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# Glyphosate negatively affects pollen viability but not pollination and seed set in glyphosate-resistant corn

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Experiments were conducted in the North Carolina State University Phytotron greenhouse and field locations in Clayton, Rocky Mount, and Lewiston-Woodville, NC, in 2002 to determine the effect of glyphosate on pollen viability and seed set in glyphosate-resistant (GR) corn. Varieties representing both currently commercial GR corn events, GA21 and NK603, were used in phytotron and field studies. All glyphosate treatments were applied at 1.12 kg ai $ha^{-1}$ at various growth stages. Regardless of hybrid, pollen viability was reduced in phytotron and field studies with glyphosate treatments applied at the V6 stage or later. Scanning electron microscopy of pollen from affected treatments showed distinct morphological alterations correlating with reduced pollen viability as determined by Alexander stain. Transmission electron microscopy showed pollen anatomy alterations including large vacuoles and lower starch accumulation with these same glyphosate treatments. Although pollen viability and pollen production were reduced in glyphosate treatments after V6, no effect on kernel set or yield was found among any of the reciprocal crosses in the phytotron or field studies. There were also no yield differences among any of the hand self-pollinated (nontreated male × nontreated female, etc.) crosses. Using enzyme-linked immunosorbent assay to examine CP4-5-enolpyruvlshikimate-3-phosphate synthase expression in DKC 64-10RR (NK603) at anthesis, we found the highest expression in pollen with progressively less in brace roots, ear leaf, anthers, roots, ovaries, silks, stem, flag leaf, and husk.

**Nomenclature:** Glyphosate; corn, *Zea mays* L.; 'DK 662RR'; 'DK 687RR'; 'DKC 64-10RR/SIL'.

**Key words:** Alexander stain, fluorochromatic reaction, pollen viability, GA21, NK603, mEPSPS, CP4-EPSPS, ELISA.

Glyphosate is a broad spectrum, nonselective herbicide that is registered in more than 50 crops and is used widely for vegetation control in nonagricultural situations (Duke 1988). Because glyphosate resistance was commercialized in canola (*Brassica napus* L.), corn, cotton (*Gossypium hirsutum* L), and soybean [*Glycine max* L. (Merr.)], its use has greatly increased in agricultural systems (Shaner 2000). Nearly 11, 58, and 75% of corn, cotton, and soybean, respectively, grown in the United States in 2002 were herbicide resistant (Anonymous 2002a).

Two different glyphosate-resistance events, GA21 and NK603, are commercially available in corn. The GA21 and the NK603 events were released for commercial production in the United States in 1998 and 2001, respectively (Anonymous 2001, 2002b). The GA21 event uses the plasmid pDPG434 as the plant transformation vector to express a modified corn 5-enolpyruvlshikimate-3-phosphate synthase (EPSPS) (mEPSPS) protein to confer glyphosate resistance (Anonymous 2001). The mEPSPS gene is regulated by the rice (*Oryza sativa* L.) actin promoter and rice actin intron (Anonymous 2001). The mEPSPS is fused with an optimized transit peptide that directs the mEPSPS protein to the chloroplast (Anonymous 2001). The mEPSPS used in GA21 varieties has two amino acid changes, threonine 102 replaced with isoleucine, and proline 106 replaced with serine, from the native corn mEPSPS protein (Anonymous 2001). The NK603 event used a gene that encodes for a CP4-EPSPS protein from common soil bacterium *Agrobacterium* sp. strain *CP4* to provide glyphosate resistance. The plasmid PV-ZMGT32 was the plant transformation vector for NK603 (Anonymous 2002b). Two copies of the CP4-EPSPS are controlled by either the rice actin promoter or the enhanced 35S promoter from cauliflower mosaic virus (CaMV) (Anonymous 2002b). The copy of CP4-EPSPS under regulation by the 35S promoter has the amino acid leucine 214 substituted with proline (Anonymous 2002b). Both cassettes were fused to a chloroplast transit peptide (Anonymous 2002b). Because the chloroplast is the location of native EPSPS and the site of amino acid synthesis (Kishore and Shah 1988), the GA21 and NK603 plasmids target the chloroplast using an optimized transit peptide and chloroplast transit peptide, respectively (Anonymous 2001, 2002b).

Glyphosate inhibits the activity of EPSPS [EC 2.5.1.19], an enzyme in the shikimic acid pathway (Duke 1988). This specific site of action inhibits the biosynthesis of the aromatic acids of tryptophan, tyrosine, and phenylalanine (Siehl 1997). As a secondary effect, the inhibition of EPSPS may affect the biosynthesis of proteins, auxins, pathogen defense compounds, phytoalexins, folic acid, precursors of lignins, flavonoids, plastoquinone, and hundreds of other phenolic and alkaloid compounds through the inhibition of aromatic acids (Bentley 1990).

Yield data from glyphosate-resistant (GR) corn and soybean indicate no significant yield losses in response to registered glyphosate treatments (Elmore et al. 2001a; Ferrell

and Witt 2002; Johnson et al. 2000; Nolte and Young 2002). Comparing conventional corn weed control systems with GR systems in Illinois, Nolte and Young (2002) showed that GR corn grain production was either equivalent or greater than conventional corn varieties, depending on the year. When comparing GR soybean with their corresponding nontransgenic isoline, Elmore et al. (2001b) found a 5% reduction in yield and lower seed weight for GR lines. However, Elmore et al. (2001b) concluded that these differences were not due to glyphosate applications. GR cotton has been shown to have boll retention problems after glyphosate treatments (Jones and Snipes 1999). Further investigations into this problem have shown reductions in pollen viability and number of seeds per boll and reduced anther filament length in response to glyphosate applications (Pline et al. 2002b, 2003a).

Therefore, our objectives were to investigate the effects of registered and nonregistered glyphosate treatments on GR corn with respect to pollen viability using light and electron microscopy techniques, anther and pollen production, pollination, and seed set.

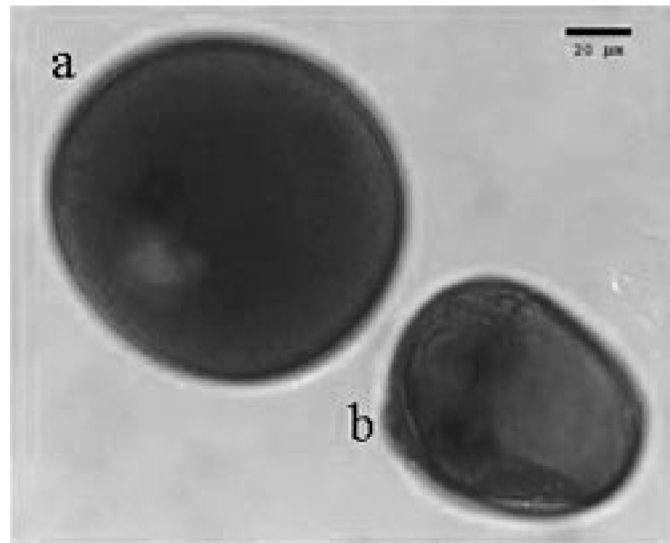


FIGURE 1. Viable (a) and nonviable (b) corn pollen grains from DK 662RR treated with glyphosate postemergence at the V4 followed by V8, stained using Alexander stain (Alexander 1969). Images were taken on a compound light microscope[27] with a digital camera[28].

## Materials and Methods

### Phytotron Experiments

Corn plants were grown in the North Carolina State University Phytotron greenhouse with a 26/22 C day/night temperature regime. DK[1] 662 RR (GA21 event) and DKC[1] 64-10RR (NK 603) varieties were planted in 25-cm (6-L-volume) pots containing a gravel-metro mix soil combination (Anonymous 1991). Plants were thinned to one plant per pot and received individual drip irrigation with standard nutrient solution twice daily (Anonymous 1991). All glyphosate[2] treatments were made at 1.12 kg ai ha$^{-1}$ using a $CO_2$-pressurized sprayer equipped with 11002 flat fan nozzles and calibrated to deliver 137 L ha$^{-1}$ at 207 kPa. Glyphosate was applied postemergence (POST) to DK 662RR at V6, V10, and V6 followed by (fb) V10 and to DKC 64-10RR at V4, V4 fb V8, and V4 fb V10. A nontreated check was included with both varieties. Glyphosate can be applied up to the V8 stage or a height of 76 cm according to the supplemental label (Anonymous 1999a). Immediately after tassel emergence from the whorl, a paper tassel bag[3] was placed over the entire tassel and secured to collect pollen, and prevent its uncontrolled shed (J. B. Holland, personal communication).

On the first day of anthesis, anthers and pollen that had shed from each tassel were collected and weighed. After the initial collection, additional samples were taken 2, 4, and 11 d after initial anthesis (Struik and Makonnen 1992). All pollen samples were collected between 9:00 and 11:00 A.M. Anther and pollen samples were weighed immediately after collection, and the bag was replaced onto the tassel. A subsample of this pollen was subjected to a viability test. Two pollen viability tests were initiated on the first day of sampling. The fluorochromatic reaction uses fluoroscein diacetate to examine the integrity of the plasma membrane and the presence of nonspecific esterases in the pollen cytoplasm (J. Heslop-Harrison and Y. Heslop-Harrison 1970; Kearns and Inouye 1993). Alexander stain was also used to estimate viability (Alexander 1969; Pline et al. 2002a). This nonvital stainability evaluation tests for a mature pollen wall and cytoplasm differentiation (Alexander 1969; Kearns and Inouye 1993). Initial evaluation of each method showed no significant difference in viability measurements. Therefore for simplification of pollen viability estimation, Alexander stain was used for the remainder of the study. Viable and nonviable corn pollen grains as determined by Alexander stain are shown in Figure 1.

To evaluate yield potential from these glyphosate treatments, reciprocal crosses of all possible male and female combinations of treatments were made. Plants were subjected to an identical growing environment for pollen viability and anther and pollen production studies. For the DKC 64-10RR hybrid, glyphosate was applied POST at the V4, V4 fb V8, and V4 fb V10. Whereas for the DK 662RR hybrid, glyphosate treatments were applied POST at V4 fb V8, V4 fb V10, and V4 fb V10 POST-directed (PDS). A nontreated check was included for both hybrids. Glyphosate was applied at 1.12 kg ha$^{-1}$ with a $CO_2$ pressurized sprayer as described previously. All crosses were made using 0.02 g pollen collected from the male parent on the second day after anthesis. Pollen viability was assessed for each cross. Ear shoots were covered with bags[4] to prevent pollination from an unintended source. Silks were also cut the day before crossing to ensure even pollination. Ears were harvested after a minimum of 45 d after fertilization (J. B. Holland, personal communication). Ears were allowed to dry using a forced air drier at 30 C. Kernels were removed with an automatic corn sheller.[5] Percent moisture was measured for each ear using a grain moisture analyzer.[6] Total kernel weight per ear was adjusted to 15.5% moisture. The number of kernels produced per ear was counted using an automated seed counter.[7] All phytotron experiments had a minimum of three replications of treatments and were repeated in time.

### Field Experiments

Field experiments were conducted at the Central Crops Research Station near Clayton, NC, at the Upper Coastal

Table 1. The effect of glyphosate on pollen viability, averaged over sampling times, and pollen and anther production on DK 662RR in phytotron studies.[a]

| Glyphosate treatments | Pollen viability | Fresh weight of pollen and anthers | | | | |
|---|---|---|---|---|---|---|
| | | Initial anthesis | 2 DAIA[b] | 4 DAIA | 11 DAIA | Total |
| | % | g plant$^{-1}$ | | | | |
| Nontreated | 99 a | 0.929 a | 1.725 a | 1.544 a | 0.069 b | 4.888 a |
| V6 | 98 a | 0.274 ab | 1.075 b | 1.271 a | 1.233 a | 3.853 b |
| V10 | 60 b | 0.295 ab | 0.664 b | 0.844 b | 0.601 b | 2.404 b |
| V6 + V10 | 57 b | 0.018 b | 0.980 b | 0.625 b | 0.698 b | 2.320 b |

[a] Means within a column followed by the same letter are not different according to Fisher's Protected LSD test at P = 0.05.
[b] Abbreviation: DAIA, days after initial anthesis.

Plain Research Station near Rocky Mount, NC, and at the Peanut Belt Research Station near Lewiston-Woodville, NC, in 2002. Studies were arranged in split blocks with complete randomization within blocks. Four replications of treatments were included at each location. DK 687RR (GA21) and DKC 64-10RR (NK 603) were planted on April 24, 2002, and April 23, 2002, in Rocky Mount and Lewiston-Woodville, respectively. Whereas only DKC 64-10RR was planted at the Clayton location on April 22, 2002. Soils were Norfolk (fine-loamy, kaolinitic, thermic Typic Kandiudults) and Goldsboro (fine-loamy, siliceous, subactive, thermic Aquic Paleudults) sandy loams at Rocky Mount, Goldsboro and Lynchburg (fine-loamy, siliceous, semiactive, thermic Aeric Paleaquults) sandy loams at Lewiston, and Dothan (fine-loamy, kaolinitic, thermic Plinthic Kandiudults) at Clayton. Percent organic matter and pH ranged from 1.1 to 1.5 and from 5.9 to 6.1, respectively.

A preemergence treatment of *S*-metolachlor[8] at 1.12 kg ai ha$^{-1}$ and atrazine[9] at 1.12 kg ai ha$^{-1}$ was applied on the respective planting date at each location. POST herbicide treatments included no glyphosate or glyphosate applied POST at the V4, V8, V4 fb V8, and V4 fb V10. A PDS treatment of glyphosate at the V10 stage was also made after a V4 POST. Glyphosate was applied at 1.12 kg ha$^{-1}$ with a $CO_2$-pressurized sprayer as described previously. The PDS treatment contacted the leaves on the lower half of the plant, simulating a late-season application with drop nozzles. Plots were kept weed free with hand weeding as needed.

At each location, pollen viability was determined using methodology and Alexander stain as described earlier. Reciprocal crosses with all possible combinations of male and female treatments were made to determine yield potential. Ear shoots were covered with bags to prevent pollination from an unintended source. Tassels were covered using tassel bags the day before crosses were made to collect pollen. All pollen collected was used for each cross. To obtain even pollination, silks were cut at the tip of the husks without cutting the cob the day before crossing. Ears were harvested after a minimum of 45 d after fertilization. Kernels were removed, counted, and weighed as previously described.

## Electron Microscopy Procedures

Pollen samples were prepared for examination using scanning electron microscopy (SEM) (Bozzola and Russell 1999a) and transmission electron microscopy (TEM) (Bozzola and Russell 1999b). Pollen samples were collected in one replication of each phytotron study and from the second replication at each of the field locations. Samples for SEM were fixed in 3% glutaraldehyde[10] with 0.05 M potassium phosphate buffer (pH 7.3). After complete dehydration using a series of graded (30, 50, 70, 95, and 100%) ethanol solutions, samples were critical point dried.[11] Pollen from all treatments was mounted on microscope stubs[12] using sticky tabs[13] surrounded with silver paint.[14] Samples were then sputter coated[15] with 25 to 30 nm of gold palladium. All samples were viewed between ×1,000 and ×1,300 using a scanning electron microscope.[16]

For TEM examination, a subsample of all pollen samples taken from the field was removed from the 3% glutaraldehyde with 0.05 M potassium phosphate buffer (pH 7.3) and postfixed using 2% osmium tetraoxide[17] with 0.05 M potassium phosphate buffer (pH 7.3). Samples were dehydrated using a series of graded (30, 50, 70, 95, and 100%)

Table 2. The effect of glyphosate on pollen viability, averaged over sampling times, and pollen and anther production on DK 64-10RR in phytotron studies.[a]

| Glyphosate treatments | Pollen viability | Fresh weight of pollen and anthers | | | | |
|---|---|---|---|---|---|---|
| | | Initial anthesis | 2 DAIA[b] | 4 DAIA | 11 DAIA | Total |
| | % | g plant$^{-1}$ | | | | |
| Nontreated | 99 a | 0.344 a | 1.141 a | 1.032 a | 0.418 a | 2.774 a |
| V4 | 91 b | 0.189 b | 0.893 b | 0.970 a | 0.471 a | 2.549 a |
| V4 + V8 | 63 c | 0.126 b | 0.649 c | 0.496 b | 0.294 b | 1.466 b |
| V4 + V10 | 63 c | 0.150 b | 0.727 c | 0.630 b | 0.311 b | 1.819 b |

[a] Means within a column followed by the same letter are not different according to Fisher's Protected LSD test at P = 0.05.
[b] Abbreviation: DAIA, days after initial anthesis.

Table 3. Effect of glyphosate on kernel set and weight of DK 662RR in phytotron trials.[a]

| Male | Female | N[b] | Kernel Weight | Kernel Number |
|---|---|---|---|---|
| | | | g | # |
| Nontreated | Nontreated | 4 | 146.5 | 399 |
| V4 + V8 POST[c] | V4 + V8 POST | 4 | 125.2 | 362 |
| V4 + V10 POST | V4 + V10 POST | 5 | 92.6 | 269 |
| V4 + V10 PDS[c] | V4 + V10 PDS | 5 | 85.3 | 218 |

[a] No comparisons were significantly different where P = 0.05.
[b] The number of samples per treatment.
[c] Abbreviations: PDS, postemergence-directed; POST, postemergence.

ethanol washes. After dehydration, pollen samples were embedded in capsules[18] using resin[19] and allowed to cure at 70 C. Thick (1 to 2 μm) and thin (80 nm) sections of each sample were made using an ultramicrotome.[20] Thick sections were placed on slides for viewing in a compound light microscope. After thin sections were placed on either 150 or 200 mesh grids,[21] they were stained using 4% uranyl acetate[22] for 1 h and with lead citrate[23] for 4 min. Mesh screens were then placed in the transmission electron microscope[24] for viewing. All TEM images were taken at ×5,000 magnification.

## Quantification of CP4-EPSPS in DKC 64-10RR

Corn was grown in the North Carolina State University Phytotron greenhouse as described previously. Roots, brace roots, stems, leaf subtending the dominant ear, silks, husks, ovaries, the flag leaf, anthers, and pollen were collected from three plants at anthesis. Sampled plants were considered replicates. The study was repeated in time. Because pollen has autofluorescence characteristics, pollen from nontransgenic corn was used as positive control. CP4-EPSPS was extracted from 100 mg freshly ground tissue, diluted 5,000 fold, and analyzed by enzyme-linked immunosorbent assay (ELISA)[25] designed for quantification of CP4-EPSPS and included directions for quantification of CP4-EPSPS in food products (Anonymous 1999b; Pline et al. 2002b).

## Statistical Analysis

Data variance retrieved from phytotron, field, and ELISA studies was inspected visually by plotting residuals to confirm homogeneity of variance before statistical analysis. Both nontransformed and arcsine-transformed data were examined, and transformation did not improve homogeneity.

*Phytotron Trials*

Analysis of variance (ANOVA) was performed on nontransformed data using SAS.[26] Anther and pollen production and pollen viability for both varieties were measured four times during pollen shed. Linear and quadratic effects of pollen viability and pollen and anther production with time from phytotron trials were tested by partitioning sums of squares (Draper and Smith 1981). Because no significant linear and quadratic effects were found with pollen viability data, data were pooled over sampling times and experimental replications and separated using Fisher's Protected LSD using $\alpha = 0.05$. Anther and pollen production data were pooled over experimental replication. For grain yield, ANOVA revealed no significant differences for any experimental replication. Grain yield was separated using Fisher's Protected LSD using $\alpha = 0.05$.

*Field Trials*

Pollen viability was averaged over locations because of lack of location and treatment interaction. Because only DKC 64-10RR was planted at the Clayton location, means were separated using the least square means function in SAS to account for unbalanced data. For grain yield, ANOVA revealed a significant location interaction. Therefore, grain yield is presented by hybrid, location, and treatment combination. Means for grain yield were separated using least significant means function in SAS. ELISA data were separated using Fisher's Protected LSD using $\alpha = 0.05$.

# Results and Discussion

## Phytotron Experiments

Any glyphosate treatment applied after the V6 (registered and nonregistered), presumably after tassel initiation (Kies-

Table 4. Effect of glyphosate on kernel set and weight of DKC 64-10RR in phytotron trials.[a]

| Male | Female | N[b] | Kernel Weight | Kernel Number |
|---|---|---|---|---|
| | | | g | |
| Nontreated | Nontreated | 5 | 171.5 | 344 |
| V4 POST[c] | V4 POST | 5 | 166.5 | 334 |
| V4 + V8 POST | V4 + V8 POST | 5 | 90.5 | 177 |
| V4 + V10 POST | V4 + V10 POST | 5 | 118.4 | 225 |

[a] No comparisons were significantly different where P = 0.05.
[b] The number of samples per treatment.
[c] Abbreviation: POST, postemergence.

Table 5. The effect of glyphosate on pollen viability of DK 687RR and DKC 64-10RR in field trials averaged over locations. Contrast statements were used to compare pollen viability between varieties averaged over locations.[a]

| Glyphosate treatments | Pollen viability | |
|---|---|---|
| | DK 687RR | DKC 64-10RR |
| | % | |
| Nontreated | 99 a | 98 a |
| V4 POST[b] | 99 a | 97 a |
| V8 POST[c] | 76 b | 68 b |
| V4 + V8 POST[c] | 74 bc | 70 b |
| V4 + V10 POST[c] | 69 c | 65 b |
| V4 + V10 PDS[b] | 78 b | 72 b |

[a] Means within a column followed by the same letter are not different according to Fisher's Protected LSD test at P = 0.05.
[b] Abbreviations: PDS, postemergence-directed; POST, postemergence.
[c] Mean pollen viability of hybrids are significantly different at P = 0.05 for V8 POST, V4 + V8 POST, and V4 + V10 POST.

selbach 1992), caused reductions in pollen viability, regardless of variety. Pollen viability in DK 662RR (GA21 event) was reduced by 39 and 41.9 percentage points with nonregistered (V10) glyphosate POST treatments at the V10 and V6 fb V10, respectively, compared with the nontreated check (Table 1). No pollen viability reductions in DK 662RR were seen with the registered V6 glyphosate treatment. Pollen viability in DKC 64-10RR (NK 603 event) was reduced by 8, 36, and 36 percentage points with glyphosate applied POST at V4, V4 fb V8, and V4 fb V10 compared with the nontreated check, respectively (Table 2).

Pollen and anther production were also negatively influenced by glyphosate treatments at various timings. Total anther and pollen production for DK 662RR was reduced 21.2, 50.8, and 52.5% by glyphosate POST treatments at V6, V10, and V6 fb V10, respectively (Table 1). Whereas DKC 64-10RR showed total anther and pollen production reductions of 47.2 and 34.4% with glyphosate applied POST at V4 fb V8 and V4 fb V10 compared with the nontreated, respectively (Table 2). Anther and pollen production of DKC 64-10RR with glyphosate applied at V4 POST fb V8 POST and V4 POST fb V10 POST was also lower than the nontreated check and V4 POST treatments at all sampling times (Table 2). Glyphosate-induced pollen viability reductions in corn are not transitory as in cotton, perhaps because of the short duration of pollen shed (approximately 7 d) and fertilization of corn (approximately 3 d) (Kiesselbach 1992; Pline et al. 2003a). The reductions in anther and pollen production may be because of delayed reproductive maturity caused by glyphosate. Pline et al. (2003a, 2003b) found that glyphosate treatments to GR cotton delayed flowering by 3 to 4 d. In GR cotton, glyphosate-treated plants at anthesis showed either no pollen or malformed pollen compared with the pollen-covered anthers of nontreated cotton plants (Pline et al. 2002b; Yasuor et al. 2000). Furthermore, Pline et al. (2002b) found 42.2 to 42.5% less loose pollen per stigma from glyphosate-treated plants than nontreated plants. In field-grown GR cotton, pollen from cotton treated with glyphosate at four-leaf POST and four-leaf POST fb eight-leaf PDS was 70 and 38% viable, respectively compared with 90 to 92% viability in the nontreated check (Pline et al. 2003a). However, the reduction in cotton pollen viability in response to glyphosate treatments was transitory (Pline et al. 2003a). Cotton has the reproductive ability to produce multiple flowers per plant. Contrary to cotton, corn is monoecious which limits the ability of corn to compensate reproductively. In addition to its reproductive characteristics, glyphosate translocation and accumulation in GR corn may be altered because of difference in the numbers and duration of reproductive sinks compared with GR cotton.

Regardless of treatment to either hybrid, kernel set and weight were not reduced by any glyphosate treatment (data not shown). Further analysis showed no difference in hand self-pollinated crosses (nontreated male × nontreated female, etc.) (Tables 3 and 4). Even though our data is based on single ear yields, it corresponds with previous research showing no grain yield reductions in response to glyphosate treatments (Ferrell and Witt 2002; Johnson et al. 2000; Nolte and Young 2000). However, Young (2002) did report lower grain yields in 1999 with a GR system, but attributed these lower yields to environmental conditions and a late planting date. Johnson et al. (2000) showed equivalent grain yields comparing hand-weeded checks and plots receiving sequential glyphosate applications. Furthermore, Ferrell and Witt (2002) found no differences in grain yield with any herbicide (with and without glyphosate) system in any year or location.

## Field Experiments

Regardless of hybrid, any glyphosate treatment applied after the V4 stage reduced pollen viability (Table 5). Pollen

Table 6. Effect of glyphosate on kernel set and weight of DK 687RR in 2002 field trials.[a]

| Male | Female | Rocky Mount, NC Kernel | | | Lewiston-Woodville, NC Kernel | | |
|---|---|---|---|---|---|---|---|
| | | N[b] | Weight | Number | N[b] | Weight | Number |
| | | | g | | | g | |
| Nontreated | Nontreated | 18 | 136.9 | 409 | 10 | 59.7 | 157 |
| V4 POST[c] | V4 POST | 10 | 123.3 | 368 | 8 | 45.5 | 117 |
| V8 POST | V8 POST | 13 | 139.9 | 410 | 8 | 55.5 | 139 |
| V4 POST + V8 POST | V4 POST + V8 POST | 14 | 132.0 | 407 | 7 | 16.6 | 45 |
| V4 POST + V10 POST | V4 POST + V10 POST | 11 | 111.6 | 356 | 5 | 31.2 | 74 |
| V4 POST + V10 PDS[c] | V4 POST + V10 PDS | 14 | 125.4 | 364 | 7 | 42.7 | 115 |

[a] No comparisons were significantly different where P = 0.05.
[b] The number of samples per treatment.
[c] Abbreviations: PDS, postemergence-directed; POST, postemergence.

TABLE 7. Effect of glyphosate on kernel set and weight of DKC 64-10RR in 2002 field trials.[a]

| Male | Female | Clayton, NC Kernel | | | Rocky Mount, NC Kernel | | | Lewiston-Woodville, NC Kernel | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | N[b] | Weight | Number | N[b] | Weight | Number | N[b] | Weight | Number |
| | | | g | | | g | | | g | |
| Nontreated | Nontreated | 14 | 172.6 | 459 | — | — | — | 7 | 75.2 | 229 |
| V4 POST[c] | V4 POST | 13 | 173.4 | 403 | — | — | — | 8 | 53.4 | 173 |
| V8 POST | V8 POST | 13 | 158.2 | 379 | 3 | 73.3 | 205 | 7 | 23.3 | 69 |
| V4 POST + V8 POST | V4 POST + V8 POST | 13 | 147.5 | 385 | 2 | 122.8 | 380 | 7 | 24.8 | 105 |
| V4 POST + V10 POST | V4 POST + V10 POST | 13 | 162.2 | 374 | 3 | 126.0 | 356 | 7 | 24.9 | 73 |
| V4 POST + V10 PDS[c] | V4 POST + V10 PDS | 14 | 192.6 | 456 | 2 | 144.0 | 373 | 7 | 42.9 | 121 |

[a] No comparisons were significantly different where P = 0.05.
[b] The number of samples per treatment.
[c] Abbreviations: PDS, postemergence-directed; POST, postemergence.

viability of DK 687RR hybrid was lowest in the V4 POST fb V10 POST treatment compared with all other treatments. The reduction in pollen viability in the V4 POST fb V10 POST may be due to the complete spray coverage of the emerged leaves at the V10 stage. When only the lower half of the plants at the V10 stage (V4 POST fb V10 PDS) were treated with glyphosate, pollen viability was higher than the V4 POST fb V10 POST treatment (Table 5). A comparison of the means of pollen viability from each hybrid showed higher viability in DK 687RR hybrid with glyphosate POST at the V8, V4 fb V8, and V4 fb V10 compared with DKC 64-10RR (Table 5).

All crosses were not made at each location. Because a significant location interaction was found, locations were analyzed separately. No significant yield effects were found among any glyphosate treatment combination (data not shown). After further statistical analysis using the hand self-pollinated crosses (nontreated male × nontreated female, etc.), no significant yield effects were found regardless of location or hybrid (Tables 6 and 7). Because no grain yield reductions were evident in field trials or phytotron trials, our data supports previous research showing no grain yield reductions in response to glyphosate treatments (Ferrell and Witt 2002; Johnson et al. 2000; Nolte and Young 2000).

## Electron Microscopy

SEM and TEM techniques were used to examine pollen grains from all treatments in each hybrid. SEM images indicated distinct morphological alterations in corresponding treatments with reduced pollen viability. Comparative SEM images of DK 662RR pollen from a nontreated check and from a plant treated with glyphosate POST at the V10 stage (nonregistered treatment) are shown in Figure 2. In Figures 2a and 2b, pollen grains from the nontreated check and V10, respectively, have a spherical shape with the characteristic single pollen aperture (Chang and Neuffer 1989; Kiesselbach 1992; Mandaron et al. 1990). However, Figure 2b shows collapsed regions of a pollen grain from a glyphosate-treated corn plant. Similarly, Pline et al. (2002b) found collapsed pollen grains from GR cotton plants treated with registered glyphosate applications.

TEM micrographs showed pollen anatomy alterations corresponding with glyphosate treatments that reduced pollen viability. In Figure 3, pollen from DK 687RR and DKC 64-10RR hybrids treated with glyphosate V4 POST and V4 POST fb V10 POST are shown. Regardless of hybrid, pollen from plants receiving the V4 POST fb V10 POST has large vacuoles and lower starch accumulation than those receiving the V4 POST treatment. No significant differences were seen between pollen from the nontreated and V4 POST-treated plants (data not shown). Researchers have described the various stages of corn microsporogenesis including the uninucleate stages, first pollen mitosis, second pollen mitosis, and mature pollen (Chang and Neuffer 1989) and the respective size of pollen after the tetrad stage (Mandaron et al. 1990). Because the germ pore, exine, and intine have already formed (Figures 2 and 3), all pollen grains examined were after the uninucleate stage (Chang and Neuffer 1989). The level of starch accumulation and size of the vacuole is indicative of the stages of microsporogenesis after the uninucleate stage (Chang and Neuffer 1989; Mandaron et al. 1990). Because the locations of the nuclei were unidentifiable in our TEM micrographs, we were unable to distinguish between the first and second pollen mitosis. Yet Mandaron et al. (1990) have shown that pollen is 60, 90, and 90 to 110 μm in diameter for the vacuolated microspore, late starch accumulation, and pollen at dehiscence, respectively. Pollen sizes in SEM micrographs of characteristic and abnormal pollen grains (Figure 2) are not visually different. Therefore, microsporogenesis was inhibited after the uninucleate stage, but most probably during the later stages of starch accumulation because of the size of the vacuole and level of starch accumulation (S2 in Mandaron et al. [1990] and second pollen mitosis in Chang and Neuffer [1989]). Pollen from GR cotton has shown similar developmental alterations from glyphosate treatments. Pline et al. (2002b) has shown alterations at the conversions between the early vacuolated microspore and vacuolated microspore, the vacuolated microspore and vacuolated microgamete, and vacuolated microgamete and mature binucleate pollen in GR cotton treated with glyphosate.

## Quantification of CP4-EPSPS in DKC 64-10RR

Analysis of CP4-EPSPS in various corn tissues showed variable levels of CP4-EPSPS with the highest expression in pollen with progressively less in brace roots, ear leaf, anthers, roots, ovaries, silks, stem, flag leaf, and husk (Figure 4). Various vegetative tissues in corn have been shown to accumulate silica, reducing the amount of leaf tissue available to contain CP4-EPSPS compared with pollen in the extrac-

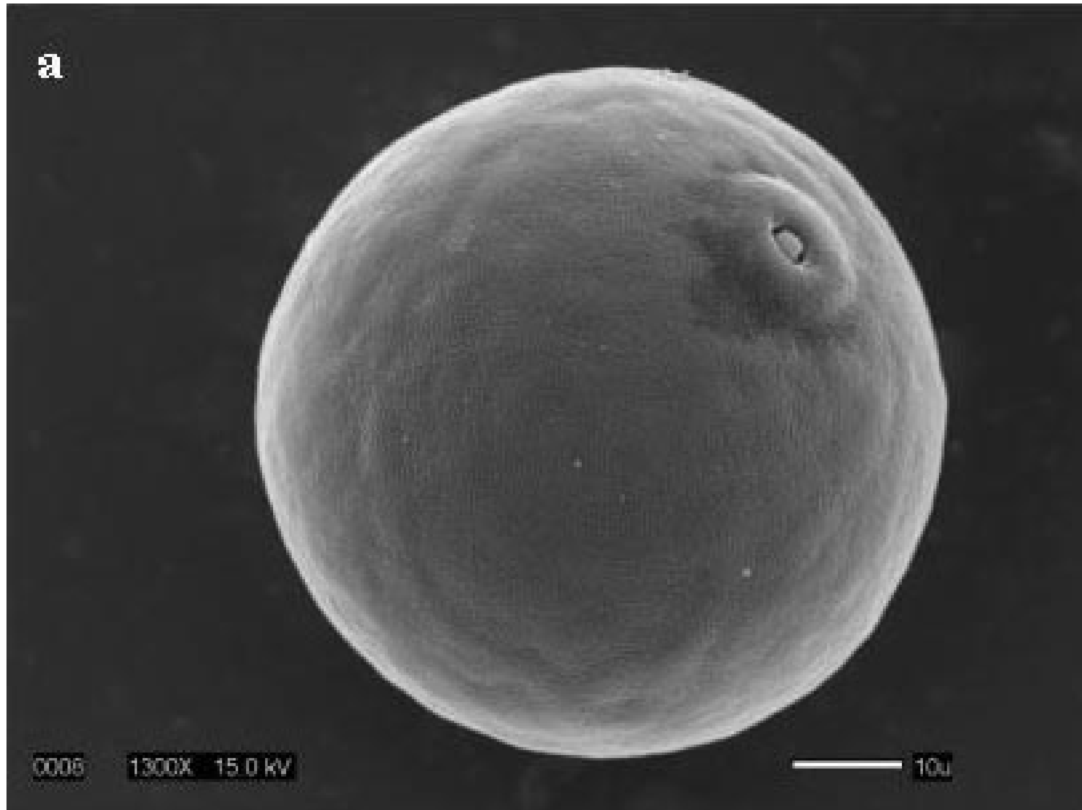

Figure 2. Scanning electron micrographs of pollen from DK 662RR treated with glyphosate postemergence at V10 (b) with the nontreated check (a) included for comparison. The pollen grain on the left (b) would be considered viable whereas the pollen grain on the right would be considered not viable.

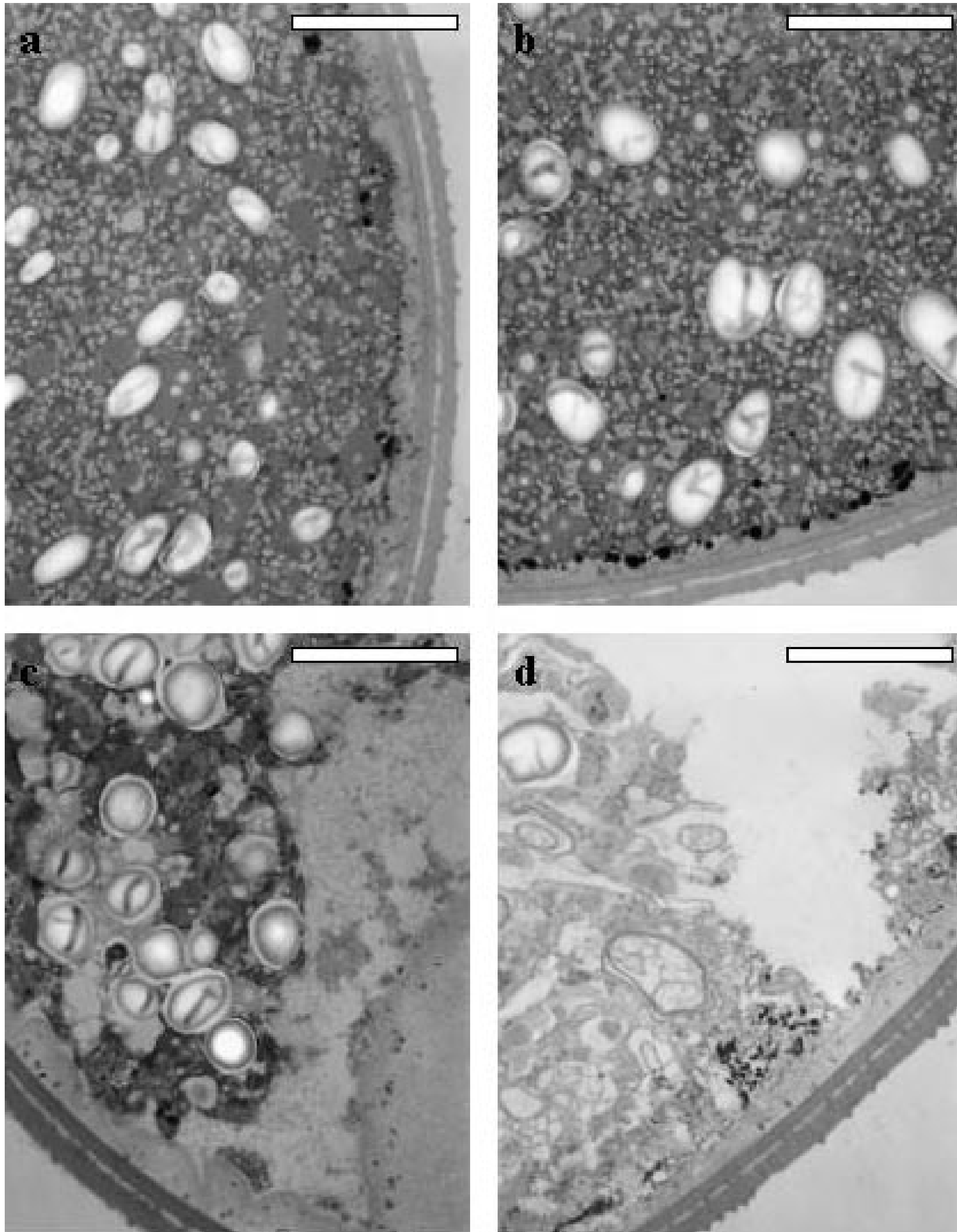


FIGURE 3. Transmission electron micrographs of pollen from DK 687RR and DKC 64-10RR varieties treated at the V4 postemergence (POST) (a, b) and V4 POST followed by V10 POST (c, d), respectively. All images were taken at ×5,000 magnification. All magnification bars are equal to 5 μm.

tion process (Lanning et al. 1980). Lanning et al. (1980) also found measurable silica amounts in the stem pith, stem epidermis, tassel, and roots. Therefore, it is difficult to directly compare CP4-EPSPS levels in different tissues because the composition of tissues is so variable.

Although in this study, CP4-EPSPS levels in pollen are greater than any other tissue, pollen development was negatively affected by glyphosate treatment. As in GR cotton, corn pollen development appears to be slowed or arrested, possibly because of the dose and timing of glyphosate during pollen development.

Pollen viability reductions were evident with glyphosate treatments after the V6 stage in phytotron experiments and the V4 stage in field experiments. Because tassel initiation can begin as early as 2 wk after emergence, it is probable that any glyphosate treatment made to GR corn before tassel initiation will not have an effect on pollen viability or anther and pollen production (Kiesselbach 1992). Because many

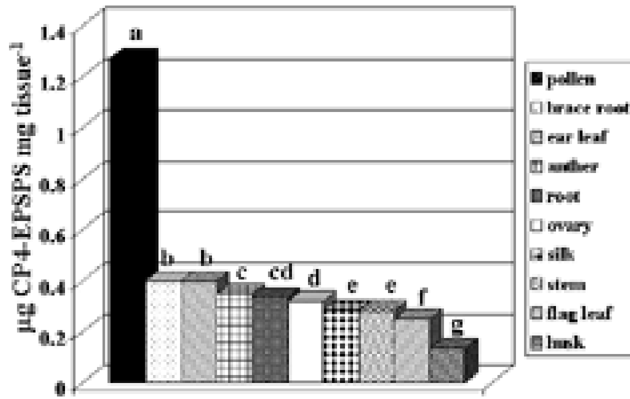


FIGURE 4. Levels of CP4-EPSPS in NK603 hybrid DKC 64-10RR in various vegetative and reproductive tissues at anthesis.

researchers have shown glyphosate accumulates in meristematic regions and reproductive organs (Gougler and Geiger 1981; Hetherington et al. 1999; Pline et al. 2001; Sandberg et al. 1980; Viator et al. 2003), the tassel and ear may accumulate glyphosate. If accumulation does occur, then this accumulation may explain the resulting pollen damage and reduction in pollen and anther production.

Kiesselbach (1992) has shown that corn can produce up to 25,000,000 pollen grains per plant. Assuming each ear had 1,000 silks, Kiesselbach (1992) estimated that 25,000 pollen grains would be shed from a single tassel for each silk on one ear. Kiesselbach (1992) calculates further that 42,500 pollen grains were shed per square inch and 170 grains per silk. Bassetti and Westgate (1994) and Otegui et al. (1995) have shown that pollen production would not limit kernel set. Uribelarrea et al. (2002) designed experiments to evaluate pollen production and pollen thresholds for kernel set under various planting densities and a 50% detasseling treatment. Using an exponential model, two and three pollen grains per exposed silk could account for 95 and 99% percent kernel set, respectively. Additional data from Uribelarrea et al. (2002) found that pollen production could be reduced by half in the 50% detasseling treatments with no expected changes in kernel set. Therefore, on the basis of our results, registered glyphosate treatments to GR corn should not result in reduced kernel set nor reduced grain yields.

## Sources of Materials

[1] Dekalb, 800 North Lindburg Boulevard, Monsanto Co., St. Louis, MO 63167.

[2] Roundup UltraMax, 800 North Lindburg Boulevard, Monsanto Co., St. Louis, MO 63167.

[3] Tassel bag #217, Lawson Pollinating Bags, P.O. Box 8577, Northfield, IL 60093.

[4] Ear shoot bag #402, Lawson Pollinating Bags, P.O. Box 8577, Northfield, IL 60093.

[5] Agriculex SCS-2 corn sheller, Agriculex Inc., 1-59 Avenue, Guelph, ON N1E 6B4, Canada.

[6] Moisture chek SW16060, John Deere Company, 2001 Deere Drive, Conyers, GA 30208.

[7] Seed counter model 850-2, International Marketing & Design, 13802 Lookout Road, San Antonio, TX 78233.

[8] Dual Magnum, Syngenta, P.O. Box 8353, Wilmington, DE 19803-8353.

[9] Aatrex 4L, Syngenta, P.O. Box 8353, Wilmington, DE 19803-8353.

[10] Glutaraldehyde #20105, Ladd Research, 83 Holly Court, Williston, VT 05495.

[11] Samdri 795, Tousimis Research Corp., Rockville, MD 20852.

[12] Microscope stubs #60065, Ladd Research, 83 Holly Court, Williston, VT 05495.

[13] Sticky tabs #14445, Ladd Research, 83 Holly Court, Williston, VT 05495.

[14] Sliver paint #60810, Ladd Research, 83 Holly Court, Williston, VT 05495.

[15] Hummer V, Anatech LTD, 6621-F Electronic Road, Springfield, VA 22153.

[16] Phillips 505T, FEI Company, 7451 NW Evergreen Parkway, Hillsboro, OR 97124.

[17] Osmium tetraoxide #55091, Ladd Research, 83 Holly Court, Williston, VT 05495.

[18] BEEM capsules #21600, Ladd Research, 83 Holly Court, Williston, VT 05495.

[19] Spurr's resin #21230, Ladd Research, 83 Holly Court, Williston, VT 05495.

[20] LKB NOVA, Leica, 2345 Waukegan Road, Bannockburn, IL 60015.

[21] Mesh grids #150CP #200CP, Ladd Research, 83 Holly Court, Williston, VT 05495.

[22] Uranyl acetate #23620, Ladd research, 83 Holly Court, Williston, VT 05495.

[23] Lead citrate #23605, Ladd Research, 83 Holly Court, Williston, VT 05495.

[24] JEOL 100S, JEOL USA, 11 Dearborn Road, Peabody, MA 01960.

[25] Soya Trait Check kit #7100000, Strategic Diagnostics, Inc., 111 Pencader Drive, Newark, DE 19702.

[26] SAS ver. 8.0, SAS Institute, Inc., 100 SAS Campus Drive, Cary, NC 27513-2414.

[27] Nikon Biophot Biological Microscope, Southern Micro Instruments Inc., 1700 Enterprise Way, STE 112, Marietta, GA 30067.

[28] Spot camera model 1.4.0, Diagnostic Instruments, 6540 Burroughs Street, Sterling Heights, MI 48310.

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