

Monitoring the Cry1Ab Susceptibility of European Corn Borer in Germany

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ABSTRACT The European corn borer, *Ostrinia nubilalis* (Hübner), is one of the most important insect pests in corn, *Zea mays* L. Transgenic corn cultivars expressing *Bacillus thuringiensis* (Bt) toxin provide a promising crop protection strategy against European corn borer; however, management is needed to avoid resistance development of the target pest species. The aim of this work was to establish the baseline susceptibility of different European corn borer populations in Germany to be able to forecast a possible development of resistance at an early stage. To standardize test procedures for future resistance management, the efficiency of Cry1Ab toxins from different suppliers and different production was assessed. Furthermore, two different test methods, surface treatment and the incorporation method, were compared with regard to their practicability and efficiency. Neither method provided significant differences in the baseline susceptibility of populations from different German regions. Overall, the data suggested little differentiation among German populations in terms of their susceptibility to Bt toxin and their genetic background. Future monitoring could therefore use a single European corn borer population as a representative for southwestern Germany. However, toxins from different suppliers and different production batches produced a vast range of LC₅₀ values. Changes because of different toxin batches may be mistaken as a change in baseline susceptibility or even as the start of a resistance development. Thus, it is important throughout insect resistance management that the same toxin batches will be available for baseline susceptibility bioassays and for future tests.

KEY WORDS *Ostrinia nubilalis*, European corn borer, baseline susceptibility, Bt corn

During the last decade, *Bacillus thuringiensis* (Bt) genes of *B. thuringiensis* variety *kurstaki* (Berliner) encoding lepidopteran-specific toxins were engineered into corn for protection against the European corn borer, *Ostrinia nubilalis* (Hübner). In the European Union (EU), a total of 50,000 ha of Bt corn, *Zea mays* L., was grown in 2004, which represented only 0.5% of the worldwide area of corn cultivation. Currently, the commercial cultivation of Bt corn is concentrated to Spain (Devos et al. 2005). It is to be expected for the near future that more Bt corn and even new corn varieties will be grown in the EU in areas with high infestation of European corn borer (Demont and Tollens 2004). European corn borer is the most important insect pest in corn and can reduce yields up to 20%. However, because of the season-long, constitutive expression of the Bt toxin during the vegetation period, a selection of resistant genotypes is more likely than by conventional Bt sprays. Hence, transgenic corn cultivars expressing Bt toxin need management to avoid resistance development of the target pest species. So far, much effort has been put

into establishing insect resistance management (IRM) strategies to delay or avoid this effect (Alstad and Andow 1995, Siegfried et al. 2000). Insect resistance to Bt toxins has occurred in Lepidoptera but mostly in laboratory strains exposed to Bt toxin. So far, only the diamondback moth, *Plutella xylostella* (L.), showed resistance in field populations (Ferre et al. 1991). The lack of field-developed resistance of European corn borer to Bt toxins suggested that a reasonable resistance management may be able to detect resistance within a sufficient time span and be an efficient tool to manage a possible resistance development. Hence, the estimation of the baseline susceptibility of European corn borer to Cry toxins is discussed and favored as an important part of a proactive resistance management in Europe. This has to be performed in advance, before Bt corn is cultivated to a greater extent. Such prior estimates of the baseline susceptibility might help to detect changes in the susceptibility in response to selection against Cry toxins after the introduction of transgenic Bt corn in Germany. Integrated pest management (IPM) and monitoring also must be adapted to German and European agricultural structures with relatively small fields, which may be close to or even within nature conservation areas.

The aim of this study was to 1) establish the baseline susceptibility of European corn borer populations from different geographical regions in Germany

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against Cry1Ab toxins; 2) compare the efficiency of Cry1Ab toxins from different suppliers and different production (protoxin and truncated version) with regard to future resistance management; and 3) compare different susceptibility test methods: surface treatment and the incorporation method.

Materials and Methods

Egg Collection. In four subsequent years (2000–2003), we collected European corn borer egg masses in four different geographic regions along the Rhine Valley: east of Lake Constance, west of Freiburg (Upper Rhine Valley), north of Karlsruhe (Central Rhine Valley), and west of Bonn (Lower Rhine Valley). The distances between the populations were 160 miles (Bonn to Karlsruhe) and \approx 100 miles (Karlsruhe to Freiburg and Freiburg to Lake Constance). All of these regions have high infestation levels of European corn borer and are therefore potential regions for a future cultivation of Bt corn. None of the four populations have ever been exposed to Bt toxin, not even as Bt spray. Farming practices in these areas are similar and represent the standard cultivation procedures throughout Germany.

For egg collection, we used light traps linked to cages (2 by 1.50 by 2 m), which contained eight to 10 corn plants. In each sampling region, the light traps were placed at three different locations within a radius of 20 km. During the flight period of European corn borer, adults were caught alive and started egg laying on the corn plants within the cage. After oviposition, the egg masses could easily be collected from the plants and were brought to the laboratory. Collected egg masses from traps in the same region were pooled. The number of egg masses collected for each geographic region was between 300 and 350, except for Bonn (1,000 egg masses).

Insect Rearing. European corn borer rearing procedures were based on Wyniger (1974) and modified for our purposes. Leaf sections containing the collected egg masses were cut out and placed in plastic petri dishes without vents (three to four egg masses per dish) together with a block of diet. The diet consisted of crunched corn, wheat germ and yeast, to which a vitamin mixture, antibiotics, and preserving agents were added. Agar powder was used for the solidification of the diet. After hatching, larvae were kept at $25 \pm 1^\circ\text{C}$ at a photoperiod of 16:8 (L:D) h and 70% RH in a climate chamber. At the onset of pupation, insects were moved to plastic boxes (7 cm in diameter, 6 cm in height) provided with filter paper. Emerging adults were transferred to mating cages and maintained at $22 \pm 1^\circ\text{C}$ at a photoperiod of 16:8 (L:D) h and 70% RH. For oviposition, a transparent plastic foil was placed at the back of the cage, which was changed each day. To maximize longevity and egg production adults were provided with cotton soaked with a solution of 10% honey in water (Leahy and Andow 1994, Fadamiro and Baker 1998). Cages were

daily misted with water to increase humidity. In addition to lines from the field populations, long-term laboratory strains from different populations were reared and kept at the conditions described above. Infestation by microsporidia was checked twice a year in all strains.

Bt Toxin. Three different Cry1Ab toxins were used for the bioassays. Native Cry1Ab crystals, isolated from a bacterial culture of *Bacillus thuringiensis* ssp. *kurstaki* strain were provided by Syngenta Biotechnology Inc. (Research Triangle Park, NC). This was the full-length form (protoxin) of the Bt protein, which needs to be split in the gut of European corn borer to express its toxicity. The toxin was provided as a lyophilized powder and was resuspended at pH 10.5 in cyclohexyl aminopropene sulfonic acid (CAPS) 50 mM pH 10.5 buffer. The other two Cry1Ab toxins were truncated forms of the protein; one form was provided by Monsanto (St. Louis, MO), and the other form was from DLR (Neustadt, Germany). Both toxins were trypsinated and dissolved in CAPS buffer at pH 10.5. The commercially used Bt corn plants express a truncated version of Cry1Ab toxin (Koziel et al. 1993, Mendelsohn et al. 2003).

Bioassays. For the bioassays, neonate F_1 larvae from each geographical population were used that originated from the collected egg masses. When egg numbers of a population were too low to provide sufficient F_1 offspring, neonates from the next generation were used.

To test whether laboratory strains could be used for bioassays in case of insufficient field samples, additional tests were performed with insects reared in the laboratory for more than 10 generations. For that purpose an existing laboratory strain was used, which at that time had reached the 42nd generation. This served the additional purpose of testing the effects of long-term rearing on susceptibility. These results were compared with the susceptibility values obtained for the different field populations.

Two different bioassay methods were tested: the incorporation method and the surface overlay treatment, which are described below. The bioassays were performed in 128-well trays (Bio-Ba-128, Color-Dec, Italy). Each well was supplied with a single neonate larva. The wells were sealed with vented covers provided with the bioassay trays (Bio-Cv-16). In total, 32 larvae were tested at each toxin concentration plus a control (CAPS buffer without toxin) of 64 larvae. All bioassays were conducted in a climate chamber at 25°C in constant dark. After 7 d, larval mortality was recorded.

Incorporation Method. For the incorporation method, the toxin was evenly mixed with the diet. All three Cry1Ab toxins (one protoxin and two truncated toxins from different suppliers) were used for this method. Serial dilutions of each protein were prepared in CAPS buffer whereby 4 ml of each toxin concentration was mixed with 36 g of artificial diet. The toxin was incorporated into the diet after cooling down to below 50°C and thoroughly homogenized with a mixer. For the protoxin, five concentrations were used (0.2, 2, 4, 6, and 8 $\mu\text{g/g}$ diet), and seven

Table 1. Results of a probit analysis for different German European corn borer populations tested with the incorporation method indicating susceptibility against different Cry1Ab toxins provided by different suppliers

Pop	<i>n</i>	Slope ± SE	LC ₅₀ (95% CL)	LC ₉₀ (95% CL)	χ ²	df	<i>P</i>
Natives Cry1Ab toxin /protoxin (provided by Syngenta)							
Karlsruhe	1,440	1.45 ± 0.06	1.63 (1.12–2.42)a	12.42 (7.32–21.08)a	12.88	3	0.07
Freiburg	864	1.55 ± 0.08	1.60 (1.05–2.44)a	10.67 (6.46–17.63)a	10.54	3	0.06
Lake Constance	1,152	1.91 ± 0.12	1.23 (0.76–1.95)a	5.70 (4.03–8.06)a	3.18	3	0.36
Truncated Monsanto toxin							
Bonn	2,880	1.15 ± 0.62	0.34 (0.21–0.54)b	4.45 (2.31–12.44)b	0.15	5	0.97
Karlsruhe	2,880	1.35 ± 0.96	0.11 (0.09–0.13)a	0.93 (0.70–1.31)a	0.03	5	0.89
Freiburg (field pop)	1,152	0.87 ± 0.02	0.11 (0.07–0.19)a	3.33 (1.13–9.81)ab	2.18	5	0.82
Lake Constance	2,880	1.30 ± 0.98	0.10 (0.06–0.14)a	0.92 (0.56–1.88)a	0.09	5	0.91
Freiburg (labstrain generation 9–11)	2,880	1.00 ± 0.74	0.10 (0.05–0.16)a	1.84 (0.89–6.14)ab	0.09	5	0.97
Freiburg (labstrain generation 42)	256	0.98 ± 0.03	0.19 (0.11–0.32)a	3.94 (1.65–9.44)ab	0.14	5	0.84
Truncated DLR toxin							
Bonn	1,728	1.22 ± 0.04	2.12 (1.40–3.21)a	23.99 (10.34–55.68)a	0.80	5	0.98
Freiburg	1,728	1.02 ± 0.03	1.54 (0.97–2.42)a	28.01 (10.87–72.16)a	3.75	5	0.59
Lake Constance	1,728	1.23 ± 0.03	1.64 (1.13–2.39)a	17.98 (8.87–36.43)a	2.74	5	0.74

Data presented are number of larvae analyzed (*n*); the LC₅₀ and LC₉₀ values, with confidence limits (CL) followed by different letters indicating statistically significant differences; the slope with SE of the probit model; and the χ² values of the LC₅₀ values. Doses are expressed as micrograms of truncated toxin per gram of artificial diet.

concentrations for both the truncated Monsanto toxin (0.012, 0.025, 0.05, 0.1, 0.5, 1, and 2 μg/g diet) and the truncated DLR toxin (0.2, 0.5, 1, 2, 4, 8, and 16 μg/g diet). One milliliter of the diet with incorporated toxin was filled into each well. The incorporation bioassay was repeated four to seven times (protoxin, Syngenta Biotechnology Inc.), six times (truncated DLR toxin) and 10 times (truncated Monsanto toxin) for each population, depending on the availability of both larvae and toxin.

Surface Treatment. For the overlay technique, the toxin was applied to the surface of the solidified diet. Both truncated Cry1Ab toxins were used with seven different concentrations (Monsanto toxin: 0.02, 0.04, 0.08, 0.156, 0.3, 0.625, and 2.5 μg/g diet; and DLR toxin: 0.08, 0.156, 0.3, 0.625, 1.125, 2.5, and 5 μg/g diet). Thereby, 1 ml of diet was dispensed into each well, and 100 μl of each toxin solution was applied onto the surface of the diet and allowed to dry. Each bioassay was replicated five times per population. Bioassays performed with truncated Monsanto toxin were only used as an additional comparison between the two different test methods.

Statistical Analysis. Mortality estimates for each population were analyzed by probit analysis by using ToxRat (2003). LC₅₀ and LC₉₀ values of the baseline susceptibility with their 95% confidence intervals were calculated. Additionally, we tested for the linearity of dose–mortality curves and determined the slope of each dose–mortality line.

The significance of differences in the susceptibility of different populations was tested by pairwise comparison using nonoverlapping 95% CIs. When the confidence intervals of one population did not overlap, they were considered to be significantly different from one another (*P* < 0.05; Robertson and Preisler 1992, Payton et al. 2003).

Results

Geographical Differences in Susceptibility. Susceptibility estimates for the different German *O. nubilalis* populations based on the incorporation method are listed in Table 1. For the protoxin provided by Syngenta Biotechnology Inc., LC₅₀ values ranged from 1.23 to 1.63 μg/g. There was no significant difference in the susceptibility of the three analyzed populations to the protoxin as indicated by the nonoverlapping confidence intervals.

For the truncated Monsanto toxin, LC₅₀ values ranged from 0.10 to 0.34 μg of Cry1Ab per gram of diet. The most northern population from Bonn had a significantly higher LC₅₀ value than all other populations (Table 1). On the basis of the LC₉₀ value, the population from Bonn is only significantly higher than that from Lake Constance and Karlsruhe.

The LC₅₀ values for the second truncated toxin (DLR toxin) were considerably higher and varied from 1.54 to 2.12 μg/g (Table 1). Consistently, the most northern population from Bonn had the lowest susceptibility. The difference, however, was not statistically significant.

Only the truncated Monsanto toxin was used to compare the susceptibility of the long-term laboratory strain with its origin field population from Freiburg. LC₅₀ values of the two different strains (Table 1) were very similar (0.10 and 0.11 μg/g) with no statistically significant difference (*t*-test: *t* = 0.55, *P* = 0.957).

Effectiveness of Different Toxins. Overall, the two different truncated toxin batches showed big differences in their effectiveness. Depending on the population LC₅₀ differences ranged from 6- to 16-fold (Table 1).

A comparison between the two types of toxins, the protoxin and the truncated toxins, showed a higher variability in the LC₅₀ values of different populations

Table 2. Probit analysis of mortality of European corn borer exposed to truncated Cry1Ab toxin (DLR toxin, Monsanto toxin) by using the surface treatment

Pop	Toxin supplier	n	Slope \pm SE	LC ₅₀ (95% CL)	LC ₉₀ (95% CL)	χ^2	df	P
Bonn	DLR	1,440	1.45 \pm 0.05	28.04 (19.74–39.84)a	213.37 (102.3–445.08)a	0.89	5	0.97
Karlsruhe	DLR	1,440	1.38 \pm 0.03	21.62 (15.63–29.90)a	183.15 (104.9–324.60)a	4.17	6	0.65
Freiburg	DLR	1,440	1.18 \pm 0.03	30.79 (21.26–44.60)a	372.89 (171.34–811.51)a	1.04	5	0.96
Lake Constance	DLR	1,440	1.33 \pm 0.04	17.17 (11.96–23.90)a	156.66 (77.35–317.28)a	1.65	5	0.90
Freiburg	Monsanto	1,280	1.24 \pm 0.03	3.61 (2.41–5.41)b	38.67 (20.37–73.43)b	0.81	5	0.97

Doses are expressed as nanograms of truncated Cry1Ab toxin per square centimeter of diet surface area.

when tested with the protoxin. Furthermore, the protoxin yielded much higher LC₅₀ values (1.23–1.63 μ g/g) than the bioassays with truncated Monsanto toxin (0.10–0.34 μ g/g; Table 1).

Comparison of Different Methods. Surface treatment susceptibility data for European corn borer populations exposed to both truncated Cry1Ab toxins are presented in Table 2. LC₅₀ values ranged from 17.17 to 30.79 ng/cm² for the DLR toxin, with no significant difference between the different populations.

As a reference, the population from Freiburg was tested in addition with the truncated Monsanto toxin. The mean LC₅₀ value for this population was 3.61 ng/cm² (Table 2). Thus, the difference between the LC₅₀ values of the same population effected by toxins from different producers was ninefold (30.79 ng/cm² with truncated DLR toxin and 3.61 ng/cm² with truncated Monsanto toxin) and highly statistically significant.

There were no major differences between the two test methods in terms of the ranges of the confidence intervals relative to the size of the lethal concentration values. Comparing, for example, the tests of the Lake Constance European corn borer population the 95% CL represented 77% of the mean LC₅₀ value for the incorporation method and 70% for the surface treatment. On average, the relative range of the confidence intervals achieved with the surface treatment was slightly narrower than for the incorporation method. Similarities also can be seen for the SE of slopes, which were between 0.03 (incorporation method) and 0.04 (surface treatment). For populations from Karlsruhe and Freiburg, the SE of slopes was the same for both methods (0.03).

Discussion

Before a large-scale commercialization of Bt corn in Germany and other European countries, it will be necessary to develop a cost-effective monitoring program (Bolin et al. 1998) to provide a proactive resistance management plan for Europe. To maintain the effectiveness of Bt corn, it is critical to detect changes in susceptibility through regular monitoring. This can be accomplished by periodical replications of bioassays, where the baseline susceptibility has been established previously.

Our population samples encompassed the geographic region of southwestern Germany, where there are high infestation levels of European corn borer. Overall, the data suggested only small genetic differ-

entiation of European corn borer populations in terms of their susceptibility to Bt toxin. This confirmed previous studies in Europe (Gonzales-Nunez et al. 2000, Farinos et al. 2004) and the United States (Marcon et al. 1999) in which no or very small differences in susceptibility to Cry1Ab toxin of European corn borer populations from different geographic regions were observed. In our study, only European corn borer from Bonn, which is located at the Lower Rhine Valley, showed a significantly lower susceptibility compared with other tested populations. But, even this difference was only evident for one of the truncated Cry1Ab toxins. Tests with the second truncated Cry1Ab toxin showed no statistically significant difference.

The comparison with a long-term laboratory strain reared from one of the field populations did not show any differences in susceptibility. Multiple tests up to the 11th and even the 42nd generation of the laboratory strain did not indicate any marked changes in LC₅₀ values. This suggested that rather than testing larvae directly from field populations, it may be possible to rear larvae for one or two generations in the laboratory and use those for testing (Reed and Halliday 2001). This approach is particularly useful when the amount of egg masses or larvae is too small for a meaningful number of bioassays and independent replications. Because the reliability and significance of a test increases with the number of individuals per bioassay, using laboratory-reared individuals in sufficient numbers may improve the overall quality of susceptibility testing.

The two different methods for bioassays, the incorporation and surface treatment, yielded almost the same range of variation for the two truncated toxin batches. Only small susceptibility differences were observed between the populations. By using both methods, their advantages and disadvantages could be evaluated. An important advantage of the surface treatment is the lower amount of toxin required for each test. However, compared with the surface treatment, the incorporation method allowed a more homogenous distribution of the toxin solution in the diet and thus a persistently higher exposition of each larva. This method is more time-consuming in test preparation and evaluation, and needs larger amounts of toxin. For populations tested with both methods, a conversion factor was calculated. For the truncated DLR toxin, the mean conversion factor for the LC₅₀ values was \approx 14 (10.5–20.0), and for the truncated Monsanto toxin, a factor of 33 was determined. However, this can

only serve as a rough estimate for a comparison of the results obtained with different methods.

The comparison of the baseline susceptibility data from bioassays performed with the same method but with different toxin batches (e.g., in this case, from different producers) was not straightforward. The use of the same toxin, here a truncated version of Cry1Ab, from different batches resulted in different LC_{50} values. These differences were 7- to 16-fold for the incorporation method and up to eightfold for tests performed with the surface treatment. The LC_{50} values indicated a reduced activity for the second truncated toxin (DLR toxin). Most of the susceptibility data published so far were based on the surface treatment and are therefore not comparable with LC_{50} values resulting from the incorporation method. Marcon et al. (1999) established baseline data for European corn borer with a LC_{50} value of 3 $\mu\text{g}/\text{ml}$ (2.13–4.27) for Cry1Ab toxin. This is within the range of the truncated DLR toxin (1.54–2.12 $\mu\text{g}/\text{g}$) and the protoxin (1.23–1.60 $\mu\text{g}/\text{g}$) used for this study. Previous studies and now our study indicated that different toxin batches and methods can produce vastly different values of susceptibility. For American European corn borer populations LC_{50} values ranged from 4.11 ng/cm^2 to 11.95 ng/cm^2 for bioassays performed with native purified Cry1Ab toxin (Siegfried et al. 2001). Applying the same toxin, the LC_{50} values of Spanish European corn borer populations ranged from 3 to 4 ng/cm^2 (Farinos et al. 2004) to 104 to 109 ng/cm^2 (Gonzales-Nunez et al. 2000), depending on the toxin batch used. For a French-Swiss European corn borer population, the LC_{50} value was $\approx 20 \text{ ng}/\text{cm}^2$ (Chaufaux et al. 2001) and is similar to the LC_{50} values obtained for the German population with truncated DLR toxin (17–31 ng/cm^2). Because of this enormous variability in LC_{50} values depending on the applied toxin, it is very difficult to interpret susceptibility results. Significant differences, which are actually because of different toxin batches, may be mistaken as a change in baseline susceptibility or even as the start of a resistance development. As a consequence, it is important throughout IRM that the same toxin batches will be available for baseline susceptibility bioassays and for future tests. Only then a developing resistance could be identified unequivocally. One problem arising is that sufficient amounts of toxin for a long-term susceptibility monitoring must be produced and stored for future testing, when it is not even clear how long toxins can be stored without losing their activity. This problem has already occurred in other studies (Farinos et al. 2004) and needs to be considered when comparing baseline susceptibility data from different laboratories using various toxin sources.

One possible solution could be the use of a toxin standard to which every new toxin batch could be compared. A laboratory strain with a well-known (already established) baseline susceptibility could then be used for overlapping bioassays with different toxin batches. This would provide one possible way of assessing the efficacy of each toxin batch. Only based on

this knowledge predictions about a possible change in susceptibility can be made.

Differences between strains, assay methods and Bt toxin batches complicate the assessment of Bt toxicity to European corn borer. The development of resistance against Bt toxins in field populations can be influenced by a high variation in the susceptibility against these toxins. Previous studies on the baseline susceptibility of European corn borer showed great differences and ranges (Siegfried et al. 1995, Marcon et al. 1999). Interpopulation variation in susceptibility to Cry1A toxins among Northern American populations of corn earworm, *Helicoverpa zea* (Boddie), and tobacco budworm, *Heliothis virescens*, were found by Stone and Sims (1993). Such variation can be a potential for the developing resistance (Rossiter et al. 1990). In addition, high variation in susceptibility between populations can result in problems detecting a developing resistance and distinguish between natural variation and a low resistance (Koziel et al. 1993, Glare and O'Callaghan 2000).

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