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Immune system challenge in a host-parasitoid-pathogen system: interaction between *Cotesia plutellae* (Hym.: Braconidae) and *Bacillus thuringiensis* influences parasitism and phenoloxidase cascade of *Plutella xylostella* (Lep.: Plutellidae)

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Abstract

We investigated the effects of interaction between *Cotesia plutellae* (Kurdjumov) and *Bacillus thuringiensis* Berliner on parasitism and an immune effector (phenoloxidase activity) of a *Bt*-susceptible and a *Bt*-resistant population of *Plutella xylostella* (Linnaeus) in laboratory. Parasitism success of *C. plutellae* varied depending on the use of *B. thuringiensis* or its toxin, and the timing of application. Percentage parasitism was significantly greater on Cry1Ac-treated hosts than *B. thuringiensis* var. *kurstaki*-treated hosts (the susceptible population), and greater when hosts were treated with *B. thuringiensis* var. *kurstaki* before parasitism compared to that after parasitism (the resistant population). Specific phenoloxidase activity was significantly reduced in Cry1Ac-treated or parasitized hosts, but combined effect of the toxin and the parasitoid caused a greater reduction in phenoloxidase activity. The lower phenoloxidase activity in unparasitized resistant population of *P. xylostella* compared with the susceptible one is likely to be due to fitness costs, manifesting a possible trade-off between pathogen resistance and parasitoid resistance. However, *C. plutellae* overwhelmingly suppressed phenoloxidase activity of both the susceptible and resistant populations of *P. xylostella*. We found that the interaction between *B. thuringiensis* and *C. plutellae* was synergistic, which is promising for integration of the pathogen and the parasitoid in management of *P. xylostella* populations.

Key words: *Plutella xylostella*, parasitism success, phenoloxidase activity, pathogen resistance, parasitoid resistance, *Cotesia plutellae*, trade-off, *Bacillus thuringiensis*, synergistic

چکیده

در این پژوهش، اثرات برهم‌کنش بین زنبور پارازیتوئید *Cotesia plutellae* (Kurdjumov) و باکتری *Bacillus thuringiensis* Berliner بر روی پارازیتیسم و یک مجری سیستم ایمنی (فعالیت آنزیم فنول اکسیداز) جمعیت‌های بید کلم، *Plutella xylostella* (Linnaeus)، حساس و مقاوم به *Bt* در آزمایشگاه بررسی گردید. موفقیت پارازیتیسم، بسته به کاربرد *Bt* یا توکسین آن (Cry1Ac) و همچنین زمان کاربرد آن‌ها، متغیر بود. در جمعیت حساس، درصد پارازیتیسم میزبان‌های تیمار شده با Cry1Ac بیشتر از میزبان‌های تیمار شده با *Bt* var. *kurstaki* (*Btk*) بود. در جمعیت مقاوم، درصد پارازیتیسم میزبان‌های تیمار شده با *Btk* قبل از پارازیتیسم بیشتر از میزبان‌های تیمار شده با *Btk* بعد از پارازیتیسم بود. فعالیت اختصاصی فنول‌اکسیداز در میزبان‌های تیمار شده با Cry1Ac یا پارازیته‌شده به طور معنی‌داری کاهش یافت ولی اثر تلفیقی توکسین و پارازیتوئید کاهش بیشتری در فعالیت فنول‌اکسیداز را سبب شد. فعالیت پایین فنول‌اکسیداز در جمعیت مقاوم نسبت به جمعیت حساس می‌تواند به علت هزینه‌های سازگاری (fitness costs) باشد. هرچند، *C. plutellae* فعالیت فنول‌اکسیداز را در هر دو جمعیت حساس و مقاوم بید کلم به‌شدت کاهش داد. در این بررسی مشخص گردید که برهم‌کنش بین *Bt* و *C. plutellae* حالت سینرژیستی دارد که نشان‌دهنده‌ی چشم‌اندازی نویدبخش برای تلفیق این دو عامل کنترل بیولوژیک در مدیریت پایدار بید کلم است. یافته‌های مربوط به واکنش ایمنی بدیع هستند و می‌توانند در مطالعات مزرعه‌ای با هدف مدیریت مطلوب‌تر جمعیت‌های بید کلم تأثیرگذار باشند. به‌علاوه، به علت هزینه‌های سازگاری ناشی از مقاومت، محتمل است که جمعیت‌های بید کلم مقاوم به *Bt* حساسیت بیشتری در مقابل حمله‌ی پارازیتوئیدها از خود نشان

دهند. یک جانشینی (trade-off) احتمالی بین مقاومت به عامل بیماری‌زا و مقاومت به پارازیتوئید پیامد بسیار جالبی از چنین هزینه‌های سازگاری خواهد بود.

واژگان کلیدی: *Plutella xylostella*، موفقیت پارازیتیسم، فعالیت فنول‌اکسیداز، مقاومت به پاتوژن، مقاومت به پارازیتوئید، *Cotesia plutellae*، جانشینی، *Bacillus thuringiensis*، سینرژیستی

Introduction

Insects defend themselves against foreign invaders by an efficient immune reaction despite their lack of adaptive immunity (Hultmark, 1993; Strand & Pech, 1995). The insect innate immune reaction involves humoral responses, such as synthesis of antimicrobial peptides and the prophenoloxidase activation system, and cellular responses, such as phagocytosis and encapsulation (Bulet *et al.*, 1999; Lavine & Strand, 2002; Cerenius & Soderhall, 2004).

The insect principal immune defences against endoparasitoids are encapsulation and melanization (Schmidt *et al.*, 2001). The best known enzyme associated with the encapsulation response is phenoloxidase (PO; Cerenius & Soderhall, 2004). The phenoloxidase in insect haemolymph is present as a pro-enzyme, prophenoloxidase, which is activated in response to traumatic stress and microbial invasion (Ashida & Brey, 1997; Cerenius & Soderhall, 2004). The prophenoloxidase cascade is proposed to play an important role in the encapsulation of parasitoid eggs and is also a part of the insect recognition system of foreign invader (Ashida & Yamazaki, 1990; Ashida & Brey, 1997; Schmidt *et al.*, 2001). Variation in phenoloxidase activity has been linked to the ability to mount a successful defence against a foreign invader (Moreau *et al.*, 2000; Siva-Jothy & Thompson, 2002; Tucker & Stevens, 2003).

The overuse of insecticides against *Plutella xylostella* (Linnaeus), a worldwide pest species of crucifers, has resulted in several problems such as insecticide resistance in many field populations (Iqbal & Wright, 1997; Mohan & Gujar, 2003). Resistance to synthetic pesticides has led to the development of integrated pest management system based on more sustainable technologies including parasitoid wasps and pathogens such as *Bacillus thuringiensis* Berliner (*Bt*) (Chilcutt & Tabashnik, 1997a; Ivey & Johnson, 1998; Schuler *et al.*, 2003). The solitary, koinobiont, larval endoparasitoid *Cotesia plutellae* (Kurdjumov) is a possible biological control agent in regulating *P. xylostella* populations (Talekar & Shelton, 1993). More recently, interactions between parasitoids and *Bt* are becoming increasingly important as sustainable pest management strategies are more frequently being used in agro-

ecosystems (Groot & Dicke, 2002; Sharma *et al.*, 2004). These interactions can range from synergistic to competitive depending on environmental conditions and timing of interactions (Chilcutt & Tabashnik, 1997a, 1997b). For example, infection with *Bt* may influence body size and developmental time of an insect, such that *Bt*-infected hosts develop slower or smaller, rendering them more susceptible to attack by parasitoids (Mascarenhas & Luttrell, 1997). On the other hand, insect larvae parasitized before contact with *Bt* often are deficient in the feeding stimulus required to ingest a lethal dose, whereas unparasitized larvae feed normally and are more likely to ingest lethal doses (Nealis *et al.*, 1992).

Here, we examined the interactions between a herbivore (*P. xylostella*), a pathogen (*Bt*) and a parasitic wasp (*C. plutellae*) to evaluate the influence of *Bt* infection on parasitism success by *C. plutellae*, and to investigate the combined effects of the parasitoid and the pathogen on immune defence of *Bt*-resistant and *Bt*-susceptible populations of *P. xylostella*.

Materials and methods

Plant-insect rearing protocols

Brassica pekinensis (Loureiro) (Chinese cabbage) cv. F_1 One Kilo SB (Suttons Seeds, Devon, UK) was grown under glasshouse conditions (25 ± 5°C; L: D 16: 18 h) without the application of any pesticide. An insecticide-susceptible strain of *P. xylostella* (Roth population) and *C. plutellae* were both obtained from Rothamsted Research (Harpenden, UK). A highly resistant strain of *P. xylostella* to *Bt* (SERD4 population; originally from Lowland Malaysia) had been maintained on Cry1Ac from 1999 onwards (Sayyed & Wright, 2001).

Populations of *P. xylostella* were cultured on 4-week-old Chinese cabbage in ventilated Perspex cages (35 × 35 × 35 cm). The cultures of *C. plutellae*, in turn, were maintained on the susceptible *P. xylostella* larvae in ventilated Perspex oviposition cages (35 × 35 × 35 cm). Both cultures were kept in a standard constant environment (25 ± 2°C; 70 ± 5% RH; L: D 16: 8 h) (Karimzadeh *et al.*, 2004).

Bacillus thuringiensis treatments

Plutella xylostella larvae were treated with either *B. thuringiensis* var. *kurstaki* (*Btk*) or *Bt* toxin (Cry1Ac). Formulated *Btk* strain HD-1 (Dipel1) was obtained from Abbott Laboratories (Chicago, USA) and stored at room temperature. Cry1Ac was supplied by Juan Ferré (University of València) and stored at -20°C. The test products were freshly prepared in distilled water with Triton X-100 (50 μg ml^{-1}) as a surfactant.

Leaf discs (4.8 cm dia.) of Chinese cabbage were immersed in the test solution for 10 s, and then kept on a corrugated sheet of aluminium foil with the adaxial leaf surface uppermost for 1 to 2 h at room temperature to dry up. Control leaf discs were immersed in distilled water containing Triton X-100 (50 μg ml^{-1}). The leaf discs were then transferred to individual plastic Petri dish (5 cm dia.) containing a moistened filter paper. Ten two-day-old second instar larvae of *P. xylostella* were then placed on each leaf disc. The concentrations *Btk* or Cry1Ac used in experiments were 0.1 and 10 μg ml^{-1} for susceptible and resistant *P. xylostella* larvae, respectively (Sayyed *et al.*, 2000; Sayyed & Wright, 2001).

Parasitism success

To test the hypothesis that *Bt* treatment and its application timing influence parasitism of *P. xylostella* by *C. plutellae*, two tests were conducted. 1) Parasitism test of susceptible *P. xylostella* population, where the effects of *Btk* or Cry1Ac were tested on parasitism success. 2) Parasitism test of resistant *P. xylostella* population, where the timing of *Btk* application was tested on parasitism success; pre-parasitism treatment with *Bt* may affect the behaviour of parasitoid larva and adult, whereas post-parasitism treatment with *Bt* may only influence the behaviour of parasitoid larva (Erb *et al.*, 2001). *Bt* inoculation was performed one day before (pre-parasitism treatment) or after (post-parasitism treatment) parasitization.

To perform parasitism test a single, mated two-day-old female *C. plutellae* was released into a Petri dish (5 cm dia.) containing ten two-day-old second instar larvae of *P. xylostella* for 1 h. All *P. xylostella* larvae were then reared on the leaves of Chinese cabbage until either the moths pupated or the parasitoid cocoons formed (Karimzadeh *et al.*, 2004). Each treatment was replicated 12 times. The proportion of hosts that formed parasitoid cocoons was used as a measurement of percentage parasitism (with exclusion of host mortality from unknown factors).

Phenoloxidase assay

Plutella xylostella larvae were placed in individual Petri dishes (5 cm dia.) and reared on leaf discs from Chinese cabbage (as described above) until the late fourth instar larval stage but prior to cessation of feeding. PO activity was determined in haemolymph samples (2 μl) collected from late fourth instar larvae of *P. xylostella* by cutting off one or more prolegs and drawing up exuded haemolymph bubbles with a pulled 1-μl microcapillary tube. The haemolymph sample was quickly added to phosphate-buffered saline (PBS), pH 7.4 (30 μl) in

a 0.5 ml Eppendorf tube on ice and immediately frozen to -80°C to disrupt haemocyte membranes. Frozen samples were then thawed to 4°C and centrifuged at 12000 g at 4°C for 10 min. An aliquot (2 µl) of the supernatant was taken for protein assay (Bradford, 1976). A second aliquot (20 µl) was incubated for 20 min at 20°C to activate PO activity, which was then assayed spectrophotometrically using 1 ml of 3,4-D-L-dihydroxyphenylalanine (10 mM) in PBS as a substrate (Siva-Jothy & Thompson, 2002). Negative controls contained 10% (w/v) 1-phenyl-2-thiourea, a known inhibitor of PO (Reeson *et al.*, 1998).

This experiment was carried out with different treatments, including control (untreated with Cry1Ac and unparasitized) hosts, Cry1Ac-treated (but unparasitized) hosts, parasitized (but untreated with Cry1Ac) hosts and parasitized, Cry1Ac-treated hosts. Each treatment was replicated 10 times.

Statistical analyses

Differences in the levels of percentage parasitism and percentage mortality between treatments were analysed using logistic analysis of deviance (binomial error). In case of overdispersion, it was compensated by refitting the model using quasibinomial rather than binomial error. To achieve the minimal adequate model, non-significant terms were removed through model simplification in normal way but the models were compared by *F*-test instead of a Chi-squared test (Crawley, 2005). Linear regression analysis was used to calculate protein concentrations. PO data were analysed using one-way ANOVA. Pairwise comparisons were performed using Student's t-test (Crawley, 2002). All statistical analyses were completed in S-Plus 6.1 (Insightful Corp., Seattle).

Results

When the susceptible population were tested, there was a significantly different percentage parasitism by *C. plutellae* between treatments (*z-value* = 2.531, *d.f.* = 33, $P < 0.05$) (table 1). The proportion of Cry1Ac-treated hosts parasitized by *C. plutellae* was significantly greater than for *Btk*-treated hosts (0.862 vs. 0.667). However, there was no significant difference in the level of parasitism either between untreated and *Btk*-treated hosts or between untreated and Cry1Ac-treated hosts. In addition, there was no significant difference in percentage mortality between treatments ($F_{2, 33} = 0.045$, $P = 0.96$) (table 1).

Similarly, with the resistant population a significant difference in percentage parasitism between the treatments was found (*z-value* = 5.009, *d.f.* = 30, $P < 0.001$) (table 1). The hosts

treated with *Btk* preparasitism revealed significantly higher level of parasitism compared with the hosts treated with *Btk* postparasitism (0.897 vs. 0.650). On the contrary, there was no significant difference in parasitism levels between *Btk*-treated and untreated hosts. Furthermore, no significant difference in percentage mortality between treatments was found ($F_{2, 30}$ = 1.901, P = 0.15) (table 1).

Table 1. Effects of *B. thuringiensis* or its toxin and timing of application on percentage parasitism by *C. plutellae* of *Bt*-susceptible and *Bt*-resistant *P. xylostella* larvae (n = 12).

Experiment (host larval type used)	Treatment (in time order)	Percentage parasitism (Mean ± SE) *	Percentage mortality (Mean ± SE) **
1 (*Bt*-susceptible *P. xylostella*)			
	parasitism	62.1 ± 14.2 ab	25.8 ± 5.1
	Btk + parasitism	66.7 ± 6.8 a	25.0 ± 3.9
	Cry1Ac + parasitism	86.2 ± 11.1 b	25.8 ± 5.9
	P	< 0.05	0.96
2 (*Bt*-resistant *P. xylostella*)			
	parasitism	76.8 ± 9.3 ab	22.0 ± 3.4
	parasitism + *Btk*	65.0 ± 10.5 a	26.9 ± 3.6
	Btk + parasitism	89.7 ± 6.3 b	21.0 ± 4.0
	P	< 0.001	0.15

* Survived hosts that produced wasps.
** Hosts that neither pupated nor produced wasps (i.e., dead from unknown factors).
Values marked with different letters show significantly (P < 0.05) different means (Student's t-test).

The PO specific activity of the susceptible population varied significantly between treatments ($F_{3, 36}$ = 4.566, P < 0.01) (fig. 1). PO specific activity was greatest (152.5 and 149.1 units per mg protein), intermediate (55.2 units per mg protein) and lowest (15.8 units per mg protein) for control and Cry1Ac-treated hosts, parasitized hosts and parasitized, Cry1Ac-treated hosts, respectively.

The PO specific activity of the resistant population also showed a significant difference between treatments ($F_{3, 36}$ = 4.792, P < 0.01) (fig. 1). PO specific activity of control hosts (98.1 units per mg protein) was significantly greater compared with Cry1Ac-treated (59.0 units per mg protein) or parasitized (52.8 units per mg protein) hosts, which in turn, showed significantly higher PO specific activities than parasitized, Cry1Ac-treated hosts (27.1 units per mg protein).

Furthermore, when two populations of *P. xylostella* were compared, unparasitized susceptible population showed a significantly greater PO specific activity than unparasitized resistant population (control hosts: 152.5 vs. 98.1 units per mg protein; Cry1Ac-treated hosts:

149.1 vs. 59.0 units per mg protein). On the contrary, there was no significant difference between susceptible and resistant populations for PO specific activity of parasitized hosts (55.2 vs. 52.8 units per mg protein). Interestingly, when hosts were both parasitized and treated with Cry1Ac, resistant population revealed a significantly greater PO specific activity compared with susceptible population (27.1 vs. 15.8 units per mg protein).

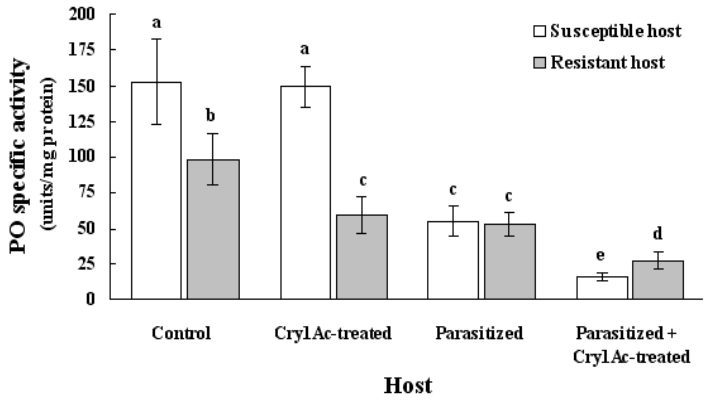

Figure 1. The effects of Cry1AC and parasitism by *C. plutellae* on specific phenoloxidase activity (mean ± SE) of *Bt*-susceptible and *Bt*-resistant *P. xylostella* larvae (n = 10). Different letters show significantly ($P < 0.05$) different means. One unit represents 0.001 absorbance at 490 nm per min.

Discussion

In the present study, the greater parasitism success on Cry1Ac-treated compared to *Btk*-treated susceptible *P. xylostella* population indicated a better compatibility of the parasitoid with Cry1Ac compared to *Btk*. Given this and the fact that the main insecticidal property of *Bt* resides in the proteinaceous crystal consisting of *d*-endotoxins (Cry toxins; Schnepf *et al.*, 1998) point to a promising management of *P. xylostella* populations using combining such sustainable strategies. In their studies using Dipel 2X (a commercial formulation of the HD-1 strain of *Btk* that contains live spores and insecticidal crystal proteins), Chilcutt & Tabashnik (1997a, 1997b) also found that host mortality caused by interactions between *C. plutellae* and *B. thuringiensis* depended on the susceptibility of the hosts to the pathogen; such that increasing colony resistance reduced the effects of *B.*

thuringiensis but it had no effects on *C. plutellae* performance. Their results indicated that *Bt*-highly resistant hosts provide a refuge from *B. thuringiensis*, in which *C. plutellae* can complete its development without competition for the host. In addition, the greater parasitism success on resistant *P. xylostella* population when treated with *Btk* preparasitism in comparison with that postparasitism (as found in the present study) definitely has implications for combining the pathogen and the parasitoid on the basis of application timing.

The results demonstrated that fatal concentrations of Cry1Ac (used for the resistant population) markedly suppressed PO activity, which may benefit a parasitoid larvae developing in the host haemolymph. On the contrary, sublethal concentrations of Cry1Ac (used for the susceptible population) might cause little damage to gut epithelium, which is sufficient for microbial elicitors to reach the haemolymph and trigger phenoloxidase cascade (Ashida & Brey, 1997; Cerenius & Soderhall, 2004).

The reduced PO activity in the resistant *P. xylostella* population compared with the susceptible one clearly has implications for potential fitness costs through the manifestation of trade-off between the pathogen resistance and other life history traits (Boots & Begon, 1993; Schmid-Hempel, 2003; Graham *et al.*, 2005); such fitness costs may result in less capability of mounting an efficient immune defence against parasitoids. This imply possible trade-off between the two immune effectors (antibacterial activity and PO activity), or in other words, between pathogen resistance and parasitoid resistance in insects (Moret & Schmid-Hempel, 2001; Moret & Siva-Jothy, 2003; Cotter *et al.*, 2004) and remains an area for future work. This hypothesis, however, can be challenged by the fact that *C. plutellae* overwhelmingly suppressed PO activity of both the susceptible and resistant populations of *P. xylostella*. This has implications with the resistance-virulence coevolution; endoparasitoid wasps have evolved various mechanisms to ensure successful development of their progeny, including injection of venom, calyx fluid, and polydnaviruses into the host haemocoel (Asgari, 2006; Karimzadeh & Wright, 2008). Here, it was found that the interaction between *Bt* and *C. plutellae* was not competitive; *Bt* had no adverse effect on the parasitism success by *C. plutellae*, which is in agreement with the previous findings on *C. plutellae* and *Bt*-plants interactions (Schuler *et al.*, 2004). In addition, *C. plutellae* may act better on *Bt*-treated hosts (both the susceptible and resistant populations) compared with untreated hosts, as the interaction of the pathogen and the parasitoid resulted in more suppression of the host immune function, implying a synergistic effect. From a pest management viewpoint, this may

be promising for integration of the pathogen and the parasitoid against *P. xylostella* in field studies.

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