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ARAŞTIRMA MAKALESİ / RESEARCH ARTICLE

PERSISTENCE OF TWO BACTERIAL BIOPESTICIDES ON *Bombus terrestris* L. AND TOMATO BLOSSOMS

Bombus terrestris L. ve Domates Çiçekleri Üzerinde İki Bakteriyel Biyopestisitlerin Kalıcılığı

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ABSTRACT

In the present study, the persistence of the biological control agent bacteria *Bacillus thuringiensis* var. *kurstaki* (Rebound, Hektaş) and *Bacillus velezensis* QST 713 (Serenade SC, Bayer) on tomato flowers and *B*ombus *terrestris* worker bees was determined after application. This study consists of two parts. In the first part, these bacterial agents were applied to tomato flowers under greenhouse conditions. Suspensions prepared at the recommended field dose of each bacterial isolate were applied by spraying the ten flowering tomato plants under greenhouse conditions. Ten flowers from each treated tomato plant were collected 24, 48, and 72 hours after application. Surface sterilization was performed using 70% alcohol on tomato flowers collected from the greenhouse at various observation times. These flowers were then transferred separately to petri dishes containing Nutrient Agar (NA) and Sabouraud Dextrose Agar (SDA). In the second part, bacterial agents were applied to *B. terrestris* worker bees under laboratory conditions and then transferred to hives under field conditions. The field dose of each bacterial isolate was applied to 50 *B. terrestris* workers for 20 seconds (0.5 ml) using a system capable of spraying at 4 atm pressure. These workers were then transferred to a separate hive for each bacterial isolate. Re-isolation was carried out by mechanically killing ten worker bees taken from these hives at the 24th, 48^{th,} and 72nd hours. During the reisolation phase, worker bees were individually dissected in sterile distilled water after surface sterilization for each observation time. A drop of haemolymph obtained from the dissected insect was diluted 100 times with sterile water and then spread on Nutrient Agar. According to the results obtained from the study, it was determined that *B. thuringiensis* var. *kurstaki* and *B.velezensis* QST 713 were found in both tomato flowers and the haemolymph of *B. terrestris* worker bees at 24, 48, and 72 hours after application.

Key Words: Entomopathogen, Spore viability, Biocontrol agent, Bumblebee

ÖZ

Çalışmada biyolojik mücadele etmeni olan bakterilerden *Bacillus thuringiensis* var. *kurstaki* (Rebound, Hektaş) ve *Bacillus velezensis* QST 713 (Serenade SC, Bayer)'in uygulandıktan sonra domates çiçekleri ve *B. terrestris* işçi arılarında kalıcılığı belirlenmiştir. Çalışma iki kısımdan oluşmaktadır. Birinci

kısımda bu bakteriyel etmenler domates çiçeklerine sera koşullarında uygulanmıştır. Sera koşullarında 10 adet çiçekli domates bitkisine her bir bakteri izolatına ait arazi tavsiye dozunda hazırlanmış süspansiyonlar püskürtme yöntemi ile uygulanmıştır. Uygulamadan sonra 24., 48. ve 72. saatlerde uygulama yapılmış domates bitkilerinden her bir izolat için onar adet çiçek toplanmıştır. Seradan farklı gözlem zamanlarında alınan domates çiçeklerine %70'lik alkol ile yüzey sterilizasyonu uygulanmıştır. Daha sonra bu çiçekler Besin Agarı (NA) ve Sabouraud Dekstroz Agarı (SDA) içeren petri kaplarına ayrı ayrı aktarılmıştır. İkinci kısımda ise, *B. terrestris* işçi arılarına laboratuvar koşullarında uygulama yapıldıktan sonra işçi arılar arazi koşullarında kovan içerisine aktarılmıştır. Her bir bakteri izolatının arazi dozu 50 adet *B. terrestris* işçi bireyine 4 atm basınçta püskürtme yapabilen sistem yardımıyla 20 sn süreyle (0,5 ml) uygulanmıştır. Bu işçi arılar her bir bakteri izolatı için ayrı birer kovan içerisine aktarılmıştır. Bu kovanlardan 24., 48. ve 72. saatlerde alınan onar adet işçi arı mekanik yöntemle öldürülerek re-izolasyon gerçekleştirilmiştir. Re-izolasyon aşamasında, her bir gözlem zamanı için işçi arılar ayrı ayrı yüzey sterilizasyonu yapıldıktan sonra steril damıtılmış suda disekte edilmiştir. Disekte edilen böcekten elde edilen bir damla hemolenf 100 kez steril su ile seyreltildikten sonra Nutrient Agar'a yayılmıştır. Çalışmadan elde edilen sonuçlara göre; *B. thuringiensis* var. *kurstaki* ve *B. velezensis* QST 713'in uygulandıktan 24, 48 ve 72 saat sonra hem domates çiçeklerinde hem de *B. terrestris* işçi arılarının hemolenfinde bulunduğu saptanmıştır.

Anahtar kelimeler: Entomopatojen, Spor canlılığı, Biyokontrol etmeni, Bombus arısı

GENİŞLETİLMİŞ ÖZET

Amaç: Bu çalışma kapsamında örtü altı üretim alanlarında domates üretiminde ekonomik kayıplara neden olan zararlı ve hastalık etmenlerine karşı kullanılan *B. thuringiensis* var. *kurstaki* ve *B. velezensis* QST 713'ün çiçek ve bu çiçekleri ziyaret eden ve maruziyet yaşayabilecek tarlacı işçi arılar üzerinde canlı kalma gücünün belirlenmesi hedeflenmiştir.

Gereç ve Yöntem: Çalışmada biyolojik kontrol etmeni olan bakterilerden 16000 IU/mg *Bacillus thuringiensis* var. *kurstaki* (Rebound, Hektaş) ve % 1.34 *Bacillus velezensis* QST 713 (Serenade SC, Bayer) kullanılmıştır. *Bombus terrestris* kolonileri özel bir firmadan temin edilmiş ve bu kolonilerden deneylerde yer alan bireylerin yetiştiricilikleri ile ilgili uygulamalar Arıcılık Araştırma ve Uygulama Laboratuvarında (Isparta Uygulamalı Bilimler Üniversitesi, Ziraat Fakültesi, Zootekni Bölümü) 27-28 °C ve oransal nemi %50-60 olan bombus arısı yetiştirme kabinlerinde gerçekleştirilmiştir. Bu çalışma sera ve açık arazi koşullarında olmak üzere iki aşamadan oluşmaktadır. Sera koşullarında yetiştirilen domates bitkileri çiçeklendiğinde Rebound (150 g /100 L su) ve Serenade (1400 mL /da) arazi tavsiye dozunda hazırlanmış ve süspansiyonlar çiçekli domates bitkilerine püskürtme yöntemi ile uygulanmıştır. Her bir biyopestisit için onar adet bitkiye ayrı ayrı uygulama yapılmıştır. Uygulamadan sonra 24., 48. ve 72. saatlerde bakteriyel biyopestisitlerin uygulandıktan sonra kalıcılığının belirlenmesi amacıyla uygulama yapılmış domates bitkilerinden her bir biyopestisit için onar adet çiçek toplanmıştır. Her bir gözlem zamanı için alınan domates çiçekleri %70 alkol içinde yüzeysel sterilizasyonu yapılarak ve alkol kalıntılarını gidermek için üç kez steril su ile yıkanmıştır. Daha sonra sırasıyla Nutrient agar (NA) ve Sabouraud Dextrose Agar (SDA) içeren petrilere ayrı ayrı inokule edilmiştir. Çalışmanın ikinci aşamasında, her bir biyopestisit 50 adet *B. terrestris* işçi bireyine 4 atm basınçta püskürtme yapabilen sistem yardımıyla 20 sn süreyle (0,5 ml) uygulanarak uygulama yapılan bireyler ayrı ticari kovanlara aktarılmıştır. Uygulama yapılmış bireylerin yer aldığı Bombus kovanlarının açık arazi (alan) koşullarında kalması sağlanmıştır. Bu kovanlardan 24., 48. ve 72. saatlerde onar adet işçi arı alınarak mekanik yöntemle ölmesi sağlandıktan sonra re-izolasyon protokolü uygulanmıştır.

Bulgular ve Sonuç: Seranade ve Rebound adlı bakteri içerikli ticari biyo-preparatların uygulandıkları ortamlarındaki canlılıkları incelenmiş ve her iki biyopestisitin uygulanmalarının ardından 72. saate kadar domates çiçeklerinde ve doğrudan üzerine uygulandıkları işçi arıların hemolenfinde bakterilere ait sporların canlılığını korudukları saptanmıştır. Elde edilen sonuçların, tarımsal üretim koşullarında çevre dostu, sürdürülebilir ve kalıntı problemi oluşturmayan yöntemlerin uygulamadaki etkililiğinin ortaya çıkarılması yönünde katkılar sağlayacağı düşünülmektedir.

INTRODUCTION

Bombus terrestris L. (Hymenoptera: Apidae) is native to temperate Eurasia and has spread worldwide since the 1800s. The spread of this species accelerated in the 1980s, when bees were artificially reared in Europe and provided commercially for greenhouse pollination services (Dafni 2010, Goulson et al. 2015). Under greenhouse conditions, bumblebees are more effective pollinators than other species for many Solanaceae plants, including tomatoes, eggplants, hot peppers, and bell peppers. This is attributed to their buzz-pollinating behavior, larger body surface area, more hair, and tolerance to abiotic conditions such as low temperature and light (Kraus et al. 2011, Zhang et al. 2022). *B. terrestris* is also an effective pollinator of many crops, particularly greenhouse crops, and is widely utilized commercially (Zhou et al. 2024). Given the well-documented role of synthetic pesticides in decimating pollinator populations worldwide (Brittain et al. 2010, Ndakidemi et al. 2016, Sponsler et al. 2019), the adoption of more sustainable strategies, such as biological control and integrated pest management (IPM) approaches in plant protection practices, has gained prominence. However, IPM strategies are not always clearly 'pollinator-friendly', and a new approach described as 'integrated pest and pollinator management' (IPPM) has recently been introduced. IPPM encompasses all strategies that minimize negative impacts on pollinators while promoting synergy between crop pollination, pest control practices, and ecosystem services (Cappa et al. 2022, Egan et al. 2020).

Ensuring the sustainability of agroecosystems during agricultural production has become an important issue worldwide. Therefore, the need to identify and develop biopesticides that can protect crops against harmful organisms while being both cost-effective and environmentally friendly is noteworthy. In the development of biocontrol agents, entomopathogenic microorganisms play an important role as environmentally friendly biopesticides and are considered an alternative to synthetic pesticides (Soumia et al. 2021). Declining pollinator populations are expected to increase in many world regions due to the higher Total Applied Toxicity (TAT) of newer-generation pesticides. These increased toxicities have been attributed to the use of insecticides such as pyrethrins and neonicotinoids (Basu et al. 2024, Schulz et al. 2021).

Since synthetic pesticides play a significant role in the decline of pollinator populations worldwide, there is growing interest in using biopesticides for more sustainable pest management. These biological control agents are generally considered safe for non-target species such as pollinators. (Cappa et al. 2022). Bacterial biopesticides based on *B. thrungiensis*, *B. velezensis* (formerly *B. amyloliquefaciens*), and their toxins are widely used for the biological control of insect pests and fungal diseases (Cappa et al. 2022). The intensive use of plant protection products containing *the B. velezensis strain QST 713 in various crops has raised concerns about the safety of these products* for non-target pollinators (Sabo et al. 2020).

The biopesticide exposure of bees in their habitats is almost always expected to be lower than the recommended maximum land-use dose of the applied pesticide. Additionally, it is thought that bees will experience shorter-term exposure to bacterial biopesticides, such as *B. thuringiensis*, compared to those of other chemical origins due to factors like temperature, humidity, and UV radiation (D'urso et al. 2017). It is reported that temperature negatively affects the spore viability and activity of biological control bacteria, thus leading to a decrease in the expected pesticide activity (Ignoffo 1992, Krebs et al. 1998). *Bacillus velezensis* is known to have antagonistic effects against various fungal phytopathogens (Abdalla et al. 2014, Jangir et al. 2019, Morsy et al. 2009). *Fusarium oxysporum* f. sp. *lycopersici*, also known as fusarium wilt in tomato, causes significant yield losses. *Bacillus velezensis* EPCO16 has been reported to produce volatile compounds that effectively inhibit the growth of *F. oxysporum* f. sp., reducing mycelial development of the pathogen by 46.04% (Ramyabharathi and Raguchander 2014).

The effectiveness of different strains of *B. velezensis* against this pathogen has been supported by numerous studies (Jangira et al. 2018, Ramyabharathi and Raguchander 2014, Shanmugam and Kanoujia 2011). *Bacillus thuringiensis* var. *kurstaki* is a safe biopesticide for controlling *Tuta absoluta*, one of the most significant pests affecting tomatoes (Alsaedi et al. 2017, Doğanlar et al. 2015, Topakçı and Keçeci 2017). Furthermore, significant reductions in the population of this pest and resulting yield losses have been achieved with Bt formulations in Spain (Alsaedi et al. 2017, Gonzalez-Cabrera et al. 2011). In this study, the viability of *B. thuringiensis* var. *kurstaki* and *B.*

velezensis QST 713, which are used to control diseases and pests that cause economic losses in tomato greenhouses, was evaluated on tomato flowers and worker bees that may be exposed to these flowers.

MATERIALS AND METHODS

In this study, *Bacillus thuringiensis* var. *kurstaki* (16000 IU / mg, 150 g /100 L water/ da) (Rebound®, Hektaş) and *Bacillus velezensis* QST 713 ($1x10^9$ cfu / mL, 1400 mL/ da) (Serenade® SC, Bayer) were used as biological control agents. *Bombus terrestris* colonies were obtained from a private company that commercially produces and markets them. The practices related to the breeding of the individuals included in the experiments from these colonies were carried out in bumble bee rearing cabins with a temperature of 27-28 °C and a relative humidity of 50-60% in the Beekeeping Research and Application Laboratory in the Department of Animal Science, Faculty of Agriculture, Isparta University of Applied Sciences.

Application of Biopesticide to Tomato Plants

Tomato plants were grown in the greenhouse of the Plant Protection Department of Isparta University of Applied Sciences. When the tomato plants grown under greenhouse conditions bloomed, Rebound (150 g/100 L water) and Serenade (1400 mL/da) suspensions were prepared at the recommended field dose and applied to the flowering tomato plants using spraying. For each biopesticide, bacterial suspensions were applied to ten randomly selected tomato plants in full bloom. To determine the persistence of bacterial biopesticides after application, ten flowers/each representing a different biopesticide, were randomly distributed among the appropriate numbers of tomato plants in the greenhouse and collected at 24, 48, and 72 hours after application. Tomato flowers were randomly collected from the greenhouse for each observation time, superficially sterilized in 70% alcohol in the laboratory, and then washed three times with sterile water. Then, they were inoculated separately into petri dishes containing Nutrient agar (NA) and Sabouraud Dextrose Agar (SDA), respectively. The inoculated NA medium was incubated at 37 °C for 48 hours, and the SDA medium was incubated at 25 °C for 48 hours (Basit et al. 2014).

Application of Biopesticide on *Bombus terrestris*

At this stage of the study, the bacterial suspension of each biopesticide was sprayed onto 50 *B. terrestris* workers at 4 atm for 20 seconds (0.5 mL). The bacterial suspension-treated worker bees were then transferred to field-maintained commercial hives for both biopesticides (one hive each). Ten worker bees were randomly removed from these hives 24, 48, and 72 hours after the treatment. A re-isolation protocol was followed, ensuring that these worker bees were mechanically killed.. According to this protocol, these worker bees were transferred individually to Falcon (50 mL) tubes containing 70% ethanol and gently shaken for 3 minutes. These worker bees were then washed three times with sterile distilled water (Kuzina et al. 2001, Yaman et al. 2002). According to this protocol, these worker bees were transferred individually to Falcon tubes (50 mL) containing 70% ethanol and gently shaken for 3 minutes. These worker bees were then washed three times with sterile distilled water (Kuzina et al. 2001, Yaman et al. 2002). One drop (50 µL) of haemolymph was collected from each dissected worker bee in sterile distilled water. This haemolymph was diluted 100-fold with sterile water and inoculated into NA and SDA. These media prepared for bacterial growth were incubated at 25-36°C for 24-48 hours (Thiery and Frachon, 1997). Methylene blue staining was used to visualize the bacteria under a microscope (Benzina et al. 2017, Larpent 1997, Singleton 2005)..

RESULTS

In this study, the life span of commercial biopreparations containing bacteria, called Seranade and Rebound, was examined in the environments where they were applied, and it was determined that the spores of the bacteria contained in them maintained their viability in tomato flowers and in worker bees to which they were directly applied until the 72nd hour after the application of both biopesticides (Table 1).

Table 1. Development status of biological control agent bacteria in the environment and the organisms where they were applied

Developmental status of the applied environment and organism						
Observation Hours	***Bacillus thuringiensis* var. *kurstaki***		***Bacillus velezensis* QST 713**		**Temperature (ºC)-Humidity (%)**	
	worker bee	tomato flower	worker bee	tomato flower	Greenhouse	Field
24th	●	●	●	●	28.8-69	26-53
48th	●	●	●	●	25.8-74	25-55
72nd	●	●	●	●	26.5-76	20-76

●: Bacterial growth is present

It was obtained from tomato flowers (60 flowers) collected in a tomato greenhouse at temperatures ranging from 25.8 to 28.8 °C and humidity levels of 69-76% (Figures 1, 2, 3).

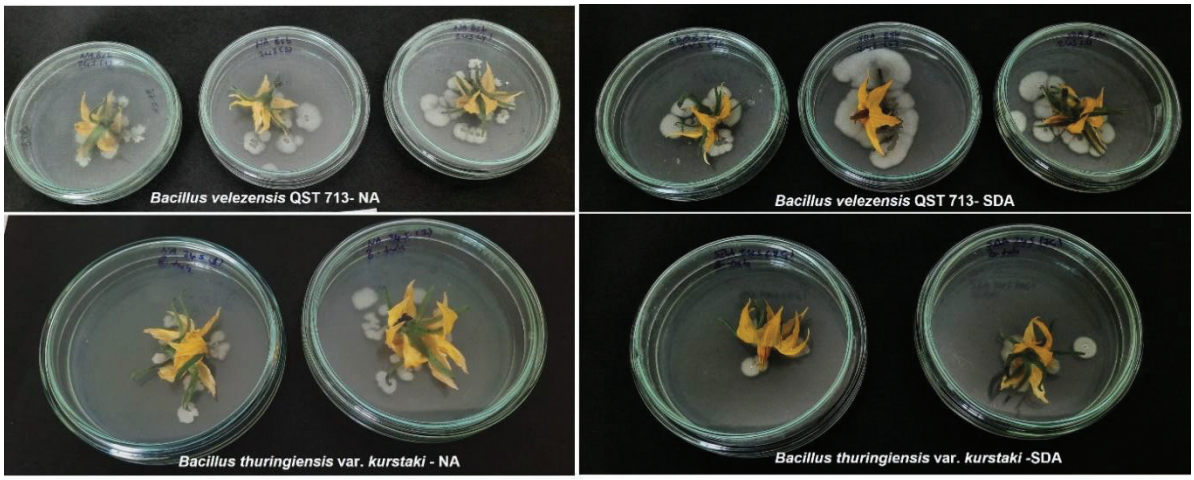

Figure 1. Growth of Gram +ve bacterium of *Bacillus thuringiensis* var *kurstaki* and Gram +ve bacterium of *Bacillus velezensis* QST 713 in tomato flowers sampled 24 hours after application.

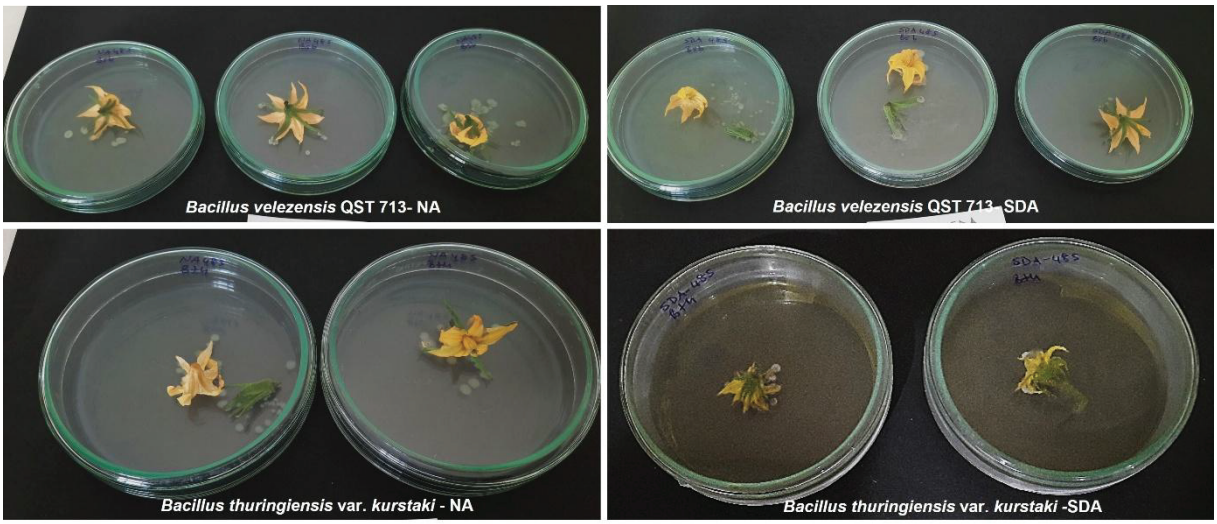

Figure 2. Growth of Gram +ve bacterium of *Bacillus thuringiensis* var *kurstaki* and Gram +ve bacterium of *Bacillus velezensis* QST 713 in tomato flowers sampled 48 hours after application.

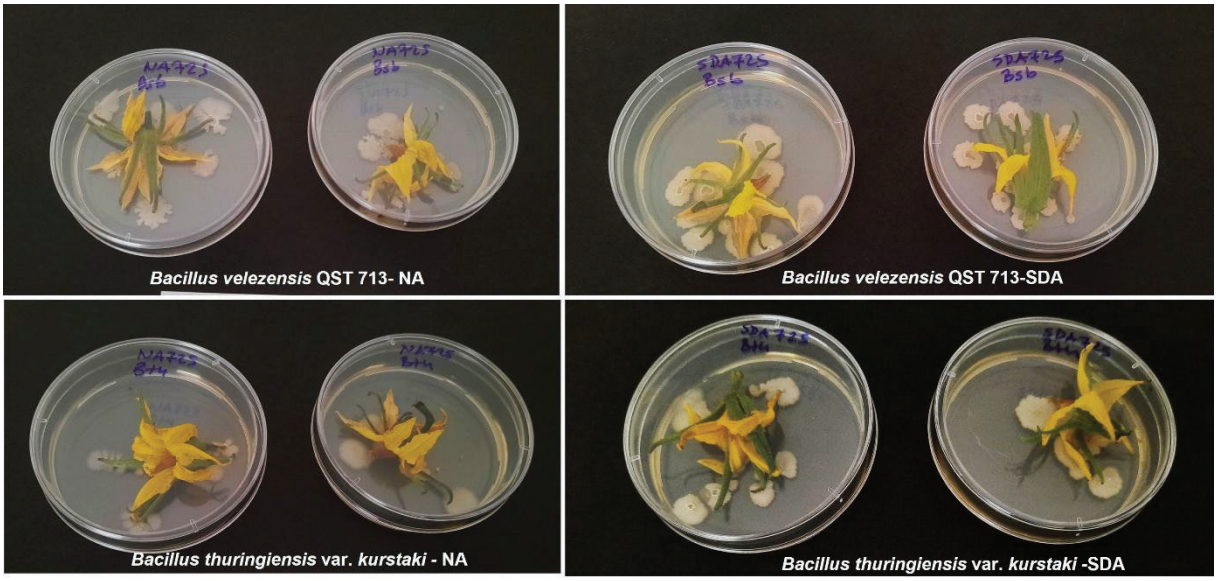

Figure 3. Growth of Gram +ve bacterium of *Bacillus thuringiensis* var *kurstaki* and Gram +ve bacterium of *Bacillus velezensis* QST 713 in tomato flowers sampled 72 hours after application.

Bacterial isolates were re-isolated from the haemolymph obtained from all samples taken from *B. terrestris* worker bees kept in separate hives for each bacterial isolate in the field, under temperature conditions of 20-26 °C and humidity levels of 53-76% (Figure 4).

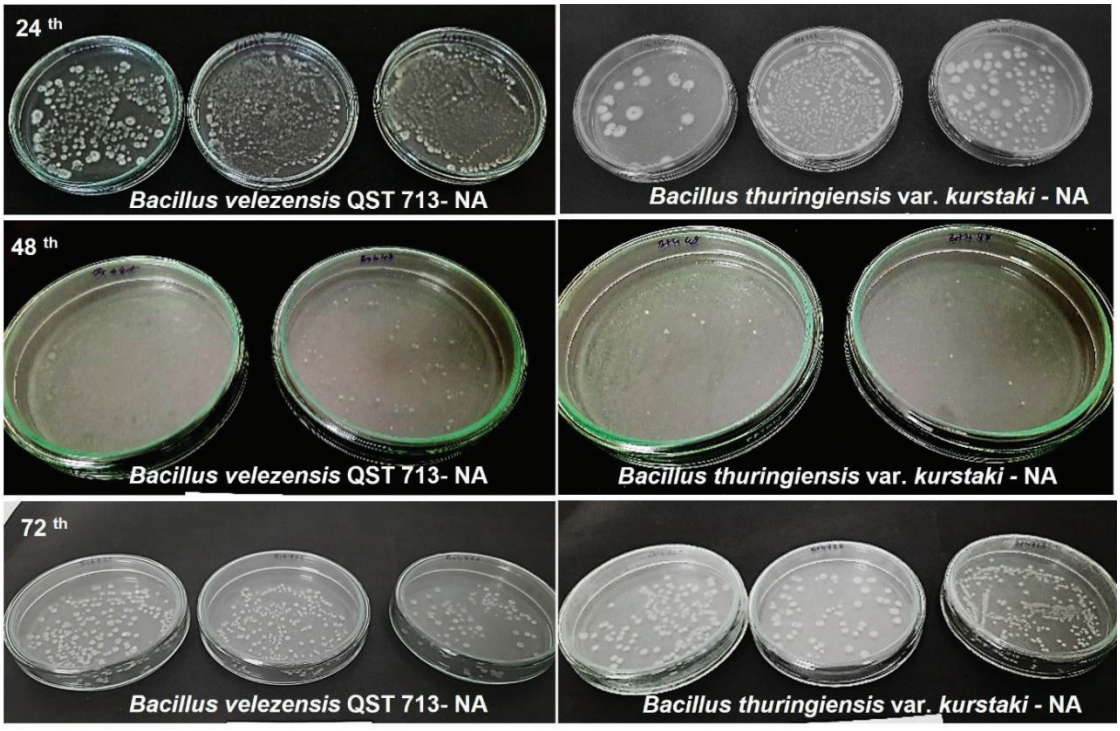

Figure 4. Growth of Gram +ve bacterium of *Bacillus thuringiensis* var *kurstaki* and Gram +ve bacterium of *Bacillus velezensis* QST 713 in the haemolymph of *Bombus terrestris* worker bees taken 24, 48, and 72 hours after application

It was determined that both bacterial isolates remained on flowers and *B. terrestris* workers until the 72nd hour (Table 1).

Spores and vegetative cells of *B. velezensis* QST 713 and *B. thuringiensis* var. *kurstaki* re-isolated from worker bees are given in Figure 5.

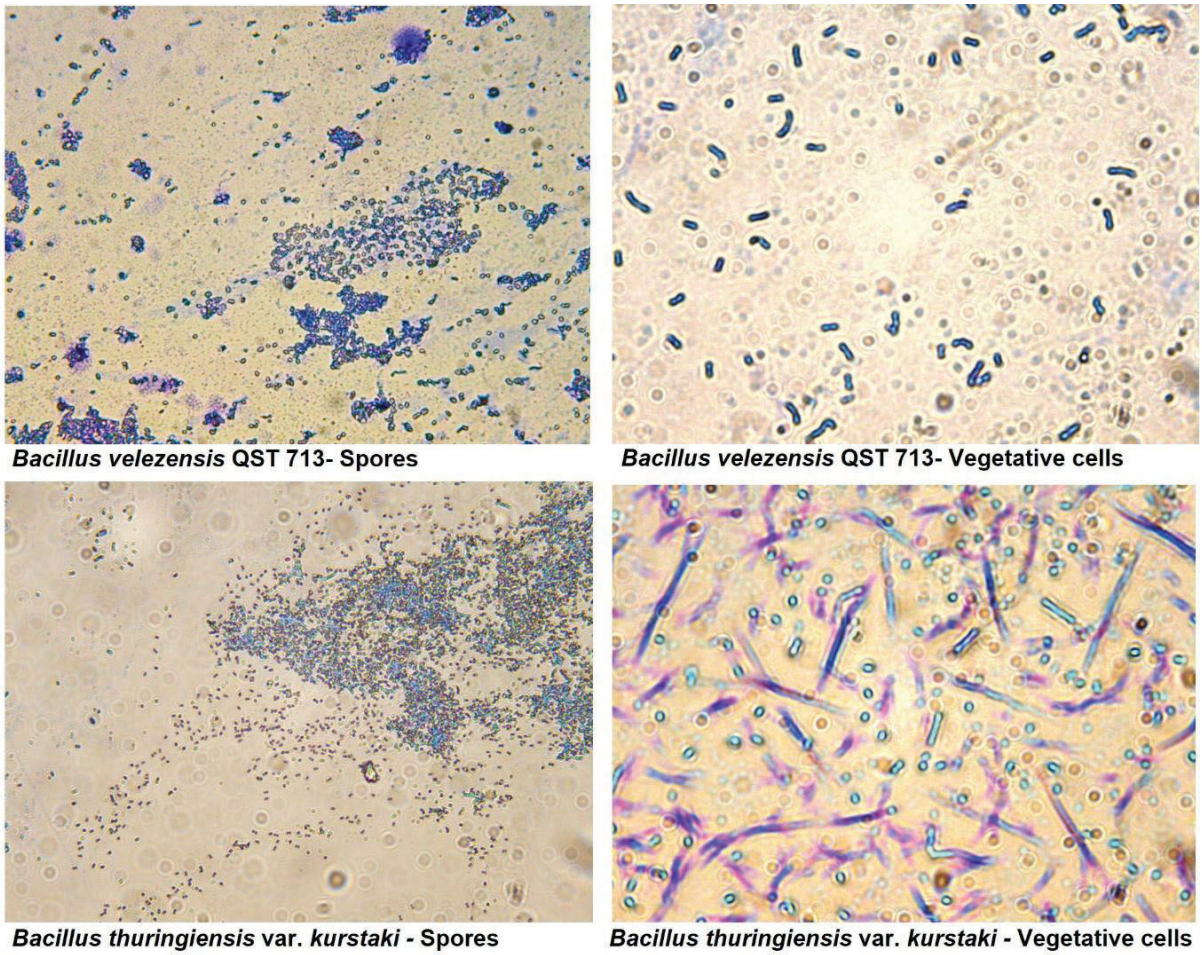

Figure 5. Spores and vegetative cells of Gram +ve bacterium of *Bacillus velezensis* QST 713 and Gram +ve bacterium of *Bacillus thuringiensis* var. *kurstaki* re-isolated from *Bombus terrestris* worker bees

DISCUSSION

All classes of biopesticides are known to cause less harmful effects on pollinator species, in addition to their direct toxicity. The current risk assessment approach is inadequate to accurately assess the potential adverse effects of these control agents on pollinators (Cappa et al. 2022). Exposure to *B. amyloliquefaciens* caused high mortality and reduced colony growth in *B. terrestris* after several weeks (Mommaerts et al. 2009b). The lethal or sublethal effects of Bt-derived Cry proteins (Cry1Ab and Cry1Ac) on *B. terrestris* were not determined (Babendreier et al. 2008, Morandin and Winston 2003). In addition, worker bee weights, pollen consumption, colony size, brood size, and the number of sexual bees produced were not affected by exposure (Babendreier et al. 2008). Commercial formulations based on different isolates of *B. thuringiensis* subsp. Bt. *kurstaki* (Btk) and Bt subsp. *aizawai* (Bta) are the most preferred biopesticides in organic and conventional agriculture to control Lepidoptera larvae (Bravo et al. 2011).

In practice, reapplication of these products is recommended at intervals of 3-8 days due to the

sensitivity of toxin crystals or spores to abiotic conditions such as UV (EFSA Biohaz Panel (EFSA Panel on Biological Hazards) 2016). Therefore, during field activities, bumblebees may be exposed to these biopesticides and may carry Bt-contaminated nectar and pollen to the colony. Pesticide formulations contain different compounds to protect spores and toxin crystals, especially in biopreparations (Brar et al. 2006). Previous studies have reported that Btk can still be detected on the leaf surface 72 hours after application (Bizzarri and Bishop, 2008, Raymond et al. 2010). The conditions inside the bee hive are quite different from those in the field, with no greater UV effect on spores and/or products, and no high humidity conditions (Alkassab et al. 2022). In the current study, it was determined that both *B. velezensis* QST 713 and *B. thuringiensis* var. *kurstaki* survived in tomato flowers for up to 72 hours under greenhouse conditions. Recently, the distribution of Bt spores within the colony was evaluated after an artificial in-hive feeding experiment, and it was found that Bt was present at different concentrations for 2-3 weeks (Steinigeweg et al. 2021).

Although the widespread use of Bt-based plant protection products has been approved worldwide, few studies show the persistence of Bt spores after applying Bt-containing microbial control agents in the environment. These plant protection products are considered to have low persistence on leaves under field conditions, where the half-life of viable Bt spores is assumed to be between a few hours and up to 2 days (Pinnock et al. 1971, Ignoffo and Garcia 1978, Pedersen et al. 1995, Haddad et al. 2005). It is reported that the rapid degradation of Bt spores is associated with various abiotic factors, including UV radiation, temperature, and humidity (Dunkle and Shasha 1989, Ignoffo et al. 1974, Khorramvatan et al. 2014, Sansinenea et al. 2015).

Sunlight is probably the most important environmental factor that can negatively affect the persistence of entomopathogenic bacteria and commercial microbial pesticides. Temperatures ranging from approximately 10 to 40 °C, which are common in most agricultural ecosystems, generally do not adversely affect entomopathogens. It was reported that when *B. thuringiensis* was exposed to natural sunlight, spore viability decreased by up to 80% in 1 day, and its insecticidal activity varied between 1 and 5 days (Ignoffo 1992). In general, Btk loses 50% of its insecticidal activity within 1-3 days and has been reported to have longer residual activity (10 days) (Surgeoner and Farkas 1990). This enables repeated and targeted applications to manage pest populations, potentially reducing exposure time for non-target organisms (Young 2023). The lethal and sublethal effects of biological control agents of bacterial origin on non-target organisms, such as *B. terrestris*, have been supported by numerous studies (Cappa et al. 2022, Demirözer et al. 2023, Malone et al. 2007, Mommaerts et al. 2009a,b, Mommaerts et al. 2010, Ramanaidu and Cutler 2013).

Within the scope of this study, it was determined that bacterial preparations maintained their viability in *B. terrestris* worker bees exposed to bacterial preparations for up to 72 hours in the hive environment under field conditions. In addition, since bacterial preparations are used as pollinators in tomato greenhouses, considering that they feed on nectar in tomato flowers, the persistence of these bacteria in tomato flowers was investigated for up to 72 hours. It was determined that both bacterial control agents were present in tomato flowers taken from the greenhouse environment and *B. terrestris* workers taken from the hive environment 72 hours after the application.

Conclusion

While the persistence of these bacterial factors in greenhouse production areas under controlled conditions is desired to increase the effect of these bacterial factors on the target harmful organisms by remaining on the plant, the possibility of exposure of non-target *B. terrestris* worker bees that will visit these plants is one of the undesirable situations, considering that it may negatively affect the beneficial organism and harm pollination. It is thought that the obtained results will contribute to the effectiveness of environmentally friendly, sustainable, and residue-free methods in agricultural production conditions. It is believed that determining the effects of these plant protection products on non-target organisms will be an important resource in their safe use in practice when biological pesticides are preferred as an alternative to chemical pesticides.

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and AG; data collection was performed by AUY and IYB; data evaluation was performed by AUY. The manuscript was written by AUY. All authors read and contributed to the manuscript.

Conflict of interest: The authors declare that they have no conflict of interest.

Data availability: All data generated or evaluated during this study are included in this article.

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