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Asunto : Solicitud de patente para **PLANTA EVENTO DE ALGODÓN
MON15985 Y COMPOSICIONES Y MÉTODOS PARA
DETECCIÓN DEL MISMO**

Solicitante : MONSANTO TECHNOLOGY LLC

Fecha de solicitud : 11 de junio de 2002.

1. Yo, Emilio FERRERO, obrando como apoderado del solicitante en el asunto antes identificado, me refiero al Oficio No. **12665** notificado por fijación en lista del 02 de noviembre de 2006, y atentamente presento junto con este escrito los siguientes artículos:

- ADAMCZYK, J.J., ET AL.: "Evaluation of BollgardII (cv. DP50BII) in the mississippi delta: field efficacy against various lepidoptera while profiling season-long expression of Cry1Ac and Cry2Ab" PROCEEDINGS OF THE BELTWIDE COTTON CONFERENCE, HELD JAN. 9-13, 2001, vol. 2, 2001 pages 835-836, XP008054531.
- AKIN, D.S., ET AL.: "Field efficacy of cotton expressing two Insecticidal proteins of Bacillus thuringiensis" PROCEEDINGS OF THE BELTWIDE COTTON CONFERENCE, HELD JAN. 9-13, 2001, pages 1041-1043 XP008054529.

- NORMAN, J.W., ET AL: "Performance of bollgard II cotton against lepidopterous pests in the lower rio grande valley of texas" PROCEEDINGS OF THE BELTWIDE COTTON CONFERENCE, HELD JAN. 9-13 2001, vol. 2, 2001, pages 833-835, XP008054530.
- JACKSON, R.E., ET AL: "Efficacy of bollgard and bollgard II cottons against bollworm, *Helicoverpa zea* (Boddie) in field and greenhouse studies" PROCEEDINGS OF THE BELTWIDE COTTON CONFERENCE, HELD JAN. 9-13, 2001 vol. 2, 2001, pages.815-818, XP008054533.
- STEWART S D ET AL: "Impact of Bt cottons expressing one or two insecticidal proteins of *Bacillus thuringiensis* Berliner on growth and survival of noctuid (Lepidoptera) larvae" JOURNAL OF ECONOMIC ENTOMOLOGY, vol. 94, no. 3, June 2001 (2001-06), pages 752-760, XP008054502 ISSN: 0022-0493.
- WINDELS P ET AL: "DEVELOPMENT OF A LINE SPECIFIC GMO DETECTION METHOD A CASE STUDY" MEDEDELINGEN VAN DE FACULTEIT LANDBOUWWETENSCHAPPEN UNIVERSITEIT GENT, GENT, BE, vol. 64, no. 5B, 22 September 1999 (1999-09-22), pages 459-462, XP001032975 ISSN: 0368-9697.

Respetuosamente le informo al Despacho, que de acuerdo con la información recibida de nuestro cliente, el artículo de ADAMCZYK, J.J., ET AL. y el de JACKSON, R.E., ET AL. cuenta con dos (2) hojas y con cuatro (4) hojas respectivamente; ya que las páginas faltantes corresponden a publicidad.

Con el fin de demostrar que los documentos requeridos por su Despacho no afectan la patentabilidad de la presente invención, considero fundamental poner de presente inicialmente cuál es el objeto de esta última.

Así se debe tener en cuenta que la presente invención reivindica (i) células transgénicas de una planta de algodón que comprenden un inserte que codifica Cry2Ab y las secuencias de unión de las SEQ ID NOs: 14-18; (ii) las sondas y los iniciadores que son útiles para detectar tal evento de inserción; (iii) los métodos para detectar tal evento de inserción; (iv) y los kits de detección de ADN que comprenden las sondas y los iniciadores reivindicados. Vale la pena resaltar que las características técnicas esenciales de la presente invención son las secuencias de unión únicas, más específicamente las secuencias de amplificación únicas que son diagnósticas para el evento de inserción objetivo.

Así entonces, toda vez que tal evento de inserción involucra secuencias únicas que no están descritas ni sugeridas en el estado de la técnica como se demostrará a continuación, la patentabilidad de la presente invención, que específicamente reivindica las secuencias únicas anteriormente mencionadas, no se ve afectada por los artículos citados. Veamos;

Los artículos de **ADAMCZYK ET AL., AKIN ET AL., NORMAN ET AL., y JACKSON ET AL.** no revelan secuencias que sean diagnósticas para la inserción MON15985. Adicionalmente, no suministran ninguna descripción de las secuencias de unión SEQ ID NOs: 14-18 o las secuencias de amplificación de las SEQ ID NOs: 11 y 12. Aunque, los artículos anteriores emplean términos como BollgardII®, DP50BII, MON15985, DPL15985 y/o DP50BX, los mismos son simplemente términos arbitrarios exentos de cualquier característica técnica.

Por lo tanto, toda vez que las características técnicas únicas para la inserción MON15985 no son divulgadas en ninguno de los artículos anteriores, los mismos no afectan la patentabilidad de la presente invención.

Por su parte, el artículo de **STEWART ET AL.,** describe el impacto del algodón transgénico que expresa tanto las endotoxinas Cry1Ac y Cry2Ab de *B. thuringiensis* sobre el crecimiento y supervivencia de las larvas de Lepidóptera. Aunque menciona que el MON15985 es uno de los cultivos primarios utilizados en sus ensayos, este artículo no describe ninguna secuencia que sea diagnóstica para la inserción MON15985.

Así entonces, al no divulgar ninguna descripción sobre las secuencias de unión de las SEQ ID NOs. 14-18 o las secuencias de amplificación de la SEQ ID NOs: 11 y 12, ni ninguna de las características únicas para la inserción MON15985, este artículo tampoco afecta la patentabilidad de la presente invención.

194
290

Finalmente, el artículo de **WINDELS ET AL.**, describe un método PCR para detectar el ADN de frijol de soya RoundUP Ready™ en una forma de línea específica. Este artículo no enseña ni sugiere un método para detectar un evento de inserción de algodón que involucre las secuencias de unión de las SEQ ID N°s: 14-18, o las secuencias de amplicón de las SEQ ID N°s: 11 y 12. Así entonces, toda vez que las características técnicas específicas anteriormente mencionadas para la inserción MON15985 no son divulgadas en este artículo, aunque el mismo suministre un método PCR para detectar una línea específica de organismos genéticamente modificados (OGM), y toda vez que el mismo no enseña ni sugiere un método para detectar el evento de inserción de algodón, tal y como se reivindica en la presente solicitud, el mismo no puede tampoco afectar la patentabilidad de la presente invención.

3. Puesto que se han atendido a cabalidad las peticiones hechas por su Despacho sobre la presente solicitud de patente, atentamente solicito que se continúe su tramitación.

Bogotá,
Señora Jefe,



EMILIO FERRERO

T.P.A. No. 31.929

195 291

EVALUATION OF BOLLGARD® (CV. DP50B) IN THE MISSISSIPPI DELTA: FIELD EFFICACY AGAINST VARIOUS LEPIDOPTERA WHILE PROFILING SEASON-LONG EXPRESSION OF CRY1Ac AND CRY2Ab

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Abstract

Transgenic cotton varieties (Bollgard® and BollgardII®) were evaluated in Stennisville, MS in 2000. It was apparent that the two protein product (BollgardII®) had increased activity against a multitude of lepidopteran pests compared to Bollgard®. Studies profiling the season-long expression of both proteins in BollgardII® indicated that the new proteins (Cry2Ab) was expressed at a much higher level than Cry1Ac in Bollgard® and BollgardII®.

Introduction

Since transgenic Cry1Ac *Bacillus thuringiensis* Berliner (Bt) cotton (Bollgard®, Monsanto Co., St. Louis, Mo.) became widely commercialized in the US in 1996, growers and researchers have noted that many lepidopteran pests are not controlled with this technology alone. The addition of a second Bt protein (Cry2Ab) to Bollgard® (=BollgardII®) may offer additional control of various occasional and secondary Lepidoptera in cotton. The purpose of this research was to evaluate the efficacy of experimental transgenic cotton (BollgardII®) against various lepidopteran pests of cotton as well as to profile season-long expression of both proteins in aid in developing resistance-management strategies for this forthcoming technology.

Materials and Methods

Field Plots

Three varieties (conventional, cv. DP50; transgenic Cry1Ac Bollgard®, cv. DP50B; and transgenic Cry1Ac+Cry2Ab BollgardII®, cv. DP50BII) were planted in experimental plots on 23 May 2000 near Ellabuck, MS. Plots consisted of 4 rows (1.5 m centers) x 9.15 m treatments arranged in a randomized complete block design with each variety replicated twice in each block. Only insecticides not active on Lepidoptera were applied to all plots throughout the season as dictated by local management practices. Plots were irrigated twice.

Insects

To ensure that data would be generated for occasional lepidopterous pests of cotton, two colonies of armyworms were established. Larvae (ca. 300) of the fall armyworm (FAW), *Spodoptera frugiperda* (J. E. Smith), were collected from second-stage field corn near Stennisville, MS in April 2000 and reared for one complete generation in the laboratory as described by Adamczyk et al. (1998). Adult male beet armyworms (BAW), *Spodoptera exigua* (Hübner), were collected using pheromone traps located near Napaoca, MS in December 2000 and mated with a laboratory colony of females to establish a new colony suitable for field inoculations.

Inoculations of beet and fall armyworm egg masses to plants for all varieties were conducted in late July 2000. In brief, egg masses of either species were deposited on nylon cloth in the laboratory. An egg mass of equal size (ca. 2.54 cm² cloth sample) was stapled to the underside of a mature leaf with 4 replicates/variety. For fall armyworms, each infested plot received 24 egg masses over a two-week period. For beet armyworms, each plot received 10 egg masses on July 17, 2000.

Natural infestations of many lepidopteran species (BAW; FAW; tobacco budworm (TBW), *Heliothis virescens* (H.); soybean looper (SBL), *Parasplanesia incarnatus* (Walker); ash trash caterpillar (SMARSH), *Erioponema ceras* (Drury); bollworm (BW), *Heliothis zea* (Lindley) occurred in all plots. Sampling of whole plants and individual plant parts was conducted only in one replicated plot/block (4 replicates/variety). Cotton was mechanically harvested for all rows in all plots and weighed.

Biological Assays

For each sample date, a single terminal leaf was randomly harvested from 10 plants/plot for all varieties (4 replicates/variety). Leaves were transported to the laboratory and within a few hours after being harvested, one sample (ca. 5-8 mg) was taken from each leaf using a standard 6-mm paper disk punch. The leaf samples were weighed to accurately determine the amount of starting material and combined for each variety into a 1.5 ml microcentrifuge tube containing extraction buffer (BioViroLogic, Inc., Portland, ME). The tissue was then homogenized using a mini-beadbeater-87 (Biospec Products, Inc., Butlerville, Ok.) using 6.4 mm steel ball bearings. To quantify the amount of Bt present for each variety (either Cry1Ac in DP50B or Cry1Ac and Cry2Ab in DP50BII), a commercial quantification plate kit was utilized as described in Adamczyk et al. (2000). For all sample dates, varieties were always identically compared in a side-by-side experiment. The proper standard curve, dilution factors, positive and negative controls, and calculations were conducted as dictated in the kit protocol.

Statistics

All means were log-transformed and analyzed using REML-ANOVA. The means were then arcsinized using the LSMRANS option of PROC MIXED (Littell et al. 1996).

Results and Discussion

Drop cloth samples indicated that many lepidopterous pests were reduced in BollgardII® plots (cv. DP50BII) compared to Bollgard® (cv. DP50B) or conventional cotton (cv. DP50) (Table 1). In addition, very few fall armyworms were found in BollgardII® plots compared to Bollgard® or conventional cotton (Table 2). Because fall armyworms are primarily associated with flower damage and subsequent boll damage in Bollgard® cotton, BollgardII® may have increased efficacy against certain Lepidoptera that primarily feed on fruiting structures. The addition of Cry2Ab to Cry1Ac transgenic cotton could increase lepidopteran activity because of increased expression of reproductive tissues (Greenplate 2000). In the present study, the addition of Cry2Ab to transgenic cotton containing Cry1Ac significantly increased activity against pests such as SBL and to a lesser extent SMARSH, FAW, BAW, and BW. Activity against TBW remained superior (Table 3). However, no yield increase was observed with cv. DP50BII compared to cv. DP50B (Table 4).

The lepidopteran bioactivity and protein expression levels of Cry2Ab appear to be quite different than Cry1Ac. Stein (1997) noted that soybean looper were highly sensitive (LC₅₀ = 0.06 µg/ml) to purified Cry2A incorporated into artificial diet, while other studies have shown that they are not as sensitive to Cry1Ac in Bollgard® (Sumnerford and Solomon 2000). Greenplate (2000) showed that Cry2Ab is less potent than Cry1Ac against TBW, but overall expression of Cry2Ab over Cry1Ac is 10 times higher. In our study, we also observed that Cry2Ab is expressed at a higher level than Cry1Ac throughout the growing season (Figure 1). Thus, depending on the species, the increased activity of BollgardII® compared to Bollgard® can be due to increased potency of Cry2Ab, increased overall expression level of Cry2Ab, or possibly a synergistic combination.

Achieving season-long disease-free activity in BollgardII® will be a critical element in resistance management strategies for TBW and BW. Expression profiles of both proteins in BollgardII® appeared to be independent and

another (Figure 1). Our data further indicated that the addition of Cry2Ab has no deleterious effect on levels of Cry1Ac in Bollgard®. Because cv. DP50B1 results from cloned plants regenerated from transformed tissue of cv. DP300 (Bollgard®), backcrosses with elite variation will have to be conducted before commercialization. Thus, future research must be conducted to ensure that expression profiles of delta proteins are adequate to provide season-long dual-protein activity against a multitude of Lepidoptera.

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Disclaimer

Mention of a commercial or proprietary product does not constitute an endorsement by the U.S. Department of Agriculture for its use.

Table 1. Mean number of Lepidoptera found in Bollgard® and Bollgard® plots using a 1.25 m² drop cloth.

Variety	Mean for 3 drops/plot				
	BAW	PAW	TBW	SBL	SMARSH
DP 50	42.00 a	3.00 a	7.00 a	32.00 a	8.75 a
DP 50B	33.50 a	1.75 a	0.25 b	37.75 a	2.75 a
DP 50B11	2.50 b	0.75 a	0 b	0.25 b	0.25 a
F-value	56.11	1.33	9.92	116.77	1.90
df	2, 9	2, 9	2, 9	2, 9	2, 9
P > F	<0.001	0.153	0.045	<0.001	0.205

Table 2. Mean number of fall armyworms found in Bollgard® and Bollgard® flower and whole plants.

Variety	25 Flowers/plot		16 Whole Plants
	White Flowers	Pink Flowers	Plants
DP 50	1.50 a	2.25 a	2.75 a
DP 50B	0.25 a	0.75 ab	2.50 ab
DP 50B11	0.25 a	0 b	0.25 b
F-value	1.13	6.54	4.25
df	2, 9	2, 9	2, 9
P > F	0.348	0.021	0.050

Table 3. Cumulative percent reduction, relative to DP50, of larvae found in field plots of Bollgard® and Bollgard® cotton in Stoneville, MS 2000.

Species	DP50R	DP50B11
DW	57.0	86.0
TBW	99.9	99.9
SBL	0	99.0
PAW	20.0	87.5
BAW	30.0	86.0
SMARSH	29.0	97.0

Table 4. Mean number of damaged plant parts and yield in Bollgard® and Bollgard® plots.

Variety	25 Pink Flowers		16 Whole Plants	Yield (lb/A)
	Burgundy	Flowers	Bolls	
DP 50	7.75 a	7.75 a	22.5 a	872 c
DP 50B	6.25 a	6.50 a	1.0 b	1182 c
DP 50B11	0.75 a	1.50 b	1.25 b	1173 b
F-value	2.17	5.18	54.55	2.10
df	2, 9	2, 9	2, 9	2, 21
P > F	0.171	0.032	<0.001	0.001

¹Damage significant to cause fruit loss, 11 whole plants/plot.
²Lepidopteran feeding on bracts or petals, 35 flowers/plot.
³Damage significant to cause fruit loss, 20 whole plants/plot.
⁴Based on 35% gin turnout.



Figure 1. Season-long expression of Bt protein(s) in Bollgard® and Bollgard®.

EFFICACY OF BOLIGARD AND BOLIGARD II COTTONS AGAINST BOLLWORM, *HELIOverPA ZEA* (BODDIE): 2N FIELD AND GREENHOUSE STUDIES

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ABSTRACT

Boligard™ and Boligard II™ cottons, along with the conventional sister line, were evaluated in field and greenhouse experiments with respect to efficacy against bollworm in North Carolina in 2000. Field studies were prepared to resolve the question of increased efficacy of Boligard II over Boligard on bollworm, as well as to determine survivorship parameters of bollworm on each cotton genotype. The impact of supplemental bollworm control on yield was evaluated at field sites. A greenhouse study was designed to determine the efficacy of three *Bacillus thuringiensis* Berliner (*Bt*) cotton genotypes on a field-collected bollworm strain and a CryI Ac-tolerant bollworm strain that had been released for resistance to the CryI Ac toxin in the laboratory for two generations. Results from field studies indicated that although DP50BX (Boligard II) illustrated no significant effects on larval survival beyond that of DP50B (Boligard), a significant reduction in the level of fruit damage sustained by DP50 and DP50B was observed for the stacked-gene line. Yield response to supplemental pyrethroid overage varied among test sites due to differing bollworm infestation levels and moderate levels of boll rot. Greenhouse study results suggested that DP50BX was equally effective in reducing numbers of both the CryI Ac-tolerant and susceptible bollworm larvae, as well as surface-damaged and penetrated fruit below that of DP50B.

Introduction

The commercialization of Boligard™ (Monsanto Agric. Co., St. Louis, MO) cottons has provided growers with an excellent alternative for controlling lepidopteran pests. However, reports from Stone and Shree (1993) indicated that *B. thuringiensis* was much less effective in controlling bollworm, *Heliothis zea* (Boddie), than the tobacco budworm, *Heliothis virescens* (Fen.). Results from field trials conducted in North Carolina verify that Boligard cottons could sustain significant fruit damage and yield losses, thus creating the need for supplemental insecticide overage for bollworm (Burd et al., 1999; Lambert et al., 1996, 1997; Mainfroy et al., 1994, 1995). This is most likely caused by the observed drop in average levels of CryI Ac endotoxin (Greenplate 1999; Greenplate et al., 1998) which coincides with the major bollworm flight in North Carolina. With the bollworm population receiving a sublethal dose of CryI Ac, the "high dose strategy" for insect resistance evolution is violated by Boligard cottons, which initiates a need for new technological developments.

Boligard II™ cottons produce two proteins, CryI Ac and Cry2Ab, that are active against bollworm. The novel dual-gene line illustrates approximately the same level of expression of the CryI Ac endotoxin as the Boligard variation (Greenplate et al., 2000). Greenplate also reported that the Cry2Ab endotoxin is expressed at a much higher level than CryI Ac in the Boligard II line. The dual-gene construct, therefore, would more appropriately fit the "high dose strategy" for insect resistance evolution than would the currently utilized single-gene construct. Reports from field and greenhouse trials conducted in North Carolina demonstrated that Boligard II genotypes significantly reduced numbers of susceptible and CryI Ac-tolerant bollworm larvae below that of the Boligard cottons (Jackson et al., 2000). This suggests that the implementation of Boligard II cottons could possibly extend the time frame for bollworm resistance evolution beyond that of current Boligard varieties.

Results from field and greenhouse trials evaluating the efficacy of Boligard and Boligard II cottons and the conventional sister line by measuring bollworm numbers, fruit damage, and yield under pyrethroid treated and untreated systems are reported herein.

Materials and Methods

Field Studies

Experiments were conducted at the Central Cross Research Station, Johnston Co., NC, the Upper Coastal Plain Research Station, Edgemont Co., NC, and the Tidewater Research Station, Washington Co., NC, in 2000. Each test site consisted of a randomized complete split-plot design with four replicates. Within plots were 16, 20, and 24 rows by 60 feet for DP50, DP50B, and DP50BX, respectively, at each location. Different plot sizes were chosen for determination of the survival of bollworm in each genotype through mass sampling procedures in which large numbers of bolls were examined for each genotype to estimate bollworm survival on a per acre basis for making resistance management decisions and estimating refuge requirements. Subplots contained 12, 16, and 20 untreated rows for DP50, DP50B, and DP50BX, respectively, and 4 rows that were treated with a pyrethroid as required for supplemental bollworm control. Yield differences between pyrethroid-treated and untreated subplots were determined for each site.

Cotton genotypes DP50, DP50B, and DP50BX were planted on 15 May in Edgemont Co., 17 May in Johnston Co., and 18 May in Washington Co. Alectra (Temik 3G, Aventis CropScience, Research Triangle Park, NC) was applied in-furrow at planting at 3.0 lb. a.i./acre for control of early season insect pests. Acephate (Orthene 97, Valent USA Corp., Walnut Creek, CA) was applied at 0.75 lb. a.i./acre as a mid-season overpray for sucking bugs and plant bugs, as well as to eliminate arthropod predators. Two applications of lambda cyhalothrin (Sarcos 2.38 CS, Zeneca Inc., Wilmington, DE) were made to suppress aphid populations for supplemental bollworm control at Johnston and Edgemont counties on 16 July and 7 August, as well as at Washington Co. on 27 July and 1 August. Fertilization, plant growth regulation, weed control, and defoliation were accomplished as recommended by North Carolina State University.

Terminal region measurements of bollworm eggs, live larvae, and damage were made the weeks of 30 July and 6 August by the random sampling of 25 bolls/plant per plot. Square evaluations for live larvae and damage were made the weeks of 6 and 13 August by examination of 30 squares per treatment per replicate. Boll measurements for live larvae and damage were made the weeks of 13, 20, and 27 August, as well as 3 September by examination of 30 bolls per treatment per replicate. Bollworm egg, live larvae, and damage numbers per treatment per replicate were converted to percentages prior to analysis. Yields were determined by harvesting two center rows of each subplot with a mechanical cotton picker on 30 October at Edgemont Co. and 7 November at Johnston and Washington counties. Yields were converted to lb. seed cotton per acre prior to analysis.

The total numbers of fourth to fifth instar larvae, harvestable bolls, and damaged harvestable bolls were counted in a randomly selected area of five rows feet per treatment per replicate at the Edgemont and Washington county sites on three sample dates (24 and 31 August and 7 September). Large larvae, fourth and fifth instars, were transported to the laboratory and reared on bolls from the respective genotypes until the prepupal stage. Prepupae were then placed into 30 ml plastic cups containing 50% ± artificial diet which served as a substrate to detect toxin for pupation. Numbers of successfully emerged adults from each genotype were counted and converted to a per acre basis prior to analysis along with total numbers of harvestable bolls, damaged bolls, and live larvae. These numbers provide an estimation of survival parameters of the bollworm, which is important in resistance management.

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Greenhouse Study

An experiment was conducted in a greenhouse chamber at the Tidewater Research Station, Washington Co., NC. The test was a randomized complete block design with seven replications. Each replicate consisted of 12 plants, two plants per treatment combination of cotton genotype and bollworm strain. Plants within blocks were separated by a distance of two feet, whereas, blocks were separated by a 3 foot space on tables.

Cotton genotypes DP50B, DP50X, and DP50BX were planted in three gallon pots at one plant per plot on 27 June. Arthropod natural enemies, as well as aphids and whiteflies, were eliminated by side-drenching cotton plants two weeks prior to bollworm infestation with Aldicarb to achieve a rate of 0.75 lb. a.i./acre. Fertilization and plant growth regulation were provided as recommended for greenhouse cotton raises.

Local bollworm adults were collected from light traps and sent to the laboratory for egg collection. Nontoxic larvae from the eggs were used as the strain in the experiment. A Cry1Ac-tolerant strain of bollworm was originally collected from light traps and selected for tolerance to the Cry1Ac toxin (MVP) in artificial diet for two generations. Infestation of five female larvae onto fruiting structures on respective plants from each genotype using a fine-mist sprayer at 1000 hours occurred when cotton plants reached approximately 100 days. Assessments of surviving larvae, superficial fruit damage, and fruit penetration were made by whole plant examination at 14 and 21 days after infestation. Surviving larvae from the same evaluation were taken to the laboratory and placed into 70 ml plastic cups containing 100% artificial diet which served as a medium for larvae to mature into pupae. Numbers of bollworm that successfully pupated and those that successfully emerged as adults were recorded and converted to percentages.

Data Analysis

All data were subjected to ANOVA using PROC GLM (SAS Institute 1990), and means for each treatment were separated ($P < 0.05$) using Fisher's Least Significant Difference test or LSMEANS in SAS.

Results

Field Studies

No differences were found among the three cotton genotypes with distribution of bollworm eggs in cotton terminals being consistent across field sites (Table 1). No significant differences were evident between DP50BX and DP50B with respect to percent live bollworm larvae in cotton terminals, and both Bollgard genotypes reduced percent bollworm larvae below that of the conventional variety (Table 1). Percent terminal damage was significantly lessened by both Bollgard genotypes compared to the conventional variety with DP50BX further reducing percent terminal damage below that of DP50B (Table 1). Square evaluations revealed that Bollgard cottons did not differ with respect to the percentage of squares containing live bollworm larvae, but both sustained a significantly lower percentage of live larvae than the conventional variety (Table 2). Data in Table 2 also show a significant reduction in percent bollworm damaged squares by both Bollgard genotypes compared to the conventional variety with DP50BX further reducing square damage below that of DP50B. Boll evaluations indicated that Bollgard cottons did not differ with respect to percent live bollworm larvae in bolls but significantly lessened percent live bollworm larvae compared to the conventional variety (Table 2). However, Table 3 indicates that DP50BX sustained less boll damage than DP50B at each test site with both Bollgard cottons having less boll damage than the conventional variety.

Field evaluations for determination of total harvestable fruit on a per acre basis revealed that the conventional variety had a significantly lower number of bolls than the Bollgard cottons (Table 4). Results in Table 4 also reflect that the Bollgard cottons had significantly lower numbers of

damaged bolls than the conventional variety in which greater than half the bolls were bollworm damaged. Table 5 illustrates that no differences were evident between Bollgard genotypes with respect to the number of surviving fourth to fifth instar bollworm larvae per acre. However, only DP50BX contained a significantly lower number of larvae per acre compared to the conventional variety. The number of large larvae per acre directly relates to the number of successfully emerged bollworm adults on a per acre basis which was much lower in the Bollgard genotypes than in the conventional variety (Table 5). Differences in number of successfully emerged bollworm adults were not evident among Bollgard cottons even though successful emergence was much lower numerically in DP50BX than in DP50B.

Yield differences measured in pounds of seed cotton per acre between pyrethroid-treated and untreated subplots in three cotton genotypes varied among test sites. Pyrethroid-treated and untreated subplots did not differ significantly with respect to yield in lb. seed cotton per acre at the Central Crops Research Station (Table 6). Cotton genotype also had no impact on yield at this site. Data in Table 7 reveal a genotype $\times$ insecticide interaction at the Upper Coastal Plain Research Station in which all treatment combinations were compared. Yield benefits from pyrethroid applications were only evident in the conventional variety. Both untreated Bollgard genotypes illustrated significantly improved yields compared to the untreated conventional variety. Among pyrethroid-treated subplots, DP50B produced more seed cotton than DP50BX. Neither pyrethroid-treated Bollgard variety differed from the treated conventional variety. At the Tidewater Research Station, pyrethroid-treated subplots averaged across cotton genotypes produced significantly higher yields than untreated subplots (Table 8). Yields in lb. seed cotton per acre averaged across subplots were higher in DP50BX than DP50B, which also out-yielded the conventional variety.

Greenhouse Study

The Cry1Ac-tolerant bollworm strain displayed a significantly higher percent larval survival than the susceptible bollworm strain on DP50B, but no differences among larval strains were evident for the DP50X and DP50BX genotypes (Table 9). As with larval survival, Table 10 indicates that the Cry1Ac-tolerant bollworm strain caused a significantly higher percent surface-damaged fruit than did the susceptible bollworm strain in DP50B only. Bollworm strains did not differ with respect to percent surface damaged fruit within genotypes DP50X and DP50BX. Data in Table 11 indicate that the percentage of penetrated fruit averaged across cotton genotypes was significantly higher for the Cry1Ac-tolerant strain compared to the susceptible strain. Although no differences in percent penetrated fruit existed between DP50X and DP50BX, only DP50BX significantly reduced the percentage of penetrated fruit below that of DP50B. The percentage of larvae from the Cry1Ac-tolerant bollworm strain that developed on DP50B and successfully pupated was 4.29%. No other larvae on any genotype survived until pupation. Of those that pupated, 60.7% successfully emerged as adults.

Discussion

Field Studies

Bollworm populations varied spatially in 2000, with the most characteristic low populations at the Central Crops Research Station and moderate to high infestations at the Upper Coastal Plain and Tidewater Research Stations. In field studies, both DP50B and DP50BX provided similar levels of bollworm control with respect to percent live bollworm larvae in terminals, squares, and bolls with both transgenic cultivars reducing larval numbers below that of the conventional variety. The DP50BX line, however, was more effective in reducing leaf fruit damage than DP50B in these plant regions. Significantly less fruit damage should have equated to higher yields, but moderate levels of loss not partitioned the best treatment, reducing source available yield differences imperceptible. Both transgenic

cotton out-performed DP50 with respect to production of total and damaged harvestable bolls per acre, as well as successfully emerged bollworm adults per acre. Only DP50BX contained significantly lower numbers of large live larvae than the conventional variety. Cotton genotypes DP50B and DP50BX also performed similarly with respect to production of total and damaged harvestable bolls per acre, as well as numbers of large live larvae and successfully emerged adults per acre. However, numerical reductions made by DP50BX in numbers of bollworms that successfully completed development may extend the time frame for bollworm resistance evaluation, but possibly for a shorter time period than originally expected.

Greenhouse Study

The CryI δ -tolerant bollworm strain out-performed the susceptible strain with respect to larval survival and surface damage inflicted onto feeding structures, which was largely due to the increased performance of the CryI δ -tolerant strain compared to the susceptible strain or DP50B. Averaged across genotypes, increased fruit penetration by the CryI δ -tolerant bollworm strain over the susceptible strain was caused by the increased success of the CryI δ -tolerant strain on genotypes DP50B and DP50X. Fruit penetration by the CryI δ -tolerant strain on DP50BX was approximately 10X less than that of DP50X and DP50B, which is likely due to some additional effect of the two-gene construct versus that of the single-gene. Averaged across bollworm strains, DP50BX sustained less fruit penetration than DP50B. These results support reports from Jackson et al. (2000) indicating the increased bollworm control provided by Bollgard II lines over the commercial Bollgard variety with respect to larval survival and fruit penetration. In addition to larval survival and fruit penetration, the percentage of the CryI δ -tolerant bollworm strain that successfully pupated and emerged as adults was higher in DP50B (4.39 and 2.86%, respectively) than in DP50BX (0.07%) and DP50X (0.00%). These greenhouse data and those from field studies suggest that the commercialization of the dual-gene construct (DP50BX) would reduce bollworm damage over that experienced by Bollgard varieties, as well as eliminate the need for supplemental insecticide application for bollworm control. With the incidence of field resistance to Bollgard entices in North Carolina (Jackson et al. 2000; Burd et al. 1999; Lambert et al. 1997, 1998; McChaffey et al. 1994, 1995), the implementation of Bollgard II lines may also be necessary to provide control for that portion of the bollworm population already expressing resistance traits to Bollgard cottons.

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Table 1. Mean (SE) percent bollworm egg, live larvae, and damage in the terminal region of three cotton genotypes averaged across three locations and two sample dates in North Carolina in 2000.

Genotype	Percent Egg	Percent Larvae	Percent Damage
DP50	8.00 (.018) a	4.25 (.006) a	5.92 (.019) c
DP50B	11.33 (.020) a	0.67 (.002) b	6.42 (.021) b
DP50BX	7.50 (.017) a	0.25 (.002) b	3.33 (.010) c

Means within the same column followed by the same letter are not significantly different, Fisher's LSD ($P \leq 0.05$).

Table 2. Mean (SE) percent live bollworm larvae in squares, square damage, and live bollworm larvae in bolls averaged across three locations and respective sample dates in North Carolina in 2000.

Genotype	Square Evaluations		Boll Evaluations	
	Percent Larvae	Percent Damage	Percent Larvae	Percent Damage
DP50	4.92 (.005) a	16.25 (.003) a	4.92 (.008) a	1.30 (.003) b
DP50B	0.42 (.003) a	3.33 (.004) b	1.30 (.003) b	6.17 (.002) b
DP50BX	0.00 (.000) b	0.25 (.001) c	0.00 (.000) c	0.00 (.000) c

Means within the same column followed by the same letter are not significantly different, Fisher's LSD ($P \leq 0.05$).

296

Table 3. Mean (SE) percent boll damage for each cotton genotype averaged across three sample dates for each test site in North Carolina in 2000.

Genotype	Upper Coastal		
	Central Crops Research Station	Plein Research Station	Tidewater Research Station
DP50	12.50 (.008) a	49.25 (.030) a	44.25 (.037) a
DP50B	0.86 (.003) b	10.25 (.010) b	7.75 (.013) b
DP50BX	0.00 (.000) c	1.38 (.005) c	0.75 (.000) c

Means within the same column followed by the same letter are not significantly different, Fisher's LSD ($P \leq 0.05$).

Table 4. Mean (SE) number of total bolls and damaged bolls per acre for each cotton genotype averaged across three sample dates and two locations in North Carolina in 2000.

Genotype	Number of Bolls Per Acre	Number of Damaged Bolls Per Acre
DP50	111,817 (23,319) a	156,695 (24,328) a
DP50B	458,832 (1,2637) a	27,830 (3,185) b
DP50BX	145,668 (8,047) a	7,501 (3,018) b

Means within the same column followed by the same letter are not significantly different, Fisher's LSD ($P \leq 0.05$).

Table 5. Mean (SE) number of live large bollworm larvae and successfully emerged adults per acre for each cotton genotype averaged across three sample dates and two locations in North Carolina in 2000.

Genotype	Number of Larvae Per Acre	Number of Emerged Adults Per Acre
DP50	6993 (1619) a	6093 (2,165) a
DP50B	3155 (816) ab	528 (197) b
DP50BX	1989 (1601) b	72 (72) b

Means within the same column or row followed by the same letter are not significantly different, Fisher's LSD ($P \leq 0.05$).

Table 6. Mean (SE) weight of seed cotton in pounds per acre for pyrethroid-treated and untreated subsplots of each genotype at the Central Crops Research Station in North Carolina in 2000.

Genotype	Untreated	Treated
DP50	3,722 (193) a	3,618 (165) a
DP50B	4,107 (90) a	4,076 (84) a
DP50BX	4,053 (110) a	3,846 (135) a

Means within the same column or row followed by the same letter are not significantly different, Fisher's LSD ($P \leq 0.05$).

Table 7. Mean (SE) weight of seed cotton in pounds per acre for pyrethroid-treated and untreated subsplots of each cotton genotype at the Upper Coastal Plain Research Station in North Carolina in 2000.

Genotype	Insecticide	Pounds of Seed Cotton Per Acre
DP50B	Untreated	3,902 (340) a
DP50B	Treated	3,808 (108) ab
DP50BX	Untreated	3,624 (90) abc
DP50	Treated	3,452 (258) bc
DP50BX	Treated	3,210 (146) c
DP50	Untreated	3,440 (229) d

Means within the same column followed by the same letter are not significantly different, LSMEANS ($P \leq 0.05$).

Table 8. Mean (SE) weight of seed cotton in pounds per acre for pyrethroid-treated and untreated subsplots of each genotype at the Tidewater Research Station in North Carolina in 2000.

Genotype	Untreated	Treated	Mean
DP50	1,399 (27)	2,083 (193)	1,741 (157) c
DP50B	2,435 (199)	2,474 (100)	2,465 (103) a
DP50BX	2,846 (131)	3,057 (90)	2,952 (114) a
Mean	2,233 (188) b	2,537 (140) a	

Means within the same column followed by the same letter are not significantly different, Fisher's LSD ($P \leq 0.05$).

Table 9. Mean (SE) percent surviving larvae for each bollworm strain within each cotton genotype averaged across two evaluation dates in the greenhouse in North Carolina in 2000.

Strain	DP50B	DP50X	DP50BX
Cry1Ac-tolerant Strain	5.00 (.023) a	0.71 (.007) c	0.00 (.000) c
Susceptible Strain	0.00 (.000) b	0.00 (.000) a	0.00 (.000) a

Means within the same column followed by the same letter are not significantly different, Fisher's LSD ($P \leq 0.05$).

Table 10. Mean (SE) percent surface-damaged fruit for each bollworm strain within each cotton genotype averaged across two evaluation dates in the greenhouse in North Carolina in 2000.

Strain	DP50B	DP50X	DP50BX
Cry1Ac-tolerant Strain	4.36 (.000) a	0.45 (.005) a	1.36 (.006) c
Susceptible Strain	1.19 (.006) b	0.00 (.000) a	1.36 (.006) a

Means within the same column followed by the same letter are not significantly different, Fisher's LSD ($P \leq 0.05$).

Table 11. Mean (SE) percent penetrated fruit for each cotton genotype and each bollworm strain averaged across two evaluation dates in the greenhouse in North Carolina in 2000.

Genotype	Cry1Ac-tolerant		Susceptible	Mean
	Strain	Strain		
DP50B	4.92 (.017)	0.45 (.005)	2.89 (.000) a	
DP50X	4.10 (.020)	0.00 (.000)	2.05 (.010) ab	
DP50BX	0.45 (.004)	0.00 (.000)	0.22 (.000) b	
Mean	3.16 (.019) a	0.28 (.002) b		

Means within the same column or row followed by the same letter are not significantly different, Fisher's LSD ($P \leq 0.05$).

Impact of Bt Cottons Expressing One or Two Insecticidal Proteins of *Bacillus thuringiensis* Berliner on Growth and Survival of Noctuid (Lepidoptera) Larvae

297

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ABSTRACT A series of laboratory assays were performed to compare the relative impact of commercial and experimental cultivars of cotton, *Gossypium hirsutum* (L.), expressing zero, one, or two insecticidal proteins of *Bacillus thuringiensis* Berliner, on several lepidopteran pests. Assays in which larvae were fed fresh plant tissue indicated that dual-toxin *B. thuringiensis* (Bt) cultivars, expressing both Cry1Ac and Cry2Ab endotoxins of *B. thuringiensis*, were more toxic to bollworms, *Heliothis virescens* (Boddie), fall armyworms, *Spodoptera frugiperda* (J. E. Smith), and beet armyworms, *Spodoptera exigua* (Hübner), than single-toxin cultivars expressing Cry1Ac. Assays in which lyophilized plant tissue was incorporated into artificial diet also indicated improved activity of the dual-toxin Bt cultivar compared with single-toxin plants. Both bollworm and tobacco budworm, *Heliothis virescens* (F.), growth was reduced by Bt cotton, particularly the dual-toxin cultivar. Although assays with lyophilized tissues were done using largely sublethal doses, bollworm survival was reduced by the dual-toxin cultivar. It appears that this newly developed Bt cotton expressing two toxins will be more effective and have a wider range of activity on these lepidopteran pests.

KEY WORDS transgenic *Bacillus thuringiensis* cotton, Cry1Ac, Cry2Ab, Lepidoptera, toxicity

TRANSGENIC BT COTTON, *Gossypium hirsutum* (L.), expressing the Cry1Ac insecticidal δ -endotoxin of *Bacillus thuringiensis* Berliner, has been commercially available in the United States since 1996. Since then, *B. thuringiensis* (Bt) cotton has demonstrated remarkable control of some lepidopteran pests, particularly the tobacco budworm, *Heliothis virescens* (F.), and the pink bollworm, *Pectinophora gossypiella* (Saunders). Since its release into commercial markets, Bt cotton seldom, if ever, has required supplemental insecticide control for these two pests (Williams 2000). Control of the bollworm, *Heliothis virescens* (Boddie), has been less dependable, and economically damaging infestations of this pest can occur on Bt cotton (Mahaffey et al. 1995), particularly after plants have begun flowering and when insecticides have disrupted populations of predators and parasites (Lambert et al. 1996, Turnipseed and Sullivan 1999). The bollworm is inherently more tolerant than tobacco budworm to the Cry1Ac δ -endotoxin expressed in currently available Bt cultivars (Luttrell et al. 1999). Additionally, low expression of the toxin in some plant parts, such as in pollen and petals, has been implicated in increased survival of the bollworms on Bt cotton (Adamczyk et al. 2000). Other common lepidopteran pests [e.g., fall armyworms, *Spodoptera frugiperda* (J. E. Smith); beet armyworms, *Spodoptera exigua* (Hübner); and soybean loopers,

Pseudoplusia includens (Walker)] are even more tolerant than bollworms to Cry1Ac (Luttrell et al. 1998, 1999).

One advantage of Bt cotton is that it greatly reduces injury and insecticide use associated with control of tobacco budworms and bollworms (Stewart et al. 1998). However, in low-spray environments, pests like soybean loopers, beet armyworms, and fall armyworms can potentially thrive, even in Bt cotton (Adamczyk et al. 1998, Ashfaq and Young 1999, Stewart et al. 2000). The insertion of an additional Bt gene encoding for a different toxin (Cry2Ab; Monsanto, St. Louis, MO) into current Bt cotton cultivars containing Cry1Ac may provide additional activity on certain lepidopteran pests and would improve the pest-control value of Bt cotton.

The potential impact on resistance management of cotton that expresses multiple toxins has been addressed (Tabashnik 1994, Sachs et al. 1996, Gould 1998). Mandating a refuge of non-Bt cotton as a source of susceptible individuals to mate with resistant insects has been the primary approach for preventing resistance to Bt cotton (Caprio 1994). This strategy relies on Bt cotton expressing a "high dose" (e.g., > 25 fold the LD₅₀) to lepidopteran pests so that survivors in Bt cotton are rare and heterozygous individuals are rendered functionally recessive (Gould 1998). This tactic has been criticized because commercially available Bt cottons do not appear to meet the high-dose criterion for bollworm as it presumably does for tobacco bud-

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worm and pink bollworm. A potential advantage of having two Bt toxins expressed in cotton is that the risk of insect pests developing resistance to Bt cotton may be reduced, especially if resistance to one toxin does not confer resistance to the other.

Although not yet commercially available, transgenic cotton lines expressing two Bt toxins are under development. This article reports the results of laboratory assays with a dual-toxin Bt cotton (Cry1Ac and Cry2Ab) compared with single-toxin Bt cottons (Cry1Ac) and non-Bt cottons. Our primary objective was to compare the relative toxicity of single- and dual-toxin Bt cotton cultivars on tobacco budworm, fall and beet armyworm, and particularly bollworm. A secondary objective was to determine assay methods that are best suited for quantifying differences in the relative toxicity of Bt cottons.

Materials and Methods

Source of Plant Materials and Larvae Used in Assays. DPL50 (non-Bt), DPL50B (expressing Cry1Ac), and MON15985 and MON15813 (expressing Cry1Ac and Cry2Ab) were the primary, near-isogenic cultivars used in our assays. Other cultivars were used in some comparisons as indicated below. All cottons were Delta and Pine Land (Scott, MS) cultivars provided to the researchers in 1999 by the Monsanto. All plant materials used in fresh tissue assays were collected from field plots during 1999.

Fresh tissue assays with neonate and third-instar bollworms were done at the USDA-ARS-SIMRU (Southern Insect Management Research Unit, Stoneville, MS). All bollworms used in testing were from a colony created in the spring of 1999 when ~300 individuals were collected from field corn near Stoneville, MS. The laboratory rearing of bollworms was conducted as described in Adamczyk et al. (1998), and the G₁ or G₂ generation was used in assays. Replicated field plots (Shelby, MS) containing DPL50, DPL50B and NuCOTN 33B (expressing Cry1Ac), and MON15985 and MON15813 (expressing Cry1Ac and Cry2Ab) were the source of plant tissue used in these experiments. In addition, replicated field plots (Elizabeth, MS) containing eight non-Bt and 17 Bt cotton cultivars were also used to make comparisons with dual-toxin Bt cotton. Field plots were maintained by standard agronomic practices for the region, including herbicide and nitrogen applications.

Assays using second-instar bollworm, beet armyworm, and fall armyworm were done at Mississippi State University (Mississippi State, MS). Additional experiments with neonate bollworm and tobacco budworm, using lyophilized leaf tissue, were also done at Mississippi State University. Larvae used were from the USDA-ARS-CHPRRU (Corn Host Plant Resistance Research Unit, Mississippi State, MS). These are long established colonies maintained as described by Davis (1988). Unless indicated, replicated field plots of DPL50, DPL50B, and MON15985 at Mississippi State University were used as a source of plant tissue in

these assays. Field plots were established and maintained as described by Stewart et al. (2000).

When field plots were used as a source of plant material for assays, plant tissues were collected equally from insecticide-untreated replicates. Throughout all experiments, larvae were maintained at 25 ± 2°C, 70–80% RH, and a photoperiod of 14:10 (L:D) h.

Fresh Tissue Assays (Neonate Bollworm). To examine if dual-toxin Bt cotton (MON15813 and MON15985) was more toxic than single-toxin Bt cotton (17 different commercial cultivars expressing Cry1Ac) to neonate bollworms, leaves from plants in the fourth true leaf stages were harvested and fed to larvae in the laboratory. In addition, we used non-B commercial cultivars (8) as controls. Fully expanded leaves (15) were harvested from each replicate (four replicates per cultivar) with ~0.5 cm of petiole remaining to prevent desiccation and transported to the laboratory in a cooler containing ice. Within 2 h of harvesting, a single leaf was placed into individual rearing cups (30 ml, JetPlastics, Hattisburg, PA), and a neonate bollworm was placed into each cup, which was then capped (60 neonates/cup). All cups were placed inside an autoclave bag (model 12 by 24 inches (30.5 by 61.0 cm), clear polyethylene; Fisher, Norcross, GA) to reduce leaf desiccation. Two days after exposure to cotton leaves, survival was recorded (i.e., movement present after disturbed with a camel's hair brush). Fresh leaves, collected as before, were provided to larvae and this time. Survival was reassessed after two additional days.

Cry1Ac expression in flower parts is low compared with other plant parts (Adamczyk et al. 2000), and bollworms are commonly associated with flowers (J.J.A., unpublished data). Thus, a study was designed to examine if flower parts from the dual-toxin Bt cultivars were more toxic to neonate bollworms than flowers from single-toxin Bt cultivars. During the second week of flowering (peak flowering period), first position pink flowers were harvested from plots in Shelby, MS (three replicates), of NuCOTN 33B (expressing Cry1Ac) and MON15985 (expressing Cry1Ac and Cry2Ab) and transported to the laboratory within a cooler containing ice. To help make finding larvae easier, the petals were removed from the flowers. Within 2 h after harvesting, a single petal-less pink flower was placed into individual cups (30 ml), and two neonate bollworms were placed into each cup (6 flowers per cultivar, 120 neonates/cultivar). Survival was recorded at 2 d after larvae were infested onto the plant parts, and flowers were changed in cups containing live larvae, using flowers collected before. Survival was reassessed after two additional days. For the leaf and pink flower assays, mean survivorship was analyzed using analysis of variance (PROC REML-ANOVA, SAS Institute 1998), and means were separated via the lsmeans option of PROC Mixed (Littel et al. 1996). Numbers of live larvae were log transformed before analysis because treatment variances were not homogeneous.

Because it is widely believed that larvae move from flowers to bolls once flowers begin to desiccate, a study was designed to examine if increased efficacy on bollworm neonates was observed with dual-toxin Bt cotton on 1-d-old bolls. First position white flowers (30) from DPL50, NuCOTN 33B, and MON15985 plants were randomly tagged (Slant 'N Lock; A.M. Leonard, Fiqua, OH [as described in Adamczyk et al. 1998]) in field plots at Shelby, MS. After 24 h, the 1-d-old bolls were removed from the plant and transported to the laboratory within a cooler containing ice. All remaining flower parts, including bracts and stems, were removed from the bolls, and the bolls were washed with distilled water and allowed to air dry. The bolls were then placed into individual cups (30 ml), and a single neonate was introduced. Survival of neonates was rated 4 d after they were infested onto plant parts and analyzed using PROC Freq with likelihood ratio chi-square (SAS Institute 1998). Survival differences among cultivars were separated using Fisher exact test (two-tailed) (Steel and Torrie 1980).

Fresh Tissue Assays (Second-Instar Bollworm, Beet Armyworm, and Fall Armyworm). Two similar methods were used to evaluate the relative toxicity of DPL50, DPL50B, and MON15985 on bollworm, fall armyworm, and beet armyworm. The two assay methods differed only in that in one method the insects were allowed to feed on plant tissue for 48 h and were then transferred to artificial diet (King and Hartley 1985), and in the other method, new plant tissue was provided to larvae every 48 h. Plant parts were collected from field plots, returned to the laboratory, washed with tap water, and air-dried before use in assays.

Second-instar larvae previously maintained on artificial diet (King and Hartley 1985) for 48 h post-hatch were fed various plant parts. Bollworms and fall armyworms were given freshly picked white flowers because they primarily feed on fruiting structures. Beet armyworms were fed fully expanded leaves from the four to sixth node below the terminal because they are primarily leaf-feeders in cotton. Our assay method was similar to that of Chang et al. (2000). Approximately 0.5 cm of 2.0% sterile agar was poured into petri dishes (9 cm diameter), and autoclaved paper toweling was placed over the agar. This paper was cut to fit the bottom of the dishes and served as a wick for the moisture in the agar. The plant material was trimmed so that it would fit into the dishes and maintain contact with the toweling. The plant parts and one larva were placed in each dish, with the larvae placed on top of the plant tissue. Dishes were covered with corresponding lids. For each of the three species and cultivars, 80 larvae were tested. A few larvae escaped or were lost during the study, and these were omitted from analyses.

After 48 h, survival and the relative amounts of feeding damage (0-5 scale, no damage to total consumption) to the plant parts were recorded for each individual. Approximately one-half of the surviving insects were transferred to 30-ml plastic rearing cups (JetPlastica Co., Harrisburg, PA) containing ~8.0 ml

artificial diet. The remaining larvae were provided additional plant material at 48-h intervals until death or pupation. Survival was again determined 24 h later and subsequently at 48-h intervals until pupation. The lengths of larvae (mm) were recorded 5 and 7 d after their initial placement on plant tissue. Dates of pupation were also recorded for all surviving insects.

Our primary interest was to determine cultivar effects on the survival and growth of common lepidopteran pests such as bollworm, beet armyworm, and fall armyworm. Statistical comparison about how different plant parts influenced insect survival and development were not done because these experiments were not always done at the same time or with the same cohort of insects. Also, no attempt was made to statistically compare across species because different plant parts were used for testing. Besides cultivar effects, we also were interested in comparing the two assay methods (i.e., larvae transferred to artificial diet and those continually fed plant material). Thus, cultivar and assay methods were the two factors included in analysis. Each larva represented a replicate. Data for survival and the relative amount of feeding on plant tissue were categorical; therefore, we used categorical data analysis and protected means for separation of treatment effects (PROC Genmod, SAS Institute 1998). Cultivar effects on larval length and time to pupation were analyzed with ANOVA and using Student-Newman-Keuls for mean separations (PROC GLM, SAS Institute 1998).

Fresh Tissue Assays (Third-Instar Bollworm). Because bollworms may have the ability to survive on Bt cotton plant parts expressing low levels of Bt (i.e., flowers) or immigrate from non-Bt cotton, an experiment was designed to test the effect dual-toxin Bt cotton had on older bollworm larvae when fed 4-d-old bolls. First position white flowers were tagged in field plots at Shelby, MS, as previously described. After 4 d, the corresponding bolls (15, 25, and 35 for DPL50, NuCOTN 33B, and MON15985, respectively) were harvested. Bioassays were done as described for pink flowers except larvae were reared initially to the third instar on artificial diet (King and Hartley 1985), and only one larva was placed in each cup. Survival of neonates as well as number of bolls penetrated (i.e., penetration of boll wall to seed/fiber) were rated at 2, 4, and 6 d after infestation on plant tissue and analyzed using PROC Freq with likelihood ratio chi-square (SAS Institute 1998). Differences in survival among cultivars were separated using Fisher's exact test (two-tailed) (Steel and Torrie 1980).

Assays with Lyophilized Leaf Tissue (Neonate Bollworm and Tobacco Budworm). Bioassays using lyophilized leaf tissue that was incorporated into artificial diet were done with neonate tobacco budworms and bollworms to compare the sensitivity of these species to DPL50, DPL50B, and MON15985. In the first repetition of this experiment, done in 1999, 100 leaves located four to five nodes below the terminals were collected from the field plots at Mississippi State University. These leaves were collected when plants were near physiologic cutout (Harris et al. 1997). At

vae were provided intervals until death determined 24 h later until pupation. The 5 and 7 d after issue. Dates of pupa- nving insects. stermine cultivar ef- of common lepidopt- armyworm, and fall n about how differ- survival and devel- : these experiments ne time or with the attempt was made to es because different . Besides cultivar ef- comparing the two rred to artificial diet material). Thus, cul- two factors included i a replicate. Data for : of feeding on plant , we used categorical ans for separation of mod, SAS Institute length and time to IOVA and using Stu- separations (PROC star Bollworm). Be- ability to survive on Bt w levels of Bt (i.e., -Bt cotton, an exper- effect dual-toxin -Bt vae when fed 4-d-old ; were tagged in field -described. After 4 d, , and 35 for DPL50, , respectively) were as described for pink d initially to the third d Hartley 1985), and ach cup. Survival of olls penetrated (i.e., iber) were rated at 2, it tissue and analyzed od ratio chi-square es in survival among har's exact test (two- ssue (Neonate Boll- . Bioassays using ly- incorporated into arti- is tobacco budworms ; sensitivity of these d MON15985. In the nt, done in 1999, 100 i below the terminals its at Mississippi State collected when plants Harris et al. 1997). At

Table 1. Proportion of neonate bollworms surviving 2 and 4 d when fed cotton containing zero, one (Cry1Ac), or two Bt proteins (Cry1Ac and Cry2Ab)

Plant part	Cultivar	Survival		n
		2 d	4 d	
Leaves ^a	Non-Bt ^b	0.98 ± 0.01a	0.98 ± 0.01a	48
	Bt ^c	0.67 ± 0.03b	0.34 ± 0.03b	1,081
	Bt ^d	0.37 ± 0.07c	0.07 ± 0.08c	18
Pink flowers ^e	NuCOTN 33B ^f	0.72 ± 0.05a	0.48 ± 0.04a	18
	MON15985 ^g	0.55 ± 0.05a	0.18 ± 0.03b	18
1-d-old bolls ^h	DPL50	—	0.83a	3
	NuCOTN 33B ⁱ	—	0.10b	3
	MON15985 ^j	—	0.00b	3

Analyses for leaves and pink flowers were done on number of surviving insects per replicate following log transformation (PROC Mixed Littell et al. 1998). Means ± SE within columns and plant parts not followed by a common letter are significantly different (lameans, $P < 0.01$). Data for 1-d-old bolls were analyzed with PROC Freq (SAS Institute 1998). Numbers not followed by a common letter are significantly different (Fisher's exact test (two-tailed), $P < 0.05$, Steel and Torrie 1980). a, Number of insects tested.
^a ANOVA results: 2d- $F = 44.4$; $df = 2, 108$; $P < 0.01$. 4d- $F = 88.8$; $df = 2, 108$; $P < 0.01$.
^b Mean from eight commercial non-Bt cultivars, Elizabeth, MS.
^c Mean from 17 commercial Bt cultivars (Cry1Ac), Elizabeth, MS.
^d Mean from two experimental Bt cultivars, MON15813 and MON15985 (Cry1Ac and Cry2Ab), Shelby, MS.
^e ANOVA results: 2d- $F = 8.3$; $df = 1, 4$; $P = 0.01$. 4d- $F = 9.3$; $df = 1, 4$; $P < 0.01$.
^f Commercial Bt cultivar (Cry1Ac).
^g Experimental Bt cultivar (Cry1Ac and Cry2Ab).
^h Likelihood ratio chi-square (PROC Freq, SAS Institute 1998). $\chi^2 = 65.1$, $df = 2$, $P < 0.01$.

physiological cutout, plants have a near maximum fruit load, resulting in slowed vegetative growth. Greenhouse-raised plants were used during the second repetition of the experiment, which was done in April 2000. Greenhouse plants, two per pot, were watered twice weekly and kept at 20–30°C without supplemental lighting. Plants were fertilized once (Osmocote 14–14–14, N:P:K, Scotts, Columbus, OH). Fifty fully expanded leaves per cultivar were collected from ~50 preflowering plants. All leaves were returned to the laboratory, freeze dried, and stored at –80°C until use.
 Lyophilized leaves were ground into a powder using a mortar and pestle. For each of the three cultivars, the tissue was incorporated into artificial diet using a high-speed blender. Sublethal doses (1.124, 11.24, 112.4, and 1124 µg plant tissue/ml diet) were used to compensate for the high susceptibility of these insects, particularly tobacco budworms, to Bt cotton. For each dose, and for both repetitions of the experiment, 96 tobacco budworm and bollworm neonates were individually placed in 5.0-ml wells containing ~2 ml of treated diet. Trays containing 24 wells were covered with shrink wrap plastic and heat sealed. Survival of larvae was determined 3 and 7 d after infestation on diet. Larval length (mm) was measured at 7 d for six randomly selected larvae from each tray. Survival data were analyzed using PROC Genmod and lameans for separation of treatment effects (SAS Institute 1998). ANOVA was used to determine treatment effects on larval length, and Student-Newman-Keuls was used for mean separations (PROC GLM, SAS Institute 1998).
Results
Fresh Tissue Assays (Neonate Bollworm). Neonate bollworms were more susceptible to leaves and pink

flowers from dual-toxin Bt cultivars (Cry1Ac and Cry2Ab) than those from non-Bt or single-toxin Bt cultivars (Cry1Ac), when mortality was assessed after 4 d (Table 1). After 2 and 4 d, survival of neonates fed leaves of dual-toxin Bt cultivars was less than those fed single-toxin Bt or non-Bt cultivars (Table 1). Higher survival rates were also found for larvae fed leaves of non-Bt cotton compared with single-toxin Bt cultivars. Although there was not a significant difference after 2 d, more larvae were alive after 4 d when fed pink flowers of a single-toxin Bt cultivar (NuCOTN 33B) compared with a dual-toxin Bt cultivar (MON15985).
 A higher survival rate (Table 1) was observed for neonates fed 1-d-old bolls from non-Bt cotton (DPL50) compared with either Bt cultivar (NuCOTN 33B and MON15985). However, no significant differences were observed between the single and dual-toxin Bt cultivar.
Fresh Tissue Assays (Second Instar Bollworm, Beet Armyworm, and Fall Armyworm). Our results indicate that dual-toxin Bt cotton, compared with the non-Bt and the single-toxin cultivars, reduced the survival of second-instar larvae. Across all species and assays, cultivar effects on survival were highly significant ($\chi^2 = 60.7$, $df = 2$, $P < 0.01$). The fewest larvae (36%) survived to the pupal stage when fed MON15985 than when fed plant material from DPL50B (64%) or DPL50 (77%). DPL50B had less of an impact on survival than MON15985 ($\chi^2 = 26.7$, $df = 1$, $P < 0.01$). The greater effect of the dual-toxin cultivar on survival was especially apparent for beet armyworms (Table 2). Only 36% of the beet armyworms fed tissue from the MON15985 cultivar survived to pupation, but 93% of larvae survived when fed tissue from DPL50B. At 3, 7, and 15 d, the survival of both bollworm and fall armyworm was statistically less when fed MON15985 than when fed DPL50B. However, it should be noted that fall armyworm survival or

Table 2. Relative feeding, survival, mean larval lengths (mm at 48 h), and delays in pupation when second-instar bollworms, fall armyworms, and beet armyworms were fed plant parts from cotton seedlings grown (DPT-50), non (DPT-50), Cry1Aa, or two Bt proteins (MON15906, Cry1Aa and Cry2Ab).

Species	Plant part	Cultivar	n	Relative feeding ^a	Proportion of larvae surviving				Larval length (mm) after			Pupation delay (d) ^b
					3 d	7 d	10 d	To pupa	3 d	7 d		
Bollworm	Flower ^c	DPT-50	20	1.75 ± 0.17 ^a	0.00 ^a	0.00 ^a	0.00 ^a	0.00 ^a	15.8 ± 0.6 ^a	94.1 ± 0.6 ^a	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a
		DPT-50	20	0.47 ± 0.07 ^a	0.00 ^a	0.00 ^a	0.00 ^a	0.00 ^a	8.5 ± 1.0 ^a	16.8 ± 1.7 ^a	0.00 ± 1.10 ^a	0.00 ± 1.10 ^a
		MON15906	20	0.49 ± 0.08 ^a	0.00 ^a	0.00 ^a	0.00 ^a	0.00 ^a	8.9 ± 1.0 ^a	17.8 ± 1.8 ^a	0.00 ± 1.10 ^a	0.00 ± 1.10 ^a
		MON15906	20	0.49 ± 0.08 ^a	0.00 ^a	0.00 ^a	0.00 ^a	0.00 ^a	8.9 ± 1.0 ^a	17.8 ± 1.8 ^a	0.00 ± 1.10 ^a	0.00 ± 1.10 ^a
Fall armyworm	Flower ^c	DPT-50	20	1.25 ± 0.07 ^a	0.00 ^a	0.00 ^a	0.00 ^a	0.00 ^a	14.5 ± 0.6 ^a	17.0 ± 0.6 ^a	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a
		DPT-50	20	1.25 ± 0.07 ^a	0.00 ^a	0.00 ^a	0.00 ^a	0.00 ^a	14.5 ± 0.6 ^a	17.0 ± 0.6 ^a	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a
		MON15906	20	1.25 ± 0.07 ^a	0.00 ^a	0.00 ^a	0.00 ^a	0.00 ^a	14.5 ± 0.6 ^a	17.0 ± 0.6 ^a	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a
		MON15906	20	1.25 ± 0.07 ^a	0.00 ^a	0.00 ^a	0.00 ^a	0.00 ^a	14.5 ± 0.6 ^a	17.0 ± 0.6 ^a	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a
Beet armyworm	Leaf ^d	DPT-50	20	1.00 ± 0.07 ^a	1.00 ^a	1.00 ^a	1.00 ^a	1.00 ^a	19.1 ± 0.6 ^a	91.1 ± 0.6 ^a	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a
		DPT-50	20	1.00 ± 0.07 ^a	1.00 ^a	1.00 ^a	1.00 ^a	1.00 ^a	19.1 ± 0.6 ^a	91.1 ± 0.6 ^a	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a
		MON15906	20	0.87 ± 0.08 ^a	0.00 ^a	0.00 ^a	0.00 ^a	0.00 ^a	17.7 ± 0.6 ^a	19.7 ± 0.6 ^a	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a
		MON15906	20	0.87 ± 0.08 ^a	0.00 ^a	0.00 ^a	0.00 ^a	0.00 ^a	17.7 ± 0.6 ^a	19.7 ± 0.6 ^a	0.00 ± 0.00 ^a	0.00 ± 0.00 ^a

Numbers within columns and species not followed by a common letter are significantly different ($P < 0.05$, FREQ G-test) or FREQ CLM, [Student-Newman-Keuls] ANS Institute 1998). n, Number of larval instars, including appropriate days post hatch which were transferred to artificial diet after 48 h. Non-bolled uppermost leaves indicate significant effects of feeding method for indicated species (n) or on an interaction (n) between cultivars and feeding methods.

^a Relative feeding (0-5 scale, 0-5) at 48 h after plant parts were infested.

^b Pupation delay = delay in days relative to DPT-50. No bollworm larvae survived to the pupal stage on MON15906.

^c Entire white flowers, including buds and all floral parts.

^d Fully expanded leaves 4-6 nodes below the apical node.

MON15906 was only consumed from leaves on any of the 48 h survey, the amount of feeding on the 48-h cultivar (DPT-50) was higher than on the cultivars. Both fall armyworms and beet armyworms fed less on leaves from MON15906 than on leaves from DPT-50. The data from cultivars also had greater relative effects than did the single-diet cultivar (Table 2). After 6 and 7 d, there was a reduction in the size of beet and fall armyworm larvae that were fed MON15906 versus DPT-50 and DPT-50, and a corresponding delay in pupation was observed. Bollworms also were reduced by feeding on either of the Bt cultivars. No bollworms survived when fed MON15906, so the effect of the feeding on time to pupation could not be determined. However, DPT-50 caused a significant delay in bollworm pupation (3.6 d) relative to DPT-50. Generally, cultivar effects on survival and larval development were greater when larvae were fed plant material continuously than when transferred to diet after 48 h. Across all comparisons, the many methods had a significant impact on survival to the pupal stage ($\chi^2 = 14.4$, $df = 1$, $P < 0.01$) and on other variables measured in our experiments. Fewer larvae survived to the pupal stage when continuously fed plant material (19%) than when transferred to diet (48%). Similarly, 5 d after originally infested on plant leaves, larvae that had been transferred to diet were larger (17.8 ± 0.3 mm) than those kept on leaves (14.5 ± 0.3 mm) ($\chi^2 = 70.6$, $df = 1$, $P < 0.01$).

Interactions between cultivar effects and many methods were detected for survival (e.g., survival to pupal stage across all comparisons, $\chi^2 = 7.2$, $df = 2$, $P = 0.03$) (Table 2). These interactions were related to the recovery of larvae, after exposure to toxic plant tissue, when they were transferred to artificial diet. For plant tissue that was relatively nontoxic, transferring larvae to diet had little effect on survival. For example, beet armyworm survival to pupation was only 30% when larvae were continuously fed plant material from MON15906. Survival to the MON15906 treatment was higher (70%) when beet armyworms were transferred to diet after 48 h ($\chi^2 = 10.0$, $df = 1$, $P < 0.01$), whereas the many methods did not affect beet armyworm survival for DPT-50 and DPT-506 cultivars ($\chi^2 = 0.10$ and 0.01 , $df = 1$, $P = 0.73$ and 0.91 , respectively). Similarly, treatment interactions on larval length and pupation delay were detected for beet and fall armyworms. For example, fall armyworm continuously fed leaves of MON15906 and DPT-506 were 13.0 ± 0.6 and 14.0 ± 0.6 mm long after 5 d, respectively. Larvae that had been transferred to diet after 48 h were larger (18.4 ± 0.4 and 18.8 ± 0.7 mm long on MON15906 and DPT-506, respectively). In contrast, 5-d larval lengths were similar for fall armyworms continuously fed DPT-50 and for those transferred to diet (18.7 ± 0.7 and 18.8 ± 0.6 mm, respectively).

From these assays (Tables 2 and 3), it appears that if bollworm larvae survive to the third instar by feeding on relatively nontoxic buds or plant parts, they may then be able to survive and damage young buds from dead-leaves Bt cultivars (Table 2). For any cultivar, no mortality was observed for larvae

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larvae were fed plant tissue and transferred to diet 1a, the assay method was the same as that used for the pupal stage. No differences were observed on other variables. Larvae that were continuously fed plant material survived and molted to diet (86%). Larvae that were fed plant tissue, then transferred to diet 1a, survived and molted to diet (14.9 $\pm$ 0.3%, $P < 0.01$).

effects and assay val (e.g., survival to $x^2 = 7.8$, $df = 2$, P tions were related to posure to toxic plant ed to artificial diet y nontoxic, transfer- fect on survival. For val to pupation was ntinuously fed plant val in the MON15985 an beet armyworms h ($x^2 = 18.0$, $df = 1$, ethod did not affect .50 and DPL50B cot- 1, $P = 0.72$ and 0.91, interactions on larval detected for beet and 1 armyworms contin- 35 and DPL50B were ng after 5 d, respec- nferred to diet after 4 19.9 ± 0.7 mm long ectively). In con- ar for fall armyworms r those transferred to mm, respectively). nstar Bollworm). It survive to the third ontoxic hosts or plant ; survive and damage ultivars (Table 3). For bserved for larvae

TABLE 3. Proportion of third instar bollworms that survived and penetrated 4-6 old boll bolls when fed cotton containing one (DPL50), one (NCOTN 338, CryIAc), or two Bt proteins (MON15985, CryIAc and Cry2Ab)

Cultivars	Surviving larvae, Boll penetration		
	2 d.	4 d.	6 d.
DPL60	1.00a, 1.00a	1.00a, 1.00a	0.83a, 1.00a
NuCOTN 33B	1.00a, 0.88ab	0.74b, 0.88a	0.63b, 0.88a
MON15935	1.00a, 0.88b	0.75b, 0.80a	0.54ab, 0.80a
χ^2 (df = 2)*	0.00, 0.79	8.90, 5.17	6.98, 8.17
F	1.00, 0.01	0.03, 0.06	0.04, 0.09

Data were analysed using PROC FREQ (SAS Institute 1996). Numbers within columns not followed by a common lower letter are significant different (Fisher's exact test [two-tailed]; $P < 0.05$; Steel and Torrie 1980). n , Number of insects tested.

* Likelihood ratio chi-square (PROC FREQ, SAS Institute 1996).

feeding on bolls for 2 d, and the ability to successfully penetrate the external boll wall was high ($\geq 68.0\%$). While there was no difference in boll penetration between single and dual-toxin Bt cultivars, boll penetration was significantly reduced for the dual-toxin cultivar compared with the non-Bt cultivar. There were no significant differences in boll penetration between cultivars at 4 d, but larvae on both Bt cultivars had lower survival rates than the non-Bt cultivar. After 6 d, larval survival on Bt cultivars was similar and remained somewhat lower than survival on non-Bt cotton, but there was no difference in boll penetration among cultivars ($P > 0.05$).

Assays with Lyophilized Leaf Tissue (Neonate Bollworm and Tobacco Budworm). For all doses of plant material incorporated into the diet, average mortality of tobacco budworm and bollworm never exceeded 26% at 7 d. Thus, the doses used in this experiment were largely sublethal for this time frame. Across all cultivars and for both species, mortality only increased by ~5% between 3 and 7 d (bollworm: $\chi^2 = 4.0$, $df = 1$, $P < 0.05$; budworm: $\chi^2 = 4.7$, $df = 1$, $P < 0.05$).

Despite the fact that the doses chosen were mostly sublethal, cultivar and dose significantly influenced the mortality of bollworm at 3 d ($\chi^2 = 34.1$, $df = 2$, $P < 0.01$ and $\chi^2 = 30.5$, $df = 3$, $P < 0.01$, respectively) and 7 d ($\chi^2 = 44.1$, $df = 2$, $P < 0.01$ and $\chi^2 = 28.4$, $df = 3$, $P < 0.01$, respectively). Bollworm mortality for MON15985 was about 9–13% higher than for the other cultivars on days 3 and 7. No significant difference in mortality was detected between DPL50 and DPL50B (Table 4). Lower dosages (1.124 and 11.24 $\mu\text{g}/\text{ml}$ diet) tended to cause less mortality of bollworms at both three and 7 d. An interaction between cultivar and dose was detected 3 d after bollworm larvae were infested on treated diet ($\chi^2 = 16.8$, $df = 8$, $P = 0.02$), but not at 7 d ($\chi^2 = 11.7$, $df = 8$, $P > 0.07$). At 3 d, survival of bollworms fed 1.124, 11.24, 112.4, and 1124 μg of DPL50 plant tissue/ml of diet was 93.1, 91.4, 83.8, and 80.5%, respectively. For DPL50B, survival at these same dosages was 93.6, 91.4, 81.7, and 85.3%, respectively. So higher dosages tended to cause more mortality for these varieties, even for the non-Bt cultivar. Bollworm survival on MON15985 was 84.5, 73.2, 75.9, and 80.6% as dosages were increased. Thus, a clear dose-response pattern was not evident for the MON15985 cultivar, with greater mortality actually occurring at intermediate dosages.

Tobacco budworm survival was not significantly influenced by cultivar on either day 3 or 7 ($\chi^2 = 5.3$, $df = 2$, $P > 0.07$ and $\chi^2 = 2.81$, $df = 2$, $P > 0.24$ respectively), and no interactions were found between treatment and dose for either day ($\chi^2 = 6.9$, $df = 6$, $P > 0.33$ and $\chi^2 = 5.7$, $df = 6$, $P > 0.46$ respectively). Despite the lack of cultivar effects on survival, there was a significant dose-response on day 7 ($\chi^2 = 9.8$, $df = 3$, $P = 0.03$). There was 5% more mortality occurring at the highest dose compared with the lowest dose (Table 4).

Sublethal effects on larval development (i.e. length) were greatly influenced by cultivar and dose for both species. Cultivar effects on bollworm ($F = 197$; $df = 2, 563$; $P < 0.01$) and budworm ($F = 100$; $df = 2, 563$; $P < 0.01$) were similar in that larvae fed MON15985 were typically smaller than larvae fed DPL50B, and larvae fed non-Bt cotton were larger than those fed either of the Bt cultivars (Table 5). The dose-response for bollworm and budworm also were similar (bollworm: $F = 62.9$; $df = 3, 563$; $P < 0.01$, and budworm: $F = 76.0$; $df = 3, 563$; $P < 0.01$) in that larvae fed higher doses were generally smaller than those fed lower doses. However, there was an obvious interaction between cultivar and dose for both species (bollworm: $F = 6.81$; $df = 6, 563$; $P < 0.01$, and budworm:

Table 4. Proportion of nonstar-bellworm and tobacco budworm that survived after being fed *Bt*-hybridized leaf tissue from cotton containing zero (DPL50), one (DPL50B, CryIAc), or two *Bt* proteins (MONT8985, CryIAc and Cry2Ab)

Analysis variable	Bollworm survival ^a		Burdworm survival	
	3 d ^b		3 d	7 d
Caliver DFL60	0.57a	0.57a	0.58a	0.78a
DFLAB	0.68a	0.68a	0.78a	0.78a
MCN15805	0.76b	0.73b	0.88a	0.78a
Dose ^b				
1194	0.60a	0.65a	0.63a	0.81a
11.94	0.65b	0.61ab	0.60a	0.78ab
119.4	0.60c	0.75c	0.81a	0.78ab
1194	0.68bc	0.76c	0.79a	0.73b

Means without columns not followed by a common letter are significantly different ($P < 0.05$, PROC GLM, protected means; SAS Institute 1989).

* Indicates a significant interaction, for bollworm, between cultivar and dose on day 3 (see text).

^b 10% of plant tissue per ml of artificial diet.

Table 5. Larval length (mm $\pm$ SE) of armyworm and tobacco budworm larvae after 1 d when continuously fed on diet containing lyophilized leaf tissue at four different doses

Dose*	Bollworm length			Bollworm length		
	DPL50	DPL50B	MON15985	DPL50	DPL50B	MON15985
L154	15.7 $\pm$ 0.3Aa	14.9 $\pm$ 0.3Ba	11.0 $\pm$ 0.7Ca	13.5 $\pm$ 0.3Aa	13.0 $\pm$ 0.4Aa	13.0 $\pm$ 0.6Aa
L154	15.6 $\pm$ 0.4Aa	15.6 $\pm$ 0.3Ba	11.5 $\pm$ 0.8Ca	13.9 $\pm$ 0.4Aa	13.0 $\pm$ 0.4Aa	12.4 $\pm$ 0.5Ba
L154	14.3 $\pm$ 0.4Ab	13.1 $\pm$ 0.5Bb	8.1 $\pm$ 0.5Cb	13.3 $\pm$ 0.4Aa	13.4 $\pm$ 0.3Ba	8.7 $\pm$ 0.4Cb
L154	13.9 $\pm$ 0.5Ab	8.5 $\pm$ 0.5Bc	8.1 $\pm$ 0.5Cc	13.2 $\pm$ 0.4Aa	10.2 $\pm$ 0.5Bb	4.4 $\pm$ 0.5Cc

Larvae were from cotton containing none (DPL50), one (DPL50B, CryIAC), or two Bt proteins (MON15985, CryIAC and Cry2Ab). Means within a row and species not followed by a common uppercase letter are significantly different, and means within columns not followed by a common lower letter are significantly different ($P < 0.05$, PROC GLM, Student-Newman-Keuls, SAS Institute 1989).
* μ g of plant tissue per ml of artificial diet.

$F = 34.5$; $df = 6, 583$; $P < 0.01$). Compared with DPL50B and MON15985, increasing the amount of DPL50 tissue incorporated into diet had a relatively small impact on larval growth. The dose-response for MON15985 was particularly evident for both species compared with the other varieties.

Discussion

It is uncertain how much additional protection from insects the insertion of this second Bt toxin-producing gene will give to cotton until it is introduced and used on a large scale. However, our data suggest that dual-toxin Bt cottons will provide substantially better control of lepidopteran pests compared with the existing single-toxin Bt cultivars. In field studies, Stewart et al. (2000; unpublished data) have shown that the dual-toxin Bt cultivar (MON15985) provided a significant improvement in control of bollworm, soybean looper, and fall armyworm populations relative to the single-toxin cultivar (DPL50B). The superiority of cultivars expressing both CryIAC and Cry2Ab should be most apparent for soybean loopers, beet armyworms, and fall armyworms because these species are less affected than bollworm by the CryIAC single-toxin Bt cultivars that are presently available. The available, single-toxin Bt cottons sometimes require applications of foliar insecticides to control soybean looper, beet armyworm, fall armyworm, and bollworm. Based on these results and that of Stewart et al. (2000; unpublished data), the dual-toxin cultivars (expressing CryIAC and Cry2Ab) may not require supplemental insecticide applications for these pests.

Relative to the single-toxin Bt cultivar, the dual-toxin cultivar had similar and negative impacts on growth of both bollworm and tobacco budworm (Tables 4 and 5). This will have clear implications in managing resistance to Bt cotton for these pests (see Tabashnik 1994, Sachs et al. 1998, Gould 1998). Interestingly, a relatively small but significant dose-response was observed for bollworm when fed tissue from non-Bt cotton. Naturally occurring toxins in cotton leaves, such as gossypol, may have caused this effect (Lukafahr et al. 1976).

The interactions we detected between cultivar effects and assay method, for fresh tissue assays (Table 2), could indicate that one of the assay methods was not as reliable in separating treatment effects. These

interactions resulted from the recovery of larvae after they were transferred from Bt-cotton tissue to diet (particularly MON15985), whereas recovery did not occur when insects were continuously fed plant material. Despite the interactions, similar conclusions concerning cultivar effects on larval survival and development would generally be made based on the results of either assay method. However, cultivar effects were more dramatic when larvae were kept on plant tissue throughout development. Thus, maintaining insects on plant material, rather than transferring to diet, appears the best approach for increasing the sensitivity of fresh-tissue assays.

Because we did not test a cultivar expressing Cry2Ab alone, we cannot conclude that Cry2Ab had greater activity on armyworms than the CryIAC toxin in DPL50B. Also, the amount of Bt toxins, CryIAC and Cry2Ab, expressed in the plant tissue was not determined. Greenplate et al. (2000) reported similar levels of CryIAC in near-isogenic lines of cotton expressing either CryIAC alone or CryIAC and Cry2Ab (referred to as "CryX" in his article). However, the level of Cry2Ab expression in plant tissue was ~ 10 times that of CryIAC. The potency of CryIAC versus Cry2Ab, at equivalent doses, varies for different lepidopteran species (Sims 1997). Furthermore, the dose received by an insect was self-regulated in our assays. Nonpreference for the various toxin plant tissues may influence the dose ingested by individuals. Insects fed higher doses of Bt toxins may actually feed less as a result of gut paralysis (Sikorowski and Lawrence 1997), and thus, actually consume a smaller dose of plant tissue. Reduced feeding on Bt tissue was obvious in assays using fresh plant parts. So for our assays, these factors make it difficult to compare the sensitivity of different species to the various Bt toxins (or compare among 'doses' within a given assay) because the ingested dose was not known. However, assays with fresh tissue or lyophilized tissue were well suited for comparing the relative toxicity of plant tissues.

Reductions in larval length and pupation delays observed in fresh-tissue and lyophilized-tissue assays were most diagnostic of increased toxicity in the dual-toxin cultivars. Besides indicating the direct effects of the Bt toxins, these sublethal variables may also reflect the indirect impact of reduced feeding on toxic plant tissue. Assays using lyophilized plant tissue incorporated into diet have some advantages because plant

304

length	MON1805
1a	11.6 ± 0.8Aa
1Ba	12.4 ± 0.8Ba
2a	8.7 ± 0.4Cb
2b	4.4 ± 0.3Cc

ry1Ac and Cry2Ab).
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cultivar expressing
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nt lepidopteran spe-
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issues may influence
Insects fed higher
ed less as a result of
erence 1997), and
dose of plant tissue.
as obvious in assays
assays, these factors
maltivity of different
(or compare among
we the ingested dose
with fresh tissue or
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and pupation delays
skilled-tissue assays
i toxicity in the dual-
the direct effects of
bles may also reflect
eding on toxic plant
plant tissue incorpo-
tages because plant

tissues could be stored until needed. This allows for comparison among plant tissues collected at different times (e.g., early versus late season). Also, there is no need to periodically give fresh plant material to the insects. An additional advantage is that dosages can be adjusted so that excessive mortality caused by toxic plant tissue does not occur, and hence neonates can be used in assays. With fresh plant tissue, particularly with neonates, excessive mortality may make separating treatment effects (i.e., Bt-cultivars) statistically difficult.

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305

PERFORMANCE OF BOLLGARD II COTTON AGAINST LEPIDOPTEROUS PESTS IN THE LOWER RIO GRANDE VALLEY OF TEXAS

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Abstract

The Bollgard II variety, which has not been licensed for use by commercial agriculture as of this report date was tested against several lepidopterous pest species. Results indicated that as the cotton leaf aged, increased larval mortality was recorded. The results show promise for improved control of the various tested species with Bollgard II compared to a currently registered Bollgard variety, DPL 50B and a non-Bollgard variety, DPL 50.

Introduction

The introduction of genetically altered cotton varieties throughout the U.S. cotton belt in 1995 has resulted in some significant changes in insect management. Growers have had to change their decision making process about insect management since the now-insect resistant varieties provide control, but not 100 percent control of various pests. As new varieties of the genetically altered varieties come into the market, growers need current research data to assist them in making variety choices. The objective of this study was to evaluate the relative efficacy of Bollgard II against lepidopterous pests compared to its parental Bollgard line.

Methods and Materials

A small plot trial was planted at the Texas A&M Research and Extension Center Miller Farm to evaluate the efficacy of Bollgard II against lepidopterous esterpiians. In conjunction with this field trial, a series of laboratory bioassays were conducted to evaluate the efficacy of Bollgard II against the beet armyworm (*Spodoptera exigua*) (BAW) and the cotton bollworm (*Heliothis virescens*), also known as the corn earworm (CEW). These bioassays also included evaluation of the effects of leaf age on efficacy.

The field portion of this test consisted of a randomized complete block design with four replications of six treatments. The treatments were three cotton varieties, with two plots of each variety; one designated for treatment with an insecticide as needed and the other designated for non-treatment. Varieties included DPL 50, DPL 50B (Bollgard), and DPL 15985 (Bollgard II). Plot size was 8 rows (40 feet center) by 30 feet. The data presented in this study was collected from non-treated plots only.

The laboratory bioassays were conducted with larvae (CEW and BAW) provided by the USDA-ARS laboratory in Weslaco, Texas. For each bioassay, leaves were collected from each plot and transported to the laboratory. Leaf disks (7/8 inch diameter) were cut with a cork borer and placed in a 34 ounce diet cup which contained a 7/8 inch disk of filter paper moistened with R2 water. A single larva was placed on each leaf disk, with ten larvae per treatment per replication. Mortality was checked every two days for 10 days, and leaf disks were replaced with freshly cut disks every two days. At 10 days after initial exposure, any surviving larvae were counted and weighed.

Bioassays were initiated on three dates: On May 22, bioassays were conducted against BAW and CEW using the third leaf from the terminal of each plant. Plants at this time consisted of approximately 12 nodes. These bioassays were initiated with teenage larvae of both species.

On May 23, a single bioassay was conducted against BAW. This bioassay was initiated with 1 day old larvae. This bioassay included evaluation of the third and sixth leaf from the terminal. Plants at this point contained approximately 12 nodes.

On June 3, both species of caterpillars were bioassayed against the third, sixth, and ninth leaf of each variety. Nonteen larvae were used to initiate the bioassays. Plants contained 14 to 15 nodes at this point in the season.

Field populations of lepidopterous larvae remained low until fairly late in the season. Insecticidal treatments were applied to the wrong plots shortly after larvae first appeared, resulting in a single date of field larval counts for this study. On July 3, drop cloth samples were taken in the field plots to determine the number of lepidopterous larvae present in a 6 feet of row. Larvae were identified and counted by species.

Seed cotton was harvested for fiber quality analysis and ginned in a G view portable gin. Fiber quality was determined by selecting seed cotton, ginning same and sending it to the International Textile Center in Lubbock, Texas for analysis.

All data were analyzed with the PROC GLM procedure of PC-SAS. Where significant differences were indicated ($P < 0.05$), means were separated with DMRT ($P = 0.05$).

Results and Discussion

Bollgard provided increased mortality and decreased larval weights in surviving larvae of CEW as compared to DPL 50 (Tables 1, 3 and 5), but generally showed minor impact on BAW (Table 2, 3, and 6). Bollgard II generally showed increased activity on both species as compared to Bollgard, with increased mortality and greatly decreased survivor weights (Tables 1 - 6). All insect bioassays on leaf age generally showed decreased survival on older leaves (Tables 4 - 6).

Bioassays were also conducted with square bracts (data not presented). These bioassays showed near 100% mortality of CEW on all three varieties, while BAW data closely resembled the leaf bioassay data.

Bullworm, beet armyworm, cabbage looper (*Trichoplusia ni*) and fall armyworm (*Spodoptera frugiperda*) (FAW) appeared in the test field very late in the season. Larval feeding was primarily confined to leaves due to severe loss of fruit from prior boll weevil damage. Field counts of larvae in this test indicated that Bollgard II was superior in protection from the various species encountered since all counts in the Bollgard II were zero and statistically different from Bollgard and DPL 50 (Table 7). Previous research conducted by the authors (unpublished data) indicated that tobacco budworm were significantly reduced by Bollgard II compared to the standard non-Bollgard variety DPL 50 and equal to DPL 50B.

Yield data were not taken because boll weevil damage was so severe as to make harvest not economic. No significant differences in fiber quality were detected among the varieties (Table 8).

Table 1. Bollgard test mortality of neonate CEW larvae reared on leaf disks of selected cotton varieties. Leaf disks were cut from the third leaf from the terminal of plants with 12 nodes, initiated May 22, 2000.

Variety	Percent mortality at specified days after exposure				
	1 DAE	4 DAE	6 DAE	8 DAE	10 DAE
BG,II	31.7 a	73.3 a	75.0 a	80.0 a	81.7 a
DPL	5.6 b	16.7 b	18.3 b	18.3 b	20.0 b
DPL 50	1.7 b	1.7 b	1.7 b	1.7 c	1.7 c

Numbers within a column followed by the same letter are not significantly different (DMRT).

Table 2. Buligard test mortality of neonate BAW larvae reared on leaf disks of selected cotton varieties. Leaf disks were cut from the third leaf from the terminal of plants with 12 nodes. Initiated May 22, 2000.

Variety	Percent mortality at specified days after exposure				
	2 DAE	4 DAE	6 DAE	8 DAE	10 DAE
BG II	11.7 c	44.7 a	63.0 c	68.3 a	73.3 c
DPL 50B	0.0 b	0.0 b	0.0 b	0.0 a	8.3 b
DPL 50	0.0 a	0.0 b	0.0 b	0.0 b	5.0 c

Numbers within a column followed by the same letter are not significantly different (DMRT).

Table 3. Buligard test surviving larvae weights of neonate CEW and BAW larvae reared for 10 days on leaf disks of selected cotton varieties. Leaf disks were cut from the third leaf from the terminal of plants with 12 nodes. Initiated May 22, 2000.

Variety	Mean Larval Weights	
	CEW	BAW
BG II	0.003 c	0.001 c
DPL 50B	0.008 b	0.016 a
DPL 50	0.002 c	0.007 a

Numbers within a column followed by the same letter are not significantly different (DMRT).

Table 4. Buligard test mortality of one day old BAW larvae reared on leaf disks of selected cotton varieties. Leaf disks were cut from the third and sixth leaf from the terminal of plants with 12 nodes. Initiated May 23, 2000.

Variety	Percent mortality at specified days after exposure			
	2 DAE		4 DAE	
	Leaf 3	Leaf 6	Leaf 3	Leaf 6
	45.0 a	97.5 a*	72.5 a	100.0 a
DPL 50B	5.0 b	37.5 b*	7.5 b*	32.5 b*
DPL 50	2.5 b	15.0 a	7.5 a	27.5 b
Variety	6 DAE		8 DAE	
	Leaf 3	Leaf 6	Leaf 3	Leaf 6
	85.0 a	100.0 a	92.5 c	100.0 a
	7.5 b	47.5 b*	7.5 b	37.5 b*
DPL 50	7.5 b	27.5 b	7.5 b	27.5 b
Variety	Mean Larval Weights (g) at 10 DAE			
	Leaf 3	Leaf 6	Leaf 3	Leaf 6
	95.0 a	100.0 a	---	---
	10.0 b	47.5 b*	0.008 b	0.007 b
DPL 50	7.5 b	27.5 b	0.010 a	0.010 a

Numbers within a column followed by the same letter are not significantly different (DMRT).

* following a value indicates that the mortalities on the 3rd versus 6th leaf were significantly different for the indicated DAE and variety.

Table 5. Buligard test mortality and surviving larvae weights and sizes of neonate CEW larvae reared for 10 days on leaf disks of selected cotton varieties. Leaf disks were cut from the third, sixth and ninth leaf from the terminal of plants with 14 to 15 nodes. Initiated June 5, 2000.

Variety	Percent mortality at specified days after exposure		
	2 DAE		
	Leaf 3	Leaf 6	Leaf 9
BG II	17.5 Aa	17.5 Aa	10.0 Aa
DPL 50B	7.5 Aa	12.5 Aa	20.0 Aa
DPL 50	5.0 Aa	0.0 Aa	3.5 Aa
Variety	4 DAE		
	Leaf 3	Leaf 6	Leaf 9
BG II	72.5 Ab	67.5 Aa	57.5 Aa
DPL 50B	30.0 Ba	25.0 Ba	22.5 Ba
DPL 50	7.5 Bc	2.5 Cc	2.5 Cc
Variety	6 DAE		
	Leaf 3	Leaf 6	Leaf 9
BG II	80.0 Aa	97.5 Aa	100.0 Aa
DPL 50B	32.5 Bc	32.5 Ba	37.5 Bc
DPL 50	20.0 Ca	5.0 Cc	10.0 Cc
Variety	8 DAE		
	Leaf 3	Leaf 6	Leaf 9
BG II	95.0 Aa	100.0 Aa	100.0 Aa
DPL 50B	67.5 Ba	82.5 Ba	90.0 Aa
DPL 50	45.0 Cc	27.5 Cc	22.5 Ba
Variety	10 DAE		
	Leaf 3	Leaf 6	Leaf 9
BG II	100.0 Aa	100.0 Aa	100.0 Aa
DPL 50B	87.5 Aa	97.5 Aa	95.0 Aa
DPL 50	52.5 Ba	30.0 Ba	37.5 Ba
Variety	Mean larval weights (g) at 10 DAE		
	Leaf 3	Leaf 6	Leaf 9
BG II	---	---	---
DPL 50B	0.001 B	0.001	0.003
DPL 50	0.002 Aa	0.003 a	0.007 a

Numbers within a data category and leaf age (column) followed by the same UPPER CASE letter are not significantly different (DMRT).

Numbers within a data category and variety (row) followed by the same LOWER CASE letter are not significantly different (DMRT).

Table 6. Bollgard test mortality and surviving larvae weights and sizes of neonate BAW larvae reared for 10 days on leaf disks of selected cotton varieties. Leaf disks were cut from the third, sixth and ninth leaf from the terminal of plants with 14 to 15 nodes. Inhabant June 5, 2000.

Percent mortality at specified days after exposure			
Variety	2 DAE		
	Leaf 3	Leaf 6	Leaf 9
BGI	35.1: Aa	42.5 Aa	92.5 Aa
DPL 50B	0.0 Ba	0.0 Ba	0.0 Ba
DPL 50	0.0 Ba	0.0 Ba	0.0 Ba
	4 DAE		
	Leaf 3	Leaf 6	Leaf 9
BGI	40.0 Aa	67.5 Ab	95.0 Aa
DPL 50B	0.0 Ba	0.0 Ba	2.5 Ba
DPL 50	2.5 Ba	3.0 Ba	0.0 Ba
	6 DAE		
	Leaf 3	Leaf 6	Leaf 9
BGI	47.5 Aa	72.5 Ab	100.0 Aa
DPL 50B	0.0 Ba	0.0 Ba	2.5 Ba
DPL 50	2.5 Ba	3.0 Ba	2.5 Ba
	8 DAE		
	Leaf 3	Leaf 6	Leaf 9
BGI	57.5 Ab	72.5 Ab	100.0 Aa
DPL 50B	0.0 Ba	2.5 Ba	2.5 Ba
DPL 50	2.5 Ba	3.0 Ba	7.5 Ba
	10 DAE		
	Leaf 3	Leaf 6	Leaf 9
BGI	60.0 Ab	73.0 Ab	100.0 Aa
DPL 50B	0.0 Ba	2.5 Ba	7.5 Ba
DPL 50	2.5 Ba	7.5 Ba	7.5 Ba
Mean Larval Weights (g) at 10 DAE			
	Leaf 3	Leaf 6	Leaf 9
BGI	0.001 Ca	0.001 Ca	---
DPL 50B	0.012 Bh	0.017 Ba	0.008 Ac
DPL 50	0.014 Ab	0.020 Aa	0.008 Ac

Numbers within a data category and leaf age (column) followed by the same UPPER CASE letter are not significantly different (DMRT). Numbers within a data category and variety (row) followed by the same LOWER CASE letter are not significantly different (DMRT).

Table 7. Lepidopteran larval counts in Bollgard test cotton varieties, TAMU "Hiler" farm, Weslaco, Texas, July 3, 2000.

Variety	Number of larvae/6 feet row				
	Loopers	BAW	CSW	FAW	Total Loops
DPL 50	54.6 a	2.3 a	3.3 a	4.3 a	64.5 a
DP 50B	63.3 a	1.6 a	4.4 a	4.4 a	73.6 a
DP BGI	0.0 b	0.0 b	0.0 b	0.0 b	0.0 b

Table 8. Bollgard test cotton variety fiber quality results. TAMU "Hiler" farm, Weslaco, Texas 2000.

Variety	Microaire	Staple	Strength
BGI	3.7	1.20	31.0
DPL 50	3.5	1.18	31.5
DPL 50B	3.5	1.22	33.4
BGI	3.9	1.22	30.6
DPL 50	3.5	1.17	31.3
DPL 50 B	3.4	1.20	32.8

FIELD EFFICACY OF COTTON EXPRESSING TWO INSECTICIDAL PROTEINS OF *BACILLUS THURINGIENSIS*

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Abstract

Field studies were conducted in 1999 and 2000 to evaluate field efficacy of cotton (*Gossypium hirsutum* L.) expressing two insecticidal proteins of *Bacillus thuringiensis* Berliner (Bt) on various lepidopteran pests. Near-isogenic varieties including non-transgenic, single-toxin, and dual-toxin cotton were compared for bollworm numbers, numbers of noctuid/lepidopteran pests, damaged terminals, bolls, and squares, and seed cotton yield. Relative to the non-Bt cotton cultivar, both single and dual-toxin cotton caused a significant reduction in the numbers of heliothine larvae, damaged terminals, and damaged squares and bolls found during visual sampling. Generally, lower heliothine were found in MON5963 than in DPL508 or DPL50 (Table 1, Figure 1). Additionally, fewer beetarmies were found in unsprayed subplots with cotton expressing both Cry1Ac and Cry2Ab than non-Bt subplots treated with insecticide. There was no significant difference in damaged terminals, squares, or bolls (predominantly due to bollworm) between single and dual-toxin varieties, but both Bt cultivars exhibited less damage than non-Bt cotton. Cotton expressing one and two insecticidal proteins reduced cumulative bollworm populations 74% and 94% for both years, respectively. Although single-toxin cotton only yielded a 14% cumulative reduction of fall armyworm, populations were reduced by 96% in cotton expressing two Bt toxins compared with non-Bt cotton and cotton expressing Cry1Ac alone. Dual-toxin Bt cotton caused a 94% cumulative reduction in soybean loopers, when compared to non-Bt cotton. More soybean loopers were found in the single-toxin Bt cotton than the non-Bt cultivar. Although both single and dual toxin varieties met cotton yield over non-Bt cotton, there was no significant difference between the two varieties.

These data suggest that the insertion of the Cry2Ab gene will provide substantially better control of a wide range of lepidopteran pests when compared to cotton expressing Cry1Ac alone. Since armyworms and loopers are not as affected by the single-toxin cultivar as heliothines, the true benefit of this second toxin-producing gene will likely be more apparent for these occasional pests. However, control of bollworms should also be improved.

Introduction

For many years, lepidopteran pests have been a major source of economic damage to cotton throughout the United States. Much of the total economic damage induced by insects and control costs involved can be attributed to the tobacco budworm (*Heliothis virescens*), bollworm (*Helioverpa zea*), fall armyworm (*Spodoptera frugiperda*), beet armyworm (*Spodoptera exiguus*), and soybean looper (*Parasplanthis lachrymans*) (Williams 2000).

The use of insecticides has been an effective method of controlling caterpillar pests in cotton. The issues of insecticide resistance (Holland et al. 1990, Graves et al. 1994) and the negative impact on non-target beneficial arthropod populations, however, has spawned an interest in alternate control methods for these pests. In 1996, transgenic cotton expressing the Cry1Ac δ -endotoxin of *Bacillus thuringiensis* var. *kurstaki* was introduced for commercial production (i.e., Bollgard[®]; Monsanto Company, St. Louis, MO). This technology provides season-long control of lepidopteran pests, particularly the heliothine complex. Although this

new technology has proven to be quite effective, some weaknesses have been noted, such as lack of efficacy against occasional pests (e.g., armyworms, loopers) and high populations of bollworm.

Cotton expressing two insecticidal proteins of *Bacillus thuringiensis* (i.e., Bollgard II[®]) has recently been developed by Monsanto Company. This transgenic cotton not only expresses the Cry1Ac toxin, but an additional gene expressing Cry2Ab toxin has been added to aid in control of occasional pests and high populations of bollworm. The addition of this second toxin to the original Bt cotton variety can potentially provide additional control of occasional pests as well as bollworm present in high populations.

Materials and Methods

In 1999 and 2000, field studies were conducted to compare the relative efficacy of near-isogenic lines of cotton expressing one, two, or two insecticide proteins of *Bacillus thuringiensis* Berliner on certain lepidopteran pests. Three varieties of cotton were planted: DPL50 (non-Bt), DPL508 (expresses Cry1Ac), and MON5963 (expresses Cry1Ac and Cry2Ab). Four main plots of each cultivar were planted on 20 May, 1999 and 16 May, 2000 in a randomized complete block design at the Plant Science Research Center, Mississippi State, MS. Both years, cotton was planted at 11.7 seeds/m using a cone planter. Each plot was 12 rows wide (97 cm row spacing) and 12-m long and was divided into a four-row sprayed subplot and an eight-row unsprayed subplot. Sprayer subplots were treated with insecticide for control of lepidopteran pests based on average insect counts made in the non-Bt cultivar such that whenever the DPL50 plots exceeded a treatment threshold for any lepidopteran pests, insecticide was applied in all varieties. After the initial insecticide application, only insect samples in sprayed subplots were used to trigger subsequent applications. Insecticide treatments consisted 76 g a.i./ha spinosad + 37 g a.i./ha cyfluthrin. All insecticide applications were made with a high-clearance, small plot tractor calibrated to deliver a spray volume of 68 l/ha. Unsprayed subplots were not treated for lepidopteran pests with insecticide.

No insecticide applications were used either year, not 227 g a.i./ha acephate was applied to all plots approximately one week after plant emergence. Additionally, several applications of JUV emulsion (22g a.i./ha) were made to all plots during the course of the season as part of the existing boll weevil eradication program. Applications for the control of caterpillar pests (primarily bollworm and tobacco budworm) were made in sprayed subplots on 29 July and 4, 13, and 27 August of 1999 (later application for soybean loopers). In 2000, insecticide applications were made on 12 and 29 July and 5 August. Based on insect control recommendations for Mississippi cotton (Layton 1999, 2000) no other insecticide applications were necessary, either in 1999 or 2000. All plots were furrow irrigated four times in 1999 and five times in 2000.

In order to determine relative efficacy of the different cultivars under field conditions, naturally occurring populations were monitored twice weekly via visual, sweep-net, and drop-cloth samples. Prior to the initiation of insecticide applications for heliothines (29 July in 1999 and 12 July in 2000), samples were taken randomly throughout each main plot. Following the first application, samples were taken in the four sprayed rows and the four adjacent unsprayed rows. The remaining unsprayed rows were used as a source of plant material for use in laboratory assays.

Visual samples consisted of examining the top five nodes of fifteen plant terminals in sprayed and unsprayed subplots for heliothines. Also examined were fifteen half-grown or larger squares and fifteen half-grown or smaller bolls for damage and the presence of lepidopteran pests, particularly bollworms, tobacco budworms, or fall armyworms. On time sampling dates, fifteen sweeps with a 38-cm diameter sweep net were made

in each subplot in 1999 and 2000. For drop cloth samples, one sample (1 ft. x 1 ft. of row) was taken in each subplot in 1999 and two were taken in each subplot in 2000. For both years, counts of soybean loopers and bollworms were recorded. Seed cotton yields were estimated by harvesting the center two rows of each subplot on 8 October in 1999, and 29 September in 2000.

Data were pooled in both years and were analyzed using split-plot analysis of variance procedures and using linear contrasts for main comparison ($\alpha=0.05$, Proc GLM; Computers, SAS 1998).

Results

Relative to the non-Bt control cultivar, our data indicated that both single and dual-toxin cultivars caused a significant reduction in the numbers of heliothine larvae, damaged terminals, and damaged squares and bolls found during visual sampling. There was no interaction between cultivar and insecticide in either year. Generally, fewer heliothines were found in transgenic cotton than in single-toxin or non-Bt cotton (Table 1, Figure 1). Fewer heliothines were also found in unsprayed subplots of MON15985 compared to DPL50 subplots treated with insecticide (Table 1). For damaged terminals, squares, and bolls (predominantly caused by bollworm), less damage was noted in the single and dual-toxin varieties than in the non-Bt variety (Table 2).

For other lepidopteran pests, only soybean loopers were present in high numbers during August of 1999, and moderate levels of full armyworm were found in 2000. Cumulative counts represent numbers of total insects in visual, sweep net, and drop cloth samples in sprayed and unsprayed subplots for both years. Cumulative counts showed a 79% and 96% reduction of bollworm larvae in Btgard II plots, compared to those with original Btgard and non-Bt, respectively (Figure 1). In both years, only one bollworm >1/4 inch was found in MON15985 as opposed to >10 that were found in DPL50B. For full armyworm, plots containing dual-toxin cotton reduced larvae by 96% over both non-Bt and single-toxin cotton in 1999 and 2000 (Figure 2). Populations of soybean loopers were also significantly reduced in plots containing dual-toxin cotton (96%). There were 41% more soybean loopers in the single toxin variety than the non Bt cotton (Figure 3).

Seed cotton yield for both 1999 and 2000 showed an increase by at least 15% in single and dual-toxin cotton, compared to the non-Bt variety (Figure 4). However, unsprayed subplots not-yielded cotton treated with insecticide by 8% (Figure 5). This is due to unusually high yield in unsprayed subplots in 1999. There was no interaction between cultivar and insecticide in either year.

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Table 1. Percent heliothine infestation in non-Bt, single-toxin, and dual-toxin transgenic cotton varieties in 1999 and 2000.

		% Heliothine-infested terminals	% Heliothine-infested squares/bolls	% Heliothine in all
DPL50	unsprayed	2.39 a	2.22 a	2.19 a
	sprayed	1.16 b	0.74 b	0.68 b
DPL50B	unsprayed	0.39 bc	0.35 bc	0.33 bc
	sprayed	0.47 bc	0.28 bc	0.24 cd
15985	unsprayed	0.04 c	0.08 c	0.06 d
	sprayed	0.30 bc	0.19 bc	0.15 cd

Table 2. Percent damaged terminals, squares, and bolls in non-Bt, single toxin, and dual-toxin transgenic cotton varieties in 1999 and 2000.

	% Damaged terminals	% Damaged squares	% Damaged bolls
DPL50	9.44 a	0.52 a	0.83 a
DPL50B	1.76 b	0.18 b	0.13 b
15985	1.14 b	0.14 b	0.05 b

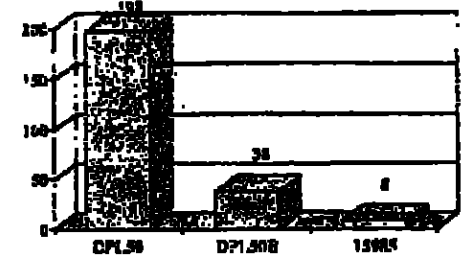


Figure 1. Cumulative counts for bollworm in non-Bt, single toxin, and dual-toxin transgenic cotton varieties in 1999 and 2000.

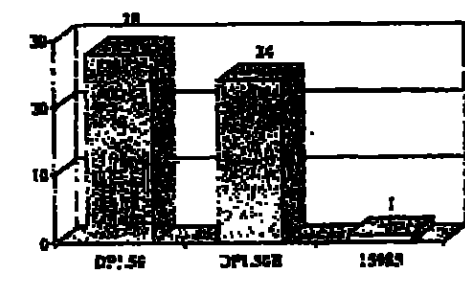
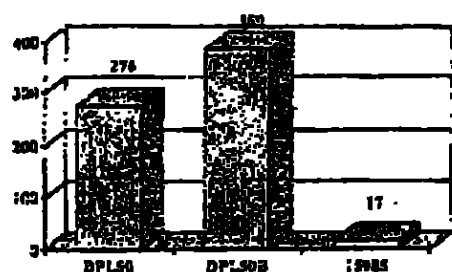
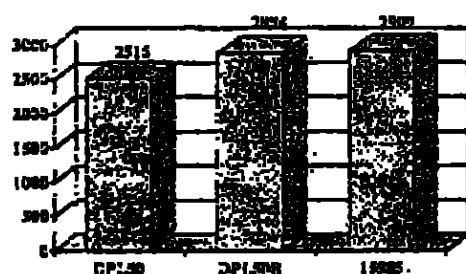


Figure 2. Cumulative counts for full armyworm in non-Bt, single toxin, and dual-toxin transgenic cotton varieties in 1999 and 2000.



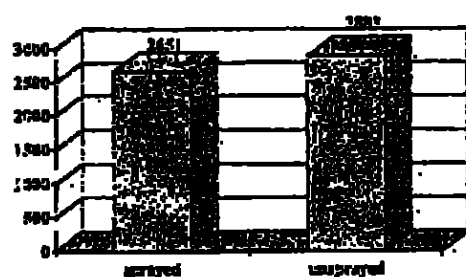
Means not followed by the same letter are significantly different (Duncan's $P < 0.05$).

Figure 3. Cumulative counts for soybean looper in non-Bt, single toxin, and dual-toxin transgenic cotton varieties in 1999 and 2000.



Means not followed by the same letter are significantly different (Duncan's $P < 0.05$, Proc GLM (LSmeans, p007), SAS Institute 1998).

Figure 4. Seed cotton yield from non-Bt, single toxin, and dual-toxin transgenic cotton varieties in 1999 and 2000.



Means not followed by the same letter are significantly different (Duncan's $P < 0.05$, Proc GLM (LSmeans, p007), SAS Institute 1998).

Figure 5. Seed cotton yield from sprayed and unsprayed subplots in 1999 and 2000.

DEVELOPMENT OF A LINE SPECIFIC GMO DETECTION METHOD: A CASE STUDY

pp. 459-462 (4)
312

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Summary

A strategy for a line specific GMO detection is presented. The method makes use of the anchored-PCR and can be used to screen foods for unauthorised GMOs. It is demonstrated that the proposed strategy is useful to develop a primer pair that can detect RoundUp Ready™ (Monsanto) soybean DNA in a line specific way.

Introduction

More and more foods are containing genetically modified organisms (GMOs) or GMO derived products. In the European Union such foods are regulated by regulation 90/220/EC and the directive Novel Food. These regulations stipulate a premarket approval and labelling requirements for foods derived from or containing GMOs. In this context, it is obvious that the authorities involved should have an adequate methodology for the identification of GMOs.

Up to now most DNA-based detection methods use the Polymerase Chain Reaction (PCR) to amplify parts of the inserted transgene. These PCR-based detection methods focus on frequently used genetic elements in GMOs, such as promoters, terminators, marker genes, etc. (Meyer, 1995; Pietsch *et al.*, 1997; Hupfer *et al.*, 1998). The disadvantage of such detection methods is that they are not line specific. This implies that it is impossible to discriminate between events that were obtained, using the same or similar transformation vectors.

Here we present a strategy to develop a line specific GMO detection procedure without preknowledge of the DNA sequence of the inserted fragment. The technique is based on the anchored-PCR (Theuns *et al.*, in preparation). This transgene display, used to amplify the junction between the insert and the plant-DNA, starts with digestion of the isolated DNA. By using a radioactively labelled anchor-primer, that is homologous with the border region of the inserted DNA, and an adapter-primer, that is specific for the adapter that is ligated at the end of each restriction fragment, the junction is specifically amplified. Subsequently the junctions are separated on a polyacrylamide gel after which the desired junction can be isolated from the gel. A method for the rapid isolation and subsequent sequencing of insert junctions is described. The obtained sequence data of the flanking plant-DNA of a particular T-DNA enabled us to design event-specific primers. Thereupon the newly designed event-specific primer together with the above mentioned anchor-primer is used in a regular PCR reaction to amplify an unique fragment spanning the junction between plant-DNA and the inserted DNA.

The technique is successfully being used to isolate and characterise the insert junction with plant-DNA of Monsanto's soybean line 40-3-2, a glyphosate tolerant soybean line, commercialised as RoundUp Ready (RR) soybeans. A line specific detection for RR soybeans is developed.

Materials and Methods

Anchored-PCR

The anchored-PCR was conducted as described by Theuns *et al.* (in preparation), using 5 different restriction enzymes – 1 hexacutter (EcoRI) and 4 tetracutters (MseI, MboI, BfaI and TaqI)

homologous to the 35S promoter region (35S-primer) and one homologous to the nos 3' terminator region (3' nos-1-primer).

313

Isolation of junctions

In order to isolate the correct junction from the dried polyacrylamide gel, we superimposed the autoradiogram upon the gel. In this way the autoradiogram could be used as a template to find the fragment of interest on the gel. The desired junction was excised out of the gel using a sharp scalpel. The junction-DNA was eluted out of the gelslice by incubating the slice in 200 μ l of water during 1 hour on ice. During this incubation the solution was mixed 3 to 4 times. After a centrifugation of 10 min. at maximum speed, 150 μ l of the eluate was collected and could be used for further processing.

Reamplification and sequencing of junctions

Before reamplification the eluate had to be purified. For this purification the QIAquick PCR Purification Kit (Qiagen) was used.

The reamplification of each junction was done using the same primers as the primers that were used during anchored-PCR to generate the junction. The PCR was conducted using following conditions : 1 μ l of purified eluate, 1X PCR-buffer (Perkin Elmer, USA), 250 μ M dNTP's (Pharmacia Biotech, USA), 0.4 μ M anchor-primer and 0.4 μ M adapter-primer. Water was added to a final volume of 50 μ l. The PCR cycle profile used was the same as used during anchored-PCR. Cycle 1: 25s at 93°C, 30s at 65°C and 60s at 72°C; cycle 2-13: 25s at 93°C, 30s at an annealing temperature 0.7°C lower than for each previous cycle, starting at 64.3°C, 55s at 72°C; cycle 14-37: 25s at 93°C, 30s at 56°C and 55s at 72°C.

After completion of the PCR programme, the 50 μ l reaction volume was loaded on a 1.5 % agarose gel. After electrophoresis the gel was stained with ethidium bromide and the reamplified fragments were visualised using an UV transilluminator. The reamplified fragments were excised from the agarose gel and the junctions were eluted from the gelslice using the QIAquick Gel Extraction Kit (Qiagen). The concentration of the excised fragments was determined on a 1.5 % agarose gel, and the solution was concentrated to 25 ng/ μ l by means of ethanol precipitation.

Subsequently the junctions were sequenced using the ABI Prism™ Big Dye Terminator Cycle Sequencing Kit (PE Applied Biosystems) according to the handbook provided with the kit. The junctions were sequenced in both directions by using on the one hand the anchor-primer and on the other hand the adapter-primer as the sequencing primers. An ABI Prism 377 DNA Sequencer and matching software were used to separate the sequencing reactions and to analyse the sequence information.

Recent results obtained with other junction fragments, show that it is possible to reamplify the junctions without performing a pre-purification step on the eluate.

Line specific RR soybean PCR

The line specific amplification of the DNA was carried out in a total volume of 25 μ l using following conditions: 1X PCR-buffer (Perkin Elmer, USA), 200 μ M dNTP's (Pharmacia Biotech, USA), 0.5 μ M 35S-primer (5' GATAGTGGGATTGTGCGTCA 3'), 0.5 μ M event-specific-primer (5' CCTTCAATTTAACCGATGC 3') and 25 ng of DNA. The PCR reactions were performed using the following cycle profile. Step 1 : initial denaturation at 95°C for 3 min. ; step 2 (34 cycles) : 20s at 95°C, 40s at 53°C and 40s at 72°C ; step 3 : 3 min. at 72°C. After the amplification 5 μ l of the reaction mix was separated on a 1.5 % agarose gel and stained with ethidium bromide and visualised using a UV transilluminator.

Results and discussion

The proposed strategy was tested on a glyphosate tolerant soybean line that was constructed by Monsanto using the particle acceleration method. This RoundUp Ready soybean line 40-3-2 was

showed that line 40-3-2 contains part of the CaMV 35S promoter, the EPSPS chloroplast transit peptide from *Petunia hybrida*, the CP4 EPSPS gene and a portion of the 3' nontranslated region of the nopaline synthase gene terminator. Line 40-3-2 was selected because this line carried a single genetic insert (Fig. 1).

314

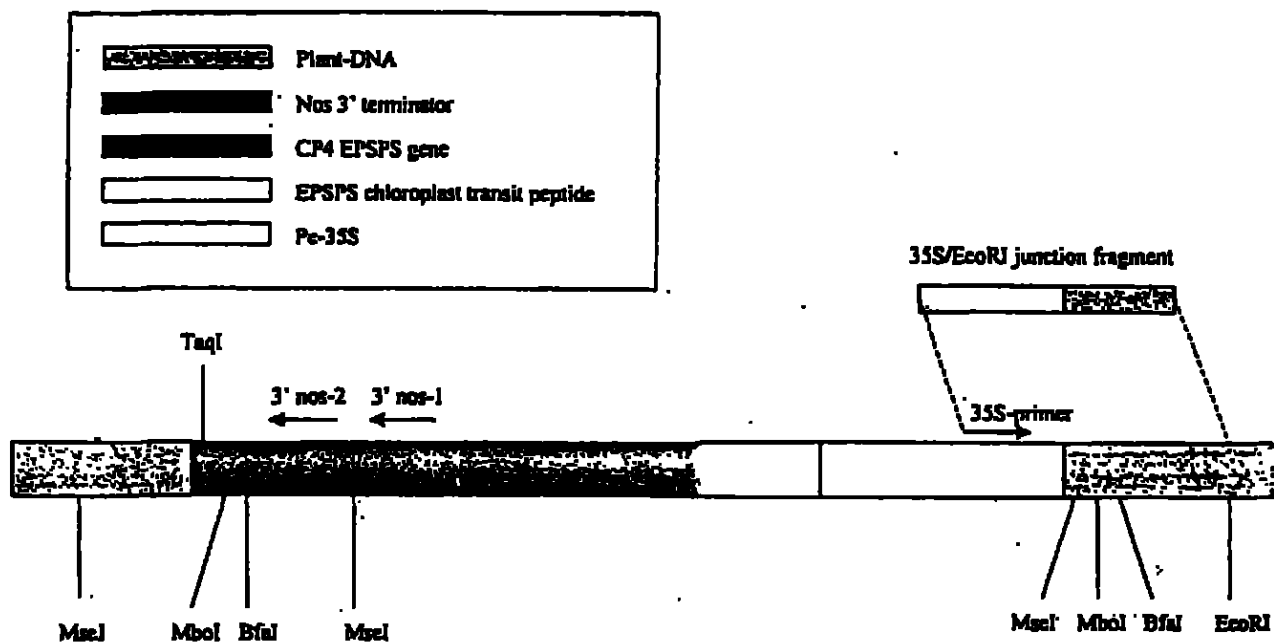


Fig. 1. Schematic diagram of inserted DNA present in RR soybeans (Padgett *et al.*, 1995).

To test our strategy we focussed on the 35S promoter region and the nos 3' terminator region because these two genetic elements flank the transgene and form the boundaries with the plant-DNA. In this way anchor-primers homologous to these two regions in combination with one of the 5 restriction enzymes and the matching adapter-primers would in all probability give us an anchored-PCR fragment that spans the border plant-DNA/inserted DNA. Because all tetracutters used had a recognition site between the 3'nos-1-primer and the insert border our work initially concentrated on the 35S junction fragment. Eventually the isolation of 4 different junction fragments spanning the junction 35S promoter/plant-DNA: 35S/MseI, 35S/MboI, 35S/BfaI and 35S/EcoRI, was successful. As could be expected the 35S/EcoRI fragment was the longest fragment. This 383 bp long 35S/EcoRI fragment was made up of 173 bp plant-DNA and 210 bp insert-DNA. We used this sequence information to design a primer that was situated in the plant-DNA. This newly designed event-specific primer and the 35S-primer make up a primer pair that should give a 360 bp amplicon when used to identify RR soybean DNA. Indeed the new primer pair was tested and gave a 360 bp fragment (Fig.2).

In the future this new primer pair will be tested on different foods containing soy (Messens *et al.*, in this volume). Also, the sequence of the 35S/EcoRI fragment will be used to develop a new line specific primer pair that will give a shorter amplicon (+/- 180 bp) to be able to identify RR soy DNA in processed foods in which the existing DNA could be fragmented (Hupfer *et al.*, 1998).

In the near future we will characterise and sequence also the 3'nos plant junction fragment. A new primer, the 3'nos-2 primer, homologous to the 3'nos terminator region, is being designed. As there is no MseI restriction site between the border and this new primer it should be possible to

299 315

amplify a 3' nos junction fragment. Once this junction fragment is isolated and sequenced, a primer specific for the plant-DNA adjacent to the nos 3' terminator will be developed. A combination of both event-specific primers will be used in the future to amplify the pre-insertion locus in the wild-type soybeans. A comparative analysis between the pre-insertion locus and the locus structure of the transgene locus could give us valuable information concerning the substantial equivalence between wild type and GMO.

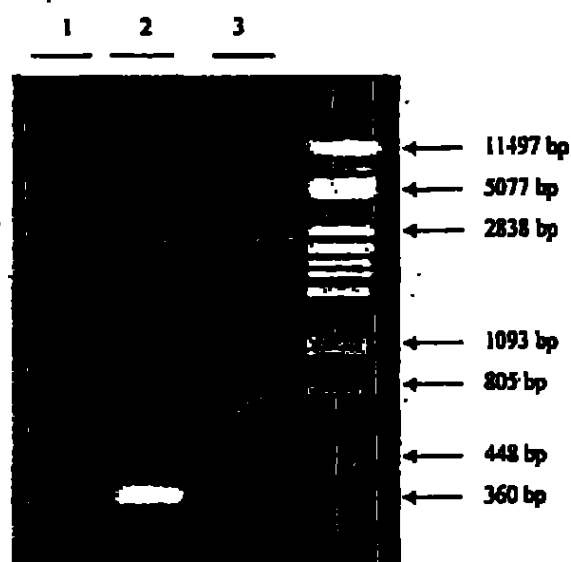


Fig. 2. Agarose gel analysis of line specific RR soy PCR products. Lane 1, line specific RR soy PCR on non-transgenic soy DNA. Lane 2, line specific RR soy PCR on transgenic RR soy DNA. Lane 3, negative control: line specific RR soy PCR on sample without DNA.

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220
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**SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIVISIÓN DE NUEVAS CREACIONES**

2020
Bogotá, D.C.,

Doctor
EMILIO FERRERO
Monsanto Technology
(Apoderado)

ASUNTO

Radicación 02-49630
Trámite 002
Evento 001
Actuación 430
Oficio - 2757
Folios

Como resultado del examen de fondo, realizado con fundamento en el artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le comunica:

Documentos estudiados:

Los textos estudiados fueron la descripción (Folios: 66 a 148) y las reivindicaciones 1 a 17 (folios: 182 a 186).

La presente solicitud reivindica prioridad Americana N° 60/297,406 de 11 de junio de 2001 y cumple con lo establecido en los artículos 9, 10 y 11 de la Decisión 486 de la Comisión de la Comunidad Andina.

Objeto de invención:

La presente solicitud se refiere a una célula vegetal resistente a insectos, derivada de una planta de algodón evento MON 15985 depositada como ATCC PTA-2516, y que comprende un inserto Cry2Ab y por lo menos una secuencia nucleotídica seleccionada a partir del grupo de SEC ID:14, SEC ID No 15, SEC ID No 17 y SEC ID No: 18 o CryA2b más las secuencias SEQ ID No 11 y SEQ ID No 12.

Así mismo se reclama la célula de planta en la que se puede obtener un amplicón al emplear los cebadores SEQ ID 34, o SEQ ID 36. También se reivindica el método para producir una planta de algodón resistente a herbicidas que comprende cruzar una planta resistente a herbicidas con una no resistente y seleccionar la progenie.

También hacen parte de la invención las secuencias de ADN aisladas que comprenden las SEC ID:14, SEC ID No 15, SEC ID No 17 y SEC ID No: 18. Además se reivindican métodos para detectar la presencia de la inserción MON15985 en el tejido de algodón que comprende la selección de cebadores que hibridizan sobre secuencias que flanquean la secuencia de inserción MON15985 y la amplificación del segmento de interés. Por último se reclaman los kits de detección de ADN que comprenden las sondas que hibridizan con las secuencias SEC ID No 14, SEC ID No 15, SEC ID No 17 y SEC ID No: 18 o que contienen los cebadores que hibridizan con las secuencias flanqueantes de MON 15985.

El objeto de la solicitud definido anteriormente se clasificó en la IPC A01H 1/006

En el concepto técnico 720, en virtud del artículo 45 de la D.486 se le indicó al solicitante que debía dar respuesta dentro del término de sesenta días contados a partir de la fecha de la notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Claridad de la solicitud:

El artículo 30 de la Decisión 486 de la Comisión de la Comunidad Andina establece que: "Las reivindicaciones definirán la materia que se desea proteger mediante la patente. Deben ser claras y concisas y estar enteramente sustentadas por la descripción.

Las reivindicaciones podrán ser independientes o dependientes. Una reivindicación será independiente cuando defina la materia que se desea proteger sin referencia a otra reivindicación anterior. Una reivindicación será dependiente cuando defina la materia que se desea proteger refiriéndose a una reivindicación anterior. Una reivindicación que se refiera a dos o más reivindicaciones anteriores se considerará una reivindicación dependiente múltiple."

En todo el capítulo reivindicatorio se emplean términos imprecisos como al menos o una fracción de..., que no limitan el alcance de la protección solicitada.

La reivindicación 1 reclama una célula derivada de la planta PTA-2516. Sin embargo, como se mencionó en el concepto técnico previo, las plantas no son patentables a la luz de la D.486, por lo que una célula caracterizada por un elemento no patentable no estaría correctamente definida.

La reivindicación 4 reclama un Inserto de una célula vegetal resistente a Insecto definiéndolo por que es capaz de producir un amplicón empleando determinados cebadores. Un inserto de ADN debe definirse única y exclusivamente por su secuencia de nucleótidos, no por el resultado que se espera alcanzar con el. Lo mismo sucede con la reivindicación 9 que reclama una molécula aislada de ADN que cuando se utiliza en un procedimiento se diagnostica MON15985, caracterizando la molécula reclamada no por su secuencia sino por el resultado esperado. Las reivindicaciones 10-11 reclaman una molécula aislada (o fragmento) que comprende la secuencia de flaqueo del evento MON15985.

Todas las anteriores cláusulas además de no definir claramente el objeto reclamado mediante sus características técnicas, pueden estar reivindicando moléculas completamente diferentes y que no guardan unidad de invención entre ellas. El ser usadas con un mismo objetivo no las relaciona estructuralmente y por ende no pueden ser consideradas como un único objeto de invención.

El kit reclamado en la cláusula 16 no define los elementos que lo componen mediante sus características propias si no por el resultado esperado cómo es que se unan a determinadas secuencias. . Así que la reclamación de un kit con elementos indefinidos y caracterizados por el resultado a alcanzar es amplia, especulativa y no permite determinar con exactitud cual es la materia a proteger. Esto ya se había mencionado con anterioridad.

De acuerdo con lo anterior, las reivindicaciones 1,4 ,9-11,16 en mención no cumplen

con el requisito de claridad definido en el artículo 30 de la Decisión 486 de la Comisión de la Comunidad Andina de Naciones.

Evaluación de la Unidad de Invención:

El artículo 25 de la Decisión 486 de la Comisión de la Comunidad Andina establece: "La solicitud de patente sólo podrá comprender una invención o un grupo de invenciones relacionadas entre sí, de manera que conformen un único concepto inventivo."

Las reivindicaciones 2 a 3 reclaman una célula vegetal resistente a insecto que comprende una secuencia que codifica Cry2Ab + ADN que puede estar representado por cualquiera de las secuencias SEQ ID No 14, SEQ ID No 15, SEQ ID No 16, SEQ ID No 17, SEQ ID No 18, SEQ ID No 11, SEQ ID No 12.

El elemento común entre las secuencias SEQ ID No 14, SEQ ID No 15, SEQ ID No 16, SEQ ID No 17, SEQ ID No 18, SEQ ID No 11, SEQ ID No 12 es que todas ellas pueden acompañar al gen Cry2Ab en el inserto empleado para la transformación de las plantas de algodón. Sin embargo, este gen ya es bien conocido en la obtención de plantas transgénicas resistentes a insectos y por lo tanto no puede ser considerado como el elemento común inventivo que vincule a todos los insertos de ADN con los que se pueden transformar las células reivindicadas y en ese sentido las diferentes células caracterizadas mediante distintos insertos posibles no guardan unidad de invención entre ellas.

Por lo tanto, es importante resaltar que además de estar ligadas al gen de la endotoxina, las secuencias mencionadas arriba deberían presentar tanto una estructura común como una misma actividad, para poder considerarlas como un único objeto de invención. En este sentido, sería estrictamente necesario que dicha estructura común representara verdaderamente un salto cualitativo sobre la formulación de la regla técnica. De lo contrario, no existiría una característica común nueva e inventiva que pudiese vincular a todas las posibles invenciones aquí reivindicadas, pero sobretodo ninguna de las combinaciones posibles Crya2b-SEQID No 14 a 17 sería patentable, por que no cumpliría con el requisito de nivel inventivo.

De acuerdo con lo anterior las cláusulas 2 a 3 y dependientes no cumplirían con el requisito de Unidad de Invención exigido en el artículo 25 de la Decisión 486.

Es una Invención:

El artículo 15 de la Decisión 486 de la Comisión de la Comunidad Andina establece: "No se consideran invenciones: a) los descubrimientos, las teorías científicas y los métodos matemáticos; b) el todo o parte de seres vivos tal como se encuentran en la naturaleza, los procesos biológicos naturales, el material biológico existente en la naturaleza o aquel que pueda ser aislado, inclusive genoma o germoplasma de cualquier ser vivo natural; c) las obras literarias y artísticas o cualquier otra protegida por el derecho de autor; d) los planes, reglas y métodos para el ejercicio de actividades intelectuales, juegos o actividades económico-comerciales; e) los programas de ordenadores o el soporte lógico, como tales; y, f) las formas de presentar información."

La reivindicación 7 reclama el método de producción de una planta resistente a insectos que comprende las etapas de: a) el cruce de una planta resistente con una no resistente, b) contener al menos una planta derivada del cruce y c) seleccionar la progenie.. Es claro que el cruce entre plantas implica necesariamente la etapa de reproducción, que constituye indiscutiblemente un proceso esencialmente biológico, independientemente si este se efectúa entre organismos modificados genéticamente o no; de igual forma que las plantas y sus células se generan mediante procesos

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biológicos naturales en donde los resultados a obtener no son inesperados. De acuerdo con lo anterior se conceptúa que método reclamado al depender fundamentalmente de procedimientos esencialmente biológicos que pueden presentarse espontáneamente en la naturaleza sin la intervención del hombre, se encuentra dentro del alcance del artículo 15 de la Decisión 486 de la Comisión de la Comunidad Andina de Naciones y por lo tanto no puede ser considerado como una invención.

Por otra parte, las reivindicación 8 reclaman secuencias de ADN aisladas, que constituirían materia existente en la naturaleza, no modificada por el hombre y por ende que no puede ser considerada invención a la luz del literal b) del artículo 15 de la Decisión 486.

Determinación del estado de la técnica:

El artículo 16 de la Decisión 486 de la Comisión de la Comunidad Andina establece que "El estado de la técnica comprenderá todo lo que haya sido accesible al público por una descripción escrita u oral, utilización, comercialización o cualquier otro medio antes de la fecha de presentación de la solicitud de Patente o, en su caso, de la prioridad reconocida.

Sólo para el efecto de la determinación de la novedad, también se considerará dentro del estado de la técnica, el contenido de una solicitud de patente en trámite ante la oficina nacional competente, cuya fecha de presentación o de prioridad fuese anterior a la fecha de presentación o de prioridad de la solicitud de patente que se estuviese examinando, siempre que dicho contenido esté incluido en la solicitud de fecha anterior cuando ella se publique o hubiese transcurrido el plazo previsto en el artículo 40".

Considerando que la fecha de presentación de la primera solicitud de patente de la cual se reivindica prioridad es: 11 de junio de 2001, se buscaron documentos del estado de la técnica relacionados, con fecha de publicación anterior a esta.

Se hizo la búsqueda de documentos relevantes en el estado de la técnica, entendiendo como estado de la técnica la definición provista en el artículo 16 de la Decisión 486 de la Comisión de la Comunidad Andina y las excepciones fijadas en el artículo 17 de la misma.

Se encontró como anterioridades (documentos cuya fecha de publicación es anterior a la fecha de presentación de la primera solicitud de patente sobre la cual se reivindica prioridad o de la fecha de presentación de esta solicitud) que pueden afectar la patentabilidad del objeto de la presente solicitud:

Nº	Documento	Título	Fecha de Publicación
1	ADAMCZYK, J.J., ET AL. Proceedings of the beltwide cotton conference, vol. 2, 2001, pages 835-837	Evaluation of BollgardII (cv. DP50BII) in the mississippi delta: field efficacy against various lepidoptera while profiling season-long expression of Cry1Ac and Cry2Ab"	JAN. 9-13, 2001,
2	AKIN, D.S., ET AL. Proceedings of the Beltwide cotton conference, vol. 2, 2001, pages 1041-1043	Field efficacy of cotton expressing tow insecticidal proteins of Bacillus thuringiensis"	JAN. 9-13, 2001,

N°	Documento	Título	Fecha de Publicación
3	NORMAN, J.W., ET AL Proceedings of the Beltwide cotton conference, vol. 2, 2001 pages 833-835.	"Performance of bollgard II cotton against lepidopterous pests in the lower rio grande valley of texas"	JAN. 9-13, 2001
4	ACKSON, R.E., ET AL Proceedings of the Beltwide cotton conference, vol. 2, 2001 pages 815-819	Efficacy of bollgard and bollgard II cottons against bollworm , <i>Helicoverpa zea</i> (Boddie) in field and greenhouse studies"	JAN. 9-13, 2001
5	STEWART S D ET AL: Journal Of Economic Entomology, vol. 94, no. 3, pages 752-760	"Impact of Bt cottons expressing one or two insecticidal proteins of <i>Bacillus thuringiensis</i> Berliner on growth and survival of noctuid (Lepidoptera) larvae" (2001-08), .	June 2001
6	WINDELS P ET AL. Mededelingen Van De Faculteit Landbouwwetenschappen Universiteit Gent, Gent, BE, vol. 64, no. 5B, 22. pages 459-462.	: "Development Of A Line Specific GMO Detection Method A Case Study"	1999-09-22
7	WO0026371	Methods For Transforming Plants To Express <i>Bacillus Thuringiensis</i> Delta-Endotoxins.	11/05/2000

Evaluación de los requisitos de Patentabilidad:

El Artículo 14 de la Decisión 486 de la Comisión de la Comunidad Andina el cual establece: "Los Países Miembros otorgarán Patentes para las invenciones, sean de producto o de procedimiento en todos los campos de la tecnología siempre que sean nuevas, tengan nivel inventivo y sean susceptibles de aplicación industrial".

Se procedió a evaluar los requisitos de novedad, nivel inventivo y aplicación industrial con el fin de establecer la patentabilidad de la invención reivindicada en la solicitud en estudio.

Evaluación de Nivel Inventivo:

El artículo 18 de la Decisión 486 de la Comisión de la Comunidad Andina establece que "Se considerará que una invención tiene nivel inventivo, si para una persona del oficio normalmente versada en la materia técnica correspondiente, esa invención no hubiese resultado obvia ni se hubiese derivado de manera evidente del estado de la técnica".

El Nivel Inventivo se evaluó por comparación entre lo solicitado y encontrado en el estado de la técnica y que se relaciona con el objeto reivindicado y de acuerdo con la definición de nivel Inventivo del Artículo 18 de la Decisión 486 de la Comisión de la Comunidad Andina.

Las reivindicaciones 1 a 3 y dependientes reclaman una célula vegetal derivada de una planta resistente a insectos (PTA 2526 - evento MON 15985) caracterizada en que comprende un inserto del gen CryA2b acompañado de las secuencias SEQ ID No 14, SEQ ID No 15, SEQ ID No 16, SEQ ID No 17, SEQ ID No 18, SEQ ID No 11, SEQ ID No 12.

El problema técnico a resolver con la presente invención es la generación de plantas de algodón resistentes a insectos incluidas las células de plantas transformadas con genes codificantes para proteínas con actividad insecticida. Los documentos D1 a D5 divulgan el evento MON15985, elemento central de la presente invención, bajo diferentes nombres (DP50BII, Bolgard II, DPL15985, DP50Bx) que corresponden específicamente a plantas transgénicas de algodón resistentes a insectos que expresan las endotoxinas de *Bacillus thuringiensis*. En esos mismos documentos se enseña que dichas plantas pueden expresar una o más proteínas insecticidas de *B. Thuringiensis* (D5), dentro de las que son bien conocidas Cry1Ac y Cry2Ab. D7 resalta la combinación de las dos endotoxinas en un mismo OGM.

La presente solicitud difiere de las anterioridades en que indica un evento de inserto particular para el gen Crya2b en algodón. Sin embargo, de la lectura de la presente solicitud no se encuentra evidencia que dicho inserto posea un efecto técnico que supere los eventos conocidos en el estado del arte. Entonces, el problema a resolver con la presente solicitud sería generar plantas de algodón alternativas que comprendan los genes CryAb y CryAc para conferir resistencia a insectos. No obstante, este evento de inserción representa una modificación que no es significativamente superior al estado del arte, dado que es una selección arbitraria de una inserción aleatoria a la cual hubiera podido llegar cualquier persona versada en la materia sin ninguna actividad inventiva (la posición de la inserción no fue planeada con el objeto de obtener resultados inesperados)

Las reivindicaciones 12 a 15 reclaman métodos para detectar la inserción de MON15985, que consisten en la amplificación simple de determinados segmentos del genoma empleando cebadores seleccionados y examinando el amplicón obtenido; así como métodos que emplean el principio de hibridización para detectar ciertas regiones del genoma complementarios a ciertas sondas escogidas.

El documento D6 divulga emplea un procedimiento basado en PCR empleando primers contra las secuencias que flanquean el inserto. Apartir del anterior documento la selección de primers que representan una selección arbitraria entre un número de alternativas y el uso de los mismos para identificar secuencias de diagnóstico y su empleo en la identificación del evento por hibridización no representa un salto cualitativo en la formulación de la regla técnica.

Así que, a pesar que se efectuó una selección de determinados cebadores o sondas, el método no va a diferir de los empleados constantemente en los ensayos de caracterización molecular. De hecho, cualquier técnico versado en la materia, como actividad de rutina selecciona primers y sondas que puedan hibridizar con sus secuencias target. De esta forma, los métodos reclamados más bien representan el uso de las sondas y cebadores seleccionados en métodos corrientes, pero que no le atribuyen ninguna ventaja técnica sobre los que ya existen en el estado de la técnica.

Como se observa, el objeto de esta solicitud podría resultar obvio para una persona del oficio normalmente versada en la materia y derivarse de manera evidente del estado de la técnica.

En conclusión los requisitos de patentabilidad de la presente solicitud se encuentran afectados así:

7322

Nº	Documento	Reivindicaciones afectadas	Requisito que afecta
1	ADAMCZYK, J.J., ET AL. Proceedings of the beltwide cotton conference, vol. 2, 2001, pages 835-837	1 a 3 y dependientes	Nivel Inventivo
2	AKIN, D.S., ET AL. Proceedings of the Beltwide cotton conference, vol. 2, 2001, pages 1041-1043	1 a 3 y dependientes	Nivel Inventivo
3	NORMAN, J.W., ET AL. Proceedings of the Beltwide cotton conference, vol. 2, 2001 pages 833-835.	1 a 3 y dependientes	Nivel Inventivo
4	ACKSON, R.E., ET AL. Proceedings of the Beltwide cotton conference, vol. 2, 2001 pages 815-819	1 a 3 y dependientes	Nivel Inventivo
5	STEWART S D ET AL: Journal Of Economic Entomology, vol. 94, no. 3, pages 752-760	1 a 3 y dependientes	Nivel Inventivo
6	WINDELS P ET AL Mededelingen Van De Faculteit Landbouwwetenschappen Universiteit Gent, Gent, BE, vol. 64, no. 5B, 22, pages 459-462.	12-15 y dependientes	Nivel Inventivo
7	WO0026371	1 a 3 y dependientes	Nivel Inventivo

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta días contados a partir de la fecha de la notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única N° 10 de 2001.

ALIX CARMENZA CESPEDES DE VERGEL
Jefe de la División de Nuevas Creaciones.

El anterior requerimiento fue notificado por fijación en lista, de fecha 01 MAR. 2007

CONCEPTO TÉCNICO Nº: 321	EXAMINADOR TÉCNICO Código N°: 202063	2007-02-22
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