



## Non-Target Effect of *Bacillus subtilis* on the Parasitoid *Drino inconspicua* (Meigen, 1830) (Diptera: Tachinidae)

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### MAKALE BİLGİSİ

Alınış tarihi:26/05/2025

Kabul tarihi:04/08/2025

**Anahtar Kelimeler:** *Bacillus subtilis*,  
*Biological control*, *Drino inconspicua*,  
*Neodiprionsertifer*, *Non-target effect*

DOI: 10.55979/tjse.1706739

### ABSTRACT

This study was conducted to inform those working on sustainable production and pest control that biological control agents may also have side effects on non-target organisms. The study area was pine forests of Burdur, Türkiye. The *in vitro* effects of *Bacillus subtilis* on *Drino inconspicua* was determined by applying different concentrations of bacterial suspensions on its host (*Neodiprion sertifer* larvae). This bacterial treatment managed to control 58% of the *N. sertifer* larvae on average. Some of the larvae survived because they stopped feeding before pupariation. All the detected *D. inconspicua* larvae died within the lowest concentration of bacterial treatment. The effects of biological agents must be evaluated in light of the undesirable outcome to non-target organisms on an ecosystem. Although biological control is the sustainable method with the least undesirable effects among pest and disease control methods, since nature has a very complex structure, the undesired effects must be investigated in detail. In this way, we will have the chance to be more sensitive towards living things that benefit humanity.

## *Bacillus subtilis*'in Parazitoid *Drino inconspicua* (Meigen, 1830) (Diptera: Tachinidae) Üzerindeki Hedef Dışı Etkisi

### ARTICLE INFO

Received: 26/05/2025

Accepted: 04/08/2025

**Keywords:** *Bacillus subtilis*, *Biyolojik mücadele*, *Drino inconspicua*,  
*Neodiprionsertifer*, *Hedef dışı etki*

DOI: 10.55979/tjse.1706739

### ÖZET

Bu çalışma, sürdürülebilir üretim ve zararlı kontrolü konusunda çalışanları, biyolojik mücadele etmenlerinin hedef dışı organizmalar üzerinde de olumsuz etkilere sahip olabileceği konusunda bilgilendirmek için gerçekleştirilmiştir. Burdur ili sınırlarında bulunan çam ormanları çalışma materyallerinin bir kısmının temin edildiği alanlardır. *Bacillus subtilis*'in *Drino inconspicua* üzerindeki *in vitro* etkileri, konukçu (*Neodiprion sertifer* larvaları) üzerine farklı konsantrasyonlarda bakteri süspansiyonları uygulanarak belirlenmiştir. Uygulama sonucunda, biyolojik mücadele etmeni bakteri, ortalama olarak *N. sertifer* larvalarının %58'ini öldürmüştür. Larvaların bir kısmı, pupa olmadan önce bakteri uygulanan çam iğneleriyle beslenmeyi bıraktıkları için hayatta kalmıştır. Tespit edilen tüm *D. inconspicua* larvaları, bakteriyel uygulamanın en düşük konsantrasyonunda ölmüştür. Biyolojik mücadele etmenlerinin etkileri, zararlı ile aynı ekosistemdeki hedef dışı organizmalara yönelik istenmeyen sonuçlar ışığında değerlendirilmelidir. Biyolojik mücadele, zararlı ve hastalık kontrol yöntemleri arasında en az istenmeyen etkiye sahip sürdürülebilir bir yöntem olmasına rağmen, doğada çok karmaşık ilişkiler bulunduğundan, istenmeyen etkilerin detaylı bir şekilde araştırılması önemlidir. Bu şekilde insanlığa fayda sağlayan canlılara karşı daha duyarlı olma şansına sahip olabiliriz.

### 1. Introduction

Application of synthetic pesticides has accelerated since their invention, promoting improved agricultural productivity (Popp et al., 2013). The variety of insect populations drastically decreased as a result, and synthetic chemicals gained popularity throughout time because of their toxicity spectrum and residual activity.

These pesticides were applied during entire growing season as well as throughout the whole storage period to deal with all insects. The promiscuous use of these

compounds leads to a rebound of pests, for elimination of their enemies and emergence of synthetic insecticide resistant pest populations. Subsequently, significant health and environmental problems caused by chemical residues in food, water, and air started to be identified (Moazami, 2019). The efficacy of these pesticides on beneficial organisms like pollinators, parasitoids and predators represent a certain part of the problems (Godfray et al., 2015; IPBES, 2016; Sánchez-Bayo et al., 2016; Guedes et al., 2016).

The demand for biocontrol solutions has increased recently, coinciding with the wide spread acceptance of integrated pest management (IPM) as the paradigm for crop protection of the future (Stenberg, 2017). Biocontrol solutions are mainly specific for targeted pests unlike most insecticides. Biopesticides offer less dangerous impacts on the environment, water quality and they are more environment friendly alternative in comparison to chemical pesticides (Moazami, 2019).

Biopesticides are used to control pest populations to acceptable levels rather than completely wipe targeted pest. Biopesticides should be quickly biodegradable, safe and less toxic, have more specific mode of action and target (USEPA, 2024).

In regions such as forests, where chemical control is difficult and application costs are high, the integration of biological control agents against pests increases the chance of success in control of pests over extended periods of time.

Pine sawflies (Hymenoptera: Diprionidae) represents the most persistent threat to conifers as population surges can result in extensive defoliation (Davis et al., 2023). Defoliation means removing not only a portion of the photosynthetic material but also chemicals such as growth hormones and this affects many vital functions (Çatal, 2011), causing substantial decreases in tree growth parameters (Carroll et al., 1993; Carus & Avcı, 2005; Carus, 2009).

It is almost impossible to prevent such a pest with only chemical control economically. This is where biological control comes into play. In their natural habitats, living things continue their lives in an orderly manner. However, today's environmental and climatic changes have partially disrupted this balance. In order to maintain the balance and prevent pest populations from causing epidemics, the natural enemies of harmful organisms must be supported. Tachinidae, with approximately 8,500 identified species (O'Hara, 2013) all are parasitoids of at least 15 orders of Arthropoda (Arnaud Jr, 1978; Stireman et al., 2006). Majority of tachinids parasitize holometabolous insect larvae, adult beetles alongside true bugs (Stireman et al., 2006; Cerretti, 2010; Cerretti et al., 2014; Stireman et al., 2019). *Drino inconspicua* is an ovolarviparous koinobiont endoparasitoid tachinid fly (Stireman et al., 2006). This bivoltine species is common in Europe and northern Africa. It parasitizes sawfly larvae in addition to various lepidopteran larvae feeding on coniferous and deciduous trees (Webber, 1932; Herting, 1960). *D. inconspicua*, a natural biological control agent of pine sawfly, recorded for the Turkish fauna by Avcı & Akıncı in 2016.

Another possible control method against pests in forest habitat is the introduction of entomopathogenic organisms. Positive results have been obtained in many studies (Mesquita & Lacey, 2001; Roy et al., 2008; Aqueel & Leather, 2013; Al-Momani et al., 2013; Portilla et al., 2017; Miranda-Fuentes et al., 2020; Miranda-Fuentes et al., 2021). *Bacillus subtilis* is one of the most well-known model organisms in green microbiology and is valued for its role in promoting sustainable practices and researching

ecological processes (Errington & Wu, 2017). Across diverse environments such as soil, plants, and animal digestive tracts *B. subtilis* is widely present by using various carbon sources for energy and playing an important part in essential ecological processes like nutrient cycling, bioremediation, waste management, biosynthesis of macromolecules, medicine, animal feeding and as entomopathogen in agriculture (Harwood et al., 2013; Mol et al., 2017; Su et al., 2020; Miljaković et al., 2020; Kalaycı Kara et al., 2021; Noa et al., 2021; Nordgaard et al., 2022; Bremer et al., 2023). In addition to these interesting features of this bacterial species, it should not be overlooked that many more different features may emerge.

The aim of this study was to find out the non-target effect of biological control agent species *B. subtilis*.

## 2. Material and Methods

The main material of the study was collected in May 2024, consisted of *Neodiprion sertifer* larvae and pine needles from forests around Burdur province. They were brought to the laboratory and placed in petri dishes (90 mm diameter) in groups of 10 individuals of sawfly along with some pine needles.

Bioassays carried out by exposing *N. sertifer* larvae to bacterial spore suspensions sprayed to pine needles, in order to simulate pest's natural intake of bacterial spores. Thus, all pest larvae were forced to consume bacterial spores with their natural diet. The bioassays performed under laboratory conditions at 25 °C, 55% relative humidity and natural photoperiod. Three replicates performed for each bacterial concentration and one control group is selected for each replication.

Isolates of the *B. subtilis* obtained from the roots of eggplant were first characterized according to their morphologic and biochemical parameters by the authors, then purified and identified by MALDI-TOF MS analyses in Hatay Mustafa Kemal University. The strain was cultured on LB broth (Lysogeny Broth is a rich medium containing peptone, yeast extract, NaCl and agar) in an orbital shaker (Biosan PSU 20i, Latvia) 180 rpm for 48 hours.

Bacterial suspensions administered into plastic petri dishes each containing 10 larvae and pine needles. Each one checked daily to assess mortality. The following range of bacterial spore concentrations tested:  $1 \times 10^8$ ,  $1 \times 10^7$ ,  $1 \times 10^6$ , spores/ml. Control group insects fed with untreated pine needles.

The encountered tachinid *D. inconspicua* larvae (Figure 1), which died after the death of its host, identified by comparing larvae of the stock populations, which has adults of *D. inconspicua* species. Larvae subjected to superficial sterilization. Then dissection of gut was performed under a binocular microscope (Leica EZ4, Germany). The gut placed in nutrient agar medium to clarify whether the cause of the deaths was *B. subtilis*. The bacterial strain confirmed according to its morphologic and biochemical parameters.



Figure 1. Dead parasitoid tachinid *D. inconspicua* larva (indicated by blue arrow) in *N. sertifer* larva (indicated by black arrow)

Şekil 1. *N. sertifer* larvası içerisinde (siyah okla gösterilmiştir) ölü parazitoid tachinid *D. inconspicua* larvası (mavi okla gösterilmiştir)

### 3. Results and Discussion

The majority of bacterial pathogens of arthropod-pests belong to Bacillaceae family. *Bacillus thuringiensis* (Bt), *B. sphaericus*, *B. cereus* and *B. popilliaere* received most attention. (Bt) and its toxins, the most successful microbial pesticides, which has dominated the biopesticide market worldwide up to date. Most studies indicated that the entomopathogenic bacterial activity were associated with production of metabolites acting as powerful insecticides such as vegetative proteins, toxins, enzymes and antibiotics (Oulebsir-MohandKaci et al., 2021).

Several studies have emphasized the entomopathogenicity of various *B. subtilis* strains, and this bacterial strain is an important source for potential biopesticides against dipteran species (Assié et al., 2002; Das & Mukherjee, 2006; Geetha & Manonmani, 2010; Manonmani et al., 2011; Torres et al., 2022).

As a result of the study, out of 120 *N. sertifer* larvae, 3 *D. inconspicua* larvae emerged after the hosts died. Continued to feed with inner body parts of *N. sertifer* larvae including head. While feeding within the head of the larvae of *N. sertifer*, all the 3 parasites died unexpectedly. The normal developmental period of the parasite larvae may have been interrupted because of the death of host larva in the last stages of development. The sources for parasite were not enough to complete its life cycle. The fact that the bacterial strains isolated from the digestive system of the parasite match the bacteria that kill the host larvae indicates that it is the main factor causing the death of the parasite. Unlike the feeding habits of other parasites in the same genus, it was determined that this species consume all the organs and even the structures inside the head in addition to the fatty tissue in order to complete its developmental processes. This feeding behavior may be triggered by the influence of bacterial treatment to host species.

Insecticidal activity of the *B. subtilis* strains on some dipteran species is known. Although an entomopathogen may be effective for one or a few orders of insects, targeted investigations must be conducted to more accurately evaluate its activity within each taxon. Diptera exemplifies an insect order characterized by species living in distinct habitats and establishing significantly various relationships with the communities inhabiting the same ecosystems (Pape et al., 2011). So, we applied *B. subtilis* strain to another taxon *N. sertifer* and achieved success. This bacterial treatment managed to control over 58% of the *N. sertifer* larvae on average.

However, remaining uninfected sawfly larvae became pupae, then adult sawflies emerge after 2 months (Table 1).

Table 1. Total, eclosion and dead *N. sertifer* pupae numbers after treatment

Çizelge 1. Uygulama sonrası toplam, ergin çıkışı ve pupada ölen *N. sertifer* sayıları

Groups	Total Pupae	Male and Female Eclosion	Dead Pupae
$1 \times 10^6$	9	6♂ 2♀	1
$1 \times 10^7$	13	9♂ 2♀	2
$1 \times 10^8$	15	7♂ 2♀	6
Control	12	5♂ 5♀	2

We can conclude that bacterial treatment didn't affect the pupae or the larvae that didn't feed with *B. subtilis* just before pupariation. Timing of treatments must be evaluated according to life tables of the pest.

Undoubtedly there is positive effect of *D. inconspicua* within this success (%0,4). However, the host immune evasion strategies of the *D. inconspicuoides* against bacterial treatment (Yamashita et al., 2019) seems to be different in *D. inconspicua*. This may be due to different feeding behaviors between species. It is clear that *D. inconspicua* consumed all of the host's internal organs and therefore suffered from the effects of bacterial treatment. *D. inconspicua* larvae emerged only within *N. sertifer* larvae treated with  $1 \times 10^6$  concentration. We conclude that the higher dose of applications may have greater effect on the larvae of parasite so they couldn't even emerge.

The safety and environmental effects of entomopathogenic bacteria must be evaluated in light of the danger to non-target organisms on an ecosystem (Lacey & Siegel, 2000). Further research is needed to understand the specific mechanisms that lead to different degrees of susceptibility of non-target insect species to pathogens. As we can conclude, non-target effects may occur in biological control methods too.

#### 4. Acknowledgements

We would like to thank Assoc. Prof. Dr. Fatma Gül GÖZE ÖZDEMİR and Dr. Şükran OĞUZOĞLU for encouraging us.

#### Conflict of Interest

The authors declared that there is no conflict of interest.

#### Author Contributions

The authors declare that they have contributed equally to the article.

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