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Original article (Orijinal araştırma)

Seasonal occurrence and prevalence of the invasive spider mite *Eutetranychus orientalis* (Klein, 1936) and the citrus red mite *Panonychus citri* (McGregor, 1916) (Prostigmata: Tetranychidae) in citrus-growing and recreational areas of Adana (Türkiye)[1]

Adana ili (Türkiye) turunçgil yetiştirilen ve rekreasyon alanlarında istilacı akar türü *Eutetranychus orientalis* (Klein, 1936) ve turunçgil kırmızı örümceği *Panonychus citri* (McGregor, 1916) (Prostigmata: Tetranychidae)'nin mevsimsel görülme sıklığı ve yaygınlığı

Kemal YALÇIN[2] **Cengiz KAZAK**[3*]

Abstract

In this study, surveys were conducted every two or three weeks to evaluate the prevalence of the invasive spider mite *Eutetranychus orientalis* (Klein, 1936) (Prostigmata: Tetranychidae) and the citrus red mite *Panonychus citri* (McGregor, 1916) (Prostigmata: Tetranychidae), as well as their population developments in citrus and sour orange (*Citrus aurantium* L.) growing in recreational (non agricultural - residential) areas in Adana between 2019 and 2022. The host preference of *E. orientalis* varied among citrus species, with the pest predominantly found on lemons (*C. limon* (L.) Burm. f.) (62%), followed by oranges (*C. sinensis* (L.) Osbeck) (26%) and mandarins (*C. reticulata* Blanco) (12%), but no infestations were recorded on grapefruits (*C. paradisi* Macf.). Similarly, *P. citri* was most commonly observed on lemons (58%), followed by mandarins (19%), oranges (17%), and grapefruits (6%). The highest *E. orientalis* population densities were observed in December, while the pest was either absent or at low densities during the summer. Regardless of citrus species, *P. citri* did not establish noticeable populations in any of the orchards where *E. orientalis* was detected. On sour orange, *E. orientalis* showed a similar seasonal abundance to those observed in other citrus species, whereas *P. citri* was unable to establish a population. Likewise, the majority of *E. orientalis* preferred to feed and colonize on the adaxial side of the leaf in all citrus species. Predatory mite populations remained at low densities across all sampling sites and did not exhibit a consistent population pattern. Regarding the population developments of *E. orientalis*, the most frequent Phytoseiidae species were *Amblyseius swirskii* Athias-Henriot, 1962, *Euseius scutalis* (Athias-Henriot, 1958) and *Typhlodromus athiasae* Porath & Swirski, 1965 (Mesostigmata: Phytoseiidae).

Keywords: Citrus, *Eutetranychus orientalis*, *Panonychus citri*, population dynamics, seasonal abundance

Öz

Bu çalışmada, Adana ilinde turunçgil yetiştiriciliği yapılan bahçeler ve rekreasyon amaçlı turunç (*Citrus aurantium* L.) yetiştirilen alanlarda *Eutetranychus orientalis* (Klein, 1936) (Prostigmata: Tetranychidae) ve turunçgil kırmızı örümceği *Panonychus citri* (McGregor, 1916) (Prostigmata: Tetranychidae)'nin yaygınlığı ve popülasyon gelişmelerinin belirlenmesi için 2019 - 2022 yılları arasında iki–üç haftada bir alınan örnekler ile periyodik surveyler gerçekleştirilmiştir. *E. orientalis*'in konukçu tercihi turunçgil türlerine göre değişiklik göstermiş, zararlının bulaşıklığı en çok limonlarda (*C. limon* (L.) Burm. f.) (%62) saptanmış, bunu portakal (*C. sinensis* (L.) Osbeck) (%26) ve mandalina (*C. reticulata* Blanco) (%12) izlemiş; greyfurt (*C. paradisi* Macf.) bahçelerinde ise zararlıya rastlanmamıştır. Benzer şekilde, *P. citri* bulaşıklığı da en yüksek limonda (%58) gerçekleşmiş, bunu mandalina (%19), portakal (%17) ve greyfurt (%6) izlemiştir. *Eutetranychus orientalis* popülasyon yoğunlukları en yüksek Aralık ayında gözlenirken yaz aylarında zararlı popülasyonuna ya hiç rastlanmamış ya da düşük yoğunluklarda bulunmuştur. Turunçgil türlerinden bağımsız olarak *P. citri*, *E. orientalis* saptanan bahçelerin hiçbirinde yüksek popülasyonlar oluşturmamıştır. *Eutetranychus orientalis* turunçta diğer turunçgil türlerine benzer bir populasyon gelişmesi göstermiş, *P. citri* popülasyon oluşturamamıştır. Benzer şekilde tüm turunçgil türlerinde *E. orientalis* popülasyonunun çoğunluğu yaprak üst yüzeyinde beslenmeyi ve koloni oluşturmayı tercih etmiştir. Avcı akar popülasyonları tüm örnekleme alanlarında düşük yoğunluklarda kalarak tutarlı bir popülasyon gerlişmesi göstermemiştir. *Eutetranychus orientalis* popülasyon gelişmesinin izlendiği bahçelerde en sık görülen Phytoseiidae türleri *Amblyseius swirskii* Athias-Henriot, 1962, *Euseius scutalis* (Athias-Henriot, 1958) ve *Typhlodromus athiasae* Porath & Swirski, 1965 (Mesostigmata: Phytoseiidae) olmuştur.

Anahtar sözcükler: Turunçgil, *Eutetranychus orientalis*, *Panonychus citri*, popülasyon dinamiği, sezonsal bolluk

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Introduction

Citrus, *Citrus* spp. (Sapindalaes: Rutaceae) is one of the most widely cultivated and consumed fruit globally. Türkiye ranks eighth in global citrus production, with most of it concentrated in the eastern Mediterranean region, particularly around Adana (TEBGE, 2024; USDA, 2024). In addition to insect pests, numerous mite species have been documented in association with citrus across global citrus-growing regions. However, only a limited number of these species are recognized as causing significant economic damage (McMurtry, 1977; Vacante, 2010). Recently, several mite species that were once not considered economically significant have expanded their range due to factors such as extensive pesticide use, the pronounced effects of climate change, and the international movement of plant material (Yalçın et al., 2022). One such species, the citrus brown mite, *Eutetranychus orientalis* (Klein, 1936) (Prostigmata: Tetranychidae), has become a major invasive pest, posing a substantial threat to citrus production in over 40 countries (Migeon & Dorkeld, 2021).

Eutetranychus orientalis, a phytophagous mite, was first described in the Middle East by Klein in 1936 (Klein, 1936). Although its presence in Turkish citrus orchards was initially noted by Jeppson et al. (1975), detailed information about the pest remained scarce until Çobanoğlu & Can (2014) confirmed its establishment in Türkiye based on samples from citrus orchards in the Kumluca district of Antalya. Subsequent surveys across the Eastern Mediterranean have highlighted *E. orientalis* among the most prevalent mite species affecting citrus plantations. This species frequently coexists with various citrus pests, such as the citrus bud mite *Aceria sheldoni* (Ewing, 1937), rust mite *Phyllocoptruta oleivora* (Ashmead, 1879) (Prostigmata: Eriophyidae) and citrus red mite *Panonychus* citri (McGregor, 1916) (Prostigmata: Tetranychidae).

Although *E. orientalis* primarily infests citrus, it is capable of developing on a broad spectrum of host plant species, including soft and stone fruits, various forest trees, and ornamental plants (Dhooria, 2003; Yeşilayer & Çobanoğlu, 2010; Migeon & Dorkeld, 2014; Elhalawany, 2019; Amer, 2020). At high population densities, *E. orientalis* may also damage fruits and young shoots, leading to fruit drop. If control measures are not taken in a timely manner, this will eventually lead to plant death (Jeppson et al., 1975; Ferragut et al., 2013; El-Sharabasy, 2015). Vela et al. (2017) reported that the population of *E. orientalis* in Spain reached its highest density once a year, exclusively during the autumn season. Likewise, the pest population in orange, grapefruit, and lemon orchards peaked annually between August and September in Egypt (Halawa et al. 2020). In contrast, Chouikhi et al. (2022) found that in Tunisia, the population of *E. orientalis* showed four distinct peak periods each year, occurring in February, March, April, and May.

Although several methods are available for controlling *E. orientalis*, chemical control remains the most commonly employed strategy among citrus growers in Türkiye and the rest of the world (Halawa et al., 2014; Heikal et al., 2019). Among these methods, biological control holds significant potential as an environmentally sustainable and eco-friendly approach to mite management. Mites belonging to the Phytoseiidae family (Mesostigmata) are essential natural enemies contributing significantly to managing mite pest populations in agricultural and horticultural systems. Research has demonstrated that species such as *Amblyseius swirskii* Athias-Henriot, 1962 and *Euseius scutalis* (Athias-Henriot, 1958) can effectively manage *E. orientalis* under controlled conditions (Ali & Zaher, 2007; Momen & Abdel-Khalek, 2009; Nawar, 2017; Al-Azzazy & Alhewairini, 2020). Research on the field efficacy of these species in controlling *E. orientalis* remains scarce (Yalçın et al., 2023).

Panonychus citri is considered to be another major pest in citrus-growing regions worldwide, including the Eastern Mediterranean region of Türkiye (McMurtry, 1969; Jeppson et al., 1975). This species was first recorded in 1952 in Türkiye, but it was initially not considered a significant pest. However, recent outbreaks associated with ecosystem disturbances, often caused by the use of broad-spectrum pesticides that reduce natural enemy populations, have increased its importance as a primary pest (Düzgüneş, 1977; Kasap et al., 2009; Schmidt-Jeffris, 2023). The mite primarily infests citrus species such as lemons, oranges, grapefruits and mandarins, causing leaf bronzing during peak population periods on spring and autumn (Kasap, 2005). According to Furuhashi (1980), *P. citri* populations on citrus trees in Japan generally display a bimodal pattern, with population peaks occurring in June–July and October–November, while maintaining low densities during the midsummer and winter months. Similarly, Vela et al. (2017) observed that in Spain, the population of *P. citri* in citrus orchards reached its peak twice annually, specifically in June and September. *P. citri* population development is closely linked to temperature, with peaks in early summer and autumn during shoot growth and declines in mid-summer and winter (Jeppson et al., 1975; Zanardi et al., 2015).

Recent preliminary studies have indicated an increasing population density of *E. orientalis* in citrus- growing areas of the Eastern Mediterranean region of Türkiye. However, there is a lack of detailed information on the pest's prevalence on its primary host, citrus, as well as its species-specific distribution and seasonal occurrences in Türkiye. Therefore, this study aims to assess the prevalence of the pest in citrus within Adana province and its districts, analyze the infestation rates by citrus species, and to monitor the population development of three citrus species in four different areas with minimal to no pesticide application in comparison to the seasonal occurrence of *P. citri.*

Material and Methods

Prevalence of *Eutetranychus orientalis* and *Panonychus citri*

In this study, the prevalence of *E. orientalis* and *P. citri* on lemon, *Citrus limon* (L.) Osbeck; oranges, *Citrus sinensis* (L.) Osbeck; mandarins, *Citrus reticulata* Blanco and grapefruits, *Citrus paradisi* Macf. (Sapindales: Rutaceae) in various districts of Adana was assessed in relation to the infestation rates of these pests, with a primary focus on commercial citrus orchards. To achieve this aim, extensive surveys were conducted in the districts of Ceyhan, Sarıçam, Yüreğir, Karataş, Kozan, Seyhan, and Yumurtalık regions renowned for their substantial citrus production. The surveys were conducted between November 2019 and January 2022, during which a total of 190 citrus orchards were sampled, representing a broad range of citrus species regardless of variety. The highest number of samples was collected from Yüreğir district, with 80 orchards, followed by Seyhan, Kozan, Karataş, Ceyhan, Sarıçam, and Yumurtalık, based on the distribution of citrus orchards on each area. The total number of trees sampled was determined to represent at least 0.01% of the citrus trees in each district, following the methodology of Lazarov & Grigov (1961).

During sampling, leaves were randomly collected from citrus trees, with four specimens obtained from the outer canopy across different orientations and one from the inner canopy, resulting in five leaves per tree. Within each sampling unit (orchard), 100 leaf samples were collected from 20 trees. The samples were then stored in labeled polyethylene bags and transported to the laboratory using an ice box. The counts were conducted directly under stereo binocular microscope using a 6 cm² template, which included three marked 1 cm² sections on both the adaxial and abaxial leaf surfaces. During the counts, the egg and motile stages (larvae, protonymph, deutonymph, male and females) of *E. orientalis* and *P. citri*, as well as any predatory mites present, were recorded separately. The orchard was considered infested if any stage of *E. orientalis* or *P. citri* occurred in a sample. Additionally, the GPS coordinates of each sampled orchard were documented during the survey.

Seasonal occurrance of *Eutetranychus orientalis*, *Panonychus citri* and phytoseiid predatory mites

The seasonal occurrence of *E. orientalis* and *P. citri* was monitored in four different areas between November 2019 and January 2022. The first two studies conducted in an experimental citrus orchard at Çukurova University (Ç.Ü.), Adana. Monitoring focused on lemon (*Citrus limon* (L.) Burm cv. Interdonato) and orange (*C. sinensis* (L.) Osbeck cv. Yafa) species in this area. The third and fourth study sites consisted of sour oranges (*C. aurantium* L.) planted for recreational purposes in both Seyhan district of Adana and Ç.Ü campus areas, respectively. The studies prioritized areas where pesticides were either not used or applied sparingly. In this regard, only a single application of abamectin combined with petroleum oil was applied in May 2021 at the experimental orchard (Table 1).

The seasonal occurrance of both mite species on orange and lemon was monitored in 1 and 2 decare (da) of >20-year-old orchards with an area of 8 da, in Balcalı-Adana. Additionally, the population of the pests on sour orange was monitored on 20 trees in a 1 km-long row of trees in Seyhan district and Çukurova University campus area (Table 1). Based on the biology of *E. orientalis*, leaf samples were collected at 10-15 day intervals during the fall and winter months, when the pests were more abundant, and at 15-20 day intervals during the spring and summer months, when their presence was less frequent (Yalçın et al, 2022; Viola et al, 2023). A total of 100 leaf samples were taken from 20 trees on each of the sampling date in each orchard.

Table 1. Sampling sites information for monitoring the seasonal occurrence of *Eutetranychus orientalis* and *Panonychus citri*

Orchard	No. of trees	Vegetation	Coordination
Lemon (2 da) (>20 yr)	60	Single	37°01'39.2"N 35°21'43.0"E
Orange (1 da) (>20 yr)	30	Single	37°01'39.2"N 35°21'43.0"E
Sour orange (≈15 yr)	200 (km^{-1})	High vegetation	37°03'35.3"N 35°21'35.2"E
Sour orange (≈15 yr)	200 (km^{-1})	Road side	37°01'46.5"N 35°18'39.6"E

The sampling method and the number of samples taken were consistent with those used in the studies on the prevalence of *E. orientalis* and *P. citri*. The results are presented as the mean number of eggs and motile stages per 1 cm² on both sides of the leaves, including the adaxial and abaxial surfaces. During the counts, naturally ocurring predatory mites from the Phytoseiidae family, which were encountered and potentially associated with the pests, were preserved in 70% alcohol for identification purposes, as in the survey. Permanent preparations of the predatory mites were made using Hoyer's medium. The identification of species in both studies was based on Chant & McMurtry (2007) and Döker et al. (2016). Predatory mite densities are given per leaf due to their very low populations. In order to determine the statistical significance of the differences in mite population densities observed on the adaxial and abaxial leaf surfaces of all citrus species, the data were first subjected to homogeneity and normality tests. Subsequent analyses were performed with the non-parametric Mann-Whitney *U* test *(p*<0.05) (SPSS version 23).

Results

Prevalence of *Eutetranychus orientalis* and *Panonychus citri*

A comprehensive survey was conducted across 190 commercial citrus orchards in Adana province and its districts from 2019 to 2021. The results showed that *E. orientalis* was present in 38 (20%) orchards, *P. citri* in 65 (34.2%) orchards, and mixed infestations of both pests in 6 (3.15%) orchards. Additionally, 81 (42.63%) orchards were free from tetranychid mite infestations (Figure 1).

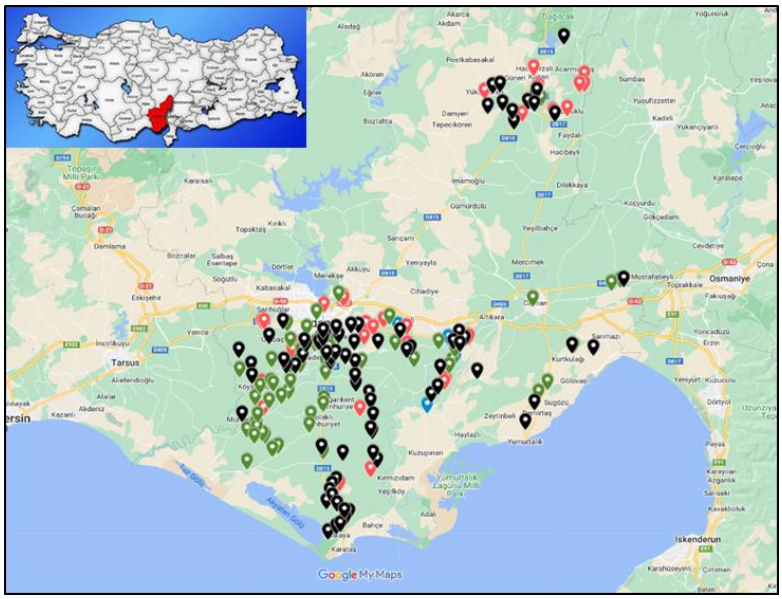

Figure 1. Distribution of *Eutetranychus orientalis* (red), *Panonychus citri* (green), both species coexisting (blue) and pest-free zones (black) in citrus orchards surveyed in Adana between 2019 and 2021.

Infestation rates by citrus species revealed that lemons had the highest infestation rate for *E.* orientalis. Out of the 65 lemon orchards surveyed, 17 were solely infested with *E. orientalis*, while 5 had mixed infestations with *P. citri*, resulting in a total of 22 affected orchards and an infestation rate of 33.84%. Oranges had the second-highest infestation rate, with 16 out of 51 orchards affected, 15 were infested solely with *E. orientalis*, and 1 exhibited mixed infestations. The infestation rate in oranges was 31.37%, nearly identical to that of lemons. In mandarins, *E. orientalis* was found in 6 out of the 69 (8.69%) sampled orchards. However, no biological stages of *E. orientalis* were observed in grapefruit orchards (Table 2).

The presence of *P. citri*, either alone or in combined with *E. orientalis*, was detected in 40 (61.53%) of the 65 lemon orchards surveyed. Oranges displayed the second-highest infestation rate, with 21 out of 51 orchards (41.17%) impacted. In mandarins, *P. citri* was present in 8 out of 69 orchards (11.59%). In contrast to *E. orientalis*, *P. citri* was observed in a single grapefruit orchard (Table 2).

The distribution of *E. orientalis* and *P. citri* infestations across various citrus species and districts exhibited significant regional variation. The highest infestation rates of *E. orientalis* were recorded in Yüreğir, Kozan, and Seyhan districts on lemons, oranges and mandarin, respectively. In contrast, *P. citri* infestations were most prevalent in Yüreğir district across all the three citrus species: lemons, oranges, and mandarins. Notably, *E. orientalis* was absent in grapefruit orchards, while *P. citri* was detected in only one orchard in Seyhan district (Table 2).

Table 2. Occurrence and infestation rates of *Eutetranychus orientalis* and *Panonychus citri* on various citrus species across districts of Adana Province

Citrus species	Total number of orchards (n*)	*E. orientalis* infested orchards (n)	*E. orientalis* infestation rate (%)	*P. citri* infested orchards	*P. citri* infestation rate (%)	Orchards infested with both species (n)	Orchards infested with both species (%)	Number of uninfested orchards (n)	Uninfested orchards (%)
Yüreğir (Districts)									
Lemon	31	8	25.8	17	54.8	4	12.9	2	6.4
Orange	16	3	18.7	9	56.2	-	-	4	25.0
Mandarin	30	2	6.6	6	20.0	-	-	22	73.3
Grapefruit	3	-	-	-	-	-	-	3	100
Total	80	13	16.2	32	40.0	4	5.0	31	38.7
Seyhan									
Lemon	10	-	-	9	90.0	-	-	1	10.0
Orange	12	3	25.0	6	50.0	-	-	3	25.0
Mandarin	15	3	20.0	1	6.6	-	-	11	73.3
Grapefruit	1	-	-	1	100	-	-	0	0.0
Total	38	6	15.7	17	44.7	-	-	15	39.4
Kozan									
Lemon	10	5	50.0	1	10.0	1	10.0	3	30.0
Orange	11	7	63.6	-	-	1	9.0	3	27.2
Mandarin	4	-	-	-	-	-	-	4	100
Grapefruit	1	-	-	-	-	-	-	1	100
Total	26	12	46.1	1	3.8	2	7.6	11	42.3
Karataş									
Lemon	9	2	22.2	5	55.5	-	-	2	22.2
Orange	3	-	-	-	-	-	-	3	100
Mandarin	10	-	-	1	10.0	-	-	9	90.0
Total	22	2	9.0	6	27.2	-	-	14	63.6
Ceyhan									
Lemon	2	1	50.0	1	50.0	-	-	0	0.0
Orange	5	1	20.0	4	80.0	-	-	0	0.0
Mandarin	7	-	-	-	-	-	-	7	100
Total	14	2	14.2	5	35.7	-	-	7	50.0
Sarıçam									
Lemon	2	1	50.0	1	50.0	-	-	0	0.0
Orange	2	1	50.0	1	50.0	-	-	0	0.0
Mandarin	1	1	100	-	-	-	-	0	0.0
Total	5	3	60.0	2	40.0	-	-	0	0.0
Yumurtalık									
Lemon	1	-	-	1	100	-	-	0	0.0
Orange	2	-	-	1	50.0	-	-	1	50.0
Mandarin	2	-	-	-	-	-	-	2	100
Total	5	-	-	2	40.0	-	-	3	60.0
Total	190	38	20	65	34.2	6	3.1	81	42.6

*n: number of surveyed orchards.

Seasonal occurrence of *Eutetranychus orientalis, Panonychus citri* and phytoseiid predatory mites

Seasonal occurrence on lemon

The seasonal abundance of *E. orientalis*, *P. citri*, and the phytoseiid predatory mites on lemon between 2019 and 2022, are presented in Figures 2a, b. The majority of the *E. orientalis* population preferred the adaxial side of leaf for feeding and colonization (Figure 2a). The mean total densities of *E. orientalis* on the abaxial and adaxial leaf surfaces across the entire sampling period were 2.31 and 0.39 all stages/cm², respectively and the difference was found to be statistically significant (Z=-2.936; $p<0.01$). In contrast, the same densities of *P. citri* on the abaxial and adaxial leaf surfaces were 0.05 and 0.12 all stages/cm², respectively, with no significant difference observed (Z=-0.741; $p>0.05$).

Eutetranychus orientalis was first observed in the first week of November in 2019. The pest population increased as ofthis date, with the highest density on the adaxial side of the leaf recorded on 15 December, showing a mean of 3.31 eggs and 0.83 motile stages/cm². Subsequently, the population density decreased to zero by March 2020 (Figure 2a). In 2021, the pest first began to establish a population on 11 October, reaching its peak in early January 2021 with a mean of 7.16 eggs and 2.73 motile stages/cm² on the adaxial side of the leaf. The last individuals were observed in March of the same year. The pest exhibited population development for the second time at the beginning of July 2021, achieving the highest density on November 1, 2021, with a mean of 6.08 eggs and 3.22 active stages/cm², after which it showed a decreasing trend (Figure 2a).

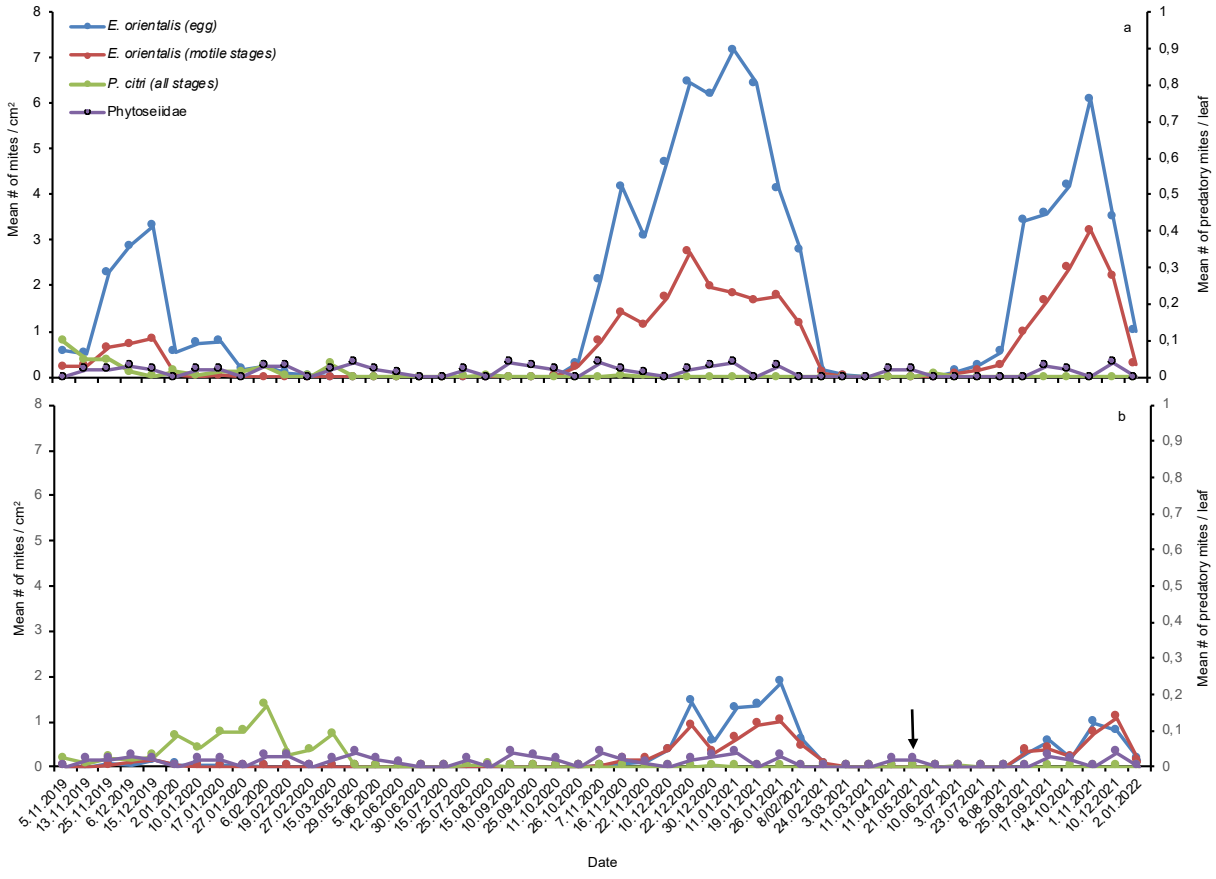

Figure 2. Seasonal occurrence of *Eutetrancyhus orientalis*, *Panonychus citri* and predatory mites on the adaxial (a) and abaxial (b) sides of leaf in lemon (*Citrus lemon* cv. Interdonato) in Adana (arrow: spraying).

The seasonal occurrence of *P. citri* was assessed concurrently with *E. orientalis*, which exhibited its highest density on 5 November 2019, recording 0.77 eggs and motile stages/cm² on the adaxial leaf surface. Following this peak, the population of *P. citri* remained at low levels until March 2020 (Figures 2a, b).

Eutetranychus orientalis exhibited significantly lower population development on the abaxial leaf surface compared to the adaxial side. The highest density on the abaxial side was recorded on 26 January 2021, with a mean of 1.87 eggs and 1.01 motile stages/cm² (Figure 2b). Subsequently, the pest's population remained consistently low, with a secondary peak observed in November 2021, with values of 0.97 eggs and 1.09 motile stages/cm² (Figure 2b). Throughout the sampling period, the total mean densities of phytoseiid predatory mites observed on both the abaxial and adaxial sides of lemon peaked at 0.04 individuals per leaf.

Seasonal occurrence on orange

As in lemon, the primary *E. orientalis* population on orange developed on the adaxial side of the leaves (Figures 3a, b). The mean total densities of *E. orientalis* on the abaxial and adaxial leaf surfaces across the entire sampling period were 1.15 and 0.14 all stages/cm², respectively, with this difference being statistically significant (Z=-2.727; p<0.01). In contrary, the mean total densities of *P. citri* on the same leaf surfaces were 0.007 and 0.006 all stages/cm², respectively, showing no statistically significant difference (Z=-0.281; p>0.05).

The highest population density of the pest, recorded at the beginning of sampling on November 13, 2019, was 3.47 eggs and 1.08 motile stages/cm². Following this peak, the population decreased to its lowest level in March 2020, before increasing again in October. It reached another peak of 3.1 eggs and 1.58 motile stages/ cm² on December 10, 2020. By March 2021, the population had dropped to zero but began to rise again in July. After peaking in December, the population declined once more (Figure 3a).

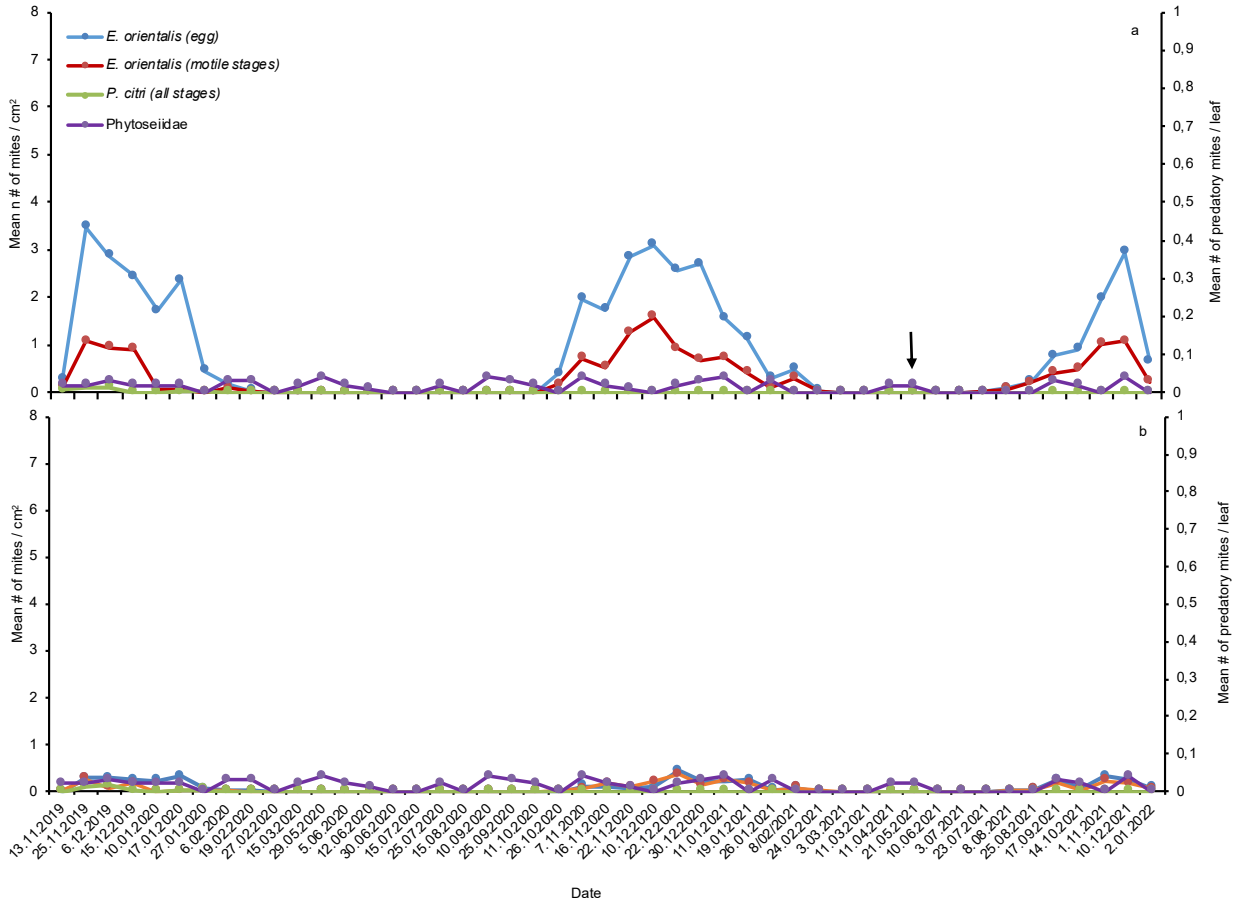

Figure 3. Seasonal occurrence of *Eutetrancyhus orientalis*, *Panonychus citri* and predatory mites on the adaxial (a) and abaxial (b) sides of leaf in orange (*Citrus sinensis* cv. Yafa) in Adana (arrow: spraying).

In contrast, the pest showed no significant population development on the abaxial side of the leaves; it remained at very low levels throughout the study (Figure 3b). Similarly, low populations of *P. citri* were observed on both citrus species and leaf surfaces (Figure 3a, b). During the sampling period, *Amblyseius swirskii* (Athias-Henriot) constituted 95% of the predatory mite species, while the remainder were *Tyhplodromus athiasae* Porath and Swirski (Mesostigmata: Phytoseiidae) (Figure 3a). In both citrus species, the populations of predatory mites remained low and did not exhibit stable development in response to pest density.

Seasonal occurrence on sour orange

Seasonal occurrence of *E. orientalis* and phytoseiid predatory mites on the adaxial and abaxial leaf surfaces of sour orange trees are presented in Figures 4a, b, in Seyhan, Adana. Similar to the lemon and orange, the density of *E. orientalis* determined on the abaxial side of the leaf was higher and statistically different from the adaxial side (Z=-3.595; p<0.001).

In this area, the pest population was detected from the first sampling date, reaching its highest density in mid-December, with an average of 2.82 eggs and 1.39 motile stages/cm² (Figure 4a). After this peak, the population density began to decrease, reaching its lowest level on February 10, 2020. The *E. orientalis* population was first recorded on the 1st of August in the same year, reaching another peak at the end of November, with 4.00 eggs and 2.17 motile stages/cm². Following these dates, the pest population began to decrease again, reaching zero by March 2021. The population resurged on May 21, 2021, and peaked on December 10, with 3.04 eggs and 1.41 motile stages/cm² on the adaxial leaf surface, before decreasing again (Figure 4a). On the abaxial leaf surface, the pest population remained significantly lower compared to the adaxial leaf surface (Figure 4b).

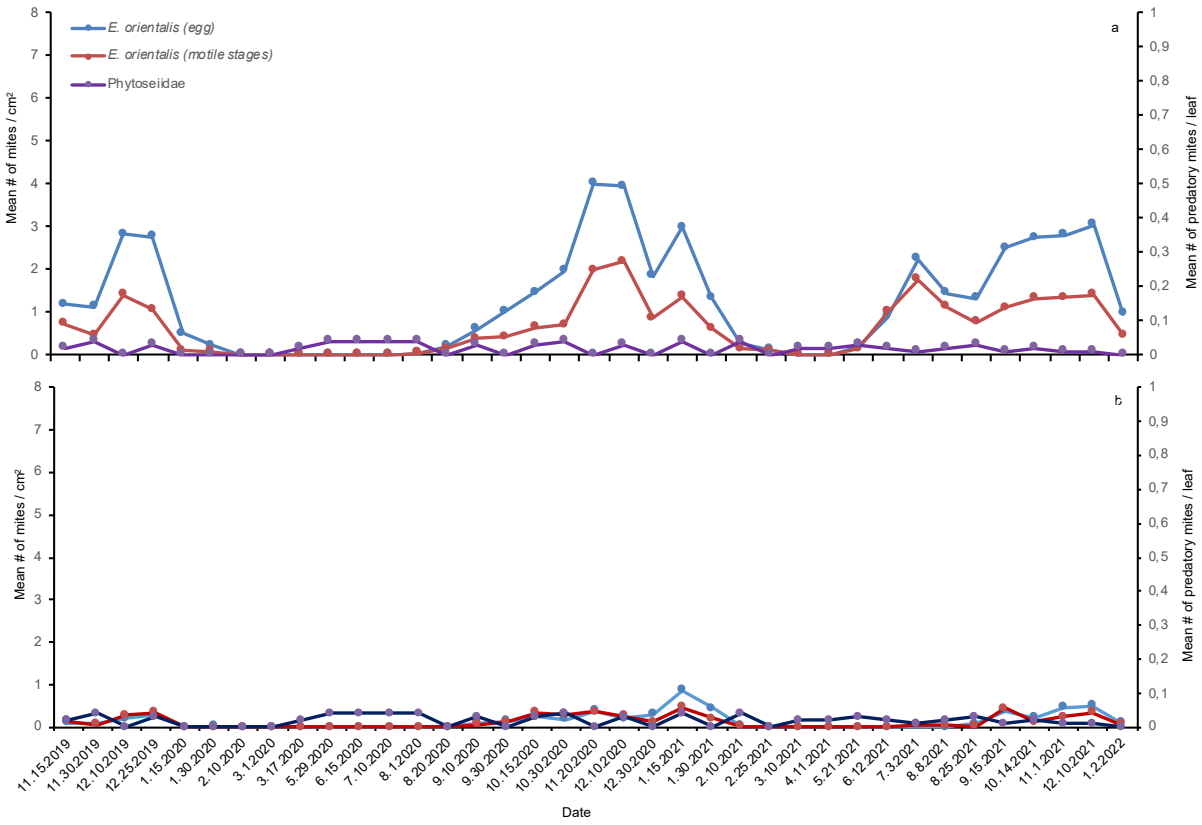

Figure 4. Seasonal occurrence of *Eutetrancyhus orientalis*, *Panonychus citri* and predatory mites on the adaxial (a) and abaxial (b) sides of leaf in sour orange (*Citrus aurantium*) in Seyhan, Adana.

Throughout the study, no biological stages of *P. citri* were observed in the samples. The predatory mite population persisted at a low level and did not increase in response to the increasing pest population (Figure 4a, b). Among the sampled phytoseiid predatory mite species, 57% were identified as *E. scutalis*, while 42% comprised *T. athiasae*, and the remaining portion consisted of *T. intercalaris* Livshitz & Kuznetsov (Figure 4a, b). Consistent with the densities recorded in lemons and oranges, the total density of phytoseiid predator mites across all samples varied between 0.01 and 0.04 per leaf for all stages.

Seasonal occurrence of *E. orientalis* on the adaxial and abaxial sides of the leaves of sour oranges in the Ç.Ü. campus area between 2019 and 2022 is shown in Figures 5a, b. On the first sampling date, the pest's density on the adaxial surface of the leaves was found to be 2.38 eggs and 1.01 motile stages/cm². Subsequently, the pest reached its highest density on December 10, with 3.76 eggs and 1.76 motile stages/cm² on the leaf surface. After this peak, the population began to decrease and had completely disappeared by early February 2020. *E. orientalis* re-emerged for the second time on August 20, 2020, reaching its peak on November 17, with a mean of 3.18 eggs and 1.75 motile stages/cm². After this date, the pest population began to decline again, maintaining low levels until March-April 2021 (Figure 5b). From May onwards, the population began to rise once more, reaching its highest density again in November 2021. On the abaxial leaf surface, the pest population remained significantly lower compared to the adaxial leaf surface (Figure 5b) ($Z=-3.634$; $p<0.001$). No eggs or motile stages of *P.* citri were observed on the orange at Balcalı campus during these surveys. The predatory mite population remained at very low levels ($p<0.05$ all stages/leaf).

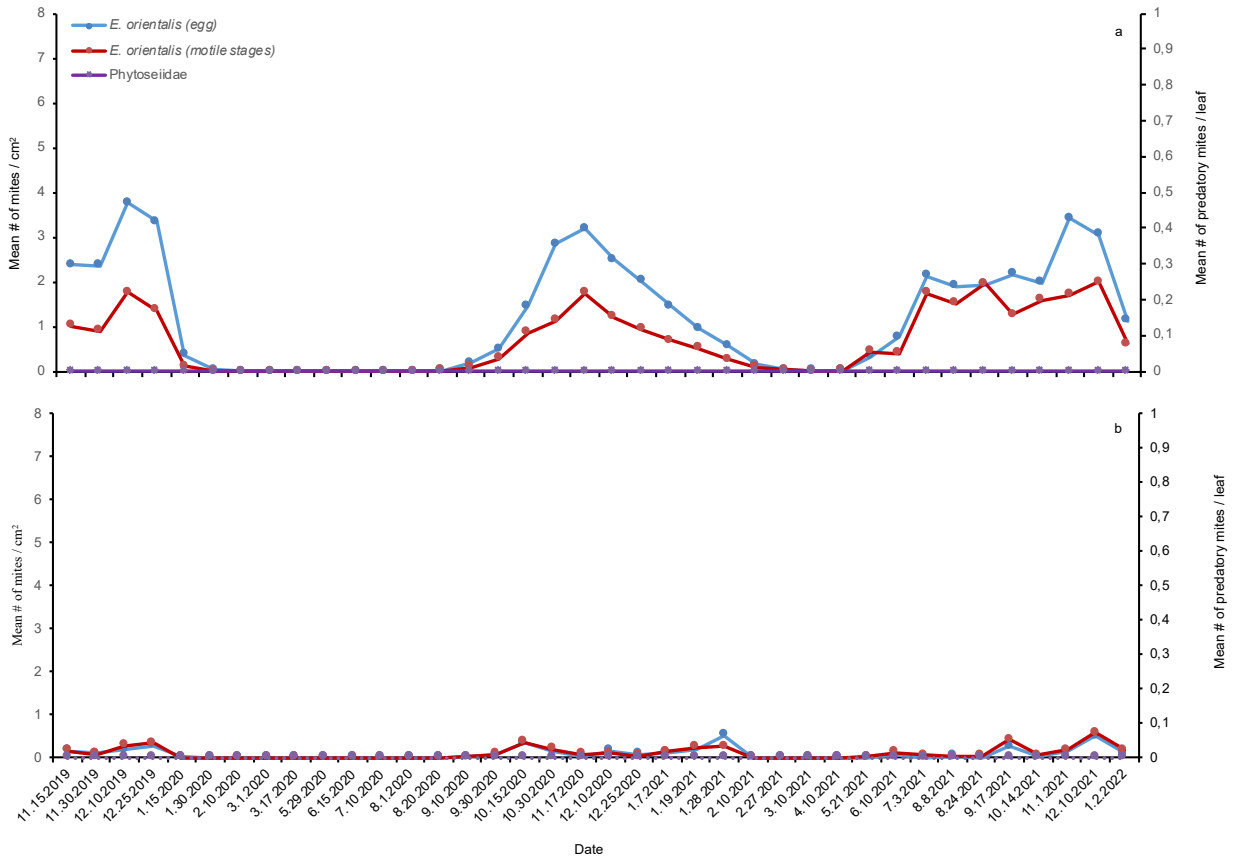

Figure 5. Seasonal occurrence of *Eutetrancyhus orientalis*, *Panonychus citri* and predatory mites on the adaxial (a) and abaxial (b) sides of leaf in sour orange (*Citrus aurantium*) in Ç.Ü. campus area, Adana.

Throughout the years of following the pest population development, the minimum and maximum temperatures were recorded at 6ºC and 37ºC, respectively, while the average humidity varied between 42% and 74%. The highest rainfall was recorded in December 2019, reaching 13 kg/m². The total rainfall varied between 1 and 7 kg/m² (Figure 6).

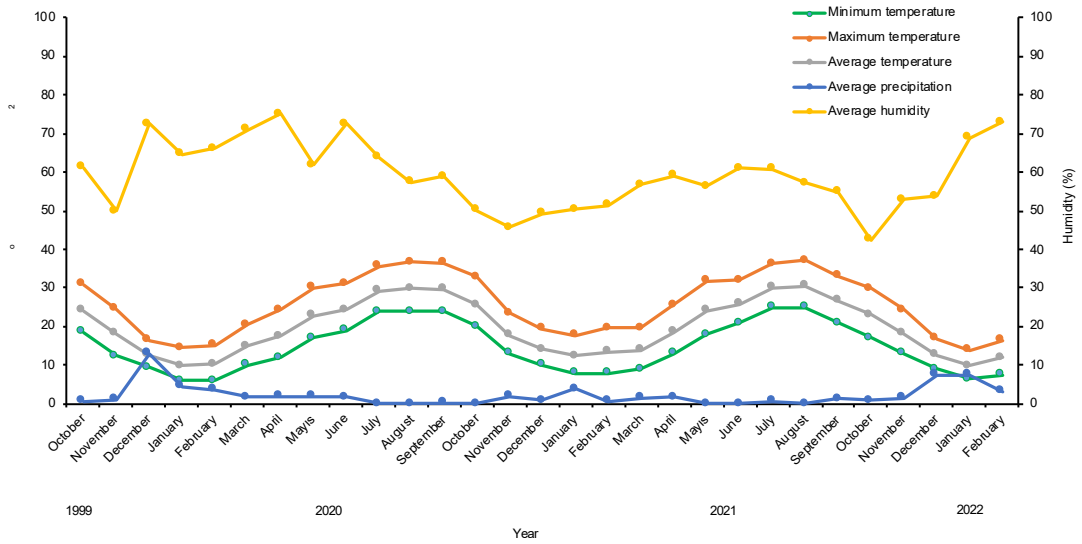

Figure 6. Meteorological data for Adana province during the 2019-2022 period.

Discussion

The presence of *E. orientalis* in Türkiye was first reported in the 1970s, with the first significant population density observed on citrus in 2014 (Jeppson et al., 1975; Çobanoğlu & Can, 2014). Currently, *E. orientalis* is among the most frequently found mites in citrus orchards of the Eastern Mediterranean region (Yalçın et al., 2022). No resistance to commonly used acaricides in the region has been observed on this pest (Akbay et al., 2024). In this context, potential factors contributing to the widespread presence of *E. orientalis* in the region include the impacts of long-term global climate change, as well as the negative effects of intensive pesticide use which may suppress natural enemies that could otherwise control citrus pest populations (Döker et al., 2021). Similarly, Ferragut et al. (2013) reported that *E. orientalis* rapidly became a major pest of citrus in Portugal shortly after its detection in 1999, and by 2001, it had formed dense populations in southern Spain. The pest has also been reported to reach remarkably high population densities in a short period in citrus in Sicily (Garzia et al., 2025). In addition, the capacity of *E. orientalis* to feed and reproduce on a range of host plants beyond citrus is likely a significant factor contributing to its widespread distribution (Beard, 2018; Garzia et al., 2025).

Surveys on lemon, orange, mandarin, and grapefruit orchards revealed that *E. orientalis* infestation rates and host plant preferences varied depending on the citrus species. In this study, *E. orientalis* was the most prevalent on lemon, followed by orange, and mandarin. No infestations were observed in grapefruit orchards. The host plant preference of *E. orientalis* among citrus species has been partially established in previous studies (Ledesma et al., 2011; Vela et al., 2017; Halawa et al., 2020; Chouikhi et al., 2022). These findings align with those of Ledesma et al. (2011), in which the highest infestation rate was on lemon, followed by orange, and the lowest on mandarin in Spain. Similarly, in another study conducted in Spain, *E. orientalis* was identified as the most prevalent tetranychid mite species on orange and lemon trees (Ferragut et al., 2013).

Morphological characteristics of leaves and secondary plant metabolites have significant effects on the feeding, development, and reproduction of phytophagous species. The quantity of tannic acids and the density of glandular structures in the host plant leaves have been found to positively influence mite population growth, while the thickness of the leaf cuticle has a negative impact on the development of certain *Eutetranychus* species (Mohamed, 1965). Overall, the biochemical properties of host plants significantly impact the population development of phytophagous mites. While oil content has been linked to the development duration of *Tetranychus urticae* Koch, 1836 (Prostigmata: Tetranychidae) no such correlation exists for carbohydrate and protein levels (Puspitarini et al., 2021). In contrast, the protein concentration in *C. limon* has been shown to positively influence the development of *E. orientalis* compared to other citrus species (Jyotika & Mandeep, 2003). Additionally,

secondary plant metabolites appear to have a negative effect on mite development (Luczynski et al., 1990; Jyotika & Mandeep, 2003). Yalçın et al. (2022) reported the net reproductive rate (r_m) of *E. orientalis* on lemon, orange, mandarin and grapefruit as 0.167, 0.163, 0.130 and 0.120 females/female/day, respectively. Therefore, it is plausible that the differences observed in the biological characteristics of *E. orientalis* across the three citrus species studied in this study may be attributed to the favorable contents of amino acids, proteins, carbohydrates, and oils found in lemon, which likely support the biological characteristics of the pest (Yalçın et al., 2022).

The seasonal occurrence of *P. citri* exhibited much abaxial density on lemon and orange compared to *E. orientalis*, and no individuals of *P. citri* were found on sour orange. Overall, when the *E. orientalis* population was low on lemon, the density of *P. citri* tended to increase. Rode et al. (2024) reported that *Panonychus ulmi* (Koch, 1836) which belongs to the same genus as *P. citri*, deposited significantly fewer eggs on leaves infested with *Aculus schlechtendali* (Nalepa, 1890) (Prostigmata: Eriophyidae) or *T. urticae*. This finding, alongside the higher population density of *E. orientalis* compared to *P. citri*, and the relatively rare occurrence of both pests coexisting in the same area, suggests the interspecific competition between *P. citri.* In addition, the preference of certain mite species for colonizing the abaxial or adaxial side of leaves is a multifaceted issue influenced by leaf surface characteristics, microhabitat conditions, and interactions with plant structures such as trichomes and domatia (Schmidt, 2014). Interactions between predatory and herbivorous mites also play a role in surface preference. Such behaviors suggest that mites actively choose leaf surfaces that balance environmental conditions and predator avoidance (Sudo & Masahiro, 2013).

In this study, *E. orientalis* populations reached the highest densities between November (mean temp. 18ºC) and January (mean temp. 10ºC), regardless of citrus species. The population declined in early spring and remained low throughout the summer. Similarly, Fathi (2018) reported that *E. orientalis* peaked in winter, especially when rainfall was low and temperatures exceeded seasonal averages in Morocco. In this study, the mean temperature did not fall below 10ºC, including in January, and rainfall was very low. Abdellah et al. (2021), also in Morocco, found two population peaks in the autumn, with low densities in the summer. Ledesma et al. (2011) observed the highest population densities between October and December in southern Spain. In Egypt, *E. orientalis* populations peaked in August and September, with significantly higher density on fruits than leaves (Halawa et al., 2020). Chouikhi et al. (2022) reported four population peaks for the pest in Tunisia between February and May. The results of this study indicate that the temporal patterns of *E. orientalis* population peaks differ from those previously reported for Tunisia and Egypt. These two countries exhibit comparable seasonal dynamics, characterized solely by dry and wet periods, in contrast to the more diverse climatic conditions observed in Turkey, Spain, and Morocco. The development of *E. orientalis* populations in Tunisia and Egypt primarily occurs outside the dry season, which may explain the observed discrepancies in population trends.

The *P. citri* population is generally found at a higher density on the abaxial side of lemon and orange leaves compared to *E. orientalis*, but at much lower densities overall, and no population development was observed on sour orange. Due to the very low population density, it could have not been possible to precisely determine the seasonal population development of the pest related to population density increases. Kasap (2009) reported that *P. citri* exhibited population peaks twice a year, in the spring and fall, depending on the new shoot production in citrus trees. These findings suggest that interspecific competition between these two pests warrants further investigation. Accordingly, Ledesma et al. (2011) reported that *E. orientalis* was the dominant species in lemon and orange orchards in Spain, while *P. citri* and *T. urticae* were present at low densities.

In light of these findings, further data collection is necessary to understand *E. orientalis* seasonal occurrence in detail with a particular concern on lemon, orange, and mandarin orchards. Furthermore, studies are required to determine the dominant species in the competition between *E. orientalis* and *P. citri* within infested orchards. Although preliminary results indicate that *A. swirskii* and *E. scutalis*, two predatory mites commonly found in the Eastern Mediterranean, exhibit predatory efficacy against the pest under controlled conditions, no positive association was detected between these predators and *E. orientalis* populations in untreated citrus orchards. Similarly, Gonzalez-Zamora et al. (2011) reported that *E. scutalis* and *E. stipulatus* showed no response to the population increase of *E. orientalis* in citrus orchards in Spain. Consequently, future studies should focus on evaluating the effectiveness of other potential natural predators, including various phytoseiid predatory mites and insect species, for pest control.

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Mortality effect of wettable powder formulations containing entomopathogenic fungal spores against some stored-product pests[1]

Entomopatojen fungus sporları içeren ıslanabilir toz formülasyonların bazı depolanmış ürün zararlılarına karşı ölüm etkisi

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Abstract

In this study, the effectiveness of wettable powder formulations containing entomopathogenic fungal spores of *Beauveria bassiana* Vuillemin (Hypocreales: Cordycipitaceae) and *Metarhizium robertsii* Sorokin (Hypocreales: Clavicipitaceae) was examined against major stored-product pests in 2021 in Bioinsecticide Mass Production Laboratory of Plant Protection Department, Faculty of Agriculture, Kahramanmaraş Sütçü İmam University. Twelve formulations were developed: six of which were based on *B. bassiana* isolate 5-4 and the other six on *M. robertsii* isolate S3. The formulations were applied on both wheat and concrete surfaces at predetermined dosages. Bioassays were conducted using *Plodia interpunctella* (Hübner, 1813) (Lepidoptera: Pyralidae) third-instar larvae, *Rhyzopertha dominica* (F.,1792) (Coleoptera: Bostrichidae) and *Sitophilus oryzae* L.,1763 (Coleoptera: Curculionidae) adults, under controlled conditions at 30±2°C, 65±5% relative humidity, and in darkness. When applied to wheat, all formulations exhibited high mortality rates against *R. dominica* and *P. interpunctella*, while showing limited efficacy against *S. oryzae*. Conversely, applications on concrete surfaces demonstrated higher efficacy to all pests, particularly against *S. oryzae*. Amongst the formulations, those containing *B. bassiana* outperformed *M. robertsii*-based formulations in terms of efficacy, both on concrete surfaces and wheat. The findings suggest that the developed formulations have significant potential as an effective alternative for pest management in empty storage facilities. Notably, surface applications provided superior results compared to applications directly on stored products.

Keywords: Bioinsecticide, entomopathogenic fungi, mortality, stored product pests

Öz

Bu çalışmada, entomopatojen fungus *Beauveria bassiana* Vuillemin (Hypocreales: Cordycipitaceae) ve *Metarhizium robertsii* Sorokin (Hypocreales: Clavicipitaceae) sporlarını içeren ıslanabilir toz formülasyonların depolanmış ürün zararlılarına karşı etkinliği 2021 yılında Kahramanmaraş Sütçü İmam Üniversitesi Ziraat Fakültesi Bitki Koruma Bölümü Biyoinsektisit Kitlesel Araştırma Laboratuvarında incelenmiştir. *B. bassiana*'nın 5-4 nolu izolatından 6 adet ve *M. robertsii*'nin S3 nolu izolatından 6 adet olmak üzere toplam 12 formülasyon hazırlanmış ve belirlenen dozlarda hem buğdaya hem de beton yüzeye uygulanmıştır. Biyolojik testlerde, *Plodia interpunctella* (Hübner, 1813) (Lepidoptera: Pyralidae) 3. dönem larvaları ile *Rhyzopertha dominica* (F., 1792) (Coleoptera: Bostrichidae) ve *Sitophilus oryzae* L., 1763 (Coleoptera: Curculionidae) erginleri kullanılmıştır. Testler, 30±2°C sıcaklık ve %65±5 nispi nemde karanlık ortam şartlarında gerçekleştirilmiştir. Tüm formülasyonlar ürüne uygulandığında, *R. dominica* ve *P. interpunctella* üzerinde yüksek ölüm etkisi gösterirken, *S. oryzae*'ye karşı daha yüksek etki göstermiştir. Beton yüzeye uygulandığında ise tüm zararlılara, özellikle *S. oryzae*' üzerinde etkisi düşük kalmıştır. Formülasyonlar arasında, *B. bassiana* içerenlerin üç zararlı türüne karşı hem yüzeyde hem de üründe, *M. robertsii* içerenlere kıyasla daha etkili olduğu tespit edilmiştir. Bu sonuçlar, hazırlanan ıslanabilir toz formülasyonların, boş depolarda zararlılara karşı mücadelede etkili ve önemli bir alternatif olabileceğini göstermektedir. Özellikle beton yüzeye uygulamanın, ürüne uygulamaya kıyasla daha başarılı sonuçlar sunduğu belirlenmiştir.

Anahtar sözcükler: Biyoinsektisit, entomopatojen fungus, ölüm etkisi, depolanmış ürün zararlıları

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INTRODUCTION

Cereals are subjected to both direct and indirect damage by a variety of pest species during the post-harvest storage process. These pests contribute to both qualitative and quantitative losses in stored grains. The damage caused by feeding includes reductions in grain weight and nutritional value, seed germination loss, accumulation of excreta, presence of webbing, and insect body fragments, all of which result in a decline in market value (Abdel-Raheem et al., 2015). These losses can rise to as much as 9% in developed countries and 20% or higher in developing countries (Barra et al., 2013). Therefore, effective management of stored product pests is of critical importance.

Synthetic chemicals have been widely and intensively used in pest management of stored products. However, the use of chemicals in the control of stored-product pests has led to concerns regarding resistance development, residue problems, toxic effects on non-target organisms and environmental contamination (Khan & Khan, 2023). As a result, alternative pest control strategies have been sought. Among these alternatives, entomopathogenic fungi stand out as a promising solution. Notably, *Beauveria* Vuill. (Hypocreales: Cordycipitaceae) and *Metarhizium* Sorokin (Hypocreales: Clavicipitaceae) have been shown, through numerous studies, to be significantly effective against major pests of stored products. Studies have reported the effectiveness of *Beauveria bassiana* Vuillemin (Hypocreales: Cordycipitaceae) in controlling pests such as *Rhyzopertha dominica* (F.,1792) (Coleoptera: Bostrichidae) (the lesser grain borer), *Sitophilus oryzae* (L., 1763) (Coleoptera: Dryophthoridae) (the rice weevil), and *Plodia interpunctella* (Hübner, 1813) (Lepidoptera: Pyralidae) (the Indian meal moth) (Moino et al., 1998; Rice & Cogburn, 1999; Padin et al., 2002; Vassilakos et al., 2006; Lord, 2007; Batta, 2008; Mahdneshin et al., 2009; Sabbour et al., 2012; Kavallieratos et al., 2014; Wakil & Schmitt, 2015; Er et al., 2016, 2018; Barış & Er, 2021). Similarly, *Metarhizium* spp. have demonstrated high efficacy against the same pest species, with supportive evidence from numerous studies (Dal Bello et al., 2001; Batta, 2005; Kavallieratos et al., 2006; Athanassiou et al., 2008; Mahdneshin et al., 2009; Sewify et al., 2014). *Metarhizium robertsii* (Metchnikoff) Sorokin (Hypocreales: Clavicipitaceae) among *Metarhizium* species has the ability to infect over 200 insect species across various orders, including Lepidoptera, Coleoptera, Hemiptera, Diptera, Dermaptera, and Orthoptera (Brunner-Mendoza et al., 2019).

In literature, the use of entomopathogenic fungi as a dust application on stored-product is commonly tested for the control of stored-product pests. While numerous studies have focused on the application of chemicals on structural surfaces, there are very few studies regarding the use of entomopathogenic fungi with the same approach (Athanassiou et al., 2017; Wakil et al. 2023). Since entomopathogenic fungal spores can be acquired by insects from treated surfaces, much like insects feeding on fungus-treated foliage, applying fungal spore suspensions onto surfaces seems to be a feasible approach in treatments of empty storage and processing facilities of stored commodities. It has been suggested that formulations of entomopathogenic fungi, which can be applied in water, may be effective in reducing the use of chemicals for pest control in empty warehouses. The potential of entomopathogenic fungal isolates, which has been demonstrated in numerous studies against stored product pests, raises interest regarding their performance once formulated.

Unlike formulations that are used in powder form, there is no research on the development of formulations that will be mixed in water prior spraying for the control of pests in stored products. This study was conducted to demonstrate the mortality effects of *B. bassiana* and *M. robertsii* on three insects, *R. dominica, S. oryzae* and *P. interpunctella* by application on both commodity and concrete surface, and to explore probable enhancement of the effects by formulating the spores of these entomopathogenic fungi as wettable powders.

Materials and Methods

Test Insects and their cultures

In the Entomology Laboratory of the Department of Plant Protection at Faculty of Agriculture, Kahramanmaraş Sütçü İmam University, insect species used in bioassay tests were reared under controlled laboratory conditions. Rearing of *R. dominica* and *S. oryzae* was conducted using wheat as substrate. For the preparation of the rearing medium of *P. interpunctella*,, a mixture consisting of 2 kg of wheat bran, 350 g of corn flour, 350 ml of glycerin, and one teaspoon of yeast was utilized (Henteş, 2020). Cultures of *S. oryzae* were maintained at 26±2°C and 65±5% relative humidity (r.h.) in complete darkness. The cultures of *P. interpunctella* and *R. dominica* were kept at 30±2°C and 65±5% r.h. For the tests, one-week-old *R. dominica* and *S. oryzae* adults, third-instar larvae of *P. interpunctella*, were selected to ensure uniformity in developmental stage and age.

Entomopathogen fungi culture

Beauveria bassiana and *M. robertsii* used in this study were obtained from the collection of the Department of Plant Protection at Faculty of Agriculture, Kahramanmaraş Sütçü İmam University. The *B. bassiana* isolate 5-4 (single spore culture of isolate 151138) was originally obtained from a field-infected *R. dominica* adult (Er et al., 2016), while *the M. robertsii* isolate S3 (single spore culture of isolate F17-2-1) was derived using the *Galleria mellonella* trap method. Spore production was conducted following the protocol described by Barış & Er (2021). Rice (100 g) was soaked overnight, drained, and then mixed with 1.5 g of $CaSO_4$ and $CaCO_3$ each. The mixture was autoclaved and allowed to cool. Under sterile conditions, the cooled rice was inoculated with 10 ml of a spore suspension containing $2x10^7$ spores/ml. The inoculated rice was sealed in bags and incubated at 25±2°C with a 12/12 hour light/dark photoperiod for 14 days. Post-incubation, the bags were opened, and the rice was dried at 25±2°C. Dried spores were separated using a 38 µm sieve and collected in glass bottles for formulation studies.

Germination test

Prior to the bioassay tests, the germination rate of the fungal spores was assessed to ensure their viability. A diluted spore suspension was prepared and inoculated onto potato dextrose agar (PDA). The plates were then incubated at 25±2°C for 24 hours. Spore germination was evaluated under a light microscope. Germinated spores were defined as those exhibiting germination tubes at least equal in length to the diameter of the spore. The analysis revealed a germination rate exceeding 98%, indicating high viability of the spores and their suitability for use in bioassays.

Test surface

A concrete surface was used to test the insecticidal effectiveness of wettable powder (WP) formulations against *P. interpunctella, R. dominica,* and *S. oryzae.* Plastic Petri dishes with a surface area of 100.0 cm² were used for the bioassays and each one had a bottom covered with ordinary concrete (Teknoflex, Türkiye) commercially available for multiple purposes. Concrete and tap water were combined in a 5:1 ratio to create a thick, long-lasting paste. Approximately 20 ml of this concrete mixture was applied to each Petri dish, and the mixture was allowed to set and dry for a period of two weeks.

WP formulations

All solid materials used in the wettable powder formulation, including fillers and additives, were dried at 70±2°C, while fungal spores were dried at 25±2°C. All materials were then sieved through a 38 µm mesh. When preparing the formulations, blank formulations were first prepared for each different formulation. Then, fungal spores were added to the prepared blank formulations and mixed homogeneously. Details of the formulation composition per 1 kg of product are provided in Tables 1 and 2. The formulation includes carboxymethylcellulose and a non-ionic, organosilicon-based surfactant.

Table 1. Ingredients of wettable powder formulations containing *Beauveria bassiana*

Formulation names	Fungal spores	Filler	Adjuvant	Other substances
BbWP1	25% *B. bassiana* (3×10^{13})	68% Diatomaceous Earth (Detech)	5% Silicon dioxide	2% Surfactants
BbWP2	50% *B. bassiana* (6×10^{13})	43% Diatomaceous Earth (Detech)	5% Silicon dioxide	2% Surfactants
BbWP3	75% *B. bassiana* (9×10^{13})	18% Diatomaceous Earth (Detech)	5% Silicon dioxide	2% Surfactants
BbWP4	25% *B. bassiana* (3×10^{13})	68% Kaolin	5% Silicon dioxide	2% Surfactants
BbWP5	50% *B. bassiana* (6×10^{13})	43% Kaolin	5% Silicon dioxide	2% Surfactants
BbWP6	75% *B. bassiana* (9×10^{13})	18% Kaolin	5% Silicon dioxide	2% Surfactants

Table 2. Ingredients of wettable powder formulations containing *Metarhizium robertsii*

Formulation names	Fungal spores	Filler	Adjuvant	Other substances
MrWP1	50% *M. robertsii* (2×10^{13})	45.33% Diatomaceous Earth (Detech)	3.33% Silicon dioxide	1.34% Surfactants
MrWP2	75% *M. robertsii* (3×10^{13})	21.5% Diatomaceous Earth (Detech	2.5% Silicon dioxide	1% Surfactants
MrWP3	90% *M. robertsii* (3.6×10^{13})	7.2% Diatomaceous Earth (Detech)	2% Silicon dioxide	0.8% Surfactants
MrWP4	50% *M. robertsii* (2×10^{13})	45.33% Kaolin	3.33% Silicon dioxide	1.34% Surfactants
MrWP5	75% *M. robertsii* (3×10^{13})	21.5% Kaolin	2.5% Silicon dioxide	1% Surfactants
MrWP6	90% *M. robertsii* (3.6×10^{13})	7.2% Kaolin	2% Silicon dioxide	0.8% Surfactants

Bioassays

Grain treatments

Formulations containing *B. bassiana* were applied at the concentration of 1 g/kg wheat. *M. robertsii* formulations were applied at concentrations that will deposit equivalent number of spores of the formulations containing *B. bassiana*; MrWP1 and MrWP4 at 1.5 g/kg wheat, MrWP2 and MrWP5 at 2.0 g/kg wheat, MrWP3 and MrWP6 at 2.5 g/kg wheat. In addition to these concentrations (1X), all the formulations were also tested doubling the dosage (2X). Additionally, pure fungal spore suspensions ($3x10^{10}$, $6x10^{10}$, $9x10^{10}$, $12x10^{10}$, and $18x10^{10}$ spores/kg) were tested to demonstrate the effect of formulating spores. Furthermore, all three insects were exposed to blank formulations with the highest filler contents (WP1 and WP4) as well. Each treatment was prepared with 10 mL of water and uniformly applied to 1 kg of wheat using a compressor-equipped HSENG Airbrush AS18. Control wheat was treated with 1% Tween 80 solution. *S. oryzae* and *R. dominica* bioassays were conducted in 50 mL Falcon tubes containing 40 g of treated wheat per replicate (five replicates). *P. interpunctella* assays were conducted using 1-liter glass jars with 300 g of treated wheat per replicate (three replicates). Twenty insects were added to each replicate. Experimental units were maintained at 30±2°C, 65±5% relative humidity (regulated using saturated $NaNO_2$ solutions), and in darkness. Mortality was recorded on days 3, 5, and 7 for *P. interpunctella* larvae, and on days 7 and 14 for *S. oryzae* and *R. dominica* adults.

Surface treatments

Formulations containing *B. bassiana* were applied at the concentration of 1 g/m² of surface area. The concentrations of *M. robertsii* formulations were determined using the same approach as that used for grain treatments. The concentrations were 1.5 g/m² for MrWP1 and MrWP4, 2.0 g/m² for MrWP2 and MrWP5, 2.5 g/m² for MrWP3 and MrWP6. Fungal spore suspensions ($3x10^{10}$, $6x10^{10}$, and $9x10^{10}$ spores/m²) were also tested. Blank formulations with the highest filler content (WP1 and WP4) were applied as fungus-free formulation. Each treatment was applied using 250 µL of water and a compressor-equipped HSENG Airbrush AS18 for uniform coverage. Control surfaces were treated with 1% Tween 80 solution. Twenty insects and three wheat grains were placed on each treated surface (five replicates per treatment). Bioassays were conducted at 30±2°C, 65±5% RH (maintained using saturated $NaNO_2$ solutions), and in darkness. Mortality was recorded on days 3, 5, 7, 9, 11, and 13 post-exposures.

Statistically analysis

One-way ANOVA was used to analyze the mortality rates of the wettable powder formulations against pests on both the product and the surface. Tukey's multiple comparison test ($p \leq 0.05$) was used to determine significant differences between means. For grain treatments (bioassay), an independent t-test ($p \leq 0.05$) was used to compare mortality rates between the two application doses (1X and 2X). Mortality data were arcsine-transformed prior to statistical analysis.

Results

Grain treatments

Insect mortalities after application of unformulated *B. bassiana* 5-4 and *M. robertsii* spores are illustrated in Figure 1. Both fungi showed quite high mortalities on *R. dominica* and *P. interpunctella* depending on spore concentration and time. However, *S. oryzae* adult mortalities were rather low exceedingly only 20% after 14 days at the highest concentrations. These data set a baseline to recognize the effects of formulations on mortalities.

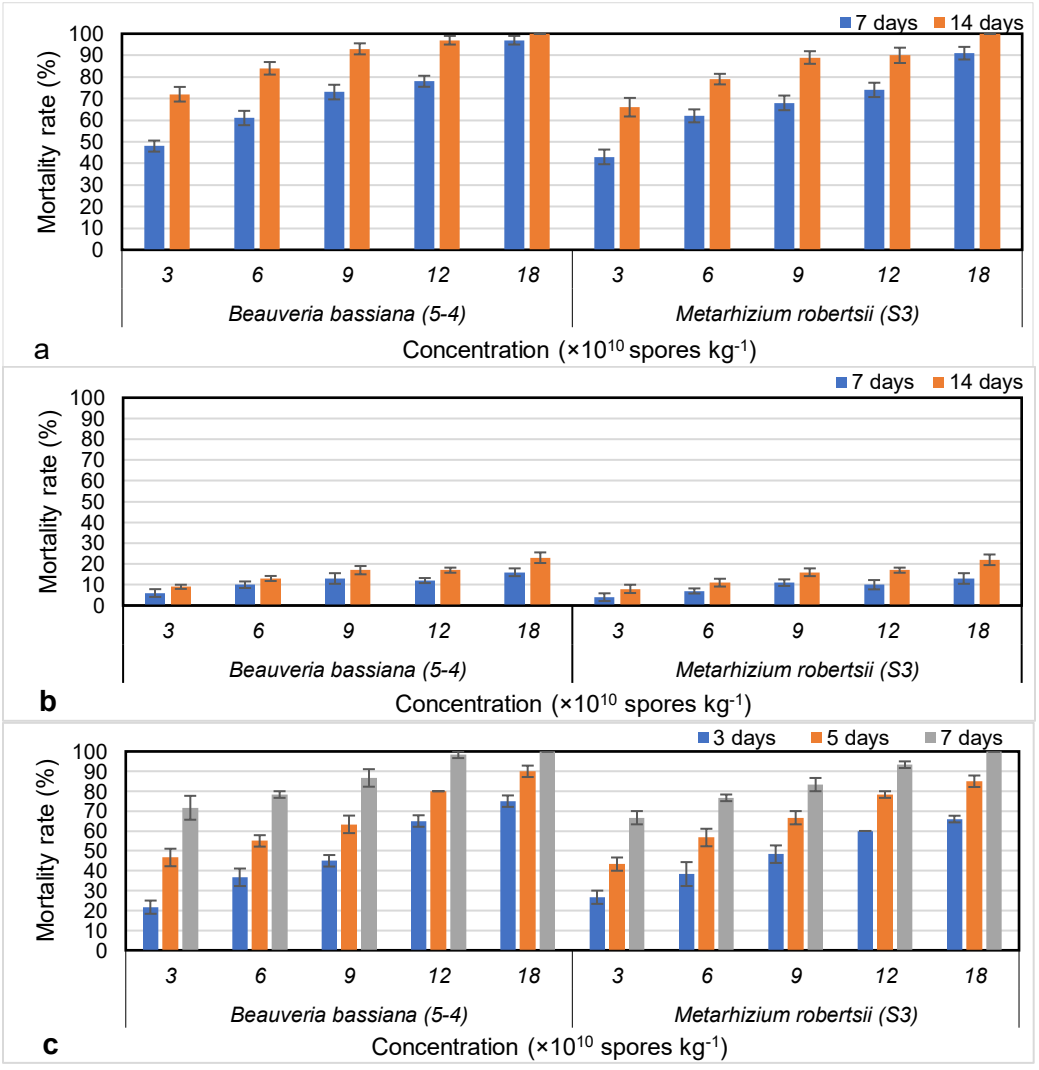

Figure 1. Mean (%)±SE mortality of a) *Rhyzopertha dominica*, b) *Sitophilus oryzae* and c) *Plodia interpunctella* on wheat treated with *Beauveria bassiana* and *Metarhizium robertsii* spores at five different concentrations.

Rhyzopertha dominica adult mortalities after applying formulations are presented in Table 3. Statistically significant differences in mortality were observed 7 and 14 days after application of wettable powder formulations at the standard dose (1X). Formulations BbWP2, BbWP3, BbWP5, BbWP6, MrWP2, MrWP3, MrWP5, and MrWP6 exhibited higher mortality than other formulations on both days. BbWP3 showed the highest mortality (87%) at 1X on day 7, while both BbWP2 and BbWP3 reached 100% mortality by day 14. All formulations achieved 100% mortality at the double dose (2X) on both days. Significant differences in mortality between the 1X and 2X doses were observed for all formulations on day 7 and for BbWP1, BbWP4, MrWP1, MrWP2, and MrWP4 on day 14. The blank formulations (WP1 and WP4) caused no mortality by day 14. Incorporation into wettable powder formulations significantly enhanced the insecticidal activity of both fungal isolates (5-4 and S3) across all tested applications. This demonstrates the improved efficacy of formulated entomopathogenic fungi against *R. dominica* under laboratory conditions.

Table 3. Mean (%)±SE mortality of *Rhyzopertha dominica* adults after 7 and 14 days of exposure to wheat treated with two different doses (1X and 2X) of wettable powder formulations

Exposure	7th day				14th days			
Treatment	1X		2X		1X		2X	
Control	0.0±0.0		0.0±0.0	-	0.0±0.0		0.0±0.0	-
BbWP1	59.0±4.30	bc	100.0±0.0	***	83.0±3.74	bc	100.0±0.0	**
BbWP2	81.0±2.91	a	100.0±0.0	***	100.0±0.0	a	100.0±0.0	-
BbWP3	87.0±2.00	a	100.0±0.0	***	100.0±0.0	a	100.0±0.0	-
BbWP4	57.0±4.10	c	100.0±0.0	***	76.0±4.0	c	100.0±0.0	***
BbWP5	77.0±2.55	a	100.0±0.0	***	97.0±2.0	a	100.0±0.0	-
BbWP6	81.0±4.00	a	100.0±0.0	**	99.0±1.0	a	100.0±0.0	-
MrWP1	49.0±2.45	c	100.0±0.0	***	72.0±2.55	c	100.0±0.0	***
MrWP2	76.0±3.32	ab	100.0±0.0	***	93.0±2.55	ab	100.0±0.0	*
MrWP3	81.0±3.67	a	100.0±0.0	***	97.0±2.0	a	100.0±0.0	-
MrWP4	56.0±1.87	c	100.0±0.0	***	77.0±2.0	c	100.0±0.0	***
MrWP5	77.0±3.90	a	100.0±0.0	***	96.0±1.87	a	100.0±0.0	-
MrWP6	84.0±2.91	a	100.0±0.0	**	97.0±2.0	a	100.0±0.0	-
F and *p* values	$F_{11,59}$=14.403 *p*<0.0001		-		$F_{11,59}$=16.442 *p*<0.0001		-	

- Within each column, different letters indicate significant differences according to Tukey's multiple comparison test ($p<0.05$).
* $p<0.05$, ** $p<0.001$, and ***$p<0.0001$ indicate differences according to the significance level and T-test.

The mortality effects of formulations on *S. oryzae* adults are given in Table 4. Statistically significant differences were observed in the mortality effects at 1X and 2X doses on day 14. However, by day 7, a significant difference occurred at the 2X dose, while no significant difference was detected at the 1X dose. For all the formulations, the mortalities significantly increased by doubling the application dosage. Among the formulations, BbWP1 at the 2X dose exhibited the highest mortality rate against *S. oryzae* on day 14, reaching 65%. The blank formulations, WP1 and WP4, showed mortality rates of 24±1.87% and 18±1.22%, respectively, against *S. oryzae* on day 14. Comparing the mortalities caused by unformulated and formulated spores (Figure 1), formulating both isolates enhanced their efficacy against *S. oryzae* adults.

According to *P. interpunctella* larval mortalities (Table 5), statistically significant differences were observed between the formulations at the 1X dose on days 3 and 5. On both days, mortalities increased with the increasing number of spores in formulations. At the 1X dose, MrWP6 showed the highest mortality (90%) on day 3, reaching 100% by day 5. At the 2X dose, all formulations achieved 100% mortality by day 3. A significant difference in mortality between the 1X and 2X doses was observed across all formulations on day 3. The blank formulations (WP1 and WP4) resulted in 15±2.89% and 10±0.0% mortality, respectively, against *P. interpunctella* on day 7. The incorporation of entomopathogenic fungal isolates (5-4 and S3) into the wettable powder formulations significantly increased mortality in *P. interpunctella*.

Table 4. Mean (%)±SE mortality of *Sitophilus oryzae* adults after 7 and 14 days of exposure to wheat treated with two different doses (1X and 2X) of wettable powder formulations

Exposure	7 days				14 days			
Treatment	1X	2X			1X	2X		
Control	0.0±0.0	0.0±0.0	-		0.0±0.0	0.0±0.0	-	
BbWP1	21.0±2.91	47.0±2.55 a	***		43.0±2.55 a	65.0±2.74 a	***	
BbWP2	23.0±2.00	41.0±2.91 ab	**		39.0±1.87 ab	62.0±3.00 ab	**	
BbWP3	21.0±2.91	41.0±4.85 ab	*		39.0±2.91 ab	60.0±4.18 ab	**	
BbWP4	18.0±3.00	30.0±2.24 b	*		35.0±3.16 ab	49.0±1.87 ab	**	
BbWP5	15.0±1.58	33.0±3.00 ab	**		29.0±1.87 ab	51.0±2.91 ab	***	
BbWP6	13.0±2.55	30.0±4.47 b	*		25.0±3.53 b	48.0±4.64 ab	**	
MrWP1	21.0±1.87	44.0±5.79 a	**		37.0±2.55 ab	62.0±5.78 ab	**	
MrWP2	19.0±2.91	43.0±5.39 a	**		34.0±4.58 ab	57.0±6.44 ab	*	
MrWP3	19.0±2.91	40.0±5.39 ab	**		33.0±3.39 ab	58.0±4.64 ab	**	
MrWP4	22.0±1.22	34.0±3.32 ab	*		36.0±1.87 ab	49.0±2.91 ab	*	
MrWP5	17.0±3.39	30.0±3.54 b	*		31.0±2.92 ab	47.0±3.39 ab	*	
MrWP6	18.0±3.00	31.0±3.67 b	*		31.0±4.30 ab	45.0±3.87 b	*	
F and *p* values	$F_{11,59}$=1.40 *p*=0.204	$F_{11,59}$=2.499 *p*<0.05			$F_{11,59}$=2.628 *p*<0.05	$F_{11,59}$=2.906 *p*<0.05		

- Within each column, different letters indicate significant differences according to Tukey's multiple comparison test (p<0.05).
* p<0.05, ** p<0.001, and *** p<0.0001 indicate differences according to the significance level and T-test.

Table 5. Mean (%)±SE mortality of *Plodia interpunctella* 3rd instar after 3, 5, and 7 days of exposure to wheat treated with two different doses (1X and 2X) of wettable powder formulations

Exposure	3rd day				5th day			7th day	
Treatment	1X	2X			1X	2X		1X	2X
Control	0.0±0.0	0.0±0.0	-		0.0±0.0	0.0±0.0		0.0±0.0	0.0±0.0
BbWP1	65.0±2.89 d	100.0±0.0	***		83.33±1.67 c	100.0±0.0	***	100.0±0.0	100.0±0.0
BbWP2	71.0±3.33 bcd	100.0±0.0	***		90.0±0.00 abc	100.0±0.0	**	100.0±0.0	100.0±0.0
BbWP3	85.0±0.00 abc	100.0±0.0	***		98.33±1.66 ab	100.0±0.0	*	100.0±0.0	100.0±0.0
BbWP4	66.67±3.33 cd	100.0±0.0	***		83.33±3.33 bc	100.0±0.0	**	100.0±0.0	100.0±0.0
BbWP5	76.67±4.41 abc	100.0±0.0	**		93.33±4.41 abc	100.0±0.0		100.0±0.0	100.0±0.0
BbWP6	83.33±3.33 abc	100.0±0.0	**		95.0±2.89 abc	100.0±0.0		100.0±0.0	100.0±0.0
MrWP1	70.0±5.00 cd	100.0±0.0	***		90.0±2.89 abc	100.0±0.0	*	100.0±0.0	100.0±0.0
MrWP2	78.33±4.41 abc	100.0±0.0	**		95.0±2.89 abc	100.0±0.0		100.0±0.0	100.0±0.0
MrWP3	88.33±1.67 ab	100.0±0.0	***		98.33±1.67 ab	100.0±0.0		100.0±0.0	100.0±0.0
MrWP4	70.0±2.89 cd	100.0±0.0	***		86.67±1.67 bc	100.0±0.0	***	100.0±0.0	100.0±0.0
MrWP5	80.0±5.00 abc	100.0±0.0	*		96.66±1.67 abc	100.0±0.0		100.0±0.0	100.0±0.0
MrWP6	90.0±2.89 a	100.0±0.0	*		100.0±0.0 a	100.0±0.0		100.0±0.0	100.0±0.0
F and *p* values	$F_{11,35}$=6.038 *p*<0.0001				$F_{11,35}$=4.399 *p*<0.001				

Within each column, different letters indicate significant differences according to Tukey's multiple comparison test (p<0.05).
* p<0.05, ** p<0.001, and *** p<0.0001 indicate differences according to the significance level and T-test.

Surface treatments

Insect mortalities after exposure to concrete surface sprayed with unformulated *B. bassiana* 5-4 and *M. robertsii* spores are illustrated in Figure 2. Similar to grain treatment results (Figure 1), both fungi were quite effective against *R. dominica* and *P. interpunctella* depending on spore concentration and time. *Sitophilus oryzae* adult mortalities were still lower but not as low as those in grain treatments. Except for one formulation, mortalities were above 40% reaching up to 64%. The data set a baseline to understand the effects of formulations on mortalities.

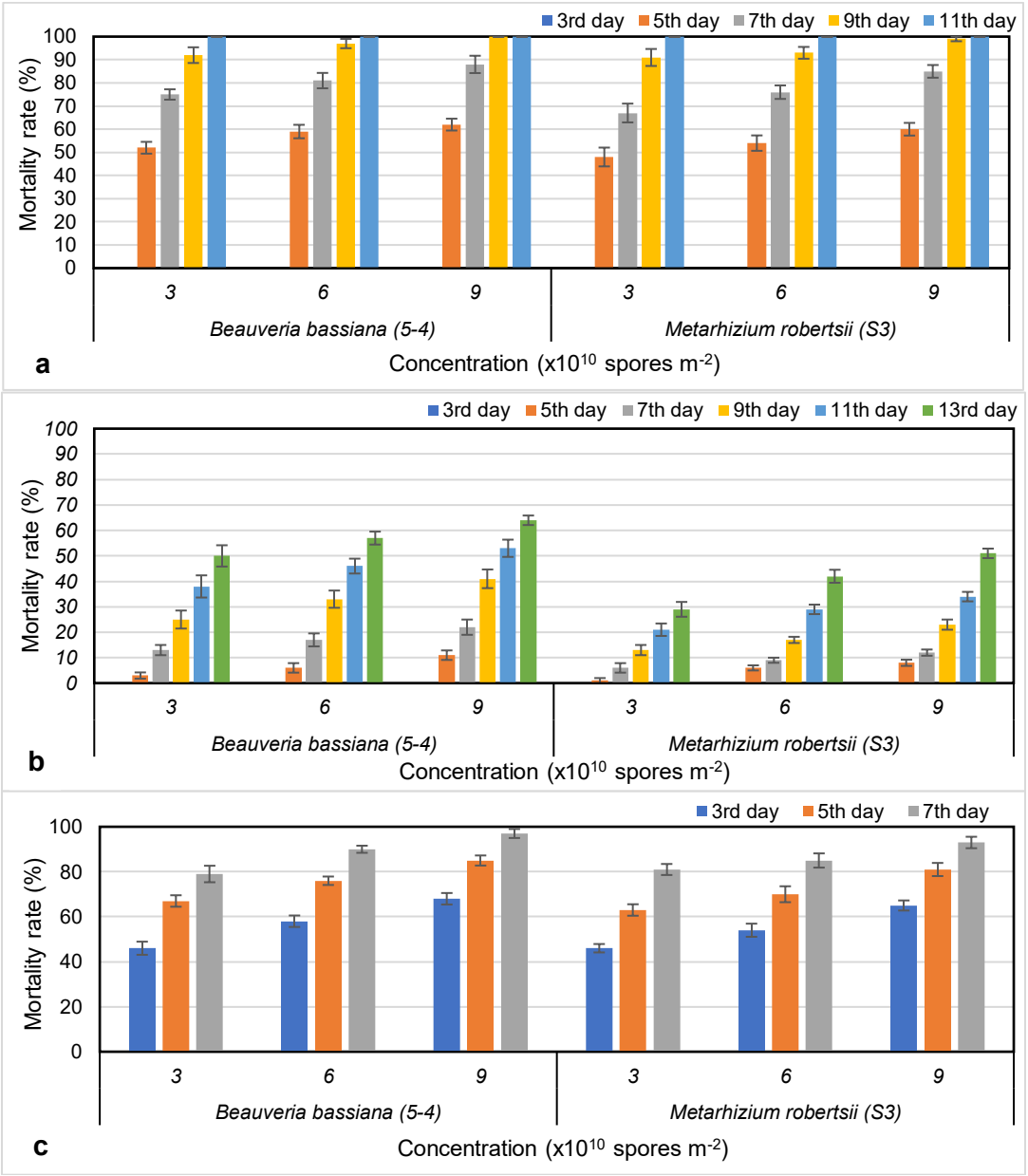

Figure 2. Mean (%)±SE mortality of (a) *Rhyzopertha dominica*, (b) *Sitophilus oryzae* and (c) *Plodia interpunctella* on concrete surfaces treated with *Beauveria bassiana* and *Metarhizium robertsii* spores at three different concentrations.

Table 6 shows the *R. dominica* adult mortalities after exposure to treated surface. None of the wettable powder formulations exhibited lethal effects on *R. dominica* after 3 days of exposure. Statistically significant differences in mortality were observed between the formulations on days 5, 7, and 9. BbWP3 showed the highest mortality, with 75% on day 5 and 94% on day 7. Most of the formulations caused quite high mortalities reaching 100% by day 9. On day 11, all the formulations achieved 100% mortality. The blank formulations had no measurable effect on *R. dominica* adults.

Table 6. Mean (%)±SE mortality of *Rhyzopertha dominica* adults after 3, 5, 7, 9, and 11 days of exposure to concrete surfaces treated with wettable powder formulations

Exposure / Treatment	3rd day	5th day		7th day		9th day		11th day
BbWP1	0.0±0.0	62.0±3.39	ab	83.0±3.00	ab	96.0±1.87	abc	100.0±0.0
BbWP2	0.0±0.0	70.0±2.74	a	89.0±2.91	ab	99.0±1.00	ab	100.0±0.0
BbWP3	0.0±0.0	75.0±2.24	a	94.0±1.87	a	100.0±0.0	a	100.0±0.0
BbWP4	0.0±0.0	60.0±2.74	ab	79.0±2.91	ab	93.0±2.55	bc	100.0±0.0
BbWP5	0.0±0.0	69.0±2.92	a	88.0±2.55	ab	100.0±0.0	a	100.0±0.0
BbWP6	0.0±0.0	74.0±3.67	a	93.0±2.55	a	100.0±0.0	a	100.0±0.0
MrWP1	0.0±0.0	50.0±2.74	b	75.0±2.74	b	91.0±2.91	bc	100.0±0.0
MrWP2	0.0±0.0	67.0±4.36	a	87.0±4.36	ab	100.0±0.0	a	100.0±0.0
MrWP3	0.0±0.0	73.0±2.00	a	90.0±2.74	ab	100.0±0.0	a	100.0±0.0
MrWP4	0.0±0.0	50.0±3.53	b	73.0±3.00	b	91.0±1.87	c	100.0±0.0
MrWP5	0.0±0.0	65.0±3.16	ab	87.0±2.55	ab	99.0±1.00	ab	100.0±0.0
MrWP6	0.0±0.0	71.0±3.67	a	88.0±4.64	ab	96.0±2.45	abc	100.0±0.0
F	-	$F_{11,59}$=7.102		$F_{11,59}$=3.219		$F_{11,59}$=6.196		-
p	-	<0.0001		<0.005		<0.0001		-

-Within each column, different letters indicate significant differences according to Tukey's multiple comparison test (p<0.05).

The wettable powder formulations showed varying degrees of effectiveness against *S. oryzae* (Table 7). While no mortality was observed on day 3, significant differences emerged from day 5 onwards. BbWP1 consistently performed better than the rest, achieving 100% mortality by day 13. BbWP2 also reached 100% mortality on day 13. The blank formulations, WP1 and WP4, demonstrated lower mortality rates of 32±2.55% and 12±1.22%, respectively, by day 13. These results highlight the superior efficacy of BbWP1 and BbWP2 against *S. oryzae* compared to the other formulations and the controls.

Table 7. Mean (%)±SE mortality of *Sitophilus oryzae* adults after 3, 5, 7, 9, 11, and 13 days of exposure to concrete surfaces treated with wettable powder formulations

Exposure / Treatment	3rd day	5th day		7th day		9th day		11th day		13rd day	
BbWP1	0.0±0.0	38.0±3.39	abc	61.0±4.00	a	79.0±3.67	a	92.0±3.00	a	100.0±0.0	a
BbWP2	0.0±0.0	36.0±3.32	ab	53.0±4.10	ab	67.0±3.74	ab	89.0±1.87	ab	100.0±0.0	a
BbWP3	0.0±0.0	33.0±2.55	abc	47.0±4.36	abc	58.0±3.39	bc	73.0 ±3.39	bcd	87.0±3.39	bc
BbWP4	0.0±0.0	17.0±3.74	cd	30.0±3.53	cd	41.0±4.85	c	52.0±4.06	de	67.0±3.00	d
BbWP5	0.0±0.0	16.0±4.45	cd	32.0±3.00	cd	46.0±2.45	c	57.0±3.39	de	72.0±3.74	cd
BbWP6	0.0±0.0	18.0±2.55	cd	34.0±4.00	bcd	49.0±4.30	c	62.0 ±4.35	cde	76.0±3.67	cd
MrWP1	0.0±0.0	23.0±4.36	abcd	34.0±3.67	bcd	48.0±2.55	bc	62.0 ±3.39	cde	74.0±2.45	cd
MrWP2	0.0±0.0	39.0±4.30	a	52.0±5.15	ab	67.0±4.36	ab	80.0 ±4.18	abc	94.0±2.92	ab
MrWP3	0.0±0.0	32.0±4.67	abc	44.0±4.00	abcd	56.0±4.30	bc	66.0±4.30	cde	77.0±5.61	cd
MrWP4	0.0±0.0	21.0±3.32	abcd	31.0±3.32	cd	45.0±4.74	c	56.0±5.10	de	69.0±4.30	cd
MrWP5	0.0±0.0	20.0±3.53	bcd	32.0±3.74	cd	46.0±5.79	c	59.0±5.57	de	73.0±3.74	cd
MrWP6	0.0±0.0	15.0±3.16	d	26.0±3.32	d	38.0±4.10	c	48.0±4.10	e	62.0±4.63	d
F	-	$F_{11,59}$=6.523		$F_{11,59}$=8.184		$F_{11,59}$=9.033		$F_{11,59}$=13.347		$F_{11,59}$=24.099	
p	-	<0.0001		<0.0001		<0.0001		<0.0001		<0.0001	

-Within each column, different letters indicate significant differences according to Tukey's multiple comparison test (p<0.05).

Larval mortalities of *P. interpunctella* are given in Table 8 Formulations BbWP3 and BbWP6 showed the greatest efficacy against *P. interpunctella*. BbWP3 achieved 86% mortality after three days and 100% mortality after five. BbWP6 reached 99% mortality on day 5 and 100% on day 7. By day 7, most formulations (except MrWP1 and MrWP4) achieved over 90% mortality. The blank formulations had no effect. These results suggest that BbWP3 and BbWP6 are the most promising formulations for controlling *P. interpunctella* infestations.

Table 8. Mean (%)±SE mortality of *Plodia interpunctella* 3rd instar after 3, 5, and 7 days of exposure to concrete surfaces treated with wettable powder formulations

Exposure / Treatment	3rd day		5th day		7th day	
BbWP1	59.0±4.00	cde	77.0±4.06	bc	91.0±3.32	ab
BbWP2	60.0±2.74	cde	81.0±2.45	bc	97.0±1.22	ab
BbWP3	86.0±2.92	a	100.0±0.0	a	100.0±0.0	a
BbWP4	58.0±4.06	cde	81.0±2.92	bc	93.0±2.55	ab
BbWP5	56.0±4.30	de	79.0±4.58	bc	90.0±4.58	ab
BbWP6	82.0±3.39	ab	99.0±1.00	a	100.0±0.0	a
MrWP1	53.0±4.06	e	67.0±3.39	c	86.0±3.67	b
MrWP2	58.0±3.39	cde	74.0±2.92	bc	90.0±3.54	ab
MrWP3	74.0±2.45	abc	88.0±2.55	b	100.0±0.0	a
MrWP4	53.0±4.36	e	69.0±3.67	c	84.0±4.58	b
MrWP5	61.0±2.92	cde	76.0±3.32	bc	90.0±2.24	b
MrWP6	71.0±2.92	bcd	87.0±4.06	b	98.0±2.00	ab
F	$F_{11,59}$=10.833		$F_{11,59}$=21.281		$F_{11,59}$=5.113	
p	<0.0001		<0.0001		<0.0001	

-Within each column, different letters indicate significant differences according to Tukey's multiple comparison test (p<0.05).

DISCUSSION

This study assessed the efficacy of wettable powder formulations of entomopathogenic fungi against stored product pests *R. dominica*, *P. interpunctella*, and *S. oryzae*. The formulations were tested both on wheat grains and on a concrete surface.

The formulations exhibited significantly different mortality effects depending on the pest species and application method. *Rhyzopertha dominica* and *P. interpunctella* were notably more susceptible to the formulations than *S. oryzae*, regardless of the application method. This observation aligns with previous studies showing greater susceptibility of *R. dominica* and higher resistance of *S. oryzae* to entomopathogenic fungi (Moino et al., 1988; Rice & Cogburn, 1999; Dal Bello et al., 2001; Vassillakos et al., 2006; Kavallieratos et al., 2014). Formulations with a high spore content were more effective against *R. dominica* and *P. interpunctella*. Conversely, formulations with lower fungal content, but containing more fillers like diatomaceous earth and kaolin, were more effective against *S. oryzae*. The impact of these fillers on stored product pests has been documented in various studies. Abdelgaleil et al. (2021) specifically suggested the effect of kaolin against certain stored product pests. The efficacy of diatomaceous earth against stored product pests is well-established, as highlighted by numerous studies (Adane et al., 1996; Hidalgo et al., 1998; Storm et al., 2016; Lord, 2001; Dal Bello et al., 2001; Vassilakos et al., 2006; Kavallieratos et al., 2006; Sağlam et al., 2022). Mostly the effect of diatomaceous earth was found higher on *S. oryzae* adults and lower on *R. dominica* adults. These findings explain the results presented in this study. Although diatomaceous earth has an effect on *S. oryzae* mortality, blank formulations could only kill 32% of the adults in surface treatment, while fungal formulations reached up to 100%. For the other two tested insects, blank formulations caused much less mortalities, highlighting the efficacy of entomopathogenic fungi in the final formulations. Even though unformulated spores resulted in high mortalities for *R. dominica* and *P. interpunctella*, mortalities increased when spores were formulated. All the findings support that the tested formulations were suitable both for the fungal spores and for the targeted pests, as no adverse effects were noticed, and elevated efficacy was achieved. Overall, formulations containing *Beauveria bassiana* demonstrated greater efficacy against the tested stored product pests compared to formulations containing *M. robertsii*. The latter only showed better efficacy against *P. interpunctella* larvae when applied to wheat grains.

Effective concentration ranges for entomopathogenic fungal spores against stored product pests are typically between 500-1000 ppm equivalent to $1x10^{10}$-$1x10^{11}$ spores/kg grains according to the literature. The concentrations in our experiments were within this range.

The formulations were more effective against pests on concrete surfaces compared to grain applications. While many studies investigated applications of entomopathogenic fungi as powder on surfaces (Athanassiou et al., 2017; George et al., 2018), research on applying entomopathogenic fungi as suspensions in liquid is limited for stored-product pests. Existing surface application studies are primarily on chemical treatments. Wakil et al. (2023) demonstrated significant mortality effects against *T. castaneum* larvae and adults using *B. bassiana* and *M. anisopliae* by surface application. Further research on liquid surface applications of entomopathogenic fungi is needed. The findings presented here demonstrated that WP formulations of tested fungi can be considered for empty storage treatments against stored-product pests.

A commercially available diatomaceous earth-based insecticide, Protect-It, used for empty warehouses utilizes silicon dioxide and silica aerogel at concentrations of 70 mg/100 cm² (liquid) and 50 mg/100 cm² (powder). In the present study, significant pest mortality on concrete surfaces was achieved using formulations including considerably lower concentrations of diatomaceous earth.

Considering all the findings, the prepared wettable powder formulations are found to be appropriate for entomopathogenic fungi and may be more effective in empty storage areas. Specifically, the BbWP1 formulation demonstrated superior efficacy in general. Continued development and refinement of these formulations, incorporating new ingredients and technologies, can lead to further development for enhancing this pest control strategy.

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Original article (Orijinal araştırma)

Investigation of insecticide residues in the soil of agricultural areas and around water resources and associated risk assessment[1]

Tarımsal alanlar ve su kaynakları çevresindeki topraklarda insektisit kalıntılarının araştırılması ve ilgili risk değerlendirmesi

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Abstract

Pesticides are the important contaminants for the environment. In this study, insecticide residues of the soils, taken from agricultural lands and around water resources in the Çanakkale-Central district were investigated. The Quick-Easy-Cheap-Efficient-Rugged-Safe (QuEChERS) method was employed to determine residues. Method verification was performed by spiking blank samples at 1 and 8 times the limit of quantification. 54 soil samples were taken from study area in 2020 and subjected to residue analyses. Of these samples, 44 had insecticides at various concentrations. Twenty insecticides were detected at various frequencies. Insecticide residue levels were between 1.01 and 760.01 µg/kg. Maximum etoxazole was detected as 760.01 µg/kg in one sample. This sample was sampled from the nearby fields where wastes were seen. In addition, 17 insecticides were found at various concentrations in the same sample. Risk assessments revealed low hazard for children and adults. In terms of hazard quotient (HQ) levels, maximum values were encountered for pyridaben ($445.00*10^{-7}$ for children and $59.33*10^{-7}$ for adults). The sum of HQs for all insecticides was $1310.00*10^{-7}$ for children and $174.67*10^{-7}$ for adults. It was concluded that farmers should be encouraged to use insecticides with low HQ values to mitigate soil contamination in places where insecticides are detected.

Keywords: Hazard quotient, health risk assessment, neonicotinoid, persistence of insecticide

Öz

Pestisitler çevre için önemli kirleticilerdir. Bu çalışmada Çanakkale-Merkez ilçedeki tarımsal alanlardan ve su kaynakları çevresinden alınan topraklarda insektisit kalıntıları araştırılmıştır. Kalıntıları belirlemek için Hızlı-Kolay-Ucuz-Etkili-Sağlam-Güvenli (QuEChERS) yöntemi kullanılmıştır. Metot doğrulaması, hesaplama limitinin 1 ve 8 katı seviyelerinde pestisit standardı eklenmesi ile yapılmıştır. Çalışma alanlarından 2020 yılında 54 toprak örneği alınmış ve kalıntı analizine tabi tutulmuştur. Bu örneklerden 44'ü çeşitli konsantrasyonlarda insektisit kalıntısı içermiştir. Farklı sıklıklarda 20 insektisit tespit edilmiştir. İnsektisit kalıntı seviyeleri 1.01 ila 760.01 µg/kg arasında değişmektedir. Maksimum etoxazole bir örnekte 760.01 µg/kg olarak tespit edilmiştir. Bu örnek pestisit atıklarının görüldüğü tarlaların yakınından alınmıştır. Ayrıca, aynı numunede çeşitli konsantrasyonlarda 17 insektisit bulunmuştur. Risk değerlendirmeleri, çocuklar ve yetişkinler için düşük düzeyde tehlike ortaya koymuştur. Tehlike katsayısı (HQ) seviyeleri açısından, pyridaben için maksimum değerlere rastlanmıştır (çocuklar için $445.00*10^{-7}$ ve yetişkinler için $59.33*10^{-7}$). Tüm insektisitler için toplam HQ değerleri çocuklar için $1310.00*10^{-7}$ ve yetişkinler için $174.67*10^{-7}$'dir. İnsektisit tespit edilen yerlerde toprak kirliliğini azaltmak için çiftçilerin düşük HQ değerlerine sahip insektisitlerin kullanmaya teşvik edilmesi gerektiği sonucuna varılmıştır.

Anahtar sözcükler: Tehlike katsayısı, sağlık risk değerlendirmesi, neonikotinoid, insektisit kalıcılığı

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Introduction

Pesticides are an important part of intensive agriculture. Pesticides potentially reduce pests-induced yield losses and increase production levels. Despite various advantages, pesticides also have some disadvantages, since only a small proportion of these pesticides reach the target organisms and the rest pollute the environment, affecting animals and humans (Kaur et al., 2023). Excessive and unintentional use of pesticides cause soil and water pollution and toxicity to living organisms. Pesticide residues may also generate serious damage on ecosystems and penetrate into the food chain (Tiryaki & Temur, 2010; Lewis et al., 2016; Kaur et al., 2024).

Soils around agricultural fields, orchards and animal drinking water sources can easily be contaminated with pesticides. Direct applications, runoff from pesticide-treated surfaces, accidental spills and the incorporation of plant residues treated with pesticides into the soil may result in soil contamination. The fate of pesticides in soil is affected by various physico-chemical, dynamic and biological processes. Such processes include adsorption-desorption, runoff, leaching, evaporation and degradation. Previous literature revealed that 14- 80% of applied chemicals reach the soil depending on the method and rate of application, plant growth stages and varieties (Çılgı & Jepson, 1992; Temur et al., 2012). Furthermore, Pimentel (1995) indicated that only 0.3% of applied pesticides reached the targeted pest and the rest (99.7%) dispersed into the surrounding environment. Just because of soil-environment and soil-plant interactions, soils constitute an important source of pesticides for plants (Li, 2025). Pesticides can also bioaccumulate in the soil due to previous years' applications, leading to greater environmental risk. Soil health is very important for sustainable agriculture. Therefore, the pesticide content of agricultural soils should regularly be assessed. Appropriate measures should be taken if there is a risk of pesticide contamination or accumulation (Karasali et al., 2016). Introducing restrictions on fertilizer or pesticide use without providing alternatives can destabilase food security (Futa et al., 2024). Persistent agrochemicals may exert serious health risks for both humans and the environment. Unconscious uses of these agrochemicals potentially destruct biodiversity, soil health and ecological processes (Liu et al., 2016; Bhandari et al., 2019). Therefore, several agrochemicals have been banned. These prohibitions have already raised an awareness of pesticide residues on foodstuffs.

Soil contamination with agrochemicals adversely affects agricultural fields. The widespread use of pesticides in agriculture could affect many non-target organisms and their succession in the ecosystem by altering the interaction between soil microbes and plants (Liu et al., 2025). The overuse and misuse of pesticides may result in contamination of agricultural fields (Balderacchi et al., 2014). These contaminants then exert serious health risks on humans, soils and groundwater. Such contaminations require integrated management and monitoring systems (Mariappan & Tamilarasan, 2025). Measurement of pesticide residues in agricultural soils is important to maintain environmental health standards and thereby minimize the harmful effects of pesticides on soil and water resources (Faraj et al., 2024).

The fate and behavior of a pesticide is designated by its solubility, degradation, half-life and partition coefficient (Li et al., 2025). Manufacturers usually provide half-life (DT_{50} days) values to indicate persistence of pesticides (Mangold et al., 2024). DT_{50} indicates whether pesticide tends to accumulate in the soil. Pesticides can be divided into 4 groups according to their half-life (days): <20 - readily degradable, 20-60 - fairly degradable, 60-180 - slightly degradable and >180 - very slightly degradable. Those with shorter DT_{50} tend to accumulate less in soil. Those with longer DT_{50} pose a greater risk in agricultural areas (Anonymous, 2025). Organochlorines and neonicotinoids with long half-lives can pose a serious contamination risk, especially in soils. Although organophosphate insecticides are highly toxic, they usually have DT_{50} values of <30 and therefore do not exert a long-term risk to soils and agricultural lands (Seagraves and Lundgren, 2012; Di Bartolomeis et al., 2019). However, residues of persistent pesticides can generate a source of chronic soil contamination (Mangold et al., 2024).

Total annual pesticide use in Türkiye is 57.7 kt in 2023 (TUIK, 2024). Pesticides constitute about 12.3 kt of this sum. The average pesticide consumption is about 2300 g a.i. per hectare. Fresh fruits and vegetables of different varieties are cultivated in Central District of Çanakkale province, Türkiye. Pesticides (especially insecticides) are used intensively to protect crops from pests. In 2023, 2.2 kt pesticides (243 t insecticides) were utilized on agricultural fields of Çanakkale province. Of this sum, 27% was utilized in Central District (Anonymous, 2024).

Various studies have confirmed that the Quick-Easy-Cheap-Efficient-Rugged-Safe (QuEChERS) method could also be safely used for pesticide residue analysis of soils (Nagel, 2009; Temur et al, 2012; Balkan, 2021; Polat & Tiryaki, 2022; Top et al., 2023). In the borders of Troia National Park, captan, endosulfan, ethion, cypermethrin, trifluralin, mancozeb, pesticides were detected with the range of 100-230 ppb, 16.7-230 ppb, 1-6 ppb, 20-80 ppb, 20 ppb and 2 ppb, respectively (Yıldırım & Özcan, 2007). In another study, the insecticide load of Troia was determined using the QuEChERS method. Detected pesticides were ordered as chlorantraniliprole > imidacloprid > pyridaben > clothianidin > indoxacarb (Polat & Tiryaki, 2022).

Insecticide residue levels of the soil samples, taken from agricultural lands and around water resources in the Çanakkale - Central district were investigated in present study. Health risk assessments were also performed for adults and children.

Materials and Methods

Chemicals and reagents

Pesticides were supplied by Chem Service (USA) and Dr. Ehrenstorfer GmbH (Germany). Chemicals and reagents used in present experiments included QuEChERS cleanup and extraction kits and nylon syringe filters. Other reagents and solvents, such as acetic acid (AcOH), acetonitrile (ACN), ammonium acetate, methanol and sodium chloride (NaCl) were supplied by Merck in Darmstadt, Germany. All reagents and solvents were analytical grade with a purity of 99%.

Equipment and chromatography

Insecticide detection was performed with the use of an LC-MS/MS instrument equipped with an Acquity UPLC BEH C_{18} column (1.7 µm, 2.1 mm x 100 mm). The flow rate, injection volume and the total run time were 0.35 mL/min, 1 µL, and 15 minutes, respectively. The current gradient programme consists of 10 mM ammonium acetate ($NH_4CH_3CO_2$) in pH 5 water (A) and 10 mM $NH_4CH_3CO_2$ in methanol (B). Fragment and precursor ions and retention times (t_R) are listed in Table 1.

Sampling and analysis of the soil

Soils were sampled from a depth of 5 to 25 cm in agricultural areas in Çanakkale Central District. In Autumn of 2020, 54 samples were taken from the study area, located between 39°57’24″ N latitudes and 26°14’48″E longitudes. The samples were transported to the laboratory in an ice box and kept frozen (-20°C) until the analyses were performed (Zaidon et al., 2019; Polat & Tiryaki, 2022). The soils were dried in the air and sieved through 2 mm sieves (EPA, 2007). Insecticide-free samples (confirmed by chromatographic analysis) were also taken from the same sites. Modified QuEChERS method was employed for analyses of blank and spiked samples (Adeyinka et al., 2019; Polat, 2021; Vickneswaran et al., 2021; Polat & Tiryaki, 2022). Approximately 10 g air-dried sample was supplemented with 100 µL AcOH, then with 15 mL ACN and mixed thoroughly for 15 seconds. Extraction kits were added to the mixture and vortexed for 1 min. The mixture was centrifuged at 3000 rpm for 10 min. After centrifugation, the supernatant was taken into a 50 mL centrifuge tube (including QuEChERS Cleanup Kits), vortexed for 15 s and centrifuged again at 3000 rpm for 10 min. The supernatant was then filtered through a 0.22 µm syringe into 2 mL vials and analysed by LC-MS/MS instrument.

Table 1. LC-MS/MS parameters and calibration parameters (5 -point calibration levels) for the insecticide

Insecticide	t_R*	Precursor ion m/z	Fragment ion, m/z (CE) **	Calibration range (ppb)	Calibration curve equation***	R^2
Acetamiprid	5.05	223.09	125.95 (21)	1-200	y=-71.6832 x^2 + 108657 x + 1294.52	0.9997
Bifenthrin	11.97	440.08	181.05 (20)	1-200	y=-1.75921 x^2 + 10550.5x + 745.791	0.9999
Chlorantraniliprole	8.19	482.00	283.94 (10)	1-200	y= -9.73986 x^2+14384 x- 708.385	0.9999
Clofentezine	9.99	302.98	137.96 (12)	1-200	y=-37.7542 x^2+ 33418.1 x + -61.8424	0.9998
Clothianidin	4.58	250.04	131.93 (16)	1-200	y= -11.008 x^2+ 15197.3 x + 2078.26	0.9996
Cyhalothrin-lambda	11.26	467.22	225.04 (10)	10-2000	y= -0.03035 x^2 +1418.16 x +1139.43	0.9999
Deltamethrin	11.39	523.04	280.92 (15)	1-200	y= -0.274504 x^2 + 3382.47 x+-515.595	0.9998
E. benzoate	4.02	886.60	158.11(36)	1-200	y = -44.8434 x^2 + 114086 x +15623.8	0.9999
Etoxazole	11.09	360.19	140.99 (48)	1-200	y= -175.839 x^2 + 237612 x + 16003.1	0.9997
Flubendiamide	9.60	680.99	253.99 (30)	1-200	y=-11.112x^2+11290.5x+ 1704.8	0.9988
Hexythiazox	10.93	353.08	227.99 (16)	1-200	y=-19.6547 x^2 + 37913 x + 926.07	0.9997
Imidacloprid	4.57	256.03	175.05 (15)	1-200	y= -5.76398 x^2+ 12261.9 x + -35.9641	0.9998
Indoxacarb	10.27	528.04	202.99 (24)	1-200	y= -7.27211 x^2+ 7726.08 x + -571.225	0.9999
Metaflumizone	10.69	507.13	178.04 (36)	10-2000	y= 0.106122 x^2 + 3653.8 x + 511.94	0.9999
Methoxyfenozide	8.87	369.19	149.02 (16)	1-200	y= -297.829 x^2+ 82874.9 x + -3970.18	0.9999
Novaluron	10.40	493.05	158.01 (18)	1-200	y= -0.704415 x^2+ 6529.42 x + 323.143	0.9997
Pirimicarb	7.58	239.15	71.99 (22)	1-200	y= -86.9902 x^2 + 180575 x + -2330.9	0.9999
Pymetrozine	3.80	218.09	104.94 (24)	1-200	y= -53.4778 x^2+ 123182 x + -6110.02	0.9999
Pyridaben	11.46	365.14	147.08 (28)	1-200	y= -127.957 x^2+ 116395 x + 8582.84	0.9997
Thiamethoxam	3.86	292.01	211.04 (13)	1-200	y= -12.4825 x^2 + 32248.7 x + -634.857	0.9999

* t_R, retention time (minutes); ** CE, collision power (V); *** matrix matched calibration; R^2, Correlation coefficients.

Method verification

Method verification was conducted to prove the method employed for a specific sample provided reliable outcomes (Aysal et al., 2007; Yolci Omeroğlu et al., 2015; Balkan & Karaağaçlı, 2023). Recovery, linearity, precision and LOQ were used to verify the method (Yolci Omeroğlu et al., 2013; SANTE, 2021). For recovery testing, 100 µL of insecticide fortification solutions were added to blank samples (10 g) at 1 and 8 times of LOQ levels. Analyses were performed in five repetitions. Calibration ranges of insecticides were provided in Table 1. A matrix-matched calibration curve was utilized for insecticide quantification.

Health risk assessment

Humans are exposed to insecticides by ingestion, inhalation and direct contact. The health risks of insecticides were assessed based on the residue levels found in insecticide-contaminated areas. Health risks were estimated based on previously described methods (EPA, 1998; Chen et al., 2011; Sadeghi-Yarandi et al., 2020; Polat & Tiryaki, 2023). LADD (life- time average daily dose, mg/kg bw day), HI (hazard index) and HQ (hazard quotient) were estimated using the below formulas (EFSA, 2007; Jing et al.,2021; Tadesse, 2021).

$$LADD=\frac{Cs \text{ X } IR \text{ X } CF \text{ X } EF \text{ X } ED}{BW \text{ X } AT} \quad (1)$$

$$HQ=\frac{CDI}{RfD} \quad (2)$$

$$HI=\sum HQ \quad (3)$$

where Cs is insecticide concentration (mg/kg or mg/L), IR is ingestion rate (for soil: 200 mg/day for children and 100 mg/day for adults; for water: 0.87 L/ day for children and 1.4 L/day for adults, CF is conversion factor (10^{-6} kg/mg). ED is exposure duration, EF is exposure frequency (350 days), BW is body weight, AT is averaging time (EF × ED days) and RfD is reference dose. CDI also known LADD is chronic daily intake (mg/ kg day) for a single compound.

The LADD values less than 10^{-6} for insecticides in soil indicate an acceptable risk limit (EPA, 1998). The HQ was calculated for each insecticide using the LADD and the reference dose (RfD) value. The HQ≥1 indicates a potential risk to human health and the HQ<1.0 indicates an insignificant hazard (EFSA, 2007; Jing et al., 2021). The total exposure to all insecticides was estimated using an HI. HI values less than 1 indicate that the consumer is protected, whereas HI values greater than 1 represent an unreasonable health risk (Yeladi et al., 2024). This comment gives an indication of which compound contributes most to the hazard. Together with the HQ for the individual insecticide, the HI values provide an indication of which pesticides would be most appropriate to reduce the risk from the insecticide (EFSA, 2007).

Results and Discussion

Method verification

Calibration curves of 20 insecticides over the various concentration ranges, retention times and equations for matrix-matched calibration lines are provided in Table 1. Correlation coefficients (R^2) were all greater than 0.999. Insecticides were quantified with the use of relevant analytical functions (Tiryaki et al., 2008). Recovery rates and LOQs are provided in Table 2.

Table 2. Recoveries with RSDs values, and LOQs

Insecticide	LOQ (µg/kg)	Spiking level						Mean	
		1×LOQ			8×LOQ				
		Found (µg/kg)	Recovery (%)*	RSD (%)	Found (µg/kg)	Recovery (%)*	RSD (%)	Recovery (%) (As a tool for trueness)	RSD (%) (As a tool for precision, repeatability)
Acetamiprid	1	0.88	87.79	4.5	8.30	103.76	1.58	95.77	9.28
Bifenthrin	1	0.65	64.56	3.27	7.48	93.50	4.70	79.03	19.73
Chlorantraniliprole	1	0.89	88.64	6.39	7.91	98.84	1.13	93.74	7.05
Clofentezine	1	0.80	80.12	4.98	7.46	93.25	14.34	86.69	13.37
Clothianidin	1	0.92	92.48	5.39	7.99	99.91	0.83	96.19	5.37
Cyhalothrin- L.	10	8.95	89.48	5.33	46.43	116.07	3.38	102.77	14.21
Deltamethrin	1	1.49	76.40	13.42	9.94	62.14	2.25	68.37	13.76
E. benzoate	1	0.52	51.95	15.60	3.59	48.85	3.97	48.40	13.79
Etoxazole	1	0.88	87.80	2.60	6.63	82.86	1.10	85.33	3.60
Flubendiamide	1	0.79	78.83	5.12	7.91	96.40	1.52	87.61	11.06
Hexythiazox	1	0.80	80.32	1.51	8.89	111.10	3.88	95.71	17.23
Imidacloprid	1	0.95	94.61	7.55	8.09	101.13	2.47	97.87	6.24
Indoxacarb	1	0.66	65.60	11.19	6.65	83.11	3.25	74,35	14.25
Metaflumizone	10	7.38	73.81	10.32	83.67	104.59	2.28	89.2	19.14
Methoxyfenozide	1	0.86	85.97	5.50	7.85	98.15	2.36	92.06	7.94
Novaluron	1	0.86	85.87	10.65	7.77	97.07	3.65	91.32	9.75
Pirimicarb	1	0.83	83.31	5.57	7.84	98.04	2.71	90.67	9.42
Pymetrozine	1	0.61	60.59	8.20	5.90	73.71	3.91	67.15	11.97
Pyridaben	1	0.94	93.88	4.42	8.55	106.92	8.88	100.40	9.71
Thiamethoxam	1	0.79	89.92	3.88	8.53	106.62	3.11	92.71	16.72
Method overall recovery (accuracy): 86.77 % (n=200; SD=16.54; RSD%=19.06)									

* Average of 5 repetitions.

Insecticide recovery rates ranged from 60.59 to 115.50 % with RSDs of between 3.6 - 19.73%, all of the values were within the acceptable range for SANTE (2021) (60-140%). Overall recovery ratio was determined to be 86.77% with an RSD of 19.06 % (SD= 16.54; n=200). LOQ values showed that present method could detect insecticide residues lower than the MRL set by the EU (EU, 2025; Polat & Tiryaki, 2023). The modified QuEChERS method has proven to be an accurate, reliable and rapid tool for the detection of insecticide residues in soil. In previous studies (Lesueur et al., 2008; González-Curbelo et al., 2022), the QuEChERS method for pesticide residue analyses in soil has been compared with other extraction methods such as accelerated solvent extraction (ASE), pressurized liquid extraction (PLE), microwave-assisted extraction (MAE), solid-liquid extraction (SLE), solid-phase extraction (SPE), Soxhlet extraction and ultrasonic solvent extraction (USE). Although MAE, ASE and USE have been developed as

faster, more practical and environmentally friendly methods than the Soxhlet method, the QuEChERS method is the first choice because of its high performance and ease of modification for specific pesticide and matrix combinations. The QuEChERS method provides the best recoveries with reliability on a QA/QC basis.

Residues in samples

An LC-MS/MS system was used for analyses of insecticides and detected residues above the LOQ were assessed. The max-min and mean insecticide residues are shown in Table 3. Of 54 samples collected from the study area, 44 samples (81.48%) contained different concentrations of insecticide and residues of 20 insecticides were detected at different frequencies. The environmental risk characteristics and hazard classifications of the pesticides are shown in Table 4. The most frequent insecticides were in the following order: chlorantraniliprole (27 samples) > pyridaben (22 samples) > clothianidin and imidacloprid (19 samples) > thiamethoxam (14 samples) > indoxacarb (13 samples) > flubendiamide (10 samples) > deltamethrin and methoxyfenozide (9 samples). The other insecticides were found in less than 9 samples. The concentration of insecticide residues varied from 1.01 µg/kg for deltamethrin, imidacloprid and flubendiamide to 760.01 µg/kg for etoxazole. Etoxazole was detected at a concentration of 760.01 µg/kg in the sample with the highest level. This sample was collected from the nearby fields where pesticide waste was encountered. In addition, 17 insecticides were detected at various concentrations in the same sample. Thiamethoxam was also detected (230.30 µg/kg) in this sample.

Chlorantraniliprole residues ranged from 1.10 to 153.53 µg/kg, pyridaben residues varied between 1.02 - 104.61 µg/kg, clothianidin between 1.02 - 14.07 µg/kg and imidacloprid between 1.01 - 32.37 (Table 3). Clothianidin was banned in Türkiye on 31 July 2019 (PPPD, 2024). EFSA has not ruled out a high risk for clothianidin (EFSA, 2016).

Table 3. Concentrations of insecticides (in triplicate analysis) in soil samples from different cropping areas

Insecticide	Residue, µg/kg											
	Agricultural land (empty)			Vegetable			Orchard			Around water source		
	Min.	Max.	Mean/ F.D*	Min.	Max.	Mean/ F.D*	Min.	Max.	Mean/ F.D*	Min.	Max.	Mean/ F.D*
Acetamiprid	2.10	47.60	24.40 / 2							1.69	1.81	1.70 / 1
Bifenthrin	13.13	14.36	13.60 / 1									
Chlorantraniliprole	1.10	153.53	14.10 / 18	1.57	73.29	28.9/4	2.00	12.80	6.45 / 2	2.66	4.52	3.50 / 3
Clofentezine	3.98	65.55	20.50 / 5							1.15	2.63	1.70 / 1
Clothianidin	1.02	14.07	3.70 / 14	1.56	4.08	2.4 / 4				5.70	6.32	6.02 / 1
Cyhalothrin-L.	106.10	113.39	108.80 /1							113.67	129.48	121.70 / 1
Deltamethrin	1.01	71.90	16.80 / 5	3.77	5.75	4.8 / 2	1.28	1.38	1.34 / 1	7.29	13.22	9.70 / 1
E. benzoate	3.15	5.16	3.90 / 1									
Etoxazole	159.00	760.01	376.40 /1	3.14	7.89	5.3 / 1						
Flubendiamide	1.02	117.81	23.44 / 8							1.01	12.18	6.60 / 2
Hexythiazox	1.44	73.43	25.30 / 3							1.05	1.29	1.10 / 1
Imidacloprid	1.01	12.29	3.55 / 13	1.49	32.37	11.7 / 3				1.86	6.36	4.77 / 3
Indoxacarb	1.02	256.86	25.50 / 9	5.13	9.36	7.5 / 2				1.20	13.16	3.50 / 2
Metaflumizone	9.90	25.25	15.80 / 2	12.95	19.43	15.7 / 1						
Methoxyfenozide	3.54	194.51	44.10 / 6	1.25	3.24	2.2 / 2				5.19	7.32	6.20 / 1
Novaluron	1.29	149.00	38.90 / 4							2.55	3.42	3.10 / 1
Pirimicarb	1.47	42.89	18.72 / 2									
Pymetrozine	58.64	65.60	63.27 / 1									
Pyridaben	1.02	104.61	19.41 /14	2.12	39.84	15.2 / 3	16.25	39.63	26.50 / 2	1.14	56.76	10.10 / 3
Thiamethoxam	1.08	230.30	32.72 / 9	1.65	34.11	15.8 / 3	15.54	34.61	24.30 / 2			

* Frequency of detection.

Table 4. Hazard classifications and environmental risk characteristics (decimal digit point)

Insecticide	Chemical category*	Persistency*	DT_{50} (field), day*	WHO Classification**
Acetamiprid	Neonicotinoid	Non-persistent	3.0	II
Bifenthrin	Pyrethroid	Moderately persistent	86.8	II
Chlorantraniliprole	Diamide	Persistent	204.0	U
Clofentezine	Tetrazine	Moderately persistent	63.0	III
Clothianidin	Neonicotinoid	Persistent	121.2	II
Cyhalothrin-lambda	Pyrethroid	Non-persistent	26.9	II
Deltamethrin	Pyrethroid	Non-persistent	21.0	II
E. benzoate	Micro-organism derived substance	Non-persistent	1.1	II
Etoxazole	Diphenyl oxazoline	Non-persistent	7.3	III
Flubendiamide	Phthalamide	-	-	III
Hexythiazox	Carboxamide	Non-persistent	17.7	U
Imidacloprid	Neonicotinoid	Persistent	174.0	II
Indoxacarb	Oxadiazine	Non-persistent	5.9	II
Methoxyfenozide	Carbohydrazide compound	Moderately persistent	68.0	U
Metaflumizone	Semicarbazone compound	Non-persistent	13.8	U
Novaluron	Benzoylurea	Moderately persistent	96.5	U
Pirimicarb	Carbamate	Non-persistent	9.0	II
Pyridaben	Pyridazinone	Non-persistent	29.0	II
Pymetrozine	Pyridine	Non-persistent	22.6	III
Thiamethoxam	Neonicotinoid	Moderately persistent	39.0	II

* From the IUPAC-PPDB (PPDB, 2024) **III, low hazardous; II, modera hazard; U, probably not an acute health hazard; (WHO, 2019).

A total of 4 neonicotinoid and 3 pyrethroid insecticides were detected in soil samples (Tables 3 & 4). According to the WHO classification (WHO, 2019), 11 out of 20 insecticides were moderately hazardous (Class II). Neonicotinoids, which can be used against aphids, whiteflies and thrips, kill the insects with their neurotoxic effect by binding to acetylcholine receptors (PPDB, 2024; IRAC, 2025). Pyrethroid pesticides are bound to the sodium channel, immobilize and paralyze insects (Ahamad & Kumar, 2023). Pyrethroid pesticides are also linked to neurologic and cardiovascular diseases of humans (Bao et al., 2020; Xue et al., 2021; Ahamad & Kumar, 2023). Pyrethroid pesticides include cypermethrin, deltamethrin and cyhalothrin-lambda (Yang et al., 2020). The mean insecticide residues of bifenthrin, cyhalothrin- lambda, deltamethrin and were found to be 14 µg/kg, 115 µg/kg, 8 µg/kg, respectively (Figure 1).

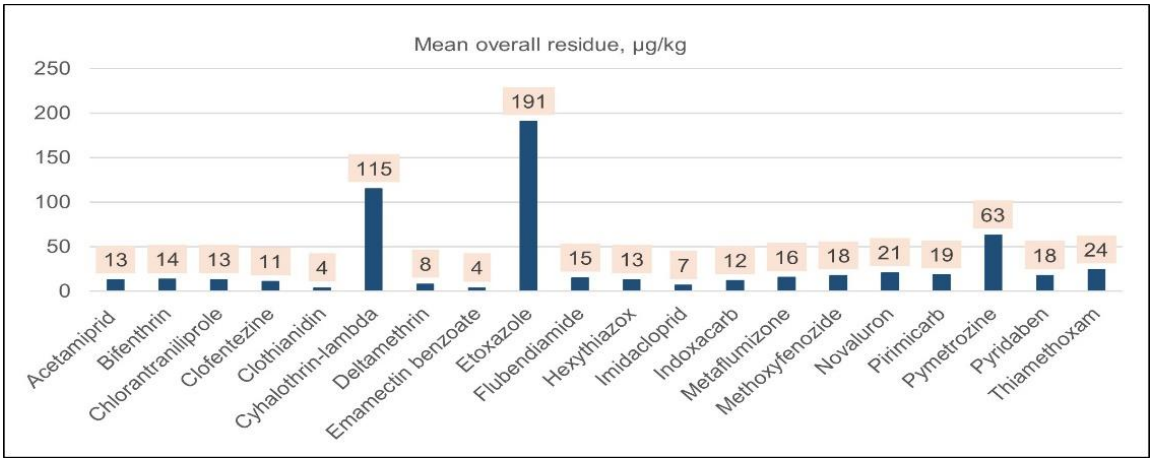

Figure 1. The mean insecticide residues.

The half-life (DT_{50}) of insecticides provides information on the persistence of insecticides in soil and the environment. Chlorantraniliprole (DT_{50}=204 days), clothianidin (DT_{50}=121.2 days) and imidacloprid (DT_{50}=174 days) were detected in 65 samples as persistent insecticides. These persistent insecticides and pyridaben (DT_{50}=29 days) were found in almost all of the agricultural lands (Table 3). The relationship between DT_{50} and frequency of detection of insecticides is shown in Figure 2. Present DT_{50} values varied from 3 days (acetamiprid) to 204 days (chlorantraniliprole).

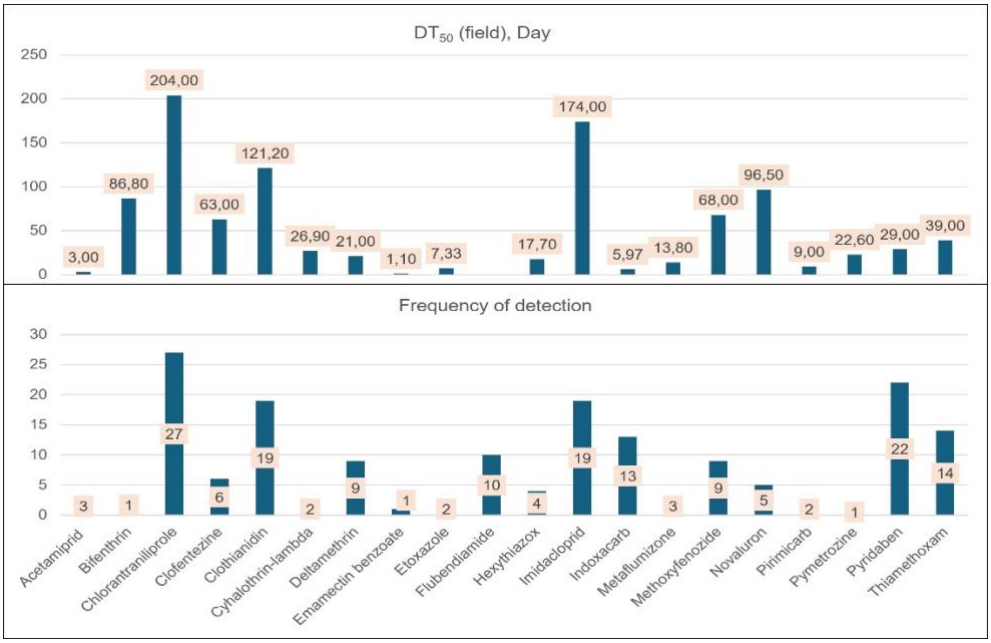

Figure 2. The relationship between half-lives and detection frequencies of insecticides.

The study identified the insecticides emamectin benzoate, acetamiprid, indoxacarb, etoxazole and pirimicarb with DT_{50} values less than 10 days. Mariappan & Tamilarasan (2025) showed that indoxacarb (DT_{50}= 5.97 days) was readily degradable and therefore did not exert a risk of contamination for groundwater. Indoxacarb had a limited mobility in soil, thus posed a slight risk of groundwater contamination.

Health risk assessment

Health risk assessments were performed for insecticides (10 insecticides in total) that were most frequently detected (more than 5 samples) in soil samples. The HQs of individual insecticides, calculated with LADD (mg/ kg bw per day) and RfD values, and the cumulative hazard of all insecticides (HI) for soil samples are presented for children and adults in Table 5. Current HQ and HI values for soil samples were < 1, indicating insignificant hazards. The highest mean HQ value for pyridaben was identified as $445.00*10^{-7}$ (with a range of $2.55\text{-}2615.25*10^{-7}$) for children and $59.33*10^{-7}$ (with a range of $3.40\text{-}348.7*10^{-7}$) for adults (Table 5). Similarly, the lowest mean HQ values for chlorantraniliprole were found to be $1.05*10^{-7}$ (with a range of $0.09\text{-}12.15*10^{-7}$) and $0.14*10^{-7}$ (with a range of $0.01\text{-}1.62*10^{-7}$) for children and adults, respectively. The mean HQs for both children and adults were ordered as pyridaben > thiamethoxam > novaluron > deltamethrin > indoxacarb > clothianidin. The sum of hazard ratios (HQ) for all the insecticides was $1310.00*10^{-7}$ (with a range of $100.54\text{-}10689.00*10^{-7}$) for children and $174.67*10^{-7}$ (with a range of $13.405\text{-}1425.28*10^{-7}$) for adults. But HQ for an individual insecticide indicates which insecticide should be used to decrease the risks for a specific location. In present cases, pyridaben (FD=22) had greater risk than clothianidin and imidacloprid. Pyridaben is non-persistent with a DT_{50}= 29 days. Clothianidin and imidacloprid (FD=19) are persistent with a DT_{50}= 121.2 and 174 days, respectively. Pyridaben is used to control spider mite and whiteflies insects. Clothianidin and imidacloprid are used against the aphids, thrips

and plant hopper insects, respectively. In this case, farmers should prefer insecticides with less environmental risk. The risks of human exposure to insecticides in the study soils were within acceptable limits. However, the levels of insecticides in agricultural soils should be monitored regularly, especially for environmental risks arising from their transfer to the surface waters.

Table 5. Hazard Quotients [HQ ($x10^{-7}$)] and Hazard Indexes [HI ($x10^{-7}$)] for children and adults in soil

Insecticide	F.D*	Children				Adult			
		HQ**		HI***		HQ**		HI***	
		Range	Average	Range	Average	Range	Average	Range	Average
Chlorantraniliprole	27	0.09-12.15	1.05	100.54-10689.00	1310.00	0.01-1.62	0.14	13.405-1425.28	174.67
Pyridaben	22	2.55-2615.25	445.00			3.40-348.7	59.33		
Clothianidin	19	13.01-179.46	51.28			1.73-23.93	6.84		
Imidacloprid	19	2.21-70.99	14.61			0.30-9.46	1.95		
Thiamethoxam	14	11.25-2398-96	252.71			1.50-319.86	33.69		
Indoxacarb	13	6.38-1605.38	75.98			8.50-214.05	10.13		
Flubendiamide	10	0.63-73.63	9.38			0.08-9.81	1.25		
Deltamethrin	9	2.53-1797.50	200.00			3.37-239.66	26.66		
Methoxyfenozide	9	1.56-243.14	21.38			0.21-32.42	2.92		
Novaluron	5	14.66-1693.18	238.13			1.95-225.76	31.75		

* Frequency of detection; **Hazard quotient (HQ); ***Hazard index (HI).

Conclusion

Experimental findings showed that the QuEChERS combined with an LC-MS/MS device could provide a reliable, accurate and rapid tool for insecticide analysis in soils. Twenty insecticides were detected at various concentrations in soil samples. The most abundant insecticides were in the following order: chlorantraniliprole (27 samples) > pyridaben (22 samples) > clothianidin and imidacloprid (19 samples) > thiamethoxam (14 samples) > indoxacarb (13 samples) > flubendiamide (10 samples) > deltamethrin and methoxyfenozide (9 samples). The DT_{50} values of insecticides were high, indicating different persistence classes. The maximum residue of etoxazole was found in one sample at 760.01 µg/kg. This sample was taken from the nearby fields with pesticide wastes. HIs and HQs were mostly < 1.0 for both adults and children. Despite safe levels of existing insecticides, precautions might be taken against potential toxicity of insecticides. The environmental and human health impacts of pesticide residues in soil, rather than degradation products, should not be neglected. These residues can remain in the environment for long periods of time, potentially leading to bioaccumulation and trophic transfer along the food chain. Furthermore, the behavior of agricultural producers also plays a critical role in determining residue levels. Therefore, targeted education programs and awareness campaigns are essential to promote responsible pesticide use and reduce soil contamination.

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Original article (Orijinal araştırma)

Effect of different processing techniques on the residue levels of some acaricides and insecticides in gherkin pickles[1]

Farklı işleme tekniklerinin kornişon turşularındaki bazı akarisit ve insektisitlerin kalıntı seviyeleri üzerindeki etkisi

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Abstract

Gherkin plants grown in the greenhouse of Bursa Uludağ University were sprayed with different acaricides and insecticides (spiromesifen, etoxazole, deltamethrin, chlorantraniliprole, acetamiprid) at the legal field application doses in 2023. Fruits that were harvested after the pre-harvest intervals of the applied test pesticides were processed for pickle making. Pickles were produced by fermentation and canning (fresh pack) techniques. Changes in pesticide residue levels were monitored at each processing step. Processing factors for each pesticide were calculated for fermentation and canning techniques. No significant reductions were observed in the concentrations of all pesticides in raw material following harvest. On the other hand, changes in the concentrations of spiromesifen, chlorantraniliprole and acetamiprid were significant throughout both canning and natural fermentation processes. However, neither process affected the concentrations of deltamethrin and etoxazole. The stability of deltamethrin residues may be related to low pH in both types of processes, but this explanation is not suitable for etoxazole due to its increased stability under high pH conditions. Processing factors of all the tested pesticides were lower than 1 for both treatments but varied depending on the processing method and chemical characteristics and degradation mechanisms of the pesticides.

Keywords: Acaricide, canning, gherkins, fermentation, food safety, insecticide, processing factor

Öz

Bu çalışmada, Bursa Uludağ Üniversitesinin serasında yetiştirilen kornişon tipi hıyar bitkilerine, 2023 yılında önerilen uygulama dozlarında farklı akarisit ve insektisitler (spiromesifen, etoxazole, deltamethrin, chlorantraniliprole, acetamiprid) uygulanmıştır. Tüm pestisitler için hasat öncesi aralık süreleri tamamlandıktan sonra hasat edilen meyveler, turşu işleme için hazırlanmıştır. Turşular, fermantasyon ve konserve teknikleri kullanılarak üretilmiştir. Her işleme aşamasında pestisit kalıntılarındaki değişiklikler izlenmiş ve her bir pestisit için fermente ve konserve yöntemleri için işleme faktörleri hesaplanmıştır. Hasat sonrasında ham maddede uygulanan tüm pestisitlerin konsantrasyonlarında önemli bir azalma bulunmamıştır. Öte yandan, spiromesifen, chlorantraniliprole ve acetamiprid konsantrasyonlarındaki değişiklikler hem konserve hem de doğal fermantasyon işlemleri boyunca önemli bulunmuştur. Ancak, her iki işlem türü de deltametrin ve etoxazole konsantrasyonlarını etkilememiştir. Deltametrindeki bu kararlılığın, her iki işlem türünde de düşük pH ile ilişkili olabileceği düşünülse de etoxazole için bu açıklama uygun bulunmamıştır. Çünkü yüksek pH koşullarında etoxazole'ün kararlılığının arttığı bilinmektedir. Her iki işlem için de tüm pestisitlerin işleme faktörleri 1'den düşük olmakla birlikte, işleme yöntemi, pestisitlerin kimyasal yapısı ve bozunma mekanizmalarına bağlı olarak değişiklikler göstermiştir.

Anahtar sözcükler: Akarisit, konserve, kornişon, fermantasyon, gıda güvenliği, insektisit, işleme faktörü

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Introduction

Pesticide use is generally unavoidable in agricultural practices to prevent the losses due to pests. The use of agrochemicals is considered safe for humans and other non-target organisms when applied at the approved dosages and in accordance with pre-harvest interval regulations (Banshtu et al., 2018). However, improper uses of these chemicals or harvesting before the recomended pre-harvest interval (PHI), generally result in pesticide residues in the fresh commodities (Gonzalez-Rodriguez et al., 2011). Pesticide residue levels may change depending on the pH, light exposure, temperature and moisture content of the environment, and the degradation levels may vary depending on the structure and the formulation of the active compound (Regueiro et al., 2015; Hepsag & Kizildeniz, 2021). Additionally, food processing techniques have a significant effect on the pesticide residue levels of the processed products (Maden & Yildirim Kumral, 2020). Alteration in pesticide levels affected by the processing method is measured by processing factor (PF). PF is determined by calculating the ratio of pesticide residue concentration in the processed product to that in the corresponding raw material (EC, 2005). PF is additionally used for the interpretation of the initial pesticide residue level of the processed food product and helps to assess its compliance with the legal requirements. It is also a necessary tool for the assessment of acute and chronic health risks that may occur with the exposure to the processed foods contaminated with pesticide residues (BFR, 2023). For an accurate and reliable evaluation, processing factor calculations must be performed for all active compounds and for each process type separately.

Pickling is one of the oldest and widely used food preservation method that allows long-term conservation of foods under acidic conditions (Montano et al., 2016; Behera et al., 2020). Industrially or homemade pickles are commonly categorised into two groups and named as fermented and canned (fresh pack) pickles (Stankus, 2014; Zincke et al., 2022). Despite the differences in the processing method, inhibition of pathogenic and/or spoilage microorganisms is the main target in both type of pickles. Canned pickles are not fermented, and they are immediately pasteurised by heating for the long-term conservation of the product. Fermented pickles are obtained by spontaneous or controlled fermentation by naturally occurring lactic acid bacteria (LAB). In the later processing, the pickles are firstly fermented for 2-6 weeks, and then pasteurised by heating (BFR, 2023). In both methods, low pH environment is desired and obtained with addition of vinegar in canned pickles and produced by LAB in the fermented ones. Both processing types involve a heat treatment (pasteurisation) at different steps of the production for the prolonged preservation of the products without refrigeration (BFR, 2023).

Gherkin fruits are widely used for pickle production and their pickled forms are extensively consumed worldwide in different food preparations. Many pesticides (acaricides and insecticides) are registered for cucumber/gherkin cultivation, and their residue alteration during pickle processes is not clarified with the current knowledge. Scientific data related to the impact of pickle processing on pesticide residue changes is still limited. The half-life (DT_{50}) value of a pesticide helps to estimate its degradation duration, and it is usually affected by the pH sensitivity and the chemical structure (Luyinda & Yildirim Kumral, 2023; PPDB, 2024). It has been previously demonstrated that low pH levels of different pickled commodities (olives, cabbages, tomatoes) slowed down the degradation or induced the stabilization of certain pesticides (Maden & Yildirim Kumral, 2020; Yildirim Kumral et al., 2020a; Luyinda & Yildirim Kumral, 2023). Recent studies showed that pesticide degradation might also be affected during fermentation by the activity of LAB that can metabolise pesticides (Behera et al., 2020; Yildirim Kumral et al., 2020b).

Gaps in knowledge about pesticide degradation mechanisms and limited scientific data about the transformation of pesticides during food processing sometimes hinder research efforts. Currently, only a limited number of PFs for pickles were determined and declared by the food safety authorities. The progress is so slow, and new pesticides are coming into use each passing day. However, there is still limited data on the impact of the pickling process on newly registered (for cucumber/gherkin cultivation) pesticides. This study aimed to explain the changes in the residues of extensively used pesticides (acaricides and insecticides) during the pickling of gherkins and to designate the PFs for each processing technique and the specific pesticide.

Materials and Methods

Chemicals and reagents

Pesticide standards and sample extraction-cleanup kits were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany) and Lab Instruments (Castellana Grotte, Italy) respectively. Other chemicals used during the experiments were of analytical grade. Names of the active chemicals, their trade names, application doses, PHIs, maximum residue limits (MRLs) and toxicological properties are given in Table 1 and Table 2.

Table 1. Names, application doses, pre-harvest interval and maximum residue limits of pesticides

Active substance	Commercial name	Application dose	Pre-harvest interval (days)	Maximum residue limits (mg kg^{-1})
Acetamiprid (20%)	Effore	0.30 g L^{-1}	3	0.60
Etoxazole (110 g/L)	Eurogold	0.35 mL L^{-1}	3	0.01
Spiromesifen (240 g/L)	Oberon	0.50 mL L^{-1}	3	0.30
Deltamethrin (25 g/L)	Dentis	0.50 mL L^{-1}	3	0.20
Chlorantraniliprole (20%)	Coragen	0.07 g L^{-1}	1	0.30

Table 2. Toxicological features of pesticides (Pesticide Properties Database, 2023)

Chemical name	Chemical group	Mode of action	Solubility -in water at 20°C (mg l^{-1})	Boiling point (°C)	Degradation point (°C)	Octanol-water partition coefficient at pH 7, 20°C Log P	pH sensivity [DT_{50} (days) under low pH conditions]
Acetamiprid	Neonicotinoid	Nicotinic acetylcholine receptor (nAChR) competitive modulators	2950	DBB*	200	0.80	stable
Chlorantraniliprole	Diamide	Ryanodine receptor modulators	0.88	DBB	330	2.86	stable
Deltamethrin	Synthetic Pyrethroid	Sodium channel modulators	0.0002	DBB	-	4.60	stable
Etoxazole	Diphenyl oxazolin	Mite growth inhibitors affecting CHS1	0.07	DBB	293	5.52	9.60
Spiromesifen	Tetronic acid	Inhibitors of acetyl CoA carboxylase	0.13	DBB	375	4.55	107.30

*DBB: Decomposes before boiling; ** npH= No pH sensitive.

Pesticide application

Active chemicals used in the experiments were selected according to the findings of a preliminary market survey conducted during the year 2021-2022 (Hazarhun et al., 2022). The most prevalent 5 pesticides (survey study results, data not shown) detected in commercial pickled gherkin samples collected from markets were used as research material. Gherkin plants, *Cucumis sativus* L. (Cucurbitales: Cucurbitaceae) used during these experiments were grown by the research team in an experimental greenhouse at Bursa Uludağ University. The fruit samples were collected from experimental area and stored frozen (-24°C) until analysis (OECD, 2008). Pesticides were homogenously sprayed on gherkin plants at the legal application doses using an electrical atomizer (Table 1) and harvested after the specified PHI (Table 1) for all chemicals tested.

Pickling process and experimental design

Pickle processing methods were applied based on the "database of processing techniques and processing factors compatible with the EFSA food classification and description system FoodEx 2" (Scholz et al., 2018; Zincke et al., 2022) (Figure 1). In addition to pickling treatments, raw gherkin fruits were stored at chilled conditions concurrently to discriminate the effects of processing from the self-degradation of the active compounds. Details of the experiments and processing methods are summarized in Table 3. All experiments were planned and conducted in triplicate.

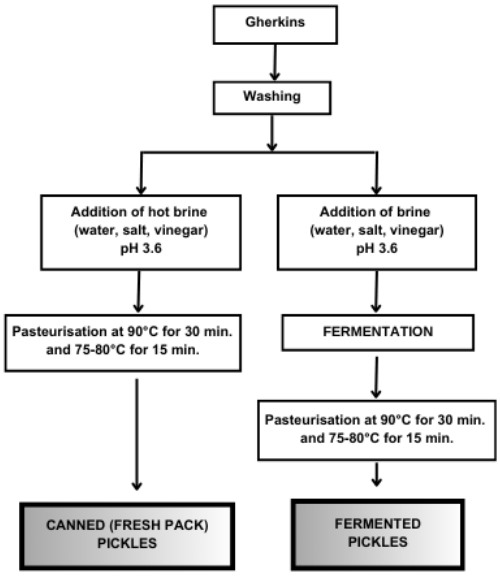

Figure 1. Experimental design and treatments.

Table 3. Details of the experiments and processing methods

Treatment	Application
Chilled storage (control)	Fruits were harvested and then kept at 10±2°C during pickling treatments.
Fresh pack gherkins	Fruits were harvested, washed and transferred into glass containers. Containers were filled with brine (8% salt and 10% vinegar) and pasteurised (30 min. at 90°C, and 15 min. at 75-80°C). Stored at 22±2°C until analysis.
Fermented gherkins	Fruits were harvested, washed and transferred into glass containers. Containers were filled with brine (8% salt and 10% vinegar) and fermented for 4 weeks at 22±2°C. Pasteurised after fermentation (30 min. at 90°C, and 15 min. at 75-80°C). Stored at 22±2°C until analysis.
Fresh gherkins	Fruits were harvested and transferred into glass containers. Stored in the refrigerator (+4°C) until analysis.

Pesticide analysis

Pesticide extraction and cleaning procedures

Samples of fresh and pickled gherkins fruits were homogenised with a laboratory grinder (Retsch Knife Mill Grindomix GM300, Germany) and prepared according to Quick Easy Cheap Effective Rugged Safe (QuEChERS) method recommended for the pesticide analysis of fresh fruit and vegetables (Lehotay, 2007). Sligtly modified QuEChERS steps for extraction and cleaning are given in Figure 2 (Hazarhun et al., 2022).

Instrumental analysis

Samples prepared for the detection of pesticide concentrations were subjected to LC-MS/MS analyses. The specifications and conditions of the instrument are shown in Table 4. Information on tested pesticides is listed in Table 5.

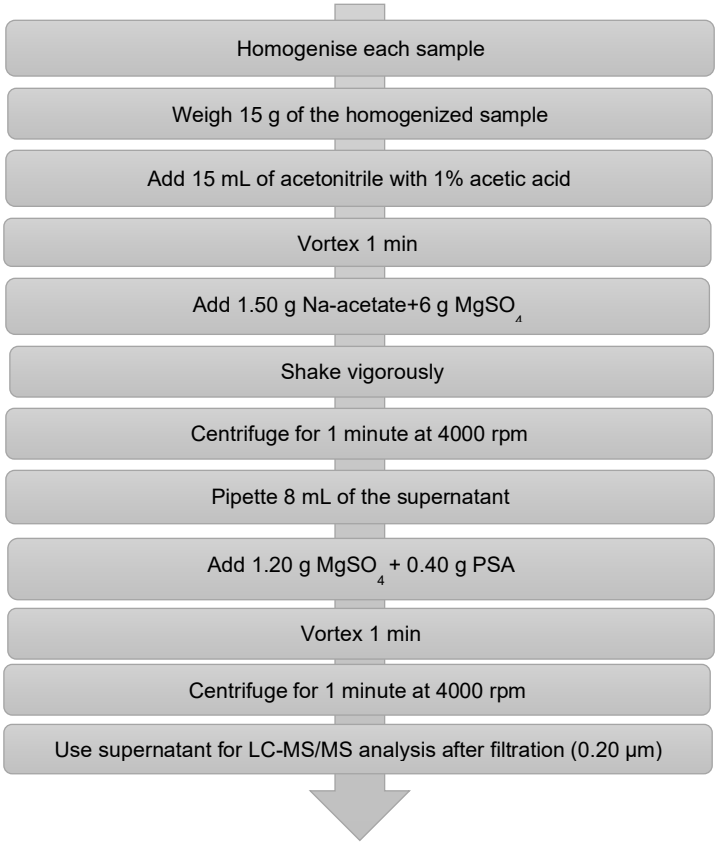

Figure 2. Analytical steps for extraction and cleaning (QuEChERS-AOAC Official Method 2007.01).

Table 4. LC-MS/MS and GC-MS conditions

LC-MS/MS system	Agilent 1260 Infinity II HPLC and Agilent 6470 Triple Quad Liquid-Mass Spectrometry
Column	Agilent Poroshell SB-C18 Column (3x100 mm x 2.7 mm)
Ionisation mode	Electrospray ionization
Acquisition mode	Multiple-reaction monitoring (Negative and positive)
Mobile phase	A: 0.1% formic acid and 1 mM ammonium format in water B: Methanol
Gradient	0-0.5 min 70% A, 8 min 5% A, 8-12.5 min 5% A, 12.6 min. 70% A, 12.6-15 min %70 A
Flow rate	0.50 mL /min
Column temperature	45ºC
Injection volume	1 µL
Run time	15 min.
GC system	GCMS-TQ8040 NX
Column	Restek GC Column (Rtx-624, 30 m., 0.25 mmID, 1.4 µm df
Column temperature	120ºC
Flow rate	1.50 mL/min
Injection mode	(AOC-20i Plus) Splitless
Injection temperature	250.0ºC
Carrier gas	Helyum (%99.9)
Carrier gas temprature	120.0ºC
Carrier gas pressure	121.9 kPa
Total flow	19.5 mL/min
Column flow	1.50 mL/min
Column oven temprature program	0-2 min 120ºC, 2-8 min 230ºC, 8-12 min 300ºC, 12-16 min 300ºC

Table 5. Pesticide information

Chemical name	CAS number	Molecular weight	Molecular formula	Ionization	Precursor Ion	Product Ion	Collision energy (V)	Retention time (min.)
Acetamiprid	135410-20-7	222.67	$C_{10}H_{11}ClN_4$	[M+H]+	223.1	126.1, 56.2	17, 11	3.14
Chlorantraniliprole	500008-45-7	483.15	$C_{18}H_{14}BrCl_2N_5O_2$	[M+H]+	484, 482	285.9, 283.9	21, 21	6.62
Deltamethrin	52918-63-5	505.20	$C_{22}H_{19}Br_2NO_3$	[M+NH4]+	522.8	505.8, 280.6	6, 12	9.62
Etoxazole	153233-91-1	359.42	$C_{21}H_{23}F_2N_{02}$	[M+H]+	360	141, 113	15, 23	9.37
Spiromesifen	283594-90-1	370.48	$C_{23}H_{30}O_4$	[EI]	272	254, 209	6, 14	10.32

Method validation

Pesticide analysis method was validated as per the direction of Analytical Quality Control and Method Validation Procedures for Pesticide Residues Analysis in Food and Feed SANTE 11312/2021 (EURL, 2021; Hazarhun et al., 2022). Tested validation parameters for each pesticide were given in Table 6. For the validation studies pesticide-free gherkin fruits and pickles were used. The pesticide free gherkin fruits were obtained from the experimental greenhouse and a part of these fruits were processed as pickles for further validation studies. Linearity was checked with seven concentrations (2.50-250 µg kg^{-1}). Calculations of recovery rates and precision parameters were performed using the test results obtained by two analysts, at two different concentrations (10 and 50 µg kg^{-1}) and across five different time points.

Table 6. Validation parameters

Chemical name	Concentration range for calibration (µg kg^{-1})	Correlation coefficient (R^2)	LOQ (µg kg^{-1})	Spike level (µg kg^{-1})	RSDr (%)	RSDwr (%)	Mean recovery (%)
Acetamiprid	2.50-250	0.99	3.97	10 50	0.77-0.99 0.74-0.85	5.72 5.16	111.82 112.16
Chlorantraniliprole	2.50-250	0.99	3.87	10 50	5.54-13.19 2.58-4.74	13.07 10.27	99.02 106.36
Deltamethrin	2.50-250	0.99	3.00	10 50	3.75-11.68 6.86-6.88	7.93 4.72	100.49 102.05
Etoxazole	2.50-250	0.99	3.95	10 50	1.35-5.01 3.62-3.75	8.29 7.24	108.61 105.38
Spiromesifen	2.50-250	0.99	7.11	10 50	5.45-6.21 2.88-4.46	13.70 14.11	89.09 93.03

Calculation of the processing factors and residue reduction rates

Processing factors were calculated for each pesticide active compound and the processing method by dividing the residue concentration of processed product to the residue concentration of the relevant raw material (EC, 2005). Pickled samples firstly drained and separated from brine and then prepared for pesticide residue test and then used for the calculation of the PFs.

Statistical analysis

Experimental trials and analyses were performed in triplicate. The data were first evaluated for normal distribution using the Shapiro-Wilk's test. After normality test, pesticide concentration values determined at each processing stage were subjected to analysis of variance (ANOVA). To identify significant differences between groups, Tukey's multiple comparison test was subsequently applied (α=0.05). All statistical analyses were performed using JMP 7.0 software (SAS, Cary, NC).

Results and Discussion

Pesticide residue changes

Changes in pesticide concentrations during chilled storage of fresh fruits are demonstrated in Table 7. Analysis samples were taken 3 hours after pesticide application, on the harvest day and at the end of 30 days. During this period, no significant changes were observed in the levels of most pesticide residues, including acetamiprid, chlorantraniliprole, deltamethrin, and etoxazole, with the exception of spiromesifen. The significant change detected in spiromesifen levels may be attributed to the half-life of this pesticide (PPDB, 2024).

Changes in the residue levels and related reduction rates during fresh pack and fermented pickling processes are given in Tables 8 and 9. Pesticide reduction rates were calculated at the end of processing and storage for fresh pack trials and before and after fermentation for fermentation trials. Reductions in residues of spiromesifen, chlorantraniliprole and acetamiprid were significant across both canning and natural fermentation processes. When pickled trials are compared with the chilled stored raw commodities, it is observed that pickling processes significantly accelerated the degradation of acetamiprid and chlorantraniliprole (Tables 7, 8 & 9). In both pickling processes, pH levels were 3.60 at the beginning of processes due to vinegar addition (Figure 1). The effects of fermentation process on pesticide degredation have been shown in previous studies (Dordevic et al., 2013; Bajwa & Sandhu, 2014; Regueiro et al., 2015; Maden & Yildirim Kumral, 2020; Yildirim Kumral et al., 2020a; Luyinda & Yildirim Kumral, 2023). In addition, the photodegradation of pesticides were disregarded owing to the storage of the samples in the dark conditions during the experiments. In this context, the degradation of the pesticides could be one of the consequences of microbial activities (Maden & Yildirim Kumral, 2023). On the other hand, deltamethrin and etoxazole concentrations were not affected by either of the pickling processes (Table 8 and 9). This could be related with the stability of deltamethrin under acidic conditions but can not be explained with the same mechanism for etoxazole because of its high degradability under low pH conditions (PPDB, 2024; Table 2). Low water solubility (0.07 mg L^{-1}) and higher fat solubility (octanol-water partition coefficient, Log P: 5.52) of etoxazole could be the fact for non degradation of its residues (Borcakli et al., 1993; Kiai & Hafidi, 2014; Featherstone, 2016; PPDB, 2024) (Table 2). In fermentation trials, washing and brine addition steps decreased the residue levels at varying rates (17.09-49.35%) (Table 9). In the fresh pack trials, it is quite difficult to see the effects of washing because both washing, brine addition and pasteurization steps were done at the same time. However, significant degradation was observed immediately after the pasteurization step on the third day during the fresh pack trials. In this step, acetamiprid, chlorantraniliprole, and spiromesifen residues were significantly reduced by 70, 83, and 97 percent, respectively.

Table 7. Pesticide residue changes in the fresh gherkin fruits (mg kg^{-1})

Active compound	Application day** (day 0) (mg kg^{-1})	Harvest day** (day 3) (mg kg^{-1})	Storage** (day 30) (mg kg^{-1})	F_{df}; p^b
Acetamiprid	0.24±0.05 a*	0.28±0.03 a	0.24±0.02 a	$F_{2,8}$=0.47, p=0.64
Chlorantraniliprole	0.32±0.07 a	0.41±0.08 a	0.25±0.06 a	$F_{2,8}$=1.34, p=0.33
Deltamethrin	0.06±0.01 a	0.09±0.03 a	0.04±0.01 a	$F_{2,8}$=2.16, p=0.20
Etoxazole	0.08±0.02 a	0.08±0.03 a	0.03±0.01 a	$F_{2,8}$=1.97, p=0.22
Spiromesifen	0.55±0.08 ab	0.70±0.10 a	0.25±0.04 b	$F_{2,8}$=8.16, p=0.02*

* Significant at 0.05 level;

**means±standard errors followed by different letters in a row are significantly different.

Table 8. Pesticide residue changes during fresh pack trials (mg kg^{-1})

Active compound	Harvest day** (day 3) (mg kg^{-1})	Washing, brine addition and pasteurization** (day 3) (mg kg^{-1})	Reduction rate*** (%)	Storage** (day 30) (mg kg^{-1})	Reduction rate*** (%)	F_{df}; p
Acetamiprid	0.42±0.13 a	0.13±0.03 ab	69.62	0.09±0.01 b	32.28	$F_{2,8}$=5.63; p=0.04*
Chlorantraniliprole	0.45±0.15 a	0.08±0.02 b	83.15	0.07±0.01 b	10.53	$F_{2,8}$=6.46; p=0.03*
Deltamethrin	0.181±0.07 a	0.05±0.01 a	72.38	0.04±0.01 a	20.00	$F_{2,8}$=3.82; p=0.08
Etoxazole	0.11±0.03 a	0.05±0.02 a	54.21	0.06±0.02 a	-	$F_{2,8}$=1.72; p=0.26
Spiromesifen	0.70±0.10 a	0.02±0.02 b	96.69	0.01±0.00 b	69.56	$F_{2,8}$=41.74; p<0.01*

* Significant at 0.05 level;

** means±standard errors followed by different letters in a row are significantly different;

*** Reduction rates were calculated in comparison with the previous process.

Table 9. Pesticide residue changes during fermentation trials (mg kg^{-1})

Active compound	Harvest day** (day 3) (mg kg^{-1})	Washing and brine addition** (day 3) (mg kg^{-1})	Reduction rate*** (%)	After fermentation and pasteurization** (day 30) (mg kg^{-1})	Reduction rate*** (%)	F_{df}; p
Acetamiprid	0.28±0.03 a	0.22±0.05 ab	20.07	0.14±0.01 b	39.46	$F_{2,8}$=5.16; p=0.05*
Chlorantraniliprole	0.41±0.08 a	0.31±0.06 ab	25.43	0.11±0.02 b	64.59	$F_{2,8}$=7.53; p=0.02*
Deltamethrin	0.09±0.03 a	0.08±0.00 a	20.21	0.03±0.00 a	64.00	$F_{2,8}$=3.95; p=0.08
Etoxazole	0.08±0.03 a	0.04±0.00 a	49.35	0.01±0.00 a	81.18	$F_{2,8}$=4.31; p=0.07
Spiromesifen	0.70±0.10 a	0.58±0.05 a	17.09	0.01±0.00 b	98.96	$F_{2,8}$=29.82; p<0.01*

* Significant at 0.05 level;

** means±standard errors followed by different letters in a row are significantly different;

*** Reduction rates were calculated in comparison with the previous process.

Consistent with our findings, previous studies demonstrated that pasteurisation reduced pesticide residue levels to a varied extent depending on the chemical structure of each compound (Bajwa & Sandhu, 2014; Hrynko et al., 2023). No significant change in pesticide residues was detected after 30 days of storage following pasteurisation (Table 8). In fact, the storage period under dark conditions less affected pesticide residue amounts (from 0 to 32.28%) except for spiromesifen (69.56%). It is explained by the faster degradation of spiromesifen (DT_{50}= 4.1 days under laboratory conditions at 20ºC) (PPDB 2024). Thus, residual concentrations of most of the test pesticides generally remained unchanged during post-pasteurisation storage. On the other hand, higher pesticide degradation rates (39.46 to 98.96%) were detected in fermented samples compared to canned ones during 30 days of fermentation period (Table 9). Besides, spiromesifen residues decreased significantly during fermentation step compared to brine addition step. These high reductions in natural fermentation could be related to microbial activity and the pasteurisation procees. Stabilization of pH-sensitive pesticides at low pH levels reached during fermentation processes have been previously reported (Li et al., 2008; Maden & Yildirim Kumral, 2020; Yildirim Kumral et al., 2020a; Lucinda & Yildirim Kumral, 2023). Lactic acid production is a desired activity of LAB in fermented pickles for the product's microbial safety and long-term preservation (Aljahani, 2020). pH levels below 4.6 are targeted in optimum fermentation processes (Borcakli et al., 1993; Kiai & Hafidi, 2014; Featherstone, 2016). LAB's designated with generally recognised as safe (GRAS) status produce acid during fermentation which is required for the prevention of the pathogen and spoilage microorganisms by lowering the pH (Behera et al., 2020). The pH of a food also has a critical impact on the way pesticide residues change during processing, preparation and storage of the food product (Maden & Yildirim Kumral, 2020; Yildirim Kumral et al, 2020a; Luyinda & Yildirim Kumral, 2023).

Processing factors

The processing factors for all pesticides were determined as the ratio of the residue levels detected on day 30 (after pasteurisation in fermented trials and at end of storage in canned trials) to the residue levels detected on the day of harvest (Table 10). PF lower than 1 implied a reduction, whereas PF higher than 1 implied a concentration in the pesticide level of the pickles (Zhang et al., 2020). During the experiments, the PF values obtained for all of the tested pesticides and process methods were lower than 1, demonstrating that all treatments applied during the experiments caused significant degradations of the compounds (Zhang et al., 2020). But the effects of canning and fermentation processes on the concentrations of each compound showed variations depending on different degradation mechanisms (Bai et al., 2021). For instance, PFs of acetamiprid and chlorantraniliprole were lower for canning process where as PF of etoxazole was lower for fermentation process. Additionally, contradictory results about the effects of fermentation were reported by different researchers previously. Regarding the effects of fermentation on pesticide degradation, there are several research papers denoting the acceleration of the pesticide degradation (Dordevic & Durovic-Pejcev, 2015; Kong et al., 2016; Dusek et al., 2018; Xu et al., 2020), as well as others reporting the stabilisation and/or deceleration of the degradation (Maden & Yildirim Kumral, 2020; Yildirim Kumral et al., 2020a; Luyinda & Yildirim Kumral, 2023). These variations were primarily influenced by the pH of the food product as well as the chemical structure of the pesticide (Maden & Yildirim Kumral, 2020; Yildirim Kumral et al., 2020a; Luyinda & Yildirim Kumral, 2023).

Table 10. Process factors for selected pestiicdes in gherkin

	Fresh pack (canned) gherkins	Fermented gherkins
Acetamiprid	0.21	0.48
Chlorantraniliprole	0.15	0.26
Deltamethrin	0.22	0.28
Etoxazole	0.54	0.07
Spiromesifen	0.01	0.01

Conclusion

In conclusion, the results of the current study showed that different pickling methods caused diverse changes in the residue levels of the pesticides applied at the recommended doses. This provides us with at least a small amount of knowledge about the behaviour of a limited number of registered pesticides applied under acceptable conditions and concentrations. However, there is still a lack of information about the fate of many extensively used pesticides at concentrations exceeding the recommended limits. Further studies are needed to display the effects of food processing technologies on the residues of different chemicals and to generate reliable information to estimate risks that consumers may face associated with pesticide residues.

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Original article (Orijinal araştırma)

Physicochemical properties of local diatomaceous earths and their effects of water-suspension treatments against *Callosobruchus maculatus* (Fabricius, 1775) (Coleoptera: Chrysomelidae) on chickpeas

Yerel diatom toprakların fiziko-kimyasal özellikleri ve nohutlarda sulu süspansiyon uygulamalarına *Callobruchus maculatus* (Fabricius, 1775) (Coleoptera: Chrysomelidae)'a karşı etkileri

Hüseyin BOZKURT[1*]

Abstract

This study evaluated the chemical and physical characteristics of two locally sourced diatomaceous earth (DE) formulations (Detech®-s and Detech®-m) alongside the commercial product SilicoSec® to assess their effects on adult mortality, lethal time (LT_{99}), and progeny reduction of the cowpea weevil, *Callosobruchus maculatus* (Fabricius, 1775) (Coleoptera: Chrysomelidae) in stored chickpeas. Conducted in 2023 at the Entomology Laboratory of Kahramanmaraş Sütçü İmam University, the research found Detech®-m to be the most effective formulation. It achieved 99% mortality in the shortest time (LT_{99}=70.65 hours at 1000 ppm) and the highest progeny suppression (98.1%). This superior performance was attributed to its finer particle size (d_{50}=7.34 µm) and larger surface area (60.45 m²/g), which enhanced adsorption and cuticle disruption. Although SilicoSec® contained a higher percentage of silicon dioxide (85.7% SiO_2) than Detech® (80.6% SiO_2), its broader particle size distribution and lower Brunauer-Emmett-Teller (BET) surface area reduced its effectiveness. These findings highlight the critical role of particle size and surface area in optimizing DE formulations for *C. maculatus* control. Overall, our results suggest that refining DE formulations for water-suspension applications can improve stored-legume pest management by enhancing adhesion, uniformity, and persistence while minimizing dust-related health risks.

Keywords: Cowpea weevil, diatom powder, particle size, slurry application, surface area

Öz

Bu çalışmada, yerel olarak temin edilen iki diatom toprağı (DE) formülasyonunun (Detech®-s ve Detech®-m) ve ticari formülasyon SilicoSec®'in kimyasal ve fiziksel özelliklerini değerlendirerek, bunların depolanan nohutlardaki börülce tohum böceği, *Callosobruchus maculatus* (Fabricius, 1775) (Coleoptera: Chrysomelidae) üzerindeki ergin ölümleri, lethal süre (LT_{99}) ve yeni nesil ergin sayısındaki azaltmasına etkileri incelenmiştir. Çalışma, 2023 yılında Kahramanmaraş Sütçü İmam Üniversitesi'nin Entomoloji Laboratuvarı'nda gerçekleştirilmiştir. Sonuçlar, Detech®-m'nin en etkili olduğunu, en kısa sürede (LT_{99}=1000 ppm'de 70.65 saat) %100 ölüm oranına ve en yüksek yeni nesil ergin baskılamasına (%98.1) ulaştığını göstermiştir. Daha ince partikül boyutu (d_{50}=7.34 µm) ve yüksek yüzey alanı (60.45 m²/g), Detech®-m formülasyonun adsorpsiyon ve böcek kütikülasını bozma özelliklerini artırarak üstün etkinlik göstermesine katkıda bulunmuştur. SilicoSec®'in daha yüksek silisyum dioksit içeriğine (%85.7) sahip olmasına rağmen, Detech®-m (%80.6) ile karşılaştırıldığında daha geniş partikül boyutu dağılımı ve daha düşük Brunauer-Emmett-Teller (BET) yüzey alanı nedeniyle daha düşük bir etki göstermiştir. Bu bulgular, *C. maculatus* mücadelesinde DE formülasyonlarının etkinliğini optimize etmede partikül boyutunun ve yüzey alanının kritik rolünü vurgulamaktadır. Sonuç olarak, bu çalışmada elde edilen bulgular su süspansiyon uygulamaları için DE formülasyonların yapışma, homojenlik ve kalıcılığı artırarak optimize edilmesi depolanmış tahıl zararlılarının yönetimini iyileştirebileceğini ve toz kaynaklı sağlık risklerini en aza indirebileceğini göstermektedir.

Anahtar sözcükler: Börülce tohum böceği, diatom tozu, partikül büyüklüğü, sulu bulamaç uygulaması, yüzey alanı

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Introduction

Stored-product insect pests pose a significant threat to global food security by causing substantial post-harvest losses in cereals, legumes, and other agricultural commodities (Phillips & Throne, 2010). These pests, including beetles (Coleoptera) and moths (Lepidoptera), inflict direct grain damage, reduce weight, and contaminate products with feces, webbing, and frass, ultimately diminishing quality and marketability (Lorini et al., 2007). To mitigate these losses, conventional chemical insecticides such as organophosphates and pyrethroids, have been widely used (Arthur, 1996; Kljajic & Peric, 2007; Agrafioti et al., 2015; Vassilakos et al., 2015; Rumbos et al., 2018; Mutlu et al., 2019). However, concerns over insecticide resistance, residue accumulation, and environmental toxicity have driven the search for alternative control methods (Arthur, 1996; Athanassiou et al., 2007a). Among these eco-friendly alternatives, diatomaceous earth (DE) has gained attention as a promising insecticidal agent due to its physical mode of action rather than chemical toxicity (Korunić, 1998). DE formulations are considered as safe, effective, and sustainable for stored-grain pest management, making them a viable substitute for synthetic pesticides (Subramanyam & Roesli, 2000; Athanassiou et al., 2011). However, their efficacy depends on physicochemical properties such as particle size, surface area, chemical composition, and hydrophobicity (Korunić, 1997, 2013; Korunić et al., 2021; Mewis & Ulrichs, 2001; Ogreten et al., 2023; Henteş & Işıkber, 2024). Understanding how these factors influence insecticidal activity is essential for optimizing DE-based formulations for practical use.

Diatomaceous earth (DE) is a naturally occurring sedimentary material composed primarily of fossilized diatom remains, with a high silica (SiO_2) content ranging from 80% to 93% (Korunić, 1998). Depending on the deposit source, it may also contain clay minerals, organic matter, quartz, calcium carbonate, and magnesium oxide (Stathers et al., 2004). The unique porous structure of DE particles provides a high surface area, enhancing their strong adsorption properties, which contribute to their insecticidal action (Mewis & Ulrichs, 2001, Sağlam et al., 2017). DE's effectiveness against insect pests stems from its ability to disrupt the cuticle, leading to desiccation and death. The particles adhere to the exoskeleton and absorb lipid layers from the epicuticle, causing excessive water loss and dehydration (Ebeling, 1971; Subramanyam & Roesli, 2000). Additionally, its abrasive nature creates micro abrasions on the insect's body, further accelerating water loss (Korunić, 2013). Unlike synthetic insecticides, which act through neurotoxic mechanisms, DE remains effective against insect populations regardless of resistance development (Athanassiou et al., 2007b).

While DE is widely recognized as an effective non-chemical insecticide, its efficacy varies significantly based on multiple physicochemical factors. Understanding these properties is essential for optimizing DE formulations for stored-product protection. Particle size plays a crucial role in DE's ability to adhere to the insect cuticle and remove its protective wax layer (Athanassiou et al., 2003). Smaller particles generally exhibit higher insecticidal activity due to their greater surface area and enhanced adsorption capacity (Korunić, 2013). However, excessively fine particles can pose dust hazards and increase inhalation risks, raising concerns about worker safety in grain storage facilities (Subramanyam et al., 1994). The chemical composition of natural DE deposits also influences insecticidal properties. A higher amorphous silica content is generally associated with greater efficacy, whereas impurities such as clay and quartz may reduce effectiveness by diluting the active components (Stathers et al., 2004). Additionally, studies indicate that calcined DE, which undergoes high-temperature treatment, differs in physical characteristics from natural DE, affecting both its adsorptive capacity and insecticidal activity (Athanassiou et al., 2005).

In recent years, researchers have compared the effectiveness of aqueous and powder formulations of various commercial DE products against stored-grain pests. Studies have shown that dust applications, where DE is sprayed onto grain, are more effective at controlling insect and mite pests than wet applications (Field & Korunić, 2000; Athanassiou et al., 2006; Collins & Cook, 2006a; Wakil et al., 2006; Athanassiou & Korunić, 2007c). Similarly, research indicates that when DE formulations are applied to different surfaces,

dust formulations exhibit higher efficacy than aqueous formulations (Bridgeman, 1994; McLaughlin, 1994; Collins & Cook, 2006b). A study comparing the reduced effectiveness of aqueous DE formulations on wheat to dust formulations found that diatom particles adhere more readily to cereal grains in wet applications (Johnson et al., 2014). This phenomenon is attributed to the formation of capillary bridges between DE particles and the grain surface due to residual surface water (Israelachvili, 2011). The adhesion force resulting from capillary bridging is primarily driven by water's surface tension. Additionally, studies have shown that DE particles fail to adhere effectively to the insect cuticle upon contact, also due to water's high surface tension.

Although Türkiye has substantial diatom deposits, the potential use of locally derived DE for insect control has only recently been evaluated. Most studies have tested DE preparations in powder form against various pests in grain storage (Doğanay, 2013; Alkan et al., 2019; Şen et al., 2019; Ertürk et al., 2020; Öztekin & Mutlu, 2020; Kılıç, 2022; Sağlam et al., 2022a; Henteş & Işıkber, 2024). However, powder applications often present challenges, including difficulty achieving homogeneous distribution, poor adherence to surfaces, and powder drift during application. In many cases, DE is applied as water suspension, but its effectiveness against stored-grain pests is known to be somewhat reduced compared to powder applications (Field & Korunić, 2000; Athanassiou et al., 2006; Collins & Cook, 2006b; Wakil et al., 2006; Athanassiou & Korunić, 2007c). While the challenges of powder application are well-documented, research on the effects of mixing locally sourced DE with surfactants for spray applications remains limited (Ertürk et al., 2020; Kılıç, 2022). Therefore, there is a need for a locally sourced DE formulation with a flowable, easy-to-apply suspension suitable for spray application on insect-infested products. This study aimed to characterize the physicochemical properties of two Turkish-origin DE formulations (Detech®-s and Detech®-m) and the commercial product SilicoSec® while evaluating their insecticidal efficacy in water-suspension treatments against the cowpea weevil, *Callosobruchus maculatus* (Fabricius, 1775) (Coleoptera: Chrysomelidae: Bruchinae) in stored chickpeas. Additionally, the study sought to identify key formulation properties influencing DE performance in aqueous applications.

Materials and Methods

Test insect rearing

In the biological tests, adult cowpea seed beetles, *C. maculatus* were used. The initial insect population was obtained from chickpea samples collected from legume warehouses in the Mersin and Adana provinces. The adults were reared in the Laboratory of the Plant Protection Department, Faculty of Agriculture, Kahramanmaraş Sütçü İmam University, where a stock culture was established for routine experiments. The "Azkan" variety of chickpeas, *Cicer arietinum* L. (Fabales: Fabaceae), with a moisture content of 10±1%, served as both the culture medium and the test diet. Before use in experiments, chickpeas were stored at -20°C for 7-10 days to eliminate potential contamination. For rearing, 300 g of chickpeas were placed in 1-liter glass jars, and 100-150 mixed-sex adults (1-2 days old) were introduced. The jars were covered with mesh to allow airflow. The jars were kept in a cooled incubator (Lovibond TC-135S, Germany) under controlled conditions: 65±5% relative humidity, 30±1°C temperature, and complete darkness. To facilitate mating and egg-laying, adults remained in the jars for two days before being removed using a 2 mm mesh metal sieve (Retsch®, Germany). The eggs laid on the chickpeas hatched, and the emerging larvae to penetrate the grains. The larvae and pupae completed their development inside the chickpeas, and the cultures were maintained under the same incubator conditions until the new generation of adults emerged. Once the new adults emerged, they were collected from the culture jars using a 2 mm mesh metal sieve and used in subsequent biological tests.

Diatomaceous earth formulations

Two forms of the local diatomaceous earth (DE) formulation, Detech®, were used in the biological tests: the standard (Detech®-s) and the micronized (Detech®-m) powder formulations. The standard formulation (Detech®-s), characterized by a relatively coarse particle size distribution, was produced by grinding DE deposits using a laboratory-scale ball mill (RAM1107; Rantek Test Solutions, Ankara). In contrast, the micronized formulation (Detech®-m), distinguished by its finer particle size distribution, was prepared using a lab-scale fluidized bed opposed jet mill (AFG 100, Hosokawa Alpine AG, Germany). Detech® is a blend of freshwater DE deposits sourced from three reserves in Middle Anatolia, Türkiye. It primarily consists of naturally occurring amorphous silica, has a white-grey color (Figure 1a), and is formulated by Entoteam R&D Food Agriculture Co. as a pest control-grade insecticide. For comparison, the commercial DE formulation SilicoSec® was used as a positive control in the biological tests. SilicoSec®, a freshwater diatomaceous earth formulation manufactured by Biofa GmbH (Münsingen, Germany), is derived from fossilized single-celled diatoms and has a white color (Figure 1b).

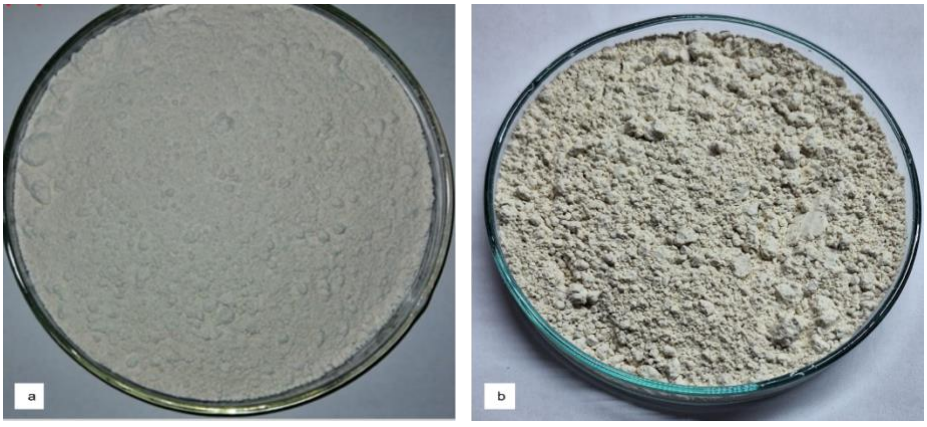

Figure 1. Powder formulations of a) the local standard diatomaceous earth (Detech®), and b) the commercial diatomaceous earth (SilicoSec®) used in biological tests.

Physicochemical characterization of tested diatomaceous earth powder formulations

Quantitative mineral analyses of three diatomaceous earth (DE) powder formulations (Detech®-s, Detech®-m, and SilicoSec®) were conducted at the Accredited Mineralogy and Petrography Laboratory of the General Directorate of Mineral Research and Exploration of Türkiye (MTA). Elemental composition was determined using atomic absorption spectroscopy (AAS) following a melting and acid removal process. Particle size and distribution analyses were performed using a Mastersizer 2000 laser diffraction particle size analyzer (Malvern Instruments) with wet dispersion. Measurements were taken in triplicate using a micro-volume (200 mg) sample feeder at 2-second intervals, with obscuration limits set between 5% and 15%. The Brunauer-Emmett-Teller (BET) method was used to assess the specific surface area by measuring nitrogen multilayer adsorption relative to pressure. This technique evaluates both external and pore surface areas, providing crucial data on total specific surface area (m^2/g) and offering insights into factors affecting particle size and surface porosity in various applications. BET surface area measurements were conducted using a Quantachrome® ASiQwin™ (version 5.23) instrument (Quantachrome Instruments, Boynton Beach, FL, USA) at the MTA laboratory. Before measurement, samples were dried at 150°C for 24 hours and degassed at 200°C for 5 hours under vacuum.

To examine the morphological and structural characteristics of the silica-based remains of diatoms—single-celled algae with intricate, species-specific silica frustules—Scanning Electron Microscopy (SEM) analyses were conducted at the Scientific and Technological Research Implementation and Research Center of Tekirdağ Namık Kemal University (NABILTEM). Water and oil absorption capacities were

determined using the gravimetric method. Before testing, DE samples were conditioned at 80°C for 24 hours in an air-circulating oven. Oil absorption capacity was measured according to the ASTM D1483 standard, in which linseed oil was titrated onto a 1 g DE sample until free oil appeared (ASTM, 2012). Similarly, water absorption capacity was measured following the ASTM D570 standard, where water was titrated onto a 1 g DE sample until free water appeared (ASTM, 2012). Tapped bulk density analysis was performed by placing 10 g of DE in a 100 mL graduated cylinder. The cylinder was tapped 100 times on a hard rubber surface, and the final volume was recorded. Results were expressed in grams per liter (g/L) (Korunić, 1997). All measurements were conducted in triplicate. The pH was determined by stirring 10 g of DE with 90 mL of double-distilled water for six hours, followed by measurement using a Hanna pH-211 meter. pH measurements were also performed in triplicate.

Commodity

For biological tests and insect cultures, the Azkan variety of edible chickpea, widely used in Türkiye, was selected. The moisture content of the Azkan chickpeas used in the experiments was measured at 10%±1% using a KETT PM-600 portable moisture analyzer (Kett Electric Laboratory, Japan).

Biological test with diatomaceous earth (DE) formulations

In the biological tests, diatomaceous earth (DE) formulations (Detech®-s, Detech®-m, and SilicoSec®) were applied to chickpea grains as water suspensions at concentrations of 500 ppm and 1000 ppm (mg DE/kg chickpea). The DE suspensions were prepared by mixing 0.5 g and 1 g of DE with 10 mL of water, respectively, in a 250 mL beaker using a magnetic stirrer. One kilogram of chickpea grains was spread in a single layer on a flat surface, and 10 mL of DE suspension at 500 ppm or 1000 ppm was evenly sprayed onto the grains using an HSENG Airbrush AS18 spray compressor (Ningbo Haosheng Pneumatic Machinery Co., Zhejiang, China). As a control treatment, only pure water was applied. After spraying, the chickpeas were left to dry at room temperature for one hour. Sub-samples of 50 g of DE-treated chickpeas were taken from the 1 kg batch and transferred to 100 mL glass vials (8.3 cm × 4.5 cm). Thirty mixed-sex adult insects (aged 1-2 days) were introduced into each vial. The vial openings were covered with gauze to prevent insect escape while ensuring airflow. Each DE treatment was replicated four times, with the vials placed inside 80 L plastic storage containers (26 cm × 36,5 cm × 15 cm) with lockable lids. To maintain 65±3% relative humidity, the containers were conditioned with a saturated sodium nitrate solution (Greenspan, 1976), and plastic grids were placed at the bottom. The containers were then stored in a refrigerated incubator set at 25±1°C.

The biological tests followed a randomized parcel design, with mortality assessed at 24-hour intervals over 6- and 7-day exposure to DE treatments. During each assessment, chickpea grains were sieved using a 2 mm sieve, and the number of dead and live adults was recorded. To evaluate *C. maculatus* F_1 progeny emergence, the glass vials were stored in a dark, climate-controlled room at 25±1°C and 65±5% relative humidity for 45 days. At the end of this period, the grains from both DE-treated and control samples were sieved using a 2 mm sieve, and the number of newly emerged adults was recorded.

Data processing and analysis

In the biological tests, mortality rates in DE treatments were corrected using Abbott's correction formula when mortality occurred in the control group by the end of the exposure period (Abbott, 1925). Percentage mortality at the end of each exposure period for spray applications was arcsine-transformed to normalize heteroscedastic treatment variances and analyzed using one-way analysis of variance (ANOVA), with DE formulation as the main factor. Transformed data were used for the analysis, whereas raw data were used in the figures. The analysis was performed using Minitab 21 (Minitab Inc., USA), and differences between means were determined using Tukey's Honestly Significant Difference (HSD) test at a 5% significance level.

Differences in the number of adult emergences in the new generation compared to the control were evaluated using Dunnett's test (p=0.05). Adult progeny counts for each concentration-DE formulation combination were compared to those in the control vials using Dunnett's test at a 5% significance level. Percentage reduction in progeny emergence for each concentration was analyzed using one-way ANOVA, with DE formulation as the main factor. Mean percentage reductions in new-generation adult emergence for each concentration and DE formulation were compared using Tukey's HSD test at a 5% significance level.

Percentage mortality data over different exposure times for each DE formulation were subjected to probit analysis using the PC-POLO program (Leora Software, 1994). Based on the probit analysis, lethal time (LT_{50}, LT_{90}, and LT_{99}) values were calculated for each DE formulation at 500 and 1000 ppm concentrations. Differences among LT_{50}, LT_{90}, and LT_{99} values were determined by examining the overlap of their lower and upper confidence intervals.

Results

Physicochemical characterization of tested diatomaceous earth powder formulations

Scanning Electron Microscopy (SEM) images of the Detech® formulation (Figure 2a) reveal numerous cylindrical and rod-shaped diatom skeletons, whereas the commercial DE formulation SilicoSec® consists of fossilized single-celled diatoms with a rod-like structure (Figure 2b).

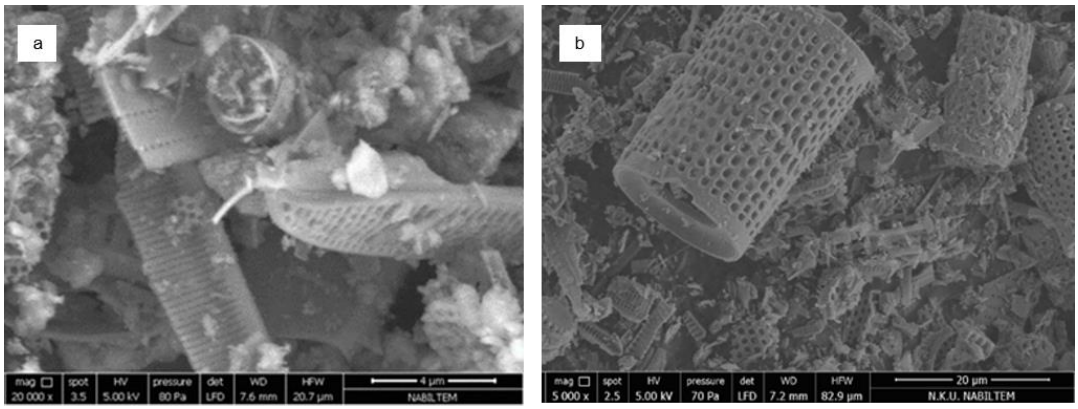

Figure 2. Scanning Electron Microscopy (SEM) images of diatom frustules in a) the local standard DE formulation (Detech®) at 20,000x magnification, and b) the commercial DE formulation (SilicoSec®) at 5,000x magnification.

The chemical composition of the diatomaceous earth formulations used in biological tests is provided (Table 1). Notably, SilicoSec® has a higher silicon dioxide (SiO_2) content (85.7%) than Detech® (80.6%). Figure 3 presents the particle size distributions (by volume) of the three DE formulations: Detech®-s, Detech®-m, and SilicoSec®. The particle sizes (in µm) at the 10^{th}, 50^{th}, 90^{th}, and 99^{th} percentiles of the cumulative size distribution (d_{10}, d_{50}, d_{90}, and d_{99}) are provided in Table 2. Detech®-m and SilicoSec® are relatively fine powders, with d_{50} values of approximately 7.34 µm and 12.69 µm, respectively, and d_{99} values ranging from 41.82 to 42.79 µm. In contrast, Detech®-s consists of coarser particles, as indicated by its cumulative frequency distribution, where 50% of particles measure 24.89 µm and 99% reach 280.39 µm. Uniformity analysis showed that Detech®-s (1.20) and Detech®-m (0.69) had similar uniformity values, while SilicoSec® (0.53) exhibited a much lower uniformity value, indicating a narrower particle size distribution with more consistent particle sizes.

Table1. Chemical composition of the diatomaceous earths used in biological tests

DE samples	Compound weight percentage (%)							
	SiO_2	Al_2O_3	Fe_2O_3	CaO	MgO	Na_2O	K_2O	TiO_2
Detech®	80.6	4.7	1.5	4.75	0.85	0.4	0.5	<0.01
SilicoSec®	85.7	4.45	1.35	0.45	0.25	0.35	0,35	0.2

Table 2. Particle size distribution and Brunauer-Emmett-Teller (BET) specific surface area of three diatomaceous earth formulations (Detech®-s, Detech®-m, and SilicoSec®)*

DE formulations	Particle size distribution					BET specific surface area (m^2/g)
	d (10) (µm)	d (50) (µm)	d (90) (µm)	d (99) (µm)	Uniformity	
Detech®-s	3.22±0.11ab	24.89±0.42 a	135.84±1.61 a	280.39±5.13 a	1.20±0.072 a	40.35±1.88 b
Detech®-m	2.08±0.18 b	7.34±0.69 c	20.83±1.74 b	41.82±2,59 b	0.69±0.13 a	60.45±2.30 a
Silicosec®	4.07±0.56 a	12.69±0.95 b	26.05±1.50 b	42.79±2.45 b	0.53±0.067 b	43.81±2.09 b
F and *p* value*	$F_{2,6}$=8.44 p=0.018	$F_{2,6}$=24.28 p=0.001	$F_{2,6}$=175.62 p< 0.001	$F_{2,6}$=155.34 p< 0.001	$F_{2,6}$=12.61 p=0.007	$F_{2,6}$=24.50 p=0.001

* A one-way analysis of variance (ANOVA) was conducted, and differences between means were determined using Tukey's HSD test at a 5% significance level. The different letters in the column indicate statistically significant differences.

The Brunauer-Emmett-Teller (BET) analysis revealed that Detech®-m had a significantly greater specific surface area (SSA) (60.45 m²/g) compared to Detech®-s and SilicoSec® ($F_{2,6}$=24.50, p=0.001). Detech®-s (43.81 m²/g) and SilicoSec® (40.35 m²/g) had nearly identical BET specific surface area values.

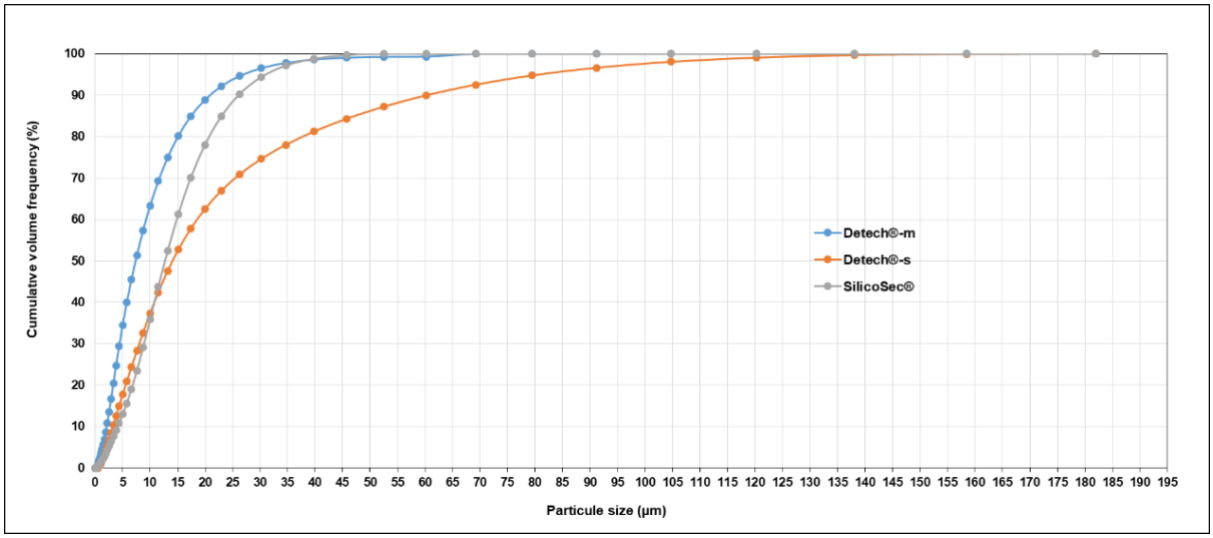

Figure 3. Particle size distribution (by volume) of three diatomaceous earth formulations (Detech®-s, Detech®-m, and SilicoSec®).

There were significant differences in oil and water absorption values among the tested DE formulations ($F_{2,6}$=293.1, p< 0.0001; $F_{2,6}$=68.5, p< 0.0001, respectively). SilicoSec® exhibited the highest oil absorption (173.6 g/mL), which was significantly greater than that of both Detech® formulations (Table 3).

Table 3. Physical properties of three diatomaceous earth formulations (Detech®-s, Detech®-m, and SilicoSec®)*

DE formulation	Oil absorption value (g/mL)	Water absorption value (g/mL)	Mean pH (n=3)	Compressed density (g/L)
Detech®-s	127.5±1.3 c	119.6±0.8 b	8.6±0.0 a	441.3±6.5 b
Detech®-m	141.6±1.2 b	124.0±1.5 b	7.8±0.0 b	340.9±3.8 a
Silicosec®	173.6±1.5 a	147.0±2.5 a	7.7±0.0 b	337.1±3.8 a
F and *p* value*	$F_{2,6}$=293.1 p< 0.0001	$F_{2,6}$=68.5 p< 0.0001	$F_{2,6}$=124.4 p< 0.0001	$F_{2,6}$=143.9 p< 0.0001

* A one-way analysis of variance (ANOVA) was conducted, and differences between means were determined using Tukey's HSD test at a 5% significance level.

Detech®-m had an intermediate absorption value (141.6 g/mL), while Detech®-s had the lowest (127.5 g/mL). Similarly, SilicoSec® had the highest water absorption (147 g/mL), followed by Detech®-m (124 g/mL) and Detech®-s (119.6 g/mL). In terms of pH, Detech®-s had the highest value (8.66), which was significantly different from both Detech®-m and SilicoSec® ($F_{2,6}$=124.4, p< 0.0001). Detech®-m (7.88) and

SilicoSec® (7.72) had similar pH values but remained statistically distinct from Detech®-s. While SilicoSec® and Detech®-m were mildly alkaline and close to neutral, Detech®-s was more alkaline, suggesting a higher potential for distinct chemical and biological interactions. Detech®-s also had the highest compressed density (441.3 g/L), whereas Detech®-m (340.9 g/L) and SilicoSec® (337.1 g/L) had significantly lower densities ($F_{2,6}$=143.9, p< 0.0001).

Mortality

The mean percentage mortality (± standard error) of *C. maculatus* adults exposed to 500 ppm of three DE formulations (Detech®-s, Detech®-m, and SilicoSec®) over a 7-day period is presented in Figure 4.

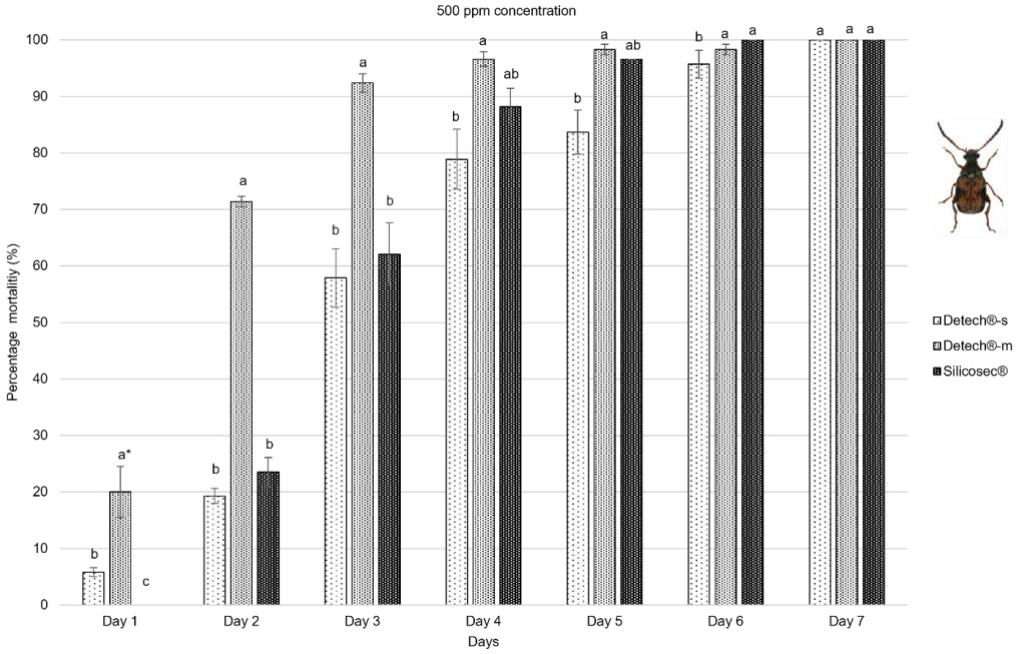

Figure 4. Mean percentage mortality (± standard error)* of *Callosobruchus maculatus* adults exposed to chickpeas treated with a water suspension of three diatomaceous earth (DE) formulations (Detech®-s, Detech®-m, and SilicoSec®) at 500 ppm over a 7-day period.

* A one-way analysis of variance (ANOVA) was conducted, and differences between means were determined using Tukey's HSD test at a 5% significance level. Bars with different letters indicate statistically significant differences and each day is compared within each day.

On Day 1, Detech®-m exhibited the highest initial mortality (20%), which was significantly higher than Detech®-s (5.8%) and SilicoSec® (0%) ($F_{2,9}$=33.8, p< 0.0001). Detech®-s was significantly more effective than SilicoSec®, while Detech®-m remained the most effective overall. By Day 2, Detech®-m showed a sharp increase in mortality (71.4%), whereas Detech®-s (19.3%) and SilicoSec® (23.5%) had significantly lower mortality rates ($F_{2,9}$=218.6, p< 0.0001). On Day 3, Detech®-m maintained the highest mortality (92.4%), followed by Detech®-s (57.9%) and SilicoSec® (62.1%). Significant differences persisted between Detech®-m and the other two DE formulations ($F_{2,9}$=22.8, p< 0.0001). On Day 4, Detech®-m approached 100% mortality, while Detech®-s and SilicoSec® continued increasing (80-88%). However, Detech®-m still trended slightly higher. By Day 5, Detech®-s reached at least 85% mortality, while Detech®-m and SilicoSec® achieved nearly complete mortality (>96%). By Day 6, all DE formulations had reached 100% or nearly 100% mortality. On Day 7, no significant differences remained between the formulations, as all achieved complete mortality. Overall, Detech®-m was significantly more effective than Detech®-s and SilicoSec® during the early exposure period (Days 1-3), while SilicoSec® was the least effective. However, by the end of the study, all DE formulations reached 100% mortality (Figure 4).

The mean percentage mortality (± standard error) of *C. maculatus* adults exposed to 1000 ppm of three DE formulations (Detech®-s, Detech®-m, and SilicoSec®) over a 6-day period is presented in Figure 5. On Day 1, Detech®-m exhibited the highest initial mortality (66.6%), which was significantly higher than SilicoSec® (2.5%). However, there was no significant difference between Detech®-m and Detech®-s, despite Detech®-s resulting in a slightly lower mortality rate (46.6%) ($F_{2,9}$=35.3, p< 0.0001). By Day 2, mortality remained high for both Detech®-m (94.1%) and Detech®-s (76.4%), while SilicoSec® had a significantly lower mortality rate (32.7%) ($F_{2,9}$=21.9, p< 0.0001). On Day 3, SilicoSec® showed a sharp increase in mortality (77.3%), but its mortality rate was still significantly lower than that of Detech®-m (98.3%) ($F_{2,9}$=8.9, p< 0.0001). By Day 4, Detech®-m achieved 100% mortality of *C. maculatus* adults, while SilicoSec® and Detech®-s reached complete mortality on Days 5 and 6, respectively. Overall, Detech®-m was significantly more effective than SilicoSec® during the early exposure period (Days 1-3). However, by Days 5 and 6, all DE formulations achieved 100% mortality.

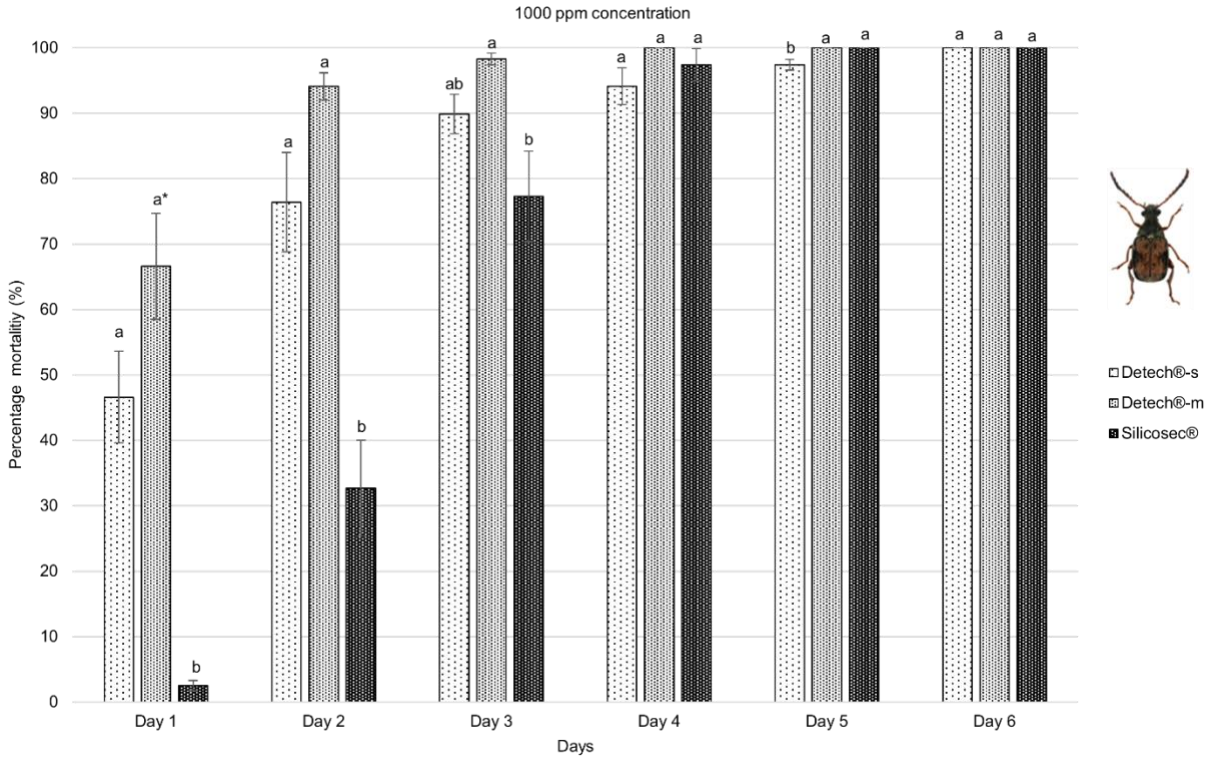

Figure 5. Mean percentage mortality (± standard error)* of *Callosobruchus maculatus* adults exposed to chickpeas treated with a water suspension of three diatomaceous earth (DE) formulations (Detech®-s, Detech®-m, and SilicoSec®) at 1000 ppm over a 6-day period.

* A one-way analysis of variance (ANOVA) was conducted, and differences between means were determined using Tukey's HSD test at a 5% significance level. Bars with different letters indicate statistically significant differences and each day is compared within each day.

Lethal time values

The lethal times (LT_{50} and LT_{99}) and probit analysis parameters for three DE formulations—Detech®-s, Detech®-m, and SilicoSec®—when applied to *C. maculatus* adults at 500 ppm are presented in Table 4. In all formulations, the results of the probit analysis indicate that the χ^2 tests demonstrated a good fit to the data (p > 0.05). There were statistically significant differences in the exposure times required to kill 50% (LT_{50}) and 99% (LT_{99}) of *C. maculatus* adults on chickpeas treated with water suspensions of the three DE formulations. Among them, Detech®-m exhibited the fastest action, with the lowest LT_{50} (36.55 h) and LT_{99}

(105.22 h). Importantly, its confidence intervals (CIs) did not overlap with those of Detech®-s (LT_{50}=72.80 h, LT_{99}=154.66 h) or SilicoSec® (LT_{50}=67.31 h, LT_{99}=122.73 h), confirming the statistical significance of the differences. These findings indicate that Detech®-m acts significantly faster than the other formulations, while Detech®-s is the slowest, and SilicoSec® exhibits an intermediate rate of mortality progression. Furthermore, SilicoSec® demonstrated the best probit model fit (χ^2=24.26, p< 0.05, H=1.10), indicating higher reliability in its mortality response predictions. While Detech®-m showed moderate variability (χ^2=37.19, p< 0.05, H=1.49), its model still provided a good fit. Regarding the rate of mortality progression, SilicoSec® had the steepest slope, meaning its mortality rate increased more sharply after exposure. In contrast, Detech®-m had a moderate slope, aligning with its faster LT_{50} compared to SilicoSec®. These results highlight the distinct modes of action of the tested DE formulations, with Detech®-m being the most rapid, SilicoSec® acting moderately fast, and Detech®-s being the slowest.

Table 4. Lethal time (LT_{50} and LT_{99}) values and probit analysis parameters for *Callosobruchus maculatus* adults exposed to a water suspension of three diatomaceous earth formulations (Detech®-s, Detech®-m, and SilicoSec®) at 500 ppm

DE formulation	n[a]	Slope±SE	Intercept	*t* value	LT_{50} (hour) (Lower-upper confidence interval)[b]	LT_{99} (hour) (Lower-upper confidence interval)[b]	χ^{2c} (df)[d] *p value*	*H*[e]
Detech®-s	840	0.028±0.002	-2.069	16.934	72.80 (67.40 - 77.96)	154.66 (143.61- 169.22)	39.47 (26) p< 0.05	1.52
Detech®-m	840	0.034±0.003	-1.239	11.363	36.55 (29.00 - 42.44)	105.22 (94.50 - 121.33)	37.19 (25) p< 0.05	1.49
Silicosec®	840	0.042±0.003	-2.826	15.397	67.31 (63.67 - 70.88)	122.73 (115.30 - 132.35)	24.26 (22) p< 0.05	1.10

[a]: Total number of individuals tested, SE: Standart error [b]: Lower-upper confidence interval (at 5% significance level), [c]: Chi-square value, [d]: Degree of freedom, [e]: Heterogeneity value.

The lethal times (LT_{50} and LT_{99}) and probit analysis parameters for three DE formulations—Detech®-s, Detech®-m, and SilicoSec®—when applied to *C. maculatus* adults at 1000 ppm are presented in Table 5. There were statistically significant differences in the exposure times required to kill 50% (LT_{50}) and 99% (LT_{99}) of *C. maculatus* on chickpeas treated with water suspensions of the three DE formulations. Among them, Detech®-m exhibited the fastest action, with the lowest LT_{50} (12.42 h) and LT_{99} (70.65 h). Importantly, its confidence intervals (CIs) did not overlap with those of SilicoSec® (LT_{50}=57.53 h, LT_{99}=101.02 h) or Detech®-s (LT_{50}=22.59 h, LT_{99}=123.50 h), except for a partial overlap in LT_{50} CIs between Detech®-s and Detech®-m. This confirms that the difference in LT_{99} values is statistically significant, while the LT_{50} difference between Detech®-s and Detech®-m requires careful interpretation due to overlapping confidence intervals. These findings indicate that Detech®-m acts significantly faster than the other formulations, while SilicoSec® is the slowest, and Detech®-s exhibits an intermediate rate of mortality progression based on LT_{50} values. Furthermore, Detech®-m demonstrated the best probit model fit (χ^2=18.73, p< 0.05, H=0.85), indicating higher reliability in its mortality response predictions. In contrast, SilicoSec® showed high variability (χ^2=32.16, p > 0.05, H=3.43), suggesting a less consistent mortality response. Regarding mortality progression rates, SilicoSec® had the steepest slope, meaning its mortality rate increased more sharply after exposure. In contrast, Detech®-m had a moderate slope, aligning with its faster LT_{50} compared to SilicoSec®. These results highlight the distinct modes of action of the tested DE formulations, with Detech®-m being the most rapid, Detech®-s acting moderately fast, and SilicoSec® being the slowest.

Table 5. Lethal time (LT_{50} and LT_{99}) values and probit analysis parameters for *Callosobruchus maculatus* adults exposed to a water suspension of three diatomaceous earth formulations (Detech®-s, Detech®-m, and SilicoSec®) at 1000 ppm

DE formulation	n[a]	Slope±SE	intercept	*t* value	LT_{50} (hour) (Lower-upper confidence interval)[b]	LT_{99} (hour) (Lower-upper confidence interval)[b]	X^{2c} (df)[d] *p* value	H[e]
Detech®-s	720	0.023±0.002	-0.521	10.391	22.59 (8.82 - 32.03)	123.50 (108.20 - 147.98)	34.69 (22) $p< 0.05$	1.58
Detech®-m	720	0.040±0.006	-0.496	6.734	12.42 (1.65 - 18.99)	70.65 (61.67- 86.21)	18.73 (22) $p< 0.05$	0.85
Silicosec®	720	0.053±0.004	-3.066	13.820	57.53 (53.34 - 61.65)	101.18 (93.03 - 113.02)	32.16 (18) $p > 0.05$	3.43

[a]: Total number of individuals tested, SE: Standart error [b]: Lower-upper confidence interval (at 5% significance level), [c]: Chi-square value, [d]: Degree of freedom, [e]: Heterogeneity value.

Progeny production

Dunnett's test showed that *C. maculatus* progeny production in the control group was significantly higher than in all DE-treated chickpea formulations at 500 ppm (Table 6). Among the treatments, Detech®-m had the lowest adult progeny emergence (17.6), indicating the highest suppression of reproduction. In contrast, Detech®-s was the least effective, with the highest progeny emergence (181.7), though still significantly lower than the control. Progeny emergence in SilicoSec® (109.7) was higher than in Detech®-m but lower than in Detech®-s, confirming its intermediate effectiveness. Detech®-m provided the highest suppression rate (96.7%), significantly reducing progeny production. Meanwhile, Detech®-s was the least effective (64.9% suppression), allowing significantly more progeny to emerge. SilicoSec® showed a suppression rate of 78.8%, which was significantly higher than Detech®-s but lower than Detech®-m ($F_{2,9}$=49.72, p< 0.0001).

Table 6. Mean number of progeny emergence (± S.E.) and percentage reduction in progeny production for Callosobruchus maculatus 45 days after the removal of parental adults from chickpea grains treated with 500 ppm of three diatomaceous earth (DE) formulations (Detech®-s, Detech®-m, and SilicoSec®)

F_1 progeny emergence *Callosobruchus maculatus* (500 ppm)*			
DE Formulation	Number of progeny emergence±S.E	*p* value***	Percentage reduction in progeny production (%)±S. E.
Detech®-s	181.7±18.7 (518.7±39.3)**	0.0001	64.9±3.6 C
Detech®-m	17.6±3.6 (518.7±39.3)	0.0001	96.7±0.7 A
Silicosec®	109.7±6.8 (518.7±39.3)	0.0001	78.8±1.3 B
F and *p* value*	-	-	$F_{2,9}$=49.72 $p< 0.0001$

* A one-way analysis of variance (ANOVA) was conducted, and differences between means were determined using Tukey's HSD test at a 5% significance level. Different letters indicate statistically significant differences.

** The values in parentheses represent the average number of new-generation adults obtained from the control treatment.

*** Dunnett's test was used to compare the number of new-generation adults from the biological tests with the control group's average.

According to Dunnett's test, *C. maculatus* progeny production in the control group was significantly higher than in all DE-treated chickpea formulations at 1000 ppm (Table 7). Among the treatments, Detech®-m had the lowest adult progeny emergence (9.5), indicating the highest suppression of reproduction. In contrast, Detech®-s was the least effective, with the highest progeny emergence (107), though still significantly lower than the control. Progeny emergence in SilicoSec® (87.5) was higher than in Detech®-m but lower than in Detech®-s, confirming its intermediate effectiveness. Detech®-m provided the highest suppression rate (98.1%), significantly reducing progeny production. Meanwhile, Detech®-s was the least effective (79.3% suppression), allowing significantly more progeny to emerge. SilicoSec® showed a suppression rate of 83.1%, which was statistically in the same group as Detech®-s but significantly lower than Detech®-m ($F_{2,9}$=8.46, p=0.009).

Table 7. Mean number of progeny emergence (± S.E.) and percentage reduction in progeny production for Callosobruchus maculatus 45 days after the removal of parental adults from chickpea grains treated with 1000 ppm of three diatomaceous earth (DE) formulations (Detech®-s, Detech®-m, and SilicoSec®)

F_1 progeny emergence *Callosobruchus maculatus (*1000 ppm)*			
DE formulation	Number of adult emergence±S. E	*p* value***	Percentage reduction in progeny production (%)±S. E
Detech®-s	107±30.4 (518.7±39.3)**	0.000	79.3±5.8 B
Detech®-m	9.5±3.5 (518.7±39.3)	0.000	98.1±0.7 A
Silicosec®	87.5±1.8 (518.7±39.3)	0.000	83.1±0.3 B
F and *p* value*	-	-	$F_{2,9=}$8.46 *p*=0.009

* A one-way analysis of variance (ANOVA) was conducted, and differences between means were determined using Tukey's HSD test at a 5% significance level. Different letters indicate statistically significant differences.

** The values in parentheses represent the average number of new-generation adults obtained from the control treatment.

*** Dunnett's test was used to compare the number of new-generation adults from the biological tests with the control group's average.

Discussion

The mode of application—water suspension (slurry) vs. dry dust—significantly affects the effectiveness of diatomaceous earth (DE) formulations. Dry DE relies on direct contact with insect exoskeletons, where its fine abrasive particles attach to the cuticle, absorb lipids, and cause dehydration (Athanassiou et al., 2003). However, dust applications present challenges such as displacement, reduced adhesion to grains, and inhalation risks for workers (Fields & Korunić, 2000). In contrast, slurry applications involve suspending DE in water and spraying it onto stored products. As the water evaporates, a uniform DE layer remains on the surface, ensuring better adhesion to grains and insect bodies (Ertürk et al., 2020). This method minimizes dust-related health concerns and may enhance insecticidal activity by providing broader coverage and longer persistence (Stathers et al., 2004). In this study, the water suspension of Detech®-m at 1000 ppm resulted in complete mortality of *C. maculatus* by the fourth day and reduced progeny production by 98.1%, indicating near-total reproductive inhibition. A previous study reported that dry application of Detech® at 1000 ppm achieved 100% mortality by the fifth day and reduced progeny production by 88.7% (Sağlam et al., 2022b). The efficacy of Detech® and SilicoSec® in controlling *C. maculatus* varied depending on whether they were applied as dry powder or water suspension. Similarly, in the same study, dry SilicoSec® at 1000 ppm achieved full mortality by day five, but at 500 ppm, mortality was only 77% after seven days (Sağlam et al., 2022a). In the present study, water-suspension application of SilicoSec® at 500 ppm required six days to reach complete mortality, while at 1000 ppm, full mortality was achieved in a shorter time. With dry application, SilicoSec® at 500 ppm reduced progeny production by 48.7%, whereas at 1000 ppm and above, suppression exceeded 80.6%. However, in the water-suspension treatment, SilicoSec® at 500 ppm provided 78.8% progeny suppression, and at 1000 ppm, it achieved 83.1%. Overall, comparing dry and water-suspension applications suggests that water suspensions result in higher mortality, particularly at lower concentrations, and require slightly less exposure time. Additionally, water suspensions provide better progeny suppression at lower concentrations, likely due to more uniform grain coverage. This application method improves coverage and adhesion to grain surfaces, reducing dust displacement and increasing persistence (Korunić, 1998; Stathers et al., 2004). Our findings align with previous research demonstrating that slurry-applied DE formulations prolong insecticidal activity due to better adhesion to treated surfaces (Fields & Korunić, 2000).

This study also examined the relationship between the chemical and physical properties of different diatomaceous earth (DE) formulations and their effects on adult mortality, lethal time (LT_{99}), and progeny reduction of *C. maculatus*. The primary component of DE is silicon dioxide (SiO_2), which constitutes a significant portion of its composition. Among the formulations analyzed, SilicoSec® contains 85.7% SiO_2,

while Detech® contains 80,6% SiO_2. The higher SiO_2 content in SilicoSec® suggests a greater abundance of diatom frustules, which are critical for the abrasive action that causes insect desiccation. Studies indicate that the insecticidal properties of DE are influenced by its chemical composition. Formulations with higher SiO_2 concentrations have been associated with greater efficacy against pests such as *C. maculatus* (Korunić, 1998), as well as other stored-product insects, including the rice weevil, *Sitophilus oryzae* (L., 1763) and the red flour beetle, *Tribolium castaneum* (Herbst, 1797) (Athanassiou et al., 2005). However, DE efficacy is not determined solely by SiO_2 concentration. A study by Arnaud et al. (2005) assessed the insecticidal efficacy of various DE formulations with differing oxide compositions, including SiO_2, Al_2O_3, Fe_2O_3, CaO, and MgO. The findings revealed that higher SiO_2 content did not always correlate with increased insecticidal activity. Instead, the presence and proportions of other oxides, such as Al_2O_3 and Fe_2O_3, influenced the hydrophilic properties and abrasiveness of DE particles, ultimately affecting their overall efficacy against stored-product insects. These results suggest that insecticidal performance is a multifaceted trait influenced by the complete chemical composition of DE, rather than SiO_2 concentration alone.

The physical attributes of DE, particularly particle size distribution and specific surface area (SSA), play a crucial role in its effectiveness as an insecticide (Korunić, 1997; Robinson, 2005; Vayias et al., 2009; Ziaee et al., 2013; Henteş & Işıkber, 2024). Detech®-m, with a median particle size (d_{50}) of approximately 7.34 µm and a specific surface area of 60.45 m²/g, exhibited superior insecticidal performance. Several studies have also demonstrated that smaller DE particles enhance insecticidal activity. For instance, Vayias et al. (2009) found that DE formulations with particles smaller than 45 µm were more effective against *Rhyzopertha dominica* (Fabricius, 1792) (Coleoptera: Bostrichidae), *Sitophilus oryzae* A.Hustache, 1930 (Coleoptera: Dryophthoridae), and *Cryptolestes ferrugineus* (Stephens, 1831) (Coleoptera: Laemophloeidae) compared to formulations with larger particles. The increased efficacy of finer particles is attributed to their greater adherence to the insect cuticle, leading to enhanced desiccation and higher mortality rates. However, particle size alone does not solely determine efficacy. Baliota & Athanassiou (2020) reported that particle shape also plays a crucial role, with certain shapes enhancing DE's insecticidal properties. Their research suggests that while smaller particles tend to be more effective, overall shape and structure are also critical factors influencing DE's performance as an insecticide. Moreover, a higher specific surface area (SSA) provides a greater interaction area between DE particles and insect exoskeletons. This increased contact enhances the abrasive action of DE, effectively disrupting the insect's protective waxy cuticle and accelerating desiccation. In contrast, Detech®-s, with a coarser particle size (d_{50}=24.89 µm) and a lower specific surface area (40.35 m²/g), was less effective. The larger particle size and reduced surface area likely resulted in diminished contact with the insect cuticle, reducing desiccation efficiency. This finding aligns with previous research, which indicates that finer DE particles with higher surface areas are more effective for stored-product insect pest control (Athanassiou et al., 2004).

The efficacy of DE formulations is often assessed by measuring the time required to achieve a specific mortality rate, such as LT_{99} (the time needed to kill 99% of the target population). Unlike many other stored-product insect pests, adult *C. maculatus* have a short lifespan, typically surviving only 5 to 10 days in stored pulses under tropical conditions (Rees, 2004). During this period, they do not feed or directly damage stored grains; instead, their primary function is egg-laying. Fast-acting DE formulations with high SSA and optimal particle size can kill adults before oviposition, preventing infestation without chemical pesticides (Arthur, 2000). The key objective is to minimize LT_{50} and LT_{99} values, ensuring *C. maculatus* adults perish before significant egg-laying occurs. In this study, at 500 ppm, Detech®-m exhibited a significantly lower LT_{99} (105.22 hours) than SilicoSec® (122.73 hours) and Detech®-s (154.66 hours). At 1000 ppm, the same trend persisted, with Detech®-m (70.65 hours) acting more rapidly than Detech®-s (123.50 hours) and SilicoSec® (101.18 hours). These results confirm that Detech®-m provides faster and more effective mortality against *C. maculatus* adults due to its fine particle size, higher BET surface area, and superior adsorption properties. Studies have shown that DE products with enhanced SSA and finer particles induce higher mortality in stored-product pests like *R. dominica* and *C. maculatus* within shorter time frames than

formulations with lower SSA (Athanassiou et al., 2005; Kabir & Wulgo, 2014). Although SilicoSec® contains a higher SiO_2 content, it exhibited a longer LT_{99} (101.18 hours) under similar conditions. This disparity may be due to its broader particle size distribution and lower BET surface area, which could reduce consistent contact with the insect cuticle. These findings highlight the importance of optimizing physical properties, such as SSA, particle size and distribution, to enhance the insecticidal efficiency of DE formulations.

Beyond immediate adult mortality, an effective DE formulation should also disrupt the reproductive cycle of pests to prevent population resurgence (Arthur, 2000; Subramanyam & Roesli, 2000; Athanassiou et al., 2005). Detech®-m exhibited a substantial reduction in progeny production, with a suppression rate of 98.1% at 1000 ppm. This result suggests that exposure to Detech®-m not only causes high adult mortality but also adversely affects the reproductive capabilities of surviving individuals, possibly by interfering with oviposition or egg viability. In contrast, Detech®-s, with its coarser particles and lower surface area, achieved a lower progeny suppression rate (79.3%) under the same conditions. The reduced efficacy in progeny reduction may result from insufficient disruption of the insect's protective cuticular layer, allowing some individuals to survive and reproduce. These findings align with Stathers et al. (2004), who reported that DE treatments significantly reduce oviposition and inhibit larval development in *C. maculatus*. Furthermore, DE water-suspension applications may enhance efficacy by ensuring more uniform grain coverage, preventing larvae from burrowing and developing (Athanassiou et al., 2003).

Other physical characteristics, such as oil and water absorption capacities, pH, and compressed density, can influence the performance of DE formulations (Korunić, 1997 & 1998; Losic & Korunić, 2017). SilicoSec® exhibited the highest oil absorption (173.6 g/mL) and water absorption (147 g/mL), which may enhance its desiccating effect on insects. However, its lower uniformity value (0.53) suggests a narrower particle size distribution, which might limit its overall efficacy due to reduced coverage and contact with the insect cuticle. The pH of DE formulations can also affect their insecticidal activity. Detech®-s had the highest pH value (8.66), making it more alkaline compared to Detech®-m (7.88) and SilicoSec® (7.72). The pH of DE may influence its interaction with insect cuticles, as a neutral to slightly alkaline pH is considered optimal for maintaining stability, dispersion, and effectiveness of DE particles (Athanassiou et al., 2005). However, the direct impact of DE pH on insect mortality remains unclear and requires further investigation.

Conclusion

This study clearly demonstrates that water-suspension (slurry) application significantly enhances the insecticidal efficacy of diatomaceous earth (DE) formulations against *C. maculatus* in stored chickpeas compared to dry dust applications. Slurry treatments improved DE adhesion, reduced dust-related health risks, and achieved higher mortality and progeny suppression at lower concentrations. Among the evaluated formulations, Detech®-m exhibited the highest effectiveness, achieving 99% adult mortality and 98.1% progeny suppression, attributable to its fine particle size (7.34 µm) and elevated specific surface area (60.45 m^2/g), which enhanced cuticular abrasion and desiccation. Although SilicoSec® contained a higher SiO_2 content, its broader particle size distribution and lower BET surface area diminished its performance relative to Detech®-m. These findings reinforce that the physical characteristics of DE, particularly particle size and surface area, are more critical determinants of efficacy than chemical composition alone. Moreover, slurry applications further augmented these properties by ensuring more uniform coverage and prolonged persistence on treated grains.

The results advocate for refining DE formulations by targeting their physicochemical attributes to maximize efficacy and minimize health risks. Water-suspension methods emerge as a promising strategy to develop sustainable, non-chemical pest management practices for stored legumes. Future research should investigate the interaction between DE formulations and storage environment variables, such as humidity and temperature, to optimize their practical application under diverse conditions. The strategic optimization of diatomaceous earth formulations and application techniques thus represents a pivotal advancement toward sustainable, non-chemical preservation of global food security.

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Original article (Orijinal araştırma)

Insect growth regulators as chemosterilants: a study on house fly, *Musca domestica* L., 1758 (Diptera: Muscidae) populations in Türkiye[1]

Kemosterilant olarak böcek büyüme düzenleyicileri: Türkiye'deki ev sineği, *Musca domestica* L., 1758 (Diptera: Muscidae) popülasyonları üzerine bir çalışma

Gökhan ERDOĞAN[2*] **Hüseyin ÇETİN[3]**

Abstract

The house fly, *Musca domestica* L., 1758 (Diptera: Muscidae), is a public health pest commonly found on animal farms, manure heaps, and garbage dumps. In agricultural and livestock areas, house flies are frequently exposed to pesticides used against various pests, which leads to the development of insecticide resistance. This resistance complicates their control and has prompted researchers and insecticide manufacturers to explore alternative control strategies and methods. This study examines the effects of insect growth regulators (IGRs), specifically diflubenzuron and pyriproxyfen, used as larvicides, on egg yield, egg-laying index, and egg-to-adult transition rates in six different house fly populations. These populations were collected and cultured from five Turkish provinces (Antalya, Bursa, Edirne, Gaziantep, and İzmir) between June 2020 and August 2021, as well as from a susceptible population provided by the World Health Organization (WHO). The adult house flies were fed sugar solutions (40%) containing either 5% or 10% concentrations of diflubenzuron and pyriproxyfen. Our findings show an 80% reduction in egg yield and a 90% reduction in adult emergence rates across all populations compared to the control group. Although exposure to IGRs significantly decreased egg area indices, it did not affect the egg to adult transition rate.

Keywords: Chemosterilant, diflubenzuron, *Musca domestica*, pyriproxyfen

Öz

Ev sineği, *Musca domestica* L., 1758 (Diptera: Muscidae), hayvan çiftliklerinde, gübre yığınlarında ve çöp alanlarında yaygın olarak bulunan bir halk sağlığı zararlısıdır. Tarım ve hayvancılık alanlarında, ev sinekleri çeşitli zararlılara yönelik kullanılan pestisitlere sıkça maruz kalmakta ve bu durum insektisit direncinin gelişmesine yol açmaktadır. Gelişen direnç, bu zararlının kontrolünü zorlaştırmakta ve araştırmacılar ile insektisit üreticilerini alternatif mücadele stratejileri ve yöntemleri aramaya yönlendirmektedir. Bu çalışma, larvasit olarak kullanılan böcek gelişim düzenleyicilerin (diflubenzuron ve pyriproxyfen) altı farklı ev sineği popülasyonunda yumurta verimi, yumurtlama indeksi ve yumurtadan ergin döneme geçiş oranı üzerindeki etkilerini incelemektedir. Bu popülasyonlar, Haziran 2020 ve Ağustos 2021 tarihleri arasında Türkiye'nin beş ilinden (Antalya, Bursa, Edirne, Gaziantep ve İzmir) toplanmış ve kültüre alınmış popülasyonlar ile Dünya Sağlık Örgütü (WHO) duyarlı popülasyonundan oluşmaktadır. Ergin ev sinekleri, %40 şeker içeren ve %5 ya da %10 oranında diflubenzuron ve pyriproxyfen içeren çözeltilerle beslenmiştir. Bulgularımız, kontrol grubuna kıyasla tüm popülasyonlarda yumurta veriminde %80 ve ergin hale geçme oranlarında %90 azalma olduğunu göstermektedir. Böcek gelişim düzenleyicilere maruz kalma, yumurta alan indeksini anlamlı düzeyde azaltmış olsa da yumurtadan ergine geçiş oranını etkilememiştir.

Anahtar sözcükler: Kemosterilant, diflubenzuron, *Musca domestica*, pyriproxyfen

[1] This study is based on the doctoral dissertation of the first author.

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Introduction

The house fly *Musca domestica* L., 1758 (Diptera: Muscidae) is a widespread vector of numerous pathogens, including viruses, bacteria, and fungi in human-inhabited environments. House flies are found in manure heaps, garbage dumps, and livestock-raising areas (Sudagidan et al., 2022). This insect transmits pathogens that can cause significant diseases, such as cholera, dysentery, hepatitis, and tuberculosis, in humans and animals. In agricultural settings, such as barns and poultry houses, high densities of house flies can also negatively impact the production of meat, eggs, and milk (Cheng & Kesler, 1961). Furthermore, house flies contribute to the spread of *Escherichia coli* Escherich (Enterobacteriaceae: Enterobacteriales) bacteria, which can infect both animals and humans and potentially promote antibiotic resistance (Bakry et al., 2022).

Recent research has shown that house fly populations have developed significant resistance to various insecticides, including synthetic pyrethroids and neonicotinoids (Koc et al., 2012; Erdogan & Cetin, 2020; Abobakr et al., 2022; Polat, 2022). This rapid increase in resistance poses a significant challenge for effective management of these pests. Therefore, scientists are exploring various insecticides and alternative methods that are effective against house flies. Among these, insect growth regulators (IGRs) are particularly notable. IGRs are classified into four main groups: ecdysone agonists (e.g., Tebufenozide and Methoxyfenozide), classic IGRs (e.g., diflubenzuron, cyromazine, and novaluron), juvenile hormone analogs (JHA) (e.g., methoprene and pyriproxyfen), and anti-juvenile hormone agents (e.g., terpenoid imidazoles and fluoromevalonate). IGRs and JHAs are commonly used in vector control, while ecdysone agonists are mainly applied against agricultural pests, particularly larvae of the Lepidoptera and Coleoptera. However, despite their effectiveness, anti-juvenile hormone agents are rarely used in pest control due to their toxicity to non-target organisms (Tunaz & Uygun, 2004; Oz et al., 2024). IGRs containing JHA and other IGR elements are the most frequently used insecticides in this category.

Juvenile hormones (JHs) significantly influence various physiological and biochemical processes in insects during different life stages. In the larval stage, JHs suppress metamorphosis by delaying development, thus preventing the transition to the pupal and adult stages. In adult insects, JHs are essential for pheromone production, the development of accessory glands, and notably, in females for egg and ovarian maturation (Hu et al., 2019). Chitin, a polymer of N-acetylglucosamine, is an essential component of the cuticle and exoskeleton of insects. Exposure to IGRs, commonly used as larvicides in vector control, disrupts insect development by inhibiting the polymerization of N-acetylglucosamine, a process facilitated by chitin synthase enzymes. These developmental disruptions are typically evident during molting (Göktay & Kısmalı, 1990; Özparlak, 2003; Sankar & Kumar, 2023). The severity of developmental and molting disorders depends on the timing of IGRs exposure as well as the extent and duration of exposure and dosage. Mortality usually occurs during the larval or pupal stage. Late-stage larvae exposed to IGRs may survive to adulthood but often suffer from deformities in body structures, such as wings and legs, leading to reduced survival and mating success (Post et al., 1974; Merzendorfer, 2013; Ser & Çetin, 2016).

One of the integrated pest management strategies used against vector insects is the Sterile Insect Technique (SIT). SIT is an eco-friendly method employed in area-wide pest management to suppress or eliminate detrimental insect populations. This technique involves rearing large quantities of the target pest, sterilizing them, and releasing them into the environment to reduce their reproductive capacity. Sterility in insects can be induced by genetic modification, chemical means, or ionizing radiation (Parker & Metha, 2007). The first study on chemical sterilization was conducted by Goldsmith & Frank (1952), who used aminopterin, a folate antagonist, on *Drosophila melanogaster* Meigen, 1830 (Diptera: Drosophilidae), resulting in infertility by slowing ovarian development in females. Motivated by these findings, scientists and manufacturers have since then tested the chemosterilant effects of over 400 chemicals on various insect species, including IGRs.

In this study, we investigated the sterilizing, oviposition-reducing, and egg-to-adult transition inhibiting effects of two IGRs: diflubenzuron, a chitin synthesis inhibitor, and pyriproxyfen, a juvenile hormone analog, used as a larvicide in house flies, with the goal of assessing their chemosterilant and toxic effects on some Turkish house fly populations.

Materials and Methods

House fly populations

To collect house fly specimens for research, field collections were conducted between June 2020 and August 2021 in five Turkish provinces (Antalya, Bursa, Edirne, Gaziantep, and İzmir) spanning four geographical regions: the Mediterranean, Aegean, Southeastern Anatolia, and Marmara (including Southern Marmara and Marmara-Thrace) (Figures 1-2, Table 1). The five collection sites were selected for their diverse geographic locations as well as their distinct climatic and agricultural characteristics. This ecological variation enabled us to evaluate the efficacy of IGRs across a broad range of vector breeding habitats representative of different regions in Türkiye. In each province, house flies were captured from different areas of a cattle farm using sweep nets. Over 200 house flies were placed in 20x20x20 cm mesh cages and transported to the laboratory. During transport, their survival was ensured by providing moisture with water-soaked cotton balls, nourishment with milk-soaked cotton, and additional energy with sugar.

Figure 1. A map showing the provinces where house flies were collected from the field.

Figure 2. House fly collection using a sweep net on cattle farm.

Table 1. Locations of house fly collections from the field

City	District	Neighborhood	Location
Antalya	Kepez	Karşıyaka (Varsak)	36,99272ºN-30,71738ºE
Bursa	Nilüfer	Fadıllı	40,16571ºN-28,72822ºE
Edirne	Merkez	Bosna	41,62572ºN-26,56585ºE
Gaziantep	Oğuzeli	Çaybaşı	37,03459ºN-37,52870ºE
İzmir	Çeşme	Germiyan	38,31391ºN-26,47018ºE

House flies collected from different locations were transported to the Vector Ecology and Research Laboratory in the Biology Department at the Faculty of Science at Akdeniz University. There, they were housed in mesh cages for culturing. To promote healthy development and ensure the sustainability of the cultures, flies were provided with ample food (Wheat bran, milk, and sugar) and water. The laboratory conditions were meticulously controlled, maintaining a temperature of 26±2°C, humidity levels of 60±5%, and a photoperiod of 12 hours of light followed by 12 hours of darkness.

Chemosterilant activity tests

Pupae from field collections and WHO population were individually placed in Falcon tubes lined with cotton soaked in sugary water to prevent mating and facilitate sex determination upon reaching adulthood. We meticulously monitored the emergence of adult flies from the pupae daily, recording the emergence dates on the Falcon tubes to classify individuals as 1, 3, or 5 days old. Adults were grouped by their days post-emergence, with sex determined for each group. For the experiments, two virgin males and two virgin females from each group were placed into 20×20×20 cm mesh cages to evaluate the effects of diflubenzuron and pyriproxyfen at final concentrations of 5% and 10%. The feeding solution was prepared by initially mixing 40% sugar water, into which the appropriate volume of IGR stock solution was added to achieve the desired concentrations. Cotton pads soaked in this IGR-sugar solution were placed in petri dishes inside the treatment cages (Howard & Wall 1995b; 1996b). A separate petri dish containing cotton soaked in milk was also placed in each cage. In the control cages, cotton pads soaked in 40% sugar water (without IGRs) and milk were similarly provided, ensuring that the only variable between groups was the presence of the active ingredients. All feeding materials were freshly prepared and renewed daily to ensure consistent and continuous exposure throughout the experimental period. Each treatment condition (including different age groups and concentrations) was replicated three times, as were the control groups, to ensure statistical robustness. The flies had continuous access to the test solutions, which were freshly prepared and renewed daily throughout the experimental period. This setup aimed to mimic potential field exposure through bait-based delivery methods.

Figure 3. General view of the 20x20x20 cm cages used in laboratory experiments.

Table 2. Information on insect growth regulators used in the study (PubChem, 2019)

Name	Mode of action	CAS Number	IUPAC Name
Diflubenzuron	Chitin Synthesis Inhibitor	35367-38-5	*N*-[(4 chlorophenyl)carbamoyl]-2,6-difluorobenzamide
Pyriproxyfen	Juvenile Hormone Analog	95737-68-1	2-[1- (4-phenoxyphenoxy)propan-2-yloxy]pyridine

Egg counts for both the experimental and control groups were conducted using a digital stereo microscope and analysis software. We measured the dimensions-length and width-of the eggs to compute the egg area index. After counting and measuring, the eggs were placed into culture media composed of milk and bran to observe the transition rates from egg to adult. House flies that reached adulthood were then relocated to mesh cages equipped with cotton soaked in sugar, water, and milk. This setup facilitated the examination of sterility effects in the subsequent generation.

To closely mimic field conditions, large mesh cages measuring 120×120×120 cm were established for population monitoring over a 45-day period (Figure 4). Each cage was populated with 100 virgin male and 100 virgin female *M. domestica*. In the treatment cage (IGR group), flies were provided with a potential oviposition site consisting of a 5×10×20 cm plastic container filled with milk and Wheat bran mixture. In addition, separate plastic cups containing plain water, 40% sugar water, and 40% sugar water supplemented with 10% diflubenzuron or pyriproxyfen were placed inside the cage. In the control cage, the same setup was used, except that the sugar solution did not contain any IGRs. Each day, all containers and feeding materials were checked, and fresh solutions and food were added as needed to maintain surface moisture and ensure continuous exposure. Population densities in each cage were recorded at the end of the 45-day trial period to evaluate the effects of the treatments.

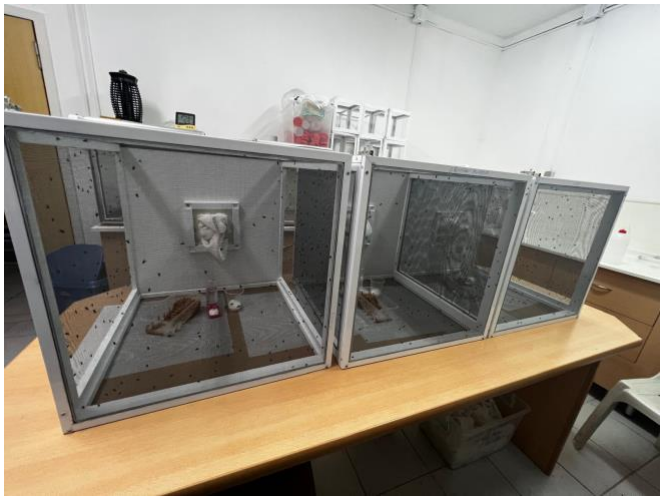

Figure 4. General view of the 120x120x120 cm cages used in field simulation experiments.

Data analysis

The evaluation of the data obtained from the tests was conducted using the following methods (Abbott 1987):

1. For the effect on egg production rates, the data were transformed into an index using the following equation:

$$\text{Egg production decrease rate (\%)} = \left(\frac{N_c - N_t}{N_c}\right) \text{x}100$$

N_c: Number of eggs in control N_t: Number of eggs in treatment

2. For the effect on emergence from eggs to adults, the data were transformed into an index using the following equation:

$$\text{Emergence decrease rate (\%)} = 100 - \left(\frac{E_c - E_t}{E_c} \times 100\right)$$

E_c: Emergence rate from eggs of control individuals E_t: Emergence rate from eggs of individuals exposed to insecticide

3. For the effect on egg area index, the data were transformed into an index using the following equation:

$$\text{Egg area index decrease rate (\%)} = 100 - \left(\frac{A_c - A_t}{A_c} \times 100\right)$$

A_c: Egg area index of control individuals A_t: Egg area index of individuals exposed to insecticide

The data garnered from both the field-collected populations and the WHO population were analyzed through variance analysis (ANOVA), focusing on percentage values and egg area index data to compare these two sets comprehensively. Additionally, the percentage data collected across various doses and different age groups were further examined using the Duncan multiple comparison test, with a significance level set at $p \leq 0.05$. This analysis was conducted using SPSS Statistics Base version 23 for Windows, facilitating a thorough and statistically sound comparison of the effects observed in the different experimental conditions.

The outcomes from the field simulation experiment were categorized according to the population density observed in the test cages after 45 days. The classification for chemosterilant efficacy based on the number of emerged individuals per cage was developed specifically for this study, without reference to any existing publication. It was designed using our own emergence data and population benchmarks established under controlled conditions. According to this classification: a population density of 0-500 individuals indicated very high chemosterilant efficacy; 500-1000 individuals represented high efficacy; 1000-1500 individuals indicated moderate efficacy; 1500-2500 individuals reflected low efficacy; and more than 2500 individuals corresponded to very low efficacy. This customized classification system enabled a clear and quantifiable assessment of the chemosterilant's impact on *M. domestica* population dynamics within the experimental framework.

Results and Discussion

Effects of diflubenzuron and pyriproxyfen on egg yield of house fly populations

Diflubenzuron and pyriproxyfen significantly reduced egg yields across all populations and age groups studied, at various doses. Specifically, a 5% concentration of diflubenzuron resulted in a reduction in egg yield ranging from 37% to 100%. Increasing concentration to 10% led to similar reductions, between 35% and 100%. For pyriproxyfen, a 5% concentration decreased egg yields by 31% to 100%, while 10% concentration resulted in reductions from 50% to 100%. Gaziantep population showed relatively lower inhibition effects compared to other populations. However, no formal resistance tests were performed, and thus, no definitive conclusions on resistance can be made. Further analysis showed that neither the age of the insects nor the dosage significantly affected the reduction in egg yield caused by either diflubenzuron or pyriproxyfen (Table 3).

In studies utilizing a 5% concentration of diflubenzuron, the transition from egg to adult stage showed a significant decrease, ranging from 56.84% to 100% in comparison to the control group across all tested populations. Similarly, trials with a 10% diflubenzuron concentration exhibited statistically significant reductions in adult emergence rates, from 91.96% to 100% compared to control groups across all populations (Table 4).

Trials involving a 5% concentration of pyriproxyfen led to reductions in the emergence from egg to adult stage ranging from 67.08% to 96.52%. In cases where no egg yield occurred, the emergence rates from egg to adult stage were not evaluated. The examination of the 10% concentration of pyriproxyfen on emergence rates revealed significant decreases, ranging from 83.95% to 98.06% compared to control groups across all populations (Table 4).

Table 3. Effects of diflubenzuron and pyriproxyfen on egg production

Insect Age (Days)	Chemical	Populations											
		Antalya		Bursa		Edirne		Gaziantep		İzmir		WHO	
		NEPF±SE	IR (%)	NEPF±SE	IR (%)	NEPF±SE	IR (%)	NEPF±SE	IR (%)	NEPF±SE	IR (%)	NEPF±SE	IR (%)
1	Diflu %5	15.67±6.44 AB a	87	50.33±2.76 C a	59	0.00±0.00 A a	100	26.00±5.44 B a	64	29.67±8.83 B a	71	30.50±4.09 B a	72
	Diflu %10	8.50±6.95 AB a	94	43.50±8.12 C a	64	0.00±0.00 A a	100	31.50±9.20 BC a	57	24.83±10.57 BC a	75	0.00±0.00 A a	100
	Pyri %5	12.83±10.49 AB a	91	61.67±14.26 D a	50	0.00±0.00 A a	100	23.67±6.16 BC a	68	32.83±2.99 C a	68	10.17±4.20 AB a	91
	Pyri %10	5.66±4.63 A a	96	40.16±10.07 B a	67	0.00±0.00 A a	100	36.67±7.22 B a	50	21.33±3.08 AB a	79	0.00±0.00 A a	100
	Control	138.33±6.30 B a		122.33±3.78 B a		108.83±6.78 AB a		73.00±2.96 A a		101.16±4.33 AB a		110.50±2.49 AB a	
3	Diflu %5	15.66±11.17 A a	88	49.66±5.20 B a	62	1.83±1.50 A a	98	49.66±7.99 A a	58	35.33±3.19 B b	59	7.83±6.40 A b	91
	Diflu %10	9.50±5.02 A a	64	27.33±7.67 B a	79	0.00±0.00 A a	100	6.83±5.59 A b	94	25.33±2.36 B a	70	4.66±3.81 A b	95
	Pyri %5	10.50±8.58 AB a	90	24.50±1.44 B b	81	0.00±0.00 A a	100	82.50±4.65 C b	31	23.50±7.18 B a	73	0.00±0.00 A a	100
	Pyri %10	4.00±3.27 A a	96	23.17±9.48 B a	82	0.00±0.00 A a	100	24.33±6.68 B a	80	16.33±7.00 AB a	81	0.00±0.00 A a	100
	Control	110.00±8.30 A a		131.33±3.31 A a		84.5±3.93 A a		119.00±11.96 A a		85.50±6.99 A a		91.16±2.12 A a	
5	Diflu %5	27.50±7.77 AB a	70	34.50±9.04 AB a	60	21.17±9.39 A b	71	28.00±11.47 AB a	56	62.16±3.61 B c	37	8.83±7.22 A b	90
	Diflu %10	22.16±1.83 AB a	100	16.33±6.69 A a	81	8.53±3.61 A b	88	41.17±6.33 B a	35	9.00±7.36 A b	91	4.33±3.54 A b	95
	Pyri %5	26.17±2.33 B a	71	33.67±9.53 BC b	61	10.83±6.73 A a	89	23.17±9.85 B a	63	51.50±6.49 C a	47	0.00±0.00 A a	100
	Pyri %10	23.83±4.37 AB a	73	29.00±5.41 AB a	66	0.00±0.00 A a	100	18.17±7.58 AB a	71	33.50±3.49 B a	66	0.00±0.00 A a	100
	Control	89.83±12.0 A a		86.33±7.08 A b		73.83±4.90 A a		63.17±3.89 A a		98.66±9.20 A a		84.33±5.60 A a	

Abbreviations: NEPF: Number of eggs per female; IR: Egg production inhibition rate compared to control; Diflu: Diflubenzuron; Pyri: Pyriproxyfen; SE: Standard Error.

[x] If the capital letters in a column are different, there is a statistical difference between the IGRs and doses on the same day ($p \leq 0.05$);

[y] If the lower-case letters in a column are the different, there is a statistical difference between populations ($p \leq 0.05$).

Table 4. The effect of pyriproxyfen and diflubenzuron on the transition from egg to adult stage

Insect Age (Days)	Chemical	Populations											
		Antalya		Bursa		Edirne		Gaziantep		İzmir		WHO	
		EATR±SE (%)	RR (%)	EATR±SE (%)	RR (%)	EATR±SE (%)	RR (%)	EATR±SE (%)	RR (%)	EATR±SE (%)	RR (%)	EATR±SE (%)	RR (%)
1	Diflu %5	8.42±2.81 A a	90.42	12.41±6.12 A a	85.84	-	-	1.93±0.86 A.a	97.10	0.00±0.00 A a	100	4.17±0.81 A a	92.52
	Diflu %10	0.00±0.00 A a	100	2.29±0.12 B a	97.39	-	-	0.77±0.72 A a	99.08	0.00±0.00 A a	100	-	-
	Pyri %5	8.33±2.83 A a	90.52	6.67±0.41 A a	92.39	-	-	2.85±1.20 A a	95.69	4.17±2.8 A a	95.15	8.00±0.00 A	91.03
	Pyri %10	6.67±0.00 BC a	92.41	3.87±1.36 AB a	95.58	-	-	10.50±1.05 C a	83.95	1.67±0.46 A a	98.06	-	-
	Control	87.87±2.25 B a		87.62±3.20 B a		84.29±3.25 B a		66.20±2.55 A a		86.00±5.26 B a		89.23±2.29 B a	
3	Diflu %5	4.00±0.00 A a	95.56	4.62±1.30 A a	94.24	6.67±0.00 A	92.28	25.60±1.56 B b	56.84	4.80±2.86 A a	94.67	8.88±0.50 A a	90.51
	Diflu %10	3.50±1.69 B a	96.11	0.00±0.00 A a	100	-	-	2.50±1.77 AB a	98.90	2.00±0.00 AB a	97.78	4.00±0.00 B a	96.21
	Pyri %5	6.25±2.31 A a	93.06	5.00±0.00 A a	93.97	-	-	4.44±1.44 A a	92.51	14.74±5.05 B a	83.62	-	-
	Pyri %10	0.00±0.00 A a	100	0.00±0.00 A a	100	-	-	4.00±1 89 B.a	93 26	5.56±1.41 B a	93.82	-	-
	Control	90.00±1.10 C a		82.86±5.80 B a		86.36±0.39 C a		59.31±3.54 A a		90.00±1.56 C a		87.80±2.20 C a	
5	Diflu %5	7.78±2.32 AB a	91.54	2.86±1.20 A a	96.68	9.1±4.33 B	89.82	0.00±0.00 A a	100	5.00±1.16 A a	94.60	0.00±0.00 A a	100
	Diflu %10	1.66±0.79 A a	98.20	6.86±2.79 A a	92.04	0.00±0.00 A a	100	5.60±1.66 A a	91.96	0.00±0.00 A a	100	0.00±0.00 A a	100
	Pyri %5	3.20±0.60 A a	96.52	3.63±1.13 A a	95.79	11.11±2.48 AB	87.57	22.66±6.73 B b	67.08	8.85±2.95 A ab	90.45	-	-
	Pyri %10	2.86±1.20 A a	96.89	1.82±1.36 A a	97.89	-	-	3.08±0.76 A b	95.33	4.50±4.59 A a	95.14	-	-
	Control	92.00±0.62 B a		86.15±3.41 B a		89.41±0.24 B a		68.87±2.64 A a		92.67±1.01 B a		88.40±0.28 B a	

Abbreviations: EATR: Egg to adult transition rate; RR: Egg to adult transition reduction rate; Diflu: Diflubenzuron; Pyri: Pyriproxyfen; SE: Standard Error.

[x] If the capital letters in a column are different, there is a statistical difference between the IGRs and doses on the same day ($p \leq 0.05$);

[y] If the lower-case letters in a column are the different, there is a statistical difference between populations ($p \leq 0.05$).

Effects of diflubenzuron and pyriproxyfen on adult emergence rates

The investigation into the egg-to-adult transition involved analyzing emergence rates, focusing on the inhibitory effects of diflubenzuron and pyriproxyfen. Our findings revealed that both substances significantly reduced the rates of emergence in all populations and age groups, with statistically significant reductions ranging from 56.84% to 100% compared to control groups. The analysis further indicated that the age of the insects and the dosage applied did not statistically impact the reduction in emergence rates to the adult stage across the studied populations.

Analysis of emergence rates from eggs of control group individuals revealed rates spanning from 59.31% to 92.67% across all populations. The Gaziantep population showed the lowest emergence rate, while the highest rates were found in both the Antalya and Izmir populations. In contrast, within the experimental groups treated with diflubenzuron and pyriproxyfen, emergence rates ranged markedly lower, from 0% to 25.60%. This substantial difference underscores that diflubenzuron and pyriproxyfen, across all concentrations and populations, averaged an efficacy in reducing the egg-to-adult emergence rate by over 80% (Table 4).

The eggs collected were assessed for their area indices by calculating the product of their lengths and widths. Our research identified statistically significant reductions in egg area index in 49 of the 72 experimental groups, encompassing six populations, two types of IGRs, two concentrations, and three age

groups. Meanwhile, no statistically significant difference in egg area index was noted in nine groups. In 14 of the test groups, egg yield did not occur, preventing the measurement of egg area index. The observed reduction in egg area index varied between 1% and 34%. Notably, this decrease in egg areas was found not to impact the rate of adult emergence.

For the field trial simulation, new custom cages measuring 120x120x120 cm were prepared, into which 100 male and 100 female adult house flies, confirmed as unmated, were released for a duration of one month to observe population development. The cages received consistent supplementation with a nutrient solution and active ingredients. To mimic breeding sites more accurately, milk-soaked wheat bran was layered over the existing nutrient medium. The outcomes of this trial, detailed in Table 5, showed that in cages treated with either diflubenzuron or pyriproxyfen, there was an approximate 90% decrease in population density relative to the control group. This significant reduction corroborates the findings from our laboratory experiments.

Table 5. Number of individuals in cages after diflubenzuron and pyriproxyfen application in field simulation trial

IGR	F_1	F_2	F_3	Chemosterilant effect
Control	>1000	>2000	>5000	
Diflubenzuron %10+sugar water	< 500	100-250	<100	Very high
Diflubenzuron %10	< 250	<100	0	Very high
Pyriproxyfen %10+sugar water	<1000	250-500	<250	Very high
Pyriproxyfen %10	<500	100-250	<100	Very high

In the experiments conducted, eggs collected from various populations were incubated in a nutrient medium to observe their development into adult house flies. Upon emergence, the adults were transferred to mesh cages provided with milk, water, and sugar to assess their egg-laying capabilities. When egg-laying was observed, the new set of eggs was again placed in the nutrient medium to monitor hatching rates.

A noteworthy observation from this study was the performance of eggs from 5-day-old individuals of the İzmir population treated with 5% pyriproxyfen. Out of 50 eggs incubated in the nutrient medium, 5 adults emerged, collectively producing 69 eggs. However, to precisely evaluate their hatching rates, these eggs were initially placed on milk-soaked cotton to observe larval emergence, resulting in none of the 69 eggs hatching. Additionally, in the WHO population, eggs from 1-day-old individuals treated with 5% diflubenzuron transitioned to the larval stage without the need for the nutrient medium but subsequently perished within a day. In the case of the Antalya population, it was recorded that individuals treated with 5% pyriproxyfen at 1 day old and those treated with 10% pyriproxyfen at 3 days old failed to lay eggs upon reaching adult form.

Discussion and Conclusion

Recent research has shown that house flies have evolved resistance to many insecticides that target the nervous system (Khan et al., 2013; Ma et al., 2017; Khan et al., 2017; Khan, 2019; Abbas & Hafez, 2023). This resistance has prompted a shift among scientists and practitioners towards alternative pest control methods, resulting in a resurgence of interest in utilizing IGRs as a viable and effective strategy in recent times. Our literature review has revealed that research on the chemosterilant effects of IGRs on insects has predominantly focused on a single insect population (Howard & Wall 1995a, b,1996a, b; Knapp & Cilek, 1988; Alam & Motoyoma, 2000; Caimi et al., 2002; Charmillot et al., 2002; Myers & Hull, 2003; Moya et al., 2010; Singh & Kaumar, 2015; Rhyne & Richards, 2020; Hasnain et al., 2023). In contrast, our study is distinctive in its approach, as it investigates house fly populations collected from five different provinces across four unique geographical regions of Türkiye. The inclusion of samples from such diverse regions broadens the geographical scope of this study to encompass five distinct areas. The insights gained from these varied regions are expected to offer valuable contributions to practical pest management.

The administration of diflubenzuron and pyriproxyfen at concentrations of 5% and 10% through food led to a statistically significant reduction in egg production compared to control groups across all studied populations and age categories. Notably, the Edirne, WHO, and Antalya populations exhibited significant declines in egg production, with rates of decrease surpassing those reported in prior research. Our review of the literature reveals that global studies in this domain have predominantly utilized trap-based methods to monitor population density, rather than direct egg counting, to evaluate the impact on egg production. Nonetheless, the outcomes of these international studies employing IGRs align with our findings, reinforcing the efficacy of IGRs in significantly reducing egg production among house fly populations.

Alam & Motoyoma (2000) explored the effects of cyromazine on house flies by incorporating concentrations of 500 and 1000 ppm into their drinking water and concluded that cyromazine did not significantly influence egg production, hatchability, pupal formation, or adult emergence. In contrast, our research demonstrated that diflubenzuron and pyriproxyfen markedly decreased both egg production and egg-to-adult transition rate. A key factor underlying this discrepancy is believed to be the dosages used; whereas Alam and Motoyoma administered cyromazine at 500 ppm (0.05%) and 1000 ppm (0.1%), our study employed considerably higher concentrations of 5% and 10%, respectively. Moreover, in our experiment, the IGRs were dissolved in a 40% sugar-water solution, diverging from Alam and Motoyoma's method of using tap water. This variation in solvent is also presumed to influence the attractiveness of house flies to the treated solutions.

In studies conducted by Myers & Hull (2003) on adult *Platynota idaeusalis* (Walker, 1859) (Lepidoptera: Tortricidae), significant inhibition of egg production and hatchability was observed when 90 ppm tebufenozide and 45 ppm methoxyfenozide were used as contact agents, with no notable difference in the chemosterilant effects between the two substances. This finding was echoed by Nisar et al. (2020), who treated adult *Bactrocera zonata* (Saunders, 1842) (Diptera: Tephritidae) with methoxyfenozide, fenoxycarb, lufenuron, pyriproxyfen, and buprofezin via food, with reductions in egg production by 37.5%, 34.8%, 30.9%, 25.1%, and 22.4%, respectively, and decreased egg hatchability of 65.9%, 67.3%, 67.8%, 72.2%, and 72.9%, respectively. They also observed decreases in sperm count of 29.4%, 25.8%, 22.2%, 17.6%, and 16.1% and in egg densities by 36.2%, 32.2%, 27.8%, 20.8%, and 19.6%. These findings corroborate our results, which also showed significant declines in egg production, egg size, and egg-to-adult transitions. Furthermore, Öz et al. (2024) examined the effects of diflubenzuron at 0.5%, 1%, and 2% concentrations on adult German cockroaches, *Blattella germanica* (L., 1767) (Blattodea: Ectobiidae) via both solid and liquid food, revealing decreased egg production and lower survival rates of nymphs from the treated eggs. This body of research supports the efficacy of IGRs in significantly affecting the reproduction and development of various insect species.

In research conducted by Hasnain et al. (2023) on the peach or melon fruit fly, *Bactrocera cucurbitae* (Coquillett, 1899) (Diptera: Tephritidae), egg production was analyzed using methodologies comparable to those in the present study. Exposure of *B. zonata* to concentrations ranging from 50 to 300 ppm/5 ml of pyriproxyfen, novaluron, lufenuron, buprofezin, and flubendiamide resulted in a 15% to 55% reduction in egg production compared to the control. In contrast, our study recorded inhibition rates ranging from 31% to 100% across various concentrations in four distinct populations. A major factor contributing to this discrepancy is the differing ecological roles of the target pests: *B. zonata* is primarily an agricultural pest, while house flies are considered public health pests. The use of chemical insecticides, such as fenoxycarb and related compounds, is substantially more widespread in agriculture than in public health pest management.

When evaluating the impact of diflubenzuron and pyriproxyfen concentrations on egg production across all studied populations, establishing a clear and statistically significant relationship between increasing concentrations and reduced egg output proved challenging. A review of the existing literature supports our findings, indicating that even a single exposure to insecticide can led to reduced egg production. For example,

Alam & Motoyama (2000) observed no concentration-dependent effect on egg production in house flies orally exposed to 500 and 1000 ppm of cyromazine. Similarly, Hasnain et al. (2023) reported no direct correlation between increasing concentrations of various IGRs (pyriproxyfen, lufenuron, novaluron, buprofezin, and flubendiamide) and reduced egg production in *B. zonata*; intriguingly, higher concentrations often resulted in more pronounced chemosterilant effects.

Conversely, some studies have indicated that the chemosterilant effects may intensify at higher concentrations. For instance, Charmillot et al. (2002) reported a reduction in egg production in *Cydia pomonella* L. (Lepidoptera: Tortricidae) with prolonged exposure to fenoxycarb and found that even female-only contact with the IGR was sufficient to suppress egg laying. Such discrepancies in concentration-related outcomes may be attributed to the species-specific responses observed in different studies. Given the limited number of investigations on the effects of orally administered IGRs on egg production, our review of the literature suggests that a straightforward correlation between increasing concentrations and inhibition of egg production cannot be universally established. This variability likely depends on both the species involved and the specific active ingredients used.

In a study by Singh & Kaumar (2015), virgin female *Sarcophaga ruficornis* (Fabricius, 1794) (Diptera: Sarcophagidae) were topically treated with pyriproxyfen at concentrations of 50 and 100 µg/5 µl to evaluate its reproductive effects and impact on the F_1 generation. The treatment resulted in increased adult mortality, a significant reduction in fertility, and a 90% decrease in larval transition rates. In the F_1 generation, elevated mortality, reduced pupariation, and an increase in deformed adult emergence were observed, with a positive correlation between concentration and morphological abnormalities. These findings highlight the complexity of the relationship between IGR concentration and biological effects, suggesting that outcomes can vary considerably depending on both the species and the specific insecticide used.

In our investigation of the effects of diflubenzuron and pyriproxyfen on insect egg-laying capacity, we carefully designed the experimental setup by placing Falcon tubes containing cotton soaked in a 40% sugar solution individually for each group to prevent mating prior to their introduction into the test cages. The study included 72 experimental sets, covering six populations, two IGR types, two concentrations, and three age groups. Among these, 13 sets recorded the highest egg production from 5-day-old insects, while 5 sets involving the same age group showed the lowest production. No egg production was observed in 4 sets, and the remaining 2 sets showed no statistically significant difference among insect ages. Delayed exposure (associated with increased insect age) correlated with higher egg yields in 5-day-old individuals. When analyzing egg yield inhibition rates relative to the control group, the highest levels were observed in experiments with insects aged 3, 1, and 5 days, in that order. This pattern likely reflects the biological cycle of house flies, which reach sexual maturity within 24-48 hours and typically begin oviposition 3-4 days after emergence. These results suggest that the potential for reproductive system damage diminishes with delayed exposure. Our findings are consistent with previous studies, including those by Knapp & Cilek (1998), Howard & Wall (1995b), Alam & Motoyama (2000), and Caimi et al. (2002), which collectively support the observed trends.

In this study, egg area indices were calculated by multiplying egg length by their maximum width. The effect of IGRs on reducing the egg area index was assessed by statistically comparing values from experimental groups to those of the control groups. Among the 72 experimental groups established (covering six populations, two IGR types, two concentrations, and three age groups) statistically significant reductions in egg area index were observed in 49 groups. In contrast, 9 groups showed no significant differences, and in 14 groups, no egg production was recorded, making it impossible to calculate the egg area index. The analysis indicated that neither IGR concentration, insect age, nor duration of exposure had a statistically significant effect on the egg area index. Although some trial sets showed egg area indices higher than those of the control group, these increases were not statistically significant.

Furthermore, when examining individuals that transitioned from the egg stage to the adult stage from the measured eggs, it was noted that smaller adults emerged from eggs with a lower egg area index. Nonetheless, the egg area index, whether low or high, did not affect the transition to the adult stage, indicating a similar inhibition rate across transitions to the adult stage. This study's findings contrast with those of other studies indicating a positive correlation between body size and egg production under normal conditions. For instance, Barnard et al. (1995) found that in female house flies at low population densities, a 1 mg increase in average pupal mass led to an estimated 10.6±1.5 additional eggs laid. This discrepancy highlights the complexity of understanding the effects of IGRs on insect development and reproduction and suggests that while IGRs can reduce egg production and modify egg size, these changes may not directly correlate with reproductive capacity or adult size under controlled conditions.

In our study, adult house flies were fed diflubenzuron and pyriproxyfen at concentrations of 5% and 10%, and the emergence of adults from the eggs they laid was monitored by seeding the eggs onto a diet of milk and Wheat bran. The emergence rates were then compared to those of the control groups to assess the transition from egg to adult stage. The results revealed statistically significant reductions in transition rates across all concentrations and age groups by the end of the experiments. When data from all trial sets were analyzed collectively, both IGRs were found to inhibit the transition from egg to adult by an average of over 90%, regardless of concentration or insect age. It was noted that the age of the insects and the duration of their exposure had no discernible impact on the inhibition of the transition. While the inhibition rates seemed to increase with higher concentrations, this trend was not statistically significant. Thus, administering adult house flies with food containing 5% or higher concentrations of diflubenzuron or pyriproxyfen led to a 90% reduction in the transition from egg to adult stage. Upon examining the milk and bran diet on which the eggs were laid, no unemerged pupae were found in the experimental groups, indicating that the chemosterilant effects primarily target the egg and larval stages. The suppression of pupation and adult emergence, especially at higher concentrations of JHA and CSI, suggests a transovarial effect, in which parental exposure to these IGRs adversely affects the developmental stages of their offspring, resulting in a marked disruption of population continuity.

The transovarial effects of novaluron, pyriproxyfen, azadirachtin, and buprofezin on adults of *Stephanitis pyrioides* (Scott, 1874) (Hemiptera: Tingidae) and *Teleonemia scrupulosa* Stål, 1873 (Hemiptera: Tingidae), as explored in studies by Joseph (2019; 2022), respectively, highlight the potential for IGRs to reduce offspring viability in insects significantly. These studies demonstrated that topical application of these substances could effectively decrease nymph emergence in a manner that is not concentration-dependent, indicating that even low concentrations can be as effective as the label concentration in producing transovarial effects.

An interesting aspect of IGR activity involves the behavior of insects such as house flies, which often deposit feces near their oviposition sites. This behavior may result in eggs and early larval stages being exposed to IGRs through fecal matter, potentially inhibiting egg hatching or adult emergence. Supporting this hypothesis, Ivei & Wright (1978) reported negligible levels of diflubenzuron in the eggs of treated *M. domestica* and *Stomoxys calcitrans* (L., 1758) (Diptera: Muscidae). In contrast, Medina et al. (2002) detected diflubenzuron in 20% of feces and 1% of eggs following topical application to *Chrysoperla carnea* Stephens, 1836) (Neuroptera: Chrysopidae). Additionally, Trostanetsky et al. (2015) observed inhibited egg hatching in *Tribolium castaneum* (Herbst, 1797) (Coleoptera: Tenebrionidae) after adults were treated with novaluron attributing the effect to the presence of the compound in feces. When the eggs were transferred to a novaluron-free environment, hatching rates returned to normal. Our research, which involved seeding eggs onto a sterile milk and bran diet, suggests that the transovarial transfer of IGRs may play a significant role in inhibiting adult emergence rather than direct contamination of eggs or larvae via feces. This hypothesis is supported by Casana-Giner et al. (1999), who investigated the chemosterilant effects of ten IGRs on Mediterranean fruit fly, *Ceratitis capitata* (Widemann, 1824) (Diptera: Tephritidae), demonstrating

that even a 1000 ppm concentration of lufenuron could completely inhibit egg hatching when administered to adult females, and similar sterilizing effects were also observed at varying concentrations and exposure durations across different IGRs and sexes. These findings collectively underscore the complex mechanisms by which IGRs can affect insect reproduction, suggesting that both direct exposure and environmental contamination via fecal matter can contribute to the observed reduction in offspring viability. Furthermore, they highlight the importance of considering both the method of IGR application and the behaviors of target insect species in pest management strategies.

To simulate field conditions, we designed large custom-made cages (120×120×120 cm) and introduced 100 virgin male and 100 virgin female *M. domestica* individuals into each cage. Throughout the month-long experiment, flies were provided with a consistent supply of food, water, and IGRs (diflubenzuron and pyriproxyfen). To mimic natural breeding conditions, a mixture of milk and bran was layered onto the existing diet. Our findings revealed a dramatic reduction (approximately 90%) in population density in treated cages compared to controls. This suppressive effect persisted in subsequent generations, with the F_2 population reduced by 90%, and near elimination observed by the F_3 generation. Although our trials were conducted in controlled settings, these results suggest strong chemosterilant potential in real-world applications as well. Supporting this, Howard and Wall (1996a) reported that traps containing a 50% sucrose, and 10% triflumuron solution achieved significant population suppression of house flies in Indian barns, reducing egg-to-adult emergence to below 1%. However, once traps were removed, fly populations quickly rebounded, highlighting the importance of sustained treatment in field environments. Taken together, our laboratory outcomes and previous field studies suggest that diflubenzuron and pyriproxyfen may be similarly effective in outdoor settings. We believe that further open-field evaluations are warranted to validate the long-term applicability of these IGRs in integrated house fly control programs.

Several studies have explored the chemosterilant potential of various IGRs against agricultural pests, highlighting differences in efficacy based on compound type, dosage, and delivery method. In a recent study on *B. cucurbitae*, Kainat et al. (2025) tested multiple IGRs, including lufenuron, pyriproxyfen, novaluron, buprofezin, and flubendiamide and found that lufenuron exhibited the highest efficacy, reducing fecundity by 68.4% and adult emergence by 70.97% at 300 ppm under laboratory conditions. Its performance was further validated in field trials using bait trap applications that led to substantial reductions in fruit fly damage. Similar success was reported in *C. capitata* control: Navarro-Llopis et al. (2004) achieved up to 80% population reduction in orchards using lufenuro n-based protein bait traps, and Alemany et al. (2008) documented over 63% reduction in female capture rates across a 300-hectare area with lufenuron traps. These findings emphasize not only the broader chemosterilant utility of IGRs but also position lufenuron as particularly effective among them. In line with this growing body of evidence, our study confirms that other IGRs, diflubenzuron and pyriproxyfen, also suppress reproductive parameters in *M. domestica*, supporting their potential role in integrated control strategies for synanthropic pests.

In conclusion, our findings clearly demonstrate the strong chemosterilant potential of diflubenzuron and pyriproxyfen against *M. domestica* under controlled laboratory conditions. Significant reductions were observed in egg production, egg area index, and the transition rate from egg to adult stage, with inhibition rates exceeding 90% across various concentrations, age groups, and populations. The consistency of these results across multiple experimental sets reinforces our confidence in the efficacy of these IGRs. While traditionally used as larvicides in both agricultural and public health settings, IGRs have recently attracted attention for their chemosterilant effects as well. To our knowledge, this is the first study in Türkiye to demonstrate the chemosterilant efficacy of these two IGRs specifically against house flies. Additionally, evidence pointing to transovarial effects and indirect exposure through fecal matter suggests that these compounds can disrupt population continuity beyond direct contact. While our cage system provided a reliable simulation of population dynamics, we acknowledge that the behavioral flexibility and ecological adaptability of house flies may be more pronounced in natural settings. Therefore, we believe that further open-field trials are needed to validate our results, which may pave the way for the broader application of IGRs in integrated house fly management programs.

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Original article (Orijinal araştırma)

Effect of *Trichoderma harzianum* Rifai and *Trichoderma viride* Pers. (Ascomycota: Hypocreales) on demographic parameters of *Myzus persicae* (Sulzer, 1776) (Hemiptera: Aphididae) feeding on bell pepper plant

Trichoderma harzianum Rifai ve *Trichoderma viride* Pers. (Ascomycota: Hypocreales)'nin dolmalık biber bitkileri üstünde beslenen *Myzus persicae* (Sulzer, 1776) (Hemiptera: Aphididae)'nin demografik parametreleri üzerine etkisi

Hilmi KARA[1*]

Abstract

In this study, the indirect effects of *Trichoderma harzianum* Rifai and *Trichoderma viride* Pers. (Ascomycota: Hypocreales) on *Myzus persicae* (Sulzer, 1776) (Hemiptera: Aphididae) via the bell pepper, *Capsicum annuum* L. (Solanales: Solanaceae) variety were determined using age and stage-specific two-sex life table. Cartesian product was used in the comparison tests. The study was conducted at Van Yüzüncü Yıl University, Faculty of Agriculture, Department of Plant Protection between May and July 2024. The differences in the intrinsic rate of increase (*r*), finite rate of increase (*λ*), and doubling time (*DT*) *Trichoderma* spp. treatments were statistically significant compared to the control. The intrinsic rate of increase for *M. persicae* on *T. harzianum*-treated plants (0.3321 d^{-1}) was significantly lower than on *T. viride* (0.3462 d^{-1}) and the mixture treatment (0.3583 d^{-1}). In conclusion, it was determined that both *Trichoderma* spp. negatively affected the fitness of *M. persicae* through the pepper plant, with *T. harzianum* being more effective than *T. viride*. Testing beneficial microorganisms in different plant-pest combinations in future studies will enhance the understanding of this mechanism and provide significant contributions to integrated pest management.

Keywords: Bell pepper, *Myzus persicae*, *Trichoderma harzianum*, *Trichoderma viride*, two-sex life table

Öz

Bu çalışmada, dolmalık biber, *Capsicum annuum* L. (Solanales: Solanaceae) çeşidi aracılığıyla *Trichoderma harzianum* Rifai ve *Trichoderma viride* Pers. (Ascomycota: Hypocreales)'nin *Myzus persicae* (Sulzer, 1776) (Hemiptera: Aphididae) üzerindeki dolaylı etkileri yaş ve döneme özgü iki eşeyli yaşam çizelgesi kullanılarak belirlenmiştir. Karşılaştırma testi olarak kartezyen çarpım kullanılmıştır. Çalışma, Van Yüzüncü Yıl Üniversitesi, Ziraat Fakültesi, Bitki Koruma Bölümü'nde 2024 yılı Mayıs-Temmuz ayları arasında yürütülmüştür. *Trichoderma* spp. uygulamalarında kalıtsal üreme yeteneği (*r*), üreme gücü sınırı (*λ*) ve popülasyonu ikiye katlama süresi (*DT*) parametreleri kontrole kıyasla istatistiksel olarak anlamlı bulunmuştur. *Trichoderma harzianum* ile muamele edilmiş bitkilerle beslenen *M. persicae* bireylerinin kalıtsal üreme yeteneği (0.3321 d^{-1}), diğer muamelelere, (*T. viride*, 0.3462 d^{-1} ve her iki *Trichoderma* spp. karışımı 0.3583 d^{-1}) kıyasla önemli ölçüde daha düşük olduğu tespit edilmiştir. Çalışmanın sonunda, her iki *Trichoderma* spp. de biber bitkisi aracılığıyla *M. persicae*'nin biyolojisi üzerinde olumsuz etkiye sahip olduğu ve *T. harzianum*'un *T. viride*'den daha etkili olduğu belirlenmiştir. İlerleyen çalışmalarda faydalı mikroorganizmaların farklı bitki ve zararlı kombinasyonlarında test edilmesi, bu mekanizmanın daha iyi anlaşılmasını sağlayarak entegre zararlı yönetimine önemli katkılar sunacaktır.

Anahtar sözcükler: Dolmalık biber, *Myzus persicae*, *Trichoderma harzianum*, *Trichoderma viride*, iki eşeyli yaşam tablosu

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Introduction

While plants grow and develop under the influence of suitable soil, water, nutrients and other environmental factors, they also interact with many organisms both above and below the ground. These interactions may be beneficial or harmful to their health and productivity. Plant interactions with below- and above-ground organisms have begun to be carefully monitored in recent years, with particular focus on some interactions that benefit plant health (Pieterse et al., 2014; Pineda et al., 2015). The importance of developing more natural, environmentally friendly, healthy, and cost-effective approaches in pest control has increased. In this context, in addition to increasing the use of natural enemies in pest control, the use of soil-borne microorganisms that reduce the performance of pests and attract natural enemies has made plants more tolerant to herbivorous insects feeding on them (Noman et al., 2020). There are various studies in which positive results were obtained by utilizing plant-pathogen-microorganism interaction in the control of some disease agents in plants (Adeleke & Babalola, 2021; Alınç et al., 2021; Grabka et al., 2022; Hagh-Doust et al., 2022). Likewise, studies have been conducted to test the changes in the development and reproduction values of herbivorous insects (Fernandez-Conradi et al., 2018; Grabka et al., 2022). Beneficial microorganisms such as endophytic fungi, mycorrhizae, rhizobia, and rhizobacteria, which are naturally present in the subsoil, are reported to be involved in plant defense against diseases and pests (Pieterse et al., 2014; Pineda et al., 2015; Wielkopolan & Obrępalska, 2016; Verma et al., 2019; Sheridan et al., 2023).

Trichoderma spp. (teleomorph *Hypocrea* spp.) (Ascomycota: Hypocreales) are microorganisms found in almost all natural environments, with some strains free-living and others showing close relationships with plants (Chaverri et al., 2003; Sheridan et al., 2023). *Trichoderma* spp. are considered safe and environmentally friendly biocontrol agents because they are non-pathogenic to plants and animals, do not produce harmful residues or secondary metabolites, and do not cause environmental pollution or resistance in target pathogens (Bardin et al., 2015). *Trichoderma* spp. provide important contributions to the plant in improving many parameters important for plant development as well as making nutrients in the soil useful to the plant through various mechanisms. It has been reported to improve parameters such as total soluble carbohydrate, protein content, total free amino acids in plants and to increase the mineral and nutrient content of the plant (Metwally, 2020). *Trichoderma* spp. are reported to stimulate induced systemic resistance (ISR) in plants and increase resistance to abiotic and biotic stress conditions (Harman, 2006; Verma et al., 2019; Macías-Rodríguez et al., 2020; Noman et al., 2020; Adeleke & Babalola, 2021).

Myzus persicae (Sulzer, 1776) (Hemiptera: Aphididae) (The green peach aphid) is a phytophagous pest that feeds on more than 400 host plant species belonging to over 40 families and is a vector for more than 100 plant viruses (Tang et al., 2019). In the control of this pest, which is frequently encountered in pepper cultivation, the frequency and irregularity of chemical applications due to the short reproductive period brings problems such as resistance development and significant ecological and economic losses are encountered (Bass et al., 2014). *Myzus persicae* is the most economically important aphid pest in the world due to its high host diversity, the damage mechanism it causes to the plant, its life cycle, its ability to spread rapidly, its vectoring of virus diseases and its ability to easily develop resistance to insecticides.

This study investigated the effects of pepper plant-mediated *Trichoderma* spp. on *M. persicae* populations. While studies on the reducing effects of *Trichoderma harzianum* Rifai on some plant-mediated phytophagous insect species are more frequently encountered, this study focused on the effects of *Trichoderma viride* Pers. alone and in combination with *T. harzianum* on *M. persicae*. Using the age-stage, two-sex life table, a powerful tool for evaluating quantitative data on the biology of organisms, the indirect plant-mediated effects of *Trichoderma* spp. and thus pest-plant-microorganism interactions were examined at the tritrophic level.

Materials and Methods

Trichoderma spp.

Trichoderma harzianum and *T. viride* were obtained from stock cultures in the Mycology Laboratory of Faculty of Agriculture (Van Yüzüncü Yıl University). Solutions containing $1x10^8$ spores/ml were prepared by hemocytometer from one-week-old colonies of these fungi on potato dextrose agar (PDA; Merck Ltd., Darmstadt, Germany). The roots of the pepper seedlings at the planting stage were placed in the prepared solutions using the deep method, providing an environment for the microorganisms to settle into the roots.

Pepper plants

Bell pepper plants of Kasırga F1 variety (Anamas Seed, Antalya) were used in the experiment. The pepper seedlings were planted in 4 liter pots (20 cm diameter, 25 cm height) in a mixture of peat (Klassman TS1): perlite (local producer) (2:1). At the start of the experiment, 45 days were allowed for vegetative growth in order to reach the appropriate plant size.

Myzus persicae

Individuals obtained from the *M. persicae* population produced on pepper plants for one winter season in the laboratory of the Plant Protection Department (Van Yüzüncü Yıl University) to be used in the experiment were produced on the pepper plants in the study for at least 2 generations. Then, newly mature individuals from this culture were taken into the plastic clips and the experiment was started with the nymphs of the first stage that these adults had just left. The climate chambers used for insect production were set at 25±1°C, 65± 5% relative humidity and 16:8 light: dark photoperiod.

Life table study

The experiment was started by taking the nymphs of the first stage, which were born alive from the new adult individuals, into the clips with a diameter of 2 cm and a height of 2 cm, which were attached to the pepper leaves with metal pliers, one end of which was covered with gauze, the mouth part of which was in contact with the leaf and could be compressed with the other arm of the metal pliers from underneath. A total of 4 groups were created in the study: control, *T. harzianum*, *T. viride*, and a mixture application consisting of both *Trichoderma* species. The experiment was initiated with 40 replicates per treatment, resulting in a total of 160 individuals (40 individuals per group). Each clip was checked daily, and the individuals were observed one by one to monitor and record their survival and developmental processes. From the observation of the first juvenile to the death of the last adult individual, the process was conducted in climate chambers adjusted to 65±5% RH, 25±2°C and 16:8 light:dark periods. The study was conducted in controlled laboratory conditions at Department of Plant Protection, Faculty of Agriculture (Van Yüzüncü Yıl University), between May and July 2024.

Statistical analysis

Life table parameters were analyzed using the age-stage, two-sex life table theory (Chi & Liu, 1985; Chi, 1988) and calculated with the Two-Sex MSChart software (Chi, 2024a). From the raw experimental data, we derived key life table metrics, including the intrinsic rate of increase (r), finite rate of increase (λ), net reproductive rate (R_0), mean generation time (T), and doubling time (DT), along with relevant biological parameters. [age specific survival rate (l_x), fecundity (m_x), age-stage specific survival rate (s_{xj}; age, x; stage, j), life expectancy (e_{xj}), and reproductive value (v_{xj})] were calculated (Goodman, 1982; Chi & Su, 2006; Tuan et al., 2014).

The Cartesian product was used to compare the biological parameters gathered from the experiments. The Cartesian product ensures that all possible combinations of life table parameters are considered, enhancing the robustness of demographic analyses (Chi et al., 2022). By generating all possible combinations

of individuals from different cohorts, it provides comprehensive coverage of sampling probabilities, thus allowing every possible difference between groups to be captured (Chi et al., 2022). The variances and standard errors (SE) of the stage development time, reproductive traits, population parameters, and stage predation rate were estimated by the bootstrap resampling method with 2,000 resampling (B = 2,000) (Tibshirani & Efron, 1993; Huang & Chi, 2012). The Cartesian product analysis was applied to all possible combinations of life table parameters. The bootstrap resampling method (B = 2,000) was used separately to estimate variances and standard errors, and the Cartesian product was subsequently applied to these bootstrapped estimates to compare parameter differences comprehensively.

Cartesian products can be used to include all possible paired comparisons between two parameters (r, λ, R_0 e.g.) (Chi et al., 2022). The Cartesian product of two sets, e.g., rA and rB, is the set consisting of all ordered pairs whose first element belongs to rA and whose second element belongs to rB (Chartrand et al., 2008). It can be expressed as:

$$r,A \times r,B = \{(r,Ax, r,By): r,Ax \in rA \text{ and } rB,y \in rB \}$$

The differences between all pairs of the Cartesian products (CPT:4,000,000) will definitely include all possible differences of the bootstrap results of the two parameters. To determine the statistical differences among the groups in this study, pairwise comparisons of group means were performed using the Cartesian product method. All graphics were created using SigmaPlot 12.0 (Systat Software Inc., San Jose, CA, USA).

Population projection

Population growth was projected based on life table data encompassing development, survival, and fecundity, using the TIMING-MSChart software (Chi, 2024b). To better understand the temporal variations in population growth differences of *M. persicae* subjected to different treatments, an initial population of 10 newly laid nymphs was selected for observation, and their population increase was assessed over a 60-day period.

Results

Development duration, survival, longevity and fecundity of *M. persicae*

When the developmental periods of the pest were examined, it was seen that *M. persicae* feeding on *T. harzianum*-treated plants became adults in a longer period than the control and other treatments (p<0.05) (Table 1).

Table 1. Effects of pepper-mediated *Trichoderma* spp. treated on developmental times, oviposition days, fecundity and survival rate of *Myzus persicae* (mean ± SE)

	Control		*T. harzianum*		*T. viride*		Mixture	
	n	Mean±SE*	n	Mean±SE	n	Mean±SE	n	Mean±SE
N1 (d)	40	1.12±0.05b	40	1.32±0.08a	40	1.38±0.08a	40	1.45±0.08a
N2 (d)	40	1.75±0.09a	40	1.55±0.09ab	40	1.62±0.08ab	40	1.43±0.08b
N3 (d)	40	1.57±0.08a	40	1.55±0.09a	40	1.55±0.09a	40	1.38±0.10a
N4 (d)	40	1.82±0.08b	40	2.42±0.12a	40	1.93±0.10b	40	2.02±0.11b
Preadult duration (d)	40	6.28±0.09b	40	6.85±0.12a	40	6.47±0.13b	40	6.28±0.14b
Total longevity (d)	40	29.43±1.03b	40	33.0±0.72a	40	32.75±0.94a	40	31.7±0.74ab
TPRP (d)	40	6.28±0.09b	40	6.85±0.12a	40	6.47±0.13b	40	6.28±0.14b
F (nymph/female)	40	70.67±2.39a	40	62.35±2.38b	40	65.62±2.35ab	40	65.88±2.18ab
Oviposition days (d)	40	18.32±0.72a	40	18.12±0.46a	40	19.12±0.70a	40	18.77±0.59a
Survival rate (%)	40	100	40	100	40	100	40	100

* Values in each row followed by the same letter are not significantly different from each other at the 0.05 level, based on pairwise comparisons of group means conducted using the Cartesian product approach.

Total longevity values of *M. persicae* individuals fed on *T. harzianum* and *T. viride* treated plants were longer than the control. *Trichoderma* spp. treatment did not have any effects on the survival rate and number of oviposition days of *M. persicae*. However, fecundity value of individuals feeding on *T. harzianum*-treated plants was lower than the control (p<0.05). The longest total pre-reproduction period (TPRP) (6.85 d) was observed in *T. harzianum*-treated plants, while the difference between the other treatments and the control was insignificant.

Age-stage-specific survival rates of different treatments are presented in Figure 1. Age-stage survival rate plots (s_{xj}) show the survival rate of a newborn nymph at age x and period j and the transition between stages. In the study, the images of the curves overlap in the sense that all of the populations of different treatments reached the adult stage and started to die after living for a certain period of time. The first deaths were observed in the control from the 14th day onwards, while the deaths in *T. harzianum* and *T. viride* treated plants started from the 25th day onwards (Figure 1).

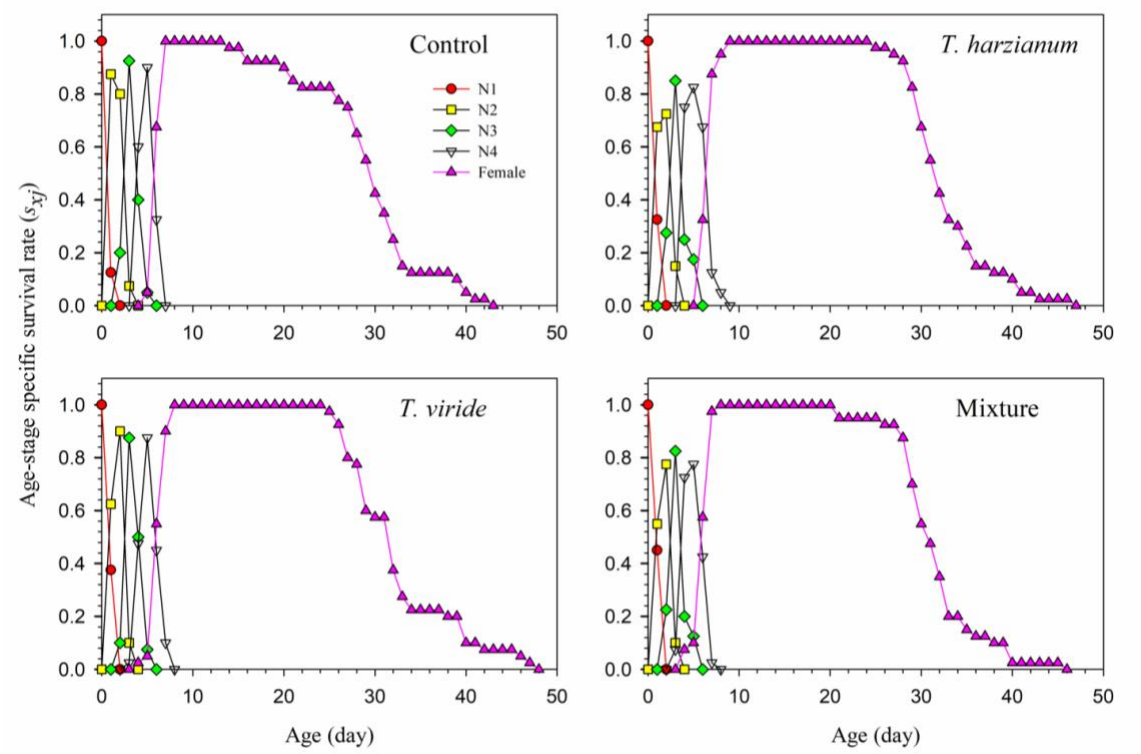

Figure 1. Age- stage specific survival rate of *Myzus persicae* feeding on *Trichoderma* spp.-treated pepper plants.

Age-specific survival rate (l_x), age-specific fecundity (m_x) and age-specific maternity (l_xm_x) of *M. persicae* in different treatments are presented in Figure 2. The age-specific survival rate (l_x) is a graph showing the overall survival rate without taking into account differences in age. In general appearance, it can be seen that the survival rate in each graph continued for a long time without mortality, with the first dramatic decline occurring in the control condition (day 26), followed by *T. viride* (day 27), mixture (day 29), *T. harzianum* (day 30), respectively (Figure 2). The highest fecundity (m_x) and maternity (l_xm_x) were observed in individuals fed on the control plant, while the lowest values were recorded on the plant with mixture treatment (Figure 2).

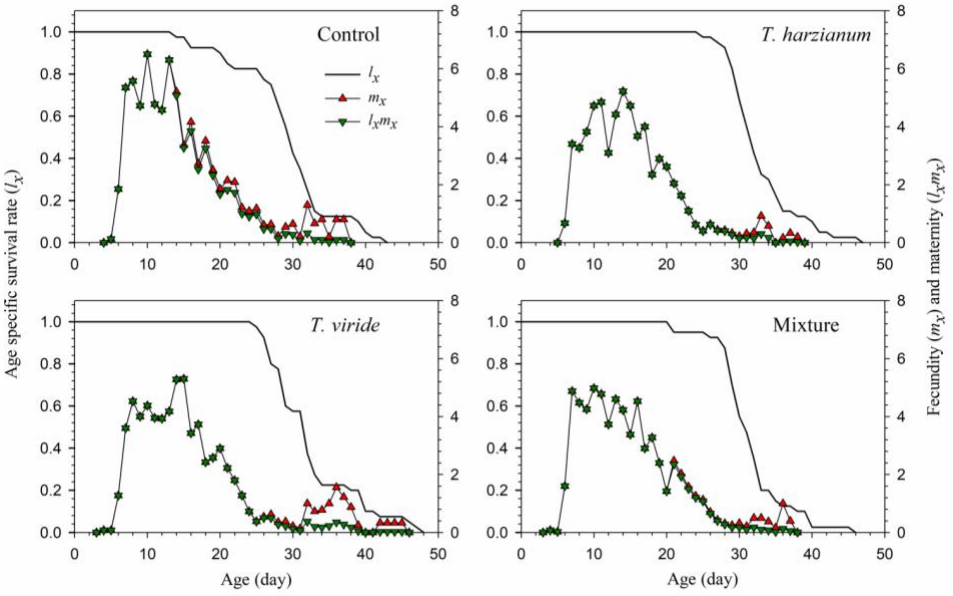

Figure 2. Age-specific survival rate (*lx*), fecundity (*mx*) and maternity (*lxmx*) of *Myzus persicae* feeding on *Trichoderma* spp.-treated pepper plants.

Life expectancy (e_{xj}) and reproductive value (v_{xj})

The life expectancy graph of *M. persicae* shows the total life expectancy of an individual at age x and period j as a result of different treatments. Life expectancy graphs for different treatments are given in Figure 3. Accordingly, the longest expected life span was recorded in individuals feeding on *T. harzianum*-treated plants (33 days), while the shortest was recorded in individuals feeding on control plants (29.43 days) (Figure 3). Age-stage-specific reproductive value indicates the future contribution of an individual at age x and stage j to population growth (Figure 4). The highest reproductive value was observed in the control plant (16.85 at age 7 d), while the other treatments were mixture (15.14 at age 7 d), *T. harzianum* (15.13 at age 10 d), *T. viride* (14.20 at age 7 d) (Figure 4).

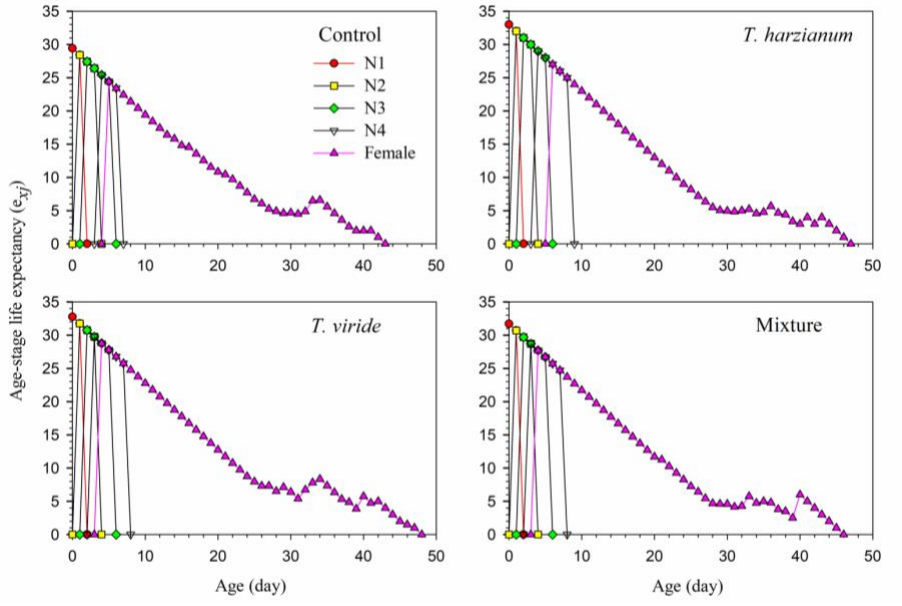

Figure 3. Age-stage life expectancy (exj) of *Myzus persicae* feeding on *Trichoderma* spp.-treated pepper plants.

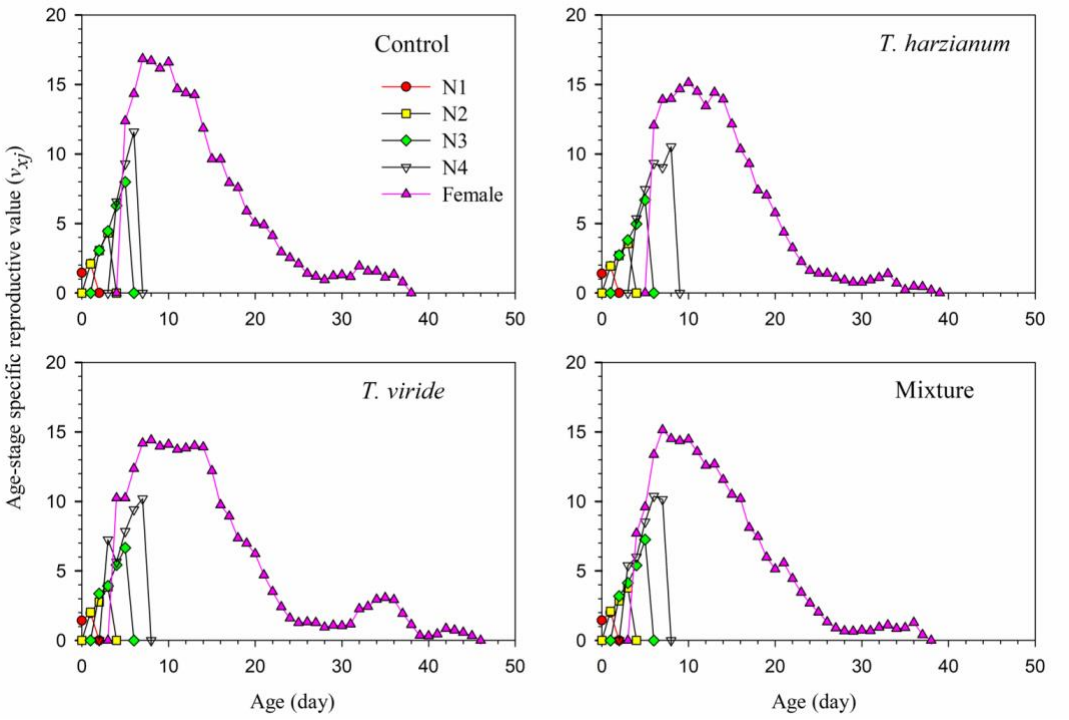

Figure 4. Age-stage specific reproductive value (vxj) of *Myzus persicae* feeding on *Trichoderma* spp.-treated pepper plants.

Life table parameters

Age-stage, two-sex life table parameters of *M. persicae* on different treatments are shown in Table 2. Based on the findings, population growth parameters (R_0, r, λ and T) were affected significantly by the *Trichoderma* spp. treatments. The highest values of r, λ and R_0 were observed in *T. harzianum*-treated plant populations compared to the control. The lowest mean generation time (T) was observed in the populations of *T. harzianum*-treated plants in the same direction compared to the control. Doubling time (DT) is the time required for the population to double. This value was highest in the plant treated with *T. harzianum* (2.09 d) and lowest in the control (1.86 d) (Table 2).

Table 2. Population parameters of *Myzus persicae* feeding on pepper plants treated with *Trichoderma* spp., (mean ± SE)

	Control		*T. harzianum*		*T. viride*		Mixture	
	n	Mean±SE*	n	Mean±SE	n	Mean±SE	n	Mean±SE
r (d^{-1})	40	0.37±0.00a	40	0.33±0.01c	40	0.35±0.00b	40	0.36±0.01b
λ (d^{-1})	40	1.45±0.01a	40	1.39±0.01c	40	1.41±0.01b	40	1.43±0.01b
R_0 (offspring)	40	70.68±2.35a	40	62.35±2.35b	40	65.63±2.27ab	40	65.88±2.17ab
T (d)	40	11.41±0.12c	40	12.45±0.16a	40	12.09±0.17ab	40	11.69±0.15bc
DT (d)	40	1.86c	40	2.09a	40	2.00b	40	1.93b

r: intrinsic rate of increase, λ: the finite rate of increase, R_0:net reproductive rate, T: mean generation time, DT: doubling time.

* Values in each row followed by the same letter are not significantly different from each other at the 0.05 level, based on pairwise comparisons of group means conducted using the Cartesian product approach.

Population projection of *M. persicae*

The dynamics of the predicted population of aphids in different treatments at the end of 60 days and their stage differences are given in Figure 5. At the end of 60 days of the analysis started with the same initial population (ten newly laid nymphs), the highest population growth was observed in the control (11,107,273,232 individuals, all stage) and the lowest in the *T. harzianum*-treated plants (1,530,995,430 individuals, all stage) (Table 3).

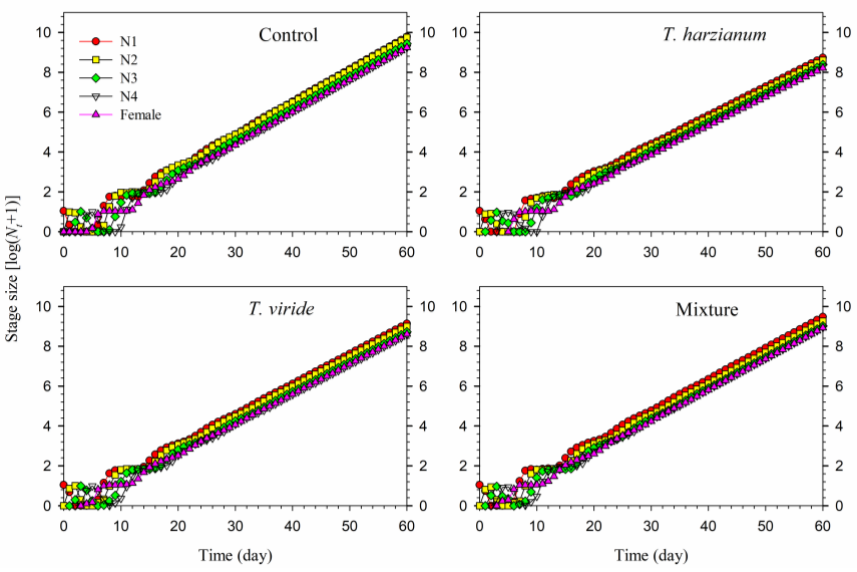

Figure 5. Population projection of *Myzus persicae* feeding on *Trichoderma* spp.-treated pepper plants.

Table 3. Population projection of *Myzus persicae* feeding on pepper plants treated with *Trichoderma* spp. on the 60th day

	N1	N2	N3	N4	Female	Total
Control	6,055,008,393	5,591,221,681	2,739,649,712	1,714,992,982	1,753,492,959	11,107,273,232
T. harzianum	532,846,554	394,917,186	240,485,761	200,850,483	161,895,446	1,530,995,430
T. viride	1,343,813,715	983,771,821	528,022,324	369,695,632	401,793,847	3,627,097,339
Mixture	1,970,587,364	995,850,546	134,590,641	854,775,348	836,515,407	4,792,319,309

Discussion

The green peach aphid, *M. persicae*, is an important polyphagous pest that needs to be controlled in agricultural areas. In the last century, chemical insecticides have been used predominantly to control agricultural pests. However, intensive and irregular use of these substances for a long time leads to the development of resistance in many insect species (Bras et al., 2022) and causes environmental and health problems (Mottet et al., 2024). This study is designed to develop more environmentally friendly and innovative solutions to deal with these problems.

In recent years, the effects of using beneficial microorganisms as plant-mediated biocontrol agents against herbivorous insects have been frequently investigated (Pieterse et al., 2014; Pineda et al., 2015; Wielkopolan & Obrępalska, 2016; Verma et al., 2019; Shafiei et al., 2024). Among the beneficial microorganisms, *Trichoderma* spp. is one of the important microorganisms with high success in the control against harmful insects with its direct and indirect mechanism of action (Poveda, 2021; Islam et al., 2022). Among these organisms, a large body of research has focused on the successful effects of *T. harzianum* in particular. In this study, the biological and life table parameters of the insect were investigated in detail by analyzing the survival and reproduction data of *Myzus persicae* feeding on pepper plants treated with *T. viride* as well as *T. harzianum* with two-sex life table program. It was observed that all of the insects in all treatments passed to the adult stage, and that there was a hundred percent survival rate. The growth period of *M. persicae* individuals fed on the plants whose roots were treated with *T. harzianum* was longer than the other treatments and the control. From this result, it is understood that the pest spends more time to complete the feeding it needs to become an adult. Similarly, there are studies in which prolongations were determined in the development periods of herbivorous insects feeding on *Trichoderma* spp. plant-mediated (Ağırtmış, 2021; Gültekin, 2022; Rişvanlı, 2022). It was reported that *Tuta absoluta* feeding on tomato plants treated with arbuscular mycorrhizal fungi (AMF), another beneficial microorganism with similar effects on the plant, had a longer developmental period compared to the control (Shafiei et al., 2024). The total

longevity of aphids feeding on *T. harzianum*-treated plants was longer than the control, while the decrease in the number of offspring left by adult individuals was found to be statistically significant compared to the control plant. The fecundity values of aphids feeding on *T. viride* treated plants were found insignificant compared to the control. In the plants where both *Trichoderma* sp. were applied together, it was observed that the difference in the fecundity value was insignificant compared to the control. In previous studies, Ağırtmış (2021) reported that the difference in the fecundity value of *M. persicae* individuals feeding on *T. harzianum*-treated hot long pepper was statistically significant compared to the control. Rişvanlı (2022) determined that the fecundity values of *S. exigua* feeding on potato and cotton plants treated with *T. harzianum* were lower than the control and the difference was statistically significant. Shafei et. al. (2024) reported that total longevity of *Tuta absoluta* feeding on AMF treated tomato plants was longer than the control, while fecundity was lower than the control and the difference was statistically significant. These results indicate that the pests can live longer on the plant but have a lower fecundity value, which may be advantageous in terms of providing nutrients to natural enemies. In this study, the increase in the total preoviposition period (TPRP) of *Myzus persicae* following the application of *T. harzianum* was consistent with previous findings (Gültekin, 2022; Osmanoğlu, 2022). An extended TPRP suggests a reduction in the number of generations produced annually and a decline in fecundity compared to the control, ultimately indicating a potential decrease in population size.

The results of this study showed that pepper-mediated feeding on *T. harzianum* and *T. viride* significantly affect developmental time, survival rate, fecundity and, consequently, population parameters of *Myzus persicae*. It was observed that *M. persicae* individuals fed on pepper plants treated with *T. harzianum* constituted the most unfavorable plant in terms of nutrition, with the lowest life table parameters of the intrinsic rate of increase, finite rate of increase, net reproductive rate, and the highest mean generation time. Although the life table parameter results of *T. viride* application were lower than those of *T. harzianum* application, the difference between them compared to the control was found to be statistically significant. There are studies in which different species of *Trichoderma* spp. were tested for various plants and pests and different results were obtained (Gültekin, 2022; Osmanoğlu, 2022; Rişvanlı, 2022). Doubling time refers to the time required for the population to double. From the results obtained, it was understood that *Trichoderma* spp. applications were a significant reducing factor in the population growth of *M. persicae*. Similar results were obtained for the *T. absoluta* pest, which feeds on tomatoes with beneficial microorganisms settling on their roots, and it became a reducing factor in the increase of the population (Shafiei et al., 2024).

Population projection analysis is a program that simulates how much the population will grow in the future, based on basic life table parameters. It was recorded that there was a 7.3-fold difference between the aphids feeding on the control plants and the populations feeding on the plants treated with *T harzianum*. Likewise, other treatments resulted in 2.4 and 3.1-fold lower population numbers for *T. viride* and mixture, respectively. At the end of the research, it was determined that *T. viride* also affected the biology of *M. persicae* significantly, although *T. harzianum* was more prevalent. In the 60-day population size estimates alone, while *M. persicae* formed a population of 11 billion individuals in the control group, a population of 1.5 billion in *T. harzianum*-treated plants and a population of 3.6 billion in *T. viride*-treated plants are quite impressive results. These results have highlighted the necessity of addressing the effectiveness of beneficial microorganisms not only in plant health and productivity but also in pest management, and the need to consider such trophic relationships in a multidimensional manner.

In order to determine in detail, the mechanism of the plant-insect-microorganism multitrophic interaction tested here, different interactions need to be examined. Furthermore, in order to reveal this mechanism more comprehensively, investigating the changes in the amounts of enzymes and hormones of plants and insects at the molecular level will provide a more in-depth scientific perspective.

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Original article (Orijinal araştırma)

Optimization of a *Bacillus*-based bioproduct for *Meloidogyne incognita* (Kofoid & White, 1919) Chitwood, 1949 (Tylenchida: Meloidogynidae) control using response surface methodology[1]

Meloidogyne incognita (Kofoid & White, 1919) Chitwood, 1949 (Tylenchida: Meloidogynidae) kontrolü için *Bacillus* bazlı bir biyoürünün yanıt yüzeyi metodolojisi kullanılarak optimizasyonu

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Abstract

Root-knot nematodes (RKNs) cause significant yield losses in agriculture. Environmentally friendly bioproducts are important components of sustainable nematode management. This study evaluated the efficacy of two commercial *Bacillus* bioproducts, *Bacillus amyloliquefaciens* MBI 600 (Bioproduct-I) and *Bacillus subtilis* QST 713 (Bioproduct-II), against *Meloidogyne incognita* (Kofoid & White, 1919) Chitwood, 1949 (Tylenchida: Meloidogynidae), a major RKN species, on tomato plants. The trial, conducted in 2024 at Bolu Abant İzzet Baysal University, Faculty of Agriculture, Department of Plant Protection, assessed root gall index, number of second-stage juveniles (J2) and number of egg masses in pot experiments at seed and seedling stages. The dose of Bioproduct-I was optimized using response surface methodology (RSM) and design of experiments (DOE), with results visualized using Pareto and normal plots. The 1000 ml/ha dose of Bioproduct-I was characterized by the lowest root gall index (2.37 in seed, 2.75 in seedling) and the lowest number of J2 (382.5 in seed, 415.0 in seedling). However, higher doses showed reduced efficacy, indicating that increasing concentrations did not increase biological activity. This study highlights the potential of *Bacillus* spp. for biological control and demonstrates the usefulness of statistical tools in optimizing Bioproduct applications against RKN.

Keywords: *Bacillus*, bioproduct, response surface method (RSM), root-knot nematode, tomato

Öz

Kök-ur nematodları tarımda önemli verim kayıplarına yol açmaktadır. Çevre dostu biyolojik ürünler, sürdürülebilir nematod yönetiminde önemli bir rol oynamaktadır. Bu çalışmada, iki ticari *Bacillus* tabanlı biyolojik ürünün, *Bacillus amyloliquefaciens* MBI 600 (Bioproduct-I) ve *Bacillus subtilis* QST 713 (Bioproduct-II), domates bitkilerinde önemli bir kök-ur nematodu türü olan *Meloidogyne incognita*'ya (Kofoid & White, 1919) Chitwood, 1949 (Tylenchida: Meloidogynidae) karşı etkinliği değerlendirilmiştir. Bolu Abant İzzet Baysal Üniversitesi, Ziraat Fakültesi, Bitki Koruma Bölümü'nde 2024 yılında yapılan denemelerde, tohum ve fide aşamalarındaki saksı deneylerinde kök ur indeksi, ikinci dönem larva (J2) sayısı ve yumurta paketi sayısı değerlendirilmiştir. Bioproduct-I dozu, yanıt yüzeyi metodolojisi (YYM) ve deney tasarımı (DT) kullanılarak optimize edilmiş ve sonuçlar Pareto ve normal plot grafikleri ile görselleştirilmiştir. Bioproduct-I'in 1000 ml/ha dozu, en düşük kök gal indeksi (tohumda 2.37, fidede 2.75) ve en düşük ikinci dönem larva (J2) sayısı (tohumda 382.5, fidede 415.0) ile belirlenmiştir. Daha yüksek dozlar, biyolojik aktiviteyi artırmamış, daha düşük etkinlik göstermiştir. Bu çalışma, *Bacillus* spp. türlerinin biyolojik mücadele potansiyelini ve kök-ur nematodlarına karşı biyolojik ürün uygulamalarının optimize edilmesinde istatistiksel araçların yararlılığını ortaya koymaktadır.

Anahtar sözcükler: *Bacillus*, biyolojik ürün, yanıt yüzey metodu (YYM), kök-ur nematodu, domates

[1] This study is derived from the MSc thesis of the first author.

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Introduction

Nematodes are found in every ecosystem and are one of the most common in animal taxa being thought to number over 250.000 species (Mitreva et al., 2005). In addition, over 4100 species of nematodes have been identified that are associated with plants (Decraemer et al., 2006). Plant-parasitic nematodes (PPNs) are one of the most destructive soil-borne pathogens, causing devastating economic losses in agriculture (Imren et al., 2017; Pires et al., 2022). They cause significant losses to agriculture worldwide, estimated at more than $80 billion per year (Nicol et al., 2011; İmren et al., 2019; Abd-Elgawad, 2024).

Among PPNs, the most important nematodes are the root-knot nematodes (RKNs; *Meloidogyne* spp.), which are responsible for most of the major agricultural losses worldwide (Elling, 2013). RKNs are found in almost all regions of the world and parasitize all vascular plants in greenhouses and in the field. They are among the most economically important nematodes at the genus level, as there are approximately 100 species in the genus *Meloidogyne* (Sikandar et al., 2020). *Meloidogyne incognita* (Kofoid & White, 1919) Chitwood, 1949, *Meloidogyne arenaria* (Neal, 1889) Chitwood, 1949, *Meloidogyne javanica* (Treub, 1885) Chitwood, 1949 and *Meloidogyne hapla* Chitwood, 1949 (Tylenchida: Meloidogynidae) are the most common species of RKNs (Wesemael et al., 2011; Jones et al., 2013; Coyne et al., 2018).

Meloidogyne incognita is the most important *Meloidogyne* species due to its economic importance. Although common in the tropics, it is typically restricted to greenhouses in temperate regions (Karssen & Moens 2006). *Meloidogyne incognita* placed in as eggs or second-stage juveniles (J2) in the soil, and J2s infect roots and creates feeding sites. This results in root gall and reduced water and nutrient uptake, resulting in loss of quality and yield (Talwana et al., 2016). Yield losses due to *M. incognita* infection range from 20% to 60% in tomato (Li et al., 2020).

Current RKN control practices are predominantly based on chemical nematicides. Unfortunately, indiscriminate use of these chemicals and prolonged exposure lead to critical adverse effects on both human health and the environment (Riga, 2011). Therefore, it became essential to develop alternative means using a multi-pronged and environmentally friendly strategy, such as biological control strategies, to reduce nematode damage without causing previous adverse effects (Singh & Mathur 2010).

Biological control of PPNs is a promising area for nematode control in sustainable agriculture. To date, rhizosphere microorganisms have been identified as very good biological control agents for PPN management (Ye et al., 2022; Shi et al., 2024). Among antagonistic bacteria, *Bacillus* spp., have been shown to be effective against RKNs due to their ability to rapid colonize the rhizosphere and direct the nematicidal production of secondary metabolites (Tian et al., 2022; Bhat et al., 2023). The bionematicidal activity of *Bacillus* is based on its ability to induce a systemic plant defense mechanism of nematode resistance. Proteases, chitinases, antibiotics, crystalline proteins and several secondary metabolites are formed by *Bacillus* species to perform this function (Engelbrecht et al., 2018).

Design of experiments (DOE) is one of the main tools used in plant biology, providing plant scientists with a systematic method to study various aspects of plant biology, plant ecology, and even plant agronomy (Timmusk et al., 2017). DOE provides an organized means of initiating and conducting research on plants and the environment in terms of growth, development, and other responses. The use of modern DOE techniques, such as factorial design, response surface methodology (RSM), and Taguchi techniques, provides further information on plant physiology and plant adaptation (Swain et al., 2021). RSM is a statistical technique that uses designed experiments to analyze the behavior of complex systems (Nwabueze, 2010). This method addresses the problem of parameter optimization in a variety of processes driven by more than one input variable, which can be optimized through a series of statistically validated model estimates (Baçaoui et al., 2001). RSM provides an efficient approach to optimizing parameters by approximating relationships using a quadratic surface. In addition, RSM helps us to analyze the interaction between several parameters (Azargohar & Dalai, 2005).

Although many studies have been conducted on the efficacy of bioproducts against nematodes, there are very few comparative studies on the efficacy and dose optimization of *Bacillus*-based bioproducts against the root-knot nematode *M. incognita*. In this context, dose optimization comparisons with RSM are an important tool to better understand the efficacy of bioproduct applications and to determine the most efficient treatment conditions. Therefore, the aim of this study was to investigate the effects of *Bacillus*-based bioproducts on *M. incognita* and to optimize bioproduct dosages using RSM. In this way, the efficacy of bioproducts can be increased and the most appropriate application conditions can be determined.

Materials and Methods

Nematode population

The RKN species *M. incognita* was used for the experiment. RKN pure cultures were established from single egg masses on tomato cultivar in a growth room (25±2ºC, > 60% humidity) at the Bolu Abant Baysal University. Infected roots were carefully cleaned to remove soil particles. Egg masses from the infected plants were collected with care and immersed in distilled water and then placed in a BOD incubator at 28±2°C to obtain the J2. The juvenile suspension was calibrated to a final concentration of 100 juveniles per milliliter of distilled water.

Bioproduct and nematicides

The following active ingredients were used in the study: Abamectin 20 g/l, abbreviated as 'Abamectin'; Bioproduct-I containing *Bacillus amyloliquefaciens* MBI 600; and Bioproduct-II containing *Bacillus subtilis* QST 713. The commercial bioproducts and nematicides used in the study are listed in Table 1.

Table 1. Bioproducts and nematicides used in the experiment

Code	Active substance	Trade name of product	Company
Nematicide	Abamectin 20 g/l	TERVIGO 20 SC	Syngenta Agriculture Ind. & Trade JSC
Bioproduct-I	*Bacillus amyloliquefaciens* MBI 600	SERIFEL WP	BASF Türk Chemistry Ind. Ltd. Co.
Bioproduct-II	*Bacillus subtilis* QST 713	SERENADE SC	Bayer Türk Chemistry Ind. Ltd. Co.

Experimental design and set up

The experiment was established as a completely randomized design, including six treatments with four replications each. All experiments were performed twice to ensure consistency and reliability of results. The experiments were conducted in 500 cc plastic pots. The soil mixture, composed of 75% sand and 25% peat, was sterilized in an autoclave at 121°C. Three-week-old susceptible tomato seedlings were planted in each pot for every treatment.

In the study of investigating the effect of some bioproducts on the reproduction of the RKN species *M. incognita*, the treatments were applied at different times to assess their effect on various stages of the nematode's life cycle (Table 2). The J2s of RKN were inoculated into two wells near the roots immediately prior to treatments on the planting days for treatment code A at a rate of 1000 J2 per pot using a 5 ml micropipette according to the application times listed in (Table 2). Simultaneously with the J2, 10 g of infected roots were embedded within the soil in each pot.

Table 2. Application code timing

Code	Time
A	at seeding or transplanting by drench
B	28 days after at seeding or transplanting by drench

The commercial bioproducts and nematicides were applied at the recommended doses, considering that tomato seedlings must be planted at a density of 1500 plants per decare. After application, the pots were irrigated to enhance the effect. Table 3 presents the doses and experimental design.

Table 3. Application treatments and rates used in trials

No	Treatments	Application on seed or seedling	Formulation Type	Rate (ml/ha or ml/da)	Time
1	Bioproduct-I	Seed- seedling	WP	250 mL/ha	AB
2	Bioproduct-I	Seed- seedling	WP	500 mL/ha	AB
3	Bioproduct-I	Seed- seedling	WP	750 mL/ha	AB
4	Bioproduct-I	Seed- seedling	WP	1000 mL/ha	AB
5	Bioproduct-I	Seed - seedling	WP	1250 mL/ha	AB
6	Bioproduct-I	Seed- seedling	WP	1500 mL/ha	AB
7	Bioproduct-II	Seed- seedling	SC	1000 mL/ha	AB
8	Nematicide	Seed- seedling	SC	400 ml/da	AB
9	Control (+)	Seed- seedling	NA	NA	NA
10	Control (-)	Seed- seedling	NA	NA	NA

* Control (+) refers to the condition with nematodes applied; NA refers to the condition with no application.

** Bioproduct-I, recommended at a dosage of 50 g/100 L, is in WP formulation and prepared for application at different dosages (250, 500, 750, 1000, 1250 and 1500 ml) for 1 ha area.

Evaluation of the trial

Overall, 56 days after the start of the experiment, tomato plants were ready for harvesting. The plants were harvested by cutting at soil level, the roots were carefully removed from the soil and gently washed under running water to remove adhering soil particles. The fresh and dry weights of the roots were then carefully measured and recorded. For dry weight measurement, the roots were dried in an oven at a constant temperature of 60°C.

The severity of root galling indices was assessed using the 0-10 scale described by Zeck (1971) and the degree of damage caused by *M. incognita* infestation was determined. The modified Baermann funnel technique (Hooper, 1986) was applied to determine the nematode population density and the J2 were counted under Zeiss light microscope with coverslip under 100x magnification.

Statistical analysis

The experimental data were evaluated using Minitab software, version 21.1.0. Analysis of variance (ANOVA) was used to analyze the significance of variance in different parameters of the experiment. Post hoc analysis was performed using the Tukey test at a 95% confidence level. In addition, RSM was used to optimize the experiment and study the dependencies and interactions between the independent and dependent variables, in order to obtain the optimum values of the different factors affecting the root gall index and the population of J2s. The variables were Pareto charts and normal plots, which revealed the effects of the variables and deepened the trustworthiness of the results.

Results

The treatments effect on nematode reproductive parameters

The analysis of the effect of different bioproduct and nematicide treatments on root gall index found the lowest values in Bioproduct-II and Nematicide treatments. Extremely low root gall index was recorded for both the seed 2.25 and seedling 2.37 treatments at the 1000 ml/ha dose of Bioproduct-II. The nematicide treatments had similarly low root gall index values of 2.37 for seed and 2.25 for seedling. In the case of the Bioproduct-I treatment, the root gall index was found to be low with increasing dose. The minimum root gall index was found to be 2.37 for seed at 1000 ml/ha dose and 3.00 for seedling at 750 ml/ha dose. However, an increase in root gall index was observed at 1250 ml/ha and 1500 ml/ha. The maximum root gall index of the positive control remained at 7.00 and 7.12 in seed and seedling respectively. The above results indicate that Bioproduct-I and Bioproduct-II inhibit the growth of root gall index (Table 4).

Table 4. The effects of treatments on reproduction parameters in *Meloidogyne incognita*-infested plants

Run No.	Treatment	Root gall index Mean±SD (95% CI)*	Second-stage juveniles (J2) Mean±SD (95% CI)	Number of egg masses Mean±SD (95% CI)
1	Bioproduct-I (Seed, 250 ml/ha)	6.62±0.47^{abc} (6.067; 7.183)	1725.0±64.5^{ab} (1628.3; 1821.7)	187.0±9.83^{e} (174.65; 199.35)
2	Bioproduct-I (Seedling, 250 ml/ha)	7.00±0.70^{ab} (6.442; 7.558)	1637.5±179.7^{b} (1540.8; 1734.2)	275.25±9.14^{bd} (262.90; 287.60)
3	Bioproduct-I (Seed, 500 ml/ha)	4.25±0.50^{efg} (3.692; 4.808)	1062.5±110.9^{d} (965.8; 1159.2)	171.25±11.12^{ef} (158.90; 183.60)
4	Bioproduct-I (Seedling, 500 ml/ha)	5.62±0.47^{bcde} (5.067; 6.183)	1362.5±110.9^{c} (1265.8; 1459.2)	230.25±13.33^{d} (217.90; 242.60)
5	Bioproduct-I (Seed, 750 ml/ha)	2.75±0.95^{hi} (2.192; 3.308)	707.5±29.9^{e} (610.8; 804.2)	33.50±10.79^{h} (21.15; 45.85)
6	Bioproduct-I (Seedling, 750 ml/ha)	3.00±0.81^{ghi} (2.442; 3.558)	652.5±114.7^{ef} (555.8; 749.2)	70.25±12.28^{g} (57.90; 82.60)
7	Bioproduct-I (Seed, 1000 ml/ha)	2.37±0.47^{i} (1.817; 2.933)	382.5±104.4^{gh} (285.8; 479.2)	41.50±9.33^{gh} (29.15; 53.85)
8	Bioproduct-I (Seedling, 1000 ml/ha)	2.75±0.50^{hi} (2.192; 3.308)	415.0±50.0^{fgh} (318.3; 511.7)	51.25±9.81^{gh} (38.90; 63.60)
9	Bioproduct-I (Seed, 1250 ml/ha)	5.12±0.25^{def} (4.567; 5.683)	1052.5±38.6^{d} (955.8; 1149.2)	186.5±21.5^{e} (174.2; 198.8)
10	Bioproduct-I (Seedling, 1250 ml/ha)	5.25±0.28^{cdef} (4.692; 5.808)	1145.0±137.0^{cd} (1048.3; 1241.7)	246.25±12.53^{cd} (233.90; 258.60)
11	Bioproduct-I (Seed, 1500 ml/ha)	4.12±0.85^{fgh} (3.567; 4.683)	630.0±235^{efg} (533; 727)	151.75±16.01^{f} (139.40; 164.10)
12	Bioproduct-I (Seedling, 1500 ml/ha)	5.87±0.25^{abcd} (5.317; 6.433)	1092.5±69.9^{d} (995.8; 1189.2)	291.25±7.97^{b} (278.90; 303.60)
13	Bioproduct-II (Seed, 1000 ml/ha)	2.25±0.50^{i} (1.692; 2.808)	255.0±31.1^{h} (158.3; 351.7)	28.25±3.10^{hi} (15.90; 40.60)
14	Bioproduct-II (Seedling, 1000 ml/ha)	2.37±0.47^{i} (1.817; 2.933)	292.5±58.0^{h} (195.8; 389.2)	28.00±6.88^{hi} (15.65; 40.35)
15	Nematicide (Seed)	2.37±0.47^{i} (1.817; 2.933)	285.0±23.8^{h} (188.3; 381.7)	22.50±2.65^{hi} (10.15; 34.85)
16	Nematicide (Seedling)	2.25±0.50^{i} (1.692; 2.808)	267.5±17.08^{h} (170.82; 364.18)	24.75±4.03^{hi} (12.40; 37.10)
17	Control (+) (Seed)	7.00±0.81^{ab} (6.442; 7.558)	1735.0±107.5^{ab} (1638.3; 1831.7)	287.0±21.9^{b} (274.7; 299.3)
18	Control (+) (Seedling)	7.12±0.62^{a} (6.567; 7.683)	1905.0±38.7^{a} (1808.3; 2001.7)	369.3±24.9^{a} (356.9; 381.6)
19	Control (-) (Seed)	0.00±0.00^{j} (-0.00; 0.00)	0.00±0.00^{i} (-0.00; 0.00)	0.00±0.00^{i} (-0.00; 0.00)
20	Control (-) (Seedling)	0.00±0.00^{j} (-0.00; 0.00)	0.00±0.00^{i} (-0.000; 0.000)	0.00±0.00^{i} (-0.000; 0.000)

* Each value represents the mean of four replicates. Means followed by the same letter within a column are not significantly different at the 0.05 probability level, according to Tukey's HSD test.

When the number of J2 was analyzed, the lowest values were recorded in Bioproduct-II and Nematicide treatments. The 1000 ml/ha dose of Bioproduct-II provided the lowest values with 255.0 J2 counts in seed treatment and 292.5 J2 counts in seedling treatment. Nematicide treatment gave similarly low J2 numbers (285.0 in seed and 267.5 in seedling). In Bioproduct-I treatments, a decreasing trend in J2 numbers was observed as the dose increased. At 1000 ml/ha dose, 382.5 and 415.0 J2 numbers were recorded in seed and seedling applications, respectively. However, at 1250 ml/ha and 1500 ml/ha doses, J2 numbers increased again. In the positive control group, J2 numbers were quite high and recorded as 1735.0 in seed and 1905.0 in seedling. These results indicate that Bioproduct-II and Nematicide are effective in suppressing J2 population (Table 4).

The effects of different bioproduct and nematicide doses applied in the trial on number of egg masses were clearly observed. The nematicide treatments gave the lowest number of egg masses, 22.50 in the seed treatment and 24.75 in the seedling treatment. Similarly low number of egg masses were recorded in

the Bioproduct-II treatments, with values of 28.25 in the seed treatment and 28.00 in the seedling treatment. Seed application of Bioproduct-I at a dose of 750 ml/ha gave a low value of 33.50 number of egg masses, while this number was measured as 70.25 in the seedling treatment. The highest number of egg masses were recorded in the positive control groups; 287.0 number of egg masses in the seed treatment and 369.3 number of egg masses in the seedling treatment. These data indicate that Nematicide and Bioproduct-II are effective in preventing egg mass formation (Table 4).

The impact of the treatments on plant growth parameters

When analyzing the plant height data, the highest value of 34.63 cm was measured in the negative control group in the seedling treatment. Bioproduct-II applications had a positive effect on plant height, reaching a value of 28.38 cm in the seedling treatment. In Bioproduct-I applications, the plant height reached 32.97 cm in the seedling application at a dose of 1000 ml/ha and provided a remarkable development (Table 5).

Table 5. The effects of treatments on plant development parameters in *Meloidogyne incognita*-infested plants

Run No.	Treatment	Plant height* Mean±SD (95% CI)	Root fresh wt. Mean±SD (95% CI)	Root dry wt. Mean±SD (95% CI)
1	Bioproduct-I (Application on seed) 250 ml/ha	14.75±1.19^{d} (12.76; 16.74)	17.65±0.47^{i} (15.99; 19.31)	1.79±0.10^{g} (1.61; 1.97)
2	Bioproduct-I (Application on seedling) 250 ml/ha	26.38±2.06^{d} (24.39; 28.36)	26.78±1.67^{def} (25.12; 28.44)	2.64±0.19^{de} (2.46; 2.81)
3	Bioproduct-I (Application on seed) 500 ml/ha	15.23±1.34^{d} (13.24; 17.21)	19.21±1.17^{hi} (17.55; 20.87)	1.97±0.09^{fg} (1.79; 2.14)
4	Bioproduct-I (Application on seedling) 500 ml/ha	26.88±2.25^{c} (24.89; 28.86)	28.34±1.80^{cd} (26.67; 30.00)	2.75±0.19^{de} (2.57; 2.93)
5	Bioproduct-I (Application on seed) 750 ml/ha	18.90±3.76^{d} (16.91; 20.89)	23.95±3.42^{efg} (22.29; 25.61)	2.37±0.34^{ef} (2.20; 2.55)
6	Bioproduct-I (Application on seedling) 750 ml/ha	26.70±2.90^{c} (24.71; 28.69)	32.54±1.02^{bc} (30.88; 34.20)	3.27±0.15^{bc} (3.09; 3.44)
7	Bioproduct-I (Application on seed) 1000 ml/ha	18.38±0.95^{d} (16.39; 20.36)	19.74±1.18^{ghi} (18.08; 21.40)	1.92±0.17^{fg} (1.74; 2.09)
8	Bioproduct-I (Application on seedling) 1000 ml/ha	32.98±1.61^{ab} (30.99; 34.96)	31.75±1.91^{bc} (30.09; 33.41)	3.23±0.16^{bc} (3.05; 3.40)
9	Bioproduct-I (Application on seed) 1250 ml/ha	16.63±0.75^{d} (14.64; 18.61)	17.13±1.13^{i} (15.46; 18.79)	1.78±0.07^{g} (1.60; 1.95)
10	Bioproduct-I (Application on seedling) 1250 ml/ha	26.75±2.63^{c} (24.76; 28.74)	26.05±2.44^{def} (24.39; 27.71)	2.71±0.28^{de} (2.54; 2.89)
11	Bioproduct-I (Application on seed) 1500 ml/ha	16.75±0.65^{d} (14.76; 18.74)	17.63±1.67^{i} (15.97; 19.29)	1.84±0.13^{g} (1.66; 2.01)
12	Bioproduct-I (Application on seedling) 1500 ml/ha	25.38±2.25^{c} (23.39; 27.36)	28.25±0.86^{cde} (26.59; 29.91)	2.92±0.16^{cd} (2.74; 3.09)
13	Bioproduct-II (Application on seed) 1000 ml/ha	17.75±1.85^{d} (15.76; 19.74)	18.56±0.79^{i} (16.90; 20.22)	1.86±0.09^{g} (1.68; 2.03)
14	Bioproduct-II (Application on seedling) 1000 ml/ha	28.38±1.11^{bc} (26.39; 30.36)	33.73±1.33^{b} (32.07; 35.39)	3.43±0.14^{b} (3.26; 3.61)
15	Nematicide (Application on seed)	18.75±2.78^{d} (16.76; 20.74)	22.94±1.37^{fgh} (21.28; 24.60)	2.34±0.15^{ef} (2.16; 2.52)
16	Nematicide (Application on seedling)	33.88±2.50^{a} (31.89; 35.86)	33.30±2.62^{b} (31.63; 34.96)	3.45±0.31^{b} (3.27; 3.62)
17	Control (+) (Application on seed)	14.63±1.11^{d} (12.64; 16.61)	16.62±1.97^{i} (14.96; 18.28)	1.75±0.17^{g} (1.57; 1.93)
18	Control (+) (Application on seedling)	25.38±1.70^{c} (23.39; 27.36)	25.84±1.25^{def} (24.18; 27.50)	2.64±0.11^{de} (2.46; 2.81)
19	Control (-) (Application on seed)	18.25±0.87^{d} (16.26; 20.24)	18.34±0.98^{i} (16.67; 19.99)	1.92±0.12^{fg} (1.75; 2.10)
20	Control (-) (Application on seedling)	34.63±1.97^{a} (32.64; 36.61)	45.57±1.20^{a} (43.91; 47.23)	4.65±0.11^{a} (4.48; 4.83)

* Each value represents the mean of four replicates. Means followed by the same letter within a column are not significantly different at the 0.05 probability level, according to Tukey's HSD test.

The nematicide applications supported plant growth with a plant height of 33.88 cm in the seedling application. In the positive control groups, plant height values were lower and measured as 14.62 cm in seed and 25.37 cm in seedling. When evaluating the seed treatments, it was observed that some bioproduct doses had the potential to increase plant height. In particular, the seed treatment with 1250 ml/ha dose of Bioproduct-I achieved a plant height of 18.75 cm, which showed a positive effect compared to the seedling treatments. These results showed that bioproducts can have different effects on plant growth depending on the method of application (Table 5).

In terms of root fresh weight, the highest value was obtained in the seedling application of the negative control group and this value was measured as 45.57 g. Seedling application of Bioproduct-II at 1000 ml/ha showed positive effects on root development and root fresh wt. was recorded as 33.73 g. Seedling application of nematicide also gave a high value in root development and reached 33.30 g root fresh wt. In Bioproducts-I seedling applications supported root development; in particular, seedling application at 750 ml/ha dose stood out with 32.54 g root fresh wt. Although root growth was generally lower in seed treatments than in seedling treatments, significant results were obtained at some doses. For example, 23.95 g root fresh wt. was measured in the seed treatment with the 750 ml/ha dose of Bioproduct-I and this value stood out among other seed treatments (Table 5).

In root dry weight measurements, the highest value was observed in the seedling treatment in the negative control group and this value was recorded as 4.65 g. In Bioproduct-II applications, the seedling application gave favorable results in terms of root dry wt. and a value of 3.43 g was obtained. The nematicide seedling application also increased the root dry wt. and reached a value of 3.45 g. In the Bioproduct-I applications, the seedling application at a dose of 750 ml/ha gave a root dry weight of 3.26 g. In the seed treatments, the root dry weights remained at lower levels compared to the seedling treatments. However, the root dry wt. of 2.37 g obtained with the 750 ml/ha seed treatment of Bioproduct-I was higher than the other seed treatments. These results showed that seed treatments can be effective especially in the early development of the root system (Table 5).

Response surface analysis (RSM)

The experimental data were analyzed using the surface regression model (Table 6), which is an important component of RSM and allows 10 independent variables to be optimized simultaneously (Ali and Aasim 2024). For the nematode reproduction parameters, the square effect of dose (p<0.001), application method (p<0.001) and dose (p<0.001) on root gall index and number of J2 were significant at 1% level. This indicates a non-linear dose-response relationship, suggesting that bioproduct efficacy increases at certain dose intervals, but this effect may tend to decrease at extreme doses. However, the method of application to seed or seedling showed no significant effect on root gall index (p= 0.386) and J2 number (p= 0.123). This result indicates that the type of application and dose are the main determinants of bioproduct efficacy, not the method of application (Table 6).

When examining the interaction analyzes, although the Dose ml/ha × Application on seed or seedling interaction was not significant overall, a trend approaching significance at the 5% level was observed for the J2 number (p= 0.072). This finding suggests that different treatment combinations may have potential effects on nematode control and warrants further research in the future.

The effect of factors on plant growth was more pronounced. Plant height, fresh weight and dry weight parameters were significantly affected by treatment type (p<0.001), application on seed or seedling (p<0.001) and the square effect of dose (p<0.001) at the 1% level. This indicates that the method of application has a direct effect on plant growth and that seedling treatments generally favored plant growth more. Furthermore, the interaction of treatment type × application on seed or seedling was significant at the 1% level for plant height (p= 0.006), fresh weight (p<0.001) and dry weight (p<0.001), indicating that different treatment combinations can have strong effects on plant growth (Table 6). In general, bioproduct dose and application method were the most critical factors in nematode control, while application method was the

main factor directly affecting plant growth. These results highlight the importance of strategic application plans in biological control and provide guidance for determining optimal application combinations that both reduce nematode pressure and support plant growth. In this context, it is recommended that interactions, especially those of borderline significance, should be re-examined with a larger sample in the future.

Table 6. Response surface regression analysis of *Bacillus*-based bioproducts on *Meloidogyne incognita*

Source	F-Value	*p*-Value	F-Value	*p*-Value
	Root gall index		Second-stage juveniles (J2)	
Model	43,08	0,000**	51,41	0,000**
Linear	84,69	0,000**	101,32	0,000**
Dose ml/ha	24,06	0,000**	80,18	0,000**
Treatment	123,37	0,000**	128,83	0,000**
Application on seed or seedling	0,76	0,386	2,44	0,123
Square	104,45	0,000**	83,86	0,000**
Dose ml/ha×Dose ml/ha	104,45	0,000**	83,86	0,000**
2-Way Interaction	0,77	0,576	1,01	0,421
Dose ml/ha×Application on seed or seedling	0,45	0,506	3,33	0,072
Treatment×Application on seed or seedling	0,89	0,476	1,12	0,354
	Number of egg packs		Plant height	
Model	39,37	0,000**	50,88	0,000**
Linear	72,21	0,000**	65,69	0,000**
Dose ml/ha	2,01	0,161	2,51	0,118
Treatment	100,71	0,000**	15,57	0,000**
Application on seed or seedling	6,19	0,015*	325,48	0,000**
Square	101,51	0,000**	14,72	0,000**
Dose ml/ha×Dose ml/ha	101,51	0,000**	14,72	0,000**
2-Way Interaction	2,12	0,074	3,31	0,010**
Dose ml/ha×Application on seed or seedling	0,77	0,382	0,89	0,350
Treatment×Application on seed or seedling	2,65	0,041*	4,00	0,006**
	Fresh wt. (g)		Dry wt. (g)	
Model	77,02	0,000**	75,92	0,000**
Linear	110,47	0,000**	110,54	0,000**
Dose ml/ha	0,02	0,877	0,92	0,341
Treatment	40,01	0,000**	39,41	0,000**
Application on seed or seedling	502,76	0,000**	505,12	0,000**
Square	26,40	0,000**	20,45	0,000**
Dose ml/ha×Dose ml/ha	26,40	0,000**	20,45	0,000**
2-Way Interaction	24,97	0,000**	24,74	0,000**
Dose ml/ha×Application on seed or seedling	0,66	0,418	2,50	0,118
Treatment×Application on seed or seedling	27,47	0,000**	28,74	0,000

* Significant at p=0.05; ** significant at p=0.01.

Pareto charts and normal plots analysis

The Pareto charts from the surface regression (Figure 1) rank the factors in order of importance: dose (A), treatment (B), and application method (C). The quadratic effect of dose (AA) significantly reduced root gall development, indicating a non-linear effect. Treatment (B) also had a notable impact, while treatment method (C) had minimal effect. Dose (A), the quadratic effect of dose (AA), and treatment (B) had significant effects on the J2 population, while treatment method (C) was insignificant (Figure 1).

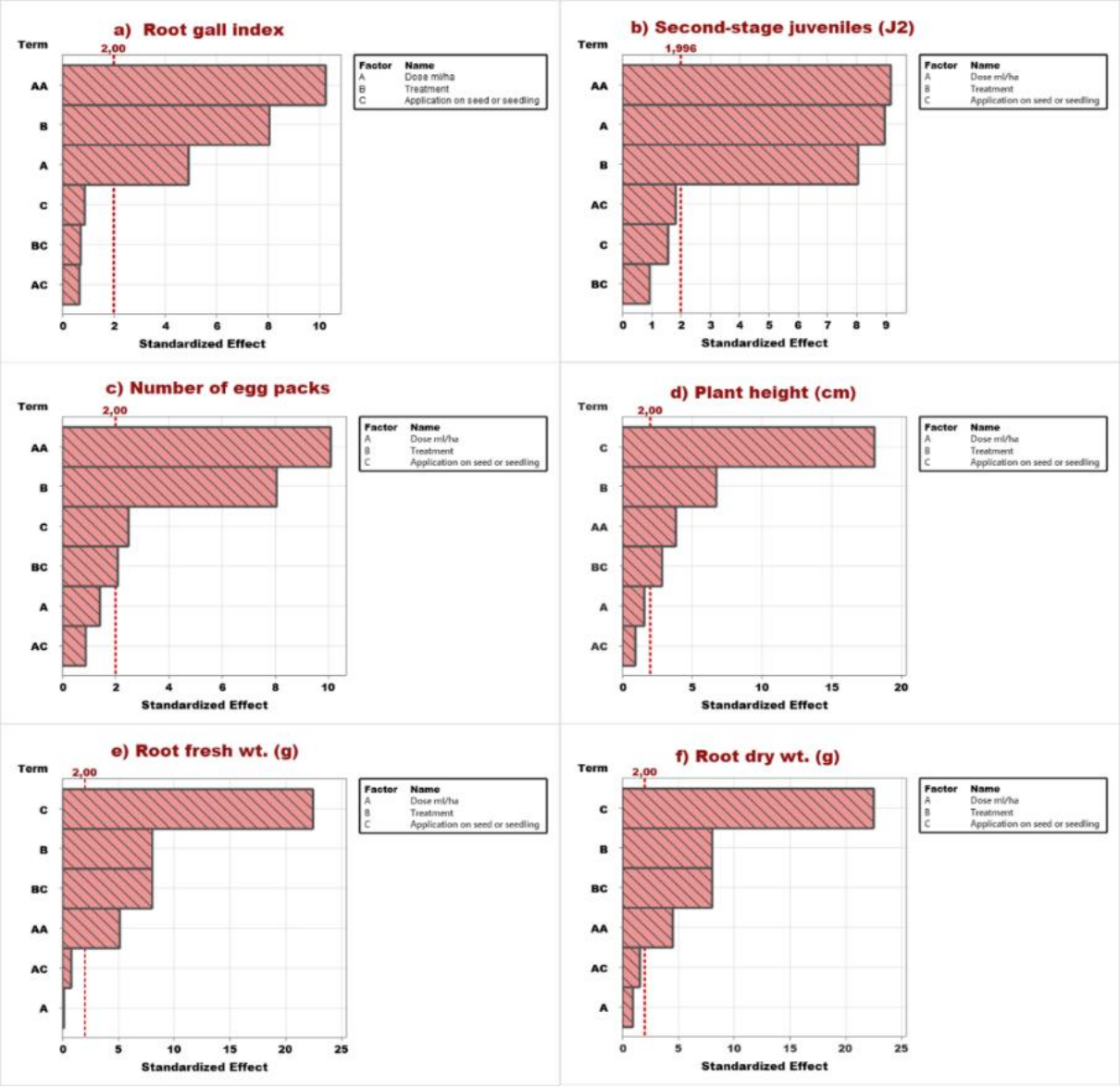

Figure 1. Pareto charts analysis of *Bacillus*-based bioproduct effects on *Meloidogyne incognita*.

AA was the most influential factor on egg production, with both high and low doses affecting it differently. Treatment (B) and method (C) also reduced egg production. Application method (C) had the greatest effect on plant height and root weight, with treatment (B) also contributing. Overall, dose (AA) was key for nematode control, and method (C) was crucial for plant growth, underscoring the need for optimal dose, application method, and timing for effective results (Figure 1).

In addition, normal plots indicate significant input factors with red squares and insignificant ones with blue circles. Factors to the right of the median line show a positive relationship with output (Xu et al., 2019). The dose (A) significantly reduced root gall index and J2 highlighting the importance of dose level. Application to seed or seedling (C) negatively impacted root weight, while treatment (B) benefited plant height and root growth, with no effect on J2 population. The BC interaction showed optimal results for plant growth. These findings suggest the need to optimize dose rates and application methods for effective nematode control and plant growth (Figure 2).

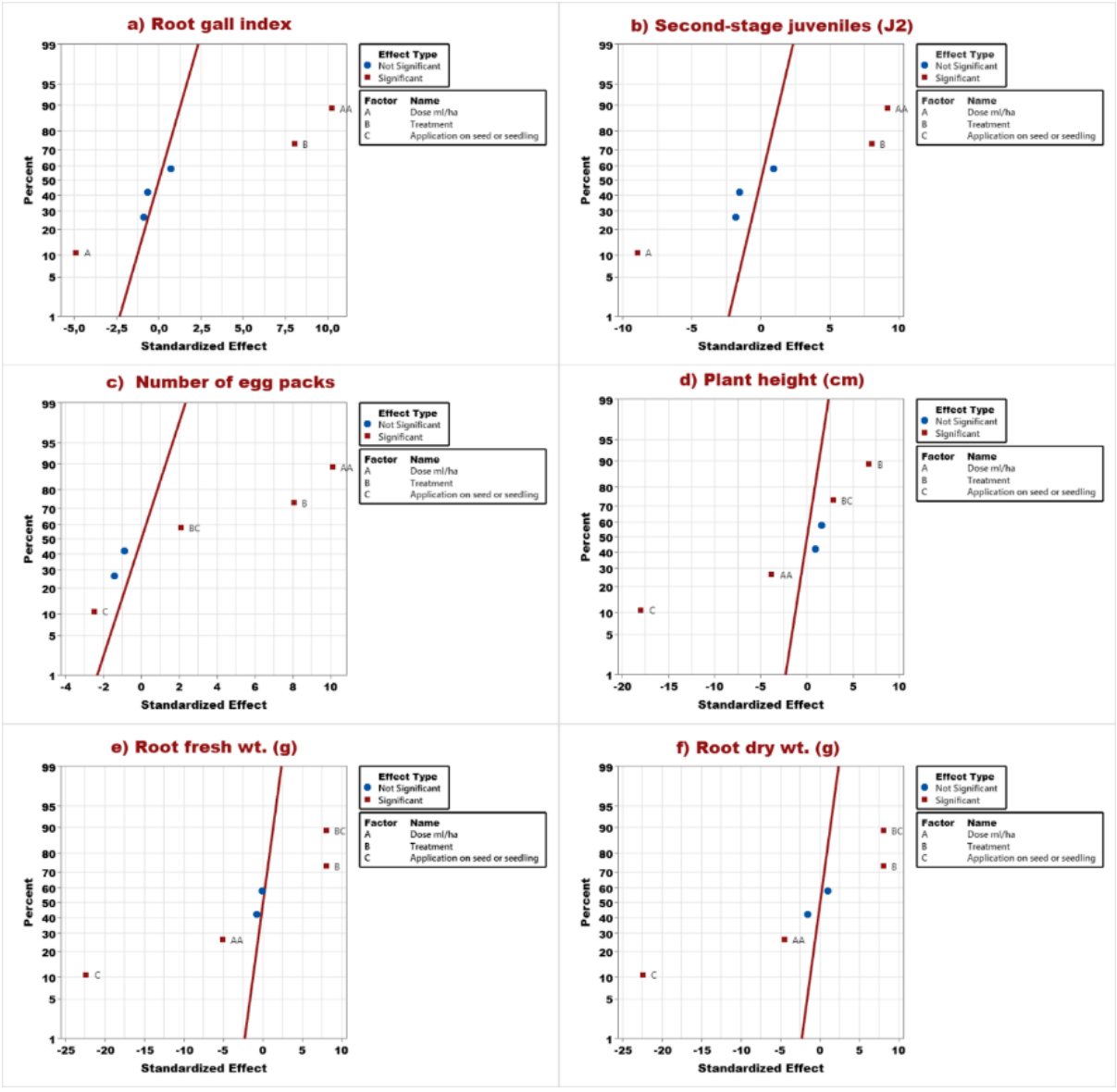

Figure 2. Normal plots analysis of *Bacillus*-based bioproduct effects on *Meloidogyne incognita.*

Discussion

The RKN species *M. incognita* causes significant soil-borne diseases by attacking a wide range of hosts (Kavitha et al., 2012). Although conventional chemical pesticides are widely used, their environmental damage and adverse effects on human health have increased interest in biological control (Yılmaz et al., 2025). Biocontrol agents colonize the rhizosphere, suppress pathogens through mechanisms such as antibiosis, competition, mycoparasitism and cell wall disruption, develop resistance in plants and promote growth (Junaid et al., 2013). Against plant-parasitic nematodes (PPNs), antagonistic bacteria provide an important line of defense in the rhizosphere, protecting roots from nematode attack (Yang et al., 2013). *Bacillus* species are particularly noted for their ability to colonize the rhizosphere, promote plant growth and reduce nematode populations. These bacteria produce toxins and enzymes that inhibit nematode reproduction, suppress egg hatching and reduce juvenile survival (Siddiqui & Mahmood, 1999).

In the current study, the efficacy of two bioproducts, *B. amyloliquefaciens* MBI 600 and *Bacillus subtilis* QST 713, as commercial formulations, in suppressing *M. incognita* infection was evaluated in pot tests in a growth chamber. In addition, the effect of different bioproduct doses on root gall index, J2 population and egg masses was approximated using Design of Experiment (DOE) and Response Surface Methodology (RSM). These statistical tools allowed the performance of the bioproducts to be analyzed in detail. Such as pareto and normal plots were used to improve the accuracy of the results and identify the variables that made the greatest contribution to the outcomes.

The results show that the application of *B. subtilis* QST 713 (Bioproduct-II) was the most effective biological agent on root gall index, J2 population and egg masses. The lowest root gall index (2.25 in seed, 2.37 in seedling) and J2 numbers (255.0 in seed, 292.5 in seedling) were recorded with Bioproduct-II, especially at the 1000 ml/ha dose. *B. amyloliquefaciens* MBI 600 (Bioproduct-I) also performed well, with the lowest root gall index (2.37) observed at 1000 ml/ha, although a slight increase was noted at 1250 ml/ha and 1500 ml/ha. Similarly, J2 populations decreased at moderate doses but increased again at the highest doses (Table 4), indicating that while the bioproduct suppresses nematode populations up to a point, additional dosage offers no further benefit. This finding highlights the importance of applying the correct dose when using bioproducts.

The ability of *Bacillus* species to control nematode infestations has been recognized in several previous studies. For example, *B. subtilis* has been considered a preventive agent against soil-borne infections, with demonstrated efficacy against nematodes and fungal species (Abd-Elgawad et al., 2010). Khalil et al. (2012) and Khalil (2013) found that root gall formation and egg masses of *M. incognita* in cotton were inhibited by *B. subtilis*, reducing nematode density in the soil. Xiang et al. (2017) similarly demonstrated that *B. subtilis* strains reduced cotton egg numbers by 73.63% to 80.72% and increased cotton yield in microplot studies. In pot trials, *B. subtilis* DTBS 5, *Pantoea agglomerans*, and *B. amyloliquefaciens* DSBA 11 significantly reduced root galls, egg masses, the egg/egg mass ratio, and reproductive factor (RF) following soil irrigation with PGPR isolates (Aballay et al., 2020). Gattoni et al. (2023) also reported over 75% mortality of second-stage juveniles (J2) of *M. incognita* with *B. firmus* I-1582 and its metabolite extracts.

In greenhouse trials, *B. amyloliquefaciens* QST 713 and *B. firmus* I-1582 were as effective against *M. incognita* as the chemical nematicide fluopyram. However, the efficacy of *Bacillus* species is strongly influenced by external factors such as temperature, light, and humidity (Brar et al., 2006). Differences between pot trials and field performance can be attributed to variations in soil structure, climatic conditions and microbial competition (Meyer, 2003; Tian et al., 2007). Therefore, developing stable and effective bioproduct formulations with long shelf-life is crucial for sustainable agriculture (Zhang et al., 2023).

Process and dose optimization is critical for enhancing efficiency and ensuring stability of promising microorganisms in bioproduct development (Kumar & Banerjee, 2013). Optimizing the rate and timing of application can influence the growth stage at which the target pest is most vulnerable (Hynes & Boyetchko, 2006). Factors such as nutrients, pH, temperature, inoculum volume, and inducers significantly affect the production of secondary metabolites and enzyme output (Kumar & Banerjee, 2013; Fayad et al., 2022). Overall, production efficiency is controlled by physicochemical parameters like temperature, carbon, nitrogen, aeration and pH (Kumar & Banerjee, 2019; dos Santos et al., 2024).

In this study, Bioproduct-I (*B. amyloliquefaciens* MBI 600) was optimized using DOE and RSM. The results showed a dose-dependent decrease in J2 population and root gall index, with the lowest values observed at 1000 ml/ha (2.37 in seed, 2.75 in seedling for root gall index; 382.5 in seed, 415.0 in seedling for J2 population). However, a significant increase in these parameters was noted at higher doses (1250 and 1500 ml/ha), once again indicating that higher concentrations do not necessarily enhance performance. RSM provided valuable insights into optimal application rates based on precise modelling of bioproduct effects.

These results underscore the importance of robust statistical methods such as DOE and RSM in developing biological control strategies. Furthermore, validation of laboratory results with field data will improve the success rate of bioproducts under real agricultural conditions. The market success of bioproducts relies not only on their efficacy in controlled environments but also on their consistency in the field. Future research should focus on evaluating the performance of bioproducts under various environmental conditions and refining their formulations accordingly.

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Original article (Orijinal araştırma)

Distribution of entomopathogenic fungi inducing natural infections in *Orosanga japonica* (Melichar, 1898) (Hemiptera: Ricaniidae) in the Eastern Black Sea Region of Türkiye

Türkiye'nin Doğu Karadeniz Bölgesi'nde *Orosanga japonica* (Melichar, 1898) (Hemiptera: Ricaniidae)'da doğal enfeksiyona neden olan entomopatojenik fungusların dağılımı

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Abstract

Orosanga japonica (Melichar, 1898) (Hemiptera: Ricaniidae) is an important pest in the Black Sea Region in Türkiye. Despite the use of various methods and microbial factors from different sources to control this pest until recently, several pathogens have been detected. Entomopathogenic fungi are important microbial agents in pest control. In the present study, we isolated eighteen fungal strains from nymphs and adults of *O. japonica* with fungal infection collected in Rize, Trabzon and Artvin provinces of Türkiye in 2021-2022. According to morphological and molecular characterization based on internal transcribed spacer (ITS) sequences, all strains were identified as *Beauveria bassiana* (Bals.-Criv.) Vuill. (Ascomycotina: Hypocreales) (OJ1-OJ18). A screening test at 1×10^7 conidia/ml revealed that all the strains caused 75–100% mortality in nymphs and adults after 7 days. Further experiments were performed with the three most effective strains, all belonging to *B. bassiana* (OJ3, OJ7 and OJ15). The dose–response tests of the three fungal strains were carried out on *O. japonica* nymphs and adults at concentrations of $1 \times 10^{4–8}$ conidia/ml. As a result of the concentration-response, the *B. bassiana* OJ3, OJ7 and OJ15 strains presented mortality rates of 100, 98.84 and 100%, respectively, at the highest dose (10^9 conidia/ml) on the 10th day, and the calculated LC_{50} values of OJ3, OJ7 and OJ15 were determined to be 1×10^4, 1.4×10^4 and 0.3×10^3 conidia/ml against nymphs and 1.6×10^4, 1.4×10^4 and 0.4×10^3 conidia/ml against adults, respectively. The presence of *B. bassiana* detected natural infections and the efficacy trials conducted in the laboratory revealed that *B. bassiana* has potential for use against *O. japonica*.

Keywords: *Beauveria bassiana*, biological control, entomopathogenic fungi, *Orosanga japonica*

Öz

Orosanga japonica (Melichar, 1898) (Hemiptera: Ricaniidae), Türkiye'nin Karadeniz Bölgesi'nde önemli bir zararlıdır. Yakın zamana kadar bu zararlının mücadelesinde çeşitli yöntemler ve farklı kaynaklardan elde edilen mikrobiyal etmenler kullanılmasına rağmen, birkaç patojen tespit edilmiştir. Entomopatojenik funguslar, zararlı mücadelesinde önemli mikrobiyal etmenlerdir. Bu çalışmada, 2021-2022 yıllarında Türkiye'nin Rize, Trabzon ve Artvin illerinden mantar enfeksiyonu taşıyan *O. japonica* nimf ve erginlerinden on sekiz fungus izolasyonu yapılmıştır. Morfolojik ve iç transkripsiyon aralayıcı (ITS) dizilerine dayanan moleküler karakterizasyon sonucunda, tüm suşların *Beauveria bassiana* (Bals.-Criv.) Vuill. (Ascomycotina: Hypocreales) (OJ1-OJ18) olduğu tanımlanmıştır. Tarama testlerinde, 1×10^7 konidi/ml konsantrasyonunda tüm izolatların 7 gün sonunda nimf ve erginlerde %75-100 oranında öldürücülük sağladığı gözlemlenmiştir. En etkili üç izolat olan OJ3, OJ7 ve OJ15 ile gerçekleştirilen konsantrasyon-cevap testlerinde, *O. japonica* nimf ve erginleri üzerinde 1×10^4-10^9 konidi/ml konsantrasyon aralığında uygulamalar yapılmıştır. Konsantrasyon-cevap testleri sonucunda, 10. günde en yüksek dozda (10^9 konidi/ml) OJ3, OJ7 ve OJ15 izolatları sırasıyla %100, %98.84 ve %100 oranında öldürücülük göstermiştir. OJ3, OJ7 ve OJ15 izolatlarının LC_{50} değerleri nimfler için sırasıyla 1×10^4, 1.4×10^4 ve 0.3×10^3 konidi/ml; erginler için ise 1.6×10^4, 1.4×10^4 ve 0.4×10^3 konidi/ml olarak belirlenmiştir. Bu çalışmada, *B. bassiana* doğal enfeksiyonlarının tespit edilmesi ve laboratuvarda gerçekleştirilen etkinlik denemeleri, *B. bassiana*'nın *O. japonica*'ya karşı kullanım potansiyeline sahip olduğunu ortaya koymuştur.

Anahtar sözcükler: *Beauveria bassiana*, biyolojik mücadele, entomopatojenik fungus, *Orosanga japonica*

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Introduction

Orosanga japonica (Melichar, 1898) (Hemiptera: Ricaniidae), commonly known as invasive polyphagous, has emerged as a significant pest in Türkiye (Demir, 2009; Arslangündoğdu & Hizal, 2019). This invasive insect species, native to East Asia, has found its way to Turkish shores, causing concern among agricultural experts and farmers alike. Nymphs and adults are intensively found on many plant species including vegetables, fruits, and ornamental plants (Gjonov, 2011; Göktürk, 2018; Karataş et al., 2020). Its rapid reproduction rate and voracious feeding habits make it particularly destructive, leading to significant economic losses in agricultural areas. The pest, which was first detected in our country in 2006, reached a very significant population density in the next few years and spread throughout the Black Sea Region (Göktürk & Aksu, 2014; Ak et al., 2015; Göktürk & Mıhlı, 2015).

Entomopathogenic fungi are part of the fungal phylum Ascomycota and the order Hypocreales, which includes several notable genera such as *Beauveria* spp. and *Metarhizium* spp. These fungi have evolved specialized mechanisms to invade and colonize their insect hosts, leading to host mortality through a combination of direct damage and the production of potent toxins. They stand out as an environmentally friendly option instead of pesticides. The increasing application of integrated pest management (IPM) is motivated by its specificity, low toxicity to non-target beneficial organisms, and minimal environmental impact. Furthermore, their ability to persist in the environment and adapt to different ecological niches makes them attractive candidates for long-term pest control.

Numerous studies have been conducted in Türkiye on the identification, biology, distribution, and management of this pest (Demir, 2009, 2018; Güçlü et al., 2010; Ak et al., 2013, 2014; Göktürk & Mıhlı, 2015, 2016; Alev & Sezen, 2016; Göktürk, 2018, 2019, 2020; Göktürk et al., 2018; Akıner et al., 2019, 2020, 2021, 2022; Altaş & Ak, 2019; Arslangündoğdu & Hizal, 2019; Güney et al., 2020; Erper et al., 2022). Microbial natural enemies have significant effects on controlling pest populations in nature (Biryol et al., 2021; Sevim et al., 2012; Sönmez et al., 2016; Gençer et al., 2023). In support of this, several studies have demonstrated that entomopathogenic fungi exert highly lethal effects on their host organisms from which they are isolated (Yücel et al., 2018; Biryol et al., 2020, 2021). Despite these findings, research on the distribution of *O. japonica* cadavers infected with fungi in the Eastern Black Sea Region, the isolation of fungal strains from those cadavers, and investigations into the potential use of these fungi for biological control of the pest is extremely rare. In this study, we isolated eighteen fungal strains from *O. japonica* nymphs and adults with fungal infections provided from Rize (Ardeşen, Pazar, and İyidere), Trabzon (Of), and Artvin (Hopa) provinces in Türkiye, characterized them, and analyzed the pathogenicity of strains isolated from dead *O. japonica* collected from various fields in Türkiye.

Materials and Methods

Collection of insect samples

Orosanga japonica nymph and adult cadavers were collected from Rize (Ardeşen, Pazar and İyidere), Trabzon (Of) and Artvin (Hopa) in Türkiye, where insects are very densely transferred to the laboratory in sterile plastic tubes and stored at 4ºC until use. Individuals infected with mycosis from *O. japonica* nymphs and adults were used for isolating entomopathogenic fungi.

Healthy *O. japonica* nymphs were collected from tea plantation fields in Rize (Pazar) Province and transported to the laboratory in plastic containers with proper ventilation and nutrient content. They were then fed fresh tea leaves and shoots under laboratory conditions until the insecticidal activity tests were conducted.

Isolation and purification of fungal isolates

Fungal strains were obtained from mycosed *O. japonica* nymphs and adults using an inoculation loop and cultured on PDAY (potato dextrose agar + 1% yeast extract; Merck, Darmstadt, Germany). To suppress bacterial contamination, chloramphenicol was added to the medium at a concentration of 40 µg/ml. The inoculated Petri dishes were kept waiting at 28ºC until fungal colonies developed. Pure isolates were obtained by selecting morphologically distinct single colonies based on their infection patterns (Yücel et al., 2018). A total of eighteen isolates were purified and labeled with local codes (e.g., OJ1–OJ18). Stock spore suspensions were prepared from purified cultures and kept at - 80ºC.

Morphological characterization

The purified isolates were first assessed for colony morphology on Sabouraud-CAF agar (Liofilchem s.r.l., Italy). Microscopic features were examined using the slide culture method, which involved placing small fragments of the medium inoculated with fungal spores on glass slides and covering them with coverslips (Senthilkumar, et al., 2021). Following 4-5 days of incubation, fungal structures were directly observed under a light microscope. Additional morphological details, including conidial structures, were examined under phase-contrast microscopy after staining with lactophenol cotton blue. Identification was performed following the diagnostic guidelines of Humber (1997).

Molecular characterization

To genetically identify the isolates, the internal transcribed spacer (ITS) regions-comprising ITS1, 5.8S, and ITS2 were amplified. Spores from pure cultures were cultivated on Sabouraud CAF agar at 28ºC for seven days. Mycelial biomass was collected using sterile spatulas and transferred to microtubes. Genomic DNA was extracted from ~50 mg of fungal material using the ZR Fungal/Bacterial DNA MiniPrep kit (Zymo Research), following the manufacturer's instructions.

PCR sample was conducted using the primers ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′), as detailed by White et al., (1990). Each 50 µl PCR reaction included genomic DNA, reaction buffer, $MgCl_2$, dNTPs, Taq polymerase, and primers. Amplification was performed using the thermal cycling protocol recommended for the kit. The reaction conditions were after a denaturation step at 98ºC for 30 s, 35 cycles of denaturation at 98ºC for 30 s, annealing at 50ºC for 30 s, extension at 72ºC for 1 min and a final extension at 72ºC for 10 min. PCR products were separated using 1.0% agarose gels containing ethidium bromide and visualized under UV illumination.

Following purification, Sanger sequencing was performed by ETKA Biotechnology (Samsun, Türkiye). Resulting sequences were analyzed for similarity using BLAST in the GenBank database (Benson et al., 2012). Multiple sequence alignments were performed using ClustalW2 in BIOEDIT v.7.2.5 (Hall, 1999). Phylogenetic relationships were inferred with the Maximum Likelihood (ML) method in MEGA v.11.0 (Tamura et al., 2013), and the results were compared to related taxa. The ITS sequences of all isolates were submitted to the NCBI database and assigned unique accession numbers.

Screening tests

Eighteen fungal strains isolated from *O. japonica* nymphs and adult cadavers were tested for efficacy against *O. japonica* nymphs and adults. Fungal samples were incubated on agar plate at 25ºC for 3 weeks. Fungal conidia were collected using a 0.1% Tween 80 (AppliChem) solution and subsequently counted under a microscope to determine concentrations. Fungal suspension was applied using a sprayer at 1×10^7 conidia/ml concentration. Thirty individuals were used for the whole treatment, and tests were conducted in triplicate at different times. Only 0.1% Tween 80 was utilized for the control tests. Mortality was monitored for 7 days following treatment.

Concentration-response tests

The isolates OJ3, OJ7, and OJ15, which showed the highest insecticidal activity in the screening trials, were assessed in concentration-response assays. In this study, conidial suspensions from fungal strains were diluted in a 0.1% Tween 80 solution to obtain five different concentrations ranging from 10^8 to 10^4 conidia/ml. These concentrations were applied to third-instar nymphs and adults of *O. japonica*. The experiments were conducted following the methodology outlined in the screening tests described above (Biryol et al., 2021).

Statistical analysis

Mortality rates were adjusted using the formula developed by Abbott (1925). The study data were examined using a one-way analysis of variance (ANOVA) and a post-hoc comparison with the least significant difference (LSD) test to identify differences between the means. Probit regression analysis was performed on the concentration–mortality data to estimate the median lethal concentration (LC50) (Finney, 1971). Statistical analyses were conducted with SPSS software version 25.0 for all experiments (IBM Corp., Armonk, NY, USA).

Results

A total of 18 fungal isolates were obtained from 263 (6.84%) *O. japonica* infected individuals (Table 1). These isolates were considered infected when mycelial growth extended beyond the cadavers. These isolates were obtained from *O. japonica* nymphs and adults naturally infected by fungi and collected from areas exposed to insect infestation (Figure 1). Morphological observations revealed that all fungal isolates exhibited characteristics consistent with *Beauveria* species. Identification was based on the morphological characteristics, specifically the appearance and size of spores grown on agar medium, following the criteria outlined by Humber (2012). The colonies appeared round, characterized by a white, powdery surface with subtly downy, concentric rings. Phase-contrast microscopy images of the fungal isolates and slide cultures are presented in Figure 2. The spores were globose to subglobose in structure.

Table 1. Information on sampling locations and individual counts of adults and nymphs

Location	Nymph individual number	Adult individual number	Fungal isolates
Rize (Ardeşen)	12	19	2 (OJ6 and OJ7)
Rize (Pazar)	17	26	5 (OJ1-OJ5)
Rize (İyidere)	37	41	3 (OJ9-11)
Trabzon (Of)	48	52	7 (OJ12-18)
Artvin (Hopa)	4	7	1 (OJ8)

Figure 1. *Orosanga japonica* adult with natural *Beauveria bassiana* infection.

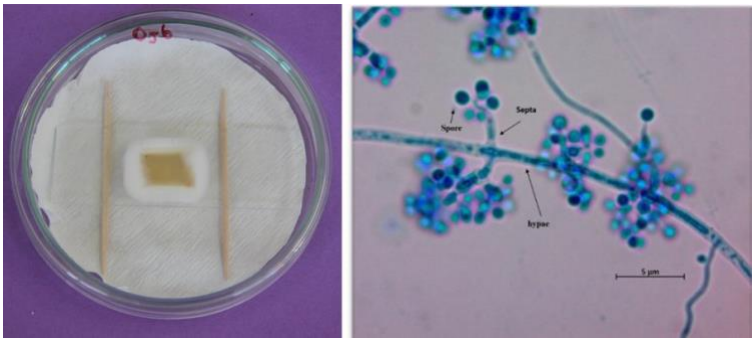

Figure 2. *Beauveria bassiana* morphological identification using the Bloc culture method (left) and light microscope image stained with lactophenol blue (right).

PCR amplification targeted the ITS1-5.8S-ITS2 gene region, and total DNA was extracted from all fungal isolates. The DNA fragments, approximately 530 base pairs in length, were successfully amplified and subsequently visualized using gel electrophoresis. The ITS sequences of the isolates were >99% similar to *B. bassiana* as a result of BLAST. In the phylogenetic analysis, all isolates were observed to be closely related to *B. bassiana* (Bals.-Criv.) Vuill. (Ascomycotina: Hypocreales) (Figure 3). The sequences were submitted to GenBank with accession numbers OR835539–OR835556.

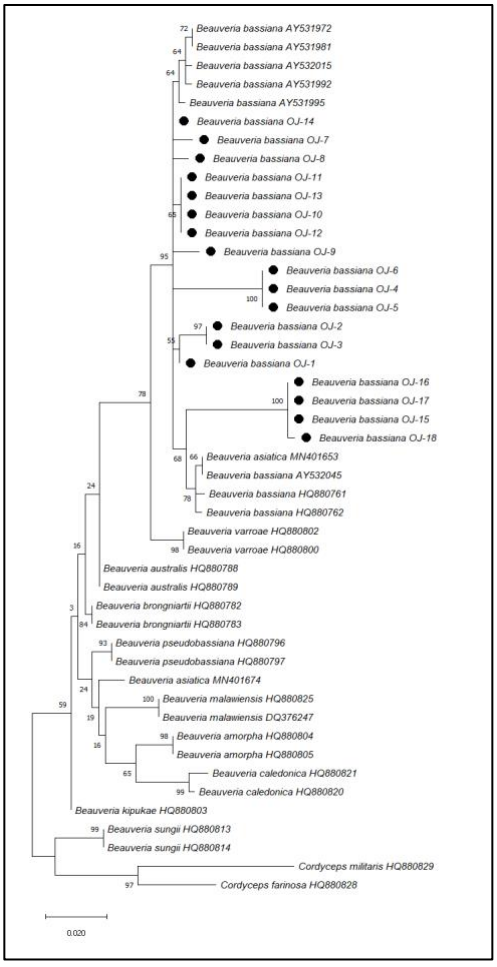

Figure 3. Phylogenetic relationships among *Beauveria bassiana* isolates and related taxa were inferred via Maximum Likelihood analysis using ITS1-5.8S-ITS2 sequences. Bootstrap values (1,000 replicates) are shown at nodes, and individual isolates are marked with black dots.

In a screening test conducted with 1×10^7 conidia/mL, all strains resulted in mortality rates between 75% and 100% in both nymphs and adults after 10 days. Furthermore, experiments were carried out with the three most effective strains, all of which were *B. bassiana* species (OJ3, OJ7, and OJ15) (Figure 4). These strains were selected based on their spread on petri dishes and cadavers.

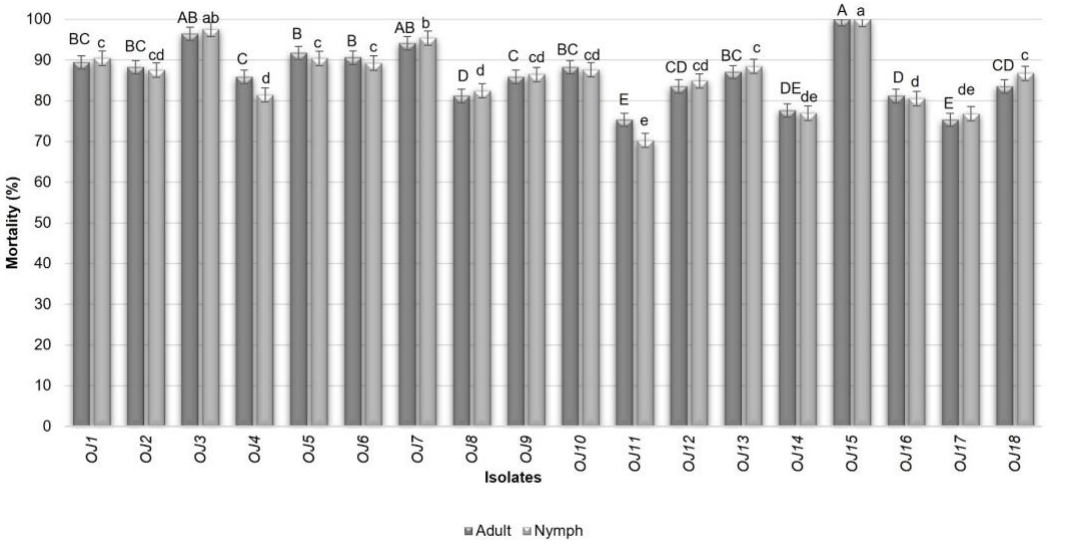

Figure 4. Insecticidal activities of *Beauveria bassiana* OJ1-18 isolates at 1×10^7 conidia/ml on nymphs and adults of *Orosanga japonica* within 10 days. Capital letters represent data for nymphs; lower case letters represent data for adults. Bars show standard error.

Eighteen strains of entomopathogenic fungi exhibited pathogenicity against *O. japonica* within seven days of inoculation, with mortality rates ranging from 75% to 100%. While all strains demonstrated varying mycosis rates, strains OJ3, OJ7, and OJ15 recorded the highest mortality rates (>94%, $p< 0.05$; Figure 4). However, these values were not significantly different from those of several other strains. The mortality rates of all tested strains significantly exceeded those of the control group within seven days ($F = 79.4$; $df = 17, 36$; $p< 0.05$). *B. bassiana* OJ3, OJ7, and OJ15 (the most virulent strains), resulted in mortality rates of 97.52%, 95.42%, and 100% in nymphs, and 96.47%, 94.12%, and 100% in adults, respectively.

In the dose-response assessment, these strains exhibited mortality rates of 100%, 98.84%, and 100%, respectively, at the highest concentration (10^9 conidia/ml) by the 10th day (Figure 5). The calculated LC50 values for OJ3, OJ7, and OJ15 were 0.3×10^5, 0.2×10^5, and 0.2×10^4 conidia/ml, respectively (Table 1).

Table 1. Concentration mortality rates of *Beauveria bassiana* strains on nymphs and adults of *Orosanga japonica*

Isolate	LC_{50} (conidia ml^{-1}) (FL 95%)	Intercept	(*Slope* ± *SE*)	X^2	*df*	*n*
		Nymph				
OJ3	1×10^4 ($0.06\text{-}15.3\times10^4$)	3.492	0.37±0.60	0.992	3	90
OJ7	1.4×10^4 ($0.11\text{-}18.3\times10^4$)	3.291	0.41±0.56	0.995	3	90
OJ15	0.3×10^3 ($0.0009\text{-}11.5\times10^3$)	4.132	0.33±0.78	0.996	3	90
		Adult				
OJ3	1.6×10^4 ($0.12\text{-}21.5\times10^4$)	3.334	0.39±0.56	0.990	3	90
OJ7	1.4×10^4 ($0.11\text{-}17.6\times10^4$)	3.274	0.41±0.55	0.994	3	90
OJ15	0.4×10^3 ($0.001\text{-}14.8\times10^3$)	4.082	0.33±0.76	0.998	3	90

Abbreviations: *df*: degrees of freedom; *FL*: fiducial limit; *SE*: standard error; X^2: chi-square; *n*: Number of individuals.

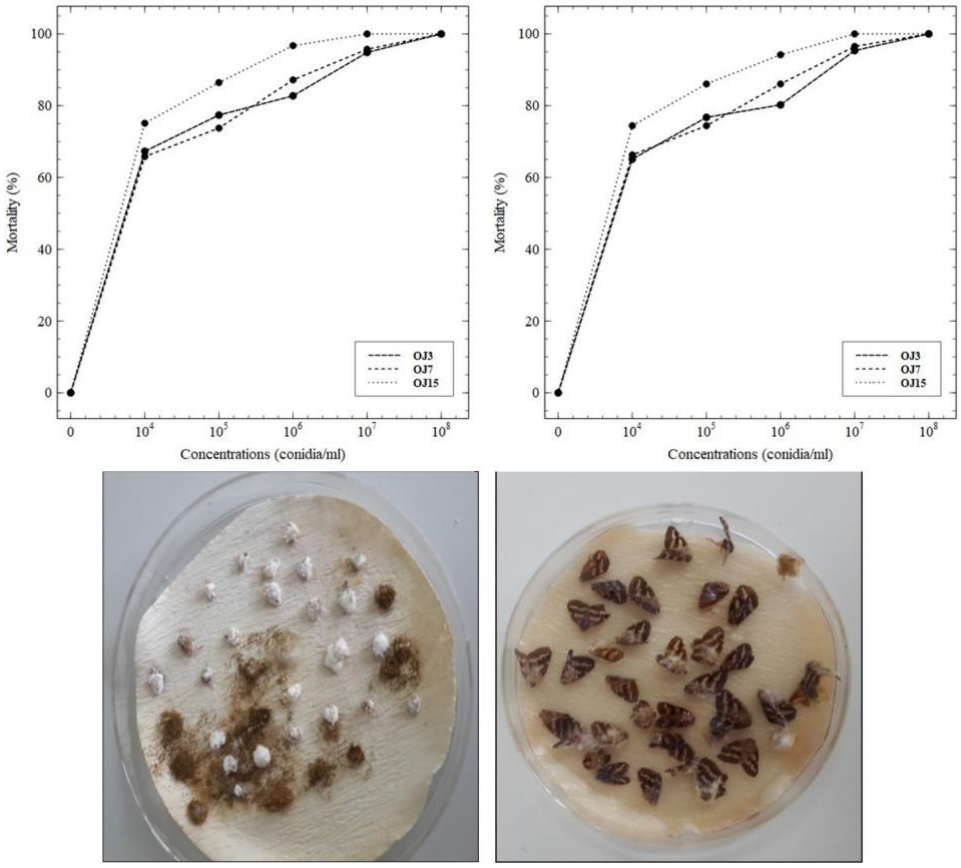

Figure 5. The insecticidal effects of the newly isolated *Beauveria bassiana* strains OJ3, OJ7, and OJ15 on *Orosanga japonica* nymphs and adults over a 10-day period. The fungal conidia concentrations tested ranged from 1 × 10^4 to 1 × 10^8 conidia/mL (above). Images of *Orosanga japonica* nymphs (bottom left) and adults (bottom right) exhibiting mycosis following infection with the *Beauveria bassiana* isolates in the bioassay.

Discussion

In recent years, a consistent increase in the population of the invasive species *O. japonica* was observed in the Eastern Black Sea region, leading to significant damage to various wild and cultivated plant species (Ak et al., 2015). The climate in the region where fungal infection samples are found is characterized by heavy rainfall, low temperature, and high humidity. These climatic features are highly conducive to the improvement of entomopathogenic fungi (EPFs) that can infect insects such as *B. bassiana* (Akıner et al., 2020). EPF conidia require moisture for germination and sporulation, with certain species specifically dependent on elevated humidity levels to initiate infection. Furthermore, rainfall plays a critical role in the dissemination of EPFs across insect populations (Goettel et al., 2005).

Beauveria bassiana, an entomopathogenic fungus, has shown considerable promise as a biological control agent for various insect pests. The pathogenicity of this fungus, when isolated from insect pests, varies between different strains, which affects its efficacy in pest control. Ribosomal RNA genes exhibit varying degrees of conservation, with spacer regions showing considerable divergence. The internal transcribed spacer (ITS) sequence is widely used to classify and identify fungi (Driver et al., 2000; Cai et al., 2013). Sevim et al., (2010) indicated that 23.3% of *B. bassiana* isolates from the eastern Black Sea region of Türkiye were obtained from various soil sources, and their identity was confirmed by analyzing the ITS1-5.8S-ITS2 region. Eighteen distinct *B. bassiana* isolates were identified in the current study, differing from those previously found in the eastern Black Sea region. These findings suggest that the region possesses a diverse range of entomopathogenic fungal strains, likely attributed to the high humidity levels.

In the present study, 18 entomopathogenic fungi were isolated. *B. bassiana* was found to be effective against a wide range of orders and pest species (citation). The results of studies on *O. japonica* indicate that various native isolates of *B. bassiana* exhibit high virulence against *O. japonica*, with laboratory tests revealing lethal effects at concentrations as low as 1×10^6 conidia/ml, resulting in 100% mortality within five days (Akıner et al., 2020; Erper et al., 2022). Akıner et al. (2020) reported that the leaf dipping method at the concentration of 1×10^6 conidia\ml yielded the most effective results, with LT_{50} values decreasing to 2.56 days for isolate 2. Similarly, for isolate 1, the LT_{50} values ranged from 6.634 days at 1×10^4 conidia/ml to 2.92 days at 1×10^6 conidia/ml, indicating a dose-dependent increase in virulence. In contrast, the dose-response assessment presented in the current study demonstrates a pronounced efficacy at higher concentrations, with mortality rates reaching 100%, 98.84%, and 100% at 1×10^9 conidia/ml for isolates OJ3, OJ7, and OJ15, respectively, by the 10th day. The calculated LC_{50} values for OJ3, OJ7, and OJ15 were significantly lower, ranging from 0.3×10^5, 0.2×10^5, and 0.2×10^4 conidia/ml, indicating higher virulence at lower concentrations when compared to Akıner et al. (2020). The discrepancies between the two sets of findings may be attributed to variations in experimental methodologies, isolate virulence, and application techniques.

Akıner et al. (2020) reported that the optimal concentration for the potential use of *B. bassiana* isolates as a biological control agent ranged from 1×10^6 to 1×10^8 conidia/ml, which aligns with the findings of the current study. Göktürk et al., (2018) observed that the biopesticidal efficacy of *B. bassiana* against nymphs and adults of *O. japonica* was relatively low, with mortality rates below 20%. In contrast, the 18 *B. bassiana* strains isolated in this study demonstrated a high level of efficacy against both nymphs and adults.

In this study, the fungal strains OJ3, OJ7, and OJ15 caused mortality rates of 100%, 98.84%, and 100%, respectively, when applied at the highest concentration of 1×10^9 conidia/ml by day 10. The corresponding LC_{50} values were estimated as 0.3×10^5, 0.2×10^5, and 0.2×10^4 conidia/ml for OJ3, OJ7, and OJ15, respectively. Gençer & Bayramoğlu (2022) similarly reported high insecticidal activity of *B. bassiana* isolates obtained to *Galleria mellonella* L. larvae collected from beehives, with mortality rates of 96.54% and 89.66% at 1×10^9 conidia/ml. The LC_{50} values for the G-A and G-B strains were calculated to be between 0.2×10^6 (0.03–1.6) and 0.6×10^6 (0.07–6.1) conidia/ml. Comparable bio efficacy has been reported in other studies evaluating *B. bassiana* isolates against various insect pests, confirming their potential as effective biological control agents.

Sevim et al. (2013) indicated that entomopathogenic fungal isolates tested, the *B. bassiana* isolate KTU-24, originating from *Thaumetopoea pityocampa* (Den. & Schiff., 1775) (Lepidoptera: Thaumetopoeidae), exhibited the highest mortality rate, causing 86% mortality in both adult and nymph stages of *Corythucha ciliata* (Say, 1832) (Hemiptera: Tingidae) within 14 days at a concentration of 1×10^7 conidia/ml. The same isolate reached 100% mortality at a higher concentration of 1×10^8 conidia/ml.

Utilizing *B. bassiana* as a biopesticide provides an eco-friendly alternative to chemical pesticides, decreasing ecological damage and supporting sustainable agriculture (El-Maraghy et al., 2023; Eko et al., 2024). While *B. bassiana* shows promise, its interaction with the gut microbiota can influence its virulence, suggesting a complex relationship that warrants further investigation (Peng et al., 2023).

Eighteen natives *B. bassiana* strains were isolated from *O. japonica* cadavers. Phylogenetic analysis using ITS confirmed their identification and classified them alongside with reference *B. bassiana* isolates from GenBank. The presence of *B. bassiana* in the detected natural infections and the efficacy trials conducted in the laboratory revealed that *B. bassiana* has insecticidal effects on *O. japonica*. These results may have contributed to the decrease in the population of this pest in the Eastern Black Sea Region in recent years.

This study was conducted under laboratory conditions using both nymphs and adults of the target pest. Future research should extend to greenhouse and field trials to further validate these findings. Additionally, the most effective isolate identified in this study could be developed as a mycoinsecticide, potentially contributing to the biological control strategy against the pest.

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Original article (Orijinal araştırma)

Determination of tannin activity and *Paenibacillus alvei* (Cheshire & Cheyne) Ash et al. (Bacillii: Paenibacillaceae) on the biocontrol of tannin-tolerant *Agelastica alni* L., 1758 (Coleoptera: Chrysomelidae) larvae[1]

Tanen toleranslı *Agelastica alni* L., 1758 (Coleoptera: Chrysomelidae) larvalarının biyolojik mücadelesinde *Paenibacillus alvei* (Cheshire & Cheyne) Ash et al. (Bacillii: Paenibacillaceae) ve tanen aktivitesinin belirlenmesi

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Abstract

Tannins are among the most abundant secondary metabolites synthesized by plants. *Agelastica alni* L., 1758 (Coleoptera: Chrysomelidae) is a critical forest pest. This study investigated the effect of *Paenibacillus alvei* (Cheshire & Cheyne) Ash et al. (Bacillii: Paenibacillaceae) and tannins against *A. alni* larvae. The larvae were collected from the Çayeli district of Rize province in 2022. In the feeding experiments, artificial diets containing 1.25%, 2.5% and 5% tannins were prepared. 100 and 200 µl of *P. alvei* were applied to the infected groups. Nutritional indices, pupal masses, phenoloxidase activities, antioxidant enzyme activities and mortality rates of larvae fed with different diets were studied. Relative consumption rate (RCR) increased with tannin concentration in all groups. Relative growth rate (RGR) increased with rising tannin concentrations across all groups. In the infected groups, the increase in tannin concentration caused a decrease in developmental time. While superoxide dismutase and phenoloxidase activities of uninfected larvae decreased with tannin concentration, catalase and glutathione peroxidase activities of larvae increased. In infected larvae, catalase activity decreased with increasing tannin concentration. The dose of *P. alvei* caused an increase in superoxide dismutase and phenoloxidase activities, but did not affect catalase and glutathione peroxidase activities. The diet containing 5% tannic acid had the lowest mortality rate.

Keywords: *Agelastica alni*, antioxidant system, biocontrol, *Paenibacillus alvei*, tannin-tolerant

Öz

Tanenler, bitkiler tarafından sentezlenen en bol ikincil metabolitler arasındadır. *Agelastica alni* L., 1758 (Coleoptera: Chrysomelidae) önemli bir orman zararlısıdır. Bu çalışmada, *Paenibacillus alvei* (Cheshire & Cheyne) Ash et al. (Bacillii: Paenibacillaceae) ve tanenlerin *A. alni* larvaları üzerindeki etkisi araştırılmıştır. Larvalar 2022 yılında Rize ili Çayeli ilçesinden toplanmıştır. Besleme deneylerinde, %1.25, %2.5 ve %5 tanen içeren yapay diyetler hazırlanmıştır. Enfekte gruplara 100 ve 200 µl *P. alvei* uygulanmıştır. Farklı diyetlerle beslenen larvaların beslenme indeksleri, pupal kütleleri, fenoloksidaz aktiviteleri, antioksidan enzim aktiviteleri ve ölüm oranları incelenmiştir. Nisbi tüketim oranı (RCR), tüm gruplarda tanen konsantrasyonu ile artmıştır. Nisbi büyüme oranı (RGR), tüm gruplarda tanen konsantrasyonunun artmasıyla yükselmiştir. Enfekte gruplarda tanen konsantrasyonundaki artış, gelişim süresinde azalmaya neden olmuştur. Enfekte olmayan larvalarda süperoksit dismutaz ve fenoloksidaz aktiviteleri tanen konsantrasyonu ile azalırken, katalaz ve glutatyon peroksidaz aktiviteleri artmıştır. Enfekte larvalarda ise tanen konsantrasyonundaki artış katalaz aktivitesinin azalmasına neden olmuştur. *P. alvei* dozu, süperoksit dismutaz ve fenoloksidaz aktivitelerinde artışa yol açarken, katalaz ve glutatyon peroksidaz aktivitelerini etkilememiştir. %5 tanik asit içeren diyet, en düşük ölüm oranına sahip olmuştur.

Anahtar sözcükler: *Agelastica alni*, antioksidan sistem, biyolojik mücadele, *Paenibacillus alvei*, tanen tolerant

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Introduction

Agelastica alni (L., 1758) (Coleoptera: Chrysomelidae), is an important pest of alder, hazel and willow trees. It has a wide distribution in Europe, the Caucasus, Siberia, north-eastern Kazakhstan and the USA. Its high reproductive rate causes defoliation of plants in natural habitats and parks, resulting in both economic and aesthetic damage. This condition also facilitates the invasion of host plants by other pests. The significant economic and aesthetic damage caused by *A. alni* is a cause for concern.

Chemical insecticides interfere with the physiological activities of target organisms. Pesticide residues also cause significant damage to environmental factors such as water and soil (Jayaraj et al., 2016). Due to the need for safer control methods, microbial insecticides, entomobacteria and their secondary metabolites are being used to control harmful insects (Yaman & Demirbağ, 2000; Sezen & Demirbağ, 2006; Isayama et al., 2021). *Paenibacillus* spp. Ash et al. (Bacillii: Paenibacillaceae) are widely distributed in the soil and rhizosphere (Atanasova-Pancevska & Kungulovski, 2018). Many *Paenibacillus* species can directly promote plant growth through biological nitrogen fixation, phosphate solubilization, production of the phytohormone indole-3-acetic acid (IAA), and release of siderophores that promote iron uptake. In this way, plants can protect themselves against herbivorous insects and phytopathogens, including bacteria, fungi, nematodes and viruses (Grady et al., 2016). It is also used as a biocontrol agent against many phytopathogenic fungi (Atanasova-Pancevska & Kungulovski, 2018). *Paenibacillus alvei* (Cheshire & Cheyne) Ash et al. (Bacillii: Paenibacillaceae) is a secondary pathogen of American Foulbrood (AFB) in honeybees (Djukic et al., 2012). AFB is a fatal enteric disease. Spores germinate in the midgut lumen and vegetative bacteria multiply there before eventually attacking and penetrating the midgut epithelium, killing the larva (Poppinga & Genersch, 2015). It is effective in bees at low doses and lethal at high doses. However, there are few studies on the insecticidal activity of *Paenibacillus* species against other pests (Neung et al., 2014). Therefore, the insecticidal activity of *P. alvei* remains a gap in the literature.

Plants produce direct or indirect defense strategies against herbivore attack. Phytochemicals in direct defense strategies can influence herbivore preference and performance (Corry & Hoover, 2006). Tannins are one of the most abundant groups of phytochemicals in all vascular plants. They protect plants from insects by acting as deterrents or poisons. They are polyphenolic compounds of two types: hydrolysable tannins between 500-3000 Da and condensate tannins. Plant tannins act as protease inhibitors and can interact with digestive enzymes to precipitate proteins. This reduces protein utilization in herbivorous insects and impairs digestion (Pizzi et al., 2009). In the highly acidic environment of the insect gut, tannins undergo oxidation and bind to various enzymes, further disrupting digestive processes. They also contribute to food loss by binding to lipids and reducing their digestibility. Tannins also negatively affect insect development by inducing midgut lesions, which ultimately inhibit growth. They also act as a food deterrent due to their bitter taste (Price et al., 2019). In some plants, tannins also protect plants from harmful insects. Because of these properties, they can be used as an insecticide (Isayama et al., 2011, 2021; Mostafa et al., 2012; Djilali et al., 2021). In addition, studies show that the resistance of insects to viral and bacterial pathogens decreases with increasing tannin content in the leaves (Keating et al., 1989; Young et al., 1995; Lindroth et al., 1999). Host plants can modify the relationships between herbivorous insects and their pathogens. Changes in plant chemistry and structure can lead to differences in pathogen infection of insects. Hydrolysable tannins and condensate tannins have been shown to reduce viral mortality in Lymantria dispar and cotton bollworm (Corry & Hoover, 2006).

Various physiological responses, such as immune responses and intermediary metabolism, are present in insect haemolymph. Enzymatic changes in infected larvae would presume the metabolic stress experienced by insects during the development of the pathogen (Ibrahim et al., 2019). These enzymes control ROS (Reactive Oxygen Species) generated by biotic and abiotic stress in insects. ROS include free radicals, oxygen ions, and organic molecules. These radicals cause protein oxidation, lipid peroxidation,

nucleic acid damage, and activation of the immune system (Meşe et al., 2022). An increase in ROS elements is seen with exposure to pathogens and cell damage. Many studies have shown that the antioxidant system is a defense mechanism against pathogen-induced ROS production. The major antioxidant enzymes in insects are catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) (Vengateswari et al., 2020). SOD is the first detoxifying enzyme produced in cells and is a powerful antioxidant.

Insufficient degradation of superoxide anions or H_2O_2 can lead to the formation of hydroxyl radicals, which will cause the oxidation of sulfur residues in polypeptides or proteins and their degradation (Zhang & Feng, 2018). GPx is also essential in preventing lipid peroxidation and protecting cells from oxidative stress (Meşe et al., 2022). Another enzyme involved in humoral immunity is phenoloxidase. Phenoloxidase (PO) is a key enzyme that is activated by the prophenoloxidase (PPO) cascade in the cuticle or haemolymph of many insects as a defense response to immune challenges (Gillespie et al., 1997). During this process, the peptidoglycan recognition protein recognizes the appropriate elicitor and triggers the production of inactive prophenoloxidase. Phenoloxidase plays a crucial role in the conversion of phenols to quinones, which subsequently leads to melanin synthesis. Melanin is essential for defense against bacterial (Gram + and -) and fungal infections (Ibrahim et al., 2019).

In forest ecosystems, secondary metabolites can act against insect herbivores in two ways. They can either have a toxic effect on the larvae themselves or affect entomopathogens (Fernandez-Conradi et al., 2021). Therefore, the effects of both factors should be evaluated together to control forest pests. Plants of the genus *Alnus* are rich in tannins (Janceva et al., 2011). In the process of co-evolution, phytophagous insects have developed behavioral and physiological defense mechanisms to protect themselves from the harmful effects of secondary metabolites and to continue their population development under selective pressure. Behavioral mechanisms are designed to adapt feeding strategies and developmental rhythms. Physiological defense mechanisms are designed to reduce the toxicity of food and protect against oxidative damage (Jiang et al., 2021). *Agelastica alni* larvae feeding on alder leaves have also adapted to the tannins in the host plant during the coevolutionary process. The aim of our study is to investigate whether the tannins in *Alnus* leaves reduce their immune resistance when exposed to the entomopathogen *P. alvei*, even though they have adapted to feeding on host plants. In addition, this study aims to answer the question of whether *P. alvei* can be used as an entomopathogen to control other insect pests.

Materials and Methods

Obtaining larvae and preparation of artificial diets

The larvae were collected while feeding on alder leaves in the Çayeli district of Rize in 2022 and brought to the laboratory. They were fed on an artificial diet as a group in the laboratory. A total of four different artificial diets were used in the feeding experiment. The composition of the artificial diet, developed by Yamamoto (1969), is as follows: wheat germ, casein, sucrose, torula yeast, a vitamin mixture, cholesterol, sorbic acid, methyl paraben, linseed oil, agar, and distilled water. Diets containing tannic acid were prepared by adding tannic acid at concentrations of 1.25%, 2.5%, and 5%. The control diet did not contain tannic acid.

Preparing bacterial suspension

Paenibacillus alvei bacterial cultures were obtained from stock cultures at the Recep Tayyip Erdoğan University Microbiology Research Laboratory (deposited at DDBJ/ENA/GenBank under the accession number MZ673473). MYPGP (Mueller Hinton Broth, Yeast Extract, Potassium Phosphate, Glucose, Na-Pyruvate, Agar-agar) medium was used to prepare the cultures. An isolate of *Paenibacillus alvei,* collected from a deep freezer, was grown in MYPGP medium at 5% CO_2 and 36ºC temperature for 24-48 hours. The plate culture was inoculated as a single colony into 10 ml of MYPGP broth and incubated overnight at 36ºC in a shaking incubator at 120 rpm. Before use, the culture was diluted to 1×10^6 (cfu/ml) with sterile saline water.

Larval rearing

In the feeding trials, groups were established for all diets (no tannin, 1.25%, 2.5% and 5% TA) with no bacteria, and with 100 µl and 200 µl bacterial applications. A total of 12 experimental groups were set up. Larvae were placed in plastic petri dishes (10 cm x 3 cm) for each group. Fresh food was given every other day until the last larval stage, and the unconsumed food was replaced with fresh food. Bacteria were added using an automatic pipette inside a sterile cabinet. The procedure was repeated on each food replacement day.

Nutritional indices

When the larvae reached the last instar, as determined by the exuviae, 30 insects per experimental group were weighed and placed in plastic cups for individual feeding. The number of larvae used for the experiments was in accordance with the literature (Truzi et al., 2021). The last instar larvae were weighed on a balance with an accuracy of 0.001 mg at the beginning of the feeding, and their weight and the amount of food given were recorded. The diets were changed every two days, the remaining diets were packaged, and the weight gain of the larvae was recorded. The remaining diets were dried in an oven at 50ºC until they reached a constant weight. This process was continued until the larvae became pupae. Pupa mass was determined by drying the pupae at 50ºC until they reached a constant weight (Lee et al., 2002). Their developmental time was defined as the time required to reach the pupal stage.

According to Waldbauer (1968), consumption measured on the basis of the dry weight of the food reflects the rate at which nutrients enter the digestive system of an insect, whereas consumption calculated on the basis of the wet weight indicates the insect's behavioral response to the food. In this study, nutritional indices were assessed for the final instar larvae. The nutritional indices RCR and RGR were determined from fresh weight as described by Stochoff (1992). RGR is influenced by the quality of the host plant, the physiological condition of the insect and environmental factors. When calculations were based on dry weight, leaves, faeces and larvae were dried to a constant weight before measurement.

$$RCR\ (relative\ consumption\ sate) = \frac{dry\ weight\ of\ food\ consumed}{(insect\ weight\ at\ start\ of\ experiment)(time\ a)}$$

$$RGR\ (relative\ growth\ rate) = \frac{insect\ wet\ weight\ gain}{(insect\ wet\ weight\ at\ beginning\ of\ experiment)(time)}$$

Nutritional assays for enzyme activities

Larvae in the enzyme activity feeding groups continued to be fed together. As in the nutritional indices test group, the diets were changed every two days, and in the bacterial application groups, the application was made on the days when the nutrients were changed. Haemolymph samples for enzyme activities were collected two days before pupation. Hemolymph samples were collected from the larvae two days before pupation. For this purpose, the larvae were first sterilized with 95% ethanol. They were then collected by piercing the last proleg with a sterile needle. The haemolymph was extracted into Eppendorf tubes and stored at -27ºC until needed (Lee et al., 2008).

Haemolymph preparation for antioxidant enzyme activities

The haemolymph samples used in all enzyme activity assays were prepared as follows: 8 µL of hemolymph and 400 µL of ice-cold phosphate-buffered saline (PBS; pH 7.4) were mixed and vortexed. Samples were then frozen at -20ºC to disrupt haemocyte membranes (Wilson et al., 2001). The Bradford method was used to quantify the protein concentration of the haemolymph samples (Bradford, 1976).

Phenoloxidase activity assay

Phenoloxidase activity in haemolymph samples was assessed spectrophotometrically by following the formation of dopachrome (2-carboxy-2,3-dihydroindole-5,6-quinone) from L-dihydroxyphenylalanine (L-DOPA). For this purpose, 100 µL of 10 mM L-DOPA (substrate) was combined with 100 µL of ice-cold phosphate-buffered saline (PBS, pH 7.4) haemolymph and incubated at 25ºC for 20 minutes (Wilson et al., 2001). After incubation, the absorbance of the mixture was measured at 492 nm.

Antioxidant enzyme activity assays

The superoxide dismutase activity of haemolymph samples was determined using a method based on the inhibition of nitroblue tetrazolium (NBT) reduction to formazan, which is then detected spectrophotometrically at a wavelength of 560 nm. A critical aspect of this method is the use of the xanthine-xanthine oxidase system as the superoxide generator. This system, a technique established by Beauchamp and Fridovich in 1971, is important because it provides a controlled and reliable source of superoxide ions for the assay. The reaction mixture, which was adjusted to a pH of 7.40 in a 10 mM phosphate buffer solution, contained 100 µM NBT and 50 µM xanthine. The concentration of xanthine oxidase in the reaction mixture was adjusted to produce an absorbance change of 0.025 min^{-1} at 560 nm.

According to Aebi (1984), the catalase activity of haemolymph samples was determined by measuring the decrease in H_2O_2 concentration spectrophotometrically at 240 nm. This reliable method involved mixing a freshly prepared hydrogen peroxide solution (0.036% (w/w). 2.80 mL of hydrogen peroxide solution in a quartz cuvette) with 20 µL of haemolymph samples and monitoring the change in absorbance (Shobha et al. 2016).

Glutathione peroxidase activity was measured spectrophotometrically using H_2O_2 as substrate according to Drotar et al (1985). The reaction mixture (250) contained 2 mM glutathione (GSH), 1 mM EDTA, 0.1 mM NADPH, 2.5 units of glutathione reductase, and 90 µM H_2O_2. Glutathione peroxidase activity was calculated from the rate of peroxide removal by following the change in absorbance at 340 nm resulting from the oxidation of NADPH in the reaction. The rate of NADPH oxidation was measured at 348 nm.

Determination of mortality rate

Confirmation of mortality rates in groups treated with different diets and different doses of *P. alvei* was performed using the Schneider-Orelli's formula (Hristov et al., 2022).

Statistical analyses

Statistical analyses of nutritional indices, developmental time, pupal mass, antioxidant enzyme activity, phenoloxidase activity and mortality rates were performed using SPSS version 23. Only the mortality rate follows a normal distribution. Two-Way ANOVA test was used to assess differences between groups. Tukey's Honestly Significant Difference (Tukey's HSD) test was used as a post hoc test to evaluate whether there were statistically significant changes. However, as the other parameters did not follow a normal distribution, the Kruskal-Wallis H test was used to assess group differences. Post hoc analysis was conducted with Dunn's test to observe statistically significant changes (p <0.05). Correlation tests were also performed to assess the relationships between the parameters.

Results

The effects of tannin concentrations and biocontrol agent application doses on the nutritional indices RCR and RGR, development time, and pupal mass were investigated. According to the results of the Kruskal-Wallis analysis, there are differences in the values of RCR, RGR, pupal mass, and development time according to the diets and *P. alvei* application dose (Table 1).

Table 1. Pairwise comparison results of nutritional indices, pupa mass, development duration indices, and antioxidant activities as a result of *Paenibacillus alvei* administration in different diets and at different doses

Parameters	N	X^2	df	*p*
RCR	180	168.37	11	0.00
RGR	180	158.56	11	0.00
Pupal Mass	180	163.99	11	0.00
Development Duration	180	173.88	11	0.00
Phenoloxidase Activity	180	165,07	11	0.00
SOD Activity	180	173,32	11	0.00
Catalase Activity	180	176,29	11	0.00
GPx Activity	180	177.09	11	0.00

Among all the diets, the highest RCR value was found in larvae fed on an uninfected 5% tannic acid diet. The lowest value was found in uninfected larvae fed on the control diet (no tannic acid) (Figure 1). When each tannic acid concentration was evaluated in isolation, infection had no significant effect except on the 2.5 % TA diet (Figure 1). As the tannin concentration increased in the uninfected groups, the RCR value also increased (r=0.29, $p<0.05$). In the infected groups, RCR increased with tannin concentration (r=0.92, $p<0.01$), but *P. alvei* dose had no effect on RCR ($p>0.05$). As the RCR increased in the non-infected groups, development time was prolonged (r=0.93, $p<0.01$) and pupal mass increased (r=780.52, $p<0.01$). Development time was shortened in the infected groups as RCR increased (r=-0.62, $p<0.01$).

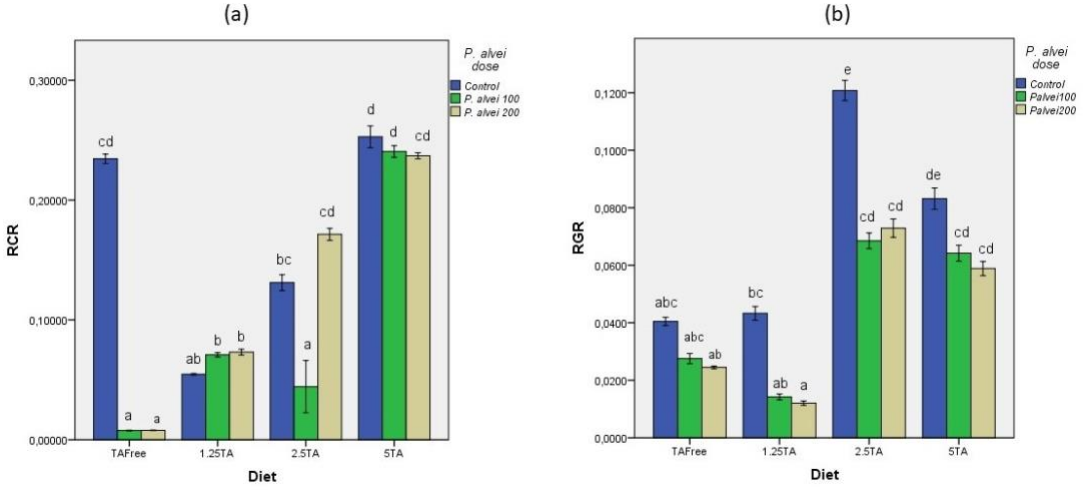

Figure 1. a) RCR values according to different tannin concentrations and different doses of *Paenibacillus alvei* applications (mean±standard deviation) b) RGR values according to different tannin concentrations and different doses of *Paenibacillus alvei* applications (mean±standard deviation).

* According to Dunn's test, no statistically significant difference is indicated by values with the same letter.

In uninfected larvae, the highest RGR was observed in the 2.5 % TA diet (Figure 1). When the diet was examined separately at each tannic acid concentration, it was found that the RGR decreased with bacterial application (Figure 1). As the tannin concentration increased, the RGR increased in both the infected and uninfected groups (r=0.26, $p<0.01$; r=0.58, $p<0.01$). In the uninfected groups, as the RGR increased, the developmental period shortened (r=-0.29, $p<0.05$) and the pupal mass decreased (r=-0.47, $p<0.01$). In the infected groups, the pupal mass increased with increasing RGR (r=0.18, $p<0.05$).

The highest pupal mass value was recorded in the 5TA % diet, while the lowest pupal mass value was recorded in the group containing 200 µl of *P. alvei* with 1.25 % TA (Figure 2). The bacterial dose applied in the tannin-free diets had no effect on pupal mass (Figure 2). No relationship was found between tannin concentration and pupal mass in infected larvae ($p>0.05$). No correlation was found between pupal mass and *P. alvei* dose in infected groups ($p>0.05$). Pupal mass also increased with prolongation of development time in infected and non-infected groups (r=0.71, $p<0.01$; r=0.73, $p<0.01$).

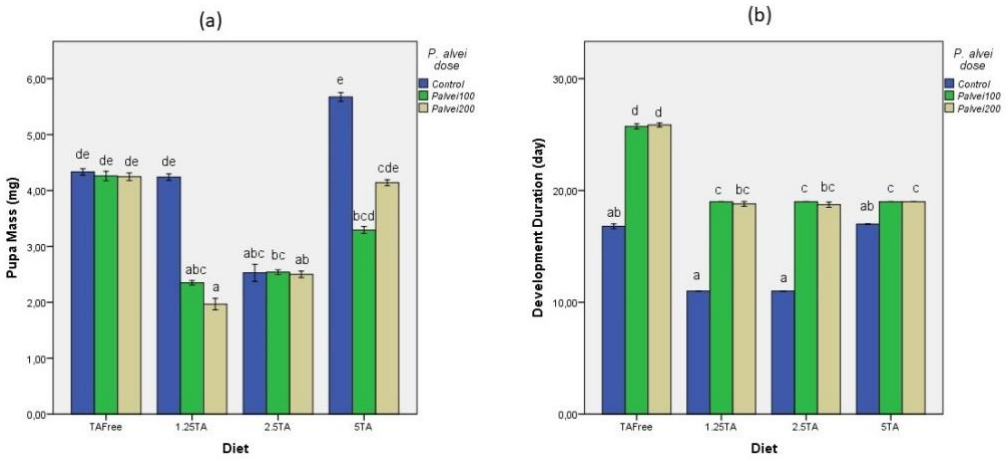

Figure 2. a) Pupal mass values (mean±standard deviation) according to different tannin concentrations and different doses of *Paenibacillus alvei* applications; b) Development times (mean±standard deviation) according to different doses of *Paenibacillus alvei* in diets containing different tannin concentrations.

* According to Dunn's test, no statistically significant difference is indicated by values with the same letter.

The longest development times were observed in tannic acid-free diets with 100 and 200 µl of *P. alvei.* The shortest development times were observed in 1.25 % TA and 2.5 % TA diets without *P. alvei* application (Figure 2). There was no correlation between tannin concentration and development time in the non-infected groups (p>0.05). In the infected groups, an increase in tannin concentration caused a decrease in the development time (r=-0.69, p<0.01). The application of *P. alvei* caused a prolonged development time (Figure 2). However, there was no correlation between dose and development time (p>0.05).

The enzyme activities of fhenoloxidase, catalase, SOD, and GSH-Px were examined. According to Kruskal-Wallis analysis, differences in enzyme activities were observed between groups (Table 1). The highest phenoloxidase activity was found when 200 µl of *P. alvei* was applied to the larvae fed the diet at 2.5 % TA concentration. The lowest activity was found in the larvae of the group treated with 100 µl of *P. alvei* on the 5 TA diet (Figure 3). Tannin concentration caused a decrease in phenoloxidase activity in the non-infected groups (r=-0.70, p<0.01). While the tannin concentration did not affect the infected groups (p>0.05), the dose of *P. alvei* caused an increase in phenoloxidase activity (r=0.76, p<0.01). In all the diets, especially the application of 200 µl in particular caused a significant increase in activity (Figure 3).

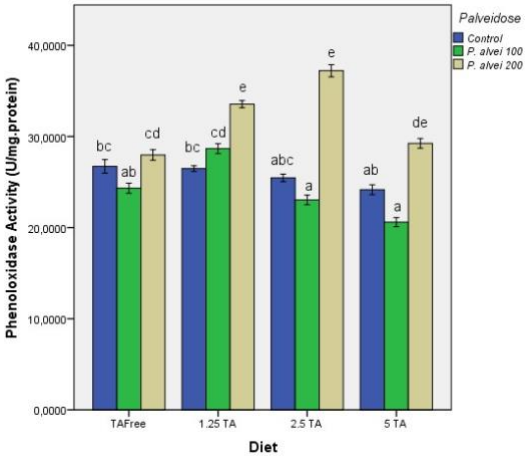

Figure 3. Phenoloxidase activities (mean±standard deviation) according to different tannin concentrations and different doses of*Paenibacillus alvei* applications.

* According to Dunn's test, no statistically significant difference is indicated by values with the same letter.

The highest SOD activity was detected in larvae fed on a 2.5% TA diet treated with 200 µl of *P. alvei*. The lowest SOD activity was found in the 5% TA diet without *P. alvei* application (Figure 4). SOD activity decreased with tannin concentration in the non-infected groups (r=-0.97, p<0.01). While tannin concentration did not affect SOD activity in infected groups (p>0.05), *P. alvei* dose caused an increase in activity (r=0.18, p<0.05). In contrast, this increase was seen in the tannin groups (Figure 4).

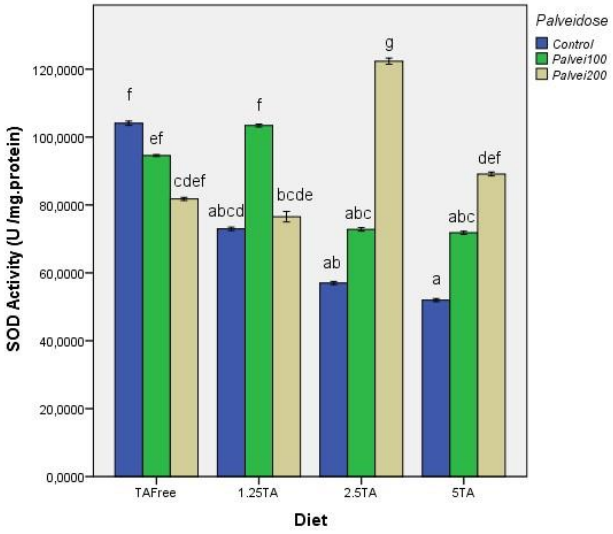

Figure 4. Superoxide dismutase activities (mean±standard deviation) according to different tannin concentrations and different doses of *Paenibacillus alvei* applications.

* According to Dunn's test, no statistically significant difference is indicated by values with the same letter.

The lowest catalase activity was found in the tannin-free diet treated with 100 µl of *P. alvei*. The highest activity was found in the larvae fed on the 1.25 TA diet treated with 100 µl of *P. alvei* (Figure 5). In the non-infected groups, enzyme activity increased with tannin concentration (r=0.74, p<0.01). In the infected groups, a decrease in activity with tannin concentration was observed (r=-0.26, p<0.01). However, the dose of *P. alvei* had no effect on catalase activity (p>0.05).

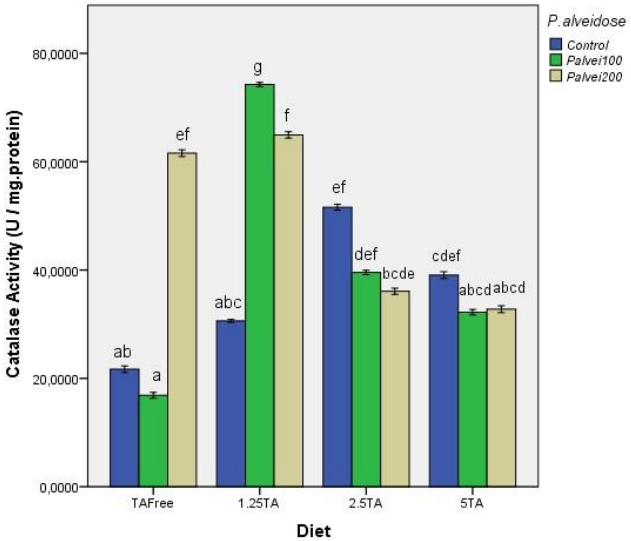

Figure 5. Catalase activities (mean±standard deviation) according to different tannin concentration and different dose *Paenibacillus alvei* applications.

* According to Dunn's test, no statistically significant difference is indicated by values with the same letter.

The lowest GPx activity was found in the diet containing no tannin to which 100 µl of *P. alvei* was applied. The highest activity was found in the diet containing 1.25% TA to which 100 µl of *P. alvei* was applied (Figure 6). Tannin concentration increased GSH-Px activity in the non-infected groups (r=0.77, p<0.01). In the infected groups, tannin concentration and *P. alvei* had no effect on GPx activity (p>0.05).

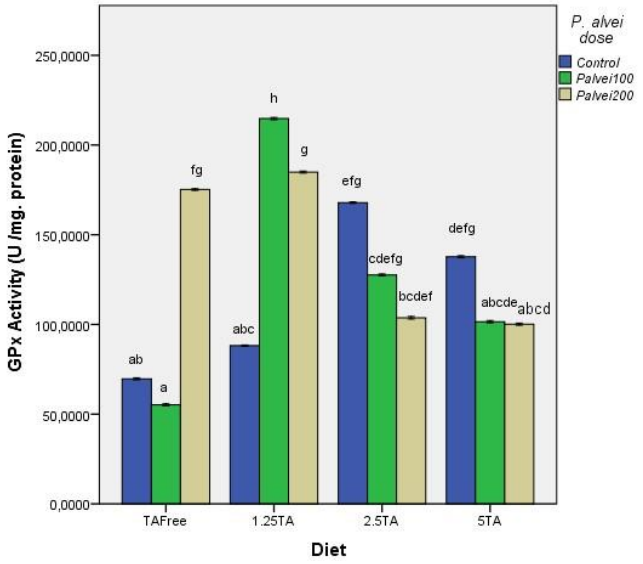

Figure 6. Glutathione peroxidase (mean±standard deviation) activities according to different tannin concentrations and different doses of *Paenibacillus alvei* applications.

* According to Dunn's test, no statistically significant difference is indicated by values with the same letter.

Correlation and regression analyses were performed to determine whether there was a relationship between RCR and RGR values and enzyme activity. The increase in the RCR in the uninfected groups caused only a decrease in phenoloxidase activity (r=-0.31, p<0.05). The increase in the RCR in the infected groups caused a decrease in catalase and GPx activities (r=-0.30, p<0.01; r=-0.31, p<0.01). In comparison, the increase in RGR in the uninfected groups caused an increase in catalase and GPx activities (respectively; r=0.95, p<0.01; r=0.97, p<0.01). It caused a decrease in SOD and phenoloxidase activities (respectively; r=-0.74, p<0.01; r=-0.4, p<0.01).

SOD activity increased with the increase in phenoloxidase activity (r=0.59, p<0.01), whereas CAT and GPx activity decreased (respectively; r=-0.42, p<0.01; r=-0.52, p<0.01) in the uninfected groups. While SOD activity increases, CAT and GPx activities decrease (respectively; r=-0.84, p<0.01; r=-0.86, p<0.01). Catalase and GPx activities are also directly positive (r=0.98, p<0.01). In the infected groups, while phenoloxidase activity increased, SOD, CAT, and GPx activities also increased (respectively; r=0.64, p<0.01; r=0.33, p<0.01; r=0.28, p<0.01). Catalase and GPx activities were positive (r=0.99, p<0.01).

Mortality rates have been corrected using the Schneider-Orelli formula. Mortality rates and standard errors are presented in Table 2. The highest mortality rate among the diets treated with *P. alvei* was observed in the larvae fed the diet without tannin and treated with 200 µl of *P. alvei*. The lowest mortality was observed in larvae fed on diets containing 5 % TA and diets treated with 100 µl of *P. alvei* (Table 2).

According to the results of the TUKEY test, the mortality rates of all diets were different (df11,24, F=302,797, p<0.01). As the data were normally distributed, the effect of *P. alvei* dose and dietary tannin concentration on mortality rate was determined by two-way ANOVA. While the effect of *P. alvei* alone was significant (F=14.73, p<0.01), no effect of tannin concentration alone on mortality was detected (p>0.05). However, the effect of *P. alvei* dose and tannin concentration on mortality was significant (F=168.22, p<0.01).

Table 2. Adjusted mortality rates using Schenider-Orelli's formula. No statistically significant difference is indicated by values with the same letter according to Tukey's HSD (0.05)

Diet	*Paenibacillus alvei* dose (µl)	Mortality (%)±Standard deviation
Control	-	12.37±0.12^{a}
	100	59.65±0.55^{b}
	200	85.73±1.29^{c}
1.25 TA	-	13.3±0.30^{d}
	100	57.05±1.03^{e}
	200	70.3±0.53^{f}
2.5 TA	-	40.63±0.60^{g}
	100	44.44±0.75^{h}
	200	58.96±0.49^{m}
5 TA	-	12.73±0.25^{n}
	100	42.99±0.37^{p}
	200	56.30±5.45^{r}

Discussion

Plants and pests are always in a relationship. In this relationship, plants develop physical and chemical defenses against herbivores. Secondary metabolites such as alkaloids, terpenoids, and tannins defend against pathogens and pests (Djilali et al., 2021). Tannins have larvicidal or deterrent properties in several insect orders, including Lepidoptera, Coleoptera, and Hemiptera (Hafeez et al., 2019; Tan et al., 2022). Tannins affect the development and fecundity of phytophagous insects. Ingestion through food or fumigation disrupts the integrity of the digestive system, resulting in death and malformation of the offspring (Djilali et al., 2021). In addition, phytophagous insects may reduce their food intake and activate their antioxidant and defense systems to protect their populations (Tan et al., 2022). The relative consumption rate (RCR) is often calculated as the weight of food consumed divided by the average body weight over the entire feeding period (Farrar et al., 1989). The highest RCR values of *A. alni* larvae were found in the larvae fed with 5% tannin and in the control group. The concentration of tannin in the larvae affected the RCR value of the larvae in the uninfected larvae. Similarly, the RGR values of the larvae increased with the tannin concentration. In herbivorous insects that cannot adapt to plant phenolics, toxic phenolics cause a decrease in RGR due to an inability to use the nutrient effectively, an inability to transform it, and intestinal paralysis (Deml et al., 1999). Although the RCR increased in the control diet containing no tannin, the RGR was low. However, in the diet containing 5% TA, the RGR and the RCR values are high. This result shows that the larvae are adapted to tannin and can use it. Larval mass and RGR values of gypsy moth larvae fed on tannin-containing oak and Robinia plants also decreased. Larvae use energy for development at the expense of the metabolic processes of tannins. Even if they are fed diets high in tannins for about 50 generations, their growth is negatively affected by tannins (Mrdakovic et al., 2013). The fact that the developmental time of larvae in the uninfected groups is not affected by the tannin concentration can be explained by adaptation.

There may be an antagonistic interaction between plant allelochemicals and the insecticidal activity of microorganisms. In this interaction, the feeding of herbivorous insects decreases, they ingest fewer microorganisms, and the insecticidal activity may decrease. Therefore, allelochemicals may reduce mortality. Conversely, allelochemicals may increase the insecticidal activity of microorganisms (Navon, 1992). In our study, in applications where tannin and *P. alvei* are present, RCR and RGR values increase with rising tannin concentrations and mortality decreases. Although similar to the results of Navon (1992) in terms of decreasing mortality and insecticidal activity of microorganisms, it differs in terms of decreasing consumption and RGR. The increase in the metabolic cost of immunity due to the application of *P. alvei* (Ardia et al., 2012) caused an increase in the protein and carbohydrate intake of the larvae. As a result, consumption and the RCR may have increased. As the larvae adapted to tannin, their convertibility increased and the RGR increased. The increase in pupal mass with the increase in RGR is also an indicator of usability. However, as pupal mass is an indicator of fecundity (Czypionka & Hill, 2007), the increased yield may suggest that biocontrol should not be applied during periods of high tannin concentrations.

When *P. larvae*, the causative agent of American foulbrood disease, enter the bodies of *Apis mellifera* L., 1758 (Hymenoptera: Apidae) larvae, they multiply in the midgut cells of the larvae and cause intestinal infection (Krongdan et al., 2019). During the invasive phase of the infection, the peritrophic matrix is degraded, and *P. larvae* penetrate the midgut epithelium. The bacteria enter the larval hemocoel, resulting in the larval death (Müller et al., 2015). *Paenibacillus alvei* is also a secondary pathogen of American Foulbrood. Therefore, *P. alvei* cause damage to the intestine. Krongdang et al. (2019) suggested that the reason why the disease is less common in *Apis cerana* Fabricius, 1793 (Hymenoptera: Apidae) may be more resistance due to the substances they ingest with their diet. Mortality of *A. alni* larvae is also lower in the % 5TA diet. Barbehenn and Martin (1992) found that the peritrophic membranes of tannin-tolerant *Orgyia leucostigma* Smith, 1797 (Lepidoptera: Erebidae) larvae were not permeable to high molecular weight tannins, and less than 1% of the grain in the diet was adsorbed. Again, Barbehenn et al (2001) found that the midgut of *O. leucostigma* larvae had higher concentrations of ascorbate and glutathione against oxidative stress than tannin-sensitive *Malacosoma disstria* Hübner, 1820 (Lepidoptera: Lasiocampidae) larvae. In addition, Summers and Felton (1996) stated that the protection of the midgut epithelium is a result of the antioxidant properties of the peritrophic membrane. *P. alvei* germinate and multiply in the midgut of larvae. In cases where tannin concentration is high, the excess of ascorbate and glutathione concentration in tannin-adapted larvae protects the midgut lumen from oxidative stress. This reduces the toxic effect of the microorganism. Therefore, mortality may be lower in 5TA diets.

Our study has opened up new avenues for further research in this area. Phenolic compounds can induce the antioxidant system. In our study, while tannin concentration caused a decrease in SOD and PO activities in the uninfected groups, an increase in CAT and GPx activities was observed. This result differs from the literature (Korayem et al., 2012; Tanet al., 2022). According to Korayem et al. (2012), *A. mellifera* increased SOD activity with phenolic compounds. Tannin stress inhibits antioxidant capacity in *Hyphantria cunea* Drury, 1793 (Lepidoptera: Erebidae) larvae. Catalase activity decreases with tannin stress (Tan et al., 2022). The presence of ROS and the deterioration of the antioxidant system indicate the presence of plant secondary metabolites with toxic effects. These findings not only contribute to our understanding of the effects of tannins and microorganisms on herbivorous insect larvae but also point to the need for further research in this area to fully understand the complex interactions involved.

Superoxide dismutase (SOD) is the first enzymatic defense against ROS. SOD enzymes convert negatively charged oxygen molecules to hydrogen peroxide, which is subsequently destroyed by catalase (Parker et al., 2004). Induction of SOD activity which rapidly destroys superoxide radicals appears to be the primary response to dietary pro-oxidant exposure (Ahmad & Pardini, 1990). Consumption of pro-oxidant compounds increases SOD and catalase activity. Tannins are also considered to be pro-oxidants. Tannin pro-oxidant activity and toxicity in herbivores is likely when tannins oxidise to form high levels of semiquinone radicals and quinones (Barbahenn & Constabel, 2011). In addition, the uptake of secondary metabolites is based on adaptation to a secondary metabolite involving antioxidant and detoxification enzymes (Tan et al., 2022). The decrease in SOD activity with tannin exposure in *A. aln*i larvae indicates that tannin is not a pro-oxidant for *A. alni* larvae and that they adapt to tannins. As catalase is one of the detoxification enzymes, the increase in catalase activity also shows the adaptation of larvae to secondary metabolites. An increase in tannin concentration and a decrease in phenoloxidase activity were observed in uninfected larvae. This result was reported by Sagona et al. (2021) and is in agreement with the present results. On the other hand, according to Sagona et al. (2021), tannin concentration decreased phenoloxidase activity in *A. mellifera*. Liu et al. (2010) found that tannin inhibited the phenoloxidase activity of *Spodoptera exigua* Hübner, 1808 (Lepidoptera: Noctuidae) larvae.

Enzyme activity increased with both presence and dose of *P. alvei* in infected groups. In addition, the mortality rate of larvae increased as the applied dose of *P. alvei* increased. The increase in enzyme activity also indicates that the immune system is stimulated. These results support the literature. Karthi et al. (2018) reported a significant increase in antioxidant enzymes of *Spodoptera litura* Fabricius, 1775 (Lepidoptera: Noctuidae) larvae infected with *Aspergillus flavus* Link (Eurotiales: Aspergillaceae).

Similarly, Ali et al. (2013) reported increased superoxide dismutase, catalase, and glutathione peroxidase activities in *S. exigua* larvae infected with *Isaria fumosorosea* Wize (Hyporeales: Cordycipitaceae). The antioxidant system is a defense mechanism against ROS production induced by pathogens and tannins. Various toxins released by microorganisms after bacterial or fungal infection cause activation of immune responses in insects or insect deaths (Lalitha et al., 2018). SOD is located in the mitochondrial intermembrane space and is the first enzyme to play a role in antioxidant defense. Baud et al. (2004) found that the high activity of catalase and low activity of GPx work in concert. However, catalase and GPx activities are directly proportional in both infected and uninfected groups of *A. alni* larvae. Jovanovic´-Galovic et al. (2004) found that catalase and GPx activities are directly proportional in *Ostrinia nubilalis* Hübner, 1796 (Lepidoptera: Pyralidae) larvae. Hydrogen peroxide is converted to water by either catalase or GPx enzymes (Baudet al., 2004). These enzymes are the cell's defense mechanism against hydrogen peroxide (Ali et al., 2013). Catalase is sensitive to high levels of H_2O_2, but GPx activity can also increase to maintain catalase activity. To overcome the cytotoxic effects of hydroxyl radicals and H_2O_2 under stressful growth conditions, both enzyme activities can be increased (Huarte-Bonnet et al., 2015).

Phenoloxidase is present in the haemolymph as an inactive zymogen. The increase in prophenoloxidase and phenoloxidase activity following the microbial or metazoan invasion is a stress response (Ashida & Brey, 1997). PO converts phenols to quinones and the subsequent production of melanin, which is essential for defense against bacterial (Gram + and −) and fungal infections (Eleftherianos & Revenis, 2011). In our study, the increase in *P. alvei* infection and the increase in phenoloxidase activity with its dosage is consistent with the literature.

In conclusion, the present study investigated the effect of *P. alvei* on the biocontrol of tannin-tolerant *A. alni* larvae. This research highlights the importance of considering secondary metabolites and tolerance to them in the biological control of pests, a crucial aspect that should be valued and respected in the fields of entomology, ecology and biological control. The results show that tannins, although generally considered toxic and repellent, can be tolerated or even utilized by adapted insect species such as *A. alni*, enhancing their growth and metabolic efficiency under certain conditions. Conversely, the entomopathogenic *P. alvei* significantly increases mortality and induces antioxidant and immune responses in larvae, the effects of which are modulated by the presence of tannins. The results suggest that the dual exposure to plant allelochemicals and microbial pathogens can have synergistic or antagonistic effects, depending on the physiological adaptations of the host insect. Furthermore, the observed changes in enzymatic activity highlight the importance of antioxidant and immune defenses in mediating insect responses to biotic stressors. These findings contribute to a better understanding of plant-insect-pathogen interactions and may inform future integrated pest management strategies, particularly regarding the timing and compatibility of botanical and microbial biocontrol agents.

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Türkiye Entomoloji Dergisi Yayın İlkeleri

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

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

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