Parasitoids and entomopathogens allied in biocontrol: the case of *Tuta absoluta*

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Abstract

Biological pest control is an environmentally-friendly alternative to synthetic pesticides, using organisms such as viruses, bacteria, fungi, and parasitoids. However, efficacy is variable and combining different biocontrol agents could improve success rates. To date, little is known about the compatibility and the efficacy of parasitoid wasps and microbial agents when used in combination. To fill this gap, we conducted a systematic review of studies combining a parasitoid with an entomopathogenic virus, bacteria or fungus. We hypothesised that the effects could be positive and that more efficient pest control could be achieved through combining agents. We searched in Web of Science and extracted data from 49 publications matching the pre-defined inclusion criteria. Combinations of 36 hymenopteran parasitoids with 17 entomopathogenic microorganisms used to control 31 target pests were found. *Trichogramma pretiosum* and *Encarsia formosa* were the most frequently studied parasitoids, while *Beauveria bassiana*, *Metarhizium anisopliae*, *Lecanicillium muscarium*, *Bacillus thuringiensis* var. *kurstaki*, the Spodoptera exigua multiple nucleopolyhedrovirus, and the Spodoptera frugiperda multiple nucleopolyhedrovirus were the main microbial agents assessed. Out of 49 parasitoid-microorganism combinations assessed in the laboratory experiments, 38 were reported as compatible and six as incompatible. Timing and dosage of biopesticides played a crucial role, with later application and appropriate dosage minimizing adverse effects on parasitoid development. Combinations, including parasitoids and fungi, were well represented in the literature. In contrast, few experiments combined bacteria (11%) and viruses (9%) with parasitoids highlighting a need for further research into these types of combinations. Moreover, most of the reviewed experiments (84%) were carried out in the laboratory.

In the light of these results, we then looked more closely at the case of the South American tomato leafminer. *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) is a major threat to worldwide tomato production, and sustainable management methods are needed. Phthorimaea operculella granulovirus (PhopGV) and the parasitoid wasp *Necremnus tutae* Ribes & Bernardo (Hymenoptera: Eulophidae) were identified as promising biocontrol agents of this pest. Since the virus is most effective against first instar larvae, whereas the parasitoid targets older ones, we hypothesised that combining both agents could result in increased pest control. We conducted a replicated semi-field experiment to evaluate the control efficacy of *N. tutae* and PhopGV when used alone and in combination against *T. absoluta* and their compatibility over an entire growth period. Four treatments were compared: 1) *T. absoluta* only (control, CO); 2) *T. absoluta* + *N. tutae* (PA); 3) *T. absoluta* + PhopGV (VI); 4) *T. absoluta* + *N. tutae* + PhopGV (VP). On day 0, five potted tomato plants cv. Admiro (De Ruiter) were placed in 28 walk-in cages and 50 *T. absoluta* pupae were released in each cage to build an initial population. The tomato plants in treatments VI and VP were sprayed at a high PhopGV dosage (0.015%, $\geq 3 \times 10^9$ OB/l) upon their introduction, on day 4 and on day 11, and at a low dosage (0.0015%, $\geq 3 \times 10^8$ OB/l) on days 28 and 42. All further plants introduced into these treatments were sprayed at high dosage on the day of their introduction. Plants in treatments CO and PA were sprayed with the equivalent amount of water. On day 16, thirty couples of adult parasitoids were released in each plot of treatments PA and VP. A second release of 30 parasitoid couples was conducted on day 41. During the 9 weeks long assay, we systematically recorded the number of *T. absoluta* larvae, and measured plant damage on a weekly basis. The number of adult *T. absoluta* and *N. tutae* was recorded once at the end of the assay.

Combining the two agents achieved the highest larval (-24%) and crop damage (-29%) reduction cumulated over the entire experiment. However, there was no significant additive effect of the two agents. At the end of the trial, which coincided with the emergence of the third generation of *T. absoluta* adults, using the virus alone resulted in the strongest reduction of adult density (-78%), followed by the virus-parasitoid combination (-59%). No negative effect of the virus was recorded on the parasitoid population. Our results demonstrate the compatibility and potential of *N. tutae* and PhopGV to reduce population growth and crop damage of *T. absoluta* under semi-field conditions. Predictive models show that biocontrol intervention timing and intensity are crucial for success and cost efficiency. Therefore, further research should be conducted to determine the appropriate doses of *N. tutae* and PhopGV when applied at different times and on different population densities of *T. absoluta*. Moreover, new experiments under field conditions are necessary to include agronomic and financial parameters in the final compatibility assessment. While more research is needed, our findings provide important insights into an innovative combination of biocontrol agents, thereby contributing to more sustainable agriculture.

Keywords: biological control, integrated pest management, compatibility, baculovirus, *Phthorimaea absoluta*

1 General introduction

Demographic growth and climate change pose serious challenges for the agricultural sector: global food demand rises, while warmer temperatures lead to increased pest pressure in agricultural crops (Skendžić et al. 2021; van Dijk et al. 2021). Simultaneously, synthetic pesticides, largely used for crop protection, face growing controversy due to adverse effects on the environment and human health (Yadav and Devi 2017; Sharma et al. 2020). Alternative sustainable methods for pest control are sought. Biological control is an alternative to synthetic pesticides with fewer adverse environmental effects (Collatz et al. 2021). Stenberg et al. (2021) define it as "the exploitation of living agents (incl. viruses) to combat pestilential organisms (incl. pathogens, pests, and weeds) for diverse purposes to provide human benefits". Among the most used organisms in biological control are microorganisms such as entomopathogenic bacteria, fungi, and viruses, and macroorganisms such as parasitoids (Eggleton and Belshaw 1992; Lacey et al. 2015; Qu et al. 2022). The success of biological control relies on multiple biotic and abiotic factors (Gillespie et al. 2016; Sabbahi et al. 2022). As some of these are difficult to manage, biocontrol efficacy and reliability can be variable. Combining different biocontrol agents could help to minimize that problem (Roy and Pell 2000; Cossentine 2009; Quesada-Moraga et al. 2022). To explore this topic, the case study of the South American tomato leafminer was considered.

About 190 million tons of tomatoes (*Solanum lycopersicum* L.) are produced annually on five million hectares worldwide (FAO 2023). *Tuta absoluta* Meyrick (Lepidoptera: Gelechiidae) is a major threat to this essential cash crop, relied on by many people (Bergougnoux 2014). It causes up to 100% yield loss in all tomato production systems, ranging from soil grown open-field crops to high-tech greenhouse crops (Desneux et al. 2010; Desneux et al. 2011; Biondi et al. 2018). Pest control with synthetic insecticides, largely used to control *T. absoluta* (Desneux et al. 2010; Desneux et al. 2022), is unsustainable as the moth species rapidly develops high levels of resistance to many chemical compounds (Guedes et al. 2019). Moreover, synthetic insecticides harm key beneficial arthropods and disrupt existing integrated pest management programmes (Abbes et al. 2015; Soares et al. 2019). Phthorimaea operculella granulovirus (PhopGV, Baculoviridae: Betabaculovirus) and the parasitoid *Necremnus tutae* Ribes & Bernardo (Hymenoptera: Eulophidae) were identified as interesting biocontrol agents against *T. absoluta*. PhopGV has a good level of pathogenicity against the first instars of *T. absoluta* larvae and causes sublethal effects such as retarded larval growth and pupation failure in the older larval instars (Mascarin et al. 2010; Gómez Valderrama et al. 2018). *N. tutae* parasitizes and feeds on second to fourth instar larvae of the pest with a distinct preference for the third instar (Calvo et al. 2013). It causes additional mortality by host-killing (i. e. host-feeding, host-stinging). Augmentative releases of *N. tutae* alone seem insufficient to keep *T. absoluta* damage on tomato plants below the economic threshold (Cocco et al. 2015a; Calvo et al. 2016; Desneux et al. 2022). Regarding PhopGV, high doses (weekly application of the highest recommended concentration) are required to lethally infect *T. absoluta* larvae. In addition, the most damaging older larvae are difficult to reach due to their location inside the mines, so complementary control measures are required (Gonthier et al. 2023a). Combining both biocontrol agents could increase pest control against *T. absoluta*. The virus is more effective on young larvae, while the parasitoid targets the later instars. In addition, no adverse effects of PhopGV were detected on the development of *N. tutae* under controlled conditions, indicating that both agents are compatible for combined use (Gonthier et al. 2023a).

We first conducted a systematic review of literature aiming to synthesize the current state of the art when combining a parasitoid with an entomopathogenic microorganism. Combining parasitoids and microorganisms may be either positive, negative or with no effect on pest control compared with their use alone. We hypothesised that effects can be positive and more efficient pest control can be achieved through combining agents. We therefore assessed: 1) Which are the most studied combinations of parasitoids and microorganisms? 2) Which combinations of microorganisms and parasitoids are compatible? 3) How do microorganisms influence the life table parameters of parasitoids? 4) Which key factors influence the compatibility of microorganisms and parasitoids? 5) Can more efficient pest control be achieved by combining a parasitoid with a microorganism instead of using them individually? In the light of the results of this systematic review, we then conducted a replicated semi-field experiment to assess the efficacy of *N. tutae*, and PhopGV used alone and in combination for the biocontrol of *T. absoluta*. More precisely, we addressed the following research questions: 1) Do *N. tutae* and PhopGV influence the population development of *T. absoluta* when used alone or in combination? 2) Do *N. tutae* and PhopGV influence the level of crop damage caused by *T. absoluta* when used alone or in combination? 3) Is the *N. tutae* population influenced by PhopGV under semi-field conditions (effects on population size and sex ratio).

2 State of research: “Entomopathogens and parasitoids allied in biocontrol: a systematic review”

To date, numerous studies have been conducted to assess the efficacy of parasitoid wasps and entomopathogenic microorganisms for pest biocontrol. However, few is known about the compatibility and the efficacy of these two types of biocontrol agents when used combined. To fill this gap, we conducted a systematic review of studies combining a parasitoid with an entomopathogenic virus, bacteria or fungus. We published the following article on 20th July 2023 in the special issue “Application of insect viruses as biopesticides” in Pathogens. This journal focuses on pathogens and pathogen-host interactions and is published monthly online by MDPI. The article can be found under the following link: https://www.mdpi.com/2076-0817/12/7/957. Author guidelines can be found in Annex 1 and original data in Annex 2.

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Systematic Review

Entomopathogens and Parasitoids Allied in Biocontrol: A Systematic Review

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Abstract: Biological pest control is an environmentally friendly alternative to synthetic pesticides, using organisms such as viruses, bacteria, fungi, and parasitoids. However, efficacy is variable and combining different biocontrol agents could improve success rates. We conducted a systematic review of studies combining a parasitoid with an entomopathogenic microorganism, the first of its kind. We searched in Web of Science and extracted data from 49 publications matching the pre-defined inclusion criteria. Combinations of 36 hymenopteran parasitoids with 17 entomopathogenic microorganisms used to control 31 target pests were found. *Trichogramma pretiosum* and *Encarsia formosa* were the most frequently studied parasitoids, while *Beauveria bassiana*, *Metarhizium anisopliae*, *Lecanicillium muscarium*, *Bacillus thuringiensis* var. *kurstaki*, the Spodoptera exigua multiple nucleopolyhedrovirus, and the Spodoptera frugiperda multiple nucleopolyhedrovirus were the main microbial agents assessed. Out of 49 parasitoid–microorganism combinations assessed in the laboratory experiments, thirty-eight were reported as compatible and six as incompatible. Timing and dosage of biopesticides played a crucial role, with later application and appropriate dosage minimizing adverse effects on parasitoid development. More research is needed to assess compatibility and efficacy under real-world conditions. Our review provides valuable insights for researchers and practitioners to optimize the combined use of micro- and macroorganisms for effective pest control.

Keywords: pest management; microbial pesticide; parasitoid wasp; compatibility; synergism; antagonism

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1. Introduction

Biological pest control is an alternative to synthetic pesticides with fewer adverse environmental effects [1]. Among the most used organisms in biological control are microorganisms such as entomopathogenic bacteria, fungi, and viruses. *Bacillus thuringiensis* sp. (Bt) are the most widely used bacteria to control pests in agriculture, forestry, and public health [2]. They release toxins that cause cell lysis and death after binding with specific receptors in the insect midgut [3]. Entomopathogenic bacteria have a wide host range, mainly lepidopteran, dipteran, and coleopteran, but were reported to have minimal to no negative effects on beneficial organisms [3]. *Beauveria bassiana* (Balsamo) Vuillemin, *Metarhizium* sp., *Paecilomyces farinosus* (Holm ex S.F. Gray) Brown & Smith, and *Lecanicillium muscarium* Zare & Gams (previously known as *Verticillium lecanii* (Zimmermann) Viegas) are the most used fungi for insect biocontrol. They attack pests by damaging their integument or gut epithelium, using nutrients in their hemocoel, or releasing toxins [2,3]. They are ubiquitous in the environment, have a broad range of arthropod hosts, and often cause epizootics in insect populations. Compatibility with arthropod predators and parasitoids should be tested to ensure compatibility and maximize efficacy [3]. Approximately a dozen viral bioinsecticides are commercially available, and currently only nucleopolyhedroviruses and granuloviruses specifically target lepidopteran pests. Viruses enter the host cells, replicating in the nuclei or cytoplasm before causing cell lysis and sometimes enzootics [2,3]. Another key group for all types of biocontrol are parasitoids. Most of them belong to the orders of the Hymenoptera and Diptera, fewer to the orders of the Coleoptera, Lepidoptera and, Neuroptera [4]. Their larvae develop on or in the body of other arthropods and usually kill them by their feeding. Some parasitoids parasitize eggs, while others parasitize larvae, pupae, or even adults. *Trichogramma* sp. are the commercially most used parasitoids, which develop in the eggs of Lepidoptera [5,6]. *Encarsia formosa* is commonly used against whiteflies and *Aphidius colemani* against aphids [7].

The success of biological control relies on multiple biotic and abiotic factors. For example, entomopathogenic microorganisms are susceptible to climatic conditions such as temperature, humidity, and UV radiation [8]. Likewise, parasitoid longevity and efficacy rely on factors such as host density, nectar and pollen sources, habitat composition, and climatic conditions [9]. With some of these factors being difficult to manage, biological control can be of variable efficacy and reliability. Combining different biocontrol agents could minimize that problem (Figure 1). Roy and Pell [10] conducted a narrative review on interactions between entomopathogenic fungi (EF) and other natural enemies. They found that predators and parasitoids may foster the development of epizootics by vectoring EF and causing increased movement of infected hosts. Several key factors that influence potential antagonistic effects when combining parasitoids with EF were mentioned: (i) fungal dosage, (ii) relative timing of parasitism and fungal infection, and (iii) fungal identity. More recently, Quesada-Moraga et al. [11] reviewed the compatibility between EF and parasitoids with mixed results. Some studies concluded that parasitoids serve as vectors of EF, even showing synergistic interactions. Other studies found that previous inoculation with EF can impact the fitness of parasitoids, shortening their lifetime yet increasing oviposition rates. Most studies concluded that the combination is beneficial when, as underlined by Roy and Pell [10], release times are adjusted appropriately, with the timing and order of agent administration being crucial. Cossentine [12] reviewed the interactions between baculoviruses and parasitoids. In laboratory experiments, parasitoids could reduce the pathogenicity of baculoviruses in hosts. Yet, in field trials, parasitoids did not reduce the overall mortality caused by an applied baculovirus. Indeed, parasitoids can spread or accelerate the spread of the virus within hosts, increasing efficacy under field conditions. Baculoviral infections can lower parasitoid population densities, but many parasitoids can avoid or reduce their use of virus-infected hosts, and a strategically timed baculoviral biopesticide should have a low impact on host–parasitoid populations. The impact of Bt-bioinsecticides on parasitoids has been reviewed recently [13] with the conclusion that combining parasitoids with Bt-bioinsecticides could significantly increase crop yield and improve pest control. However, the impact of Bt on beneficial arthropods is still being studied due to the high number of Cry toxins untested against them. It is particularly important to consider indirect impacts of these products on parasitoid physiology and behaviour [13].

Given the increasing interest in biological pest control in the past decade and the growing importance of entomopathogen agents [14,15], a new and systematic review of the literature combining all different entomopathogens is timely. We aimed to synthesize the state-of-the-art when combining a parasitoid with an entomopathogenic microorganism. Combining parasitoids and microorganisms may be positive, negative, or with no effect on pest control compared to their use alone. We hypothesized that the effects could be positive and that more efficient pest control could be achieved through combining agents. We, therefore, assessed: (1) Which are the most studied combinations of parasitoids and microorganisms? (2) Which combinations of microorganisms and parasitoids are compatible? (3) How do microorganisms influence the life table parameters of parasitoids? (4) Which key factors influence the compatibility of microorganisms and parasitoids?

(5) Can more efficient pest control be achieved by combining a parasitoid with a microorganism instead of using them individually?

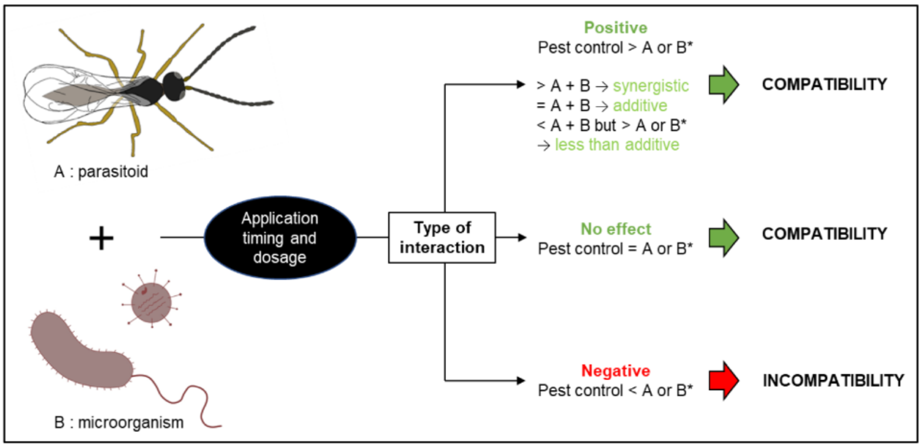

Figure 1. Types of interactions and factors influencing the compatibility of entomopathogenic microorganisms and parasitoids. * Comparison made with the more effective of agents **A** or **B**.

2. Materials and Methods

2.1. Search Criteria

We used the following search string in Web of Science Core Collection on 2 March 2023: ((fung* OR vir* OR entomopathog* OR "vir*-based insecticid*" OR "fung*-based insecticid*" OR "biological insecticid*" OR "microbial insecticid*" OR "natural insecticid*") AND (biocontrol OR biological control)) AND (*parasit* AND (biocontrol OR biological control)) AND (combin* OR interaction OR substitut* OR synergist* OR antagonist*) AND ("integrated pest control" OR "biological pest control" OR "pest biocontrol" OR "pest populations" OR pest OR "pest management" OR IPM). We did not use any date limitation but confined our search to journal articles in English.

2.2. Data Inclusion and Exclusion Criteria

We assessed articles by analysing abstracts following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA, [16]) (Figure S1). We obtained 547 initial hits, of which 121 were excluded as they were review articles. Based on other predefined exclusion criteria, 377 further articles were excluded. Inclusion of a publication was based on three criteria: (1) the study was an experiment concerning the biological control of a pest; (2) the experiment combined at least a parasitoid and an entomopathogenic fungus, bacterium, or virus; and (3) the study design included at least a no-treatment control. Studies combining entomopathogenic nematodes with parasitoids were excluded. Laboratory, semi-field, field, and greenhouse experiments were considered, but modelling and simulations were excluded.

2.3. Data Extraction

We used Citavi software (version 6.11.0.0) to import the included studies. We extracted data on (1) pest, (2) control agents, (3) crop, (4) location, (5) study design, (6) evolution of pest and biocontrol agent populations, (7) crop damage, (8) crop yield, and (9) compatibility of biocontrol agents from the 49 selected articles [17–65]. Each combination of biocontrol agents was considered an experiment, leading to 100 distinct experiments, as some publications studied multiple combinations simultaneously. Different strains of the same microorganism combined with one parasitoid were also counted as distinct experiments. Data from experiments assessing multiple dosages, timing of application, and types of exposition of the parasitoid to the microbial biocontrol agent were extracted as several observations. Each different treatment was considered as a single observation leading to the extraction of 484 individual observations.

2.4. Data Synthesis and Analysis

Most studies reported the effect of entomopathogenic microorganisms on the life table parameters of parasitoids. In these studies, parasitoids were the dependent variable. The impact of a treatment with entomopathogenic microorganisms was usually compared with a "no-treatment control" (parasitoids only). Data were synthesized by "vote counting" of the numbers of positive, neutral, and negative effects caused by the entomopathogen on each studied parameter of the parasitoid's fitness. We used "positive" when the microorganism treatment significantly improved the development of the parasitoid in comparison to the no-treatment control. "Negative" was used when the microorganism treatment significantly hindered the development of the parasitoid compared to the no-treatment control. "No effect" was used when no significant difference was found between the treatment and the parasitoid-only control. In a simplified way, combinations with positive or no effect were defined as compatible. However, in the reviewed papers, compatibility was assessed by an overall analysis of all the studied parameters. Being unable to synthesize all interconnected parameters for all reviewed papers, we reported compatibility based on the authors' conclusions. For example, if they mentioned that two biocontrol agents could be combined at a defined timing and dosage despite observed antagonistic effects under other conditions, we reported those as compatible.

When the effect on pest control was reported, "positive" was used to categorize when the pest reduction achieved by the combined biocontrol agents was higher than the reduction achieved by the strongest agent applied individually. Positive effect can be either "synergistic" when the pest reduction achieved by the combined biocontrol agents is higher than the addition of that achieved by each agent applied individually, "additive" when the pest reduction achieved by the combination is equal to the addition of that achieved by each agent used alone, or "less than additive" when the pest reduction achieved by the combined biocontrol agents is significantly higher than that achieved by each agent used alone, yet lower than additive. "No effect" was used when no significant difference was observed between the pest reduction achieved by the combined agents and that achieved by the strongest agent applied individually. "Negative" was used when the combined biocontrol agents achieved a lower pest reduction than the strongest agent used individually.

Among the publications selected for this review, reports on laboratory combination experiments were predominant. From those publications, we extracted life-history data on sixteen parameters for parasitoids, four parameters for pests, and five parameters for entomopathogenic microorganisms. For further analysis, we focused on the parasitism rate, emergence rate, mortality, sex ratio, and longevity of parasitoids, as these were the most documented parameters.

The packages ggplot2 [66], tidyverse [67], and webr [68] in RStudio (version 4.1.2), as well as Microsoft Excel (version 2208), were used to obtain descriptive statistics and to visualize data.

3. Results and Discussion

3.1. Scope of the Publications

Forty-nine studies detailing one hundred combination experiments were conducted from 2000 to 2022. Eighty-four were laboratory experiments and thus formed the focus of our results. In addition, nine were field experiments, five were semi-field experiments, and two were greenhouse experiments. All combination experiments included a no-treatment control as it was an inclusion criterion. Four experiments included additional controls with either the parasitoid (3) or microorganism (1) alone, and fourteen included both types of controls. Six experiments included a synthetic insecticide control in addition to the no-treatment control.

The reviewed studies dealt with 31 target pests. Approximately half of these were Lepidoptera, of which 52% were Noctuidae. The remaining were Hemiptera, Diptera, and Coleoptera (Figure 2). The studied biocontrol agents included 36 parasitoids and 17 entomopathogenic microorganisms. All parasitoids were Hymenoptera, and the most represented families were Braconidae (44%) and Trichogrammatidae (18%) (Figure 3). Most combination experiments were conducted with fungi (80%), followed by bacteria (11%) and viruses (9%) (Figure 4).

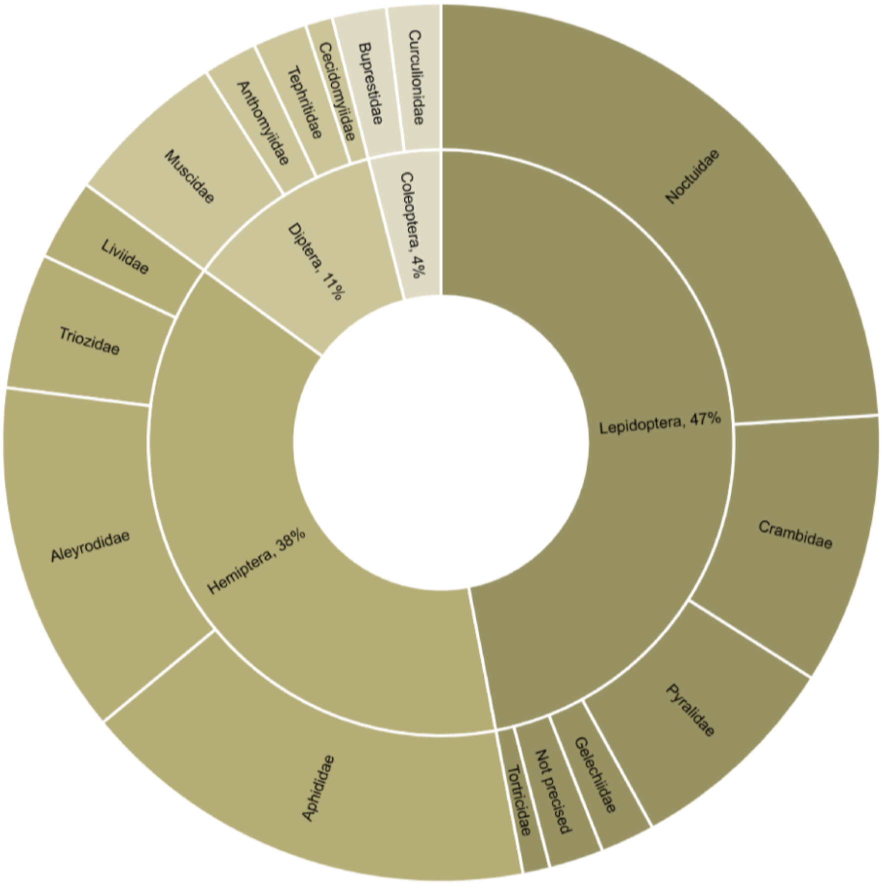

Figure 2. Order and family of target pests where combined biocontrol agents were used (n = 100 combination experiments from 49 studies).

3.2. Assessed Combinations of Biocontrol Agents in Laboratory Experiments

The conducted laboratory experiments reported entomopathogenic microorganisms' effects on parasitoid's life table parameters. In total, 49 combinations were tested in 84 laboratory experiments. While many experiments were conducted with fungi, few analysed the compatibility of viral and bacterial biocontrol agents with parasitoids (Table 1). *B. bassiana* was part of all the most frequent combinations with *Trichogramma pretiosum* [24,52], *Tamarixia triozae* [63,64], *E. formosa* [40,51], and *Trichogramma atopovirilia* [24]. *Metarhizium anisopliae* (Metschnikoff) used with *Cotesia flavipes* [55,58] was the next most assessed combination. All other combinations appeared in one to three laboratory experiments each. *Trichogramma pretiosum* [24,47,52] and *E. formosa* [29,38,40,51] were the most researched parasitoids, followed by *A. colemani* [23,30,32,35,46], *C. flavipes* [55,58], and *Diaeretiella rapae* [18,43]. In terms of microorganisms, *B. bassiana* [17,18,24,26,32,33,35,40,42,43,48,51,52,54,55,57–61,63–65] was the most studied fungus, followed by *M. aniso-*

pliae [30,34,36,37,48–51,53,55,57,58] and *L. muscarium* [18,23,38,41,46]. *Bacillus thuringiensis* var. *kurstaki* (Btk) [19,21,22,47,56] was the most frequently assessed bacterium. Among viruses, the *Spodoptera exigua* multiple nucleopolyhedrovirus (SeMNPV) [25,39,62] and the *Spodoptera frugiperda* multiple nucleopolyhedrovirus (SfMNPV) [27,28] were the most frequently tested in combination with parasitoids.

Figure 3. Family and species of hymenopteran parasitoids used in combination with an entomopathogenic microorganism (n = 100 combination experiments from 49 studies).

3.3. Reported Compatibility of Biocontrol Agents Assessed in Laboratory Experiments

Thirty-eight out of forty-nine combinations of biocontrol agents were reported as compatible [18–20,22,24–26,29,32,34–37,39,41,43–45,47,49,50,52–60,62–65] (Table 1). Six combinations were reported as incompatible [17,27,28,42,51], often due to a lower emergence rate caused either by bad timing, direct infection, or too high dosage. No answer about compatibility was given for the five remaining ones [30,38,54,56]. Divergent results reported from the combination of *L. muscarium* with *A. colemani*. Aqueel and Leather [23] found that these biocontrol agents interacted negatively. In contrast, Mohammed and Hatcher [46] reported them as compatible as long as the fungus was applied more than five days after parasitoid release.

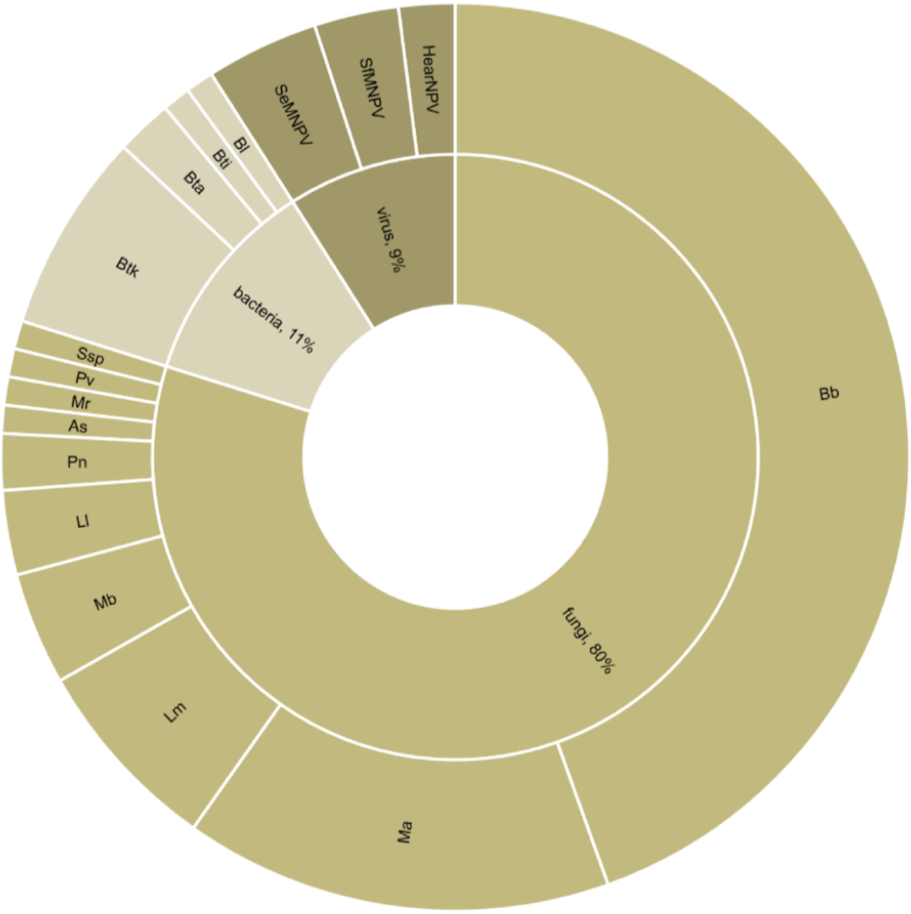

Figure 4. Type and species of entomopathogenic microorganisms used in combination with a parasitoid (*n* = 100 combination experiments from 49 studies). Bb = *Beauveria bassiana*; Ma = *Metarhizium anisopliae*; Lm = *Lecanicillium muscarium*; Mb = *Metarhizium brunneum*; Ll = *Lecanicillium longisporum*; Pn = *Pandora neoaphidis*; As = *Acremonium sclerotigenum*; Mr = *Metarhizium robertsii*; Pv = *Paecilomyces variotii*; Ssp. = *Simplicillium* sp.; Btk = *Bacillus thuringiensis* var. *kurstaki*; Bta = *Bacillus thuringiensis* var. *aizawai*; Bti = Bt var. *israelensis*; Bl = *Brevibacillus laterosporus*; SeMNPV = Spodoptera exigua multiple nucleopolyhedrovirus; SfMNPV = Spodoptera frugiperda multiple nucleopolyhedrovirus; HearNPV = Helicoverpa armigera nuclopolyhedrovirus.

The influences of combined biocontrol agents on pest mortality were analysed in ten laboratory experiments extracted from nine studies [25,28,38,44,45,50,57,62,65]. Out of 41 observations made in these experiments, 14 reported a positive effect, with significantly higher pest mortality when biocontrol agents were combined compared to the strongest agent used alone. In 11 of these, the interaction was less than additive [25,28,57,65]. In two further observations, it was additive [50], and in one, it was synergistic [50]. Twenty-six further observations reported that the combination had no effect on pest mortality [25,38,44,45,62], and one single study reported a negative effect [25].

Table 1. Compatibility of 49 combinations of biocontrol agents extracted from laboratory experiments in the reviewed studies; green = combination reported as compatible; red = combination reported as incompatible; no fill = no report of compatibility; ^{t} = at least one paper mentioning application timing as important; ^{d} = at least one paper mentioning dosage as important; FI. = Figitidae; ICHN. = Ichneumonidae; PTEROM. = Pteromalidae; TRICHOGRAMM. = Trichogrammatidae; NPV = nucleopolyhedrovirus; MNPV = multiple nucleopolyhedrovirus (*n* = 84 experiments from 43 studies [17–20,22–30,32,34–39,41–47,49–60,62–65]).

Combinations of Parasitoids and Entomopathogenic Microorganisms		**Aphelinidae**				**Braconidae**										**Eulophidae**				**FI.**	**ICHN.**		**PTEROM.**			**TRICHOGRAMM.**					**Total**
		A. abdominalis	*E. formosa*	*E. furuhashii*	*E. mundus*	*A. colemani*	*C. insularis*	*C. flavipes*	*D. longicaudata*	*D. rapae*	*D. gelechiidivoris*	*H. hebetor*	*M. bicoloratus*	*M. pallidipes*	*S. agrili*	*E. plathypenae*	*T. radiata*	*T. triozae*	*T. planipennisi*	*T. rapae*	*C. sonorensis*	*H. didymator*	*M. raptor*	*S. cameroni*	*S. endius*	*T. atopovirilia*	*T. brassicae*	*T. chilonis*	*T. dendrolimi*	*T. pretiosum*	
Bacteria	*Bacillus thuringiensis* var. *aizawai*																													🟩 2	2
	Bacillus thuringiensis var. *israelensis*																						1								1
	Bacillus thuringiensis var. *kurstaki*											🟩 1^{d}											1					🟩 2^{d}		🟩 2	6
	Brevibacillus laterosporus																						🟩 1								1
Fungi	*Acremonium sclerotigenum*									🟩 1																					1
	Beauveria bassiana	🟩 1^{td}	🟥 5^{t}		🟩 1	🟩 2		🟩 3		🟩 2^{t}					🟩 1		🟩 2^{t}	🟩 5^{td}	🟩 1	1^{d}					🟥 2	🟩 4^{td}			🟩 1^{td}	🟩 6^{td}	37
	Lecanicillium longisporum		🟩 3^{td}																												3
	Lecanicillium muscarium		1^{t}	🟩 1^{td}		🟩 1^{t} 🟥 2				🟩 1																					6
	Metarhizium anisopliae		🟥 1^{t}			1		🟩 4	🟩 1		🟩 1^{t}	🟩 2^{t}					🟩 1^{t}							🟩 1^{d}			🟩 1^{t}				13
	Metarhizium brunneum					🟩 1														🟩 1^{d}		🟩 2									4
	Metarhizium robertsii								🟩 1																						1
	Paecilomyces variotii									🟩 1																					1
	Simplicillium sp.									🟩 1																					1
Viruses	*Helicoverpa armigera* NPV											🟩 1^{td}																			1
	Spodoptera exigua MNPV												🟩 1^{td}	🟩 1^{td}		🟩 1^{td}															3
	Spodoptera frugiperda MNPV						🟥 2^{t}														🟥 1^{d}										3
Total		1	10	1	1	7	2	7	2	6	1	4	1	1	1	1	3	5	1	2	1	2	3	1	2	4	1	2	1	10	84

Out of 266 observations, 83 (31%) reported a negative effect of entomopathogenic microorganisms on parasitism rate [17,24,37–39,42–46,51,52,55,60,65] (Table 2). This parameter was positively influenced in four observations (2%) [20,24,52,54]. The other studies observed no significant differences between the treatment and control [19,20,22–24,28,32,35,36,39,43–47,52–55,58,60,63,65]. The parasitism rate was often related to the ability of the parasitoid to discriminate against infected hosts. Females appeared to avoid ovipositing on treated hosts mainly when they had a choice between treated and healthy hosts. Under no-choice conditions, females only laid fewer eggs on treated hosts than on healthy ones in one out of seventy observations (~1%) [19,20,38,57,63]. In contrast, when females had a choice, they avoided treated hosts for oviposition in 15 out of 35 observations (43%) [19,20,32,37,39,44–47,54,65]. It is important to stress that sixty-three out of the seventy observations made under no-choice conditions were extracted from a single study reporting three experiments [63]. Therefore, further research needs to be done to confirm the above statements. Discrimination of infected hosts would be beneficial under field conditions. Indeed, parasitoids could complement the effects of entomopathogenic microorganisms on the pest while avoiding the negative effects of the latter on themselves.

A reduced emergence rate of parasitoids combined with a microbial biocontrol agent was reported in 144 of 257 observations (56%) [17–20,23–30,34,36–39,42–46,49–60,62–65]. Entomopathogenic microorganisms had no significant influence on this parameter in all other observations [19,20,22,24,28,29,32,35,39,41,43,46,47,52,53,55–57,60,65]. Parasitoids combined with microbial agents had higher mortality in seventy-one out of one hundred ninety-four observations (37%) [18,24,26–29,39,49,54–57,59,65] and lower mortality in four further observations (2%) [55]. No significant effect was reported in the remaining ones [18,24,26,28,39,41,47,49,53–57,63,65]. The contact with entomopathogenic microorganisms reduced the female offspring sex ratio of parasitoids in 17 out of 92 observations (18%) [23,24,46,52]. No significant change of this parameter was observed in all other cases [17,24,28,32,35,41,43,46,52,53,55,57,65]. Female parasitoids combined with microbial biocontrol agents had shorter longevity in 67 out of 130 observations (52%) [17,19,20,24,42,43,52,55–57,64]. A single observation (1%) reported higher longevity of female *Trichogramma chilonis* when fed with a mixture of honey and Btk in comparison with females fed pure honey [22]. No significant difference in female longevity was reported in all other observations [19,20,22,24,29,34,35,42,46,50,52,53,55,56]. When combined with microbial biocontrol agents, male parasitoids had shorter longevity in 55 out of 125 observations (44%) [17,19,24,42,52,55,64] (Table 2). This parameter remained unchanged in all other observations [17,19,20,24,29,34,35,41,42,50,52,53,55,56].

Timing of application and dosage of biopesticides were important factors influencing the compatibility of entomopathogenic microorganisms with parasitoids. In total, the importance of the timing of application was emphasized in 44 out of the 84 laboratory experiments (52%) [17,20,21,24,25,27–29,34,36,38,39,41,43,45,46,50–52,57,60,62–65] (Table 1). The importance of dosage was highlighted in 25 out of these 84 experiments (30%) [19,20,22,24,25,29,39,41,49,54,60,62,64,65]. For example, *B. bassiana* was reported as compatible with parasitoids in 34 out of 37 experiments [17,18,24,26,32,34,35,42,43,51,52,54,55,58–60,63–65], but the importance of 1) an adapted application timing and 2) dosage was mentioned in 25 (68%) [17,24,34,43,51,52,60,63–65] and 13 (35%) [24,54,60,64,65] of these, respectively. The optimal dosage differed according to the target pest and the combination of biocontrol agents used. It must be sufficient to kill the pest without negatively affecting the parasitoid. The interval length between parasitoid release and infection also differed and needed to be defined for each pair of biocontrol agents.

Table 2. Effect of entomopathogenic microorganisms on the life cycle of parasitoid wasps; N = significantly negative effect; NE = no significant effect; P = significantly positive effect (*n* = 468 observations extracted from 84 experiments from 43 studies [17–20,22–30,32,34–39,41–47,49–60,62–65]).

Effect of Entomopathogenic Microorganisms on Different Parameters of Parasitoid Wasps.		Parasitism Rate			Emergence Rate			Parasitoid Mortality			Female Sex Ratio			Female Longevity			Male Longevity		
		N	**NE**	**P**	**N**	**NE**	**P**	**N**	**NE**	**P**	**N**	**NE**	**P**	**N**	**NE**	**P**	**N**	**NE**	**P**
Bacteria	*Bacillus thuringiensis* var. *aizawai*		2			2			2										
	Bacillus thuringiensis var. *israelensis*				2			1						1	1			2	
	Bacillus thuringiensis var. *kurstaki*		10		2	5			3					2	2	1	2	1	
	Brevibacillus laterosporus					2		1						1	1			2	
Fungi	*Acremonium sclerotigenum*				1			1											
	Beauveria bassiana	53	124	2	77	50		13	97	1	5	48		50	30		45	29	
	Lecanicillium longisporum				9	9		36							9			9	
	Lecanicillium muscarium	11	7		15	13			6		12	10			1			5	
	Metarhizium anisopliae	8	11		13	14		5	5	3		10		11	14		8	16	
	Metarhizium brunneum	6	14	1		1			1			1			1			1	
	Metarhizium robertsii		1			2			1			2			2			2	
	Paecilomyces variotii				1			1											
	Pandora neoaphidis																		
	Simplicillium sp.				1				1										
Viruses	*Helicoverpa armigera* NPV		3	1	1	2								1	2			3	
	Spodoptera exigua MNPV	4	2		11	9		2	3										
	Spodoptera frugiperda MNPV		4		10	1		10	1			4							
	Total	82	178	4	143	110	0	70	120	4	17	75	0	66	63	1	55	70	0

In most reviewed publications, applying the entomopathogenic microorganism after parasitism was recommended to reduce its negative effects on parasitoid development. Waiting for 24 h after the emergence of *T. pretiosum* before applying *B. bassiana* reduced the negative effects of the entomopathogenic fungus on the parasitoid [52]. *Beauveria bassiana* and *T. trizoae* were assessed as compatible if applied at different times [63]. Infection rate of this parasitoid by the fungus was significantly higher in early instars than in more advanced developmental stages [64]. Therefore, the parasitoid should be released before applying the fungus [17,64]. The same conclusion was obtained for *B. bassiana* and *M. anisopliae* used in combination with *T. trizoae* [34]. When combined with *Aphelinus abdominalis*, *B. bassiana* should be applied only when most parasitoids already transformed into pupae and are less susceptible to the fungus [60].

Similarly, the first application of *Lecanicillium longisporum* (Petch) Zare & Gams should be conducted one day after *E. formosa* enters the pupal stage to reduce competition between the biocontrol agents [29]. Detrimental effects of *B. bassiana* and *M. anisopliae* on the development of *E. formosa* could be reduced by waiting at least four days after parasitoid release to spray the microbial agents [51]. Post-parasitism application of *Metarhizium brunneum* Petch also appeared to be best suited for *Hyposoter didymator* as it limited negative effects on the parasitoid due to direct contact with the fungus [45]. A spatial separation of the microbial treatment and the parasitoid release is also possible to avoid these kinds of effects [53]. Fewer *A. colemani* with a lower rate of females emerged from aphids treated with *L. muscarium* within five days of parasitization. In contrast, fungal application six or seven days after aphids had been parasitized did not significantly affect the development, emergence rate, or sex ratio of the parasitoid [46]. Four different time intervals between parasitism by *Campoletis sonorensis* and application of the SfMNPV were tested. Decreasing parasitoid mortality was observed with increasing time interval. The virus did not affect the survival of *C. sonorensis* when applied six days after parasitization [28]. In an experiment combining *Euplectrus plathypenae* and the SeMNPV, the parasitoid was only able to complete its development when the viral infection occurred at least two days after parasitization [46].

In contrast, few recommendations to apply the entomopathogenic microorganism prior to the parasitoid release were found in the reviewed publications. *Bacillus thuringiensis* var. *kurstaki* and the *Helicoverpa armigera* nucleopolyhedrovirus (HearNPV) were recommended to be applied two days before releasing *H. hebetor* to control *Helicoverpa armigera* on chickpeas [20]. Similarly, it was recommended to apply *B. bassiana* before releasing *Trichogramma dendrolimi* so that pest eggs unaffected by the fungal treatment become parasitized [65].

Here, we show for the first time that most studied combinations of biocontrol agents are compatible under controlled conditions. In the best cases, parasitoids are outside the field of action of entomopathogenic microorganisms and remain unaffected when combined with the latter. If not, the timing of application of the biocontrol agents and the biopesticide dose must be carefully determined. Mathematical models such as the one created by Gonthier et al. [69] for the combined use of *Necremnus tutae* and Phthorimaea operculella granulovirus against *Tuta absoluta* can be helpful tools for this purpose. If the dose required to control a specific pest is higher than that tolerated by the parasitoid, the compatibility of the two biocontrol agents is compromised. In terms of pest control, combined biocontrol agents had a positive influence in most cases compared with each agent used alone. However, in their narrative review, Roy and Pell [10] highlighted the importance of conducting field experiments in addition to laboratory bioassays to assess the physiological and ecological susceptibility of natural enemies in a realistic environment. In the field, unpredictable climatic conditions could modify the dynamics of the biocontrol agents observed in the laboratory. Furthermore, less precise application of biopesticides and broader spatial dispersion of pests and parasitoids could significantly influence the compatibility and efficacy of the combinations of biocontrol agents. The presence of other insect species could also influence the level of pest control achieved by parasitoids and entomopathogenic microorganisms that have a wide host range.

3.4. Parasitoid Life History and Susceptibility to Entomopathogens

Parasitoid life history (e.g., generation time, population structure) can influence their susceptibility to entomopathogens [70,71]. As many entomopathogens target the larval stage of the pest, egg parasitoids are less likely to be in contact or compete with entomopathogens, making them de facto more compatible. The development strategies of larval parasitoids can strongly influence their susceptibility to entomopathogens. Endoparasitoids, which lay their eggs inside the host insect's body, may be less exposed than ectoparasitoids, which lay their eggs on the surface of the host, sometimes in open environments where the microorganisms can directly reach the parasitoid larvae [72]. On the other hand, endoparasitoids typically have a longer development time than ectoparasitoids, which can also influence their susceptibility to entomopathogens. As entomopathogens have a slower mode of action, taking longer to kill the host insect, parasitoids with shorter life cycles may emerge from the host before the entomopathogen has a chance to kill it, reducing their exposure to the pathogen [72].

In fact, endoparasitoids are generally considered to be more susceptible to entomopathogens than ectoparasitoids [73], as the pathogen has more time to act on the immature parasitoid during its extended development period inside the host insect [74]. Additionally, endoparasitoids are more likely to be exposed to entomopathogens that are ingested by the host insect, as the pathogen can spread throughout the host's body and affect the parasitoid's physiological function [75].

3.5. Investigated Combinations and Reported Compatibility of Biocontrol Agents in Field, Semi-Field and Greenhouse Experiments

Nine field, five semi-field, and two greenhouse experiments reported the effects of fifteen combinations of parasitoids and entomopathogenic microorganisms on pest control. Thirteen experiments included fungi, one included a bacterium, and the two remaining ones were conducted with viruses [21,31,33,39,40,46,48,61]. As in the laboratory experiments, *B. bassiana* was the most studied microorganism. It was tested in combination with *E. formosa* [65], *Chelonus bifoveolatus*, *Coccygidium luteum* and *Cotesia* sp. [48], *Anisopteromalus calandrae* and *Lariophagus distinguendus* [33], and *Macroglenes penetrans* [61] in one experiment, each. In their greenhouse experiment, Labbé et al. [40] found that *B. bassiana* used in addition to *E. formosa* resulted in a higher pest reduction than the parasitoid alone without harming the development of the latter. In contrast, the fungus was reported as incompatible with *A. calandrae* and *L. distinguendus* [33]. Indeed, in semi-field experiments, *B. bassiana* affected both parasitoids negatively, resulting in lower pest control of the fungus–parasitoid combinations compared with the parasitoid released alone. No answer about the compatibility of this fungus with the other parasitoids mentioned above was given.

In the field experiments conducted by Ngangambe and Mwatawala [48], *M. anisopliae* was tested in combination with *C. bifoveolatus*, *C. luteum*, and *Cotesia* sp., also in one experiment each. In this study, biopesticides based on *B. bassiana* and *M. anisopliae* were reported as less harmful to natural parasitoids than synthetic insecticides based on flubendamide. Fuentes-Contreras and Niemeyer [31] assessed *Pandora neoaphidis* and *Aphidius rhopalosiphi* as compatible in two semi-field experiments. Combining these biocontrol agents resulted in more efficient pest control than each agent used alone. It significantly reduced the growth rate of the pest population. In a greenhouse experiment, the SeMNPV and *Microplitis pallidipes* were found to be compatible [39]. The parasitoid vectored the virus, and their combined use resulted in a significantly higher pest control. Thus, it was recommended to expose the parasitoid to the virus before releasing it.

H. hebetor was tested in combination with Btk and the HearNPV in one field experiment each [21]. These combinations were reported as compatible. Both parasitoid–microbe combinations significantly reduced pest density and crop damage in comparison with each biocontrol agent alone. Crop yield was significantly increased, but so were the control costs. Yield gain was insufficient to cover the additional treatment costs, meaning that combining the biocontrol agents negatively affected the crop's profitability. The remaining investigated combinations were *L. muscarium* with *A. colemani* in a semi-field experiment [46] and the SeMNPV with *M. pallidipes* in a greenhouse experiment [39]. Both were reported as compatible and significantly reduced pest density when deployed together.

Here, we show that combining entomopathogenic microorganisms with parasitoids in the field appears beneficial for pest control; however, profitability may be reduced due to increased control costs. However, the interaction of biocontrol agents on key aspects, namely crop damage, crop yield, and treatment costs, were assessed in only two out of the hundred reviewed experiments. These agronomic and financial parameters must be considered in future research assessing the compatibility of parasitoids and entomopathogenic microorganisms.

4. Conclusions

Environmental pollution, loss of biodiversity, pest resistances, and risks to human health are among the controversial effects of synthetic pesticides. Alternative methods for pest control are sought after. Combining biocontrol agents can improve pest control and reduce harmful effects on the environment. In this systematic review, we show for the first time that many combinations of parasitoids and entomopathogenic microorganisms are compatible and can be deployed together. Eighty percent of the biocontrol agent combinations included in the reviewed papers were deemed compatible. Combinations, including parasitoids and fungi, were well represented in the literature. In contrast, few experiments were found combining bacteria with parasitoids, despite the large number of Bt-biopesticides. Further research on combining bacterial or viral biopesticides with parasitoids is required. The most studied microorganisms of each category, i.e., *B. bassiana* and *M. anisopliae* for fungi, Btk for bacteria, as well as the SeMNPV and the SfMNPV for viruses, were found compatible with many different parasitoids. However, most of the studies were conducted in the laboratory, and new experiments under field conditions are necessary to include agronomic and financial parameters in the final compatibility assessment. Moreover, most of the reviewed studies focused on the impacts of entomopathogenic microorganisms on parasitoids. Few examined the effects of parasitoids on the development and dissemination of microbial biocontrol agents. Further research is required to analyse how both types of biocontrol agents influence each other in the field. Such an assessment should be conducted on more than one generation of parasitoids to highlight possible long-term effects. Appropriate timing of application and dosage must be defined individually for each combination of biocontrol agents against each specific pest, as these are key success factors. Combining biocontrol agents has the potential for pest control, yet interactions between parasitoids and entomopathogenic microorganisms should be further researched. To develop innovative methods, interdisciplinary work should be fostered. Finally, pest biocontrol methods must be viable. Therefore, the availability and the production costs of biocontrol agents should be assessed and further improved.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pathogens12070957/s1, Figure S1: PRISMA flow diagram of studies looking at combinations of biocontrol agents.

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Entomopathogens and parasitoids allied in biocontrol: a systematic review

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PRISMA 2020 Flow diagram

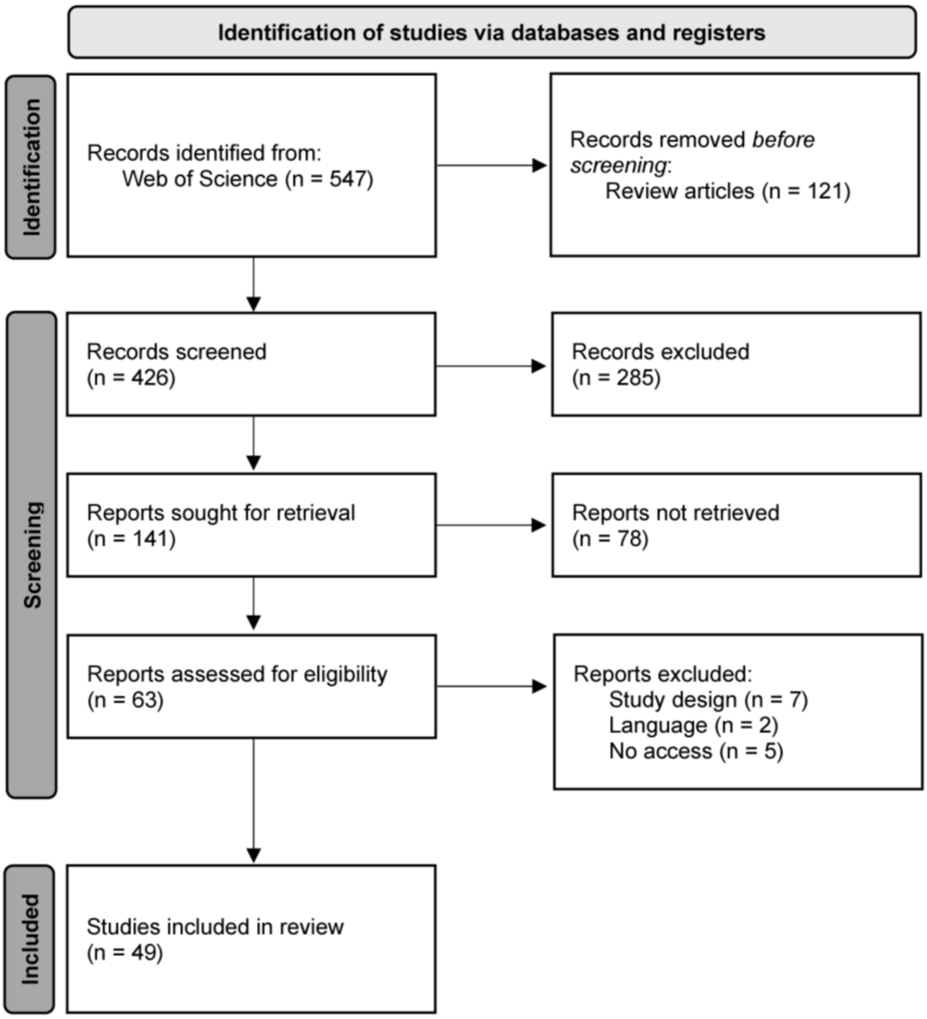

Figure S1 PRISMA flow diagram detailing screening process of articles included in meta-analysis. Procedure adapted from Page et al. 2021.

3 Semi-field experiment: “A parasitoid wasp allied with an entomopathogenic virus to control *Tuta absoluta*”

The following manuscript was submitted on 28th June 2023 for consideration as a research article in the special issue “Entomopathogens: from laboratory to the field” in Crop Protection. This journal focuses on the development and description of control strategies and their integration into practical pest management programs worldwide. In this manuscript, results of a semi-field experiment carried out on the site of Agroscope in Conthey (Valais, Switzerland) from June to August 2021 are reported. The aims of the study were: 1) to evaluate the control efficacy of the parasitoid wasp *Necremnus tutae* and the Phthorimaea operculella granulovirus when used alone and in combination against *Tuta absoluta*; 2) to assess the compatibility of the two biocontrol agents under semi-field conditions over an entire growth period. Authors guidelines can be found in Annex 3, original data in Annex 4 and statistical analyses in Annex 5.

A parasitoid wasp allied with an entomopathogenic virus to control *Tuta absoluta*

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Highlights

- *Necremnus tutae* and PhopGV are compatible biocontrol agents.
- Both agents, used alone or combined, showed potential against *Tuta absoluta*.
- Release intensity of parasitoids is a critical factor for effective control.
- Repetitive viral applications are necessary to restrain population growth.

Abstract

Tuta absoluta (Meyrick) (Lepidoptera: Gelechiidae) is a major threat to worldwide tomato production, and sustainable management methods are needed. Phthorimaea operculella granulovirus (PhopGV) and the parasitoid wasp *Necremnus tutae* Ribes & Bernardo (Hymenoptera: Eulophidae) were identified as promising biocontrol agents of this pest. Since the virus is most effective against first instar larvae, whereas the parasitoid targets older ones, combining both agents could result in increased pest control. We conducted a replicated semi-field experiment to evaluate the control efficacy of *N. tutae* and PhopGV when used alone and in combination against *T. absoluta* and their compatibility over an entire growth period. Combining the two agents achieved the highest larval (-24%) and crop damage (-29%) reduction cumulated over the entire experiment. However, there was no significant additive effect of the two agents. At the end of the trial, which coincided with the emergence of the third generation of

T. absoluta adults, using the virus alone resulted in the strongest reduction of adult density (-78%), followed by the virus-parasitoid combination (-59%). No negative effect of the virus was recorded on the parasitoid population. Our results demonstrate the compatibility and potential of *N. tutae* and PhopGV to reduce population growth and crop damage of *T. absoluta* under semi-field conditions. While more research is needed, our findings provide important insights into an innovative combination of biocontrol agents, thereby contributing to more sustainable agriculture.

Keywords

Baculovirus, combination, tomato leafminer, integrated pest management, biological control, *Phthorimaea absoluta*

1 Introduction

About 190 million tons of tomatoes (*Solanum lycopersicum* L.) are produced yearly on five million hectares worldwide (Food and Agriculture Organization of the United Nations, 2023). The South American tomato leafminer, *Tuta absoluta* Meyrick (Lepidoptera: Gelechiidae), is a major threat to this essential cash crop, relied on by many people (Bergougnoux, 2014). The pest can cause up to 100% damage in all tomato production systems, ranging from soil grown open-field crops to high-tech greenhouse crops (Biondi et al., 2018; Desneux et al., 2011; Desneux et al., 2010). *Tuta absoluta* is native to South America and has, since its arrival in Spain in 2006, spread to nearly 100 countries outside its endemic region (European and Mediterranean Plant Protection Organization, 2023). Females lay their eggs individually on leaves, stems, and petioles, preferentially on apical and median plant parts (Cocco et al., 2015b; Torres et al., 2001). The leaf-mining larvae penetrate the leaves and feed on the mesophyll throughout their four-instar development. Larvae mainly damage leaves and attack fruits when the population density is high (Cocco et al., 2015b; Desneux et al., 2010). Mature larvae usually drop to the soil and pupate on the ground. *T. absoluta* has a high reproduction potential and can complete up to 12 generations per year under favourable environmental conditions (Desneux et al., 2022; Desneux et al., 2010).

Pest control with synthetic insecticides, largely used to control *T. absoluta* (Desneux et al., 2022; Desneux et al., 2010), is unsustainable as the moth rapidly develops high levels of resistance to many chemical compounds (Guedes et al., 2019). Moreover, synthetic insecticides harm key beneficial arthropods, disrupt existing integrated pest management (IPM) programmes and cause environmental and human health concerns (Abbes et al., 2015; Soares et al., 2019; Yadav and Devi, 2017). Therefore, alternative sustainable management methods to control *T. absoluta* are required to ensure the economic viability of tomato production in the invaded regions. Biological control methods involving egg parasitoids, mirid predators, and microbial agents have been integrated into pest management programmes with variable success against *T. absoluta* in various regions, such as South America, Europe, Africa, and Asia (Desneux et al., 2022). Using multiple pest control methods, including diverse biological control agents, provides numerous advantages compared to relying solely on one single agent. One benefit is the possibility of enhancing effectiveness, as different control methods have advantages and disadvantages, and combining them can help overcome each method's limitations. For instance, combining various natural enemies may be more effective at controlling a pest population as each species can have distinct foraging behavior or target different pest life stages (Dainese et al., 2017). Regarding *T. absoluta*, most studies on combining different biological control agents focused on combining *Trichogramma* egg parasitoids with other methods (Bueno et al., 2023; Chailleux et al., 2013; Mansour and Biondi, 2021), and the ones testing the efficacy under semi-field conditions are scarce.

Phthorimaea operculella granulovirus (PhopGV, Baculoviridae: Betabaculovirus) is a granule-shaped virus highly specific to Lepidoptera and infectious to larvae of different Gelechiidae. It was first developed for the biocontrol of the potato moths *Phthorimaea operculella* (Zeller) and *Tecia solanivora* (Povolný), which are closely related to *T. absoluta* (Gómez Valderrama et al., 2018; Mascarin et al., 2010). Infection and replication of baculoviruses occur exclusively in the larval stage. Larvae become infected when they feed on plants contaminated with occlusion bodies (OBs) containing occlusion-derived viruses (ODVs). After ingestion, OBs are dissolved in the insect midgut, releasing ODVs that infect the midgut epithelial cells. Budded viruses (BVs) then disperse and replicate in other susceptible tissues. The larval tegument ruptures upon death, releasing new OBs that contaminate the plant surface (Slack and Arif, 2007; Williams et al., 2017). The PhopGV has a good level of pathogenicity against the first instars of *T. absoluta* larvae and also causes sublethal effects such as retarded larval growth and pupation failure in the older larval instars (Gómez Valderrama et al., 2018; Mascarin et al., 2010).

Necremnus tutae Ribes & Bernardo (Hymenoptera: Eulophidae), previously classified as *Necremnus artynes* (Walker), is native to the Mediterranean region and is the most abundant and widespread larval parasitoid of *T. absoluta* in that area (Desneux et al., 2022; Ferracini et al., 2019). This synovigenic idiobiont ectoparasitoid parasitizes and feeds on second to fourth instar larvae of the pest with a distinct preference for the third instar (Calvo et al., 2013). *N. tutae* causes additional mortality by host-killing (i. e. host-feeding, host-stinging). It is currently not commercially available but occurs spontaneously in tomato production fields in different regions (Abbes et al., 2014; Arnó et al., 2021; Crisol-Martínez and van der Blom, 2019; Desneux et al., 2022).

Augmentative releases of *N. tutae* alone seem insufficient to keep damage caused by *T. absoluta* on tomato plants below the economic threshold (Calvo et al., 2016; Cocco et al., 2015a; Desneux et al., 2022). Regarding PhopGV, high doses (weekly application of the highest recommended concentration) are required to lethally infect *T. absoluta* larvae. In addition, the most damaging older larvae are difficult to reach due to their location inside the mines, so complementary control measures are required (Gonthier et al., 2023a). Combining both biocontrol agents could increase pest control against *T. absoluta*. The virus is more effective on young larvae, while the parasitoid targets the later instars. In addition, no adverse effects of PhopGV were detected on the development of *N. tutae* under controlled conditions, indicating that both agents are compatible for combined use (Gonthier et al., 2023a).

We conducted a replicated semi-field experiment to assess the efficacy of *N. tutae*, and PhopGV used alone and in combination for the biocontrol of *T. absoluta*. More precisely, we addressed the following research questions: Q1 Do *N. tutae* and PhopGV influence the population development of *T. absoluta* when used alone or in combination? Q2 Do *N. tutae* and PhopGV influence the level of crop damage caused by *T. absoluta* when used alone or in combination? Q3 Is the *N. tutae* population influenced by PhopGV under semi-field conditions (effects on population size and sex ratio)?

2 Material and methods

2.1 Study area and greenhouse

The replicated semi-field experiment was conducted from June to August 2021 in the facilities of Agroscope in Conthey (Valais, Switzerland). Twenty-eight walk-in cages (Diatex SAS, France) of 2.6 m^2 surface area and 2.5 m height with thrips-proof mesh (300 x 300 μm^2) were set up in two identical 90 m^2 glass greenhouses. The floor below the cages was covered with a white woven ground cloth (100 g/m^2). Temperature, relative humidity, and irrigation were managed using a remote-control system.

2.2 Biological material

2.2.1 Tomato plants

Ungrafted single-stemmed tomato plantlets cv. Admiro (De Ruiter) 30 cm in height and with five fully expanded leaves with leaflets were repotted in 10-litre pots, fertilized with Osmocote (NPK 15/9/12), a slow release fertilizer providing enough nutrients for four months, and moved into the walk-in cages. No plant protection interventions, other than those detailed in 2.2.2 and 2.2.3, were realised. The average temperature during the experiment was 25°C (max. 41°C, min. 17°C). Four plants were placed in each of the 2.6 m^2 walk-in cages. The main stem of each plant was trellised on a string attached to a wire above the cage and passed through an insect-proof hole in the ceiling of the latter. Secondary shoots were removed weekly, and plants were watered daily through drip irrigation.

2.2.2 Insects

Pupae of *T. absoluta* were provided by Andermatt Biocontrol Switzerland. These were originally collected in Switzerland and reared in the company's facilities, and a sample was checked to ensure a 1:1 sex ratio.

N. tutae parasitoids were collected from commercial tomato fields in El Maresme county, Barcelona, Spain, and a colony was established at Agroscope, Switzerland. Adult parasitoids were kept in cages (50 × 50 × 50 cm) (bug dorm; MegaView Science Co. Ltd., Taiwan) and provided with honey-water (10% v/v) soaked cotton and tomato plants with *T. absoluta* larvae in their second or third instar for parasitization. After emerging, adult parasitoids were gathered and kept at 12 °C with honey and water.

Every ten days, a new generation of parasitoids was started. Only naive, mated (stored with males for at least two days), and less than one-week-old female parasitoids were used in the experiments. Adult parasitoids were briefly cooled in a cold room (4 °C) for counting (sex ratio 1:1). They were then placed in a box for 24 h to feed and mate before being released in the middle of each walk-in cage. Honey was supplied twice a week for one month to the parasitoids to ensure their proper establishment.

2.2.3 Baculovirus

Tutavir®, a biopesticide containing a 2×10^{13} OB/l of PhopGV, was produced and provided by Andermatt Biocontrol Switzerland. The product was applied with an electrical backpack sprayer (Fox Motori, Cod. 5-19-180) following the supplier's instructions specific to the trial (see 2.3 Experimental setup). A Teejet noozle (TXA8001vk) was used and pressure was set at 2.5 bar. Good coverage of the underside of the leaves was achieved by using a sprayer with the nozzle pointing upwards. A plastic sheeting was hung between the cages before each application of PhopGV to avoid cross-contamination.

2.3 Experimental setup

The four following treatments were compared in a completely randomized block design with seven replicates: 1) Control with *T. absoluta* only (CO), 2) *T. absoluta* and *N. tutae* (PA), 3) *T. absoluta* and PhopGV (VI), and 4) *T. absoluta, N. tutae* and PhopGV (VP). To provide *T. absoluta* with food at libitum, four batches of plants were transplanted in the walk-in cage throughout the trial. A first set of four potted plants was inserted on day one, followed by a second batch on day 45 (**Table 1**). Ten plants cut in a neighbouring greenhouse were placed in each cage on day 48, and two potted plants were added on day 58. All batches of plants were sprayed with water (CO and PA) or with PhopGV (VI and VP) at standard dosage (0.015%, $\geq 3 \times 10^9$ OB/l) on the day of their introduction (**Table 1Figure 1**). Additionally, the first batch of plants was sprayed at a standard dosage on days 3 and 10 and at a low dosage (0.0015 %; $\geq 3 \times 10^8$ OB/l) on days 28 and 42. Fifty *T. absoluta* pupae were released in each of the 28 plots on day one. *N. tutae* neither parasitizes nor feeds on first instar *T. absoluta* larvae (Calvo et al., 2013). Thus, the parasitoid was first released on day 16, coinciding with the first availability of the second instar *T. absoluta* larvae (**Table 1**). At least two studies have conducted semi-field experiments with *T. absoluta* and *N. tutae* previously (Calvo et al., 2016; Chailleux et al., 2014; De Campos et al., 2020). We chose a similar setup to the one described by Calvo et al. (2016) since they used similar cages. Thirty couples of adult *N. tutae* were released in each plot of treatments PA and VP. A second release of thirty couples was done on day 41.

Table 1. Experimental setup of the semi-field experiment. Numbers in the cells below indicate the exact day each action occurred (1 = start of the trial, release of *Tuta absoluta*). ^{50} = 50 *T. absoluta* pupae, ^{30} = 30 couples of *Necremnus tutae*, ^{H} = application of Phthorimaea operculella granulovirus (PhopGV) at standard dosage (0.015%, $\geq 3 \times 10^9$ OB/l), ^{L} = application of PhopGV at low dosage (0.0015 %; $\geq 3 \times 10^8$ OB/l).

Description	**Week Number Year 2021**											
	23	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**	**33**	**34**
Tomato plants	**1**						**45**	**48**	**58**			
***T. absoluta* release**	**1^{50}**											
PhopGV application	**1^{H}** **3^{H}**	**10^{H}**			**28^{L}**		**42^{L}** **45^{H}**	**48^{H}**	**58^{H}**			
***N. tutae* release**			**16^{30}**				**41^{30}**					
Pest monitoring			**16**	**23**	**30**	**37**	**44**	**51**	**58**	**65**		
Yellow sticky traps										**62**	**to**	**79**

2.4 Monitoring

One plant per cage was sampled weekly, starting two weeks after *T. absoluta* release (**Table 1**). Each week, a different plant was sampled. Two leaflets of leaves 6, 8, 10, 12, and 14 (apex =1) were collected systematically on each selected plant, resulting in a sample of ten leaflets per cage. The number of healthy *T. absoluta* larvae in each leaflet was counted using a binocular. After counting, leaflets were placed in a transparent sheet and scanned using a standard printer, and the percentage of surface mined by the larvae of *T. absoluta* was assessed using the software ImageJ (Version 1.53j). Total area of leaflets and the damaged area in pixels were measured using different hues, saturation, and brightness of the red threshold colour in the HSB colour space. The percentage of crop damage was calculated by dividing the damaged area by the total area of the leaflets and multiplying the result by 100. Leaflets were put back in their original cage at the latest 36 h after sampling to avoid bias due to the removal of insect individuals.

From day 62 to day 79, batches of two yellow sticky traps (25 x 40 cm^2) (yellow catch-it, Andermatt Biocontrol Switzerland) with a synthetic *T. absoluta* female pheromone dispenser (pheromone dispenser for Delta-trap, Andermatt Biocontrol Switzerland) were hung in each cage to catch all alive adults of *T. absoluta* and *N. tutae*. Once removed from the cages, the traps were wrapped in plastic film, scanned on both sides, and placed in the refrigerator. All scans were sent to Trapview B. V. in the Netherlands, where the numbers of adult *T. absoluta* on the sticky traps were counted with a faster Rcnn Object Detection Network using the Tensorflow Object Detection API. In addition, the numbers of *N. tutae* males and females were counted visually using a binocular.

2.5 Statistical analysis

Statistical analyses of the dataset (Koller et al., 2023a) were performed with R 4.2.3 (R Core Team, 2023). First, the blocking factor (spatial distribution of cage in the greenhouse) was tested as a fixed effect for all response values, and as there was no significant effect, it was excluded in the further modelling process. To assess whether the biocontrol agents influenced the population development of *T. absoluta* (Q1), two linear models with treatment as a fixed factor and cumulative larvae and adults as response variables, respectively, were fitted using the nlme package (version 3.1-162). To assess whether the biocontrol agents influenced the level of crop damage caused by *T. absoluta* (Q2), the average percentage of crop damage recorded over the trial was fitted as a response value in a linear model with treatment as a fixed effect. To assess whether PhopGV influenced the *N. tutae* population (Q3), the total number of parasitoid adults caught on the yellow traps at the end of the trial were sqrt(x) transformed to normalize distribution before fitting a linear model with treatment as the explanatory variable. To detect potential effects of the virus on the sex ratio of the parasitoid, a generalized linear model with binomial error structure and treatment as a fixed factor was fitted.

Linear models were analysed using two-way ANOVA. To find significant differences between treatment levels, we compared them pairwise using Tukey's HSD test in linear models that were globally significant. Inference from the generalized linear model of parasitoid sex ratio was drawn using a chi-square test. P values lower than 0.05 were considered statistically significant. For all models, assumptions were checked according to the graphical validation procedures recommended by Zuur et al. (2009).

3 Results

T. absoluta completed almost three generations during the semi-field experiment (**Figure 1**). The first generation lasted from day 1 to day 30, while the second spanned from day 30 to day 60. The third generation started around day 60. The start and end of each generation were determined based on the larval peak populations.

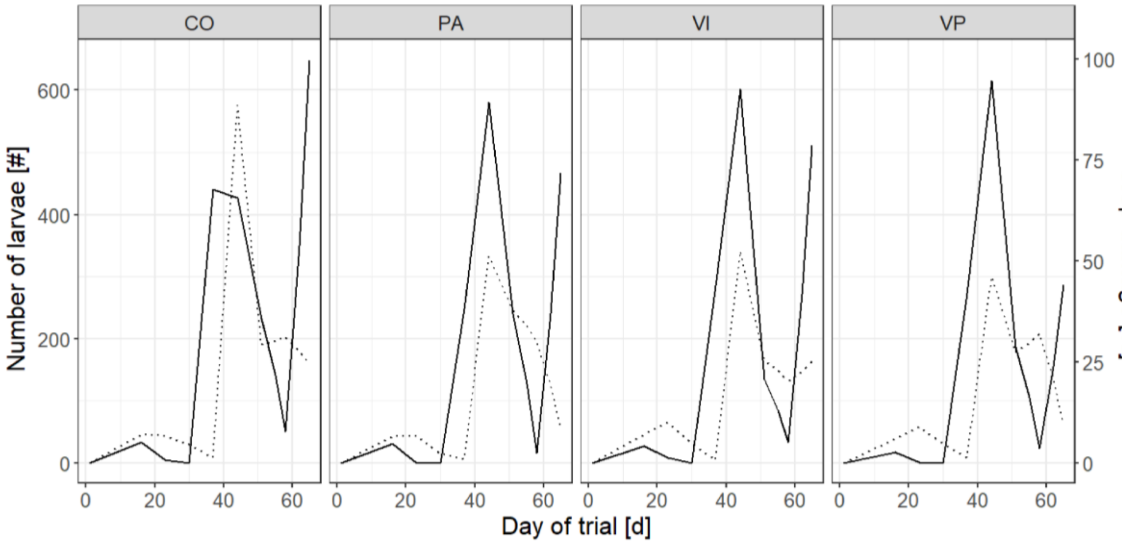

Figure 1. Population dynamic of healthy *Tuta absoluta* larvae and crop damage (percentage of leaf area mined by the larvae) monitored on tomato plants over a three-month semi-field experiment comparing different experimental treatments (CO = control without treatment (only *Tuta absoluta*), PA = parasitoid (N*ecremnus tutae*), VI = virus (PhopGV), VP = virus + parasitoid). The solid line represents the number of larvae and is connected to the y-axis on the left. The dotted line represents the percentage of crop damage and is connected to the y-axis on the right.

3.1 Effect of the biocontrol agents on the population growth of *T. absoluta*

PhopGV and *N. tutae* reduced the number of *T. absoluta* larvae cumulated over the trial by 13% and 14%, respectively, compared to the control (**Figure 2**). Both biocontrol agents applied together decreased the number of larvae by 24%. However, the differences between the four treatments were non-significant (ANOVA, $F_3 = 1.508$, $p = 0.238$).

Regarding the number of adult *T. absoluta* caught on the sticky yellow traps at the end of the trial, the treatment with the lowest adult density was the virus treatment, followed by the virus-parasitoid combination (**Figure 2**). PhopGV alone significantly reduced the third generation of adults by 78% compared to the control and the parasitoid treatment, respectively (ANOVA, $F_3 = 9.545$, $p = < 0.001$; Tukey's test: VI-CO $p = 0.001$, VI-PA $p = 0.002$). Similarly, the virus-parasitoid combination caused a reduction of 59% and 58% of adults compared to the control and the parasitoid treatment, respectively (VP-CO $p = 0.018$, VP-PA $p = 0.022$). With a reduction of 2% only, the parasitoid treatment was similar to the control (PA-CO $p = 0.999$). No significant difference was found between PhopGV used alone and in combination (VI-VP $p = 0.713$).

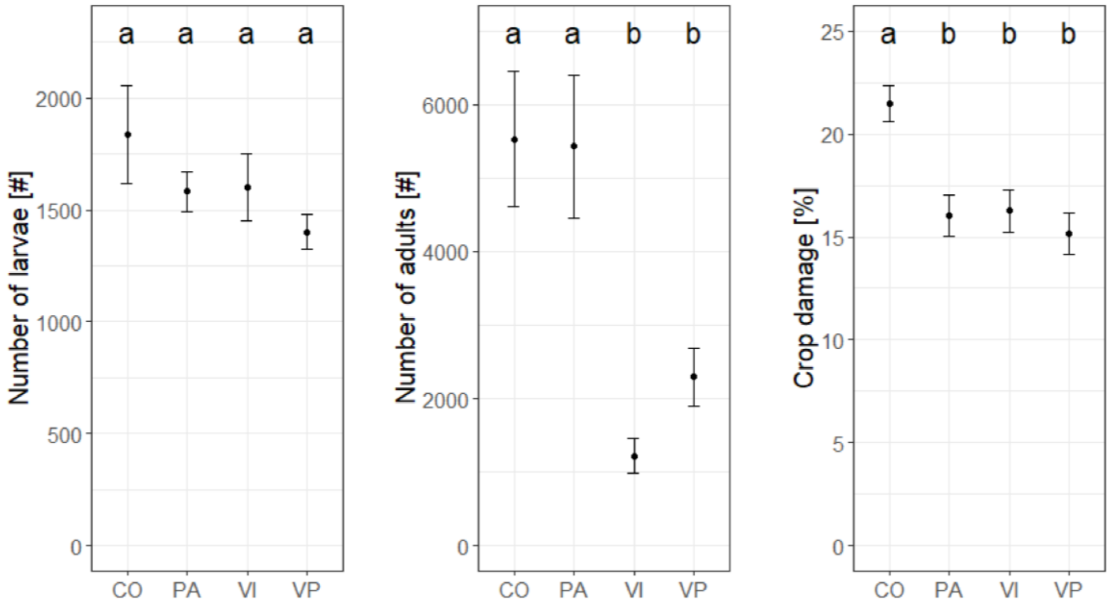

Figure 2. Cumulated number of healthy *Tuta absoluta* larvae (left), total number of adult *T. absoluta* (middle), and average percentage of crop damage (leaf area mined by the larvae) (right) monitored over a three-month semi-field experiment comparing different experimental treatments (CO = control without treatment, PA = parasitoid (*Necremnus tutae*), VI = virus (PhopGV), VP = virus + parasitoid). Larvae and crop damage were monitored weekly on tomato plants, and third-generation adults were caught on sticky yellow traps at the end of the trial. Error bars represent the standard error. Different lowercase letters indicate significant differences between the experimental treatments according to Tukey's HSD.

3.2 Effect of the biocontrol agents on crop damage caused by *T. absoluta*

PhopGV and *N. tutae*, both alone and combined, caused a significant and similar damage reduction cumulated over the trial (ANOVA, $F_3 = 8.524$, $p < 0.001$; Tukey's test: PA-CO $p = 0.003$, VI-CO $p =$

0.005, VP-CO $p < 0.001$, **Figure 2**). PhopGV and *N. tutae* reduced crop damage by 24% and 25% compared to the control. The combined biocontrol agents reduced crop damage by 29%. No significant differences were found between the biocontrol agents used alone or in combination (all P values > 0.855).

3.3 Compatibility of the biocontrol agents under semi-field conditions

N. tutae and PhopGV were found to be compatible under semi-field conditions. Similar numbers of parasitoids were recorded at the end of the trial in the parasitoid and the virus-parasitoid treatments (ANOVA, $F_1 = 0.229$ $p = 0.641$). Similar sex ratios were found in both treatments, indicating no adverse effect of PhopGV on *N. tutae* (Chi-square, $LRT_1 = 0.020$, $p = 0.888$).

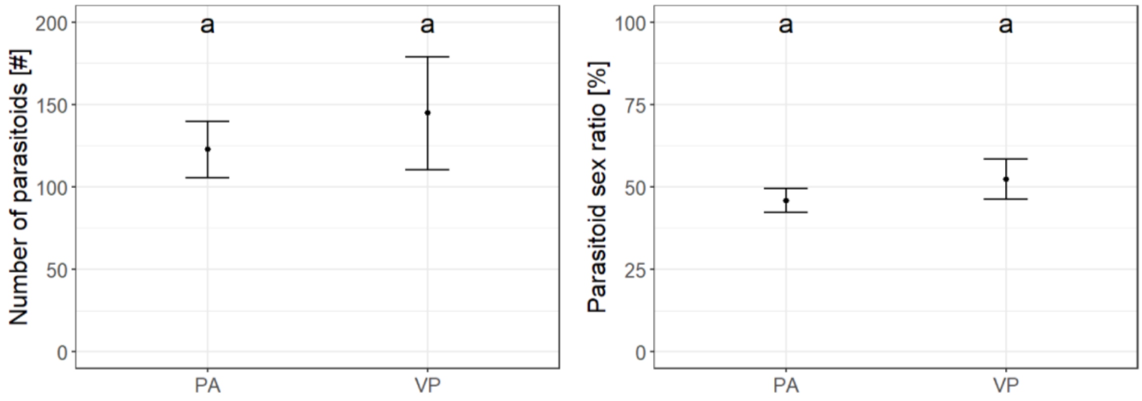

Figure 3. *Necremnus tutae* adults (left) and mean percentage of *N. tutae* females (right) recorded on sticky yellow traps at the end of a three-month semi-field experiment including different experimental treatments (CO = control without treatment, PA = parasitoid (*N tutae*), VI = virus (PhopGV), VP = virus + parasitoid). Error bars represent the standard error. Different lowercase letters indicate significant differences between the experimental treatments according to Tukey's HSD (adults) and chi-square test (sex ratio).

4 Discussion

This research demonstrates the potential of the baculovirus PhopGV and the parasitoid *N. tutae* when used alone and combined against *T. absoluta* under semi-field conditions in the greenhouse. Both the virus and the parasitoid have previously displayed potential against this pest when used separately in laboratory and semi-field experiments (Ben Tiba et al., 2019; Calvo et al., 2016; De Campos et al., 2020). We found that using both agents in combination resulted in the lowest numbers of pest larvae and the least crop damage cumulated over the entire experiment, yet the additive reduction compared to the use of each agent alone was statistically non-significant. The virus-only treatment, followed by the combined biocontrol agents, resulted in the strongest reduction of adult density recorded on the yellow traps at the end of the experiment (corresponding to the third generation of *T. absoluta* adults).

Both biocontrol agents, alone and combined, significantly reduced crop damage compared to the control. Larval density in the presence of *N. tutae* displayed a cumulative reduction of 14% compared to the control, yet this effect was not significant. It must be noted that the number of larvae recorded in the control on day 44, at the larval peak of the second generation, may have been unrepresentative of the number of larvae present in the cages (**Figure 1**). The percentage of crop damage measured that day was nearly 100%, and the larvae may have already left the leaflets sampled during the monitoring to seek food elsewhere in the cages. In addition, larval numbers displayed high variance within treatments. According to Cocco et al. (2015b), counting mines on the median proportion of the tomato canopy is best suited to provide reliable estimates of the total infestation. We monitored specifically this part of the plant; however, it is possible that high levels of infestation may have caused an altered distribution of larvae on the plant and thus masked differences that would be visible otherwise.

Two similar semi-field studies using the same parasitoid found a significant reduction in larval density by the end of the experiment (Calvo et al., 2016; De Campos et al., 2020). As in these studies, we used a parasitoid-pest ratio of 1.2 but applied a different release sequence. Calvo et al. (2016) progressively released 48 adult *T. absoluta* per week over three consecutive weeks and 24 adult *N. tutae* per week over seven consecutive weeks starting two weeks after the first pest release. Campos et al. (2020) made two releases, each of 40 adult *T. absoluta,* with two weeks between releases. This was followed two weeks later by a release of 50 adult *N. tutae*. We released 50 adult *T. absoluta* on the first day, 30 adult *N. tutae* on day 16 and, again 30 adult *N. tutae* on day 41 (**Table 1**). According to Bodino et al. (2019), a single *N. tutae* individual is able to attack a maximum of 10 host larvae per day. Given the high adult density of the pest recorded in the parasitoid-only treatment at the end of the trial, it appears that *N. tutae* was outnumbered and, therefore, unable to keep *T. absoluta* under control. Releasing a sufficient number of parasitoids is crucial at the beginning of the pest infestation. Moreover, progressive and frequent parasitoid releases are recommended to ensure good establishment of *N. tutae* in a more realistic setup with continuous pest infestation.

While PhopGV doubled the natural mortality of *T. absoluta* larvae in the laboratory (Gonthier et al., 2023a), no significant effect of the baculovirus on pest larval density was visible during the eight weeks of the semi-field experiment. Weekly application of the high dose (0.015%, $\geq 3 \times 10^9$ OB/l) is usually recommended for controlling *T. absoluta* (Andermatt Biocontrol, personal communication). In this trial, we assessed the efficacy of PhopGV sprayed at high and low doses (0.0015%) to unveil the potential long-term effects of the virus. Even at low doses, baculoviruses can have a long-term impact on the population, possibly via covert infection (Burden et al., 2003) or sublethal infection, such as reduced feeding activity (Larem et al., 2019). The effect might be visible only later in the pest population or plant damage reduction. Following this, we found a strong reduction in crop damage in the virus treatments. Sublethally affected larvae might have produced fewer and smaller mines than healthy ones. Adult *T. absoluta* caught on the yellow sticky traps at the end of the trial represented the third pest generation. Larvae of this generation developed on plants sprayed with the high dosage of PhopGV on days 45, 48, and 58, respectively (**Table 1**). Adult density was significantly reduced by 78% and 59% in the virus-only and virus-parasitoid treatments, respectively, highlighting the virus's potency when sprayed repetitively at high doses.

Our semi-field trial showed that combining both agents tended to result in the strongest pest and damage reduction cumulated over the entire experiment; however, compared to using both agents alone, the effect was insufficient to be significant. PhopGV showed its full potential in the third generation of pest adults after plants were sprayed at the recommended high dose. The discrepancy between the larval population of the third generation and the adult population emerging from it indicates a high rate of pupation failure in the third pest generation since no effect of the virus was recorded on larval density over the experiment. So far, few studies have addressed the combination of entomopathogenic viruses with parasitoids beyond the laboratory scale. In a greenhouse trial, damage to tomato plants by *Lacanobia oleracea* (Lepidoptera: Noctuidae) was reduced by the Lacanobia oleracea baculovirus (LoGV) and the parasitoid *Meteorus gyrator* (Hymenoptera: Braconidae). Similar to our study, combining the two agents further reduced damage, but the difference was not significant (Matthews et al., 2004). When Spodoptera exigua nucleopolyhedrovirus (SeNPV) and *Microplitis pallidipes* (Hymenoptera: Braconidae) were used on cabbage grown in commercial greenhouses, the reduction in the *Spodoptera exigua* (Lepidoptera: Noctuidae) population was greater when using *M. pallidipes*-carrying SeNPV than with parasitoids without virus (Jiang et al., 2011). In this case, the parasitoid could transmit the virus, for example, when emerging from an infected host.

Timing and sequence are crucial parameters for successfully combining entomopathogens with parasitoids (Koller et al., 2023b). Parasitization after virus application was detrimental for *Euplectrus plathypenae* (Hymenoptera: Eulophidae), whereas infesting *S. exigua* with Spodoptera exigua multiple nucleopolyhedrovirus (SeMNPV) two days post parasitism had no significant effects on the parasitoid (Stoianova et al., 2012). Likewise, *Campoletis sonorensis* (Hymenoptera: Ichneumonidae) survival increased with a greater delay between parasitism and the infection with the Spodoptera frugiperda multiple-enveloped nucleopolyhedrovirus (SfMNPV) of the host (Escribano et al., 2000). Since the PhopGV becomes less effective with the increasing age of *T. absoluta* larvae (Larem et al., 2019) and *N. tutae* targets older larvae (Chailleux et al., 2014), both agents would best complement each other for pest control when the virus is applied several days before the parasitoid. Previous research has shown that prior parasitism can reduce the pathogenicity of baculoviruses in hosts (Abbes et al., 2014; Cossentine, 2009). We are unsure whether this played a role in the limited positive effect of the combination in our study, and further investigation is needed to explore this aspect of the interaction. However, since the number and sex ratio of parasitoids found in the PhopGV-treated plots was similar to that in the plots without the virus at the end of the trial, we can confirm that infection with PhopGV after parasitization has no negative impact on the population of *N. tutae* (Gonthier et al., 2023a). Consequently, PhopGV should also be compatible with naturally occurring populations of *N. tutae*, which is a significant advantage for its use in conservation biocontrol due to the wide distribution of this parasitoid.

The semi-field setup of our study allowed for precise application, dosage, and release timing. Yet such a controlled environment cannot perfectly represent field conditions. Factors such as temperature, low aeration, and lack of shading screens can significantly impact plant growth and the biological cycle of the pest and the parasitoid. Additionally, environmental factors such as UV radiation, heat, and desiccation may increase the degradation of PhopGV, and inferior leaf coverage due to spraying larger surfaces may reduce the effectiveness of the virus, further affecting the interaction outcome. The infestation was set artificially high in our experiment and conducted in a single day. Such a high pest density may have led to an underestimation of the efficacy of *N. tutae* and PhopGV. Real-world populations of *T. absoluta* are more complex, with individuals immigrating and emigrating, aggregated spatial distribution, and multiple developmental stages present simultaneously (Cocco et al., 2015b). Under those conditions, where precise control may be challenging, combining the two biocontrol agents can offer greater flexibility in the timing and dosage of application. Predictive models show that biocontrol intervention timing and intensity are crucial for success and cost efficiency (Gonthier et al., 2023b). Further research should be conducted to better understand the effectiveness of biocontrol agent combinations in real-world settings and determine whether and how parasitoids can assist in spreading or accelerating the spread of the virus within the pest population (Jiang et al., 2011).

The difficulties linked with the mass rearing of *N. tutae* may challenge the widespread implementation of this strategy (Desneux et al., 2022). Combining PhopGV with the larval parasitoid *Dolichogenidea gelechiidivoris* (Marsh) (Hymenoptera: Braconidae), which is also compatible with PhopGV (Gonthier et al., 2023a), could be an interesting alternative. The parasitoid has the potential to reach an 86 % parasitism rate on *T. absoluta* and is easy to mass rear (Morales et al., 2013). Moreover, its combined use with the entomopathogenic *Metarhizium anisopliae* (Metschnikoff) (Hypocreales: Clavicipitaceae) in the laboratory resulted in additive control effects despite the reduction of parasitoid fitness by the fungus (Mama Sambo et al., 2022).

5 Conclusions

Our results demonstrate the compatibility and the potential of *N. tutae* and PhopGV to reduce population growth and crop damage of *T. absoluta* under semi-field conditions. *N. tutae* significantly restrained population growth and crop damage cumulated over the entire experiment but appeared to be outnumbered at the end of the trial. Intensity and timing of parasitoid releases are crucial and must be adjusted precisely according to the time and level of pest infestation to ensure effective biological control under field conditions. PhopGV reduced crop damage to the same extent as *N. tutae* but strongly reduced adult density in the third pest generation, whereas the parasitoid did not. Under the conditions of our semi-field experiment, the combined use of PhopGV and *N. tutae* had no additive effect compared to each biocontrol agent used alone. However, the interaction outcome could be affected by altered population dynamics and environmental factors in real-world settings, as well as by reduced leaf coverage during application of the virus under field conditions. Further investigations are warranted to optimize the timing of application and dosage. The system should be studied under various environmental conditions to advance this research area further, fully understand its potential, and estimate possible benefits under real-world field or greenhouse conditions. Additionally, a cost-benefit assessment of this control strategy could provide valuable information for smallholders, organic growers, and large-scale productions. It would also be essential to study the seasonal and infestation level variations in the efficacy of this control strategy. In summary, while more research is needed to fully evaluate the potential of combining PhopGV and *N. tutae* for *T. absoluta* control, our findings provide interesting insight into an innovative combination of biological control agents.

6 Authors contributions

JK, JG, JC and LS conceived the study. JK, JG and LS collected the data. JK and LS analysed the data. JK led the writing of the manuscript. JK, JG, JC wrote the manuscript. LN, JA, JG, JC, LS critically revised the study concept and manuscript. All authors contributed to the writing of the manuscript and gave final approval for its publication.

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9 Conflict of Interest Declaration

The authors have no conflicts of interest to declare that are relevant to the content of this article.

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4 General conclusions

4.1 Systematic review on combinations of biocontrol agents

Environmental pollution, loss of biodiversity, pest resistances, and risks to human health are among the controversial effects of synthetic pesticides. Alternative methods for pest control are sought. Combining biocontrol agents can improve pest control and reduce harmful effects on the environment. In our systematic review, we showed that many combinations of parasitoids and entomopathogenic microorganisms are compatible and can be deployed together. Eighty percent of the biocontrol agent combinations included in the reviewed papers were deemed compatible. Combinations, including parasitoids and fungi, were well represented in the literature. In contrast, few experiments were found combining bacteria with parasitoids, despite the large number of Bt-biopesticides. Further research on combining bacterial or viral biopesticides with parasitoids is required. The most studied microorganisms of each category, i.e., *B. bassiana* and *M. anisopliae* for fungi, Btk for bacteria, as well as the SeMNPV and the SfMNPV for viruses, were found compatible with many different parasitoids. However, most of the studies were conducted in the laboratory, and new experiments under field conditions are necessary to include agronomic and financial parameters in the final compatibility assessment. Moreover, most of the reviewed studies focused on the impacts of entomopathogenic microorganisms on parasitoids. Few examined the effects of parasitoids on the development and dissemination of microbial biocontrol agents. Further research is required to analyse how both types of biocontrol agents influence each other in the field. Such an assessment should be conducted on more than one generation of parasitoids to highlight possible long-term effects. Appropriate timing of application and dosage must be defined individually for each combination of biocontrol agents against each specific pest, as these are key success factors. Combining biocontrol agents has the potential for pest control, yet interactions between parasitoids and entomopathogenic microorganisms should be further researched. To develop innovative methods, interdisciplinary work should be fostered. Pest biocontrol methods must be viable. Therefore, the availability and the production costs of biocontrol agents should be assessed and further improved.

4.2 Semi-field experiment on the biological control of *Tuta absoluta*

The results of our semi-field experiment demonstrate the compatibility and the potential of *N. tutae* and PhopGV to reduce population growth and crop damage of *T. absoluta* under semi-field conditions. *N. tutae* significantly restrained crop damage cumulated over the entire experiment but appeared to be outnumbered at the end of the trial. Intensity and timing of parasitoid releases are crucial and must be adjusted precisely according to the time and level of pest infestation to ensure effective biological control under field conditions. PhopGV reduced crop damage to the same extent as *N. tutae* but strongly reduced adult density in the third pest generation, whereas the parasitoid did not. Under the conditions of our semi-field experiment, the combined use of PhopGV and *N. tutae* had no additive effect compared to each biocontrol agent used alone. However, the interaction outcome could be affected by altered population dynamics and environmental factors in real-world settings, as well as by reduced leaf coverage during application of the virus under field conditions. Further investigations are warranted to optimize the timing of application and dosage. The system should be studied under various environmental conditions to advance this research area further, fully understand its potential, and estimate possible benefits under real-world field or greenhouse conditions. Additionally, a cost-benefit assessment of this control strategy could provide valuable information for smallholders, organic growers, and large-scale productions. It would also be essential to study the seasonal and infestation level variations in the efficacy of this control strategy. In summary, while more research is needed to fully evaluate the potential of combining PhopGV and *N. tutae* for *T. absoluta* control, our findings provide interesting insight into an innovative combination of biological control agents.

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Annexes

1. **Guidelines for authors of *Pathogens* – see digital annex 1**

2. **Original data of the systematic review – see digital annexes 2a and 2b**

3. **Guidelines for authors of *Crop protection* – see digital annex 3**

4. **Original data of the semi-field experiment – see digital annexes 4a and 4b**

5. **Statistical analysis of the data from the semi-field experiment – see digital annex 5**

6. **Master Thesis Project Definition**

Bern University of Applied Sciences
School of Agricultural, Forest and
Food Sciences HAFL

Master Thesis Project Definition

Student's name and contact details	**Janique Studer** **+41 79 716 85 49, janiquestephanie.studer@students.bfh.ch**
Principal advisor[1]	Lindsey Norgrove
Co-advisor's name and contact details[2]	Louis Sutter +41 58 463 23 99, louis.sutter@agroscope.admin.ch
Working Title of the master's thesis	**Does *Necremnus tutae* perform better against *Tuta absoluta* when deployed with the granulovirus, *Phthorimae operculella* ?**
Background[3] and state of knowledge	The South American tomato leafminer (*Tuta absoluta* Meyrick, Lepidoptera: Gelechiidae) is a major threat to field and greenhouse tomato production worldwide. Native to South America, it has spread rapidly across Europe, Africa and Asia since it was first recorded in Spain in 2006. Adult moths lay their eggs on the underside of the leaves and larvae penetrate the leaves, stems and fruit to feed on the parenchyma throughout their development. *T. absoluta* causes up to 80-100% damage in tomato crops. The pest has already developed resistance to multiple chemical insecticides. Currently, pest control based on the use of pheromones and natural enemies provides the best results. However, the success of these practices is highly dependent on the time of intervention. Moreover, pheromone-based control only works if there is sexual reproduction of the pest. Cases of deuteroparthenogenesis have been observed in populations of *T. absoluta* under laboratory and greenhouse conditions questioning the efficacy of this method. New biocontrol methods and biopesticides need to be developed to ensure sustainable management of the pest. Biocontrol is based on the use of living organisms, e.g. predators and parasitoids, to reduce pest populations. For example, *Necremnus tutae* (Reuter, Hymenoptera: Eulophidae) is considered a parasitoid with the potential to control *T. absoluta.* Biopesticides are plant protection products containing microorganisms (e.g. fungus, bacteria, virus) as their active substance. Andermatt Biocontrol AG has developed a product called Tutavir based on the granulovirus *Phthorimaea operculella* (PhopGV). *T. absoluta* larvae become infected while feeding on contaminated tomato leaves.
Objective of the thesis	To assess the efficacy of the combination of the parasitoid wasp, *Necremnus tutae,* and the granulovirus, *Phthorimaea operculella* (PhopGV), for the biocontrol of *Tuta absoluta*.
Expected outcomes[4]	Combining a parasitoid wasp with a microorganism (entomopathogenic fungus, virus or bacterium) could be better than agents applied separately. For example, pest impaired by parasitoids might be more susceptible to microbial infection. Alternatively, parasitoids might transmit the microorganism between individuals. Conversely, there might also be antagonistic effects, for example, if parasitoid wasps avoided ovipositing on diseased hosts.
Research questions or hypotheses	• Can *N. tutae*, the PhopGV AND/OR the combination of both reduce plant damage caused by *Tuta absoluta*?

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[1] This must be a member of the academic staff within the specialisation programme, either a senior lecturer or a research associate with a PhD or an MSc with a minimum of three years' research experience.

[2] If the thesis is carried out in collaboration with a partner organisation, a substantial part of the content supervision may be delegated to a scientist at the partner organisation (co-advisor). Also, research associates involved in the specialisation programme or working at BFH-HAFL may act as a co-advisor. The co-advisor will not necessarily be the expert for the oral examination. It is not mandatory to define a co-advisor.

[3] If necessary include a description of the project in which the thesis is embedded in an annex

[4] Break down the objective into several partial objectives (expected outcomes or expected results or expected outputs)

	• Does the parasitoid-virus combination have interactive effects (synergistic/antagonistic) on the population development and the plant damage caused by *Tuta absoluta*? • Does the effect of the virus onthe population growth of *T. absoluta* increase over the generations? • Does the virus, the parasitoid AND/OR the combination of both break the exponential population growth of *T. absoluta*?
Approach and methodology	A semi-field experiment will be set up in 28 walk-in thrips-proof cages placed in two identical 60m^2 greenhouses. Four tomato plants (cv. Admiro) with five fully expanded leaves with leaflets will be transferred to the cages at the beginning of the trial and additional ones will be added according to need to provide ad libitum food to *T. absoluta*. Climate and irrigation will be managed with a remote-control system. According to results of a pre-test, 50 couples of *T. absoluta* pupae (sex ratio 1:1) will be released in each cage on day 1. Using calculations based on the literature, two releases of 30 couples of *Necremnus tutae* adults (sex ratio 1:1) were defined as the adequate number of parasitoid wasps to release. The first release will be done as soon as second instar larvae of the moth will be available. The second release will occur 3 weeks later. The plants will be sprayed according to a plan provided by Andermatt Biocontrol AG. The trial will be organised in a randomized complete block design with 7 replicates of the four treatments: (1) *T. absoluta* only (control), (2) *T. absoluta* and *N. tutae, (3) T. absoluta* and PhopGV and (4) *T. absoluta*, *N. tutae* and PhopGV. The blocking factor is position in the greenhouse. One different plant per cage will monitored every week from the emergence of the first generation of *T. absoluta* until the end of the 3rd generation of the pest. The following data will be monitored: number of *T. absoluta* adults per cage, number of *T. absoluta* larvae per cage, percentage of leaf damage per cage. At the end of the trial, yellow traps will be hung in the cages to assess the number of remaining *T. absoluta* adults and of remaining *N. tutae* males and females. If needed, monitored data will be transformed before being analysed using a two-way ANOVA or linear mixed-effects models including the two factors (virus and parasitoid treatments) each with two levels (+/-) as fixed effects as well as their interaction term. Block and time (week of recording) will probably be included as random intercept and/or slope.
Place(s) of research	Agroscope, Conthey

Date of master's thesis colloquium	16.02.2022
Language of the master's thesis	☒ English ☐ German ☐ French ☐ Spanish ☐ Italian
Confidential master's thesis	☒ no ☐ yes ⇨ ☐ agreement[5] has been signed
Assessment matrix	☐ standard HAFL, *Type 1 Desk-work focus* ☒ standard HAFL, *Type 2 Practical focus* ☐ other (add to annex)
Extensive application of a social-scientific method (especially surveys, interviews)	☒ no ☐ yes
Submission of MSc thesis in the form of a scientific publication	☐ no ☒ yes

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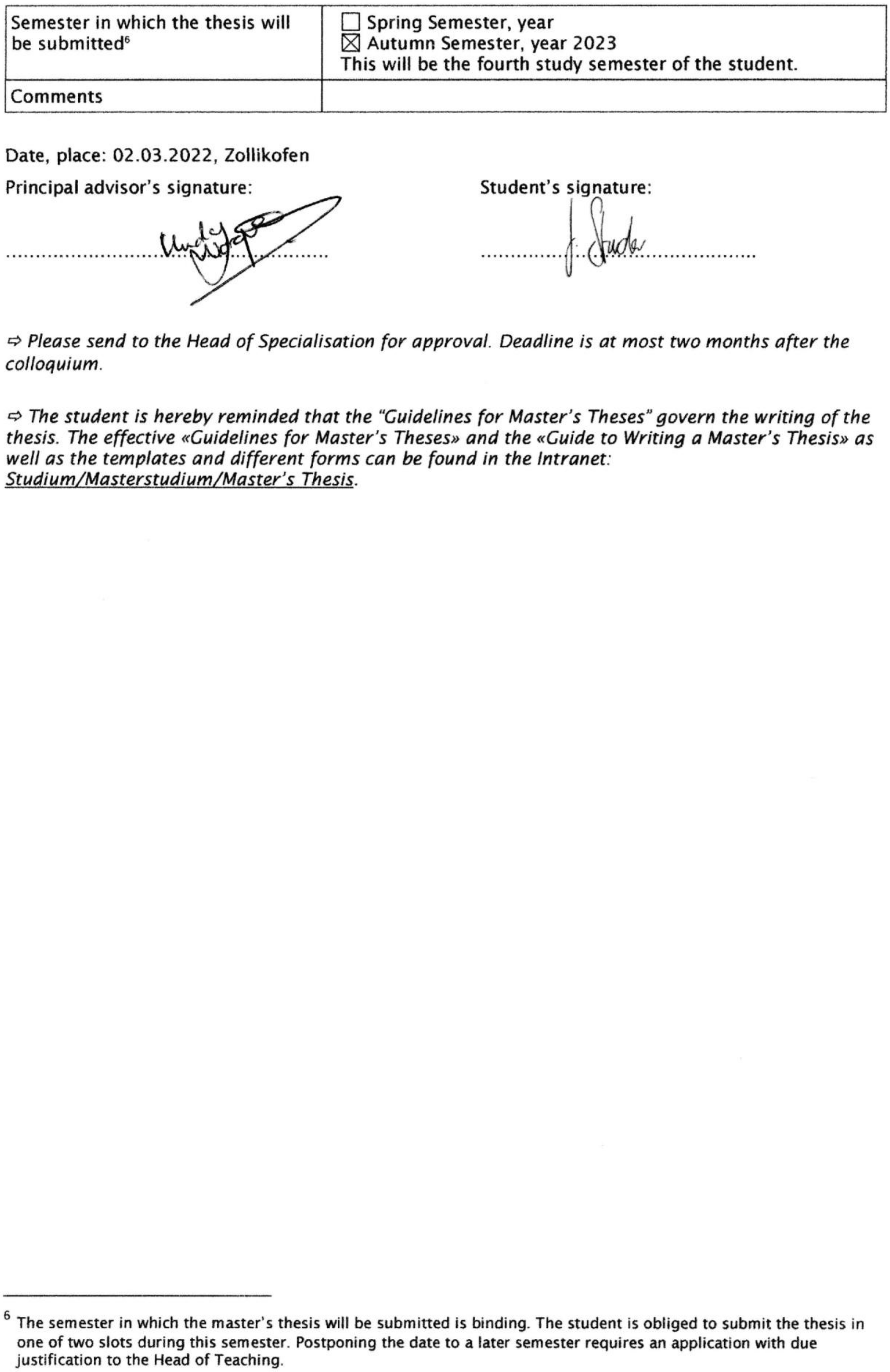
[5] Student Work Agreement - Confidentiality Agreement between BFH-HAFL and student



Semester in which the thesis will be submitted[6]	☐ Spring Semester, year ☒ Autumn Semester, year 2023 This will be the fourth study semester of the student.
Comments	

Date, place: 02.03.2022, Zollikofen

Principal advisor's signature: ....

Student's signature: ....

⇨ *Please send to the Head of Specialisation for approval. Deadline is at most two months after the colloquium.*

⇨ *The student is hereby reminded that the "Guidelines for Master's Theses" govern the writing of the thesis. The effective «Guidelines for Master's Theses» and the «Guide to Writing a Master's Thesis» as well as the templates and different forms can be found in the Intranet: Studium/Masterstudium/Master's Thesis.*

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[6] The semester in which the master's thesis will be submitted is binding. The student is obliged to submit the thesis in one of two slots during this semester. Postponing the date to a later semester requires an application with due justification to the Head of Teaching.