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Prey mediated effects of Bt maize on fitness and digestive physiology of the red spider mite predator *Stethorus punctillum* Weise (Coleoptera: Coccinellidae)

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Abstract The present study investigated prey-mediated effects of two maize varieties expressing a truncated Cry1Ab, Compa CB (event Bt176) and DKC7565 (event MON810), on the biology of the ladybird *Stethorus punctillum*. Although immunoassays demonstrated the presence of Cry1Ab in both prey and predator collected from commercial maize-growing fields, neither transgenic variety had any negative effects on survival of the predator, nor on the developmental time through to adulthood. Furthermore, no subsequent effects on ladybird fecundity were observed. As a prerequisite to studying the interaction of ladybird proteases with Cry1Ab, proteases were characterised using a range of natural and synthetic substrates with diagnostic inhibitors. These results demonstrated that this predator utilises both serine and cysteine proteases for digestion. In vitro studies demonstrated that *T. urticae* were not able to process or hydrolyze Cry1Ab, suggesting that the toxin passes through the prey to the third trophic level undegraded, thus presumably retaining its insecticidal properties. In contrast, *S. punctillum* was able to activate the 130 kDa protoxin into the 65 kDa fragment; a fragment of similar size was also obtained with bovine trypsin, which is known to cleave the protoxin to the active form. Thus, despite a potential hazard to the ladybird of Bt-expressing maize (since the predator was both exposed to, and able to proteolytically cleave the toxin, at least in vitro), no deleterious effects were observed.

Keywords Non-target arthropods · Bt maize · Cry1Ab · Digestive proteases · Ladybird *Stethorus punctillum* · Tetranychid mites *Tetranychus urticae*

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Introduction

In 2006, genetically modified maize expressing δ-endotoxins from *Bacillus thuringiensis* (Bt maize) was commercially planted in thirteen countries, occupying a global area of 21.1 million ha (James 2006). In Spain, Bt maize has been planted since 1998 and, in 2007, approximately 75,000 ha were grown (MON810 event), representing around 21% of

the total maize acreage; currently Spain is the principal grower of this biotechnology crop in the European Union. Bt maize has been shown to be effective for control of the corn borers *Ostrinia nubilalis* (Hübner) and *Sesamia nonagrioides* (Lefèbvre), the most damaging maize pests in Spain and the Mediterranean area (Castañera 1986). However, one of the major environmental concerns determining whether transgenic crops will have a sustainable role in agriculture is their possible effects on non-target entomophagous arthropods (predators and parasitoids) (Cowgill and Atkinson 2003). A number of studies, both field and laboratory, have been conducted to assess the impact of transgenic Bt plants on non-target organisms (Pilcher et al. 1997; Lozzia 1999; Al-Deeb et al. 2001; Zwahlen et al. 2003; Romeis et al. 2004; De la Poza et al. 2005; Ludy and Lang 2006) and whilst most studies have demonstrated little effect on beneficial insects, particularly natural enemies, a few studies have suggested that such plants will have negative effects (Hilbeck et al. 1998; Dutton et al. 2002; Meissle et al. 2005).

Recent field studies in the Northeast of Spain, carried out at different periods over the season, demonstrated that Cry1Ab was present at concentrations 3-fold greater in the non-target herbivore *Tetranychus urticae* Koch than those found to be present in Bt maize (Event Bt176) leaves (Obrist et al. 2006a). In this particular study, the presence of the toxin was also demonstrated in higher trophic levels, including the predators *Stethorus punctillum* Weise, *Chrysoperla carnea* (Stephens) and *Orius* species. Similarly, Harwood et al. (2005) reported significant levels of Cry1Ab in other non-target herbivores and arthropod predators (Coccinellidae, Araneae, and Nabidae) collected from a transgenic maize (event MON810) agroecosystem.

Transgenic Bt maize varieties derived from Bt176 and MON810 events express truncated forms of the Cry1Ab toxin. Event 176 expresses a truncated form of the toxin that corresponds to the first 648 aa (about 65–70 kDa) of the 1155 aa (about 130 kDa) of the native Cry1Ab protoxin (Koziel et al. 1993), whilst MON810 expresses a truncated protein with a molecular weight of approx. 90 kDa (http://www.agbios.com/dbase.php?action=Submit&evidx=9). Activation of the protoxin is believed to occur by the removal of a few residues at the N-terminal and a large fragment at the C-terminal end, resulting in an active toxin (residues 29–35 to 599–607 of the protoxin sequence) (Schnepf et al. 1998; Rukmini et al. 2000). Thus, the truncated form expressed by the event MON810 requires activation by digestion at both ends, whereas removal of the N-terminal peptide (about 30 aa) is most likely necessary for the activation of the truncated form expressed by the event 176. In lepidopteran species, trypsin- and chymotrypsin-like proteases seem to be the principal enzymes implicated in Cry1Ab activation by digestion at both ends to form an active toxin of 60–70 kDa (Oppert 1999; Miranda et al. 2001; Díaz-Mendoza et al. 2007). Once the toxin is activated, it passes through the peritrophic membrane and binds to specific receptors located in the epithelial cells of the midgut (De Maagd et al. 2003). However, it is not known whether the toxin reaches the midgut of the predators as a truncated toxin, or whether the toxin has been activated by proteolytic enzymes present in prey species, prior to consumption by these predators. Furthermore, it is possible for the toxin to be degraded by proteases present within the prey gut, as has been shown in resistant lines of target species (Forcada et al. 1996) and in non-target lepidopteran species (Miranda et al. 2001). It is also not known as to the fate of the toxin once present in the predator gut, and in particular the subsequent effects of digestive proteases present in the predators.

The ladybird *S. punctillum* is a specialist predator of tetranychid mites (Rott and Ponsonby 2000). Both larvae and adults of this predator are very voracious, have a high capacity for dispersion (Congdon et al. 1993) and are being used as biological control agents of spider mites in agricultural crops (Hull et al. 1977; Roy et al. 1999). *S. punctillum* was one of the most abundant predators found in commercial plots in two Spanish maize growing areas (De la Poza et al. 2005). In spite of the importance of *S. punctillum* as a biological control agent, the potential effects of Cry1Ab expressing transgenic maize on this specialist predator have never been investigated. Furthermore, it is not known whether proteases present in the spider mite *T. urticae* are able to process or degrade the Bt toxin, neither is there any information available regarding the possible interactions of this toxin with the proteolytic enzymes of the ladybird *S. punctillum*, once they reach the midgut.

Thus, the aim of the present study was to establish the impact of transgenic Bt maize varieties derived

from the events Bt176 and MON810, on development, survival and fecundity of the beneficial predator *S. punctillum* via fed prey. Moreover, it reports the characterization of the proteolytic enzymes of this ladybird as a prerequisite step to investigate the interaction of the digestive proteases of *S. punctillum* and its prey, the red spider mite *T. urticae*, with the Cry1Ab toxin expressed by the Bt maize.

Material and methods

Insects

Adults of *Stethorus punctillum* were purchased from Applied Bionomics (Canada) and placed in boxes (11.5 cm diameter, 5 cm high) with maize leaves infested with *Tetranychus urticae* of various stages. Eggs were collected three times a week with a small brush. Adults, larvae and eggs were kept in a climatic chamber at 26 ± 0.3°C, 80 ± 5% RH and L:D 16:8 h photoperiod. *T. urticae* were provided by Dr. Vicente Marco (Universidad de La Rioja, Spain) in 2006 to start a laboratory colony. Spider mites were maintained on maize plants at 25 ± 0.3°C, 70 ± 5% RH and L:D 16:8 h photoperiod.

Plant material

Commercial cultivars of transgenic Bt maize (*Zea mays* L.) (Event Bt176, Compa CB and event MON810, DKC7565) (designated Bt+) expressing a gene encoding a truncated, synthetic version of the Cry1Ab gene from *Bacillus thuringiensis* var. *kurstaki* and the corresponding non-transformed near-isogenic varieties (Brasco and Tiétar) (designated Bt−) as controls were used for experiments. All plants were planted in plastic pots and cultivated in a growth chamber at 25 ± 0.3°C, 70 ± 5% RH, and L:D 16:8 h photoperiod. Plants were used when they had reached the seven-leaf stage.

Cry1Ab detection in maize plants, *T. urticae* and *S. punctillum* from field samples

Maize leaves, spider mites and ladybird adults were collected in commercial Bt maize (event MON810) and non-transgenic maize fields located in Central Spain, on two dates in August 2006. All samples were collected individually, transferred into 1.5 ml Eppendorf tubes and frozen at −20°C in a portable freezer immediately after collection.

Cry1Ab protein levels in plants and arthropods were determined using a double sandwich ELISA kit (Agdia, USA). Briefly, samples (5 for both leaves and *S. punctillum;* 3 for *T. urticae*) were homogenised in 0.5 ml phosphate buffered saline pH 7.4 (PBS), centrifuged for 5 min at 12,000 × *g* and total protein determined according to the method of Bradford (see manufacturer's instructions). Spectrophotometric measurements were conducted in a microtitre plate reader at 450 nm, using 8 µg of protein (in PBS) per plant sample and 20 µg of protein per arthropod sample. Cry1Ab standards at concentrations 0, 0.125, 0.25, 0.5, 1, 2, 4, 8 and 16 ng were used as calibrators.

Characterization of *S. punctillum* proteolytic enzymes

S. punctillum adults were homogenized in 0.15 M NaCl, centrifuged at 16,000 × *g* for 5 min, and the supernatants pooled and stored frozen (−20°C) until required. All assays were carried out in triplicate and appropriate blanks were used. A series of overlapping buffers were used to generate a pH gradient from 2 to 11: 0.1 M citric acid-NaOH (pH 2–pH 3), 0.1 M citrate (pH 5–pH 6.5), 0.1 M Tris–HCl (pH 6.5–pH 9) and 0.1 M glyicine–NaOH (pH 9–pH 11). All buffers contained 0.15 M NaCl and 5 mM $MgCl_2$.

All protease activities were performed at 30°C at their optimum pH of activity, incubating for 24 h in 1 ml of reaction mixture containing 20 µl of homogenate. Non-specific protease activity was assayed with 0.1% sulfanilamide-azocasein as substrate. Other protease assays were as follows: trypsin-like activity using 1 mM BApNa (Nα-benzoyl-DL-arginine p-nitroanilide), chymotrypsin-like activity with 0.25 mM Sa_2PPpNa (N-succinyl-$(alanine)_2$-proline-phenylalanine-p-nitroanilide), and elastase-like activity with 0.25 mM SA_3pNa (N-succinyl-$(alanine)_3$-p-nitroanilide), as describe by Ortego et al. (1996). Cathepsin D-like activity was measured with 0.2% haemoglobin as substrate, and cathepsin B-like activity was assayed with 50 µM ZAA_2MNA (N-carbobenzoxy-alanine-arginine-arginine 4-methoxy-β-naphthyl amide), as described by Novillo et al. (1997). Total protein in adult extracts was

determined according to the method of Bradford (1976) using bovine serum albumin as the standard.

The proteolytic activities of adult extracts were further characterised using the following specific protease inhibitors: the serine protease inhibitor SBBI (Soybean Bowman-Birk inhibitor); the cysteine protease inhibitor E-64 (L-trans-epoxysuccinyl-leucylamido-(4-guanidino)-butane); IAA (Iodoacetamide); and the aspartic protease inhibitor pepstatin-A. The cysteine protease activators L-cysteine and DTT (dithiothreitol) were also tested. Protease inhibitors and activators were pre-incubated at 30°C with the adult extract for 15 min, prior to addition of the substrate. All compounds were added in 100 µl of 0.15 M NaCl, except pepstatin-A, which was added in 20 µl of DMSO. The doses tested were selected according to the effective concentrations recommended by Beynon and Salvesen (1989).

All substrates as well as protease inhibitors and activators were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Spectrophotometric measurements were made using a Hitachi U-2000 spectrophotometer.

Digestion of Cry1Ab protoxin by prey and predator proteases

Cry1Ab toxin

Cry1Ab protoxin crystals (81% purity), produced by *B. thuringiensis* ssp. *kurstaki* HD1-9 strain (Carlton and González 1985), were provided by Syngenta. Lyophilised crystals were re-suspended in 0.1% (w/w) Triton X-100 in a 0.1 M glycine–NaOH, 0.15 M NaCl and 5 mM $MgCl_2$, pH 10.5 buffer. Because autoprocessing of the solubilized native protoxin was detected after long incubation periods at 30°C, the Cry1Ab solution was treated at 62°C for 30 min to deactivate putative *B. thuringiensis* proteases. This treatment did not alter the toxicity of Cry1Ab protoxin. Activated Cry1Ab toxin was obtained by expression in *E. coli* of a sequence of the Cry1Ab protoxin originally obtained from the *Bacillus* genetic stock centre and activated by proteolytic cleavage using bovine trypsin.

Digestion assays in vitro

T. urticae and *S. punctillum* adults were homogenized in PBS, centrifuged at 13,000 × *g* for 5 min and the supernatant collected. Total soluble protein was determined by Bradford assay according to the method of Bradford (1976) using bovine serum albumin as the standard.

In vitro digestion assays were performed at 30°C at pH 5.0 for *T. urticae* and at pH 5.0 and 10.0 for *S. punctillum*. Prey/predator extracts were incubated with Cry1Ab toxin for 1 and 24 h at a 1:25 protoxin:extract ratio. The reactions were terminated by addition of 5 µl of electrophoresis buffer (tris–HCl 60 mM, pH 6.8; 10% SDS (w/v); 33.3% 2-mercaptoethanol (v/v); 33.3% glycerol (w/v); 0.06% bromophenol blue) followed by boiling for 5 min. Furthermore, protoxin was incubated with bovine trypsin at pH 10.0 and commercial papain at pH 5.0 for 1 h at a 1:8 protoxin:protease ratio. Samples were separated by SDS-PAGE (12.5%) and subsequently electrophoretically transferred to 0.45 µm nitrocellulose membranes (Schleicher & Schuell, BA83). The membranes were developed for immunoassay by Western blotting using antibodies raised against Cry1Ab as the primary antibody, and HRP-conjugated goat anti-rabbit IgG as the secondary antibody, as described by Gatehouse et al. (1996). Cry1Ab was detected by enhanced chemiluminescence (ECL) according to the manufacturer's instructions.

Effects of Bt maize on predator development and reproduction via the tritrophic interaction

Experimental arenas consisted of 4 cm^2 inverted maize leaf discs in individual dishes covered with lids ventilated with a fine mesh; each contained moist filter paper to prevent desiccation. Ladybirds were transferred to the leaf discs with a small brush. Assays were conducted in a growth chamber at 26 ± 0.3°C, 80 ± 5% RH, and L:D 16:8 h photoperiod. Observations were made with a stereomicroscope, provided with a cold light source.

Effects of Bt maize on predator survival and development

Neonate larvae of *S. punctillum* were placed individually on leaf discs from the following maize varieties: Compa CB (Bt+, $n = 79$), Brasco (Bt−, $n = 83$), DKC7565 (Bt+, $n = 63$) and Tietar (Bt−, $n = 61$). Larvae were fed daily ad libitum with *T. urticae* of

various stages reared on either transgenic or non-transformed control maize. Immatures were transferred onto fresh leaf discs every 2–3 days until pupation. Survival of *S. punctillum* larvae and pupae was monitored on a daily basis and developmental time (time to each instar, time to pupation, time to adult emergence) was recorded throughout.

Effects of Bt maize on predator fecundity

On emergence (see above), the adult ladybirds from each sibling group were sexed and assigned to mating pairs for 14 days. During this period, the number of eggs laid per pair was recorded daily. Adult female fecundity was estimated by counts of number of eggs produced per individual. The assay with the varieties Compa CB and Brasco was carried out with 25 pairs, while 23 pairs were used for assay with the varieties DKC7565 and Tiétar.

Statistical analysis

Larval and pupal development and female fecundity were analyzed by means of Mann–Whitney U-test as data were not normally distributed. Survivorship of immature stages was compared by a chi-square test. Differences between treatments were considered significant at the $P < 0.05$ level.

Results

Cry1Ab accumulation in *T. urticae* and *S. punctillum* under field conditions

The concentration of Cry1Ab toxin detected in maize leaves, *T. urticae* and *S. punctillum* collected from a commercial Bt maize field is shown in Fig. 1. A mean concentration of 90.2 ± 30.1 ng Cry1Ab toxin per mg of total soluble protein (TSP) was detected in Bt maize leaves. *T. urticae* contained on average 413.2 ± 14.9 ng mg^{-1} TSP, whilst a significantly lower concentration of toxin was detected in *S. puntillum* adults (47.5 ± 12.35 ng mg^{-1} TSP). As expected, Cry1Ab toxin was not detected in non-transformed maize leaves (control), nor in prey and predators collected from fields growing non-transgenic maize.

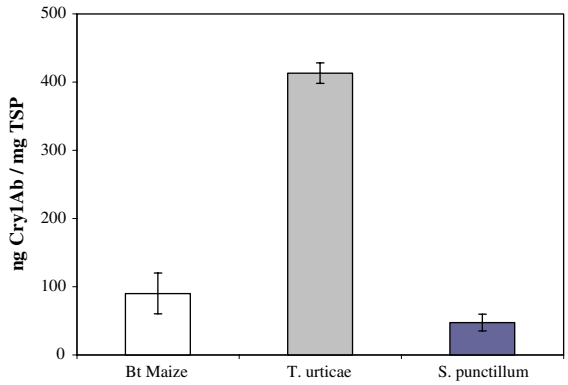

Fig. 1 Mean concentrations (±SE) of Cry1Ab toxin in Bt maize leaves, *T. urticae* and *S. punctillum* collected in a transgenic maize field. Corresponding samples were collected from a non-transgenic maize field as controls

Characterization of *S. punctillum* proteolytic enzymes

The pH optima and the specific activities of enzyme extracts from *S. punctillum* against general and specific protease substrates are presented in Table 1. The results clearly show that general proteolysis, with azocasein as substrate, occurred over a broad range of pH, with two peaks of optimum activity at pH 5 and pH 10 (Fig. 2). Maximal hydrolysis of BApNa occurred at pH 10.5, whereas maximal activity with SA_2PPpNa was observed at pH 9.5, suggesting the presence of trypsin-like and chymotrypsin-like activity, respectively. The pH optima for both ZAA_2MNA and haemoglobin occurred in the acidic range with optima at pH 6.5 and pH 3.5 respectively, indicative of Cathepsin B-like and D-like activity. However, no hydrolytic activity of SA_3pNa occurred, even after 24 h of incubation, indicating the absence of elastase-like activity.

The proteolytic activity of *S. punctillum* was further characterized using specific diagnostic protease inhibitors (Table 1). Hydrolysis of haemoglobin was inhibited by pepstatin-A, whilst ZAA_2MNA hydrolysis was inhibited both by E-64 and IAA, but activated by DTT and L-cysteine. The hydrolysis of BApNa and SA_2PPpNa was inhibited by SBBI. These studies indicate the presence of proteases from two mechanistic classes i.e. serine proteases and cysteine proteases.

Table 1 Proteolytic activity of *S. punctillum* adult extracts against general and specific substrates; effects of protease inhibitors and activators

Substrate	Optimum pH	Specific activity[a]	% relative activity[b]					
			IAA	SBBI	E-64	Pepstatin-A	DTT	L-cysteine
BApNa	10.5	2.1 ± 0.4	nd	7 ± 1	ne	ne	ne	ne
SA2PppNa	9.5	1.5 ± 0.3	nd	56 ± 2	ne	ne	ne	ne
ZAA2MNA	6.5	0.5 ± 0.1	42 ± 2	ne	25 ± 1	ne	123 ± 3	172 ± 5
Haemoglobin	3.5	9.4 ± 0.1	nd	ne	ne	9 ± 3	ne	ne

[a] Specific activities as nmoles of substrate hydrolysed/(min mg protein), except for proteolytic activity against haemoglobin as mU Δ Abs 280 nm/(min mg protein). Figures are mean ± SE of triplicate measurements

[b] Values are mean ± SE of triplicate measurements from a pool of adult extracts treated in the presence/absence of inhibitor or activator relative to their corresponding controls. No effect (ne) was considered for activities between 80% and 120%; nd: not determined

Substrates: BApNa, (Nα-benzoyl-DL-arginine p-nitroanilide); SA2PPpNa, (N-succinyl-(alanine)$_2$-proline-phenylalanin-p-nitroanilide); ZAA2MNA, (N-carbobenzoxy-alanine-arginine-arginine 4-methoxy-β-naphthyl amide)

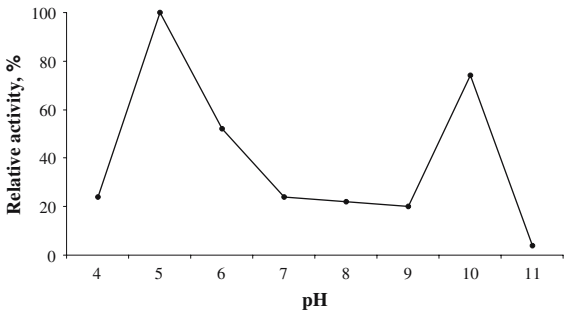

Fig. 2 pH optima of *S. punctillum* adult proteolytic activity with azocasein as substrate, using a pH range of 2–11. All assays were carried out in triplicate

Digestion of Cry1Ab protoxin by prey and predator proteases

The in vitro digestion of Cry1Ab was carried out in order to study the interaction between Cry1Ab toxin and the proteases of both the spider mite, *T. urticae* and its predator, the ladybird *S. punctillum*. Assays with *T. urticae* were carried out at a single pH value of pH 5.0, but at two different times of incubation; the results showed that the Cry1Ab protoxin was not processed or hydrolysed by the spider mite proteases, since the 130 kDa fragment belonging to the protoxin was still present and the activated toxin could not be detected, even after 24 h of incubation (Fig. 3a).

Digestion of Cry1Ab protoxin by digestive proteases of *S. punctillum* was also carried out for different time intervals (1 and 24 h) but at two different values of pH (5.0 and 10.0). At acidic pH, proteases were not able to process the Cry1Ab toxin; the fragment of 130 kDa belonging to the protoxin was still present after 24 h of incubation (Fig. 3b). In contrast, at pH 10.0, the proteolytic enzymes of the ladybird were able to process the protoxin into its active form; after 24 h of incubation the 130 kDa fragment disappeared and a fragment of about 65 kDa was generated (Fig. 3c).

The digestion of Cry1Ab protoxin with commercial bovine trypsin and papain were also performed as positive controls. After 1 h of incubation, a fragment of 65 kDa was readily visible, suggesting that both commercial proteases are able to process the Bt toxin (Fig. 3d).

Prey-mediated effects of Bt maize on predator development and reproduction

A tritrophic assay was carried out to investigate the effects of transgenic Bt expressing maize (commercial varieties derived from events Bt176 and MON810) on *S. punctillum* via dosed prey. The results demonstrated that exposure to Cry1Ab had no effect on the survival of neonate ladybird larvae through to adulthood (Figs. 4, 5). Statistical analysis were performed at two time points within the trial, midway through (day 8), and at day 16, when all adults had emerged. Chi-square tests at both these time points showed no significant differences in survival between the transgenic varieties (Compa CB and DKC7565) and their respective controls (Brasco and Tiétar). Furthermore, no significant differences in

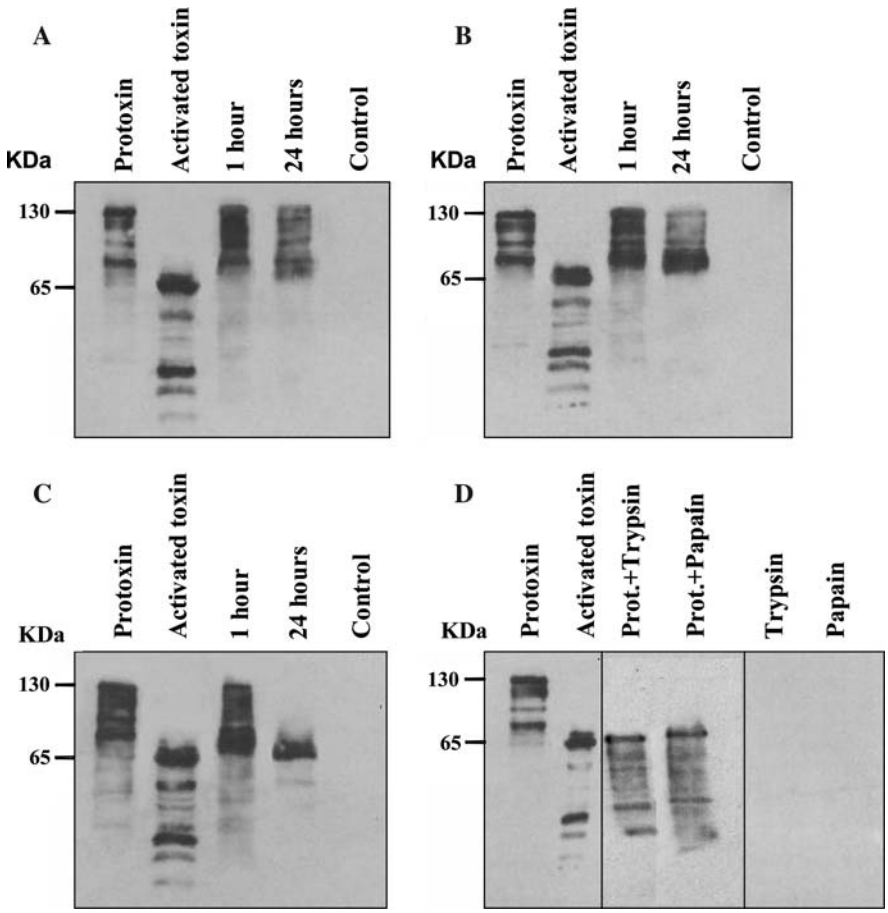

Fig. 3 Digestion of Cry1Ab protoxin in vitro by: (**a**) *T. urticae* at pH 5.0, after 1 and 24 h; (**b**) *S. punctillum* at pH 5.0, after 1 and 24 h; (**c**) *S. punctillum* at pH 10.0, after 1 and 24 h; and (**d**) bovine trypsin and papain after 1 h. Digestion products were visualized by western blotting using antibodies raised against Cry1Ab. Numbers within the gel refer to the estimated molecular mass of protoxin (130 kDa) and activated toxin (65 kDa). Controls are midgut extracts of *S. punctillum* and *T. urticae* without incubation with Cry1Ab protoxin

developmental time of immature stages was found between Compa CB (Bt+, event Bt176) and Brasco (Bt−) (Table 2). However, the duration of the fourth instar was significantly different between DKC7565 (Bt+, event MON810) and Tiétar (Bt−) (Mann–Whitney U-test; $P = 0.04$), being 2.5 and 2.3 days for Bt+ and Bt−, respectively (Table 3). However, there were no significant differences for the developmental time for the other larval instars, nor for time to pupation or time to adult emergence.

Adult *S. punctillum* emerging from the feeding trials were assigned to breeding pairs, and allowed to consume either transgenic-fed or control-fed prey for 14 days. The number of eggs laid per female was used as a measure of relative fecundity. The results showed that the Bt maize had no significant effect on mean cumulative ladybird fecundity for either event (Fig. 6). Females from the Brasco treatment (Bt−) laid a total of 58.2 ± 5.4 eggs, whereas females that had consumed Compa CB (Bt+)-fed prey, laid 53.9 ± 3.7 eggs. Likewise, the total number of eggs laid per female was 59.6 ± 7.0 in the DKC7565 (Bt+) treatment, and 54.6 ± 4.2 in those that had consumed Tiétar (Bt−)-fed prey.

Discussion

All known species of the genus *Stethorus* are predators of spider mites (McMurtry et al. 1970) and *S. punctillum* has been suggested to have potential as a biological control agent of spider mites in agricultural crops (Roy et al. 1999). In spite of being one of the most abundant predators found in maize fields in Spain, to date, no study has evaluated the impact of transgenic Bt maize on this agronomically important ladybird species.

In the present study the presence of Cry1Ab toxin was readily detected in samples of both *T. urticae* and *S. punctillum* collected in a commercial Bt maize

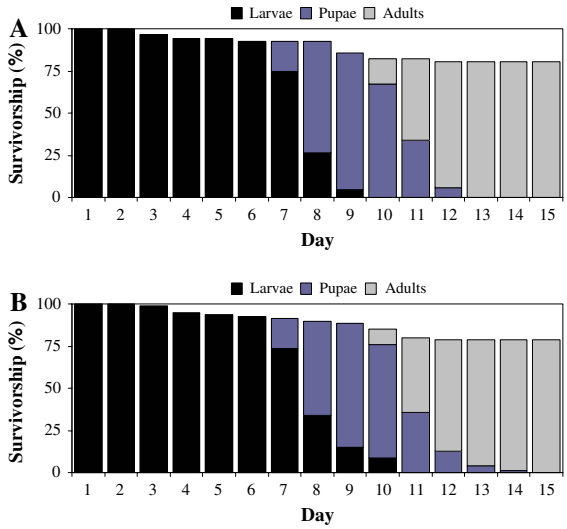

Fig. 4 Effects of transgenic Bt maize (event Bt176) on survival and development of *S. punctillum* from neonate through to adulthood when fed (**a**) Bt maize fed prey and (**b**) control. Survival was compared by Chi-square test. $n = 79$ for Bt+ and 83 for Bt−

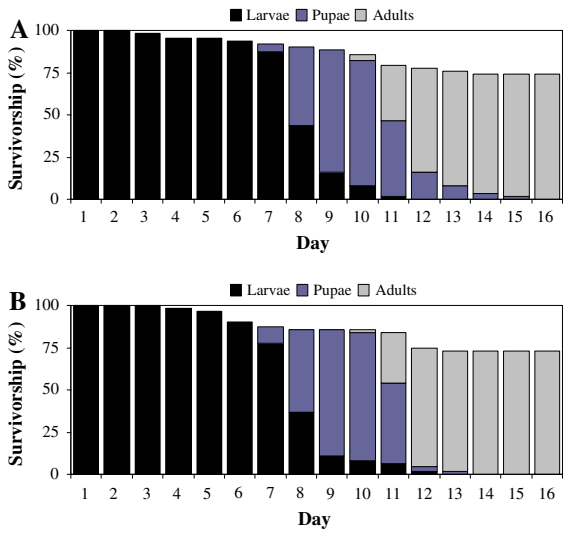

Fig. 5 Effects of transgenic Bt maize (event MON810) on survival and development of *S. punctillum* from neonate through to adulthood when fed (**a**) Bt maize fed prey and (**b**) control. Survival was compared by Chi-square test. $n = 63$ for Bt+ and 61 for Bt−

field (event MON810) located in Central Spain. Obrist et al. (2006a) similarly demonstrated the passage of the toxin to the spider mite and to larvae and adults of this ladybird species in the field (event Bt176). From these studies it was clear that Cry1Ab toxin is transferred through the trophic chain, although the amount of toxin accumulated was reduced from the second to the third trophic level. However, what was not previously known was whether the toxin reaches the midgut of the predator as a truncated toxin (i.e. as it is expressed by the transgenic maize), or whether it has been previously activated or hydrolyzed by digestive proteases present in the prey. It was also unclear as to the role of the proteolytic enzymes of the predator once they come into contact with the Bt protein. Native Cry1 proteins are produced by *B. thuringiensis* as protoxins and thus need to be processed by proteolytic digestion to obtain the active form (Rukmini et al. 2000). This processing of the Cry1Ab protoxin is believed to occur by the removal of a few residues at the N-terminal and removal of a large fragment at the C terminal end, resulting in an active toxin of 60–70 kDa (Schnepf et al. 1998). Since this processing is an essential step for subsequent toxicity of Cry proteins, study of the interaction between proteases and Cry proteins could provide valuable information in risk assessment studies for non-target arthropods.

In target species, trypsin-like and chymotrypsin-like proteases (Díaz-Mendoza et al. 2007; Oppert 1999) are responsible for proteolytic cleavage, and hence activation, of the native toxin; this cleavage is known to occur at both ends of the molecule (Mohan and Gujar 2003). In *T. urticae*, the digestive proteases are predominantly cysteine and aspartyl, whilst serine proteases do not appear to be present in the digestive extracts of this spider mite (Michaud et al. 1996; Nisbet and Billingsley 2000). Despite the potential importance of *S. punctillum* in biological control, no previous studies have been carried out to investigate protein digestion in this species. One of the objectives of the present study was therefore to characterize the proteolytic enzymes present in the predator as a prerequisite to studying the interaction of those enzymes with Cry1Ab toxin. The results demonstrated that *S. punctillum* could readily hydrolyze the general substrate azocasein, with two pH optima, one in the acidic region and the other in the alkaline region, suggesting the presence of proteases of different mechanistic classes. The ability of extracts to hydrolyze specific diagnostic synthetic substrates, the elucidation of the pH at which maximal hydrolysis occurs, and their subsequent sensitivity to a range of protease inhibitors demonstrated that adults

Table 2 Development of *S. punctillum* through the immature stages and time to adult emergence (mean number of days ± SE) when fed either non Bt maize (Control; Brasco, Bt−) or Bt maize (Compa CB, Bt+) fed prey

	L1	L2	L3	L4	Pupae	Larvae-Adult
Bt−	1.7 ± 0.1 a (79)	1.6 ± 0.1 a (79)	1.6 ± 0.1 a (77)	2.3 ± 0.1 a (74)	3.2 ± 0.1 a (64)	10.4 ± 0.1 a (64)
Bt+	1.9 ± 0.1 a (75)	1.5 ± 0.1 a (71)	1.5 ± 0.1 a (71)	2.3 ± 0.1 a (65)	3.3 ± 0.1 a (60)	10.56 ± 0.1 a (60)

Values followed by different letters in the same row represent significant differences ($P < 0.05$; Mann–Whitney U-test)

(n), number of individuals at each developmental stage

Table 3 Development of *S. punctillum* through the immature stages and time to adult emergence (mean number of days ± SE) when fed either non Bt maize (Control; Tiétar, Bt−) or Bt maize (DKC7565, Bt+) fed prey

	L1	L2	L3	L4	Pupae	Larvae-Adult
Bt−	2.0 ± 0.1 a (60)	1.5 ± 0.1 a (56)	1.6 ± 0.1 a (56)	2.5 ± 0.1 a (52)	3.3 ± 0.1 a (47)	10.8 ± 0.2 a (47)
Bt+	1.9 ± 0.1 a (62)	1.6 ± 0.1 a (58)	1.6 ± 0.1 a (55)	2.3 ± 0.1 b (49)	3.4 ± 0.1 a (44)	10.6 ± 0.1 a (44)

Values followed by different letters in the same row represent significant differences ($P < 0.05$; Mann–Whitney U-test)

(n), number of individuals at each developmental stage

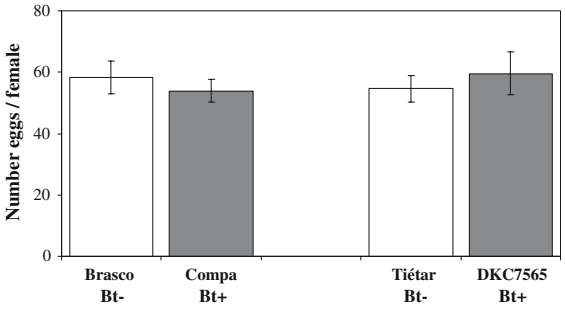

Fig. 6 Effect of transgenic Bt maize ingestion on *S. punctillum* fecundity (mean number of eggs laid by a single female *S. punctillum* adult) when fed either Bt maize fed or control fed prey. Numbers of eggs laid were compared using Mann Whitney U-test. $n = 25$ for the event Bt176 and 23 for the event MON810

predominantly rely on two mechanistic classes of protease, i.e. serine proteases (trypsin-like and chymotrypsin-like) and cysteine proteases (cathepsin B-like), with a minor contribution from aspartyl proteases (cathepsin D-like), for protein digestion. Other ladybirds, such as *Harmonia axyridis* (Ferry et al. 2003), *Adalia bipunctata* (Walker et al. 1998) and *Epilachna varivestis* (Murdock et al. 1987) rely mainly upon cysteine proteases. Such proteases appear to be common in the family of Coccinellidae and very frequent in other members of the Coleoptera (Terra and Ferreira 1994). However, the presence of trypsin-like proteases has not previously been reported in ladybirds.

In vitro digestion studies demonstrated that red spider mite was not able to activate the Cry 1Ab protoxin into its active form, even after 24 h of incubation, the longest incubation time studied. The absence of serine proteases could explain the inability of the prey to activate the native protein. Furthermore, the absence of any digestion products would suggest that the protoxin was resistant to proteolysis by this pest species, at least in vitro. These findings are supported by the report that under laboratory conditions, *T. urticae* ingested large amounts of toxin when feeding on transgenic maize leaves, but that its performance was not negatively affected by the presence of the Bt toxin (Dutton et al. 2002). In feeding bioassays using larvae of the target pest *O. nubilalis*, Obrist et al. (2006b) confirmed that Cry1Ab toxin remains biologically active after ingestion by *T. urticae*. Hence, it would appear that the Cry1Ab toxin is transferred to the next trophic level in its biologically active state. Interestingly, in similar in vitro digestion studies, proteases of *S. punctillum* were also unable to process the native toxin at pH 5.0, where cysteine and aspartyl proteases are active; in contrast, however, the proteases of the predator could process the protoxin at an alkaline pH i.e. under conditions where serine proteases (trypsin-like and chymotrypsin-like) are active. In target species these serine proteases are known to be responsible for the activation of the native toxin (Oppert 1999; Díaz-Mendoza et al. 2007). The

resulting band of about 60–70 kDa was similar to the fragment obtained with commercial bovine trypsin and papain. Activation of Cry1A protoxins is commonly achieved by commercial trypsin. However, it has been reported that other proteases such as papain may also produce the active toxin and smaller polypeptides (Bietlot et al. 1989; Choma et al. 1990), when used at high concentrations. The fact that commercial papain processed the toxin, whereas the potentially active cysteine proteases of both arthropods were not able to cleave it, may be the result of the different evolution of cysteine proteases in plants and arthropods.

In the present study laboratory assays were carried out to evaluate the impact of Bt maize varieties Compa CB (derived from event Bt176 and cultivated in Spain from 1998 to 2005) and DKC6575 (derived from event MON810) on the biology and reproduction of *S. punctillum* via the non-susceptible prey *T. urticae*. Events Bt176 and MON810 are characterized for expressing truncated forms of the Cry1Ab protein; however, the expression of Cry1Ab in leaves is three times higher in event MON810 compared to event Bt176 (EPA 2000). The results from feeding trials with immature stages and adults of the ladybird showed that neither variety caused any negative effects on any of the parameters investigated. Since binding to the midgut is a prerequisite for toxicity of Cry proteins to known target species, all these findings suggest that, although *S. punctillum* is able to process the Cry1Ab protoxin, the predator midgut lacks specific receptors in the brush border membrane of the midgut epithelial cells for the active toxin to bind to. Results from tritrophic studies conducted here are consistent with other laboratory studies that assessed the potential effects of Bt toxins on coccinellids. Cry1Ab toxin expressed in Bt pollen from two transgenic rice lines did not have any negative impact on the performance of larvae and adults of the generalist ladybird *Propylea japonica*, an important predator of insect pests of rice (Bai et al. 2005). Larvae and adults of *Coleomegilla maculata*, a polyphagous predator that is important for suppressing pest populations in corn, were similarly unaffected in a tritrophic system via its herbivorous prey, *Leptinotarsa decemlineata*, previously reared on potato plants expressing the coleopteran specific Cry3A toxin (Riddick and Barbosa 1998). When *C. maculata* consumed Cry3Bb-expressing transgenic maize pollen, no detrimental effects on fitness parameters were observed on either the larvae or pupae of this polyphagous ladybird (Lundgren and Wiedenmann 2002).

Field experiments to determine the impact of transgenic Bt maize on the abundance of larvae and adults of *S. punctillum* corroborate the results obtained under laboratory conditions. De la Poza et al. (2005) carried out a farm-scale study over three consecutive years at two Spanish growing areas (Lleida and Madrid) by comparing the abundance of larvae and adults of *S. punctillum* in transgenic (cv. Compa CB) and non-transgenic plots (its near-isogenic hybrid). The results of the visual surveys did not show any significant difference between treatments. Three years later, and after eight years of continuous cultivation of Bt maize, similar results have been obtained from the same plots in Madrid (unpublished results).

In conclusion, no negative effects of the Bt expressing varieties derived from the events Bt176 and MON810 on the fitness of larvae and adults of *S. punctillum* via *T. urticae* were observed, and thus it would appear highly unlikely that either event would pose any risk to this beneficial predator. Nevertheless, it would be interesting to determine whether Bt maize expressing coleopteran specific Cry proteins, such as event MON863 that produces the Cry3Bb1 toxin and that has been cultivated in the USA since 2003 to control the chrysomelid *Diabrotica virgifera virgifera* (Vaughn et al. 2005), similarly had no deleterious effects on the predator *S. punctillum*.

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