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The combined use of *Bacillus thuringiensis* and *Nesidiocoris tenuis* against the tomato borer *Tuta absoluta*

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Abstract Since *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) was first detected at the end of 2006 in the Mediterranean Basin, several endemic natural enemies have been reported to prey on this exotic pest. The predator *Nesidiocoris tenuis* Reuter (Hemiptera: Miridae) can regulate *T. absoluta* populations, because it is able to prey efficiently on *T. absoluta* eggs. Furthermore, previous studies have demonstrated that first-instar larvae of *T. absoluta* are highly susceptible to *Bacillus thuringiensis* (Bt) treatments. In this work, we tested the combination of both approaches under greenhouse conditions. *B. thuringiensis* formulations were sprayed weekly for two months, three months or throughout the growing cycle, and in all cases, one *N. tenuis* per plant was also released. Control plants were completely destroyed by the infestation levels reached by *T. absoluta*. In contrast, all treatments based on *B. thuringiensis* treatments and releases of *N. tenuis* reduced leaf damage by more than 97% when compared to the untreated control, with no significant differences among them. Furthermore, yield in the control plants was significantly reduced when compared with all Bt–*N. tenuis* treatments. Our results demonstrate that when *B. thuringiensis* treatments are applied immediately after the initial detection of *T. absoluta* on plants, they do not interfere with *N. tenuis* establishment in the crop because *T. absoluta* eggs are available. According to our data, treatments with *B. thuringiensis* later in the growing season would no longer be necessary because mirids alone would control the pest.

Keywords Invasive species · Endemic natural enemies · Conservation biological control · Miridae · IPM

Introduction

The tomato borer *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) is originally from South America, where it is considered one of the most devastating pests of tomato crops (Barrientos et al. 1998; Estay 2000; EPPO 2006). This pest is present throughout the crop growing cycle. Larvae can infest leaves, flowers, stems and fruits, causing important losses in tomatoes (López 1991; Apablaza 1992). At the end of 2006, *T. absoluta* was detected in northern Castellón de la Plana (Spain) (Urbaneja et al. 2007). It was subsequently found in Algeria, Canary Islands, France, Italy, Morocco and Tunisia in 2008 and in Albania, Bulgaria, Cyprus, Germany, Malta, Portugal, Switzerland,

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The Netherlands and the United Kingdom in 2009 (Desneux et al. 2010; EPPO 2010).

Since *T. absoluta* was detected in the Mediterranean Basin, the most common control practice has been based on the use of chemical insecticides (Bielza 2010). However, these treatments may disrupt the existing integrated pest management programs in tomato crops also based on biological control (van der Blom et al. 2009) since pesticides may have effects on natural enemies (Croft 1990; Desneux et al. 2007) and they may lead to resistance (Devonshire and Field 1991; Bielza 2010), as documented in the area of origin of this pest (Siqueira et al. 2000, 2001; Lietti et al. 2005). Therefore, implementation of environmentally safe measures to manage *T. absoluta* while limiting the use of chemical insecticides is urgently necessary.

As an alternatives to chemicals, commercial formulations based on *Bacillus thuringiensis* (Berliner) have been used to control insect pests for decades. These formulations are used because they are environmentally friendly, harmless to humans and other vertebrates, and highly compatible with the use of natural enemies (Entwistle et al. 1993; McClintock et al. 1995; IPSC-WHO 2000). Furthermore, they are recommended for pre-harvest treatments and in cases where the insect populations have developed resistance to other products. However, few studies have evaluated the efficacy of *B. thuringiensis* treatments on *T. absoluta* and most of these studies were performed in the region of origin of this pest (Giustolin et al. 2001; Theoduloz et al. 2003; Niedmann and Meza-Basso 2006). In Spain, commercially available formulations based on *B. thuringiensis* have been tested against *T. absoluta* in laboratory, greenhouse and open staked-tomato field experiments (González-Cabrera et al. 2011). These studies demonstrated that *B. thuringiensis* can be highly efficient in controlling *T. absoluta*, with a reduction in damage close to 90% when sprayed regularly at 90.4 MIU l^{-1} (millions of international units per liter). First-instar larvae were the most susceptible.

Predators such as *Macrolophus pygmaeus* (Rambur), *Nesidiocoris tenuis* Reuter and *Dicyphus marrocannus* Wagner (Hemiptera: Miridae) were detected preying upon *T. absoluta* immediately after its detection in the Spanish Mediterranean coastal region (Arnó et al. 2009a, b; Mollá et al. 2009, 2010; Urbaneja et al. 2009; Desneux et al. 2010). *Macrolophus pygmaeus* and *N. tenuis* have already been tested under laboratory conditions to assess their suitability as *T. absoluta* predators (Arnó et al. 2009b; Urbaneja et al. 2009). Adults of both predator species were able to prey on more than 100 eggs per individual per day with no significant differences between them. In contrast, nymphs of *M. pygmaeus* consumed significantly fewer eggs than nymphs of *N. tenuis* (Arnó et al. 2009b). Furthermore, both species preyed on all larval stages, preferring first-instar larvae (Urbaneja et al. 2009), although the number of larvae preyed on was significantly lower that the number of eggs preyed on. Mollá et al. (2009) showed that when *M. pygmaeus* and *N. tenuis* were established in the crop, they were able to reduce up to 75% and 97% of leaflet infestations or 56% and 100% of fruit infestations, respectively. Additionally, Arnó et al. (2009b) observed in 281 tomato plots of northwest Spain (either under greenhouse or open field conditions), that the level of fruit damage caused by *T. absoluta* remained below 4% in those plots where mirid populations were established at a rate of above 4.5 mirids per plant. Hence, these predators might be able to regulate *T. absoluta* populations under field conditions, when a certain number is present in the crop.

The integration of *B. thuringiensis* treatments with the release or conservation of these predators may provide a clean and safe strategy to manage *T. absoluta* because *B. thuringiensis* targets larvae and mirids prey preferentially on *T. absoluta* eggs. Therefore, we hypothesize that *B. thuringiensis* treatments applied immediately after the initial detection of *T. absoluta* on plants do not interfere with mirid establishment in the crop since *T. absoluta* eggs are available. Afterwards, treatments with *B. thuringiensis* could be terminated, allowing mirids alone to control the pest. The capacity of inoculative releases of *N. tenuis* to control *T. absoluta* was tested when they were combined with *B. thuringiensis* treatments at three different intervals under greenhouse conditions.

Materials and methods

Test facilities

The experiment was conducted in a 40 × 10 m greenhouse equipped with a drip irrigation system located at IVIA in Moncada (Valencia, Spain). The greenhouse was accessed through a double door and divided into 12 experimental cages. Cages were screened with "anti-thrips" polyethylene mesh with 220 × 331 μm interstices, and the floor was covered with a 2 mm-thick woven white polyethylene ground cloth. Each experimental cage was 2.5 × 2 × 2.5 m (l × w × h) and was accessed by a separate door secured with a zipper.

Environmental conditions

One Datalogger (model TESTO 175-H2, Amidata S.A. Pozuelo de Alarcón, Madrid, Spain) was placed in the central cage to record temperature and relative humidity. The average temperature during the experiment ranged between 30.9°C, on 23 July 2009, to 18.7°C, on 28 September 2009, with an absolute minimum and maximum of 13.1°C and 41°C, respectively, during the test period. Average relative humidity ranged from 98.5%, on 6 September 2009, to 52.3%, on 18 July 2009, with absolute values of 99.9% and 23.7%, respectively.

Crop management

To obtain homogenous and pesticide-free plants, seeds of the TYLCV-tolerant tomato variety 'Virgilio' (Clause Vegetable Seeds, UK) were sown on 31 March 2009. Seeds were deposited in 5.4 cm^2 cells in expanded polystyrene trays of 70 × 45 cm filled with a mixture of sand and peat (1:2 w:w) and left undisturbed inside a plant growth chamber (CMP, Conviron Europe Ltd. Cambridgeshire, UK) at 25 ± 2°C, 60 ± 10% RH, 16:8 h (L:D) photoperiod. On 15 May 2009, seedlings were transplanted into individual polyethylene 20-l pots filled with the peat growing medium described above and placed inside the experimental cages in the greenhouse in two rows of five plants each (1.25 plants m^{-2}).

Crop cultivation techniques typical of tomato greenhouse cultivation in Spain were followed: a trellis of one wire-guide for each plant, to which the main stem was trained tied with green polyethylene string, weekly pruning of secondary shoots, application of a standard nutrient solution for tomato by means of an automated-irrigation system with an irrigation frequency adjusted to the environmental conditions and an irrigation time of 15 min.

Tuta absoluta and commercial supplies

Individuals of *T. absoluta* were collected in tomato fields located in the Castellón de la Plana region and reared on tomato plants in a climatic chamber [25 ± 1°C, 60 ± 5% RH and 16:8 h (L:D) photoperiod]. Weekly, a group of six tomato plants measuring 30 cm in height were placed inside the screened cage (120 × 70 × 125 cm) (l × w × h) where the rearing took place. Plants were left undisturbed for five weeks. Such a long period enabled moths to emerge within the cage so that artificial re-infestation was unnecessary. When needed, adults were collected and used in our assays.

Nesidiocoris tenuis individuals (NESIBUG®) were purchased from Koppert Biological Systems, S.L., Águilas, Murcia, Spain. The commercial formulation based on *B. thuringiensis* used in our assay was Costar® (*B. thuringiensis* var. *kurstaki*, 90.4 MIU g^{-1}, WG) distributed in Spain by Syngenta Agro. S.A. (Madrid, Spain). This commercial formulation demonstrated high efficacy against *T. absoluta* in both greenhouse and open fields of staked tomatoes during the 2009 tomato growing season (González-Cabrera et al. 2011).

Experimental design

Combined *B. thuringiensis* and *N. tenuis* treatments were tested in 12 cages (ten plants each, see above) using a randomized complete block design with four treatments replicated three times. Three treatments received weekly sprays of *B. thuringiensis* at 180.8 MIU l^{-1} (2 g l^{-1}) for a duration of two months (hereafter Bt-1), three months (hereafter Bt-2) and throughout the experiment (hereafter Bt-3), respectively, and a control was sprayed with water. Sprays were applied with a backpack sprayer (SuperAgro 20, Matabi®, Antzuola, Guipuzcua, Spain).

To simulate a heavy and constant *T. absoluta* attack on the crop, each cage was infested once weekly with ten *T. absoluta* pairs, starting on 21 May 2009. It is crucial to start the *B. thuringiensis* treatments at the correct time in order to control *T. absoluta* (González-Cabrera et al. 2011). Under field conditions, it is recommended to begin these treatments immediately after detecting the first captures of *T. absoluta* in pheromone traps. Because we could not place pheromone traps inside the cages in our assay, we decided to check all plants in the greenhouse carefully. Two weeks later, the first eggs and first-instar larvae of *T. absoluta* were detected (4 June 2009), and weekly *B. thuringiensis* treatments were therefore initiated. On 9 June 2009, one *N. tenuis* per plant was released in all cages receiving *B. thuringiensis* treatments. One *N. tenuis* per plant is the recommended release rate in tomato (Calvo et al. 2009, 2010). *Bacillus thuringiensis* treatments were terminated on 7 August 2009 for the Bt-1 treatment and on 4 September 2009 for Bt-2. The crop was terminated on 16 October 2009. On 3 July 2009, all cages were treated with spiromesifen (Oberon® 24% w/v; Bayer Cropscience, Spain) to control an accidental infestation of *Aculops lycopersici* (Masse) (Acari: Eriophydae). Spiromesifen was selected for its high efficacy on *A. lycopersici* (Elbert et al. 2005), and low toxicity to non-target organisms, including bacteria and *N. tenuis* (Nicolaus et al. 2005; Bielza et al. 2009; Bayer CropScience 2010).

Evaluations

Four randomly chosen plants per cage were sampled weekly over 18 weeks, beginning on 17 June 2009. First, the number of leaves, leaflets and fruits infested per plant were counted. Then *N. tenuis* (adults and nymphs) and necrotic rings were counted in the whole apical third of the plant (leaves, flowers and shoots). In addition, harvested tomatoes were weighed. To reduce the risk of accidental contamination among treatments, special care was always taken to enter the control cages first and the cages with *N. tenuis* releases afterwards.

Statistical analysis

Treatment effects on plant damage, expressed as the number of infested leaflets, variations in mirid populations (adults and nymphs) and number of necrotic rings were analyzed using a linear mixed model with repeated measures with time as a random factor. Once significant differences were exhibited, pairwise comparisons of the fixed factor levels were performed with a Bonferroni post-test for separation of means ($P < 0.05$). At the end of the experiment, data on the accumulated fruit weight and the percentage of infested fruits were subjected to a one-way analysis of variance together with a Bonferroni post-test for mean separation ($P < 0.05$). When required, angular transformation of percentage data was performed to satisfy the assumptions of normality and homogeneity of variance.

Results

Plant damage

Tomato plant infestation with ten *T. absoluta* couples per week resulted in the complete destruction of control plants at the end of the experiment. On 22 July 364 ± 27 leaflets per plant were found damaged by *T. absoluta* larvae (Fig. 1). The number of damaged leaflets per week was subsequently decreased and reached a "plateau" of approximately 200–230 per week that persisted until the end of the experiment (Fig. 1). On the other hand, damage in plants infested in the same way but treated with Bt/mirids was more than 97% lower than in the control treatment ($F_{3,195} = 264.2, P < 0.0001$). The protection afforded by Bt/mirids was almost the same for all treatments, and it did not differ significantly (for all three pairwise comparisons, Bt-1 *vs.* Bt-2, Bt-1 *vs.* Bt-3 and Bt-2 *vs.* Bt-3: df = 1, 195 and $P = 1.000$) (Fig. 1).

N. tenuis populations

Releases of one *N. tenuis* per plant proved to be sufficient for mirid establishment in the experimental cages. The numbers of both, nymphs and adults,

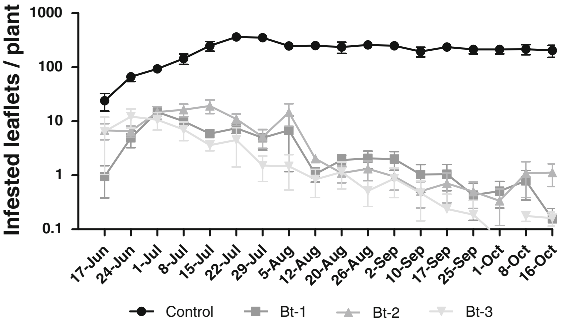

Fig. 1 Infested leaflets per plant per week (mean ± SE) for plants infested with *T. absoluta* and sprayed weekly with *B. thuringiensis*. One *N. tenuis* was released per plant three weeks after infestation

increased regularly from their release until the end of the experiment (Fig. 2). There were significant differences between the control treatment and treatments with released *N. tenuis* (adults: $F_{3,195} = 20.472$, $P < 0.0001$; nymphs: $F_{3,195} = 18.662$, $P < 0.0001$). The numbers of both adults and nymphs did not differ significantly among Bt/mirids treatments (for all three pairwise comparisons for adults and nymphs, Bt-1 *vs.* Bt-2, Bt-1 *vs.* Bt-3 and Bt-2 *vs.* Bt-3: df = 1, 195 and $P = 1.000$).

In two treatments (Bt-1 and Bt-2), Bt spray treatments were stopped before the experiment ended. Thus, in these cases, *T. absoluta* was controlled by *N. tenuis* alone. A total of 0.75 ± 0.25 individuals were present in the apical part of the plant when the sprays were stopped in treatment Bt-1 and 1.25 ± 0.25 individuals were present under the same conditions in treatment Bt-2 (Fig. 2).

Necrotic rings

As *N. tenuis* exhibits zoophytophagous behavior under certain conditions, plants were checked weekly for the presence of necrotic rings. Necrotic rings were first detected when the experiment was about to end (from 25 September on) (Fig. 3). As expected, no necrotic rings occurred in control plants. The maximum value of necrotic rings per plant in treatment Bt-1, 5.25 ± 1.8 (Mean ± SE) rings per plant, occurred on 16 October. The numbers of necrotic rings differed significantly among treatments with released *N. tenuis* ($F_{3,41} = 17.822$, $P < 0.0001$). The number of rings was lower in the treatment Bt-3, compared with Bt-1 (Bt-3 *vs.* Bt-1: df = 1, 41 and $P = 0.007$; Bt-3 *vs.* Bt-2: df = 1, 41 and $P = 0.1195$; and Bt-1 *vs.* Bt-2: df = 1, 41 and $P = 1.000$). Despite the presence of necrotic rings, no flower abortion was observed in any of the treatments with released *N. tenuis* throughout the experiment.

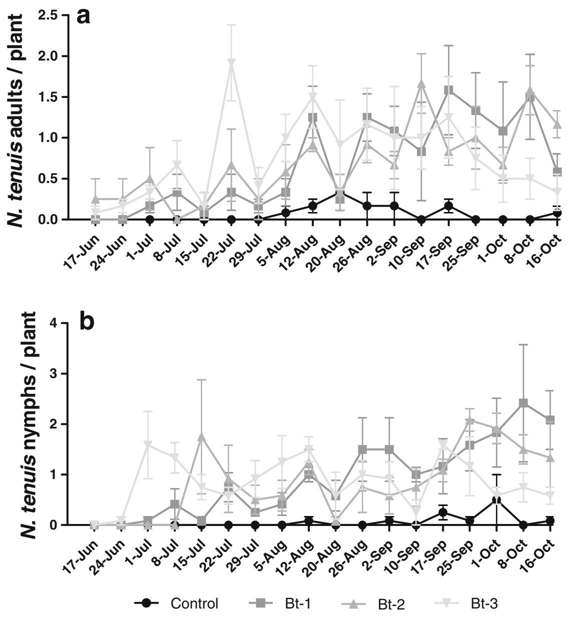

Fig. 2 Number of *N. tenuis* adults (**a**) and nymphs (**b**) (mean ± SE) in the apical third of the plants infested with *T. absoluta* and sprayed weekly with *B. thuringiensis*. One *N. tenuis* was released per plant three weeks after infestation

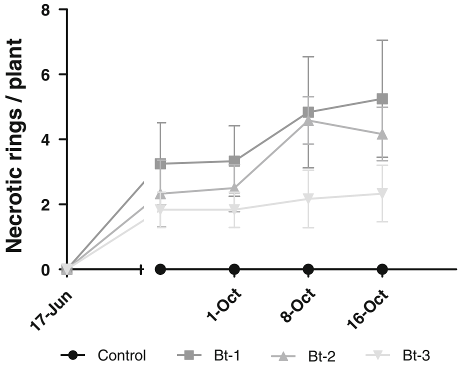

Fig. 3 Number of necrotic rings (mean ± SE) in plants infested with *T. absoluta* and sprayed weekly with *B. thuringiensis*. One *N. tenuis* was released per plant three weeks after infestation

Effect of Bt/mirid treatments on yield

The impact of *T. absoluta* infestation on tomato yield was very high. The weight of fruit harvested from control plants was strongly and significantly less than the weight of fruit harvested from Bt/mirid-treated plants ($F_{3,11} = 17.99$, $P = 0.0006$) (Fig. 4a). Moreover, yield did not differ significantly among Bt/mirid treatments ($P > 0.05$). The percentage of infested fruits was significantly higher in the control treatment than in the Bt/mirid treatments ($F_{3,11} = 386.3$, $P < 0.0001$). No significant differences were found among the Bt/mirid treatments ($P > 0.05$) (Fig. 4b).

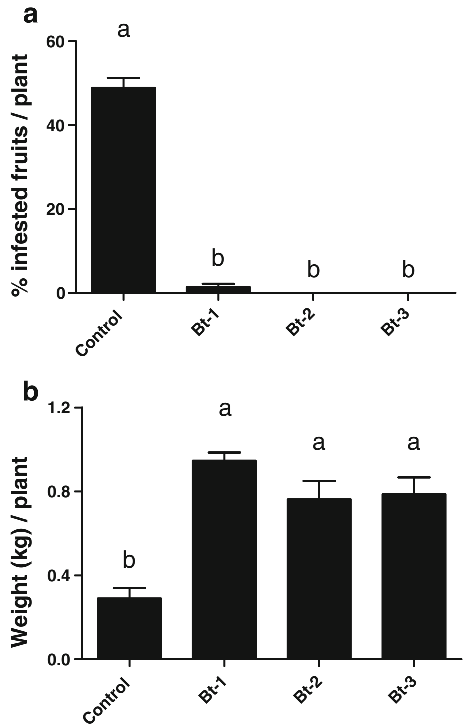

Fig. 4 Percentage of infested fruits (**a**) and weight (**b**) (mean + SE) for plants infested with *T. absoluta* and sprayed weekly with *B. thuringiensis*. One *N. tenuis* was released per plant three weeks after infestation. Different letters above the bars indicate significant differences ($P < 0.05$; Bonferroni's test)

Discussion

Combined use of inoculative releases of the predator *N. tenuis* and *B. thuringiensis* treatments has proven highly effective in reducing the damage caused by *T. absoluta*. Regardless of the time at which *B. thuringiensis* treatments were stopped (two or three months), plants receiving *B. thuringiensis* treatments and *N. tenuis* showed no fruit damage and a 97% reduction in leaflet damage. Treatment effects among these plants did not differ significantly. Therefore, the established populations of *N. tenuis* were able to control the pest by themselves after *B. thuringiensis* treatments ended. These results are consistent with previous findings that have shown *N. tenuis* to be highly effective in controlling *T. absoluta* once it is established in the crop (Arnó et al. 2009b; Mollá et al. 2009; Gabarra and Arnó 2010).

The hypothesis tested in this work was based on the finding that good control of young *T. absoluta* larvae can be achieved with *B. thuringiensis* (González-Cabrera et al. 2011). Such successful control favors the increase of *N. tenuis* populations, which prey on *T. absoluta* eggs. However, in practice, the implementation of this strategy may not be so simple, because the choice of the moment at which *B. thuringiensis* treatments are interrupted will logically depend on the numbers of predators present in the crop. In turn, infestation levels depend on other factors such as plant physiological stage, temperature, pest pressure or the availability of alternative prey. Our experience leads us to believe that, in a commercial scenario, proper crop monitoring is required so that decisions are made only after accurate evaluation of the aforementioned factors. These factors should be considered in terms of their dynamics over time and not only at certain moments in the growing season.

The zoophytophagous behavior of *N. tenuis* also makes it necessary to assess the potential damage it may cause to the crop. When this predator feeds directly from vegetative and reproductive parts of the plant, it produces necrotic rings on stems and leaf petioles, it produces flower abortion, and can also puncture tomato fruits, possibly reducing yields (Sánchez 2008). The intensity of plant feeding by *N. tenuis* on tomato crops under Mediterranean conditions has been addressed by several authors (Sanchez and Lacasa 2008; Calvo et al. 2009; Sanchez 2009; Arnó et al. 2010), who concluded that damage caused by this mirid on tomato plants was directly related to the abundance of *N. tenuis* and inversely related to the interaction between the number of *N. tenuis* and the number of prey. All these studies were conducted using the sweet potato whitefly *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae) as prey. Therefore, the presence of a new abundant prey such as *T. absoluta* in the tomato agrosystem may modify *N. tenuis* behavior. This consideration makes necessary to update the density thresholds previously established for this zoophytophagous predator.

In our study, no flower abortion was observed, although a maximum of 5.25 ± 1.8 necrotic rings per plant were observed 18 weeks after the release of one *N. tenuis* per plant. Nevertheless, comparisons with previous work are difficult because *T. absoluta* eggs were always available (ten couples were released weekly in the experimental cages).

The yield in the control treatment was lower than that in other treatments. This difference obviously resulted from the fact that *T. absoluta* damaged the tomato leaflets and thereby reduced their photosynthetic capacity. In addition, half of the fruits collected had to be rejected, owing to the presence of larval damage. In contrast, there was no fruit infestation in plants treated with *B. thuringiensis* and/or releases of *N. tenuis*, probably owing to the high mortality of young larvae. As a result of this high mortality, the late larval instars were present in much lower numbers. These instars are primarily responsible for fruit infestation (Desneux et al. 2010).

As expected, *B. thuringiensis* proved to be harmless and compatible with *N. tenuis*. However, commercial formulations of *B. thuringiensis* may impact on *N. tenuis* populations indirectly in two different ways. First sublethal side effects may result not from *B. thuringiensis* itself but from the adjuvants present in the commercial formulation. Some adjuvants may have the potential to disturb (and/or kill) arthropods by suffocation at the time they are sprayed (Acheampong and Stark 2004; Desneux et al. 2006; Purcell and Schroeder 1996). Also, *B. thuringiensis* treatment may impact *N. tenuis* through a demographic effect on its prey in the agroecosystem (and may consequently lead to extinction of *N. tenuis* populations in the system). These two factors could explain the lower number of *N. tenuis* and necrotic rings observed in the Bt-3 (weekly applications of *B. thuringiensis*) treatment during the last weeks of the experiment. Further research would be needed to confirm the validity of this explanation.

The high reproductive rates of *T. absoluta,* along with previous reports on resistance developed by other insect species to *B. thuringiensis*-based formulations (Ferré and Van Rie 2002; Gassmann et al. 2009) indicate that it is highly desirable to alternate *B. thuringiensis* treatments with the use of other active ingredients. Such ingredients should be selective for mirid bugs and effective against *T. absoluta* larvae. This strategy may also include the alternative use of different *B. thuringiensis* formulations based on subspecies commonly bearing different toxin profiles (i.e. subsp. *kurstaki* or *aizawai*) (Schnepf et al. 1998). These formulations can be more than 95% effective when used at the proper concentration (González-Cabrera et al. 2011).

In summary, our results suggest that the combination of *N. tenuis* and *B. thuringiensis* may result in a cost-effective strategy for tomato crop pest management because the number of treatments (either with *B. thuringiensis* or with other selective pesticides) can be minimized. These two control agents can act together to control *T. absoluta* infestations. Once *N. tenuis* is established in the crop, it will help control other pests such as whiteflies (Mollá et al. 2009), whereas *B. thuringiensis* treatments will prevent damage by other lepidopteran pests such as *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) (Avilla et al. 2005). Overall, this strategy is environmentally safe and may provide a clean tomato yield without chemical residues, thereby increasing fruit safety and quality.

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