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Señor

Jefe de la División de Nuevas Creaciones

Superintendencia de Industria y Comercio

Ministerio de Comercio, Desarrollo, Industria y Turismo

E. S. D.

Re.: Expediente No. **05.090.937**

Solicitud de Patente de Invención titulada: **"EVENTO DE ALGODON MON88913 Y COMPOSICIONES Y METODOS PARA SU DETECCION"**

Solicitante: **MONSANTO TECHNOLOGY LLC**

RESPUESTA AL EXAMEN DE BUSQUEDA DE ANTERIORIDADES

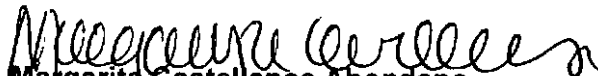
MARGARITA CASTELLANOS ABONDANO, mayor de edad, identificada y domiciliada como aparece al pie de mi firma, en atención a su oficio No. 2256 notificado el 11 de febrero de 2011, atentamente me permito dar repuesta al mismo de la siguiente manera:

Presento copia completa del artículo requeridos por este Despacho como resultado de la búsqueda de anterioridades:

- **PLINE WENDY A ET AL:** "Reproductive abnormalities in glyphosate-resistant cotton caused by lower CP4-EPSPS levels in the male reproductive tissue". **WEED SCIENCE**, vol. 50, no. 4, July 2002 (2002-07), pages 438-447.
- **MICHAEL A. JONES AND CHARLES E. SNIPES:** "Tolerance of Transgenic cotton to Tropical Application of Glyphosate". **JOURNAL OF COTTON SCIENCE**, vol. 3, 1999, pages 19-26, XP002292746
- **WINDELS P. ET AL:** "Development of a line specific GMO detection method a case study". **FACULTEIT LANDBOUWWETENSCHAPPEN UNIVERSITEIT GENT, GENT, BE**, vol. 64, no.5B, 22 September 1999 (1999-09-22), pages 459-462.

Cumplido así con lo dispuesto, atentamente solicito a Ud. se continúe con el trámite correspondiente a esta solicitud de Patente de Invención.

Del Señor Jefe,


Margarita Castellanos Abondano

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Reproductive abnormalities in glyphosate-resistant cotton caused by lower CP4-EPSPS levels in the male reproductive tissue

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Glyphosate treatments to glyphosate-resistant (GR) cotton have been associated with poor pollination and increased boll abortion. Anatomical studies were conducted to characterize the effect of glyphosate treatments on the development of male and female reproductive organs of cotton flowers at anthesis. In comparison with nontreated plants, glyphosate applied at both the four-leaf stage postemergence (POST) and at the eight-leaf stage POST directed inhibited the elongation of the staminal column and filament, which increased the distance from the anthers to the receptive stigma tip by 4.9 to 5.7 mm during the first week of flowering. The increased distance from the anthers to the stigma resulted in 42% less pollen deposited on stigmas of glyphosate-treated plants than in nontreated plants. Moreover, pollen from glyphosate-treated plants showed numerous morphological abnormalities. Transmission electron microscopy showed the presence of large vacuoles, numerous starch grains, and less organized pockets of the endoplasmic reticulum containing fewer ribosomes in pollen from glyphosate-treated plants than from nontreated plants. Pollen development in glyphosate-treated plants is likely inhibited or aborted at the vacuolate microspore and vacuolate microgamete stages of microgametogenesis, resulting in immature pollen at anthesis. Although stigmas from glyphosate-treated plants were 1.2 to 1.4 mm longer than those from nontreated plants, no other anatomical differences in stigmas were visibly evident. The presence of the GR 5-enolpyruvylshikimate-3-phosphate synthase (CP4-EPSPS) enzyme from *Agrobacterium* sp. strain CP4 was quantified in reproductive and vegetative tissues using enzyme-linked immunosorbent assay. The content of CP4-EPSPS in the stigma, anther, preanthesis floral bud (square), and flower petals was significantly less than that in the vegetative leaf tissue. Glyphosate effects on the male reproductive development resulting in poor pollen deposition on the stigma, as well as production of aborted pollen with reduced viability, provide a likely explanation for reports of increased boll abortion and pollination problems in glyphosate-treated GR cotton.

Nomenclature: Glyphosate; cotton, *Gossypium hirsutum* L., 'Delta Pine & Land 5415RR', 'Delta Pine & Land 50', 'Delta Pine & Land 90', 'SureGrow 125RR'.

Key words: Herbicide-resistant crops, transgenic crops, gametogenesis, male sterile, ELISA, pollen, anther.

Glyphosate-resistant (GR) crops, such as cotton, allow growers greater flexibility in the timing of herbicide applications, as well as a broader spectrum of weed control than that offered by other herbicide systems (Askew and Wilcut 1999; Culpepper and York 1999; Scott et al. 2001). Since its commercialization in 1997, however, concern over the reproductive tolerance of GR cotton to glyphosate has been raised. Numerous reports of increased boll abscission and pollination problems in response to glyphosate applications have occurred in cotton, occasionally leading to yield loss and a modified fruiting pattern (Jones and Snipes 1999; Vargas et al. 1998). A similar slight yield reduction has been reported in GR soybean [*Glycine max* (L.) Merr.], but this was attributed to differences between the GR and conventional isolines and not to the glyphosate applications (Elmore et al. 2001). Such reports have not surfaced in other GR crops such as corn (*Zea mays* L.), suggesting that either problems with reproductive sensitivity to glyphosate are limited to GR cotton or that reproductive sensitivity to glyphosate of corn and soybean does not incur a noticeable yield penalty.

Glyphosate resistance has been conferred to cotton by the incorporation of a GR 5-enolpyruvylshikimate-3-phosphate synthase (CP4-EPSPS) gene cloned from *Agrobacterium* sp. strain CP4. The expression of the CP4-EPSPS gene produces a GR EPSPS enzyme, which can bypass the inhibition of the native EPSPS in the presence of glyphosate, allowing sufficient production of aromatic amino acids and secondary metabolites (Nida et al. 1996).

Glyphosate applications on GR cotton in North Carolina are typically made before the five-leaf stage (approximately 25 d after emergence) as a postemergence (POST) application and again as either a postemergence-directed spray (approximately 40 d after emergence) or as a late POST-directed treatment (>40 d after emergence). Reproductive development begins relatively early in the cotton life cycle. Mauney (1966) observed differentiation of the first floral bud as early as 10 to 14 d after emergence. From the time the first reproductive bud (square) is visible, anthesis typically occurs 25 to 30 d later, whereas the actual stimulus to flower is thought to start 10 to 15 d before this stage. In

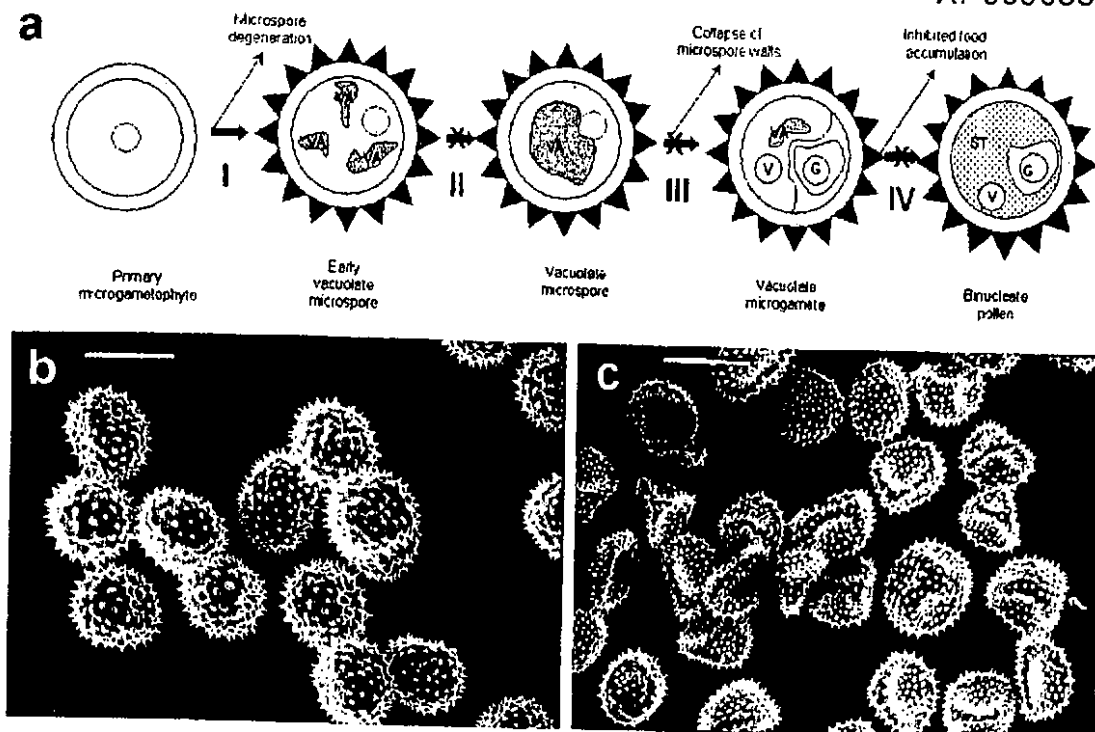


FIGURE 1. Pollen development and glyphosate effects on pollen morphology. (a) Development and maturation of cotton (*Gossypium hirsutum* L.) pollen from a primary microgametophyte (postmeiosis) to a mature binucleate pollen at anthesis. Conversion from a primary microgametophyte to an early vacuolate microspore, I. Conversion from an early vacuolate microspore to a vacuolate microspore, II. Conversion from a vacuolate microspore to a vacuolate microgamete, III. Conversion from a vacuolate microgamete to a mature binucleate pollen, IV. Labels: X, points of proposed glyphosate inhibition; VA, vacuoles; V, vegetative nucleus; G, generative nucleus; ST, storage material. Thin arrows represent the resulting phenotype if the conversion step is inhibited. Figure derived from Frankel and Galun (1977), Jensen et al. (1968), and Wetzel and Jensen (1992). Scanning electron microscopic images of pollen from (b) nontreated (c) and glyphosate-treated glyphosate-resistant cotton plants. Scale bars = 100 μ M.

cotton squares, anther development and microspore meiosis occur earlier than in ovules, about 22 d before anthesis (Quintanilha et al. 1962).

The timing of glyphosate treatments to GR cotton relative to the development of reproductive organs may lead to damage if glyphosate accumulation in these organs exceeds a tolerance threshold. Glyphosate accumulates in tissues such as reproductive organs, which act as metabolic sinks (Gougler and Geiger 1981; Sandberg et al. 1980). Pline et al. (2001) found that 3.7% of glyphosate applied to the stem tissue of GR cotton at 2 wk after the first bloom was translocated to developing squares. Viator et al. (2000) reported that concentrations of 126.4 μ g glyphosate g^{-1} boll tissue promoted boll abscission. Therefore, if the reproductive tissue is not sufficiently tolerant to glyphosate, herbicidal damage or impaired reproductive development may occur.

Reduced reproductive tolerance to glyphosate has been reported in transgenic GR tobacco (*Nicotinia tabacum* L.) plants containing different constructs of the CP4-EPSPS gene encoded in either the chloroplast or nuclear genomes (Ye et al. 2001). In all constructs the amount of glyphosate needed to reach 50% injury of the reproductive tissues (50% reduction in seed set) was less than that for vegetative tissues. It is also possible, therefore, that the expression of the CP4-EPSPS gene in cotton reproductive tissues is less than that in vegetative tissue. Pline et al. (2002b) reported that squares from GR cotton accumulate 11 times more shikimic acid (a metabolite which accumulates in response to EPSPS inhibition) per micromolar translocated glyphosate than do

leaf tissues, further suggesting reproductive sensitivity in GR cotton.

The male reproductive organ development in many crop species is more sensitive than the female reproductive organ development to environmental factors (Frankel and Galun 1977). Male sterility caused by temperature extremes, genetics, or incompatibility has been reported in numerous crops including cotton. Flowers from some male sterile cotton plants exhibit shortened or missing anthers that do not properly dehisce (Allison and Fisher 1964). In another case, the dominant mutant gene, *ms10*, induces abortion of mature cotton pollen (Bowman et al. 1978). Inhibition of stamen initials early in development often leads to stamenless or antherless phenotypes. Such mutants exist in a wide range of species including cotton (Allison and Fisher 1964). The presence of meiocytes or tetrads of microspores at anthesis, instead of mature pollen, indicate the abortion of microsporangocytes during microsporogenesis (Frankel and Galun 1977). Inhibition of development at later stages, for example, failure of the tapetum to degenerate and provide nutrients to the developing pollen may arrest pollen maturation. Finally, male sterile plants may also fail to shed mature pollen at anther dehiscence because of the developmental inhibition of the anther epidermis (Frankel and Galun 1977).

Pollen development and maturation is a multistep process of synchronized events occurring between the anther tapetum and the developing sporocyte. Breakdown at any point in the developmental pattern can lead to pollen abortion. A summary of the phenotypes of pollen aborted at different stages of microgametogenesis is presented in Figure 1a. As-

suming normal development of the free microspores (no breakdown in microsporogenesis), the primary microgametophyte is still susceptible to developmental inhibition during microgametogenesis. During the initial step of microgametogenesis, the primary microgametophyte is converted to an early vacuolate microspore, which contains several vacuoles. When this conversion is inhibited, a general degeneration of the microspore occurs (Figure 1a, I). The second step in microspore development consists of the conversion of the early vacuolate microspore to a vacuolate microspore, characterized by the formation of a large central vacuole (Figure 1a, II). The vacuolate microspore stage occurs approximately 9 d before anthesis (Wetzel and Jensen 1992). The third step in microgametogenesis is the conversion of the vacuolate microspore to a vacuolate microgamete (Figure 1a, III). The major feature of this step is the mitotic division of the vegetative nucleus to form a generative nucleus. From this point forward the microgamete is binucleate, and it is during this conversion that the large vacuole shrinks, eventually filling with storage material. If the conversion from vacuolate microspore to vacuolate microgamete was inhibited, the large vacuole and a collapse of the microspore walls would be evident in the aborted microspore (Frankel and Galun 1977). The fourth step in postmeiotic pollen maturation is the conversion of the vacuolate microgamete into a mature binucleate pollen grain. The main feature of this conversion is the accumulation and organization of food storage materials. If the conversion from vacuolate microgamete to mature binucleate pollen was inhibited, food accumulation in the pollen grain would be inhibited, and the microgamete would retain a smaller vacuole.

Wetzel and Jensen (1992) described the time course of events of cotton pollen development from the vacuolate microspore stage (approximately 9 d before anthesis) to the mature binucleate pollen grain (anthesis). Nine days before anthesis, large plastids containing starch begin to accumulate. Lipids begin to accumulate 6 d before anthesis and correlate with a decrease in size of the starch-containing plastids. Ribosomes on the endoplasmic reticulum (ER) increase from 6 d before anthesis to a peak in mature pollen at anthesis. A unique feature of cotton pollen is its arrangement of the inflated ER into spherical pockets covered with ribosomes (Jensen et al. 1968; Wetzel and Jensen 1992). These ER pockets contain an organized arrangement of lipid droplets and small vesicles containing carbohydrates and organize only a few hours before anthesis (Figure 3e). Upon pollen germination, these ER pockets open, and the lipid droplets and carbohydrate-containing vesicles mix in the cytoplasm and are likely used for pollen tube construction and elongation (Jensen et al. 1968).

The objectives of the current study were to characterize differences in floral anatomy, pollen deposition, and pollen morphology between glyphosate-treated and nontreated GR cotton and conventional cotton and to quantify the level of CP4-EPSPS protein in reproductive and vegetative tissues of GR cotton.

Materials and Methods

Plant Material and Growth Conditions

Pollen and reproductive tissue samples were obtained from cotton plants grown in an air-conditioned greenhouse

at the North Carolina State University Phytotron with a 26 and 22 °C day and night temperature regime, respectively. Delta Pine & Land cultivars 'DP 50', 'DP 90', and 'DP 5415RR' and SureGrow cultivar 'SG 125RR' seeds were planted in 25.4-cm pots containing a gravel-peat combination substrate. Plants were thinned to one plant per pot and were watered with the standard Phytotron nutrient mixture twice daily. Glyphosate-treated plants received 1,120 g ai ha⁻¹ glyphosate (Roundup Ultra) at the four-leaf stage (foliar application) and at the eight-leaf stage (postdirected application to stem), according to the Roundup Ultra supplemental label for GR cotton (Anonymous 2001). The growth regulator mepiquat-chloride was applied to the upper leaves at the rate of 840 g ai ha⁻¹ at the 10-leaf stage to control vegetative growth, according to the North Carolina Cooperative Extension Service guidelines (Edmisten 2001).

Pollen and Tissue Sampling

All pollen and anther samples were collected between 9:00 and 11:00 A.M. Brightfield images of pollen and reproductive organs were captured with a digital camera. Measurements of floral anatomy (height of the stigma, height of the staminal column, distance from the highest anther to the tip of the stigma, and anther filament length in millimeters) were taken on all flowers three times weekly on separate days. Stigma height and staminal column height were measured from the lowest portion of the inner flower, where the petals converge with the anther-containing sheath to either the stigma tip for stigma heights or the top of the staminal column where the style is visible (Figure 2a). The anther-stigma distance was measured from the tip of the stigma to the top anther, where anthers reaching above the stigma would give positive values, and anthers below the tip of the stigma would give negative values (Figure 2a). The anther filament length was measured for the uppermost anther in the flower (Figure 2a).

Pollen and tissue samples for transmission electron microscopy were fixed in McDowell and Trump's 4F:1G fixative (McDowell and Trump 1976). Samples were rinsed three times with 0.1 M phosphate buffer, pH 7.2 to 7.4, within a 15-min period. Tissues were then subjected to an ethanol dehydration series, embedded in Spurr resin (Spurr 1969), and polymerized overnight at 70 °C. Ultrathin sections (90 nm thick) of the embedded tissue were cut, placed on grids, and poststained using methanolic uranyl acetate followed by Reynold's lead citrate (Reynolds 1963). Grids were viewed using a transmission electron microscope at 80 kV. Pollen and anther samples for scanning electron microscopy were placed on a double-stick tape, which was then affixed to a stub and sputter coated with gold-palladium. Samples were viewed using a scanning electron microscope at 17 kV.

Pollen Deposition on the Stigma

Stigmas from flowers used for pollen viability assessment were harvested 24 h after anthesis by cutting at the stigma base. The sheath containing anthers was removed from the stigma, and stigmas were placed into microfuge tubes containing a 1-ml solution of ethanol-chloroform-acetic acid (6:3:1). Stigmas were vortexed to remove loose pollen grains

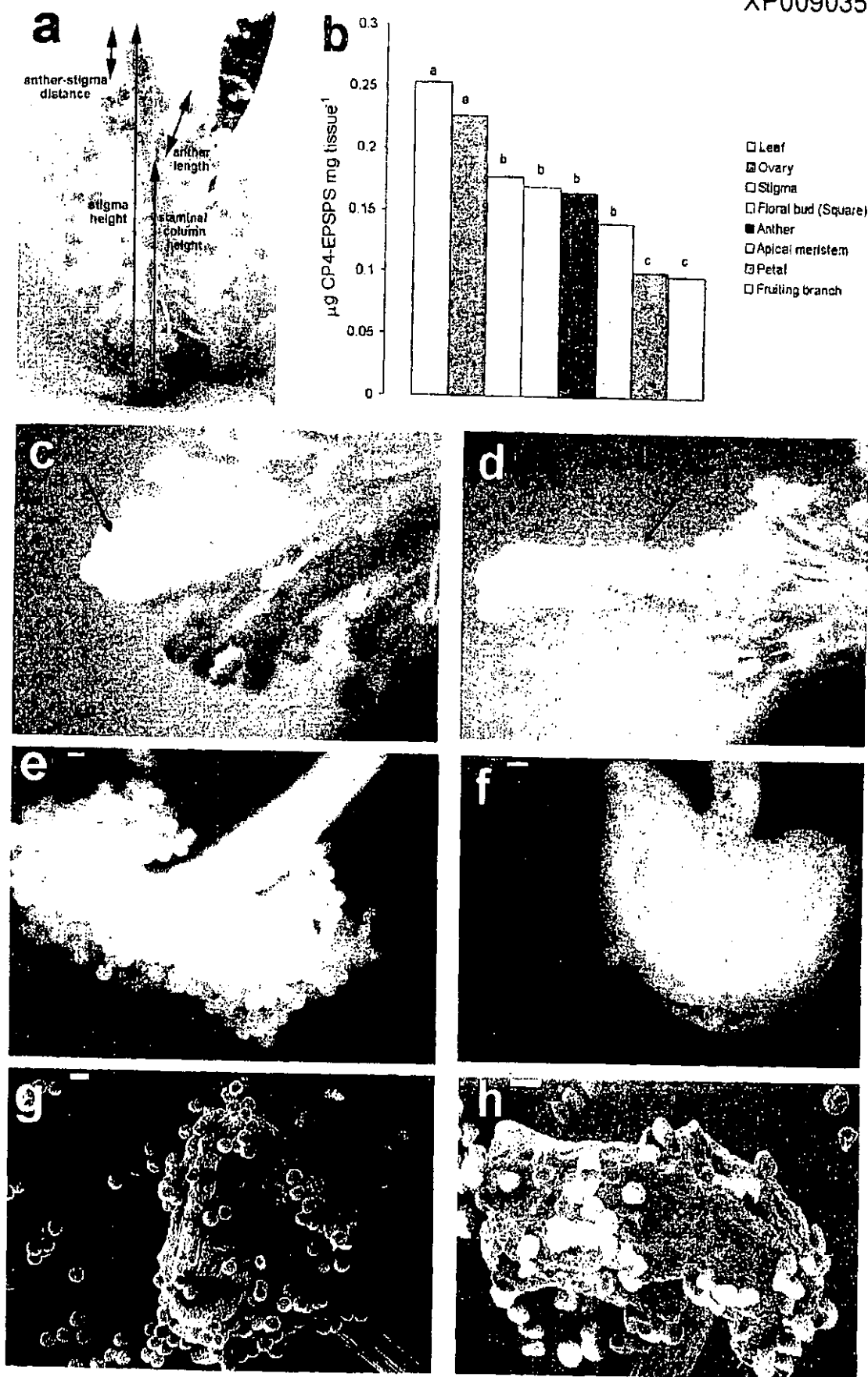


FIGURE 2. Cotton (*Gossypium hirsutum* L.) flower anatomy, quantity of CP4-EPSPS in tissues, and effect of glyphosate on floral morphology. (a) Anatomy of the cotton flower at anthesis. Arrows represent measurements taken for Tables 1 and 2. (b) Amount of CP4-EPSPS ($\mu\text{g CP4-EPSPS mg}^{-1}$ fresh weight) in vegetative and reproductive tissues of glyphosate-resistant (GR) cotton. Morphological characteristics of (c) nontreated and (d) glyphosate-treated GR cotton stigmas, anthers, and pollen grains. Points of pollen coverage indicated by arrows. Anthers from (e and g) nontreated plants and (f and h) glyphosate-treated plants using (e and f) light and (g and h) scanning electron microscopy. Scale bars = 100 μM (e-h).

TABLE 1. Heights of the staminal column and the stigma and length of the anther for conventional, glyphosate-resistant (GR), and glyphosate-treated GR cotton (*Gossypium hirsutum* L.) cultivars at 1–4 wk of flowering.

Cultivar treatment	Staminal column height				Stigma height	Anther length
	1 wk	2 wk	3 wk	4 wk	1–4 wk	1–4 wk
	mm					
DP 50	15.3 a*	13.9 bc	14.5 bc	14.9 bc	23.2 cd	7.4 a
DP 90	12.8 ab	15.5 a	15.5 a	14.7 c	21.3 e	7.2 a
DP 5415RR	13.2 ab	14.4 abc	14.8 abc	14.7 c	22.2 de	6.3 bc
DP 5415RR Trt.	10.5 c	12.4 d	13.9 c	14.8 c	23.4 cd	5.4 d
SG 125RR	13.8 ab	15.3 ab	15.2 ab	15.7 a	24.8 b	6.9 ab
SG 125RR Trt.	11.5 bc	13.7 cd	14.9 ab	15.5 ab	26.2 a	6.1 cd

* Means followed by the same letter in each column are not significantly different at $\alpha = 0.05$, according to Fisher's Protected LSD test.

from the stigma surface. The stigma was removed from the microfuge tube, and pollen was pelleted by gently centrifuging and then resuspended in the 1-ml solution remaining in the microfuge tube. The number of pollen grains in a 25- μ l subsample was then counted on a microscope slide under a compound light microscope.

Quantification of CP4-EPSPS

Mature leaves, fruiting branches, apical meristems, reproductive buds, and flowers were gathered from DP 5415RR (GR) and 'DP 5415' (conventional, glyphosate sensitive) cotton plants grown at two separate field sites in Clayton and Lewiston-Woodville, NC, in July 2001. Anthesis flowers were dissected into anthers (filament, anther, and pollen), stigma, ovary (capsule and ovules), and petal. CP4-EPSPS was extracted from 100 mg freshly ground tissue, diluted 5,000-fold, and analyzed by enzyme-linked immunosorbent assay (ELISA) using the Strategic Diagnostic, Inc. Trait Check™ kit and included directions (Anonymous 1999) for the quantification of CP4-EPSPS in food products.

Experimental Design

Cotton plants were arranged in a randomized complete block design with four replications and four repeated measurements over time (Weeks 1–4). Floral morphology measurement means from each of the three separate sampling days within a week were combined to create a mean for each week of sampling (Weeks 1–4). The entire study was repeated. Data were subjected to analysis of variance using SAS (1990). Because the run main effect and interactions were not significant ($P > 0.05$), data from both runs were combined. The variety-glyphosate treatments and the week of flowering main effects were significant for pollen deposition, anther–stigma distance, stigma height, column height, and anther length measurements. The interactions between the variety-glyphosate treatment and the week of flowering were only significant for the anther–stigma distance and the column height, thus variety-glyphosate treatment means were compared by Fisher's Protected LSD for each week individually.

Tissues for CP4-EPSPS quantification were collected from a single plant per replication per site in each of the two field sites in North Carolina. Tissue extract from each sample was analyzed in duplicate on ELISA plates. The absorbance means for each tissue were compared with a stan-

dard curve derived from soya protein extracts with known CP4-EPSPS concentrations in order to calculate the CP4-EPSPS concentration in each sample. Means from both runs (locations) were combined because of insignificant tissue by location interactions. Tissue means were compared by Fisher's Protected LSD at $P = 0.05$.

Results and Discussion

Glyphosate Effects on Floral Morphology

Differences in floral morphology between glyphosate-treated and nontreated GR cotton cultivars may explain potential differences in pollination. The height of the cotton stigma differed with cultivar and glyphosate treatment. Stigmas from glyphosate-treated plants were 1.2 to 1.4 mm longer than those from nontreated plants (Table 1); however, no other visual physical differences were evident between the stigmas of glyphosate-treated and nontreated GR cultivars or conventional cultivars. Differences in the male reproductive organs of glyphosate-treated and nontreated GR cultivars were also evident. The column to which the anther filaments are attached were 2.7 and 2.0 mm shorter in DP 5415RR glyphosate-treated GR plants than in nontreated GR plants in the first and second week of flowering, respectively (Table 1). In the first 4 wk of flowering, the anther filaments were 0.8 to 0.9 mm shorter than those from nontreated plants (Table 1). The shorter anthers and staminal columns, along with slightly elongated stigmas, resulted in a 4.9- to 5.7-mm greater distance from the top anther to the tip of the stigma in glyphosate-treated than in nontreated plants in the first week of flowering (Figures 2c and 2d; Table 2). Nontreated and conventional cotton plants had anther–stigma distances of 0.4 mm (anthers above the stigma) to –2.9 mm (anthers below the stigma). Although anthers in nontreated and conventional cotton plants often have anthers below the stigma, the small distances from the anthers to the tip of the stigma allow pollen deposition on the receptive stigma. Close proximity of anthers and the stigma would allow adequate pollen coverage of the receptive stigma. Increased distances between the anthers and the stigma have been shown to cause reduced seed set in introgressive *Lythum* hybrids, presumably because of poor pollen deposition on the stigma (Anderson and Ascher 1994).

Glyphosate Effects on Pollen Deposition

Differences in floral morphology between glyphosate-treated and nontreated GR cotton cultivars explain differ-

TABLE 2. Distance from the top anther to the tip of the stigma (negative values indicate anthers below the tip of the stigma, positive values indicate anthers above the tip of the stigma) and number of loose pollen grains per stigma from conventional, nontreated glyphosate-resistant (GR), and glyphosate-treated GR cotton (*Gossypium hirsutum* L.) varieties at 1–4 wk of flowering.

Cultivar treatment	Anther to stigma distance				Loose pollen grains per stigma
	1 wk	2 wk	3 wk	4 wk	1-4 wk
	mm				pollen grains
DP 50	- 2.0 b ^a	0.2 c	0.4 e		
DP 90	0.4 c	1.9 d	2.0 f	-0.1 c	1,323 b
DP 5415RR	-1.8 b	-1.3 b	-0.6 d	1.4 d	1,843 a
DP 5415RR Trr.	-7.5 a	-4.6 a	-3.0 b	-1.0 b	1,120 bc
SG 125RR	-2.9 b	-1.9 b	-1.8 c	-2.3 a	647 d
SG 125RR Trr.	-7.8 a	-4.8 a	-3.6 a	-2.4 a	857 cd
				-2.7 a	493 d

^a Means followed by the same letter in each column are not significantly different at $\alpha = 0.05$, according to Fisher's Protected LSD test.

ences in pollination, boll retention, and seed set. Male reproductive organs in GR cotton were highly affected by glyphosate applications. Anthers from nontreated GR cotton plants were covered with pollen grains at anthesis (Figures 2e and 2g), whereas those from glyphosate-treated GR cotton plants either exposed no pollen (Figure 2f) or malformed pollen (Figure 2h). Glyphosate-treated plants had 42.2 to 42.5% less loose pollen per stigma when compared with nontreated GR plants (Table 2). Pollen deposition on the stigma of glyphosate-treated GR cotton was likely reduced because of the greater distance between the anthers and the stigma (Table 2). There was a highly negative correlation (-0.98) between the deposition of pollen, as measured by the removal of loose pollen, and anther–stigma distances, demonstrating the association of greater anther–stigma distances with lower amounts of pollen deposition on the stigma. Pollen from glyphosate-treated GR plants was typically deposited only on the lower portion of the stigma (Figure 2d). Iyengar (1938) reported that pollen placed on the lower portion of the cotton stigma germinated much less readily than did pollen placed at the top portion of the stigma, which may lead to an even lower number of pollen tubes and seed set in treated plants.

Cotton anthers produce heavy, sticky pollen, which is normally not well dispersed by wind, accounting for its status as a primarily self-pollinated plant (McGregor 1976). Pollinating insects, by their movement within a flower, may deposit pollen on stigmas that may not receive pollen by anther–stigma contact alone (McGregor 1976). In areas where the insect pollinator concentration is high, the effects of increased distances between anthers and stigmas on pollination in glyphosate-treated GR cotton may be reduced. However, in areas where pest insect pressure is high and frequent insecticide applications are made, pollinator numbers may be reduced. Because glyphosate causes reduced anther contact with the receptive uppermost portion of the stigma, an additional reduction in the availability of pollinators who may often supplement pollination in these circumstances could lead to reductions in pollination and seed set.

Glyphosate Effects on Pollen Morphology

Mature, viable pollen is essential for proper pollen tube growth and embryo fertilization. Glyphosate applications at

the four-leaf (POST) and eight-leaf (POST directed) stages have previously been reported to reduce pollen viability between 45 to 62% over the first 4 wk of flowering (Plinc et al. 2002a). This work also noted that between 46 and 55% of the pollen grains from glyphosate-treated plants did not fluoresce uniformly when stained with fluorescein diacetate, a viability stain, whereas pollen grains obtained from nontreated GR plants showed uniform fluorescence in approximately 95% of the pollen grains (Plinc et al. 2002a). The current study compares the morphology of the pollen grains from the first bloom on glyphosate-treated and nontreated DP 5415RR cotton plants using light microscopy, scanning electron microscopy, and transmission electron microscopy.

Scanning electron microscopy confirmed the presence of collapsed pollen grains from glyphosate-treated plants (Figure 1c). In contrast, pollen from nontreated plants appeared fully turgid (Figure 1b). The exine wall of the pollen from both glyphosate-treated and nontreated plants appeared intact, suggesting that either glyphosate does not affect exine formation or glyphosate effects occurred after exine formation in pollen development.

Glyphosate inhibited normal pollen maturation at various stages of development. Pollen grains from the same anthers showed inhibition at the conversions from early vacuolate microspore to vacuolate microspore, vacuolate microspore to vacuolate microgamete, and vacuolate microgamete to mature binucleate pollen. Glyphosate did not inhibit pollen development of all developing grains at the same stage of development or with the same level of severity. Because developing pollen is dependent on the anther tapetum to supply necessary resources for maturation, glyphosate may translocate from the tapetum tissue into the developing pollen grains. Differences in the stage of inhibition may result from the pollen grains proximity to the tapetum and the corresponding dose of glyphosate during a stage of development. Because some features of pollen development, such as the organization of the ER into pockets, occur within only a few hours (Wetzel and Jensen 1992), time differences in glyphosate translocation may influence pollen development at different developmental stages. Sawhney and Bhadula (1988) described the development of anthers and pollen in the male-sterile *stamenless-2* mutant of tomato (*Lycopersicon esculentum* Mill.), which has delayed tapetum degeneration. Pollen near the tapetum appeared normal, whereas the development of pollen oriented toward the inner portion

of the anther locule was severely delayed. Because the flow of nutrients from the tapetum wall to the pollen forms a gradient relative to proximity, the translocation of glyphosate may also form this gradient, thereby affecting pollen near the tapetum earlier than the pollen in the inner locule. This action could account for the inhibition at different stages in pollen development (Raghavan 2000).

The earliest stage in which pollen development was inhibited or delayed by glyphosate was at the early vacuolar microspore stage (Figures 1a [II] and 3c). At this stage, numerous vacuoles and a general unorganized cytoplasmic array of small lipid and starch bodies were present. The ER was not inflated and showed no organization into the storage pockets present in mature pollen at anthesis.

The next stage of pollen development affected by glyphosate was the conversion from the vacuolar microspore to the vacuolar microgamete (Figures 1a [III] and 3d). In these pollen grains, the large central vacuole remained intact, and mitosis of the microspore nucleus to form a vegetative and generative nucleus did not occur. The organization of the ER into storage pockets containing vesicles begins to occur, but only in the area surrounding the central vacuole (Figure 3d). Frankel and Galun (1977) reported that the arrest of pollen grains at the vacuolar stage resulted in collapsed outer walls, which are clearly evident by scanning electron microscopy in pollen from glyphosate-treated plants (Figure 1c).

The latest stage in which pollen maturation was inhibited by glyphosate was at the conversion of the vacuolate microgamete to the mature binucleate pollen grain (Figures 1a [IV], 3a, 3b, 3e, and 3f). In this stage, occurring in the final 1 to 2 d before anthesis (Wetzel and Jensen 1992), the ER of pollen from nontreated plants was fully inflated, covered with ribosomes, and organized into storage pockets (Figure 3e). Starch grains in these storage pockets were hydrolyzed and replaced with lipid bodies and small carbohydrate-containing vesicles. Vacuoles disappeared and were filled with storage reserves. However, the arrangement of the ER in pollen from glyphosate-treated cotton plants differed from that of nontreated plants. The ER in nontreated pollen inflated with clearly defined borders because of the presence of ribosomes (Figures 3a and 3e), but in glyphosate-treated plants the pollen ER was severely distended, did not form walls surrounding these pockets, and had a lower density of ER-bound ribosomes (Figures 3b and 3f). The ER from glyphosate-treated cotton pollen resembled that of the mature pollen following germination described by Jensen et al. (1968). At 3 to 6 d before anthesis in normal pollen, the ER is also in a distended form, suggesting that glyphosate may interfere with or delay the ER arrangement (Wetzel and Jensen 1992). Starch grains in pollen from glyphosate-treated plants were larger and more numerous than in pollen from nontreated plants, suggesting inhibited or slowed starch hydrolysis (Figures 3b and 3f). Large grains of starch in pollen from glyphosate-treated plants remained within storage pockets, whereas starch in nontreated pollen was found only outside these pockets (Figures 3a and 3e).

Glyphosate has previously been shown to affect plastid integrity by causing plastids to swell or burst (Mollenhauer et al. 1987; Uotila et al. 1980) or to have abnormal chloroplast grana (Pihakaski S. and Pihakaski K. 1980; Vaughn and Duke 1986). Other cellular organelles are likewise sen-

sitive to glyphosate disruption. Vaughn and Duke (1986) reported abnormal nuclei in glyphosate-treated soybean seedlings; whereas Uotila et al. (1980) found decreases in starch content, increases in dictyosome and mitochondria number, and disruption of tonoplasts in glyphosate-treated white mustard (*Sinapis alba* L. = *Brassica hirta* Moench). Deterioration of oil bodies, ER, and ribosomes, along with increases in lipid bodies and cytoplasmic vacuolation, were reported in glyphosate-treated *Pellia epiphylla* (L.) Lindb. (Pihakaski S. and Pihakaski K. 1980). In glyphosate-treated GR cotton plants, the reductions in anther and staminal column lengths may be caused by glyphosate-induced inhibition of elongation. Glyphosate has been reported to cause swollen "club" morphology at the root tips because of microtubule loss and an arrest of mitosis and cell division (Vaughn and Duke 1986). If anthers were sensitive to glyphosate, similar elongation inhibition may occur.

CP4-EPSPS in Tissues

Analysis of the CP4-EPSPS content in various tissues of GR cotton revealed that the CP4-EPSPS content in stigma, anther, preanthesis floral bud (square), and flower petals were significantly less than that in the vegetative leaf tissue (Figure 2b). The CP4-EPSPS content in the ovary of anthesis GR cotton blooms was similar to that in the vegetative leaves. Reduced reproductive tolerance to glyphosate has also been shown in experimental, noncommercial GR tobacco plants containing different constructs of the CP4-EPSPS gene encoded in the chloroplast genome (Ye et al. 2001). Western blot analysis of these tobacco plants indicated that CP4-EPSPS protein levels were 10-fold lower in flower petals and 50-fold lower in immature anthers and ovaries than in the mature leaf tissue (Ye et al. 2001).

From the current work, male reproductive organs in GR cotton appear to be only partially resistant to the effects of glyphosate, presumably because of the insufficient presence of CP4-EPSPS. However, affected pollen does not seem to degenerate as has been reported for chloroplasts in glyphosate-treated plants (Mollenhauer et al. 1987; Uotila et al. 1980). Membranes are intact, and no unusual organelle swelling is observed, except for the distended ER. Instead, pollen development appears to be slowed or arrested at different maturation stages, likely because of the localized dose and timing of glyphosate during pollen development. At anthesis, pollen from glyphosate-treated plants shows a range of phenotypes closely resembling arrested pollen from reported male-sterile mutants as well as from cotton pollen analyzed at intervals from 12 d before anthesis to anthesis.

Reduced levels of amino acids have been associated with the male-sterile pollen in other plants (Frankel and Galun 1977). In addition, aromatic compounds such as flavonoids and sporopollenin, derived downstream from the shikimic acid pathway, are needed for pollen development and wall formation. Absence of these compounds can cause male sterility (Niester-Nyveld et al. 1997; Vandermeer et al. 1992). Reproductive organs of glyphosate-treated GR cotton may still produce some aromatic amino acids and secondary metabolites even though there is less CP4-EPSPS in these tissues than in the vegetative tissues. The presence of suboptimal quantities of these compounds may slow, instead of completely inhibit, pollen maturation. A slower maturation of pollen in glyphosate-treated GR plants would explain the

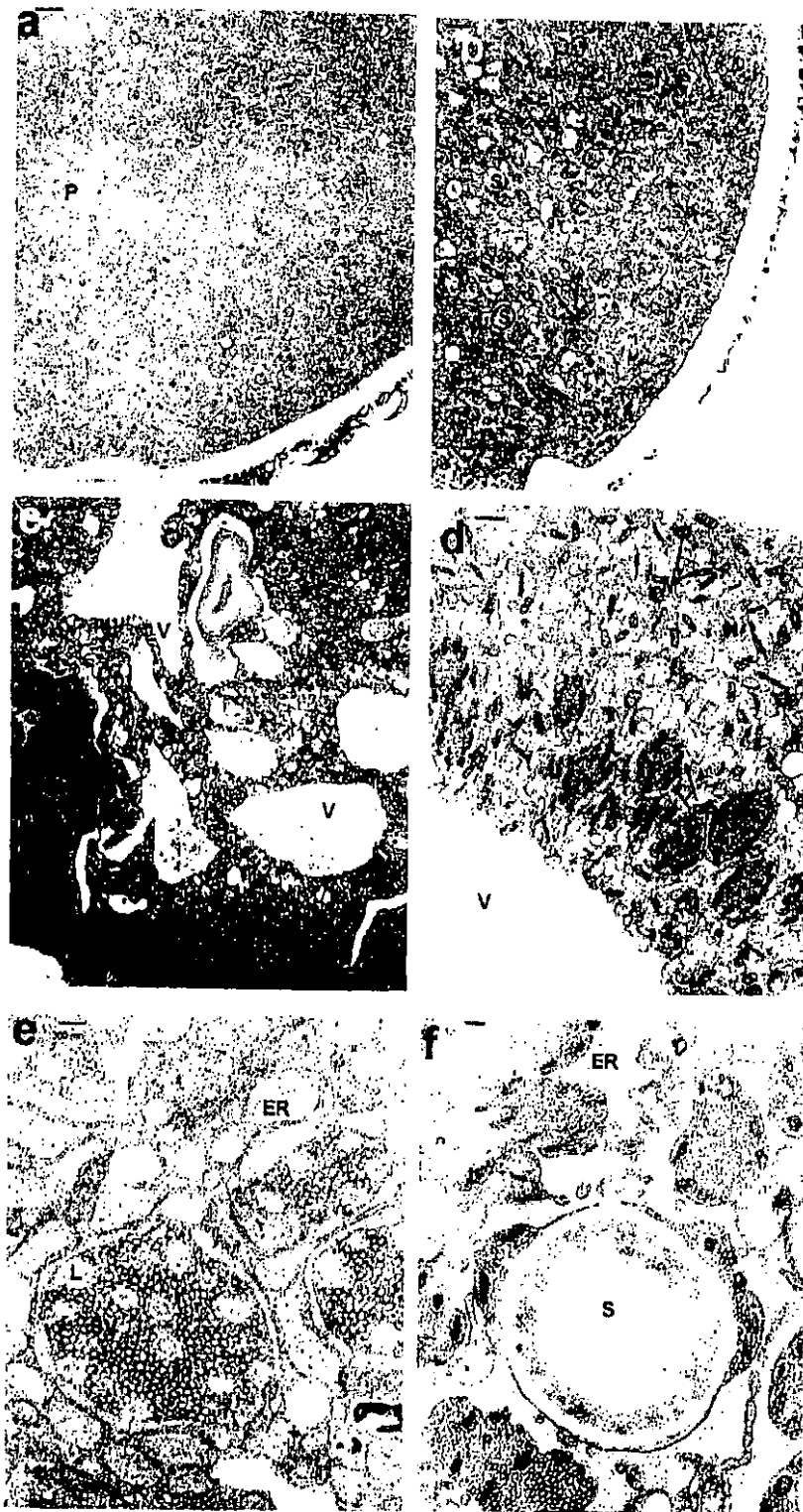


Figure 3. Pollen from glyphosate-treated and nontreated glyphosate-resistant (GR) cotton (*Gossypium hirsutum* L.). (a) Pollen grain at anthesis from nontreated GR cotton flower. P, storage pocket. Scale bar = 3 μ M. (b) Pollen grain at anthesis from glyphosate-treated GR cotton flower, exhibiting inhibited food accumulation. P, storage pocket; S, starch-containing plastids. Scale bar = 3 μ M. (c) Glyphosate-treated GR cotton pollen inhibited at the early vacuolate microspore developmental stage. V, vacuoles. Scale bar = 3 μ M. (d) Pollen from glyphosate-treated GR cotton inhibited at the vacuolate microspore developmental stage. V, vacuole; M, mitochondria; ER, endoplasmic reticulum. Scale bar = 1 μ M. (e) Storage pocket in pollen grain from nontreated GR cotton at anthesis. L, lipid; SV, small vesicles; ER, inflated endoplasmic reticulum. Scale bar = 300 nm. (f) Storage pocket from pollen grain from glyphosate-treated GR cotton plant at anthesis. L, lipid; ER, distended endoplasmic reticulum; SV, small vesicles; S, starch-containing plastid. Scale bar = 300 nm.

apparent presence of pollen grains from earlier developmental stages at anthesis.

The effect of glyphosate on floral morphology, as well as on pollen morphology, is transitory with the most pronounced effect during the first week of flowering (Tables 1 and 2). The first flowers on a cotton plant likely receive the highest dose of glyphosate because they are the strongest metabolic sinks at the time of glyphosate application. Subsequently, as the plants increase in biomass, glyphosate may be diluted or lost from the system as early floral organs abscise. Therefore, later-developing squares may not receive an inhibitory dose of glyphosate, and the flowers may appear similar to those of the nontreated plants.

In conclusion, our findings demonstrate that floral morphology is altered in GR cotton plants treated with recommended rates of glyphosate, presumably because of lower reproductive tissue CP4-EPSPS content. The anthers and the staminal columns are less elongated, whereas the stigma length increases slightly. These morphological differences result in less pollen deposition on the stigmas of glyphosate-treated GR plants. The majority of pollen from glyphosate-treated GR plants is arrested at different developmental stages, resulting in immature or aborted pollen at anthesis. The combination of reduced pollen deposition on the stigma along with pollen development inhibition provide a plausible explanation for the reports on increased boll abscission in glyphosate-treated GR cotton.

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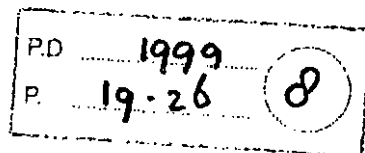
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WEED SCIENCE

Tolerance of Transgenic Cotton to Topical Applications of Glyphosate

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INTERPRETIVE SUMMARY

Transgenic cotton varieties expressing vegetative tolerance to over-the-top applications of glyphosate herbicide are now commercially available to producers. Weed management programs using glyphosate-tolerant cotton varieties allow growers a new option to control weeds in conventional tillage systems, as well as in reduced-tillage systems where cultivation is not used. However, limitations exist with glyphosate-tolerant cotton management systems with regard to the proper timing of over-the-top applications to cotton. Glyphosate is labeled only for over-the-top applications up to the four-leaf stage of development. This label restriction appears to be related to fruit shedding when over-the-top applications are made past the four-leaf stage of development.

Field studies were conducted to evaluate the effect of applying glyphosate over-the-top of glyphosate-tolerant cotton varieties. In these studies, all glyphosate-tolerant varieties appeared to respond similarly to delayed over-the-top applications of glyphosate. Plant mapping data revealed a sequential decrease in boll retention at early-season fruiting sites as glyphosate applications were delayed from the three-leaf to the six-leaf stage. The loss of these early-season fruiting structures did not reduce total lint yields. However, there appeared to be a delay in maturity as a result of the later over-the-top glyphosate applications. It appeared that considerable compensation for the loss of early-season fruit occurred after the topical applications of

glyphosate. The ability of glyphosate-tolerant cotton plants to recover from delayed over-the-top applications of glyphosate in most years will be, in many cases, directly related to environmental conditions that allow extended boll development later in the season.

ABSTRACT

Transgenic cotton (*Gossypium hirsutum* L.) cultivars tolerant to topical applications of glyphosate herbicide are now commercially available to producers. However, little information exists with regard to the tolerance of various cotton cultivars as affected by the timing of glyphosate applications. Therefore, the response of several glyphosate-tolerant transgenic cotton cultivars to various application timings of glyphosate was investigated. Two separate studies were conducted in 1995 and one in 1996 at the Delta Research and Extension Center located near Stoneville, MS. Treatments for both studies in 1995 consisted of seven glyphosate-tolerant cultivars (Coker 312-RR, Hartz 1215RR, Hartz 1220RR, Hartz 1244RR, Hartz 1330RR, Hartz 1380RR, and Hartz 1560RR) that were either unsprayed (that is, untreated control) or sprayed topically with 1.0 kg a.e. ha⁻¹ glyphosate plus a non-ionic surfactant at 0.5% v v⁻¹ at one of three different growth stages (four-, five-, and six-leaf stage). A different strain of each glyphosate-tolerant cultivar was evaluated in the two studies in 1995. Treatments in 1996 consisted of four glyphosate-tolerant cultivars (Deltapine 9683RR, Deltapine 9685RR, Deltapine 9687RR, and Coker 312RR) that were either unsprayed (i.e., untreated control) or sprayed topically with glyphosate at 1.0 kg a.e. ha⁻¹ plus a non-ionic surfactant at 0.5% v v⁻¹ at one of three different growth stages (three-, four-, and five-leaf stage) and two unsprayed conventional cultivars (Deltapine 5415 and 90). All transgenic cultivars responded similarly to glyphosate, and no differences in vegetative growth parameters or in total lint yield were found among any of the application timings in either year. However, mapping data revealed a sequential decrease in first position sympodia boll retention of the first three fruiting

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branches as topical applications of glyphosate were delayed. Retention values were 61, 44, 34, and 19% for the untreated, four-leaf stage, five-leaf stage, and six-leaf stage in one study in 1995 and 62, 55, 41, and 21%, respectively, for the same treatments in the second study. In 1996 retention values were 58, 45, 38, and 25% for the untreated, three-leaf stage, four-leaf stage, and five-leaf stage applications, respectively. The loss of early-season fruit caused by topical glyphosate applications resulted in a slight delay in maturity as measured by nodes above white flower counts. Although vegetative growth and total lint yield were unaffected in this study, it appeared that early-season fruit retention was negatively correlated and maturity was delayed as the timing of topical applications of glyphosate herbicide was delayed from the third true-leaf stage to the sixth true-leaf stage.

Weed management programs incorporating cotton genetically engineered for tolerance to topical applications of specific nonselective herbicides provide producers more weed control options in conventional- and reduced-tillage systems. These programs may also provide greater flexibility in application timing, allow a broader spectrum of weeds to be controlled, and allow weeds to be controlled more economically and in a more environmentally sound manner. Since the mid-1980s, the Monsanto Company has been developing genes to confer crop tolerance to glyphosate [*N*-(phosphonomethyl)glycine] in many crops, including cotton, corn (*Zea mays* L.), and soybean (*Glycine max* L. Merr.) (Johnson, 1996). Glyphosate is a competitive inhibitor of the 5-enolpyruvylshikimate-3-phosphate synthase enzyme in the shikimic acid pathway, which is needed to synthesize the essential aromatic amino acids tryptophan, tyrosine, and phenylalanine (Comai and Stalker, 1986). Tolerance to glyphosate has been achieved by placing a cloned gene for 5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium* spp. strain CP4 into transgenic plants under transcriptional control of a strong promoter (Johnson, 1996). This gene produces a 5-enolpyruvylshikimate-3-phosphate synthase enzyme with reduced sensitivity to glyphosate. Therefore, cotton plants containing this gene are tolerant to topical or post-directed applications of glyphosate.

Postemergence glyphosate applications in transgenic cotton will be a valuable tool for cotton

management because glyphosate offers the potential for postemergence control of a broader spectrum of weeds compared to other cotton herbicides (Snipes, 1995). Applying postemergence herbicides in cotton can be more economical than current standards (Snipes, 1995), and glyphosate is effective at controlling herbicide-resistant weed biotypes (Smeda et al., 1997). However, limitations appear to exist with regard to proper timing of topical glyphosate applications. Glyphosate is labeled for topical applications up to the four-leaf stage of development and thereafter post-directed applications are required (Roundup Ultra label, 1997, The Monsanto Co., 800 N. Lindbergh Blvd., St. Louis, MO 63167). These label restrictions appear to be related to the potential for fruit abortion following applications just prior to or during reproductive development. Since this technology is relatively new to researchers and producers, many questions exist regarding the optimum timing of topical applications of glyphosate to transgenic cotton and the subsequent effects of these applications on cotton growth, maturity, and yield. Recent complaints of high fruit abortion rates and the development of misshapen bolls in glyphosate-tolerant transgenic cotton cultivars by producers in the Mississippi Delta and surrounding states further reiterates the need for research evaluating transgenic cottons. Therefore, this study tested the hypothesis that delaying topical applications of glyphosate to glyphosate-tolerant cotton increases loss of early-season fruiting structures. Additional objectives were to determine the effects of these treatments on cotton maturity and yield.

MATERIALS AND METHODS

Two field studies were conducted in 1995 and one was conducted in 1996 at the Delta Research and Extension Center near Stoneville, MS, on a Bosket very fine sandy loam (fine-loamy, mixed, thermic Mollic Hapludalfs). Treatments for both studies in 1995 included seven glyphosate-tolerant cultivars (Coker 312-RR, Hartz 1215RR, Hartz 1220RR, Hartz 1244RR, Hartz 1330RR, Hartz 1380RR, and Hartz 1560RR) that were either unsprayed (i.e., untreated control) or sprayed topically with glyphosate (MON2139) at 1.0 kg a.e. ha⁻¹ plus a non-ionic surfactant [Induce nonionic low

foam wetter/spreader adjuvant (alkyl aryl polyoxyalkane ether free fatty acids), Helena Chem. Co., 6075 Poplar Ave., Suite 500, Memphis, TN 38119] at 0.5% v v⁻¹ at one of three different growth stages (four-, five-, and six-leaf stage).

A different strain of each glyphosate-tolerant cultivar was evaluated in the two studies in 1995. Treatments in 1996 included four glyphosate-tolerant cultivars (Deltapine 9683RR, Deltapine 9685RR, Deltapine 9687RR, and Coker 312RR) that were either unsprayed (i.e., untreated controls) or sprayed topically with 1.0 kg a.i. ha⁻¹ glyphosate plus a non-ionic surfactant at 0.5% v v⁻¹ at one of three different growth stages (three-, four-, and five-leaf stage) and two unsprayed conventional cultivars (Deltapine 5415 and 90).

Cotton was planted on 10 May 1995 and 3 May 1996 at a rate of approximately 14 seed m⁻² of row on a 1.0 m row spacing. Final plant population was 9 to 10 plants m⁻². Plots were four rows wide by 9.1 m-long in 1995 and four rows wide by 12.2 m-long in 1996. Treatments were arranged in a randomized complete block design with four replications in 1995 and six replications in 1996.

Approximately 8 weeks before planting, 0.6 kg a.i. ha⁻¹ trifluralin (α, α, α -trifluoro-2,6-dinitro-*N,N*-dipropyl-*p*-toluidine) and 0.3 kg a.i. ha⁻¹ norflurazon [4-chloro-5-(methylamino)-2-(α, α, α -trifluoro-*m*-tolyl)-3(2*H*)-pyridazinone] were applied and incorporated. At planting, 0.6 kg a.i. ha⁻¹ fluometuron [1,1-dimethyl-3-(α, α, α -trifluoro-*m*-tolyl) urea] and 0.3 kg a.i. ha⁻¹ norflurazon [4-chloro-5-(methylamino)-2-(α, α, α -trifluoro-*m*-tolyl)-3(2*H*)-pyridazinone] were applied.

About 6 weeks after planting, 1.0 kg a.i. ha⁻¹ cyanazine {2[[4-chloro-6-(ethylamino)-*s*-triazin-2-yl]amino]-2-methylpropionitrile} and 2.2 kg a.i. ha⁻¹ MSMA (monosodium acid methanearsonate) were postdirected in 1995 and 0.2 kg a.i. ha⁻¹ lactofen {1-(carboethoxy)ethyl 5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-nitrobenzoate} was postdirected in 1996. About 2 weeks later in both years, a layby application of diuron [3-(3,4-dichlorophenyl)-1,1-dimethylurea] was applied.

Cultivation and hand-removal of weeds were used to ensure all plots were weed free. Other production practices and insect control measures were used in an attempt to optimize yields. Plots were furrow-irrigated (7.5 cm) on 26 July 1995 and

17 July 1996 to alleviate potential moisture stress. Nitrogen was applied at 134 kg ha⁻¹ as a 32% anhydrous urea-ammonium nitrate solution injected beside the row using fertilizer knives before planting on 23 March in both years.

Glyphosate was applied to the two middle rows of each four row plot on 5 June (four-leaf stage), 8 June (five-leaf stage), and 13 June (six-leaf stage) with a CO₂-pressurized backpack sprayer (XR11003 spray tips) calibrated to deliver 190 L ha⁻¹ at 26 kPa in 1995. Glyphosate was applied to the two middle rows of each four row plot on 25 May (three-leaf stage), 31 May (four-leaf stage), and 3 June (five-leaf stage) with a CO₂-pressurized backpack sprayer (XR11002 spray tips) calibrated to deliver 80 L ha⁻¹ at 21 kPa in 1996.

Prior to each application, the actual leaf stage at the time of glyphosate applications was determined for each plot by taking counts from 10 consecutive plants from the second row of each four row plot. Leaf counts were taken using the cotyledons as a reference point (node 0) and counting each node that had a leaf 2.5 cm in diameter or larger. These observations were averaged and recorded as actual leaf number for use in regression analysis. Plant height, node development, first fruiting branch, and nodes above white flower were determined by mapping five consecutive plants from the second row of each four row plot on 3 Aug. 1995 and on 14 June, 9 July, and 30 July in 1996.

Before harvest each year (3 Aug. 1995 and 19 Aug. 1996), percent boll retention at first position sympodial locations was determined for the first three sympodial branches by measuring five consecutive plants from the second row of each four row plot. Total boll retention for these three branches was determined by summing the boll retention for each individual branch and dividing by three. Seed cotton yield was determined by machine-harvesting the two middle rows of each plot with a spindle-picker modified for plot harvest on 5 Oct. 1995 and 10 Oct. 1996. A subsample consisting of 0.5 to 1.0 kg of seed cotton from each plot was used to determine gin turnout and percent lint. Lint yield was determined by multiplying gin turnout by seed cotton weight.

Data were evaluated using analysis of variance and regression analysis (SAS Institute, Inc., Cary, NC). Glyphosate application timings were averaged

across cultivars when treatment by cultivar interactions were not significant. Since leaf stages for application timings differed in the 2 years, the data were analyzed separately for each year. In 1996, the two conventional cultivars (Deltapine 5415 and 90) were combined with the untreated control treatments for data analysis. Treatment means were separated using Fisher's Protected LSD at $P < 0.05$. The actual measured leaf-stages for each plot at the time of glyphosate applications were regressed against total fruit retention or nodes above white flower counts.

RESULTS AND DISCUSSION

There were no differences in yield, boll retention, and most measured plant growth parameters among

cultivars in both years (data not shown). The glyphosate treatment by cultivar interaction was not significant in either year. Since the effects of topical applications of glyphosate were the same irrespective of cultivar in either year, means for measured parameters were averaged across cultivars by year.

The timing of glyphosate applications did not affect lint yield or the location of the first fruiting branch, but did cause decreases in boll retention. In 1995, lint yields from the untreated control plots and from applications made to cotton at the four-, five-, and six-leaf stage averaged 1113, 1093, 1101, and 1099 kg ha^{-1} , respectively, when combined over both studies (Tables 1 and 2). In 1996, lint yields for the untreated control plots averaged 1264 kg ha^{-1} , while lint yields for the three-, four-, and five-leaf treatments averaged 1248, 1260, and 1101 kg ha^{-1} .

Table 1. Lint yield, first fruiting branch, and boll retention at first sympodial branch locations in response to glyphosate applied at various stages of cotton development in 1995 at Stoneville, MS. Values are averaged over seven cultivars (Strain one).

Glyphosate: treatment	Lint yield kg ha^{-1}	First fruiting branch‡ no.	Boll retention at first position sympodia			
			First§	Second	Third	Total
Untreated	1155	6.3	65.7	57.9	60.0	61.2
Four leaf stage	1133	6.2	45.7	42.9	43.6	44.3
Five leaf stage	1143	6.1	21.4	37.9	43.6	34.3
Six leaf stage	1143	6.1	12.9	16.4	27.9	19.1
LSD(0.05)¶	NS	NS	11.3	12.1	12.0	8.3

† Glyphosate applied at 1.0 a.e. kg ha^{-1} plus Induce at 0.5% v v⁻¹.

‡ First fruiting branch measured in ascending order with the cotyledonary node as 0.

§ First, second, and third refer to boll retention on the first, second, and third fruiting branches, respectively. Total is the mean boll retention averaged over the first three fruiting branches.

¶ Least significant difference at the 0.05 level of probability. NS = not significant at the 0.05 level of probability.

Table 2. Lint yield, first fruiting branch, and boll retention at first sympodial branch locations in response to glyphosate applied at various stages of cotton development in 1995 at Stoneville, MS. Values are averaged over seven cultivars (Strain 2).

Glyphosate†	Lint yield kg ha^{-1}	First fruiting branch‡ no.	Boll retention at first position sympodia			
			First§	Second	Third	Total
Untreated	1070	5.9	61.4	67.9	57.1	62.1
Four leaf stage	1053	5.8	57.1	60.0	47.9	55.2
Five leaf stage	1057	5.8	35.7	45.7	40.0	40.5
Six leaf stage	1054	6.0	15.7	17.9	29.3	21.0
LSD(0.05)¶	NS	NS	13.1	12.7	12.7	8.9

† Glyphosate applied at 1.0 kg a.e. ha^{-1} plus Induce at 0.5% v v⁻¹.

‡ First fruiting branch measured in ascending order with the cotyledonary node as 0.

§ First, second, and third refer to boll retention on the first, second, and third fruiting branches, respectively. Total is the mean boll retention averaged over the first three fruiting branches.

¶ Least significant difference at the 0.05 level of probability. NS = not significant at the 0.05 level of probability.

Table 3. Lint yield, first fruiting branch, and boll retention at first sympodial branch locations in response to glyphosate applied at various stages of cotton development in 1996 at Stoneville, MS. Values are averaged over six cultivars.

Glyphosate [†] treatment	Lint yield kg ha ⁻¹	First fruiting branch [‡] no.	Boll retention at first position sympodia			
			First [§]	Second	Third	Total
Untreated	1264	5.1	54.2	60.0	59.2	57.8
Three leaf stage	1248	5.2	36.7	46.7	51.7	45.0
Four leaf stage	1260	5.0	25.0	44.2	43.4	37.5
Five leaf stage	1101	5.0	18.3	23.4	32.5	24.7
LSD(0.05) [¶]	NS	NS	12.8	10.4	12.8	10.2

[†] Glyphosate applied at 1.0 kg a.e. ha⁻¹ plus Induce at 0.5% v v⁻¹.

[‡] First fruiting branch measured in ascending order with the cotyledonary node as 0.

[§] First, second, and third refer to boll retention on the first, second, and third fruiting branches, respectively. Total is the mean boll retention averaged over the first three fruiting branches.

[¶] Least significant difference at the 0.05 level of probability. NS = not significant at the 0.05 level of probability.

respectively (Table 3). The location of the first fruiting branch ranged between nodes 5.0 and 6.3 over the 2 years.

Delayed glyphosate applications decreased boll retention at first position sympodial fruiting sites (Tables 1, 2, and 3). Untreated plants retained 65.7, 61.4, and 54.2% of their first position bolls located on the first fruiting branch in the two 1995 studies and the 1996 study, respectively. Percent boll retention on the first fruiting branch declined incrementally as the timing of glyphosate application was delayed from the three-leaf to the six-leaf stage. The greatest decrease in boll retention occurred on plants treated topically with glyphosate at the six-leaf stage, with average boll retentions of 12.9 and 15.7% on the first fruiting branch in 1995 (six-leaf treatments were not evaluated in 1996).

Reductions in boll retention at first position sympodial locations similar to that observed on the first fruiting branch occurred on the second and third fruiting branches in both years (Tables 1, 2, and 3). Total boll retention for the first three fruiting branches for plants treated topically at the three-, four-, five-, and six-leaf stages was reduced by 25.5, 24.4, 45.1, and 67.8%, respectively, compared to the untreated control plants. Total boll retention for the first three fruiting branches displayed a negative linear relationship with the actual leaf-stage when glyphosate was topically-applied ($r^2 = -0.74$), regardless of cultivar or year (Fig. 1). Therefore, it appeared that the reproductive development of glyphosate-tolerant cotton was highly sensitive to delayed topical applications of glyphosate. Since fruit retention and boll numbers are highly correlated with lint yield (Jones et al., 1996a; Wells and

Meredith, 1984), caution should be exercised by producers when considering mid- and late-season (i.e., after the four-leaf stage to pre-harvest) over-the-top salvage applications of glyphosate, especially after reproductive development has initiated.

The potential of cotton to compensate for early-season boll loss may be important in deciding whether or not to use glyphosate over-the-top after square development has occurred. There were no differences in lint yield (Tables 1, 2, and 3) among treatments in this study despite reductions in early-season fruit retention, indicating that considerable compensation following early-season fruit loss occurred. Because of their indeterminate, perennial growth habit, cotton plants have several mechanisms

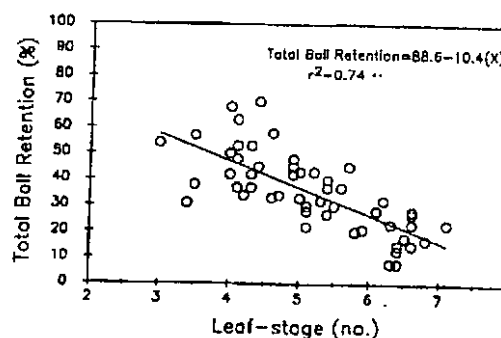


Fig. 1. Relationship of total boll retention on the first three fruiting branches at the first position sympodial locations to leaf-stage at the time of topical glyphosate applications. Each data point represents the actual measured leaf-stages at the time of glyphosate application for each sprayed plot for two studies (seven glyphosate-tolerant cultivars) in 1995 and one study (four glyphosate-tolerant cultivars) in 1996 averaged across replications. ** Indicates that the slope is significant at $P = 0.01$.

that promote compensatory late-season boll production. Early fruit removal studies have shown that cotton plants can compensate for early-season fruit loss with increased flowering (Ehlig and LeMert, 1973; Kletter and Wallach, 1982; Patterson et al., 1978; Ungar et al., 1987), flowering rates (Kennedy et al., 1986), fruit retention (Ehlig and LeMert, 1973; Kletter and Wallach, 1982; Patterson et al., 1978) and boll setting rates (Kennedy et al., 1986). Moreover, cotton plants exposed to early-season fruit removal shift their boll development locations to positions higher on the main stem and at more distal fruiting branch positions (Jones et al., 1996a).

The loss of early-season fruit in these studies may have also delayed maturity as evidenced by a slight increase in nodes above white flower values as the timing of topical glyphosate applications was delayed from the third leaf-stage to the sixth leaf-stage (Fig. 2, Tables 4 and 5). Early maturity is often associated with low first fruiting branch nodal positions, rapid early node production, and greater fruit retention on these early fruiting sites (Kerby et al., 1990). Although it is possible for considerable

reproductive compensation to occur in cotton, the amount and duration of reproductive compensation after early-season fruit loss is highly dependent on a sufficiently long growing season with adequate environmental conditions to mature later-season fruit development.

There are numerous studies documenting yield losses when early-season square loss occurs (Brown, 1965; Kennedy et al., 1986; Pettigrew et al., 1992; Ungar et al., 1987). Therefore, some caution should be used when dealing with situations that may induce early-season square loss. Also, the slight delay in maturity associated with delayed applications in this study could increase production costs associated with prolonged insect and weed management, as well as reduce the effectiveness of harvest-aid materials and the harvesting process. In Mississippi, the number of days suitable for field work declines from 10.3 to 3.3 days for each 15 day period beginning 26 September and continuing until 9 December (Cooke et al., 1972). Delays in maturity jeopardize harvesting operations and contribute to late-season weather related yield and quality losses.

No differences in plant height, number of main

Table 4. Height (ht), number of nodes, first fruiting branch (FFB), and nodes above white flower (NAWF) at 85 days after planting in response to glyphosate applied at various stages of cotton development in 1995 at Stoneville, MS. Values are averaged over seven cultivars.

Glyphosate†	Strain one				Strain two			
	Ht	Nodes	NAWF	FFB‡	Ht	Nodes	NAWF	FFB
treatment	cm	no.	no.	no.	cm	no.	no.	no.
Untreated	104	20.2	5.8	6.3	99	19.6	5.1	5.9
Four leaf stage	103	20.4	5.9	6.2	101	20.0	5.4	5.8
Five leaf stage	107	20.5	5.7	6.1	103	20.2	5.4	5.8
Six leaf stage	106	20.5	6.0	6.1	106	20.4	5.8	6.0
LSD(0.05)§	3	NS	0.3	NS	4	NS	0.4	NS

† Glyphosate applied at 1.0 kg a.e. ha⁻¹ plus Induce at 0.5% v v⁻¹.

‡ First fruiting branch measured in ascending order with the cotyledonary node as 0.

§ Least significant difference at the 0.05 level of probability. NS = not significant at the 0.05 level of probability.

Table 5. Height, number of nodes, first fruiting branch, and nodes above white flower in response to glyphosate applied at various stages of cotton development in 1996 at Stoneville, MS. Values are averaged over six cultivars.

Glyphosate†											
treatment	38 DAP			63 DAP				84 DAP			
	Ht	Nodes	FFB‡	Ht	Nodes	FFB	NAWF	Ht	Nodes	FFB	NAWF
	cm	no.		cm	no.			cm	no.		
Untreated	33	8.8	5.1	84	14.4	5.3	7.1	100	18.3	5.5	3.7
Three leaf stage	34	8.7	5.2	91	15.1	5.2	7.4	111	19.2	5.8	3.8
Four leaf stage	35	8.7	5.0	90	15.2	5.3	7.2	105	18.5	5.7	3.5
Five leaf stage	33	8.6	5.0	87	15.0	5.4	7.5	109	19.3	5.8	4.2
LSD(0.05 %)	NS	NS	NS	4	NS	NS	0.4	5	0.6	0.3	0.5

† Glyphosate applied at 1.0 kg a.e. ha⁻¹ plus Induce at 0.5% v v⁻¹.

‡ First fruiting branch measured in ascending order with the cotyledonary node as 0.

§ Least significant difference at the 0.05 level of probability. NS = not significant at the 0.05 level of probability.

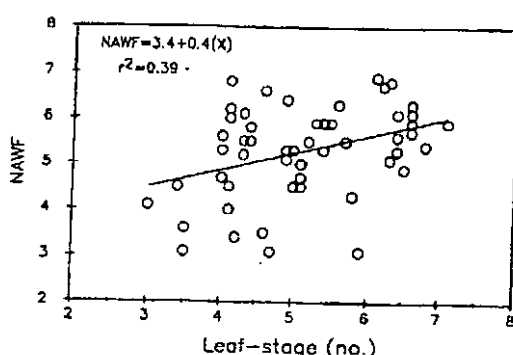


Fig. 2. Relationship of nodes above white flower counts to leaf-stage at the time of topical glyphosate applications. Each data point represents the actual measured leaf stages at the time of glyphosate application for each sprayed plot for two studies (seven glyphosate-tolerant cultivars) in 1995 and one study (four glyphosate-tolerant cultivars) in 1996 averaged across replications. *Indicates that the slope is significant at $P = 0.05$.

stem nodes, or location of the first fruiting branch were found among cultivars or application timings at 38 days after planting in 1996 (Table 5). Also, no differences in visual injury symptoms were observed among cultivars or application timings (data not shown). Glyphosate-tolerant cultivars appeared to be resistant to vegetative injury from topical applications of glyphosate. Plants that received topical glyphosate applications showed increased plant height at 85 days after planting in 1995 and at 63 and 84 days after planting in 1996 and showed increased node numbers at 84 days after planting in 1996 compared to the untreated plants (Tables 4 and 5). It appeared that the loss of early fruit caused by the topical glyphosate applications may have redirected assimilates within the plant toward vegetative development at the expense of reproductive structures. Similar increases in vegetative plant development after loss of early-season fruit were also reported by Jones et al. (1996b) after hand-removal of early-season flowers.

CONCLUSIONS

Topical applications of glyphosate herbicide to glyphosate-tolerant cotton cultivars decreased early-season boll retention at first position sympodia. Leaf-stage was negatively correlated with boll retention and positively correlated with nodes above

white flower values; however, topical applications of glyphosate to transgenic cotton did not significantly reduce total lint yield. The ability of cotton plants to compensate for the loss of early-season fruit after topical applications of glyphosate appeared to be extremely important in the ability of this new technology to work effectively in cotton production systems. The loss of early-season fruit can be tolerated in most years when the environment permits reproductive development to be extended later into the season. However, harvesting and weather related yield losses may be encountered as a result of delays in maturity.

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

pp. 459-462 (4)

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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)

- and the matching adapters and adapter-primers in combination with two anchor-primers: one homologous to the 35S promoter region (35S-primer) and one homologous to the nos 3' terminator region (3' nos-1-primer).

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 µl 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 µl 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 µl of purified eluate, 1X PCR-buffer (Perkin Elmer, USA), 250 µM dNTP's (Pharmacia Biotech, USA), 0.4 µM anchor-primer and 0.4 µM adapter-primer. Water was added to a final volume of 50 µl. 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 µl 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/µl 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 µl using following conditions: 1X PCR-buffer (Perkin Elmer, USA), 200 µM dNTP's (Pharmacia Biotech, USA), 0.5 µM 35S-primer (5' GATAGTGGGATTGTGCGTCA 3'), 0.5 µM event-specific-primer (5' CCTTCAATTAAACCGATGC 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 µl 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

characterised at the molecular level (Padgett *et al.*, 1995). The results of this characterisation 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).

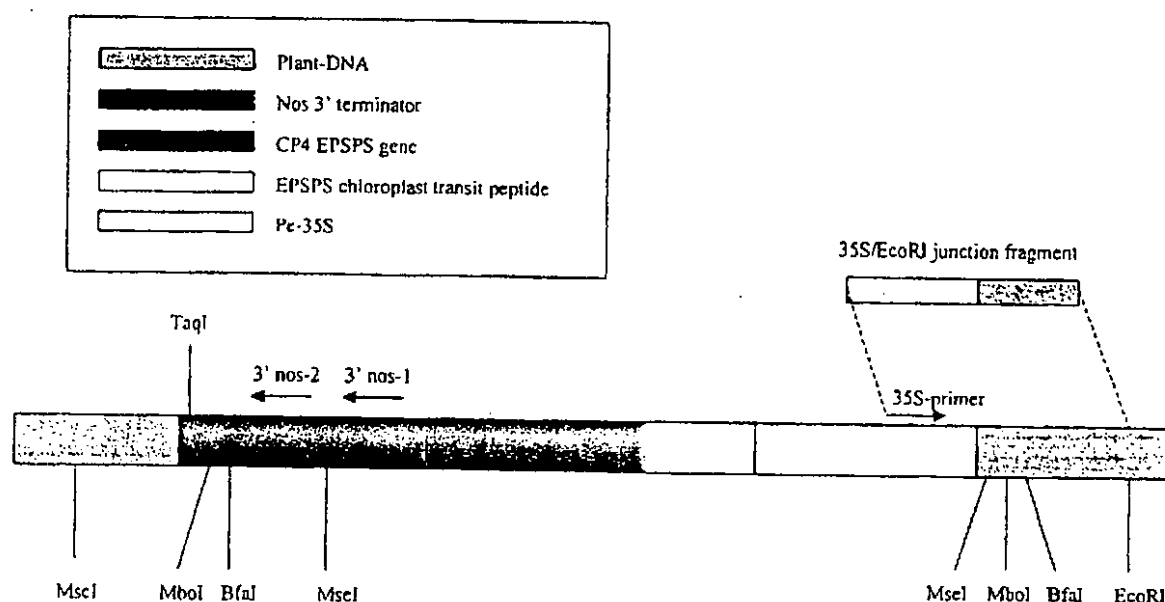


Fig. 1. Schematic diagram of inserted DNA present in RR soybeans (Padgett *et al.*, 1995).

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

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.

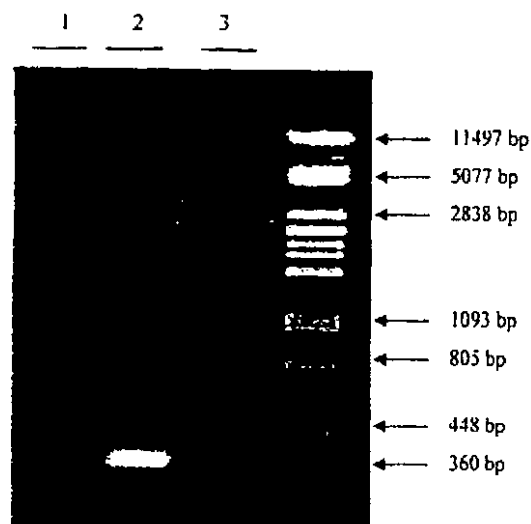


Fig. 2. Agarose gel analysis of line specific RR soy PCR products. Lane 1, line specific RR soy PCR on non-transgenic soy DNA. Lane 2, line specific RR soy PCR on transgenic RR soy DNA. Lane 3, negative control: line specific RR soy PCR on sample without DNA.

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