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Original article (Orijinal araştırma)

Assessing the antiparasitic potential of *Bifidobacterium* sp. Ersapi20 isolated from the cuticle of honeybees against *Varroa destructor* Oudemans, 1904 (Acari: Varroidae)[1]

Bal arılarının kutikulasından izole edilen *Bifidobacterium* sp. ERSapi20 suşunun *Varroa destructor* Oudemans, 1904 (Acari: Varroidae)'a karşı antiparazitik potansiyelinin değerlendirilmesi

Mustafa ERSAL[2] **Kemal KARABAĞ[3]** **Hüsniye Tansel YALÇIN[4*]**

Abstract

The mite *Varroa destructor* Oudemans, 1904 (Acari: Varroidae) is a major contributor to honeybee colony losses worldwide. Actinobacteria are known for their wide distribution and production of biologically active compounds effective against various pathogens, including bacteria and parasites. This study aims to investigate the bioactive compounds produced by actinobacterial symbionts associated with *Apis mellifera* L., 1758 (Hymenoptera: Apidae) and evaluate their acaricidal potential against *V. destructor*. In 2022, a strain of *Bifidobacterium* sp. ERSapi20 was isolated from worker bees in Antalya, Türkiye, using a culture-based method. The antiparasitic activity of the extracellular solution and intracellular extract of the strain was assessed through direct spraying on *V. destructor* at varying concentrations of insect Ringer solution (0%, 25%, 50%, 75%, and 100%). The extracellular solution achieved 90% mite mortality within 16 hours, while the intracellular extract resulted in 100% mite mortality within 18 hours. This is the first report demonstrating acaricidal activity against *V. destructor* using intracellular extracts of actinobacterial strains. These findings support the hypothesis that honeybee-associated actinobacteria produce compounds effective against varroa mites, highlighting the potential of natural products for controlling *V. destructor* in honeybee colonies.

Keywords: Actinobacteria, antiparasitic activity, *Apis mellifera*, secondary metabolite, *Varroa destructor*

Öz

Varroa destructor Oudemans, 1904 (Acari: Varroidae), bal arısı kolonilerinin kaybına ciddi katkıda bulunduğu bilinmektedir. Aktinobakteri türleri ise geniş bir dağılıma sahiptir ve bakteriler ve parazitler gibi çeşitli patojenlere karşı biyolojik olarak aktif bileşikler üretirler. Bu temele dayanarak, bu çalışma *Apis mellifera* L., 1758 (Hymenoptera: Apidae) ile ilişkili aktinobakteri simbiyontları tarafından üretilen biyolojik olarak aktif bileşikleri araştırmayı ve bunların *V. destructor*'a karşı akarisit potansiyelini değerlendirmeyi amaçlamaktadır. Kültür bazlı bir yöntem kullanılarak işçi arılardan *Bifidobacterium* sp. ERSapi20 suşu Antalya, Türkiye lokasyonundan 2022 yılında izole edilmiş ve tanımlanmıştır. Antiparazitik aktiviteyi belirlemek için, suşun ekstraselüler ve intraselüler ekstraktı, böcek Ringer çözeltisinin değişen konsantrasyonlarında (%0, %25, %50, %75 ve %100) püskürtme yöntemi kullanılarak *V. destructor*'a uygulanmıştır. Ekstraselüler çözeltinin 16 saat içinde %90 oranında akar ölümüne yol açtığı, intraselüler ekstraktın ise 18 saat içinde %100 oranında akar ölümüne neden olduğu belirlenmiştir. Bu, aktinobakteriyel suşların intraselüler ekstraktı kullanılarak *V. destructor*'a karşı akarisit aktivitesinin rapor edildiği ilk çalışmadır. Bu araştırma, bal arılarının varroa akarlarına karşı etkili bileşikler üreten aktinobakterilerle ilişki kurduğu hipotezini desteklemekte ve *V. destructor*'u kolonilerde kontrol etmek için doğal ürünlerin kullanılmasına dair bilgiler sunmaktadır.

Anahtar sözcükler: Aktinobakteri, antiparazitik aktivite, *Apis mellifera*, sekonder metabolit, *Varroa destructor*

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Introduction

Honeybee, *Apis mellifera* L., 1758 (Hymenoptera: Apidae), is one of the most important invertebrates in the agriculture, playing a crucial role in pollination and the production of honey and other bee products. However, honeybee colonies have been experiencing significant losses globally (Simon-Delso et al., 2014). Various factors contribute to these declines, including habitat loss, inadequate nutrition, numerous pathogens and parasites, reduced genetic diversity, climate change, introduction of alien species, and intensive agricultural practices (Meixner, 2010; Crotti et al., 2013; Goulson et al., 2015; DeGrandi-Hoffman et al., 2017; Muñoz-Colmenero et al., 2020). Among these factors, parasitic infections, particularly by the mite *Varroa destructor* Oudemans, 1904 (Acari: Varroidae), are highly detrimental, affecting honeybee performance, productivity, and overall colony health (Evans, 2003; Zemene et al., 2015; Tutun et al., 2018). This mite not only compromises honeybee health but also acts as a biological vector for various viruses, thereby exacerbating colony losses worldwide (Shen et al., 2005; Hussain et al., 2018; Reyes-Quintana et al., 2019). Efforts to control *V. destructor* over the last few decades have included physical, chemical, and biological methods. However, none of the current mite mitigation strategies are fully effective, and the varroa mite remains a significant challenge for beekeeping (Rosenkranz et al., 2010).

The increasing resistance of varroa mites to commonly used acaricides, such as flumethrin, coumaphos, fluvalinate, and amitraz, has further complicated control efforts, leading to chemical residues in bee products and necessitating alternative management approaches (Martin, 2004; Pettis, 2004; Lodesani & Costa, 2005; Maggi et al., 2010; Rosenkranz et al., 2010; Higes et al., 2020).Consequently, there is an immediate necessity for alternative management approaches, including microbial control. Among them, actinobacteria may offer promise for microbial control of the varroa mite because they are capable of producing a variety of bioactive molecules and are known to be sources of various metabolites with their complex biochemical processes (Khasabuli & Kibera, 2014; ul Hassan & Shaikh, 2017). In this regard, actinobacteria may be the critical bacterial group to resolve varroa mite control.

Actinobacteria are gram-positive, filamentous bacteria with high guanine-cytosine (G+C) content in their genomes, characterized by their ability to produce a wide array of bioactive molecules (Barka et al., 2016). Actinobacterial genomes display heterogeneity, which is presumed to contribute to their biodiversity (Daffe et al., 1990). Generally, actinobacteria are free-living microorganisms that are widely distributed in various ecosystems and are able to utilize numerous nutritional sources, including complex polysaccharides (Zimmermann, 1990). These phyla are a crucial bacterial group in the field of biotechnology (Běhal, 2000; Barka et al., 2016). These microorganisms are well-known for their production of secondary metabolites with diverse biological activities, including antibacterial, antifungal, anticancer, antitumor, cytotoxic, cytostatic, anti-inflammatory, antiparasitic, antimalarial, antiviral, antioxidant, antiangiogenetic, immunosuppressive and enzyme effects (Kanbe et al., 1992; Chaudhary et al., 2013; Manivasagan et al., 2014). In this regard, actinobacteria, particularly the genus Bifidobacterium, have shown potential as biological control agents against arthropod pests, including mites.

Specifically, Bifidobacterium species have been highlighted for their acaricidal potential against *V. destructor*. For instance, Saccà & Lodesani (2020) reported that *Bifidobacterium asteroides* strains were 90-95% effective as acaricides against varroa mites. This study underscores the potential of Bifidobacterium species in managing varroa populations, providing a foundation for further exploration of these bacteria as viable biocontrol agents. Given the critical need for alternative and sustainable varroa control strategies, exploring the efficacy of Bifidobacterium strains, such as ERSapi20 isolated from honeybee surfaces, is of great importance.

To fully assess the antiparasitic potential of bacteria such as *Bifidobacterium* sp., it is essential to evaluate both extracellular and intracellular extracts. Extracellular extracts can contain secreted bioactive molecules, including enzymes, toxins, and secondary metabolites, which may directly impact parasite viability. In contrast, intracellular extracts may unveil additional bioactive compounds that are stored within the cells and not secreted into the surrounding environment. Analyzing both types of extracts helps in developing a comprehensive understanding of the mechanisms through which these bacteria exert their antiparasitic effects. This approach ensures that all relevant bioactive components, whether secreted or retained within the cell, are accounted for in the assessment of antiparasitic efficacy. This comprehensive evaluation is crucial, as the variability in bacterial efficacy against varroa mites can be influenced by the specific metabolites present in different extracts.

This study aims to build on these findings by investigating the antiparasitic potential of *Bifidobacterium* sp. ERSapi20 isolated from honeybee surfaces. By assessing the impact of this strain on varroa mite mortality under laboratory conditions, this research seeks to contribute to the development of novel microbial control strategies that can effectively mitigate varroa mite infestations and support honeybee health.

Materials and Methods

Sample collection

Worker honeybees (*Apis mellifera*) were collected individually from different beehives of an apiary in the spring of 2022. All bees were selected based on their vitality and collected separately to ensure the consistency of the bacterial source. Sampling was conducted in the middle-west of Antalya (Latitude: 36.8919 N, Longitude: 30.5666 E), located in the southwest of Türkiye. Five worker honeybees were selected from each beehive, resulting in a total of 20 bees used as bacterial sources. Each bee was placed in a separate sterile container to avoid cross-contamination before being transferred to a combined peptone water solution (0.95%) and stored at +4ºC under aseptic conditions. The parasitic mite *V. destructor* samples were selected according to physical and morphologic features, and were collected from different beehives located in the same apiary as described above.

Selective isolation and preliminary identification of actinobacteria

The surfaces of freshly collected honeybees were subjected to a 0.95% peptone water solution to selectively isolate actinobacteria. Sampling was incubated overnight in International Streptomyces Project-II (ISP-2) broth at 27ºC in a shaker at 150 rpm. ISP-2 broth was prepared as follows: Yeast extract (4.0 g/l), malt extract (10.0 g/l), dextrose (4.0 g/l), pH 7.3±0.2 (Shirling & Gottlieb, 1966). Then the fluid was inoculated on the petri dish containing Actinomycetes Isolation Agar (AIA, BD DIFCO) supplemented with cycloheximide (50 µg/ml) and nalidixic acid (75 µg/ml). The inoculated plates were incubated at 27ºC for 14 days (Roshan, 2013). All experiments were conducted in triplicate.

The isolates were selected according to their colony morphology, the color of mycelium, and diffusible pigments and subcultured three times to obtain pure isolates (Shirling & Gottlieb, 1966). The Gram staining method was used to determine for gram reactions of isolates (Beveridge, 2001). The Gram-positive isolate was selected and used for further analyses. The selected isolate was stored at -20ºC in Luria-Bertani broth (LB broth, Merck) with 20% (v/v) glycerol.

DNA extraction, amplification, and identification of actinobacteria.

Based on morphological characterization and anti-parasitic potential, the selected isolate was identified based on the 16S rRNA sequencing. Genomic DNA was extracted using the GeneJET Genomic DNA Purification Kit following the manufacturer's instructions (Thermo Scientific, USA). The purity of the product resulting from genomic DNA isolation was determined using a nanodrop device (NanoDrop 200c, Thermo Scientific). The 16S rRNA gene was amplified with 5'-AGAGTTTGATCCTGGCTCAG-3' and 5'-GGCTACCTTGTTACGACTT-3' universal primer set (Hong et al., 2014). PCR amplification was performed using the 5X PCR Dye Master Mix II (GeneMarkBio) following the manufacturer's instructions. Thermal cycling was programmed as follows: initial denaturation at 95ºC for 5 min, 40 cycles of denaturation at 95ºC for 1 min, annealing at 57ºC for 1 min, extension at 72ºC for 1 min, and a final extension at 72ºC for 10 min. Thermal cycling was performed in a final volume of 25 µl using a GeneAmp PCR System 9700 (Thermo Fisher Scientific, USA). The amplified products were determined by 2% (w/v) agarose gel electrophoresis. The PCR products (around 1,300 bp lenght) were sequenced by Aquatayf Biotechnology LLC (Türkiye). The sequencing results were analyzed using the GENEIOUS software (https://www.geneious.com/). The software-generated 16S rRNA consensus sequences were analyzed for homology to type strains in the database via NCBI BLAST (https://blast.ncbi.nlm.nih.gov). The phylogenetic tree was constructed using neighbour-joining method (Saitou & Nei, 1987) by MEGA11 software and was bootstrapped using 1000 replicates for each sequence (Stecher et al., 2020; Tamura et al., 2021).

Bacterial growth curve

Growth curve of *Bifidobacterium* sp. ERSapi20 was performed for the characterization of the death phase or the phase of decline (Monod, 1949; Zwietering et al., 1990). The strain was incubated in 250 ml flasks with 100 ml

of LB broth at 27ºC with aeration in a shaker at 150 rpm. Each 24 h of incubation, 1.5 ml of fluid was taken from flasks, and transferred to eppendorf tubes. The fluids were washed using distilled water and centrifuged at 10.000 rpm for 10 minutes. The growth phases of the isolate were tracked by measuring optical density (OD) at 600 nm under aerobic conditions. The distilled water serves as the control. All experiments were conducted in triplicate.

Screening for antiparasitic activity

In this step, the antiparasitic activity test was conducted using the extracellular and intracellular solutions of the actinobacterial isolate. The approach was modified based on the study by Tsagou et al. (2004). Adult female *Varroa destructor* mites were collected aseptically from capped brood cells and placed in petri dishes (90 mm × 10 mm), with each dish containing ten mites and five bee pupae (two mites per pupa). To prepare the bacterial solutions, the extracellular and intracellular extracts of *Bifidobacterium* sp. ERSapi20 were obtained based on the bacterial growth curve analysis. Solutions were prepared in varying concentrations (250 µl, 500 µl, 750 µl, and 1000 µl) and diluted with 1 ml of insect Ringer solution (130 mM NaCl, 5 mM KCl, and 1 mM $CaCl_2$). For the spray application, a fine mist sprayer calibrated to deliver a consistent 1 ml volume was used. Each petri dish containing the mites and pupae was placed on a clean, sterile surface, and the lid was partially opened to avoid contamination while allowing access for spraying. The sprayer nozzle was held approximately 15 cm above the mites to ensure even coverage without causing physical disturbance to the mites or pupae.

The solution was sprayed gently in a sweeping motion across the surface of the petri dish to uniformly coat the mites and pupae. After spraying, the petri dishes were immediately covered to minimize evaporation and prevent external contamination. The sprayed petri dishes were then placed in an incubator set at 31±1.0°C. Mite survival and activity were monitored every two hours for 24 hours post-application. For the extracellular solution test, Amitraz was used as a positive control, and insect Ringer solution served as a negative control. In the intracellular solution test, sterilized LB broth and Tris/HCl buffer were used as controls, sprayed in the same manner as the test solutions. Each assay was conducted in triplicate to ensure consistency and reliability of the results.

For the experiment estimating the effect of the extracellular broth, the strain was cultivated in 250 ml flasks with 100 ml of LB broth at 27ºC in a shaker at 150 rpm for 6 d. The cultures (45 ml) were transferred to sterilized falcon tubes and centrifuged at 5000 rpm for 30 min at 4ºC. Then, the supernatant was filtered using Whatman No.1 filter paper. The filtered supernatant was sprayed at a dose of 1 ml per dish containing 10 mites. For analyzing the effect of the intracellular extract, the harvested bacterial cells (corresponding to 121.1 mg of dried biomass) were suspended in 5 ml of Tris/HCl buffer, pH 7.5, and were ruptured by four 45 s sonic bursts (20 kHz) on ice using a sonicator (Bandelin Sonopuls HD 2070, Bandelin electronic, Berlin, Germany). Sonicated solution was centrifuged at 5000 rpm for 30 min at 4ºC. Afterward, the supernatant was passed through Whatman No.1 filter paper and utilized in the bioassay (1 ml per petri dish), as outlined above. The bioassays were performed at 31±1ºC in the dark. Varroa mite mortality was recorded per 2 hours for 24 hours. Varroa mites treated with fermentation medium (LB broth), sterilized insect ringer solution, and Tris/HCl buffer served as negative controls. Amitraz was used in this study as a positive control (data not shown). The assay was replicated 3 times.

Assessing the ımpact of extracellular and ıntracellular *Bifidobacterium* sp. ERSapi20 solutions on honeybee mortality

To determine the effect of extracellular and intracellular solution of *Bifidobacterium* sp. ERSapi20 strain on *A. mellifera*, bacterial solutions were sprayed on the bees in laboratory cages. The honeybees were housed in standard laboratory cages constructed with wire mesh to prevent escape, ensure ventilation, and equipped with feeders containing sugar syrup for nutrition. The bees (20-25 individuals) were collected from different beehives located in the same apiary described above. The groups of 20 bees were sprayed with 1 ml extracellular and intracellular solution, separately. The extracellular and intracellular solutions obtained from the isolate were prepared at varying concentrations (0%, 25%, 50%, 75%, and 100%) by mixing with 1 ml of Insect Ringer solution and were applied to the bees using a spraying method (De Guzman et al., 1993; Tsagou et al., 2004). The Insect Ringer solution used in the study was composed of 130 mM NaCl, 5 mM KCl, and 1 mM CaCl2 (Gliński & Jarosz, 1990). The bee mortality was recorded per 2 hours for 24 hours, as described above. The bees in the control laboratory cages were sprayed with bacteria-free mediums.

Data analysis

The sequence of *Bifidobacterium* sp. ERSapi20, along with sequences from closely related species obtained from the GenBank database (NCBI), was aligned using multiple alignment in Clustal W through GENEIOUS (https://www.geneious.com). Pairwise distances were computed using the Tamura-Nei model, and a phylogenetic tree was constructed using the neighbor-joining method as implemented in MEGA11 software (Tamura et al., 2021). The tree was bootstrapped with 1000 replicates, using resampled alignments of the 16S rDNA sequences.

All the bioassays were conducted in triplicate and subjected to statistical analysis ($p < 0.05$). The differences in the lethal effects of intracellular and extracellular solutions of ERSapi20 at different concentrations on Varroa were analyzed using R-Studio. (https://www.rstudio.com/). The impact of bioactive compounds produced by *Bifidobacterium* sp. ERSapi20 on varroa mite survival was assessed using a survival probability test conducted in R Studio (https://CRAN.R-project.org/view=Survival), a package utilized for data analysis. The analysis involved estimating the survival function using the Kaplan-Meier method, generating survival curves, and determining the statistical significance between the negative control groups. Statistical analysis further involved conducting log-rank tests or Cox proportional hazards models to assess differences in survival rates between treatment and control groups (Weerahandi & Yu, 2020).

RESULTS

Isolation and identification of ERSapi20

The strain was grown on the AIA medium. The colony of the strain is regular, circular, milky, and smooth (Figure 1a). The gram staining result showed that the isolate is a gram-positive bacteria and a curved shape in purple (Figure 1b).

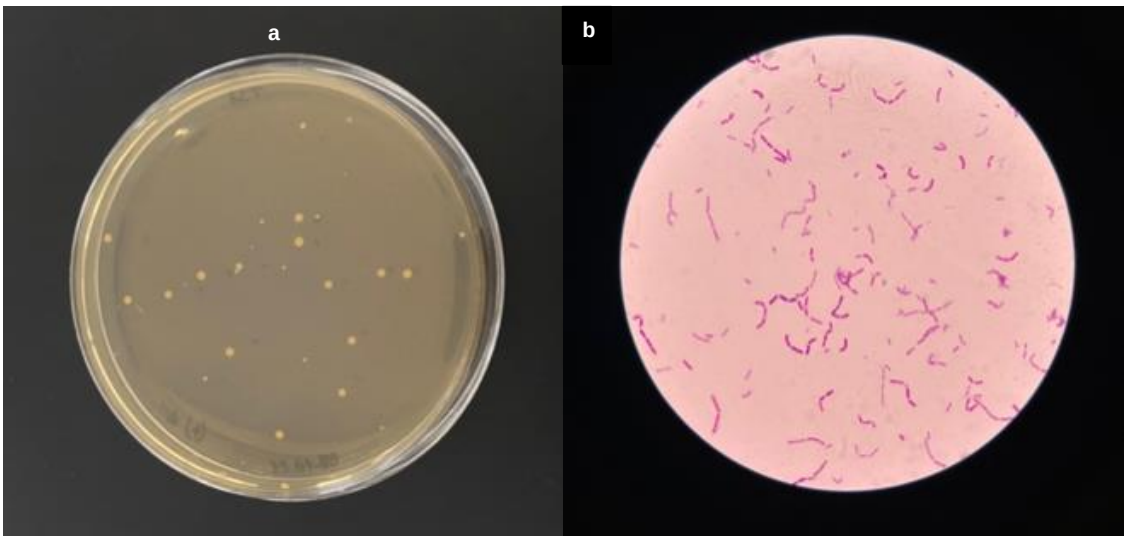


Figure 1. Morphological characterization of *Bifidobacterium* sp. ERSapi20: a) colonizing form of strain on Actinomycete Isolation Agar, b) gram staining result.

The partial 16S rDNA sequences of the isolate have been deposited in the GenBank database under the following accession number: OQ073505. The sequence size of the strain was 1397 bp in length, possessing an average G + C% content of 60.56%. The 16S rDNA sequence of the strain ERSapi20, is having a 99.57% similarity with *Bifidobacterium asteroides* DSM 20089, as analyzed with NCBI. Phylogenetic analysis with the neighbour joining algorithm using the Tamura-Nei model shows that the isolate originated from the same ancestor as *B. asteroides* strains. However, based on the branch length with *Bifidobacterium* sp. strains, it can be assumed that the isolate might be quite different from its closest relative (Figure 2).

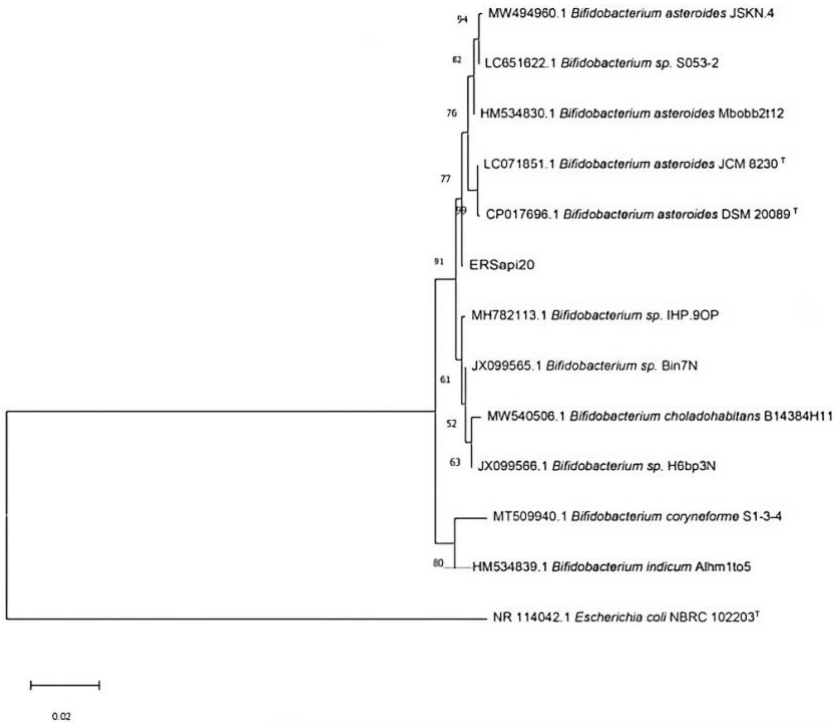


Figure 2. Phylogenetic tree of actinobacteria isolated from honeybee surfaces and their closest type strains, constructed using the neighbour joining method based on 16S rDNA sequences with the Tamura-Nei model. Bootstrap values, indicating the confidence level of each branch, were derived from 1,000 replicates and are shown at the branch nodes.

Antiparasitic activity of ERSapi20 against varroa mites

The production of secondary metabolites occurs during the stationary phase (Duquesne et al., 2007). Thus, bacterial growth was observed for ERSapi20 strain in LB broth at 27 ºC. The observation was performed once every 24 h until the strain entered the death phase. The strain entered the stationary phase after 48 h, with OD600 values of 2.5122, whereas it entered the death phase after six days with OD600 values of 2.3241 (Figure 3). Varroa mite mortality was determined after treatment with fermentation solution of ERSapi20 strain isolated from the surface of worker honeybees (Figure 4). For each experiment mite susceptibility to the fermentation solutions of six-day-old strain (according to growth curve result) within different concentrations were determined by calculating the time for 50% and 90% mortality of the varroa mites (LT50 and LT90, respectively) (Table 1). At 31±1ºC in the dark, mite mortality in the treatments of intracellular extract of ERSapi20 strain reached 100% within 18 h, whereas 90% mortality of the mites occurred using the extracellular solution at 16 h.

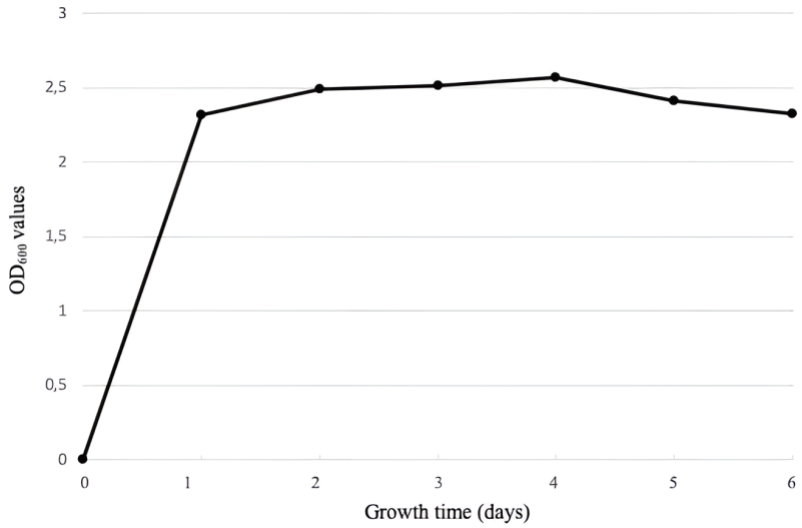
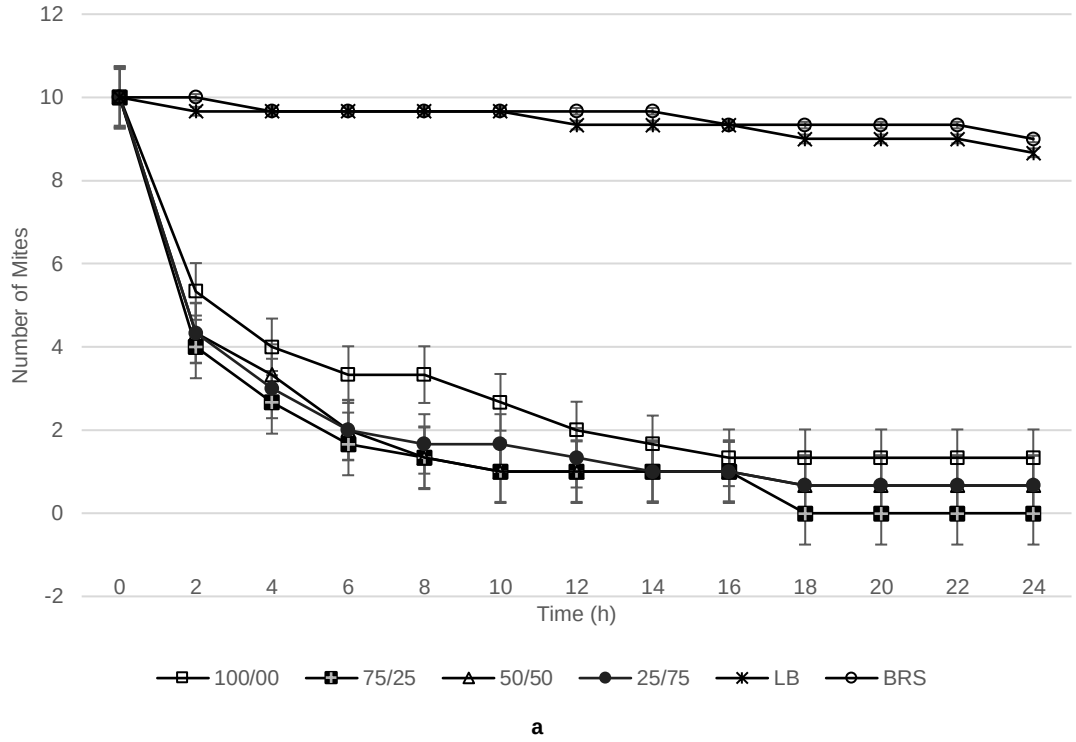
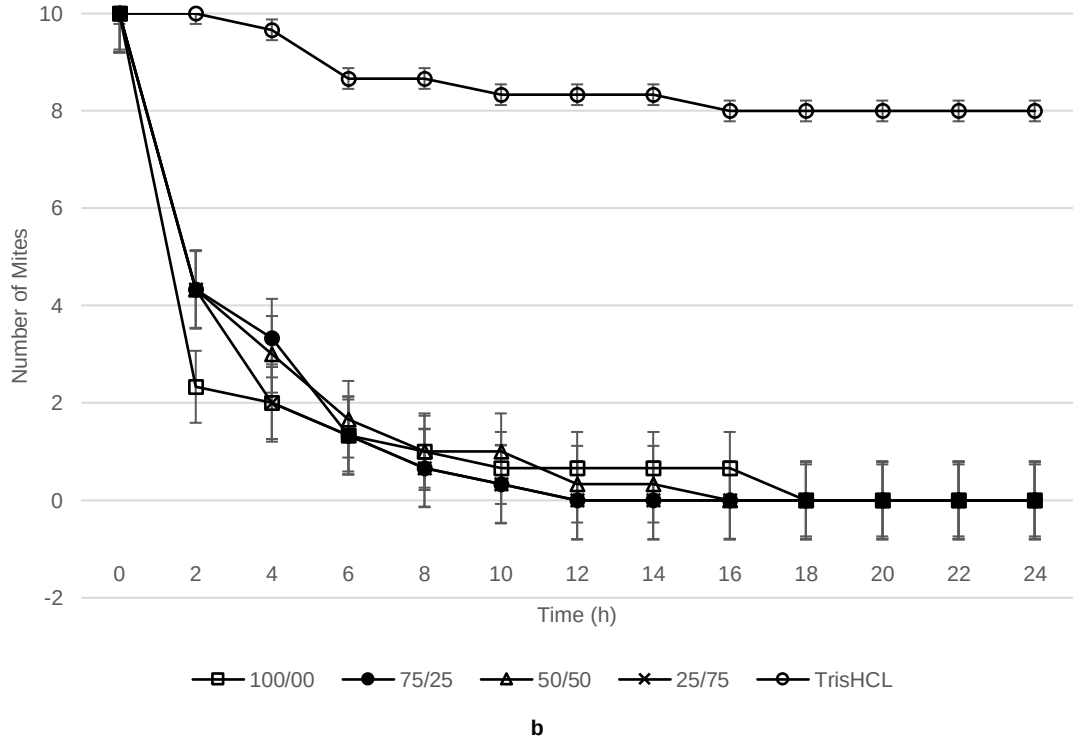


Figure 3. Growth curve of bacterial culture showing OD_{600} values over time.



a



b

Figure 4. Effect of *Bifidobacterium* sp. ERSapi20 on the survival of *V. destructor* mites over time. The graph presents the mean number of surviving *V. destructor* mites, with standard error values included for each concentration tested: a) effect of extracellular solution of ERSapi20 at different concentrations on the number of living mites. Concentrations were prepared by mixing extracellular solution with insect ringer solution (BRS) in different ratios (extracellular solution / insect ringer solution, v/v). Mites sprayed both sterilized LB broth and BRS. Also, LB and BRS served as negative controls; b) effect of intracellular extract of ERSapi20 on the number of living mites using different concentrations of extract. Concentrations were prepared by mixing intracellular extract with Tris-HCl buffer (intracellular solution / Tris-HCl buffer v/v). Tris-HCl buffer was used as a negative control.

Table 1. Effect of ERSapi20 strain on the*Varroa destructor**

Sprayed agents	LT_{50}[a] (h)	LT_{90}[b] (h)
ERSapi20e*	3.0 ± 1.0	16.0 ± 0.5
ERSapi20e : IRS** (75:25, v/v)	1.5 ± 0.5	10.0 ± 0.5
ERSapi20e : IRS (50:50, v/v)	1.5 ± 0.5	10.0 ± 0.5
ERSapi20e : IRS (25:75, v/v)	2.0 ± 0.5	14.0 ± 0.5
LB broth control	ND	ND
IRS control	ND	ND
ERSapi20i***	1.5 ± 0.25	8.0 ± 0.5
ERSapi20i : TrisHCl (75:25, v/v)	2.0 ± 0.5	4.0 ± 0.5
ERSapi20i : TrisHCl (50:50, v/v)	2.0 ± 0.5	4.0 ± 0.5
ERSapi20i : TrisHCl (25:75, v/v)	2.0 ± 0.5	4.0 ± 0.5
TrisHCl control	ND	ND

[a]: Times for 50% mortality of mites; [b]:Times for 90% mortality of mites.

ND: Not detected. *: extracellular solution of ERSapi20; **: insect ringer solution; ***: intracellular extract of ERSapi20.

The survival probability for all concentrations was found to be close to 1.0 within a 24-hour timeframe. This suggests that the survival rate of the bees exposed to the different concentrations of the bacterial solution was similar to that of the control group. In other words, the bacterial solution did not have a significant adverse effect on the bees' survival within the tested time frame. These findings have important implications, as they indicate that the use of the bacterial solution, even at different concentrations, did not lead to a significant reduction in the bees' survival rate. It supports the potential safety and effectiveness of the bacterial solution as an intervention method against varroa mites without harming the honeybees.

The survival probability test and ggplot revealed significant differences in mite mortality rates between the control group and treatment groups exposed to the extracellular solution and intracellular extract of *Bifidobacterium* sp. ERSapi20 (Figures 5 & 6). These findings suggest that the bioactive compounds derived from the strain have a substantial impact on varroa mite survival ($p < 0.05$).

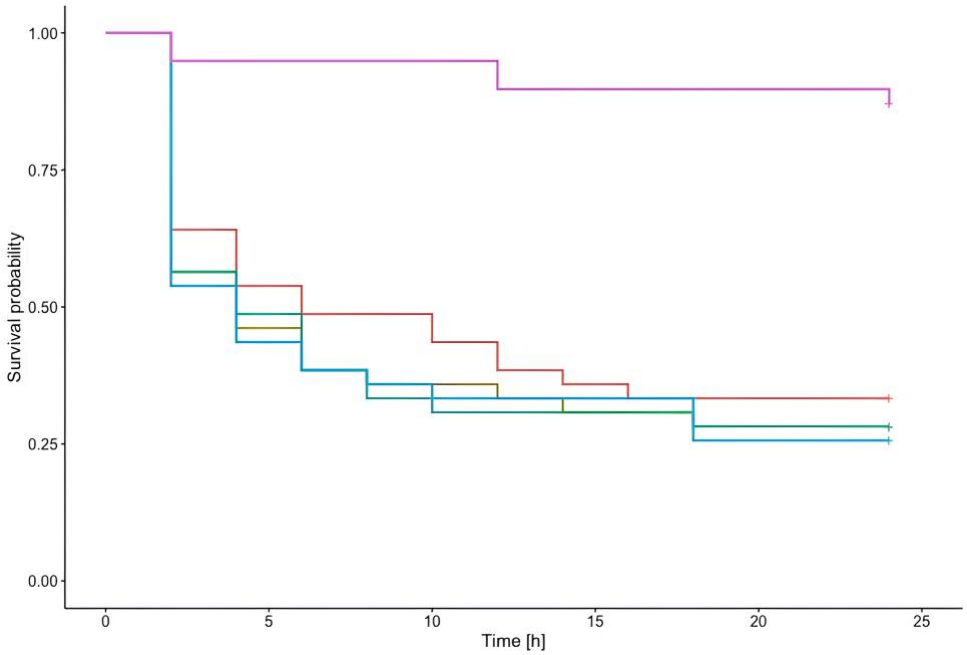


Figure 5. Survival analysis of Varroa mites exposed to varying concentrations of *Bifidobacterium* sp. ERSapi20. The Kaplan-Meier survival curves show no statistically significant differences among the different bacterial solution concentrations tested. Concentrations are represented by different colors: red (100% concentration), brownish (75% concentration), green (50% concentration), blue (25% concentration), and pink (control group).

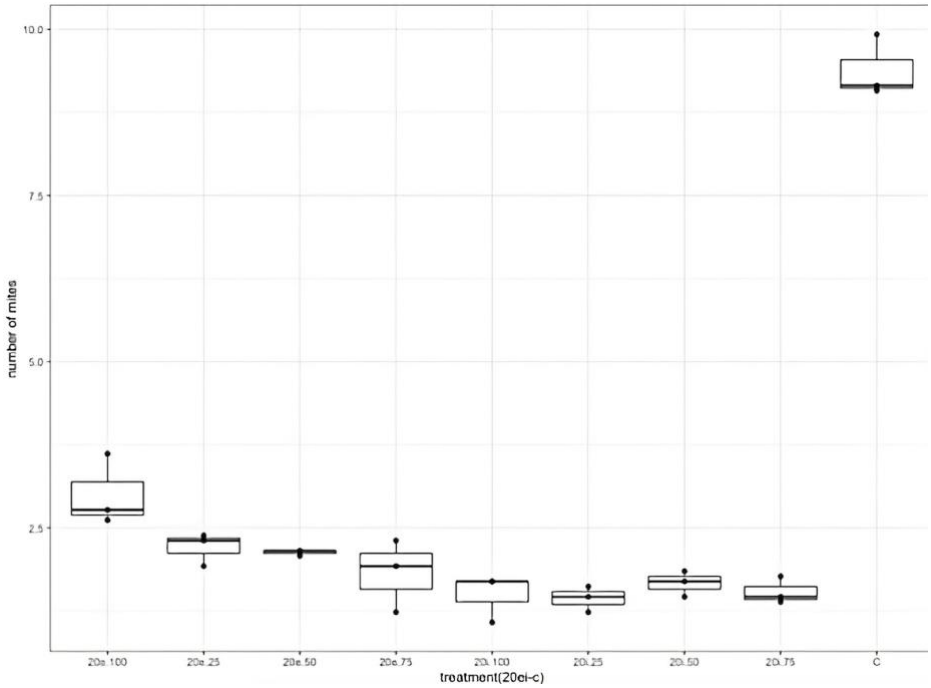


Figure 6. Acaricidal activity of intracellular and extracellular solutions of ERSapi20 against *Varroa destructor* analyzed using ggplot. The figure shows the acaricidal efficacy of different concentrations of intracellular and extracellular solutions compared to the control group. The extracellular solution at 75% concentration exhibited the highest acaricidal activity, significantly reducing mite numbers compared to other tested concentrations and controls.

Discussion

The findings of this study provide a thorough analysis of the actinobacterial strains colonizing the honeybee surface in Türkiye. The findings showed that a strain of *Bifidobacterium* sp. ERSapi20 isolated from worker bees using a culture-based method produced bioactive compounds that exhibited strong acaricidal activity against *Varroa destructor* Oudemans, 1904 (Acari: Varroidae). Moreover, 90% mite mortality rate was achieved by the extracellular solution of the strain within 16 hours, whereas complete mite mortality was observed within 18 hours with the intracellular extract. This is the first report of the acaricidal activity against *V. destructor* using the intracellular extract of actinobacterial strains in Türkiye. This publication also represents the first use of a culture-dependent approach to investigate *Bifidobacterium* strain, making it a significant contribution to the field of bee health regulation. Thus, it was aimed to find new solutions for controlling the varroa mites that harm honeybees. The acaricidal potential of *Bifidobacterium* sp. ERSapi20 (GenBank accession no. OQ073505) was evaluated, focusing on its ability to produce bioactive metabolites effective against mites.

The bacterial pre-cultivation step is an important stage to isolate specific groups of microorganisms. In this context, peptone water was the most commonly used diluent for the enumeration of *Bifidobacteria* sp. (Roy, 2001). Therefore, in our laboratory, peptone water (0.95%) was used for the isolation of Bifidobacteria from the surface of honeybees. Additionally, to mimic of bee-keeping field conditions, the incubation temperature was fixed to 31±1ºC in the dark during this study, minimizing the effects of environmental factors on varroa mites. This bioassay strategy was reported for the first time in the literature. In the growth of bacterial cultures, a succession of phases, characterized by a growth curve, may be conveniently distinguished (Monod, 1949). Moreover, certain environmental stresses, among which is starvation, induce toxin–antitoxin (TA) production. During the stationary phase, a range of secondary metabolites, including antibiotics and toxins, are synthesized (Engelberg-Kulka et al., 2006; Duquesne et al., 2007). In this regard, ERSapi20 growth curve was determined to achieve the end of the stationary phase and the beginning of the death phase, which is the most common stage for releasing secondary metabolites into the exhausted media.

Varroa mite has been reported to be susceptible to the entomopathogenic fungi, *Metarhizium anisopliae*, *Beauveria bassiana*, *Verticillium lecanii*, *Hirsutella* spp., and *Hirsutella thompsonii* (Chandler et al., 2000, 2001; Peng et al., 2002; Shaw et al., 2002; Hamiduzzaman et al., 2012) Moreover, most of the literature has focused

on the bacterial communities of the gut microbiome (Crotti et al., 2013), the gastrointestinal tract or stored food (Anderson et al., 2013; Asama et al., 2015; Kwong & Moran, 2016), and the acquisition of bacteria from the environment (Aizenberg-Gershtein et al., 2013). The present study resulted in a microbial collection of strain belonging to the *Actinobacteria* phyla, which have been frequently found in the pollination environment, alimentary tract, and stored food of honeybees (Martinson et al., 2011; Ganeshprasad et al., 2022).

Although the genera *Bifidobacterium* were commonly found in bees' gut (Ganeshprasad et al., 2022), the isolation of *Bifidobacterium* from the surface of bees was also confirmed in this study, as previously reported by Saccà and Lodesani (2020). Both extra- and intracellular solutions of ERSapi20 caused a significant increase in mite mortality (Figure 4). Strains of the genus *Bifidobacterium* have already been reported to be pathogens of varroa mites. Bioassays done by Saccà & Lodesani (2020) showed strain of *B. asteroides* isolated from an apiary located in Italy. By comparison, our study result was found to be more effective than the finding published by Saccà & Lodesani (2020). This result disclosed that the same species isolated from different regions might have different effects on mites' mortality. On the other hand, some studies have shown the effectiveness of lactic acid bacteria in the biocontrol of varroosis in bees. Tejerina et al. (2020) reported a 50-80% reduction in varroosis levels with the use of *Lactobacillus salivarius* A3iob, and de Piano et al. (2020) found that strains of *L. johnsonii* AJ5 and *Enterococcus faecium SM21* improved bee survival and increased bee protein levels. Saccà & Lodesani (2020) conducted in vitro experiments which showed that bacterial cultures of *L. kunkeei* BO-G12 caused a 95-100% mortality rate of the mite within a period of three days. Nevertheless, the present study results show that members of the actinobacteria group have promising potential in terms of duration and effect of toxicity against the mite.

The present study indicated that intracellular extract of ERSapi20 was more rapidly affected varroa mites than the extracellular solution. This study has shown that adult honeybees inoculated with bacterial suspensions of ERSapi20 by directly applying them onto their bodies had not affected under laboratory conditions. In addition, the survival probability test conducted in R Studio highlighted the significant acaricidal activity of *Bifidobacterium* sp. ERSapi20 against varroa mites. These findings underscore the importance of this research in the development of bee health products and integrated pest management strategies. However, additional research is necessary to validate these results, address limitations, and explore potential avenues for further investigation. Conversely, previous studies have shown that adult honeybees inoculated with spores of entomopathogenic fungi onto their bodies had higher mortality than non-inoculated bees (Hamiduzzaman et al., 2012).

Interestingly, the similar mean and standard deviation values observed for different concentrations of ERSapi20 mixed with Tris-HCl buffer (75:25, 50:50, and 25:75 v/v) suggest that the acaricidal effect reaches a plateau within this concentration range. This indicates that the efficacy of ERSapi20 may not significantly increase beyond a certain concentration threshold. The consistent results imply stability and reproducibility, but further investigation is needed to understand the underlying mechanisms. Additional studies exploring a broader concentration range could help clarify whether these trends hold at different levels and identify the optimal formulation for practical use.

In summary, this study is the first to utilize a culture-dependent approach to investigate actinobacterial strains colonizing the honeybee microbiota in Türkiye, highlighting the uniqueness of our findings in this region. The identification of *Bifidobacterium* sp. ERSapi20 and its acaricidal activity against varroa mites is a significant step forward in the development of novel bee health products and integrated pest management strategies. As similar studies have not been conducted in Türkiye before, our research fills a crucial gap in understanding the potential of actinobacterial strains for controlling varroa mites. Further research is necessary to confirm these findings and address any potential limitations of the study.

Conclusion

Beekeepers constantly look for high-quality products combined with a good quality/price ratio (Alberoni et al., 2016). Therefore, this study may be an opportunity to find a new solution to *V. destructor*. The virulence of *Bifidobacterium* sp. ERSapi20 against varroa mites needs to be reassessed by additional tests conducted under bee-keeping conditions similar to those, as the physical environment of the honeybee colony will be a major factor ensuring the success and sustainability of any prospective biological control agent. This strain holds potential for integration into pest management strategies or development of bee health products. The study findings are

promising, but it is important to address potential limitations and further explore the scope of this research. One potential limitation could be the relatively small sample size used in the study, which may affect the generalizability of the results. Potential confounding factors and limitations of the experimental design that relate to this research could be further investigated in future studies.

Even if this study proved that the bacterial solution of ERSapi20 is harmless to the honeybees, several experiments should be investigated, such as effects on the fecundity of queen. To resolute method against the mite, a comprehensive approach that emphasizes understanding *V. destructor* biology and its interaction with honeybees is essential. This approach should involve in silico, in vivo, semi-field, and field-scale (Vilarem et al., 2021). Further research is necessary to ascertain the prolonged impacts of microbial solutions on bees, both in controlled laboratory settings and field conditions.

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Original article (Orijinal araştırma)

Investigation of biodegradable insecticidal properties of different plant lectins on *Tropinota* (*Epicometis*) *hirta* (Poda, 1761) (Coleoptera: Cetoniidae)[1]

Tropinota (*Epicometis*) *hirta* (Poda, 1761) (Coleoptera: Cetoniidae) üzerinde farklı bitki lektinlerinin biyolojik olarak parçalanabilir insektisit özelliklerinin araştırılması

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Abstract

Tropinota (*Epicometis*) *hirta* (Poda, 1761) (Coleoptera: Cetoniidae), a polyphagous agricultural pest that causes significant economic damage to fruits, vegetables and cereals. Due to its presence during the same period as bees, chemical control methods are not recommended. This study aims to evaluate the effectiveness of plant lectins as bioinsecticides in comparison to synthetic insecticides. Adult insects were collected from various locations in İvrindi district of Balıkesir, Türkiye, between March 25 and June 6, 2024. The insects were fed *in vivo* with lectins from *Wisteria floribunda* (Willd.) DC. (Fabales: Fabaceae*)* (WF), *Phaseolus vulgaris* L. (Fabales: Fabaceae) (PV), *Triticum vulgaris* L. (Poales: Poaceae) (TV) and *Phytolacca americana* L. (Caryophyllales: Phytolaccaceae) (PA) at concentrations of 200, 250 and 300 mg/g for 7 days. The highest lethal effect among the applied lectin doses was detected at the PA300 dose. Upon examining the insect gut tissues, it was found that the TV300 lectin dose contained higher levels of oxidants compared to the synthetic insecticide. Additionally, the applied lectins exhibited inhibitory effects on the activity of various digestive enzyme in insects. Our findings suggest that lectins can be used as a biodegradable alternative to chemical insecticides for controlling *T. hirta*.

Keywords: Bioinsecticides, digestive enzymes, lectins, oxidative stress, apple blossom beetle

Öz

Tropinota (*Epicometis*) *hirta* (Poda, 1761) (Coleoptera: Cetoniidae), meyve, sebze ve tahıllara önemli ekonomik zararlar veren polifag bir tarımsal zararlıdır. Arılarla aynı dönemde bulunması nedeniyle kimyasal kontrol yöntemleri önerilmemektedir. Bu çalışma, sentetik insektisitlere kıyasla biyoinsektisit olarak bitki lektinlerinin etkinliğini değerlendirmeyi amaçlamaktadır. Ergin böcekler, 25 Mart-06 Haziran 2024 tarihleri arasında Türkiye'nin İvrindi (Balıkesir) ilçesinin çeşitli lokasyonlarından toplanmıştır. Böcekler, 7 gün boyunca *Wisteria floribunda* (Willd.) DC. (Fabales: Fabaceae*)* (WF), *Phaseolus vulgaris* L. (Fabales: Fabaceae) (PV), *Triticum vulgaris* L. (Poales: Poaceae) (TV) ve *Phytolacca americana* L. (Caryophyllales: Phytolaccaceae) (PA) lektinlerin 200, 250 ve 300 mg/g konsantrasyonuyla *in vivo* olarak beslenmiştir. Uygulanan lektin dozları arasında en yüksek öldürücü etki PA300 dozunda tespit edilmiştir. Böcek bağırsak dokuları incelendiğinde, TV300 lektin dozunun sentetik insektisit ile karşılaştırıldığında daha yüksek düzeyde oksidan içerdiği tespit edilmiştir. Ek olarak, uygulanan lektinler böceklerdeki çeşitli sindirim enzimlerinin aktivitesi üzerinde inhibitör etki göstermiştir. Bulgularımız, lektinlerin *T. hirta*'nın kontrolünde kimyasal pestisitlere alternatif biyolojik olarak parçalanabilen bir insektisit olarak kullanılabileceğini göstermektedir.

Anahtar sözcükler: Biyo-insektisit, sindirim enzimleri, lektinler, oksidatif stres, çiçek zınnı

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Introduction

Tropinota (*Epicometis*) *hirta* (Poda, 1761) (Coleoptera: Cetoniidae), commonly known as the Apple Blossom Beetle, is a polyphagous agricultural pest (Kara, 1995). This species, which has a widespread global distribution, has been reported primarily from Europe, North Africa and a large part of Northern Asia (Çelik & Yaşar, 2021). It also has a widespread distribution across Turkey (Lodos et al., 1999; Şenyüz et al., 2015). It has been reported to damage over 70 plant species, including fruits such as apples, cherries, quinces, strawberries and peaches, as well as grains, ornamental plants and various weeds (Buşmachiu & Toderaş, 2014). Adult insects attack the pistils, leaves, buds and fruits of plants, leading to economic losses (Kara, 1995). It has been reported to cause a 70% reduction in flowers in Bulgarian cherry orchards (Kutinkova & Andreev, 2004), 90%-100% damage in pear orchards around Tokat (Kara, 1995), and 50% damage in blueberry orchards (Slav et al., 2018). Adult insects, which begin flying in April due to rising temperature, remain active until mid-July (Oltean et al., 2015).

Chemical control is not recommended because the insects, whose populations increase during the flowering period, coexist with bees and other beneficial insects. This drawback of chemical control has prompted researchers to explore biological and biotechnical control methods (Yaşar & Dahham, 2019). Traps of different colors and various attractants added to them are frequently used in the control of *T. hirta* (Schmera et al., 2004). Efforts are ongoing to develop biological agents against *T. hirta* using entomopathogenic nematodes (Akpınar et al., 2020) and entomopathogenic fungus isolates (Atmaca et al., 2018). While these studies have been effective in reducing the population, they are not yet sufficient for definitive control. As a result, local producers continue to rely on chemical pesticides to manage the increasing population.

Lectins are essential carbohydrate-binding glycoproteins that serve as one of the defense mechanisms of insect-resistant plants (Upadhyay & Singh, 2012). The fact that lectins are generally destructive in the digestive tract is because it is the first site of action of orally ingested nutrients. Therefore, it is quite possible that lectins with specific carbohydrate-binding properties bind to these sites where activities such as enzyme production, secretion, and nutrient absorption take place. In addition, lectins deform epithelial cells and cause them to swell. This leads to the absorption of toxic substances in the gastrointestinal system. As a result, toxic substances that enter the insect's circulatory system bind to various sites and exert harmful effects (Gatehouse et al., 1984; Peumans & Van Damme, 1995; Jaber et al., 2010; Vandenborre et al., 2011; Douglas, 2012). Therefore, in this study the effects of the lectins on important digestive enzymes (protease, trypsin, acid phosphatase, alkaline phosphatase, exoglucanase, endoglucanase, β-glucosidase, and α-amylase) were examined to be able to correlate the mechanisms by which lectins kill *T. hirta. In addition,* it has been reported that antioxidant enzyme activities increase due to lectin intake in insects and this increase leads to hydrogen peroxide accumulation. Thus, free radicals can be produced, and oxidative stress can be induced in insects (Lima et al., 2016; Rahimi et al., 2018; Khoobdel et al., 2022). *Phaseolus vulgaris* L. (Fabales: Fabaceae) lectin showed toxic effects on *Callosobruchus maculatus* (Fabricius, 1775) (Coleoptera: Bruchidae). Lectins isolated from *P. vulgaris* and *Triticum vulgaris* L. (Poales: Poaceae) have shown strong effects on Coleoptera and Lepidoptera orders (Czapla & Lang, 1990; Sauvion et al., 2004). Lima et al. (2016) found that the lectin they applied caused a decrease in the number of digestive cells and that this lectin caused cell death. Additionally, the findings observed in the peroxidase staining analysis indicated the presence of oxidative stress. Thus, the total oxidant status (TOS) values in the intestinal tissues were measured in this study to assess the oxidative stress by lectins in *T. hirta.*

A number of entomotoxic effects of plant lectins on insects have been demonstrated in the literature. These include food deterrence, enzyme inhibition due to deformation of the digestive tract, reduced oviposition, impaired larval development, induction of oxidative stress and death. These effects provide important data in the field of pest control (Czapla & Lang, 1990; Macedo et al., 2002; Sauvion et al., 2004; Napoleão et al., 2013; Lima et al., 2016). Khoobdel et al. (2022), noted that some digestive enzymes were

inhibited in adult *Sitophilus oryzae* (L., 1763) (Coleoptera: Curculionidae), fed lectin. It has been reported that lectin binding to epithelial cells may result in cytotoxicity, decrease the number of cells secreting digestive enzymes, or inhibit enzymes by binding to enzyme receptors. There are no study investigating the use of plant lectins for the control of *T. hirta.* This study aims to explore alternative methods to reduce the reliance on chemical pesticides in managing *T. hirta*. Additionally, it seeks to determine the entomotoxic effects of plant lectins on this pest and to provide preliminary data for their potential use as biopesticides in future research. Therefore, mortality rates of *T. hirta* fed with lectins extracted from *Wisteria floribunda* (Willd.) DC. (Fabales: Fabaceae*)*, *P. vulgaris*, *T. vulgaris* and *Phytolacca americana* L. (Caryophyllales: Phytolaccaceae) were calculated. Furthermore, the total oxidant status (TOS) values in the intestinal tissues were measured to assess the oxidative stress by lectins, given the known effects of insecticides on insects' oxidative balance. Many of the natural plant compounds and organic compounds used in the control of insect pests are known to affect digestive enzymes. To evaluate how lectins influence digestive physiology, their effects on the activity of key enzymes -protease, trypsin, acid phosphatase, alkaline phosphatase, exoglucanase, endoglucanase, β-glucosidase and α-amylase- within insect intestines were also investigated.

Materials and Methods

Insects

Adults of *T. hirta* were manually collected from wild mustard, *Sinapis arvensis* L. (Brassicales: Brassicaceae) plants at different locations in Balıkesir/İvrindi at regular intervals between March 25 and June 6, 2024. The collected insects were transported to the laboratory in 1.2 liter plastic containers. Fresh wild mustard plants were added to the containers and the insects fed on them until the study was carried out. To ensure that the insects remained alive for the experiments, the containers were covered with a tulle to allow airflow. The insects were kept at 28±2ºC, %65±5 humidity and 16:8 photoperiod in the rearing room for seven days. Each experimental group consisted of 12 adults that were in the colony for 1-2 months of mixed sex. The study was conducted in 3 replications and 6 male- 6 female insects were used for each experimental group. In each set of experiments, 168 individuals were used, totaling 504 individuals for 3 replicates.

Lectins

Lectins from *Wisteria floribunda* (Sigma L8258-1MG), *Phaseolus vulgaris* (Sigma L2646-25MG), *Triticum vulgaris* (Sigma 61767-5MG), and *Phytolacca americana* (pokeweed) (Sigma L9379-5MG) were purchased from Sigma-Aldrich.

Chemicals

P-nitrophenyl phosphate, azocasein, trichloroacetic acid solution, Triton X-100, Nα-Benzoyl-L-arginine 4-nitroanilide hydrochloride (BApNA) and avicel were purchased from Sigma-Aldrich. *P*-nitrophenyl-β-D-glucopyranoside and 3,5-dinitrosalicylic acid (DNS) were purchased from Bostonchem. Carboxymethyl cellulose was purchased from BLDpharm, while cellulose was acquired from Central Drug House. Starch was purchased from ISOLAB chemicals. Other chemicals were procured from local vendors and met the required purity standards.

Growth media preparation

100 g of wild mustard flowers were blended with 100 mL of water creating a porridge mixture (Napoleão et al., 2013). This mixture was distributed equally among 14 experimental groups. The negative control group received only the water and flowers while the positive control group was treated with 5 mg/mL Bayer K-othrine SC50 (Deltamethrin) insecticide. Four distinct lectins were introduced in the nutrient medium of the other experimental groups, each applied *in vivo* at three different doses (200, 250 and 300 mg/g). Lectins, isolated from Wisteria plant (*W. floribunda*), kidney bean (*P. vulgaris*), wheat (*T. vulgaris*) and sugar cane plant (*P. americana*), were coded as outlined in Table 1.

Table 1. The types of plant lectins and their respective doses used in the experiments

Code	Plant Source	Dose (mg/g)
PA200	*Phytolacca americana*	200
PA250	*Phytolacca americana*	250
PA300	*Phytolacca americana*	300
PV200	*Phaseolus vulgaris*	200
PV250	*Phaseolus vulgaris*	250
PV300	*Phaseolus vulgaris*	300
TV200	*Triticum vulgaris*	200
TV250	*Triticum vulgaris*	250
TV300	*Triticum vulgaris*	300
WF200	*Wisteria floribunda*	200
WF250	*Wisteria floribunda*	250
WF300	*Wisteria floribunda*	300

Death rates

Experiments were conducted at 28±2ºC over a period of seven days with daily monitoring of the insects. At the end of the 7th day, live and dead insects were counted and placed in separate tubes. The insects were then prepared for intestinal dissection.

At the end of the experiments, the mortality rate (%) was calculated using the following formula.

Mortality rate (%) = (Survivors / Total Number of Individuals tested) * 100

Intestinal extracts

Insects fed for 7 days with growth media containing lectin extracts (200-250-300 mg/g), insecticide (Bayer K-othrine SC 50) and pure water (control), then kept inactive at -20ºC for 10 minutes (Napoleão et al., 2013). The intestines of each insect were manually separated and homogenized in appropriate buffer solutions (1 mL tris buffer (0.1 M tris HCl, pH 8.0, 0.02 M $CaCl_2$ and 0.15 M NaCl), 1 mL acetate buffer (0.1 M sodium acetate, pH 5.5, 0.02 M $CaCl_2$ and 0.15 M NaCl) or 1 mL sodium phosphate buffer (0.02 M sodium phosphate, pH 7.0, 0.15 M NaCl). Intestinal samples were centrifuged at 9157 rpm for 15 minutes at 4ºC. Extracts were sampled and assayed for enzyme activity.

Total oxidant status (TOS) analysis in intestinal extract

Total oxidant levels (TOS) were measured in the intestinal tissues of *T. hirta* adults after a seven-day interaction with different lectin proteins *in vivo*. TOS assay kits (Rel Assay Diagnostics, Türkiye) were used, which operate on the principle of Fe^{+2} the conversion to Fe^{+3} by different oxygen species in an acidic environment. This reaction results in the color change due to the xylenol orange and Fe^{+3}, measured at 530 nm. Measurements were taken in a microplate spectrophotometer. The standard concentration was 10 µmol H_2O_2 equivalent/L with results expressed as µmol H_2O_2 equivalent/L (Erdem et al., 2021).

Enzyme experiments

Protease enzyme activity

The protease activity of the gut extract in Tris buffer was assayed following the method of Napoleão et al. (2013), with azocasein as the substrate. The assay mixture consisted of 50 µL of gut extract, 300 µL of 0.1 M sodium phosphate (pH 7.5) containing 50 µL of 0.6% (w/v) azocasein and 100 µL of 0.1% (w/v) Triton X-100. The mixture was incubated at 37ºC for 3 hours, after which the reaction was stopped by the addition of 200 µL of 10% (v/v) trichloroacetic acid. The sample was then incubated at 4ºC for 30 minutes, followed by centrifugation at 9157 rpm for 10 minutes. The absorbance of the supernatant was measured at 366 nm using a spectrophotometer (Napoleão et al., 2013).

Trypsin-like enzyme activity

Enzyme activity in the intestinal samples in Tris buffer was measured in a 96-well microplate using BApNA as the substrate (Napoleão et al., 2013). Intestinal homogenate (30 µL), BApNA (15 µL), Tris buffer (55 µL) were mixed in a microplate (100 µL in total). This mixture was incubated at 37ºC for 30 minutes, and enzyme activity was measured at 405 nm using a microplate reader.

Acid and alkaline phosphase activity

In accordance with the methodology proposed by Napoleão et al. (2013), 50 µL of intestinal extract in sodium phosphate buffer was combined with 450 µL of either 0.05 M sodium acetate buffer (pH 4.0) for an acid phosphatase assay, or 0.05 M Tris-HCl buffer (pH 8.0) for an alkaline phosphatase assay. To each mixture, 500 µL of 12.5 mM p-nitrophenyl phosphate, prepared separately in the respective buffers, was added. The mixture was incubated at 37ºC for 15 minutes in a water bath. To complete the reaction, 100 µL of 0.5 M sodium hydroxide was added to the mixture. Intestinal samples were centrifuged at 6105 rpm for 5 minutes, and the absorbance of the resulting supernatant was measured at 410 nm using a spectrophotometer (Thermo Scientific Multiskan Go, Type: 1510, Finland).

Endoglucanase and exoglucanase activity

To determine endoglucanase enzyme activity, 100 µl of intestinal extract in acetate buffer was incubated with a 1% (w/v) carboxymethylcellulose solution sodium acetate buffer (pH 5.5) containing 400 µl of 0.15 M NaCl at 50ºC for 10 minutes. For exoglucanase activity, 1% (w/v) sodium hydroxide solution was used. After the incubation period, 500 µL of DNS was added to terminate the reaction. The mixture was heated at 100ºC for six minutes and subsequently cooled on ice for 15 minutes. Subsequently, the absorbance was measured at 540 nm using a spectrophotometer (Napoleão et al., 2013).

Betaglucosidase enzyme activity

Intestinal homogenate (50 µL) in acetate buffer and 400 µl of *p*-nitrophenyl-b-D-glucopyranoside were incubated in an eppendorf tube at 50ºC for 10 minutes. To stop the reaction, 500 µL of 10% sodium bicarbonate was added to the tubes. 300 µL of mixture was added to each well for measurements, and absorbance was read 410 nm using a spectrophotometer (Napoleão et al., 2013).

Alpha amylase enzyme activity

Enzyme activity was determined following the method of Napoleão et al. (2013). A mixture of 100 µL of intestine in acetate buffer and 400 µL of starch solution were incubated at 50ºC for 10 minutes. The reaction was stopped by adding 500 µL of DNS solution. The mixture was heated at 100ºC water for 6 minutes in a water bath and then cooled on ice for 15 minutes. Afterward, 300 µL of the mixture was added to each well in the microplate in triplicate. Absorbance measurements were taken at 540 nm using a spectrophotometer (Napoleão et al., 2013).

Statistical analysis

All experiments were performed in three biological replicates. Standard error values and statistical analysis for mortality rates and survival rates were calculated using the one-way Anova and Tukey's tests in SPSS. One-way Anova and Dunnett's post-hoc tests were used to calculate total oxidant status and enzyme activity experiments. Values of $p < 0.05$ were considered statistically significant. Enzyme unit was defined as the change of 0.001 in absorbance in 1 min for 1 mL of enzyme solution.

Results and Discussion

Mortality and survival rates

The toxic effects of plant lectins on insects suggest they are effective control proteins that can be used as biopesticides (Mantzoukas et al., 2020). Lectins targeting coleopterans, flies, butterflies, hemipterans, termites, bees and neuropterans decrease the number of egg laying, act as feeding deterrents, inhibit or stimulate enzyme activities in the digestive system, and cause mortality, highlighting their potential of lectins in combating pests (Powell, 2001; Carlini & Grossi-De-Sá, 2002; Jaber et al., 2010; Napoleão et al., 2013; Mishra et al., 2019).

The aim of this study was to evaluate the insecticidal activity of plant lectins against *T. hirta* and to provide preliminary data for the use of lectins as biopesticides. Developing effective toxicokinetic and toxicodynamic models needs comprehensive understanding of the processes that can cause mortality of individuals when exposed to toxic chemicals (Sowa et al., 2024). Mortality studies on insects provide important insights into the nature of the vital question whether the physiological changes at the individual level influence the limited time/lifetime patterns of cohort mortality (Carey & Liedo, 1999). Therefore, in this study, over a 7-day study period, daily and cumulative mortality rates were calculated, with daily survival rates illustrated in Figure 1 and cumulative mortality at 7 days detailed in Table 2.

Table 2. Post-experimental mortality rates of *Tropinota (Epicometis) hirta* adults fed with various plant lectins at varying concentrations

Experimental groups	Mean Mortality rate (%)±S. error	p values
NC	0.0 [a]	
TV200	47.8±7.8 [ab]	,172
TV250	51.1±10.6 [ab]	,113
TV300	60.6±16.4 [b]	,029
PA200	52.2±6.1 [ab]	,097
PA250	61.1±5.6 [b]	,027
PA300	79.4±2.4 [b]	,001
PV200	65.0±12.3 [b]	,015
PV250	57.8±13.1 [b]	,044
PV300	67.2±17.2 [b]	,010
WF200	51.1±10.6 [ab]	,113
WF250	66.7±17.4 [b]	,011
WF300	77.8±12.1 [b]	,002
PC	100.0±0.0 [b]	,000

NC: Negative control, PC: Positive control. $p < 0.05$ values are significant for one-way ANOVA and Tukey's tests. a,b : Different letters in the same column indicate differences between groups.

Our findings indicate that there was no mortality in the negative control (NC) group, while all insects in the positive control (PC) group died. In the lectin-fed experimental groups, mortality occurred in relation to the dose applied, with higher lectin doses leading to proportionally higher mortality rates ($p<0.05$) (Table 2). Additionally, an independent experimental group was established where the insects were starved and given no food. In this experimental group, no mortality was observed by day 7; the first mortality occurred on day 12. These results demonstrate that the insect mortality observed in our study was due to the toxic effects of the lectins rather than starvation alone. The highest dose of *T. vulgaris* lectin used increased insect mortality by 60.6% compared to the control group (Table 2). In previous studies, *T. vulgaris* lectin added in a 2% solution showed a 100% lethal effect on *Ostrinia nubilalis* (Hübner, 1796) (Lepidoptera: Crambidae) larvae. Additionally, *P. americana* lectin resulted in 25% mortality of *Diabrotica undecimpunctata* howardi Barber, 1947 (Coleoptera: Chrysomelidae) larvae (Czapla & Lang, 1990). In our study, this lectin was found to be the most lethal lectin, causing 79.4% mortality in adults at the highest dose tested (Table 2). Adult mortality

rates for PV lectin ranged from 57.8% to 67.2% (Table 2). For *C. maculatus* larvae treated with *P. vulgaris* lectin, a mortality rate of 40% was reported (De Sá et al., 2014). In a study conducted with *Trogoderma granarium* Everts, 1898 (Coleoptera: Dermestidae) larvae, *P. vulgaris* caused 40% and 86.7% mortality at the PV25 and PV100 dose respectively (Mantzoukas et al., 2020). Wheat germ agglutinin (WGA) has been reported as the lectin with the highest entomotoxic property against *C. maculatus* with insect mortality increasing proportionally with dose (Murdock et al., 1990). Additionally, *Myracrodruon urundeuva* Allemão (Sapindales: Anacardiaceae) extract has been shown to cause significant insect mortality in *Sitophilus zeamais* Motchulsky, 1855 (Coleoptera: Curculionidae) adults at the highest doses (100-150mg/g). Lectins not only cause food rejection, but also release the toxic substances that can be lethal to insects. High doses of extracts and lectins cause deformation in the digestive and absorption activities of target organism (Napoleão et al., 2013). Studies indicate that plant-derived pesticides, when applied at appropriate doses, exhibit a high lethal potential against insects at both adult and larval stages.

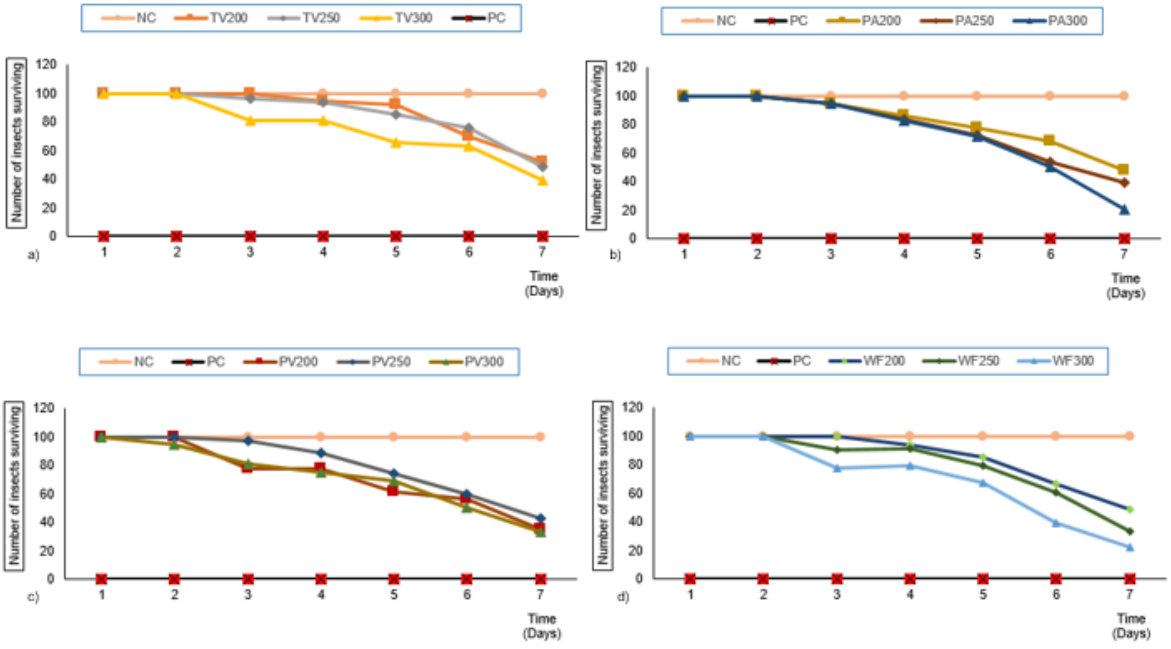

Figure 1. The mean number of insects surviving observed over to the incubation days for each lectin type: a) *Triticum vulgaris* lectin, b) *Phytolacca americana* lectin, c) *Phaseolus vulgaris* lectin, d) *Wisteria floribunda* lectin. Each lectin dose, along with the negative control (NC) and positive control (PC) groups, is represented in the graph.

Insect mortality was recorded daily. After 7 days of feeding, the first lethal effect was noted on day 2 for the PV300 dose and on day 3 for the other lectins (Figure 1). The PV300 dose caused 5.6% mortality of the insects on day 2, with mortality rates continuing to rise each day. On day 3, the WF300, PA300, and TV300 dose killed 22.8%, 5.6%, and 19.4% of the insects, respectively Although the first insect death was caused by the PV300 dose, the PA300 dose produced the highest statistically significant lethal effect by the end of day 7 (Figure 1). It has been reported that *P. vulgaris* increased aphid mortality within 24 hours of the experiment's start (Sprawka, 2008). In another study, *P. vulgaris* was observed to cause the earliest insect mortality among the lectins tested at the lowest dose. With a higher dose, this lectin was reported to kill the majority of insects in 2 days (Habibi et al., 1993). The effectiveness of lectins in insect mortality may be linked to their ability to spread early in the digestive tract. Indeed, the results of this study align with this hypothesis.

Total oxidant status (TOS) analysis

Pesticides are known to cause significant oxidative stress across a wide variety of animal species, including insects (Chakrabarti et al., 2015). In our study, as expected, the negative control group had the lowest oxidant levels, while the positive insecticide-treated group had higher TOS levels ($p<0.05$) (Tables 3). The dose of TV300 lectin used in this study induced oxidative stress in insects by increasing oxidant levels.

Table 3. Mean TOS values determined after 7 days of feeding with growth media containing lectins and pesticides

Lectin dose (mg/g)	Mean H_2O_2 concentration±. error (mmol/L)	P values
NC	0.82±0.5	
TV200	6.10±1.6	,181
TV250	6.70±1.7	,107
TV300	7.51±2.9*	,049
PA200	4.80±0.2	,475
PA250	4.72±0.6	,630
PA300	5.49±1.7	,296
PV200	5.47±1.4	,300
PV250	6.51±2.2	,127
PV300	6.09±1.5	,183
WF200	4.94±1.0	,436
WF250	5.13±0.4	,383
WF300	4.92±1.4	,440
PC	5.65±2.2	,261

* $p < 0.05$ values are significant for one-way ANOVA and Dunnett's post-hoc tests (*), NC: Negative control, PC: Positive control

Many phytophagous insects exhibit detoxification enzymes that reduce oxidative radicals generated by plant pesticides. Research has shown that both leaf extract and synthetic insecticides used in studies on aphids yield similar lethal effects, resulting in peroxide and malondialdehyde accumulation. Elevated levels of oxidative damage have been linked to increased insect mortality (Quandahor et al., 2022). Furthermore, *M. urundeuva* lectin has been found to causes more intense peroxidase staining in the midgut epithelium of termites compared to control groups (Lima et al., 2016). Another study demonstrated that the lectin form *Polygonum persicaria* L. (Caryophyllales: Polygonaceae) (PPA) induced physiological disorders in *Sitophilus oryzae* leading to digestive system deformation and oxidative stress (Khoobdel et al., 2022). Literature findings also suggest that a varied diet can trigger oxidative stress by increasing oxidative radicals in insects (Krishnan & Sehnal, 2006). The findings in our study show that changes in insect feeding and exposure to lectins may contribute to heightened oxidative stress.

Enzyme Activity Results

It is a well-known fact that many of the natural compounds used in the control of insect pests are known to affect digestive enzymes (Senthil-Nathan et al., 2006a). One of those compounds are the lectin glycoproteins having globular protein subunits which contain one or more carbohydrate-binding sites. As a result of their molecular structure, when they are consumed frequent exposure begins in the digestive tract, because they are relatively stable against heat denaturation and proteolytic digestion (Muramoto, 2017). Considering the economic and environmental advantages compared to the traditional chemical insecticides, it is clear that new control methods are needed such as digestive enzyme inhibitors (Mehrabadi et al., 2012).Therefore, in this study the effects of different plant lectins on the digestive enzymes were analyzed.

Protease enzyme activity

The protease activity determined in the course of this study is illustrated in Figure 2a. Our results show that the dose with the highest effectiveness in reducing protease enzyme activity compared to the control group was TV200 (90.1%) ($p<0.05$) (Figure 2a). In the PC group, activity decreased by 77.3%. TV200 and TV300 doses were more effective at reducing enzyme activity than the synthetic insecticide. Some of the lectin doses used in the study showed inhibitory effects on protease enzyme activity.

The binding of plant lectins to different regions of the digestive system of insects can inhibit digestive enzymes (Macedo et al., 2002; Camaroti et al., 2018; Lima et al., 2018). Amino acids, released by the breakdown of proteins by proteases, play vital roles in insect growth, development and energy production (Gholamzadeh Chitgar et al., 2013). Studies have shown that the protease activity in *S. zeamais* adults exposed to *M. urundeuva* lectin (MuLL) decreased by 69.7% compared to the control, with lectin proving effective in reducing the activity of enzymes involved in insect protein metabolism (Napoleão et al., 2013). Lectins used in our study showed dose-dependent effects on protease activity, reducing it by 70.7% to 90.1% (Figure 2a). Additionally, the protease-inhibiting effects of *P. vulgaris* lectin were also observed in *C. maculatus* larvae (De Sá et al., 2014), and similiar inhibitory effects were comfirmed in our study (Figure 2a). Two different lectins from *M. urundeuva*, MuBL and MuLL,), have been reported to reduce protease activity in insects by 40.4% and 27%, respectively (Lima et al., 2018). Additionally, *Schinus terebinthifolius* Raddi (Sapindales: Anacardiaceae) leaf lectin (SteLL), when applied to *S. zeamais* adults, significantly reduces protease activity due to its interaction with the peritrophic membrane, which suppresses nutrient absorption (Camaroti et al., 2018). In contrast, protease activity is stimulated in insects exposed to *Opuntia ficus - indica* L. (Caryophyllales: Cactaceae) lectin, potentially leading to unbalanced hydrolysis of proteins during digestion (Souza et al., 2018). A reduction in protease activity can hinder the conversion of dietary proteins into amino acids, thus impairing the insect's ability to perform essential activities. Consequently, targeting digestive enzymes in insects could be valuable strategy for pest control.

Trypsin-like enzyme activity

Trypsin-like enzymes play an essential role in physiological processes in insects, including molting, diapause, tissue regeneration, reproduction and development. Control methods targeting the trypsin enzyme have been promise in pest management (Lazarević & Janković-Tomanić, 2015). As shown in Figure 2b, most doses tested in the trypsin-like enzyme activity experiment were highly effective in reducing the enzyme activity. Compared with the negative control, the most effective dose in reducing the activity was PA300 (81.13 %) ($p<0.05$) (Figure 2b). In this study, lectin applications resılted in strong inhibitory effects on trypsin-like activity than chemical pesticides, pronounced disruptions.

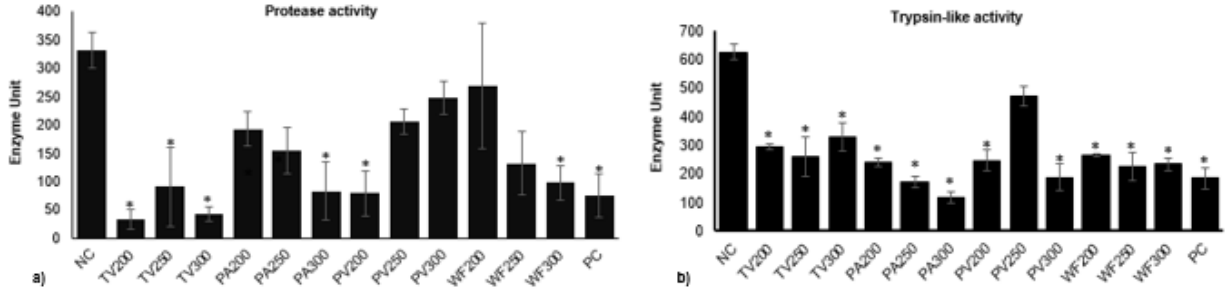

Figure 2. a) Protease and b) trypsin-like enzyme activities after 7 days of feeding with growth media containing various lectins and insecticide. $p < 0.05$ values are significant for one-way ANOVA and Dunnett's post-hoc tests (*).

The binding of lectin to the peritrophic membrane, which is composed of protein, proteoglycan and chitin, may disrupt the porous structure of this membrane, consequently affecting the enzyme regulatory

system (Martinez et al., 2012). Studies have shown that the activity of adult *Sitophilus zeamais* (Motschulsky, 1855) (Coleoptera: Curculionidae) treated with MuLL decreased by 55% (Napoleão et al., 2013). Furthermore, *Annona coriacea* Mart. (Magnoliales: Annonaceae) lectin reduced trypsin activity in *Anagasta kuehniella* (Zeller, 1879) (Lepidoptera: Pyralidae) larvae by 34%. The disruption of the peritrophic membrane caused by lectins is thought to result in the reduction in trypsin activity (Coelho et al., 2007; Camaroti et al., 2018). In our study, the lowest reduction in trypsin-like activity (47.5%) was observed at the TV300 dose, while the highest (81.1%) occurred at the PA300 dose (Figure 2b). Variations in these reduction ratios may arise from the specific binding sites and enzyme inhibitory properties of lectins. Lectins bind to sugars on glycosylated enzymes, while, in non-glycosylated enzymes, they attach to areas outside the substrate-binding site, thereby inhibiting enzyme activity. Lectin binding action hinders digestion and limits the metabolic activities essential for the insect (Macedo et al. 2007).

Acid phosphatase and alkaline phosphatase enzyme activities

Acid phosphatase (ACP) and alkaline phosphatase (ALP) are hydrolase enzymes that play crucial roles in insect physiological processes, including reproduction and growth, tissue cytolysis, the last stage of digestion, molting and maturation of reproductive cells (Zibaee & Bandani, 2010; Hamadah et al., 2016). ACP and ALP enzyme activities observed in this study are shown in Figure 3. According to our results, all doses, except PA200 and PV200, inhibited ACP activity by 43.63% to 82.51% compared to the negative control. In positive control, ACP activity decreased by 57.84% ($p<0.05$) (Figure 3a). Similarly, ALP activity was affected by some of the lectin doses applied, with significant reductions observed at the highest doses (PA300: 73.73%, TV300: 73.31%, WF300: 77.60%). In the positive control group, ALP activity decreased by 38.77%, but this result was not statistically significant (Figure 3b).

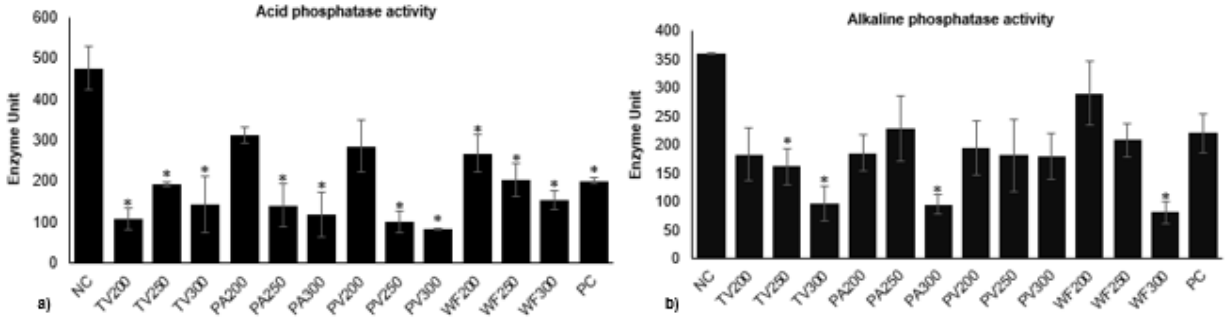

Figure 3. a) Acid and b) alkaline phosphatase enzyme activities after 7 days of feeding with growth media containing various lectins and insecticide. $p < 0.05$ values are significant for one-way ANOVA and Dunnett's post-hoc tests (*).

A significant decrease in acid and alkaline phosphatase activities was reported in *Pieris brassicae* (L., 1758) (Lepidoptera: Pieridae) larvae fed high concentrations of PPA. It was reported that the administered lectin was reported to affect enzymes involved in lipid digestion (Zibaee et al., 2014). Similarly, 2% dose of *Melia azedarach* L. (Rutales: Meliaceae) seed extract caused 69% and 71% decrease in acid and alkaline phosphatase activity in the insect, respectively. Changes in ALP and ACP activities adversely affect the intestinal physiology of insects (Senthil-Nathan, 2006b). Kaur et al. (2006) reported that *Arisaema helleborifolium* Schott (Alismatales: Araceae) lectin used against *Bactrocera cucurbitae* (Coquillett, 1899) (Diptera: Tephritidae) decreased acid and alkaline phosphatase activities, findings that were similarly noted by Sprawka et al. (2011). The inhibitory effect of lectin on phosphatase activities was observed in aphids treated with *P. vulgaris* lectin. Our study's results align with these findings, indicating that most of the lectins we used have an inhibitory effect on phosphatase enzyme activities. This is because plant-derived substances can inhibit these enzymes, limiting nutrient utilization, and the inability to convert substances essential to insect life leads to mortality (Senthil-Nathan, 2006b).

Endoglucanase and exoglucanase enzyme activities

When cellulose is broken down, large amounts of free glucose molecules are released, making cellulose a significant of carbohydrate source for phytophagous insects. The enzymes endoglucanase, exoglucanase and betaglucosidase are involved in the hydrolysis of cellulose (Douglas, 2012). The enzyme activities for endoglucanase, exoglucanase and β-glucosidase observed in this study are shown in Figure 4. Results indicate that in the positive group the endoglucanase activity decreased by 73.9%. This effect was not significant in exoglucanase activity. Among the TV lectin doses, TV300 significantly reduced endoglucanase and TV200 significantly reduced exoglucanase enzyme activity ($p<0.05$) (Figure 4a, 4b). Compared to the negative group, lectin uptake reduced insect endoglucanase and exoglucanase activity, depending on the dose and specificity of the lectin.

A study reported no change in endoglucanase activity observed in *Nasutitermes corniger* (Motschulsky, 1855) (Blattodea: Termitidae) insects following exposure to MuBL and MuLL although exoglucanase enzyme activity was stimulated (Lima et al., 2018). In contrast, *S. zeamais* treated with MuLL showed no change in exoglucanase activity, but endoglucanase activity was decreased (Napoleão et al., 2013). Administration of *Moringa oleifera* Lam. (Brassicales: Moringaceae) lectin (WSMoLc) to *N. corniger* workers and soldiers, beginning with a low dose, led to decreased exogluconase and endogluconase activities (Oliveira et al., 2023). The lectins used in this study also varied in their effects on the enzymes involved in cellulose digestion. Our results suggest that the specificity of each lectin arises from the variability of their mechanisms of action across different insect species. The enzymes involved in cellulose digestion work in a delicate balance; any alteration within this system disrupts the mechanism, thereby hindering digestion (Lima et al., 2018).

Betaglucosidase enzyme activity

The enzyme β-glucosidase is involved in the hydrolysis of cellobiose to glucose in insect digestion, aiding in the breakdown of cellulose and hemicellulose (Huber et al., 2021). The highest glucosidase activity has been observed in phytophagous insects (Douglas, 2012). In our study, β-glucosidase activity is shown in Figure 4c where most lectin doses significantly reduced this enzyme activity, ranging from 55.3% to 84.0% (Figure 4c).

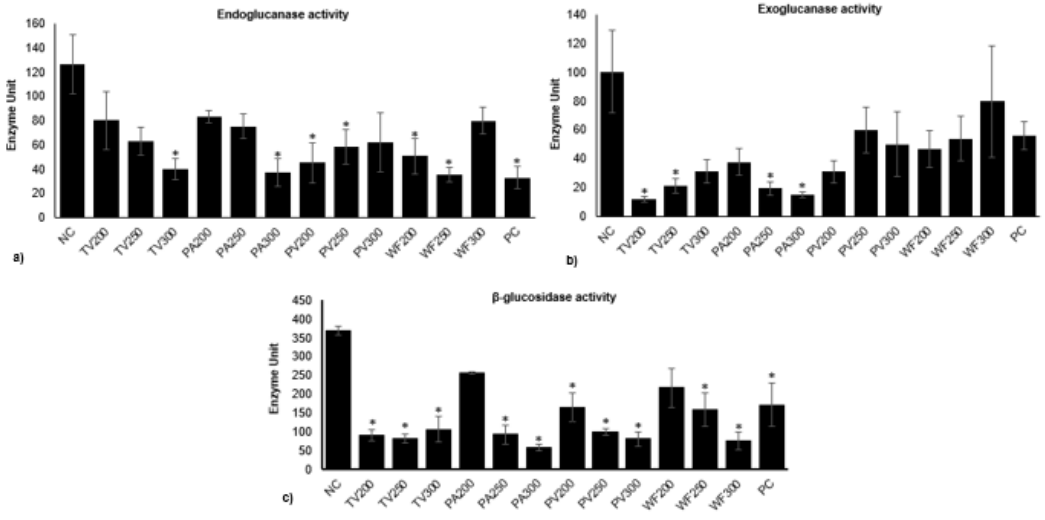

Figure 4. a) Endoglucanase, b) exoglucanase and c) β-glucosidase enzyme activities after 7 days of feeding with growth media containing various lectins and insecticide. $p < 0.05$ values are significant for one-way ANOVA and Dunnett's post-hoc tests (*).

The results demonstrated that significant lectin doses were more effective in reducing enzyme activity than the PC. Numerous studies have also reported that lectins affect β-glucosidase activity, with changes in this enzyme activity leading to impaired digestion. When *N. corniger* termites were fed *Microgramma vacciniifolia* (Langsd. & Fisch.) Copel. (Polypodiales: Polypodiaceae) rhizome (MvRL), β-glucosidase activity was inhibited (Lima et al., 2018). The similar results were observed in *S. zeamais* adults, where MvRL was reported to reduce β-glucosidase activity (Albuquerque et al., 2020). Additionally, Sprawka et al. (2011) reported that phytohemagglutinin (PHA) suppressed β-glucosidase enzyme at high doses, while at low doses, it stimulated enzyme activity. According to the researchers, this stimulation may suggest that the lectin used plays a role in detoxification.

Alpha-amylase enzyme activity

Amylase enzyme activity observed in this study is shown in Figure 5. Some of the doses used in the study significantly inhibited α-amylase activity, with rates ranging from 61.74% to 87.52% compared to the control ($p<0.05$).

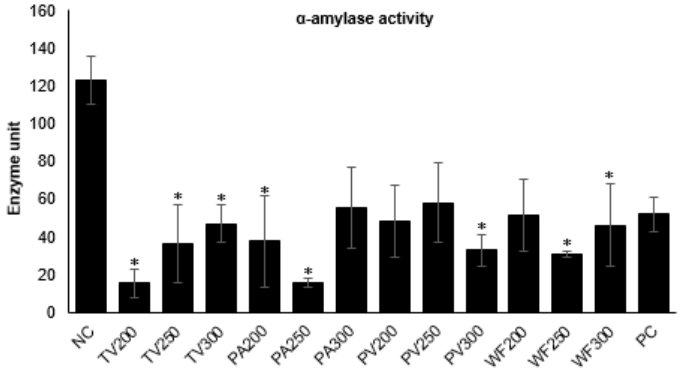

Figure 5. α-amylase enzyme activity after 7 days of feeding with growth media containing various lectins and insecticide. $p < 0.05$ values are significant for one-way ANOVA and Dunnett's post-hoc tests (*).

The α-amylase enzyme is particularly sensitive to grain-feeding insects (Terra & Ferreira, 1994). The result of our study supports the idea that lectins, which can bind to cells in the digestive system, cause cellular damage and reduce enzymes-secreting cells, effectively reducing enzyme activity (Zibaee et al., 2014). *Microgramma vacciniifolia* rhizome lectin (MvRL) inhibited α-amylase activity in *S. zeamais*, likely due to lectins interfering with carbohydrate digestion and resulting in inefficient biomass conversion (Albuquerque et al., 2020). *Moringa oleifera* lectin inhibited amylase activity in worker termites while stimulating it in soldier termites. Enzymatic differences, such as inhibition or stimulation have negative effects on carbohydrate digestion (Oliveira et al., 2023). *Polygonum persicaria* lectin reduced alpha-amylase activity in adult *S. oryzae* by 63% (alpha-amylase in PPA: 1.58, U/mg protein, control 4.28, U/mg protein) (Khoobdel et al., 2022). Similarly, lectins from *Glycine max* (L.) Merr. (Fabales: Fabaceae) and *P. vulgaris* plants caused a 58.8% to 66% reduction in amylase activity in *Earias insulana* (Boisduval, 1833) (Lepidoptera: Nolidae) larvae (Metayi et al., 2024). Conversely, lectin has been reported to stimulate amylase activity, increasing enzyme levels in *S. zeamais* adults fed with SteLL (Camaroti et al., 2018). These effects show that while lectin effects vary among insect species, they ultimately disrupt the normal function of digestive enzymes.

This study found that mortality rates in *T. hirta* increased significantly with higher lectin doses. Additionally, the inhibitory effect of plant lectins on digestive enzymes restricts developmental and reproductive activities of insects. The increase in oxidant levels, leading to oxidative stress, further supports these entomotoxic effects. By highlighting lectins as environmentally safe biopesticides, this study contributes to agricultural pest management. To maximize the practical application of these findings, field testing is recommended, with a particular focus on controlling insects that undergo their larval and pupal stages underground.

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Original article (Orijinal araştırma)

Determination of insecticide resistance levels of *Leptinotarsa decemlineata* (Say, 1824) (Coleoptera: Chrysomelidae) in potato fields of Niğde, Konya, and Afyonkarahisar provinces[1]

Niğde, Konya ve Afyonkarahisar İlleri patates ekim alanlarında *Leptinotarsa decemlineata* (Say, 1824) (Coleoptera: Chrysomelidae)'nın insektisitlere direnç seviyelerinin belirlenmesi

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Abstract

In our research, acetamiprid, spinosad, and deltamethrin resistance levels were determined in *Leptinotarsa decemlineata* (Say, 1824) (Coleoptera: Chrysomelidae) populations collected from two different locations in potato cultivation areas of Niğde, Afyonkarahisar, and Konya provinces, where 34.8% of the amount was produced. The insecticides were applied using a spray tower at different concentrations. 3-4th instar larvae were subjected to residual effect tests. The susceptible population was obtained from Niğde Ömer Halisdemir University. Field populations were collected starting from June 2023. In the residual effect tests, at the end of the 24-hour exposure period, the LC_{50} values obtained from the susceptible population, Sandıklı, Şuhut, Konaklı, Çiftlik, Çumra, Selçuklu populations were determined as 1.507, 111.964, 89.617, 81.977, 73.563, 38.062, 7.409 mg ai l^{-1} for acetamiprid; 1.105, 21.035, 16.218, 11.982, 15.841, 8.183, 8.945 mg ai l^{-1} for deltamethrin and 4.738, 104.224, 96.404, 70.211, 64.147, 56.863, 37.639 mg ai l^{-1} for spinosad, respectively. The highest resistance rate was detected in the Sandıklı population as 74.30-fold, 19.04-fold, and 22-fold for acetamiprid, deltamethrin, and spinosad, respectively. Considering insecticide resistance, it is important to give priority to insecticides to which the pest has developed less resistance in chemical control of *L. decemlineata*.

Keywords: Acetamiprid, deltamethrin, insecticide resistance, *Leptinotarsa decemlineata*, spinosad

Özet

Araştırmamızda üretiminin %34.8'inin yapıldığı Niğde, Afyonkarahisar ve Konya illeri patates ekim alanlarındaki ikişer farklı lokasyondan toplanan *Leptinotarsa decemlineata* (Say, 1824) (Coleoptera: Chrysomelidae) popülasyonlarında acetamiprid, spinosad ve deltamethrin direnç seviyeleri belirlenmiştir. İnsektisitler farklı konsantrasyonlarda püskürtme kulesi kullanılarak uygulanmıştır. 3-4. dönem larvalar rezidüel etki testlerine tabi tutulmuştur. Hassas popülasyon Niğde Ömer Halisdemir Üniversitesinden elde edilmiştir. Arazi popülasyonları 2023 yılı haziran ayından itibaren toplanmıştır. Rezidüel etki testlerinde 24 saat maruz bırakma süresi sonunda hassas popülasyon, Sandıklı, Şuhut, Konaklı, Çiftlik, Çumra, Selçuklu, popülasyonlarından elde edilen LC_{50} değerleri sırasıyla acetamiprid için 1.507, 111.964, 89.617, 81.977, 73.563, 38.062, 7.409 mg ai l^{-1}; deltamethrin için 1.105, 21.035, 16.218, 11.982, 15.841, 8.183, 8.945 mg ai l^{-1} ve spinosad için 4.738, 104.224, 96.404, 70.211, 64.147, 56.863, 37.639 mg ai l^{-1} olarak belirlenmiştir. En yüksek direnç oranı acetamiprid, deltamethrin ve spinosad için sırasıyla 74.30 kat, 19.04 kat, 22 kat olarak Sandıklı popülasyonunda tespit edilmiştir. İnsektisit direnci dikkate alındığında *L. decemlineata* ile kimyasal mücadelede zararlının daha az direnç geliştirdiği insektisitlere öncelik verilmesi önem arz etmektedir.

Anahtar sözcükler: Acetamiprid, deltamethrin, insektisit direnci, *Leptinotarsa decemlineata*, spinosad

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Introduction

Potatoes (*Solanum tuberosum* L. 1753 (Solanales**:** Solanaceae)) rank 4th in agricultural production after maize, rice, and wheat (Kumar & Pandey, 2008). According to FAO reports, approximately 18.2 million hectares of potato were planted worldwide in 2021. Turkey contributes 1.4% of the world's potato production and ranks 16th in terms of production volume (Anonymous, 2023). Potatoes play a significant role in Turkey's economic growth (approximately 3% of Gross Domestic Product) and are the most consumed vegetable in the country (Günel et al., 2010).

In 2022, Turkey's total potato production was 5.200.000 tons, with an average yield of 3.739 kg/da. Niğde leads in potato production with 176.300 da (12.7%) of planting area and 679.653 tons (13.1%) of production. Afyonkarahisar ranks second with 149.968 da (10.8%) of planting area and 607.539 tons (11.7%) of production, while Konya ranks third with 120.491 da (8.7%) of planting area and 518.677 tons (10%) of production (Anonymous, 2023).

In potato cultivation, factors such as insects, diseases, and weeds adversely affect the quality and quantity of the crop. It has been reported that 270 insect and 17 mite species are harmful to potato worldwide (Alkan et al., 2017).

In many parts of the world, *Leptinotarsa decemlineata* (Say, 1824) (Coleoptera: Chrysomelidae), is a major pest of potato. Yield losses due to this pest range from 30-50% annually, with losses up to 100% in dense populations (Zhou et al., 2012). The leaf area consumed by a single individual over its lifetime can reach 100 cm^2 (Ferro et al., 1985). The most damaging period for the Colorada potato beetle is the mature larval stage, during which 90% of the total leaf consumption occurs (Hare, 1990).

The first record of resistance in the *L. decemlineata* was reported in 1952 for DDT (Quinton, 1955), and later in 1968 for dieldrin (Gauthier et al., 1981). It has been noted that populations susceptible to carbofuran can develop resistance within a single growing season (Ioannidis et al., 1992). Similarly, resistance to endosulfan was detected in the year of its registration (Sharif et al., 2007). The extensive use of chemical control methods in the control of *L. decemlineata* has caused the pest to become resistant to many insecticide classes. This has made resistance management necessary (Gökçe et al., 2018). Today resistance has been observed to 56 different active ingredients from 13 insecticide groups (Özdemir et al., 2021).

In our country, the first study was conducted in 1974-1975. It was found that the *L. decemlineata* did not develop resistance to azinphosmethyl, trichlorphon, and propoxur (Atak & Atak, 1977). This was explained by the pest's recent introduction to the country and limited use of insecticides. In 1980, chemical control was applied to 19% of potato fields, while this rate increased to 54% by 1988 (Ünal & Kılıç, 1997). Resistance to azinphosmethyl was detected in Bolu, Nevşehir, and Tekirdağ populations at 11.24, 8.99, and 9.04-fold, respectively, and for endosulfan and carbofuran at 5.29, 3.82, 6.83-fold, and for deltamethrin at 225.92, 58.83, 90.42-fold, and for endosulfan at 15.24, 17.58, and 45.46-fold (Erdoğan & Gürkan, 1997).

Resistance may be defined as 'a heritable change in the sensitivity of a pest population that is reflected in the repeated failure of a product to achieve the expected level of control when used according to the label recommendation for that pest species' (IRAC 2024). There are 5 types of resistance in harmful insects: behavioral, morphological, physiological, multi-directional and cross-resistance (Yu, 2008). There are some enzymes that are effective when insecticides enter the insect body. These enzymes, such as P450, glutathione-S-transferase (GST) and hydrolase, help eliminate the toxicity of chemicals in insecticides (Tsagkarakou et al., 2009).

The significant procreative potential of *L. decemlineata* accelerates the development of mutations that lead to resistance (Bishop & Grafius, 1996). The beetle, which feeds on Solanaceae family plants, has developed physiological capabilities to detoxify toxic glycoalkaloids found in the leaves (Ferro, 1993). The

L. decemlineata, which can feed on tobacco (*Nicotiana tabacum* L., 1753 (Solanales: Solanaceae)), has developed resistance to neonicotinoids, analogs of nicotine found in this plant. Resistance of 100-fold was detected 2 years after the registration of imidacloprid (Zhao et al., 2000) and soon reached 300-fold (Mota-Sanchez et al., 2006).

Resistance mechanisms in the *L. decemlineata* include increased secretion of esterases, carboxylesterases, and monooxygenases, target site insensitivity, and increased secretion of detoxification enzymes (Clark et al., 2001). The most common resistance mechanism involves monooxygenases, which also play a significant role in neonicotinoid resistance. These insecticides cause desensitization due to mutations in the target AChE receptors (Stanković et al., 2004).

Desensitization is not limited to AChE receptors; mutations in sodium channels, as seen with permethrin, can also affect imidacloprid sensitivity (Tan et al., 2005).

Resistance in pests is determined by proportional increases in LD_{50} or LC_{50} values in laboratory studies. Despite increases in LD_{50} and LC_{50} values, there may not be a noticeable reduction in field control results (Grafius & Douches, 2008). Increases detected in bioassay studies contribute significantly to early warning systems for producers, delay the development of resistance, and support resistance management.

Studies have found that resistance ratios can increase to 220-fold for endosulfan (Sharif et al., 2007), 252-fold for phasolone (Mohamadi et al., 2010), 300- fold for imidacloprid (Mota-Sanchez et al., 2006), and 645-fold for bensultap (Sladan et al., 2012). Resistance ratios also vary regionally and locally (Sharif et al., 2007), depending on application methods and across different pest life stages.

In this study, the resistance ratios of the 3rd and 4th instar larvae of the *L. decemlineata* gathered from the top three potato-producing provinces of Niğde, Afyon, and Konya were determined against acetamiprid, deltamethrin, and spinosad using residual effect tests with different concentrations and exposure durations.

Materials and Methods

Materials

Leptinotarsa decemlineata populations used in the study were collected from potato fields in two locations each in Niğde, Konya, and Afyonkarahisar provinces starting from June 2023 (Table 1). Insects in the larval stage were collected from the fields. The collected larvae were brought to the laboratory and fed on potato leaves in a climate chamber for 24 hours. During this time, individuals damaged during collection and transportation were eliminated (Huseth & Groves, 2013), and healthy 3rd-4th stage larvae were selected and used in the tests. The susceptible population was obtained from the Department of Plant Production and Technology, Faculty of Agricultural Sciences and Technologies, Niğde Ömer Halisdemir University. They were brought to the climate chamber in plastic containers covered with mesh and fed with potato plant leaves that we had previously grown.

Table 1. Provinces and districts where *Leptinotarsa decemlineata* populations were collected

Province	District	Coordinates
Afyon	Şuhut	38°31'03"N 30°32'57"E
	Sandı	38°28'36"N 30°16'12"E
Niğde	Konakl	38°09'34"N 34°49'40"E
	Çiftlik	38°12'26"N 34°31'11"E
Konya	Çumra	37°34'05"N 32°38'33"E
	Selçuk	38°02'02"N 32°30'55"E

A mixture of sandy soil, perlite, and peat was prepared and planted to grow the potato plants. Plantings were made at regular intervals to ensure the continuation of the cultures. The plants and insects were grown in the climate room at 26±1ºC, 60±10% relative humidity, and 16-8 hours (light-dark) photoperiod conditions.

In the study, commercial insecticides often used for *L. decemlineata* control were used: Decis 2.5 EC (containing 2.5% (w/v) deltamethrin from Bayer CropScience), Phomidan 480 SC (containing 480 g/L spinosad from Koruma Tarım) and Goldplan 20 SP (containing 20% acetamiprid from Agrobest Group) (Table 2). A spray tower (Burkard Scientific Laboratory Spray Tower) was used for insecticide applications, and Petri dishes containing larvae were placed in a climate chamber (Nüve TK 120).

Table 2. Information about the insecticides used in the study

Active ingredient name	Trade name	Formulation	Mechanism of Action	Dosage	Action mode	Chemical group
Acetamiprid (% 20)	Goldplan	SP	4A	6 g/da	Nicotinic acetylcholine receptor agonists/antagonists	Neonicotinoids
Deltamethrin (25 g/l)	Decis	EC	3A	30 ml/da	Sodium channel modulators	Pyrethroids, Pyrethrins
Spinosad (480g/L)	Phomidan	SC	5	10 ml/da	Nicotinic Acetylcholine receptor agonists	Spinosyns

Methods

Bioassays

The dose determination study was conducted using a reference susceptible population. Residual effect tests were performed using 3rd-4th instar larvae of *L. decemlineata* Six effective doses causing mortality ranging from 15% to 99% were selected for each active substance in preliminary experiments.

Insecticide concentrations were prepared by diluting with distilled water 50% at each step. Distilled water was used as a control. The prepared insecticides were sprayed into petri dishes (2 ml) using spray tower at 1-atmosphere pressure.

The petri dish (12 cm in diameter, 2.5 cm in height) was left to dry 5 minutes before ten healthy 3rd-4th instar larvae were placed in each using soft forceps (according to the contact and dry film method) (Gokulakrishnaa & Thirunavukkarasu, 2023). No food was added to the petri dishes. The dishes were closed with lid. To prevent larvae from dying due to lack of air, a small rectangular piece of paper was placed between the Petri dish and its lid to avoid the lid closing completely. The experiments were carried out in the climate chamber under conditions of 26±1ºC warmth, 60±10% proportional humidity, and 16-8 hours (light-dark) photoperiod. After 24, 48, and 72 hours of exposure, larvae were touched with a soft brush on various parts of their bodies, and immobile larvae were considered dead (Alkan et al., 2017). Experiments were conducted in a completely randomized design with three replications. One Petri dish was used for each replication.

Data Analysis

The data were subjected to probit analysis, and LC_{50} value was determined using the POLO computer package program (LeOra, 2002). The resistance ratio (RR) was calculated by dividing the LC_{50} values of the resistant group by the LC_{50} values of the susceptible group. Resistance levels were assessed using the scale suggested by Lee et al. (1999): RR <2: Not or minimal, RR = 2-5: Little , RR = 5-10: Medium, RR >10: High resistance.

Results and Discussion

Rate of acetamiprid resistance

The highest resistance rates in the 24-hour exposure period were found to be 74.30-fold in the Sandıklı population, 59.47-fold in the Şuhut population, 54.40-fold in the Konaklı population, 48.81-fold in the Çiftlik population, 31.46-fold in the Selçuklu population and 25.26-fold in the Çumra population, respectively (Table 3). The highest LC_{50} values were found in the Sandıklı population: 111.964 mg ai l^{-1} at 24 hours, 68.804 mg ai l^{-1} at 48 hours, and 29.080 mg ai l^{-1} at 72 hours (Tables 3, 4 & 5).

Table 3. Residual toxicity of acetamiprid to 3rd-4th instar larvae of *Leptinotarsa decemlineata* after a 24-hour exposure period

Population	n	LC_{50} (mg ai l^{-1}) (%95 CL)	Slope ± SEM	λ^2	DF	H	p value	RR
Susceptible	180	1.507 1.045-2.280	1.255± 0.209	13.212	16	0.826	0.830	-
Sandıklı	180	111.964 73.563-177.976	1.414±0.216	21.131	16	1.320	0.470	74.30
Şuhut	180	89.617 61.602-129.358	1.296±0.210	7.547	16	0.472	0.512	59.47
Konaklı	180	81.977 55.312-118.474	1.268±0.210	10.058	16	0.629	0.528	54.40
Çiftlik	180	73.563 50.951-102.649	1.404 ± 0.218	10.325	16	0.645	0.551	48.81
Çumra	180	38.062 26.783-52.654	1.454±0.219	6.193	16	0.387	0.545	25.26
Selçuklu	180	47.409 34.046-66.186	1.461±0.218	5.841	16	0.365	0.502	31.46

n: the count of larvae; SEM.: standart error of the mean; λ^2: chi square; DF: degrees of freedom; H: heterogeneity; RR: resistance rate.

Table 4. Residual toxicity of acetamiprid to 3rd-4th instar larvae of *Leptinotarsa decemlineata* after a 48-hour exposure period

Population	n	LC_{50} (mg ai l^{-1}) (%95 CL)	Slope ± SEM	λ^2	DF	H	p value	RR
Susceptible	180	0.856 0.548-1.258	1.184±0.207	7.241	16	0.453	0.558	-
Sandıklı	180	68.804 44.529-100.427	1.209±0.209	15.250	16	0.953	0.558	80.38
Şuhut	180	58.710 36.444-85.991	1.190±0.209	7.948	16	0.497	0.584	68.59
Konaklı	180	38.609 21.768-57.238	1.359±0.235	8.917	16	0.557	0.678	45.10
Çiftlik	180	37.619 22.034-54.608	1.465±0.246	5.876	16	0.367	0.689	43.95
Çumra	180	27.318 17.203-39.101	1.468± 0.238	7.567	16	0.473	0.622	31.91
Selçuklu	180	29.324 17.668-43.191	1.367±0.235	6.962	16	0.435	0.607	34.26

n: the count of larvae; SEM.: standart error of the mean; λ^2: chi square; DF: degrees of freedom; H: heterogeneity; RR: resistance rate.

Similar studies have shown that the *L. decemlineata* has developed varying resistance levels to imidacloprid, a neonicotinoid insecticides. Baker et al. (2014) detected imidacloprid resistance in *L. decemlineata* larvae in three divergent populations in the USA, ranging from 7.6 to 71-fold. In a study conducted between 1995 and 1998 across the United States, Canada, Germany, France, and Poland, the lowest resistance to imidacloprid was reported as 29-fold (Olson et al., 2000). Crossley & Rondon (2018) reported a resistance increase of 9-340-fold among populations in 10 different regions of the USA. Keskin & Yorulmaz Salman (2020) found that populations from the districts of Afyonkarahisar displayed resistance to imidacloprid ranging from 3.96 to 27.31 times. In our study, resistance levels of 59.47-fold and 74.30-fold were determined in Şuhut and Sandıklı districts of Afyonkarahisar, respectively. Given the time elapsed and cross-resistance, it is normal to observe increased resistance in pests due to intensive insecticide applications (both in terms of number of spraying and dosage). It is known that insecticide sensitivity in *L. decemlineata* is highest in young larvae, decreases in mature larvae, and is lowest in adults (Sayıncı et al.,

2013). The resistance rates for imidacloprid in Çumra, Karapınar and Seydişehir, Güneysınır, Doğanhisar populations were determined as 4.63, 7.01, 4.16, 2.84 and 2.44-fold, respectively in 2020 (Çağırgan & Çetin, 2021). They reported that imidacloprid was used for treating tubers, and during the growing season, green part applications were also made with imidacloprid (EC formulations) or acetamiprid from the same group. In a study conducted in Serbia, imidacloprid resistance in adult *L. decemlineata* was reported as 82.90-fold (Sladan et al., 2012), while a study in New York reported 29-fold resistance to acetamiprid in adults (Mota Sanchez et al., 2006). Jeschke & Nauen (2008) noted that cross-resistance developed to other neonicotinoid insecticides when one neonicotinoid was used. In our study, the highest resistance ratio was determined for acetamiprid. Considering that neonicotinoids are frequently used for both seed and green part spraying in the control of *L. decemlineata*, acetamiprid resistance is expected to be high.

Table 5. Residual toxicity of acetamiprid to 3rd-4th instar larvae of *Leptinotarsa decemlineata* after a 72-hour exposure period

Population	n	LC_{50} (mg ai l^{-1}) (%95 CL)	Slope ± SEM	λ^2	DF	H	p value	RR
Susceptible	180	0.461 0.217-0.736	1.132±0.225	10.065	16	0.629	0.668	-
Sandıklı	180	29.080 13.535-45.951	1.225±0.236	5.001	16	0.313	0.712	63.08
Şuhut	180	27.012 12.203-43.035	1.226±0.237	10.491	16	0.656	0.723	58.59
Konaklı	180	23.708 12.502-35.175	1.566±0.279	4.181	16	0.261	0.772	51.43
Çiftlik	180	22.168 11.367-33.110	1.578±0.287	4.946	16	0.309	0.783	48.09
Çumra	180	18.125 9.762-27.302	1.460±0.259	4.995	16	0.312	0.706	39.32
Selçuklu	180	16.733 9.020-25.065	1.515±0.268	2.618	16	0.164	0.723	36.30

n: the count of larvae; SEM.: standart error of the mean; λ^2: chi square; DF: Degrees of Freedom; H: heterogeneity; RR: resistance rate.

Probit analysis indicates that populations with a slope value less than 2 are heterogeneous (Yu, 2015) and that resistance development is faster in heterogeneous populations (Keskin & Yorulmaz-Salman, 2020). Table 3 shows that heterogeneity values are less than 2.

Rate of deltamethrin resistance

The highest resistance rates in the 24-hour period were determined as 19.04-fold in the Sandıklı population, 14.68-fold in the Şuhut population, 14.34-fold in the Çiftlik population, 10.84-fold in the Konaklı population, 8.10-fold in the Selçuklu population and 7.41-fold in the Çumra population, respectively (Table 6). The highest LC_{50} values were found in the Sandıklı population: 21.035 mg ai l^{-1} at 24 hours, 12.177 mg ai l^{-1} at 48 hours, and 4.492 mg ai l^{-1} at 72 hours (Tables 6, 7 & 8).

Table 6. Residual toxicity of deltamethrin to 3rd-4th instar larvae of *Leptinotarsa decemlineata* after a 24-hour exposure period

Population	n	LC_{50} (mg ai l^{-1}) (%95 CL)	Slope ± SEM	λ^2	DF	H	p value	RR
Susceptible	180	1.105 0.768-1.713	1.255±0.210	12.144	16	0.759	0.431	-
Sandıklı	180	21.035 14.097-32.557	1.153±0.205	5.303	16	0.331	0.474	19.04
Şuhut	180	16.218 10.772-24.023	1.196±0.206	9.730	16	0.608	0.518	14.68
Konaklı	180	11.982 7.206-18.044	1.098±0.204	11.482	16	0.718	0.566	10.84
Çiftlik	180	15.841 10.917-22.611	1.326±0.213	10.342	16	0.646	0.523	14.34
Çumra	180	8.183 5.729-11.566	1.376±0.214	5.051	16	0.316	0.518	7.41
Selçuklu	180	8.945 6.249-12.823	1.339±0.212	3.571	16	0.223	0.501	8.10

n: the count of larvae; SEM.: standart error of the mean; λ^2: chi square; DF: degrees of freedom; H: heterogeneity; RR: resistance rate.

Table 7. Residual toxicity of deltamethrin to 3rd-4th instar larvae of *Leptinotarsa decemlineata* after a 48-hour exposure period

Population	n	LC_{50} (mg ai l^{-1}) (%95 CL)	Slope ± SEM	λ^2	DF	H	p value	RR
Susceptible	180	0.470 0.274-0.718	1.057±0.204	12.372	16	0.773	0.574	-
Sandıklı	180	12.177 6.754-19.442	0.954±0.199	9.012	16	0.563	0.557	25.91
Şuhut	180	8.829 4.545-13.869	0.997±0.203	5.533	16	0.346	0.607	18.79
Konaklı	180	6.288 3.243-9.624	1.277±0.232	4.536	16	0.283	0.694	13.38
Çiftlik	180	6.434 3.287-9.898	1.256±0.231	9.004	16	0.563	0.689	13.69
Çumra	180	4.531 2.394-7.046	1.164±0.221	6.118	16	0.382	0.629	9.64
Selçuklu	180	5.209 3.168-7.604	1.403±0.237	4.262	16	0.266	0.618	11.08

n: the count of larvae; SEM.: standart error of the mean; λ^2: chi square; DF: degrees of freedom; H: heterogeneity; RR: resistance rate.

Table 8. Residual toxicity of deltamethrin to 3rd-4th instar larvae of *Leptinotarsa decemlineata* after a 72-hour exposure period

Population	n	LC_{50} (mg ai l^{-1}) (%95 CL)	Slope ± SEM	λ^2	DF	H	p value	RR
Susceptible	180	0.280 0.158-0.413	1.435±0.249	8.600	16	0.538	0.696	-
Sandıklı	180	4.492 1.442-7.951	0.994±0.224	10.535	16	0.658	0.712	16.04
Şuhut	180	4.057 1.268-7.215	1.021±0.228	9.538	16	0.596	0.729	14.49
Konaklı	180	4.188 2.028-6.422	1.454±0.269	7.104	16	0.444	0.772	14.96
Çiftlik	180	4.073 1.966-6.245	1.472±0.273	10.984	16	0.687	0.778	14.55
Çumra	180	2.382 0.947-3.972	1.208±0.247	7.407	16	0.463	0.739	8.51
Selçuklu	180	2.264 1.124-3.443	1.575±0.294	4.060	16	0.254	0.779	8.09

n: the count of larvae; SEM.: standart error of the mean; λ^2: chi square; DF: degrees of freedom; H: heterogeneity; RR: resistance rate.

Keskin & Yorulmaz Salman (2020) reported that populations from Afyonkarahisar's districts exhibited 9.41-77.17-fold resistance to deltamethrin. Sladan et al. (2012) found up to 60-fold resistance to cypermethrin in adult *L. decemlineata* in Serbia. In a study investigating deltamethrin resistance in 4th instar larvae of *L. decemlineata* collected from 4 different populations from Urumqi, Changji, Qitai and Qapqal (China) in 2009 and 2010, 1.7- to 42.7-fold resistance to deltamethrin was detected (Jiang et al., 2011). Jörg et al. (2007) reported that the effectiveness of lambda-cyhalothrin decreased from 92% in 2003 to 73% in 2006 due to sequential applications. Sladan et al. (2012) found up to 60-fold resistance to cypermethrin, another pyrethroid, in a study conducted in Serbia. Erdoğan & Gürkan (1997) found resistance levels to deltamethrin in *L. decemlineata* adults and 3rd instar larvae to be 225.92, 58.83, and 90.42-fold for Bolu, Nevşehir, and Tekirdağ provinces, respectively. Resistance rates for lambda-cyhalothrin in the Çumra, Karapınar and Seydişehir populations were determined as 2.98, 2.63 and 2.40-fold, respectively in 2020 (Çağırgan & Çetin, 2021). In our study, resistance ratios to deltamethrin in six populations ranged from 7.41 to 19.04-fold. These resistance ratios are lower compared to other studies.

Rate of spinosad resistance

The highest resistance rates in the 24-hour period were determined as 22-fold in the Sandıklı population, 20.35-fold in the Şuhut population, 14.82-fold in the Konaklı population, 13.54-fold in the Çiftlik population, 12-fold in the Çumra population, and 7.94-fold in the Selçuklu population, respectively (Table 9). The highest LC_{50} values were found in the Sandıklı population: 104.224 mg ai l^{-1} at 24 hours, 56.777 mg ai l^{-1} at 48 hours, and 34.410 mg ai l^{-1} at 72 hours (Tables 9, 10 & 11).

Table 9. Residual toxicity of spinosad to 3rd-4th instar larvae of *Leptinotarsa decemlineata* after a 24-hour exposure period

Population	n	LC_{50} (mg ai l^{-1}) (%95 CL)	Slope ± SEM	λ^2	DF	H	p value	RR
Susceptible	180	4.738 3.274-6.877	1.294±0.210	8.223	16	0.514	0.502	-
Sandıklı	180	104.224 71.255-150.526	1.286±0.210	8.915	16	0.557	0.518	22.00
Şuhut	180	96.404 60.802-147.107	1.091±0.202	7.464	16	0.466	0.529	20.35
Konaklı	180	70.211 45.000-100.637	1.275±0.213	10.978	16	0.686	0.589	14.82
Çiftlik	180	64.147 42.359-89.534	1.408± 0.222	8.356	16	0.522	0.611	13.54
Çumra	180	56.863 40.130-80.802	1.379±0.213	4.578	16	0.286	0.502	12.00
Selçuklu	180	37.639 26.212-51.535	1.502±0.223	2.274	16	0.142	0.584	7.94

n: the count of larvae; SEM.: standart error of the mean; λ^2: chi square; DF: degrees of freedom; H: heterogeneity; RR: resistance rate.

Table 10. Residual toxicity of spinosad to 3rd-4th instar larvae of *Leptinotarsa decemlineata* after a 48-hour exposure period

Population	n	LC_{50} (mg ai l^{-1}) (%95 CL)	Slope ± SEM	λ^2	DF	H	p value	RR
Susceptible	180	2.501 1.454-3.732	1.136±0.210	13.952	16	0.872	0.608	-
Sandıklı	180	56.777 33.961-82.914	1.225±0.214	7.983	16	0.499	0.623	22.70
Şuhut	180	41.753 21.667-63.596	1.159±0.216	3.713	16	0.232	0.667	16.69
Konaklı	180	35.475 16.974-55.507	1.249±0.237	9.628	16	0.602	0.712	14.18
Çiftlik	180	34.077 19.996-48.610	1.682±0.283	5.693	16	0.356	0.751	13.63
Çumra	180	29.907 17.665-44.091	1.346±0.230	3.290	16	0.206	0.634	11.96
Selçuklu	180	26.615 16.849-37.480	1.607±0.253	4.540	16	0.284	0.667	10.64

n: the count of larvae; SEM.: standart error of the mean; λ^2: chi square; DF: degrees of freedom; H: heterogeneity; RR: resistance rate.

Table 11. Residual toxicity of spinosad to 3rd-4th instar larvae of *Leptinotarsa decemlineata* after a 72-hour exposure period

Population	n	LC_{50} (mg ai l^{-1}) (%95 CL)	Slope ± SEM	λ^2	DF	H	p value	RR
Susceptible	180	1.605 0.795-2.484	1.272±0.240	9.679	16	0.605	0.702	-
Sandıklı	180	34.410 16.332-53.906	1.262±0.240	9.504	16	0.594	0.718	21.44
Şuhut	180	25.375 10.430-41.368	1.260±0.250	4.774	16	0.298	0.761	15.81
Konaklı	180	26.067 12.577-39.967	1.472±0.273	3.782	16	0.236	0.778	16.24
Çiftlik	180	24.119 12.742-35.244	1.764±0.324	5.470	16	0.342	0.811	15.03
Çumra	180	18.313 9.136-28.341	1.384±0.253	3.581	16	0.224	0.728	11.41
Selçuklu	180	14.043 7.654-20.321	1.866±0.347	4.549	16	0.284	0.801	8.75

n: the count of larvae; SEM.: standart error of the mean; λ^2: chi square; DF: degrees of freedom; H: heterogeneity; RR: resistance rate.

Sayıncı et al. (2013) reported that in Erzurum, *L. decemlineata* larvae and adults showed increased resistance to spinosad as their developmental stage progressed. They found that spinosad had 93.3% effectiveness on 2nd instar larvae and 50% effectiveness on 4th instar larvae and adults after 72 hours of application in the laboratory. Çağırgan & Çetin (2021) found spinosad effectiveness to be 75.33% and 76.05% during the first applications in 2019 and 2020, respectively. Osman (2010) noted that in Russia, the effectiveness of spinosad on mature larvae increased over time, with mortality rates rising from 57.78%

on the 3rd day to 95.56% on the 7th day. Previous studies have reported that spinosad's mode of action is both through ingestion and contact (Kowalska, 2010), with effects beginning to be noticeable 4-5 hours after application (Azimi et al., 2009) and providing high protection for up to 20 days (Igrc et al., 1999). In Croatia, spinosad's effectiveness on larvae was reported as 99.9% on the 2nd day, 99.7% on the 7th day, 98.4% on the 14th day, and decreasing to 80.6% by the 21st day (Igrc et al., 1999). Mota-Sanchez et al. (2006) found up to 7.6-fold resistance in adults to spinosad. In the USA, research on four different populations of *L. decemlineata* larvae showed spinosad resistance ranging from 17.5 to 40.6-fold (Schnaars-Uvino & Baker, 2021). Klein (2019) detected spinosad resistance in 2nd instar larvae of three different *L. decemlineata* populations in Long Island (USA). In this study, two susceptible populations, one reared in a laboratory and one collected from organic fields, were used. When the laboratory susceptible population was used, the resistance rates were found to be 52.4, 38.43 and 33.99 times, and when the field susceptible population was used, the resistance rates were 213.33, 156.46 and 138.37 times. These results showed that the resistance rates were higher when the laboratory-reared susceptible population was used and lower when the field-collected susceptible population was used. In our study, spinosad resistance rates in *L. decemlineata* larvae ranged from 7.94 to 22.00 times.

In the context of potato production in Turkey, insecticides have been ranked as follows based on resistance levels in the Niğde, Afyonkarahisar, and Konya populations: acetamiprid, deltamethrin, and spinosad. This ranking is consistent with the frequency and dose of insecticide applications during the potato production season.

Compared to deltamethrin and spinosad, the higher resistance to acetamiprid is due to the use of neonicotinoids in both foliar and tuber applications, which promote cross-resistance and increase resistance to acetamiprid. Monitoring the number of insecticides used, their doses (especially when recommended doses are exceeded) and their frequency is essential for effective pest control. Continuous monitoring of resistance levels in *L. decemlineata* and updating pest management programs are vital to improving the effectiveness of chemical control. Detailed studies of the annual increase in resistance and cross-resistance are also essential.

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Original article (Orijinal araştırma)

Determination of plant-parasitic nematode fauna and evaluation of soil quality in apple orchards of Çanakkale province (Türkiye)[1]

Çanakkale ili (Türkiye) elma bahçelerinde bitki paraziti nematod faunasının belirlenmesi ve toprak kalitesinin değerlendirilmesi

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Abstract

In September 2023, a total of 130 soil samples were collected from five different districts in the province of Çanakkale (Türkiye) and its surroundings to identify plant-parasitic nematode communities in apple orchards and to create distribution maps of these species. The density and distribution of nematode communities were examined. In total, 14.090 nematode individuals were analyzed, and 30 genera were identified. The nematodes were classified into six different orders, with the order Rhabditida standing out as the most dominant (53.87%). The order Tylenchida ranked second (23.22%). Among the most common plant-parasitic nematodes identified in the study were *Paratylenchus* spp. Micoletzky, 1922 (Tylenchida: Tylenchulidae) (3.46%), *Merlinius* spp. Siddiqi, 1970 (Tylenchida: Dolichodoridae) (3.36%), and *Pratylenchus* spp. Filipjev, 1936 (Tylenchida: Pratylenchidae) (3.02%). The results of the study indicate that the c-p 2 group is prevalent under disturbed soil conditions, and the dominance of the p-p 2 and p-p 3 groups poses a significant threat to apple orchards. These findings highlight that nematode c-p series are important bioindicators in the assessment of soil quality, and such analyses should be expanded through regional studies.

Keywords: Apple orchards, biodiversity, bioindicators, plant-parasitic nematodes, soil quality

Öz

Çanakkale (Türkiye) ili ve ilçelerindeki elma bahçelerinde bitki paraziti nematod toplulukların tespiti ve bu türlerin dağılım haritalarının oluşturulması için 2023 yılı Eylül ayında beş farklı ilçeden toplam 130 toprak örneği toplanmış ve nematod toplulukların yoğunluğu ve yayılımı incelenmiştir. Çalışmada toplam 14.090 nematod bireyi incelenmiş ve 30 cins tanımlanmıştır. Nematodlar altı farklı takımda yer almakta olup, Rhabditida takımı (53.87%) ile en baskın takım olarak öne çıkmıştır. Tylenchida takımı (23.22%) ile ikinci sırada yer almıştır. Araştırmada en fazla bulunan bitki paraziti nematodlar arasında *Paratylenchus* spp. Micoletzky, 1922 (Tylenchida: Tylenchulidae) (3.46%), *Merlinius* spp. Siddiqi, 1970 (Tylenchida: Dolichodoridae) (3.36%), ve *Pratylenchus* spp. Filipjev, 1936 (Tylenchida: Pratylenchidae) (3.02%) yer almaktadır. Çalışmada elde edilen sonuçlar c-p 2 grubunun bozulmuş toprak koşullarında yaygın olduğunu ve p-p 2 ile p-p 3 gruplarının baskınlığı ile elma bahçelerinde önemli bir tehdit oluşturduğunu göstermektedir. Bu bulgular nematodların c-p serilerinin toprak kalitesinin değerlendirilmesinde önemli bir biyoindikatör olduğunu ve bölgesel çalışmalarla bu tür analizlerin yaygınlaştırılması gerektiğini ortaya koymaktadır.

Anahtar sözcükler: Elma bahçeleri, biyoçeşitlilik, biyoindikatörler, bitki paraziti nematodlar, toprak kalitesi

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Introduction

Nematodes represent a large group of invertebrates and are organisms belonging to the phylum Nematoda. This phylum is divided into three main classes, which include approximately 32 orders, 3.030 genera, and 28.537 species (Hodda, 2022). Nematodes are unsegmented roundworms, most of which are microscopic in size. They have adapted to various habitats (soil, water, marine, freshwater). There are both free-living and parasitic species. Parasitic species can cause significant damage to both plants and animals (Bhat et al., 2022). Globally, approximately 4.100 plant-parasitic nematode species have been identified (Decraemer & Hunt, 2006).

Plant-parasitic nematodes are microscopic organisms, typically ranging in size from 0.5 mm to 3 mm, that are commonly found worldwide and cause significant economic losses in agricultural production. These nematodes penetrate plant parts such as roots, stems, and leaves, feeding and causing damage to plant tissues. Additionally, the damage they inflict weakens the plant's defense mechanisms, making it more susceptible to infections by other pathogens (Sato et al., 2019). *Pratylenchus* spp. Filipjev, 1936 (Tylenchida: Pratylenchidae), *Meloidogyne* spp. Goeldi, 1892 (Tylenchida: Meloidogynidae), *Paratylenchus* spp. Micoletzky, 1922 (Tylenchida: Tylenchulidae) and *Helicotylenchus* spp. Steiner, 1945 (Tylenchida: Hoplolaimidae) are the primary nematodes causing damage in apple orchards (Askary et al., 2012). The economic losses caused by these nematodes are substantial. Globally, the economic damage to agricultural production caused by plant-parasitic nematodes is estimated to be $215 billion annually (Abu-Elgawad & Askary, 2015).

Türkiye holds a significant position in fruit cultivation due to its diverse climate and fertile soils. The fruit-growing sector not only plays an important role in human nutrition but also adds economic value to Türkiye (Niyaz & Demirbaş, 2011). Among the most widely cultivated fruits in Türkiye are figs, grapes, apricots, apples, hazelnuts, pomegranates, and cherries. In 2022, the total fruit production in Türkiye amounted to 26.794.706 tons, with apple orchards covering an area of 1.709.408 decares and total production reaching 4.817.500 tons (TUİK, 2022).

Türkiye plays a crucial role in apple cultivation, and apples hold a significant share in the country's agricultural production. Globally, Türkiye ranks second among the top apple-producing countries with an annual production of approximately 4.8 million tons (FAO, 2024). Apple cultivation provides an important economic contribution both for the domestic market and for export.

Nematodes stand out as important microorganisms for assessing the health of soil ecosystems. The diversity and distribution of nematode populations play a critical role in determining soil health and ecosystem functions (Bongers & Ferris, 1999). The use of nematodes, particularly for monitoring the impacts of agricultural practices and pollution, serves as a valuable method for measuring soil health (Neher, 2001). For these reasons, the commercial potential of using nematodes as bioindicators is noteworthy, highlighting the significance of their role in environmental assessment programs (Trett et al., 2009).

The primary objective of this study is to identify the plant-parasitic nematodes in apple orchards within the province of Çanakkale (Türkiye) and its districts, as well as to determine their distribution maps. Additionally, the study aims to understand the ecological roles of nematodes in apple orchards and to examine the effects of different environmental and climatic conditions on nematode populations.

Materials and Methods

Survey

Soil sampling was conducted in apple orchards within the province of Çanakkale and its districts during the period leading up to harvest with the aim of identifying plant-parasitic nematodes and determining their densities. Since nematodes generally exhibit clustered distributions, samples were collected in a

manner that best represents the region (Southey, 1986). Soil samples were taken from a depth of 15-30 cm under the canopy projection of the trees. The samples were placed in polyethylene bags with label information and stored under appropriate conditions at the Nematology Laboratory of Çanakkale Onsekiz Mart University. A total of 130 soil samples were collected from five different districts throughout September 2023 (Figure 1).

Figure 1. Map of sampling locations from five different districts in Çanakkale.

Nematode extraction from soil

The Modified Baermann Funnel Method, which is used for the extraction of mobile nematodes from the soil through separation using water, was employed (Hooper, 1986). In the Modified Baermann funnel method, plastic Petri dishes with a diameter of 12 cm and a height of 2 cm were used. In each Petri dish, 100 g of soil was placed with filter paper, allowing the active nematodes to migrate into the water. After keeping the Petri dishes in this state for 48 hours, the water they contained was transferred into 100 ml glass cylinders, and after 24 hours, it was transferred into 10 ml glass tubes. Subsequently, these glass tubes were stored under appropriate conditions at the Çanakkale Onsekiz Mart University Nematology Laboratory.

Diagnosis at the genus level under light microscopy

The water in the preserved glass tubes was allowed to settle for 6-8 hours and then diluted to 1 ml. After the dilution process, the glass tubes were mixed using a vortex device, and 100 µl of water was taken with a micropipette and placed between a slide and a coverslip. To immobilize individuals and ensure accurate identification, the specimens were processed on a heated plate operating at a specific temperature. Samples were identified at the genus level under a Leica DM 1000 light microscope using the Leica Application Suite v4 software.

Analysis of nematode communities

Taxonomic keys were used as the basis for the classification of nematodes. For the classification of plant-parasitic nematodes in particular, the book Plant-parasitic Nematodes: A Pictorial Key to Genera was used (Mai et al., 1996). The Coloniser-persister (c-p) classification categorizes nematodes on a scale of 1 to 5 based on their life cycles (Bongers, 1990). Nematodes were evaluated according to this classification

system by Yeates et al. (1993) and Du Preez et al. (2022). The Maturity Index (MI) was used to assess ecosystem disturbances in nematode communities (Ferris & Bongers, 2009). The online calculation tool Nematode Indicator Joint Analysis (NINJA) was used to perform the specified analyses (Sieriebriennikov et al., 2014).

Result

Among the total number of 14,090 nematodes examined, six different orders and 30 genera were identified (Table 1). The order Rhabditida, with 7.590 individuals, constituted the majority of the population (53.87%), making it the most dominant order. This was followed by the order Tylenchida, with 3.273 individuals (23.22%). The Aphelenchida order represented 2.888 individuals, accounting for 20.50 % of the total population. Orders represented in lower proportions included Mononchida with 215 individuals (1.53%), Dorylaimida with 100 individuals (0.71%), and Triplonchida with 24 individuals (0.17%).

Table 1. The prevalence rates, cp series, and feeding types of nematode communities

Genus Name	Order: Family	Prevalence Rate (%)	C-p Class	P-p Class	Feeding Type
Aglenchus Andrassy, 1954	Tylenchida: Tylenchidae	0.92	0	2	Herbivores
Aphelenchoides Fischer, 1894	Aphelenchida: Aphelenchoididae	13.61	2	0	Fungivores
Aphelenchus Bastian, 1865	Aphelenchida: Aphelenchidae	6.89	2	0	Fungivores
Boleodorus Thorne, 1941	Tylenchida: Tylenchidae	0.09	0	2	Herbivores
Criconema Hofmanner & Menzel, 1914	Tylenchida: Criconematidae	0.01	0	3	Herbivores
Discocriconemella De Grisse & Loof, 1965	Tylenchida: Criconematidae	0.04	0	3	Herbivores
Ditylenchus Filipjev, 1936	Tylenchida: Anguinidae	2.78	2	0	Fungivores
Dorylaimus Dujardin, 1845	Dorylaimida: Dorylaimidae	0.33	4	0	Omnivores
Eucephalobus Steiner, 1936	Rhabditida: Cephalobidae	46.83	2	0	Bacterivores
Filenchus Andrassy, 1954	Tylenchida: Tylenchidae	2.16	2	0	Fungivores
Helicotylenchus Steiner, 1945	Tylenchida: Hoplolaimidae	1.21	0	3	Herbivores
Hoplolaimus von Daday, 1905	Tylenchida: Hoplolaimidae	0.14	0	3	Herbivores
Longidorus Micoletzky, 1922	Dorylaimida: Longidoridae	0.03	0	5	Herbivores
Malenchus Andrassy, 1968	Tylenchida: Tylenchidae	0.25	0	2	Herbivores
Meloidogyne Goeldi, 1892	Tylenchida: Meloidogynidae	0.20	0	3	Herbivores
Merlinius Siddiqi, 1970	Tylenchida: Dolichodoridae	3.36	0	3	Herbivores
Mononchus Bastian, 1865	Mononchida: Mononchoidea	1.53	4	0	Predators
Paratylenchus Micoletzky, 1922	Tylenchida: Tylenchulidae	3.46	0	2	Herbivores
Pratylenchoides Winslow, 1958	Tylenchida: Pratylenchidae	0.47	0	3	Herbivores
Pratylenchus Filipjev, 1936	Tylenchida: Pratylenchidae	3.02	0	3	Herbivores
Psilenchus de Man, 1921	Tylenchida: Psilenchidae	0.84	0	2	Herbivores
Rhabditis Dujardin, 1844	Rhabditida: Rhabditidae	7.04	1	0	Bacterivores
Rotylenchus Filipjev, 1936	Tylenchida: Hoplolaimidae	0.06	0	3	Herbivores
Scutellonema (Steiner, 1937) Andrassy, 1958	Tylenchida: Hoplolaimidae	0.31	0	3	Herbivores
Trichodorus Cobb, 1913	Triplonchida: Trichodoridae	0.17	0	4	Herbivores
Trophurus Loof, 1956	Tylenchida: Telotylenchidae	0.34	0	3	Herbivores
Tylencholaimus De Man, 1876	Dorylaimida: Tylencholaimoidea	0.04	4	0	Fungivores
Tylenchorhynchus Cobb, 1913	Tylenchida: Telotylenchidae	0.81	0	3	Herbivores
Tylenchus Bastian, 1865	Tylenchida: Tylenchidae	2.75	0	2	Herbivores
Xiphinema Cobb, 1913	Dorylaimida: Longidoridae	0.31	0	5	Herbivores

Classification of nematodes according to their feeding type

The distribution of nematode communities based on feeding types showed significant differences between districts (Figure 2). Generally, bacterivores (nematodes feeding on bacteria) were the dominant group in all districts, with the highest rate observed in the center district (62.90%). Herbivores (plant-parasitic nematodes) were more prominent in Lapseki (27.80% and Bayramiç (20.90%), while they were absent in Biga. Fungivores (fungus-feeding nematodes) stand out with a high proportion in Biga (45.70%) and were more evenly distributed in other districts. Predator nematodes were found in low proportions in some districts, but slightly higher in Lapseki and Biga (around 5.00%). These results suggest that organic matter cycling in the soils of these districts was more related to bacteria and fungi, with variations in biodiversity.

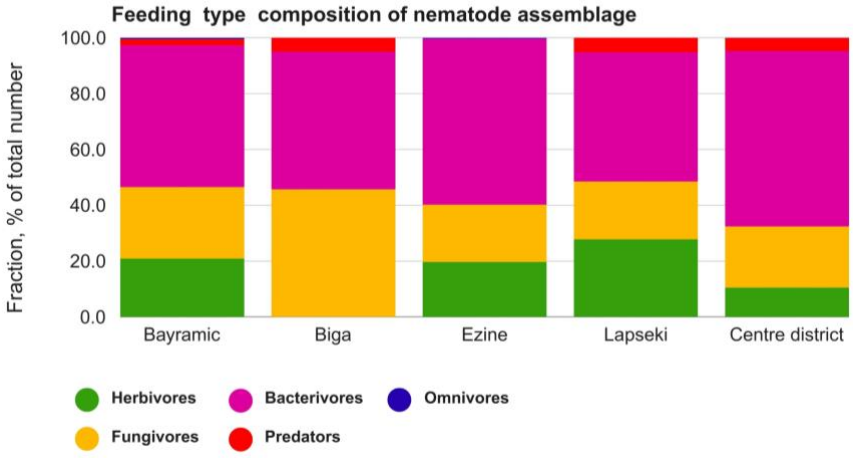

Figure 2. Distribution of nematode communities based on feeding types.

The distribution of free-living nematodes based on feeding types indicates that bacterivores were dominant in all districts (Figure 3). The highest rate was found in Ezine (74.10%), while the lowest was in Biga (49.30%). Fungivores were especially prominent in Biga (45.70%). Predator nematodes had higher rates in Lapseki (7.20%) and Biga (5.00%), whereas omnivores generally had low levels, with the highest rate observed in Bayramiç at 0.70%. Unicellular eukaryote feeders were recorded as 0% in all districts. These distributions indicate that bacterial and fungal activity varies between districts, with possibly greater biodiversity in Lapseki and Biga.

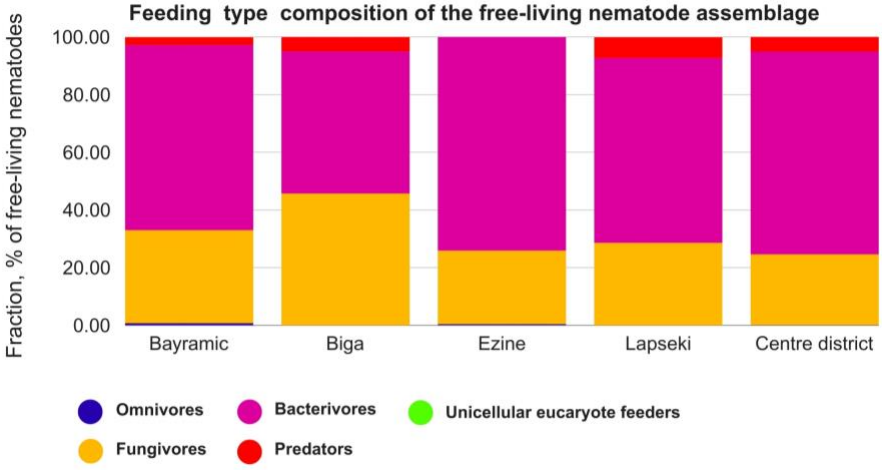

Figure 3. Distribution of free-living nematode communities based on feeding types.

The distribution of plant-parasitic nematodes based on feeding types varies between districts (Figure 4). In Bayramiç, the most dominant group was epidermal/root hair feeders at 32.50%, while in Ezine, it was the ectoparasites group at 43.30%. In Lapseki, ectoparasites and semi-endoparasites were almost equally present (38.80% and 38.09%), whereas in the center district, ectoparasites had the highest proportion at 63.70%. Sedentary parasites were either absent or found in very low proportions in most districts. These results indicate that the feeding strategies of plant-parasitic nematodes vary according to the ecological conditions of each district.

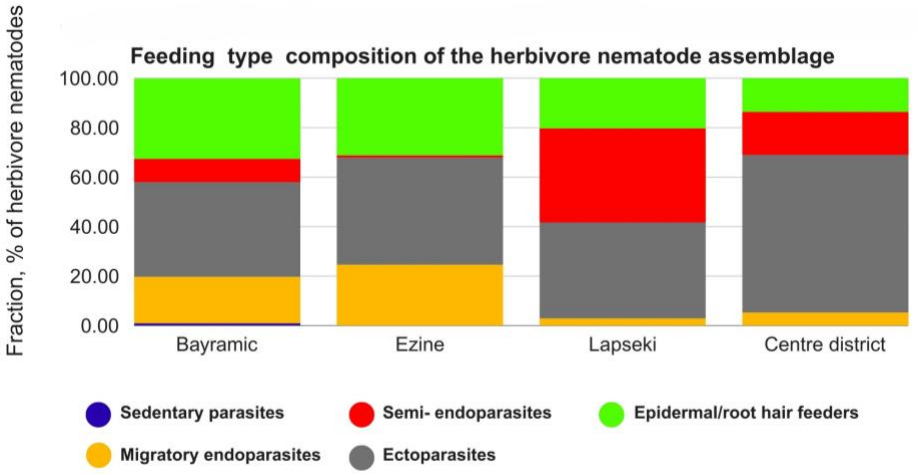

Figure 4. Distribution of plant-parasitic nematode communities based on feeding types.

In the food web analysis, soil samples from different regions (Bayramiç, Biga, Ezine, Lapseki, Center district) were evaluated based on enrichment and structure indices (Ferris et al., 2001). Most points had low enrichment and low structure indices, indicating that the soils in these regions were depleted or degraded in terms of organic matter (Figure 5). However, in some regions, particularly in the center district, Bayramiç, and Lapseki, the points shifted towards the middle and upper right of the graph, indicating more structured and enriched soils, which mean that these soils were more balanced and fertile.

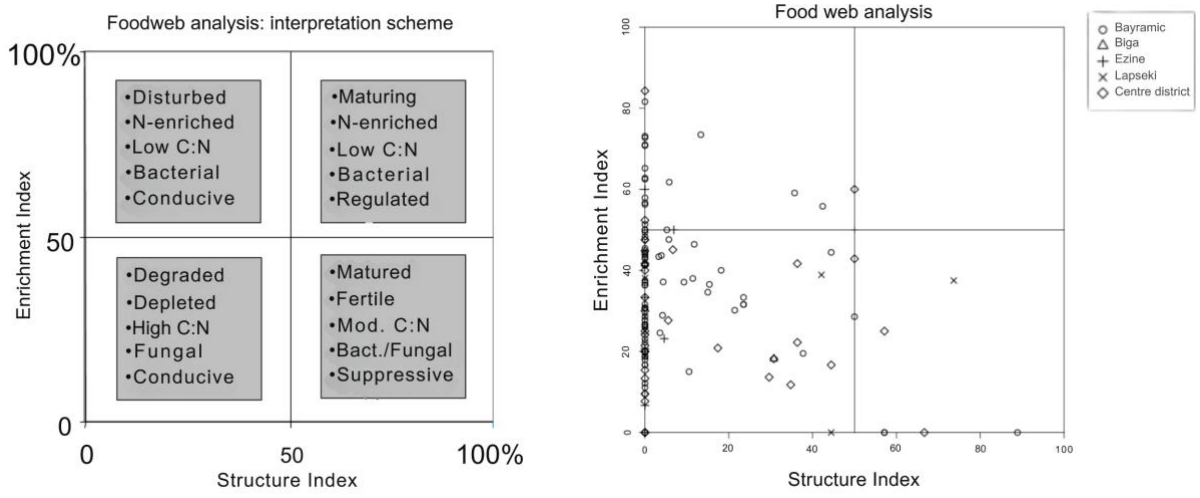

Figure 5. Overall food web analysis of the districts.

Index analysis and classification according to c-p series

According to the Maturity Index (MI) analysis, values showed significant variation between districts (Figure 6). The MI values were found to be 1.98 for Bayramiç, 2.10 for Biga, 1.96 for Ezine, 2.20 for Lapseki, and 2.06 for the center district. MI values between 2-5 reflected the maturity levels of free-living nematode communities more specifically and exclude c-p 1 nematodes (Bongers and Korthals, 1993). The values for Bayramiç were recorded as 2.06, Biga as 2.10, Ezine as 2.01, Lapseki as 2.21, and the center district as 2.11. Sigma MI values were also analyzed, with 2.11 for Bayramiç, 2.10 for Biga, 2.10 for Ezine, 2.32 for Lapseki, and 2.15 for the center district. These data suggest that Lapseki had a more mature ecosystem compared to the other regions. Plant-parasitic Index (PPI) values were also evaluated, with 2.59 for Bayramiç, 2.73 for Ezine, 2.64 for Lapseki, and 2.94 for the center district. These results reflected the plant-parasitic potential of nematode communities, with a particularly higher pressure in the center district (Ferris and Bongers, 2009).

According to the Enrichment Index (EI) analysis results, nutrient enrichment levels varied between districts (Figure 5). Bayramiç had the highest EI value at 37.10, while Biga had 29.92, Ezine 26.73, Lapseki 26.69, and the center district 27.38, indicating lower enrichment levels. These results showed that ecosystems in Bayramiç were characterized by a higher accumulation of organic matter and nutrients compared to other regions. In the Structure Index (SI) analysis, the structural complexity of nematode communities was assessed (Figure 5). The SI value for Bayramiç was relatively low at 7.98, while it was 15.38 for Biga, 1.44 for Ezine, 22.89 for Lapseki, and 14.56 for the center district. Lapseki stood out as the region with the highest structural diversity in this context. These differences could be interpreted as indicators of the stability of regional ecosystems and the complexity of nematode communities.

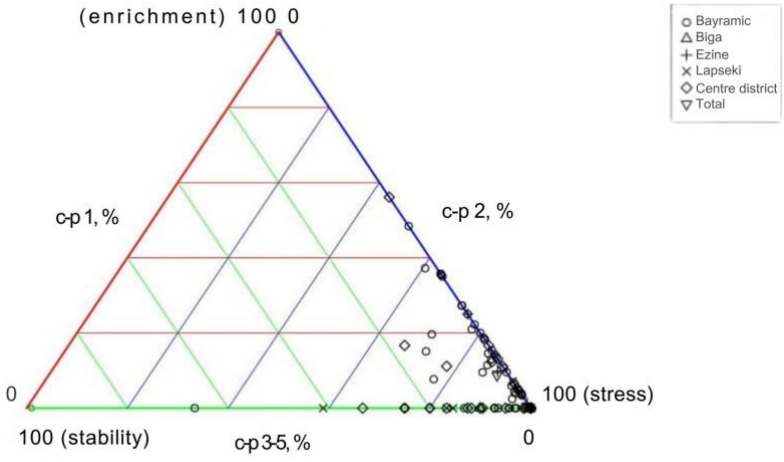

Figure 6. Triangular diagram representing the structure of nematode communities.

This ternary diagram represents the responses of nematodes to environmental conditions (enrichment, stress, stability) according to their life strategies, displayed as a maturity index graph (Figure 6). It also shows the distribution of samples from different regions (Bayramiç, Biga, Ezine, Lapseki, Center district) based on their c-p (colonizer-persister) groups. Most samples were located between c-p 2 and c-p 3-5 groups. Nematodes in the c-p 2 group represent species that thrive in nutrient-rich but more stressful conditions, while the c-p 3-5 group represents species found in soils with lower nutrient levels but lower environmental stress, indicating more stable conditions. Overall, these results reveal that the examined nematode communities had adapted to higher stress conditions and unstable environments, reflecting these characteristics in the soils of these regions.

In the analysis of free-living nematodes based on the colonizer-persister (c-p) structure, the dominance of the c-p 2 group was observed in all districts (Figure 7). The dominance of colonizer species was evident in Bayramiç (88.60%), Biga (95.00%), Ezine (95.30%), Lapseki (91.00%), and the center district (90.00%). Resilient species (c-p 4) were detected at the highest rates in Lapseki (7.80%) and the center district (5.20%). Additionally, c-p 1 species were found above 4% in Ezine and the center district, while in Bayramiç, they were recorded at a higher level of 8% but remained at lower levels in other districts. C-p 3 species were not detected in any district. These results indicate that c-p 2 species dominate ecosystems, although resilient species were also present in some regions.

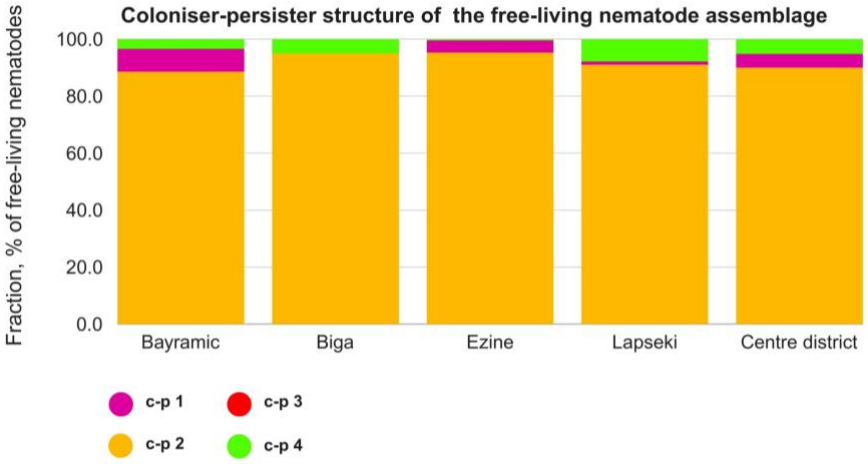

Figure 7. C-p structure of free-living nematode communities.

In the analysis of plant-parasitic nematodes based on their life strategies, the p-p 2 and p-p 3 groups predominantly distributed among the districts (Figure 8). In Bayramiç, the p-p 2 ratio was recorded as 47.00%, p-p 3 as 51.10%, p-p 4 as 0.60%, and p-p 5 as 1.40%. In Ezine, the p-p 2 ratio was 36.40% and the p-p 3 ratio was 63.60%, with no observation of p-p 4 and p-p 5 groups. In Lapseki, the p-p 2 ratio was 23.3% and p-p 3 was 76.70%, with no p-p 4 and p-p 5 groups observed here either. In the center district, the p-p 2 ratio was 27.80%, p-p 3 was 59.10%, p-p 4 was 5.40%, and p-p 5 was 7.70%. Generally, the p-p 3 group was dominant across all districts, with particularly higher proportions of p-p 4 and p-p 5 groups in the center district.

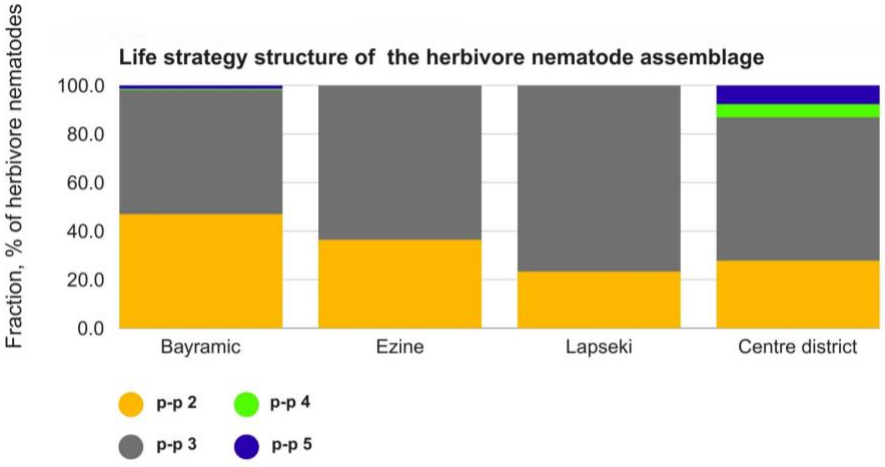

Figure 8. P-p structure of plant-parasitic nematode communities.

Discussion

In this study, a total of 30 nematode genera were identified, with *Pratylenchus* species being predominant, and root-knot nematodes were detected in three samples. In a study by Yüksel et al. (2023), 8 nematode genera were identified in samples from two provinces, with root-knot nematodes found in only one sample. This difference may be attributed to regional environmental conditions, variations in sampling methods, or differences in the number of samples. In the first study conducted in apple orchards in Türkiye by Kepenekçi & Öztürk (2002), 13 genera belonging to the order Tylenchida were identified, while Gözel & Yıldız (2015) reported that root-knot nematodes were found at low levels in apple nurseries in Ödemiş. In contrast, the identification of a greater number of nematode genera in this study suggests that it could provide more information about the distribution of nematode populations. The dominance of the *Pratylenchus* genus in this and other studies emphasizes the need to develop sustainable management strategies against this harmful species in apple orchards.

There are no studies in Türkiye evaluating soil quality through the examination of c-p series in apple orchards. However, a study by Pokharel et al. (2015) in apple orchards in the United States found that the c-p 2 group was predominant among free-living nematodes. This finding aligns with the results obtained in this study, confirming that the c-p 2 group is prevalent in soil ecosystems under stress or degraded soil conditions. Additionally, plant-parasitic nematodes (herbivores) were predominantly of the p-p 3 type. In present study, similar findings of predominant p-p 2 and p-p 3 groups indicate that these groups pose a significant threat to plant health and should be considered in soil management strategies. These findings highlight the importance of using c-p series as bioindicators for evaluating soil quality and suggest that such analyses should be extended through regional studies.

The Plant-parasitic Index (PPI) values recorded in the districts indicate the impact of plant-parasitic nematodes on the soil ecosystem. The moderate PPI values in these regions suggest that soil health is threatened by plant-parasitic nematodes. A similar study conducted in China by Yin et al. (2014) examined the effects of nematode communities on soil health and found that low PPI values did not pose a serious threat to apple orchards. However, the PPI values obtained in this study, especially in the center district (PPI: 2.94), are higher, indicating that regional differences should be considered when developing management strategies for apple orchards (Bongers et al., 1997).

The differences in nematode communities' feeding types among the districts suggest that nematodes contribute differently to bacterial and fungal cycles. Studies examining the contributions of nematodes to bacterial and fungal cycles emphasize that in soils dominated by bacterivores, bacteria play a central role in organic matter decomposition (Ferris et al., 2001). In this study, bacterivores are observed to be dominant. The high proportions of plant-parasitic nematodes in Lapseki and Bayramiç suggest that they could pose a threat to agricultural areas. The high proportions of fungivores in Biga support studies suggesting that fungus-based organic matter cycles are more dominant in soil ecosystems in this region (Neher, 1999). These findings underscore that soil ecosystems vary in biological diversity across districts and that management strategies should be adapted to these differences.

The study reveals the distribution of nematode populations in soil ecosystems and the impact of plant-parasitic nematodes on ecosystem health. The observed differences in nematode communities' feeding types and various index values among districts highlight how regional environmental conditions influence the distribution of nematodes and soil health. The dominance of *Pratylenchus* species and moderate PPI values emphasize the need to develop sustainable management strategies against these harmful species in apple orchards. Since studies on soil quality evaluation based on c-p series in Türkiye are limited, the impact of c-p and p-p groups on nematode fauna should be used as bioindicators. It is recommended to expand agricultural soil management and nematode population monitoring programs, considering regional differences. In regions like the center district, where PPI is high, adopting integrated management strategies specific to the region against harmful plant-parasitic nematodes will contribute to the preservation of soil health.

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Original article (Orijinal araştırma)

Abundance and diversity of natural enemies of aphids (Hemiptera: Aphidomorpha) on different host trees in forest habitats[1]

Orman habitatlarında farklı ağaç türlerindeki yaprak bitlerinin (Hemiptera: Aphidomorpha) doğal düşmanlarının bolluk ve çeşitliliği

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Abstract

Forests are ecosystems with high biological diversity, where various groups of organisms live. In the province of Isparta, during the years 2019-2020, systematic sampling in forest areas yielded data on aphids and their natural enemies. The tree species studied included [*Pinus nigra* Arnold. subsp. *pallasiana* (Lamb.) Holmboe., *Pinus brutia* Ten., *Cedrus libani* A. Rich., *Abies cilicica* (Antoine & Kotschy) Carrière (Pinaceae), *Juniperus* spp. (Cupressaceae), *Quercus* spp. (Fagaceae), and *Robinia pseudoacacia* L. (Leguminosae)]. The study identified 34 aphid species and 42 natural enemy species across 14 host tree species. The distribution of these species among orders was as follows: 54% Coleoptera, 15% Neuroptera, 13% Hemiptera, 13% Hymenoptera, 3% Diptera, and 3% Raphidioptera. The most abundant natural enemy species were *Oenopia lyncea* (Olivier, 1808) (19.0%), *Harmonia quadripunctata* (Pontoppidan, 1763) (11.3%), and *Adalia decempunctata* (L., 1758) (9.2%) in 2019 and *H. quadripunctata* (14.3%), *Coccinula quatuordecimguttata* (L., 1758) (12.5%), and *Hippodamia variegata* (Goeze, 1777) and *Stethorus gilvifrons* (Mulsant, 1850) (8.9%) in 2020. The tree species with the highest number of aphid species were *P. nigra* (15 species) and *C. libani* (13 species). In total, 123 interactions were identified among the 34 aphid species and 42 natural enemy species across 14 host tree species, representing tri-trophic levels.

Keywords: Forest trees, predator, parasitoid, sap-sucker pest

Öz

Ormanlar, çeşitli canlı gruplarının yaşadığı ve biyolojik çeşitliliğin yüksek olduğu ekosistemlerdir. Isparta ilinde 2019-2020 yıllarında orman alanlarında [*Pinus nigra* Arnold. subsp. *pallasiana* (Lamb.) Holmboe., *Pinus brutia* Ten., *Cedrus libani* A. Rich., *Abies cilicica* (Antoine & Kotschy) Carrière (Pinaceae), *Juniperus* spp. (Cupressaceae), *Quercus* spp. (Fagaceae) ve *Robinia pseudoacacia* L. (Leguminosae)] sistematik örnekleme ile yaprak biti ve doğal düşman türleri elde edilmiştir. Çalışmada 14 konukçu ağaç türünde 34 yaprak biti türü ve 42 doğal düşman türü bulunmuştur. Türlerin takımlara göre dağılımı %54'ü Coleoptera, %15'i Neuroptera, %13'ü Hemiptera, %13'ü Hymenoptera, %3'ü Diptera ve %3'ü Raphidioptera şeklindedir. Doğal düşmanlardan en fazla bireye sahip olan türler 2019 yılında *Oenopia lyncea* (Olivier, 1808) (%19,0), *Harmonia quadripunctata* (Pontoppidan, 1763) (%11,3) ve *Adalia decempunctata* (L., 1758) (%9,2), 2020 yılında ise *H. quadripunctata* (%14,3), *Coccinula quatuordecimguttata* (L., 1758) (%12,5), *Hippodamia variegata* (Goeze, 1777) ve *Stethorus gilvifrons* (Mulsant, 1850) (%8,9) olmuştur. Tür sayısı en fazla *P. nigra* (15 tür) ve *C. libani'*de (13 tür) görülmüştür. Çalışmada 14 konukçu ağaç türünde 34 yaprak biti türü 42 doğal düşman türü ile bu türler arasında üçlü trofik düzeyde 123 etkileşim tespit edilmiştir.

Anahtar sözcükler: Orman ağacı, predatör, parazitoit, öz suyu zararlısı

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Introduction

The forest ecosystem includes a diverse array of living creatures that inhabit various ecological conditions and habitats. These organisms sustain their existence in an intricate structure by adapting to different ecological conditions both within their own groups and with other living groups with which they share their space (Brockerhoff et al., 2017). The stronger and more diverse this structure is in terms of biotic factors, the more robust the ecosystem's balance is, and the more resistant it is to biotic and abiotic conditions (Fischbein & Corley, 2022). Yet, when the existing intricate structure in the ecosystem loses its strength due to various reasons such as rapid population growth, increased construction, and habitat degradation, the ecological balance is disrupted (Tscharntke et al., 2002; Hisano et al., 2018). Consequently, especially rare species living in a sensitive habitat may face extinction, while some species with high populations may become the dominant species in their habitat (Aerts & Honnay, 2011; Brockerhoff & Liebhold, 2017). For example, *Harmonia axyridis* (Pallas, 1773) (Coleoptera: Coccinellidae) is a successful species in the control of aphids, but due to its superiority in competition and feeding, it negatively affects the presence of native natural enemy species and is considered an invasive species (Kenis et al., 2008; Pell et al., 2008; Roy & Wajnberg, 2008; Roy & Brown, 2015).

Especially relationships such as competition and nutritional status between living things in the ecosystem can determine the course of the process in the ecosystem. Phytophagous species that increase in population or enter the ecosystem and become dominant (alien invasive species) can cause significant economic or ecological damage. Upon the failure of the population of these pest species to reach equilibrium in a certain period of time, many ecological relationships such as biodiversity and nutrient cycling within the ecosystem, can be negatively affected (Ayres & Lombardero, 2000; Gullan & Cranston, 2012). In recent years, it has been reported that some alien invasive species have a negative impact on the presence of natural enemies due to their pressure on natural habitats (Brockerhoff & Liebhold, 2017). For instance, the aphid species *Adelges tsugae* (Annand, 1928), which was introduced by mistake from Japan to North America, is reported to have reached dense populations on *Tsuga canadensis* (L.) Carr. trees in the Eastern USA since the 1950s (Havill & Montgomery, 2008).

The presence of natural enemies is the most important factor in stabilizing the populations of species whose populations increase and are considered pests. The diversity of natural enemies can suppress pest populations with features such as the increase in species-rich living communities and the formation of different feeding niches. Prey diversity, on the other hand, does not always lead to natural enemy diversity, and the predominance of some polyphagous species may keep natural enemy species diversity low. If the number of prey species is low in a certain area, natural enemy species may feed on the same prey. In addition, the amount of prey consumption of natural enemy species also affects the dominance over prey (Rosenheim et al., 2004; Schmitz, 2009; Krey et al., 2021).

Aphids are species that generally do not cause direct tree death in forest trees but weaken the tree due to feeding. Owing to their high ecological tolerance, they are among the living groups that can reach dense populations in a short time (Dixon, 2012; Wieczorek et al., 2019; Blackman & Eastop, 2024). However, it can be said that the richness of natural enemy species is effective in the lower incidence of aphid damage in natural forests (Oğuzoğlu & Avcı, 2019, 2023) although it occasionally causes significant damage to trees, especially in plantation and monoculture forests (Straw et al., 2005; Kebede & Mulugeta, 2021).

This study aimed to determine predator and parasitoid species of aphids and trophic relations (host plant-aphid-natural enemies) in different forest trees in Isparta.

Materials and Methods

Study areas and design

The study area is Isparta province located in the Mediterranean Region (Figure 1). The elevation of the area varies between 247 and 2985 m. The forest area of Isparta province is approximately 386 thousand hectares. The most widespread forest tree species in Isparta province are *Pinus nigra* Arnold. subsp. *pallasiana* (Lamb.) Holmboe., *Pinus brutia* Ten., *Cedrus libani* A. Rich., *Abies cilicica* (Antoine & Kotschy) Carrière (Pinaceae), *Juniperus* spp. (Cupressaceae), *Quercus* spp. (Fagaceae), and *Robinia pseudoacacia* L. (Leguminosae). Tree species with the largest spatial distribution area are *Juniperus* spp. (97,173.1 hectares) and *P. nigra* subsp. *pallasiana* (66,825.5 hectares) (IOBM, 2020).

Sample areas were selected according to the most widespread tree species. The samples were collected via systematic sampling method in a total of 34 sample areas over 7 months (April-October) in the years 2019-2020 (Table 1).

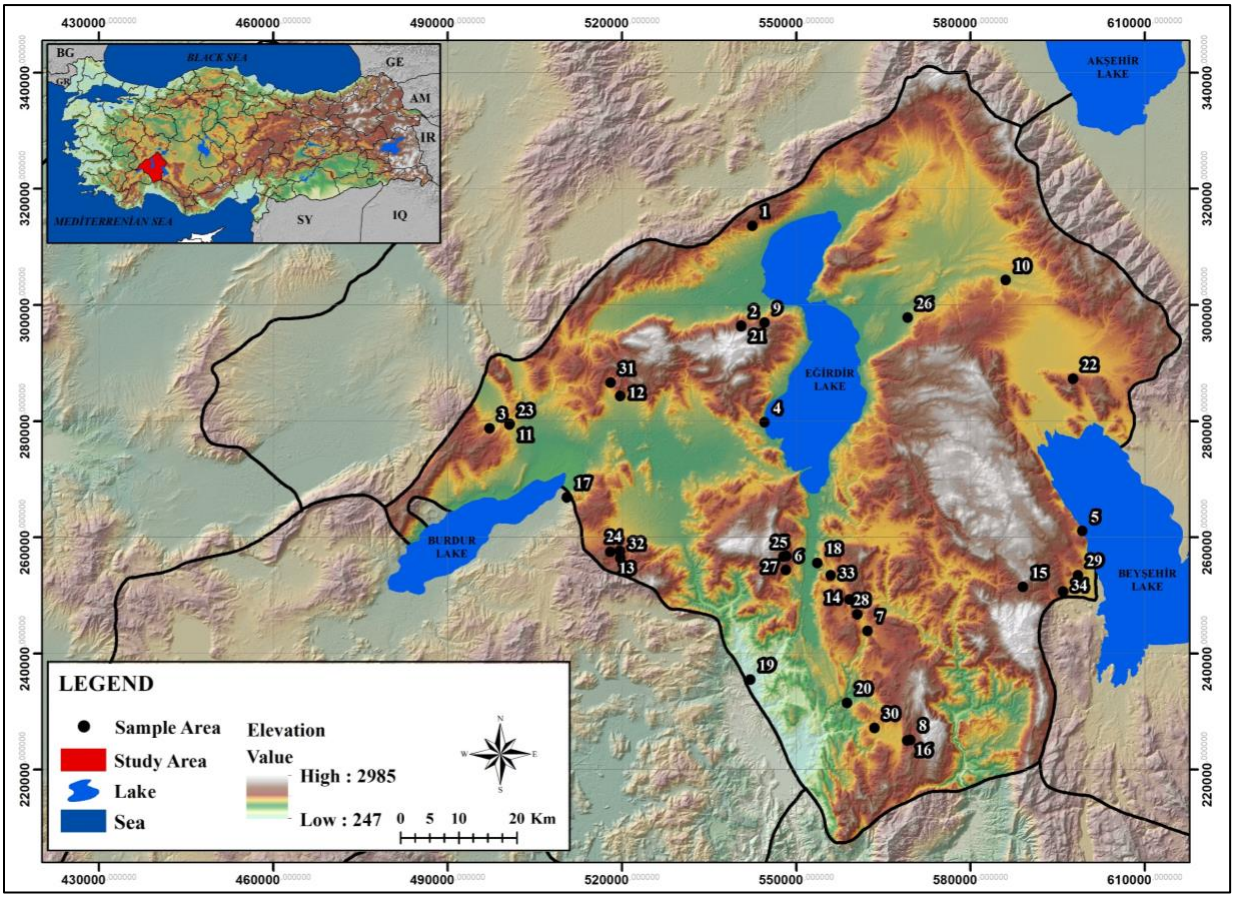

Figure 1. Distribution of sample areas.

Data collection

The circle-shaped sample areas with a radius of nearly 25 m (approximately 2000 m^2) were taken. The shoot (about 30 cm) of the nearest 10 trees in four main directions (North, South, East, and West) was sampled by selecting the central point of each sample area. On each sampling date, samples were collected from 10 trees near the previously sampled trees (Stekolshchikov & Kozlov, 2012). The samples were collected after they were preserved in tubes that contained 70% ethanol. The preparation of the aphids was

done according to the research by Martin (1983). The identification of the specimens followed Blackman & Eastop (2024) and assistance was received from Prof. Dr. Gazi Görür and Dr. Özhan Şenol (Niğde Ömer Halisdemir University, Faculty of Arts and Sciences) for the diagnosis. The species names and synonyms were checked according to the research by Favret (2021). Predator and parasitoid samples were separated into families and species. Those that could not be identified were sent to experts. The samples were stored at the Entomology Museum of the Forestry Faculty at the Isparta University of Applied Sciences.

Table 1. Some properties of sample areas

Areas	Coordinates	Altitude (m)	Dominant tree species
A1	38°15'04"N-30°43'30"E	1364	*Juniperus excelsa, J. oxycedrus*
A2	38°05'54"N-30°42'49"E	1458	*Juniperus excelsa, J. oxycedrus, J. foetidissima*
A3	37°54'27"N-30°13'33"E	1235	*Juniperus excelsa, J. oxycedrus, J. foetidissima*
A4	37°58'24"N-30°46'30"E	971	*Juniperus excelsa, J. oxycedrus*
A5	37°48'29"N-31°24'17"E	1140	*Juniperus excelsa, J. oxycedrus, J. foetidissima*
A6	37°43'13"N-30°49'44"E	1330	*Juniperus excelsa, J. oxycedrus*
A7	37°37'59"N-30°59'43"E	1207	*Juniperus excelsa, J. oxycedrus*
A8	37°28'05"N-31°05'25"E	1324	*Juniperus excelsa, J. oxycedrus*
A9	38°11'16"N-31°13'38"E	1128	*Pinus nigra*
A10	38°06'08"N-30°45'36"E	1320	*Pinus nigra*
A11	37°55'15"N-30°16'21"E	1184	*Pinus nigra*
A12	37°58'32"N-30°29'07"E	1202	*Pinus nigra*
A13	37°43'50"N-30°29'08"E	1415	*Pinus nigra*
A14	37°41'37"N-30°56'51"E	1247	*Pinus nigra*
A15	37°42'45"N-31°17'40"E	1808	*Pinus nigra*
A16	37°28'00"N-31°05'05"E	1314	*Pinus nigra*
A17	37°49'00"N-30°23'37"E	928	*Pinus brutia*
A18	37°44'05"N-30°53'17"E	1113	*Pinus brutia*
A19	37°32'49"N-30°46'16"E	356	*Pinus brutia*
A20	37°31'13"N-30°57'47"E	966	*Pinus brutia*
A21	38°05'52"N-30°42'50"E	1458	*Cedrus libani*
A22	38°02'16"N-31°22'13"E	1471	*Cedrus libani*
A23	37°55'11"N-30°16'19"E	1171	*Cedrus libani*
A24	37°44'07"N-30°30'20"E	1491	*Cedrus libani*
A25	37°44'29"N-30°49'37"E	1558	*Cedrus libani*
A26	38°07'18"N-31°02'28"E	1034	*Quercus ithaburensis, Q. infectoria, Q. cerris, Q. trojana*
A27	37°44'34"N-30°49'43"E	1555	*Quercus vulcanica, Q. trojana*
A28	37°39'31"N-30°58'23"E	1218	*Quercus cerris*
A29	37°44'15"N-31°23'55"E	1181	*Quercus cerris*
A30	37°28'57"N-31°01'09"E	1191	*Quercus cerris, Q. infectoria*
A31	37°59'41"N-30°27'55"E	1230	*Robinia pseudoacacia*
A32	37°43'17"N-30°30'05"E	1418	*Robinia pseudoacacia*
A33	37°42'58"N-30°54'58"E	1167	*Abies cilicica*
A34	37°42'38"N-31°22'16"E	1431	*Abies cilicica*

Results and Discussions

In the study, 197 individuals belonging to 42 species of Coleoptera (Cantharidae and Coccinellidae), Neuroptera (Chrysopidae and Hemerobiidae), Raphidioptera (Raphididae), Hemiptera (Anthocoridae, Reduviidae, and Miridae), Diptera (Sryphidae, and Hymenoptera (Braconidae, Megaspilidae, and Pteromalidae) were obtained (Table 2).

Table 2. The predators and parasitoids species in the study area in 2019 and 2020

Species	2019		2020		Total	
	No	%	No	%	No	%
Coleoptera: Cantharidae						
Boveycantharis tokatensis (Pic, 1898)	1	0.7	0	0.0	1	0.5
Cantharis livida (L., 1758)	1	0.7	0	0.0	1	0.5
Cantharis sp.	1	0.7	0	0.0	1	0.5
Coleoptera: Coccinellidae						
Adalia (*Adalia*) *bipunctata* (L., 1758)	5	3.5	1	1.8	6	3.0
Adalia (*Adalia*) *decempunctata* (L., 1758)	13	9.2	0	0.0	13	6.6
Adalia (*Adalia*) *fasciatopunctata revelierei* Mulsant, 1866	0	0.0	1	1.8	1	0.5
Anatis ocellata (L., 1758)	6	4.2	1	1.8	7	3.6
Chilocorus bipustulatus (L., 1758)	3	2.1	0	0.0	3	1.5
Coccinella septempunctata (L., 1758)	11	7.7	1	1.8	12	6.1
Coccinula quatuordecimguttata (L., 1758)	3	2.1	7	12.5	10	5.1
Exochomus (*Parexochomus*) *nigromaculatus* Goeze, 1777	1	0.7	2	3.6	3	1.5
Exochomus quadripustulatus (L., 1758)	9	6.3	2	3.6	11	5.6
Harmonia axyridis (Pallas, 1773)	2	1.4	2	3.6	4	2.0
Harmonia quadripunctata (Pontoppidan, 1763)	16	11.3	8	14.3	24	12.2
Hippodamia (*Hippodamia*) *variegata* (Goeze, 1777)	7	4.9	5	8.9	12	6.1
Myrrha (*Myrrha*) *octodecimguttata* (L., 1758)	4	2.8	2	3.6	6	3.0
Nephus nigricans Weise, 1879	0	0.0	1	1.8	1	0.5
Oenopia conglobata (L., 1758)	0	0.0	3	5.4	3	1.5
Oenopia lyncea (Olivier, 1808)	27	19.0	2	3.6	29	14.7
Scymnus apetzi Mulsant, 1846	0	0.0	1	1.8	1	0.5
Scymnus (*Scymnus*) *rubromaculatus* (Goeze, 1778)	1	0.7	0	0.0	1	0.5
Scymnus (*Pullus*) *subvillosus* (Goeze, 1777)	4	2.8	0	0.0	4	2.0
Scymnus suturalis Thunberg, 1795	0	0.0	1	1.8	1	0.5
Stethorus gilvifrons (Mulsant, 1850)	2	1.4	5	8.9	7	3.6
Neuroptera: Chrysopidae						
Peyerimhoffina gracilis (Schneider, 1851)	1	0.7	1	1.8	2	1.0
Suarius nanus (McLachlan, 1893)	1	0.7	1	1.8	2	1.0
Chrysoperla carnea (Stephens, 1836)	1	0.7	3	5.4	4	2.0
Neuroptera: Hemerobiidae						
Hemerobius (*Hemerobius*) *micans* Olivier, 1792	2	1.4	0	0.0	2	1.0
Wesmaelius (*Kimminsia*) *mortoni mortoni* (McLachlan, 1899)	1	0.7	0	0.0	1	0.5
Sympherobius (*Sympherobius*) *elegans* (Stephens, 1836)	0	0.0	2	3.6	2	1.0
Raphidioptera: Raphididae						
Raphidia (*Raphidia*) *ambigua* Aspöck & Aspöck, 1964	1	0.7	0	0.0	1	0.5
Hemiptera: Anthocoridae						
Orius (*Heterorius*) *minutus* (L., 1758)	1	0.7	0	0.0	1	0.5
Orius (*Heterorius*) *laticollis* (Reuter, 1884)	0	0.0	1	1.8	1	0.5
Orius niger (Wolff, 1811)	0	0.0	2	3.6	2	1.0
Hemiptera: Reduviidae						
Nagusta goedelii (Kolenati, 1857)	1	0.7	0	0.0	1	0.5
Hemiptera: Miridae						
Deraeocoris sp.	1	0.7	0	0.0	1	0.5
Diptera: Syrphidae						
Eupeodes corollae (Fabricius, 1794)	2	1.4	0	0.0	2	1.0
Hymenoptera: Braconidae						
Aphidius ervi Haliday,1834	3	2.1	0	0.0	3	1.5
Praon volucre (Haliday,1833)	2	1.4	0	0.0	2	1.0
Praon dorsale (Haliday,1833)	1	0.7	1	1.8	2	1.0
Hymenoptera: Megaspilidae						
Dendrocerus sp.	4	2.8	0	0.0	4	2.0
Hymenoptera: Pteromalidae						
Pachyneuron aphidis (Bouché, 1834)	3	2.1	0	0.0	2	1.0
Total	**142**	**100**	**56**	**100**	**197**	**100**

Cantharis sp., *Deraeocoris* sp., and *Dendrocerus* sp. could be identified at the genus level. Five (12%) of the species were parasitoids, and 37 (88%) were predators. The highest number of species was seen in the Coccinellidae family, with 21 species (50%). Looking at the species distribution by year, 34 species were found in 2019 and 19 species in 2020. Fourteen species were detected in both years. The number of individuals varied according to the years, as 142 in 2019 and 56 in 2020. In the analysis of the number of individuals of the species, the highest number of individuals was seen in *Oenopia lyncea* (Olivier, 1808) with 29 individuals*, Harmonia quadripunctata* (Pontoppidan, 1763) with 24 individuals, and *Adalia decempunctata* (L., 1758) with 13 individuals. Parasitoid species made up five of the natural enemies. All of the species belonged to the order Hymenoptera; 3 of them belonged to Braconidae, one to Megaspilidae, and one to Pteromalidae. *Aphidius ervi* Haliday,1834; *Praon dorsale* (Haliday,1833) and *Praon volucre* (Haliday,1833) species were identified from Braconidae family. These species are reported to have a wide prey spectrum. *Dendrocerus sp.* (Mikó et al., 2011; Satar et al., 2014; Apak & Akşit, 2016; Mackauer & Chow, 2016; Nakashima et al., 2016) from the family Megaspilidae and *Pachyneuron aphidis* (Bouché, 1834) from the family Pteromalidae (Kılınçer, 1982; Müller et al., 1997; Chai et al., 2008; Chen et al., 2014) are reported to be hyperparasitoids.

Studies have been conducted to investigate the natural enemy species of aphids in various regions of Türkiye. In Kahramanmaraş province, 11 species of Sryphidae (Aslan & Uygun, 2007), 19 species of Braconidae and Aphelinidae (Aslan et al., 2004), 33 species of Coccinellidae (Aslan & Uygun, 2005) were identified in agricultural and non-agricultural areas. In the same province, 18 aphid species were identified from 13 forest tree species, 17 predators (14 coccinellids and 3 syrphids) and 6 parasitoids were found (Aslan, 2014). The species distribution of the natural enemies of nine aphid groups in popular areas of Konya included 2 species from the Neuroptera group, 2 species from the Hemiptera group, 5 species from the Diptera group, and 16 species from the family Coccinellidae (Şahbaz & Uysal, 2006). In Çanakkale and Balıkesir, a total of 58 natural enemy species, including 21 Coccinellidae, 15 Aphididae, 13 Sryphidae, 4 Forficulidae, 2 Chrysopidae, 1 Nabidae, 1 Anthocoridae, and 1 Miridae, were found in trees, shrubs, and herbaceous plants (Kök et al., 2020). A total of 29 predator species from Coccinellidae (24), Cantharidae (1), Nabidae (1), Miridae (1), Sryphidae (1), and Forficulidae (1) families were found in urban trees in Burdur province (Patlar et al., 2021). Eight coccinelid species feeding on aphids were recorded in the urban areas of Çanakkale (Doğan & Kök, 2023). In the same province, 5 species of Sryphidae related to aphids were identified in Çardak Lagoon (Kök & Kasap, 2023). In a study conducted in *C. libani* forests in and around Isparta, 28 natural enemy species from Coccinellidae (18), Syrphidae (5), Braconidae (2), Pteromalidae (1), Chrysopidae (1), and Raphididae (1) families were reported (Oğuzoğlu & Avcı, 2019). Studies conducted in different locations and plant species reveal that coccinellid species stand out among the natural enemies of aphids.

Coccinellid species were followed by syrphid species in terms of the highest number of species and prevalence. In this study, one species from the family Sryphidae was detected, and species belonging to the Neuroptera and Hemiptera groups were found to be more abundant. The distribution of the number of natural enemy species according to host tree species is given in Figure 2. The highest number of species was observed in *P. nigra* (26%), *Quercus* spp. (23%), and *C. libani* (22%). Fewer natural enemies were found in *A. cilicica* (4%) and *P. brutia* (3%).

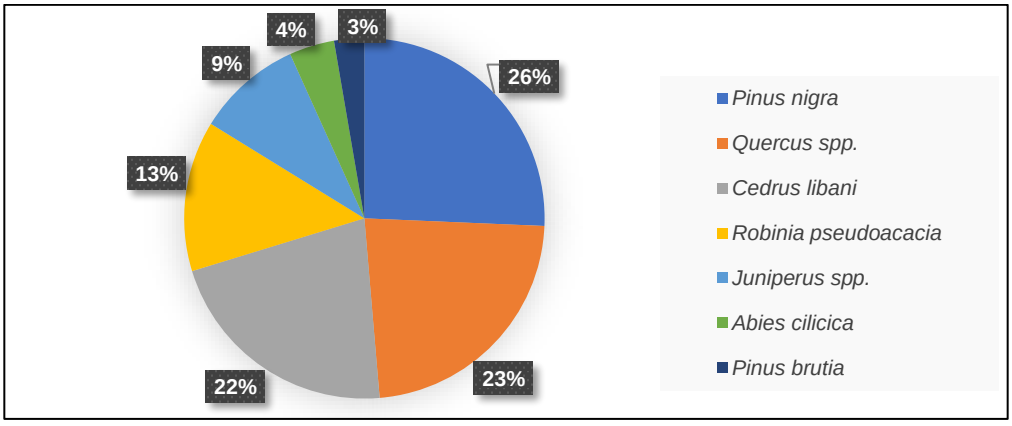

Figure 2. Distribution of natural enemies' species according to host plant trees.

Figure 3 presents the distribution of the number of natural enemies according to aphid species. Accordingly, *Cinara cedri* with 14 species, *Myzocallis boerneri* with 10 species, and *Eulachnus rileyi* with 8 species, had the highest number of natural enemies. The aphid species with the highest number of natural enemies was *C. cedri*. The natural enemy species of *Cinara cedri* were identified as follows: *Adalia bipunctata, Anatis ocellata, Coccinella septempunctata, Exochomus nigromaculatus, E. quadripustulatus, Harmonia axyridis, H. quadripunctata, Hippodamia variegata, Scymnus subvillosus, Stethorus gilvifrons, Chrysoperla carnea, Orius minutus, Nagusta goedelii,* and *Praon volucre*. The natural enemy species of *C. cedri* were identified as *Episyrphus baltaeus* and *Meliscaeva aurallis* (Diptera: Sryphidae) (Aslan & Uygun, 2007), *Pauesia* sp. (Hymenoptera: Braconidae) (Aslan et al., 2004), *Exochomus quadripustulatus* (Coleoptera: Coccinellidae) (Patlar et al., 2021) in Türkiye. It was reported that *Pauesia anatolica* Michelena, Assael & Mendel, 2005 (Hymenoptera: Braconidae) is a parasitoid of *C. cedri* and is found in Adana, Ankara, Antalya, Konya, Karaman, Karaman, Kahramanmaraş, Mersin, Niğde, and Osmaniye provinces (Aytar, 2006). Oğuzoğlu & Avcı (2019) reported that the species with the highest abundance value among the natural enemies of *C. cedri* was *Pauesia anatolica*. However, in this study, *Praon volucre* was found to be the parasitoid of *C. cedri*, but *P. anatolica* was not identified.

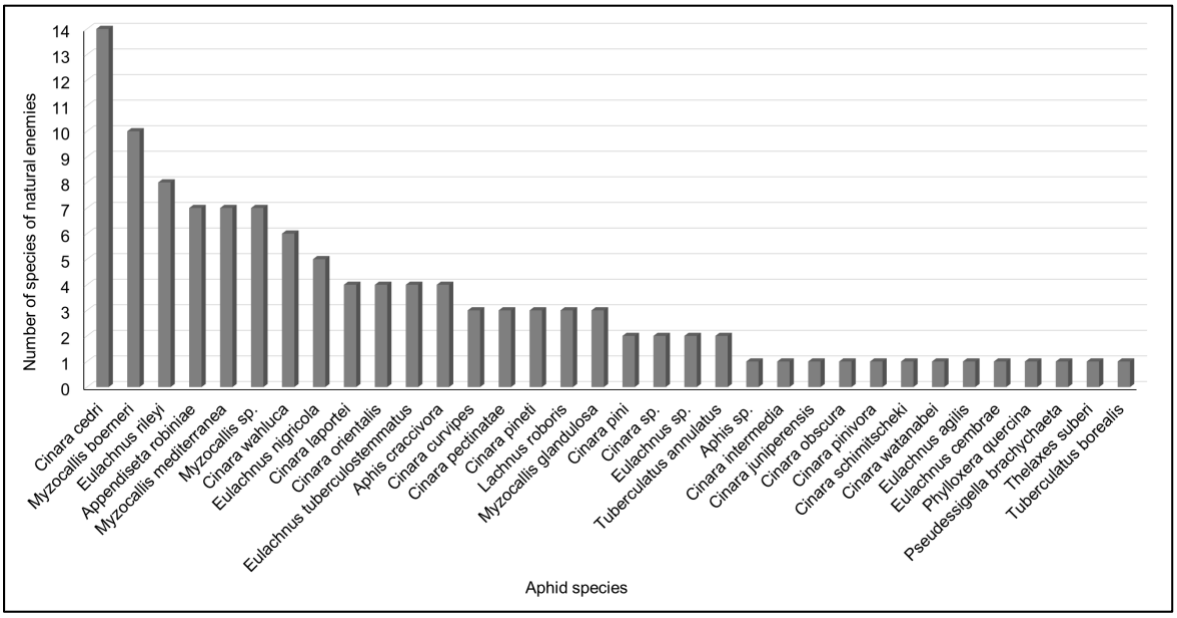

Figure 3. Distribution of number of natural enemies' species according to aphid species.

Figure 4 illustrates the number of natural enemies and aphids in the sampling areas according to the years. In the sampling areas with *Pinus nigra* (A9-A16), *Quercus* spp. (A26-A30), *Cedrus libani* (A21-A25) and *Robinia pseudoacacia* (A31-A32), aphid and natural enemy numbers were high. In the sample areas with *Juniperus* spp. trees (A1-A8), both aphid and natural enemy numbers were low in both years. In the *Abies cilicica* (A33) sampling area, while the number of aphids was low in 2019, it increased in 2020. Natural enemies were found in the same area in 2019, but not in 2020. Over the years, an evaluation shows that the number of aphids and natural enemies decreased in 2020.

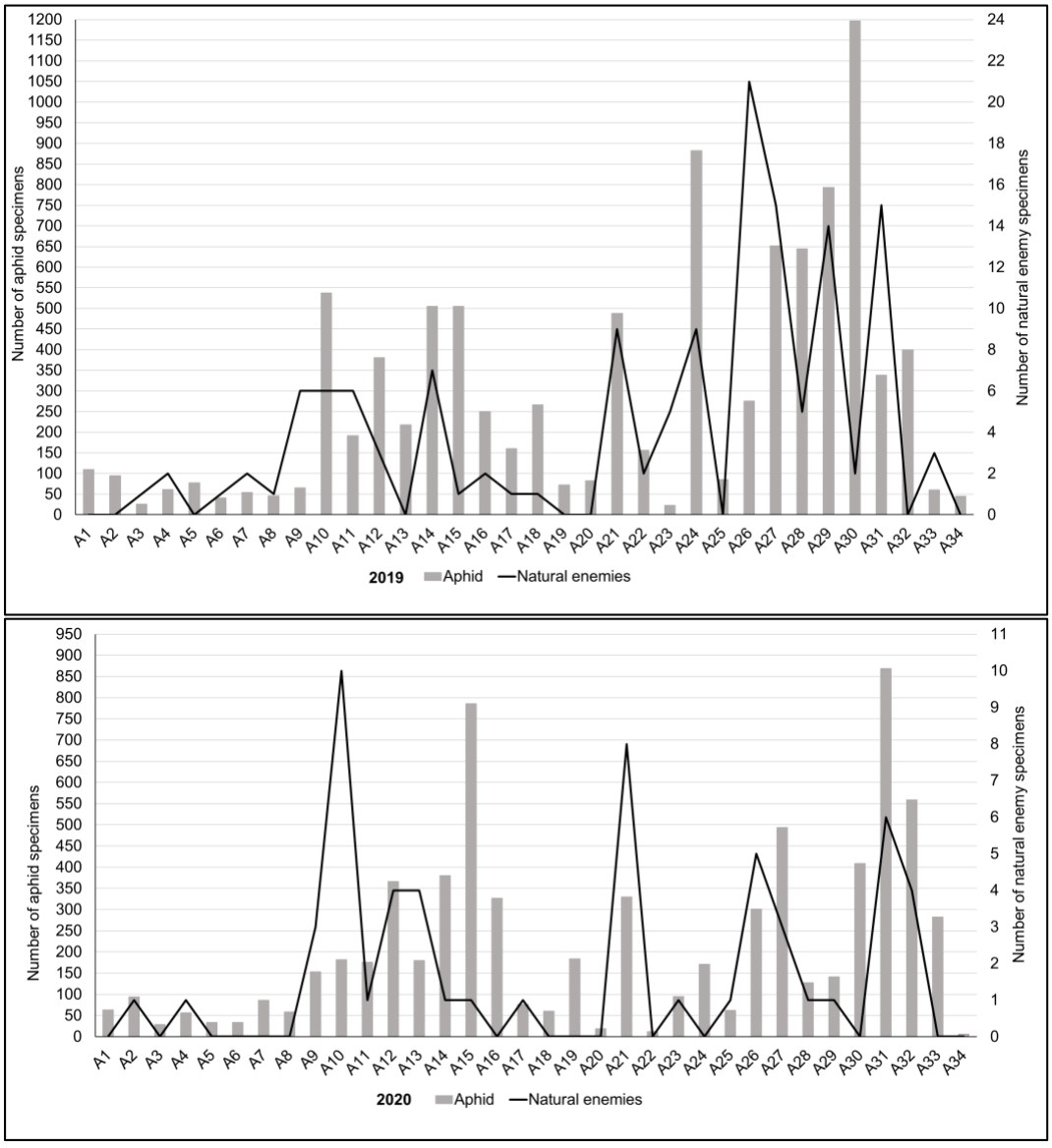

Figure 4. The number of natural enemies and aphids in sample areas (A1-A8: *Juniperus* spp.; A9-A16: *Pinus nigra*; A17-A20: *Pinus brutia*; A21-A25: *Cedrus libani*; A26-A30: *Quercus* spp.; A31-A32: *Robinia pseudoacacia*; A33-A34: *Abies cilicica*).

In the study, 34 aphid species and 42 natural enemy species were found in 14 host tree species, and 123 triple trophic interactions were detected between these species (Table 3). In Türkiye, Kök et al. (2020) reported 43 aphid species and 58 natural enemy species on 58 plant species in Çanakkale-Balıkesir provinces and 173 triple trophic interactions. In a study conducted in urban areas of Burdur province, 48 aphid and 29 predator species were found on 34 plant species (Patlar et al., 2021). Despite the study's

reduction in the overall number of aphid and natural enemy species on the host plant species, it still maintained a significant presence. A random sampling of *Cinara cedri* on *Cedrus libani* trees in Isparta and its environs resulted in identification of 28 natural enemy species (Oğuzoğlu & Avcı, 2019). However, despite the similarity of the study area, the high number of natural enemy species for one host species and one aphid species may be due to the sampling method.

Table 3. The associations of natural enemies-aphid species-host plants

Natural enemies	**Aphid species**	**Host plants**
Boveycantharis tokatensis	*Cinara watanabei*	*Pinus nigra*
Cantharis livida	*Myzocallis boerneri*	*Quercus cerris*
Cantharis sp.	*Myzocallis* sp.	*Quercus ithaburensis*
Adalia bipunctata	*Cinara cedri*	*Cedrus libani*
	Appendiseta robiniae	*Robinia pseudoacacia*
	Tuberculatus borealis	*Quercus cerris*
	Myzocallis sp.	*Quercus vulcanica*
	Myzocallis mediterranea	
Adalia decempunctata	*Myzocallis* sp.	*Quercus cerris*
		Quercus vulcanica
		Quercus infectoria
	Myzocallis mediterranea	*Quercus vulcanica*
	Myzocallis boerneri	*Quercus cerris*
		Quercus vulcanica
	Myzocallis glandulosa	*Quercus vulcanica*
	Tuberculatus annulatus	
Adalia fasciatopunctata revelierei	*Eulachnus* sp.	*Pinus nigra*
Anatis ocellata	*Cinara pectinatae*	*Abies cilicica*
	Cinara cedri	*Cedrus libani*
	Cinara curvipes	
Chilocorus bipustulatus	*Eulachnus rileyi*	*Pinus nigra*
	Eulachnus nigricola	
	Cinara pineti	
Coccinella septempunctata	*Myzocallis* sp.	*Quercus ithaburensis*
	Myzocallis boerneri	*Quercus ithaburensis*
		Quercus cerris
	Cinara laportei	*Cedrus libani*
	Cinara cedri	
	Cinara orientalis	*Pinus nigra*
	Cinara obscura	
	Eulachnus nigricola	*Pinus brutia*
Coccinula quatuordecimguttata	*Eulachnus tuberculostemmatus*	*Pinus nigra*
	Eulachnus cembrae	
	Cinara orientalis	
	Cinara wahluca	*Juniperus foetidissima*
Exochomus (*Parexochomus*) *nigromaculatus*	*Cinara cedri*	*Cedrus libani*
	Eulachnus rileyi	*Pinus nigra*
	Lachnus roboris	*Quercus coccifera*
Exochomus quadripustulatus	*Lachnus roboris*	*Quercus cerris*
	Myzocallis mediterranea	
	Myzocallis boerneri	
	Myzocallis mediterranea	*Quercus ithaburensis*
	Cinara wahluca	*Juniperus excelsa*
	Cinara juniperensis	*Juniperus oxycedrus*
	Cinara cedri	*Cedrus libani*
	Appendiseta robiniae	*Robinia pseudoacacia*
	Aphis craccivora	

Table 3. Continued

Natural enemies	Aphid species	Host plants
Harmonia axyridis	*Cinara cedri*	*Cedrus libani*
	Cinara laportei	
	Appendiseta robiniae	*Robinia pseudoacacia*
	Aphis sp.	
Harmonia quadripunctata	*Cinara pectinatae*	*Abies cilicica*
	Cinara cedri	*Cedrus libani*
	Cinara pineti	*Pinus nigra*
	Cinara orientalis	
	Cinara schimitscheki	
	Eulachnus sp.	
	Eulachnus rileyi	
	Eulachnus tuberculostemmatus	
	Myzocallis mediterranea	*Quercus vulcanica*
Hippodamia (*Hippodamia*) *variegata*	*Cinara cedri*	*Cedrus libani*
	Cinara curvipes	
	Eulachnus tuberculostemmatus	*Pinus nigra*
	Myzocallis sp.	*Quercus ithaburensis*
	Myzocallis boerneri	
	Appendiseta robiniae	*Robinia pseudoacacia*
Myrrha (*Myrrha*) *octodecimguttata*	*Cinara pineti*	*Pinus nigra*
	Cinara orientalis	
	Cinara intermedia	
	Eulachnus agilis	
	Eulachnus rileyi	
Nephus nigricans	*Cinara wahluca*	*Juniperus excelsa*
Oenopia conglobata	*Appendiseta robiniae*	*Robinia pseudoacacia*
	Myzocallis sp.	*Quercus ithaburensis*
Oenopia lyncea	*Myzocallis boerneri*	*Quercus cerris*
		Quercus vulcanica
		Quercus ithaburensis
	Myzocallis mediterranea	*Quercus cerris*
		Quercus vulcanica
	Myzocallis sp.	*Quercus ithaburensis*
		Quercus cerris
	Phylloxera quercina	*Quercus cerris*
	Thelaxes suberi	
	Lachnus roboris	
	Eulachnus rileyi	*Pinus nigra*
Scymnus apetzi	*Eulachnus tuberculostemmatus*	*Pinus nigra*
Scymnus rubromaculatus	*Cinara* sp.	*Juniperus foetidissima*
Scymnus subvillosus	*Aphis craccivora*	*Robinia pseudoacacia*
	Cinara cedri	*Cedrus libani*
Scymnus suturalis	*Myzocallis glandulosa*	*Quercus robur*
Stethorus gilvifrons	*Eulachnus rileyi*	*Pinus nigra*
	Eulachnus nigricola	*Pinus nigra*
	Pseudessigella brachychaeta	*Pinus brutia*
	Myzocallis boerneri	*Quercus vulcanica*
	Cinara cedri	*Cedrus libani*
	Cinara wahluca	*Juniperus excelsa*

Table 3. Continued

Natural enemies	Aphid species	Host plants
Peyerimhoffina gracilis	*Cinara pectinatae*	*Abies cilicica*
	Cinara curvipes	*Cedrus libani*
Suarius nanus	*Cinara wahluca*	*Juniperus excelsa*
	Appendiseta robiniae	*Robinia pseudoacacia*
Chrysoperla carnea	*Cinara cedri*	*Cedrus libani*
Hemerobius micans	*Myzocallis glandulosa*	*Quercus cerris*
	Myzocallis mediterranea	*Quercus ithaburensis*
Wesmaelius mortoni mortoni	*Eulachnus nigricola*	*Pinus nigra*
Sympherobius elegans	*Eulachnus rileyi*	*Pinus nigra*
	Cinara laportei	*Cedrus libani*
Raphidia ambigua	*Myzocallis boerneri*	*Quercus ithaburensis*
Orius minutus	*Cinara cedri*	*Cedrus libani*
Orius niger	*Cinara laportei*	*Cedrus libani*
Orius laticollis	*Appendiseta robiniae*	*Robinia pseudoacacia*
Nagusta goedelii	*Cinara cedri*	*Cedrus libani*
Deraeocoris sp.	*Eulachnus nigricola*	*Pinus nigra*
Eupeodes corollae	*Cinara pini*	*Pinus nigra*
	Myzocallis boerneri	*Quercus cerris*
Aphidius ervi	*Aphis craccivora*	*Robinia pseudoacacia*
	Cinara sp.	*Pinus nigra*
Praon volucre	*Cinara cedri*	*Cedrus libani*
Praon dorsale	*Cinara pini*	*Pinus nigra*
	Aphis craccivora	*Robinia pseudoacacia*
Dendrocerus sp.	*Cinara wahluca*	*Juniperus oxycedrus*
		Juniperus excelsa
	Cinara sp.	*Pinus nigra*
Pachyneuron aphidis	*Cinara pinivora*	*Pinus nigra*

In the study, *Adalia bipunctata, Coccinella septempunctata*, *Adalia fasciatopunctata revelierei*, *Scymnus subvillosus, Stethorus gilvifrons*, *Chrysoperla carnea, and Orius niger* species were generally *found on Cedrus*, *Pinus* and *Quercus* trees. *Pinus nigra* and *Quercus* species were the tree species with the highest diversity of natural enemies. *Adalia decempunctata* and *Oenopia lyncea* were generally found on the *Quercus* species. Düzgüneş et al. (1980) found *Exochomus quadripustulatus* (*Myzocallis* sp., *Lachnus* sp, *L. roboris*, and *Tuberculatus* sp.), *Adalia decempunctata* (*Myzocallis* sp.), *Coccinella septempunctata* (*E. rileyi* and *E. nigricola*), *Coccinella quattuordecimpustulata* (*L. roboris* and *Tuberculatus* sp.), *Harmonia quadripunctata* (*E. rileyi*, *Cinara schimitscheki*), *Myzia oblongoguttata*, *Hyperaspis pseudopustulata* and *Myrrha octodecimguttata* (*E. rileyi* and *C. schimitscheki*), *Hyperaspis quadrimaculata* (*E. nigricola*), *Scymnus auritus* (*Myzocallis* sp., *L. roboris* and *Tuberculatus* sp.), *Scymnus subvillosus* (*E. rileyi* and *C. schimitscheki*), and *Pauesia unilachni* (*S. pineti*). Usta & Keskin (1992) reported that *Hemorobis micans* (Neuroptera: Hemerobiidae) was the predator of *Cinara cedri*.

Harmonia axyridis and *Anatis ocellata* species have been identified in our country in recent years (Aysal & Kıvan, 2014; Oğuzoğlu et al., 2017; Oğuzoğlu & Avcı, 2019). *H. axyridis*, a species originating from the Far East, was seen for the first time in 2014, and its distribution has been determined in many provinces until today (Karataş et al., 2021). The prey of *H. axyridis* in Türkiye are *Cinara curvipes* (Görür et al., 2015), *Macrosiphum rosae* (Öztemiz & Yayla, 2018), *Cinara cedri* (Oğuzoğlu & Avcı, 2019), *Aphis spiraecola, Myzus cerasi, Cinara tujafilina,* and *Liosomaphis berberidis* (Kök et al., 2020), and *Cavariella aegopodii* (Patlar et al., 2021). This species was associated with *Cinara cedri, Cinara laportei*, *Appendiseta robiniae* and *Aphis* sp. in *Cedrus libani*, and *Robinia pseudoacacia* trees. *A. robiniae* is also among the

newly recorded species in the fauna of Türkiye (Oğuzoğlu et al., 2023). This species was found to be the prey of *Adalia bipunctata*, *Suarius nanus, Hippodamia variegata, Oenopia conglobate, and Orius laticollis* species besides *H. axyridis*. The first predator records of *A. robiniae* in Türkiye are presented in this study. *Anatis ocellata* was found to be a predator of *Cinara cedri* and C*. curvipes* on *Cedrus libani* trees and *C. pectinatae* on *Abies cilicica* trees. *A. ocellata* was found for the first time in our country on *Cedrus libani* and *Pinus nigra* trees in Isparta and Bilecik provinces (Oğuzoğlu et al., 2017). It was reported to be a predator of *Cinara cedri* in Isparta province (Oğuzoğlu & Avcı, 2019). In this study, *A. ocellata* was detected for the first time in *A. cilicica*, an endemic forest tree species of our country.

Aphidius ervi was reported to be a parasitoid of *Acyrthosiphon pisum*, *Rhopalosiphum padi*, *Aulacorthum solani* and *Macrosiphum euphorbiae* in our country (Say, 2019; Kök et al., 2020). This species, known to be a parasitoid of many species (Kavallieratos et al., 2004; Güz & Kılınçer, 2005), was obtained from *Aphis craccivora* on *Robinia pseudoacacia* and *Cinara* sp. on *Pinus nigra* in this study. *Praon volucre*, the other parasitoid species detected in the study, is reported to be a parasitoid of *Aphis fabae*, *A. craccivora*, *A. spiraecola*, *Acyrtosiphum pisum*, *Brachycaudus cardui*, *Cinara pinea*, *C. tujafilina*, *Macrosiphum rosae,* and *Uroleucon erigeronense* (Wiacowska et al., 2001; Kavallieratos et al., 2004; Güz & Kılınçer, 2005; Güleç, 2011; Say, 2019). This study determined that *Cinara cedri* was parasitized in *Cedrus libani*. *Praon dorsale*, one of the parasitoid species, was found to be associated with *Cinara pini* in *Pinus nigra* and *Aphis craccivora* in *Robinia pseudoacacia*. *P. dorsale* has been reported to be a parasitoid of many aphid species, such as *Cinara juniperi* and *Metopolophium dirhodum* (Jaskiewicz, 2003; Güz & Kılınçer, 2005).

Dendrocerus sp. of the Megasiphilidae family and *Pachyneuron aphidis* of the Pteromalidae family are generally known as hyperparasitoid species (Kılınçer, 1982; Chen et al., 2014; Satar et al., 2014). In Türkiye, *P. aphidis* was reported to be the hyperparasitoid of *Aphis pomi* on apple trees (Aslan & Karaca, 2005), *Brevicoryne brassicae* on cabbage plants (Kılınçer, 1982), and *Cryptomyzus ribis* on blackcurrants (Alaoğlu, 1994). In this study, *Dendrocerus* sp. was associated with *Juniperus oxycedrus*, *J. excelsa*, *Cinara* sp., and *C. wahluca* on *P. nigra,* and *P. aphidis* was associated with *Cinara pinivora* on *P. nigra*.

As with living communities or individuals, there are different interactions, such as predation-feeding, competition, and mutualism between aphids and related species. In aphids, some species feed on a single plant species, while others feed on many plants. Host preference and distribution of aphids are influenced by the plant species composition and diversity in the habitat, and natural enemies can also find their prey in the presence of host plants (Brodeur & Rosenheim, 2000). This study revealed interactions at the triple trophic level (host plant-aphid-natural enemy).

As there is a difference in the number of aphid species distributed across tree species, the presence of natural enemies also varies. In this study, the abundance of aphid and natural enemy was high in *Quercus* spp. and *Pinus nigra* forests, but low in *Juniperus* spp. sampling areas.

Detection and monitoring of pest species and biological control of these species with appropriate organisms are important for protecting and conserving ecological balance in forested areas. Furthermore, aphids are the food source for many organisms, such as Coleoptera, Diptera, and Hymenoptera, and contribute to biodiversity. The presence of these species will ensure the continuity of forest health and biodiversity and protect the biological balance. Conducted in different forest habitats of Isparta province, the study discussed host tree-aphid-natural enemy relationships and revealed the diversity of natural enemy species.

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Original article (Orijinal araştırma)

Investigation of the dissipation kinetics of lufenuron in pepper grown under field conditions

Tarla koşullarında yetiştirilen biberlerde lufenuron'un parçalanma kinetiğinin araştırılması

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Abstract

Pepper, *Capsicum annuum* L. (Solanales: Solanaceae) production is widely cultivated worldwide, with Türkiye ranks third in global pepper production. However, pests in pepper cultivation often necessitate pesticide use, leading to concerns about pesticide residue levels and their potential impact on food safety. This study investigated the dissipation behavior of lufenuron in pepper under field conditions in Tokat, Türkiye in 2022. Liquid chromatography-tandem mass spectrometry coupled with the quick, easy, cheap, effective, rugged, and safe technique was used to analyze residues of lufenuron in pepper. The average recoveries varied from 77% to 97%, with relative standard deviations of 13% for lufenuron. Lufenuron residues administered as recommended dose and double dose degraded in pepper following a first-order kinetic model, with an estimated half-life ($t_{1/2}$) of 4.33 and 6.42 days in Tokat, 6.80 and 7.45 days in Niksar, respectively. Furthermore, a health risk assessment was conducted, which showed that the chronic risk quotient for lufenuron was much lower than 1. The present results indicated that the health risks posed for consumers by the lufenuron residues were negligible at the recommended dosages.

Keywords: Chronic risk, degradation kinetics, method verification, QuEChERS, pesticide residue

Öz

Biber, *Capsicum annuum* L. (Solanales: Solanaceae), dünyada üretimi son derece yaygın olan bir kültür bitkisi olup, biber üretiminde Türkiye global ölçekte üçüncü sırada yer almaktadır. Biber yetiştiriciliğinde üretimi sınırlayan zararlılar genellikle pestisit kullanımını gerekli kılmakta, bu durum pestisit kalıntıları ve gıda güvenliği ile ilgili endişelere yol açmaktadır. Bu çalışmada, 2022 yılında Türkiye'nin Tokat ilinde tarla koşullarında biberde lufenuron'un parçalanma davranışı araştırılmıştır. Analizler hızlı, kolay, ucuz, etkili, sağlam ve güvenilir bir yöntem olan QuEChERS tekniği ile sıvı kromatografisi-tandem kütle spektrometresi kullanılarak gerçekleştirilmiştir. Lufenuron için ortalama geri kazanım oranları %77 ile %97 arasında değişirken, bağıl standart sapma değeri %13 olarak kaydedilmiştir. Lufenuron kalıntıları, tek doz ve çift doz uygulamaları sonrasında, biberde birinci dereceden kinetik modele uygun olarak bozunmuş ve yarılanma ömrü Tokat'ta sırasıyla 4,33 ve 6,42 gün, Niksar'da ise 6,80 ve 7,45 gün olarak hesaplanmıştır. Ayrıca yapılan sağlık risk değerlendirmesi yapılmış ve lufenuron için akut ve kronik risk katsayı değerleri 1'den çok daha düşük olarak belirlenmiştir. Ayrıca yapılan sağlık risk değerlendirmesi sonucunda lufenuron için kronik risk katsayısının 1'den çok daha düşük olduğu görülmüştür. Elde edilen sonuçlara göre, önerilen dozlarda lufenuron kullanılmasının tüketiciler için herhangi bir sağlık riski oluşturmadığı ortaya konulmuştur.

Anahtar sözcükler: Kronik risk, parçalanma kinetiği, metot doğrulama, QuEChERS, pestisit kalıntısı

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Introduction

The pepper, *Capsicum annuum* L. (Solanales: Solanaceae), native to Central and South America (Mexico, Chile, Peru), is a significant crop with numerous varieties cultivated in many countries (Yıldırım & Çiftçi, 2022). Pepper is a vegetable species with vitamin content rich in human nutrition (Çınar, 2022). Green sweet bell peppers are rich in vitamins A, B1, B2, and C, and elements such as P and K, and alkaloids called capsaicin. Pepper is among the plants most resistant to drought stress due to its large leaf surface, high stoma conductivity and superficial root system (Tezcan & Kaman, 2018). Türkiye is a suitable country for growing peppers and is the world's third-largest producer of peppers (Altuntas et al., 2021; FAOSTAT, 2023). In Türkiye, peppers are consumed freshly and processed into various forms such as canned products, paste, pickles, sauces, roasted peppers, crushed peppers, and powdered peppers (Şeker, 2018). According to Turkish Statistical Institute data, Türkiye's pepper production amounts to 3018 ktons, with 172 tons exported (TUIK, 2022, 2024a).

Pests in pepper cultivation cause significant losses when conditions are favorable. Farmers typically prefer to use pesticides to control these pests. Although these toxic chemicals are effective in protecting the product, they have environmental impacts and potential risks to human health due to the residue. In this context, research on the dissipation kinetics, half-lives, and pre-harvest interval (PHI) of pesticides used during the crop growth period is of great importance (Li et al., 2020; Vijay et al., 2024).

Excessive pesticide use may lead to residue levels surpassing European Union (EU) or international Maximum Residue Levels (MRLs). The pesticide dissipation rate is a key indicator for assessing residue dynamics after application. Additionally, residue dissipation kinetics provide a scientific basis for estimating the timeframe required for pesticide levels to decline below established MRLs. Therefore, commercial pesticide formulations specify a PHI, defining the required time between the final pesticide application and harvest to ensure residue levels comply with safety standards. However, PHI is not a fixed value and may vary based on ecological parameters (MacLachlan & Hamilton, 2010). Therefore, to set appropriate PHI standards for maintaining food safety and promoting sustainable agricultural practices, pesticide residues should be evaluated under the actual climatic conditions where the pesticide is used (Bletsou et al., 2013; Cheng et al., 2022).

In Türkiye, there are 26 insecticides licensed for pepper pests, one of which is lufenuron (21 formulations). Lufenuron controls *Dialeurodes citri* (Ashmead, 1885) (Hemiptera: Aleyrodidae), rust mites, and manages Lepidoptera and Coleoptera larvae on cotton, grains, and vegetables (Tomlin, 2004). This compound is also utilized for the control of the cotton leafworm, *Spodoptera littoralis* (Boisduval, 1833) (Lepidoptera: Noctuidae), in *Capsicum* species. Lufenuron, as a member of the benzoylurea insecticide group, is an effective insect growth regulator used to destroy pests by inhibiting insect molting via acting on chitin synthesis (Rachid et al., 2008). The mechanism of action in insects has been reported by Insecticide Resistance Action Committee as "Inhibitors of chitin biosynthesis affecting CHS1" (IRAC, 2024).

The Joint FAO/WHO Expert Committee on Food Additives (JECFA) has concluded that setting an acute reference dose (ARfD) for lufenuron is not required, indicating a low risk of acute toxicity from short-term dietary exposure. JECFA has established an acceptable daily intake (ADI) for lufenuron at 0-0.02 mg kg^{-1} body weight, based on the no observed adverse effect level (NOAEL) from chronic studies and applying appropriate uncertainty factors. Chronic exposure studies on lufenuron have revealed potential adverse effects at higher doses. Higher doses resulted in tonic-clonic seizures and histopathological changes in the lungs, liver, gastrointestinal tract, and urinary tract (WHO, 2006). Lufenuron causes profound histological and histochemical damage in mammals (Farrag & Shalby, 2007).

Food safety policies are progressively influencing research into developing and applying advanced methodologies for quantitatively determining pesticide residues (Xu et al., 2022). High-sensitivity methods

provide significant advantages in detecting residues at trace levels, which is essential for meeting global pesticide residue standards. In this study, lufenuron detection was conducted using the QuEChERS-LC-MS/MS methodology.

Each pesticide has unique properties. The fate of pesticides after application depends on their chemical and physical characteristics. The residue levels of a pesticide are influenced by its chemistry and environmental factors such as temperature, rainfall, and soil pH. Investigating the fate of pesticide residue in crops is crucial to preventing their adverse effects (Osman et al., 2010). Therefore, rigorous surveillance of pesticide residues in agricultural environments and accurately assessing the optimal harvest period are critical for maintaining food safety and minimizing potential health risks (Cheng et al., 2022). Despite few studies have been conducted on the dissipation kinetics of lufenuron (Khay et al., 2008; Malhat et al., 2012; Bletsou et al., 2013; Cheng et al., 2022; Li et al., 2022; Feng et al., 2022; Álvarez-Vilca et al., 2023; Tang et al., 2023; Vijay et al., 2024), no published data exist regarding its fate in sweet bell peppers. This study examines the dissipation behavior of lufenuron in peppers cultivated under field conditions in Türkiye. Furthermore, Turkish consumers' health risks associated with lufenuron residues were assessed.

Materials and Methods

Chemicals and reagents

The pesticide reference material of lufenuron (with 98.51% purity) was procured from Dr. Ehrenstorfer GmbH in Augsburg, Germany. The commercial formulation of lufenuron, an emulsion concentrate containing 50 g/l, was obtained from Hektaş in Kocaeli, Türkiye. Chemicals such as acetonitrile, methanol, anhydrous magnesium sulfate, anhydrous sodium acetate, ammonium formate with purity over 99.0%, and acetic acid were supplied by Merck in Darmstadt, Germany. Additionally, PSA (Primary Secondary Amine) with a particle size of 40 µm was provided by Supelco Analytical in Bellefonte, PA, USA.

Field trials

Field studies were conducted at the Agricultural Applications and Research Center of Gaziosmanpaşa University and Niksar, Türkiye in 2022. The Anemon F1 pepper variety was used, and the total experimental area spanned 500 m², with plants spaced 40 cm apart within rows and 1.4 m apart between rows. Pepper plants were grown without pesticide applications, following recommended agronomic practices. Drip irrigation was used to cultivate experimental plants. Pesticides were applied according to established guidelines for 'On data requirements for setting maximum residue levels, comparability of residue trials and extrapolation of residue data on products from plant and animal origin' (SANTE, 2019). The pesticide was applied using a knapsack sprayer at concentrations of 30 mL da^{-1} (recommended dose, RD) and 60 mL da^{-1} (double dose, DD). A randomized block design with three replications was used, with each plot containing 20 pepper plants. Pepper samples were harvested and analyzed 24 hours before the application of the pesticide confirming the absence of residues. Spraying occurred at the early fruit ripening stage, one week before the expected harvest. During the study, Tokat recorded an average relative humidity of 69.9% (ranging from 65.0% to 74.7%) and an average temperature of 24.5°C (ranging from 22.8°C to 26.9°C), while Niksar had an average relative humidity of 58.2% (ranging from 50.1% to 63.5%) and an average temperature of 25.6°C (ranging from 23.6°C to 27.4°C). There was no precipitation during the study period.

Sample collection and storage

Pepper samples were collected according to the Commission's 2002/63/EC regulation, which outlines the protocols for the formal sampling of pesticide residues in plant and animal products. Samples, each weighing approximately 1 kg (EC, 2002), were gathered at specific intervals: 0 day (2 hours post-spraying, zero-time sample), 1st, 3rd, 7th, 10th, and 14th day after pesticide application (OECD, 2021). To mitigate contamination risks during harvesting, disposable latex gloves and polyethylene bags were utilized. Upon

collection, the samples were rapidly transported to the laboratory and analyzed immediately. The QuEChERS AOAC Method 2007.01 was applied to the extraction and clean-up procedures (Lehotay, 2007).

Sample preparation, extraction, and clean-up

The extraction and clean-up steps were performed according to the QuEChERS AOAC Method 2007.01, as described by Association of Official Agricultural Chemists (AOAC, 2007). During the QuEChERS extraction, 6 g of anhydrous magnesium sulfate and 1.5 g of anhydrous sodium acetate were used. For the clean-up step, 1.2 g of magnesium sulfate and 0.4 mg of PSA were utilized. A 4-blade blender (Groupe SEB, France) was used to homogenize the pepper samples. Lufenuron residue analyses of peppers were conducted in triplicate using LC-MS/MS.

Analytical instruments and conditions

The analyses were conducted using a Shimadzu® LC-MS 8050 system, renowned for its advanced UPLC and MS/MS capabilities. Chromatographic separation was executed on a Synergi Fusion-RP 100A HPLC column (2.0 mm x 50 mm, 2.5 µm particle size) from Phenomenex™ (California, USA). The mobile phase comprised 10 mmol L^{-1} ammonium formate in distilled water (A) and methanol (B). The mobile phase gradient initiated at 70% B, ramped up to 95% B over 10 minutes, returned to 40% B at 10.01 minutes, and was maintained at 40% B from 10.01 to 12.0 minutes. Each sample injection volume was precisely 10 µL. The mobile phase flow rate was consistently maintained at 0.4 mL min^{-1}, with the column temperature regulated at 40°C. LabSolution® software (version 5.118) was used to precisely manage all instrument parameters.

Method verification

The analytical method was subject to rigorous in-house verification following European SANTE parameters, which cover a variety of critical metrics such as linearity, mean recovery, limit of determination (LOD), limit of quantitation (LOQ), accuracy, precision (repeability and reproducibility in the laboratory) and measurement uncertainty (SANTE, 2021). Linearity was evaluated using matrix-matched calibration standards, with concentrations ranging from 5 to 200 µg kg^{-1}. The recovery of lufenuron from the matrix was assessed by analyzing blank samples that were fortified at three concentration levels (10, 50, and 100 µg kg^{-1}).

Statistical analysis

The dissipation kinetics of pesticides in pepper over a period were characterized by a single first-order kinetic model. Determining half-life ($t_{1/2}$) has been executed according to the following equations 1 and 2 (EPA, 2015).

$$C_t = C_0 \times e^{(-kt)} \quad (1)$$

$$t_{1/2} = \ln 2/k \quad (2)$$

where, C_0 is the initial concentration of pesticide residues obtained from field experiments, while Ct is the pesticide residue concentration at a given time, k is the dissipation coefficient, $t_{1/2}$ is the time interval required for the pesticide residue concentration to decline to half of its initial value (C_0) after application.

In assessing the acute and chronic risks, the estimated dietary exposure was compared to ARfD, expressed in mg kg^{-1} bw day^{-1} and ADI, expressed in mg kg^{-1} bw day^{-1}. ADI values were set at 0.015 mg kg^{-1} bw day^{-1} and ARfD (None allocated) (IUPAC, 2024). The acute hazard quantity (HQ_a), which represents an acute or short-term consumer health risk, is calculated by dividing the estimated short-term intake (ESTI, expressed in mg kg^{-1} day^{-1}) by the ARfD. On the other hand, chronic hazard quantities (HQ_c), which pose chronic or long-term consumer health risks, were determined by dividing the estimated daily intake (EDI is expressed in mg kg^{-1} $days^{-1}$) by ADI (EFSA, 2015). For the Turkish population, the average adult body weight was assumed to be 73.7 kg (TUIK, 2024b; Yelaldı et al., 2024), with a reported daily pepper

consumption (FC) of 0.077 kg per person (TUIK, 2022). Additionally, median pesticide residue (MR) and high pesticide residue (HR) observed for 7, 10 and 14 days (mg kg^{-1}) was taken into account. The following formulas were used for these calculations.

$$ESTI=HR\times FC/body\ weight \quad (3)$$

$$HQ_a=ESTI/ARfD \quad (4)$$

$$EDI=MR\times FC/body\ weight \quad (5)$$

$$HQ_c=EDI/ADI \quad (6)$$

HQ_a and HQ_c values exceeding 1 were categorized as indicative of unacceptable risk. Higher values were associated with elevated levels of risk.

Result and Discussion

Method verification

Matrix-matched calibration solutions with the concentrations of 5, 10, 25, 50, 100, and 200 µg L^{-1} were rigorously prepared and analyzed as triplicate by Liquid chromatography-tandem mass spectrometry. The calibration curve exhibited a linearity by a coefficient of correlation (R^2) exceeding 0.997. LODs and LOQs of 2.26 and 7.55 µg kg^{-1}, respectively have been determined to be lower than the MRLs set by the EU for pepper (0.8 mg kg^{-1}) (EU-MRL, 2024). The mean recovery ranged from 77.14% to 97.23%, with a maximum relative standard deviation (RSD) of 13.45% (Table 1). The expanded measurement uncertainty (U') is 36.96% and does not exceed the default value (50%) (SANTE, 2021). Recovery results across all spiking levels confirmed compliance with the method's performance criteria for pesticide residue analysis, thereby reaffirming its reliability and efficacy.

Table 1. Method optimization and verification data of lufenuron

Precursor ion m/z	Product ions, m/z (CE, eV)	RT (min)	Linear regression equation	Correlation coefficient (r^2)	LOD (µg kg^{-1})	LOQ (µg kg^{-1})	Spike level (µg kg^{-1})	Repeatability Recovery, % (RSD, %)	Reproducibility Recovery, % (RSD, %)	U'%
509.0	175.1 (37)	8.669	Y= 99942.5X + 624807	0.99655	2.26	7.55	10	77.14 (9.13)	78.62 (9.12)	36.96
	201.8 (24)						50	97.23 (8.51)	89.51 (13.45)	
	325.8 (18)						100	86.31 (8.84)	82.88 (9.92)	

CE, Collision energy; RT, Retention time; LOD, limit of detection; LOQ, limit of quantification; U', expanded measurement uncertainty.

Dissipation kinetics

Pesticide residues pose a significant threat to food safety, making it crucial to understand the residue behavior of pesticides after application in agricultural fields. Research on the dissipation of lufenuron in peppers is limited. Most existing studies focus on the behavior of this compound in other plant species.

Table 2 provides detailed information regarding the kinetic equations, R^2 and $t_{1/2}$ of lufenuron, facilitating a quantitative comparison of its dissipation dynamics across different plant species. Additionally, Figure 1 visually depicts the dissipation curve of lufenuron specifically in pepper under field conditions, offering a graphical representation of its temporal behavior post-application. Through these analytical tools, we aim to provide a comprehensive understanding of the dissipation kinetics of lufenuron in pepper plants.

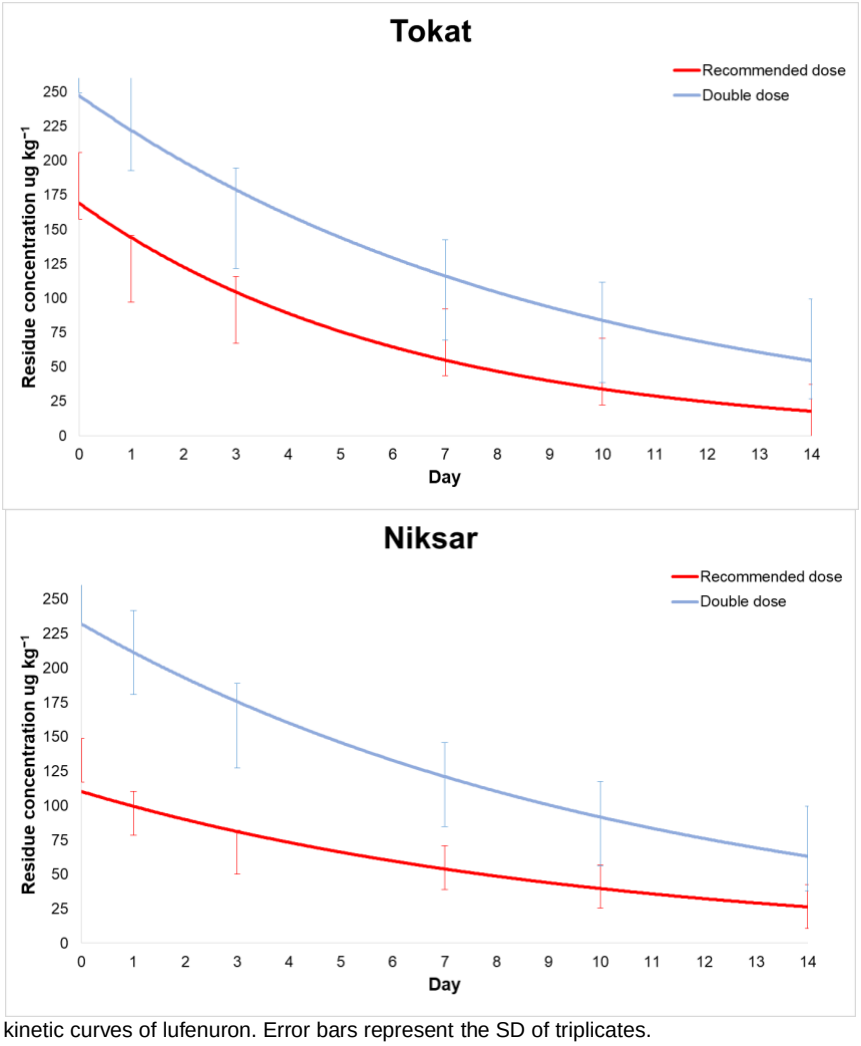

Figure. 1. Dissipation kinetic curves of lufenuron. Error bars represent the SD of triplicates.

Table 2. The parameters for the single first-order kinetic model of lufenuron, along with the residue concentrations at the PHI

Pesticide	Experiment site	Treatment	Kinetics equation	k (day^{-1})	Determination coefficient (R^2)	$t_{1/2}$ (day)	Residue at PHI (7^{th} day)
Lufenuron	Tokat	Recommended dose	$C_t = 169.31e^{-0,160x}$	0.160	0.9375	4.33a	67.97±7.58
	Tokat	Double dose	$C_t = 247.47e^{-0,108x}$	0.108	0.9568	6.42b	105.96±3.31
	Niksar	Recommended dose	$C_t = 110.22e^{-0,102x}$	0.102	0.9039	6.80c	54.98±1.35
	Niksar	Double dose	$C_t = 231.99e^{-0,093x}$	0.093	0.9599	7.45d	115.29±2.82

a-d—means in the column marked by the same letters they do not differ significantly p=0.05; level of significance (Tukey test).

For recommended dose (RD) and double dose (DD) applications, the initial concentrations of lufenuron were 181.58 and 286.07 μg kg^{-1} in Tokat, with corresponding half-lives of 4.33 and 6.42 days. In Niksar, the initial concentrations for RD and DD were 133.10 and 262.35 μg kg^{-1}, with half-lives of 6.80 and 7.45 days, respectively. These findings indicate that lufenuron dissipated more quickly in Tokat compared to Niksar. Analysis of climatic conditions revealed that Niksar had an average relative humidity of 58.2% and an average temperature of 25.6°C, whereas Tokat experienced a higher average relative humidity of 69.9% and a slightly lower average temperature of 24.5°C. The observed differences in dissipation rates between the two locations are likely influenced by these climatic factors, underscoring the impact of environmental conditions on the degradation kinetics of lufenuron.

To the best of our knowledge, there are no papers on the dissipation kinetics of lufenuron in peppers. However, the dissipation kinetics of lufenuron in some agricultural products other than peppers has been studied. Dong et al. (2015) found half-lives ranging from 1.74 to 5.04 days in cabbage. Wang et al. (2023) reported in their studies in Anhui and Guangdong that the half-life of lufenuron in cabbage was 3.24 and 6.80 days, respectively. Cheng et al. (2022) investigated the effect of different formulations of lufenuron on half-life in maize plants and found that lufenuron in commercial formulation (3.00 days) had a longer half-life compared to nano-microemulsion formulation (2.51 days). In a study on the dissipation kinetics of lufenuron in *Pisum sativum* L., Alvarez-Vilca et al. (2023) found that the dissipation varied with the application dose and frequency. Half-lives were found to range between 1.67 and 4.84 days in pea pods and 1.88 and 13.35 days in pods. The highest half-life values were observed at a dose of 24 g a.i. ha^{-1} with an 8-day frequency. Applications with a 4-day frequency resulted in higher residue levels but shorter half-lives. At higher doses (30 g a.i. ha^{-1}), the residue levels increased, yet the dissipation kinetics remained similar. Vijay et al. (2024) found the half-life of lufenuron in cauliflower to be 3.27 days. When the half-lives in the studies are examined, it is seen that there are various values. The reasons for this difference are seen to be locations with different climate characteristics, different doses, and plant varieties. The findings of this study align with those reported in previous research.

Pesticide dissipation kinetics have demonstrated that elevated temperatures and high humidity can significantly impact pesticide residue levels and metabolites. Furthermore, residue levels vary due to various factors, including crop species, variety, developmental phase, pesticide chemical formulation, application method, timing, dosage, light exposure, microbial activity, and other environmental conditions (Lee et al., 2018; Balkan & Kara, 2023; Balkan et al., 2024). A study conducted in greenhouses at two different locations highlighted that the half-life of azoxystrobin in Swiss chard is related to changes in temperature and humidity (Kabir et al. 2018). Similarly, Sun et al. (2019) revealed in their study that environmental factors such as light, temperature, humidity and plant species (especially growth dilution factors) affect the half-life of methoxyfenozide. Their findings indicated higher temperature, humidity, or precipitation levels were associated with a reduced pesticide half-life.

Health risk assessment

Pesticide hazard evaluations have attracted considerable consumer attention in recent years, particularly in Türkiye (Çatak & Tiryaki, 2020; Balkan & Kara, 2022; Balkan & Yılmaz, 2022; Serbes & Tiryaki, 2023; Yelaldı et al., 2024). In this study, health risk assessment of lufenuron residues in pepper was conducted and the results are shown in Table 3. As a result of the risk assessment, the chronic health risk values were below 1 and there was no health risk.

Table 3. The results of long-term and short-term risk assessments of lufenuron

Experiment Site	Treatment	MR (mg kg^{-1})	HR (mg kg^{-1})	EDI (mg $kg^{-1}day^{-1}$)	ESTI (mg $kg^{-1}day^{-1}$)	HQ_c
Tokat, Niksar	RD	0.042	0.055	0.00004	0.00006	0.003
	DD	0.086	0.115	0.00009	0.00014	0.006

RD, Recommended dose; DD, Double dose ; MR, Median pesticide residue; HR, High pesticide residue; EDI, estimated daily intake; ESTI, estimated short-term intake; HQ_c, chronic hazard quantities.

Under recommended dose application, the EDI and ESTI values ranged from 0.00004 to 0.00009 mg kg^{-1} bw day^{-1}, whereas for double dose (DD) application, these values were 0.00006 and 0.00014 mg kg^{-1} bw day^{-1}, respectively. The Chronic Hazard Quotient (HQ_c) remained below 1 for both applications, indicating acceptable risk levels. Notably, the HQ_c for DD application was twice as high as that for RD application. The health risk assessment determined that the risk value due to lufenuron residues was less than one and did not pose a health risk. Studies investigating the health risks of lufenuron residues in different plants have similar results to our study (Cheng et al., 2022; Mujahid et al., 2022; Wang et al., 2023). Based on existing studies, lufenuron, when administered at appropriate dosages, does not exhibit any health risks.

Conclusion

In this study, an LC-MS/MS-QuEChERS method was verified for the detection of lufenuron residues in peppers. The dissipation kinetics of lufenuron were investigated in peppers grown in two different regions, Tokat and Niksar. Additionally, the health risk assessment of lufenuron residues was performed. This study emphasizes the critical impact of environmental factors on the dissipation of lufenuron in peppers. Faster degradation was observed in Tokat due to its higher relative humidity (69.9%) and lower temperature (24.5°C) compared to Niksar (58.2% humidity, 25.6°C). These results underscore the critical role of climate in the pesticide degradation process, highlighting the importance of environmental conditions in evaluating agricultural pesticide applications. Health risk assessments for both recommended and double-dose applications of lufenuron revealed that the HQ_c values were well below safety thresholds, suggesting a minimal risk to human health. The fact that double dosing causes a twofold increase in HQc indicates that overdoses potentially increase the health risk. Therefore, it is important to use lufenuron at recommended doses.

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Original article (Orijinal araştırma)

Distribution and infestation rates of *Phenacoccus solenopsis* Tinsley, 1898 (Hemiptera: Pseudococcidae) in cotton fields in Aydın province (Türkiye) and its alternative host plants[1]

Aydın ilinde (Türkiye) pamuk tarlalarında *Phenaoccus solenopsis* Tinsley, 1898 (Hemiptera: Pseudococcidae)'in yayılışı, bulaşma oranları ve diğer konukçu bitkileri

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Abstract

Intense infestations of a mealybug species were observed on cotton plants in Aydın province (Türkiye) between 2022 and 2023. Insect samples were identified as the cotton mealybug, *Phenacoccus solenopsis* Tinsley, 1898 (Hemiptera: Pseudococcidae). In 2024, surveys were conducted to determine the distribution, infestation rates and alternative hosts of the cotton mealybug in cotton cultivation areas in Aydın. As a result of the surveys, it was determined that the cotton mealybug was distributed in 12 districts of Aydın province and 44.37% of total cotton cultivation areas was infested in Aydın. The infestation rates per field varied between 0.00% and 100%, and for each district changed between 0.00% and 46.13%. In and around cotton fields, 27 alternative host plants were found. Of these, four species are for the first time recorded as hosts of cotton mealybug, of which the most common host plants were *Conyza* spp. (Asterales: Asteraceae).

Keywords: Cotton mealybug, first record, *Gossypium hirsutum*, insect pest, invasive species, Türkiye

Öz

Aydın ilinde (Türkiye) 2022 ve 2023 yıllarında pamuk bitkisinde yoğun bulaşmalar gözlenmiştir. Alınan örnekler pamuk unlubiti, *Phenacoccus solenopsis* Tinsley, 1898 (Hemiptera: Pseudococcidae) olarak teşhis edilmiştir. Pamuk unlubitinin, 2024 yılında Aydın'daki pamuk yetiştirilen alanlarda yaygınlığı, bulaşma oranlarını ve alternatif konukçularını belirlemek amacıyla sürveyler gerçekleştirilmiştir. Sürveyler sonucunda pamuk unlubitinin Aydın ilinin 12 ilçesine ve pamuk üretim alanlarının %44,37'sine yayıldığı belirlenmiştir. Tarla başına bulaşma oranları %0,00 ile%100 arasında değişmektedir. İlçelere göre bulaşma oranlarının %0,00 ile %46,13 arasında değiştiği saptanmıştır. Pamuk alanları ve çevresinde 27 alternatif konukçu bitki tespit edilmiştir. Bunlardan dört tür pamuk unlubitinin konukçusu olarak ilk kez kaydedilmiş olup, en yaygın görülen alternatif konukçu bitkiler *Conyza* spp. (Asterales: Asteraceae) olarak belirlenmiştir.

Anahtar sözcükler: Pamuk unlubiti, ilk kayıt, *Gossypium hirsutum*, zararlı böcek, istilacı türler, Türkiye

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Introduction

The cotton mealybug, *Phenacoccus solenopsis* Tinsley, 1898 (Hemiptera: Pseudococcidae) was first detected in New Mexico, USA in 1898 by Tinsley (1898), and in the following years it has been recorded in many different countries in Africa, Asia, North America, South America and the Caribbean countries (García Morales et al., 2016). *Phenacoccus solenopsis* is a polyphagous pest with the ability of spreading rapidly in a short time, and it has been detected in 212 different plants belonging to 64 families (García Morales et al., 2016). The first invasive species record of *P. solenopsis* in Türkiye was in Adana in 2012 in ornamental plants (Kaydan et al., 2013), and the species is now widespread in the Mediterranean region of Türkiye. In a study conducted in agricultural fields and ornamental plants in Aydın province, *P. solenopsis* was found on 45 different host plants belonging to 25 families (Yerlikaya et al., 2023).

The mealybugs not only damage the host plant by sucking plant sap, but in general they are also responsible for the transmission of viral diseases (Bertin et al., 2010). In addition, the honeydew secreted by mealybugs provides an environment for the development of sooty molds, which also disrupts the photosynthesis process of the plant (Williams & Granara de Willink, 1992). This pest is often overlooked due to the very small nymphal stages. In recent years, as a result of the increase in agricultural products and ornamental plant trade worldwide and the increased mobility of plant material carried by people, many pest species living on these plant parts are entering other countries from where they are found. Thus, as in many other pest groups, species of the family Pseudococcidae are rapidly spreading on earth (Hodgson et al., 2008; Chong, 2009; Zhu et al., 2011; Kaydan et al., 2012; Ibrahim et al., 2015; Waqas et al., 2021). In addition, due to global warming, milder winter months and the expansion of the distribution areas of their host plants contribute to mealybug species becoming invasive species (Yerlikaya, 2022). The cotton mealybug has up to 15 generations per year in tropical climates, with a generation time of 25-30 days; females are ovoviviparous, laying 150-600 eggs per brood, and have a temperature tolerance of 0-45°C, indicating the pest's population can increase rapidly (Sharma, 2007; Kondo & Watson, 2022). In 2012, eleven species were announced as dangerous pest species in the invasive species list of CABI (Center for International Agriculture and Bioscience) and it was reported that *P. solenopsis* was one of the most important pest species among these. *Phenacoccus solenopsis* is adaptable to various climatic conditions and has many host plants. Therefore, the expected distribution of this species has a great potential of threat to agricultural areas (Wang et al., 2010). *Phenacoccus solenopsis* can easily become a problem in the areas where it spreads due to its wide host range and high reproductive potential (Kahya, 2020). Since *P. solenopsis* has a high-risk to become an invasive species, it is as quarantine pest in Argentina, China, Chile, Japan, Peru and many EPPO (European and Mediterranean Plant Protection Organization) member countries (Kondo & Watson, 2022).

Phenacoccus solenopsis causes significant problems in Pakistan and India, which account for 75% of the world cotton production, and this situation negatively affects the cotton trade at a global basis (Kahya, 2020). In fact, a study conducted in Pakistan reported a yield loss of 44% in cotton production due to *P. solenopsis* damage (Dhawan et al., 2009). In studies conducted in India, 30-80% of yield losses in cotton have been reported (Nagrare et al., 2009; Nalwar et al., 2009; Vennila et al., 2010). The impact of global climate change on the distribution and population density of *P. solenopsis* was estimated in a study in India. As a result of the study, it was determined that in 80% of the cotton cultivated areas, the mealybug can produce 4 or more times offspring, and cause significant economic losses (Fand et al., 2014). It was reported that this invasive species could spread over large areas and caused significant economic damages in China, and was therefore accepted as a high-risk invasive species (Wang et al., 2009).

Aydın province is a significant contributor to Türkiye's cotton production, ranking third with 12% of the national output (MAF, 2022). In recent years, meteorological records show that the relative humidity has increased in Aydın province due to reasons such as the increase in the number of dams, geothermal

energy facilities and the increase in irrigation due to expanding corn production areas (Table 1). It is thought that these conditions provide positive ecological conditions for the reproduction and multiplication of many insect species and are also an important factor in terms of the increase in the population of mealybugs.

Table 1. Some meteorological data of cotton growing periods (April-October) in Aydin province over years (DGM, 2023)

Meteorological parameters	1991-2021 (average)	2019	2020	2021	2022
Average temperature (°C)	23.93	23.90	24.34	24.60	24.65
Average maximum temperature (°C)	30.10	31.96	33.30	32.82	32.83
Average minimum temperature (°C)	17.67	17.20	17.34	17.47	17.89
Average relative humidity (%)	46.86	53.58	53.12	46.00	48.73
Total precipitation (mm)	210.00	192.80	121.40	50.40	85.40

Most of the research conducted in Türkiye on the invasive cotton mealybug, which causes significant problems in many countries, has been limited to non-agricultural areas and laboratory studies (Kaydan et al., 2013; Çalışkan et al., 2016; Çalışkan Keçe, 2019; Kahya et al., 2019; Kahya, 2020; Görür & Karut, 2021). Therefore, it is essential to determine the damage potential of this invasive pest in cottonfields. Additionally, complaints have been received from the Nazilli Cotton Research Institute and cotton producers that the spreading areas and population density of the mealybug have increased. For these reasons, this study aimed to determine the distribution and infestation rates of *P. solenopsis* in cotton fields in Aydın province, and surveys were conducted. In addition, other host plants infested with *P. solenopsis* in and around the cotton fields were determined.

Materials and Methods

Field surveys and sampling methods

The main material of the study was *P. solenopsis* in cotton fields and other host plants in and around cotton fields in Aydın province. In the preliminary study, samples were taken from cotton fields in Nazilli, Söke and Koçarlı districts between 2022 and 2023. The mealybugs species in the samples were identified as *P. solenopsis*. During the cotton growing season in 2024, surveys were conducted in all districts, especially in the districts where cotton cultivation was more extensive, and the distribution and infestation rates of *P. solenopsis* were determined. In addition, other plants in and around the cotton fields were also examined and plants infested with *P. solenopsis* were recorded as host plants.

In order to determine the distribution and infestation rates of *P. solenopsis*, surveys and sampling were conducted in cotton fields covering 0.01% of the cotton cultivation areas in the province (Bora & Karaca, 1970). The surveys started in June and continued until the end of September, the period when mealybug found in cotton fields. Adapted from MAF (2017) protocols, in each cotton field up to 50 decares (da), 50 cotton plants were randomly examined from different points in the field. The plant was considered infested if an individual in any biological stage of *P. solenopsis* was observed on the plant. The number of infested plants was divided by the total number of plants examined to determine the percentage (%) of infestation rate in the field. For fields larger than 50 da, this process was repeated for every 50 da. To determine the distribution rate, a cotton field was considered to be infested if even one cotton plant was found to be infested with *P. solenopsis.* At the same time, samples were obtained from mealybug infested fields for the identification of *P. solenopsis*. In addition, weeds and other plants in and around the cotton fields were examined, and if *P. solenopsis* was observed on these plants, samples were collected, and the host plant was recorded. Weed samples were identified by the authors and their identification was confirmed by relevant researchers. The infestation rate of *P. solenopsis* was calculated according to the formula below (Bertin et al., 2010):

IP : Infestation percentage

$IP=(X\div T)\times 100$ X : Number of plants infested with mealybug

T : Total number of plants examined

The infestation rate of *P. solenopsis* in provinces and districts was calculated according to the weighted average (Bora & Karaca, 1970).

The distribution rate was calculated according to the formula below:

DP: Distribution percentage

$DP=(A\div Y)\times 100$ A : Mealybug infested area

Y : Total area surveyed

Laboratory studies and morphological identification

Identification of mealybugs is made by observing the morphological characters of adult female individuals. For this reason, mealybug samples were examined under a stereo-binocular microscope and about 20 adult females from each sample were preserved in vials containing 70% ethyl alcohol. Female adult mealybugs were slide-mounted at the Sultanhisar Vocational School, Aydın, Türkiye, using the methods proposed by Kosztarab & Kozár (1988) with modifications (using sterile water after KOH to clean the specimens with a fine brush). The specimens were identified using the keys of Williams (2004) and Hodgson et al. (2008). A Leica DM2500 phase-contrast binocular microscope was used for identification of mealybugs. In addition, weeds and other plants in and around the cotton field infested with mealybug were prepared for the identification and preserved in a herbarium. The herbarium is in Sultanhisar Vocational School, Aydın, Türkiye. For the floristic catalogue, Güner et al. (2012) was followed.

Results and Discussion

***Phenacoccus* Cockerell, 1893 (Hemiptera: Pseudococcidae)**

***Phenacoccus solenopsis* Tinsley, 1898**

Material examined. Türkiye, Aydın: Buharkent, 144 m, 15.VIII.2024, 18 ♀♀; Didim, 7 m, 9.VIII.2024; 12 ♀♀; Efeler, 30 m, 28.VI.2024; 9 ♀♀; Efeler, 38 m, 25.VII.2024, 24 ♀♀; Efeler, 34 m, 2.VII.2024, 6 ♀♀; Efeler, 33 m, 4.VIII.2024, 15 ♀♀; Efeler, 32 m, 10.IX.2024, 12 ♀♀; Germencik, 27 m, 05.VII.2024, 3 ♀♀; Germencik, 14 m, 26.VII.2024, 6 ♀♀; Germencik, 32 m, 24.IX.2024, 78 ♀♀; İncirliova, 42 m, 25.VII.2024, 6 ♀♀; İncirliova, 40 m, 24.IX.2024, 21 ♀♀; Koçarlı, 26 m, 14.VIII.2022, 9 ♀♀; Koçarlı, 28 m, 19.VII.2023, 12 ♀♀; Koçarlı, 35 m, 8.VIII.2024, 3 ♀♀; Koçarlı, 40 m, 9.VIII.2024, 18 ♀♀; Koçarlı, 32, 10.IX.2024, 39 ♀♀; Köşk, 55 m, 17.IX.2024, 15 ♀♀; Kuyucak, 108 m, 15.VIII.2024, 30 ♀♀; Nazilli, 60 m, 10.IX.2022; 12 ♀♀; Nazilli, 61 m, 7.VII.2023, 15 ♀♀; Nazilli, 62 m, 10.VI.2024, 6 ♀♀; Nazilli, 60 m, 26.VI.2024, 12 ♀♀; Nazilli, 55 m, 02.VII.2024, 6 ♀♀; Nazilli, 86 m, 15.VIII.2024, 3 ♀♀; Söke, 15 m, 12.VIII.2022, 6 ♀♀; Söke, 10 m, 22.VIII.2023, 6 ♀♀; Söke, 6 m, 28.VI.2024, 3 ♀♀; Söke, 10 m, 22.VII.2024, 3 ♀♀; Söke, 5 m, 26.VII.2024, 6 ♀♀; Söke, 13 m, 9.VIII.2024, 60 ♀♀; Sultanhisar, 49 m , 27.VI.2024, 3 ♀♀; Yenipazar, 52 m, 17.IX.2024, 9 ♀♀.

Phenacoccus solenopsis, which was previously reported in non-agricultural areas and ornamental plants in Aydın by Yerlikaya et al. (2023), is for the first time reported in cotton plants in this study. *Phenacoccus solenopsis* infests all above-ground parts of the plant during the cotton growing season (Figure 1a,1b,1c). The cotton mealybug reached high populations and caused the death of plants especially in the edge rows of the fields (Figure 1d). *Phenacoccus solenopsis* was also observed infesting many weeds in and around the cotton field and reached to high populations on these plants. It is possible to state that the first infestations on cotton plants are due to mealybugs that move from weeds.

Figure 1. *Phenacoccus solenopsis* on cotton plants: a) on the top shoots; b) on the stem of the flowers; c) on the leaves; d) on the edge rows of the field.

Distribution and infestation rates of *Phenacoccus solenopsis* Tinsley, 1898 (Hemiptera: Pseudococcidae)

According to the Farmer Registration System data from the Turkish Ministry of Agriculture and Forestry, 560 343 da of cotton is cultivated in Aydın province. The biggest cotton area in the province is located in Söke district with 321 031 da, followed by Koçarlı and Efeler districts with 72 655 da and 41 633 da, respectively (Table 2).

As a result of the surveys conducted in Aydın province, 13 908.60 da (280 fields) cotton fields were observed and 6 171.50 da (142 fields) cotton fields were found to be infested with *P. solenopsis* (Table 2). In this case, the distribution rate of *P. solenopsis* in Aydın province was determined as 44.37%.

In the fields, infestation rates varied between 0.00% and 100%. The highest infestation rate was 100% in a cotton field in Yenipazar district (Figure 3c). This was followed by Germencik and Buharkent with 84.00 % and 82.00% infestation rates, respectively (Table 3).

Table 2. Cotton cultivation areas and survey results in different districts of Aydın province

Districts	Cotton cultivated areas (decare)	Surveyed areas (decare)	Infested areas (decare)	Number of survey fields	Number of infested fields
Söke	321 031	4 928.50	862.80	59	24
Koçarlı	72 655	1 631.70	1 041.70	43	20
Efeler	41 633	1 540.40	913.70	44	22
Germencik	39 663	2 006.00	1 505.20	39	29
İncirliova	27 420	451.30	308.90	13	9
Didim	18 630	734.20	232.80	8	4
Nazilli	16 150	631.80	479.40	16	9
Yenipazar	11 615	269.90	44.90	13	3
Köşk	4 007	549.00	307.20	12	5
Sultanhisar	3 955	224.00	11.00	6	1
Kuyucak	1 324	508.40	354.90	15	10
Çine	962	310.20	0,00	4	0
Buharkent	903	109.00	109.00	6	6
Bozdoğan	395	14.20	0.00	2	0
Total	560 343	13 908.60	6171.50	280	142

Table 3. Infestation and distribution rates of *Phenacoccus solenopsis* in cotton fields in different districts in Aydın province

District	Minimum infestation rates per field (%)	Maximum infestation rates per field (%)	Average infestation rates (%)	Average distribution rates (%)
Söke	0	80	7.53	17.51
Koçarlı	0	80	10.65	63.84
Efeler	0	66	8.91	59.32
Germencik	0	84	30.10	75.03
İncirliova	0	56	19.38	68.45
Didim	0	54	13.00	31.71
Nazilli	0	36	9.38	75.88
Yenipazar	0	100	17.69	16.64
Köşk	0	70	21.50	55.96
Sultanhisar	0	4	0.67	4.91
Kuyucak	0	56	46.13	69.81
Çine	0	0	0.00	0.00
Buharkent	8	82	35.33	100.00
Bozdoğan	0	0	000	0.00

Considering the average infestation rates by districts, the highest infestation was found in Kuyucak with 46.13%. This was followed by Buharkent and Germencik with 35.33% and 30.10% infestation, respectively. No infestation was observed in Bozdoğan and Çine districts (Table 3).

Considering the distribution rates by districts, Buharkent ranked first with 100%, followed by Nazilli and Germencik with 75.88% and 75.03%, respectively (Table 3).

Other host plants of *Phenacoccus solenopsis* Tinsley, 1898 (Hemiptera: Pseudococcidae)

In the study, 27 species of host plants infested with *P. solenopsis* were recorded in and around the cotton field (Figure 2). The most prevalent host plants were *Conyza* spp. (Asterales: Asteraceae) with 72 samples, followed by *Portulaca oleracea* L. (Caryophyllales: Portulacaceae) with 37 samples and *Convolvulus arvensis* L. (Solanales: Convolvulaceae) with 33 samples (Table 4). According to ScaleNet data (García

Morales et al., 2016), four species were first recorded as hosts of *P. solenopsis*: *Alhagi maurorum* Medik. subsp. *maurorum* (Fabales: Fabaceae), *Glycyrrhiza glabra* L. (Fabales: Fabaceae), *Vigna unguiculata* (L.) Walp. (Fabales: Fabaceae), *Xanthium spinosum* L. (Asterales: Asteraceae).

Table 4. Other hosts of *Phenacoccus solenopsis* in cotton fields and number of samples

Host Plant	Family	Samples
Abelmoschus esculentus (L.) Moench	Malvaceae	4
Alhagi maurorum Medik. subsp. *maurorum*	Fabaceae	1
Amaranthus viridis L.	Amaranthaceae	29
Capparis sp.	Capparaceae	1
Chenopodium album L.	Amaranthaceae	22
Citrullus lanatus (Thunb.) Matsum. & Nakai	Cucurbitaceae	1
Conyza bonariensis (L.) Cronquist	Asteraceae	49
Conyza canadensis (L.) Cronquist	Asteraceae	23
Convolvulus arvensis L.	Convolvulaceae	33
Cucurbita sp.	Cucurbitaceae	1
Cuscuta sp.	Convolvulaceae	2
Cyperus rotundus L.	Cyperaceae	1
Datura stramonium L.	Solanaceae	4
Daucus carota L.	Apiaceae	2
Ecballium elaterium (L.) A. Rich.	Cucurbitaceae	1
Glycyrrhiza glabra L.	Fabaceae	6
Heliotropium europaeum L.	Boraginaceae	2
Malva parviflora L.	Malvaceae	9
Physalis alkekengi L.	Solanaceae	1
Portulaca oleracea L.	Portulacaceae	37
Solanum melongena L.	Solanaceae	1
Solanum nigrum L.	Solanaceae	9
Sonchus oleraceus L.	Asteraceae	4
Tribulus terrestris L.	Zygophyllaceae	16
Vigna unguiculata L. Walp.	Fabaceae	3
Xanthium spinosum L.	Asteraceae	2
Xanthium strumarium L.	Asteraceae	16

If *Conyza* spp. at the border of the cotton field were infested with *P. solenopsis*, it was observed that the cotton plants were also infested. This situation was also noticed by the farmers. In fact, some of the farmers we encountered during the surveys stated that mealybugs were in *Conyza* spp. at the beginning of the season and then they moved from this weed to cotton fields by wind and other means. Our observations also supported this report. As a matter of fact, it was observed that some farmers used methods such as ploughing, burning and spraying herbicides to control *Conyza* spp. at the edge of the field. While heavy infestations *P. solenopsis* was determined on other weed hosts only from time to time, but *Conyza* spp. were observed to be infested always with *P. solenopsis* throughout the cotton production season. In support of all these observations, it was determined that *P. solenopsis* overwintered on weeds at the edges of the field and increased its populations in the following year and then moved to cotton plants. The presence of weed hosts that act as alternate hosts around cotton fields was reported to be an important factor in the distribution of this mealybug (Jhala et al., 2008). In order to prevent *P. solenopsis* infestations, it is necessary to control the weeds effectively in the early season, especially at the edge of the fields.

Figure 2. Host plants infested with *Phenacoccus solenopsis* in and around the cotton field: a) *Amaranthus viridis*; b) *Chenopodium album*; c) *Ecballium elaterium*; d) *Ecballium elaterium*; e) *Heliotropium europaeum*; f) *Malva neglecta*; g) *Physalis peruviana*; h) *Portulaca oleracea*; I) *Solanum nigrum*; j) *Sonchus oleraceus*; k) *Xanthium spinosum*; l) *Xanthium strumarium*.

Due to the damage caused to cotton plant by *P. solenopsis*, it has been observed that the plants grow poorly, wither and may even die. Mealybugs could infest all above-ground parts of the plant and caused damage by forming dense populations in the stem part of the bolls. This damage results in the bolls to be weak, remain small and wilt without completing its development. It has been also reported in many studies that mealybugs caused indirect damage to plants by sooty molds that grow on the honeydew they produce (Williams & Granara de Willink, 1992). Sooty mold damage was observed both in the early period and after the bolls opened. At an early stage, sooty mold on the green parts of the plant reduces the area of photosynthesis and causes poor development of the plant, while sooty mold on cotton after the bolls have opened significantly decreases quality.

Figure 3. Damage caused by *Phenacoccus solenopsis* on cotton: a) on the stem of a cotton boll; b) sooty mold on cotton; c) up to 100% damage in the cotton field; d) colony of *Phenacoccus solenopsis* on cotton stalks.

As a result, all districts of Aydın province where cotton is cultivated were found to be infested with *P. solenopsis*, except Bozdoğan (the district with the least cotton area in Aydın province) and Çine. In some cotton fields, high levels of infestation was observed and this was expected to reduce cotton yields significantly. The cotton mealybug was reported to cause yield losses up to 80% in Pakistan and India, which produce most of the world's cotton (Nagrare et al., 2009; Nalwar et al., 2009; Vennila et al., 2010). It is known that with the increase in the population density of cotton mealybug, yield, quality. will drop dramatically and on the other hand, it can be expected that economic losses in cotton fields will increase (Fand et al., 2014). In fact, it should be taken into account that no marketable crop could be obtained due to infestations up to 100% in some fields. During the study, economic production was observed to become almost impossible because of the intense infestations in some fields. Therefore, it can be suggested that studies on the bio-ecology and control of the cotton mealybug in this area should be supported as a priority.

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Original article (Orijinal araştırma)

Efficacy of native entomopathogenic fungus *Beauveria bassiana* (Balsamo) Vuillemin (Hypocreales: Cordycipitaceae) against *Helicoverpa armigera* (Hübner, 1808) (Lepidoptera: Noctuidae)

Yerel entomopatojen fungus *Beauveria bassiana* (Balsamo) Vuillemin (Hypocreales: Cordycipitaceae)'nın *Helicoverpa armigera* (Hübner, 1808) (Lepidoptera: Noctuidae) üzerindeki etkinliği

Abdurrahman Sami KOCA[1*]

Abstract

Helicoverpa armigera (Hübner, 1808) (Lepidoptera: Noctuidae) is a significant agricultural pest with resistance to conventional synthetic insecticides. The present study, conducted in 2024 at Bolu Abant Izzet Baysal University, Biological Control Laboratory, investigated the pathogenicity of *Beauveria bassiana* (Balsamo) Vuillemin (Hypocreales: Cordycipitaceae) against six larval stages of *H. armigera*. Three conidial concentrations (1×10^6, 1×10^7, and 1×10^8 conidia ml^{-1}) were evaluated. This study analyzed the effects of varying spore concentrations of *B. bassiana* on the mortality of *H. armigera* larvae, considering days post-application, dose, and larval stage. Lethal concentrations (LC_{50} and LC_{90}) and lethal time values (LT_{50} and LT_{90}) were calculated using probit analysis. Mortality increased with dose and was highest in early instars, particularly at 1×10^8 conidia ml^{-1}, where LT_{50} and LT_{90} values for first instars were 4.05 and 8.10 days, respectively. Older instars exhibited lower mortality rates. LC_{50} and LC_{90} decreased with increasing concentrations, achieving 100% mortality in the first and second instars across all doses. Third instars displayed dose-dependent mortality, with LC_{50} and LC_{90} values of 6.88×10^8 and 1.94×10^4 conidia ml^{-1}, respectively. Higher spore concentrations reduced LT_{50} and LT_{90}, enhancing mortality rates in younger larvae. These findings underscore the efficacy of *B. bassiana* against early larval stages, emphasizing the importance of application timing for effective biological control and its potential role in integrated pest management strategies.

Keywords: *Beauveria bassiana,* biological control, biopesticide, entomopathogenic fungus, *Helicoverpa armigera*

Öz

Helicoverpa armigera (Hübner, 1808) (Lepidoptera: Noctuidae), sentetik insektisitlere karşı direnç geliştirmiş önemli bir tarımsal zararlıdır. Bu çalışmada, entomopatojenik fungus *Beauveria bassiana* (Balsamo) Vuillemin (Hypocreales: Cordycipitaceae)'ın *H. armigera*'nın altı larva dönemine karşı etkinliği 2024 yılında Bolu Abant Izzet Baysal Üniversitesi, Biyolojik Mücadele Laboratuvarı'nda incelenmiştir. Üç farklı konidiyal konsantrasyon (1×10^6, 1×10^7 ve 1×10^8 konidi ml^{-1}) kullanılarak ölüm oranları değerlendirilmiştir. Uygulama sonrası günler, doz ve larva dönemleri göz önünde bulundurularak, *B. bassiana*'nın farklı spor konsantrasyonlarının etkileri analiz edilmiştir. Probit analizi ile öldürücü konsantrasyonlar (LC_{50} ve LC_{90}) ve öldürücü zaman değerleri (LT_{50} ve LT_{90}) hesaplanmıştır. Ölüm oranlarının, artan dozlarla yükseldiği ve özellikle genç larva dönemlerinde belirgin olup, özellikle birinci larva döneminde 1×10^8 konidi ml^{-1} konsantrasyonunda, LT_{50} ve LT_{90} değerleri sırasıyla 4,05 ve 8,10 gün olarak kaydedilmiştir. Daha ileri dönemlerde ise ölüm oranlarının düştüğü tespit edilmiştir. LC_{50} ve LC_{90} değerleri, artan konsantrasyonlarla azalarak birinci ve ikinci dönem larvalarda tüm dozlarda %100 ölüm sağlamıştır. Üçüncü dönem larvalar doz-bağımlı bir tepki göstermiş ve LC_{50} ile LC_{90} değerleri sırasıyla 6.88×10^8 ve 1.94×10^4 konidi ml^{-1} olarak bulunmuştur. Daha yüksek spor konsantrasyonları, LT_{50} ve LT_{90} sürelerini kısaltarak özellikle genç larvalarda ölüm oranlarını artırmıştır. Bu bulgular, *B. bassiana*'nın genç larva dönemlerine karşı etkinliğini, etkili biyolojik mücadele için uygulama zamanlamasının önemini ve entegre zararlı yönetimi stratejilerinde potansiyel rolünü vurgulamaktadır.

Anahtar sözcükler: *Beauveria bassiana,* biyolojik mücadele, biyopestisit, entomopatojen fungus, *Helicoverpa armigera*

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Introduction

Helicoverpa armigera (Hübner, 1808) (Lepidoptera: Noctuidae) is a highly destructive, polyphagous pest that affects over 100 plant species, including several economically significant crops such as maize, common bean, cotton, tobacco, soybean, chickpea, and various vegetables (Talekar et al., 2006; Cunningham & Zalucki, 2014; Koca & Kaçar, 2024). Over the past few decades, the pest has significantly expanded its geographic range, with reports from regions across Europe, Africa, South America, Asia, and Australasia (Kriticos et al., 2015; Arnemann et al., 2016). Adult *H. armigera* demonstrates notable migratory potential, with recorded long-distance dispersals extending up to 2.000 kilometers (Feng et al., 2009; Jones et al., 2015; Riaz et al., 2021). These moths are particularly attracted to the reproductive stages of host plants, with the larvae predominantly feeding on reproductive parts such as flowers and pods (Liu et al., 2010). This feeding behavior, focused on yield-forming organs, contributes to the severe economic losses observed in a wide range of crops affected by *H. armigera* (Zalucki et al., 1986). Therefore, its infestations are responsible for substantial yield losses, often necessitating costly control measures. Additionally, the species exhibits a remarkable ability to develop resistance to chemical insecticides, further complicating management strategies (Fitt, 1989; Walsh et al., 2022). The extensive use of broad-spectrum chemical insecticides has led to the rapid development of resistance in *H. armigera* populations, particularly against pyrethroids, endosulfan, carbamates, and organophosphates (Ahmad et al., 1999, 2003). This resistance poses significant challenges for sustainable pest control and highlights the need for alternative approaches.

The management of *H. armigera* involves multiple strategies, including transgenic insect-resistant crops, pheromone traps, light traps, chemical pesticides, and biological control agents (Riaz et al., 2021). Among these methods, biological control plays a critical role within Integrated Pest Management (IPM) programs, as it reduces the reliance on synthetic pesticides, particularly due to its eco-friendly and sustainable application (Kaçar et al., 2023). In IPM programs, biological control agents like entomopathogenic fungus (EPF) are integrated with other strategies such as crop rotation, resistant varieties, and selective pesticide use. This multi-faceted approach ensures a reduction in chemical pesticide applications and delays the development of resistance in pest populations (Bale et al., 2008). Entomopathogenic fungi, especially *Beauveria bassiana* (Balsamo) Vuillemin (Hypocreales: Cordycipitaceae) have emerged as highly promising biological control agents due to their ability to infect and kill the pest at various larval stages effectively (Wagner & Lewis, 2000; Younas et al., 2017; Altinok et al., 2019). As a widespread entomopathogenic fungus and obligate parasite, *B. bassiana* is the causal agent of white muscardine disease in insects (Deans & Krischik, 2023). These fungi are known to infect and kill *H. armigera* larvae by adhering to and penetrating the insect's cuticle through their conidial spores, ultimately leading to the host's death via internal colonization and degradation of the hemolymph (Nguyen et al., 2007; Goettel & Glare, 2010).

Considering the potential of entomopathogenic fungi as biocontrol agents, they present an effective alternative to traditional synthetic insecticides within an integrated pest management framework aimed at controlling field populations of *H. armigera*. These biocontrol agents disrupt the physiology, behavior, and development of their host insects, providing a strategic advantage in managing resistant pest species (Michereff-Filho et al., 2008). Unlike chemical pesticides, which can lead to non-target effects and environmental contamination, entomopathogenic fungi like *B. bassiana* offer an environmentally friendly alternative. They are host-specific, leave minimal residues, and contribute to the conservation of natural enemy populations (Vega et al., 2009). Moreover, their compatibility with other IPM tactics highlights their strategic importance in sustainable agriculture (Kaçar et al., 2023).

The present study aimed to evaluate the effects of the entomopathogenic fungus *B. bassiana* as a biocontrol agent against *H. armigera,* investigating the effects across different larval instars and spore concentrations. It is hypothesized that the native strain of *B. bassiana* will significantly reduce *H. armigera* larval populations in vitro, showcasing its potential for broader field applications in an IPM framework. The results of this study will contribute to the ongoing efforts to integrate EPFs into pest management frameworks, reducing the reliance on chemical insecticides and promoting sustainable crop protection.

Materials and Methods

Mass rearing of *Helicoverpa armigera*

The mass rearing of *H. armigera* was conducted under controlled laboratory conditions, with the temperature maintained at 25±1°C, relative humidity (RH) at 65±5%, and 14:10 h light/dark (L/D) photoperiod. Larvae were provided with an artificial diet specifically formulated to support optimal growth (Southland Products Inc., USA). The diet consists of toasted soybean flour, stabilized wheat germ, sugar, vitamins, and mineral salts. Cannibalism among the larvae was mitigated by rearing individuals from the third instar in separate plastic vials. After pupae had developed, they were transferred to ventilated containers to allow adults to emerge. Newly emerged adults were fed a 20% honey solution and maintained under the same conditions. The overall rearing protocol was adapted from Armes et al., (1992), with some modifications. The six larval instars of *H. armigera* (L1, L2, L3, L4, L5, and L6) were determined based on head capsule width and overall size (Queiroz-Santos et al., 2018).

Isolation and identification of entomopathogenic fungi

Insect cadavers of *H. armigera* larvae suspected to be infected with entomopathogenic fungi were collected in maize fields and surface sterilized to eliminate external contaminants. Initially, each cadaver was submerged in 1% sodium hypochlorite solution for 2 minutes, followed by rinsing in 70% ethanol for 1 minute. Subsequently, cadavers were rinsed three times in sterile distilled water to remove any chemical residues. Once cleaned, the insect cadavers were placed individually on sterile filter paper within Petri dishes containing Potato Dextrose Agar (PDA) medium (Sigma Aldrich, St. Louis, MO, USA) supplemented with 0.01% streptomycin to prevent bacterial contamination. The Petri dishes were incubated at 25±2°C with 65±5% RH in darkness for 7–10 days to allow fungal growth. Fungal colonies emerging from the insect tissues were regularly monitored, and those displaying typical morphological characteristics of *B. bassiana.* Once isolated, individual colonies were transferred to fresh PDA plates for further purification and maintenance. Purified isolates were stored at 4°C until further identification and use in bioassays (Inglis et al., 2012; Zimmermann, 2007). To confirm the identification, the internal transcribed spacer (ITS) and the partial *β-tubulin* (*tub2*) loci were amplified and sequenced using primers ITS1/ITS4 (White et al., 1990) and BT2a/BT2b (Glass & Donaldson, 1995) according to Güney et al. (2022). The sequence homology searches were conducted using the Basic Local Alignment Search Tool (BLAST: http://blast.ncbi.nlm.nih.gov/) against the nucleotide database of the NCBI GenBank to identify the closest available reference sequences.

Conidial suspension of *Beauveria bassiana*

Beauveria bassiana isolate used in this study was mass-cultured PDA medium, supplemented with 1% yeast extract to enhance fungal growth. Plates were incubated at 25±2°C with 65±5% RH under a 14:10 h L/D photoperiod for 14 days, allowing for conidial production. Once mature, conidia were harvested by gently scraping the surface of the culture plates with a sterilized spatula. The collected conidia were then air-dried at room temperature and stored at 4°C in sterile containers until further use. To prepare the bioassay, the dry conidial powder was suspended in sterile distilled water supplemented with 0.01% Tween-80 (Merck, Darmstadt, Germany) to achieve uniform spore dispersion. The suspension was passed with two layers of sterile cheesecloth to remove any mycelial fragments, ensuring a uniform spore suspension.

The resulting suspension was thoroughly mixed using a vortex mixer for 1 minute to break any spore clumps. Spore concentrations were adjusted by performing serial dilutions to achieve the desired concentrations of 1×10^6, 1×10^7, and 1×10^8 conidia ml^{-1}. Spore viability was assessed by incubating a small aliquot of the suspension on fresh PDA plates at 25°C for 24 hours, and the germination rate was confirmed to be above 90% prior to each bioassay. The Bb_Dzc01 isolate was selected among the isolates tested for further assays.

Insect bioassay

The entomopathogenic fungus, *B. bassiana* was utilized for this study, and spore suspensions were prepared in sterile distilled water containing 0.01% Tween 80 as a surfactant. Three spore concentrations were tested: 1×10^6, 1×10^7, and 1×10^8 conidia ml^{-1}. These concentrations were freshly prepared prior to each bioassay to ensure viability and consistency. The larvae were exposed to the fungal conidial suspensions through immersion. Each larva was individually immersed in a 100 ml conidial suspension of the respective concentration for 10 seconds, ensuring uniform exposure. After immersion, larvae were allowed to dry air on sterile filter paper for 30 minutes before being transferred to experimental places. The bioassay protocol was adapted from Tahir et al. (2019) and Alwaneen et al. (2024). For the L1 and L2 larval instar, each treatment group consisted of 5 larvae per Petri dish (90 mm diameter), with five replicates per concentration. For the L3, L4, L5, and L6 larvae, due to cannibalistic behavior (Kakimoto et al., 2003), only one larva was placed per Petri dish, with five replicates per concentration. Each replicate was repeated five times, ensuring a total of 25 larvae per concentration for each instar. Control groups consisted of larvae immersed in a solution containing 0.01% Tween 80 without fungal conidia. After treatment, all Petri dishes were sealed with a parafilm and maintained in a climate-controlled chamber at 25±1°C and 65±5% RH under a 14:10 h L/D photoperiod. The larvae were provided with a fresh artificial diet every two days to ensure adequate nutrition. Mortality was recorded after 1, 3, 5 ,7, 10 and 14 days, and dead larvae were removed and transferred to moist filter paper in a separate Petri dish to encourage fungal outgrowth and confirm cause of death.

Statistical analysis

Mortality rates were adjusted for control mortality using Abbott's formula (Abbott 1925), to account for natural death rates in the control groups. Corrected mortality rates were then used for further analysis. Before performing analysis of variance (ANOVA), the normality and homogeneity of variances were checked using the Shapiro-Wilk and Levene's tests, respectively. All data was not normally distributed. The percentage of mortality data collected at various time intervals was subjected to arcsine square root transformation to normalize the data. To statistically compare the effects of EPF applications on mortality, a three-way ANOVA was conducted, followed by Tukey's HSD test for multiple comparisons among the spore concentrations (1×10^6, 1×10^7, and 1×10^8 conidia ml^{-1}). Probit analysis was performed to estimate the lethal concentrations (LC_{50} and LC_{90}) and lethal times (LT_{50} and LT_{90}) for each larval instar and spore concentration. The fiducial limits for the LC and LT values were calculated with a 95% confidence interval. All statistical analyses were conducted using SPSS software version 17.0 for Windows (SPSS Inc, Chicago, IL, USA). For effective visualization of the data, graphical representations were generated using GraphPad Prism version 6.04 (GraphPad Software, San Diego, CA, United States). All statistical analyses were conducted on arcsine-transformed data, while graphs represent raw (untransformed) data for easy interpretation.

Result

In this study, the Bb_Dzc01 isolate obtained from *H. armigera* larvae in Düzce was identified as *B. bassiana* with 100% identity for the ITS region (541bp), and *tub2* (291 bp). The resulting sequences were deposited in GenBank under the accession numbers PQ826424 for ITS and PQ857545 for *tub2*. The effects of different spore concentrations of *B. bassiana* on the mortality of *H. armigera* larvae were assessed by

considering factors such as day, dose, and larval stage. The analysis results indicate that each factor (day, dose, and larval stage) and its interactions (day×dose, day×larval stage, dose×larval stage, and day×dose×larval stage) significantly impacted mortality rates (Table 1).

Table 1. Results of the factorial analysis for mortality of all larval stages of *Helicoverpa armigera* exposed to *Beauveria bassiana*

Source	*df*	Sum of Squares	*F* Ratio	*p*
Day	5	2543.421	216.255	<0.0001
Dose	3	1669.359	236.563	<0.0001
Larval stage	5	1439.679	122.409	<0.0001
Day * Dose	15	870.991	24.685	<0.0001
Day * Larval stage	25	576.879	9.809	<0.0001
Dose * Larval stage	15	549.150	15.564	<0.0001
Day * Dose * Larval stage	75	512.369	2.904	<0.0001
Error	576	1354.892	-	-
Total	719	9516.741	-	-

Mortality rates showed significant variation across days post-application ($F_{5, 576}$ = 216.255, p < 0.0001), doses applied ($F_{3, 576}$ = 236.563, p< 0.0001), and larval stages ($F_{5, 576}$ = 122.409, p < 0.0001), with significant interactions observed among these factors. A significant day-by-dose interaction ($F_{15, 576}$ = 24.685, p < 0.0001) revealed changes in dose effectiveness over time. The interaction between the day and larval stage ($F_{25, 576}$ = 9.809, p < 0.0001) suggested that the impact of time on mortality varied by developmental stage. Similarly, a dose-by-larval stage interaction ($F_{15, 576}$ = 15.564, p < 0.0001) indicated dose effectiveness differences across stages. A three-way interaction among the day, dose, and larval stage ($F_{75, 576}$ = 2.904, p < 0.0001) highlighted the complex interaction in which timing, spore concentration, and developmental stage collectively shaped mortality outcomes. These findings demonstrate the significant influence of dose, application timing, and larval stage on *H. armigera* mortality under *B. bassiana* exposure, with combined interactions among these factors.

Figure 1 presents the mortality rates of *H. armigera* larvae at different instars when exposed to varying spore concentrations (1×10^6, 1×10^7, and 1×10^8 conidia ml^{-1}) of *B. bassiana*. Tukey's HSD test results revealed statistically significant differences among treatments and larval stages. Data analysis showed a clear pattern of higher mortality in younger instars compared to older instars, indicating that susceptibility to *B. bassiana* decreases as larvae progress through their developmental stages. At the lowest concentration of 1×10^6 conidia ml^{-1}, mortality rates were notably higher in the first and second instars, with a marked decline in effectiveness observed in the later stages, particularly in the fifth and sixth instars. Although overall mortality rates were slightly higher than at the lower concentration, this trend was still visible at 1×10^7 conidia ml^{-1}, where early instars were maintained to show higher mortality than more mature stages. At the highest concentration, mortality was consistently high in early instars yet still reduced considerably in the fifth and sixth instars, underscoring the reduced susceptibility of older larvae. The results indicated that increasing the spore concentration enhanced the overall mortality rate, though the effect was particularly remarkable in the younger instars.

The results in Table 2 demonstrate the lethal concentration (LC_{50} and LC_{90}) values of *B. bassiana* applied to different larval stages of *H. armigera* over time. For each larval stage, LC_{50} and LC_{90} values decreased with an increase in days post-application, indicating a time-dependent increase in fungal pathogenicity. In particular, younger instars (L1, L2, and L3) were more susceptible to *B. bassiana*, as reflected by the relatively lower LC_{50} and LC_{90} values compared to older instars (L4, L5, and L6). For instance, mortality rates reached 100% at the 1×10^7 and 1×10^8 concentrations 10 days after treatment in first-instar larvae. At the lowest concentration, a mortality rate of 96% was observed by the end of two weeks (Figure 2). For first-instar larvae (L1), the estimated LC_{50} and LC_{90} values at the end of the two

weeks were 3.65×10^3 and 2.03×10^5, respectively. In second-instar larvae (L2), mortality rates reached 100% by 14 days across all concentrations, with no statistically significant differences observed ($\chi2 = 7.93$, df = 13, $p = 0.848$). 10 days post-application, mortality rates were 96% (1×10^8), 72% (1×10^7), and 60% (1×10^6), respectively (Figure 2). For second-instar larvae, the estimated LC_{50} and LC_{90} values after 10 days were 5.86×10^5 and 5.13×10^7. For third-instar larvae (L3), mortality rates by the end of 14 days were 100% at 1×10^8, 84% at 1×10^7, and 72% at 1×10^6 (Figure 2). The estimated LC_{50} and LC_{90} values for the third instar were 6.88×10^8 and 1.94×10^4. In fourth-instar larvae (L4), mortality rates at two weeks post-application were 84%, 48%, and 32% from the highest to the lowest concentrations (Figure 2). The average LC_{50} and LC_{90} values at the end of 14 days were 6.32×10^6 and 3.88×10^8, respectively. Finally, the mortality rates of older (L5 and L6) instar larvae were significantly lower than those of earlier stages and were statistically grouped as similar.

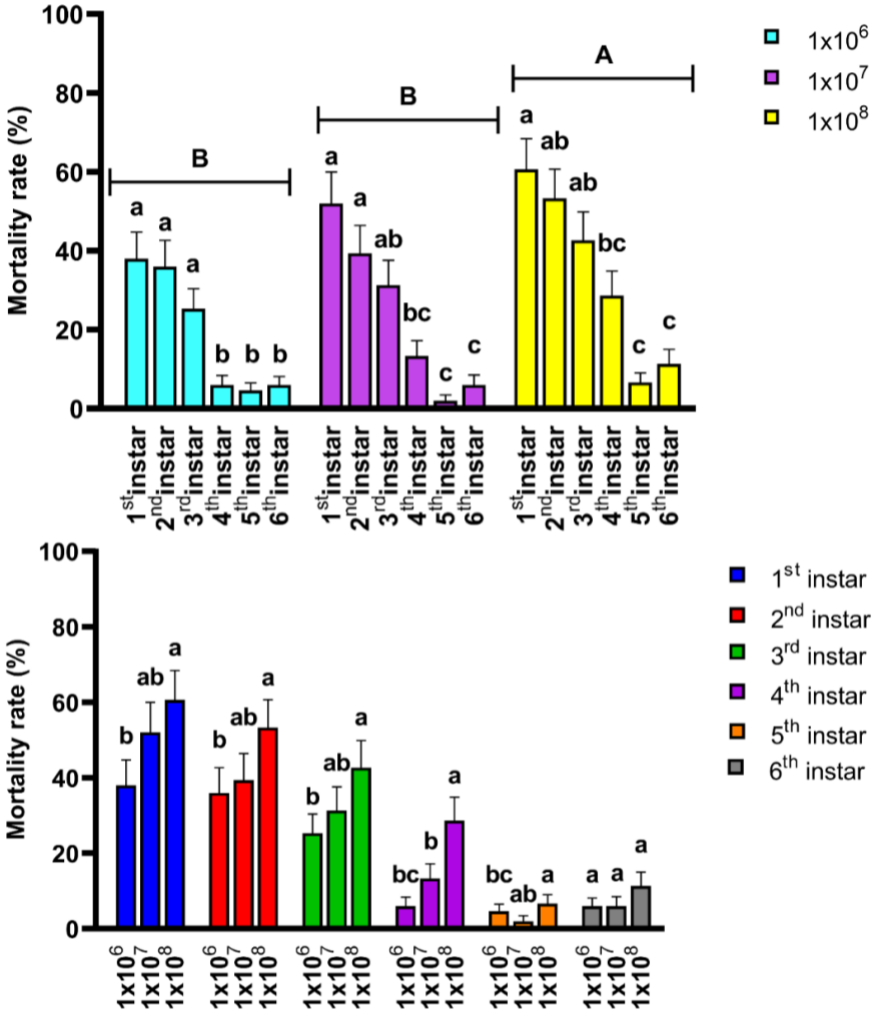

Figure 1. Mortality rates (±SE) of *Helicoverpa armigera* larvae across six instars treated with different concentrations of *Beauveria bassiana*. Significant differences between concentrations and larval instars as indicated by different lowercase letters. Significant differences between fungal concentrations were indicated by the uppercase letters (Tukey's HSD at $p < 0.05$).

Table 2. Lethal concentration (LC_{50} and LC_{90}) values of *Beauveria bassiana* tested against larval stages of *Helicoverpa armigera*

Larval Stage	Days	LC_{50} (conidia ml^{-1})	LC_{90} (conidia ml^{-1})	Slope±SE	Intercept±SE	$\chi 2$ (*df* = 13)	*p*
L1	3	6.62×10^{15}	5.61×10^{22}	0.18±0.07	-2.93±0.47	233.46	0.0001
	5	9.58×10^{7}	2.13×10^{11}	0.38±0.04	-3.06±0.29	221.40	0.0001
	7	9.81×10^{5}	3.82×10^{7}	0.80±0.05	-4.82±0.35	156.39	0.0001
	10	9.63×10^{5}	2.55×10^{6}	3.02±0.43	-18.10±2.60	147.96	0.0001
	14	3.65×10^{3}	2.03×10^{5}	0.74±0.19	-2.61±1.19	93.95	0.0001
L2	3	2.85×10^{8}	1.10×10^{9}	2.17±0.49	-18.46±3.98	83.61	0.0001
	5	5.09×10^{9}	6.68×10^{14}	0.25±0.04	-2.43±0.30	272.46	0.0001
	7	1.10×10^{7}	1.41×10^{10}	0.41±0.04	-2.90±0.28	92.48	0.0001
	10	5.86×10^{5}	5.13×10^{7}	0.66±0.05	-3.80±0.34	103.91	0.0001
	14	6.77×10^{1}	2.56×10^{2}	0.50±0.76	-0.08±4.73	7.93	0.848
L3	3	1.30×10^{8}	3.28×10^{8}	3.18±0.86	-25.81±6.90	326.49	0.0001
	5	6.53×10^{8}	1.43×10^{12}	0.38±.044	-3.38±0.31	562.24	0.0001
	7	3.94×10^{8}	1.62×10^{12}	0.35±0.04	-3.04±0.30	538.04	0.0001
	10	4.64×10^{6}	1.70×10^{12}	0.05±0.04	-0.33±0.27	314.86	0.0001
	14	6.88×10^{8}	1.94×10^{4}	0.28±0.04	-2.49±0.29	368.82	0.0001
L4	3	-	-	-	-	-	-
	5	2.07×10^{12}	4.86×10^{15}	0.38±0.09	-4.68±0.72	231.48	0.0001
	7	2.96×10^{8}	3.32×10^{9}	1.22±0.11	-10.34±0.82	155.52	0.0001
	10	6.53×10^{7}	1.57×10^{9}	0.93±0.05	-7.26±0.39	184.09	0.0001
	14	6.32×10^{6}	3.88×10^{8}	0.72±0.04	-4.87±0.30	147.55	0.0001
L5	3	-	-	-	-	-	-
	5	-	-	-	-	-	-
	7	-	-	-	-	-	-
	10	5.80×10^{6}	1.04×10^{7}	0.63±0.06	-1.61±0.44	232.39	0.0001
	14	9.62×10^{10}	2.09×10^{17}	0.20±0.04	-2.22±0.31	198.87	0.0001
L6	3	-	-	-	-	-	-
	5	-	-	-	-	-	-
	7	-	-	-	-	-	-
	10	1.29×10^{18}	1.29×10^{28}	0.12±0.05	-2.16±0.39	176.30	0.0001
	14	1.39×10^{8}	1.26×10^{12}	0.32±0.04	-2.64±0.29	155.02	0.0001

* Each concentration was tested using 25 larvae, divided into five replicates, each consisting of five larvae.

** Cells left blank in the table indicate instances where no or fewer mortality events were observed, and thus values could not be calculated for the respective days post-treatment.

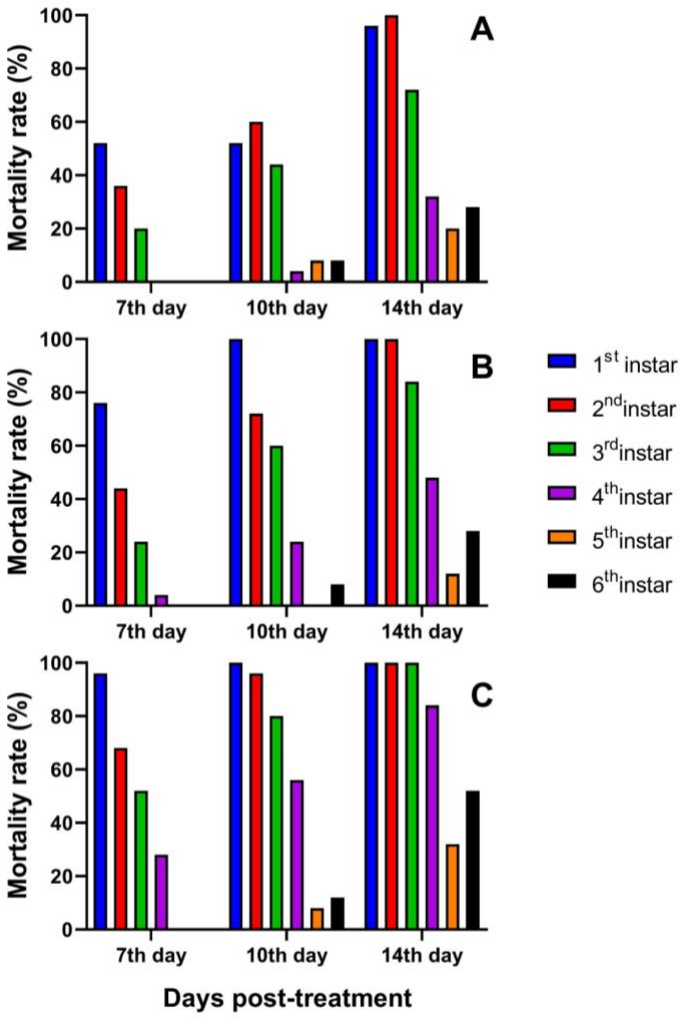

Figure 2. Mortality rates of *Helicoverpa armigera* larvae for six instars at three doses of *Beauveria bassiana* on the 7th, 10th, and 14th days post-treatment (A: $1x10^6$, B: $1x10^7$, C: $1x10^8$ conidia ml^{-1}).

The estimated LT_{50} and LT_{90} values based on the mortality trends across different concentrations were presented in Table 3. Increasing conidial concentrations reduced the LT_{50} and LT_{90} estimates and increased the larval mortality percentages (Figure 3). The LT_{50} of *B. bassiana* against larval instars of *H. armigera* ranged from 7.53 to 20.87 days at $1×10^6$ conidia ml^{-1}, while at the highest concentration, values varied from 4.05 to 18.13 days. Likewise, LT_{90} values at the lowest concentration ranged from 13.06 to 39.01 days, compared to 8.10 to 32.79 days at the highest concentration. The results revealed a concentration-dependent effect on the time required to achieve 50% (LT_{50}) and 90% (LT_{90}) mortality, with shorter LT values observed at higher concentrations for each larval stage. For instance, in the first instar (L1), LT_{50} decreased from 7.53 days at $1×10^6$ conidia ml^{-1} to 4.05 days at $1×10^8$ conidia ml^{-1}, while LT_{90} reduced from 14.99 days to 8.10 days over the same concentration range, indicating a rapid mortality effect at higher fungal doses. Similarly, in the second instar (L2), LT_{50} values decreased from 7.84 to 4.99 days with increasing concentration, showing that younger larvae respond more quickly to higher fungal doses. LT_{90} values for this instar ranged from 10.45 to 13.06 days. In the case of third-instar larvae, LT_{50} values for the $1×10^6$, $1×10^7$, and $1×10^8$ concentrations were 10.43, 8.82, and 7.50 days, respectively, while the LT_{90} values were 21.65, 16.57, and 13.80 days.

Table 3. Estimates of lethal time (LT_{50} and LT_{90}) values of *Beauveria bassiana* tested against larval stages of *Helicoverpa armigera*

Larval Stage	Concentration (conidia ml^{-1})	LT_{50} (days) (CI)	LT_{90} (days) (CI)	Slope±SE	Intercept±SE	χ^2 (*df*=28)	*p*
L1	1×10^6	7.53 (6.50-8.77)	14.99 (12.10-21.91)	4.28±0.15	-3.75±0.13	535.61	0.0001
	1×10^7	5.50 (5.09-6.01)	8.09 (7.32-9.39)	7.80±0.28	-5.81±0.22	328.27	0.0001
	1×10^8	4.05 (2.66-5.51)	8.10 (5.89-16.69)	4.25±0.13	-2.58± 0.09	1885.68	0.0001
L2	1×10^6	7.84 (7.31-8.42)	13.06 (11.75-15.09)	5.78±0.20	-5.17±0.18	194.13	0.0001
	1×10^7	7.32 (6.81-7.87)	11.76 (10.63-13.52)	6.23±0.21	-5.38±0.19	222.53	0.0001
	1×10^8	4.99 (3.34-6.79)	10.45 (7.51-24.39)	3.99±0.12	-2.79± 0.10	1868.96	0.0001
L3	1×10^6	10.43 (9.37-11.92)	21.65 (17.56-30.25)	4.03±0.16	-4.11±0.15	241.69	0.0001
	1×10^7	8.82 (7.87-10.01)	16.57 (13.78-22.44)	4.68±0.17	-4.42±0.16	376.48	0.0001
	1×10^8	7.50 (7.00–8.10)	13.80 (12.90–14.75)	4.75±0.15	-4.30± 0.04	1846.92	0.0001
L4	1×10^6	18.90 (16.47-24.14)	34.46 (26.31-57.29)	4.91±0.40	-6.27±0.43	98.02	0.0001
	1×10^7	14.52 (12.35-19.72)	28.05 (20.41-57.69)	4.48±0.23	-5.21±0.24	385.96	0.0001
	1×10^8	9.31 (8.66-10.07)	15.44 (13.73-18.27)	5.83±0.21	-5.65± 0.20	211.39	0.0001
L5	1×10^6	20.87 (17.16-32.60)	39.01 (26.96-94.69)	4.71±0.43	-6.22±0.46	135.87	0.0001
	1×10^7	27.77 (19.09-37.82)	53.46 (27.97-57.45)	4.50±0.66	-6.50±0.73	175.55	0.0001
	1×10^8	18.13 (16.02-22.29)	32.79 (25.66-50.74)	4.98±0.38	-6.26± 0.41	93.16	0.0001
L6	1×10^6	18.92 (16.48-24.14)	34.54 (26.37-57.21)	4.90±0.40	-6.25±0.42	96.31	0.0001
	1×10^7	18.92 (15.87-28.02)	34.54 (24.58-78.52)	4.90±0.40	-6.25±0.42	179.31	0.0001
	1×10^8	14.70 (12.24-23.04)	25.67 (18.33-74.94)	5.29±0.28	-6.18±0.29	675.44	0.0001

* Each concentration was tested using 25 larvae, divided into five replicates, each consisting of five larvae.

The trend continues across all instars, though older larval stages (L4, L5, and L6) generally exhibited higher LT_{50} and LT_{90} values, reflecting their enhanced resistance to fungal infection compared to younger instars. For example, in fourth-instar larvae (L4), LT_{50} ranged from 9.31 days at 1×10^8 conidia ml^{-1} to 18.90 days at 1×10^6 conidia ml^{-1}, demonstrating the extended time required to induce mortality in more mature larvae. Finally, for fifth (L5) and sixth-instar larvae (L6), where no statistically significant differences were observed, LT_{50} values at the 1×10^6 conidia ml^{-1} ranged from 18.92 to 20.87 days, with LT90 values ranging from 34.54 to 39.01 days. At the highest concentration, LT_{50} values varied between 14.70 and 18.92 days, while LT_{90} values ranged from 25.67 to 32.79 days. In summary, the first instar larvae exhibited the shortest LT_{50} and LT_{90} periods at the highest concentration (1×10^8 conidia ml^{-1}), with values of 4.05 and 8.10 days, respectively. Larval mortality rates increased while both LT_{50} and LT_{90} periods decreased as the concentration increased (Table 3). The dose effect was particularly significant in the third and fourth instar larvae, with the highest dose resulting in LT_{50} and LT_{90} values of 7.50 and 13.80 days for the third instar and 9.31 and 15.44 days for the fourth instar, respectively (Table 3, Figure 3).

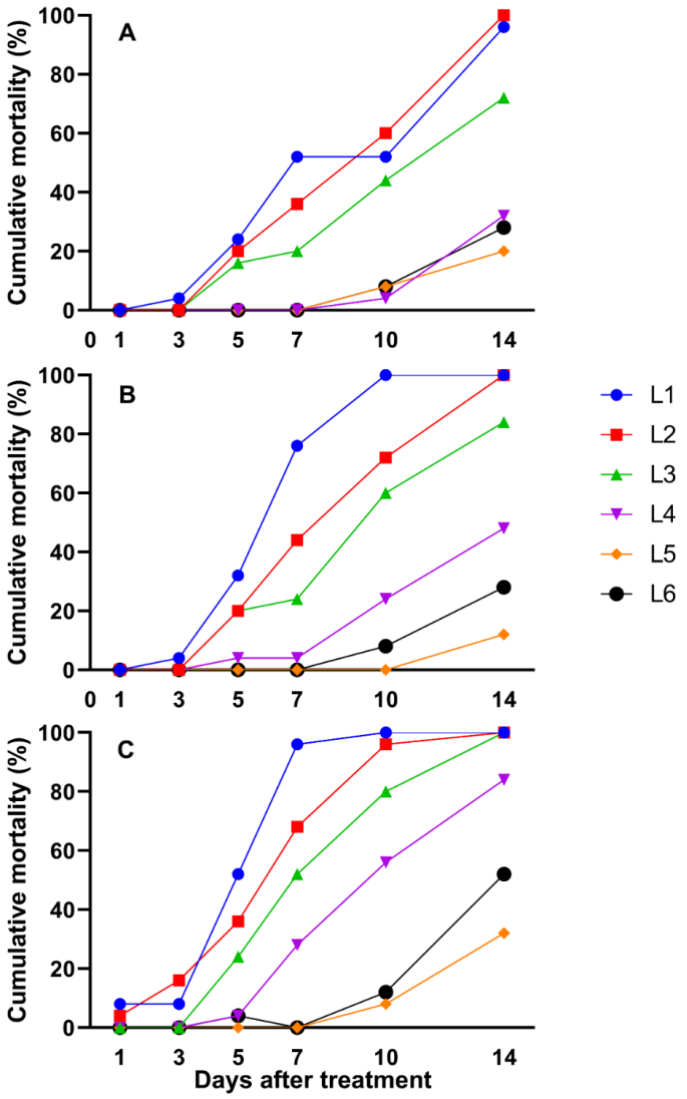

Figure 3. The efficacy of *Beauveria bassiana* against larval stages (L1, L2, L3, L4, L5, L6) of *Helicoverpa armigera* (A: 1×10^6 conidia ml^{-1}, B: 1×10^7 conidia ml^{-1}, 1×10^8 conidia ml^{-1}).

Discussion

The present study highlights the effectiveness of the entomopathogenic fungus *B. bassiana* against various larval stages of the highly destructive polyphagous pest, *H. armigera*. Our findings from the screening study demonstrated *B. bassiana* as pathogenic to the larvae of *H. armigera*. Previous studies have indicated the potential of entomopathogenic fungi, particularly *B. bassiana*, in controlling *H. armigera*. (Sandhu et al., 2001; Nguyen et al., 2007; Alwaneen et al., 2024). However, exotic strains of entomopathogenic fungi developed for pest control may not exhibit effectiveness against insect pests, influenced by the ecological and genetic conditions of the fungal strain (Bidochka et al., 1998). Therefore, it is essential to study the spatial distribution and prevalence of local entomopathogenic fungi to integrate them into pest management programs effectively (Tahir et al., 2019). Differences in virulence among fungal strains of the same species can result from genetic variations associated with their geographical distribution (Coates et al., 2002). Conducting laboratory screenings is crucial to identify virulent strains before their application in field conditions (Cherry et al., 2005).

In our study, *B. bassiana* infected all larval stages of *H. armigera*. However, the younger instars (L1, L2, L3) were particularly susceptible to fungal infection. These larval stages reached 100% mortality at a concentration of 1×10^8 conidia ml^{-1}, while the fourth larval stage (L4) was less susceptible (%84) compared to the first three larval instars. In the older larval stages (L5 and L6), the mortality rates remained low percentages. Insects exhibit varying susceptibility to infection by entomopathogenic fungi of the Hyphomycetes class across different developmental stages (Inglis et al., 2001). Wilson et al. (2001) reported that the level of resistance in caterpillars against pathogens and parasites is positively associated with the occurrence of melanism in the cuticle and midgut. Melanin protects the insect's cuticle, thereby preventing the penetration of parasites and pathogens. Previous studies consistently highlight the higher efficacy of *B. bassiana* against younger instars across various insect species. For example, melanin levels, which contribute to cuticle severity, vary significantly across the larval stages of the cotton leafworm, *Spodoptera littoralis* Boisd*.* (Lepidoptera: Noctuidae), with a notable increase from the late fourth to mid-sixth instar, especially when larvae were reared individually (Lee & Wilson 2006). This increase in cuticle melanin can correlate with greater resistance to fungal infections in older stages. Similarly, Hafez et al. (1997) reported that the younger instars of the potato tuber moth, *Phthorimaea operculella* (Zeller, 1873) (Lepidoptera: Gelechiidae), exhibited greater susceptibility to *B. bassiana* compared to the later larval stages, while Vandenberg et al. (1998) observed that third and fourth larval stages of the *Plutella xylostella* (L., 1767) (Lepidoptera: Plutellidae) were more vulnerable than second instars. In the case of *H. armigera* larvae, Nguyen et al. (2009) reported high mortality in second to fifth instars when treated with 1×10^7 conidia ml^{-1}, with second instars showing the shortest lethal time, possibly due to their brief developmental duration of about two days. Similarly, Tahir et al. (2019) found that the *B. bassiana* isolate WG-18 was highly effective against younger instars, though fourth instars exhibited moderately lower mortality compared to second instars. Alwaneen et al. (2024) also noted reduced pupation and adult emergence rates in second instar *H. armigera* treated with *B. bassiana* compared to treatments at the fourth instar, further supporting the trend of higher susceptibility in earlier stages. Increasing spore concentrations not only improved the mortality rate but also accelerated the speed of action, as reflected by reduced LT_{50} and LT_{90} values at higher doses. This indicates that higher fungal doses can provide more rapid control, particularly in the early larval stages where *B. bassiana* susceptibility is greatest (Altinok et al., 2019). However, older instars showed greater resistance, suggesting that biological control may be less effective as larvae mature. The composition of their outer integument becomes less penetrable to fungal infections as larvae mature. This reduced susceptibility may explain the higher mortality observed in early instars, where the fungus can invade more effectively (Idrees et al., 2021; Bosa et al., 2024). Targeting these younger stages could thus maximize the impact of *B. bassiana* in integrated pest management programs.

The results further underscore the potential of entomopathogenic fungi as sustainable alternatives to chemical pesticides. Excessive chemical pesticide has led to environmental contamination, harm to non-target species, and increased pest resistance (Ahmad et al., 2003; Walsh et al., 2022). In contrast, *B. bassiana* offers an eco-friendly approach that preserves natural predators and promotes agroecosystem biodiversity (Meyling & Eilenberg, 2007). Integrating biological agents into IPM strategies contributes to reducing pest resistance and decrease dependence on chemical controls (Bale et al., 2008). Entomopathogenic fungi such as *B. bassiana* were increasingly recognized for their role in pest management due to their ability to infect and kill target insects through natural infection processes (Goettel & Glare, 2010; Inglis et al., 2012). Additionally, they can be applied in various formulations (e.g., sprays, wettable powder, or even seed treatments) to control pest populations effectively within IPM frameworks (García-Estrada et al., 2016; Mascarin & Jaronski, 2016; Darsouei et al., 2024). Furthermore, various plants have been shown to be colonized by *B. bassiana* as an endophyte, a relationship linked with decreased herbivore damage. For example, endophytic *B. bassiana* has been linked to lower feeding by pests such as *Ostrinia nubilalis* Hübner, 1796 (Lepidoptera: Crambidae) in maize, *Helicoverpa zea* Boddie, 1850 (Lepidoptera: Noctuidae) in tomato (Jaber & Ownley, 2018). Fungal endophyte infections in plants

negatively impact insect development (Christian et al., 2020; Silva et al., 2020) but also deter pest feeding. For instance, the larvae of *Helicoverpa gelotopoeon* Dyar, 1921 (Lepidoptera: Noctuidae) and *H. zea* prefer uninfected plants over those harboring fungal endophytes (Russo et al., 2019; Castillo Lopez & Sword, 2015). Likewise, *B. bassiana*-infected maize shows reduced feeding by *Sesamia calamistis* Hampson, 1910 (Lepidoptera: Noctuidae) (Cherry et al., 2004) and *O. nubilalis* (Bing & Lewis, 1991). Overall, this study provides key insights for incorporating *B. bassiana* into IPM strategies, particularly through targeted application in the early larval stages to improve pest control. Future research should explore the efficacy of *B. bassiana* across diverse environmental conditions and crop systems, supporting its broader adoption as a fungal biopesticide in sustainable agriculture.

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

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4. Daha önce Kongre/Sempozyum vs. de sözlü/poster bildiri olarak sunulmuş ancak sadece kısa özet olarak basılmış eserler, dipnotta belirtilmesi koşuşuyla kabul edilir.
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