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[Short-form communication]

Effects of *Bacillus thuringiensis* ∂-endotoxins on the pea aphid, *Acyrthosiphon pisum*

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

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ABSTRACT

Four *Bacillus thuringiensis* ∂-endotoxins, Cry3A, Cry4Aa, Cry11Aa, and Cyt1Aa, were found to exhibit low to moderate toxicity on the pea aphid, *Acyrthosiphon 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.

Aphids belong to an important group of ecologically related insects that feed on plant vascular fluids. Their feeding mechanism makes these insects excellent vectors of many plant pathogens, especially viruses, yet less amenable to standard, non-systemic, chemical control by insecticides. Minor effects on survival and fecundity of aphids reared on Bt crops have been noted in some studies, but not in others (1-3). However, the sensitivity of aphids to Bt toxins, or lack thereof, has not been previously tested through artificial diet bioassays with exhaustive buffer-based controls.

Bt ∂-endotoxins Cyt1A, Cry4A/Cry4B, and Cry11, obtained from three recombinant strains of *B. thuringiensis* subsp. *israelensis* as well as Cry1Ab and Cry3A, obtained from recombinant *Escherichia coli,* were purified by ultracentrifugation in a sucrose discontinuous gradient as described previously (4). Cry proteins were solubilized in solubilization buffer (50 mM Na_2CO_3, 100 mM NaCl, adjusted at pH 10) with DTT added before use (dithiothreitol, 10 mM). Cyt1A was first solubilized on 10 mM Na_2CO_3 (pH 11) buffer and then neutralized at pH 7.5-8 with 10 µl HCl 1N. Both solubilised as well as trypsin-digested aliquots (1:30 over toxin weight) were used at different concentrations (32 µg/ml, 125 µg/ml and 500 µg/ml) to supplement AP3 aphid synthetic diet (5) used to feed *Acyrthosiphon pisum* (LL01 green clone). Ampicillin (100 µg/ml), an ineffective antibiotic for *A. pisum* or its obligate symbiont *Buchnera*, was added to the medium to avoid bacterial growth. For each concentration (10 nymphs/box and 3 repetitions) thirty nymphs were bioassayed at 20 °C, and under a 16:8 (light:dark) photoperiod. Survival time was calculated from aphid deposition on test diet (day 0). Mortality was surveyed daily and body weight noted at day 7 in survivors. ST_{50} (median survival time after challenge) was calculated by using an

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

All Cry ∂-endotoxins tested produced mortality in *Acyrthosiphon pisum*, and retarded growth in survivors (Figures 1 and 2). Mortalities ranged from only 25% (Cry1Ab) to 100% (Cry4 and Cry11) after 3 to 6 days of exposure to 500 µg/ml of solubilized protein (Figure 1). Trypsin activation enhanced toxicity, particularly for Cry4, since activation at the intermediate concentration tested (125 µg/ml) resulted in a two-fold increase in mortality (Figure 1D). Median survival times (ST_{50}s) were calculated for both solubilized protoxins and activated Cry3A, Cry4 and Cry11. The ST_{50}s values (at 500 µg/ml) ranged from 1.8 ± 0.14 days for solubilized Cry4 and Cry11, to 3.7 ± 1.2 days for the trypsin-activated Cry3A (Table1). Control aphids fed buffer all survived for longer than eight days. The LC_{50}s for trypsinized Cry1Ab and Cry4, respectively, the least and the most toxic proteins, were calculated after seven days. The LC_{50} for Cry1Ab was estimated to be >800 µg/ml and that of Cry4, 70-100 µg/ml (data not shown).

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

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

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

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

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

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FIGURE LEGENDS

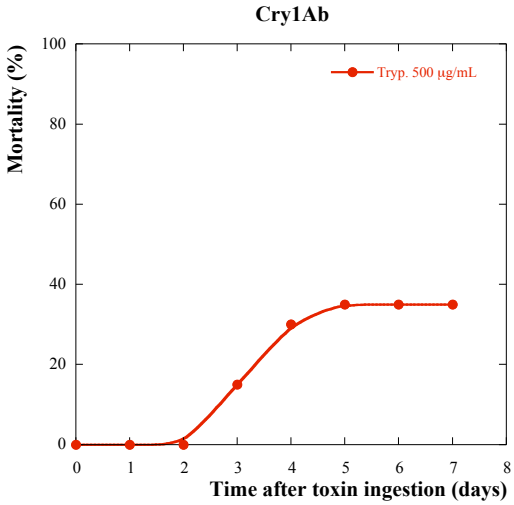
FIGURE 1**.** Mortality assays over the nymphal life of the pea aphid, *Acyrthosiphon pisum*, upon ingestion of artificial diets containing purified toxins of *Bacillus thuringiensis*, after either solubilization (dotted lines) or solubilization and trypsin activation (solid lines). The toxins used were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

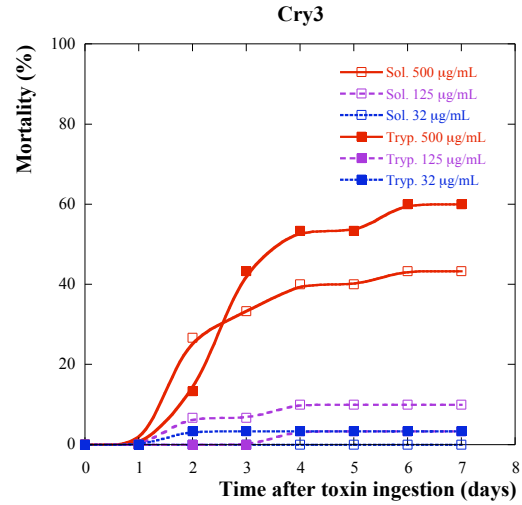
FIGURE 2. Growth inhibition assays with purified *B. thuringiensis* toxins Cry3A, Cry4, and Cry 11 (A), and Cry1Ab and Cyt1A (B) on the pea aphid *A. pisum*. Toxins were added to the diet either after solubilization (dotted lines) or after solubilization and trypsin activation (solid lines). Error bars 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).

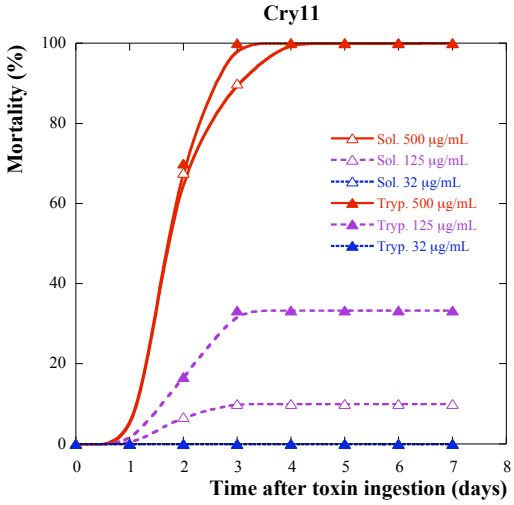
TABLE 1. Mean survival times in days (± SE) of pea aphids feeding on solubilized Cry toxins and solubilized Cry toxins activated with trypsin.

		Δοσε (μg / ml)		
Toxin		32	125	500
Cry1Ab	*Sol.*	nl	> 8	> 8
	Tryp.	nl	> 8	> 8
Cry3A	*Sol.*	nl	> 8	> 8
	Tryp.	nl	> 8	3.7 ± 1.2
Cry4A	*Sol.*	nl	> 8	1.8 ± 0.14
	Tryp.	> 8	1.8 ± 0.15	1.9 ± 0.17
Cry11A	*Sol.*	nl	> 8	1.8 ± 0.14
	Tryp.	nl	> 8	2.5 ± 0.10

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


Cry1Ab
Mortality (%)
Time after toxin ingestion (days)
Tryp. 500 µg/mL


Cry3
Mortality (%)
Time after toxin ingestion (days)
Sol. 500 µg/mL
Sol. 125 µg/mL
Sol. 32 µg/mL
Tryp. 500 µg/mL
Tryp. 125 µg/mL
Tryp. 32 µg/mL


Cry11
Mortality (%)
Time after toxin ingestion (days)
Sol. 500 µg/mL
Sol. 125 µg/mL
Sol. 32 µg/mL
Tryp. 500 µg/mL
Tryp. 125 µg/mL
Tryp. 32 µg/mL

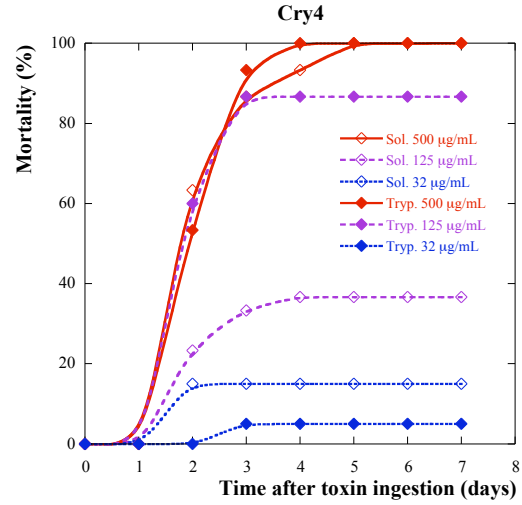

Cry4
Mortality (%)
Time after toxin ingestion (days)
Sol. 500 µg/mL
Sol. 125 µg/mL
Sol. 32 µg/mL
Tryp. 500 µg/mL
Tryp. 125 µg/mL
Tryp. 32 µg/mL

Figure 1

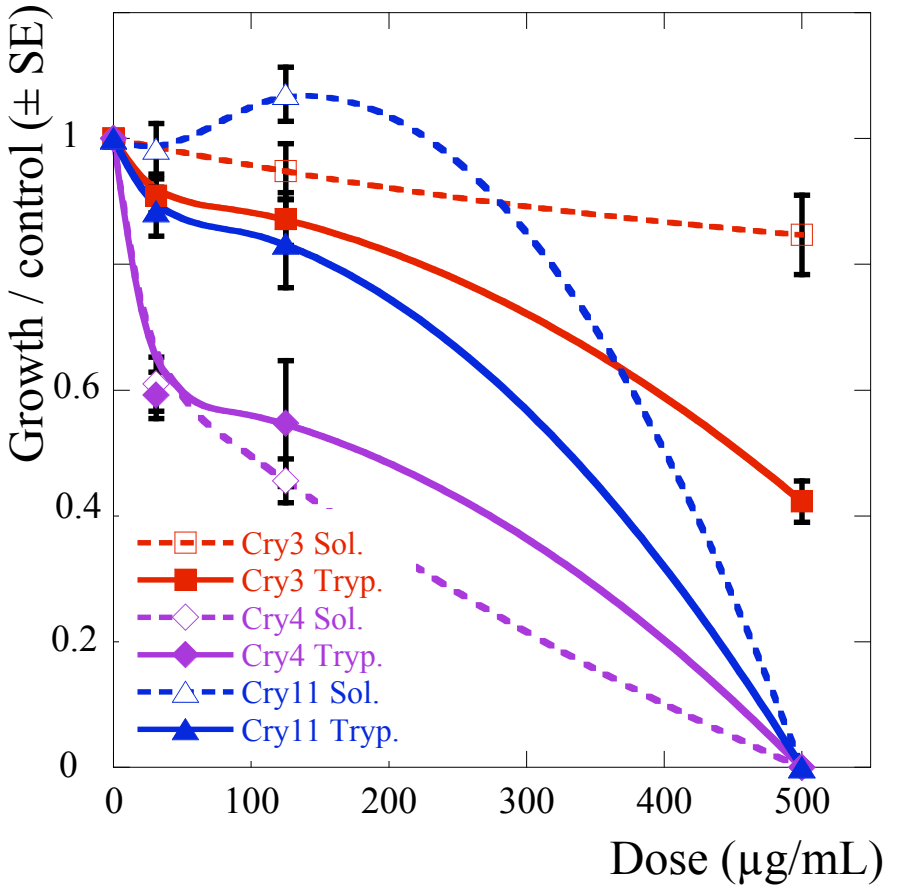

Growth / control (± SE)
1
0.6
0.4
0.2
0
0
100
200
300
400
500
Dose (µg/mL)
Cry3 Sol.
Cry3 Tryp.
Cry4 Sol.
Cry4 Tryp.
Cry11 Sol.
Cry11 Tryp.

Figure 2a

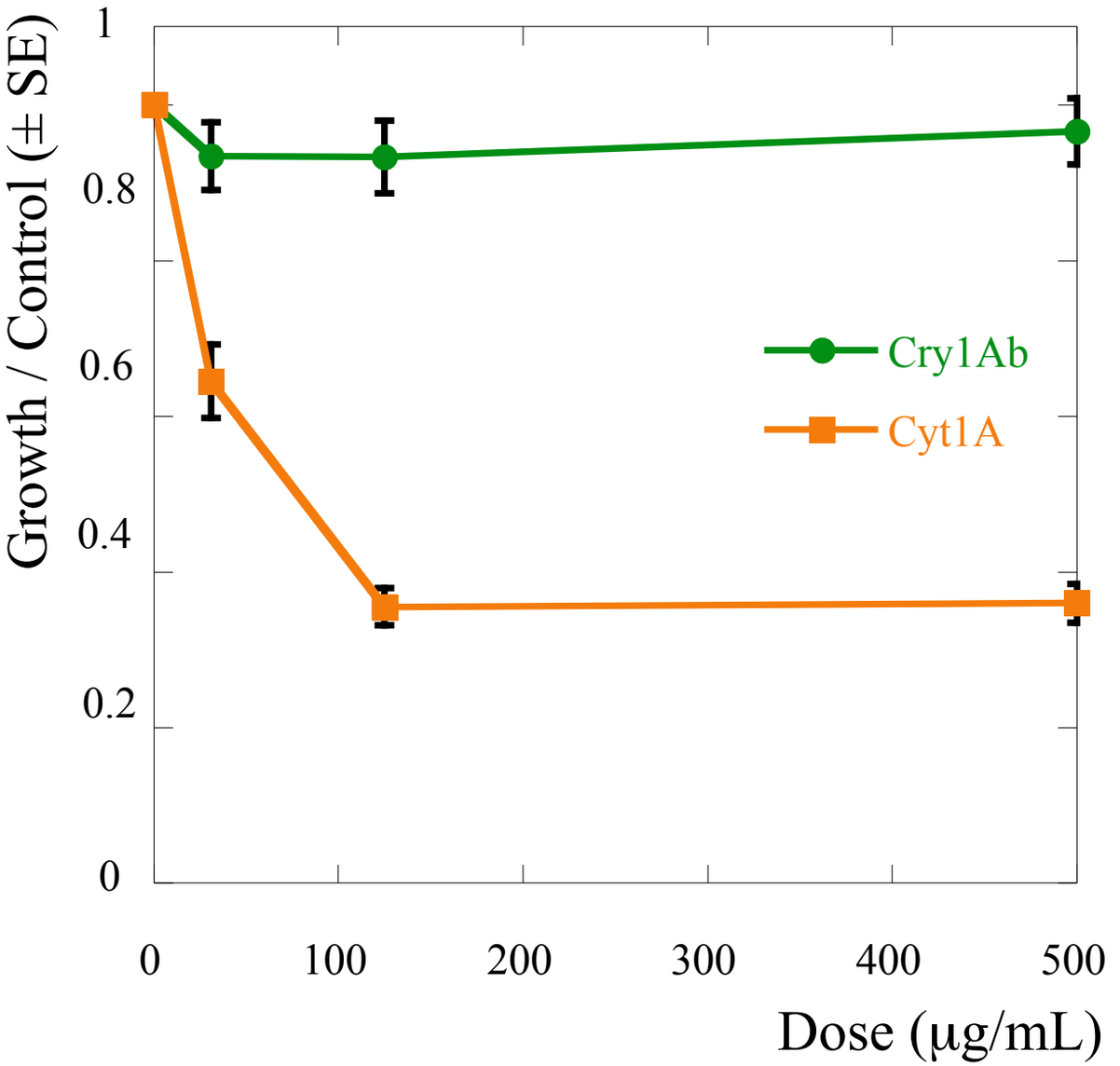

Growth / Control (± SE)
1
0.8
0.6
0.4
0.2
0
0
100
200
300
400
500
Dose (µg/mL)
Cry1Ab
Cyt1A

Figure 2a