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Effects of a Salicylic Acid Analog on *Aphis gossypii* and Its Predator *Chrysoperla carnea* on Melon Plants

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Abstract: The salicylic acid analog BTH (benzo-(1,2,3)-thiadiazole-7-carbothioic-acid S-methyl ester) induces systemic acquired resistance by promoting plant resistance against numerous plant pathogens and some insect pests. The objective of the research was to evaluate the activation of plant defenses with BTH on melon (*Cucumis melo* L., Cucurbitaceae) and its effects on the herbivore *Aphis gossypii* Glover, 1877 (Hemiptera: Aphididae) and on the aphid predator *Chrysoperla carnea* (Stephens, 1836) (Neuroptera: Chrysopidae). Under laboratory conditions, plants were sprayed with BTH (50 g/ha) zero (B0), four (B4), and seven (B7) days prior exposure to insects. B0 treatment resulted in 100% mortality of aphid nymphs and disrupted adult feeding behavior (recorded by electrical-penetration-graphs technique), by prolonging the time to reach the phloem, requiring more probes to first salivation and reducing ingestion activities. There were no effects on feeding behavior of *A. gossypii* fed on B4 plants but on its life history because fewer nymphs were born, intrinsic rate of natural growth decreased, and mortality increased. There were no effects on biological parameters of aphids reared on B7 plants. Prey consumption by *C. carnea* larvae when predated *A. gossypii* fed on BTH-treated plants was not different among treatments. Therefore, BTH enhances the suppression of *A. gossypii* in the short term, without negative effects on the predatory larva *C. carnea*, which makes this plant strengthener a useful tool to be considered in integrated pest management programs.

Keywords: biological control; electrical penetration graphs (EPG) technique; elicitors; feeding efficiency; integrated pest management (IPM); pest control; plant defenses; plant strengtheners

1. Introduction

Plants respond to herbivores and pathogens with a wide range of physical and chemical defenses [1,2]. The last can be constitutive, inducible, and even indirect through the use of nectars and volatile organic compounds (VOCs) that attract natural enemies of pests [3,4]. Chemical defenses involve complex processes with cascading reactions that use molecules, signals, and regulators with an initial short-term response at membrane level when the plant receptors detect the presence of phytophagous pests and the subsequent trigger of induced direct and indirect defenses [4]. Systemic acquired resistance (SAR) is a type of induced long-lasting defense response [5], very effective against biotrophic and hemibiotrophic pathogens and some insect herbivores [6]. The induction of SAR is signaled by the phytohormone salicylic acid (SA) and it is associated with pathogenesis-related proteins

accumulation [7,8]. Elicitors are conserved molecular patterns of pathogens and pests recognized by plant transmembrane receptors that activate defense events termed pattern-trigged immunity (PTI). Besides, plant intracellular receptors identify attacker virulence molecules known as effectors inducing effector-trigged immunity (ETI). Both defense responses, PTI and ETI, are mediated by a complex hormonal crosstalk [9,10]. Generally, the SA signaling pathway is activated by biotrophs and some sucking insects (aphids and thrips) while necrotrophs, chewing insects, and cell-sucking acari induce jasmonic acid (JA) cascade reactions to regulate defenses [4,11].

The active ingredient benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH), also called acibenzolar-S-methyl (ASM), a SA analog, can trigger a SAR response promoting plant resistance against viruses, fungi, and bacteria [6,12–14]; even conferring resistance against aphids and other herbivorous insects [15–17]; and moreover, increasing parasitoid attraction to herbivore-damaged plants [18–21]. This plant strengthener has minimal negative effects on human health and environment, although it could have an "allocation fitness cost", as SAR activation might reduce plant growth [5,7,22]. Despite this negative effect, the inclusion of elicitors within integrated pest management (IPM) practices will probably allow the reduction of pesticide use and/or to enhance the biological control, for example by changing the emission of VOCs or increasing nectar production that attracts parasitoids [1,3,16,20].

To our knowledge, limited information has been reported about the effect of BTH on melon (*Cucumis melo* L., Cucurbitaceae), an important fruit crop worldwide, with 1.05×10^6 ha and 2.7×10^7 tonnes production in 2018 [23]. It is known that BTH could induce melon defense responses against pathogens of soil-borne diseases and could also prevent postharvest fruit infection [24–26]. However, no information has been noticed about the effect of BTH on piercing-sucking pests, which could be a vector of melon pathogens, and on predators as biocontrol agents. Furthermore, it is not clear from literature how much earlier plant defenses must be activated to observe an effect of the elicitor since it seems to be dependent on the specific plant-pathogen/pest system studied, the kind of elicitor, the type of induction, and the means of activation of defenses [5,22].

Therefore, in order to better understand BTH-effects, this study was performed in a tritrophic system commonly found in melon crops. We chose the cotton-melon aphid, *Aphis gossypii* Glover, 1877 (Hemiptera: Aphididae), one of its principal aphid pest species, with an overall economic impact as a vector of plant viruses affecting melon and other important crops [27,28]. The natural enemy selected was the cosmopolitan and polyphagous predator *Chrysoperla carnea* (Stephens, 1836) (Neuroptera: Chrysopidae), which is commonly found in natural and agricultural ecosystems all over the world and is commercially available in many countries for the control of aphids and other soft-body herbivore insects in greenhouse crops [29,30]. The stated hypothesis was that the potential activation of plant defenses with BTH could lead to changes in the development, reproduction, and feeding behavior of *A. gossypii* on melon plants, and subsequently could affect the feeding efficiency (i.e., prey consumption) of the predatory larva *C. carnea*. The effects of this elicitor at different application periods on the tritrophic system are discussed.

2. Materials and Methods

2.1. Chemical and Treatments

Melon plants were sprayed to the point of run-off with BION® [acibenzolar-S-methyl (BTH) 50% *w/w*, WG, Syngenta Crop Protection AG, Basel, Switzerland] at 50 g of active ingredient/ha and 300 L/ha (0.167 g BION®/L and 0.023 L/plant), as recommended by the manufacturer. Aqueous fresh solution was prepared with distilled water immediately prior to the application with a hand sprayer.

Three different application times were chosen to detect the potential activation of melon plants' defenses by BTH: zero (B0), four (B4), and seven (B7) days before first aphid contact with plants. Control plants were sprayed with distilled water the day of aphid exposure. Insects were placed on melon plants as soon as BTH residues or water had dried (3–4 h later).

2.2. Plants and Insects: General Aspects

Melon cv. Sancho (Syngenta Seeds B.V., Enkhuizen, The Netherlands), a hybrid melon cultivar (toad skin type) widely planted in Central Spain was used for the experiments.

The clonal *A. gossypii* colony was originally started from a single virginoparous apterae collected from melon in El Ejido, Spain, in 1998. Laboratory colony was reared on melon plants and renewed every fortnight inside plastic aphid-proof cages in walk-in environmental chambers (23:18 °C temperature, 75% relative humidity (RH), and 16:8 light:dark (L:D) photoperiod).

Both plants and aphids were maintained at the Crop Protection Unit, Escuela Técnica Superior de Ingeniería Agronómica, Alimentaria y de Biosistemas—Universidad Politécnica de Madrid (ETSIAAB-UPM, Madrid, Spain), and at the Insect Vectors of Plant Pathogens laboratory, Institute of Agricultural Sciences-Spanish National Research Council (ICA-CSIC), where the different experiments were performed.

Chrysoperla carnea larvae were initially purchased from Agrobio (La Mojonera, Almería, Spain) and maintained for several generations in a climatic chamber (25 ± 2 °C, 75 ± 5% RH and 16:8 (L:D) photoperiod) at the ETSIAAB-UPM laboratory, following standard rearing procedures [31]. To obtain third larval stage L3 (<24 h) for the experiments, a gauze was put on top of the adult rearing plastic cage to allow mated females to lay eggs for 24 h. Then, the gauze with the eggs was placed in a new cage with zig-zag filter paper and *Ephestia kuehniella* (Zeller) eggs ad libitum (as food for newly-emerged larvae). Once larvae reached L2 stage, they were individually placed with *E. kuehniella* eggs ad libitum to avoid cannibalism. Change in the larval stage was verified with the molt presence.

2.3. Aphis gossypii Individual Fitness

Melon was transplanted twice in 1:1 mixture of soil substrate (Projar S.A., Valencia, Spain) and vermiculite (No. 3, Projar S.A., Valencia, Spain), first from germination in darkness (20 seeds/15 cm glass Petri dish with wet filter paper on the bottom) to 7 × 7 cm cell seedling trays at seven-days-old (BBCH stage = 10) [32], and from there to the definite potting in 10.5 cm diameter containers at BBCH stage = 14. Plants were maintained in a walk-in environmental chamber, at 23.35 ± 0.03 °C, 87.17 ± 0.37% RH, and 16:8 (L:D) h photoperiod. Temperature and humidity data were recorded every hour with a data logger (Tinytag Ultra 2, Gemini Data Loggers, Chichester, UK). Once a week, plants were fertilized (multifunctional fertilizer COMPO NPK 7 + 5 + 6 + microelements, COMPO EXPERT GmbH, Münster, Germany). Five-week-old melon plants (BBCH stage = 15) were used in the experiment, performed at ETSIAAB-UPM laboratory.

Aphids were reared in a controlled environmental chamber (SANYO-MLR 351, Osaka, Japan) at 23–19 °C, 75% RH, and 16:8 h (L:D) photoperiod and synchronized for the experiment. Then, to guarantee same-aged aphids, adult females from the laboratory colony were placed on 5-week-old melon plants. Aphids were maintained for 24 h inside clip-cages (3 cm diameter) with a mesh at the top to allow ventilation, then adults were removed with a wet paintbrush and the new nymphs were left on plants to develop into adults until the bioassay.

To analyze the effect of BTH on the biological parameters of *A. gossypii*, a single adult apterae female (8–9 days old since birth) was placed on the adaxial side of the second true-leaf of 5-week-old melon plants. This day was considered the beginning of the bioassay. Aphid females were confined for 24 h to generate offspring in a 3 cm diameter clip-cage. Afterward, only one neonate nymph was left per plant, which was monitored until adulthood. Then, adult offspring were counted by removing its nymphs daily until adults complete a period equal to the pre-reproductive period (number of days from birth to the onset of its reproduction) [33,34]. Aphid survivorship, duration of each nymphal instar (presence of exuvia was indicative of molting), pre-reproductive period (d), effective fecundity (offspring produced during period equal to pre-reproductive period) (Md), intrinsic rate of natural increase ($rm = 0.738 (\ln Md)/d$) [35,36], mean generation time ($Td = d/0.738$), and mean relative growth rate ($RGR = rm/0.86$) [37] were calculated. Plants' position inside the environmental chamber was randomized every day to avoid any positional effect. Each replicate consisted of a single aphid and

plant. Due to the limited space inside the environmental chamber, in the first experiment, control, and B0 treatments were compared (n = 34 replicates, respectively), and in a second one, control, B4 and B7 treatments (n = 22, 25, and 25 respectively). For the B0 treatment, only aphid survival was analyzed as all nymphs died before the sixth day of monitoring.

2.4. *Aphis gossypii* Feeding Behavior

Five-week-old melon plants were used in this experiment conducted at ICA-CSIC. Melon was seeded every week to have enough plants for the trials. Seven-day-old seedlings were transplanted into 1:1 mixture of soil substrate (GoV4, Jiffy International, A.S., Stange, Norway) and vermiculite (No. 3, Asfaltex S.A., Barcelona, Spain) in 8 × 8 × 8 cm pots. Plants were placed in the growth chamber at 24:20 °C (L:D), 60–100% RH, and 16:8 h (L:D) photoperiod. Plants were watered on alternate days with 50 mL/plant and fertilized with 20-20-20 N-P-K (Miller Chemical and Fertilizer Corp., Hanover, PA, USA) added to the irrigation water (1 g/L).

Aphis gossypii colonies were reared on melon plants inside rearing cages placed in an environmental chamber at 23:18 °C (L:D), 75% HR, and 16:8 h (L:D) photoperiod. Aphids were synchronized to guarantee age homogeneity at the time of the electrical penetration graph (EPG) recording.

To determine whether the application of BTH to melon plants affected *A. gossypii* feeding behavior, the EPG technique [38] was used to monitor in real-time the plant penetration activities of the aphid pierce-sucking mouthparts, adapting the method previously described for *A. gossypii* on cucumber (*Cucumis sativus* L.) [33]. Plants from the three treatments (control, B0, and B4), treated as described above, were used every time EPGs were performed. Because there were no statistical differences between aphid fitness on control plants and on B7, this treatment was not included in the feeding behavior experiment.

To facilitate wiring, apterous adult *A. gossypii* (11–12 days old since birth) was immobilized using a vacuum-operated plate (Eyela Aspirator A3S, Tokyo Rikakikai Co. Ltd., Tokyo, Japan). Then, an extra thin gold wire (2 cm length, 18.5 µm in diameter) was attached to the aphid dorsum with a small droplet of water-based silver-conducting glue (EPG-Systems, Wageningen, The Netherlands). The opposite extreme of the gold wire was glued to a thin copper wire (2-cm length), attached to a brass pin, which was inserted into the input connector of the primary amplifier of the 4-channel Gigaohm DC-EPG device (EPG Systems, Wageningen, The Netherlands). Another copper electrode (10-cm length, 2-mm diameter) was inserted into the soil of the plant container. Aphids were placed on the abaxial side of the third of four-leaf growth-stage melon plants. Each single aphid and melon plant was used only once for EPG recordings. The probing and feeding behavior was recorded during a 6-h period, which started directly after aphids were placed on the melon leaves. The EPGs acquisition was carried out inside a Faraday cage to avoid electrical noises in an air-conditioned room (24 °C). Data acquisition and analysis were conducted by Stylet + v01.25 software for Windows (EPG Systems, Wageningen, The Netherlands). Finally, 20 replicates per control treatment and 24 replicates per B0 and B4 were analyzed.

The aphid associated EPG waveforms [38] analyzed in this study were: (non-probe) non-probing behavior, i.e., no stylet contact with the leaf tissue; (C) intercellular apoplastic stylet pathway where insects show a cyclic activity of mechanical stylet penetration and secretion of saliva; (E1) salivation into phloem sieve elements at the beginning of the phloem phase; and (E2) passive phloem sap uptake from the sieve elements. The term "probe" refers to any type of event during the period in which the stylets of an individual insect were in contact with plant tissue, whereas "non-probe" refers to the event with no contact between stylets and plant tissue [39]. EPG sequential and non-sequential variables related to the probing and feeding behavior, described by the different waveforms, were calculated for each EPG recording using the MS Excel workbook for automatic parameter calculation of EPG data (version 5.0) [40]. The mean ± standard error of the 32 selected EPG sequential and non-sequential variables was calculated and organized as described in Backus et al. [41]: PPW, the proportion of individuals that produced a specific waveform type; NWEI, number of waveform events per insect,

that is the sum of the number of events of a particular waveform divided by the total number of insects under each treatment; WDI, waveform duration per insect, calculated using the sum of the duration of each event of a particular waveform made by each individual insect that produced the waveform divided by the total number of insects under each treatment; and WDE, waveform duration (min) per event, that is the sum of the duration of the events for a particular waveform divided by the total number of events of that particular waveform under each treatment (this parameter provides information on behavior at the population level).

2.5. Feeding Efficiency of Chrysoperla carnea

A protocol adapted from Wanumen et al. [42] was used to analyze whether BTH had a repellent or anti-feeding action on the predator *C. carnea* when the prey *A. gossypii* fed on treated plants, as some alterations in aphid feeding behavior on BTH-treated plants had been observed previously. The L3 *C. carnea* was fed for three consecutive days with four instar *A. gossypii* nymphs (N4), previously fed over 6 h on BTH-treated plants, as changes in aphid feeding behavior had been already observed over this period in the prior EPG experiment. This instar was used as prey for *C. carnea* instead of adults, in order to know the exact number of prey ingested/day, because the latter could generate offspring. Accordingly, the aphid laboratory colony reared in ETSIAAB-UPM facilities was synchronized to have enough 5-day-old nymphs (N4) for the bioassay.

Melon seedlings were transplanted in 9 × 9 × 10 cm pots and maintained in the growth chamber at ICA-CSIC facilities as described in *A. gossypii* feeding behavior experiment until bioassay in ETSIAAB-UPM laboratory when plants were five weeks old. Thereafter, plants were maintained in the environmental chamber (SANYO-MLR 351) at the same environmental conditions as in ICA-CSIC. Plants from the three treatments (control, B0, and B4) were used in the bioassay. Three plots of plants per treatment, one for each day of consumption experiment, were sprayed at the same time (21 plants/treatment). The fourth true-leaf of each plant was placed in 12 cm cylindrical cages, with a hole with foam between the lid and the cage to protect and allow the petiole insertion. In this way, BTH could continue relocating through the plant as cutting the leaf was avoided. Filter paper was placed at the bottom of the cage to absorb moisture and prevent water condensation.

Three consecutive aphid synchronizations were performed to get enough nymphs for the experiment (300 adults to get ≈ 2400 N4/day, to use ≈ 1400). Daily, 21 lots of 60 N4 aphids (the two first days) or 65 (the third day), as L3 were bigger and could need to ingest more food supply, were placed in 4 cm cages with ventilation (7 replicates × 3 treatments), where they were fasting until passed to the treated plants. Aphids were transferred to the 12 cm cage and allowed to settle on the leaf. Nymphs were feeding on the plant for 6 h. After this period, one L3 *C. carnea* (<24 h), that had been starved for 24 h to assure it had a voracious appetite, was placed on the cage and left 24 h to predate the nymphs. After 24 and 48 h later, the same procedure was repeated with new plants and aphids but transferring the same *C. carnea* larvae.

Every 24 h, once larvae had been transferred to new experimental units, leaves with aphids (predated or not) were excised and aphids counted. The fourth day, *C. carnea* larvae were placed in 2.5 cm diameter cages and fed with untreated *E. kuehniella* eggs *ad libitum* until larvae pupated. Larvae were checked daily until pupation and adult emergence. Furthermore, *C. carnea* larvae were weighed immediately before the beginning of the bioassay (0 h) and 72 h after feeding on aphid nymphs.

The parameters evaluated were: L3 *C. carnea* larval development time until pupation (days), pupation length (days), daily (after 24, 48, and 72 h) and total consumption of aphids (number of aphids ingested/offered), and total increase in weight after three days of contaminated prey consumption (mg). The experiment was repeated three times.

2.6. Statistical Analysis

Aphid survivorship data were analyzed by Kaplan–Meier survival analysis with global and pairwise multiple comparison procedures in order to compare survival curves among treatments

(Log-Rank test, $p < 0.05$). Censored observations were those subjects that had not died at the end of the study and those that disappeared midway in the study (aphids that escaped from clip-cages) [43].

Aphid biological parameters were studied comparing B4, B7, and control treatments for the second trial. Aphid probing and feeding behavior as well as feeding efficiency of *C. carnea* were studied comparing B4, B0, and control treatments. All data sets that followed the assumptions of normality and homoscedasticity were analyzed by one-way analysis of variance (ANOVA). When necessary, data were transformed using the SQRT (X + 1) to reduce heteroscedasticity. The transformed data that also followed a Gaussian distribution were analyzed by one-way ANOVA test, while the non-parametric Kruskal–Wallis test was used for variables that did not follow a normal distribution or remained heteroscedastic. Subsequently, when significant differences were found ($p < 0.05$) in the ANOVA, a post-hoc Tukey test was performed for pairwise comparisons; likewise, Kruskal–Wallis test was followed by post-hoc Dunn's pairwise comparison test with Bonferroni correction. Statistical tests were conducted using the IBM SPSS Statistics 24.0 software (package for Windows, 64-bit edition, Chicago, IL, USA) at a 0.05 significance level.

A chi-squared test (if expected values were higher than 5) or a Fisher's exact test if expected values were lower than 5 (Stat View 2, Abacus Concepts, Berkeley, CA, USA) were used to analyze the PPW among treatments.

3. Results

3.1. *Aphis gossypii* Individual Fitness

Survival curves of *A. gossypii* varied significantly among treatments (Log rank test, global comparisons: $\chi^2 = 112.643$, df = 4, $p \leq 0.001$) and that of B0 plants (BTH fresh residue) significantly differed from the rest. This treatment caused 100% mortality of *A. gossypii* nymphs in the first six days of evaluation and the individual fitness parameters could not be analyzed. The survival curve of *A. gossypii* reared on B4 plants (treated with BTH four days before the bioassay when the initial aphids were placed on treated plants) was significantly different from that of B7 plants. Survivorship of aphids on B4 and B7 plants was similar to that of the control of its trial (C2) (Log rank test, pairwise multiple comparison; Figure 1, Supplementary Table S1). Although no significantly different, C2 survival curve was lower than B7, this could be explained as there were some censored observations in C2 treatment, as some aphids disappeared from clip-cages.

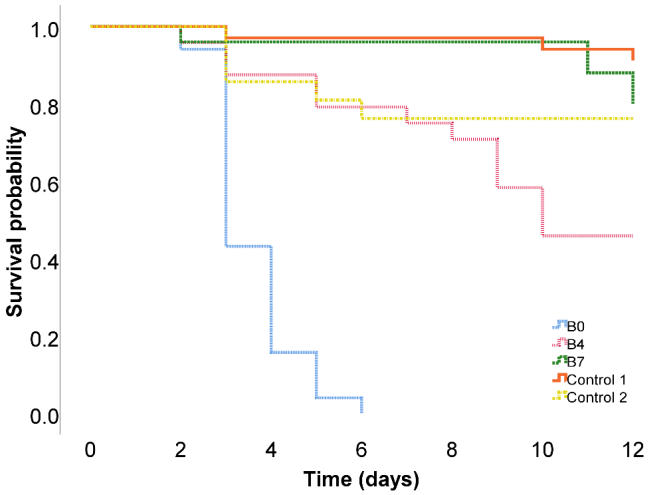

Figure 1. Survival curves of *Aphis gossypii* reared on melon plants sprayed with BTH (benzo-(1,2,3)-thiadiazole-7-carbothioic-acid S-methyl ester) to the point of run-off, zero (B0), four (B4), and seven (B7) days prior to aphid introduction (Log rank test, *p*-value < 0.05). Control plants were sprayed with distilled water (Control 1: first trial control, Control 2: second trial control) the day of aphid introduction.

The development time of each nymphal instar, the pre-reproductive period as well as the mean generation time of *A. gossypii* were not affected by the application of BTH in B4 and B7 plants ($p > 0.05$ in all cases, Table 1). The effective fecundity (*Md*) of aphids grown on B4 plants significantly decreased (23.16%) compared to the control, but the effect disappeared when aphids were reared on B7 plants (*Md*: H = 7.565, $p = 0.023$) (Table 1).

There was a significant reduction in the intrinsic rate of natural increase (*rm*) of aphids grown on B4 plants compared to the control and to aphids grown on B7 plants. There were no differences in *rm* between aphids reared on B7 plants and the control. Because of the mean relative growth rate (*RGR*) is a related to *rm* by the formula ($rm = 0.86\ RGR$), the same trend was observed (*rm* and *RGR*: $F_{2,42} = 8.371$, $p = 0.001$) (Table 1).

Table 1. Biological parameters of *Aphis gossypii* reared on melon plants treated with BTH (benzo-(1,2,3)-thiadiazole-7-carbothioic-acid S-methyl ester) to the point of run-off, four (B4), and seven (B7) days prior to aphid introduction. Control plants were sprayed with distilled water the day of aphid introduction.

Aphid Parameter	Treatment	Mean ± SE	Statistic Test	*p*-Value
N1	Control	1.13 ± 0.09	H = 2.413	0.299
	B4	1.36 ± 0.15		
	B7	1.16 ± 0.09		
N2	Control	1.00 ± 0.00	H = 3.091	0.213
	B4	1.09 ± 0.09		
	B7	1.00 ± 0.00		
N3	Control	1.07 ± 0.07	H = 4.452	0.108
	B4	1.18 ± 0.12		
	B7	1.37 ± 0.11		
N4	Control	1.60 ± 0.13	H = 2.914	0.233
	B4	1.36 ± 0.15		
	B7	1.32 ± 0.11		
d	Control	5.80 ± 0.11	H = 2.316	0.314
	B4	6.00 ± 0.00		
	B7	5.84 ± 0.09		
Md	Control	59.867 ± 1.693 a	H = 7.565	0.023 *
	B4	46.000 ± 4.665 b		
	B7	52.158 ± 2.367 ab		
rm	Control	0.522 ± 0.008 a	$F_{2,42} = 8.371$	0.001 ***
	B4	0.463 ± 0.015 b		
	B7	0.498 ± 0.007 a		
Td	Control	7.859 ± 0.145	H = 2.316	0.314
	B4	8.130 ± 0.000		
	B7	7.916 ± 0.116		
RGR	Control	0.607 ± 0.010 a	$F_{2,42} = 8.371$	0.001 ***
	B4	0.538 ± 0.017 b		
	B7	0.580 ± 0.008 a		

N1–N4: first-forth instar nymphs (days); *d*: pre-reproductive period (days); *Md*: effective fecundity; *rm*: intrinsic rate of natural increase; *Td*: mean generation time (days); *RGR*: mean relative growth rate. Different lower case letters in means within an aphid parameter indicate significant differences among treatments (* $p < 0.05$, *** $p < 0.001$) according to ANOVA test followed by post-hoc Tukey test for normal and homoscedastic variables; or according to Kruskal–Wallis test followed by post-hoc Dunn's pairwise comparison test with Bonferroni correction, for non-normal and/or heteroscedastic variables.

3.2. *Aphis gossypii* Feeding Behavior

The effect of BTH on the feeding behavior of aphids fed on B0 and B4 plants was analyzed because mortality of aphids exposed to the first treatment was very high and fecundity and growth rates were

affected on B4 plants. However, as there were no differences in the biological parameters of *A. gossypii* reared on B7 plants compared to the control, this treatment was not considered.

In general, for the majority of variables studied, feeding behavior of aphids exposed to control plants (C) did not differ significantly from the other two treatments (B0–B4) (Table 2). The probing activities measured by the number of waveform events per insect (NWEI) of "probe", intercellular stylet pathway "C" and "short probes" (C < 3 min), were significantly more numerous in aphids feeding in B0 plants compared to B4 ones ($p < 0.05$ in all cases, Table 2). The duration of the "probe" waveform per insect (WDI) was significantly shorter while the duration of "C" was significantly longer on aphids feeding in B0 plants compared to B4 ones ($p < 0.05$ in both cases, Table 2). No differences were observed in the duration of the "probe" event as well as in the "C" event (WDE) among treatments (WDE, $p > 0.05$ in both cases, Table 2). The percentage of probing time spent in intercellular stylet pathway activities ("C") was significantly higher in aphids feeding in B0 plants compared to those feeding in B4 ones ($p < 0.05$, Figure 2). There were significantly more "non-probe" events (NWEI) and its durations per aphid (WDI) and per event (WDE) were significantly longer in aphids feeding in B0 plants compared to those feeding in B4 plants ($p < 0.05$ in all cases, Table 2).

Regarding phloem-related activities, the number of phloem salivation ("E1"), ingestion ("E2"), and "E1 that was not followed by E2" ("single E1"), did not differ significantly among treatments (NWEI, $p > 0.05$ in all cases, Table 2). However, the number of "sustained E2" (longer than 10 min) (sE2) was significantly lower in aphids feeding in B0 plants compared with those feeding in B4 plants (NWEI, $p < 0.05$, Table 2). The proportion of aphids fed on B0 plants (PPW = 14/24) that produced "sustained E2" was significantly lower than those fed on B4 plants (PPW = 21/24) or control plants (PPW = 18/20) (B0–B4: χ^2: 5.169, $p = 0.023$; B0-C: χ^2: 5.515, $p = 0.019$; B4-C: Fisher's Exact $p > 0.999$) (Table 2). There was a 50% significant reduction in the duration of ingestion ("E2") phase (WDI) in aphids feeding in B0 plants compared to B4 and control plants, and these two treatments were not significantly different (WDI, $p < 0.05$, Table 2). The percentage of probing time spent in ingestion activities ("E2") was significantly lower in aphids feeding in B0 plants than in B4 or control plants and the two last treatments did not differ between them ($p < 0.05$, Figure 2).

No differences were observed in the duration of "E1" or "single E1" per insect among treatments (WDI, $p > 0.05$, Table 2), neither in the duration per event of phloem-related activities "E1", "single E1" and "E2" (WDE, $p > 0.05$, Table 2). No differences in the percentage of probing time spent in salivation ("E1") were observed among treatments ($p > 0.05$, Figure 2).

Table 2. Mean ± standard error (min) of non-sequential electrical penetration graph (EPG) variables and their ranges in parenthesis for the feeding behavior of *Aphis gossypii* exposed to melon plants at 0 (fresh residue) (B0) and 4 days (B4) after sprayed BTH (benzo-(1,2,3)-thiadiazole-7-carbothioic-acid S-methyl ester), using water-sprayed plants as control, during a six-hour recording.

Variables	Treatment	PPW	NWEI	Test Statistic	*p*-Value	WDI	Test Statistic	*p*-Value	WDE	Test Statistic	*p*-Value
Non-probe	Control	20/20	16.15 ± 1.62 (5.00–32.00) ab	$F_{2,65}$ = 4.725	0.012 *	26.90 ± 3.68 (6.23–63.04) ab	H = 10.461	0.005 **	1.68 ± 0.10 (0.10–16.82) ab	H = 6.876	0.032 *
	B0	24/24	22.38 ± 2.22 (3.00–41.00) a			43.44 ± 5.04 (2.83–98.84) a			1.95 ± 0.10 (0.03–28.34) a		
	B4	24/24	13.96 ± 1.72 (4.00–33.00) b			23.58 ± 4.27 (1.87–96.76) b			1.70 ± 0.10 (0.06–13.66) b		
Probe	Control	20/20	16.15 ± 1.62 (5.00–32.00) ab	H = 8.526	0.014 *	333.10 ± 3.68 (296.96–353.77) ab	H = 10.918	0.004 **	20.63 ± 3.03 (0.19–331.00)	H = 5.883	0.053
	B0	24/24	22.29 ± 2.21 (3.00–40.00) a			316.56 ± 5.04 (261.16–357.17) b			14.20 ± 1.72 (0.17–340.44)		
	B4	24/24	13.96 ± 1.72 (4.00–33.00) b			337.89 ± 4.65 (263.24–381.28) a			24.14 ± 3.44 (0.17–351.50)		
C	Control	20/20	17.55 ± 1.81 (5.00–35.00) ab	H = 8.061	0.018 *	157.68 ± 18.36 (44.61–313.89) ab	H = 9.413	0.009 **	8.98 ± 0.65 (0.19–65.52)	$F_{2,1328}$ = 0.100	0.905
	B0	24/24	25.13 ± 2.65 (3.00–51.00) a			221.99 ± 17.94 (58.63–321.45) a			8.84 ± 0.67 (0.17–161.44)		
	B4	24/24	15.67 ± 1.92 (5.00–39.00) b			145.58 ± 18.81 (16.38–336.68) b			9.27 ± 0.70 (0.14–99.43)		
short probes (C < 3 min)	Control	20/20	7.95 ± 0.93 (2.00–20.00) ab	H = 7.983	0.018 *						
	B0	24/24	12.71 ± 1.61 (1.00–30.00) a								
	B4	24/24	7.17 ± 1.12 (1.00–24.00) b								
E1	Control	20/20	2.25 ± 0.31 (1.00–5.00)	H = 0.105	0.949	1.38 ± 0.32 (0.42–5.67)	H = 2.205	0.332	0.61 ± 0.13 (0.11–5.00)	H = 3.535	0.171
	B0	23/24	3.17 ± 0.90 (0.00–17.00)			3.45 ± 1.71 (0.00–41.60)			1.09 ± 0.38 (0.08–27.82)		
	B4	23/24	2.29 ± 0.38 (0.00–8.00)			5.06 ± 1.58 (0.00–28.16)			2.21 ± 0.73 (0.06–28.16)		
single E1	Control	4/20	0.25 ± 0.12 (0.00–2.00)	H = 3.542	0.170	0.33 ± 0.23 (0.00–4.51)	H = 3.753	0.153			
	B0	8/24	1.46 ± 0.78 (0.00–15.00)			2.77 ± 1.70 (0.00–40.75)					
	B4	3/24	0.13 ± 0.07 (0.00–1.00)			0.15 ± 0.08 (0.00–1.38)					
E2	Control	20/20	1.95 ± 0.29 (1.00–5.00)	H = 1.390	0.499	170.65 ± 22.28 (5.90–308.33) a	H = 10.081	0.006 **	87.51 ± 17.72 (0.14–308.33)	H = 2.860	0.239
	B0	20/24	1.71 ± 0.30 (0.00–5.00)			88.31 ± 20.79 (0.00–282.87) b			51.69 ± 13.60 (0.24–278.36)		
	B4	23/24	2.13 ± 0.34 (0.00–7.00)			174.76 ± 23.17 (0.00–334.86) a			82.24 ± 15.70 (0.15–318.71)		
sE2	Control	18/20	0.95 ± 0.09 (0.00–2.00) ab	H = 7.930	0.019 *				177.81 ± 21.84 (14.07–308.33)	H = 1.050	0.592
	B0	14/24	0.63 ± 0.12 (0.00–2.00) b						139.13 ± 24.16 (19.54–278.36)		
	B4	21/24	1.04 ± 0.11 (0.00–2.00) a						165.62 ± 21.90 (10.56–318.71)		

PPW, proportion of individuals that produced the waveform type; NWEI, number of waveform events per insect; WDI, waveform duration (min) per insect; WDE, waveform duration (min) per event. Waveforms: C, intercellular stylet pathway; E shows phloem-related activities, E1: correlates with salivation into phloem elements; single E1: E1 not followed by E2; E2: regards as phloem ingestion; sE2: sustained E2 (>10 min). Different lower case letters within variables indicate significant differences among treatments (* $p < 0.05$, ** $p < 0.01$) according to ANOVA test followed by post-hoc Tukey test for normal and homoscedastic variables, transformed by SQRT (X + 1) when needed; or to Kruskal–Wallis test followed by post-hoc Dunn's pairwise comparison test with Bonferroni correction, for non-normal and/or heteroscedastic variables.

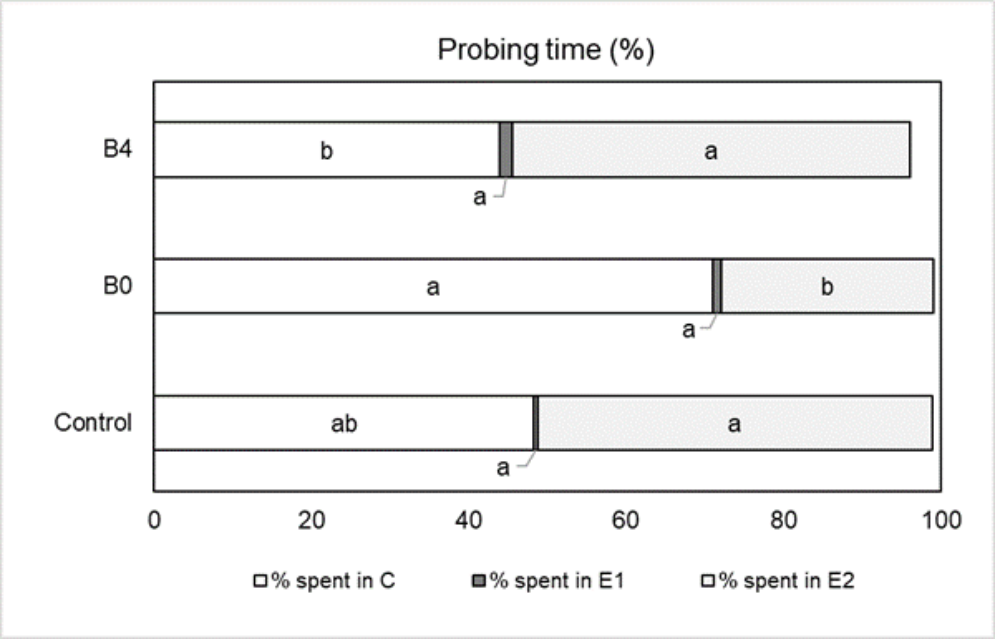

Figure 2. Percentage of probing time spent in a specific waveform when *Aphis gossypii* was fed on melon plants sprayed with BTH (benzo-(1,2,3)-thiadiazole-7-carbothioic-acid S-methyl ester) to the point of run-off, zero (B0) and four (B4) days prior to aphid first contact, using water-sprayed plants as control. Six-hour electrical penetration graph (EPG) recording. Waveforms: C, intercellular stylet pathway; E shows phloem-related activities: E1, correlates with salivation into phloem sieve elements; E2, regards as phloem ingestion [44]. Statistical comparisons among treatments for each parameter were made by non-parametric Kruskal–Wallis test. Means within variables followed by different lower-case letters are significantly different (p-value < 0.05).

Sequential EPG variables that describe the sequence of events related to each other during the six hours of recording are shown in Table 3. Time spent from first probe to first E, time from first probe to first E2, time from first probe to first E2 and number of probes to the first E1 were significantly higher in aphids feeding in B0 plants than in the other two treatments, not observing differences in these variables between aphids feeding on B4 and control plants ($p < 0.05$ in all cases, Table 3). Total duration of non-probe before the first E was significantly longer in aphids feeding in B0 plants than in B4 plants, while aphids exposed to control plants did not significantly differ from the other two treatments ($p < 0.05$, Table 3). Time to first probe from start of EPG, time from the beginning of that probe to first E, time from the beginning of that probe to first E2 and time from the beginning of that probe to first sE2 were not significantly different among treatments ($p > 0.05$ in all cases, Table 3).

Table 3. Mean ± standard error (min) of sequential electrical penetration graph (EPG) variables and their ranges in parenthesis for the feeding behavior of *Aphis gossypii* exposed to melon plants at 0 (fresh residue) (B0) and 4 days (B4) after sprayed BTH (benzo-(1,2,3)-thiadiazole-7-carbothioic-acid S-methyl ester), using water-sprayed plants as control, during a six-hour recording.

	Sequential EPG Variables	Treatment	PPW	Mean ± SE (Range)	Test Statistic	*p*-Value
WDI	Time to 1st probe from start of EPG	Control	20/20	2.19 ± 0.86 (0.00–16.82)	H = 0.821	0.663
		B0	24/24	1.59 ± 0.61 (0.00–14.32)		
		B4	24/24	0.85 ± 0.20 (0.00–3.44)		
	Time from 1st probe to 1st E	Control	20/20	110.98 ± 16.14 (20.53–343.71) b	H = 9.586	0.008 **
		B0	23/24	190.26 ± 19.76 (43.85–345.68) a		
		B4	23/24	119.60 ± 17.58 (18.99–359.94) b		
	Time from the beginning of that probe to 1st E	Control	20/20	33.75 ± 3.90 (13.59–65.52)	H = 1.267	0.531
		B0	23/24	46.03 ± 7.91 (8.98–161.44)		
		B4	23/24	38.89 ± 8.52 (10.21–206.26)		
	Time from 1st probe to 1st sE2 (>10 min)	Control	18/20	172.16 ± 20.36 (51.45–358.11) b	H = 8.426	0.015 *
		B0	14/24	257.93 ± 22.03 (73.44–360.00) a		
		B4	21/24	170.96 ± 22.74 (19.72–359.94) b		
	Time from the beginning of that probe to 1st sE2 (>10 min)	Control	18/20	32.41 ± 3.51 (12.87–64.21)	H = 3.630	0.163
		B0	14/24	58.39 ± 11.39 (14.62–162.19)		
		B4	21/24	47.50 ± 9.47 (11.31–206.95)		
	Time from 1st probe to 1st E2	Control	20/20	125.03 ± 17.68 (20.73–344.86) b	$F_{2,65}$ = 7.157	0.002 **
		B0	20/24	214.66 ± 21.48 (57.96–360.00) a		
		B4	23/24	123.28 ± 17.75 (19.72–359.94) b		
	Time from the beginning of that probe to 1st E2	Control	20/20	33.97 ± 3.93 (14.08–66.68)	H = 0.728	0.695
		B0	20/24	46.83 ± 8.78 (14.35–162.19)		
		B4	23/24	42.73 ± 8.43 (10.42–206.95)		
	Total duration of non-probe before the 1st E	Control	20/20	18.19 ± 3.06 (0.20–55.34) ab	H = 8.425	0.015 *
		B0	23/24	30.89 ± 4.12 (2.83–83.58) a		
		B4	23/24	16.54 ± 2.47 (1.51–48.80) b		
NWEI	Number of probes to the 1st E1	Control	20/20	10.95 ± 1.23 (1.00–22.00) b	$F_{2,65}$ = 4.88	0.011 *
		B0	23/24	16.88 ± 1.73 (3.00–32.00) a		
		B4	23/24	10.79 ± 1.25 (3.00–22.00) b		

PPW, proportion of individuals that produced the waveform type; WDI, waveform duration (min) per insect; NWEI, number of waveform events per insect. Waveforms: E shows phloem-related activities: E1, correlates with salivation into phloem elements; E2, regards as phloem ingestion; sE2: sustained E2 (>10 min). Different lower case letters within variables indicate significant differences among treatments (* $p < 0.05$, ** $p < 0.01$) according to ANOVA test followed by post-hoc Tukey test for normal and homoscedastic variables, transformed by SQRT (X + 1) when needed; or to Kruskal–Wallis test followed by post-hoc Dunn's pairwise comparison test with Bonferroni correction, for non-normal and/or heteroscedastic variables.

3.3. Feeding Efficiency of *Chrysoperla carnea*

All *C. carnea* biology- and feeding-related parameters analyzed were similar among treatments ($p > 0.05$ in all cases, Table 4).

Table 4. *Chrysoperla carnea* biology and consumption parameters when third instar larvae (L3) predated forth instar nymphs of *Aphis gossypii* fed on melon plants treated with BTH (benzo-(1,2,3)-thiadiazole-7-carbothioic-acid S-methyl ester) the day of aphid introduction (B0) or 4 days before (B4), using water-sprayed plants as control.

***Chrysoperla carnea* Parameter**		**Treatment**	**Mean ± SE**	**Test Statistic**	***p*-Value [a]**
Development time (d)	L3	Control	6.53 ± 0.46	H = 0.040	0.980
		B0	6.88 ± 0.76		
		B4	6.70 ± 0.50		
	Pupae	Control	10.83 ± 0.17	H = 3.123	0.210
		B0	10.41 ± 0.15		
		B4	10.65 ± 0.11		
Consumption (%) [b]	24 h	Control	42.51 ± 4.61	$F_{2,55} = 0.159$	0.854
		B0	43.88 ± 4.64		
		B4	40.49 ± 3.64		
	48 h	Control	46.42 ± 5.05	$F_{2,55} = 1.222$	0.303
		B0	53.61 ± 5.26		
		B4	42.75 ± 4.71		
	72 h	Control	39.37 ± 3.63	H = 1.038	0.595
		B0	46.29 ± 5.02		
		B4	42.20 ± 6.40		
	Total	control	42.73 ± 3.29	$F_{2,53} = 0.562$	0.573
		B0	47.13 ± 4.07		
		B4	41.53 ± 4.28		
Weight (mg) [c]	0 h	Control	3.72 ± 0.29	$F_{2,30} = 0.364$	0.698
		B0	3.83 ± 0.35		
		B4	4.07 ± 0.21		
	72 h	Control	11.65 ± 0.48	$F_{2,47} = 0.210$	0.811
		B0	11.99 ± 0.50		
		B4	12.16 ± 0.69		
	Total increase [d]	Control	7.17 ± 0.29	$F_{2,30} = 3.049$	0.062
		B0	8.10 ± 0.51		
		B4	8.91 ± 0.69		

[a] All data sets among each parameter were not significantly different (ANOVA, Kruskal–Wallis; (p-value > 0.05)). [b] Number of *Aphis gossypii* nymphs, previously fed over 6 h on BTH-treated plants, consumed by L3 *C. carnea* (ingested/offered, %) counted after 24, 48, and 72 h. [c] L3 *C. carnea* were weighed immediately before the beginning of the bioassay (0 h) and 72 h after being fed with aphid nymphs (contaminated prey). [d] Total increase in weight after three days of contaminated prey consumption.

4. Discussion

Aphis gossypii has evolved resistance to the principal families of synthetic insecticides [27,45]. Owing to the fact that the application of pesticides must be reduced to achieve sustainable agriculture, the use of elicitors as plant strengtheners that could induce herbivore resistance at the same time that enhance the biological control of these pests could be an interesting strategy to be included in IPM. The BTH promotes pathogen resistance in a wide range of crops [6,14], but just a few studies have explored the susceptibility of herbivore insects and mites to this elicitor [15–17,46]. It is known that BTH improves indirectly the biological control since it can increase the attractiveness of herbivore-damaged plants to parasitic wasps due to qualitative and quantitative changes in the induced volatiles emissions [19–21], but information on BTH-effect on predators is insufficient.

There is great heterogeneity in scientific literature about how and when is necessary to apply BTH to induce plant resistance, and the time and mode of application chosen influence plants and herbivorous insects differently. BTH might be applied soaking seeds [47], spraying leaves or the whole plant [20], or as a soil drench [21]. Some researchers apply the product in young plants (1–2 weeks old) while others in older plants and just a few hours/days before the starting of the bioassays [16,18,46,48]. In this study, the pest was exposed to three different application periods of BTH to discern if the effect on insects' performance was due to an induction of defenses or due to a direct toxicity of the product.

A reduction in foliar thickness that finally generated necrotic lesions on BTH-sprayed leaves was observed in this experiment in agreement with phytotoxic effects detected in different plant species after application of elicitors [16,49]. Even if SA analogs are less phytotoxic than the application of SA [6,7], BTH may cause an allocation fitness cost [5]. This compound sprayed on seedlings of melon var. Orange Flesh caused an inhibition in growth from 6 days after treatment onwards and this could be related to the peroxidase activity increase. This defense-related enzyme is associated with the lignification of the plant cell wall that could increase resistance to pathogen attack, therefore, even if BTH could reduce melon growth, it finally induces its plant defenses [24]. Further biochemical research could elucidate if the BTH-effects observed in our trophic system may be related to the possible phytotoxicity.

In the present work, the EPG technique reveals that there was not a contact effect of BTH on the initial probing behavior (time from the start of the EPG recording until the first probe) of *A. gossypii* exposed to the different BTH-treated plants compared to the control plants. This EPG variable is associated with pre-phloem resistant factors present in epidermis or mesophyll that reduce attractiveness of the plant to the insect [50] and is used to evaluate the deterrent effect or delays in aphid probing on insecticide-treated plants [51]. In our case, BTH neither inhibited nor delayed *A. gossypii* initial probe on treated plants.

In agreement with Cooper et al. [17], who reported that BTH could reduce the population growth of *Macrosiphum euphorbiae* Thomas, 1878 on a susceptible tomato cultivar and enhance the aphid resistance in a resistant cultivar, the present research showed that fresh residues of BTH (B0 plants) prevented the population growth of *A. gossypii* in melon cv. Sancho because nymphs did not develop to maturity. The B0 plants modified probing and feeding behavior of *A. gossypii* adults thus, aphids spent more time in non-probe activities and although they could reach the phloem, they took longer to contact the sieve elements, needed more probes to salivate for the first time and finally spent less time in ingestion activities (i.e., passive phloem sap uptake). Therefore, B0 plants could have activated their defense mechanisms at phloem level in a short term, as *A. gossypii* cannot initiate ingestion from the phloem as when fed on control plants or B4 plants [52]. Using the same EPG recording technique, Powell and Hodge [53] observed that phloem sap ingestion was also reduced when *Acyrthosiphon pisum* Harris, 1776 fed on tic bean (*Vicia faba* var. *minor* L.) treated with the elicitor β-aminobutyric acid (BABA), but only when there has been a previous aphid infestation on the plants. In our study, the reduction in *A. gossypii* individual fitness in addition to the impairing in its feeding behavior on B0 plants could be more related to a direct toxic effect of fresh residue of BTH or to the increase in levels of pathogenesis-related proteins gene expression (immediate-early gene induction) [11,14,54].

In B4 plants, there was no effect on *A. gossypii* feeding behavior compared to the control. However, there was a clear effect on aphid life history traits because fewer nymphs were born, the intrinsic rate of natural growth decreased (also, the *RGR*), and the mortality increased compared to those on control plants. Therefore, an antibiosis more than an antixenosis mechanism could have reduced *A. gossypii* population growth when fed on these plants. Despite using different methodologies, similar effects on aphid fitness were reported in other elicitor-plant-aphid systems [16,55].

BTH can induce a long-lasting resistance against some aphids [7,20], but this was not the case in the present study because *A. gossypii* had similar performance and survival when reared on B7 plants compared to the control. Overall, the BTH effects observed could be due to an activation of the

defense mechanism only in the short term. For reasons still unknown, BTH does not induce prolonged resistance mechanisms against *A. gossypii.* On the other hand, BTH might be toxic to these aphids.

In this study, spray application of BTH to melon plants did not affect the feeding efficiency of *C. carnea* when predated *A. gossypii* fed on treated plants, independently of the application day. Similar to the results exposed here, the elicitor methyl jasmonate when applied to tomato seeds did not alter the behavior of *Chrysoperla externa* (Hagen, 1861) although reduced the performance of its pest, the leaf miner *Tuta absoluta* (Meyrick, 1917) [56]. In tritrophic systems involving elicitors and parasitoids from genus *Aphidius*, slight effects on the wasps' emergence were observed depending on the concentration of the compound, so specific studies case by case should be performed [49,57].

Alterations in aphid feeding behavior on BTH-treated plants could also change acquisition, retention, latency, and inoculation of aphid-borne plant viruses. The greater number of short superficial probes and intracellular stylet punctures increased the transmission of a non-persistent virus [33]. On the other hand, circulative virus particles are usually ingested from phloem sieve elements [58]. Therefore, stylets penetration behavior could help to determine the effect of elicitors on the transmission efficiency of plant viruses by piercing-sucking insects [59].

5. Conclusions

The effects of BTH applied at fresh residue could be due to a direct toxicity of the product on the aphid that undermines its fitness and feeding behavior, causing total mortality of *A. gossypii* nymphs. When the product is applied four days before *A. gossypii* exposure, BTH impairs aphid fitness reducing the population growth. A short-term but not long-lasting activation of plant defenses could be pointed for seven days after BTH application the product seems to have degraded regarding the effect on the aphid. The direct effect of BTH to *A. gossypii* in the short term, together with the lack of effect of the elicitor on *C. carnea* feeding efficiency, are indicative that this plant strengthener contributes to enhance the suppression of the phloem-feeding insect in the trophic system studied without harming the beneficial chrysopid. However, in order to consider BTH as an interesting strategy to control aphid pests within IPM for melon crops, further research should be addressed to ascertain the molecular and biochemical mechanisms behind the observed effects on melon and aphids caused by the elicitor BTH. Thus, a more efficient use of the product could be implemented.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/10/11/1830/s1, Table S1: Kaplan–Meier survival analysis of *Aphis gossypii* reared on melon plants sprayed with BTH to the point of run-off, zero (B0), four (B4), and seven (B7) days prior to aphid introduction. Control plants were sprayed with distilled water (C1: first trial control, compared to B0; C2: second trial control, compared to B4 and B7) the day of aphid introduction.

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