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ARTHROPOD MANAGEMENT

The Impact of Transgenic Cottons Expressing One or Two Proteins from *Bacillus thuringiensis* on Survival and Damage Potential of First and Second Instars of *Ostrinia nubilalis* (Lepidoptera: Crambidae)

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ABSTRACT

European corn borer, *Ostrinia nubilalis* (Hübner), is susceptible to toxins produced by *Bacillus thuringiensis* (Berliner), but damage to Bollgard (Bt) cotton (*Gossypium hirsutum* L.), which produces the cry1Ac δ–endotoxin, has been observed in the field. Laboratory studies investigated the comparative survival and damage potential of first and second instar larvae of *O. nubilalis* on transgenic cotton bolls in 1993 and 1994. After 6 d, a significantly higher percentage of second instars survived on both non-Bt and Bt bolls compared with first instars. A similar trend, greater damage by second instars than first instars on non-Bt and Bt bolls, was detected for the percentage of damaged bolls for both genotypes. An additional experiment was performed in 2004 that included non-Bt, Bollgard, and Bollgard II cotton cultivars. Bollgard II was included because it expresses two Bt proteins, cry1Ac and cry2Ab. Percentage survival of second instars was significantly higher than that of first instars after 6 d on non-Bt and Bollgard bolls. A similar trend was observed for Bollgard II bolls, but the differences in larval survival between instars was not significant. Second instar larvae damaged a significantly higher proportion of bolls of non-Bt, Bollgard, and Bollgard II cultivars than first instars. These data suggest that the presence weed hosts of *O. nubilalis* in cotton fields may lead to economic injury in Bt cottons. Results from this study also indicate that boll damage by *O. nubilalis* may be higher for Bt cottons due to row-to-row movement in embedded refuge plantings.

The European corn borer [*Ostrinia nubilalis* (Hübner)] is a well-documented pest of cotton in North Carolina (Gourd and Gouger, 1983; Savinelli et al., 1986). Larvae of *O. nubilalis* cause economic damage to cotton by boring into leaf petioles, stems, and bolls. Fiber and seed may be destroyed within damaged bolls. In addition, various boll rot fungi (Simpson et al., 1973; Palmateer et al., 2004) gain entrance to bolls after the carpel wall has been penetrated by *O. nubilalis* and destroy the entire boll. Prior to the widespread adoption of Bollgard cotton, damage to cotton by *O. nubilalis* exceeded that of bollworm [*Helicoverpa zea* (Boddie)] and tobacco budworm [*Heliothis virescens* (F.)], in certain years (Head, 1990; Bacheler, 1991). One reason for the high level of damage was that larvae of *O. nubilalis* could penetrate bolls that were not susceptible to attack from the bollworm complex (Ellsworth and Bradley, 1992). Damage to bolls of Bollgard cotton has been minimal because larvae of *O. nubilalis* are highly susceptible to the cry1Ac δ–endotoxin, even across geographic locations, pheromone races, and voltine ecotypes (Marçon et al., 1999).

Aside from cotton, *O. nubilalis* has over 200 host plants, including both crop and weed hosts (Caffrey and Worthley, 1927; Hodgson, 1928; Mason et al., 1996). Many of these weed hosts can be found in and adjacent to cotton fields, although the duration of weed presence may be shorter in cottons containing Roundup Ready technology. While oviposition of *O. nubilalis* occurs on cotton, weed hosts of *O. nubilalis* can serve as the primary innoculum source of first instar and larger larvae for eventual infestation of cotton plants. Coupled with the observation that second instars of *O. nubilalis* had a greater propensity to penetrate non-Bt bolls than first instars (Ellsworth and Bradley 1992), the survival and damage potential of first and second instars of *O. nubilalis* in Bollgard cotton needs to be addressed.

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Although Bollgard/Roundup Ready cotton cultivars have been extensively planted for 8 yr, natal weed hosts of *O. nubilalis* can be found within and/or adjacent to many of these fields. Two hosts that are commonly observed in cotton fields because of tolerance to glyphosate are horseweed (*Conyza canadensis* L. Cronq.) and pigweed (*Amaranthus* spp.). Other weed hosts of *O. nubilalis* can be present in cotton because of untimely application of glyphosate. Adults of *O. nubilalis* deposit eggs onto existing weeds where larval development begins. Once glyphosate has killed the weeds, first instar or larger larvae may move to adjacent cotton plants. Consequently, damage to Bollgard cottons by *O. nubilalis* has occurred (J. W. Van Duyn, personal communication). The presence of a few weeds can produce large numbers of larvae that may move to Bt plants. Cocklebur (*Xanthium strumarium* L.), barnyard grass [*Echinochloa crus-galli* (L.) Beauv.], knotweed (*Polygonum aviculare* L.), and pigweed (*Amaranthus* spp.) have been reported to produce 200, 150, 98, and 48 larvae of *O. nubilalis* from a single plant, respectively (Hodgson, 1928). Other sources of inoculum of *O. nubilalis*, such as wheat (*Triticum aestivum* L.), may mature when cotton seedlings are available. The larvae may move from mature wheat to cotton seedlings to complete development.

Bollgard cotton produces the Cry1Ac δ-endotoxin, which is toxic to the primary lepidopterous pests of cotton. Bollgard II cottons produce two Bt endotoxins, cry1Ac and cry2Ab, which are active against lepidopterous pests. Because Bollgard II cottons have shown greater efficacy than Bollgard against caterpillar pests other than *O. nubilalis* (Jackson et al., 2003; Stewart et al., 2001), the survival and damage potential of first and second instars of *O. nubilalis* was measured in Bollgard and Bollgard II cotton cultivars.

This research evaluated the survival and damage potential of first and second instars of *O. nubilalis* on non-Bt and cry1Ac-producing Bt cotton genotypes. First and second instar survival and damage potential were also assessed on non-Bt, Bollgard, and Bollgard II cultivars.

MATERIALS AND METHODS

Plant tissues. Field plots were established at the Tidewater Research Station in Washington County, NC, in 1993 and 1994. Plots were planted to two cotton genotypes, non-Bt Coker 312 and its cry1Ac-producing sister line event 531, and replicated four times in a randomized complete block design. In 2004, field plots were planted to three cotton cultivars, Fibermax 991R (non-Bt; FM 991R; Bayer Cropscience, Research Triangle Park, NC), Fibermax 991BR (Bollgard; FM991BR), and Fibermax 991B2R (Bollgard II; FM991B2R). Each treatment was replicated four times in a randomized complete block design. Agronomic practices applied to field plots were conducted according to North Carolina Extension Service recommendations. Approximately 3 to 4 wk after first bloom, 100 bolls that were 7 to14 d old were collected from each plot. Bolls of this age were used because they are phenologically susceptible to attack from *O. nubilalis*. To determine boll age, white blooms from each treatment were tagged. After 7 d, white blooms were again tagged. Approximately 7 d after the second tagging, bolls smaller than those first tagged and larger than the last tagged were collected from each plot for testing. Bolls were collected by cutting the fruiting branch approximately 7.5 cm from the boll toward the main stem with pruning shears. Excised bolls were immediately placed into coolers with ice packs and transported to the laboratory where they were immediately placed into water picks.

Test insects. During late May 1993 and 1994, larvae of *O. nubilalis* were collected from field-grown potato plants on the Tidewater Research Station. Larvae were placed onto artificial diet (Burton, 1970) in 30 ml plastic cups and transported to the laboratory. Larvae were reared at 25 °C under a 16:8 light:dark photoperiod regime. Upon eclosion, adults were mass mated and eggs were collected to establish a test colony. In late July of 2004, egg masses of *O. nubilalis* were collected from corn on the Tidewater Research Station. Neonates that hatched within 24 h were placed onto artificial diet and reared until second instar. Neonates that hatched at 72 h served as first instar test insects and were infested directly onto bolls. The populations of *O. nubilalis* used in these experiments were most likely the E-pheromone strain that predominates in eastern North Carolina and are known to use cotton as a host (Sorenson et al., 1992).

Experimental procedures. Cup cages as described by Ellsworth and Bradley (1992) were used in these experiments. A single boll was placed into each cage, and each boll was infested with a single first or second instar larva of *O. nubilalis*. Larvae were held in cages at 25 °C for 6 d. After 6 d, each boll was

removed from its cage and scored for the presence of a live larva and/or feeding damage (penetration of the carpel wall of the boll). Four treatment combinations were assayed with this procedure in 1993 and 1994; Coker 312 bolls infested with a first instar larva, Coker 312 bolls with a second instar larva, Bt event 531 with a first instar larva, and Bt event 531 with a second instar larva. Four hundred bolls of each treatment combination (100 bolls per replicate) were assessed during 1993 and 1994. Six treatment combinations were evaluated in 2004; non-Bt, Bollgard, and Bollgard II bolls each infested with either a first or second instar larva. One hundred bolls of each treatment combination (25 bolls per replicate) were evaluated in this study.

Statistical analysis. Numbers of live larvae of *O. nubilalis* and damaged bolls were converted to percentages and subjected to arcsine square root transformation prior to analysis. These data were then subjected to ANOVA using PROC GLM (ver. 9.1.3, SAS Institute; Cary, NC). Treatments were compared using Fisher's Protected LSD test ($P \leq 0.05$). Results for data transformed before analysis are reported as untransformed arithmetic means and standard deviations. Because *O. nubilalis* is known to be susceptible to Bollgard cottons, analyses were conducted to determine whether larval survival and damage potential were different between larval instars within each genotype.

RESULTS

Averaged across years (1993-1994), survival of second instar larvae of *O. nubilalis* was significantly higher than first instars after 6 d on non-Bt (F = 35.08, df = 1, 6; P = 0.001) and Bt (F = 21.30, df = 1, 6; P = 0.004) bolls. The percentage survival of first and second instar larvae on non-Bt bolls was 57.8% and 77%, respectively. Larval survival on Bt bolls for first and second instars was 2.5% and 22.3%, respectively.

A similar trend existed for penetration of the boll wall by first and second instars of *O. nubilalis*. First instar damage to non-Bt bolls averaged 54.5%, which was significantly less than the 74.3% damage from second instars (F = 22.86, df = 1, 6; P = 0.003). On Bt bolls, second instars damaged a significantly higher percentage of bolls than first instars (F = 102.81, df = 1, 6; $P < 0.001$) For Bt bolls, 7.5% were damaged by first instars and 44% were damage by second instars.

Survival of larvae of *O. nubilalis* and associated boll damage were similar for all cultivars tested in 2004. Survival of second instars was significantly higher than first instars after 6 d on non-Bt (F = 46.09, df = 1, 3; P = 0.007) and Bollgard (F = 15.70, df = 1, 3; P = 0.029) bolls; however, survival between instars was not different on Bollgard II (F = 9, df = 1, 3; P = 0.0577) bolls. First instar survival averaged 48% compared with 61% for second instars on non-Bt bolls. On Bollgard bolls, larval survival for first and second instars was 3% and 18%, respectively. Survival of *O. nubilalis* on Bollgard II bolls was 1% and 4% for first and second instars, respectively.

The percentage of bolls penetrated by second instars of *O. nubilalis* was higher than first instars on non-Bt (F = 30.94, df = 1, 3; P = 0.012), Bollgard (F = 26.41, df = 1, 3; P = 0.014), and Bollgard II (F = 26.68, df = 1, 3; P = 0.014) bolls. Non-Bt bolls sustained 63% damage by first instars compared with 82% caused by second instars. First instars penetrated only 10% of Bollgard bolls, whereas 48% of the bolls were damaged by second instars. Boll damage caused by first and second instars of *O. nubilalis* on Bollgard II bolls was 1% and 15%, respectively.

DISCUSSION

Survival and damage potential of first and second instars of *O. nubilalis* on non-Bt bolls in this study are similar to that reported by Ellsworth and Bradley (1992), who described the increased ability of second instars to survive and to penetrate the carpel wall of non-Bt bolls compared with first instars. Although larvae of *O. nubilalis* are susceptible to the cry1Ac toxin in the laboratory and field (Marçon et al., 1999; Huang et al., 2002), these data demonstrate that second instars are less susceptible than first instars to the cry1Ac protein expressed in Bollgard cotton. These results also agree with those of Huang et al. (1999) in which first instars of *O. nubilalis* were more susceptible than second instars to artificial diet containing commercial formulations of Bt toxin. The greater survival for second instars was directly related to more boll damage on Bollgard cottons. Second to third instar larvae of other cotton pests, such as the heliothine complex, were also less susceptible to the cry1Ac toxin than first instars (Parker and Luttrell, 1999; Parker et al., 2000).

Bollgard II cultivars provide increased control of various lepidopteran pests of cotton compared with Bollgard cotton (Jackson et al., 2003; Stewart

et al., 2001). In this study, survival of larvae of *O. nubilalis* and damage on Bollgard II bolls was lower than on Bollgard bolls. With the additional production of the Cry2Ab endotoxin, Bollgard II expresses a higher protein titer than Bollgard (Greenplate et al., 2000). Survival and damage on Bollgard II was lower than on Bollgard, and second instars remained less susceptible than first instars on Bollgard II. The increased activity of Bollgard II cottons on *O. nubilalis* provides an added value to farmers.

From an economic standpoint, it is important to note that although most larvae of *O. nubilalis* on Bt bolls died by 6 d, some first instars and a higher proportion of second instars were able to penetrate the carpel wall of the boll before death. Even if larvae do not directly damage fiber and seed, boll rot organisms typically destroy the entire boll in the Southeastern USA when the carpel wall is breeched. Alternate hosts of *O. nubilalis* within the field, such as *Amaranthus* spp. and *C. canadensis*, that may serve as potential inoculum sources of *O. nubilalis* must be removed in a timely manner to avoid the risk of economic injury. If inoculum sources remain, both Bollgard and Bollgard II cottons may require supplemental insecticide applications that must be well-timed because of the boring behavior of *O. nubilalis*. Inoculum sources of *O. nubilalis* within the field will also increase as glyphosate-tolerant weeds continue to emerge.

While these data provide inference to the importance of timely weed control practices with respect to survival of *O. nubilalis* on and damage to Bt cotton, implications also exist for Bt resistance management of *O. nubilalis*. Seed mixtures consisting of non-Bt and Bt cottonseed have been suggested as a possible refuge option for Bt cotton; however, these data indicate that a mixed seed approach would potentially hasten resistance development through movement of larger, less susceptible larvae from non-Bt to Bt plants, as observed for heliothines (Parker et al., 2000; Mallet and Porter, 1992). The potential for accelerating the evolution of resistance also exists in systems using the embedded refuge option due to row-to-row movement of larvae from non-Bt plants to Bt plants. These data do not document the ability of larvae *O. nubilalis* to complete development on Bt cottons and indeed suggest that few can complete development on these transgenic lines. Implications can only be made as to the possible impact of increased larval survival with increased larval instar on Bt resistance evolution in *O. nubilalis*.

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