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F_2 screen for resistance to a *Bacillus thuringiensis*-maize hybrid in the sugarcane borer (Lepidoptera: Crambidae)

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Abstract

A novel F_2 screening technique was developed for detecting resistance in sugarcane borer, *Diatraea saccharalis* (F.), to transgenic *Bacillus thuringiensis* (Bt)-maize expressing the Cry1Ab insecticidal protein. The F_2 screening method involved (i) collecting larvae from maize fields; (ii) establishing two-parent families; (iii) screening F_2 neonates for survival on Bt-maize leaf tissues; and (iv) confirming resistance on commercial Bt-maize plants. With the F_2 screening method, 213 isoline families of *D. saccharalis* were established from field collections in northeast Louisiana, USA and were screened for Bt resistance. One family was confirmed to carry a major Bt resistance allele(s). In a laboratory bioassay, larval mortality of the Bt-resistant *D. saccharalis* on Bt-maize leaf tissues was significantly lower than that of a Bt-susceptible strain. This Bt-resistant *D. saccharalis* population is the first corn stalk borer species that has completed larval development on commercial Bt-maize. The F_2 screening protocol developed in this study could be modified for detecting Bt resistance alleles in other similar corn stalk borers, such as the European corn borer, *Ostrinia nubilalis* (Hübner), and the southwestern corn borer, *D. grandiosella* Dyar.

Keywords: *Diatraea saccharalis*, Bt-maize, resistance monitoring, insecticide resistance management

Introduction

Transgenic crops expressing *Bacillus thuringiensis* (Bt) endotoxins have become one of the most important tools for managing maize and cotton insect pests in the US and other countries (James, 2004; NASS, 2005). The widespread adoption of transgenic Bt crops could place a high selection pressure on the target insect populations and accelerate development of resistance, raising concerns about the long-term durability of Bt plants (Ostlie *et al*., 1997; Gould, 1998). One of the key factors for a successful resistance management plan to prolong the durability of Bt plants is to have a cost-effective resistance monitoring system. The monitoring system should provide information about the initial Bt resistance allele frequencies at low levels. Early shifts in the resistance allele frequencies of field insect populations should be detected so that proactive measures for managing resistance can be deployed before a field control failure occurs.

Several methods have been used, or suggested, to detect and monitor insecticide resistance in field insect populations (Andow & Alstad, 1998; Huang, 2006). Among these, the

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dose-response technique has often been suggested because it is a relatively simple procedure (Bolin *et al*., 1998; Marçon *et al*., 1999; Reed & Halliday, 2001; Trisyono & Chippendale, 2002; Wu *et al*., 2002; Farinós *et al*., 2004). However, the dose-response method is not suitable for monitoring resistance for Bt crops because it is inefficient (Roush & Miller, 1986) and not sufficiently sensitive to detect resistance alleles before field resistance occurs (Andow & Alstad, 1998).

A more common method used to detect and monitor resistance alleles is the diagnostic dose technique (Marçon *et al*., 2000; Tabashnik *et al*., 2000). The diagnostic dose method is a more powerful technique than the dose-response method (Roush & Miller, 1986) because all sample insects are phenotypically characterized individually at a single diagnostic dose rather than as a population for dose-response bioassays. The diagnostic dose method is very effective for detecting dominant resistance alleles (Plapp *et al*., 1990) or when recessive resistance allele frequencies in pest populations are at high levels. The diagnostic dose method was modified to screen F_1 iso-female lines for non-recessive Bt resistance alleles in bollworm, *Helicoverpa zea* (Boddie), in the US (Burd *et al*., 2003) and in cotton bollworm, *H. armigera*, in China (Li *et al*., 2004). However, the diagnostic dose method cannot detect heterozygous individuals if resistance is recessive. Most insect resistance to Bt-maize is genetically or functionally recessive (Tabashnik *et al*., 2003). Screening a large number (e.g. >1,000,000) of field-collected individuals at the diagnostic dose is necessary to estimate the frequency of a rare recessive allele (e.g. <0.001) in a field population (Andow & Alstad, 1998). Therefore, the diagnostic dose method by itself is usually not a feasible technique for detecting rare recessive Bt resistance.

Andow and Alstad (1998) introduced an F_2 screening technique. As originally proposed, this technique involves inbreeding iso-female families and screening their F_2 progeny on Bt plants or exposure to a diagnostic dose. Theoretically, this technique can detect heterozygous individuals even if the resistance is recessive. The F_2 screen, therefore, should be more powerful than the diagnostic dose method alone, provided the resistance gene is recessive. Since 1998, more than 2000 iso-line families of the European corn borer, *Ostrinia nubilalis* (Hübner), have been screened for Bt resistance by using the F_2 screening method, but no major resistance alleles that allowed larval development on high dose commercial Bt plants were detected (Andow *et al*., 1998, 2000; Bourguet *et al*., 2003; Stodola *et al*., 2006).

Based on the principles of the F_2 screen proposed by Andow and Alstad (1998), a cost-effective modification of the F_2 screening method was developed to detect Bt resistance in sugarcane borer, *Diatraea saccharalis* (F.). *Diatraea saccharalis* is a corn pest and a primary target of Bt-maize in Louisiana (Castro *et al*., 2004). This paper details the use of the F_2 screening technique for detecting a major Bt resistance in a population of *D. saccharalis* collected from northeast Louisiana.

Materials and methods

The F_2 screening method for detecting Bt resistance in *D. saccharalis* involved modifications in (i) collecting larvae from maize field; (ii) establishing two-parent families from the field-collected insects; (iii) screening F_2 neonates of each two-parent family for survival on Bt-maize leaf tissues; and (iv) confirming Bt resistance on commercial Bt-maize plants in a greenhouse and on Bt-maize leaf tissues in the laboratory (fig. 1).

Insect collection

The F_2 screen originally proposed by Andow and Alstad (1998) was initiated with mated feral adult females. In the present study, feral larvae of *D. saccharalis* were collected from maize fields near Winnsboro, Louisiana during the first and second field generations. Field-collected larvae were reared individually until the pupal stage in 30-ml plastic cups (Fill-Rite, Newark, NJ) each containing approximately 10 ml of a meridic diet (Bio-Serv, Frenchtown, NJ). The larval-rearing cups were held on 30-well trays (Bio-Serv, Frenchtown, NJ) and placed in environmental growth chambers (Percival Scientific, Perry, IA) maintained at 27°C, 50% RH and a 16 : 8 h (L : D) photoperiod.

Establishment of two-parent families

Pupae collected from the larval-rearing cups were sexed using characters described by Butt and Cantu (1962). To establish two-parent families, a pair of newly-eclosed virgin male and female adults was introduced into a 450-ml paper container (Huhtamaki Foodservice, De Soto, Kansas). A 30-ml plastic cup containing cotton wadding saturated with a 10% sugar water solution was placed in the centre of the adult container. For insects collected in the second generation, approximately 10 g of vermiculite (Sun Gro, Pine Bluff, AR) were also added to each of the containers. The inside wall of each container was covered with wax paper. The open end of the container was covered with cotton gauze. The wax paper served as an adult oviposition substrate. Progeny from each pair comprised an iso-line family. F_1 neonates hatched from eggs of each two-parent family were reared on a meridic diet as previously described. The F_1 adults were sib-mated within each family in a 3.8-l cardboard carton (Neptune Paper Products, Newark, NJ) containing approximately 100 g of vermiculite (Huang *et al*., 2006). The cardboard cartons were placed in environmental chambers maintained at the same conditions as mentioned above.

Screening on maize leaf tissues – negative controls

To establish the validity of using maize leaf tissue for screening F_2 neonates, we evaluated larval survival on negative controls of non-Bt-maize leaf tissue. Five seeds of DK697 (Monsanto, St Louis, MO), a commercial non-Bt-maize cultivar, were planted in 18.9-l pots containing approximately 5000 g of a standard potting soil mixture (Perfect Mix™, Expert Gardener Products, St. Louis, MO) in a greenhouse on the campus of the Louisiana State University AgCenter in Baton Rouge, Louisiana. Plants were thinned to three plants per pot after two weeks. When plants reached the V6–V8 stages (Ritchie *et al*., 1993), fully-expanded maize leaves were removed from the plants and cut into approximately 7-cm lengths across the entire leaf width. Six pieces of the leaf tissues were placed in each well of an 8-well C-D international tray (Bio-Smart-8, C-D International, Pitman, NJ). Each well contained approximately 10 ml of 3.5% solidified agar solution to maintain high moisture (Huang *et al*., 2006). Thirty neonates (<24 h old) of a susceptible *D. saccharalis* colony (LA-NE-A) derived

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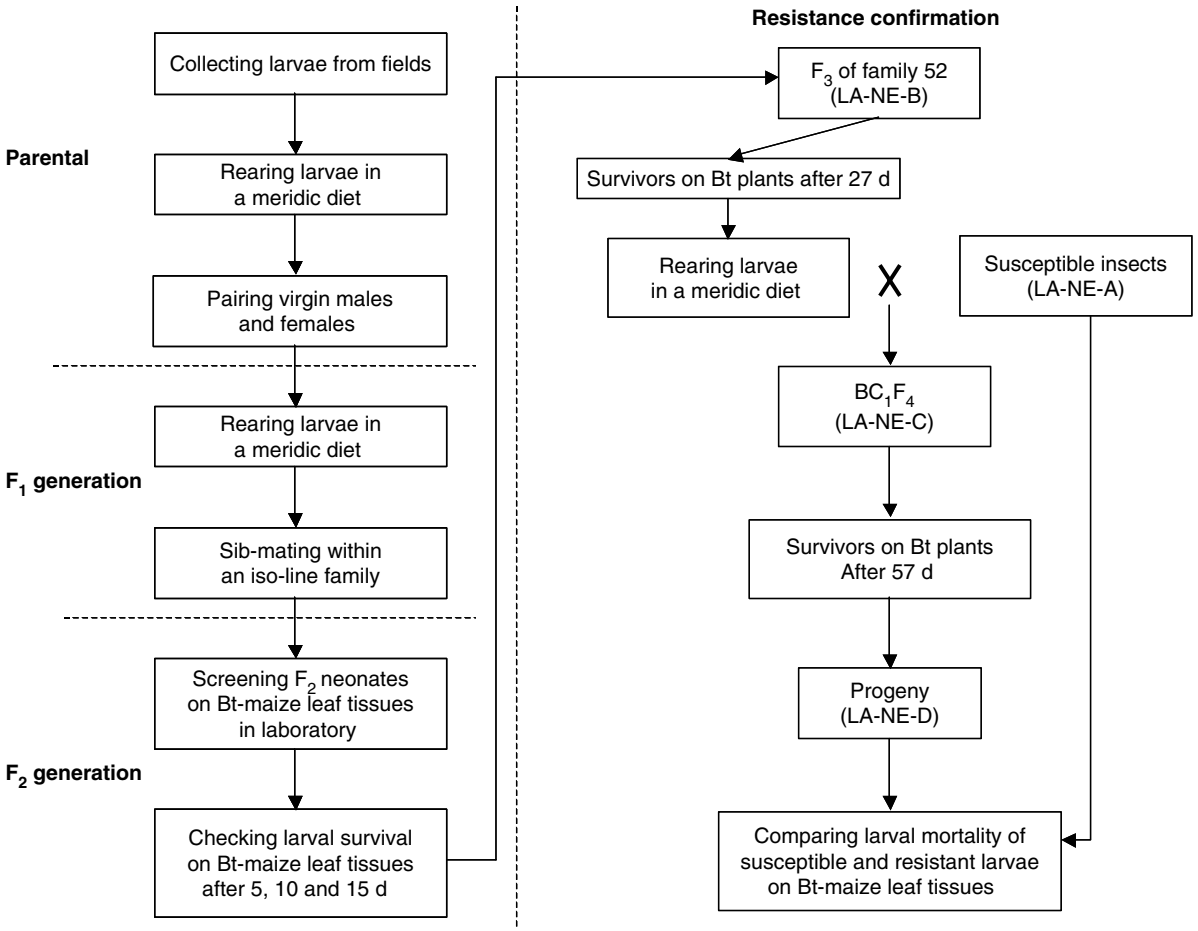

Fig. 1. Illustration of F_2 screening procedures for detecting Bt resistance alleles in *Diatraea saccharalis*.

from a collection near Winnsboro, Louisiana in 2004 were released on the leaf tissues in each well. The trays were placed in an environmental chamber and maintained at the same conditions as described above. Preliminary tests showed that most larvae of *D. saccharalis* were late 2nd instars or 3rd instars after 10 days on non-Bt-maize leaf tissues. Large larvae (≥3rd instar) of *D. saccharalis* usually bore into plant stalks and do not feed on fully-expanded leaves in the field. Thus, bioassays with non-Bt-maize leaf tissues were terminated 10 days after larval release. Larval survival was evaluated at 5 days and 10 days after inoculation. Leaf tissues were replaced with fresh leaf tissues every 3 days. For each test duration, there were five replications ($N = 150$).

Screening F_2 neonates on Bt-maize leaf tissues

Seeds of the DKC69-70 hybrid (Monsanto, St Louis, MO) were planted in a greenhouse as described above. DKC69-70 is a Bt-maize hybrid expressing the Cry1Ab protein. This cultivar is commonly planted in Louisiana to control a corn borer complex of *D. saccharalis* and the southwestern corn borer, *D. grandiosella* Dyar (Castro *et al*., 2004). It is genetically similar to the DK697 non-Bt-maize hybrid. F_2 neonates from each two-parent family were screened in 8-well trays (C-D International) containing Bt-maize leaf tissues dissected from fully-expanded leaves of V6–V8 greenhouse Bt plants. The F_2 screening trays were maintained at the same environmental conditions as used for assessing larval survivorship on non-Bt-maize leaf tissues, except the non-Bt leaf tissues were replaced with Bt-maize leaf tissues. In each family, approximately 60 F_2 neonates were screened in each of five wells (300 larvae per family). Previous laboratory bioassays showed that susceptible larvae of *D. saccharalis* survived on Bt-maize leaf tissues for more than 4 days (Huang *et al*., 2006). However, larval growth and development of *D. saccharalis* on Bt-maize leaf tissues were delayed considerably compared to on non-Bt-maize leaf tissues. Therefore, to increase the ability for detecting Bt resistance in *D. saccharalis*, F_2 neonates of each family were screened on Bt-maize leaf tissues for 15 days, 5 days longer than the time that larvae were exposed to non-Bt-maize leaf tissues. The number of surviving larvae was recorded at 5 days, 10 days and 15 days after inoculation. Leaf tissues were replaced every 3 days. Surplus eggs (if any) for each family were held in an environmental chamber maintained at 21°C, 50% RH and a 16:8 h (L:D) photoperiod. These eggs (or larvae hatched from the eggs) were discarded if no F_2 larvae survived in the F_2 screen. If any larvae survived, the surplus larvae of the family (if available) were continuously

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reared on a meridic diet and used as a backup strain for that family.

Resistance confirmation on Bt plants

Data from the F_2 screen indicated at least one family (family 52) might possess a major Bt resistance allele. F_3 larvae (LA-NE-B) of this family were selected on Bt-maize plants in a greenhouse. DKC69-70 was planted and thinned to one plant per pot two weeks later. At the V12–V13 stages, ten F_3 neonates (<24 h old) of family 52 (LA-NE-B strain) were manually placed into the whorl of each of 27 plants. After 27 days, all live F_3 larvae recovered from Bt plants were transferred into 30-ml plastic cups containing 10 ml of a meridic diet, and survivors were reared to the pupal stage. Pupae were removed from the larval rearing cups and sexed. Newly-emerged virgin moths from the F_3 survivors were individually transferred into 450-ml paper containers (Huhtamaki Foodservice, De Soto, KS), one moth per container. The design of the moth containers was similar to that used in the moth pairing for establishing two-parent families. To facilitate colony establishment, each of the four earliest emerged moths derived from the F_3 survivors was immediately provided with three newly emerged virgin males from the susceptible LA-NE-A strain if it was a female or three newly emerged virgin females of the LA-NE-A strain if it was a male (backcross, BC_1). The pooled backcross neonates (BC_1F_3) produced from the four backcrosses were reared on a meridic diet. The BC_1F_3 adults were mated in 3.8-l cardboard cartons (Neptune Paper Products, Newark, NJ) under the same conditions as described above. BC_1F_4 eggs of the backcross were harvested from wax papers attached against the inside wall of the cartons.

To confirm resistance, survival of BC_1F_4 neonates (LA-NE-C) was evaluated on Bt plants in the greenhouse. Five Bt-maize seeds were planted in each pot (18.9 l) within the greenhouse. The plants were thinned to three plants per pot after two weeks. At V12–V13 stages, approximately 80 neonates (<12 h) of LA-NE-C strain were placed into the whorl of each Bt plant using the same methods as described above. A total of approximately 6000 larvae were exposed to 75 Bt plants in 25 pots. In addition, 21 Bt plants (seven pots) were infested with neonates of the susceptible LA-NE-A strain (control). All Bt plants were tested for the Cry1A expression with Cry1Ab/Cry1Ac Lateral Flow QuickStix™ Strip Kit (Envirologix, Portland, ME) before insect infestations. To determine the termination time of these tests, larval development on non-Bt-maize plants was monitored by infestation of 10 LA-NE-C neonates on each of 12 non-Bt plants in four pots in the same greenhouse room. To reduce the risk of larvae moving from non-Bt plants to Bt plants, the four pots with non-Bt plants were placed in one corner of the greenhouse and separated from Bt plants by >3 m. During the initial screen of family 52 (LA-NE-B strain), larval development on Bt-maize plants was significantly delayed compared to the larvae on non-Bt plants. Therefore, during the resistance confirmation tests, larval development was monitored on Bt plants. After the first pupa was observed on non-Bt-maize plants, all remaining non-Bt-maize plants were removed from the greenhouse. Larval development on Bt plants was then monitored twice a week (Monday and Friday) by recording the number of surviving larvae on three Bt-maize plants in three pots, one plant from each pot. The greenhouse tests were terminated 57 days after inoculation, when pupae were first observed on Bt plants. At the point of test termination, 55 Bt plants were still available for data collection. These Bt plants were dissected to record number and locations of surviving insects.

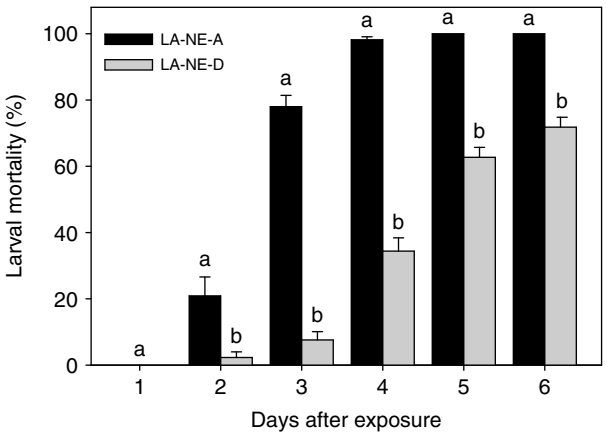

Fig. 2. Larval mortality (%) of Bt-susceptible (LA-NE-A) and Bt-resistant (LA-NE-D) strains of *Diatraea saccharalis* on DKC69-70 Bt-maize leaf tissues. Mean values within a specific test period followed by a different letter are significantly different ($P < 0.05$; Cochran and Cox's *t*-test).

Resistance confirmation on Bt leaf tissues

Pupae and pre-pupae (mature larvae) of BC_1F_4 recovered from Bt plants after 57 days were transferred into cardboard cartons (Neptune Paper Products, Newark, NJ) for adult emergence, mating and oviposition. A Bt 'resistant' insect strain (LA-NE-D) was established from the eggs produced from these survivors. Larval mortality of the susceptible LA-NE-A and the resistant LA-NE-D were assayed on leaf tissues of non-Bt and Bt-maize plants in the 8-well trays using a similar method as described above. In the bioassays, 20 neonates were released on the maize leaf tissues in each well of the 8-well trays. Larval mortality was recorded every day for 6 days. There were four replications for each combination of insect strain, maize cultivar and test duration, and each replicate included 80 neonates in four wells ($N = 320$). Each replication was treated separately.

Larval mortality of *D. saccharalis* on Bt-maize leaf tissues was corrected using the method of Abbott (1925). The corrected mortality data were transformed using arcsine ($x^{0.5}$) (Zar, 1984). Differences in larval mortality between LA-NE-A and LA-NE-D strains at a specific observation time were analyzed using *t*-tests (SAS Institute, 1999). Treatment means were separated using the conservative Cochran and Cox test at the $\alpha = 0.05$ level (Cochran & Cox, 1950). Untransformed data are presented in fig. 2.

Results

Establishment of two-parent families

A total of 228 pairs of *D. saccharalis* adults were established from a collection of 629 larvae during the first field generation. However, only 45 families (19.7%) produced enough F_2 progeny (>300 neonates) for the F_2 screen. An average of 44 ± 3 (mean ± SEM) F_1 pupae for each family

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Table 1. Summary of an F_2 screen searching for Bt resistance alleles in a Louisiana population of *Diatraea saccharalis*.

Generation	No. larvae	No. pairs of adults	No. families	Survival 5 DAE[1]		Survival 10 DAE[1]		Survival 15 DAE[1]	
				No. families	No. larvae	No. families	No. larvae	No. families	No. larvae
1st	629	228	45	5	40	0	0	0	0
2nd	558	197	168	45	608	4	37	3	26
Total	1,187	425	213	50	648	4	37	3	26

[1] Days after exposure.

were used to produce the F_2 progeny for the insects collected in the first generation. The success rate was higher for insects collected during the second generation. A total of 168 iso-line families from 197 adult pairs (85.3%) produced enough F_2 neonates for the F_2 screen. An average of 41 ± 2 F_1 pupae per family were used to produce F_2 larvae for the insects collected in the second generation.

Larval survivorship of D. saccharalis *on non-Bt-maize leaf tissues*

D. saccharalis neonates feeding on non-Bt-maize leaf tissues survived well in the laboratory. The larval survival rate (mean ± SEM) was $94 \pm 1\%$ after 5 days and $91 \pm 1\%$ after 10 days.

Screening F_2 neonates on Bt-maize leaf tissues

All F_2 larvae of 163 families out of the 213 iso-line families were killed on Bt-maize leaf tissues after 5 days (table 1). Only 40 F_2 larvae from five families survived after 5 days, for the insects collected during the first field generation, and all of these larvae were dead after 10 days. A total of 608 larvae in 45 families, established from insects collected during the second field generation, survived the Bt-maize leaf tissues for ≥5 days. Among these, 37 larvae in four families and 26 larvae in three families survived after 10 days and 15 days, respectively. Twenty of the 26 survivors after 15 days were from one family (family 52). The other six survivors were from families 146 and 176. Three larvae of family 147 also were alive after 10 days, but died after 15 days. No success in establishing colonies from these survivors was achieved because of the limited number of larvae and differences in larval development and moth emergence time.

Resistance confirmation on Bt-maize plants

All Bt-maize plants in the greenhouse tested positively for Cry1A endotoxin expression. No surviving larvae were found on the Bt plants 57 days after inoculation with neonates of the susceptible LA-NE-A strain. A total of 14 F_3 larvae of family 52 (LA-NE-B) were recovered from nine Bt plants after 27 days. A large number (>10,000) of BC_1F_4 progeny (LA-NE-C) were produced from the backcross. On the 57th day after larval inoculations, a total of 351 larvae and pupae of the LA-NE-C strain were recovered from 51 out of the 55 Bt plants tested. These survivors included 20 pupae, 48 large larvae (pre-pupae or 5th instars) and 283 small larvae (2nd–4th instars). The pupae and larvae were viable and were located within the stalks (182 insects), on the ears (79 insects) and in the ear shanks (90 insects). All moths derived from the pupae and pre-pupae emerged normally as observed for those reared on the meridic diet. The emerged moths laid viable eggs (see below).

Resistance confirmation on Bt-maize leaf tissues

Larval mortality on non-Bt-maize leaf tissues during the 6 day test period ranged from $4 \pm 1\%$ (mean ± SEM) to $13 \pm 1\%$ for the LA-NE-A susceptible strain and from $3 \pm 1\%$ to $9 \pm 3\%$ for the LA-NE-D resistant strain. The difference in larval mortality on non-Bt leaf tissues between the two *D. saccharalis* strains was not significant ($t \leq 1.16$, $df = 3$, $P \geq 0.3316$).

Neonates of the LA-NE-D resistant strain were more tolerant to Bt-maize leaf tissues than were those of the LA-NE-A susceptible strain (fig. 2). No mortality occurred after 1 day for either strain on Bt-maize leaf tissues. However, significant mortality, $21 \pm 6\%$ (mean ± SEM), of the LA-NE-A strain was observed after 2 days. Mortality of the susceptible strain on Bt leaf tissues was $98 \pm 1\%$ after 4 days and 100% after 5 days. Larval mortality of the resistant LA-NE-D on Bt leaf tissues was significantly lower ($t < 3.12$, $df = 3$, $P < 0.05$) than that of the LA-NE-A strain across the test periods except at 1 day. Significant mortality of LA-NE-D did not occur on Bt leaf tissues until 5 days ($63 \pm 3\%$) and 6 days ($72 \pm 3\%$).

Discussion

Since the F_2 screen was proposed as a method for identifying Bt resistance alleles in *O. nubilalis* by Andow and Alstad (1998), several modifications of the screening protocol have been evaluated to detect Bt resistance in *O. nubilalis* (Andow *et al*., 1998, 2000; Bourguet *et al*., 2003; Stodola & Andow, 2004); *D. grandiosella* (Huang *et al*., 2007); a rice stem borer, *Scirpophage incertulas* (Walker) (Bentur *et al*., 2000); a coleopterous poplar leaf beetle, *Chrysomela tremulae* F. (Génissel *et al*., 2003); and diamondback moth, *Plutella xylostella* (L.) (Zhao *et al*., 2002). Indeed, Stodola and Andow (2004) indicated that many potential modifications could be made to the F_2 screen to adapt it to other species and to improve its efficiency.

The present study used a new method to develop two-parent families for the F_2 screen. The families of *D. saccharalis* were established from field-collected larvae rather than field-collected adult males or mated females. Starting with field-collected larvae of *D. saccharalis* was cost-effective for several reasons. Larvae of *D. saccharalis* can be easily collected from maize plants, particularly from late maize plantings. For example, in 2004, >700 1st generation larvae of *D. saccharalis* and >600 2nd generation larvae were collected from late-planted maize fields near Winnsboro,

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Louisiana, in only a few hours. In addition, there is no sex pheromone available for trapping male *D. saccharalis*, and adult moths of *D. saccharalis* are calm during the daytime so that they are easy to pair mate.

Although the success rate (<20%) of screening two-parent families was low for samples from the first field generation, success (>85%) was substantially higher for pairs from the second field generation. The reasons for the considerable improvement in the success rate are unknown. The addition of a small amount (10 g) of vermiculite in each adult container for the insects derived from the 2nd generation collection might have improved the environmental conditions for adult mating and oviposition.

Another notable modification of the F_2 screen developed in the present study was the use of Bt-maize leaf tissues for screening F_2 neonates rather than directly screening F_2 larvae on Bt plants (Andow *et al*., 1998, 2000; Bourguet *et al*., 2003) or on a diet containing Bt protoxins (Zhao *et al*., 2002; Bourguet *et al*., 2003). The use of Bt-maize leaf tissues may have several advantages above screening on whole Bt plants. First, when screening a large number of iso-line families, using Bt-maize leaf tissues saves considerable space and can be conducted in growth chambers in the laboratory. If whole Bt plants are used, it must be done in the field or in large greenhouses. This also reduces the cost and labour involved in an F_2 screen. Secondly, field collections of corn borers (e.g. *O. nubilalis* or *D. grandiosella*) are often done during the second field generation, which typically occurs in June to October, depending on geographical location. Because the insects are reared in a laboratory for two generations, screens would most likely be conducted during the winter season. In the northern US, whole plant assays could only be done in greenhouses at considerable expense compared to leaf tissue assays, which require significantly fewer plants. Thirdly, it is much easier to recover survivors from maize leaf tissues in small bioassay trays than from whole Bt plants. The high larval survival and recovery rates of *D. saccharalis* on non-Bt-maize leaf tissues observed in the present study also suggest that the use of maize leaf tissues is an effective method to assay larval mortality of *D. saccharalis*. Finally, it minimizes the effect of any potential Bt concentration variations that might occur within plants. Although this has not been demonstrated, research with Bt-broccoli whole plant assays has suggested that a whole plant assay may produce more false negatives than is desirable (Zhao *et al*., 2002).

In addition, the method has some advantages over diet assays. Firstly, it does not require synthesis, purification and quantification of Cry toxin. Secondly, it does not require determination and validation of a diagnostic dose for detecting resistance alleles. Thirdly, it is unnecessary to use a toxin model when it is difficult to purify the toxin as expressed in the plant. For example, use of more readily obtained Bt protoxin, trypsinized Bt toxin or microbial formulations, such as Dipel, may not accurately reflect the status of insect resistance to Bt-maize because the Bt proteins expressed in Bt plants can be different from these model systems (National Research Council, 2002).

Selection of properly aged leaves is important in the F_2 screen with Bt-maize leaf tissue. In preliminary tests, leaves removed from older maize plants (V12–V13) could be maintained longer in the laboratory than younger leaves (data not shown). However, these data also showed that old leave tissues excised from non-Bt-maize plants could cause higher larval mortality to *D. saccharalis* than younger leaves. In our study, mortality of *D. saccharalis* larvae feeding on non-Bt-maize leaf tissues excised from fully expanded leaves of V6–V8 plants was low, <9% after 10 days. These tissues can be maintained in the screening trays for at least 3 days, suggesting that fully expanded leaves of V6–V8 Bt plants are suitable for assays for Bt resistance alleles. *D. saccharalis* has three overlapping generations a year on maize in the mid-southern region of the US (Castro *et al*., 2004). Neonates of first generation typically occur during V8–V12 maize plant stages, while the second and third generations occur during reproductive plant stages. Therefore, any potential resistance identified in the laboratory bioassays with leaf tissues needs to be further evaluated in intact whole Bt plants to confirm the resistance, because Bt concentration variations might occur in different plant stages and Bt expression in leaf tissues might be different compared to whole plants.

In this study, we were unable to establish colonies directly from the F_2 survivors after screening on Bt-maize leaf tissues to confirm resistance because of the limited number of survivors and differences in developmental times. Instead, the insect population was increased by rearing the surplus F_2 larvae of family 52 to F_3 generation. F_3 progeny of family 52 were screened on greenhouse Bt-maize plants. The F_3 survivors recovered from Bt plants were then backcrossed with a susceptible family. Finally, the F_2 offspring of this backcross were selected again on Bt-maize plants in the greenhouse to verify and isolate resistance in a single resistant line (LA-NE-D). However, if the F_2 screen survivors were immediately backcrossed to a susceptible strain and the F_2 progeny of the backcross were screened and selected on Bt plants, resistance could be verified and isolated at a lower cost compared to the present method.

Bt resistance confirmed in family 52 using intact Bt-maize plants in the greenhouse indicates that the 15 day screening procedure was effective and feasible for detecting Bt resistance alleles in *D. saccharalis*. In contrast, the 5 day screen apparently was not sufficiently severe for detecting Bt resistance in *D. saccharalis* because many families (23%) survived on Bt-maize leaf tissues for ≥5 days, but most of these survivors died on Bt-maize leaf tissues after 10 days. Data from the present study are not sufficient to conclude that the 10 day screening procedure is a valid approach for detecting Bt resistance in *D. saccharalis*. Three of the four families (including family 52) that survived the Bt-maize leaf tissues in the 10 day screen procedure also survived after 15 days. However, except family 52, survival rates of the other three families were very low, ≤1.7% after 10 days and ≤1.3% after 15 days. The results suggest that the 10 day screening procedure might be used to detect both major and partial resistance alleles in *D. saccharalis*. However, additional laboratory bioassays are needed to validate such abilities for the 10 day screening procedure. In summary, we recommend, at this point, the 15 day procedure for screening Bt resistance alleles in *D. saccharalis*.

One disadvantage associated with the use of Bt-maize leaf tissues for the F_2 screen, compared to the use of whole Bt plants, is that the concentration or biological activity of the Bt toxin (Cry1Ab) in leaf tissues may change after removal from the whole plants. Preliminary tests showed that insecticidal activity of Bt-maize leaf tissues did not significantly change for at least 4 days after removal from whole plants unless the leaf tissues were chlorotic yellow (FH, BY & BRL., unpublished data). Laboratory bioassays have shown that leaves removed from transgenic Bt poplar

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trees were lethal to larvae of *C. tremulae*, regardless of leaf age, and maintained the insecticidal activity for at least 3 days in the laboratory conditions (Génissel *et al*., 2003). In addition, dissociated leaf tissues may produce additional defense mechanisms that may have affected the survival of Bt-resistant larvae. However, bioassays with neonates of *D. saccharalis* and two other Bt-susceptible maize borer species, *O. nubilalis* and *D. grandiosella*, did not show significant mortality on dissociated non-Bt-maize leaf tissues (Huang *et al*., 2006), suggesting such effect, if any, should be limited and not be practically important in the F_2 screen.

The F_2 screen technique developed in this study could be easily modified for detecting rare Bt resistance alleles in other similar corn stalk borers, such as *O. nubilalis* and *D. grandiosella* (Huang *et al*., 2007). In fact, the protocol may be even more suitable for screening these two corn borer species than for *D. saccharalis*. Larvae of *O. nubilalis* and *D. grandiosella* are more susceptible to Bt-maize leaf tissues than *D. saccharalis* (Huang *et al*., 2006). For example, under the same environmental conditions as used in the present study, 100% of larvae of *O. nubilalis* and *D. grandiosella* were killed on DKC69-70 Bt-maize leaf tissues within 4 days (Huang *et al*., 2006), suggesting that a 4 day screening period could be used for these two species instead of the 5 day screening used for *D. saccharalis*. As discussed above, a 4 day screening period implies that changes of leaf tissues in the screen trays may not be necessary. Thus, cost of screening *O. nubilalis* and *D. grandiosella* would be further reduced.

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