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Side effects of *Bacillus thuringiensis* var. kurstaki on the hymenopterous parasitic wasp *Trichogramma chilonis*

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Abstract Most of the detrimental effects of using conventional insecticides to control crop pests are now well identified and are nowadays major arguments for replacing such compounds by the use of biological control agents. In this respect, the bacterium *Bacillus thuringiensis* var. *kurstaki* and *Trichogramma* (Hymenoptera: Trichogrammatidae) parasitic wasp species are both effective against lepidopterous pests and can actually be used concomitantly. In this work, we studied the potential side effects of *B. thuringiensis* var. *kurstaki* on *Trichogramma chilonis* females. We first evidenced an acute toxicity of *B. thuringiensis* on *T. chilonis*. Then, after ingestion of *B. thuringiensis* at sublethal doses, we focused on life history traits of *T. chilonis* such as longevity, reproductive success and the time spent on host eggs patches. The reproductive success of *T. chilonis* was not modified by *B. thuringiensis* while a significant effect was observed on longevity and the time spent on host eggs patches. The physiological and ecological meanings of the results obtained are discussed.

Keywords *Bacillus thuringiensis* · *Trichogramma chilonis* · Life history traits · Interaction · Biological control · Bioinsecticide

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Introduction

Conventional chemical pesticides used to control crop pests are the object of numerous studies demonstrating their impact on the environment and human health. This includes, for instance, the induction of resistance in the targeted pest species, disruption in the endocrine system of several non-target species or reduction of their life span (more information can be found herein: http://toxnet.nlm.nih.gov/; http://www.pesticideinfo.org/). To overcome these problems, the use of natural products or beneficial organisms through biological control programs is promoted as an alternative pest control strategy. In this respect, projections of the phytoprotection market predict that biological control will account for 15 % of the sales in the 2015–2020 period (The world of organic agriculture–Statistics and emerging trends 2015, FiBL and IFOAM). Biological control can rely on either micro- or macroorganisms.

Among microorganisms, bacteria are widely used and many species present a good potential to be used as bioinsecticide (Ruiu 2015). Among the microorganisms, the bacterium *Bacillus thuringiensis* (Bt) is the major bioinsecticide used in biological control programs (50 % of the biopesticide market, (Consultants 2010)) and was the first organism used at a large scale to control different lepidopterous pests (for a review about bioinsecticides based on bacteria, see (Ruiu 2015)). Bt is a Gram-positive bacterium naturally found in the soil, at the surface of leaves (Raymond et al. 2010), or even within the plant (Monnerat et al. 2009), that sporulates under stressful conditions. Interestingly, during the process of sporulation, the bacterium yields toxins that aggregate in a crystal, thereby the toxins are called Cry toxins. The Cry toxins differ by their primary sequence and their acute toxicity covers a wide range of animal species, although each single Cry toxin has a limited number of target species (van

Frankenhuyzen 2009; 2013). So far, more than 300 Cry toxins produced by many subspecies of Bt have been identified and the toxins are named relative to their sequences. To cope with the number of toxins, families and sub-families have been created (Crickmore et al. 2014).

Since the late 1960s, several strains have been selected for their insecticidal properties. These insecticidal properties are associated with the production of a cocktail of Cry toxins. A Bt strain can be characterized by the number and type of Cry toxins produced. The most popular Bt strain is the Bt var. *kurstaki* (Btk) which is efficient in killing the larvae of a number of lepidopterous species (van Frankenhuyzen 2009; van Frankenhuyzen 2013). The commercial preparations are composed of a mixture of sporangia (bacterial envelopes containing a spore and a crystal), spores, toxins-containing crystals, fermentation solids and co-formulants. The latter are mentioned on the label but covered by trade secret. Upon ingestion, the Cry toxins are solubilized from the crystals. In susceptible species, upon sequential binding to specific receptors (E-cadherin and N-amino-peptidases or Alkaline Phosphatases, respectively), the Cry toxins form pores in the membranes of enterocytes promoting cytoplasm leaks (Bravo et al. 2011). This results in the destroying of gut lining, septicaemia, and the animal dies in a couple of days (Adang et al. 2014).

Another biological pest control strategy involves the use of parasitoid insects that are released on the crop to be protected. Especially against lepidopterous pests, species of the *Trichogramma* genus (Hymenoptera: Trichogrammatidae) are tiny hymenopterous wasps that are used efficiently against different crop pests on different cropping systems worldwide (Wajnberg and Hassan 1994). Females are <1-mm-long insects that lay their eggs in the egg of their hosts and, after killing them, an adult emerges after about 10 days (at 25 °C). As a result of an important number of accurate ecological works done on these insects over several decades, these insects are mass-produced at the industrial scale in an important number of countries, and used nowadays on millions of hectares for biological control programs throughout the world (Cônsoli et al. 2010).

Although Btk exerts its acute toxicity against lepidopterous pests, we cannot rule out that Btk may have side effects on other insects such as hymenopterous as both lepidopterous and hymenopterous can be used concomitantly to control the same pests on the same crops. The goal of the present work was to study the potential side effects of Btk spores (Dipel® commercial preparations) on several biological parameters of the parasitic wasps *Trichogramma chilonis*. Although some studies previously addressed the effects of Btk on parasitic wasps (i.e. Sedaratian et al. 2013), our study is original as we address the direct effects of Btk on the wasps. Indeed, wasps are contaminated through their food whereas, in the previous studies, the source of Btk for the wasps is what have been ingested, and possibly transformed, by the host. Furthermore, we tested the effects of Btk on the adult wasp whereas the previous studies were done necessarily on the larvae. Several life history and behavioural traits of *T. chilonis* females after Btk ingestion were compared to control animals fed with honey only. Our results demonstrate that, although Btk is used to control lepidopterous species, side effects can also be observed on hymenopterous wasps at doses that are used under field release conditions.

Materials and methods

Bacillus thuringiensis preparation

All the strains used in this work belong to the serovar *kurstaki*. The bioinsecticide preparation was purchased as commercial formulations (known as Dipel®) and includes the HD1 strain of Btk (4D1 strain according to the Bacillus Genetic Stock Center catalogue). We titrated the batch of bioinsecticide we used at 5×10^4 colony forming unit/μg by plating serial dilutions of the preparation and cfus counting. The 4D22 strain was obtained from the *Bacillus* Genetics Stock Centre (http://www.bgsc.org) and does not produce any Cry toxins (González et al. 1982). Therefore, this strain was used to address the effect of the spores themselves. First experiments were based on longevity testing and were done under four conditions: control, 4D22, 4D1 and Dipel®. We obtained indiscernible results between Dipel® and 4D1 preparations (data not shown). Thus, the putative co-formulants of Dipel® had no or negligible effects and we have done all the experiments with Dipel® only. The lack of available information about co-formulants prevented us from setting up a suitable control to confirm this hypothesis. To produce spores, we inoculated 4 mL of LB-erythromycine (50 μg/mL) (LB contains tryptone 1 %; yeast extract 0.5 %; NaCl 170 mM; pH=7.5) with a single colony obtained from a glycerol stock and the culture was incubated for 8 h at 30 °C under agitation (200 rpm). Two milliliters of this pre-culture were then used to inoculate 1 L of HCT-erythromycine (50 μg/mL)-glucose (0.3 %) medium (HCT contains tryptone 1 %; casamino acids 2 %; KH_2PO_4 25 mM; $MgSO_4$, $7H_2O$ 0.5 mM; $MnSO_4$, H_2O 0.01 mM; $ZnSO_4$, $7H_2O$ 0.05 mM; Fe_2SO_4 0,05 mM; H_2SO_4 1mN; $CaCl_2$, $2H_2O$ 1 mM; pH=7.25). This culture was then separated in three Erlenmeyer flasks (2 L) and incubated for 15 days at 30 °C under agitation (200 rpm). To kill the remaining vegetative cells, the culture was heated at 70 °C during 20 min before being centrifuged at 6000*g* for 15 min at 4 °C. The pellet was re-suspended in NaCl 0.1 M and centrifuged as previously described. The process was repeated once more with NaCl 0.1 M and then twice with water. The spores were then lyophilized for further use.

Trichogramma rearing

The *T. chilonis* strain used in the experiments originates from several females (more than ten) trapped in China in 2013. These females were not virgin so that we ensure that the genetic variability was sufficient and correctly represents what can be found under natural conditions. Its specific identity was verified using both standard morphological traits (Pintureau 1987) and molecular markers, especially using polymerase chain reaction of a 710-pb fragment of the mitochondrial cytochrome *c* oxidase subunit I gene (Folmer et al. 1994) (data not shown). The strain has been maintained under laboratory conditions on the eggs of one of its factitious hosts: *Ephestia kuehniella* (Lepidoptera: Pyralidae), at 25 °C, 70 % relative humidity, light:dark 16:8, and all experiments described below were done under these laboratory conditions with virgin and naive (i.e., without previous experience with hosts) females.

Acute toxicity assessment

Dipel® preparation or 4D22 spores were incorporated into the insect food. For calculating the LC_{50}, (the concentration that kills 50 % of the population) we offered to the *T. chilonis* females 0.5 μL of honey containing different amounts of Btk : 0 (control), 4, 10, 20, 40, 80 and 200 μg/μL. For each dose and for each Btk strain, we scored the mortality after a 24-h period. Toxicological responses of the *T. chilonis* females were analyzed using a probit model (PriProbit 1.63 © Masayuki Sakuma). When possible, the LC_{50} and LC_{10} were calculated. Then, for the remaining experiments (see below), we offered to each wasp female tested 0.5 μL of honey/Btk mixtures containing 4 μg/μL of Btk preparation, i.e. 2 μg per insect.

Longevity

To measure the impact of Btk on *T. chilonis* female lifespan, isolated insects were fed with honey supplemented or not with 4 μg/μL of Btk preparation. The living insects were scored each day and the mean survival time was calculated (n=59, 58 and 42, for the control, 4D22 and Dipel®, respectively). Longevities for each condition were compared using a log-rank test (Statview 4.5 SAS).

Host-feeding behavior

Female *T. chilonis* that were <12-h-old and fed during one or three consecutive days with the two Btk strains or with honey alone for the control were monitored. Such host-feeding behavior almost always appears on the first host egg encountered (Ferracini et al. 2006). Hence, *E. kuehniella* eggs were offered ad libitum to isolated *T. chilonis* females (1-day treatment: n=20, 23, 24, for the control, 4D22 and Dipel®, respectively; 3-day treatment: n=18 in all cases) up to the moment the first attacked egg is left. The frequency of host-feeding behaviors observed was recorded and compared using a logistic regression with a logit link function, a special regression method to handle binomial data (MacCullagh and Nelder 1989).

Fecundity and parasitism success

The overall fecundity and parasitism success was also measured and compared among <2-h old *T. chilonis* females fed during one or 3 days with the Btk strains or with honey for the control. For this, isolated females (1-day treatment: n=27, 26 and 24, for the control, 4D22 and Dipel®, respectively; 3-day treatment: n=16, 15 and 14, for the control, 4D22 and Dipel®, respectively) were offered *E. kuehniella* eggs ad libitum for 24 h after being exposed to each condition. At 25 °C, attacked hosts turn black after 4–5 days and adults emerged after 9 days (Wajnberg et al. 2012). Hence, the numbers of black eggs were counted 5–6 days after being offered to the females and the number of adults emerging was counted 4–5 days later. The different situations were statistically compared with an analysis of variance (ANOVA).

Patch time allocation

To measure the time *T. chilonis* females spend on eggs patches, we used the protocol described in (Wajnberg 2000). Rapidly, each female was offered a single patch of nine UV-killed *E. kuehniella* eggs, arranged on a 3×3 regular square grid pattern, with a distance of 1 mm between the hosts aligned in rows and columns. Females were used only once and were free to leave the patch whenever they wanted. Hosts were not replaced during the observation, so patches may have suffered a continuous depletion. The behaviour of each female was observed continuously from the moment the parasitoid entered the patch for the first time up to the moment it left the patch for more than 60 s. Females might sometimes leave the patch and then return to the hosts. These short excursions were included in the computation of the patch residence time. Patch residence times were compared using log-rank tests. The number of replicates were, on 1-day treatment: n=18, 19 and 19, for the control, 4D22 and Dipel®, respectively; and on 3-day treatment: n=18, 18 and 18, for the control, 4D22 and Dipel®, respectively.

Results

Btk toxicity

At any of the doses we used, we observed no acute toxicity of the 4D22 strain on *T. chilonis* females so that neither the LC_{50}

nor the LC_{10} could be calculated. However, the wasp females were actually observed eating honey supplemented with the spores. By contrast, mortality was observed when females were fed with Dipel® spores (Fig. 1). The LC_{50} and LC_{10} values were 84.2 (9.5–288.5) µg/µL and 22.0 (9.5–47.4) µg/µL, respectively (95 % confidence intervals, $n=198$). As a first result, Dipel® is acutely toxic to *T. chilonis* females but not its variant devoid of Cry toxins. The following experiments were done with 4 µg/µL for each insect (corresponding to 2 µg per female), thus representing about five times less than the LC_{10} value.

Longevity

The longevity of females fed with Btk spores, whatever the strain, at low dose (i.e. 2 µg per female) was significantly longer than the one obtained on the control (Fig. 2). The average survival durations were 11.35±0.46, 12.34±0.46 and 13.02±0.50 days (±SE) for the control, 4D22-fed, and Dipel®-fed females, respectively. The differences between average survival durations of the Dipel®-fed females and the control are statistically significant ($Chi^2=4.128$, df=1, $p<0.05$) but not for the 4D22-fed insects ($Chi^2=2.746$, df=1, $p>0.05$) although these insects tend to live longer than control. Surprisingly, while a high dose of Dipel® was lethal, feeding *T. chilonis* females with lower doses increased their longevity compared to control females fed only with honey.

Host-feeding behavior

Whether after 1 day or 3 days of exposure to 4D22 or Dipel® strains, *T. chilonis* females performed a host-feeding behaviour on the first host they encounter as frequently as the control animals fed with pure honey ($Chi^2=1.798$, df=2, $p>0.05$). Noteworthy, the rate of such behaviour was significantly reduced for younger females after only 1 day of treatment (see Fig. 3, $Chi^2=11.828$, df=1, $p<0.001$).

Fecundity and parasitism success

We did not record any differences between the conditions tested and the feeding duration with Btk preparations for both the number of hosts attacked (effect of treatment: $F_{2,116}=0.88$, $p>0.05$; effect of feeding duration: $F_{1,116}=1.38$; $p>0.05$) and the number of adult *T. chilonis* emerging from these hosts (effect of treatment: $F_{2,116}=1.78$, $p>0.05$; effect of feeding duration: $F_{1,116}=0.89$; $p>0.05$) (Fig. 4).

Time on host patches

Females that were fed with only honey (i.e. control) during three days spent significantly a longer time on host patches (1485.12±93.48 s; ±SE) than younger females fed during one day only with honey (984.99±95.71 s; ±SE; log-rank test: $Chi^2=11.997$, df=1, $p<0.001$) (Fig. 5). Such a difference is not observed any more when females were fed with Btk preparations, whatever the strain used.

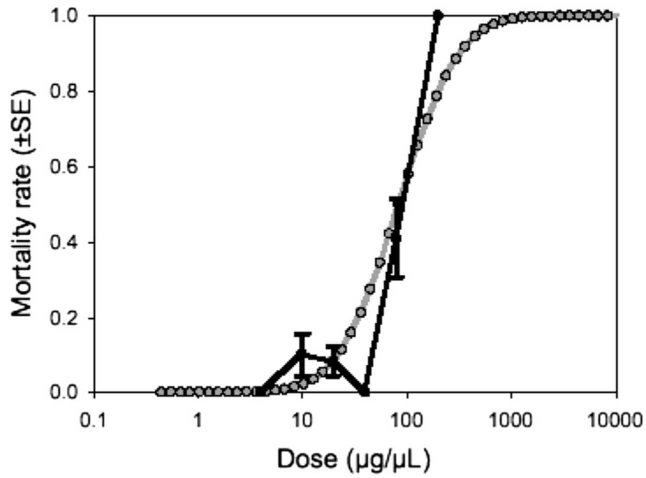

Fig. 1 Dose vs. mortality curve of *Trichogramma chilonis* females for Dipel®. The curve relative to the actual results is presented (*black circles*) as well as the regression curve (*grey circles*) built for the LC_{50} calculation

Discussion

As biocontrol agents, Dipel® spores and *Trichogramma* parasitoid females can be used concomitantly to control lepidopterous pests on crops. While some data are available about acute toxicity of Cry toxins on insects (van Frankenhuyzen 2013), much less exists regarding Dipel® spores side effects. Our aim in this work was to test the effects of Btk spores on several life history traits of the parasitic wasp *T. chilonis*.

Our first goal was to assess the acute toxicity Btk may exert on *T. chilonis* females. Spores of the 4D22 strain, devoid of Cry toxins, have no acute toxicity against the females at all the doses we tested. By contrast, the Dipel® preparations, thus including Cry toxins, do exert an acute toxicity against wasp females with a LC_{50} calculated at 84.2 (9.5–288.5) µg/µL of diet. Even if, from the results of our preliminary experiments, we can deduce a non-significant effect of co-formulants, we cannot exclude that some part of the effects we observed might be due to these components of the commercial formulation, although it is expected for them to be neutral.

Longevity was augmented when Dipel® was offered to the wasp females. We wondered then whether this increased longevity could be due to an improved nutritional quality of the spores/honey mixed food. It has been previously described that *Trichogramma* females can perform a host-feeding behaviour (Ferracini et al. 2006). Hence, if Dipel® improves the nutritive quality of the honey, the animals should reduce the frequency of such behaviour. As a consequence, the host-feeding behaviour is expected to be less frequent for Btk-fed

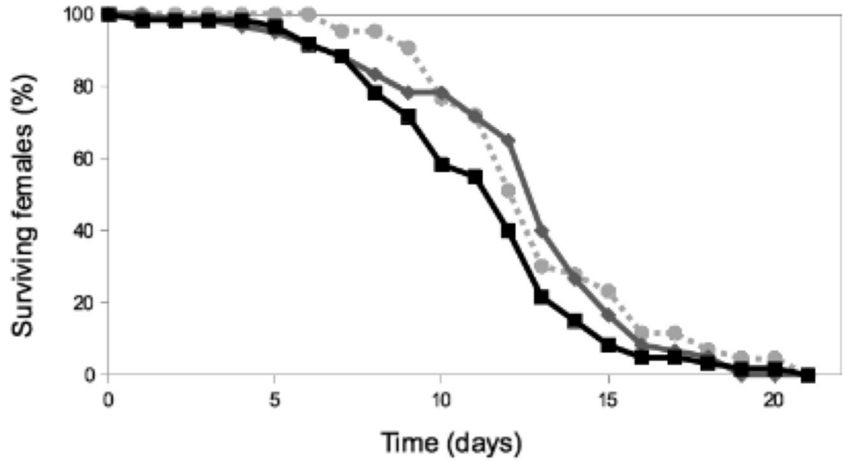

Fig. 2 Longevity of 4D22-fed and Dipel®-fed or honey-fed *Trichogramma chilonis* females. The longevity is expressed as the percentage of females still alive as a function of time. *Black squares* and *plain line,* control wasp females; *grey diamonds* and *plain line,* 4D22-fed wasp females; *light grey circles* and *dotted line*, Btk-fed wasp females

T. chilonis females. This was not what we observed but the host-feeding behaviour measured in our experiment actually represents a short-term event. It is possible that the putative nutritive advantage of the Btk-added honey cannot be quantified in such a short time but requires a longer period to be noticeable as we did with longevity. The impact of Btk on longevity on parasitoids is an intriguing feature that needs further investigations relative to its origin and its specificity.

Parasitism success and fecundity of *T. chilonis* were not affected by Btk spores, whether they produce Cry toxins or not. This is a key point of the study as these parameters are most likely of utmost importance for the efficiency of *Trichogramma* females, and more generally of parasitoids, as biocontrol agents.

Interestingly, previous works were designed like ours, i.e. parasitoid adults fed with sugar solutions containing Btk spore preparations, and gave results sometimes different from what we present here. Ruiu et al. (2007) working on the association *Muscidifurax raptor*/*Musca domestica* found that Btk spores (HD1strain) had no effect on survival and reproduction parameters of parasitoids. On the other hand, two other studies done on the associations *Bracon brevicornis*/*Plodia interpunctella* or *Cardiochiles nigriceps*/*Heliotis virescens* evidenced both a reduced lifespan and a decrease in the fecundity of parasitoids (Salama et al. 1991; Dunbar and Johnson 1975). It is useful to note that *M. raptor* and *T. chilonis* belong to the Chalcidoidae super-family and undergo little or no effects on lifespan and fecundity when exposed to Btk whereas *C. nigriceps* and *B. brevicornis*, belonging to the Ichneumonoidae super-family, undergo marked effects on both longevity and fecundity when fed with Btk. Furthermore, for another Ichneumonoidae species *Habrobracon hebetor*, the same effects have been observed when the larvae develop in a contaminated *Helicoverpa armigera* host (Sedaratian et al. 2013). Therefore, all these results strongly suggest that some of the side effects of Btk are submitted to specificity just as what is known about acute toxicity.

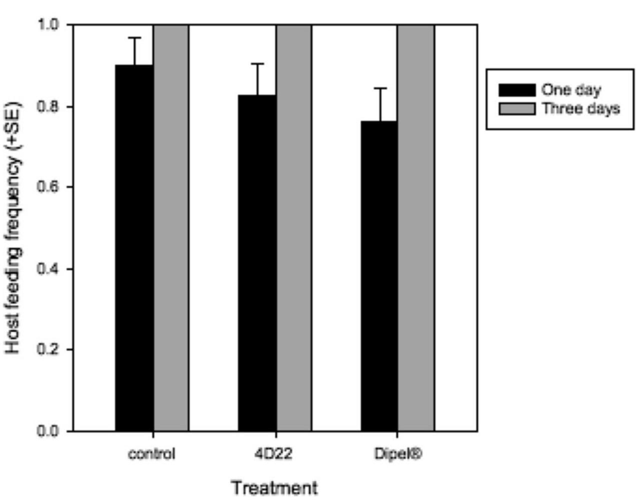

Fig. 3 Rate of host-feeding behaviours (±SE) by 4D22- and Dipel®- or honey-fed *Trichogramma chilonis* females

In the present study, we also wanted to test whether the treatment with Btk strains at a low dose might affect the foraging behaviour of *T. chilonis* females. An important behaviour for parasitic wasps concerns the way the females organize the time they remain on patches of their hosts (Wajnberg 2006) which is an essential parameter for the efficiency of *Trichogramma* as a biocontrol agent (Wajnberg 2000). We thus decided to compare this behaviour among <12-h-old isolated *T. chilonis* females fed during 1 or 3 days with the different Btk strains or with honey for the control. In this respect, we observed that, when fed with pure honey only, older females stayed longer on eggs patches of their hosts than younger females. This corresponds to the results described in (Wajnberg 2006) on another egg parasitoid species, i.e. *Anaphes victus* (Hymenoptera: Mymaridae) exploiting egg patches of its host, the carrot weevil *Litronotus oregonensis* (Coleoptera: Curculionidae). These authors demonstrated theoretically that such difference was actually an adaptive

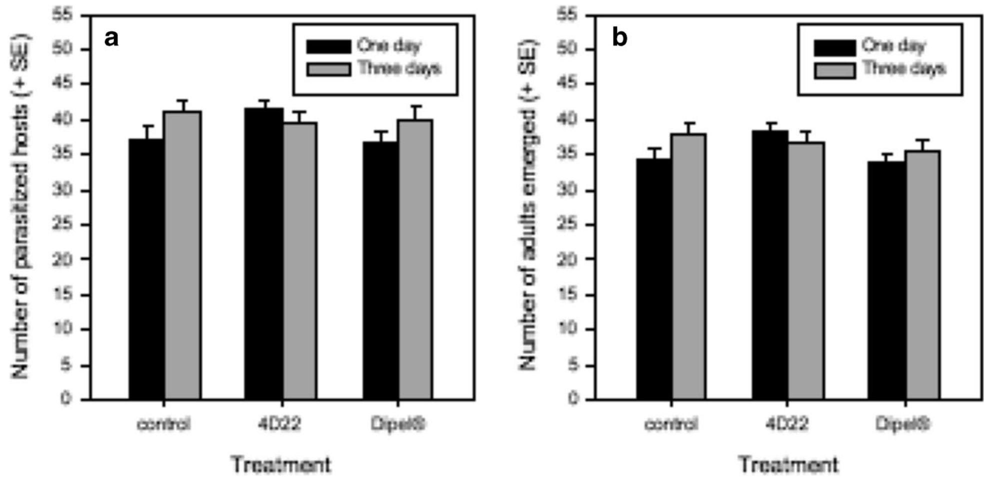

Fig. 4 Average (±SE) Reproductive success of 4D22-fed and Dipel®-fed or honey-fed *Trichogramma chilonis* females expressed as the number of hosts attacked during a 24-h period after treatment (**a**), and the number of adult wasps emerging from these hosts (**b**)

strategy since older females would not have the necessary available time to reach another patch of hosts in their life and should thus better remain on the patch on which they are currently foraging in order to increase the number of progeny produced as much as they can. Interestingly, results obtained here demonstrate that this effect apparently disappears when the *T. chilonis* were fed with Btk preparations. Most likely, these results suggest that ingestion of Btk spores could lead to alteration of the nervous system function. Nevertheless, such a neuroethological aspect deserves further experiments to determine which of these functions is affected by the spores: detection of the eggs, motility, signal treatment in the central nervous system etc. Nevertheless, we can thus consider this effect of Btk as being deleterious. Another

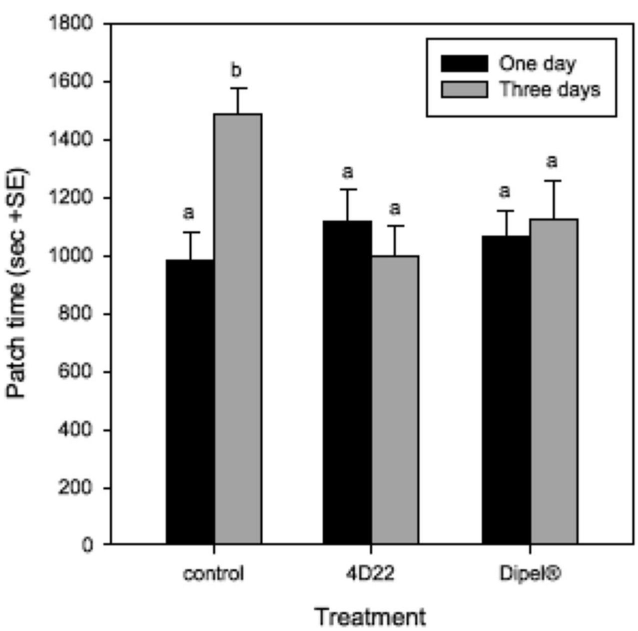

Fig. 5 Average (±SE) time spent by *T. chilonis* females fed during 1 or 3 days with different Bt strains of with honey for the control on patches of nine hosts. Values with *different letters* significantly differ according to a log-rank test ($p<0.001$)

alternative would be that as females fed with Btk live longer, at the same age (e.g. 3 days old), the females fed with Btk feel better than the females fed only with honey. Consequently, they feel capable of exploring a new patch and as a consequence spend less time on the previous patch.

All the experiments, apart the acute toxicity assessment, were performed with 2 μg of the Btk preparations (0.5 μL of a 4 μg/μL solution, see the Materials and methods section) offered to the *T. chilonis* females. Our Dipel® preparation titrated at 5×10^4 cfu/μg so that we offered to the females about 10^5 cfu. After spraying, it has been demonstrated that Btk spores can reach a density of more than 10^5 cfu/cm^2 in the environment for one month (Raymond et al. 2010). This is actually about the amount of spores we gave to *T. chilonis* females in our experiments. Hence, although the doses necessary to observe acute toxicity should be hardly reached in a Dipel®-sprayed area, *Trichogramma* females may be harmed by the bioinsecticide. In other words, the efficiency of *Trichogramma* as a biocontrol agent could be altered by a concomitant treatment by Dipel®.

In this paper, we observed three kinds of results as follows: (1) either no effect (host-feeding behavior, fecundity and parasitism success), (2) effects linked to the spores regardless of the Cry toxins (reduced time spent on host patches for older females) and (3) effects associated with the presence of Cry toxins (acute toxicity and extended longevity). Thus we conclude that both the spores and the Cry toxins contained in the Btk spores have some side effects on the *T. chilonis* females. When comparing our work to other studies it appears that Btk can have different side effects most likely according to the phylogeny of the insects tested (see above). We consider these similarities and differences as a demonstration for the need of further experiments to address the side effects of biocontrol methods on non-target organisms.

Taken together, all these effects exemplify the difficulty to address ecological risks of an active compound, especially when it is a living organism with complex modes of action.

Indeed, depending on the parameters measured, a product can appear to be neutral, beneficial or harmful. The ecotoxicological status of the compound depends on the balance between these different effects. Many bacteria species are already or are planned to be used as bioinsecticides (Ruiu 2015) and some can present similarities in their strategies: weakening of the host that then allows the multiplication of the parasite in the insect (Castagnola & Stock 2014). Thus, we can be apprehensive of the existence of other side effects on non-target organisms. Therefore, the search of such side effects appears necessary for a safer use of microorganism-based bioinsecticides.

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