

Article

The Potential of a Novel Concept of an Integrated Bio and Chemical Formulate Based on an Entomopathogenic Bacteria, *Bacillus thuringiensis*, and a Chemical Insecticide to Control Tomato Leafminer, *Tuta absoluta* '(Meyrick)' (Lepidoptera: Gelechiidae)

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Abstract: Tomato leafminer, *Tuta absoluta* (Meyrick), (Lepidoptera: Gelechiidae), poses severe threats to sustainable tomato production globally with a high capacity to develop resistance to pesticides. Recently, the management programs for this cosmopolitan pest have relied on combinations of chemical insecticides which exacerbate the problems of cross-resistance and adverse environmental effects. A novel concept of integrated formulates through combining a chemical insecticide model of lambda-cyhalothrin with *Bacillus thuringiensis* (*Bt*) products as the formulation core was explored and evaluated. The susceptibility of the second instar larvae of tomato leafminer to the original formulation of lambda-cyhalothrin, Dipel, XenTari, and Agree products was assessed and compared to the combined formulations. The positive and negative impacts of lambda-cyhalothrin on the viability and pathogenicity of *Bt* strains were tracked and documented. The physicochemical properties of the combined formulates were examined and compared to the international standards of pesticide formulations. The proposed integrated combined formulates of Dipel, XenTari, and Agree biopesticides with lambda-cyhalothrin showed an enhancing effect and had a higher potential than the originally manufactured formulations alone for about a 3.67–10.08-fold impact on larval mortality. Lambda-cyhalothrin had no significant adverse effect on either the viability of *Bt* strains or the physicochemical properties of combined co-formulates. Therefore, such integrated combined formulates would have the potential to be involved as an alternative or a complementary approach in pest management and pest resistance management tools for sustainable pest management.

Keywords: biorational insecticides; *Bacillus thuringiensis*; lambda-cyhalothrin; tomato leafminer; *Tuta absoluta*; chemical and bio co-formulated; sustainable pest management

1. Introduction

Tomato leafminer, *Tuta absoluta* (Meyrick), is one of the most destructive pests and has a tremendous economic impact on sustainable tomato production globally. This insect, originally from South America, invaded Egypt, Saudi Arabia, the United Arab Emirates, and Syria from 2009 to 2012 [1–4]. The nature of damage of this super climate-adapted insect is cryptic and very aggressive to tomato plants. Larvae attack the whole tomato plants on three main levels; they mine the young leaves, penetrate the stems and new

branches, pierce the flowers, and invade the non-ripe and ripe fruits. This endophytic behavior of feeding renders the most commonly used contact insecticide ineffective [5]. To date, few formulations of chemical synthetic systemic insecticides have been developed to constitute the most adopted method in managing this insect. However, the indiscriminate use of them resulted in several negative impacts such as: insecticide resistance, yield loss, negative effects on non-target organisms, widespread environmental contamination, and pesticide residues on tomato fruits [6].

The larvae of *T. absoluta* have acquired resistance to most of the marketed insecticides worldwide. Different levels of resistance to deltamethrin, permethrin, abamectin, methamidophos, cartap, and spinosad were found in Brazil, Argentina, and other South American countries [7–11]. Furthermore, resistance to diamide insecticides such as chlorantraniliprole and flubendiamide and oxadiazine insecticides such as indoxacarb has been reported in Italy, Greece, Cyprus, Spain, Israel, and Brazil [12,13]. Meanwhile, in the UK, high levels of resistance were observed to spinosad and chlorantraniliprole in field strains of tomato leafminer [14,15]. Meanwhile, other field populations of *T. absoluta* from Europe and Brazil showed high levels of resistance to lambda-cyhalothrin and tau-fluvalinate due to the existence of three mutations of the para-type sodium channel-kdr/super-kdr-type (M918T, T929I and L1014F) with high frequencies at the IIS4-IIS6 region of the para gene in tested larvae [16]. In Northern Nigeria, the field population of *T. absoluta* larvae was susceptible to abamectin, while at the same time it exhibited resistance to lambda-cyhalothrin, propoxur, chlorpyrifos-methyl [17]. Thus, special attention has been drawn to the resistance management of this insect by shifting to insecticide mixtures with different modes of action, and utilizing synergistic agents, enhancers, and biorational pesticides [18,19].

A compatible synthetic insecticides mixture (with different modes of action) has become the proposed tool for resistance management due to the fact it has a higher efficacy than the rotational use of insecticides [20,21]. Pyrethroid, carbamate, and organophosphate insecticides have constituted the predominant core in insecticide mixtures in the last two decades [22,23]. However, avermectins, pyrethroids, anthranilic diamides, and neonicotinoids have been used most recently in insecticide mixtures under the approval and authorization of the United States Environmental Protection Agency (US-EPA) for manufacturing such co-formulations [24,25].

Biopesticides based on *Bacillus thuringiensis* (*Bt*) have proved their potential for decades as alternative or complementary eco-friendly insecticides on Lepidopteran, Dipteran, Orthopteran, and Coleopteran insects [26–29]. The *Bt*-products are environmentally safe for humans, beneficial vertebrates, and arthropods in addition to having no pre-harvest intervals on agricultural products [30,31]. In general, *B. thuringiensis* is a spore-forming bacteria known for its capability for synthesizing different types of toxic substances such as crystal protein (δ -endotoxin), vegetative insecticidal protein (Vip), and secreted insecticidal protein (Sip) [32,33]. The crystal protein is made up of three domains of Cry toxins (3-d Cry) that bind onto specific receptors in the midgut apical microvilli membrane, after they have been cleaved and oligomerized, causing pore formation that leads to epithelial disruption in terms of ion leakage, cell lysis, septicemia, and death [34,35]. The commonly known receptors for three d-Cry toxins are Cadherin-like proteins (CAD), aminopeptidase N (APN), alkaline phosphatase (ALP), glycolipids, and V-ATPase subunit A intracellular proteins [36].

Although few studies have investigated the potential of *Bt*-products for *T. absoluta* control, *B. thuringiensis* sub spp kurstaki (*Btk*) and aizawai (*Bta*) have proved effective versus tomato leafminer in Malta, Italy exhibiting a synergistic/additive effect in controlling resistant genotypes [37–39]. However, in another study, *B. thuringiensis* spp. were highly effective on the first instar larvae of *T. absoluta*, while the second and third instars had a lower susceptibility [40]. Simultaneously, the potency of *Btk* and *Bta* in Dipel and XenTari was enhanced by the combination with other strains of *Bacillus* sp. and *Pseudomonas* sp. isolated from northern masked chafer larvae midgut versus Coleopteran and Lepidopteran larvae [41]. Three different strains of *Bt* were chosen in this study due to their varied

composite mix of cry toxins; the *Btk* strain ABTS-351, found in the Dipel DF formulation, has a unique balanced mix of Cry1Aa, Cry1Ab, Cry1Ac, and Cry 2A [42]. Similarly, the *Bta* strain ABTS-1857 found in XenTari DF formulation has a different composition mix of Cry1 toxin proteins in terms of Cry1Aa, Cry1Ab, Cry1C, and Cry1D [43]. Moreover, the *Bta* strain GC-91 found in the Agree WG formulation has a single major toxin protein (Cry1Ac) along with two minor toxins at very low levels (Cry1C and Cry 1D) [44].

Therefore, the integration of biorational insecticides together or with synthetic insecticides in a co-formulate may improve control. Hence, a shift toward a holistic approach consisting of chemical and biological agents suggested by the European and Mediterranean Plant Protection Organization may prove effective [45,46].

The present study aimed to explore the possibility of combining *Bt*-products with lambda-cyhalothrin, evaluate the positive and negative impacts of this combination, and assess its potential to be involved as an alternative or complementary approach in the management programs of the invasive resistant strain of *T. absoluta*.

2. Materials and Methods

2.1. Insects

Tomato leafminer larvae were collected from infested tomato plants cultivated under greenhouse conditions at Al-Kharj province 58QV + G2, Riyadh region, Saudi Arabia as a field population. A colony of *T. absoluta* were established and maintained in a small, air-conditioned greenhouse sized 54 m² (9 m × 6 m) at the National Agriculture and Animal Resources Research Center, Riyadh, Saudi Arabia. Larvae were transferred to a controlled laboratory (25 ± 2 °C, 60–70% RH, 16 L: 8 D) using tomato plantlets as a preferred host. Leaves of Romaine lettuce, *Lactuca sativa* L. var. longifolia, grown in a hydroponic greenhouse free of pesticide treatments, were offered as a food source after preliminary palatability tests suggested that they can be used for rearing larvae. Insects were reared in the laboratory at least for the first generation to obtain a homogeneous cohort and to make sure that they were not contaminated from the field before they were subjected to susceptibility evaluations as recommended by the Insecticide Resistance Action Committee (IRAC) [47].

2.2. Insecticides

ICON[®] 10 WP, lambda-cyhalothrin (Syngenta Crop Protection, Greensboro, NC, USA), a synthetic contact wide spectrum pyrethroid and three common biorational insecticides based on *B. thuringiensis* bacteria; Dipel pro[®] DF 54% (*Bt* subsp kurstaki (*Btk*) strain ABTS-351, Valent BioScience—Libertyville, IL, USA), XenTari[®] DF 54% (*Bt* subsp aizawai (*Bta*) strain ABTS-1857, Valent BioScience—Libertyville, IL, USA) and Agree[®] WG 50% (*Bt* subsp aizawai (*Bta*) strain GC-91, CERTIS-USA) were investigated individually and in the lab-combined formulates for their potential versus *T. absoluta* larvae considering the evaluation of the impact of lambda-cyhalothrin on bacteria.

2.3. Bio and Chemical Insecticide Combinations

Lab-made combinations of Icon[®] 10 WP and each of the tested biopesticides, Dipel pro[®] DF, XenTari[®] DF, and Agree[®] WG, were conducted at three different rates; 1:1; 1:2, and 1:3 (Chemical: Bio w/w). Five grams of the lambda-cyhalothrin formulation was used as a core consistent weight to prepare the combined formulates in 50 mL sterile Blucapp centrifuge tubes (Nordhausen, Germany) using Thermo Scientific™ Digital Vortex Mixer (ThermoFisher Scientific© Inc., Waltham, MA, USA) at consistent 1500 rpm/3 min for median agitation and then transferred to Petri dishes (100 mm × 15 mm) in three replicates. Amounts of 5, 10, and 15 g used for combinations of Dipel, XenTari, and Agree were vortexed at 1500 rpm/3 min and then kept in separate Petri dishes as controls. All Petri dishes were kept at 28 ± 2 °C under aseptic conditions for 3 months.

2.4. Bacterial Viability

Bacterial viability was tracked and examined by the traditional plate counting and turbidity measurement techniques and confirmed using BacLight bacterial viability kit (ThermoFisher Scientific© Inc., Waltham, MA, USA) after 5, 10, 15, 30, 45, 60, 75, and 90 days to evaluate the positive and negative impact of lambda-cyhalothrin on the viability of *Bt* and to determine the proper mixing rate for the combinations.

2.5. Plate Counting and Turbidity Measurement Techniques

In brief, fifty milliliters of Tryptone Soy Broth (TSB) media in a 250 mL Erlenmeyer flask were inoculated individually with 200 µL of 5% suspension (0.5 g/10 mL sterile double distilled water) of each original *Bt*-formulation and combined formulates. Flasks were incubated for 24 h/30 °C with a rotary agitation of 160 rpm. Bacterial growth was centrifuged at 5000 rpm/15 min/4 °C and the supernatant was discarded. Pellets were washed individually by a sterile phosphate buffer saline (PBS) and then suspended in 30 mL of PBS. Turbidity was measured, for each stock suspension and its four serial dilutions, at 600 nm using NANOCOLOR® UV/VIS II (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany). A plate count for each original and combined formulate was conducted by inoculating 100 µL of each serial dilution on Trypton Soy Agar (TSA) Plates and incubating them for 48 h/28 °C in three replicates. The mean values of the counted colonies were used in generating colony-forming units (cfu's) that corresponded to the turbidity absorption of each tested formulate.

2.6. Baclight Viability Technique

Samples of the original *Bt*-products and the lab-made combined formulates were transferred to the Microbiological Research Unit belonging to the Animal Health Department at the National Agriculture and Animal Resources Research Center, Riyadh, to determine the viability of *Btk* and *Bta*. Live/dead assays were carried out as per the manufacturer's instructions (Invitrogen, 2004 using the Live/Dead BacLight™ bacterial kit for fluorescent quantitative assays (L7012, Molecular Probes, Invitrogen, ThermoFisher Scientific© Inc., Waltham, MA, USA). *Escherichia coli* K12 strain MG1655, obtained from New England Biolabs® Inc., was used as a positive control.

2.7. Preparation of Live/Dead Cultures of Bacteria

A pure colony of *Btk*, *Bta*, and *E. coli* were picked up individually to inoculate 100 mL of TSB in a 250 mL Erlenmeyer flask for 24 h/30 °C/160 rpm (*Btk* and *Bta*), for 24 h/37 °C/200 rpm for *E. coli*. Dilutions of 1:20 were made up in fresh TSB and incubated at the same conditions for 30–45 min to grow until the turbidity range (OD600 of 3.0–6.0) was reached. Subcultures were centrifuged at 5000 rpm/10 min and half of the pellets for each strain were diluted in 10-fold of saline (0.85% (*w/v*)) to prepare live cells. However, the rest of the pellets of each strain were diluted 10-fold in isopropanol 70% (*v/v*) for dead cells. Live and dead cells were incubated for 30 min/200 rpm then harvested at 5000 rpm/10 min. Pellets were resuspended in saline and the turbidity was measured and cfus of each were adjusted to approximate 1×10^8 CFU/mL. The ratio of live: dead cells was adjusted by mixing volumes of live and dead suspensions and subjected to live/dead assay in a fluorescence microplate reader for standard curves.

2.8. Staining and Fluorescence Measurement

Briefly, *B. thuringiensis* strains in the original and combined formulate were cultured on TSB for 24 h/30 °C/160 rpm then centrifuged at 5000 rpm/15 min. Pellets were washed twice before being resuspended in 0.85% (*w/v*) of NaCl buffer and subjected to BacLight Stain. The BacLight viability kit contains two different fluorescent dyes, SYTO 9 and Propidium iodide (PI). Each dye has a special ability to penetrate bacterial cell membranes, whereas live cells with intact membranes stain green and dead cells with damaged membranes stain red [48,49].

A 2× stain working solution was prepared by adding 6 µL of component A (SYTO9 dye, 3.34 mM) and 6 µL of component B (Propidium iodide, 20 mM) to 2.0 mL of filter-sterilized double-distilled H₂O in Eppendorf tube and vortexed well. A total of 100 µL of each *Bt* and *E. coli* strain suspension was added in a 96-well black, flat-bottomed microplate (ThermoFisher Scientific© Inc., Waltham, MA, USA). One hundred microliters of the 2× stain solution were pipetted on each bacterial cell suspension and mixed thoroughly by pipetting up and down. All samples were conducted in triplicate and the first and last columns and rows were not used as recommended by the manufacture to avoid interference or false reading. The microplate was incubated in the dark at 27 °C for 15 min. A fluorescent parameter was adjusted to the excitation wavelength at 485 nm, the fluorescence intensities at 530 nm (green emission) and 630 nm (red emission) to analyze the fluorescent ratio of green/red and calculated the viability % of each bacterial sample.

2.9. Physicochemical Properties of the Original and the Highest Mixing Ratio of Lab-Made Combined Formulate

2.9.1. Wettability Test–CIPAC F-MT 15.2

A representative sample (5 ± 0.1 g) of the original formulations and the highest mixing ratio 1:1 of lab-made combined formulates was added into 100 ± 1 mL of working solution of CIPAC Standard Water D (Hardness 342 ppm, pH 6.0–7.0) purchased from CHEMOS, GmbH & Co. KG, Altdorf, Germany) at once without agitation. The consumed time was recorded and justified to the nearest second until complete wetting [50].

2.9.2. Suspensibility and Persistent Foam Test–CIPAC-F-MT15, MT184, and MT172

A known amount (6.25 g) of the original formulations, Icon WP, Dipel DF, XenTari DF, Agree WG, and the highest mixing ratio of lab-made combined formulates were added to 250-mL of CIPAC Standard Water D in borosilicate graduated cylinders with a toppler. The suspension was inverted at 180 ± 1 degrees 30 times and then helped upright in a water bath at $30 \pm 1/30$ min without vibration or direct sunlight exposure. Nine-tenths of the suspension were discarded using a polyethylene transfer pipette. The remaining tenth of the suspension was quantitatively transferred using double-distilled H₂O onto Whatman® Grade 1 filter paper. The filter paper with the deposit was dried then reweighted and the suspensibility % was calculated using the following equation according to Collaborative International Pesticide Analytical Council (CIPAC-F) [50].

$$\text{Suspensibility} = 10/9 \times 100 (C - Q)/C = 111(C - Q)/C\%$$

where:

C = the mass of active ingredient in the actual sample (g)

Q = the mass of active ingredient in the remaining 1/10 (25 mL) in the cylinder (g).

The persistent foam of each suspension was measured after 1 min of the 30 inversions while it was in an upright position in the water bath according to CIPAC-F, MT 47.1 [50].

2.10. Bioassay

Susceptibility of the second instar larvae of *T. absoluta* to the original tested insecticide formulations, Icon, Dipel, XenTari and Agree was evaluated using the leaf-dip bioassay technique as described by IRAC method No. 022 with a little modification [47].

2.10.1. Original Insecticide Suspensions

Stock suspensions (4000 µg/mL) of the original biopesticides were accurately prepared in double-distilled water and serial seven dilutions (3500, 3000, 2500, 2000, 1500, 1000, 500 µg/mL) were accurately adjusted. The lambda-cyhalothrin stock suspension (1500 µg/mL) was accurately prepared in double-distilled water and five serial dilutions (1000, 800, 600, 400, 200 µg/mL) were conducted. The potency of the stock suspensions and serial dilutions of all tested insecticides were evaluated on the second instar larvae of tomato leafminer. Those concentrations were used after the preliminary bioassay proposed

that they would produce LC₅₀s of each original formulation. The preliminary trials were conducted using a wide range of concentrations of 100 µg/mL up to 5000 µg/mL for Dipel pro[®] DF 54%, XenTari[®] DF 54%, and Agree[®] WG 50%. The preliminary experiment for ICON[®] 10 WP, lambda-cyhalothrin was carried out using a wide range of concentrations of 50 µg/mL up to 2000 µg/mL. All the prepared concentrations were conducted based on the active ingredient % of each bio and chemical insecticide.

2.10.2. Suspensions of the Lab-Made Fortified Formulates

A preliminary bioassay was conducted using a wide range of *Bt* concentrations of 100, 250, 500, 750, 1000, 1250, 1500, 1750, and 2000 µg/mL fortified individually with different lambda-cyhalothrin concentrations of 50, 200, 350, 500, and 650 µg/mL, in order to screen for the proper mixing rates that would produce LC₅₀ on the second instar larvae of *T. absoluta*. An effective range of *B. thuringiensis* dilutions was suggested from the preliminary experiments with a proposed efficient chemical fortification. A stock suspension (1500 µg/mL) of each original *Bt* product was prepared in double-distilled H₂O. Dilution rates of 750, 500, 250, 100 µg/mL of Dipel pro[®] DF, XenTari[®] DF and 1000, 750, 500, 250, 100 µg/mL of Agree[®] WG were accurately adjusted and then fortified individually with a proposed consistent concentration (350 µg/mL) of lambda-cyhalothrin 10 WP. Our preliminary tests suggested this combination rate would enhance the toxicity of both *Bt* and lambda-cyhalothrin formulations to tomato leafminer larvae.

2.10.3. Leaf-Dip Bioassay

Adequate fresh uniform leaves of romaine lettuce were immersed individually for 3 s with gentle agitation, ensuring that the entire surface was perfectly immersed in the original and lab-made fortified suspensions and in double-distilled sterilized water as the untreated control. Treated leaves of the same suspension and untreated were allowed to dry at an ambient temperature for 20 min before they were offered to three replicates of 30 (second instar) larvae of *T. absoluta* as a sole food source in a plastic Petri dish (100 mm × 15 mm) with a slightly moistened filter paper at the bottom, taking into account the change in the introduced food with fresh treated or untreated ones every 24 h for either treatments and control. Replicates were kept under controlled environmental conditions (25 ± 2 °C, 60–70% RH, photoperiod 16L: 8D). Observations were conducted daily, and mortality rates were recorded after 72 h according to IRAC [47]. Larvae were counted as dead if either they did not move, or if they were moving in an uncoordinated manner for a distance not equal to double their body length. All bioassay treatments were repeated three times on different days and mortality rates were recorded and compared.

2.11. Statistical Analysis

The normality of the data was examined firstly using SAS proc univariate to perform the Kolmogorov–Smirnov test which proved that our data were normally distributed with a significant *p*-Value > 0.05 whereas, the test statistic was 0.1067 and the corresponding *p*-value was >0.142. The analysis of variance (ANOVA) was conducted to the bacterial viability data using the Proc GLM procedure of SAS at *p* < 0.05. Mortalities of replicate experiments were pooled together and subjected to probit analysis using PROC PROBIT of SAS [51]. Significant differences between LC_{50,s} were determined based on the lack of overlap in fiducial limits values at the 95% level. The data were adequately conformed to the probit model depending on the Likelihood ratio (L.R.) chi-square goodness-of-fit values of treatments [52].

3. Results

3.1. Influence of Lambda-Cyhalothrin on the Viability of *B. thuringiensis* Strains in the Lab-Made Combined Formulates

The potential effect of lambda-cyhalothrin on the viability of *Bt*-based biopesticides was investigated through traditional plate counting and the turbidity technique and

confirmed by staining the bacterial nucleic acid using a BacLight bacterial viability kit. Live/dead bacterial cells were distinguished via a green stain for live and a red stain for the compromised membrane that was considered as dead. Through 90 days of the lab-made combination the survival of *Btk* strain ABTS-351, *Bta* strain ABTS-1857, and *Bta* strain GC-91 were in a gradual decline depending on the combination rate. The lowest survival was 76% compared to the control of 87% after 90 days in the lambda-cyhalothrin/*Bta* strain GC-91 combination (Figure 1C), while the mean values of live ratio of *Btk* in the lab-made (lambda-cyhalothrin/Dipel) combined formulate were 89.17%, 88.69%, and 85.62% with the corresponding dead ratio of 10.8%, 11.3%, and 14.4% for 1:3, 1:2, and 1:1 mixing rates, respectively (Figure 2).

Similarly, the mean values of viability % of *Bta* strain ABTS-1857 in the lab-made combined formulate (lambda-cyhalothrin/XenTari) were 85.39%, 81.38%, and 80.67% with the corresponding dead ratios of 14.6%, 18.6% and 19.3% for 1:3, 1:2, and 1:1 mixing rate, respectively (Figure 3). Meanwhile, the mean values of the viable *Bta* strain GC-91 in the lab-made combined formulate (lambda-cyhalothrin/Agree) were 86.1%, 82.2%, 80.7% with corresponding non-viable ratios of 13.9%, 17.8%, and 19.3% for 1:3, 1:2, and 1:1 mixing rates, respectively through 90 days of the combination (Figure 4). However, for the control treatments (original *Bt*-product), the mean values of viability % were 90.3%, 88.6%, and 89.1% with the corresponding non-viability values of 9.7%, 11.4%, and 10.9% for Dipel®, XenTari®, and Agree®, as an original manufactured formulation. It is noteworthy that in the high rate of combination 1:1 (lambda-cyhalothrin: *B. thuringiensis*), around 80.7–85.6% of bacterial cells were able to survive and succeeded in re-growth, whereas, the *Btk* strain ABTS-351 (Dipel pro® DF) was the most viable at 85.6% followed by the *Bta* strain ABTS-1857 (XenTari® DF) at 81.4% while the *Bta* strain GC-91 (Agree® WG) recorded the lowest viability at 80.7% as shown in Figures 2–4.

The analysis of variance between the three rates of combination of lambda-cyhalothrin/*Bt* strains and the control showed that no significant differences were found between the viability of the bacterial strains at different mixing rates. Therefore, lambda-cyhalothrin had no significant adverse effect on *Bt* strains (Table 1).

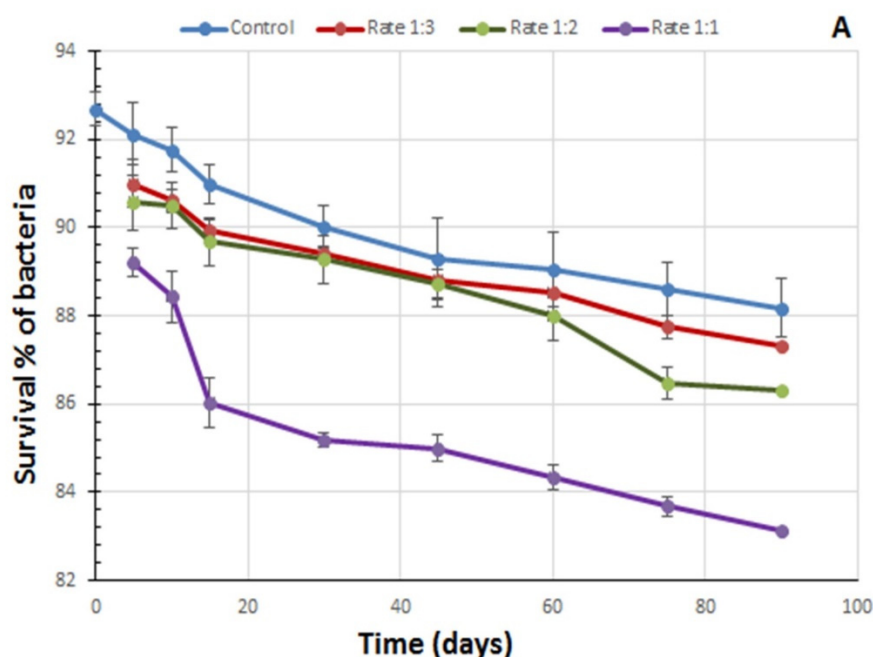


Figure 1. Cont.

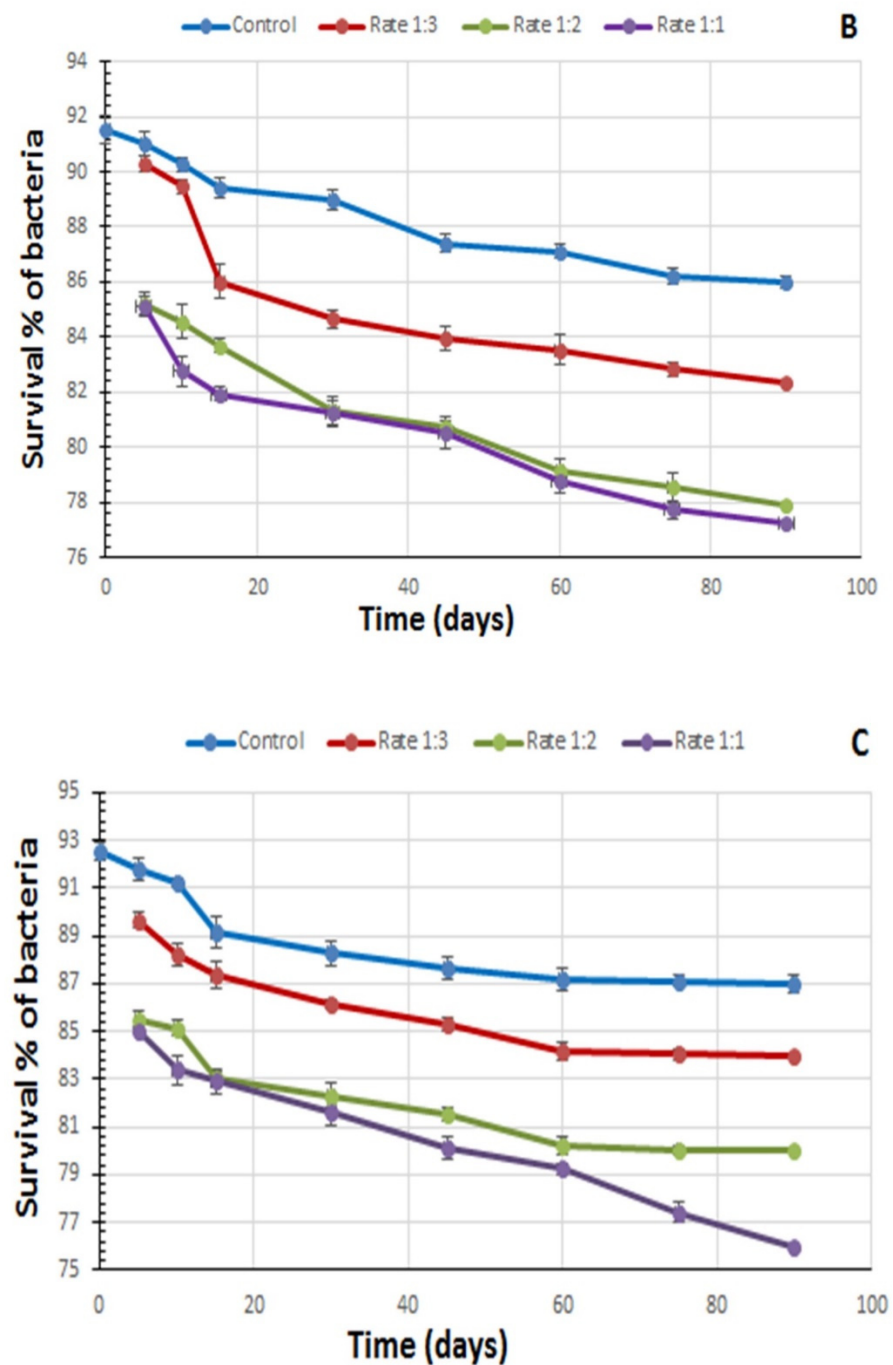


Figure 1. Survival % of *B. thuringiensis* (*Bt*) strains in the three lab-made combined formulates using three rates of combination (lambda-cyhalothrin/*Bt*) 1:1, 1:2, and 1:3 after 5, 10, 15, 30, 45, 60, 75, and 90 days of the combination and in the control treatments at zero time, 5, 10, 15, 30, 45, 60, 75, and 90 days. (A) Combined formulate of lambda-cyhalothrin/Dipel (*Btk* strain ABTS-351). (B) Combined formulate of lambda-cyhalothrin/XenTari (*Bta* strain ABTS-1857). (C) Combined formulate of lambda-cyhalothrin/Agree (*Bta* strain GC-91).

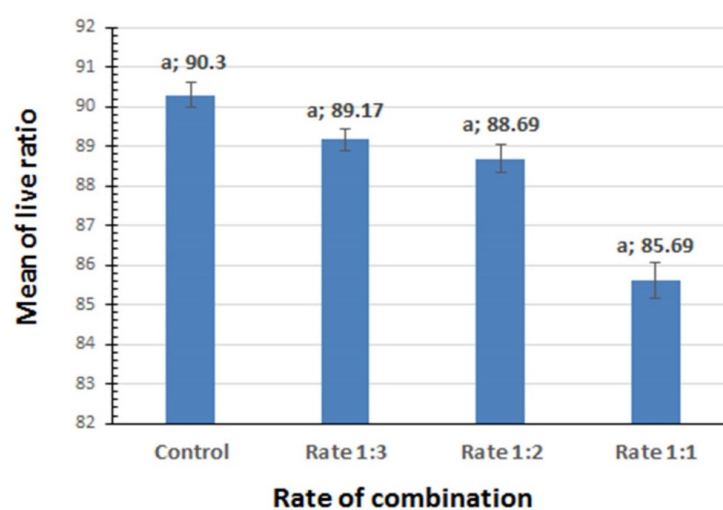


Figure 2. Mean values of the live ratio for the *B. thuringiensis kurstaki* strain (*Btk*) in the lab-made combined formulate of lambda-cyhalothrin/Dipel pro[®] DF at three different rates of the combination through 90 days.

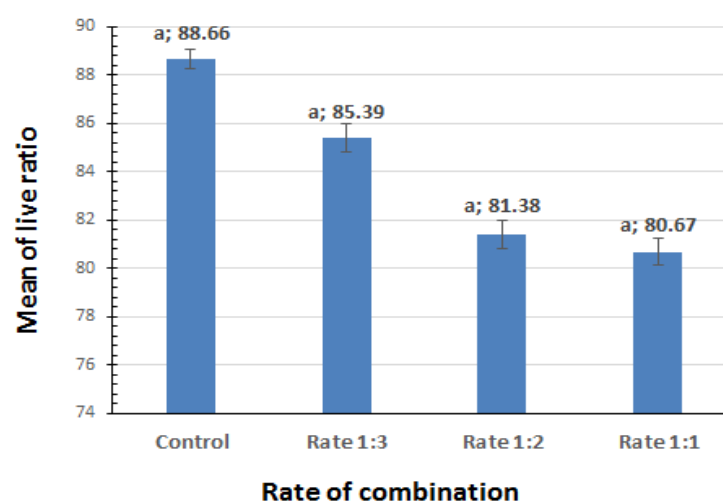


Figure 3. Mean values of the live ratio for the *B. thuringiensis aizawai* strain (*Bta*) in the lab-made combined formulate lambda-cyhalothrin/XenTari[®] at different rates of the combination through 90 days.

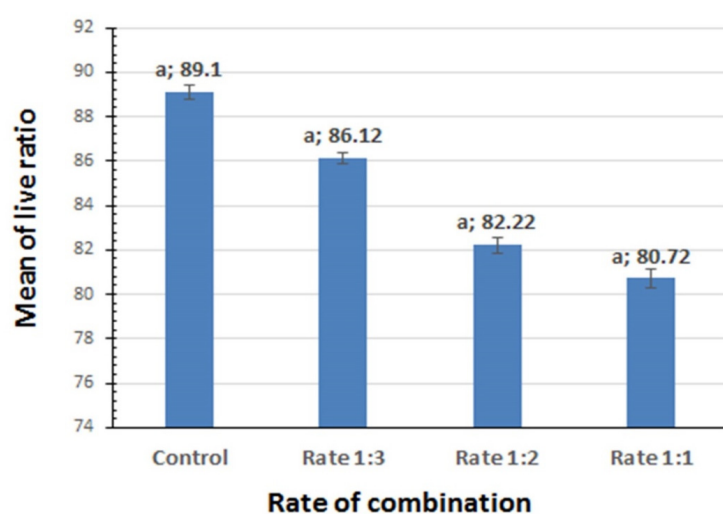


Figure 4. Mean values of the live ratio for the *B. thuringiensis aizawai* strain (*Bta*) in the lab-made combined formulate lambda-cyhalothrin/Agree[®] at different rates of the combination through 90 days.

Table 1. Analysis of variance between the four rates of combinations of lambda-cyhalothrin/*B. thuringiensis* in the lab-made combined formulates depending on the bacterial viability.

Source	df	MS	F-Statistic	p	Effect
The three rates of combination + Control (Lambda-cyhalothrin/ <i>B. thuringiensis</i>)	3	27.8	3.7	0.0612	Not Significant
Error	8	7.5			
Total	11				

df: degrees of freedom, MS: Means of Square, p-value at $p \leq 0.05$.

3.2. Effect of the Proposed Combination on the Physicochemical Properties of the Tested Formulation

The physicochemical properties of the original and combined formulations were tested. The data in Table 2 show that the originally tested formulations (Icon® 10 WP, Dipel pro® DF, XenTari® DF, Agree® WG and their lab-made combined mixtures at the highest rates (Icon: Dipel 1:1 w/w; Icon: XenTari 1:1 w/w; Icon: Agree 1:1 w/w) were completely wetted in 1 min, without swirling. The measured persistent foam for all formulations was less than the maximum limit (60 mL) after 1 min. The originally tested formulations passed the minimum limits for suspensibility test whereas the values were 50, 90, 90, and 90% for Icon, Dipel, XenTari, and Agree, respectively; hence, they were valid as described by FAO/WHO [53]. The physicochemical properties of the lab-made combined formulates were in accordance with the specifications of FAO/WHO 2018 and WHO 2012 [53,54] for wettability and persistent foam. However, suspensibility % exceeded the minimum limits for all the originally tested formulations. Meanwhile, the combined formulates did not have a minimum limit specified.

Table 2. Physicochemical properties of Icon, Dipel, XenTari, and Agree products (original formulation) and their lab-made combined formulations.

Parameter Tests	Original Formulations				Lab-Made Combined Formulations		
	Icon®	Dipel®	XenTari®	Agree®	Icon:Dipel (1:1)	Icon:XenTari (1:1)	Icon:Agree (1:1)
Wettability at 1 min.	Completely wetted	Completely wetted	Completely wetted	Completely wetted	Completely wetted	Completely wetted	Completely wetted
Persistent Foam after 1 min	40 (+) (60 mL) †	20 (+) (60 mL) †	26 (+) (60 mL) †	22 (+) (60 mL) †	28 (+) (60 mL) †	30 (+) (60 mL) †	28 (+) (60 mL) †
Suspensibility%	81 (ML 50) ‡	96 (ML 90) ‡	95 (ML 90) ‡	91 (ML 90) ‡	83 (NA) §	80 (NA) §	78 (NA) §

(+) means Valid. Where: Valid means (Tested value < maximum limit). † Maximum limit according to (FAO/WHO 2018) [53]. ‡ Minimum limit of suspensibility % for original formulations according to WHO 2012 and 2015 [54,55]. § Minimum Limit for suspensibility % for co-formulations not available.

3.3. Potency of the Original Insecticide Formulations on the Second Instar Larvae of *T. absoluta*

The potency of the four original insecticides; Dipel, XenTari, Agree, and Icon were experimentally evaluated under the laboratory conditions on the second instar larvae of *T. absoluta*. Regardless of the high concentrations used, lambda-cyhalothrin (Icon) proved potent on the tomato leafminer larvae with an LC₅₀ of 826.9 µg/mL followed by the *Btk* strain ABTS-351 (Dipel DF) with an LC₅₀ of 1566.8 µg/mL and then the *Bta* strain ABTS-1857 (XenTari DF) with an LC₅₀ of 2119.1 µg/mL. However, the *Bta* strain GC-91 (Agree WG) was the least potent with an LC₅₀ of 2694.4 µg/mL after 72 h of treatment (Table 3). Significant differences were shown between the original tested formulations at the LC₅₀ level depending on the 95% fiducial limits. The calculated relative potency revealed that the potency of lambda-cyhalothrin was 3.25× that of Agree (the least potent), while the potencies of Dipel and XenTari were 1.72 and 1.27× that of Agree according to Finney [56] as shown in Table 3.

Table 3. Statistical toxicity values of three *Bacillus thuringiensis*-based formulations; Dipel pro[®] DF, XenTari[®] DF, Agree[®] WG, and one chemical insecticide; Icon[®] 10 WP (Lambda-Cyhalothrin); on the second instar larvae of tomato leafminer, *Tuta absoluta* after 72 h.

Treatments	N	Slope (SE)	LC ₅₀ ^{ab}	95% FL	$\chi^2(df)$ ^c	RP ^d	CM ^e
Dipel Pro [®] 54 DF	720	3.59 (0.38)	1566.8 ^b	1196.81–1734.03	3.64(6)	1.72	4.4
XenTari [®] 54 DF	720	2.73 (0.29)	2119.1 ^c	1782.23–2352.19	2.41(6)	1.27	2.2
Agree [®] 50 WDG	720	2.49 (0.23)	2694.4 ^d	2432.44–2868.12	5.09(6)	1	2.2
Icon [®] 10 WP	540	2.62 (0.22)	826.9 ^a	709.6–1043.5	3.32(4)	3.25	3.3

N refers to the total tested insect in each treatment. ^a LC₅₀'s reported as µg/mL. ^b LC₅₀'s followed by the same letter are not significantly different at $p < 0.05$ based on overlap of their 95% fiducial limits. ^c L.R. chi-square goodness-of-fit values. Tabular values at $p = 0.05$ for 4 df = 9.496, 6 df = 12.59. ^d Relative Potency = LC₅₀ of Agree (highest value) as standard/LC₅₀ of tested insecticides (Finney, 1971) [56]. ^e Percentage of control mortality.

3.4. Potency of the Lab-Made Fortified Formulates on the Second Instar Larvae of *T. absoluta*

The efficiency of the lab-made combined formulates was evaluated under laboratory conditions on the second instar larvae of tomato leafminer. Our previous preliminary bracketing experiments revealed that the fortification of the applied rate of *Bt* product with a 350 ppm of lambda-cyhalothrin would enhance the potency on the second instar larvae of *Tuta absoluta*. The fortified formulate of the *Bta* strain ABTS-1857 (XenTari DF)/lambda-cyhalothrin showed a high potential on the second instar larvae with an LC₅₀ of 267.3 µg/mL and was not significantly different from the following fortified formulate of the *Btk* strain ABTS-351 (Dipel DF)/lambda-cyhalothrin with an LC₅₀ of 344.9 µg/mL. Both of them showed significant differences with the fortified formulate of *Bta* strain GC-91 (Agree WG)/lambda-cyhalothrin at LC₅₀ of 733.5 µg/mL (Table 4). The calculated toxicity index according to Sun [57] for each fortified formulate demonstrated the superiority of the combined XenTari/lambda-cyhalothrin over other formulates. The toxicity indexes of the combined Dipel/lambda-cyhalothrin and combined Agree/lambda-cyhalothrin were 77.5% and 36.4% of the toxicity index of the combined XenTari/lambda-cyhalothrin at 100%.

Table 4. Statistical toxicity values of the three lab-made combined formulates on the second instar larvae of tomato leafminer, *Tuta absoluta* after 72 h.

Treatments (Lab-Made Combined Formulates)	N	Slope (SE)	LC ₅₀ ^{ab}	95% FL	$\chi^2(df)$ ^c	Toxicity Index ^d	RP ^e	CM ^f
Dipel (<i>Btk</i>) + Icon (lambda cyhalothrin)	450	2.24 (0.22)	344.9 ^a	296.11–423.55	2.31(3)	77.5	7.81	3.3
XenTari (<i>Bta</i>) + Icon (lambda cyhalothrin)	450	2.49 (0.27)	267.3 ^a	206.16–354.07	2.62(3)	100	10.08	3.3
Agree (<i>Bta</i>) + Icon (lambda cyhalothrin)	540	2.52(0.32)	733.5 ^b	610.05–782.21	3.43(4)	36.4	3.67	2.2

N refers to the total tested insect in each treatment. ^a LC₅₀'s reported as µg/mL. ^b LC₅₀'s followed by the same letter are not significantly different at $p < 0.05$ based on overlap of their 95% fiducial limits. ^c L.R. chi-square goodness-of-fit values. Tabular values at $p = 0.05$ for 3 df = 7.81, 4 df = 9.49. ^d Toxicity index = (LC₅₀ of the most efficient compound (as Standard)/LC₅₀ of the other tested compound) 100 [57]. ^e Relative Potency = LC₅₀ Agree/LC₅₀ of tested insecticides (Finney, 1971) [56]. ^f Percentage of control mortality.

Subsequently, the relative potency of the combined formulates showed an accelerated increase over the original formulations. For instance, the relative potency of the combined Agree/lambda-cyhalothrin was 3.67× of the relative potency of Agree alone. Similarly, the relative potency of the combined formulate Dipel/lambda-cyhalothrin and XenTari/lambda-cyhalothrin were 7.81× and 10.08× of the least potent original insecticide (Agree) (Table 4). In control treatments, mortality rates did not exceed 4.4%.

4. Discussion

Tomato leafminer, *T. absoluta*, is one of the most invasive and severe threats to sustainable tomato production globally, causing a tremendous reduction in tomato yield 60–100% [1–4]. Larvae of *T. absoluta* have acquired resistance to most of the marketed insecticides worldwide due to the heavy use of insecticides [7–17]. Critical attention has been drawn to the resistance management of this insect by shifting to insecticide mixtures of different modes of action including chemical and synthetic reduced risk pesticides and diverse biorational agents [18–20]. For the last two decades, the pyrethroid insecticides have usually been exhibited in the proposed and marketed synthetic insecticides mixtures [21–25]. Although *Bt*-based biopesticides have proven that they have the potential to act as alternative or complementary eco-friendly insecticides on about 3000 insect species, they still need enhancers or synergistic agents to beat the resistant strains of tomato leafminer [29,30]. The integration of biorational insecticides with chemicals is imperative for the successful control of this insect and for the sustainable protection of the environment. In this study, we explored the possibility of combining *B. thuringiensis*-based bio-insecticides with one of the most commonly used pyrethroids with special reference to the positive and negative impacts beyond this proposed combination. The evaluated concentrations of each original insecticide alone on the larvae of *T. absoluta* exceeded the recommended dose by the manufactures for other pests, suggesting a lower susceptibility of the larvae to lambda-cyhalothrin, Dipel, XenTari, and Agree formulations. The resulted LC_{50s} for the four tested original formulations emphasized that none of them could be used individually to reduce the damage of second instar larvae on tomato crop, while few studies have documented the potential of the combination between biorational pesticides and chemical insecticides in the field as a tank-mix or integrity use in periodic rotation as well [42,43]. Indeed, to our knowledge, this is the first report demonstrating the impacts beyond the combination between *B. thuringiensis* and chemical insecticide in such a co-formulation and its potential on the invasive unsusceptible or resistant pests.

Data in Figure 1 and Table 1 reveal that the possible combination between *B. thuringiensis* and the chemical insecticide in such a co-formulate was achieved with no significant impact on the viability of the bacterial cells or their ability to survive and re-grow perfectly. Meanwhile, the rate of combination had little effect on the live and dead ratio of each strain. However, in the highest combination rate (1:1 of lambda-cyhalothrin: *Bt*) the percentage of the accumulative reduction in viability did not exceed 10% in 3 months (Figures 2–4) which is good as a preliminary consistent test for the shelf life of the combined formulations. In addition, further industrial formulation technologies would help in maintaining the combined formulations for a long shelf life. Considering the potential impact of the proposed combination on the physicochemical properties, this is a limiting factor for the field application. The lab-made combined formulations showed a relative completion in accordance with the specifications of FAO/WHO [53] for wettability and persistent foam. Although there are no minimum limits of suspensibility for the combined formula that have been specified yet, the percentage of suspensibility of each combined formula was higher than the minimum limit of lambda-cyhalothrin (50%), a little lower than the minimum limit for any of the tested *Bt* formulations (90%). However, it was still higher than the average of suspensibility between them (Table 2). Subsequently, we believe that in the near future, sustainable agriculture for food production will push further interest regarding the specific criteria for the physicochemical properties of the bio and chemical co-formulations due to the need to increase their use. In fact, for decades many researchers working on pest management strategies using either chemical synthetic insecticides or bio-insecticides have expressed huge concern about the negative impacts on the bacterial strains, resulting in the combination. Actually, in the last few decades pest management strategies have highlighted the possibility of combining chemical synthetic insecticides and bio-insecticides based on bacterial fermentation derivatives such as Abamectin, Emamectin, and Spinosad to be produced in a co-formulated mixture to overcome the pest resistance spread to insecticides. However, huge concerns were expressed about the negative impacts of the chemical syn-

thetic insecticides on the biopesticide based on bacteria which encouraged and motivated us to investigate, track, document, and be the first to report the possibility of combining *Bt* products with a chemical insecticide in one formulate ready to use without any negative concerns about the efficacy. Lambda-cyhalothrin is a wide-spectrum pyrethroid insecticide that was first reported in 1984 and introduced to central America and the Far East in 1985. To date, it is still approved for use in all 27 EU countries and UK, USA, Australia, and Russia as well [58]. Such a commonly used insecticide has been proven to be an effective contribution in managing key pests alone and has been predominant in many commercial mixture formulates. In the USA, the annual estimation for pesticide use revealed that lambda-cyhalothrin is used on most economic crops such as cotton, wheat, oats, corn, rice, sorghum, barley, alfalfa, canola, tomato, cucumber, lettuce, onion, broccoli, cabbage, and cauliflower; thus, it is one of the most highly used insecticides annually [59,60]. The virulence of *B. thuringiensis* was not adversely affected by interfering with lambda-cyhalothrin in the combined formula even in the high mixing rate. Moreover, such combinations proved to have higher mortalities than the originally manufactured formulations alone for about 3.67–10.08 fold. However, significant differences occurred among lambda-cyhalothrin, Dipel, XenTari, and Agree when examined in the original manufactured formula individually with high concentrations against the second instar larvae of *T. absoluta*. The significant difference disappeared between the lab-made combined formulate of Dipel and XenTari with lambda-cyhalothrin. Meanwhile, significant differences were predominant between the combined Agree©/lambda-cyhalothrin formulate and that of the Dipel or XenTari combined formulate, suggesting that their potency is affected by the composite cry toxins in each bacterial strain. The *Btk* strain ABTS-351, found in the Dipel DF formulate, has a unique balanced mix of Cry1Aa, Cry1Ab, Cry1Ac, and Cry 2A that gives the potent superiority [42]. Likewise, the *Bta* strain ABTS-1857 found in the XenTari DF formulate has a balanced mix of Cry1 toxin proteins in terms of Cry1Aa, Cry1Ab, Cry1C, and Cry1D that gives the proficiency in killing larvae [43]. On the other hand, the *Bta* strain GC-91 found in the Agree WG formulate has a single major toxin protein (Cry1Ac) along with two minor toxins at very low levels (Cry1C and Cry 1D) that gives a restrictive and insufficient control over insects which increases the potential for developing resistance [44].

Hence, it is clear that lambda-cyhalothrin does not adversely affect the viability or the potential pathogenicity of *B. thuringiensis*. Our obtained results highlight the high potency of the co-formulation of *B. thuringiensis* with lambda-cyhalothrin on *T. absoluta*. Moreover, the combined formulates were distinguished by gathering two different dimensional modes of action that provided a potential higher efficacy to tomato leafminer larvae and other lepidopteran insects. In general, the first component, *B. thuringiensis*, produces three domains of Cry protein toxins that bind to specific receptors in the midgut apical microvilli membrane causing a pore formation that leads to ion leakage, cell lysis, septicemia, and death [34–36]. The second component, lambda-cyhalothrin, affects the nervous system of treated insects by disrupting the sodium channels' activation gate resulting in the prolonged excitation of neuronal cells causing rapid paralysis and then death [61,62]. In our proposed combination, *Btk* and *Bta* causes damage and disrupts the gut functions of caterpillars thereby reducing the detoxification rate of the ingested pyrethroid, lambda-cyhalothrin [63–65]. Therefore, the bacteria in such combinations would act as toxicants, protectants for the chemical insecticide, and enhancers at the same time. We believe that such a co-formulate pattern could break or mitigate the evolution of insect resistance. Therefore, the combined toxicity in this study would have the potential to be involved as an alternative or a complementary approach in pest management strategies and pest resistance management tools that would be successful in slowing, delaying, and overcoming pest resistance to pesticides [64]. We believe such an integrated formulation based on entomopathogenic bacteria would be one of the main inputs of a sustainable production system for food security that would maximize the reliance of biopesticides while rationalizing the use of chemical pesticides for the sustainable protection of biotic and abiotic environmental factors, livelihood, livestock, and wildlife.

5. Conclusions

Tomato leafminer, *Tuta absoluta* is an invasive cosmopolitan threat to sustainable tomato production with a tremendous capability of acquiring resistance to most pesticides used. To date, neither chemical synthetic nor biorational insecticides have proven to be highly effective in managing this insect. A shift toward insecticide mixtures has been documented as a necessary strategy to overcome the pest threat with a high concern about their adverse effects on the biotic and abiotic environmental factors. In this study, the impacts beyond the combination of *B. thuringiensis* and chemical insecticide, lambda-cyhalothrin in such a co-formulation and its potential on the invasive unsusceptible or resistant pests was documented. The proposed combined formulations of Dipel, XenTari, and Agree biopesticides with lambda-cyhalothrin showed an enhancing effect and was proven to have a higher potential than the originally manufactured formulations alone for about 3.67–10.08 fold on larval mortality. Additionally, lambda-cyhalothrin had no significant adverse effect on either the viability of *Bt* strains or the physicochemical properties of combined co-formulates. Therefore, such combined formulations would have the potential to be involved as an alternative or a complementary approach in pest management and pest resistance management tools. However, further work is required to investigate the potential impacts of lambda-cyhalothrin on the structures of Cry toxins, the biochemical and toxicological interactions between bio and chemical insecticides in the insect midgut, and the combination with other entomopathogenic bacterial strain. Further work will also be required to evaluate their potential in relation to other insect pests under laboratory and field conditions.

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