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4 **Effects of *Bacillus thuringiensis*  $\delta$ -endotoxins on**

5 **the pea aphid, *Acyrtosiphon pisum***

6 Running title: **Effects of Cry and Cyt proteins on the pea aphid**

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## ABSTRACT

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Four *Bacillus thuringiensis*  $\delta$ -endotoxins, Cry3A, Cry4Aa, Cry11Aa, and Cyt1Aa, were found to exhibit low to moderate toxicity on the pea aphid, *Acyrtosiphon pisum*, in terms both of mortality and growth rate. Cry1Ab was essentially non-toxic except at high rates. To demonstrate these effects, we had to use exhaustive buffer-based controls.

1  
2       Aphids belong to an important group of ecologically related insects that feed on  
3 plant vascular fluids. Their feeding mechanism makes these insects excellent vectors of  
4 many plant pathogens, especially viruses, yet less amenable to standard, non-systemic,  
5 chemical control by insecticides. Minor effects on survival and fecundity of aphids  
6 reared on Bt crops have been noted in some studies, but not in others (1-3). However,  
7 the sensitivity of aphids to Bt toxins, or lack thereof, has not been previously tested  
8 through artificial diet bioassays with exhaustive buffer-based controls.  
9  
10 Bt  $\delta$ -endotoxins Cyt1A, Cry4A/Cry4B, and Cry11, obtained from three recombinant  
11 strains of *B. thuringiensis* subsp. *israelensis* as well as Cry1Ab and Cry3A, obtained  
12 from recombinant *Escherichia coli*, were purified by ultracentrifugation in a sucrose  
13 discontinuous gradient as described previously (4). Cry proteins were solubilized in  
14 solubilization buffer (50 mM Na<sub>2</sub>CO<sub>3</sub>, 100 mM NaCl, adjusted at pH 10) with DTT  
15 added before use (dithiothreitol, 10 mM). Cyt1A was first solubilized on 10 mM  
16 Na<sub>2</sub>CO<sub>3</sub> (pH 11) buffer and then neutralized at pH 7.5-8 with 10  $\mu$ l HCl 1N. Both  
17 solubilised as well as trypsin-digested aliquots (1:30 over toxin weight) were used at  
18 different concentrations (32  $\mu$ g/ml, 125  $\mu$ g/ml and 500  $\mu$ g/ml) to supplement AP3 aphid  
19 synthetic diet (5) used to feed *Acyrtosiphon pisum* (LL01 green clone). Ampicillin  
20 (100  $\mu$ g/ml), an ineffective antibiotic for *A. pisum* or its obligate symbiont *Buchnera*,  
21 was added to the medium to avoid bacterial growth. For each concentration (10  
22 nymphs/box and 3 repetitions) thirty nymphs were bioassayed at 20 °C, and under a  
23 16:8 (light:dark) photoperiod. Survival time was calculated from aphid deposition on  
24 test diet (day 0). Mortality was surveyed daily and body weight noted at day 7 in  
25 survivors. ST<sub>50</sub> (median survival time after challenge) was calculated by using an

1 actuarial survival analysis (Statview) with censoring values of survivors at the end of  
2 experiment.

3

4 All Cry  $\delta$ -endotoxins tested produced mortality in *Acyrtosiphon pisum*, and  
5 retarded growth in survivors (Figures 1 and 2). Mortalities ranged from only 25%  
6 (Cry1Ab) to 100% (Cry4 and Cry11) after 3 to 6 days of exposure to 500  $\mu\text{g/ml}$  of  
7 solubilized protein (Figure 1). Trypsin activation enhanced toxicity, particularly for  
8 Cry4, since activation at the intermediate concentration tested (125  $\mu\text{g/ml}$ ) resulted in a  
9 two-fold increase in mortality (Figure 1D). Median survival times ( $\text{ST}_{50\text{s}}$ ) were  
10 calculated for both solubilized protoxins and activated Cry3A, Cry4 and Cry11. The  
11  $\text{ST}_{50\text{s}}$  values (at 500  $\mu\text{g/ml}$ ) ranged from  $1.8 \pm 0.14$  days for solubilized Cry4 and  
12 Cry11, to  $3.7 \pm 1.2$  days for the trypsin-activated Cry3A (Table1). Control aphids fed  
13 buffer all survived for longer than eight days. The  $\text{LC}_{50\text{s}}$  for trypsinized Cry1Ab and  
14 Cry4, respectively, the least and the most toxic proteins, were calculated after seven  
15 days. The  $\text{LC}_{50}$  for Cry1Ab was estimated to be  $>800 \mu\text{g/ml}$  and that of Cry4, 70-100  
16  $\mu\text{g/ml}$  (data not shown).

17 Aphids that survived ingestion of Cry and Cyt proteins in the bioassays showed  
18 a marked reduction in growth rates compared to those of the control group (Figure 2).  
19 Growth inhibition of each Cry protein correlated with mortality. Cry4 inhibited growth  
20 the most (Fig. 2A), whereas Cry1Ab inhibited growth the least (Fig. 2B). The  
21 concentration resulting in a 50% decrease in mean body weight ( $\text{IC}_{50}$ ) was calculated  
22 for Cry1Ab as well as for Cry4. The  $\text{IC}_{50}$  for Cry1Ab was calculated to be  $>800 \mu\text{g/ml}$   
23 and that of Cry4 about 135  $\mu\text{g/ml}$ . Growth of aphids surviving Cyt1A ingestion was  
24 strongly inhibited, with an average weight at the end of the assay, for doses of 125  
25  $\mu\text{g/ml}$  or higher, corresponding to less than 40% of that of the control group (Fig. 2B).  
26 This decrease in aphid weight associated with the ingestion of Cyt1A is in contrast to

1 the low mortality (about 10%) produced by the same dose of this protein. Most  
2 surviving insects did not reach adulthood under Cyt1A intoxication, while control  
3 insects completed their nymphal development at the end of the bioassay.

4 There are two previous studies (6, 7), reporting the sensitivity of another aphid,  
5 *Macrosiphum euphorbiae*, suspensions of Cry2, Cry3A and Cry4 crystals, but not to the  
6 solubilized endotoxins. This may be explained by the lack of complete solubilization of  
7 the Bt crystals (6), and because control groups were fed water-based artificial diet  
8 instead of diets containing the buffer used to solubilize the crystals. Our bioassays,  
9 performed with buffer-based controls show that *A. pisum* is indeed sensitive to Bt  $\delta$ -  
10 endotoxins, although to a low extent. In fact, LC<sub>50</sub> we calculated are very high  
11 compared to those of highly susceptible targets of Bt  
12 (<http://www.glfrc.forestry.ca/bacillus/>). This low activity of Bt endotoxins on aphids  
13 suggests that these proteins have not evolved to kill aphids. In fact, the ecological  
14 niches of Bt and these insects are very different and it is unlikely that aphids, feeding on  
15 a virtually germ-free environment such as plant phloem, come in contact with bacteria  
16 living either in other susceptible insects or on the plant surface. It might be  
17 hypothesized that the sensitivity of pea aphid to these Bt endotoxins is a consequence of  
18 similarities among midgut microvillar proteins and lipids, especially the surface  
19 molecules that compose the sugar residues known to serve as the initial binding sites for  
20 Bt toxins (8), rather than as a result of direct selection for aphid sensitivity.

21 The low susceptibility of aphids to Bt toxins is not in contrast with reports on  
22 lack of deleterious effects of GM crops on aphid population. A recent report confirms  
23 the presence of Cry1Ac in the phloem of transgenic oilseed rape, and in aphids feeding  
24 on these plants (9). But the concentration of Cry1Ac in phloem, being low, is  
25 compatible with the absence of deleterious effects of transgenic oilseed rape on aphids,  
26 as well as with previous studies reporting no detectable levels of Cry toxins in phloem

1 translocated through sieves of commercial transgenic plants (10). The susceptibility of  
2 aphids to Bt we report here could theoretically lead to the development of effective  
3 strategies for controlling these and other sucking insect pests with GM crops expressing  
4 appropriate toxins. However, two conditions should concur: (i) toxins must be present  
5 in the plant phloem to be accessible to these pests and vectors and (ii) more effective  
6 toxins should be found, and thus screening programs with a range of natural and  
7 engineered toxins should be performed in order to determine their activity on sucking  
8 insects. Although a wide range of further studies are still needed to assess the potential  
9 of Bt crops for controlling aphids and other sucking insect pests, the substantial  
10 economic losses sucking insects cause to agriculture worldwide clearly merit  
11 exploration of the possibilities our results suggest.

12 .....  
13 Manuel Porcar has a Ramón y Cajal research contract from the Spanish Ministerio de  
14 Educación y Ciencia.

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## FIGURE LEGENDS

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FIGURE 1. Mortality assays over the nymphal life of the pea aphid, *Acyrtosiphon pisum*, upon ingestion of artificial diets containing purified toxins of *Bacillus thuringiensis*, after either solubilization (dotted lines) or solubilization and trypsin activation (solid lines). The toxins used were Cry1Ab (circles), Cry3A (squares), a mixture of Cry4A and Cry4B (losanges), and Cry11A (triangles). Soluble toxin doses vary from low 32  $\mu\text{g/ml}$  (blue) to 125  $\mu\text{g/ml}$  (violet) and high 500  $\mu\text{g/ml}$  (red). Assays were carried out on 30 initial neonate insects in 3 batches of 10 individuals.

FIGURE 2. Growth inhibition assays with purified *B. thuringiensis* toxins Cry3A, Cry4, and Cry 11 (A), and Cry1Ab and Cyt1A (B) on the pea aphid *A. pisum*. Toxins were added to the diet either after solubilization (dotted lines) or after solubilization and trypsin activation (solid lines). Error bars are shown as SE of individual weights at day 7 of experiments, standardized by control group mean weight (toxin dose = 0; initial numbers n=30). Color coding of toxins: Cry1Ab (circles, green), Cry3A (squares, red), Cry4A and Cry4B mixture (losanges, violet) and Cry11A (triangles, blue). In this experiment with Cry1Ab (B), the toxin was HPLC-purified, and activated toxin provided as a salt-free lyophilisate by W. Moar (Auburn University, Alabama, USA).

1 TABLE 1. Mean survival times in days ( $\pm$  SE) of pea aphids feeding on solubilized Cry  
 2 toxins and solubilized Cry toxins activated with trypsin.

3

Toxin		$\Delta$ οσε ( $\mu$ g / ml)		
		32	125	500
Cry1Ab	<i>Sol.</i>	nl	> 8	> 8
	<i>Tryp.</i>	nl	> 8	> 8
Cry3A	<i>Sol.</i>	nl	> 8	> 8
	<i>Tryp.</i>	nl	> 8	3.7 $\pm$ 1.2
Cry4A	<i>Sol.</i>	nl	> 8	1.8 $\pm$ 0.14
	<i>Tryp.</i>	> 8	1.8 $\pm$ 0.15	1.9 $\pm$ 0.17
Cry11A	<i>Sol.</i>	nl	> 8	1.8 $\pm$ 0.14
	<i>Tryp.</i>	nl	> 8	2.5 $\pm$ 0.10

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5 nl = non-lethal; > 8 = survival longer than 8 days.

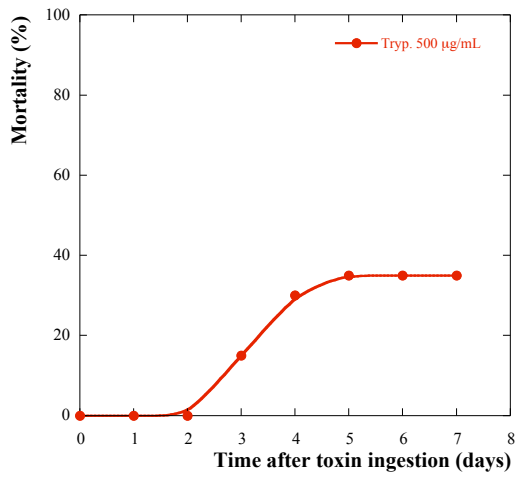
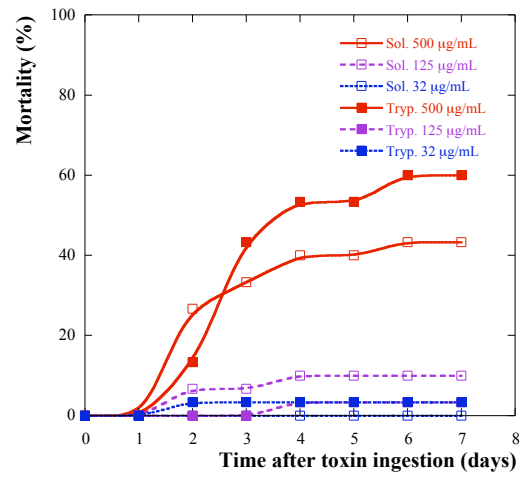
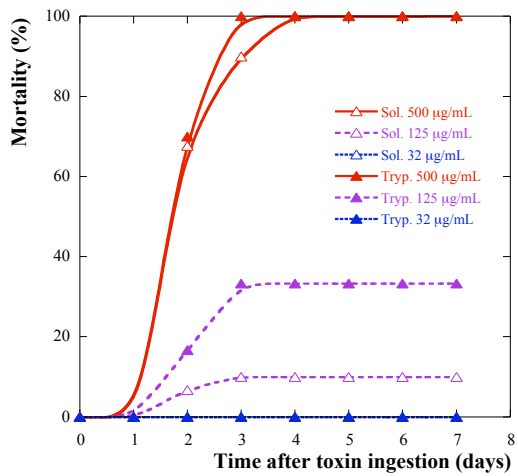
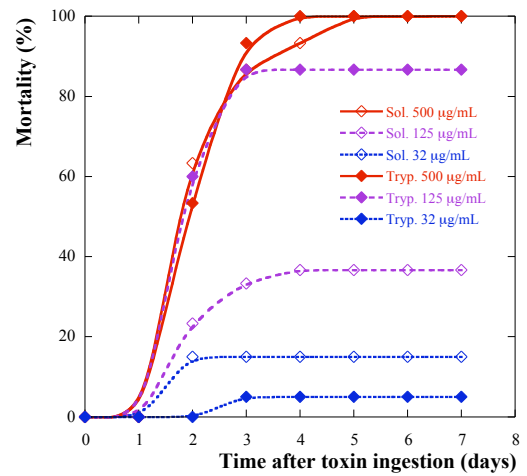
**Cry1Ab****Cry3****Cry11****Cry4**

Figure 1

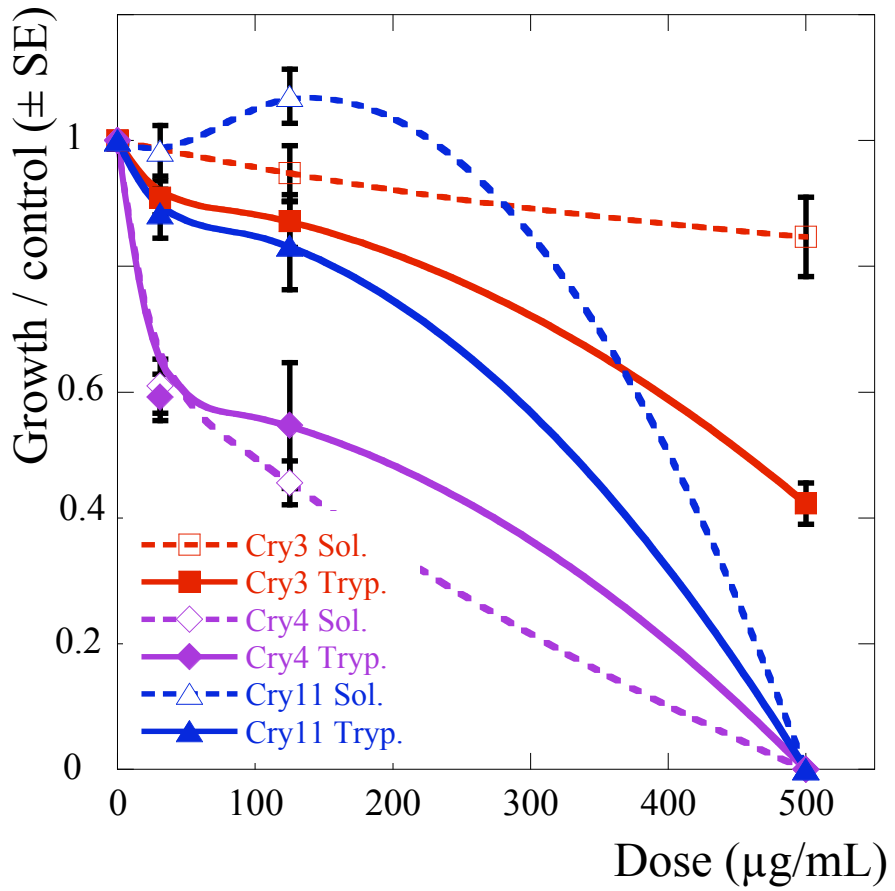


Figure 2a

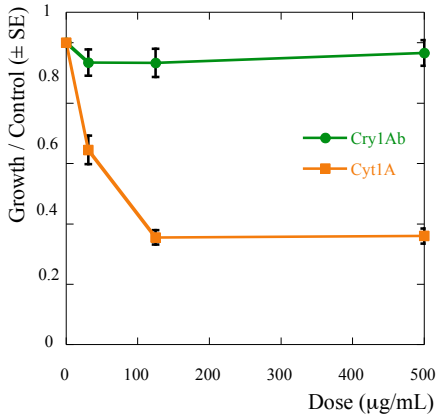


Figure 2a