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

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

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

1. Introduction

The tomato potato psyllid (TPP) *Bactericera cockerelli* (Šulc) (Hemiptera: Triozidae) is a widely recognised pest of several solanaceous crops [1–4]. All TPP nymphal stages and adults can damage host plants by injecting salivary toxins that lead to foliar symptoms, such as leaf curling and yellowing. This condition was designated by Munyaneza [5] as "psyllid yellows". Moreover, TPP is also a vector of the bacterial pathogen *Candidatus* Liberibacter solanacearum (*C*Lso), which is responsible for zebra chip disease in potatoes [6,7]. *C*lso can cause the decline and death of infected plants [5], reducing yields and costing growers millions of dollars each year [8,9].

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

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

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

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

2. Materials and Methods

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

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

When the plants were 15–20 cm high, a total of 42 plants (21 grown from *T. atroviride* coated seeds and 21 from non-coated seeds) were randomly selected and each transplanted into a 6 L pot containing the growing medium. The medium was obtained from a 500 L mix that comprised 400 L of composted bark, 100 L of pumice, 2 kg of *Osmocote*® NPK fertiliser (www.growwithosmocote.com, accessed on September 2023), and 500 g of horticultural lime. During the transplanting process for *T. atroviridae*-treated plants, pellets containing the four *T. atroviride* strains manufactured by Agrimm Technologies Ltd. were mixed into the growing medium at a rate of 0.3 g/6 L pot (equivalent to 15 kg/ha). For the duration of the greenhouse experiment, single plants were kept in 60 cm × 60 cm × 180 cm cages (BugDorm 6E630; www.bugdorm.com, accessed on September 2023) and trickle-irrigated daily such that each plant received 0.25 L of water. The mean ambient greenhouse temperature was 21.9 °C (max 38 °C; min 15 °C), and mean relative humidity (RH) was 61.6% (max 90.5%; min 30%)

2.2. Experimental Design

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

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

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

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

2.4. Weekly Data Collection

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

2.5. VOC Sampling

Volatile organic compounds (VOCs) were sampled between the 7 and 10 of December 2021, using a dynamic push–pull headspace sampling technique as described by Effah et al. [44,45]. For this experiment, six treatments were used with seven replicates each, as described in the experimental design section. One individual leaf per treatment was enclosed in a multi-purpose 50 cm × 30 cm cooking bag (AWZ Products Inc., China) with both ends fastened using a cable tie. Using a portable PVAS22 pump (Volatile Assay Systems, Rensselaer, NY, US), carbon-filtered air was pushed into the bags through a PTFE tube (0.9 L/min) and simultaneously pulled out through another tube (0.8 L/min), creating a slight positive pressure to reduce external contaminants.

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

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

2.6. Statistical Analyses

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

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

3. Results

3.1. Average Rates of TPP Suppression

The average rates of TPP suppression per treatment across the experiment are shown in Figure 1. Across all four measured TPP variables, each of the three treatments significantly reduced the mean TPP counts relative to the control. However, *T. atroviride* alone was significantly less effective than either *E. nicotianae* or the combined treatment. Moreover, the combined treatment was no better overall than *E. nicotianae* alone (i.e., *T. atroviride* did not improve the average performance of the *E. nicotianae* treatment across the sampling period).

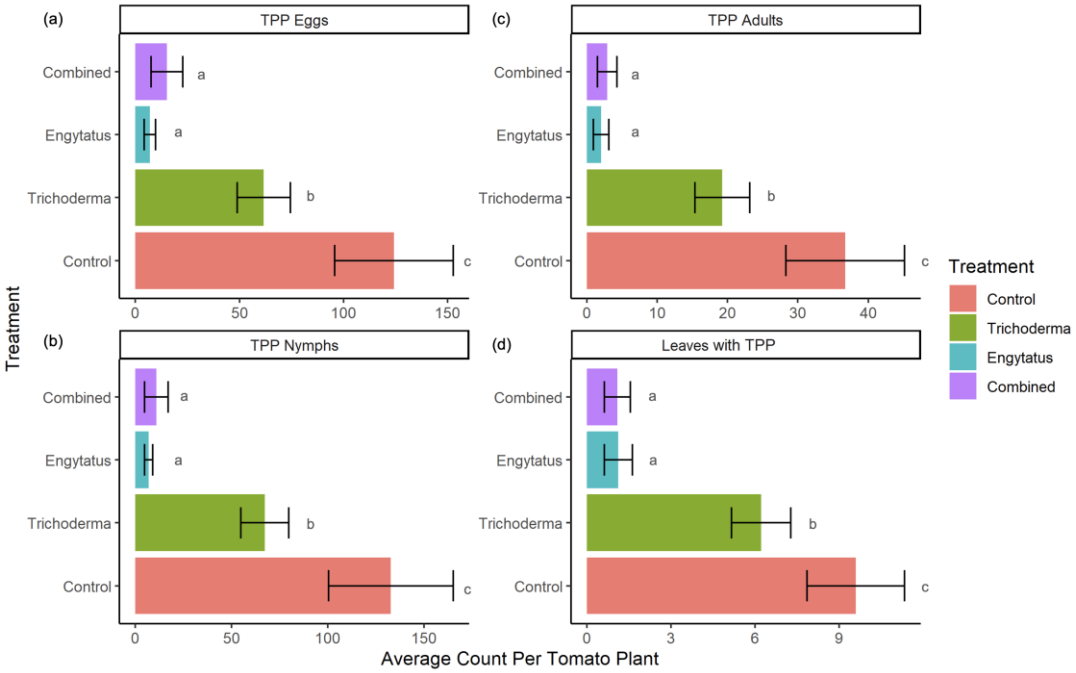

Figure 1. Average counts of *Bactericera cockerelli* (TPP) (**a**) eggs, (**b**) nymphs, (**c**) adults, and (**d**) number of leaves infested with TPP per tomato plant by treatment group. Error bars indicate ± SEM. Control = plants infested with TPP without a biocontrol agent; Trichoderma = *T. atroviride*;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

3.2. Comparison of TPP Growth Rates

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

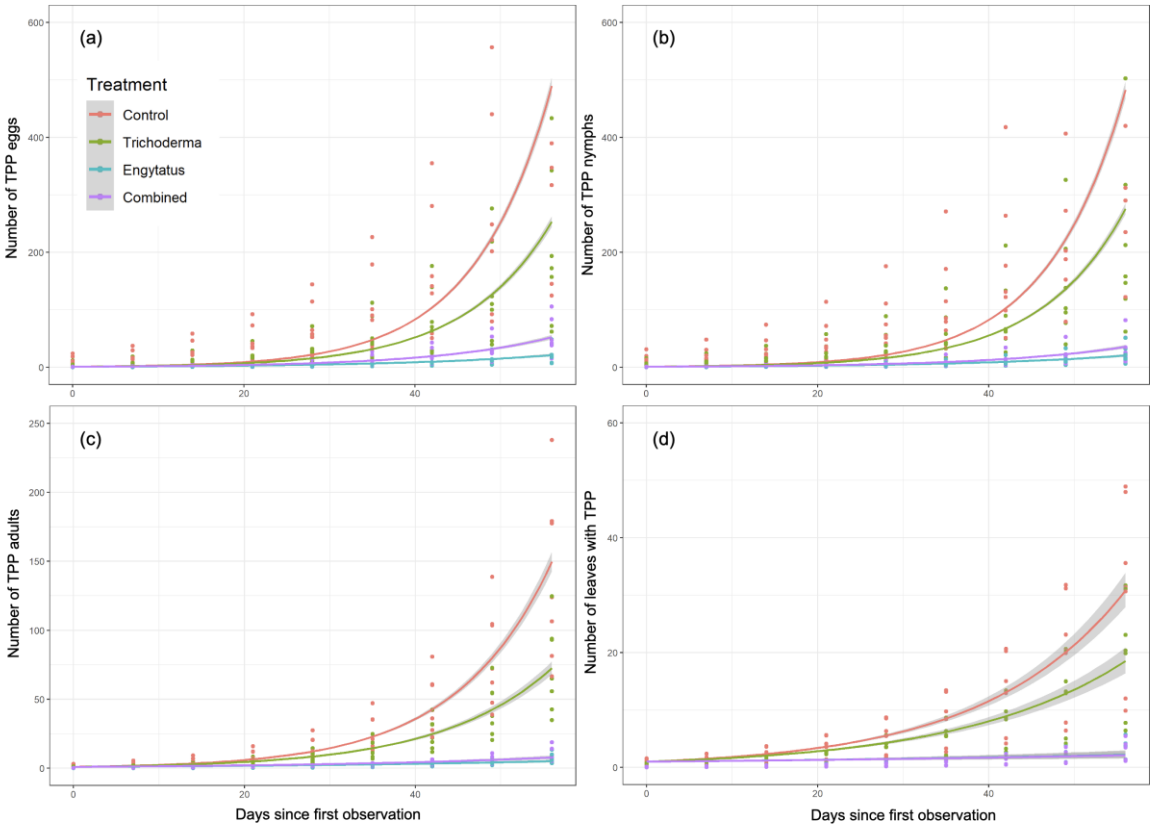

Figure 2. Poisson-distributed counts for *Bactericera cockerelli* (TPP) (**a**) eggs, (**b**) nymphs, (**c**) adults, and (**d**) number of leaves infested with TPP per treatment over the observation period. Control = plants infested with TPP without a biocontrol agent; Trichoderma = *T. atroviride*;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

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

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

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

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

3.3. Treatment Effect of on Plant VOC Emissions

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

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

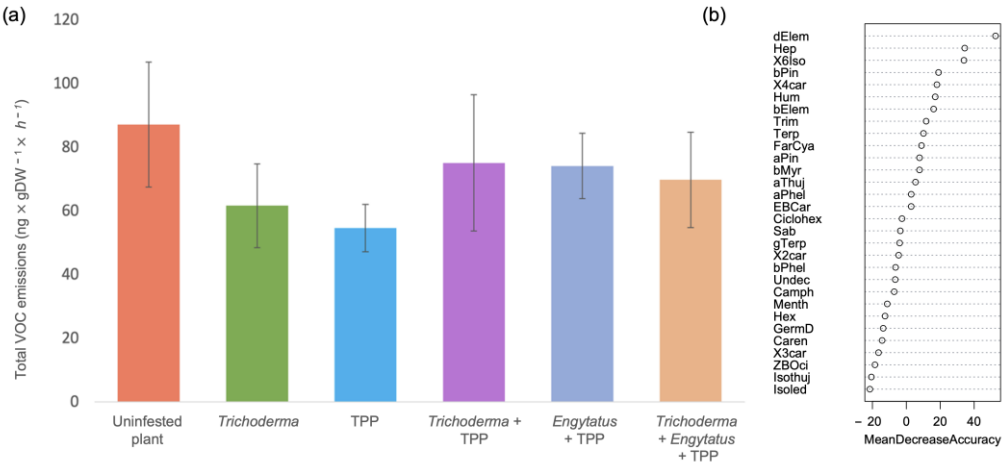

Figure 3. (**a**) Total volatile organic compound (VOC) emissions from tomato plants under different treatments. Error bars indicate ± SEM. Uninfested plant = healthy plant without TPP or a biocontrol agent (salmon); Trichoderma = *Trichoderma atroviride* (green); TPP = *Bactericera cockerelli* (blue);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

4. Discussion

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

Previous studies have shown the potential of the predatory bug *E. nicotianae* in controlling TPP under greenhouse conditions [26–29]. However, its use alone may not be enough to manage established populations. Therefore, it was suggested that it could be used in combination with another biocontrol agent to enhance protection against TPP [28] but simultaneous use of biocontrol agents is not always positive and can result in interference, e.g., [48–51]. In this study, we observed excellent results when the predator was used in early phases of TPP establishment, and it retained its effect, even when a fungal

biocontrol agent was applied simultaneously, suggesting both agents can be safely used together to reduce TPP populations. However, there seems to be no added benefit in their simultaneous use to control TPP.

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

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

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

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

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

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

5. Conclusions

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

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

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Data Availability Statement: Data will be provided by the corresponding authors upon reasonable request.

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