been reported in studies with several DE deposits against *S. oryzae* (White et al., 1966; Mohan & Fields, 2002; Nwaubani et al., 2014). Nwaubani et al. (2014) reported that adults of *S. oryzae* avoid contact with wheat treated with two DEs obtained from Nigeria, and therefore, there were significantly fewer adults of both insect species in DE-treated wheat, whereas there were more adults in the untreated wheat. However, in our study the numbers of the insects with *R. dominica* had approximately equal distribution in arena with DE-treated and untreated wheat, and therefore, there was no preference for arena with DE-treated and untreated wheat. Hence, Detech on soft wheat cultivar has no or limited repellency to *R. dominica* adults. Similar to the results obtained for *R. dominica* adults in the present study, Vardeman et al. (2007) reported that although adult deaths were directly related to DE concentration, the movement of *R. dominica* in the product did not diminish with the presence of DE and therefore, DE did not show a clear repellency to *R. dominica* adults. In contrast to these results for *R. dominica*, Nwaubani et al. (2014) reported that adults of *S. oryzae* avoid contact with wheat treated with two DEs obtained from Nigeria. This difference could be due to different physical and chemical properties of the tested DEs, wheat cultivars or repellency test methods. Mohan & Fields (2002) reported that one positive effect of DE repellency would be that there could be reduced insect immigration into a grain mass

and greater insect emigration out of a grain mass treated with DEs, hence, reducing insect populations. In present study, it appeared that high repellency to *S. oryzae* and *T. confusum* could be substantially correlated with their high mortality rates. This could be due to the fact that DE application could potentially increase the rate of *S. oryzae* and *T. confusum* movement in grain mass, which therefore, could cause picking up significantly more DE dust particles from the surface of grain.

In conclusion, the present study indicates that novel Turkish DE formulation (Detech) which consists of a mixture of three DEs from different DE reserves in different regions of Turkey, has potential as a stored grain protectant given its toxicity and repellency to the insect species studied here. Additional studies are required to determine the effects of biotic and abiotic factors on efficacy of Detech against other stored grain insects and then to evaluate its insecticidal performance under commercial storage conditions.

Acknowledgements

The authors would like to thanks Entoteam R&D Food Agriculture Co., Turkey for supplying the Detech test materials. This work was not funded by any of the specific agencies in the public, commercial, or not-for-profit sector.

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Türk. entomol. derg., 2022, 46 (1): 89-98
DOI: http://dx.doi.org/10.16970/entoted.1022988

ISSN 1010-6960
E-ISSN 2536-491X

Original article (Orijinal araştırma)

Development of an *in vivo* bioassay to identify Turkish chickpea genotypes resistance to *Ditylenchus dipsaci* (Kühn, 1857) (Tylenchida: Anguinidae)[1]

Ditylenchus dipsaci (Kühn, 1857) (Tylenchida: Anguinidae)'ye karşı dayanıklı Türk nohut genotiplerini belirlemek için *in vivo* biyolojik testlerin geliştirilmesi

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Abstract

Stem and bulb nematode, *Ditylenchus dipsaci* (Kühn, 1857) (Tylenchida: Anguinidae) is one of the most damaging plant-parasitic nematodes and damage most legume crops such as chickpea. The aim of the study was to develop a protocol for *in-vivo* bioassay to investigate *D. dipsaci* interaction with chickpea. Nine accessions of wild and domesticated *Cicer* spp. including three *Cicer reticulatum* Ladiz., three *Cicer echinospermum* P.H.Davis and three *Cicer arietinum* L. (Fabales: Fabaceae) were used to evaluate chickpea genotype for resistance to *D. dipsaci* between 2019 and 2020 at Çukurova University, Nematology laboratory. The incubation time and inoculum density were determined to correlate with the size of the produced nematode population. It was concluded that the highest reproduction of *D. dipsaci* on carrot discs occurred between 20 and 25ºC at 45-60 days and an initial inoculum density were determined 100 nematodes per carrot disc. Also, the initial inoculum density of 300 and the growing time of 16 weeks was the best practice to identify reaction of chickpea genotypes to *D. dipsaci*. These methods provided that quick screening for resistance studies in chickpea against *D. dipsaci* to observe information of highest reproduction period of *D. dipsaci* as time, temperature, inoculum density.

Keywords: Carrot culture, chickpea, *Ditylenchus dipsaci*, inoculum density**,** screening methods

Öz

Soğan sak nematodu, *Ditylenchus dipsaci* (Kühn, 1857) (Tylenchida: Anguinidae) en önemli zarar yapan bitki paraziti nematodlarından biridir ve nohut gibi birçok baklagillere zarar vermektedir. Çalışmanın amacı, *D. dipsaci*' nin nohut ile etkileşimini araştırmak için bir *in-vivo* biyolojik test protokolü geliştirmektir. *Ditylenchus dipsaci*'ye dayanıklılık açısından nohut genotipini değerlendirmek için üçü *Cicer reticulatum* Ladiz. (Fabales: Fabaceae), üçü *Cicer echinospermum* P. H. Davis (Fabales: Fabaceae) ve üçü *Cicer arietinum* L. (Fabales: Fabaceae) dahil olmak üzere yabani ve yerli *Cicer* türlerinin dokuz genotipi 2019-2020 yılları arasında Çukurova Üniversitesi Nematoloji laboratuvarında test edilmiştir. İnkübasyon zamanı ve inokulum yoğunluğunun, üretilen nematod popülasyonu miktarıyla bağlantılı olduğu belirlenmiştir. *Ditylenchus dipsaci*'nin havuç disklerinde en yüksek üremesinin, 20ºC ile 25ºC arasında 45-60 günde olduğu ve başlangıç inokulum yoğunluğunun havuç disk başına 100 nematod olarak saptanmıştır. Ayrıca, 300 adet bireyden oluşan inokulum yoğunluğu ve 16 hafta inkübasyon süresi nohut genotiplerinde *D. dipsaci* 'ye dayanıklılık için en iyi uygulama olmuştur. Bu yöntemler *D. dipsaci*'ye karşı dayanıklılık çalışmalarında hızlı testleme yapılması için; *D. dipsaci* en yüksek üreme periyodunun zaman, sıcaklık ve inokulum yoğunluğu olarak gözlenmesini sağlamıştır.

Anahtar sözcükler: Havuç kültürü, nohut, *Ditylenchus dipsaci*, inokulum yoğunluğu, testleme yöntemleri

[1] This study was financially supported by the Grains Research and Development Corporation as part of the Australian Coordinated Chickpea Improvement Program, Grant Project No:CSP 00185.

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Received (Alınış): 26.11.2021 Accepted (Kabul ediliş): 11.03.2022 Published Online (Çevrimiçi Yayın Tarihi): 22.03.2022

Introduction

Legume crops are an important the nutrition of millions of people globally. Chickpea, *Cicer arietinum* L. (Fabales: Fabaceae), is one of the most important legume crops. Australia, Canada, Ethiopia, India, Iran, Mexico, Myanmar, Pakistan, Russia, Turkey and the USA are among the largest chickpea producing countries in the world (FAO, 2019). *Ditylenchus dipsaci* (Kühn, 1857) (Tylenchida: Anguinidae) is an important migratory endoparasitic nematodes with the fourth-stage juveniles (J4) that have an important economic impact on agriculture crops, causing damage in below and above parts of the plant (Sikora et al., 2005). The number of wild and cultivated plant hosts for *D. dipsaci* is more than 1 200, and it is listed as an A2 quarantine nematode in the world (EPPO, 2015). In addition, it is widely observed in chickpea growing areas in Turkey. Recently, study from chickpea growing areas in Turkey indicated that 45% of the soil samples collected were infected with *D. dipsaci* (Behmand et al., 2019). Symptoms of *D. dipsaci* nematode infection in plants cause swelling and distortion of part plants such stem and leaves (Sturhan & Brzeski, 1991). However, the symptom of nematode damage depends on the plant species. For example, legume crops infected with *D. dipsaci* show symptoms such as swelling and deformation of stems and distortion on the surface plant (Sikora et al., 2005; Bridge & Starr, 2007; CABI, 2015). Thompson et al. (2000) showed that an integrated pest management strategy including rotation with non-host crops or fallow, and use of resistant cultivars is the best method to keep the nematode population below the economic threshold level in legume crops and cereals. A similar study by Trudgill, (1992) demonstrated that the use of tolerant cultivars is one of the best-integrated pest management strategies that can grow and yield well in the extremely infested region with plant-parasitic nematodes. Some sources of resistance to *D. dipsaci* are known for bean, clover, lucerne, oat and rye but none has been described for chickpea (Plowright et al., 2002). Providing useful information such as the improvement of an *in vivo* bioassay technique and clear source initial inoculum density of nematode can help the breeding program to make new resistance cultivars to the plant-parasitic nematode (Kühnhold et al., 2006). However, a chickpea improvement program for resistance cultivar to plant-parasitic nematodes is not easy because of the high genetic diversity of chickpeas. Generally, wild *Cicer* spp., *Cicer reticulatum* Ladiz. and *Cicer echinospermum* P. H. Davis (Fabales: Fabaceae), are more resistant genotypes to disease and are used in breeding programs (Rebecca et al., 2019). The levels of resistance to plant-parasitic nematodes have been diagnostic in wild *Cicer* spp. that are present in cultivated *C. arietinum* (Basandrai et al., 2011).

The effect of increasing population density of *D. dipsaci* and resistant chickpea cultivars was investigated. Investigation of factors such as the initial inoculum density of nematodes and harvest time to keep the nematode population below damage threshold levels is important in the development of a screening study in nematology. Estimating threshold levels and calculating economic thresholds for most nematodes/crops depend on the reproduction factor (RF). RF, the ratio of final and initial population density (Pf/Pi), is commonly used in nematology (Oostenbrink, 1966) as an indicator of the suitability of a host plant for a particular nematode. Thus, susceptible plants have RF > 1 and resistant or non-hosts, RF < 1 (Seinhorst, 1967). In addition, evaluation the relationship of Pi and Pf is basic to being able to predict yield reductions. Therefore, providing information about the relationship between these population densities of nematodes and crop performance is essential.

In addition, most plant-parasitic nematodes can develop in fresh root tissue. Nematodes can feed as migratory endoparasites and ectoparasites from plant tissue. Laboratory production of endoparasitic nematodes like *D. dipsaci* can be done in three ways: (1) on plants include alfalfa callus (Acosta & Malek, 1979; Draper & Smith, 1981), (2) root explants (Chitambar & Raski, 1985), and (3) carrot discs (Coolen, 1979). The latter method can give better production of endoparasitic nematodes such as *D. dipsaci* on carrot discs in Petri dishes in an incubator greater than root plants under laboratory conditions (Kühnhold et al., 2006). These nematodes reproduce easily on plant tissue like carrot discs and is the best and most practical way to reproduce migratory endoparasitic nematodes such as *D. dipsaci* in vitro on carrot discs. (O'Bannon & Taylor, 1968; Kühnhold et al., 2006). Likewise, it is one of the best ways the production of

root-lesion nematodes (Huettel, 1985). There are various studies on the reproduction of root-lesion nematodes on carrot discs (Chitambar & Raski, 1985; Kaplan & Davis, 1990; Verdejo-Lucas & Pinochet, 1992). Reproduction of *D. dipsaci* on carrot culture discs can be influenced by factors such as incubation time, temperature, and inoculum density. Abiotic factors like temperature are important for the development of *D dipsaci* on alfalfa and white clover plants (Griffith et al., 1997; Williams-Woodward & Gray, 1999). However, Blake (1962b) showed that the development population of this nematode also can affect by the growth of the host plant. Tenente & Evans (1998) indicated that the best optimum temperature for the development of this nematode is between 15 and 20ºC. It completes its life cycle (J1 to J4) in 21 to 28 days at 20ºC and between 28 to 34 days at 15ºC.

The first part of this study focused on establishing a reliable methodology for identification the reaction of wild and domesticated chickpea genotypes to *D. dipsaci* using different initial inoculation densities and times of assessment, and the second part aimed to determine (1) the effect of incubation time on the population density of *D. dipsaci*, (2) the effect of temperature on nematode reproduction, and (3) the effect of initial inoculum density on nematode reproduction on carrot discs. The second part of the study was designed to develop a protocol for carrot culture of *D. dipsaci* screening studies under sterile conditions to help attach a clean, identical and pure source of inoculum.

Materials and Methods

General Methods

Collection of nematodes and species determination

A population of *D. dipsaci* from a chickpea field in Şanlıurfa, Turkey was used in this study with nematodes extracted from 100 g of soil and 5 g roots by using a modified Baermann funnel (Hooper et al., 2005). The source population density of 2-70 nematodes/100 g of soil and 10-50 nematodes/5 g of the root. Nematodes were collected with a 5-µl micropipette using a stereomicroscope at 40× magnification and placed in a concave glass block. Standard morphological methods were to identify the plant-parasitic nematodes (Hooper, 1972; Fortuner, 1982; Brzeski, 1991). The extracted nematodes were placed in sterile falcon centrifuge tubes containing 0.1% streptomycin sulfate (w/v) and 0.1% amphotericin B (w/v) and allowed to stand at room temperature for 10 min and then rinsed three times with sterile water. The nematode suspensions were then kept at 4ºC until used.

In vivo inoculation system

Nine accessions of Turkish domesticated and wild *Cicer* spp. including three *C. arietinum* (Azkan, Çağatay and Gökçe), three *C. echinospermum* (Karabahçe, Ortance and Destek), and three *C. reticulatum* (Fabales: Fabaceae) (Şırnak, Kallen and Eğil) were assessed for resistance to *D. dipsaci* under laboratory conditions. Genotypes used in this study were collected from three sites in Diyarbakır, Şanlıurfa and Şırnak provinces of Turkey. The seeds were scarified by making a small cut in the seed coat before germinating to improve water absorption and germination in the wild *Cicer* spp. The individual chickpea seeds were disinfected with hypochlorite (4%) and ethanol (30%). Also, to enhance seed germination, about 30 seeds of each accession were placed on the surface of wet filter paper at 4ºC for 3 days in sterile Petri dishes (Garcia et al., 2006). We incubated seeds at room temperature for 16 h before planting. The study was conducted as a completely randomized block design with four replicates. A germinated seed was then planted in the open-ended standard small tube (16 cm high and 2.5 cm in diameter) that contained 60 g of autoclaved (15 min at 121ºC) field soil, (73% clay, 17% silt and 10% sand) and supported by a frame. One week after planting, the nematodes were transferred at 25ºC, and plants were inoculated with either 150, 300 and 400 nematodes/tube in 1 ml water. The experiment was conducted concurrently with all accessions and grown in a growth room at 25ºC and 50% RH under a 16:8 h L:D photoperiod provided by high-pressure sodium lamps. After a further 16 and 20 weeks, the plants were harvested and nematodes from both soil and roots extracted using a Baermann funnel and counted (Hooper et al., 2005). RF was calculated as described above.

In vitro culturing

The second part study examined the effect of incubation time, temperature and inoculum density on *D. dipsaci*) rearing on carrot cultures at Çukurova University, Nematology laboratory between 2019 and 2020. All equipment and materials were sterilized by autoclaving at 121ºC for 15 min. Also, all the working surfaces were cleaned with 70% ethanol. Moderately thick carrots were selected without cracks and washed in distilled water, then tap water, and surface sterilized in a 6% (v/v) NaOCl solution for 2 min, peeled, and soaked in 95% ethanol for 15 min. Then, 5 mm thick sections 3-4 cm in diameter were cut and transferred to sterilize Petri dishes (5-6 cm in diameter) with sterilize forceps. These were incubated in the dark for 3-4 weeks at 19-23ºC for 12-18 days until callus formed on the disc surface. The cultures were examined every week. Nematodes surface sterilized in glass measuring cylinders with 10 ml of water containing 6 mg of streptomycin with gentle mixing. After 1 h, this solution was changed to sterilized distilled water and reduced in volume, replenished with sterilized distilled water to 10 ml and left for 1 h. Fifty surface-sterilized females and males (1:1) were added to a drop of distilled water on carrot disc using a sterile needle. The discs were then incubated at 19 ± 1ºC. When the first nematodes egressed from the discs, the number of days after inoculation was recorded. The effect of time of incubation period on the development population density of *D. dipsaci* nematode was assessed at 20, 45, 60 and 90 days. Also, the effect of temperature on nematode reproduction was assessed at 15, 20, 25 and 30 ± 1ºC for 35 days. To determine the optimum number of *D. dipsaci* as initial inoculum carrot calluses were inoculated with 50, 100, 200, 300 and 500 nematodes per disc. Nematodes were inoculated in 100 µl sterile water with three drops of suspension were added to carrot disc. The average composition of life stage in the inoculum was 16% male, 57% juveniles, and 27% female. All the inoculated carrot discs were then incubated at 20 ± 1ºC in the dark for 35 days. Finally, carrot discs were cut into small pieces, and nematodes were extracted to determine the effect of time, temperature, and inoculum density on the reproduction of *D. dipsaci* nematodes. A completely randomized design with 10 replicates was conducted for the determination of these experiments.

Experimental design and analysis

The data (numbers of nematodes per tube and carrot discs) were analyzed using ANOVA in Genstat (V13). Significant differences between treatment and replication of data were calculated at $P < 0.05$. Outliers and variance distribution was assessed using residual plots. The data transformed to log10(x+1) values to provide normality.

Results

In vivo inoculation

The screening study for resistance to *D. dipsaci* indicated that there was no significant difference between growing time (16 and 20 weeks; P = 0.539) and *Cicer* spp. (P = 0.601; Table 1 and Figure 1). However, the population density of *D. dipsaci* in *C. arietinum* was higher than the any *Cicer* spp. (Figure 1). Also, the lowest population density was in *C. echinospermum* except for 16 weeks with 400 nematodes as the initial population after 16 and 20 weeks (Table 1 and Figure 1). Analysis of variance showed that initial nematode density has a large effect on final numbers (P linear = 0.009) and that there is some indication of an interaction between species and the linear effect of nematode density P = 0.086 (Table 1 and Figure 1). Figure 1 shows the RF increasing with 150-300, but not 400 nematodes applied, and that *C. echinospermum* is more responsive to the initial nematode density because it responds more steeply to the 150-300 change P = 0.086 (Table 1 and Figure 1). Thus at 150 nematode/plant *C. echinospermum* had lower final nematode counts than the other two species, while at 300 and 400 nematodes there is no significant difference. The data indicate that the initial inoculum of 300 nematodes/plant was optimal. The development of *D. dipsaci* was higher than at 150 nematodes in all species (Figure 1). The highest

population density of *D. dipsaci* in the inoculation density of 150 nematodes was observed in *C. arietinum* (Gökçe RF = 2.7, Çağatay RF = 2.6 and Menemen RF = 2.5) and the lowest population density observed in *C. echinospermum* (Karabahçe RF = 2.2, Ortance RF = 2.3 and Destek RF = 2.4).

Table 1. Analysis of Variance for chickpea resistant to *Ditylenchus dipsaci*

Source of variation	Degree of Freedom (df)	Sum Square (SS)	Mean Square (MS)	F Ratio	P Value
Period	1	0.088	0.088	0.07	0.539
Density	2	12.154	5.077	4.55	0.016
Linear	1	10.959	10.959	9.81	0.009
Species	1	0.690	0.690	0.30	0.601
Period.Density	2	0.331	0.165	0.14	0.316
Period.Linear	1	0.101	0.101	0.09	0.587
Period.Species	2	5.791	2.895	2.59	0.137
Linear.Species	2	5.447	2.723	2.44	0.086
Residual	18	20.074	1.115		
Total	30	55.634			

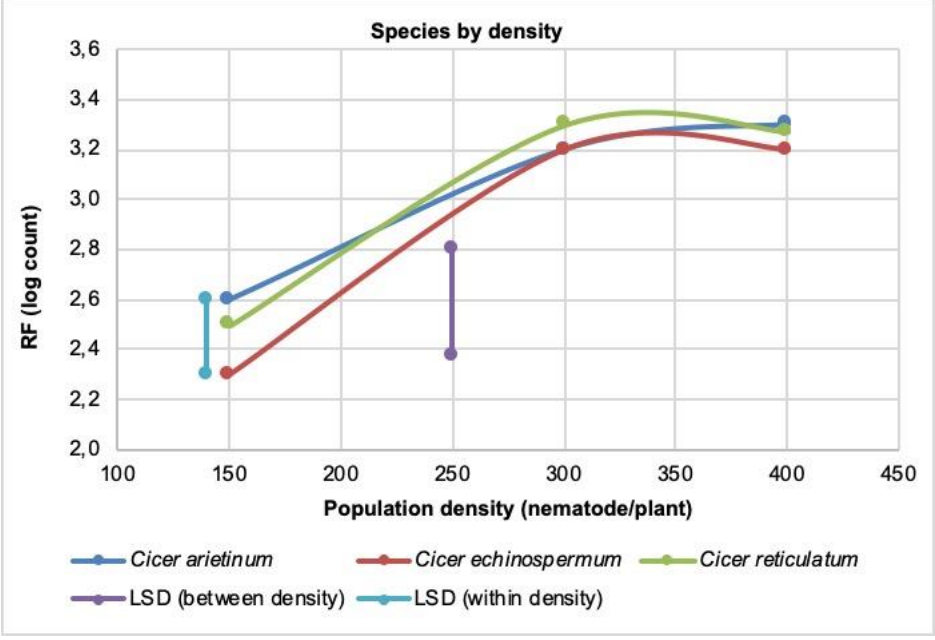

Figure 1. Effect of inoculation density on the reproduction factor (RF) of *Ditylenchus dipsaci* on chickpea species.

In vitro culturing

The population density and indicates of *D. dipsaci* increased with time 20 days after incubation at 19 ± 1ºC (Table 2 and Figure 2). The highest and lowest RF (RF = 644 and 7.4) occurred after 90 and 20 days after incubation respectively. The RF of *D. dipsaci* was 600 after 45-60 days, compared to 7.4 after 20 and 59.2 after 40 days. A higher number of nematodes and RF occurred after 45 and 60 days than after 20 and 40 days, and there were no differences observed between 60 and 90 or 45 and 60 days (Table 2 and Figure 2).

Table 2. Effect of incubation period on reproduction of *Ditylenchus dipsaci* on carrot discs after incubation with 50 nematodes

	Number of nematodes per carrot disk				
Incubation period (day)	Female	Juvenile	Male	Pf	RF
20	67	220	30	370	7.4
45	860	1 200	100	2 960	59.2
60	4 800	1 300	12 200	30 000	600.0
90	5 200	15 500	11 500	32 200	644.0
$LSD_{0.05}$	1.353	5.645	4.127	11.126	222.5

Pf, final population; Pi, initial population; and RF, reproduction factor as Pf/Pi.

In carrot cultures development population density of *D. dipsaci* was greater at 19 and 25ºC than 15 and 30ºC. There were no other significant differences observed i between 20 and 25ºC or between 15 and 30ºC (Table 3). The highest and lowest numbers of *D. dipsaci* were observed with inoculum of 100 and 500 *D. dipsaci* per disc (Table 4 and Figure 2). The maximum numbers were obtained after 35 days at 20ºC with 100 nematodes per disc. Also, it was found that by increasing nematode inoculum density from 100 to 500 the reproduction rate of *D. dipsaci* declined.

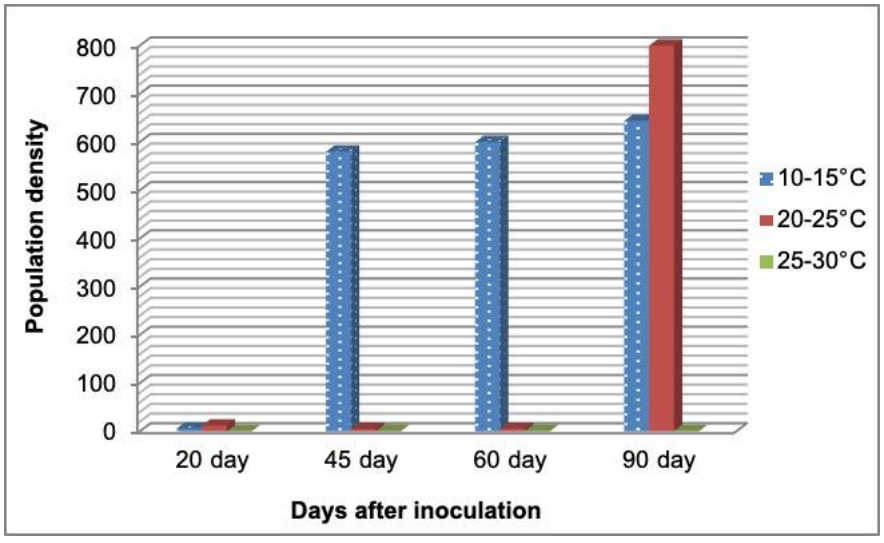

Figure 2. Effect of incubation time (20, 45, 60 and 90 days) and temperature (10, 15, 20, 25 and 30ºC) on the population density of *Ditylenchus dipsaci* nematode on carrot discs.

Table 3. The effect of temperature on reproduction of *Ditylenchus dipsaci* on carrot discs after incubation 50 nematodes

	Number of nematodes per carrot disk				
Temperature (ºC)	Female	Juvenile	Male	PF	RF
15	15	9	30	54	1.1
20	68	120	80	268	5.4
25	60	92	72	224	4.5
30	8	4	3	15	0.3
$LSD_{0.05}$	12.5	35.7	22.8	71.0	1.4

Pf, final population; Pi, initial population; and RF, reproduction factor as Pf/Pi.

Table 4. Effect of inoculum density on reproduction of *Ditylenchus dipsaci* on carrot discs at 20 ± 1ºC

	Number of nematodes per carrot disk				
Inoculum density	Female	Juvenile	Male	PF	RF
50	137	260	290	687	13.74
100	240	1 580	960	2 780	27.80
200	540	2 200	1 880	4 620	23.10
300	885	3 200	2 200	6 285	21.60
500	1 100	5 100	3 420	9 620	19.24
$LSD_{0.05}$	390.000	1.902	880	3.172	4.26

Pf, final population; Pi, initial population; and RF, reproduction factor as Pf/Pi.

Discussion

The present study was design to develop a standard protocol for culture *D. dipsaci* for use in a screening study. This method allows providing a clean, uniform, and pure source of high numbers of *D. dipsaci in vitro* rearing under sterile conditions. Kagoda et al. (2010) reported that the carrot discs provided as a food source for reproduction high number of population nematodes that used in screening study.

Overall, our results indicate that the carrot disc culture can be used to produce *D. dipsaci* obtained from chickpea. The maximum population density of *D. dipsaci* in carrot disc cultures was after 45 and 60 days incubation with an initial inoculum density of 100 nematodes at 20ºC. These results indicated that the reproduction of *D. dipsaci* between 20 and 25ºC at 45-60 days after inoculation was nearly 4 times the rate after 20 days inoculation at 20ºC. The population density of plant-parasitic nematodes depends on their complete life cycle. In plant-parasitic nematodes, passing through ecdysis (molting) from the first stage juvenile (J1) to the second stage depends on the temperature and period time (Trudgill, 1995). The study found that the population of males was more than females in carrot cultures at 25ºC indicating that a temperature rise may impact male number. A similar study by Tenente & Evans (1998) showed that the population density of males was more than females in onion tissues at 26ºC 28 days after inoculation. Laughlin et al. (1969) showed that the temperature could affect the number of females to males during the mating process. Blake (1962a) showed that the reproduction of *D. dipsaci* was highly affected by some factors such as temperature and moisture. Griffin (1974) showed that the temperature range can affect the activity and reproduction rate of *D. dipsaci* on alfalfa. Similarly, studies indicated the optimum temperature for development was between 15 and 20ºC and there was minimal activity of this nematode between 3 and 5ºC (Yüksel, 1960; Griffith et al., 1997). The initial inoculum density of nematodes, different sizes of carrots could affect food sources and cause nutrients to become depleted and initiate nematode migration or not reproduce from the carrot (Coyne et al., 2014). Similarly, Castillo et al. (1995) showed that the population density of root-lesion nematodes, *Pratylenchus thornei* Sher & Allen, 1953 (Tylenchida: Pratylenchidae) decreased with an increasing initial inoculum density of 100 nematodes on chickpea under laboratory conditions.

Interaction between initial and final population density of nematodes is important on resistance or susceptibility of different cultivars. Toktay et al. (2012) showed that optimization of these factors is necessary for the development methodology for resistance screening and to keep the population density of nematode below the damage threshold level in the wheat breeding programs. It was reported the effect of temperature on egg production, hatching and the life cycle of *Ditylenchus destructor* Thorne, 1945 (Tylenchida: Anguinidae) isolated from groundnut *in vitro* (Waele & Wilken, 1990). Also, Schomaker & Been (2006) indicated that the information on the initial population density of nematodes and predication of damage under specific conditions for specific crops is essential for an integrated pest management strategy. The screening of the different chickpea genotypes did not show a significant difference in *D. dipsaci* resistance at 16 and 20 weeks and between the initial inoculum densities of 300 and 400 nematodes/tube. However, accessions of *C. echinospermum* had more resistance than *C. arietinum* and

C. reticulatum. Thompson et al. (2011) observed that the response of the root lesion nematodes for resistance was the difference between *Cicer* spp. A similar study by Behmand et al. (2019) indicated that there were differences between *Cicer* spp. to *P. thornei* sensitivity, and both of *C. arietinum* and *C. reticulatum* accessions were more susceptible than any of the *C. echinospermum* accessions.

Based on the presented data, the standard protocol now implemented in our laboratory for production of *D. dipsaci* uses an initial inoculum density of 300 nematodes per tube with harvesting after 16 weeks. The results are similar to those obtained study by Behmand et al. (2019), who reported that there was no significant difference observed between growing times 16 and 20 weeks and between the initial inoculum density of 225 and 300 nematodes/tube to identify chickpea resistance to *P. thornei*. Chickpea is cool-season crop and requires a growing time of 100 days to reach maturity (Singh & Ocampo, 1997). Reen & Thompson (2009) indicated that the maximum difference in population density of *P. thornei* among cultivars of chickpea was observed after 18 to 20 weeks under laboratory conditions. This study was the first to assess chickpea genotypes collected from Turkey for resistance to *D. dipsaci*, some of which offer new sources of *D. dipsaci* resistance and genetic diversity useful.

These days, genomic tools and standard plant breeding technologies make it possible to study the genotypes associated with desirable phenotypes. Standard breeding methods have been highly successful for developing new cultivars. The use of standard pre-genomic breeding methods has yielded improvements to modern cultivars that allow for a dramatic increase in staple crop yields. These standard methods can help future breeding efforts aimed at *D. dipsaci* prevention. The identification of resistant *D, dipsaci* germplasm to nematode diseases is a fundamental task for breeding nematode resistant cultivars.

Acknowledgments

We thank Dr. Abdullah Kahraman (Department of Field Crops, Agriculture Faculty, Harran University) and Dr. Jens Berger (Commonwealth Scientific and Industrial Research Organization, Agriculture and Food, Perth, Australia) for helping and provision of chickpea seeds.

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Türk. entomol. derg., 2022, 46 (1): 99-114
DOI: http://dx.doi.org/10.16970/entoted.1022151

ISSN 1010-6960
E-ISSN 2536-491X

Original article (Orijinal araştırma)

Invasion history of *Orosanga japonica* (Melichar, 1898) (Hemiptera: Ricaniidae) in Turkey, comparisons with other Ricaniidae family members using molecular tools and modeling of potential global distribution

Orosanga japonica (Melichar, 1898) (Hemiptera: Ricaniidae)'nin Türkiye'deki yayılma geçmişi, diğer Ricaniidae familyası üyelerinin moleküler araçlarla karşılaştırılması ve dünyadaki potansiyel dağılımı

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Abstract

Orosanga japonica (Melichar, 1898) (Hemiptera: Ricaniidae) is an invasive species in Turkey and Caucasian area of the Palearctic. Seventeen localities were selected for molecular studies and 163 samples from Black Sea and Marmara coasts collected between 2019-2020 were evaluated for potential distribution and habitat suitability with the maximum entropy method and CORINE Land Cover (CLC) index. Molecular analysis revealed two haplotypes for mitochondrial cytochrome b and three for cytochrome oxidase I gene regions. The phylogenetic trees showed similarities for the tested gene regions and samples stated the *Ricania* and *Pochazia* samples. Trabzon population, which is showed to be the main population for Giresun, Sinop, Düzce and Zonguldak populations. Hap 3 was found only Rize, Trabzon and İstanbul populations. The results indicated that movement of the species was caused by human activity. Precipitation and temperature were found to be the most important parameters for the distribution of *O. japonica.* The whole level of CLC index indicated the distribution of *O. japonica* had significant differences between the Marmara and three Black Sea areas. The results indicated that agricultural areas are important for the distribution *O. japonica* at CLC level 1. Past and present records of the host plants indicated that *O. japonica* threatens wide range of plants along the Black Sea and Marmara coasts of Turkey.

Keywords: Genetic structure, habitat suitability, mtCOI, mtCyt-b, *Orosanga japonica*

Öz

Orosanga japonica (Melichar, 1898) (Hemiptera: Ricaniidae) Türkiye ve Palearktik bölge ile birlikte Kafkasya bölgesinde istilacı bir türdür. Moleküler çalışmalar için 17 nokta seçilmiş ve 2019-2020 yılları arasında Karadeniz ve Marmara sahil hattında 163 kayıt noktasına göre, maxent model ve CORINE arazi örtüsü indeksi (CLC) ile potansiyel dağılım ve habitat tercihleri açısından değerlendirilmiştir. Moleküler analiz sonuçları, mt-cytb bölgesi için iki, mt-COI bölgesi için üç haplotip olduğunu göstermiştir. Filogenetik ağaçlar her iki bölge için birbirine benzer yapı ortaya çıkarmış, örnekler *Ricania* ve *Pochazia* cinsi arasında yer almıştır. Trabzon popülasyonunun Giresun, Sinop, Düzce ve Zonguldak popülasyonları için ana popülasyon olduğu görülmüştür. Hap 3 sadece Rize, Trabzon ve İstanbul popülasyonlarında gözlenmiştir. Sonuçlar türün taşınımının insan eliyle olduğunu göstermiştir. Elde edilen sonuca göre, türün dağılımı için en önemli parametrelerin nem ve sıcaklık olduğu bulunmuştur. CLC indeksinin tüm seviyesine göre, tür dağılımı Marmara ve Karadeniz'deki üç bölge arasında önemli farklılık göstermektedir. Sonuçlar, CLC seviye 1'deki tür dağılımında tarımsal alanların önemli olduğuna işaret etmektedir. Konukçu bitki tercihlerinin geçmişteki ve günümüzdeki kayıtları, Türkiye'nin Karadeniz ve Marmara kıyılarında geniş bir bitki yelpazesinin tehlike altında olduğunu ve türün ekonomik açıdan önemli bitki türleri için ciddi bir tehdit oluşturduğuna işaret etmektedir.

Anahtar sözcükler: Genetik yapı, habitat tercihleri, mt-COI, mt-cytb, *Orosanga japonica*

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Received (Alınış): 11.11.2021 Accepted (Kabul ediliş): 21.03.2022 Published Online (Çevrimiçi Yayın Tarihi): 28.03.2022

Introduction

Orosanga japonica (Melichar, 1898) (Hemiptera: Ricaniidae), is an invasive species in Turkey. Even though the species was described from the Oriental Region (Melichar, 1898), then it was reported that the species was distributed in some Western Palearctic countries such as Georgia, Russia (Krasnodar) and Ukraine (Crimea) after 1950s because of climate change and developing trade (Nast, 1987; Demir, 2009, 2018; Gnezdilov & Sugonyaev, 2009; Gjonov, 2011; Gjonov & Shishiniova, 2014; EPPO, 2016; Hayashi & Fujinuma, 2016; Bourgoin, 2017; Demir, 2018; Mozaffarian, 2018). *Orosanga japonica* introduced into coastal areas of northeastern Turkey from Georgia and it was first detected from Rize in 2007 (Demir, 2009). In a short time, its distribution expanded to nearby provinces (Artvin and Trabzon) (Güçlü et al., 2010; Ak et al., 2013, 2015; Göktürk & Mıhlı, 2015). Recent studies have shown that the species has invaded western areas of Black Sea Region of Turkey (Düzce, İstanbul, Giresun, Bartın, Kocaeli, Kırklareli, Ordu, Samsun and Sinop) (Arslangündoğdu & Hızal, 2018; Demir, 2018; Öztemiz, 2018; Akıner et al., 2019; Karataş et al., 2020).

It can damage host plants by sucking, egg-laying on the young branches, and by spreading some plant parasites (Tayutivutikul & Kusigemati, 1992; Bourgoin, 2017; Mozaffarian, 2018). *Orosanga japonica* feeds on 18 species including economic important species such as kiwifruit (*Actinidia deliciosa* (Chev.) C.F.Liang & A.R.Ferguson), bean (*Phaseolus vulgaris* L.), fig (*Ficus carica* L.), cucumber (*Cucumis sativus* L.), tomato (*Lycopersicum esculentum* Mill.), grape (*Vitis vinifera* L.) and blackberry (*Rubus* spp.), belonging to 12 families that have include agriculturally important crops and fruit trees (Demir, 2009; Mozaffarian, 2018; Karataş et al., 2020). It has been assumed that the main factors in the rapid spread of *O. japonica* may have been the favorable vegetation and climate of the areas (summers are warm and humid, and winters are cool and dam in coastal lands). Also, these cultivated plants, carrying eggs, might easily transfer the pest from one region to another through human activity. It is of great importance to determine the potential distribution of introduced species, such as *O. japonica*, that can spread rapidly. For this purpose, a number of models have been developed to predict suitable habitats and areas that a species may occupy in the future.

Orosanga japonica was first described as *Ricania episcopalis* Stål, 1865 (Hemiptera: Ricaniidae) and the name of this species was changed to *Ricania japonica* Melichar, 1898 (Hemiptera: Ricaniidae) (Melichar, 1898; Hayashi & Fujinuma, 2016). However, in recent publication, the species has been described as *O. japonica* both morphologically and molecularly (Akıner et al., 2019). During the initial invasion period in Turkey, its naming was controversial. Some authors used the species name as *Ricania simulans* (Walker, 1851), while others used the species name as *R. japonica* (Demir, 2009; Ak et al., 2015). Some researchers have reported recently that the genus name of the species specified as *R. japonica* should be changed to *Orosanga* Melichar, (1898) (Bourgoin, 2017; Demir, 2018; Mozaffarian, 2018). Akıner et al. (2019) revealed that the species name *O. japonica* with using 28S rDNA gene region.

Understanding the origin and distribution of invasive species is important to better understand the species behavior in the newly invaded areas (e.g., geographical distribution, habitats and host plants). Genetic markers are tools used in both determinations of the origin and identification of species. However, the most important problem is the lack of data and literature on genetic studies. Relevant studies with *O. japonica* in Turkey have been increasing in recent years. These studies include control, identification, population status, distribution, and host plants of the species (Demir, 2009, 2018; Güçlü et al., 2010; Ak et al., 2013, 2015; Göktürk & Aksu, 2014; Göktürk & Mıhlı, 2015; Akıner et al., 2019, 2020; Karataş et al., 2020). Investigation of the past and future status are important for better understanding distribution, invasion route and difficulties for control. Genetic markers and statistical methodology may allow us to understand the invasion history and population characteristics (Garg & Mishra, 2018). Mitochondrial cytochrome b (mtCyt-b) and cytochrome oxidase I (mtCOI) gene regions are the preferred mitochondrial genes to study genetic diversity between species (Lavagnini et al., 2015; Kwon et al., 2017; Garg & Mishra, 2018). Also, the mt-COI gene region is used to investigate intraspecies genetic diversity (Kwon et al., 2015, 2017).

There are various methods for species distribution models which use the correlation of climatic and land surface within the distribution of species (Seo et al., 2008). The maximum entropy method (MaxEnt) is an algorithm program that generates habitat suitability estimates by comparing the conditional density of predictors in existing regions with the marginal density of predictors in the study area. MaxEnt raw output represents the possibility of habitat compatibility (Phillips et al., 2006). The program is both useful and popular due to the accuracy and convenience (Phillips et al., 2006; Ortega-Huerta & Peterson, 2008). MaxEnt not only measures entropy but also characterizes probability distributions of incomplete information using the presence data of species. Thus, this approach predicts the closest realistic distribution of the species by making all environmental data uniform (Phillips et al., 2006). The predictive maps of the ecological niche model that it is created using the MaxEnt environmental conditions have on a scale of 0 to 1 (lowest to highest suitability). If the area under the curve (AUC) is ≤0.5, the model's explanatory and predictive power weakens and was not used.

This study aimed to investigate the genetic structure and invasion history in Turkey with the molecular data of *O. japonica*. Secondly, determine new potential distribution areas and suitable habitats in Turkey, Europe and globally based on occurrence data for Turkey.

Material and Methods

Collection of data on *Orosanga japonica*

A total of 163 recorded localities were used of occurrence data of adults and nymphs *O. japonica* from Black Sea and Marmara Regions of Turkey in different manuscripts and our field study during 2018-2020 (Demir, 2009, 2018; Ak et al., 2015; Arslangündoğdu & Hızal, 2018; Öztemiz, 2018; Akıner et al., 2019; Karataş et al., 2020) (Figure 1).

For molecular studies, 17 localities were selected from the provinces of Rize, Trabzon, Artvin, Giresun, Sinop, Zonguldak, Düzce and İstanbul in 2018-2019 years. Selected areas were given in Table 1. Morphological identifications of all collected samples were made under computer compatible Leica EZ4 D branded stereomicroscope according to the species identification key prepared by previous studies (Rahman et al., 2012; Mozaffarian 2018). Samples were stored at −20ºC until DNA extraction in Recep Tayyip Erdoğan University Biology Department Vector Ecology and Control Laboratory (Rize, Turkey).

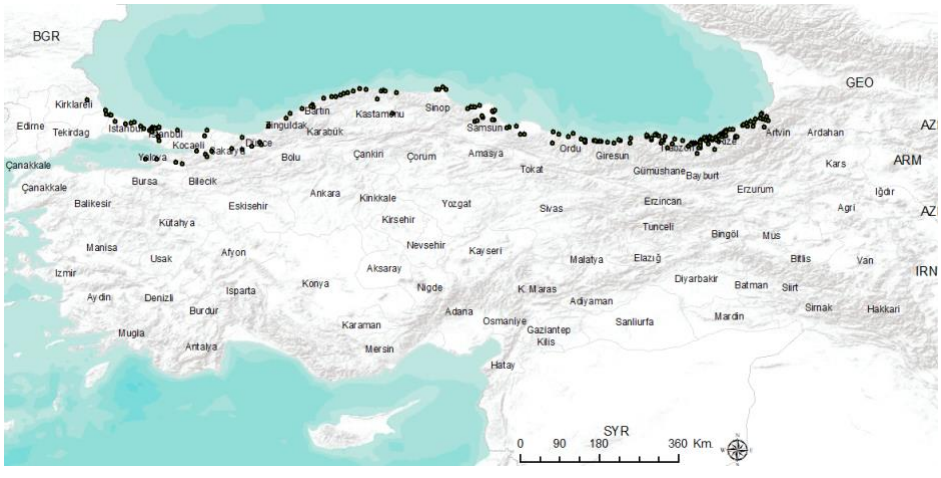

Figure 1. Recorded sites of *Orosanga japonica* from Black Sea and Marmara coast of Turkey according to the Demir (2009, 2018), Ak et al. (2015), Arslangündoğdu & Hızal (2018), Öztemiz (2018), Akıner et al. (2019), Karataş et al. (2020) and this study.

Table 1. Basic information of *O. japonica* used for molecular studies

City	Town	Sample code	Collection date	Host plant	Stage	Number of individuals
Artvin	Arhavi	AA	August 2018	*Actinidia deliciosa* (kiwifruit)	Adult	3
	Hopa	AH	August 2018	*Actinidia deliciosa* (kiwifruit)	Adult	3
	Kemalpaşa	AK	Augus2019	*Actinidia deliciosa* (kiwifruit)	Adult	3
	Sarp	AS	August 2018	*Phaseolus vulgaris* (bean)	Adult	3
Düzce	Düzce	DD	July 2019	*Rubus spp.* (blackberry)	Adult	2
Giresun	Giresun	GG	August 2018	*Cucumis sativus* (cucumber)	Adult	3
İstanbul	İstanbul	II	July 2019	*Ficus* spp. (fig)	Adult	3
Rize	Ardeşen	RA	August 2018	*Actinidia deliciosa* (kiwifruit)	Adult	3
	Çayeli	RÇ	August 2018	*Rubus* spp. (blackberry)	Adult	3
	Derepazarı	RD	August 2018	*Ficus* spp. (fig)	Adult	3
	İyidere	RI	August 2018	*Rubus* spp. (blackberry)	Adult	3
	Pazar	RP	August 2018	*Rubus* spp. (blackberry)	Adult	3
	Rize	RR	August 2018	*Alnus glutinosa* (alder)	Adult	3
Sinop	Sinop	SS	July 2019	*Rubus* spp. (blackberry)	Adult	3
Trabzon	Arsin	TA	August 2018	*Zea mays* (maize)	Adult	3
	Sürmene	TS	August 2018	*Rubus* spp. (blackberry)	Adult	3
Zonguldak	Zonguldak	ZZ	July 2019	*Zea mays* (maize)	Adult	3

DNA extraction, amplification, and sequencing

DNA isolation was performed using the GeneJET Gel Extraction Kit (Thermo Fisher Scientific, Massachusets, USA), according to the manufacturer's instructions. The whole sample was used in DNA isolation. They were stored at −20ºC until to use for PCR amplification. DNA isolation was made 10 samples for each locality and three samples were used sequencing after performing the PCR.

DNA samples were used as templates for the amplification of specific fragments of mtDNA: a 576-bp fragment for Cyt-b and a 615-bp fragment for COI. Two sets of primers that were used are given in Table 2.

Table 2. Primers used in this study

Name	Oligonucleotide sequence (5′→3′)	Reference
Full_cytb_F	GTTCTACCTTGAGGTCAAATATC	Song & Liang, 2013
Full_cytb_R	TTCTACTGGTCGTGCTCCAATTCA	Song & Liang, 2013
LCO-1490 F	GGTCAACAAATCATAAAGATATTGG	Folmer et al., 1994
HCO-2198 R	TAAACTTCAGGGTGACCAAAAAATCA	Folmer et al., 1994

Each reaction was performed in a T100™ Thermal Cycler (Bio-Rad,California, USA), in a final volume of 30 ml. For Cyt-b and COI, the PCR mixture contained 100 ng genomic DNA, 1 x buffer, 2.5 mM $MgCl_2$, 250 mM each dNTP, 100 nM each primer, and 1-unit Biolabs *Taq* polymerase. Amplification was achieved by heating at 94°C for 5 min and then subjecting the mixture to 35 cycles of 94 xC for 50 s, annealing temperature (53°C for Cyt-b and 40°C for COI) for 45 s, and 72°C for 1.5 min. The mixture was then subjected to a final extension step at 72°C for 10 min. These PCR products were analyzed by 1.5% agarose gel electrophoresis. PCR products were directly sequenced at Macrogen Inc (Amsterdam).

Phylogenetic tree construction

The nucleotide sequences were aligned by using MEGA 7 (Kumar et al., 1994) software and all sequences are clipped to the same length. The sequences were blasted at the National Center for Biotechnology using the website (www.ncbi.nlm.nih.gov/blast). Cyt-b and COI nucleotide sequences of the Fulgoridae members were obtained from the GenBank database and used on the topology of each phylogenetic tree. In the topology of the phylogenetic trees, the haplotype with the highest frequency (Hap 1 for mtCOI, Hap 1 for mtCyt-b) of the haplotype data was used to avoid data confusion. The haplotype identification was done according to Librado & Rozas (2009). The list of the haplotype of the Cyt-b and COI gene regions is given in Table 3.

Table 3. Haplotype of the mitochondrial cytochrome b (mtCyt-b) and cytochrome oxidase I (COI) gene regions

Gene Region	Haplotypes	N	Sample Code (Frequency)	GenBank ID
mtCOI	Hap 1	21	RA(3), RÇ(3), RP(3), AA(3), AH(3), AS(3), AK(3)	MW832512
	Hap 2	14	TA(3), GG(3), SS(3), DD(2), ZZ(3)	MW832511
mtCyt_B	Hap 1	15	RD(3), RI(3), RR(3), TS(3), II(3)	MW832510
	Hap 2	32	RA(3), RÇ(3), RP(3), AA(3), AH(3), AS(3), AK(3), GG(3), SS(3), DD(2), ZZ(3)	MW854830
	Hap 3	18	RD(3), RI(3), RR(3), TA(3), TS(3), II(3)	MW854831

Phylogenetic analyses for the mtCyt-b and COI were performed by maximum likelihood method (ML) analysis. The best-fitting nucleotide substitution model selection was selected using Akaike information criterion (AIC) applied using the JModeltest2 (Darriba et al., 2012). ML analysis was performed with a GTR + G + I (for Cyt-b) and GTR + I (for COI) substitution model using MEGA 7 software. Bootstrap analysis was also performed with 1000 replicates to estimate the support of nodes.

Analysis of molecular variance

Molecular parameters of genetic diversity (number of segregation sites, nucleotide diversity. haplotype diversity and number of haplotypes) of COI nucleotides were calculated with DNASP version 5.0 software (Librado & Rozas, 2009). Pairwise difference and their significance were tested using the non-parametric permutation approach described in Excoffier et al. (1992). The genetic distance of populations was calculated Arlequin v 3.5.1.2 software (Excoffier & Lischer, 2010).

Habitat suitability

Proportional differences of presence and abundance in the different habitat type of *O. japonica* in Black Sea coast and Marmara Region of Turkey were evaluated using a Williams-corrected likelihood ratio test (G-test) (Sokal & Rholf, 2012). Sampling sites were classified to habitat types according to CORINE Land Cover (CLC) database (EEA, 2018) for analyze habitat type suitability.

Environmental variables related to *Orosanga japonica*

Nineteen bioclimatic variables and elevation thought to severely constrain the distribution of the species, were used to predicting of current potential distribution maps. Variables were obtained from the WorldClim (Fick & Hijmans, 2017) global climate database, which is often used for species distribution modeling and have data that includes the entire world, at 30 s (1 km^2) spatial resolution as the most recent version 1970-2000 (30 years). In ArcGIS 10.1, the variables were converted to ASCII file format using a species distribution model tool (Brown et al., 2017). Three prediction maps were used in the study. The first and second maps including to global and European potential distribution of the species using Turkey presence data and 19 bioclimatic variables and elevation. The setting was including to linear, quadratic and product features (auto features) and analysis was run on 30 replicates.

The third map was covering to only Turkey (Table 4). The variables were clipped to represent the country, and then the variables reprocessed again to remove possible inaccurate from caused by high correlation (Byers et al., 2013). Therefore, all variables to use were tested in ArcGIS using the multivariate band acquisition statistics tool for correlation relations. Highly correlated pairs of variables ($R > 0.80$) were removed from the analysis by making a Pearson's correlation matrix for all pairs of variables. Therefore, 12 variables were used for the model construction. The codes and explanations of used variables are given in Table 4. Settings were selected as automatic features (sample size > 80). Replicate run type was included in the analysis as cross validate to include 100 repetitions.

Modeling performance evaluation was made using the AUC value. The AUC shows the precision of the model and is used in the training field to test the performance of the model with real observations. The

AUC value takes a value between 0 and 1. If this value is below 0.5, the model predicts randomly, and a value close to 1 indicates the optimum model performance with high model performance. The relative contributions of the environmental variables used in the MaxEnt model were estimated using permutation significance estimation.

Table 4. Environmental variables used in MaxEnt model (source: WorldClim)

Code	Variable	Unit
Alt	Altitude	m
Bio 1	Annual mean temperature	°C
Bio 2*	Mean diurnal range (mean of monthly max temp - min temp)	°C
Bio 3*	Isothermality (BIO2/BIO7) (×100)	-
Bio 4*	Temperature seasonality (SD ×100)	-
Bio 5*	Max temperature of warmest month	°C
Bio 6*	Min temperature of coldest month	°C
Bio 7	Temperature annual range (BIO5-BIO6)	
Bio 8*	Mean temperature of wettest quarter	°C
Bio 9*	Mean temperature of driest quarter	°C
Bio 10	Mean temperature of warmest quarter	°C
Bio 11	Mean temperature of coldest quarter	°C
Bio 12*	Annual precipitation	mm
Bio 13	Precipitation of wettest month	mm
Bio 14	Precipitation of driest month	mm
Bio 15*	Precipitation seasonality (CV)	-
Bio 16	Precipitation of wettest quarter	mm
Bio 17*	Precipitation of driest quarter	mm
Bio 18	Precipitation of warmest quarter	mm
Bio 19*	Precipitation of coldest quarter	mm

* variables used in prediction maps for Turkey.

Results

Construction of a molecular phylogenetic tree

Mitochondrial Cyt-b gene was used for the molecular phylogenetic analysis of *O. japonica*; with 10 species Fulgoroidea members given in Table 5 and the largest haplotype (Hap 1) obtained from the samples. *Orosanga japonica* samples were located between *Ricania* and *Pochazia* genus species (Figure 2).

Table 5. Access numbers and other information of the GenBank samples for mitochondrial cytochrome b (mtCyt-b) and cytochrome oxidase I (COI) gene regions

mtCyt-b		mtCOI	
Access number	Species	Access number	Species
KX371891	*Ricania speculum*	KX371891	*Ricania speculum*
JX556854	*Ricania marginalis*	JN242415	*Ricania marginalis*
KU377157	*Ricania shantungensis*	KX721251	*Ricania shantungensis*
KX702898	*Metcalfa pruinosa*	KR043727	*Metcalfa pruinosa*
MN607209	*Lycorma delicatula*	KX721251	*Lycorma delicatula*
JX556843	*Magadha flovisigna*	KX721251	*Magadha taibaishanensis*
JX556855	*Ricania simulans*		
JX556852	*Pochazia confusa*		
KC517496	*Pochazia guttifera*		

The mtCOI gene region was used for molecular phylogenetic analysis of *O. japonica*. The largest haplotype obtained from the haplotype analysis (Hap 1) and six species of Fulgoroidea members were used in the tree structure. The species used are given in Table 5. *Pochazia* spp. samples could not use in the tree construction due to the lack of relevant GenBank accessions. The *O. japonica* specimens were located close to the *Ricania* specimens and the structure of the phylogenetic tree was almost identical to that of the tree made with the Cyt-b gene region.

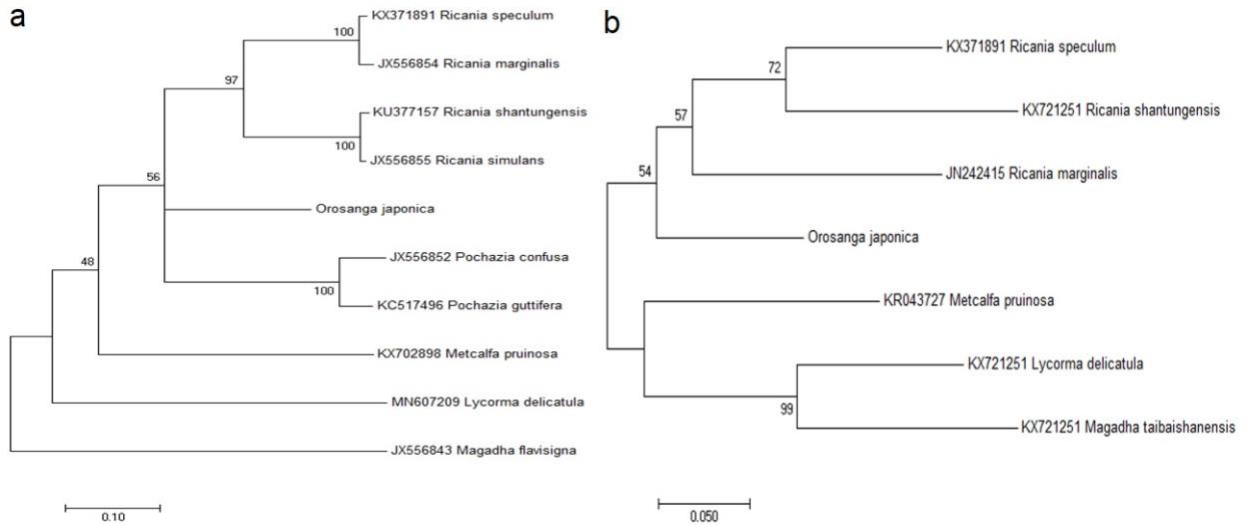

Figure 2. Phylogenetic trees according to the maximum likelihood analysis with a) mitochondrial cytochrome b sequence data, and b) cytochrome oxidase I sequence data.

Genetic diversities and haplotype distribution of *Orosanga japonica*

Since the mtCOI gene region is more variant than the mtCyt-b gene region, it was thought that it would give more sensitive results. Therefore, the mtCOI gene region was used in genetic diversity and genetic relationships among populations studies. In total, three haplotypes were identified for mtCOI. Hap 1 included a total of 21 samples and consisted of samples from the Artvin and Rize populations. Hap 2; It included 14 samples containing populations from Trabzon, Giresun, Sinop, Düzce and Zonguldak. Hap 3; It included 15 samples of the Rize and Trabzon and İstanbul populations (Table 6).

Table 6. Haplotype frequencies in populations of COI

Haplotype	Rize (n = 18)	Artvin (n = 12)	Trabzon (n = 6)	Giresun (n = 3)	Sinop (n = 3)	Düzce (n = 2)	Zonguldak (n = 3)	Istanbul (n = 3)
Hap 1	9	12	0	0	0	0	0	0
Hap 2	0	0	3	3	3	2	3	0
Hap 3	9	0	3	0	0	0	0	3

Nucleotide sequences of mtCOI (615 bp) were used for haplotype analysis. A total of 50 nucleotide sequences for mtCOI were used. For mtCOI, there were three haplotypes and three segregating sites were observed. The haplotype Hap 1 was the haplotype with the highest frequency, accounting for 54% of the total samples, and was found only in the Artvin and Rize populations. The haplotype Hap 2 made up about 31% of the total samples, and it was the haplotype found in different geographical regions (Trabzon, Giresun, Sinop, Düzce, Zonguldak). The haplotype Hap 3 was found in 15.4% of the samples and was only in the Artvin and Trabzon populations. The haplotype and nucleotide diversity values of all sequences are 0.669 and 0.001372, respectively. As a population, the highest haplotype and nucleotide diversity were observed in the Trabzon population as 0.6000 and 0.00195, respectively. The second highest haplotype and nucleotide diversity were observed in the Rize population as 0.529 and 0.00086, respectively. For other populations, haplotype and nucleotide diversity were calculated as 0 (Table 7).

Table 7. Number of segregation sites, nucleotide diversity, haplotype diversity, number of the haplotypes for cytochrome oxidase I gene region

Population	N[1]	S[2]	π[3]	Hd[4]	H[5]
Rize	18	1	0.00086	0.529	2
Artvin	12	0	0	0	1
Trabzon	6	2	0.00195	0.600	2
Giresun	3	0	0	0	1
Sinop	3	0	0	0	1
İstanbul	3	0	0	0	1
Zonguldak	3	0	0	0	1
Düzce	2	0	0	0	1
Total	50	3	0.00137	0.669	3

[1] Number of the individuals; [2] number of segregation sites; [3] nucleotide diversity; [4] haplotype diversity; and [5] number of haplotypes.

Genetic relationships among the seven local populations

The genetic distance among the eight populations was determined by the paired FST and significant results ($p < 0.05$) ranged from 0.41 to 1.0 (Table 8). While the pair between Artvin and Giresun, Sinop, Zonguldak, Düzce, and Istanbul exhibited the highest value (1.0), Rize and Artvin are the lowest value (0.41) (Table 8).

Table 8. Pairwise FST using partial sequences of mitochondrial cytochrome oxidase I from eight populations of *Orosanga japonica* and its statistical significance

	Rize	Trabzon	Artvin	Giresun	Sinop	Zonguldak	Düzce	İstanbul
Rize		NS	***	***	***	**	***	NS
Trabzon	0.204		***	NS	NS	NS	NS	NS
Artvin	0.415	0.547		**	***	***	***	***
Giresun	0.710	0.250	1.000		NS	NS	NS	NS
Sinop	0.710	0.250	1.000	0.000		NS	NS	NS
Zonguldak	0.710	0.250	1.000	0.000	0.000		NS	NS
Düzce	0.690	0.143	1.000	0.000	0.000	0.000		NS
İstanbul	0.250	0.250	1.000	1.000	1.000	1.000	1.000	

*** $p < 0.001$; ** $p < 0.01$; NS, not significant.

Prediction of *Orosanga japonica* global distribution

Prediction of *O. japonica* global distribution was made using 19 variables and elevation and presence of the species in Turkey show that the Palearctic, Oriental and Nearctic biogeographic regions of located at 20°-60° north latitude are suitable areas for the species (Figure 3a). The model was the average of 30 replicates and the AUC and SD values were 0.988 and 0.013, respectively. The model was corrected that presence of the species in China, Iranian, Georgia and Japan, Korean, Russia (Krasnodar) and Ukraine. In addition, it was seen that there are large suitable areas for the species in the European continent (especially the Mediterranean coastal areas) (Figure 3b). The maps are categorized on a four-color scale according to the suitability; red indicates high probability, green medium-high probability, yellow low-probability and blue unsuitable.

Turkey prediction of *Orosanga japonica*

As expected, prediction maps using 12 variables showed that the northern areas of Turkey near the Black Sea and the Marmara are suitable for *O. japonica*. In addition, the Mediterranean coasts, especially the Amanos Mountains and the interior of the Aegean Region are potentially suitable areas for the species. The potential geographical distribution of the species is given in Figure 3 based on current data. In our analysis, the AUC value for training data was 0.981 ± 0.017. According to the result of the analysis, variables Bio 19, Bio 9, Bio 14 and Bio 4 (in that order) were the most important determinants the distribution of the species (Table 9 and Figure 3).

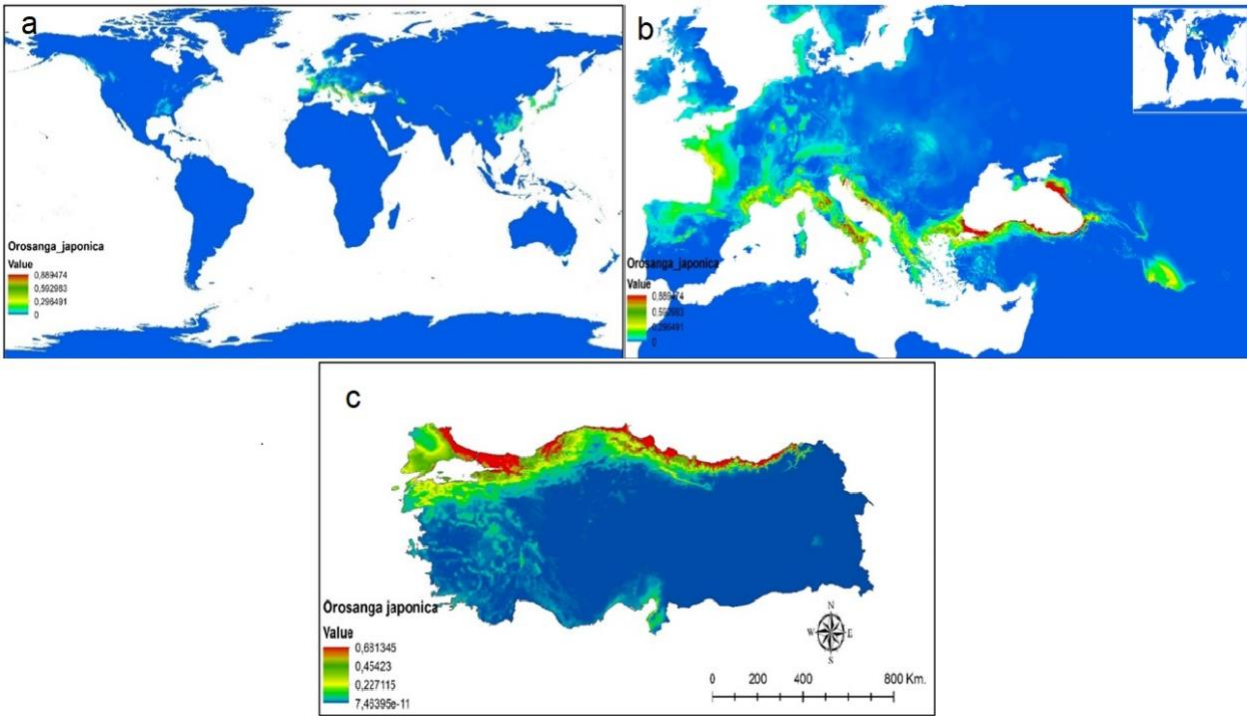

Figure 3. Potential distributions of *Orosanga japonica*: a) global, b) Europe, and c) Turkey.

Table 9. Selected environmental variables, Percent contribution and their percent contribution in Maxent model

Variable	Percent contribution (%)	Permutation importance
Bio 19	31.7	2.4
Bio 9	16.7	1.7
Bio 14	15.6	3.7
Bio 4	13.4	35.7
Bio 2	7.4	5.8
Bio 8	6.9	3.1
Bio 6	2.5	0
Bio 15	1.7	0.3
Bio 10	1.6	0.4
Bio 3	0.8	1.4
Bio 7	0.5	0.1
Bio 1	0.4	37.8
Alt	0.3	0
Bio 5	0.3	7.5
Bio 13	0.1	0.1
Bio 12	0	0.1
Bio 16	0	0.1
Bio 18	0	0
Bio 17	0	0
Bio 11	0	0

In modeling, the response curves of thees variables (Bio 19, Bio 9, Bio 14 and Bio 4) contributed more than 10% to the model as shown in Figure 4. Bio 19 (precipitation of coldest quarter) had a positive relationship to the distribution of the species up to about 200 mm and after that it was negatively associated. Bio 9 (mean temperature of driest quarter) was positively associated with the distribution of the species between -10 and 20ºC, with a negative association above 20ºC. Bio 14 (precipitation of driest quarter) had positive association to about 70 mm and negative above that value. The response relationship for Bio 4 (temperature seasonality) was quite narrow and positively between 400 to 800.

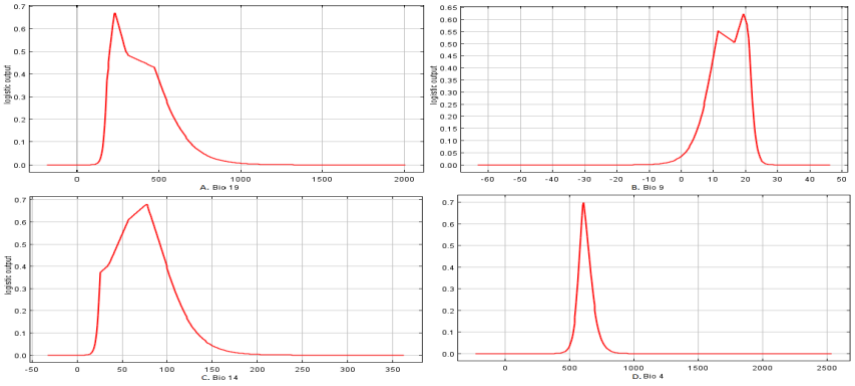

Figure 4. Response curve of most contribute variables (A. Bio 19, B. Bio 9, C. Bio 14 and D. Bio 4).

In addition, the tolerance range of the species was calculated from the response curves. While the lowest temperature tolerance of the species was -8.6ºC (Bio 6), the highest temperature tolerance was calculated as 29.1ºC (Bio 5). The precipitation tolerance range of the species was calculated as 0 mm (Bio 13) to 2200 mm (Bio 18) (Table 10).

Table 10. *Orosanga japonica* tolerance range and ideal range values

Code	Maximum tolerance range	~≥ 50% logistic output	unit
Alt	0-1000	0-250	m
Bio 1	5-28	12-18	°C
Bio 2*	2-13	6-8	°C
Bio 3*	22-50	27-33	
Bio 4*	566.1-770.3	550-650	
Bio 5*	23.7-29.1	24-27	°C
Bio 6*	-8.6 -3.9	2-4	°C
Bio 7	15-35	22-25	
Bio 8*	4.1-13.7	10-13	°C
Bio 9*	-2.4-22.7	4-15	°C
Bio 10	5-25	20-23	mm^3
Bio 11	-5-17	7-8	mm^3
Bio 12*	500-5000	600-1200	mm^3
Bio 13	0-700	100-200	mm^3
Bio 14	0-200	40-80	mm^3
Bio 15*	18-75	20-30	-
Bio 16	100-2000	300-500	mm^3
Bio 17*	50-750	150-300	mm^3
Bio 18	100-2200	120-250	mm^3
Bio 19*	100-1100	200-300	mm^3

Habitat suitability

Analysis results of the distribution in habitat types of CLC level 1 habitat types showed significant differences (G = 19.8, d, df= 6, p = 0.003). Species predominately in agricultural (Black Sea Region), forest and semi natural areas (Marmara Region) in CLC level 1. Significant differences were found in CLC level 2 habitat types (G = 33.6, d, df = 15, p=0.003). While the species is distributed in permanent crop areas in Eastern and Middle Black Sea Regions, also it is distributed heterogenous agricultural areas in western Black Sea and Marmara Regions. Similarly, significant differences were found CLC level 3 habitat types between the regions (G = 59.9, d, df = 27, p = 0.0002). The species is exclusively found in fruit trees and berry plantation areas in Eastern and Middle Black Sea area. The predominate distribution pattern of the species in western Black Sea area is areas principally occupied by agriculture with significant areas of natural vegetation. Marmara Region distribution had a different pattern than in the other areas with CLC level 3 habitat types. The species predominate distribution pattern was similar in three different habitat

types (discontinuous urban fabric, land principally occupied by agriculture with significant areas of natural vegetation, mixed forest) (Table 11).

Table 11. Habitat suitability for *Orosanga japonica* and their frequency (%)

		Black Sea Area			Marmara
Level	CORINE Land Cover nomenclature	Eastern	Middle	Western	Marmara
1	Artificial surface	17	8	15	28
	Agricultural areas	72	56	53	36
	Forest and natural areas	10	36	27	40
2	Urban Fabric	16	8	15	24
	Industrial, commercial and transport unit	1	0	0	4
	Permanent crop	39	32	12	8
	Heterogenous agricultural areas	28	24	42	28
	Forest	8	24	27	28
	Scrub and/or herbaceous vegetation associations	2	12	0	12
3	Continuous urban fabric	0	0	0	4
	Discontinuous urban fabric	16	8	15	20
	Industrial or commercial units	1	0	0	4
	Fruit trees and berry plantations	39	32	12	8
	Complex cultivation patterns	18	16	4	8
	Land principally occupied by agriculture, with significant	15	8	39	20
	Broad-leaved forest	7	12	15	4
	Coniferous forest	1	8	7	4
	Mixed forest	0	4	4	20
	Transitional woodland/shrub	2	12	0	3

Discussion

Orosanga japonica is native to West Asia. It was first detected in Turkey in Rize Province, in 2007 and has continued to spread along Black Sea coastal areas. The most recent studies on this species revealed that it was distributed in western Palearctic as *O. japonica* but had previously been identified as *R. japonica* and *R. simulans* (Demir, 2009; Gjonov, 2011; Ak et al., 2013, 2015; Mozaffarian, 2018; Akıner et al., 2019). Also, ecological, genetic studies and molecular phylogenetic studies on this genus indicate that its distribution in the western Palearctic is limited. Therefore, the present study aimed to contribute to the population genetic analysis and phylogeny using mitochondrial markers and determine the current distribution and potential habitat suitability of *O. japonica* using MaxEnt to help with management strategies and national long-term agricultural plans for this important pest in Turkey, Palearctic and the world.

The phylogenetic trees were prepared using the Cyt-b gene region from our samples and Genbank. Our studied samples located separate branches from other Ricaniidae samples generally known to be large and widespread genera (*Pochaiza* and *Ricania*). Kwon et al. (2017) considered that *Ricania* and *Pochazia* are congeneric and misidentified in many morphological studies. To clarify the species situation, we conducted the molecular phylogenetic analysis with ML. Our results are similar to those of Song & Liang (2013) with 65 taxa of Fulgoridae ITS and Cyt-b regions our comparing groups. However, Song & Liang (2013) did not included *Orosanga* or *O. japonica* samples. Tree topologies of Song & Liang (2013) and Kwon et al. (2017) showed similarities between the genera *Ricania* and *Euricania*. Our samples are located between *Pochazia* and *Ricania* genus and closer *Pochazia* than *Ricania*. Therefore, identification made using morphological characters may be erroneous within this group for *Pochazia* and *Ricania*.

The structure of the phylogenetic trees prepared using the COI gene region showed almost the same results as the structure of the tree made using the mtCyt-b gene region. However, the tree obtained using this region is not as detailed as the tree made with the mtCyt-b gene region. This is due to the lack of sequence data of the close groups of this gene region. In addition, *R. simulans* samples from Turkey (e.g., CGAEC029 accession number sequence) from Barcoding of Life Data System (www.boldsystems.org) had high similarity (99%) with *O. japonica*. These are probably misidentified records. The species has been given as *R. simulans* in some publications (Güçlü et al., 2010; Ak et al., 2013, 2015; Göktürk & Aksu, 2014;

Göktürk & Mıhlı, 2015). However, recent molecular and morphological studies have accepted that the species is *O. japonica* (Demir, 2018; Arslangündoğdu & Hızal, 2018; Akıner et al., 2019; Karataş et al., 2020).

Three haplotypes were found using the mtCOI gene region and two haplotypes for mtCyt-b. This result indicates a lack of genetic diversity of the species perhaps due to a bottleneck or founder effect of. However, the latter scenario is more likely. Akiner et al. (2019) reported the existence of six haplotypes belonging to the species in the Black Sea Region. This situation is not consistent with the results obtained in the present study. This is probably due to the difference in gene regions used. This may be due to the existence of the site showing more variation in a 28S-rDNA region than Cyt-b and COI. Given that low haplotype diversity is common in invasive species (Kwon et al., 2015; Kim et al., 2020). According to the results obtained in the data of the COI gene region, it was determined that Trabzon and Rize populations, which are very close to each other geographically, contain all haplotypes. This is a clear indication that this species first established in these areas. The definition of *O. japonica* in Rize for the first time also supports this conclusion (Demir, 2009). Likewise, Akıner et al. (2019) found six haplotypes in their study including Artvin, Rize, Trabzon, and Giresun Provinces, and the main haplotype consisting of 22 samples included Artvin, Rize, and Trabzon Provinces. The fact that Hap 2 was in Trabzon, Giresun, Sinop, Düzce and Zonguldak Provinces is possibly because they originated from the Trabzon population, which is considered to be the primary population. Also, the Trabzon population was found not to be significantly different between the Giresun, Sinop, Düzce and Zonguldak populations ($p > 0.05$). In contrast, the population of Istanbul Province had Hap 3. This haplotype was found only in the Rize and Trabzon populations. This situation indicates that the species was transported to this region by human activities. Among these regions, human movement is quite intense. However, there was no significant difference between the populations ($p > 0.05$).

Karataş et al. (2020) published the most recent and comprehensive distribution of the species. However, the present study included new records from the provinces of Kastamonu, Zonguldak, Sakarya, Yalova, Bursa (Asiatic part of Turkey) and Tekirdağ (Thracian area of Turkey). At the national level, the modeling of potential distribution of *O. japonica*, AUC (area under the curve) score, showed a substantially high value for global and Turkey prediction maps (0.988 and 0.981 respectively). This indicates that the result obtained is highly reliable. The potential distribution model of the species revealed that it can establish in the Black Sea, Marmara (including Thrace) and Aegean (inland areas) Regions, and Mediterranean coast, especially the Amanos Mountains. It has been shown that the species can reach damaging population densities in these areas, especially along the Black Sea coast, which contains suitable habitats for suitable of the species. The results obtained by niche modeling analysis were consistent with the field observations published by Karataş et al. (2020). Also, past and present records of the species are mostly located coastal areas of the Black Sea and Caspian Sea (Nast, 1987; Gnezdilov & Sugonyaev, 2009; Gjonov, 2011; Hayashi & Fujinuma, 2016; Mozaffarian, 2018). The occurrence of *O. japonica* is known in the Black Sea and Marmara Regions in Turkey (Karataş et al., 2020 and this study). The present results revealed that the species can also expand to the Mediterranean and Aegean Regions. Although, it was determined that the Aegean Region was mostly not suitable for the species, the Amanos Mountains in the Mediterranean Region were suitable. In fact, considering the known host range and ecological (high humidity and suitable temperature) demands of the species, it is logical that the species does not pose a serious threat to the Aegean Region because this region has less humid and suitable vegetation compared to the Black Sea coastal areas. In addition, while only the coastal area of the Black Sea Region is suitable for the species, interior parts of the Aegean Region have been found to be more suitable for the species. The reason for this situation is thought to be due to the fact that humidity can be maintained in the interior parts of the Aegean Region. The reason why the Amanos Mountains are suitable for the species may be related to the geographical structure of this region, with it being ecologically similar to the Black Sea Region.

The global potential distribution areas revealed the suitability of the native range of the species and in other invaded areas (Nast, 1987; Gnezdilov & Sugonyaev, 2009; EPPO, 2016; Hayashi & Fujinuma, 2016; Bourgoin, 2017). In addition, the species has a wide distribution potential in the European continent.

Research of the *O. japonica* revealed the spread of the species from east to west in Turkey (Demir, 2018; Akıner et al., 2019, 2020). The occurrence of the species in Bulgaria is also reported (Bourgoin, 2017). This result reveals that the species is likely to spread to other European countries in the future.

The most important climatic determinants of the geographical distribution of the species were basically temperature and precipitation, especially model variables Bio 19, Bio 9, Bio 14 and Bio 4 are important. These results suggest that the humidity-related variables (e.g., Bio 19) in the distribution of the species are highly predictive. Species distribution is also related to the occurrence of host plants for the species. It is reported that this species mostly deposits eggs on humidity-loving plants such as tea, black alder, blackberry and kiwifruit. Kim et al. (2017) reported for a closely related species, *Pochazia shantungensis* (Chou & Lu, 1977) that their eggs had greater survival success in areas with high humidity during the winter months. Another important variable (Bio 9), which was shown to be highly predictive for the distribution of the species, is related to the temperature. Baek et al. (2019) reported that the temperature related variables positively affected the distribution of the *R. shantungensis* in the Korea. Bradie & Leung (2017) indicated that temperature and precipitation are most important environmental variables related to the target species occurrence when reviewing results from the Maxent models from different studies.

The potential distribution of *O. japonica* determined in the present study indicated that the agricultural areas are important at CLC level 1. Also, the differentiation of the habitat suitability from the east to west at CLC level 3 was important. Although the species mainly occurs in fruits tree and berry plantation areas in eastern and middle Black Sea areas, its distribution in the western Black Sea and Marmara Region included areas of agriculture and natural vegetation. Also, it was predicted for discontinuous urban areas in the Marmara Region. Potential distribution is most probably related to te host plant abundances but the species has a wide range of known host plants and news are possible in newly invaded areas. Wilson & O'Brien (1987) reported the economically-important hosts of *O. japonica* with *O. japonica* most commonly found on *Phaseolus sp.*, *Morus sp*., *Camellia sinensis* and *Cannabis sativa*. Tayutivutikul & Kusigemati (1992) reported that the host for *O. japonica* in Japan, Taiwan and Korea with *Morus austrialis* Poir., *Wisteria brachybotrya* Siebold & Zucc., *Vigna angularis* (Willd.) Ohwi & H.Ohashi, *P. vulgaris*, *Glycine max* (L.) Merr., *Pueraria montana* (Lour.) Merr, *Citrus* spp. and *Camellia sinensis* (L.) Kuntze as common hosts. Ak et al. (2015) reported that the hosts after the initial invasion in Turkey were *Sambucus* sp., bean, kiwifruit, wild blackberry, bigleaf hydrangea, fig, alder, common laurel, tea and grapevine. Karataş et al. (2020) also reported 18 other plant species in the 12 families as hosts, excluding those given above. This situation indicates that *O. japonica* threatens wide range of plants in newly invaded areas in the Black Sea and Marmara Region of Turkey. Host range in the original areas and newly invaded areas are different so may be a shift host plant association that differ from the original areas. This shows that *O. japonica* can be serious threat to economically important plant species in these areas considering the field dynamics and plant growth profiles of the Black Sea and Marmara Regions. It raises the concern that the lack of active pest control strategies may least to serious damage, especially in agricultural and forests regions. However, it is reassuring that in the areas where the species has spread, native predators or biological control agents belonging to the species have emerged over time (Akıner et al., 2020; Karataş et al., 2020). During this period its spread and damage to host plants should be carefully monitored and control strategies developed according to the field data.

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Türk. entomol. derg., 2022, 46 (1): 115-127
DOI: http://dx.doi.org/10.16970/entoted.1055181

ISSN 1010-6960
E-ISSN 2536-491X

Original article (Orijinal araştırma)

Biochemical and molecular identification of root-knot nematodes in greenhouse vegetable areas of Eastern Mediterranean Region (Turkey)[1]

Doğu Akdeniz Bölgesi (Türkiye) örtüaltı sebze alanlarında kök-ur nematodlarının biyokimyasal ve moleküler tanımlanması

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Abstract

Root-knot nematode surveys were conducted during the growing seasons of 2017 and 2018 to cover the greenhouse vegetable areas in Mersin, Hatay and Adana Provinces in the Eastern Mediterranean Region (Turkey). A total of 46 root-knot nematode populations were characterized using biochemical and molecular diagnostic techniques. DNA extraction was done from second-stage juvenile and in molecular tests using SCAR primers, *Meloidogyne arenaria* (Neal, 1889) Chitwood, 1949, *Meloidogyne javanica* (Treub) Chitwood, 1949, *Meloidogyne incognita* (Kofoid & White, 1919) Chitwood, 1949, *Meloidogyne hapla* Chitwood, 1949 and *Meloidogyne ethiopica* Whitehead, 1968 (Tylenchida: Meloidogynidae) species were screened. Samples obtained from thirty-nine regions were identified as *M. incognita* (85%) and seven as *M. javanica* (15%). In addition, young females obtained from all regions were biochemically analyzed using the polyacrylamide gel electrophoresis diagnostic method. The esterase enzyme profile was examined to identify the *M. incognita, M. javanica* and *M. ethiopica* groups. Esterase phenotype I1 band was observed in 62% of *M. incognita* populations and esterase phenotype I2 band was observed in 39%. Esterase phenotype J3 band was detected in all *M. javanica* populations. *M. javanica* and *M. incognita* were verified and also supported by molecular and biochemical methods in the Eastern Mediterranean Region.

Keywords: *Meloidogyne*, PAGE, PCR, root-knot nematodes, vegetable

Öz

Kök ur nematodu sürvey çalışmaları Doğu Akdeniz Bölgesi (Türkiye)'nde bulunan Mersin, Hatay ve Adana illerinde yoğun olarak sebze üretimi yapılan sera alanlarını kapsayacak şekilde 2017 ve 2018 yılı üretim sezonu boyunca yapılmıştır. Biyokimyasal ve moleküler tanılama teknikleri kullanılarak toplam 46 kök-ur nematodu popülasyonu karakterize edilmiştir. DNA ekstraksiyonu ikinci dönem larvalardan yapılmış olup, SCAR primerleri kullanılarak yapılan moleküler analizde *Meloidogyne arenaria* (Neal, 1889) Chitwood, 1949, *Meloidogyne javanica* (Treub) Chitwood, 1949, *Meloidogyne incognita* (Kofoid & White, 1919) Chitwood, 1949, *Meloidogyne hapla* Chitwood, 1949 ve *Meloidogyne ethiopica* Whitehead, 1968 (Nematoda: Heteroderidae) türleri taranmıştır. Sonuçlar incelendiğinde, otuz dokuz bölgeden elde edilen örnekler *M. incognita* (%85) olarak, yedi örnek ise *M. javanica* (%15) olarak tespit edilmiştir. Ek olarak, polyacrylamide jel elektroforez tanılama yöntemi kullanılarak tüm bölgelerden elde edilen genç dişi bireyler biyokimyasal olarak analiz edilmiştir. *Meloidogyne incognita, M. javanica* ve *M. ethiopica* grubu tanılamak için esteraz enzim profiline bakılmıştır. *Meloidogyne incognita* popülasyonlarında %62 oranında esteraz fenotip I1 bandı, %39 oranında ise esteraz fenotip I2 bandı gözlenmiştir. Bütün *M. javanica* popülasyonlarında esteraz fenotip J3 bandı tespit edilmiştir. *Meloidogyne javanica* ve *M. incognita*, Doğu Akdeniz Bölgesi'nde moleküler ve biyokimyasal yöntemlerle tanılanmış olup, bu iki yöntemden elde edilen sonuçlar birbirini desteklemektedir.

Anahtar sözcükler: *Meloidogyn*e, PAGE, PCR, kök-ur nematodları, sebze

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Received (Alınış): 26.01.2022 Accepted (Kabul ediliş): 06.04.2022 Published Online (Çevrimiçi Yayın Tarihi): 13.04.2022

Introduction

The total greenhouse vegetable production area in Turkey is about 80 kHa. Adana, Hatay and Mersin Provinces in the Eastern Mediterranean Region have about 30 kHa of greenhouse vegetable area and are one of the most important centers of Turkey in this respect (TUIK, 2021).

More than half of the world's agricultural lands are infested with root-knot nematodes characterized by forming galls on the roots of host plants (Taylor, 1987; Trudgill & Blok, 2001). The size of the damage caused by root-knot nematode varies according to the type of host and climatic conditions (Netscher & Sikora, 1990; Karssen & Moens, 2006; Greco & Di Vito, 2009; Collange et al., 2011).

Root-knot nematodes inhibit plant growth by reducing nutrient and water uptake. They also increase plant susceptibility to fungal and bacterial disease due to the lesions they caused (Netscher & Sikora, 1990; Trudgill & Blok, 2001). The correct identification of the species is the most important for determining the economic control methods. There has been a considerable increase in the number of root-knot nematode species recognized through the development of diagnostic techniques in recent years. Hunt & Handoo (2009) and Jones et al. (2013) list more than 100 root-knot nematode species across the world.

With the development of diagnostic techniques, molecular and biochemical methods can make specific, reliable and accurate diagnoses in a short time. Markers such as SCAR, mtDNA, SSR and ISSR are currently used in molecular diagnosis of *Meloidogyne* spp. (Gözel et al., 2016). Also, root knot nematode ethiopica group [*Meloidogyne ethiopica* Whitehead, 1968, *Meloidogyne luci* Carneiro et al., 2014 (Nematoda: Heteroderidae)] which has been identified in recent years in Turkey and also some other European countries, can be distinguished only by its biochemical esterase enzyme profile (Aydınlı et al., 2013; Aydınlı & Mennan, 2016; Gerič Stare et al., 2017, 2018, 2019; Aydınlı, 2018; Gürkan et al., 2019). Esterase enzyme profiles are used as species-specific markers and support other identification methods (Butler et al*.,* 1981; Leslie et al., 1982; Barker, 1985; Esbenshade & Triantaphyllou, 1986; McGhee & Cottrell, 1986).

In studies conducted in Turkey, ten *Meloidogyne* spp., viz., *M. incognita, M. javanica, M. arenaria, M. hapla, M. chitwoodi, M. artiellia, M. acrita, M. luci, M. exiqua* and *M. thamesi*, have been recorded (Diker, 1959; Yüksel, 1966; Öztüzün, 1970; Ertürk & Özkut, 1973; Yüksel, 1974; Gürdemir & Ağdacı, 1975; Hekimoğlu, 1975; Pehlivan & Kaşkavalcı, 1993; Di Vito et al., 1994; Elekçioğlu & Uygun, 1994; Elekcioglu et al., 1994; Mennan & Ecevit, 1996; Kaşkavalcı & Öncüer, 1999; Söğüt & Elekçioğlu, 2000; Kepenekci et al., 2002; Devran et al., 2009; Devran & Söğüt, 2009; Özarslandan et al., 2009; Özarslandan & Elekçioğlu, 2010; İmren et al., 2014; Çetintaş & Çakmak, 2016; Gürkan, 2017; Uysal et al., 2017; Aydınlı, 2018; Gürkan et al., 2019).

The Eastern Mediterranean Region is one of the most important greenhouse vegetable production areas of Turkey (Elekcioğlu & Uygun, 1994; Elekcioglu et al., 1994). Söğüt & Elekcioğlu (2000) previously identified root-knot nematodes by morphological and North Carolina host test methods in this region. It was reported that *Meloidogyne incognita* (Kofoid & White, 1919) Chitwood*,* 1949 races 2 and 4, and *Meloidogyne javanica* (Treub, 1885) Chitwood*,* 1949 race 1 were common species, and *Meloidogyne arenaria* (Neal, 1889) Chitwood, 1949 and *Meloidogyne hapla* Chitwood, 1949 were rare species.

The fact that *M. luci* was found for the first time in Samsun and later found in Osmaniye and Gaziantep, provinces close to the Eastern Mediterranean Region where the study was conducted, revealed the necessity of revision by making detailed scanning in the region (Aydınlı, 2018; Gürkan et al., 2019). The aim of this study was to update root-knot nematode species and current species distributions in the Eastern Mediterranean region by using molecular scar primer and biochemical esterase enzyme.

Materials and Methods

Survey

One hundred root samples (20 from Adana, 75 from Mersin and 5 from Hatay) were collected in April to June 2018 in the areas where greenhouse of tomato, pepper, cucumber and eggplant vegetables were

grown in the Eastern Mediterranean Region (Bora & Karaca, 1970) (Figure 1). Root-knot nematode adult female, egg mass and second-stage juvenile were isolated from 46 samples (Table 3). The other 54 samples were not infected.

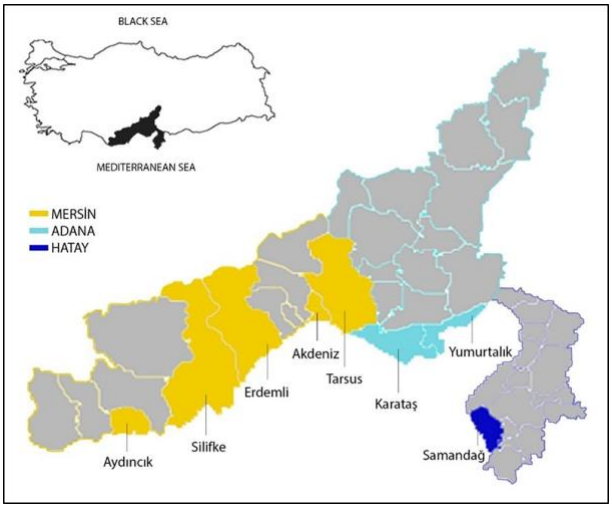

Figure 1. Districts surveyed in the greenhouse vegetable areas of the Eastern Mediterranean Region.

DNA isolation

DNA was extracted from second-stage juvenile of root-knot nematode populations according to the CTAB method stated by Ahrens & Seemüller (1992).

PCR studies

In the identification of root-knot nematodes, PCR was performed using METH F-METH R, MIF-MIR, FJAV-RJAV, FAR-RAR, and JMV1-JMV2-JMVhapla species-specific primers given in Table 1.

Table 1. Species-specific primers used in PCR studies to identify *Meloidogyne* spp.

Primers	Primer sequences (5'-3')	Fragments (bp)	*Meloidogyne* sp.	Reference
FJAV	GGTGCGCGATTGAACTGAGC	670	*M. javanica*	Zijlstra et al., 2000
RJAV	CAGGCCCTTCAGTGGAACTATAC			
FAR	TCGGCGATAGAGGTAAATGAC	420	*M. arenaria*	Zijlstra et al., 2000
RAR	TCGGCGATAGACACTACAAACT			
METH F	ATGCAGCCGCAGGGAACGTAGTTG	350	*M. ethiopica*	Correa et al., 2014
METH R	TGTTGTTTCATGTGCTTCGGCATC			
MIF	GTGAGGATTCACCTCCCCAG	999	*M. incognita*	Meng et al., 2004
MIR	ACGAGGAACATACTTCTCCGTCC			
JMV1	GGATGGCGTGCTTTCAAC	540, 670 and 440	*M. chitwoodi, M. fallax* and *M. hapla*	Wishart et al., 2002
JMV2	TTTCCCCTTATGATGTTTACCC			
JMVhapla	AAAAATCCCCTCGAAAAATCCACC			

In the PCR mix, 1 µl dNTPs (10 mM), 5 µl Dream Taq green buffer (10X), 2 µl DNA, 1 µl reverse primer (10 pmol), 0.25 µl Dream Taq DNA polymerase (5 u/µl), 1 µl forward primer (10 pmol) and 39.75 µl ddH_2O were added to make up the mixture to 50 µl. Different PCR cycles were used according to relevant literature for each primer pair (Table 2).

Table 2. Thermo-cycles applied for species-specific primer pairs

MIF-MIR & FJAV-RJAV	JMV1-JMV2-JMVhapla	FAR / RAR	METH F / METH R	
95°C 3 min	95°C 3 min	95°C 3 min	95°C 3 min	
95°C 60 s 59°C 60 s 72°C 60 s	95°C 45 s 53°C 45 s 72°C 45 s	95°C 30 s 56°C 30 s 72°C 45 s	95°C 45 s 60°C 45 s 72°C 45 s	} 35 cycles
72°C 7 min	72°C 7 min	72°C 7 min	72°C 7 min	

Agarose gel electrophoresis studies were conducted according to the method proposed by Galitelli & Minafra (1994). Agarose gel concentrations of 1% for *M. incognita*, 1.5% for *M. javanica* and 2% for *M. arenaria, M. hapla, M. chitwoodi* and *M. fallax* were adjusted according to the expected PCR product sizes by used primers and has been visualized in a UV transilluminator after stained with ethidium bromide.

Polyacrylamide gel electrophoresis

The study was performed on young females obtained from root samples with galls under a stereo binocular microscope. One young female was collected from each sample and Bio-Rad Mini-Protein II electrophoresis was run with 10 µl of extraction buffer according to the polyacrylamide gel electrophoresis (PAGE) method developed by Esbenshade & Triantaphyllou (1985). Five young females of *M. javanica* from pure culture were used as a control. The voltage was maintained at 80 v for the first 13 min and then increased to 200 v for the next 45 min. To visualize the esterase phenotype, gels was taken from electrophoresis and placed in the dying solution and kept in the dark for 30 min. After that, the gels were transferred to the solution containing 10% glycerol, 20% ethanol and 70% distilled H_2O fixative, and esterase phenotype bands were visualized in white light (Esbenshade & Triantaphyllou, 1985; Fargette 1987). The proportional mobilities of the bands were calculated and the esterase phenotype bands were designed according to Esbenshade & Triantaphyllou (1985) (Figure 6).

Results

In the sampling made from greenhouse vegetable production areas in the Eastern Mediterranean Region, the prevalence of root-knot nematodes was 46%. In the study, all samples were screened with the SCAR primer indicated in Table 2 and only *M. incognita* and *M. javanica* species were identified. Esterase enzyme profiles of 46 populations identified by PCR from root-knot nematode samples collected from the Eastern Mediterranean Region were monitored by the PAGE method. The identification key of the esterase profiles of the reference isolates used as a result of the tests was designed according to Esbenshade & Triantaphyllou (1985) (Figure 2).

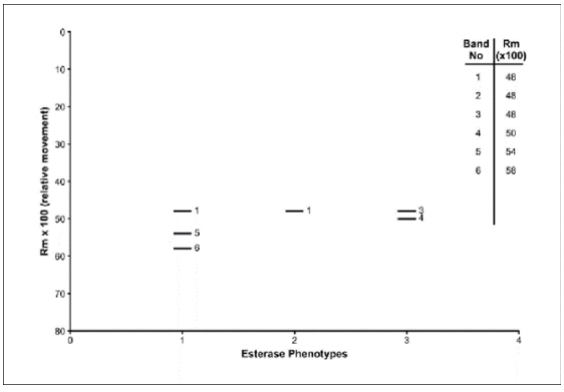

Figure 2. *Meloidogyne* spp. collected from vegetable greenhouses in the Eastern Mediterranean Region. The esterase enzyme phenotypes identification key was designed according to Esbenshade & Triantaphyllou (1985). Two esterase phenotypes of *M. incognita*, I1(1) and I2 (3 and 4); and *M. javanica*, J3 (1, 5 and 6).

Meloidogyne incognita

Meloidogyne incognita was identified in 39 samples collected from Karataş, Tarsus, Adanalıoğlu, Kazanlı, Erdemli, Aydıncık and Samandağ, and the prevalence was 85% (Table 3). For the *M. incognita* population, after the PCR study using the MIF-MIR primer pair developed by Meng et al. (2004), a DNA band with a length of 999 bp was visualized (Figure 3). It was supported by the PAGE method that 39 *M. incognita* isolates, which were identified as a result of molecular studies, also had I1 or I2 esterase profiles and that these isolates *were M. incognita* (Figure 4). It was found that I1 esterase enzyme phenotype was 62% and I2 esterase enzyme phenotype was 39%.

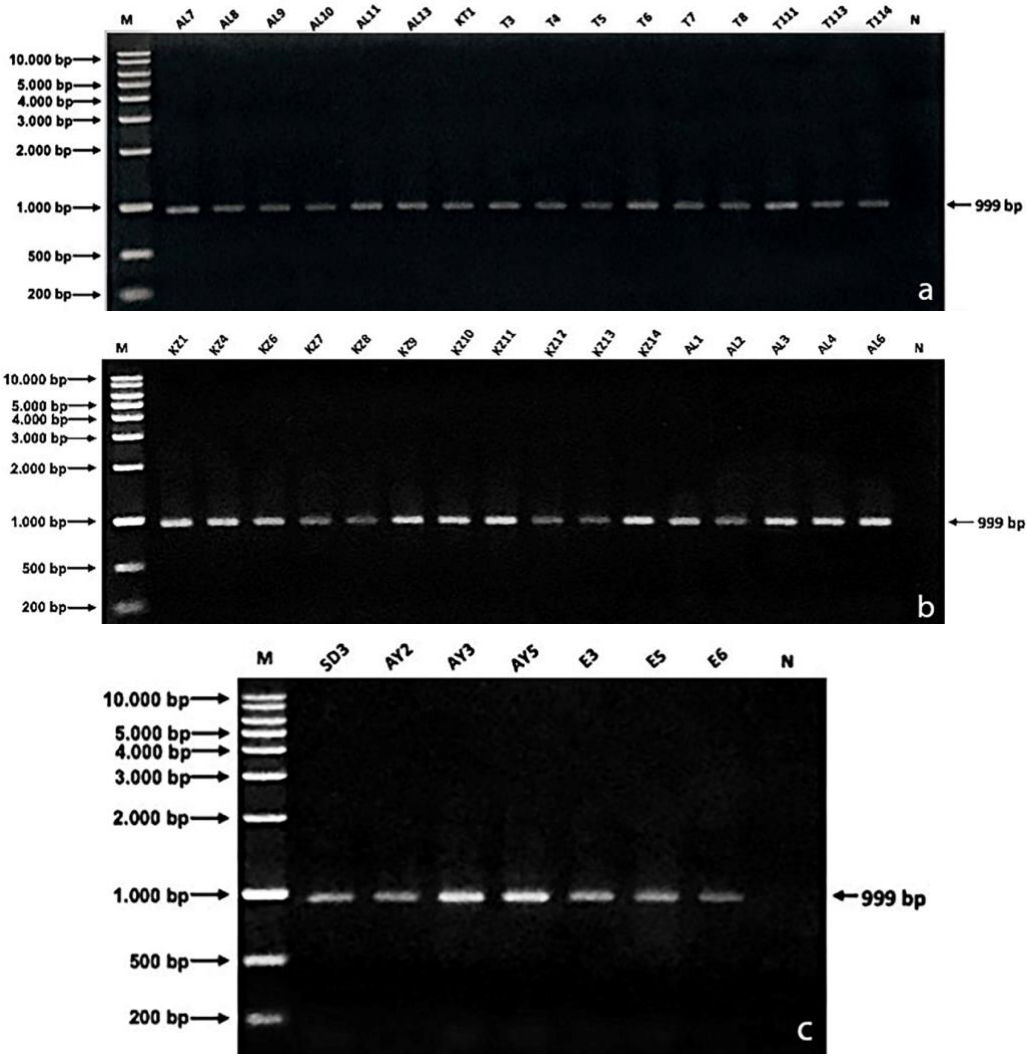

Figure 3. PCR products (999 bp) of *Meloidogyne incognita* species-specific MIR-MIF primers in AL7-E6 samples. (M, 1000 bp DNA marker; and N, dd-water).

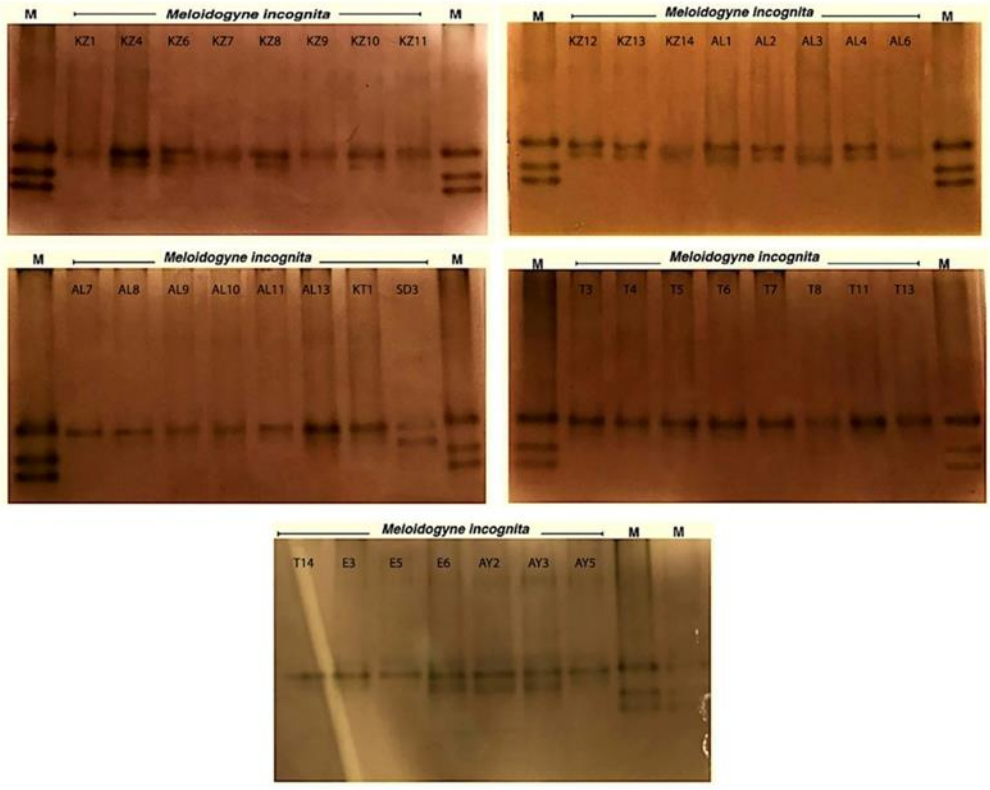

Figure 4. *Meloidogyne incognita* 24 population I1 esterase enzyme phenotypes: KZ1, KZ7, KZ9, KZ11, KZ14, AL6, AL7, AL8, AL9, AL10, AL11, AL13, KT1, T3, T4, T5, T6, T7, T8, T11, T13, T14, E3, E5. 15 population I2 esterase enzyme phenotypes: KZ4, KZ6, KZ8, KZ10, KZ12, KZ13, AL1, AL2, AL3, AL4, SD3, E6, AY2, AY3, AY5. M, marker-reference population (*Meloidogyne javanica*).

Meloidogyne javanica

Meloidogyne javanica was diagnosed in 7 of 46 samples collected from Kazanlı, Aydıncık, Erdemli and Samandağ and the prevalence was 15%. To identify *M. javanica*, after the PCR study using the FJAV/RJAV primer pair developed by Zijlstra et al. (2000), the expected DNA band of 670 bp was displayed as a result of the PCR study (Figure 5).

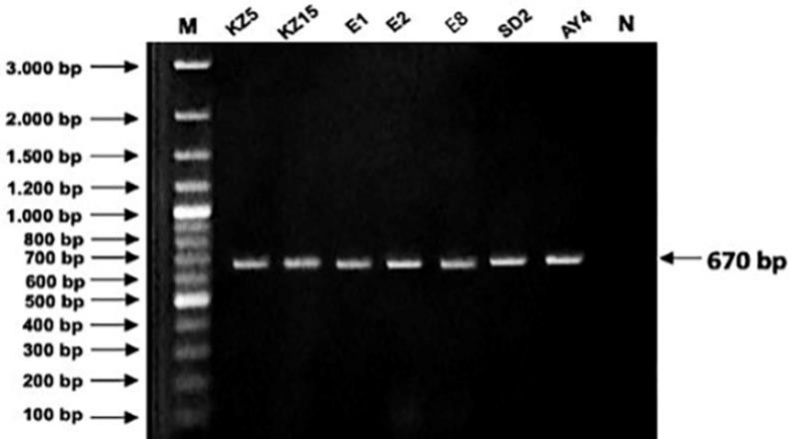

Figure 5. PCR products of KZ5-AY4 samples at 670 bp with the *Meloidogyne javanica* FJAV-RJAV primers. M, 100 bp DNA ladder; and N, dd-water.

This result was supported by the PAGE method that 7 *M. javanica* isolates, which were identified as a result of molecular studies, had J3 esterase profile and that these isolates were *M. javanica* as a result (Figure 6).

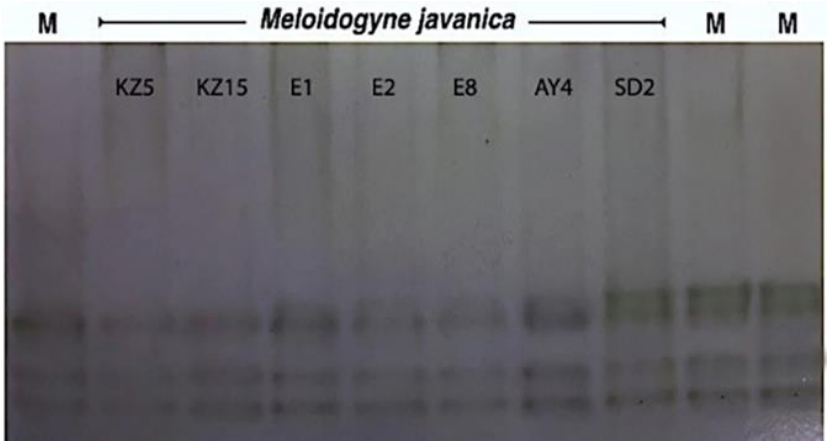

Figure 6. J3 phenotype of esterase enzyme profile of *Meloidogyne javanica* isolates in polyacrylamide gel. M, marker-reference population (*Meloidogyne javanica*).

The results obtained from molecular and biochemical analyzes are presented in Table 3.

Table 3. Comparison of the molecular and biochemical analysis results (as positives and negatives) for *Meloidogyne* spp. of the isolates collected from the Eastern Mediterranean Region

Location	Code	Host	Esterase			Primers		*Meloidogyne* sp.	Coordinates	
			I1	I2	J3	MIF-MIR	FJAV-RJAV		N	E
Adana, Karataş	KT1	Tomato	+	-	-	+	-	*M. incognita*	36°71'18"	35°14'51"
	KZ1	Pepper	+	-	-	+	-	*M. incognita*	36°82'57"	34°76'68"
	KZ4	Pepper	-	+	-	+	-	*M. incognita*	36°82'78"	34°73'37"
	KZ5	Pepper	-	-	+	-	+	*M. javanica*	36°82'23"	34°77'56"
	KZ6	Pepper	-	+	-	+	-	*M. incognita*	36°82'15"	34°77'41"
	KZ7	Pepper	+	-	-	+	-	*M. incognita*	36°82'23"	34°76'50"
	KZ8	Pepper	-	+	-	+	-	*M. incognita*	36°83'26"	34°74'19"
Mersin, Kazanlı	KZ9	Pepper	+	-	-	+	-	*M. incognita*	36°81'39"	34°75'42"
	KZ10	Pepper	-	+	-	+	-	*M. incognita*	36°83'85"	34°74'18"
	KZ11	Pepper	+	-	-	+	-	*M. incognita*	36°81'37"	34°78'24"
	KZ12	Pepper	-	+	-	+	-	*M. incognita*	36°81'37"	34°78'24"
	KZ13	Pepper	-	+	-	+	-	*M. incognita*	36°81'04"	34°78'90"
	KZ14	Pepper	+	-	-	+	-	*M. incognita*	36°82'48"	34°76'73"
	KZ15	Pepper	-	-	+	-	+	*M. javanica*	36°82'23"	34°77'56"

Table 3. Continued

Location	Code	Host	Esterase			Primers		*Meloidogyne* sp.	Coordinates	
			I1	I2	J3	MIF-MIR	FJAV-RJAV		N	E
Mersin, Adanalıoğlu	AL1	Pepper	-	+	-	+	-	*M. incognita*	36°83'63"	34°80'90"
	AL2	Pepper	-	+	-	+	-	*M. incognita*	36°83'63"	34°81'00"
	AL3	Pepper	-	+	-	+	-	*M. incognita*	36°83'56"	34°81'09"
	AL4	Pepper	-	+	-	+	-	*M. incognita*	36°83'69"	34°81'29"
	AL6	Pepper	+	-	-	+	-	*M. incognita*	36°83'40"	34°81'74"
	AL7	Pepper	+	-	-	+	-	*M. incognita*	36°83'15"	34°81'50"
	AL8	Pepper	+	-	-	+	-	*M. incognita*	36°83'13"	34°81'28"
	AL9	Pepper	+	-	-	+	-	*M. incognita*	36°83'07"	34°81'62"
	AL10	Pepper	+	-	-	+	-	*M. incognita*	36°82'79"	34°81'92"
	AL11	Pepper	+	-	-	+	-	*M. incognita*	36°82'73"	34°82'58"
	AL13	Pepper	+	-	-	+	-	*M. incognita*	36°81'26"	34°81'55"
Mersin, Tarsus	T3	Eggplant	+	-	-	+	-	*M. incognita*	36°79'85"	34°87'90"
	T4	Pepper	+	-	-	+	-	*M. incognita*	36°79'77"	34°87'87"
	T5	Eggplant	+	-	-	+	-	*M. incognita*	36°79'77"	34°87'81"
	T6	Eggplant	+	-	-	+	-	*M. incognita*	36°79'67"	34°87'81"
	T7	Eggplant	+	-	-	+	-	*M. incognita*	36°79'63"	34°87'83"
	T8	Pepper	+	-	-	+	-	*M. incognita*	36°79'70"	34°87'87"
	T11	Pepper	+	-	-	+	-	*M. incognita*	36°80'27"	34°86'63"
	T13	Pepper	+	-	-	+	-	*M. incognita*	36°80'26"	34°86'67"
	T14	Pepper	+	-	-	+	-	*M. incognita*	36°80'30"	34°86'64"
Mersin, Erdemli	E1	Tomato	-	-	+	-	+	*M. javanica*	36°60'46"	34°26'14"
	E2	Tomato	-	-	+	-	+	*M. javanica*	36°60'55"	34°26'22"
	E3	Tomato	+	-	-	+	-	*M. incognita*	36°60'16"	34°27'37"
	E5	Tomato	+	-	-	+	-	*M. incognita*	36°60'26"	34°26'90"
	E6	Tomato	-	+	-	+	-	*M. incognita*	36°60'58"	34°26'42"
	E8	Tomato	-	-	-	-	+	*M. javanica*	36°60'54"	34°25'26"
Mersin, Aydıncık	AY2	Cucumber	-	+	-	+	-	*M. incognita*	36°16'13"	33°33'84"
	AY3	Cucumber	-	+	-	+	-	*M. incognita*	36°16'08"	33°36'31"
	AY4	Cucumber	-	-	+	-	+	*M. javanica*	36°16'25"	33°38'28"
	AY5	Eggplant	-	+	-	+	-	*M. incognita*	36°15'90"	33°37'55"
Hatay, Samandağ	SD2	Tomato	-	-	+	-	+	*M. javanica*	36°07'30"	35°98'57"
	SD3	Tomato	-	+	-	+	-	*M. incognita*	36°06'92"	35°98'80"

Discussion

Overall, 46 of 100 root samples collected from vegetable growing greenhouses in the Eastern Mediterranean Region were found to be heavily infested with root-knot nematodes. *Meloidogyne incognita*

was determined as the dominant species in the region. *Meloidogyne incognita* was identified as the common species in Adanalıoğlu, Kazanlı and Tarsus districts in Mersin, where pepper cultivation is intense. Also, *M. incognita* was detected in Aydıncık, Erdemli (Mersin), Karataş (Adana) and Samandağ (Hatay) regions where cucumber, tomato and eggplant cultivation are intense. In the Eastern Mediterranean Region, the prevalence of *M. incognita* was 42% in the Söğüt & Elekçioğlu (2000) and as 62% in the Özarslandan & Elekcioğlu (2010). In this study, *M. incognita* found as the dominant species with a very high rate of 85%. Similarly, Gürkan et al. (2019) determined that the prevalence of *M. incognita* was 93% in Gaziantep and 90% in Osmaniye.

The rate of *M. incognita* infestation in greenhouse vegetable cultivation in the Western Mediterranean Region was previously reported as 64% (Devran & Söğüt, 2009) and 37% in the Lakes Region (Uysal et al., 2017). Kaşkavalcı & Öncüer (1999) report the prevalence of *M. incognita* in the Aegean Region as 80% whereas Aydınlı & Mennan (2016) in the Central Black Sea vegetable areas as 4%. It is thought that the reason for the low prevalence in the latter case may be the identification of the species previously identified as *M. incognita or M. ethiopica as M. ethiopica* group (*M. ethiopica, M. luci* and *Meloidogyne inornata* Lordello, 1956).

Meloidogyne javanica was identified in only seven samples collected from Kazanlı, Aydıncık, Erdemli (Mersin) and Samandağ (Hatay). The prevalence of *M. javanica* in the Eastern Mediterranean Region was 15%. In the same region, Söğüt & Elekçioğlu (2000) found the prevalence of *M. javanica* to be 55% and Özarslandan & Elekcioğlu (2010) as 39%. Söğüt & Elekçioğlu (2000), Devran & Söğüt (2011), Uysal et al. (2017), Gürkan et al. (2019) and Dinçer (2021) determined that *M. javanica* race 1 was dominant, but different races of *M. javanica* were reported both in the region and in different regions in the same studies. In this study, a race test was not conducted, but *M. javanica* was detected in the samples taken from the pepper fields of the Kazanlı district of Mersin.

In the present study, the prevalence of *M. javanica* in the Eastern Mediterranean Region was found to have decreased considerably. Similarly, Gürkan et al. (2019) found low rates of *M. javanica* infestation in Gaziantep and Osmaniye were 7% and 10%, respectively. Devran & Söğüt (2009) reported the prevalence of *M. javanica* on the coastline of the Western Mediterranean Region was 28% and Uysal et al. (2017) reported it as 37% in the Lakes Region. The prevalence of *M. javanica* in the Central Black Sea vegetable areas was 15% and in the Aegean Region it was 12% (Aydınlı & Mennan, 2016). In the present study, 62% I1 esterase bands in 24 populations and 39% I2 esterase bands in 15 populations were detected in the esterase enzyme phenotypes of 39 *M. incognita* samples identified by PAGE. In other PAGE studies conducted in Turkey, it was reported that *M. incognita* I2 esterase enzyme phenotype is more common (Aydınlı & Mennan, 2016; Cetintas & Cakmak, 2016; Gürkan et al., 2019). Esterase enzyme phenotypes were reported by Pais & Abrantes (1989), Carneiro et al. (1996, 2000), Castro et al. (2003), Cofcewicz et al. (2005) and Cetintas & Cakmak (2016). The two esterase enzyme phenotypes found in 2017 were similar to I1, I2 and J3 and were consistent those studies.

Conclusion

In recent years, *M. luci* have been identified in studies conducted with PAGE, esterase phenotype differences in the Central Black Sea and Eastern Mediterranean Regions and in Osmaniye and Gaziantep Provinces (Aydınlı & Mennan, 2016; Gürkan et al., 2019). Both nematodes show high similarity at the morphological and genetic level, and it was been reported that the best way to distinguish them is the esterase enzyme phenotype (Gerič Stare et al., 2018, 2019). Similarly, *M. ethiopica* has been redefined as *M. luci* in Slovenia, Greece and Italy. It has also been reported that *M. ethiopica* populations in Turkey, reclassified as *M. luci* (Gerič Stare et al., 2017).

Root-knot nematodes cause great economic yield losses, especially in vegetable growing areas. In addition, because they are soil-borne and their hosts range is wide, it is difficult and costly to control in and eradicate from soil. Accurate identification is of great importance, especially in resistance cultivar studies and commercial use of developed cultivars. The use of plant species resistant to root-knot nematode in the region is important in terms of determining control strategies of this pest.

In the present study, *M luci* was not found in the region using PAGE method. Rather, the previously identified species, *M incognita*, was found to be a highly dominant species. This research showed that invasive pests with complex host-pathogen relationships, such as root-knot nematodes, need to be regularly surveyed with the best available diagnostic methods.

Acknowledgement

Assoc. Prof. Dr. Behcet Kemal Çağlar (University of Çukurova, Adana, Turkey) and Dr. Betül Gürkan (East Mediterranean Transitional Zone Agricultural Research of Institute, Kahramanmaraş, Turkey) are thanked for their support in molecular studies and PAGE studies. Also, Dr. Dilek DİNÇER (Republic of Türkiye, Ministry of Agriculture and Forestry, Biological Control Research Institute, Adana, Turkey) is thanked for her assistance with the pure culture of *M. javanica*.

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