

## Effectiveness of *Bacillus thuringiensis*-Transgenic Chickpeas and the Entomopathogenic Fungus *Metarhizium anisopliae* in Controlling *Helicoverpa armigera* (Lepidoptera: Noctuidae)<sup>∇</sup>

N. C. Lawo,<sup>1,4</sup> R. J. Mahon,<sup>2</sup> R. J. Milner,<sup>2</sup> B. K. Sarmah,<sup>3</sup> T. J. V. Higgins,<sup>4</sup> and J. Romeis<sup>1\*</sup>

Agroscope Reckenholz-Tänikon Research Station ART, Reckenholzstr. 191, 8046 Zurich, Switzerland<sup>1</sup>; CSIRO Entomology, GPO Box 1700, Canberra, ACT 2601, Australia<sup>2</sup>; Department of Agricultural Biotechnology, Assam Agricultural University, Jorhat 785 013, India<sup>3</sup>; and CSIRO Plant Industry, GPO Box 1600, Canberra, ACT 2601, Australia<sup>4</sup>

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The use of genetically modified (Bt) crops expressing lepidopteran-specific Cry proteins derived from the soil bacterium *Bacillus thuringiensis* is an effective method to control the polyphagous pest *Helicoverpa armigera*. As *H. armigera* potentially develops resistance to Cry proteins, Bt crops should be regarded as one tool in integrated pest management. Therefore, they should be compatible with biological control. Bioassays were conducted to understand the interactions between a Cry2Aa-expressing chickpea line, either a susceptible or a Cry2A-resistant *H. armigera* strain, and the entomopathogenic fungus *Metarhizium anisopliae*. In a first concentration-response assay, Cry2A-resistant larvae were more tolerant of *M. anisopliae* than susceptible larvae, while in a second bioassay, the fungus caused similar mortalities in the two strains fed control chickpea leaves. Thus, resistance to Cry2A did not cause any fitness costs that became visible as increased susceptibility to the fungus. On Bt chickpea leaves, susceptible *H. armigera* larvae were more sensitive to *M. anisopliae* than on control leaves. It appeared that sublethal damage induced by the *B. thuringiensis* toxin enhanced the effectiveness of *M. anisopliae*. For Cry2A-resistant larvae, the mortalities caused by the fungus were similar when they were fed either food source. To examine which strain would be more likely to be exposed to the fungus, their movements on control and Bt chickpea plants were compared. Movement did not appear to differ among larvae on Bt or conventional chickpeas, as indicated by the number of leaflets damaged per leaf. The findings suggest that Bt chickpeas and *M. anisopliae* are compatible to control *H. armigera*.

*Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) is one of the most important insect pests in the Old World due to its mobility, high polyphagy, short generation duration, and high reproductive rate (11, 45). Currently, the application of chemical spray insecticides is the most common method of controlling this pest on crops, including cotton (7, 22) and chickpea (44, 46). *H. armigera* is known to develop resistance to almost all the insecticides used for its control (14, 23). These chemical sprays are also of environmental concern and are responsible for human health problems (34, 35). Thus, alternative control methods are increasingly being employed. The use of genetically modified (GM) crops that express insecticidal genes, such as those derived from the soil bacterium *Bacillus thuringiensis* (Bt crops), provide a powerful option to control pest Lepidoptera (48). This technology, for example, is applied to protect cotton plants by the expression of *B. thuringiensis* cry genes, i.e., *cry1Ac* and *cry2Ab*, either alone or in combination, from damage by the budworm/bollworm complex [*Helicoverpa/Heliopsis* spp. and *Pectinophora gossypiella* (Saunders)]. These *B. thuringiensis*-transgenic cotton plants are highly resistant to damage by lepidopteran pests, and consequently, the application of chemical insecticides has been greatly reduced (12, 32). This makes Bt cotton a valuable

component of integrated pest management programs, with many environmental, economic, and health benefits (34, 35).

As with cotton, the expression of *B. thuringiensis* cry genes is an option to protect chickpeas from damage by *H. armigera* (41). Chickpea plants that express either Cry1Ac or Cry2Aa, or both proteins, are currently under development and could become commercially available in the future (28, 43).

The deployment of insect-resistant GM plants poses two potential problems. First, the target pest may develop resistance to the expressed insecticidal protein(s) due to the strong and continued selection pressure imposed on the insect populations (16, 48, 49). This is particularly the case for *H. armigera*, for which populations resistant to single Cry proteins have been selected in the laboratory (9). To manage insect resistance development, the use of high-dose-expressing Bt plants, along with an adjacent refuge of non-Bt plants, is considered to be the most effective strategy (9, 49). Most resistance alleles are recessive, and the frequency of such alleles in pest populations is generally very low before resistance becomes evident (9). However, recently, a relatively high baseline frequency of resistance alleles for Cry2Ab (0.0033) has been reported in an Australian *H. armigera* population prior to the widespread adoption of Bollgard II (Monsanto Company, St. Louis, MO) cotton, which expresses this protein in combination (pyramided) with Cry1Ac (26, 27).

The second area of concern is the possible effect of insect-resistant GM crops on nontarget organisms, especially those that provide important ecological services, such as biological control (39, 42). These organisms are important, since they

\* Corresponding author. Mailing address: Agroscope Reckenholz-Tänikon Research Station ART, Reckenholzstr. 191, 8046 Zurich, Switzerland. Phone: 41-44-377 72 99. Fax: 41-44-377 72 01. E-mail: joerg.romeis@art.admin.ch.

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help to keep other herbivores that are not affected by the insecticidal GM protein under their economic thresholds, but also because they potentially help to kill target insects that have developed resistance against the GM trait. Biological control of arthropods is thus considered during the environmental-risk assessment of insecticidal GM crops (42), and a great deal of research has been conducted to assess the impact of *B. thuringiensis*-transgenic crops on arthropod predators and parasitoids (40). Overall, studies have not revealed any direct effects of the *B. thuringiensis* Cry proteins on natural enemies. In contrast to arthropod natural enemies, insect pathogens have received little attention. This needs to be addressed, since it is known that the activity of entomopathogens is affected by host plant resistance factors. Hare (19) has compiled a comprehensive literature review of the interactions between host plants, herbivores, and pathogens. Additive effects of these interactions were reported in over half of the published studies, while approximately one-third reported synergistic effects. However, little attention has been given to the interactions of Bt plants with entomopathogenic fungi, despite evidence that *H. armigera* is attacked by a variety of entomopathogens, such as *Nomuraea rileyi*, *Beauveria bassiana*, and *Metarhizium anisopliae* (18). Since the application of *M. anisopliae* is a promising method to control *H. armigera* in India (29, 30, 31), it was selected for our investigations.

To evaluate the complementarity of a pathogen and a Bt plant for insect pest control, we studied the interaction of the entomopathogenic fungus *M. anisopliae*, a susceptible and a Cry2A-resistant strain of *H. armigera*, and Cry2Aa-expressing chickpea plants (transformation line BS 5A). Previous bioassays have shown that this line caused approximately 36% mortality among neonate *H. armigera* larvae (S. Acharjee, B. K. Sarmah, P. A. Kumar, K. M. Olsen, R. J. Mahon, W. J. Moar, A. Moore, and T. J. V. Higgins, unpublished data). This low-cry2Aa-expressing line was chosen in order to examine the combined effects of *M. anisopliae* and Bt chickpea plants on the susceptible *H. armigera* strain. To determine the responses of susceptible and Cry2A-resistant *H. armigera* larvae to *M. anisopliae* on control (non-Bt) plants, a concentration-response curve was established. Using this knowledge, we examined whether the fungus could complement the mortality induced by the toxin in the Bt plant in laboratory and greenhouse studies. Subsequently, we evaluated the feeding behavior of susceptible and Cry2A-resistant *H. armigera* larvae when exposed to control and Bt chickpeas.

#### MATERIALS AND METHODS

**Plants.** *B. thuringiensis*-transgenic chickpea (*Cicer arietinum* var. Desi) plants (transformation line BS 5A; families, 5A.6.14.1, 5A.6.17.3, and 5A.6.17.4), (Acharjee et al., unpublished) expressing a full-length Cry2Aa toxin (Bt plants) and the corresponding nontransformed near-isoline cv. Semsen (control plants) were used in the bioassays. Seeds were germinated in a climate chamber at 24°C ± 1°C before being planted. The plants were grown individually in sandy soil (70% compost, 15% sand, 15% perlite) in plastic pots (15 cm in diameter; 14 cm high) at a temperature of 25 to 32°C during the day and 15 to 20°C at night with an ~12-h photoperiod in the greenhouse. Plants 5 to 8 weeks old were used in all bioassays.

**Expression of Cry2Aa protein in plants.** To ensure that presumptive transgenic chickpea plants were expressing the Cry2Aa protein, Western blot analyses were conducted on a total of 152 plants.

In the laboratory bioassay examining the complementarity of Bt chickpeas and *M. anisopliae*, 47 presumptive transgenic plants of the families used in the

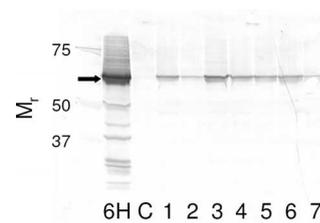


FIG. 1. Western blot of chickpea leaf proteins (40  $\mu$ g per lane). Lane 6H contained protein from a high-expressing line that caused 98% mortality in *H. armigera* larvae, lane C contained protein from a nontransgenic chickpea leaf, and lanes 1 to 7 contained proteins from individual Bt chickpea plants used in the bioassays. The numbers on the y axis ( $M_r$ ) refer to the relative molecular masses of markers ( $10^3$ ). The arrow indicates the position of full-length Cry2Aa protein.

bioassay were analyzed. In 6% of the plants, no Cry2Aa protein could be detected. Because of the presence of nonexpressors, all transgenic plants were tested before use in subsequent bioassays. A two- to threefold variation in expression of the Cry2Aa toxin among expressors was also observed (Fig. 1).

Leaves of untransformed chickpea plants were used as a negative control, while a high-expressing line, BS 6H, which caused 98% mortality in *H. armigera* larvae (Acharjee et al., unpublished) was used as a positive control. Protein was extracted from 80 to 100 mg of a young, fully expanded leaf into 400  $\mu$ l of extraction buffer {0.1 M TES (*N*-tris[hydroxymethyl]methyl-2-aminoethane-sulfonic acid) (Sigma catalog no. T-5691) pH 7.6, 0.2 M NaCl, 1 mM PMSF, 1 mM EDTA}. The suspension was centrifuged at 13,000 rpm for 5 min, and the resultant supernatant was used for protein determination (6). Forty micrograms of protein (with disulfide bonds reduced in the presence of  $\beta$ -mercaptoethanol) from each sample was separated by size fractionation in a NuPage precast 10% Bis-Tris polyacrylamide gel system (Invitrogen catalog no. NP0315), using a MOPS (morpholinepropanesulfonic acid)-sodium dodecyl sulfate running buffer (50 mM MOPS, 50 mM Tris base, 3.5 mM sodium dodecyl sulfate, 1 mM EDTA). The protein was transferred electrophoretically to a nitrocellulose membrane (200 mA for 90 min) using transfer buffer (25 mM Bicine, 25 mM Bis-Tris, 1 mM EDTA, 10% methanol). The nitrocellulose membrane was blocked in Tris-buffered saline solution (20 mM Tris, pH 7.5, 0.5 M NaCl) and 5% skim milk powder for 1 h. The membrane was washed in Tris-buffered saline solution and 0.1% Tween 20 (TTBS). The primary anti-Cry2Aa antibody, raised in rabbit, was diluted in TTBS and incubated with the membrane for 1 h before being washed briefly with TTBS. The secondary antibody, anti-rabbit immunoglobulin G (Fc)-alkaline phosphatase conjugate (Promega catalog no. S3731), was diluted in TTBS and incubated with the membrane for 1 h before being washed briefly in TTBS. Cry2Aa protein bands were detected by the addition of BCIP (5-bromo-4-chloro-3-indolylphosphate)/Nitro Blue Tetrazolium substrate (Sigma catalog no. B5655).

**Insect material.** Strains of susceptible and Cry2A-resistant *H. armigera* were provided by CSIRO Entomology, Canberra, Australia. The Cry2A-resistant strain (SP15) was established from a single *H. armigera* pair collected as eggs on corn near Griffith, NSW (27). To maintain fitness vigor, the Cry2A-resistant strain was outcrossed with a susceptible strain (GR). After three outcrosses to the susceptible strain, the Cry2A-resistant colony was genetically very similar (87% isogenic) to the susceptible strain (with the exception of the linkage group containing the gene conferring resistance) (27).

Larvae were reared as described by Teakle and Jensen (50), except that three or four neonates were kept in each well (3 cm by 3 cm by 2 cm) of a 32-well plastic tray (Oliver Products Company, Grand Rapids, MI) until they reached the third instar. Subsequently, the larvae were separated and kept in individual wells on a fresh diet. Larvae used to evaluate the sensitivity of *H. armigera* to *M. anisopliae* were treated as described above. For bioassays involving Bt chickpea plants, ~50 neonates were reared in plastic boxes (12 cm in diameter; 6 cm high) on control chickpea leaves (10 to 12 weeks old) until they reached the third instar. Two pieces (ca. 5 ml) of 2% agar were included in the box to raise the humidity in order to limit leaf desiccation. The larvae were refed after 2 or 3 days if necessary. Early-third-instar larvae were used in all bioassays. Adults were housed as described by Mahon et al. (26).

**Fungus.** *M. anisopliae* var. *anisopliae* (FI-1248) from the CSIRO Insect Pathogen Culture collection was used in the experiments. The strain was originally isolated from a termite, *Mastotermes darwiniensis*, collected near Darwin, Northern Territory, Australia, in 1997. *M. anisopliae* was grown on Oxoid Sabouraud's

dextrose agar plus 1% yeast extract for 3 weeks at  $\sim 24^{\circ}\text{C}$  under natural daylight conditions. Spores were harvested by scraping them from the agar surface using a loop and stored at  $4^{\circ}\text{C}$  until they were used. Clumps of spores were then dispersed in 0.5% Tween 80 using a magnetic stirrer for 1 h. The concentration of conidia was estimated using a Petroff-Hausser counting chamber (Hausser Scientific Partnership, Horsham, PA; 1/400 mm<sup>2</sup>; 0.02 mm deep). The initial suspension was serially diluted with 0.5% Tween 80 to the concentrations used in the experiments.

Prior to each bioassay, a sample of spores was taken to determine viability by germinating conidia on thin plates of Sabouraud's dextrose agar with 0.1% chloramphenicol. A droplet of spore suspension ( $\sim 10^7$  spores/ml) was pipetted onto the plate, covered with a thin coverslip, and incubated at  $\sim 28^{\circ}\text{C}$  in the dark for 24 h. The plate was examined using a phase-contrast microscope (Leitz, Wetzlar, Germany) at  $\times 400$  magnification. One hundred spores were examined at three locations on each plate and scored as either germinated (viable) or not germinated (dead). A spore was considered to have germinated if the germ tube was clearly visible. Germination was  $>90\%$  in all bioassays.

**Sensitivities of susceptible and Cry2A-resistant *H. armigera* larvae to *M. anisopliae*: first laboratory bioassay.** Control chickpea leaves (8 leaflets each) were dipped into six *M. anisopliae* Tween 80-based spore suspensions prepared as fivefold serial dilutions ranging from  $9.6 \times 10^5$  to  $3 \times 10^9$  spores/ml. A 0.5% Tween 80 solution was used as a control. The dipped chickpea leaves were placed on the surface of 4 to 5 ml cooled 2% agar in wells of 32-well plastic trays. After 2 to 3 h of exposure to air (to allow the leaves to dry), a single early-third-instar *H. armigera* larva was placed on each leaf. The trays were then heat sealed with a vented acetate cover and maintained at  $28^{\circ}\text{C} \pm 1^{\circ}\text{C}$ . To give the fungus optimal conditions for germination, the trays were wrapped in damp tissues and enclosed in a plastic bag to provide a humid environment for the first 24 h. On days 2, 4, and 6, dead larvae were removed and fresh untreated leaves (8 to 10 leaflets each) were provided to each living larva. On day 8, survivors from each treatment were pooled, and their fresh weight and larval stage were recorded. Dead larvae were incubated at  $28^{\circ}\text{C}$  and  $>90\%$  rH for up to 10 days and examined regularly for evidence of conidial growth. The bioassay was repeated twice, resulting in the exposure of a total of 55 to 61 larvae to each spore concentration. Slopes, intercepts, and 50% lethal concentration ( $\text{LC}_{50}$ ) estimates for the two *H. armigera* strains were calculated using the software package POLO-PC (LeOra Software, Berkeley, CA).

**Bt chickpeas and *M. anisopliae* in combination to control *H. armigera*. (i) Second laboratory bioassay.** Control and Bt chickpea leaves (eight leaflets each) were dipped either in a Tween 80-based suspension of a "low" (L) spore suspension ( $1.2 \times 10^8$  spores/ml), in a "medium" (M) spore suspension ( $5.7 \times 10^8$  spores/ml), or in 0.5% Tween 80 (O) as a control. The L and M spore suspensions were chosen to lie approximately midway between the  $\text{LC}_{30\text{s}}$  (L) or  $\text{LC}_{50\text{s}}$  (M) of the two *H. armigera* strains, respectively, as determined in the concentration-response assay. The bioassays were set up as described above, except that the larvae used in the experiments were reared on control chickpea leaves until they reached the third instar. The larvae were fed with eight leaflets each (four leaflets each from two plants to provide a mixture of plants that might have different expression levels). The leaves were changed on days 2, 4, and 6. The parameters assessed were larval survival, weight, and instar after 8 days and the proportion of larvae producing *M. anisopliae* spores. The bioassay was repeated four times, with 30 to 32 larvae tested at each spore concentration.

Larval survival was analyzed using the Cox proportional-hazard model. Bonferroni-Holm correction was performed when required. Each run was analyzed separately, comparing the fungus treatments (L and M) and the controls (susceptible/Cry2A-resistant strain on control/Bt leaves with no fungus application). Where no larvae died in a control group, one additional dead larva was added to each treatment to enable statistical analysis. This procedure was necessary for susceptible larvae, run B on control plants, and for Cry2A-resistant larvae, runs C and D on Bt plants. Data on larval weights after 8 days of feeding were checked for normality and homogeneity of variances prior to analysis. Since all assumptions were met, the data were analyzed for all repeated experiments (runs), together with a three-way analysis of variance (ANOVA) (factors: plant, fungus, and *H. armigera* strain;  $n = 125$  to 128). For all tests, the  $\alpha$ -level was set at 5%. Statistical analyses were conducted using the software package Statistica (version 6; StatSoft Inc., Tulsa, OK).

**(ii) Greenhouse bioassay.** Half of the available control and Bt chickpea plants were sprayed until runoff with an *M. anisopliae* spore suspension containing  $5.4 \times 10^8$  spores/ml. The remainder were sprayed with 0.5% Tween 80 as a control. After the plants were allowed to dry for 1 h, 10 susceptible early-third-instar larvae were placed on each plant, each on a separate leaf. The plants were then enclosed in a cloth bag, which was sealed to the pot to ensure that the larvae could not escape. To provide humid conditions, the plants were enclosed in a

plastic bag for the first 24 h. The plants were watered every 2 days by placing the pots into water-filled dishes (17 cm in diameter; 2 cm deep) for 2 to 3 h. The mortality of the larvae was evaluated after 10 days. In total, three to five plants were used for each of the four treatments. The plants were placed at randomized positions in the greenhouse. During the experiment, the greenhouse temperature varied between  $15^{\circ}\text{C}$  and  $35^{\circ}\text{C}$  with an  $\sim 12$ -h photoperiod and  $\sim 40\%$  rH.

**Feeding behavior of susceptible and Cry2A-resistant *H. armigera* larvae on control and Bt chickpeas.** Control or Bt chickpea leaves of similar sizes and structures (10 leaflets each) were placed in petri dishes (9 cm in diameter; 2 cm high). Subsequently, one early-third-instar *H. armigera* larva, either susceptible or Cry2A resistant, was placed on the lowest leaflet of either control or Bt leaves. The petri dishes were stored at  $25 \pm 1^{\circ}\text{C}$ ,  $40 \pm 5\%$  rH, and a 14-h photoperiod. After 24 h, the larvae were removed and leaf feeding activity was evaluated using a nine-category scale according to the damage inflicted by the feeding larvae (categories: 0, 0% damage; 1,  $<1\%$ ; 2, 2 to 5%; 3, 5 to 10%; 4, 10 to 20%; 5, 20 to 30%; 6, 30 to 50%; 7, 50 to 70%; 8, 70 to 80%; 9,  $>80\%$ ). Feces produced during the exposure period by each larva were collected and stored in a desiccator containing silica gel for at least 24 h before storage at  $-80^{\circ}\text{C}$ . Samples were dried further at  $50^{\circ}\text{C}$  in an oven for at least 4 days before being weighed on a microbalance (Mettler Toledo MX5; division, 1  $\mu\text{g}$ ; tolerance,  $\pm 2 \mu\text{g}$ ). The experiment was repeated twice, resulting in a total of 32 to 43 larvae per treatment. The data on feeding damage and feces weight were evaluated by Kruskal-Wallis ANOVA, followed by pairwise comparisons using the Mann-Whitney U test adjusted for ties and Bonferroni-Holm correction. The importance of two factors, plant (control or Bt) and strain (susceptible or Cry2A-resistant), was evaluated.

## RESULTS

**Sensitivities of susceptible and Cry2A-resistant *H. armigera* larvae to *M. anisopliae*: first laboratory bioassay.** The concentration response of the two *H. armigera* strains to *M. anisopliae* is given in Fig. 2, and details of the mortality, sporulation, weight, and larval instar of survivors are shown in Table 1. The  $\text{LC}_{50}$  for the susceptible strain was determined to be  $1.9 \times 10^8$  spores/ml (95% confidence interval [CI],  $1.5 \times 10^7$ ,  $8.1 \times 10^8$ ), while for the Cry2A-resistant strain, it was  $7.8 \times 10^8$  spores/ml (95% CI,  $4.5 \times 10^8$ ,  $1.3 \times 10^9$ ). The slope of the line for susceptible larvae ( $0.95 \pm 0.187$ ) differed significantly from that of the Cry2A-resistant strain ( $1.94 \pm 0.370$ ;  $P = 0.002$ ).

**Bt chickpeas and *M. anisopliae* in combination to control *H. armigera*. (i) Second laboratory bioassay.** The performances of larvae on control and Bt chickpea leaves at different concentrations of fungal spores are shown in Fig. 3. For the control chickpea leaves, mortality rates in the *H. armigera* strains were similar ( $P > 0.05$ ); however, a marked fungus effect ( $P < 0.0001$ ) was observed. For the Bt chickpea leaves, both strain ( $P < 0.0001$ ) and fungus ( $P < 0.0001$ ) effects were recorded. The data were thus analyzed separately for each *H. armigera* strain. Since larval survival differed significantly between bioassay runs for control and Bt leaves ( $P = 0.003$ ;  $P = 0.018$ ), each run was evaluated separately (runs A to D in Fig. 3).

While the L spore concentration did not increase mortality among susceptible *H. armigera* larvae feeding on control leaves ( $P > 0.05$ ), a significant increase was observed in two of the four runs when the larvae were fed Bt leaves (run A,  $P = 0.006$ ; run C,  $P < 0.0001$ ). In contrast, the M spore concentration caused a significant level of mortality in susceptible *H. armigera* larvae on control leaves in three of the four runs (run A,  $P = 0.009$ ; run B,  $P < 0.001$ ; run D,  $P = 0.033$ ) and in all four runs on Bt leaves (run A,  $P = 0.035$ ; run B,  $P = 0.022$ ; run C,  $P < 0.0001$ ; run D,  $P = 0.002$ ). Mortality was not increased at the L spore concentration on control plants in the Cry2A-resistant strain in any of the runs ( $P > 0.05$ ). The M spore concentration

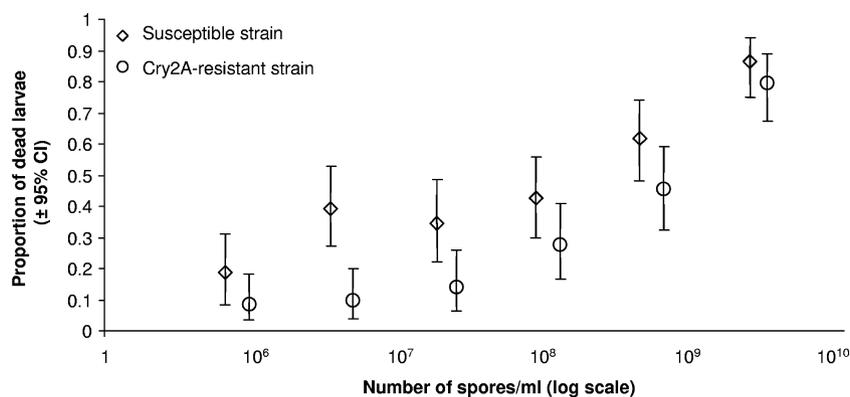


FIG. 2. Proportions of dead susceptible and Cry2A-resistant *H. armigera* larvae ( $\pm 95\%$  CI) fed on control chickpea leaves treated with six different spore concentrations of *M. anisopliae* differing fivefold ( $n = 55$  to 61). Third-instar larvae were treated with the fungus.

caused significant mortality among Cry2A-resistant larvae in two runs (run A,  $P = 0.009$ ; run B,  $P = 0.005$ ) when control leaves were fed and in one run (run B,  $P = 0.014$ ) when the larvae were feeding on Bt leaves.

A three-way ANOVA evaluating the factors fungus (0, L, or M), *H. armigera* strain (susceptible or Cry2A resistant), and plant (control or Bt) indicated that a significant decrease in larval weight occurred after 8 days due to the factors strain and plant ( $P < 0.0001$ ). However, the factor fungus did not significantly contribute to this decrease ( $P > 0.05$ ) (Table 2).

(ii) **Greenhouse bioassay.** The greenhouse bioassay was conducted only with susceptible *H. armigera* larvae, since the second laboratory bioassays revealed no interaction of Bt plants and fungal efficacy for the Cry2A-resistant *H. armigera* strain. Hardly any larval mortality was observed on the untreated

control plants. Approximately 50% of the *H. armigera* larvae died on untreated Bt plants and fungus-treated control plants. Combining the *B. thuringiensis* protein and the fungus caused a mortality rate of 89%. Consequently, the greenhouse bioassay suggested an additive effect of the *B. thuringiensis* toxin and the fungus on the mortality of susceptible *H. armigera* larvae (Fig. 4). Of the larvae that had fed on fungus-treated leaves, 25 to 100% of the larvae from the control plants and 20 to 86% of the larvae from the Bt plants produced fungal spores. None of the dead larvae on the control treatments produced *M. anisopliae* spores.

Toward the end of the experimental period, an incursion of an unknown insect pathogen, or perhaps insecticide use in neighboring glasshouses, caused mortality to insects in all treatments, including controls. This prevented planned repli-

TABLE 1. Evaluation of the sensitivities of susceptible and Cry2A-resistant *H. armigera* larvae to *M. anisopliae*<sup>a</sup>

Spore concn (no. of spores/ml)	<i>H. armigera</i> strain	No. of larvae exposed	No. of dead larvae (no. of larvae producing spores)	Mortality (%)	Mean wt (mg)	Distribution of larval instars (%)		
						L3	L4	L5
0	Susceptible	60	6 (0)	10.0	68.0	0	48.2	51.9
	Cry2A resistant	60	6 (0)	10.0	77.7	0	37.0	63.0
$9.6 \times 10^5$	Susceptible	59	11 (0)	18.6	68.1	0	51.1	48.9
	Cry2A resistant	59	5 (0)	8.5	67.2	0	59.3	40.7
$4.8 \times 10^6$	Susceptible	61	24 (6)	39.3	66.7	0	75.7	24.3
	Cry2A resistant	61	6 (1)	9.8	75.9	0	65.5	34.6
$2.4 \times 10^7$	Susceptible	55	19 (6)	34.5	63.3	0	81.1	18.9
	Cry2A resistant	57	8 (2)	17.0	83.1	0	69.4	30.6
$1.2 \times 10^8$	Susceptible	59	25 (15)	42.4	59.3	0	88.2	11.8
	Cry2A resistant	58	16 (8)	27.6	76.9	0	63.4	36.6
$6 \times 10^8$	Susceptible	60	37 (27)	61.7	63.2	12.0	48.0	40.0
	Cry2A resistant	57	26 (24)	45.6	72.4	0	56.7	43.3
$3 \times 10^9$	Susceptible	59	51 (48)	86.4	51.2	0	100	0
	Cry2A resistant	59	47 (46)	79.7	57.9	9.1	72.7	18.2

<sup>a</sup> Larvae were exposed to chickpea leaves treated with six different spore concentrations for 2 days, differing fivefold in their spore concentrations. Subsequently, the larvae were provided with untreated chickpea leaves every other day until day 8. Weight is shown as the mean weight of all survivors per treatment. The larval instar reached by surviving larvae at the completion of the experiment is given as the percentage of survivors.  $n = 55$  to 61.

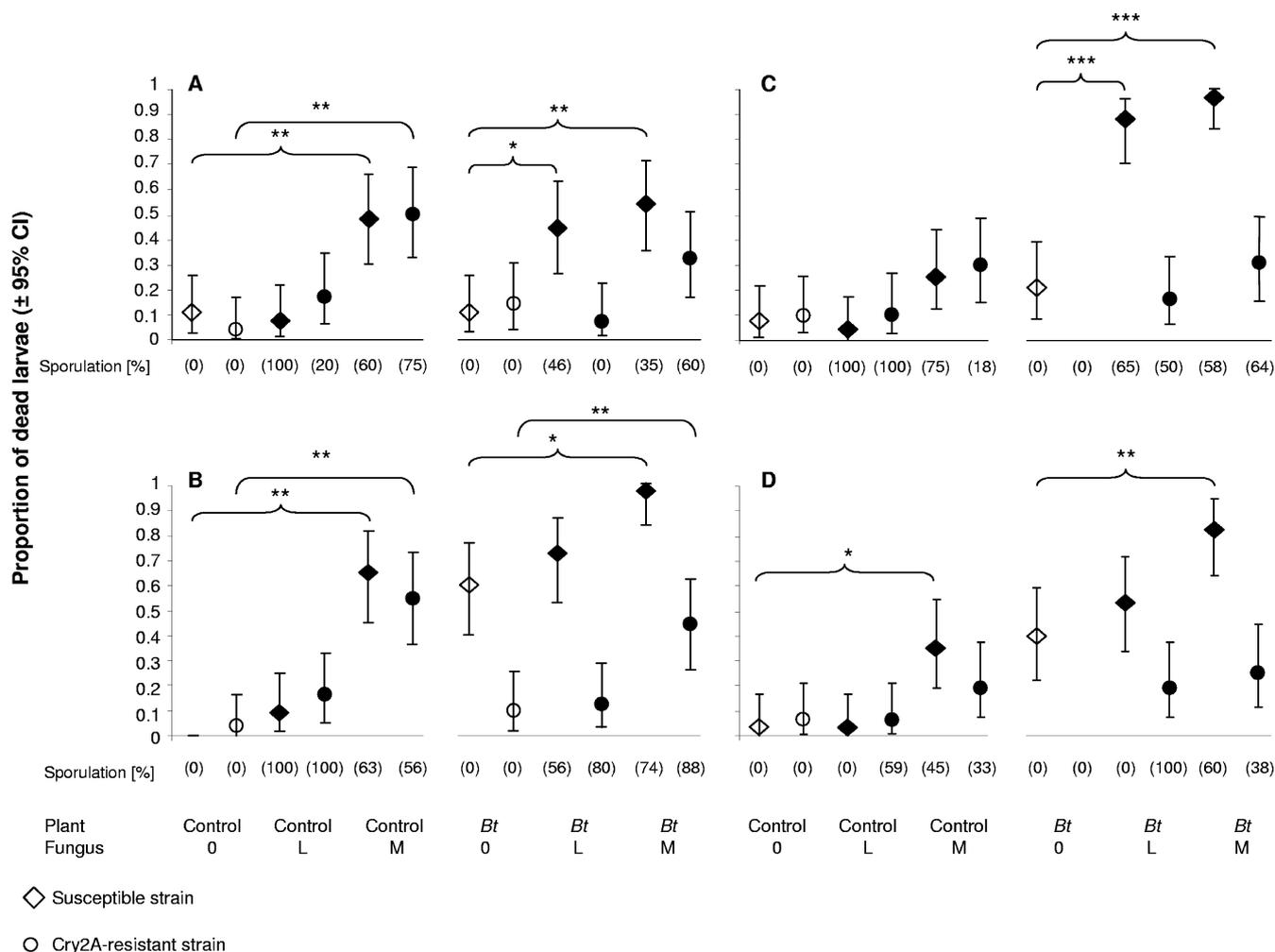


FIG. 3. Proportions of dead susceptible and Cry2A-resistant *H. armigera* larvae ( $\pm 95\%$  CI) fed on control or Bt chickpea leaves treated with different concentrations of *M. anisopliae* spores [0 (0.5% Tween 80), L ( $1.2 \times 10^8$  spores/ml), and M ( $5.7 \times 10^8$  spores/ml)]. The experiment was repeated four times (runs A to D) with an *n* of 30 to 32 per run. Statistical comparisons were made separately for each *H. armigera* strain and for control or Bt chickpea leaves. Statistical significances are shown between the fungus treatments (L and M) and the controls (susceptible/Cry2A-resistant strains on control/Bt leaves with no fungus application). No control mortality occurred for susceptible larvae in run B and on Bt leaves for Cry2A-resistant larvae in runs C and D. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; Cox proportional-hazard model. Sporulation (percent) in dead larvae is given for each treatment. Open symbols refer to the control treatments; filled symbols refer to the fungus treatments.

cation of this bioassay. Thus, no statistical analyses were performed.

**Feeding behavior of susceptible and Cry2A-resistant *H. armigera* larvae on control and Bt chickpeas.** There was a high correlation between feeding damage caused by *H. armigera* larvae on the chickpea leaves and the weight of feces they excreted ( $R^2 = 0.716$ ). For the susceptible *H. armigera* strain, leaf damage was significantly higher for control leaves than for Bt chickpea leaves after 24 h of feeding ( $P < 0.001$ ) (Fig. 5A). The difference in feeding activities on the two plant types was also evident in the feces weight measurements ( $P < 0.001$ ) (Fig. 5B). In contrast, the Cry2A-resistant strain inflicted similar levels of feeding damage ( $P > 0.05$ ) and produced similar amounts of feces ( $P > 0.05$ ). On control plants, the two strains inflicted similar levels of leaf damage; however, significantly more feces were produced by susceptible larvae ( $P = 0.007$ ).

Data for the two experimental runs were combined for the analysis, since they revealed similar patterns.

No significant difference was observed in the number of leaflets damaged per leaf provided to the two strains on either plant type during 24 h (means for the susceptible strain, 8.1 to 9.3 leaflets damaged/leaf; Cry2A-resistant strain, 8.7 to 9.1 leaflets damaged/leaf).

## DISCUSSION

Our studies revealed that *M. anisopliae* is effective at killing both susceptible and Cry2A-resistant *H. armigera* larvae on control and Bt chickpeas and that the number of larvae producing *M. anisopliae* spores did not differ between the two *H. armigera* strains. In some treatments, 20 to 100% of larvae that were apparently killed by the fungus did not produce fungal

TABLE 2. Weights and developmental stages of susceptible and Cry2A-resistant *H. armigera* larvae after 8 days of feeding on either control or Bt chickpea leaves treated with different spore concentrations of *M. anisopliae*<sup>a</sup>

Plant	Fungus <sup>b</sup>	<i>H. armigera</i> strain	Mean wt (mg) ± SE <sup>c</sup>	Distribution of larval instars (%) <sup>d</sup>		
				L3	L4	L5
Control	0	Susceptible	42.0 ± 0.7 a	2.5	41.0	56.6
		Cry2A resistant	45.1 ± 0.5 a		47.1	52.9
	L	Susceptible	38.6 ± 0.7 a	5.0	57.9	37.2
		Cry2A resistant	35.8 ± 0.6 a		63.1	36.9
	M	Susceptible	34.3 ± 0.7 a	12.3	53.4	34.2
		Cry2A resistant	35.6 ± 0.4 a	5.1	73.1	21.8
Bt	0	Susceptible	16.8 ± 0.8 b	41.2	48.2	10.6
		Cry2A resistant	42.3 ± 0.9 a	1.7	46.6	51.7
	L	Susceptible	28.0 ± 0.2 b	37.8	53.3	8.9
		Cry2A resistant	41.7 ± 1.1 a	4.5	53.6	41.8
	M	Susceptible	16.0 ± 0.7 b	17.4	60.9	21.7
		Cry2A resistant	37.8 ± 1.1 a	3.4	50.6	46.0

<sup>a</sup> Data from the four runs (Fig. 3) were pooled ( $n = 125$  to  $128$ ).

<sup>b</sup> 0, control; L,  $1.2 \times 10^8$  spores/ml; M,  $5.7 \times 10^8$  spores/ml.

<sup>c</sup> Weight is shown as the mean weight of all surviving larvae per treatment. Different letters indicate significant differences ( $P < 0.05$ ) between means of a group (i.e., a certain "plant" and "fungus" treatment).

<sup>d</sup> The larval instar reached after 8 days is given as a percentage of the survivors.

spores. It is known that entomopathogenic fungi can cause high mortality with little sporulation (47). It was suggested in earlier studies that the insects were killed by toxins, such as destruxins, produced by *M. anisopliae* (21, 51).

The Cry2A-resistant strain of *H. armigera* appeared to be more tolerant of *M. anisopliae* than the susceptible strain in the concentration-response bioassay in which larvae were fed control chickpea leaves treated with various spore concentrations of the entomopathogenic fungus. However, this finding was not confirmed in the second laboratory bioassay, in which larvae received control or Bt chickpea leaves treated with no fungus or an L or an M spore concentration. In this bioassay, no difference in larval susceptibility to *M. anisopliae* was observed between the two *H. armigera* strains while feeding on fungus-treated control chickpea leaves.

A number of studies have shown significant fitness costs with some laboratory-selected *B. thuringiensis*-resistant strains of different species of Lepidoptera (2). Fitness costs may be ex-

pressed in a variety of forms, e.g., reduced survival rates; diminished fertility, fecundity, and mating ability; and increased overwintering mortality and developmental rates. For larvae of a Cry1Ac-resistant strain of *H. armigera*, a reduced survival rate and an increased development time on different host plants were observed (1, 5). These fitness costs can also be expressed as an increased susceptibility to natural enemies, such as entomopathogenic nematodes (15) or insect viruses (38). However, we did not detect a higher susceptibility of Cry2A-resistant *H. armigera* larvae to infection by *M. anisopliae*, which is supported by the fact that previous studies had revealed that the two *H. armigera* strains used in our study are indistinguishable in a number of life table parameters (R. J. Mahon, unpublished data). Our findings are consistent with the study by Johnson et al. (20), who did not find a higher fungal infection rate with *N. rileyi* in *B. thuringiensis*-resistant *Heliothis virescens* larvae. Likewise, susceptibility to nucleopolyhedrovirus infection was not increased in a *B. thuringiensis*-resistant *Plutella xylostella* strain (37) and *B. thuringiensis* resistance in larvae of the flour moth *Ephestia kuehniella* had no effect on parasitism by an endoparasitoid (36).

Interestingly, a greater susceptibility to *M. anisopliae* occurred when susceptible *H. armigera* larvae fed on Bt chickpea leaves than when they fed on control leaves. On control leaves, the L spore concentration of the fungus did not cause mortality, while significantly increased mortality (31 to 65%) due to the M spore concentration was observed in three out of four bioassays (Fig. 3A, B, and D) (the L spore concentration refers to the approximate LC<sub>30</sub> calculated in the concentration-response curve and the M spore concentration refers to the LC<sub>50</sub>). When susceptible *H. armigera* larvae fed on Bt chickpea leaves treated with *M. anisopliae*, an additive effect occurred at an M spore concentration, with larval mortalities between 53 and 97%, whereas at an L spore concentration, the effect was more than additive in two out of four bioassays, resulting in 72 and 87% mortality (Fig. 3A and C). Interestingly, this was observed when the untreated Bt leaves caused little mortality

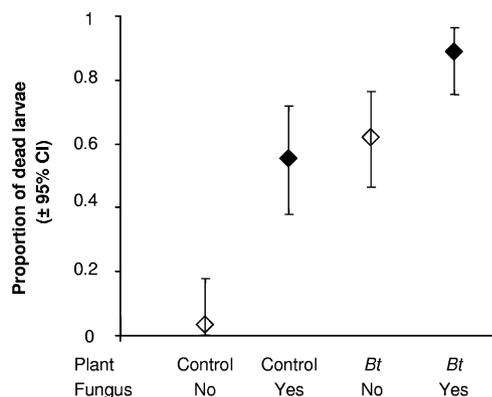


FIG. 4. Proportions of dead susceptible *H. armigera* larvae ( $\pm 95\%$  CI) fed on control or Bt chickpea leaves treated with *M. anisopliae* ( $5.4 \times 10^8$  spores/ml) or 0.5% Tween 80 in the greenhouse. The mortality of susceptible *H. armigera* larvae was recorded after 10 days.  $n = 3$  to 5 plants per treatment.

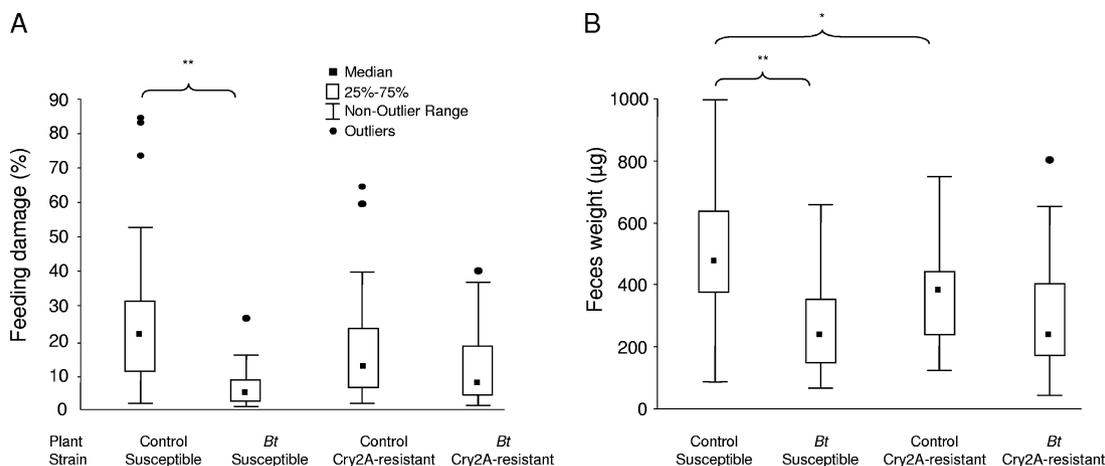


FIG. 5. Boxplots showing the distribution of feeding damage (percent;  $n = 32$  to  $43$ ) (A) and feces weights ( $\mu\text{g}$ ;  $n = 27$  to  $40$ ) (B) for susceptible and Cry2A-resistant *H. armigera* larvae fed for 24 h on control or Bt chickpea leaves. \*,  $P < 0.01$ ; \*\*,  $P < 0.001$ . The nonoutlier range is the range of values that fall above the upper outlier limit ( $+1.5 \times$  the height of the box) and above the below-outlier limit ( $-1.5 \times$  the height of the box).

to susceptible *H. armigera* larvae (9 and 20%). In the two runs (runs B and D) in which larval mortality was already substantial on the Bt leaves (39 and 59%), no significant increase in larval mortality due to an *L. M. anisopliae* spore concentration was observed (52 and 72%). Varying levels of mortality on Bt chickpea leaves in susceptible *H. armigera* larvae probably reflected varying expression levels in the Bt chickpea plants to which they were exposed. The variation may be due to segregation of at least two copies of the gene present at different loci (Acharjee et al., unpublished). As the leaves provided to the *H. armigera* larvae were always taken from two different plants during each feeding regime and a weight reduction in survivors was measured in each experimental run, it can be concluded that larvae always ingested at least low doses of *B. thuringiensis* toxin, which caused sublethal damage that subsequently resulted in the enhanced efficacy of the entomopathogenic fungus. The Cry2A-resistant larvae showed no indication of deleterious effects of very high levels of Cry2Ab toxin and were cross-resistant to Cry2Aa (27). Therefore, it was not surprising that mortalities of resistant insects induced by *M. anisopliae* did not differ when larvae fed on either control or Bt chickpea leaves. Once higher-expressing Bt chickpeas are available that are appropriate for *H. armigera* control in the field, additional studies should examine the effect of the entomopathogenic fungus on Cry2A-resistant larvae in more detail. Those data would also be valuable in the context of resistance management.

The application of pathogens as a biopesticide in combination with *B. thuringiensis*-transgenic plants to control pest Lepidoptera has previously been examined in the laboratory. For susceptible *H. virescens* larvae, a synergistic effect was observed between Cry1Ab-expressing tobacco plants and the entomopathogenic fungus *N. rileyi* (20). Unlike bacteria and viruses, fungi can infect insects not only through the gut, but also through spiracles and, in particular, through the surface of the integument (10). This leads to the possibility of infecting insects independently of their feeding activity. A previous study reported that susceptible larvae of *H. virescens* moved more than resistant larvae on *B. thuringiensis* (Cry1Ab)-expressing

tobacco plants (20). Consequently, when the plants were treated with a pathogen, susceptible larvae were more likely to be infected than *B. thuringiensis*-resistant larvae. Similarly, a higher level of activity was reported for *B. thuringiensis*-susceptible *Spodoptera exigua* larvae when feeding on a *B. thuringiensis*-containing diet than on a control diet (4). Interestingly, in some cases of orally active pathogens, such as nucleopolyhydrovirus, antagonistic effects by *B. thuringiensis* were reported (8, 25, 37). This could be due to the feeding-deterrent effect of the *B. thuringiensis* toxin, which reduces the consumption rate of plant material and thus ingestion of the virus.

In our studies, the movements of larvae of both *H. armigera* strains were similar on both Bt and non-Bt chickpea leaves, as indicated by the number of leaflets damaged per leaf. This finding is supported by behavioral observations made over an 11-h period of the movements of susceptible and Cry2A-resistant larvae on either control or Bt chickpea leaves, which did not reveal any obvious differences (data not presented). One possible explanation for the lack of activity differences observed in this study is that the *B. thuringiensis* expression level in the chickpea plants employed may have been simply too low to cause behavioral effects in *H. armigera*, as has been suggested for *H. virescens* on low-expressing Bt cotton lines (3). However, the chickpea plants were clearly expressing at some level, as susceptible *H. armigera* larvae caused significantly less damage to Bt chickpea leaves than to control leaves. In the case of the Cry2A-resistant strain, no difference in feeding activity between the plant types was observed. In contrast to the leaf damage data, our study revealed a difference in feces production within 24 h by both *H. armigera* strains while feeding on control chickpea leaves. A reason for this difference is not obvious, but it could be a discrepancy in food utilization between strains. If food utilization differences were responsible, one would expect to see differential larval and pupal weights. However, such differences were not seen when the two genotypes were fed an artificial diet, cotton or pigeonpea plants (Mahon, unpublished).

Our laboratory/glasshouse studies with low-expressing Bt chickpea plants and *M. anisopliae* have shown that the two

control methods are generally complementary for the control of *H. armigera*. Bt chickpea plants that are developed for commercial release will need to provide much greater control than the plants used in our study. Furthermore, they are likely to express two Cry proteins that are sufficiently different that insects resistant to one would still be susceptible to the other. Such a pyramid of cry genes should provide good control, as well as reduce the likelihood of the development of resistance by the target pest (2, 9). Nevertheless, it is likely that some *H. armigera* larvae will survive in a Bt chickpea crop. First, we do not expect that the Bt technology will provide 100% control, and therefore, occasional susceptible larvae will survive. This could occur, for example, through selective feeding on lower-expressing tissues, or the toxin concentration could decline in chickpea plants after flowering, as has been documented in cotton crops (13, 24, 33). Secondly, larvae might survive on Bt chickpeas through possession of a level of tolerance or resistance to the expressed Cry proteins. In both cases, the impact of natural enemies, such as *M. anisopliae*, will help to kill survivors and potentially decrease the speed of resistance development (17, 38).

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