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Original article (Orijinal araştırma)

Determination of the efficacy of some entomopathogenic nematodes on *Spodoptera littoralis* (Boisduval, 1833) (Lepidoptera: Noctuidae) in laboratory conditions[1]

Bazı entomopatojen nematodların *Spodoptera littoralis* (Boisduval, 1833) (Lepidoptera: Noctuidae)'e etkinliklerinin laboratuvar koşullarında belirlenmesi

Esra PARTAL[2*] **Galip KAŞKAVALCI[3]**

Abstract

Spodoptera littoralis (Boisduval, 1833) (Lepidoptera: Noctuidae) is a polyfag pest causing losses all over the world. Entomopathogenic nematodes (EPN) have a regulatory role on insect populations in the soil ecosystem. The aim of this study was to determine the effects of commercial biopreparations of some EPNs on fifth instar larvae of *S. littoralis* at different doses and days. Biopreparations containing *Heterorhabditis bacteriophora* (Poinar, 1975) (Rhabditida: Heterorhabditidae), *Steinernema carpocapsae* (Weiser, 1955) and *Steinernema feltiae* (Filipjev, 1934) (Rhabditida: Steinernematidae) were used in the study. These biopreparations were adjusted at doses of 50, 100, 200 and 400 IJs/5ml of tap water and applied to 50 g of sterilized sandy soil in a plastic container, and one individual of *S. littoralis* larvae was released on it. The study was conducted in 2022 at 27±2°C under laboratory conditions for 5 days. According to the results of the study, the highest average mortality rates were 100 and 95% in the *S. carpocapsae* preparation application at 400 and 200 IJs doses on the 5th day, respectively. In the application of *S. feltiae* preparation, 90% mortality rate on average was observed at 200 and 400 IJs doses on the 5th day. *Heterorhabditis bacteriophora* preparation treatment showed the highest mean mortality rate of 75% at 400 IJs dose and on the 5th day. This study is an acceptable step in determining the possibilities of using EPNs in the control of *S. littoralis*.

Keywords: *Heterorhabditis bacteriophora, Spodoptera littoralis, Steinernema carpocapsae, Steinernema feltiae*

Öz

Pamuk yaprak kurdu, *Spodoptera littoralis* (Boisduval, 1833) (Lepidoptera: Noctuidae) tüm dünyada kayıplara sebep olan polifag bir zararlıdır. Entomopatojen nematodlar (EPN), toprak ekosistemi içinde böcek popülasyonlarını düzenleyici role sahiptirler. Bu çalışmanın amacı, bazı EPN'lerin ticari biyopreparatlarının, farklı dozlarda ve günlerde *S. littoralis*'in beşinci dönem larvaları üzerindeki etkilerinin belirlenmesidir. Çalışmada, *Heterorhabditis bacteriophora* (Poinar, 1975) (Rhabditida: Heterorhabditidae), *Steinernema carpocapsae* (Weiser, 1955) ve *Steinernema feltiae* (Filipjev, 1934) (Rhabditida: Steinernematidae) türlerini içeren biyopreparatlar kullanılmıştır. Bu biyopreparatlar, 50, 100, 200 ve 400 IJs/5ml su dozlarında ayarlanarak, plastik kap içerisindeki 50 g sterilize edilmiş kumlu toprağa verilmiş, üzerine beşinci dönem *S. littoralis* larvalarından birer birey bırakılmıştır. Çalışma, 2022 yılında 27±2°C'de laboratuvar koşullarında 5 gün boyunca kontrolleri yapılarak gerçekleştirilmiştir. Çalışma sonucunda en yüksek ortalama ölüm oranları *S. carpocapsae* preparatı uygulamasında, 5. günde 400 ve 200 IJs dozlarında sırası ile %100 ve 95 olarak görülmüştür. *Steinernerma feltiae* preparatı uygulamasında 5. günde, 200 ve 400 IJs dozunda %90 ortalama ölüm oranı görülmüştür. *Heterorhabditis bacteriophora* preparatı uygulaması ise en yüksek ortalama ölüm oranını, %75 ile 400 IJs dozunda ve 5. günde göstermiştir. Bu çalışma, *S. littoralis* ile mücadelede EPN'lerin kullanım olanaklarının belirlenmesinde kabul edilebilir bir adımdır.

Anahtar sözcükler: *Heterorhabditis bacteriophora, Spodoptera littoralis, Steinernema carpocapsae, Steinernema feltiae*

[1] This article has been drawn up from the first author's Master Science Thesis.

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Introduction

Spodoptera littoralis (Boisduval, 1833) (Lepidoptera: Noctuidae) adults are grey brown with yellow stripes on the forewings. They lay eggs on the back surface of cotton leaves in packets and covered with buff-colored hairs (TAGEM, 2008). Fourth and fifth instar larvae display cannibalism behavior and the last instar larvae descend to the soil and pupate. The pest can be found in cotton from July onwards. *Spodoptera littoralis* starts to cause damage right after hatching. The newly hatched larva first eats the eggshell, then gnaws the lower surface of the leaf and eats the epidermis and leaves it like a membrane. In this case, the leaf looks like a sieve. In later stages, the larva moves to other leaves and feeds and riddles the leaves with holes.

The search for alternative methods to chemical-based insect control began in the 1960s along with the determination of the negative effects of chemical pesticides on the environment, human, and animal health (Altıkat et al., 2009). Bans, strict rules, and sanctions in chemical control have accelerated the transition to biological control and it has become a necessity to conduct agricultural control by considering agro-ecosystem and sustainable agricultural production. This requires prioritizing alternative methods to chemical control, especially cultural measures, and, if necessary, integrating them with each other (TAGEM, 2011). Biological control is the use of parasitoids, predators, pathogens, antagonists, or competitive populations to suppress the pest population, reduce its density and damage level (Waterhouse & Norris, 1987). Therefore, biological control is the basis of integrated pest control (Gaugler et al., 1997).

Nematodes are called "Entomopathogenic Nematodes (EPN)", which are natural enemies of many insect species in the soil ecosystem and have a regulatory role in insect populations. The application of these nematodes as biological control agents in certain habitats provides effective and reliable control of harmful insect species (Adams & Nguyen, 2002). Current studies have focused on 8 families in terms of their potential to be used in biological control of insect pests with nematodes (Kaya & Stock, 1997; Stock, 2005). Nematodes belonging to the families Steinernematidae and Heterorhabditidae have a wide host distribution (Liu et al., 2000).

The third juvenile (J3) stage of EPNs, which is free-living in the soil and can search for and find their host, is called the 'infective juvenile' (IJs) stage, during which the larvae can remain alive in the soil for more than one year (Burnell & Stock, 2000; Koppenhöfer et al., 2000; Susurluk & Ehlers, 2008). Nematodes do not feed and develop during this period (Kaya & Gaugler, 1993). When infective juveniles find a suitable host in the soil, they enter the haemocoel of the insect using the insect's natural openings (stigma, mouth, anus) or in some cases directly through the cuticle (only in *Heterorhabditis*) (Bedding & Molyneux, 1982; Wang & Gaugler, 1998). When the infective juveniles reach the haemolymph of the host, they release the bacteria that they carry into the haemocoel of the host. The bacteria released into the haemocoel of the insect start to multiply rapidly and cause the host to die of septicemia within 48 hours with the extracellular enzymes and toxins they secrete (Hazır et al., 2003). Nematodes belonging to the genus *Heterorhabditis* spp. are mutualistically related to *Photorhabdus* spp. and nematodes belonging to the genus *Steinernema* spp. are mutualistically related to *Xenorhabdus* spp.

Considering that 90% of insect pests in agricultural areas complete at least one biological period in the soil, the host list of EPNs is very large (Klein, 1993). The virulence of nematodes on lepidopterans varies significantly (Mbata & Shapiro-Ilan, 2005).

Within the scope of biological control of *Spodoptera littoralis*, which causes extensive damage in agricultural areas, although there is no EPN-containing biopreparation licensed for this pest in Türkiye, preparations containing *Steinernema carpocapsae* (Weiser, 1955) and *Steinernema feltiae* (Filipjev, 1934) (Rhabditida: Steinernematidae) have been recommended by some companies and *Heterorhabditis bacteriophora* (Poinar, 1975) (Rhabditida: Heterorhabditidae) was found to be highly effective in the literature. In this study, it was aimed to determine the efficacy of preparations containing these species on *S. littoralis* at different doses (50, 100, 200 and 400 IJs) under laboratory conditions of 27±2°C and 50-65% relative humidity.

Materials and Methods

Preparations containing Entomopathogenic nematodes

The biopreparations of *Steinernema carpocapsae* (Ekobioset®), *Steinernema feltiae* (Nematac®), *Heterorhabditis bacteriophora* (Bioteam®) obtained from Bioglobal Company (Türkiye). Entomopathogenic nematode biopreparations used in the experiment were diluted with tap water and adjusted at dose levels of 50, 100, 200 and 400 IJ/5 ml tap water.

Mass rearing of *Spodoptera littoralis* (Boisduval, 1833) individuals

For the artificial food used for feeding *Spodoptera littoralis* larvae, tap water (800 ml), ascorbic acid (4 g), sorbic acid (1.25 g), streptomycin sulfate (1 g), wheat germ (3 g), yeast (35 g), agar (14 g) and beans (266.5 g) were used by modifying the diet formulation of Doğan et al. (2021).

Spodoptera littoralis larvae were mass reared with artificial diet in plastic petri dishes with blotting paper placed inside. The blotting papers were changed, and the diet was refreshed every day until pupation. Pupated *S. littoralis* individuals were transferred to a separate petri dish and the adult individuals were expected to emerge. Cotton wool soaked with 14% honey water was used as adult diet. After the first adult emergence was observed, they were allowed to lay eggs in the area formed with 2 pieces of oviposition paper shaped as a cylinder. The egg clusters laid on the paper were cut and collected every other day. Mass rearing of *S. littoralis* individuals was performed under laboratory conditions at 27±2°C and 50-65% relative humidity.

Mass rearing of *Galleria mellonella* (L., 1758)

In mass rearing of *Galleria mellonella* (L., 1758) (Lepidoptera: Pyralidae) larvae, artificial diet used to include honey (20%), glycerin (20%), wheat bran (20%), maize flour (15%), soya flour (10%), milk powder (10%), yeast (5%). Mass rearing of *G. mellonella* larvae was realized in jars containing food and in incubator at 60±5% relative humidity and 29±2°C. To prevent the larvae from escaping from the culture jars, the tops of the jars were covered with blotting paper, chrome screen wire and metal clamp. After the first adult individual was seen in the jars, filter paper was placed on the jars and the adults were allowed to lay their eggs there. The filter papers on which the eggs were laid were collected and placed in another jar and the eggs were removed.

Experimental design

The biopreparations containing 3 different species of EPN were added separately to plastic containers of 60 mm diameter, 70 mm height and 100 ml volume. The biopreparations were added separately to each container containing 50 g of sterilized sandy soil at 4 different dose levels (50, 100, 200, and 400 IJs/5 ml of tap water). For the control character, only 5 ml of tap water without nematodes was added. One individual of 5th instar *S. littoralis* larvae was placed in each container and placed in the climate chamber (27±2°C) of Ege University, Faculty of Agriculture, Department of Plant Protection in 2022. During five days, the larvae were checked daily, and the dead individuals were removed.

The experiment was set up according to the random plots experimental design with 5 replications repeated 4 times and *S. littoralis* larvae were controlled at 24, 48, 72, 96, and 120 hours. At 24, 48 and, 72 hours, the larvae that were observed to die were taken into the White traps. The individuals whose death was observed at 96 and 120 hours were examined under stereomicroscope and light microscope by disrupting the body integrity with the help of forceps and scalpel, and the presence of EPNs inside were determined. The preparation containing the relevant entomopathogenic nematode species was recorded as the cause of death of individuals in whom the presence of entomopathogenic nematodes was detected.

Re-isolation was conducted to check the activity of the observed EPNs. The activity of the EPNs transferred to the tap water in the traps that is obtained from dead *S. littoralis* larvae was checked on the last stage *G. mellonella* larvae.

Statistical analysis

The data obtained from the experiment were analyzed using IBM SPSS (Version 25.0) statistical software. Biological activity of EPNs was calculated using Abbott (1925)'s formula and square root transformation was applied. Mean mortality rates were compared by analysis of variance (ANOVA) and groups were evaluated according to Duncan's Multiple Range Test ($p<0.05$).

Results

The effects of the biopreparations of three different EPNs (*S. carpocapsae*, *S. feltiae* and *H. bacteriophora*) at 4 different doses ranging from 50 to 400 IJs on *S. littoralis*, which progressed from 5th instar larvae to pupate under the soil were determined at 24, 48, 72, 96, and 120 hours.

Evaluation of the efficacy of entomopathogenic nematode bio-preparation

In all EPN-containing preparations used in the application, mortality increased in direct proportion to the dose from the third day onwards. Only in the *S. feltiae* preparation, the mortality rate at the dose of 200 and 400 IJs on day 5 was the same. In the preparation containing *H. bacteriophora* species, the highest mortality rate of 75% was reached at the highest dose and on the last controlled day. In the preparation containing *S. carpocapsae* EPN, the highest mortality rate was observed on the 5th day (100%) at a dose of 400 IJs. In *S. feltiae* treatment, the highest mortality rate was 90% at 400 IJs dose on day 4 and 5 and 200 IJs dose on day 5 (Figure 1).

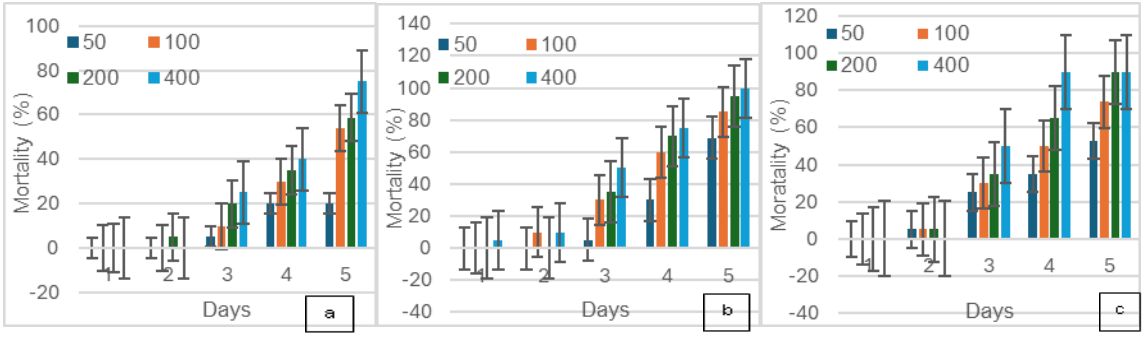

Figure 1. Mortality rates (X±SD) of different species on *Spodoptera littoralis* (Boisduval, 1833) larvae on different days and doses: a) mortality rates of larvae treated with *Heterorhabditis bacteriophora* (Poinar, 1975) (F_{dose}= 0,610; df_{dose}= 3: 16; sig_{dose}= 0,618) (F_{day}= 13,779; df_{day}= 4: 15; sig_{day}= 0,000); b) mortality rates of larvae treated with *Steinernema carpocapsae* (Weiser, 1955) (F_{dose}= 1,056; df_{dose}= 4: 15; sig_{dose}= 0,412) (F_{day}= 27,091; df_{day}= 4: 15; sig_{day}= 0,000); c) mortality rates of larvae treated with *Steinernema feltiae* (Filipjev, 1934) (F_{dose}= 0,375; df_{dose}= 3: 16; sig_{dose}= 0,772) (F_{day}= 23,144; df_{day}= 4: 15; sig_{day}= 0,000).

Evaluation of efficacy in terms of application doses

At the doses used in the application (50, 100, 200, and 400 IJs), the mortality rate increased in direct proportion to the dose, except for the 400 IJs dose of the biopreparation containing *S. feltiae* species from the 3rd day onwards (Figure 2).

At 50 IJs dose, the highest mortality rate was observed on the 5th day in the preparation containing *S. carpocapsae* species with 68.75%. From day 3 onwards, the mortality rates in *S. carpocapsae* and *S. feltiae* treatments increased in direct proportion and the difference between these averages was statistically significant. On day 5, the difference in mortality rates between *S. carpocapsae* and *S. feltiae* was not statistically significant (Figure 2).

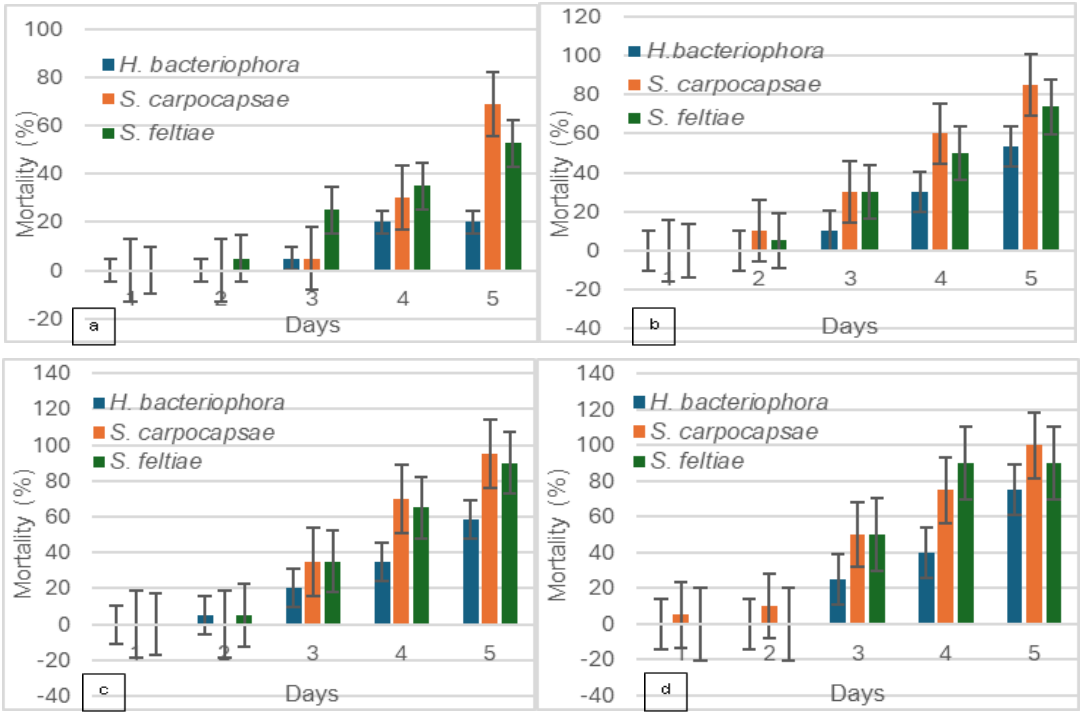

Figure 2. Mortality rates (X±SD) of different doses on *Spodoptera littoralis* (Boisduval, 1833) larvae on different species and days: a) mortality rates of 50 IJs dose ($F_{species}$= 0,616; $df_{species}$= 2: 12; $sig_{species}$= 0,556) (F_{day}= 7,285; df_{day}= 4: 10; sig_{day}= 0,005); b) mortality rates of 100 IJs dose ($F_{species}$= 0,484; $df_{species}$= 2: 12; $sig_{species}$= 0,628) (F_{day}= 20,503; df_{day}= 4: 10; sig_{day}= 0,000); c) mortality rates of 200 IJs dose($F_{species}$= 0,323; $df_{species}$= 2: 12; $sig_{species}$= 0,730) (F_{day}= 22,014; df_{day}= 4: 10; sig_{day}= 0,000); d) mortality rates of 400 IJs dose ($F_{species}$= 0,388; $df_{species}$= 2: 12; $sig_{species}$= 0,687) (F_{day}= 20,930; df_{day}= 4: 10; sig_{day}= 0,000).

At 100 IJs dose, the highest mortality rate was observed on the 5th day in the preparation containing *S. carpocapsae* species with 85%. At 100 IJs dose, the difference between the EPN treatments was not statistically significant in the first 4 days. On day 5, *S. carpocapsae* was the most effective treatment followed by *S. feltiae* (73.75%) and *H. bacteriophora* (53.75%), the difference between these species was statistically significant (Figure 2).

At 200 IJs dose, the highest mortality rate was observed on the 5th day in the preparation containing *S. carpocapsae* species with 95%. This mortality rate was followed by *S. feltiae* with 90% and statistically the same effect was observed. The difference between the average mortality rates observed from day 3 in *H. bacteriophora* and *S. carpocapsae* treatments was statistically significant, while the difference between the mortality rates between day 4 and day 5 in *S. feltiae* treatment was not significant (Figure 2).

At 400 IJs, the highest mortality rate of 100% was observed on day 5 in the preparation containing *S. carpocapsae* species. While the difference in mortality rates after the 2nd day was statistically significant in *H. bacteriophora* and *S. carpocapsae* treatments, there was no difference in the mortality rate of *S. feltiae* since no mortality was observed after the 4th day (Figure 2).

Evaluation of activities in terms of days

In the first 2 days of the application, no mortality in any preparation and at any dose was statistically significant. On the 3rd day of EPN application, *S. carpocapsae* and *S. feltiae* treatments at a dose of 400 IJs killed half of the population *of S. littoralis* individuals with a mortality rate of 50%. At 50 IJs dose, except for the mortality rate in *S. feltiae*, the other doses (100, 200, and 400 IJs) were statistically the same for all species (Figure 3).

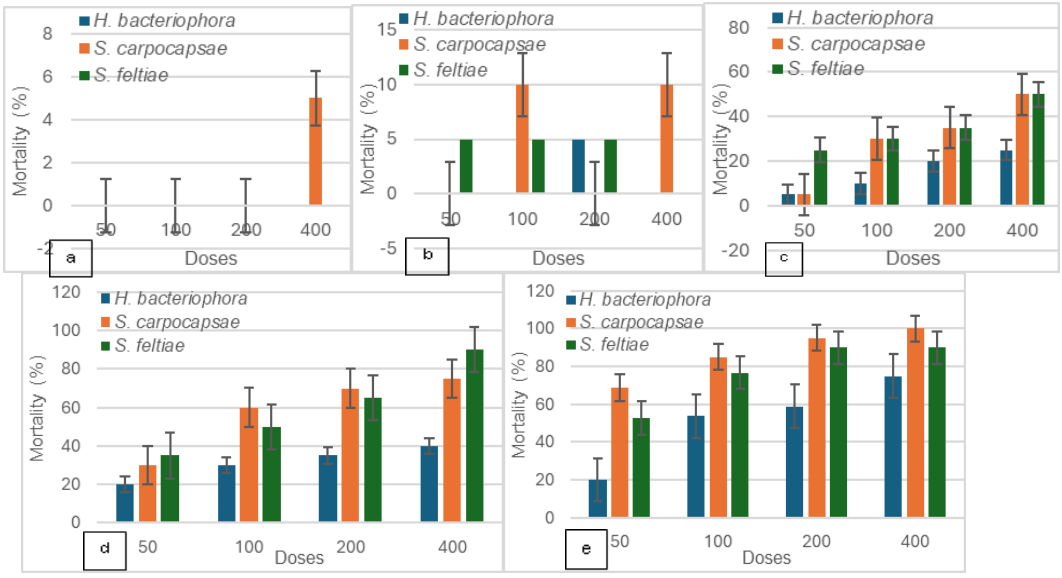

Figure 3. Mortality rates (X±SD) of different days on *Spodoptera littoralis* (Boisduval, 1833) larvae on different species and doses:
a) at the end of the first day ($F_{species}$= 1,000; $df_{species}$= 2: 9; $sig_{species}$= 0,405) (F_{dose}= 1,000; df_{dose}= 3: 8; sig_{dose}= 0,441);
b) at the end of the second day ($F_{species}$= 0,955; $df_{species}$= 2: 9; $sig_{species}$= 0,421) (F_{dose}= 0,296; df_{dose}= 3: 8; sig_{dose}= 0,827);
c) at the end of the third day($F_{species}$= 2,364; $df_{species}$= 2: 9; $sig_{species}$= 0,150) (F_{dose}= 3,434; df_{dose}= 3: 8; sig_{dose}= 0,072);
d) at the end of the fourth day($F_{species}$= 3,079; $df_{species}$= 2: 9; $sig_{species}$= 0,096) (F_{dose}= 2,633; df_{dose}= 3: 8; sig_{dose}= 0,122);
e) at the end of the fifth day ($F_{species}$= 3,862; $df_{species}$= 2: 9; $sig_{species}$= 0,062) (F_{dose}= 2,722; df_{dose}= 3: 8; sig_{dose}= 0,114).

On the 4th day of EPN application, the highest mortality rate belonged to *S. feltiae* treatment with 90% at the highest dose used (400 IJs). *S. carpocapsae* was the second most effective treatment with 75% mortality rate, but these two preparations had statistically the same average mortality rate (Figure 3).

On the last day of the experiment, *S. carpocapsae* had the two highest mortality rates with 100% at 400 IJs dose and 95% at 200 IJs dose. The mortality rates at 100 and 400 IJs are statistically different between the preparations (Figure 3).

Discussion

Cotton leafworm, *S. littoralis*, which can produce 6-7 progeny per year in regions where climatic conditions are suitable for its biology, can cause up to 100% losses when this species is not controlled (Aydın, 2002; Ünlü & Kornoşor, 2003; Hadim & Gürkan, 2007; TAGEM, 2008; Yıldırım & Başpınar, 2008).

With the restriction of pesticides commonly used for chemical control, new control methods are being encouraged and emphasized (Lu et al., 2012; Barzman et al., 2015). Biological control in agriculture is one of the alternatives to chemical control (Stern et al., 1959; Waage & Greathead, 1988; Baker et al., 2020). Entomopathogenic nematodes are among the most important organisms successfully used in biological control (Ehlers, 1996).

The virulence of EPN on lepidopterans varies significantly (Mbata & Shapiro-Ilan, 2005). Studies with EPNs have concentrated on the families Steinernematidae and Heterorhabditidae. Kaya & Gaugler (1993) emphasized the need for more in-depth basic information on the biology of EPNs, including ecology, behavior, and genetics, to help understand the reasons underlying their success and failure as biological control agents. Selection of the most appropriate nematode species and/or strain is important in terms of efficacy and abiotic factors such as soil type, soil temperature and humidity. Appropriate matching of the nematode to the host requires virulence, host finding and ecological factors to be essential before application to the field.

Campos-Herrera et al. (2009) infected *S. littoralis* with *S. feltiae* and *S. carpocapsae* and observed that the mortality rate of *S. littoralis* infected with *S. feltiae* varied between 74-100%, while the mortality rate of individuals infected with *S. carpocapsae* was 71-82%. These studies were deemed worthy of further research on this subject.

Atwa & Hassan (2014) observed the activity of *H. bacteriophora* and *Steinernema glaseri* (Steiner, 1929) (Rhabditida: Steinernematidae) entomopathogenic nematodes on *S. littoralis* and *Temnorhynchus baal* (Reiche & Saulcy, 1856) (Coleoptera: Scarabaeidae) in 50, 100, 200 and 400 IJ doses on filter paper and 50 g soil. In 50 g of soil, *H. bacteriophora* nematode showed a mortality rate of 80-100% and *S. glaseri* nematode showed a mortality rate of 17-92% on *S. littoralis* and it was stated that the infection of EPNs may very highly depending on the experimental conditions.

Acharya et al. (2020a) applied entomopathogenic nematodes at a dose of 250 IJs to all larval stages of the species *Spodoptera frugiperda* (Smith, 1797) (Lepidoptera: Noctuidae) and the 5th and 6th instar larvae of *S. frugiperda* were 100% killed by *Heterorhabditis indica* (Poinar, Karanukar and David, 1992) (Rhabditida: Heterorhabditidae), *Steinernema arenarium* (Artyukhovsky, 1967), *S. carpocapsae* and *Steinernema longicaudum* (Shen & Wang, 1992) (Rhabditida: Steinernematidae) killed 100% of the entomopathogenic nematodes. As a similar result to current study, in this study, *H. bacteriophora* species lagged behind the activities of other species with an average mortality rate of 53%.

Acharya et al. (2020b) conducted a similar study on *Spodoptera litura* (Fabricius, 1775) (Lepidoptera: Noctuidae) with *H. indica*, *H. bacteriophora*, *S. carpocapsae* and *S. longicaudum* testing the activity of entomopathogenic nematodes and found a similar result to the results of current study, with *S. carpocapsae* having an average mortality rate of 100% and *H. bacteriophora* having the lowest mortality rate of 67%.

Yağcı et al. (2022) used *H. bacteriophora*, *S. carpocapsae* and *S. feltiae* species on the late larvae of *S. littoralis* at 4 different doses (0, 250, 500, and 1000 IJs/ml). The mortality rates of larvae were calculated at 48, 72, and 96 hours. The highest mortality rates of fifth instar larvae were found to be caused by *S. carpocapsae* (Tokat-Bakışlı 05) with 100% and *S. feltiae* (Tokat-Emir) with 92.12%, in parallel with our study, while *H. bacteriophora* (11KG) and *H. bacteriophora* (TOK-20) were found to have 87.62 and 49.28% mortality rates, respectively.

In another similar study, Nouh (2022) applied *H. bacteriophora*, *S. glaseri* and *S. carpocapsae* species at doses of 15, 30, 60, 120, and 240 IJs to the 3rd and 5th larval stages of *S. littoralis* and according to the results obtained at the end of the 6th day, the highest mortality rate was reached by *H. bacteriophora* species at 240 IJs dose with 100% in the 5th larval stage, followed by *S. carpocapsae* with 94% mortality rate. Unlike the findings of this study, the results of our study have indicated that at a dose of 200 IJs and at the end of 5 days, *H. bacteriophora* species displayed a mortality rate of 60% while *S. carpocapsae* species showed a mortality rate of 95%.

The results of this study clearly show that 5th instar larvae of *S. littoralis* are highly susceptible to the tested EPNs and that these beneficial organisms can be used as effective biological control agents against *S. littoralis*. All three EPN preparations tested showed different levels of efficacy against *S. littoralis*. In this study, the infectivity and mortality rate of *H. bacteriophora* species entomopathogen nematode was lower than the mortality rate of other EPNs used in this study, which may be due to the fact that symbiotic bacteria (*Xenorhabdus* spp.) may be due to the fact that symbiotic bacteria (*Photorhabdus* spp.) in Heterorhabditidae species EPNs settle in the host more successfully than symbiotic bacteria (*Photorhabdus* spp.) (Atwa & Hassan, 2014). Due to the difference in the host-finding behavior of *H. bacteriophora* in the application of EPNs in the present study, it is thought that the efficacy success may have been lower than other species.

In the current study, differences in mortality rates were observed between the species, and all of the EPNs which were used in the applications under laboratory conditions were highly effective on *S. littoralis*. Yet more studies need to be conducted in greenhouses and open areas by investigating the combined use of EPN species.

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Physiological reactions of some entomopathogenic nematodes to long-term storage

Bazı entomopatojen nematodların uzun süreli depolamaya fizyolojik tepkileri

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Abstract

Entomopathogenic nematodes (EPNs) are commonly used for pest control. Determining the optimal storage duration for EPNs is crucial for their effective utilization. The aim of this study is to determine the efficacy and reproductive capacities of some EPNs stored for different durations. *Heterorhabditis bacteriophora* Poinar, 1976 (Rhabditida: Heterorhabditidae) HBH Hybrid Strain, HBNL, and HB4 isolates, as well as *Steinernema feltiae* Weiser, 1955 (Rhabditida: Steinernematidae) SADIÇ and ST5 isolates, were used in the study. The Infective Juveniles (IJs) stored at 4ºC for 6, 12, 18, and 24 months were assessed for their efficacy and reproductive capacities on last instar larvae of *Galleria mellonella* L., 1758 (Lepidoptera: Pyralidae) at the end of the periods. This study was conducted at Bursa Uludağ University, Plant Protection Department, Nematology Laboratory. The highest mortality rate observed on *G. mellonella* larvae was 86.67% on the *H. bacteriophora* HBH Hybrid Strain stored for 6 months. Similarly, the highest reproductive capacity was determined to be 153 000 IJs/*G. mellonella* larva, also on the *H. bacteriophora* HBH Hybrid Strain stored for 6 months. This study showed significant results in determining the effects of storage durations on the efficacy and reproductive capacity of the EPNs.

Keywords: *Heterorhabditis bacteriophora,* reproductive capacity, *Steinernema feltiae*, storage duration

Öz

Entomopatojen nematodlar (EPN) zararlıların mücadelesinde yaygınlıkla kullanılmaktadır. EPN'lerin özellikle depolama süresinin uzunluğunun belirlenmesi EPN'lerin etkili bir şekilde kullanılması açısından önemlidir. Bu çalışmanın amacı, farklı süreler boyunca depolanmış olan bazı EPN'lerin stok süreleri sonunda etkinlikleri ve üreme kapasitelerinin belirlenmesidir. Çalışmada *Heterorhabditis bacteriophora* Poinar, 1976 (Rhabditida: Heterorhabditidae) HBH Hibrit Irkı, HBNL ve HB4 izolatı ve *Steinernema feltiae* Weiser, 1955 (Rhabditida: Steinernematidae) SADIÇ ve ST5 izolatları kullanılmıştır. 4ºC'de 6, 12, 18 ve 24 ay boyunca inkübe edilmiş olan infektif jüvenillerin (IJs) belirtilen süreler sonunda *Galleria mellonella* L., 1758 (Lepidoptera: Pyralidae) son dönem larvaları üzerinde etkinlikleri ve üreme güçleri belirlenmiştir. Bu çalışma 2024 yılında Bursa Uludağ Üniversitesi, Bitki Koruma Bölümü, Nematoloji Laboratuvarında gerçekleştirilmiştir. Sonuç olarak, *G. mellonella* larvaları üzerinde görülen en yüksek ölüm oranı 6 ay depolanmış olan *H. bacteriophora* HBH Hibrit Irkında %86.67 olarak belirlenmiştir. En yüksek üreme kapasitesi de aynı şekilde 6 ay depolanmış olan *H. bacteriophora* HBH Hibrit Irkında 153 000 IJs/ *G. mellonella* larva şeklinde belirlenmiştir. Bu çalışma, EPN'nin depolanma süresinin EPN etkinliği ve üreme kapasitesi üzerindeki etkilerinin belirlenmesi açısından önemli sonuçlar taşımaktadır.

Anahtar sözcükler: *Heterorhabditis bacteriophora,* üreme kapasitesi, *Steinernema feltiae*, depolama süresi

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Introduction

With the recent regulations implemented by the European Union, restrictions have been imposed on the use of pesticides in areas where agricultural production takes place, due to the toxic effects of pesticides on non-target organisms (Jess et al., 2014; Lechelet et al., 2017; Marchand, 2023; Yang et al., 2024). This situation has brought other control methods such as biological control to the forefront in the potential pest managements (Bale et al., 2008; Ulu et al., 2016; Filgueiras et al., 2023).

Entomopathogenic nematodes (EPNs) are widely used in agricultural fields for pest control within the scope of biological control (Gaugler, 1988; Ehlers, 1996; Campos-Herrera et al., 2012; Lacey et al., 2015; Baker et al., 2020; Ulu & Erdoğan, 2023). These EPNs, belonging to the families Heterorhabditidae and Steinernematidae of the class Secernentea, are endoparasitic organisms that require a host to complete their life cycle and spend almost their entire lives underground in the soil (Kaya & Koppenhöfer, 1996; Ehlers, 2001; Susurluk, 2008; Dillman & Sternberg, 2012). When their life cycle is examined, the biological stages consist of egg, juvenile 1, juvenile 2, 3 juvenile (Infective Juvenile), juvenile 4, and adults. Additionally, only during the Infective Juvenile (IJ) stage, these organisms possess the ability to infect their hosts (Lewis et al., 2006; Shapiro-Ilan et al., 2006; Susurluk & Ehlers, 2008; Koppenhöfer et al., 2020; Dede et al., 2022). They can locate their hosts by following various volatile compounds emitted by either other nematodes or the hosts themselves (Erdogan et al., 2021; Stevens et al., 2023). Upon encountering their hosts, they enter the host organism through natural openings such as the mouth or anus (Kaya & Koppenhöfer, 1996; Ehlers, 2001; Vashisth et al., 2013; Tarasco et al., 2023; Susurluk & Bütüner, 2024). Once inside the host organism, EPNs release gram-negative bacteria belonging to the Enterobacteriaceae family, with which they live symbiotically, into the host. Following this, the host typically succumbs to septicemia and dies approximately 24-72 hours later. Members of the Heterorhabditidae family carry *Photorhabdus* spp. in a dispersed state within their bodies symbiotically, whereas members of the Steinernematidae family are in a symbiotic relationship with *Xenorhabdus* spp. (Gaugler et al., 1992; Ciche et al., 2006; Ulu & Susurluk, 2014; Şahin et al., 2018; Bütüner et al., 2024; Ulu & Susurluk, 2024).

With the determination of the high efficacy of EPNs in pest control, research efforts have become crucial in identifying the conditions required for the mass production and long-term shelf life of EPNs (Ehlers, 2001; Gaugler, 2002; Sharma et al., 2011; Maru et al., 2016). Various factors such as temperature, humidity, heat, and light play significant roles in determining the shelf life of EPNs. Additionally, studies have suggested that the shelf life of EPNs may have an impact on their physiological characteristics, such as their effectiveness and reproductive capacity on hosts (Ulu & Susurluk, 2014; Susurluk & Ulu, 2015; Bütüner et al., 2023; Bütüner & Susurluk, 2023; Ulu, 2023).

The aim of this study is to determine the reproductive capacity and efficacy of different isolates including *Heterorhabditis bacteriophora* Poinar, 1976 (Rhabditida: Heterorhabditidae) HBH Hybrid Strain, HBNL, and HB4 isolates, as well as *Steinernema feltiae* Weiser, 1955 (Rhabditida: Steinernematidae) SADIÇ and ST5 isolates, stored for various durations.

Materials and Methods

Entomopathogenic Nematode Species

In this study, the patented (TPMK Patent No: TR 2013 06141 B) hybrid strain HBH of *H. bacteriophora*, along with two different isolates, HBNL and HB4, and the isolates of *S. feltiae*, SADIÇ and ST5, were used. The hybrid strain and isolates were harvested using last instar larvae (6th instar) of *Galleria mellonella* L., 1758 (Lepidoptera: Pyralidae), then stored at 4ºC for 6, 12, 18, and 24 months until use. Subsequently, the reproductive capacity and efficacy of the EPNs stored for different durations were determined using last instar larvae of *G. mellonella*.

Experimental design

In the study, the hybrid strain and isolates were stored in 250 ml culture containers with filter capped, containing 50 ml of Ringer solution (Ringer, 1882) with a capacity of 1 000±20 IJs. The EPNs were maintained at 4ºC to preserve their viability and activity. Last instar larvae (6th instar) of *G. mellonella* were used to determine the reproductive capacity and efficacy of the EPNs. After placing the larvae into 24-well tissue culture plates at a depth of 3-3.5 cm and a diameter of 1.5-2 cm, the plates were covered with 10% moist alluvial soil and inoculated with EPNs.

Determination of efficacy

After placing the larvae into 24-well tissue culture plates at a depth of 3-3.5 cm and a diameter of 1.5-2 cm, the plates were covered with 10% moist alluvial soil, and EPNs stored at 4ºC for 6, 12, 18, and 24 months were inoculated in the form of 20 IJs. The tissue culture plates were then incubated at 25ºC for 3 days after inoculation to ensure accurate results, with a low dose of IJs determined for inoculation. Subsequently, infected and dead larvae were identified to determine the efficacy of EPNs. Moreover, we carefully dissected the dead larvae to determine whether their death was caused by the EPNs or other factors. This process was repeated three times, with 10 larvae used in each repetition. As a control, newly harvested infective juveniles (IJs), aged 3-4-day, and stored at 4ºC, were utilized.

Determination of reproduction capacity

The EPNs were stored at 4ºC for the specified durations (6, 12, 18, and 24 months), and their reproductive capacities on *G. mellonella* were determined. The research was conducted as follows. Initially, EPNs used in the study were inoculated into 24-well tissue culture plates containing *G. mellonella* larvae at the 6th instar stage. Infected *G. mellonella* larvae were then transferred to a White Trap (White, 1927), and after 10-12 days, their reproductive capacity was determined. The reproductive capacity was evaluated using last instar *G. mellonella* larvae with a length of approximately 1.5-2 cm and an average weight of approximately 280±20 mg. The number of IJs obtained was determined as the total number obtained from these larvae. This process was repeated ten times, with one larva used in each repetition. As a control, newly harvested 3-4-day old IJs kept at 4ºC was used.

Statistical analyses

Efficiency and reproductive capacity were analyzed by analysis of variance (ANOVA) using JMP® Pro 16.0.1 software, followed by Student-T test using the least significant difference (LSD) test ($p < 0.05$) to determine mean differences.

Results

Efficiency of *H. bacteriophora* HBH hybrid strain

The highest mortality rate exhibited by the HBH hybrid strain on *G. mellonella* larvae was observed in larvae treated with IJs stored at 4ºC for 6 months. This value was obtained as 86.67%. These values were determined as 33.33, 43.33, and 73.33%, respectively, in individuals treated with IJs stored at 4ºC for 24, 18, and 12 months. In the control group, this rate was obtained as 96.67%. Statistically significant differences we found among the results (Table 1).

Efficiency of *H. bacteriophora* HBNL Isolate

The HBNL isolate demonstrated the highest mortality rate of 83.33% on *G. mellonella* larvae when treated with IJs stored at 4ºC for 6 months. These values were determined as 26.67, 46.67, and 70%, respectively, in individuals treated with IJs stored at 4ºC for 24, 18, and 12 months. In the control group, this rate was obtained as 93.33%. Statistically significant differences were observed among the results (Table 1).

Efficiency of *H. bacteriophora* HB4 Isolate

The HB4 isolate showed the highest mortality rate of 83.33% on *G. mellonella* larvae when treated with IJs stored at 4ºC for 6 months. These values were determined as 23.33, 40, and 70%, respectively, in individuals treated with IJs stored at 4ºC for 24, 18, and 12 months. In the control group, where IJs were applied to larvae, this rate was obtained as 90%. Statistically significant differences were detected among the results (Table 1).

Efficiency of *S. feltiae* SADIÇ Isolate

The SADIÇ isolate exhibited the highest mortality rate of 73.33% on *G. mellonella* larvae when treated with IJs stored at 4ºC for 6 months. These values were determined as 26.67, 43.33, and 66.67%, respectively, in individuals treated with IJs stored at 4ºC for 24, 18, and 12 months. In the control group, where IJs were applied to larvae, this rate was obtained as 83.33%. Statistically significant differences were found among the results (Table 1).

Efficiency of *S. feltiae* ST5 Isolate

The highest mortality rate observed on *G. mellonella* larvae with the ST5 isolate was determined as 70% in larvae treated with IJs stored at 4ºC for 6 months. These values were determined as 26.67, 40, and 63.33%, respectively, in individuals treated with IJs stored at 4ºC for 24, 18, and 12 months. In the control group, where IJs were applied to larvae, this rate was obtained as 83.33%. Statistically significant differences were seen among the results (Table 1).

Table 1 Mortality of *Galleria mellonella* larvae that were treated with EPN was analyzed separately

EPN Species	Time (Month)	Mortality Rate ± S.E.*	F (df); p
Heterorhabditis bacteriophora HBH Hybrid Strain	Control	96.67 ± 3.33 a	F (4, 10) = 42.18; p <0.0001
	6	86.67 ± 3.33 a	
	12	73.33 ± 3.33 b	
	18	43.33 ± 6.67 c	
	24	33.33 ± 3.33 c	
Heterorhabditis bacteriophora HBNL Isolate	Control	93.33 ± 3.33 a	F (4, 10) = 33.35; p <0.0001
	6	83.33 ± 3.33 ab	
	12	70.00 ± 5.77 b	
	18	46.67 ± 6.67 c	
	24	26.67 ± 3.33 d	
Heterorhabditis bacteriophora HB4 Isolate	Control	90.00 ± 5.77 a	F (4, 10) = 20; p <0.0001
	6	83.33 ± 3.33 ab	
	12	70.00 ± 5.77 b	
	18	40.00 ± 10.00 c	
	24	23.33 ± 3.33 c	
Steinernema feltiae SADIÇ Isolate	Control	83.33 ± 3.33 a	F (4, 10) = 14.20; p =0.0004
	6	73.33 ± 6.67 a	
	12	66.67 ± 8.82 a	
	18	43.33 ± 6.67 b	
	24	26.67 ± 3.33 b	
Steinernema feltiae ST5 Isolate	Control	83.33 ± 3.33 a	F (4, 10) = 15.83; p =0.0003
	6	70.00 ± 5.77 ab	
	12	63.33 ± 8.82 b	
	18	40.00 ± 5.77 c	
	24	26.67 ± 3.33 c	

* Means in columns followed by the same letters are not significantly different.

The reproductive capacity of *H. bacteriophora* HBH hybrid strain

The IJs of the HBH hybrid strain were stored at 4ºC for the specified months. Subsequently, the reproductive capacities of the individuals were evaluated on last instar *G. mellonella* larvae. According to the results obtained, the highest reproductive capacity was observed in individuals stored at 4ºC for 6 months. This value was determined as 146 500 IJs per *G. mellonella* larva. These values were determined as 51 500, 52 500, and 107 000 IJs respectively, for IJs stored at 4ºC for 24, 18, and 12 months. When examining the reproductive capacity of the infective juveniles (IJs) in the control group, which consisted of newly harvested IJs aged 3-4 days and stored at 4ºC, this value was determined as 153 000 IJs. Statistically significant differences were obtained among the results (Table 2).

Table 2 The reproductive capacity of EPNs were obtained from the incubation of IJs at the specified months

EPN Species	Time (Month)	Reproductive Capacity IJs/ *G. mellonella* Larva ± S.E.*	F (df); p
Heterorhabditis bacteriophora HBH Hybrid Strain	Control	153 000 ± 4 027.68 a	F (4, 45) = 78.10; p <0.0001
	6	146 500 ± 4 475.24 a	
	12	107 000 ± 8 793.94 b	
	18	52 500 ± 4 297.93 c	
	24	51 500 ± 4 657.73 c	
Heterorhabditis bacteriophora HBNL Isolate	Control	149 000 ± 4 459.69 a	F (4, 45) = 90.24; p <0.0001
	6	142 500 ± 7 001.98 a	
	12	103 500 ± 5 002.77 b	
	18	51 500 ± 3 655.28 c	
	24	47 500 ± 4 669.64 c	
Heterorhabditis bacteriophora HB4 Isolate	Control	143 500 ± 3 419.71 a	F (4, 45) = 119.24; p <0.0001
	6	118 500 ± 4 412.73 b	
	12	64 500 ± 4 044.89 c	
	18	53 500 ± 5 273.10 c	
	24	36 000 ± 3 480.10 d	
Steinernema feltiae SADIÇ Isolate	Control	137 500 ± 3 670.45 a	F (4, 45) = 145.53; p <0.0001
	6	127 500 ± 6 247.22 a	
	12	69 000 ± 4 000.00 b	
	18	38 000 ± 3 511.88 c	
	24	27 500 ± 2 608.74 c	
Steinernema feltiae ST5 Isolate	Control	142 000 ± 3 091.21 a	F (4, 45) = 148.66; p <0.0001
	6	132 500 ± 4 549.11 a	
	12	79 000 ± 6 046.12 b	
	18	48 000 ± 3 958.11 c	
	24	22 500 ± 2 910.71 d	

* Means in columns followed by the same letters are not significantly different.

The reproductive capacity of *H. bacteriophora* HBNL isolate

According to the results obtained for the individuals of the HBNL isolate, the highest reproductive capacity was observed in individuals stored at 4ºC for 6 months. This value was determined as 142 500 IJs per *G. mellonella* larva. These values were determined as 47 500, 51 500, and 103 500 IJs, respectively, for IJs stored at 4ºC for 24, 18, and 12 months. When the reproductive capacity obtained from the IJs in the control group was examined, this value was determined as 149 000 IJs. Statistically significant differences were obtained among the results (Table 2).

The reproductive capacity of *H. bacteriophora* HB4 isolate

For the individuals of the HB4 isolate, the highest reproductive capacity was observed in individuals stored at 4ºC for 6 months. This value was determined as 118 500 IJs per *G. mellonella* larva. These values were determined as 36 000, 53 500, and 64 500 IJs, respectively, for IJs stored at 4ºC for 24, 18, and 12 months. When the reproductive capacity obtained from the IJs in the control group was examined, this value was determined as 143 500 IJs. Statistically significant differences were obtained among the results (Table 2).

The reproductive capacity of *S. feltiae* SADIÇ isolate

For the individuals of the SADIÇ isolate, the highest reproductive capacity was observed in individuals stored at 4ºC for 6 months. This value was determined as 127 500 IJs per *G. mellonella* larva. These values were determined as 27 500, 38 000, and 69 000 IJs, respectively, for IJs stored at 4ºC for 24, 18, and 12 months. When the reproductive capacity obtained from the IJs in the control group was examined, this value was determined as 137 500 IJs. Statistically significant differences were seen among the results (Table 2).

The reproductive capacity of *S. feltiae* ST5 isolate

For the individuals of the ST5 isolate, the highest reproductive capacity was observed in individuals stored at 4ºC for 6 months. This value was determined as 132 500 IJs per *G. mellonella* larva. These values were determined as 22 500, 48 000, and 79 000 IJs, respectively, for IJs stored at 4ºC for 24, 18, and 12 months. When the reproductive capacity obtained from the IJs in the control group was examined, this value was determined as 142 000 IJs. Statistically significant differences were observed among the results (Table 2).

In this study, the efficacy of *H. bacteriophora* HBH Hybrid Strain, HBNL and HB4 isolates, as well as *S. feltiae* SADIÇ and ST5 isolates, was examined on *G. mellonella* larvae following storage for varying durations. The results revealed a decrease in efficacy of EPNs with prolonged storage periods. For instance, the highest mortality rate exhibited by *H. bacteriophora* HBH Hybrid Strain on *G. mellonella* larvae was observed at 96.67% when applied with IJs stored for 6 months, whereas this rate decreased to 33.33% when applied with IJs stored for 24 months. Furthermore, the reproductive abilities of the specified EPN isolates were determined after storage for the indicated months. Similarly, an increase in storage duration led to a decrease in reproductive capacity for all EPN isolates and the hybrid strain. Likewise, the highest reproductive rate for *H. bacteriophora* HBH Hybrid Strain was determined to be 153 000 individuals in specimens stored for 6 months, whereas this value decreased to 51 500 individuals in specimens stored for 24 months. These findings underscore the potential of the EPN isolates used in the study to maintain their efficacy and reproductive capacities over time.

Discussion

This study tested the effectiveness and reproductive capacity of *H. bacteriophora* HBH Hybrid Strain, HBNL and HB4 isolates, as well as *S. feltiae* SADIÇ and ST5 isolates, on *G. mellonella* larvae after varied storage periods. The findings demonstrated that EPN effectiveness decreased with longer storage.

After being stored under indicated conditions for specified months, the efficacy, and reproductive capacities of EPNs were investigated on *G. mellonella* larvae. Additionally, the use of different isolates in this study further strengthens the findings. For instance, in the study conducted by Boff et al. (2000), isolates belonging to the species *Heterorhabditis megidis* Poinar, Jackson & Klein, 1987 (Rhabditida: Heterorhabditidae) were stored for up to 70 days at different temperatures, followed by an evaluation of the reproductive capacity and efficacy of these isolates. Upon examination of the results, it was observed that an increase in storage duration led to a decrease in the reproductive capacity and efficacy of the isolates. Similarly, in their study, Sharmila & Subramanian (2016) stored isolates belonging to the species of *Heterorhabditis* and *Steinernema* at different temperatures for 100 days, followed by an assessment of the isolates' efficacy on *Corcyra cephalonica* (Stainton, 1866) (Lepidoptera: Pyralidae) larvae. The results indicated a decrease in efficacy with an increase in storage duration. In the study conducted by Katti et al. (2006), isolates belonging to the species *Oscheius* sp. Andrássy, 1976 (Nematoda: Rhabditidae) and *Steinernema thermophilum* Sudershan & Singh, 2000 (Rhabditida: Steinernematidae) were maintained at room temperature for durations ranging from 5 to 150 days. Subsequently, the efficacy of these isolates was assessed on *G. mellonella* and *C. cephalonica* larvae. The results indicated a decrease in efficacy of the isolates with an increase in storage duration. Thus, there is consistency between the results obtained from the present study and those from previous studies.

In another study conducted by Bütüner et al. (2023), various EPN isolates were stored at 4, 15, 25, and 35ºC for 7, 14, and 21 days. Subsequently, their efficacy was determined on *Tenebrio molitor* L., 1758 (Coleoptera: Tenebrionidae). The results indicated a decrease in efficacy of the isolates with an increase in storage duration for each temperature. Similarly, in the study by Akı et al. (2023), isolates belonging to *Heterorhabditis* and *Steinernema* species were stored in distilled water, tap water, and Ringer's solution at different temperatures and durations. Examination of the results revealed a decrease in efficacy of the isolates with an increase in storage duration. Likewise, in the study conducted by Bütüner & Susurluk (2023), different EPN isolates were stored for 7, 14, and 21 days at 15, 25, and 35ºC, and their reproductive capacities were evaluated. The results indicated a decrease in reproductive capacity with an increase in storage duration for all temperatures. Thus, there is consistency between the results of the current study and previous studies.

According to the results obtained from the study, it has been revealed that long-term storage has adverse effects on the efficacy and reproductive capacities of IJs. While studies in this field generally cover different temperatures, this study examines the effects of long-term storage of EPN isolates at the recommended temperature on the efficacy and reproductive capacities of EPNs. Thus, this study will serve as a reference for future studies or ongoing research in this area.

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Original article (Orijinal araştırma)

The response of the pepper with and without *Me1* gene to *Mi-1.2*-virulent *Meloidogyne incognita* (Kofoid & White, 1919) Chitwood, 1949 (Tylenchida: Meloidogynidae) isolates[1]

Me1 geni taşıyan ve taşımayan biberlerin *Mi-1.2*-virülent *Meloidogyne incognita* (Kofoid & White, 1919) Chitwood, 1949 (Tylenchida: Meloidogynidae) izolatlarına tepkisi

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Abstract

Root-knot nematodes are important organisms that infect vegetables. Due to the intense use of *Mi-1.2*, virulent populations that break resistance have become widespread and have become an important factor limiting the use of this gene. *Me1* resistance gene on pepper provides resistance against *Meloidogyne arenaria* (Neal, 1889) Chitwood, 1949 *Meloidogyne incognita* (Kofoid & White, 1919) Chitwood, 1949 and *Meloidogyne javanica* (Treub, 1885) Chitwood, 1949 (Tylenchida: Meloidogynidae) species. However, there is limited information on the effectiveness of the *Me1* gene against *Mi-1.2* virulent populations. Therefore, it is important to know the reaction of pepper cultivars carrying the *Me1* resistance gene against *Mi-1.2* virulent populations. In this study, the response of resistant pepper cultivar MT-01 F1 (bearing *Me1*) and susceptible pepper cultivar Safran F1 against both *Mi-1.2* natural virulent *M. incognita* isolates and *Mi-1.2* selected virulent *M. incognita* isolate was investigated under controlled conditions. This study was conducted in Akdeniz University Faculty of Agriculture Department of Plant Protection Nematology Laboratory in 2021. All isolates caused many egg masses and galls on the resistant tomato cultivar Seval F1 as expected, and the susceptible pepper cultivar Safran F1. Five isolates were found to cause egg masses and gall formation, while the V3 isolate did not multiply on the resistant pepper cultivar MT-01 F1. The results showed that pepper cultivars carrying the *Me1* gene exhibited different responses against *Mi-1.2* virulent isolates.

Keywords: *Capsicum annum,* resistance, RKN, root-knot nematode

Öz

Kök-ur nematodları sebzelerde zarar yapan önemli organizmalardır. *Mi-1.2* geninin yoğun kullanımı nedeniyle, bu genin sağladığı dayanıklılığı kıran virülent popülasyonlar yaygınlaşmış ve bu genin kullanımını sınırlayan önemli bir faktör haline gelmiştir. Biberdeki *Me1* dayanıklılık geni, *Meloidogyne arenaria* (Neal, 1889) Chitwood, 1949; *Meloidogyne incognita* (Kofoid & White, 1919) Chitwood, 1949 ve *Meloidogyne javanica* (Treub, 1885) Chitwood, 1949 (Tylenchida: Meloidogynidae) türlerine koruma sağlamaktadır. Ancak, *Me1* geninin *Mi-1.2* virülent popülasyonlarına karşı performansı hakkında detaylı bilgi bulunmamaktadır. Bu yüzden, *Me1* genini taşıyan biberlerin *Mi-1.2* virülent popülasyonlarına tepkisinin bilinmesi önemlidir. Bu çalışmada, *Mi-1.2* doğal virülent *M. incognita* izolatlarına ve *Mi-1.2* seçilmiş virülent *M. incognita* izolatına karşı *Me1* geni taşıyan dayanıklı biber çeşidi MT-01 F1 ve duyarlı biber çeşidi Safran F1'in tepkisi kontrollü koşullar altında araştırılmıştır. Bu çalışma 2021 yılında Akdeniz Üniversitesi Ziraat Fakültesi Bitki Koruma Bölümü Nematoloji laboratuvarında yürütülmüştür. Tüm izolatlar, beklendiği gibi dayanıklı domates çeşidi Seval F1 ve duyarlı biber çeşidi Safran F1 üzerinde çok sayıda yumurta kümesi ve ur oluşturmuştur. Beş izolatın dayanıklı biber çeşidi MT-01 F1 üzerinde yumurta kümesi ve ur oluşumuna neden olduğu, ancak V3 izolatının çoğalmadığı tespit edilmiştir. *Me1* geni taşıyan biber çeşidinin *Mi-1.2* virülent izolatlarına karşı farklı tepkiler gösterdiği belirlenmiştir.

Anahtar sözcükler: *Capsicum annum,* dayanıklılık, RKN, kök-ur nematodu

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Introduction

Pepper, *Capsicum* spp. (L.) (Solanales: Solanaceae) is an economically important vegetable species. However, Root-knot nematodes (RKNs) cause economic losses in pepper production areas (Talavera-Rubia et al. 2022). One of the most effective control methods against RKNs is using resistant cultivars which prevent nematode feeding and reproduction on plant roots (Lopez-Perez et al., 2006). *Mi-1.2* in tomatoes, which is one of the most commonly used genes to plant, provides resistance against the major RKN species, *Meloidogyne arenaria* (Neal, 1889) Chitwood, 1949, *Meloidogyne incognita* (Kofoid & White, 1919) Chitwood, 1949, *Meloidogyne javanica* (Treub, 1885) Chitwood, 1949 (Tylenchida: Meloidogynidae) (Williamson & Hussey, 1996). However, gene inactivation at high soil temperatures (Dropkin, 1969; Özalp & Devran, 2018) and the ability of *Mi-1.2* virulent populations to break resistance are factors limiting its use (Roberts, 1990). *Mi-1.2* virulent RKN populations are becoming increasingly common. Virulent populations breaking *Mi-1* resistance have been reported in many countries around the world, including the USA (Riggs & Wingstead, 1959), India (Sikora et al., 1973), Senegal (Berthou et al., 1989); Japan (Narabu & Momota, 1992), Tunisia (Eddaoudi et al., 1997), Spain (Ornat et al., 2001), France (Jacquet et al., 2005), Greece (Tzortzakakis et al., 2005), Türkiye (Devran & Söğüt, 2010), Israel (Iberkleid et al., 2014), Brazil (Silva et al., 2019). Therefore, using different resistance genes in fields where *Mi-1.2* virulent populations are detected is recommended. In addition, knowing the reproductive potential of *Mi-1.2* virulent RKNs in vegetables other than tomatoes would also be valuable information for crop rotation.

Many resistance genes against RKNs have been detected in pepper, making it a vegetable species with high economic value that can be used in crop rotation. The *Me* and *N* genes are used in breeding programs against RKNs in pepper. *Me1, Me3, Me7* and *N* genes are effective against *M. arenaria, M. incognita,* and *M. javanica*, which are common species in pepper production areas (Djian-Caporalino et al., 1999, 2001, 2007; Changkwian et al., 2019). In addition, it has been shown that *N, Me1* and *Me3* genes provide resistance against *Meloidogyne haplanaria* Eisenback et al., 2003 (Tylenchida: Meloidogynidae) as well as the three major species (Hajihassani et al., 2019). The *N* gene carrying pepper cultivar Carolina Wonder was recently tested against *Meloidogyne luci* Carneiro et al., 2014 (Tylenchida: Meloidogynidae), and it was found that the *N* gene does not confer resistance to *M. luci* (Özalp et al., 2024). Because of the potential of resistance genes in pepper, it is essential to know the responses of resistance genes against *Mi-1.2* virulent RKN populations. Therefore, studies have been conducted on the responses of some genes conferring resistance to RKNs in pepper against *Mi-1.2* virulent populations (Castagnone-Sereno et al., 1992, 2001; Tzortzakakis & Blok, 2007; Djian-Caporalino et al., 2011; Özalp et al., 2024).

However, to better understand the responses of pepper cultivars carrying the resistance gene against RKNs, further studies and determination of the host status of these peppers against virulent RKN isolates obtained from different locations are required. Therefore, this study aims to investigate the response of a pepper cultivar carrying the *Me1* gene against the selected and natural *Mi-1.2* virulent *M. incognita* isolates from Türkiye.

Materials and Methods

Plant materials

The susceptible pepper cv. Safran F1 and resistant pepper cv. MT-01 F1 carrying the *Me1* gene were used in this study. In addition, the resistant tomato cv. Seval F1, bearing the *Mi-1.2* gene, was used as control in the experiments. Safran F1, MT-01 F1 and Seval F1 seeds were provided from vegetable seed companies, which are Yuksel Seeds and Multi Seeds, Antalya, Türkiye.

Nematode populations

In the study, *Mi-1.2* virulent *M. incognita* isolates (V3, V6, V13, V15, V18 and V30) were used, five of which were naturally *Mi-1.2* virulent, and one (V30) was selected for virulence in the laboratory. The *Mi-1.2* natural virulent isolates were collected from protected greenhouses with sandy soil types growing resistant tomato cultivars in Kepez district of Antalya province of Türkiye. The *Mi-1.2* natural virulent isolates V6 and V18 were used in previous studies (Mıstanoğlu et al., 2020; Sargın & Devran, 2021). Pure cultures were multiplied from a single egg mass. In addition, the *Mi-1.2* selected virulent V30 isolate was obtained from the continuous selection of an avirulent *M. incognita* isolate on a resistant tomato cv. under controlled conditions (Mıstanoğlu, 2020). For this purpose, 1000 J2s avirulent *M. incognita* from the S6 isolate was inoculated onto cv. Seval F1. After the first inoculation, a very few egg masses (2 to 5) were obtained from plant roots. All juveniles obtained from these egg masses were re-inoculated until the population became *Mi-1.2* virulent, which took approximately five generations. By the time the experiment in this paper was set up, the V30 isolate had been multiplied at least 10 times on the resistant tomato.

Culturing of the *Mi-1.2* virulent isolates

The *Mi-1.2* virulent isolates were maintained on resistant tomato cv. Seval F1. For this purpose, the Seval F1 seeds were sown in vials and maintained in a controlled condition at 25±1ºC. Then, for each isolate, tomato seedlings were planted in 250 ml plastic pots. For the multiplication of each isolate, twenty plants were separately inoculated with egg masses of each isolate after 7 days from planting. Plants were cultivated in a controlled growth chamber as in previous studies (Öçal et al., 2018; Özalp & Devran, 2018). Following nematode inoculation, plants were harvested from their pots 60 days later and their roots were carefully washed under running tap water to remove any adhering soil. Subsequently, individual egg masses were meticulously extracted from visibly galled roots using a sterile needle. To promote hatching, these egg masses were then incubated within a sieve apparatus maintained at 25°C room temperature for 24 hours. Subsequently, the hatched J2s were treated using established methodologies outlined in previous studies (Öçal et al., 2018; Özalp & Devran, 2018)

Confirmation of *Mi-1.2* virulent isolates

In order to compare the reproduction of isolates on Seval F1, nematode testing was conducted under controlled conditions. For this purpose, Seval F1 seedlings with the four-leaf stage were transplanted into 250 ml pots using autoclaved sandy soil. After one week, each plant was inoculated with 1000 J2s. Each isolate was inoculated to five plants. Therefore, there were five replicates for each isolate. The experiment was performed as two repeats according to a completely randomized design. The plants were grown in a controlled climate cabin with a temperature of 25±1ºC, a humidity of 60±5%, and 16:8 hours light-dark cycle. Plants were uprooted eight weeks after inoculation.

The testing of pepper plants

The resistant pepper MT-01 F1 which carries the *Me1* gene and susceptible pepper Safran F1 were used in this experiment. For this purpose, both pepper cultivar seeds were sown in vials and maintained in an environment condition at 25±5°C. Each cultivar seedling at the four-leaf stage were transplanted into 250 cc plastic pots containing sterile soil. One week after transplanting the plants, 1000 J2s per plant were inoculated for each isolate. There were five replicates for each combination (isolate x cultivar) according to a completely randomized design. The experiment was repeated two times (10 replications in total). The plants were grown in a growth chamber with a temperature of 24±1°C and humidity of 60±5%, and 16:8 hours light-dark cycle.

Data evaluation

Approximately eight weeks after inoculation, the experiment was finalized, and plants were uprooted. The roots of the uprooted pepper plants were washed, and their root weights were saved. Then, the roots were stained with Pholexine B (Merck) (0.15 g/L) for easier counting of egg masses (Öçal et al., 2018). Egg masses and galls on the roots were counted, and the resistant status of the plants was scored according to the 0-5 index (Hartman & Sasser 1985). Egg mass index and gall index were evaluated according to all root system. The reproduction factor [RF= number of J2 in soil at the final + number of eggs in egg masses (Pf) / initial population density of nematodes (Pi)] value of the *M. İncognita* V3 isolate was calculated to determine the multiplication status in resistant and susceptible peppers (Oostenbrink, 1966; Proite et al., 2008). Egg masses picked from the root systems were submerged in 2% NaOCl for approximately 5 minutes, and then counted under the microscope (Hussey & Barker, 1973). J2s were obtained from soil (100 g) using the Modified Baermann Funnel Method (Hooper, 1986) and counted under the microscope. The RF values of V3 isolate in peppers were calculated as mentioned above.

Data analyses

The differences between the egg mass index, gall index, eggs and galls per gram of fresh roots and RF values were analyzed statistically. Initially, the log10(x+1) transformation was performed for data. Then, an analysis of variance (ANOVA) was applied, and the differences between the means were compared with Tukey multiple comparison test ($p \leq 0.05$) using the SAS (SAS Institute Inc., Cary, NC). The effects of plant variety, nematode isolate, and plant variety*nematode isolate interaction on the number of egg masses and galls per gram of root were analysed by two-way ANOVA. Standard errors are given with the means in the tables.

Results and Discussion

All *Mi-1.2* virulent *M. incognita* isolates used in the tests caused galls, multiplied and produced egg masses on resistant tomato Seval F1. Thus, it was confirmed that all isolates were *Mi-1.2* virulent. However, the number of egg masses and galls on resistant tomatoes caused by some isolates was significantly different ($p \leq 0.05$). V6 isolates caused the most egg masses and galls. Results showed that the virulence levels of isolates were different (Table 1).

Table 1. Mean of egg masses per g root, egg masses index, galls per g root and gall index caused by *Mi-1.2* virulent *Meloidogyne incognita* isolates on resistant Seval F1 tomato cultivar

Isolate	Egg masses/g root		Egg mass index		Galls/g root		Gall index	
V3	62.51 ± 7.43	AB	4.9 ± 0.10	A	75.37 ± 4.54	AB	5.0 ± 0.00	A
V6	76.02 ± 9.41	A	4.9 ± 0.10	A	85.78 ± 9.51	A	5.0 ± 0.00	A
V13	41.21 ± 8.45	BC	4.1 ± 0.20	A	71.67 ± 8.45	ABC	4.8 ± 0.15	A
V15	49.27 ± 5.22	AB	4.6 ± 0.16	A	51.69 ± 3.82	C	4.8 ± 0.13	A
V18	54.17 ± 6.83	AB	4.9 ± 0.15	A	60.41 ± 5.78	ABC	4.9 ± 0.10	A
V30	20.88 ± 2.00	C	4.3 ± 0.22	A	52.79 ± 4.32	BC	5.0 ± 0.00	A

Values within a column followed by a different uppercase letter are significantly different ($p \leq 0,05$) according to Tukey's multiple range test. ± indicates standard error. Untransformed data is presented in the table, all statistical analyses were conducted on the log10(x+1) transformed data.

All *Mi-1.2* virulent *M. incognita* isolates caused galls, multiplied, and reproduced egg masses on susceptible pepper cultivar Safran F1. However, the number of egg masses and galls in susceptible pepper caused by some isolates were significantly different from each other ($p \leq 0.05$). V3 isolate caused the least egg masses and galls. Results showed that the pathogenicity capacity of *Mi-1.2* virulent *M. incognita* isolates was different on susceptible pepper cultivar Safran F1 (Table 2).

Table 2. Mean of egg masses per g root, egg masses index, galls per g root and gall index caused by *Mi-1.2* virulent *Meloidogyne incognita* isolates on Safran F1 pepper cultivar

Isolate	Egg masses/g root		Egg mass index		Galls/g root		Gall index	
V3	10.39 ± 1.99	B	3.4 ± 0.17	A	13.91 ± 2.25	C	3.8 ± 0.15	B
V6	41.80 ± 7.34	A	4.5 ± 0.27	A	45.17 ± 5.66	A	4.9 ± 0.12	A
V13	33.19 ± 7.47	A	4.1 ± 0.31	A	47.38 ± 4.89	A	4.7 ± 0.17	A
V15	18.51 ± 3.77	AB	4.1 ± 0.23	A	19.28 ± 3.53	C	4.1 ± 0.20	AB
V18	17.55 ± 2.87	AB	3.9 ± 0.18	A	19.64 ± 2.38	BC	3.9 ± 0.10	B
V30	22.70 ± 6.15	AB	4.0 ± 0.29	A	35.00 ± 5.09	AB	4.3 ± 0.23	AB

Values within a column followed by a different uppercase letter are significantly different (P≤0,05) according to Tukey's multiple range test. ± indicates standard error. Untransformed data is presented in the table, all statistical analyses were conducted on the log10(x+1) transformed data.

All *Mi-1.2* virulent *M. incognita* isolates caused galls and reproduced egg masses on resistant pepper cultivar MT-01 F1. However, the number of egg masses and galls in resistant pepper caused by some isolates were significantly different from each other (p≤0.05). V3 isolate caused the least egg masses and galls. Results showed that the virulence levels of isolates were different (Table 3).

Table 3. Mean of egg masses per g root, egg masses index, galls per g root and gall index caused by *Mi-1.2* virulent *Meloidogyne incognita* isolates on MT-01 F1 pepper cultivar

Isolate	Egg masses/g root		Egg mass index		Galls/g root		Gall index	
V3	0.35 ± 0.17	C	0.6 ± 0.22	C	0.35 ± 0.17	C	0.6 ± 0.22	B
V6	88.58 ± 16.99	A	5.0 ± 0.00	A	80.44 ± 13.24	A	5.0 ± 0.00	A
V13	56.51 ± 10.51	A	4.8 ± 0.15	A	62.37 ± 5.44	A	4.9 ± 0.11	A
V15	20.72 ± 3.14	B	3.8 ± 0,13	B	25.26 ± 3.31	B	4.0 ± 0.15	A
V18	15.33 ± 2.68	B	3.6 ± 0.16	B	21.42 ± 3.81	B	3.7 ± 0.15	A
V30	12.25 ± 0.90	B	4.0 ± 0.00	AB	23.87 ± 2.09	B	4.5 ± 0.17	A

Values within a column followed by a different uppercase letter are significantly different (p≤0,05) according to Tukey's multiple range test. ± indicates standard error. ± indicates standard error. Untransformed data is presented in the table, all statistical analyses were conducted on the log10(x+1) transformed data.

In the conducted study, it was determined that *Mi-1.2* virulent *M. incognita* isolates showed different virulence on susceptible and resistant pepper carrying *Me1* cultivars. Differences in egg mass and gall numbers were found on susceptible and resistant pepper cultivars. *Mi-1.2* natural virulent V3 caused numerically fewer egg masses and galls on the susceptible pepper Safran F1 than the other isolates (Table 2). V3 isolate had about 75% fewer egg masses/g root than V6, which had the highest egg masses/g root value. Similarly, it produced few egg masses and galls on the resistant pepper cultivar MT-01 F1. It was observed that the gall/g root value of V3 was 83.7% of the value of V18, which is the closest to V3 (Table 3).

Since the *Mi-1.2* virulent *M. incognita* V3 isolate could not reproduce as much as the other isolates on the resistant pepper cultivar, the reproduction factor (RF) was calculated. For this purpose, the resistant pepper cultivar MT-01 F1 and susceptible pepper cultivar Safran F1 were retested, and the RF of the isolate on both varieties were compared. The RF of the V3 isolate was 13.32±4.4 (RF>1) on the susceptible pepper cultivar Safran F1, and 0.19±0.08 (RF<1) on the resistant pepper cultivar MT-01 F1. These results confirmed that the *Mi-1.2* virulent V3 isolate of *M. incognita* did not grow on the resistant pepper cultivar MT-01 F1 and the cultivar was resistant to V3 isolate.

In this study, plant variety (F value= 77.2; df=2; p<0.0001), nematode isolate (F value= 28.4; df=5; p<0.0001), and plant variety-nematode isolate interaction (F value=5,8; df= 10; p<0.0001) were found to have statistically significant effects on the number of galls formed per gram of root. Similarly, plant variety (F value= 26.3; df=2; p<0.0001), nematode isolate (F value= 21.9; df=5; p<0.0001), and plant variety-nematode isolate interaction (F value=6.2; df=10; p<0.0001) were found to have statistically significant effects on the number of egg masses formed per gram of root.

The proliferation of *Mi-1.2* virulent populations has become a major challenge in tomato production areas (Verdejo-Lucas et al., 2012). This situation underscores the importance of investigating the responses of different plants to *Mi-1.2* virulent isolates. The response of different resistance genes in pepper (Castagnone-Sereno et al., 2001; Djian-Caporalino et al., 2011), cucurbit rootstock *Cucumis metuliferus* E. Mey. ex Schrad. (Cucurbitales: Cucurbitaceae) (Exposito et al., 2018) and eggplant rootstock *Solanum torvum* Sw. (Solanales: Solanaceae) (Öçal et al., 2018), against *Mi-1* virulent isolates was investigated. The results show that the reproductive potential of virulent populations in different plants might vary.

The ability of five isolates tested in this study to grow and form galls in peppers carrying the *Me1* resistance gene may limit the potential of using resistant pepper varieties carrying the *Me1* gene instead of tomatoes in crop rotation in areas infected with *Mi-1.2* virulent *M. incognita*. On the other hand, further studies are needed regarding host-nematode interaction to determine why an isolate (V3) used in the study, unlike other isolates, produced many galls and egg masses in susceptible pepper plants but could not reproduce in resistant plants. In addition, testing the V3 isolate on pepper cultivars carrying the *Me1* gene with different genetic backgrounds might contribute to understanding host-nematode interaction. Previous study has indicated that a plant variety's response to different nematode isolates can differ statistically (Özalp et al., 2024). It is also anticipated that a nematode isolate may exhibit varying feeding and reproduction rates on different varieties (Nas et al., 2022). Interestingly, in this study, one of the *Mi-1* virulent isolates reproduced very limitedly in the resistant variety carrying the *Mi-1* gene, while other virulent isolates were able to form a high number of egg masses.

Castagnone-Sereno et al. (1992) reported that *Mi-1.2* virulent RKNs lost their ability to reproduce on susceptible pepper varieties. Similarly, Castagnone-Sereno et al. (2001) observed that the reproduction of *Mi-1.2* virulent populations decreased on susceptible peppers with few exceptions. Tzortzakakis et al. (1999) investigated the responses of *M. javanica* (*Mi-1.2* virulent and avirulent) and *M. incognita* (*Mi-1.2* avirulent) populations on resistant tomato and susceptible pepper varieties. They stated that *M. javanica* populations did not produce on susceptible pepper varieties, but all *M. incognita* populations produced. Tzortzakakis & Blok (2007) examined the response of *Mi-1.2* (a)virulent isolates of *M. incognita* in 10 different pepper cultivars. They found that the avirulent isolate reproduced on all plants, while *Mi-1.2* virulent isolate could not reproduce in any of them. In another study, it was determined that while none of the four *Mi-1.2* virulent *M. javanica* populations developed on susceptible pepper cultivars, one of the two *Mi-1.2* virulent *M. incognita* populations reproduced while the other did not (Tzortzakakis et al., 2016). Similarly, in this study, it was determined that five *Mi-1.2* virulent isolates were able to grow in susceptible pepper. However, V3 isolate grew less on susceptible pepper than other isolates.

Previous studies have reported that the *Me1* gene confers resistance to *Mi-1.2* virulent populations (Castagnone-Sereno et al., 1992, 1996, 2001). On the other hand, in this study, pepper bearing *Me1* gene showed resistance to only one (V3) of six *Mi-1.2* virulent *M. incognita* isolates. Özalp et al. (2024) investigated the response of *Mi-1.2* virulent and avirulent *Meloidogyne* spp. on eight pepper genotypes. As a result of testing, they reported that *Mi-1.2* virulent *M. javanica* isolates did not cause egg masses or galls on roots. However, the same study reported that *Mi-1.2* virulent *M. incognita* caused many egg masses and galls on susceptible peppers. In contrast, no egg masses and galls were observed on resistant peppers carrying *N* gene. In the present study, similar results were obtained on susceptible pepper cultivar. However, the

response of *N* gene to the *Mi-1.2* virulent isolates and the response of the *Me1* investigated in this study to the *Mi-1.2* virulent isolates were not similar. These results showed that there is variability in infection ability and pathogenicity ability within the population of *Mi-1.2* virulent *M. incognita* isolates, depending on the plant variety and whether the plant carries a resistance gene.

Knowing how the resistance provided by the *Me1* gene in pepper will perform against *Mi-1.2* virulent RKNs is particularly important for the effectiveness of the resistance, durability of the resistance, and sustainability of crop rotation. In this study, five isolates were found to cause egg masses and galls, while one isolate did not multiply on the resistant pepper cultivar bearing *Me1* gene. Resistant and susceptible pepper cultivars exhibited different responses against *Mi-1.2* virulent *M. incognita* isolates. It should be investigated whether inoculum density influences the multiplication of *Mi-1.2* virulent isolates on the peppers bearing *Me1* gene in the future studies. The results obtained might contribute to preventing product loss due to RKNs in areas with intensive vegetable production or taking precautions against virulent populations. Since the response of nematodes can vary in resistant plants with different genetic backgrounds, it will be important to investigate the response of pepper varieties with different genotypes carrying the *Me1* gene to *Mi-1* virulent RKNs in future studies for the effective use and performance of resistant varieties.

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Original article (Orijinal araştırma)

Biology of Citrus long-horned beetle, *Anoplophora chinensis* (Forster, 1771) (Coleoptera: Cerambycidae) on hazelnut[1]

Turunçgil uzunantenli böceği, *Anoplophora chinensis* (Forster, 1771) (Coleoptera: Cerambycidae)'in fındık bitkisindeki biyolojisi

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Abstract

Anoplophora chinensis (Forster, 1771) (Coleoptera: Cerambycidae) is an important quarantine pest causing significant economic losses. It was detected in hazelnut orchards in Trabzon province in 2017 and an eradication program continues. In this study, the population level and biology of the pest were determined by rearing it and conducting periodic surveys in hazelnut orchards in Trabzon province Maçka district between 2020 and 2021. It has been revealed that the adult emergence of *A. chinensis* takes place in the last weeks of June and adults are found in nature until the end of August. The oviposition period continued from the end of June to the beginning of September and the larval stage of *A. chinensis* was observed throughout the year. The pupal stage was observed in May and June. With this study, the biology of *A. chinensis* in Türkiye has been revealed in detail for the first time and obtained data guides the eradication and surveillance studies.

Keywords: *Anoplophora chinensis*, *Coryllus avellana,* hazelnut, invasive insects

Öz

Anoplophora chinensis (Forster, 1771) (Coleoptera: Cerambycidae) ekonomik kayıplara neden olan, önemli bir karantina zararlısıdır. 2017 yılında Trabzon ili fındık bahçelerinde saptanmış ve eradikasyon çalışmaları devam etmektedir. Bu çalışmada 2020 ve 2021 yıllarında Trabzon ili Maçka ilçesinde fındık bahçelerinde yürütülen periyodik sürveylerle zararlının popülasyon durumu ve kültüre alınarak biyolojisi ortaya konulmuştur. Ergin çıkışlarının haziran ayının son haftalarında gerçekleştiği ve erginlere doğada ağustos ayının sonlarına kadar rastlandığı, ovipozisyon süresinin haziran ayı sonundan eylül ayı başına kadar devam ettiği belirlenmiştir. *A. chinensis*'in larva dönemlerine yıl boyunca, pupa dönemine ise mayıs ve haziran aylarında rastlanmıştır. Bu çalışma ile Türkiye'de *A. chinensis*'in biyolojisi ilk kez detaylı olarak ortaya konmuş olup, elde edilen veriler eradikasyon ve sürvey çalışmaları için yol gösterici nitelik taşımaktadır.

Anahtar sözcükler: *Anoplophora chinensis, Coryllus avellana*, fındık, istilacı böcekler

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Introduction

Citrus long-horned beetle, *Anoplophora chinensis* (Forster, 1771) (Coleoptera: Cerambycidae) is a polyphagous wood boring invasive insect that its native range is Asia, including China, Korea, Japan with occasional records from Indonesia, Malaysia, Philippines, Taiwan and Vietnam (Haack et al., 2010). Lingafelter & Hoebeke (2002) stated that *Anoplophora malasiaca* (Thomson, 1865) is a synonym of *A. chinensis* due to the similarity of the morphological features of its reproductive system. Besides, because of the significant differences in terms of life cycle, behavior and developmental characteristics of *A. chinensis* and *A. malasiaca* (Makihara, 2007), Fujiwara-Tsujii et al. (2016) describe the Japanese population of *A. chinensis* as *A. malasiaca*.

Wang et al. (1996) report *A. chinensis* as an important pest of fruit and ornamental trees in East and Southeast Asia, especially in China, causing serious damage to citrus trees and causing substantial economic losses. Larvae bore into the trunk and root resulting in the decay and death of the tree (Komazaki & Sakagami, 1989). *A. chinensis* has serious potential to invade other areas of the world through worldwide commerce in woody plants and wood products (Hansen et al., 2015). *A. chinensis* was given as a new record to Turkish invasive alien insect species fauna by Hızal et al. (2015). It was first recorded on *Acer palmatum* Thunberg*, Acer saccharum* Marshall (Sapindales: Aceraceae) and *Salix caprea* L. (Salicales: Salicaceae) trees in a nursey in Istanbul. Following the first record in Istanbul, *A. chinensis* was reported on *Acer negundo* L. (Sapindales: Aceraceae) trees in Antalya in 2016 (Topakçı et al. 2017), later reported on *Acer palmatum purperea* T., *Acer negundo flamingo* L. and *Acer platanoides* L. (Sapindales: Aceraceae) plants imported from China in Bartın province by Yıldız (2017). In the same year Eroğlu et al. (2017) reported *A. chinensis* on *A. palmatum* in Trabzon province. After the detection on ornamental trees in Trabzon, *A. chinensis* began to spread in hazelnut orchards surrounding the first infested area. Recently, this invasive pest was detected in Diyarbakır and Rize provinces in 2021 and 2022, respectively. Eradication programs are underway in Trabzon, Sakarya, Diyarbakır and Rize provinces. In addition to the eradication, determining the biology of *A. chinensis* is a very important data for the control measures. Hızal & Arslangündoğdu (2017) determined the brief life cycle and host plants of *A. chinensis* in Istanbul between 2014-2016. Eroğlu et al. (2017) determined the morphology, brief biology and damage of *A. chinensis* on *A. palmatum* and *A. negundo* plants in laboratory and a nursery. The biology and behaviours of *A. chinensis* on hazelnut *Coryllus avellana* L. (Fagales: Betulaceae) in the field conditions in Türkiye have been determined in detail for the first time in this study.

Materials and Methods

The biology of the Citrus long-horned beetle, *A. chinensis* was studied in hazelnut orchards of the Maçka District in Trabzon province and in the quarantine area established in Hazelnut Research Institute garden in 2020-2021.

Determination of adult stage and population level

Adult longevity, population level and temporal fluctuation of *A. chinensis* adult emergence were followed in hazelnut orchards in the Maçka district in Trabzon province in Türkiye. Surveys were conducted once every two weeks in April-May and once a week as of June in both years. In the hazelnut orchards, the adult emergence holes, sawdust debris caused by the larvae and adults on the trunk and all green parts of 100 hazelnut plants were examined by visual inspection method. Adults were recorded by their sexes in each followed garden every week for determining the population level. The adult stage was determined as the date between the first adult was seen in nature and the date when the last adult was observed in the surveys.

In order to determine the time-dependent variation of adult emergence, suckers of 60 hazelnut ocaks (traditional planting system of hazelnut) corresponding to 0.1 hectar area of different orchards in 2020 and 2021 were removed and the former adult emergence holes were marked with the help of red spray paint.

After the beginning of the adult emergence, new adult emergence holes in this area were determined and recorded weekly and then they were marked with a different color of spray paint. Some properties of adult emergence holes such as diameter, height from the ground level and the diameter of the stem where the emergence hole was found were determined.

Determination of pre-adult stage

Field studies to determine the pre-adult stages of *A. chinensis* were implemented once a month between January and April, once every two weeks between May and September and once a month between October and December in both years. In the studies on the larval and pupal stages, hazelnut branches showing wilting symptoms and sawdust on the ground level were cut with the help of saw or wood motor and larvae/pupae were searched in the galleries. The biological stages found in the galleries were recorded according to the survey date. In order to obtain information about larval stages, the larvae found in the surveys were brought to the laboratory and the larval length and head capsule width were determined by digital caliper.

In order to determine egg stage, T-shaped egg laying scars on the hazelnut branches were investigated by visual inspection method. In addition to the egg stage, oviposition and hatching periods were determined in the quarantine area constructed in the garden of the institute. Adults collected from the field in the first days of adult emergence were brought to the quarantine area in 5 lt plastic containers with ventilation holes. Fresh hazelnut shoots were placed in the containers to feed the adults during transportation. Adults brought to the quarantine area were grouped as 1 female + 1 male and placed in 5 lt plastic containers. Adults were reared with 5-6 hazelnut shoots with a length of 20-25 cm for feeding and a hazelnut branch with a diameter of about 5 cm and a length of 20-25 cm for laying eggs. In order to prevent moisture loss of branches and shoots, sterile perlite of 5 cm height was placed on the bottom of the plastic containers and moistened with distilled water in the controls made every 2 days. The hazelnut branches placed for oviposition were renewed once a week and the shoots were renewed every 2 days. In the controls performed every two days, the viability of the male and female individuals and the number of eggs laid were recorded. The *A. chinensis* eggs were removed from the branches with the help of a scalpel, taken into petri dishes and the dates of oviposition were recorded. To determine the egg stage longevity of *A. chinensis*, they were taken into the incubator (26±1^{o}C, %70 RH) according to their oviposition dates. The eggs were checked daily and hatched eggs were recorded by the hatching date and removed from the petries. Hatching rates of *A. chinensis* eggs were determined according to the oviposition date of the egg. Egg hatching rates were determined by the ratio of the number of hatched eggs to the total number of eggs laid on that date.

Results

Determination of adult stage and population fluctuations

In order to obtain data on the adult stage of *A. chinensis* and monitor the population fluctuations, surveys were conducted weekly in three selected hazelnut orchards in Esiroğlu town of Maçka District (Trabzon, Türkiye) in 2020 (Table 1).

Adult emergence of *A. chinensis* began in mid-June in 2020. It was observed that males were predominant in this period and this rate changed at the end of June. It was determined that the population started to decline from the beginning of August and the adults were last seen on 13 August 2020. According to the survey results, adult longevity was determined 58 days under field conditions.

Adult stage of *A. chinensis* was checked weekly in two orchards in Akmescit and Durali towns of Maçka District in 2021 (Table 2).

Table 1. *Anoplophora chinensis* adult population fluctuation over time in 2020

Survey dates	Location					
	Esiroğlu 1		Esiroğlu 2		Esiroğlu 3	
	♂	♀	♂	♀	♂	♀
16.06.2020	Emergence					
24.06.2020	1	0	6	1	10	3
26.06.2020	0	0	11	3	5	2
30.06.2020	0	0	2	4	8	8
03.07.2020	2	0	9	8	11	13
07.07.2020	4	3	3	1	4	5
16.07.2020	5	9	1	4	9	7
21.07.2020	5	4	2	5	6	4
04.08.2020	6	2	1	0	0	0
13.08.2020	1	0	0	0	0	0
20.08.2020	0	0	0	0	0	0
31.08.2020	0	0	0	0	0	0
Total	24	18	35	26	53	42

Table 2. *Anoplophora chinensis* adult population fluctuation over time in 2021

Survey dates	Location			
	Akmescit		Durali	
	♂	♀	♂	♀
11.06.2021	0	0	0	0
19.06.2021	0	0	0	0
22.06.2021	0	0	0	0
30.06.2021	4	1	0	1
06.07.2021	1	0	1	3
13.07.2021	0	4	1	3
27.07.2021	3	2	1	0
03.08.2021	0	0	0	0
12.08.2021	0	0	0	0
01.09.2021	0	0	0	0
19.09.2021	0	0	0	0
26.09.2021	0	0	0	0
Total	8	7	3	7

In the second year of the study, the first adult emergence took place on 25.06.2021, the last adult was seen on 12.08.2021 and the adult longevity was determined as 49 days in the field conditions.

In order to determine *A. chinensis* adult emergence fluctuations over time, adult emergence holes in an area of 0.1 ha were recorded weekly in one orchard whose population was observed every two years. Weekly fluctuation of adult emergence in 2020 is given in Figure 1 and 2021 is shown in Figure 2.

In 2020, a total of 84 adult emergence holes were found in an area of 0.1 ha where the weekly emergence of *A. chinensis* was followed. In the whole field (0.6 ha) a total of 42 adults (24 males and 18 females) were found during 2020 surveys. Adult emergence increased in the first week of July and continued by a decline until the last week of August.

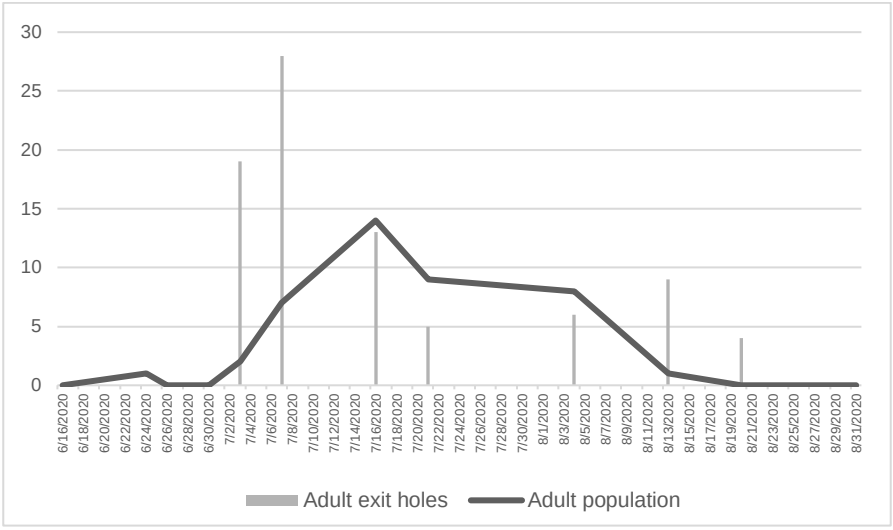

Figure 1. Weekly fluctuations of *Anoplophora chinensis* adult emergence hole and adult population in 2020.

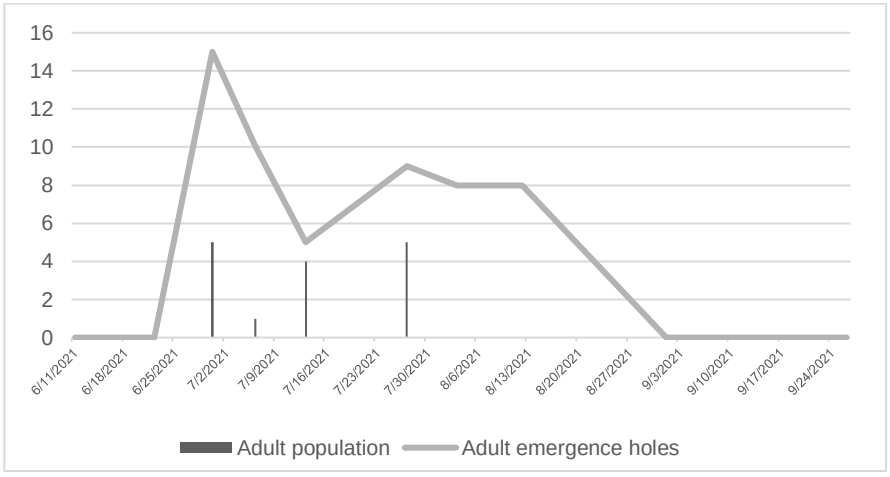

Figure 2. Weekly fluctuations of *Anoplophora chinensis* adult emergence hole and adult population in 2021.

A total of 55 adult emergence holes were found in the 0.1 ha area where the weekly adult emergence of *A. chinensis* were examined in 2021. A total of 15 adults (8 male and 7 female) were found in the whole garden. In 2021, adult emergence increased at the beginning of July and continued until mid-August. When the two-year data are evaluated, it is seen that the adult emergence begins towards the end of June, is highest in the beginning of July and the adult emergence begins to decline from the middle of August. The results obtained show that the population follow-up performed over the adult exit holes gives trusting information about the population of *A. chinensis*.

Determination of pre-adult stage

Surveys to determine the developmental stages of *A. chinensis* were conducted between 2020 and 2021. In the surveys, the larval stage was observed between January and June and both larval and pupal stages were observed in May, June and July. The lengths and head capsule widths of the larvae detected in the surveys were recorded according to the dates they were found (Table 3).

The newly hatched *A. chinensis* larvae are 5-6 mm in length and the width of the head capsule varies between 0.5-1 mm (Figure 3).

Table 3. Body length and head capsule widths of *Anoplophora chinensis* larvae at different dates between 2020 and 2021 (mm)

	Survey date	Length of larvae (mm)	Head capsule width (mm)	Survey date	Length of larvae (mm)	Head capsule width (mm)
1	13.03.2020	35.73	4.55	09.02.2021	55.35	6.23
2	13.03.2020	46.00	5.55	09.02.2021	45.37	5.57
3	22.04.2020	46.80	4.80	09.02.2021	51.03	5.49
4	24.06.2020	50.00	4.60	09.02.2021	40.74	5.40
5	24.06.2020	50.00	4.20	09.02.2021	45.79	5.73
6	24.06.2020	41.00	4.00	09.02.2021	52.38	5.36
7	24.06.2020	45.00	3.90	09.02.2021	48.65	5.30
8	20.08.2020	50.00	4.61	09.02.2021	52.69	5.40
9	04.11.2020	50.00	5.63	09.02.2021	44.66	5.44
10	04.11.2020	50.00	5.45	09.02.2021	50.50	4.74
11	04.11.2020	40.00	4.28	09.02.2021	50.10	4.71
12	04.11.2020	48.00	5.53	22.02.2021	41.35	4.44
13	04.11.2020	43.00	4.53	22.02.2021	54.11	5.32
14	04.11.2020	45.00	4.72	22.02.2021	39.90	5.22
15	04.11.2020	45.00	4.16	22.02.2021	44.14	4.66
16	04.11.2020	45.00	4.70	22.02.2021	49.55	5.03
17	04.11.2020	45.00	4.83	22.02.2021	46.31	5.11
18	04.11.2020	50.00	5.18	22.02.2021	37.80	4.45
19	04.11.2020	43.00	3.90	22.02.2021	27.85	3.21
20	04.11.2020	33.00	2.97	22.02.2021	30.69	3.91
21	04.11.2020	39.00	4.13	22.02.2021	45.04	4.50
22	04.11.2020	40.00	5.08	22.02.2021	39.90	4.87
23	04.11.2020	44.00	4.50	22.02.2021	35.20	4.58
24	04.11.2020	47.00	4.00	22.02.2021	40.11	4.16
25	04.11.2020	40.00	4.48	22.02.2021	39.51	4.24
26	04.11.2020	35.00	3.55	22.02.2021	45.84	5.25
27	04.11.2020	30.00	3.74	22.02.2021	40.59	4.39
28				22.02.2021	49.51	5.67
29				22.02.2021	58.79	5.96
30				22.02.2021	36.57	3.99
31				22.02.2021	54.32	5.69
32				22.02.2021	40.90	4.83
33				22.02.2021	36.95	5.25
34				22.02.2021	39.93	5.57
35				22.02.2021	37.18	3.74
Mean		43.58	4.50		44.27	4.95

Figure 3. Newly hatched *Anoplophora chinensis* larvae.

The maximum length of mature larva found in the galleries was 50 mm and the maximum width of head capsule was determined as 5.63 mm. While *A. chinensis* galleries were mostly found at lower parts of hazelnut stems near soil level, larger larvae were found in galleries that take place in the root parts. It was determined that the first instar initially fed under the bark for a while after hatching and then opened galleries into the wood tissue (Figure 4). The galleries are formed downwards, and mature larvae are usually found in the lower parts of the trunk close to the ground and in the roots.

Figure 4. *Anoplophora chinensis* larvae feed under bark tissue of hazelnut branch and frass.

Oviposition period of *A. chinensis*, egg stage duration and hatching rates of the laid eggs were determined in the quarantine area. The number of eggs laid by 17 *A. chinensis* females reared in 2020, hatching rate and egg stage duration are given in Table 4. Since the eggs are adhered to the tissue with a liquid and compressed between wood and bark tissues, they are damaged while being removed from the branch (Figure 5). For this reason, it is thought that the hatching rate under natural conditions is higher than the rates obtained in Tables 4 & 5.

Figure 5. Eggs of *Anoplophora chinensis* laid under the bark tissue of hazelnut branch and damaged eggs when the bark is removed.

Table 4. Total number of eggs, hatching rate (%) and egg stage duration (days) of 17 pairs of *Anoplophora chinensis* reared in quarantine area in 2020

Oviposition date	Total number of eggs/17 pairs	Hatching rate (%)	Egg stage duration (days)
29.06.2020	3	0	
01.07.2020	12	0	
04.07.2020	66	89.39	8-13
06.07.2020	46	91.30	7-11
08.07.2020	45	73.33	9-16
10.07.2020	41	46.34	7-14
13.07.2020	51	43.13	4-14
17.07.2020	49	18.36	10-12
20.07.2020	38	5.26	4-14
22.07.2020	26	3.84	14
24.07.2020	66	21.21	9-17
27.07.2020	48	14.58	11-14
29.07.2020	28	14.28	14-16
01.08.2020	53	22.64	9-14
03.08.2020	11	0	
05.08.2020	10	0	
07.08.2020	11	0	
10.08.2020	15	0	
12.08.2020	8	0	
14.08.2020	3	0	
17.08.2020	4	0	
19.08.2020	1	0	
21.08.2020	1	0	
26.08.2020	6	0	
28.08.2020	7	0	
04.09.2020	4	0	

In 2020, *A. chinensis* females began to lay eggs on 29.06.2020, and oviposition continued until 4.09.2020. The oviposition period was determined as 66 days. No hatching was observed in the eggs laid at the beginning of oviposition period. The egg stage duration was determined minimum 4 and maximum 17 days. The highest rate of hatching was recorded mostly on the 14th day. The highest egg hatching rate occurred in the first week of July. The eggs laid after the beginning of August have not been hatched.

The total number of eggs laid by 27 *A. chinensis* females reared in 2021, the hatching rate and egg longevity are given in Table 5.

In 2021, the oviposition period of *A. chinensis* began on 02.07.2021, and female individuals continued to lay eggs until 3.09.2021. The oviposition period lasted 63 days. Egg stage duration was determined minimum 5 and maximum 19 days. The highest hatching rate was generally recorded on the 14th day. Hatching rates were generally low and increased in the second week of August.

In the study, aimed to reveal the biology of *A. chinensis* on filbert, as a result of the observations that started in March 2020, only larvae of the pest was recorded in March and April while larvae and pupae were observed in May, and larvae, pupae and adults were observed in June. Adults, eggs and larvae were observed in July and August. Since September, only larvae have been encountered. The studies for 2021 started in January and only the larvae of *A. chinensis* were observed in January-April. In May and June, the larvae and pupae were recorded, as well as the adults began to emerge at the end of June. The adult, egg and larval stages were observed in July and August and the egg period continued until the first week of September. Larvae were recorded until the end of the year (Figure 6).

Table 5. Total number of eggs, hatching rate (%) and egg stage duration (days) of 27 pairs of *Anoplophora chinensis* reared in quarantine area in 2021

Oviposition date	Total number of eggs/27 pairs	Hatching rate (%)	Egg stage duration (days)
02.07.2021	31	35.48	10-17
05.07.2021	58	22.41	12-18
07.07.2021	41	58.54	10-15
10.07.2021	238	49.58	8-20
12.07.2021	78	70.51	7-10
14.07.2021	68	27.94	**5**-11
16.07.2021	31	58.06	9-13
19.07.2021	156	32.69	7-16
21.07.2021	49	30.61	**5**-11
23.07.2021	31	29.03	9-13
26.07.2021	33	6.06	11
28.07.2021	41	12.20	11-16
30.07.2021	34	23.53	9-17
02.08.2021	97	14.43	8-16
04.08.2021	49	10.20	12-14
06.08.2021	29	31.03	10-15
08.08.2021	86	69.77	9-19
11.08.2021	48	77.08	10-14
13.08.2021	14	64.29	10-14
16.08.2021	36	75.00	9-15
18.08.2021	38	44.74	9-13
20.08.2021	15	53.33	10-14
23.08.2021	26	34.62	11-14
25.08.2021	15	20.00	12-16
27.08.2021	4	0.00	0
31.08.2021	23	0.00	0
03.09.2021	4	0.00	0

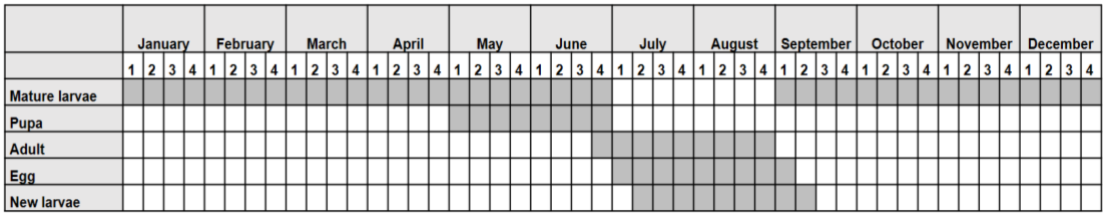

Figure 6. Biological periods of *Anoplophora chinensis* on hazelnut by months.

Discussion

Wang et al. (1996) report that *A. chinensis* gives offspring once a year and the adult stage is seen between April and June in China with reference to Hua (1982). Adachi has studied the biology of *A. malasiaca,* the synonym of *A. chinensis*. Adachi & Korenaga (1989) report that adult emergence begins in late May and lasts until late July and females survive for more than two months in Japan. Adachi (1990) reports that adult emergence starts in the beginning of June, reaches the highest level towards the end of June, and then starts to decline gradually. Iwaizumi et al. (2014) report that adults of *A. malasiaca* are seen between May and October. According to studies based on one-year observations on the biology of *A. chinensis* in Türkiye, Eroğlu et al. (2017) stated that adult emergence of *A. chinensis* starts in the second half of May and most of the adult emergence takes place in June-July in Trabzon. In this study, according to two-year survey results, the first adult emergence took place in June in accordance with Hizal & Arslangündoğdu

(2017) under the conditions of the Eastern Black Sea Region and the most intense adult emergence was observed in July. It was determined by gallery inspections that the pupae in the galleries have turned into adults since the end of May, but the adults did not leave the gallery before the last week of June. Hizal & Arslangündoğdu (2017) report as a result of the surveys they conducted in Istanbul that *A. chinensis* gives offspring once a year and the adults are seen from mid-June to mid-August. Results of this study showed that the adult stage of *A. chinensis* begins at the end of June and proceeds till the end of August in accordance with Eroğlu et al. (2017) in hazelnut orchards. Adult emergence mostly takes place in July and starts to decline as of August.

In the surveys conducted in hazelnut orchards, the adult lifespan of *A. chinensis* was determined as 49 and 58 days respectively between 2020 and 2021. If we look at the studies conducted where this pest is native, Adachi (1988) reports that female individuals of *A. malasiaca* reared on citrus plants survived between 47-109 days. Fujiwara-Tsujii et al. (2016) report the lifespan of *A. malasiaca* females cultured on mandarin, willow, and blueberry, respectively; 117±31 days, 82±19 days, and 32±9.2 days. It is known that the host plant affects the lifespan (Keena, 2002). Keena et al. (2021) showed that *A. chinensis* females from Italy and China, cultured with *Acer rubum* L. (Sapindales: Aceraceae) lived 272.7-5.1 days between 10-40ºC and reported that the survival time increased at low temperatures. Temperature and the origin of the insect were both significantly effective during this period. Considering the lifespan results of the adults obtained in this study, *A. chinensis* adults survive shorter on hazelnut than citrus, mandarin, willow and maple. Apart from the results obtained in this study, there are only observational data on the adult lifespan of *A. chinensis* in Türkiye.

Anoplophora species have obligatory sexual maturation feeding before oviposition. Adachi (1988) reports *A. malasiaca*'s maturation feeding is 10 days and Haack et al. (2010) report that *A. chinensis* and *Anoplophora glabripennis* Motschulsky, 1854 (Coleoptera: Cerambycidae) perform 10-15 days maturation feeding. On the other hand, Maspero (2015) reports this period as 5-7 days for *A. chinensis* and *A. glabripennis*. In the quarantine area, it was determined that the maturation eating period of *A. chinensis* was 4-5 days in both years, closer to Maspero (2015). Out of the results obtained in this study, there is no other information about the maturation feeding period of *A. chinensis* in Türkiye.

Lieu (1945) reports that oviposition period of *A. chinensis* takes place between June and August and according to the different time of oviposition during this period, development level of larvae can be very different before overwintering. It is reported that the larvae complete their development earlier or later in the following year or can not complete until the next year according to the level of development they reach before overwintering, therefore the larvae can be seen throughout the year. In both years of the study oviposition period of *A. chinensis* took place between the beginning of July and the beginning of September under Eastern Black Sea Region conditions. It is seen that adults laid most of the eggs in July and after the beginning of August oviposition declined.

Fujiwara-Tsujii et al. (2016) report that *A. malasiaca* eggs hatch in 7-10 days at 24ºC and 16:8 LD conditions. Ali et al. (2017) report *A. glabripennis* eggs hatch in 10-15 days at 25ºC, %55±8 conditions. Keena et al. (2021) determined that hatching occurred at 15, 20, 25, 30 and 35ºC in 31, 9, 5, 5 and 11 days respectively and 50% and 90% of *A. chinensis* eggs need 125.5 and 165.6 days.degrees to hatch respectively. In this study, at 26ºC and 16:8 LD, 65% humidity conditions, hatching took place between 4 and 20 days and the maximum hatching was recorded on the 14th day. These results are parallel with those of Fujiwara-Tsujii et al. (2016) and Ali et al. (2017).

Adachi (1988) stated that the hatching rate of *A. malasiaca* eggs was generally above 78% and an average of 90%. The most important factor in not hatching is unfertilization due to his observations. Keena et al. (2021) revealed that temperature is effective on hatching rate of *A. chinensis* eggs. Maximum hatching rate was observed respectively for the Italy and China population at 20ºC and 25ºC. In Türkiye there has

not been any study about hatching rate. We recorded maximum hatching 91.30% and 77.08% respectively in 2020 and 2021. As stated by Adachi (1988), unfertilized eggs are thought to play a role in low hatching rates, because eggs were kept at 25±1ºC, 65±5% RH in this study as Keena et al. (2021) revealed that the highest hatching rate of *A. chinensis* was 25ºC. It was observed that eggs of abnormal shape and color were laid on days when the daily temperatures were above the average and these eggs did not hatch.

Haack et al. (2010) report that the larvae of *A. chinensis* are legless, cream-colored and 30-50 mm long in the mature stage. In the surveys conducted, larvae were found between January and December. The maximum width of the head capsule in mature larvae was determined as 5.63 mm and the maximum larval length was determined as 50 mm in consistent with Haack et al. (2010). Head capsule widths of newly hatched larvae ranged between 0.5-1 mm and lengths of larvae varied between 5-6 mm. Keena & Richards (2022) state that head capsule widths of first instar larvae vary between 0.99-1.07 mm in individuals kept at different temperatures. Larval length and head capsule width values obtained from larvae of different stages in hazelnut orchards were found to be compatible with the literature. In this study, *A. chinensis* larvae were found in the surveys every month and detailed research needed about larval stages.

Anoplophora chinensis pupae are whitish and 27-38 mm long (Haack et al., 2010). Pupal stage lasts for 2-3 weeks depending on local temperatures and the color changes to light yellow towards the end of the pupal period (Maspero, 2015). In this study, *A. chinensis* pupae were encountered in May and June, and the length were 30-40 mm long. Shouping et al. (2016) reported that some of the *A. chinensis* individuals from Japan and China populations, kept at constant temperatures of 20-28ºC, could pupate without the need for a chilling period, while both populations could not enter the pupal stage without chilling period according to Keena & Richards (2022) and they thought this probably depend on the content of the food that the larva feeds on. Considering that the larvae were not fed during the last larval stage and that in some previous studies (Adachi 1994, Shouping et al., 2016) *A. chinensis* larvae could enter the pupal stage without chilling, Keena & Richards (2022) state that the temperature is not the only factor controlling the pupal stage. The factors controlling the pupal stage have not been clearly explained yet.

Keena (2005) and Haack et al. (2010) report that *A. chinensis* usually completes a generation within a year, and the larvae must reach a certain weight in order to pupate the next spring, otherwise completes one offspring in two years. In the light of the data obtained, it is seen that *A. chinensis* can complete one generation per year on filbert in the conditions of the Eastern Black Sea Region. The adult period starts at the end of June and continues until the end of August. It is observed that the adult period is shorter on filbert compared to other host plants. In order to decide whether hazelnut is a suitable host for *A. chinensis* as much as citrus or maple, further studies on the reproductive properties of individuals fed on hazelnut plants are needed.

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Original article (Orijinal araştırma)

New and additional records of Cryptinae and Phygadeuontinae (Hymenoptera: Ichneumonidae) Ağrı province and Mount Ararat in Türkiye[1]

Türkiye'de Ağrı ili ve Ağrı Dağı Cryptinae ve Phygadeuontinae (Hymenoptera: Ichneumonidae) için yeni ve ek kayıtlar

Gamze AYHAN[2] **Saliha ÇORUH[2*]**

Abstract

The study was conducted to detect the Cryptinae and Phygadeuontinae (Ichneumonidae) species of Ağrı province and Mount Ararat between 2022 and 2023. Adult samples were collected in the different habitats and altitudes using sweeping nets. The specimens were then pinned and properly labelled for identification. New faunistic data for ten species are provided. *Enclisis vindex* (Tschek, 1871), and *Endasys analis* (Thomson, 1883) are recorded for the first time from Türkiye. Also, present *Gelis vicinus* (Gravenhorst, 1829) and *Phygadeuon hercynicus* Gravenhorst, 1829 are new for East Anatolia. Additionally, collecting locality, date, altitude individual and sex counts, hosts, associated plants, distribution in Türkiye and general geographic distribution of the species are given.

Keywords: Cryptinae, Darwin wasps, Ichneumonidae, new records, Phygadeuontinae

Öz

Çalışma, Ağrı ili ve Ağrı Dağı'ndaki Cryptinae ve Phygadeuontinae (Ichneumonidae) türlerinin tespiti amacıyla 2022 ve 2023 yılları arasında gerçekleştirilmiştir. Yetişkin örnekleri, atrap kullanılarak farklı habitatlarda ve rakımlarda toplanmış, daha sonra iğnelenmiş, ve teşhis çin uygun hale getirilmiştir. On tür için yeni faunistik veriler sağlanmıştır. *Enclisis vindex (*Tschek, 1871) ve *Endasys analis* (Thomson, 1883) Türkiye'den ilk kez kaydedilmiştir. Ayrıca mevcut *Gelis vicinus* (Gravenhorst, 1829) ve *Phygadeuon hercynicus* Gravenhorst, 1829 Doğu Anadolu Bölgesi için yenidir. Ayrıca türlerin toplanma yeri, tarihi, rakım, birey ve cinsiyet sayıları, konukçuları, ilişkili bitkileri, Türkiye'deki yayılışları ve genel coğrafi dağılımları da verilmiştir.

Anahtar sözcükler: Cryptinae, Darwin arıları, Ichneumonidae, yeni kayıtlar, Phygadeuontinae

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Introduction

The Ichneumonidae family, represent the most diverse group within Hymenoptera, with more than 40,000 taxa describes, and more than 25,000 species considered valid as of 2016 (Yu et al., 2016).

Cryptinae and Phygadeuontinae are subfamilies of Ichneumonidae. These two subfamilies are the most diverse subfamily of Ichneumonidae, with nearly 6,000 valid species (Yu et al., 2016) distributed worldwide. Most adults of these subfamilies have been described as idiobiont ectoparasitoids. Among these, their most common hosts are endopterygote pupae or prepupae enclosed in cocoons or plant tissue. Besides, there are some endoparasitic species in the Hedycryptina, Phygadeuontina, and Stilpnina. A few species may be koinobionts. Furthermore, some species parasitize the egg sacs of Pseudoscorpionida and Araneae, and many can develop as secondary parasitoids (Goulet & Huber, 1993).

Until Kolarov, (1995), only 66 species from these subfamilies, belonging to 44 genera have been documented in Türkiye (Sedivy, 1959; Tuatay et al., 1972; Soydanbay, 1976; Düzgüneş et al., 1982; Erkin, 1983; Kolarov, 1989; Öncüer, 1991). At present day, approximately 302 species from these subfamilies are known to occur in Türkiye (Yu et al., 2016; Çoruh et al., 2016, 2018, 2022a, b; Çoruh & Çalmaşur, 2016; Çoruh & Kolarov, 2016; Özdan & Gürbüz, 2016; Sarı & Çoruh, 2018; Çoruh, 2019; Çaylak & Çoruh, 2020; Kıraç & Gürbüz, 2020; Yurtcan et al., 2021; Barik, 2022; Birol, 2022; Doğru, 2022; İnecikli oğlu, 2022; Ataş & Çoruh, 2022; Kaplan & Riedel, 2022; Korkmaz & Çoruh, 2022; Kaplan, 2023, 2024; Barik & Çoruh, 2023).

This study was performed to determine Ichneumonidae from Türkiye (Mount Ararat, and Ağrı province) and evaluate their plant associations and distributions.

Materials and Methods

Data sampling

The specimens were collected from different altitudes from the foothills of Mount Ararat and from different localities in the province of Ağrı during 2022–2023 (Figures 1 & 2). All examined material was collected and photographed by Gamze Ayhan and determined by Dr. Janko Kolarov (Bulgaria) and Dr. Saliha Çoruh. All specimens are deposited in the collection of Department of Entomology, Atatürk University, Erzurum, Türkiye. After identification, each species was photographed by the digital shooting unit (Canon EOS 1100 D, Canon EF 100 mm, f/2.8L Macro lens, Kaiser digital), Lenovo brand computer and Helicon focus 6.7.1. using. New records of species from Türkiye are marked by an asterisk (*). Genera and species are listed in alphabetic order, and nomenclature follows Yu et al. (2016). Also, general distribution of species, hosts and associated plants are given from Yu et al. (2016).

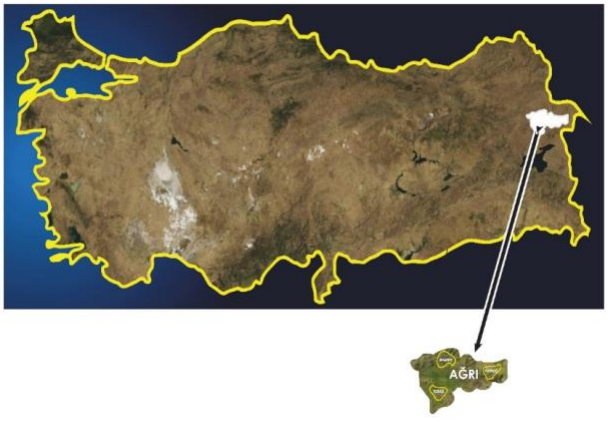


Figure 1. Map of study area.

Figure 2. Pictures of study area.

Study area

Ağrı province is extending from the Eastern Anatolia Region to the Iranian region. It is one of the highest regions in Türkiye because of its mountainous nature. Its old name was Karaköse until 1946, and before that, it was known as Karakilise.

Mount Ararat, with its height of 5137 m, is not only the highest peak in the country, but also the only mountain with a current ice cap of 10 km² (Anonymous, 2023). This Mount is a volcanic mountain made of basalt, which changes to andesite lava around 4000 m. Mount Ararat consists of two peaks. These are the 5137 m Atatürk Peak (Big Ararat) and the 3,898-meter İnönü Peak (Little Ağrı) (Anonymous, 2023).

It has a legendary significance, as is believed to be the final resting place of Noah's Ark. It is also mentioned in the Bible and has many names in different languages Ararat, Kuh-I Nuh and Jebel ul Harist. Mount Ararat has a fascinating appearance with its glaciers, geological formations and always snow-covered mountain meadows.

Results

Here we report 53 specimens belonging to 10 species of Cryptinae and Phygadeuontinae from Ağrı province and Mount Ararat. Among them, namely *Enclisis vindex* (Tschek, 1871), and *Endasys analis* (Thomson, 1883), are new records for the Turkish fauna. With the results presented here, the number of species of Ichneumonidae has reached around 1.460 in Türkiye. All species are new records for Ağrı Province. The provinces where each species is distributed in Türkiye are given in Table 1.

Table 1. Province-level records of Cryptinae and Phygadeuontinae species collected in Ağrı (Central and Mount Ararat), in Türkiye

Names of Taxa	Distributions in Türkiye
CRYPTINAE	
Genus *Cryptus*	
Cryptus viduatorius	Afyonkarahisar, Ağrı, Bilecik, Bitlis, Bolu, Burdur, Bursa, Diyarbakır, Erzurum, Isparta, İstanbul, Kayseri, Kırklareli, Konya, Mersin, Nevşehir, Rize, Sivas, Trabzon, Van
Genus *Enclisis*	
**Enclisis vindex*	New for Türkiye
Genus *Mesostenus*	
Mesostenus transfuga	Adana, Antalya, Aydın, Burdur, Bursa, Diyarbakır, Edirne, Erzurum, Hatay, Isparta, Kırklareli, Mersin, Tekirdağ
Genus *Trychosis*	
Trychosis ambigua	Bingöl, Çanakkale, Diyarbakır
Trychosis legator	Adana, Ankara, Antalya, Bingöl, Burdur, Çanakkale, Diyarbakır, Edirne, Erzurum, Gaziantep, Gümüşhane, Isparta, Istanbul, Kırklareli, Rize, Tekirdağ, Tunceli
Trychosis tristator	Çanakkale, Diyarbakır, Edirne, Isparta, Kırklareli, Tunceli
PHYGADEUONTINAE	
Genus *Blapsidotes*	
Gelis vicinus	Antalya, Burdur, Denizli, Isparta, Kastamonu
Genus *Endasys*	
**Endasys analis*	New for Türkiye
Genus *Mesoleptus*	
Mesoleptus vigilatorius	Erzurum
Genus *Phygadeuon*	
Phygadeuon hercynicus	Bursa

*New for Türkiye.

Cryptinae Kirby, 1837

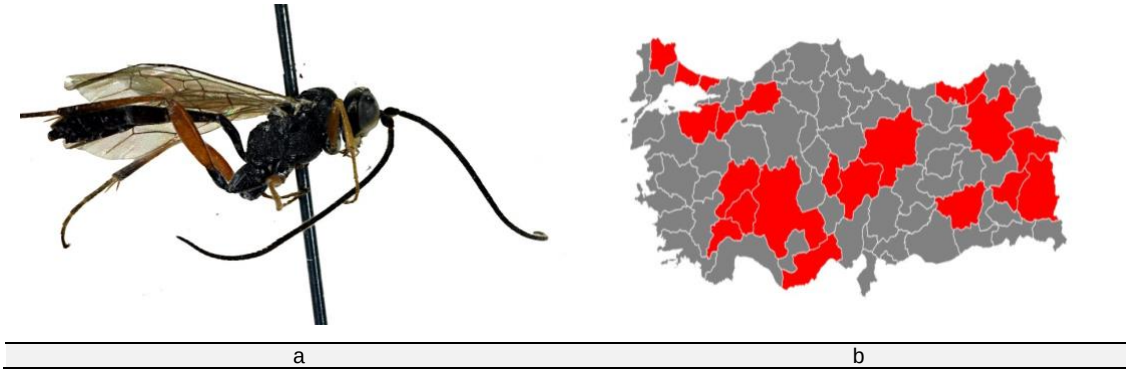
Cryptus viduatorius Fabricius, 1804 (Figure 3a)

Material examined. Ağrı: Hamur, Havalimanı, 39°38’47,69" N, 43°00’36.70" E, 1619 m, 3.VII.2022, ♀; 39°77’45,91" N, 43°11’77,86" E, 1639 m, 23.VII.2023, 2 ♂♂; Hamur, Derekenarı, 39°61’95,14" N, 43°00’34,20" E, 1680 m, 01.VI.2023, 2 ♂♂; Tutak, 39°26’32.68" N, 42°45’55.83" E, 21.VII.2022, 1679 m, 6 ♀♀.

Distribution. Andorra, Armenia, Austria, Azerbaijan, Belarus, Belgium, Bulgaria, Croatia, Cyprus, Czech Republic, Czechoslovakia, Denmark, Finland, France, Georgia, Germany, Greece, Hungary, Iran, Ireland, Italy, Kazakhstan, Kyrgyzstan, Latvia, Lithuania, Macedonia, Moldova, Mongolia, Morocco, Netherlands, Norway, Poland, Romania, Russia, Slovakia, Slovenia, Spain, Sweden, Switzerland, Tajikistan, Turkmenistan, Ukraine, United Kingdom, Uzbekistan, Yugoslavia, Yugoslavia Serbia (Yu et al., 2016); known from Türkiye (Kohl, 1905; Szepligeti,1916; Kolarov, 1995; Beyarslan & Kolarov, 1994; Öncüer, 1991; Kolarov et al., 1997a, 2016; Gürbüz & Kolarov, 2008; Çoruh & Çoruh, 2008; Gürbüz et al., 2009a; Çoruh & Çoruh, 2012; Özdan, 2014; Çoruh et al., 2014a, b, 2016, 2018; Schwarz, 2015; Çoruh & Kolarov, 2016; Özdan & Gürbüz, 2016; Sarı & Çoruh, 2018; Çoruh, 2019; Quicke et al., 2009; Yılmaz, 2020; Barik, 2022; Kaplan & Riedel, 2022; Barik & Çoruh, 2023) (Figure 3b).

Hosts. *Alsophila aescularia* Den. & Sch., 1775 (Lepidoptera: Geometridae), *Loxostege sticticalis* (L., 1761) (Lepidoptera: Crambidae), *Phlogophora meticulosa* (L., 1758) (Lepidoptera: Noctuidae), *Saperda populnea* (L., 1758) (Coleoptera: Cerambycidae), *Sparganothis pilleriana* Den. & Sch., 1775 (Lepidoptera: Tortricidae).

Associate plants. Associate plants. *Anethum graveolens* L. (Apiales: Apiaceae), *Angelica sylvestris* L. (Apiales: Apiaceae), *Daucus carota* L. (Apiales: Apiaceae), *Euphorbia nicaeensis* All. (Malpighiales: Euphorbiaceae), *Euphorbia virgata* Waldst & Kit. (Malpighiales: Euphorbiaceae), *Ferula communis* L. (Apiales: Apiaceae), *Heracleum sphondylium* L. (Apiales: Apiaceae), *Medicago sativa* L. (Fabales: Fabaceae), *Peucedanum oreoselinum* Moench (Apiales: Apiaceae).



a b

Figure 3. *Cryptus viduatorius* Fabricius, 1804: a) Habitus, b) distribution of Türkiye.

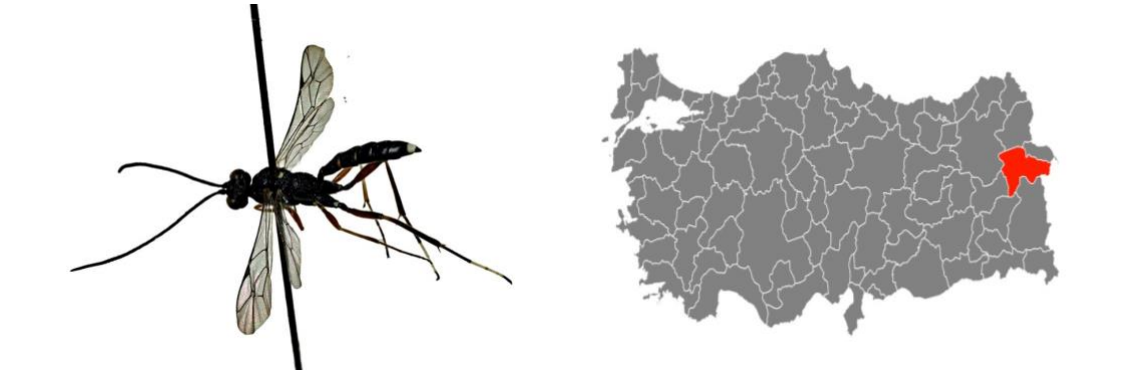
**Enclisis vindex* (Tschek, 1871) (Figure 4a)

Material examined. Ağrı: Mount Ararat, 39°68'23.31" N, 44°08'67.86" E, 1520 m, 23.VII.2023, 3 ♀♀; Hamur, Derekenarı, 39°61'95,14" K, 43°00'34,20" D, 1680, 01.VI.2023, 2 ♂♂.

Distribution. Austria, Belgium, Bulgaria, Czech Republic, Czechoslovakia, Denmark, Finland, France, Germany, Hungary, Italy, Latvia, Lithuania, Luxembourg, Norway, Poland, Romania, Spain, Sweden, Switzerland, United Kingdom, Yugoslavia (Yu et al., 2016); new for Türkiye (Figure 4b).

Hosts. *Diprion simile* (Hartig, 1834) (Hymenoptera: Diprionidae), *Epinotia nanana* (Treit., 1835), (Lepidoptera: Tortricidae), *Schizopleurus balteatus* (De Geer, 1776) (Diptera: Syrphidae), *Thanasimus formicarius* (L., 1758) (Coleoptera: Cleridae).

Associate plants. *Daucus carota*, *H. sphondylium*, *Picea excelsa* (Lam.) Link. (Pinales: Pinaceae).



a b

Figure 4. *Enclisis vindex* (Tschek, 1871): a) Habitus, b) distribution of Türkiye.

Mesostenus transfuga (Gravenhorst, 1829) (Figure 5a)

Material examined. Ağrı: 39°46’29.85" N, 43°07’30.83" E, 1677 m, 28.07.2022, ♀; Hamur, Derekenarı, 40°30’32" N, 41°27’38" E, 1680, 01.VI.2023, 2 ♂♂.

Distribution. Algeria, Austria, Azerbaijan, Azores, Belgium, Bulgaria, Canary Islands, Czech Republic, Czechoslovakia, Egypt, Finland, France, Germany, Greece, Hungary, Iran, Ireland, Israel, Italy Kazakhstan, Latvia, Lithuania, Moldova, Mongolia, Poland, Portugal, Romania, Russia, Spain, Sweden, Switzerland, Turkmenistan, Ukraine, United Kingdom (Yu et al., 2016); known from Türkiye (Soydanbay, 1976; Öncüer, 1991; Kolarov, 1995; Beyarslan & Kolarov, 1994, Kolarov et al., 1997a; Gürbüz & Kolarov, 2008; Gürbüz et al., 2009b; Çoruh & Çoruh, 2008; Çoruh, 2019; Barik, 2022; Kaplan & Riedel, 2022; Barik & Çoruh, 2023) (Figure 5b).

Hosts. *Cydia funebrana* (Treit., 1835) (Lepidoptera: Tortricidae), *Ephestia elutella* (Hübner, 1796), (Lepidoptera: Pyralidae), *Ephestia kuehniella* Zeller, 1879 (Lepidoptera: Pyralidae), *Phyllocolpa leucapsis* (Tisch., 1846) (Hymenoptera: Tenthredinidae), *Pontania gallarum* (Hartig, 1837) (Hymenoptera: Tenthredinidae), *Trichiosoma betuleti* (Klug, 1834) (Hymenoptera: Cimbicidae).

Associate plants. *Euphorbia seguieriana* Neck. (Malpighiales: Euphorbiaceae) *E. virgata*, *Fraxinus excelsior* L. (Lamiales: Oleaceae), *Pimpinella tragium* Vill. (Apiales: Apiaceae), *Seseli libanotis* (L.) W.D.J.Koch (Apiales: Apiaceae).

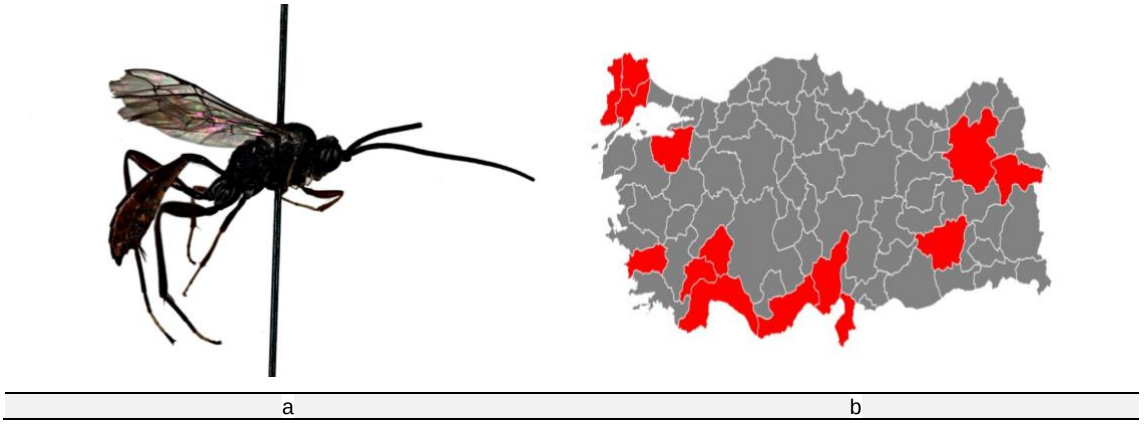


Figure 5. *Mesostenus transfuga* (Gravenhorst, 1829): a) Habitus, b) distribution of Türkiye.

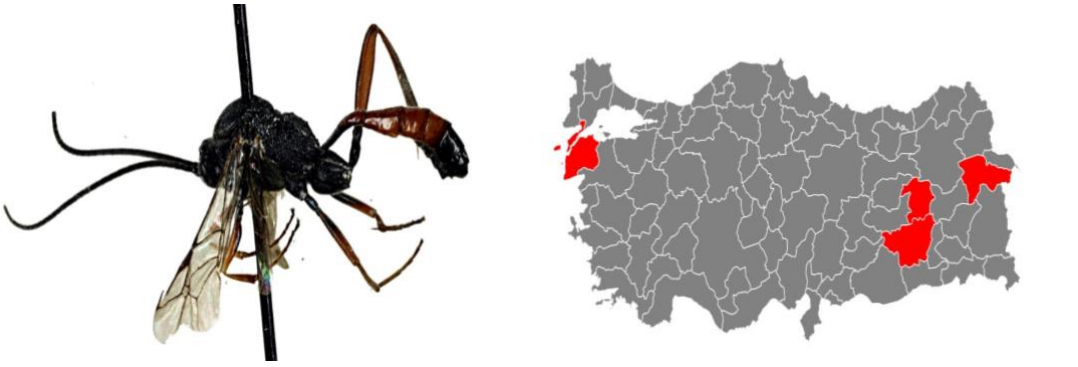
Trychosis ambigua (Tschek, 1871) (Figure 6a).

Material examined. Ağrı: 40°29’40" N, 41°30’07" E, 1249 m, 23.07.2023, 2 ♀♀; Patnos, Derekenarı, 41°31’31" N, 41°32’26" E, 1650 m, 27.VI.2023, 3 ♂♂, 2 ♀♀.

Distribution. Austria, Azerbaijan, Belgium, Bulgaria, Czech Republic, Czechoslovakia, Finland, France, Germany, Hungary, Israel, Moldova, Norway, Poland, Romania, Spain, Sweden, Yugoslavia (Yu et al., 2016); known from Türkiye (Kolarov et al., 1997a, Kaplan, 2023) (Figure 6b).

Associate plants. *Chaerophyllum* sp. L. (Apiales: Apiaceae), *D. carota*, *E. seguieriana*, *H. sphondylium*, *Quercus* spp. L. (Fagales: Fagaceae).

Remark: Fourth exact locality from Türkiye.



a b

Figure 6. *Trychosis ambigua* (Tschek, 1871): a) Habitus, b) distribution of Türkiye.

Trychosis legator (Thunberg, 1822) (Figure 7a).

Material examined. Ağrı: 39°46’29.85" N, 43°07’30.83" E, 1677 m, 28.07.2022, 3 ♂♂, 40°29’40" N, 41°30’07" E, 1249 m, 23.07.2023, 2 ♂♂; Tutak, 39°26’32.68" N, 42°45’55.83" E, 1679 m, 24.VIII.2022, 3 ♀♀.

Distribution. Albania, Algeria, Austria, Belgium, Bulgaria, Canary Islands, Croatia, Czech Republic, Czechoslovakia, Denmark, Finland, France, Germany, Greece, Hungary, Iran, Iraq, Ireland, Israel, Italy, Korea, Latvia, Lithuania, Macedonia, Madeira Islands, Moldova, Netherlands, Norway, Poland, Romania, Russia, Slovenia, Spain, Sweden, Switzerland, Tajikistan, Tunisia, United Kingdom, Yugoslavia (Yu et al., 2016); known from Türkiye (Öncüer, 1991; Beyarslan & Kolarov, 1994; Kolarov, 1995; Kolarov et al., 1997b, 2014; Gürbüz & Kolarov, 2008; Çoruh et al., 2014b, 2016; Çoruh, 2019; Barik, 2022; Kaplan & Riedel, 2022; Barik & Çoruh, 2023) (Figure 7b).

Hosts. *Eupithecia tantillaria* Boisduval, 1840 (Lepidoptera: Geometridae), *L. sticticalis*, *Xysticus cristatus* (Clerck, 1757) (Araneae:Thomisidae)

Associate plants. *Anethum graveolens*, *Chaerophyllum bulbosum* L. (Apiales: Apiaceae), *Cornus sanguinea* L. (Cornales: Cornaceae), *D. carota*, *Euphorbia cyparissias* L. (Malpighiales: Euphorbiaceae), *E. nicaeensis*, *E. seguieriana*, *E. virgata*, *F. excelsior*, *H. sphondylium*, *Pastinaca* spp. Dekay (Apiales: Apiaceae), *P. oreoselinum*, *Quercus* spp.

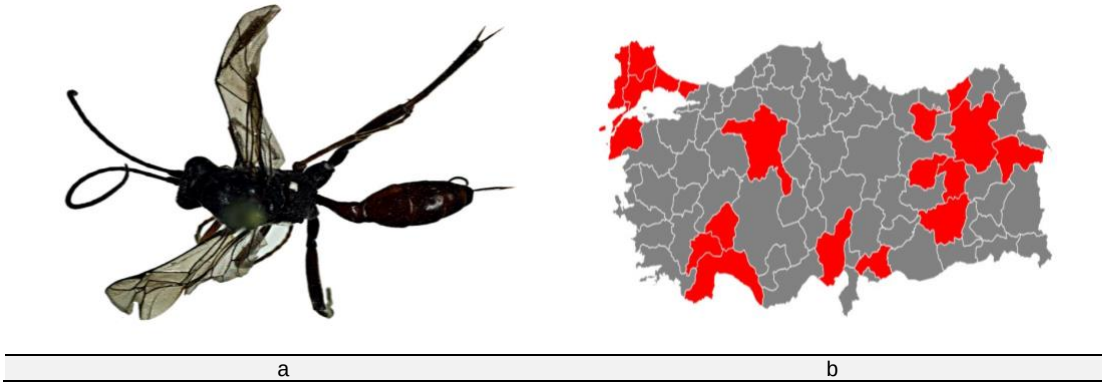


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Figure 7. *Trychosis legator* (Thunberg, 1822): a) Habitus, b) Distribution of Türkiye.

Trychosis tristator (Tschek, 1871) (Figure 8a).

Material examined. Ağrı: 40°29’40" N, 41°30’07" E, 1249 m, 23.VII.2023, 2 ♂♂; Mount Ararat, 39°39’13.39" N, 43°0’53.98" E, 1613 m, 03.VII.2022, ♂; Patnos, Derekenarı, 41°31’31" N, 41°32’26" E, 1650 m, 27.VI.2023, 2 ♂♂, ♀.

Distribution. Austria, Azerbaijan, Belarus, Belgium, Bulgaria, Croatia, Czech Republic, Czechoslovakia, Denmark, Finland, France, Germany, Greece, Hungary, Lithuania, Moldova, Netherlands, Norway, Poland, Romania, Spain, Sweden, Switzerland, United Kingdom (Yu et al., 2016); known from Türkiye (Beyarslan & Kolarov, 1994; Kolarov et al., 1997b, 2014; Gürbüz & Kolarov, 2008; Çoruh et al., 2014b; Çoruh, 2019; Kaplan & Riedel, 2022) (Figure 8b).

Hosts. *Pisaura mirabilis* (Clerck, 1757) (Araneae: Pisauridae), *Tibellus oblongus* (Walckenaer, 1802), (Araneae: Philodromidae), *X. cristatus*, *Xysticus ulmi* (Hahn, 1831) (Arachnida: Thomisidae).

Associate plants. *Daucus carota*, *E. nicaeensis*, *E. seguieriana*, *H. sphondylium*, *Prunus cerasifera* Ehrh. (Rosales: Rosaceae).

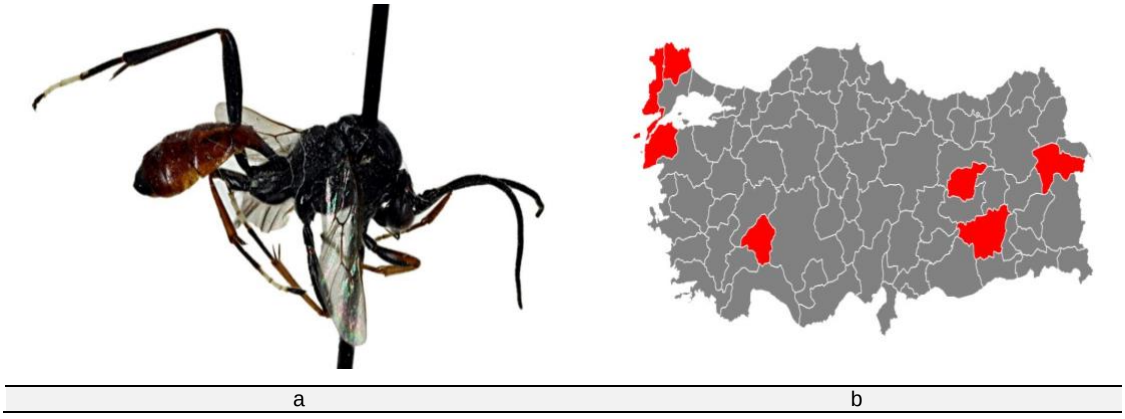

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Figure 8. *Trychosis tristator* (Tschek, 1871): a) Habitus, b) Distribution of Türkiye.

Phygadeuontinae Förster, 1869

Gelis vicinus (Gravenhorst, 1829) (Figure 9a)

Material examined. Ağrı: Patnos, Derekenarı, 39°17’04,35" N, 42°47’14,20" E, 27.VI.2023, 1650 m, 3 ♂♂, 2 ♀♀.

Distribution. Austria, Bulgaria, Croatia, Czech Republic, Czechoslovakia, Denmark, Finland, France, Germany, Hungary, Iran, Ireland, Latvia, Macedonia, Netherlands, Norway, Poland, Romania, Russia, Spain, Sweden, Switzerland, United Kingdom, Yugoslavia (Yu et al., 2016); known from Türkiye (Kolarov & Gürbüz, 2007; Kolarov & Yurtcan, 2008; Çoruh, 2019; Kıraç, 2012, Kıraç & Gürbüz, 2020) (Figure 9b).

Hosts. *Aglais urticae* (L., 1758) (Lepidoptera: Nymphalidae), *Aporia crataegi* (L., 1758) (Lepidoptera: Lycaenidae), *Argynnis paphia* (L., 1758) (Lepidoptera: Nymphalidae), *Blastophagus piniperda* (L., 1758) (Coleoptera: Scolytidae), *Coleophora hemerobiella* (Scop., 1763), *Coleophora valesianella* Zeller, 1849 (Lepidoptera: Coleophoridae), *Cotesia glomerata* L. 1758 (Hymenoptera: Braconidae), *Cynips collari* Hartig, 1843 (Hymenoptera: Cynipidae), *Exoteleia dodecella* (L., 1758) (Lepidoptera: Gelechiidae), *Hylurgus ligniperda* (J.C. Fabricius, 1787) (Curculionidae: Scolytinae), *Inachis io* (L., 1758) Lepidoptera: Nymphalidae), *Issoria lathonia* (L., 1758) (Lepidoptera: Nymphalidae), *Lobesia botrana* (Den. & Schif., 1775) (Lepidoptera: Tortricidae), *Magdalis phlegmatica* (Herbst, 1797), *Magdalis ruficornis* (L., 1758), *Magdalis violacea* (L., 1758) (Coleoptera: Curculionidae), *Microgaster subcompleta* Nees, 1834 (Hymenoptera: Braconidae),

Nymphalis antiopa (L., 1758) (Lepidoptera: Nymphalidae), *Pieris brassicae* L., 1758, *Pieris napi* (L., 1758), *Pieris rapae* L. 1758, (Lepidoptera: Pieridae), *Pissodes notatus* (De Geer, 1775) (Coleoptera, Curculionidae), *Pogonocherus fasciculatus* (De Geer, 1775) (Coleoptera: Cerambycidae), *Polygonia c-album* (L., 1758), (Lepidoptera: Nymphalidae), *S. populnea*, *Scolytus scotylus* (F., 1775) (Coleoptera: Curculionidae), *Taleporia triquetrella* (Fischer von Röslerstamm, 1837) (Lepidoptera: Psychidae), *Vanessa atalanta* (L., 1758) (Lepidoptera: Nymphalidae), *Zygaena lonicerae* (Scheven, 1777) (Lepidoptera: Zygaenida).

Remark: This species is new for East Anatolia.

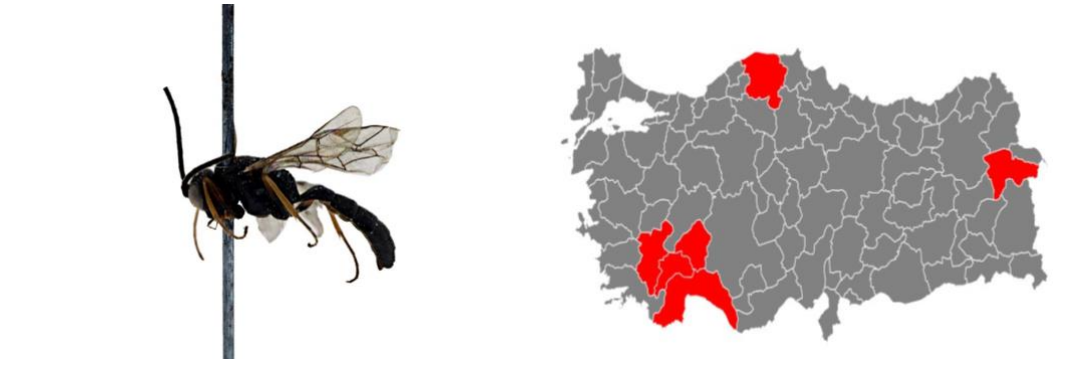

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Figure 9. *Blapsidotes vicinus* (Gravenhorst, 1829): a) Habitus, b) distribution of Türkiye.

**Endasys analis* (Thomson, 1883) (Figure 10a)

Material examined. Ağrı: 39°77’45,91" N, 43°11’77,86" K, 1639 m, 23.VII.2023, ♂.

Distribution. Austria, Azerbaijan, Bulgaria, Czechoslovakia, Denmark, Finland, France, Germany, Hungary, Latvia, Lithuania, Norway, Poland, Romania, Russia, Sweden (Yu et al., 2016); new for Türkiye (Figure 10b).

Host. *Pristiphora abietina* (Christ, 1791) (Hyrnenoptera: Tenthredinidae).

Associate plant. *Listera ovata* (L.) R.Br. (Asparagales: Orchidaceae).

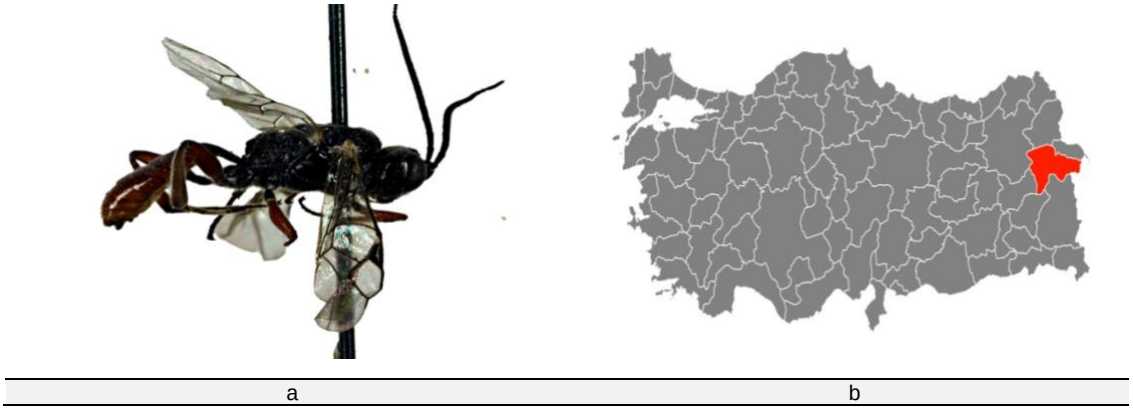

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Figure 10. *Endasys analis* (Thomson, 1883) a) Habitus, b) distribution of Türkiye.

Mesoleptus vigilatorius (Förster, 1876) (Figure 11a)

Material examined. Ağrı: 40°29’40" N, 41°30’07" E, 1249 m, 23.07.2023, 2 ♂♂. Mount Ararat, 39°68’23.31" N, 44°08’67.86" E, 1520 m, 23.VII.2023, 2 ♀♀.

Distribution. Bulgaria, Czechoslovakia, Finland, France, Germany, Italy, Latvia, Norway, Poland, Russia, Switzerland, United Kingdom (Yu et al., 2016); known from Türkiye (Barik & Çoruh, 2023) (Figure 11b)

Remark: Second exact locality from Türkiye.

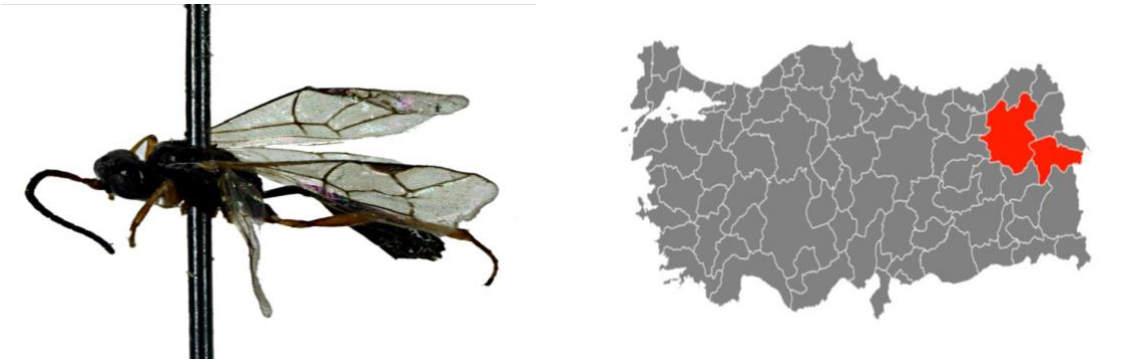

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Figure 11. *Mesoleptus vigilatorius* (Förster, 1876): a) Habitus, b) distribution of Türkiye.

Phygadeuon hercynicus Gravenhorst, 1829 (Figure 12a)

Material examined. Ağrı: Tutak, 39°26’32.68" N, 42°45’55.83" E,1679 m, 21.07.2022, ♀; Oğlaksuyu, 40°02’46" N, 41°20’23" E, 1788 m, 07.VI.2023, 2 ♂♂, 2 ♀♀.

Distribution. Austria, Azerbaijan, Belgium, Czechoslovakia, Finland, Germany, Ireland, Norway, Poland, Russia, Sweden, United Kingdom (Yu et al., 2016); known from Türkiye (Çaylak & Çoruh, 2020) (Figure 12b).

Associate plant: *Angelica sylvestris*.

Remark: This species is new for East Anatolia.

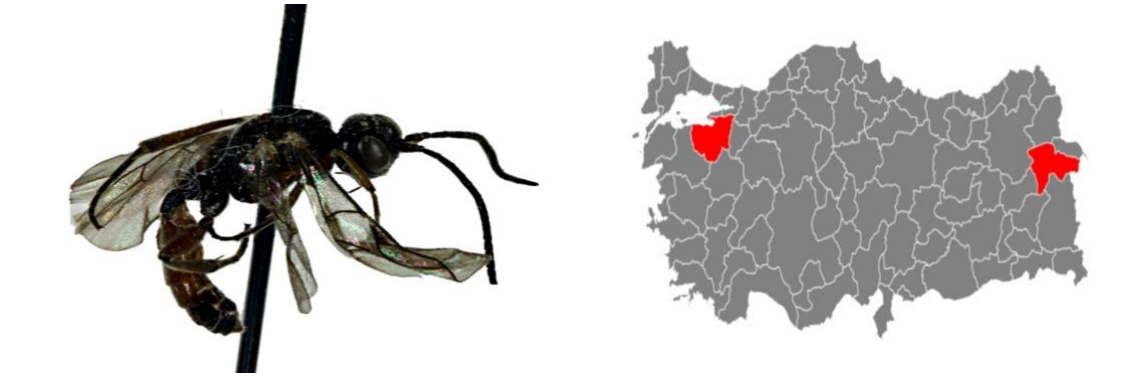

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Figure 12. *Phygadeuon hercynicus* Gravenhorst, 1829: a) Habitus, b) Distribution of Türkiye.

Discussion

A total of 53 specimens belonging to eight genus and 10 species are recorded. Among them, *Enclisis vindex*, and *Endasys analis* are recorded for the first time from Türkiye. Also, present *Gelis vicinus* and *Phygadeuon hercynicus* are new for East Anatolia.

When evaluation is made according to the number of species and individuals possessed by the existing genera we see that one species (11 samples) belong to the genus *Cryptus* Fabricius, one species (five samples) belong to genus *Enclisis* Townes, one species (three samples) belong to *Mesostenus* Gravenhorst, three species (21 samples) belong to *Trychosis* Förster, one species (five samples) belong to *Blapsidotes* Förster, one species (one sample) belong to *Endasys* Förster, one species (four samples) belong to *Mesoleptus* Gravenhorst, one species (five samples) belong to *Phygadeuon* Gravenhorst. *Cryptus* is the genus with the most species in the world (Yu et al., 2016; Çoruh, 2019; Schwarz, 2015.).

As a result, when all genuses are compared, we can see that the number of individuals of the *Trychosis is* more dense than other genus. Among the species determined, *Cryptus viduatorius* (with 11 individuals), *Trychosis legator* (with 8 individuals) and *Trychosis ambigua* (with 7 individuals) were the most abundant in research areas. In contrast, *Endasys analis* (with 1 individual) was rarely found in this study.

Adult specimens were collected from different altitudes in collection areas in this study. Among the 10 species, all species were collected from Ağrı center and districts, while *Enclisis vindex*, *Trychosis tristator*, and *Mesoleptus vigilatorius* were collected from the foothills of Mount Ararat at the same time. *E. vindex* is the first species recorded as new in Mount Ararat.

When the species are analyzed in terms of geographic regions of Türkiye, it is seen that, *Cryptus viduatorius* and *Trychosis legator* were collected from seven regions (Çoruh et al., 2014 a, b, 2016; Schwarz, 2015), *Mesostenus transfuga* was collected from five regions (Çoruh, 2019; Barik, 2022; Kaplan & Riedel, 2022; Barik & Çoruh, 2023), *Trychosis tristator* was collected from four regions (Kolarov et al., 2014; Çoruh, 2019; Kaplan & Riedel, 2022) Besides, *Mesoleptus vigilatorius* and *Phygadeuon hercynicus* were collected from a single region (Çaylak & Çoruh, 2020).

According to these results, Palaearctic Region have the highest number of species. Among these species, *Cryptus viduatorius* showed distribution in 67 different countries (Yu et al. 2016; Çoruh, 2019). This species is almost a cosmopolitan species, similarly, *Trychosis legator* showed distribution in 49 different countries (Yu et al., 2016) and *Mesostenus transfuga* showed distribution in 41 different countries (Yu et al., 2016; Çoruh, 2019) in zoogeographical regions. These species, which are common worldwide, are also widely found in our country. Also, *Phygadeuon hercynicus* has the least widespread area among the species (12 countries) (Yu et al., 2016). In the same vein, this species was previously detected only in Bursa province (Çaylak & Çoruh, 2020). Ağrı province is the second locality for the species.

As a result, while Ağrı province was the new locality for 9 other species except *Cryptus viduatorius*, it became the second locality for *Phygadeuon hercynicus* and *Mesoleptus vigilatorius* and the fourth locality for *Trychosis ambigua*. The Eastern Anatolia region, which includes the province of Ağrı, is also a new record for *Phygadeuon hercynicus* and *Gelis vicinus*.

Every faunistic data study conducted will contribute to the diversity of Türkiye's Ichneumonidae and set an example for new studies

Acknowledgements

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Original article (Orijinal araştırma)

Evaluating the role of insect pollinators in the viability of true seeds of shallot in tropical agroecosystems[1]

Tropikal tarım ekosistemlerinde arpacık soğanı gerçek tohumlarının canlılığında böcek tozlayıcılarının rolünün değerlendirilmesi

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Abstract

Pollen transfer in tropical agroecosystems to increase seed production has received limited research, notably on shallot pollination and insect pollinators. This study evaluated how insect pollinators optimize shallot seed production in tropical agroecosystems, i.e. Batu and Malang Districts in Indonesia. This study was conducted from June to October in 2023. We examined pollinator diversity, foraging behavior, and the effectiveness of dominating insect pollinators during umbel flower anthesis, as well as visiting insect foraging and visiting patterns. We assessed pollinator effectiveness by comparing visitation rates under four treatments. A total of 21 insect species belonging to three orders visited the shallot flowers, of which 14 species have the potential to act as pollinators. *Apis cerana* Fabricius, 1793 (Hymenoptera: Apidae) and *Lucilia sericata* (Meigen, 1826) (Diptera: Calliphoridae) emerged as potentially effective pollinators. Air temperature significantly influenced pollinator activity in visiting anthesis umbels. Evaluations of pollinator efficiency showed that *A. cerana* was more efficient than *L. sericata* in promoting the production of true shallot seed and has a significant role in ensuring high-quality pollination. This highlights the necessity of comprehending the specialized contributions of pollinators for shallot seed production.

Keywords: *Allium cepa* var. *aggregatum*, cross pollination, insect pollinator, tropical agroecosystem, true shallot seed

Öz

Polen transferi, tropik tarımsal ekosistemlerde tohum üretimini artırmak için özellikle arpacık soğanı tozlaşması ve böcek tozlaştırıcılar üzerine sınırlı araştırmalara konu olmuştur. Bu çalışma, böcek tozlaştırıcıların Endonezya'nın Batu ve Malang Bölgelerinde tropik tarımsal ekosistemlerde arpacık soğanı tohum üretimini nasıl optimize ettiğini değerlendirmiştir. Bu çalışma Haziran-Ekim 2023 tarihleri arasında gerçekleştirilmiştir. Tozlaştırıcı çeşitliliğini, beslenme davranışlarını ve çiçeklenme dönemi boyunca baskın böcek tozlaştırıcıların etkinliğini, ayrıca ziyaret eden böceklerin beslenme ve ziyaret desenlerini incelenmiştir. Ziyaret oranlarını dört farklı uygulamada karşılaştırarak tozlaştırıcı etkinliğini değerlendirilmiştir. Arpacık soğanı çiçeklerini ziyaret eden, üç gruba ait toplamda 21 böcek türü tespit edildi ve bu türlerin 14'ünün potansiyel tozlaştırıcı olabileceği belirlenmiştir. *Apis cerana* Fabricius, 1793 (Hymenoptera: Apidae) ve *Lucilia sericata* (Meigen, 1826) (Diptera: Calliphoridae) potansiyel olarak etkili tozlaştırıcılar olarak ortaya çıkmıştır. Hava sıcaklığı, çiçeklenme döneminde tozlaştırıcı aktivitesini önemli ölçüde etkiledi. Tozlaştırıcı verimliliğinin değerlendirilmesi, *A. cerana*'nın gerçek arpacık soğanı tohumu üretimini teşvik etmede *L. sericata*'dan daha verimli olduğunu ve yüksek kaliteli tozlaşmayı sağlamakta önemli bir rol oynadığını göstermiştir. Bu durum, arpacık soğanı tohum üretimi için tozlaştırıcıların özel sorumluluklarını anlamanın gerekliliğini vurgulamaktadır.

Anahtar sözcükler: *Allium cepa* var. *agregatum*, çapraz tozlaşma, böcek tozlaştırıcı, tropikal tarımsal ekosistem, arpacık tohumu

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Introduction

Shallot, *Allium cepa*. var. *aggregatum* G.Don (Asparagales: Amaryllicidaceae) is a globally significant agricultural crop due to its substantial contribution to food security and socioeconomic development, especially in tropical areas. Originally native to subtropical regions, shallots are extensively cultivated, including in tropical areas. Shallot seed production as known True Shallot Seeds (TSS), is a critical aspect of shallot cultivation for bulb formation. TSS production technology plays a pivotal role in the plant's life cycle, as shallot plants rely on cross-pollination to produce high-quality seeds. Utilizing TSS as seeds offers various benefits, such as increased productivity, reduced production costs, and enhanced market acceptance, making it appealing to farmers (Askari-Khorasgani & Pessarakli, 2019). Insect pollinators are critical in the TSS production process because they facilitate pollen transfer from male to female flowers, ensuring the formation of quality seeds.

In tropical agroecosystems, research on the effectiveness of insect pollinators of shallot in TSS formation is still limited, unlike in subtropical agroecosystems, where much more research has been conducted (Monasterio et al., 2023). Tropical agroecosystems tend to have more extreme climatic characteristics, with higher temperatures and greater humidity throughout the year, often experiencing more unpredictable rainfall patterns and higher intensity. These climatic factors can affect pollinator activity and TSS formation. On the other hand, subtropical agroecosystems tend to have more regular seasonal variations, with more stable temperatures and rainfall. The differences in environmental conditions between tropical and subtropical regions can directly impact pollinator diversity and behavior, as well as their effectiveness in seed formation.

Tropical regions such as Indonesia cultivate shallots using monoculture systems and extensive insecticide applications (Nurjati et al., 2018). Monoculture practices result in reduced plant diversity, altering habitats, and affecting pollinator populations (Tarakini et al., 2021; Bukhari et al., 2024). Intensive insecticide use disrupts insect community, diminishing the essential pollinator presence for effective pollination (Kumari & Rana, 2018; Buszewski et al., 2019). Consequently, the reliance on insect pollinators directly impacts the efficacy of true shallot seed formation. These environmental shifts highlight the necessity for a deeper comprehension of the interplay between prevalent agricultural practices in tropical agroecosystems and the dynamics of pollinator populations, including their behaviors, and their consequent effects on true shallot seed production. This understanding is critical for supporting Indonesian shallot cultivation's sustainability.

Previous research has highlighted the crucial role of specific insect species in shallot pollination. Palupi et al. (2015) demonstrated that *Apis cerana* Fabricius, 1793, *Trigona* sp. Jurine, 1807 (Hymenoptera: Apidae), and *Lucilia* sp. Robineau-Desvoidy, 1830 (Diptera: Calliphoridae) significantly contribute to shallot pollination, enhancing both the production and quality of true shallot seeds (TSS). In subtropical regions, various bee species [*A. cerana*, *Apis dorsata* Fabricius, 1793, and *Apis florea* Fabricius, 1787 (Hymenoptera: Apidae)] and fly species [*Episyrphus balteatus* (De Geer, 1776), *Eristalinus aeneus* (Scopoli, 1763), (Diptera: Syrphidae) and *Calliphora vicina* Robineau-Desvoidy, 1830) (Diptera: Calliphoridae)] have been identified as pollinators for *Allium* spp. (Abrol, 2006; Devi et al., 2015). Pangestuti et al. (2023) confirmed the significance of *A. cerana*, *Trigona* sp., and *Lucilia* sp. in shallot pollination in tropical regions. However, the specific contributions of each pollinator to TSS quality remain understudied.

In tropical regions, the Calliphoridae family (Diptera) plays a notable role in shallot flower pollination. Understanding the diversity and foraging behavior of these pollinators provides valuable insights into tropical ecosystem biodiversity and has significant implications for agricultural productivity in tropical agroecosystems. Investigating the composition and foraging behavior of flower-visiting insects, particularly potential shallot pollinators, is crucial for developing effective pollinator conservation strategies and improving crop yields. Therefore, conducting comprehensive studies on flower-visiting insect diversity is essential for strengthening the foundation of pollinator conservation and enhancing agricultural productivity in tropical agroecosystems.

This study aims to comprehensively examine the role of insect pollinators in shallot pollination success and their impact on TSS production and quality in tropical regions. The research seeks to assess pollinator diversity through various indicators such as diversity indices, evenness, and dominance of both visitor and pollinator insects on shallot umbels. It also analyzes the foraging behavior of key insect pollinators by examining their visitation patterns during shallot flower anthesis. Furthermore, the study evaluates the effectiveness of different pollinator species in facilitating successful pollination of shallot umbels and investigates the relationship between pollinator activity and the quantity and quality of TSS produced. By adopting this multifaceted approach, the research endeavors to advance scientific understanding of the intricate interaction between insect pollinators and shallots in tropical agroecosystems. Additionally, this study aims to provide crucial insights for developing targeted pollinator conservation strategies and enhancing shallot agricultural productivity, with a specific focus on improving TSS production and quality in tropical regions.

Materials and Methods

This study comprised several components: documenting all insects visiting shallot flowers during anthesis, analyzing the pollinators, and evaluating the effectiveness of these pollinators in enhancing the production of high-quality TSS.

Diversity and composition of insect species visiting shallot flowers

The inventory of insects visiting shallots and identification of potential insect pollinators during flower anthesis were conducted at two different altitudes, namely Batu and Ngantang, Indonesia, from June to September 2023. Both observation locations were in the shallot cultivation centers in Batu and Ngantang. The agroecosystems in both observation locations were similar, consisting of monoculture shallot plants of the “Tajuk” variety, with intensified pesticide spraying every 2-5 days when the plants were 1-5 weeks after planting (WAP). The observation plots, measuring 4 m by 1 m, contained 2100 plants. The observation plots received no pesticide spraying (Table 1).

Table 1. Geographical description of research locations for the inventory of insects visiting shallot flowers

Location	Coordinate	Altitude (m asl)	Altitude
Batu, Batu City	7°52'31"S,112°31'20"E	848.5	High
Ngantang, Malang	7°52'49"S,112°34'22"E	625.7	Moderate

We conducted the inventory of insects visiting the shallot flowers (umbels) during flower anthesis, when the plants were 9-10 WAP. The term "flower anthesis" refers to the period when a flower is fully open and functional, often marked by the expansion and blooming of petals or florets arranged in an umbel. We documented the types and numbers of insects visiting the umbels during anthesis. These observations occurred at specific time intervals: (06:00-08:00), (08:00-10:00), (10:00-12:00), (12:00-14:00), (16:00-16:00), and (16:00-17:00), over a period of six consecutive days. These observations revealed that 30-70% of the florets in each umbel had fully opened.

We sampled a total of 10 randomly selected plants representing bloomed umbels. The observation time was 5 minutes per umbel. We collected insects visiting umbels using a sweep net (diameter 30 cm). We pinned and dried the captured insects for further storage and identification. We recorded the air temperature and humidity during each observation. Identification of visiting insects was done by separating them by Order, then grouped into families using insect identification guidelines such as Borror et al. (1954) and Goulet et al. (1993). Identification to the species level was conducted through morphological observation, comparing specimens with the insect collection reference at the Museum Zoologicum Bogoriense, National Research and Innovation Agency in Cibinong, Bogor. For pollinator analysis, all captured visiting insects underwent microscopic examination to detect the presence of pollen grains on their bodies.

Assessment of the effectiveness of shallot pollinators

To assess effective pollinators, we conducted a pollinator analysis at the Laboratory of Entomological Research Group in Malang, Indonesia, from August to October 2023. This analysis involved microscopic examination of insect visitors to identify potential pollinators based on the presence of pollen grains on their bodies. We focused on pollen found on body parts other than specialized pollen-carrying structures like pollen baskets. This approach allowed us to distinguish between insects that might incidentally carry pollen and those more likely to contribute to pollination. Our analysis identified two species, *Apis cerana* and *Lucilia sericata* (Meigen, 1826), as potential pollinators due to the presence of pollen grains on their bodies.

We calculated the diversity and composition of insect species visiting shallot flowers using the Shannon-Wiener diversity indices, which include the evenness index, species richness index, and dominance index (Odum, 1971).

The research locations were conducted at shallot planting centers of the “Tajuk variety in Batu (7°52'56", 112°32'35"). We selected shallot plants in 3 plots (4 m x 1 m) for this assessment, ensuring each plant had two umbels. Ten plants were observed, for a total of 20 umbels per treatment. We specifically selected two pollinator species for this study: *A. cerana* and *L. sericata*, which were determined to be the most often visited pollinators based on our previous analysis. The objective of this experiment was to assess the efficacy of pollinators by introducing several pollinator species to enclosed shallot plants, with the assumption that only the chosen pollinator species would facilitate pollination. The cage's dimensions were 50 cm x 50 cm x 100 cm, and it was equipped with a 50 mesh screen. The treatments applied were:

1. Shallot plants were caged; 2 individuals *A. cerana* were introduced into the cage for 6 days when 70% of the florets per umbel had bloomed (Caged + *A. cerana* - CAc).
2. Shallot plants were caged; 2 individuals were *L. sericata* introduced into the cage for 6 days when 70% of the florets per umbel had bloomed (Caged + *L. sericata* - CLs.).
3. Shallot plants in open condition/without cage (Control-opened- CO).
4. Shallot plants were caged without pollinator introduction (Control-closed-CC).

For the caged treatments and pollinator release experiment, we used specially designed cages that enclosed individual shallot plants. Each cage contained two umbels undergoing anthesis to ensure sufficient floral resources. We introduced two individual pollinators of the same species (either *A.cerana* or *L. sericata*) into each cage at the beginning of each day's observation period.

The pollinator release was conducted daily from 7 am to 2 pm, coinciding with the peak foraging activity of these species. This process was repeated for six consecutive days to cover the full anthesis period of the shallot umbels. Importantly, we used new, naive insects each day to prevent habituation or exhaustion. These fresh pollinators had not previously visited shallot flowers, ensuring unbiased foraging behavior. At the end of each day's 6-hour observation period, the insects were removed from the cages. This approach allowed us to control exposure time precisely while maintaining the insects' well-being. The use of fresh pollinators each day eliminated concerns about long-term confinement effects on insect behavior or survival.

Observations were made on various parameters, including the number of florets per umbel, the number of capsules per umbel, the number of TSS per umbel, the weight of 100 seeds, and TSS viability as qualified seeds. Seed viability tests were conducted using 100 seeds per treatment with six replicates. Observations of TSS viability included TSS germination rate, and the proportion of germinated seeds that grew normally and abnormally.

Data analysis

The diversity of visiting insects and potential pollinators was analyzed using Shannon's formula, diversity indices, evenness index, species richness index, and dominance index (Odum, 1971). To evaluate the relationship between temperature and relative air humidity and the number of pollinators visited to umbels during anthesis, linear regression analysis was done for each environmental condition factor. The effectiveness test of pollinator performance was conducted using analysis of variance (ANOVA), followed by Fisher's PLSD test for each observation parameter. We used linear regression analysis to look at the link between seed weight, germination capacity, or seed germination rate, and the percentage of germinated seeds that grew normally for the TSS viability test as qualified seeds. All statistical analyses were performed using Minitab 19.2 Statistical Software (Minitab, LLC, 2020).

Results and Discussion

Results

Diversity and composition of insect species visiting shallot flowers

This study examined the variety and composition of insect species visiting shallot umbels during anthesis. A total of 21 insect species from 11 families and three orders (Hymenoptera, Diptera, and Lepidoptera) were identified at two different elevations (Table 2 and 3). The Hymenoptera order, particularly the Apidae family, exhibited the highest species diversity and the greatest number of individual visitors at both locations. Among the 21 species, 14 were classified as potential pollinators, while 7 were categorized as mere visitors (Table 3).

Table 2. Number of species and individual of Pollinators on Shallot Umbel During Anthesis at High Altitude (Batu) and Moderate Altitude (Ngantang) Agroecosystems

No	Order	Family	Number of Species	Number of individuals at	
				Batu	Ngantang
1	Diptera	Calliphoridae	2	124	120
2		Phoridae	2	5	1
3		Syrphidae	1	6	51
4	Hymenoptera	Apidae	6	151	78
5		Halictidae	2	7	3
6		Chalcididea	1	2	0
7		Formicidae	1	4	2
8		Vespidae	2	2	11
9	Lepidoptera	Erebridae	1	2	1
10		Noctuidae	1	24	6
11		Nymphalidae	1	2	0

Table 3 shows that the species of insects that visited shallot flowers during anthesis were different in the agroecosystems at high altitude (Batu) and mid-altitude (Ngantang). The diversity index for the family of visitors and species of pollinators in the agroecosystem at high altitude (Batu) and mid-altitude (Ngantang) was moderate. However, the diversity index for the family of pollinators was low in both agroecosystems. This indicates that in both agroecosystems, the number of pollinator insect families is low, or their diversity is limited (Table 4).

The composition of pollinators observed visiting shallot flowers during the anthesis period revealed distinctive patterns across different agroecosystems. *Lucilia sericata* appeared to be a prevalent visitor to both medium (Ngantang) and highland (Batu) agroecosystems, exhibiting high frequency across both environments (Figure 1). Following this trend, *A. cerana* also demonstrated notable activity in these agroecosystems. Conversely, *T. iridipennis* exhibited a preference for visiting shallot agroecosystems in highland regions, while *C. megacephala* and *E. balteatus* displayed a greater inclination towards shallot agroecosystems situated in medium agroecosystems. Interestingly, pollinators from the Apidae family consistently exhibited intensive visitation patterns across both types of agroecosystems. This description

of how pollinators behave helps us understand the complex dynamics that affect pollination interactions in shallot agroecosystems in a range of environmental conditions. Based on these observations, we can conclude that *L. sericata* and the three Apidae species are frequent visitors to shallot umbels and have significant potential as effective pollinators.

Table 3. List of visitor insects and potential pollinators in shallot flowers during anthesis at high altitude (Batu) and moderate altitude (Ngantang) agroecosystems

No	Species	Ordo:Family	V/P	Number of individuals at	
				Batu	Ngantang
1	*Apis cerana* F.	Hymenoptera: Apidae	P	34	53
2	*Apis florea* F.	Hymenoptera: Apidae	P	57	11
3	*Tetragonula iridipennis* (Smith)	Hymenoptera: Apidae	P	37	21
4	*Trigonula* sp.	Hymenoptera: Apidae	P	0	0
5	*Amegila zonata*	Hymenoptera: Apidae	P	6	6
6	*Brachymeria lasus* (Walker)	Hymenoptera: Chalcididae	V	2	0
7	*Polyrachis* sp	Hymenoptera: Formicidae	V	4	2
8	*Nomia* sp1	Hymenoptera: Halticidae	P	4	2
9	*Nomia* sp2	Hymenoptera: Halticidae	P	3	1
10	*Sceliphon javanum* (Lepeletier de S. F)	Hymenoptera: Sphecidae	V	1	1
11	*Cerceris* sp.	Hymenoptera: Sphecidae	V	1	1
12	*Euodynerus* sp	Hymenoptera: Vespidae	V	1	1
13	*Delta campaniforme* (F.)	Hymenoptera: Vespidae	V	2	1
14	*Lucilia sericata* (Meigen)	Diptera: Calliphoridae	P	124	120
15	*Chrysomya megacephala* (F.)	Diptera: Calliphoridae	P	20	15
16	*Episyrphus balteatus*	Diptera: Syrphidae	P	6	51
17	*Graptomyza* sp.	Diptera: Syrphidae	P	1	0
18	*Musca domestica* L.	Diptera: Muscidae	V	16	10
19	*Amata huebneri* (Boisduval)	Lepidoptera: Erebidae	V	2	2
20	*Spodoptera exigua* Hübner	Lepidoptera: Noctuidae	V	24	6
21	*Ariadne ariadne* (L.)	Lepidoptera: Nymphalid	V	2	2

Note: P: Insects that visit and potentially act as pollinators; V: Insects that only visit and did not potentially act as pollinators based on pollinator analysis.

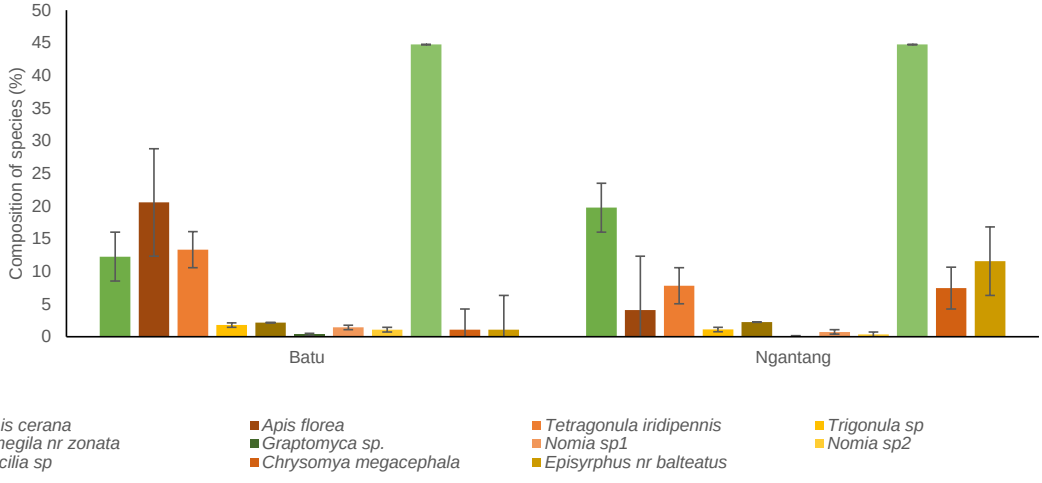

Figure 1. Composition (Means ± Standard error) of shallot pollinator species in shallot agroecosystems at high- (Batu) and moderate- (Ngantang) altitudes.

The values of the evenness index for the visitor and pollinator families, and species of pollinators were all less than 0.4, indicating low evenness in the distribution of visitor and pollinator families and species based on the Shannon-Wiener evenness index category (Table 4). This suggested a relatively uniform distribution of pollinators and visitors within the agroecosystem. The richness indices for the family of all visitors and species of pollinators in both agroecosystems showed higher values compared to the family of pollinators. This indicates that while there were fewer pollinator families compared to visitor families, there was a relatively high number of pollinator species. However, the richness index values for all three parameters were relatively low in both highland and midland agroecosystems. In both midland and highland agroecosystems, the dominance index for all three parameters was low, indicating no dominant species among visitors or pollinators in the shallot agroecosystem.

Table 4. Diversity of visiting insects during shallot flower anthesis in agroecosystems at high altitude, B (Batu) and moderate altitude, N (Ngantang) based on Shannon-Wiener indices

Parameters	Diversity index (H')		Evenness index (E)		Richness Index (R)		Dominancy Index (C)	
	B	N	B	N	B	N	B	N
Family of Visitors	1.312	1.373	0.119	0.125	1.725	1.783	0.359	0.312
Family of Pollinators	0.780	0.452	0.130	0.041	0.176	0.185	0.452	0.278
Species of Pollinator	1.000	1.556	0.100	0.141	1.762	1.805	0.271	0.290

We observed a clear pattern of high pollinator activity at regular times, especially from 6:00 a.m. to 12:00 p.m., in the tropical shallot agroecosystems in the highlands (800 m) and midlands (600 m). Specifically, at the highland site, the number of Dipteran pollinators exceeds that of Hymenopteran pollinators between 12:00 and 17:00 (Figures 2A and 2B). The pollinating insects that visit shallot umbels on a regular basis come from three Hymenopteran families and two Dipteran families. Among the Hymenopteran pollinators, individuals from the Apidae family and Dipteran pollinators from the Calliphoridae family dominated in abundance compared to other families (Figure 2C). The Apidae consisted of three species, namely *A. cerana, A. florea*, and *T. iridipennis*, and two species, *L*. *sericata* and *C. megachepala*, comprised the Calliphoridae family (Table 3).

The regression analysis revealed that temperature significantly influenced *L*. *sericata* and *C. megacephala*, contributing 38% and 31%, respectively, at both agroecosystem sites, while humidity did not affect their activity at the midland site. However, it is linearly related, contributing only 11% to the highland agroecosystem.

Assessment of the effectiveness of shallot pollinators

A quantitative assessment of successful pollination, based on the number of fruit capsules formed, demonstrated the effective transfer of pollen grains to the stigmas of shallot flowers. When comparing treatments, the CO and CAc treatments significantly exhibited higher rates of fruit capsule formation than the CC and CLc treatments (Table 5). This suggests that the presence of *A. cerana* in the CAc treatment closely replicates natural pollination processes. Furthermore, the proportion of seed formation reinforced the effectiveness of pollination. Among all treatments, the OC treatment produced the most seeds, followed by the CAc treatment with *A. cerana*, the CLs treatment with *L. sericata*, and the CC treatment. These results highlight the importance of both pollination effectiveness and the treatment environment in determining the reproductive success of shallot plants. In the CC treatment, the percentage of successful pollination was very low and significantly different from other treatments. This indicates that pollination cannot occur optimally in conditions where environmental factors and insect pollinators do not have access to the flowers.

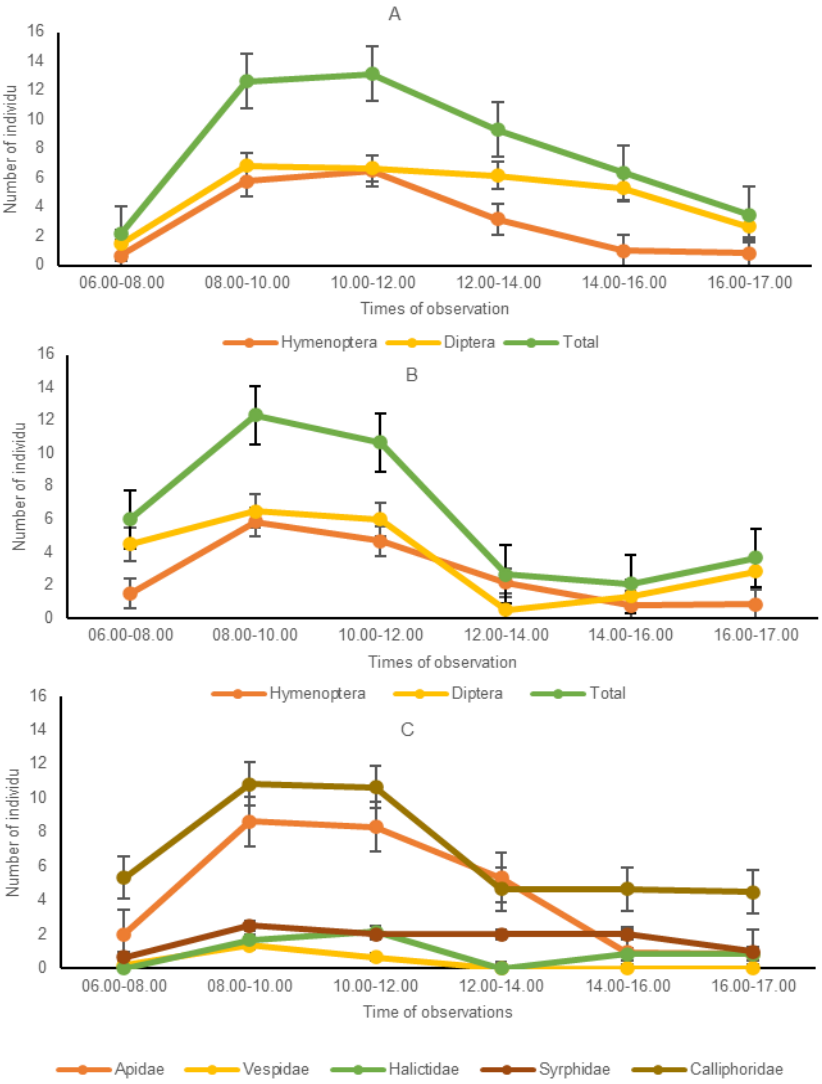

Figure 2. Diel pattern of shallot flower pollinator activity in Batu and Ngantang (Mean number of individuals ± Standard Error) during umbel anthesis. A. Diel pattern of pollinators in Ngantang; B. Diel pattern of pollinators in Batu; C. Diel pattern of Hymenopteran and Dipteran families.

Table 5. Means (± standard error) of number of florets per umbel, number and percentage of fruit capsules' formation per umbel, number of seeds formed per umbel, and percentage of successful pollination in the treatments: non-caged (Open control), caged (Closed control), caged with the introduction of *A. cerana*, and caged with the introduction of *L. sericata*. in shallot plants

Treatment	Number of florets/umbels	Number of fruit capsules/umbel	% Capsule formation[1]	Number of seeds/umbels	% successful fertilization[2]
Caged+*A. cerana* (CAc)	83.3 ± 4.0 ab	65.5 ± 3.3 a	80.2 ± 5.3 a	50.6 ± 6.7 a	63.6 ± 7.5 b
Caged+*L. sericata* (CLs)	71.6 ± 5.5 b	28.7 ± 1.9 b	41.9 + 4.2 b	15.8 ± 1.8 b	38.9 ± 4.4 c
Closed Control (CC)	74.7 + 2.8 b	11.5 ± 1.4 c	15.6 + 2.0 c	0.7 ± 0.3 c	5.6 ± 2.5 d
Open control (OC)	90.5 ± 7.1 a	62.9 ± 5.7 a	70.2 ± 3.8 a	57.1 ± 4.1 a	82.7 ± 5.5 a

[1] The percentage of capsule formation indicates the plant's ability to successfully generate fruit following fertilization.
[2] The percentage of successful fertilization is an indicator of the successful conversion of fertilization into the development of fruit and seeds.
Different letters on values corresponding to each response variable of treatments indicated significant differences between the treatments according to Fisher's PLSD test ($p < 0.05$).

Viability test of the true shallot seeds (TSS)

This test aimed to determine whether pollination from the plots in the pollinator effectiveness test produced TSS suitable for use as high-quality seeds. The weight of TSS obtained from the CAc treatment was not significantly different from the open control (OC) and closed control (CC), with values ranging between 0.29 g and 0.32 g. However, the weight of TSS from the CLs treatment was lower than that of the CAc treatment and even OC, measuring 0.24 g (Table 6). This indicates that pollination in the CLs treatment was less optimal compared to the CA and OC treatments, resulting in the formation of lighter seeds

Table 6. Parameters indicating the viability of True Shallot Seeds (TSS) (Mean ± Standard Deviation) obtained from the effectiveness test on shallot agroecosystems in tropical regions

Parameter	Treatments			
	Caged+*A. cerana*) (CAc)	Caged+*Lucilia sericata*) (CLc)	Opened Control (OC)	Closed Control (CC)
Weight of 100 seeds (g)	0.32 ± 0.04 a	0.24 ± 0.04 b	0.30 ± 0.05 a	0.29 ± 0.02 ab
Seed germination rate (%)	52.29 ± 5.3 a	44.14 ± 10.7 b	35.57 ± 4.5 c	58.71 ± 5.4 a
Normal germination (%)	90.48 ± 3.3 a	59.05 ± 19.05 b	81.45 ± 5.70 a	42.48 ± 4.43 c
Abnormal germination (%)	9.52 ± 3.3 b	40.95 ± 19.05 a	18.55 ± 5.70 b	49.44 ± 5.45 a

Notes: Values in the same rows (parameters) followed by different letters indicate significantly different ($p < 0,05$) based on Fisher's PLSD tests.
Germination capacity indicates the seed's ability to grow normally and produce normally under optimal environmental conditions.
Abnormal seedling growth refers to seeds that are capable of germination but fail to develop further, such as those without root growth or with irregular stem development.

The germination rate of TSS in the CAc and CC treatments was higher and significantly different from that in the CLs and OC treatments. The caged treatment, with the introduction of *A. cerana* (CAc), had an average total of 50.6 seeds per umbel, indicating successful pollination, whereas the OC treatment had an average of 57.1 seeds per umbel. There was no significant difference between these two treatments. The CC treatment and the caged treatment with *L. sericata*, on the other hand, had lower average TSS values, with 0.7 seeds per umbel for the CC treatment and 14.6 seeds per umbel for the caged treatment (Table 6).

We calculated the percentage of successful pollination by dividing the average TSS per umbel by the total number of fruit capsules per umbel. The pollination success rate in the OC and CC treatments showed no significant difference, whereas the CAc treatment exhibited a lower success rate compared to both controls. Furthermore, the caged treatment, which included *L. sericata*, demonstrated the lowest success rate. The high percentage of successful pollination in CC resulted from the low number of fruit capsules formed per umbel (Table 5). Conversely, in the OC treatment, where pollination occurred with the aid of pollinators and wind, the percentage of successful pollination was optimal, reaching 82.7% (Table 5). In CAc, only 39% of pollinations were successful, which is almost half of the open control. However, this was still a higher and significantly different success rate than in the caged treatment with *L. sericata* (treatment CLs). Therefore, we can conclude that *A. cerana* outperformed *L. sericata* as a superior pollinator in facilitating the formation of TSS.

The linear regression model did not reveal a significant relationship between the rate of germination and the TSS weight ($p > 0.05$), indicating that the relationship might be non-linear. The TSS weight accounted for 19.68% of normal seed growth (Table 7). In the CC and CLs treatments, TSS weight was lower compared to the open control and CAc treatments (Table 6), resulting in reduced normal germination growth. Although the germination rate in the CC and CLs treatments was not significantly different from that in the CAc treatment and was higher than the OC, it did not lead to optimal growth. Therefore, we concluded that *A. cerana* significantly influences pollination, resulting in the production of high-quality TSS. Conversely, *L. sericata* was less efficient at pollinating shallots and producing TSS of comparable quality. Natural cross-pollination, *i.e.* OC treatment, also produced TSS with an ideal weight and low germination rate (Table 6); however, there was a significantly higher likelihood that this successful germination would result in the emergence of a healthy and prosperous plant.

Table 7. Linear regression analysis between tss weight, germination rate, and normal seed growth

Regression	p value	S	R-sq	R-sq(adj)	R-sq(pred)
TSS weight vs germination rate	0.379	0.05091	2.99%	0.00%	0.00%
TSS weight vs normal seed growth	0.010	0.04546	22.66%	19.68%	10.64%

Note: - S (Standard Error of the Estimate): This is an estimate of the average deviation of the observed values from the values predicted by the model.
- R-squared (R-sq): This is the coefficient of determination, indicating the proportion of variation in the dependent variable that can be explained by the independent variable in the regression model.
- Adjusted R-squared (R-sq(adj)): This indicates the estimated accuracy and quality of the model.
- Predicted R-squared (R-sq(pred)): This provides an indication of how well the model can generalize patterns from the training data to the test data

Discussion

Our study revealed a diverse array of insect visitors to senescing shallot flowers, with the Hymenoptera order, particularly the Apidae family, exhibiting the highest species diversity and visitation frequency across both high and moderate altitude agroecosystems. This aligns with previous research highlighting the importance of Apidae in shallot pollination (Palupi et al., 2015; Davidar & Carr 2015). Among the 14 species classified as potential pollinators, *L. sericata* and three Apidae species (*Apis cerana, Apis florea*, and *Tetragonula iridipennis*) emerged as frequent visitors, suggesting their potential as effective pollinators. The consistent presence of these species across different altitudes indicates their adaptability to various environmental conditions, a characteristic crucial for reliable pollination services in diverse shallot-growing regions (Garibaldi et al., 2019).

The moderate diversity of pollinator species, coupled with low family-level diversity and evenness, suggests a concentration of pollination services among a few key species. This finding highlights the potential vulnerability of shallot pollination to declines in these specific pollinator populations, a concern reverberated in recent studies on crop pollination systems (Reilly et al., 2020). The frequent visits of *L. sericata, A. cerana, T. iridipennis,* and *A. florea* during anthesis likely contribute significantly to pollen transfer and subsequent TSS formation. However, as Mallinger et al. (2021) emphasize, visitation frequency alone does not guarantee pollination efficiency; factors such as pollen load, foraging behavior, and morphological compatibility also play crucial roles in determining a species' effectiveness as a pollinator and its impact on TSS formation and quality.

Several interconnected factors influence the low diversity of visitors and pollinators observed in this research, particularly in heavily sprayed shallot plantations' centers. Heavy insecticide application may directly kill or indirectly affect pollinators by reducing their abundance, diversity, and foraging behavior (Vanbergen et al., 2013; Bloom et al., 2021). Pesticides may also disrupt pollinator foraging behavior by altering floral scent, nectar availability, and flower attractiveness. Furthermore, intensive agricultural practices such as monoculture cropping systems common in large-scale plantations can lead to habitat loss and fragmentation, limiting suitable nesting sites, food resources, and shelter for pollinators. Moreover, monoculture cropping systems often provide a limited range of flowering plants for pollinators, especially during non-crop blooming periods. Thus, the complexity of relationships between farm management, pesticide use, changing habitats, and pollinator movements in tropical agroecosystems contributes to the observed lack of diversity in visitors and pollinators in heavily sprayed shallot plantations.

Balancing agricultural productivity with biodiversity conservation is essential for promoting sustainable farming practices that support pollinator health and ecosystem resilience. Comparative research on insect community diversity indices in these agroecosystems can provide insights into what promotes insect community diversity and how they interact with crops like shallot. The abundance and diversity of Hymenoptera insects, particularly bees, underscore their importance in upholding biodiversity and ecosystem functioning, with extensive research documenting their effectiveness in pollinating a wide range of plant species, particularly *Allium* spp. (Anoosha et al., 2020; Divija & Jayanthi, 2022; Soto et al., 2023).

The study findings suggest that hymenopteran pollinators, particularly members of the Apidae family, especially *A. cerana*, significantly influence pollination dynamics and subsequent TSS production within tropical agroecosystems. Research on the feasibility and perception of TSS technology further reinforces the benefits and practicality of utilizing it for shallot farming (Rahayu et al., 2019). Additionally, TSS offers advantages over traditional seed bulbs, including lower volume requirements, simpler storage and transportation, and healthier plant yields (Hasanah et al., 2022; Marpaung et al., 2023).

Understanding the specific contributions of pollinators to the TSS pollination process is crucial for increasing both productivity and quality. Conservation initiatives should prioritize preserving efficient pollinator species and implementing management strategies that promote their proliferation and activity, reinforcing crop yields and seed quality in onion cultivation. Recent studies have highlighted the effectiveness of floral plantings in bolstering wild bee populations and improving pollination services, particularly for crops reliant on insect pollination, emphasizing the importance of meticulous planning concerning planting dimensions, geographic positioning, and landscape attributes to maximize crop pollination efficacy (Blaauw & Isaacs, 2014).

Solitary bees and other native pollinators are vital components of agricultural ecosystems, conferring significant economic and ecological advantages to agricultural sectors and indigenous habitats. Consequently, activities to mitigate habitat degradation and loss are imperative to preserve environments crucial for sustaining these invaluable pollinator populations (Kline & Joshi, 2020).

Conclusion

Insect pollinators play a vital role in ensuring the viability of true shallot seeds in tropical agroecosystems. Their activity directly influences both the quantity and quality of TSS produced. Among the diverse pollinator community, *Apis cerana* stands out as a particularly effective pollinator, suggesting its potential for managed pollination services. However, the study also highlights the vulnerability of the pollination system to agricultural intensification and the need for conservation efforts to maintain pollinator diversity. These findings have significant implications for shallot cultivation practices and pollinator management strategies in tropical regions, emphasizing the need to integrate pollinator conservation into agricultural systems to ensure sustainable and high-quality TSS production.

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Original article (Orijinal araştırma)

Investigation of insecticide residues in fig and health risk assessment[1]

İncirlerde insektisit kalıntılarının araştırılması ve sağlık risk değerlendirmesi

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Abstract

This study aimed to detect insecticide, acaricide, and nematicide residues in 92 fresh and 50 dried fig samples collected from locations with intensive fig, *Ficus carica* L. (Rosales: Moraceae) production in Aydın, Türkiye in 2022. The analysis method was validated according to SANTE 11312/2021 guidelines. The recoveries ranged between 70% and 120%, with repeatability (RSDr) and within-laboratory reproducibility (RSDwR) ≤ 20% and expanded measurement uncertainties below 50% for all insecticides indicating satisfactory analytical performance. In total, 114 different insecticides were screened, revealing residues in 27 samples. Bifenazate was detected in 13 samples, etoxazole in 3 samples, spiromesifen in 5 samples, and both bifenazate and spiromesifen in 6 samples. No detectable residues were found in the dried fig samples. All samples in which bifenazate was detected were above the European Union Maximum Residue Limits (EU-MRL). Etoxazole and spiromesifen, unauthorized in figs, showed etoxazole residues above EU-MRLs, while spiromesifen residues were below EU-MRLs. The acute and chronic health risk indices for the insecticides were found to be below 1, indicating low health risks associated with fig consumption. The risk assessment suggests that fig consumption is safe for consumers.

Keywords: Dried fig, fig, LC-MS/MS, pesticide residue, QuEChERS

Öz

Bu çalışma 2022 yılında Aydın ilinde yoğun incir, *Ficus carica* L. (Rosales: Moraceae) üretiminin yapıldığı lokasyonlardan alınan 92 yaş ve 50 kuru incir numunesinde insektisit, akarisit ve nematisit kalıntılarının tespiti için gerçekleştirilmiştir. Analiz metodu SANTE 11312/2021'e göre doğrulanmıştır. Ortalama geri kazanımlar %73,2 ile %119,6 arasında olup yöntemin tekrarlanabilirliği (RSDr) ve laboratuvar içi tekrar üretilebilirliği (RSDwR) için ≤ %20 ve genişletilmiş ölçüm belirsizlikleri %50'nin altındadır. Bu veriler tatmin edici analitik performansı göstermektedir. Toplamda 114 farklı insektisitin tarandığı çalışmada, 92 yaş incir numunesinin 13'ünde bifenazate, 3'ünde etoxazole, 5'inde spiromesifen, 6'sında ise hem bifenazate hem spiromesifen olmak üzere toplam 27 numunede pestisit kalıntısı kaydedilmiştir. Kuru incir örneklerinde tespit edilebilir kalıntıya rastlanmamıştır. Bifenazate tespit edilen örneklerin tamamı Avrupa Birliği Maksimum Kalıntı Limitlerinin (AB-MRL) üzerindedir. Etoxazole ve spiromesifen etken maddeleri incirde ruhsatsız olup, numunelerde tespit edilen etoxazole kalıntıları AB-MRL üzeri, spiromesifen ise AB-MRL altıdır. Tespit edilen insektisitlerin akut ve kronik sağlık risk indeksi 1'den düşük bulunmuştur. Risk değerlendirmesi incir tüketiminin tüketiciler için güvenli olduğunu göstermiştir.

Anahtar sözcükler: Kuru incir, incir, LC-MS/MS, pestisit kalıntısı, QuEChERS

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Introduction

Fig, *Ficus carica* L. (Rosales: Moraceae) is an important agricultural product with a trademark value for Türkiye, and in 2006, it obtained a geographical indication specific to the province of Aydın. Figs are fruits that are characteristic of the Mediterranean climate, spreading from Anatolia to the whole world. Global fig production amounted to 1.321 million tons in 296 thousand hectares in 2021. Türkiye ranks first in fig production with 320 thousand tons, followed by Egypt with 211 thousand tons, and Morocco holds the third position with 144 thousand tons of production (FAO, 2021). Of the 148 thousand tons of dried figs produced worldwide in 2020/2021, 85.5 thousand (58%) were produced in Türkiye, 25 thousand tons (17%) in Iran, and 10 thousand tons (7%) in Spain. Türkiye holds the largest share of dried fig exports in Europe. The export of dried figs in 2020 amounted to about 43 thousand tons, with revenues reaching $158 million. Approximately 68 thousand tons of dried figs were exported in 2021, generating $263 million, while in 2022, exports increased to 70 thousand tons, contributing $278 million in foreign exchange earnings (Hasdemir, 2022).

The fig is affected by several important pests such as *Ceroplastes rusci* (L., 1758) (Hemiptera: Coccidae), *Tetranychus urticae* Koch,1836 (Acari: Tetranychidae), *Carpophilus* spp. Stephens, 1829 (Coleoptera: Nitidulidae), *Drosophila* spp. Fallen, 1823 (Diptera: Drosophilidae), *Otiorhynchus dauricus* Stierlin, 1862 (Coleoptera: Curculionidae), and *Poecilimon sanctipauli* Brunner von Wattenwyl, 1878 (Orthoptera: Tettigoniidae) (Akşit et al., 2003). Due to constantly changing climate and weather conditions in recent years, intensive pesticide applications are sometimes necessary to combat these pests. Although licensed (pyriproxyfen and spinosad) or temporarily licensed (azadirachtin-a, cyantraniliprole, deltamethrin, and pyrethrin) insecticides are applied against the pests (Anonymous, 2024), some producers prefer unlicensed pesticides. The use of these toxic chemicals leads to residue problems in products and can cause health issues for both applicators and consumers in the long term. Hence, different organizations regulate pesticide residues by setting maximum residue limits (MRLs), aiming to mitigate health risks (EU-MRL, 2022; TGK-MRL, 2022). The regulatory framework governing pesticide residue levels in food products within European Union countries is defined by European Parliament and Council Regulation No. 396/2005 (EC, 2005), which establishes MRLs. Türkiye has implemented the Turkish Food Codex Regulation to regulate MRLs in food products, aligning with EU regulations as part of efforts to harmonize with European Union legislation (Anonymous, 2023).

Studies on monitoring of pesticide residue in fig, are important for food safety, and these studies also help to evaluate the potential risks on consumers' health. The Rapid Alert System for Food and Feed (RASFF) reported that chlorpyrifos-methyl and cypermethrin were detected in dried figs (RASFF, 2024). Yet studies reporting on risk assessment and pesticide residues in figs are limited (Aydin & Ulvi, 2019; Soydan et al., 2021; Rosas-Sánchez et al. 2023).

This study aims to evaluate the presence of insecticide, acaricide, and nematicide residues in fig sampled in Aydın, Türkiye. For this purpose, method verification was conducted following the SANTE guidelines (SANTE/11312/2021) to detect and quantify 114 active ingredients in samples prepared for analysis with the QuEChERS (quick, easy, cheap, effective, rugged, and safe) sample preparation method. The validated method was successfully applied to analyze 92 fresh and 50 dried fig samples collected in Aydın provinces in September and October 2022. In addition, the potential health risks posed by pesticide residues in figs were evaluated.

Materials and Methods

Chemicals and reagents

The pesticide reference standards were supplied by Dr. Ehrenstorfer GmbH (Augsburg, Germany). Methanol (CH_4O) and acetonitrile (C_2H_3N), both of gradient grade for liquid chromatography (≥99.9% purity), acetic acid ($C_2H_4O_2$ >99% purity), and ammonium formate (CH_5NO_2 ≥99% purity) were procured

from Merck (Darmstadt, Germany). QuEChERS materials were purchased from Restek (Bellefonte, PA, USA). QuEChERS extraction [6 g magnesium sulfate anhydrous ($MgSO_4$ >98% purity); 1.5 g sodium acetate anhydrous ($C_2H_3NaO_2$ ≥99% purity)] and clean-up kits [1.2 g $MgSO_4$, 400 mg primary and secondary amines (PSA, 40 µm particle size) and 400 mg C_{18}] were used.

Sample collection and storage

Insecticide, acaricide, and nematicide residue analysis was conducted on 92 fresh and 50 dried figs collected from Aydın, Türkiye in 2022. Harvest-ripe fresh and sun-dried figs were gathered from five locations (Nazilli, Germencik, İncirliova, Köşk, and Bozdoğan) known for their high commercial production. The number of fresh and dried fig samples taken from Nazilli, Germencik, incirliova, Köşk and Bozdoğan are 38-19, 24-12, 12-8, 10-6 and 8-5, respectively. According to the Commission Directive 2002/63/EC, fresh fig samples from the medium-sized fruit and berry fruit category were collected weighing 1 kg (at least 10 units), while dried fig samples from the processed plant products category were collected weighing 0.5 kg (EC, 2002). Samples were promptly stored in the vehicle's refrigerator under appropriate conditions and delivered to the laboratory in polyethylene bags within a maximum of 24 hours. Upon arrival, the samples were kept in a deep freeze at -18°C until they were ready for analysis.

Sample preparation, extraction, and clean-up

The extraction and clean-up steps were performed following the QuEChERS AOAC Method 2007.01, as described by AOAC (2007). A 4-blade blender (Groupe SEB, France) was used to homogenize the fig samples. Figure 1 provides an illustration of the QuEChERS protocols. Analyses of both fresh figs and dried figs were conducted in triplicate using LC-MS/MS.

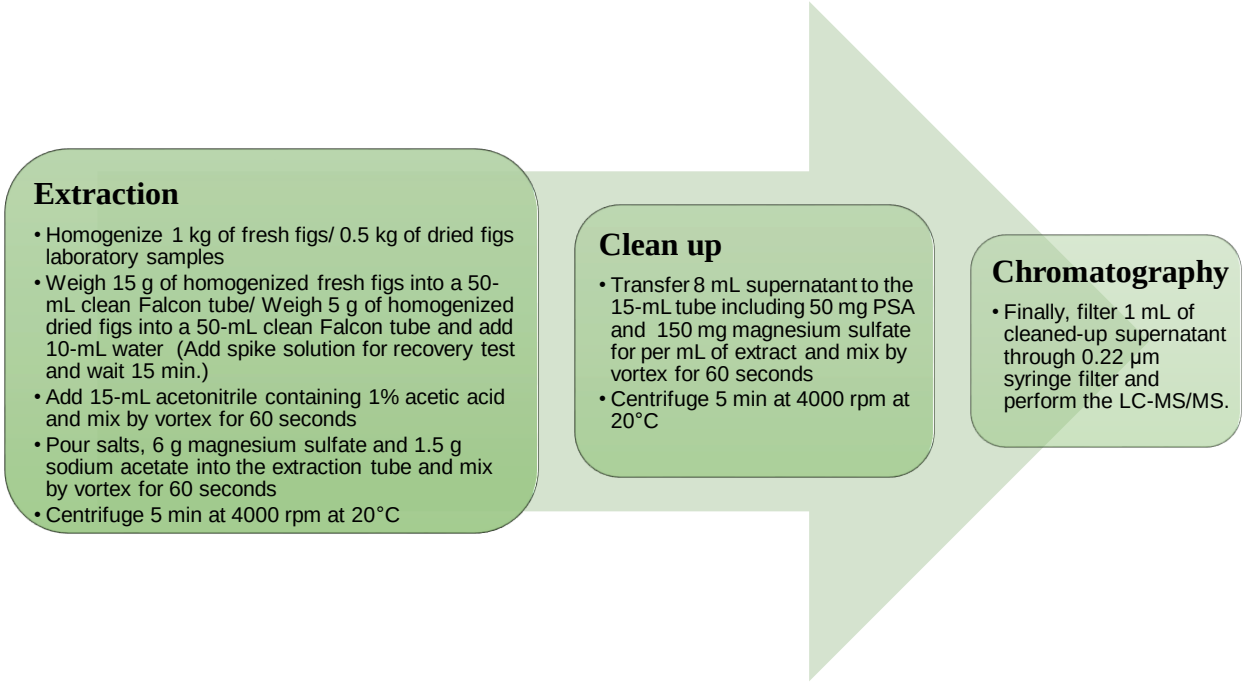

Figure 1. Analytical steps of the QuEChERS-AOAC Official Method 2007.01.

Instrumentation and optimization for LC-MS/MS

A Shimadzu UHPLC Nexera X2 system coupled with an LCMS-8050 triple quadrupole mass spectrometer equipped with electrospray ionization (ESI) was employed for the analyses. The LC column was C_{18} Inertsil (ODS-IV) column (2.1 mm × 150 mm, 3 µm particle size) (GL Sciences, Japonya). The mobile phase comprised 10 mmol/L CH_5NO_2 in distilled H_2O (A) and CH_4O (B), with a flow rate of 0.4 mL/min. Throughout the analysis, the oven temperature remained steady at 50°C, and each injection volume was set at 10 µL. Supplementary operational parameters encompassed collision-induced dissociation (CID) gas pressure set at 230 kPa, a nitrogen (N_2) drying gas flow rate of 10 L/min, nebulizer gas flow rate of 3 L/min, heating gas flow rate of 10 L/min, desolvation line temperature maintained at 250°C, interface temperature set to 300°C, block heater temperature of 400°C, capillary voltage adjusted to 4.5 kV, and dwell time ranging from 1 to 33 ms.

A total of 114 insecticides, acaricides, nematicides, and their metabolites were screened. Optimization parameters for 111 of these compounds were meticulously provided by Balkan & Yılmaz (2022a), whereas supplementary optimization parameters concerning three additional agents are delineated in Table 1. LC-MS/MS optimization employed multiple reaction monitoring (MRM).

Table 1. Optimization of LC-MS/MS parameters of 3 pesticides in the MRM mode

Analyte	Type of pesticide*	Substance group*	Molecular formula*	Retention time (min)	Ion mode	Precursor ion (m/z)	Product ion (m/z)	Dwell time (msec)	Q1 Pre Bias (V)	CE (V)	Q3 Pre Bias (V)
Bifenazate	Insecticide, Acaricide	Carbazate	$C_{17}H_{20}N_2O_3$	8.437	pos	301.10	170.0	3.0	-15.0	-14.0	-18.0
						301.10	152.1	3.0	-15.0	-38.0	-29.0
						301.10	198.1	3.0	-15.0	-6.0	-20.0
Etoxazole	Acaricide	Diphenyl oxazoline	$C_{21}H_{23}F_2NO_2$	10.753	pos	360.10	141.0	1.0	-27.0	-27.0	-25.0
						360.10	304.1	1.0	-27.0	-17.0	-20.0
						360.10	57.0	1.0	-27.0	-28.0	-22.0
Spiromesifen	Insecticide, Acaricide	Tetronic acid	$C_{23}H_{30}O_4$	10.679	pos	371.20	273.2	2.0	-18.0	-10.0	-28.0
						371.20	255.2	2.0	-25.0	-23.0	-26.0

*IUPAC Pesticides Properties DataBase (IUPAC, 2024).

Method verification

To assess recovery, 15 g of pesticide-free fig samples were spiked with mixed pesticide solutions at concentrations of 0.01 and 0.05 mg/kg, each replicated five times. This experiment was conducted weekly for five consecutive weeks with the participation of two analysts.

The analytical methods were verified following internationally recognized guidelines (EURACHEM, 2014; SANTE, 2021). Key parameters assessed included limit of detection and quantification (LOD / LOQ), linearity, recovery efficiency, precision (both repeatability; RSDr and within-laboratory reproducibility; RSDwR), measurement uncertainty (U'). The expanded U' was computed for all insecticides using Approach 2, which involves estimating a generic measurement uncertainty based on proficiency test data (PT), as recommended in the SANTE Guideline (SANTE, 2021). Further are provided in the study by Balkan & Yılmaz (2022b).

Pesticide residues in figs

One hundred and fourteen pesticides were analyzed in 92 fresh fig and 50 dried fig samples using LC-MS/MS. The detection of pesticides in these samples was confirmed based on retention time and ion ratio, as defined in the identification criteria outlined in the SANTE guidelines (SANTE, 2021).

To evaluate the potential health risks, we compared the estimated dietary exposure (per unit of body weight, bw) to established toxicological benchmarks such as the 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

assessed using the estimated short-term intake (ESTI, mg kg^{-1} d^{-1}) divided by the ARfD. Conversely, the chronic/long-term consumer health risk (chronic hazard index, cHI) was determined by comparing the estimated daily intake (EDI, mg kg^{-1} d^{-1}) to the ADI (EFSA, 2015). Following equations were used for these calculations (Liu et al.,2016);

$$ESTI = \text{the highest residue level} \times \text{food consumption} / bw \quad (1)$$

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

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

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

The average body weight of an adult was taken as 73.7 kg (TUIK, 2023). Daily fig consumption for the general population in Türkiye was reported as 19 g per day (TUIK, 2022). In risk assessment, when the Hazard Index (HI) exceeds one, it indicates potential health risks from pesticide residues to consumers (Akoto et al., 2015; Soydan et al., 2021).

Result and Discussion

Method verification

The outcomes of the method verification studies pertaining to the identified pesticides were delineated in Table 2. Satisfactory linearities were observed with correlation coefficients (R^2) from 0.990 to 0.998. The LOD and LOQ values of the analytical method were calculated in accordance with the Eurachem Guide (EURACHEM, 2014). Blank fig samples were spiked with an insecticide mixture solution at the level of 10 µg/kg and 10 replicate analyses were performed. The LOQ were found to be below the maximum residue limits (MRLs) established by the EU for figs. With the two-level spiking, the recovery rate of 70-120%, and repeatability (RSDr) and intra-laboratory reproducibility (RSDwR) ≤ 20% for insecticides, were found. Furthermore, expanded measurement uncertainties were below 50% for all insecticides. These findings underscore the efficacy of the QuEChERS method as a rapid and accurate technique for insecticide residue analysis in figs.

Table 2. Method verification parameters of detected active ingredients in fig samples

Active ingredient	LOD (µg/kg)	LOQ (µg/kg)	R^2	Repeatability (n=10)				Reproducibility (n=10)				U' %
				10 µg/kg		50 µg/kg		10 µg/kg		50 µg/kg		
				Rec.%	RSDr%	Rec.%	RSDr%	Rec.%	RSDwR%	Rec.%	RSDwR%	
Bifenazate	1.23	4.11	0.995	102.17	16.01	99.91	4.80	105.37	10.91	99.00	4.49	20.03
Etoxazole	1.68	5.60	0.998	84.93	3.75	105.34	2.01	74.55	4.32	95.28	2.94	15.58
Spiromesifen	2.09	6.97	0.990	113.26	16.78	107.17	8.72	104.02	14.03	108.86	6.41	27.04

Rec: Recovery; LOD: limit of detection; LOQ: limit of quantification; U': expanded measurement uncertainty.

Residue analyses in figs

Insecticide residue analysis was conducted on fig samples collected from the districts of Nazilli, Germencik, İncirliova, Köşk, and Bozdoğan in Aydın province. A total of 142 fig samples were analyzed. In the study, a total of 114 different insecticides were monitored, and no detectable residues were found in dried fig samples. However, insecticide residues exceeding the limit of quantification (LOQ) were detected in 27 out of 92 samples. The number of fresh fig samples exceeding the EU-MRLs was 16 (Table 3).

Table 3. Pesticides residues (mg/kg) detected in figs

Food commodity	Number of samples >LOQ and percentage (%)	Number of samples >MRL and percentage (%)	Pesticide	Frequency of detection	Pesticide residue range (mg kg^{-1})	Number of samples >MRL	MRL* (mg kg^{-1})
Fig	27 (29.3)	16 (17.4)	Bifenazate	19	0.013-0.153	13	0.02
			Etoxazole	3	0.063-0.031	3	0.01
			Spiromesifen	11	0.010-0.011	-	0.02
Dried fig	-	-	-				

*EU-MRL.

During the period from August 2 to September 30, 2022, bifenazate received temporary approval from the Ministry of Agriculture and Forestry of the Republic of Türkiye for use in figs (Hasdemir, 2022). However, etoxazole and spiromesifen are not authorized for figs. Residue levels exceeding the EU-MRL were detected for bifenazate in 13 samples and for etoxazole in 3 samples (Table 3). These findings were evaluated in accordance with EU-MRL standards. These results were assessed based on EU-MRL. The occurrence of bifenazate residues above the MRL may be attributed to factors such as repeated applications, excessive dosages, or non-compliance with the time interval between the last pesticide application and harvest. Nevertheless, the use of unauthorized pesticides may also be attributed to producers' unawareness and a solution-focused approach.

The studies regarding pesticide residue in figs are quite limited. Tatlı (2006) investigated pesticide residues in 5 fresh and 10 dried fig samples produced in the Aegean Region and reported that no pesticide residues were found in the fresh and dried fig samples. Aydin & Ulvi (2019) determined only two insecticides, chlorpyriphos and malathion, in 2 of 14 dried fig samples. Soydan et al. (2021) investigated pesticide residues in 900 dried fig samples in their study in the Aegean Region. They reported that no residues above LOD were found in 846 of these figs. They found residues exceeding EU-MRLs in 38 samples, while 16 samples had residue levels equal to or below the MRLs. Their residue analysis identified various active ingredients, including α-cypermethrin, α-endosulfan, bromopropylate, carbendazim, chlorpyrifos, chlorpyrifos-methyl, cypermethrin, deltamethrin, dichlorvos, dimethoate, λ-cyhalothrin, lufenuron, malathion, metalaxyl m, methoxychlor, permethrin, and triadimefon. Additionally, they found one pesticide in 48 samples, two pesticides in three samples, and five or more pesticides in three samples. However, in our study, no pesticide residues above the limit of detection (LOD) were found in dried figs.

Health risk assessment

Pesticide hazard evaluations have attracted considerable consumer attention in recent years, particularly in Türkiye (Çatak & Tiryaki, 2020; Soydan et al., 2021; Balkan & Kara, 2022; Serbes & Tiryaki, 2023). Health risk analyses were performed for three pesticides (Table 4). The calculations revealed that both acute (aHI) and chronic (cHI) health risk values were below 1, indicating the absence of health risks.

Table 4. Health risk estimation of insecticides residues in figs 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
Bifenazate	0.01	0.1	3.98E-06	0.00398	1.16E-06	0.0116
Etoxazole	0.04	/	8.14E-06	/	3.95E-06	0.0099
Spiromesifen	0.03	2	2.86E-07	0.00001	2.60E-07	0.0009

* ADI and ARfD values are from the IUPAC Pesticides Properties DataBase (IUPAC, 2024).

Literature regarding health risk assessment in figs is extremely limited. Rosas-Sánchez et al. (2023) conducted a study and did not find any health risk associated with the insecticides cypermethrin and permethrin in figs in Mexico.

Conclusion

This study investigates the presence of insecticide, acaricide, nematicide, and their metabolite residues in figs sourced from local markets in Aydın, Türkiye, in 2022. Furthermore, it evaluates the health risk posed by these residues to consumers. The research highlights that implementing conscientious and sustainable agricultural practices to reduce pesticide residues in figs. This encompasses the promotion of integrated pest management (IPM) strategies and the exploration of alternative pest control methodologies. The study underscores the necessity of strict adherence to pesticide residue regulations and standards within the agricultural sector. It encourages the agricultural industry to prioritize consumer safety and comply with established maximum residue limits (MRLs) for pesticides. The findings highlight the importance of ongoing research and monitoring of pesticide residues in figs and other agricultural products. This supports the continuous improvement of agricultural practices and ensures the safety of food products for consumers. Overall, the research has implications for promoting consumer awareness, encouraging responsible agricultural practices, and fostering regulatory compliance within the fig industry in Aydın, Türkiye.

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Original article (Orijinal araştırma)

Environmental persistence of the conidia of native entomopathogenic fungi and their efficiency on *Ceratitis capitata* (Wiedemann, 1824) (Diptera: Tephritidae)[1]

Yerel entomopatojen fungus konidialarının çevresel kalıcılığı ve *Ceratitis capitata* (Wiedemann, 1824) (Diptera: Tephritidae) üzerindeki etkinliği

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Abstract

This study aimed to investigate the effects of local *Beauveria bassiana* (Balsamo) Vuillemin and *Cordyceps fumosorosea* (Wize) (Hypocreales: Cordycipitaceae) isolates on *Ceratitis capitata* (Wiedemann, 1824) (Diptera: Tephritidae) prepupae, during 2020 and 2021 in the Mediterranean Region of Türkiye. In petri dishes, mortality rates varied with doses: for LD.2016 (*B. bassiana*), between 30 and 60%, for M6-4 (*B. bassiana*), 30-46%, of IFR (*C. fumosorosea*), 65-100%. Additionally, differences were detected between the prepupa and pupa periods of the larvae depending on the isolate. In sterile soil studies, the IFR caused mortality rates of 53-93%, while those for the LD.2016 isolate were 20-55%, depending on the doses. In conidial survival studies, the IFR caused mortality rates of 70-93% at 0 months and 44-60% in the 12th month. The LD.2016 mortality was among 37-55% at 0 months, while mortality rates decreased to 2% in the 12th month. The reproductive capacity of adult individuals that emerged alive after infection was negatively affected depending on time and fungal isolate. In semi-controlled field conditions, the IFR isolate had mortality rates varied between 40 and 65%, and LD.2016 mortality rates varied between 34 and 62%, depending on doses. It was observed that the fungi used in the study had potential in biological control of *C. capitata*.

Keywords: *Beauveria bassiana*, *Cordyceps fumosorosea*, reproductive capacity, soil surface application, sublethal effects

Öz

Bu çalışmada, 2020-2021 yıllarında Türkiye Akdeniz Bölgesi'nde yerel *Beauveria bassiana* (Balsamo) Vuillemin ve *Cordyceps fumosorosea* (Wize) (Hypocreales: Cordycipitaceae) izolatlarının *Ceratitis capitata* (Wiedemann, 1824) (Diptera: Tephritidae) prepupaları üzerindeki etkilerinin araştırılması amaçlanmıştır. Petri kaplarında ölüm oranları dozlara göre; LD.2016 (*B. bassiana*) için %30-60 arasında, M6-4 (*B. bassiana*) için %30-46 arasında, IFR (*C. fumosorosea*) için %65-100 arasında değişmiştir. Ayrıca, izolatlara bağlı olarak larvaların prepupa ve pupa dönemleri arasında farklılıklar tespit edilmiştir. Steril toprak çalışmalarında IFR %53-93 arasında mortaliteye neden olurken, LD.2016 izolatı için bu oran doza bağlı olarak %20-55 olmuştur. Konidial hayatta kalma çalışmalarında IFR 0. ayda %70-93, 12. ayda ise %44-60 arasında mortaliteye neden olmuştur. LD.2016 mortalitesi 0. ayda %37-55 arasında iken, 12. ayda mortalite oranları %2'ye düşmüştür. Enfeksiyondan sonra canlı çıkan ergin bireylerin üreme kapasitesi zamana ve fungal izolata bağlı olarak olumsuz etkilenmiştir. Yarı kontrollü arazi koşullarında IFR izolatında mortalite oranları doza bağlı olarak %40-65 arasında, LD.2016 mortalite oranları ise %34-62 arasında değişmiştir. Çalışmada kullanılan fungusların *C. capitata*'nın biyolojik mücadelesinde potansiyel taşıdığı görülmüştür.

Anahtar sözcükler: *Beauveria bassiana*, *Cordyceps fumosorosea*, üreme kapasitesi, toprak yüzey uygulaması, ölümcül olmayan etkiler

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Introduction

Terrestrial ecosystems are characterized by a wide variety of species and the diversity of interactions between these species (Tscharntke & Hawking, 2000). The fungi kingdom, which is referred to as the 'communication networks of the forest' in the book 'Entangled Life' due to their biology (Sheldrake, 2020), contains an important group called 'entomopathogenic fungi (EPF)' within the ecosystem. The majority of EPF are pathogenic to insects in nature (Shah & Pell, 2003; Scholte et al., 2004; Vega et al., 2009; Dash et al., 2018) and they exhibit a high degree of efficiency in infecting their hosts to manage the pest population (Ortiz-Urquiza & Keyhani, 2013; Vidal & Jaber; 2015; Lu & St Leger, 2016). EPF have attracted attention in multitrophic studies in recent years because of their many positive aspects such as contribution to plant nutrition and soil improvement, support to the plant kingdom in the competitive/antagonistic relationships between plant pathogenic fungi, and lack of toxic effects (O'Callaghan et al., 2022; Quesada-Moraga et al., 2022). EPF not only kill arthropods in nature, but also contribute to natural regulation by changing their reproductive abilities (Castrillo et al., 2000; Quesada-Moraga et al., 2006; Ullah & Lim, 2017) and their spores and toxins have been used in biological control for many years (Villaseñor et al., 2019).

Beauveria bassiana (Balsamo) Vuillemin (Hypocreales: Cordycipitaceae) has been the most studied entomopathogenic fungus species as a biological control agent for many years after it was first detected on silkworms (Muscardine diseases) (Feng et al., 1994; Zimmermann, 2007). *Beauveria bassiana* is very common in soil in agricultural ecosystems. It has a wide host range and is widespread throughout the world (Roberts & St. Leger, 2004; Rehner & Buckley, 2005). While *Cordyceps fumosorosea* (Wize) (formerly *Isaria fumosorosea* or *Paecilomyces fumosoroseus*) (Hypocreales: Cordycipitaceae), which is in the same family with *B. bassiana*, was not mentioned among the important species in the past (Zimmermann, 2008). It has been included in the list as an important microbial control agent due to its effectiveness in recent years (Zimmermann, 2008; Villaseñor et al., 2019). Commercial preparations of both species are used as biological control agents nowadays.

Entomopathogenic fungi can be found in every layer of the ecosystem (Behie et al., 2015). The fungi are shielded from UV radiation and are able to withstand biotic and abiotic stresses thanks to the protective qualities of the soil environment, which prolongs their viability (Samson et al., 1988; Keller & Zimmermann, 1989). In general terms, soil is the natural reservoir of entomopathogens, where bacteria and fungi can live as saprophytes and can colonize insect cadaver parts and organic materials (Solter et al., 2017). This situation supports the idea that applying fungal propagules to the soil surface may be a good strategy in the biological control of subsoil pests that are difficult to control and harmful insects that spend any biological stage in the soil (Jackson et al., 2000). The persistence of fungal spores in the environment is important for the success of the management. Conidial environmental persistence is essential for the spread of fungi in ecosystems and also serves as a determinant for the biocontrol effectiveness of EPF during integrated pest management (Ding et al., 2023).

While research continues on the possibilities of management of many pests with EPF; researchers, in addition to chemical control studies (Yeşilırmak et al., 2024), have reported that EPF has a lethal effect on different biological stages of an important quarantine pest, *Ceratitis capitata* (Wiedemann, 1824) (Diptera: Tephritidae), which spends its pupation period in the soil. In addition to the studies focused on the pupa and adult stages of *C. capitata* (Castrillo et al., 2000; Dimbi et al., 2003; Quesada-Moraga et al., 2006; Ekesi et al., 2010; Beris et al., 2013; Qazzaz et al., 2015; Chergui et al., 2020; Gava et al., 2020; Soliman et al., 2020; Hallouti et al., 2021), in some other articles it was mentioned that 3rd larval instar was sensitive to *B. bassiana* (Chergui et al., 2020; Gava et al., 2020; Soliman et al., 2020). It was also determined that the fungus changed their flight activities, feeding and sexual behavior, (Dimbi et al., 2009; Bernardo & Singer, 2017), and decreased fertility (Quesada-Moraga et al., 2006) when it was applied to adult individuals. It shows the versatile effects of EPF in the biological control of *C. capitata*. Although melanization and sclerotization in pupae pose major challenges to infection by entomopathogens, larvae and pupae of *C. capitata*

are subject to EPF infection in soil (Dias et al., 2018). The biological period delay ultimately contributes to reducing the population and is an important support for the success of the pest management. For this reason, microbial pathogens are evaluated as an alternative to chemical insecticides due to their high pathogenicity and the fact that they cause delays in biological periods. Therefore, mortality alone should not be used to evaluate effective control (Hussain et al., 2009).

The efficiency rate of each EPF isolate may differ from each other. The sensitivity rates of late-stage larvae may vary compared to pupae and adults at the first moment when *C. capitata* comes into contact with soil, which is an important fungal reserve. However, there is no practical soil applications using EPF in the management of *C. capitata*. The ability of fungi to persist in soil affects the persistence of fungal pathogenicity and this is important for sustainable pest management. For this reason, it is necessary to determine the level of response of pests that visit the application area at different times by the EPF propagules in the environment (Gava et al., 2020).

The present study was initiated at the Department of Plant Health in Fruit Research Institute, Isparta, Türkiye, in order to investigate the influence of native isolates *Beauveria bassiana* and *Cordyceps fumosorosea*, on 3rd instar larvae of *C. capitata* by determining indices of growth times and mortality rates. We investigated whether fungal infection with different densities of conidiospores affect the duration of prepupa and pupation period and survival rate of third instar larvae in the *C. capitata*. Additionally, the objective of this work was not only to select virulent EPF isolates among local strains to control *C. capitata* during the cryptic life cycle in soil, but also to investigate the effects of exposure to EPF at different times, on postemergence survival of adults and oviposition of them under controlled conditions. In addition, lethal effect of EPF under field (semi-controlled) conditions was studied for two years.

Materials and Methods

Ceratitis capitata culture

Ceratitis capitata culture was maintained in climatized rooms with 25±1ºC temperature, 65±10% humidity and 16: 8 hours light and dark conditions in the Fruit Research Institute. Golden delicious apple fruits were used for the mass rearing of *C. capitata*.

Entomopathogenic fungi culture

Beauveria bassiana (Bals.) Vuill (BMAUM M6-4 and BMAUM LD.2016) isolates, which were found to be highly pathogenic, as a result of the preliminary pathogenicity test, were provided from the investigation of Baydar et al. (2016). *Cordyceps fumosorasea* (IFR) isolate were also obtained from stock culture of Center of Biological Management Research and Apply (BMAUM) at University of Applied Science. Entomopathogenic fungus isolates used in the experiment were cultured on Potato Dextrose Agar (PDA) medium in the Phytopathology laboratory using a sterile cabinet and an incubator and the isolates stored-80ºC conditions.

Preparation of fungal suspensions

Conidiospores were extracted using a scraper from the 10 to 14 days old fungal cultures in Petri dishes with PDA incubated at 25ºC. Conidiospores of each isolate were suspended in 50 mL sterile distilled water amended with 0.01 mL Tween 80. In order to achieve densities of $1x10^7$, $1x10^6$, $1x10^5$, and $1x10^4$ conidia/mL, the initial suspension with a density of $1x10^8$ conidiospore/mL was diluted by one-tenth for each new concentration. Spore counts were checked by using a Thoma hemacytometer.

Petri dishes bioassay

The experiment was set up in 9 cm diameter petri dishes to assess the percentage of lethality and duration of lethality of *B. bassiana* (LD.2016 & M6-4) and *C. fumosorosea* (IFR) on 3rd instar larvae of *C. capitata*.

A double layer of filter paper was placed on the inner surface of each petri dish, leaving no gap at the bottom. 2 mL of the suspensions with densities of $1x10^8$, $1x10^7$, $1x10^6$, $1x10^5$, $1x10^4$ conidia/mL were applied on filter papers. For control group, the same volume of distilled water with 0.01 mL Tween 80 was used. All treatments were applied with a hand spray from a 20-cm distance. The prepared insect diet (*Cydia pomonella* diet-Southland Products® Arkansas, USA), a piece of roughly 1 cm^3, was placed in the center of the filter paper to prevent the larvae from dying of malnutrition. The last instar larvae, which emerged from the fruit to enter the pupa period, were placed on an insect diet. The treatment consisted of 5 replications for each density, with 20 late-stage larvae were used in each replication. The treatment was conducted in climatic chambers at 25ºC, 65% R.H, and 16: 8 (L: D). As a result of determining the mortality rates in bioassay studies larval and pupal development periods of surviving individuals were monitored and recorded daily in the same conditions.

Conidial persistence studies

The soil used in the planting of saplings in Eğirdir Fruit Research Institute was sterilized in an oven at 105ºC for 48 hours in case it contained live microorganisms before the first treatment. The lids of 10x12x5 cm -sized plastic boxes were cut and covered with tulle, and 50 g of sterile soil was placed into these plastic containers. As a result of petri bioassay, the most effective two EPF isolates' $1x10^8$, $1x10^7$, $1x10^6$ conidia/mL of the spore suspensions were applied to soil with 5 replicates and 1cm^3 insect diet was placed at the center of the plastic container on the soil. The last instar larvae, which were emerging from the fruit for enter the pupa period, were placed on the insect diet in the trial container. 20 third instar larvae were used for each dose and replicates. In all replicates, containers were checked until all individuals reached adult stage and the data were recorded.

This experiment was named '0^{th} month' after application of EPF spores to the soil surface. EPF was not applied to these plastic containers again for subsequent trials for determination the conidial persistence, but 2 mL of sterile water was sprayed to provide moisture before the treatments. The experiment was repeated at 3^{rd}, 6^{th}, 9^{th} and 12^{th} months. This was done by adding 20 late-stage larvae to each replication container at 3-month intervals. The containers were checked daily until all individuals reached the adult stage and data were recorded to determine conidial persistence. The treatment was conducted in climatic chambers at 25ºC, 65% R.H, and 16: 8 (L: D).

Detecting adult infertility

Individuals that became adults in the plastic containers used in the sterile soil application experiments were transferred to different insectariums for each application, to examine their reproduction. In the experiment, number of adult emergence and dates were recorded starting from the emergence day of the first individual. Golden delicious fruits were given to the individuals in the population every day so that they could lay eggs, and those given the previous day were taken from the insectarium. Fruits where eggs were thought to have been laid were placed into double layer insect netted insectarium of different sizes which intertwined to prevent contamination from flies outside (if present) to monitor larval development in climate chambers.

Field trials (semi-controlled conditions)

During field trials, apertures were cut into the lids of 2-liter plastic sealed containers with a utility knife and closed with tulle. The soil taken from the Antalya region was put in the plastic containers up to a height of 4-5 cm without any application. The suspensions of IFR and LD.2016 isolates with $1x10^8$, $1x10^7$, $1x10^6$ conidia/mL densities prepared in the laboratory the day before and 6 ml of these suspensions were applied to the soil surface from a distance of 20 cm with the help of a hand spray for each container. 1 cm^3 insect diet was placed in the center of the boxes and 20 late-stage larvae were added to the containers. After the emergence of adults from the soil, the soil used in the experiments was examined and the numbers of empty pupal capsules and dead *C. capitata* adults were evaluated to obtain the mortality rates. The experiment was conducted with 5 replications for each concentration and control group in field conditions of Antalya province.

Statistical analyses

The obtained data were used to determine the actual mortality values (% effect) by applying the Abbott formula (Abbott, 1925);

$$\text{Corrected mortality rate (\%)} = \frac{\text{Mortality rate of treated larvae} - \text{Mortality rate of control}}{100\text{x Mortality rate of control}}$$

The mortality rates were first subjected to Levene's homogeneity test, Shapiro-Wilk's normality test was used and then one-way analysis of variance (One-Way ANOVA) was applied. Then, Tukey multiple comparison test (Tukey, 1949) was performed to determine the source of differences. Differences were considered significant at $p<0.05$. Statistical analyses were performed with the help of IBM SPSS® Statistics (Version 23.0, 2015, IBM SPSS, Armonk, New York, USA) (IBM SPSS, 2015).

Results and Discussion

Petri dishes bioassay result

Mortality data obtained at the end of the trial are shown in Figure 1. Although doses of $1x10^8$ conidia/mL and $1x10^7$ conidia/mL of IFR caused 100 percent mortality, the mortality rates in subsequent doses were 73%, 65% and 65%, respectively. The mortality of the doses of the LD.2016 isolate was 60%, 53%, 46%, 40% and 30%, respectively. The mortality rates of the M6-4 isolate according to the doses were 46%, 40%, 40%, 30% and 30% from highest to lowest. In the control group, all individuals remained alive at the end of the experiment.

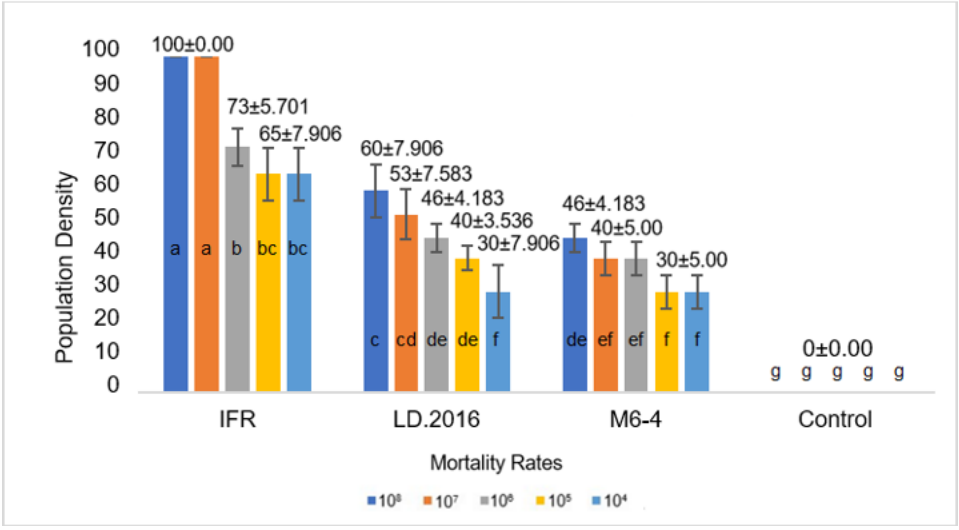

Figure 1. Mortality rates (±std. dev.) of *Cordyceps fumosorosea* (IFR) and *Beauveria bassiana* (LD.2016 & M6-4) isolates on 3rd larva of *Ceratitis capitata*. *According to Tukey's multiple comparison test, different letters are statistically different ($p<0.05$). (F: 110,493; df:15,64; p<000.1).

The petri dishes bioassay was designed to be studied in petri dishes to clearance whether the larval period in which the 3rd instar larvae that spend their pupa period in the soil, are most sensitive to pathogens, is delayed. Because the soil wasn't suitable for visible the development stages, however, it is easier to observe the biological process in petri dishes. Chergui et al. (2020), reported that petri dishes bioassays had higher mortality compared to the sterile soil experiment with their study of *C. capitata* larvae-EPF interaction bioassays both petri dishes and sterile soil. Previous sterile soil studies reported high mortality rates on *C. capitata*. The findings were consistent with the findings of other research (Ekesi et al., 2005; Quesada-Moraga et al., 2006; Chergui et al., 2020) that found that the larval stage of *C. capitata* was the most susceptible to EPF.

The mortality times of larvae in petri dishes and the effects of the isolates on the pupal stages of surviving individuals were determined. In the application of $1x10^8$ conidia/mL dose of IFR isolate, larval deaths due to infection occurred from the 3rd day to the 7th day after the treatment. On the 3rd day, 25 individuals died totally and reducing the population to 75 individuals; on the 5th day of IFR application, the population were 5 individuals and on the 7th day, all individuals died.

After the treatment of $1x10^8$ conidia/mL dose of LD.2016 isolate, larval deaths occurred from the 3rd day to the 13th day after the treatment. However, all of the individuals (40 individuals) that will emerge as adults on the 13th day have entered the pupation period (Figure 2). These individuals completed their pupation period in 2 days, becoming 27 pupae on the 12th day and 40 pupae on the 13th day. The first adult emergence was seen on the 25th day of the experiment, and emergence continued until the 31st day (Figure 3). In the application of $1x10^8$ conidia/mL dose of M6-4 isolate, infection-related deaths started on the 11th day and continued until the 17th day, and 54 individuals entered the pupal stage (Figure 2). The pupation process, which started on the 14th day of the experiment, ended on the 17th day, taking a total of 3 days. The pupal stage lasted until the 29th day, while adult emergence ended on the 35th day of the experiment (Figure 3).

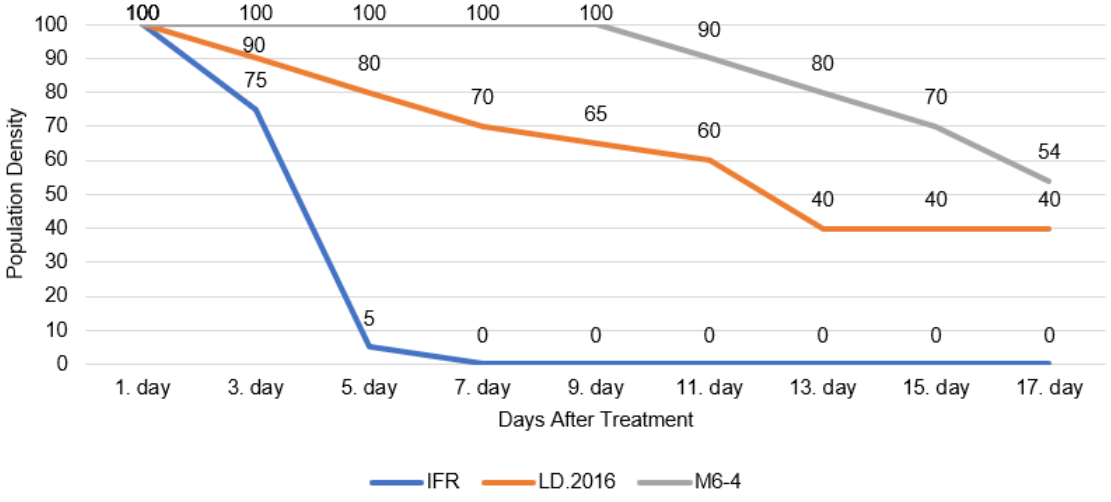

Figure 2. Time-dependent change of the *Ceratitis capitata* population applied with $1x10^8$ conidia/mL.

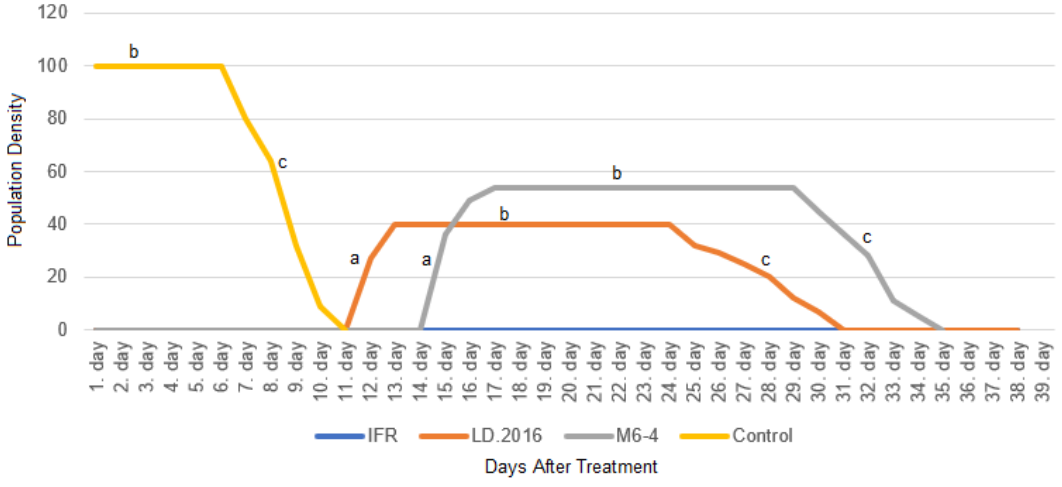

Figure 3. Time-dependent population fluctuation of the pupal stages of individuals that emerged as adults after $1x10^8$ conidia/mL applications of all isolates. (a: entry into the pupal stage, b: the entire population is in the pupal stage, c: adult *Ceratitis capitata* emergence).

Individuals exposed to the IFR dose of 10^7 conidia/mL died within 7 days, similar to the dose of $1x10^8$ conidia/mL. After the application of 10^7 conidia/mL dose of LD.2016 isolate, larval deaths due to infection occurred from the 3rd day to the 17th day. However, on the 17th day, all of the individuals (47 individuals) that will emerge as adults entered the pupal period (Figure 4). These individuals completed their pupation period from in 1 day. The first adult emergence was seen on the 23rd day of the treatment, and adult emergence continued until the 29th day (Figure 5). In the M6-4 isolate, infection-related deaths started on the 9th day and continued until the 17th day, and 60 individuals entered the pupal stage (Figure 4). The pupation process, which started on the 16th day of the experiment, ended on the 17th day, taking a total of 1 day. The pupal stage lasted until the 29th day and adult emergence ended on the 35th day, which took 6 days in total (Figure 5).

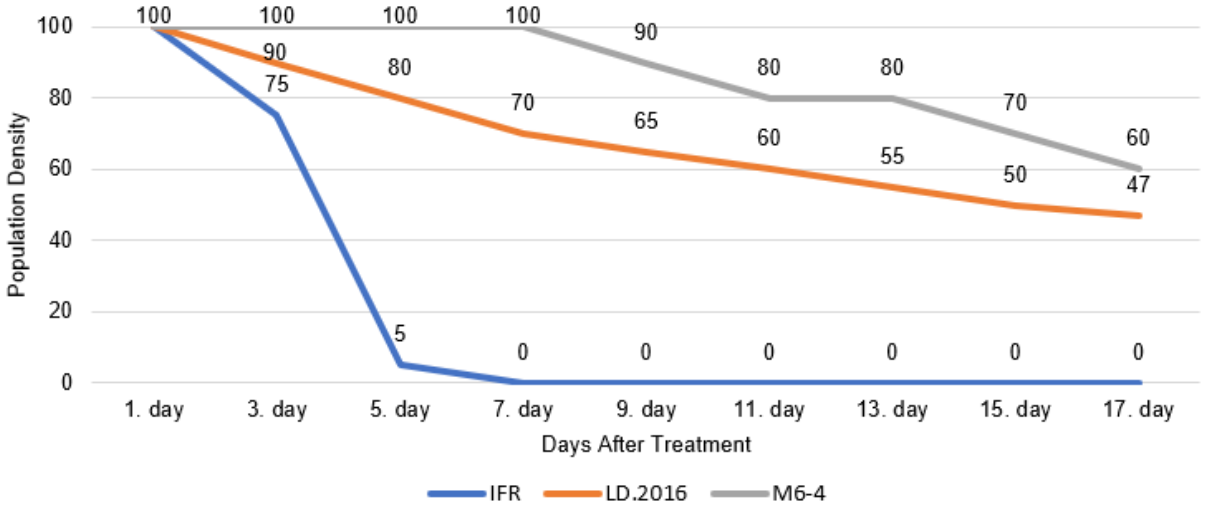

Figure 4. Time-dependent change of *Ceratitis capitata* population applied with $1x10^7$ conidia/mL.

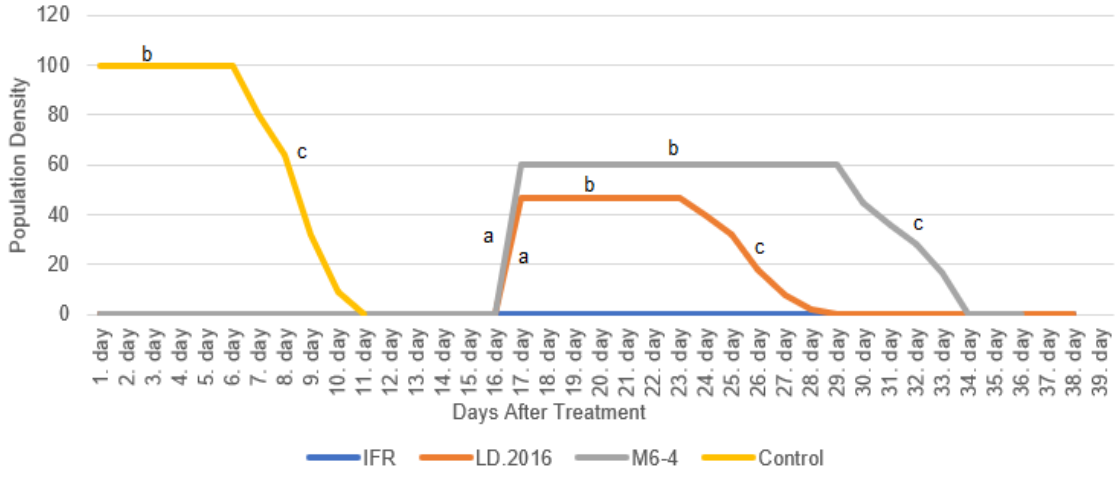

Figure 5. Time-dependent population fluctuation of the pupal stages of individuals that emerged as adults after $1x10^7$ conidia/mL applications of all isolates. (a: entry into the pupal stage, b: the entire population is in the pupal stage, c: adult *Ceratitis capitata* flights).

After applying a dose of $1x10^6$ conidia/mL of the IFR isolate, larval deaths due to infection occurred from the 5th day to the 11th day (Figure 6). Pupation started on the 10th day and was completed on the 13th day with 27 individuals. Adult emergence continued from the 17th to the 20th day (Figure 7). After the application of $1x10^6$ conidia/mL dose of LD.2016 isolate, larval deaths due to infection occurred from the 11th day to the 17th day. However, all of the individuals (54 individuals) that will emerge as adults on the 17th day have

entered the pupal period (Figure 6). These individuals completed their pupation period from day 16 to day 19 in 3 days. The first adult emergence was seen on the 23rd day of the experiment, and flights continued until the 29th day (Figure 7). In the M6-4 isolate, infection-related deaths started on the 11th day and continued until the 15th day, and 60 individuals entered the pupal stage (Figure 6). The pupation process, which started on the 16th day of the experiment, ended on the 19th day, taking a total of 3 days. Adult emergence from the pupal stage, which lasted until the 24th day, ended on the 32nd day, a total of 6 days (Figure 7).

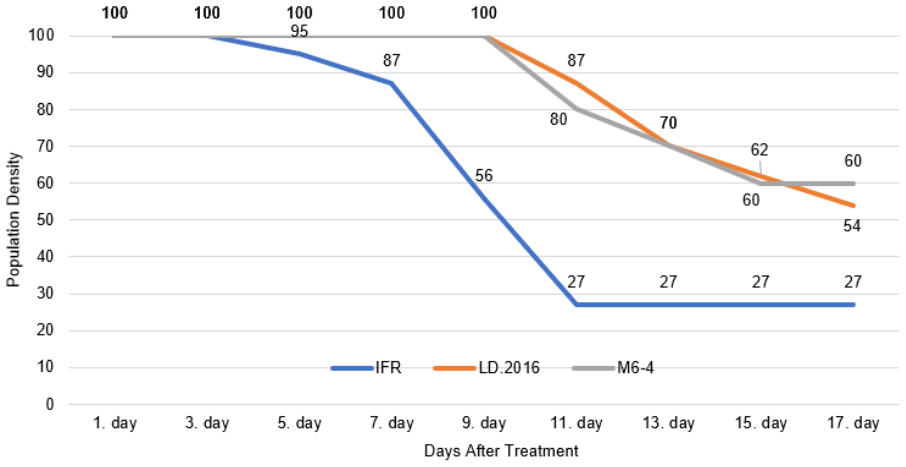

Figure 6. Time-dependent change of the *Ceratitis capitata* population applied with 1x10^6 conidia/mL.

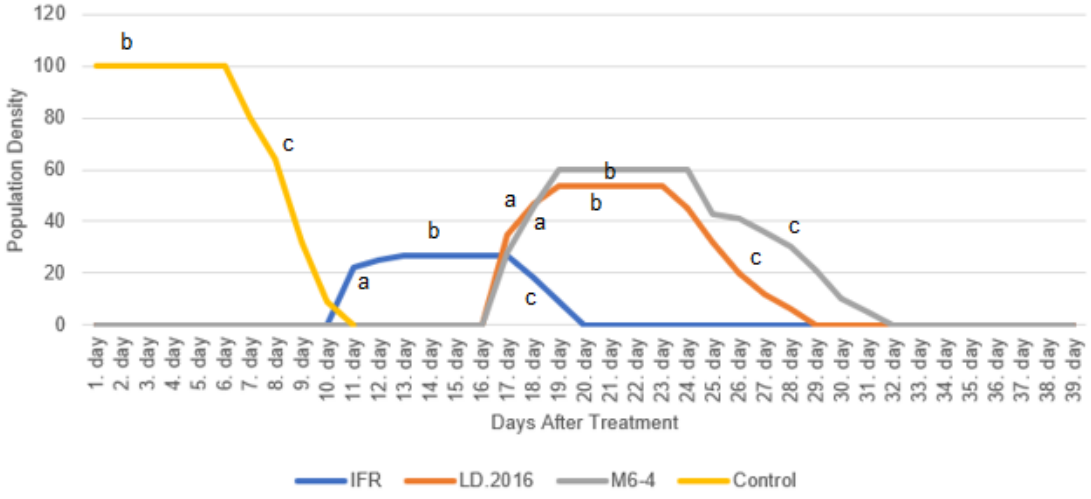

Figure 7. Time-dependent population fluctuation of the pupal stages of individuals that emerged as adults after 1x10^6 conidia/mL applications of all isolates. (a: entry into the pupal stage, b: the entire population is in the pupal stage, c: adult *Ceratitis capitata* flights).

In the control group of the experiment, all larvae treated with water became pupae within the first two hours of the experiment. Adult flights were recorded from the 7th to the 11th day. The pupal period was determined as 7 days for 20 individuals, 8 days for 16 individuals, 9 days for 32 individuals, 10 days for 23 individuals and 11 days for 9 individuals. In total, the pupal period of the population ended in 11 days. Sevinç & Karaca (2024) reported the differences between the developmental periods of individuals laid on the same day. According to the study, the period from egg to adult varied between 18 and 55 days. In this study, only the pupal period lasted between 7 and 11 days in the control group and these differences were thought to be possible. In addition, all individuals in the control group survived. As seen in Figures 3, 5 & 7, pupae formed on the first day of the experiment and their pupal periods and adult emergence times were completed earlier than those of individuals exposed to EPF.

Conidial persistence bioassay results

The lethal effects of 1x10^8, 1x10^7, 1x10^6 conidia/mL doses of the IFR isolate (*C. fumosorosea*) on 3rd instar *C. capitata* at different time intervals are shown in Figure 8. According to the Figure 8, mortality rates of larvae added to the plastic containers 0, 3, 6, 9 and 12 months after IFR 1x10^8 conidia/mL dose applications to the sterile soils were 93%, 65%, 60%, 60% and 60%, respectively. In soils where 1x10^7 conidia/mL IFR suspension was applied, larvae mortality rates were 82%, 55%, 52%, 52% and 52% at 0, 3, 6, 9 and 12 months, respectively. Larval mortality rates in soil where 1x10^6 conidia/mL dose was applied are as follows; 70%, 52%, 45%, 45% and 44%. When the mortality rates of all conidia densities in same months were compared, it was seen that the highest effect was obtained from 1 X10^8 conidi/ mL, conidia densities affected the mortality rates and statistical differences emerged. However, in the first application, different conidia densities were included in different groups.

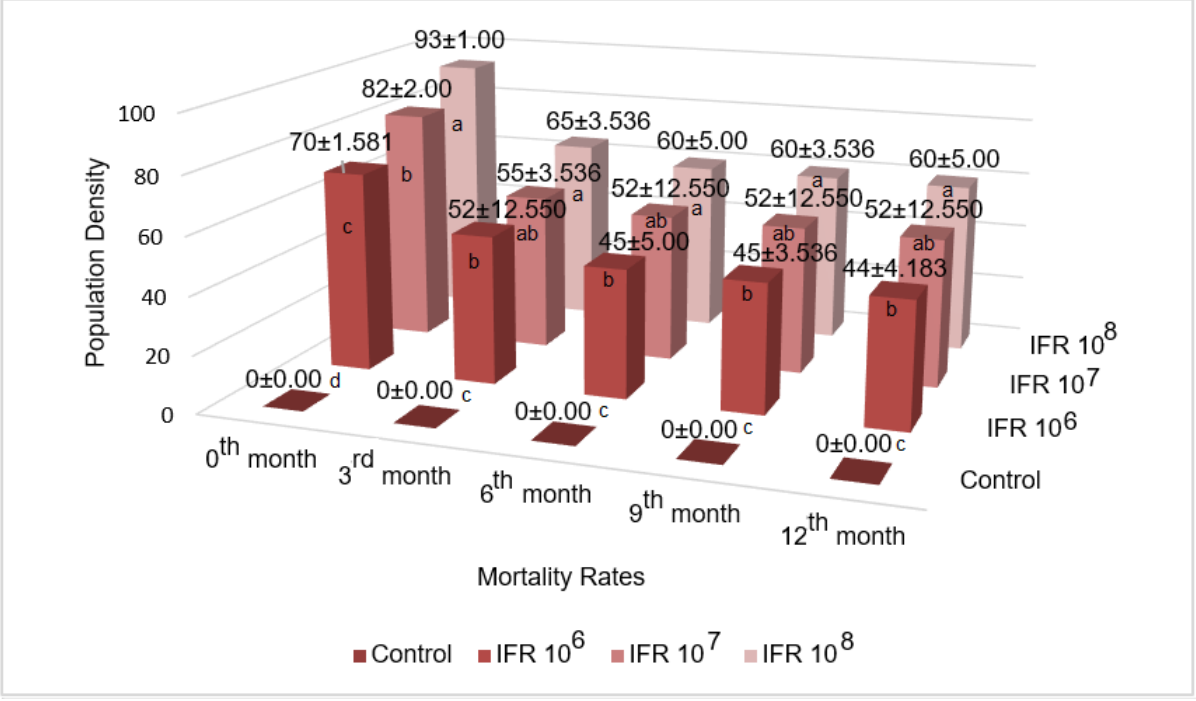

Figure 8. Percentage mortality rates (±std. deviation) of larvae released after 0, 3, 6, 9 and 12 months into sterile soil treated with 1x10^8 conidia/mL, 1x10^7 conidia/mL, 1x10^6 conidia/mL doses of the IFR isolate. *According to Tukey's multiple comparison test, different letters in the same month application are statistically different ($p<0.05$) (0th month's F value: 4681,556; 3rd month's F value: 93,443; 6th month's F value: 69,614; 9th month's F value: 79,151; 12th month's F value: 71,867). (df:3, 16; p<000.1).

The mortality rates of 1x10^8 conidia/mL, 1x10^7 conidia/mL, 1x10^6 conidia/mL doses of LD.2016 isolate (*Beauveria bassiana*) on 3rd instar of *C. capitata* in different 3-month time periods are given in Figure 9. According to Figure 9, the mortality rates of larvae left to soils treated with LD.2016 1x10^8 conidia/mL dose after 0, 3, 6, 9 and 12 months were; 55%, 40%, 40%, 12% and 2%, respectively. with IFR 1x10^7 conidia/mL dose after 0, 3, 6, 9 and 12 months were; 46%, 12%, 4%, 2% and 2%, respectively. IFR 1x10^6 conidia/mL dose after 0, 3, 6, 9 and 12 months were; 37%, 4%, 3%, 2% and 2%, respectively. Similarly, when the mortality data of the studies conducted in different months with different conidia densities were evaluated statistically within each trial month, the highest effect was seen in the first application of 1x10^8 conidia/mL of LD.2016, followed by 1x10^7 conidia/mL and 1x10^8 conidia/mL in the 3rd and 6th months, respectively. When different letters were taken into account in the relevant application month, it was determined that the 10^8 conidia/mL dose was statistically different compared to the other doses and the control in the 0th, 3rd, 6th and 9th months, but no statistical difference was observed between the 10^8 conidia/mL dose and the other doses and the control group in the 12th month.

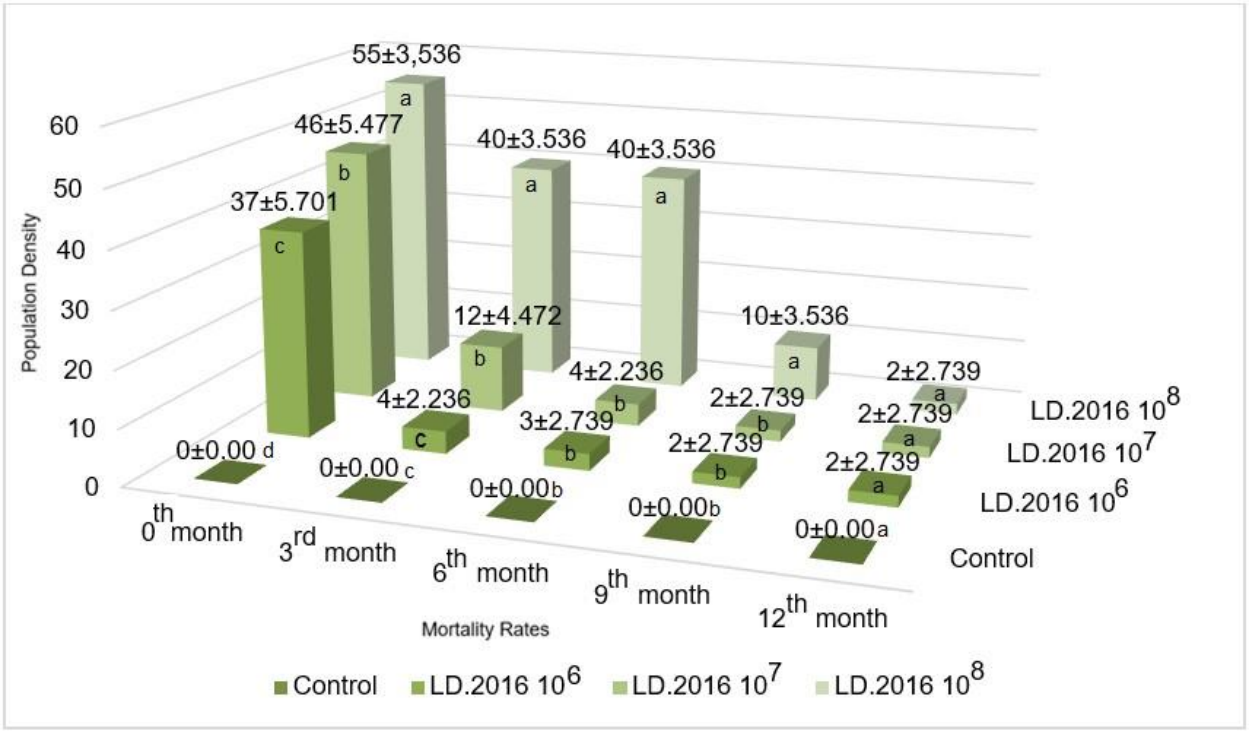

Figure 9. Percentage mortality rates (±std. deviation) of larvae released after 0, 3, 6, 9 and 12 months into sterile soil treated with $1x10^8$ conidia/mL, $1x10^7$ conidia/mL, $1x10^6$ conidia/mL doses of the LD.2016 isolate. *According to Tukey's multiple comparison test, different letters in the same month application are statistically different ($p<0.05$) (0^{th} month's F value: 155,467; 3^{rd} month's F value: 173,511; 6^{th} month's F value: 286,067; 9^{th} month's F value: 14,303; 12^{th} month's F value: 0,889). (df:3, 16; p<000.1).

Detecting adult infertility

The time-dependent population fluctuation of individuals reaching adulthood from the first EPF application (0^{th} month) is shown in Figures 10 & 11. Adult flight was detected in individuals in the control group earlier than in the EPF-contaminated groups, and a difference was observed between the isolates. There was variability in the time it took for individuals in each replication to reach adulthood. On the first day, 38 individuals from the control group were transferred to the rearing insectarium. After 13 days, the population reached 98 individuals. It remained at its peak until the 32^{nd} day, and while the 38^{th} day continued with 95 individuals, the experiment was terminated when there were no individuals left in the other experimental characters.

At the IFR dose of $1x10^8$ conidia/mL, the first adult flight started with 5 individuals on the 7^{th} day, reached its peak with 7 individuals on the 8^{th} day, remained at the peak until the 10th day, and then the population was completely exhausted on the 19^{th} day. At IFR $1x10^7$ conidia/mL, the first adult flight started on the 4^{th} day with 10 individuals, reached its peak on the 6^{th} day with 18 individuals, and the population was exhausted by 20 days At IFR $1x10^6$ conidia/mL, the first adult flight started on the 3^{rd} day with 6 individuals, reached its peak on the 7^{th} day with 30 individuals, and the population became extinct on the 20^{th} day. At the IFR dose of $1x10^5$ conidia/mL, the first adult flight started with 12 individuals on the 2^{nd} day, reached its peak with 38 individuals on the 5^{th} day, remained at the peak until the 7^{th} day, and then the population was completely exhausted on the 26^{th} day. At the IFR dose of $1x10^4$ conidia/mL, the first adult flight started with 18 individuals on the 2^{nd} day, reached its peak with 47 individuals on the 8^{th} day, and the population was completely exhausted on the 34^{th} day (Figure 10).

According to Figure 11, the first adult flight of the LD.2016 isolate at a dose of $1x10^8$ conidia/mL started with 5 individuals on the 11^{th} day, reached its peak with 45 individuals on the 21^{st} day, and the

population was completely exhausted on the 26th day. At the dose of LD.2016 1x10^7 conidia/mL, the first adult flight started on the 10th day with 22 individuals, reached its peak on the 16th day with 54 individuals, and the population was exhausted by 23 days. At a dose of 1x10^6 conidia/mL, the first adult flight started on the 5th day with 26 individuals, reached its peak on the 12th day with 63 individuals, and the population became extinct on the 31st day. At a dose of 1x10^5 conidia/mL, the first adult flight started with 27 individuals on the 4th day, reached its peak with 35 individuals on the 10th day, and the population was completely exhausted on the 30th day. At a dose of 1x10^4 conidia/mL, the first adult flight started with 25 individuals on the 1st day, reached its peak with 80 individuals on the 14th day, and the population was completely exhausted on the 38th day (Figure 11).

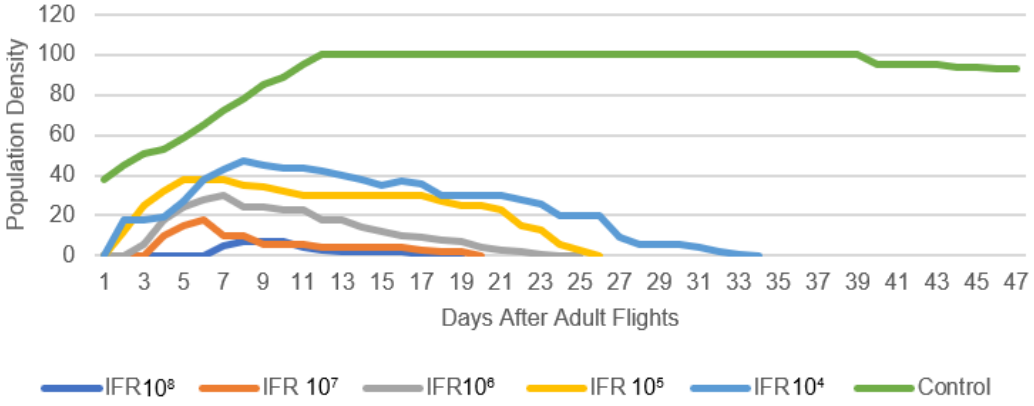

Figure 10. Population fluctuation of adult individuals emerging alive as a result of application of different IFR doses.

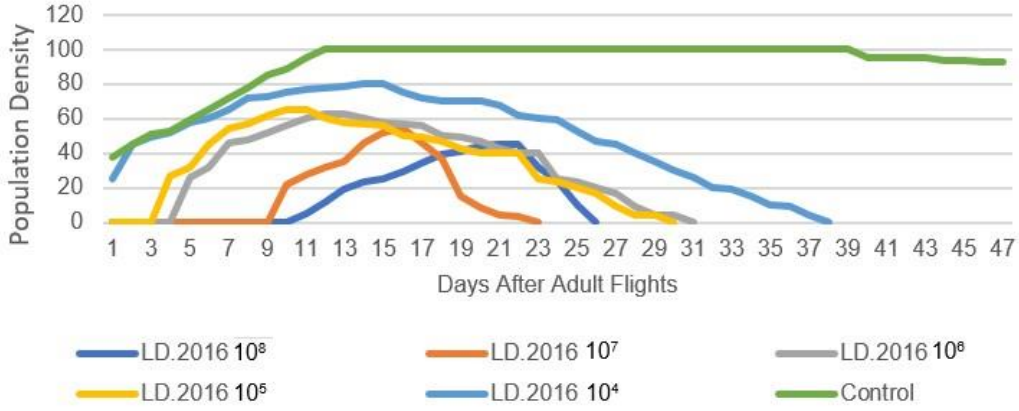

Figure 11. Population fluctuation of adult individuals emerging alive as a result of application of different LD.2016 doses.

The reproductive abilities of individuals were determined by checking the larval development in the Golden delicious apple fruit. The results are shown as present or absent in Table 1. As seen in Table 1, no larval development was observed at the IFR dose of 1x10^8 conidia/mL. In the remaining doses, larval development was observed, and adults were obtained. No larvae or adult development was observed at the doses of 1x10^8 conidia/mL, 1x10^7 conidia/mL and 1x10^6 conidia/mL of LD.2016. At other doses, there was larval development and adults were obtained. Additionally, individuals in groups with no larval development were observed to exhibit passive behavior and die before mating after a while in the 0th month. In the 12th month, in the adult individuals developed from the larvae left to the 1x10^8 conidia/mL dose application trial of the IFR isolate, no fertility was observed, while larval development was observed in all other trial characters.

Table 1. Larval development in apples given to individuals that survived after EPF application

EPF isolates	$1x10^8$	$1x10^7$	$1x10^6$	$1x10^5$	$1x10^4$
IFR (0th Month)	Absent	Present	Present	Present	Present
IFR (12th Month)	Absent	Present	Present	Present	Present
LD.2016 (0th Month)	Absent	Absent	Absent	Present	Present
LD. 2016 (12th Month)	Present	Present	Present	Present	Present

Field trials result

The mortality rates of IFR and LD.2016 isolate doses on *C. capitata* in the field studies conducted in Antalya province in July-August 2020 and 2021 are shown in Figure 5. *C. capitata* larvae that died in soil experiments disintegrated and disappeared in a very short time. For this reason, counts were made by comparing emerging adult individuals and empty pupal capsules. In 2020, the mortality rates of IFR and LD.2016 isolates at a dose of $1x10^8$ conidia/mL were 65% and 62%, and the mortality rates at a dose of $1x10^7$ conidia/mL were 60%, 55% and 42% and 35% at a dose of $1x10^6$ conidia/mL, respectively. In 2021, the mortality rates of IFR and LD.2016 isolates at a dose of $1x10^8$ conidia/mL were 60% and 60%, and the mortality rates at a dose of $1x10^7$ conidia/mL were 56%, 55%, and the mortality rates at a dose of $1x10^7$ conidia/mL were 40% and 34%, respectively. In the control, the number of empty pupal capsules and dead adults was same in all replicates. The mortality rate in the control group was 0%. While IFR $1x10^8$ conidia/mL had the highest effect in 2020, in 2021 the mortality rates of the $1x10^8$ and $1x10^7$ conidia/mL doses of both isolates were statistically in the same group. In both years, all conidia concentrations were found to be different compared to the control group (Figure 12).

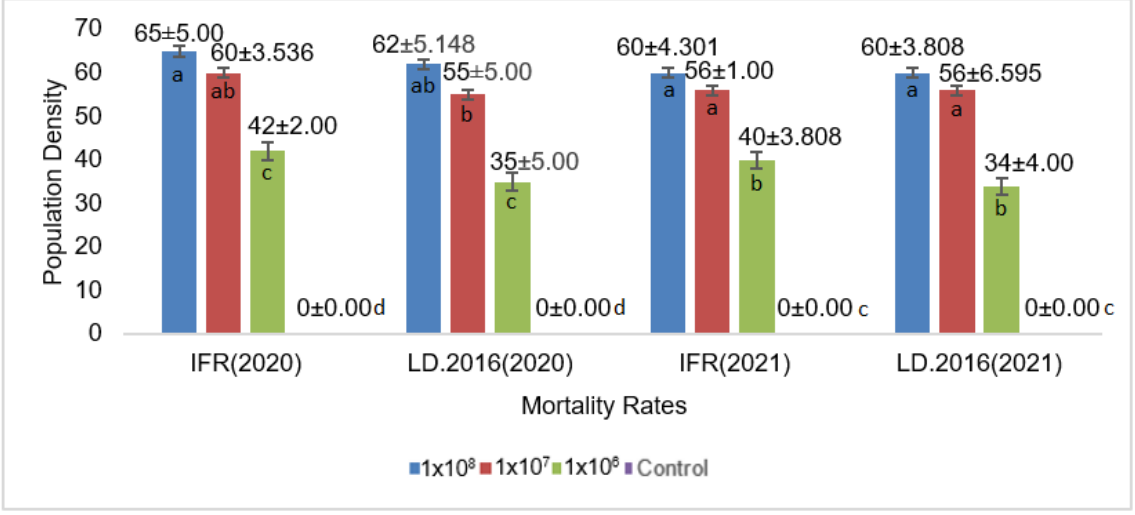

Figure 12. Mortality percentages (±std. deviation) on *Ceratitis capitata* of IFR and LD.2016 isolates in Antalya Province in 2020 and 2021.* Statistical analyses were made separately for 2020 and 2021. *According to Tukey's multiple comparison test, different letters are statistically different ($p<0.05$). (2020; F: 155,508; Between groups df:7; Within groups df: 32; p<000.1), (2021; F: 154,012; Between groups df:7; Within groups df: 32; p<000.1).

In this study conducted with *Beauveria bassiana* and *Cordyceps fumosorosea*, the change in dose-related mortality rates seems to be compatible with previous studies targeting different biological stages of *C. capitata* (Castrillo et al., 2000; Dimbi et al., 2003; Quesada-Moraga et al., 2006; Ekesi et al., 2010; Beris et al., 2013; Qazzaz et al., 2015; Chergui et al., 2020; Soliman et al., 2020; Hallouti et al., 2021). Studies targeting late-stage larvae of *C. capitata*, as in our study; In 2020, Cherqui et al. (2020) studied the *Beauveria bassiana* isolate with a dose of $1x10^7$ conidia/mL and reported the mortality rate as 44%. Gava et al. (2020) and Soliman et al. (2020) obtained similar results as our study in their study on late-stage larvae, which reported death at varying rates depending on the dose. The success of infection and the development of

fungal structures are affected by the pathogenicity of the disease, spore density, and the immune response of the host (Islam et al., 2021). For this reason, it is acceptable to obtain different results from other studies. Continuity of fungal populations is essential for sustainable management of pests. The formation of the fungal population is based on the transformation of resources within the host cadaver because infective spores spread from the cadaver (Meyling and Eilenberg, 2007). One reason why mortality rates are decreasing over time in our conidial persistence studies (Figures 8 & 9) can be attributed to the small size of *C. capitata* larvae and their inability to provide sufficient nutrients for a larger amount of spore production.

However, it is important to note that EPFs help minimize damage to crops (below the economic threshold) by triggering infection of pests, which ultimately leads to a reduction in feeding, oviposition, development, mating and other physiological characteristics of insects (Thomas et al., 1997). As seen in our study results, the negative effects on larval development and the fertility of individuals who emerged as adults after infection, as well as the shortening of the lifespan of adults, were parallel to some previous studies. Although not on the same biological period comparison to present study (Table 1), Quesada-Moraga et al. (2006) stated that the fertility of *C. capitata* adults decreased 6, 8, and 10 days after the applications. Castrillo et al. (2000) reported that fertility was reduced over 53% compared to control group. Another study was by Gava et al. (2020), who reported that EPF applications shortened adult lifespan. Although there was a difference in application, similar results were seen in our study (Figures 10 & 11). Although the delay or prolongation of a biological period of the target organism is a negative feature for the target organism, it is an advantage in terms of management.

In studies conducted with non-sterile field soil under semi-controlled conditions, it is seen that the mortality rates caused by IFR and LD.2016 isolate were close to each other in both years, unlike climate chamber studies (Figure 12). This may be caused by climatic conditions or the influence of other organisms in the soil environment. Additionally, the soil environment has a diverse microbiota with antagonistic fungi and bacteria that may inhibit EPF survival or reduce biocontrol activity (Jaronski, 2007).

In the light of all these results and evaluations, it is thought that the *C. fumosorosea* and *B. bassiana* isolates used in the experiment have a significant potential to be used at the soil level to apply to late-stage larvae in the management against *C. capitata*. For this reason, the application of fungal soil inoculations to the tree crown projection, including rhizosphere, in fruit cultivation may be a good strategy for both pest control and benefiting from its other contributions. In addition, it is necessary to continue detailed research to better understand the interactions of fungal species in the soil with the entomopathogenic fungi to be used and to define their effects on the reproductive capacity of the target organism for sustainable pest management strategies.

Acknowledgement

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Original article (Orijinal araştırma)

Prodigiosin, a promising biocontrol agent against *Thaumetopoea wilkinsoni* (Tams, 1926) (Lepidoptera: Notodontidae)

Prodigiosin, *Thaumetopoea wilkinsoni* (Tams, 1926) (Lepidoptera: Notodontidae)'ya karşı umut verici bir biyolojik kontrol ajanı

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Abstract

Serratia marcescens Bizio (Enterobacteriaceae: Serratia) is an entomopathogenic bacterium that produces hydrolytic enzymes and toxins. It also produces a pigment with various biological properties called prodigiosin. The study was conducted at Bilecik Seyh Edebali University in 2023. In this study, the effects of medium, incubation temperature and time on the process of prodigiosin production by *S. marcescens* strain Se9 and the extraction efficiency of different solvents were optimized for the first time using the orthogonal Taguchi array design. The optimal yield of pigment was achieved by methanol extraction from bacteria grown in tyriptic soy broth medium at 30ºC for 96 hours. The yield of prodigiosin pigment was 83.4±1.7 mg/L in the validation experiment conducted under the optimum conditions determined. The insecticidal potential of pigment against the larvae of *Thaumetopoea wilkinsoni* (Tams, 1926) (Lepidoptera, Notodontidae) was demonstrated for the first time. While the mortality rate in larvae exposed to 1000 ppm of the pigment was only 40%, it was observed that doubling the applied concentration led to a significant increase in larval mortality, reaching 91%. The LC_{50} value of the pigment for the fourth larval stage of *T. wilkinsoni* was determined to be 1192 ppm. The study showed that the pigment prodigiosin may be a promising biocontrol agent for the control of *T. wilkinsoni.*

Keywords: Insecticidal activity, microbial control, pigment, Serratia, *Thaumetopoea wilkinsoni*

Öz

Serratia marcescens Bizio (Enterobacteriaceae: Serratia) hidrolitik enzimler ve toksinler üreten entomopatojenik bir bakteridir. Ayrıca, prodigiosin adı verilen çeşitli biyolojik özelliklere sahip bir pigment de üretir. Çalışma, 2023 yılında Bilecik Şeyh Edebali Üniversite'sinde gerçekleştirilmiştir. Bu çalışmada, besiyeri, inkübasyon sıcaklığı ve süresinin *S. marcescens* Se9'un prodigiosin üretim sürecine etkileri ve farklı çözücülerin ekstraksiyon verimliliği ilk kez ortogonal Taguchi dizi tasarımı kullanılarak optimize edilmiştir. Optimum pigment verimi, 30ºC'de 96 saat boyunca triptik soya sıvı besiyeri ortamında büyütülen bakterilerden metanol ekstraksiyonuyla elde edilmiştir. Belirlenen optimum koşullar altında yapılan validasyon deneyinde prodigiosin pigment verimi 83,4±1,7 mg/L olarak belirlenmiştir. Prodigiosin pigmentinin *Thaumetopoea wilkinsoni* (Tams, 1926) (Lepidoptera, Notodontidae) larvaları üzerindeki insektisidal potansiyeli ilk kez gösterilmiştir. 1000 ppm pigmente maruz kalan larvalarda ölüm oranı sadece %40 iken, uygulanan konsantrasyonun iki katına çıkarılmasının larva ölümlerinde önemli bir artışa neden olarak %91'e ulaştığı gözlenmiştir. *T. wilkinsoni'*nin dördüncü larva dönemi için pigmentin LC_{50} değeri 1192 ppm olarak belirlenmiştir. Çalışma, prodigiosin pigmentinin *T. wilkinsoni'*nin kontrolü için umut verici bir biyokontrol ajanı olabileceğini göstermiştir.

Anahtar sözcükler: İnsektisidal aktivite, mikrobiyal kontrol, pigment, Serratia, *Thaumetopoea wilkinsoni*

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Introduction

The pine processionary moths (PPM), represented in Europe and North Africa by *Thaumetopoea pityocampa* (Denis & Schiffemüller, 1775) and in Asia and the Middle East by *Thaumetopoea wilkinsoni* (Tams, 1926) (Lepidoptera: Notodontidae), are the most damaging defoliating pests of pine forests in the coastal zone of Mediterranean countries. In most previous studies on *Thaumetopoea* sp. in Turkey, the species was referred to as *T. pityocampa* (Kanat & Alma, 2004; Er et al., 2007; Ince et al., 2008; Sevim et al., 2010). However, recent studies using molecular techniques have shown that the populations in Turkey are *T. wilkinsoni* (İpekdal et al., 2020).

Thaumetopoea wilkinsoni affects the yield of trees by feeding on the needles of various pine species [*Pinus brutia* Ten., *Pinus nigra* Arnold., *Pinus sylvestris* L., *Pinus pinea* L., *Pinus halepensis* Mill. (Pinales: Pinaceae)] during the larval stages in autumn and winter (İpekdal, 2005; Castagneyrol et. al., 2016). The pest causes deformities in seedlings and stunting and even tree mortality in young stands (Kanat et al., 2005). The larval stages cause the pines to shed their leaves and allow the invasion of other opportunistic pine pests, resulting in reduced viability of the pines and a lower yield of edible pine nuts (Faria, 2021). In addition to its serious impact on biodiversity and the environment, the pest also has a dangerous effect due to the stinging hairs it produces from the third larval stage onwards, which cause severe allergic reactions on contact with humans and animals (Olivieri et al., 2023). The stinging hairs are produced in large quantities in special abdominal pouches of the larvae and contain a protein-like toxin, thaumetopoein, which causes allergic reactions to the skin, respiratory tract, mouth and eyes (Bonamonte et al., 2013).

Various methods are used to control PPM, including the use of sex pheromones and mass trapping programs (Salvato et al., 2005). The nests are physically removed and destroyed, or insecticide-soaked tape is placed around the trees. Commercial tree boot barrier traps show high effectiveness in trapping larvae in the last larval stage during their migration and prevent them from reaching the ground to pupate (Colacci et al., 2018).

Chemical insecticides, especially insect growth inhibitors such as diflubenzuron, dimilin and deltamethrin, have been used mainly to control PPM (Demolin & Martin, 1986; Halperin, 1980). However, these agents have potentially undesirable side effects, particularly on predators and parasites of the pest, humans, plants and other non-target animals. Microbial control with entomopathogenic bacteria and bacterial secondary metabolites is an excellent alternative to conventional insecticides because it is cost-effective and high-yielding, does not harm beneficial organisms and releases fewer chemical residues into the fields.

Serratia marcescens Bizio (Enterobacteriaceae: Serratia) is a rod-shaped gram negative bacterium. It infects both invertebrates and vertebrates but is primarily known as an important pathogen of insects, causing rapid insect death (Mohan et al., 2011; Zhang et al., 2021). In addition, *S. marcescens* synthesizes a red pigment known as prodigiosin, which has biological properties including antibacterial (Lapenda et al., 2015), antifungal (John et al., 2021), antimalarial (Arifiyanto et al., 2022), nematicidal (Gomez Valdez et al., 2022), immunosuppressive (D'Alessio et al., 2000) and anticancer (Li et al., 2018). Its toxicity is mainly attributed to its secondary metabolite prodigiosin (Suryawanshi et al., 2015). Just as the culture conditions influence the production of prodigiosin, the extraction of the pigment produced has a considerable influence on the amount of pigment obtained. For these reasons, the production and extraction process of the pigment must be optimized.

Thus, the aim of the present study was to optimize the prodigiosin production of the local *S. marcescens* strain Se9 using Taguchi's orthogonal array method and to demonstrate its insecticidal potential against the field population of *T. wilkinsoni* larvae. This is the first study to show that the pigment prodigiosin is a promising biocontrol agent against *T. wilkinsoni*.

Materials and Methods

Culture of *Serratia marcescens*

Serratia marcescens strain (Se9), a red pigmented bacterium was kindly provided by Laboratory of the Department of Biology at Karadeniz Technical University. 100 µl of the bacteria spread onto tryptic soy agar (TSA) medium and incubated overnight at 30ºC. A single colony was then transferred to fresh TSA medium and used as a seed culture in experiments.

Optimization of prodigiosin production

The Taguchi method was used here to determine the optimal process conditions for the production of prodigiosin from *S. marcescens* strain Se9. The critical fermentation factors such as the medium, incubation temperature and incubation time as well as the solvent to be used for extraction were selected for the optimization process. The factors and their levels are listed in Table 1. A total of 9 experiments were performed based on the orthogonal array method of Taguchi's L9 design using the four target parameters (Table 2). Shaking flask experiments were performed according to the experimental design listed in Table 2. Erlenmeyer containing 90 ml medium was inoculated with 10 ml of overnight culture of *S. marcescens* strain Se9 and incubated on a rotary shaker at 200 rpm for pigment production. Each experiment was repeated three times.

Table 1. Factors and their levels used in optimization process

Factors	Level 1	Level 2	Level 3
Medium	Nutrient broth	Tryptic soy broth	Peptone glycerol broth
Incubation temperature (ºC)	25	30	37
Incubation time (hour)	48	72	96
Solvent	Acetone	Methanol	Petroleum ether

Table 2. Taguchi L9 orthogonal array and experiments to be performed

Experiments	Process parameters			
	Medium	Temperature (ºC)	Incubation time (hour)	Solvent
1	1	1	1	1
2	1	2	2	2
3	1	3	3	3
4	2	1	2	3
5	2	2	3	1
6	2	3	1	2
7	3	1	3	2
8	3	2	1	3
9	3	3	2	1

Extraction of solvent

After incubation, the pigmented culture was centrifuged at 10,000 rpm for 15 minutes at 4ºC and the supernatant discarded. Following the method of Suryawanshi et al (2015) with some modifications, the pellet was resuspended with the solvent indicated for each experiment and vortexed vigorously for at least 10 minutes. The mixture was then centrifuged again at 10,000 rpm for 15 minutes and the supernatant, which contained prodigiosin, was carefully transferred to a sterile Falcon tube. The solvent was removed under vacuum using a rotary evaporator (Bibby Scientific Ltd, Staffordshire, UK) at 50ºC, keeping the cooling temperature below 10ºC, until a dry red pigment was obtained. The crude pigment was collected, quantified on a dry weight basis and stored at 4ºC until use in bioassays.

Validation of the model

The prodigiosin yield was considered as a quality characteristic using the "the larger is the better approach" and the signal-to-noise ratio was determined. In addition, the effect of the selected factors on the pigment production was determined using analysis of variance (ANOVA). All analyses were carried out using Minitab statistical software. A validation experiment was then performed under optimal conditions, which were determined using the Taguchi method. The results were compared with the estimated values obtained with the Minitab software.

Bioassay

The PPM larvae used for the bioassay were collected between October and November 2023 in *Pinus brutia* forests in Bilecik, Türkiye (40.1587ºN, 29.9758ºE and altitude of 850 m above sea level). The silk nests collected with the help of ligature scissors were placed in disinfected containers and brought to the laboratory. Also, it has been confirmed that the pest is *T. wilkinsoni* (K. İpekdal, personal communication). Fourth instar larvae were selected for use in bioassays based on the size of the head capsule and body morphology (EPPO/CABI 1997).

After resuspending ten mg of the dry pigment in 5 ml of methanol, different concentrations (2000, 1000, 500, 250 and 125 ppm) were prepared by 1:2 serial dilution with methanol. The pigment (5 ml) was sprayed onto the needle using a mini hand sprayer. After evaporation of the solvent, the field collected twenty larvae (4th instar) were placed on pine needles and transferred to boxes (30×20×15 cm) with perforated covers to allow air flow. The boxes were incubated in a climate-controlled room at 25±1ºC, 60% relative humidity and a photoperiod of 16:8 hours light: dark. The control group was sprayed with the solvent and twenty larvae were placed on pine needles after evaporation of the solvent. Bioassays were repeated three times for each concentration. Mortality was recorded daily for 14 days.

Statistical analysis

The mortality data were corrected using the Abbott formula (Abbott, 1925). Cumulative mortality was analyzed with survival analysis using Kaplan-Meier estimates of survival probabilities and pairwise comparisons were performed using the log-rank test at 0.05 probability (Mantel-Cox). Concentration-response data were subjected to probit regression analysis and lethal concentrations (LC_{50} and LC_{95}) of the pigment were estimated. Statistical analyses were performed using the IBM SPSS version 25 (IBM, 2017).

Results

Optimization of pigment production

The effectiveness of culture conditions, incubation time and temperature, differences in medium and solvent used in extraction on the production of prodigiosin by *S. marcescens* strain Se9 was tested in 9 experiments using the Taguchi experimental design. The results showed significant variations in the yield of prodigiosin, and it was found that the production was highly dependent on the culture conditions and solvent. The yield of prodigiosin ranged from 0 to 88.7 mg/L (Table 3).

The optimal conditions for each factor were determined by calculating the signal-to-noise ratio of the tested factors. The signal-to-noise ratio should have a maximum value according to the Taguchi method in order to achieve optimal prodigiosin production. It was found that the optimum yield of pigment can be obtained by methanol extraction from bacteria incubated in TSB medium at 30ºC for 96 hours (Figure 1).

In addition, an analysis of variance (ANOVA) was performed to determine the contribution of the individual factors to the variation. The results are shown in Table 6. While the incubation temperature had the greatest positive influence on prodigiosin production among the tested factors with 56.54%, the medium

had the least influence with 1.22% (Table 4). The model obtained from ANOVA showed that the multiple correlation coefficient R2 is 1.0, i.e. the model can explain 100% of the variation in the response.

Table 3. Experiments carried out for optimization and the pigment yield obtained

Experiments	Medium	Incubation temperature (ºC)	Incubation time (hour)	Solvent	Pigment yield (mg/L)±SD
1	Nutrient broth	25	48	Acetone	13.0±0.75
2	Nutrient broth	30	72	Methanol	88.7±0.67
3	Nutrient broth	37	96	Petroleum ether	1.20±0.12
4	Tryptic soy broth	25	72	Petroleum ether	22.7±0.02
5	Tryptic soy broth	30	96	Acetone	52.5±0.70
6	Tryptic soy broth	37	48	Methanol	3.70±0.25
7	Peptone glycerol broth	25	96	Methanol	65.7±0.22
8	Peptone glycerol broth	30	48	Petroleum ether	27.5±0.10
9	Peptone glycerol broth	37	72	Acetone	0.0±0.0

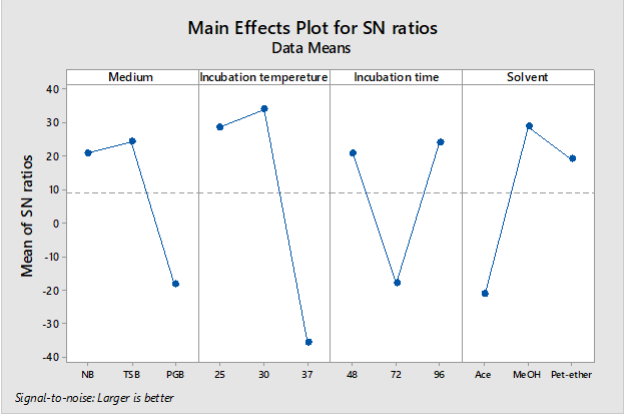

Figure 1. The signal-to-noise ratio plot of tested factors for prodigiosin production. Ace: acetone, MeOH: Methanol, Pet-eter: petroleum ether.

Table 4. Analysis of variance (ANOVA) for the yield of prodigiosin

Factors	DF	Contribution (%)	Adj SS	Adj MS	F-value	P-value
Medium	2	1.22	97.18	48.59	12.86	0.044
Incubation temperature	2	56.54	4519.11	2259.55	46.74	0.021
Incubation time	2	14.23	1137.21	568.6	18.56	0.042
Solvent	2	28.02	2239.83	1119.91	30.06	0.032
Error	0	-	-	-		
Total	8	100				

DF: degree of freedom, Seq SS: sequential sums of squares, Adj SS: adjusted sum of squares, Adj MS: Adjusted mean squares, R2=100%.

The validation experiment was performed with the optimized factors at the recommended levels. The result was a prodigiosin production of 83.93±1.75 mg/L, which is comparable to the predicted value (83.41 mg/L). Therefore, the Taguchi method is validated for the production of prodigiosin pigments.

Insecticidal properties of pigment

The insecticidal potential of the pigment prodigiosin was investigated on the larvae of *T. wilkinsoni.* No mortality was observed at a concentration of 125 ppm, but the mortality rate increased with increasing concentration, reaching 92% at a concentration of 2000 ppm. The log-rank analysis showed that the survival rates of *T. wilkinsoni* larvae treated with the pigment at concentrations of 2000, 1000 and 500 ppm differed from those of the control group (Table 5). However, it was found that the survival rates of larvae treated with the pigment at concentrations of 250 and 125 ppm did not differ from those of the control group ($p>0.05$).

Table 5. Pairwise comparison using Log-Rank (Mantel-Cox) test for *Thaumetopea wilkinsoni* larvae exposed to different concentration of prodigiosin

Concentrations (ppm)	125		250		500		1000		2000		Control	
	X^2	*p*	X^2	*p*	X^2	*p*	X^2	*p*	X^2	*p*	X^2	*p*
125			1.02	0,31	11.11	0.00	47.29	0.00	184.24	0.00	1.00	0.31
250	1.02	0.31			6.71	0.01	40.55	0.00	174.51	0.00	3.03	0.08
500	11.11	0.00	6.71	0.01			19.18	0.00	145.70	0.00	13.84	0.00
1000	47.29	0.00	40.55	0.00	19.18	0.00			75.00	0.00	50.17	0.00
2000	184.24	0.00	174.51	0.00	145.70	0.00	75.00	0.00			185.42	0.00
Control	1.00	0.31	3.03	0.08	13.84	0.00	50.17	0.00	185.42	0.00		

X^2: Chi-square, *p:* significance at 0.05**.**

Figure 2 illustrates the cumulative survival rates of *T. wilkinsoni* larvae exposed to various concentrations of prodigiosin. While larvae exposed to 125 and 250 ppm of the pigment showed relatively high survival rates, those exposed to 2000 ppm had a survival rate of just 8%. The LC_{50} value for prodigiosin, which is the concentration at which 50% of the larvae are expected to die, was determined to be 1192 ppm (Table 6).

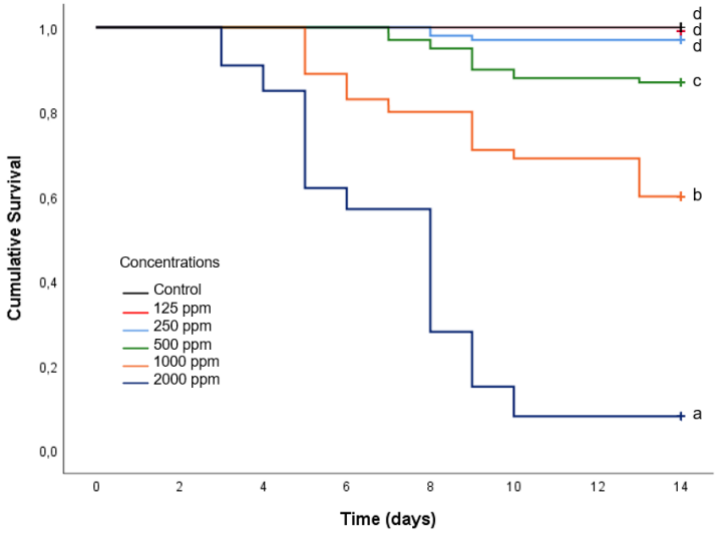

Figure 2. Kaplan-Meier plot showing survival of *Thaumetopea wilkinsoni* larvae after exposure with different concentration of prodigiosin pigment. Curves bearing different letters are significantly different in survival mode (Log-rank test, p<0.05).

Table 6. Lethal concentrations (LC_{50} and LC_{95}) of prodigiosin pigment for the larvae of *Thaumetopea wilkinsoni*

Pigment	LC_{50} (ppm)	95% *FL*		Slope ± SE	LC_{95} (ppm)	95% *FL*		*df*	X^2
		Lower bound	Upper bound			Lower bound	Upper bound		
Prodigiosin	1192	1103	1291	0.02 ±0.00	2064	1901	2275	3	5.06

A total of 360 individuals, 60 for each concentration and controls, were used.

Discussion

The emergence of resistance to conventional insecticides has revived interest in the search for and development of pesticides. Microbial control agents and their products offer alternatives to conventional insecticides as they can be more selective. The insecticidal potential of *S. marcescens*, an entomopathogenic bacterium, has already been reported, but the use of the whole microorganism is limited due to its pathogenicity for animals and humans. Therefore, the active compounds produced by the bacterium could be more useful than the bacterium itself.

The pigment prodigiosin produced by *S. marcescens* is one of the secondary metabolites that confer insecticidal properties to the bacterium. As with many secondary metabolites, the biosynthesis of prodigiosin is dependent on the growth phase and reaches its maximum when the cell enters the stationary phase. Prodigiosin synthesis depends on cultural conditions such as media, incubation time and temperature. This is the first report describing the application of the Taguchi method to prodigiosin production in an entomopathogenic bacterium.

The type of culture medium is an important factor for the production of prodigiosin, with tryptic soy broth proving to be the most effective compared to other media in our experiments The differences in the amount of prodigiosin are directly related to the composition of the media. Since each microorganism has its own nutrient requirements for growth, the tryptic soy broth, which is favorable for the production of prodigiosin in the present study, could contain the necessary nutrients. Peptone from casein, peptone from soymeal, glucose monohydrate, sodium chloride and di-potassium hydrogen phosphate are the main components of the tryptic soy broth. According to Paul et al (2024), peptone is an excellent source of nitrogen as it provides essential fatty acids and amino acids that are important for bacterial growth. However, there is no single nitrogen source that is specifically optimized for the production of prodigiosin in *S. marcescens*, as different nitrogen sources provide different amounts of amino acids and growth-promoting compounds. Furthermore, Giri et al. (2004) point out that the choice of carbon source is crucial for the production of prodigiosin in *Serratia marcescens* strains. In our study, a TSB medium containing 2.5% (w:v) glucose proved to be the most effective for maximizing prodigiosin production. This finding contrasts with previous research by Su et al. (2011), which indicated that glucose and lactose can inhibit prodigiosin production.

In addition to the composition of the medium, the incubation temperature and duration are crucial factors that influence the production of prodigiosin. Paul et al. (2024) reported that the highest prodigiosin yield (15 mg/L) occurred at 30ºC when *S. marcescens* was grown in nutrient broth. Temperatures above 30ºC resulted in a decrease in pigment production, a finding consistent with our results. We found that 30ºC is the optimal temperature for prodigiosin synthesis, with production decreasing significantly at 37ºC. At temperatures above 30ºC, *S. marcescens* ceases pigment production and colonies appear creamy white, which is due to the fact that temperatures below or above the optimal temperature (30ºC) can lead to deactivation of ribosomes and enzymes involved in the prodigiosin synthesis pathway (de Araujo et al., 2010). Incubation time is also an important factor for pigment production. In our study, the optimal incubation time for the production of prodigiosin was determined as 96 hours. Koyun et al (2022) found that the strain *S. marcescens* MB703 showed maximum production of prodigiosin pigments on the 6th day in NB medium. Similarly, maximum production of prodigiosin by *S. marcescens* strain MN5 was observed 6 days after incubation (Elkenawy et al., 2017). Interestingly, Su et al. (2011) achieved high prodigiosin yields from a 1-day culture, although it is generally assumed that microorganisms must reach stationary phase to produce secondary metabolites like prodigiosin.

In addition to the culture conditions for the production of prodigiosin, the extraction of the pigment produced by the bacterium is also an important task. Typically, solvent-assisted extraction is employed, and the choice of organic solvent significantly affects the yield. Park et al. (2012) reported that ethanol is the best solvent in terms of extraction efficiency and that acetone has an extraction efficiency of 98.5% compared to ethanol. However, methanol has high extraction efficiency and unlike Park et al. (2012), acetone showed low extraction efficiency in our study. Overall, highly polar solvents such as ethanol, methanol, acetone, and acetonitrile generally offer high extraction efficiency for prodigiosin (Park et al., 2012).

Prodigiosin is a promising biomolecule with many potential applications. So far, many studies have reported the antibacterial, antifungal, antiviral, anticancer and algicidal effects of prodigiosin extracted from various microorganisms (Islan et al., 2022). In addition, the insecticidal property of prodigiosin was first

determined in three different species of lepidoptera (Asano et al., 1999). They were administered to the larvae of *Spodoptera litura* Fab., 1775 (Lepidoptera: Noctuidae), *Plodia xylostella* L., 1758 (Lepidoptera, Pyralidae) larvae and *Adoxophyes honmai* Yasuda, 1998 (Lepidoptera Tortricidae) by the ingestion method at a dosage of 8 µg/g diet. The mortality rate of the larvae of *P. xylostella* reached up to 96%, while very low mortality rates were observed in the larvae of *S. litura* and *A. honmai*. However, Patil et al. (2013) reported 100% mortality in larvae of *S. litura* treated with high concentrations (30 mg/mL) of prodigiosin. In this study, prodigiosin caused 92% mortality in another lepidopteran, *T. wilkinsoni*, at a concentration of 2000 ppm. This shows that the insecticidal effect of prodigiosin is concentration dependent. Similarly, Eski & Özdemir (2022) reported that at low concentrations (125 and 250 ppm), prodigiosin caused less than 10% mortality in *Tenebrio molitor* L., 1758 (Coleoptera: Tenebrionidae) larvae, while at high concentrations (1000 and 2000 ppm), the mortality rate was over 90%. They also reported an LC_{50} value of prodigiosin for *T. molitor* larvae of 924 ppm. In this study, the LC_{50} value for *T. wilkinsoni* larvae was determined to be 1192 ppm. Compared to previous studies, the LC_{50} value was high. Wang et al. (2012) reported the LC_{50} value of the pigment prodigiosin extracted from *S. marcescens* TKU011 as 230 ppm for the larvae of *Drosophila melanogaster* Meigen, 1830 (Diptera: Drosophilidae). Similarly, the LC_{50} value of prodigiosin extracted from *S. marcescens* NMCC46 was determined to be 103.95 and 105.52 ppm for *Aedes aegypti* L., 1762 (Diptera: Culicidae) and *Anopheles stephensi* Liston, 1901 (Diptera: Culicidae) larvae, respectively. (Patil et al., 2011). The difference in prodigiosin activity in different insects can be explained by the detoxification mechanism through high activity of esterases (Suryawanshi et al., 2015). Therefore, the susceptibility of insects to the pigment prodigiosin can vary. Although there are many studies showing that prodigiosin has an insecticidal effect, Zhou et al. (2016) tested the pigmented strain and its non-pigmented mutant on *Bombyx mori* L., 1758 (Lepidoptera: Bombycidae) and found no difference between the LC_{50} values. Therefore, it was reported that prodigiosin is not a necessary virulence factor. However, in a study conducted by Suryawanshi et al. (2015) to understand the mode of action of the prodigiosin, it was found that immune system enzymes such as protease, acetylcholinesterase, esterase and phosphatase change significantly when mosquito larvae are exposed to the pigment. In addition, nutrient absorption decreases, and the insect dies due to the decreasing pH value in the insect's midgut.

In conclusion, it has been demonstrated that the pigment prodigiosin has the potential to be used in the control of *T. wilkinsoni*, an important pest in pine forests. The efficacy of the pigment should be tested under field conditions. Furthermore, the usability of the pigment together with other control agents should be investigated as part of integrated pest management strategies for this pest.

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Original article (Orijinal araştırma)

Insecticidal efficacy of local diatomaceous earth compositions with different particle sizes against stored grain pests

Farklı partikül boyutlarına sahip yerel diatom toprağı kompozisyonların depolanmış tahıl zararlılarına karşı insektisidal etkinliği

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Abstract

In this study, the efficacy of a mixture of local diatomaceous earth (DE) with different diatomite compositions and particle sizes against stored-grain pests, *Sitophilus oryzae* L., 1763 (Coleoptera: Curculionidae), *Tribolium confusum* du Val, 1863 (Coleoptera: Tenebrionidae), and *Rhyzopertha dominica* (F., 1792) (Coleoptera: Bostrichidae) adults was investigated in 2023 in the Entomology Laboratory of the Department of Plant Protection, Faculty of Agriculture, KSU. In biological tests, 500 and 1000 ppm concentrations (mg DE/kg wheat) of different particle sizes (≤ 20, 20-75, 75-150, and ≥ 150 µm) of DE mixture and original DE (≤ 250 µm) were applied to wheat. The highest mortality rate for all insect species tested at 500 ppm was obtained from DE treatments of ≤20 µm. At 1000 ppm, 100% mortality was observed for *T. confusum* and *S. oryzae* in almost all particle size DE-treatments, whereas no local DE treatments resulted in 100% mortality for *R. dom*inica adults. Moreover, the greatest reduction in the number of F1 generation adults for *S. oryzae* and *R. dominica* was obtained in the DE treatment of ≤20 µm at 500 and 1000 ppm. In conclusion, local DE mixture treatments with ≤ 20 µm particle size were found to be highly effective in the control of stored-grain pests.

Keywords: Diatomite composition, local diatomaceous earth, particle size, stored-grain insect pests

Öz

Bu çalışmada, farklı diyatomit bileşimlerine ve partikül boyutlarına sahip yerel diyatom toprağı (DE) karışımının depolanmış tahıl zararlıları *Sitophilus oryzae* L, 1763 (Coleoptera: Curculionidae), *Tribolium confusum* du Val, 1863 (Coleoptera: Tenebrionidae) ve *Rhyzopertha dominica* (F., 1792) (Coleoptera: Bostrichidae) erginlerine karşı etkinliği 2023 yılında KSÜ Ziraat Fakültesi Bitki Koruma Bölümü Entomoloji Laboratuvarında araştırılmıştır. Biyolojik testlerde, DE karışımı ve orijinal DE'nin (≤ 250 µm) farklı partikül boyutlarının (≤ 20, 20-75, 75-150 ve ≥ 150 µm) 500 ve 1000 ppm konsantrasyonları (mg DE/kg buğday) buğdaya uygulanmıştır. Test edilen tüm böcek türleri için 500 ppm'de en yüksek ölüm yüzdesi ≤20 µm DE uygulamalarından elde edilmiştir. 1000 ppm'de, *T. confusum* ve *S. oryzae* için neredeyse tüm partikül boyutu DE uygulamalarında %100 ölüm gözlenirken, hiçbir yerel DE uygulaması *R. dominica* erginleri için %100 ölümle sonuçlanmamıştır. Ayrıca, *S. oryzae* ve *R. dominica* için F1 nesli ergin sayısındaki en büyük azalma 500 ve 1000 ppm'de ≤20 µm DE uygulamasında elde edilmiştir. Sonuç olarak, ≤ 20 µm partikül boyutuna sahip yerel DE karışımı uygulamalarının depolanmış tahıl zararlılarının kontrolünde oldukça etkili olduğu bulunmuştur.

Anahtar sözcükler: Diatomit kompozisyonu, yerel diatomit toprağı, partikül büyüklüğü, depolanmış tahıl zararlıları

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Introduction

Cereals are among the most important food sources of plant products. In 2023, global cereal production increased by about 3.8 million metric tons, reaching a total of 2,819 million metric tons. Turkey's cereal production was approximately 42.2 million metric tons (FAOSTAT, 2024). These cereals are stored in warehouses for periods that can range from a few weeks to several years, making them susceptible to infestation by various insect pests. Post-harvest insect infestations can lead to substantial losses in grain production (Reichmuth et al., 2007; Mason & McDonough, 2012). Each year, 10-30% of global grain production is lost due to storage insects, which compromise both the quantity and quality of the grain during the post-harvest period (Wijayaratne & Rajapakse, 2018). In many parts of the world, chemical control is the most commonly used method against stored grain pests. Currently, residual contact insecticides (chlorpyriphos-methyl, pirimiphos-methyl, and deltamethrin + piperonyl butoxide (PBO)) and fumigants (phosphine and methyl bromide) are widely used in the chemical control of stored-grain insects. These synthetic insecticides have a rapid lethal effect on insects; however, the use of synthetic insecticides and fumigants in the control of stored grain insect pests causes yearly resistance problems (Zettler & Cuperus 1990; Benhalima et al., 2004; Pimentel et al., 2010). There is a growing need to develop environmentally friendly insect pest control methods, which are considered safer or less harmful (Arthur et al., 2007). This need is driven by concerns over health risks from toxic chemicals (Arthur, 1996), potential side effects on non-target organisms (Fields, 1992), and detrimental impacts on the environment (Zettler & Keever, 1994; Benhalima et al., 2004; Phillips & Throne, 2010; Pimentel et al., 2010; Alkan & Gökçe, 2012).

Diatomaceous earth (DE) is sediment formed from fossilized siliceous shells of unicellular microscopic algae of the algae class. The cell walls of the diatomite are composed of amorphous silica ($SiO_2 + H_2O$). DE is a natural source of dry matter that can be used as an insecticide (Korunic, 1998). The physical mode of action of DE in insects involves acting on the insect's cuticle, causing rapid drying, ultimately resulting in death due to dehydration (Ebeling, 1971; Korunic, 1998). DE is safe for mammals, demonstrated by an oral LD_{50} value greater than 5000 mg/kg body weight in rats, and it does not leave toxic residues in products (Anonymous, 1991; Quarles, 1992). As a result, it is classified as a food additive and falls within the GRAS (Generally Recognized as Safe) category according to the US EPA (FDA, 1995). DEs can be used as an alternative to conventional insecticides, particularly in protecting stored grains from insect pests where extensive research on a wide range of stored-product insect species has been done, for extended periods due to their ability to remain on treated crops for an extended period (Quarles, 1992; Korunic, 1998; Subramanyam & Roesli, 2000; Kavallieratos et al., 2007). Numerous studies have been also conducted to evaluate the efficacy of local DEs in Turkey against various stored-grain pests and as a result, some micronized local DE deposits showed high efficacy against various stored-grain insect pests at a concentration of 1000 ppm (1 g DE /1 kg wheat) (Doğanay, 2013; Alagöz, 2016; Bayram, 2019; Bağrıaçık, 2020; Sağlam et al., 2022).

The effectiveness of DE in controlling insect pests is influenced by several biotic and abiotic factors, including the diatomite shell morphology, DE's physicochemical properties, the type of grain treated, the specific insect species, as well as environmental conditions like temperature and relative humidity (Korunic, 1998). One crucial physicochemical property of DEs that affect its efficacy against insect pests is DE particle size. Ideally, DE should be composed of high-purity amorphous silica with uniformly small particles, minimal clay content, and less than 1% crystalline silica (Quarles, 1992; McLaughlin, 1994; Vayias et al., 2009). DE rocks should be finely ground, ensuring that diatoms are well separated and ideally remain physically intact (Quarles, 1992). The effectiveness of DE in absorbing lipids and killing insects is also affected by the size, shape, and surface characteristics of the diatom species, along with the uniformity of the particles and the formulation's purity (Korunic, 1997, 2013). DE rocks should be finely milled to ensure that diatoms are well separated and, if feasible, maintain their physical integrity (Quarles, 1992). Additionally, the effectiveness of DE in lipid absorption and insect control depends on the diatom size, shape, and

surface features, as well as the uniformity of the particles and the purity of the formulation (Korunic, 1997, 2013). However, Baliota & Athanassiou (2020) reported that the insecticidal properties of DEs depend on particle shape rather than particle size, and that DE particle size does not necessarily mean higher efficacy. Research has shown that modifying diatomite composition and particle size can improve the insecticidal effectiveness of DE against stored-grain pests (Vayias et al., 2009; Ziaee et al., 2013; Baliota & Athanassiou, 2020). It is important to study whether adjusting the physicochemical properties, such as diatom composition and particle size, of local DEs can enhance their effectiveness against stored grain pests at lower concentrations. This study aimed to evaluate how different compositions and particle sizes of local DE affect its insecticidal efficacy against stored-grain pests *Sitophilus oryzae* L., 1763 (Coleoptera: Curculionidae) (rice weevil), *Tribolium confusum* Jacquelin du Val., 1863 (Coleoptera: Tenebrionidae) (confused flour beetle), and *Rhyzopertha dominica* (F., 1792) (Coleoptera: Bostrichidae) (lesser grain borer).

Materials and Methods

Test Insect species and their culture

Insect species used in biological tests were obtained from stock cultures in the Entomology Laboratory of the Department of Plant Protection of Kahramanmaraş Sütçü Imam University. Elbistan soft bread wheat with 11%-12% moisture content was used as food for *S. oryzae* and *R. dominica* cultures and wheat flour was used as food for *T. confusum*. Whole wheat and wheat flour used for the culture of test insects were kept in a -20ºC deep freezer for one week in case of insect contamination. For *S. oryzae*, jars were filled one-third full with wheat, and adult insects were added. The jars were kept in a dark climate chamber at 26±1ºC and 65±5% relative humidity for one week to allow the adults to lay eggs. After this period, the adults were removed using 70 mesh (210 µm) metal sieves (Retsch™ Test Sieves, Germany). The mouths of the jars were covered with fine muslin cloth and placed in the climate chamber for the emergence of the new generation. Two-week-old, mixed-sex (1:1 sex ratio) adults from this new generation were used in the biological tests. For *R. dominica*, 300-400 adults were initially placed in 200 g of wheat flour and allowed to lay eggs for two days. Afterward, the adults and eggs were separated using 35 mesh (500 µm) and 70 mesh (212 µm) sieves (Retsch™, Germany). Adults and eggs were collected using the 500 µm and 212 µm sieves, respectively, and the flour was transferred into a collection container. The eggs collected from the 212 µm sieve were placed in 1-liter sterile glass jars containing 250 g of wheat, and the jar mouths were covered with fine muslin cloth. The cultures were maintained at 30±1ºC and 65±5% relative humidity under dark conditions. Two-week-old new generation mixed-sex (1:1) *R. dominica* adults were used in the biological tests. For *T. confusum* culture, 250 g of wheat flour and 5% dry yeast (inactive) were added to 1-liter sterile glass jars, and 250-300 mixed-sex (1:1) *T. confusum* adults were introduced into the jars. The jars were covered with fine muslin cloth and stored in a completely dark climate chamber at 26±1ºC and 65±5% relative humidity for one week to allow the adults to lay eggs. After one week, the adults were removed using metal sieves, and two-week-old new-generation adults were used in the biological tests. All insect culture jars were covered with fine muslin cloth to ensure proper ventilation.

Preparation of binary mixture of local diatomaceous earths

The local DEs used in the biological tests were obtained from diatomite rocks with BHN-1 and CBN-1 codes collected from two DE reserves in Turkey. To ensure a representative sample, 10 random splits were made at different points within each DE reserve, and groove DE samples were collected. Raw DE samples, weighing at least 5 kg, were collected from each DE reserve and transported to the laboratory for processing. After coarse grinding (2 mm) of the natural raw DE rocks in a laboratory-scale jaw crusher (Alfa Test Equipment, Ankara), the coarse ground DE samples were dried in an oven (MEMMERT UN 75 Memmert Gmb, Germany) at 100±10ºC for three days until 3-5% moisture content of DE samples were reduced. The dried DEs were micronized using a laboratory-scale ball mill (RAM1107; Rantek Test Solutions, Ankara, Turkey). Powder formulations of the micronized BHN-1 and CBN-1 local DEs were obtained after sieving

them using an automatic oscillating metal sieve with a mesh size of 250 µm (Retsch, Germany). The resulting powder formulations are shown in Figure 1. Scanning electron microscopy (SEM) images and physicochemical properties of the powder formulations of BHN-1 and CBN-1 local DEs are presented in Figure 2 and Table 1, respectively. To prepare a binary mixture of BHN-1 and CBN-1 local DEs at a 1:1 ratio, each local DE was weighed and mixed using a high-shear powder mixer (PRMG-5, Prism Pharma Machinery, India) at 1500 rpm for 15 min to ensure uniform mixing. The resulting local DE mixture was placed in 1 L capacity glass bottles with tightly closed metal caps and stored at room temperature until use in biological tests. This mixture was used as the original DE in biological tests. Metal sieves of three different sizes (20, 75, and 150 µm) were used to separate the original DE mixtures into four different particle sizes. By sieving the original DE mixture (≤ 250 µm) through a metal sieve with three different mesh sizes, a local DE mixture with four different particle sizes (≤20, 20-75, 75-150 and ≥150 µm) was obtained.

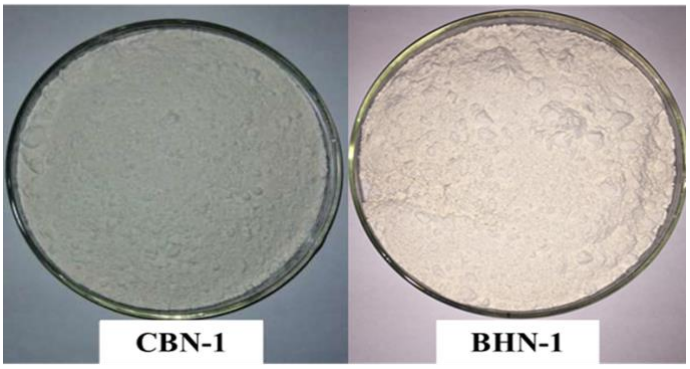

Figure 1. Micronized powder formulations of CBN-1 and BHN-1 diatomaceous earth to form local diatomaceous earth binary mixture used in biological tests.

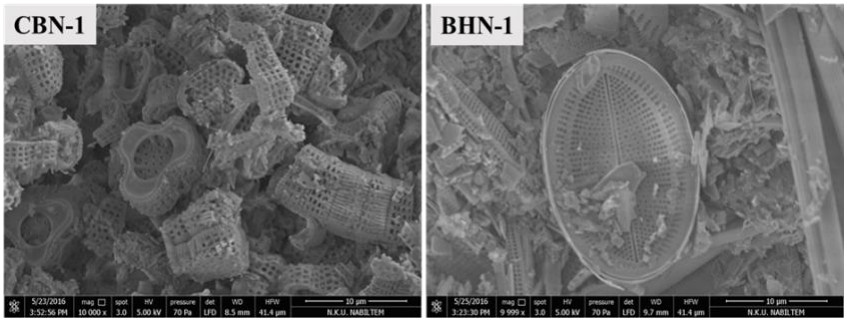

Figure 2. Scanning electron microscopy (SEM) images of CBN-1 and BHN-1 diatomaceous earth at 10000 x magnification [Scanning Electron Microscope (SEM) (FEI, QUANTA FEG 250) analysis of local DE samples was performed at Tekirdağ Namık Kemal University, Scientific and Technological Research Application and Research Centre Centre (NABILTEM].

Table 1. Silicon dioxide (SiO_2) content, median particle sizes, pH values, and adhesion rates on wheat of CBN-1 and BHN-1 diatomaceous earths to constitute the local diatomaceous earth binary mixture used in biological tests

Diatomaceous earth	SiO_2 ratio (%)[1]	Median particle size (µm)[2]	Mean pH ±S.E	Adhesion rate (%) ± S.E[3]
CBN-1	80.75	13.09	8.1 ± 0.01	75.0 ± 1.3
BHN-1	91.40	20.05	7.8 ± 0.06	89.3 ± 0.4

[1] Quantitative chemical analysis was conducted by the Accredited Mineralogy and Petrography Laboratory of General Directorate of Mineral, Research and Exploration of Turkey (MTA), and atomic absorption spectroscopy (AAS) was also used to analyze the elements following melting and acid removal processes.

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

[3] Adherence rate of DE on wheat kernels were determined using the method described by Korunic (1997) at the Stored Product Insects Laboratory of Kahramanmaraş Sütçü Imam University.

Biological tests

In the biological tests, 500 and 1000 ppm (mg DE/kg wheat) concentrations of the binary mixture of local DE with particle sizes ≤ 20 µm, 20 -75 µm, 75-150 µm, ≥ 150 µm, and original DE (≤ 250 µm) were applied in powder form to wheat. For each concentration and particle size used in the biological tests, 1 kg of wheat was weighed using a balance (EGE-M-30 BARTES, Turkey) and placed in 3 L jars. Then, for the desired concentrations, 500 and 1000 ppm local DE mixtures were weighed using a precision microbalance (AUW220D, Shimadzu Corporation, Japan) and added to 1 kg of wheat in 3 L jars. This was done separately for each of the tested particle sizes of the DE mixture. To ensure a homogenous distribution of the weighed local DE mixture on wheat, the jars were tightly closed and shaken by hand for 3 min. Wheat that was not treated with DE was used as the control. The DE treatment was repeated three times for each insect species. Sub-samples of 50 g DE-treated wheat were randomly collected from each lot (1 kg of DE-treated wheat) and transferred into 100 mL (8.3 cm x 4.5 cm) glass vials. Subsequently, 30 mixed-sex and 14-days-old adults of each insect species were transferred to glass vials containing the DE-treated wheat. The openings of the vial were tightly covered with muslin cloth, which allowed for an air inlet and outlet. Each DE treatment with three replicates was placed in 80 L (26 × 36.5 × 15 cm) lockable plastic storage containers with plastic grids underneath and 65±3% r.h. which was prepared with saturated Sodium nitrate ($Na\ NO_2$) solution (Greenspan, 1977). Plastic containers were placed in an incubator at 25±1ºC. After 14 d of treatment, live and dead adults were counted. Subsequently, all live and dead individuals were removed from the vials, and the glass vials were kept in the dark in a climate chamber of 26 ± 1ºC and 65 ± 5 % r.h. for additional 45 days to determine the number of F1 generation adults of *S. oryzae* and *R. dominica*. After 45 days, the grains in the treatment vials were sieved and the number of F1 progeny was counted.

Data evaluation and statistical analysis

When mortality was observed in the control group, Abbott's formula (Abbott, 1925) was applied to adjust the mortality rates. All mortality data for each DE treatment were normalized using the arcsine square root transformation and then subjected to three-way analysis of variance (ANOVA) with main factors: insect species, DE concentration, and DE particle size by using SAS 9 (SAS Inst., 2009) statistical software. Mean percentage mortalities for each species DE particle size, and DE concentrations were separated by using the Tukey's HSD test (Tukey's Honestly Significant Difference Test) at a 5% significance level. The differences in number of adult emergences of the new generation from the control were tested using Dunnett's test ($p = 0.005$). Adult progeny in each insect species-DE particle size combination was compared with adult progeny in the control vials using Dunnett's test at a 5% significant level. The formula was used to determine the percentage reduction in the number of adults in new generation: ((Nc-Nt) / Nc) x 100, where, Nc and Nt were the number of adult progenies in the control and DE treatments, respectively. The data on percentage reduction in the progeny adult emergence data for each insect species was evaluated using one-way analysis of variance (ANOVA) with main factor of DE particle size. The means of percentage reduction in the number of adults of new generation for each insect species and DE particle size were separated by using the Tukey's HSD test at a 5% significance level.

Results

Insect mortality

Particle size, insect species and DE concentration of the local DE mixture had a significant effect on mortality rate as shown in the statistical analysis ($p<0.0001$) Statistically significant interactions were observed between insect species and DE particle size, insect species and DE concentration, DE particle size and concentration ($p<0.0001$). However, the interaction effect between insect species, DE particle size and concentration was not significant ($p=0.468$) (Table 2).

Table 2. Results of three-factor analysis of variance applied to the percentage mortalities of *Sitophilus oryzae, Tribolium confusum*, and *Rhyzopertha dominica* adults exposed to 500 and 1000 ppm concentrations of local diatomaceous earth mixture with different particle sizes for 14 days in biological tests conducted on wheat

Sources	Degrees of freedom	Sum of squares	Mean squares	F value	p value
Insect species (B)	2	10826.6	5413.28	270.50	<0.0001
Particle size (P)	4	3382.7	845.68	42.26	<0.0001
DE dose (D)	1	7946.2	7946.24	397.07	<0.0001
B * P	8	1777.3	222.16	11.10	<0.0001
B * D	2	851.8	425.89	21.28	<0.0001
P * D	4	999.1	249.77	12.48	<0.0001
B * P * D	8	155.4	19.42	0.97	0.468
Error	60	1200.7	20.01		
Total	89	27139.8			

None of the local DE treatments with different particle sizes at 500 ppm for 14 d resulted in 100% mortality of *S. oryzae*, *T. confusum*, and *R. dominica* adults (Figure 3). For *T. confusum*, the highest percentage of death (93.1%) was obtained at a particle size ≤ 20 µm of local DE, whereas the lowest percentage of mortality (67.2%) was achieved in the local-origin DE treatment. Percentage of death did not differ significantly between the ≤ 20 µm and 20-75 µm particle sizes of local DE treatments. However, the mortality rate for the ≤ 20 µm particle size was significantly higher than those for the 75-150 µm, ≥ 150 µm, and original DE (≤ 250 µm) treatments. For *S. oryzae*, the highest percentage of death (86.5%) was obtained at the local DE particle size of ≤ 20 µm, whereas the lowest mortality rate (65.1%) was achieved at the local DE particle size of ≥ 150 µm (Figure 3). Although there was no statistical difference between the percentage of deaths obtained from ≤ 20 µm, 20-75 µm and 75-150 µm particle sizes of the local DE and original DE (≤ 250 µm) treatments, the percentage of mortality obtained from the local DE ≤ 20 µm particle size was significantly higher than that obtained from the local DE particle of ≥ 150 µm. For *R. dominica*, the highest mortality rate (80.9%) was obtained at a local DE particle size of ≤ 20 µm, whereas the lowest percentage (43.9%) was achieved at the local DE of ≥ 150 µm. The mortality rate in the ≤ 20 µm particle size of the local DE treatment was significantly higher than those obtained in the 20-75 µm and 75-150 µm particle sizes of the local DE and original DE (≤ 250 µm) treatments (Figure 3). However, there was no statistical difference between the percentage of mortalities in the 20-75 µm and 75-150 µm particle sizes of the local DE and original DE (≤ 250 µm) treatment.

Local DE with all particle sizes at 1000 ppm resulted in 100% mortality for *S. oryzae* and *T. confusum*, whereas 100% adult mortality for *R. dominica* was not achieved in any of the DE treatments. At 1000 ppm, the highest percentage mortality for *R. dominica* (85.3%) was obtained in the local DE with a particle size ≤ 20 µm, whereas the lowest percentage mortality (56.1%) was achieved in the local DE with a particle size ≥ 150 µm. The mortality rate for *R. dominica* obtained from local DE with ≤ 20 µm particle size was significantly higher than that obtained from local DE with 75-150 µm and ≥ 150 µm particles, whereas there was no significant difference between those obtained from local DE with ≤ 20 µm, 20-75 µm particle size, and original DE (Figure 3).

Significant differences in mortality rate between the insect species tested were detected at both 500 and 1000 ppm (Figure 4). At 500 ppm, no significant difference was observed in the mortality rates for *T. confusum* and *S. oryzae* adults exposed to all DE treatments, whereas the mortality rates for *T. confusum* and *S. oryzae* adults were significantly higher than those of *R. dominica.* At 1000 ppm, no significant difference was observed in the mortality rates for *T. confusum* and *S. oryzae* adults exposed to all DE treatments, whereas the percentage of death for *T. confusum* and *S. oryzae* adults were significantly higher than that for *R. dominica*. (Figure 4).

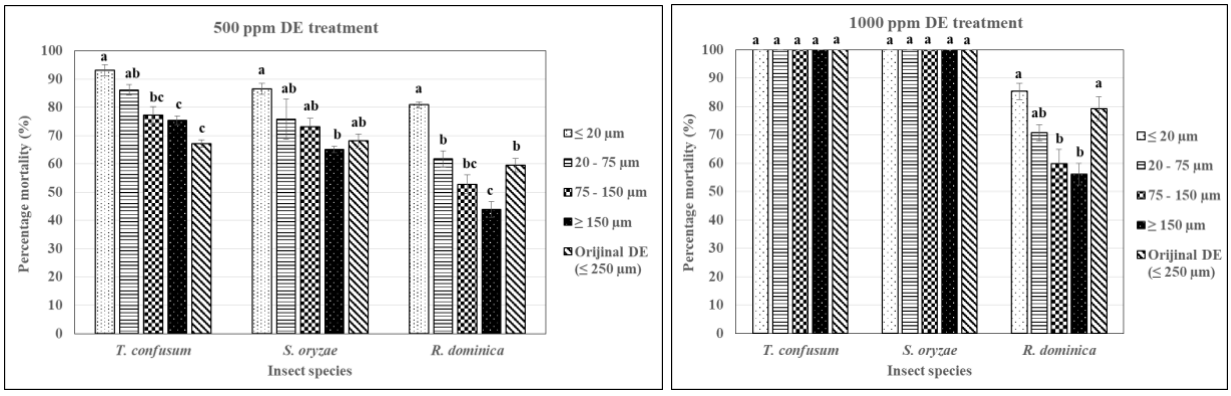

Figure 3. The percentage mortality (mean±S.E) of *Sitophilus oryzae*, *Tribolium confusum*, and *Rhyzopertha dominica* adults exposed to 500 and 1000 ppm concentrations of local diatomaceous earth mixture with different particle sizes on wheat for 14 days (Differences between means are presented according to Tukey's HSD test at 5% significance level. For a given species, means with same lowercase letters are not significantly different).

The mortality rates (mean±S.E) for *S. oryzae*, *T. confusum*, and *R. dominica* adults exposed to 500 and 1000 ppm concentrations of local diatomaceous earth mixture with different particle sizes on wheat for 14 days (Differences between means are presented according to Tukey's HSD test at a 5% significance level. For a given species, means with same lowercase letters are not significantly different) (Figure 4).

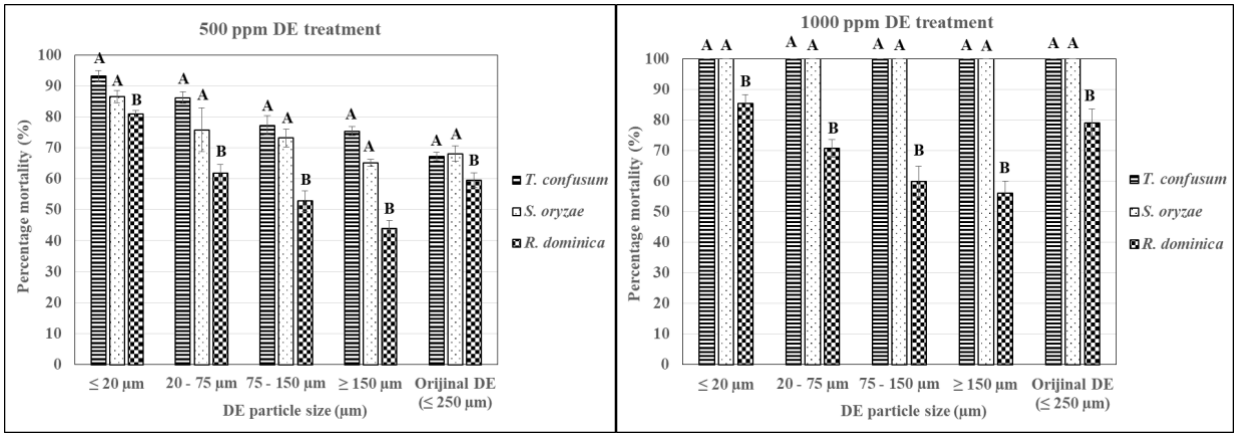

Figure 4. Effect of local diatomaceous earth mixture with different particle sizes at 500 and 1000 ppm concentrations on the percentage mortality (mean±S.E) of *Sitophilus oryzae*, *Tribolium confusum*, and *Rhyzopertha dominica* adults in wheat (differences between means are presented according to Tukey's HSD test at the 5% significance and any statistically significant differences between the species were indicated by different capital letters above the bars). For a given DE particle size, means with same uppercase letters are not significantly different)

The DE concentration had a significant effect on percentage mortality of *T. confusum* and *S. oryzae*. For both species, the mortality rates at 1000 ppm were higher than those at 500 ppm for all DE treatments with varying particle sizes, except for the local DE treatment with ≤ 20 µm particle size for *T. confusum* (Figure 5). However, for *R. dominica*, no significant differences were found among the DE concentrations in all DE treatments, except for the original DE treatment. In the original local DE treatment, the mortality rates at 1000 ppm were higher than that at 500 ppm (Figure 5).

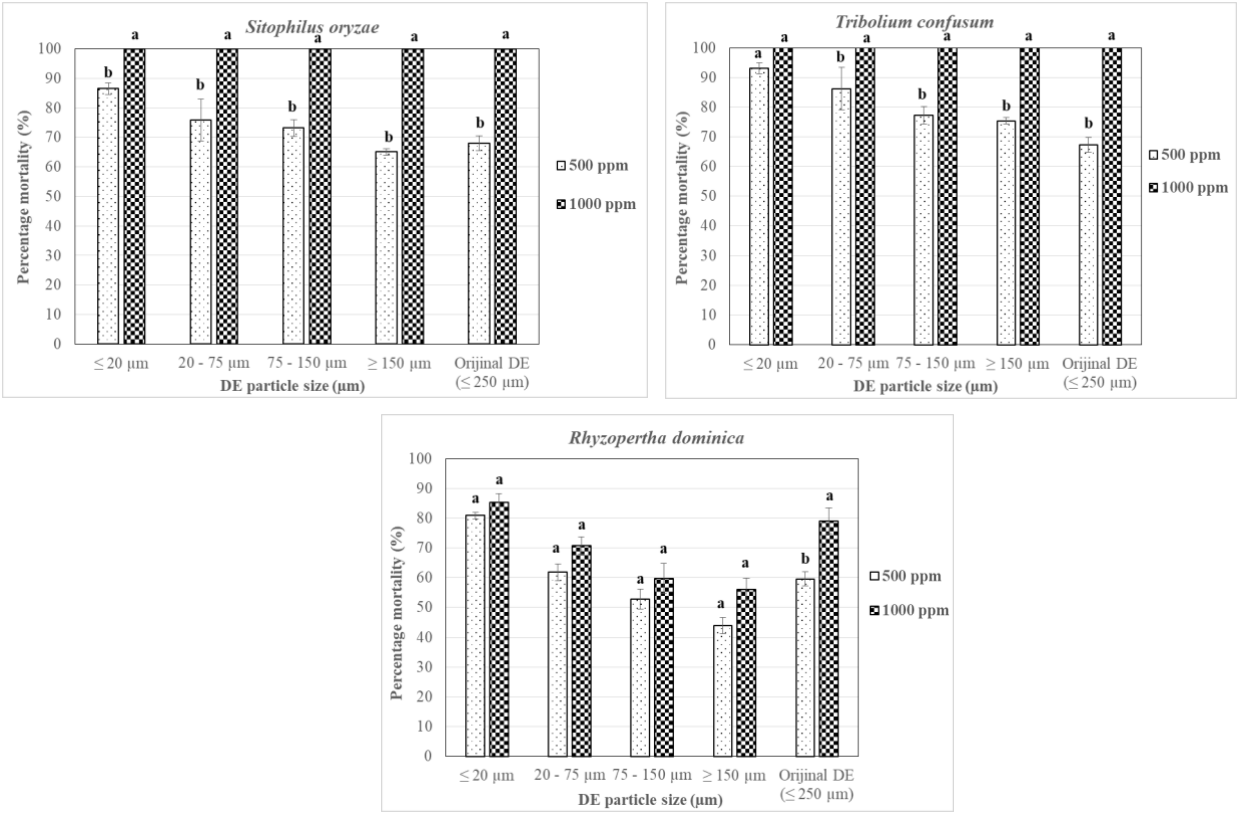

Figure 5. Effect of application concentration of local diatomaceous earth with different particle sizes on the percentage mortality (mean±S.E) of *Sitophilus oryzae, Tribolium confusum*, and *Rhyzopertha dominica* adults in wheat (differences between means are presented according to Tukey's HSD test at a 5% significance level. For a given DE particle size, means with same lowercase letters are not significantly different)

F1 progeny

According to Dunnett's test for *R. dominica*, there were significant differences between the number of new-generation adults obtained from each DE treatment at 500 and 1000 ppm, and the control treatment (Table 3). At both 500 and 1000 ppm, the lowest number of new-generation adults was obtained in the local DE with particle size ≤ 20 µm, whereas the highest number of new-generation adults was observed in the local DE with particle size ≥ 150 µm. At 500 ppm concentration, the highest reduction rate in the number of new-generation adults (75%) was obtained with the local DE ≤ 20 µm particle-size treatment, whereas the lowest reduction rate (42.7%) was observed with the local DE ≥ 150 µm particle-size treatment. The reduction rates in the number of new-generation adults in the local DE with ≤ 20 µm particle size treatment were significantly higher than those in all the other DE treatments. Similarly, the reduction rates in the number of new-generation adults in the local DE with 20-75 µm particle size treatment were significantly higher than those in the local DE with 75-150 µm, ≥ 150 µm, and local-origin DE treatments. The lowest rates of reduction in the number of new-generation adults were obtained in the local DE with ≥ 150 µm and original DE treatments. For the 1000 ppm concentration, the highest reduction rate in the number of new-generation adults (94%) was achieved in the local DE with ≤ 20 µm particle size treatment, whereas the lowest reduction rate (88.6%) was observed in the DE with ≥ 150 µm particle size treatment. The reduction rates in the number of new-generation adults in the local DE with ≤ 20 µm particle size treatment were significantly higher than those in all the other DE treatments. Similarly, the reduction rates in the number of new-generation adults in the local DE with 20-75 µm particle size treatment were significantly higher than those in the local DE with 75-150 µm, ≥ 150 µm particle size, and original DE treatments. The lowest rates

of reduction in the number of new-generation adults were achieved in the local DE with ≥ 150 µm and original DE treatments. In addition, there was no significant difference between the rates of decrease in the number of new-generation adults in the local DE with 75-150 µm, ≥ 150 µm particle size, and the original DE.

Table 3. Mean numbers of new generation of *Rhyzopertha dominica* adults and reduction rates (%) in the number of adults as a result of biological tests at 500 and 1000 ppm concentration of local diatomaceous earth mixture with varying particle sizes on wheat

DE particle size (µm))	500 ppm DE treatment			1000 ppm DE treatment		
	Number of new generation adults ± S.E	p value**	Reduction rate in the number of adults (%) ± S.E***	Number of new generation adults ± S.E	p value**	Reduction rate in the number of adults (%) ± S.E***
≤ 20 µm	53.3±4.03 (216± 1.4)*	<0.0001	75.4±1.8 A	11.6±2.18 (216 ±1.4)*	<0.0001	94.6±0.5 A
20- 75 µm	74.3±3.05 (216 ± 1.4)	<0.0001	66.1±1.4 B	21.2±2.51 (216 ± 1.4)	<0.0001	90.1±1.1 AB
75-150µm	95.6±4.8 (216 ± 1.4)	<0.0001	55.7±2.2 C	23.6±1.45 (216 ± 1.4)	<0.0001	89.07±0.6 B
≥ 150 µm	123.6±1.8 (216 ± 1.4)	<0.0001	42.7±0.8 D	24.6±1.85 (216 ± 1.4)	<0.0001	88.6±0.8 B
Original DE	108.3±4.4 (216 ± 1.4)	<0.0001	49.8.±0.3 DC	22.1±0.57 (216 ± 1.4)	<0.0001	89.7.±0.8 B
	F and p value		$F_{4,10}$ = 59.82; p<0.0001	F and p value		$F_{4,10}$ = 6.45; p=0.008

* Values in parentheses are the average number of new generation adults obtained from the control treatment.
** Dunnett's test at a 5% significance level was applied to compare the number of new generation adults in DE treatments with that in the control.
*** One-way analysis of variance (ANOVA) was applied to the data, and the differences between the means were determined according to Tukey's HSD test at a 5% significance level. Means with same uppercase letters are not significantly different.

According to Dunnett's test for *S. oryzae*, there were significant differences between the number of new-generation adults in each DE treatment and the control treatments at 500 and 1000 ppm (Table 4). At 500 ppm, the lowest number of new-generation adults was obtained in local DE with ≤ 20 µm particle size, whereas the highest number of new-generation adults was achieved in local DE with 75-150 µm, ≥ 150 µm particle size, and original DE treatments. At 500 ppm, the highest reduction rate in the number of new-generation adults (91%) was obtained in local DE with ≤ 20 µm particle size, whereas the lowest reduction rate (85%) was observed in local DE with ≥ 150 µm particle size and original DE treatments. At 500 ppm, the reduction rates in the number of new-generation adults in the local DE with ≤ 20 µm particle size treatment were significantly higher than those in all the other DE treatments. Similarly, the reduction rates in the number of new-generation adults in the local DE with 20-75 µm particle size treatment were significantly higher than those in the local DE with 75-150 µm, ≥ 150 µm, and original DE treatments. At 500 ppm, the lowest rate of reduction in the number of new-generation adults were obtained in the original DE treatment (85.1%). At 1000 ppm, the local DE with ≤ 20 µm and 20-75 µm particle size treatments resulted in the complete suppression of F1 progeny production. No significant difference in reduction rates for the number of new generations in the local DE ≤ 20 µm, 20-75 µm, 75-150 µm, and ≥ 150 µm particle size treatment were detected, whereas, the reduction rates in the number of new-generation adults for the original DE treatment were significantly lower than those of the other DE treatments.

Table 4. Mean numbers of new generation of *Sitophilus oryzae* adults and reduction rates (%) in the number of adults as a result of biological tests conducted at 500 and 1000 ppm concentration of local diatomaceous earth mixture with varying particle sizes on wheat

DE particle size (µm)	500 ppm DE treatment			1000 ppm DE treatment		
	Number of new generation adults ± S.E	p value**	Reduction rate in the number of adults (%) ±S.E.***	Number of new generation adults ± S.E	p value**	Reduction rate in the number of adults (%) ±S.E.***
≤ 20 µm	23.1±1.52 (280 ±1.3)*	<0.0001	91.7.3±0.7 A	0±0 (280 ± 1.3)*	<0.0001	100±0 A
20- 75 µm	29.6±2.60 (280 ± 1.3)	<0.0001	89.4.2±1.2 B	0±0 (280 ± 1.3)	<0.0001	100±0 A
75-150 µm	38.2±3.21 (280 ± 1.3)	<0.0001	86.3±1.4 C	3.1±0.57 (280 ± 1.3)	<0.0001	98.8±0.2 A
≥ 150 µm	42±0.57 (280 ± 1.3)	<0.0001	85.5±0.2 C	5±0.58 (280 ± 1.3)	<0.0001	98.2±0.4 A
Original DE	41.6±1.20 (280 ± 1.3)	<00001	85.1±0.7 C	8.3±0.8 (280 ± 1.3)	<0.0001	97.03±1.7 B
	F and p value		$F_{4,10}$=39.70; p<0.0001	F and p value		$F_{4,10}$ = 44.73; p<0.0001

* Values in parentheses are the average number of new generation adults obtained from the control treatment.
** Dunnett's test at a 5% significance level was applied to compare the number of new generation adults in DE treatments with that in the control.
*** One-way analysis of variance (ANOVA) was applied to the data, and the differences between the means were determined according to Tukey's HSD test at a 5% significance level. Means with same uppercase letters are not significantly different.

Discussion

In the present study, the DE particle size had a significant effect on insect mortality. Therefore, the efficiency of the local DE formulation increases with decreasing DE particle size. Local DE formulations with small particle sizes (generally ≤ 20 µm) were more effective than those with large particle sizes. Similarly, several previous studies concluded that DE treatments with low particle sizes are more effective against various stored grain pest species. For instance, DE formulations with particle sizes ≤ 10 µm exhibited high insecticidal activity against *Sitophilus granarius* (L., 1758) (Coleoptera, Curculionidae) (Aldryhim, 1990; McLaughlin, 1994; Korunic & Fields, 2006; Saez & Fuentes Mora, 2007). Another study tested DEs with particle sizes ≤ 45 µm, 45-150 µm, and ≤ 150 µm against *R. dominica*, *Chriptolestes ferrugineus* (Stephens, 1831) (Coleoptera, Cucujoidea: Laemophloeidae*)*, and *S. oryzae* adults, and found that the smallest particle size was the most effective (Vayias et al., 2009). Ziaee et al. (2013) also found that DE treatment with particle sizes ≤ 37 µm was more efficacious against *S. granarius* than treatment with larger particle sizes. These results are consistent with the findings of the present study.

Particle size distribution is a factor that greatly affects the insecticidal efficacy of inert dusts, particularly DE (Korunic, 1997). DE with smaller particles has a higher toxicity against the tested insect species compared to those with higher particles, mainly because of three reasons: (a) larger surface area and higher particle number per mass unit, (b) better and more uniform dispersity or coverage on the grains, and (c) increased contact with insect cuticles. Previous studies have highlighted the significance of particle size in DEs, indicating that smaller particles can adhere more effectively to insect bodies, leading to faster degradation of the waxy cuticular layer and increased water loss (Robinson, 2005; Ziaee et al., 2013). Consistent with these findings, Vayias et al. (2009) reported that DE efficacy is generally inversely proportional to the particle size, with smaller particles providing a larger contact area with insects. Ziaee et al. (2013) also found that particles in the 0-37 µm range successfully adhered to insect bodies. These studies collectively suggest that larger surface areas and higher surface-to-volume ratios enhance the reactivity of DE particles, significantly impacting their toxicity to insect species.

Local DE particles ≤ 45 µm exhibited stronger insecticidal properties against the tested insect species than larger particles. In our study, the presence of larger particles (≥ 150 µm), such as rocks, sand, and large diatoms, negatively impacted DE performance. Consequently, it is important to eliminate these larger particles during DE processing and purification. However, Korunic & Ormesher (1998) found no correlation between mean particle size < 15 µm and diatom shape with insecticidal activity. In some cases, DE formulations from Elassona, Greece, with particle sizes of 45-150 µm proved to be more effective than DE samples with smaller particles (Vayias et al., 2009). Baliota & Athanassiou (2020) observed that particle shape affects insecticidal value of DE, where smaller particles do not necessarily increase efficacy. This suggests that insecticidal value of DE depends on factors beyond particle size, such as active surface area, oil adsorption, inner pore diameter, moisture content, SiO_2 content, and tapped density (Korunic, 1997).

The effectiveness of local DE treatments with varying particle sizes differed among the insect species tested. At concentrations of 500 and 1000 ppm, there were no significant differences in the mortality rates of *T. confusum* and *S. oryzae* adults across all local DE treatments. However, the mortality rates for T. confusum and *S. oryzae* were significantly higher compared to *R. dominica*. At 1000 ppm, all local DE treatments achieved complete mortality for *T. confusum* and *S. oryzae*, while *R. dominica* did not reach 100% mortality with any of the local DE treatments. At 500 ppm, none of the local DE treatments with all particle sizes achieved complete mortality for any species, whereas the highest mortality rates of *T. confusum (*93%*), S. oryzae (*86%), and *R. dominica* (80%) were observed in local DE treatments with the smallest particle size (≤ 20 µm). Our findings suggest that the local DE mixture is highly effective against *T. confusum*, which is one of the most tolerant stored-grain insects to DE (Korunic, 1998; Arthur, 2000). Kavallieratos et al. (2007) noted that three commercially available modified DE formulations at 500 ppm

with 7 days exposure resulted in low mortality rates of *T. confusum* adults, ranging from 5% to 39%. According to these results, our local DE mixture appeared to be more effective against *T. confusum* adults than PyriSec, Insecto, or Protect-It. Athanassiou et al. (2007) reported that PyriSec, Insecto and Protect-It on wheat at 500 ppm resulted in mortality rates of *S. oryzae* and *R. dominica* adults ranging from 92% to 99% after 7 days exposure. Based on these mortality results, it appears that our local DE mixture is slightly less effective against *S. oryzae* and *R. dominica* than PyriSec, Insecto and Protect-It. These findings clearly indicate that the blends of local DEs can be a potential source for the development of commercial products, despite the slightly lower mortality rates in *S. oryzae* and *R. dominica* compared to the three commercial DE formulations.

Subramanyam and Roesli (2000) suggest that, under practical cereal storage conditions, it is often more critical to prevent the formation of progeny than to focus exclusively on achieving direct lethal effects of DE on adult insects. In our study, aligned with the mortality rates observed at 500 and 1000 ppm, there was a statistically significant difference in the number of new-generation adults between the control treatments and those with different particle sizes for all insect species. Specifically, *S. oryzae* showed complete progeny inhibition with local DE at ≤ 20 µm and 20-75 µm particle sizes only at 1000 ppm, whereas for *R. dominica*, progeny production was not inhibited in any of the local DE treatments at either concentration. These results are consistent with those of previous studies. Similar findings in controlling *S. oryzae* progeny have been observed with several commercially available DE formulations, such as SilicoSec, Insecto, and Protect-It (Subramanyam & Roesli, 2000; Athanassiou et al., 2003, 2005, 2008). In our study, *R. dominica* offspring were present at all intervals, regardless of DE particle size and dose. Similarly, Athanassiou et al. (2011) observed that the commercial DEs tested could not completely eliminate *R. dominica* progeny production in wheat, maize, rice, and barley.

Diatom geometric properties vary between species, with an increase in pore number, size, and surface distribution enhancing insecticidal capability and cuticular wax absorption. Diatom particles can erode insect cuticles, resulting in increased water loss and death (Ebeling, 1971; Subramanyam & Roesli, 2000). Small particles, when they come into contact with pests, limit their movement and lead to death (Robinson, 2005). Our findings confirm that smaller particles are more effective, particularly against stored grain pests. Diatom particles are prepared for commercial use by quarrying, drying, and milling, processes that mainly decrease moisture content and the average particle size. As a result, milling could play a key role in defining the insecticidal effectiveness of DE formulations against stored-grain pests. In conclusion, this study emphasizes the critical role of particle size in affecting the insecticidal effectiveness of DE formulations and stresses the need to account for these factors during the DE production process.

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