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Original article (Orijinal araştırma)

Oxidative effects of boric acid on different developmental stages of *Drosophila melanogaster* Meigen, 1830 (Diptera: Drosophilidae)[1]

Drosophila melanogaster Meigen, 1830 (Diptera: Drosophilidae)'in farklı gelişme dönemleri üzerine Borik asitin oksidatif etkileri

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Summary

Synthetic organic insecticides are widely used to combat agricultural pests. Boric acid has a great importance in pest management because it has less toxic effect on non-target organisms compared to other organic chemical insecticides. For this purpose, the fruit fly *Drosophila melanogaster* Meigen, 1830 (Diptera: Drosophilidae) was reared from first stage larvae on an artificial diet containing boric acid at 10, 100, 200 or 300 mg/L to adult stage. The effect of boric acid on important oxidative stress indicators such as lipid peroxidation product of malondialdehyde contents (MDA) and protein oxidation products of protein carbonyl contents (PCO) and detoxification enzyme activity of glutathione S-transferase (GST) in the third stage larvae, pupae, adults and eggs of *D. melanogaster* were investigated. All boric acid concentrations significantly increased MDA content in third stage larva. When the adults from the larvae reared on 300 mg/L of dietary BA were also fed with high BA concentration for a 10-day period, MDA and PCO contents of male and female adults were considerably went up in comparison to control. MDA and PCO content in the eggs of these females were hugely increased. The rise in PCO content of the eggs was 31-fold relative to control. Our results indicate that BA feeding at high concentrations in all developmental stages of *D. melanogaster* is more effective on oxidative stress indicators and detoxification enzyme.

Keywords: Boric acid, *Drosophila melanogaster*, oxidative stress

Özet

Tarımsal zararlılar ile mücadelede sentetik organik insektisitler yoğun olarak kullanılmaktadır. Borik asit organik kimyasal insektisitlere göre hedef olmayan organizmalara karşı daha düşük toksisiteye sahip olması nedeniyle önem taşımaktadır. Bu amaçla çalışmamızda meyve sineği *Drosophila melanogaster* Meigen,1830 (Diptera: Drosophilidae)'in birinci dönem larvaları borik asitin farklı konsantrasyonlarını (10, 100, 200 ve 300 mg/L) içeren yapay besinler ile yetiştirilmiştir. *Drosophila melanogaster*' in üçüncü dönem larva, pupa, ergin dönemleri ve yumurtalarında oksidatif stresin önemli indikatörleri olan lipid peroksidasyonu ürünü malondialdehid (MDA) ve protein oksidasyon ürünü protein karbonil miktarları (PCO) ile detoksifikasyon enzimi glutatyon-S-transferaz (GST) aktivitesi üzerine etkisi incelenmiştir. Borik asitin denenen konsantrasyonlarını içeren yapay besinler ile yetiştirilen *D. melanogaster*' in üçüncü dönem larvalarının MDA miktarı önemli derecede artmıştır. Yüksek borik asit ile yetiştirilen erginlerin 10 gün süreyle 300 mg/L borik asit içeren besin ile beslenilmesi sonucunda kontrol besinine göre dişi ve erkek bireylerde MDA ve PCO miktarları önemli derecede artmıştır. Bu dişilerin yumurtalarındaki MDA, PCO miktarları ve GST aktivitesi önemli derecede artarken, PCO miktarındaki artış yaklaşık 31 katı oranında olmuştur. Sonuçlarımız, borik asitin yüksek konsantrasyonlarıyla beslenen *D. melanogaster*'in tüm gelişme dönemlerindeki oksidatif stres indikatörleri ve detoksifikasyon enzimi üzerine oldukça etkili olduğunu göstermiştir.

Anahtar sözcükler: Borik asit, *Drosophila melanogaster*, oksidatif stres

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Introduction

The use of chemical insecticides and their synthetic analogs to fight insects harmful agriculture is gradually increasing. Excessive and misdirected use of these substances harm the environment having negative effects on both target and non-target organisms. Innovative studies try to develop alternative control methods centering on the use of compounds and elements (e.g. B), which have lower toxicity to the environment, humans and other species, and do not have direct impacts on organisms.

Boron, a beneficial bioactive element for many organisms, is classified in a different group (Group D) than boron oxide and boric acid (BA, H_3BO_3) (Anonymous, 2004). According to the results of toxicity studies, BA used for various medical and agricultural purposes has negative effects on animals, but is less toxic to honey bees, birds, fish, aquatic organisms, beneficial insects that are biological control agents and mammals when used in low doses (EFSA, 2004). BA is used on organisms such as ants, cockroaches and mosquitos as a registered inorganic insecticide having a sterilizing effect and also affecting digestion acting as a stomach toxin (Cochran, 1995). BA increases in the amount of consumption stimulating nutrient intake and forms complexes with carbohydrates, nucleotides and vitamins causing insufficient uptake of these nutrients (Xue & Barnard, 2003). For a long time BA has been used alone or in complex for the control of vermin (Xue & Barnard, 2003). The use of substances which have effects on a relatively narrow group and are less toxic with different activity for controlling insects is important for organisms in the food chain. Having knowledge of the effects of chemicals used will allow minimization of negative effects on beneficial non-target insects, biological control agents humans, and the environment. The oxidative effect of BA on biochemical parameters and detoxification capacity of *Drosophila melanogaster* Meigen, 1830 (Diptera: Drosophilidae) has not been determined. Also, aging mechanisms subject to nutrient-induced oxidative stress in organisms exposed to pesticides have not been studied.

Reactive oxygen species may be generated in organisms as byproducts during normal metabolic activities or as a result of exposure to the substances causing various chemical or environmental pollution (Felton & Summers, 1995). These radicals impede metabolic processes and cause oxidative damage in organisms damaging cellular components, such as fatty acids, proteins, carbohydrates, enzymes, nucleic acids, hormones and neurotransmitters (Hermes-Lima & Zenteno-Savin, 2002). For instance, they cause damage changing structures of the enzymes which produce or annihilate reactive oxygen species (ROS) (Giordano et al., 2007). Toxic or harmful chemical substances cause oxidative damage leading to lipid peroxidation, protein and enzyme oxidation and increased level of cellular glutathione in insect tissues (Ahmad, 1995). The increase in the level of malondialdehyde (MDA), which is an important aldehyde derivative being a final product of the reactions known as lipid peroxidation reactions, and the generation of protein carbonyl (PCO) products as a result of oxidation of proteins by ROS and covalent modification of proteins are used as indicators in the assessment of oxidative effect (Evans et al., 1999; Gülbahar, 2007).

There is an antioxidant defense system which is responsible for annihilating or counteracting endogenous or exogenous reactive chemicals in organisms. The antioxidant mechanism also activates in insect tissues in response to stress and allows insects to survive under chemically unsuitable environmental conditions (Hyršl et al., 2007). There is a tissue-specific antioxidant defense mechanism which consists of antioxidant enzymes and substances in adipose tissue and midgut of insects (Barbehenn & Stannard, 2004). Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione S-transferase (GST), glutathione reductase (GR), ascorbate peroxidase, thioredoxin peroxidase, disulfide reductase, and methionine sulfoxide reductase are the antioxidant enzymes found in insects (Missirlis et al., 2003).

Organisms protect themselves from harmful effects of environmental stress depending on their detoxification capacities (Vasseur & Leguille, 2004). GST which is responsible for insecticide resistance and detoxication of xenobiotics functions as an antioxidant enzyme with its peroxidase-like activity (Krishnan & Kodrik, 2006). GST enzymes in invertebrates and vertebrates constitute a group of multifunctional detoxification enzymes neutralizing toxic effects of electrophilic substances by means of phase II detoxification system of reactive metabolites which are produced by microsomal oxidation and

which conjugate xenobiotic with glutathione in order to transform them into their less toxic forms (Vontas et al., 2001). Researches have shown that insects have GST and may increase their GPx-like activity as a physiological adaptation against toxic substances. Also, there are studies showing that this activity varies between different stages of insects (Peric-Mataruga et al., 1997).

In this research, assessed how antioxidant defense systems of *D. melanogaster*, an organism not directly harmful to agriculture (Uysal & Şişman, 2003), is affected when feed on BA and if BA has an effect on the antioxidant defense system in association with aging. Previous studies were conducted in order to determine the effects of BA and other B-containing compounds on survival and development of insects in terms of stimulation of nutrient intake (Hyršl et al., 2007; Durmuş & Büyükgüzel, 2008). For this purpose, *D. melanogaster* was studied because it is an ectodermic organism with known quantitative nutrient needs, a short life span and is a model organism used for biochemical and physiological studies (Keser, 2010). In the study, changes in the activities of three important indicators for oxidative stress, MDA (a product of lipid peroxidation), PCO (a product of protein oxidation) and GST (a detoxification enzyme), were examined in third stage larvae, pupae and adults of insects fed on larval nutrient containing BA, which has a low toxicity to non-target organisms.

Materials and Methods

Drosophila melanogaster culture

Culture of *D. melanogaster* (W^{1118}, wild type) was allowed to feed on an artificial nutrient (8 g agar, 20 g D-sucrose, 11.8 g dry yeast, 0.8 g L-ascorbic acid, nipagin 3.5% solution, 36 g mashed potatoes, 1000 ml of distilled water, heated to dissolve the agar) containing potato and sucrose under non-sterile conditions in 250 ml glass bottles (Lesch et al., 2007). The insect culture was exposed to 25 ± 2°C, 60-70% relative humidity, and 12 h photoperiod in a Nüve cooled incubator.

Boric acid treatment

BA (99%, H_3BO_3) provided by the National Boron Research Institute, Ankara, Turkey was used in feeding experiments. BA was added to 1000 ml of the artificial nutrient in 10, 100, 200, 300 mg doses dissolved in water before the agar set. This concentration range was based on the studies conducted on various organisms using B and BA (Massie, 1994; Yang et al., 2000; Cisneros et al., 2002; Gore et al., 2004; Ali et al., 2006; Xue et al., 2006; Espinoza-Navarro et al., 2009), and because it would allow *D. melanogaster* to complete its development until the adult stage in pre-feeding experiments.

Feeding experiments

Newly hatched insects were raised until the adult stage with nutrients containing specified concentrations of BA. MDA and PCO levels, and GST activity were recorded during the last (third) larva, pupa and adult stages. Following this process, the adult individuals raised with various concentrations of BA from their larva stage were allowed to feed on various BA concentrations for 10 d in a second experimental setup, and the adult individuals raised with various BA concentrations from their larva stage were allowed to feed on BA-free nutrient for 10 d in a third experimental setup (Figure 1). MDA and PCO levels, and GST activity in adults and in the eggs of the females raised with adjusted concentrations of BA and in adults allowed to feed on BA-free nutrient for 10 d after they reach the adult stage (male and female) were determined. The experiments were conducted for 10 d of the adult stage because that it is the ideal period for reproductive efficiency (Kaya et al., 2009).

Specimen preparation and biochemical analysis

The specimens were extracted using cold homogenization buffer (1.15% potassium chloride, 25 mM dipotassium hydrogen phosphate, 5 mM ethylene diamine tetraacetic acid, 2 mM phenylmethylsulfonyl, 2 mM dithiothreitol, pH 7.4) in +4°C in an ultrasonic homogenizer (Bandelin Sonoplus, HD2070, Berlin, Germany). The specimens were kept at -80°C until analysed. The experiments were repeated four times using 20 larvae, 20 pupae, 20 females and 20 male adults (Taşkın et al., 2007), and 200 eggs for biochemical analysis.

The levels of MDA which is a final product of lipid peroxidation in the specimens reacted with thiobarbituric acid at 532 nm were measured based on the method used by Jain & Levine (1995). The coefficient of 1.56 x 10^5 $M^{-1}cm^{-1}$ was used and the level of MDA was calculated as nmol/mg protein.

Determination of protein carbonyl followed the method of Levine et al. (1994) with minor modifications (Krishnan & Kodrik, 2006): 2,4-dinitrophenylhydrazone, a stable compound formed by carbonyl groups in proteins, and 2,4-dinitrophenylhydrazine were calculated as nmol/mg protein at 370 nm using the coefficient of 22.0 $M^{-1}cm^{-1}$ in a strong acidic medium.

Glutathione S-transferase (EC 2.5.1.18) activity was determined by the method of Habig et al. (1974), as the amount of thioethers produced per 1 mg of total protein in the supernatant at 340 nm (ε_{340}: 9.6 mM/cm) for 1 min.

The enzyme specific activity was calculated as µmol/mg protein/min. Total protein was determined at 600 nm by the Folin-Lowry method (Lowry et al., 1951). A Shimadzu 1700, UV/VIS (Kyoto, Japan) spectrophotometer was used for the determination of MDA and PCO levels, the total amount of protein, and GST activity. All chemicals used in the experiments where analytical grade.

Data evaluation

In order to determine the effects of BA in various concentrations on *D. melanogaster*; one-way analysis of variance was used for MDA and PCO levels and GST activity, and LSD test was used to compare means (SPSS, 1997). The Kruskal-Wallis test was used to determine differences between respective MDA and PCO levels and GST activities of larva-pupa-adult stages at each BA concentration, and the Mann Whitney U test for differences between male and female adults at each BA concentration. The significance level used was $P \leq 0.05$.

Results and Discussion

For *D. melanogaster* raised until the adult stage with BA concentrations added to the nutrient medium, the effects of different BA concentrations on MDA and PCO levels and GST activity in last (third) stage larvae, pupae and adults (male and female) are shown in Tables 1 to 3.

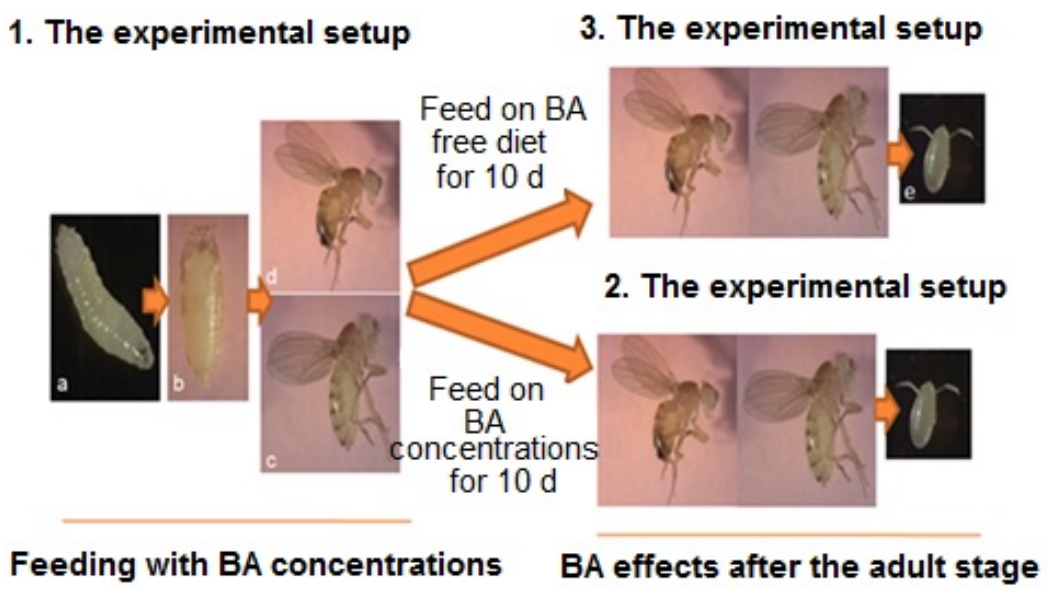

Figure 1. The insects with different BA concentrations before and after feeding experiments; a. last larval stage b. pupae, c. female, d. male e. egg.

Table 1. Malondialdehyde (MDA) levels in various developmental stages of *Drosophila melanogaster* raised with artificial nutrient containing various concentrations of boric acid

Boric acid (mg/Liter)	MDA (nmol/mg protein) (Mean* ± S.E)†#			
	Third stage larvae	Pupae	Adult	
			Female	Male
0.0§	0.13 ± 0.08 aA	0.06 ± 0.01 aAB	0.04 ± 0.03 aB	0.04 ± 0.01 aB
10	0.29 ± 0.02 bAB	0.04 ± 0.01 aA	0.48 ± 0.12 bB	0.04 ± 0.01 aA
100	0.26 ± 0.56 bAB	0.05 ± 0.02 aA	0.37 ± 0.07 bB	0.12 ± 0.03 abAB
200	0.34 ± 0.03 bcAB	0.07 ± 0.02 aA	0.16 ± 0.02 abAB	0.30 ± 0.05 bB
300	0.41 ± 0.05 bcA	0.11 ± 0.03 aA	0.31 ± 0.10 bA	0.38 ± 0.14 bA

* The mean of four experimental runs, 20 insects in various developmental stages were used for each run;
† The values containing the same lower case letter in the same column are not different from each other, P≤ 0.05 (LSD test);
# The values containing the same upper case letter in the same row are not different from each other, P≤ 0.05 (Kruskal-Wallis test);
§ Control nutrient (boric acid free).

While B and B-containing compounds do not have a repellent effect (Maistrello et al., 2002), it is known that they can be toxic depending on the the rate of consumption and interaction with nutrients (Büyükgüzel & İçen, 2004; Büyükgüzel & Kalender, 2007). As a result of the pre-feeding experiments, it was found that 300 mg/L is the most effective BA concentration for *D. melanogaster* to complete its development and reach the adult stage.

Table 2. Protein carbonyl (PCO) levels in various developmental stages of *Drosophila melanogaster* raised with artificial nutrient containing various concentrations of boric acid

Boric acid (mg/Liter)	PCO (nmol/mg protein) (Mean* ± S.E)†#			
	Third stage larvae	Pupae	Adult	
			Female	Male
0.0§	2.96 ± 0.79 aA	6.35 ± 1.23 aAB	11.27 ± 2.56 aAB	31.85 ± 8.63 aB
10	27.33 ± 7.00 abA	14.45 ± 4.50 aA	19.46 ± 3.75 aA	10.67 ± 6.34 abA
100	25.74 ± 9.16 abAB	9.80 ± 0.60 aA	37.40 ± 5.32 aB	13.99 ± 1.69 abAB
200	39.11 ± 11.96 abA	24.81 ± 12.79 aA	28.88 ± 9.10 aA	13.37 ± 3.12 abA
300	52.50 ± 17.61 bA	19.26 ± 3.44 aA	120.88 ± 34.15 bB	69.11 ± 21.47 cA

* The mean of four experimental runs, 20 insects in various developmental stages were used for each run;
† The values containing the same lower case letter in the same column are not different from each other, P≤ 0.05 (LSD test);
# The values containing the same upper case letter in the same row are not different from each other, P≤ 0.05 (Kruskal-Wallis test);
§ Control nutrient (boric acid free).

Radiation, viruses, ultraviolet light, products of fossil fuels combustion, cigarette smoke, infection, stress, byproducts or final products generated during normal metabolic activity, some chemicals and substances such as insecticides are known to be free radical sources (Sarıkaya et al., 2012). It is known that MDA and PCO levels change in insects due to free radicals and overall oxidative stress (Wang et al., 2001). MDA and PCO levels in midgut cells and adipose tissue of *Galleria mellonella* L., 1758 increased and the insect underwent oxidative stress in the studies conducted using substances such as α-solanine, ornidazole, gemifloxacin and niclosamide which is an anthelmintic antibiotic (Büyükgüzel et al., 2013; Erdem et al., 2013; Vuran et al., 2013; Büyükgüzel & Kayaoğlu, 2014). Furthermore, nutrient components induce ROS generation in insects interacting with each other or substances added to the nutrient. Sugars added to *Drosophila*'s nutrient such as galactose increase the level of MDA and decrease SOD activity (Jordens et al., 1999). The ROS produced in nutrients change toxicity depending on nutrient consumption of insect larvae (Cohen & Crittenden, 2004). It has been observed that xanthotoxin, which is an allelochemical, increases MDA and PCO levels and decreases GST activity in *G. mellonella* (Erdem & Büyükgüzel, 2015). While the mechanism of pathophysiological action of BA and some of B-containing compounds in insects is not known exactly, it is known that they induce the production of $O_2^{\cdot-}$ radicals transferring electron to the molecular oxygen (Jolly, 1991). In this experiment, MDA level increased four times in third stage larvae raised with nutrient containing 300 mg/L BA compared to the control group and reached 0.41 ± 0.05 nmol/mg, and the level of PCO increased about 17 times and reached 52 ± 18 nmol/mg protein. A similar situation was observed in females and males raised with nutrients containing high concentrations of BA; the level of MDA increased nine times in males and seven times in females compared to the control group and the level of PCO increased to 120 ± 34 and 69 ± 21 nmol/mg of protein, respectively (Tables 1 & 2). The accumulation of B added to the nutrient in tissues varies depending on the development stage and age (Massie, 1994). The decrease observed in the level of MDA during the development process from larva stage to adult stage in the study shows similarity to previous studies. *Drosophila* larvae stop feeding towards the end of third stage (Lozinsky et al., 2012). The decrease in the level of MDA in the pupae indicates that BA accumulation is at the lowest level in this stage due to feeding cessation and perhaps due to the decrease in potential oxidative damage. A similar situation is observed in the level of PCO, a variation was seen between the developmental stages of insects. It is known that insecticides in particular cause oxidative stress effecting lipid, carbohydrate and protein levels in insects (Damien et al., 2004). For instance, the level of PCO is higher in males compared to females of *Drosophila* which were allowed to feed on high concentrations of S-nitrosoglutathione (Lozinsky et al., 2012). It is believed that BA used as an insecticide might cause changes in nutritional physiology of insects in association with excessive chemical intake due to the stimulation of nutrient intake, and oxidative damage in tissues. In this study, the increased levels of MDA and PCO proves that BA can cause cell damage in insects.

Table 3. Glutathione S-transferase (GST) activities in various developmental stages of *Drosophila melanogaster* raised with artificial nutrient containing various concentrations of boric acid

Boric acid (mg/Liter)	GST (nmol/mg protein/min) (Mean* ± S.E)†#			
	Third stage larvae	Pupae	Adult	
			Female	Male
0.0§	0.36 ± 0.01 aA	0.52 ± 0.28 aA	0.15 ± 0.02 aA	0.21 ± 0.03 aA
10	0.54 ± 0.03 bA	0.44 ± 0.16 aAB	1.17 ± 0.21 bA	0.15 ± 0.01 aB
100	0.56 ± 0.05 bA	0.35 ± 0.13 aA	0.45 ± 0.14 acA	0.33 ± 0.04 aA
200	0.64 ± 0.04 bA	1.05 ± 0.20 aA	0.68 ± 0.11 cA	0.57 ± 0.07 abA
300	0.50 ± 0.05 abA	1.80 ± 0.74 bB	0.75 ± 0.10 bcAB	0.87 ± 0.32 bAB

* The mean of four experimental runs, 20 insects in various developmental stages were used for each run;

† The values containing the same lower case letter in the same column are not different from each other, P≤ 0.05 (LSD test);

The values containing the same upper case letter in the same row are not different from each other, P≤ 0.05 (Kruskal-Wallis test);

§ Control nutrient (boric acid free).

The basic mechanism which enables insects to adapt to the environment is the common phenomenon of enzyme induction (Wu & Miyata, 2005). GST, a detoxification enzyme, is an important indicator of oxidative stress (Otitoju & Onwurah 2007). It is known that BA causes oxidative damage in midgut epithelial tissues of *Blatella germanica* L., 1767 adults and thus GST activity increases (Habes et al., 2006). It was found that the high resistance to synthetic pyrethroid observed is associated with the increase in GST activity in *Spodoptera littoralis* Boisduval, 1833 (Lagadic et al., 1993). Also, it was found that the activities of GST enzyme increase in direct proportion to increased resistance to insecticides such as chlorpyrifos, carbamate, and deltamethrin in *S. littoralis* populations (Hadim, 2008). It was observed that the antioxidant defense system of *D. melanogaster* is highly responsive increasing the activities of reduced glutathione (GSH), SOD, CAT, GR and GST (Özata, 2006). Studies of *G. mellonella* showed that the activities of antioxidant systems of SOD, CAT, GST and GPx significantly change in parallel with the increased levels of MDA and PCO in hemolymph and adipose tissue due to increased BA concentrations (Hyršl et al., 2007; Büyükgüzel et al., 2013; Büyükgüzel & Kayaoğlu, 2014). In our study, GST activity in insects raised with nutrient containing high concentrations of BA significantly increased compared to the control group (Table 3). It is determined that the increase in GST activity is associated with insecticide resistance in studies conducted on cockroaches, domestic mosquitos and agricultural vermin. Particularly in studies conducted using chlorinated hydrocarbon insecticides (e.g. DDT), it was determined that GST activity increases in insects (Vontas et al., 2000; Rakotondravelo et al., 2006). It was found that there are different GST classes responsible for the elimination of oxidative stress in midgut (delta, epsilon, sigma, theta and omega zeta) of the third stage *D. melanogaster* larva, and most of the GST in midgut belongs to the delta and epsilon classes (Li et al., 2008). An increase was observed in GST activity against oxidative stress in pupa and adult stages of *D. melanogaster* when oxadiazole and 1-chloro-2,4-dinitrobenzene are used as herbicides (Scott et al., 1990). In our study, the highest BA concentrations tested on *D. melanogaster* significantly increased (three times) GST activity in pupae compared to the control. Although more detailed experiments are required on this point, the activity of this enzyme might have increased in parallel with the decrease in the level of GSH. GSH is the cofactor of this enzyme, and eliminates harmful effects of other free radicals and some of the organic and inorganic peroxides conjugating them (Dandapat et al., 2003). The increase in the level of this enzyme might be due to BA or metabolic products acting as oxidants and accelerating the production of free radicals thus causing oxidative stress in larvae. Particularly 200 and 300 mg/L BA increased the GST activity approximately 2.5 and four times in adult males compared the control nutrient, respectively. Relatively resistant individuals might have been produced due to the resistance mechanism in *Drosophila* males. Also, the fact that the level of MDA increased in pupae and adult males raised with this nutrient about 10 times more than females corroborates this opinion. It is clearly seen in our study that BA causes changes in the levels of MDA and PCO, which are indicators of oxidative stress, and in the activity of GST, which is an important detoxification enzyme.

Table 4. Malondialdehyde (MDA) and protein carbonyl (PCO) levels and glutathione S-transferase (GST) activity in females and men raised with boric acid and then allowed to feed on boric acid for 10 d

Boric acid (mg/Liter)	MDA (nmol/mg protein) (Mean* ± S.E)†#		PCO (nmol/mg protein) (Mean* ± S.E)†#		GST (nmol/mg protein/min) (Mean* ± S.E)†#	
	Female¥	Male¥	Female¥	Male¥	Female¥	Male¥
0.0§	0.03 ± 0.01 aA	0.05 ± 0.02 aA	3.08 ± 0.88 aA	2.89 ± 1.04 aA	0.67 ± 0.26 aA	1.00 ± 0.39 aA
10	0.08 ± 0.03 abA	0.07 ± 0.02 abA	4.47 ± 0.47 aA	2.14 ± 0.16 aB	2.14 ± 0.42 bA	1.83 ± 0.39 aA
100	0.04 ± 0.02 aA	0.26 ± 0.11 bA	8.97 ± 2.85 aA	2.22 ± 0.22 aB	1.18 ± 0.12 abA	5.78 ± 1.26 bB
200	0.07 ± 0.02 abA	0.17 ± 0.05 abA	8.85 ± 2.56 aA	8.67 ± 2.13 aA	1.90 ± 0.11 bA	2.41 ± 0.40 aA
300	0.12 ± 0.01 bA	0.08 ± 0.02 abA	29.01 ± 6.86 bA	20.91 ± 5.10 bA	1.65 ± 0.15 bA	1.75 ± 0.26 aA

* The mean of four experimental runs, 20 insects in various developmental stages were used for each run;

† The values containing the same lower case letter in the same column are not different from each other, P≤ 0.05 (LSD test);

For each parameter: The values containing the same upper case letter in the same row are not different from each other, P≤ 0.05 (Mann-Whitney test);

§ Control nutrient (boric acid free);

¥ Adults raised with boric acid and then allowed to feed on boric acid for 10 d.

It has been reported that tissue resistance to spontaneous autoxidation decreases with age, peroxidation more frequently occurs in old tissues than normal tissues and antioxidants may decrease the frequency but cannot delay the aging process. However, it has been found that the levels of endogenous antioxidants acting as a means of defense against antioxidant damage does not change, decrease or increase with age (Yüzüak, 2008). It has been reported that high gravity (3 and 5 g) does not affect enzyme activities (SOD and CAT) in two, four and six-week-old *D. melanogaster* (Le Bourg et al., 2000). In our study, no significant difference was observed between males and females (10 d) in terms of the level of MDA in the feeding experiments conducted using the control nutrient and nutrient containing various concentrations of BA after reaching adult stage (Table 4). Prooxidative changes during the aging process may not induce pathology as in our study (Büyükgüzel & Akın, 2014). It was seen that continuing intake of the nutrient containing 300 mg/L BA after reaching the adult stage significantly (nine times in females and seven times in males) increases the level of PCO in adults compared to the control. It was shown in the previous studies that the level of oxidatively modified proteins increase with age (Büyükgüzel, 2013), which is similar to our findings. The GST activity in *Drosophila* is associated with the length of life and nutrient intake (Toba & Aigaki, 2000). The GST activity in *Drosophila* males and females rose as S-nitrosoglutathione increased starting from the larva stage (Lozinsky et al., 2012). In our study, the GST activity increased to 1.65 ± 0.15 nmol/mg protein/min in females and 1.75 ± 0.26 in males. The increased enzyme activity is believed to have a protective role in insects. This statistically non-significant increase in males indicates that oxidative stress might have reached the level the enzyme cannot counteract and the enzyme itself might have been damaged.

Table 5. MDA and PCO levels and GST activity in females and men raised with boric acid and then allowed to feed on boric acid-free diet for 10 d

Boric acid (mg/Liter)	MDA (nmol/mg protein) (Mean* ± S.E)†#		PCO (nmol/mg protein) (Mean* ± S.E)†#		GST (nmol/mg protein/min) (Mean* ± S.E)†#	
	Female¥	Male¥	Female¥	Male¥	Female¥	Male¥
0.0§	0.03 ± 0.01 aA	0.05 ± 0.01 aA	3.08 ± 0.88 aA	2.89 ± 1.04 aA	0.67 ± 0.26 aA	1.00 ± 0.39 aA
10	0.10 ± 0.02 aA	0.04 ± 0.01 aA	4.31 ± 0.32 abA	2.28 ± 0.48 aB	1.41 ± 0.16 abA	1.92 ± 0.61 abA
100	0.10 ± 0.02 aA	0.11 ± 0.03 aA	2.92 ± 0.51 aA	2.54 ± 0.54 aA	3.35 ± 1.24 bA	3.62 ± 1.03 bA
200	0.10 ± 0.02 aA	0.09 ± 0.03 aA	4.82 ± 1.31 abA	4.64 ± 0.54 aA	2.79 ± 0.71 abA	1.50 ± 0.36 abA
300	0.07 ± 0.02 aA	0.22 ± 0.12 aA	7.75 ± 2.36 bA	13.16 ± 4.07 bA	2.28 ± 0.41 abA	4.12 ± 0.89 bA

* The mean of four experimental runs, 20 insects in various developmental stages were used for each run;
† The values containing the same lower case letter in the same column are not different from each other, P≤ 0.05 (LSD test);
# For each parameter: The values containing the same upper case letter in the same row are not different from each other, P≤ 0.05 (Mann-Whitney test);
§ Control nutrient (boric acid free);
¥ Adults raised with boric acid and then allowed to feed on boric acid for 10 d.

Antioxidant enzyme activity changes in females and males after the *Drosophila* larvae, exposed to xenobiotics such as sodium nitroprusside, S-nitrosoglutathione and potassium ferrocyanide, reached the adult stage (Lozinsky, 2013). Another study determined that wild type *Drosophila* Oregon males (85 d) lived longer than the males with atrophied wings (56 d) and age-related changes in their CAT activity were similar, and GR activity (between 10 and 40 d) and the level of total glutathione first increased then decreased suddenly (Fışkın et al., 1994). Ay & Yorulmaz (2008) determined that the levels of esterase and GST increased in the bifenthrin resistant *Tetranychus urticae* C. L. Koch, 1836 population compared to the initial population. Our study showed that feeding on high concentrations of BA until the adult stage and then control nutrient for 10d significantly increased the level of PCO (2.5 times in females and 4.5 times in male) and the activity of GST (2.28 ± 0.41 in females; 4.12 ± 0.89 nmol/mg protein/min in males) (Table 5). The change in the accumulation of B in *Drosophila* depending on age (Massie, 1994) and the amount of nutrient observed in our study indicates that antioxidant enzyme activity will improve due to decreased oxidation by the elimination of BA after reaching the adult stage.

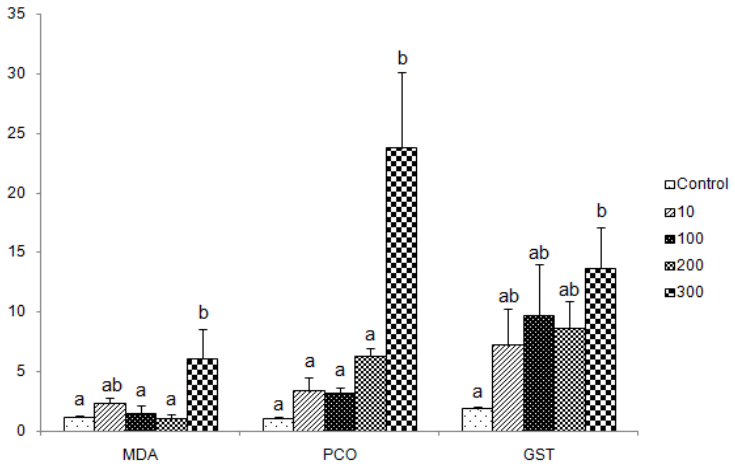

Figure 2. MDA and PCO levels and GST activity in eggs of adult females raised with boric acid and then allowed to feed on boric acid for 10 d.

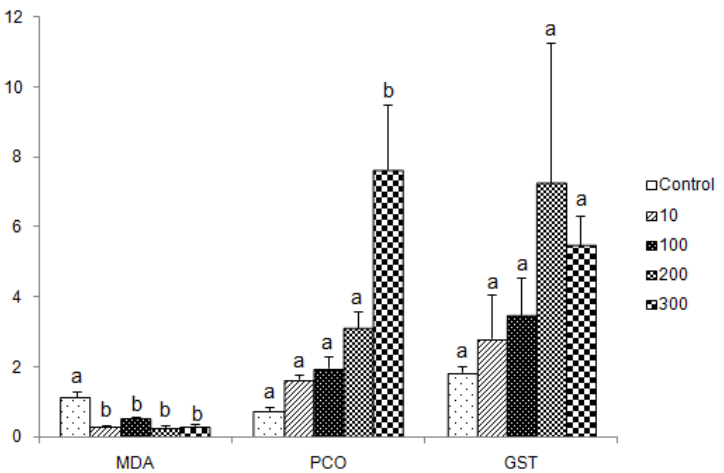

Figure 3. MDA and PCO levels and GST activity in eggs of adult females raised with boric acid and then allowed to feed on boric acid-free diet for 10 d.

Drosophila melanogaster adjusts the number of its eggs depending on its nutrition in order not to endanger the species (Partridge et al., 1987). Vitellogenin (egg yolk protein) and adipose tissue used for larva and adult development are synthesized in hemolymph and stored in oocyte in *Drosophila* (De Man et al., 1981). Changes in the protein content of hemolymph, adipose tissue and ovary of *Pyrrhocoris apterus* L., 1758 can be caused by some nucleoside-derived synthetic insecticides (Gelbič &Šula, 1990). BA is known to decrease protein, lipid and carbohydrate content of the ovary in cockroaches (Kilani-Morakchi et al., 2005). The best period for egg production in *Drosophila* is its first 10d as an adult (Yeşilada & Bozcuk, 1995). In our study, feeding on the highest concentration of BA during both larva and adult stages increased the level of MDA by about 5 times, the level of PCO about 31 times, and the activity of GST about 7 times in the eggs of females compared to the control (Figures 2 and 3). It is known that the maximum B accumulation occurs in the egg (Massie, 1994). The accumulation due to exposure to BA may have caused an increase in the levels of MDA and PCO which are indicators of oxidation in both larva and adult stages. It is believed that exposure to BA increases oxidative stress effecting biomolecules of insects, inhibiting vitellogenin synthesis, or due to oxidation of proteins in eggs. It is shown that these types of effects are associated with prooxidant and antioxidant systems in insects (Büyükgüzel, 2006).

According to these findings, the increase in the levels of MDA and PCO in *D. melanogaster* due to BA exposure indicates that free oxygen radicals induce oxidative stress in cells. While low concentrations of BA were tolerated and the antioxidant defense mechanism was activated in a shorter time trying to prevent oxidative stress, the mechanism was not able to prevent oxidative stress in case of high concentrations of BA. It can be clearly seen that the effects of BA on the oxidant and antioxidant reactions in insects varies depending on the concentration of BA. It is shown that feeding on high concentrations of BA from the larva stage negatively effects insects and its detoxification capacity. Also, the use of low concentrations of BA as in the adult diet increased the levels of MDA and PCO and the activity of GST thus indicating that the antioxidant defense mechanism works. According to these results, low concentrations of BA may be added to the adult nutrient as an ingredient to improve the capacity of *D. melanogaster* adults, however, potential cell damages due to oxidative stress should be considered. This result is significant in terms of fighting target insects without harming the environment and non-target organisms, suggesting the use of low concentrations of BA, which is an inorganic insecticide less harmful than currently used insecticides. However, this oral feeding study was carried out in the laboratory, futher studies are needed to examine the use of BA in the field.

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Original article (Orijinal araştırma)

Attraction responses of ladybird beetle *Hippodamia variegata* (Goeze, 1777) (Coleoptera: Coccinellidae) to single and binary mixture of synthetic herbivore-induced plant volatiles in laboratory tests[1]

Laboratuvar koşullarında zararlılar tarafından teşvik edilen sentetik bitki kokularının tekli ve ikili karışımlarına uğurböceği *Hippodamia variegata* (Goeze, 1777) (Coleoptera: Coccinellidae)'nın yönelim cevabı

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Melis SEİDİ[3] **Bilgi PEHLEVAN[2]**

Summary

The chemoreception response of an aphidophagous coccinellid predators [*Hippodamia variegate* (Goeze, 1777) (Coleoptera: Coccinellidae)] to the odors from four different synthetic HIPVs [methyl salicylate (MeSA), (E)-2-hexenal (E(2)H), farnesene (F) and benzaldehyde (Be)] was tested using two different doses (0.001 and 1 g/L) of the HIPVs, both alone and in five binary combinations [MeSA+F, MeSA+E(2)H, MeSA+Be, E(2)H+F and Be+F]. Insect responses were evaluated using two-choice experiments with a Y-tube olfactometer in laboratory conditions. The low single dose of MeSA attracted significantly more adults of *H. variegata* (71%) towards tubes containing the volatile source compared with the control volatile containing pure n-hexane. Adults of *H. variegata* did not significantly prefer single forms of either Be, E(2)H or F compared with MeSA alone. Additionally, this study showed that binary blends of MeSA with Be or F had significantly more attractiveness for *H. variegata* adults than controls. Thus, the compounds, Be and F, used together with MeSA were observed to increase adult attraction. In the future, additional studies that monitor the preferences of field populations of these predators treated with the attractive HIPV combinations should be conducted to confirm these findings.

Keywords: Attraction, biological control, *Hippodamia variegata*, predator, synthetic HIPV, Y-tube olfactometer

Özet

Yaprakbiti avcısı coccinellid türü *Hippodamia variegata* (Goeze, 1777) (Coleoptera: Coccinellidae)'nın zararlılar tarafından teşvik edilen (HIPV) kokularının dört farklı sentetik formunun [methyl salicylate (MeSA), (E)-2-hexenal (E(2)H), farnesene (F) ve benzaldehyde (Be)] tek başına ve ikili kombinasyonlarının [MeSA+F, MeSA+E(2)H, MeSA+Be, E(2)H+F ve Be+F] iki farklı dozuna (0.001 ve 1 g/L) olan kimyasal algılama cevapları test edilmiştir. Böceğin cevapları laboratuvar koşullarında iki seçenekli Y-tüp olfaktometre ile değerlendirilmiştir. Kontrol olarak kullanılan saf hekzana göre, MeSA'nın düşük dozu önemli bir şekilde daha fazla *H. variegata* erginini (%71) cezbetmiştir. Ayrıca, *H. variegata* erginleri Be, E(2)H ve F'nin tekli kombinasyonları ile önemli düzeyde cezbedilememiştir. Diğer taraftan, MeSA'nın Be ve F ile ikili kombinasyonlarının kontrole göre önemli bir şekilde daha fazla *H. variegata* dişisini çektiği belirlenmiştir. Sonuç olarak, bu bileşikler (Be ve F) MeSA ile birlikte kullanıldığında erginlerin cezbedilmesini arttırmışlardır. Gelecekte, bu bulguların desteklenmesi için bu avcı böceğin arazi koşullarında cezbedildiği kokulara yöneliminin izleneceği ek çalışmaların yapılması gerekmektedir.

Anahtar sözcükler: Çekicilik, biyolojik mücadele, *Hippodamia variegata*, avcı, sentetik HIPVler, Y-tüp olfactometre

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Introduction

Ladybird beetles are of great economic importance in agricultural production and have been used successfully for biological control of spider mites, aphids, coccids and other soft bodied insects (Hippa et al., 1978; Kring et al., 1985; Agarwala & Dixon, 1992; William, 2002). The coccinellid species, *Hippodamia variegata* (Goeze, 1777) (Coleoptera: Coccinellidae), is commonly found in Turkey (Bastug & Kasap, 2015; Bugday et al., 2015). This species feeds mainly on aphids such as the green apple aphid, *Aphis pomi* de Geer, 1773 (Homoptera: Aphididae) as well as psyllids, whiteflies, various lepidopterans and mealy bugs (Franzman, 2002; Khan & Mir, 2008).

Different blends of herbivore-induced plant volatiles (HIPVs) are released whenever a plant is fed upon by any herbivorous arthropod (Gaquerel et al., 2009; Kaplan, 2012). These volatiles are signals of anti-herbivore defense mechanisms in plants (Dicke, 1999; Pare & Tumlinson, 1999; Mumm & Dicke, 2010). Synthetic formulations of these HIPVs could have an important role as signals in tritrophic interactions among plants, pests and predators and might serve to aggregate members of the predatory species to locations with plants infested with mites and/or aphids (Rodriguez-Saona et al., 2011).

To date, although the attractant effects of some synthetic HIPVs that might be attractive for predatory and parasitoid species have been studied, often in both laboratory and field conditions, the attraction responses of *H. variegata* to these volatiles and their binary blends has not been studied. In recent years, methyl salicylate (MeSA) has been shown to attract some coccinellid species in vineyards, hop fields and sweet corn fields (James & Price, 2004; James, 2003b, 2005; James & Castle, 2005; Zhu & Park, 2005; Woods et al., 2011; Gadino et al., 2012; Maeda et al., 2015). MeSA has also been launched commercially as Predalure™, aimed at attracting ladybird beetles as well as lacewings and syrphids, *Orius* spp. (Anonymous, 2016). The attractiveness of benzaldehyde to some coccinellid species (i.e., *Coccinella septempunctata* (L., 1758), *Stethorus punctum picipes* (Casey, 1899)*, Stethorus gilvifrons* (Muls.,1850) has been demonstrated via electroantennogram and olfactometer laboratory and field tests (Han & Chen, 2002; James, 2005). It has also been revealed that farnesene, the aphid alarm pheromone, showed a kairomonic effect on *C. septempunctata, Hippodamia convergens* Guérin-Méneville, 1842, *Harmonia axyridis* (Pallas, 1773)*,* and *Adalia bipunctata* (L., 1758) (Coleoptera: Coccinellidae) (Abassi et al., 2000; Acar et al., 2001; Francis et al., 2004; Verheggen et al., 2007). Positive responses of *C. septempunctata* to (E)-2-hexenal, which is one of the nine components of tea aphid/tea shoot complex volatiles were shown using electroantennograms and wind tunnel bioassays by Han & Chen (2002). Furthermore, synthetic HIPVs have been verified to attract coccinellids under some laboratory and field conditions; thus, their potential for use in biological control strategies has been recognized (Yu et al., 2008; Lee, 2010). Although knowledge is limited, Y-tube olfactometer tests showed that *H. variegata* was attracted by olfactory stimuli from herbivore-infested plants in the laboratory (Tapia et al., 2010; Li et al., 2013). Still, information about the effectiveness of the attraction of synthetic HIPVs to this aphidophagous predatory species is lacking.

In a number of previous studies, each HIPV was considered individually to evaluate which exerts the strongest attraction to predators and parasitoids (James, 2003a, b; Yu et al., 2008; Simpson et al., 2011). However, plants damaged by herbivores release a complex of highly specific volatile blends consisting of multiple HIPVs. We hypothesized that instead of using only standard single synthetic HIPVs, blends consisting of two or more chemicals might be more attractive to insects (Szendrei & Rodriguez-Saona, 2010; Kaplan, 2012).

HIPVs attractive effects are usually evaluated by simple laboratory tests such as olfactometers and wind tunnels. Such tests can obtain prior knowledge about the potential role of HIPV regarding their attractiveness to any natural enemy species (Gols et al., 2003). Therefore, a Y-tube olfactometer setup was used in the laboratory tests in this study to better understand the response of *H. variegata* to some synthetic HIPVs that may have a role in attracting predators. Specifically, we compared the differences between two different doses (low and high) of single and binary combinations of four synthetic HIPVs, namely, MeSA, (E)-2-hexenal, farnesene and benzaldehyde.

Materials and Methods

Insects

Hippodamia variegata adults were obtained in early August 2012 from apple orchards of Uludag University, Bursa, Turkey.

Chemicals

Synthetic formulations of four HIPVs, benzaldehyde (Be) (99.5% purity; Merck, Kenilworth, NJ, USA), methyl salicylate (MeSA) (99% purity) (Acros Organics, Geel, Belgium), farnesene (F) (95% purity; Sigma-Aldrich, St. Louis, MO, USA), (E)-2-hexenal (E(2)H) (98% purity; Sigma-Aldrich) and a solvent that was mainly n-hexane (95% purity; Carlo Erba, Peypin, France) were used in this study.

Experimental design

The single and binary combinations of above mentioned synthetic agents used for olfactory tests were Be, E(2)H, F, MeSA, MeSA+F, MeSA+E(2)H, MeSA+Be, E(2)H+F and Be+F. Each synthetic HIPV was prepared at both low and high doses (0.001 and 1 g/L, respectively) in n-hexane. A total of 10 µl of each volatile source was dropped on two-layer filter paper (4-inch rectangles) using a micropipette. The n-hexane-diluted synthetic HIPVs were mixed in binary combinations in a microcentrifuge tube. A separate filter paper on which 10 µl of pure n-hexane was dropped was used as a control treatment. Before beginning the test, for provide odor flow stability, the olfactometer arms were prepared by forced airflow through the filter paper volatile sources for 5 min. The volatile sources (filter paper) were renewed for every test replicate or after every 30 min. The positions of the tube containing the HIPVs and the n-hexane were exchanged in each test. After each test, the Y-tube was cleaned with ethanol (≤100% purity) (Merck) and left to dry for 5 min in an incubator at 90°C.

Olfactometer set-up

The responses of *H. variegata* to the HIPV sources were tested using two-choice tests with a closed system Y-tube olfactometer as described in Gencer et al. (2009), which was a slight modification of Takabayashi & Dicke (1992). Distinctively, in the olfactometer, two Pyrex tubes (2.5 cm in diameter) connected to the Y-tube were used as volatile containers. These containers had an air inlet and outlet (0.8 cm in diameter) at opposite walls. An air pump was used to create an airflow from each container through the olfactometer arms; the airflow was adjusted with a flowmeter to 1.5 L/min and humidified with deionized water. The air passed through activated charcoal before reaching the cylinders. Airflow was measured in the entry arm. At the base of the Y-tube olfactometer, air was removed by a vacuum system.

Olfactometer bioassays

Adults of *H. variegata* that had been fed on green aphids (*A. pomi*) as prey were used in this study. The adults were kept in a small box (14 x 14 x 18 cm) without prey but with a small amount of water for 2-4 h prior to the test. The experiments were carried out in a climate controlled room (25°C, 60±5% RH). A single beetle was introduced into the tube and monitored until it had walked at least 7 cm up one of the arms. The behavior of each individual was observed for a maximum of 5 min. Adults that did not choose one of the side arms within 5 min were recorded as having made no choice and excluded from the statistical analysis (Takabayashi & Dicke, 1992; Cakmak et al., 2006). All olfactometer experiments for each HIPV alone and their blends were carried out on three different days. For each replicate, a total of 15 adult ladybirds were tested. Each individual insect was used only once.

Data analysis

Differences in the proportions of *H. variegata* adults' attraction by moving toward one of the HIPV sources or toward the n-hexane control were analyzed using the replicated goodness of fit test at a 5% critical level (Sokal & Rohlf, 1995). The null hypothesis was that the predator would exhibit a 1:1 distribution across the two odor sources for each replicate. Insects that did not make a choice were excluded from the statistical analysis.

Results

The response of *H. variegata* to different single synthetic HIPVs and their different doses is shown in Figure 1. *Hippodamia variegata* adults showed significant preference for MeSA at low dose compared to pure n-hexane (control) in one out of three replicates ($P < 0.01$, Figure 1). Pooled results (GP) also showed significant preference for MeSA at low dose ($P < 0.05$; Table 1). *Hippodamia variegata* did not show a preference between MeSA at high dose and control, and between other single synthetic HIPVs at both doses and control ($P > 0.05$, Figure 1, Table 1).

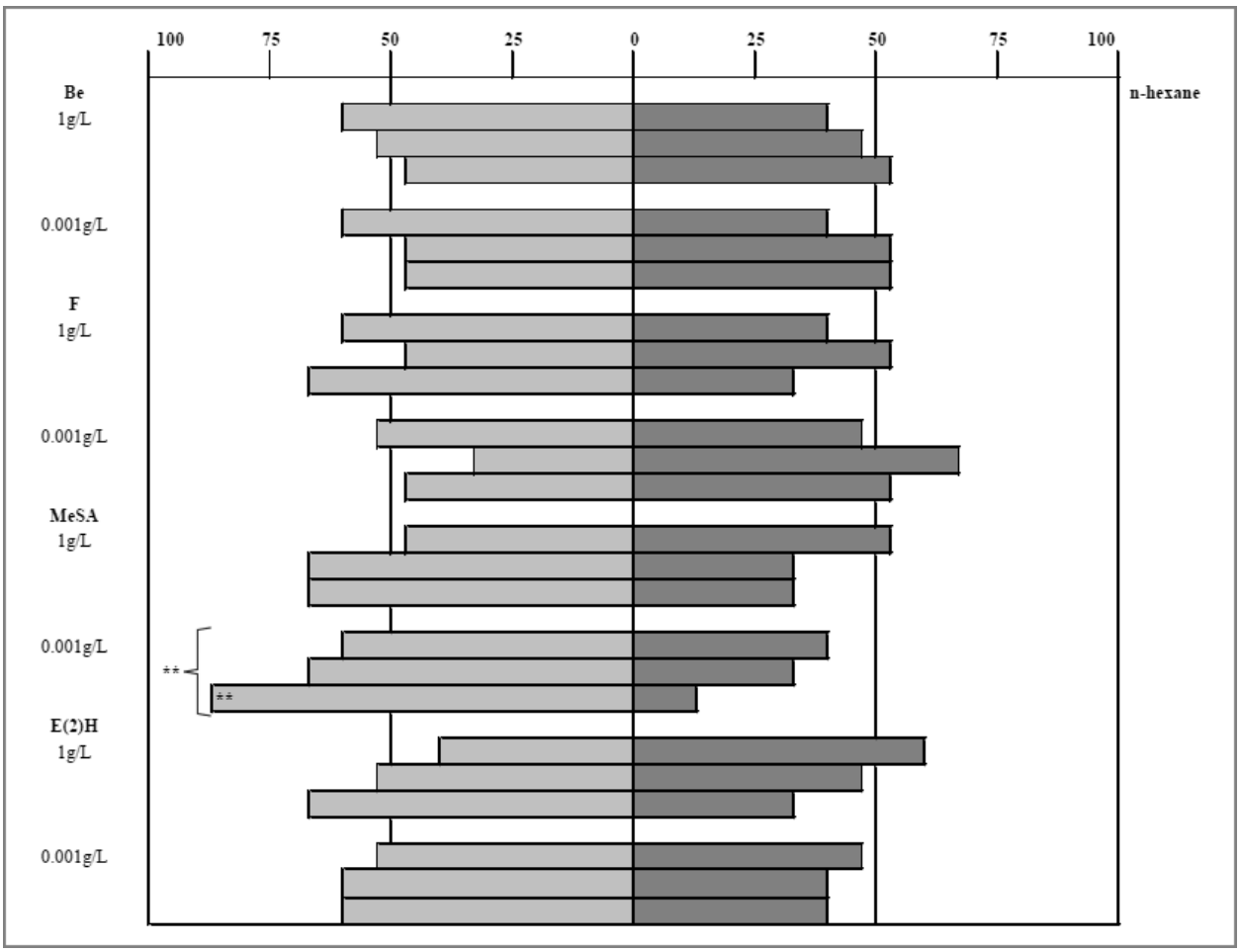

Figure 1. The response of *Hippodamia variegata* when offered high and low doses of four single synthetic HIPVs. Be, benzaldehyde; MeSA, methyl salicylate; F, farnesene; E(2)H, (E)-2-hexenal; *,**, significant P-values of 0.05 and 0.01, respectively, based on replicated goodness of fit test for G per replicate and G_P.

The response of *H. variegata* to the five binary combinations of the synthetic HIPVs and their different doses is shown in Figure 2. *Hippodamia variegata* showed a significant preference for the high dose of MeSA+F in one replicate and for the high dose of MeSA+Be in two replicates. Pooled results indicated that both doses of MeSA+F and the high dose of MeSA+Be significantly attracted *H. variegata* adults toward the volatile source ($P<0.05$, Table 1). However, pooled results indicated that *H. variegata* showed no preference for E(2)H+F, MeSA+E(2)H, Be+F at both doses and MeSA+Be at low dose compared to control, whereas it showed a significant preference in one out of three replicates for MeSA+E(2)H at high dose and Be+F at low dose (Table 1 and Figure 2). *Hippodamia variegata* showed the highest preference for the high dose of the MeSA+Be binary combination (overall, 87%), followed by MeSA alone at low dose (71%) and MeSA+F blend at high dose (69%) (Figures 1, 2).

Table 1. The results of replicated goodness of fit test on response of *Hippodamia variegata* to alone and five binary combination of four synthetic HIPVs

HIPVS	Doses (g/L)	n	G_H, df=2 P	G_P, df=1 P	G_T, df=3 P
Be	1	45	0.537 0.764	0.200 0.655	0.737 0.864
	0.001	45	0.715 0.699	0.022 0.881	0.737 0.864
E(2)H	1	45	2.170 0.338	0.200 0.655	2.370 0.499
	0.001	45	0.182 0.913	1.093 0.296	1.275 0.735
F	1	45	1.276 0.528	1.093 0.296	2.369 0.499
	0.001	45	1.276 0.528	0.557 0.456	1.833 0,608
MeSA	1	45	1.653 0.438	1.812 0.178	3.465 0.325
	0.001	45	3.038 0.219	8.279* 0.040	11.317 <0.01
MeSA+F	1	45	4.195 0.123	6.584** 0.010	10.779 0.013
	0.001	45	1.764 0.414	5.097* 0.024	6.861 0.076
E(2)H+F	1	45	0.537 0.764	0.200 0.655	0.737 0.864
	0.001	45	0.191 0.909	2.716 0.099	2.907 0.406
MeSA+E(2)H	1	45	2.644 0.267	3.810 0.05	6.554 0.088
	0.001	45	0.191 0.909	2.716 0.099	2.907 0.406
MeSA+Be	1	45	6.163* 0.046	27.043** <0.01	33.206 <0.01
	0.001	45	0.795 0.672	3.810 0.05	4.605 >0.05
Be+F	1	45	5.360 0.069	1.093 0.296	6.453 0.092
	0.001	45	1.353 0.508	3.810 0.050	5.163 0.160

Be, benzaldehyde; MeSA, methyl salicylate; F, farnesene; E(2)H, (E)-2-hexenal; G, replicated goodness of fit test; G_H, G for heterogeneity; G_P, pooled G; G_T, total G; P, probability; df, degree of freedom; n, 3 replicates x 15 individuals; *,**, significant *P*-values of 0.05 and 0.01, respectively.

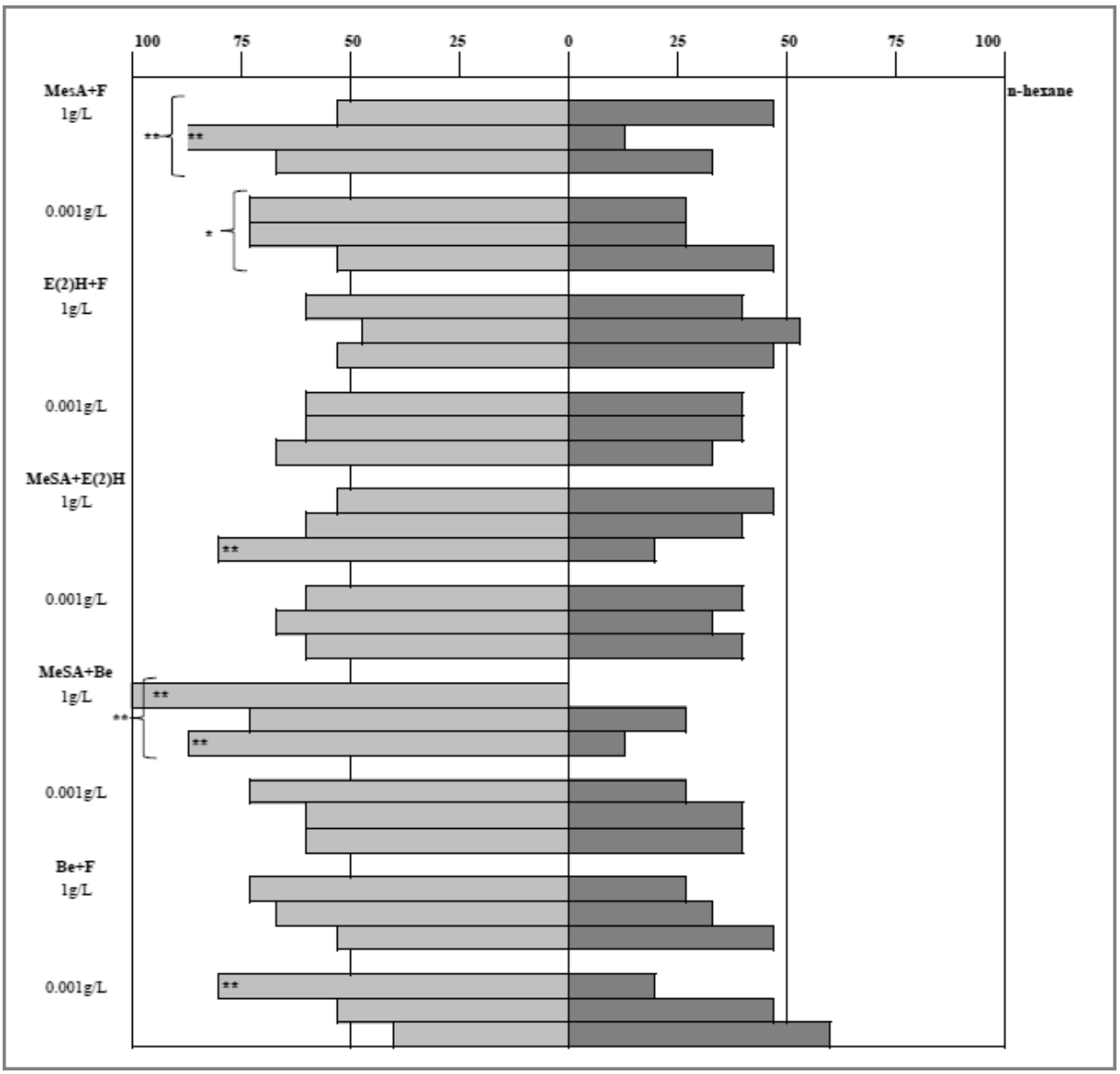

Figure 2. The response of *Hippodamia variegata* when offered high and low doses of five binary combination of four synthetic HIPVs. Be, benzaldehyde; MeSA, methyl salicylate; F, farnesene; E(2)H, (E)-2-hexenal; *,**, significant P-values of 0.05 and 0.01, respectively, based on replicated goodness of fit test for G per replicate and G_P.

Discussion

The current study demonstrated that MeSA among experimental synthetic HIPVs was significantly more attractive for *H. variegata* adults than the control. Similarly, some ladybird beetle species such as *Cycloneda polita* Casey, 1899, *C. septempunctata, H. axyridis, Propylea japonica* (Thunberg, 1780) and *Stethorus* spp., were attracted to MeSA-baited traps in hop fields (James, 2003b, 2005; Woods et al., 2011), vineyards (James & Price, 2004; Gadino et al., 2012), soybean fields (Zhu & Park, 2005; Mallinger et al., 2011), tea fields (Qi et al., 2008) and cranberry fields (Rodriguez-Sauna et al., 2011). In fact, MeSA lures have already been used as a commercial product to attract ladybird beetles (Lee, 2010; Anonymous, 2016). The findings obtained from this study are the first controlled study of HIPV attraction to *H. variegata*. Our results showed that *H. variegata* is one of the predators attracted by MeSA as a broad-spectrum lure.

Of the other tested HIPVs, farnesene is known as an important aphid alarm pheromone (Joachim et al., 2015). Furthermore, attractiveness of farnesene has been reported for two other ladybird species, *H. convergens* and *H. axyridis* (Colburn & Asquith, 1970). In addition, Li et al. (2013) showed that farnesene, one of the main components in volatiles released by *Aphis gossypii* Glover, 1877 (Hemiptera: Aphididae), elicited behavioral effects from *H. variegata.* Additionally, some researchers have shown that farnesene is attractive to some other ladybird species including *Coleomegilla maculata* (De Geer, 1877) (Coleoptera: Coccinellidae) and *P. japonica* in both laboratory and field tests (Zhu et al., 1999; Liu et al., 2014). In recent years, the attractant property of benzaldehyde has been demonstrated in some coccinellids (*C. septempunctata*, *S. gilvifrons* and *S. punctum picipes*) with olfactometer tests and in field studies (Han & Chen, 2002; James, 2003b; Sachin et al., 2008). In contrast, our test results were contradictory for pure farnesene and benzaldehyde. In the last pure synthetic HIPV (E(2)H) experiments, no evidence was obtained that ladybirds tended to be attracted to this compound due to no significant effect was found. This finding supports another study which found that the compound had no effect on females of *H. axyridis* even though it attracted males (Leroy et al., 2012).

Additionally, this study showed that binary blends of MeSA with Be or F had significantly more attractiveness for *H. variegata* adults than controls. Thus, compounds used together with MeSA were observed to have to increase adult attraction. The advantages of using mixtures instead of single compounds have been demonstrated in both field and laboratory studies in recent years (Maeda et al. 2015). Our mixture results are consistent with the findings of Szendrei & Rodriguez-Saona (2010), who revealed that individual volatiles were attractive overall, but that increased blend complexity corresponded with stronger attraction. Similarly, Jones et al. (2010) reported that the attractiveness of iridodial (a male-produced aggregation pheromone) increased when MeSA was added, doubling the number of lacewings captured in traps in apple orchards. Toth et al. (2009) achieved similar results for lacewings by adding MeSA to a blend of phenylacetaldehyde and acetic acid. Here, we demonstrated the attraction of MeSA+Be and MeSA+F combinations to coccinellids, especially *H. variegata.* This study constitutes a first report because there is no prior record in the literature.

Additionally, the binary combination (MeSA+E(2)H) were not attractive for the predator insect, though we obtained significant results concerning insect attraction to MeSA with Be or F. Our results are also in accordance with some results about the differences in olfactory responses in other predator and parasitoid species to various blends of HIPVs (Simpson et al., 2011; Van Wijk et al., 2011; Maeda et al., 2015). It seems likely that ecological differences may be the cause of such variations in the olfactory responses of different coccinellid species. The discrepancies in ladybird species attraction to different synthetic HIPVs or their combinations may depend on a number of different factors. Insects learn different volatile cues in their environments and have different experiences. In this study, we collected ladybirds from apple orchards, where their foraging host was *A. pomi*. Our data suggest that specific HIPV combinations should be tested for their effect on specially targeted organisms as well as particular agroecosystems. Our results are agreement with the results of Gencer et al. (2009), who showed that the olfactory responses of *S. gilvifrons* changed when its host plant or prey were altered. The volatile blends released by injured plants can vary between different combinations of plant and herbivore and between different herbivores on the same plant species as well as the same herbivore on different genotypes of the same plant species (Degen et al., 2004; Van Den Boom et al., 2004; Leitner et al., 2005). Our results lead us to two separate conclusions concerning the deployment of synthetic HIPVs for the purpose of conservation biological control. First, our data suggest that attraction of predator arthropods to synthetic volatiles is potentially dependent upon factors specific to the crop system (context dependency), to the local arthropod community, and to the specific field studied, which makes the implementation of HIPVs across systems challenging. Second, our results asserted that the effectiveness of HIPVs for attracting beneficial arthropods may be increased using different doses and combinations of synthetic HIPVs when a single compound is not sufficiently attractive. Our results are consistent with Qi et al. (2008) who noted that different dosages (10^{-6} to 10^{-2} g/ml) of HIPVs such as benzaldehyde, methyl salicylate, significantly affected the choices of *P. japonica* in Y-tube olfactometer tests. Furthermore, studies need to determine the most effective combination of HIPVs to more consistently and reliably attract predatory arthropods to these plant volatiles in the field.

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Original article (Orijinal araştırma)

Inhibition of egg development by hypercarbia and hypoxia in almond moth, *Ephestia cautella* (Walker, 1863) (Lepidoptera: Pyralidae)

İncir kurdu *Ephestia cautella* (Walker, 1863) (Lepidoptera: Pyralidae)'nın yumurta gelişiminin yüksek karbondioksit ve düşük oksijenle engellenmesi

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Summary

Hypercarbia-induced delay in the development of eggs was investigated in almond moth, *Ephestia cautella* (Walker, 1863) (Lepidoptera: Pyralidae), using two controlled atmospheres (CAs), 85% CO_2 + 3% O_2 (balance N_2) and 95% CO_2 + 1% O_2 (balance N_2) between 2012 and 2014 in Stored Products Pests Laboratory, Agricultural Faculty, Ankara University. Eggs of *E. cautella* (1-3 day-old) were exposed to both CAs for a wide a range of exposure periods of up to 104 h at three temperatures of 20±1, 25±1 and 30±1°C at 65±5% RH. In general, both CAs caused delay in egg development by 1 to 8 d. Inhibitory effects were more pronounced at lower temperatures. A maximum delay of 8 d was recorded at 20°C for the three-day-old eggs exposed to 95% CO_2 plus 1% O_2 for 88 h. Short exposure periods caused short term delays in development. Four h exposure caused 1d delay in three-day-old eggs exposed to 95% CO_2 plus 1% O_2 at 25°C. In practice, total egg hatch including delays lasted 5 d at 30°C, 8 d at 25°C, and 12 d at 20°C, which must be taken into account for successful CAs applications.

Keywords: Delayed development, egg hatching, *Ephestia cautella*, high carbon dioxide, low oxygen

Özet

İncir kurdu olarak bilinen *Ephestia cautella* (Walker, 1863) (Lepidoptera: Pyralidae) isimli zararlıya %85 CO_2 + %3 O_2 (denge gaz N_2) ve %95 CO_2 + %1 O_2 (denge gaz N_2) kompozisyonundaki iki farklı kontrollü atmosferin (KA) uygulanmasıyla yüksek karbondioksitli atmosferlerin yumurta gelişiminde oluşturduğu gecikme 2012-2014 yılları arasında Ankara Üniversitesi Ziraat Fakültesi Depolanmış Ürün Zararlıları Laboratuvarında yapılan çalışmada incelenmiştir. *Ephestia cautella*' nın 1-3 gün-yaşlı yumurtaları 20±1, 25±1 ve 30±1°C sıcaklık ve %65±5 orantılı nem koşullarında her iki KA kompozisyonuna 104 saate kadar varan değişik sürelerde maruz bırakılmıştır. Genel olarak, her iki KA 1-8 gün aralığında yumurta gelişiminde gecikmeye yol açmıştır. Gecikme düşük sıcaklıklarda daha dikkate değer bulunmuştur. Maksimum gecikme 20°C sıcaklıkta %95 CO_2 + %1 O_2 konsantrasyonuna 88 saat süreyle maruz kalan üç-gün-yaşlı yumurtalarda sekiz gün olarak tespit edilmiştir. Kısa uygulama süresi gelişmede kısa süreli gecikmeye neden olmuştur. Dört saatlik uygulama süresi %95 CO_2 + %1 O_2 konsantrasyonuna 25°C sıcaklıkta maruz kalan üç-gün-yaşlı yumurtada bir günlük gecikmeye neden olmuştur. Gecikmeyi de içeren yumurta açılımı toplam süresi 30°C de 5 gün, 25°C de 8 gün ve 20°C de 12 gündür. Dolayısıyla KA uygulamalarını başarıyla uygulayabilmek için bu sürelerin dikkate alınması önemlidir.

Anahtar sözcükler: Gelişimde gecikme, yumurta açılımı, *Ephestia cautella*, yüksek karbondioksit, düşük oksijen

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Introduction

Almond moth, *Ephestia cautella* (Walker, 1863) (Lepidoptera: Pyralidae), is not only an important international pest of stored cereals and various food commodities, but also the most detrimental pest of dried figs, which are of significant export value for Turkey. Turkey is the biggest producer and exporter of dried figs in the world. Turkish dried fig export had revenue of 274 M USD (about 69 kt) in 2014-2015. After the ban on methyl bromide, which was the only fumigant used in the dried fig sector in Turkey, various alternative methods have been studied, primarily phosphine fumigation, sulfuryl fluoride, high CO_2, high CO_2 at elevated temperatures, high pressure CO_2, irradiation and ozonation (Tütüncü et al., 2004; Cetinkaya et al., 2006; Işıkber et al., 2006; Uslu et al., 2006; Sen et al., 2009; Akan & Ferizli, 2010; Tütüncü & Emekci, 2014). Also, high temperature, irradiation, controlled atmosphere, low pressure applications and numerous fumigants have been considered as alternatives in other studies (Fields & White, 2002; Baltaci et al., 2006; Navarro et al., 2006; Campabadal, 2007; Small, 2007; Ducom, 2012).

Among the methyl bromide alternatives, controlled atmospheres (CAs) have been increasingly adopted worldwide (Adler et al., 2000; Navarro, 2012). The major constraint of CAs applications for the disinfestation of dried figs in Turkey is the length of exposure periods. Exposure of 24 h to methyl bromide at normal atmospheric pressure was the typical disinfestation practice before its ban in 2015. Development of resistance to phosphine resulted with ineffective treatments at the label exposure periods of minimum 3 d (personal communication, S. Navarro), which means CA applications to compete with phosphine in term of exposure periods in dried fig pest management are now favored.

CAs in sublethal doses can cause several physiological and behavioral changes in insects. Among physiological responses; impaired metamorphosis (Ali-Niazee, 1971, 1972; Storey, 1977, 1978), reduced mating frequency and fecundity (Shorey, 1964; Lum & Flaherty, 1972), opening the spiracles continuously in hypercarbic conditions (Navarro, 2012), increased cell membrane permeability, (Hochachka, 1986; Zhou et al., 2001), as well as decreased respiration rate, metabolic rate and ATP production (Ali-Niazee, 1971; Friedlander & Navarro, 1979; Zhou et al., 2000; Carpenter et al., 2001) have been reported. For the behavioral ones, increasing in egg laying close to odor of wheat with in presence of CO_2 (Barrer & Jay, 1980), and immobilization (Ali-Niazee, 1972; Edwards & Batten, 1973) have also been reported.

CAs and toxic fumigants treatments against the arthropod pests of stored products can also lead to prolonged development in various life stages, including eggs and pupal stages of stored products pests (Ali-Niazee & Lindgren, 1970; Ali-Niazee,1971, 1972; Storey, 1977, 1978; Spratt, 1979; Rajendran, 2000; Nayak et al., 2003), and this is of practical importance in making right decision on the length of fumigation treatments to ensure maximum efficacy with the least chance of resistance development in insect pests. There is a limited number of studies available in regard to prolonged egg development caused by CAs treatments for *E. cautella*. Retardation in embryonal development as a sublethal effect of high CO_2 atmospheres in *E. cautella* was reported, especially when exposed to 100% CO_2 and 100% N_2 in CAs that cause 1-2 d of delay in hatching of *E. cautella* and *Ephestia kuehniella* Zeller, 1879 (Lepidoptera: Pyralidae) eggs (Bell et al., 1980).

The response of insects to modified atmospheres applications differs among life stages and even among age groups of the same life stages. Egg stage, in particular, has the ability to survive in hypoxic conditions and this ability gives insects an opportunity to extend their survivorship (Bell, 2012). Eggs and pupae of *E. cautella* are found to be more tolerant to hypercarbic or hypoxic environments than the larvae and adults (Storey, 1975; Jay, 1984). Thus, this study was undertaken to evaluate if there is any change in delayed hatching among different age groups of eggs of *E. cautella* which were exposed to combination of distinctive hypercarbic and anoxic atmospheres at various temperatures.

Materials and Methods

Age of eggs

Ephestia cautella cultures were reared in 1-L glass vials containing a mix of 200 g of coarsely grounded soft wheat grain, glycerin and brewer's yeast in a ratio of 14:2:1 (by wt) as described by Bell (1975) with the slight modifications in the rate of food ingredients. Insect cultures were held at 25°C and 65% RH in a constant temperature room. To obtain eggs of similar age, newly emerged adults were transferred to egg laying cages by suction apparatus made from PVC mesh.

Twenty-four h after the adults were transferred to egg laying cages, eggs of 0-24 h old were collected from the cages. To obtain eggs 24-48 and 48-72 h old, eggs 0-24 h old were kept at insect rearing room for additional 24 and 48 h, respectively. Studies were conducted between 2012 and 2014 in laboratory of Stored Products Pests, Plant Protection Department, Faculty of Agriculture, Ankara University.

Gas composition

In the experiments, cylinders containing 85% CO_2 + 3% O_2 +12 % N_2 and 95% CO_2 + 1% O_2 + 4% N_2 supplied by Linde Gas (Ankara) were used as the gas source. For brevity, these gas compositions are called 85% CO_2 and 95% CO_2 below.

Experimental equipment and design

Plexiglass vials (10mL) were used to contain eggs of different age groups. A 10-mm round hole was drilled in the lid of each vial, and a piece of wire mesh (125 micron) was hot glued to the inner surface of the lid to allow air passage. Eggs counted under stereomicroscope at 20 X magnification and transferred to plexiglass vials as 50 eggs per vial. A small amount of food was also introduced into each vial as the food source for the emerging larvae.

The CA treatments were performed in Dreshel flasks of 550 mL capacity as described by Hashem et al. (2012). The flasks inlet valves were connected to the premixed gas cylinder and outlet valves connected with oxygen meter (OxyCheq Expedition O2 Analyzer, OA-01-01, OxyCheck, Marianna, FL, USA).

Gas application

Plexiglass vials containing eggs of different ages were put into Dreshel flask, and 15 min of gas purging period with flow rate of 100 mL/min was applied to reach the desired gas composition inside the flask. The humidity inside the Dreshel flask was controlled using plexiglass vials containing 50 mL of KOH solution (70% RH) inside the flask. During the whole gas purging period O_2 concentration inside the flask was continuously monitored by O_2 meter connected to the outlet tube of the Dreshel flask by a short hose. After the end of gas purging process, inlet and outlet valves were closed and the Dreshel flasks were put into incubators (Binder KB-720, Tuttlingen, Germany). Vials containing control groups were treated similarly except they were exposed to normal atmospheric air only.

Experiments were conducted at 20, 25 and 30°C at 65±5% RH at different exposure periods ranging from 4 to 104 h with three or more replicates.

Post treatment observations

After the end of each exposure periods, vials containing eggs were taken from Dreshel flasks and transferred to insect rearing room adjusted at 25°C temperature and 65±5% RH. Egg hatching were then checked under the stereomicroscope twice daily for 20 d.

Statistical analysis

Differences in exposure periods were statistically evaluated using the general linear model procedure for one way ANOVA (Stat Soft Inc., Tulsa, OK, USA). Duncan's multiple range test was used to compare daily hatching eggs against the control group. A 95% confidence level was applied for all statistical analysis.

Results

At 30°C, eggs of different age groups exposed to 85% CO_2 for short exposure times such as 4-12 h started hatching at the same day as the controls. However, in comparing with controls, the hatch of eggs 24-48 h and 48-72 h-old was statistically different ($P \leq 0.05$). Mostly, the delayed hatching in all of the three age groups were statistically different at various exposure periods when compared to control (Table 1).

At 30°C, disregarding exposure periods or age groups, 85% CO_2 exposure caused 1-3 d delays in egg hatch. With exposure of 16 and 20 h, a 1-d delay in hatch occurred in eggs 0-24 and 24-48 h old, whereas in eggs 48-72 h old, a 2-d delay in hatch was observed. At 95% CO_2 and 30°C, similar results were obtained at 85% CO_2 exposures. At these two CO_2 levels, there were extended delays of hatching simultaneously with exposure periods (Tables 1 & 2).

Table 1. Hatch of eggs of *Ephestia cautella* exposed to 85% CO_2 + 3% O_2 + 12% N_2 at 30°C

	Daily egg hatch (number)														
	eggs 0-24 h old				eggs 24-48 h old					eggs 48-72 h old					
Exposure period (h)	n	R	D3[a]	D4	n	R	D2[a]	D3	D4	n	R	D1[a]	D2	D3	D4
Control	546	7	284	92	646	10	430	129	0	445	6	213	172	0	0
4	300	5	209	46	250	4	164	54	0	260	3	64*	103	29*	0
8	347	5	182	76	250	4	97*	111*	0	241	3	0*	100	24*	0
12	380	5	123*	36	210	3	0*	117*	32*	210	3	0*	0*	61*	35*
16	396	5	0*	119*	387	6	0*	245*	39*	150	3	0*	0*	21*	2*
20	452	6	0*	42	266	4	0*	79	11*	280	4	0*	0*	36*	7*
24	-	-	-	-	211	3	0*	38	10*	206	3	0*	0*	10*	4*
28	-	-	-	-	262	4	0*	0*	10*	258	4	0*	0*	0	2*
32	-	-	-	-	260	4	0*	0*	1*	-	-	-	-	-	-

[a] First day of hatch after exposure; R, replicate; D1-D4: Days 1 to 4;
* In the same column differences in comparing with control is significant $p \leq 0.05$ (Duncan).

At 25°C and 85% CO_2, delayed hatching occurred with exposure of 4 h and longer. Delayed hatch of eggs 0-24 h old was observed with exposures lasting longer than 1 d. The increase of exposure periods did not cause any delay of hatching in eggs 24-48 and 48-72 h old. For eggs 24-48 h old exposed for 4-42 h, a 2-d delay in hatch occurred, and a 3-d delay in egg hatch for eggs 48-72 h old exposed for 4-36 h (Table 3).

At 95% CO_2 and 25°C, a hatching delay of 1-3 d occurred in eggs 0-24 h old as the exposure period increased. For short exposures, such as 2 and 4 h, egg hatch started at the same day as the control, while other exposure times starting from 8 h caused hatching delays. Exposures lasting longer than 12 h caused delays in egg hatch in eggs 24-48 h old, and up to a 2-d delay occurred following 32 h exposure. Unlike eggs 0-24 h and 24-48 h old, egg hatching delays in eggs 48-72 h old occurred with the exposure of 4 h and longer, and with a delay of up to 2 d as exposure time increased (Table 4).

At 20°C and 85% CO_2, egg hatch started 4-7 d later than controls with long exposures of 80, 88 and 96 h. Delayed hatch was positively correlated with increased in exposure period. Hatching delay in eggs 0-24 h old started after 24 h of exposure, but started at 8 h for eggs 24-48 and 48-72 h old (Table 5).

Table 2. Hatch of eggs of *Ephestia cautella* exposed to 95% CO_2 + 1% O_2 + 4% N_2 at 30°C

	Daily egg hatch (number)															
	eggs 0-24 h old					eggs 24-48 h old					eggs 48-72 h old					
Exposure period (h)	n	R	D3[a]	D4	D5	n	R	D2[a]	D3	D4	n	R	D1[a]	D2	D3	D4
Control	547	9	322	115	0	644	10	387	152	0	590	9	294	217	0	0
8	-	-	-	-		-	-	-	-	-	200	3	0*	129	31*	0
12	250	4	22*	99*	5*	250	4	5*	155*	10*	250	4	0*	98	47*	0
16	314	5	0*	109	37*	250	4	0*	73	22*	200	3	0*	0*	49*	22*
20	443	7	0*	114	50*	759	11	0*	74	46*	379	6	0*	0*	39*	16*
24	402	5	0*	0*	16*	257	4	0*	0*	19*	242	3	0*	0*	0	26*
28	365	6	0*	0*	3*	369	6	0*	0*	4*	611	6	0*	0*	0	3*
32	271	5	0*	0*	6*	265	5	0*	0*	3*	-	-	-	-	-	-

[a] First day of hatching after exposure; R, replicate; D1-D5: Days 1 to 5;
* In the same column differences in comparing with control is significant $p \leq 0.05$ (Duncan).

Table 3. Hatch of eggs of *Ephestia cautella* exposed to 85% CO_2 + 3% O_2 + 12% N_2 at 25°C

	Daily egg hatch (number)																						
	eggs 0-24 h old								eggs 24-48 h old							eggs 48-72 h old							
Exposure period (h)	n	R	D3[a]	D4	D5	D6	D7	D8	n	R	D2[a]	D3	D4	D5	D6	n	R	D1[a]	D2	D3	D4	D5	
Control	506	8	330	89	12	0	0	0	403	7	248	94	3	0	0	404	7	222	126	14	0	0	
4	250	4	0*	112*	5	0	0	0	240	4	0*	0*	191*	0	0	189	3	0*	0*	0*	130*	0	
12	250	4	0*	54	54*	0	0	0	150	3	0*	0*	99*	0	0	157	3	0*	0*	0*	100*	0	
16	150	3	0*	10	99*	2*	0	0	150	3	0*	0*	99*	0	0	150	3	0*	0*	0*	67*	0	
20	375	5	0*	35	17	3*	0	0	220	4	0*	0*	118*	18*	0	319	5	0*	0*	0*	91*	10*	
24	469	6	0*	3*	5	2*	0	0	193	3	0*	0*	115*	18*	0	458	4	0*	0*	0*	136*	25*	
28	405	6	0*	0*	0*	7*	11*	1*	210	4	0*	0*	83*	43*	0	208	3	0*	0*	0*	73*	3*	
32	150	3	0*	0*	0*	1*	6*	1*	250	3	0*	0*	42*	49*	0	197	3	0*	0*	0*	17*	0	
36	422	8	0*	0*	23	49*	2*	0	251	4	0*	0*	61*	10*	0	381	6	0*	0*	0*	8*	0	
42	150	3	0*	0*	0*	0	17*	0	361	5	0*	0*	11	14*	2*	-	-	-	-	-	-	-	
48	-	-	-	-	-	-	-	-	219	4	0*	0*	0**	1*	0	-	-	-	-	-	-	-	

[a] First day of hatching after exposure; R, replicate; D1-D8: Days 1 to 8;
* In the same column differences in comparing with control is significant $p \leq 0.05$ (Duncan).

Table 4. Hatch of eggs of *Ephestia cautella* exposed to 95% CO_2 + 1% O_2 + 4% N_2 at 25 ^{0}C

Exposure period (h)	Daily egg hatch (number)																			
	eggs 0-24 h old							eggs 24-48 h old							eggs 48-72 h old						
	n	R	D3[a]	D4	D5	D6	D7	n	R	D2[a]	D3	D4	D5	D6	n	R	D1[a]	D2	D3	D4	D5
Control	401	6	235	94	3	0	0	587	8	245	259	27	0	0	591	8	195	314	28	0	0
2	150	3	43*	35*	0*	0	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	150	3	11*	94*	7	0	0	150	3	1*	108*	9	0	0	150	3	0*	110*	28	0	0
8	150	3	0*	03*	9	1*	0	192	3	12*	130*	10	3*	0	434	6	0*	256	106*	11*	0
12	287	6	0*	72*	50*	2*	0	475	7	20*	248	98*	0	0	243	4	0*	31*	90*	1*	0
16	250	5	2*	16*	72*	2*	0	290	6	0*	167	94*	3*	0	331	5	0*	8*	122*	57*	0
20	350	7	0*	99	110*	4*	0	505	9	0*	174	13*	14*	1*	322	5	0*	0*	102*	7*	0
24	257	5	0*	13	84*	12*	0	223	4	0*	18*	05*	9*	3*	303	6	0*	0*	86*	15*	0
28	216	4	0*	0*	31	7*	0	212	4	0*	0*	45*	16*	0	220	4	0*	0*	19	9*	0
32	158	3	0*	0*	9	5*	0	155	3	0*	0*	24	2*	0	200	3	0*	0*	3	12*	1*
36	720	9	0*	0*	12	34*	7*	-	-	-	-	-	-	-	424	7	0*	0*	5	0	0

[a] First day of hatching after exposure; R, replicate; D1-D7, Days 1 to 7;
* In the same column differences in comparing with control is significant $p \leq 0.05$ (Duncan).

Table 5. Hatch of eggs of *Ephestia cautella* exposed to 85% CO_2 + 3% O_2 + 12% N_2 at 20°C

	Daily egg hatch (number)																											
	eggs 0-24 h old									eggs 24-48 h old										eggs 48-72 h old									
E (h)	n	R	D6[a]	D7	D8	D9	D10	D11	D12	n	R	D4[a]	D5	D6	D7	D8	D9	D10	D11	n	R	D3[a]	D4	D5	D6	D7	D8	D9	D10
C	200	4	54	98	27	2	0	0	0	350	7	36	131	130	12	1	1	0	0	402	8	34	109	128	100	1	1	0	0
8	150	3	4*	54	39	7	0	0	0	250	5	0*	118	56	31	5	1	0	0	250	5	0*	113	73	23	1	0	0	0
16	150	3	1*	45	59*	16	1*	1*	0	250	5	0*	81	81	22	4	1	0	0	250	5	0*	79	57	26	4	0	0	0
24	150	3	0*	7*	45	19	1*	0	0	250	5	0*	13*	136	43	19	2	0	0	250	5	0*	68	66	37	9	0	0	0
32	150	3	0*	0*	18	28*	4*	0	0	300	6	0*	0*	117	81*	26	16	0	0	250	5	0*	38	84	43	15	1	0	0
40	150	3	0*	1*	23	47*	4*	1*	0	250	5	0*	0*	77	71*	21	15	0	1*	250	5	0*	0*	42	34	23	2	0	0
48	150	3	0*	0*	0*	14	4*	0	0	250	5	0*	0*	0*	70*	35	23	6*	0	250	5	0*	0*	60	38	25	7	2*	0
56	150	3	0*	0*	0*	10	12*	5*	0	250	5	0*	0*	0*	69*	39	34*	8*	1*	150	3	0*	0*	1	19	31*	7	9*	0
64	150	3	0*	0*	0*	1	16*	12*	0	150	3	0*	0*	0*	22	13	20*	9*	0	150	3	0*	0*	0*	0*	18	17*	4*	0
72	150	3	0*	0*	0*	0*	1*	1*	0	150	3	0*	0*	0*	1	27*	4	4*	0	150	3	0*	0*	0*	0*	10	20*	5*	0
80	150	3	0*	0*	0*	0*	1*	7*	1*	150	3	0*	0*	0*	0*	0*	0*	28*	0	150	3	0*	0*	0*	0*	0*	0*	0	14*
88	150	3	0*	0*	0*	0*	1*	0	2*	150	3	0*	0*	0*	0*	0*	0*	13*	0	150	3	0*	0*	0*	0*	0*	0*	0	3*
96	-	-	-	-	-	-	-	-	-	200	4	0*	0*	0*	0*	0*	11	9*	1*	200	4	0*	0*	0*	0*	0*	0*	5*	1*

[a] First day of hatching after exposure; D3-D12, Days 3 to 12; E (h), Exposure period (h); C, control; R, replicate;
* In the same column differences in comparing with control is significant $p \leq 0.05$ (Duncan).

At 95% CO_2 and 20°C, hatching delays at short exposures, such as 8 h onwards, occurred in eggs 0-24 and 48-72 h old, but from 24 h onwards in eggs 24-48 h old. Delays in egg hatch increased to 7 d for eggs 0-24 h old, and 6 and 8 d for eggs 24-48 and 48-72 h old, respectively (Tables 6 & 7).

Table 6. Hatch of eggs of *Ephestia cautella* 0-24 h old exposed to 95% CO_2 + 1% O_2 + 4% N_2 at 20°C

Exposure period (h)	n	Replicate	Daily egg hatch (number)							
			D5[a]	D6	D7	D8	D9	D10	D11	D12
Control	256	6	5	98	103	43	4	0	0	0
8	150	3	0*	3*	27	59*	6	0	0	0
16	150	3	1	19	24	53*	19	0	0	0
24	150	3	0*	0*	21	20	31*	10*	1*	1*
32	150	3	0*	0*	1*	13	31*	10*	2*	0
40	150	3	0*	0*	2*	33	32*	7*	0	0
48	150	3	0*	0*	0*	3	23	7*	0	1*
56	150	3	0*	0*	0*	0*	9	10*	2*	3*
64	150	3	0*	0*	0*	0*	4	5*	3*	1*
72	150	3	0*	0*	0*	0*	0*	5*	0	1*
80	150	3	0*	0*	0*	0*	0*	0	8*	7*
88	150	3	0*	0*	0*	0*	0*	0	0	1*

[a] First day of hatching after exposure; D5-D12, Days 5 to 12;
* In the same column differences in comparing with control is important $p \leq 0.05$ (Duncan).

Table 7. Hatch of eggs of *Ephestia cautella* exposed to 95% CO_2 + 1% O_2 + 4% N_2 at 20°C

	Daily egg hatch (number)																				
	eggs 24-48 h old										eggs 48-72 h old										
Exposure period (h)	n	R	D4[a]	D5	D6	D7	D8	D9	D10	D11	n	R	D2[a]	D3	D4	D5	D6	D7	D8	D9	D10
Control	406	8	29	143	160	29	3	0	1	0	253	5	8	57	68	31	75	4	0	0	0
8	150	3	9	41	25	41	9	0	0*	0	150	3	0*	37	34	18	39	2	0	0	0
16	150	3	5	45	31	25	18	2*	0*	0	150	3	0*	4	67	5	36	11	1*	0	0
24	150	3	0*	15	40	18	28	7*	3	0	200	4	0*	0*	50	37	27	22	3*	0	0
32	200	4	0*	1*	49	37	37	22*	2	1*	150	3	0*	0*	18	43	13	31	7*	2*	0
40	150	3	0*	0*	32*	24	10	19*	3	0	150	3	0*	0*	0*	25	22	18	16*	3*	0
48	150	3	0*	0*	8*	38	7	18*	7	2*	150	3	0*	0*	0*	8	29	12	18*	4*	0
56	150	3	0*	0*	6*	24	10	8*	6	1*	150	3	0*	0*	0*	5	10	12	13*	8*	2*
64	150	3	0*	0*	0*	9	1	2*	2	1*	200	4	0*	0*	0*	0*	0*	11	18*	6*	0
72	150	3	0*	0*	0*	1	9	3*	0*	1*	150	3	0*	0*	0*	0*	1	12	13*	5*	2*
80	200	4	0*	0*	0*	0*	0*	0	31*	0	150	3	0*	0*	0*	0*	0*	0*	0	0	9*
88	150	3	0*	0*	0*	0*	0*	0	15	0	150	3	0*	0*	0*	0*	0*	0*	0	0	3*
96	150	3	0*	0*	0*	0*	0*	6*	8	0	150	3	0*	0*	0*	0*	0*	0*	1*	0	1*
104	250	5	0*	0*	0*	0*	0*	3*	0*	0	-	-	-	-	-	-	-	-	-	-	-

[a] First day of hatching after exposure; D2-D11, Days 2 to 11; R, replicate;
* In the same column differences in comparing with control is important $p \leq 0.05$ (Duncan).

Discussion

As found in the earlier studies, both low O_2 and high CO_2 cause mortality by disrupting the metabolic balance (Banks & Annis, 1990; Fleurat-Lessard, 1990). However, there are numerous metabolic factors that lead to mortality (Mitcham et al., 2006). The reason that the insects react to high CO_2 more than to low O_2 lies in the difference in the permeability of insect tissues to these gases. The permeability to CO_2 is six times more than that to O_2. Regulation of respiration which is largely dependent on the brain receptors is another reason, since the brain receptors are more susceptible to increasing CO_2 than to low O_2 levels (Fleurat-Lessard, 1990).

In CA applications, 3% O_2 is the critical concentration and any CO_2 atmosphere containing O_2 below this concentration should not be considered as a high CO_2 atmosphere (Banks & Annis, 1990). On the other hand, some researchers believe that low O_2 and high CO_2 act together synergistically in low O_2 atmospheres (2-5%) containing some amount of CO_2 (5-40%) (Ali-Niazee, 1971; Calderon & Navarro, 1979; Calderon & Navarro, 1980; Krishnamurthy et al., 1986). Gas compositions used in this study fall into both low O_2 and high CO_2 categories and therefore they are considered to act synergistically to induce mortality.

Egg hatch in *E. cautella* is completed in 5 d and most embryonic development in the first 2 d (Bell, 1975). Embryonic development in insects starts with the first formative period (larval development phase) and lasts for 1 d in most insects. In this period, metabolic rate is slow. This slow period is followed by an increased metabolic rate (Fink, 1925). It is reported that during anoxia development is arrested and the longevity is largely dependent on the capacity of both accumulating glycolytic products and lowering the metabolic demands (Fleurat-Lessard, 1990). Both low O_2 and high CO_2 cause depleted metabolic rate and elevated cell permeability. Decreased metabolic rate means reduced ATP production, which implies insufficient energy supply to the tissues (Hochachka, 1986; Carpenter et al., 2001; Ofuya & Reichmuth, 2002). In these conditions delayed embryonal development is an inevitable result.

In the present study, eggs 0-24, 24-48 and 48-72 h old were exposed to various conditions that caused delayed hatch in response to both high CO_2 and low O_2 atmospheres. In 85% CO_2 atmosphere hatching delay in eggs 0-24 h old was found to be 1 d with short exposure periods and ≥2 d in long exposure periods (Tables 1, 3 & 5). Due to the lowered metabolic rate in eggs 24 h old, it is thought that the inhibitory effect of high CO_2 atmospheres for short exposures is minimal and its inhibitory effects can only be marked during long exposure periods. Fink (1925) proposed that metabolic rate increases when eggs are at an age closer to hatching. In the present study, in agreement with Fink (1925), there was a greater delay in eggs 48-72 h old (2-6 d at 25 and 20°C, respectively) than in eggs 24-48 h old (3-7 d at 25 and 20°C, respectively) (Tables 3 & 5). Due to the increased metabolic rate, we assume that older eggs when compared to younger ones respire more and absorb greater amounts of CO_2, which presumably resulted in an increased hatching delays in older eggs. Similarly, at 30°C, egg hatching delay extended to 3 d in eggs 48-72- h old, which was 2 d in eggs 24-48 h old (Table 1).

In a 95% CO_2 atmosphere, delays in eggs 48-72 h old were seen earlier than the two other age groups. Delays at different temperatures that occurred after 4-8 h exposures in eggs 48-72 h old started with 16 or 24 h of exposure in the other two age groups (Tables 2, 4, 6 & 7).

High temperatures cause an increase in insect metabolism which accelerates the toxic effect of CO_2 (White et al., 1995). In the present study at 85% CO_2 atmosphere, delays observed in different age groups and in different exposure periods were between 1-3 d at 30°C, 1-4 d at 25°C and 1-7 d at 20°C (Tables 1, 3 & 5).

At 30°C, delay in egg hatch increased to 3 d. Decreasing temperature to 25 and 20°C, increased the hatching delay to 4 and 7 d, respectively. These differences in hatching demonstrated the effect of temperature on the metabolic rate under elevated CO_2 and depleted O_2 conditions.

The respiration rate of low O_2 conditions reported to be decreased similarly with high CO_2 atmospheres (Zhou et al., 2000). In *Tribolium castaneum* (Herbst, 1797) (Coleoptera: Tenebrionidae), egg and adult respiration rate showed a significant decrease at O_2 below 5% (Yang et al., 2008a). In another study with *T. castaneum*, $O_2 \leq 5\%$ was found to be effective in lowering the respiration rate, particularly in eggs and young larvae (Emekci et al., 2002). Similarly, Guiqiang et al. (2008) reported that 10% O_2 caused delay in development in eggs and larvae of *T. castaneum*. In *T. castaneum, Oryzaephilus surinamensis* (Linnaeus, 1758) (Coleoptera: Silvanidae) and *Sitophilus zeamais* (Motschulsky), 1855 (Coleoptera: Curculionidae), a low O_2 atmosphere (15%) was reported to increase the egg development period (Yang et al., 2008b). In *S. zeamais*, egg development increased by up to 10-11 d after exposure to a modified atmosphere consisting of O_2, CO_2 and N_2 gases in a ratio of 1:1:8 (by vol.) (Spratt, 1979). In *Rhyzopertha dominica* (Fabricius, 1792) (Coleoptera: Bostrichidae), respiration of one-day-old eggs was suppressed by O_2 below 2% (Emekci et al., 2004). *Callosobruchus maculatus* (Fabricius, 1775) (Coleoptera: Chrysomelidae) eggs exposed to a gas composition of 10% CO_2 + 10% O_2 completed their development more slowly than the control (Cheng et al., 2012). According to Ali-Niazee & Lindgren (1970), high CO_2 in *Tribolium confusum* Jacquelin du Val, 1863 (Coleoptera: Tenebrionidae) and *T. castaneum* causes delayed or failed egg development through interfering with the embryo's nervous system by narcosis and with egg growth. Another important issue relating with synergistic effect of high CO_2 and low O_2 conditions is that threshold concentration of susceptibility for high CO_2 toxicity generally decreases in hypoxic conditions (Fleurat-Lessard, 1990). In the present study, delayed egg hatch was found to be statistically significant and longer exposure times caused greater delays, which is in agreement with previous studies. In the present study, there were eggs that hatched at 20^{o}C after 12 d following an 88-h treatment. Similarly, at 25 and 30°C, live larvae were found after 8 d following 42-h exposure and after 5 d following 32-h exposure. In practice, potential delays in insect development in CA applications should be considered carefully when making decision on suitable fumigation times for dried fruits. Fumigation time should be extended until there are no live insects, otherwise infestation by storage pests can easily build up.

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Original article (Orijinal araştırma)

Modeling the distribution of the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann, 1824) (Diptera, Tephritidae) in Turkey and its range expansion in Black Sea Region[1]

Akdeniz meyve sineği, *Ceratitis capitata* (Wiedemann, 1824) (Diptera, Tephritidae)' nin Türkiye'deki dağılımının modellenmesi ve yayılış alanında Karadeniz Bölgesi'ndeki genişleme

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Summary

Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann, 1824) (Diptera, Tephritidae), an important fruit pest, is not expected to expand its range under current climatic conditions with an exception of the Black Sea coasts that was hypothesized to be suitable for *C. capitata* invasion. After finding a *C. capitata*-infested mandarin in western Black Sea Region of Turkey, we tested this hypothesis. We first used ecological niche modeling approach to determine suitable places for *C. capitata* survival in Turkey; and then visited eastern Black Sea region of Turkey where the model predicted to be suitable. For the model, we collected fruit samples from 49 localities in southern and western Turkey, where *C. capitata* presence has already been known, and one locality from northern Turkey, where its presence has not been known, in November 2014 and 2015 and transferred them to laboratory to observe adult emergence. *Ceratitis capitata* adults emerged from 44 localities. These localities were used as the presence data for the model. After obtaining the model results, we visited eastern Black Sea Region, and collected fruit samples from 11 different localities, and transferred them to laboratory to observe adult emergence. In total, 49 *C. capitata* adults emerged. Thus, presence of *C. capitata* was shown in the eastern Black Sea for the first time. The model also predicted that suitable areas in the Black Sea basin for *C. capitata* survival tend to expand in future.

Keywords: Mediterranean fruit fly, *Ceratitis capitata*, ecological niche modeling, Eastern Black Sea, range expansion

Özet

Önemli bir meyve zararlısı olan Akdeniz meyve sineği, *Ceratitis capitata* (Wiedemann, 1824) (Diptera, Tephritidae)'nın yayılış alanını mevcut iklimsel koşullar altında genişletmeyeceği düşünülmekte; ancak, *C. capitata* istilası için uygun görünen Karadeniz kıyılarının bu genellemenin dışında olduğu varsayılmaktadır. Batı Karadeniz Bölgesi'nde *C. capitata* ile enfekte olmuş bir mandalina bulunduktan sonra bu varsayım sınanmıştır. Öncelikle Türkiye'de *C. capitata*'nın yaşamasına uygun olan alanların tespiti için ekolojik niş modellemesi yaklaşımı kullanılmış, daha sonra da modelin uygun olarak tahmin ettiği Doğu Karadeniz Bölgesi'nde arazi çalışmaları yapılmıştır. Modelleme için *C. capitata*'nın varlığı bilinen Akdeniz ve Ege Bölgesi'nden 49 noktadan ve *C. capitata*'nın varlığı bilinmeyen Karadeniz Bölgesi'nden bir noktadan 2014 ve 2015 yılları Kasım ayında meyve örneği toplanmış ve laboratuvara getirilerek ergin çıkışları izlenmiştir. Toplamda 44 farklı yöreden ergin *C. capitata* elde edilmiştir. Bu noktalar model için veri olarak kullanılmıştır. Modelleme sonuçlarına göre Karadeniz Bölgesi'nde arazi çalışmaları gerçekleştirilmiştir. Meyve örnekleri 11 noktadan toplanarak laboratuvara getirilmiş ve ergin çıkışları izlenmiştir. Bu örneklerden toplam 49 ergin *C. capitata* çıkmıştır. Böylece Doğu Karadeniz'de Akdeniz meyve sineğinin ilk kez varlığı gösterilmiştir. Model ayrıca gelecekte Karadeniz havzasında *C. capitata* için uygun olan alanların oluşacağını da göstermektedir.

Anahtar sözcükler: Akdeniz meyve sineği, *Ceratitis capitata*, ekolojik niş modellemesi, Doğu Karadeniz, yayılış alanı genişlemesi

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Introduction

Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann, 1824) (Diptera: Tephritidae), is a cosmopolitan species distributed in the Middle East, southern Europe, Africa, South America and Australia (Liquido et al., 1990; Bergsten et al., 1999; USDA, 2003; Bayrak & Hayat, 2012). Its host range consists more than 400 plant species, including several economically valuable fruits. This fact along with its capacity for long-distance flight makes *C. capitata* one of the most important fruit pests in many countries worldwide (Liquido et al., 1990; Harris & Olalquiaga, 1991; Bergsten et al., 1999; Israely et al., 2005; Meats & Smallridge, 2007).

Ceratitis capitata adult females generally prefer mature fruit with thin skin for oviposition. Citrus fruits except lemon, kiwi and persimmon are among the highly effected hosts of *C. capitata*. Banana, eggplant, grape, tomato, papaya, and pepper are among its other important hosts with relatively high damage. However, *C. capitata* was also found to attack cacti, cucumber and zucchini in the laboratory and almond, bean and pineapple in the field, but the economic level of damage in these hosts is unknown (Elekçioğlu, 2013; UF-IFAS, 2016). Economically important plants that have been reported being attacked in Turkey by *C. capitata* are citrus except apple, avocado, fig, lemon, peach, persimmon, pomegranate, and quince (Elekçioğlu, 2009, 2013).

Damage caused by *C. capitata* occurs immediately after eggs are laid in fruit or soft tissues of the host plant, and emerging larvae start feeding. Moreover, saprophytic bacteria transmitted during oviposition cause fruit to decay (Bergsten et al., 1999). Bacterial activity leads to color deterioration in fruit which starts from the point of oviposition and expands to the entire fruit over time; and eventually the fruit turns yellow and falls off earlier (Elekçioğlu, 2013). *Ceratitis capitata* has been included in the EPPO A2 quarantine list (EPPO, 1981) because of the damage it causes to fruit.

Turkey is a major fruit-producing country (e.g. annual production of citrus, peach, pomegranate, and fig is about 3,976, 650, 450 and 300 kt, respectively) (TUIK, 2016). *Ceratitis capitata* is one of the most important insect pests of fruit in Turkey (Hendrichs et al., 1995; Elekçioğlu, 2009). Due to its quarantine status, it can also cause issues in fruit export (Elekçioğlu, 2009).

According to the Turkish Ministry of Agriculture, *C. capitata* is mainly present along the southern and western coast areas of Turkey (Kaya, 2013 and references cited therein). EPPO (2015) claims that considering seasonal and geographic conditions that *C. capitata* needs to survive, it has reached the limits of its possible range, and expansion of its range is not expected with an exception of the Black Sea basin. Thus, the potential invasion of the Black Sea basin by *C. capitata* rests as a hypothesis to test. A recent article in local newspapers reported that *C. capitata* was present in Samsun (central Black Sea Region), therefore this hypothesis needs to be seriously considered. In a previous field trip to Bartın in the western Black Sea Region of Turkey in 2015, we found *C. capitata* larvae in mandarin which further strengthened the need to test this hypothesis.

Ecological niche modeling (ENM) is widely used to predict the potential geographic distribution of species using presence data and environmental variables (Guisan & Thuiller, 2005). Based on the assumptions of species–climate equilibrium and stability of ecological niches through time (Nogués-Bravo, 2009), ENM can also be used to predict past or future geographic distribution of species (Peterson et al., 2002; Hijmans & Graham, 2006; Waltari et al., 2007; Waltari & Guralnick, 2009; Gür, 2013). These projections can be useful in planning conservation and pest management programs (Sen et al., 2016).

In this study, we tested the hypothesis described above through a region wide survey. For this purpose, (1) we determined localities where *C. capitata* can survive in Turkey by modeling its ecological niches, and (2) we conducted field work along the Black Sea coastal line in Turkey. We also built ENM-based future distribution maps which can be useful in planning pest management strategies.

Materials and Methods

To use as the species presence data, we collected fruit samples from 49 localities in southern and western Turkey where the occurrence of *C. capitata* was already known and from one locality (Bartın) in the Black Sea Region where no presence data has been reported before this study (Table 1, Figure 1). Collection of fruit samples were carried out in November 2014 and 2015.

Table 1. *Ceratitis capitata* (Wiedemann, 1824) presence data collected in Turkey to use in ecological niche modeling

	Coordinates / Altitude (m)	Locality	Host Fruit
1	39° 31' N, 26° 30' E / 5	Behram, Çanakkale	mandarin
2	39° 34' N, 27° 00' E / 9	Edremit, Balıkesir	mandarin
3	39° 12' N, 26° 47' E / 10	Altınova, Balıkesir	orange
4	39° 05' N, 26° 54' E / 20	Dikili, İzmir	mandarin
5	38° 51' N, 27° 02' E / 10	Yenişakran, İzmir	mandarin
6	38° 37' N, 27° 08' E / 23	Emiralem, İzmir	mandarin
7	38° 27' N, 27° 14' E / 63	Bornova, İzmir	mandarin
8	38° 09' N, 27° 22' E / 32	Torbalı, İzmir	mandarin
9	38° 14' N, 27° 58' E / 138	Ödemiş, İzmir	mandarin, orange
10	37° 57' N, 27° 23' E / 17	Selçuk, İzmir	mandarin
11	37° 48' N, 28° 27' E / 200	Aydın	mandarin
12	37° 43' N, 27° 17' E / 62	Kuşadası, Aydın	mandarin, orange
13	37° 48' N, 27° 17' E / 26	Kuşadası-Söke, Aydın	mandarin
14	37° 54' N, 28° 19' E / 75	Nazilli, Aydın	mandarin
15	37° 46' N, 27° 37' E / 28	Söke-Koçarlı, Aydın	mandarin
16	37° 41' N, 27° 59' E / 70	Çine, Aydın	mandarin
17	37° 34' N, 27° 49' E / 85	Karpuzlu, Aydın	mandarin
18	37° 20' N, 27° 47' E / 15	Milas, Muğla	mandarin
19	37° 19' N, 27° 46' E / 78	Milas, Muğla	mandarin, orange
20	37° 03' N, 28° 21' E / 10	Marmaris, Muğla	mandarin
21	36° 58' N, 28° 41' E / 28	Köyceğiz, Muğla	mandarin
22	37° 00' N, 28° 30' E / 83	Marmaris-Köyceğiz, Muğla	mandarin, orange
23	36° 47' N, 28° 50' E / 2	Muğla-Fethiye, Muğla	orange
24	36° 38' N, 29° 18' E / 177	Fethiye, Muğla	mandarin, orange
25	36° 19' N, 29° 19' E / 6	Kınık, Antalya	mandarin, orange
26	36° 12' N, 29° 38' E / 2	Kaş, Antalya	orange
27	36° 14' N, 30° 01' E / 10	Demre, Antalya	mandarin, orange
28	36° 19' N, 30° 11' E / 2	Finike, Antalya	mandarin, orange
29	36° 21' N, 30° 17' E / 3	Kumluca, Antalya	mandarin, orange
30	36° 24' N, 30° 28' E / 5	Çıralı, Antalya	fig
31	36° 35' N, 30° 32' E / 22	Kemer, Antalya	mandarin
32	36° 59' N, 30° 36' E / 292	Döşemealtı, Antalya	mandarin

Table 1. (Continued)

	Coordinates / Altitude (m)	Locality	Host Fruit
33	36° 49' N, 31° 22' E / 60	Manavgat, Antalya	mandarin
34	36° 33' N, 32° 03' E / 20	Alanya, Antalya	mandarin
35	36° 09' N, 33° 40' E / 10	Silifke, Mersin	fig
36	36° 33' N, 34° 09' E / 505	Çamlıgöz, Mersin	fig, mandarin, orange
37	36° 37' N, 34° 20' E / 2	Alata, Mersin	fig, mandarin, orange
38	36° 51' N, 34° 34' E / 281	Aslanköy, Mersin	fig, mandarin, orange
39	36° 52' N, 34° 35' E / 281	Bulluklu, Mersin	mandarin
40	36° 54' N, 34° 49' E / 93	Bağlarbaşı, Mersin	fig, mandarin, orange
41	36° 55' N, 34° 54' E / 14	Tarsus, Mersin	fig, mandarin, orange
42	36° 59' N, 35° 02' E / 39	Yenice, Mersin	fig, mandarin, orange
43	37° 00' N, 35° 20' E / 33	Adana	mandarin, orange
44	36° 55' N, 35° 20' E / 21	Havutlu, Adana	mandarin, orange
45	36° 50' N, 36° 11' E / 29	Dörtyol-Erzin, Hatay	mandarin, orange
46	36° 35' N, 36° 10' E / 8	İskenderun, Hatay	fig, mandarin, orange
47	36° 12' N, 36° 07' E / 131	Çekmece, Hatay	fig, mandarin, orange
48	36° 07' N, 36° 00' E / 85	Mızraklı, Hatay	fig, mandarin, orange
49	36° 06' N, 35° 56' E / 17	Samandağ, Hatay	fig, mandarin, orange
50	41° 38' N, 32° 12' E / 249	Güzelcehisar, Bartın	mandarin

Fruit samples were collected either directly from trees or among the fallen fruit on the ground around the trees visited. Samples were transferred to the laboratory in 750ml glass jars with covers having a net with a mesh size 0.5mm fixed on a ventilation hole with a diameter of 2.5mm. In the laboratory, fruit samples were transferred separately to 1.5 l plastic pupation boxes with covers having a net with a mesh size 0.5mm fixed on a ventilation hole with a diameter of 50mm. The fruit samples were placed on sterilized sand poured in the pupation box to the height of 20mm. These boxes were checked daily for 20d in order to observe larvae jumping out of the decaying fruit to pupate in the sand. After 20d, pupae in the sand were collected using a sieve and transferred to Petri dishes for rearing. Adult emergence from the pupae were checked and recorded daily. Emerging adults were identified under a dissection microscope. Only the localities from infested fruit were collected were used as presence data for the ENM.

We mostly collected presence data from the western and southern Turkey. In order to correct this sampling bias, a Gaussian kernel density of presence records was created by a sampling bias distance of two decimal degree (Elith et al., 2010; Fourcade et al., 2014).

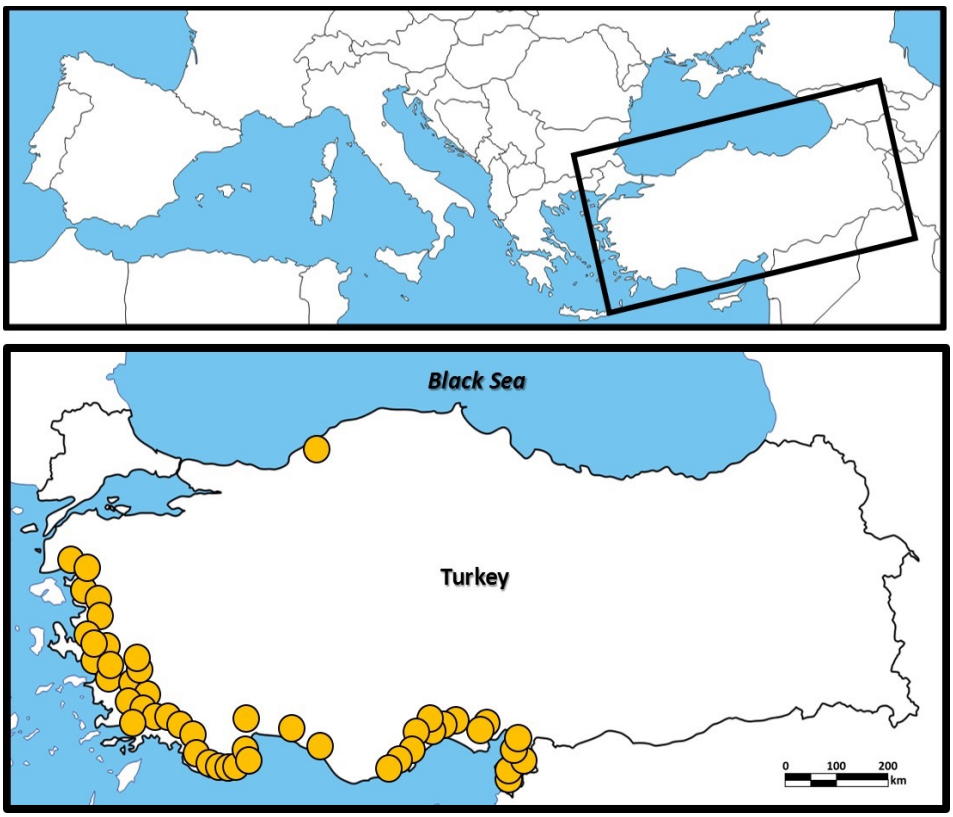

Figure 1. Distribution of the presence data for the Mediterranean fruit fly in Turkey used in ecological niche modeling.

Current (1950 to 2000) and projected future (2050 and 2070) bioclimatic variables (BIO1-19) were downloaded from the WorldClim database (Hijmans et al., 2005) at a resolution of 2.5 arc min. The 2050 bioclimatic data was the average for 2041 to 2060 and the 2070 data was the average for 2061 to 2080. Both future bioclimatic variables are based on the CCSM4 general circulation model simulation (see Coupled Model Intercomparison Project Phase 5; cmip-pcmdi.llnl.gov/cmip5). Future bioclimatic variables are available in four different representative concentration pathways (RCPs) at WorldClim which are rcp26, rcp45, rcp60 and rcp85 (Moss et al., 2008). In this study, we used bioclimatic variables that were adopted by rcp60 scenario only. The variables were masked by following coordinates: 34^{o} to 43^{o} N and 24^{o} to 47^{o} E. All of 19 bioclimatic variables were used for modeling.

To predict the potential distribution of *C. capitata* in Turkey under current and projected future bioclimatic conditions, we used the maximum entropy machine learning algorithm in the software MAXENT 3.3.3k (Phillips et al., 2006). All GIS operations were conducted using the software SDMtoolbox 1.1c (Brown, 2014) implemented in the ArcGIS version 10.2.2. MAXENT was run with default settings. Modeling was performed with ten-fold cross-validation runs. 80% of the presence records were used to train the modeling and 20% were used to test it for each run. For future projections, the modeling was repeated for the 2050 and 2070 data sets separately.

The area under curve (AUC) was used to evaluate the model performance (Fielding & Bell, 1997). An AUC > 0.5 indicates that the model performs better than a random prediction (i.e. AUC ≥ 0.9 = very good, 0.9 > AUC ≥ 0.8 = good, and AUC < 0.8 = poor) (Gassó et al., 2012). The model outputs were generated in logistic format.

After obtaining the results from the ENM study described above, we visited the localities along the Black Sea coastal line where the model expected relatively higher probability of *C. capitata* occurrence. We collected fruit samples from 11 localities in November 2014 and 2015, and transferred them to laboratory (Table 2, Figure 2). Details related to field and laboratory procedures were as described above.

Table 2. Fruit sampling localities visited to search *Ceratitis capitata* (Wiedemann, 1824) presence along the Black Sea Region of Turkey

	Coordinates / Altitude (m)	Locality	Host Fruit
1	41° 14' N, 36° 15' E / 200	İlkadım, Samsun	apple, mandarin, medlar, pear, pomegranate
2	41° 12' N, 36° 57' E / 9	Terme, Samsun	apple, pear, persimmon, quince
3	41° 03' N, 37° 28' E / 15	Fatsa, Ordu	apple, orange, persimmon
4	41° 03' N, 37° 46' E / 10	Perşembe, Ordu	apple , mandarin, pear, persimmon
5	41° 01' N, 38° 56' E / 200	Görele, Giresun	mandarin
6	40° 56' N, 38° 42' E /13	Espiye, Giresun	apple, medlar, persimmon
7	40° 56' N, 38° 14' E / 10	Bulancak, Giresun	apple, mandarin, persimmon
8	41° 02' N, 39° 13' E / 40	Beşikdüzü, Trabzon	apple
9	41° 01' N, 29° 33' E / 55	Akçaabat, Trabzon	mandarin
10	41° 10' N, 40° 53' E / 28	Pazar, Rize	mandarin
11	41° 04' N, 41° 01' E / 350	Çamlıhemşin, Rize	fig, mandarin

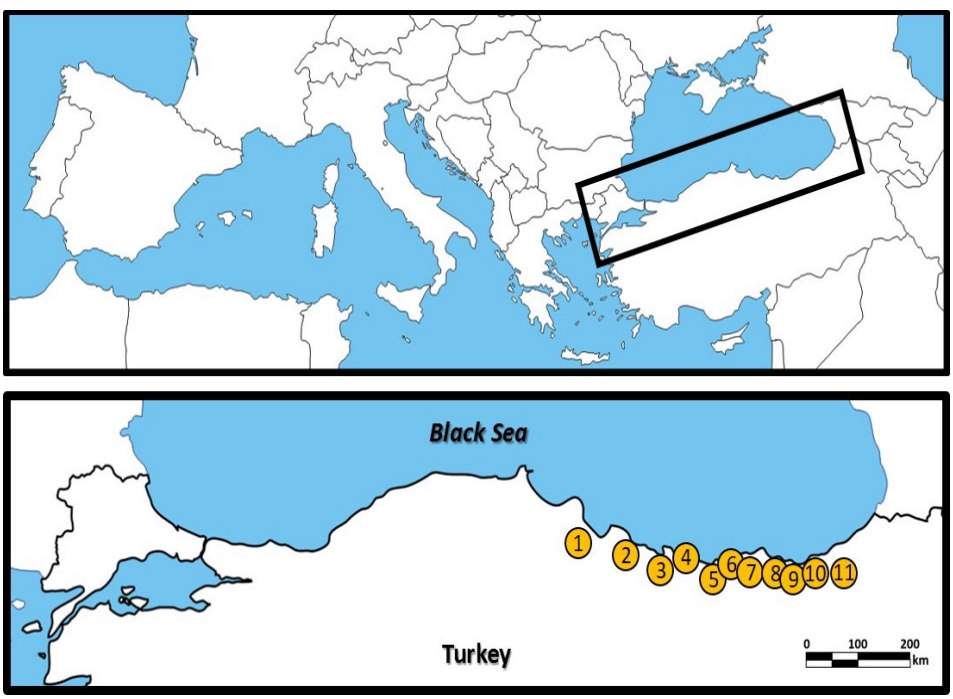

Figure 2. Localities visited to collect fruit samples to search *Ceratitis capitata* (Wiedemann, 1824) presence along the Black Sea Region of Turkey.

Results and Discussion

In total, more than 500 adults emerged from the fruit collected from 50 different sites. The ENM performed better than a random prediction (AUC > 0.5). The average test AUC for the replicate runs was 0.957, and the standard deviation was 0.054. These results indicate that performance of the model was very good (Gassó et al., 2012). The prediction of current bioclimatic conditions largely matched the known geographic distribution of *C. capitata* which is very near to equilibrium with climate and inhabits coastal areas of Turkey. The percentage contribution suggested that 'mean temperature of coldest quarter' (BIO11) (45%), 'precipitation of coldest quarter' (BIO19) (32.6%), and 'precipitation of driest quarter' (BIO17) (6.5%) were the most significant bioclimatic variables in predicting the present potential distribution of *C. capitata*.

According to our model, *C. capitata* is potentially present along the Black Sea coast, particularly in the eastern part (Figure 3a). Additionally, the potential distribution of *C. capitata* under projected future bioclimatic conditions tends to expand as a response to future climatic change (Figure 3b, c).

From 136 of 170 (80%) fruit samples collected from the localities where the model predicted a relatively higher probability of *C. capitata* occurrence (Eastern Black Sea Region, Figure 2), a total of 49 adult *C. capitata* emerged from pupae. Localities where *C. capitata* adults emerged were in Samsun, Ordu and Trabzon (localities 1, 2, 3, 4, 8 and 9, Table 2, Figure 2). Thus, in parallel to the predictions of the ENM and for the first time, we demonstrated that *C. capitata* has invaded the Black Sea Region of Turkey.

The number of generations produced by the pest is 4-5 in the Aegean Region and 7-8 in the Mediterranean Region, and oviposition ceases when the temperature drops below 17°C (Bergsten et al., 1999; USDA, 2003; BKU, 2016; UF-IFAS, 2016). The minimum and optimum temperatures for larval emergence are 11 and 25°C, respectively. The minimum and maximum temperatures for the pupal stage are 9.7 and 35°C, respectively, whereas the optimum temperature is between 22 and 30°C (Shoukry & Hafez, 1979; Bergsten et al., 1999; USDA, 2003). Development of egg, larva and pupa stages slows down or ceases below 10°C (Bergsten et al., 1999; USDA, 2003; BKU, 2016). Thus, temperature is one of the most significant limiting factors and our results suggested the same. This is not a surprising result for insect species in general because their metabolic rates are directly tied to environmental temperature (Taylor, 1981; Pedigo & Rice, 2009; O'Connor et al., 2011).

Additionally, our model suggested that the precipitation can be responsible of the current distribution pattern of *C. capitata*. This result can be explained by the high correlation between temperature and precipitation in the Mediterranean (Tanarhte et al., 2012).

We visited the localities in the eastern Black Sea Region that were expected to be invaded by the Mediterranean fruit fly according to the ENM and we found the pest in these localities. To the best of our knowledge, this study is the first attempt to predict current and future distribution of *C. capitata* in Turkey. We cannot conclude that the model used was adequate to predict the exact distribution of the pest in Turkey; however, it successfully predicted its distribution in eastern Black Sea Region. Wider sampling is needed to test total success of the model.

This study is a first record of *C. capitata* range expansion through northern Turkey. We did not conduct any study to detect the size of the populations or the damage ratio in the region, but we found that *C. capitata* is widely established in the Black Sea Region, which suggests that invasion is not recent. Dispersal speed of *C. capitata* is reported to be 20 km/yr (Harris & Olalquiaga, 1991; Israely et al., 2005; Meats & Smallridge, 2007). Therefore, if this has not already happened, *C. capitata* is likely to spread to other countries in the eastern Black Sea basin in the near future.

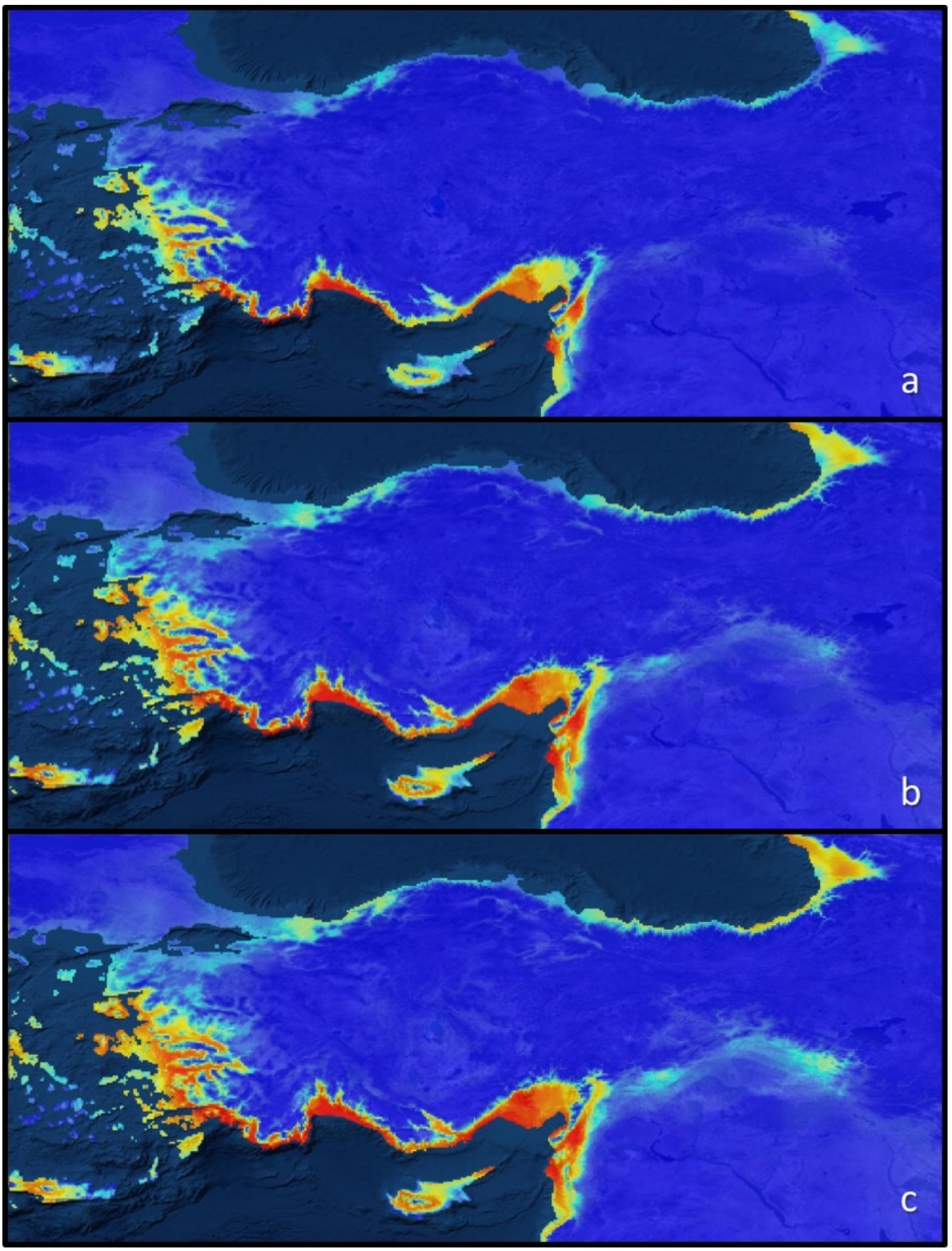

Figure 3. Distribution of *Ceratitis capitata* (Wiedemann, 1824) in (a) present day, (b) 2050, (c) 2070 according to the ecological niche model used. Warmer colors indicate areas of relatively higher suitability.

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Original article (Orijinal araştırma)

Effects of artificial diets and floral nectar on parasitization performance of *Trichogramma brassicae* Bezdenko, 1968 (Hymenoptera: Trichogrammatidae)[1]

Yapay besin ve bitki nektarının *Trichogramma brassicae* Bezdenko, 1968 (Hymenoptera: Trichogrammatidae)'nin parazitleme performansına etkileri

Nihal ÖZDER[2*] **Şeyda DEMİRTAŞ[2]**

Summary

This study was conducted to determine whether various food resources enhanced the longevity and fecundity of the egg parasitoid *Trichogramma brassicae* Bezdenko, 1968 (Hymenoptera: Trichogrammatidae) under laboratory conditions (25°C, 65% RH, 16L:8D h photoperiod) at Laboratory of Biological Control, Department of Plant Protection, Agriculture Faculty, Namık Kemal University in 2014. Newly hatched female wasps were fed on *Ephestia kuehniella* Zeller, 1879 (Lepidoptera: Pyralidae) eggs with either honey, grape molasses and royal jelly as a main food, alone or double combination of this main foods or supplemented with resin (derived from plants), acacia nectar, *Paulownia* nectar, red tulip nectar, yellow asphodel nectar, apple syrup, liquid of *E. kuehniella* eggs or mashed *E. kuehniella* larvae. *Trichogramma brassicae*, females that were fed on honey and acacia nectar (17.47 d), honey + apple syrup (17.20 d), honey (16.93 d) and honey + *Paulownia* nectar (16.60 d) lived significantly longer than females that fed on other floral nectars and artificial diets. Females were fed on royal jelly + mashed *E. kuehniella* larvae (1.40 d) had the shortest longevity. *Trichogramma brassicae* females that were fed on honey (106.8 eggs), honey + acacia nectar (105.4 eggs), *Paulownia* nectar (103.13 eggs) parasitized significantly more hosts than females that fed on other floral nectars and artificial diets. Females fed on royal jelly were had the lowest parasitizing ability (3.33 eggs). These results showed that providing *T. brassicae* with honey, honey + acacia nectar, honey + apple syrup resulted in greater longevity and total fecundity than other food resources.

Keywords: *Trichogramma brassicae, Ephestia kuehniella,* floral nectar, food, fecundity, longevity

Özet

Bu çalışma, çeşitli besin kaynaklarının yumurta parazitoiti *Trichogramma brassicae* Bezdenko, 1968 (Hymenoptera: Trichogrammatidae)'e etkilerinin araştırılması amacıyla Namık Kemal Üniversitesi, Ziraat Fakültesi, Bitki Koruma Bölümü, Biyolojik Mücadele Laboratuvarı'nda laboratuvar koşullarında (25°C sıcaklık, %65 nem, 16:8 saat (aydınlık: karanlık) aydınlanma periyodu) 2014 yılında yürütülmüştür. Ergin dişi bireyler, değirmen güvesi *Ephestia kuehniella* Zeller, 1879 (Lepidoptera: Pyralidae) yumurtaları üzerinde ana besin (bal, üzüm pekmezi ve arı sütü) ve ara besin (reçine (bitkilerden salgılanan), akasya nektarı, çin kavağı nektarı, kırmızı lale nektarı, sarızambak nektarı, elma şurubu, *E. kuehniella* yumurta sıvısı ve ezilmiş *E. kuehniella* larvası) ve bu ana besinlerin ikili kombinasyonları ile beslenmiştir. Bal + akasya nektarı (17.47 gün), bal + elma şurubu (17.20 gün), bal + çin kavağı nektarı (16.60 gün) ile beslenen bireylerin diğer besin ve nektar ile beslenen bireylere göre daha uzun yaşadığı belirlenmiştir. En kısa ömür ise arı sütü + ezilmiş *E. kuehniella* larvası (1.40 gün) ile beslenen bireylerde görülmüştür. Çalışma sonucunda toplam parazitlenen yumurta sayısı, bal (106.8 yumurta), bal + akasya nektarı (105.4 yumurta), çin kavağı nektarı (103.13 yumurta) ile beslenen bireylerde ve belirgin olarak diğer besin ile beslenen bireylerin parazitledikleri yumurta sayılarından fazla bulunmuştur. Arı sütü ile beslenen bireylerin parazitledikleri yumurta sayısı (3.33 yumurta) en düşük olarak belirlenmiştir. Bu sonuçlar, *T. brassicae*'ye bal, bal + akasya nektarı, bal + elma şurubu verilmesinin diğer gıda kaynaklarına göre daha uzun ömür ve toplam doğurganlık sağladığını göstermiştir.

Anahtar sözcükler: *Trichogramma evanescens, Ephestia kuehniella,* bitki nektarı, besin, yumurta verimi, ömür

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Introduction

Egg parasitoids are most important parasitoids for biological control programs. *Trichogramma* species are the most widely used biological control agent. *Trichogramma* wasps are used against lepidopterans in biological control programs around the world (Li, 1994; Smith, 1996).

Food has a significant effect on parasitoid performance, such as developmental time, survival, fecundity and longevity (Hohmann et al., 1988; Fuchsberg et al., 2007; Özder & Kara, 2010, Tunçbilek et al., 2012). Parasitization performance of *Trichogramma* spp. (longevity, fecundity, adult emergence and female emergence) is known to be influenced by extrafloral nectar, pollen, honey, carbohydrate and protein (Ashley & Gonzalez, 1974; Hohmann et al., 1988; Baggen et al., 1999; Jervis et al., 2004; Lee et al., 2004, Shearer & Atanassov, 2004; Zhang et al., 2004; Wäckers, 2005; Witting-Bissinger, 2008).

Field release requires a large number of *Trichogramma* individuals (Stinner et al.,1974; Jalali & Singh, 1992). Many studies have focused on the mass rearing and storage of *Trichogramma* species (Jalali & Singh, 1992; Özpınar & Kornoşor, 1993; Özpınar, 1994; Karabörklü & Ayvaz, 2007; Yılmaz et al., 2007; Ayvaz et al., 2008).

Study on the mass rearing of parasitoids has focused primarily on food. Both longevity and fecundity of parasitoids can increase with of certain kinds of food (Ashley & Gonzalez, 1974; Özkan, 2007; Tunçbilek et al., 2012; Çınar et al., 2015) In the field parasitoids can obtain carbohydrates in homopteran honeydew, floral and extrafloral nectar (Wäckers et al., 2008). A number of studies have provided considerable evidence for increased parasitoid abundance and parasitism level when flowering plants are present (Berndt et al., 2006; Diaz et al., 2012, Masetti et al., 2010; Zhu et al., 2013).

Acacia nectar, *Paulownia* nectar, red tulip nectar, yellow asphodel nectar, honey, grape molasses and royal jelly were chosen for this study because of their floral morphology and they are widely planted in in gardens. Also, several laboratory studies have shown that hymenopteran parasitoids exhibit increased longevity and fecundity when provided with honey.

The aim of this study was to determine the effects of food sources on *Trichogramma brassicae* Bezdenko, 1968 (Hymenoptera: Trichogrammatidae) longevity, parasitism rate, progeny production and progeny sex ratio for mass rearing and in the field.

Materials and Methods

Trichogramma brassicae used in this experiment had been continuously reared on eggs of the Mediterranean flour moth, *Ephestia kuehniella* Zeller, 1879 (Lepidoptera: Pyralidae) since 1998 in the laboratory at 25 ± 1°C and 70 ± 5% RH with a 16L:8D h photoperiod. Fresh (less than 24 h old*) E. kuehniella* eggs were glued on pieces of white cardboard (2 x 4 cm) and were then placed in glass vials (7.5 x 2 cm). These eggs were offered to single *T. brassicae* females for 24 h and then were discarded. Females used in the experiments were newly emerged, mated and lacked egg laying experience. Sex ratio was calculated by using the form of the antenna to distinguish the adult females (Özder, 2006).

Eggs of *E. kuehniella* were used for rearing and as host eggs in the experiment. *Ephestia kuehniella* were reared on wheat bran. Throughout the rearing, cultures were kept in a rearing room, equipped with a control system, at 25 ± 1°C, 70 ± 5% RH. To obtained eggs, approximately 100 mated females of Mediterranean flour moth were collected from stock cultures and released in plastic jars (Özder & Kara, 2010).

Twenty-seven diet treatments were assessed (Table 1). Mature females were fed with either honey, grape molasses or royal jelly as a main food, alone or supplemented with resin (derived from plants), acacia nectar, *Paulownia* nectar, red tulip nectar, yellow asphodel nectar, apple syrup, liquid of *E. kuehniella* egg and mashed *E. kuehniella* larvae. All of the female parasitoids were fed daily until all female parasitoids died.

About 50 fresh *E. kuehniella* eggs were placed in glass vials (10 x 3 cm) and newly emerged (< 24 h) *T. brassicae* females were introduced and held for 24 h. After exposure, the adults were removed and the number of parasitized eggs counted. Fresh eggs of *E. kuehniella* were exposed to *T. brassicae* adults in glass vials until all female parasitoids died. All experiments were carried out at 25 ± 1°C and 70 ± 5% RH with a 16L:8D h photoperiod. Treatments were replicated 15 times. The number of parasitized eggs, adults and sex ratio were determined after the larval and pupal development of the parasitoids. For each sample sheet, the number of host eggs parasitized (blackened eggs) was counted daily for 5 d following exposure. To assess the effect of the diets on the longevity of *T. brassicae*, the flowers and other diets were offered simultaneously to single *T. brassicae* females. Treatments were replicated 15 times and the food sources and cardboard sheets were replaced daily until all parasitoids died. The longevity of each female was recorded. The flowers were brought to the laboratory and cleaned of any plant parts and insects that may have fallen into the collection cylinder. Different whole flower with nectar were offered to *T. brassicae* females in glass tubes (vials). Honey, molasses and the other diets were dotted onto the paper with a sharpened dissecting probe to provide four dots no larger than 2 mm in diameter. Flowers were collected daily and spread on white paper under a lamp to check for insects and then offered to the parasitoids.

Data were analyzed using SPSS 8 Windows. A one-way analysis of variance (ANOVA) was used to study the effects of the food sources applied as a factor and the number of parasitized eggs, adult emergence and female emergence as parasitization efficiency dependent. Means were compared Duncan's Multiple Range test was applied as a means of separation.

Results

Longevity

The longevity of *T. brassicae* females was influenced by their diet (Table 1). When royal jelly + *E. kuehniella* larvae were given, *T. brassicae* individuals lived for only 1.40 ± 0.50 d. Honey + acacia nectar significantly increased the longevity of females ($P < 0.05$) of *T. brassicae*. Females lived the longest when fed with honey + acacia nectar (17.46 ± 6.52 d), honey + apple syrup (17.20±4.44 d), honey (16.93±3.91 d) and honey + *Paulownia* nectar (16.60 ± 4.54 d).

Fecundity

Diet had a significant effect on fecundity ($P < 0.05$). The percentage of fecundity was significantly greater on honey (106.8 ± 30.26 eggs), honey + acacia nectar (105.4 ± 12.26 eggs) and honey + *Paulownia* nectar (103.13 ± 15.34 eggs), than royal jelly + red tulip nectar (3.33 ± 1.34) eggs) (Table 1). A large variation was found within the diets as regards fecundity. The mean parasitism decreased dramatically, especially for females on fed royal jelly and royal jelly + resin.

Adult emergence

The parasitoid completed development on all diets tested (Table 1). The greatest adult emergence was obtained on royal jelly + red tulip nectar (100%) and royal jelly + *E. kuehniella* larvae (100%).

Female emergence

The numbers of females that emerged were significantly affected by the food given. The numbers of female progeny which emerged were different from the pattern shown in the number of parasitized eggs. The highest female emergence was obtained royal jell and combinations (Table 1).

Table 1. Mean longevity, fecundity and percentage of adult and female emergence of *Trichogramma brassicae* Bezdenko [(X±SD) (min-max)]

Diets	Longevity (d)*	Fecundity*	Adult emergence (%)*	Female emergence (%)*
Honey	16.93 ± 3.91 (9-23) H	106.8 ± 30.26 (55-149) L	98.86 ± 2.35 (91-100) BCD	85.00 ± 3.81 (78-95) CD
Molasses	11.60 ± 5.17 (4-22) G	91.60 ± 24.03 (55-131) KL	97.93 ± 2.76 (92-100) ABC	85.13 ± 2.87 (80-90) CD
Royal jelly	3.00 ± 0.92 (2-5) CD	7.40 ± 4.5 (2-15) AB	97.20 ± 5.63 (80-100) ABCD	89.53 ± 10.84 (73-100) F
Honey + acacia nectar	17.46 ± 6.52 (7-26) H	105.40 ± 12.26 (78-121) L	99.33 ± 1.04 (97-100) BCD	85.46 ± 1.92 (83-89) CD
Honey + *Paulownia* nectar	16.60 ± 4.54 (8-23) H	103.13 ± 15.34 (79-126) L	99.26 ± 1.16 (97-100) BCD	83.60 ± 3.56 (75-89) C
Honey + apple syrup	17.20 ± 4.44 (10-24) H	80.20 ± 19.84 (36-110) JK	98.33 ± 1.49 (95-100) ABC	83.00 ± 3.22 (78-90) C
Honey + yellow asphodel nectar	7.66 ± 3.69 (4-17) F	67.93 ± 40.70 (28-151) HI	98.13 ± 1.80 (95-100) ABC	79.13 ± 5.48 (71-86) BC
Honey + red tulip nectar	14.60 ± 3.83 (8-19) H	74.80 ± 29.11 (33-125) IJ	99.06 ± 1.43 (96-100) BCD	80.53 ± 2.87 (75-86) C
Honey + *E. kuehniella* larvae	6.66 ± 2.49 (4-11) EF	51.93 ± 23.01 (23-97) G	98.06 ± 1.98 (94-100) ABC	79.20 ± 6.57 (65-87) BC
Honey + *E. kuehniella* eggs	9.66 ± 2.55 (5-14) G	74.40 ± 19.87 (32-97) IJ	98.93 ± 1.53 (96-100) BCD	84.93 ± 4.78 (72-89) CD
Honey + resin	10.13 ± 2.13 (6-13) G	57.66 ± 13.60 (39-89) GH	98.06 ± 1.83 (96-100) ABC	81.93 ± 2.40 (78-85) C
Molasses + yellow asphodel nectar	7.00 ± 1.30 (5-10) EF	32.20 ± 10.59 (16-59) F	98.53 ± 3.06 (89-100) BCD	69.20 ± 3.66 (63-75) A
Molasses + red tulip nectar	5.73 ± 1.90 (2-9) E	29.73 ± 16.28 (12-66) F	98.40 ± 3.92 (86-100) BCD	70.26 ± 4.78 (57-77) AB
Molasses + apple syrup	10.13 ± 2.66 (5-13) G	65.73 ± 19.07 (37-100) IJ	99.53 ± 1.24 (96-100) CD	80.73 ± 3.41 (75-86) C
Molasses + *Paulownia* nectar	9.93 ± 2.18 (6-13) G	56.60 ±13.38 (38-89) GH	99.20 ± 1.69 (95-100) CD	78.86 ±3.92 (71-84) BC
Molasses + Acacia Nectar	5.60 ± 2.16 (3-10) E	36.26 ± 18.22 (7-77) F	98.26 ± 2.08 (94-100) ABCD	78.26 ± 7.78 (67-100) BC
Molasses + *E. kuehniella* larvae	3.06 ± 1.16 (2-5) CD	16.86 ± 8.45 (7-37) DC	96.06 ± 4.21 (87-100) AB	83.00 ± 6.64 (73-100) C
Molasses + *E. kuehniella* eggs	5.60 ± 2.09 (2-9) E	23.80 ± 7.39 (9-33) EF	97.00 ± 4.14 (86-100) ABC	77.73 ± 5.50 (67-83) ABC
Molasses + resin	3.53 ± 1.18 (2-6) CD	12.80 ± 5.01 (5-23) BC	94.73 ± 5.92 (85-100) A	83.60 ± 10.28 (70-100) CD
Royal jelly + *Paulownia* nectar	3.06 ± 0.70 (2-4) CD	13.13 ± 6.94 (6-34) BC	97.00 ± 5.25 (87-100) ABCD	80.40 ± 7.56 (67-100) C
Royal jelly + red tulip nectar	2.40 ± 0.50 (2-3) BC	3.33 ± 1.34 (2-7) A	100.0 ± 0.00 (100-100) D	97.73 ± 6.08 (80-100) G

Table 1. (Continued)

Diets	Longevity (d)*	Fecundity*	Adult emergence (%)*	Female emergence (%)*
Royal jelly + acacia nectar	3.66 ± 0.72 (2-5) D	20.00 ± 8.16 (11-46) DE	98.00 ± 2.64 (93-100) ABCD	94.20 ± 4.17 (88-100) EF
Royal jelly + resin	2.93 ± 0.70 (2-4) CD	9.53 ± 4.30 (3-18) B	97.80 ± 4.84 (86-100) BCD	96.46 ± 4.58 (88-100) G
Royal jelly + apple syrup	3.06 ± 0.88 (2-5) CD	9.06 ± 4.80 (3-21) B	97.73 ± 4.11 (88-100) ABCD	97.20 ± 4.87 (87-100) G
Royal jelly + *E. kuehniella* larvae	1.40 ± 0.50 (1-2) A	8.86 ± 3.75 (5-18) B	100.0 ± 0.00 (100-100) D	97.60 ± 4.18 (89-100) G
Royal jelly + *E. kuehniella* eggs	1.86 ± 0.74 (1-3) AB	9.26 ± 3.51 (4-16) B	96.46 ± 4.59 (88-100) ABC	89.60 ± 9.25 (71-100) F
Royal jelly + yellow asphodel nectar	1.93 ± 1.03 (1-4) AB	11.13 ± 6.17 (3-21) BC	96.46 ± 4.77 (88-100) ABC	83.26 ± 12.15 (50-100) CD

* Mean in a column the same letters are not significantly different ($P<0.05$)

Discussion

All natural floral nectars and artificial diets allowed *T. brassicae* to complete development. This indicates that there is a potential for rearing this parasitoid on artificial and floral nectars. Fecundity of *T. brassicae* females was greater honey, honey + acacia nectar and honey + *Paulownia* poplar nectar and this value were significantly higher from those obtained on others. The number of parasitization was lowest royal jelly + red tulip nectar. Cruden & Hermann (1983) found that cut flowers may produce less nectar that intact flowers. Our results correspond with the finding of (Witting-Bissinger, 2008) where *Trichogramma exiguum* Pinto & Platner, 1978 longevity and fecundity were increased significantly when wasps were provided honey and honey + buckwheat. Our results are supported by other studies which found and increase in longevity and fecundity with honey or flowers as a food sources compared with water or no food (Özkan, 2007; Tunçbilek et al., 2012; Çınar et al., 2015).

In this study, with honey as a food source, mean female longevity was ten times longer, and the mean fecundity was 100 times greater than with royal jelly. A number of studies have shown that an increase in fecundity can occur when parasitoid wasps are provided particular food (Leius, 1961; Ashley & Gonzalez, 1974; Yu et al., 1984; Leatemia et al., 1995; Aydin Özder & Kılınçer, 1996 a, b; Blanche et al., 1996; Gurr & Nicol, 2000; Johanowicz & Mitchell, 2000; Costamagna & Landis, 2004; Shearrer & Atanassov, 2004; Fuchsberg et al., 2007; Özkan, 2007; Tunçbilek et al., 2012; Lessard & Boivin, 2013; Çınar et al., 2015). Saljoqi & Khattak (2007) reported that adult *Trichogramma chilonis* Ishii, 1941 females provided with 50% honey and water lived significantly longer than unfed females or those provided with some other kind of food. Feeding has been shown to increase the longevity of *Trichogramma platneri* Nagarkatti, 1975 (Hohmann et al., 1989). Although the availability of honey markedly affected the longevity of *T. platneri,* it did not increase fecundity (Hohmann et al., 1989). Özder et al. (2011) reported that *Trichogramma evanescens* Westwood, 1833 longevity increased significantly when wasps were provided corn pollen + honey compared to pollen alone. In other studies, the mean fecundity of mated females fed with was similar to unfed females (Hohmann et al., 1989, Özder, 2006; Özder & Kara, 2010).

Nectar is a good food for insects, and nectar and pollen have positive effects on longevity, fecundity, adult emergence and female emergence (Lewis & Takasu, 1990; Wäckers, 1994; Patt et al., 1999; Thompson & Hagen, 1999; Wäckers, 2003). Zhang et al. (2004) showed that *T. brassicae* females fed on corn pollen plus water had significantly increased longevity and fecundity compared with those fed on water alone. Also, Zhu et al. (2015) reported that *T. chilonis* females provided *Sesamum indicum* L. flowers lived significantly longer and had significantly increased fecundity than when on provided water.

Trichogrammatid egg parasitoids have been used to reduce egg hatching and subsequent damage caused by larval pests. The success of biological control program may largely depend on potential reproductive rate (the number of adult female progeny produced by a female parasitoid in the presence unlimited prey). In our study, a higher ratio of female to male offspring was observed in *T. brassicae* females provided royal jelly and royal jelly combination. This was probably due to the special food. Royal jelly is a honey bee secretion that is used in nutrition of larvae as well as adult queens and includes water, protein, fat, enzyme, hormone, vitamin and some micronutrients. When worker bees make a new queen, they choose small larvae and feed them with royal jelly. This type of feeding triggers the development of queen morphology including the fully developed ovaries needed to lay eggs (Doğaroğlu & Doğaroğlu, 2015).

Food quality is important in determining the effectiveness of parasitoids as control agents. We conclude that artificial and natural diets are effective for rearing *T. brassicae*, based on parasitization, adult emergence and female longevity. In the current study, artificial diets proved to be suitable foods for sustaining the development and reproduction of *T. brassicae.* Floral nectar qualities may be of importance to parasitoid longevity when selecting floral resources for conservation biological control. Nectar sugar composition may also be crucial in determining its nutritional suitability.

Our results suggest that honey, grape molasses, acacia nectar and *Paulownia* nectar food sources could serve as food sources for *T. brassicae* in stores, warehouses and in the field. While information on the effect of food resources on longevity and reproduction output of parasitoids are important to the study of biological control, additional studies on royal jelly and feeding behavior of parasitoids in the field are needed.

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Original article (Orijinal araştırma)

Comparative study of the sex pheromone of carob moth, *Apomyelois ceratoniae* (Zeller, 1839) (Lepidoptera: Pyralidae) from four regions of Iran using headspace solid phase micro extraction - gas chromatography/mass spectrometry

İran'ın dört bölgesinden toplanan Harnup güvesi *Apomyelois ceratoniae* (Zeller, 1839) (Lepidoptera: Pyralidae)'nde eşeysel feromonun üst katman katı faz mikro ekstraksiyonu - gaz kromatografisi / kütle spektrometresi kullanarak karşılaştırılması

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Summary

Carob moth, *Apomyelois ceratoniae* (Zeller, 1839) (Lepidoptera: Pyralidae) is the most important pest of pomegranate in Iran as well as in most other countries. There is no suitable chemical method available for controlling this pest. The sex pheromone components, emitted by virgin female of *A. ceratoniae* were characterized by headspace solid phase micro extraction (HS-SPME) and subsequently analyzed by gas chromatography/mass spectrometry. The low rate of release of pheromone from the gland, common to the most of the lepidopteran insects, is one of the limiting factors in pheromone research studies. As a result, sex pheromone components of the insect from four different geographical regions of Iran were analyzed comparatively by HS-SPME in 2015. The major component, (*Z,E*)-9,11, 13-tetradecatrienal, and minor components, (*Z,E*)-9,11-tetradecadienal and (*Z*)-9-tetradecenal, were identified and compared to reference samples. Compared to gland extraction, the simplicity of HS-SPME technique revealed its suitability for identification of the pheromone components.

Keywords: *Apomyelois ceratoniae,* GC/MS, micro extraction, (*Z,E*)-9,11-tetradecadienal

Özet

Harnup güvesi, *Apomyelois ceratoniae* (Zeller, 1839) (Lepidoptera: Pyralidae) diğer ülkelerde olduğu gibi İran'da da narın en önemli zararlısıdır. Zararlıların mücadelesi için uygun herhangi bir kimyasal yöntem bulunmamaktadır. *Apomyelois ceratoniae*'nin çiftleşmemiş dişisi tarafından yayılan eşeysel feromon bileşenleri, üst katman katı fazlı mikro ekstraksiyon (HS-SPME) yöntemi ile karakterize edilmiş ve daha sonra gaz kromatografisi / kütle spektrometresi ile analiz edilmiştir. Lepidopterlerin çoğunda olduğu gibi feromon araştırmalarında sınırlayıcı faktörlerden biri, salgı bezinden feromon salım oranının düşük olmasıdır. Sonuç olarak, İran'ın dört farklı coğrafi bölgesindeki böceklerin cinsiyet feromon bileşenleri HS-SPME yöntemiyle karşılaştırmalı olarak 2015 yılında analiz edilmiştir. Ana bileşen olan (*Z,E*)-9,11,13-tetradekatriyenal ve iz bileşenler, (*Z,E*)-9,11-tetradekadienal ve (*Z*)-9-tetradekenal tanımlanmış ve referans örneklerle karşılaştırılmıştır. Bez çıkarma ile karşılaştırıldığında, HS-SPME tekniğinin basitliği, feromon bileşenlerin tanımlanması için uygunluğunu ortaya koymuştur.

Anahtar sözcükler: *Apomyelois ceratoniae,* GC/MS, mikro ekstraksiyon, (*Z,E*)-9,11-tetradekadienal

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Introduction

Insect pest management, monitoring and control programs, utilizing sex pheromones as behavior modifying chemicals, have become important for several insect groups, particularly moths. Established methods of analyzing insect pheromones involve extraction by solvents. The low rate of release from the glands, common to most of the Lepidoptera, is one of the limiting factors. These methods often require tedious and solvent consumptive procedures. Also, before analytical studies can be undertaken, large quantities of insects are needed for extraction of pheromone. Recently, solid phase micro extraction (SPME) has been used widely for the analysis of traces of organic compounds by trapping or rubbing insect glands. SPME is a viable alternative to solvent extraction and offers a convenient, solvent-free and time saving method (Miklas et al., 2000; Gago et al., 2013; Kühbandner & Ruther, 2015). An optimized SPME method coupled with gas chromatography/mass spectrometry (GC/MS) has been developed for the determination of the sex pheromone of *Eucosma notanthes* Meyrick, 1936 (Lepidoptera: Tortricidae). Compared to solvent extraction methods, the optimized SPME method is easier, faster and more efficient. Moreover, it consumes no solvent, and is less prone to contamination from living tissues (Chu et al., 2005). Frerot et al. (1997) used this method to extract (*Z*)-11-hexadecan-1-ol pheromone by rubbing SPME fiber on the gland surface and gland washes. SPME trapped a larger amount of each identified pheromone components than gland washes; about 120 ng per gland with SPME compared to 60 ng per gland by the wash technique. In addition, it is worth to noting that the isolation of the *Thyrinteina arnobia* Stoll, 1782 (Lepidoptera: Geometridae) pheromone components has also been achieved by two different techniques: gland extract and SPME of virgin females (Heath et al., 1983; Delisle & Royer 1994; Jardel et al., 2013).

The carob moth, *Apomyelois* (=*Ectomyelois*) *ceratoniae* (Zeller, 1839) (Lepidoptera: Pyralidae), is a worldwide pest of several nuts and fruits including carobs, almonds, and dates. Its range has been expanding to the new parts of the world. For instance, in the USA, this species is a primary pest of dates in the desert valleys of southern California and has the potential to expand north to threaten vast almond and pistachio groves of the San Joaquin Valley (Gothilf 1984; Warner 1988; Cosse et al., 1994). Three pheromone components including (*Z,E*)-9,11,13-tetradecatrienal, (*Z,E*)-9,11-tetradecadienal, (*Z*)-9-tetradecenal have been extracted by solvent (Baker et al., 1991; Todd et al., 1992). In Iran, pomegranates are grown in several climate regions and the carob moth is the most important pest, causing serious damage to fruit. However, no chemical method is available to control this key pest. Furthermore, the commercial pheromone lure did not attract any male insects in field tests (Farazmand, 2011). In order to evaluate the pheromone of carob moth for pest control in Iran, SPME was used to compare pheromone from Iranian populations of the moth with that reported by Baker (1991). Populations of *A. ceratoniae* were sampled form four regions of Iran, viz. Isfahan, Sistan, Lorestan and South Khorasan, and volatile substances emitted by virgin females collected, analyzed and compared them to a reference samples (Millar, 1990, Baker et al., 1991).

Materials and Methods

Insect culture: Infested pomegranates, containing the pupal stage of *A. ceratoniae*, were collected from Isfahan, Sistan, Lorestan, and South Khorasan in 2015. Fruits were kept at room temperature (25-28°C). Moths were allowed to emerge under a 14L:10D h photoperiod regime and female moths sexed while in pupal stage.

Collection of pheromones by dynamic SPME: Headspace solid phase micro extraction (SPME; Supelco, Sigma-Aldrich Corporation, St. Louis, MO, United States) was used to collect pheromone compounds emitted by virgin female carob moths. The SPME fiber (70 µm polydimethylsiloxane coating) was conditioned in a GC injector at 250°C for 10 min before use. Five virgin female moths were placed in a vial (3.0 × 2.5 cm) sealed with a cap, and left under laboratory conditions at 25±1°C. The SPME fiber was located in the outlet tube of the vial to collect the emitted volatiles for several days, then the loaded fiber was immediately analyzed by coupled GC/MS.

Chemical analysis: All samples were analyzed by GC/MS on a fused capillary column HP5-MS (30 m × 0.25 mm I.D., 0.25 µm film thickness, Agilent, Technologies, Palo Alto, CA, USA) in an Agilent mod. The 6890 chromatograph was equipped with the mass selective detector Agilent 5973 under the following conditions: the injector temperature was held at 250°C; it as carrier gas at l ml/min.; the sample was injected in the split less mode, oven temperature program: 5 min isotherm at 45°C followed by a linear temperature increase of 4ºC/min up to 300°C, which was held for 10 min. All chemicals and reagents were obtained from Merck (Kenilworth, NJ, USA) and Sigma-Aldrich. ^{1}H-NMR spectra were measured using a Bruker 500 MHz spectrometer (Bruker, Rheinstetten, Germany), and chemical shifts were expressed as δ (ppm) with tetramethylsilane as internal standard. The infrared (IR) spectra were obtained on a Shimadzu IRPrestige-21 (Tokyo, Japan). The purity of all compounds was confirmed by the thin-layer chromatography (TLC) using different mobile phases. The elemental analysis was performed with an Elementar Analysensysteme GmbH (Langenselbold, Germany) VarioEL in CHNS mode, which was within 0.4% of theoretical values for C, H and N.

Chemicals

(*Z,E*)-9,11-tetradecadienal and (*Z*)-9-tetradecenal were obtained from Agrisense-BCS Ltd (Pontypridd, UK). (*Z,E*)-9,11,13-tetradecatrienal was synthesized (Figure 1) by modification of the method of Millar (1990). Tetrahydrofuran (THF) was distilled from sodium/benzophenone ketyl under N_2. Prepared solutions were dried over anhydrous Na_2SO_4, and concentrated by rotary evaporation under reduced pressure. Crude products were purified by flash or vacuum flash chromatography on silica gel (230-400 mesh). Reactions with air- or water-sensitive reagents were done in dried glassware under N_2 atmosphere.

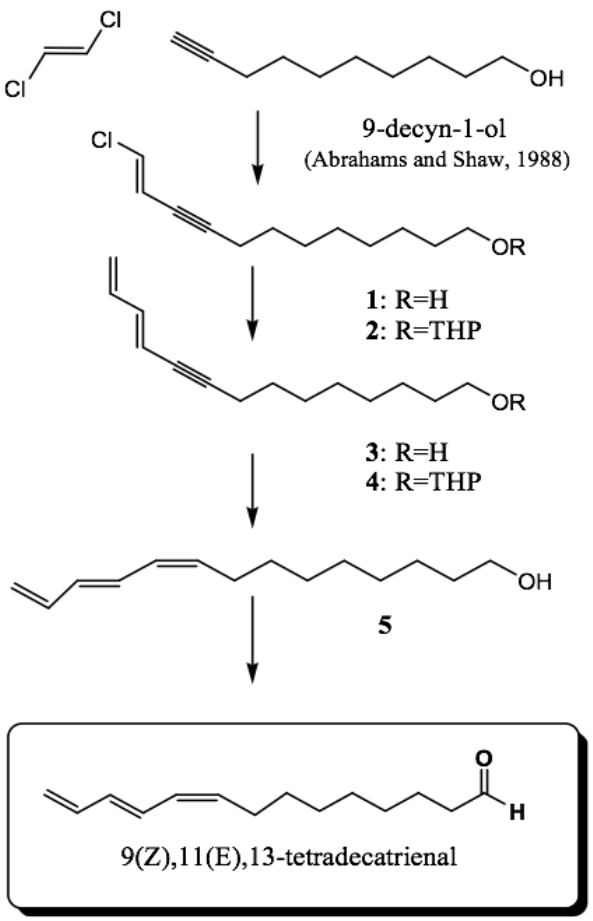

Figure 1. Synthesis of (*Z,E*)-9,11,13-tetradecatrienal.

Synthesis of (*Z,E*)-9,11,13-tetradecatrienal as the major sex pheromone component:

1. (*E*)-1-chloro-dodec-l-en-3-yn-12-ol (1)

A dry 50-ml flask was loaded with bis (triphenylphosphine) palladium (II) chloride (300 mg, 0.45 mmol) and CuI (160 mg, 0.8 mmol) flash with Ar. 9-Decyn-1-ol (1 g, 7.5 mmol) prepared by Zipper reaction (Abrahams & Shaw, 1988), trans-1,2-dichloroethylene (0.13 ml, 1.7 mmol) and THF (3 ml) were added to the reaction flask. Diisopropylamine (0.18 ml, 1.3 mmol) was added dropwise to the stirred mixture, and the initially pale yellow solution rapidly turned into brown then black. The mixture was stirred up at room temperature for 1 day. Hexane (20 ml) was then added, and the mixture filtered. The filtrate was extracted by saturated aqueous NH_4Cl (2× 3 ml), dried and passed through a column of silica gel, and eluted with 20% EtOAc in hexane. The eluate was concentrated, yielding 1.2 mg, 5.6 mmol (85% yield) of chloroalcohol (1). ^{1}NMR δ*:* 1.21-1.44 (m, 8H, H7, 8, 9, 10), 1.45-1.6 (m, 5H, H6, 11, OH), 2.28 (td, 2H, *J* = 6.8, 2.2 Hz, H5), 3.65 (t, 2H, *J* = 6.7 Hz*,* H12), 5.92 (dt, 1H, *J* = 13.5, 2.3 Hz, H2), 643 (d, 1H*, J* = 13.5 Hz, H1). MS *m/z:* 179 (4, M-Cl), 114 (37), 105 (39), 91 (70), 79 (100), 67 (40), 55 (52). *Anal.* Calcd for $C_{17}H_{27}ClO_2$: C, 68.32; H, 9.11, Found: C, 68.12; H, 9.32.

2. THP ether of (*E*)-l-chloro-dodec-l-en-3-yn-12-ol (2)

Compound 1 (1.2 g, 5.4 mmol) was protected as the THP ether by treatment with dihydropyran (1 ml) and a few crystals of p-toluenesulfonic acid in the ether overnight. The mixture was prepared by extraction with sat. aq. $NaHCO_3$ and brine, dried, concentrated and removed solvent traces under vacuum. The protected alcohol 2 (1.3 g, 4.4 mmol) 80% gave one spot on TLC (5% EtOAc in hexane) and was used for next reaction without further purification. ^{1}HNMR δ*:* 1.25-1.45 (m, 8H, CH_2), 1.45-1.65 (m, 8H, CH_2), 1.65-1.9 (m, 2H, H12), 2.28 (td, 2H, *J* = 6.8*,* 2.3 Hz, H5), 3.38 (dt, 1H, *J* = 9.6, 6.7 Hz, H12), 3.46-3.54 (m, 1H, THP), 3.73 (dt, 1H, *J* = 9.6*,* 6.9 Hz, H12'), 3.84-3.91 (m, 1H, THP), 4.57 (br. t, 1H, *J* = 2.7 Hz*,* THP), 5.90 (dt, 1H, *J* = 13.6, 2.3 Hz, H2), 6.43 (d, IH, *J* = 13.6 Hz, H1). IR cm^{-1}: 3075 (w), 2915 (s). MS *m/z:* 299 (M^+), 101 (14), 85 (100), 79 (16), 55 (20). *Anal.* Calcd for $C_{17}H_{26}ClO_2$: C, 68.32; H, 9.11. Found: C, 68.47; H, 9.01.

3. 11(*E*),13-tetradecadien-9-yn-1-ol (4)

Tetrakis (triphenylphosphine) palladium (0.3 g, 0.26 mmol) and chloride 2 (1.3 g, 4.4 mmol) were added to 10 ml of toluene under N_2 at room temperature. The mixture was cooled in an ice bath and vinyl magnesium bromide (10 ml of a 1 M solution in THF) was added dropwise over 5 min. The mixture warmed to room temperature while being stirred overnight. The reaction mixture was poured into hexane (30 ml), extracted thoroughly with 2 M NH_4Cl and brine, dried and concentrated. The residue (**3**) was dissolved in MeOH (10 ml) and catalytic amount of *p*-toluenesulfonic acid, and stirred at room temperature. After the completion of the reaction (checked by TLC), $NaHCO_3$ (0.3 g) was added and the mixture concentrated on a rotary evaporator to remove MeOH. The residue was partitioned between water and hexane (30 ml each). The hexane layer was washed with brine, dried and partially fractioned by passing through a column of silica gel, eluting with 20% EtOAc in hexane. The fraction containing the purified product was concentrated and pumped under vacuum, giving dienynol 4 as a yellow oil (0.7 g, 3.3 mmol) 76% yielded over 3 steps. ^{1}NMR δ*:* 1.24-1.46 (m, 8H, H3, 4,5,6),1.46-1.65 (m, 5H, H2, 7, OH), 2.33 (td, 2H, *J* = 6.9*,* 2.2 Hz, H8), 3.65 (t, 2H, *J* = 6.6 Hz*,* H1), 5.12 (d, 1H, *J* = 9.7 Hz*,* H14), 5.25 (br.d, 1H, *J* = 16.5 Hz*,* H14), 5.62 (dt, IH, *J* = 15.6*,* 2.0 Hz, H11), 6.35 (dt, 1H, *J* = 16.5*,* 10.0 Hz, H13), 6.51 (dd, 1H, *J* = 10.9*,*

15.4 Hz). IR cm^{-1}: 3333 (s, br.), 2932 (s). MS *m/z:* 206 (2, M^+), 105 (29), 91 (100), 79 (37), 65 (31), 41 (37). *Anal.* Calcd for $C_{14}H_{22}O$: C, 80.50; H, 10.75. Found: C, 80.77; H, 10.86.

4. (*Z,E*)-9,11,13-tetradecatrienol (5)

Zinc dust (2.39 g, 46.50 mmol) was stirred with 6 ml × 3 HCl (3%) for 2 min under N_2. The acid was decanted and the zinc was rinsed twice with distilled water twice. Cu(II)OAc (0.2 g, 1.02 mmol) in hot water (2.6 ml) was slowly added, then $AgNO_3$ (0.24 g, 1.41 mmol) in H_2O (2.6 ml) was added to the solution and it was stirred for 15 min. The mixture was filtered and the filtrate was added into the flask containing MeOH (5 ml) and H_2O (7 ml), followed by the solution of dienynol 4 (0.3 g, 1.45 mmol) in 2 ml MeOH. The mixture was stirred for 11 h at 50°C under N_2, then filtered, and washed with MeOH 3 ml, MeOH (10 ml) and HCl 10% (1.2 ml). The filtrate was concentrated and the residue was extracted with Et_2O: hexane (1:1), aq. Sat. NH_4Cl, which was dried over $MgSO_4$, and concentrated, and purified by vacuum flash chromatography on silica gel (hexane: EtOAc, 95:5) gave 0.16 g (0.32 g, 1.45 mmol, quantitative yield) of olefin 5 as a colorless oil. ^{1}NMR (Figure 2) *δ*: 1.2-1.45 (m, 8H, H3, 4, 5, 6), 1.5-1.65 (m, 4H, H2, 7), 2.19 (br. quartet, 2H, *J* = 6.8 Hz*,* H8), 2.37 (s, lH, OH), 3.64 (t, 2H, *J* = 6.6 Hz*,* Hl), 5.08 (d, lH, *J* = 10.2 Hz, H14), 5.21 (d, lH, *J* = 15.6 Hz, Hl4'), 5.48 (dt, lH, *J* = 10.7, 7.7 Hz, H9), 6.02 (br. t, lH, *J* = 11.0 Hz, H10), 6.20 (dd, lH, *J* = 14.9, 10.7 Hz, H12), 6.41 (dt, lH, *J* = 16.8,10.3 Hz, H13), 6.51 (dd, lH, *J* = 14.8*,* 11.3 Hz, H11). IR cm^{-1}: 3345 (s), 2940 (s), 1005 (s). MS *m/z:* 208 (25, M^+), 107 (13), 91 (51), 79 (100), 67 (25). *Anal.* Calcd for $C_{14}H_{24}O$: C, 80.71; H, 11.61. Found: C, 80.87; H, 11.53.

5. (*Z,E*)-9,11,13-tetradecatrienal

The trienal alcohol 5 was converted to corresponding aldehyde by pyridinium chlorochromate (PCC) oxidation (Moreira 2006). Flask was charged with CH_2Cl_2 (10 ml) and PCC (0.36 g, 1.68 mmol), and powdered molecular sieve. Alcohol (0.18 g, 0.84 mmol) in CH_2Cl_2 (2 ml) was added to this solution, and it was stirred up for 3 h. Furthermore, hexane was added, and the mixture was stirred up for 10 min and then filtered. As the filtrate was dried, concentrated and then flash chromatogramed (SiO_2), it gave 9(*Z*),11(*E*),13-tetradecatrienal as a colorless oil (0.16 g, 0.77 mmol, 91%). ^{1}H-NMR (500 MHz, CDCl3, Figure 2) δ: 1.24- 1.31 (m, 8H, H4, 5, 6, 7), 1.55- 1.62 (m, 2H, H3), 2.18 (brq, 2H, *J* = 6.8 Hz, H8), 2.42 (td, 2H, *J* = 7.0, 2.0 Hz, H2), 5.09 (d, 1H, *J* = 10.0 Hz, H14), 5.22 (d, 1H, *J* = 16.8 Hz, H14`), 5.47 (dt, 1H, *J* = 10.8, 7.6 Hz, H9), 6.02 (brt, 1H, *J* = 10.8 Hz, H10), 6.20 (dd, 1H, *J* = 15.2, 10.8 Hz, H12), 6.41 (dt, 1H, *J* = 16.8, 10.4 Hz, H13), 6.41 (dt, 1H, *J* = 16.8, 10.4 Hz, H13), 6.50 (dd, 1H, *J* = 15.2, 11.6 Hz, H11), 9.78 (t, 1H, *J* = 2 Hz, aldehyde H). 3040 (w), 2940 (s). IR cm^{-1}: 2747 (m), 1730 (s), 1008 (s), 945 (m). MS m/z: 207 (13), 206 (M^+, 86), 178 (4), 135 (7) 121 (5), 107 (15), 94 (20), 93 (52), 91 (65), 79 (100), 77 (60), 67 (22), 41 (22). *Anal*. Calcd for $C_{14}H_{22}O$: C, 81.50; H, 10.75 Found: C, 81.66; H, 10.56.

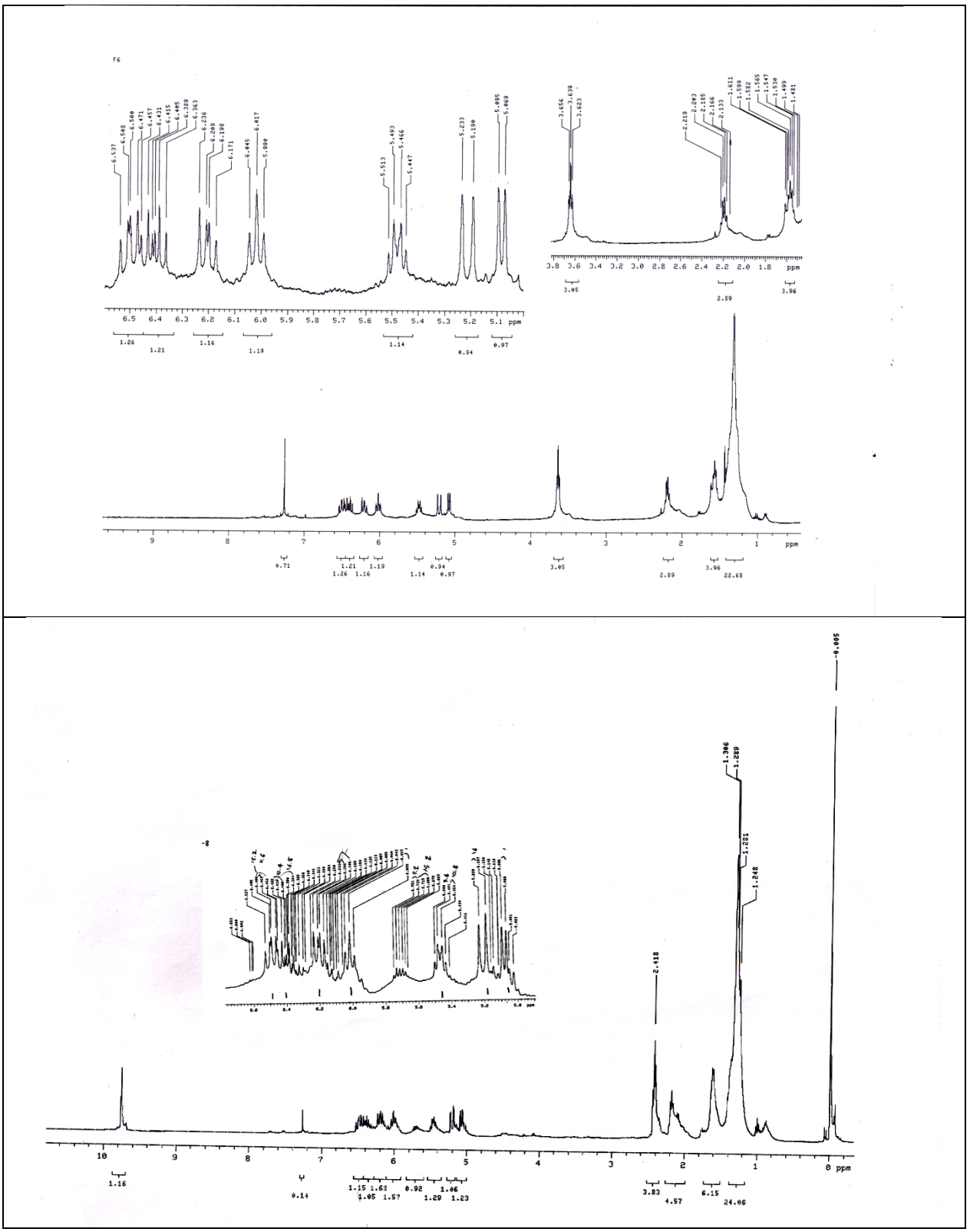

Figure 2. H-NMR of alcohol **5** (top), (Z,E)-9,11,13-tetradecatrienal (bottom).

Results and Discussion

In the present study, four pomegranate regions with different climate conditions were selected for the isolation of the volatile compounds of the sex pheromone of carob moth. This solvent-free technique was reliable, greatly sensitive and fast. Also, it did not damage insects and was applied on the same sample over several consecutive days. Isolation of the pheromone components was carried out using solid phase micro extraction (SPME) of virgin females. (*Z,E*)-9,11,13-tetradecatrienal, (*Z,E*)-9,11-tetradecadienal, and (*Z*)-9-tetradecenal were assigned according to mass analytical data and retention time (RT) standard. On the basis of careful comparison of synthetic compounds from the Isfahan, Sistan, Lorestan, and South Khorasan samples with the MS data from the literature and the RT, three sex pheromone components were assigned in Iran (Figure 3). Comparison of diagnostic ions (79, 67, 55, 206, and 91) with each other indicated the peak at RT =12.56± 0.01, belonging to (*Z*,*E*)-9,11,13-tetradecatrienal (Figures 3, 4). In addition, comparison of the ions 67 and 55, as the base peak, for two other components resulted in the assignment of two peaks at RT =11.45±0.01 and 10.86±0.01, for compounds (*Z,E*)-9,11-tetradecadienal and (*Z*)-9-tetradecenal, respectively (Figure 6). As well, Frerot et al. (1997) isolated three components which were similar to those of *A. ceratoniae* from *Sesamia nonagrioides* (Lefebvre, 1827), including *Z* (11)-hexadecen-1-ol, acetate form and 16:OAc by rubbing SPME on the gland surfaces. The consequence revealed that, compared to gland washes, the SPME technique appeared to be more efficient in trapping both synthetic and natural pheromone. Baker et al. (1991) isolated three components from date fruits which included (*Z,E*)-9,11,13-tetradecatrienal, (*Z,E*)-9,11-tetradecadienal and (*Z*)-9-tetradecenal carob moth sex pheromone in the ratio of 10:1:1. As shown in Table 1, the ratio of three sex pheromone components in four regions of Iran are different from each other; that is, while this ratio was 10:0.45:0.43 in Isfahan sample, it was 10:1.1:0.9 and 10:0.9:0.9 in South Khorasan and Sistan, respectively. This is similar to the data ratio of Baker et al. (1991). Also, the Lorestan sample had a different ratio of 10:2.5:2.1. Considering the discrepancies in these ratios, it seems that there might be a conspecific relationship between carob moth species in Iran. Moreover, in an unpublished field study, a trap cage with a virgin female was utilized, and notably, it revealed that only males form the same regional population were captured; that is, males from the other regions were not captured. Furthermore, commercial pheromone of carob moth lure did not attract male insects. It seems that there is regional variability among the sexual behaviors of carob moths in different climatic areas of Iran. Similar sexual behaviors were also reported in the males of *Nezara viridula* (L., 1758) (Heteroptera: Pentatomidae). It is worth noting that the sexually mature males specifically release a pheromone which is attractive in the field to conspecific females. As the author explained, the pheromone strains of *N. viridula* from Florida were different from those form Hawaii (Aldrich et al., 1987).

Four chromatograms of volatile compounds of carob moth were extracted by SPME (Figures 3, 4) in Isfahan, Sistan, Lorestan and South Khorasan. Ions 79, 67, 55, 206 and 91 of the peak at (RT) =12.56± 0.01 belong to (*Z*,*E*)-9,11,13-tetradecatrienal (Figure 5). Ions 67 and 55 as base peaks at (RT) =11.45 ± 0.01 and 10.86 45 ± 0.01, belong to (*Z*,*E*)-9,11-tetradecadienal and (*Z*)-9-tetradecenal, respectively (Figure 6). Baker (1989) isolated CS_2 extraction solvent and identified sex pheromone components of the carob moth by solvent extraction method and upon comparison with a synthetic samples, GC/MS analysis, three fragment ions: M^+ = 79, 91, 206 for (*Z*,*E*)-9,11,13-tetradecatrienal. Rodstein et al. (2009) identified 3,5,9-trimethylundecanoic acid as Females sex pheromone of cerambycid beetle by SPME technique and characterized the fragment ions of the mass spectrum, a base peak at m/z 74, m/z 143 and 185, which was the same fragment ions analogous to the synthetic compound and also with the same retention time.

The retention times and ratios of the sex pheromone components of *A. ceratoniae* in Isfahan, Sistan, Lorestan and South Khorasan are shown in Table 1. The RT for (*Z*,*E*)-9,11,13-tetradecatrienal, (*Z*,*E*)-9,11-tetradecadienal and (*Z*)-9-tetradecenal were 12.56±0.01, 11.45±0.01 10.86 45 ± 0.01, respectively. Baker et al. (1991) reported Rt for these three components of 8.56, 8.93, and 9.28 min, respectively, with GC/EAG with 30-m DB-1 column.

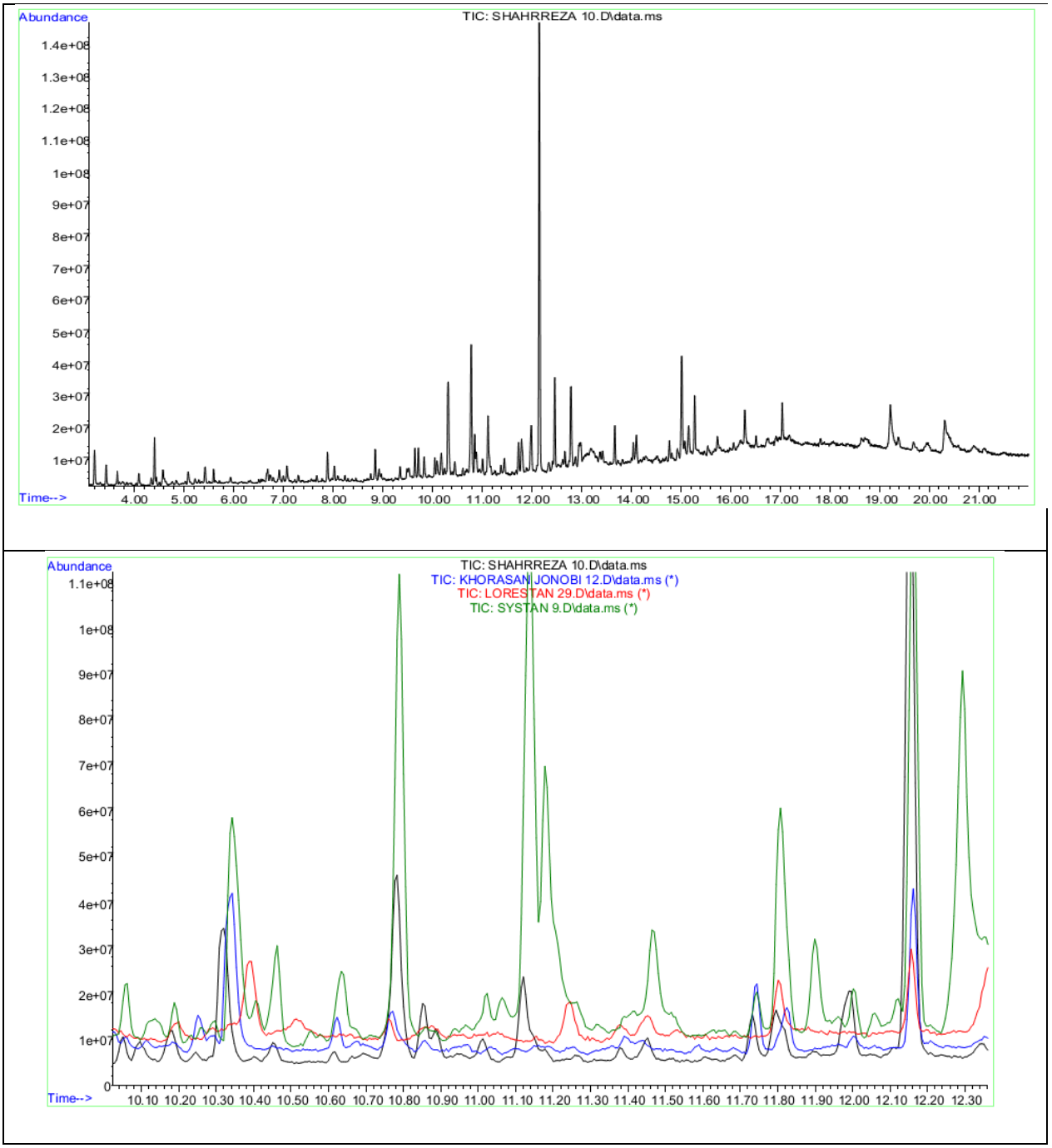

Figure 3. Chromatogram obtained by SPME on pheromone glands of *Apomyelois ceratoniae* in Isfahan, Iran (top), and chromatogram expansion at 10.60-12.20 min of four regions (bottom).

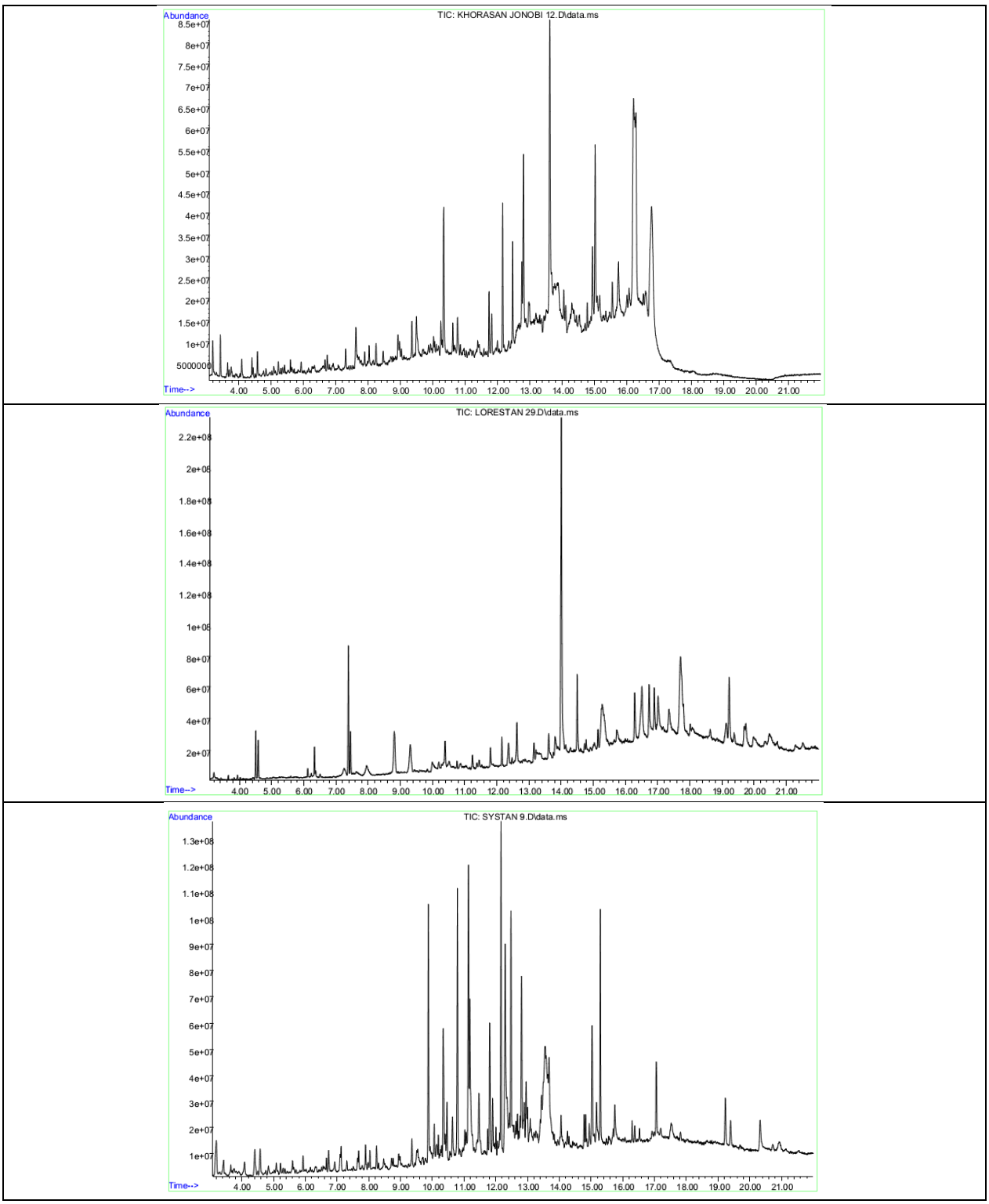

Figure 4. Chromatogram obtained by SPME on pheromone glands of *Apomyelois ceratoniae* in Khorasan, Lorestan and Sistan province.

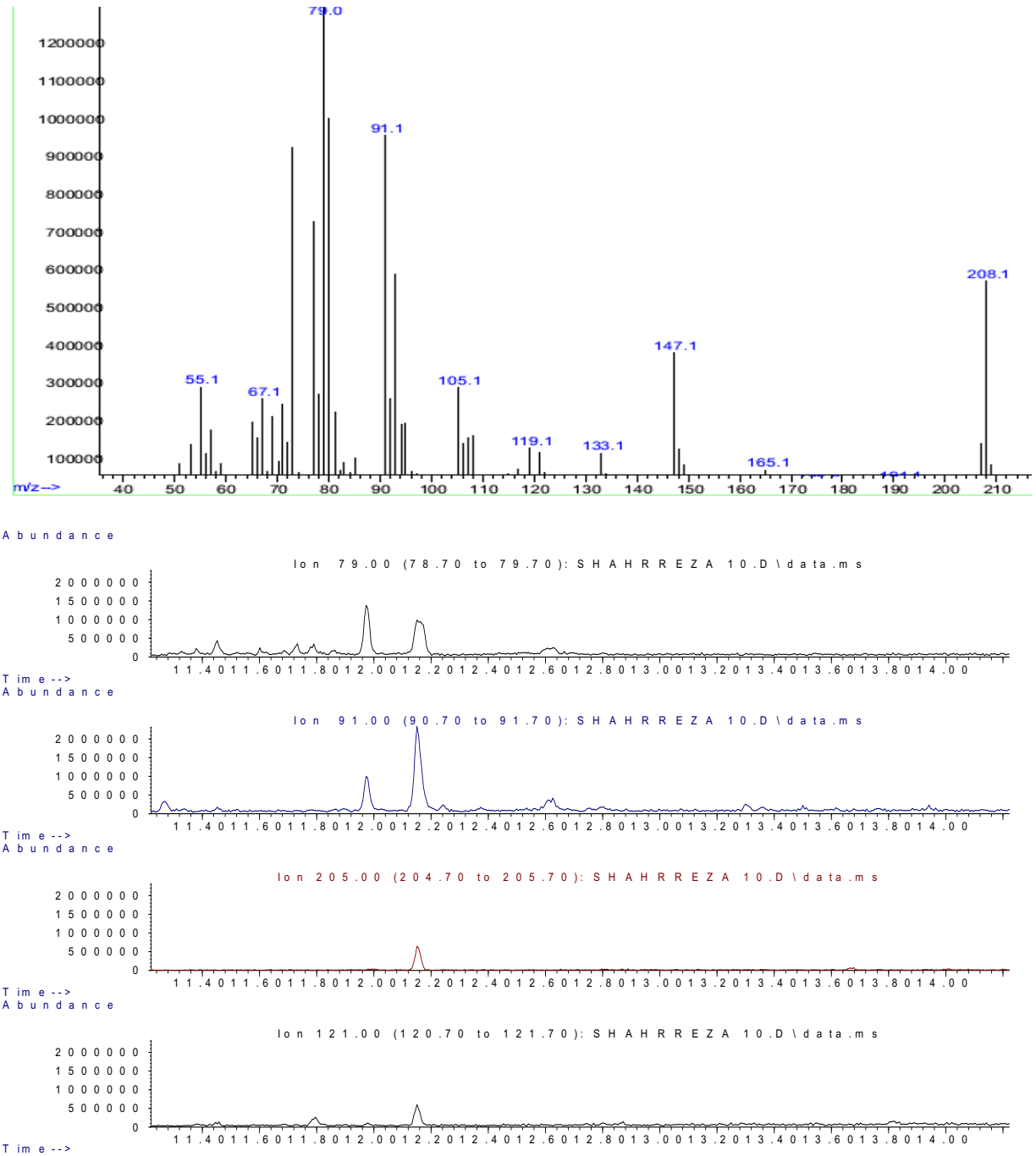

Figure 5. Results of the GC/MS of component (*Z*,*E*)-9,11,13-tetradecatrienal (top) and extracted ion chromatograms with diagnostic ions: i.e., 79, 91, 121 and 206 (bottom).

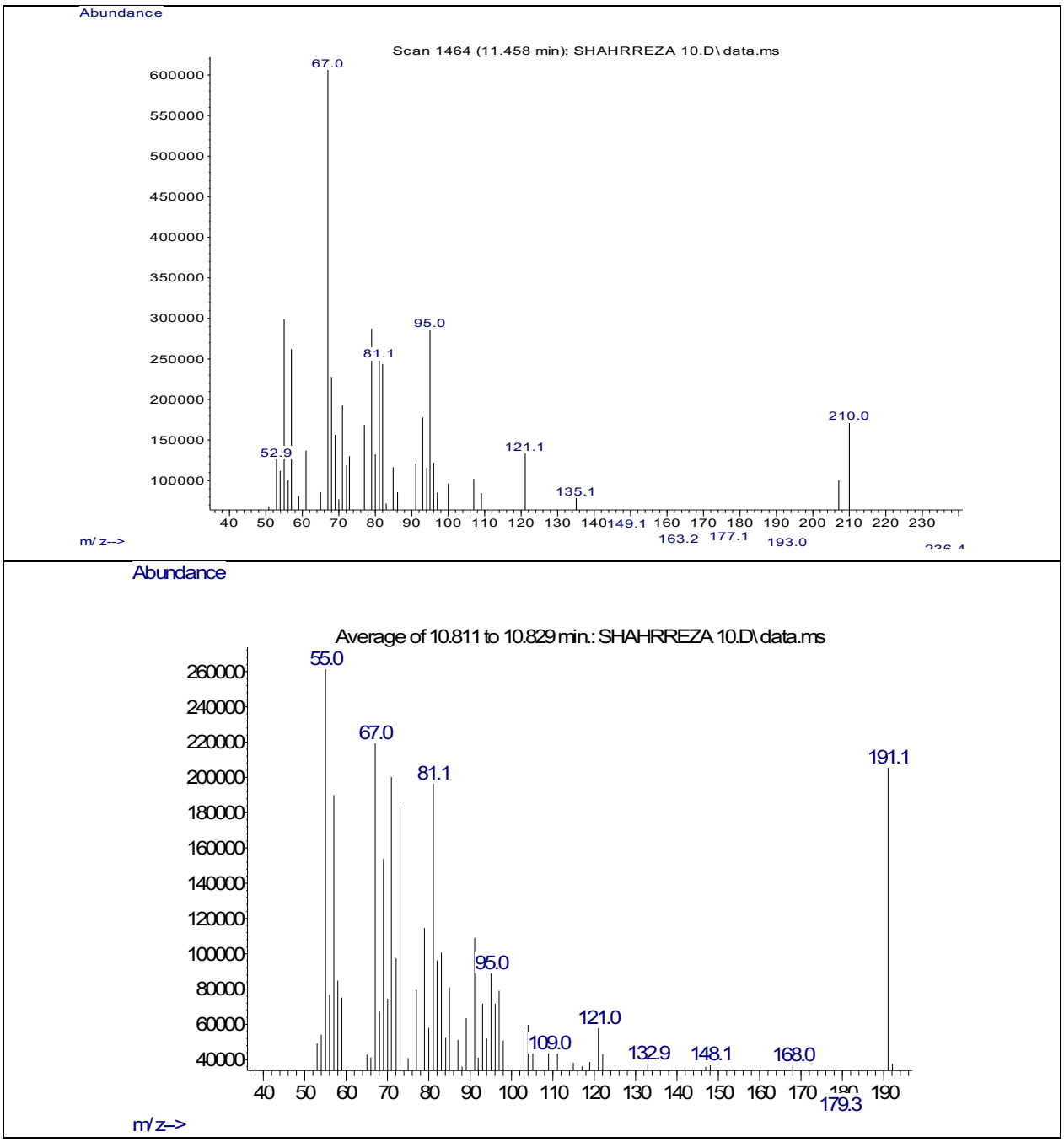

Figure 6. EI mass spectra of the compound from the SPME extract of female ovipositor 11.45 min (*Z,E*)- 9,11-tetradecadienal (top); 10.82 min (Z)-9-tetradecenal (bottom).

The ratios of the three components were 10:0.45:0.43 in Isfahan, 10:1.1:0.9 in South Khorasan, 10:0.9:0.9 in Sistan, and 10:2.5:2.1 in Lorestan, respectively. In addition, Frerot et al. (1997) used SPME for the isolation of Z11-16:Ac and Z11-16:OH from gland female *S. nonagrioides*, and identified their structures by GC/MS analyses of ions *m/z* 43, 55, 67, 82, 96, 222 and 41, 55,67, 82, 96, 222 and their ratio was found to be 9:0.5.

Mozaffarian et al. (2007) observed geometric and morphometric differences among carob moths from different populations in Iran due to the genetic changes in the population. These changes were considered to have resulted from the natural selection and adaptation to environmental conditions (Girling & Carde, 2006). Ziaaddini et al. (2010) indicated that there were differences in foraging behaviors of males and calling behaviors of females in different populations of carob moth in Saveh, Kerman and Arsanjan under the same conditions. However, the differences between populations did not prevent cross attraction and mating was not be prevented between the different populations (Phelan & Baker, 1986; Gemeno et al., 2000). Nevertheless, differences among the pheromones were evident for the populations used. In addition, the ratios of these components in from the different geographical regions differed. In response to the studies of Mozaffarian et al. (2007) and Ziaaddini et al. (2010), the authors of the present study decided to investigate these pheromone compounds from different parts of Iran.

In summary, SPME followed by GC/MS was an excellent technique for the analysis and study of volatiles of *A. ceratoniae* as an important pest of pomegranate. Three pheromone components (*Z,E*)-9,11,13-tetradecatrienal, (*Z,E*)-9,11-tetradecadienal and (*Z*)-9-tetradecenal were identified. The ratios of these components were characterized and found to vary among the carob moth populations from different geographical regions of Iran. Further investigations are ongoing to prepare various ratios of three pheromone components of *A. ceratoniae* to be tested in the field in other regions of Iran.

Table 1. Variation of the retention time and the ratio of sex pheromone components of *Apomyelois ceratoniae* in four regions of Iran

	(*Z,E*)-9,11,13-tetradecatrienal Ratio (RT)	(*Z,E*)-9,11-tetradecadienal Ratio (RT)	(*Z*)-9-tetradecenal Ratio (RT)
Isfahan	10 (12.15)	0.45 (11.45)	0.43 (10.88)
South khorasan	10 (12.16)	1.1 (11.44)	0.9 (10.86)
Lorestan	10 (12.15)	2.5 (11.53)	2.1 (10.95)
Sistan	10 (12.16)	0.9 (11.57)	0.9 (10.86)

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Original article (Orijinal araştırma)

Using degree-day and nonlinear regression models to predict seasonal flights of *Adoxophyes orana* (Fischer von Röslerstamm, 1834) (Lepidoptera: Tortricidae) in plum orchards

Erik bahçelerinde *Adoxophyes orana* (Fischer von Röslerstamm, 1834) (Lepidoptera: Tortricidae)'nın mevsimsel uçuşlarını tahmin etmek için gün-derece ve doğrusal olmayan regresyon modellerinin kullanılması

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Summary

Adoxophyes orana (Fischer von Röslerstamm, 1834) is a major pest of pome and stone fruits in Europe and Asia. This study reports the first record of *A. orana* in plum orchards in Turkey. Moth flight activity was monitored using pheromone traps in Bursa during 2011 to 2013. Also, cumulative degree-days (DD) were calculated to predict moth emergence time and flight peaks in plum orchards. *Adoxophyes orana* had three flight peaks. Depending on the year, the first, second and third flight of moths began between 2-25 May, 20 June-13 July, and 7 August-14 September, respectively. Emergence dates of each flight coincided with 325-391, 957-1065 and 1797-1943 DD in the same order. Richards' function and logistic regression models were applied to forecast seasonal flights of this pest. The 50% moth emergence of the first, second and third flight of *A. orana* was predicted at 606, 1407 and 2169 DD using the Richards' function, and 636, 1427 and 2341 DD using the logistic model as compared with observed values at 626, 1393 and 2110 DD, respectively. Previously developed forecasting models may help apply timely control and thus reducing the number of pesticide applications in plum orchards.

Keywords: *Adoxophyes orana*, logistic model, nonlinear regression, Richards' function, summer fruit tortrix moth

Özet

Adoxophyes orana (Fischer von Röslerstamm, 1834) Avrupa'da ve Asya'da yumuşak çekirdekli ve sert çekirdekli meyvelerde önemli bir zararlıdır. Bu çalışma, Türkiye'de erik bahçelerinde *A. orana*' nın ilk defa kaydedildiğini bildirmektedir. Bursa'da 2011-2013 yılları arasında feromon tuzakları kullanılarak güvenin uçuş zamanı izlenmiştir. Ayrıca, erik bahçelerinde güve çıkış zamanını ve uçuş piklerini tahmin etmek için gün-derece (GD) toplamları hesaplanmıştır. *Adoxophyes orana*' nın üç uçuş piki vardır. Yıla bağlı olarak, birinci, ikinci ve üçüncü güve uçuşu sırasıyla 2-25 Mayıs, 20 Haziran-13 Temmuz ve 7 Ağustos-14 Eylül tarihleri arasında gerçekleştirilmiştir. Her uçuşun başlangıç tarihi aynı sırayla 325-391, 957-1065 ve 1797-1943 GD toplamları ile çakışmıştır. Richards'ın fonksiyonu ve lojistik regresyon modeli bu zararlının mevsimsel uçuşlarını tahmin için uygulanmıştır. *Adoxophyes orana*' nın birinci, ikinci ve üçüncü uçuş dönemlerinde %50 güve çıkışı, gözlemlenen 626, 1393 ve 2110 GD değerleri ile karşılaştırıldığında Richards'ın fonksiyonu kullanılarak sırasıyla 606, 1407 ve 2169 GD ve lojistik model kullanılarak ise 636, 1427 ve 2341 GD değerleri tahmin edilmiştir. Daha önce geliştirilen tahmin modelleri zamanında mücadele yapılmasını sağlayarak erik bahçelerinde pestisit uygulamalarının sayısını azaltabilir.

Anahtar sözcükler: *Adoxophyes orana*, lojistik model, doğrusal olmayan regresyon analizi, Richards'ın fonksiyonu, yaprak yapıştıran

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Introduction

The summer fruit tortrix moth, *Adoxophyes orana* (Fischer von Röslerstamm, 1834) (Lepidoptera: Tortricidae), is a major pest of pome and stone fruits in Europe and Asia, particularly during the summer (Kocourek & Stara, 2005). *Adoxophyes orana* was first recorded in northwestern Turkey in 2010. Since then, it has become a key pest of pears and peaches in the Marmara Region. In addition, *A. orana* larvae have been observed to feed on leaves of sweet cherry and apple in Turkey (Pehlevan & Kovancı, 2014).

Larvae prefer to feed on young shoots and leaves of stone and pome fruits in spring. Leaf and shoot damage has no economic importance, unless leaf roller densities are high (Dickler, 1991). Larvae can cause extensive and costly damage to fruit. Fruit damage is usually superficial, but the external damage may also create a suitable environment for pathogens to establish. Therefore, *A. orana* has a low damage threshold, and it should be monitored with pheromone traps (Kocourek & Stara, 2005). The summer fruit tortrix moth may have two to three generations per year in peach and apple orchards of Turkey (Pehlevan & Kovancı, 2014), whereas it has three generations in peach orchards of Greece and two generations in apple orchards in Europe (Milonas & Savopoulou-Soultani, 2006). Under laboratory conditions, the mean generation time of *A. orana* was found to be the longest on plum (35.7 d) compared with apple, peach and apricot (Lina et al., 2015). However, no specific information is available on the number of *A. orana* generations produced annually on plum grown in the field.

Pehlevan & Kovancı (2014) reported that emergence time and flight period of *A. orana* adults were different between host plants. The first moth flight was caught in apple and peach orchards in early or mid-May, coinciding with an average cumulative degree-days (DD) of 350-356 DD in Turkey. The second moth flight started at the end of June or at the beginning of July (1003-1027 DD) while the third moth flight began in early August (1600-1690 DD). Moth flights lasted for an average of 2305 DD until late September in Turkey (Pehlevan & Kovancı, 2014). However, it is not known if *A. orana* populations in plum orchards have similar flight patterns to those in apple and peach orchards. The ability to detect and predict the activity of *A. orana* in plum orchards may help farmers appropriately schedule pesticide application to correspond with the period of each flight peak of the pest.

Degree-day models are considered as a useful tool for forecasting the seasonal flight activity of many lepidopterous pests (Hrdý et al., 1996; Del Tio et al., 2001). These models have been used more effectively for making pest control decisions (Akotsen-Mensah et al., 2011). For example, models based on linear and nonlinear functions may help predict insect phenology such as emergence time of the codling moth *Cydia pomonella* (L., 1758) in the field (Demir & Kovancı, 2015). Similarly, Kocourek & Stara (2005) and Damos & Savopoulou-Soultani (2010) constructed different phenological models to predict the flight activity of *A. orana* in apple and peach orchards, respectively. To validate their temperature-based models in plum orchards, both Richards' function and logistic regression to predict flight emergence patterns of *A. orana* during the growing season were used in this study.

The main objective of this study was to monitor the population fluctuations of the summer fruit tortrix moth using pheromone traps in plum orchards in Turkey. In addition, moth phenology and cumulative catch data in plum orchards obtained between 2011 and 2013 were used to check and compare the reliability of previously developed degree-day models for *A. orana* adults in apple and peach orchards (Kocourek & Stara, 2005; Damos & Savopoulou-Soultani, 2010).

Materials and Methods

Study sites

Studies were carried out in plum orchards in İnegöl, Bursa (40°19´ N, 29°06´ E), northwestern Turkey in 2011, 2012 and 2013. Trials were conducted in three separate locations, where the occurrence of *A. orana* in plum orchards recorded for the first time in Turkey with this study. Each trial site consisted of a 0.5 ha orchard with three plots (0.17 ha each) of the late-ripening plum cultivar, President. Trees were 10 years old and about 2.5 m tall.

Three insecticide applications were made against insect pests each year. Chlorpyrifos-ethyl was applied once in early May at 100 ml in 100 l of water per 0.1 ha to control European fruit lecanium, *Parthenolecanium corni* (Bouché, 1844), and plum scale, *Sphaerolecanium prunastri* (Boyer de Fonscolombe, 1834). Aphid nymphs and adults were controlled with one application of imidacloprid at 20 ml in 100 l of water per 0.1 ha in early May. Spirodiclofen was used twice at a rate of 25 ml in 100 l of water per 0.1 ha to control spider mites in early May and mid-June.

Flight monitoring

Delta type traps loaded with a synthetic sex pheromone to monitor seasonal population fluctuation of the male *A. orana* moths. Each year, three traps per orchard were deployed at about 2 m above from the soil and at a distance of 45 m from each other to avoid trap interaction (Milonas & Savopoulou-Soultani, 2006). The traps were deployed on 1 May before night temperatures exceed 13-14°C, being the temperature threshold for the initiation of moth flight (Whittle, 1985). Traps were checked every day until the first flight was detected. The number of moths was recorded and moths were removed from traps every week. Pheromone capsules and the sticky surface were changed at six-week intervals (Pehlevan & Kovancı, 2013). At the end of the season, moth flight activity was considered to have ended after three consecutive zero captures.

Degree-days and phenological models

Weekly average temperature data for the trial season periods during 2011-2013 in İnegöl, Bursa is given in Figure 1. Weather data was acquired from the meteorological station. DD were calculated based on the following equation of Baskerville & Emin (1969):

$$\frac{t_{min} + t_{max}}{2} - \mathrm{MTT}$$

where t_{min} and t_{max} for lower and higher day temperature and MTT is the minimum temperature threshold of the insect development. According to the results of previous studies, a developmental threshold of 7.2°C was adopted to calculate DD (Pehlevan & Kovancı, 2013). Temperatures above the degree of developmental threshold were accumulated from 1 February when larval diapause was found to be terminated by Milonas & Savopoulou-Soultani (2006).

The fitness of Richards' function model by Kocourek & Stara (2005) for predicting flight emergence patterns of *A. orana* was compared with that of logistic regression model by Damos & Savopoulou-Soultani (2010). The cumulative percentages of the flights of each generation and DD were fitted to a curve using three-parameter Richards' function and four-parameter logistic regression function. Both equations are S-shaped cumulative distribution functions. Three-parameter Richards' function:

$$y = \frac{100}{(1 + c_3 \,\mathrm{X}\, e^{-c_1(x-c_2)}) \,\mathrm{X}\, 1/c_3}$$

where y is the cumulative percentage, x is the cumulative DD *and* c_1, c_2, and c_3 are parameters. c_1 is the slope of the curve, c_2 is the time when 50% of the population were caught, and c_3 is the upper limit of the curve (Kocourek & Stara, 2005). Four-parameter logistic regression function:

$$g(x) = d + \frac{a}{1 + \left(\frac{x}{c}\right)^b}$$

where g is the cumulative percentage, x is the cumulative DD, and a, b, c and d are constant numerical parameters that regulate the shape of the curve. Moreover, c indicates the DD at which 50% moth emergence occurs (Damos & Savopoulou-Soultani, 2010).

Statistical analysis

Moth catch data were transformed using √(x+1) before analysis of variance to normalize the data distribution. Least squares means comparisons were used to determine interaction effects. Parameter estimation values both in the Richards' function and in the logistic model were obtained through JMP (Schlotzhauer, 2007). Computations of the percent emergence of populations were calculated with Microsoft Excel. The accuracy of each model was tested based on the adjusted coefficient of determination (Adj. R^2), as well as the Akaike's information criteria (Damos & Savopoulou-Soultani, 2010).

$$\text{Adj } R^2 = 1 - \frac{(\frac{\text{RSS}}{n} - (Q+1))}{\frac{\text{SS}}{n} - 1}$$

$$\text{AIC} = n[\text{In}(\text{RSS})] - [n - 2(Q-1)] - n\ln(n)$$

where RSS is the residual sum of squares, SS is the total sum of squares, Q is the number of parameters, and n the number of observations. To find out the probability of the ith model that minimizes the information loss, we used the following formula, $\exp((\text{AIC}_{\text{min}} - \text{AIC}_i)/2)$ (Burnham & Anderson, 2002).

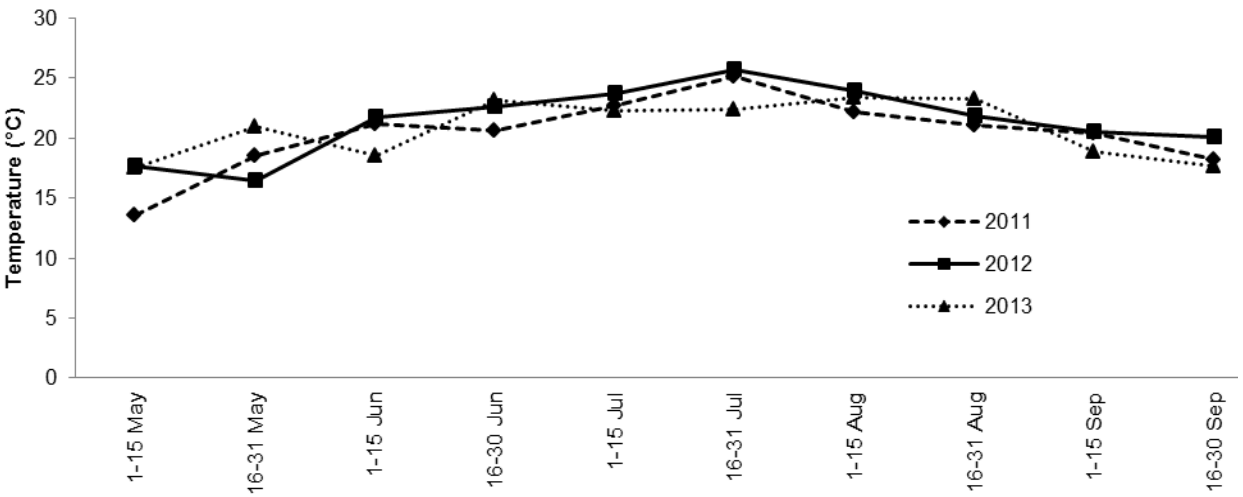

Figure 1. Weekly average temperature data in İnegöl, Bursa, Turkey during 2011-2013.

Results

Moth flight activity in plum fruits

A total of 1835 adults of *A. orana* was caught in traps during the three-year study period. There were significant differences in total moth catches between the 2011 (172), 2012 (887) and 2013 (776) growing seasons ($F_{2,\ 681} = 17.7$, $P < 0.01$). Moth pressure was low during 2011. The peak catches of moths occurred on 27 July, 8 July and 27 June in 2011 to 2013, respectively (Figure 2). Significant differences were observed between weeks in the number of moths caught in 2011 ($F_{16,\ 187} = 6.44$, $P < 0.01$), 2012 ($F_{19,\ 220} = 12.0$, $P < 0.01$), and 2013 ($F_{19,\ 220} = 4.16$, $P < 0.01$) in all orchards. The total number of moths caught in traps did not significantly differ between locations ($F_{2,\ 675} = 1.54$, $P = 0.22$), but they were significantly different between years ($F_{2,\ 675} = 17.8$, $P < 0.01$). Numbers of captured moths during the first, second and third flights were significantly different in 2011 ($F_{2,\ 152} = 2.60$, $P = 0.08$), 2012 ($F_{2,\ 213} = 51.0$, $P < 0.01$) and 2013 ($F_{2,\ 213} = 8.50$, $P < 0.01$).

Adult emergence times and total flight period showed significant variation among years. The first adults were caught on 25 May, 6 May and 2 May in 2011 to 2013, respectively (Figure 2), corresponding to the accumulation of 391, 325 and 377 DD, respectively, starting from 1 February. The second flight began on 13 July (1065 DD), 24 June (957 DD) and 20 June (976 DD) in 2011 to 2013, respectively. The third flight began on 7 September (1943 DD), 12 August (1806 DD) and 15 August (1767 DD) in 2011 to 2013, respectively. Moth captures continued until mid-September, and the total flight period ended at 1955, 2316 and 2311 DD in 2011 to 2013, respectively (Figures 2 & 3).

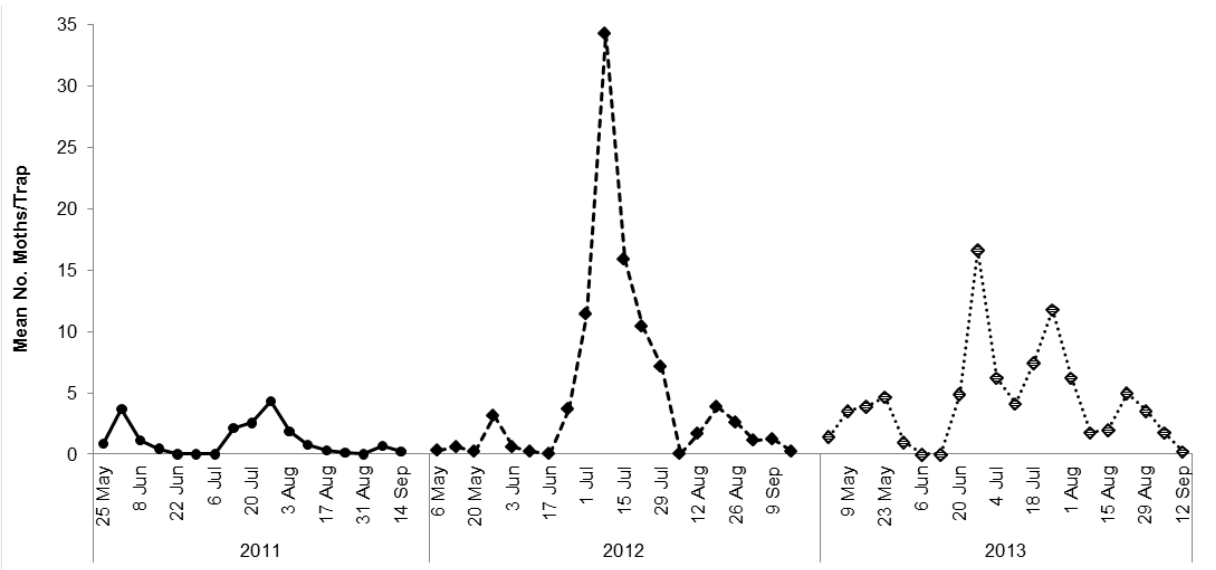

Figure 2. Seasonal flight patterns of *Adoxophyes orana* in three respective plum production regions of Turkey in 2011, 2012 and 2013.

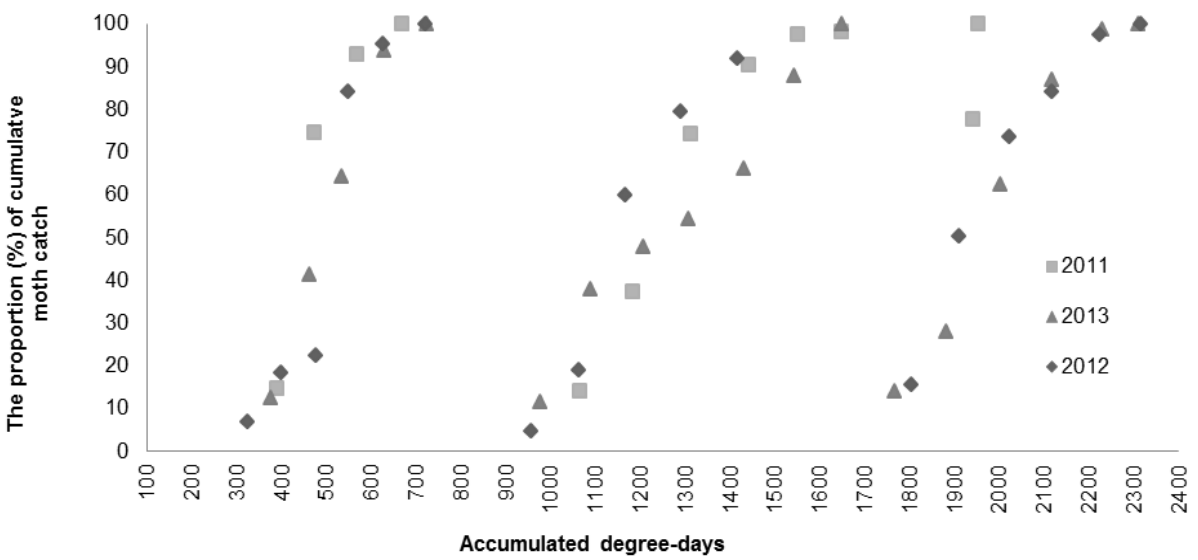

Figure 3. The proportion (%) of cumulative male moths caught in pheromone traps and cumulative degree-days during the first, second, and the third flight of *Adoxophyes orana* in 2011, 2012 and 2013 in plum orchards in Bursa, Turkey.

Forecasting models

Data on the cumulative proportion of *A. orana* adults caught in plum orchards during 2011-2013 were subjected to nonlinear regression analysis. Generated models using the relationship between cumulative catches of the first, second and third flight of *A. orana* males and DD are shown in Figure 4.

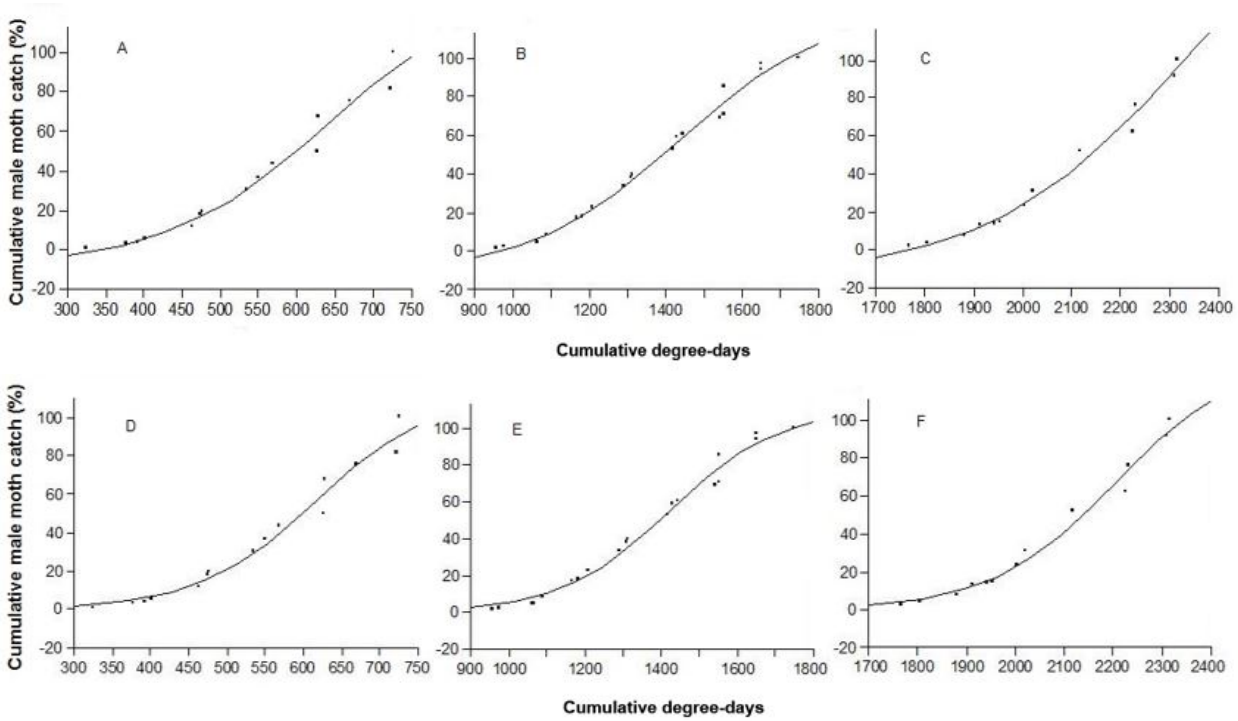

Figure 4. Developed models for logistic regression function (A, B and C) and Richards' function (D, E and F) in describing first, second and third flight cumulative male moth catches of *Adoxophyes orana* in relation to degree-days.

The flight curve of the first, second and third flight was fitted into a plot using both Richards' function and logistic regression function. There was a significant correlation between logistic regression function and Richards' function (Figure 5).

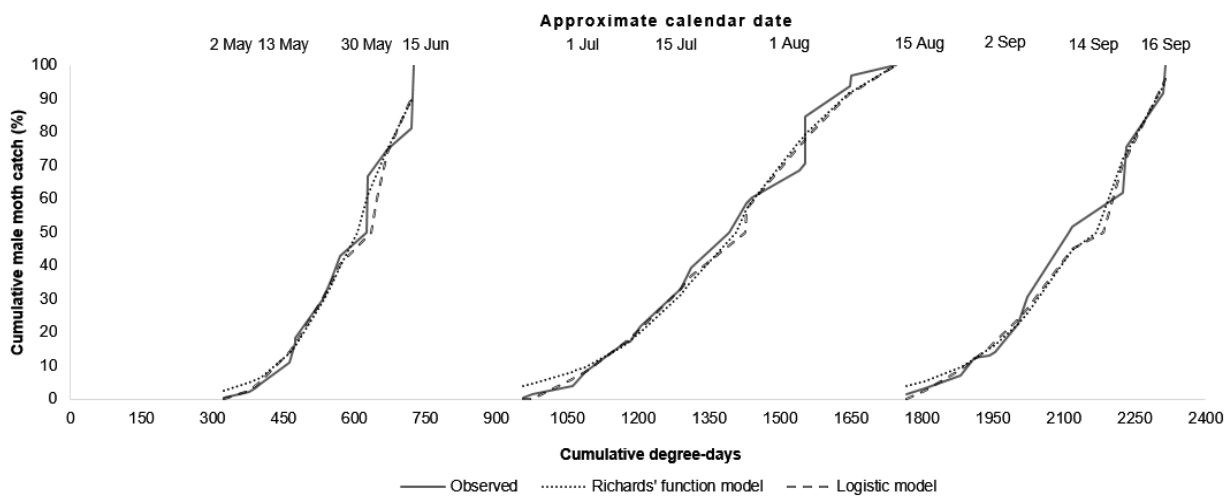

Figure 5. Observed and predicted cumulative male moth catches of *Adoxophyes orana* relative to cumulative degree-days averaged over years according to the adjusted logistic and Richards' function model in plum orchards in Bursa, Turkey.

Both models were good at estimating the first emergence time and each flight period of *A. orana*. Parameter *b* of the Richards' model and parameter *m* of the logistic model represented the 50% of cumulative moth emergence. Parameters in the Richards' function (606 DD) and the logistic model (636 DD) were similar to each other. Estimated regression parameters of the models for all three years are listed in Table 1. Across years, the first male moths were caught between 325 and 391 DD. The second moth flight began between 957 and 1065 DD, while third moth flight occurred between 1767 and 1943 DD. Total flight periods lasted between 1955 and 2316 DD for all three years, starting from 1 February. Despite the standard errors that lead to deviations in model predictions, predicted moth phenology remained within acceptable limits.

Table 1. Parameter estimates (SE) and coefficient of determinations of Richards' and logistic- regression function models in defining moth phenology of *Adoxophyes orana* in plum orchards

Flight	Parameter Estimates			
	Richards' function		Logistic regression function	
First	*a*	0.013 (0.002)	*a*	-7.8 (10.8)
	b	606 (15.2)	*b*	0.01 (0.004)
	c	1.10 (0.20)	*c*	141 (52.8)
	-	-	*m*	636 (60.4)
	R^2	0.975	R^2	0.977
	Adjusted R^2	0.973	Adjusted R^2	0.975
	Regression equation	y = 1.81 + 0.96x	Regression equation	y = 0.82 + 0.98x
	F	508	F	554
	AIC*	60.0	AIC	62.2
	df	12	df	11
Second	*a*	0.007 (0.001)	*a*	-12.3 (7.90)
	b	1407 (14.3)	*b*	0.005 (0.001)
	c	1.10 (0.10)	*c*	138 (20.5)
	-	-	*m*	1427 (33.0)
	R^2	0.986	R^2	0.989
	Adjusted R^2	0.985	Adjusted R^2	0.989
	Regression equation	y = 1.88 + 0.97x	Regression equation	y = 0.44 + 0.99x
	F	1333	F	1769
	AIC	65.4	AIC	62.6
	df	17	df	16
Third	*a*	0.008 (0.001)	*a*	-13.3 (16.3)
	b	2169 (28.7)	*b*	0.005 (0.003)
	c	1.40 (0.30)	*c*	233 (217)
	-	-	*m*	2341 (317)
	R^2	0.983	R^2	0.986
	Adjusted R^2	0.982	Adjusted R^2	0.985
	Regression equation	y = 1.5 + 0.97x	Regression equation	y = 0.5 + 0.99x
	F	674	F	816
	AIC	50.7	AIC	53.6
	df	10	df	9

* Akaike's information criterion (AIC) was used to compare the quality of each model relative to each other.

Models for estimating flight times were tested according to the adjusted R^2 (Table 1). Both models defined first, second and third flights of *A. orana* with high accuracy. Based on the adjusted coefficient of determination values (>0.97) for all flights, there were only minor differences between the two models. Moreover, these models were assessed based on the Akaike's information criteria. Logistic regression model was 0.33 and 0.24 times as probable as the Richards' function model to minimize the information loss in predicting the first and third moth flights. In contrast, the probability of estimated information loss for the second flight by Richards' function model was 0.25 times the logistic regression model. Thus, both models yielded similar predictions despite subtle differences between flights. The differences in the accuracy of each model for predicting each moth flight can be explained by the climate variables such as temperature, directly affecting insect phenology (Damos & Savopoulou-Soultani, 2010).

First emergence time and flight periods of *A. orana* across over the years were forecasted by both models. Estimated regression equations of the two nonlinear models for all three years are given in Table 2. The adjusted R^2 values were used to evaluate the model performance of each function (Table 2). Good accuracy in describing the first, second and third flights of *A. orana* was obtained by both models

Table 2. Estimated regression equations of the two nonlinear models for all years and regression statistics of moth phenology for *Adoxophyes orana*

			Richards' function			Logistic regression function		
Flight	Year	*N*	Regression equation	R^2	Adj R^2	Regression equation	R^2	Adj R^2
First	2011	4	y = 0.57 + 0.99*x*	0.995	0.993	y = 20.1 + 0.67*x*	0.948	0.922
	2012	6	y = -6.64 + 1.07*x*	0.979	0.974	y = 0.43 + 0.99*x*	0.992	0.990
	2013	5	y = 0.67 + 0.99*x*	0.996	0.995	y = 0.17 + 1.00*x*	0.997	0.996
Second	2011	7	y = -0.02 + 1.00*x*	0.999	0.999	y = 0.06 + 1.00*x*	0.999	0.999
	2012	6	y = 1.89 + 0.97*x*	0.989	0.986	y = 0.54 + 0.99*x*	0.990	0.988
	2013	7	y = 3.32 + 0.95*x*	0.961	0.953	y = 1.03 + 0.97*x*	0.971	0.966
Third	2011*	-	-	-	-	-	-	-
	2012	6	y = 2.70 + 0.97*x*	0.986	0.982	y = 2.57 + 0.96*x*	0.998	0.997
	2013	6	y = -0.75 + 1.00*x*	0.998	0.997	y = 0.02 + 1.00*x*	0.999	0.999

*Not calculated.

The parameters of the Richards' and logistic equation used to fit the curve are shown in Table 3. Observed and predicted data revealed a good fit for *A. orana* moth phenology for all three flight periods. Combined regression statistics were also calculated for predicted and observed cumulative percentages of moth catches in each generation.

Table 3. Observed and predicted times (month and day) of 50% moth emergence of first, second and third *Adoxophyes orana* flight relative to degree-days using Richards' function and logistic regression function in plum orchards in 2011, 2012 and 2013

			Richards' function	Logistic regression function
Flight	Year	Observed	Predicted	Predicted
First	2011	391 (25.5)	389 (23.5)	283 (15.5)
	2012	325 (06.5)	335 (06.5)	323 (05.5)
	2013	377 (02.5)	375 (01.5)	376 (01.5)
First	Pooled data for all years	325	316	319
First flight 50%		626	606	636
Second	2011	1065 (13.7)	1065 (13.7)	1064 (13.7)
	2012	957 (24.6)	936 (22.6)	950 (23.6)
	2013	1008 (20.6)	930 (14.6)	945 (15.6)
Second	Pooled data for all years	957	930	948
Second flight 50%		1393	1407	1427
Third	2011*	1943 (07.9)	-	-
	2012	1806 (12.8)	1746 (08.8)	1744 (08.8)
	2013	1797 (08.8)	1783 (08.8)	1765 (05.8)
Third	Pooled data for all years	1767	1716	1745
Third flight 50%		2110	2169	2341

*Not calculated.

Discussion

Flight activity

In this study, the presence of *A. orana* in plum orchards in Turkey were reported for the first time. Previously the only known hosts of this introduced pest in Turkey were apple, pear, peach and sweet cherry (Pehlevan & Kovancı, 2014). *Adoxophyes orana* larvae have been recorded to feed on pome fruit such as pear and apple in Europe (Stamenkovic et al., 1999), whereas stone fruit like peach and sweet cherry were more heavily attacked in southern Europe (Savopoulou-Soultani et al., 1985). The high levels of moth catch in plum orchards in this study clearly showed that stone fruit are suitable alternative hosts of the summer fruit tortrix moth.

Pheromone traps were successfully used to detect and monitor the flight activity of male moths during the growing season as in other countries such as Greece and the Czech Republic (Kocourek & Stara, 2005; Damos & Savopoulou-Soultani, 2010). However, degree-day and nonlinear regression models developed by Kocourek & Stara (2005) in apple orchards and by Damos & Savopoulou-Soultani (2010) in peach orchards to predict emergence time and flight activity periods of *A. orana* adults may improve the success of monitoring activities in plum orchards as well, enabling more appropriately timed sprays.

There were three flight peaks in plum orchards in May, late June or mid-July and mid-August. The number of flight peaks recorded in this study is consistent with the results of Pehlevan & Kovancı (2014) in apple, pear, peach and sweet cherry orchards. Nevertheless, there were subtle differences between the emergence time of *A. orana* males in plum orchards compared with pome and other stone fruit. However, emergence time and flight peak periods were possibly unrepresentative in 2011 due to the low population density in that year. Temperatures above the upper larval development threshold of 30°C in August may have been responsible for the late detection of summer generation moths in 2011 (Milonas & Savopoulou-Soultani, 2000).

Adoxophyes orana completed three generations in plum orchards in Turkey based on flight monitoring data in all years. This finding is consistent with earlier field studies conducted in Thessaloniki, northern Greece, where three to four generations were reported in peach orchards (Charmillot & Brunner, 1990; Milonas & Savopoulou-Soultani, 2006). Unlike southern Europe, two generations of *A. orana* occurred in apple orchards in central and northern Europe (Charmillot & Brunner, 1990; Cross et al., 1997).

In this study, the first flight of moths emerged between 2 and 25 May, the second between 20 June and 13 July and the third between 7 August and 14 September depending on the year. These flight periods differed markedly between years due to weather conditions. In the Czech Republic, the time interval for the first capture of the first flight of moths differed by more than one month from 11 May to 15 June during 1992 to 2003. Similarly, there was also one month difference at the beginning of the flight of the second flight varying from 8 July to 7 August between years (Kocourek & Stara, 2005). Two flight periods observed from late May to early July, and from early August to mid-September in apple orchards in western Serbia (Stamenkovic et al., 1999). Over the same time span, *A. orana* had three flights in Turkey. This variation may be caused by an adaptation to phenology of different hosts, as well as by the colder temperatures and shorter photoperiods in central and northern Europe than in southern Europe. Particularly, day lengths below a critical photoperiod of 12 h can stimulate diapause in the third instar larvae (Ankersmit, 1968).

Degree-day calculations

Estimation of first moth emergence is crucial for successful control of *A. orana*. Hence, the seasonal flight activity of *A. orana* was monitored using pheromone traps.

DD accumulations can be used as a tool for forecasting emergence time of adults in each flight period (Damos & Savopoulou-Soultani, 2010). For this purpose, we examined the interaction among temperature data and moth catch in plum orchards in 2011, 2012 and 2013. DD calculations for the first flight of *A. orana* were similar in all years at each locality. However, they were significantly different for the second and third flights according to year and locality. Based on these results, the first adult emergence began between 325 and 391 DD, the second between 957 and 1065 DD and the third between 1797 and 1943 DD during 2011 to 2013 (Table 3). Except for the delayed onset of the third flight, these findings were consistent with those of Pehlevan & Kovancı (2014) in apple, pear, peach and sweet cherry. Apparently, it takes more time for *A. orana* to develop on plum in the field as previously observed by Lina et al. (2015) under laboratory conditions.

We recorded 50% moth emergence of the first, second and third flights of *A. orana* at 626, 1393 and 2110 DD, respectively, in plum orchards. Whereas, Damos & Savopoulou-Soultani (2010), who used the same threshold value, reported 406, 1260 and 2141 DD for the first, second and third flights of *A. orana* in peach orchards in Greece. Apart from the third flight, there was a considerable delay in 50% moth emergence period in plum orchards when compared with DD calculations in peach orchards in Greece.

Nonlinear regression models

DD values have been used to test nonlinear regression models to forecast moth phenology in a more accurate way (Kocourek & Stara, 2005; Damos & Savopoulou-Soultani, 2010). Richards' function and logistic regression models were applied to compare predicted and observed DD values. Also, the predictions obtained by Richards' function and logistic regression models were compared with each other.

First emergence and subsequent flight activity of *A. orana* for each flight were well described by both models. For the first *A. orana* flight, parameter *b* in the Richards' function (606 DD) and parameter *m* in the logistic model (636 DD), which defines the 50% of cumulative moth emergence, were very similar to each other. Both models also provided a similar prediction for the second *A. orana* flight of 1407 and 1427 DD in the same order. Whereas, divergence in predictions were higher for the third flight, 2169 and 2341 DD, respectively. Kocourek & Stara (2005) also applied Richards' function to estimate 50% of moth catch for the first flight in apple orchards based on the sum of DD above an 8°C development threshold. Unlike our prediction of 606 DD, this model with a higher base temperature predicted 300 DD in the

Czech Republic. Damos & Savopoulou-Soultani (2010) used a three-parameter Boltzmann-type and a four-parameter logistic regression function to predict 50% of moth catch of the first, second and third flights of *A. orana* in peach orchards in Greece. However, their models gave predictions of 404 and 406 DD for the first flight, 1253 and 1260 DD for the second, and 2136 and 2141 DD for the third, which distinctly differs from our findings in plum orchards.

In all three flight periods, the observed and predicted data fit well with *A. orana* moth phenology. The coefficient of determination (R^2) showed that both models had very high prediction competence, although variation increased in succeeding generations.

Clearly, this study indicated three distinct flights of *A. orana* in plum orchards, with the last flight occurring later than in pome and other stone fruits orchards. Previously developed forecasting models for the summer fruit tortrix moth in other crops can offer a means of achieving timely pest control, thus reducing the number of pesticide applications in plum orchards. Finally, further field studies in plum orchards are needed to validate the parameter estimates of the Richards' function and logistic model for *A. orana* flight activity in other regions of Turkey as well in other parts of the world.

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Original article (Orijinal araştırma)

Contributions to the fauna of Elateridae (Coleoptera) of Turkey with a description of a new species and two new records[1]

Türkiye Elateridae (Coleoptera) faunasına bir yeni tür ve iki yeni kayıt ile katkılar

Osman SERT[2] **Mahmut KABALAK[2]***

Summary

A new species, *Athous* (*Orthathous*) *savsatensis* n. sp., was discovered in Artvin province and two new species for Turkish fauna, *Agriotes bogatschevi* Dolin, 1969 and *Athous* (*Athous*) *kobachidzei* Dolin & Chantladze, 1982, were recorded from Giresun, Gümüşhane, Rize and Trabzon provinces within comprehensive field studies carried out in 2013, 2014 and 2015 on the Elateridae family of the Eastern Black Sea Region of Turkey. The morphology of the new species is described. Photographs of habitus, drawings of aedeagi and the distribution map of the new species, its closely related species and new records are given.

Keywords: *Athous*, *Agriotes*, Eastern Black Sea Region, new records, new species

Özet

Türkiye'nin Doğu Karadeniz Bölgesi Elateridae familyası üzerinde 2013, 2014 ve 2015 yıllarında gerçekleştirilen kapsamlı arazi çalışmalarında Artvin ilinden *Athous* (*Orthathous*) *savsatensis* n. sp. yeni türü keşfedilmiş ve Giresun, Gümüşhane, Rize ve Trabzon illerinden Türkiye faunası için iki yeni kayıt olan *Agriotes bogatschevi* Dolin, 1969 ve *Athous* (*Athous*) *kobachidzei* Dolin & Chantladze, 1982 türleri tespit edilmiştir. Yeni türün morfolojisi betimlenmiştir. Yeni türün, yeni türe yakın türün ve yeni kayıtların ergin fotoğrafları, erkek üreme organlarının çizimleri ve yayılış haritası verilmiştir.

Anahtar sözcükler: *Athous*, *Agriotes*, Doğu Karadeniz Bölgesi, yeni kayıtlar, yeni tür

[1] This study is a part of Turkish Scientific and Research Council (TÜBİTAK) (212T103) Project "Systematical Studies on the family Elateridae (Coleoptera), the subfamilies Aleocharinae and Steninae (Coleoptera: Staphylinidae) in Eastern Blacksea Region".
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Introduction

The family Elateridae is the ninth biggest family of Coleoptera and belongs to the superfamily Elateroidea (Lawrence, 1982). According to various authors (Lawrence, 1982; Booth et al., 1990; Lodos, 1998; Demirsoy, 1999; Laibner, 2000), the family Elateridae has 6,000-10,000 species. The elaterid fauna (Coleoptera) of Turkey includes seven subfamilies, 65 genera and 483 species (Mertlik & Platia, 2008; Kabalak & Sert, 2009, 2010a, b, 2011, 2012a, b, 2013; Platia & Gudenzi, 2009; Platia et al., 2009; Schimmel et al., 2009; Gülperçin & Tezcan, 2010, 2015; Platia, 2010a, b, 2011a, b, 2012, 2014, 2015, 2016; Platia & Nemeth, 2011; Platia et al., 2011; Kabalak et al., 2013a, b; Nemeth & Platia, 2014; Platia & Kakiopoulos, 2015). Among them, 85 species of the genus Agriotes and 53 species of the genus Athous have been recorded from Turkey to date.

In this study, a new species and two new records are given from Eastern Blacksea Region of Turkey.

Materials and Methods

Field methods

The materials were collected by using insect nets from Artvin, Giresun, Gümüşhane, Rize and Trabzon provinces of Eastern Blacksea Region of Turkey in 2013, 2014 and 2015.

Examination of specimens

The body length and width of holotype and paratypes of the new species were measured along the midline from the anterior margin of the frons to the apices of the elytra and across the broadest part of the elytra, respectively. Photographs of imago and aedeagi were taken using a stereoscopic microscope system. Holotype and paratypes of the new species were compared with paratype of *Athous* (*Orthathous*) *fragariae* Platia & Kovanci, 2005. Aedeagi of the new species and newly recorded species were drawn in detail, and aedeagus of *Athous* (*O.*) *fragariae* (Figures 2 & 6) was redrawn from the literature (Platia & Kovancı, 2005). The distribution map (Figure 9) was prepared with CFF 2.0 (Carto Fauna-Flora) (Barbier & Rasmont, 1996, 2000). The aedeagi were extracted using standard methods, dipped in Canada balsam, and placed on a celluloid plate.

Results

As a result of this study, a new species was discovered and two new records were detected. The description of a new species, locality data, photographs of habitus, drawings of aedeagi and the distribution map of the new species, its closely related species and new records are given below.

Athous (Orthathous) savsatensis n. sp. (Figures 1 & 5)

Type material: Holotype, male, Artvin province, Şavşat county, Karagöl-Şavşat road, 1572 m, 41°17'36.6" N, 42°26'51,7" E, 15.VII.2014, leg. M. Kabalak & O. Sert.

Paratype: 3 males, Artvin province, Şavşat county, Karagöl-Şavşat road, 1572 m, 41°17'36.6" N, 42°26'51,7" E, 15.VII.2014, leg. M. Kabalak & O. Sert.

Holotype and paratype are deposited in Hacettepe University Zoology Museum of the Biology Department, Hacettepe University, Ankara.

Holotype: Male, length 8.14 mm, width 2.13 mm. Body light brown except dark brown head, pronotum and scutellum, covered with long and grayish yellow hairs.

Antenna exceed apices of posterior angles of pronotum by about 2.5 segments, third segment two times as long as second segment.

Pronotum 1.1 times as long as wide, punctuation generally umbilicate and sparse, posterior margin of pronotum narrower than basal part of elytra.

Scutellum V-shaped, 1.18 times as long as wide.

Elytra 2.6 times as long as wide, elytral striae distinct and bearing umbilicate punctures, almost parallel sided from basal part to medial part, gradually narrowing from medial part to apical part, truncated at apex, elytra 2.28 times as long as pronotum.

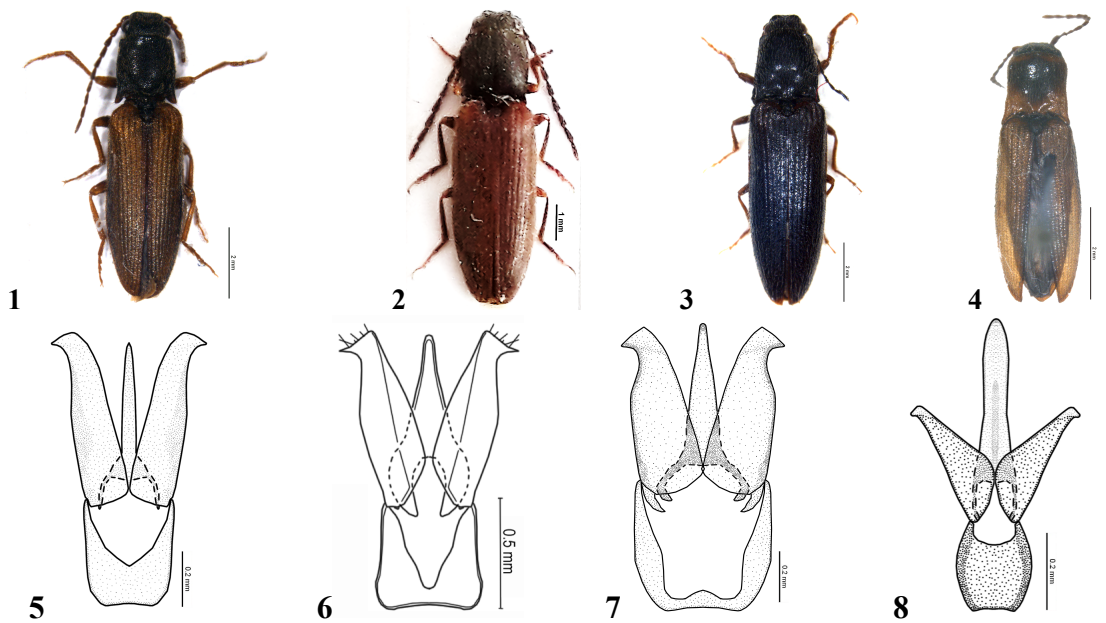
Length of aedeagus almost 1 mm, with morphology typical for the genus (Figure 4), parameres triangularly dentate and apex slightly angled.

Female: Unknown.

Paratype: Length 7.32-8.58 mm, width 1.97-2.3 mm.

Etymology: The name is derived from the name of the Şavşat county, Artvin province.

Habitat: Specimens were collected from herbaceous plants on the forest floor using an insect net.



Figures 1-8. 1. A*thous* (*Orthathous*) *savsatensis* n. sp., habitus. 2. A*thous (Orthathous) fragariae,* habitus. 3. *Athous* (*Athous*) *kobachidzei,* habitus. 4. *Agriotes bogatschevi,* habitus. 5. A*thous (Orthathous) savsatensis* n. sp., aedeagus (dorsal view). 6. A*thous (Orthathous) fragariae,* aedeagus (dorsal view, redrawn from Platia & Kovanci, 2005). 7. *Athous* (*Athous*) *kobachidzei,* aedeagus (dorsal view). 8. *Agriotes bogatschevi,* aedeagus (dorsal view).

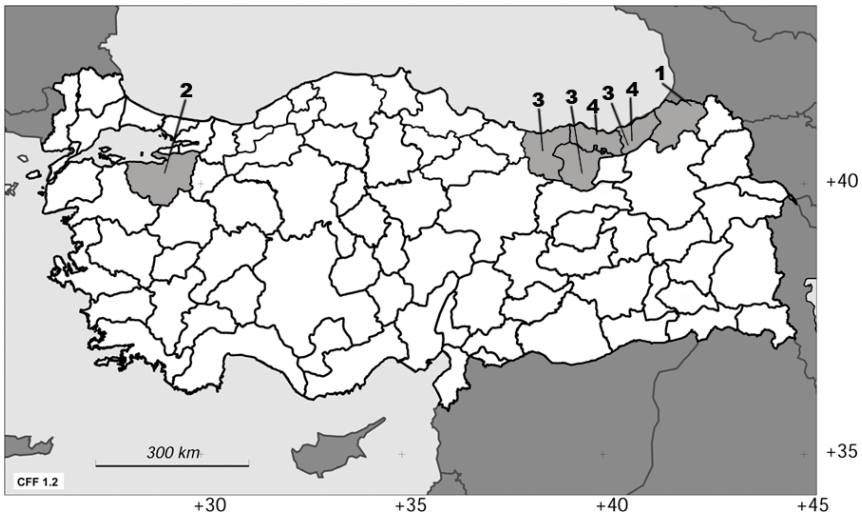


Figure 9. Distribution map of the new species, its closely related species and new records: (1) *Athous* (*Orthathous*) *savsatensis*, (2) *Athous* (*Orthathous*) *fragariae*, (3) *Athous* (*Athous*) *kobachidzei*, (4) *Agriotes bogatschevi*.

Athous (*Athous*) *kobachidzei* Dolin & Chantladze, 1982 (Figures 3 & 7)

Material examined: Giresun province, Espiye county, Ericek road, 1048 m, 40°47'30.7" N, 38°43'31.5" E, 10.VI.2014, ♂, and Dereli county, Şebinkarahisar road, 1569 m, 40°31'21" N, 38°21'16.8" E, 03.VI.2015, 2 ♂♂, leg. M. Kabalak, Y. Turan; Gümüşhane province, Kelkit county, 1991 m, 40°15'41.7" N, 39°28'28.4" E, 04.VI.2014, 2 ♂♂, and Rize province, İkizdere county, Çilekli village, 2037 m, 40°37'27.7" N, 32°31'2" E, 23.VI.2013, ♂, leg. M. Kabalak, Y. Turan & O. Sert.

Habitat: Specimens were collected from herbaceous plants near a stream using an insect net.

Turkey distribution: New record for Turkish fauna.

World distribution: Azerbaijan, Georgia, and Southern European territory of Russia (Cate, 2007).

Agriotes bogatschevi Dolin, 1969 (Figures 4 & 8)

Material examined: Rize province, İkizdere county, Güneyce Entrance, 242 m, 40°49'42.9" N, 40°28'31,4" E, 30.VII.2013, ♀, ♂, and Trabzon province, Yomra county, Yomra-Demirciler road, 888 m, 40°48'07.41" N, 39°48'40.98" E, 26.VI.2013, ♂ leg. M. Kabalak, Y. Turan & O. Sert.

Habitat: Specimens were collected from herbaceous plants near a stream using an insect net.

Turkey distribution: New record for Turkish fauna.

World distribution: Azerbaijan, Georgia, and Southern European territory of Russia (Cate, 2007).

Discussion

Athous (O.) savsatensis n. sp. is closely allied to *At. (O.) fragariae*, but can be separated by the following characters; *At.* (*O.*) *savsatensis* n. sp. is shorter and slender, while *At.* (*O.*) *fragariae* is longer and thicker; antennae of *At.* (*O.*) *fragariae* are longer and exceeding about 3.5 segments the apices of posterior angles of pronotum, while antennae of the new species is shorter and exceeding the apices of posterior angles of pronotum by almost 2.5 segments; anterior corners of pronotum are protruding in *At.* (*O.*) *savsatensis* n. sp., while anterior corners of pronotum are not protruding in *At.* (*O.*) *fragariae*; and elytra of the new species is lighter brown, while elytra of *At.* (*O.*) *fragariae* is brown.

Morphology of aedeagi of the new species and its closely related species are compared in Table 1. The median lobe is slim, distinctly shorter than parameres, pointed at apex and with short, slim and pointed arms in *At.* (*O.*) *savsatensis* n. sp., while the median lobe is gradually thickening, slightly shorter than parameres, finger shaped at apex and with long, thick and slightly pointed arms in *At.* (*O.*) *fragariae*. Paramere has short and slightly pointed tooth and slightly rounded at apex in *At.* (*O.*) *savsatensis* n. sp., while it is long and strongly pointed and angled at apex in *At.* (*O.*) *fragariae*.

Table 1. Morphology of aedeagus, collection months, collection localities and zoogeographical distribution comparisons of *Athous (Orthathous) savsatensis* n. sp. and *Athous (Orthathous) fragariae*

Character	*Athous (Orthathous)savsatensis* n. sp.	*Athous (Orthathous) fragariae* (Figure 6)
Median lobe	Slim	Gradually thick
Median lobe length	Distinctly shorter than parameres	Slightly shorter than parameres
Arms of median lobe	Short and slim; apex pointed	Long and thick; apex slightly pointed
Apex of median lobe	Pointed	Finger shaped
Distal tooth of paramere	Short, slightly pointed and directed laterally	Long, strongly pointed and slightly directed laterally
Lateral sides of paramere	Slightly sinuate	Sinuate
Apex of paramere	Slightly rounded	Angled
Collection month	July	June and July
Collection localities	Artvin province	Bursa province
Zoogeographical distribution	Turkey	Turkey

According to the literature (Platia & Kovancı 2005) and data of this study, collection months, collection localities of Turkey and zoogeographical distributions of the new species and its closely related species are given (Table 1). *Athous* (*O.*) *fragariae* is collected in June and July, while *At.* (*O.*) *savsatensis* n. sp. was only collected in July. *Athous* (*O.*) *fragariae* was recorded from Bursa province (Platia & Kovancı, 2005), while *At.* (*O.*) *savsatensis* n. sp. was collected from Artvin province (Figure 9). According to the current status, both species are only known to occur in Turkey and they could reasonably be considered to be endemic to Turkey. On the other hand, the collection locality of the new species was found quite close to border of Georgia, therefore it is possible that it also occurs in Georgia.

Athous (*A.*) *kobachidzei* was described by Dolin & Chantladze (1982). It is clearly distinguishable from *Athous* (*Athous*) *vittatus* Reitter, 1890, which also occurs in Turkey, by the structure of antennae, scutellum and aedeagus. According to Cate (2007), *At.* (*A.*) *kobachidzei* occurs in Azerbaijan, Georgia and Southern European territory of Russia. These areas are close to the collection areas in Turkey (i.e., in Giresun, Gümüşhane and Rize provinces; Figure 9). There are floristic and topographic similarities between its collection area and its known distribution, which could explain the presence of *At.* (*A.*) *kobachidzei* in Turkey.

Agriotes bogatschevi was described by Dolin (1969). Drawings of aedeagi of genus *Agriotes* in the study of Gurjeva (1972) showed that *Ag. bogatschevi* is clearly distinguishable from other species by morphology of the aedeagus (Figure 8), which has a long median lobe and short parameres. In Mardjanian (1987), *Ag. bogatschevi* is given as a synonym of *Agriotes integricollis* Reitter, 1911, whereas, Cate (2007) gave *Agriotes bogatschevi bogatschevi* Dolin, 1969 and *Agriotes bogatschevi rugosus* Gurjeva, 1979. *Agriotes bogatschevi bogatschevi* occurs in Georgia and Southern European

territory of Russia, and *Ag. bogatschevi rugosus* occurs in Azerbaijan and Southern European territory of Russia (Cate, 2007). In this study, it was recorded it in Rize and Trabzon provinces (Figure 9), which could be accepted as a continuation of the Caucasus Region, because floristic and topographic properties of collection area are very similar with the rest of its known distribution.

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Original article (Orijinal araştırma)

Laboratory assessment for biological control of *Tribolium confusum* du Val., 1863 (Coleoptera: Tenebrionidae) by entomopathogenic fungi

Tribolium confusum du Val., 1863 (Coleoptera: Tenebrionidae)'un biyolojik mücadelesinde entomopatojen fungusların kullanımının laboratuvar ortamında değerlendirilmesi

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Summary

This research was carried out at the Plant Protection Department, Agricultural Faculty, Ataturk University (Erzurum, Turkey) in 2016. The objective of this study is to determine using as biological control agent against *Tribolium confusum* du Val., 1863 (Coleoptera: Tenebrionidae) adults of seven entomopathogenic fungal treatments, *Beauveria bassiana*, *Paecilomyces farinosus*, *Isaria fumosorosea*, *Isaria farinosa*, *Lecanicillium muscarium* (2 isolates) and an extract of *L. muscarium*, under laboratory conditions (25±1°C and 75±1% RH). Fungal isolates at two different concentrations ($1x10^5$ and $1×10^7$ conidia/mL) were sprayed on the tested adult insects in Petri dishes. The results demonstrated that the mortality rates of *T. confusum* adults treated with seven entomopathogenic fungi varied from 34.6 to 100% after 10 days of treatment. The entomopathogenic fungi isolates at both $1×10^5$ and $1×10^7$ conidia concentration caused in high mortality levels of *T. confusum* adults. In conclusion, it was observed that tested seven entomopathogenic fungi isolates might have a potential effect to biological control of this stored-product pest.

Keywords: Biological control, entomopathogenic fungi, stored-product insect, *Tribolium confusum*

Özet

Bu çalışma, 2016 yılında Atatürk Üniversitesi, Ziraat Fakültesi, Bitki Koruma Bölümü (Erzurum, Türkiye)'nde yürütülmüştür. Çalışmanın amacı, yedi entomopatojen fungal (*Beauveria bassiana*, *Paecilomyces farinosus*, *Isaria fumosorosea*, *Isaria farinosa*, *Lecanicillium muscarium* (2 izolat) ve *L. muscarium* ekstraktı) izolatlarının, laboratuvar şartlarında **(**25±1°C and 75±1% RH**).** *Tribolium confusum* du Val., 1863 (Coleoptera: Tenebrionidae) erginlerine karşı biyolojik kontrol ajanı olarak kullanımlarını tespit etmektir. Fungus izolatları petri kaplarında test edilen ergin böceklere karşı iki farklı konsantrasyonda ($1x10^5$ ve $1×10^7$ konidi/mL) sprey şekilde uygulanmıştır. Elde edilen sonuçlar, uygulamadan 10 gün sonra yedi entomopatojen fungus izolatı uygulaması ile *T. confusum* erginlerinin ölüm oranlarının %34.6'dan %100'e kadar değiştiğini göstermiştir. Entomopatojen fungus izolatları $1×10^5$ ve $1×10^7$ konsantrasyonlarında *T. confusum* erginlerinde yüksek seviyede ölüme neden olmuştur. Sonuç olarak, test edilen yedi fungal entomopatojenin depolanmış ürün zararlılarının biyolojik mücadelesi için potansiyel etkiye sahip olabileceği gözlemlenmiştir.

Anahtar sözcükler: Biyolojik mücadele, entomopatojen funguslar, depolanmış ürün zararları, *Tribolium confusum*

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Introduction

Each year throughout the world about 10 to 40% of stored cereal grain is qualitatively and quantitatively damaged by insect pests, especially in tropical and subtropical regions of developing or undeveloped countries (Madrid et al., 1990; Shaaya et al., 1997; Tripathi et al., 2009). Stored foods are destroyed by different groups of insect pests, especially by beetles, moths and mites (Rajendran, 2002). Protecting stored grain and seeds against insect pests is a major challenge in post-harvest processes. Since stored-grain insect pests become widespread throughout the world through human activity and seed transportation, they are considered to have evolved adaptations to different stored foodstuffs. One of the most important common and destructive stored-product insects worldwide is confused flour beetles, *Tribolium confusum* du Val., 1863 (Coleoptera: Tenebrionidae) (Aitken, 1975; Hodges et al., 1996). Confused flour beetles have an extremely large appetite for a variety of foods, such as food products stored in soils, warehouses, grocery stores, and houses including meal, crackers, beans, spices, pasta, dried pet food, dried flowers, chocolate, nuts and seeds, and even dried museum specimens (Via, 1999; Weston & Rattlingourd, 2000). Also, they are particularly abundant in cereal products, in wheat and flour (Aitken, 1975; Hodges et al., 1996). When they occur in large number, confused flour beetles secrete a chemical mixture that includes quinones, which are carcinogenic, thereby affecting product quality (Hodges et al., 1996). Generally, the control of this pest species relies on fumigants, phosphine and residual grain protectants. However, fumigation, by far the most effective method of grain and grain-product disinfestation, has serious limitations (Mills, 1983; Taylor, 1989; Bell & Wilson, 1995; Bell, 2000; Caddick, 2004).

Increased concern by consumers over grain protectant (organophosphorus and pyrethroid insecticides and fumigants) residues in processed cereal products, the occurrence of insecticide resistant insect strains (Champ & Dyte, 1976; Zettler, 1991; Arthur & Zettler, 1992; Arthur, 1996; Zettler & Arthur, 1997; APRD, 2016) and the precautions necessary to work with chemical insecticides, call for new approaches to control stored-product insect pests.

Entomopathogenic fungi, as both biological control agents and sources of bioactive compounds active against the insect pests, could provide an alternative to chemical pesticides (Isaka et al., 2005; Monlar et al., 2010), since they have low mammalian toxicity, high effectiveness and a natural origin (Moore et al., 2000). Entomopathogenic fungi as natural enemies of insect pests in different ecosystems have high potential to control pests in agroecosystems (Altieri, 1999; Gurr et al., 2003; Tscharntke et al., 2005; Fiedler & Sosnowska, 2007; Jaronski, 2010; Jaber, 2015). There are approximately 90 genera and 700 species of entomopathogenic fungi known (Roberts & Humber, 1981) and the common species of *Beauveria*, *Metarhizium*, *Lecanicillium* and *Isaria* are quite amenable for mass production. Previous studies have mostly focused on *Beauveria bassiana* (Balsamo) Vuillemin and *Metarhizium anisopliae* (Metschnikoff) Sorokin (Rice & Cogburn, 1999; Moore et al., 2000; Dal Bello et al., 2001; Lord, 2001, 2005; Akbar et al., 2004; Batta, 2004, 2005; Kavallieratos et al., 2006; Michalaki et al., 2006; Vassilakos et al., 2006).

The anamorphic entomopathogenic fungi such as *B. bassiana*, *M. anisopliae*, *Lecanicillium muscarium* (Petch) Zare & W. Gams, *Isaria farinosa* (Holmsk.) Fr. (formerly *Paecilomyces farinosus* (Holmsk.) A.H.S. Br. & G. Sm.), *Isaria fumosorosea* Wize (formerly *Paecilomyces fumosoroseus* (Wize) A.H.S. Br. & G. Sm.), and *Lecanicillium muscarium* (Zimm.) Zare & W. Gams from the order Hypocreales (Ascomycota) are natural enemies of wide range of insect pests, and these fungi may produce enormous numbers of conidia over many asexual life cycles in a single cultivation season (Roberts & St. Leger, 2004; Rehner, 2005; Gurulingappa et al., 2010). Some of the entomopathogenic fungi (e.g., *B. bassiana*) are endophytic symbionts in maize, potato, cotton, date palm, banana and coffee (Jones, 1994; Wagner & Lewis, 2000; Leckie, 2002; Ownley et al., 2004; Arnold & Lewis, 2005; Gómez-Vidal et al., 2006; Akello et al., 2007; Posada et al., 2007), and could control the insect pests after feeding. The grain loss by *Tribolium castaneum* (Herbst, 1797) treated with *B. bassiana* has been studied by Padin et al. (2002). In their study, *B. bassiana* do not show effective control against the *T. castaneum*. *Beauveria bassiana* mixed with diatomaceous earth as a desiccant insecticide had a synergistic effect on the adults of *Rhyzopertha dominica* (Fabricius, 1792) (Lord, 2001).

The objective of this study was to assess the effectiveness of entomopathogenic fungi (*B. bassiana*, *I. farinosa*, *I. fumosorosea*, *L. muscarium*, *P. farinosus*), collected from different locations and infected insects, against *T. confusum* adults under laboratory conditions. A Mycotal extract of *L. muscarium* was used as a positive control.

Materials and methods

Rearing of test insect

Tribolium confusum adults used as test insects were obtained from a laboratory culture maintained at the Plant Protection Department, Agricultural Faculty, Ataturk University, Erzurum, Turkey, which were initially collected from hard wheat (cv. Seval in grain storage) in 2016 and were reared on cracked wheat grains. The adults were kept in cracked wheat grain under laboratory conditions in cloth mesh covered plastic pots (15 cm diameter, 20 cm high) until used in the experiments as newly emerged adults with mixed sex. Each experiment was conducted with three replicates and 25 adults were used for each replicate. The adults were fed with wheat grains in plastic Petri dishes (9 cm) during laboratory bioassay of entomopathogenic fungi.

Entomopathogenic isolates and preparation

Seven entomopathogenic fungi isolates (*Beauveria bassiana* (ARSEF-4984); *Paecilomyces farinosus* (ARSEF-2538); *Isaria fumosorosea* (ARSEF-4501); *Isaria farinosa* (ARSEF-3580); *Lecanicillium muscarium* (ARSEF-972 and ARSEF-5128), Mycotal extract of *Lecanicillium muscarium* (as positive control) and distilled sterile water with Tween 20 (as negative control) were tested against *T. confusum* adults in this study. Fungal isolates were cultivated in potato dextrose agar (PDA, Oxoid, CM0139) medium at 25°C for two weeks before being used to spray *T. confusum* adults. Conidia harvested from 14-day-old cultures were thoroughly mixed in 3 mL distilled sterile water with 12 µL Tween 20 in screw capped bottles. The suspensions were sieved, diluted and 1 mL sprayed on each replicate consisting of the insects, wheat grains and filter paper in Petri dishes. The sprayed Petri dishes were incubated at 25°C and the alive and dead adults were counted every 48 h for 10 days.

Bioassays

Fungal entomopathogenic treatments were applied at 1×10^5 and 1×10^7 conidia/mL sterile distilled water using PET plastic spray bottles. In each Petri dish, 25 adults of *T. confusum* were fed by wheat grains (30 wheat grains/dish) and incubated at 25±1°C and 75±1% RH in a completely dark growth chamber. The mortality of the adults was evaluated at 48-h intervals for 10 days.

Statistical analysis

The differences among insecticidal activities of the seven tested entomopathogenic fungi isolates were determined according to analysis of variance using the SPSS 17.0 software package. Duncan's test was used for comparison between means. The significance of differences between means were determined at $p < 0.05$.

Results

The seven entomopathogenic fungi isolates were tested against *T. confusum* at two concentrations (1×10^5 and 1×10^7 conidia/mL) and compared with controls. The mortality of *T. confusum* adults varied from 34.6% to 100% 10 days after treatment (Table 1). The mortalities of *T. confusum* adults for positive control (Mycotal extract of *L. muscarium*) and negative control (distilled sterile water with Tween 20) were 34.6% and 4% 10 days after treatment, respectively. There were not significant differences in mortality of *T. confusum* adults 6, 8 and 10 days after treatment. The highest mortalities of *T. confusum* adults were observed for *P. farinosus* (ARSEF-2538) with 100% mortality at 1×10^7 conidia/mL and *I. farinosa* (ARSEF-3580) with 97.3% mortality at 1×10^7 conidia/mL, followed by *I. fumosorosea* (ARSEF-4501), *B. bassiana* (ARSEF-4984) and *L. muscarium* (ARSEF-5128) with 94.6% mortality (Table 1). The lowest mortalities were observed for *I. farinosa* (ARSEF-3580) with 37.3% mortality at 1×10^5 conidia/mL and the

positive control with 34.6% mortality. The mortality of *T. confusum* adults differed between the different spore concentrations for one isolate only, *I. farinosa* (ARSEF-3580). However, the mortality rates at 1×10^5 conidia/mL were generally lower than those at 1×10^7 conidia/mL. All the entomopathogenic fungi caused high levels of mortality of *T. confusum* adults (Table 1).

More than 80% mortality of *T. confusum* adults was observed with 1×10^5 conidia/mL of *P. farinosus* (ARSEF-2538), *B. bassiana* (ARSEF-4984), *L. muscarium* (ARSEF-5180) and *L. muscarium* (ARSEF-972) (Figure 1), while *P. farinosus* (PAF-2538), *I. fumosorosea* (ARSEF-4501), *B. bassiana* (ARSEF-4984), *I. farinosa* (ARSEF-3580) and *L. muscarium* (ARSEF-972) at 1×10^7 conidia/mL caused more than 90% mortality of *T. confusum* adults (Figure 2).

Table 1. Mortality of *Tribolium confusum* exposed to two concentrations of six entomopathogenic fungi isolates and controls over 10 days from treatment (DAT)

Entomopathogenic fungi treatment	Dose	Cumulative mortality (%)[a*]				
		2 DAT	4 DAT	6 DAT	8 DAT	10 DAT
Paecilomyces farinosus (ARSEF-2538)	$1x10^7$	69.3 ± 11.3 cba	73.3 ± 11.3 a	92.0 ± 6.11 a	98.6 ± 1.33 a	100 ± 0.0 a
	$1x10^5$	50.6 ± 15.3 dc	70.6 ± 16.2 a	78.6 ± 9.33 a	81.3 ± 9.61 ba	85.3 ± 7.42 ba
Isaria fumosorosea (ARSEF-4501)	$1x10^7$	86.6 ± 13.3 a	90.6 ± 9.33 a	90.6 ± 9.33 a	93.3 ± 6.66 ba	94.6 ± 5.33 a
	$1x10^5$	66.6 ± 10.6 cba	68.0 ± 10.0 a	70.6 ± 8.74 a	72.0 ± 8.0 cb	72.0 ± 8.0 cb
Beauveria bassiana (ARSEF-4984)	$1x10^7$	76.0 ± 14.4 cba	90.6 ± 3.52 a	92.0 ± 4.00 a	94.6 ± 5.33 ba	100 ± 0.0 a
	$1x10^5$	69.3 ± 17.3 cba	78.6 ± 13.5 a	81.3 ± 12.7 a	81.3 ± 12.7 ba	89.3 ± 8.74 ba
Lecanicillium muscarium (ARSEF-972)	$1x10^7$	81.3 ± 10.6 ba	88.0 ± 10.0 a	88.0 ± 10.0 a	89.3 ± 10.6 ba	90.6 ± 9.33 ba
	$1x10^5$	82.6 ± 11.8 ba	88.0 ± 12.0 a	88.0 ± 12.0 a	88.0 ± 10.0 ba	88.0 ± 10.0 ba
Isaria farinosa (ARSEF-3580)	$1x10^7$	90.6 ± 1.33 a	92.0 ± 0.0 a	93.3 ± 1.33 a	94.6 ± 1.33 ba	97.3 ± 2.66 a
	$1x10^5$	10.6 ± 2.66 fe	13.3 ± 2.66 c	30.6 ± 14.8 cb	90.6 ± 1.33 a	37.3 ± 15.3 d
Lecanicillium muscarium (ARSEF-5128)	$1x10^7$	58.6 ± 16.7 cb	85.3 ± 12.8 a	89.3 ± 5.81 a	90.6 ± 1.33 a	94.6 ± 5.33 a
	$1x10^5$	28.0 ± 2.30 ed	68.0 ± 14.0 a	76.0 ± 10.0 a	81.3 ± 7.42 ba	86.6 ± 7.05 ba
Positive control (*L. muscarium* extract)	$1x10^7$	22.6 ± 3.52 fe	38.6 ± 14.8 b	49.3 ± 11.3 b	58.6 ± 13.10 c	62.6 ± 13.9 c
	$1x10^5$	6.66 ± 2.66 fe	17.3 ± 2.66 cb	26.6 ± 7.05 c	29.3 ± 7.42 d	34.6 ± 7.05 d
Negative control (Tween20+sterile water)	-	0.0 ± 0.0 f	0.0 ± 0.0 c	1.33 ± 1.11 d	3.5 ± 0.78 e	4.0 ± 0.0 e

[a] Mean ± SE of three replicates, each consisting of 25 adults.
[*] Values followed by different letters in the same column differ significantly at $p < 0.05$ according to Duncan Multiple test.

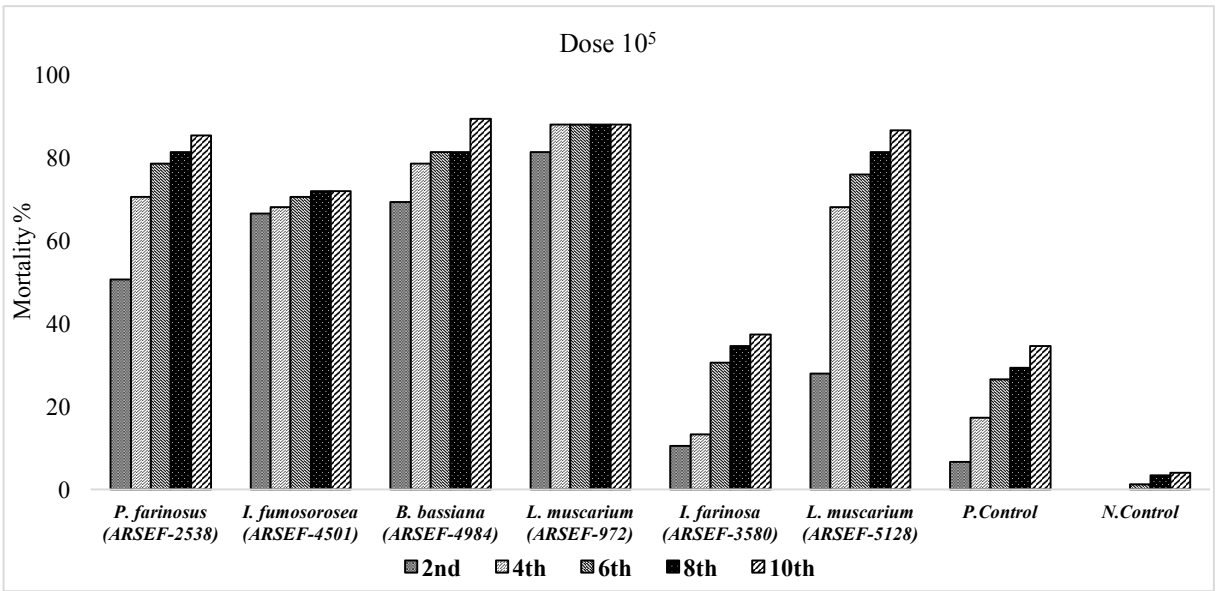

Figure 1. Mortality (%) of *Tribolium confusum* adults exposed to different entomopathogenic fungi isolates at 1×10^5 conidia/mL 2, 4, 6, 8 and 10 days of treatment (ANOVA; $p < 0.05$). The negative control was sterile distilled water with Tween 20 and the positive control a Mycotal extract of *Lecanicillium muscarium*.

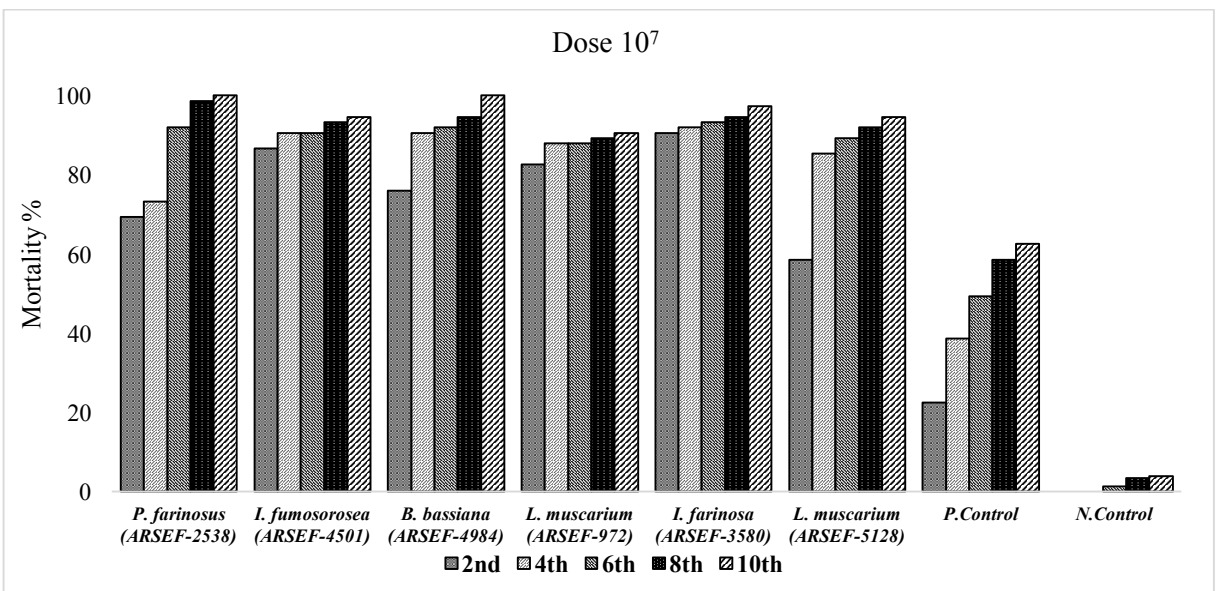

Figure 2. Mortality (%) of *Tribolium confusum* exposed to different entomopathogenic fungi isolates at 1×10^7 conidia/mL 2, 4, 6, 8 and 10 days of treatment (ANOVA test; $p < 0.05$). The negative control was sterile distilled water with Tween 20 and the positive control a Mycotal extract of *Lecanicillium muscarium*.

Discussion

This study determined the mortality of *T. confusum* adults 10 days after exposure to five species of different entomopathogenic fungi. The pathogenicity of *B. bassiana*, *P. farinosus*, *I. fumosorosea*, *I. farinosa*, *L. muscarium* (2 isolates) to beetles was demonstrated by spraying *T. confusum* adults with conidia under laboratory conditions. Mortality of *T. confusum* adults was high, ranging from 37% to 100% across the different entomopathogenic fungi. Specifically, the adult mortalities were 37% with *I. farinosa* (ARSEF-3580) and 89.3% with *B. bassiana* (ARSEF-4984) at $1x10^5$ conidia/mL, and 90.6% with *L. muscarium* (ARSEF-972) and 100% with *B. bassiana* (ARSEF-4984) and *P. farinosus* (ARSEF-2538) at $1x10^7$ conidia/mL.

Many studies indicate that entomopathogens which occupy plant tissues and insects have the potential to interact with insect pest in diverse ways. Entomopathogenic fungi may produce conidia on the plants, where they may contact insects. The fungal metabolites via consumption of plant materials or on the leaf surfaces have the potential to control pest insects. Thus, the role of entomopathogenic fungi as biological control agents in pest management requires further consideration. Inclusion of entomopathogens in IPM appears to be an obvious approach to take advantage of the potential of these fungi. While a number of questions remain to be clarified, published research has demonstrated the potential for the use of entomopathogens in IPM (Padin et al., 1997; Barra et al., 2013).

Recently the use of fungal entomopathogens against grain pests has been gained increasing attention throughout the world and researchers continue to seek highly pathogenic fungal isolates for controlling stored-product insects. In this regard, *Tribolium* species appear as a particularly good candidate for biocontrol by entomopathogenic fungi as was indicated by the survey of Wakil et al., (2014). *Metarhizium anisopliae* inhibited *Sitophilus oryzae* (L., 1763) (Coleoptera: Dryophthoridae) by 73.3% to 86.7% (Batta, 2004). Padin et al. (2002) investigated the insecticidal effects of *B. bassiana* on *T. castaneum, Acanthoscelides obtectus* (Say, 1831) (Coleoptera: Chrysomelidae) and *S. oryzae* by exposing pest-infested wheat and bean seeds to conidia of *B. bassiana* over a long period. In that study, *S. oryzae* was significantly affected from *B. bassiana*, but the other species were not significantly affected after four months. In the present study, *B. bassiana* (ARSEF-4984) had up to 94% mortality four days after treatments with no subsequent increase in mortality increase, which may indicate a rapid decline in efficiency of *B. bassiana* conidia. Contrary to current findings, Rice & Cogburn (1999), recorded a lower efficiency with another *B. bassiana* isolate (22292A); only 31.5% mortality, of *T. castaneum* adult was achieved on 14 days after treatment. Although these differences may be attributed to differences in methods used, there is also likely variation in pathogenicity of different isolates of the fungus was a contributing factor (Zettler, 1991).

Based on the findings of the present study, all isolates performed better at higher dosage 10 days after treatment causing mortality of over 90%. The increasing trend observed in mortalities (with the exception of *B. bassiana*) throughout the experiment is also considered as a good indication of preserved pathogenicity. Among the isolates tested, *I. farinosa* (ARSEF-3580) and *I. fumosorosea* (ARSEF-4501) particularly gave high mortalities from the beginning of experiment. Similarly, *P. farinosus* gave a consistent increase in mortality and had kill all adults by 10 days after treatment. In conclusion, based on their high pathogenicity, these three isolates are considered as good candidates for biocontrol agents against *T. confusum* adults.

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Original article (Orijinal araştırma)

Identification and distribution of root-knot nematode species (*Meloidogyne* spp.) in vegetable growing areas of Lakes Region in Turkey[1]

Türkiye Göller Bölgesi sebze üretim alanlarında kök-ur nematodu türleri (*Meloidogyne* spp.)'nin tanılanması ve yaygınlıkları

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Summary

In this study, the distribution and characterization of root-knot nematode species collected from intensively vegetable growing areas of Lakes Region were determined by morphological, molecular and North Carolina Differential Host Test between September 2014 and December 2015. A total of 160 samples were collected and 83 (51.8%) were found to be infested with root-knot nematodes. Each population was cultured from a single egg mass taken from galled roots and multiplied on the susceptible tomato cv. Tueza F1. Sixty-eight populations were morphologically identified based on perineal patterns and morphometrics of second stage juveniles, and molecularly determined using species specific primers. Of the 68 populations analyzed, 66 were identified as *Meloidogyne incognita* (25), *M. hapla* (22), *M. javanica* (18) and *M. arenaria* (1), and two populations were not identified. The incidence of *M. incognita*, *M. hapla*, *M. javanica* and *M. arenaria* was 36.7, 32.3, 36.5 and 1.5%, respectively. According to the differential host test, *M. incognita* races 2, 4 and 6 and *M. javanica* races 1 and 3 were determined. This was the first detection of *Meloidogyne javanica* race 3 in Turkey. Eighty four percent of the *M. incognita* populations were found in microclimatic areas with altitudes of up to 800 m, while 16% were found at altitudes between 800 and 1035 m. Some *M. javanica* populations (17%) were found in high plateau fields in this region, whereas most (83%) were found in lowlands. In contrast, the large majority of *M. hapla* populations (91%) of were found in cool, high altitude areas with sandy soils, whereas only 9% of *M. hapla* populations were in lowlands.

Keywords: Lakes Region, *Meloidogyne* spp., molecular identification, morphological identification, PCR, race

Özet

Çalışmada, Göller Bölgesi'nde yoğun sebze üretimi yapılan alanlarda, Eylül 2014 – Aralık 2015 yılları arasında toplanan kök-ur nematodu türleri morfolojik, moleküler ve Kuzey Karolina Konukçu Testi yöntemleri kullanılarak karakterize edilmiş ve yayılışları belirlenmiştir. Toplam 160 adet örnek alınmış ve 83 tanesinin (%51.8) kök-ur nematodu ile bulaşık olduğu bulunmuştur. Urlu kök örneklerinden tek yumurta paketi alınarak duyarlı 'Tueza F1' domates çeşidinde her popülasyonun saf kültürleri oluşturulmuş ve kitle üretimleri yapılmıştır. Altmış sekiz popülasyonun tür tanımlamaları morfolojik olarak dişi bireylerin perineal bölge modelleri ve ikinci dönem larva ölçümlerinden ve moleküler olarak türe özgü spesifik primerler kullanılarak yapılmıştır. Tanımlanan 68 kök-ur nematodu popülasyonundan 25 adedi *M. incognita*, 22 adedi *M. hapla*, 18 adedi *M. javanica* ve 1 adedi de *M. arenaria* olarak tespit edilmiş, iki popülasyonun ise tanılamaları yapılamamıştır. Türlerin yaygınlık oranları sırasıyla, %36.7, %32.3, %26.5 ve %1.5 olarak belirlenmiştir. Konukçu testine göre, *M. incognita*'nın ırk 2, ırk 4 ve ırk 6, *M. javanica*'nın ise ırk 1 ve ırk 3'ü belirlenmiş ve *M. javanica* ırk 3 Türkiye'de ilk kez rapor edilmiştir. *Meloidogyne incognita* popülasyonlarının %84'ü 800 m yükseltiye sahip mikroklima bölgelerde bulunurken, %16'sı 800-1035 m yükseltiye sahip örtü altı alanlarda tespit edilmiştir. Bazı *M. javanica* populasyonları (%17) yüksek yayla bölgelerinde tespit edilirken, çoğu (%84) alçak yükseltiye sahip sebze alanlarında bulunmuştur. Buna karşılık *M. hapla* popülasyonlarının büyük çoğunluğu (%91) daha yüksek serin bölgelerde ve kumsal toprak yapısına sahip alanlarda bulunurken, sadece %9 'u alçak yükseltiye sahip bölgelerde bulunmuştur.

Anahtar sözcükler: Göller Bölgesi, *Meloidogyne* spp., moleküler tanılama, morfolojik tanılama, PCR, ırk

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Introduction

Root-knot nematodes are one of the most devastating plant parasitic nematodes, affecting yield and quality of many crops, particularly causing economically significant losses in vegetable production. Blok et al. (2008) reported that the annual damage to vegetables from root-knot nematodes amounts to more than €80 billion. Lamberti (1978) and Davis & May (2005) reported that in tropical and subtropical climatic regions, root-knot nematodes caused yield loss of 47 29, 23, 22 and 15% in tobacco, tomato, eggplant, okra and pepper, respectively. Similarly, Netscher & Sikora (1990) reported that root-knot nematodes caused yields losses of 42-54% in tomatoes and 30-60% in eggplants. These nematodes also cause significantly economic loses in Turkey. Ağdacı (1978) found root-knot nematodes causing 17-47% loss in cucumber greenhouses in Antalya and Adana. Söğüt & Elekcioğlu (2007) reported that yield losses of about 80% in pepper greenhouses in Adana.

More than 90 root-knot nematode species have been identified across the world (Hunt & Handoo, 2009; Moens et al., 2009). Johnson & Fassuliotis (1984) that across 75 countries, 1000 root-knot nematode populations were 52% *Meloidogyne incognita* (Kofoid & White, 1919) Chitwood, 1949, 30% *Meloidogyne javanica* (Treub, 1885) Chitwood, 1949, 8% *Meloidogyne arenaria* (Neal, 1889) Chitwood, 1949, 8% *Meloidogyne hapla* Chitwood, 1949 and the remaining 2% other species. Similarly, root-knot nematodes are one of main groups of pests in the vegetable growing areas of the Mediterranean, Aegean and Black Sea Regions of Turkey. *Meloidogyne incognita* and *M. javanica* were dominant species in coastal regions of Turkey. Also, *M. arenaria, M hapla , Meloidogyne chitwoodi* Golden, O'Bannon, Santo & Finley, 1980, *Meloidogyne ethiopica* Whitehead, 1968, *Meloidogyne artiellia* Franklin, 1961 and *Meloidogyne exigua* Goeldi, 1892 have been found in Turkey (Elekcioğlu et al., 1994; Mennan & Ecevit, 1996; Kaşkavalcı & Öncüer, 1999; Söğüt & Elekçioğlu, 2000; Devran & Söğüt, 2009; Özarslandan et al., 2009; Akyazı & Ecevit, 2010; Yıldız & Elekçioğlu, 2011; Aydınlı et al., 2013; İmren et al., 2014; Kepenekçi et al., 2014). In the recent years, *M. chitwoodi* has been identified in potato producing areas in the Central Anatolia Region as an invasive species and *M. ethiopica* was identified in vegetable crops in the Black Sea Region (Aydınlı & Mennan, 2016; Evlice & Bayram, 2016). Particular *Meloidogyne* species have races distinguishable by a standardized set of differential hosts according to North Carolina Differential Host Test (Sasser & Triantaphyllou, 1977). This differential host test distinguishs four races of *M. incognita,* two races of *M. javanica* and two races of *M. arenaria* (Hartman & Sasser, 1985; Decker & Fritzsche, 1991). Carneiro et al. (2004) and Robertson et al. (2009) found new races of these common species in Brazil and Spain. Devran & Söğüt (2011) determined the new races of *M. incognita* (race 6) and *M. arenaria* (races 2 and 3) in the western coastal areas of the Mediterranean Region of Turkey.

Accurate identification of root-knot nematode species and races is important for management and control of these pests through host resistance, biological management and crop rotation. The Lakes Region of Turkey has agricultural areas with different geographic characteristics and altitudes from 275 to 1430 m in Burdur and Isparta Provinces. Vegetables can be grown twice a year in locations such as Çandır, Yeşilyurt, Elsazı and Çamlık with relatively low altitudes. Vegetables are also grown in other microclimatic areas at higher altitudes. The total production of vegetables, including bean, cucumber, eggplant, okra, sweet pepper and tomato, is about 283 kt/yr (TUIK, 2014). Thus, this region has a significant role for vegetables production in Turkey, particularly in of summer and early autumn. Root-knot nematodes infest crops in the region and are a major concern for farmers.

The purpose of study was to identify root-knot nematode species in vegetable growing areas of Lakes Region of Turkey using morphology, molecular methods and differential host test and to determine their distribution.

Material and Methods

Sampling and culturing of root-knot nematode populations

A total of 160 samples were collected from vegetable growing areas of Lakes Region between September and October, 2014. Samples consisted of about 19% open fields and 81% greenhouses. Between five and 20 plants were uprooted and average five root systems and soil samples were collected at each location. The number of samples each town were determined according to the size of the vegetable production areas reported by TUIK (2014). Locations infested with root-knot nematodes host plants, geographic coordinates, and altitudes are given in Table 1.

Table 1. Root-knot nematode populations and their host plants, production system, location, coordinates and altitudes from samples collected in Lakes Region of Turkey

Code	Host Plant	Production system	Location	Latitude (N)	Longitude (E)	Altitude (m)
B6	Eggplant (*Solanum melongena* L.)	Greenhouse	Askeriye/Burdur	37° 45' 54.2"	30° 21' 22.3"	960
B7	Pepper (*Capsicum annuum* L.	Greenhouse	Askeriye/Burdur	37° 45' 30.5"	30° 19' 54.0"	923
B10	Tomato (*Lycopersicon esculentum* Mill.)	Greenhouse	Askeriye/Burdur	37° 45' 18.2"	30° 19' 30.9"	925
B11	Pepper (*C. annuum*)	Greenhouse	Askeriye/Burdur	37° 45' 24.2"	30° 17' 21.4"	877
B12	Eggplant (*S. melongena*)	Greenhouse	Askeriye/Burdur	37° 45' 24.4"	30° 17' 19.2"	875
B13	Tomato (*L. esculentum*)	Greenhouse	Askeriye/Burdur	37° 45' 24.4"	30° 17' 19.2"	875
B15	Pepper (*C. annuum*)	Field	Elsazı/Burdur	37° 26' 34.0"	30° 47' 10.5"	276
B16	Cucumber (*Cucumis sativus* L.)	Greenhouse	Elsazı/Burdur	37° 26' 34.0"	30° 47' 10.5"	276
B18	Cucumber (*C. sativus*)	Greenhouse	Elsazı/Burdur	37° 26' 35.5"	30° 47' 09.6"	275
B19	Eggplant (*S. melongena*)	Field	Elsazı/Burdur	37° 27' 14.2"	30° 48' 46.4"	308
B22	Cucumber (*C. sativus*)	Greenhouse	Elsazı/Burdur	37° 26' 51.8"	30° 47' 12,8"	276
B23	Cucumber (*C. sativus*)	Greenhouse	Elsazı/Burdur	37° 26' 54.4"	30° 48' 32.5"	284
ISP28	Eggplant (*S. melongena*)	Greenhouse	Elsazı/Burdur	37° 27' 55.8"	30° 47' 45.1"	288
ISP30	Eggplant (*S. melongena*)	Greenhouse	Elsazı/Burdur	37° 26' 55.8"	30° 48' 31.8"	287
B24	Tomato (*L. esculentum*)	Greenhouse	Söğüt/Burdur	37° 01' 13.9"	29° 49' 13.1"	1430
B25	Tomato (*L. esculentum*)	Greenhouse	Söğüt/Burdur	37° 01' 03.9"	29°49' 24.6"	1433
B26	Tomato (*L. esculentum*)	Greenhouse	Söğüt/Burdur	37° 01' 05.4"	29°49' 20.5"	1434
B27	Tomato (*L. esculentum*)	Greenhouse	Söğüt/Burdur	37° 00' 54.0"	29° 49' 24.5"	1436
Ç4	Cucumber (*C. sativus*)	Greenhouse	Çamlık/Burdur	37° 29' 24.3"	30° 45' 26.4"	366
Ç5	Cucumber (*C. sativus*)	Greenhouse	Çamlık/Burdur	37° 29' 08.4"	30° 45' 30.4"	350
Ç7	Eggplant (*S. melongena*)	Field	Çamlık/Burdur	37° 29' 00.6"	30° 45' 37.8"	341
Ç8	Bean (*Phaseolus vulgaris* L.)	Greenhouse	Çamlık/Burdur	37° 28' 19.8"	30° 45' 34.7"	327
Ç9	Lettuce (*Lactuca sativa* L.)	Greenhouse	Çamlık/Burdur	37° 28' 38.3"	30° 44' 57.2"	360
Ç11	Cucumber (*C. sativus*)	Greenhouse	Çamlık/Burdur	37° 29' 01.0"	30° 45' 46.0"	338
Ç12	Cucumber (*C. sativus*)	Greenhouse	Çamlık/Burdur	37° 28' 57.9"	30° 45' 53.1"	339
ISP29	Tomato (*L. esculentum*)	Greenhouse	Çamlık/Burdur	37° 28' 37.5"	30° 45' 39.5"	325
ISP31	Cucumber (*C. sativus*)	Greenhouse	Çamlık/Burdur	37° 28' 18.5"	30° 45' 17.3"	353
ISP32	Tomato (*L. esculentum*)	Greenhouse	Çamlık/Burdur	37° 28' 55.7"	30° 45' 36.3"	350
E1	Tomato (*L. esculentum*)	Field	Eğirdir/Isparta	37° 55' 14.1"	30° 46' 25.0"	930
ISP1	Pepper (*C. annuum*)	Field	Yeşilyurt/Isparta	37° 31' 57.2"	30° 51' 42.1"	602
ISP3	Bean (*P. vulgaris*)	Greenhouse	Yeşilyurt/Isparta	37° 31' 58.8"	30° 51' 42.0"	607
ISP5	Eggplant (*S. melongena*)	Greenhouse	Yeşilyurt/Isparta	37° 31' 54.6"	30° 51' 46.7"	595
ISP6	Okra (*Abelmoschus esculentus* (L.) Moench)	Field	Yeşilyurt/Isparta	37° 31' 54.6"	30° 51' 45.2"	595
ISP40	Pepper (*C. annuum*)	Greenhouse	Yeşilyurt/Isparta	37° 29' 14.7"	30° 52' 44.1"	451

Table 1. (Continued)

Code	Host Plant	Production system	Location	Latitude (N)	Longitude (E)	Altitude (m)
ISP44	Eggplant (*S. melongena*)	Greenhouse	Yeşilyurt/Isparta	37° 32' 00.5"	30° 51' 46.7"	676
ISP55	Tomato (*L. esculentum*)	Greenhouse	Yeşilyurt/Isparta	37° 31' 54.6"	30° 51' 46.7"	596
ISP77	Cucumber (*C. sativus*)	Greenhouse	Yeşilyurt/Isparta	37° 31' 53.3"	30° 51' 31.8"	599
ISP78	Tomato (*L. esculentum*)	Greenhouse	Yeşilyurt/Isparta	37° 31' 53.3"	30° 51' 31.8"	600
ISP15	Cucumber (*C. sativus*)	Greenhouse	Çandır/Isparta	37° 27' 12.4"	30° 53' 12.3"	289
ISP16	Tomato (*L. esculentum*)	Greenhouse	Çandır/Isparta	37° 26' 13.6"	30° 53' 35.7"	275
ISP17	Cucumber (*C. sativus*)	Greenhouse	Çandır/Isparta	37° 26' 13.6"	30° 53' 35.7"	275
ISP18	Okra (*A. esculentus*)	Greenhouse	Çandır/Isparta	37° 26' 01.3"	30° 53' 40.4"	286
ISP21	Okra (*A. esculentus*)	Field	Çandır/Isparta	37° 26' 02.3"	30° 53' 40.0"	286
ISP22	Pepper (*C. annuum*)	Field	Çandır/Isparta	37° 26' 03.5"	30° 53' 44.7"	302
ISP151	Pepper (*C. annuum*)	Greenhouse	Çandır/Isparta	37° 27' 12.4"	30° 53' 12.3"	357
ISP11	Eggplant (*S. melongena*)	Greenhouse	Şeyhler/Isparta	37° 28' 05.2"	30° 52' 43.0"	321
ISP14	Eggplant (*S. melongena*)	Greenhouse	Şeyhler/Isparta	37° 27' 49.4"	30° 53' 00.2"	303
ISP141	Tomato (*L. esculentum*)	Greenhouse	Şeyhler /Isparta	37° 27' 49.4"	30° 53' 00.2"	359
ISP41	Cucumber (*C. sativus*)	Greenhouse	Şeyhler/Isparta	37° 27' 49.4"	30° 53' 00.2"	303
ISP42	Tomato (*L. esculentum*)	Field	Kuleönü/Isparta	37° 53' 06.8"	30° 38' 53.4"	941
ISP43	Tomato (*L. esculentum*)	Field	Atabey/Isparta	37° 56' 19.1"	30° 40' 50.3"	1036
ISP45	Eggplant (*S. melongena*)	Greenhouse	Atabey /Isparta	37° 56' 19.1"	30° 40' 50.3"	993
ISP47	Eggplant (*S. melongena*)	Field	Atabey /Isparta	37° 56' 24.9"	30° 40' 04.4"	993
ISP23	Tomato (*L. esculentum*)	Greenhouse	Deregümü/Isparta	37° 46' 55.8"	30° 30' 26.6"	1110
DR2	Tomato (*L. esculentum*)	Greenhouse	Deregümü/Isparta	37° 47' 49.4"	30° 30' 35.6"	1080
DR8	Tomato (*L. esculentum*)	Greenhouse	Deregümü/Isparta	37° 47’ 35.5"	30° 30’ 16.9"	1077
DR14	Tomato (*L. esculentum*)	Greenhouse	Deregümü/Isparta	37° 47' 49.4"	30° 30' 35.6"	1066
DR15	Tomato (*L. esculentum*)	Greenhouse	Deregümü/Isparta	37° 47' 46.6"	30° 30' 40.1"	1074
DR16	Tomato (*L. esculentum*)	Greenhouse	Deregümü/Isparta	37° 47' 42.9"	30° 30' 43.5"	1075
DR17	Eggplant (*S. melongena*)	Greenhouse	Deregümü/Isparta	37° 47' 43.5"	30° 30' 47.2"	1075
DR20	Eggplant (*S. melongena*)	Field	Deregümü/Isparta	37° 47' 35.6"	30° 30' 59.5"	1074
DR21	Tomato (*L. esculentum*)	Greenhouse	Deregümü/Isparta	37° 47' 35.6"	30° 30' 59.5"	1074
DR23	Tomato (*L. esculentum*)	Greenhouse	Deregümü/Isparta	37° 47' 31.6"	30° 31' 06.7"	1071
DR29	Tomato (*L. esculentum*)	Greenhouse	Deregümü/Isparta	37° 47' 41.7"	30° 31' 26.3"	1055
DR30	Tomato (*L. esculentum*)	Greenhouse	Deregümü/Isparta	37° 47' 28.1"	30° 30' 57.9"	1076
DR31	Tomato (*L. esculentum*)	Greenhouse	Deregümü/Isparta	37° 47' 30.0"	30° 30' 25.5"	1090
DR33	Tomato (*L. esculentum*)	Greenhouse	Deregümü/Isparta	37° 47' 27.8"	30° 30' 24.5"	1090
DR35	Pepper (*C. annuum*)	Field	Deregümü/Isparta	37° 47' 39.4"	30° 30' 30.3"	1083

Galled roots were gently washed with tap water and an egg mass was collected using needle and placed in Eppendorf tubes under a stereomicroscope. These egg masses were surface-sterilized for a short period in 0.5% NaOCl, rinsed in tap water three times and prepared for inoculation.

Susceptible tomato seedlings with 5-6 true leaves (cv. Tueza F1; Multi Tohum, Antalya Turkey) were transplanted into 250 ml pots containing a mixture of 68% sand, 21% silt and 11% clay soil autoclaved at 121°C for 40 min. Single egg masses were inoculated in to a hole 2-3 cm deep near each tomato seedlings five days after the transplantation. The assay was conducted at 25±1°C and 65±5% RH, with a 16:8 h L:D photoperiod in a controlled environment chamber. Five single-egg-mass cultures were established for each population. Eight weeks after the inoculation, plants were uprooted and the most developed selected for multiplication in pure culture.

Morphological identification

Perineal patterns: Mature root-knot nematode females were removed from galled tomato roots using needles and forceps under a stereomicroscope. Perineal regions were cut in 45% lactic acid and permanently mounted in glycerin (Hooper, 1986). Species level identification was made by one of us (İHE) according to Jepson (1987) and Karssen (2002).

Morphometric measurements of second stage juveniles: Second stage juveniles (J2) from the pure culture populations were fixed in TAF fixative and made permanently mounted according to Seinhorst (1959). About 15-20 J2s were placed on each slide and measured morphometrically according to Karssen (2002).

Microscopic examination and image analysis were done with a Leica DM 2500 light microscope and Leica Application Suite Software Version 4.1.0 program.

Differential host test: The host races was determined according to North Carolina Differential Host Test (Sasser & Triantaphyllou, 1977). Each of five standardized host cultivars were inoculated with an average of 2000 J2 and eggs from each pure cultured population: *Nicotiana tabacum* L. cv. NC 95; *Gossypium hirsutum* L. cv. Deltapine 61, *Capsicum frutescens* L. cv. California Wonder, *Arachis hypogaea* L. cv. Florunner and *Lycopersicon esculentum* Mill. cv. Tueza F1. Experiments were conducted with four replicates of each differential host. The plants were maintained in a growth room at 25±1°C with a 16:8 h L:D photoperiod for after 60 days from inoculation. Plants were harvested and roots washed with tap water. Galls and egg mass indices were determined on a 0 to 5 scale according to Hartman & Sasser (1985). Each host cultivar was classified as resistant or susceptible, when the average number of galls and egg masses per root system was 0-2 or 3-5, respectively.

Molecular identification

DNA isolation of root-knot nematode populations was obtained from twenty egg masses using a DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). The final DNA samples extracted from each sample were in 100 µl of AE buffer and kept -20°C until PCR procedures for identification.

For molecular identification of root-knot nematode species, the primer pairs INCK 14R/INCK 14F, FJAV/RJAV, FAR/RAR were optimized by Devran & Söğüt (2009) with major root-knot nematode populations in the western Mediterranean Region of Turkey, and *M. hapla* specific primers, JMV1, JMV2 and JMV hapla, were used for *M. incognita*, *M. javanica*, *M. arenaria* and *M. hapla*, respectively (Table 2). A total of 25 µl PCR reaction was conducted by thermocycler (Veriti Thermal cycler, Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA). Reaction mixture consisted of 10 ng DNA (5 µl), PCR buffer (2.5 µl), 2 mM $MgCl_2$ (1 µl), 0.2 mM dNTP (1 µl), 10 mM Primer F (1 µl), 10 mM Primer R (1 µl), 1 unit Taq DNA polymerase (GenEon, San Antonio, TX, USA) (0.25 µl) and ddH_2O (13.25 µl).

The PCR cycles are described in Table 3. PCR products were visualized by agarose electrophoresis in 2% gel (Agarose Type I, Sigma-Aldrich, St. Louis, MO, USA) using EtBr as a fluorescent dye at 90 V for 2 h. The size of PCR fragments giving the DNA bands corresponding to different *Meloidogyne* spp. are listed in Table 2.

Table 2. Species specific primers of root-knot nematodes (*Meloidogyne* spp.) for molecular identification in the study

Species	Primers	Primer sequences (5'-3')	Fragments (bp)	Reference
M. arenaria	Far	TCGGCGATAGAGGTAAATGAC	420	Zijlstra et al., 2000
	Rar	TCGGCGATAGACACTACAACT		
M. javanica	Fjav	GGTGCGCGATTGAACTGAGC	670	Zijlstra et al., 2000
	Rjav	CAGGCCCTTCAGTGGAACTATAC		
M. incognita	INCK14R	CCCGCTACACCCTCAACTTC	399	Randig et al., 2002
	INCK14F	GGGATGTGTAAATGCTCCTG		
M. hapla	JMV1	GGATGGCGTGCTTTCAAC	440	Wishart et al., 2002
	JMV2	TTTCCCCTTATGATGTTTACCC		
	JMV hapla	AAAAATCCCCTCGAAAAATCCACC		

Table 3. Root-knot nematode primers and their PCR cycles

INCK14F/INCK14R and Fjav/Rjav	Far/Rar	JMV1/JMV2/JMV hapla	
94°C 3 min	94°C 3 min	94°C 3 min	
94°C 30 s 60°C 30 s 72°C 60 s	94°C 30 s 56°C 30 s 72°C 60 s	94°C 30 s 48°C 30 s 72°C 2 min	35 cycles
72°C 7 min	72°C 7 min	72°C 7 min	

Results and Discussion

Morphological identification

Root-knot nematodes were detected in 83 soil and root samples and 68 populations were cultured and multiplied. Fifteen populations did not multiply on the tomatoes. Of the 68 root-knot nematode populations, 25 were identified as *M. incognita*, 18 as *M. javanica*, 22 as *M. hapla* and one as *M. arenaria* by perineal patterns. However, the two pure cultures could not be identified by their perineal patterns.

Tail length, hyaline terminus length, stylet length, distance of DGO from the stylet base of J2 are the most important morphometric characters for identification of *Meloidogyne* spp. (Whitehead, 1968; Eisenback et al., 1981; Jepson, 1987; Karssen, 2002). However, Kaur & Attri (2013) showed that body length, stylet length, head to median bulb length, tail length, c and c' ratios of J2 for *M. incognita* were highly variable from different host plants and district in India. Table 4 shows means of morphometric measurements of J2 for three root-knot nematode species in the Lakes region. J2 of *M. arenaria* could not measure because no population of this species was cultured.

Meloidogyne incognita: Although there were small differences between some isolates, all perineal patterns of the 25 populations showed typical *M. incognita* features. The perineal region generally had an angularly oval structure with a high dorsal arch in a typical pyriform and typically inverted-V shape formed by striae in the dorsal to the tail. Striae were in distinct waves which bent towards the lateral lines and were not interrupted. Lateral fields were not distinct. Striae were straighter with an oval appearance in ventral region (Figure 1). All perineal pattern features for *M. incognita* isolates were similar to those described as Jepson (1987).

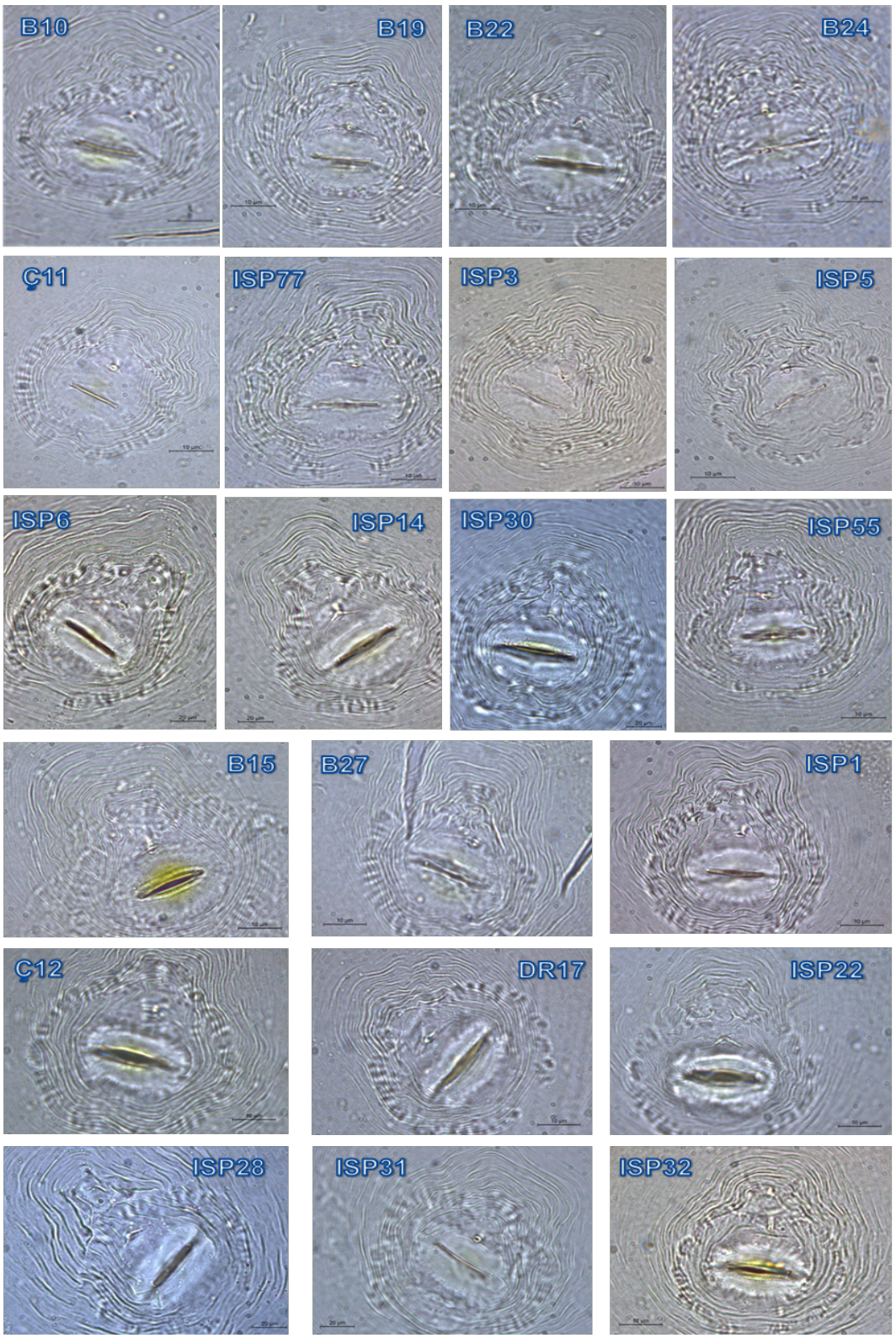

Figure 1. Perineal patterns of *Meloidogyne incognita* isolates collected from Lakes Region of Turkey. Bar: 10 µm.

Table 4 give some morphometric measurements of J2 of *M. incognita* in the study. J2 had the longest tail length (57.6 µm) compared to *M. javanica* and *M. hapla* (Table 4). This value was longer than in reported by Jepson (1987), Özarslandan (2009) and Kaur & Attri (2013). Hyaline terminus length was the smallest among three root-knot nematode species (Table 4) and similar to measurements of Jepson (1987) and Özarslandan (2009). The stylet length (13.3 µm) and DGO to stylet knob distance (3.4 µm) of the J2 were longer than in described by Whitehead (1968) and Özarslandan (2009), but, stylet length was shorter than Indian populations in described by Kaur & Attri (2013).

Meloidogyne javanica: Distinct lateral fields formed by double incisures are typically clear in the perineal patterns of *M. javanica* (Eisenback et al., 1981; Jepson, 1987). Similarly, perineal patterns of all *M. javanica* populations in our study show clear double lateral lines separating dorsal and ventral regions (Figure 2). Also, *M. javanica* had a general oval or oval to pyriform with a medium height and occasionally compressed dorsal arch in perineal regions. There was no transverse striae between vulva and anus in our patterns of populations (Figure 2).

Hyaline terminus length of J2 (14.40 µm) was markedly longer than the other two species and tail length was moderately long (55.2 µm) (Table 4). These lengths were in agreement with Jepson (1987), but longer than reported by Özarslandan (2009). In our study, stylet length was markedly longer than in measured by Özarslandan (2009), whereas DGO to stylet knob distance (3.4 µm) was similar to that reported by Özarslandan (2009) (Table 4).

Meloidogyne hapla: The concentration of punctuations between anus and tail terminus is the most characteristic feature of the *M. hapla* perineal pattern (Eisenback et al., 1981; Jepson, 1987). Likewise, in our study, there were punctuations with a stippled area between the anus and tail terminus in the patterns of *M. hapla* isolates (Figure 3). Some specimens (DR21 and ISP78) in Figure 3 had punctuations entirely in tail terminal area, which might have been an artifact of the fixation or preparation process. All cross-sectioned perineal patterns were roughly oval, regularly spaced with smooth and softly waved striae and with low dorsal arch. Lateral lines clearly appeared in softly irregular lined structure leading to outward from the punctuations as stated by Jepson (1987). The striae of the ventral and lateral regions intersected on one or both sides to become elongated and have wing shaped structure (Figure 3). Additionally, in our study, the physical appearance and position of galls on roots can helped in the diagnosis for *M. hapla*. Similarly, the relatively small and irregular galls of *M. hapla* often had lateral roots as described by Hunt & Handoo (2009).

The body length of the J2 (380.5 µm) was shorter than for *M. incognita* and *M. javanica* J2 (Table 4). In our study, tail length and hyaline terminus length of the J2 were relatively short compared to those reported by Chitwood (1949) and Jepson (1987). The DGO to stylet knob distance of *M. hapla* J2 (4.8 µm) was extremely long compare to the J2 of *M. incognita* and *M. javanica* and the measurements reported by Chitwood (1949) (Table 4).

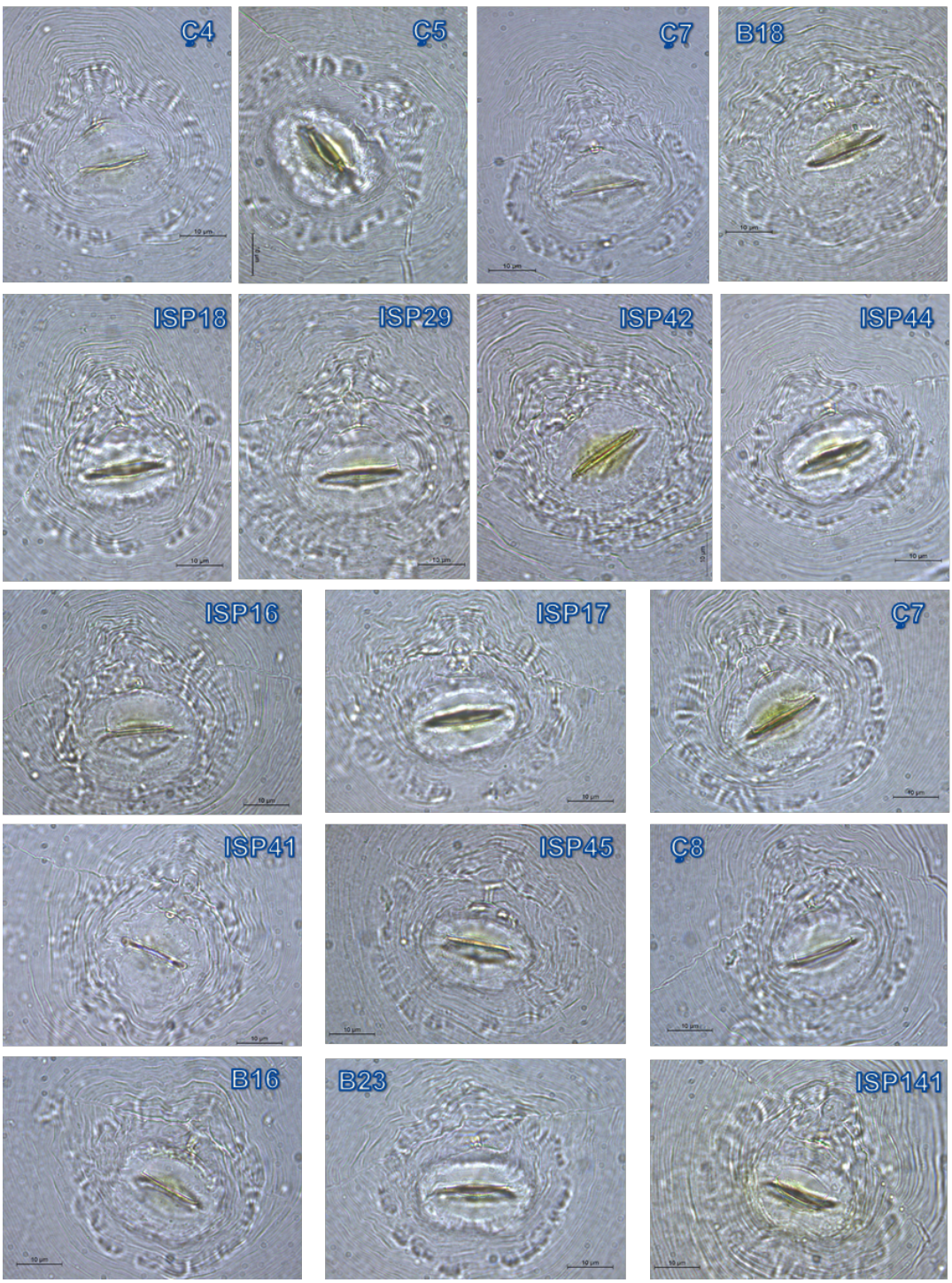

Figure 2. Perineal patterns of *Meloidogyne javanica* isolates collected from Lakes Region of Turkey. Bar: 10 µm.

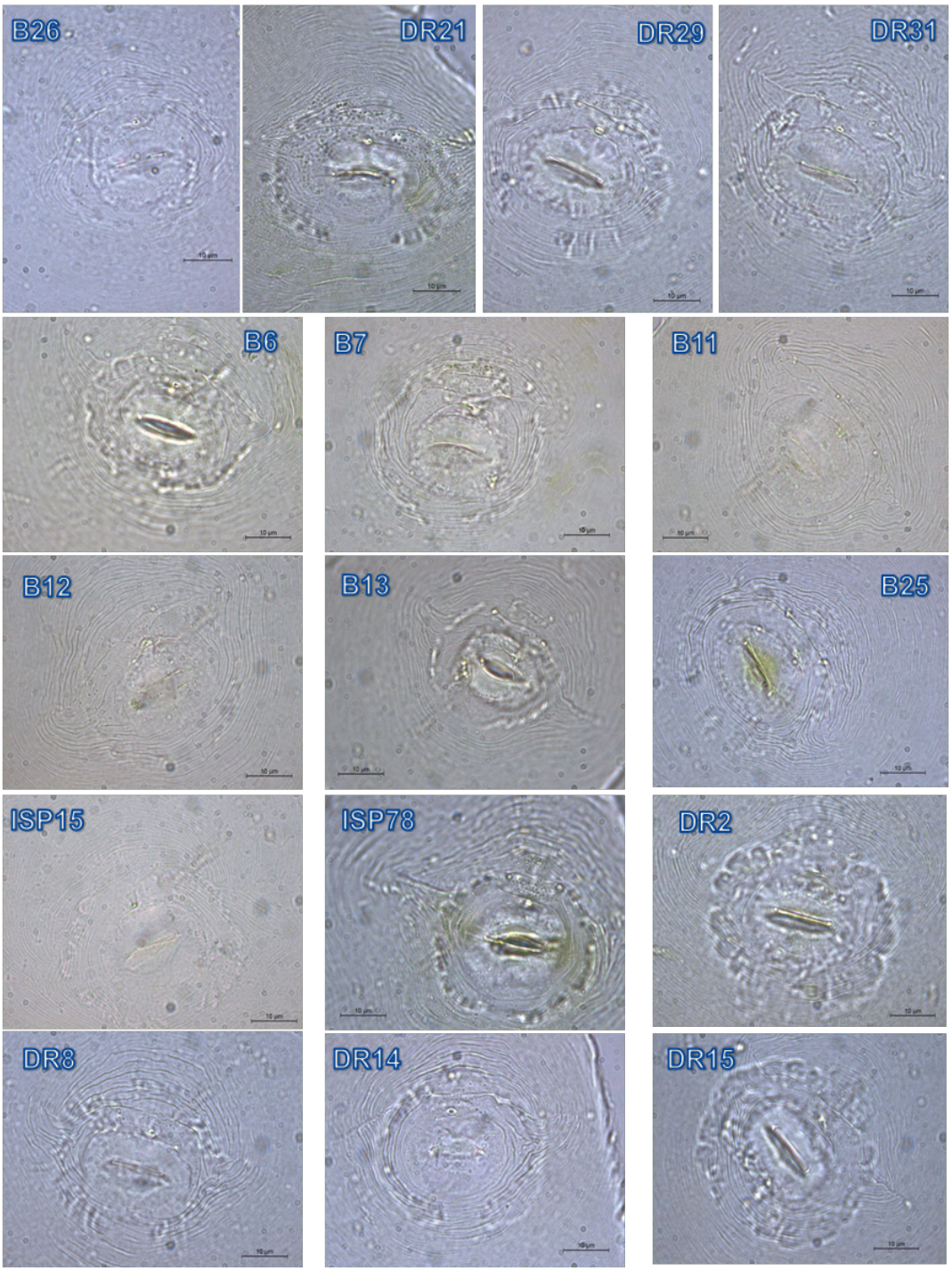

Figure 3. Perineal patterns of *Meloidogyne hapla* isolates collected from Lakes Region of Turkey. Bar: 10 µm.

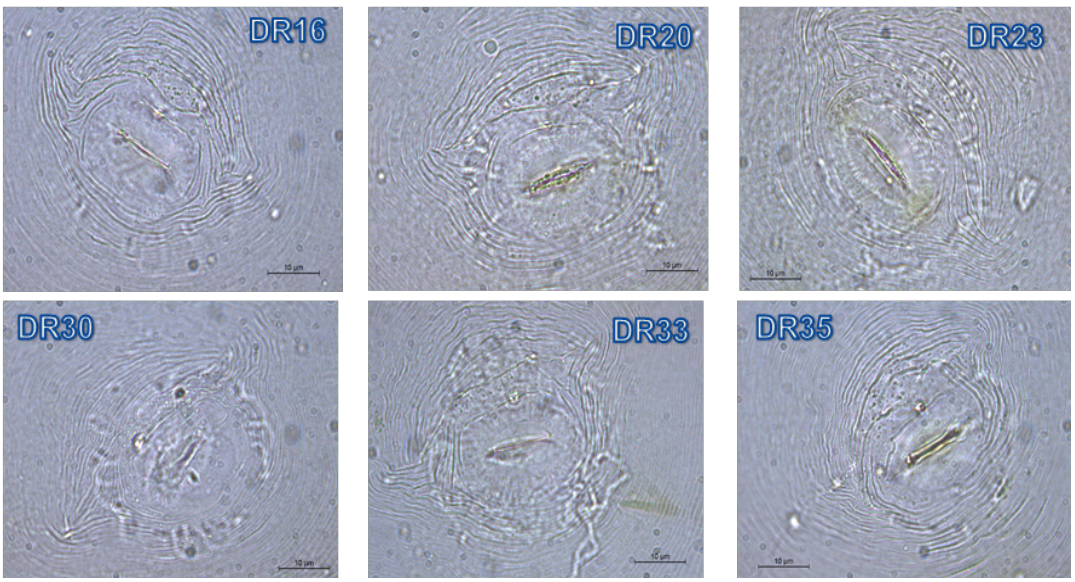

Figure 3. (continued).

Only one population was identified as *M. arenaria*. The lateral field was forked and the broken striae within lateral line region were curving with a striational winged form as described by Chitwood (1949) and Jepson (1987). Striae were distinctly separated and smoother in the ventral region (Figure 4). The dorsal arch was low and prevulval region was free of striae.

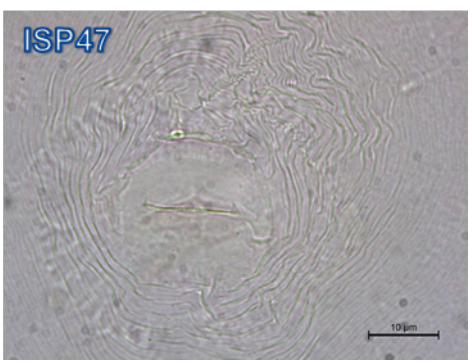

Figure 4. Perineal patterns of *Meloidogyne arenaria* isolates collected from Lakes Region of Turkey. Bar: 10 µm.

Table 4. Morphometric data for second stage juveniles of root-knot nematode species collected from Lakes Region of Turkey

Morphometric characters	J2 measurements (µm)		
Meloidogyne incognita	This study (n = 15)	Özarslandan (2009)	Whitehead (1968)
Body length (L)	409.7[x] (360-441.6)[y] [22.4][z]	407.60 [x]± 4.7[w] (387.8-428.8)[y]	360-393[y]
Tail length	57.6 (50.4-68.8)[5.5]	47.0±1.5 (38.4-52.8)	
Hyaline terminus length	11.6 (6.4-16)[2.2]	10.10± 0.4(8.0-11.2)	
DGO – Stylet knob distance	3.4 (3.2-4.8)[0.5]	2.56 ±0.1(2.4-2.9)	2-2.25
Stylet length	13.3 (12-14.4)[0.7]	11.40±0.4(9.6-12.8)	10
a	29.5	33.17	29-33
c	7.1	8.77	8-9.4
Meloidogyne javanica	This study (n:15)	Özarslandan (2009)	Whitehead (1968)
Body length (L)	448 (427.2-465.6) [16.3]	426.56 ±4.4 (408.0-454.4)	387-459
Tail length	55.2 (52.8-60.8)[3.81]	51.44 ±1.1(46.40-59.20)	36-56
Hyaline terminus length	14.40 (12.8-17.6)[2.3]	12.96 ±0.4(11.20-15.20)	
DGO – Stylet knob distance	3.4 (3.2-4)[0.4]	3.36 ±0.1(3.2-4.0)	4
Stylet length	14.0 (13.6-14.4)[0.5]	13.36 ±0.4(11.20-14.40)	9.4-11.4
a	32	30.33	27.1-35.9
c	8.1	8.31	7.3-11.1
Meloidogyne hapla	This study (n:20)	-	Chitwood (1949)
Body length (L)	380.5 (328-412.8) [23.7]		357-467
Tail length	49.5 (44.8-56)[2.9]		46-58
Hyaline terminus length	13.1 (11.2-17.6)[2.1]		12-19
DGO – Stylet knob distance	4.8 (3.2-6.4)[1.7]		3-4
Stylet length	12.4 (11.2-13.6)[0.8]		10-12
a	29.2		
c	7.7		

[x] mean, [y] max-min value; [z] standard deviation; [w] standard error.

Molecular identification

For 25 *M. incognita* populations, a PCR was conducted using primer set INCK14F/INCK14R (Randig et al., 2002) and a DNA band of 399 bp was obtained for all *M. incognita* populations. This result was in agreement with the results of Tesarova et al. (2003) and Devran & Söğüt (2009). For *M. arenaria* and *M. javanica*, specific SCAR primers (Zijlstra et al., 2000) produced 420 bp and 670 bp DNA bands, respectively, and this result was in agreement with previous studies in Turkey (Devran & Söğüt, 2009; Özarslandan & Elekçioğlu, 2010; Akyazı et al., 2012; Aydınlı & Mennan, 2016). All *M. hapla* populations were identified using primer set JMV1/JMV2/JMV hapla and a 440 bp DNA bands was obtained as described by Wishart et al. (2002) and this result was in agreement with previous a study in Turkey (Akyazı et al., 2012) (Figure 5).

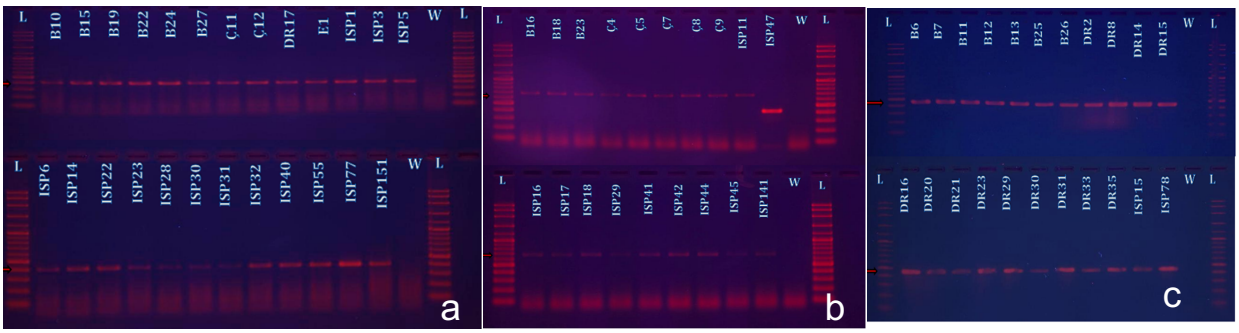

Figure 5. Amplification products with the *Meloidogyne* spp. collected from Lakes Region of Turkey a) *M. incognita* populations (B10-ISP151); b) *M. javanica* populations (B16-ISP141) and *M. arenaria* (ISP47); c) *M. hapla* populations (B6-ISP78). L: 100 bp DNA ladder, W: Water.

Root-knot nematode races

Meloidogyne incognita races 2, 4 and 6 and *M. javanica* races 1 and 3 were determined. *Meloidogyne arenaria* races could not be determined because the species was not mass cultured. Table 5 shows *M. incognita* and *M. javanica* races and their reaction on differential host plants and the altitude at which they were collected.

Seventeen *M. incognita* populations reproduced well on tomato, pepper and tobacco cultivars, but did not on cotton and peanut, so these populations were identified as race 2. Six populations developed well on tobacco, however there were no galls or egg masses on the other differential host cultivars. Therefore, these were identified as race 6. The other two populations reproduced on tomato, cotton, pepper and tobacco, but not on peanut and were identified as race 4. *Meloidogyne incognita* race 2 was found to be the most common (68% of samples) in this study. Similarly, race 2 was found to be as widespread in the Mediterranean and Black Sea Regions (Söğüt & Elekçioğlu, 2000; Mennan & Ecevit, 2001; Devran & Söğüt, 2011). *Meloidogyne incognita* race 6 was first reported in western coastal areas of the Mediterranean Region of Turkey by Devran & Söğüt (2011). In the current study, *M. incognita* race 6 was more common (24% of samples) than in the western coastal areas of the Mediterranean Region (3% of samples). In addition, Kaçar (2011) reported the occurrence of *M. incognita* races 5 and 6 in Turkey and Robertson et al. (2009) reported *M. incognita* races 6 and 5 in vegetable growing areas of Spain. *Meloidogyne incognita* race 4 was not common (8% of samples) in the Lakes region. Similarly, Söğüt & Elekçioğlu (2000) reported *M. incognita* race 4 in several vegetables growing areas in eastern areas of the Mediterranean Region of Turkey, whereas, Akyazı & Ecevit (2010) reported that *M. incognita* race 1 was more common in Tokat than race 2 in the Black Sea Region of Turkey.

Fifteen *M. javanica* populations developed well and formed egg masses on the roots of tobacco and tomato but not on cotton, pepper and peanut. Thus, these were identified as *M. javanica* race 1. Only one *M. javanica* population (B16) formed galls and egg masses on the roots of tobacco, tomato and peanut, but not on cotton and pepper. This *M. javanica* population was determined as race 3 and represents the first detection of this race in Turkey. The other two *M. javanica* populations (ISP41 and ISP45) were not tested with differential host because of they were not cultured. In a previous study, *M. javanica* race 1 was reported to be widespread in eastern and western areas of the Mediterranean Region of Turkey (Söğüt & Elekçioğlu, 2000; Devran & Söğüt, 2011). *Meloidogyne javanica* races 1 and 3 did not reproduce on pepper, however, the other races of *M. javanica* develop well in pepper and have been reported from different parts of the world. *Meloidogyne javanica* races 2 and 3 were reported by Rammah & Hirschmann (1990). Carneiro et al. (2004) identified *M. javanica* race 4 in Parana State, Brazil and Robertson et al. (2009) reported *M. javanica* race 1 and 5 from vegetable growing areas of Spain.

Table 5. Differential host test to classifying races of *Meloidogyne incognita* and *M. javanica* populations and the altitude at which they were collected from Lakes Region of Turkey

Meloidogyne incognita						*Meloidogyne javanica*					
Code	Race	Tobacco	Pepper	Cotton	Altitude (m)	Code	Race	Tobacco	Pepper	Peanut	Altitude (m)
B22	2	3.5±0.5*	2.7±0.5	0.0±0.0	276	B18	1	4.2±0.2	0.0±0.0	0.0±0.0	275
ISP14	2	4.2±0.5	2.7±0.2	0.0±0.0	303	B23	1	3.7±0.5	1.2±0.5	0.0±0.0	284
ISP31	2	2.2±1.3	4.2±0.2	1.2±0.2	353	Ç4	1	4.5±0.3	0.0±0.0	0.0±0.0	366
Ç11	2	3.5±0.3	2.2±0.8	0.0±0.0	338	Ç5	1	4.0±0.6	0.0±0.0	0.0±0.0	350
Ç12	2	4.0±0.4	4.0±0.6	0.0±0.0	339	Ç7	1	3.7±0.5	0.0±0.0	0.0±0.0	341
ISP151	2	3.5±0.6	2.7±0.2	0.0±0.0	357	Ç8	1	4.5±0.5	0.0±0.0	0.0±0.0	327
ISP40	2	3.7±0.5	2.7±0.2	0.0±0.0	451	Ç9	1	4.2±0.2	0.0±0.0	0.0±0.0	360
ISP55	2	3.5±0.3	3.2±0.2	0.0±0.0	596	ISP11	1	3.2±0.3	0.0±0.0	0.0±0.0	321
ISP5	2	3.0±0.4	4.2±0.5	0.0±0.0	595	ISP16	1	3.2±0.5	1.0±0.6	0.0±0.0	275
ISP6	2	3.0±0.4	5.0±0.0	0.5±0.5	595	ISP17	1	4.5±0.3	0.2±0.2	0.0±0.0	275
ISP1	2	3.7±0.5	3.5±0.3	0.0±0.0	602	ISP18	1	4.2±0.2	0.0±0.0	0.0±0.0	286
ISP3	2	4.0±0.4	2.7±0.2	0.0±0.0	607	ISP29	1	4.0±0.4	0.0±0.0	0.0±0.0	325
B10	2	4.2±0.2	4.0±0.0	0.5±0.5	925	ISP141	1	5.0±0.0	0.0±0.0	0.0±0.0	359
E1	2	3.7±0.3	3.0±0.4	0.0±0.0	930	ISP44	1	3.7±0.5	0.0±0.0	0.0±0.0	676
DR17	2	2.7±0.9	3.0±0.3	0.0±0.0	1075	ISP42	1	4.7±0.2	0.0±0.0	0.0±0.0	941
B24	2	3.7±0.2	3.5±0.3	0.2±0.2	1430	B16	3	4.2±0.2	0.0±0.0	3.2±0.2	276
B27	2	4.5±0.3	4.2±0.2	0.0±0.0	1436	ISP41**	nt				303
B27	4	3.2±1.1	4.5±0.3	3.2±0.2	350	ISP45**	nt				993
ISP23	4	3.7±0.5	4.2±0.2	2.7±0.5	1110						
B15	6	3.5±0.3	0.0±0.0	0.0±0.0	276						
B19	6	3.0±0.4	0.7±0.5	0.0±0.0	308						
ISP22	6	4.5±0.3	0.0±0.0	0.0±0.0	302						
ISP28	6	5.0±0.0	0.7±0.2	0.0±0.0	288						
ISP30	6	3.7±0.2	0.0±0.0	0.0±0.0	287						
ISP77	6	4.0±0.5	0.0±0.0	0.0±0.0	599						

* Mean ± standard errors of four replicates;
** nt: not tested.

Distribution of root-knot nematodes in Lakes Region of Turkey

Root-knot nematodes infested 52% of the samples taken from open fields and greenhouses in the Lakes region. Major root-knot nematode species, *M. incognita*, *M. javanica* and *M. hapla* were common in vegetable growing areas at different altitudes at 37, 27 and 32% of samples, respectively. Only one population was identified as *M. arenaria* collected from eggplant in Atabey District of Isparta Province at an altitude of about 1000 m. Two populations were not identified from their perineal patterns model and molecular assays. *Meloidogyne incognita* populations were found on tomato, eggplant, pepper, cucumber and bean. *Meloidogyne javanica* populations were detected on tomato, eggplant and cucumber, and *M. hapla* populations were predominantly detected on tomato and eggplant crops at higher altitudes. *Meloidogyne incognita* and *M. javanica,* as thermophilic species, were found to be more prevalent at lower altitudes. Six *M. incognita* isolates (ISP23, DR17, E1, B10, B24 and B27) were found at locations higher than 900 m. All *M. incognita* race 6 isolates were detected at lower altitudes. Similarly, 12 *M. incognita* race 2 isolates were found at lower altitudes. However, five race 2 isolates were found at higher altitudes. One of two *M. incognita* race 4 isolates was found at a lower altitude, whereas the other was found at over 1000 m. Also, 20% of *M. incognita* populations were detected in open fields and 80% in greenhouses. Only one *M. javanica* isolate (ISP42, race 1) was found on a high plateau area, while the others were found in vegetable growing areas across the lower plateau areas, which have warmer climatic conditions. *Meloidogyne javanica* race 3 was found in a cucumber greenhouse in Elsazı Village of Burdur Province at 276 m. Also, 17% of *M. javanica* populations were found in open fields and 83% in greenhouses. One *M. arenaria* population (ISP47) was found in an open field at 993 m. *Meloidogyne hapla* was observed in the cooler areas. About 91% of *M. hapla* populations were from high altitude sites with sandy soil. Greenhouses in Deregümü District of Isparta Province were commonly infested with *M. hapla* at altitudes over 900 m. The other 9% of populations were found at lower altitudes. Excluding two populations (DR20 and DR35), all *M. hapla* populations were collected from greenhouses.

Researchers have studied the identification and distribution of root-knot nematodes in different regions of Turkey. Özarslandan & Elekçioğlu (2010) reported that *M. incognita*, *M. arenaria*, *M. javanica* and *M. chitwoodi* were represented 28, 27 35 and 10%, respectively, of 79 root-knot nematode populations collected from all over Turkey. Devran & Söğüt (2009) showed that soils where the vegetables are grown along the western coastal areas of the Mediterranean Region were infested by *M. incognita* (63%), *M. javanica* (30%) and *M. arenaria* (7%). Elekcioğlu & Uygun (1994) were the first to to identify root-knot nematodes in the Mediterranean Region, with *M. incognita* and *M. javanica* the most commonly species detected. In the eastern Mediterranean Region, *M. javanica*, *M. incognita* and *M. hapla* were found in 55, 42 and 3%, respectively, of 38 samples collected from protected vegetable areas (Söğüt & Elekçioğlu, 2000). In the other regions of the Turkey, various studies have found root-knot nematode species. Yüksel (1974) reported that *M. incognita*, *M. javanica*, *M. arenaria* and *M. hapla* occurred in vegetable growing soils of the Marmara Region. Important vegetables growing areas of Aydın Province of the Aegen Region were found to have *M. incognita*, *M. javanica* and *M. hapla* when sampled in summer (Kaşkavalcı & Öncüer, 1999). Studies in the Black Sea Region have reported that Bafra and Çarşamba Plains are infested with *M. incognita*, Tokat vegetable areas with *M. incognita*, Ordu and Samsun Provinces with *M. arenaria* and *M. hapla* (Mennan & Ecevit, 2001; Akyazı & Ecevit, 2010; Akyazı et al., 2012). In recently studies, *M. arenaria* (42%), *M. ethiopica* (41%), *M. javanica* (12%) and *M. incognita* (4%) were reported in the central areas of the Black Sea Region (Aydınlı & Mennan, 2016) and *M. incognita* in Kahramanmaraş Province in the eastern Mediterranean Region (Çetintaş & Çakmak, 2016).

In conclusion, for vegetables grown during summer and early autumn in the Lakes region, fumigants, such as metam sodium or Dazomet, and soil solarization are not sufficient for control of root-knot nematodes. Thus, it is of great importance for root-knot nematodes species are identified so that appropriate available and alternative control methods can be chosen based on the species present and regional conditions. *Meloidogyne incognita* and *M. javanica* were the most common species on cultivated

plants in low altitude areas of the Lakes region, just as in the coastal areas of the Mediterranean Region. Importantly, three host races (2, 4 and 6) of *M. incognita* were detected. Race composition of these two-major species are important for plant resistance and breeding, and in crop rotation strategies, so this is a significant outcome of this study. In contrast, *M. hapla* was found to be the prevalent species in higher high altitude areas in the Lakes region, which is also an important finding as there are no suitable resistant cultivar available for vegetable crops grown in these areas. Given that *M. hapla* induced spherical galls on the proliferating or branching small roots of tomatoes and eggplants, and egg masses were clearly visible on the outside of the small branching roots, this symptomology could facilitate control by egg parasitizing fungi or bacteria, or nematicide treatments. *Meloidogyne hapla* has a wide host range, however, crop rotation with grasses and grain crops can significantly decrease its population density. Therefore, it is important to consider geographic and species distribution of root-knot nematode species when implementing control programs. This study has provided such data for the Lake region of Turkey, which should prove to be a valuable resource for future research and for developing effective control and management strategies for the region.

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