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Original article (Orijinal araştırma)

Effectiveness of various insecticides and predatory bug, *Orius laevigatus* (Fieber, 1860) (Hemiptera: Anthocoridae) releases on *Thrips hawaiiensis* (Morgan, 1913) (Thysanoptera: Thripidae) in lemon, *Citrus limon* (L.) (Rutales: Rutaceae), orchard in Mersin (Türkiye)

Mersin (Türkiye)'de limon, *Citrus limon* (L.) (Rutales: Rutaceae) bahçesinde farklı insektisitlerin ve avcı böcek *Orius laevigatus* (Fieber, 1860) (Hemiptera: Anthocoridae) salımının *Thrips hawaiiensis* (Morgan,1913) (Thysanoptera: Thripidae)'e karşı etkinliği

Adalet HAZIR[1*] Miraç YAYLA[1] Doğancan KAHYA[1] Ekrem ATAKAN[2]

Abstract

The important invasive thrips species Hawaiian flower thrips, *Thrips hawaiiensis* (Morgan, 1913) (Thysanoptera: Thripidae) was first reported in Türkiye in 2015. Since then, it has been causing damage in lemon orchards. This study was conducted to reveal the most effective insecticides, the most effective spraying time and the efficacy of biological control. For this purpose, the effectiveness of five insecticides (480 g/l spinosad, 25% spinetoram, 100 g/l spirotetramat, 50% flonicamid, 240 g/l tau-fluvalinate), effect of three spray programs and effectiveness of predatory bug *Orius laevigatus* (Fieber, 1860) (Hemiptera: Anthocoridae) were tested in a lemon orchard [*Citrus limon* (L.) (Rutales: Rutaceae)] in Erdemli district of Mersin province in 2018 and 2019. In order to determine the most effective spraying time, three spray programs were tested. In Program 1, two sprays during the flowering period were applied. In Program 2, two sprays were applied, one at petal fall and the other at the small fruiting stage. In Program 3, one spray at petal fall and two sprays in the fruiting stages were applied. According to the results, 240 g/l tau-fluvalinate and 50% flonicamid showed the lowest efficacy of the insecticides in the three programs. Spinetoram was found the most effective of the others. Insecticide applications to control *T. hawaiiensis* during the flowering period (Program 1) had low efficacy. Program 3 was found to be the most effective. Predatory bug *O. laevigatus*, as a biological control agent was found to have a potential efficacy for suppressing *T. hawaiiensis* populations.

Keywords: Biological control, insecticide, lemon, *Thrips hawaiiensis*

Öz

Önemli bir istilacı thrips türü olan Hawai çiçek tripsi *Thrips hawaiiensis* (Morgan, 1913) (Thysanoptera: Thripidae) Türkiye'de ilk defa 2015 yılında rapor edilmiştir. O zamandan beri, limon bahçelerinde zarara neden olmaktadır. Bu çalışma, limon bahçelerinde sorun olan *T. hawaiiensis*'in mücadelesinde en etkili insektisiti, en etkili ilaçlama zamanını ve biyolojik mücadelenin etkinliğini ortaya koymak için yürütülmüştür. Bu amaçla, 2018 ve 2019 yıllarında, Mersin ili Erdemli ilçesinde bir limon bahçesinde [*Citrus limon* (L.) (Rutales: Rutaceae)] 5 farklı insektisitin (480 g/l spinosad, %25 spinetoram, 100 g/l spirotetramat, %50 flonicamid, 240 g/l Tau-fluvalinate), üç farklı uygulama programının ve avcı böcek *Orius laevigatus* (Fieber, 1860) (Hemiptera: Anthocoridae)'un etkinliği denemeye alınmıştır. En etkili ilaçlama zamanını belirlemek için 3 ilaçlama programı denenmiştir. Birinci programda çiçeklenme döneminde iki ilaçlama test edilmiştir. İkinci programda, biri taç yaprak dökümü diğeri küçük meyve dönemi olmak üzere 2 ilaçlama test edilmiştir. Üçüncü programda ise taç yaprak dökümünde bir, meyve döneminde 2 ilaçlama test edilmiştir. Elde edilen sonuçlara göre, 240 g/l tau-fluvalinate ve %50 flonicamid üç programın hepsinde ilaçlar arasında en düşük etkiyi göstermişlerdir. Spinetoram etkili maddeli ilacın, diğerlerine göre en etkili preparat olduğu tespit edilmiştir. *Thrips hawaiiensis*'e karşı çiçek döneminde yapılan insektisit uygulamalarının (program 1) etkinliği düşük bulunmuştur. Üçüncü program en etkili program olarak bulunmuştur. Biyolojik mücadele ajanı olarak avcı böcek *Orius laevigatus*'un *T. hawaiiensis*'i baskı altına alabilme potansiyeli olduğu belirlenmiştir.

Anahtar sözcükler: Biyolojik mücadele, insektisit, limon, *Thrips hawaiiensis*

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Introduction

Citrus is an important crop for Türkiye for both domestic consumption and export. The major citrus plantations are in Adana, Antalya, Hatay and Mersin Provinces in the Mediterranean Region of Türkiye. Insect pests, diseases and weeds are the main problems during citrus production. About 90 pest species, 17 of which are known to be economically important pests, have been identified in Türkiye (Uygun, 2001; Anonymous, 2021). Thrips species are known as one of the most important pest group causing economic losses by feeding on the sap of citrus flowers, fruit and leaves (Yiğit et al., 1991; Childers & Beshear, 1992; Tunç, 1992; Childers & Achor, 1995; Tekşam & Tunç, 2007). This pest group cause spot and scar damage on young fruit which leads to high negative effects on market and export value of citrus fruit (Jeppson et al., 1975).

There are many thrips species recorded as a pest on citrus (Blank & Gill, 1997; Froud et al., 2001; Childers & Nakahara, 2006; Costa et al., 2006; Navarro et al., 2008; Tekşam & Tunç, 2009). *Heliothrips haemorrhoidalis* (Bouché, 1833) and *Pezothrips kellyanus* (Bagnall, 1916) are important thrips species recorded as a pest on citrus in the Mediterranean countries (Tekşam & Tunç, 2009; Navarro et al., 2008; Jacas et al., 2010; Vassiliou, 2010; Navarro-Campos et al., 2012). Aguilar-Fenollosa & Jacas (2013) revealed that citrus species were more attractive to thrips in the period that starting from petal fall until fruit reach to 4 cm size. Thrips species on citrus are listed by Tunç (1989, 1996). Another thrips species, *Frankliniella occidentalis* (Pergande, 1895) was first recorded in Türkiye in 1993 and spread to different regions causing damage to different host plant species (Tunç & Göçmen, 1994; Atakan & Tunç, 2004; Atakan, 2007a, b; Nas et al., 2007; Hazır et al., 2011; Hazır & Ulusoy, 2012).

In Türkiye, *Thrips hawaiiensis* (Morgan, 1913) (Thysanoptera: Thripidae) was found as a first record in 2015 in Mersin in the eastern Mediterranean Region (Atakan et al., 2015). Adults are nearly 1.3 mm, abdomen is brownish, thorax and head are orange-brown, legs are yellow or yellowish-brown (Atakan et al., 2015) (Figure 3). The first instars are white or nearly transparent in the beginning while second instars are white to yellow-white without wings (Figure 4) (Mau & Martin, 1993). Murai (2001) conducted a study on the biology of *T. hawaiiensis* and showed that this pest completed its life cycle from egg to adult in about 37 days at 10ºC, 10 days at 25ºC and 8 days at 30ºC. The main damage of this pest appears on the fruit. The pest causes silver-brown spotting, necrosis and deformation of fruit (Figure 5) (Goldaranzena, 2011; Atakan & Pehlivan, 2020a, b). *Thrips hawaiiensis* is a polyphagous flower thrips and occurs in Asia, the Pacific Region, North America and southern Europe (CABI, 1983; Sakimura, 1986; Nakahara, 1994; Reynaud et al., 2008; Goldaranzena, 2011).

Limited studies were conducted on the chemical control of *T. hawaiiensis.* Fu et al. (2020) studied the effectiveness of insecticides against *T. hawaiiensis* in the laboratory and field conditions. Under the field conditions, spinetoram, spirotetramat and cyantraniliprole were found to be more effective. Atakan & Pehlivan (2020b) suggested that chemical applications should be applied 1 month after petal fall and when a few flowers remain on the lemon trees. In addition, spinosad with summer mineral oil was found to be quite effective against *T. hawaiiensis* according to their field observations.

Orius laevigatus (Fieber, 1860) (Hemiptera: Anthocoridae) is one of the most commonly used commercial predatory bugs in various agroecosystems against aphids, whiteflies and thrips in biological control programs (Frescata & Mexia, 1996; Hernández & Stonedahl, 1999; Venzon et al., 2002; van Lenteren & Bueno, 2003). *Orius laevigatus* is commonly used against *F. occidentalis* in greenhouse pepper in the Mediterranean countries (Sanchez & Lacasa, 2002).

This study aimed to contribute for developing chemical and biological control strategies against *T. hawaiiensis* in lemon, *Citrus limon* (L.) (Rutales: Rutaceae), plantations. For this purpose, experiments were conducted to determine the efficacy of five insecticides (spinetoram, spirotetramat, spinosad, tau-

fluvalinate, and flonicamid), the efficacy of biological control by *O. laevigatus* releases and efficacy of three management programs in a lemon orchard in Mersin in 2018 and 2019.

Materials and Methods

Study area and materials

This study was conducted in a 5-ha lemon orchard (36.618° N, 34.326° E) of the Alata Horticultural Research Institute located in Mersin Province, Türkiye in 2018 and 2019 (Figure 1). Lemon cv. Kütdiken trees were planted at 6 x 8 m and were 12 years old. Trial area was surrounded by orange and grapefruit orchards. The insecticide applications were done by using garden sprayer at a pressure of 5-7 bar. Figure 2 shows the insecticide applications in the trial area. An *O. laevigatus* stock culture was obtained from Biological Control Research Institute, Adana and mass rearing of the predator was conducted in the insectarium of the Institute.

Figure 1. Aerial view of trial area.

Figure 2. Insecticide applications during this study.

Methods

The thrips adults were collected during flowering (Figure 3) and fruiting (Figure 4) periods in the experimental orchard. The adults were preserved in alcohol in Eppendorf tubes and identified to species by one of us (EK) with the needed expertise. The prevalence of *T. hawaiiensis* in all species was 70-80% in samples from flowers in April-May and 95-100% in samples from fruit in June-July.

Figure 3 *Thrips hawaiiensis* individuals on lemon a) buds, and b, c) flowers.

Figure 4. a) *Thrips hawaiiensis* larvae on an unripe fruit, b) adult and larvae on a ripe fruit.

The experimental design was a randomized block design with six characters (4 insecticides + predatory bug release + control). Each treatment was applied to four replicates consisting of six trees each. One row was left as buffer between each treated plot. The insecticides tested in the first year were 480 g/l spinosad, 25% spinetoram, 100 g/l spirotetramat and 50% flonicamid, and in the second year, 480 g/l spinosad, 25% spinetoram, 100 g/l spirotetramat, 240 g/l tau-fluvalinate. The biological control agent used was the predatory bug, *O. laevigatus*. The predator adults were released at least a week after spraying to prevent them being exposed to the toxic effects of insecticides. The predators were released using packages of 20 adults up to 24 h old. Seven releases were made starting from full bloom until the fruit were 26-42 mm diameter.

Table 1 shows the applied rates of the insecticides and release of predatory bug.

Table 1. Active ingredients of insecticides, application rates and release number of *Orius laevigatus* in 2018 and 2019 spray programs

2018 spray programs		2019 spray programs	
Active Ingredient	Application rate	Active ingredient	Application rate
Spinetoram 25% WG	50 g/100 l	Spinetoram 25% WG	50 g/100 l
Spirotetramat 100 g/l SC	100 ml/100 l	Spirotetramat 100 g/l SC	100 ml/100 l
Spinosad 480 g/l SC	50 ml/100l	Spinosad 480 g/l SC	50 ml/100 l
Flonicamid 50% WG	15 g/100 l	Tau-fluvalinate 240 g/l	50 g/100 l
Predator (*Orius laevigatus*)	20 adults/tree	Predator (*Orius laevigatus*)	20 adults/tree
Control	No application	Control	No application

Spray programs in the various phenological periods

Three spray programs were tested in the trial in order to determine the best timing for chemical control. Each program was started at a particular phenological period of the lemon trees. In Program 1, two insecticide sprays were applied; the first at 50% flowering, and the second at 100% flowering (full bloom). In Program 2, two insecticide sprays were applied; the first at 20% petal fall, and the second when the fruit were about 18-33 mm in diameter. In Program 3, three insecticide sprays were applied; the first at 20% petal fall, the second when the fruit were about 18-33 mm in diameter and the third when the fruit were about 26-42 mm in diameter. In 2019, first spray in Program 1 was not be done at 50% flowering because there was no *T. hawaiiensis* present in the flowers therefore the treatments started at full bloom. Table 2 shows the phenology and dates of applications in each program in 2018 and 2019.

Table 2. Phenology and treatment application dates in three spray programs in 2018 and 2019

Phenology	2018 spray programs				2019 spray programs			
	Date	1	2	3	Date	1	2	3
50% flowering	3 April	++	--	--	--	--	--	--
100% flowering (full bloom)	10 April	++	--	--	13 May	++	--	--
20% petal fall + fruit (5 mm)	17 April	--	++	++	20 May	--	++	++
Small fruit (18-33 mm)	24 April	--	++	++	11June	--	++	++
Large fruit (26-42 mm)	15 May	--	--	++	27 June	--	--	++

Assessments

Assessments were made 1 month after last application in each year. The scarring and the silvering damage larger than 2 mm (shown in Figure 5) were recorded. The amount of damaged fruit was determined by examining 100 randomly selected fruit on the inward-facing branches of six trees in each plot. The assessment of biological control was made 2 weeks after last predator release. The results for both years are given in Tables 3 and 4. The effect of treatments relative to the control using Abbott formula (Abbott, 1925) are given in Table 5. Abbott's formula was used to determine relative effects of insecticides and *O. laevigatus* in Table 4 and 5 with below formula.

$$\%\text{Relative effect} = 1 - \left(\frac{Damaged\ number\ of\ fruit\ after\ treatment}{Damaged\ number\ of\ fruit\ in\ Control}\right) * 100$$

Figure 5. Damage symptoms of *Thrips hawaiiensis* on lemon fruit in the experimental area.

Identification of thrips species

To determine the Thysanoptera (thrips) species in the trial area, the flower and the fruit samples taken from the experimental plots were brought to Çukurova University Faculty of Agriculture Plant Protection Department Industrial Plant Pests laboratory in Eppendorf tubes (50 ml). Thrips were identified according to Atakan et al. (2015). The samples were extracted from flowers and fruit in Petri dishes and placed in 60% ethanol. These were the transferred to AGA medium (10:1:1 60% ethyl alcohol, glycerin and glacial acetic acid) for 2 days in order to facilitate their preparation and for this purpose to soften their bodies before returning them to 60% alcohol. Samples were placed separately into glass Petri dishes and kept in 10% KOH for approximately 1 h at 48°C. Body contents of thrips specimens were evacuated by entering the hind leg bases of thrips individuals with a very fine-tipped needle (maceration). The samples were cleaned by passing through alcohol series and transferred to Hoyer medium to prepare their microscopic slides.

Statistical analysis

Data were tested for normality and homogeneity of variance. One-way ANOVA and Duncan multiple comparison tests were performed with the SPSS 23 statistic program.

Results and Discussion

Results for 2018 spray programs

In Program 1, the results showed that there is no significant difference between control and 50% flonicamid for the number of damaged fruit (Table 3). The other insecticides gave control that was statistically different from control but these were not significantly different from each other. The number of damaged fruit was high in Program 1 compared to the other programs. Even with spinetoram, as the most effective insecticide for reducing damage in Program 1, its effect relative to the control was low (Table 5). This indicates that insecticides applied at 50-100% flowering may be unable to protect the fruit from thrips damage.

In Program 2, flonicamid was not statistically different from control (Table 3). The other insecticides provided statistically significant control but these were not significantly different from each other. The relative effects of the insecticides were higher than with Program 1 (Table 5). Spinetoram had the highest relative effect.

Although the results of Program 3 were similar to Program 2, the effect of spinetoram was statistically greater than spirotetramat and spinosad (Table 3). Again, spinetoram had the highest relative effect (Table 5).

Flonicamid failed to lower the amount of damaged fruit in all programs in 2018. For this reason, it was excluded from the trials of 2019. Overall is concluded from the 2018 insecticide data that spraying

during the fruiting period suppress *T. hawaiiensis* population more effectively than spraying during the flowering period.

Release of *O. laevigatus* in 2018 was different from control (Table 4) which shows that it can reduce damage. However, the relative effect of this application was lower than the insecticides (Table 5).

Table 3. Number of damaged fruit with chemical control in 2018 and 2019 spray programs

Treatment	2018 spray programs			2019 spray programs		
	1	2	3	1	2	3
Control	7.52 ± 0.91 a	7.52 ± 0.91 a	7.52 ± 0.91 a	30.9 ± 0.91 a	30.9 ± 0.91 a	30.9 ± 0.91 a
50% Flonicamid	7.85 ± 1.29 a	6.65 ± 1.29 a	7.45 ± 1.29 a	-	-	-
240 g/l Tau-fluvalinate	-	-	-	15.8 ± 0.16 b	15.6 ± 0.85 b	14.8 ± 0.89 b
25% Spinetoram	4.65 ± 0.92 b	3.60 ± 0.92 b	2.50 ± 0.92b	8.56 ± 0.34 c	7.81 ± 0.69 c	7.43 ± 0.27 c
100 g/l Spirotetramat	5.40 ± 1.15 b	4.45 ± 1.15 b	4.40 ± 1.15c	10.9 ± 0.59 d	12.3 ± 0.14 d	12.0 ± 0.57 d
480 g/l Spinosad	5.35 ± 0.96 b	4.30 ± 0.96 b	5.20 ± 0.96 c	8.59 ± 0.87 c	9.96 ± 0.58 e	10.2 ± 0.49 e

Means followed by same letter within columns are no statistically different according to Duncan multiple comparison test ($p < 0.05$).

Table 4. Number of damaged fruit and relative effect of biological control in 2018 and 2019 spray programs

Treatment	Damaged Fruit	Relative effect (% of control)	Damaged Fruit	Relative effect (% of control)
Control	7.5 ± 2.5 a	44.0	30.9 ± 0.91 a	45.6
Releases of *Orius laevigatus*	4.2 ± 1.2 b		16.8 ± 0.81 b	

Abbott's formula was used to determine relative effects of biological control.

Table 5. Relative effect (% of control) of chemical control in preventing damage to fruit in 2018 and 2019 spray programs

Treatment	2018 spray programs			2019 spray programs		
	1	2	3	1	2	3
50% Flonicamid	0	11.6	0.93	-	-	-
240 g/l Tau- fluvalinate	-	-	-	49.0	49.6	52.2
25% Spinetoram	38.2	52.1	66.8	72.3	74.7	75.9
100 g/l Spirotetramat	28.2	40.8	41.5	64.7	60.1	61.1
480 g/l Spinosad	28.9	42.8	30.9	72.2	67.7	67.1

Abbott's formula was used to determine relative effects of insecticides.

Results for 2019 spray programs

In 2019, thrips population was higher than in 2018. Table 3 shows the average number of damaged fruit in the three programs in 2019. With Program 1, spinetoram, spirotetramat and spinosad were more effective than tau-fluvalinate, with spinetoram have the highest relative effect of 72.3% and tau-fluvalinate the lowest at 49.0% (Table 5).

In Program 2, all insecticides were significantly different from the control and each other (Table 3). The greatest control was obtained with spinetoram. The relative effect was also the greatest with spinetoram at 74.7% followed by spinosad at 67.7% (Table 5).

In Program 3, as in Program 2, spinetoram, spirotetramat and spinosad were significantly different from the control and each other (Table 3). The lowest damage was obtained with spinetoram. The greatest relative effect was again with spinetoram at 75.9% followed by Spinosad at 67.1% (Table 5). Of the treatments, Tau-fluvalinate had the lowest relative effect.

The effect of *Orius* releases was limited (Table 4) but still promising because it is an environmentally friendly method.

Combined results and observations

When both years are considered, it was seen that spinetoram was the most efficacious insecticide in lowering the thrips damage in lemon fruit. Program 3 was found to be the most effective program for the timing of the sprays.

During the study, it was observed that *T. hawaiiensis* populations first developed in the flowers of various weed species such as *Capsella bursa pastoris* (L.) (Brassicales: Brassicaceae) in and around the orchard. The thrips adults moved from weeds to the lemon trees at the beginning of flowering to form populations on lemon flower buds. It was observed that the continuous presence of even a small number of flowers on lemon trees in the fruiting period was a factor to support the thrips population and to increase the damage levels.

There are limited studies on the effectiveness of insecticides against *T. hawaiiensis*. Fu et al. (2020) studied the efficacy of imidacloprid and spirotetramat via injection in banana flowers, and this was effective under the field conditions and there were no negative effects on fruit yield.

Srivasta et al. (2008) studied the effectiveness of spinetoram against thrips in pepper in field conditions in Florida, USA and found that spinetoram 61 g ai/ha was as effective as spinosad 140 g ai/ha against *F. occidentalis*, *Frankliniella tritici* (Fitch, 1855) and *Frankliniella bispinosa* (Morgan, 1913) (Thysanoptera: Thripidae). In addition, the *Orius insidiosus* (Say, 1832) (Hemiptera: Anthocoridae) population and predation was higher and sufficient in the experiments.

Palumbo & Richardson (2008) conducted a study to determine the efficacy of spinetoram and spinosad on Romaine lettuce against *F. occidentalis* under field conditions with spinetoram found to be more effective than spinosad. However, spinetoram and spinosad should not be used rotationally because these active ingredients have the same mode of action and may their frequent use may cause resistance problems.

Jones et al. (2005) conducted a study on the effectiveness of spinosad against *F. occidentalis*, and effects of spinosad on some biological control agents in cucumber in southern Ontario, USA and found that this active ingredient had moderate toxicity to *O. insidiosus,* high toxicity to *Encarisa formosa* Gahan, 1924 (Hymenoptera: Aphelinidae) but low toxicity to *Amblyseius cucumeris* (Oudemans, 1930) (Acarina: Phytoseiidae).

Siebert et al. (2016) studied to compare the efficacy of spinetoram and spinosad against thrips on cotton and results showed that *Frankliniella fusca* (Hinds, 1902) (Thysanoptera: Thripidae) is more sensitive to spinetoram. In addition, spinetoram was not found to be adequately effective when the thrips population was high.

Conclusions

This study was conducted not only to find out the most suitable and effective insecticide against *T. hawaiiensis* but also to find out the best timing to initiate and maintain the chemical control. Flower application (Program 1) was ineffective and therefore not economical. However, in Programs 2 and 3, when the fruit were 18-33 mm and 26-42 mm in diameter respectively, were found to be effective. Similarly,

Atakan et al. (2021) determined the critical period to control of *T. hawaiiensis* was 3-5 weeks after petal fall in lemon orchards. The insecticide 25% spinetoram was the most successful insecticide in all programs with Spinosad (480 g/l) the second most effective in both years.

In the present study, the biological control potential of *O. laevigatus* was also studied. Although the predator was less efficacious than the insecticides, it should be considered as an option because this pest can easily gain resistance to the insecticides, therefore, the potential value of the predator should not be ignored. It might be possible to increase the effectiveness of the predator by releasing more than 20 adults per tree, an option that should be tested in the field. In summary, for the biological control of *T. hawaiiensis,* it is concluded that *O. laevigatus* has potential and can be used in low population orchards where 10% of flowers are infested with the pest. For orchards with higher pest populations, the predator releases would be more effective when integrated with narrow-spectrum and environmental-friendly insecticides like those in the spinosyn group or higher application rates of *O. laevigatus*. Similarly, Srivastava et al. (2018) conducted field experiments in 2005 and 2006 in northern Florida to evaluate the various rates of spinetoram for control of thrips and to determine the impact on natural populations of *O. insidiosus*. In that study, the mean numbers of the predator were quite high in all treatments, and their numbers relative to the numbers of thrips indicated that predation was sufficient to suppress thrips populations in all treatments.

There are no registered insecticides for *T. hawaiiensis* in citrus in Türkiye. The insecticides that are registered against thrips in other crops are very expensive so the growers avoid using them because of high costs. The usage of ineffective products results in failure in thrips management and preventing this may be possible with the use of the natural enemy, *O. laevigatus*.

Besides chemical and biological control of this pest, cultural measures are also of importance. During our study, we observed that *T. hawaiiensis* adults form a colony and lay eggs on the fruit that remained on the trees after harvest (Figure 4b). The thrips adults and larvae feed on these orange/yellow lemon fruit and support a population in the orchard before the trees commence flowering. Consequently, fruit that are not picked during harvest and left on the tree, act as a reservoir of thrips that reinvest flowers, so removal and appropriate disposal of these kinds of fruit is recommended. In addition, the flowers that develop during the fruiting period that have no economic importance, act as a reservoir of thrips that can move to and damage fruit and, therefore, should be picked and removed from the orchard.

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Original article (Orijinal araştırma)

Determination of potential insect vectors and subgroups of aster yellows phytoplasma in the carrot (*Daucus carota* L.) (Apiaceae) cultivation areas of Ankara and Konya Provinces, Türkiye[1]

Ankara ve Konya (Türkiye) illeri havuç (*Daucus carota* L.) (Apiaceae) ekim alanlarında aster yellows fitoplazmasının altgruplarının ve potansiyel böcek vektörlerinin belirlenmesi

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Abstract

Aster yellows phytoplasma (16Sr-I, AYp) is a widespread plant pathogen affecting a wide range of economically important crops. AYp can be distributed widely via insect vectors and is associated with severe redness and yellowing in carrot leaves. The presence of potential insect vectors of aster yellows phytoplasma was investigated in the Ankara and Konya Provinces, the largest carrot production areas in Türkiye. Forty-five insect samples were collected during the field studies between March and September 2020. Morphological and molecular studies have shown that *Neophilaenus campestris* (Fallén, 1805) (Hemiptera: Aphrophoridae), *Empoasca decipiens* Paoli, 1930 (Hemiptera: Cicadellidae) and *Psammotettix striatus* (L., 1758) (Hemiptera: Cicadellidae) carried 16SrI-F phytoplasma. *Psammotettix striatus* collected from different locations contained subgroup 16SrI-R, as well. In addition, subgroup 16SrI-B was determined in *Cicadula divaricata* Ribaut, 1952 (Hemiptera: Cicadellidae) and in a psyllid (Psylloidea: Psyllidae) species. *Empoasca* sp., *Anaceratagallia* sp. (Hemiptera: Cicadellidae) and *Psammotettix confinis* (Dahlbom, 1850) (Hemiptera: Cicadellidae) were determined as potential phytoplasma vectors. Phytoplasma 16Sr rRNA and insect cytochrome oxidase gene nucleotide sequences were used for phylogenetic analysis. The results will contribute to vector-based control of aster yellows phytoplasmas in carrot cultivation areas.

Keywords: Aster yellows, carrot, insect vector, PCR, phylogenetic

Öz

Aster yellows fitoplazma (AYp), ekonomik açıdan önemli farklı tarım ürünlerini etkileyen yaygın bir bitki patojenidir. AYp, böcek vektörleri aracılığıyla geniş alanlara yayılabilir ve havuç yapraklarında şiddetli kızarıklık ve sararma ile ilişkilendirilir. Türkiye'nin en büyük havuç üretim alanları olan Ankara ve Konya illerinde aster yellows fitoplazmasının potansiyel böcek vektörlerinin varlığı araştırılmıştır. Mart-Eylül 2020 tarihleri arasında arazi çalışmalarında 45 böcek örneği toplanmıştır. Morfolojik ve moleküler çalışmalar *Neophilaenus campestris* (Fallén, 1805) (Hemiptera: Aphrophoridae), *Empoasca decipiens* Paoli, 1930 (Hemiptera: Cicadellidae) ve *Psammotettix striatus* (L., 1758) (Hemiptera: Cicadellidae) türlerinin 16SrI-F altgrubu ile bulaşık olduğunu göstermiştir. Bununla birlikte, farklı lokasyonlardan toplanan *P. striatus* türü ise 16SrI-R altgrubu ile bulaşıktı. *Cicadula divaricata* Ribaut, 1952 (Hemiptera: Cicadellidae) ve bir psyllid (Psylloidea: Psyllidae) türünde 16SrI-B altgrubu saptanmıştır. *Empoasca* sp., *Anaceratagallia* sp. (Hemiptera: Cicadellidae) ve *Psammotettix confinis* (Dahlbom, 1850) (Hemiptera: Cicadellidae) ise potansiyel fitoplazma vektörleri olarak belirlenmiştir. Elde edilen fitoplazma 16S rRNA ve böcek sitokrom oksidaz genlerinin nükleotit dizileri filogenetik çalışmalarda kullanılmıştır. Bu sonuçlar, havuç ekim alanlarında aster yellows fitoplazmalarının vektör kontrolüne katkıda bulunacaktır.

Anahtar sözcükler: Aster yellows, havuç, böcek vektör, PCR, filogenetik

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Introduction

The carrot (*Daucus carota* L.), a member of the family Apiaceae, has a nearly 5000-year history of domestication and continues to be widely produced and consumed (Stolarczyk & Janick, 2011). The largest carrot cultivation areas in Türkiye are the Ankara and Konya Provinces with 578 kt of production annually (TUIK, 2021).

Carrot cultivation has been adversely affected by several phytopathogens and agricultural pests resulting in decreased yield quality and quantity. Among the phytopathogens, phytoplasma strains, *Candidatus Liberibacter solanacearum* Liefting et al. (Bacteria: Phyllobacteriaceae), and *Spiroplasma citri* Saglio et al. (Bacteria: Mycoplasmataceae), in particular, have been associated with significant carrot yield losses as a result of single or mixed infections (Lee et al., 2006; Cebrián et al., 2010; Satta et al., 2017). In addition to the transmission through seeds (Bertolini et al., 2015; Alfaro-Fernández et al., 2017; Carminati et al., 2019; Randa-Zelyüt et al., 2022), insect vectors are also responsible for the spread of phytoplasmas.

Phytoplasmas, Gram-positive bacteria, require host plant cells and insect vectors to maintain their unique life cycles and replication (Hogenhout & Loria, 2008). Effective pathogen-host-vector interactions can have a critical impact on plants, causing significant symptoms and yield losses in their hosts (Hogenhout et al., 2008). These mollicutes, which are found in almost every region of the world and infect more than a thousand plant species, lack a cell wall and are transmitted and spread by phloem insects of the order Hemiptera (Weintraub & Beanland, 2006; Harrison et al., 2014). More specifically, phytoplasmas are mainly spread by insects of the families Cicadellidae and Psyllidae and the superfamily Fulgoroidea, which feed on the phloem sap of infected plants. Therefore, the host range depends on the feeding habits of the insect vectors (Bertaccini, 2007).

Phytoplasmas have been reported to infect various vegetable crops in 47 countries throughout five continents (Kumari et al., 2019). Among them, aster yellows (16SrI) phytoplasmas are the most common across all genera, followed by the peanut witches' broom (16SrII), clover proliferation (16SrVI), and stolbur (16SrXII-A) phytoplasmas (Kumari et al., 2019). Moreover, phytoplasmas belonging to diverse subgroups of the aster yellows (AY) phytoplasma group (16SrI) have recently been related to diseases in carrots, including red leaves, shoot growth, and poor tap root quality (Duduk et al., 2007). In nature, phloem-feeding leafhoppers (Hemiptera: Cicadellidae) and planthoppers transmit AY group phytoplasmas persistently (Hemiptera: Cixiidae) (Weintraub & Beanland, 2006).

Since there is no effective control option directly against phytoplasmas during the cultivation period, the determination of potential insect vectors is necessary to design robust control programs. Therefore, in the present study, potential insect vectors of phytoplasmas collected from carrot cultivation areas in the Ankara and Konya Provinces of Türkiye have been morphologically and molecularly identified. In addition, the phytoplasma groups and subgroups contained by these insect vectors have been determined molecularly and phylogenetic tree and computer-simulated PCR-RFLP analyses were performed.

Materials and Methods

Sampling potential insect vectors

Field surveys were undertaken in carrot fields to collect insects in the Ankara and Konya Provinces of Türkiye in March-September in 2020. Only the areas that have carrot plants showing phytoplasma symptoms such as severe reddening and yellowing were sampled. The collected insects were directly transferred to 96% ethanol and stored at -20°C until used.

Morphological identification

All specimens were gently separated to avoid damaging key morphological characters for accurate identification. Morphological identification of potential vector insects was performed by Prof. Dr. Emine Demir-Özden under a stereo zoom microscope according to Ribaut (1952), Dlabola (1957), Emeljanov (1964), Ossiannilsson (1981) and Holzinger et al. (2003).

DNA isolation and PCR amplifications

Genomic DNA was extracted from insect specimens individually (n = 27) and three individuals as pooled samples (a total of six pools) using Qiagen DNeasy Blood & Tissue Kit following the manufacturer's instructions. Purity, concentration, and quality controls of the extracted DNAs were measured using a spectrophotometer (Nano-Drop 2000, ThermoFisher Scientific, Waltham, MA, USA). Then, DNA extracts were stored at −20°C until used in PCR amplification.

Molecular identification has been performed to verify/support the morphological identification and also to determine the insect species that cannot be identified morphologically due to damaged insect bodies.

The mitochondrial cytochrome oxidase I gene (*COI*) was used to identify insect specimens. *COI* has been known to provide species-level identification and is thus widely used across the animal kingdom (Hebert et al., 2003). Using the universal *COI* primers HCO2198-(5'-TAAACTTCAGGGTGACCAAAAAATCA-3') and LCO1490-(5'-GGTCAACAAATCATAAAGATATTGG-3') designed by Folmer et al. (1994), PCR amplifications were performed in a total reaction volume of 25 µl including 1.25 µl of Taq DNA polymerase (5 U/µl) (Ampliqon, Denmark) and 100-150 ng/µl of DNA. PCR conditions were according to İnak et al. (2021).

A nested PCR was performed to investigate the presence of phytoplasmas in potential insect vectors. The first step of nested-PCR was conducted using the 16S rRNA gene region amplifying P1-(5'-AAGAATTTGATCCTGGCTCAGGATT-3') /P7-(5'-CGTCCTTCATCGGCTCTT-3') universal primers (Deng & Hiruki, 1991). Before being used as a template in the second step of PCR reactions, the PCR products obtained from the first step were diluted at 1:30 with nuclease-free water. In the second step, universal primer pair R16F2n-(5'-GAAACGACTGCTAAGACTGG-3') -R2-(5'-TGACGGGCGGTGTGTACAAACCCCG-3') (Gundersen & Lee, 1996) or fU5-(5'-CGGCAATGGAGGAAACT-3') -rU3-(5'-TTCAGCTACTCTTTGTAACA-3') (Lorenz et al., 1995) were used. The nested-PCR condition was performed as described by Gundersan & Lee (1996). PCR products were separated on a 1% agarose gel with 100 V for 45 minutes.

The nested PCR reactions were performed using 30-50 ng/µl of genomic DNA (or 1 µl of diluted PCR product for the second step), 2.5 µl of 10X PCR buffer, 0.25 µl of 25 mM MgCl2, 1 µl of 10 mM dNTPs, 1 µl of 10 mM of each primer, and 1.25 U of Taq DNA polymerase (5 U/µl) (Ampliqon, Denmark) in a total reaction volume of 25 µl.

Sequencing and phylogenetic analysis

PCR products of the 16S rRNA gene of phytoplasmas and *COI* gene regions of insects obtained through molecular amplification were sequenced bidirectionally (BMlabsis, Ankara, Türkiye). The quality of sequence chromatographs was manually checked using BioEdit v7.0.5 (Hall, 1999).

BLAST analyses were performed to validate the identification of insects. The sequences were submitted to NCBI (National Center for Biotechnology Information). In addition, the similarity ratios of the nucleotide sequences belonging to phytoplasmas were obtained using F2n/R2 primer pair, and their subgroup classifications were determined using the *i*PhyClassifier software (Zhao et al., 2013).

Phylogenetic analyses were performed using the sequences herein obtained and some retrieved from the public GenBank to reveal the positioning of insect *COI* and phytoplasma 16SrRNA genes. All the sequences were aligned using MAFFT (Katoh et al., 2019) and trimmed using BioEdit v7.0.5 (Hall, 1999).

Next, a neighbor-joining phylogenetic tree has been constructed using MEGA X (Kumar et al., 2018) with 1000 bootstraps. The Tamura-3 (T92) (Tamura, 1992) parameter model has been identified to be the best-fit substitution model by MEGA X (Kumar et al., 2018). *Spiroplasma citri* (accession no AM157769) was used as an outgroup.

PCR-RFLP analysis

Restriction fragment length polymorphism (RFLP) analyses were performed using endonuclease enzymes to determine the genetic profiles of phytoplasma PCR products obtained with the F2n/R2 primer pair. The PCR products were digested with 6 U of restriction endonuclease *Alu*I and *Taq*I enzymes separately (Eurx, Estonia). The digested products were separated on a 1.7% agarose gel with 80 V for 2 h. The agarose gel was treated with ethidium bromide and visualized on a UV transilluminator (Genegenius, England).

Computer-simulated PCR-RFLP analyses for nucleotide sequences obtained from amplicons amplified with the F2n/R2 primer pair were visualized with the *i*PhyClassifier software using *Mse*I and *Alu*I endonuclease enzymes (https://plantpathology.ba.ars.usda.gov; Zhao et al., 2013).

Results

Field surveys and morphological identification of insects

During the field surveys, symptoms such as severe reddening and yellowing which were previously associated with phytoplasma infections were observed in the carrot plants. In addition, the roots of plants exhibiting the leaf symptoms also had lateral root development and abnormal discoloration. A total of forty-five potential vector insect specimens were collected from the fields showing these symptoms.

According to morphological investigations, one species *Neophilaenus campestris* (Fallén, 1805) belonging to the Aphrophoridae family, one species belonging to the family Psyllidae, one species *Javesella* sp. of the Delphacidae family, and nine species *Macropsis* sp., *Psammotettix striatus* (L., 1758), *Empoasca* sp., *Psammotettix* sp., *Euscelis incisus* (Kirschbaum, 1858), *Anaceratagallia ribauti* (Ossiannilsson, 1938), *Empoasca decipiens* Paoli, 1930, *Anaceratagallia* sp. and *Cicadula divaricata* (Ribaut, 1952) in the family Cicadellidae were identified (Figure 1 and Table 1). A list of identified insect species is presented in Table 1.

Table 1. Aster yellows phytoplasma potential insect vectors collected from carrot fields

Region	Species	Specimen	Family
Ankara	*Neophilaenus campestris*	2♀ 1♂	Aphrophoridae
	Macropsis sp.	2 ♀	Cicadellidae
	Psammotettix striatus	2♀ 1♂	Cicadellidae
	-	1*	Psyllidae
	Empoasca sp.	6*	Cicadellidae
	Psammotettix sp.	7*	Cicadellidae
Konya	*Euscelis incisus*	1♀	Cicadellidae
	Anaceratagallia ribauti	2♀	Cicadellidae
	Anaceratagallia sp.	3*	Cicadellidae
	Javesella sp.	1♀	Delphacidae
	Psammotettix striatus	9♀ 1♂	Cicadellidae
	Cicadula divaricata	1♀	Cicadellidae
	Empoasca decipiens	2♂;3♀	Cicadellidae
Total		45	

* Individuals that cannot be fully characterized morphologically.

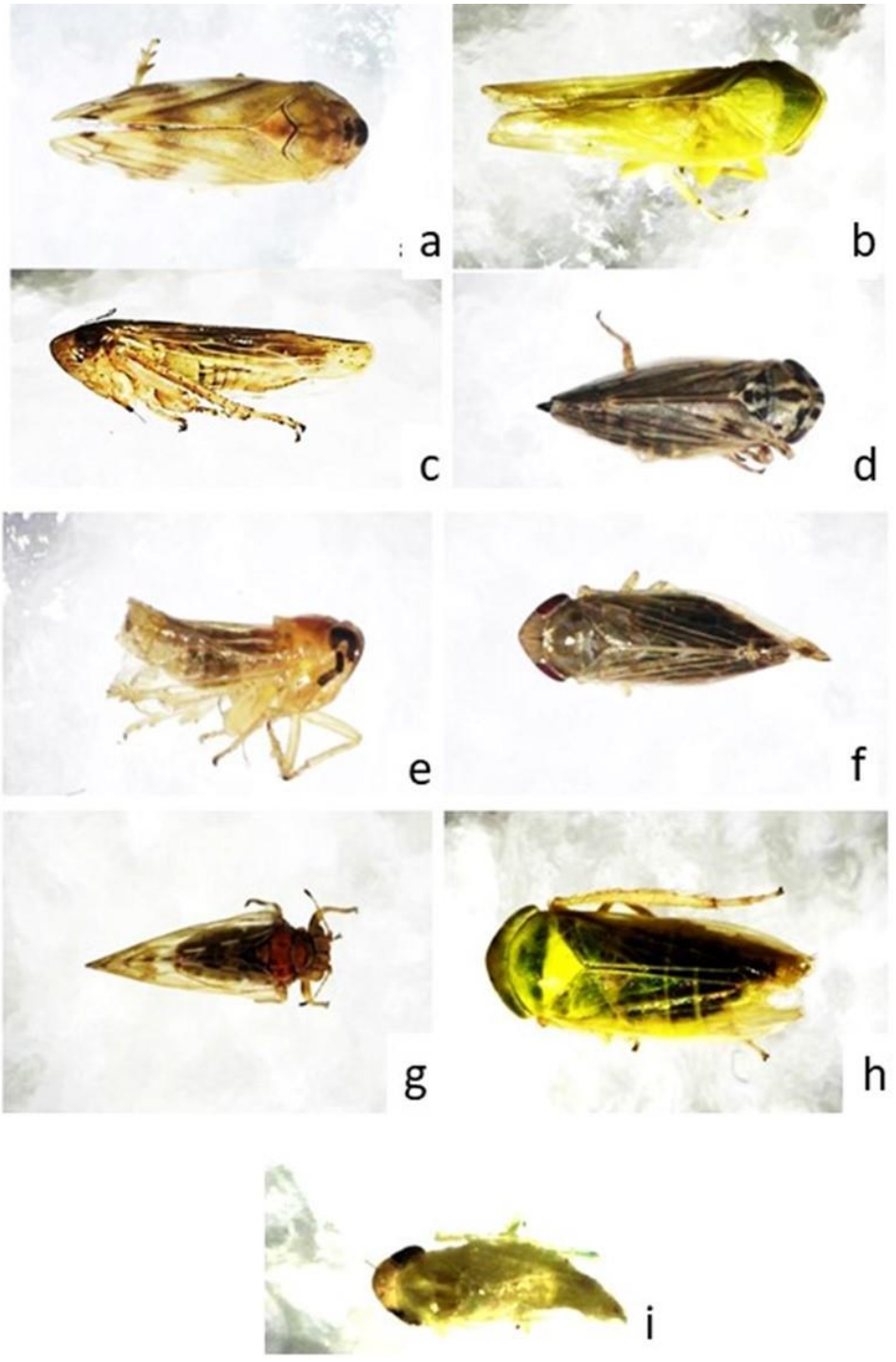

Figure 1. Aster yellows phytoplasma potential vectors collected in carrot fields: a) *Neophilaenus campestris* (Fallen); 1♀ (Aphrophoridae), b) *Macropsis* sp.; 1♀ (Cicadellidae), c) *Euscelis incisus* (Kirschbaum); 1♀ (Cicadellidae), d) *Anaceratagallia ribauti* (Ossiannilsson); 1♀ (Cicadellidae), e) *Javesella* sp.; 1♀ (Delphacidae), f) *Psammotettix striatus*; 1♀ (Cicadellidae), g) Psyllidae species, h) *Cicadula divaricata* (Ribautu); 1♀ (Cicadellidae), and i) *Empoasca decipiens* (Paoli) (Cicadellidae).

Molecular identification of insect species and associated phytoplasmas

For the molecular identification of potential phytoplasma vectors, *COI* sequences from 10 specimens in three species were obtained (accessions: MZ519869-MZ519878). Also, the *COI* gene sequence of the individual belonging to the family Psyllidae could not be obtained, despite the *COI* gene sequences of the morphologically unidentified *Psammotettix* sp., *Anaceratagallia* spp. and *Empoasca* spp. samples being obtained. Thus, all sequences obtained supported the morphological identification to genus level. Unfortunately, we were not able to identify these specimens to species level morphologically. However, BLAST analysis showed that B3, B4, K, B7 and 22B (*Psammotettix* spp.) specimens had 98.48-99.70% identity with deposited sequences of *Psammotettix confinis* from Canada and Ukraine, with accession numbers KR573169 and MW301811, respectively. Therefore, these specimens were considered to be *P. confinis* based on the high similarity of sequences. Although 7B and 9B (*Anaceratagallia* spp*.)* specimens were 96.80% similar to *Anaceratagallia ribauti* species (accession: MK188546), *Anaceratagallia* sequences herein obtained did not been clustered with *A. ribauti* in the phylogenetic tree, suggesting that they were different species. Finally, Y1, Y2 and B1 (*Empoasca* spp.) specimens were found to be 99.84% similar to *Empoasca* sp. from Pakistan (accession: HQ990703), however, the phylogenetic tree showed that they were an *Empoasca* species rather than *E. decipiens* (Table 2).

Table 2. Accession of insect specimens and similarity rates with the GenBank isolates

Insect specimen	Accession	NCBI similarity-Accession Number-Definition
B4 (*Psammotettix* spp.)	MZ519870	99.01%-KR573169-Canada-*Psammotettix confinis*
B1 (*Empoasca* spp.)	MZ519878	99.84%-HQ990703-Pakistan-*Empoasca* sp.
B7 (Psammotettix spp.)	MZ519872	99.00%-KR573169-Canada-*Psammotettix confinis*
K (*Psammotettix* spp.)	MZ519869	98.81%-MW301811-Ukraine-*Psammotettix confinis*-Rub-1
Y1 (*Empoasca* spp.)	MZ519876	99.85%-HQ990703-Pakistan-*Empoasca* sp. HOP-00013
Y2 (*Empoasca* spp.)	MZ519877	99.85%-HQ990703-Pakistan-*Empoasca* sp.
9B (*Anaceratagallia* spp.)	MZ519875	96.80%-MK188546-France-*Anaceratagallia ribauti*
7B (*Anaceratagallia* spp.)	MZ519874	96.80%-MK188546-France-*Anaceratagallia ribauti*
22B *(Psammotettix* spp.)	MZ519871	98.48%-KR573169-Canada-*Psammotettix confinis*
B3 *(Psammotettix* spp.)	MZ519873	99.70%-KR573169-Canada-*Psammotettix confinis*

The 16S rRNA gene region was amplified to determine the presence of phytoplasmas in the DNAs extracted from potential insect vectors. Overall results showing the presence of phytoplasmas in various vector species are given in Table 3. The presence of phytoplasmas was detected in nine of 27 individuals, indicating that 33.3% of screened insects were infected by various subgroups of phytoplasmas. In addition to individual testing, the presence of phytoplasmas was also investigated from pooled insect DNAs and the results showed that three of six pools were positive for phytoplasmas. This result showed that 50% of collective individuals could potentially transmit the pathogen.

All phytoplasma isolates from insect samples (based on either 883 bp or 1.2 kb) were sequenced for further analyses. Three sequences from 12 phytoplasma isolates [1.2 kb (B4-phy, B6-phy, B7-phy, K-phy and 15B-phy), 883 bp (12B-phy, 19B-phy, 21B-phy, 22B-phy, 5B-phy, B1-phy and B2-phy)] were submitted to the GenBank as accessions: MZ457919, MZ464025-MZ464031, MZ450789-MZ450792. The potential vector species and associated phytoplasma species are presented in Table 3. Isolates having 1.2 kb (B4-phy, B7-phy and K-phy isolates) sequences shared 99.92% nt identity with the NCBI isolate M30790 and the 16SrI-F *i*PhyClassifier isolate AY265211. Also, the 15B-phy isolate had 99.92% nt similarity with the NCBI isolate with accession number MN877914 and the 16SrI-B *i*PhyClassifier isolate with accession number AP006628. The B6-phy isolate; the NCBI Iran isolate with accession number MK307856; and the 16SrI-R *i*PhyClassifier isolate with accession number HM067754 had 99.20% nt identity. NCBI data was

used for nt similarity of seven other phytoplasma-infected insect isolates (883 bp). The B1-phy, B2-phy, 5B-phy, 22B-phy, 21B-phy, and 19B-phy isolates had 99.64-99.46% similarity with the Iranian isolate, Bajgah periwinkle little leaf phytoplasma, accession DQ266089. Finally, the 12B-phy isolate had 99.81% nt similarity with the rapeseed phyllody (16SrI-B) Polish isolate accession CP055264.

Table 3. Number of potential vector insects (single/collective) and number of infected specimens

Specimens	Total individuals	Infected/Individual sample	Infected/pooled sample*	16Sr group
Neophilaenus campestris	3	**(5B)** 1/3	-	16SrI **(5B-phy)**
Macropsis sp.	2	0/2	-	-
Euscelis incisus	1	0/1	-	-
Anaceratagallia ribauti	2	0/2	-	-
Javesella sp.	1	0/1	-	-
Psammotettix striatus	13	**(B6) (19B) (22B)** 3/7	0/2	16SrI-R **(B6-phy)**/16SrI **(19B-phy) (22B-phy)**
Psyllidae	1	**(12B)** 1/1	-	16SrI **(12B-phy)**
Empoasca decipiens	5	**(21B)** 1/2	0/1	16SrI **(21B-phy)**
Cicadula divaricata	1	**(15B)** 1/1	-	16SrI-B **(15B-phy)**
Anaceratagallia sp.	3	0/3	-	-
Psammotettix sp.	7	**(B7)** 1/1	**(B4) (K)** 2/2	16SrI-F **(B7-phy) (B4-phy) (K-phy)**
Empoasca sp.	6	**(B1)** 1/3	**(B2)** 1/1	16SrI **(B1-phy) (B2-phy)**
Total	45	9/27	3/6	

* Three individuals were used for each pool.

Phylogenetic analysis

The phylogenetic tree based on *COI* sequences of hemipteran species is presented in Figure 2. In general, the tree showed great resolution to genus level and allowed the genus-level identification of *Empoasca* and *Anaceratagallia* species. Also, the tree, together with BLAST analysis, suggest that the specimens which could not be identified to species level morphologically were *P. confinis* (Dahlbom, 1850), and it was supported with a confidence ratio of 100.

The phylogenetic tree was divided into several evolutionary lineage branches containing 16SrV, 16SrI, 16SrX and 16SrXII groups of phytoplasmas. All of the phytoplasma isolates obtained from potential vector insects were grouped within the 16SrI (aster yellows) main branch and the node of this cluster was supported by a confidence ratio of 99. Major cluster of 16SrI was subdivided into 16SrI-A, 16SrI-B, 16SrI-C, 16SrI-E, 16SrI-F and 16SrI-R subgroups. B1-phy (*Empoasca* sp.), B2-phy (*Empoasca* sp.), 5B-phy (*N. campestris*), 22B-phy (*P. striatus*), 21B-phy (*E. decipiens*), 19B-phy (*P. striatus*), B4-phy (*Psammotettix* sp.), B7-phy (*Psammotettix* sp.) and K-phy (*Psammotettix* sp.) isolates clustered with 16SrI-F subgroup isolates; B6-phy (*P. striatus*) isolate was within 16SrI-R subgroup; 15B-phy (*C. divaricata*) and 12B-phy (Psyllidae species) isolates clustered with 16SrI-B subgroup isolates. Although isolates with both 1.2 kb and 883 bp length sequences were located in a subcluster, these clusters were not supported by high bootstrap values (Figure 3). Moreover, it provided hypothetical information on the main groups which isolates B1-phy, B2-phy, 5B-phy, 22B-phy, 21B-phy, 19B-phy and 12B-phy included. To obtain this hypothetical information, it was necessary to include three or more large phytoplasma groups in the data set.

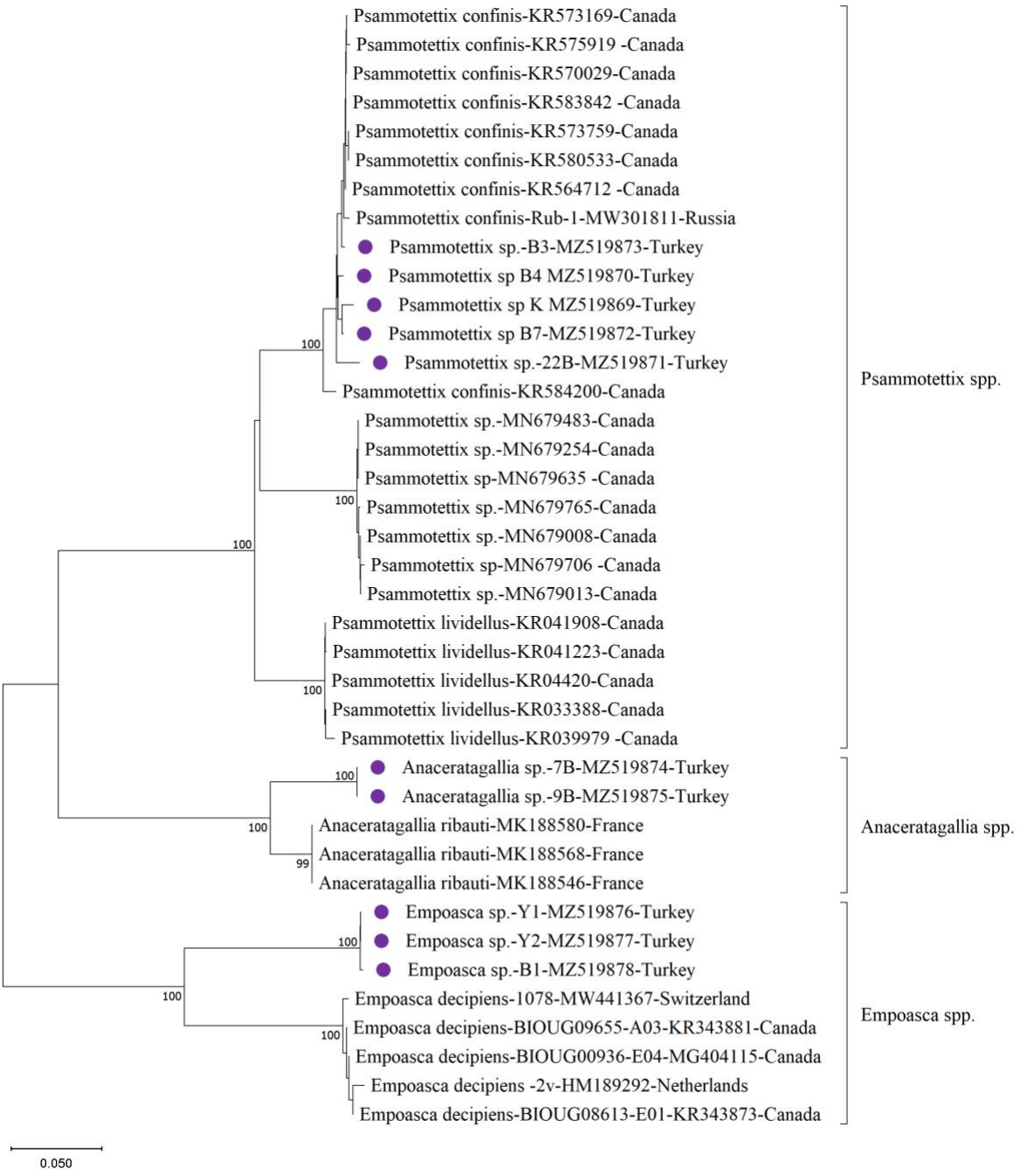

Figure 2. Phylogenetic tree generated by the Neighbor-Joining statistical method, and the substitution model Tamura-3 parameter (T92+G) of nt sequences of the insect *COI* genes. Insect specimens in this study are marked with circle symbols. Bootstrap values on each branch were supported by 1000 replicates; only values greater than 90% were shown.

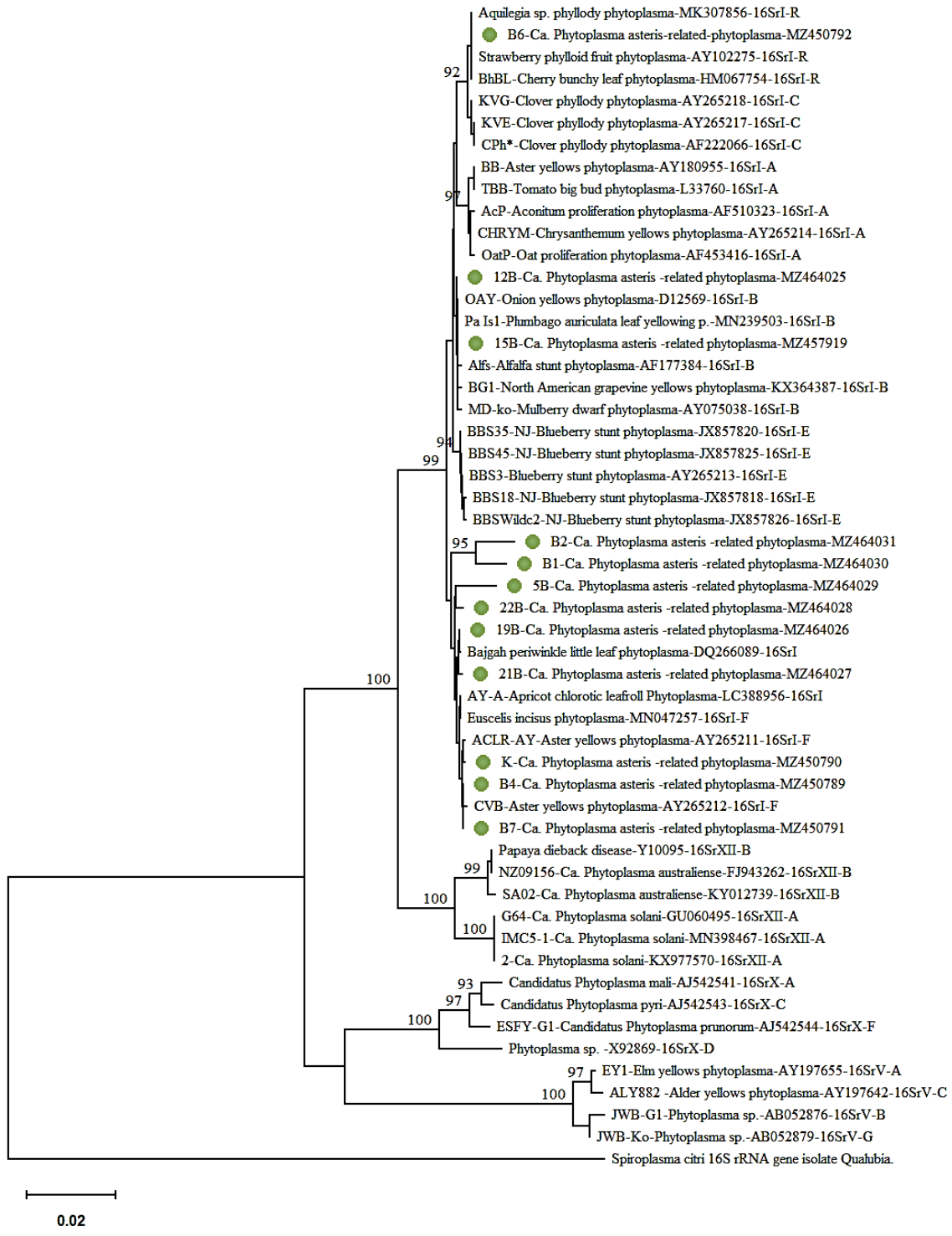

Figure 3. Phylogenetic tree generated using the Neighbor-Joining statistical method, and the substitution model Tamura-3 parameter (T92) of nucleotide sequences of the 16Sr gene of the phytoplasma isolates from insect specimens. A green circle is used to identify phytoplasma isolates in this study. Only values greater than 90% were shown in the bootstrap values on each branch, which was supported by 1000 replicates. *Spiroplasma citri* (accession no AM157769) was used as an outgroup.

In-vitro and *in-silico* PCR-RFLP analysis results

PCR products of 1.2 kb obtained from five individual insects (B4-phy, B6-phy, B7-phy, K-phy and 15B-phy isolates) which are potential phytoplasma vectors, were digested with *TaqI* and *AluI* enzymes *in vitro* conditions, as shown in Figure 4. The profiles were compared with the reference sample ‘’*Ca*. P. asteris’’ related strain cabbage chloranthy (Chlorantie du Chou, in French) CHLL.

Reference aster yellows group and its subgroup sequences were used to compare *in vitro* computer-simulated slaughter profiles of five individual putative insect vectors for which nt sequence analysis was completed. Accordingly, *AluI* and *MseI* endonuclease enzymes were used to separate subgroups in the *i*PhyClassifier (Zhao et al., 2013) software. In the digestion with the *AluI* enzyme, B4-phy, K-phy and B7-phy isolates had identical profiles with the 16SrI-F reference strain and separated from 16SrI-R-16Sr I-B subgroups. B4-phy, K-phy, B7-phy and 15B-phy isolates had the same patterns with each other in the *MseI* enzyme digestion profile. The B6-phy isolate was ideally mirrored to the profile of the 16SrI-R reference isolate and the other phytoplasma subgroups were separated from the 16SrI-B/16SrI-F subgroups (Figure 5).

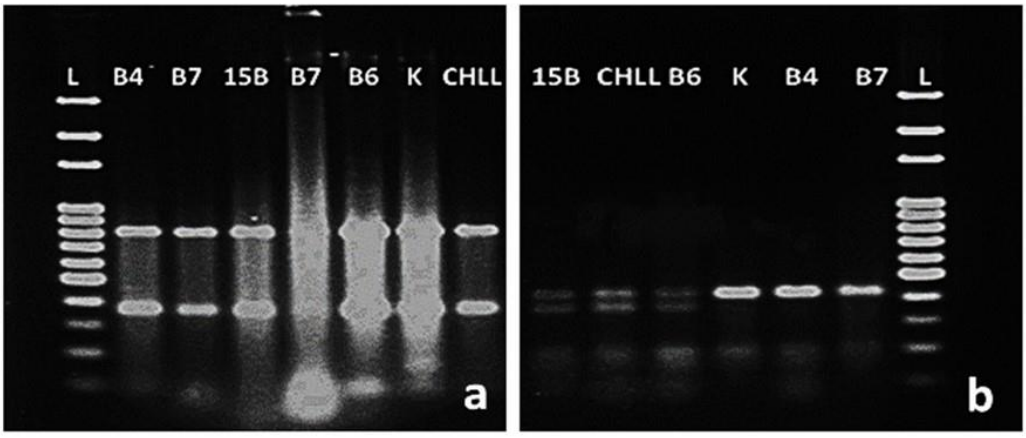

Figure 4. *TaqI* (a) and *AluI* (b) enzyme digestion profiles obtained by amplification of five phytoplasma potential vectors with R16F2n/R16R2 (1.2 kb) primers (B4-phy, *Psammotettix* sp.; B7-phy, *Psammotettix* sp.; K-phy, *Psammotettix* sp.; B6-phy, *P. striatus;* 15B-phy, *C. divaricata*; and reference strain CHLL, 16SrI).

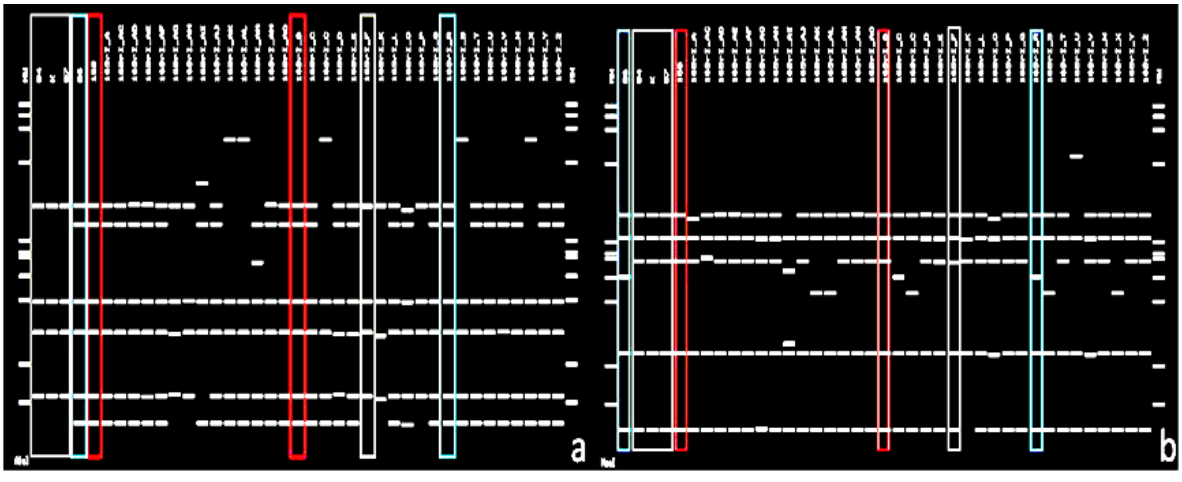

Figure 5. Digest profiles of insect isolates (B6-phy-*P. striatus*, B4-phy-*Psammotettix* sp., K-phy-*Psammotettix* sp., B7-phy-*Psammotettix* sp. and 15B-phy-*C. divaricata*) formed *in silico* with (a) *AluI* and (b) *MseI* enzymes and comparison with other 16SrI aster yellows reference subgroups. Red rectangles indicate the pattern of the 16SrI-B subgroup and the 15B-phy isolate, blue rectangles indicate the pattern of the 16SrI-R subgroup and the B6 isolate, and white rectangles indicate the pattern of the 16SrI-F subgroup with B4-phy, K-phy and B7-phy.

Discussion

Phytoplasmas can infect various economically important crop plants, and they can spread around via numerous hemipteran insect vectors, which provide a great example of tritrophic interactions: host-pathogen-vector (Weintraub & Beanland, 2006; Bertaccini & Lee, 2018). Although chemical pesticides are widely used to control insect vectors and thus prevent the spread of phytoplasmas, total eradication seems to be impossible (Kumari et al., 2019). Also, the lack of studies to determine which insect species can transmit the pathogens limits the design of robust vector control programs. In the present study, we investigated the potential insect vectors and phytoplasmas transmitted by them in carrot production areas in Ankara and Konya, which are the largest carrot-growing provinces in Türkiye.

Although more than 20 leafhopper and planthopper species (Insecta: Hemiptera) have been reported to successfully acquire and transmit AY phytoplasma (strains so far, the aster leafhopper, *Macrosteles quadrilineatus* Forbes, 1885 has been considered primary AY phytoplasma vector (Hoy et al., 1999; Frost et al., 2011). More specifically, in the northeastern USA and Canada, *M. quadrilineatus* and *Scaphytopius irroratus* Van Duzee, 1910 have been determined to be the main vector transmitting the 16SrI-A subgroup phytoplasma and 16SrI-B subgroup phytoplasma, respectively (Lee et al., 2006). Similarly, *M. quadrilineatus* was the most common vector species in carrot production areas of the USA, followed by *Empoasca fabae* (Harris, 1841), *Doratura stylata* (Boheman, 1847), and *Latalus* sp. (Stillson & Szendrei, 2020). Other *Macrosteles* species, *M. quadripunctulatus* (Kirschbaum, 1868) and *M. sexnotatus* (Fallén, 1806) species were found to be potential vectors of the 16SrI-A and 16SrI-B subgroups, while *Macrosteles laevis* (Ribaut, 1927) was identified to be a potential vector of the 16SrXII group in Serbian carrot growing areas (Duduk et al., 2008). Although we determined 13 hemipteran species (mostly belonging to the family Cicadellidae), we did not find any *Macrosteles* spp. in the surveyed areas. However, some *Macrosteles* spp. have been reported among the non-intensive pest populations in the sainfoin cultivation areas of the Ankara and Konya Provinces (Tamer et al., 1997). More specifically, the inability to reach *Macrosteles* spp. populations within the scope of this study may be related to parameters such as the frequency of surveillance, climatic changes, and the diversity of agricultural product patterns. *Psammotettix striatus* was found in both neighboring cities in the present study, however, overall vector fauna even in these two closely located areas seems to be quite different, indicating the importance of local pest control programs to prevent vectors from transmitting phytoplasmas in carrot fields.

Gera et al. (2011) collected a number of leafhopper species such as *Orosius orientalis* (Matsumura, 1914), *Circulifer* sp., *Exitianus capicola* (Stål, 1855), *Neoaliturus fenestratus* (Herrich-Schäffer, 1834) and *Hyalesthes obsoletus* Signoret, 1865 using sticky traps in a carrot field in Israel. However, we did not collect any of these species in the present study. In Serbia, several species belonging to the genera *Psammotettix* and *Anaceratagallia* (especially *P. confinis* and *A. laevis*) have been recorded in phytoplasma-infected carrot fields and the presence of AY (16SrI-A/C) and STOL (16SrXII-A) groups in these genera have been documented (Drobnjaković et al., 2010) whereas *Psammotettix* spp. (including *P. confinis*) sampled in the present study were infected with 16SrI-F/R subgroups. In parallel, *Empoasca* spp. collected from apricot-plum orchards have been reported to contain a 16SrX-B subgroup (Pastore et al. 2004) whereas the phylogenetic tree showed that the phytoplasma was isolated from *Empoasca* sp. from Türkiye carrot fields clustered with 16SrI-F subgroups. These results indicated that a single species or a certain genus can potentially transmit diverse groups of phytoplasmas. In addition, we determined that the 16SrI-B subgroup includes *C. divaricata* and a Psyllidae species, however, none of the collected samples had the 16SrXII subgroup. The 16SrI-F isolate obtained from insects sampled from carrot fields shared a high similarity (99.92%) with the ACLR-AY strain from apricot in Germany (accession: AY265211). This can be explained by the fact that aster yellows have a very wide host range (Kumari et al., 2019).

Although we initially identified *Psammotettix* to genus level based on morphology, molecular identification indicated that the species was *P. confinis* based on BLAST analysis. This clearly shows the usefulness of DNA-based vector identification which also provides early detection of potential vectors in field conditions that have crucial importance. In addition, molecular identification also allows species identification using all developmental stages of insects, contrary to morphological diagnosis which needs adults to make decisions. However, we could not identify the other two species to species level due to the lack of reference sequences, therefore, more and more studies are needed to enlarge the reference sequence database related to vectors of phytoplasma diseases.

Possible vector insects that could be a source of inoculum for phytoplasma infections in carrot-growing areas, as well as the phytoplasma groups transmitted by them, were revealed in this study. More research is needed to identify diseases caused by phytoplasmas in vegetable cultivation areas and characterize their vectors and indirectly control them. Also, an insect cannot be assumed to be a pure vector just because its body contains phytoplasma; therefore, a transmission assay is required in future studies to develop rational control strategies and to provide clear evidence of pathogen transmission in laboratory and field conditions.

Conclusion

The vector-phytoplasma-host complex can explain how they interact with their environments and how they persist, namely, how they fit into ecological niches. Although genetic variation in most phytoplasma subgroups appears to be associated with the ecological isolation of the organisms, genetic diversity in some phytoplasma subgroups (16SrI-A,B) is related to a wide diversity of host plants and insect vectors (McCoy et al., 1989). The families Aphrophoridae, Cicadellidae, Psyllidae and Delphacidae were identified as potential phytoplasma insect vectors in this study, which were sampled from carrot-growing areas. However, only members of the Cicadellidae and Psyllidae have been found to carry the phytoplasma of the aster yellows group in their natural habitat. In these regions where the continental climate is dominating, there is a greater requirement for the identification of vector insects that are effective in the transmission of phytoplasma diseases. On the other hand, identifying the phytoplasma groups and subgroup populations that can adapt to these environments and dominate their ecological niches may reveal more possibilities for developing an agricultural control strategy against these phytopathogens.

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Original article (Orijinal araştırma)

Biocontrol potential of *Heterorhabditis bacteriophora* Poinar, 1976 (Rhabditida: Heterorhabditidae) HBH hybrid strain against the beet webworm, *Loxostege sticticalis* L., 1761 (Lepidoptera: Pyralidae)[1]

Heterorhabditis bacteriophora Poinar, 1976 (Rhabditida: Heterorhabditidae) hibrit HBH ırkının ayçiçeği çayır tırtılı, *Loxostege sticticalis* L., 1761 (Lepidoptera: Pyralidae)'e karşı biyolojik mücadele potansiyeli

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Abstract

With limits on the use of pesticides, biological control has become increasingly important. Consequently, entomopathogenic nematodes (EPN) are now used widely in biological control. EPNs can potentially be used against beet webworm, *Loxostege sticticalis* L., 1761 (Lepidoptera: Pyralidae), which established in sunflower-growing areas in Türkiye in 2022. Therefore, the hybrid EPN strain, *Heterorhabditis bacteriophora* Poinar, 1976 (Rhabditida: Heterorhabditidae) HBH, was assessed for this purpose. The study was conducted in Bursa Uludağ University, Faculty of Agriculture, Plant Protection Department, Nematology Laboratory in 2022. Four nematode doses (2, 5, 10 and 20 IJs) were applied to the last instars of *L. sticticalis* at three temperatures (20, 25 and 30°C). The highest mortality was 97% with 20 IJs dose nematodes at 30°C. LD_{50} and LD_{90} of the nematode were determined at all tested temperatures. The lowest LD_{50} and LD_{90} were at 30°C; 4.37 and 11.0 IJs, respectively. These results indicated that the HBN strain has potential for control of *L. sticticalis*.

Keywords: Biological control, *Heterorhabditis bacteriophora, Loxostege sticticalis,* sunflower

Öz

Pestisit kullanımının sınırlandırılması ile birlikte buna alternatif olan biyolojik mücadele giderek daha önemli hale gelmiştir. Bu nedenle, 2022 yılında Türkiye'de ayçiçeği tarlalarında ayçiçeği yetiştirilen tarım alanlarında istilaya neden olan çayır tırtılı, *Loxostege sticticalis* L., 1761 (Lepidoptera: Pyralidae) zararlısına karşı EPN'lerin potansiyel olarak kullanılabileceği düşünülmektedir. Bu çalışmada *Heterorhabditis bacteriophora* Poinar, 1976 (Rhabditida: Heterorhabditidae) hibrit ırkı HBH kullanılmıştır. Bu çalışma 2022 yılında Bursa Uludağ Üniversitesi, Ziraat Fakültesi, Bitki Koruma Bölümü, Nematoloji Laboratuvarı'nda yürütülmüştür. Bu çalışmada 4 farklı nematod dozu (2, 5, 10 ve 20 IJs), böceğin son dönem larvası üzerine üç farklı sıcaklıkta (20, 25 ve 30°C) uygulanmıştır. Sonuçlara göre, en yüksek ölüm oranı %97 olarak 20 IJs doz nematod yoğunluğunda 30°C'de elde edilmiştir. Ayrıca, HBH hibrit ırkının LD_{50} ve LD_{90} değerleri uygulamada kullanılan tüm sıcaklık değerlerinde belirlenmiştir. En etkili LD_{50} ve LD_{90} değeri sırasıyla 4.37 ve 11.0 IJs olarak 30°C'de gözlenmiştir. Sonuçlar, bu HBH ırkın *L. sticticalis*'ye karşı potansiyel bir ajan olabileceğini göstermiştir.

Anahtar sözcükler: Biyolojik mücadele, *Heterorhabditis bacteriophora, Loxostege sticticalis,* ayçiçeği

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Introduction

Insect pests cause considerable yield and economic losses in agricultural production worldwide. Although the decrease in agricultural productivity can be attributed to various reasons, insects and diseases are the most important cause worldwide. Losses from plant pests range from 20 to 40% (Savary et al., 2012).

Chemical control methods have been used for many years in control of insect pests that cause economic losses to crop plants (Gaugler, 2002). Chemical insecticides can have adverse effects on non-target organisms such as humans, animals and natural enemies of pests (van der Blom et al., 2009). With the regulations adopted by the European Union in 2009 (Marchand & Robin, 2019), it was recommended that the use of pesticides should now be reduced (Barzman et al., 2015).

One of the best alternatives to chemical control is biological control. One of biological agents that can be used effectively is entomopathogenic nematodes (EPNs) (Gaugler, 2002) with no negative effects on beneficial and non-target organisms (Ehlers, 1996; Lacey et al., 2015).

The EPNs, belonging to the order Rhabditida in the families Heterorhabditidae and Steinernematidae, live underground and seek insects in which to complete their life cycle. Infective juveniles (IJs) of EPNs can search for a host in the soil for long periods without feeding. The IJs are third stage juveniles (Glazer, 2002).

Heterorhabditis bacteriophora Poinar, 1976 (Rhabditida: Heterorhabditidae), in particular, is effective in for control of pests in a range of crop plants and remains alive in the soil for about 2 years (Susurluk & Ehlers, 2008). Nematodes in the Heterorhabditidae and Steinernematidae have a symbiotic relationship with bacteria, *Photorhabdus* spp. and *Xenorhabdus* spp., respectively. These bacteria belonging to the family Enterobacteriaceae and are present in the digestive system of third stage juvenile and pass to the host following nematode infection of the host, causing death of the host (Boemare et al., 1996).

Heterorhabditis bacteriophora HBH strain created by hybridization and patented was used in the study. This HBH hybrid strain has superior life characteristics and has adapted to the conditions of Türkiye.

The beet webworm, *Loxostege sticticalis* L., 1761 (Lepidoptera: Pyralidae), is commonly seen in eastern and western Europe (Pepper, 1938; Lizhi et al., 2009; Kong et al., 2010). *Loxostege sticticalis* is a highly invasive species that damage to crop plants such as sunflowers and maize. High populations of this pest are found on adult migration routes (Yue & Yuan, 1983; Luo, 2004). When population explosion occurs in a particular area, this stimulates migration behavior (Tammaru et al., 2000; Kong et al., 2010). *Loxostege sticticalis* is also capable of traveling particularly long distances as a result of the morphology and physiology of their adults (Yajie & Ruilu, 1995). Despite there being many studies on this insect, there are very few studies on control methods and especially biological control (Malysh et al., 2021).

The objective of this study was to assess the effectiveness of HBH hybrid EPN strain against the beet webworm in laboratory bioassays. Three temperatures and four nematode doses were tested. The efficacy of EPNs against *L. sticticalis* has not been assessed before and therefore this study is of primary importance for determining potential options for control of *L. sticticalis*.

Materials and Methods

Beet webworm and entomopathogenic nematode

HBH, a hybrid strain of *H. bacteriophora*, which has features such as high efficiency and longevity, was used based on the results of earlier hybridization studies. This hybrid strain was patented in 2018 (Patent No: TR 2013 06141 B). HBH is a superior breed adapted to the climatic conditions of Türkiye and due to these characteristics (Ulu & Susurluk, 2014). *In vivo* production of the strain was done in the last instar of *Galleria mellonella* L., 1758 (Lepidoptera: Pyralidae) larvae in the study. 2-3-day-old IJs formed using *in vivo* methods were used (Kaya & Stock, 1997; Şahin & Susurluk, 2020).

The last instars larvae of *L. sticticalis* were collected from infested sunflower fields of Bursa Uludağ University, Agricultural Research and Application Center. Simultaneously with the collection of these pests, the experiment phase was started after the larvae were brought to laboratory.

Experimental design

The experiment was conducted in 24-well tissue culture plates (each well; 1.5 cm diameter x 3 cm deep). One larva was placed in each well, and then filled with soil at 10% moisture. Four doses (2, 5, 10 and 20 IJs) of HBH were then applied to the top of the soil (under a binocular microscope at low doses) and the plates sealed with paraffin. Each HBH does rate was incubated at 20, 25 and 30°C for 3 days. Water only controls were also included.

After 3 days, all plates were open and the mortality of larvae determined. All dead larvae were dissected and examined for EPN juveniles in order to determine if their death was a result of dead by EPN infestation. Three plates were used for each dose and temperature with 20 larvae used in each plate (n = 20) and all assessment were repeated three times.

Statistical analyses

Mortality data were analyzed by analysis of variance using JMP®7.0 software. In addition, the least significant difference test ($p < 0.05$) was also performed. Probit analysis was performed with XLSTAT® software to calculate LD values. Data were fit to a response model using a non-linear regression.

Results

With increasing dose, mortality also increased at all temperatures except for 10 and 20 IJs at 30°C. At 20°C, the mortality ranged between 8 and 73%. The differences between the mortalities in all doses were statistically significant ($F = 75.6$; $df = 3,8$; $P < 0.0001$). At 25°C, the mortality ranged between 10 and 95% and were all statistically significant ($F = 188$; $df = 3,8$; $P < 0.0001$). At 30°C, the mortalities ranged between 15 and 97%, with 2 and 5 IJs the mortality was statistically different ($F = 265$; $df = 3,8$; $P < 0.0001$) whereas between the 10 and 20 IJs there was no significant difference. No larvae used in control treatments died. HBH application gave substantive mortality even at the low dose 5 IJs with over 70% mortality at the 30°C (Figure 1).

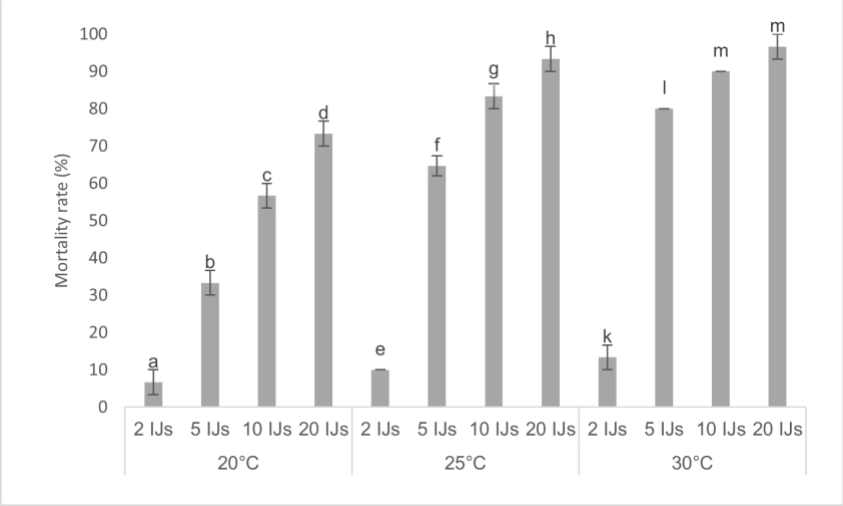

Figure 1. Mortality of *Loxostege sticticalis* was analyzed separately and statistically at each temperature value. Means in columns followed by the same letters are not significant different.

Across all temperatures, mortalities of 2, 5, 10 and 20 IJs doses ranged between 8 and 15%, 35 and 80%, 60 and 90%, 75 and 97%, respectively (Figure 1).

LD_{50} and LD_{90} values deceased with increasing temperatures as mortality increased with temperature. The LD_{50} (4.38) and LD_{90} (11.0) values at 30°C were lower than those at the other temperatures. The greatest effect of the HBH strain was at 30°C. The LD_{50} and LD_{90} values at 25 and 30°C were closer than at 20°C (Table 1). Regression analyses of the mortality values over the dose range for each temperature are presented graphically in Figure 2.

Table 1. LD_{50} and LD_{90} values of the HBH strain on *Loxostege sticticalis*

Temperature (°C)		Dose (IJs)	Std. Error	Lower	Upper
20	LD_{50}	11.2	1.26	8.97	14.3
	LD_{90}	24.6	3.21	19.8	34.2
25	LD_{50}	4.69	0.79	2.84	6.18
	LD_{90}	13.6	1.61	11.2	18.3
30	LD_{50}	4.38	0.75	2.64	5.82
	LD_{90}	11.0	1.28	9.06	14.7

Discussion

A decision was made by the European Union in 2009 to limit the use of pesticides. This has stimulated the use of biological control, which can be a highly effective method of combating pests (Marchand and Robin, 2019)).

Loxostege sticticalis is a species that can damage a wide range of field crop species. Although this varies between regions, it can can complete five life cycles per year. In addition, many larvae can invade plants at the same time, give the large number of egg-laying females (Kong et al., 2010; Frolov, 2015). *Loxostege sticticalis* has recently established in sunflower fields in Türkiye and has caused considerable damage. Therefore, this study was conducted to assess an EPN as alternative to pesticides. Entering soil to pupae after larval stage, this insect is a vulnerable host for EPNs. Therefore, there is potential to use EPNs in its control but this has not previously been assessed.

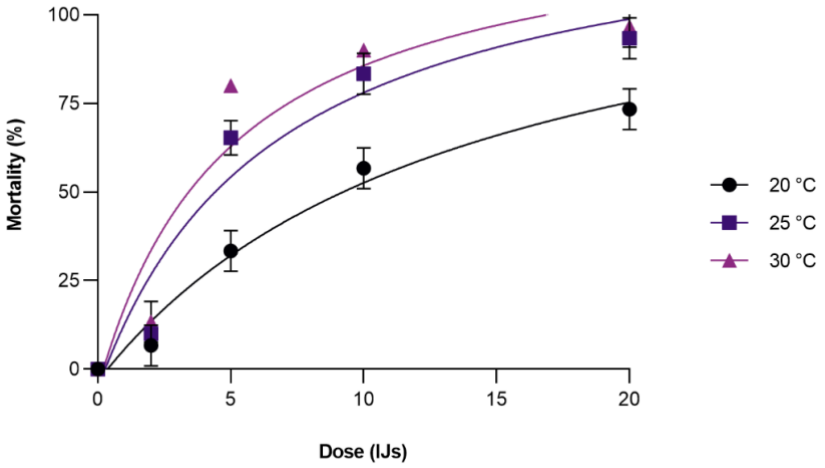

Figure 2. Regression analysis of mortality values at 20, 25 and 30°C against applied EPN doses (2, 5, 10 and 20 IJs).

Heterorhabditis bacteriophora has been widely and effectively used in the agricultural areas against many insect pests (Ehlers, 1996; Susurluk & Ehlers, 2008; Şahin et al., 2018). There are many commercial products based on this species. These are mostly used in temperate climatic regions; since they are not effective below 12°C (Boemare et al., 1996; Gaugler, 2002; Glazer, 2002). The fact that *H. bacteriophora* is cruiser is one of the reasons it is a preferred EPN species. Given that the last instar larvae of *L. sticticalis* move into soil for pupation, it is likely that EPNs could be used for its control in Türkiye. The patented hybrid strain (*H. bacteriophora* HBH) was assessed for this purpose because of its known suitability for the conditions of Türkiye and this choice was supported by the high mortalities founding in the present study.

In general, it has been found that mortality increases as the temperature increases in most EPNs efficacy trials (Fitters et al., 2001; Susurluk, 2008; Ulu & Susurluk, 2014). Consistent with previous studies, the same was found in the present study. Doses used in previous studies have generally been high, 50, 100 or 200 IJs/larva and even more. However, the usefulness of a species or strain is increase if it is effective at low doses (Ulu et al., 2015). Therefore, in this study, doses of *H. bacteriophora* HBH hybrid strain were used as low as possible, that is, 2 and 5 IJs/larva, with mortality ranging from 8 and 80%. This indicates the high the susceptibility of *L. sticticalis* and the efficacy of this EPN strain (Frolov, 2015; Cheng et al., 2016). Efficacy at low doses is critical for achieving economically acceptable commercial outcomes for EPNs in field applications.

The temperatures used in the study were those at which EPNs are most active. Many EPNs efficacy studies have been conducted in this temperature range (Susurluk, 2008; Mukuka et al., 2010). In this respect, the present study is consistent with many studies. Also, the beet webworm completes its life cycle during the summer months within the soil temperatures used in this study (Kong et al., 2010; Frolov, 2015), so the test temperatures were appropriate.

Ulu et al. (2015) found that LD_{50} and LD_{90} values for *H. bacteriophora* against the larvae of the yellow saw fly, *Hoplocampa flava* (L., 1761) (Hymenoptera: Tenthredinidae), were 6.5 and 15.5 at 25°C, respectively. These findings are consistent with the results of present study at 25°C.

There are few studies on biological control of *L. sticticalis*. One of these used a microsporidium, *Nosema pyrausta* (A.Paillot) J. Weiser, 1961 (Protozoa: Nosematidae), against the insect and it was determined to be a promising biocontrol agent for *L. sticticalis* (Malysh et al., 2018, 2021). However, this microsporidium is unlikely to be commercially viable for large scale field application. Lizhi et al. (2016) found that the parasitoid wasp, *Orgilus ischnus* Marshall, 1898 (Hymenoptera: Braconidae), is also an a

potentially effective biocontrol agent but only for early stages of larval *L. sticticalis*. Consistently, Luo et al. (2018) found that some hymenopteran wasps attack beet webworm, but their effects indicated limited potential for control at a field scale.

According to results of the present study, it is clear that the test EPN was quite effective for the control of *L. sticticalis* in a laboratory context. Therefore, this demonstrates that is a promising choice for evaluation of field control of this invasive species. If this proved successful, it would help in limiting the use of pesticides by the adoption of alternative control methods against agricultural pests.

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Original article (Orijinal araştırma)

Widespread and high levels of resistance to spinosad and spinetoram in *Frankliniella occidentalis* (Pergande, 1895) (Thysanoptera: Thripidae) populations of Antalya Province (Türkiye)[1]

Frankliniella occidentalis (Pergande, 1895) (Thysanoptera: Thripidae) Antalya ili (Türkiye) popülasyonlarında yaygın ve yüksek düzeyde spinosad ve spinetoram direnci

Badegül KAMIŞ[2] **Fatih DAĞLI[3*]**

Abstract

The western flower thrips, *Frankliniella occidentalis* (Pergande, 1895) (Thysanoptera: Thripidae) is an important agricultural pest worldwide. This invasive thrips has a significant threat to greenhouse vegetable production and export in Antalya. In this study, the prevalence of spinosad and spinetoram resistance in *F. occidentalis* populations from greenhouse locations in Antalya Province (Türkiye) was investigated. Eight *F. occidentalis* populations were taken from vegetable greenhouses in 2018-2019. A leaf dip bioassay was used to determine LC values and resistance levels. Spinosad and spinetoram resistance in the assayed populations were 19-312 and 5-170 times that of the susceptible population, respectively. The findings showed that spinosad and spinetoram resistance has reached significant levels and is now common in Antalya populations. Also, the stability of spinosad and spinetoram resistance was monitored in the most resistant population (Manavgat) for 6 months without insecticides. No significant decline in resistance was not found for both spinosad and spinetoram in this population over this period.

Keywords: *Frankliniella occidentalis*, resistance, spinosyn, stability, Türkiye

Öz

Batı çiçek thripsi, *Frankliniella occidentalis* (Pergande, 1895) (Thysanoptera: Thripidae) dünya çapında önemli bir tarımsal zararlıdır. Bu istilacı thrips, Antalya'da örtü altı sebze üretimi ve ihracatı için önemli bir tehdit oluşturmaktadır. Bu çalışmada Antalya sera üretim alanlarından alınan *F. occidentalis* popülasyonlarında spinosad ve spinetoram direncinin yaygınlık durumu araştırılmıştır. Sebze üretimi yapılan seralardan 2018-2019 yıllarında sekiz *F. occidentalis* popülasyonu toplanmıştır. LC değerlerinin ve direnç seviyelerinin belirlenmesi için yaprak daldırma test yöntemi kullanılmıştır. Test edilen popülasyonlarda spinosad ve spinetoram için direnç oranları duyarlı popülasyona göre sırasıyla 19-312 ve 5-170 kattır. Bulgular, Antalya popülasyonlarında spinosad ve spinetoram direncinin önemli düzeylere ulaştığını ve yaygın duruma geldiğini göstermiştir. Ayrıca, spinosad ve spinetoram direncinin stabiliteleri, en yüksek dirence sahip (Manavgat) popülasyonunda 6 aylık bir süre boyunca insektisit uygulanmaksızın izlenmiştir. Bu popülasyonda hem spinosad hem de spinetoram için bu süre içerisinde direnç düzeylerindeki düşüş önemli bulunmamıştır.

Anahtar sözcükler: *Frankliniella occidentalis*, direnç, spinosyn, kalıcılık, Türkiye

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Introduction

The western flower thrips, *Frankliniella occidentalis* (Pergande, 1895) (Thysanoptera: Thripidae), is a serious agricultural pest worldwide which causes economic losses in many crops both feeding on host plants and as a vector of several serious viruses including tomato spotted wilt virus (TSWV) (EPPO, 1999; Kirk & Terry, 2003; Mouden et al., 2017). *Frankliniella occidentalis*, originated in North America, and has become widespread in many countries in Africa, America, Asia, Australia and Europe (EPPO, 2022a). This species is among the most resistant pests to insecticides worldwide (Gao et al., 2012; Sparks et al., 2012; APRD, 2022).

Numerous studies associated with this issue in various countries have shown that many population of this thrips species have gained resistance to not only organic chlorine, organophosphorus, carbamate and pyrethroid classes but also new generation bioinsecticides classes such as avermectin and spinosyn (Immaraju et al., 1992; Brodsgaard, 1994; Jensen, 1998; Kontsedalov et al., 1998; Jensen, 2000; Espinosa et al., 2002; Herron & James, 2005; Bielza et al., 2007; Thalavaisundaram et al., 2008; Zhang et al., 2008; Gao et al., 2012; Sparks et al., 2012; Dong-Gang et al., 2016; Dağlı, 2018; Cubillos-Salamanca et al., 2019; APRD, 2022). *Frankliniella occidentalis* was the first detected in Türkiye in 1993 (Tunç & Göçmen, 1995). Subsequently, it spread rapidly and has already become established in a large number of crop species in most parts of the Türkiye (Tunç & Göçmen, 1995; Bulut & Göçmen, 2000; Atakan, 2003; Kılıç & Yoldaş, 2004; Özsemerci et al., 2006; Sertkaya et al., 2006; Nas et al., 2007; Atakan, 2008a, b; Doğanlar & Aydın, 2009; Tekşam & Tunç, 2009; Hazır et al. 2011; Yıldırım & Başpınar, 2013). Greenhouse cultivation is quite common and extremely valuable from an economic point of view in Antalya. In 2020, 381 kt of fresh vegetables (worth about 307 million USD) were exported from Antalya (Anonymous, 2022a). *Frankliniella occidentalis* is one of the most harmful pest insects in crop plants mainly vegetables grown in greenhouse in Antalya. In addition to the injury caused by feeding directly on the crops, it causes significant economic losses due to its transmission of TSWV, common problem in Türkiye (Şevik, 2011; Şevik & Arlı-Sökmen, 2012; Fidan, 2016; Fidan & Sarı, 2019). In addition, officials of the Antalya Directorate of Agricultural Quarantine report that this thrips sometimes prevents agricultural exports from Antalya, as it is a quarantine pest (EPPO, 2022b). The area where biological and biotechnical control is applied (1.55 kha) is still quite limited when compared to the total greenhouse cultivation area (31.2 kha) in Antalya (Anonymous, 2022a). This situation still leads to the heavily use of pesticides in the management against *F. occidentalis* and other major pests in greenhouse growing. It has been reported that 11 kt of insecticide was used in 2021 against agricultural pests in Türkiye, and about 10% of this amount was applied in Antalya (Anonymous, 2022b). Currently, spinosad and spinetoram are the main active substances used against *F. occidentalis* in Türkiye as well as worldwide (Gao et al., 2012; Bacci et al., 2016). These two insecticides are derivatives of biologically active ingredient produced by *Saccharopolyspora spinosa* Mertz & Yao, 1990, and these spinosyn compounds are considered to have a low environmental risk (Bacci et al., 2016). However, in a previous study, a high level of spinosad resistance (235 times) to *F. occidentalis* was detected in one location (Kumluca) in 2015 (Dağlı, 2018). Due to their considerable safety, use of spinosad, and spinetoram have expanded in agricultural areas in the region recently by obtaining recommendations against important pests in vegetables, industrial plants, vineyards and various fruits (Anonymous, 2022c). More widespread and frequent use of spinosad and spinetoram may lead to increased selection pressure on populations (mainly thrips) which may cause the problem of resistance to these two active ingredients to become more serious. Bioinsecticides such as spinosad and spinetoram should be used within scope of resistance management programs to extend their effective lifespan as much as possible. Periodic screening of populations at different locations and obtaining current resistance levels are necessary to recommend the correct actives and suggestions about pesticide use against pest insects including thrips species.

This study aims to reveal the prevalence of spinosad and spinetoram resistance in *F. occidentalis* populations collected from greenhouses in Antalya Province, especially coastal districts. Additionally, the stability of spinosad and spinetoram resistance in a highly resistant *F. occidentalis* population was investigated. The findings of this study could be contributed to management of *F. occidentalis* by reducing the economic and ecological losses due to insecticide resistance to a certain extent.

Materials and Methods

Collection of thrips populations

A susceptible population of the *F. occidentalis* was collected from a home garden in district Şuhut located at Afyonkarahisar Province (Türkiye) (38°31'40" N, 30°32'45" E) in 2017. Pesticides have generally not been used in that garden. Therefore, this *F. occidentalis* population was found to be quite susceptible to spinosad and spinetoram. One-fiftieth of the recommended dose of spinosad and one percent of the recommended dose of spinetoram cause 90% mortality in this susceptible population.

The greenhouses populations of *F. occidentalis* to be screened for resistance were collected from eight locations in 2018-2019 from greenhouses in districts Aksu, Alanya, Demre, Gazipaşa, Kumluca, Manavgat and Serik in Antalya (Figure 1).

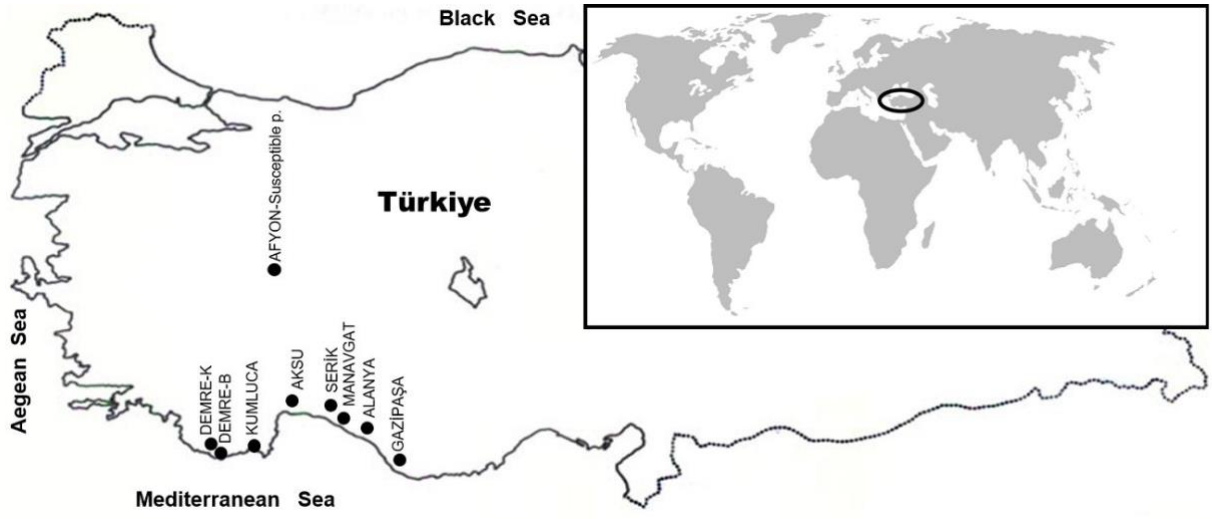

Figure 1. Sampling locations for *Frankliniella occidentalis* populations in Türkiye.

Detailed information about the hosts and locations of the populations collected is given in Table 1. At least 100 adult thrips were collected from the commercial greenhouses to represent each population, and were placed in plastic containers with ventilation openings. The samples were brought to the department laboratory on the day of collection and the thrips collected with a mouth aspirator for transfer to rearing containers with green bean pods. Species identification for the collected populations was undertaken according to Tunç & Göçmen (1995), Doğanlar & Aydın (2009) and Cluever et al. (2015).

Table 1. Locations, host plants, and coordinates of *Frankliniella occidentalis* test populations collected in 2017-2019

Population	Host	Location	Collection date	Coordinates
Aksu	Eggplant	Aksu (Hacıaliler)	08.05.2018	36°55'39" N, 30°50'13" E
Alanya	Eggplant	Alanya (Emişbeleni)	01.05.2018	36°37'25" N, 31°53'08" E
Demre-Beymelek (B)	Pepper	Demre (Beymelek)	03.05.2018	36°14'52" N, 30°01'44" E
Demre-Köşkerler (K)	Pepper	Demre (Köşkerler)	03.05.2018	36°16'14" N, 29°59'40" E
Gazipaşa	Eggplant	Gazipaşa (Macar)	01.05.2018	36°13'27" N, 32°20'31" E
Kumluca	Pepper	Kumluca (Salur)	03.05.2018	36°21'56" N, 30°14'18" E
Manavgat	Eggplant & pepper	Manavgat (Denizyaka)	01.05.2018	36°51'32" N, 31°11'12" E & 36°51'02" N, 31°11'01" E
Serik	Pepper	Serik (Çakış)	02.01.2019	36°55'18" N, 31°11'18" E
Susceptible (home garden)	Pepper	Şuhut, Afyonkarahisar	2017	38°31'40" N, 30°32'44" E

Insecticides

Spinosad and spinetoram were used in this investigation. Details of these active ingredients are given in Table 2.

Table 2. Information about active ingredients spinosad and spinetoram used in experiments

Active ingredient	Commercial name / registration date in Türkiye	Recommended dose for *F. occidentalis*	Active ingredient mg (a.i.)/L	Mode of action (IRAC, 2022)
Spinosad	Laser 480SC Dow Agro Sciences / 1998 (Anonymous, 2022d)	20 ml/decare (Anonymous, 2022d)	96	Nerve action, Nicotinic acetylcholine receptor (nAChR) allosteric modulators, (5).
Spinetoram	Radiant 120SC Dow Agro Sciences / 2014 (Anonymous, 2022e)	50 ml/decare (Anonymous, 2022e)	60	Nerve action, Nicotinic acetylcholine receptor (nAChR) allosteric modulators, (5).

Thrips rearing method

The rearing method of *F. occidentalis* populations was adapted from Steiner & Goodwin (1998), Murai & Loomans (2001) and Espinosa et al. (2002), and was given in detail in the previous study (Dağlı, 2018). Adults were collected from the inside of the vegetable plant flowers with a mouth aspirator and transferred to transparent plastic containers (2 L) covered with filter paper. Green bean fruits were left in the culture cups for feeding and egg laying of thrips. The green beans used here were disinfected with the sodium hypochlorite (6 g/L), then they were dipped in a sugar solution (5 g/L) and left to dry. Green bean fruits in the cultures were replaced every 3-4 days. All *F. occidentalis* populations were continued in a climate room at 23 ± 1°C and a 16:8 h L:D photoperiod.

Insecticide bioassay method

In this study, the leaf dipping method described by Zhang et al. (2008) for the same thrips species was used to calculation LC of the populations. The insecticide test method was presented in detail in the previous study (Dağlı, 2018). Briefly, the bioassay was as follows. First, a 4-6-step dose series was prepared in distilled water including TritonX-100 coving concentrations known to give to 5 and 95% mortality in populations. Bean leaf discs (3 cm) were dipped (5 s) into the insecticide concentrations or in distilled water (as control). After the droplets on the surface dried the discs were placed on the agar in a Petri dish (Figure 2). Adult female thrips were then collected from rearing cups using a small mouth aspirator,

anesthetized with CO_2 and poured onto the leaf discs. The Petri dishes were covered with stretch film and were perforated with an insect pin. At least three replicates were used for each tested concentration. Generally, around 20 adults female thrips (mixed-age) were used in each replicate, however, more than 20 individuals were used in some testing populations with large numbers of thrips. Control mortality did not exceed 12%. The mortality of the tested thrips was determined after 3 days. Thrips were considered as dead if they did not any respond when prodded with a brush or pin.

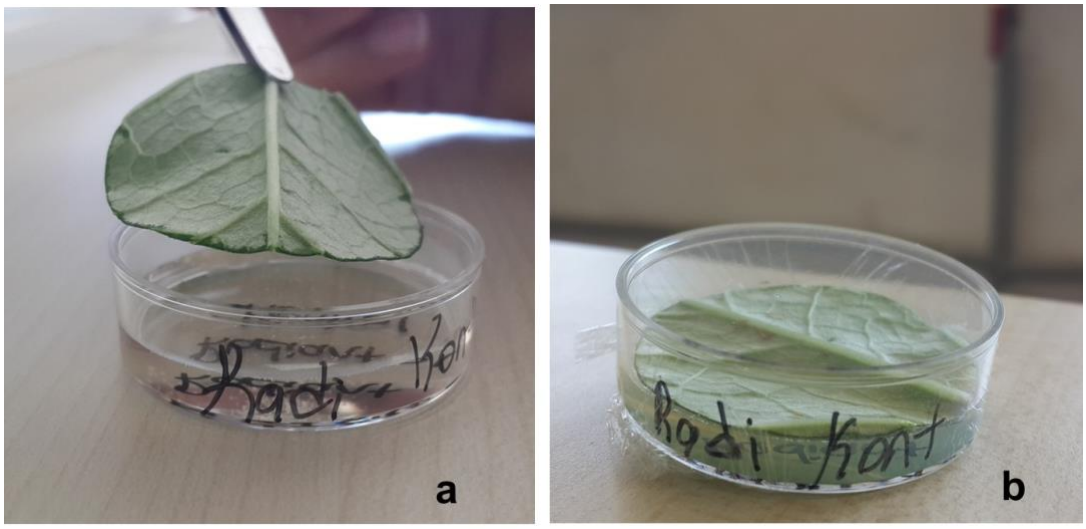

Figure 2. a) Placement of the leaf disc on agar in a petri dish, and b) forming the test cell by covering it with stretch film.

Determining resistance stability

The stability of spinosad and spinetoram resistance was determined in the Manavgat population, which showed high resistance to both insecticides. The Manavgat population, whose LC values and resistance levels were determined against these insecticides, were kept in culture cups in the climate room for about 6 months without use of pesticides, and then the same values were measured again. By comparing the LC values at the beginning and after 6 months, the rate of reversion in resistance was tested at the end of a 6-month period.

Data analysis

The numbers of alive-dead thrips obtained from insecticide bioassays were subjected to probit analysis with PoloPlus, Version 2.0 2002-2022 (LeOra Software, 2022) and LC values and confidence limits (95%) of populations were obtained. Resistance ratios of populations were calculated as LC_{50} values of the greenhouse populations divided by the LC_{50} values of the susceptible population. Confidence limits (95%) of LC values were taken into account in evaluating the significance of the differences in LC values between populations. Any two LC_{50} were considered significantly different if their respective confidence limits (95%) did not overlap.

Results

Spinosad resistance in populations

The LC_{50} values, resistance ratios, and related parameters determined for spinosad in the populations are given in Table 3. LC_{50} values for the spinosad tests for Aksu, Kumluca, Serik, Alanya, Gazipaşa, Demre (Köşkerler), Demre (Beymelek) and Manavgat populations were 7.7, 12.3, 12.7; 14.5, 17.7, 37.6, 42.5 and 125 mg a.i./L, respectively. The highest LC_{50} value was in the Manavgat population at 125 mg a.i./L. The lowest LC_{50} value was in the Aksu population at 7.7 mg a.i./L. Resistance ratios for

spinosad in Aksu, Kumluca, Serik, Alanya, Gazipaşa, Demre (Köşkerler), Demre (Beymelek) and Manavgat populations were 19.3, 30.8, 31.8, 36.3, 44.3, 94.0, 106 and 312, respectively. The LC_{90} values of all greenhouse populations were above the spinosad recommended dose of spinosad. Based on these results, spinosad could give well below 90% mortality in these populations at the recommended dose. This result indicates that spinosad applications may be not effective against the thrips in the locations where populations were collected.

Table 3. Lethal concentration (LC) values and resistance ratio (at LC_{50}) to spinosad in the *Frankliniella occidentalis* greenhouse populations collected from districts of Antalya (Türkiye) in 2017-2019

Populations	n*	Slope ± S.E	LC_{50} mg (a.i.)/l (95% CL)	Resistance Ratio**	LC_{90} mg (a.i.)/l (95% CL)	χ^2	df
Aksu	375	1.0 ± 0.1	7.7 3.8-15.4	19.3	133 57.1-474	25.4	13
Alanya	567	1.0 ± 0.1	14.5 8.1-25.2	36.3	265 130-741	22.7	13
Demre-B	493	1.1 ± 0.1	42.5 29.0-62.8	106	587 328-1340	12.8	13
Demre-K	255	0.9 ± 0.1	37.6 18.0-71.8	94.0	1100 484-3680	11.1	13
Gazipaşa	417	1.1 ± 0.1	17.7 8.9-33.1	44.3	259 124-754	22.9	13
Kumluca	400	1.0 ± 0.1	12.3 6.1-22.0	30.8	241 118-696	16.5	13
Manavgat	260	0.9 ± 0.1	125 45.0-399	312	3620 868-16,600	17.4	9
Serik	307	1.1 ± 0.1	12.7 4.0-29.9	31.8	195 77.7-895	20.2	10
Susceptible	314	1.8 ± 0.2	0.4 0.2-0.8	–	1.9 0.9-7.4	23.7	10

*n: number of adult female thrips used in bioassay;
**Resistance ratio: LC_{50} of the greenhouse populations / LC_{50} of the susceptible population.

Table 4. LC value and the resistance ratio (at LC_{50}) to spinetoram in the *Frankliniella occidentalis* greenhouse populations collected from districts of Antalya (Türkiye) in 2017-2019

Populations	n*	Slope ± S.E	LC_{50} mg (a.i.)/l (95% CL)	Resistance Ratio**	LC_{90} mg (a.i.)/l (95% CL)	χ^2	df
Aksu	507	1.9 ± 0.2	3.2 1.9-5.1	16.0	15.4 9.0-36.5	27.5	13
Alanya	389	0.8 ± 0.1	2.8 1.6-4.8	14.0	91.3 41.8-268	10.1	13
Demre-B	438	1.0 ± 0.1	10.6 5.6-21.2	53.0	206 85.7-762	25.0	13
Demre-K	299	1.3 ± 0.2	0.9 0.4-1.5	4.5	7.9 4.2-25.9	10.0	10
Gazipaşa	328	0.9 ± 0.1	7.8 3.7-14.5	39.0	185 83.5-642	11.5	11
Kumluca	363	1.0 ± 0.1	10.0 4.1-22.7	50.0	203 73.2-1,350	24.8	10
Manavgat	454	1.2 ± 0.1	34.0 20.0-56.2	170.0	389 203-1,060	19.3	13
Serik	369	1.1 ± 0.1	1.5 0.4-4.6	7.5	18.3 5.5-267	76.6	13
Susceptible	653	2.1 ± 0.2	0.2 0.1-0.2	–	0.6 0.4-1.0	5.4	10

*n: number of adult female thrips used in bioassay;
**Resistance ratio: LC_{50} of the greenhouse populations / LC_{50} of the susceptible population.

Spinetoram resistance in populations

The LC_{50} and resistance ratios determined for spinetoram in populations are given in Table 4. The LC_{50} values of Demre (Köşkerler), Serik, Alanya, Aksu, Gazipaşa, Kumluca, Demre (Beymelek) and Manavgat populations were 0.9, 1.5, 2.8, 3.2, 7.8, 10.0, 10.6 and 34.0 mg a.i./L, respectively. The resistance ratios of Demre (Köşkerler), Serik, Alanya, Aksu, Gazipaşa, Kumluca, Demre (Beymelek) and Manavgat populations against spinetoram were 4.5, 7.5, 14.0, 16.0, 39.0, 50.0, 53.0 and 170, respectively. LC_{90} values in five of the eight greenhouse populations tested in this study were above the recommended dose of the spinetoram. According to these findings, spinetoram application at the recommended dose will give less than 90% mortality in thrips collection locations of Alanya, Gazipaşa, Kumluca, Demre (Beymelek) and Manavgat populations.

Resistance stability to spinosad and spinetoram

The stabilities of spinosad and spinetoram resistances were monitored over a 6-month period in the Manavgat population, which had the highest resistance to these insecticides (Tables 5 & 6).

Table 5. Stability of spinosad resistance in spinosad-resistant Manavgat population of *Frankliniella occidentalis*

Assessment	n*	Slope ± S.E	LC_{50} mg (a.i.)/l (95% CL)	LC_{90} mg (a.i.)/l (95% CL)	χ^2	df
Initial (12.10.2018)	260	0.9 ± 0.1	125 45.0-400	3,620 868-16,600	17.4	9
After 6 months (11.04.2019)	285	1.3 ± 0.2	108 32.1-332	1,120 356-45,900	30.6	10

*n: number of adult female thrips used in bioassay.

Table 6. Stability of spinetoram resistance in spinetoram-resistant Manavgat population of *Frankliniella occidentalis*

Assessment	n*	Slope ± S.E	LC_{50} mg (a.i.)/l (95% CL)	LC_{90} mg (a.i.)/l (95% CL)	χ^2	df
Initial (07.11.2018)	454	1.2 ± 0.1	34.0 20.0-56.3	389 203-1,060	19.3	13
After 6 months (08.05.2019)	497	1.6 ± 0.2	25.6 10.1-44.6	159 88.1-483	24.2	13

*n: number of adult female thrips used in bioassay.

The Manavgat population of which initial LC values were determined for spinosad and spinetoram was maintained for about 6 months without pesticide pressure. Afterwards, LC values for these two active substances were redetermined. When the Manavgat population, which was 312 times more resistant to spinosad, was maintained without pesticides for 6 months, the LC_{50} value decreased 0.9 times the initial value, from 125 to 108 mg a.i./L (Table 5). However, this reversion was not significant because the confidence limits the initial and final assessments overlap. Similarly, in the Manavgat population, which was 170 times more resistant to spinetoram and was maintained without pesticide for 6 months, the LC_{50} value for spinetoram decreased 0.8 times compared to the initial LC_{50} value, from 34.0 to 25.6 mg a.i./L. Likewise, this reversion was not significant (Table 6). Even though LC_{90} values deceased by a greater proportion compared to the initial LC_{90} values for both insecticides, they were still well above the recommended doses of spinosad and spinetoram (Tables 5 & 6).

Discussion

In this study, resistance to spinosad and spinetoram was found to be high in eight *F. occidentalis* populations collected from greenhouses in Aksu, Alanya, Demre, Gazipaşa, Kumluca, Manavgat and Serik Districts of Antalya Province. Also, the stability of spinosad and spinetoram resistance over 6 months was monitored in the Manavgat population, which showed the highest resistance among the populations.

Greenhouse populations ranged from 19 (Aksu) to 312 (Manavgat) times more resistant to spinosad. The confidence intervals (95%) of these populations did not overlap with those of the susceptible population. Therefore, resistance to spinosad was found to be significantly elevated in all populations. The LC_{90} dose range detected for spinosad in populations (133 to 3620 mg a.i./L) was above the recommended label dose of spinosad (96 mg a.i./L). These findings showed that widespread and high levels of resistance to spinosad has developed in Antalya greenhouse populations of thrips. Therefore, spinosad may not be sufficiently effective in the locations where greenhouse populations were collected. Spinosad resistance in *F. occidentalis* population has also been reported in previous studies in Türkiye and around the world. *Frankliniella occidentalis* populations were taken from Antalya and its districts in 2007-2009, and 141 times resistance to spinosad was determined only in Kumluca from these populations (unpublished data). Spinosad resistance was found to be 235 times in the *F. occidentalis* population taken from a greenhouse in Kumluca in 2015 where pesticides were used heavily (Dağlı, 2018). While spinosad resistance was seen only in Kumluca populations in previous studies, the findings of this study showed that spinosad resistance became widespread and reached high levels in all greenhouse populations from locations Gazipaşa to Demre. Spinosad has been used against *F. occidentalis* and some other important pests for more than 20 years in Antalya (Anonymous, 2022d). It is not unexpected that resistance to this active substance was widespread and high in greenhouse populations where spinosad has been used for years without applying resistance management programs. In contrast, the susceptible population used for this study obtained in 2017 from vegetables in a home garden where almost no pesticides has been applied, approximately 300 km from the area where the greenhouse populations were collected. This indicates that susceptible populations may still exist in the areas not sprayed by insecticides. In other words, it also shows how closely the development of resistance is related to the frequency of insecticide application. All greenhouse populations tested in the study show significant levels of resistance to spinosad. However, significant differences were detected among populations in terms of resistance levels. The reason for the differences in terms of the resistance levels of the populations to spinosad may be due to frequency of spinosad applications among the location. Previous studies published and related to resistance to this thrips in other countries also indicate that spinosad resistance in *F. occidentalis* has become a serious problem worldwide. Significant levels of spinosad resistance have been reported in some *F. occidentalis* populations in USA (Loughner et al., 2005), Spain (Bielza et al., 2007), Japan (Zhang et al., 2008), Australia (Herron & James, 2005; Herron & Langfield, 2011; Herron et al., 2014), China (Dong-Gang et al., 2016; Wang et al., 2016; Zhang et al., 2022) and Mexico (Cubillos-Salamanca et al., 2019). More than 3,680 times resistance to spinosad was detected in the *F. occidentalis* Spain populations where spinosad was applied more than 10 times annually in 2004 (Bielza et al., 2007). Spinosad resistance was determined as 1,400 fold in a *F. occidentalis* population taken from ornamental plant Chrysanthemum sp. in Australia in the 2010-2011 season, and it was emphasized that this result indicates an increase in spinosad resistance in populations (Herron & Langfield, 2011). In two populations of *F. occidentalis* taken from the Shouguang and Liaocheng, China in 2014-2015, 17 and 89 times resistance was found, respectively (Dong-Gang et al., 2016). Resistance in spinosad was found in the range of 2 to 248 times in populations collected from commercial blackberries in Mexico (Cubillos-Salamanca et al., 2019). In addition to *F. occidentalis*, resistance to spinosad and spinetoram has been reported for important pest insect's species belong to order Lepidoptera, Diptera and Hymenoptera (Sparks et al., 2012).

Resistance ratios for spinetoram ranged from 4.5 (Demre-Köşkerler) to 170 times (Manavgat) in greenhouse populations. The confidence limits of these populations did not overlap with those of the susceptible population. Therefore, the resistance ratios for spinetoram in populations were found to be significant, as with spinosad resistance. LC_{90} values (91.3 to 389 mg a.i./L) for spinetoram in five of the eight greenhouse populations (Alanya, Demre-Beymelek, Gazipaşa, Kumluca and Manavgat) were above the recommended dose for spinetoram (60 mg a.i./L). Therefore, spinetoram may not be sufficiently effective in these sampling locations. The LC_{90} dose values of the other three populations (Demre-Köşkerler, Serik, Aksu) were in the range of 7.9-18.3 mg a.i./L. The recommended dose of spinetoram (60 mg a.i./L) was expected to cause over 90% mortality in these locations. However, it should be taken into account that there were resistant individuals in these three populations, albeit at a lower frequency, and frequent use of spinetoram in these locations should be avoided in order to prolong its efficacy. Results of current study shows that most of greenhouse populations of the *F. occidentalis* has developed widespread and high levels of resistance to spinetoram. As with spinosad resistance, the problem of resistance to spinetoram has become common worldwide. Spinetoram resistance was reported in China (Wang et al., 2016; Zhang et al., 2022), Australia (Langfield et al., 2018; Langfield et al., 2019; Chen et al., 2021). Resistance to spinetoram and spinosad has been found to be 17 and 15 times in *F. occidentalis* populations collected from the eggplant fields in the Shouguang and Shandong, China in 2014. Additionally, 14 times resistance to cyantraniliprole and 128 times resistance to insect growth regulator, pyriproxyfen were detected in these populations (Wang et al., 2016). The resistance ratio to spinetoram in Changping population was nearly 17,000 times (Zhang et al., 2022). In Western Australia, 17 (at LC_{50}) and -77 times (at $LC_{99.9}$) resistance to spinetoram was detected in *F. occidentalis* populations taken from stone fruits in 2017, and it was emphasized that there were failures of control at the field recommended dose (Langfield et al., 2018). Resistance to spinetoram between 6 and 56 times has been reported in *F. occidentalis* populations collected from Victoria and Queensland, Australia (Langfield et al., 2019). In addition, with PCR diagnostic test based on the G275E mutation for spinetoram resistance in Australian *F. occidentalis* populations, it was reported that spinetoram-resistant *F. occidentalis* populations collected from cotton in 2018-2019 carried the G275E mutation and resistant individuals were common (Chen et al., 2021).

Based on 12 studies on several insect species, it was reported that the most common mechanism leading to spinosad resistance in insects is target site resistance, and metabolic and other types of resistance mechanisms are more limited (Sparks et al., 2012). This general situation is also similar to resistance mechanisms in *F. occidentalis*. In most studies, spinosad resistance in *F. occidentalis* was found to be related to target site resistance (Bielza et al., 2007; Zhang et al., 2008; Gao et al., 2012). In our previous study associated with synergist and enzyme tests on a spinosad-resistant *F. occidentalis* population, it was determined that the metabolic resistance mechanism does not contribute to resistance (unpublished data). However, Herron et al. (2014) reported that metabolic resistance exists in spinosad-resistant *F. occidentalis* populations, based on the synergist PBO and esterase-based research results. Jensen (2000) investigated the resistance mechanisms in *F. occidentalis* with enzyme and synergist tests and concluded that different mechanisms may cause resistance in different *F. occidentalis* populations or those different resistance mechanisms may occur simultaneously in the same populations. Accordingly, multiple mechanisms such as target site mutation and metabolic resistance are likely to occur in resistant populations. Spinosad and spinetoram are in the same insecticide group and both are compounds that act on the nicotinic acetylcholine receptor in the insect nervous system (IRAC, 2022). The mechanism or mechanisms leading to spinosad resistance may also be expected to contribute to spinetoram resistance. Thus, several insect species such as spinosad-resistant *Drosophila melanogaster* Meigen, 1830 (Diptera: Drosophilidae), *Plutella xylostella* (Linnaeus, 1758) (Lepidoptera: Plutellidae), and *Chloridea virescens* (Fabricius, 1777) (Lepidoptera: Noctuidae) also show equal levels of cross-resistance to spinetoram as has been reported (Sparks et al., 2012).

Research findings showed that there were differences in resistance levels to spinosad and spinetoram in Antalya greenhouse populations. Resistance ratios against spinosad (19.3-312 times) were generally higher than those of spinetoram (4.5-170 times) in populations. The fact that the populations were exposed to selection pressure for a longer period to spinosad than to spinetoram may be the main reason for this situation. Spinosad has been used in Antalya more than 20 years (Anonymous, 2022d). However, spinetoram came into use in 2014 (Anonymous, 2022e). Nevertheless, a significant level of resistance to spinetoram has been detected in the majority of thrips populations, although it has been used for a short period, nearly 4 years. It is most likely that populations of *F. occidentalis* highly resistant to spinosad show lower levels of cross-resistance to spinetoram. This is also supported by the results of previous research on the *F. occidentalis* population in 2015 (Dağlı, 2018). In the study conducted on the Kumluca-2015, *F. occidentalis* population was highly resistant to spinosad (235 times) but that it was never exposed to spinetoram. Spinosad and spinetoram with recommended doses were able to kill the entire susceptible thrips population in laboratory bioassays. However, mortality rates at the recommended doses of spinosad and spinetoram in the Kumluca-2015 population were 38 and 88%, respectively (Dağlı, 2018). Spinosad-resistant populations show cross-resistance to spinetoram, albeit at lower levels. Therefore, it should be taken into account that use of insecticides with different mode of actions instead of spinosad and spinetoram at the same locations would be suitable for resistance management.

The findings in the stability tests showed that there was no significant reversion in spinosad and spinetoram resistance in the high-resistant Manavgat population, which was maintained for 6 months without pesticide exposures, and the resistance to both active substances was mostly stable. In the Manavgat population, which was continued pesticide-free for 6 months, the LC_{50} value for spinosad decreased from 125 to 108 mg a.i./L, only 0.9 times the initial value. In the same population, the LC_{50} for spinetoram decreased from 34.0 to 25.6 mg a.i./L, only 0.8 times lower than the initial LC_{50}, LC_{90} dose values after 6 months (1120 and 159 mg a.i./L) for spinosad and spinetoram in the Manavgat population, respectively were still well above the recommended doses (96 and 60 mg a.i./L) of these active substances. A similar result was obtained from a previous investigation on the stability of spinosad resistance in *F. occidentalis*. Although the highly resistant Kumluca-2015 population for spinosad was maintained without pesticide for 12 months, it was determined that there was no significant reversion in the resistance level (Dağlı, 2018). Spinosad resistance has also been reported to remain stable for 8 months in *F. occidentalis* Spanish populations (Bielza et al., 2008). Stability tests show that after high levels of resistance to spinosad and spinetoram developed in *F. occidentalis* populations, the resistance problem may not disappear in the short term, even if these insecticides are not used. For resistance management tactics to perform successfully, they must be applied before insecticides develop resistance in populations. Avoidance of frequent use of spinosad and spinetoram in locations where there are still susceptible populations of pests and alternating use of active substances with other modes of action may prolong the useful life of these insecticides.

Direct feeding damage, transmission of TSWV and being a very important quarantine pest necessitate almost a zero tolerance level for *F. occidentalis*. In recent years resistance breaking by some TSWV isolates in resistant cultivars has further increased the importance of the control of *F. occidentalis* (Fidan & Sarı, 2019). Briefly, to successfully manage this pest, it must be kept away from plant-growing areas. The use of spinosad and spinetoram in the control of *F. occidentalis* in Antalya greenhouse locations should be limited and other active substances with different modes of action should be included. However, it is not easy to come across highly effective active substances that can be recommended as alternatives in practice. The fact that carbamate and organic phosphorus active substances other than formetanate were removed from the recommendation lists in greenhouse vegetable production and the detection of high levels of resistance to the pyrethroid acrinathrin in *F. occidentalis* populations from the same locations (Toure & Dağlı, 2021) limited the number of alternative active substances that can be recommended. For

this reason, instead of using only insecticides in management, strategies should be sought to achieve the level of success required by quarantine conditions against pests by using insect nets, and an integration biological and biotechnical control methods.

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Original article (Orijinal araştırma)

Suppressive effect of seed powders of some Brassicaceae plants on *Meloidogyne incognita* (Kofoid & White, 1919) Chitwood, 1949 (Tylenchida: Meloidogynidae) in tomato and cucumber

Bazı Brassicaceae bitkilerinin tohum unlarının domates ve hıyarda *Meloidogyne incognita (*Kofoid & White, 1919) Chitwood, 1949 (Tylenchida: Meloidogynidae)'ya karşı baskılayıcı etkisi

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Abstract

The aim of the study was to investigate suppressive effect of powdered seeds of *Raphanus sativus* L. (red radish), *Lepidium sativum* L. (cress) and *Eruca vesicaria* (L.) Cav. (arugula) (Brassicales: Brassicaceae) on *Meloidogyne incognita* (Kofoid & White, 1919) Chitwood, 1949 (Tylenchida: Meloidogynidae) in tomato and cucumber. This study was conducted under controlled conditions between January and April in 2022. The experiment consisted of 14 treatments of seed powders singly, or in double or triple combinations. Nematode inoculation was made with 1 000 J2 one week following the transplanting of tomatoes and cucumbers into pots. The seed powders were mixed with the soil three days after the nematode inoculation. The root gall and egg mass were evaluated on a scale of 1-9 and the percent control effect was calculated 60 days after treatment. The highest control effect on gall and egg mass (70%) was with a triple powder treatment which consisting of radish (2 g/plant) + cress (2 g/plant) + arugula (2 g/plant) on tomato and cucumber. The control effect of double powder treatments on gall and egg masses were above 55% in tomato and cucumber. The control effect of radish (6 g/plant) in both host plants was found to be similar to double powder treatments which arugula (2 g/plant) + radish (2 g/plant), and cress (2 g/plant) + radish (2 g/plant). In single treatments, the highest control effect was obtained with radish (6 g/plant). In double powder treatments, those containing radish were found to be more effective against *M*. *incognita*. It was concluded that treatment with radish seed powder against *M. incognita* was more successful than with cress and arugula powders.

Keywords: Brassicaceae, nematicidal effect, red radish, seed powder

Öz

Bu çalışmanın amacı, *Raphanus sativus* L. (kırmızı turp), *Lepidium sativum* L. (tere) ve *Eruca vesicaria* (L.) Cav. (roka) (Brassicales: Brassicaceae)'nın toz haline getirilmiş tohumlarının domates ve salatalıkta *Meloidogyne incognita* (Kofoid & White, 1919) Chitwood, 1949 (Tylenchida: Meloidogynidae) üzerindeki baskılayıcı etkinliğinin araştırılmasıdır. Çalışma, 2022 yılı Ocak-Nisan ayları arasında kontrollü koşullarda yürütülmüştür. Çalışma, bitkilerin tohum unlarının tekli, ikili ve üçlü olmak üzere 14 uygulamasından oluşmaktadır. Domates ve salatalıkların saksılara dikilmesinden bir hafta sonra 1 000 J2 ile nematod aşılaması yapılmıştır. Tohum unları, nematod aşılamasından üç gün sonra toprakla karıştırılmıştır. Uygulamadan altmış gün sonra, köklerdeki ur ve yumurta paketi 1-9 skalasına göre değerlendirilmiştir ve yüzde kontrol etki değerleri hesaplanmıştır. Gal ve yumurta paketi üzerinde en yüksek baskılayıcı etki domates ve hıyarda turp (2 g/bitki) + tere (2 g/bitki) + roka (2 g/bitki) üçlü uygulamasında saptanmıştır. İkili uygulamaların gal ve yumurta paketi üzerindeki kontrol etkisi, domates ve hıyarda %55'in üzerinde bulunmuştur. Her iki bitkide de tek başına 6 g/bitki turp tohum unu uygulamasının kontrol etkisinin, roka (2 g/bitki) + turp (2 g/bitki) ve tere (2 g/bitki) + turp (2 g/bitki) ikili uygulamaları ile benzer olduğu bulunmuştur. Tekli uygulamada en yüksek kontrol etki 6 g/bitki ile turp tohumu unundan elde edilmiştir. İkili uygulamalarda turp içerenlerin *M*. *incognita* üzerinde daha etkili olduğu bulunmuştur. *Meloidogyne incognita* üzerinde turp tohum unu uygulamasının tere ve rokaya göre daha başarılı kontrol sağladığı belirlenmiştir.

Anahtar sözcükler: Brassicaceae, nematisidal etki, kırmızı turp, tohum unu

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Introduction

Tomato and cucumber are among the most important vegetables in terms of the economic value in Türkiye. Türkiye ranks third in the world with tomato production exceeding 12.7 Mt and ranks second in cucumber production with 2 Mt (Arslan et al., 2022). Root-knot nematodes (RKN) are a group of plant parasitic nematodes that cause significant yield losses in tomato and cucumber crops around the world (Sikora & Fernandez, 2005). They feed on roots and vascular tissues, disrupting water and nutrient flow, and cause slow growth, yellowing of leaves, wilting and early plant death of infested plants (Asaturova et al., 2022). Seid et al. (2015) reported that while the product loss due to RKN in different tomato varieties was between 25 and 100%, decrease level of yield in commercial cucumber cultivation was between 12 and 60% (Wehner et al., 1991; Sorribas et al., 1997). It has been reported that they cause 80% yield loss in tomato cultivation in the Western Anatolian Region of Türkiye (Kaşkavalcı, 2007). Although over 100 species of RKN have been described (Ghaderi & Karssen, 2020), *Meloidogyne incognita* (Kofoid & White, 1919) Chitwood, 1949, *Meloidogyne javanica* (Treub, 1885) Chitwood, 1949, *Meloidogyne arenaria* (Neal, 1889) Chitwood, 1949, *Meloidogyne chitwoodi* Golden, O'Bannon, Santo & Finley, 1980 and *Meloidogyne hapla* Chitwood, 1949 (Tylenchida: Meloidogynidae) are the most common RKN in vegetable growing areas in Türkiye (Adam et al., 2007; Evlice et al., 2022). *Meloidogyne incognita* is accepted as the most aggressive and important RKN due to its wide host spectrum and high prevalence in the world (Sikora & Fernández, 2005). This species is also common in vegetable growing areas in Türkiye (Çetintaş & Çakmak, 2016; Özarslandan, 2016; Uysal et al., 2017; Gürkan et al., 2019; Aslan & Elekcioğlu, 2022).

Management of RKN is quite expensive and difficult (Asaturova et al., 2022). To reduce the damage caused by nematodes, producers generally apply solarization and use resistant cultivars (Hajihassani et al., 2019). Many synthetic nematicides such as methyl bromide, ethylene dibromide and di-bromochloropropane have been banned due to their carcinogenic effects (Onkendi et al., 2014). The high cost of nematicides, resistance development, health and environmental hazards, residue, negative effects on soil fauna and beneficial microflora, and phytotoxic effects on plants are the limiting factors for their use (Haydock et al., 2013; Silva et al., 2019). Therefore, it has become necessary to search for alternative control methods in the control of plant parasitic nematodes. Plant-based metabolites are perhaps the most intensively researched subject in this area (Pardavella et al., 2020). Nematicidal activity of isothiocyanates, glucosides, alkaloids, ketones, aldehydes, phenolics and fixed fatty acids in plants have been demonstrated (Chitwood, 2002; Kabera et al., 2014; Shalaby et al., 2021; Stavropoulou et al., 2021).

The Brassicaceae is one of the most economically important plant families. When glucosinolate (GLS) compounds in the cell walls of plants of the Brassicaceae family react with myrosin enzyme toxic compounds are produced (Wittstock et al., 2016). As a result of this enzymatic hydrolysis, volatile and biocidal isothiocyanate compounds are produced (Zasada & Ferris, 2004; Ploeg, 2008). Isothiocyanates disrupt the protein structure and precipitate the cell contents (Mennan & Katı, 2010). In Brassicaceae plants, over 100 glucosinolate compounds have been detected, but the most known glucosinolate compounds in vegetables are neoglucobrassicin, glucobrassicanapin and glucobrassicin (Vallejo et al., 2004). Brassica plants can be used as cover or trap plants, and green manures in plant parasitic nematode control (Matthiessen & Kirkegaard, 2006; Schlaeppi et al., 2010). Recently, it seems that studies have mainly focused on Brassicaceae seeds. Brassicaceae seeds were found to have higher glucosinolate levels and were more advantageous due to lower loss of glucosinolate degradation products (Lazzeri et al., 2004). Salem et al. (2012) reported the second stage juvenile (J2) inactivity of *M. incognita* as 95 and 64%, respectively, 72 and 144 h after *Lepidium sativum* L. (Brassicales: Brassicaceae) seed extract application.

Crushing the seeds and using the seed powder have become common in the control of plant parasitic nematodes (Radwan et al., 2012). However, to the best of our knowledge, there is no study on the seed powder applications of Brassicaceae plants as an alternative control method to nematicides and fumigants in Türkiye. In this study, the effectiveness of Brassicaceae family member vegetables in the control of *M. incognita,* which is common in tomato and cucumber growing areas in Türkiye and causes serious economic losses, was evaluated. For this purpose, the suppressive effect of single and combination applications of red radish, *Raphanus sativus* L., cress, *L. sativum* and arugula, *Eruca vesicaria* (L.) Cav. (Brassicales: Brassicaceae) seeds on gall and egg mass formed by *M. incognita* in tomato and cucumber were investigated.

Materials and Methods

Materials

The red radish seed of cv. Cherry Belle, the cress seed of cv. Bahar Gülü and the arugula seed of cv. Derya were obtained from Biotek Seed Company (Konya, Türkiye). *Meloidogyne incognita* isolate DR17 was used (Uysal et al., 2017). The experiment was performed on cucumber cv. Silor F1 and tomato cv. Gülizar F1 susceptible to RKN.

Nematode inoculum

Mass production of *M. incognita* was done in tomato cv. Tueza F1 (Multi Seed) at 24 ± 1°C, 60% ± 5% RH. The tomato seedlings for mass production were transplanted into pots containing sterilized soil (68% sand, 21% silt and 11% clay) and 1 000 J2s were inoculated into the soil. Eight weeks after inoculation, tomato roots were pulled up, carefully washed in tap water and egg masses were collected under a stereomicroscope. The infective J2s from egg masses were hatched in a sterile Petri dish containing water in an oxygenated environment for 3 days and were kept in the refrigerator at 8°C until used. The 1 000 J2s were collected under the light microscope and transferred to Eppendorf tubes for use as inoculum (Lobna et al., 2017).

Preparation of seed powder

One kg of seeds of Cherry Belle, Bahar Gülü and Derya were blended until a fine powder to be applied at 2, 4 and 6 g/plant (Shalaby et al., 2021).

Effect of red radish, cress and arugula seed powder on the development of *Meloidogyne incognita* on tomato and cucumber

The study was conducted between January and April in 2022. This study was conducted under controlled conditions (24 ± 1°C, 60 ± 5% RH) in a completely randomized plot design with 5 replicates of each for the hosts Gülizar F1 tomato and Silor F1 cucumber. Treatments and doses of Brassica plants seed powder used in the experiment are given in Table 1. Plants treated only with *M. incognita* were included as controls.

Table 1. Treatments and doses of brassica plants seed powder used in the experiment

Treatments					
No	Brassica plants	Doses (g/plant)	No	Brassica plants	Doses (g/plant)
1	Arugula	2	8	Red radish	4
2	Arugula	4	9	Red radish	6
3	Arugula	6	10	Cress + arugula	2 + 2
4	Cress	2	11	Cress + red radish	2 + 2
5	Cress	4	12	Arugula + red radish	2 + 2
6	Cress	6	13	Cress + arugula + red radish	2 + 2 + 2
7	Red radish	2	14	Control	only plants treated with *M. incognita*

Gülizar F1 tomato and Silor F1 cucumber seedlings with approximately four true leaves were transplanted into 14-cm plastic pots containing ~1.5 kg of sterilized soil (68% sand, 21% silt and 11% clay). One week later, all pots were inoculated with 1 000 J2s of *M. incognita*. Three days after the nematode inoculation, slits were made around the seedlings with a spatula and seed powders were spread evenly in these slits, mixed with the soil and watered after covering (Shalaby et al., 2021). The experiment was assessed 60 days after nematode inoculation. Tomato and cucumber plants were carefully removed from the soil and their roots were washed with tap water. Evaluation procedure was made on the root gall scale of 1-9 according to Mullin et al. (1991) (1, no gall; 2, 5% root gall; 3, 6-10 % root gall; 4, 11-18 % root gall; 5, 19-25% root gall; 6, 26-50% root gall; 7, 51-65% root gall, 8, 66-75% root gall; and 9, 76-100% root gall) and egg mass production rate scale (1, no egg mass; 2, 1 or 2 egg masses; 3, 3-6 egg masses; 4, 7-10 egg masses; 5, 11-20 egg masses; 6, 21-30 egg masses; 7, 31-60 egg masses; 8, 61-100 egg masses; and 9, more than 100 egg masses) (Bozbuga et al., 2015; Göze Özdemir & Karaman, 2020). The control percentages of seed powders on gall and egg masses were calculated with the formula (Xiang et al., 2020):

$$\text{Control effect (\%)} = (\text{Control} - \text{Treatment} / \text{Control}) \times 100.$$

The averages of the gall and egg mass scale were compared by LSD test ($P \leq 0.05$) using the SAS (version 17.00) program, after arcsin transformation of the percentage control effects of seed powders on gall and egg mass.

Results

Tomato experiment

The highest galling rate was 8.6 in the control treatment. The gall indices of all treatments (1.0-5.6) were significantly lower than the control treatment ($P \leq 0.05$). Among the treatments, the highest gall index was with 2 g/plant cress (5.2) and 2 g/plant arugula (5.6). The lowest gall index (1.0) was in the triple powder treatment (2 g/plant doses of cress, arugula and radish). Gall indices of cress, arugula and radish seed powders at 6 g/plant treatments were 3.8, 3.0 and 1.6, respectively. The gall index was lower in combinations with radish in double powder treatments. The 2 g/plant cress + 2 g/plant arugula treatment of gall indices were higher than 2 g/plant cress + 2 g/plant radish, and 2 g/plant cress + 2 g/plant radish treatments (Table 2).

The percentage change in galling of roots by seed powders alone and combine treatments was between 35 and 70%. The double powder treatments reduced galling of roots by over 60%. The triple powder treatment of 2 g/plant cress + 2 g/plant arugula + 2 g/plant radish had the highest effect reducing galling of roots by 70%. This was followed by 2 g/plant arugula + 2 g/plant radish, and 2 g/plant cress + 2 g/plant radish treatments with 68%. It was determined that 6 g/plant radish treatment reduced galling roots by 65%. The control effects of 2 g/plant and 4 g/plant cress treatments, 2 g/plant arugula, and 2 g/plant radish treatments on galling roots were below 50% (Table 2).

Egg masses indices of the treatments varied between 1.0 and 5.8 and were found to be statistically significantly lower than the control treatment (9.0) ($P \leq 0.05$). The highest egg masses index was determined in the treatment of 2 g/plant seed powder of cress (5.8) and arugula (6.0) after then control. The lowest egg masses index was with 2 g/plant cress + 2 g/plant arugula + 2 g/plant radish triple powder treatment (1.0) and in double powder treatments of 2 g/plant arugula + 2 g/plant radish (1.2) and 2 g/plant cress + 2 g/plant radish (1.2). Egg masses index of 2.6 with 2 g/plant cress + 2 g/plant arugula seed powder treatment was higher than other double powder treatments. Egg masses indices of 6 g/plant cress, arugula and radish treatments were determined as 4.2, 3.6 and 2.0, respectively (Table 2).

Radish seed powder treatment at a dose of 2 g/plant decreased the egg masses formed by *M. incognita* on tomato root by 40.5%, while treatments of 4 and 6 g/plant decreased it by 52.2% and 61.9%, respectively. The control effect of 2 and 4 g/plant seed powder treatments of cress and arugula on the egg mass was below 45% whereas the control effect was above 45% in 6 g/plant treatments. The effects of radish 6 g/plant (61.9%) and 2 g/plant cress + 2 g/plant arugula (57.5%) seed powder treatments on egg mass in tomato roots were found as similar. It was determined that control effect in the same statistical group (a) in double powder treatments of 2 g/plant cress + 2 g/plant radish with 2 g/plant arugula + 2 g/plant radish and in triple powder treatments (2 g cress + 2 g arugula + 2 g radish) (Table 2).

Table 2. Effect of cress, arugula, radish plant seed powder against *Meloidogyne incognita* in tomato under controlled conditions

Treatments (plant seed powder)	Root galling index[1]		Percent effect on root galling[4]		Egg mass index[2]		Percent effect on egg masses[4]	
2 g Cress	5.2	cb[3]	39	fe	5.8	b	36	hg
4 g Cress	4.4	cd	44	de	5.2	cb	40	fg
6 g Cress	3.8	ed	48	dc	4.2	ed	47	de
2 g Arugula	5.6	b	36	f	6.0	b	35	h
4 g Arugula	3.8	ed	48	dc	4.6	cd	44	fe
6 g Arugula	3.0	e	54	c	3.6	e	51	d
2 g Radish	4.8	cb	42	fe	5.2	cb	40	fg
4 g Radish	3.0	e	54	c	3.4	e	52	cd
6 g Radish	1.6	gf	65	ba	2.0	gf	62	b
2 g Cress + 2 g arugula	2.0	f	61	b	2.6	f	58	bc
2 g Cress + 2 g radish	1.2	gf	68	a	1.2	gh	69	a
2 g Arugula + 2 g radish	1.2	gf	68	a	1.2	gh	69	a
2 g Cress + 2 g arugula + 2 g radish	1.0	g	70	a	1.0	h	70	a
Untreated Control	8.6	a	0	g	9.0	a	0	ı
LSD (%5)	0.85		6.1		0.78		5.4	
CV (%)	19.2		9.6		15.8		8.8	

[1] Scale of 1-9 root galling index; 1, no gall; 2, 5% root gall; 3, 6-10 % root gall; 4, 11-18 % root gall; 5, 19-25% root gall; 6, 26-50% root gall; 7, 51-65% root gall; 8, 66-75% root gall; and 9, 76-100% root gall (Muller et al 1991);
[2] Scale of 1-9 egg mass index;1, no egg mass; 2, 1 or 2 egg masses; 3, 3-6 egg masses; 4, 7-10 egg masses; 5, 11-20 egg masses; 6, 21-30 egg masses; 7, 31-60 egg masses; 8, 61-100 egg masses; and 9, more than 100 egg masses (Muller et al 1991);
[3] Means were compared by LSD test at P ≤ 0.05 and those followed by the same letter within columns are not significantly different.
[4] Arcsin transformation was applied before analysis.

Cucumber experiment

The gall indices for cucumber roots decreased with increasing dose in single treatments with the three seed powders. The effects of combined treatments were greater than in the single powder treatments. The lowest gall index was found 1.0 in the triple powder treatment (2 g/plant cress + 2 g/plant arugula + 2 g/plant radish). There was no difference (P ≥ 0.05) between the gall indices of cress (2 g/plant) + arugula (2 g/plant), arugula (2 g/plant) + radish (2 g/plant), and cress (2 g/plant) + radish (2 g/plant) treatments. Although, the gall index was 1.8 with 6 g/plant red radish, this was lower than the same dose of cress and arugula. The highest gall indices in cucumber roots was with 2 g/plant cress (6.0) and arugula (5.8) (Table 3).

The highest control effect on galling was 71% in the triple powder treatment of 2 g/plant cress + 2 g/plant arugula + 2 g/plant radish. Double powder treatments of cress, arugula and radish reduced galling by more than 55% with no statistically significant difference between them (P ≥ 0.05). Doses of 6 g/plant of cress, 4 and 6 g/plant of arugula and red radish reduced galling in the roots by more than 40%. The effect of 6 g/plant red radish was 55%, which was not statistically different from 2 g/plant cress + 2 g/plant arugula (59%), 2 g/plant arugula + 2 g/plant radish (60%) and 2 g/plant cress + 2 g/plant radish (60%) (Table 3).

The lowest egg masses index was 1.4 with the triple powder treatment (2 g/plant cress + 2 g/plant arugula + 2 g/plant radish) ($P \leq 0.05$) and the highest egg masses index was 9.0 in the control treatment, followed by about 6 with 2 g/plant cress and arugula. As the dose of the three seed powders increased, the egg mass index decreased. Egg mass indices were similar in double powder treatments (Table 3).

The treatments reduced egg mass formation between 32 and 67%. The greatest effect was in the triple powder treatment of 2 g/plant cress + 2 g/plant arugula + 2 g/plant radish ($P \leq 0.05$). The treatments of 2 g/plant cress + 2 g/plant arugula, 2 g/plant arugula + 2 g/plant radish, and 2 g/plant cress + 2 g/plant radish decreased the egg mass formation by 55, 58 and 56%, respectively. The 6 g/plant cress, arugula and red radish treatments reduced egg mass by 44, 51 and 62%, respectively (Table 3).

Table 3. Effect of cress, arugula, radish seed powder against *Meloidogyne incognita* in cucumber under controlled conditions

Treatments (plant seed powder)	Root galling index[1]		Percent effect on root galling[4]		Egg mass index[2]		Percent effect on egg masses[4]	
2 g Cress	6.0	b[3]	35	g	6.4	b	32	g
4 g Cress	5.0	c	42	f	5.4	c	39	ef
6 g Cress	4.0	d	48	e	4.6	d	44	e
2 g Arugula	5.8	b	37	g	6.2	b	34	fg
4 g Arugula	4.0	d	48	e	4.6	d	44	e
6 g Arugula	3.0	ef	55	cd	3.6	e	51	d
2 g Radish	5.4	bc	39	fg	6.0	bc	35	fg
4 g Radish	3.2	e	53	d	3.6	e	51	d
6 g Radish	1.8	g	64	b	2.2	g	62	ab
2 g Cress + 2 g arugula	2.4	fg	59	bc	3.0	ef	55	cd
2 g Cress + 2 g radish	2.2	g	60	b	2.8	fg	56	cd
2 g Arugula + 2 g radish	2.2	g	60	b	2.6	fg	58	bc
2 g Cress + 2 g arugula + 2 g radish	1.0	h	70	a	1.4	h	67	a
Untreated Control	9.0	a	0	h	9.0	a	0	h
LSD (%5)	0.72		5.0		0.79		5.8	
CV (%)	15.6		8.3		14.3		10.2	

[1] Scale of 1-9 root galling index; 1, no gall; 2, 5% root gall; 3, 6-10 % root gall; 4, 11-18 % root gall; 5, 19-25% root gall; 6, 26-50% root gall; 7, 51-65% root gall; 8, 66-75% root gall; and 9, 76-100% root gall (Muller et al 1991);
[2] Scale of 1-9 Egg mass index;1, no egg mass; 2, 1 or 2 egg masses; 3, 3-6 egg masses; 4, 7-10 egg masses; 5, 11-20 egg masses; 6, 21-30 egg masses; 7, 31-60 egg masses; 8, 61-100 egg masses; and 9, more than 100 egg masses (Muller et al 1991);
[3] Means were compared by LSD test at $P \leq 0.05$ and those followed by the same letter within columns are not significantly different.
[4] Archsin transformation was applied before analysis.

Discussion

In this study, it was found that soil treatment with cress, arugula and red radish seed powders had a significant nematicidal effect on *M. incognita*. This nematicidal effect increased in tomato and cucumber roots as the doses increased in single powder treatments of cress, arugula and red radish seed powders. In single powder treatments, the highest effect was with 6 g/plant powder in both tomato and cucumber. In tomato, 6 g doses of cress, arugula and radish treatments reduced root galling by 48, 54 and 65%, and egg mass formation by 47, 51 and 62%, respectively. In cucumber, 6 g doses of cress, arugula and radish treatments reduced root galling by 48, 55 and 64%, and egg mass formation by 44, 52 and 62%, respectively. The 2 and 4 g doses of cress, arugula and radish powders had much lower nematicidal effects in both plants. In contrast, Shalaby et al. (2021) in their study of peppers infested with *M. incognita*, found that the application of cress and radish seed powder at 2, 4 and 6 g/plant reduced root galling by 78, 84 and 91%, respectivelyand 85, 90 and 95% reductions in egg masses. In the same study, the researchers found that the effect of radish powder reduced root galling at the same doses by 82, 86 and 89%, respectively and egg mass formation of by 82, 89 and 90%. Salem et al. (2012) reported that *M. incognita*

gall, egg masses and J2 density in the soil were completely controlled in tomato by mixing cress seed into the soil 1 week after nematode inoculation. Aydınlı et al. (2019) reported that 4% fresh plant aqueous extracts of cress and mint in tomato, and 1% and 2% aqueous extracts of dry arugula plants significantly reduced damage caused by *M. arenaria*.

In present study, radish seed powder at 6 g/plant had a greater nematicidal effect than cress and arugula. Oka (2010) and Radwan et al. (2012) reported that there may be differences in the toxic substances or biocidal contents of dry seed powders added to the soil of different plants and their microbial degradation products. However, it has been suggested that the nematicidal effect of seed powders of allelopathic plants on nematode populations may also be due to the effect of ammonia released from the seeds, independent of the GLS content (Mazzola et al., 2007, 2009). Zasada et al. (2009) found that the pulp of mustard grass, *Brassica juncea* (L.) Czern. (Brassicales: Brassicaceae) seeds had a more nematoxic effect than white mustard, *Sinapis alba* L. (Brassicales: Brassicaceae) and that 2.5% and 10% dry w/w (corresponding to approximately 50 and 200 t/ha at an incorporation depth of 15 cm) would be required for eradication of *M. incognita* and *Pratylenchus penetrans* (Cobb, 1917) Filipjev & Schuurmans Stekhoven, 1941 (Tylenchida: Pratylenchidae), respectively, while *S. alba* is 0.5% for *B. juncea*. According to the results of study, at least 6 g/plant treatment was required for cress and arugula seed powders to have a control effect of more than 45% for *M. incognita* in tomato and cucumber roots, but only 4 g/plant radish seed powder. Radwan et al. (2012) reported that 5 g/kg radish seed powder treatment reduced root galling in tomato by 78%. In addition, it is stated that the radish plant is a very good trap for RKN and has biofumigant properties when applied to the soil as a plant and green manure (Pattison et al., 2006; Melakeberhan et al., 2008). The radish plant secretes glucosinolate into the soil or glucosinolate emerges as a result of the decomposition of plant parts, and then, as a result of the hydrolysis of glucosinolate, isothiocyanates that have biocidal effects on nematodes are formed (Vallejo et al., 2004; Zasada & Ferris, 2004; Sandler et al., 2015). Aydınlı & Mennan (2018) found that in biofumigation plots applied with radish and arugula, the number of gall and egg mass on the roots of tomatoes decreased significantly. Also, growing these 2 plants as a winter crops before susceptible plants would reduce the damage caused by *M. arenaria* and increase crop yield.

It was found that the combined applications of cress, arugula and red radish seeds powder at 2 g/plant had the highest nematicidal effect against *M. incognita* in tomatoes and cucumbers in present study. The control effects of this treatment on gall and egg masses in tomato and cucumber were both about 70%. The control effect of double powder treatments on gall and egg masses in tomato and cucumber were lower than triple powder treatment but significantly higher than the single powder treatments. In tomato and cucumber, the control effect of double powder treatments on gall and egg masses were above 55%. Gall and egg mass index of cress (2 g/plant) + arugula (2 g/plant) treatment in tomato and cucumber roots was found to be higher than arugula (2 g/plant) + radish (2 g/plant) and cress (2 g/plant) + radish (2 g/plant) treatments. The control effect increased in tomato and cucumber roots in double powder treatments with radish. In present study, the highest control effect was found in triple powder treatment of arugula (2 g/plant) + radish (2 g/plant) + cress (2 g/plant). In the study of Zanbouri & Fatemy (2014) with single and combined applications of cress, *L. sativum* and peppermint, *Mentha pulegium* L., 1753 (Lamiaceae: Mentheae), they found that the mean hatching of *Globodera rostochiensis* (Wollenweber, 1923) (Tylenchida: Heteroderidae) to be both about 0.5%, and J2 activity was blocked by 97% after 24 h in both plant extracts. Unlike the Brassicaceae, Asif et al. (2016) reported that in the application of wild spinach (Amaranthaceae) seed powder with freshly chopped leaves of different plants, *M. incognita* was significantly suppressed compared to the control.

Consequently, differences were determined in the nematicidal activity of cress, arugula and radish seeds against *M. incognita* in tomato and cucumber under controlled conditions. Radish seed powder was found to be the most effective treatment. However, combined treatments of radish, cress and arugula seed

powders successfully suppressed RKN even at low doses. As a result, plants belonging to Brassicaceae are thought to be a good alternative to chemicals both in biofumigation, with their extracts and due to the potential nematicidal and nematostatic effects contained in their seeds, and they should be supported by detailed studies in field conditions.

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Original article (Orijinal araştırma)

Comparing bioassay and diagnostic molecular marker for phosphine resistance in Turkish populations of *Rhyzopertha dominica* (F., 1792) (Coleoptera: Bostrichidae)[1]

Rhyzopertha dominica (F., 1792) (Coleoptera: Bostrichidae)'nın Türkiye popülasyonlarındaki fosfin direncinde bioassay ile moleküler markörün karşılaştırılması

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Abstract

Phosphine gas is the major pesticide applied to stored cereal grains against insects across the world and has been used in Türkiye since the 1950s. Increasing resistance to this fumigant is a problem in stored grain pests worldwide. This study determined the phosphine resistance ratios of the lesser grain borer, *Rhyzopertha dominica* (F., 1792) (Coleoptera: Bostrichidae) in 18 populations from 12 provinces of Türkiye between 2013 and 2017. Discriminating dose studies showed 3 of 15 populations comprise phosphine-resistant specimens. Dose-response bioassays established that resistance ratios were between 96 and 533-fold. The current molecular resistance marker, which detects the amino acid mutation P49S in the DLD (dihydrolipoamide dehydrogenase) gene, were assayed in phosphine-resistant populations. The R allele occurred at a high frequency (83.7%) in 15 highly resistant populations and was absent in three susceptible populations. For 324 individuals from the resistant populations the average proportion of homozygous resistant, heterozygous resistant and homozygous susceptible alleles were 62.0, 18.9 and 19.1%, respectively. The genetic marker detection results were comparable to bioassay results in relation to the resistance status of Turkish populations of *R. dominca*. So, genetic testing for phosphine resistance will simplify resistance management in Türkiye.

Keywords: Bioassay, DLD, lesser grain borer, P49S, phosphine

Öz

Fosfin gazı depolanmış hububattaki böceklere karşı dünya genelinde kullanılan ana pestisittir. Türkiye'de de 1950'lerden itibaren kullanılmaktadır. Bu fumiganta karşı dünya genelinde böceklerde direnç artışı önemli bir problemdir. Bu çalışmada ekin kambur böceği, *Rhyzoperta dominica* (F., 1792) (Coleoptera: Bostrichidae)'nın ülkemizde 12 ilden 18 popülasyonundaki fosfin dirençleri 2013-2017 yılları arasında belirlenmiştir. Ayırıcı doz çalışmaları 15 popülasyonda fosfin direnci geliştiğini göstermiştir. Bu popülasyonlarda doz-yanıt bioassayleri, direnç oranlarının 96-533 kat arasında değiştiğini göstermiştir. Ayrıca, fosfin direncine sahip bu popülasyonlarda DLD (dihydrolipoamide dehydrogenase) geninde amino asit mutasyonunu gösteren mevcut moleküler direnç markörü P49S test edilmiştir. R direnç alleli bu 15 popülasyonda yüksek frekansta (%83.7) belirlenmişken hassas olan üç popülasyonda ise belirlenmemiştir. Dirençli popülasyonlardaki 324 bireyden elde edilen genetic sonuçlara göre homozigot direnç, heterozigot direnç ve homozigot hassas allel oranları sırasıyla %62.0, 18.9 ve 19.1 olarak belirlenmiştir. Türkiye *R. dominica* popülasyonlarında genetik markör ile fosfin direncini belirleme sonuçlarının bioassay sonuçlarıyla kıyaslanabilir olduğu görülmüştür. Sonuçta, fosfin direncinin genetik olarak testlenmesi Türkiye'de direnç yönetimini kolaylaştıracaktır.

Anahtar sözcükler: Bioassay, DLD, ekin kambur biti, P49S, fosfin

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Introduction

Phosphine is a widely used fumigant insecticide for effective protection of stored products (Cato et al., 2017). After the phasing-out of methyl bromide (UNEP, 1995), the reliance on phosphine increased substantially (Nayak et al., 2010). The use of phosphine solely over several decades has led to the development of resistance in many insect species (Champ & Dyte, 1977; Collins et al., 2005; Lorini et al., 2007). The lesser grain borer, *Rhyzopertha dominica* (F., 1792) (Coleoptera: Bostrichidae) is one of the most damaging stored-product insects in Türkiye and worldwide, causing substantial economic loss to stored cereal grains. High-level phosphine resistance in *R. dominica* has been recorded in Australia (Collins et al., 2002, 2016), Bangladesh (Tyler et al., 1983; Hasan et al., 2018), Brasil (Lorini et al., 2007; Pimentel et al., 2010), Burkina Faso (Hasan et. al., 2018), China (Cao et al., 2004; Song et al., 2011), Greece (Agrafioti et al., 2019), India (Kaur et al., 2015; Muralitharan et al., 2016), Malaysia (Hasan et al., 2018), Morocco (Benhalima et al., 2004), Pakistan (Ahmad et al., 2013; Wakil et al., 2021), Philipinnes (Acda et al., 2000) and the USA (Opit et al., 2012; Cato et al., 2017; Afful et al., 2018). Nayak et al. (2015) indicated that the resistant phenotype had 100-fold or greater LD_{50}.

Phosphine has been used in Türkiye since the 1950s. About 6.3 kt of wheat were imported annually between 2012 and 2017 years in Türkiye. So, many phosphine resisted different pest species that could be entered the country. Also, poorly isolated storage and false dose applications of phosphine cause the development of resistant species. Recently, some bioassays for phosphine resistance of coleopteran insects were performed on Turkish populations of the rust-red flour beetle, *Tribolium castaneum* (Herbst, 1797) (Coleoptera: Tenebrionidae) (Koçak et al., 2015), the lesser grain borer, *R. dominica* (Yilmaz & Koçak, 2017), the rusty grain beetle, *Cryptolestes ferrugineus* (Stephens, 1831) (Coleoptera: Laemophloeidae) (Koçak et al., 2018a), the rice weevil, *Sitophilus oryzae* (L., 1763) (Coleptera Curculionidae) (Işıkber et al., 2017), the grain weevil, *Sitophilus granarius* (L., 1758) and the sawtoothed grain beetle, *Oryzaephilus surinamensis* (L., 1758) (Coleoptera: Silvanidae) (Koçak et al., 2018b). These studies have shown that phosphine resistance is more common and at high levels in Türkiye than detected previously. Phosphine resistance is a serious problem and current resistance bioassays are labor-intensive and time-consuming. So, it is important to determine the genetic resistance factors so that selection for even higher resistance levels can be avoided (Schlipalius et al., 2008). The genetic of phosphine resistance in *R. dominica* was first characterized by Schlipalius et al. (2002). Subsequently, Schlipalius et al. (2012) discovered that mutations in the gene coding for DLD in *R. dominica* is a cause of phosphine resistance at the *rph2* locus. Mau et al. (2012) showed that the same *rph2* (DLD) locus was responsible for the development of high phosphine resistance in multiple strains of *R. dominica*. Kaur et al. (2013) developed a DNA marker to determine the distribution of phosphine-resistance of *R. dominica.* Subsequently, the amino acid substitutions on the DLD gene were determined as P49S (the most frequent), P85S, G135S, K142E and N506H (Schlipalius et al., 2018). The most frequent variants have been found to be, P49S, K142E, P85S and G135S (Nayak et al., 2018, 2021) in Australia. The widespread presence of P49S in several populations of *R. dominica* across Australia (Kaur et al., 2013; Nayak et al., 2018, 2021; Schlipalius et al., 2019), the USA (Chen et al., 2015) and India (Kaur et al., 2015) have been reported recently. Molecular resistance markers have been developed for P49S (Kaur et al., 2013; Chen et al., 2015). A resistant allele in *T. castaneum*, P45S (a homolog of P49S in *R. dominica*) has also been detected in Türkiye (Koçak et al., 2015). So, we determined whether the marker for P49S was appropriate for Turkish phosphine-resistant *R. dominica* populations by determining resistance levels among field populations using discriminating dose and detailed bioassays for resistance levels. The bioassay results were compared with the resistance allele marker for determining the allele frequencies in the same populations.

Materials and Methods

Sample collection and rearing

Beetles were collected in 2013-2014 from 18 grain storage facilities in 12 provinces of Türkiye (Figure 1). They were identified according to Mason & McDonough (2012). Sampling was conducted at five locations and depths in each facility, over the conveyor belt in large silos, and from the grain stored in bulk, approximately 4 kg wheat sample was taken using a 2-m grain probe. Each sample was brought to the laboratory in nylon bags, after being labeled with the date of taking, crop type, production year and sampling place. One kg subsamples were taken from each 4-kg samples and transferred to 1-L glass jars, then mouths of the jars covered with gauze. After keeping the glass jars in climate cabinets at 27°C, 65 ± 5% RH and 16:8 h L:D photoperiod, samples were passed through metal sieves (Retsch, Haan, Germany), beetles collected, identified, counted and subsequently cultured on a mixture of 95% whole soft wheat grains and 5% cracked grain admixture (w/w) (Chen et al., 2015). The nutrient mixture (~100 g) was added to 1-L glass jars with perforated lids. To prevent contamination, the jars were placed on plastic bases in tubs filled with liquid vaseline (Pimentel et al., 2008; Opit et al., 2012). Individuals emerging from the eggs transferred to these jars completed their development in approximately 30-35 days to become adults. The 0-24-hour eggs were collected and placed in nutrient a medium containing wheat flour and yeast. Adult emergence was observed daily in jars for about 30-40 days after the addition of eggs, and the first adult emergence date was recorded. From the first adult emergence to the week 7, all adults were taken from the jars and 1-3-week-old males and females of the first generation were used as mixed in the experiments (Esin, 1971; Şayeste, 1971; FAO, 1975; Işıkber, 2005; Opit et al., 2012).

Figure 1. The provinces where collected *Rhyzopertha dominica* populations across Türkiye.

Fumigation

Phosphine gas was produced from phosphine tablets (57% $AlPH_3$) in the gas generator. The aluminum phosphide tablets were added to the water in a 1-L glass cylinder containing 5% H_2SO_4 (FAO, 1975). Glass desiccators with a volume of 3 L with closed circuit gas circulation were used for the experiments. KOH solution was placed in the desiccator in order to provide the desired humidity at a level of 60-65% before the experiment (Solomon, 1951). The phosphine gas collected in the upper part of the desiccator was sampled through a septum with a 100 ml syringe. The gas pipes in the desiccator were connected to the gas outlet and gas suction parts of the phosphine gas measuring device (Ati PortaSense, Analytical Technology Inc., Collegeville, PA, USA). The required amount of phosphine gas was given with a syringe from the desiccator gas inlet. After reaching the required gas concentration in the setup, the phosphine measuring device was turned off. In addition, after the pipes connected to the desiccator were removed from the device, the desiccators were placed in the incubator at 26°C, 60 ± 5% RH and 16:8 h L:D photoperiod (FAO, 1975; Kahraman, 2009; Opit et al., 2012). Dräger Pac 7000 gas measuring device (Draeger Arabia Co. Ltd., Riyadh, Saudi Arabia) was placed in the incubator, leak proofness was measured in the desiccators and leaky desiccators were removed. PVC containers (3 x 3 x 3 cm) with 25 adults as mixed and 1-2 g of cracked wheat were placed in each desiccator. Experiments were set up with four replicates of up to five phosphine doses. After the insects were exposed to phosphine for 20 h, they were transferred to jars containing food and kept in the incubator for 14 days and viability counts were made.

Phenotypic resistance levels were determined on the progeny of field-collected adults according to the standard method (FAO, 1975) at discriminating doses of phosphine of 20 ppm for 20 h to detect weak resistance using Ati PortaSense gas measuring device. Mortality responses to PH_3 of the resistant strains were modified from Kaur et al. (2015) and measured against a range of PH_3 concentrations, 0.025-5.0 mg/l. Fumigation was undertaken by placing 25 unsexed adults (1-3 weeks post eclosion) in a 30-ml plastic cup containing 5 g whole grain with four replicates per dose. Samples were placed inside gas-tight desiccators and PH_3 was injected through a rubber septum in the lid using a gas-tight syringe. Insects were exposed to PH_3 for 48 h, then removed from the desiccators and kept until endpoint mortality was assessed following a recovery period of 7 days at 25°C and 65% RH. Both live and dead insects from the bioassays were subsequently preserved in 70% ethanol at -20°C before DNA extraction and molecular resistance screening.

Data analysis

The mortality data were corrected using Abbott's correction for control mortality (⩽10%; Abbott, 1925) before the probit analysis (Finney, 1971). The analysis was performed using LeOra Software, PoloPlus 2002-2009 statistical package. The resistance ratio for the resistant strains was calculated by dividing the LC_{50} of the resistant strain by the LC_{50} value of a reference Australian strain, a susceptible *R. dominica*, QRD14 (Collins et al., 2002).

Genomic DNA extraction and PCR conditions

The live and dead insects from the bioassays were subsequently preserved in 70% ethanol at -20°C until the genetic study. At least 15 individuals representing each population were used in molecular studies. Insects were arbitrarily sampled proportionally from both live and dead samples to avoid bias, and insects were tested from the progeny of the field sample (Schlipalius et al., 2018). Genomic DNA was extracted from the beetle samples from the field using Qiagen (Hilden, Germany) DNeasy Blood &Tissue Kit according to the manufacturer's instructions with some revisions. One adult was crushed with tissue lysis buffer (ATL) in an Eppendorf tube, proteinase K was added and kept at 56°C for 24 h. It was shaken by adding AL buffer, and ethanol (96%) was mixed on it. This mixture was transferred to a special filtered Eppendorf, centrifuged at 8,000 rpm for 1 min. The filtered Eppendorf was placed in a new tube and AW1 was added on the filter and centrifuged at 8,000 rpm for 1 min. The plastic tube was changed and AW2 buffer was added and centrifuged at 14,000 rpm for 3 min. An Eppendorf tube (1.5 ml) was placed under filter Eppendorf tube, AE buffer was added and waited for 1 min, then centrifuged at 8,000 rpm for 1 min. The gDNAs obtained were stored at -20°C during the study. The coding region of the *R. dominica dld* gene was amplified from the DNA (12.5 µL PCR direct buffer (Mg + dNTP), 0.5 µL Taq, 6.5 µl H_2O (Gibco, Thermo Fisher Scientific, Glasgow, UK), 0.75 µL forward Rd-MM (5'-AGGTCCAAGCGTAGGGTTTT-3') and 0.75 µL reverse (5'-AACTGGGAGAATTCGGCTTT-3') RPH2 primers (Chen vet al., 2015) using the following PCR conditions: initial denaturation for 3 min at 95°C, followed by 27 cycles of 95°C for 20 s, 55°C for 20 s and 68°C for 30 s, and a final extension at 68°C for 7 min. The PCR product was visualized using 1.5% agarose gel with TAE buffer (Schlipalius et al., 2012).

Determination of *rph2* allele frequencies

Detection of the P49S allele was determined by a restriction digestion assay. A 20 µL mixture containing 10 µL PCR product, 2 µl reaction buffer, 0.2 µl restriction enzyme (MboI), and 7.8 µL ddH2O was prepared and incubated at 37°C for 12 h. The PCR product consisted of a 375 bp fragment of the *dld* gene containing the nucleotide variant corresponding to the P49S variant that has been reported to confer resistance at the *rph2* locus and gives two fragments of 236 and 139 bp long (Chen et al., 2015) when digested with MboI. The resulting digestion product was run on 1.5% agarose gel with TAE buffer at 100 V for 60 min (Schlipalius et al., 2012).

Results and discussion

Phenotype characterization of resistance

The discriminative dose assays showed that while three strains (Diyarbakır RD55, Karaman RD19, and Batman RD56) exhibited no phosphine resistance, because of dying of all individuals. The other 15 strains from the nine provinces had high resistance (Table 1). The average resistance ratio was about 325-fold for the 15 resistant populations. The LC_{50} resistance ratios of the highly resistance strains were between 96- and 537-fold. The strain RD32 showed the highest resistance among the other highly resistant strains tested. This population was collected from grain storage facility with a high frequency of phosphine use in Şanlıurfa Province, which has a dry and hot climate. In contrast, the Samsun population (RD54) had the lowest resistance ratio of 96-fold. It can be easily said that high resistance to phosphine has developed and is now common in *R. dominica* in Türkiye. High resistance ratios have been previously revealed globally as 600-fold in Australia (Collins, 1998), 595-fold (Afful et al., 2017), and 1,520-fold in the USA (Opit et al., 2012), 86-fold (Ahmad et al., 2013) and 126-fold (Wakil et al., 2021) in Pakistan, >200-fold in Brasil (Lorini & Collins, 2006) and >80-fold in Bangladesh and Burkina Faso (Hasan et al., 2018).

Table 1. Resistance ratios in *Rhyzopertha dominica* populations

Population		n	h	Slope ± SE	LC_{50} ppm (95% confidence limits)	Resistance ratio
Province	Strain					
Australian*	QRD14				1.25	
Ankara	RD46	600	2.91	3.07 ± 0.25	305 (241-362)	244
	RD47	600	2.37	5.14 ± 0.55	446 (362-509)	357
Hatay	RD13	600	3.29	9.60 ± 1.62	471 (291-541)	377
İzmir	RD37	600	2.43	4.38 ± 0.30	357 (314-400)	286
	RD36	600	2.09	5.19 ± 0.44	396 (342-442)	316
Konya	RD6	600	3.58	3.85 ± 0.36	419 (316-502)	335
	RD17	600	5.86	2.40 ± 0.25	322 (176-436)	257
Kütahya	RD45	600	3.71	1.83 ± 0.29	124 (105-143)	99
Mersin	RD7	600	2.06	8.42 ± 0.58	666 (629-704)	533
Samsun	RD54	600	4.48	5.08 ± 0.33	120 (120-213)	96
Şanlıurfa	RD32	600	2.91	13.3 ± 1.80	671 (618-721)	537
	RD33	600	5.16	3.21 ± 0.27	394 (289-491)	315
	RD38	600	3.85	6.00 ± 0.52	481 (410-540)	384
Tekirdağ	RD21	600	4.16	6.54 ± 0.73	602 (490-681)	482
	RD44	600	7.36	2.12 ± 0.22	324 (152-455)	259

Genotype characterization of resistance

We estimated the frequency of one specific variant, the P49S resistance allele, in *R. dominica* populations across Türkiye. This allele has been previously detected at high frequencies in Australia (Schlipalius et al., 2012), India (Kaur et al., 2013), the USA (Chen et al., 2015) and Türkiye (as homolog allele P45S in *T. castaneum*) (Koçak et al., 2015). Nayak et al. (2018) identified three single nucleotide variants (SNVs), P49S, G135S, and K142E in *R. dominica*. These authors found that the frequency of

resistance allele K142E was extremely dominant over the other two SNVs. In an earlier study, Kaur et al. (2013) estimated the frequency of the K142E allele was 3-26%. Schlipalius et al. (2019) found that P49S was very common and the most resistance phenotype recorded for *R. dominica* in Australia and indicated that the variant is likely to be advantaged over alternative alleles in response to selection. When we analyzed 324 individuals from the 15 resistant populations, the average ratios of homozygous resistance, heterozygous resistance and homozygous susceptible alleles were 62.0, 18.9 and 19.1%, respectively. A total of 18 populations from 12 provinces showed an average of 69.8% R allele frequency. The R allele occurred at a high frequency (average 83.7%) in the 15 highly resistant populations and it ranged between 25 and 100% (Table 2). The R allele was absent in the three susceptible populations from Diyarbakır (RD55), Karaman (RD19) and Batman (RD56) Provinces because phosphine has not been used by the farmers for an extended period. Government silos have generally high phosphine-resistant populations because of routine and frequent phosphine use. It was determined that the populations with surviving individuals after phosphine exposure have resistance alleles. The SS alleles were not found in 11 populations of the 18 populations. It was shown that the marker for P49S works for Turkish phosphine-resistant or -susceptible *R. dominica* populations (Figure 2). It should be noted that the bioassay responses are a product of both strength and frequency of the resistance alleles, it is likely that the variance in resistance ratios is due to different frequencies of the *rph2* resistance allele. All the phosphine-resistant strains exhibited high frequencies of resistance compared to the susceptible strain and the research also has shown that populations with high frequencies of resistant individuals display higher resistant phenotype responses. For example, Mersin (RD7) and Şanlıurfa (RD32) populations had only homozygous resistant alleles (RR) and their resistance ratios were both about 535-fold. It is also remarkable that Samsun (RD54) population had no RR alleles and it had the lowest resistance of the highly resistant populations. When the resistance ratio exceeded 100X, R allele frequency ratio generally become high. So, phosphine application managements like dose and exposure time increase should be applied.

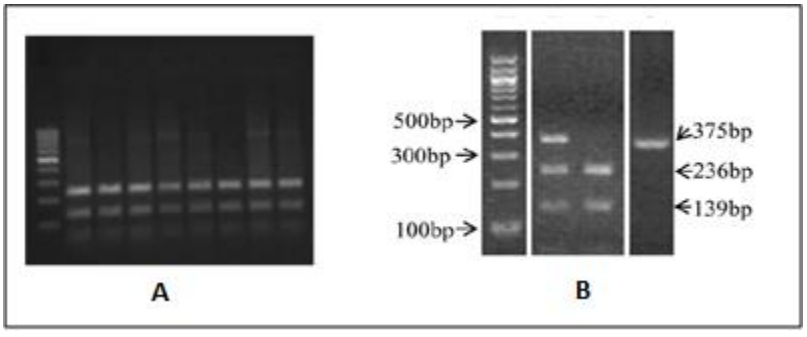

Figure 2. A) Representative gel on demonstration of utility of CAPS marker in *Rhyzopertha dominica* individuals from Türkiye; B) PCR amplicons of genomic DNA coding the DLD gene were digested with restriction enzyme, MboI. Homozygous resistant RR, 236 and 139 bp; susceptible SS, 375 bp; and heterozygous resistant, 375 and 236 bp.

No resistance alleles were found in Karaman (RD19), Diyarbakır (RD55) and Batman (RD56) populations, which were already been determined as susceptible according to discriminative dose studies (Tables 1 & 2). We found that the resistance ratios correlated with *rph2* allele frequencies in the highly resistant populations (Figure 3) and *rph2* alleles were absent in susceptible populations according to discriminative dose (Table 2). Therefore, we have demonstrated that the CAPS marker for P49S will readily detect phosphine-resistant individuals in Turkish *R. dominica* populations. This assay will inform and facilitate the implementation of phosphine resistance management strategies in Türkiye.

Table 2. Resistance related genotypes and allel frequencies in *Rhyzopertha dominica* populations

Population		n	Resistance statement			Allel frequency	
Province	Strain		RR (%)	RS (%)	SS (%)	R (%)	S (%)
Australian*	QRD14						
Ankara	RD46	19	16 (84.2)	3 (15.8)	0	92.1	7.89
	RD47	18	16 (90.0)	2 (11.1)	0	94.4	5.55
Hatay	RD13	19	10 (53.0)	8 (42.1)	1 (5.3)	73.7	23.7
İzmir	RD37	18	18 (100)	0	0	100	0
	RD36	18	15 (83.3)	3 (16.7)	0	91.7	8.33
Konya	RD6	19	15 (78.9)	4 (21.1)	0	89.5	10.5
	RD17	15	7 (46.7)	7 (46.7)	1 (6.7)	70.0	30.0
Kütahya	RD45	16	5 (31.2)	10 (62.5)	1 (6.3)	62.6	37.5
Mersin	RD7	18	18 (100)	0	0	100	0
Samsun	RD54	16	0	8 (50.0)	8 (50.0)	25.0	75.0
Şanlıurfa	RD32	18	18 (100)	0	0	100	0
	RD33	17	13 (76.5)	4 (23.5)	0	88.2	11.8
	RD38	14	12 (85.7)	2 (14.3)	0	92.8	7.14
Tekirdağ	RD21	18	15 (83.3)	3 (16.7)	0	91.7	8.33
	RD44	19	13 (68.4)	6 (31.6)	0	84.2	15.8
Batman	RD56	18	0	0	18 (100)	0	100
Diyarbakır	RD55	18	0	0	18 (100)	0	100
Karaman	RD19	18	0	0	18 (100)	0	100

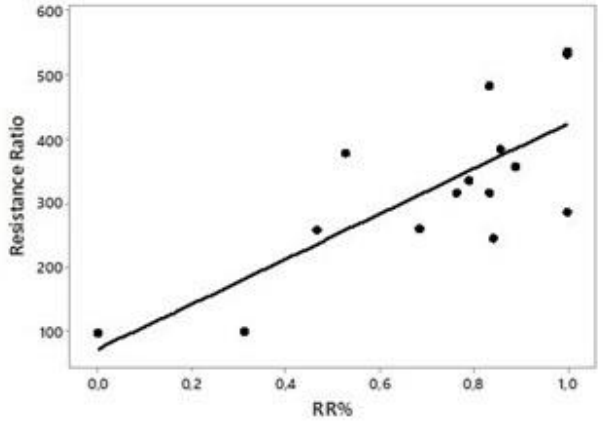

Figure 3. The regression equation is Resistance ratio = 71.4 + 352 RR% regression equation, Multiple R = 0.76, R-Sq = 0.57, R-Sq(adj) = 0.54.

This study showed the necessity of transition to phosphine use management, determination and implementation of a national phosphine resistance management strategies in order to ensure sustainable use of phosphine. In this framework, it is important to determine the factors contributing to resistance development, establish a resistance monitoring system, ensure the use of alternative control methods, evaluate the use of alternative fumigants, limit the use of phosphine according to regions, to update the phosphine usage instructions, to regulate the number of applications and to regulate the phosphine application doses according to the status of individual grain storage facilities.

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Original article (Orijinal araştırma)

Mortality, developmental biology and cellular immunity in *Achroia grisella* (Fabricius, 1794) (Lepidoptera: Pyralidae) larvae exposed to azadirachtin[1]

Azadirachtine maruz kalan *Achroia grisella* (Fabricius, 1794) (Lepidoptera: Pyralidae) larvalarında ölüm oranı, gelişim biyolojisi ve hücresel bağışıklık tepkileri

Aylin ER[2*]

Abstract

Azadirachtin, obtained from neem trees, can be a robust alternative to synthetic pesticides for the control of agricultural pests with no resistance problems. Azadirachtin-induced influences on mortality, life history traits and cellular immunity indicators of the lesser wax moth *Achroia grisella* (Fabricius, 1794) (Lepidoptera: Pyralidae) were evaluated. The experiments were conducted under controlled laboratory conditions at Balıkesir University. The topical application of azadirachtin gave an LD_{50} of 0.02 mg/ml whereas the PD_{50} (deaths without pupation) was 0.05 mg/ml. The prolongation of the larval stage and adult emergence time was significantly increased at 0.05 mg/ml and 0.1 mg/ml while the duration of the pupal stage was only significant at 0.1 mg/ml. Adult emergence ratios and longevity were reduced at all doses. Topical application of azadirachtin caused a marked decrease in the number of circulating hemocyte counts and spreading ability 24 and 48 h after treatment, however, the variations in plasmatocyte and granulocyte counts were not significant. Although azadirachtin has potential effects in the control of *A. grisella*, its effects on biological control agents such as parasitoids and predators must be determined to recommend its safe use in agroecosystems.

Keywords: *Achroia grisella*, azadirachtin, hemocyte count, toxicity

Öz

Neem ağaçlarından elde edilen Azadirachtin, direnç sorunu olmayan ve tarımsal zararlıların kontrolü için sentetik pestisitlere güçlü bir alternatif oluşturmaktadır. Küçük mum güvesi *Achroia grisella* (Fabricius, 1794) (Lepidoptera: Pyralidae)'da ölüm, gelişim biyolojisi ve hücresel bağışıklık göstergeleri üzerindeki azadirachtin kaynaklı etkiler değerlendirilmiştir. Denemeler kontrollü laboratuvar ortamında Balıkesir Üniversitesi'nde gerçekleştirilmiştir. Azadirachtinin topikal uygulamasına bağlı olarak LD_{50} 0.02 mg/ml bulunurken, PD_{50} (pupa dönemine geçmeden ölümler) 0.05 mg/ml olarak tespit edildi. 0.05 mg/ml ve 0.1 mg/ml'de larva dönemi ve ergin çıkış süresi, önemli ölçüde artarken, pupa dönemindeki uzama sadece 0.1 mg/ml'de önemli bulunmuştur. Ergin çıkış oranları ve ergin yaşam süresi, kullanılan tüm dozlarda azalmıştır. Azadirachtinin topikal uygulaması, uygulamadan 24 ve 48 saat sonra dolaşımdaki hemosit sayılarında ve hemosit yayılma davranışında önemli bir azalmaya neden olurken, plazmatosit ve granülosit sayılarındaki varyasyonlar istatistiksel olarak anlamlı bulunmamıştır. *Achroia grisella* ile mücadelede azadirachtinin potansiyel etkileri olmakla birlikte, agroekosistemlerde güvenli kullanımının önerilmesi için parazitoitler ve predatörler gibi biyolojik kontrol ajanları üzerindeki etkilerinin belirlenmesi önem arz etmektedir.

Anahtar sözcükler: *Achroia grisella*, azadirachtin, hemosit sayısı, toksisite

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Introduction

Concerns about the adverse effects of synthetic insecticides used in combating insects that damage agriculture, forestry and stored products continue to increase on the environment and human health. Short and long-term disadvantages of conventional pesticides have led to the emphasis on alternative control methods with natural origin, especially plant-derived compounds. One of the most well-known examples of biopesticides used as insect growth disruptors is azadirachtin, a limonoid tetranortriterpenoid obtained from the neem tree, *Azadirachta indica* A. Juss. (Sapindales: Meliaceae) (Mordue, 2004; Dorrah et al., 2019). Bioinsecticides with the active ingredient of azadirachtin are widely used in agriculture and integrated control programs within the scope of biological control against pests, which significantly reduce agricultural productivity (Bezzar-Bendjazia et al., 2017). The outstanding feature of these bioinsecticides depends on multiple anti-insect modes of action with no resistance problems (Mordue et al., 2005). Also, azadirachtin has also been defined as harmless for non-target organisms; however, this previous understanding has been recently reinterpreted, especially concerning pollinators, predators and parasitoids (Barbosa et al., 2015; Xavier et al., 2015; Bernardes et al., 2017).

Azadirachtin has both physiological and behavioral modes of action. The physiological effects are reported as the inhibition of insect growth, development, reproduction and synthesis of juvenile hormone (Chaudhary et al., 2017). Azadirachtin also acts as an ecdysone (molting hormone) antagonist by interacting with neural secretory cells of the insect brain-corpus cardiacum complex (Mordue et al., 2005; Bezzar-Bendjazia et al., 2017). Behavioral effects vary between different insect species defined as repellants or inhibitors of feeding (Schmutterer & Singh, 1995). In addition to these well-known and relevant influences, azadirachtin may also interact with the innate immune system of insects relying on germline-encoded factors to recognize and clear infection that is too often overlooked.

Insects have a highly conserved immune system consisting of humoral and cellular mechanisms. Cellular immune reactions are maintained by the hemocytes that phagocytose or capture non-self-invaders in multicellular layers called capsules and nodules while humoral immunity involves the synthesis of antimicrobial peptides like attacins, defensins and cecropins and a series of enzymatic cascades that regulates melanization (Lavine & Strand, 2002). In many Lepidopteran model insects, granulocytes, plasmatocytes, prohemocytes, spherulocytes and oenocytoids are the main hemocyte types in circulation (Kaya et al., 2021). Granulocytes and plasmatocytes are the most abundant hemocyte types with their ability to phagocytose, spread on foreign materials and capsule forming. Of the remaining hemocyte types that can be present in a small proportion in circulation, oenocytoids contain phenoloxidase precursors, spherulocytes are potential sources of cuticular components and prohemocytes differentiate into other hemocyte types as progenitor cells (Eleftherianos et al., 2021). It is important to point out that exposure to xenobiotics, even those of botanical origin, has the capacity of leading to stress on insects triggering immune defense reactions (Silva et al., 2020). Therefore, immune function in insects can be handled as an effective bioindicator to determine the systemic toxicity of biopesticides. Also, it can be a marker of which stage the insect will be more susceptible to infection. A limited number of studies demonstrated that azadirachtin can impact the immune functions of insect species (Azambuja et al., 1991; Sharma et al., 2003; Er et al., 2017; Silva et al., 2020). However, there appears to be no reports on the influence of azadirachtin on the biology and immune reactions of *Achroia grisella* (Fabricius, 1794) (Lepidoptera: Pyralidae). The lesser wax moth, *A. grisella*, is one of the major pests of beehives that feed on pollen, honey and wax. The pest insect is also a developing model organism frequently used to demonstrate the biological effects of xenobiotics (Uçkan et al., 2011; Çelik et al., 2017). In this study, the effects of topically applied azadirachtin on various biological parameters including mortality, development time and longevity as biological indicators and suppression of insect hemocyte counts and behavior as immune indicators were assessed.

Materials and Methods

Insect rearing

The larvae of the lesser wax moth *A. grisella* were established from adults that were obtained from apicultural regions in Balıkesir, Türkiye. Adult insects were transferred to 1-L jars containing honeycomb as an egg oviposition substrate. Insect cultures were kept in an incubator at 28 ± 2°C, 60 ± 5% RH and 12:12 h L:D photoperiod regime and were fed with blackened honeycomb to sustain their natural habitat in beehives (Er & Keskin, 2016). Last instar larvae of *A. grisella* used in all experiments. The process of establishing successive *A. grisella* cultures was continued throughout the experiments both to ensure the continuation of the culture and to obtain adults that would yield the last instar larvae used in the experiments. The experiments were conducted under laboratory conditions at Balıkesir University.

Azadirachtin treatment and toxicity bioassays

Azadirachtin used in the experimental analyses was purchased as a commercial product (NeemAzal-T/S, 10 g/L, Trifolio-M GmbH, Lahnau, Germany). Azadirachtin was diluted with distilled water to three concentrations (0.01, 0.05 and 0.1 mg/ml), accompanied by a control group was tested to search for the influences on biological and immunologic parameters. Five µl of diluted azadirachtin concentrations were applied topically (head to the abdomen) to 30 final instar larvae of *A. grisella* in three replicates for toxicity analyses and life-history traits. Control groups consisted of 30 untreated larvae. Azadirachtin-treated experimental groups along with control groups were transferred to incubators under the same conditions in Petri dishes and observed daily to determine the total and cumulative mortality. The larva that did not respond to mechanical stimulation and darkened in color were considered as dead. Based on obtained mortality data some chosen LDx and PDx concentrations were generated with probit analyses calculated by SPSS software (SPSS 22, IBM, Armonk, NY, USA).

Developmental biology

Measurement of the larval duration, the ratio of pupation (%), pupal period, duration and the ratio of adult emergence (%) was determined after azadirachtin treatment to the last instars of *A. grisella* at the same concentrations. Five µl of each concentration of azadirachtin were applied topically to *A. grisella*. Treated and control larvae were transferred to an incubator adjusted to 28 ± 2°C, 60 ± 5% RH, 12:12 h L:D photoperiod and monitored daily until adult emergence. Freshly emerged *A. grisella* adults obtained from the experimental sets were located in Petri dishes and observed daily until the adults die to record adult longevity. All the developmental indicators were determined for each replicate. For each experimental and control group, 10 arbitrarily selected last instar larvae were selected and evaluated by three replicates (n = 30).

Indicators of cellular immunity

Total and differential hemocyte counts (THC and DHC, respectively), and hemocyte spreading experiments were performed on each larva in all control and experimental groups. To determine the effects of azadirachtin on total and differential hemocyte counts and cell spreading, 5 µl of each azadirachtin concentration (0.01, 0.05 and 0.1 mg/ml) were applied topically to the last instars of *A. grisella*. Hemolymph was collected from the last instar larvae 24 and 48 h after azadirachtin treatment. *A. grisella* last instars were pierced with a 19-gauge sterile needle on the first hind leg and the hemolymph was pooled using a glass microcapillary tube (Sigma, St. Louis, MO, USA).

Total and differential hemocyte counts

To clarify the influence of azadirachtin on THCs, 4 µl hemolymph from control and azadirachtin treated groups were pooled in 36 µl ice-cold anticoagulant buffer (17 mM Na2 EDTA, 98 mM NaOH, 41 mM citric acid, 186 mM NaCl, pH 4.5) into an Eppendorf tube. Ten µl of the obtained cell suspensions were loaded

to a Neubauer hemocytometer (Neubauer Improved Hemocytometer; Superior Marienfeld, Lauda-Königshofen, Germany) and then examined under an Olympus BX51 microscope. Ten arbitrarily selected last instar larvae were assessed for every experimental and control group in three replicates (n = 30).

In order to determine DHCs, azadirachtin-treated and control last instars were bled with the aid of a 19-gauge needle and the hemolymph transferred into an Eppendorf tube containing phosphate-buffered saline (PBS, Sigma) kept on ice and mixed. Twenty µl of this dilute hemolymph was drawn onto a microscope slide and incubated in a humid chamber (Sigma) for 30 min. The hemocyte layers were examined and classified according to Er et al. (2009) under an Olympus BX51 Phase-contrast microscope (Olympus, Tokyo, Japan). Granulocytes and plasmatocytes were calculated from five arbitrarily selected fields (300 cells) and expressed as the ratio of total hemocytes.

Hemocyte spreading

Due to define the effects of azadirachtin on the spreading behavior of *A. grisella* hemocytes, 4 µl of hemolymph from control and azadirachtin-treated larvae were transferred in an Eppendorf tube containing PBS and gently mixed. Twenty µl of this suspension were spread on a slide and kept for 30 min in a humid chamber at 29 ± 1°C to allow the hemocytes to attach to the slide. Following the protocol, a total of 300 cells were considered from five arbitrarily determined fields under a light microscope and relative numbers of spreading hemocytes from 15 larvae in three replicates were recorded.

Statistics

Concentration-dependent changes in immune and developmental parameters of *A. grisella* due to azadirachtin treatment were tested for normal distribution using Levene's test. As all data were normally distributed and one-way analyses of variance were used for comparing experimental mean data. To determine the significant differences Tukey HSD test was conducted. Data for percentage values were Arcsine transformed before analyses. All statistical tests were performed with the SPSS version 22.0 software program (IBM SPSS Statistics for Windows, Armonk, NY, USA) and results were evaluated as significant when $P < 0.05$.

Results

Toxicity of azadirachtin

Mortality rates in *A. grisella* were evaluated in two categories as larval death and total death. Larval mortality rates increased from 7.5% at the lowest tested concentration of azadirachtin to 85% at 0.1 mg/ml in a dose-dependent manner. Similar results were also obtained in total mortality rates (Table 1). The topical application of azadirachtin presented an LD_{50} of 0.02 mg/ml whereas the PD_{50} (deaths without pupation) was 0.05 mg/ml (Table 2).

Table 1. Effects of topically applied azadirachtin concentrations (mg/ml) on larval and total mortality of *Achroia grisella*

Azadirachtin (mg/ml)	Larval mortality (% ± SE) *	Total mortality (% ± SE) *
0 (control)	5.0 ± 0.3 a	10.0 ± 0.8 a
0.01	7.5 ± 0.9 a	37.5 ± 3.5 a
0.05	37.5 ± 3.6 b	57.5 ± 4.2 b
0.1	85.0 ± 8.3 c	87.5 ± 7.5 c

* Groups marked with different letters are significantly different ($P < 0.05$; Tukey's HSD test). Larval mortality, $F = 17.2$, $df = 3,16$, $P = 0.001$; and total mortality, $F = 37.6$, $df = 3,16$, $P < 0.001$.

Table 2. LD (lethal doses) and PD (deaths in larval stage without pupation) of azadirachtin concentrations (mg/ml) administered to the last larval instars of *Achroia grisella*

Treatment	N#	X^2 (df)	Slope ± SE	Lethal doses (mg/ml)	
				LD (%95 CL)	
				LD_{10}	0.003
				LD_{30}	0.001
	200	8.24 (2)	1.60 ± 0.2	LD_{50}	0.020
				LD_{75}	0.050
				LD_{90}	0.130
Azadirachtin				PD (%95 CL)	
				PD_{10}	0.016
				PD_{30}	0.030
	200	8.35 (2)	2.77 ± 0.3	PD_{50}	0.047
				PD_{75}	0.082
				PD_{90}	0.136

*Total number of insects sampled in the bioassay.

Developmental biology and longevity

Duration of the larval stage was significantly elevated at 0.05 and 0.1 mg/ml azadirachtin treatment compared to control and 0.01 mg/ml doses (Table 3). Azadirachtin also prolonged the pupal period in a dose-dependent mode however the increase was only significant at 0.1 mg/ml (Table 3). Adult emergence time of azadirachtin-treated individuals increased with controls; however, significant prolongations were only observed at 0.05 and 0.1 mg/ml (Table 3). The longevity of azadirachtin-treated *A. grisella* adults was reduced significantly at all doses in comparison with the control group (Table 3).

Table 3. Effects of topically applied azadirachtin concentrations on larval, pupal period and adult emergence time of *Achroia grisella*

Azadirachtin (mg/ml)	Larval period (days ± SE) *	Pupal period (days ± SE) *	Adult emergence time (days ± SE) *	Longevity (days ± SE) *
0 (control)	7.6 ± 0.2 a	6.4 ± 0.2 a	14.1 ± 0.2 a	10.2 ± 0.6 a
0.01	7.2 ± 0.4 a	7.1 ± 0.3 a	14.2 ± 0.5 a	5.3 ± 0.4 b
0.05	14.1 ± 0.9 b	7.7 ± 0.5 ab	22.5 ± 1.0 b	3.6 ± 0.6 b
0.1	14.7 ± 1.1 b	9.2 ± 0.5 b	25.0 ± 0.7 b	4.8 ± 0.3 b

* Groups marked with different letters are significantly different ($P < 0.05$; Tukey's HSD test). Larval period, $F = 30.2$, df = 3,47, $P < 0.001$; pupal period, $F = 188$, df = 3,8, $P < 0.001$; adult emergence time, $F = 57.4$, df = 3,47, $P < 0.001$; and longevity, $F = 27.0$, df = 3,48, $P < 0.001$.

Percent pupation in control *A. grisella* larvae was 99% (Table 4). Pupation ratios decreased in all azadirachtin concentrations in a dose-related manner but were only significant at 0.05 and 0.1 mg/ml (Table 4). Similar dose-dependent reductions were also detected in adult emergence ratios. The percent adult emergence rates were 53.3, 26.6 and 5.0% at 0.01, 0.05 and 0.1 mg/ml azadirachtin treatments, respectively. The calculated adult emergence ratios were significant at all applied doses of azadirachtin (Table 4).

Table 4. Effects of topically applied azadirachtin concentrations (mg/ml) on pupation ratio (%) and adult emergence ratio (%) of *Achroia grisella*

Azadirachtin (mg/ml)	Pupation (% ± SE) *	Adult emergence (% ± SE) *
0 (control)	99.7 ± 0.5 a	99.7 ± 0.5 a
0.01	91.1 ± 7.7 a	53.3 ± 6.6 b
0.05	56.6 ± 1.3 b	26.6 ± 1.4 c
0.1	12.3 ± 1.7 c	5.0 ± 0.3 d

* Groups marked with different letters are significantly different ($P < 0.05$; Tukey's HSD test). Pupation ratios, $F = 188$, df = 3,8, $P < 0.001$; and adult emergence ratios, $F = 74.7$, df = 3,8, $P < 0.001$.

Total and differential hemocyte counts

THC in the hemolymph samples of last instar *A. grisella* larvae were 32.5 and 32.8 x 10^6 cell/ml at 24 and 48 h, respectively (Table 5). Topical application of azadirachtin caused a remarkable decrease at all doses compared to control at 24 and 48 h. The minimum count of 18.5 x 10^6 cell/ml was detected at 48 h after azadirachtin treatment at the maximum dose of 0.1 mg/ml.

Table 5. Effects of topically applied azadirachtin concentrations on total hemocyte count (x10^6 cell/ml) of *Achroia grisella*

Azadirachtin (mg/ml)	Total hemocyte count (x10^6 cell/ml) (mean ± SE) *	
	Time after treatment	
	24 h	48 h
0 (control)	32.5 ± 1.7 a	32.8 ± 0.9 a
0.01	23.6 ± 0.7 b	20.4 ± 1.5 b
0.05	23.5 ± 1.1 b	23.6 ± 1.5 b
0.1	23.1 ± 1.0 b	18.5 ± 0.8 c

* Groups marked with different letters are significantly different ($P < 0.05$; Tukey's HSD test). 24 h, $F = 14.5$; df = 3,116, $P < 0.001$; and 48 h, $F = 25.8$, df = 3,116, $P < 0.001$.

In this study, DHC was expressed as relative numbers of granulocytes and plasmatocytes that are the most prominent cell types classified in the hemocyte population of *A. grisella*. Plasmatocytes comprised 57.5 and 55.2% of the total hemocyte population of *A. grisella* last instars at 24 and 48 h control groups, respectively (Table 6). Granulocytes were the second-highest group of hemocytes in circulation with 42.0% and 44.1% in 24 and 48 h control groups. Topical application of different doses of azadirachtin caused alterations in both granulocyte and plasmatocyte ratios at 24 and 48 h after treatment however the changes were not significant ($P > 0.05$).

Table 6. Effects of topically applied azadirachtin concentrations (mg/ml) on differential hemocyte count (%) of *Achroia grisella*

Azadirachtin (mg/ml)	Granulocyte (% ± SE) *		Plasmatocyte (% ± SE) *	
	Time after treatment		Time after treatment	
	24 h	48 h	24 h	48 h
0 (control)	42.0 ± 1.7 a	44.1 ± 1.3 a	57.5 ± 1.7 a	55.2 ± 1.3 a
0.01	37.1 ± 3.2 a	46.0 ± 4.1 a	61.5 ± 3.2 a	52.1 ± 4.1 a
0.05	33.2 ± 3.1 a	45.8 ± 2.5 a	66.8 ± 3.1 a	53.4 ± 2.6 a
0.1	36.8 ± 2.6 a	39.7 ± 2.3 a	63.4 ± 2.6 a	56.7 ± 2.7 a

* Groups marked with different letters are significantly different ($P < 0.05$; Tukey's HSD test). Granulocyte 24 h, $F = 12.9$, df = 3,36, $P = 0.526$; granulocyte 48 h, $F = 21.9$, df = 3,36, $P = 0.210$; plasmatocyte 24 h, $F = 17.1$, df = 3,36, $P = 0.32$; and plasmatocyte 48 h; $F = 24.2$, df = 3,36, $P = 0.125$.

Hemocyte spreading

The ability of insect hemocytes to spread on a glass surface is commonly used as an indicator of immune fitness. Here in this study, the ratio of spreading hemocytes was 39.8% and 35.7% in control *A. grisella* larvae at 24 and 48 h period (Figure 3). The percentage of hemocytes exhibiting spreading behavior was reduced at all treated doses of azadirachtin compared to control at 24 and 48 h after treatment Table 7).

Table 7. Effects of topically applied azadirachtin concentrations on hemocyte spreading ability (%) of *Achroia grisella*

Azadirachtin (mg/ml)	Spread hemocytes (% ± SE)*	
	Time after treatment	
	24 h	48 h
0 (control)	39.8 ± 3.2 a	35.7 ± 2.3 a
0.01	23.6 ± 3.7 b	21.3 ± 3.1 b
0.05	20.5 ± 3.2 b	27.0 ± 2.0 b
0.1	21.7 ± 2.4 b	17.3 ± 2.1 b

* Groups marked with different letters are significantly different ($P < 0.05$; Tukey's HSD test). 24 h, $F = 7.89$, df = 3,36, $P < 0.001$; and 48 h, $F = 10.4$, df = 3,36, $P < 0.001$.

Discussion

Azadirachtin is one of the prominent botanical biopesticides used for agricultural pest control worldwide with more than 20 commercial products (Kilani-Morakchi et al., 2021). Existing literature reveals that the toxicity of azadirachtin varies in different insect species due to different penetration rates of pests and their physiological status. Slight to moderate toxicological effects have been reported on mortality rates (Er et al., 2017; Zhong et al., 2017; Amaral et al., 2019), growth inhibition and retardation of developmental time (Zhao et al., 2019), antifeedant activity (Qin et al., 2020), prevention of fecundity and egg viability (Amaral et al., 2018; Ferdenache et al., 2019). Also, azadirachtin is also a possible candidate to use in synergy with other microbial biocontrol agents and botanical compounds against insect pests (Konecka et al., 2019). As a basis for future studies involving such a combination with microbial control agents, more information is needed on the systemic impact of azadirachtin on the physiological state of pests, especially on insect immunity.

Topical application to last instars gave an LD_{50} of 0.02 mg/ml and led to concentration-related mortality. This finding is in accordance with a previous study demonstrating the effects of azadirachtin on the greater wax moth *Galleria mellonella* (L., 1758) (Lepidoptera: Pyralidae) (Er et al., 2017). High and dose-dependent mortality rates of azadirachtin as a bioinsecticide have also been documented in various Lepidopteran species containing *Helicoverpa armigera* (Hübner, 1808) (Lepidoptera: Noctuidae), *Spodoptera litura* Fabricius, 1775 (Lepidoptera: Noctuidae), *Schistocerca gregaria* (Forskål, 1775) (Orthoptera: Acrididae), *Tirathaba rufivena* Walker, 1864 (Lepidoptera: Pyralidae) and *Plutella xylostella* (L., 1758) (Lepidoptera: Plutellidae) (Schmutterer & Singh, 1995; Zhong et al., 2017). However, its effectiveness mostly depends on the doses, application methods and stages of insects. In a study on *T. rufivena* larvae, the contact effect of azadirachtin was found to be greater than the ingestion effect (Zhong et al., 2017). The influence of azadirachtin on insect developmental biology is due to diverse modes of action in insects (Scudeler & dos Santos, 2013). Delayed adult emergence due to azadirachtin treatment has been documented earlier in numerous lepidopteran species (Jagannadh & Nair, 1992; Adel & Sehnal, 2000; Tunca et al., 2012; Er et al., 2017). Similar results of larval, pupal and adult emergence time prolongation and high inhibition of pupal molting and adult emergence were also obtained in this study. In insects, growth and developmental processes are highly regulated by hormonal homeostasis of juvenile hormone (JH) and 20-hydroxyecdysone (Bensebaa et al., 2015; Kilani-Morakchi et al., 2021). Azadirachtin is considered to interfere with the hormonal balance by suppressing and modifying hemolymph JH and ecdysteroid titers leading to reduced pupation, failure of adult emergence, malformations and incomplete ecdysis (Bezzar-Bendjazia et al., 2017; Kilani-Morakchi et al., 2021). In a recent study, Shu et al. (2021)

identified the azadirachtin-respondent genes in *Spodoptera frugiperda* Smith & Abbot, 1797 (Lepidoptera: Noctuidae) and reported that the genes interrelated in chitin biosynthesis were mostly down-regulated by azadirachtin. The authors speculate that azadirachtin-induced suppressed expression of these genes is the molecular basis for prolonged larval molt and development inhibition (Lai et al., 2014; Shu et al., 2021). Elongated adult occurrence time of insects in the agricultural systems may give rise to greater pest mortality ratios owing to biotic and abiotic factors such as multiplied exposure to predators and pathogens (Akthar et al., 2012).

Topical application of azadirachtin on the last instars of *A. grisella* reduced adult longevity at all azadirachtin doses compared to control. The effects of azadirachtin on adult longevity have been demonstrated in a diverse array of pest insects, including *Anopheles gambiae* Giles, 19002 (Diptera: Culicidae), *Ceratitis capitata* (Wiedemann, 1824) (Diptera: Tephritidae), *Zabrotes subfasciatus* (Boheman, 1833) (Coleoptera: Bruchidae), *Amphiareus constrictus* (Stål, 1860) (Heteroptera: Anthocoridae), *G. mellonella* (Okumu et al., 2007; Silva et al., 2013; Vilca Malqui et al., 2014; Gontijo et al., 2015; Er et al., 2017). It was also mentioned that sublethal doses of azadirachtin-induced hormesis effect on adult longevity of greater wax moth *G. mellonella* (Er et al., 2017), however here in the current work longevity was decreased at all doses and not in line with the previous study. Shu et al. (2021) demonstrated azadirachtin-sensitive genes in *S. frugiperda* and postulated that there is an effect of azadirachtin on regulation of longevity. It is a known phenomenon that stress responses in insects are energetically-demanding events (Uçkan et al., 2011) and it was reported that insecticide stress although of botanical origin may cause decreases in hemolymph components such as free amino acids, protein, lipid and carbohydrates associated with energy metabolism (Sharma et al., 2012; Altuntaş et al., 2014). Previous work has demonstrated that the botanical insecticide azadirachtin also affects energy reserves, metabolism and biochemical processes of various insect species by interfering with protein synthesis and reducing protein, lipid and carbohydrate concentrations (Li et al., 1995). Most probably the reduction in energy reserves of insects terminating from azadirachtin-related stress may lead to delays in growth and development processes and also a decline in adult longevity. The shortened longevity of *A. grisella* adults may cause decreased fecundity of females in a shortened lifespan and reduce the pest abundance in subsequent generations.

The current study also demonstrated that azadirachtin interacts with the cellular immune system of *A. grisella*. Hemocytes are key components of the insect immune system and it was found that topical application of *A. grisella* last instars with azadirachtin led to a decrease in total hemocyte counts of larval hemolymph at 24 and 48 h after treatment even at low doses. The decline in the total circulating hemocyte numbers recorded in the current study has also been mentioned in other insect pests exposed to azadirachtin via various treatment methods (Azambuja et al., 1991; Sharma et al., 2003; Pandey et al., 2008; Pandey & Tiwari, 2011; Er et al., 2017; Duarte et al., 2020). Pandey et al. (2008) discussed that the decline in total hemocyte numbers as a result of azadirachtin treatment may be associated with the clustering of hemocytes in one region, the toxicological effects of azadirachtin and their inhibitor effects on endocrine glands. The most profound effect of azadirachtin at the physiological level is the inhibition of the synthesis and release of ecdysteroids from the prothoracic gland, leading to incomplete ecdysis in immature insects (Isman, 2006). It is most likely that the reduction in THC is a result of endocrine regulation of azadirachtin because it was recently reported that the cellular immunity in insects is influenced by the hormones circulating in the hemolymph, including ecdysteroids (Nunes et al., 2021). An alternative explanation could be that the reductions in THC may be due to inhibition of hematopoietic function in larvae or declined mitotic division as alterations in hemocyte counts are also influenced by these factors (Gardiner & Strand, 2000; Rajak et al., 2015). Previous studies have demonstrated the cell cycle arrest and antimitotic effects of azadirachtin in insect cell lines (Salehzadeh et al., 2003; Huang et al., 2011). To prove this hypothesis, studies of azadirachtin-induced effects on *A. grisella* hemocyte division need to be conducted. In a recent study, Zhao et al. (2019) reported that genes related to apoptosis were up-regulated in

Bactrocera dorsalis Hendel, 1912 (Diptera: Tephritidae) after azadirachtin treatment, including genes encoding cathepsins. In addition to the azadirachtin-induced factors given above, the reduced number of THC after azadirachtin treatment could also be related to apoptotic death of hemocytes non-selective to a single type of hemocyte.

Despite the decreased total hemocyte count in the current study, no significant change was observed in the ratio of granulocytes and plasmatocytes. Previous studies reported significant changes in differential hemocyte counts caused by azadirachtin in various insect species (Dorrah et al., 2019, Pandey et al., 2008, Er et al., 2017). It has been reported that the variation in granulocyte and plasmatocyte numbers may be due to the transformation of some hemocyte types into other types for the phagocytic function, combatting against abiotic and biotic factors and foreign invaders or apoptotic bodies (Dorrah et al., 2019). However, in this study, the suppression of the spreading behavior of hemocytes instead of gaining phagocytic activity as a result of azadirachtin treatment seems to have eliminated the necessity of transformation of hemocytes. Previous reports in the literature strongly suggest that the differentiation of hemocytes in insects is influenced by the secretion of hormones circulating in the hemolymph including ecdysone (Nunes et al., 2021). The fact that azadirachtin, as an ecdysone antagonist may also negatively affect hemocyte differentiation could be the reason why granulocyte and plasmatocyte ratios remain unchanged in the current study. Combine with the reduction in THC, the same azadirachtin-induced effects are thought to be non-specific to one type of hemocyte in circulation.

Hemocyte spreading behavior is also an indicator of immunity in insects that occurs prior to cellular immune responses like encapsulation, phagocytosis and nodulation as it allows plasmatocytes and granulocytes to adhere to foreign materials (Lavine & Strand, 2002). Here we detected significant reductions in the spreading ability of hemocytes at all doses compared to control 24 and 48 h after azadirachtin treatment. However, the effect was not concentration-dependent and increases in azadirachtin concentration produced no further appreciable decrease in the ratio of spreading hemocytes. Probably, the dose-response relationship for the adverse effects on spreading ability reaches a maximum of 0.01 mg/ml or at concentrations lower than the minimum tested dose. Our results are consistent with previous studies that demonstrated inhibited spreading of hemocytes in the greater wax moth *G. mellonella* on exposure to azadirachtin (Er et al., 2017) and other botanicals (Zibaee & Bandani, 2010; Zibaee et al., 2012). Based on proteomic studies, azadirachtin interfered with the regulation of cell adhesion pathways (Sun et al., 2018) and genes responsible for key steps in hormone biosynthesis (Liu et al., 2019). Considering the potent relationship between hormone signaling and the behavior of hemocytes (Nunes et al., 2021), the reduced ratio of spreading hemocytes in the current study could be related to azadirachtin-induced changes in ecdysone titers related to the regulation of immunity.

We conclude that topical application of azadirachtin has detrimental impacts on mortality, developmental biology and the cellular immune function of A. *grisella* larvae. In combination with the previous studies demonstrating the hormonal regulation of azadirachtin in insects, the current findings reveal that azadirachtin can also act as an immunotoxic agent. The interrelation of azadirachtin-like phytochemicals with insects, through regulation of hemocyte counts and immune defenses, may provide opportunities for newer methods of pest control in agroecosystems.

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Original article (*Orijinal araştırma*)

New contributions to the Turkish aphid fauna and species composition (Hemiptera: Aphididomorpha) in Isparta forests[1]

Türkiye afit faunasına yeni bir katkı ve Isparta ormanlarındaki afit türlerinin (Hemiptera: Aphididomorpha) kompozisyonu

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Abstract

A study was conducted on the aphid fauna of *Pinus nigra* Arnold. subsp. *pallasiana* (Lamb.) Holmboe., *Pinus brutia* Ten., *Cedrus libani* A. Rich., *Abies cilicica* (Antoine & Kotschy) Carrière (Pinaceae), *Juniperus* spp. (Cupressaceae), *Quercus* spp. (Fagaceae) and *Robinia pseudoacacia* L. (Leguminosae) forests in Isparta Province between 2018 and 2020. Using systematic and random sampling, 9,252 specimens in 68 species from the families Aphididae and Phylloxeridae (14 species at genus level only) were identified between 2018 and 2020. It was determined that *Appendiseta robiniae* (Gillette, 1907) collected from *R. pseudoacacia* is a Nearctic species, which was identified as a new record for the aphid fauna of Türkiye. Fifty-five species were detected in 2019, and a further 51 in 2020 using systematic sampling. The species with the highest number of specimens in 2019 were *Myzocallis boerneri* Stroyan, 1957 (16.0%), *Eulachnus rileyi* (Williams, 1911) (12.3%) and *Cinara cedri* Mimeur, 1936 (10.4%). In 2020, the species with the highest number of specimens were *E. rileyi* (10.1%), *A. robiniae* (9.3%) and *Cinara orientalis* (Takahashi, 1924) (7.2%). The highest number of aphid species was collected from *P. nigra* in the three years (2018-2020). The second highest numbers were collected from *Quercus coccifera* L. in 2018 and 2020, and *P. brutia* in 2019.

Keywords: Alien species, aphid, forest trees, Isparta, Türkiye

Öz

Bu çalışma Isparta ilinde *Pinus nigra* Arnold. subsp. *pallasiana* (Lamb.) Holmboe., *Pinus brutia* Ten., *Cedrus libani* A. Rich., *Abies cilicica* (Antoine & Kotschy) Carrière (Pinaceae), *Juniperus* spp. (Cupressaceae), *Quercus* spp. (Fagaceae) ve *Robinia pseudoacacia* L. (Leguminosae) ormanlarında afit faunasını belirlemek amacıyla gerçekleştirilmiştir. Sistematik ve rastgele örnekleme kullanılarak 2018-2020 yıllarında olmak üzere Aphididae ve Phylloxeridae familyalarından 68 türe (14'ü cins düzeyinde) ait 9252 birey toplanmıştır. Nearktik bir tür olan ve *R. pseudoacacia*'dan toplanan *Appendiseta robiniae* (Gillette, 1907)'nin Türkiye afit faunası için yeni kayıt olduğu belirlenmiştir. Sistematik örnekleme kullanılarak 55 tür 2019, 51 tür ise 2020 yılında tespit edilmiştir. En fazla birey sayısına sahip olan türler 2019 yılında *Myzocallis boerneri* Stroyan, 1957 (%16.0), *Eulachnus rileyi* (Williams, 1911) (%12.3) ve *Cinara cedri* Mimeur, 1936 (%10.4); 2020 yılında ise *E. rileyi* (%10.1), *A. robiniae* (%9.3) ve *Cinara orientalis* (Takahashi, 1924) (%7.2) olmuştur. En fazla yaprak biti tür sayısı her üç (2018-2020) yılda da *P. nigra*'dan toplanmıştır. *Pinus nigra*'nın ardından en fazla tür sayısı, 2018 ve 2020 yıllarında *Quercus coccifera* L., 2019 yılında ise *P. brutia*'dan toplanmıştır.

Anahtar sözcükler: Yabancı tür, yaprak biti, orman ağaçları, Isparta, Türkiye

[1] This study was produced by the PhD thesis of the first author accepted by Isparta University of Applied Sciences, The Institute of Graduate Education, Department of Forest Engineering.

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Introduction

Insects have high levels of species diversity and coexist with many living things in virtually all terrestrial ecosystems. Their importance is due to their various functions, such as participation in the food cycle, pollination, biological management of pests, and wood and litter decomposition, that assist conservation and ensure the continuity of ecosystems (Gullan & Cranston, 2012). Relationships like competition and nutritional status among living things specify the structure of ecosystems. The phytophagous species, which invade the ecosystem and increase their population, are called invasive species when they cause economic or ecological damage. If these harmful populations do not reach an ecological balance within a certain period, many ecological relationships such as biological diversity and the food cycle can then be negatively impacted (Ayres & Lombardero, 2000; Gullan & Cranston, 2012). Aphids (Hemiptera: Aphididomorpha), which are considered significant pests, increase their populations readily by feeding on agricultural, ornamental and forest plant species, and they cause economic loss (Wieczorek et al., 2019).

Aphids cause direct damage by feeding on phloem sap, and they also cause indirect damage by leading to sooty mold and by being a vector of plant pathogens (Uygun et al., 2000; Wieczorek et al., 2019). The sooty mold formation blocks stoma, and it prevents photosynthesis and respiration. However, due to their feeding, a loss of quality and yield was observed in plants that lead to gall formation, leaf curving, yellowing and necrosis resulting in a decrease in seed yield, sprout formation, rates of photosynthesis, chlorophyll quantity and plant nutritional elements (Görür, 2008; Wieczorek et al., 2019; Özdemir, 2020). They feed on many parts of their host plants such as the leaf, stem, root and tubers according to their mouth texture. Therefore, usually more than one type of aphid species can feed on the same host tree plant (Carter & Maslen, 1982). Particularly, the species that are members of the genus *Lachnus* Burmeister, 1835 (Hemiptera: Aphididae) feed on both leaves and stems of the broadleaved and coniferous trees (Chen et al., 2016).

In Europe, many of the invasive aphid species cause significant damage to agriculture, ornamental plants and forest trees (Coeur d'acier et al., 2010). According to Blackman & Eastop (2022), there are 5,000 aphid species in 510 genera, and according to Favret (2022) the number is 5 325 species in 534 genera. However, Simon et al. (2021) suggested that there are about 6 000 aphid species currently recognized. This number of species is estimated to be much higher due to their small body size, higher adaptability, cyclical parthenogenesis and camouflage on a different part of host plants (Blackman & Eastop, 2022; Favret, 2022). Over half of all aphids in the world feed on trees. Of the forest trees, 170, 70, 51, 225, 8 and 29 aphid species were detected on the *Pinus* spp., *Picea* spp., *Abies* spp., *Quercus* spp., *Cedrus* spp. and *Juniperus* spp. respectively (Blackman & Eastop, 2022). In North America, it was found that aphid species on coniferous trees caused major damage (Keen, 1938). Furniss & Carolin (1977) reported that aphid species cause yellowing of needles, slow down growth and cause early defoliation, especially in young trees infested with *Cinara* (*Schizolachnus*) *pineti* (Fabricius, 1781) (Carter & Maslen, 1982). Straw et al. (2005) reported that the *Elatobium abietinum* (Walker, 1849) can cause considerable loss of needles in *Picea sitchensis* (Bong.) Carrière (Pinaceae).

Çalışkan et al. (2012) reported that the North American species, *Myzocallis* (*Lineomyzocallis*) *walshii* (Monell, 1879), which was detected in Adana on *Quercus* sp., was detected for the first time in Europe (France) in 1988, and it then spread rapidly and was observed in many European countries. *Cinara curvipes* (Patch, 1912) as an invasive aphid species was reported in Türkiye in 2015 (Görür et al., 2015).

This study aimed to determine aphid species and their host plant associations in the forests of Isparta Province, Türkiye.

Materials and Methods

This study was conducted in 2018 to 2022 on *Pinus nigra* Arnold. subsp. *pallasiana* (Lamb.) Holmboe., *Pinus brutia* Ten., *Cedrus libani* A. Rich., *Abies cilicica* (Antoine & Kotschy) Carrière (Pinaceae), *Juniperus* spp., *Quercus* spp., and *Robinia pseudoacacia* L. (Leguminosae), which are the main forest trees in Isparta Province. According to the management data of the General Directorate of Forestry, the most common tree species in Isparta Province were determined and sample areas were created in the forests of these tree species. The field sample number was based on the number of areas where tree species are in pure stands, and systematic sampling was performed in 34 areas (8 areas for *P*. *nigra*, 4 for *P*. *brutia*, 5 for *C*. *libani*, 8 for *Juniperus* spp., 2 for *A*. *cilicica*, 5 for *Quercus* spp., and 2 for *R*. *pseudoacacia*) between 2019 and 2020 (Figure 1). Since there are more than one species in the oak and juniper sample areas, sample areas specified as *Juniperus* and *Quercus* genera. In addition, random sampling was carried out within the study areas.

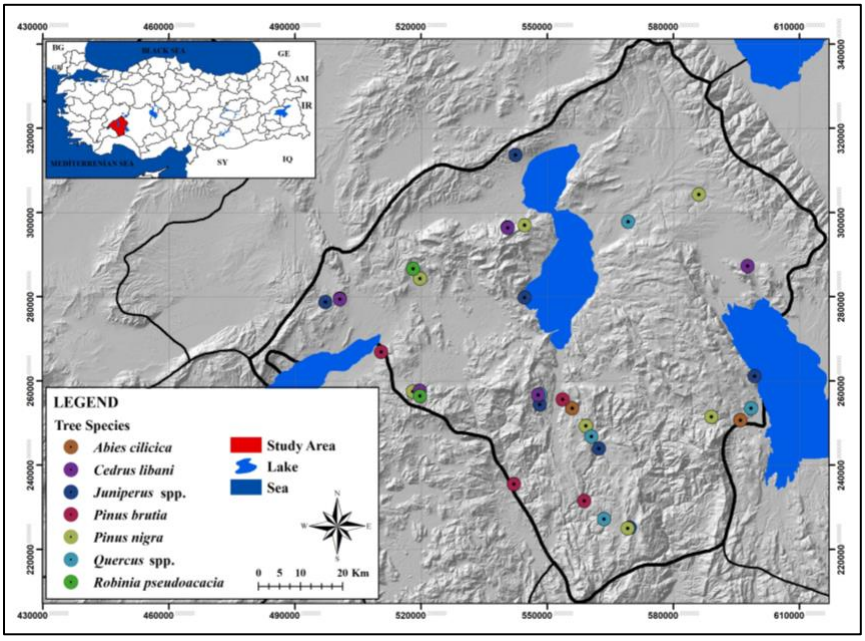

Figure 1. Distribution of sample areas according to tree species in the study area.

Circular sampling areas with a radius of about 25 m (2 000 m^2) were assessed for each tree species using systematic sampling in designated areas and the shoot of the nearest 10 trees in four cardinal directions was sampled by selecting the center point of each sample area. The samples were collected from the 10 trees adjacent to these trees were then sampled in the next month. This was to ensured that the samples were taken from all the trees in the area (Stekolshchikov & Kozlov, 2012). To eliminate any edge effect, trees found on the roadside or near gaps inside the stand were excluded as far as possible (Leather, 2005). Aphids generally feed on the young shoot so it is difficult to detect species with low population density in the canopy of trees, so 30 cm of shoot tips were taken of the sampled branches (Bryant, 1976).

The aphids were collected after they were stimulated with a fine brush (No. 0) and the samples were preserved in 96% ethanol in Eppendorf tubes. The preparation of the samples followed the methods of Martin (1983). Voucher samples were stored at the Entomology Museum of the Forestry Faculty at the Isparta University of Applied Sciences.

The identification of the specimens followed Blackman & Eastop (2022) and assistance was received from Prof. Dr. Gazi Görür (Niğde Ömer Halisdemir University, Faculty of Arts and Sciences) for the diagnosis. The species names and synonyms were checked according to Favret (2022) and de Jong et al. (2022).

Results and Discussions

The 65 species from the family Aphididae within the superfamily Aphidoidea and three species from the family Phylloxeridae within the Phylloxeroidea superfamily in the infraorder Aphididomorpha were detected by the end of the initial field work in 2018 and the systematic and random sampling between 2019 and 2020. In total, 9,252 specimens were collected during the study, with 165 of in the initial fieldwork. Fourteen of the specimens were classified at the genus level due to being alatae and immature specimens. *Thelaxes suberi* (Del Guercio, 1911) was the most abundant species in 2018 with 38 specimens and *Myzocallis* (*Myzocallis*) *boerneri* Stroyan, 1957 with 835 specimens and *Appendiseta robiniae* (Gillette, 1907) with 360 specimens were the most abundant species in 2019 and 2020 respectively (Table 1).

Table 1. Aphid species were identified in forests of Isparta Province between 2018 and 2020

No	Species	2018		2019		2020		Total	
		No	%	No	%	No	%	No	%
1	*Acyrthosiphon* (*Acyrthosiphon*) *gossypii* Mordvilko, 1914	0	0.00	1	0.02	2	0.05	3	0.03
2	*Acyrthosiphon* (*Acyrthosiphon*) *pisum* (Harris, 1776)	0	0.00	6	0.12	0	0.00	6	0.06
3	*Aphis* (*Aphis*) *craccivora* Koch, 1854	0	0.00	129	2.48	252	6.49	381	4.12
4	*Aphis* (*Aphis*) *spiraecola* Patch, 1914	0	0.00	0	0.00	13	0.33	13	0.14
5	*Aphis* sp.	0	0.00	1	0.02	86	2.22	87	0.94
6	*Appendiseta robiniae* (Gillette, 1907)	0	0.00	178	3.42	**360**	**9.27**	538	5.81
7	*Cinara* (*Cinara*) *acutirostris* Hille Ris Lamber's, 1956	0	0.00	3	0.06	0	0.00	3	0.03
8	*Cinara* (*Cinara*) *brauni* (Börner, 1940)	0	0.00	3	0.06	11	0.28	14	0.15
9	*Cinara* (*Cinara*) *cedri* Mimeur, 1936	2	1.21	**542**	**10.41**	141	3.63	**685**	**7.40**
10	*Cinara* (*Cinara*) *curvipes* (Patch, 1912)	0	0.00	31	0.6	48	1.24	79	0.85
11	*Cinara* (*Cinara*) *intermedia* (Pasek, 1954)	6	3.64	44	0.85	72	1.85	122	1.32
12	*Cinara* (*Cinara*) *juniperensis* (Gillette & Palmer, 1925)	1	0.61	79	1.52	25	0.64	105	1.13
13	*Cinara* (*Cedrobium*) *laportei* (Remaudière, 1954)	0	0.00	7	0.13	97	2.50	104	1.12
14	*Cinara* (*Cinara*) *maghrebica* Mimeur, 1934	0	0.00	51	0.98	0	0.00	51	0.55
15	*Cinara* (*Cinara*) *matsumurana* Hille Ris Lambers, 1966	0	0.00	1	0.02	13	0.33	14	0.15
16	*Cinara* (*Cinara*) *pectinatae* (Nordlinger, 1880)	0	0.00	74	1.42	0	0.00	74	0.80
17	*Cinara* (*Cinara*) *pini* (L., 1758)	0	0.00	2	0.04	0	0.00	2	0.02
18	*Cinara* (*Cinara*) *pinihabitans* (Mordvilko, 1894)	0	0.00	2	0.04	0	0.00	2	0.02
19	*Cinara* (*Cinara*) *piniphila* (Ratzeburg, 1844)	0	0.00	9	0.17	0	0.00	9	0.10
20	*Cinara* (*Cinara*) *pinivora* (Wilson, 1919)	0	0.00	30	0.58	12	0.31	42	0.45
21	*Cinara* (*Cinara*) *schimitscheki* Börner, 1940	1	0.61	7	0.13	17	0.44	25	0.27
22	*Cinara* (*Cinara*) sp. (on *Pinus nigra* and *P. brutia*)	2	1.21	37	0.71	16	0.41	55	0.59
23	*Cinara* (*Cinara*) sp. (on *Cedrus libani*)	0	0.00	16	0.31	15	0.39	31	0.34
24	*Cinara* (*Cinara*) sp. (on *Juniperus* spp.)	1	0.61	69	1.33	39	1.00	109	1.18
25	*Cinara* (*Cinara*) sp. (on *Abies cilicica*)	0	0.00	0	0.00	3	0.08	3	0.03
26	*Cinara* (*Cinara*) *wahluca* Hottes, 1952	2	1.21	251	4.82	185	4.77	438	4.73
27	*Cinara* (*Cinara*) *watanabei* Inouye, 1970	0	0.00	16	0.31	0	0.00	16	0.17
28	*Cinara* (*Cupressobium*) *tujafilina* (Del Guercio, 1909)	0	0.00	2	0.04	0	0.00	2	0.02
29	*Cinara* (*Schizolachnus*) *obscura* (Börner, 1940)	0	0.00	37	0.71	126	3.25	163	1.76
30	*Cinara* (*Schizolachnus*) *orientalis* (Takahashi, 1924)	23	13.9	163	3.13	**280**	**7.21**	466	5.04
31	*Cinara* (*Schizolachnus*) *pineti* (Fabricius, 1781)	0	0.00	192	3.69	80	2.06	272	2.94
32	*Cinara* (*Schizolachnus*) sp.	0	0.00	28	0.54	26	0.67	54	0.58
33	*Eulachnus agilis* (Kaltenbach, 1843)	0	0.00	42	0.81	6	0.15	48	0.52
34	*Eulachnus cembrae* Börner, 1950	0	0.00	7	0.13	0	0.00	7	0.08
35	*Eulachnus nigricola* (Pasek, 1953)	2	1.21	212	4.07	184	4.74	398	4.30
36	*Eulachnus pumilae* Inouye, 1939	0	0.00	19	0.37	14	0.36	33	0.36
37	*Eulachnus rileyi* (Williams, 1911)	7	4.24	**639**	**12.28**	**393**	**10.12**	**1039**	**11.23**
38	*Eulachnus* sp.	8	4.85	153	2.94	245	6.31	406	4.39
39	*Eulachnus thunbergi* Wilson, 1919	0	0.00	9	0.17	0	0.00	9	0.10

Table 1. Continued

No	Species	2018		2019		2020		Total	
		No	%	No	%	No	%	No	%
40	*Eulachnus tuberculostemmatus* Theobald, 1915	2	1.21	180	3.46	170	4.38	352	3.80
41	*Hoplocallis picta* (Ferrari, 1872)	0	0.00	1	0.02	3	0.08	4	0.04
42	*Hoplochaetaphis zachvatkini* (Aizenberg & Moravskaya, 1959)	0	0.00	0	0.00	75	1.93	75	0.81
43	*Hoplochaitophorus dicksoni* Quednau, 1999	0	0.00	5	0.10	0	0.00	5	0.05
44	*Lachnus crassicornis* Hille Ris Lambers, 1948	0	0.00	106	2.04	0	0.00	106	1.15
45	*Lachnus pallipes* (Hartig, 1841)	1	0.61	0	0.00	0	0.00	1	0.01
46	*Lachnus roboris* (L., 1758)	11	6.67	72	1.38	12	0.31	95	1.03
47	*Lachnus* sp.	0	0.00	6	0.12	8	0.21	14	0.15
48	*Lachnus swirskii* Hille Ris Lambers, 1954	13	7.88	1	0.02	0	0.00	14	0.15
49	*Lachnus tuataye* Remaudière, 2005	7	4.24	10	0.19	11	0.28	28	0.30
50	*Macrosiphum* sp.	0	0.00	0	0.00	1	0.03	1	0.01
51	*Mindarus abietinus* Koch, 1857	0	0.00	2	0.04	0	0.00	2	0.02
52	*Mindarus kinseyi* Voegtlin, 1995	0	0.00	0	0.00	114	2.94	114	1.23
53	*Mindarus* sp.	0	0.00	0	0.00	6	0.15	6	0.06
54	*Myzocallis* (*Myzocallis*) *boerneri* Stroyan, 1957	0	0.00	**835**	**16.0**	9	0.23	**844**	**9.12**
55	*Myzocallis* (*Myzocallis*) *glandulosa* Hille Ris Lambers, 1948	15	9.09	67	1.29	191	4.92	273	2.95
56	*Myzocallis* (*Pasekia*) *komareki* (Pašek. 1953)	0	0.00	0	0.00	20	0.52	20	0.22
57	*Myzocallis* (*Pasekia*) *mediterranea* Quednau & Remaudière, 1994	0	0.00	300	5.76	146	3.76	446	4.82
58	*Myzocallis* sp.	15	9.09	99	1.90	197	5.07	311	3.36
59	*Phylloxera quercina* (Ferrari, 1872)	0	0.00	22	0.42	0	0.00	22	0.24
60	*Phylloxera quercus* Boyer de Fonscolombe, 1834	0	0.00	0	0.00	6	0.15	6	0.06
61	*Phylloxera* sp.	0	0.00	0	0.00	7	0.18	7	0.08
62	*Pseudessigella brachychaeta* Hille Ris Lambers. 1966	2	1.21	11	0.21	26	0.67	39	0.42
63	*Thelaxes* sp.	1	0.61	0	0.00	1	0.03	2	0.02
64	*Thelaxes suberi* (Del Guercio, 1911)	**38**	**23.0**	302	5.80	79	2.04	419	4.53
65	*Thelaxes valtadorosi* Remaudière, 1983	0	0.00	0	0.00	6	0.15	6	0.06
66	*Tuberculatus* (*Tuberculoides*) *annulatus* (Hartig, 1841)	0	0.00	0	0.00	27	0.70	27	0.29
67	*Tuberculatus* (*Tuberculoides*) *borealis* (Krzywiec, 1971)	5	3.03	76	1.46	3	0.08	84	0.91
68	*Tuberculatus* sp.	0	0.00	18	0.35	3	0.08	21	0.23
	Total	**165**	**100**	**5205**	**100**	**3882**	**100**	**9252**	**100**

Although the most common species in 2019 were *M*. *boerneri*, *Eulachnus rileyi* (Williams, 1911) and *Cinara cedri* Mimeur, 1936 with 835 (16.0%), 639 (12.3) and 542 (10.4%) specimens, respectively, the least common species were *Acyrthosiphon gossypii* Mordvilko, 1914, *Aphis* sp., *Cinara matsumurana* Hille Ris Lambers, 1966, *Hoplocallis picta* (Ferrari, 1872) and *Lachnus tuataye* Remaudière, 2005 with only one specimen for each species. In 2020, the most common species were *E*. *rileyi*, *A*. *robiniae* and *Cinara orientalis* (Takahashi, 1924), with 393 (10.1%), 360 (9.3%) and 280 (7.2%) specimens respectively. However, at the end of the study the most common species were *E*. *rileyi* (1039), *M*. *boerneri* (844), and *C*. *cedri* (685). In 2020, it is notable that the detection of the most common species (*A*. *robiniae* with 360 specimens) on the host plant *R*. *pseudoacacia*, was from only two sampling areas using systematic sampling. It is concluded that *A*. *robiniae* was among the highest detected species as a result of its host plant-specific nature (Görür et al., 2014, 2020; Oğuzoğlu & Avcı, 2019; Kök et al., 2020, 2022; Kök & Özdemir, 2021; Patlar et al., 2021).

Studies in Türkiye have been conducted in both forest and non-forest areas, but few studies have reported aphid fauna for forest areas alone. The first studies on aphids determined in forest areas in Türkiye were made by Çanakçıoğlu (1966, 1967). Later, Tosun (1976) found five aphid species in Western Mediterranean Region. Özkazanç & Yücel (1985) species in Western Mediterranean Region. Özkazanç & Yücel (1985) detected 14 aphid species in *Pinus*, *Cedrus* and *Quercus* species in their semiarid zone plantations in Ankara. Cebeci (2003) stated that *Pineus pini* (Goeze, 1778) dried needles and shoots of

Pinus sylvestris L. in afforestation areas in Istanbul. Aytar (2006), *C*. *cedri* and *Cinara laportei* (Remaudiere, 1954) species identified on *Cedrus libani* in the forests in Eastern Mediterranean Region. Finally, Oğuzoğlu & Avcı (2019) determined the distribution, damage and natural enemies of *C*. *cedri* in the cedar forests of Isparta and Burdur Provinces. When looking at other studies, eighteen aphid species were detected in the Gölcük Nature Park in Isparta Province (Barjadze et al., 2014), 58 aphid species on *P*. *nigra*, *Quercus* spp., *Juniperus* spp., *C*. *libani* and *A*. *cilicica* were detected in Central Anatolia (Afyonkarahisar, Kütahya and Uşak Provinces) (Görür et al., 2014), 48 aphid species were detected in city parks in Burdur Province (Patlar et al., 2021) and 54 aphid species were detected in Antalya Province (Güleç, 2011). Comparing the number of aphid species to the number of host plants in the regions close to the study area, it was evident that the number of aphid species was high. Also, 68 aphid species from different host plants including some forest trees have been reported in Kahramanmaraş in Eastern Mediterranean Region (Aslan & Uygun, 2005). In Central Anatolian Region, 11 aphid species on *Pinus* spp., *Abies* spp., *Cedrus* spp. and *Picea* spp. were detected in city parks (Ülgentürk et al., 2010).

In Türkiye, to date *Aphis craccivora*, *A*. *craccivora* subsp. *pseudacaciae* Takahashi, 1966, *A*. *fabae* Scopoli, 1763, *A*. *gossypii* Glover, 1877, *A*. *spiraecola* Patch, 1914, *A*. *nasturtii* Kaltenbach, 1843, *A*. *sambuci*, *Brachycaudus* (*Brachycaudus*) *helichrysi* and *Therioaphis riehmi* (Börner, 1949) have been found on *R*. *pseudoacacia* (Schimitscheki, 1944; Yüksel, 1998; Bayhan et al., 2014; Aslan & Uygun, 2005; Toper Kaygın et al., 2008; Akyürek, 2013; Görür et al., 2014, 2018; Kök et al., 2016; Patlar et al., 2021). The feeding of *Appendiseta robiniae* on the *R*. *pseudoacacia* is a new record for Turkish aphid fauna, thus the Turkish aphid fauna reached 615 species (Figure 2) (Görür et al., 2022).

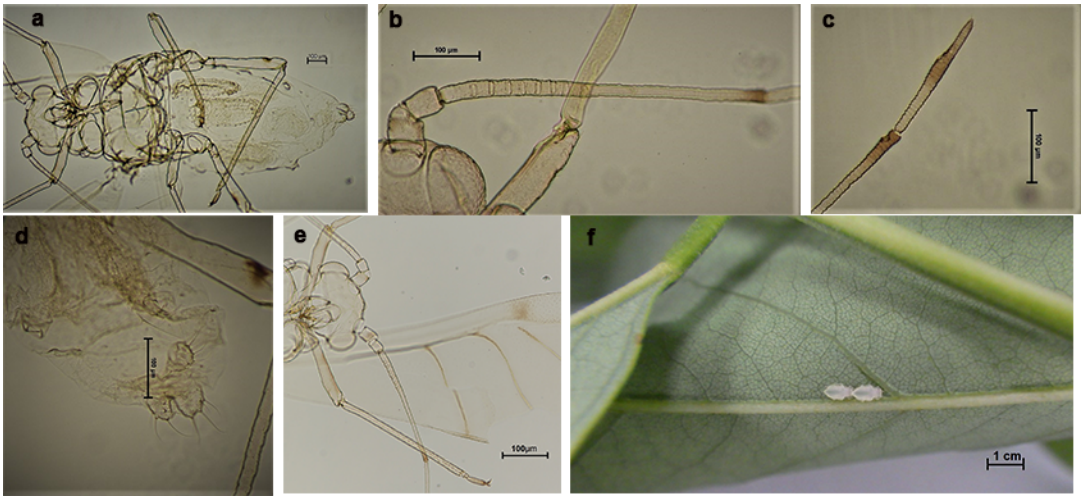

Figure 2. The taxonomic characteristics of *Appendiseta robiniae*: a) body of alate viviparous female; b) secondary rhinaria on antenna segment III; c) last segment of the antenna (base + processus terminalis); d) siphinculi and cauda; e) antenna and front wing) and f) *A*. *robiniae* alate viviparous female on the underside of a host plant leaf.

Appendiseta robiniae is an alien species in Türkiye and Europe being a Nearctic species (Borowiak-Sobkowiak & Durak, 2012). It is considered that it was introduced to the Neotropic and West Palearctic regions with host plants. It was recorded in Argentina, Chile, England, Germany, Hungary, Iran, Italy, Jordan, Netherlands, Poland, Russia, South America, Spain and Sweden (Borowiak-Sobkowiak & Durak, 2012; Entezari et al., 2016; Blackman & Eastop, 2022). This species was detected on *R*. *pseudoacacia*, *Robinia neomexicana* A. Gray, *Vitex agnus-castus* L. and *Sophora japonica* L. (Entezari et al., 2016; Blackman & Eastop, 2022). With the entry of this new alien aphid, the number of alien aphid species in Türkiye has reached 58 species (Kök & Özdemir, 2021). Also, it is assumed that this species was introduced to Türkiye some time ago given it having the highest number with a total of 538 specimens collected in 2019 and 2020. The distribution of aphid sampling numbers based on host species in the sampling area is given in Figure 3.

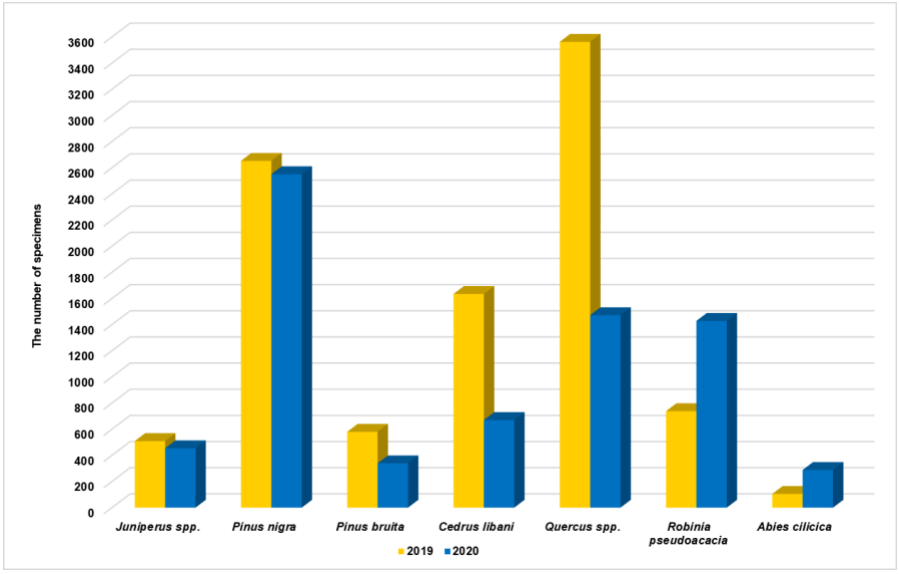

Figure 3. Number of aphid specimens collected from host trees in 2019 and 2020.

The highly infested host plants were the *C*. *libani*, *P*. *nigra*, *R*. *pseudoacacia* and *Quercus* spp. and the least infested tree species were the *A*. *cilicica*, *P*. *brutia* and *Juniperus* spp. in 2019 and 2020. Considering the aphid species distribution at genus level, the results show that the most infested host plant genus was *Cinara* with a 38% infestion rate, followed by the *Eulachnus* and *Lachnus*. However, only one aphid species was detected on the *Appendiseta*, *Hoplocallis*, *Hoplochaetaphis*, *Hoplochaitophorus*, *Macrosiphum*, and *Pseudessigella* (Figure 4).

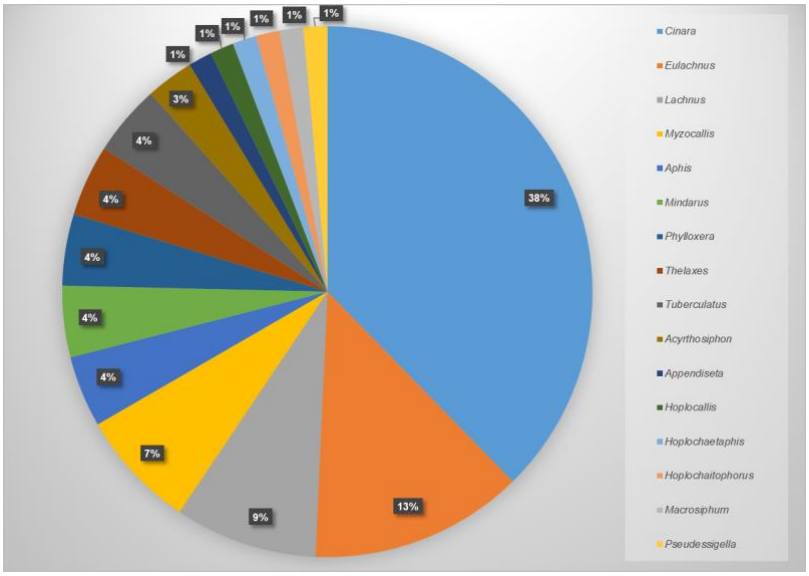

Figure 4. Aphid species distributions at genus level.

Aphids have been detected on plants in nearly 300 families and many are specific to certain host plant genera or families (Jaouannet et al., 2014; Blackman & Eastop, 2022). Therefore, aphid host-plant associations were considered in this study, and 68 aphid species were detected on 14 host species. In areas of the *Juniperus* spp., five aphid species were detected and the highest number of aphid species was detected on the most common species in this genus, namely the *Juniperus excelsa* (4 aphid species). Twenty-three aphid species were detected on the *Pinus* spp., and the highest number of aphid species

were detected on the *P*. *nigra*. Four aphid species were detected in areas of *C*. *libani* and six aphid species were detected in areas of *A*. *cilicica*. Görür et al. (2015) reported that *C*. *curvipes* feeds on both the *C*. *libani* and *A*. *cilicica*, and *C*. *curvipes* was also observed in this study on both these host plant members. In total, 22 aphid species were detected on *Quercus* with *Q*. *cerris* infested by the highest number of aphid species (15 species) and *M*. *glandulosa* was collected from all oak trees. *Robinia pseudoacacia* was included in the study due its importance as a forest tree with seven aphid species detected on this host. The aphid species composition was determined using systematic sampling in different forest habitats (Table 2).

Table 2. The relationships between aphid species and host trees

Host plants species	**Aphid species**
Robinia pseudoacacia	*Acyrthosiphon* (*Acyrthosiphon*) *gossypii*
	Acyrthosiphon (*Acyrthosiphon*) *pisum*
	Aphis (*Aphis*) *craccivora*
	Aphis (*Aphis*) *spiraecola*
	Aphis sp.
	Appendiseta robiniae
	Macrosiphum sp.
Abies cilicica	*Cinara* (*Cinara*) *matsumurana*
	Cinara (*Cinara*) *pectinatae*
	Cinara (*Cinara*) sp.
	Mindarus abietinus
	Mindarus kinseyi
	Mindarus sp.
Juniperus excelsa, *J. foetidissima*, *J. oxycedrus*	*Cinara* (*Cinara*) sp., *C*. (*Cinara*) *juniperensis*, *C*. (*Cinara*) *wahluca*
Juniperus excels	*Cinara* (*Cupressobium*) *tujafilina*
Cedrus libani	*Cinara* (*Cinara*) *cedri*
	Cinara (*Cinara*) *curvipes*
	Cinara (*Cinara*) *laportei*
	Cinara (*Cinara*) sp.
Pinus nigra	*Cinara* (*Cinara*) *acutirostris*, *C*. (*Cinara*) *brauni*, *C*. (*Cinara*) *intermedia*, *C*. (*Cinara*) *pini*, *C*. (*Cinara*) *pinihabitans*, *C*. (*Cinara*) *piniphila*, *C*. (*Cinara*) *schimitscheki*, *C*. (*Cinara*) *watanabei*, *Eulachnus agilis*, *E*. *cembrae*, *E*. *thunbergi*
Pinus nigra, *P*. *brutia*	*Cinara* (*Cinara*) sp., *C*. (*Cinara*) *maghrebica*, *C*. (*Cinara*) *pinivora*, *C*. (*Schizolachnus*) *obscura*, *C*. (*Schizolachnus*) *orientalis*, *C*. (*Schizolachnus*) *pineti*, *C*. (*Schizolachnus*) sp., *Eulachnus* sp., *E*. *nigricola*, *E*. *pumilae*, *E*. *rileyi*, *E*. *tuberculostemmatus*, *Pseudessigella brachychaeta*
Quercus cerris, *Q*. *trojana*, *Q*. *infectoria*, *Q*. *ithaburensis*	*Hoplocallis picta*
Quercus cerris, *Q*. *trojana*, *Q*. *vulcanica*	*Hoplochaetaphis zachvatkini*
Quercus ithaburensis	*Hoplochaitophorus dicksoni*
Quercus coccifera, *Q*. *vulcanica*, *Q*. *trojana*, *Q*. *cerris*	*Lachnus crassicornis*
Quercus cerris	*Lachnus pallipes*, *Phylloxera quercina*
Quercus coccifera, *Q*. *cerris*	*Lachnus roboris*, *Lachnus tuataye*
Quercus ithaburensis, *Q*. *coccifera*, *Q*. *cerris*	*Lachnus* sp.
Quercus coccifera	*Lachnus swirskii*, *Thelaxes valtadorosi*
Quercus ithaburensis, *Q*. *infectoria*, *Q*. *vulcanica*, *Q*. *trojana*, *Q*. *cerris*	*Myzocallis* (*Myzocallis*) *boerneri*
Quercus robur, *Q*. *coccifera*, *Q*. *ithaburensis*, *Q*. *infectoria*, *Q*. *vulcanica*, *Q*. *trojana*, *Q*. *cerris*	*Myzocallis* (*Myzocallis*) *glandulosa*
Quercus ithaburensis, *Q*. *infectoria*, *Q*. *cerris*	*Myzocallis* (*Pasekia*) *komareki*
Quercus robur, *Q*. *coccifera*, *Q*. *ithaburensis*, *Q*. *vulcanica*, *Q*. *trojana*, *Q*. *cerris*	*Myzocallis* (*Pasekia*) *mediterranea*
Quercus ithaburensis, *Q*. *infectoria*, *Q*. *vulcanica*, *Q*. *cerris*	*Myzocallis* sp.
Quercus infectoria	*Phylloxera quercus*, *Phylloxera* sp.
Quercus coccifera, *Q*. *infectoria*	*Thelaxes* sp.

Table 2. Continued

Host plants species	Aphid species
Quercus coccifera, *Q. ithaburensis*, *Q. infectoria*, *Q. vulcanica*, *Q. cerris*	*Thelaxes suberi*
Quercus infectoria, *Q. vulcanica*	*Tuberculatus* (*Tuberculoides*) *annulatus*
Quercus infectoria, *Q. vulcanica*, *Q. cerris*	*Tuberculatus* (*Tuberculoides*) *borealis*
Quercus vulcanica	*Tuberculatus* sp.

Considering the distribution of aphid species by host species, it was found that more than half of the species were detected on *P. nigra* (30%) and *Quercus* spp. (28%), followed by *Pinus brutia* these host with 17% of species (Figure 5). Comparing coniferous and broadleaved trees, it was observed that more than half of the aphid species were on coniferous trees (58%). The fact that coniferous tree species sampled in the study were higher than the broadleaved ones could be affected by the distribution of aphid species.

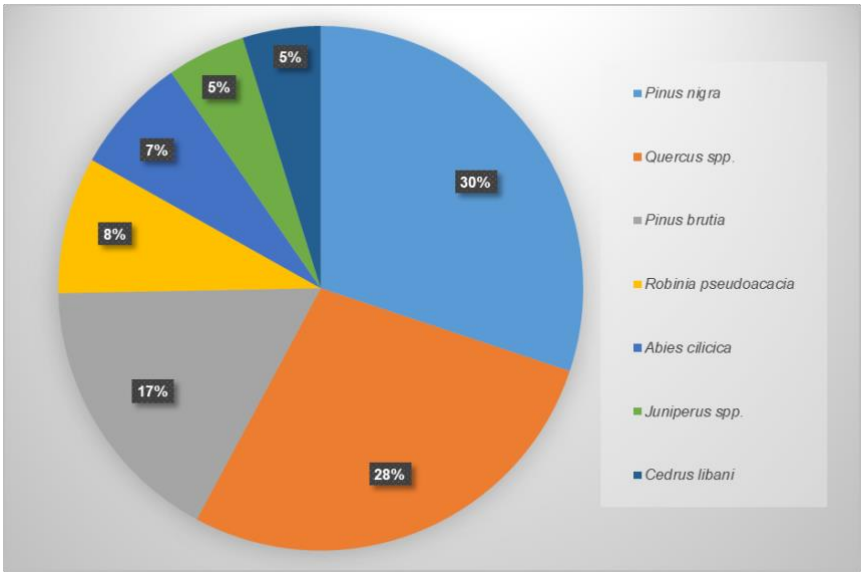

Figure 5. Distribution of aphid species detected on host trees.

During the study, the most infested host tree was *P. nigra* (Figure 6), followed by the *Quercus coccifera* and *P. brutia*. Considering the number of host species, aphids were observed on eight species in 2018 and on 15 species in 2019 and 2020. Over the three years, the greatest aphid species diversity was mostly found on *Q. cerris* with 15 aphid species. It was noted that this aphid on the endemic oak, *Q. vulcanica*, was a first record in Türkiye with 10 aphid species detected on this host. Forty-two aphid species have been detected on oak trees in Türkiye (Çanakçıoğlu, 1975; Düzgüneş et al., 1980; Özkazanç & Yücel, 1985; Tuatay, 1999; Uygun et al., 2000; Aslan & Uygun, 2005; Eser et al., 2009; Görür et al., 2009, 2014, 2018; Tepecik, 2010; Çalışkan et al., 2012; Akyürek, 2013; Kanturski et al., 2014; Öztürk, 2017; Kök, 2019; Patlar et al., 2021) and 23 (55%) of these species were detected in the present study. *Cinara curvipes*, *C. matsumurana*, *C. pectinatae* and *Mindarus kinseyi* Voegtlin, 1995 were observed on the *A. cilicica* in the present study. Also, two aphid species [*Cinara juniperensis* (Gillette & Palmer, 1925) and *C. wahluca*] on the *Juniperus foetidissima* Willd. 1806 was detected for the first time in Türkiye. Seven aphid species have previously been found on *Juniperus communis*, *J. oxycedrus*, *J. excelsa*, *J. nana*, *J. sabina* and *Juniperus* spp. in Türkiye (Çanakçıoğlu, 1975; Tosun, 1976; Tuatay, 1999; Görür et al., 2009, 2014; Ülgentürk et al., 2010; Akyürek, 2013; Şenol et al., 2015; Oğuzoğlu et al., 2021).

Sixty-eight aphid species were detected using random and systematic sampling in this study, with 65% of these detected by systematic sampling and 35% by random sampling. The highest number of species found by random sampling was in 2018 with 22 (39%) specimens, followed by 20 (36%) specimens

in 2020 and finally 14 (25%) specimens in 2019. *Lachnus swirskii* Hille Ris Lambers, 1954, *L. pallipes*, *Thelaxes valtadorosi* Remaudière, 1983 and *Phylloxera quercus* Boyer de Fonscolombe, 1834 were detected on *Quercus* spp. and *Cinara pini* was detected on *P. nigra* only by random sampling.

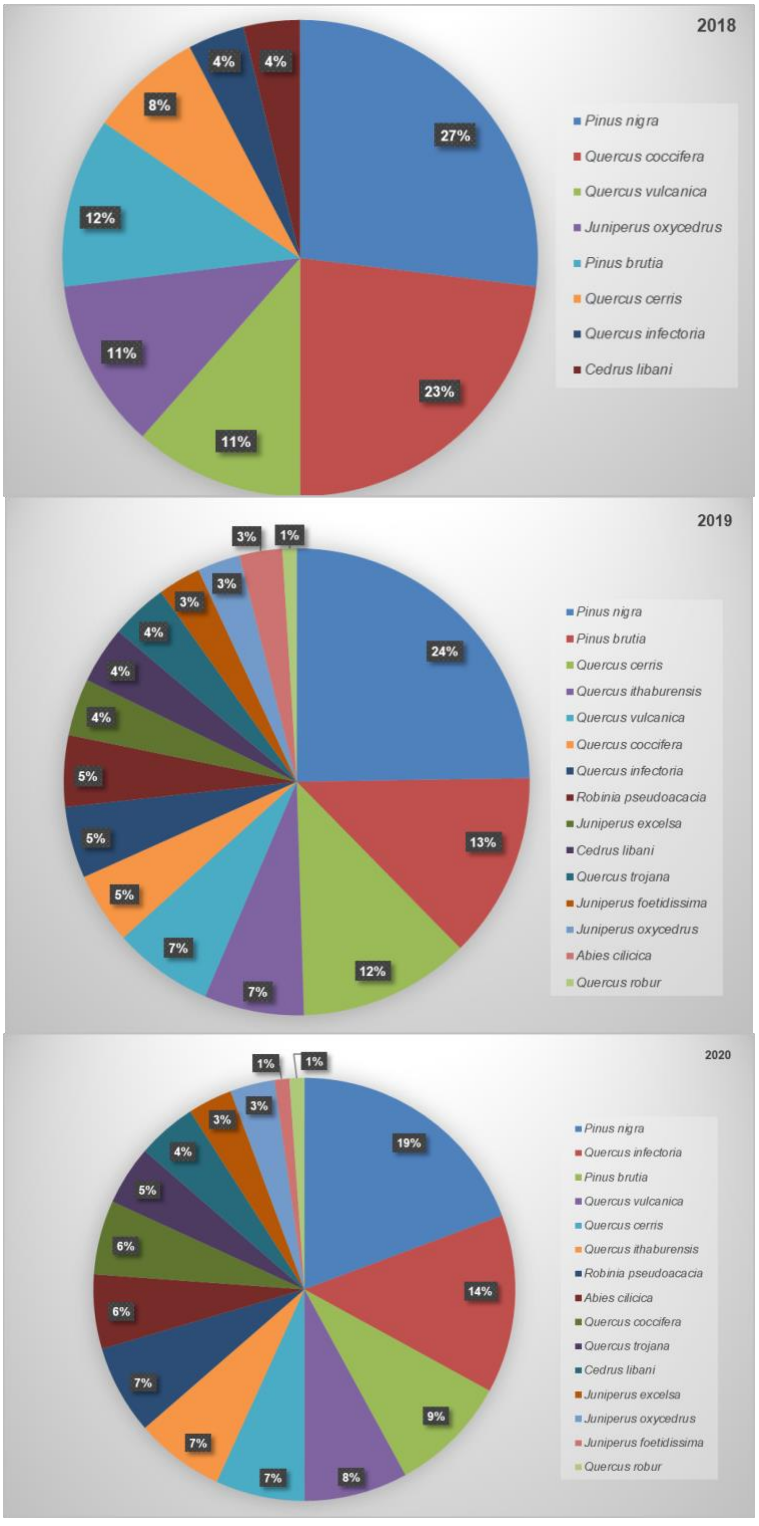

Figure 6. Associations between aphid species and host trees between 2018 and 2020.

Barjadze et al. (2014) detected *T*. *suberi* on a rock in the Gölcük Nature Park and there are no aphid records on oak trees except for this finding in study area. However, in Bitlis-Tatvan (Eastern Anatolia Region) *T*. *suberi* was recorded on *Quercus conferta* (synonym of *Quercus frainetto* Ten.) as *Thelaxes confertae* Börner, 1942 (synonym of *T*. *suberi* see Favret, 2022) (Tuatay & Remaudière, 1964). Ten aphid species on the endemic oak *Q*. *vulcanica* was recorded for the first time during the present study, so the aphid species except for the *T*. *suberi* are new records for Isparta aphid fauna. *Hoplochaitophorus dicksoni* Quednau, 1999, *Myzocallis mediterranea*, *Phylloxera quercina*, *Thelaxes valtadorosi*, *Tuberculatus borealis*, *Mindarus kinseyi*, *C*. *curvipes*, *C*. *piniphila*, *C*. *intermedia*, *C*. *orientalis*, *Eulachnus cembrae* and *E*. *thunbergii* were new records for the Turkish aphid fauna in the last 10 years (Görür et al., 2014; Görür et al., 2015; Görür et al., 2018) and were also collected in the present study. Forty-two aphid species were detected on oak trees and 35 aphid species have been detected on pine trees in Türkiye. In Isparta Province, 23 (55% of all records in Türkiye) and 21 (62% of all records in Türkiye) aphid species have been detected on oak trees and pine trees respectively (Görür et al., 2014, 2018).

Aphids do not directly cause the death of trees, but when aphid populations increase on young trees intense needle loss can be observed. In literature, it was reported that when the aphid's density, particularly with *C*. *cedri*, occasionally increase with climate changes, they can cause to death of their host plants (Çanakçıoğlu, 1975; Düzgüneş et al., 1980; Usta & Keskin, 1992; Núñez-Pérez & Tizado, 1996; Çanakçıoğlu & Mol, 1998; Tuatay, 1999; Ünal & Özcan, 2005; Binazzi et al., 2015; Mendel et al., 2016; Oğuzoğlu & Avcı, 2019). It is suggested that monitoring populations of *M*. *boerneri* and *E*. *rileyi* which had high population densities in the study area, will be useful for predicting and responding to the risk of future damage.

The detection and monitoring of harmful species are key to ensure the proper biological control of these species, the conservation of ecological balance in the forestry areas, and sustainable management. Thus, the protection and increase of biological diversity will be support continued forestry in Türkiye. Over recent years, new records of many new aphid species in Türkiye are increasing the possibility of there being other unknown aphid species in Turkish forests, which have a rich biodiversity and a high endemism rate. The fact that only a few studies on aphids have been conducted in forest areas also supports this conclusion. It has been reported that 10% of the Turkish aphid fauna consist of alien species (Akyıldırım et al., 2013; Görür et al., 2017; Oğuzoğlu et al., 2021; Kök & Özdemir, 2021). It is predicted that *A*. *robiniae*, which was detected for the first time in Türkiye, may increase in distribution due to the host distribution of this species, which is an economically important and ecologically valuable species, being frequently used in parks, gardens and forests, and for roadside plantings, erosion control and soil improvement in Türkiye (Bridgen, 1992; Li et al., 2014; Okulu, 2019).

Aphids are important in the ecosystems both directly as a prey resource of predators and parasitoids, and indirectly by secreting a honeydew, which provides nutrition for many organisms such as ants and bees. The identification of 68 aphid species on 14 host plants indicated that the species diversity was high and when considering that the aphids supply a nutrient to many organisms, this aphid diversity in the study area is significant. This study, which was conducted in Isparta Province in the Lake District, which is among the areas rich in biodiversity, concludes that the detection of aphids will contribute to forestry studies and the field of science in Türkiye.

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Original article (Orijinal araştırma)

Molecular determination of root-knot nematode species, *Meloidogyne* spp. Goeldi, 1892 (Tylenchida: Meloidogynidae) infesting weeds in kiwifruit orchards in Türkiye[1]

Kivi bahçelerindeki yabancı otlarda görülen kök-ur nematodu türlerinin, *Meloidogyne* spp. Goeldi, 1892 (Tylenchida: Meloidogynidae) moleküler yöntemlerle tespiti

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Abstract

In this investigation, the species of root-knot nematodes (RKNs) infesting weeds in kiwifruit orchards were investigated in the Ordu Province, Türkiye. A survey was conducted in 2018 and roots of weeds with RKN infestations were found in kiwifruit orchards. The infested weed samples were collected from 27 kiwifruit fruit orchards located in the Ordu Province. Identification of RKNs was performed using the molecular method based on mitochondrial DNA (*mt*DNA). The *mtDNA* region between the cytochrome oxidase II and the large subunit ribosomal RNA was amplified using two pairs of primers TRNAH/MRH106 and MORF/MTHIS. Species-specific primers previously described were used to confirm *Meloidogyne* species as the last step. *Meloidogyne incognita* (Kofoid & White, 1919)*, Meloidogyne arenaria* (Neal, 1889) and *Meloidogyne hapla* (Chitwood, 1949) (Tylenchida: Meloidogynidae) were identified from fifteen weed species (2 unidentified) in eight families. *Meloidogyne incognita* was the most frequent species with 74.1% of the samples infested, followed by *M. hapla* at 22.2% and *M. arenaria* at 3.7%. In this study found *Erigeron canadensis* L. (Asterales: Asteraceae)*, Mercurialis annua* L. (Malpighiales: Euphorbiaceae), *Oxalis pes-caprae* L. (Oxalidales: Oxalidaceae)*, Clinopodium nepeta* (L.) Kuntze (Lamiales: Lamiaceae), *Fumaria officinalis* L. (Ranunculales: Papaveraceae) and *Lycopus* spp. (Lamiales: Lamiaceae) to be previously unrecorded hosts of *M. incognita* and *Sigesbeckia orientalis* L. (Asterales: Asteraceae) and *Lythrum* spp. (Myrtales: Lythraceae) a host of *M. hapla*.

Keywords: Kiwifruit, *Meloidogyne*, mtDNA, SCAR, weeds, weed hosts

Öz

Bu araştırmada, Ordu ilinde kivi bahçelerinde yabancı otları enfekte eden kök-ur nematod türleri araştırılmıştır. 2018 yılında bir sürvey çalışması ile kivi bahçelerinde kök-ur nematodu ile bulaşık yabancı ot kökleri gözlemlenmiş ve 27 kivi bahçesinden yabancı ot örnekleri toplanmıştır. Tür teşhisleri mitokondriyal DNA'ya dayalı moleküler yöntem kullanılarak yapılmıştır. Sitokrom oksidaz II ve ribozomal RNA büyük alt birimi arasındaki *mtDNA* bölgesi, iki çift primer TRNAH/MRH106 ve MORF/MTHIS kullanılarak çoğaltılmıştır. Teşhislerin doğrulanması için türe özgü spesifik primerler kullanılmıştır. Sekiz familyaya ait 15 yabancı ot türünden (ikisi tanımlanamayan) *Meloidogyne incognita* (Kofoid & White, 1919)*, Meloidogyne arenaria* (Neal, 1889) ve *Meloidogyne hapla* (Chitwood, 1949) (Tylenchida: Meloidogynidae) türleri tespit edilmiştir. Bulaşık bahçelerde *M. incognita* en sık görülen tür olup, örneklerin %74,1'i enfekteli bulunmuş, bunu %22.2 ile *M. hapla* ve %3.7 ile *M. arenaria* izlemiştir. Bu çalışmada *Erigeron canadensis* L. (Asterales: Asteraceae)*, Mercurialis annua* L. (Malpighiales: Euphorbiaceae), *Oxalis pes-caprae* L. (Oxalidales: Oxalidaceae)*, Clinopodium nepeta* (L.) Kuntze (Lamiales: Lamiaceae), *Fumaria officinalis* L. (Ranunculales: Papaveraceae) ve *Lycopus* spp. (Lamiales: Lamiaceae) türleri *M. incognita* için, *Sigesbeckia orientalis* L. (Asterales: Asteraceae) ve *Lythrum* spp. (Myrtales: Lythraceae) ise *M. hapla* için daha önce kaydedilmemiş konukçular olarak bulunmuştur.

Anahtar sözcükler: Kivi, *Meloidogyne*, mtDNA, SCAR, yabancı otlar, yabancı ot konukçuları

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Introduction

Kiwifruit (*Actinidia* spp.) is a globally important commercial fruit crop, currently being grown in more than 20 countries, including Türkiye. Ordu Province is the second largest producer of kiwifruit in Türkiye (TUIK, 2020). Root-knot nematodes (RKNs), *Meloidogyne* spp. Goeldi, 1892 (Tylenchida: Meloidogynidae), having the wide range of host plants, is the most significant groups of the plant parasitic nematodes worldwide. The genus is known to infest more than 3,000 species of wild and cultivated plants (Hussey & Janssen, 2002; Moens & Perry, 2009; Abdellatif et al., 2016). In addition, RKNs are commonly found in kiwifruit orchards around the world. There are eight RKN species including *Meloidogyne incognita (*Kofoid & White, 1919)*, Meloidogyne arenaria* (Neal, 1889), *Meloidogyne hapla (*Chitwood, 1949), *Meloidogyne javanica* (Treub, 1885)*, Meloidogyne luci (*Carneiro et al., 2014) (as *M. ethiopica*) *Meloidogyne ethiopica (*Wheathead, 1968)*, Meloidogyne actinidiae* (Li & Yu, 1991) and *Meloidogyne aberrans* (Tao et al., 2017) (Tylenchida: Meloidogynidae) reported in kiwifruit producing areas around the world (Vovlas & Roca, 1976; Haygood et al., 1990; Le & Yu, 1991; Philippi et al., 1996; Nicotra et al., 2003; Corneiro et al., 2004; Corneiro et al., 2007; Ma et al., 2007; Ploetz, 2009; Tao et al., 2017; Shokoohi & Mashela, 2020). Eighty-six weed species in 32 families have been found in kiwifruit orchards in Ordu Province (Yonat & Kolören, 2017). Forty-nine weed species in 27 families were identified in kiwifruit orchards in Eastern Black Sea Region of Türkiye (Sezer & Kolören, 2019). Many weeds are excellent hosts of plant parasitic nematodes (Gharabadiyan et al., 2012). Such weeds can provide a reservoir of nematodes for the next season for the survival of plant parasitic nematodes in the absence of an annual crop (Rich et al., 2008). Quénéhervé et al. (2006) found 29 weed hosts of *Meloidogyne* spp. In Brazil, 24 weed species were determined to be hosts of RKNs (Belle et al., 2020). In addition, 226 weed species have been investigated for their suitability to different RKNs worldwide (Rich et al., 2009). Das et al. (1998) stated that many weeds are good hosts for RKNs and controlling weeds would be an excellent first step to reducing RKN populations. In agriculture, knowledge of the host status of weeds can be used to improve targeted weed management, especially to increase the effectiveness of the nematode management strategies used in organic farming.

When considering the high polyphagic potential of *Meloidogyne* spp., it is important to the range of host weeds in order to choose the appropriate management for these plant parasites. Therefore, the objective of this study was to determine the species RKN*s* in weeds in the kiwifruit orchards in Ordu Province, Türkiye.

Materials and Methods

Plant sampling

The survey was conducted in May-September 2018 in the Ordu Province, Türkiye. The weed samples were collected from 27 kiwifruit orchards located in the Altınordu, Fatsa, Gülyalı, İkizce, Kabadüz, Perşembe, Ulubey and Ünye districts of Ordu Province. Initially, roots of weeds were examined on site and plant samples including roots were collected from those with galls, labeled and placed in plastic bags for transport to the laboratory. Above and below parts of the samples were photographed and identified according to the Flora of Turkey (Davis, 1965-1988) and Ackerunkraeuter Europas (Hanf, 1990).

Nematode extraction and identification

Roots were examined under a stereo microscope (S8APO, Leica, Wetzlar, Germany) at magnifications of 10X after washing in tap water. Adult RKN females were carefully collected randomly from the infested roots by dissecting the roots with a needle.

Nematode Genomic DNA extraction and PCR amplification

Nematode genomic DNA was extracted from a single female using the procedure of Pagan et al. (2015). A single female was handpicked from infested roots and transferred into 10-µl AE buffer (10 mM Tris-Cl, 1 mM EDTA). Proteinase K (0.1 mg/ml) and Triton X (0.1%) were added. The females were macerated with a glass rod in a 1.5 ml tube. Samples in PCR tubes were frozen at -20°C overnight. Samples were then incubated at 56°C/1 h and 95°C/10 min, and used immediately for PCR or stored at -20°C.

To amplify the mitochondrial DNA fragments, the primers TRNAH/MRH106 or MORF/MTHIS developed by Stonton et al. (1997) were used. The primers set and sequences used for the identification of RKNs are presented in Table 1. A 25-µl PCR was performed containing 1.5 µl of DNA, 1.25 µl of each primer, 8.5 µl distilled water, and 12.5 µl DreamTaq Green PCR Master Mix (2X) (Thermo Fisher Scientific, Waltham, Massachusetts, USA). In order to determine the mitochondrial haplotype, the fragments amplified using the primer set TRNAH/MRH106 were digested with restriction enzymes, *Hin*fI and *Mnl*I, according to the manufacturer's instructions. Finally, the species of *Meloidogyne* was also determined using species-specific sequence-characterized amplified region (SCAR) primer sets Far/Rar developed for *M. arenaria (*Zijlstra et al., 2000), JMV1/JMV2/JMV *hapla* for *M. hapla* (Wishart et al., 2002) and Mi2F4/Mi1R1 for *M. incognita* (Kiewnick et al., 2013). The thermal cycler conditions for each primer set for PCR are given in Table 2.

Table 1. Primers used for *Meloidogyne* species determination for specimens collected from weeds in kiwifruit orchards

Primers	Sequence (5'-3')	Primer	Sequence (5'-3')	References
TRNAH	TGAATTTTTTATTGTGATTAA	MRH106	AATTTCTAAAGACTTTTCTTAGT	Stanton et al., 1997
MORF	ATCGGGGTTTAATAATGGG	MTHIS	AAATTCAATTGAAATTAATAGC	Stanton et al., 1997
Far	TCGGCGATAGAGGTAAATGAC	Rar	TCGGCGATAGACACTACAACT	Zijlstra et al., 2000
Mi2F4	ATGAAGCTAAGACTTTGGGCT	Mi1R1	TCCCGCTACACCCTCAACTTC	Kiewnick et al., 2013
JMV1 JMV	GGATGGCGTGCTTTCAAC	JMV2	TTTCCCCTTATGATGTTTACCC AAAAATCCCCTCGAAAAATCCACC	Wishart et al., 2002

Table 2. PCR conditions used for primer pairs in the identification of *Meloidogyne* species

Primers	Initial denaturation	Denaturation	Annealing (40 cycles)	Extension	Final extension
TRNAH/MRH106	3 min, 95°C	30 s, 95°C	30 s, 95°C	60 s, 68°C	7 min, 68°C
MORF/MTHIS	3 min, 95°C	30 s, 95°C	30 s, 95°C	60 s, 68°C	7 min, 68°C
Far/Rar	3 min, 95°C	30 s, 95°C	60 s, 95°C	60 s, 72°C	5 min, 72°C
Mi2F4/Mi1R1	3 min, 95°C	30 s, 95°C	30 s, 95°C	60 s, 72°C	1 min, 72°C
JMV1/JMV2/JMV hapla	3 min, 95°C	30 s, 95°C	30 s,95°C	2 min, 72°C	7 min, 72°C

Gel Electrophoresis

PCR products were separated using horizontal gel electrophoresis in 1.5% agarose gels containing ethidium bromide in 1X Tris-acetate EDTA (TAE) buffer. The gel was run for 25 min at 150 V then visualized and photographed under UV light using GEN-BOX imageER.

Results

In this study, the RKN species were investigated on the weeds occurring in kiwifruit orchards established in Ordu Province, Türkiye. Twenty-seven kiwifruit orchards were sampled and the 15 identified weed species (in 8 families) were found to be RKN hosts: *Solanum nigrum* L. (Solanales: Solanaceae), *Mercurialis annua* L. (Malpighiales: Euphorbiaceae), *Fumaria officinalis* L. (Ranunculales: Papaveraceae), *Clinopodium nepeta* (L.) Kuntze*, Melissa officinalis* L. (Lamiales: Lamiaceae), *Oxalis pes-caprae* L. (Oxalidales: Oxalidaceae), *Amaranthus retroflexus* L. (Caryophyllales: Amaranthaceae), *Erigeron canadensis* L.*, Sonchus asper* (L.) Hill, *Artemisia absinthium* L., *Sigesbeckia orientalis* L., *Senecio vulgaris* L., *Taraxacum officinale* L. (Asterales: Asteraceae), and two unidentified weed species, *Lycopus* spp. (Lamiales: Lamiaceae) and *Lythrum* spp. (Myrtales: Lythraceae).

Twenty-seven populations of RKNs were obtained from the infested weed plants (Table 3). The weeds were found to be infested with *M. incognita, M. hapla* and *M. arenaria* at frequencies 74.1, 22.2 and 3.7%, respectively. *Meloidogyne incognita* was the most common RKN species corresponding to 74.1%, identified in *S. nigrum*, *M. annua*, *F. officinalis*, *M. officinalis*, *O. pes-caprae*, *A. retroflexus*, *E. canadensis*, *A. absinthium*, *C. nepeta*, *S. orientalis*, *S. vulgaris* and *Lythrum* spp. Secondly, *M. hapla* was the most frequent species encountered in five sampled locations. The host weeds infested by the nematode were detected as *E. canadensis, S. orientalis, S. asper*, *T. officinale* and unidentified weed species*.* Finally, *M. arenaria* was determined at one location in *A. retroflexus*.

Identification of RKN species were made using the molecular method based on mitochondrial DNA. The polymerase chain reactions amplification with the primers TRNAH/MRH106 produced a single fragment at 556 bp for *M. hapla*, 557 bp for *M. arenaria* and *M. incognita* (Figure 1). A fragment of 214 bp in *M. arenaria* and 742 bp in *M. incognita* were produced by PCR amplification using MORF/MTHIS primers whereas *M. hapla* did not give any fragment (Figure 2). The digestion assay of TRNAH/MRH106 using *Hin*fI produced 445 and 112 bp fragments for *M. arenaria*, 446 and 110 bp fragments for *M. hapla*, and 396, 112 and 49 bp for *M. incognita* (Figure 3). The *Mnl*I digestion assay produced a single fragment of 556 bp with *M. hapla*, three fragments of 340, 140 and 77 bp with *M. arenaria*, and 340 and 217 bp with *M. incognita* (Figure 4).

Figure 1. PCR amplification products of *Meloidogyne arenaria, M. hapla* and *M. incognita* mtDNA with TRNAH and MRH106 primers. M: 100 bp ladder marker; 557 bp for *M. arenaria (*Lane 6); 556 bp for *M. hapla* (Lanes 9-10, 13, 20 and 25-26); 557 bp for *M. incognita* (Lanes 1-5, 7-8, 11-12, 14-19, 21-24 and 27).

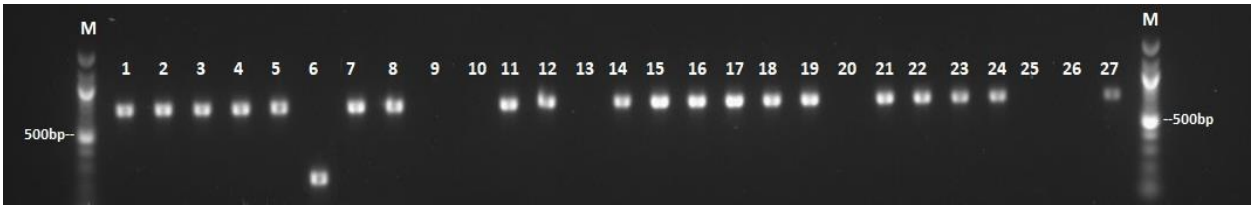

Figure 2. PCR amplification products of *Meloidogyne arenaria, M. hapla* and *M. incognita* mtDNA with MORF and MTHIS primers. M: 100 bp ladder marker; 214 bp for *M. arenaria (*Lane 6); No product for *M. hapla* (Lanes 9-10, 13, 20 and 25-26); 742 bp for *M. incognita* (Lanes 1-5, 7, 8, 11, 12, 14-19, 21-24 and 27).

Table 3. Weed species in kiwifruit orchards in Ordu Province, Türkiye that were found to be hosts of *Meloidogyne* spp.

District	Village		Common name	Family	Scientific name	*Meloidogyne*
Gülyalı	Eren	1	Black nightshade	Solanaceae	*Solanum nigrum*	*M. incognita*
Altınordu	Eyüplü	2	Annual mercury	Euphorbiaceae	*Mercurialis annua*	*M. incognita*
	Şenocak	3	Common fumitory	Fumariaceae	*Fumaria officinalis*	*M. incognita*
	Karapınar	4	Lemon balm	Lamiaceae	*Melissa officinalis*	*M. incognita*
	Burhanettin	5	Sourgrass	Oxalidaceae	*Oxalis pes-caprae*	*M. incognita*
Fatsa	Eskiordu	6 7	Redroot pigweed Black nightshade	Amaranthaceae Solanaceae	*Amaranthus retroflexus* *Solanum nigrum*	*M. arenaria* *M. incognita*
	Bozdağ	8	Annual mercury	Euphorbiaceae	*Mercurialis annua*	*M. incognita*
	Kılıçlı	9 10	Horseweed Gypyswort	Asteraceae Lamiaceae	*Erigeron canadensis* *Lycopus* spp.	*M. hapla* *M. hapla*
	Hıdırbeyli	11	Annual mercury	Euphorbiaceae	*Mercurialis annua*	*M. incognita*
	Meşebükü	12	Annual mercury	Euphorbiaceae	*Mercurialis annua*	*M. incognita*
Ünye	Cevizdere	13 14 15	Spiny sowthistle Annual mercury Redroot pigweed	Asteraceae Euphorbiaceae Amaranthaceae	*Sonchus asper* *Mercurialis annua* *Amaranthus retroflexus*	*M. hapla* *M. incognita* *M. incognita*
	Yüceler	16 17 18	Wormwood Nice mint Black nightshade	Asteraceae Lamiaceae Solanaceae	*Artemisia absinthium* *Clinopodium nepeta* *Solanum nigrum*	*M. incognita* *M. incognita* *M. incognita*
Ulubey	Güven	19	Annual mercury	Euphorbiaceae	*Mercurialis annua*	*M. incognita*
	Çatallı	20	Spiny sowthistle	Asteraceae	*Sonchus asper*	*M. hapla*
	Durak	21 22 23	Divine herb Horseweed Common groundsel	Asteraceae Asteraceae Asteraceae	*Sigesbeckia orientalis* *Erigeron canadensis* *Senecio vulgaris*	*M. incognita* *M. incognita* *M. incognita*
İkizce	Merkez	24	Loosestrife	Lythraceae	*Lythrum* spp.	*M. incognita*
Perşembe	Boğazcık	25	Divine herb	Asteraceae	*Sigesbeckia orientalis*	*M. hapla*
Kabadüz	Kabadüz	26 27	Common dandelion Annual mercury	Asteraceae Euphorbiaceae	*Taraxacum officinale* *Mercurialis annua*	*M. hapla* *M. incognita*

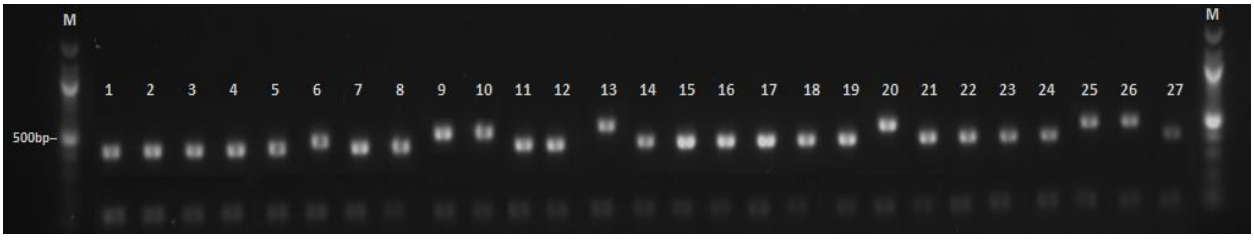

Figure 3. PCR amplification products of *Meloidogyne arenaria, M. hapla* and *M. incognita* mtDNA with TRNAH and MRH106 primers after digestion with *Hinf* I restriction enzyme. M: 100 bp ladder marker; 445 and 112 bp for *M. arenaria (*Lane 6); 446 and 110 bp for *M. hapla* (Lanes 9-10, 13, 20 and 25-26); 396, 112 and 49 bp for *M. incognita* (Lanea 1-5, 7-8, 11-12, 14-19, 21-24 and 27).

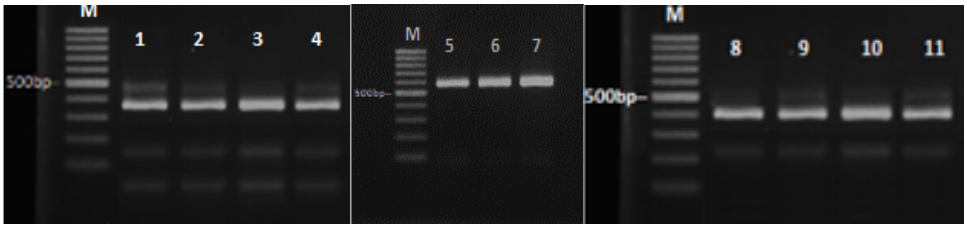

Figure 4. PCR amplification products of *Meloidogyne arenaria, M. hapla* and *M. incognita* mtDNA with TRNAH and MRH106 primers after digestion with *Mnl* I restriction enzyme. M: 100 bp ladder marker; 340, 140 and 77 bp for *M. arenaria (*Lanes 1-4); 556 bp for *M. hapla* (Lanes 5-7); 340 and 217 bp for *M. incognita* (Lanes 8-11).

Using species-specific PCR primers, the specimens were determined as *M. arenaria, M. hapla* or *M. incognita* was also confirmed. SCAR primer set Far/Rar for *Meloidogyne arenaria* gave a 420-bp fragment, JMV1/JMV2/JMV PCR primers for *M. hapla* gave a 440-bp product and Mi2F4/Mi1R1 primers for *M. incognita* gave a 300-bp product (Figure 5).

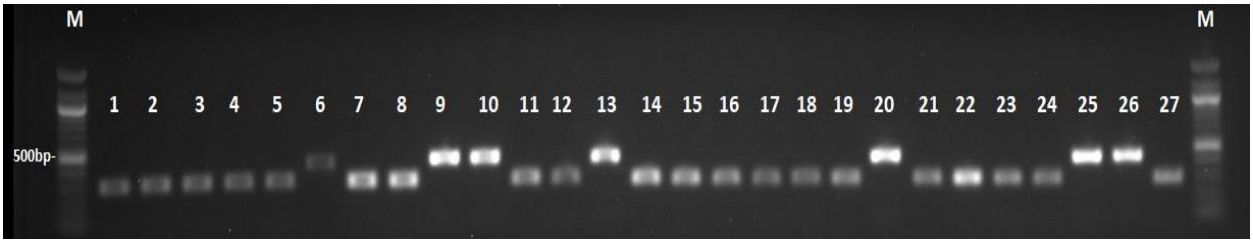

Figure 5. PCR amplification products of *Meloidogyne arenaria, M. hapla* and *M. incognita* in multiplex PCR assay using species-spesific primers Far/Rar, JMV, Mi2F4/Mi1R1 respectively. M: 100 bp ladder marker; 420 bp fragment for *M. arenaria (*Lane 6); 440 bp fragment for *M. hapla* (Lanes 9-10, 13, 20 and 25-26); 300 bp for *M. incognita* (Lane 1-5, 7, 8, 11-12, 14-19, 21-24 and 27).

Discussion

Weeds may impact the growth of the crops as competitors as well as sources of pests and pathogens, so it is important to identify both the host weed and any parasite nematodes they support. This investigation was detailed research about the identification of RKNs on the weeds occurring in kiwifruit orchards. In this study, some weeds were found to be infested by *M. arenaria, M. hapla* and *M. incognita*. Those three nematode species are considered the most common species worldwide (Taylor et al., 1982). In addition, even on a regional scale, the results were similar (Aydınlı & Mennan, 2016). In this context, the results of our investigation are consistent with the previous investigations. Fifteen weed species were found to be infested with RKNs. The most common weed host was *M. annua* at seven locations and followed by *S. nigrum* at three locations. The weed hosts, *A. retroflexus*, *E. canadensis*, *S. asper* and *S. orientalis* were found at two locations each and the least commonly detected weed host were *F. officinalis, M. officinalis*, *O. pes-caprae*, *A. absinthium*, *C. nepeta*, *S. vulgaris* and *T. officinale* at one location for each.

Mercurialis annua was infested at all sites but only with *M. incognita*. Bendixen (1986) listed this weed as a host of an unidentified *Meloidogyne* spp. Similarly, Philis & Siddiqi (1976) and Philis (1995) also listed this weed as associated with a *Meloidogyne* sp. In light of previous research, our result is the first to identify *M. incognita* in *M. annua*.

Amaranthus retroflexus was the host with the widest range of RKN species (Bendixen, 1988). In our investigation, the *M. arenaria* and *M. incognita* were found in this host. *Meloidogyne arenaria* was identified in *A. retroflexus* at all locations where it was sampled in the present study. In previous research on *A. retroflexus*, Amin (1994) reported *M. arenaria* in Hungary; Kornobis & Wolny (1997) *M. hapla* in Poland, Castillo et al. (2008) *M. incognita* in Spain, and Ercan & Elekcioğlu (2009) *M. incognita* and *M. javanica* in Türkiye. In addition, some investigations have examined the susceptibility of *A. retroflexus* to *Meloidogyne* spp. (Tedford & Fortnum, 1988; Belair & Benoit, 1996; Kaur et al., 2007; Kokalis-Burelle & Rosskopf, 2012).

In our investigation, the only RKN in *S. nigrum* was *M. incognita. Solanum nigrum* is a weed that has been a focus of previous work, and found to be as susceptible as susceptible crop cultivars *(*Zancada et al., 1998; Ehwaeti et al., 1999). In addition, as a reservoir of important RKN species such as *M. incognita*, the weed was considered as needing to be controlled (Ponce et al., 1995). The most frequently found RKN species in *S. nigrum* was *M. incognita* (Smit, 1978). In addition, Lindhardt (1963) and Whitehead (1969) found *M. hapla* and *M. javanica,* respectively, in this host, and Pajovic et al. (2007) found *M. arenaria*. In Spain (Castillo et al., 2008) and Türkiye (Ercan & Elekcioğlu, 2009), *M. incognita* and *M. javanica* have been found in *S. nigrum*. Also, *Meloidogyne exigua* (Goeldi, 1887) (Tylenchida: Meloidogynidae) and *M. ethiopica* have been found in *S. nigrum* (Curi, 1973; Aydınlı & Mennan, 2016), and *S. nigrum* and *S. vulgaris* have been reported as hosts of *Meloidogyne chitwoodi* (Golden, O'Bannon, Santo & Finley, 1980) (Tylenchida: Meloidogynidae) with galling and egg production (Kutywayo & Been, 2006).

Taraxacum officinale was found to be infested by *M. hapla* at one location in the present study. Kornobis & Wolny (1997) and Smiley et al. (2014) reported some other groups of nematodes associated with this weed, and Gaskin & Crittenden (1956) reported it as a host of *M. hapla*. Doucet et al. (2000) reported it as a good host that may enhance the spread of *M. hapla*. Mitkowski & Abawi (2002) reported successful reproduction of *M. hapla* in root culture system with *T. officinale*.

Erigeron canadensis was found to be infested with *M. incognita* and *M. hapla* at two separate locations in the present study. Bajwa et al. (2016) concluded that this weed as one of the most problematic, noxious, invasive and widespread weeds in modern-day agriculture. Kim et al. (1998) reported that *Erigeron canadensis* and *Erigeron annuus* L. (Asterales: Asteraceae) were infested by *M. hapla* under field conditions. Ijani et al. (2000) reported *M. javanica* infestion in *E. canadensis* and *Erigeron sumatrensis* Retz. (Astarales: Asteraceae).

Sonchus asper was found to be infested with *M. hapla* at two locations in the present study. In previous reports, Lindhardt (1963) identified *M. hapla* in heavily galled roots of this host. Mangat et al. (1985) obtained *M. javanica* eggs from the roots of *S. asper*. Amin (1994) reported *M. arenaria* in *S. asper* and additionally *M. incognita* and *M. arenaria* on both *Sonchus oleraceus* L. and *Sonchus arvensis* L. (Astarales: Asteraceae).

Artemisia absinthium was found to be infested with *M. incognita* at one location in the present study. Bendixen (1986) listed some species of *Artemisia* as hosts of *M. hapla,* but for *A. absinthium* the species of RKN was unidentified. In another study, Walker (1995) inoculated *A. absinthium* with *M. incognita* race 3 achieving a root galling rate between 26-51%.

Senecio vulgaris was found to be infested with *M. incognita* at one location in the present study. The host status of this weed has generally been determined in inoculation studies. Davidson & Townshend (1967) inoculated *S. vulgaris* with *M. incognita*, but no galls were observed. Townshend & Davidson (1962) conducted a similar investigation with *M. hapla* and even though they observed small galls in high numbers, no nematode reproduction was evident. Belair & Benoit (1996) inoculated *S. vulgaris* seedlings with *M. hapla* and they observed root galling but no eggs or juveniles. Thus, *S. vulgaris* has been reported as a potentially useful trap plant for RKN, especially *M. hapla* species.

Fumaria officinalis was found to be infested with *M. incognita* at one location in the present study. This weed has only previously been reported as a host of *M. javanica* (Philis & Siddiqi, 1976; Philis, 1995). Consequently, our finding indicates that *F. officinalis* can also host *M. incognita*.

Melissa officinalis was found to be infested with *M. incognita* at one location in the present study. The previous research has shown that this weed can be a host of major RKNs. Karl et al. (1997) inoculated *M. officinalis* with eggs of *M. javanica* and found that the weed as highly susceptible to this nematode. Tzortzakakis et al. (2011) reported *M. arenaria* and *M. javanica* in *M. officinalis* in Greece. Santos (2018)

reported the weed as the host of *M. hapla*. *Meloidogyne arenaria* was also found in *M. officinalis* in Greece by Karanastasi et al. (2008).

Sigesbeckia orientalis was found to be infested by *M. incognita* and *M. hapla* at separate locations in the present study. Silva et al. (2016) found *M. javanica* in *S. orientalis* in Brazil. *Sigesbeckia orientalis* is here first reported as a host of *M. hapla*.

Oxalis pes-caprae was found to be infested with *M. incognita* at one location in the present study. In previous studies, *Oxalis* spp. were found to be the hosts of the common RKNs (Martin, 1958; Oliveira & Kubo, 2006; Bellé et al., 2020). For *O. pes-caprae*, Ciancio et al. (1992) reported that *M. javanica* was the species parasitizing the weed in İtaly. Gonçalves et al. (2020) reported that *O. pes-caprae* is a potential host of *M. javanica* based on inoculation studies. Consequently, the in the present study is the first to report *O. pes-caprae* as a host of *M. incognita*.

Clinopodium nepeta was found to be infested with *M. incognita* at one location in the present study. being the first report of *M. incognita* in this weed.

In conclusion, our results showed that major RKN species, *M. arenaria, M. hapla* and *M. incognita,* can occur in many common weeds with these findings consistent with previous investigations, globally (Taylor et al., 1982) and regionally (Aydınlı & Mennan, 2016). In addition, *E. canadensis*, *M. annua*, *O. pes-caprae, C. nepeta*, *F. officinalis* and *Lythrum* spp. are reported for first time as hosts of *M. incognita*, and *S. orientalis* and *Lycopus* spp. as a host of *M. hapla*. Of the weed species, *S. nigrum*, *A. retroflexus, E. canadensis, M. annua,* and *T. officinale* were the most important species found in the present study as hosts of RKNs and as weeds in importance globally. Management practices for RKNs in the first three species must be given high priority, given they are common hosts for the major RKNs worldwide. The latter two species, *M. annua*, *T. officinale*, will be important regionally for managing *M. incognita* and *M. hapla*, respectively. Those considerations are also valid for specific management practices for both weeds and RKNs in Ordu Province. In the province, Yonat (2016) reported the weed status of kiwifruit plantations showing that *S. nigrum*, *A. retroflexus, E. canadensis, M. annua* and *T. officinale* occur in these plantations. Similarly, Sezer & Koloren (2019) determined the species and some parameters of the weeds in kiwifruit orchards in the Eastern Black Sea Region of Türkiye. They found that *S. nigrum*, *A. retroflexus, E. canadensis, M. annua* and *Taraxacum* sp. were present in the region, and that *E. canadensis* occurred frequently as at 75% in 2014 and 88% in 2015. Therefore, these five weeds must be specifically considered as nematode reservoirs in management practices in kiwifruit orchards. However, previous investigations have found that the RKN host status of weeds can be wide and variable. Although, the major RKNs were mostly reported in these investigations, some other RKNs species, including *M. ethiopica*, should also be considered (Aydınlı & Mennan, 2016). *Meloidogyne ethiopica* has been identified in Ordu Province and in other provinces to the west (Aydınlı & Mennan, 2016). Therefore, the weeds found in the surveys may affect the management practices used at a regional level in kiwifruit orchards. As a management option, trap plants can be used to reduce nematode numbers in the soil. The mechanism is that invasion and gall formation occur but the life cycle of the nematode apparently cannot be completed (Townshend & Davidson, 1962). Townshend & Davidson (1962) inoculated weeds with *M. hapla* and observed small galls in high numbers, but when inoculate with *M. incognita* (Davidson & Townshend, 1967), no galls were observed. Bélair & Benoit (1996) observed galling of the roots without eggs production with *M. hapla* inoculation. Similarly, they did not observe development of *M. hapla* on the root system of *A. retroflexus* grown in soil which when inoculated with around 18,000 juveniles. In this context, *S. vulgaris* may be the promising trap plant in kiwifruit orchards for especially *M. hapla*.

As result, there is value of knowing the host status of the weeds but it depends on many factors such as intra- and interspecies virulence of different races, different growing environments (open field or greenhouse), abiotic factors varying across regions which affect the performance of the host and nematode.

These all need to be investigated to help develop site-specific management approaches for the future. The results of the present study showed that weeds can potentially be reservoirs of RKNs and should be considered as factors affecting the success of integrated nematode management programs. Controlling the weeds would be a useful initial step in reducing RKN populations in the kiwifruit orchards.

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Original article (Orijinal araştırma)

Investigation of insecticide residues in potato grown in Türkiye by LC-MS/MS and GC-MS and health risk assessment[1]

LC-MS/MS ve GC-MS ile Türkiye menşeli patateslerde insektisit kalıntılarının araştırılması ve sağlık risk değerlendirmesi

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Abstract

Insecticide, acaricide, nematicide and metabolite residues were assayed in 104 potato samples collected from local markets in Tokat, Türkiye in 2022 and the potential health risk for consumers assessed. Analytical method verification was performed for 135 pesticide active substances in potato matrices by liquid chromatography-tandem mass spectrometry and gas chromatography-mass spectrometry. Matrix-matched calibration curves were constructed and good linearity was obtained with a coefficient of determination between 0.990 and 0.999. Average recoveries varied from 73.2 to 119.6%. Repeatability and intra-laboratory reproducibility conditions of the method expressed as %RSD were less than 20%. These figures were within the SANTE/11312/2021 recovery limits (70-120%) and the values specified for the repeatability (RSD ≤ 20%). The limits of quantification were lower than the maximum residue limits set by the European Union for the potato. No pesticide residues were found at detectable limits in 93 samples. Two samples contained residues below the maximum residue limit (MRL), while nine samples contained residues above the MRLs. Clothianidin and thiamethoxam residues detected in one sample, while acetamiprid were detected in nine samples. The health risk assessment study indicated that potato consumption was safe for consumers.

Keywords: Acute risk, chronic risk, matrix effect, method verification, pesticide residue

Öz

Bu çalışmada, 2022 yılında Tokat'ta yerel pazarlarda satılan patateslerde insektisit, akarisit, nematisit ve metabolit kalıntıları taranmış ve bu kalıntıların tüketiciler açısından potansiyel sağlık riskleri değerlendirilmiştir. Sıvı kromatografi-tandem kütle spektrometrisi ve gaz kromatografi-kütle spektrometrisi ile 135 pestisit etken madde kalıntısını belirlemek için metot doğrulaması yapılmıştır. Matris uyumlu kalibrasyon eğrileri oluşturulmuş ve 0.990 ile 0.999 arasında değişen korelasyon katsayısı ile uygun bir doğrusallık elde edilmiştir. Ortalama geri kazanımlar %73.2 ile %119.6 arasında, %RSD olarak ifade edilen yöntemin tekrarlanabilirlik koşulları ve laboratuvar içi tekrar üretilebilirlik koşulları %20'den daha düşük bulunmuştur. Bu rakamlar, SANTE/11312/2021 dokümanındaki geri kazanım limitleri (%70-120) ve tekrarlanabilirlik için belirtilen değerlere (RSD ≤ %20) uygundur. Miktar tayin limitleri, Avrupa Birliği tarafından patates için belirlenen maksimum kalıntı limitlerinden daha düşük seviyelerde bulunmuştur. 93 örnekte tespit edilebilir limitlerde pestisit kalıntısına rastlanmamıştır. İki numunede MRL değerleri altında, 9 numunede ise MRL değerleri üzerinde pestisit kalıntısı tespit edilmiştir. Bu örneklerden birinde hem clothianidin hem de thiamethoxam, dokuzunda ise acetamiprid tespit edilmiştir. Sağlık risk değerlendirmesi ise patates tüketiminin tüketiciler için güvenli olduğunu göstermiştir.

Anahtar sözcükler: Akut risk, kronik risk, matriks etkisi, metot doğrulaması, pestisit kalıntısı

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Introduction

Potato (*Solanum tuberosum* L.) is the most important crops after cereals for human nutrition. Potato is widely produced due to its adaptability to various climatic conditions and being economical, and it is consumed in many countries of the world due to high nutritional value. Potato is low in protein and high in starch, and is widely used in food and industry. Potato ranks fourth as a food in the world and it is the fifth food product in Türkiye after wheat, tomato, barley and corn (FAO, 2022; TUIK, 2022).

Potato is among the main food sources of many countries. Its consumption is also increasing rapidly in developing countries (FAO, 2008). However, in potato production, there are many biotic and abiotic factors that cause losses in potato crops. Pests are the most important ones. Insects are responsible for 16% of crop losses in potato and can cause 30-70% losses in tuber yield and quality (Weber, 2013). Key pests of potato in Türkiye are *Leptinotarsa decemlineata* (Say, 1824) (Coleoptera: Chrysomelidae), *Phthorimaea operculella* (Zeller, 1873) (Lepidoptera: Gelechiidae), *Globodera rostochiensis* (Wollenweber 1923), *Globodera pallida* (Stone, 1973) (Tylenchida: Heteroderidae) and *Meloidogyne* spp. (Tylenchida: Meloidogynidae) (TAGEM, 2017). In order to prevent the damage of these pests, insecticides, acaricides and nematicides are applied intensively from planting to the harvest.

Pesticides may remain in the harvested products and pose a health risk to consumers due to inappropriate agricultural practices, Therefore, pesticide residues are limited by various organizations with maximum residue limits (MRLs) and these approaches aim to prevent this health risk (EU-MRL, 2022; TGK-MRL, 2022). The MRLs of pesticides to be applied to foods in European Union countries are given in the European Parliament and Council Regulation No. 396/2005 (EC, 2005). In Türkiye, Turkish Food Codex Regulation on Maximum Residue Limits of Pesticides was prepared by taking the EU regulation into account within the scope of harmonizing with the European Union legislation (Anonymous, 2022).

MRL for insecticide, acaricide and nematicide residues in potato within range of 0.001-0.8 mg kg^{-1} depending on the active ingredients. The higher limits can be set in some cases (EU-MRL, 2022; TGK-MRL, 2022). Highly sensitive and accurate analytical methods are required to analyze these trace concentrations (Narenderan & Meyyanathan, 2019). Today, liquid chromatography-tandem mass spectrometry (LC-MS/MS) and gas chromatography-tandem mass spectrometry are more preferred because they provide improved sensitivity and selectivity for analyzing large numbers of pesticides with a single injection (Saha et al., 2015; Balkan, 2021). These techniques have been used for the determination of various pesticide residues in potato (Thompson et al., 2011; Lee et al., 2017; Narenderan & Meyyanathan, 2019; Reis et al., 2020).

Currently, dispersive solid phase extraction (d-SPE) is the most widely used method for cleansing in most multi-residue methods. In the d-SPE, a step of the QuEChERS (quick, easy, cheap, effective, rugged and safe) method, solid phases such as C_{18}, primary secondary amine, graphitized carbon black and zirconia-coated silica are added directly to facilitate the cleansing process. The use of the QuEChERS method has increased over the last decade due to its suitability for multiple residue analysis in various matrices (Narenderan & Meyyanathan, 2019). It is the most widely used method for detecting pesticide residues in potato (Lee et al., 2017; Reis et al., 2020; Sivaperumal et al., 2022).

In this study aimed to develop methods with high sensitivity, accuracy and precision to meet the SANTE/11312/2021 guidelines for determination of insecticide, acaricide and nematicide residues in potato by QuEChERS method using LC-MS/MS and GC-MS. The verified method was used to determine 135 pesticide residues with the QuEChERS method in potato. In addition, the health risk associated with the presence of pesticide residues in potatoes was evaluated.

Materials and Method

Chemicals and reagents

Pesticide reference standards were supplied by Dr. Ehrenstorfer GmbH (Augsburg, Germany) supplied (Tables S1 and S2). Methanol and acetonitrile gradient grade for liquid chromatography (≥99.9% purity), and acetic acid (>99% purity) were supplied by Merck (Darmstadt, Germany). The QuEChERS products were supplied by Restek (Bellefonte, PA, USA).

Preparation of standard solution

One hundred and thirty pesticide active substances were assayed, 23 (and/or their metabolites) by GC-MS analyses and 112 by LC-MS/MS analyses, for method verification and residue detection. A separate stock solution (1 mg mL^{-1}) in methanol for each pesticide was prepared and stored at -20°C. The concentrations of the matrix-matched standards were 5, 10, 25, 50, 100 and 150 µg L^{-1} of each analyte.

Sample collection and storage

Potato samples originating from Adana, Afyon, Malatya, Niğde, Nevşehir Sivas and Tokat were purchased from the supermarkets in Tokat, Türkiye in May and June 2022. Potato (at least 10 units) samples each of 1 kg were collected in sterile polythene bags for pesticide residue analysis (EC, 2002). Samples were labeled and immediately transported to the laboratory in the icebox and immediately processed within 12 h for extraction and cleansing. Blank potato samples were obtained from the tissue culture laboratory, which is known to be pesticide free, for recovery experiment and matrix-matched calibration.

Sample preparation, extraction and cleansing

Extraction and cleansing procedures in QuEChERS AOAC Method 2007.01 were performed according to (AOAC, 2007). The steps for QuEChERS process were shown in Figure 1. Potatoes were analyzed in triplicate by LC-MS/MS and gas chromatography mass spectrometry (GC-MS).

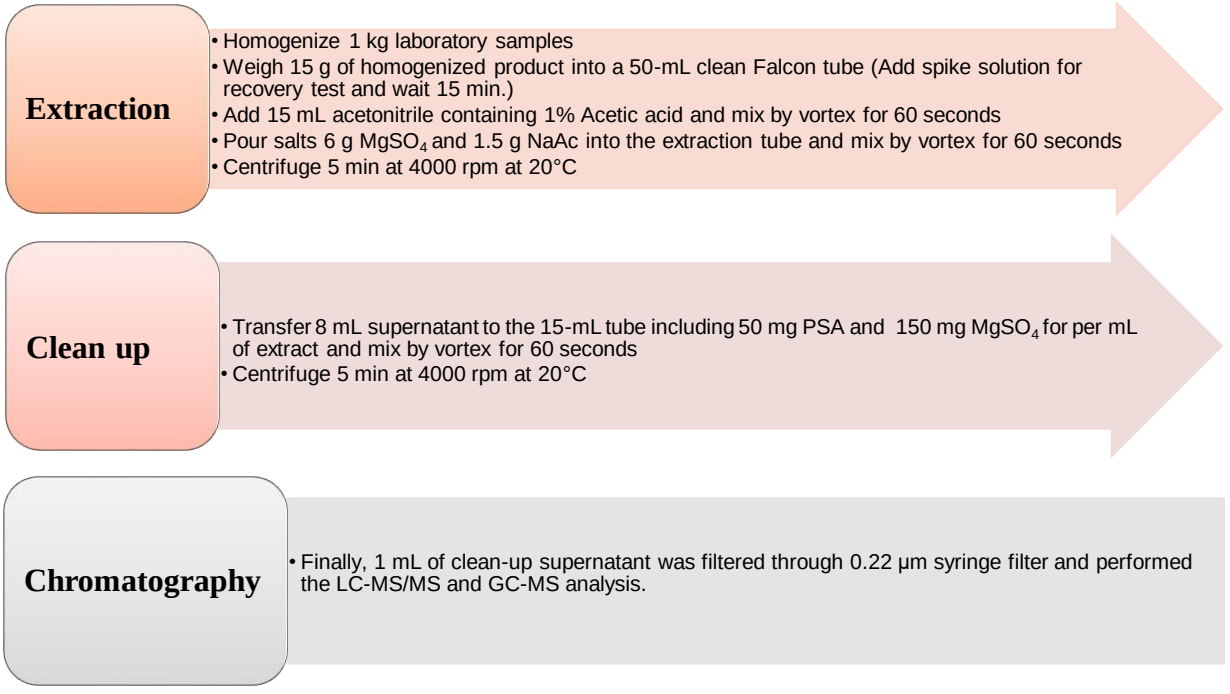

Figure 1. Analytical steps of the QuEChERS-AOAC Official Method 2007.01.

Instrumentation and optimization for LC-MS/MS and GC-MS

The analyses were performed on Shimadzu UHPLC Nexera X2, and LCMS-8050 triple quadrupole mass spectrometer with an electrospray ionization, and GC-MS QP2010 ultra model (Shimadzu) with mass spectrometry system coupled with an electron ionization. The final LC-MS/MS and GC-MS conditions are given in Table 1. Multiple reaction monitoring was used for optimization in LC-MS/MS and selected reaction monitoring for GC-MS. Optimization parameters in LC-MS/MS and GC-MS were given in Table S1 and S2, respectively.

Table 1. Analysis conditions for LC-MS/MS and GC-MS

LC Conditions (Nexera X2)		MS Conditions (LCMS-8050)	
Column	Inertsil (ODS-3), C_{18} column (2.1 mm x 150 mm, 3 µm)	Ionization mode	ESI (+/ -)
Oven temp.	40°C	Desolvation line temp.	250°C
Solvent A	10 mmol L^{-1} ammonium acetate/distilled water	Interface temp.	300°C
Solvent B	Methanol	Block heater temp.	400°C
Gradient	5% B (0 min) - 60% B (3 min) - 70% B (4 min) - 80% B (6 min) - 95% B (7 - 8.50 min) - 5% B (8.51-15 min)	Nebulizer gas flow	2.9 L/min.
Flow rate	0.4 mL min^{-1}	Drying gas flow	10.0 L min^{-1}.
Injection vol.	10 µL	Heating gas flow	15.0 L min^{-1}.
Rinse solution	R0 50% methanol/water	Dwell time	1-33 ms
GC conditions (GC 2010 Plus)		MS conditions (GC-QP2010 Ultra)	
Column	Rxi-5Sil MS column (30 m, 0.25 mm id, 0.25 µm	Ionization mode	EI
Injection temp.	250°C	Interface temp.	270°C
Gradient	90°C (1 min) - (20°C/min) - 150°C - (9°C/min) - 200°C - (12°C/min) - 300°C (5 min)	Ion source temp.	200°C
Carrier gas	Helyum	Solvent cut time	2.5 min
Linear velocity	48.1 cm s^{-1}	Data sampling time	6.3-20 min
Purge flow	3.0 mL min^{-1}	Acquisition Mode	SIM
Injection vol.	1 µL	Event time	0.3 ms

ESI, electrospray ionization; EI, electron ionization.

Method verification

For recovery, 15 g blank potato samples were spiked with the mixed pesticide solutions corresponding 0.01, 0.05 and 0.1 mg kg^{-1} levels for the five replicates. The experiment was repeated in five consecutive weeks by two analysts. Analytical methods were verified in accordance with the internationally accepted guidelines (EURACHEM, 2014; SANTE, 2021). Verified parameters were limit of detection (LOD), limit of quantification (LOQ), sensitivity/linearity, recovery, precision (repeatability; RSD_r and within-laboratory reproducibility; RSD_{wR}), measurement uncertainty, and matrix effect (ME). These parameters were described in detail by Balkan & Yılmaz, (2022).

Pesticide residues in potatoes

One hundred and thirty pesticide active substances in the 104 potato samples were analyzed in LC-MS/MS and GC-MS. The active ingredients detected in these samples were confirmed by the retention time and ion ratio defined as identification criteria according to the SANTE guidelines.

Risk assessment

Health risk assessments include estimated calculations of which extent to the health of those who consume pesticide-containing foods. Health risks for both acute and chronic exposure were assessed. Dietary exposure assessments are based on food consumption data in the relevant countries and data on the pesticide residues detected in the foods.

In assessing the acute and chronic risk of pesticide residues, estimated dietary exposure (based on body weight; BW) was compared to toxicological values known as acute reference dose (ARfD, mg kg BW^{-1} d^{-1})

and acceptable daily intake (ADI, mg kg BW^{-1} d^{-1}). The acute/short-term consumer health risk (aHI) was calculated based on the estimated short-term intake (ESTI, mg kg^{-1} d^{-1}) and the acute reference dose (ARfD). The chronic/long-term consumer health risk (chronic hazard index, cHI) was calculated based on the estimated daily intake (EDI, mg kg^{-1} d^{-1}) and the acceptable daily intake (ADI) (EFSA, 2015). The relevant formulas were given below Liu et al., 2016);

$$ESTI = \text{high residue level} \times \text{food consumption} / \text{body weight} \quad (1)$$

$$aHI = ESTI / ARfD \times 100 \quad (2)$$

$$EDI = \text{mean residue level} \times \text{food consumption} / \text{body weight} \quad (3)$$

$$cHI = EDI / ADI \times 100 \quad (4)$$

The average body weight of an adult was considered 73.5 kg (TUIK, 2019; Balkan & Kara, 2022). Daily consumption of potato for the general population in Türkiye were used as 0.14 kg^{-1} d^{-1} respectively (TUIK, 2022). When HI is greater than one, it indicates that pesticide residue could pose health risk to consumers (Akoto et al., 2015; Soydan et al., 2021).

Result and Discussion

Method verification

The results obtained from method verification studies of the detected insecticides were given in Table 2. The verification data of 135 pesticides active substances are given in Table S3. Linearity was obtained for every pesticide and showed good correlation coefficient (R^2) range between 0.990 and 0.999. For the determination of LOD and LOQ, potato blank samples were fortified with a pesticide mixture at the level of 10 µg kg^{-1} and 10 replicate analyses were performed. These values were smaller than the MRLs (except carbofuran, MRL: 1 µg kg^{-1}) for potatoes set by the EU. The recovery rate of 70-120%, and repeatability RSD_r and intra-laboratory reproducibility RSD_{wR} ≤ 20% for pesticides were acceptable. The expanded measurement uncertainties were between 18.6 and 43.2% for all pesticides. These results indicate that QuEChERS is a rapid and accurate method to analyze pesticide residues in potatoes.

MEs are classified into three types: minimal signal suppression or enhancement effects (ME range -20 to 20%), moderate effects (range, 50 to -20% or 20 to 50%) and strong matrix effects (<50% or >50%) (Szarka et al., 2022). In the LC-MS/MS analyses, minimal ME (Carbosulfan, diazinon, dicrotophos, fenthion, flubendiamide, monocrotophos, novaluron and triflumuron), moderate ME (32 pesticides) and strong ME (72 pesticides) was observed in the potato. In the GC-MS analyses, a strong matrix effect was detected in potato. Signal enhancement is generally more common in GG analyzes (Szarka et al., 2022). Signal enhancement was observed in most pesticides (Table 2).

Various degrees of ME were detected in all samples in both GG-MS and LC-MS/MS. In order to eliminate this effect, matrix-match standard solutions or other recommended approaches should be used. The use of matrix-matched calibration curves provides more precise and accurate analysis results guideline.

Table 2. Method verification parameters of detected pesticides in potato samples

Pesticide	LOD	LOQ	R^2	Repeatability (n=10)						Reproducibility (n=10)						U' %	ME %
				10 µg kg^{-1}		50 µg kg^{-1}		100 µg kg^{-1}		10 µg kg^{-1}		50 µg kg^{-1}		100 µg kg^{-1}			
	(µg kg^{-1})			Rec. %	RSD %	Rec. %	RSD %	Rec. %	RSD %	Rec. %	RSD %	Rec. %	RSD %	Rec. %	RSD %		
Acetamiprid	1.9	6.3	0.993	115	9.6	118	4.7	114	5.8	106	8.7	116	13.2	106	5.0	32.8	-28.8
Clothianidine	1.2	4.1	0.995	108	6.3	106	13.6	112	4.5	111	7.9	107	6.6	114	8.5	27.9	-78.2
Thiamethoxam	1.9	6.4	0.994	117	10.2	119	9.7	115	10.3	116	11.6	117	11.3	117	3.6	39.7	-70.4

Rec: Recovery; LOD, limit of detection; LOQ, limit of quantification; U', measurement uncertainty; ME, matrix effect.

Residue analyses in potatoes

One hundred and four potato samples were analyzed. Pesticide residues were determined equal or lower than the LODs in 93 (89%) of 104 samples. Acetamiprid was detected in nine samples, and clothianidin and thiamethoxam in two samples. Acetamiprid and thiamethoxam are currently registered for potato (PPPDA, 2022). Clothianidin has been banned in Türkiye since 31 July 2019 (Polat & Tiryaki, 2022). The results were evaluated according to European Union maximum residue limits (EU-MRL).

The LOQ value (6.3 µg kg^{-1}) determined for acetamiprid was found to be lower than the EU-MRL value (10 µg kg^{-1}). The acetamiprid residues were 60.9, 64.9, 68.7, 71.1, 78.7, 82.2, 85.0, 98.8 and 98.9 µg kg^{-1}. These values were greater than the EU-MRL. One of these samples was from Nevşehir, two from Niğde and six from Adana. The common detection of acetamiprid active in food samples indicates that farmers prefer this pesticide or they are attempting to control similar pests.

The LOQs (6.4 and 4.1 µg kg^{-1}) for thiamethoxam and clothianidin were found be lower than the EU-MRL (70 and 30 µg kg^{-1}), respectively. Clothianidin (23.2 µg kg^{-1}) was detected in one sample from Malatya, and clothianidin (21.6 µg kg^{-1}) and thiamethoxam (46.6 µg kg^{-1}) in one sample from from Niğde. The residues of clothianidin and thiamethoxam were both lower than the EU-MRL. Clothianidin detected with thiamethoxam is thought to be a metabolite of thiamethoxam. However, the detection of clothianidin in the other sample indicates that some farmers have used banned pesticides.

Bakırcı et al. (2014) reported that 4.5% of 66 potato samples contained pesticide residues above MRLs in Aegean region (Türkiye). In the present study, the pesticide residues above MRL were 8.5%. Česnik et al. (2006) detected pesticide residues above EU-MRL in 23% of 150 potato samples from Slovene. Danek et al. (2021) detected residues above EU-MRL in 8 of 15 potato samples from markets in Poland. Česnik et al. (2010) detected residues below LOQ in all 52 potato samples from Slovene. Srivastava et al., (2011) did not detect any pesticide residues in the potatoes from Lucknow City, India. Szpyrka et al. (2015) analyzed 102 unprocessed potato samples from southeastern Poland detecting pesticides under EU-MRL in only two samples. Poulsen et al. (2017) detected pesticides below EU-MRL in only 1% of a total of 669 potato samples from Denmark, France and the UK. Thompson et al. (2011) analyzed 228 fresh potatoes from 34 farmer markets in Alberta, Canada detecting pesticide residues below the Canadian maximum residue limits set for potatoes in 32 samples.

Risk assessment

The pesticide risk assessments of pesticides have attracted consumer interest in recent years, in Türkiye (Çatak & Tiryaki, 2020; Soydan et al., 2021; Balkan & Kara, 2022). Health risk analysis was conducted for three pesticides (Table 3). For acute and chronic risk assessment, the highest exposure value was obtained for acetamiprid.

Table 3. Health risk estimation of insecticides residues in potatoes in Türkiye

Insecticide	ADI* (mg kg BW^{-1} d^{-1})	ARfD* (mg kg BW^{-1} d^{-1})	ESTI (mg kg^{-1} d^{-1})	aHI (%)	EDI (mg kg^{-1} d^{-1})	cHI (%)
Acetamiprid	0.025	0.025	1.89E-04	0.755	1.51E-04	0.602
Clothianidin	0.026	0.500	4.58E-05	0.046	4.35E-05	0.045
Thiamethoxam	0.097	0.100	8.89E-05	0.018	8.89E-05	0.342

* ADI and ARfD values are from the IUPAC Pesticides Properties DataBase (IUPAC, 2022).

Earlier studies did not find any health risk for potato related to acetamiprid, clothianidin and thiamethoxam residues. Likewise, health risk assessment studies on other pesticides in potatoes in China (Wang et al., 2020; Yang et al., 2020; Sun et al., 2021) found no consumer health risk in both the short and long term.

Conclusion

This study verified the value of QuEChERS analysis for insecticide, acaricide and nematicide residue detection in potato using by LC-MS/MS and GC-MS systems. This method had acceptable specificity, linearity (R^2 > 0.99), LOD/LOQ, precision (RSD < 20%) and trueness values (70-120%) for 135 pesticide active substances in a potato matrix. This method appears to be applicable for routine analysis of pesticide residues in substrates with high water content. One hundred and four potato samples were examined using the method. Although pesticide residues higher than the LOQ were detected in 11% of potato samples, none of them exceeded the MRL values. The results supported the necessity of continuous pesticide residue monitoring in the food supply chain.

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Supplementary Tables

Table S1. Optimization of LC-MS/MS parameters of 112 insecticides, acaricides, nematicides and metabolites in the MRM mode

Analyte	Type of pesticide	Chemical group	Molecular formula	Ion mode	Retention time (min)	Precursor ion (m/z)	Product ions (m/z)	Dwell time (m sec)	Q1 Pre Bias (V)	CE (V)	Q3 Pre Bias (V)
Abamectin	Insecticide, Acaricide	Micro-organism	$C_{95}H_{142}O_{28}$	Positive	8.590	885.90	158.0	3.0	-26.0	-31.0	-30.0
						885.90	82.0	3.0	-26.0	-55.0	-16.0
						885.90	128.0	3.0	-26.0	-44.0	-24.0
Acephate	Insecticide	Organophosphate	$C_4H_{10}NO_3PS$	Positive	2.835	183.90	95.00	20.0	-19.0	-22.0	-17.0
						183.90	143.00	20.0	-19.0	-9.0	-27.0
Acetamiprid	Insecticide	Neonicotinoid	$C_{10}H_{11}ClN_4$	Positive	4.878	223.30	126.10	10.0	-17.0	-20.0	-22.0
						223.30	90.10	10.0	-16.0	-33.0	-16.0
						223.30	56.10	10.0	-16.0	-15.0	-23.0
Acrinathrin	Insecticide, Acaricide, Nematicide	Pyrethroid	$C_{26}H_{21}F_6NO_5$	Positive	8.760	559.10	208.10	5.0	-28.0	-15.0	-21.0
						559.10	181.05	5.0	-28.0	-32.0	-18.0
Aldicarb-sulfone	Insecticide, Nematicide, Metabolite	Oxime carbamate	$C_7H_{14}N_2O_4S$	Positive	3.566	240.10	86.10	13.0	-16.0	-21.0	-15.0
						240.10	148.00	13.0	-16.0	-13.0	-27.0
Aldicarb-sulfoxide	Metabolite	Oxime carbamate	$C_7H_{14}N_2O_3S$	Positive	3.415	207.10	132.00	15.0	-14.0	-8.0	-25.0
						207.10	89.00	15.0	-14.0	-13.0	-16.0
Amitraz	Insecticide, Acaricide	Amidine	$C_{19}H_{23}N_3$	Positive	4.142	294.00	163.25	10.0	-14.0	-14.0	-30.0
						294.00	122.15	10.0	-14.0	-28.0	-22.0
Benfuracarb	Insecticide	Carbamate	$C_{20}H_{30}N_2O_5S$	Positive	8.401	411.00	190.00	5.0	-29.0	-11.0	-19.0
						411.00	102.10	5.0	-28.0	-29.0	-19.0
Buprofezin	Insecticide, Acaricide	Unclassified	$C_{16}H_{23}N_3OS$	Positive	8.507	306.10	57.05	5.0	-23.0	-23.0	-22.0
						305.90	201.10	5.0	-30.0	-11.0	-21.0
Cadusafos	Insecticide	Organophosphate	$C_{10}H_{23}O_2PS_2$	Positive	8.130	270.80	131.00	3.0	-13.0	-17.0	-24.0
						270.80	97.00	3.0	-13.0	-26.0	-18.0
Carbaryl	Insecticide	Carbamate	$C_{12}H_{11}NO_2$	Positive	5.891	202.05	145.00	7.0	-13.0	-9.0	-26.0
						202.05	127.05	7.0	-13.0	-27.0	-23.0
Carbofuran	Insecticide, Nematicide, Acaricide, Metabolite	Carbamate	$C_{12}H_{15}NO_3$	Positive	5.791	222.00	165.10	7.0	-15.0	-12.0	-17.0
						222.00	123.05	7.0	-15.0	-21.0	-23.0
Carbofuran-OH	Insecticide	Carbamate. N-methyl	$C_{12}H_{15}NO_4$	Positive	4.694	255.00	163.15	5.0	-28.0	-19.0	-16.0
						255.00	220.05	5.0	-28.0	-11.0	-24.0
Carbosulfan	Insecticide, Nematicide	Carbamate	$C_{20}H_{32}N_2O_3S$	Positive	9.115	381.20	118.05	6.0	-27.0	-20.0	-21.0
						381.20	160.25	6.0	-26.0	-14.0	-30.0
Chlorantraniliprole	Insecticide	Anthranilic diamide	$C_{18}H_{14}BrCl_2N_5O_2$	Positive	6.549	483.90	452.90	4.0	-14.0	-19.0	-22.0
						483.90	285.90	4.0	-14.0	-17.0	-30.0
Chlorfenvinphos	Insecticide, Acaricide	Organophosphate	$C_{12}H_{14}Cl_3O_4P$	Positive	7.733	358.80	99.00	5.0	-17.0	-29.0	-17.0
						358.80	155.00	5.0	-17.0	-12.0	-28.0
Chlorfluazuron	Insecticide	Benzoylurea	$C_{20}H_9Cl_3F_5N_3O_3$	Positive	8.792	539.80	382.90	2.0	-38.0	-23.0	-26.0
						539.80	158.00	2.0	-38.0	-20.0	-28.0

Table S1. Cont.

Analyte	Type of pesticide	Chemical group	Molecular formula	Ion mode	Retention time (min)	Precursor ion (m/z)	Product ions (m/z)	Dwell time (m sec)	Q1 Pre Bias (V)	CE (V)	Q3 Pre Bias (V)
Chlorpyrifos	Insecticide	Organophosphate	$C_9H_{11}Cl_3NO_3PS$	Positive	8.764	349.90	97.00	5.0	-18.0	-32.0	-16.0
						349.90	197.95	5.0	-18.0	-19.0	-20.0
Clothianidine	Insecticide, Metabolite	Neonicotinoid	$C_6H_8ClN_5O_2S$	Positive	4.640	249.80	169.00	9.0	-27.0	-12.0	-17.0
						249.80	131.90	9.0	-27.0	-15.0	-23.0
Cyantraniliprole	Insecticide	Diamide	$C_{19}H_{14}BrClN_6O_2$	Positive	5.796	473.10	442.00	7.0	-17.0	-19.0	-30.0
						472.90	116.20	7.0	-13.0	-53.0	-19.0
Cyhalothrin	Insecticide, Acaricide	Pyrethroid	$C_{23}H_{19}ClF_3NO_3$	Positive	8.754	467.20	450.10	5.0	-17.0	-10.0	-30.0
						467.20	225.00	5.0	-13.0	-16.0	-23.0
Cypermethrin	Insecticide, Acaricide	Pyrethroid	$C_{22}H_{19}Cl_2NO_3$	Positive	8.847	433.20	190.95	5.0	-16.0	-15.0	-19.0
						435.20	192.85	5.0	-16.0	-15.0	-19.0
Deltamethrin	Insecticide, Metabolite	Pyrethroid	$C_{22}H_{19}Br_2NO_3$	Positive	8.875	523.00	174,20	100,0	-20,0	-31,0	-17,0
						523.00	281,10	100,0	-15,0	-15,0	-28,0
Demeton-s-methyl	Insecticide, Acaricide	Organophosphate	$C_6H_{15}O_3PS_2$	Positive	5.727	230.90	60.90	7.0	-11.0	-31.0	-22.0
						230.90	89.20	7.0	-11.0	-10.0	-15.0
Demeton-s-methyl-sulfone	Insecticide, Acaricide, Metabolite	Organophosphate	$C_6H_{15}O_5PS_2$	Positive	3.989	263.00	169.05	6.0	-30.0	-6.0	-17.0
						263.00	109.05	6.0	-30.0	-18.0	-21.0
Diafenthiuran	Insecticide, Acaricide,	Unclassified	$C_{23}H_{32}N_2OS$	Positive	8.714	385.00	278.10	3.0	-30.0	-32.0	-30.0
						385.00	186.10	3.0	-30.0	-37.0	-19.0
Diazinon	Insecticide, Acaricide	Organophosphate	$C_{12}H_{21}N_2O_3PS$	Positive	7.949	305.10	169.10	3.0	-16.0	-12.0	-17.0
						305.10	153.00	3.0	-16.0	-16.0	-29.0
Dichlorfos	Insecticide, Acaricide, Metabolite	Organophosphate	$C_4H_7Cl_2O_4P$	Positive	5.679	221.00	109.05	7.0	-15.0	-16.0	-19.0
						221.00	127.05	7.0	-15.0	-17.0	-23.0
Dicrotophos	Insecticide, Acaricide	Organophosphate	$C_8H_{16}NO_5P$	Positive	4.383	237.90	72.00	6.0	-12.0	-26.0	-30.0
						237.90	127.00	6.0	-12.0	-16.0	-22.0
Diflubenzuran	Insecticide	Benzoylurea	$C_{14}H_9ClF_2N_2O_2$	Positive	7.499	311.00	141.00	5.0	-21.0	-31.0	-27.0
						311.00	158.00	5.0	-21.0	-14.0	-30.0
Dimethoate	Insecticide, Acaricide, Metabolite	Organophosphate	$C_5H_{12}NO_3PS_2$	Positive	4.790	230.00	198.95	9.0	-15.0	-8.0	-20.0
						230.00	125.05	9.0	-15.0	-20.0	-22.0
Dioxacarb	Insecticide	Carbamate	$C_{11}H_{13}NO_4$	Positive	4.754	224.10	123.00	5.0	-25.0	-16.0	-22.0
						224.10	167.00	5.0	-25.0	-9.0	-11.0
Emamectin	Insecticide	Benzocid acid	$C_{49}H_{75}NO_{13}$	Positive	8.557	886.40	158.20	3.0	-26.0	-31.0	-30.0
						886.40	82.05	3.0	-26.0	-55.0	-16.0
Emamectin benzoat	Insecticide	Benzocid acid	$C_{56}H_{81}NO_{15}$	Positive	8.474	886.50	158.20	100.0	-30.0	-36.0	-15.0
						886.40	82.30	100.0	-30.0	-48.0	-30.0
						886.30	126.40	100.0	-26.0	-48.0	-27.0
EPN	Insecticide, Acaricide	Organophosphate	$C_{14}H_{14}NO_4PS$	Positive	8.358	324.00	157.00	5.0	-16.0	-24.0	-28.0
						324.00	296.00	5.0	-23.0	-13.0	-20.0
Ethiofencarb	Insecticide	Carbamate. N-methyl	$C_{11}H_{15}NO_2S$	Positive	5.947	226.10	107.15	7.0	-15.0	-15.0	-19.0
						226.10	164.10	7.0	-16.0	-8.0	-30.0

Table S1. Cont.

Analyte	Type of pesticide	Chemical group	Molecular formula	Ion mode	Retention time (min)	Precursor ion (m/z)	Product ions (m/z)	Dwell time (m sec)	Q1 Pre Bias (V)	CE (V)	Q3 Pre Bias (V)
Ethion	Insecticide, Acaricide, Metabolite	Organophosphate	$C_9H_{22}O_4P_2S_4$	Positive	8.773	385.00	199.00	5.0	-27.0	-10.0	-20.0
						402.00	199.00	5.0	-14.0	-15.0	-21.0
						210.20	98.10	4.0	-24.0	-22.0	-18.0
Etofenprox	Insecticide	Pyrethroid. non-ester	$C_{25}H_{28}O_3$	Positive	8.385	394.20	107.05	3.0	-20.0	-31.0	-20.0
						394.20	359.05	3.0	-20.0	-8.0	-25.0
Etoxazole	Acaricide	Diphenyl oxazoline	$C_{21}H_{23}F_2NO_2$	Positive	8.774	360.10	141.00	1.0	-27.0	-28.0	-24.0
						360.10	141.10	1.0	-27.0	-28.0	-25.0
Fenamiphos	Nematicide	Organophosphate	$C_{13}H_{22}NO_3PS$	Positive	7.332	304.20	217.00	5.0	-21.0	-23.0	-22.0
						304.20	201.90	5.0	-21.0	-35.0	-20.0
Fenamiphos-sulfone	Metabolite	Unclassified	$C_{13}H_{22}NO_5PS$	Positive	5.660	336.10	266.00	4.0	-10.0	-15.0	-28.0
						336.10	188.00	4.0	-10.0	-28.0	-20.0
Fenamiphos-sulfoxide	Metabolite	Unclassified	$C_{13}H_{22}NO_4PS$	Positive	5.596	320.10	233.00	4.0	-23.0	-21.0	-24.0
						320.10	108.00	4.0	-23.0	-41.0	-20.0
						330.80	139.00	5.0	-23.0	-36.0	-24.0
Fenazaquin	Acaricide, Insecticide	Quinazoline	$C_{20}H_{22}N_2O$	Positive	9.346	307.00	57.10	7.0	-21.0	-23.0	-22.0
						307.00	161.10	7.0	-21.0	-15.0	-29.0
Fenbutatin oxide	Acaricide, Insecticide	Organometal	$C_{60}H_{78}OSn_2$	Positive	8.933	422.50	366.00	5.0	-15.0	-19.0	-25.0
						422.30	135.00	5.0	-15.0	-34.0	-24.0
						302.05	55.05	5.0	-21.0	-40.0	-21.0
Fenoxycarb	Insecticide	Carbamate	$C_{17}H_{19}NO_4$	Positive	7.531	302.10	88.00	5.0	-15.0	-12.0	-16.0
						302.10	116.15	5.0	-15.0	-11.0	-21.0
Fenpropathrin	Insecticide, Acaricide	Pyrethroid	$C_{22}H_{23}NO_3$	Positive	8.767	349.95	125.10	5.0	-24.0	-11.0	-23.0
						349.95	57.00	5.0	-24.0	-45.0	-21.0
Fenfroxymate	Acaricide, Insecticide	Pyrazolium	$C_{24}H_{27}N_3O_4$	Positive	8.928	422.10	366.00	5.0	-15.0	-19.0	-25.0
						422.10	135.10	5.0	-15.0	-34.0	-24.0
Fenthion	Insecticide	Organophosphate	$C_{10}H_{15}O_3PS_2$	Positive	8.074	279.00	168.90	5.0	-19.0	-16.0	-30.0
						279.00	246.90	5.0	-19.0	-12.0	-26.0
Fenthion-sulfone	Insecticide, Metabolite	Unclassified	$C_{10}H_{15}O_5PS_2$	Positive	5.944	311.00	165.10	4.0	-14.0	-17.0	-17.0
						311.00	233.05	4.0	-14.0	-23.0	-24.0
Fenthion-sulfoxide	Insecticide, Metabolite	Organophosphate	$C_{10}H_{15}O_4PS_2$	Positive	5.841	295.00	279.90	4.0	-15.0	-19.0	-30.0
						295.00	109.00	4.0	-15.0	-32.0	-20.0
Fipronil	Insecticide	Phenylpyrazole	$C_{12}H_4Cl_2F_6N_4OS$	Negative	7.256	434.70	330.00	5.0	10.0	15.0	22.0
						434.70	250.00	5.0	10.0	27.0	26.0
Fipronil-sulfone	Insecticide	Unclassified	$C_{12}H_4Cl_2F_6N_4O_2S$	Negative	7.568	451.00	414.90	3.0	17.0	15.0	30.0
						451.00	282.00	3.0	17.0	26.0	30.0
Flubendiamide	Insecticide	Phthalamide; Organofluoride	$C_{23}H_{22}F_7IN_2O_4S$	Negative	7.405	680.90	254.20	5.0	20.0	29.0	16.0
						680.90	272.10	5.0	20.0	18.0	17.0
Flufenoxuron	Insecticide, Acaricide	Benzoylurea	$C_{21}H_{11}ClF_6N_2O_3$	Positive	8.654	488.80	158.00	5.0	-17.0	-20.0	-29.0
						488.80	141.00	5.0	-17.0	-43.0	-25.0
Formetanete hydrochloride	Acaricide, Insecticide	Formamidine	$C_{11}H_{16}ClN_3O_2$	Positive	5.630	222.0	93.10	100.0	-27.0	-35.0	-16.0
						222.0	120.10	100.0	-27.0	-25.0	-22.0
						222.0	165.20	100.0	-26.0	-15.0	-29.0

Table S1. Cont.

Analyte	Type of pesticide	Chemical group	Molecular formula	Ion mode	Retention time (min)	Precursor ion (m/z)	Product ions (m/z)	Dwell time (m sec)	Q1 Pre Bias (V)	CE (V)	Q3 Pre Bias (V)
Fosthiazate	Insecticide, Nematicide	Organophosphate	$C_9H_{18}NO_3PS_2$	Positive	5.973	284.10	104.10	4.0	-15.0	-11.0	-18.0
						284.10	227.85	4.0	-15.0	-6.0	-23.0
Furathiocarb	Insecticide	Carbamate	$C_{18}H_{26}N_2O_5S$	Positive	8.420	383.10	195.00	5.0	-29.0	-17.0	-20.0
						383.10	252.00	5.0	-30.0	-13.0	-26.0
Heptenophos	Insecticide, Acaricide	Organophosphate	$C_9H_{12}ClO_4P$	Positive	6.367	251.00	127.10	4.0	-13.0	-13.0	-23.0
						251.00	89.10	4.0	-13.0	-30.0	-15.0
Hexythiazox	Acaricide, Insecticide	Carboxamide	$C_{17}H_{21}ClN_2O_2S$	Positive	8.661	353.20	168.10	100.0	-24.0	-25.0	-30.0
						353.20	115.20	100.0	-30.0	-53.0	-18.0
						353.20	151.10	100.0	-29.0	-30.0	-15.0
Imidacloprid	Insecticide	Neonicotinoid	$C_9H_{10}ClN_5O_2$	Positive	4.610	255.90	209.10	21.0	-12.0	-14.0	-21.0
						255.90	174.90	21.0	−14.0	−13.0	−20.0
Indoxacarb	Insecticide	Oxadiazine	$C_{22}H_{17}ClF_3N_3O_7$	Positive	8.098	528.10	203.00	3.0	-26.0	-37.0	-21.0
						528.10	150.10	3.0	-26.0	-24.0	-27.0
Lufenuron	Insecticide, Acaricide	Benzoylurea	$C_{17}H_8Cl_2F_8N_2O_3$	Positive	8.553	509.0	175.1	100.0	34.0	37.0	18.0
						509.0	201.8	100.0	24.0	24.0	19.0
						509.0	325.8	100.0	34.0	18.0	15.0
Malaoxon	Metabolite	Organophosphate	$C_{10}H_{19}O_7PS$	Positive	5.645	314.90	127.00	7.0	-15.0	-12.0	-23.0
						314.90	99.00	7.0	-15.0	-22.0	-17.0
Malathion	Insecticide, Acaricide	Organophosphate	$C_{10}H_{19}O_6PS_2$	Positive	6.996	331.30	99.10	100.0	-13.0	-22.0	-17.0
						331.20	125.10	100.0	-28.0	-29.0	-24.0
Mecarbam	Insecticide, Acaricide	Organophosphate	$C_{10}H_{20}NO_5PS_2$	Positive	7.378	329.90	226.90	5.0	-23.0	-8.0	-23.0
						329.90	96.90	5.0	-23.0	-40.0	-17.0
Metaflumizone	Insecticide	Semicarbazon	$C_{24}H_{16}F_6N_4O_2$	Positive	8.372	507.10	178.05	3.0	-24.0	-27.0	-19.0
						507.10	287.00	3.0	-24.0	-25.0	-30.0
Methacrifos	Insecticide, Acaricide	Organophosphate	$C_7H_{13}O_5PS$	Positive	6.614	241.00	125.00	3.0	-16.0	-19.0	-22.0
						241.00	143.20	3.0	-16.0	-19.0	-26.0
Methamidophos	Insecticide, Acaricide, Metabolite	Organophosphate	$C_2H_8NO_2PS$	Positive	2.429	142.20	94.00	33.0	-27.0	-15.0	-17.0
						142.20	125.00	33.0	-15.0	-16.0	-23.0
Methidathion	Insecticide, Acaricide	Organophosphate	$C_6H_{11}N_2O_4PS_3$	Positive	6.532	303.00	144.90	6.0	-21.0	-9.0	-26.0
						303.00	85.00	6.0	-21.0	-21.0	-15.0
Methiocarb	Insecticide	Carbamate. N-methyl	$C_{11}H_{15}NO_2S$	Positive	6.778	225.90	121.10	5.0	-24.0	-17.0	-22.0
						225.90	169.10	5.0	-24.0	-9.0	-17.0
Methiocarb-sulfone	Metabolite	Carbamate. N-methyl	$C_{11}H_{15}NO_4S$	Positive	4.938	275.10	122.05	5.0	-14.0	-18.0	-23.0
						275.10	258.00	5.0	-14.0	-9.0	-27.0
Methiocarb-sulfoxide	Metabolite	Carbamate. N-methyl	$C_{11}H_{15}NO_3S$	Positive	4.689	242.10	185.05	6.0	-25.0	-3.0	-19.0
						242.10	122.10	6.0	-25.0	-23.0	-22.0
Methomyl	Insecticide, Acaricide	Oxime carbamate	$C_5H_{10}N_2O_2S$	Positive	4.054	162.90	88.00	10.0	-17.0	-9.0	-15.0
						162.90	106.00	10.0	-17.0	-10.0	-19.0
Methoxyfenozide	Insecticide	Carbohydrazide Monomethoxybenzene	$C_{22}H_{28}N_2O_3$	Positive	6.970	369.20	149.15	3.0	-19.0	-8.0	-30.0
						369.20	91.15	3.0	-19.0	-47.0	-16.0
Mevinphos	Insecticide, Acaricide	Organophosphate	$C_7H_{13}O_6P$	Positive	4.776	224.90	127.00	9.0	-24.0	-16.0	-23.0
						224.90	193.00	9.0	-25.0	-7.0	-19.0

Table S1. Cont.

Analyte	Type of pesticide	Chemical group	Molecular formula	Ion mode	Retention time (min)	Precursor ion (m/z)	Product ions (m/z)	Dwell time (m sec)	Q1 Pre Bias (V)	CE (V)	Q3 Pre Bias (V)
Monocrotophos	Insecticide, Acaricide	Organophosphate	$C_7H_{14}NO_5P$	Positive	4.199	223.90	126.90	10.0	-24.0	-14.0	-22.0
						223.90	98.00	10.0	-24.0	-11.0	-17.0
Novaluron	Insecticide	Benzoylurea	$C_{17}H_9ClF_8N_2O_4$	Positive	8.263	493.00	158.00	3.0	-15.0	-18.0	-28.0
						493.00	141.05	3.0	-15.0	-40.0	-27.0
Omethoate	Insecticide, Acaricide, Metabolite	Organophosphate	$C_5H_{12}NO_4PS$	Positive	3.188	213.90	125.00	17.0	-10.0	-21.0	-22.0
						213.90	183.10	17.0	-10.0	-10.0	-19.0
Oxamyl	Insecticide, Acaricide, Nematicide	Oxime carbamate	$C_7H_{13}N_3O_3S$	Positive	3.757	236.95	72.05	12.0	-11.0	-16.0	-29.0
						236.95	90.05	12.0	-11.0	-7.0	-16.0
Oxydemeton-methyl	Insecticide	Organophosphate	$C_6H_{15}O_4PS_2$	Positive	3.886	247.00	109.10	11.0	-18.0	-29.0	-24.0
						247.00	169.00	11.0	-18.0	-14.0	-21.0
Phenthoate	Insecticide, Acaricide	Organophosphate	$C_{12}H_{17}O_4PS_2$	Positive	7.731	321.00	135.15	5.0	-22.0	-19.0	-25.0
						321.00	163.05	5.0	-22.0	-11.0	-30.0
Phorate	Insecticide, Acaricide, Nematicide	Organophosphate	$C_7H_{17}O_2PS_3$	Positive	8.198	261.00	75.00	5.0	-18.0	-10.0	-30.0
						261.00	97.00	5.0	-18.0	-29.0	-17.0
Phosalone	Insecticide, Acaricide	Organophosphate	$C_{12}H_{15}ClNO_4PS_2$	Positive	7.987	367.95	181.95	5.0	-25.0	-15.0	-18.0
						367.95	111.00	5.0	-25.0	-39.0	-19.0
Phosphamidon	Insecticide, Acaricide	Organophosphate	$C_{10}H_{19}ClNO_5P$	Positive	5.385	299.90	174.10	8.0	-21.0	-13.0	-17.0
						299.90	227.00	8.0	-21.0	-13.0	-23.0
Pirimicarb-Desmethyl	Insecticide	Carbamate	$C_{10}H_{16}N_4O_2$	Positive	5.353	225.10	72.10	5.0	-11.0	-21.0	-29.0
						225.10	168.10	5.0	-26.0	-15.0	-30.0
Primicarb	Insecticide	Carbamate	$C_{11}H_{18}N_4O_2$	Positive	6.118	238.90	72.05	7.0	-28.0	-21.0	-29.0
						238.90	182.10	7.0	-30.0	-15.0	-18.0
Primiphos-ethyl	Insecticide, Acaricide	Organophosphate	$C_{13}H_{24}N_3O_3PS$	Positive	8.607	333.90	198.10	5.0	-23.0	-22.0	-20.0
						333.90	182.10	5.0	-23.0	-21.0	-18.0
Primiphos-methyl	Insecticide, Acaricide	Organophosphate	$C_{11}H_{20}N_3O_3PS$	Positive	8.192	305.90	67.10	5.0	-21.0	-44.0	-26.0
						305.90	108.10	5.0	-21.0	-31.0	-19.0
Profenefos	Insecticide	Organophosphate	$C_{11}H_{15}BrClO_3PS$	Positive	8.424	372.95	302.80	5.0	-13.0	-18.0	-20.0
						372.95	344.90	5.0	-13.0	-12.0	-23.0
Promecarb	Insecticide	Carbamate	$C_{12}H_{17}NO_2$	Positive	6.904	208.00	151.10	5.0	-22.0	-9.0	-29.0
						208.00	109.10	5.0	-23.0	-15.0	-20.0
Propargite	Acaricide, Insecticide	Sulphite ester	$C_{19}H_{26}O_4S$	Positive	8.700	368.15	231.10	5.0	-26.0	-10.0	-24.0
						368.15	175.10	5.0	-25.0	-16.0	-18.0
Propoxur	Insecticide, Acaricide	Carbamate	$C_{11}H_{15}NO_3$	Positive	5.569	210.10	111.10	7.0	-14.0	-13.0	-20.0
						210.10	168.00	7.0	-14.0	-8.0	-17.0
Prothiophos	Insecticide	Organophosphate	$C_{11}H_{15}Cl_2O_2PS_2$	Positive	9.185	344.80	240.80	6.0	-24.0	-19.0	-25.0
						344.80	242.80	6.0	-24.0	-19.0	-25.0
Pymetrozine	Insecticide	Pyridine	$C_{10}H_{11}N_5O$	Positive	4.228	217.90	105.00	10.0	-23.0	-20.0	-19.0
						217.90	78.00	10.0	-23.0	-41.0	-30.0
Pyridaben	Insecticide, Acaricide	Pyridazinone	$C_{19}H_{25}ClN_2OS$	Positive	8.946	365.40	309.20	25.0	-14.0	-13.0	-14.0
						365.40	147.30	25.0	-28.0	-23.0	-30.0
						365.40	132.20	25.0	-28.0	-43.0	-23.0

Table S1. Cont.

Analyte	Type of pesticide	Chemical group	Molecular formula	Ion mode	Retention time (min)	Precursor ion (m/z)	Product ions (m/z)	Dwell time (m sec)	Q1 Pre Bias (V)	CE (V)	Q3 Pre Bias (V)
Pyridaphenthion	Insecticide	Organophosphate	$C_{14}H_{17}N_2O_4PS$	Positive	7.047	340.90	189.05	5.0	-24.0	-21.0	-19.0
						340.90	92.10	5.0	-24.0	-39.0	-16.0
Pyriproxyfen	Insecticide	Juvenile hormon mimic	$C_{20}H_{19}NO_3$	Positive	8.770	321.90	78.10	2.0	-30.0	-51.0	-30.0
						321.90	96.00	2.0	-30.0	-14.0	-17.0
Quinalphos	Insecticide, Acaricide	Organophosphate	$C_{12}H_{15}N_2O_3PS$	Positive	7.930	298.90	163.00	5.0	-20.0	-20.0	-30.0
						298.90	147.00	5.0	-20.0	-21.0	-28.0
Spinosyn A	Insecticide	Micro-organism derived	$C_{41}H_{65}NO_{10}$	Positive	8.833	732.50	142.10	100.0	-28.0	-32.0	-24.0
						732.40	98.10	100.0	-24.0	-54.0	-16.0
Spinosyn D	Insecticide	Micro-organism derived	$C_{42}H_{67}NO_{10}$	Positive	9.018	746.30	142.30	100.0	-20.0	-28.0	-25.0
						746.40	98.30	100.0	-24.0	-43.0	-17.0
Spirodiclofen	Acaricide, Insecticide	Tetronic acid	$C_{21}H_{24}Cl_2O_4$	Positive	8.789	411.10	313.05	3.0	-12.0	-14.0	-22.0
						411.10	71.10	3.0	-12.0	-22.0	-27.0
Sulfoxaflor	Insecticide	Sulfoximine	$C_{10}H_{10}F_3N_3OS$	Positive	4.882	278.00	174.00	9.0	-14.0	-11.0	-30.0
						278.20	154.00	9.0	-10.0	-54.0	-25.0
Tebufenozide	Insecticide	Bishydrazide	$C_{22}H_{28}N_2O_2$	Positive	7.478	353.00	133.00	5.0	-17.0	-20.0	-23.0
						353.00	105.10	5.0	-17.0	-43.0	-18.0
Tebufenpyrad	Acaricide, Insecticide	Pyrazole	$C_{18}H_{24}ClN_3O$	Positive	8.439	333.90	171.00	2.0	-16.0	-24.0	-30.0
						333.90	147.10	2.0	-16.0	-25.0	-28.0
Tetramethrin	Insecticide	Pyrethroid	$C_{19}H_{25}NO_4$	Positive	8.486	332.20	164.10	3.0	-10.0	-24.0	-29.0
						332.20	135.10	3.0	-10.0	-18.0	-13.0
Thiacloprid	Insecticide	Neonicotinoid	$C_{10}H_9ClN_4S$	Positive	5.145	507.80	141.10	8.0	-36.0	-24.0	-27.0
						507.80	167.10	8.0	-36.0	-19.0	-17.0
Thiamethoxam	Insecticide	Neonicotinoid	$C_8H_{10}ClN_5O_3S$	Positive	4.138	292.00	211.05	10.0	-20.0	-12.0	-22.0
						292.00	181.00	10.0	-20.0	-22.0	-19.0
Thiodicarb	Insecticide	Oxime carbamate	$C_{10}H_{18}N_4O_4S_3$	Positive	6.167	354.80	88.00	7.0	-17.0	-18.0	-16.0
						354.80	107.95	7.0	-17.0	-15.0	-19.0
Tolfenpyrad	Insecticide	Pyrazolium	$C_{21}H_{22}ClN_3O_2$	Positive	8.544	384.40	197.2	5.0	-11.0	-26.0	-20.0
						384.00	116.00	5.0	-14.0	-21.0	-18.0
Triazophos	Insecticide, Acaricide, Nematicide	Organophosphate	$C_{12}H_{16}N_3O_3PS$	Positive	7.202	314.00	162.00	5.0	-22.0	-19.0	-30.0
						314.00	119.10	5.0	-21.0	-34.0	-22.0
Trichlorfon	Insecticide	Organophosphate	$C_4H_8Cl_3O_4P$	Positive	4.655	256.95	109.00	9.0	-17.0	-17.0	-19.0
						256.95	79.10	9.0	-17.0	-29.0	-14.0
Triflumuron	Insecticide	Benzoylurea	$C_{15}H_{10}ClF_3N_2O_3$	Positive	7.917	359.00	156.05	3.0	-18.0	-17.0	-28.0
						359.00	139.05	3.0	-18.0	-30.0	-26.0

Table S2. Optimization of GC-MS parameters of 23 insecticides, acaricides, nematicides and metabolites in the SRM mode

Analyte	Type of pesticide	Chemical group	Molecular formula	Retention time (min)	Quantitation ion (m/z)	Confirmation ions (m/z)	
Aldrin	Insecticide	Organochloride	$C_{12}H_8Cl_6$	15.157	66.0	207	137
Alpha HCH	Insecticide	Organochloride	$C_6H_6Cl_6$	10.278	181	264	43
Beta HCH	IMetabolite	Organochloride	$C_6H_6Cl_6$	11.055	181	286	282
Bifenthrin	Insecticide, Acaricide	Pyrethroid	$C_{23}H_{22}ClF_3O_2$	22.384	181	272	307
Bromophos-ethyl	Insecticide	Organophosphate	$C_{10}H_{12}BrCl_2O_3PS$	13.19	359	303	242
Bromophos -methyl	Insecticide, Acaricide	Organophosphate	$C_8H_8BrCl_2O_3PS$	15.844	331	174	187
Delta HCH	Metabolite	Organochloride	$C_6H_6Cl_6$	12.283	181	264	268
Dieldrin	Insecticide, Metabolite	Organochloride	$C_{12}H_8Cl_6O$	18.555	79.0	87	241
Endosulfan sulfate	Metabolite	Unclassified	$C_9H_6C_{l6}O_4S$	15.19	274	227	229
Endrin	Insecticide	Organochloride	$C_{12}H_8Cl_6O$	14.41	263	281	345
Ethoprophos	Insecticide, Nematicide	Organophosphate	$C_8H_{19}O_2PS_2$	9.187	158	261	201
Fonofos	Insecticide	Organophosphate	$C_{10}H_{15}OPS_2$	11.660	137	181	219
Heptachlor-exo-epoxide	Metabolite	Unclassified	$C_{10}H_5Cl_7O$	16.491	353	329	125
o,p'-DDD	Metabolite	Organochloride	$C_{14}H_{10}Cl_4$	18.644	237	248	318
o,p'-DDE	Metabolite	Organochloride	$C_{14}H_8Cl_4$	17.401	248	375	97
o,p'-DDT	Insecticide	Organochloride	$C_{14}H_9Cl_5$	19.820	237	159	160
p,p'-DDD	Metabolite	Organochloride	$C_{14}H_{10}Cl_4$	19.746	237	263	67
p,p'-DDE	Metabolite	Organochloride	$C_{14}H_8Cl_4$	18.455	246	329	331
Parathion ethyl	Insecticide	Organophosphate	$C_{10}H_{14}NO_5PS$	12.14	291	109	139
Tefluthrin	Insecticide, Acaricide	Pyrethroid	$C_{17}H_{14}ClF_7O_2$	12.347	177	213	183
Tetrachlorvinphos	Insecticide, Acaricide	Organophosphate	$C_{10}H_9Cl_4O_4P$	17.482	109	375	97
Tetradifon	Insecticide, Acaricide	Bridged diphenyl	$C_{12}H_6Cl_4O_2S$	23.119	159	341	166
Tetrasul	Insecticide, Acaricide, Nematicide	Bridged diphenyl	$C_{12}H_6Cl_4S$	20.176	252	165	235

Table S3. Method verification parameters of 135 pesticides

Pesticide (LC-MS/MS)	Pesticide type*	LOD (µg kg^{-1})	LOQ (µg kg^{-1})	R^2	Repeatability (n=10)						Reproducibility (n=10)						U' %	ME %
					10 µg kg^{-1}		50 µg kg^{-1}		100 µg kg^{-1}		10 µg kg^{-1}		50 µg kg^{-1}		100 µg kg^{-1}			
					Recovery %	RSD %	Recovery %	RSD %	Recovery %	RSD %	Recovery %	RSD %	Recovery %	RSD %	Recovery %	RSD %		
Abamectin	IN, AC	2.9	9.5	0.992	101.5	13.0	118.8	9.0	107.2	15.1	104.9	16.3	112.9	11.6	111.7	12.8	37.3	-56.8
Acephate	IN	2.0	6.8	0.995	114.4	11.5	101.6	14.9	100.4	11.9	96.9	17.5	115.7	16.3	87.0	9.0	34.3	-95.0
Acetamiprid	IN	1.9	6.3	0.993	115.1	9.6	118.2	4.7	113.7	5.8	106.1	8.7	115.6	13.2	105.7	5.0	32.8	-28.8
Acrinathrin	IN, AC, NE	2.2	7.4	0.990	92.8	15.6	104.2	15.5	115.6	14.6	97.1	17.5	110.0	16.6	119.5	15.0	41.6	-76.8
Aldicarb-sulfone	IN, NE, MT	1.5	5.0	0.999	115.0	6.8	118.4	5.9	108.5	5.4	117.1	8.3	117.6	11.1	105.3	3.5	33.1	-68.8
Aldicarb-sulfoxide	MT	1.5	5.0	0.994	109.4	14.8	99.6	12.4	115.3	14.6	96.5	15.0	102.1	17.0	94.7	17.2	33.7	-83.4
Amitraz	IN, AC	1.5	4.9	0.991	105.0	9.9	119.0	7.1	105.7	3.5	100.6	12.6	111.2	11.4	97.3	5.7	27.4	-72.0
Benfuracarb	IN,	2.6	8.8	0.991	118.3	11.2	103.6	16.3	95.2	18.5	116.9	9.8	97.4	13.5	81.3	15.2	38.5	-84.9
Buprofezin	IN, AC	2.4	8.0	0.998	114.5	12.1	114.8	7.0	109.5	9.8	113.2	15.7	115.9	9.5	112.9	8.0	36.6	-54.8
Cadusafos	IN	2.7	9.1	0.996	114.9	12.8	117.8	6.9	110.9	9.2	116.1	6.9	117.0	12.3	115.3	9.9	38.0	-63.4
Carbaryl	IN	0.8	2.8	0.998	92.3	7.2	101.5	7.1	86.1	8.5	98.1	14.8	105.6	7.4	104.8	12.2	27.1	-72.3
Carbofuran	IN, AC, NE, MT	1.8	6.0	0.999	117.5	8.2	110.9	3.4	109.0	6.6	118.6	5.8	114.1	6.4	106.8	4.7	30.6	-25.9
Carbofuran-OH	IN	2.1	7.1	0.994	111.6	17.3	113.9	10.4	104.1	6.9	102.2	15.1	106.7	8.8	95.9	17.1	32.1	-75.8
Carbosulfan	IN, NE	1.7	5.8	0.990	116.0	11.9	112.8	5.8	111.4	4.5	117.0	11.7	108.6	4.9	98.2	3.5	31.3	4.1
Chlorantraniliprole	IN	1.3	4.4	0.999	106.9	9.5	110.4	8.5	89.8	12.4	100.6	8.5	94.8	10.3	93.4	15.2	28.7	-26.7
Chlorfenvinphos	IN, AC	2.6	8.8	0.991	110.0	12.2	115.9	10.3	103.1	4.6	104.7	17.8	107.9	13.0	101.5	13.4	32.9	-37.2
Chlorfluazuron	IN	2.8	9.2	0.998	102.7	10.8	109.7	12.1	117.9	11.0	107.4	10.4	116.4	12.6	119.2	12.4	37.1	-78.8
Chlorpyrifos	IN	1.5	4.9	0.991	118.2	6.1	116.7	11.9	115.1	11.1	115.6	11.3	116.3	11.9	115.5	12.7	40.8	-64.3
Clothianidine	IN, MT	1.2	4.1	0.995	108.4	6.3	106.4	13.6	111.9	4.5	111.1	7.9	107.1	6.6	114.2	8.5	27.9	-78.2
Cyantraniliprole	IN	1.6	5.4	0.997	114.2	8.9	117.5	8.4	113.7	5.9	99.9	15.6	116.5	9.7	112.3	7.4	33.8	46.2
Cyhalothrin	IN, AC	2.1	7.1	0.990	91.5	19.3	115.1	18.5	109.6	11.7	91.4	14.2	108.0	15.7	110.0	13.6	38.7	-54.4
Cypermethrin	IN, AC	2.5	8.3	0.994	111.2	14.0	113.3	13.2	117.5	9.1	106.0	8.8	116.1	12.0	112.5	12.1	36.4	-77.4
Deltamethrin	IN, MT	2.6	8.7	0.991	110.0	15.4	116.4	11.5	115.2	9.7	107.9	9.4	118.1	13.7	117.8	7.0	38.7	-81.5
Demeton-s-methyl	IN, AC	2.3	7.6	0.992	100.9	10.9	104.3	12.8	101.9	9.9	102.0	13.5	110.5	8.8	96.5	9.1	23.5	-94.3
Demeton-S-methyl-sulfone	IN, AC, MT	1.9	6.3	0.992	105.1	8.7	111.8	4.0	101.4	8.2	87.2	17.2	93.2	15.6	87.3	15.5	29.9	-59.2
Diafenthiuran	IN, AC	2.4	8.1	0.997	98.6	17.7	104.4	12.0	112.2	6.7	97.1	14.9	106.6	16.4	110.1	14.3	35.6	-99.3
Diazinon	IN, AC,	2.1	7.0	0.998	117.3	4.5	108.6	6.9	101.2	6.9	113.2	13.1	112.3	10.3	103.5	14.8	30.1	10.1
Dichlorvos	IN, AC, MT	1.5	4.9	0.997	111.5	5.6	113.8	10.1	114.0	6.9	111.2	6.6	114.4	5.2	109.3	7.8	29.5	181.1
Dicrotophos	IN, AC	1.2	4.0	0.990	116.7	6.4	115.4	12.4	118.3	6.2	114.4	11.2	115.3	11.6	118.8	4.9	39.2	-15.1
Diflubenzuran	IN	2.6	8.6	0.993	117.4	17.2	114.2	8.7	108.4	6.3	110.8	18.2	118.6	12.5	104.7	8.9	38.1	129.0
Dimethoate	IN, AC, MT	1.5	4.8	0.992	106.5	9.8	117.7	13.7	116.2	6.3	108.8	12.8	116.0	11.0	117.2	4.2	36.2	-56.9
Dioxacarb	IN	2.1	6.9	0.994	105.1	9.9	117.2	5.8	101.7	6.9	103.4	9.3	115.9	3.8	97.4	6.9	25.5	-51.1
Emamectin	IN	2.7	8.9	0.996	100.3	12.4	115.3	8.9	119.2	8.1	100.6	15.8	102.6	19.2	106.0	12.6	35.7	-51.5
Emamectin benzoat	IN	1.8	6.1	0.996	96.8	14.9	108.7	13.0	109.3	15.5	91.4	18.3	114.2	16.6	111.5	13.5	37.8	-58.3
EPN	IN, AC	2.5	8.4	0.991	98.2	9.9	98.7	11.7	118.5	9.9	103.5	19.0	113.8	12.6	117.7	15.6	38.0	169.8
Ethiofencarb	IN	2.5	8.4	0.998	93.2	13.6	97.8	11.9	78.2	10.9	100.4	19.4	95.1	10.0	90.4	14.9	33.7	-55.3
Ethion	IN, AC, MT	3.0	9.9	0.990	114.0	10.0	116.7	5.8	110.7	5.8	108.9	5.5	113.5	8.2	107.5	3.4	29.2	-57.6
Etofenprox	IN	2.8	9.4	0.990	87.1	17.9	113.1	18.2	106.4	11.9	97.8	16.8	103.7	14.3	104.4	10.2	35.9	-85.0
Etoxazole	AC	2.7	8.9	0.995	109.8	6.6	118.1	4.9	116.6	7.8	111.4	6.6	119.4	7.2	116.4	6.6	34.6	-77.3

Table S3. Cont.

Pesticide (LC-MS/MS)	Pesticide type*	LOD (µg kg^{-1})	LOQ (µg kg^{-1})	R^2	Repeatability (n=10)						Reproducibility (n=10)						U' %	ME %
					10 µg kg^{-1}		50 µg kg^{-1}		100 µg kg^{-1}		10 µg kg^{-1}		50 µg kg^{-1}		100 µg kg^{-1}			
					Recovery %	RSD %	Recovery %	RSD %	Recovery %	RSD %	Recovery %	RSD %	Recovery %	RSD %	Recovery %	RSD %		
Fenamiphos	NE	2.7	9.1	0.994	99.1	16.5	114.5	11.4	108.8	7.5	101.3	12.7	117.1	10.1	101.6	10.1	31.2	-34.1
Fenamiphos-sulfone	MT	1.4	4.6	0.999	115.5	13.8	115.9	8.0	103.0	7.8	115.7	5.9	116.0	7.8	94.8	9.1	23.3	43.6
Fenamiphos-sulfoxide	MT	2.0	6.5	0.998	108.1	14.5	107.6	9.7	113.7	9.2	107.1	9.1	107.0	13.7	114.6	4.4	38.1	-29.8
Fenazaquin	IN, AC	1.8	5.9	0.992	112.3	5.9	112.5	7.1	107.6	3.4	109.2	16.4	118.6	10.9	109.5	8.4	34.0	87.1
Fenbutatin oxide	IN, AC	2.4	8.0	0.990	118.4	10.0	107.0	9.6	115.9	3.9	109.0	8.6	115.8	17.2	118.4	2.4	37.8	-87.1
Fenoxycarb	IN,	1.8	6.1	0.993	107.2	12.8	118.5	9.1	103.7	4.8	117.0	15.8	110.0	17.6	104.1	4.8	34.3	-36.6
Fenpropathrin	IN, AC	2.5	8.5	0.991	106.7	13.1	113.1	15.0	119.1	3.9	93.0	16.2	115.1	10.5	119.2	8.6	38.7	-65.8
Fenproxymate	IN, AC	2.7	8.9	0.990	109.7	7.1	112.7	12.1	118.2	7.7	94.4	11.9	116.5	13.1	115.8	7.3	36.3	-82.9
Fenthion	IN	2.7	8.9	0.991	118.5	13.2	115.3	14.4	116.2	13.9	99.5	14.4	111.6	11.9	118.8	11.7	41.2	-8.9
Fenthion-sulfone	IN, MT	1.4	4.6	0.999	106.9	9.3	79.1	10.1	90.3	11.2	99.3	16.1	88.8	12.3	85.2	11.3	33.1	-28.4
Fenthion-sulfoxide	IN, MT	1.2	4.0	0.997	103.8	5.5	110.0	8.8	94.3	11.4	100.8	11.2	113.9	10.2	100.1	11.9	26.7	-32.1
Fipronil	IN	1.3	4.4	0.991	106.3	8.6	90.1	12.6	90.8	14.4	109.7	8.0	94.2	10.1	82.3	13.5	30.0	-66.4
Fipronil-sulfone	IN	2.4	7.8	0.992	111.5	7.8	104.7	16.5	87.3	11.1	118.2	11.0	106.3	13.1	89.5	11.8	33.6	-85.2
Flubendiamide	IN	2.8	9.4	0.992	92.6	15.3	112.9	12.7	113.1	11.1	99.6	10.2	109.6	13.0	97.2	19.3	33.2	-16.4
Flufenoxuron	IN, AC	2.5	8.5	0.991	98.5	5.5	109.7	7.0	118.6	7.7	103.7	17.3	112.7	10.7	111.5	6.3	34.8	-52.5
Formetanate hydrochloride	IN, AC	1.2	4.0	0.998	106.4	10.6	108.0	7.5	95.1	3.6	112.8	9.6	109.7	10.9	99.9	5.9	24.7	-41.9
Fosthiazate	IN, NM	1.3	4.5	0.997	87.7	9.7	84.7	14.5	73.5	10.1	92.6	10.3	76.7	9.1	81.9	15.6	42.5	-66.1
Furathiocarb	IN	2.8	9.3	0.996	98.6	9.2	115.9	5.5	111.1	7.5	108.2	11.4	117.9	13.7	112.3	14.7	35.5	-62.9
Heptenophos	IN, AC	0.7	2.5	0.999	97.9	8.5	104.9	7.3	98.6	11.4	103.2	7.8	95.9	9.2	92.1	14.5	23.0	-20.3
Hexythiazox	IN, AC	2.4	8.0	0.991	103.8	15.4	117.7	9.3	115.0	8.9	116.9	18.7	112.5	11.0	119.6	8.3	41.4	-61.6
Imidacloprid	IN	0.9	2.9	0.994	108.7	11.5	116.6	6.3	102.5	6.8	97.8	12.7	115.6	10.1	100.5	5.3	28.2	-55.6
Indoxacarb	IN	2.3	7.7	0.995	115.7	10.3	114.1	13.0	111.2	7.9	109.0	16.2	116.3	13.6	96.3	14.2	37.1	-46.3
Lufenuron	IN, AC	2.7	9.1	0.992	113.3	8.4	114.1	12.0	117.2	6.1	112.8	15.4	112.0	8.7	110.2	10.0	35.7	28.0
Malathion	IN, AC	2.2	7.3	0.996	106.6	7.1	115.4	10.1	109.4	13.8	112.0	14.5	116.1	6.7	100.7	14.0	33.4	-58.4
Malaoxon	MT	2.0	6.8	0.999	112.4	11.2	117.4	8.1	105.4	7.7	110.4	7.9	115.6	4.1	100.0	3.7	28.8	-50.1
Mecarbam	IN, AC	2.6	8.5	0.991	107.2	15.6	119.5	12.8	107.7	15.1	112.8	19.2	118.9	5.2	100.2	18.4	41.1	-69.3
Metaflumizone	IN,	2.3	7.5	0.994	92.8	8.8	112.3	11.6	105.2	10.3	96.9	15.6	101.3	11.7	87.2	11.8	29.9	-22.4
Methacrifos	IN, AC	2.4	8.1	0.999	112.4	10.5	111.8	13.6	109.9	8.4	108.6	7.8	116.5	10.5	113.3	9.9	33.9	58.5
Methamidophos	IN, AC, MT	1.5	4.9	0.992	110.2	10.5	114.6	5.4	102.5	8.9	109.4	5.6	104.8	12.4	92.2	10.0	26.1	-95.7
Methidathion	IN, AC	2.5	8.4	0.996	111.1	14.7	110.8	15.8	85.7	15.4	103.3	17.5	100.7	18.0	86.5	17.7	37.7	-78.7
Methiocarb	IN	2.4	7.9	0.993	117.8	12.0	119.1	11.8	116.4	6.9	109.3	17.4	113.7	8.5	114.4	9.6	40.1	-36.9
Methiocarb-sulfone	MT	1.5	5.1	0.992	101.6	15.8	116.3	5.2	107.4	5.1	97.1	13.9	117.1	8.5	106.5	9.0	30.0	-28.0
Methiocarb-sulfoxide	MT	2.0	6.7	0.998	109.1	11.0	111.2	9.8	107.0	4.9	115.0	6.4	105.9	6.1	105.7	9.3	26.0	-43.1
Methomyl	IN, AC	1.9	6.3	0.994	118.0	9.2	116.2	13.3	108.5	8.4	116.3	10.1	118.2	7.6	110.8	4.8	36.9	-60.1
Methoxyfenozide	IN	2.7	9.0	0.991	108.3	16.1	93.9	16.2	100.8	14.1	96.1	15.8	94.2	18.6	83.1	14.1	36.3	-74.3
Mevinphos	IN, AC	2.4	8.1	0.997	99.6	8.4	112.9	11.1	108.7	9.3	86.6	14.5	107.4	4.5	96.0	9.4	27.8	-58.4
Monocrotophos	IN, AC	1.8	5.9	0.991	117.5	5.3	117.8	5.5	109.0	4.1	118.3	3.8	119.1	7.7	103.8	9.4	33.8	2.3
Novaluron	IN,	2.9	9.7	0.991	116.3	11.2	115.8	9.2	117.5	5.3	115.1	13.7	116.5	16.8	115.9	7.7	41.2	19.2
Omethoate	IN, AC, MT	1.0	3.4	0.993	91.4	15.5	98.4	15.5	83.8	16.9	96.6	16.7	98.3	17.9	96.6	12.2	36.5	-82.9
Oxamyl	IN, AC, NE	1.9	6.2	0.999	112.1	12.6	117.9	10.4	108.7	7.5	109.6	7.8	113.8	7.1	98.1	7.2	30.7	-78.6
Oxydemeton-methyl	IN	1.3	4.5	0.999	115.8	6.4	115.6	9.1	108.9	5.6	114.8	8.8	118.5	7.2	110.9	4.9	32.9	-62.9

Table S3. Cont.

Pesticide (LC-MS/MS)	Pesticide type*	LOD (µg kg^{-1})	LOQ (µg kg^{-1})	R^2	Repeatability (n=10)						Reproducibility (n=10)						U' %	ME %
					10 µg kg^{-1}		50 µg kg^{-1}		100 µg kg^{-1}		10 µg kg^{-1}		50 µg kg^{-1}		100 µg kg^{-1}			
					Recovery %	RSD %	Recovery %	RSD %	Recovery %	RSD %	Recovery %	RSD %	Recovery %	RSD %	Recovery %	RSD %		
Phenthoate	IN, AC	2.7	9.0	0.997	117.0	7.8	115.7	15.1	115.3	7.1	109.8	13.7	115.2	10.7	111.5	12.2	38.9	-26.1
Phorate	IN, AC, NE	2.0	6.8	0.996	116.6	11.7	113.5	16.3	117.6	8.7	111.7	14.9	114.1	15.4	115.3	8.7	40.8	-45.2
Phosalone	IN, AC	2.3	7.7	0.992	118.2	6.0	114.2	11.0	112.4	9.9	96.2	16.0	116.3	6.9	116.3	8.2	36.3	-43.8
Phosphamidon	IN, AC	2.0	6.7	0.999	111.3	7.8	116.8	6.7	106.0	4.5	112.7	7.7	115.9	6.7	103.6	7.0	28.5	-46.4
Pirimicarb-Desmethyl	IN	1.7	5.5	0.999	103.4	7.3	105.2	7.9	104.5	10.2	110.6	13.8	107.8	8.5	106.8	11.4	25.1	-36.6
Primicarb	IN	2.3	7.7	0.998	113.3	8.7	117.4	10.1	107.6	8.9	112.6	11.6	116.4	16.1	109.5	13.1	38.0	-91.7
Primiphos -ethyl	IN, AC	2.3	7.5	0.993	105.1	6.6	108.0	8.3	98.0	7.3	107.3	10.1	113.5	11.1	108.1	12.0	26.3	-70.3
Primiphos -methyl	IN, AC	2.4	7.9	0.990	113.6	11.6	116.9	6.2	104.3	6.1	117.4	14.5	115.7	12.4	111.7	7.7	35.9	-47.7
Profenofos	IN	2.6	8.8	0.994	112.6	6.3	117.7	13.1	116.8	5.5	111.3	13.8	109.9	11.4	107.6	6.9	35.4	-29.6
Promecarb	IN	1.5	5.2	0.996	116.5	8.9	113.0	9.5	106.6	6.5	104.8	12.5	117.8	6.2	95.3	12.0	32.4	-46.3
Propargite	IN, AC	2.6	8.5	0.994	118.1	11.5	111.6	13.5	119.1	11.6	114.4	15.1	117.3	12.5	116.3	8.7	43.2	-74.2
Propoxur	IN, AC	1.6	5.2	0.999	118.8	7.9	115.8	11.1	110.3	13.7	114.2	14.0	113.3	8.0	116.6	12.5	39.7	-25.2
Prothiophos	IN	2.7	8.8	0.991	109.9	15.1	104.5	4.9	117.4	7.4	98.3	15.0	108.1	10.0	111.1	16.3	33.5	-37.2
Pymetrozine	IN	1.6	5.4	0.998	118.1	11.8	116.3	12.5	119.4	4.8	115.6	14.1	117.4	13.4	116.3	3.6	41.8	-72.4
Pyridaben	IN, AC	1.6	5.2	0.992	116.3	13.0	106.2	13.1	115.7	6.6	97.3	9.7	118.3	15.0	119.5	8.5	39.0	-82.7
Pyridaphenthion	IN	2.0	6.6	0.991	107.5	15.5	112.0	16.1	106.0	7.5	106.5	17.6	114.4	13.6	109.4	6.8	35.0	-72.1
Pyriproxyfen	IN,	2.9	9.8	0.995	114.4	4.4	118.7	8.7	113.2	10.9	116.4	12.0	118.9	16.3	118.1	10.0	41.5	-62.8
Quinalphos	IN, AC	1.8	5.9	0.992	104.0	13.5	106.1	7.3	104.0	7.4	105.2	8.5	111.0	11.7	104.4	9.6	27.4	-58.7
Spinosyn A	IN	2.5	8.3	0.994	108.4	4.8	106.3	6.3	102.2	8.7	102.9	7.3	95.7	7.5	96.0	7.0	20.2	-83.2
Spinosyn D	IN	3.0	9.9	0.993	104.3	14.1	110.3	12.0	113.4	4.3	102.6	13.7	110.8	8.7	102.6	6.0	30.0	-66.8
Spirodiclofen	IN, AC	2.6	8.7	0.991	114.5	16.4	107.6	7.3	118.1	6.7	93.2	15.4	116.1	12.0	118.6	9.0	38.4	-82.4
Sulfoxaflor	IN	1.7	5.7	0.995	117.6	13.8	110.8	7.8	106.7	13.8	111.3	12.7	116.3	12.4	104.6	12.5	37.6	-71.7
Tebufenozide	IN	2.6	8.7	0.991	96.0	13.6	103.1	9.0	85.8	12.4	106.8	8.5	104.0	11.5	90.6	11.2	26.1	-75.4
Tebufenpyrad	AC, IN	2.8	9.3	0.994	107.3	16.1	116.4	12.8	115.1	9.4	103.3	10.0	109.3	15.7	113.8	7.9	37.0	-49.1
Tetramethrin	IN	2.8	9.5	0.993	95.0	12.3	117.4	7.3	112.2	7.0	108.3	12.8	109.8	8.7	106.6	7.6	30.1	-63.1
Thiacloprid	IN	1.5	5.1	0.993	95.8	7.9	118.2	7.3	108.8	5.3	100.7	9.1	116.0	7.3	107.8	7.5	27.2	-73.4
Thiamethoxam	IN	1.9	6.4	0.994	116.8	10.2	118.6	9.7	114.8	10.3	115.6	11.6	117.0	11.3	116.8	3.6	39.7	-70.4
Thiodicarb	IN	1.8	5.9	0.994	113.2	4.4	111.1	10.9	109.2	5.8	100.8	16.0	93.6	10.9	95.2	8.4	28.5	-91.1
Tolfenpyrad	IN	2.5	8.5	0.990	83.0	12.2	104.3	7.1	90.1	8.0	74.7	7.7	96.9	9.4	82.9	7.0	35.6	-27.2
Triazophos	IN, AC, NE	1.7	5.7	0.996	117.0	13.8	119.3	7.3	113.3	8.3	100.1	12.5	117.8	7.4	110.5	9.3	37.5	-69.6
Trichlorfon	IN	1.3	4.3	0.991	112.7	14.1	118.2	8.8	119.4	4.9	114.3	11.0	116.6	12.9	108.9	6.6	38.0	-22.9
Triflumuron	IN	2.1	7.1	0.995	104.0	11.2	108.9	8.1	100.5	5.2	106.0	12.4	115.9	7.3	96.8	8.0	27.0	3.4
Pesticide (GC-MS)																		
Aldrin	IN	0.6	2.0	0.995	100.6	1.8	84.2	4.8	107.9	9.3	104.8	5.3	109.0	5.2	109.5	3.8	23.5	374.1
Alpha HCH	IN	2.8	9.4	0.991	95.4	7.8	89.0	9.0	109.8	4.3	90.6	16.6	111.9	10.5	104.9	10.5	27.0	-90.9
Beta HCH	MT	2.1	6.9	0.998	101.1	11.9	89.9	7.4	108.6	4.4	78.3	13.7	111.6	3.9	111.8	2.6	29.8	150.3
Bifenthrin	IN, AC	2.6	8.6	0.992	93.5	13.9	105.3	7.5	100.9	11.7	98.1	14.6	103.1	10.7	100.1	9.3	24.8	236.4
Bromophos-ethyl	IN,	1.4	4.8	0.993	75.2	5.7	82.8	9.5	108.8	3.0	76.2	7.0	102.5	5.1	112.0	4.9	35.7	267.6
Bromophos -methyl	IN, AC	1.3	4.5	0.992	113.1	3.9	82.0	6.5	103.4	9.4	113.9	6.0	110.5	5.7	104.6	7.6	28.2	399.4
Delta HCH	MT	0.8	2.6	0.998	96.5	3.3	87.6	6.6	109.7	7.1	97.1	8.1	108.7	6.2	110.1	6.5	24.7	394.2

Table S3. Cont.

Pesticide (LC-MS/MS)	Pesticide type*	LOD (µg kg^{-1})	LOQ (µg kg^{-1})	R^2	Repeatability (n=10)						Reproducibility (n=10)						U' %	ME %
					10 µg kg^{-1}		50 µg kg^{-1}		100 µg kg^{-1}		10 µg kg^{-1}		50 µg kg^{-1}		100 µg kg^{-1}			
					Recovery %	RSD %	Recovery %	RSD %	Recovery %	RSD %	Recovery %	RSD %	Recovery %	RSD %	Recovery %	RSD %		
Dieldrin	IN, MT	0.8	2.5	0.991	109.4	6.0	88.0	8.8	105.1	8.2	103.2	4.9	102.3	7.9	95.9	9.3	22.2	107.7
Endosulfan sulfate	MT	2.1	6.9	0.993	84.0	10.2	93.8	9.9	108.4	7.1	74.1	2.5	104.6	10.6	105.7	10.0	31.7	208.8
Endrin	IN	2.9	9.6	0.993	96.7	5.3	83.3	3.5	97.3	5.3	98.0	7.6	93.4	5.0	98.9	6.0	19.5	99.7
Ethoprophos	IN, NE	2.3	7.5	0.998	92.4	18.3	93.8	12.6	108.6	9.4	73.8	11.8	97.2	11.2	106.8	10.3	33.6	152
Fonofos	IN	1.1	3.7	0.990	89.7	5.9	90.4	3.7	103.6	9.2	93.3	4.7	99.4	10.2	109.1	7.4	24.5	54.4
Heptachlor exo epoxide	MT	1.2	4.1	0.991	116.3	1.9	79.8	9.3	108.8	8.7	115.3	2.2	109.4	6.1	109.1	5.1	30.8	200
o.p DDD	MT	0.5	1.6	0.991	85.6	2.2	79.4	5.4	102.2	3.3	89.2	2.0	100.5	4.1	107.0	3.1	25.1	-62.2
o.p DDE	MT	0.4	1.3	0.991	88.9	2.0	86.8	8.0	87.6	2.6	87.6	2.1	87.6	3.5	94.2	3.1	27.8	61
o.p DDT	IN	2.5	8.3	0.997	109.6	4.6	92.6	5.2	103.1	10.1	97.6	17.2	97.7	4.5	109.2	7.1	24.3	-57.1
p.p DDD	MT	1.9	6.3	0.991	110.2	4.7	108.3	6.4	97.4	7.9	101.3	11.2	99.8	11.6	93.3	10.5	23.4	98.3
p.p DDE	MT	0.3	1.1	0.992	85.7	1.7	84.9	7.0	101.7	3.4	89.3	2.0	100.6	4.1	106.2	3.1	21.6	108.9
Parathion ethyl	IN, AC	0.6	1.9	0.994	86.4	2.4	81.5	3.2	100.3	2.5	88.3	2.7	104.7	2.7	106.8	1.6	23.4	-66.2
Tefluthrin	IN	0.7	2.4	0.992	84.2	3.0	83.7	5.1	106.3	4.0	85.8	2.4	106.9	4.3	111.4	3.4	26.5	253.9
Tetrachlorvinphos	IN, AC	0.7	2.2	0.993	89.2	1.7	86.5	3.1	102.3	2.8	91.5	4.4	103.5	3.0	107.8	2.1	18.6	683.7
Tetradifon	IN, AC	2.1	7.0	0.992	82.4	8.5	96.1	6.1	101.8	11.9	74,0	6.0	94.8	13.1	105.0	6.0	36.1	101.5
Tetrasul	IN, AC, NE	0.9	2.9	0.993	118.0	1.4	87.8	5.0	103.0	3.2	117.7	1.5	98.4	3.7	106.8	3.3	24.8	-25.6

* IN: Insecticide, AC: Acaricide, NE: Nematicide and MT: Metabolite

Türkiye Entomoloji Dergisi Yayın İlkeleri

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

1. Dergi, entomoloji ve tarımsal zooloji bilim dallarıyla ilişkili konulara açıktır.
2. Dergide Türkçe veya İngilizce yazılmış orijinal araştırmalar yayımlanır.
3. Yayımlanması istenilen eserlerin kısmen veya tamamen herhangi bir yerde yayınlanmamış veya yayımlanmayacak olması zorunludur.
4. Daha önce Kongre/Sempozyum vs. de sözlü/poster bildiri olarak sunulmuş ancak sadece kısa özet olarak basılmış eserler, dipnotta belirtilmesi koşuşuyla kabul edilir.
5. Lisansüstü tezleri veya TÜBİTAK, DPT, BAP gibi çeşitli kurumlarca desteklenen proje bulgularından kısımlar içeren eserler ilgililerinden gerekli izinler alındıktan sonra hazırlanmalı, ilgi durum dipnotta mutlaka belirtilmelidir.
6. Türkiye veya herhangi bir bölge için, başta karantina listesinde bulunan türler olmak üzere, yeni tür kayıtlarını içeren eserler gönderilmeden önce mutlaka ilgili kurumlara bilgi verilmiş olmalıdır.
7. Dergide yayımlanması istenilen eserler, web sayfasında sunulan “eser başvurusu” bölümünde açıklandığı gibi hazırlanarak, üst yazı, imzalı telif hakları formu ve başvuru ücreti dekontu ile dergi e-posta adresine gönderilmelidir.
8. Yayımlanması istenilen eserler web sayfasında sunulan “örnek makale taslağı” kullanılarak, gereksiz tekrar, şekil ve cetvellerden kaçınılarak, özden uzaklaşmayacak şekilde hazırlanmalı ve 16 sayfadan fazla olmamalıdır.
9. Yayın ilkelerine uygun olmayan eserler istenilen şekle göre yeniden düzenlenmek üzere yazara geri gönderilir. Detaylar için web sayfasında sunulan “eser değerlendirme süreci” ne bakınız.
10. Bir eser yayıma kabul edildiğinde, telif hakları formu tüm yazarlar tarafından imzalanıp dergimize gönderilmeden yayımlanmaz. Sorumlu yazara eserin pdf formatında hazırlanmış hali e-posta ile gönderilir, ayrıca telif ücreti ödenmez. Yayımlanan eserlere ait şekil dışı sorumluluklar yazarlarına aittir.

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