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Biological Pest Control in Agroecosystems

Edited by
Dirceu Pratissoli

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Guest Editor

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Cover image courtesy of Dirceu Pratissoli

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About the Editor

Dirceu Pratissoli

This special edition of the *Agronomy* magazine was coordinated by Professor Dirceu Pratissoli, who is a member of the staff of the Federal University of Espírito Santo, based at the Center for Agricultural Sciences and Engineering, located in the city of Alegre, Espírito Santo, Brazil. He graduated in Agronomy from the Agricultural Center and received his master's degree from the Federal University of Lavras and his doctorate from the University of São Paulo (Luiz de Queiroz School of Agronomy). He also works as a researcher for the University itself, in addition to being a researcher for the National Council for Scientific and Technological Development (CNPQ). His research expertise involves the areas of biological control and phytosanitary pest management.

Preface

The growing challenge of producing food sustainably and in harmony with the environment has driven a profound reassessment of conventional agricultural management models. In this scenario, biological pest control emerges as one of the most promising and ecologically balanced strategies to promote plant health without compromising biodiversity, human health and natural resources.

This reprint, *Biological Pest Control in the Agroecosystem*, was designed with the purpose of offering a comprehensive and updated view of the principles, practices and advances in the use of natural enemies as agents to regulate pest populations. The work is inserted in the context of agroecology, an approach that understands the agroecosystem as a whole and values the complexity of ecological interactions.

Throughout the 10 chapters, the reader will find scientific articles that address practical examples of the use of natural enemies (parasitoids, predators and entomopathogens) in different agricultural crops, as well as their characteristics and effectiveness. The articles also present the challenges faced in adopting this strategy, such as the conservation of beneficial fauna and habitat management.

This reprint is the result of a collaboration between researchers, professors, students and technicians who share a commitment to a more resilient and regenerative agriculture. It is hoped that this reading will inspire professionals and decision-makers to promote agricultural systems that respect ecological limits and strengthen natural pest control processes.

I invite you, the reader, to delve into this content with curiosity, a critical spirit and openness to new paradigms. May this work contribute not only to technical knowledge but also to the construction of a more ethical and integrated vision of agriculture.

Dirceu Pratissoli
Guest Editor

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Article

Production of *Bacillus thuringiensis* in "On Farm" Biofactories Is So Efficient Like a Commercial Product to Control *Spodoptera frugiperda* (Lepidoptera: Noctuidae)

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Abstract: The biological control of pests and diseases in Brazilian crops has increased substantially, and the large-scale multiplication of microorganisms, a practice known as "On Farm", is now being used by farmers. In this study, we aimed to produce and evaluate the quality of *Bacillus thuringiensis* var. *kurstaki* strain HD-1 in "On Farm" biofactories under three aeration rates (0.2, 0.5 and 0.8 air volume/volume of medium/min) (vvm), with regard to the production of endospores and crystals, contaminants, and to calculate the lethal concentration 50 (LC_{50}) against *Spodoptera frugiperda* larvae. After 48 h, the number of viable spores differed significantly between 0.2 (5.75×10^7), 0.5 (1.33×10^8) and 0.8 (5.40×10^8) vvm. The culture media did not show enough selectivity. A significant difference was observed for the LC_{50} calculated for 0.2 vvm (7.06×10^4), 0.5 vvm (2.07×10^4), 0.8 vvm (4.40×10^3), and the commercial product (3.79×10^3). The results of this study showed that the aeration rate affects the multiplication efficiency of *B. thuringiensis* in biofactories. In addition, post-process quality control analyses found no pathogenic contamination, reinforcing the safety and viability of the production process. The 0.8 vvm rate resulted in the greater production of spores and crystals, and statistically similar results to the commercial product for the control of *S. frugiperda*.

Keywords: aeration; biological control; entomopathogenic bacteria; large-scale multiplication

1. Introduction

The adoption of biological control of plant pests and diseases with the use of microorganisms grows every year, due to factors linked to soil and water pollution, the risk of human and animal poisoning, and the resistance of arthropod pest populations and vectors to chemical insecticides, in addition to damage caused to non-target organisms [1–5]. Several microorganisms, mainly fungi and bacteria, can be used as agricultural biodefense against pests and diseases through the synthesis of secondary metabolites, which act directly or indirectly on other organisms, as well as acting as plant growth promoters, favoring the adoption of this type of management [6–10].

In Brazil, government actions have boosted the use of biological products in the country, due, among other factors, to the proven effectiveness of biological-based products and their ecological and economic advantages over chemical inputs [11,12]. In 2009, Decree n.° 6913 was sanctioned, which established the right of farmers to produce biological inputs for their own use without the requirement for official registration [13]. Following this decree, there was a significant increase in the production of microorganisms on the farms themselves [14]. This advance reflected a change in agricultural management approaches, focusing on more sustainable solutions that are less dependent on conventional chemicals.

In this context, the production of microorganisms for use on the farm, known as "On Farm" production, has been used by farmers, enabling the large-scale multiplication of bacteria and fungi beneficial to agriculture [15].

Today there are three aspects of "On Farm" production in Brazil. The first uses low-quality inputs for multiplication, such as waste from the farm itself, untreated water, inoculum without purity and lack of final quality control [15,16]. The second utilizes automated, autoclavable equipment, temperature, pH, aeration and foam adjustment, in addition to quality control of the multiplied products; the third is where specialized companies provide "technology packages" and use biofactories made up of stainless steel tanks, electric pumps for the recirculation of the culture medium, temperature sensor, spray ball for internal cleaning of the tank, microbiological filters and air compressors, products for sanitizing equipment and water treatment, sterile culture media and inocula free of contaminants and impurities, in addition to the quality control of the cultured microorganisms [17].

One of the main multiplied microorganisms used on farms is *Bacillus thuringiensis*, a bacterium that has high efficiency and selectivity in controlling various agricultural pests [15,18]. It is a Gram-positive, aerobic bacterium that forms endospores and protein crystals, which occur naturally in soil, dead insects and plants [19,20]. The insecticidal proteins of *B. thuringiensis* are the most commonly used in insect biocontrol, due to their high degree of specificity with the target insect. These insecticidal proteins show activity against larval stages of different orders of insects, especially Lepidoptera, active even at low doses [21–23]. Among the insect pests of the order Lepidoptera, one of the most important for Brazilian agriculture is *Spodoptera frugiperda* (Lepidoptera: Noctuidae), considered the main pest in corn crops in the Americas, causing significant production losses [24]. Additionally, this insect causes damage to cotton, rice, sorghum, beans and peanuts [24–26]. Its control is carried out with the use of chemical insecticides, transgenic plants that express *B. thuringiensis* Cry toxins and biological pesticides, including commercial products based on strains of *B. thuringiensis* that present different insecticidal toxins [27–29].

The mechanism of action of *B. thuringiensis* is well described, the toxic protein specifically binds to the intestinal receptors of the target pest species, killing the larvae through starvation and septicemia [30,31]. The synthesis of Cry proteins is closely linked to the sporulation phase, which occurs when the culture enters the stationary growth phase. Mature spores and crystals are released by the lysis of the sporangia during the last few hours of fermentation [32–34]. The biomass production of *B. thuringiensis* using low-cost culture media formulated from readily available ingredients, including agricultural and food wastes, is a well-studied area [35]. Various carbon and nitrogen sources, such as glycerol discarded from biodiesel production, Saccharomyces cerevisiae waste from beer production, soybean meal and sugar cane molasses, play an important role in cell production, sporulation and the number of crystals with entomotoxic activity [30,36,37]. Other essential factors in the *B. thuringiensis* propagation process should also be considered, such as temperature, pH, and oxygen availability [38].

The supply of oxygen during the multiplication of bacteria of the genus *Bacillus* represents an extremely important factor in the sporulation process [39] and in the formation of insecticidal proteins from *B. thuringiensis* [38,40]. In studies evaluating aeration variations in the multiplication of this bacteria in bioreactors, a higher concentration of spores and crystals was found at aeration rates that vary between 0.5 and 1 vvm (air volume/volume of medium/min) than those obtained with the low availability of O_2 [41]. On an industrial production scale, one of the main objectives is to complete fermentation in bioreactors in a short period without compromising the quantity and quality of the spores and crystals produced. This same understanding can be extended to the practice of multiplication in automated biofactories of large volumes for personal use, normally 1000 to 2000 L, which require relatively high energy costs to operate.

This work aimed to evaluate the production of endospores and crystals of the *B. thuringiensis* subsp. *kurstaki* strain HD-1 in an "On Farm" biofactory system under

different aeration rates, to verify the presence of contaminants in these multiplies and to evaluate toxicity to *S. frugiperda* larvae.

2. Materials and Methods

2.1. Location of Experiments

The tests were conducted at the SoluScience research center of the company SoluBio Tecnologias Agrícolas, in Brasília, Federal District, Brazil. The biofactories, culture medium, artificial diet, *S. frugiperda* larvae and other reagents used were provided by the company.

2.2. Multiplication of Bt in Biofactories

The multiplication of *B. thuringiensis* was carried out in automated stainless steel biofactories, with a capacity of 1000 L and at different aeration rates: 0.2, 0.5 and 0.8 vvm (air volume/medium volume/min). Cultivations were carried out in triplicate. Initially, the removable parts of the biofactories were sanitized to remove possible impurities and biofilm, then the internal parts were sanitized with a product composed of peroxides (SoluBio Tecnologias Agrícolas, Jataí, GO, Brazil) at a concentration of 1% using a pump as a tool to recirculate the product, to which a spray ball is attached, which allowed the sanitizing product to reach the entire internal surface of the tank. The water used in the process was sanitized with 0.03% of the same peroxide-based product (SoluBio Tecnologias Agrícolas, Jataí, GO, Brazil). After this process, the compound was neutralized. The water temperature in the tank was adjusted to 28 ± 2 °C, 2% (*w*/*v*) sterile culture medium (SM, SoluBio Tecnologias Agrícolas, Jataí, GO, Brazil) was added and the pH was adjusted to 7.0 ± 0.2 using sodium hydroxide (NaOH, NEON, Suzano, SP, Brazil) and/or citric acid ($C_6H_8O_7$, NEON, Suzano, SP, Brazil). The last step consisted of adding 1% (*v*/*v*) of the pure bacterial inoculum previously evaluated by phase contrast microscopy (magnification 1000×). The inoculum containing *B. thuringiensis* subsp. *kurstaki*, strain HD-1 (S1450) was obtained from the company SoluBio Tecnologias Agrícolas at the minimum concentration of 1.0×10^8 spores mL^{-1}.

Throughout the process, all the necessary measures were taken to avoid contamination, such as the use of 70% ethanol (QHEMIS, Jundiaí, SP, Brazil) on the packaging and lids of the biofactories, as well as the use of all the PPE (personal protective equipment) recommended during the product handling process. A sample was taken at each stage of the process (water from the tank before cleaning, water from the clean tank, treated water and water with culture medium) to monitor the presence of contaminants and the sterility of the products used. The samples were seeded in Petri dishes (90 × 15 mm, CRAL, Cotia, SP, Brazil) containing TSA culture medium (Trypticase Soy Agar, Himedia Laboratories, Kennett Square, PA, USA) and kept at 28 °C/60% RH (relative humidity) in a growth oven (415D, Ethik Technology, Vargem Grande Paulista, SP, Brazil) for 24 h.

After 24 and 48 h of cultivation of *B. thuringiensis* in the biofactories, samples were collected in sterile Falcon tubes for use in serial dilutions and to determine the number of spores mL^{-1}. Samples taken at 48 h post multiplication were used to assess contaminants and for the mortality test against *S. frugiperda*, considering the complete sporulation of the bacteria in all systems.

Determination of Viable Spore Concentration

Qualitative assessments were performed using a phase contrast microscope at 1000× magnification (Axiolab5, Zeiss, Oberkochen, Germany) to verify the presence of vegetative cells, spores, and crystals of *B. thuringiensis* grown in each aeration system. For quantitative analyses, vegetative cells and spores were separated by heat shock of the samples and subsequent serial dilution using the spread plate technique [17,42–44]. The same procedures were applied for the commercial product containing *B. thuringiensis.*

2.3. Determination of the Presence of Contaminants

In the absence of defined criteria for assessing the presence of contaminants in bio-inputs produced in the "On Farm" system, the recommendations set out in Joint Ordinance SDA/MAPA-IBAMA-ANVISA No. 1 of 10 April 2023 [45], which establishes procedures and requirements to be adopted for the registration of industrially produced microbiological products for use in agriculture, were adopted. Table 1 shows and describes the culture media used and the contaminants assessed at the end of the multiplication process, as well as the maximum concentrations allowed, according to the Ordinance. To analyze the presence of contaminants, an established methodology was adopted [17]. Due to the limited selectivity of the available culture media, samples of non-target bacteria were sent for identification by sequencing the rpoB gene (Gogenetic Laboratories, Curitiba, PR, Brazil).

Table 1. Culture media used to detect the presence of contaminants listed in Ordinance 01/2023 MAPA/ANVISA/IBAMA in bio-inputs.

Microorganism	Specification	Culture Media [1]
Thermotolerant coliforms	$\leq 10^3$ UFC/g or mL	Violet Red Bile Glucose Agar
Salmonella spp.	Absent in 25 mL	Brilliant Green Agar
Staphylococcus coagulase-positive	$\leq 10^3$ UFC/g or mL	Baird–Parker Agar Base

[1] Not present in the current Ordinance.

2.4. Lethality Bioassay Against S. frugiperda

The insects used for the bioassay were obtained from the breeding laboratory of the company SoluBio Tecnologias Agrícolas, where the population is mass-reared [46]. Bioassays to determine the concentration to cause 50% mortality (LC_{50}) against *S. frugiperda* larvae were carried out using one sample from each biofactory after 48 h of cultivation [47]. A commercial product formulated with *B. thuringiensis* subsp. *kurstaki* strain HD-1 (S1450) was used as a positive control at a concentration of 5.0×10^8 spores mL^{-1}.

S. frugiperda mortality was observed using cell culture plates with 24 wells and acrylic lids (each well representing a replicate for the treatments), with a sterile artificial diet comprising cassava beans, brewer's yeast, wheat germ, agar and distilled water [46].

Ten serial dilutions of each sample from the biofactories were prepared. An amount of 35 µL of the diluted bacterial suspension was added to each plate containing the artificial diet and left uncovered in a laminar flow for 30 min to dry. A plate containing an uninoculated artificial diet was used as a negative control, with a maximum of 15% of mortality accepted. A second instar caterpillar (24 per replicate) was transferred to each well, using a soft bristle brush soaked in sterile distilled water to prevent damage. These plates were covered with an acrylic lid and kept inverted in a climate-controlled room at 26 °C with a 12 h photoperiod [47].

After 48 h, the surviving larvae were transferred to 50 mL plastic cups with acrylic lids containing a block of approximately 1 cm^3 of artificial diet of the same composition supplemented with preservatives and antimicrobials [46] to observe the residual effect of the treatments. After seven days, the dead larvae were counted and the percentage of final mortality in each dilution was determined [48]. The test was conducted in triplicate for each aeration rate, with *B. thuringiensis* cultivated in the biofactories and the commercial product. To calculate the mean mortality values, a total of 72 caterpillars were utilized for each aeration rate.

2.5. Statistical Analysis

Two experiments were carried out in a completely randomized design: (1) in a 3×2 factorial arrangement with three replications, where the treatments consisted of aeration rates (0.2, 0.5 and 0.8 vvm) evaluated at two fermentation times (24 h and 48 h); (2) four treatments: bacterial cultures obtained from different aeration rates (0.2, 0.5 and 0.8 vvm) and a formulated product. The viable spore mL^{-1} data were log-transformed

and subjected to analysis of variance and Tukey's test ($p < 0.05$). The LC_{50} calculation and the confidence interval for each treatment were estimated by probit analysis [49] using the "drc" statistical package in the R software (version 4.4.1, R Core Team, Vienna, Austria).

3. Results

3.1. Concentration of B. thuringiensis Spores in Biofactories with Different Aeration Rates

Aeration rates affected the multiplication of *B. thuringiensis* in biofactories. Microscopic analysis (1000× magnification) revealed bacterial cells with the presence of endospores and crystals (sporangia) in the system with an aeration rate of 0.2 vvm, whereas in the other systems the complete formation of these structures and the release of part of them into the culture medium (cell lysis) had already occurred. The size and shape of the vegetative cells and other structures were not affected by the aeration rates. On the other hand, the average number of viable spores mL^{-1} 24 h after the start of multiplication was higher at aeration rates of 0.5 vvm (1.32×10^8 spores mL^{-1}) and 0.8 vvm (1.48×10^8 spores mL^{-1}). The lowest number of viable spores was observed in the system containing 0.2 vvm, with an average of 4.07×10^7 spores mL^{-1} (Table 2). In terms of multiplication time, there was a significant difference with the 0.8 vvm aeration rate, with an average increase from 1.48×10^8 spores mL^{-1} at 24 h to 5.40×10^8 spores mL^{-1} at 48 h. After 48 h, there was a statistical difference between all treatments ($p > 0.05$), with the 0.8 vvm rate being higher than the others (5.40×10^8 spores mL^{-1}) (Table 2).

Table 2. Average number of viable spores per mL of *Bacillus thuringiensis* after 24 and 48 h of multiplication in biofactories under three aeration rates.

Aeration Rates (vvm)	Spores Count per mL^{-1}		
	24 h	**48 h**	**Coefficient of Variation (%)**
0.2	4.07×10^7 bA [1]	5.75×10^7 cA	22.93
0.5	1.32×10^8 aA	1.33×10^8 bA	3.11
0.8	1.48×10^8 aB	5.40×10^8 aA	0.83
Coefficient of variation (%)	12.28	3.29	

[1] Equal lowercase letters in the same column and equal uppercase letters in the same row do not indicate a significant difference according to Tukey's test ($p < 0.05$).

3.2. Contaminant Assessment

When assessing the presence of contaminants, no growth of thermotolerant coliforms, Salmonella spp. or coagulase positive staphylococci was observed in any of the samples taken from the cultures in the "On Farm" biofactories in this study. Bacterial colonies were observed on Violet Red Bile Glucose (VRB) and Brilliant Green (BG) agar media at all three aeration rates (Figure 1). However, the morphological characteristics of the colonies present on VRB and BG differ from those specified by the manufacturer. After sequencing and analysis of the rpoB gene, the bacteria were identified as *Enterobacter hormaechei* (Enterobacteriaceae, 99% similarity, 1293/1298 rpoB bases) and *Ralstonia pickettii* (Burkholderiaceae, 99% similarity, 1385/1395 rpoB bases), microorganisms that are not classified as contaminants according to MAPA/ANVISA/IBAMA Ordinance 01/2023. As for the selective medium Baird–Parker Agar, no bacterial growth was observed.

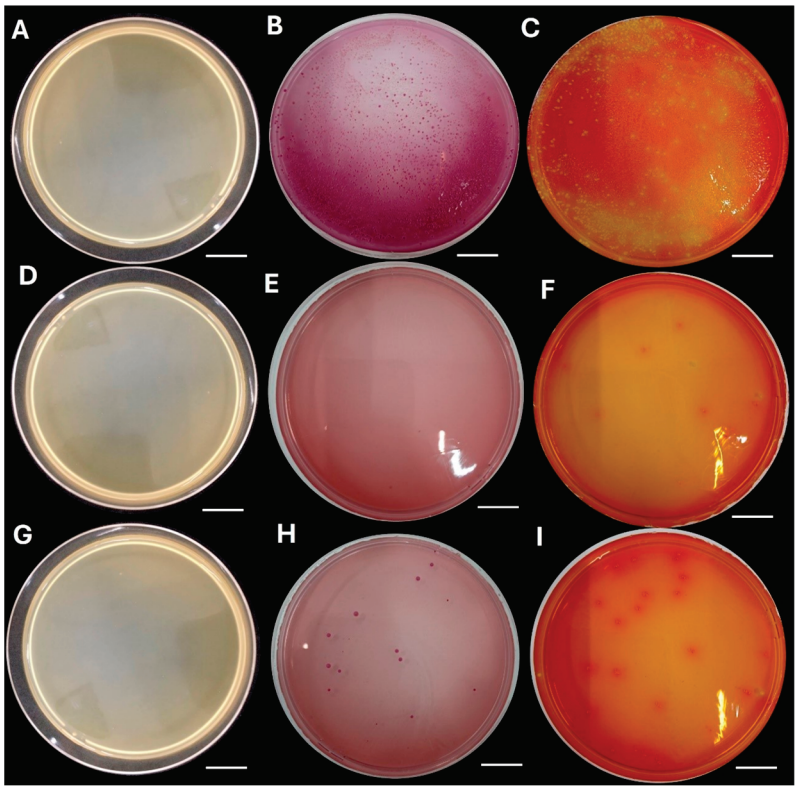

Figure 1. Detection of contaminating bacteria present in "On Farm" biofactories samples after 48 h of multiplication under different aeration rates. Baird–Parker Base Agar Medium ((**A**): 0.2 vvm; (**D**): 0.5 vvm; (**G**): 0.8 vvm); Violet Red Bile Glucose Agar Medium ((**B**): 0.2 vvm; (**E**): 0.5 vvm; (**H**): 0.8 vvm); Brilliant Green Agar ((**C**): 0.2 vvm; (**F**): 0.5 vvm; (**I**): 0.8 vvm) (bar: 1 cm).

3.3. LC_{50} Determination

All the treatments, aeration rates and the formulated product influenced the mortality of *S. frugiperda* larvae, and therefore the estimated LC_{50}, showing significant statistical differences between the multiplicates (Table 3). The highest estimated LC_{50} (7.06×10^4 spores mL^{-1}) was obtained in larvae exposed to biofactory samples containing 5.75×10^7 spores mL^{-1} (0.2 vvm aeration). In the system with a rate of 0.8 vvm, the LC_{50} value was approximately 10 times lower than those observed in the treatments with 0.2 and 0.5 vvm. The LC_{50} obtained with the 0.8 vvm rate was statistically similar to the commercial product based on the *B. thuringiensis* subsp. *kurstaki* strain HD-1 (Table 3).

Table 3. LC_{50} and confidence interval estimate for *Spodoptera frugiperda* larvae exposed to *Bacillus thuringiensis* subsp. *kurstaki* strain HD-1 (S1450) cultures multiplicated in "On Farm" biofactories.

Treatments	Spores mL^{-1}	LC_{50}	Confidence Interval
0.2 vvm [1]	5.75×10^7	7.06×10^4 a	4.61×10^4–9.51×10^4
0.5 vvm	1.33×10^8	2.07×10^4 b	1.18×10^4–2.97×10^4
0.8 vvm	5.40×10^8	4.40×10^3 c	2.81×10^3–5.99×10^3
Commercial Product	5.00×10^8	3.79×10^3 c	3.66×10^3–4.29×10^3

[1] vvm: volume of air/volume of medium/min. LC_{50} means followed by the same letter in the column do not differ from each other when the confidence interval is superimposed on the probit analysis.

4. Discussion

Few studies have been carried out on the multiplication of microorganisms "On Farm" in Brazil, the studies that has been conducted has typically focused on the evaluation of contaminating microorganisms rather than the target [16,50]. The tendency to seek safer and more efficient methods for the propagation of microorganisms for agricultural purposes

has led to an interest in elucidating the processes involved in large-scale production directly on the farm, with the aim of guaranteeing farmers' autonomy in the use of biological inputs. For this purpose, it is necessary to establish cultivation and efficiency parameters.

In *B. thuringiensis* culturing, whether in bioreactors or in "On Farm" biofactories, the aim is to complete fermentation in the shortest possible time without compromising the sporulation of the target microorganism and the number of δ-endotoxins produced. There is a high positive linear correlation between the amount of oxygen consumed to reach the maximum cell concentration [40,41,51,52]. In this study, it was possible to obtain at least 1.0×10^8 spores mL^{-1} of *B. thuringiensis* from 0.5 vvm of aeration, regardless of the growth time (Table 2), which was crucial to achieving adequate development for this strain. Achieving the ideal minimum sporulation in 24 h of multiplication in the biofactory is considered relevant, since completing the process in a shorter time can significantly reduce the energy cost of running the system and the possible appearance of unwanted microorganisms.

The two bacterial species detected in the GB and VRB media, *E. hormaechei* and *R. pickettii*, are not considered to be pathogenic contaminants. The first bacterium belongs to the family Enterobacteriaceae, and several strains of this species have already been described as symbiotic, present in the gut of houseflies [53] and in the rumen of cows [54] and can also colonize the internal tissues of plants [55]. The second, *Ralstonia pickettii* (Burkholderiaceae), is commonly found in soil, river and lake water in healthy individuals. Infections with *Ralstonia* spp. are extremely rare [56,57]. Some studies have identified a high biotechnological potential of this bacterium for use in the bioremediation of xenobiotics by degrading toxic substances such as toluene and trichloroethylene [58].

The results of the analyses for the detection of the contaminants present in this study indicate the limitations of using GB and VRB media for the analysis of multiplication in biofactories. Studies have shown that VRB medium is not selective only for *Salmonella* spp. [59,60]. The AVB medium is also not strictly selective [61], as indicated in their recommendations [62]. In this case, when microbial growth with characteristics similar to pathogenic bacteria is found, complementary tests are suggested for confirmation, such as biochemical tests [63]. It is important to consider the limit of contaminants defined by Brazilian legislation for commercial products as the minimum to be followed in this type of multiplication of biological inputs. Unwanted microorganisms can come from, for example, the water used for growth, the ambient air, the packaging of the inputs, the PPE and insects such as flies [53]. To produce these bio-inputs, it is essential to adopt strategies to reduce the potential for contamination as much as possible, especially at the beginning of the process.

Regarding the insecticidal potential of *B. thuringiensis* multiplicates, the higher oxygen rate (0.8 vvm) favored the spore yield per mL^{-1} (5.40×10^8 spores mL^{-1}), resulting in a lower LC_{50} against *S. frugiperda* (4.40×10^3 spores mL^{-1}), suggesting a directly proportional relationship between aeration and spore yield mL^{-1}, which was reflected in the mortality efficiency. Similar results were obtained when testing the insecticidal potential of *B. thuringiensis* at a concentration of 1.00×10^9 CFU mL^{-1} against *Spodoptera exigua* larvae, with an estimated LC_{50} of 7.50×10^4 CFU mL^{-1}, considered efficient for its control [64]. Seven bioinsecticides based on *B. thuringiensis* were evaluated for the control of second instar larvae of *Spodoptera albula*, and mortality rates above 80% were observed in three formulations, with a LC_{50} of 1.00×10^5 spores mL^{-1} [65], a value higher than that obtained for *S. frugiperda* at the three aeration rates evaluated in this study. These results highlight the viability and efficiency of *B. thuringiensis* growth in aerobic systems and demonstrate that industrial-scale production can be comparable in terms of efficacy to commercial products available on the market.

The control for plant pests like *Spodoptera frugiperda,* is carried out with the use of chemical insecticides, transgenic plants that express *B. thuringiensis* Cry toxins and biological pesticides, including commercial products based on strains of *B. thuringiensis* that present different insecticidal toxins [27–29] and *B. thuringiensis* multiplicated in "On Farm" biofactories (Table 3). *B. thuringiensis* strains can be successfully integrated with

pyrethroids, most organophosphorus compounds and some biological control agents within integrated pest management programs [66].

The multiplication of microorganisms must ensure several minimum microbiological procedures to guarantee that the target microorganism prevails in the culture medium and that these preparations do not carry contaminants of importance for public and environmental health [50]. This work has shown that by adopting a good infrastructure, appropriate and sterile materials, inoculum free of contaminants and at the ideal minimum concentration for cultivation, trained technical staff to carry out the multiplication, as well as an appropriate methodology to evaluate quality control, it is possible to obtain an effective and safe product for application in the field.

5. Conclusions

The results of this study showed that the aeration rate directly affects the multiplication efficiency of *B. thuringiensis* in biofactories. In addition, post-process quality control analyses found no pathogenic contamination, reinforcing the safety and viability of the production process. The 0.8 vvm rate resulted in the greater production of spores and crystals compared to the 0.2 and 0.5 vvm rates, and statistically similar results to the commercial product for the control of *S. frugiperda*, which was able to reduce the dose of "On Farm" products currently used in the field.

The production of microorganisms on farms for their own use requires infrastructure, quality control and a specialized technical team to ensure the efficiency and safety of the multiplied products. This work provides information on aeration to improve the process of multiplying *B. thuringiensis* in this system, contributing to future studies with other microorganisms and to the advancement of the use of automated biofactories in agriculture.

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Predation Efficiency and Biological Control Potential of *Micromus angulatus* Against *Aphis craccivora*

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Abstract: *Micromus angulatus* (Neuroptera: Hemerobiidae) is a widely distributed and highly effective predator that shows promise as a biological control agent against agricultural pests, particularly *Aphis craccivora*, the cowpea aphid, which threatens leguminous crops globally. This study aimed to evaluate the predation behaviour, search efficiency, and intraspecific interference of *M. angulatus* at different developmental stages, including first- to third-instar larvae and adults, in controlling adult *A. craccivora* populations. The results demonstrated that all developmental stages of *M. angulatus* exhibited predatory behaviour towards adult aphids, with the functional response fitting the Holling Type II model. The instantaneous attack rates for first-, second-, and third-instar larvae and adults were 1.0017, 1.0448, 0.9581, and 0.9508, respectively; the handling times were 0.0158, 0.0051, 0.0016, and 0.0011 days, respectively; and the theoretical maximum daily predation rates were 63.2911, 196.0784, 625, and 909.0909 aphids, respectively. The pest control efficacies were 63.3989, 204.8672, 598.8311, and 864.3192, respectively. The search efficiency at each developmental stage was negatively correlated with aphid density, which decreased as the prey density increased, with second-instar larvae showing the greatest decrease and adults the least. When the aphid density was fixed, the daily predation rate of individual *M. angulatus* decreased with increasing conspecific density, indicating that predation was affected by its own density, with the interference effect equation being $E = 0.6194P^{-0.87}$. These findings indicate that *M. angulatus*, especially in the third-instar larval and adult stages, has considerable potential as a biological control agent for managing *A. craccivora* populations in agricultural settings. This study contributes valuable insights for developing sustainable agricultural practices by decreasing reliance on chemical pesticides.

Keywords: *Micromus angulatus*; *Aphis craccivora*; Hemerobiidae; predation efficiency; biological control; brown lacewing; sustainable agriculture

1. Introduction

The cowpea aphid *Aphis craccivora* Koch, 1854, also known as the alfalfa aphid, is a pest in the order Hemiptera, family Aphididae, with a broad geographic distribution. This aphid is characterised by its rapid reproductive rate and extensive host range and affects more than 200 plant species, including important leguminous crops such as cowpea (*Vigna unguiculata* [Linnaeus, 1753]), broad bean (*Vicia faba* Linnaeus, 1753), alfalfa (*Medicago sativa* Linnaeus, 1753), peanut (*Arachis hypogaea* Linnaeus, 1753), and pea (*Pisum sativum* Linnaeus, 1753) [1]. Both the adult and nymph stages of aphids feed by piercing the tender leaves and shoots of plants, leading to leaf curling and wilting. This feeding activity significantly disrupts photosynthesis, thereby inhibiting plant growth and reducing yield. Moreover, *A. craccivora* is a vector for several plant viruses, including bean leaf roll virus (BLRV) and faba bean necrotic yellow spot virus (FBNYSV). These viruses pose severe threats to the

yield and quality of leguminous crops [2–4]. Given the extensive damage caused by this pest, effective control measures are crucial.

Traditionally, the control of *Aphis craccivora* has heavily relied on chemical pesticides. However, prolonged dependence on chemical pesticides not only poses a risk of environmental pollution but can also lead to the development of resistance to these chemicals, thereby compromising crop food safety. Furthermore, the use of chemical pesticides may negatively impact non-target organisms, including the death of beneficial insects and the disruption of ecosystem functions [5]. These issues have prompted the exploration of alternative pest management strategies that are more environmentally friendly and efficient. Biological control, as a sustainable pest management strategy, leverages natural enemies such as predatory insects, parasitic insects, and pathogens to effectively manage pest populations and reduce reliance on chemical pesticides [6]. This approach not only helps protect the environment and reduce pesticide residues but also prevents the development of pest resistance, thereby promoting sustainable agricultural practices.

In biological control research, lacewings (Neuroptera: Hemerobiidae) have received significant attention as an important group of natural enemies. Among them, the brown lacewing (*Micromus angulatus* [Stephens, 1836]) is a predatory insect of considerable economic value. Both the adults and larvae of *M. angulatus* effectively prey on various pests, including the strawberry aphid (*Chaetosiphon fragaefolii* [Cockerell, 1905]) in strawberries and the peach aphid (*Myzus persicae* [Sulzer, 1776]) in sweet peppers, demonstrating notable efficacy in controlling these pests [7,8]. *M. angulatus* is characterised by its robust predatory ability and wide adaptability and performs well under various climatic conditions [9]. However, despite its demonstrated effectiveness in multiple crops, its potential for controlling *Aphis craccivora* has not been thoroughly explored. The biological characteristics of this insect, the optimal developmental stages for release, and its performance in practical applications still require systematic research and validation [10].

Current research indicates that *Micromus angulatus* has substantial potential in biological control applications. Studies have shown that releasing first-instar larvae or female adults of *M. angulatus* can effectively control peach aphids (*Myzus persicae*) on sweet peppers within two weeks [9]. Additionally, *M. angulatus* exhibits a favourable predatory ability against various important agricultural pests, including the effective control of strawberry aphids (*Chaetosiphon fragaefolii*) [7]. Despite promising results for controlling different aphid species, its predatory function and control efficacy against *Aphis craccivora* have not been extensively studied. For example, it remains unclear which developmental stage of *M. angulatus* is most suitable for release to combat aphids. This differs from green lacewings, such as *Chrysoperla carnea* (Stephens, 1836), which are typically provided to end-users as second-instar larvae or eggs [11,12].

Therefore, this study aims to systematically evaluate the predatory ability of *Micromus angulatus* at different stages of its life cycle (first- to third-instar larvae and adults) against *Aphis craccivora*. This research explores the potential of *M. angulatus* as a biological control agent for leguminous crops. Detailed assessments will be conducted through greenhouse experiments to analyse the predatory behaviour, searching efficiency, and intraspecific interference of *M. angulatus*. These studies are intended to provide practical strategies for the biological control of *A. craccivora* and offer theoretical support and practical guidance for the biological control of other agricultural pests.

2. Materials and Methods

2.1. Materials Collection and Preparation

Vicia faba Linnaeus, 1753 was used as the host plant. Faba bean seeds were soaked one day before planting and then sown in mixed soil containing vermiculite and substrate soil (potted). After the faba bean seedlings reached 3–4 cm, each plant was inoculated with 3–5 aphids. After 10–15 days, each faba bean seedling could breed over 100 aphids, which were then used to feed *M. angulatus* in an artificial climate chamber at 25 ± 1 °C, 60 ± 5% relative humidity, and a light cycle of L:D = 14:10.

Adult samples of brown lacewing (*M. angulatus*) were collected from *Corylus mandshurica* Maximowicz, 1859 in Xianghetun, Gaojiadian Town, Xifeng County, Liaoning Province (42°67′13″ N, 124°45′92″ E). Both the larval and adult stages of the predator were maintained on a diet of cowpea aphids under controlled conditions as previously described. Detailed observations of moulting frequency and morphological characteristics allowed for the identification and differentiation of the 1st-, 2nd-, and 3rd-instar larvae (Figure 1), as well as the adult stage [13].

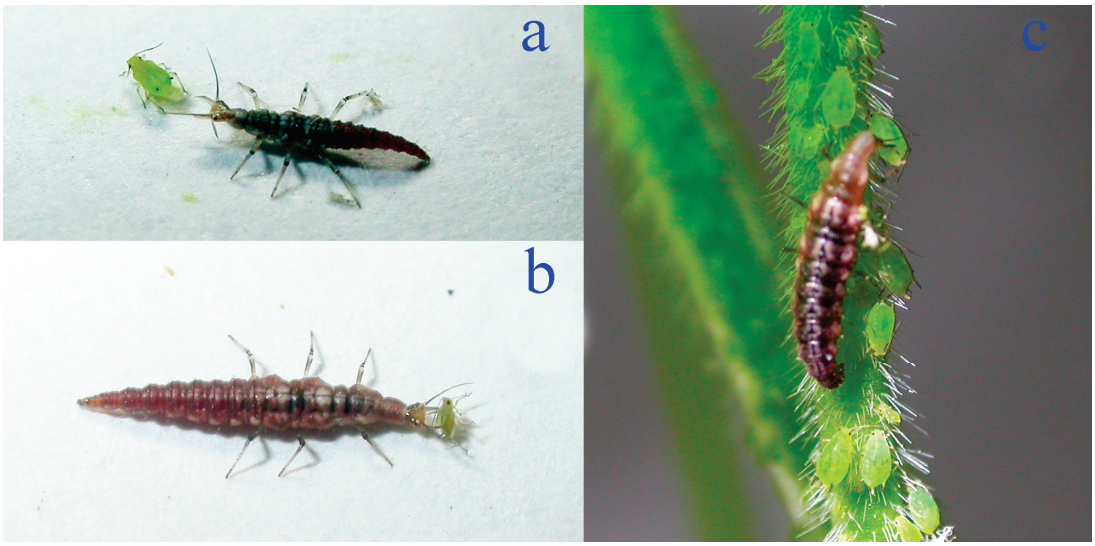

Figure 1. Predation by different developmental stages of brown lacewing: (**a**) 1st-instar larva; (**b**) 2nd-instar larva; and (**c**) 3rd-instar larva.

The following instruments were used during the experiments: SZ680 stereomicroscope, Chongqing Optech Company (Chongqing, China); and RXZ-436-type artificial climate chamber, Ningbo Jiangnan Instrument Factory (Ningbo, China).

2.2. Predation Rate Measurement of Micromus angulatus on Aphis craccivora

On the basis of preliminary experimental results, after 24 h of exposing the cowpea aphids to brown lacewings, 1st-instar larvae were starved for 12 h, while 2nd- and 3rd-instar larvae as well as adults were starved for 24 h. Each lacewing sample was then individually placed in a Petri dish (9 cm diameter and 2 cm high). Fresh 3 cm long faba bean sprouts were added to each dish, followed by the introduction of adult cowpea aphids at the required densities. The densities for the 1st-instar larvae were 10, 20, 30, 50, and 70 aphids/dish, and for the 2nd- and 3rd-instar larvae and adults, the densities were 20, 30, 50, 70, 100, and 120 aphids/dish. The dishes were sealed with cling film punched with small holes for ventilation and incubated in an artificial climate chamber at 25 ± 1 °C, 60% ± 5% relative humidity, and a light cycle of 14 L:10 D. After 24 h, the number of surviving aphids in each dish was counted, and the predation rate of *M. angulatus* was calculated. Each treatment was repeated six times, with a control group that contained only cowpea aphids at equivalent densities, and the natural mortality in the control group was used for correction [14].

2.3. Functional Response of Micromus angulatus to Aphis craccivora

On the basis of preliminary experimental results, the functional response of *M. angulatus* to adult cowpea aphids across different instars can be modelled via the Holling Type II model, with the equation $N_a = aN_0T/(1 + aT_hN_0)$ [15], where N_a is the number of aphids consumed by *M. angulatus*; a is the instantaneous attack rate of *M. angulatus* on cowpea aphids; N_0 is the initial aphid density; T is the total experimental time, in this case, 1 day; T_h is the handling time, i.e., the time taken by *M. angulatus* to consume one aphid; $1/T_h$ is

the maximum daily predation rate of *M. angulatus*; and *a/Th* is its control efficacy, which can be used to measure the predatory capacity of *M. angulatus* on cowpea aphids. The model equation was transformed via the reciprocal method to $1/N_a = 1/aTN_0 + T_h/T$, and the corresponding parameters were calculated via the least squares method. To evaluate the deviation between the measured and theoretical predation rates of *M. angulatus*, a chi-square goodness-of-fit test was conducted [16].

2.4. Searching Efficiency of Micromus angulatus on Aphis craccivora

The search efficiency of *M. angulatus* across different instars on adult cowpea aphids was analysed via the equation $S = a/(1 + aT_hN_0)$ [17], where S is the search efficiency and a, N_0, and T_h are the same as those in the Holling Type II model.

2.5. Effects of Micromus angulatus Density on Predatory Activity

In a Petri dish, 200 adult cowpea aphids and a 3 cm long fresh faba bean sprout were placed. The density of the 3rd-instar larvae of *M. angulatus* after 24 h of starvation was set at 1, 2, 3, 4, and 5 individuals/dish across the five treatments. Each treatment was replicated five times, and the dishes were sealed with cling film punched with small holes for ventilation and then incubated in an artificial climate chamber under the same conditions as above. After 24 h, the number of remaining aphids in each dish was counted to calculate the predation rate of 3rd-instar larvae of *M. angulatus* and to analyse the predation rate relative to intraspecific interference. The interference effect model was used for fitting, with the equation $E = QP^{-m}$, where E is the average predation rate calculated via the formula $E = Na/N_0P$; P is the density of predators in a given space; Q is the searching constant; and m is the interference coefficient [18]. The competitive interference intensity I of *M. angulatus* was calculated via the formula $I = (E_1 - E_P)/E_1$ [19], where E_1 is the predation rate when only one *M. angulatus* is present, and E_P is the predation rate when *M. angulatus* individuals coexist. Following Section 2.3, a chi-square goodness-of-fit test for the predation rate of a single *M. angulatus* was conducted, and the linear relationship of the interference effect equation was tested to determine the correlation between the density of *M. angulatus* and its average daily predation rate.

3. Results

3.1. Predation Rate Measurement of Micromus angulatus on Aphis craccivora

The first-, second-, and third-instar larvae and adults of *M. angulatus* exhibited predation on adult *Aphis craccivora*. Under the prey densities used in the experiment, the daily predation rates increased with increasing aphid density, but the rate of increase gradually slowed. First-instar larvae had the highest predation rate at a density of 70 aphids per dish, as it consumed 43 aphids; second- and third-instar larvae and adults had the highest predation rate at a density of 120 aphids per dish, as they consumed 85, 104, and 103 aphids, respectively (Table 1) (Figure 2). This finding indicates that the predatory capacity of older instar larvae and adults of *M. angulatus* is significantly greater than that of first-instar larvae, with third-instar larvae showing predatory capabilities similar to those of adults.

Table 1. Consumption of *Micromus angulatus* 1st- to 3rd-instar larvae and adults by *Aphis craccivora.*

1st-Instar		2nd-Instar		3rd-Instar		Adult	
Number of Hosts (N_0)	**Number of Preyed Hosts (Na) ± SD**	**Number of Hosts (N_0)**	**Number of Preyed Hosts (Na) ± SD**	**Number of Hosts (N_0)**	**Number of Preyed Hosts (Na) ± SD**	**Number of Hosts (N_0)**	**Number of Preyed Hosts (Na) ± SD**
		20	20.00 ± 0.00	20	20.00 ± 0.00	20	20.00 ± 0.00
10	9.17 ± 1.33	30	30.00 ± 0.00	30	29.83 ± 0.41	30	30.00 ± 0.00
20	13.33 ± 2.66	50	48.17 ± 1.72	50	50.00 ± 0.00	50	49.83 ± 0.41
30	23.50 ± 2.35	70	53.83 ± 8.95	70	61.50 ± 8.14	70	69.17 ± 0.75
50	36.17 ± 3.76	100	72.17 ± 9.68	100	94.67 ± 2.73	100	94.33 ± 2.58
70	42.67 ± 2.66	120	84.50 ± 6.95	120	103.83 ± 6.68	120	102.50 ± 7.28

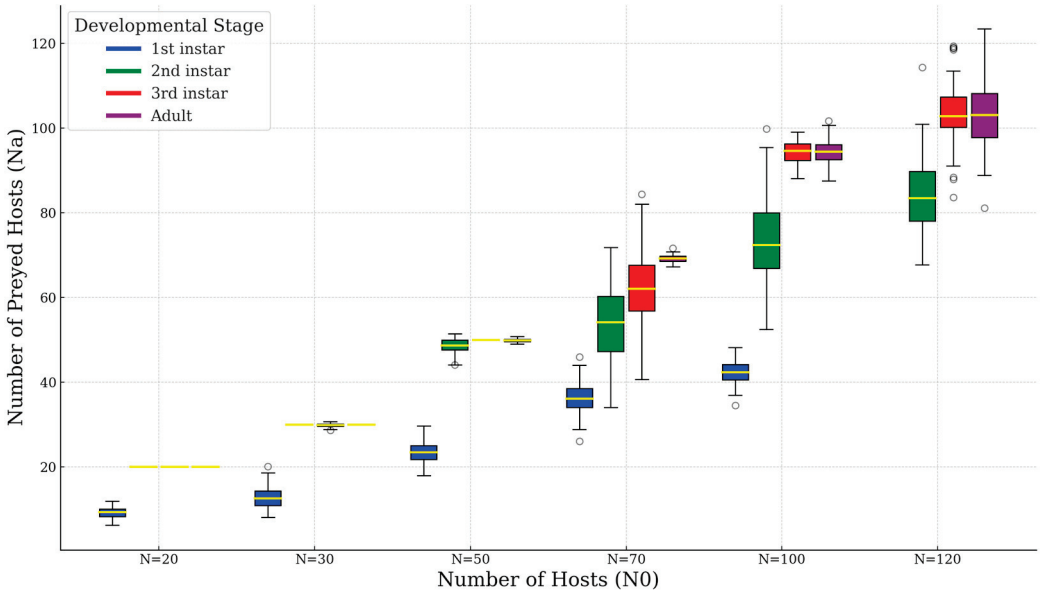

Figure 2. Predation efficiency distribution of *Micromus angulatus* on *Aphis craccivora.*

3.2. Functional Response of Micromus angulatus to Aphis craccivora

The functional response equations of the first-, second-, and third-instar larvae and adults of *M. angulatus* preying on adult *Aphis craccivora* were fitted via the Holling Type II disc equation (Table 2), and the R-squared values were 0.9319, 0.9941, 0.9938, and 0.9936, respectively. The chi-square goodness-of-fit tests between the actual and theoretical predation rates revealed chi-square values that were less than the critical value of $X^2_{0.05} = 7.81$, indicating that there was no significant difference between the observed and theoretical values. These findings confirm that the functional response equations accurately reflect the actual predation behaviour of *M. angulatus* on adult *Aphis craccivora*.

The instantaneous attack rates of first-, second-, and third-instar larvae and the adults of *M. angulatus* were 1.0017, 1.0448, 0.9581, and 0.9508, respectively. The handling times were 0.0158, 0.0051, 0.0016, and 0.0011 days, respectively. The maximum daily predation rates were 63.2911, 196.0784, 625, and 909.0909 aphids, respectively. The control efficiencies were calculated as 63.3989, 204.8672, 598.8311, and 864.3192, respectively (Table 2). The experiments indicate that the adults and third-instar larvae of *M. angulatus* exhibit significantly greater control efficiency against adult *A. craccivora* than first- and second-instar larvae do. This is due to their shorter handling times, which contribute to higher control efficiencies. Adults and third-instar larvae thus play substantial roles in controlling aphid populations. The second-instar larvae had slightly weaker predation abilities, whereas the first-instar larvae had the least control ability over adult *A. craccivora*.

Table 2. Functional responses of *Micromus angulatus* to adult *Aphis craccivora.*

Stage	Functional Response Equation	R^2	Instant Attack Rate (a)	Handling Time (T_h)/d	Predation Capacity (a/T_h)	Maximum Daily Consumption ($1/T_h$)	X^2
1st-instar	$N_a = 0.9983N_0/(1 + 0.0158N_0)$	0.9319	1.0017	0.0158	63.3989	63.2911	5.8093
2nd-instar	$N_a = 0.9571N_0/(1 + 0.0053N_0)$	0.9941	1.0448	0.0051	204.8672	196.0784	2.636
3rd-instar	$N_a = 1.0437N_0/(1 + 0.0015N_0)$	0.9938	0.9581	0.0016	598.8311	625	3.0853
Adult	$N_a = 1.0518N_0/(1 + 0.0010N_0)$	0.9936	0.9508	0.0011	864.3192	909.0909	2.4112

3.3. Searching Efficiency of Micromus angulatus on Aphis craccivora

The search efficiency of *Micromus angulatus* at different instars for adult *Aphis craccivora* decreased as the prey density increased, indicating that the difficulty and time required for searching decreased with increasing prey density. When the density of adult *A. craccivora* was 10 aphids per dish, the search efficiency of first-instar larvae reached its peak at 0.87, with a noticeable decline as the aphid density increased. At a density of 20 aphids per dish,

the search efficiency of second- and third-instar larvae peaked at 0.94 and 0.93, respectively. When the aphid density reached 30 aphids per dish, the search efficiency remained above 0.9, at 0.90 and 0.92, respectively, but subsequently declined significantly with increasing aphid density, with a more pronounced decline observed in second-instar larvae than in third-instar larvae. The highest search efficiency for adult *M. angulatus* was 0.9321 at an aphid density of 20 aphids per dish, and the lowest was 0.8489 at a density of 120 aphids per dish, indicating a decreasing trend (Figure 3).

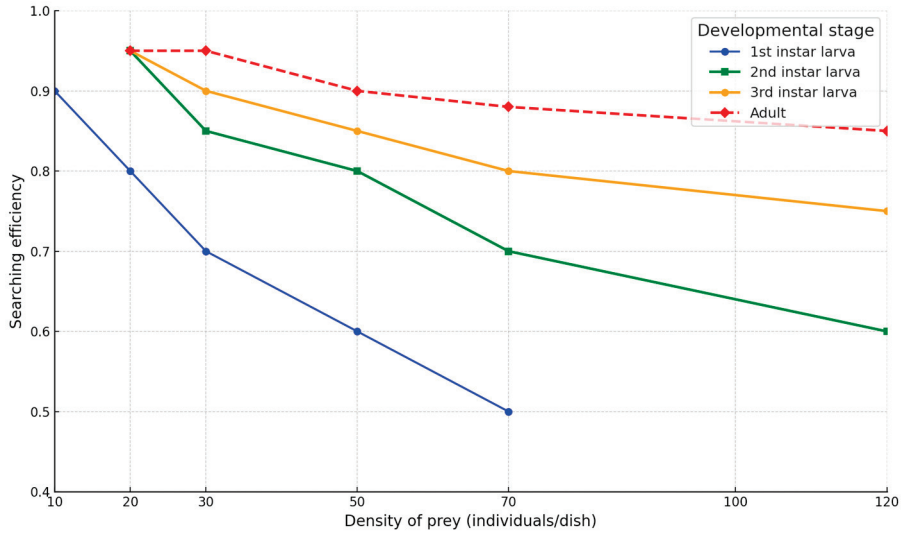

Figure 3. Searching efficiency of *Micromus angulatus* at different stages for adults of *Aphis craccivora.*

3.4. Effects of Micromus angulatus Density on Predatory Activity

Under constant prey density and predation space, the total predation by the third-instar larvae of *M. angulatus* increased with the increasing density of *M. angulatus* larvae, but the average daily predation per individual decreased from 123 to 31.48 aphids (Table 3). This finding indicates the presence of intraspecific interference and competition among individual third-instar larvae during predation. The interference effect model was fitted with a search constant Q = 0.6194 and an interference coefficient m = 0.87, resulting in the interference effect equation $E = 0.6194P^{-0.87}$. A chi-square goodness-of-fit test yielded X^2 = 1.11, which is less than $X^2_{0.05}$ = 7.81, suggesting that the difference between the actual and theoretical predation rates is not significant. The interference effect equation adequately reflects the interference effect on *M. angulatus* density, indicating a significant correlation between the density of *M. angulatus* and its average daily predation. The competitive interference intensity reached its maximum value of 0.744 when the density of third-instar larvae was five per dish (Table 3), indicating that the interference and competition among *M. angulatus* larvae were the greatest at this density.

Table 3. Interference effects of different densities of *Micromus angulatus* on the predation of adult *Aphis craccivora.*

Density of *M. angulatus* (Individuals/Disk)	Number of Preyed Hosts (*Na*)	Average Number of Preyed Hosts	Theoretical Number of Consumed Preys	X^2	Intensity of Scramble Competition
1	123.00 ± 6.708	123.00 ± 6.708	123.888	0.006	0
2	123.00 ± 6.708	61.50 ± 3.354	135.89	0.001	0.446
3	150.60 ± 14.673	50.20 ± 4.891	143.443	0.357	0.592
4	139.20 ± 5.630	34.80 ± 1.408	149.054	0.651	0.717
5	157.40 ± 10.784	31.48 ± 2.517	153.558	0.096	0.744

4. Discussion

The findings of this study demonstrate the significant potential of *Micromus angulatus* as a biological control agent against *Aphis craccivora*. The predatory behaviour, functional responses, search efficiency, and intraspecific interference exhibited by different developmental stages of *M. angulatus* are vital in understanding its potential efficacy in biological control programs. The results of this study are consistent with earlier research on lacewings and other predatory insects, providing valuable insights into the utility of *M. angulatus* in integrated pest management strategies [7–9,11,14].

The functional response of a predator is a critical component in understanding its efficacy in biological control, as it provides insights into how a predator's consumption rate changes with prey density [20,21]. Understanding the impact of predatory natural enemies on pest control is essential for maximising their control effectiveness. The Holling Type II functional response is a critical method for evaluating the pest control ability of predatory natural enemies and is widely used to assess predation efficiency and the ability of predators to control prey populations [20–22]. Our findings show that the functional responses of first- to third-instar larvae and adults of *M. angulatus* to adult *Aphis craccivora* fit the Holling II model. This finding is consistent with other predatory insects within the Neuroptera order, such as the predation of aphids and thrips by green lacewing [23–25]. Lady beetles' functional responses to cowpea aphids also follow the Holling II model [26]. Overall, the theoretical maximum daily predation rates of third-instar larvae and adults of *M. angulatus* on adult cowpea aphids are significantly higher than those of the first- and second-instars, with 625 and 909.0909 aphids, respectively. The search effect, a behavioural response exhibited by predators during predation, decreases as the prey density increases, allowing predators to complete predation more swiftly [27]. The search effect demonstrated by all instars of *M. angulatus* on adult cowpea aphids shows a continuous decline as the aphid density increases, a pattern also observed in other predators, such as lady beetles [28,29] and green lacewings [30]. The decline in the search effect is most pronounced in first-instar larvae, with the least decline observed in third-instar larvae and adults, suggesting their lesser susceptibility to changes in aphid density. When the density of adult cowpea aphids was 120 per dish, the search effect of third-instar larvae and adults was notably greater than that of other stages. Thus, when cowpea aphids are controlled with *M. angulatus*, it is advisable to utilise third-instar larvae and adults. Extensive laboratory studies have shown that, as the predator density increases, intraspecific interference occurs, leading to a decrease in predation effectiveness—a phenomenon known as intraspecific interference [31]. Such interference has been observed in various predators, including lady beetles preying on aphid nymphs and green lacewings on citrus psyllids [19]. This study confirms that intraspecific interference also occurs when *M. angulatus* preys on adult cowpea aphids. As the density of third-instar larvae increases within a constant prey density and predation space, the average daily predation per larva continuously decreases, reaching a maximum interference value of 0.74407 at a density of five per dish. Therefore, when *M. angulatus* is released in fields to control cowpea aphids, it is crucial to consider the pest population density and the intraspecific interference effects of *M. angulatus*, protect and utilise natural field populations, and release the appropriate numbers of natural enemies on the basis of field conditions for effective pest management.

By studying the predation rates, functional responses, search effects, and effects of the intraspecific interference of third-instar larvae of *M. angulatus*, this study confirmed that all instars of *M. angulatus*, especially third-instar larvae and adults, are effective in controlling cowpea aphids. While the release of third-instar larvae or adults in fields is recommended to control aphids, the natural field environment is more complex than the laboratory conditions. Predator behaviour is influenced by multiple biotic factors, such as climate conditions, predator sex, crop type, and abiotic factors. Therefore, future studies should focus on the overwintering of *M. angulatus*, artificial rearing techniques, field population dynamics, and the relationship between predator and pest densities to address

the lag effect of natural enemies and the coordination of insecticide use with biological control strategies involving *M. angulatus*.

5. Conclusions

In conclusion, this study demonstrates that under laboratory conditions, *Micromus angulatus* is a highly effective predator of *Aphis craccivora*, with significant potential for use in biological control programs. The predation efficiency, functional response, search efficiency, and intraspecific interference observed in this study provide a comprehensive understanding of the predatory behaviour of *M. angulatus*. The results support the use of third-instar larvae and adults as the most effective stages for field release. These findings will offer new perspectives for environmentally friendly pest management and contribute to the advancement of sustainable agriculture.

However, while laboratory results are promising, the influence of environmental factors, such as temperature, humidity, and the availability of alternate prey may affect the predation capacity of *M. angulatus* under field conditions. Moreover, variations in crop type, field structure, and seasonal dynamics could impact the predator's effectiveness. Therefore, future research should focus on assessing the field efficacy of *M. angulatus* under diverse environmental conditions to better understand its role in real-world integrated pest management (IPM) programs. Additionally, the mass rearing of *M. angulatus* poses another critical area for investigation. Developing efficient, cost-effective methods for producing large quantities of third-instar larvae and adults is essential for scaling up biological control operations. Moreover, exploring the predator's interactions with other natural enemies and its compatibility with various pest control methods, such as the selective use of insecticides, will ensure the successful integration of *M. angulatus* into broader IPM strategies.

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Data Availability Statement: The original contributions presented in the study are included in the article, and further inquiries can be directed to the corresponding authors.

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

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Dispersal Capacity of *Trichogramma* for the Management of *Duponchelia fovealis*

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Abstract: The European pepper moth (*Duponchelia fovealis*) is predominant in the main strawberry production areas, including Brazil, as an important invasive pest and causes substantial damage to the crop. This damage is mainly attributed to the lack of effective management strategies. A promising alternative for managing this pest is implementing biological control through releasing natural enemies. This study determined the dispersal capacity of *Trichogramma pretiosum* for the management of *D. fovealis* in a strawberry crop in a low tunnel system 24, 48, and 72 h after parasitoid release. The experiments were carried out on strawberry farms in the mountainous region of Espírito Santo. Tunnels measuring 1.20 m wide by 50 m long were selected, in which artificial infestations of 30, 60, 90, and 120 eggs of *D. fovealis* were made on both sides of the strawberry tunnel at distances of 3, 7, 11, and 15 m, respectively, from the central point where the parasitoids were released. After the initial 24 h, new eggs were placed to replace the infested ones; the replacement was repeated at 48 and 72 h. The results indicated that, in strawberry plantations, *T. pretiosum* was efficient in parasitism. There was no difference between parasitism 24 and 48 h after parasitoid release, but parasitism was substantially lower after 72 h. The dispersion capacity of *T. pretiosum* was 14.21 linear meters, corresponding to an area of 17.05 m^2. It is recommended that 93,000 *T. pretiosum* females be released per hectare every three days.

Keywords: egg parasitoid; Trichogrammatidae; biological control; strawberry

1. Introduction

Strawberry production is of great social and economic importance in Brazil. However, the crop is highly sensitive and frequently attacked by harmful agents, such as pests and diseases [1–3].

The European pepper moth, *Duponchelia fovealis* Zeller, 1847 (Lepidoptera: Crambidae), is a polyphagous insect that was introduced into Brazil in 2007, quickly established itself, and is now widespread in the country's main strawberry production fields. Adults of *D. fovealis* lay eggs on the abaxial surface of the plant's low-lying leaves close to the ground. After hatching, the caterpillars, from neonates to final instars, feed on the leaves, causing significant damage to strawberry crops, mainly due to the lack of efficient management methods [1,2,4,5].

For this reason, one way to control this pest is to integrate existing management methods. A viable management alternative is using biological control through the release

of natural enemies. Parasitoids of the genus *Trichogramma* Westwood (Hymenoptera: Trichogrammatidae) are natural enemies used with significant frequency in biological pest control and are distributed worldwide [6–8].

The origin of the species *T. pretiosum* is unknown, but it is naturally recurrent in the Americas and is the most commercialized species in Brazil for inundative release. The distribution of this species is recorded in 10 of the 12 countries of South America, and, in Brazil, it is found naturally in 15 of the 26 Brazilian states [9]. This parasitoid species is widely known as an alternative for controlling pests of agricultural importance, being reported to parasitize the eggs of *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) [10] and *Heraclides astyalus* (Godart, 1819) (Lepidoptera: Papilionidae) in *Citrus* sp. [11] and has high potential for use in *D. fovealis*.

Arguments in favor of using these parasitoids include their ease of rearing in alternative hosts at a lower cost than natural hosts, wide geographic distribution, specialization, and efficiency in parasitizing [11–13]. Several factors play a role in the efficacy of these parasitoids in controlling pests in the field [14]. These factors are mainly related to the foraging and dispersal ability of the species/strain used (which are most often influenced by the host), the number of insects released, the density of the target pest, the number and season of releases, the method of field distribution, and the phenology of the plant, which can influence the path of the parasitoid on the plant [8,12,15–18].

Dispersal ability is a particularly important factor because it directly affects the release techniques used, the potential effect of non-target pests, and the time required for *Trichogramma* to efficiently colonize the crop area [8,12,14,15].

The dispersion capacity is fundamental in inundative biological control techniques, as it determines the ideal number of parasitoid release points in the field to achieve homogeneous coverage [8].

Therefore, this study aimed to evaluate the parasitism behavior of *T. pretiosum* in the field within 72 h of release, determine the dispersal ability of *T. pretiosum* in strawberry production fields, establish the ideal release distance in low tunnel cultivation, and estimate the number of *T. pretiosum* to be released per hectare.

2. Materials and Methods

2.1. Rearing of D. fovealis

Rearing of *D. fovealis* took place in the Entomology Sector of the Center for Scientific and Technological Development in Pest and Disease Phytosanitary Management (NUDEMAFI) of the Center for Agricultural Sciences of the Federal University of Espírito Santo (CCA-UFES), Brazil.

Insects were maintained in the laboratory under conditions of 25 ± 1 °C, 70 ± 10% relative humidity, and 14 h of photophase. Newly hatched adults were transferred to cages (20 × 20 cm) made of PVC pipe, lined internally with bond paper, and sealed at the bottom with Styrofoam, which was also coated with bond paper. The end of the cage was closed with voile fabric to prevent insects from escaping. The adults were offered a 10% solution of honey soaked in cotton as food. The eggs were collected daily by changing the paper covering the cages, and they were immediately placed in Gerbox® acrylic boxes (11 × 11 × 3.5 cm).

The paper fragments containing the egg masses were transferred to flat-bottomed glass tubes (8.5 × 2.5 cm) containing an artificial diet [19]. After the third day of the pupal stage, these were removed from the tubes and transferred to rearing cages to obtain the adults.

2.2. Maintenance and Multiplication of Parasitoids

The maintenance and multiplication of parasitoids of the genus *Trichogramma* was also performed in NUDEMAFI, CCA-UFES. The species used was a commercial strain of *T. pretiosum*, Koppert®.

For the maintenance and multiplication of *Trichogramma*, eggs from stock breeding at NUDEMAFI were used, derived from the alternative host *Ephestia kuehniella* (Lepidoptera:

Pyralidae). The eggs of *E. kuehniella*, after being made non-viable under a germicidal lamp, were fixed with 20% gum arabic on blue cardboard (8.0 × 2.5 cm). These cartons were transferred to flat-bottomed glass tubes (8.5 × 2.5 cm) containing newly hatched adult parasitoids. The tubes were then sealed with PVC plastic film to prevent the escape of the parasitoids [20].

2.3. Evaluation of the Dispersal of T. Pretiosum in Strawberry Plants under Low Tunnel Cultivation for Parasitism of D. fovealis Eggs

To determine the displacement distance (dispersal ability) in linear meters and the number of release points within a low tunnel cultivated with strawberry plants, dispersal experiments were carried out on farms in the district of São João do Garrafão, municipality of Santa Maria de Jetibá, mountainous region of Espírito Santo (coordinates 1: 20°09′30″ S; 40°59′05″ W; coordinates 2: 20°08′09″ S; 40°56′53″ W). Plants of the Albion cultivars were used, planted with a spacing of 0.3 m between rows and 0.3 m between plants in beds with three planting rows during the productive period (approximately four months after planting) in a low tunnel system, with one location per tunnel. The experiment was installed under field conditions, that is, the climatic and management conditions were those typically found in the agricultural operation in question, with no additional control or monitoring of environmental conditions.

Four strawberry tunnels measuring ~1.20 m wide and 50 m long were selected. The low tunnels were built following the recommendations of Santos and Medeiros [21]. The structure was made with galvanized wire No. 6 arches at a minimum height of 80 cm, and the spacing between the arches was between 1.20 and 1.50 m. The structure was covered entirely with nonwoven fabrics (TNT). From the center of the tunnel (~25 m), plants were artificially infested at 3, 7, 11, and 15 m intervals on both the left and right sides with three, six, nine, and twelve cartons, respectively, each containing ten eggs of *D. fovealis* between 24 and 48 h old (Figure 1A,B).

The number of eggs was increased based on distance to preserve the turgidity of the more distant eggs and avoid their unavailability due to parasitism. After artificial infestation, approximately 2400 female individuals of *T. pretiosum* were released early in the day, immediately after hatching and in the middle of the bed, in each tunnel according to its treatment. The average radius of action and the dispersion area of the parasitoid were based on the methodology of Dobzhansky and Wright [22] with adaptations for the cultivation conditions found in Espírito Santo. Parasitoids were released in the tunnel's center at the recommended ratio of 1:4 (egg/parasitoid) using cartons containing the parasitoid species.

One day after the release (24 h), the cartons containing the eggs of *D. fovealis* were collected and replaced by others. This procedure was repeated for two more consecutive days (at 48 h and 72 h). All collected samples were taken to the laboratory, where the cartons were placed in plastic bags (5 × 23 cm), separated adequately according to the distance they were arranged with respect to the point of release and treatment, and placed in climate-controlled chambers at 25 ± 1 °C, relative humidity of 70 ± 10%, and photophase of 14 h, where they remained until parasitism was assessed by observing the darkening of the eggs, and confirmed after the emergence of the individuals. (Figure 1C).

A randomized block design with 4 tunnels was used. Each tunnel was considered a block, and the blocks were selected at a distance of >15 m between them. Regression analyses were performed between parasitism indices as a function of parasitoid density and species released. The mathematical relationship to obtain the average displacement, the number of release points, and the parasitoid dispersal area in strawberry cultivation in a low tunnel system was calculated based on the quadratic fit of the data; the optimal release point was determined from the derivatives, thus optimizing the intersection points between the parasitism curves.

The ExpDes. package of the R computer application [23] was used to perform the analysis.

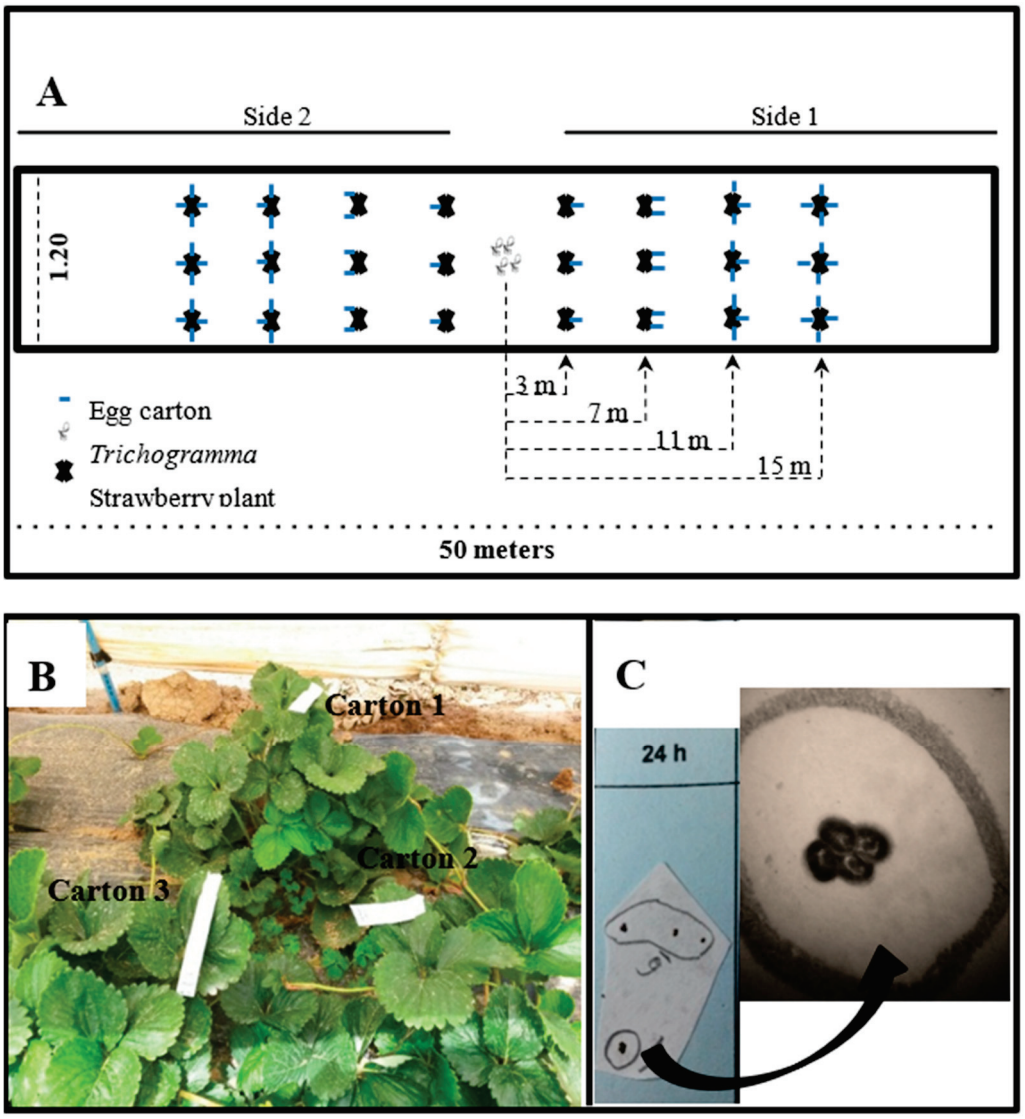

Figure 1. (**A**) The distribution of cartons and parasitoid release in a strawberry production field in a low tunnel system; (**B**) cartons with *Duponchelia fovealis* eggs distributed in the field to determine parasitoid dispersal ability; (**C**) parasitized eggs from the dispersal ability experiment after seven days.

3. Results

3.1. Release Times and Parasitism

Regarding the release time and parasitism of *T. pretiosum* in *D. fovealis* eggs, an F-test revealed a significant difference at the 5% probability level between parasitism at 24, 48, and 72 h after parasitoid release (Table 1). The efficiency of parasitism of *T. pretiosum* did not differ after 24 and 48 h. However, it was substantially lower at 72 h after release under strawberry cultivation conditions in the mountainous region of Espírito Santo (Table 2).

Table 1. Parasitism of *Trichogramma pretiosum* within 15 m from the release point, at a density of one *Duponchelia fovealis* egg for four parasitoids (1:4), at 24, 48, and 72 h after release in strawberry production fields in a low tunnel system.

FV [1]	Df [2]	SQ [3]	QM [4]	Fc [5]	P > F [6]
Block	3	1401.90	467.30	1.025	0.446
Treatment	2	13,789.40	6894.70	15.119	0.004 *
Residue	6	2736.20	456.00		
Total	11	17,927.50			
CV [7] (%)	20.33%				

[1] FV: Source of variation; [2] Df: degree of freedom; [3] SQ: sum of squares; [4] QM: mean squares; [5] Fc: observed F value; [6] P > F: F value in relation to the observed *p*-value; [7] CV: coefficient of variation. * Significant values at the 5% significance level.

Table 2. Mean accumulated parasitism of *Duponchelia fovealis* eggs by *Trichogramma pretiosum* at 1:4 density in the four replications and four distances from the release point after the initial release time in strawberry production fields in a low tunnel system.

Time after Release	Accumulated Parasitism [1]
24	129.1 a*
48	128.9 a
72	57.1 b

* Means followed by the same letter do not differ from each other at the 5% probability level using the Tukey test. [1] Mean accumulated parasitism in the proportion 1:4, in the four repetitions and four distances from the release point.

3.2. Estimation of Dispersal Ability and Number of Release Points of T. pretiosum in Strawberry Plants Cultivated in a Low Tunnel for Parasitism of D. fovealis Eggs

The results indicate that parasitism of *D. fovealis* eggs by *T. pretiosum* decreases as the sampled points move away from the parasitoid release point, with statistical differences at all distances analyzed (Table 3).

Table 3. Mean values (±standard error) of parasitism of *Duponchelia fovealis* eggs by *Trichogramma pretiosum* Koppert® commercial strain after 72 h, at different distances from the central point of release in the strawberry crop in a low tunnel system in the district of São João do Garrafão, municipality of Santa Maria de Jetibá-ES.

Distance (m)	Parasitism (%)
3	32.64 ± 2.84 a[1]
7	14.03 ± 0.08 b
11	5.74 ± 0.23 c
15	0.11 ± 0.06 d
CV [2]	20.94

[1] Means followed by the same letter do not differ from each other at the 5% probability level using the Tukey test. [2] CV: coefficient of variation.

Based on the proposed model, the mean distance of action and dispersal area of *T. pretiosum* in the strawberry crop in a low tunnel system to control *D. fovealis* eggs was 14.21 m (linear) and 17.05 m^2, respectively (Table 4). Therefore, the number of release points of *T. pretiosum* to control *D. fovealis* eggs was determined by dividing the length of tunnels by the average action of the parasitoid (14.21 linear m) (Figures 2 and 3).

Table 4. Mean distance (linear meters) and dispersal area (m^2) with the respective model and determination coefficient (R^2) for *Trichogramma pretiosum* Koppert® commercial strain in eggs of *Duponchelia fovealis* in a strawberry crop in a low tunnel system in the district of São João do Garrafão, municipality of Santa Maria de Jetibá-ES.

Parameters	Strawberry Crop
Average distance (linear m)	14.21
Dispersal area (m^2)	17.05
Mathematical model	$y = 63.267 - 8.307x + 0.275x^2$
R^2	0.9985
CV [1]	20.94

[1] CV: coefficient of variation.

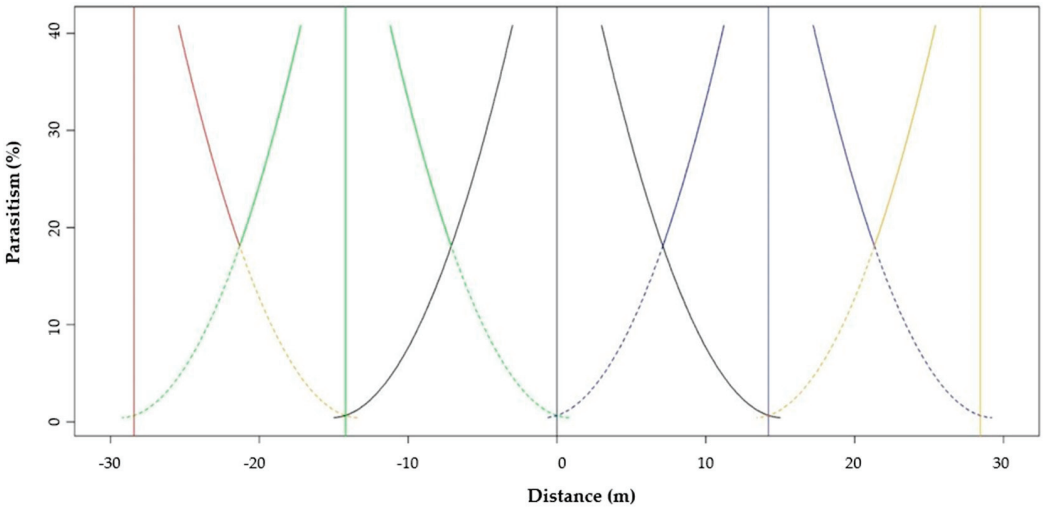

Figure 2. Estimation of the parasitism behavior of *Duponchelia fovealis* eggs by *Trichogramma pretiosum* using the model proposed for strawberry cultivation in a 50 × 1.20 m low tunnel system. Continuous vertical lines represent the release point of the parasitoids. Curves represented by solid lines correspond to the area of parasitism covered by the releases.

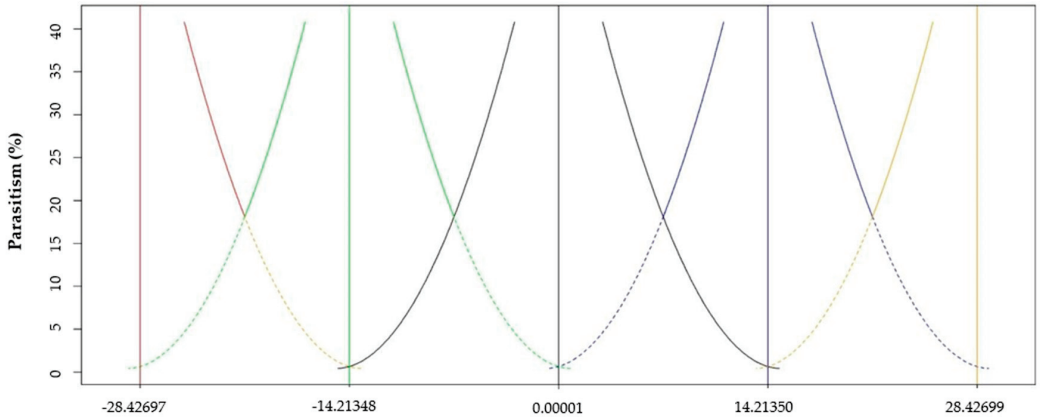

Figure 3. Parasitism of *Duponchelia fovealis* eggs by *Trichogramma pretiosum* considering the parasitoid release points every 14.21 linear meters in strawberry cultivation in a low tunnel system. Continuous vertical lines represent the release point of the parasitoids. Curves represented by solid lines correspond to the area of parasitism covered by releases.

4. Discussion

The high activity in the first 48 h after release could be due to the energetic support provided by the *T. pretiosum* eggs. After this period, with the decrease in energy supply, foraging and parasitism ability were substantially affected. A decline in parasitism over the lifetime of the parasitoid after hatching is common, whether well-fed parasitoids are involved or not [8,24].

The main factor in this decline in parasitism over time may be related to the food supply affecting *Trichogramma* activity, as the quality of the available carbohydrates is essential for increasing the parasitoid's reproductive ability. Longevity and potential for parasitism are maximized when adult *Trichogramma* are fed regularly [25–27]. Therefore, the supply in the field may have been insufficient.

Therefore, based on the data obtained, it can be concluded that the *T. pretiosum* strain used in this study did not show any difference in parasitism on *D. fovealis* eggs in the first 48 h (two days) after release, indicating that the release frequency should not be reduced.

Dispersal ability can be influenced by several factors. The lower parasitism of eggs at greater distances from the initial release point may have been influenced by the biological characteristics of the *Trichogramma* species/strain released and the crop itself. In this case, the strawberry plant was a physical barrier to parasitoid dispersal. Other authors have also studied the inverse correlation between distance and the parasitism capacity of *Trichogramma* [28–30].

In our study, the lowest rates of parasitism were associated with the biological characteristics of the parasitoid and the architectural characteristics of the plant and were observed at greater distances from the release point. These results are usually observed as there is a dependent relationship between release distance and parasitism rate, with a reduction in parasitism rate by *Trichogramma* usually observed at greater distances from the release point [30,31].

Lower parasitism rates due to greater distances from the release point have also been observed for *T. pretiosum* in soybean [15], maize [32], apple [33], tomatoes in a greenhouse [31], and tomatoes in the field [28,29]. The same pattern was observed in rice for *T. chilonis* and *T. japonicum* [34].

The turgidity of the egg of *D. fovealis* may also have affected parasitism at greater distances. Eggs located at a greater distance from the parasitoid release point may lose their turgidity because they are more exposed to adverse environmental conditions, such as high temperature and low humidity, and when found by *Trichogramma* eggs, they are in a condition unsuitable for parasitism [15]. Adverse factors may have been maximized because the tunnels remained closed throughout the period in which the experiments were conducted.

The mean radius of action and dispersal area of the parasitoid are other important parameters [22]. Based on the proposed estimates of the dispersal ability of *T. pretiosum* for control of *D. fovealis*, it is recommended that 93,000 females of the parasitoid be released at three-day intervals for each hectare of strawberry plants.

Control efficiency is directly related to the uniform distribution of the parasitoid [8,26]. Therefore, the results aimed to satisfy this condition. The calculated mean distance and the dispersal area provided greater homogeneity in the parasitoid's coverage of the area and, consequently, greater efficiency in controlling *D. fovealis* by *T. pretiosum*.

The recommendation for parasitoid release may change depending on the crop type, planting density, and intensity of pest infestation in the field [8,26,30]. For fruit trees, they can vary from 70,000 to 3.8 million parasitoids per hectare [35,36]. For staked tomatoes, the recommendation is 576,000 *Trichogramma* released every eight days [37], while in Europe, the recommendation is 150,000 to 300,000 *Trichogramma* released every seven days in maize [38].

5. Conclusions

In conclusion, in strawberry plantations, *T. pretiosum* was efficient in the parasitism of *D. fovealis* eggs. The parasitism rate was similar between 24 and 48 h after parasitoid release, but could be substantially lower after 72 h. The dispersion capacity of *T. pretiosum* was 14.21 linear meters, corresponding to an area of 17.05 m^2. It is recommended that 93,000 *T. pretiosum* females be released per hectare every three days in regions with similar climate characteristics.

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Endophytic Capacity of Entomopathogenic Fungi in a Pasture Grass and Their Potential to Control the Spittlebug *Mahanarva spectabilis* (Hemiptera: Cercopidae)

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Abstract: Pests in pastures have compromised the production of biomass for feeding livestock herds. Many strategies have been applied to sustainably solve this problem. One viable and innovative technique is the delivery of entomopathogenic fungi through endophytes. Therefore, this study aimed to (i) evaluate the endophytic capacity of two entomopathogenic fungi, *Fusarium multiceps* UFMGCB 11443 and *Metarhizium anisopliae* UFMGCB 11444, in *Urochloa brizantha* [(Hochst. ex A. Rich.) Stapf] (Poaceae) cultivar 'Marundu') via foliar inoculation or seed treatment, and (ii) measure their efficiency in controlling *Mahanarva spectabilis* Distant, 1909 (Hemiptera: Cercopidae) in *U. brizantha*. In the greenhouse, the fungi colonized the tissues of *U. brizantha* plants when inoculated via foliar spraying or seed treatment. The fungi *F. multiceps* and *M. anisopliae* caused 88% and 97.1% epizootic effects via seed inoculation, respectively, and 100% epizootic effects via foliar inoculation. In the field, the lowest fungal dose of 0.5 kg/ha had the same effect as a fourfold greater dose, with a >86% decrease in insect pest infestation observed. In summary, the fungi *F. multiceps* and *M. anisopliae* have endophytic effects and can effectively control *M. spectabilis* in *U. brizantha* pastures.

Keywords: biological control; endophytes; entomopathogenic fungi

1. Introduction

In 2021, Brazil had the largest commercial cattle herd globally [1]. In export rankings, Brazil ranks third in bovine milk production [2] and first in beef production [3–6]. Most Brazilian cattle are raised in an extensive livestock system comprising native and cultivated pastures that represent approximately 45% of the agricultural area in the country [7], and these pastures must be of good quality to serve as animal feed [8–10]. Cattle ingest a large amount of pasture biomass supplemented with salt and minerals [11,12]. However, Brazil faces several problems due to these extensive systems. Extensive *Urochloa* spp. (Hochst. ex A. Rich.) R. D. Webster (synonymous with *Brachiaria* spp. [Hochst. ex A. Rich Stapf]) [13]

monocultivation and high temperatures in the country have led to outbreaks of spittlebugs (Hemiptera: Cercopidae), the main pests in pastures in tropical America [14–18]. Nymphs and adults of this insect pest damage host plants by sucking the sap and injecting toxins that induce phototaxis and reduce photosynthetic rates [19–23], leading to a loss of biomass availability for cattle and reducing the support capacity by 60% on average [15,16,19,24–26].

Outbreaks of spittlebugs are controlled by spraying agrotoxics. This approach is economically unfeasible in large areas [21,27] and carries the risks of residues accumulating in the final product, contamination of the production chain and environment, and harm to consumer health. These pesticides leave environmental residues, leading to contamination [28]. Therefore, other strategies to address spittlebug attacks on forages in a sustainable manner are being developed. These include the induction of plant resistance to spittlebug attacks [15,21,29], the use of plant compounds as biocontrol agents [30,31], pasture diversification [15], soil fertilization [21,32] and the use of entomopathogenic fungi [17,22,33].

However, all these sustainable strategies introduce challenges in production. With regard to the use of fungi, the challenge is the instability caused by abiotic factors. Fungi are most effective at mild temperatures [34]. Therefore, it is important to consider fungal species that live in endophytes, which can alleviate this instability. Endophytic entomopathogenic fungi are plant mutualists and insect pathogens [35,36] that live within the tissues of healthy plants without causing disease [37–40]. They can colonize plants, act as endophytes for part of their life cycle [41], and resist abiotic and biotic stresses [42–45] that reduce the viability of fungal conidia [46]. Colonization by endophytic fungi can be systemic, localized in specific parts [47–50] or subdivided among plants [51,52]. Entomopathogenic fungi can potentially regulate the populations of various pest insects directly [48]. Microorganisms can be introduced into plants via seed treatment [53,54] or foliar inoculation.

The entomopathogenic fungi UFMGCB 11443 and UFMGCB 11444 were previously isolated from the spittlebug *Mahanarva spectabilis* Distant (Hemiptera: Cercopidae) in a silvopastoral system in the State of Maranhão, Brazil. Both strains caused greater insect pest mortality than did the commercial strain *Metarhizium anisopliae* (Metschn.) Sorokin [55]. Therefore, it was hypothesized that these fungi, UFMGCB 11443 and UFMGCB 11444, colonize *Urochloa brizantha* plants, making the forage an efficient vector for the biological control of *M. spectabilis* via different inoculation methods. Therefore, this study aimed to (i) evaluate the endophytic capacity of the fungi UFMGCB 11443 and UFMGCB 11444 in *U. brizantha* via foliar or seed inoculation, (ii) measure their efficiency in the sustainable control of *M. spectabilis* on *U. brizantha* in a greenhouse via different inoculation methods, and (iii) verify their efficiency in controlling *M. spectabilis* outbreaks in the field by foliar spraying.

2. Materials and Methods

2.1. Fungal Origin

The endophytic experiments were conducted at the Federal University of São João del Rei (UFSJ), CTAN Campus, in São João del Rei (MG), Brazil (21°06′13.0″ S 44°14′52.5″ W, 908 m), in a greenhouse (25 m × 7 m) without climate control, covered with transparent waterproof plastic and closed with an anti-aphid net. The average temperature inside the greenhouse was 28 °C ± 4 °C, and the relative humidity was 65% ± 30%. *Urochloa brizantha* plants were grown in 1 L plastic pots filled with oxisol fertilized with NPK (formula 4-14-8 (Agroadubo, Brazil)). Six seeds were sown per pot, and the tests were conducted 90 days after seed planting. Each experimental unit (EU) comprised a pot with plants and nymphs. *Mahanarva spectabilis* nymphs of the second generation from the rearing maintained by the Laboratory of Entomology of Embrapa Gado de Leite, which had plants of *U. brizantha* as hosts, were used with or without fungal treatment according to the description below.

Two strains of entomopathogenic fungi, UFMGCB 11443 and UFMGCB 11444, isolated in a silvopastoral system were used and were identified using molecular biological methods. Briefly, total DNA extraction was performed. The internal transcribed spacer region

(ITS1-5.8S-ITS2) of the ribosomal DNA gene was amplified using the primers ITS1 and ITS4 along with RNA polymerase II using the primers RBP2 5F and RPB2 7R. The amplicons of these marker regions (ITS and Pol II) were evaluated using 1% agarose gel electrophoresis, purified, and sequenced by the Sanger method using an ABI automated system (Applied Biosystems Life Technologies, Austin, TX, USA). The generated nucleotide sequences were submitted for BLASTn (Basic Local Alignment Search Tool) analysis through alignment and comparison of their similarities with the sequences of fungal type species deposited in GenBank and are available on the NCBI portal (http://www.ncbi.nlm.nih.gov/blast/ accessed on 15 January 2024). These fungi were deposited in the Microorganisms Collection of the Federal University of Minas Gerais, Brazil (CM-UFMG; World Data Center for Microorganisms [WDCM] 1029) with the following accession numbers: *Fusarium multiceps* UFMGCB 11443 (GenBank accession number ON831395) and *Metarhizium anisopliae* UFMGCB 11444 (GenBank accession number ON831396).

The ability of the fungi *F. multiceps* UFMGCB 11443 and *M. anisopliae* UFMGCB 11444 to persist within the tissues of *U. brizantha* was tested. Two techniques were used to inoculate the fungi in the endophytic test: seed treatment and spraying on plants before pest infestation. The batches of conidia produced were tested for viability according to the methodology proposed by Lopes et al. [56] before starting the experiments.

2.2. Production of Fungi for Bioassays

To conduct the bioassays, the *F. multiceps* UFMGCB 11443 and *M. anisopliae* UFMGCB 11444 isolates were reactivated and prepared to produce conidia for the experiments. They were subcultured in 9.0 cm $\times$ 1.5 cm Petri dishes containing potato dextrose agar (Kasvi®, Lehigh County, PA, USA) culture medium in a vertical laminar flow chamber previously sterilized with 70% alcohol. These dishes were incubated in germination chambers [to meet the biochemical oxygen demand (BOD) EletroLab®, Campinas, Brazil] at 25 $\pm$ 2 °C and 70 $\pm$ 10% RH with a 12 h photoperiod for vegetative growth and conidiogenesis. After incubation for 7–10 days, the conidia produced were removed from the surface of the culture medium with a sterile metal spatula and inoculated separately to prepare suspensions containing sterile water and an ionic surfactant (Tween 80 [0.001%], Sigma-Aldrich®, Steinheim, Germany) at a concentration of 1×10^8 conidia/mL.

2.3. Fungal Inoculation via Seed Treatment

Urochloa brizantha seeds were washed with 2.5% hypochlorite for 2 min and rinsed with distilled water for surface disinfestation, according to a methodology adapted from Carvalho et al. [57] and Ferreira et al. [58]. Then, they were inoculated in 100 mL of a suspension containing 1×10^8 conidia/mL and 0.05% Tween 80 as a surfactant for each fungus, plus the control suspension (sterile distilled water plus 0.05% Tween 80) without the presence of fungi, for a total of 3 treatments. The seeds were placed in contact with the suspension for 30 min as adapted from the methodology of Keyser et al. [59] and then sown. After seed germination, the plants were allowed to develop for approximately 45 days to evaluate the presence of fungi in the plant tissues.

2.4. Fungal Inoculation via Foliar Spraying

After germination and 30 days of growth from untreated seeds, plants were subjected to foliar spraying with either suspensions containing 1×10^8 conidia/mL of either fungus or a suspension with water and Tween without fungi using a manual sprayer with small nozzles and a calibrated spray volume of 50 L/ha. According to the methodology adapted from Ahmad et al. [60], the bases of the plants were protected to avoid contact with the spray and to prevent cross-contamination. After foliar inoculation with fungi, the plants were allowed to develop for approximately 45 days to evaluate the presence of the inoculated fungi in the plant tissues.

2.5. Analysis of Fungal Persistence in Urochloa brizantha Tissue

This bioassay used a randomized block design (DBC) with five treatments: plants from seeds treated with the fungus *F. multiceps* UFMGCB 11443; plants from seeds treated with the fungus *M. anisopliae* UFMGCB 11444; plants sprayed 30 days after planting with the fungus *F. multiceps* UFMGCB 11443; plants sprayed 30 days after planting with the fungus *M. anisopliae* UFMGCB 11444; and control plants without fungal inoculation.

Three leaves were taken randomly from each EU (one new, one young, and one senesced), totaling 150 leaf samples (3 leaves × 5 treatments × 10 replications). These leaves were placed in sterile plastic in a thermal box and taken to the DEPEB at UFSJ for testing. The surface of each leaf was disinfected by immersion in 70% ethanol (1 min) and 2% sodium hypochlorite (1 min), followed by washing with sterile distilled water (2 min) [57,58]. Disinfection was carried out to remove possible epiphyte microorganisms on the surfaces of leaves. After disinfection, a fragment of each leaf was placed in a Petri dish containing Sabouraud agar (Kasvi®) and incubated at 26 °C for approximately 15 days in a climate chamber (to meet the BOD). The macromorphologies and micromorphologies of the fungi present in these fragments were compared to those of inoculated *F. multiceps* UFMGCB 11443 and *M. anisopliae* UFMGCB 11444 and with those of the fungi isolated from the control plants (<5%). The fungal species were grouped using identification keys [34], and 45 samples were selected for subsequent molecular analysis. The experimental design used was completely randomized. For each treatment, leaves were collected randomly from ten EUs to assess the presence of the applied fungi. The presence of endophytes was confirmed when the growth of the applied fungi from leaf samples of plants for each treatment was observed according to the methodology adapted from Ahmad et al. [60].

2.6. Analysis of Fungal Persistence in the Sap of Urochloa brizantha

The EUs were taken to the DEPEB for plant sap collection. Ten repetitions of each treatment were performed (ten repetitions × five treatments = 50 samples total). Before cutting, the plants were disinfected with 2% hypochlorite and rinsed with distilled water [60]. Then, the plants were cut at the sheath, close to the first ligule and below the first node, with sterile scissors. The sap was collected with a sterile 1000 µL pipette 5 min after cutting. The collected sap was allocated and spread with Drigalski loops on a Petri dish containing Sabouraud dextrose agar. The plates were incubated at 26 °C for approximately seven days in a climate chamber (to meet the BOD), and the samples were purified by successive plating. The macromorphologies and micromorphologies of the fungi present in the fragments were compared with those of *F. multiceps* UFMGCB 11443 and *M. anisopliae* UFMGCB 11444 and of fungi isolated from control plants (<5%), and three samples from each treatment were randomly selected for subsequent molecular analysis. The experimental design used was completely randomized. Each treatment was represented by saps collected from ten random EUs to assess the presence of the applied fungi. The presence of endophytes was confirmed when we observed the growth of the applied fungi from leaf samples of plants for each treatment according to the methodology adapted from Ahmad et al. [60].

2.7. Greenhouse Bioassay with Mahanarva spectabilis

This bioassay used a randomized block design (DBC) with five treatments: plants from seeds treated with the fungus *F. multiceps* UFMGCB 11443; plants from seeds treated with the fungus *M. anisopliae* UFMGCB 11444; plants sprayed 30 days after planting with the fungus *F. multiceps* UFMGCB 11443; plants sprayed 30 days after planting with the fungus *M. anisopliae* UFMGCB 11444; and control plants without fungal inoculation. There were 20 plants or repetitions for each treatment, totaling 100 EUs (five treatments × 20 replicates). Each plant was grown in a 1 L pot previously fertilized with NPK. These plants received five third- or fourth-instar nymphs of *M. spectabilis* spittlebugs. The pots were individually covered in voile bags to avoid cross-contamination between the treatments and insect escape. Starting 24 h after release, insect mortality was evaluated daily for 15 days. The

dead nymphs were removed daily, placed in 1.5 mL Eppendorf tubes, and frozen in a −20 °C freezer to determine their cause of death [61].

2.8. Field Bioassay with Mahanarva spectabilis

The field trial was conducted in cultivated pastures with a predominance of *U. brizantha* grass (Hochst. ex A. Rich.) R.D. Webster (synonymous with *Brachiaria brizantha* [Hochst. ex A. Rich.] Stapf) in the municipality of Açailândia in the southwest of the State of Maranhão (Brazil), with the map coordinates 04°57′48.2″ S and 047°08′58.0″ W Gr and an altitude of 274 m.

This region is an ecotone between the Cerrado and the Amazon Forest. At the start of the experiment, the pasture had an average infestation density of 42.5 nymphs/m^2, which is above the minimum level of 20–25 nymphs/m^2 recommended for controlling spittlebugs [62,63]. The temperature was approximately 34.9 ± 3 °C, and the relative humidity was 89%, with winds of up to 6 km/h and solar radiation of 4 MJ/m^2. The average rainfall in February and March 2019 was 175 mm [64]. *Brachiaria* pastures were used under a rotation system for producing Nelore beef cattle, with a history of intense infestation by pasture spittlebugs.

After the start of the experiment, the height of the plants was maintained between 25 and 35 cm through continuous grazing. The experiment used a DBC with eight treatments (Table 1) and six replications each. The EUs of each trial treatment covered an area of 80 m^2 (8 × 10 m), separated from each other by 50 m wide corridors. The methodology used to count insects and foam masses [65] determined the infestation and fluctuation levels of adults and nymphs in each EU before and after fungal application. The population survey for nymphs was conducted by counting the number of nymphs per foam at the base of the plant (ground level) using a square measuring 0.25 × 0.25 m (0.0625 m^2). This square was randomly dropped within each area to define a sampling point [66], with 10 drops (sampling points) per area. The fluctuation in adults was assessed by sampling the number of adult spittlebugs using a scanning net comprising five transects in the central portion of each EU to minimize edge effects. The spittlebugs captured by the net were transferred into 2 mL Eppendorf tubes for subsequent species identification.

Table 1. *Fusarium multiceps* UFMGCB 11443 and *Metarhizium anisopliae* UFMGCB 11444 doses in kilograms per hectare in the field trials.

Treatments	Dose (kg/ha)
Fusarium multiceps	0.5
Metarhizium anisopliae	0.5
Fusarium multiceps	1.0
Metarhizium anisopliae	1.0
Fusarium multiceps	2.0
Metarhizium anisopliae	2.0
Fusarium multiceps + *Metarhizium anisopliae*	1.0 + 1.0
Control	0.0

The conidium batches were produced in parboiled rice with 1×10^{12} conidia/g, and suspensions were prepared at different concentrations for the fungi *F. multiceps* UFMGCB 11443 and *M. anisopliae* UFMGCB 11444 belonging to CM-UFMG (WDCM 1029). In all suspensions, 0.05% refined soybean oil and 0.01% neutral detergent were added as adhesives, and the fungi were added according to the treatment (Table 1).

The control area was sprayed with a pure suspension without fungi. For application, the sporulated fungi in rice were washed in water and strained through rice sieves. Then, the solution was poured into a coastal sprayer tank with low-flow fan nozzles and a volume of 50 L/ha for spraying. The application was conducted at a height of approximately 50 cm from the ground after counting the insects to allow contact between the biological pesticides with the spittlebug nymphs, the stage most susceptible to the entomopathogenic agent [67].

Zimmermann [68] emphasized that the contact-based action of the microbial insecticide is more effective at lower temperatures. Following these guidelines, the application time was between 7:00 am and 5:00 pm. Nymphs and adults were counted before and after 6, 15, 20, and 30 days of application.

2.9. Analysis of the Cause of Mahanarva spectabilis Death

From each EU, in the greenhouse, five nymph or adult spittlebug (*M. spectabilis*) samples placed in sterile Eppendorf tubes were randomly chosen (500 samples total) for the cause-of-death analysis and taken to the Microbiology Laboratory of the Department of Bioengineering (DEPEB) at UFSJ. After 24 h, these samples were incubated in culture medium to assess the presence or absence of the fungi *F. multiceps* UFMGCB 11443 and *M. anisopliae* UFMGCB 11444, which were inoculated in *U. brizantha* plants, to confirm the cause of mortality. The insects were superficially disinfected by immersion in 70% ethanol (1 min) and 2% sodium hypochlorite (1 min) and then washed with sterile distilled water (2 min) according to an adapted methodology [57,58,69] to remove external impurities. The samples were placed individually in Petri dishes containing Sabouraud agar and kept for seven days in a BOD chamber at 25 °C to allow the emergence of the fungi inside the dead insects [70]. After macroculture, microculture was conducted on a fraction of the macroculture, followed by microscopy according to a methodology adapted from Kuzhuppillymyal-Prabhakarankutty et al. [71].

The colonies of all the fungal isolates were photographed (front and back) and grouped according to their macromorphological characteristics, such as colony color (front and back), surface texture (front and back), edge appearance, and growth time. These isolates were compared with the macromorphology of the applied fungi *F. multiceps* UFMGCB 11443 and *M. anisopliae* UFMGCB 11444 [69,72]. Micromorphology was assessed by comparing the fungal reproductive structures after the samples were microcultured under an optical microscope. The fungal species were identified with the help of identification keys [34]. Afterward, molecular analysis was carried out to confirm the results, as described in the next section. The experimental design used was completely randomized. Each treatment consisted of spittlebugs randomly collected from each of the 20 EUs to assess the presence of the applied fungi.

2.10. Molecular Analysis of Fungal Samples Isolated from Mahanarva spectabilis Corpses

After macro- and micromorphological analyses, the fungal isolates from all treatments (including the control) were grouped, and from each group, a random sample was selected with characteristics similar to those of the fungi applied for molecular analysis, totaling 18 samples for molecular analysis. Filamentous fungi were inoculated on Sabouraud dextrose agar for seven days. The DNA of the isolates was extracted according to the method described by Doyle and Doyle [73] from microorganisms grown in culture medium. The extracted genomic DNA sample was subjected to polymerase chain reaction (PCR) to amplify the internal transcribed spacer (ITS) region of the rDNA using the SR6R (5′-AAGWAAAAGTCGTAACAAGG-3′) and LR1 (5′-GGTTGGTTTCTTTTCCT-3′) primer oligonucleotides [74]. The PCR mixture consisted of 1 µL of DNA, 1 µL of each primer at 10 µM, 10 µL of 5X PCR buffer, 1 µL of dNTPs at 10 mM, 0.2 µL of GoTaq DNA polymerase (5 U/µL; Promega, Madison, WI, USA), and 35.8 µL of autoclaved Milli-Q H2O, for a final volume of 50 µL. The amplification program was as follows: initial denaturation at 94 °C for 2 min, followed by 40 cycles of denaturation at 94 °C for 10 s, annealing at 54 °C for 30 s, and extension at 72 °C for 45 s and a final extension at 72 °C for 4 min. The amplified products were verified by electrophoresis on a 0.8% agarose gel stained with ethidium bromide. The amplified products were purified by precipitation with polyethylene glycol [75], subjected to sequencing by the chain termination method using Big Dye 3.1 reagent (Applied Biosystems, Waltham, MA, USA), and analyzed in a 3500 xL automatic capillary sequencer (Applied Biosystems).

2.11. Statistical Analysis

In the greenhouse bioassay evaluating the efficiency of different methods in controlling *M. spectabilis*, the treatment mortality data were corrected for natural control mortality, where there was no presence of pathogens other than those applied, using Abbott's formula [76] with 95% confidence intervals based on the average of 20 repetitions per treatment: Ma = (Mt − Mc)/(100 − Mc) × 100, where Ma is the mortality corrected for the control treatment, Mt is the mortality observed in the treatment with the insecticide, and Mc is the mortality observed in the control treatment [77]. Before the statistical analyses, all the data were tested for normality and homogeneity of variance. The data were compared among treatments using analysis of variance (ANOVA), and the Scott–Knott test was used to compare the means at a 5% ($p < 0.05$) error probability.

Data on the cause of death and the presence of fungi applied to the plant tissue and sap of *U. brizantha* plants were compared among treatments using Pearson's chi-square test. Their associations were assessed using Cramer's V.

The normality of the data was verified using the Kolmogorov–Smirnov test, and the homogeneity of variance was assessed by Bartlett's test. Data obtained in the field were extrapolated to the number of spittlebugs per m^2, tabulated, and compared between treatments using ANOVA and a nonparametric Kruskal–Wallis analysis followed by Dunn's test (5% significance level). All the statistical analyses were performed with SPSS Statistics (version 22).

3. Results

3.1. Fungal Strain Identification

BLASTn analysis revealed that the fungal strains UFMGCB 11443 and UFMGCB 11444 were *F. multiceps* and *M. anisopliae*, respectively. The fungi displayed ITS and Pol II sequences with high query cover and identified ≥99% of the respective species.

3.2. Analysis of the Persistence of Fungi in the Tissue and Sap of Urochloa brizantha

Analysis of the fungi *F. multiceps* UFMGCB 11443 and *M. anisopliae* UFMGCB 11444 in *U. brizantha* revealed no significant differences ($p = 0.880$) among cultures of fungi isolated from the leaves or sap, regardless of whether they were inoculated via the leaves or seeds. Fungi with characteristics similar to those of *F. multiceps* UFMGCB 11443 and *M. anisopliae* UFMGCB 11444 were absent in non-inoculated plants (controls) (Table 2). The degree of association among fungi present in the leaves or sap was 23.4% according to Cramer's V test, but this difference was not significant according to Pearson's chi-square test ($\chi^2 = 6.601$, df = 7, $p = 0.880$).

Table 2. The percentage of fungi present in the sap or leaves of *U. brizantha* plants treated with *Fusarium multiceps* UFMGCB 11443 or *M. anisopliae* UFMGCB 11444 via foliar or seed treatment, and percentages of fungi present in *U. brizantha* leaves at different ages.

Applied Fungi	Analyzed Site	Inoculation Site	% Presence	Chi-Square Test	Ages	% Presence	Chi-Square Test
Fusarium multiceps UFMGCB 11443	Sap	Seeds	80	$X^2 = 6.601$ $p = 0.880$ V = 0.234 GL = 7	New	80	$X^2 = 1.867$ $p = 0.867$ V = 0.176 GL = 5
		Leaves	80		Young	80	
	Leaves	Seeds	80		Senescent	60	
		Leaves	60				
Metarhizium anisopliae UFMGCB 11444	Sap	Seeds	80				
		Leaves	70		New	80	
	Leaves	Seeds	70		Young	80	
		Leaves	70		Senescent	70	
Control	Sap	Seeds	0				
		Leaves	0		New	0	
	Leaves	Seeds	0		Young	0	
		Leaves	0		Senescent	0	

Similarly, no significant differences were found in the presence of *F. multiceps* UFMGCB 11443 or *M. anisopliae* UFMGCB 11444 at different leaf ages (p = 0.867). The degree of association among leaf ages in the treatments was 17.6% according to Cramer's V test, and this was not significant according to Pearson's chi-square test (χ^2 = 1.867; df = 5, p = 0.867). Notably, ≥60% of the analyzed plants contained the inoculated fungi (Table 2), showing the endophytic capacity and persistence of *F. multiceps* UFMGCB 11443 and *M. anisopliae* UFMGCB 11444 fungi in the plants. The presence of the inoculated fungi was confirmed by molecular analysis after morphological grouping and microculture slide analysis of the *U. brizantha* plant isolates used in this study and analysis of the sequences deposited in GenBank (Supplementary Table S1).

3.3. Greenhouse Bioassay with Mahanarva spectabilis and Cause of Death

All *M. spectabilis* samples collected from plants inoculated with *F. multiceps* UFMGCB 11443 or *M. anisopliae* UFMGCB 11444 showed conidia of the inoculated fungi on their corpses. The isolates from the control plants were not used in this analysis because they did not contain any isolates from dead insects with macro- or micromorphology similar to that of the inoculated fungi, representing <5% of the samples. The degree of association between the tested fungi was 100% according to Cramer's V test and significant according to Pearson's chi-square test (χ^2 = 100.0, df = 4, $p \leq 0.001$). After grouping 205 fungal isolates extracted from spittlebug nymphs based on the macromorphological and microscopic characteristics of their microcultures, the morphotypes of the applied fungi predominated, indicating that the insect deaths were caused by the applied fungi, which was confirmed by molecular analysis and the sequence deposited in GenBank (Supplementary Table S1).

In *U. brizantha* plants inoculated via foliar spraying, *M. spectabilis* nymph mortality from the sixth day after the start of the experiment was 83% for *F. multiceps* UFMGCB 11443 and 73.6% for *M. anisopliae* UFMGCB 11444. After 10 days, both fungi showed 100% efficiency in controlling *M. spectabilis* via foliar inoculation. In *U. brizantha* plants inoculated via seed treatment, *M. anisopliae* UFMGCB 11444 and *F. multiceps* UFMGCB 11443 caused 58.2% and 65.6% epizootic effects in the spittlebugs exposed to the plants for six days, respectively, with the values increasing to 97.9% and 91.9%, respectively, by the tenth day (Table 3). These results highlight the efficiency and ability of these fungi to control spittlebug populations through the endophytic route (Figure 1).

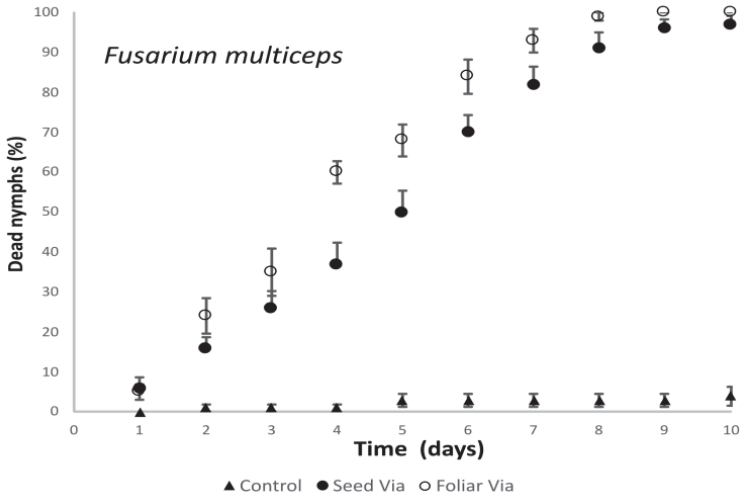

Figure 1. *Cont*.

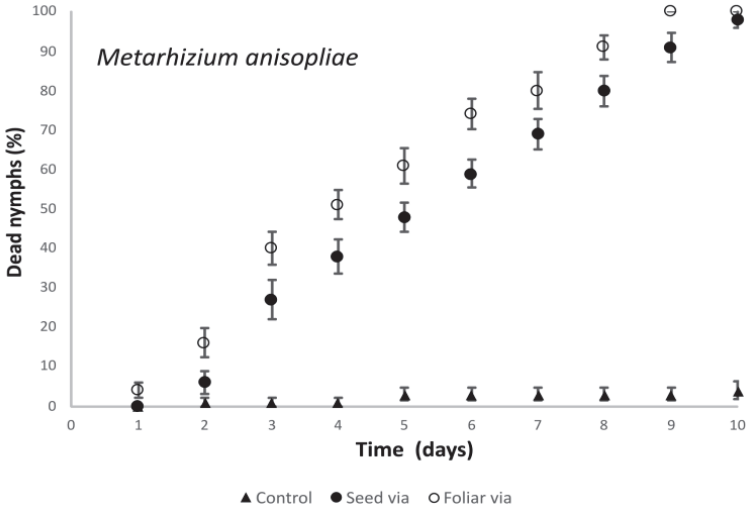

Figure 1. The cumulative percentage mortality of *Mahanarva spectabilis* nymphs fed on *Urochloa brizantha* plants inoculated via seeds or foliar spraying with the entomopathogenic fungus *Fusarium multiceps* UFMGCB 11443 or *Metarhizium anisopliae* UFMGCB 11444 or on non-inoculated control plants. The dots and bars indicate the means ± standard errors of 20 pots/fungi, with five nymphs per plant.

Table 3. The Abbott-corrected mean efficiency for the mortality of infected *M. spectabilis* nymphs in *U. brizantha* plants treated with *Fusarium multiceps* UFMGCB 11443 or *M. anisopliae* UFMGCB 11444 six and 10 days after insect contact.

Application via	*Fusariummulticeps*		*Metarhizium anisopliae*	
	6 days	10 days	6 days	10 days
Control	2.7 ^{a} ± 1.5	3.8 ^{a} ± 2.2	2.7 ^{a} ± 1.5	3.8 ^{a} ± 2.2
Seeds	65.6 ^{b} ± 5.5	91.9 ^{b} ± 5.3	58.2 ^{b} ± 3.4	97.9 ^{b,c} ± 2.1
Foliar	83.8 ^{c} ± 4.3	100.0 ^{c} ± 0.0	73.6 ^{c} ± 3.9	100.0 ^{c} ± 0.0
ANOVA	$F_{(119,5)} = 136.48$; $p \leq 0.05$		$F_{(119,5)} = 292.81$; $p \leq 0.05$	

Means with distinct letters in the columns indicate significant differences according to Tukey's test ($p < 0.05$).

3.4. Field Bioassay of Mahanarva spectabilis and Cause of Death

In the field, the applied fungi could maintain the nymphal and adult *M. spectabilis* populations at values lower than those in the control treatment group with only one spraying. A significant reduction in the insect pest population was observed after 15 days at all doses of *F. multiceps* UFMGCB 11443 and *M. anisopliae* UFMGCB 11444 used (Figure 2A–G). This period reflected the highest number of live nymphs/m^2 in the control treatment (Figure 2H). Therefore, using the lowest dose of either fungus is recommended because it is more economical, facilitates application, and is sufficient to contain the spittlebug infestation cycle in pastures.

The lowest fungal dose of 0.5 kg/ha had the same effect as the fourfold higher dose, with an appreciable decrease in insect pest infestation observed (Table 4). The effect of mixed suspension of *F. multiceps* and *M. anisopliae* at 1.0 kg/ha did not significantly differ from that of the same concentration of each fungus alone after six (p = 0.686) and 15 (p = 0.520) days of application (Table 4). Isolates extracted from dead spittlebug nymphs from the field were grouped based on the macromorphological and microscopic characteristics of their microcultures. The morphotypes of the applied fungi predominated, indicating that the insect deaths were due to the applied fungi, which was confirmed by molecular analysis and the sequence deposited in GenBank (Supplementary Table S1).

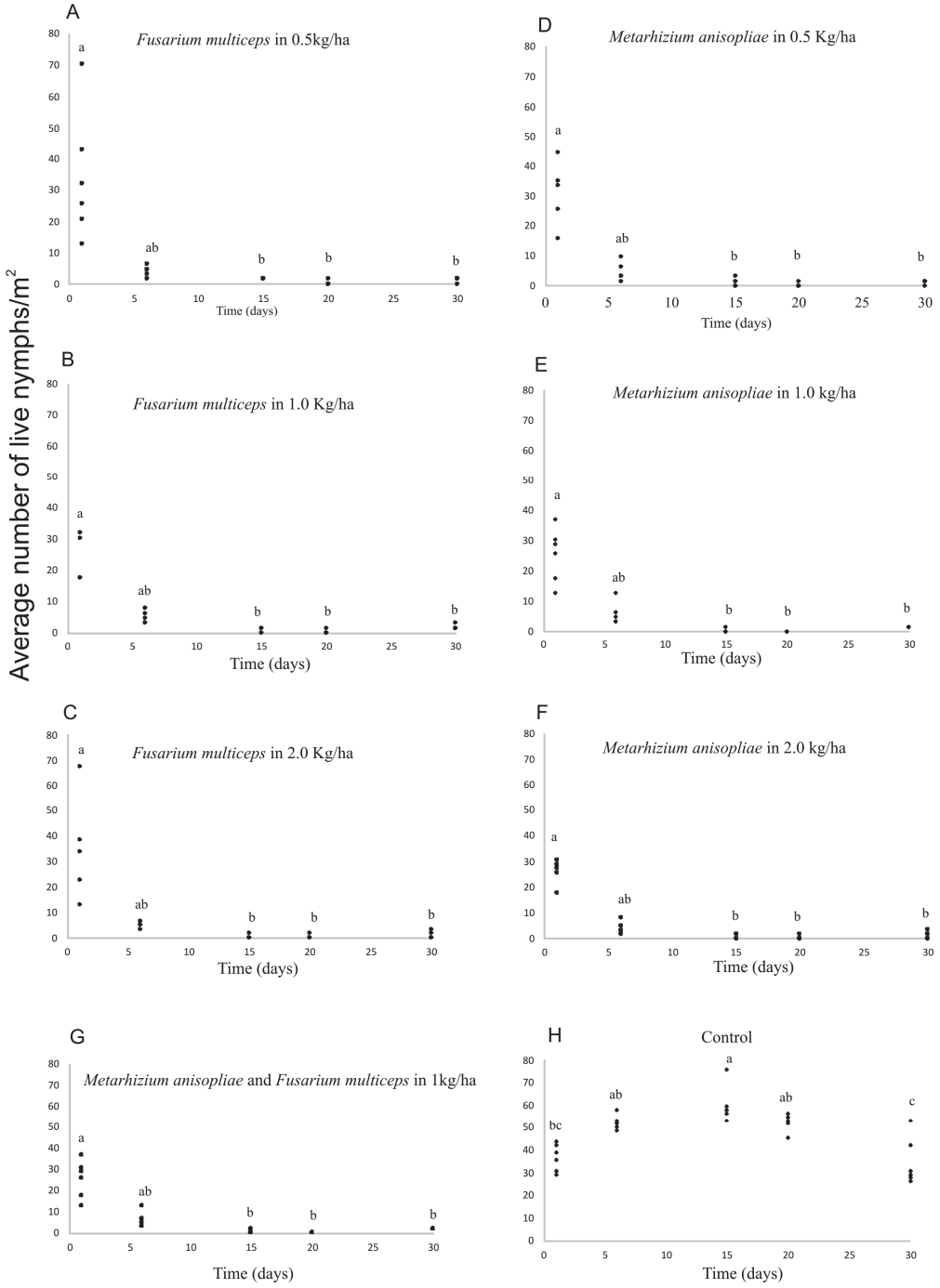

Figure 2. Average number of *Mahanarva spectabilis* nymphs/m^2 in *Urochloa brizantha* pastures sprayed with the entomopathogenic fungus *Fusarium multiceps* UFMGCB 11443 (**A**–**C**) or *Metarhizium anisopliae* UFMGCB 11444 (**D**–**F**) at different concentrations (0.5, 1.0 and 2.0 kg/ha), with both fungi combined in the same solution (**G**), or with the control solution (**H**). The numbers of nymphs/m^2 at different times with different letters were significantly different according to Dunn's test (5% significance level) following the Kruskal–Wallis test, with six replicates per time point.

Table 4. Mortality of *Mahanarva spectabilis* nymphs and adults/m^2 at six and 15 days after applying the fungus *Fusarium multiceps* UFMGCB 11443 or *Metarhizium anisopliae* UFMGCB 11444 at three different concentrations or mixed application of both fungi at 1.0 kg/ha each in *Urochloa brizantha* pastures. Kruskal–Wallis nonparametric analysis was performed.

Fungus	Dose	Median	Kruskal—Wallis
Fusarium multiceps 6 days	0.5 kg/ha	85.94	H = 4.58
	1.0 kg/ha	77.17	*df* = 2
	2.0 kg/ha	85.37	*p* = 0.101
Fusarium multiceps 15 days	0.5 kg/ha	95.31	H = 5.58
	1.0 kg/ha	93.48	*df* = 2
	2.0 kg/ha	99.80	*p* = 0.062
Metarhizium anisopliae 6 days	0.5 kg/ha	89.92	H = 3.43
	1.0 kg/ha	77.89	*df* = 2
	2.0 kg/ha	85.00	*p* = 0.180
Metarhizium anisopliae 15 days	0.5 kg/ha	97.48	H = 0.05
	1.0 kg/ha	99.00	*df* = 2
	2.0 kg/ha	97.00	*p* = 0.977
Fusarium multiceps 6 days	1.0 kg/ha	**77.17**	**H= 0.753** **df = 2** **p = 0.686**
Metarhizium anisopliae 6 days	1.0 kg/ha	**77.89**	
Fusarium multiceps + *Metarhizium anisopliae* 6 days	1.0 kg/ha/each fungus	81.63	
Fusarium multiceps 15 days	1.0 kg/ha	93.48	H= 2.81 *df* = 2 *p* = 0.52
M. anisopliae 15 days	1.0 kg/ha	99.00	
Fusarium multiceps + *Metarhizium anisopliae* 15 days	1.0 kg/ha/each fungus	96.23	

4. Discussion

Endophytic entomopathogens can influence herbivore population dynamics by reducing the populations of pests [78]. This observation corroborates other studies on the endophytic establishment of entomopathogenic fungi in a variety of cultivated plants [22,79–81]. Some of these fungi are vertically transmitted via seeds from one generation to another. Some endophyte genera form a distinct group of fungi related to the ecological requirements and discrete adaptations of grasses [82]. Various *Metarhizium* spp. naturally associate with the roots of grasses, shrubs, herbs, and trees in the field [79,83] and can control various insect pests.

This study revealed that the use of entomopathogenic fungi as endophytes in plants provides diverse opportunities that can benefit the production chain of these plants in agroecosystems at likely more affordable costs. Therefore, the inoculation of forages with the entomopathogenic fungi *F. multiceps* UFMGCB 11443 and *M. anisopliae* UFMGCB 11444 via the endophytic route has promise as a defensive strategy. Thus, these fungi could be candidates for new bioproducts. Unlike synthetic insecticide products, according to Zimmermann, the fungi likely do not affect the production chain, the environment, or human health [68]; generate less chemical waste; and add value to the final product. Reducing agrochemical use is a trend in developed countries that are increasingly using sustainable healthy agriculture. These organizations aim to participate in the competitive market for organic products that are increasingly appreciated by society.

Seed treatment with entomopathogenic fungi is a promising technology for the protection and sustainable production of crops without adverse effects on plant performance or

final products [45,79,84–86]. However, the main advantage of using biocontrol agents is their ability to regulate insect pests in a more stable manner and direct or indirect biocontrol activity. Therefore, different methods of inoculating plants with fungi that could act as endophytes can effectively reduce the spittlebug population in pastures.

This study confirmed the presence of the fungi *F. multiceps* UFMGCB 11443 and *M. anisopliae* UFMGCB 11444 as endophytes. The plants were inoculated via foliar spraying or seed treatment, and the fungi were then reisolated from the leaves or sap since they were possibly present systemically. Some fungi, such as *Metarhizium robertsii* Bischoff, Rehner & Humber and *Beauveria bassiana* (Bals.) Vuill., penetrate and colonize their hosts systemically. These proteins penetrate through the stomata and persist in the epidermal cells of *Zea mays* L., growing through the air spaces between the plant parenchymal cells [87] or roots and leaves [79].

There were no significant differences between inoculation by foliar spraying and seed treatment, indicating that both techniques are efficient for plant colonization. Fungal propagation by passive transmission leads to random diffusion into plant tissue [80]. This might explain the action of the fungi used in this study when applied via seeds. The potential of seed treatment with entomopathogenic fungi is a promising technology for the protection and sustainable production of crops without adverse effects on plant performance or final products [45,79,86]. In addition, the greatest barriers to the use of products based on entomopathogenic fungi, e.g., abiotic effects, should be mitigated. Endophytes protect fungi from these effects, as they reside within plant tissues. Therefore, different methods of inoculating plants with fungi that act as endophytes can effectively reduce the spittlebug population in pastures.

Treating seeds in conidial suspensions is an alternative artificial colonization method to induce successful endophyte colonization in many cultivated plants [88]. *Metarhizium* spp. have been studied as insect pathogens and are increasingly being investigated for their beneficial effects on host plants [79,89–93]. The seeds of corn plants were treated with this fungus, indicating that *M. robertsii* was systemically established in the maize plants [79]. Kabaluk and Ericsson [91] obtained promising results with maize seeds treated with *M. anisopliae*, including purportedly minimized damage caused by maize wireworms (*Agriotes obscurus* L.) (Coleoptera: Elateridae). Members of this genus also have effects when applied to the seeds of *U. brizantha* to control *M. spectabilis*.

Santos et al. (2019) [94] identified 29 isolates of entomopathogenic fungi of the genus *Fusarium* associated with Hemiptera. The species *F. multiceps* belongs to the *Fusarium incarnatum-equiseti* species complex (FIESC) [95]. This group is also associated with insects [96]. Several of these strains have the ability to control agricultural pests with high mortality rates of insect pests, exhibiting rapid action and abundant sporulation, making them ideal for use in microbial control [97]. Several species of the genus *Fusarium* are endophytes associated with *Rosa* (Rosaceae), many of which have not yet been identified at the species level [98]. An endophytic strain of *F. multiceps* was isolated from *Acanthus ilicifolius* L. (Acanthaceae), which produces secondary metabolites with biological characteristics suitable for use in agriculture and medicine [99]. This work is the first report of *F. multiceps* inoculated into *U. brizantha* as a vector for controlling *M. spectabilis*.

Among other genera, fungi such as *Beauveria bassiana* and *Isaria fumosorosea* (Wize) Brown & Smith Mantzoukas and Lagogiannis [85] have been reported to have high pathogenicity in insects when inoculated into plants [86]. Fungi grow systemically on all aboveground plant organs, resulting in the vertical transmission of endophytes via seeds [79,100]. Other studies have reported findings similar to those of the current study on the ability of fungi to adapt to plants and promote mutualistic interactions, inducing defense mechanisms against herbivores in plants and thereby decreasing their insect population [76,101].

For entomopathogenic fungi to successfully colonize both plants and insects, they must be able to invade and establish themselves in both organisms [35]. Our findings revealed that the fungi *F. multiceps* UFMGCB 11443 and *M. anisopliae* UFMGCB 11444 cause

epizootic effects in greenhouses and in the field. The results of this work are supported by studies demonstrating the systemic colonization of several plant species by *Metarhizium* spp. [79,102].

The mortality of spittlebugs in a greenhouse exposed to plants inoculated with *F. multiceps* UFMGCB 11443 and *M. anisopliae* UFMGCB 11444 indicates that the induction of various defense responses, which act directly as insect pest mortality factors, provides protection to plants and reduces herbivory. This fungus—plant—insect interaction was also observed by Branine et al. [36], who used *M. robertsii* as an endophyte to control cotton leafworm (*Spodoptera littoralis* Boisduval) (Lepidoptera: Noctuidae). However, few studies have examined treating other seed species with entomopathogenic fungi as endophytes [103,104]. This is the first study in which *U. brizantha* seeds were treated with entomopathogenic fungi.

This study showed that the fungi *F. multiceps* UFMGCB 11443 and *M. anisopliae* UFMGCB 11444 in the field attacked nymphs at the base of the plants when inoculated through foliar spraying. Based on these considerations, *Metarhizium brunneum* (Metchnikoff) and *B. bassiana* are widely used as bioprotectors and infest soil-dwelling or larval-dwelling insects [105,106]. Therefore, these means of infestation are ideal for reaching spittlebug nymphs living in foams at the base of *U. brizantha* plants. The population of *M. spectabilis* spittlebugs in the field was controlled by only one application of *F. multiceps* UFMGCB 11443 and *M. anisopliae* UFMGCB 11444, which, in addition to the lower dose, was more economical for the producer. Consistent with this study, a product based on *M. anisopliae* had the necessary efficiency to maintain the size of the spittlebug population below its actionable limit after *M. anisopliae*-based biopesticide application in the field [22].

The fungi *F. multiceps* UFMGCB 11443 and *M. anisopliae* UFMGCB 11444 were reisolated from the corpses of nymphs or adult spittlebugs of *M. spectabilis* from both field and greenhouse samples. Klieber and Reineke [69] also reisolated *B. bassiana* from corpses of *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) after the fungus was originally inoculated into *Solanum lycopersicum* L. cv. Harzfeuer (Solanaecae) via foliar spraying.

Bioinputs, including those based on fungi, are a trend in developed countries where integrated and ecologically friendly agriculture has already been implemented [106]. In this context, Brazil is starting to use sustainable products, such as entomopathogenic fungi, for pest control. In 2023 alone, 90 products were registered for biological control in Brazil [101]. Using grasses as hosts for entomopathogenic fungi is a low-cost innovation for producers, in addition to its benefits in promoting sustainable meat and milk production without harming the environment and generating products without toxic residues [68]. The demonstration of the efficiency of *F. multiceps* UFMGCB 11443 and *M. anisopliae* UFMGCB 11444 in the endophytic pathway of *U. brizantha* provides biological control alternatives that producers might be more willing to use, given their properties. Given the efficiencies obtained in greenhouses and in the field, and the possibility of using *U. brizantha* as a host for entomopathogenic fungi, these results provide insights for mitigating some challenges in pastures.

5. Conclusions

The entomopathogenic fungi *F. multiceps* UFMGCB 11443 and *M. anisopliae* UFMGCB 11444 are endophytic and colonize the tissues of *U. brizantha,* which makes them a viable and effective alternative for reducing the population of *M. spectabilis* spittlebugs, whether inoculated via seeds or foliar spraying. Therefore, these fungi are promising candidate bioproducts for controlling this pest.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy14050943/s1, Table S1: Confirmation of fungi applied via foliar or seed of Urochloa ruziziensis isolated from nymphs feeding on these plants in the field or greenhouse, and from plant tissue after infestation. GenBank accessions of the sequences.

Author Contributions: M.O.C. conceived and designed the study; A.M.A. identified the spittlebugs, designed the study, and reviewed and edited drafts; R.M.M. collaborated on the study and revised the manuscript; A.P.M. analyzed and interpreted the data; M.A.C. funded and provided the site for the field experiments; L.H.R. identified the fungi; M.F.A.P. collaborated on the methodology; M.M., S.R.O.S., N.B.M.S. and A.C.R.S. collaborated on the experiments; W.G.C. designed and supervised the study and analyzed and interpreted the data. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The author Michelle Oliveira Campagnani is a CNPq fellow. The other authors declare that the research was carried out in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. We confirm that all collection of plant material was conducted in accordance with relevant institutional, national, and international guidelines and legislation.

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Efficacy and Persistence of Entomopathogenic Fungi against *Rhynchophorus ferrugineus* on Date Palm: Host to Host Transmission

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Abstract: The red palm weevil (RPW), *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Dryophthoridae), is a destructive and voracious pest of palm species worldwide. Due to environmental and regulatory concerns, ecologically safe alternatives to synthetic chemical insecticides are needed to manage this cryptic insect species. Entomopathogenic fungi have the potential to manage this pest. The scope of management and effectiveness can be improved by direct control or horizontal transmission of entomopathogenic fungal isolates. We tested in the laboratory the virulence and pathogenicity of fifteen different entomopathogenic fungal isolates belonging to the following species: *Beauveria bassiana*, *Metarhizium anisopliae*, *Beauveria brongniartii* and *Purpureocillium lilacinum*. All fungal isolates were found virulent against larvae (14.9 ± 1.06 to 81.5 ± 1.48% mortality) and adults (5.6 ± 1.12 to 51.7 ± 1.51% mortality) at 12 d post-treatment. From a screening bioassay, five *M. anisopliae* (WG-08, WG-09) and *B. bassiana* (WG-23, WG-24, WG-25) isolates were tested for their concentration response mortality against larvae and adults after 7, 14 and 21 days (d) of treatment. Mortality was found positively correlated with concentration and time. At 21 d of treatment, WG-23 and WG-25 1×10^8 conidia/mL resulted in 100% mortality against larvae while only WG-25 1×10^9 conidia/mL caused 100% mortality of adults. Along with mortality, all the potential isolates have strong ovicidal effects that reduced 81.49% at 1×10^8 conidia/mL. The horizontal transmission bioassay indicated that the infected adults transmitted the disease to healthy individuals. Horizontal transmission of fungi from infected to non-infected adults not only caused significant mortality but also had a serious sublethal impact on insect development and fitness including reduced number of eggs/d fecundity, egg viability and neonate survival. Isolate WG-25 reduced oviposition (0.5 eggs/d), fecundity (11.7 eggs/female), egg viability (11.6%) along with larval survival 25.9% when infected male mated with normal female. In semi-field trials, all fungal isolates reduced survival of larvae found inside the palms and ultimately reduced infestations over a period of two months. The results of this study indicate that entomopathogenic fungi should be further tested for sustainable and efficient control of RPW in date palm production systems.

Keywords: red palm weevil; biological control; mortality; mycosis; horizontal transmission; sublethal effects

1. Introduction

Date palm, *Phoenix dactylifera* L. (Arecales: Arecaceae) is a diploid perennial plant known as one of the oldest and most common crops to Southwest Asia and North Africa. Over time, it spread to Australia, South America, southern Africa, Mexico, Pakistan and the United States [1] The red palm weevil (RPW) *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Dryophthoridae), is considered to be one of the most devastating insect pests of palm species [2]. It was first reported in India, but now it is widely distributed in Africa, America, Asia, Europe and Oceania [3,4].

In Pakistan, it is a serious pest of date palms [5,6]. The weevil has invaded a total of 69 countries over the last 30 years, and the host range has expanded from only 4 species in the mid-1950s to 40 species in 2020 [4,7]. It is an important insect pest against which quarantine and biosecurity measures should be taken by countries to try to limit its further spread, as it attacks 19 species from 15 genera [8]. It reproduces within the tree stem, damaging the vascular system which leads to the gradual death of the entire palm [9,10]. The female lays eggs in injured parts such as stems, leaf bases, offshoots, and tree crowns where larvae bore in and are usually invisible until infested palms die [11,12]. The concerns of farmers regarding economic and environmental issues of RPW are genuine and considerable. It severely affects the produce, as well as exerting negative impact on cultural and aesthetic values of palm trees [6,13].

Present management approaches against *R. ferrugineus* involve various control methods such as cultural techniques including burning of dead tree trunks to destroy immature stages inside, cutting down the infected palms, fertilization and irrigation, mass trapping, as well as monitoring, early detection, applications of acoustic devices, male sterile techniques, host plant resistance, entomopathogenic nematodes, insecticide applications, including fumigation, or application of natural substances [14–20]. Due to the high cost of pheromone traps and the prevalence of hot summers in date-producing areas, the trap strategy has not attained wide acceptance against *R. ferrugineus* [21]. Similarly, the sterile insect technique is also not an effective approach in the field as *R. ferrugineus* mates in a concealed environment, i.e., within the palm tree. Chemical insecticides have two major limitations: they harm the environment and have little or no effectiveness against the later larval stages of *R. ferrugineus* [21]. As curative and preventive treatments against *R. ferrugineus*, insecticides are usually applied on an irregular basis, which may result in environmental pollution, risks to human health and decreases in natural enemy fauna [22]. With these reservations associated with the use of traditional control approaches, there is an urgent need for novel techniques and methods for the control of invasive *R. ferrugineus*. In that vein, microbial control agents, particularly entomopathogenic fungi (EPF), may be considered as promising aids to existing control strategies against RPW [23].

Entomopathogenic fungi contribute to insect population regulation in natural habitats, and are usually harmless to non-target organisms, humans, and the environment [24,25]. EPFs are widely distributed in forest ecosystems and agricultural habitats; commercialized EPF products are used for the successful management of various insect pests [26–28]. Unlike other microbial agents (viruses, protozoa and bacteria) which need to be ingested by the host insect for the initiation of the infection process [29], fungal pathogens can directly penetrate the insect host's cuticle [30]. Entomopathogenic fungi should be considered for inclusion in IPM programs for *R. ferrugineus* [20,31–33]. EPF can affect survival and reproduction of RPW [12,34]. However, there are certain limitations in the use of EPF such as issues during their production, interactions with fungicides, the feasibility of applying them in large-scale agricultural settings, the long-term sustainability of these biological control measures, and potential environmental impacts of widespread EPF use [20]

Horizontal transmission of a pathogen within a target population can extend the potential scope of microbial control for various insect species [35]. Auto-dissemination via the horizontal transmission of pathogens within a species has been used for biocontrol of insect pests belonging to different insect orders [36,37]. Horizontal transmission of EPFs offers advantages including reduction in area treated and volume of inoculums, diminishing

harmful effects on non-target individuals [38,39] as well as providing another advantage over chemical insecticides as its impact on the pest population increases beyond the zone of direct contact [40]. Auto-dissemination in different target coleopteran hosts by *Metarhizium brunneum* Petch (Hypocreales: Clavicipitaceae) [37,41] has been previously documented.

Keeping in view the importance of EPF as potential candidates for biological control of RPW, this study was carried out to assess the efficacy of native isolates of *B. bassiana*, *M. anisopliae*, *Purpureocillium lilacinum* (Thom) Luangsa-ard, Houbraken, Hywel-Jones & Samson (Hypocereales: Ophiocordycipitaceae), recovered from soil and insect infected cadavers, to select the most virulent. This study also assessed, for the first time in RPW, the horizontal transmission of the most virulent fungal isolates and to determine the subsequent effects on oviposition, fecundity, egg hatching and larval survival among all four mating combinations of non-infected/infected males and females of *R. ferrugineus*. The efficacy of different EPF isolates was evaluated in semi-field trials, their persistence and impact on survival of *R. ferrugineus* was also assessed.

2. Materials and Methods

2.1. Insect Collection and Rearing RPW

The different developmental stages of RPW were manually collected from fallen and damaged trees of date palm, Jhang, Bahawalpur, Lodhran, Multan, Layyah and Bhakar districts of Punjab province, Pakistan. Each development stage was placed individually in boxes and transferred to the laboratory. Collected pupae were taken to the laboratory in clean perforated zip lock plastic bags.

In the laboratory, larvae were fed on sugarcane stalks depending upon the size of the larval instar. To induce larval feeding, a hole according to the size of larvae was made in the upper portion of sugarcane and larvae were released into these holes on each sugarcane stalks. The food was changed on average every three days, and larvae were allowed to feed on these pieces until they pupated. The pupae were placed in a plastic box (15 cm × 30 cm × 30 cm), which was kept moistened with distilled water using a hand sprayer; the pupae were examined daily to check adult emergence from pupae. The adults were placed in aerated plastic boxes (15 cm × 30 cm × 30 cm). Adults were fed on shredded sugarcane stems, which had been soaked in distilled water for one minute. In each box equal numbers of males and females were placed. The diet for adults was changed on alternate days and sugarcane stalks in which egg-laying occurred were moved to separate boxes for larval emergence. The sugarcane stalks were monitored daily and larvae hatching from eggs or loose in the box were transferred onto sugarcanes stalks with a camel hairbrush. The incubator was set for all the stages at 28 ± 2 °C and 65 ± 5% relative humidity (RH) and 16: 8 h (Light/Dark) photoperiod [42].

2.2. Culturing of Fungal Isolates

The virulence of fifteen isolates of different EPF including *B. bassiana* (WG-14, WG-21, WG-22, WG-23, WG-24, WG-25), *M. anisopliae* (WG-08, WG-09), *Beauveria brongniartii* (Sacc.) Petch (Hypocreales: Cordycipitaceae) (WG-26, WG-27) and *P. lilacinum* (WG-33, WG-34, WG-35, WG-36, WG-37) was evaluated against *R. ferrugineus* (Table S1). These isolates were obtained previously from insect cadavers and soil samples collected from various geographical areas (Table S1). The fungal isolates from soil samples were recovered using potato dextrose agar (PDA) (BD, Franklin Lakes, NJ, USA) with chloramphenicol (50 µg/mL), streptomycin sulphate (50 µg/mL) (Sigma, St Louis, MO, USA) and 0.5 g/L of dodine 65% *w/w* WP. All isolates were identified using taxonomic keys [43–47]). The cultures were stored at the Microbial Control Laboratory at Department of Entomology, University of Agriculture, Faisalabad (Pakistan) [48,49]. The isolates were inoculated in PDA Petri dishes (100 mm) and sealed with parafilm (Sigma Aldrich Chemie GmbH, Taufkirchen, Germany) at 25 °C with a 14: 10 h (Light/Dark) illumination period for 14 d. Conidia were harvested with sterile scalpel and suspended in a 50 mL conical tube with 30 mL of 0.05% Tween 80. The tubes were agitated with eight glass beads and vortexed

for 5 min. The required conidial concentrations were ascertained using a hemocytometer under 400× magnification. Conidial germination was determined by spreading 0.1 mL (1×10^6 conidia/mL) suspensions on two sabouraud dextrose agar (SDA) (BD, Franklin Lakes, NJ, USA) in Petri dishes (100 mm) and incubated at 25 °C with a 14:10 h (Light/Dark) illumination period for 16–18 h. Germination was recorded by placing cover slips on plates and assessing 200 spores from each plate. We averaged two counts from two plates, and the final concentration was adjusted using serial dilutions, based on germinated conidia [50]. Germination of conidia of all the EPF was assessed under 400× magnification and found to be greater than 90%.

2.3. Screening of Fungal Isolates against RPW Larvae and Adults

Fifteen isolates were evaluated against 4th instars and adults (48–72 h old) of *R. ferrugineus* at two concentrations 1×10^7 and 1×10^8 conidia/mL. These rates were selected based on preliminary concentration assays conducted in the laboratory. Larvae were dipped into either concentration for 60 s while adults were immersed for 90 s [51]. The treated insects were then separately placed on sterile filter paper to dry. After 24 h, the insects were placed individually in plastic cups (150 mL) to avoid cannibalism. Larvae were provided with artificial diet [42,52], while adults were provided with shredded sugarcane (3 cm × 3 cm) for feeding. The plastic cups were covered with perforated lids for the purpose of aeration and were placed in an incubator (MIR-254, Sanyo, Tokyo, Japan) maintained at 25 °C and 75% RH with a 16: 8 h (Light/Dark) illumination period. For the control, individuals were dipped for same period in a 0.05% Tween 80 and otherwise handled the same as the treated insects. A total of 15 larvae and adults were used per replicate, and each treatment had 3 replicates (45 individuals per treatment). The entire experiment was repeated twice with 2880 individuals used in the experiment. The experiment end point was 12 d after treatment and mortality was observed on daily basis. After the last count, the remaining live individuals (larvae and adults) were disposed of. The dead larvae and adults were individually placed on sterile moistened filter paper and observed for mycosis on cadavers. The cadavers with no visible symptoms after 24 h were surface sterilized with sodium hypochlorite (0.1%) solution followed by three rinses with distilled water. Then, cadavers were placed on sterile moistened filter paper and incubated at 25 °C, 75 RH and 16: 8 h (Light/Dark) to stimulate the fungal growth [51].

2.4. Concentration Response Bioassay against Larvae and Adults of RPW

The isolates which caused the highest larval mortalities (*M. anisopliae* WG-08 and WG-09; *B. bassiana* WG-23, WG-24, and WG-25) during preliminary screening were selected for further study against both larvae and adults at different concentrations and exposure intervals. Each fungal isolate was applied at four concentrations (1×10^6, 1×10^7, 1×10^8 and 1×10^9 conidia/mL) against 6th instars and adults (48–72 h old) by dipping. Larvae were dipped into the four different concentrations for 60 s, while adults were dipped for 90 s [51]. For post-application, insects were handled as described in Section 2.3. Mortality counts were made at 7, 14 and 21 d post-treatment. For each replication, 15 insects were used making a total of 45 individuals per treatment in three replicates. The experiment was repeated twice.

2.5. Bioassay against Eggs

The ovicidal activity of the five previously selected fungal isolates (WG-08, WG-09, WG-23, WG-24 and WG-25) was evaluated using recently laid eggs. Before the bioassay, eggs (<24 h of age) were surface sterilized by immersing them in a 3% solution of sodium hypochlorite (NaClO) and then washing with distilled water three times [53]. Fifteen eggs per replicate were immersed into two fungal concentrations (1×10^6 and 1×10^8 conidia/mL) of each isolate for 1 min by placing them in a clean stainless steel mesh basket with dimensions of 16 × 17 mm [51]. A batch of eggs immersed in distilled water containing only 0.01% Tween 80 [54] served as the control. After dipping, eggs were moved to Petri

dishes (2.5 cm diameter) lined with sterile moistened filter paper and incubated (MIR-254, Sanyo, Tokyo, Japan) at 25 °C with 75 RH and a 16: 8 h (Light/Dark) illumination period. Each treatment was replicated three times and the entire experiment was also repeated three times with new biotic and abiotic materials each time. Hatching data for eggs were recorded daily up to 5 d although most hatching occurred between 3 and 4 days; eggs not hatched by the 5th day were considered dead. After hatching, newly emerged neonates were transferred to another Petri dish and provided with clean sugarcane slices for feeding. Neonate survival times were recorded daily for 5 d.

2.6. Horizontal Transmission of Fungal Isolates among Adults and Effectiveness of Sublethal Concentrations on Reproductive Stages of RPW

The potential for horizontal transmission of five pre-selected EPF isolates (WG-08, WG-09, WG-23, WG-24, WG-25) was assessed in *R. ferrugineus* adults. EPF were applied at concentration 1×10^5 conidia/mL. Laboratory reared 48–72 h old male and female adults were immersed in a suspension of different isolates for 90 s and then allowed to mate. Four different mating pairs (10 adults with 1:1 ratio of both male and female) were arranged for each isolate: (1) non-infected females + non-infected males, (2) infected females + non-infected males, (3) non-infected females + infected males and (4) infected females + infected males. After treatment, each mating pair was transferred together into plastic boxes and provided with cotton wicks dipped into a 5% solution of sugar for feeding; the cotton wicks additionally served as a substrate for oviposition. Adult mortality and fecundity were recorded daily up to 28 d. Oviposition was recorded daily up to 7 d. Eggs laid by treated females from each combination were collected randomly and kept in Petri dishes lined with moistened filter paper. From each replication, 3 eggs/d were randomly selected up to 7 d post-treatment (21 eggs from each replicate) and hatching (%) was noted for the following 5 days (because most of hatching occurred between 3 and 4 days). The percent survival of the hatched neonates was then noted for up to 5 d. Each mating combination represented a single treatment with three replicates for individual isolates and the whole experiment was repeated three times [51].

2.7. Semi-Field Trials for RPW Survival

This experiment was conducted with 5 years old date palm plants that were purchased from a nursery with no infestation detected after careful visual observation. The plants were watered as needed and kept under a double mesh security enclosure. Each treatment consisted of six different treatments including five fungal isolates (WG-08, WG-09, WG-23, WG-24 and WG-25) and a control. Fungal isolates were suspended individually at concentration 1×10^9 conidia/mL in a 0.05% Tween 80 and 2000 mL of fungal solution. The fungal suspensions of each isolate were sprayed around the trunk with a knapsack sprayer while for the control, 2000 mL of 0.05% Tween 80 was sprayed without fungal conidia. Two hours post-application, a group of three female adults and two males were released on each plant and the trunk was wrapped with double mesh wire gauze to avoid the escape of weevils. Adults were removed 7 d post-release, and the palm plants were cut with a chainsaw at 30 d post-insect released. First, the palm plants were categorized as infested if a single larva was found inside or un-infested if no larva was found inside. The total numbers of dead and live individuals found inside were counted. The trial was arranged in a randomized complete block design (RCBD). Each treatment comprised five palm plants with four replications (total of 20 palms per treatment and total of 120 palms per trial).

2.8. Fungal Persistence over Time and Its Effectiveness against Larvae of RPW

This trial was aimed to assess the persistence of fungal isolates (WG-08, WG-09, WG-23, WG-24, WG-25) in palm plants over time. The treatment application and protocol were the same as in the previous trial. The treated and control plants were divided into five different groups that were infested with adults at 1, 15, 30, 45 and 60 d post-treatment

application. Adults remained on the plants for 7 d and then were removed. Treatment efficacy was determined by dissecting palm plants at 30 d post-insect release and the total number of larvae present inside was counted as either live or dead [51].

2.9. Statistical Analysis

The normality of the distribution of the percentages of RPW mortality and mycosis was checked by Shapiro–Wilk test [55]). All data were not normally distributed and all percentages were log-transformed before further analysis. The mortality data from different treatments were corrected using Abbott's [56]) formula and tested for comparison using Tukey HSD post hoc test [57]) at a 5% significance level. The statistical package Minitab [58] was used for analysis. Median lethal concentration (LC_{50}) and Lethal time (LT_{50}) values for fungal concentrations of isolates at different exposure intervals were calculated by Probit analysis. For semi-field trials, the mean number of live larvae per plant was analyzed (ANOVA) and significant differences were subjected to Duncan's test at 5% level of significance for comparison. The efficacy of fungal isolates against larvae on palm trees was computed following Abbott's [56] formula [51].

3. Results

3.1. Screening of Fungal Isolates against Larvae and Adults

The main effects and their possible associated interaction fungal isolates × concentration for mortality and mycosis were significant for both larvae and adults (Table S2). Larvae were more susceptible to fungal infection than adults (Figures 1 and 2) at the two different concentrations. Mortality ranged from 14.9–52.9%, at 1×10^7 conidia/mL, to 18.3–81.5% at 1×10^8 conidia/mL for larvae and 5.6–31.8%, at 1×10^7 conidia/mL, to 9.1–51.7% at 1×10^8 conidia/mL in adults.

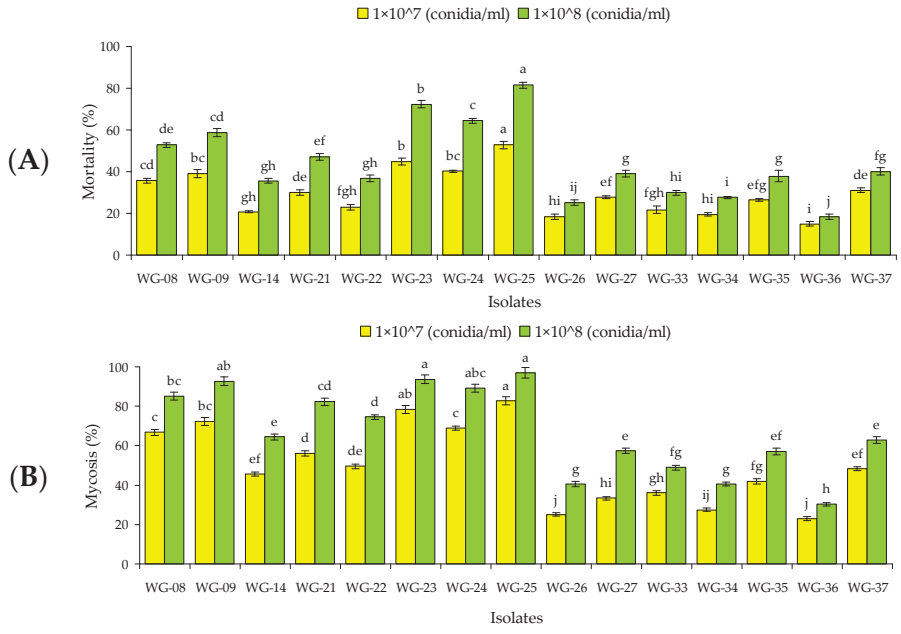

Figure 1. Mean (**A**) mortality (% ± SE) and (**B**) mycosis (% ± SE) of 15 isolates of entomopathogenic fungi against *Rhynchophorus ferrugineus* larvae 12 d post-treatment. Means followed by the same lowercase letter each for mortality (F = 11.45, df = 14, 89, $p < 0.01$ for 1×10^7 conidia/mL and F = 18.23, df = 14, 89, $p < 0.01$ for 1×10^8 conidia/mL) and mycosis (F = 21.87, df = 14, 89, $p < 0.01$ for 1×10^7 conidia/mL and F = 29.34, df = 14, 89, $p < 0.01$ for 1×10^8 conidia/mL) are not significantly different, Tukey HSD test at p = 0.05.

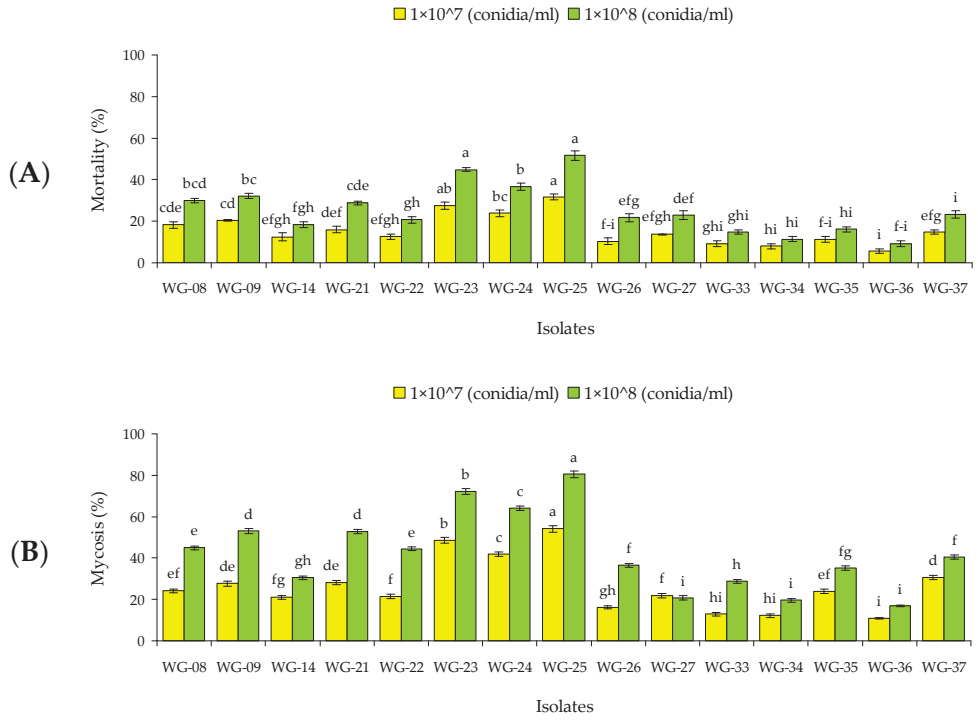

Figure 2. Mean (**A**) mortality (% ± SE) and (**B**) mycosis (% ± SE) of 15 isolates of entomopathogenic fungi against *Rhynchophorus ferrugineus* adults 12 d post-treatment. Means followed by the same lowercase letter each for mortality (F = 14.56, df = 14, 89, $p < 0.01$ for 1×10^7 conidia/mL and F = 23.48, df = 14, 89, $p < 0.01$ for 1×10^8 conidia/mL) and mycosis, F = 13.71, df = 14, 89, $p < 0.01$ for 1×10^7 conidia/mL and F = 16.12, df = 14, 89, $p < 0.01$ for 1×10^8 conidia/mL) are not significantly different, Tukey HSD test at p = 0.05.

At 1×10^8 conidia/mL, only five isolates (WG-08, WG-09, WG-23, WG-24 and WG-25) against larvae and one isolate (WG-25) against adults caused >50% mortality at 12 d post-inoculation. At the same concentration, five isolates (WG-08, WG-09, WG-23, WG-24 and WG-25) against larvae and four isolates (WG-09, WG-23, WG-24 and WG-25) against the adults produced >50% mycosis (Figures 1 and 2). Control mortality was lower than 5% against both developmental stages.

3.2. Concentration Response Bioassay against RPW Larvae and Adults

The main effects and their possible associated interactions isolate × concentration and isolate × interval, concentration × interval, and isolate × concentration × interval were significant for both larvae and adults (Table S3). Up to 7 d post-treatment, none of the isolates provided 100% mortality of larvae or adults. At 1×10^9 conidia/mL all isolates except WG-08 caused >50% mortality in larvae and only two isolates WG-23 and WG-25 caused >50% mortality in adults (Figure 3). Again, no isolate caused 100% mortality neither larval nor adult stage at 14 d post-inoculation. Maximum mortality of 98.5% and 79.3% were reported by isolate WG-25 in larvae and adults, respectively (1×10^9 conidia/mL) (Figure 3). After 21 d post-treatment, WG-23 and WG-25 caused 100% mortality of larvae at the two highest spore concentrations (1×10^8 and 1×10^9 conidia/mL) while WG-25 caused 100% mortality of adults at 1×10^9 conidia/mL (Figure 3).

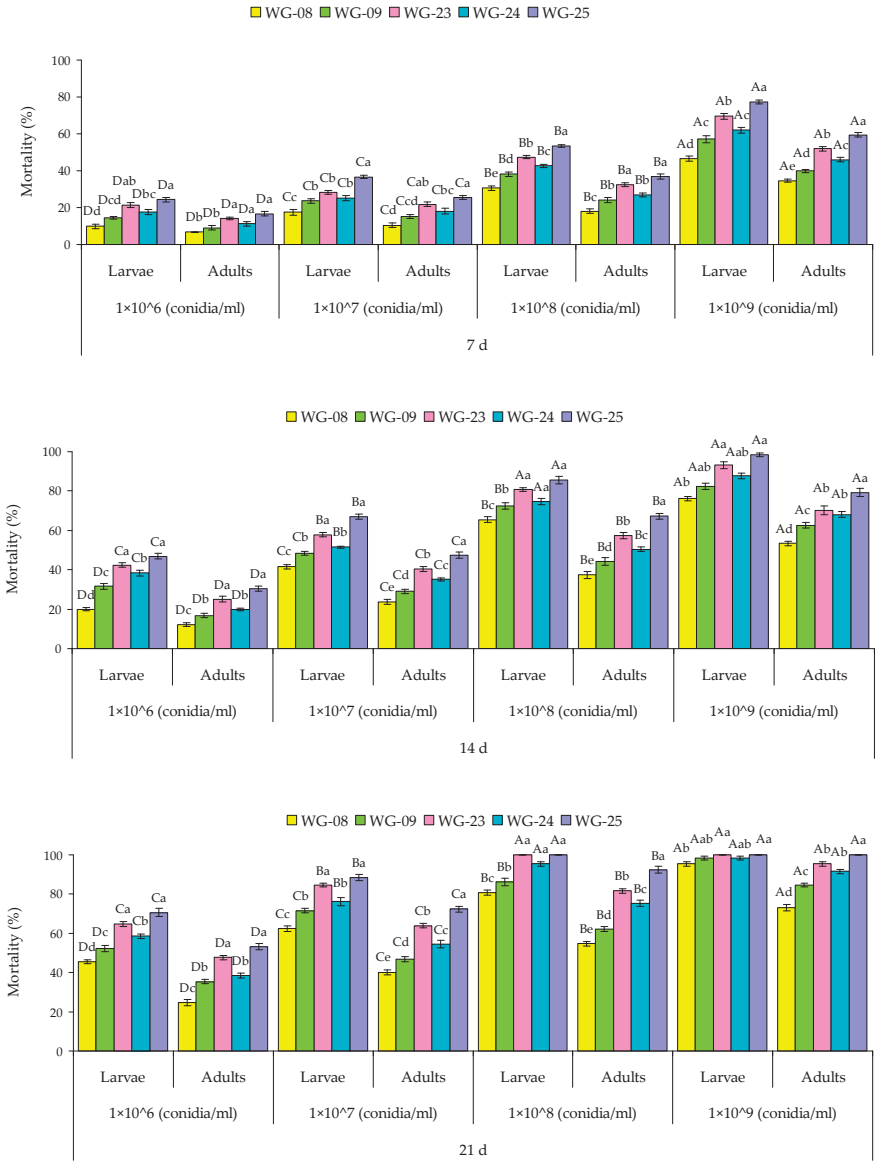

Figure 3. Mean mortality (% ± SE) of larvae and adults of *Rhynchophorus ferrugineus* 7, 14 and 21 d post-treatment with selected isolates of entomopathogenic fungi. Per exposure and life stage, within each concentration (7 d, larvae $F = 36.04$; df = 4, 44; $p < 0.01$ for 1×10^6 conidia/mL, $F = 39.70$; df = 4, 44; $p < 0.01$ for 1×10^7 conidia/mL, $F = 53.24$; df = 4, 44; $p < 0.01$ for 1×10^8 conidia/mL, and $F = 97.07$; df = 4, 44; $p < 0.01$ for 1×10^{10} conidia/mL; adults $F = 31.84$; df = 4, 44; $p < 0.01$ for 1×10^6 conidia/mL, $F = 37.28$; df = 4, 44; $p < 0.01$ for 1×10^7 conidia/mL, $F = 93.15$; df = 4, 44; $p < 0.01$ for 1×10^8 conidia/mL, and $F = 110.91$; df = 4, 44; $p < 0.01$ for 1×10^{10} conidia/mL; 14 d, larvae $F = 26.08$; df = 4, 44; $p < 0.01$ for 1×10^6 conidia/mL, $F = 77.45$; df = 4, 44; $p < 0.01$ for 1×10^7 conidia/mL, $F = 72.29$; df = 4, 44; $p < 0.01$ for 1×10^8 conidia/mL, and $F = 93.30$; df = 4, 44; $p < 0.01$ for 1×10^{10} conidia/mL; adults $F = 54.27$; df = 4, 44; $p < 0.01$ for 1×10^6 conidia/mL, $F = 66.05$; df = 4, 44; $p < 0.01$ for 1×10^7 conidia/mL, $F = 87.23$; df = 4, 44; $p < 0.01$ for 1×10^8 conidia/mL, and $F = 53.11$; df = 4, 44; $p < 0.01$ for 1×10^{10} conidia/mL; 21 d, larvae $F = 33.94$; df = 4, 44; $p < 0.01$ for 1×10^6 conidia/mL, $F = 48.26$; df = 4, 44; $p < 0.01$ for 1×10^7 conidia/mL, $F = 21.09$; df = 4, 44; $p < 0.01$ for 1×10^8 conidia/mL, and $F = 39.46$; df = 4, 44; $p < 0.01$ for 1×10^{10} conidia/mL; adults $F = 56.31$; df = 4, 44; $p < 0.01$ for 1×10^6 conidia/mL, $F = 31.09$; df = 4, 44; $p < 0.01$ for 1×10^7 conidia/mL, $F = 45.06$; df = 4, 44; $p < 0.01$ for 1×10^8 conidia/mL, and $F = 29.41$; df = 4, 44; $p < 0.01$ for 1×10^{10} conidia/mL), means followed by the same lowercase letter are not significantly

different, respectively, Tukey HSD test at $p = 0.05$. Per exposure and life stage, within each isolate (7 d, larvae $F = 41.46$; df = 3, 35; $p < 0.01$ for WG-08, $F = 54.09$; df = 3, 35; $p < 0.01$ for WG-09, $F = 68.32$; df = 3, 35; $p < 0.01$ for WG-23, $F = 61.29$; df = 3, 35; $p < 0.01$ for WG-24, $F = 88.30$; df = 3, 35; $p < 0.01$ for WG-25; adults $F = 23.34$; df = 3, 35; $p < 0.01$ for WG-08, $F = 29.91$; df = 3, 35; $p < 0.01$ for WG-09, $F = 45.05$; df = 3, 35; $p < 0.01$ for WG-23, $F = 61.29$; df = 3, 35; $p < 0.01$ for WG-24, $F = 88.30$; df = 3, 35; $p < 0.01$ for WG-25; 14 d larvae $F = 39.29$; df = 3, 35; $p < 0.01$ for WG-08, $F = 78.90$; df = 3, 35; $p < 0.01$ for WG-09, $F = 28.42$; df = 3, 35; $p < 0.01$ for WG-23, $F = 30.06$; df = 3, 35; $p < 0.01$ for WG-24, $F = 27.13$; df = 3, 35; $p < 0.01$ for WG-25; adults $F = 33.14$; df = 3, 35; $p < 0.01$ for WG-08, $F = 67.36$; df = 3, 35; $p < 0.01$ for WG-09, $F = 58.19$; df = 3, 35; $p < 0.01$ for WG-23, $F = 36.65$; df = 3, 35; $p < 0.01$ for WG-24, $F = 20.08$; df = 3, 35; $p < 0.01$ for WG-25; 21 d, larvae $F = 21.14$; df = 3, 35; $p < 0.01$ for WG-08, $F = 23.46$; df = 3, 35; $p < 0.01$ for WG-09, $F = 47.27$; df = 3, 35; $p < 0.01$ for WG-23, $F = 30.41$; df = 3, 35; $p < 0.01$ for WG-24, $F = 19.95$; df = 3, 35; $p < 0.01$ for WG-25; adults $F = 100.02$; df = 3, 35; $p < 0.01$ for WG-08, $F = 90.27$; df = 3, 35; $p < 0.01$ for WG-09, $F = 43.15$; df = 3, 35; $p < 0.01$ for WG-23, $F = 79.21$; df = 3, 35; $p < 0.01$ for WG-24, $F = 42.09$; df = 3, 35; $p < 0.01$ for WG-25), means followed by the same uppercase letter are not significantly different, respectively, Tukey HSD test at $p = 0.05$. Control mortality was <3% against both developmental stages and data were not included in analysis.

3.3. Lethal Concentration and Lethal Time

Lethal concentration (LC_{50}) values for all tested isolates ranged from 2.6×10^5–1.7×10^6 conidia/mL for larvae and 8.9×10^5–3.1×10^7 conidia/mL for adults. The most virulent isolate WG-25 caused LC_{50} 2.6×10^5 conidia/mL for larvae and 8.9×10^5 conidia/mL for larvae (Table 1).

Table 1. Lethal concentration (LC_{50}) estimates of five native entomopathogenic fungal isolates tested against *Rhynchophorus ferrugineus* larvae and adults.

Insect Stage	Isolate Code	LC_{50} (conidia/mL)	95% Fiducial Limits	Slope	X^2 (df = 2)	*p*
Larvae	WG-08	1.72×10^6	7.67×10^5–3.14×10^6	0.24 ± 0.02	1.42	0.73
	WG-09	8.25×10^5	3.30×10^5–1.58×10^6	0.25 ± 0.02	3.05	0.81
	WG-23	4.30×10^5	1.81×10^5–7.57×10^5	0.39 ± 0.05	7.19	0.63
	WG-24	4.96×10^5	1.88×10^5–9.70×10^5	0.28 ± 0.03	2.19	0.47
	WG-25	2.61×10^5	8.29×10^4–5.18×10^5	0.38 ± 0.05	5.04	0.68
Adults	WG-08	3.15×10^7	1.66×10^7–5.97×10^7	0.17 ± 0.02	0.27	0.56
	WG-09	8.20×10^6	3.92×10^6–1.50×10^7	0.18 ± 0.02	3.29	0.91
	WG-23	1.36×10^6	5.63×10^5–2.60×10^6	0.23 ± 0.02	1.94	0.61
	WG-24	3.63×10^6	1.80×10^6–6.33×10^6	0.23 ± 0.02	1.40	0.83
	WG-25	8.97×10^5	4.28×10^5–1.54×10^6	0.31 ± 0.03	5.75	0.24

Lethal time (LT_{50}) values for all five isolates ranged from 7.5–22.3 d (WG-08), 5.5–19.5 d (WG-09), 3.6–14.2 d (WG-23), 4.4–17.4 (WG-24), and 3.2–14.2 (WG-25) for larvae. Similarly, LT_{50} values for the tested isolates ranged from 12.2–31.6 d (WG-08), 9.7–25.8 d (WG-09), 7.0–21.4 d (WG-23), 8.2–24.8 d (WG-24), and 5.7–19.5 d (WG-25) for adults, against four different concentrations (Tables 2 and 3).

Table 2. Lethal time (LT_{50}) estimates of five native entomopathogenic fungal isolates tested against *Rhynchophorus ferrugineus* larvae.

Isolate Code	Concentration	LT_{50} (d)	95% Fiducial Limits	Slope	X^2 (df = 2)	*p*
WG-08	10^6	22.28	20.00–26.07	0.07 ± 0.01	0.83	0.36
	10^7	16.52	15.00–18.37	0.08 ± 0.01	0.40	0.71
	10^8	10.93	9.26–12.30	0.09 ± 0.01	2.23	0.53
	10^9	7.54	5.68–8.90	0.12 ± 0.01	0.29	0.47

Table 2. *Cont.*

Isolate Code	Concentration	LT_{50} (d)	95% Fiducial Limits	Slope	X^2 (df = 2)	*p*
WG-09	10^6	19.58	17.63–22.55	0.07 ± 0.01	0.31	0.76
	10^7	14.12	12.64–15.61	0.08 ± 0.01	0.75	0.35
	10^8	8.98	7.00–10.45	0.09 ± 0.01	1.80	0.92
	10^9	5.59	3.28–7.15	0.12 ± 0.01	1.21	0.80
WG-23	10^6	14.28	12.57–15.84	0.08 ± 0.01	0.49	0.44
	10^7	11.71	10.36–12.91	0.07 ± 0.01	0.19	0.28
	10^8	7.52	6.13–8.58	0.16 ± 0.01	5.68	0.68
	10^9	3.64	0.66 -5.39	0.15 ± 0.02	1.35	0.74
WG-24	10^6	17.49	15.81–19.75	0.08 ± 0.01	0.71	0.37
	10^7	13.21	11.78–14.57	0.09 ± 0.01	0.69	0.56
	10^8	8.24	6.63–9.48	0.12 ± 0.01	0.30	0.29
	10^9	4.49	1.75–6.24	0.12 ± 0.01	0.11	0.83
WG-25	10^6	14.28	12.75–15.84	0.08 ± 0.01	0.82	0.49
	10^7	9.67	8.00–10.99	0.10 ± 0.01	0.22	0.75
	10^8	6.50	4.86–7.68	0.16 ± 0.01	3.71	0.19
	10^9	3.24	0.308–4.91	0.20 ± 0.04	0.14	0.38

Table 3. Lethal time (LT_{50}) estimates of five native entomopathogenic fungal isolates tested against *Rhynchophorus ferrugineus* adults.

Isolate Code	Concentration	LT_{50} (d)	95% Fiducial Limits	Slope	X^2 (df = 2)	*p*
WG-08	10^6	31.65	26.03–46.38	0.05 ± 0.01	0.28	0.45
	10^7	23.63	20.84–28.80	0.06 ± 0.01	0.08	0.16
	10^8	18.46	16.59–21.19	0.07 ± 0.01	0.36	0.37
	10^9	12.18	10.10–13.92	0.07 ± 0.01	0.61	0.62
WG-09	10^6	25.81	22.48–32.43	0.06 ± 0.01	0.28	0.43
	10^7	21.35	18.96–25.50	0.06 ± 0.01	0.47	0.51
	10^8	15.91	14.17–18.00	0.07 ± 0.01	0.17	0.73
	10^9	9.76	7.73–11.28	0.09 ± 0.01	0.15	0.28
WG-23	10^6	21.40	19.17–25.09	0.07 ± 0.01	0.26	0.56
	10^7	16.25	14.67–18.17	0.08 ± 0.01	0.52	0.67
	10^8	11.56	9.97–12.92	0.09 ± 0.01	0.85	0.25
	10^9	7.07	4.81–8.64	0.10 ± 0.01	3.77	0.92
WG-24	10^6	24.85	21.65–31.16	0.06 ± 0.01	0.25	0.58
	10^7	18.73	16.84–21.52	0.07 ± 0.01	0.63	0.24
	10^8	13.34	11.86–14.75	0.09 ± 0.01	0.32	0.69
	10^9	8.24	6.16–9.75	0.10 ± 0.01	1.22	0.47
WG-25	10^6	19.58	17.63–22.57	0.07 ± 0.01	0.12	0.84
	10^7	14.11	12.63–15.60	0.08 ± 0.01	0.44	0.99
	10^8	9.78	8.35–10.96	0.12 ± 0.01	0.61	0.68
	10^9	5.74	3.49–7.25	0.13 ± 0.01	10.57	0.71

3.4. Bioassay against Eggs

All tested isolates were virulent against *R. ferrugineus* eggs. Hatching (%) was significantly lower in treated eggs compared to non-treated eggs. Egg hatching was directly correlated with application rate as hatching (%) ranged from 49.6–75.5% and 18.5–46.6% at 1×10^6 and 1×10^8 conidia/mL, respectively (Table 4). Minimum hatching was reported with WG-25, 49.6% and 18.5% using 1×10^6 and 1×10^8 conidia/mL, respectively. The lowest AST was observed in WG-25 treated eggs, at 1.7 and 0.5 d at 1×10^6 and 1×10^8 conidia/mL, respectively, and compared to the controls. The controls normally had

longer larval periods and control insects were not included in the interpretation of data (Table 4).

Table 4. Egg hatching (% ± SE) and Average Survival Time (AST) (in days) for larvae which had been exposed to five selected entomopathogenic fungi isolates at two concentration rates. Means followed by the same lowercase letter in each column are not significantly different, Tukey HSD test at $p = 0.05$. Dashes (-) mean that all controls survived after five days and data were not included in analysis.

Isolate Code	Hatching (%)		Average Survival Time (AST)	
	1×10^6 conidia/mL	1×10^8 conidia/mL	1×10^6 conidia/mL	1×10^8 conidia/mL
WG-08	75.55 ± 1.92 b	46.67 ± 1.92 b	3.02 ± 0.06 a	1.51 ± 0.05 a
WG-09	68.14 ± 1.85 b	42.96 ± 1.17 b	2.55 ± 0.07 b	1.20 ± 0.09 ab
WG-23	54.07 ± 1.73 c	27.40 ± 1.33 d	1.93 ± 0.08 cd	0.73 ± 0.08 cd
WG-24	61.48 ± 1.48 b	35.55 ± 1.11 c	2.11 ± 0.07 c	1.02 ± 0.08 bc
WG-25	49.63 ± 2.25 c	18.51 ± 0.97 e	1.71 ± 0.06 d	0.55 ± 0.06 d
Control	78.51 ± 0.97 a	72.52 ± 0.74 a	-	-
df	5, 53	5, 53	4, 44	4, 44
F	43.8	219	52.7	22.6
p	<0.01	<0.01	<0.01	<0.01

3.5. Horizontal Transmission of Fungal Isolates among Adults and Effectiveness of Sublethal Concentrations on Reproductive Stages of RPW

The results of horizontal transmission showed that mortality in non-treated pairs was significantly lower than in treated pairs. Isolate type and sex of insects significantly affected the results. For infected pairs, mortalities were 11.1–61.6% for females and 13.3–78.8% for males. Maximum mortality was observed with WG-25 against females (45.5–61.6%) and males (46.6–78.8%) (Table 5). Mortality was always observed to be less than 5% among all the non-infected pairs, not included in the analysis. Significant reductions in oviposition, fecundity, egg hatching and larval survival was observed when compared to the control (non-infected female) and were also dependent on the isolate and type of pair combination. The combination of infected females with infected males was highly significant when compared to other combinations. The results indicate that WG-25 was the most virulent isolate followed by WG-23, WG-24, WG-09 and WG-08 (Table 6).

Table 5. Mortality (% ± SE) of *Rhynchophorus ferrugineus* pairs with five selected isolates of *Beauveria bassiana* and *Metarhizium anisopliae*. Means followed by same lowercase letter for each isolate in columns were not significantly different, df = 2, 26; Tukey HSD test at $p = 0.05$.

Isolate Code	Treatment		Mortality	
	Female	Male	Female	Male
WG-08	Non-infected	Infected	11.11 ± 3.51 a	27.22 ± 3.23 a
	Infected	Non-infected	15.55 ± 2.93 a	13.33 ± 3.33 b
	Infected	Infected	20.00 ± 3.33 a	36.11 ± 2.60 a
F			29.46	21.63
p			<0.01	<0.01
WG-09	Non-infected	Infected	23.33 ± 2.20 b	43.88 ± 3.70 a
	Infected	Non-infected	29.44 ± 4.74 ab	25.00 ± 2.88 b
	Infected	Infected	36.66 ± 2.20 a	47.77 ± 3.23 a
F			33.17	24.35
p			<0.01	<0.01
WG-23	Non-infected	Infected	36.66 ± 3.99 b	54.44 ± 2.93 b
	Infected	Non-infected	48.33 ± 4.24 ab	41.11 ± 3.51 c
	Infected	Infected	53.33 ± 2.88 a	67.77 ± 4.00 a
F			27.36	21.53
p			<0.01	<0.01

Table 5. *Cont.*

Isolate Code	Treatment		Mortality	
	Female	Male	Female	Male
WG-24	Non-infected	Infected	33.88 ± 3.09 a	47.77 ± 3.23 a
	Infected	Non-infected	36.11 ± 4.23 a	33.88 ± 3.09 b
	Infected	Infected	40.55 ± 5.55 a	56.66 ± 4.08 a
F			25.93	32.69
p			<0.01	<0.01
WG-25	Non-infected	Infected	45.55 ± 2.93 b	64.44 ± 4.12 b
	Infected	Non-infected	54.44 ± 2.93 ab	46.66 ± 2.88 c
	Infected	Infected	61.66 ± 1.66 a	78.88 ± 3.41 a
F			35.78	22.53
p			<0.01	<0.01

Table 6. Oviposition rate (eggs per female per day up to 7 d), fecundity (eggs per female up to 28 d), egg hatching (% ± SE) up to 5 d and larval survival (% ± SE) 5 d after hatching of *Rhynchophorus ferrugineus* pairs infected in different combinations with five isolates of *Beauveria bassiana* and *Metarhizium anisopliae* 1×10^5 conidia/mL. Means followed by the same lowercase letter for each isolate in the columns were not significantly different, df = 3, 35; Tukey HSD test at $p = 0.05$.

Isolate Code	Treatment of Pairs		Oviposition Rate	Fecundity	Egg Hatching (%)	Larval Survival
	Female	Male				
WG-08	Control		3.28 ± 0.10 a	61.62 ± 1.68 a	70.37 ± 1.32 a	80.38 ± 0.86 a
	Non-infected	Infected	3.04 ± 0.08 ab	46.22 ± 1.32 b	67.72 ± 1.73 ab	78.85 ± 1.21 ab
	Infected	Non-infected	2.86 ± 0.10 b	41.62 ± 1.47 b	64.02 ± 1.39 b	75.27 ± 0.87 bc
	Infected	Infected	2.28 ± 0.11 c	32.26 ± 1.67 c	55.55 ± 1.12 c	71.49 ± 1.06 c
F			14.52	23.18	27.59	33.61
p			<0.01	<0.01	<0.01	<0.01
WG-09	Control		2.93 ± 0.09 a	54.11 ± 1.26 a	68.78 ± 1.15 a	86.27 ± 1.45 a
	Non-infected	Infected	2.51 ± 0.12 b	38.57 ± 1.24 b	57.67 ± 1.24 b	65.99 ± 0.86 b
	Infected	Non-infected	2.08 ± 0.10 c	33.57 ± 0.78 c	53.43 ± 1.05 b	63.31 ± 0.90 bc
	Infected	Infected	1.75 ± 0.09 c	27.02 ± 1.13 d	46.03 ± 1.12 c	58.67 ± 2.82 c
F			18.73	26.31	24.15	29.37
p			<0.01	<0.01	<0.01	<0.01
WG-23	Control		3.20 ± 0.11 a	56.02 ± 0.87 a	73.01 ± 0.79 a	84.12 ± 1.01 a
	Non-infected	Infected	1.24 ± 0.11 b	26.08 ± 1.74 b	35.97 ± 1.15 b	47.00 ± 1.68 b
	Infected	Non-infected	1.01 ± 0.06 bc	20.60 ± 1.06 c	30.15 ± 1.37 c	43.59 ± 2.32 b
	Infected	Infected	0.75 ± 0.05 c	13.00 ± 0.37 d	23.28 ± 2.16 d	41.75 ± 2.99 b
F			21.66	19.27	34.12	38.80
p			<0.01	<0.01	<0.01	<0.01
WG-24	Control		2.86 ± 0.03 a	57.28 ± 1.20 a	64.55 ± 0.83 a	77.83 ± 0.28 a
	Non-infected	Infected	2.17 ± 0.10 b	33.06 ± 0.62 b	46.03 ± 1.37 b	55.08 ± 1.50 b
	Infected	Non-infected	1.73 ± 0.08 c	25.44 ± 0.56 c	44.44 ± 1.12 b	47.77 ± 1.96 c
	Infected	Infected	1.20 ± 0.10 d	18.35 ± 0.48 d	39.68 ± 1.37 c	42.69 ± 1.67 c
F			25.84	31.36	39.04	44.59
p			<0.01	<0.01	<0.01	<0.01
WG-25	Control		3.07 ± 0.07 a	53.55 ± 1.16 a	65.07 ± 0.79 a	81.31 ± 1.23 a
	Non-infected	Infected	1.00 ± 0.07 b	17.13 ± 0.54 b	21.69 ± 0.83 b	33.33 ± 2.63 b
	Infected	Non-infected	0.86 ± 0.06 b	15.04 ± 0.69 b	16.40 ± 0.83 c	29.63 ± 1.46 b
	Infected	Infected	0.57 ± 0.04 c	11.75 ± 0.74 c	11.64 ± 0.83 d	25.92 ± 6.86 b
F			28.01	37.96	46.18	51.06
p			<0.01	<0.01	<0.01	<0.01

3.6. Semi-Field Trials for RPW Survival

Significantly lower palm infestation was observed following treatment with different fungal isolates (F = 17.78; df = 5, 23; $p < 0.01$) compared to the controls. The lowest palm infestation was observed when palms treated with WG-25 (90.50% efficacy). From the non-treated palms, all of them were found to be infested. Statistically no difference was observed between WG-08 and control palms. For palm infestation, significantly lower numbers of larvae were observed from fungus-treated palms (F = 70.57; df = 5, 23; $p < 0.01$) when compared with control palms. Among the different fungal isolates, the lowest number of larvae per palm (4.15) was observed for WG-25-treated palms followed by WG-23 (13.30 larvae per palm) (Table 7).

Table 7. Mean (% ± SE) number of infested palms, larvae of *Rhynchophorus ferrugineus* (number ± SE) and control efficacy when palms were treated with two isolates of *Metarhizium anisopliae* (WG-08 and WG-09) and three isolates of *Beauveria bassiana* (WG-23, WG-24 and WG-25) under semi-field conditions. Means followed by the same lowercase letter in the columns were not significantly different, Duncan test at p = 0.05. Dashes (-) mean that data for controls were not included in analysis and run after Abbott's formula [56].

Treatments	Palm Infested	Number of Larvae	Efficacy (%)
WG-08	80.00 ± 0.00 ab	36.05 ± 1.75 a	16.91 ± 7.47 d
WG-09	65.00 ± 9.57 bc	27.15 ± 1.94 b	37.99 ± 3.86 c
WG-23	40.00 ± 8.16 cd	13.30 ± 2.08 c	69.38 ± 5.13 b
WG-24	55.00 ± 5.00 bcd	19.45 ± 1.32 bc	55.28 ± 3.93 bc
WG-25	35.00 ± 9.57 d	4.15 ± 0.95 d	90.50 ± 2.14 a
Control	100.00 ± 0.00 a	43.85 ± 1.90 a	-
df	5, 119	5, 119	4, 99
F	17.78	70.57	43.33
p	<0.01	<0.01	<0.01

3.7. Fungal Persistence over Time and Its Effectiveness against RPW Larvae

Significant differences among all the fungal isolates (F = 70.57; df = 5, 23; $p < 0.01$) on the basis of the total number of larvae captured, with a maximum efficacy (82.21%) observed in WG-25 at 1 d post-insect release. At 15 d post-insect release, fungi persisted in a positive manner with lowest palm infestation (20.00%) and with maximum efficacy (85.77%) observed for the WG-25 treatment. From 30 to 60 d, efficacy exhibited a decreasing trend with a maximum treatment efficacy (57.65%) observed for WG-25 at 60 d. The lowest palm infestation for all the intervals was observed when using WG-23 and WG-25 (Table 8).

Table 8. Mean (% ± SE) infested palms, larvae (number ± SE) and treatment efficacy when palms were treated with two isolates of *Metarhizium anisopliae* (WG-08 and WG-09) and three isolates of *Beauveria bassiana* (WG-23, WG-24 and WG-25). Adults were released over time intervals (1, 15, 30, 45 and 60 d) under semi-field setting. Within each interval, means followed by the same lowercase letter in the columns were not significantly different, Duncan test at p = 0.05. Dashes (-) mean that data for controls were not included in analysis and run after Abbott's formula [56].

Interval (d)	Treatments	Palm Infest	Number of Larvae	Efficacy (%)
1	WG-08	100.00 ± 0.00 a	37.15 ± 2.01 b	27.62 ± 4.38 d
	WG-09	80.00 ± 8.16 ab	32.05 ± 2.61 bc	37.92 ± 3.66 cd
	WG-23	45.00 ± 15.00 bc	17.75 ± 2.20 d	65.69 ± 3.62 ab
	WG-24	60.00 ± 8.16 bc	25.00 ± 1.87 cd	51.06 ± 4.69 bc
	WG-25	30.00 ± 10.00 c	9.10 ± 0.69 e	82.21 ± 1.64 a
	Control	100.00 ± 0.00 a	51.45 ± 1.57 a	-
	df	5, 119	5, 119	4, 99
	F	12.30	65.40	32.71
	p	<0.01	<0.01	<0.01

Table 8. *Cont.*

Interval (d)	Treatments	Palm Infest	Number of Larvae	Efficacy (%)
15	WG-08	100.00 ± 0.00 a	31.70 ± 1.53 b	34.10 ± 5.45 d
	WG-09	85.00 ± 9.57 ab	26.65 ± 2.29 bc	44.89 ± 5.06 cd
	WG-23	30.00 ± 5.77 c	12.75 ± 2.35 de	72.84 ± 6.15 ab
	WG-24	50.00 ± 12.91 bc	19.50 ± 1.29 cd	59.78 ± 2.55 bc
	WG-25	20.00 ± 8.16 c	6.90 ± 0.38 e	85.77 ± 0.76 a
	Control	100.00 ± 0.00 a	48.75 ± 2.98 a	-
	df	5, 119	5, 119	4, 99
	F	18.43	55.17	37.52
	p	<0.01	<0.01	<0.01
30	WG-08	100.00 ± 0.00 a	38.50 ± 2.72 ab	22.16 ± 8.27 c
	WG-09	85.00 ± 9.57 a	29.25 ± 2.80 bc	41.52 ± 5.08 bc
	WG-23	30.00 ± 12.91 b	20.00 ± 3.17 cd	59.03 ± 8.05 ab
	WG-24	80.00 ± 8.16 a	25.50 ± 3.09 cd	49.54 ± 4.14 ab
	WG-25	40.00 ± 8.16 b	13.85 ± 1.15 d	72.27 ± 2.28 a
	Control	100.00 ± 0.00 a	50.05 ± 2.12 a	-
	df	5, 119	5, 119	4, 99
	F	12.95	23.57	10.81
	p	<0.01	<0.01	<0.01
45	WG-08	100.00 ± 0.00 a	42.30 ± 3.18 ab	18.68 ± 8.55 c
	WG-09	100.00 ± 0.00 a	36.10 ± 3.51 bc	30.18 ± 9.85 bc
	WG-23	80.00 ± 8.16 ab	24.00 ± 2.79 cd	53.55 ± 7.45 ab
	WG-24	100.00 ± 0.00 a	34.45 ± 2.25 bc	34.02 ± 5.44 bc
	WG-25	60.00 ± 14.14 b	18.50 ± 3.05 d	63.88 ± 7.54 a
	Control	100.00 ± 0.00 a	52.70 ± 2.77 a	-
	df	5, 119	5, 119	4, 99
	F	6.63	15.51	11.16
	p	<0.01	<0.01	<0.01
60	WG-08	100.00 ± 0.00 a	45.50 ± 3.61 ab	14.65 ± 7.23 c
	WG-09	100.00 ± 0.00 a	43.40 ± 3.60 ab	18.34 ± 7.96 c
	WG-23	80.00 ± 8.16 b	29.65 ± 2.69 cd	43.90 ± 7.08 ab
	WG-24	100.00 ± 0.00 a	37.80 ± 3.68 bc	29.27 ± 7.04 bc
	WG-25	60.00 ± 8.16 c	22.45 ± 2.31 d	57.65 ± 5.47 a
	Control	100.00 ± 0.00 a	53.55 ± 1.99 a	-
	5, 119	5, 119	4, 99	df
	F	15.75	14.44	10.47
	p	<0.01	<0.01	<0.01

4. Discussion

Exotic fungal isolates previously deployed against a variety of insect pests in different countries have often been unsatisfactory due to factors such as differences between hosts, isolates and climatic conditions [59,60]. Noting this constraint in the present study, we used native 15 EPF isolates of different developmental stages of *R. ferrugineus*. Prior to use under field conditions, the most important aspect is to identify highly virulent isolates through laboratory screening bioassays [61]. Results of our screening assay showed that all isolates were pathogenic to *R. ferrugineus* and further supported the findings of Sun et al. and Yang et al. [61,62] who worked with different *B. bassiana* and *M. anisopliae* isolates. Although, *R. ferrugineus* was susceptible to all isolates, the isolates varied in their virulence. This is not an unusual phenomenon as Serna-Domínguez et al. Qayyum et al. and Ullah et al. [63–65] noted variation in virulence and attributed it to genetic variations among different isolates from specific geographical origins.

The current study did not support the hypothesis that virulent isolates must be isolated from the target or closely related organisms [66,67]. Five isolates (WG-08, WG-09, WG-23, WG-24 and WG-25) that we used in virulence assay were of *B. bassiana* and *M. anisopliae*, but none was originally isolated from *R. ferrugineus* and instead they originated from

stored-grain insect pests and soil samples. Several studies [68–70] favored the concept that pathogens isolated from *R. ferrugineus* were more virulent to this invasive pest compared to non-host isolates. However, our result aligns with the findings of Liu et al. [71] who evaluated different fungal isolates against tarnished plant bug *Lygus lineolaris* (Palisot de Beauvois) (Hemiptera: Miridae) and found that the *M. anisopliae* isolate ARSEF 3540, originating from soil, was a suitable biological control agent for the mirid. Similar results were described by Qayyum et al. [72], who found that a virulent isolate of *B. bassiana* recovered from soil, caused >80% mortality. Although we did not use any isolates recovered from *R. ferrugineus*, our results indicate that strains obtained from a variety of media or non-hosts have potential to manage *R. ferrugineus* populations.

Mycosed cadavers play a key role in regulating the pest population by producing conidia, which may cause secondary infection and increase the persistence of the pathogen for a longer period [73]. We observed that some cadavers did not show any fungal outgrowth, which was similar to the studies by [74] in which no fungal outgrowth on the surface of *Lycorma delicatula* (White) (Hemiptera: Fulgoridae) living insects was observed and rarely from cadavers; however, the growth appeared when incubated under humid conditions in the laboratory. Due to their concealed nature, and suitable humid condition inside the palm tree, mycosed cadavers may play a significant role for secondary infection of *R. ferrugineus.*

Developmental stages of insects differ in their susceptibility towards different strains of EPF [75], and this was also noted in our study for *R. ferrugineus* larvae and adults. Both screening and virulence assays showed that larvae of *R. ferrugineus* were more susceptible than adults. Our results were similar to the findings of Dembilio et al., Francardi et al., Gindin et al., Güerri-Agulló et al., and Lo Verde et al. [22,51,53,68,76] in that adults were less susceptible than larvae to the fungal treatments. Güerri-Agulló et al. [76] claimed that the soft cuticle of larvae is more vulnerable to fungi than that of adults. During another investigation, Ansari and Butt [77] found that pine weevil *Hylobius abietis* (L.) (Coleoptera: Curculionidae) larvae and pupae were more susceptible than adults when treated with *B. bassiana* and *M. anisoplaie.* The reason behind the low susceptibility of adults compared to larvae is generally attributed to differences in biochemical composition of cuticle and the immune system of both stages [78], while it is suggested that during molting the shedding of the larva may get rid of infectious conidia [79]. Based on the current findings, to control *R. ferrugineus* the larval stage seems to be the best one to target, but keeping in mind their cryptic nature, it is essential to develop an effective method to infect the *R. ferrugineus* larvae with fungi.

In the screening assay, we identified five isolates with potential for development as biological control agents, three *B. bassiana* and two *M. anisopliae*. *Beauveria bassiana* performed well with higher mortality compared to *M. anisopliae* against different insect stages (larvae and adults) of *R. ferrugineus* irrespective of isolate origin (soil or insect cadaver). Lo Verde et al. [53] tested different isolates of EPF obtained from different stages of *R. ferrugineus* and found that *B. bassiana* was more effective than other fungi tested. El Kichaou et al. [80] found mortality in larvae of *R. ferrugineus* of 100% and 90% at six days post-inoculation with *B. bassiana* and *M. anisopliae*, respectively. Cherry et al. [81] tested 12 isolates of *M. anisopliae* and *B. bassiana* against cowpea weevil *Callosobruchus maculatus* (Fabricius) (Coleoptera: Chrysomelidae) and amongst the two highly effective isolates (*B. bassiana* 0362 and *M. anisopliae* 0351), *B. bassiana* 0362 was consistently more virulent against this insect. Contrary to our results Gindin et al. [68] and Francardi et al. [22] identified *M. anisopliae* as more virulent against *R. ferrugineus* compared with *B. bassiana*. We conclude that both species have considerable potential to control larvae and adults of *R. ferrugineus* with *B. bassiana* generally exhibiting higher virulence compared to *M. anisopliae*.

When eggs of *R. ferrugineus* were treated with fungi, the eggs showed a level of tolerance to infection. Previously, Gindin et al. [68] tested *M. anisopliae* against eggs of *R. ferrugineus* and obtained 43.5–80% egg mortality. Dembilio et al. [51] observed that *B. bassiana* caused a significant reduction in egg hatching compared with controls but concluded that eggs were less susceptible than 4th instar larvae, while more susceptible

than adults [51]. In our study, neonates that emerged from eggs did not survive more than three days and this was in accordance with the findings of Dembilio et al. [82], Gindin et al. [68], and Lo Verde et al. [53]. The reason behind the low average survival rate of neonates could be the inability of fungi to infect the eggs due to the presence of chorion [33] and, consequently, they can infect the neonates. Under field conditions, damaged parts of the palm are where females are attracted for egg laying; therefore, treatment of these parts with fungi may increase the death of emerged neonates resulting in reduction of *R. ferrugineus* populations.

Several studies have confirmed the efficacy of various isolates of EPF against different developmental stages of *R. ferrugineus* [28,61,62,72,83,84]. In the field, larvae due to their cryptic nature [85], are difficult to infect with EPF. Only adults are mobile and able to disperse, remaining alive for a few days after becoming infected [76]. During infection, adults have conidia on their surface, and thus horizontal transmission between adults and its effect on subsequent progeny has been investigated [38,51,86]. Horizontal transmission of *B. bassiana* and *M. anisopliae* against different insect pests has been studied previously [36,86–89]. Mortality in the present study after 28 d post-infection confirmed the findings of Gindin et al. [68] and Dembilio et al. [51], which indicated that *R. ferrugineus* can transmit the pathogen from infected to normal adults. We found that the mortality was comparatively high in the case of mating with infected adult males which agrees with studies by Quesada-Moraga et al. [38] and Dembilio et al. [51]. According to Quesada-Moraga et al. [90], when adult males of the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) were infected by inoculated adult females with dry conidia of the *M. anisopliae* isolate EAMa 01/58-Su, 100% mortality was obtained. Wai et al. [91] concluded that mated female *R. ferrugineus* consume much of their energy during their reproductive cycle. Consequently, they remain with less potential to survive in comparison to virgin females. The reason behind this observation may be because of the large number of conidia received by males from females and their mating behavior plays an important role. Contradictory to our results, Kaaya and Okech [92] reported that infected female tsetse flies, *Glossina morsitans* Wiedemann (Diptera: Glossinidae), caused higher mortality compared to males because the fly males are smaller in size and more susceptible compared to females. In another study, Quesada-Moraga et al. [88] found that mortality of the German cockroach *Blattella germanica* L. (Blattodea: Ectobiidae) was uniform irrespective of sex. The present study indicated that along with mortality against adults, fungal infection also reduced oviposition rate, fecundity and egg hatching. Dembilio et al. [51] found significant reduction in oviposition and fecundity against *R. ferrugienus* when treated with *B. bassiana* at a concentration rate of 1.5×10^9 conidia/mL. There was up to 70% reduction in fecundity when Western corn root worm *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae) was infected with *B. bassiana* [93]. Castillo et al. [94] observed reductions in fecundity of *C. capitata* of up to 65% and 40–50% when treated with *M. anisopliae* and *P. fumosoroseus*, respectively. The reduction in oviposition we observed was in line with Meadow et al. [95], who showed that reduced oviposition was related to insect pathogens that weaken the female. Along with oviposition, infection also reduced fecundity. Fragues et al. [96] observed significant reductions in fecundity when Colorado potato beetle, *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae), was treated with *B. bassiana*. Sikura et al. [97] determined that *B. bassiana* induced histological and cytological types of injuries in the ovaries of *L. decemlineata*, which prevents follicle development and caused reductions in fecundity.

In semi-field survival and persistence trials, all of the tested EPF isolates significantly reduced *R. ferrugineus* larval survival with efficacy of WG-25 being as high as 90% compared to the control. In the persistence bioassay, although persistence decreased with the passage of time, significant levels of control were achieved even after 60 d post application. Similar findings were observed by Dembilio et al. [51], indicating that EPF persisted in palm plants over 45 d.

5. Conclusions

The present study showed that a range of EPF isolates have considerable potential against certain life stages of *R. ferrugineus*. The larval stage was found to be the most susceptible stage, due to its cryptic nature and the high humidity inside the trunk that favor the infection process. But a major constraint is how these microbial agents can be delivered to larvae within the palm trunk, and so further research is needed on application methods. Adults are the only freely mobile stage, able to horizontally transmit fungi to members of their colonies and are attracted to pheromone traps. Fungus treated traps could be developed to attract and infect adults which could in turn infect other adults during mating and also infect the immature stages. Eggs were also found to be susceptible to fungi, hence cut or injured parts of the palms suitable for egg laying could be treated with EPF to reduce the pest population. There is a need for field-oriented studies to assess the efficacy of fungal isolates in different climatic conditions. The impact of temperature, humidity and post-application persistence of these fungal isolates should be tested to identify the optimum conditions for infection of the target insect pests. These virulent fungal isolates may be helpful for developing a sustainable integrated management program of the red palm weevil in date palm production systems.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy14040642/s1, Table S1: Details of native isolates of entomopathogenic fungal isolates obtained from soil samples and insect cadavers in Pakistan, Table S2: Factorial analysis for screening of fifteen fungal isolates of entomopathogenic fungi and mycosis in larvae and adults of *Rhynchophorus ferrugineus*, Table S3: Factorial analysis for virulence of five selected isolates of entomopathogenic fungi against larvae and adults of *Rhynchophorus ferrugineus.*

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Data Availability Statement: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Biocontrol Effect of *Bacillus subtilis* against *Cnaphalocrocis medinalis* (Guenèe) (Lepidoptera: Pyralidae): A Sustainable Approach to Rice Pest Management

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Abstract: Agricultural pests can be effectively controlled using microbes, providing an eco-friendly alternative to available synthetic pesticides. Suitable entomopathogenic bacterial strains were collected from agricultural fields and evaluated for their insecticidal potential against *Cnaphalocrocis medinalis*. In the four tested entomopathogenic bacteria (W1, Yc1, S1, EB01), the larval mortality ranged from 38 to 74%. Among these isolates, *Bacillus subtilis* (EB01) induced the highest mortality (74%). In greenhouse conditions, the tests confirm that the results were dosage-dependent: *B. subtilis* infection considerably delayed the overall development period, reduced pupal conversion, and decreased adult emergence with induced morphological deformities. Larvae fed *B. subtilis*-treated leaves initiate bacterial infection and broadly damage the midgut tissue, including the epithelial and peritrophic layers. The bacterial growth in the *C. medinalis* hemolymph considerably increases the activity of enzymes like α and β esterase (85.14 and 44% at 96 h) compared to the control. The isolate *B. subtilis*-treated diet significantly reduced the larval digestive α and β galactosidase enzyme activity (88.17 and 91.88% at 96 h). Furthermore, germination bioassay with strain EB01 in rice varieties (TN1 and ASD16) significantly increased both varieties' germination and biomass index. This study shows that the *B. subtilis* EB01 strain potentially inhibited the biological activity of *C. medinalis* and improved the rice seeds' germination index. It can be a potential biocontrol agent in sustainable pest-management strategies.

Keywords: entomopathogenic bacteria; insecticidal activity; leaf folder; enzyme analysis; seed emergence; paddy development

1. Introduction

Oryza sativa L. is a stable food for more than 3.5 billion people globally, particularly in Asia, where 90% of people consume rice due to its high-energy constituents [1]. However, in commercial large-scale production, rice quality and productivity are adversely affected by more pests. Different stages of pests damage the rice crops through their survival and developmental activity [2,3]. The yellow stem borer, plant hoppers, rice leaf folder, rice ear bug, etc., are potential threats that damage paddy fields [4]. Among these pests, *Cnaphalocrocis medinalis* (Guenee) Lepidoptera: Pyralidae (rice leaf folder) is a predominant foliage feeder, one of the essential pests causing agricultural loss and affecting the overall rice ecosystem in India. It is vital in pest management, since they scrap the leaf chlorophyll, leading to considerable yield losses and other saprophytic infections [5]. *Cnaphalocrocis medinalis* larvae longitudinally fold the leaves by connecting the leaf margins and scraping the

mesophyll tissue within the leaves, reducing the photosynthesis rate and leading to defects in plants' vigor and development [6]. An increasing population of rice leaf folders could complicate rice cultivation and increase the usage of synthetic pesticides [7]. Synthetic pesticides widely contribute to the commercial production of crops by inhibiting economically harmful pests, which are the leading input in recent agriculture practices. Synthetic pesticides are cheap, fast-acting, readily available for farmers, and effective against various pests [8]. For the control of the rice leaf folder, several pesticides from various synthetic categories are used [9]. Conventional synthetic pesticides against *C. medinalis* include cypermethrin, monocrotophos, fipronil, imidacloprid, and dimethoate [9,10]. However, frequent usage of these insecticides also directly and indirectly impacts the environment, non-targeted organisms, and human health [11]. In the current situation, some insects are developing resistance against synthetic insecticides, which is a significant challenge [12]. In such circumstances, the efficacy of biopesticide usage has emerged as a sustainable alternative source for controlling harmful pests. The revolution of biopesticides has focused on target pests along with agricultural production without damaging plant development [13]. Microbial biopesticides can be an effective, eco-friendly method for sustainable agriculture. Unlike chemical pesticides, the microbial biopesticides are specific in action, low cast, and are environmentally sustainable without residual effects. However, the time of application, maintenance, and temperature are the few drawbacks of biopesticides [14].

Some reports have found that some bacterial species isolated from various insects have impacts on harmful pests, and they are possible sources of entomopathogenic agents [15,16]. Entomopathogens, including *Bacillus* and *Pseudomonas* spp. are widely distributed in the environment and have virulence effects against major pests [17,18]. Entomopathogens such as *B. thuringiensis* can produce toxins, which target the lepidopteran larval species and are commercially exploited for pest control [19]. Similarly, some entomopathogens from *Pseudomonas*, *Enterobacter*, and *Bacillus* spp. produce a variety of toxins that suppress the metabolic activity of insects and induce mortality in lepidopterans, coleopterans, hymenopterans, and dipterans [20]. The present work was carried out with the aim of evaluating the insecticidal potential of entomopathogenic bacteria against *C. medinalis.*

2. Materials and Methods

2.1. Sample Collection

A field survey was conducted in the paddy fields in and around Alwarkurichi, Tenkasi district, to collect the infected and dead *C. medinalis* larvae with morphological abnormalities. Infected insects were transferred immediately to the laboratory, surface sterilized with 70% ethanol for 15 min, flamed, and air-dried in the laminar airflow chamber for 2 min. The outer layers of the larvae were removed without damage to the inner area using sterile scissors and needles. The remaining larval portion was homogenized with a phosphate-buffered solution, and 1 mL of filtered suspension was poured into Luria–Bertani (LB) agar plates. The plates were incubated at 28 $\pm$ 2 °C for 24 h. Bacterial colonies were selected based on the color and morphological appearances and it was subculturing several times to obtain purified culture. After purifications, four different bacterial colonies were selected for the pathogenicity test and denoted as W1, Yc1, S1, and EB01. The bacterial strains were maintained in LB plates and broth.

2.2. Cnaphalocrocis medinalis Culture

Cnaphalocrocis medinalis larvae were collected from the paddy fields around Tirunelveli and Tenkasi, Tamil Nadu, India. In greenhouse conditions, the rice plants were grown in earthenware pots (10 plants for each pot) for insect rearing. The collected larvae were reared in potted rice plants enclosed with mesh sleeves and maintained at 28 $\pm$ 2 °C in a 14 h dark:10 h light cycle with 80% humidity. Adults were maintained in an oviposition cage with potted plants, and the moths were fed with sucrose solution (10%). The complete life cycle of *C. medinalis* culture was maintained by following the method of Senthil-Nathan et al. [21].

2.3. Screening Bioassay

Preculture of bacterial strains (W1, Yc1, S1, and EB01) of 1 mL was inoculated into fresh LB broth and incubated at 28 ± 2 °C for two days. After incubation, each bacterial culture was centrifuged at 5000 rpm at 4 °C for 20 min. The obtained pellet was resuspended in sterile distilled water after being washed with it. The bacterial density was concentrated at 3×10^9 cfu/mL (approximately) using a hemocytometer [22]. To determine the insecticidal potential of bacterial strains, second-stage larvae (10 larvae per treatment) were taken into a laboratory to be cultured. In a greenhouse condition, the rice plants were grown in earthenware pots (10 plants for each pot) for the treated larval culture. The pathogenicity was confirmed by dipping the *C. medinalis* larvae into the 20 mL bacterial suspension from each isolate for 2 s, and the remaining suspension was sprayed into potted rice plants. Then, the bacterial-treated larvae were transferred into the same potted rice plants and were covered with mesh net sleeves. Sterile water was used for the control treatment. Treatments were maintained at 28 ± 2 °C and 80% humidity. Larval mortality was recorded daily for ten days. The bacterial strain EB01, which exhibits severe infection in *C. medinalis* larvae and has the highest mortality rate, was selected for further analysis and molecularly identified using 16s rRNA gene sequencing.

2.4. Preparation of EB01 Bacterial Suspension

The bacterial strain EB01 was prepared in two ways (active form and cell-free extract). For the live bacterial cells, four different concentrations, i.e., 1.5×10^3 cfu/mL, 2×10^5 cfu/mL, 3.5×10^7 cfu/mL, and 5×10^9 cfu/mL, were prepared. For the cell-free extracts, 1 mL of preculture EB01 strain was added to the 100 mL LB broth and incubated for seven days [23]. Simultaneously, control treatments were prepared with uninoculated broth. After centrifugation, the obtained supernatant was lyophilized and extracted using methanol (100%). The dried extracts were redissolved with methanol and concentrated with 0.1 g/mL and 1 g/mL.

2.5. Concentration-Dependent Mortality and Development Bioassay

In total, eight treatments were carried out in this experiment: T1 = 1.5×10^3 cfu/mL, T2 = 2×10^5 cfu/mL, T3 = 3.5×10^7 cfu/mL, T4 = 5×10^9 cfu/mL, T5 = 0.1 g/mL, T6 = 1 g/mL, T7 = uninoculated broth with methanol (control), and T8 = sterile water (control). Two-day-old second-stage *C. medinalis* larvae were allowed to starve for 1 h for analysis of their food intake and treatment response. Eighty-day-old rice plants were separately treated with 20 mL of different concentrations of bacterial dilution or cell-free extracts using a sprayer (Kisan Kraft—PS8000, Bangalore, India). Control treatment plants were treated with water or methanol. After 2 h, 20 larvae were transferred into treated potted rice plants covered with mesh net sleeves for each treatment. All the treatments were replicated five times. After treatment, the dead larvae were analyzed for morphological changes due to bacterial infection. Larval mortality was recorded every day. Larval duration, pupal conversion, pupal deformities, pupal duration, adult emergence, and adult deformities were recorded. Afterwards, the experiment was conducted with the surviving moths. Five treatments were carried out in this part: T (moths emerged from bacterial treatments), T5 (from cell-free extracts, 0.1 g/mL), T6 (from cell-free extracts, 1 g/mL), T7 (uninoculated broth with methanol), and T8 (sterile water), and the moths were transferred into an oviposition cage to be reared with normal moths of the opposite sex, and fed with 10% sucrose solution. Potted rice plants were placed in every treatment cage. The adult longevity, total number of eggs laid by female moths, and hatchability were recorded.

2.6. Enzyme Activity

The effect of bacterial EB01 infection on detoxification enzymes of *C. medinalis* was assessed by treating the third-stage larvae (12 days old) of the insect with a 5×10^9 cfu/mL bacterial concentration for 24, 48, 72, and 96 h. For the enzyme extraction, the treated larvae were randomly selected and were homogenized in ice with phosphate buffer (0.1 M)

containing EDTA (1 mM), DTT (1 mM), PTU (1 mM), PMSF (1 mM), and glycerol (20%). The homogenate mixture was centrifuged for 10 min at 15,000 rpm at 4 °C [24]. To measure α and β carboxylesterase activity, 20 µL extract was added to the 500 µL 0.1 M phosphate potassium buffer (0.3 mM α or β-naphthyl acetate and 1% acetone). Fast blue B (0.3%) and SDS (3.3%) were added to the reaction mixture. The reaction mixture was incubated at 29 ± 1 °C for 20 min and centrifuged. The obtained supernatant absorbance was recorded at 590 nm, generated as 1 µmol of α or β-naphthol per minute [25].

The effect of bacterial EB01 infection on digestive enzymes of *C. medinalis* was assessed by treating the fourth-stage larvae (14 days old) of the insect with 5×10^9 cfu/mL bacterial concentration for 24, 48, 72, and 96 h. Larvae were treated with sterile water for the control. Larval guts were homogenized, and 20 µL of enzyme extract was added with 50 µL of 5 mM p-nitrophenyl-α-D-galactopyranoside or p-nitrophenyl-β-D-galactopyranoside and 200 µL phosphate buffer. The reaction mixture was incubated for 10 min at 37 °C [26]. The reaction was terminated with 160 µL of 1 M sodium carbonate [27]. The absorbance was recorded at 450 nm.

2.7. Histological Analysis

The effect of EB01 infection on *C. medinalis* larvae was investigated via histology of the midgut tissue. The control and increased bacterial concentration-treated larvae were taken from the culture (10 days old from the post-treatment). The larvae were dissected aseptically, and the extracted guts were kept in 5% formalin [28]. The guts were washed with sterile water several times, and dehydration of tissue was performed using alcohol concentrations from 50 to 100%. Paraffin wax was used for tissue fixation, and a microtome (Leica, Nussloch, Germany) made tiny blocks from the embedded wax. These tiny sections were placed in a slide coated with 1% Mayer's egg albumin and kept on a hot plate at 40 °C. The slides were dewaxed using Xylene for 5 min and rehydrated with 100, 90, 80, 70, and 60% ethanol concentrations. Hematoxylin and eosin were used to stain. Then, the slides were rinsed once with 100% alcohol and twice with Xylene [29]. The observation was made using a microscope, (Nikon, Tokyo, Japan), and images were captured by connecting the microscope to the computer.

2.8. In Vitro Seed Treatments

The susceptible and moderately resistant rice varieties TN1 and ASD16 were chosen for this study, and whether the entomopathogenic bacterial strain EB01 could have any adverse effect on rice plants was analyzed. Seeds were surface-sterilized with sodium hypochlorite solution (2%), washed with sterile distilled water several times, and dried with filter paper. The bacterial culture was prepared as described above and concentrated at 1.5×10^3 cfu/mL, 2×10^5 cfu/mL, 3.5×10^7 cfu/mL, and 5×10^9 cfu/mL. For control treatments, sterile distilled water was used. Twenty seeds were taken for each experiment and were soaked with the respective bacterial concentrations for 24 h. Seed emergence was analyzed using the filter paper method for seven days [30].

2.9. In Vivo Seed Treatment under Greenhouse

Before planting, silt loam soil was autoclaved for 25 min and used to fill the pots for the treatments. Rice seeds TN1 and ASD16 were treated with separate bacterial concentrations (1.5×10^3 cfu/mL, 2×10^5 cfu/mL, 3.5×10^7 cfu/mL, and 5×10^9 cfu/mL) and sown in 1 L pots in a greenhouse (5 seeds per treatment). Seeds treated with sterile distilled water were used as a control. All the tests were performed with five replications. Rice plants were maintained for 30 days with water as needed. After 30 days, all the plants were removed from the pots and cleaned with water. The plant height and fresh weight were measured. Healthy and affected leaves were also recorded for every tested plant.

2.10. Statistical Analysis

The experiments, including larval mortality, larval duration, pupal duration, pupal deformities, adult deformities, adult emergence, and enzyme analysis, were replicated five times. The mean values were represented by comparing differences in treatments using the Tukey's family error test ($p < 0.05$) using the Minitab 17.1.0 software package.

3. Results

3.1. Insecticidal Bioassay

Effective bacterial strains were screened using mortality bioassay compared to the control; all the tested strains caused mortality (Figure 1). The EB01 strain induced a higher mortality rate among the isolates (i.e., 74%) and a morphological change in the larvae. As per the molecular analysis, the EB01 bacterial strain was identified as *Bacillus subtilis*, and its GenBank accession number is OQ071610.

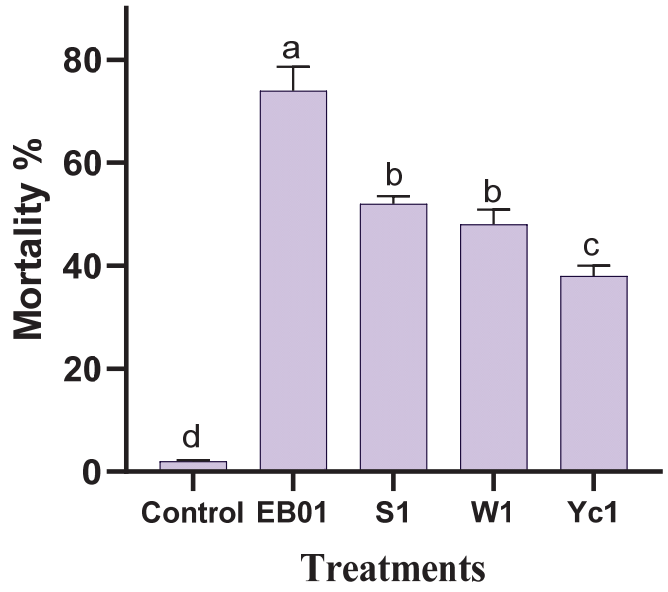

Figure 1. Mortality percentage (%) of 2nd-stage *C. medinalis* larvae treated with bacterial isolates EB01, S1, W1, and Yc1 at 3×10^9 cfu/mL concentration. Bars indicate the mean ± SE. Different letters above the bars represent significant differences at Tukey's test $p < 0.05$.

3.2. Concentration-Dependent Bioassay

The concentration response bioassay results showed that *B. subtilis* culture and its methanol extract induced toxic effects for various biological parameters of *C. medinalis* when ingested orally. The larval mortality ranged from 52 to 78% while the larvae were treated with active cell culture (T1–T4 treatments) ($p < 0.0001$). It was a concentration-dependent effect. With respect to the 3% control group, the cell-free extracts (treatments T4 and T5) also induced larval mortality in 17 and 32% (Figure 2a). During T1 treatments, the larval mortality started after 5 days and continued until the 15th day. At T4, the larvae mortality started after two days of treatments and continued until the seventh day. Compared to healthy larvae (Figure 3a), the *B. subtilis* culture-infected larvae became sluggish, stopped feeding, and their bodies ultimately turned yellow and black, leading to death (Figure 3c–f). Some larvae from the higher concentration (T4 treatment) changed their pupal stage within three days and died (Figure 3b). In the methanol-extract treatments (T5 and T6), the mortality started after eight days of treatments and continued throughout the entire stage. The growth of *B. subtilis* was observed in the infectious larvae hemolymph of treated *C. medinalis* larvae. In contrast, no growth of *B. subtilis* was detected in the extracts and control groups larvae.

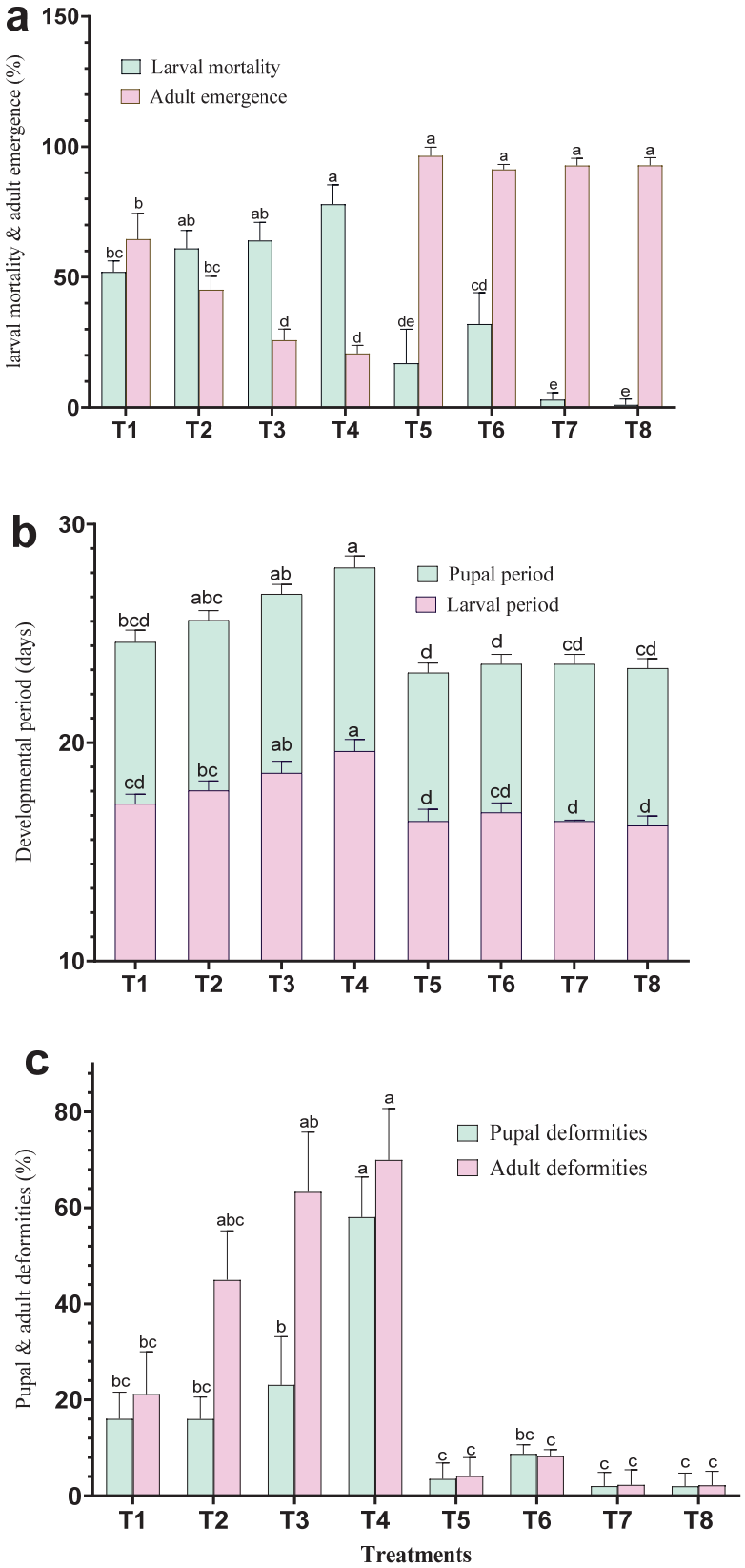

Figure 2. Treatments: T1 = 1.5 × 10^3 cfu/mL, T2 = 2 × 10^5 cfu/mL, T3 = 3.5 × 10^7 cfu/mL, T4 = 5 × 10^9 cfu/mL, T5 = 0.1 g/mL, T6 = 1 g/mL, T7 = methanol, and T8 = sterile water. Effect of *B. subtilis* on various biological parameters of *C. medinalis*. (**a**) percentage of larval mortality and adult emergence, (**b**) larval and pupal developmental duration (days), and (**c**) percentage of pupal and adult deformities. Bars indicate the mean ± SE. Different letters above the bars represent significant differences at Tukey's test $p < 0.05$.

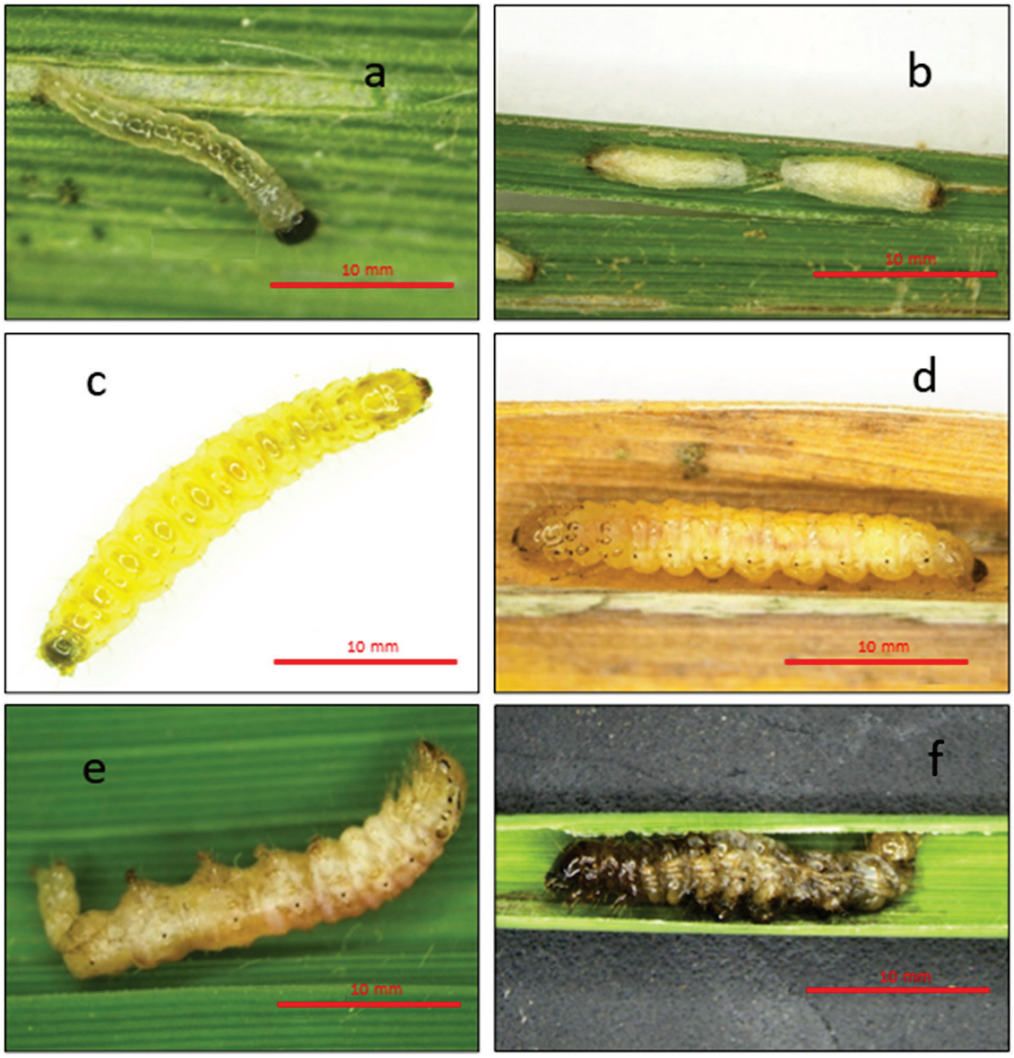

Figure 3. (**a**) Control, (**b**) cocoon formation at 2nd stage after 48 h post-treatment, (**c**) bacterial growth inside body turns yellowish after 48 h, (**d**) increased culture growth inside the body at 72 h, (**e**) the insect body swells and dies at 72 h, and (**f**) the insect body turns black and bacterial cells ooze out from the body. Scale bar—1 cm.

Oral ingestion of *B. subtilis* significantly influenced the development of *C. medinalis* larvae. Except for the bacterial extract treatments (T5 and T6), the culture of *B. subtilis* extended the larval period significantly by 1 to 3.4 days with respect to the control ($p < 0.0001$).

A significant effect was also observed during the pupal period. At the highest concentration (T4 treatment), few pupal deformities, i.e., larval–pupal intermediates, pupal–adult intermediates, and blackish body, were observed, followed by T3 and T2 treatments the notable changes were recorded (Figure 4a–e). In *B. subtilis* culture treatments (T3 and T4), only a few adults emerged, i.e., 20 and 25%, and most of the emerged adults were found to have deformities, with darkened bodies. With respect to the control groups, adults who emerged from *B. subtilis* culture treatments were found to be less active and had decreases in reproductive potential. The bacterial toxicity also induced the deformed morphological appearances of adults such as underdeveloped wings and crumpled body shapes (Figure 4f). The adverse effect of *B. subtilis* highly influenced the egg-laying capacity and larval hatchability (Figure 5) of *C. medinalis*. A significant impact was also noticed in the adult longevity that emerged from *B. subtilis* treatments, as shown in Figure 5 ($p < 0.0001$).

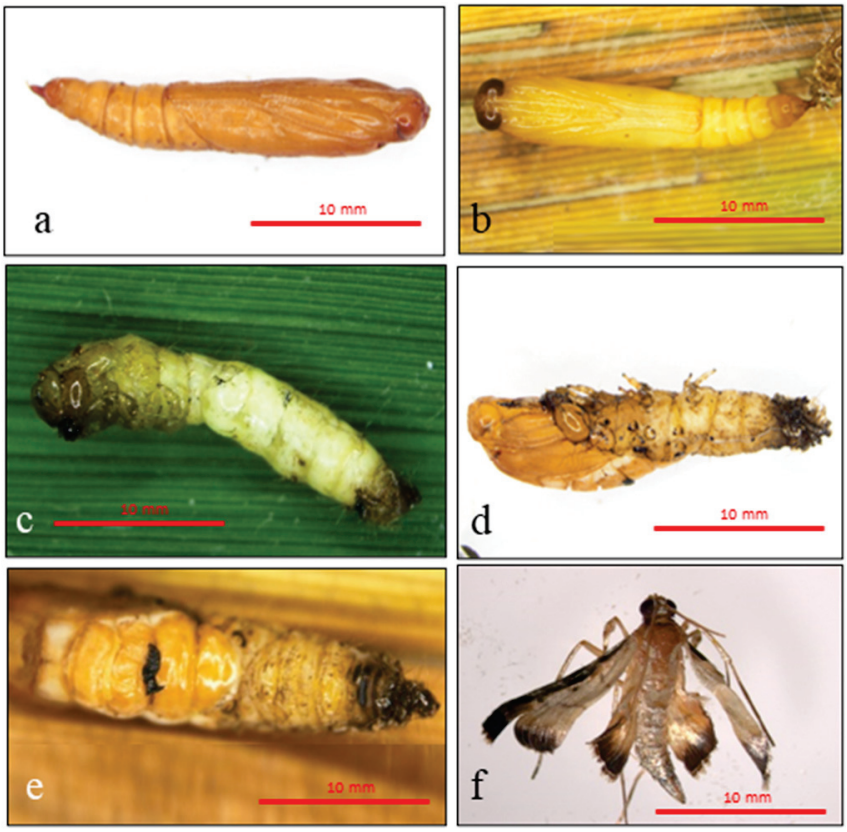

Figure 4. (**a**) Control pupae, (**b**) bacteria-infected, dead, undeveloped pupae, (**c**) underdeveloped pupae, (**d**) partially developed dead pupae, (**e**) infected unconverted larvae, and (**f**) adult deformity. Scale bar—1 cm.

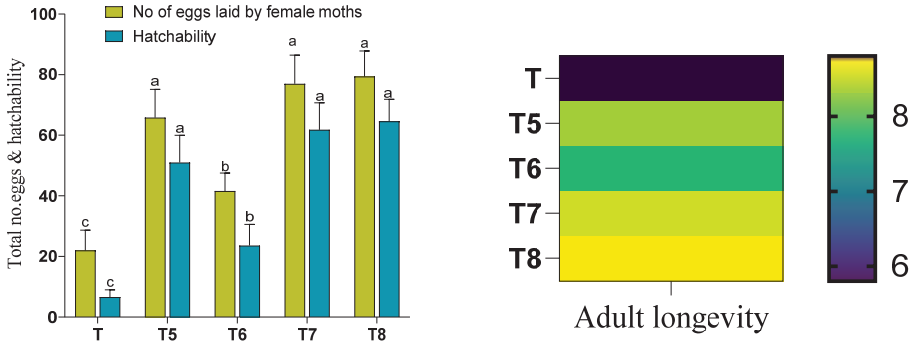

Figure 5. Effect of *B. subtilis* on reproductive potential and adult longevity of *C. medinalis* (number of eggs laid by females and total number of hatches). Bars indicate the mean ± SE. Different letters above the bars represent significant differences at Tukey's test $p < 0.05$.

3.3. Enzyme Activity

The adverse impact of *B. subtilis* on the activity of the α and β esterase activity of *C. medinalis* larvae was evident during their analysis. When *C. medinalis* larvae feed on *B. subtilis*-treated rice plants, there was a significant rise in α and β esterase 46.15 and 48.19%, 24 h; 33.87 and 27.18%, 48 h; 41.33 and 44.35%, 72 h; and 85.14 and 44.07%, 96 h, respectively, compared to control (Figure 6a,b) The effect of *B. subtilis* on the α and β galactosidases of *C. medinalis* was detected based on the reduction in activity when compared to the control. A significant drop was recorded, which showed a decreased level of 53.78 and 65.39%, 48 h; 88.17 and 91.88%, 96 h (Figure 6c,d).

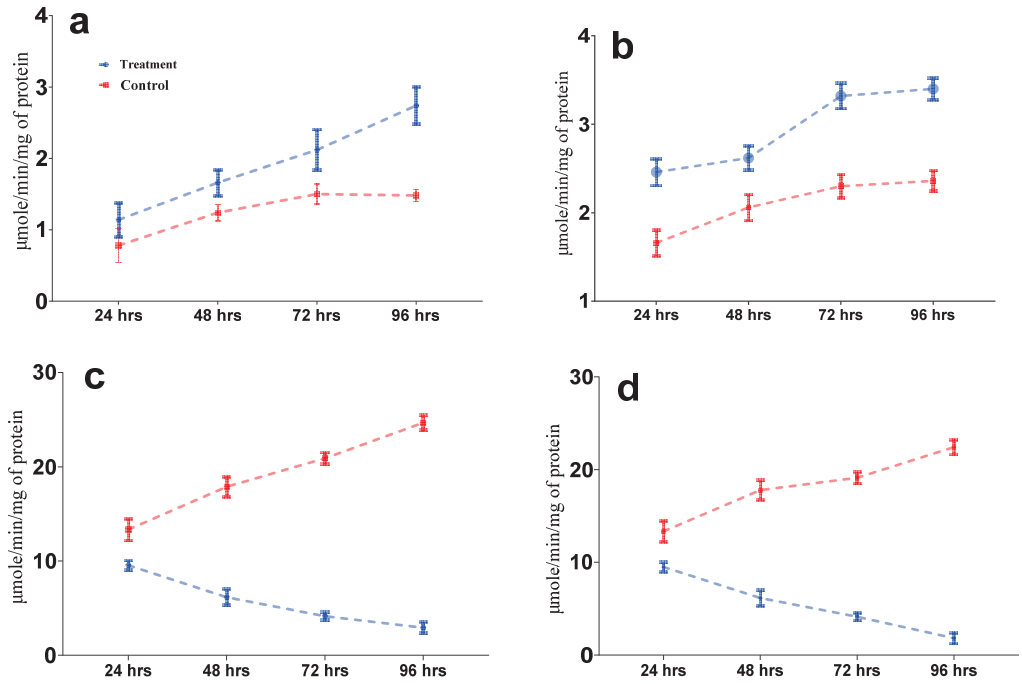

Figure 6. Effect of *B. subtilis* on the activity of (**a**) α carboxylesterase, (**b**) β carboxylesterase, (**c**) α galactosidase, and (**d**) β galactosidase enzymes of 3rd- (**a**,**b**) and 4th-stage (**c**,**d**) *C. medinalis* larvae at 5×10^9 cfu/mL. Bars indicate the mean ± SE.

3.4. Histological Analysis

We observed extensive histological alterations of midgut tissue in *B. subtilis* exposed to *C. medinalis* larvae. Infection due to *B. subtilis* disrupted the basement membrane, peritrophic, epithelial, and muscle layer of the midgut tissue of *C. medinalis*. The histology also showed that the epithelial layer was disturbed and detached from the larval midgut basement membrane (Figure 7). Control *C. medinalis* larvae displayed an excellent organization of muscle and epithelial cells.

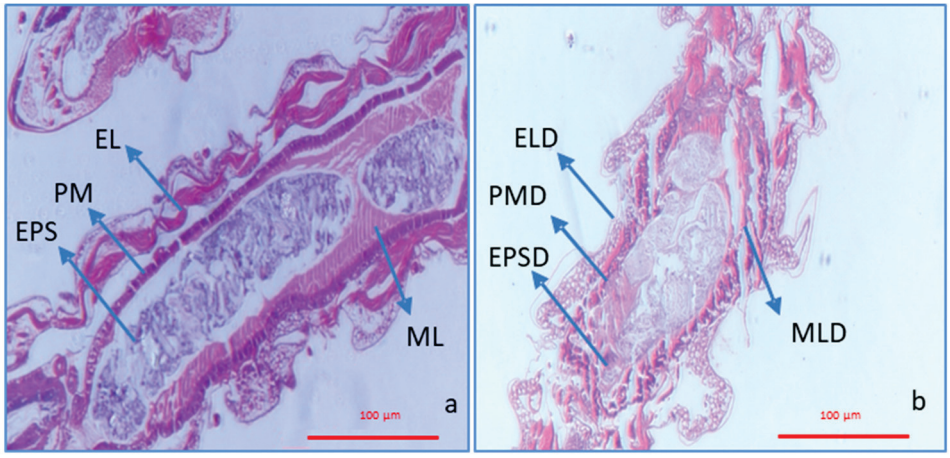

Figure 7. Longitudinal section through (**a**) midgut of fourth stage of *C. medinalis* larvae fed on control leaves showing intact peritrophic membrane (PM), endo peritrophic space (EPS), epithelial layer (EL), and muscle layer (ML). (**b**) Midgut of the larvae on treated leaves with *B. subtilis* shows disruption in the peritrophic membrane (PMD), endo peritrophic space (EPSD), epithelial layer (ELD) and muscle layer (MLD).

3.5. Plant–Bacteria Interactions

The seed treatment of *B. subtilis* (1.5×10^3 cfu/mL, 2×10^5 cfu/mL, 3.5×10^7 cfu/mL, and 5×10^9 cfu/mL) in both TN1 and ASD16 varieties of rice seeds had a positive effect on the germination rates and plant growth ($p < 0.0001$). The germination results showed a variation based on the bacterial concentrations; the germination percentages ranged from 77 to 88%, TN1; 78 to 91%, ASD16 (Figure 8a). We observed that plants grown using *B. subtilis* treatments had no adverse effects on plant biomass in both TN1 and ASD16 varieties. In the TN1 variety, *B. subtilis*-treated plants significantly increased in height from 24.96 to 30.24 cm (Figure 8b) and biomass from 237 to 264 mg. In the ASD16 variety, *B. subtilis*-treated seeds increased the plant height from 25.98 to 29.94 cm, biomass from 227 to 282 mg (Figure 8c) with respect to control plants. In both varieties, *B. subtilis*-treated seeds were germinated and grown well without any disease symptoms or stunned growth ($p < 0.0001$). The plants developed using *B. subtilis* treatment showed a healthy shoot nature compared to the control (Figure 8d).

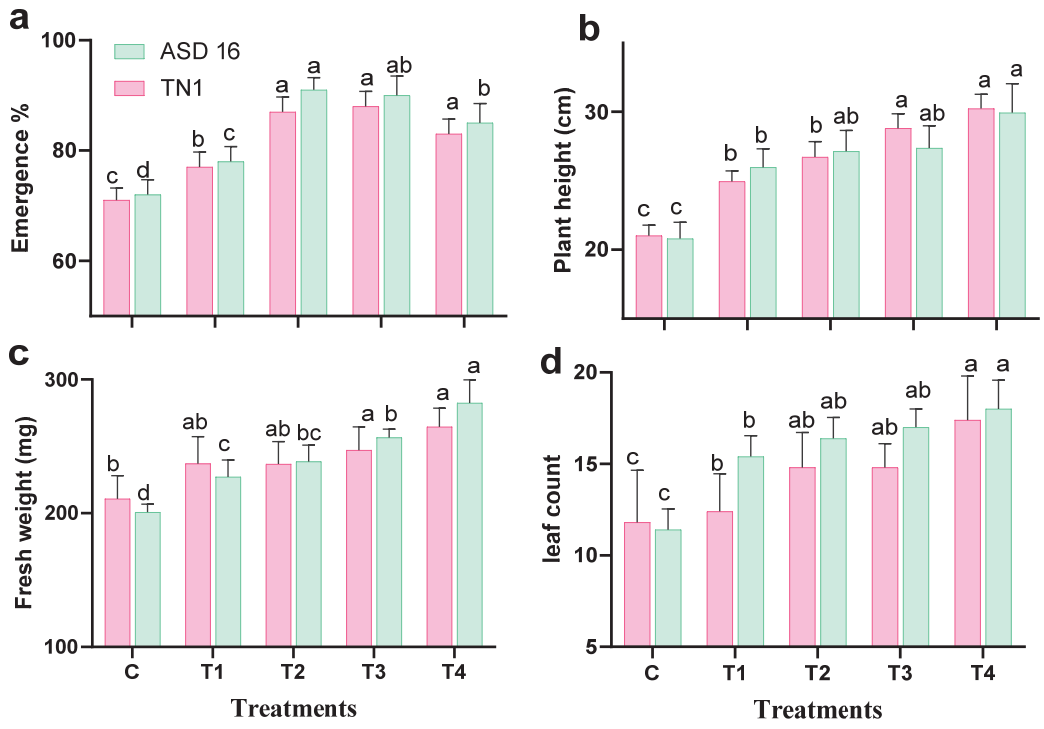

Figure 8. Efficacy of *B. subtilis* on the TN1 and ASD16 rice plants (**a**) emergence %, (**b**) plant height, (**c**) fresh weight, and (**d**) leaf count. Bars indicate the mean ± SE. Different letters above the bars represent significant differences at Tukey's test $p < 0.05$.

4. Discussion

There is a developing tendency to identify active pathogenic and efficient microbial biocontrol agents to manage effective and eco-friendly systems for controlling harmful agricultural pests. Hence, due to the demand to search for a novel biocontrol agent other than synthetic pesticides, entomopathogenic bacterial isolates from naturally infected *C. medinalis* larvae were screened for their insecticidal potential against *C. medinalis* management. Among the tested entomopathogenic bacterial strains, *B. subtilis* EB01 (OQ071610) was found to be more pathogenic, causing 74% mortality to *C. medinalis* in in vitro studies. Similarly, El-Salam et al. [31] demonstrated that the insecticidal effect of soil isolate *B. subtilis* NRC313 induced the mortality on *S. littoralis* larvae. Rizwan et al. [32] reported that entomopathogenic fungi, *B. bassiana*, controlled the growth of *C. medinalis*, causing a 74% mortality rate. Another effort of *B. subtilis* cell-free extracts also induced 32% larval mortality. The insecticidal action was slightly associated with the culture supernatant,

thus including the significant efficacy of possible soluble metabolites produced by *B. subtilis*. Larvae of *C. medinalis* infected with *B. subtilis* showed infectious symptoms such as lethargy, cessation of feeding, turning black with a flaccid body, less movement activity, decreased larval development, and, ultimately, death. A similar trend has been previously reported in many pests such as *Spodoptera litura*, *Spodoptera exigua*, and *Zophobas morio* due to infection of *Pseudomonas* sp., *B. thuringiensis*, and *Pseudomonas aeruginosa* [18,33,34]. However, Senthil-Nathan et al. [28] demonstrated that the bacterial *Btk*-toxin induced a high percentage of mortality in *C. medinalis* larvae.

The presence of bacterial proliferation in the hemolymph of *C. medinalis* larvae treated with *B. subtilis* suggests that larvae death occurs due to septicemia caused by the secretion of bacterial toxin compounds or other virulence enzymes into the hemocoel that targets the midgut epithelial barrier, breaks the body cavity, and suppresses the immune response of pests. Similarly, Sarkhandia et al. [18] reported that the mortality in *S. litura* was due to *Pseudomonas* sp. bacterial growth in hemocoel. The high proliferation of some entomopathogens in the hemolymph causes necrosis by releasing bacterial toxins [35]. Other researchers also documented mortality in *S. litura*, *Spodoptera frugiperda*, *Helicoverpa armigera*, *Plutella xylostella*, and *Delia radicum* due to the continuous proliferation of *Photorhabdus akhurstii* and *Pseudomonas protegens*.

In previous reports, several researchers found some entomopathogens from different species, such as *Bacillus popilliae*, *Bacillus lentimorbus*, *Bacillus sphaericus*, *Pseudomonas taiwanesis*, *Pseudomonas entomophila*, *Pseudomonas cedrina*, *Klebsiella pneumoniae*, *Pseudomonas paralactis*, and *Pseudomonas aeruginosa,* exhibit insecticidal activity against various pests like *Culex quinquefasciatus*, *P. xylostella*, *Drosophila melanogaster*, *Spodoptera exigua*, *S. litura*, and *Galleria mellonella* [36–40]. Some bacterial toxins like Fit toxin, exotoxin, rhizotoxins, and ExoS released from *Pseudomonas fluorescens*, *P. aeruginosa* and *P. taiwanesis* were associated with various pests causing pathogenicity that leads to death and sepsis [41–43]. Bacterial strains *P. fluorescens* and *P. protegens* contribute to the toxicity of *Drosophila* and *G. mellonella* by producing hydrolytic enzymes like chitinases, phospholipases, and proteases [44,45].

In addition to affecting mortality, treating larvae with *B. subtilis* also extended the larval developmental period and pupal period, decreased pupal conversion, and facilitated adult emergence. *B. subtilis* treatments induced deformities in pupae and adults. Delayed development of *C. medinalis* was an adverse effect of *B. subtilis*. A similar trend has been documented previously in *S. litura*, *Delia radicum*, and *H. armigera*, which emerged from *Bacillus vallismortis*, *Enterobacter cloacae*, *P. paralactis*, and *B. thuringiensis* treatments; the adults were morphologically deformed with underdeveloped and crumbled wings [14,40,46]. Similarly, several reports proved that some larval species in *H. armigera*, *D. melanogaster,* and *S. litura* have exhibited delayed development and decreases in adult emergence after being exposed to *Serratia marcescens*, *P. fluorescens*, and *B. thuringiensis* [47–49].

Our studies displayed a significant increase in the activity of α and β esterase after exposure to *B. subtilis* treatments. Other researchers also found similar enhancement in α and β esterase activity in *S. litura* after exposure to λ-cyhalothrin and insecticides [24,50]. In contrast, a significant decrease was observed in the activity of α and β galactosidases due to infection of *B. subtilis*. The suppression of digestive enzyme activity in the *B. subtilis*-treated pests may be due to effects on the efficiency of digestion [51]. A similar report documented reduced digestive enzymes in *C. medinalis*, *Diatraea saccharalis*, and *H. cunea* due to infection with *Photorhabdus temperata* and *B. thuringiensis* [52–54]. The efficiency of digestion is correlated with the histopathological impact of *C. medinalis* gut. The midgut is the major site for the digestive enzyme synthesis and secretion. In our study, histological analysis demonstrated extensive damage in midgut epithelial cells of *C. medinalis* larvae due to infection with *B. subtilis*. A bacterial toxin from *B. thuringiensis* altered the midgut epithelial cells in *S. litura* and *A. gemmatalis* [55–57]. Similar damage in the midgut tissues has been documented in *S. litura*, *S. frugiperda*, and *H. armigera* after exposure to *K. pneumoniae*, *P. akhurstii*, and *P. paralactis* [29,39].

This study aimed to develop a potential biocontrol agent without affecting crop development. The results of the plant growth promotion study show that the treatments using *B. subtilis* have increased the seed germination percentage and plant biomass in both varieties without affecting the plants' shoots and roots. In previous studies, the novel bacterial strain *B. subtilis*, isolated from soil, improved the rice plant development [58]. Javed et al. [59] reported that the bacterial entomopathogen of *Brevibacillus laterosporus* increased rice development and induced systemic resistance against *Cymothoa exigua*. Similarly, Ullah et al. [60] demonstrated that the entomopathogenic bacteria *P. temperata* promotes rice plant growth by activating gibberellins. Several studies have shown the role of entomopathogens of *Serratia marcescens* in regulating plant growth along with resistance against pests in rice [61,62].

For future implications of *B. subtilis* in integrated pest-management practices, there is a need to standardize the mass production techniques of *B. subtilis* isolate to make it cost-effective so that farmers can easily use it and, further, to evaluate its efficacy. It can be used in combination with other botanical or other control agents so as to provide effective agricultural pest control in integrated pest-management programs.

5. Conclusions

The entomopathogenic bacteria *B. subtilis* active cell culture and extract are both virulent in *C. medinalis* larvae, and this can be useful for *C. medinalis* control. Based on the analysis of *B. subtilis* culture and extract, the active cells were found to induce more pathogenicity in *C. medinalis* larvae. From the above study, we conclude that *B. subtilis* active cells are a good potential biocontrol agent for *C. medinalis* population control. In future, in order to develop good potential microbial biopesticides, the *B. subtilis* isolate will be further validated in a field evaluation.

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Data Availability Statement: The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

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Perspective

Benefiting from Complexity: Exploring Enhanced Biological Control Effectiveness via the Simultaneous Use of Various Methods for Combating Pest Pressure in Agriculture

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Abstract: Biological control, a well-established plant protection method, has garnered substantial attention in recent decades. Various approaches, including biological control agents (BCA), catch crops, biofumigation, sticky traps, and pheromones, have been extensively explored. While the effectiveness of these methods varies depending on specific circumstances, their collective significance has grown amid mounting pressures to curtail or eliminate conventional synthetic plant protection products. Previous review articles have highlighted the benefits of using two or more BCAs simultaneously, yet limited information exists regarding the concurrent use of diverse biological control methods. This comprehensive review incorporates a thorough literature search to assess the benefit of concurrently employing two or more of these methods, followed by a discussion on perspectives of holistic management and mimicking complex natural systems, shedding light on the vast potential and need for further research in this domain.

Keywords: simultaneous use; concurrent control; biological control; catch crops; biofumigation; sticky traps; pheromones; organic agriculture; agroecological crop protection; holistic management

1. Introduction

For decades now, calls for the reduction of chemicals used in agricultural production systems have been heard worldwide. The cause could be attributed both to health [1] and sustainability concerns [2]. Over the past several decades, several strategies, including organic agriculture and integrated pest management (IPM), were promoted for combating the threat, resulting in mixed success rates [3]. In efforts to reduce dependencies on unsustainable and potentially harmful practices, biological control was formulated as a plant protection system, where the use of synthetic pesticides is replaced by the use of living natural enemies of pests [4]. The approach has been adopted worldwide, and many studies have highlighted its effectiveness against various pests in different climates around the globe [3,5–7]. Despite the common appraisal of the concept and plenty of anecdotal evidence found by practitioners, it only seems to be successful in 11% of the cases [8], with researchers often struggling to replicate its effectiveness when applying it in their own circumstances [9]. The use of just one method of biological control simultaneously is the most studied, but in natural ecosystems, there is always a plethora of organisms and processes that keep the system in balance. Hence, the idea of simultaneous use of different methods seems to have an even greater potential for combating resilient pests. There are not many extant studies that have explored this concept in the past, possibly because they are difficult to conduct due to many influencing factors [10]. Some of the studies write about the use of mixed species cover crops [11], others about the use of two different biological control agents (BCAs) concurrently [12–16], or just one BCA concurrently with different inert dusts in storage facilities [17–19]. Most studies found that a combined approach improves the effectiveness of pest control, while some report otherwise, indicating that

this area of research is complex and there are many environmental and other factors contributing to the effectiveness of these approaches.

For the purpose of this review, however, we narrowed our area of interest down to the topic of simultaneous use against insect pests in farm fields. We were primarily interested in whether we could discover any studies that reported beneficial effects to plant protection from the simultaneous use of BCAs and either catch crops, biofumigation, sticky traps, or pheromones, an area of research no review studies have been written about so far but is, in our opinion, worth exploring. The term "beneficial effect" used in this study relates to the effect of reduction in pest damage to the plants caused by the simultaneous use of two of the listed methods, even though benefits to the BCAs themselves might not be present concurrently. In the first part of this review article, the collected articles are reviewed and compounded based on the general measure groups: "catch/trap/companion crops"; "sticky traps and pheromones"; and "biofumigation". For the second part, a discussion based on a wider literature search was performed in order to find articles on the wider perspectives of holistic management and mimicking complex natural systems for more effective pest management.

2. Review Methodology

Scientific articles were discovered via the use of the Google Scholar search engine and based on the following keywords: synergistic effects; simultaneous use; concurrent control; additive effect; compatible organisms; biological control agents; catch crops; biofumigation; sticky traps; pheromones; and their various combinations. Additional studies were discovered among the citations in these articles and via discussions with colleagues.

3. Catch/Trap/Companion Crops

Natural ecosystems rely on a balance of a plethora of organisms to prevent major outbreaks of a single species. The vast diversity in that case is highly functional, but in profitable agricultural systems, it is rarely found because of the complexity it demands from agricultural management. In order to artificially maintain the balance between predator and prey, the farmers rely on different solutions, including the conventional use of pesticides. As discussed, biological control can be a promising alternative, but in this case, it is crucial to provide beneficial organisms with enriched habitats, mimicking their natural environment and providing a higher likelihood of their survival and thriving. A recent study [20] has shown for the first time that "the abundance of naturally occurring enemies are directly influenced by the composition of the landscape surrounding the cultivated fields. Simple landscapes, defined as landscapes with high proportions of cropland, were positively correlated with the abundance of foliar and ground-dwelling predators (based on the control plots). In contrast to predators, parasitoids were far less abundant in simple landscapes." This is an important consideration, as it is the complex landscapes that provide shelter and mating environment for both native and introduced BCAs, thus increasing their populations. According to the authors, it is crucial to move the debate from solely "which is the best organism to use" to "what type of environment can support multiple organisms simultaneously", calling scientists to consider researching a wider context rather than just single practices.

It is very difficult to transform large-scale intensive agricultural production areas into natural or even semi-natural habitats, but studies show that the incorporation of companion crops, and also catch and trap crops, can benefit the efforts to reduce pest pressure. One study [21] found that sesame (*Sesamum indicum* L.) companion crop in tomato plantations reduced the damage to tomato that was caused by *Tuta absoluta* (Meyrick) and its natural enemies when their primary prey was not present. Damage by *Nesidiocoris tenuis* (Reuter), which would normally feed on *T. absoluta* eggs but would also target tomatoes if they were too few, was significantly reduced by providing a sesame companion crop as an alternative feed source.

Another study [22] drew attention to the importance of overall crop diversity on the landscape level and its benefits for pest control. Enhancing crop diversity provides natural enemies with a variety of food and shelter resources, possibly throughout the year. Aphid regulation in the study was reported to be up to 33% higher in high crop diversity landscapes, suggesting that even in large monoculture fields, a lot can be achieved just by altering the crop rotation in order to include more crops, which is far more acceptable for farmers than the introduction of "non-productive" buffer strips, hedges, or woodlands. The authors even suggest that natural habitats might not be the most suitable as they act as barriers to BCA migration and draw them away from the crops. A related study [23] explored the beneficial effects of ground cover and adjacent vegetation on insect pests in olive groves. While the abundance of different natural enemy groups varied depending on the species, the authors found that both forms acted together to maximize abundance. Interestingly, both studies emphasized the need for diversified ground cover and suggested it increases the abundance of natural enemies more than the small patches of woody plants if just one of them is implemented. This has important implications for the producers, as implementing woodland buffer strips and similar structures takes the land out of production, while increased ground cover also has other benefits, including water retention and weed control.

When choosing a suitable companion plant species, an important consideration is the duration of flowering and the ability to provide shelter. In a recent study [24], *Lobularia maritima* L. was used as a companion crop to shelter and feed *Orius laevigatus* (Fieber) as a BCA in strawberry plantations. The concurrent use of the companion crop and *O. laevigatus* has proven effective in controlling aphid populations, while *O. laevigatus* populations were not able to establish themselves on strawberries alone.

A similar phenomenon was described by two other studies [25,26], which used the strategy of "attract and reward" to attract the BCAs to a companion crop via the use of synthetic attractant substances. The idea behind this approach is to use a volatile attractant compound to attract a BCA to a companion crop, where it can feed on the crop itself or on the pests' populations. In the first study, buckwheat (*Fagopyrum esculentum* Moench) was the companion plant species, and in the second, it was again *L. maritima*. Both plant species are known for their pollen-rich flowers, providing a habitat for beneficial organisms. In the case of the second study, no beneficial effects were observed from the use of an attractant compound (methyl salicylate) concurrently with the *L. maritima* companion crop, but the crop itself showed promising results. The first study discovered some beneficial effects, especially the potential of buckwheat as a companion crop, but the authors warned that while this strategy is worth studying further, the attractant compounds can have very short-term effects and also attract other pests, like rodents.

One study [27] expanded on companion cropping and also explored the use of agronet covers to reduce silverleaf whitefly (*Bemisia tabaci* [Gennadius]) infestation in tomatoes. Apart from a physical barrier, agronets also provided visual disruption to the pests. When using agronets concurrently with basil (*Ocimum basilicum* L.) companion crops, *B. tabaci* infestation decreased by 62 to 72% compared to the control. The combined effect was greater than from each treatment alone. The authors also reported better growing conditions for crops under agronet cover.

4. Sticky Traps and Pheromones

Sticky traps are usually used as a tool for monitoring the presence of pests, but in some cases, they can also be used to reduce pest populations by catching them [3]. They can be used in conjunction with different volatile compounds, like attractants, infochemicals, or pheromones. These can be used as an additional strategy to attract pests to either sticky traps or trap crops or repel them from main crops. They can also be used to disrupt the activity of hyperparasitoids that can sometimes interfere with other BCAs [28].

Moreau and Isman [29] evaluated the combined effectiveness of trap crops, yellow sticky traps, and reduced-risk products against greenhouse whitefly (*Trialeurodes vaporari-*

orum [Westwood]) on sweet peppers. Reduced-risk products included insecticidal soap, capsaicin extract, olive oil, and rosemary oil. Eggplant (*Solanum melongena* L.) was used as a trap crop. The study found that the use of trap crops reduced the number of adult whiteflies by 31%, while that, in combination with the yellow sticky traps, brought the numbers down by 41%. The addition of different reduced-risk products to the combination did not show any further decrease in this study, but in another study, [30] sticky traps combined with biopesticides (spinosad, D-limonene, sodium lauryl ether sulfate) showed an effectiveness of 84–86% in decreasing *Aleurocanthus rugosa* (Singh) in betel vine (*Piper betle* L.). The results thus proved that the integrated pest management approach was even more successful than the one with the use of conventional pesticides.

Sticky traps were also used in a study [31] where they were combined with soil applications of azadirachtin, entomopathogens, and predatory natural enemies against the western flower thrips (*Frankliniella occidentalis* (Pergande)). The results showed that the combined use of the approaches was more effective at decreasing thrips numbers than when each approach was used separately.

5. Biofumigation

Biofumigation is often used in combination with other pest management approaches in order to decrease the number of pests before the main growing season starts. In one study [32], a mustard cover crop was ploughed into the soil as a fumigant agent before seeding gerberas into polyhouses, and two BCAs—*Pseudomonas fluorescens* (Rhodes) and *Trichoderma viride* (Persoon)—were used for biological control during the main crop growing season. The results show that the combined use of biofumigation and soil application of *P. fluorescens* significantly suppressed the population of *Meloidogyne hapla* (Chitwood) root nematode and increased the flower yield by over 40%. A different outcome was presented in another study [33], where biofumigation with the mustard cover crop was combined with the entomopathogenic nematodes *Steinernema feltiae* (Filipjev) and *Steinernema riobrave* (Cabanillas et al.) on root-knot nematodes and the Colorado potato beetle (*Leptinotarsa decemlineata* [Say]). Here, the use of biofumigation interfered with *Steinernema* spp. and prevented them from acting as biocontrol agents. On a wider scope, this is not an isolated case at all, as the transmission of pest entomopathogens to the natural enemies of the pest is an important topic in recent years. In our own past research [34], we found that entomopathogenic nematodes had a negative effect on the larvae of both two-spotted lady beetle (*Adalia bipunctata* L.) and lacewing (*Chrysoperla carnea* (Stephens). Other publications [35] also raise similar concerns, which indicates that interactions like these should not be neglected. The simultaneous use of a competitive combination of BCAs would definitely not be beneficial to the plant protection efforts, indicating that a broad understanding of the ecosystem interactions is crucial for success in the field of nature-based solutions.

6. Discussion

The reviewed studies seem to mostly confirm the assumption that the inclusion of several different nature-based solution methods in biological control is beneficial when compared to one-method approaches. Whether the ineffectiveness shown in single-factor research is caused by the inefficiency of the practice or the single-factor analysis design's inability to properly account for real-life complexity is hard to determine. In this section, we will further focus on exploring why this might occur and view it from the wider scope of sustainable agriculture. We will also consider the perspectives of holistic management and socio-economic evaluations for environmental studies and then conclude by discussing how to best utilize such beneficial effects in practice.

In the number of experiments concerning the beneficial effects of BCA and one of the other selected natural pest management methods, we have found that not many studies were performed specifically among the selected combinations. By searching the web for the keyword "synergistic effects", we found almost none. After trying to think outside the box, we discovered that some more similar studies exist that use different keywords,

like "concurrent use" or "simultaneous use". This seems to be a common issue in the scientific literature, where the better-known phenomena with agreed-upon definitions are much more easily found, while new niches oftentimes receive different labels, making them difficult to find, even when they describe a similar method or phenomenon. Even the use of the term "synergistic" in this sense might not be the most appropriate because, in some of the reviewed studies, detrimental effects on the BCA populations were observed, which is not synergistic to them at all. Gosnell et al. [10] discussed this topic and stressed out the importance of clearing out definitions and terminology before opening any debate on a topic to avoid conflict based on misinterpretation and miscommunication. While they were discussing this in the context of ecological debates concerning holistic management and regenerative agriculture, we feel that this still applies to our instance of searching for keywords "synergistic effects" and "concurrent use". Since the two seemingly unrelated phrases lead us to a similar topic, therefore thinking about the broader context of biological control might ultimately lead us to holistic management, regenerative agriculture or other similar ideas. As suggested by the results of our review, considering a more holistic context by incorporating more than just one pest management practice seems to improve the effectiveness compared to simple one-agent solutions. With regard to this, it might be meaningful to view complex problems like pest protection in a wider context, possibly learning from other ideas or movements, rather than focusing on single practices that may or may not be effective, depending on many factors.

Taking a step in the past, the previous century was characterized by both rapid civilizational development and growth of the human population. Agriculture was therefore confronted with unique challenges, which oftentimes seemed too complex to solve with the then-known management practices. With the introduction of mineral fertilizers and synthetic pesticides, the agricultural community started to believe that those are cheap and effective ways of submitting nature to their own will in order to feed the world. Soon after, health and sustainability concerns started to disprove that, and alternative ideas started to emerge. In the late 1900s, several alternatives, including permaculture, organic, biodynamic, conservation and regenerative agriculture, agroecology, and sustainable agriculture, began to circulate, culminating in the formulation of underlying practices and wide adoption of several of those, including organic agriculture in the last two decades [36,37]. A similar notion can be said for biological control and integrated pest management in general [3]. But despite the promotion, reported benefits, and widespread appraisal of these more sustainable practices, the area of organically farmed land in the EU is still far below the 25% goal for 2030 [38], with other alternative practices not showing much better adoption results. Furthermore, arguments are being made that the certified organic farm produce might not be as environmentally friendly as marketed, as, for example, copper- and sulfur-based fungicides (both allowed in organic production but potentially toxic) are often applied in large amounts [39,40]. Some authors [10] claim that it was the defining of strict rules and practices that prevented organic agriculture from reaching its goal of being sustainable, as this took the practices out of context of broader care for the whole ecosystem. Farmers therefore often just follow a prescribed set of practices instead of thinking how what they are doing might function in the ecosystem as a whole. This could somewhat explain why biological control with the introduction of a single BCA rarely proved effective in the past and why the presented studies that emphasize complexity tend to discover greater benefits. Providing refuge for several beneficial organisms while introducing a reliable BCA to a system certainly makes more sense than applying pesticides that not only eradicate the pest but also other beneficials [20]. Furthermore, concurrent use of BCA and biostimulants, but also other means of complex and diverse ecosystems mimicking in general, seem to improve plant health, decreasing susceptibility to pests and disease [41,42].

While alternative methods, like biological control, but also organic or, lately, regenerative agriculture, are often disregarded because of a lack of studies that could confirm their claims, there are lessons to be learned from their common underlying holistic management of ecosystems. At least theoretically, biological control capitalizes on a foundation

of mimicking the naturally occurring processes, like predation or parasitism of pests by other organisms. Albeit somewhat understandably, when practiced and studied, it is too often degraded to a very simplified set of practices since it is easier to implement or analyze the effect of a single practice in a simple system. But in doing so, the experimental design often nullifies the influences of other possibly beneficial interactions and therefore fails to exploit the full potential of such an approach. Most of the reviewed articles that focused on "the landscape context", as lucidly formulated by Perez-Alvarez et al. [20], have found improved effectiveness compared to single-practice applications [22,23,29]. Even the studies that observed antagonistic effects [33] indicate that they were oftentimes connected to weak consideration of the wider context. The issue with such complex approaches is that it becomes exceedingly difficult to conduct easily presentable scientific research with clear relationships between the many factors included. According to Redlich et al. [22], this highlights the need to study such approaches in a more general manner of ecosystem services rather than individual BCAs. To expand on this, "Depending on what values inform the weighting of the factors, an overall assessment may yield a negative or positive result in a specific context" [10]. The authors of a recently published article [36] shed more light on this by explaining that, based on the variable environmental but also social and economic conditions around the globe, comparing practices or systems can give us misleading information on comparability. They further develop the idea by proposing that "One solution to simplify the comparison of agricultural systems, and to increase independence from the products they produce, is to consider what ecosystem goods and services are needed from agricultural landscapes and to compare the ability of different agricultural systems to improve the functions that provision these over time." The question then arises about which of those ecosystem services (i.e., food, water, or biodiversity) to prioritize and on which scales (i.e., field, farm, watershed, or state), but there is no clear answer. Rather, such decisions should be made based on holistic environmental–socio-economic analyses [43] after discussions with a wide array of stakeholders.

When evaluating the effectiveness of different alternative practices, biological control included, in such holistic environmental–socio-economic analyses, the main focus shifts to evidence. In natural sciences, evidence is almost exclusively understood as a result of a one-factor analysis. That is understandable, as mentioned since this is the only definitive way of proving whether the difference actually exists and explaining its cause. But considering the importance of socio-economic studies for the holistic context of the mentioned alternative practices, it is crucial that the natural sciences also learn from the social ones. In complex systems, as seen from our review, it is sometimes difficult to pinpoint the exact cause of change, but it might be counter-productive to completely disregard such scientifically less reliable evidence [10]. In calling for a greater emphasis on "*praxis*", which is complex and qualitative, than "*scientia*", which is controlled and quantitative, Stinner et al. [44] emphasized exactly that, as the former better emulates complex, real-world conditions. But such less verified anecdotal evidence is sometimes also used to disprove the suitability of biological control methods, as demonstrated in [45]. This goes to show that acknowledging the results of some practice often comes down to agreeing on whether the experiment in question was competently carried out, which can be exceedingly more difficult to prove the more complex the experimental conditions become.

7. Conclusions

In summary, the reviewed studies strongly support the idea that combining various nature-based solutions in biological control is more effective than single-method approaches. The discussion explores the challenges of single-factor research and emphasizes the need for a broader perspective on sustainable agriculture. The scarcity of studies on beneficial effects using specific keywords underscores a common issue in scientific literature—ambiguous terminology. The gap between theoretical benefits and real-world effectiveness highlights the importance of holistic approaches that consider ecosystem dynamics. Barriers to adopting sustainable pest management practices persist, including

rigid adherence to predefined rules without the consideration of a wider context. Therefore, it would be highly beneficial for future studies to focus more on ecosystem services-based comparisons and holistic environmental–socio-economic approaches like the recently developed agroecological plant protection concept [46]. Finally, it is ever more important to consider the necessity of embracing diverse forms of evidence, even if less scientifically rigorous, to better understand the effectiveness of biological control and similar practices in the dynamic realm of agriculture.

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Biological Characteristics and Thermal Requirements of *Telenomus podisi* and *Trissolcus basalis* (Hymenoptera: Scelionidae) in Fresh and Cryopreserved Eggs of *Euschistus heros* and *Nezara viridula* (Hemiptera: Pentatomidae)

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Abstract: Brazil is one of the largest producers of pulses globally, and soybean ranks highly in terms of production. However, pests increase crop production costs and affect oilseed production and quality. Pests are primarily controlled by chemicals, leading to changes in insect pest populations. For example, secondary pests can become primary pests because of the selection of resistant insects and the elimination of natural enemies. Farmers have widely accepted biological control because of its high control efficacy and low environmental contamination risk. Two successful biological control programs in soybean used *Telenomus podisi* Ashmead (Hymenoptera: Platygastridae) to manage the Neotropical brown stink bug, *Euschistus heros* (Fabricius) (Hemiptera: Pentatomidae), and *Trissolcus basalis* (Wollaston, 1858) (Hymenoptera: Scelionidae) to manage the southern green stink bug, *Nezara viridula* (Linnaeus) (Hemiptera: Pentatomidae), when these pests were the most abundant in soybean-producing regions. To release parasitoids, rearing protocols must be developed in order to optimize production. This study evaluated the effect of temperature on the biological characteristics of *T. podisi* and *T. basalis* when fresh or frozen *E. heros* eggs were provided. Fifty fresh or previously frozen eggs were placed with parasitoids for 24 h in a climate chamber (25 ± 1 °C, 70 ± 10% RH, and 14-h photophase). At the end of this period, the eggs were transferred to other chambers and maintained at different temperatures (19, 22, 25, 28, 31, and 34 ± 1 °C, 70 ± 10% RH, with a 14-h photoperiod). The number of emerging adults from eggs parasitized by *T. basalis* was lower than that from eggs parasitized by *T. podisi*. Differences in *T. basalis* and *T. podisi* parasitism were observed depending on whether the *E. heros* eggs were frozen or fresh, but neither development nor emergence were affected. The duration of egg–adult development and the longevity of the two parasitoids were inversely proportional to the temperature increase. The sex ratio of *T. basalis* and *T. podisi* progeny was not affected by different temperatures or by the use of frozen or fresh eggs. The estimated number of both parasitoids' generations corresponded with temperature, reaching 14–15 generations/cycle of soybean for Rio Verde, GO, and 12–14 generations/cycle for Barreiras, BA. Given these characteristics, we conclude that a biological control program using *T. podisi* can benefit large areas of soybean production by controlling the eggs of phytophagous bugs in soybean crops.

Keywords: rearing protocol; parasitism rate; degree day; integrated pest management; biological control

1. Introduction

Pulse crops are grown in Brazil, with soybeans (*Glycine max* L.) being the most widely grown and important export product. In 2021/2022, Brazil produced 125.55 million tons of soybeans, leading global production [1]. If losses from soybean pests, caused mainly by Plusinae, Noctuidae, and the Pentatomidae complex, can be reduced, production would be even higher.

The most common management method used to reduce pests in soybean cultivation is chemical control, which can cause problems in the agro-ecosystem, including increased insecticide resistance, environmental contamination, and elimination of natural enemies. These changes can favor insects that were previously considered secondary pests, causing them to become primary pests [2].

Phytophagous stink bugs (Pentatomidae) include several species that are major pests of soybean crops in Brazil. They cause severe damage to both the yield and quality of the harvested product [3,4]. Among the phytophagous stink bugs that damage soybean crops, the brown stink bug, *Euschistus heros* (Fabricius, 1794) (Hemiptera: Pentatomidae), is the most abundant species in the Neotropical region, including Brazil [5], Argentina, Paraguay, Panama, Uruguay, and Bolivia [3,6,7].

Unfortunately, the management of *E. heros* populations is limited by the availability of effective insecticides. Only six active chemical ingredients registered for stink bug control are available in Brazil, most of which show low control efficacy because of insecticide resistance. Initially, the expansion of soybean plants with expression of protein crystals from *Bacillus thuringiensis* (Bt) was predicted to reduce damage from the stink bug complex. Because Bt technology requires fewer insecticide applications to control caterpillars during the vegetative period of soybean crop development, it was believed that fewer sprays would preserve the beneficial entomofauna while suppressing stink bug populations, which start to appear in the crop at the end of the vegetative stage. However, in the 2018/2019 crop, an increase in the stink bug population and crop damage was observed. This can likely be explained because, in the absence of the caterpillar complex, stink bugs benefited from reduced competition.

Ideally, modern agriculture utilizes a multidisciplinary approach to manage pests as part of an integrated pest management (IPM) program. The use of augmentative biological control, through the mass release of a pest's natural enemies, can reduce the pest population to levels below those causing economic damage [8]. In this context, the use of biological control agents at the onset of stink bug occurrence is strategic because of the limited pesticide applications, especially in areas using *Bt* soybean.

The egg parasitoid *Telenomus podisi* (Ashmead, 1893) (Hymenoptera: Scelionidae) has shown promising results when released towards the end of the soybean growing season, with *E. heros*, the most abundant bug in soybean crops, being its preferred host [9]. The release of *T. podisi* reaches a control efficacy of approximately 80% if released at the end of the vegetative period when the first stink bugs migrate to the crop and begin laying eggs (22).

Despite the potential of *T. podisi* in managing the stink bug complex in soybeans, some factors should be considered to ensure the feasibility of parasitoid use. One crucial factor is the absence of hosts for *T. podisi* during autumn and winter because there is no stink bug oviposition in the off-season. Therefore, it is necessary to develop mass-production methods for confined systems so that biological control agents are available for release at the time of pest occurrence in the field.

One method for successfully producing egg parasitoids during the off-season is to store host eggs in liquid nitrogen until needed, as demonstrated for *Nezara viridula* (Linnaeus, 1758) (Hemiptera: Pentatomidae) and *E. heros* [10,11]. Thus, parasitoids can be reared in large quantities in the off-season, to be released when stink bug oviposition occurs in soybeans. In addition to being able to mass rear parasitoids as needed, it is also necessary to determine the abiotic conditions that induce and regulate the processes of quiescence, hibernation, or diapause in egg parasitoids because manipulation of these

processes can affect the efficiency of large-scale parasitoid production for biological control purposes [12,13]. However, few studies have documented the effects of abiotic factors on biological control agents. Therefore, this study sought to determine the thermal requirements of *T. podisi* reared from frozen and fresh *E. heros* eggs to support the release of parasitoids in pulse production fields. The thermal requirements of *T. basalis* were also determined for comparison with those of *T. podisi*.

2. Materials and Methods

2.1. Stink Bug Colony and Eggs

The rearing of *E. heros* followed a methodology similar to that described in [11]. The insects were kept in climatized rooms at a temperature of 25 ± 2 °C, with relative humidity (RH) of 70 ± 10%, and a 14-h photophase. The first adult stink bugs were collected in the field and kept in a separate room for the observation of natural parasitism. Subsequently, the selected adults were kept in plastic containers with one male for each female, with a maximum capacity of 50 couples/cage. The stink bugs were fed soybean pods, groundnuts, and sunflower grains. The pods were glued onto strips of sulfite paper and suspended on the cage wall. *Ligustrum* fruits (*Ligustrum lucidum*) were provided in the cage as supplemental food [11]. Moistened cotton was placed on top of the cage to provide water and maintain environmental humidity. After the beginning of the oviposition period, eggs were collected daily. Some were returned to the breeding cage, while the rest were stored in liquid nitrogen for use in parasitoid multiplication.

2.2. Egg Parasitoid Colony

Rearing of the stink bug egg parasitoids was conducted according to the methodology described in [14]. *Telenomus podisi* was maintained on *E. heros* eggs [15], while *T. basalis* was maintained on *N. viridula* eggs, which are considered the best hosts for the development of these parasitoids.

The stink bug eggs for parasitism were provided in transparent plastic flasks of approximately 20 cm in length. One end of the flask was closed with cotton wool and the other with a fine mesh screen to allow air into the tube. A thin layer of honey was placed in the upper inner portion of the flask as a food source, and moistened cotton wool was placed in the central and upper portions of the flask.

Parasitoid adults were introduced into the flasks at the time of emergence, and subsequently, egg masses were provided, with approximately 7000 eggs per tube. Parasitism was allowed for 24 h, after which new parasitoids were introduced. After parasitism, the egg masses were placed in plastic tubes for adult emergence (brood maintenance).

2.3. Bioecological Parameters of Telenomus podisi and Trissolcus basalis on Frozen and Fresh Euschistus Heros Eggs

Fresh *E. heros* eggs with up to 24 h of embryonic development (from the broodstock and eggs previously stored in liquid nitrogen) were used to evaluate parasitoid biology. The *E. heros* eggs were glued onto white cards and then transferred to glass tubes (8 cm high × 2 cm diameter) closed with a PVC-type plastic film. Newly emerged females of *T. podisi* or *T. basalis* were introduced at a proportion of 1 parasitoid female to 50 eggs. Over a 24-h period, parasitism was allowed in a climatized chamber at 25 ± 1 °C, 70 ± 10% relative humidity, and a 14-h photophase. After parasitism, the eggs were manually removed and placed in plastic bags, and sets of 20 cards with eggs were transferred to climatized chambers, maintained at 19, 22, 25, 28, 31, or 34 ± 1 °C; 70 ± 10% relative humidity; and a 14-h photophase for observation of parasitoid development. The females remained in the tubes at the same temperatures as described above and were observed daily to determine longevity. The eggs placed in the plastic bags also remained at the same temperatures and were observed daily to determine the duration of development (egg to adult), emergence (viability), the sex ratio (given by the formula: $rs = female/(female + male)$), and the number of individuals per egg mass. To determine the duration of development

(egg to adult), daily observations of *T. podisi* emergence were made. The parasitoid emergence was evaluated under a stereoscopic microscope by counting host eggs that had an exit hole.

A completely randomized design was used in a 2 × 2 × 6 factorial arrangement (parasitoid species × fresh or previously frozen eggs × temperatures) with 20 replications. The variables parasitism (%), emergence (%), sex ratio, and duration of egg–adult development (days) were subjected to analysis of variance (ANOVA), and the experimental residuals were subjected to the Shapiro–Wilk and Bartlett's tests to verify normality and homogeneity of variances. Subsequently, the means were compared using Tukey's test ($p < 0.05$). The ExpDes. package of the R computer application (version 3.5.0) (R Core Team, 2018) was used to perform the analyses of variance and comparison of means.

2.4. Determination of the Thermal Requirements and Estimation of the Number of Generations of Stink Bug Egg Parasitoids

Calculations of the thermal requirements for parasitoid development were estimated using the base temperature (*Tb*) and the thermal constant (*K*), which were obtained from the duration of the development period (egg–adult) at the tested temperatures. The development rates of *T. basalis* and *T. podisi* as a function of temperature were analyzed using linear and nonlinear models (Table 1). The parameters included in each of the models were estimated by the Levenberg–Marquardt method, using the minpack.lm package [16] of the R computer application (version 3.5) [17]. The model with the best fit was selected based on the chi-square test of adherence (χ^2), the adjusted coefficient of determination (adjR^2), the logarithm of maximum likelihood (LogLik), the sum of squared residuals (RSS), and Akaike's information criterion (AIC) and the Bayesian information criterion (BIC) and their respective weights (wAIC and wBIC, respectively).

Table 1. Models used to relate the rate of development to room temperature.

Model	Equation	Definition of Variables	Reference
Linear	$r(T) = a + b \cdot T$	a: line intercept; b: line slope	[18]
Briere1	$r(T) = a \cdot T \cdot (T - T_{min}) \cdot (T_{max} - T)^{\frac{1}{2}}$	T_{min}: minimum temperature T_{max}: maximum temperature	[19]
Briere2	$r(T) = a \cdot T \cdot (T - T_{min}) \cdot (T_{max} - T)^{\frac{1}{b}}$	T_{min}: minimum temperature T_{max}: maximum temperature a, b: constants	
Logan6	$r(T) = \psi \cdot exp^{\rho \cdot T} - exp^{(\rho \cdot T_{max} - \frac{(T_{max} - T)}{\Delta T})}$	ψ: rate of development at temperature above the threshold of development T_{max}: maximum temperature ΔT: high temperature limit layer width ρ: constant	[20]
Lactin1	$r(T) = exp^{\rho \cdot T} - exp^{(\rho \cdot T_{max} - \frac{(T_{max} - T)}{\Delta T})}$	T_{max}: maximum temperature ΔT: high temperature limit layer width ρ, b: constants	[21]
Lactin2	$r(T) = exp^{\rho \cdot T} - exp^{(\rho \cdot T_{max} - \frac{(T_{max} - T)}{\Delta T})} + b$	T_{max}: maximum temperature ΔT: high temperature limit layer width	

The number of parasitoid generations per year in soybean-producing regions was estimated based on the average annual temperature of these locations using the equation $NG = [T(Tm - Tb)/K]$, where *K* is the thermal constant, *Tm* is the average temperature for each locality studied, *Tb* is the base temperature, and *T* is the time in days.

3. Results

3.1. Bioecological Parameters of Telenomus podisi and Trissolcus basalis Reared from Frozen and Fresh Euschistus Heros Eggs

There was a mean parasitism rate of 58.18% for *E. heros* eggs, with no relationship between parasitoid species and temperature (Figure 1A). However, host egg type had an

effect on the parasitism levels between species. Among the parasitoid species, *T. basalis* had a mean parasitism of 65.06%, and *T. podisi* had a significantly lower rate of 49.52%. A difference was also observed between fresh and frozen eggs, with parasitism being greater on fresh eggs for both species across temperatures (50.27 and 60.29% for *T. basalis* and *T. podisi*, respectively) (Figure 1B).

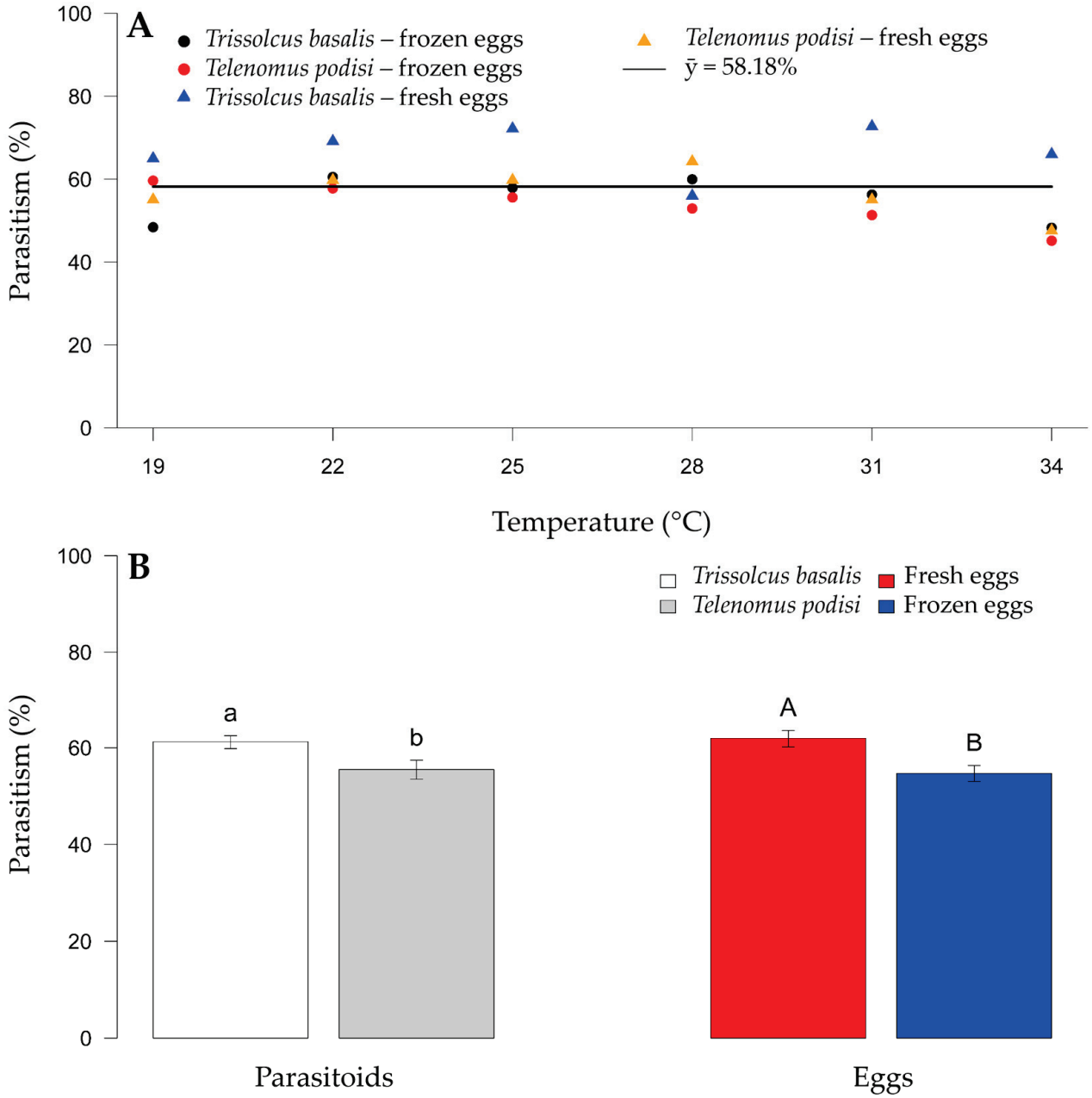

Figure 1. Parasitism of *Trissolcus basalis* and *Telenomus podisi* on frozen or fresh eggs of *Euschistus heros* at different constant temperatures, under laboratory conditions. Average for each combination of parasitoid and type of egg (**A**) (continuous line). Comparison of parasitism between parasitoids and type of egg (**B**). Bars followed by the same letter, lowercase (comparison of parasitoid species) or capital letters (comparison of egg types), do not differ statistically according to the F test ($p > 0.05$).

The duration of the egg-to-adult development of the two parasitoids was inversely proportional to the increase in temperature (Figure 2), and temperature was the only significant variable. The development of *T. basalis* and *T. podisi* on *E. heros* eggs showed no difference in any of the treatments compared at the same temperatures, showing that there was no difference in parasitoid development between fresh and frozen eggs (Figure 2).

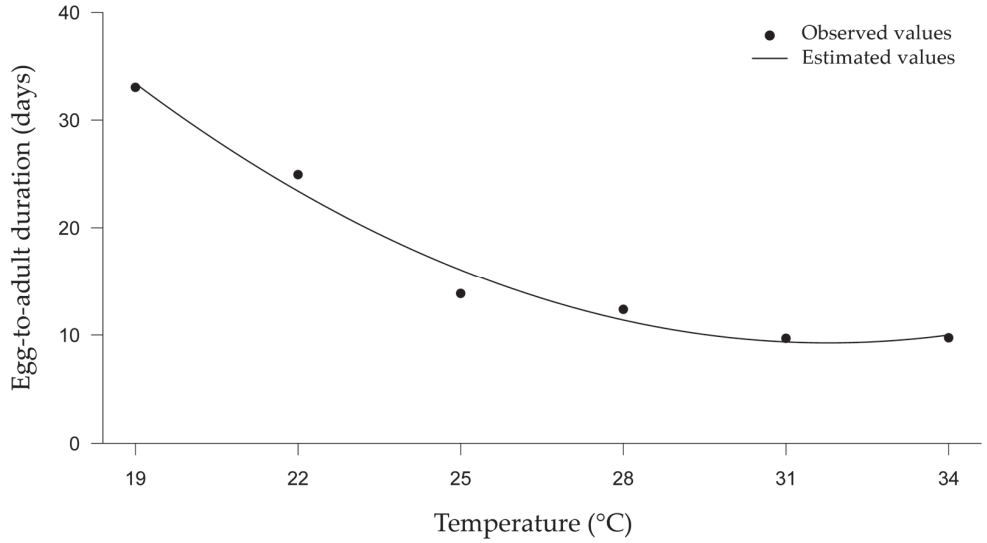

Figure 2. Duration of egg–adult development (in days) of *Trissolcus basalis* and *Telenomus podisi* parasitizing frozen or fresh eggs of *Euschistus heros* at different constant temperatures, under laboratory conditions.

The viability of the parasitized eggs was affected by parasitoid species and temperature and was not influenced by whether the eggs were frozen or fresh. For biological control programs with the release of egg parasitoids, the objective is an emergence of >80% to achieve adequate control. The viability of eggs parasitized by *T. basalis* was lower than that of eggs parasitized by *T. podisi* (Figure 3A). The viability of *T. basalis* was higher between 22 and 28 °C, with adult emergence between 58.31% and 66.54%. The viability of eggs parasitized by *T. podisi* was >81% between 22 and 31 °C, with a variation of 81.79 (31 °C) to 97.5% (28 °C) between these temperatures (Figure 3B). Thus, the viability of *T. podisi* met the criteria to be an effective biological control agent, whereas the viability of *T. basalis* was lower.

There was no interaction among the factors evaluated ($p > 0.05$) for the sex ratio of the emerging adults. When the species were evaluated separately, they also did not differ significantly ($p > 0.05$), whereas the effect of temperature was observed for all biological parameters ($p < 0.05$). Because the sex ratio of parasitoids was not affected (average greater than 0.85) by any of the factors tested, there should be sufficient parasitoids of both sexes for reproduction following release.

The longevity of *T. basalis* and *T. podisi* parasitizing frozen or fresh *E. heros* eggs at different constant temperatures was inversely proportional to the temperature increase. For *T. basalis*, only the treatments at 19 °C and 22 °C significantly differed, with longevities of 100 and 119.4 days, respectively, for frozen and fresh *E. heros* eggs. In contrast, *T. podisi* only had a difference in female longevity at 22 °C, with 116.0 and 55.00 days for frozen and fresh *E. heros* eggs, respectively (Figure 4).

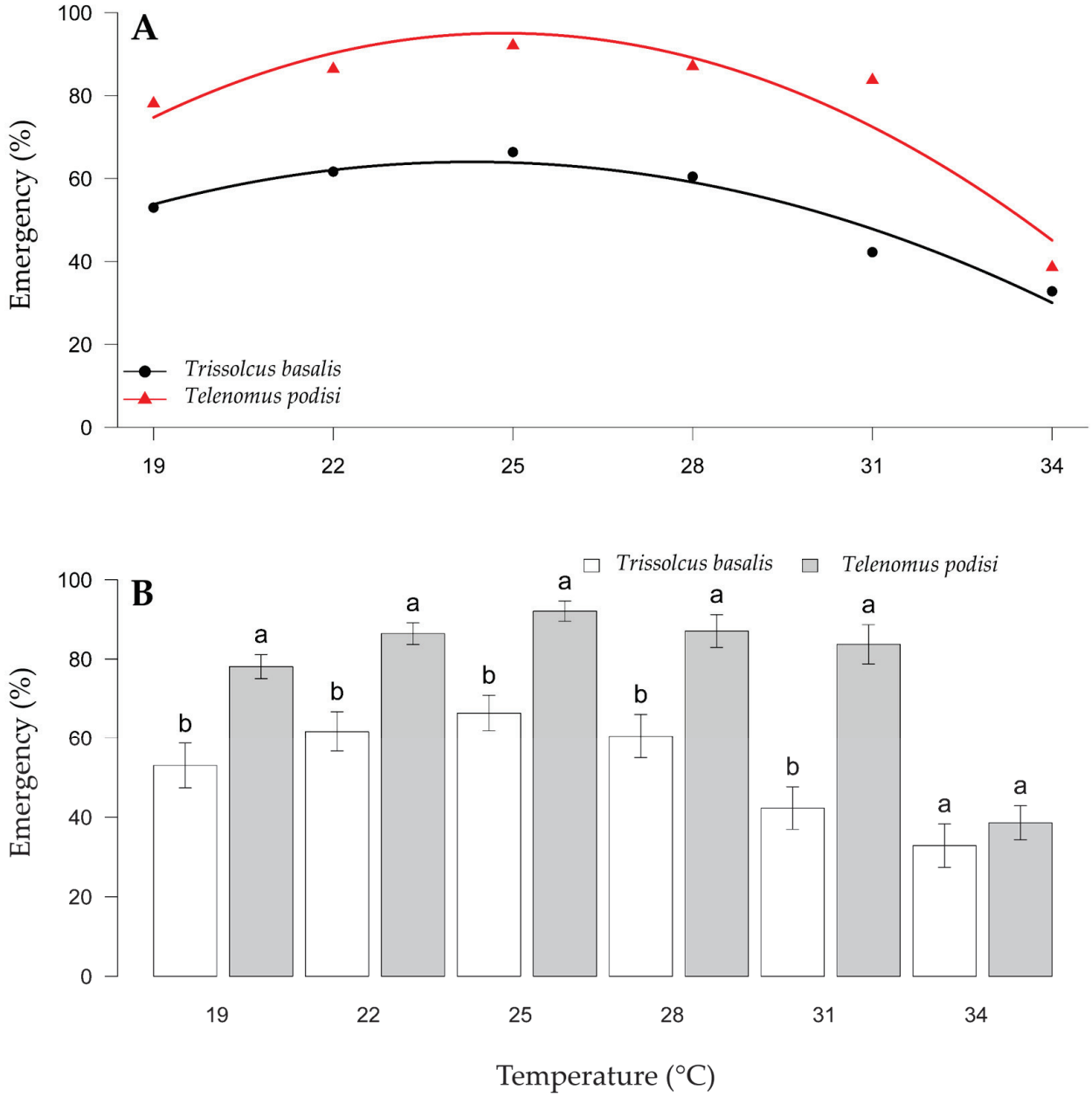

Figure 3. Viability of *Trissolcus basalis* and *Telenomus podisi* in frozen or fresh eggs of *Euschistus heros* at different constant temperatures, under laboratory conditions. Regression analysis examining the temperature effect for each parasitoid (**A**). Comparison of emergence between parasitoids within each temperature (**B**). Averages with lowercase letters (comparison within each temperature) do not differ statistically according to the F test ($p > 0.05$).

3.2. Thermal Requirements Based on Temperature-Dependent Development Ratio and Estimated Number of Generations of Stink Bug Egg Parasitoids

For the development rate from egg to adult for *T. basalis* and *T. podisi*, we determined the lower thermal threshold of development (*Tb*) and the thermal constant (*K*) for fresh and frozen *E. heros* eggs. The linear model had an adjusted R^2 higher than 0.89 for *T. basalis*, with a lower temperature threshold (*Tb*) of 16.23 °C and a thermal constant (*K*) of 145.22 °days. For *T. podisi*, the adjusted R^2 was 0.91, with a lower temperature threshold (*Tb*) of 11.96 °C and thermal constant (K) of 203.47 °days (Table 2 and Figure 5).

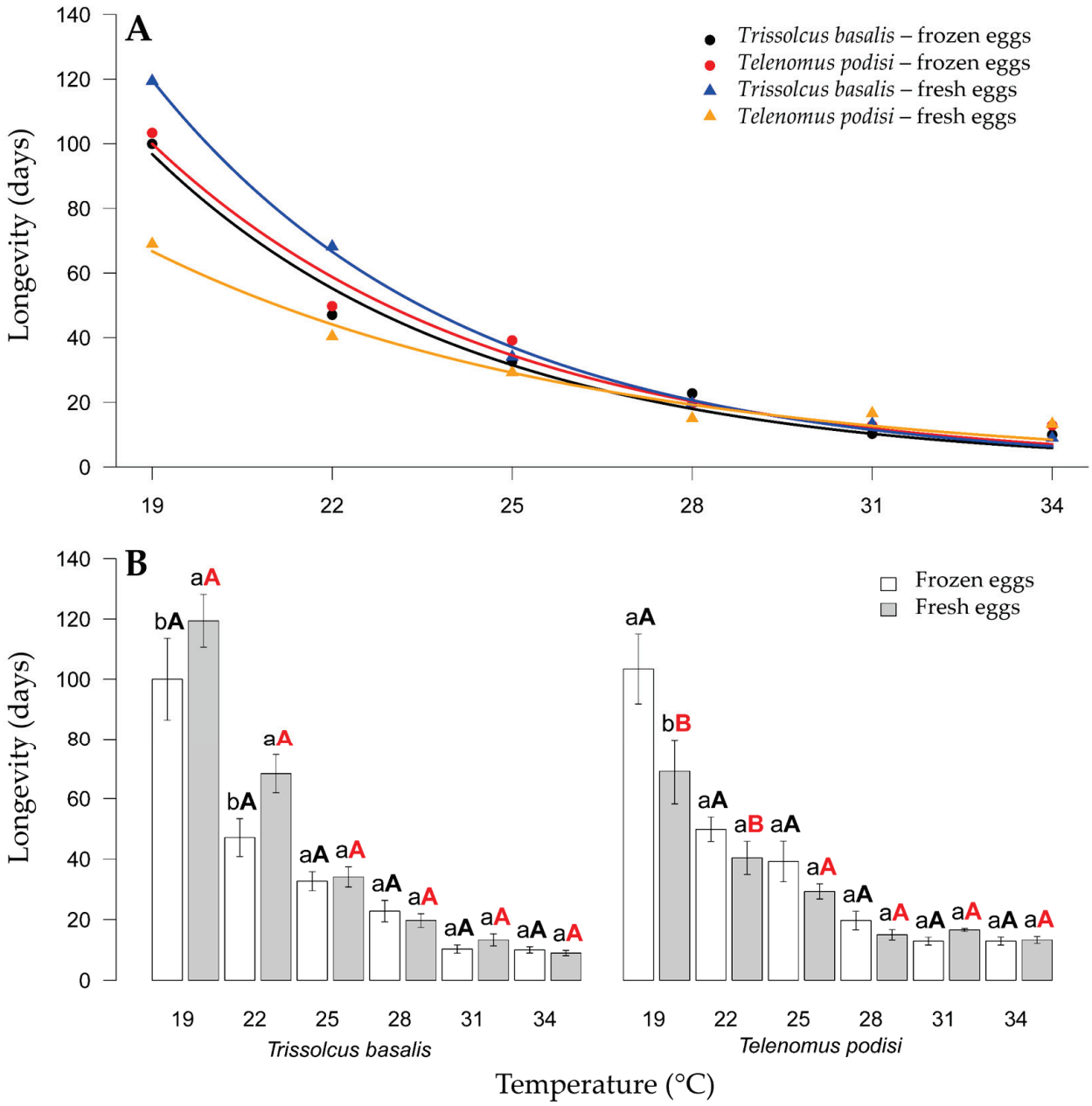

Figure 4. Longevity (days) of *Trissolcus basalis* and *Telenomus podisi* parasitizing frozen or fresh eggs of *Euschistus heros* at different constant temperatures, under laboratory conditions. Regression analysis for each combination of parasitoids and egg types (**A**). Comparison of parasitoid longevity in each combination of temperature and type of egg (**B**). Averages with lowercase letters compare eggs within species, black uppercase letters compare species parasitizing frozen eggs, and red uppercase letters compare species parasitizing fresh eggs. In all cases, the F test was applied for statistical differences ($p < 0.05$).

Table 2. Parameters of the linear regression model and adjusted R^2.

Species	a	SE	b	SE	*Tb*	*K*	R^2aj
Trissolcus basalis	−0.11179	0.030286	0.006886	0.001194	16.23	145.22	0.89
Telenomus podisi	−0.05878	0.018011	0.004915	0.000667	11.96	203.47	0.91

The R^2 was adjusted for the temperature-dependent development ratio for *Trissolcus basalis* and *Telenomus podisi* in eggs of *Euschistus heros*, under laboratory conditions.

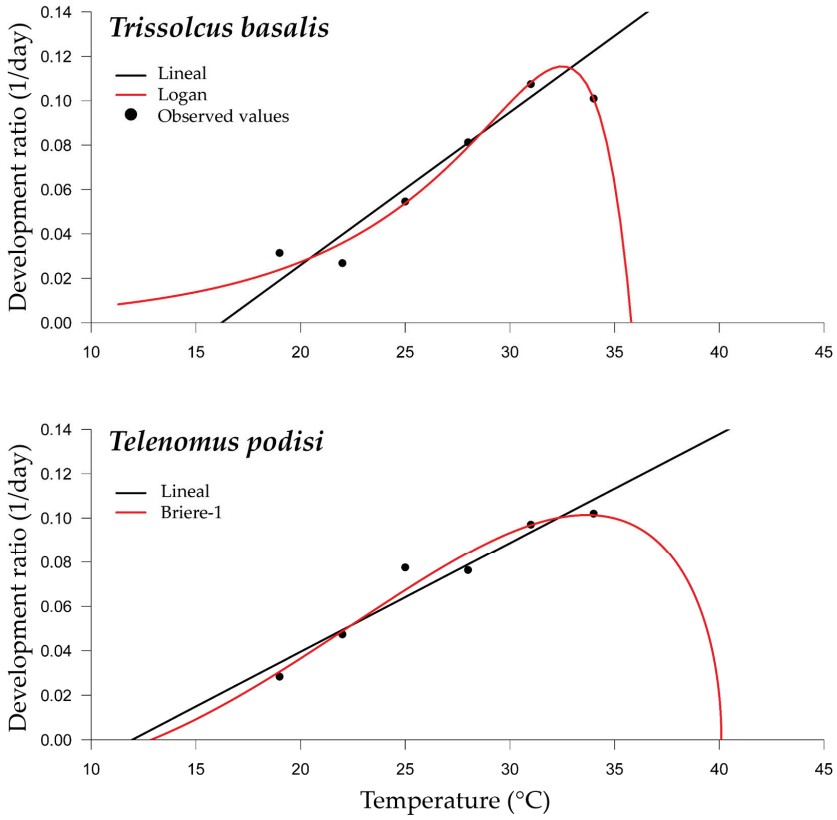

Figure 5. Temperature-dependent nonlinear developmental regression models for *Trissolcus basalis* and *Telenomus podisi* in *Euschistus heros* eggs under laboratory conditions.

Among the nonlinear models tested, the Logan model, for *T. basalis*, and Briere-1 model, for *T. podisi*, presented the best adjustments for predicting the development data (Tables 3 and 4 and Figure 5). The optimal (T_{opt}) and upper (T_{max}) thermal thresholds for development estimated for each parasitoid species were different (Table 4). For *T. basalis*, the T_{opt} estimate was 32.5 °C, while for *T. podisi*, it was 40.0 °C. The T_{max} for *T. bassalis* was 35.8 °C, lower than that of *T. podisi*, which was estimated at 40.1 °C.

Table 3. Criteria for selection of nonlinear temperature-dependent regression models for *Trissolcus basalis* and *Telenomus podisi* in eggs of *Euschistus heros*.

Species	Models	AIC	AIC Weight	LogLIK	R^2adj	RSS	χ^2
Trissolcus *basalis*	Logan6	−36.75	0.83	23.37783	0.9421	0.000145	0.002381
	Briere2	-	-	-	-	-	-
	Briere1	−31.65	0.065	19.82555	0.8718	0.000474	0.00614
	Lactin1	−32.62	0.105	20.31368	0.8388	0.000403	0.004067
	Lactin2	-	-	-	-	-	-
Telenomus podisi	Logan6	-	-	-	-	-	-
	Briere2	-	-	-	-	-	-
	Briere1	−37.70	0.65	22.8512	0.9287	0.000173	0.001205
	Lactin1	−35.43	0.21	21.71791	0.8866	0.000252	0.002162
	Lactin2	−34.55	0.14	22.27867	0.8721	0.000209	0.001573

The insects were subjected to laboratory conditions.

Table 4. Parameters of the nonlinear regression models of the temperature-dependent development ratio for *Trissolcus basalis* and *Telenomus podisi* in eggs of *Euschistus heros*, under laboratory conditions.

Species	Model	Parameters	Estimates	SE
Trissolcus basalis	Logan6	ψ	0.001757	0.0022
		ρ	0.137276	0.053434
		ΔT	1.761947	2.407383
		T_{max}	35.80	1.99
		T_{opt}	32.50	
Telenomus podisi	Briere1	a	0.0000571	0.0000192
		T_{max}	40.10	3.23
		T_{min}	12.86	2.73
		T_{opt}	40.00	

The parameters were evaluated under laboratory conditions.

4. Discussion

The development of both *T. basalis* and *T. podisi* on *E. heros* eggs occurred at all tested temperatures and increased with warmer temperatures. Thus, it is suggested that these parasitoid species can be successfully used in biological control programs throughout the temperature ranges in which crops are grown in Brazil. Although the current assessment was conducted under laboratory conditions at constant temperatures, the authors of [22] found no differences in the biological characteristics of *Trichogramma galloi* (Zucchi) (Hymenoptera: Trichogrammatidae), an egg parasitoid, when reared at constant or fluctuating temperatures. These results indicate that laboratory studies can support field releases, because the insects showed performance similar to that in the laboratory, though caution should be applied.

It is still necessary to evaluate other biological parameters that depend on temperature to project parasitoid performance in the field [15,23]. Host quality is the main factor influencing the sex ratio, which can occur in two different ways. One involves the recognition of hosts of different qualities and then the laying of eggs (male or female) according to the host quality, and the other, which occurs after oviposition, allows the sex ratio to be determined by the developing progeny in response to host quality, with both male and female eggs laid, but one sex surviving competition [24,25].

Another critical factor for parasitoids is the presence of endosymbionts in the female reproductive organs, which may limit reproduction by inducing reproductive incompatibility, feminization, and parthenogenesis [26]. However, for the egg parasitoids, *T. basalis* and *T. podisi*, this needs to be documented before sex ratio verification because the presence of Proteobacteria can lead to erroneous and conflicting results [27]. After the biological control agents are released in plant production fields, they are subjected to different abiotic factors, which may interfere with the sex ratio of the parasitoids and affect the efficacy of pest control.

The estimated number of generations for both parasitoids corresponded to the temperature in warmer areas, reaching 14 to 15 generations/cycle for Rio Verde, GO, and 12 to 14 generations/cycle for Barreiras, BA. Despite using monthly averages for these locations and considering diurnal and nocturnal thermal variations, the modeled results were very close to those obtained under field conditions. Thus, it is likely that similar simulations can be performed for any soybean-growing region of interest, where parasitoids can be released. Temperatures in the regions of Rio Verde, GO, and Barreiras, BA seldom reach the lower threshold (*Tb*) for insects and are often above 24 °C. The amplitude of variations around the averages could influence the parasitoids' biological parameters, resulting in higher development rates or lower viability than those obtained in the laboratory; therefore, additional research on the seasons of parasitoid release should be conducted.

The laboratory-based determination of biological characteristics and thermal requirements provides relevant information for implementing and maintaining a biological control program. The results suggest that released parasitoids can develop throughout the year

under field conditions. Furthermore, the number of generations of these parasitoids indicates that they could significantly impact both stink bug populations, but other variables should be further considered. The reproductive potential should be determined, and the parasitoid's ability to search for host eggs and its habitat preference should be documented because these factors may affect the functional response under field conditions. These effects may be enhanced because of the heterogeneity of areas under soybeans, especially in relation to plant architecture (number of leaves and plant height) compared with other agricultural ecosystems.

5. Conclusions

Our results for the thermal requirements were based on the lower thermal threshold of development (*Tb*) and the thermal constant (*K*) for fresh and frozen *E. heros* eggs for *T. basalis*, with a lower temperature threshold (*Tb*) of 16.23 °C and a thermal constant (*K*) of 145.22 °days. For *T. podisi*, the lower temperature threshold (*Tb*) of 11.96 °C and thermal constant (*K*) of 203.47 °days was determined. The estimated number of generations for *T. basalis* and *T. podisi* corresponded to the temperatures in warmer growing areas, reaching 14 and 15 generations/cycle for Rio Verde, GO, and 12 and 14 generations/cycle for Barreiras, BA, respectively.

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Data Availability Statement: All datasets used or analyzed during this study are included in this article.

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Conflicts of Interest: The authors declare no conflicts of interest.

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Individual and Combined Effects of Predatory Bug *Engytatus nicotianae* and *Trichoderma atroviride* in Suppressing the Tomato Potato Psyllid *Bactericera cockerelli* in Greenhouse Grown Tomatoes

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Abstract: The tomato potato psyllid (TPP) *Bactericera cockerelli* is a serious pest of the Solanaceae family. The management of this pest using synthetic pesticides is problematic because of the development of pesticide resistance and environmental concerns including impacts on non-target organisms. The predatory bug *Engytatus nicotianae* has recently been identified as a useful biocontrol agent for TPP in greenhouses. The soil fungus *Trichoderma* Pers. is commonly used as a plant growth enhancer and biocontrol agent against phytopathogenic fungi. Therefore, there could be advantages associated with the combined use of these biocontrol agents. Some reports in other systems suggest that *Trichoderma* inoculation may alter the behaviour of pests and their natural enemies by modifying plant defence metabolites such as volatile organic compounds (VOCs). For this reason, this study aimed to investigate the individual and combined efficacy of these biocontrol agents (i.e., *Trichoderma atroviride* and *E. nicotianae*) against TPP in greenhouse grown tomatoes (*Solanum lycopersicum* cv. Merlice). To this end, we compared the effect of each biocontrol agent and their combination on TPP abundance across different developmental stages (egg, nymphs, adults) and the number of infested leaves. We also investigated plant VOC emissions under the different treatments. Across all measured TPP stages, the treatments tested (*E. nicotianae* alone, *T. atrovirdae* alone, and *T. atrovirdae* + *E. nicotianae*) significantly reduced mean TPP counts relative to the control, and no significant differences were observed in VOC emissions among treatments. Overall, *T. atrovirdae* alone was less effective than *E. nicotianae* alone and its combination with *T. atrovirdae* in suppressing TPP populations. However, the combined use of *Trichoderma* + *E. nicotianae* did not show significant advantages over the use of *E. nicotianae* alone in controlling TPP. Therefore, their combined use needs to be further assessed in light of other advantages of *Trichoderma* to the crop (e.g., growth promotion or pathogen defence).

Keywords: biological control; gas chromatography–mass spectrometry; natural enemy; pest management; Solanaceae; volatile organic compounds

1. Introduction

The tomato potato psyllid (TPP) *Bactericera cockerelli* (Šulc) (Hemiptera: Triozidae) is a widely recognised pest of several solanaceous crops [1–4]. All TPP nymphal stages and

adults can damage host plants by injecting salivary toxins that lead to foliar symptoms, such as leaf curling and yellowing. This condition was designated by Munyaneza [5] as "psyllid yellows". Moreover, TPP is also a vector of the bacterial pathogen *Candidatus* Liberibacter solanacearum (CLso), which is responsible for zebra chip disease in potatoes [6,7]. Clso can cause the decline and death of infected plants [5], reducing yields and costing growers millions of dollars each year [8,9].

While there is progress towards the reduction in broad-spectrum synthetic insecticide use in New Zealand [10–12] these have not been fully phased out and are still routinely used in other countries [13–16]. Therefore, there is a need for alternative approaches, such as biological control and associated integrated pest management (IPM) strategies to reduce the development of insecticide resistance [17–19], non-target effects [20–22], environmental, and health impacts [23–25].

The predatory bug *Engytatus nicotianae* (Koningsberger) (Hemiptera: Miridae) has recently shown potential as a biological control agent to the extent that it can be used to prevent the establishment of TPP populations in caged greenhouse tomato plants [26–29]. However, it was found that such protection does not always off-set the potential physiological damage resulting from even limited TPP feeding [28].

Trichoderma species are soil-borne fungi commonly used as biocontrol agents against plant pathogens and as plant growth enhancers [30–32]. Different isolates of *Trichoderma* exhibit various mechanisms for their antagonistic effect. These include mycoparasitism, competition with pathogens (including nutrient and niche), antibiosis through fungal volatile and non-volatile compounds, enzyme activity, and changes in plant secondary metabolites with different bioactivities [30,33–36]. Some *Trichoderma* isolates have also been reported to modify the behaviour of phytophagous insects and their natural enemies through the activation of plant-defence pathways, e.g., by altering the emission of plant volatile organic compounds (VOCs) involved in host-finding and selection [37–40]. This suggests that *Trichoderma* may confer additional protection against insect pests.

Given the advantages associated with the individual use of *E. nicotianae* and *Trichoderma,* it would be of interest to explore the potential advantages of their combined use against TPP. However, the effect of *Trichoderma* on plant defence can be variable depending on the host plant, pest organism, biocontrol agent, and biotic and abiotic factors such as temperature and soil nutrients [37,40]. Likewise, VOC emission can be quite system-specific and influenced by biotic and abiotic factors [41,42]. Therefore, it is important to explore the effect of different isolates for specific plant species (or cultivars), pests, and growth conditions when developing a biocontrol and IPM strategy. To this end, the objective of this study was to explore the individual and combined efficacy of two biocontrol agents, *T. atroviride* and *E. nicotianae,* against TPP in greenhouse-grown tomato seedlings, and to explore VOC emissions under different treatments.

2. Materials and Methods

2.1. Seed Inoculation with T. atroviridae and Plant Growth Conditions

Seeds of *Solanum lycopersicum* cv. Merlice were purchased from Kings Seeds (Katikati, New Zealand). One hundred of these seeds were then sent to Agrimm Technologies Ltd., (Lincoln, New Zealand) to be commercially coated with an inert carrier containing spores of a four-strain mix of *T. atroviride* obtained from the Lincoln University Culture Collection (Karst bio-inoculant). These strains had been patented for the biological control of soil-borne plant pathogens and plant growth promotion [43]. The 100 seeds and a further 100 non-coated seeds were sown in a seedling-raising mix in separate 100-cell propagation trays that were placed in a glasshouse at Lincoln University.

When the plants were 15–20 cm high, a total of 42 plants (21 grown from *T. atroviride* coated seeds and 21 from non-coated seeds) were randomly selected and each transplanted into a 6 L pot containing the growing medium. The medium was obtained from a 500 L mix that comprised 400 L of composted bark, 100 L of pumice, 2 kg of *Osmocote*® NPK fertiliser (www.growwithosmocote.com, accessed on September 2023), and 500 g of

horticultural lime. During the transplanting process for *T. atroviridae*-treated plants, pellets containing the four *T. atroviride* strains manufactured by Agrimm Technologies Ltd. were mixed into the growing medium at a rate of 0.3 g/6 L pot (equivalent to 15 kg/ha). For the duration of the greenhouse experiment, single plants were kept in 60 cm × 60 cm × 180 cm cages (BugDorm 6E630; www.bugdorm.com, accessed on September 2023) and trickle-irrigated daily such that each plant received 0.25 L of water. The mean ambient greenhouse temperature was 21.9 °C (max 38 °C; min 15 °C), and mean relative humidity (RH) was 61.6% (max 90.5%; min 30%)

2.2. Experimental Design

The experimental design comprised a randomised complete block design with the following treatments: TPP-only (henceforth, control), TPP + *E. nicotianae*, TPP + *T. atroviride*, and TPP + *E. nicotianae* + *T. atroviride*. These were arrayed in seven blocks. For VOC collection purposes, only two additional treatments were added to each block (uninfested plant and *T. atroviride*-only) to assess the baseline emission of healthy uninfested plants and of plants inoculated with *T. atroviride* in the absence of TPP. Each block contained one cage with a single potted tomato plant for each of the treatments. The cages were laid out in two parallel rows with a main irrigation pipe down the middle lane. The distance between the cages in each block was 30 cm with 1 m between the blocks. Once the pots were placed into cages, a thin 1.8 m support stake was inserted into the centre of the pots and a drip irrigation pipe secured at soil level. A light source (16 h light: 8 h dark) was hung above each block so that the conditions for each were uniform during the experiment. After being placed in the cages, plants were then left to acclimatise for two weeks.

2.3. Infestation of Plants with TPP and Introduction of E. nicotianae

The entomological experimental methodology used in this study was based on that of [28,29] combined with unpublished data obtained from BioForce Limited (a commercial supplier of biological control agents, Karaka, New Zealand; www.bioforce.co.nz, (accessed on 2 November 2023)). All the TPP used were young adults (5–7 days old) and all *E. nicotianae* adults belonged to the same cohort (adults were c. 15 days old, nymphs were c. 7 days old).

After a one-day acclimatisation period (7 December 2021), two healthy TPP males and two healthy TPP females, obtained from a rearing cage at Lincoln University, were placed in each designated cage. *E. nicotianae* adults and nymphs purchased from BioForce, Karaka, New Zealand. *E. nicotianae* were randomly selected from a shipment of 300 individuals; one adult female and two unsexed nymphal *E. nicotianae* were then released into each of the designated cages. The *E. nicotianae* nymphs were unsexed because they are cryptic, very active, and hide when exposed; it was therefore impossible to determine their sex without risking injuring the insect. A second release of both TPP and *E. nicotianae* was made on 16 December 2021 following the same procedure as above.

2.4. Weekly Data Collection

The TPP population within each cage was assessed once per week, between 10 am and 4 pm. During this time, the numbers of TPP eggs, nymphs, adults, and TPP-infested leaves were recorded. To assess the number of TPP (eggs, nymphs, and adults), a 5 min time limit was adopted [28,29], as the exponential growth of TPP (especially in TPP-only treatments) made a full census impractical towards the latter part of the experiment.

2.5. VOC Sampling

Volatile organic compounds (VOCs) were sampled between the 7 and 10 of December 2021, using a dynamic push–pull headspace sampling technique as described by Effah et al. [44,45]. For this experiment, six treatments were used with seven replicates each, as described in the experimental design section. One individual leaf per treatment was enclosed in a multi-purpose 50 cm × 30 cm cooking bag (AWZ Products Inc.,

China) with both ends fastened using a cable tie. Using a portable PVAS22 pump (Volatile Assay Systems, Rensselaer, NY, US), carbon-filtered air was pushed into the bags through a PTFE tube (0.9 L/min) and simultaneously pulled out through another tube (0.8 L/min), creating a slight positive pressure to reduce external contaminants.

To collect the VOCs, a volatile collection trap with 30 mg HayeSep Q adsorbent (Volatile Assay Systems, Rensselaer, NY, USA) was inserted in the pull tube. Collections of the VOCs from each target plant were conducted for two hours under greenhouse conditions. Thereafter, the foliage enclosed in the bags was removed and oven-dried to measure dry weight (grams). The collection filters were subsequently eluted using 200 µL of hexane (95% purity) with 10 ng/µL of nonyl acetate (Sigma Aldrich, Merck KGaA, Darmstadt, Germany) as an internal standard.

The VOC samples were analysed using gas chromatography coupled to mass spectrometry (Shimadzu, Tokyo, Japan) with a 30 m × 250 µm × 0.25 µm TG-5MS column and helium as the carrier gas. Operating conditions were as follows: injector temperature 230 °C; split ratio of 10; initial oven temperature at 50 °C, which was held for 3 min then increased to 95 °C at a rate of 5 °C/min. Tentative identification of compounds was achieved by comparing them with target spectra in the MS library from the National Institute of Standards and Technology (NIST) and, when available, verified using authentic standards (Sigma Aldrich).

2.6. Statistical Analyses

All statistical analyses were conducted using the Stats package in R statistical software version 4.2.2. [46]. A non-parametric Kruskal–Wallis test, followed by Dunn's post-hoc tests was conducted to evaluate whether the treatments had significant effects on average TPP population numbers and infested leaves across the study and differences among treatments. Furthermore, to account for changes in TPP population numbers over time, mixed-effects models were used, where the response variables were assumed to be Poisson-distributed. These Poisson regression models accommodated the 'count' nature of the dependent variables (count of eggs, nymphs, adults, and number of infested leaves). In these models, the blocked design was accounted for by including the block number as a fixed effect. We included random intercepts for each individual plant to account for repeated-measures nature of the experimental design. The treatment groups and time (in days) were evaluated as fixed effects, and treatment *x* time interactions were calculated.

For VOC analyses, the random forest algorithm was used [47]. Random forest is a multivariate statistical tool suited to datasets with more variables than sample size and variables of autocorrelated nature, such as plant volatiles with common biosynthetic pathways. In this case, n = 100,000 bootstrap samples were drawn, with seven (variables) randomly selected at each node (the number of variables selected is based on the square root of all variables). The chance of a random sample being improperly classified is expressed as the out-of-bag (OOB) error rate. Lower OOB values indicate that the treatments differ substantially from one another, allowing the algorithm to classify samples correctly. In contrast, high OOB values suggest that there is poor discrimination among treatments leading to high error in the classification of a sample. It is further possible to identify which of the dependent variables (in this case individual compounds) contribute to separation between treatments. The importance of each compound for the distinction is expressed as the mean decrease in accuracy (MDA). However, this indicator is only relevant if adequate classification scores (low OOB values) are achieved.

3. Results

3.1. Average Rates of TPP Suppression

The average rates of TPP suppression per treatment across the experiment are shown in Figure 1. Across all four measured TPP variables, each of the three treatments significantly reduced the mean TPP counts relative to the control. However, *T. atroviride* alone was significantly less effective than either *E. nicotianae* or the combined treatment. Moreover,

the combined treatment was no better overall than *E. nicotianae* alone (i.e., *T. atroviride* did not improve the average performance of the *E. nicotianae* treatment across the sampling period).

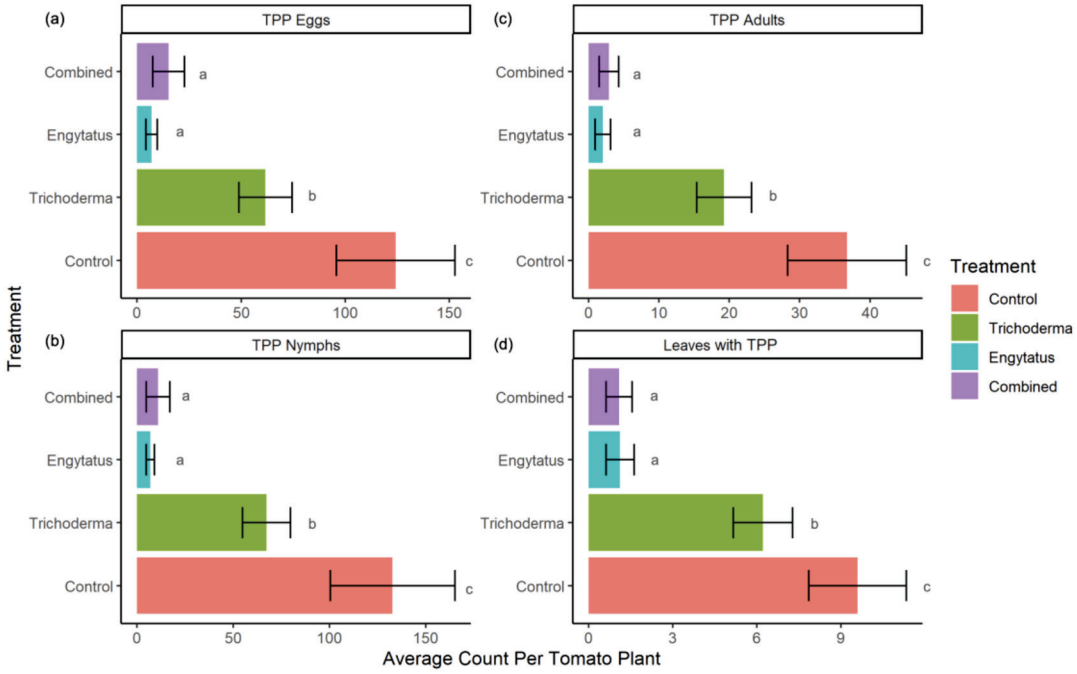

Figure 1. Average counts of *Bactericera cockerelli* (TPP) (**a**) eggs, (**b**) nymphs, (**c**) adults, and (**d**) number of leaves infested with TPP per tomato plant by treatment group. Error bars indicate ± SEM. Control = plants infested with TPP without a biocontrol agent; *Trichoderma* = *T. atroviride*; *Engytaus* = *E. nicotianae;* Combined = *T. atroviride* + *E. nicotianae*. Letters indicate significant differences ($p < 0.10$) among treatments after Kruskal–Wallis test followed by Dunn's post hoc tests.

3.2. Comparison of TPP Growth Rates

We calculated the growth rates of the TPP stages under all treatments throughout the experiment. The resulting growth curves are plotted in Figure 2. A mixed-effects model based on the Poison distribution showed the significant effects of *E. nicotianae*, the combined treatment, and time (in days) on the different growth stages of TPP vs. the control (Table S1). However, the interaction effect of treatment *x* time was variable and was only significant across all measured parameters for *E. nicotianae* (Table S1). In contrast, the use of *T. atroviride* alone showed no interaction with time (except for the nymphal stages).

We further explored the effects of the treatments on the daily population growth rates of TPP and daily percentage of TPP-infested leaves over the duration of the experiment (Table 1). Here, *E. nicotianae* was found to consistently reduce the number of infested leaves and daily TPP population growth rates of all developmental stages. *T. atroviride* alone was found to have had little suppressive effect on the growth rates of TPP eggs and adults, and TPP-infested leaves, although it caused a significant reduction in the population growth rate of TPP nymphs. In contrast, the combined treatment significantly reduced all measured parameters except daily nymph population growth.

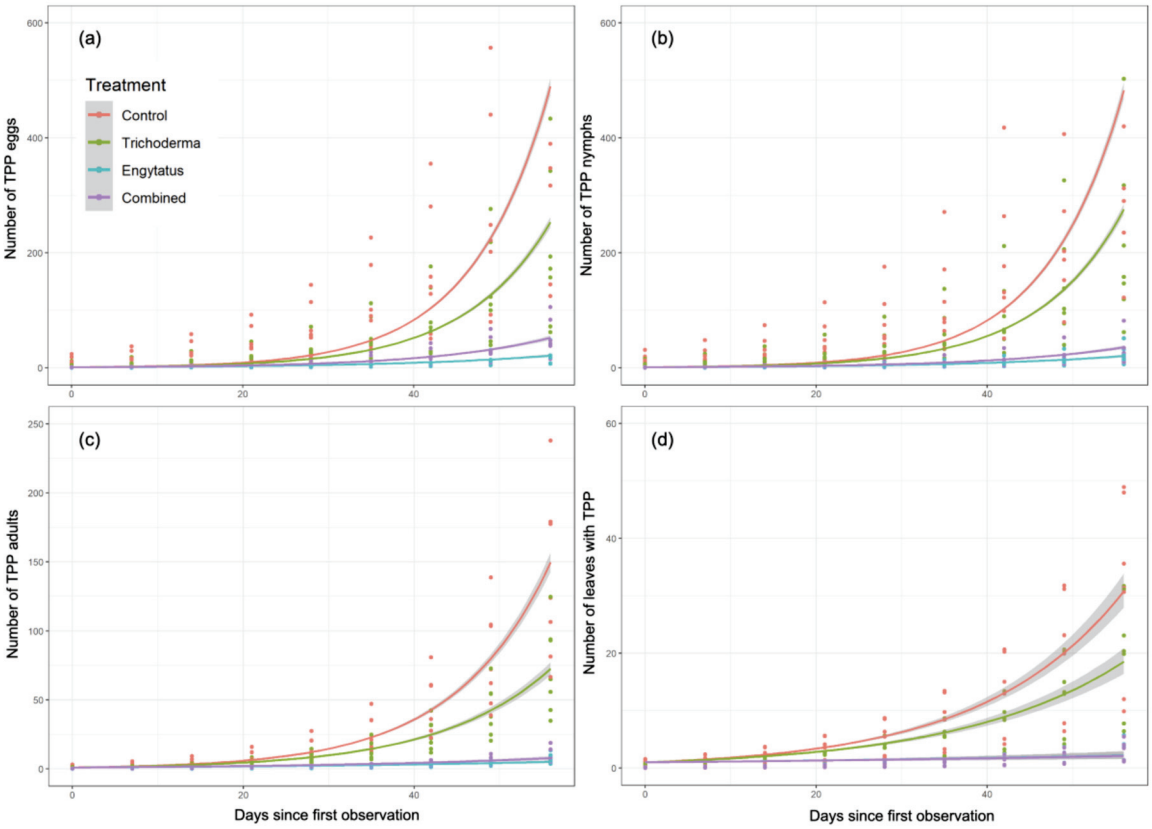

Figure 2. Poisson-distributed counts for *Bactericera cockerelli* (TPP) (**a**) eggs, (**b**) nymphs, (**c**) adults, and (**d**) number of leaves infested with TPP per treatment over the observation period. Control = plants infested with TPP without a biocontrol agent; *Trichoderma* = *T. atroviride*; *Engytaus* = *E. nicotianae;* Combined = *T. atroviride* + *E. nicotianae*.

Table 1. *Bactericera cockerelli* (TPP) daily population growth rates per developmental stage and daily percentage of TPP infested leaves per treatment.

Treatment	Eggs	Nymphs	Adults	Leaves with TPP
Control	6.82%	6.82%	8.00%	6.72%
Trichoderma	6.88%	6.18% ***	8.44%	6.61%
Engytatus	3.25% ***	1.11% ***	5.44% ***	4.50% **
Combined	5.87% ***	7.14%	6.72% *	3.98% ***

Control = plants infested with TPP without a biocontrol agent; *Trichoderma* = *T. atroviride*; *Engytaus* = *E. nicotianae;* Combined = *T. atroviride* + *E. nicotianae.* Significance asterisks are in relation to the control growth rate. * $p < 0.10$, ** $p < 0.05$, *** $p < 0.01$.

3.3. Treatment Effect of on Plant VOC Emissions

Thirty-three compounds were tentatively identified and quantified in the collected samples (Table S2). β-Phellandrene and 2-Carene were the most abundant compounds in all samples. Healthy, uninfested plants had, on average, the highest volatile organic compound (VOC) emissions, while plants infested with TPP in the absence of biocontrol agents had the lowest VOC emissions (Figure 3a). However, univariate statistical analysis of the total VOC emissions (ANOVA) showed no significant differences in the total VOC emission among treatments (N = 7, F = 0.551, $p = 0.737$).

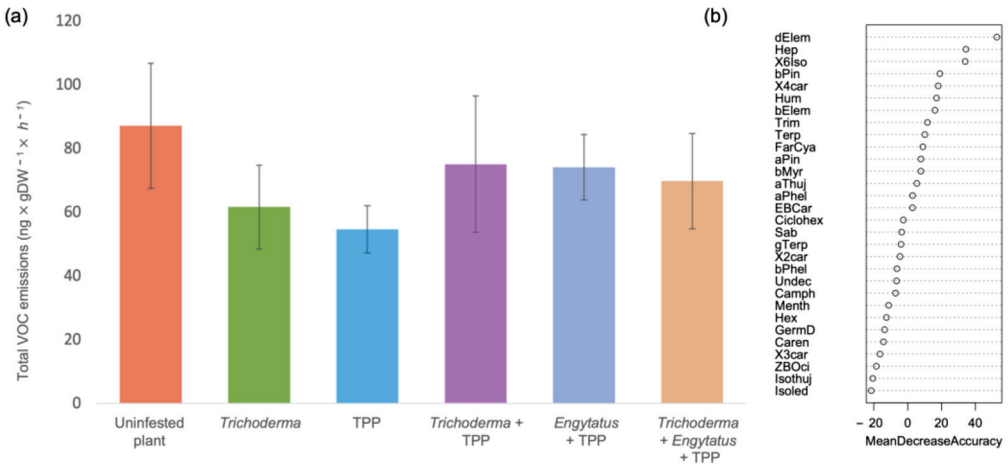

Figure 3. (**a**) Total volatile organic compound (VOC) emissions from tomato plants under different treatments. Error bars indicate ± SEM. Uninfested plant = healthy plant without TPP or a biocontrol agent (salmon); *Trichoderma* = *Trichoderma atroviride* (green); TPP = *Bactericera cockerelli* (blue); *Trichoderma* + TPP = *T. atroviride* and *B. cockerelli* (magenta); *Engytaus* + TPP = *E. nicotianae* and *B. cockerelli* (lilac); *Trichoderma* + *Engytatus* + TPP = *T. atroviride*, *E. nicotianae* and *B. cockerelli* (light orange). (**b**) Mean decrease in accuracy ranking for compounds identified on the tomato headspace samples. Top-ranked compounds: δ-eIemene (dElem), heptane (Hep) and 6-isopropylidene-1-methylbicyclo [3.1.0]hexane (X6Iso). A full list of compounds with their abbreviations is provided in Table S2.

When comparing the entire volatile blends, a random forest analysis revealed a very high out-of-bag (OOB) error rate (83.33%) showing poor separation between treatments. The mean decrease in accuracy (MDA) values (Figure 3b) suggested that δ-eiemene, heptane and 6-isopropylidene-1-methylbicyclo [3.1.0]hexane could have played a role in the separation between treatments (Figure 3b), but individual compound exploration using ANOVA did not yield significant differences among treatments for these compounds.

4. Discussion

In this study, we explored the independent and combined effect of two biocontrol agents (*T. atroviride* and *E. nicotianae*) on suppressing populations of tomato potato psyllid (TPP). Both biocontrol agents and their combination had a significant effect in reducing TPP populations at different developmental stages (egg, nymph, and adults) and the number of infested leaves when compared to the control. However, the treatments containing the predatory bug and *T. atroviride* were more effective than using *T. atroviride* alone.

Previous studies have shown the potential of the predatory bug *E. nicotianae* in controlling TPP under greenhouse conditions [26–29]. However, its use alone may not be enough to manage established populations. Therefore, it was suggested that it could be used in combination with another biocontrol agent to enhance protection against TPP [28] but simultaneous use of biocontrol agents is not always positive and can result in interference, e.g., [48–51]. In this study, we observed excellent results when the predator was used in early phases of TPP establishment, and it retained its effect, even when a fungal biocontrol agent was applied simultaneously, suggesting both agents can be safely used together to reduce TPP populations. However, there seems to be no added benefit in their simultaneous use to control TPP.

To assess whether there is an economic advantage in using both biocontrol agents, we recommend further studies using a similar experimental design taking into consideration other response variables such as plant growth and yield. Growth promotion and enhanced pathogen protection have been associated with *Trichoderma* use in other systems [30–32]. However, they have been seldom explored in a setting where *Trichoderma* is used alongside a pest insect and its natural enemy, which more closely resembles real crop conditions.

The observed reduction in the number of TPP eggs, nymphs, and adults, and decreased number of TPP infested leaves when using *T. atroviride* is consistent with other observations. For example, *Trichoderma atroviride* strain P1 was tested against two pests with different feeding habits on tomato plants, a leaf-chewing noctuid moth (*Spodoptera littoralis*) and a phloem-feeding aphid (*Macrosiphum euphorbiae*). In both cases, *Trichoderma* inoculation resulted in pest reduction. The authors suggested different mechanisms for both pests. In the case of aphids, a direct reduction was associated with the up-regulation of genes involved in the oxidative burst reaction early in the defence response, while the effect on the moth was linked to the enhanced expression of protective enzymes downstream in the defence cascade, e.g., proteinase inhibitors [38]. The authors also reported an indirect effect through increased attraction of the aphid parasitoid *Aphidius ervi* due to an increase in emission and de novo production of plant VOCs [38].

In this study, we did not observe an increase in foliar VOC emissions using *Trichoderma* that could be linked to increased attraction of the natural enemy, so we assume that the observed effects on TPP are linked mainly to direct effects on the pest (probably similar to those observed for the aphid *M. euphorbiae*). These contrasting results are not surprising, as there is evidence that the *Trichoderma* effects on plants are system-specific and depend on abiotic and biotic factors. For instance, a study on tomato using *T. afroharzianum* T22 and *T. atroviride* P1 showed differential induction of plant defence responses against *M. euphorbiae* and *S. littoralis*, and temperature-dependent effects [37]. Furthermore, biotic and abiotic factors can lead to plants producing highly plastic VOC blends [44,45,52].

Interestingly, we observed lower (albeit not significant) VOC emissions in TPP-infested plants (without biocontrol agents). While chewing herbivores often induce volatile emission, this is not always the case with phloem feeders, e.g., [53–55]. Some phloem feeders may suppress plant signalling and defence responses through their endosymbionts [56–58]. In fact, TPP is known to manipulate plant responses through its associated endosymbiont *Candidatus* Liberibacter psyllaurous [59]. Therefore, the apparent reduction in VOCs after TPP attack observed here is not surprising and requires further investigation.

The impact of *Trichoderma* on other natural enemies that can provide TPP biocontrol in this system (e.g., parasitoid *Tamarixia triozae*) must also be investigated, since natural enemies vary in their sensitivity and attraction to plant VOCs [60–62], and the possibility remains that highly sensitive parasitoid antennae may respond to minor blend variations or minor compounds in the VOC blend [63]. The role of previous experience and learning in parasitoid and predator responses to plant VOCs (and other cues) could also be further studied [64–67].

In general, it is important to note that plants grown under greenhouse conditions, as described in this contribution, are often optimally resourced. They may opt for prioritising growth, reproduction, or other forms of defence, beyond the effect of volatile emissions at low infestation densities [68–70]. To test this, further experiments could be conducted using different herbivore densities/damage levels and varying soil nutrient conditions.

5. Conclusions

Both biocontrol agents (*E. nicotianae and T. atroviride*) suppressed TPP populations respective to the control when used alone and in combination. *E. nicotianae* alone and its combination with *T. atroviride* were significantly more effective in reducing initial TPP numbers than *Trichoderma* alone, but there was no significant difference among these treatments. We found no indication of *Trichoderma*-induced changes in plant VOC emissions that could potentially lead to increased natural enemy recruitment. Therefore, at least under the conditions described here, there seems to be little advantage in combining *E. nicotianae* and *Trichoderma* to suppress TPP in greenhouse tomato crops. However, other advantages of the use of *Trichoderma* such as enhanced resistance to pathogens and growth promotion were not considered here, and these may add value to the combined use of both agents. Hence, further research considering other aspects of *Trichoderma* use in this system are needed to support its use alone or in combination with other biocontrol agents.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13123019/s1, Table S1: Mixed-effects model using Poisson regression of TPP counts for eggs, nymphs, adults, and TPP- infested leaves, including the individual effect of each treatment, time, and their interactions.; Table S2: Tentative identification and quantification of compounds present in the headspace samples of tomato plants (ng $\times$ gDW^{-1} $\times$ h^{-1}). UIP = uninfested plant, TRI = Trichoderma atroviride, TPP = tomato pPotato psyllid (Bactericera cockerelli), E= Engytatus nicotianae (predatory bug).

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agronomy

MDPI

Perspective

Natural-Enemy-Based Biocontrol of Tobacco Arthropod Pests in China

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Abstract: The devastating impact of chemical pesticides has prompted a shift towards sustainable agricultural pest management, such as biological control with natural enemies. In recent years, commercialization advancements have enabled the suppression of pest populations through augmentative releases of biological control agents, with natural enemies being a major tactic. China has successfully implemented natural-enemy-based biocontrol strategies, particularly for controlling aphids and lepidopterans. This article provides a comprehensive overview of the state-of-the-art natural-enemy-based biocontrol against arthropod pests in tobacco in China, including practical achievements in mass-rearing methods, augmentative release strategies, and the wide-scale use of natural enemies. Current and potential future challenges for natural-enemy-based biocontrol in China are also discussed.

Keywords: pest management; natural enemy; biocontrol; mass rearing; augmentative release

1. Introduction

Arthropod pests, including insects and mites, pose a major challenge to agriculture worldwide, causing substantial loss to global crop production [1]. Herbivorous arthropods, in particular, can cause yield losses of up to 20%, which can have a significant impact on sustainable crop growth [2,3]. In China, tobacco (*Nicotiana tabacum* L.) is a non-food crop of great economic importance, with a cultivation area of 9.4 billion hm^2 in 2020 [4]. However, arthropod pests have a significant impact on the yield and quality of tobacco leaves, resulting in substantial economic losses (as shown in Table 1).

Table 1. Common arthropod pest species in tobacco field in China.

Species	Damage Parts	Hazardous Degree *
Lepidoptera		
Helicoverpa assulta	leaf, bud, flower, fruit	H
Heliothis armigera	leaf, bud, flower, fruit	H
Spodoptera litura	leaf, bud	H
Mamestra brassicae	leaf	L
Spodoptera exigua	leaf	L
Mythimna separata	leaf	L
Agrotis ipsilon	stem, leaf	H
Agrotis tokionis	stem, leaf	L
Agrotis segetum	stem, leaf	L
Phthorimaea operculella	leaf	M
Scrobipalpa heliopa	stem, leaf	L
Peridroma saucia	leaf	L

Table 1. *Cont.*

Species	Damage Parts	Hazardous Degree *
Hemiptera		
† *Bemisia tabaci*	leaf	H
† *Myzus persicae*	stem, leaf, bud, flower	H
Trialeurodes vaporariorum	leaf	L
Cyrtopeltis tenius	leaf	L
Nezara viridula	leaf, stem	L
Dolycoris baccarum	leaf, stem, fruit	L
Coleoptera		
Pleonomus canaliculatus	root, stem	L
Agriotes fuscicollis	root, stem	L
Opatrum subaratum	root, stem, leaf	L
Maladera formosae	leaf, root	L
Maladera orientalis	leaf, root	L
Holotrichia oblita	root, stem, leaf	L
Holotrichia parallela	root, stem, leaf	L
Anomala corpulenta	root, stem, leaf	L
Popillia quadriguttata	root, stem, leaf	L
Henosepilachua vigintioctopunctata	leaf	L
Orthoptera		
Gryllotalpa orientalis	seed, root, stem	L
Gryllotalpa unispina	seed, root, stem	L
Thysanoptera		
Thrips flavidulus	leaf, flower	L
† *Frankliniella occidentalis*	stem, leaf, flower, fruit	M
Thrips tabaci	leaf, flower	M
Mollusca		
Agriolimax agrestis	leaf	L

† vectors of virus transmission; * hazardous degree: classification of hazardous degree according to annual yield loss caused by pests. H: high, M: medium, and L: low; information was adopted from Wang et al. (2018) [5].

In the past few decades, chemical pesticides have been the primary method of controlling arthropod pests. However, this approach has led to the development of widespread pesticide resistance, as well as the contamination of agroecosystems and negative health effects [6]. Therefore, Integrated Pest Management (IPM) should focus on more sustainable approaches, such as physical, cultural, or biological controls. While physical and cultural controls that rely on engineering techniques demand more labor and time, their implementation is often limited to specific treated areas, which restricts their effectiveness in the field-crop market [7]. As such, biological control is a main contributor to sustainable agriculture, which involves using one species or biological agent to control the population size of another species [8,9]. Predators and parasitoids are among the core components of biological control agents used in agroecosystems [10–12]. China has a rich diversity of natural enemies for arthropods, with 283 natural enemy species from 71 families known to control sugarcane pests [13,14]. For example, species from the genus *Trichogramma* have been successfully used to control Lepidopteran pests [15].

Three primary methods for manipulating predator and parasitoid densities are classical, conservation, and augmentative biological control, as outlined by Van Lenteren et al. (2006) [16]. Classical biological control involves releasing natural enemies collected from the pest's area of origin into invasive areas, resulting in permanent pest population reduction. Conservation biological control seeks to protect and enhance the performance of naturally occurring natural enemies [17]. Augmentative biological control involves mass-rearing and releasing natural enemies, either in large numbers for immediate control in crops with short production cycles (inundative biological control) or over several generations in crops

with long production cycles (seasonal inoculative biological control) [18]. This strategy is becoming an increasingly popular and important option for mitigating economic losses caused by pest damage worldwide [18]. The small populations of natural enemies in China have limited their ability to significantly control tobacco pests [19]. Moreover, the potential breakdown of current biological control agents and the predicted increase in pest outbreaks due to global climate change have further reduced the effectiveness of natural enemies in pest control [20–24]. In more detail, the occurrence of warmer winters or a decrease in the frequency of deep frosts is resulting in a rise in pest outbreaks [22–25]. Moreover, pests are spreading into regions that are lacking their natural enemies, while climate change, host plants, herbivores, and farmers' adaptive management strategies are altering the abundance and activity of natural enemies. These spatial and temporal mismatches between pests and their enemies may reduce the effectiveness of these biocontrol measures [21]. However, the augmentative releases of biological control agents partially compensate for these drawbacks and have proven to be a successful strategy in maintaining high densities of natural enemies, even in suboptimal conditions [18]. Currently, over 150 species of natural enemies are available for augmentative releases, allowing for the control of approximately 100 pest species [26]. Since the 1970s, China has made significant progress in exploring augmentative releases of biological control agents, including the use of the *Trichogramma* species [15].

In this article, we present a comprehensive review of the practical knowledge and achievements gained through the development of natural-enemy-based augmentative biological control programs in tobacco-growing regions of China. Furthermore, we highlight the successful implementation of natural enemies on a wide scale and identify the key traits that contribute to effective biological control. We believe that our findings will renew interest in natural-enemy-based methods both in China and in other countries.

2. Major Pests and Potential Natural Enemy Species on Tobacco

To date, over 700 species of arthropod pests have been identified in Chinese tobacco fields and storage facilities [27]. The common species are listed in Table 1, among which the major pests are *Helicoverpa assulta* (Guenée) (Lepidoptera: Noctuidae), *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae), *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae), *Agrotis ipsilon* (Hufnagel) (Lepidoptera: Noctuidae), *Myzus persicae* (Sulzer) (Hemiptera: Aphididae), and *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae).

A survey conducted in a tobacco-growing region of China revealed the presence of 743 natural enemy species belonging to 273 genera and 27 families [28]. Table 2 presents a list of arthropod species that have undergone successful mass-rearing for large-scale application in China. This list includes one parasite (*Aphidius gifuensis* (Ashmead) (Hymenoptera: Braconidae)) and three predators (*Arma chinensis* (Fallou) (Hemiptera: Pentatomidae), *Amblyseius cucumeris* (Oudemans) (Acari: Phytoseiidae), and *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae)), as well as predators with the potential for future development in pest management, such as: *Eocanthecona furcellata* (Heteroptera: Pentatomidae) and *Habrobracon hebetor* (Say) (Hymenoptera: Braconidae). In the following section, we will delve into several biocontrol cases that utilize natural enemies to showcase China's efforts and achievements in managing tobacco arthropod pests.

Table 2. Natural enemy species against tobacco arthropod pests.

Order	Species	Target Pests
Predators		
Arachnida	*Amblyseius cucumeris* *Amblyseius mckenziei* *Neoseiulus barkeri*	mites and thrips

Table 2. *Cont.*

Order	Species	Target Pests
Coleoptera	*Coccinella septempunctata* *Harmonia axyridis* *Propylea japonica* *Adonia variegata*	aphids and whiteflies
	Pheropsophus javanus *Microcosmodes flavospilosus* *Cylindera kaleea* *Cicindela aurulenta*	lepidopterans
Diptera	*Eupeodes corollae* *Episyrphus balteatus* *Sphaerophoria scripta* *Melanostoma scalare* *Aphidoletes aphidimyza*	aphids
	Atylotus bivittateinus	lepidopterans
Hemiptera	*Arma chinensis* *Rhynocoris fuscipes* *Eocanthecona furcellata* *Orius sauteri* *Harpactor fuscipes* *Sphedanolestes impressicollis*	lepidopterans, hemipterans, homopterans, etc.
Mantodea	*Tenodera sinensis* *Hierodula patellifera*	lepidopterans
Neuropera	*Chrysopa pallens* *Chrysoperla nipponensis* *Chrysopa formosa*	aphids, stinkbugs, lepidopterans
Parasites		
Hymenoptera	*Aphidius gifuensis* *Diaeretiella rapae*	aphids
	Habrobracon hebetor	lepidopterans (Store-product pest)
	Encarsia formosa	whiteflies
	Campoletis chlorideae	lepidopterans

3. Study of Biocontrol Cases

Over the last decade, China has witnessed a steadfast commitment to natural enemies in the control of economically significant pests on tobacco. This dedication has yielded several noteworthy successes in natural-enemy-based biocontrol against tobacco's key arthropod pests. These triumphs primarily stem from two factors: mass-rearing production and release strategies. Establishing mass production systems is a crucial prerequisite for achieving effective and cost-efficient large-scale releases in the field. Additionally, the success of release strategies is closely tied to the control effect.

3.1. Case 1: Aphidius-Gifuensis-Based Biocontrol

A. gifuensis is a versatile endoparasitoid that can parasitize various species of aphids. It is the most commonly used biological control agent for managing *M. persicae* on tobacco crops in China due to its high parasitism rate, reaching over 85% in the field [29,30]. The study and application of *A. gifuensis* for aphid control in China began in the early 1970s. Since 2010, *A. gifuensis* has been widely promoted and utilized in the tobacco system of Yunnan Province [31]. Since 2019, this biological control agent has been adopted as the main technology for aphid management, leading to a decrease in pesticide usage. Through systematic research on the biological and ecological characteristics of *A. gifuensis*, China

has accumulated extensive experience in breeding and applying this species. Furthermore, a national standard, "Code of Practice for *Myzus persicae* (Sulzer) Biological Control with *Aphidius gifuensis*," was released in 2019. This standard specifies the technical requirements for breeding *A. gifuensis* for the management of *M. persicae*, including *A. gifuensis* breeding, *M. persicae* release, investigation of control effects, seed conservation, and more.

3.1.1. Mass-Rearing Production

Most of the mass-rearing techniques for *A. gifuensis* have been mechanized and/or automated.

In certain tobacco planting areas in China, the mass-rearing of *A. fumigatus* is carried out in greenhouses located near the fields. This involves cultivating aphid host plants, propagating *M. persicae*, inoculating parasitoids, and selecting and collecting mummified aphids, *M. persicae*, and *A. gifuensis* for preservation [31,32]. This method offers the advantages of convenient transportation, detachable structures, intelligent controls of environmental conditions, and immediate release of parasitoids after production. However, the cost of mass-rearing on potted tobacco in greenhouses is high. To address this issue, a study on floating tobacco seedlings and the simultaneous inoculation method was conducted. This method is characterized by a short cycle, low cost, and high efficiency in feeding *M. persicae* [33–35]. In regions with specific climate characteristics such as seasonal rainfall and low temperature, the growth of tobacco and propagation of *A. gifuensis* may be greatly restricted. In such cases, the floating radish seedlings and simultaneous inoculation method can be a targeted solution [29]. Radish is a semi-cold-tolerant vegetable that supports the short growth and development period and the fast propagation of *M. persicae*. Moreover, the technique of simultaneous inoculation of *M. persicae* with *A. gifuensis* simplifies the breeding process to some extent [36].

A. gifuensis can be stored and transported in two forms: adult *A. gifuensis* or mummified parasitized aphids. Due to the strong activity and short life span of adults, storage and transportation conditions are more demanding and costly. Therefore, it is suggested to carry on packing transport in the form of mummified aphids. A high degree of mechanization and automation has been achieved in the separation, drying, impurity removal, collection, counting, and packaging of mummified aphids. Through multiple generations of iterative updates, a range of devices and machines have been introduced for application [37], namely the digital image recognition and counting system, an assembly line separating and washing device to collect the mummified aphids, an electrical device to dry the mummified aphids, an automatic selection machine to remove impurities, and an automatic quantitative divider to put the mummified aphids into the box with the specific packaging specifications [38–45]. To sum up, this equipment would greatly increase the mummified aphid efficiency; save money, time, and labor with a fully automatic operation; and keep the damage rate of mummified aphids at a low level.

Other key factors that restrict the industrial application of *A. gifuensis* are long-term storage and long-distance transportation. In the south and the north, the generation number of *A. gifuensis* is completely different, so the parameter setting up should be studied in different ways according to local conditions. A large number of targeted research also enables the natural enemy produce companies to master the rich experience of temperature and humidity preservation transportation [46–51].

3.1.2. Field Application

The release of the natural enemy is affected by the host crop, environment, and other factors. The amount and way of releasing are also different with different control objects. Additionally, to achieve a better aphid control effect, the augmentative release strategies of *A. gifuensis* are continuously explored. Several strategies have been developed: the release of an adult wasp *A. gifuensis*, hanging the card of mummy aphids, naturally scattering them field sheds, releasing them by the cage of *A. gifuensis*, etc. For example, the population of aphids can be controlled in a short time by the release of adult *A. gifuensis*. But it also

has some limitations, such as the high mortality rate of *A. gifuensis* during the release process and that the control effect can be achieved only after multiple releases [52]. The auto-sustained continuous release through naturally scattering them by filed *A. gifuensis* breeding sheds and releasing them by the cage of *A. gifuensis* could perform better on the persistence control of aphids. Hence, the combination of different release methods should be considered in the application to achieve a better prevention effect [53]. Moreover, suitable release strategies (the release number and times of *A. gifuensis*) for different regions or situations have been further studied (Table 3) [53–56].

Table 3. The reference standard of the quantity of released *Aphidius gifuensis* based on the occurrence degree of *Myzus persicae*.

Number of *M. persicae* per Plant	The Quantity of Released *A. gifuensis* per Hectare	The Release Mode
1–5	3000–7500	dot scope [a]
6–20	7500–15,000	planar scope [b]
21–30	15,000–18,000	regional scale [c]

[a] *A. gifuensis* was released from the *M. persicae*-infected plants. [b] *A. gifuensis* was released to the parcel of field found with *M. persicae*. [c] *A. gifuensis* was released to the region found with *M. persicae*.

Regarding field application scales, in 2018, the national promotion area reached 890,000 hm^2, covering more than 99 percent of the total tobacco planting area [57] and obtaining an excellent control effect (82.2%). Since then, the coverage of the promoted area has been kept in full tobacco field coverage, and the effect of aphid control has been continuously improved.

3.2. Case 2: Arma-Chinensis-Based Biocontrol

A. chinensis is an polyphagous predator with a broad prey range [58]. Originating from China, it is commonly utilized for controlling Lepidopteran pests such as *H. armigera*, *S. litura*, and *S. exigua* on tobacco crops [13]. Over the past three decades, extensive research has been conducted on its geographical distribution, morphology, and biological characteristics, leading to advancements in artificial breeding and field release methods.

3.2.1. Mass-Rearing Production

One of the key issues in the efficient mass-rearing production of polyphagous predators is finding the proper food. The palatability and nutrient composition of food directly affects the feeding behavior, growth and development, and reproductive characteristics of the predators. The first part is artificial breeding based on natural preys. In China, *A. chinensis* is mainly reared on the pupae of *Antheraea pernyi* (Guérin-Méneville) (Lepidoptera: Saturniidae). During the production, it was found that the inability to completely feed on pupae and the production time and cost of *A. pernyi* pupae would restrict the mass-rearing of *A. chinensis* [59]. Therefore, a study from Xie et al. (2020) compared the fitness of three preys, the larvae and pupae of *Mythimna separate* (Walker) (Lepidoptera: Noctuidae) and larvae of *Tenebrio molitor* (Coleoptera: Tenebrionidae), by monitoring the developmental duration, egg hatchability, adult body weight, egg production, and mortality of *A. chinensis*. The results showed that *M. separate* pupae performed best on each monitoring index, followed by *T. molitor* larvae and *M. separate* larvae. A similar study comparing the performance of *T. molitor*, *M. separate*, and *Prodenia litura* larvae as preys confirmed the advantages of *T. molitor* and *M. separate* as preys for the mass-rearing of *A. chinensis* [60]. In addition, Wang et al. (2020) showed that the mixed diet of *M. separate* larvae with a small amount of *H. axyridis* pupae was beneficial to the early development and reproduction of stinkbugs [61]. Compared with *M. separate* pupae, *T. molitor* pupae could improve fecundity as food during the adult mating period [62].

In mass-rearing with prey, besides the high economic and time cost, the risk of prey shortage also exists but could be effectively solved by an artificial diet. An artificial semi-

synthetic feed of a micro-encapsulated *A. pernyi* pupa reduces the feeding cost by 50% while ensuring the normal production quality by feeding *A. pernyi* pupae directly [63].

Meridic diets have been developed for rearing several hemipteran predators and are a staple of the future. The main recipes studied so far include the following three: major ingredient of Recipe 1—eggs, liver, and tuna [64]; Recipe 2—beef, beef liver, and egg yolk [65]; Recipe 3—milk and egg [66]. All formulations meet the basic nutritional requirements of *A. chinensis* growth but need to be modified to achieve the same or even better performance than preys. The nutritional analysis of preferred preys is helpful to clarify the requirement and ratio of certain nutrients for predators and to provide nutritional guidance for the development of artificial diets [67]. The growth, development, and reproduction of *A. chinensis* at different stages could be significantly and positively affected by adjusting the content of saturated fatty acids and unsaturated fatty acids in the artificial diet to increase the adult acquisition rate and population growth rate of *A. chinensis* [68].

In addition to the development of food for rearing *A. chinensis*, other constraints to its mass production were explored. For example, the process of how to adjust the parameters of density and sex ratio to obtain the optimal survival rate and fecundity and to avoid intraspecific mutilation behavior was detailed [69–71].

3.2.2. Field Application

At present, research mostly stays in the laboratory stage, and there are limited cases of large-scale field release. The year of 2018 was the first year of the national pilot promotion of 2000 hm^2 for *H. assulta* and *S. litura* management. The average control efficiency was 51.48% [57]. By 2021, the promotion area reached 35,000 hm^2 with a 57.68% control efficiency.

It is worth noting that the density of *A. chinensis* decreased quickly as individuals dispersed from the release sites, and the efficiency of pest control was largely discounted. In this scenario, early population establishment on the crop was needed to use *A. chinensis* as a biological control agent [72].

3.3. Other Cases

3.3.1. Ladybeetle-Based Biocontrol (*Coccinella septempunctata* and *Harmonia axyridis*)

Predacious ladybeetles, *Coccinella septempunctata* (ladybird) (Coleoptera: Coccinellidae) and *H. axyridis*, are widely distributed in China and have a strong predation ability on aphids. They possess the characteristics of a big appetite, long life, and strong ability to adapt to the environment. Several studies have indicated that they have broad application prospects in the control of *M. persicae* in tobacco fields. At present, many studies report on bioecological characteristics, artificial diet, artificial feeding, and field application, among which there are also related studies on *M. persicae*.

The artificial rearing of *C. septempunctata* and *H. axyridis* mainly adopts aphids as a natural prey. Attempts have also been made on male honeybee pupae, the eggs of *Anagasta kuehniella* (Zeller) (Lepidoptera: Pyralidae), the eggs of *Corcyra cephalonica* (Stainton) (Lepidoptera: Pyralidae), artificial *Trichogramma* pupae, a mix of sucrose and pig liver, and meridic diets, etc. Among them, the nutritive value of male honeybee pupae was the highest, which had no adverse effect on larval growth and development and on adult *C. septempunctata*. Artificial *Trichogramma* pupae can meet the growth and development of larvae but have effects on the fertility of an adult. But, it could be served as an alternative supplement when natural prey is insufficient. Niijima et al. (1977) originally developed a synthetic diet containing 18 amino acids (60%), 6 inorganic salts (6.9%), sucrose (32.5%), cholesterol (0.5%), and 10 vitamins (0.1%) to feed adult ladybeetles, and following chemically defined diets were generally proven to influence the production of ladybeetles [73]. Moreover, factory production of *H. axyridis* can also be realized based on the industrial production of *S. exigua* larvae [74].

Ladybeetles are selected for release as eggs, larvae, and adults. The egg card release is convenient but highly affected by weather factors. The dispersal ability of larvae is small,

which is conducive to the rapid establishment of population in the field. However, the resistance of the larvae is poor, and the larvae are not tolerant to long-distance transport. When the density of *M. persicae* was the same, both adults of *C. septempunctata* and *H. axyridis* had the largest predation [75]. Therefore, in view of the spread of adult beetles, one third of the hind wings of ladybeetles are subtracted and released after sunset on calm and sunny days in China. Next, whichever ratio of ladybugs is released, the control effect of *M. persicae* comes slower than pesticide, usually reaching an 80% control effect after 5 to 10 days. But, the ladybeetles have stronger persistence than pesticide [76,77].

Ladybeetles can also work with *A. gifuensis* to control aphids. Studies have shown that parasitoid and predatory natural enemies can complement each other to enhance predation efficiency and persistence. The joint release of *H. axyridis* and *A. gifuensis* demonstrated a significant synergistic effect on the control of *M. persicae* through intra-group predation [78]. A similar improved suppression of *B. tabaci* was found with the combination of *Eretmocerus eremicus* (Mercet) (Hymenoptera: Aphelinidae) and *Amblyseius swirskii* (Athias-Henriot) (Acari: Phytoseiidae) [79]. This suggests that a higher level of biodiversity among enemies could potentially result in a more effective pest suppression, especially when the natural enemies occupy diverse and complementary feeding niches [80]. However, the optimal proportion and technique for joint release require further investigation. Additionally, we should pay more attention to the observation that an overall increase in prey community richness could lead to trophic complexity, potentially influencing the overall suppression effectiveness [80].

3.3.2. Reduviid-Based Biocontrol (*Sphedanolestes impressicollis* and *Harpactor fuscipes*)

Reduviids (*Sphedanolestes impressicollis* (Stål) (Hemiptera: Reduviidae) and *Harpactor fuscipes* (Fabricius) (Hemiptera: Reduviidae)) are versatile predators that can feed on over 44 insect pests of economic significance [81,82]. Unlike other predacious hemipterans, they have a wider prey range and consume more prey to sustain their larger body size [81]. An overbroad diet may deprive it of the advantages used to hunt specific pests. However, in the presence of multiple pests, they can be very valuable predators. The biocontrol potential of predatory insects such as reduviids must be assessed under controlled conditions, which will form the baseline for their utilization in natural field conditions. The biocontrol potential of reduviids must be evaluated under controlled conditions, which will set the stage for their use in the natural field conditions [83].

Hence, multiple experiments were conducted on the predation function, search effect, self-density interference response, and dispersal ability of *S. impressicollis* and *H. fuscipes*. Field experiments revealed that both reduviids prey on *H. armigera*, *S. litura*, or *M. persicae*. The Y-tube was used to study the selectivity and selection preference of *H. fuscipes* for the aforementioned prey species. The result showed that *H. fuscipes* preferred to select the third-instar larvae of *S. litura* [84]. In addition, prey densities increased the predation capacity of *H. fuscipes* and *S. impressicollis*, while negatively interfering with their search efficiency [85]. Another study showed that the fifth-instar nymphs and adults of *H. fuscipes*, which have a strong dispersal ability, are not ideal for releasing time [86]. These findings provide important guidance for the widespread application in tobacco production in China.

4. Conclusions

The current artificial mass-rearing procedures of *A. gifuensis* are relatively cumbersome, especially in the host plant cultivation and *M. persicae* propagation steps, which significantly slow down the breeding speed. The same applies to natural predators. Therefore, the development of applicable artificial media is of particular importance. This artificial diet could extend beyond aphids and predators and could be used to provide the nutritional needs of *A. gifuensis* directly with artificial aphids while maintaining or even increasing survival, eclosion, and parasitic rates. If artificial materials were available, the entire large-scale reproduction process could be significantly optimized, and the disadvantages of

traditional reproduction methods—such as complicated processes, large space occupation, and high costs—could be resolved.

Through studying biocontrol cases, it becomes apparent that there is a need to increase the abundance of available commercial natural enemies. While commercial explorations are primarily focused on controlling aphids, other pests such as lepidopterans also cause significant damage. However, the development and application of corresponding natural enemies for these pests, as well as other potential candidates, like *E. formosa* against *B. tabaci* and predatory mites against thrips, is relatively lacking. Additionally, further work is needed to quantify the advantages inherent in mechanizing mass-rearing systems.

Moreover, it remains uncertain whether the over-reliance on a few particular natural enemy species could have negative environmental impacts. Despite the lack of addressed potential risks associated with the widespread mass release of natural enemies in China, the continuous manual intervention over the years calls for increased awareness of the risks, such as the superseding of native natural enemy species [16,87]. Therefore, to enhance the quality control of commercial natural enemy releases, environmental monitoring should be incorporated into the routine supervision of mass-rearing production and field application policies.

Finally, the decision regarding the production, importation, and release of biological control agents is a national matter and involves multiple authorities. The National Plant Protection Organization, responsible for implementing the guidelines outlined in the International Plant Protection Convention, oversees this process. Through advancements in homogeneous density, enemy breeding technology, automation, storage, packaging, and release techniques, a technical system was established to stabilize the production of natural enemies. Moving forward, industry standards must be established to clarify product quality indexes, inspection methods, and classification standards in order to create a healthy and sustainable application system for the natural-enemy-based biocontrol of tobacco arthropod pests. The industry is also actively promoting the implementation of biological control, especially the natural-enemy-based biocontrol and providing extensive services to farmers.

Natural-enemy-based biocontrol offers numerous advantages, including safety for humans, animals, and plants, as well as no pollution to the environment and easy development with abundant natural resources. Although, from this study, we can also see some of the drawbacks of natural enemy control, such as its slow effect, reliance on complex artificial breeding technology, limitations imposed by natural conditions, and issues with practical application. However, these limitations can be mitigated by integrating other control measures. It is worth noting that the advancement of plant genetic engineering technology has greatly facilitated the development of efficient insect-resistant crop varieties. Insecticidal Bt endotoxins have been successfully incorporated into transgenic varieties of eggplant, maize, potato, soybean, tomato, and rice, demonstrating remarkable effectiveness in insect control [88]. Nevertheless, it is essential to emphasize the necessity for long-term and systematic scientific research, along with continuous follow-up evaluations to assess the impact of insect-resistant crops on predator insects. Such evaluations are crucial for ensuring the sustainability and effectiveness of biocontrol strategies in the context of integrated pest management.

In general, the widespread use of natural enemies as a means of augmentative application has proven to be an effective way of reducing the reliance on pesticides and meeting the growing demand for sustainable agriculture and safer food. However, in comparison to pesticides, which offer a more immediate solution, the practical application of natural enemies among farmers needs to be improved. To remain competitive with other protection methods such as pesticides, the natural-enemy-based biocontrol methods used in China's agricultural industry must continue to evolve, particularly by expanding the range of targeted pests, increasing the diversity and efficiency of native natural enemies, and combining natural enemy biocontrol agents with other pest management methods to achieve additive control effects. In the future, the key to controlling arthropod pests in

China's agricultural fields will be to integrate and optimize multiple management measures based on the local ecological environment, the types of pests present, and their occurrence patterns, while also reducing the cost of natural-enemy-based biocontrol.

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