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Compositional Safety of DAS-68416-4 (AAD-12) Herbicide-Tolerant Soybean

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Abstract

The composition of transgenic DAS-68416-4 soybean seed and forage was compared with that of non-transgenic soybean. DAS-68416-4 soybean expresses the aryloxyalkanoate dioxygenase-12 enzyme from the soil bacterium, *Delftia acidovorans*, which detoxifies 2,4-dichlorophenoxyacetic acid (2,4-D), conferring tolerance to this herbicide. DAS-68416-4 also expresses the phosphinothricin acetyltransferase enzyme from *Streptomyces viridochromogenes* which confers tolerance to glufosinate-ammonium herbicides. As expected for an input trait, results indicate that DAS-68416 soybean is compositionally equivalent to non-transgenic soybean.

Introduction

Evaluation of the compositional equivalence between a transgenic crop and its conventional counterpart is a regulatory requirement for commercializing a transgenic event [1]. This requirement is designed to investigate unintentional effects due to the insertion of the transgene(s) (insertional mutagenesis) or interactions between gene products and the endogenous plant genome or metabolic pathways. For input traits such as herbicide tolerance, unintended compositional effects are expected to be less impactful compared with non-transgenic breeding methods [2], and this has been substantiated by dozens of empirical studies [3]. Considering the safe history of traditional breeding, the likelihood that safety concerns might arise due to unintended compositional changes resulting from transgenesis is negligible. Even so, compositional studies continue to be required, and regulatory requirements for composition studies are becoming increasingly complex in some regions [4].

DAS-68416-4 soybean expresses the aryloxyalkanoate dioxygenase-12 (AAD-12) enzyme from the soil bacterium, *Delftia acidovorans*, which inactivates 2,4-dichlorophenoxyacetic acid (2,4-D) conferring tolerance to this herbicide [5]. DAS-68416-4 also expresses the phosphinothricin acetyltransferase enzyme from *Streptomyces viridochromogenes* (PAT) which confers tolerance to glufosinate-ammonium herbicides [6]. Here, the composition of DAS-68416-4 soybean forage and grain are compared with non-transgenic soybean, and the safety implications of results are discussed.

Methods and Materials

Field phase

Ten field experiments were conducted in 2009 to produce soybean forage and seed for compositional analysis. Field sites were located in Lonoke, AR; Richland, IA; Carlyle and Wyoming, IL; Rockville, IN; La Plata and Dudley, MO; York, NE; and two sites in Ontario Canada. Entries in the experiments included DAS-68416-4 soybean in a Maverick-variety genetic background, a non-transgenic near-isogenic Maverick line, and six non-transgenic commercial lines (Pioneer 93M62, LG Seeds C3884N, Arise 9E394, Phillips 363, HiSOY 38C60, and Hoffman H387). Experiments at each location contained four entries of DAS-68416-4 soybean, with each entry receiving a different herbicide regime. Herbicide regimes included unsprayed (not treated with glufosinate or 2,4-D), glufosinate, 2,4-D, or glufosinate + 2,4-D. Glufosinate was applied as two post-emergence applications at the V4 and R1 growth stages at rates of 0.37 and 0.45 kg active ingredient/ha, respectively, and 2,4-D was applied as one pre-emergence application and two post-emergence applications at the V4 and R2 growth stages. The 2,4-D application rate was 1.12 kg acid equivalent/ha at each application timing. Herbicides were applied in a carrier volume of 187 L/ha, and the spray solution for 2,4-D treatments contained 0.25% v/v non-ionic surfactant. The formulations of glufosinate, 2,4-D, and non-ionic surfactant used in this research were Liberty® (200 g ai/L, Bayer CropScience), Weedar® 64 (454 g ae/L, Nufarm, Inc.), and X-77® (Loveland Products, Inc.), respectively. All entries, including the non-transgenic control, received maintenance applications of insecticides, fungicides, and conventional soybean herbicides (excluding 2,4-D and glufosinate) as necessary to protect crop health. The experimental design for the DAS-68416-4 entries and the non-transgenic near-isogenic line was a randomized complete-block. Three of the six reference lines were included at each site, and reference lines were randomized across sites in a balanced incomplete-block design. Each location contained four replicate blocks. Plots were two rows wide (76 cm apart) by 7.6 m long with an in-row spacing of approximately 7 cm. Two non-transgenic border rows surrounded each plot, and a minimum of four border rows surrounded the entire trial at each site. Samples of soybean forage (300 g) and grain (500g) were collected at the R3 and R8 growth stages, respectively, and samples were shipped frozen to the analytical laboratory (Covance Laboratories, Inc., Madison, WI) for compositional analysis.

Compositional analyses

Analyses were conducted on the soy forage and seed for key nutritional components (Covance Laboratories, Madison, WI).

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Samples were received frozen and remained frozen for the duration of the analytical phase until being removed for preparation or analysis. Samples were cryogenically ground to a homogeneous state using a blender and liquid nitrogen prior to assay. Forage analyses consisted of proximates (moisture, crude protein, carbohydrates, crude fat, and ash), acid detergent fiber, neutral detergent fiber, and minerals calcium and phosphorus. Seeds were analyzed for the same components with the addition of the following: total dietary fiber, vitamins [beta carotene, thiamine hydrochloride, riboflavin, pyridoxine hydrochloride, ascorbic acid, tocopherols (α, β, γ, δ), folic acid, pantothenic acid, and niacin]; minerals (copper, iron, magnesium, manganese, potassium, sodium, zinc); anti-nutrients and bio-active components (phytic acid, raffinose, stachyose, isoflavones, trypsin inhibitor, and lectin); fatty acid profile; and amino acid profile. In addition, carbohydrates were calculated from the proximate by difference. A brief summary of the methods employed are given below.

Proximate: Moisture content was determined gravimetrically by drying the samples in a vacuum oven at approximately 100°C [7,8]. Protein analysis was performed by Kjeldahl analysis. The protein and other organic nitrogen in the sample were converted to ammonia by digesting the sample with sulfuric acid containing a catalyst mixture. The acid digest was made alkaline. The ammonia was distilled and then titrated with a previously standardized acid. The percent nitrogen was calculated and converted to equivalent protein using the factor 6.25 [9-11]. For ash determination, the samples were placed in an electric furnace at 550°C and ignited. The residual ash was quantified gravimetrically [12]. The crude fat content of the maize seed samples was determined gravimetrically using a soxhlet extraction with pentane as a solvent [13,14]. Crude fat in the maize forage samples was determined gravimetrically using an acid hydrolysis procedure [15,16].

Fibers: Acid detergent (ADF) and neutral detergent fibers (NDF) were determined using an automated Ankom2000 fiber analyzer. For both methods, fats and pigments were removed with an acetone wash prior to analysis. For acid detergent fiber, the samples were immersed in an acidic detergent solution under controlled conditions. The fibrous residue that was primarily cellulose and lignin and insoluble protein complexes remained in the Ankom filter bag, and was determined gravimetrically [17,18]. For the NDF analysis, a neutral detergent solution and heat stable amylase were used to dissolve easily digested proteins, lipids, sugars, starches and pectins leaving a fibrous residue that is primarily cell wall components and indigestible nitrogenous matter [17,19,20]. Total dietary fiber was conducted by analysis of duplicate samples that were gelatinized with α-amylase and digested with enzymes to break down starch and protein. Ethanol was added to each sample to precipitate the soluble fiber. The samples were filtered, and the residue was rinsed with ethanol and acetone to remove starch and protein degradation products and moisture. After drying, the residue weight was determined. Protein content was determined for one of the duplicates; ash content was determined for the other. The total dietary fiber in the samples was calculated by subtracting the weight of the protein and ash from the mean residue weight [21].

Minerals: Levels of calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, and zinc were determined by inductively coupled plasma (ICP) emission spectroscopy. Samples were precharred, ashed and solubilized in a hydrochloric acid solution. Each element was determined at an appropriate wavelength against calibration solutions [22,23].

Beta carotene: The samples were saponified and extracted with hexane. The samples were then injected on a reverse phase high-performance liquid chromatography system with ultraviolet detection using an external reference standard curve [24,25].

Thiamine hydrochloride: The samples were autoclaved under weak acidic conditions to extract thiamine. The resulting solution was incubated with a buffered enzyme solution to release any bound thiamine. The solution was purified on a cation-exchange column. An aliquot was reacted with potassium ferricyanide to convert thiamine to thiochrome. The thiochrome was extracted into isobutyl alcohol, measured on a fluorometer, and quantified by comparison to a known standard [26-28].

Riboflavin: The samples were hydrolyzed with dilute hydrochloric acid and the pH was adjusted to remove interferences. The amount of riboflavin was determined by comparing the growth response of the sample, using the bacteria *Lactobacillus rhamnosus*, with the growth response of multipoint riboflavin standards. The growth response was measured turbidimetrically [29-31].

Pyridoxine hydrochloride: The samples were hydrolyzed with dilute sulfuric acid in the autoclave and the pH was adjusted to remove interferences. The amount of pyridoxine was determined by comparing the growth response of the sample, using the yeast *Saccharomyces cerevisiae,* with the growth response of a pyridoxine standard. The response was measured turbidimetrically [32,33].

Ascorbic acid (Vitamin C): The ascorbic acid in the samples were extracted, oxidized, and mixed with o-phenylenediamine to produce a fluorophor having an activation maximum at approximately 350 nm and a fluorescence maximum at 430 nm. Fluorescence was proportional to concentration. Development of the fluorescence compound with the vitamin was prevented by forming a boric acid-dehydroascorbic acid complex prior to addition of the o-phenylenediamine solution [34].

Tocopherols (Vitamin E): The samples were saponified to break down fat and release vitamin E. The saponified mixture was extracted with ethyl ether and then quantified by normal phase high-performance liquid chromatography using a silica column [35-37].

Folic acid: The samples were hydrolyzed in a potassium phosphate buffer with the addition of ascorbic acid to protect the folic acid during autoclaving. Following hydrolysis by autoclaving, the samples were treated with a chicken-pancreas enzyme and incubated approximately 18 hours to liberate the bound folic acid. The amount of folic acid was determined by comparing the growth response of the sample, using the bacteria *Lactobacillus casei*, with the growth response of a folic acid standard. This response was measured turbidimetrically [30,38,39].

Pantothenic acid: The sample was diluted with water or treated with an enzyme mixture to liberate the pantothenic acid from coenzyme A and the pH was adjusted to remove interferences. The amount of pantothenic acid was determined by comparing the growth response of the sample, using the bacteria *Lactobacillus plantarum*, with the growth response of a calcium pantothenate standard. This growth response was measured turbidimetrically [30,40].

Niacin: The samples were hydrolyzed with sulfuric acid and the pH was adjusted to remove interferences. The amount of niacin was determined by comparing the growth response of the sample, using the

bacteria *Lactobacillus plantarum,* with the growth response of a niacin standard. This response was measured turbidimetrically [30,41].

Phytic acid: The samples were extracted using 0.5M hydrochloric acid with ultrasonication. Purification and concentration were accomplished on a silica-based anion-exchange column. The samples were analyzed on a polymer high-performance liquid chromatography column PRP-1, 5µm (150 x 4.1 mm) with a refractive index detector [42,43].

Raffinose, stachyose: Sugars in the sample were extracted with a 50:50 water/methanol solution. Aliquots were taken, dried under inert gas, and then reconstituted with a hydroxylamine hydrochloride solution in pyridine containing phenyl-β-D-glucopyranoside as the internal standard. The resulting oximes were converted to silyl derivatives with hexamethyldisilazane and trifluoracetic acid and analyzed by gas chromatography using a flame ionization detector [44,45].

Isoflavones: The samples were extracted at approximately 65°C with a 80/20 methanol:water solution and the extracts were saponified with dilute sodium hydroxide solution. The extracts were then acidified, filtered, and diluted. The samples were analyzed on a high-performance liquid chromatography system with ultraviolet spectrophotometric detection and were compared against an external standard curve [46].

Trypsin inhibitor: The samples were ground and defatted with petroleum ether. A sample of matrix was extracted with 0.01N sodium hydroxide. Varying aliquots of the sample suspension were exposed to a known amount of trypsin and benzoy1-DL-arginine~p~nitroanilide hydrochloride in a timed reaction. Trypsin inhibitor activity was determined by photometrically measuring the inhibition of trypsin's reaction with benzoyl-DL-arginine~p~nitroanilide hydrochloride [47,48].

Lectin: The determination of lectin was based on the ability of lectin (a hemagglutinin) to bind to specific sugars present on the surface of red blood cells (RBCs) of different animal species resulting in the agglutination of RBCs. Samples were defatted and extracted with a saline solution. Agglutination of trypsinized rabbit RBCs was measured with a spectrophotometer at a wavelength of 620 nm [49-51].

Fatty acid profile: The lipid was extracted and saponified with 0.5N sodium hydroxide in methanol. The saponification mixture was methylated with 14% boron trifluoride in methanol. The resulting methyl esters were extracted with heptane containing an internal standard. The methyl esters of the fatty acids were analyzed by gas chromatography using external standards for quantification [52-55]

Amino acid profile: The samples were hydrolyzed in 6 N hydrochloric acid for 24 hours at approximately 110°C. Phenol is added to the 6N hydrochloric acid to prevent halogenation of tyrosine. Cystine and cysteine were converted to S-2-carboxyethylthiocysteine by the addition of dithiodipropionic acid. Tryptophan is hydrolyzed from proteins by heating at approximately 110°C in 4.2 N sodium hydroxide for 20 hours. The samples were analyzed by HPLC after pre-injection derivatization. The primary amino acids were derivatized with o-phthalaldehyde (OPA) and the secondary amino acids were derivatized with fluorenylmethyl chloroformate (FMOC) before injection [56-58].

Data interpretation: The methods used to interpret the compositional equivalence of DAS-68416-4 soybean with its conventional counterpart have been described previously [3,59]. Briefly, the main tool for interpreting the data was examination of graphs of site means and literature ranges [60-63]. Statistical analysis employed a mixed model to compare each transgenic entry with the non-transgenic near-isogenic line. Entry was considered a fixed effect, and location, block within location, and location-by-entry were designated as random effects. False Discovery Rate (FDR) procedures were used to adjust p-values for multiplicity [64] and differences were considered significant at $P < 0.05$.

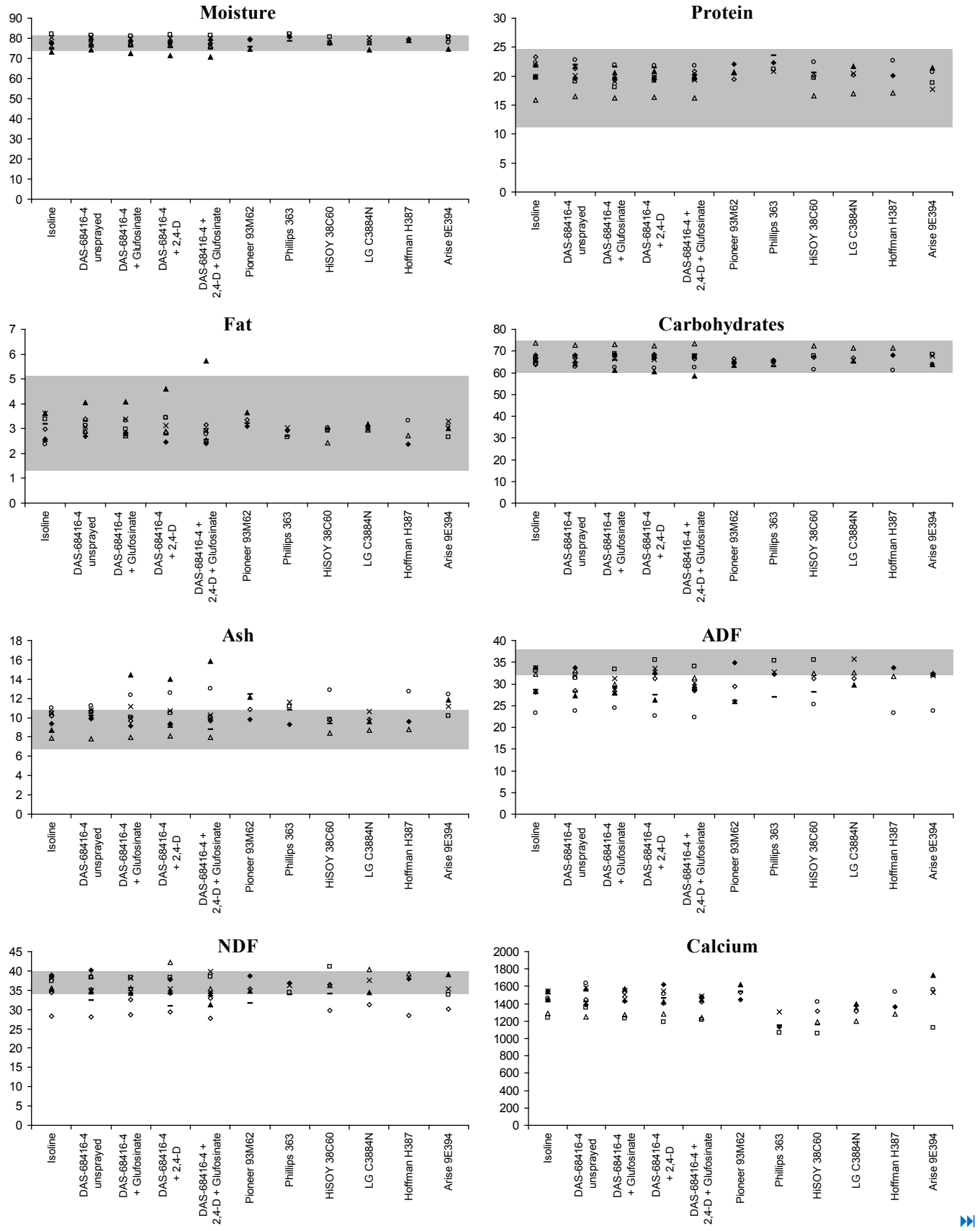
Results and Discussion

Compositional results for DAS-68416-4 soybean from a multi-site, replicated field trial were compared with the composition of non-transgenic soybean. A total of ten locations were planted, but two locations (both in Ontario, Canada) were excluded from the study because they did not mature due to an early frost. A third location (in Lonoke, AR) experienced a severe hail storm when plants were approximately 13 cm tall (V2-V3 growth stage), but data were included in the analysis although heavy foliar damage occurred. This allowed the effects of an atypical, but realistic, environmental event to be evaluated for impact on soybean composition. The remaining seven sites experienced more typical weather conditions.

The available literature ranges for soybean are somewhat limited and all entries in this study had site means for a number of analytes outside of reported ranges (Figures 1-9; values below the limit of quantitation are plotted as zero). This was true for both the transgenic entries and non-transgenic entries. In interpreting the figures, it is important to consider that each commercial non-transgenic entry was included, on average, in only half of the locations; the lower level of replication for these commercial lines is expected to reduce the spread of the data compared with the transgenic and near-isogenic entries. The most extreme values for a number of analytes occurred in samples from the Arkansas site where severe hail damage occurred (e.g. calcium in seed; Figure 3). This highlights the effects that environment can have on crop composition. This trend may have been accentuated for certain analytes when additional herbicide treatments stressed the DAS-68416-4 entries (e.g. ash in forage; Figure 1). Selective herbicides are known to stress non-transgenic crops, so this is not a unique feature of herbicide-tolerant transgenic crops [65]. In general, the levels and spread of the composition data were very similar for the transgenic and non-transgenic entries. However, a formal statistical analysis was conducted comparing each of the transgenic entries to the non-transgenic near-isogenic control.

The composition of forage samples from the four DAS-68416-4 soybean entries (each treated with a different herbicide regime), were statistically indistinguishable from the non-transgenic near-isogenic soybean entry with the exception of lower average protein levels within the DAS-68416-4 entry treated with glufosinate (19.6% dw) compared with the non-transgenic near-isogenic entry (20.6% dw). However, protein levels from the DAS-68416-4 entry were typical of that seen for non-transgenic soybean (Figure 1).

Proximate levels for grain from the DAS-68416-4 entries also did not significantly differ from the non-transgenic near-isogenic control with the exception of average protein for the transgenic entry treated


Moisture
Protein
Fat
Carbohydrates
Ash
ADF
NDF
Calcium
Isoline
DAS-68416-4 unsprayed
DAS-68416-4 + Glufosinate
DAS-68416-4 + 2,4-D
DAS-68416-4 + 2,4-D + Glufosinate
Pioneer 93M62
Phillips 363
HiSOY 38C60
LG C3884N
Hoffman H387
Arise 9E394

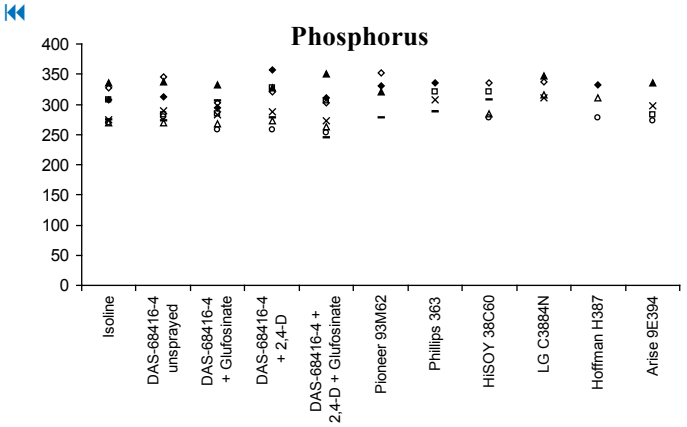
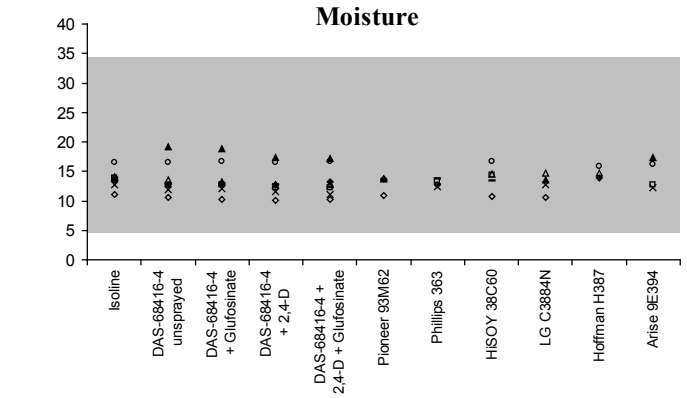
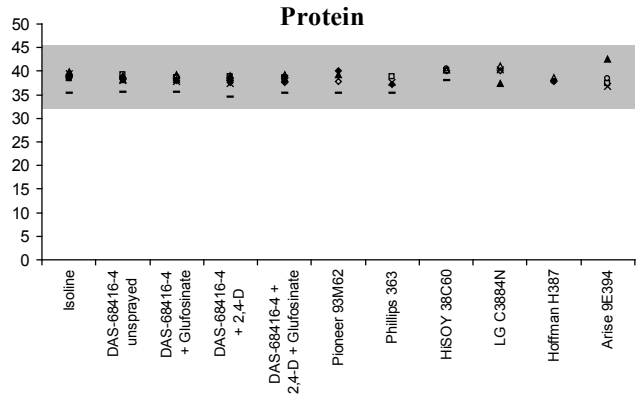
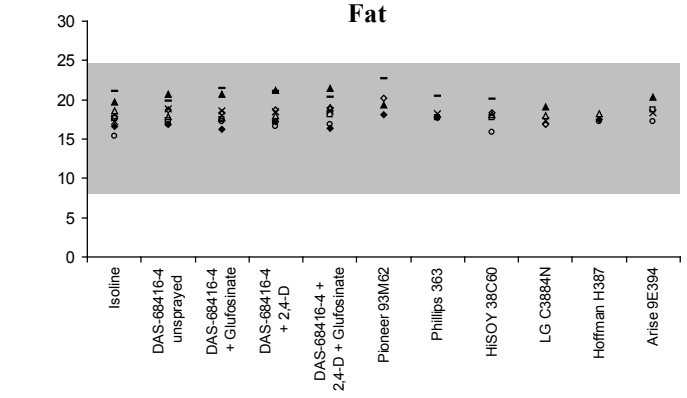
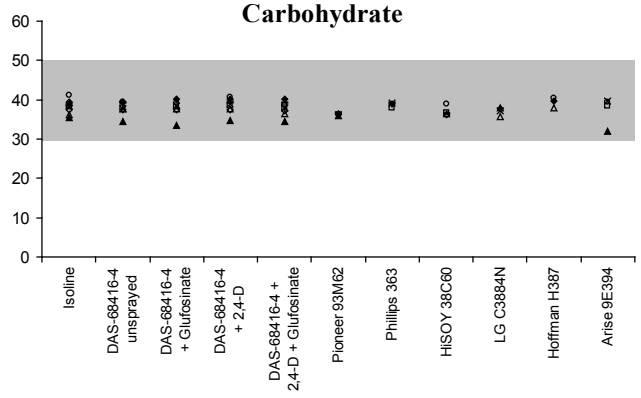


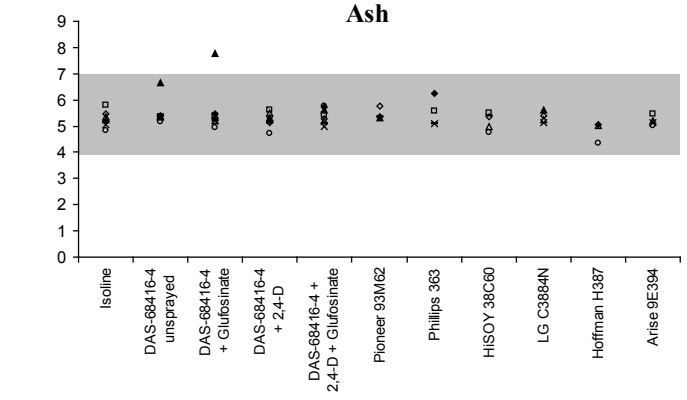
Figure 1: Proximate, fiber, and mineral levels in non-transgenic control soybean forage, unsprayed DAS-68416-4 (AAD-12), 2,4-D-treated DAS-68416-4, glufosinate-treated DAS-68416-4, 2,4-D + glufosinate-treated DAS-68416-4, Pioneer 93M62, Phillips 363, HiSOY 38C60, LG C3884N, Hoffman H387, and Arise 9E394 forage. The shaded area spans the published values for each analyte, and levels are expressed as a percentage of dry weight (Note: moisture is expressed in fresh weight). Symbols in the plots are as follows: ▲, Lonoke (AR); ×, Richland (IA); ◊, Carlyle (IL); □, Wyoming (IL); ○, Rockville (IN); ∆, La Plata (MO); **−**, Dudley (MO); ♦, York (NE).

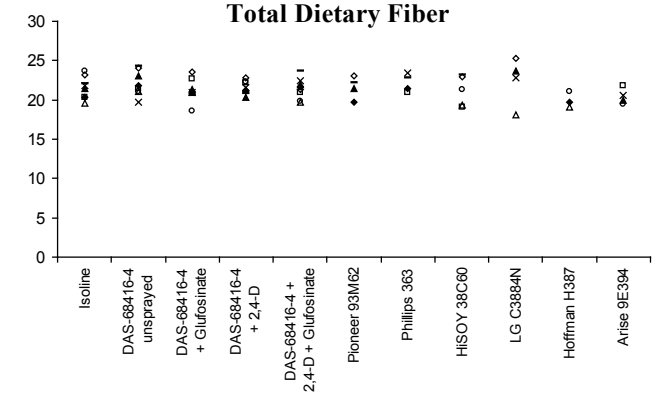












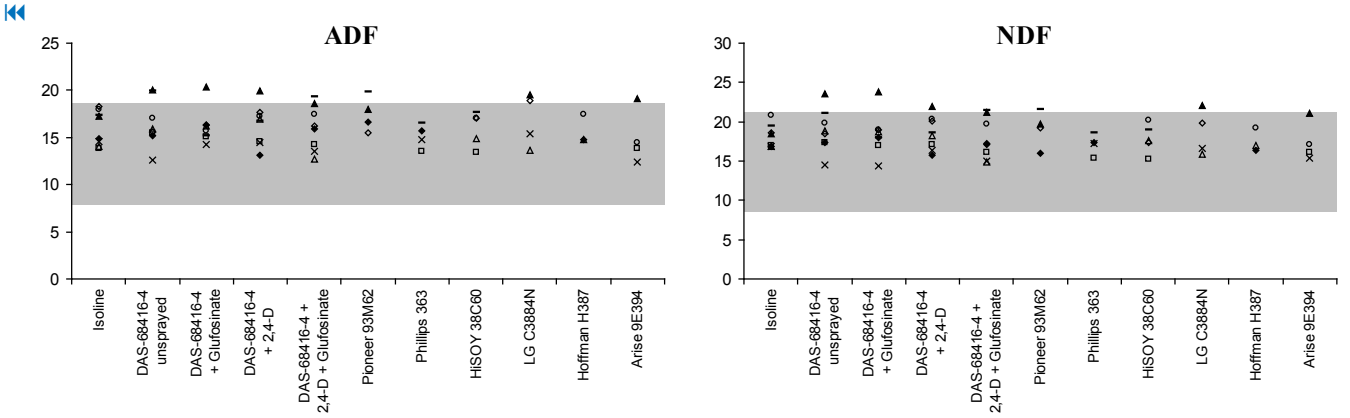
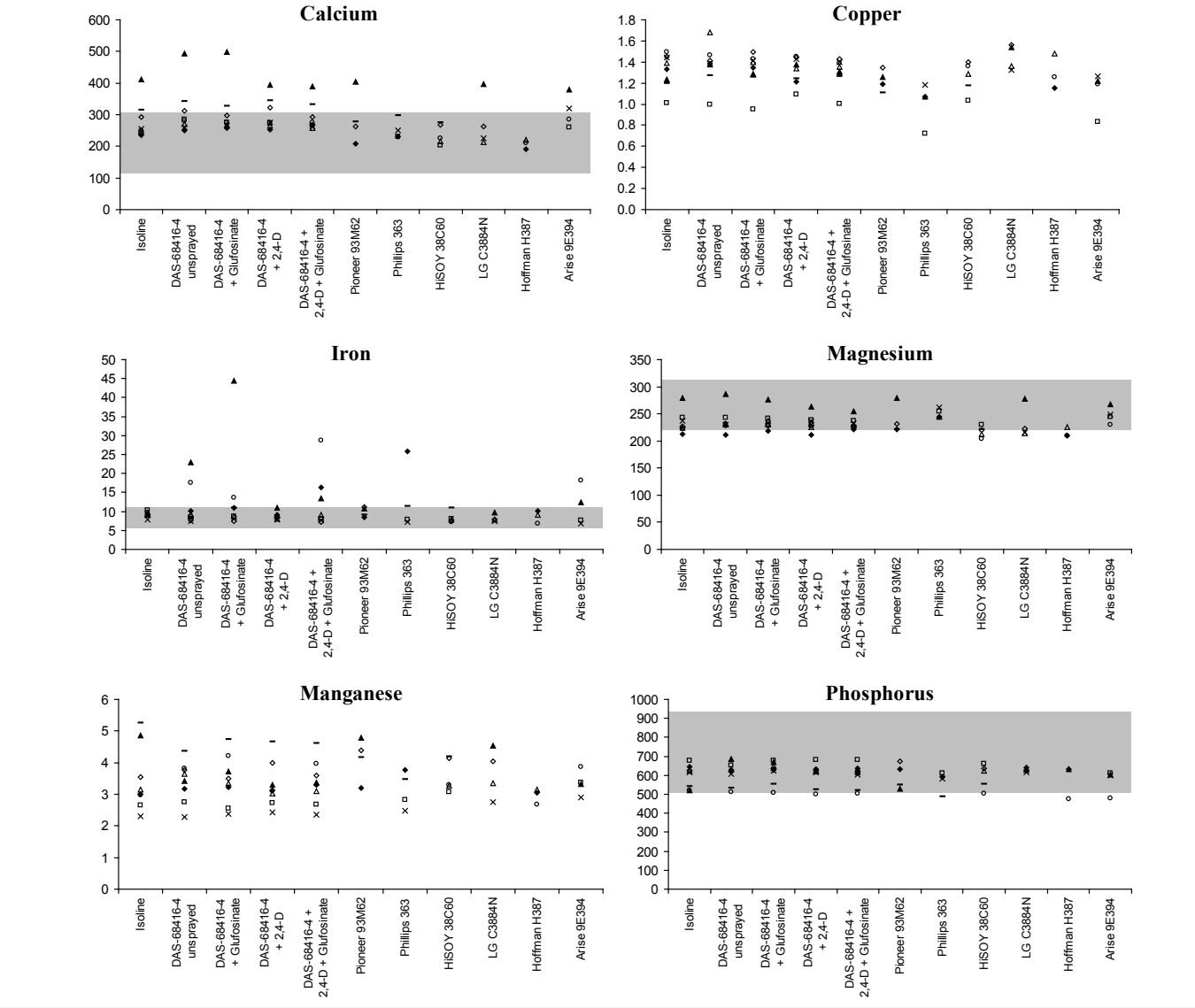
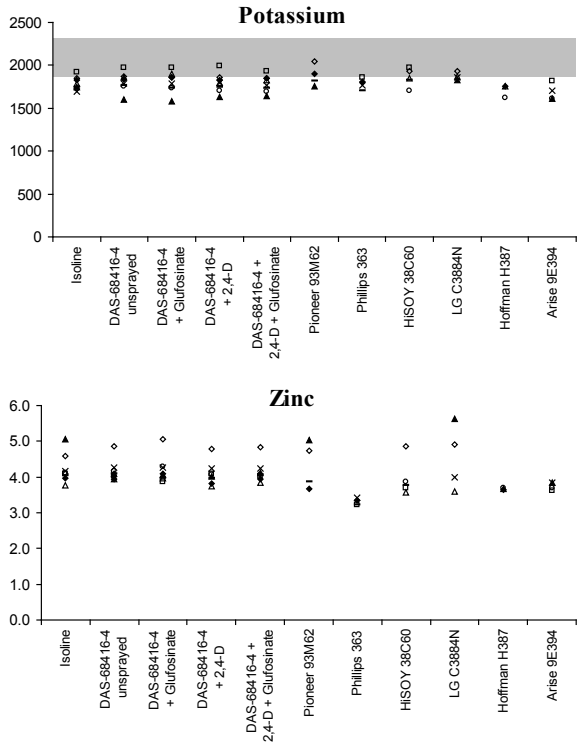


Figure 2: Proximate and fiber levels in non-transgenic control soybean seed, unsprayed DAS-68416-4 (AAD-12), 2,4-D-treated DAS-68416-4, glufosinate-treated DAS-68416-4, 2,4-D + glufosinate-treated DAS-68416-4, Pioneer 93M62, Phillips 363, HiSOY 38C60, LG C3884N, Hoffman H387, and Arise 9E394 seed. The shaded area spans the published values for each analyte, and levels are expressed as a percentage of dry weight (Note: moisture is expressed in fresh weight). Symbols in the plots are as follows: ▲, Lonoke (AR); ×, Richland (IA); ◊, Carlyle (IL); □, Wyoming (IL); ○, Rockville (IN); Δ, La Plata (MO); −, Dudley (MO); ♦, York (NE).





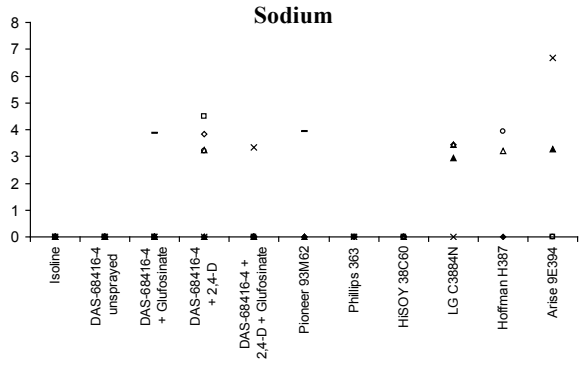
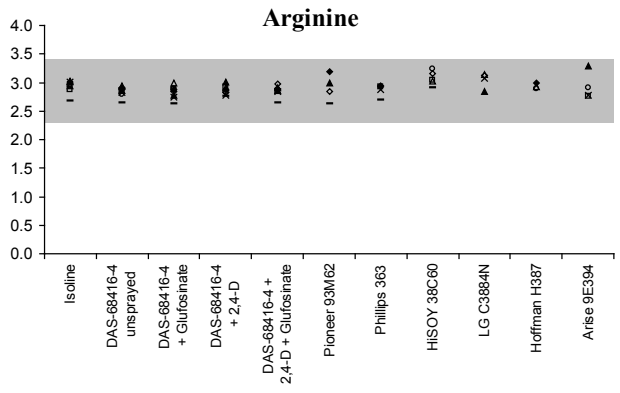
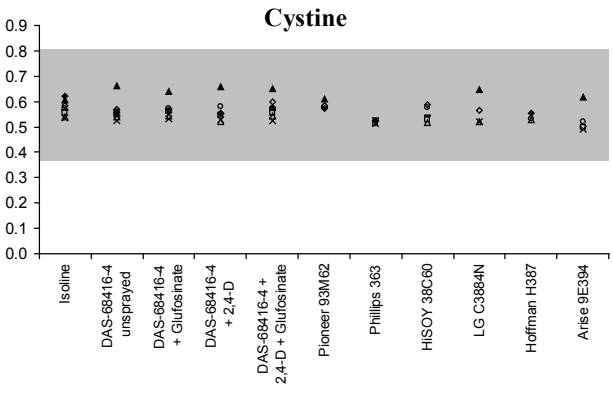
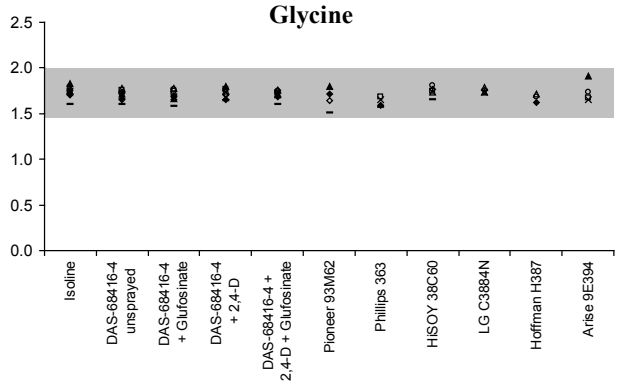
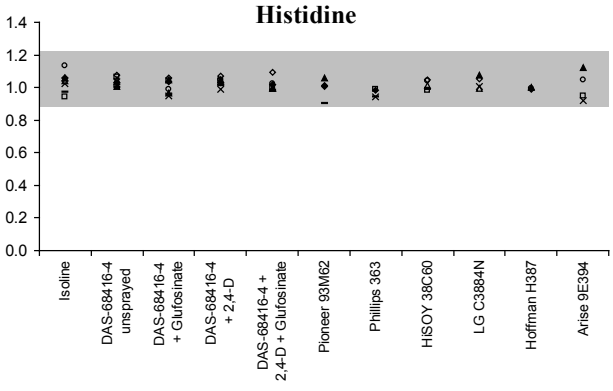


Figure 3: Mineral levels in non-transgenic control soybean seed, unsprayed DAS-68416-4 (AAD-12), 2,4-D-treated DAS-68416-4, glufosinate-treated DAS-68416-4, 2,4-D + glufosinate-treated DAS-68416-4, Pioneer 93M62, Phillips 363, HiSOY 38C60, LG C3884N, Hoffman H387, and Arise 9E394 seed. The shaded area spans the published values for each analyte, and levels are expressed as mg per 100 g dry weight. Symbols in the plots are as follows: ▲, Lonoke (AR); ×, Richland (IA); ◊, Carlyle (IL); □, Wyoming (IL); ○, Rockville (IN); △, La Plata (MO); **–**, Dudley (MO); ♦, York (NE).









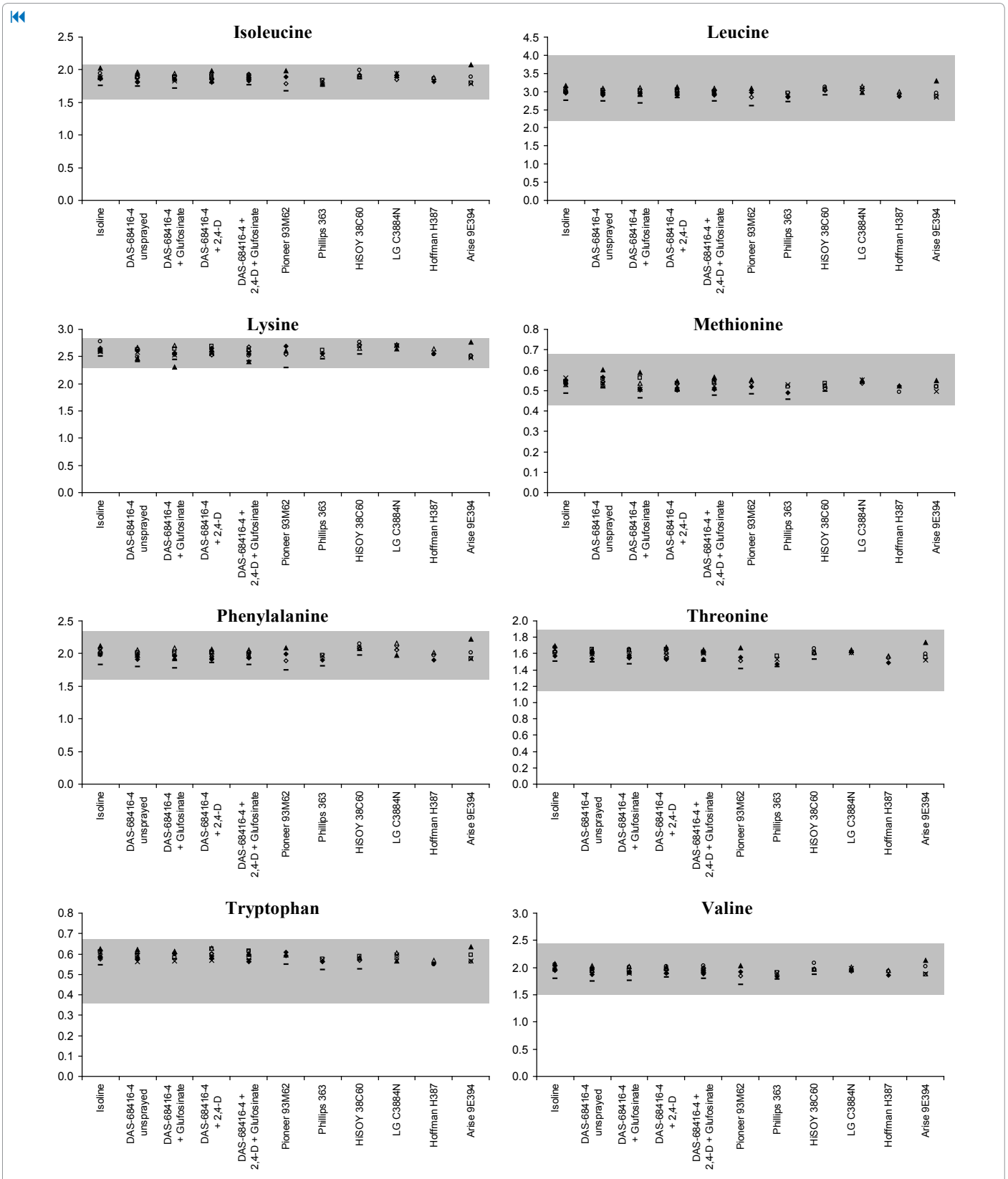


Figure 4: Essential amino acid levels in non-transgenic control soybean seed, unsprayed DAS-68416-4 (AAD-12), 2,4-D-treated DAS-68416-4, glufosinate-treated DAS-68416-4, 2,4-D + glufosinate-treated DAS-68416-4, Pioneer 93M62, Phillips 363, HiSOY 38C60, LG C3884N, Hoffman H387, and Arise 9E394 seed. The shaded area spans the published values for each analyte, and levels are expressed as a percentage of dry weight. Symbols in the plots are as follows: ▲, Lonoke (AR); ×, Richland (IA); ◊, Carlyle (IL); □, Wyoming (IL); ○, Rockville (IN); Δ, La Plata (MO); **–,** Dudley (MO); ♦, York (NE).

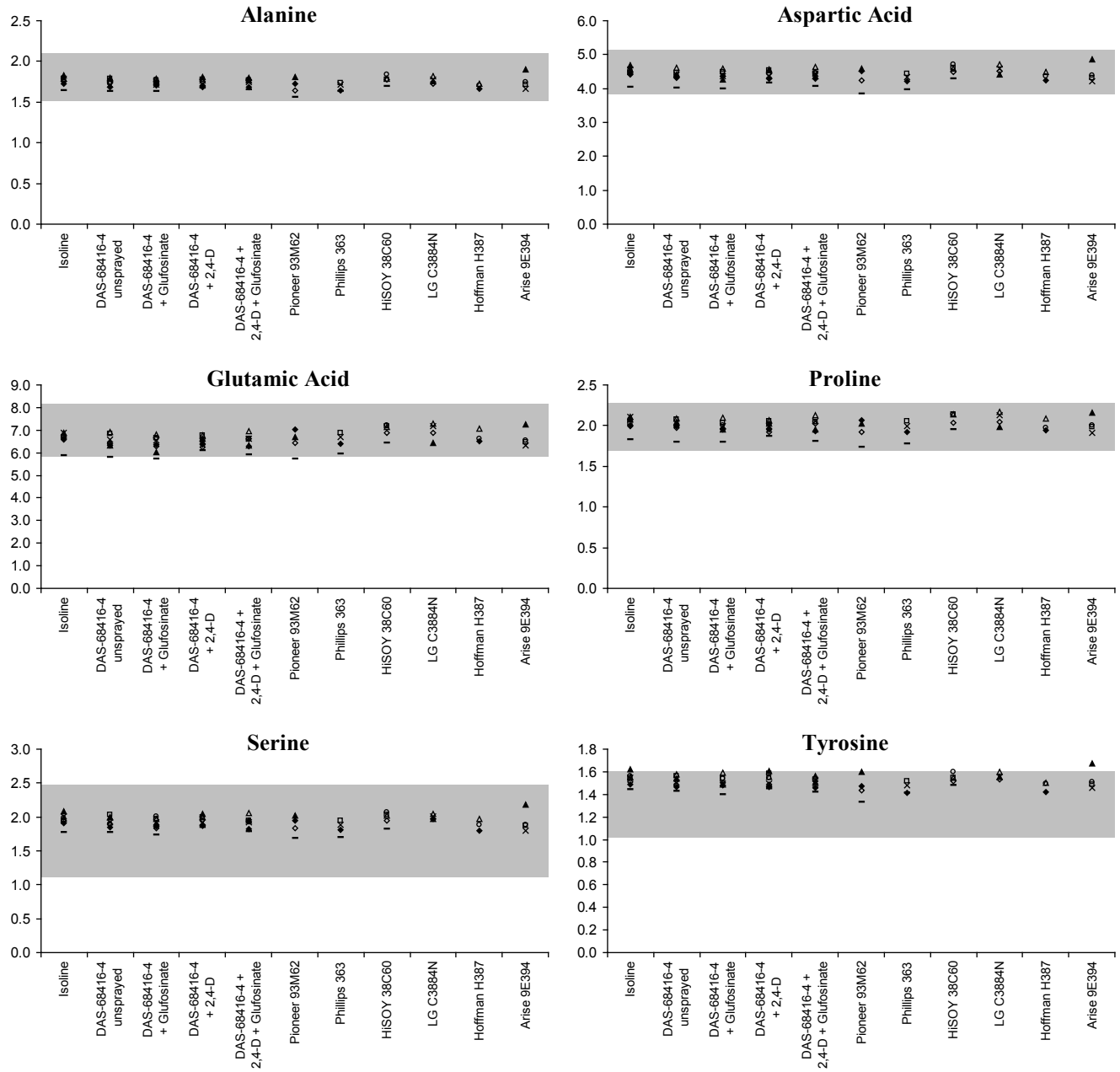
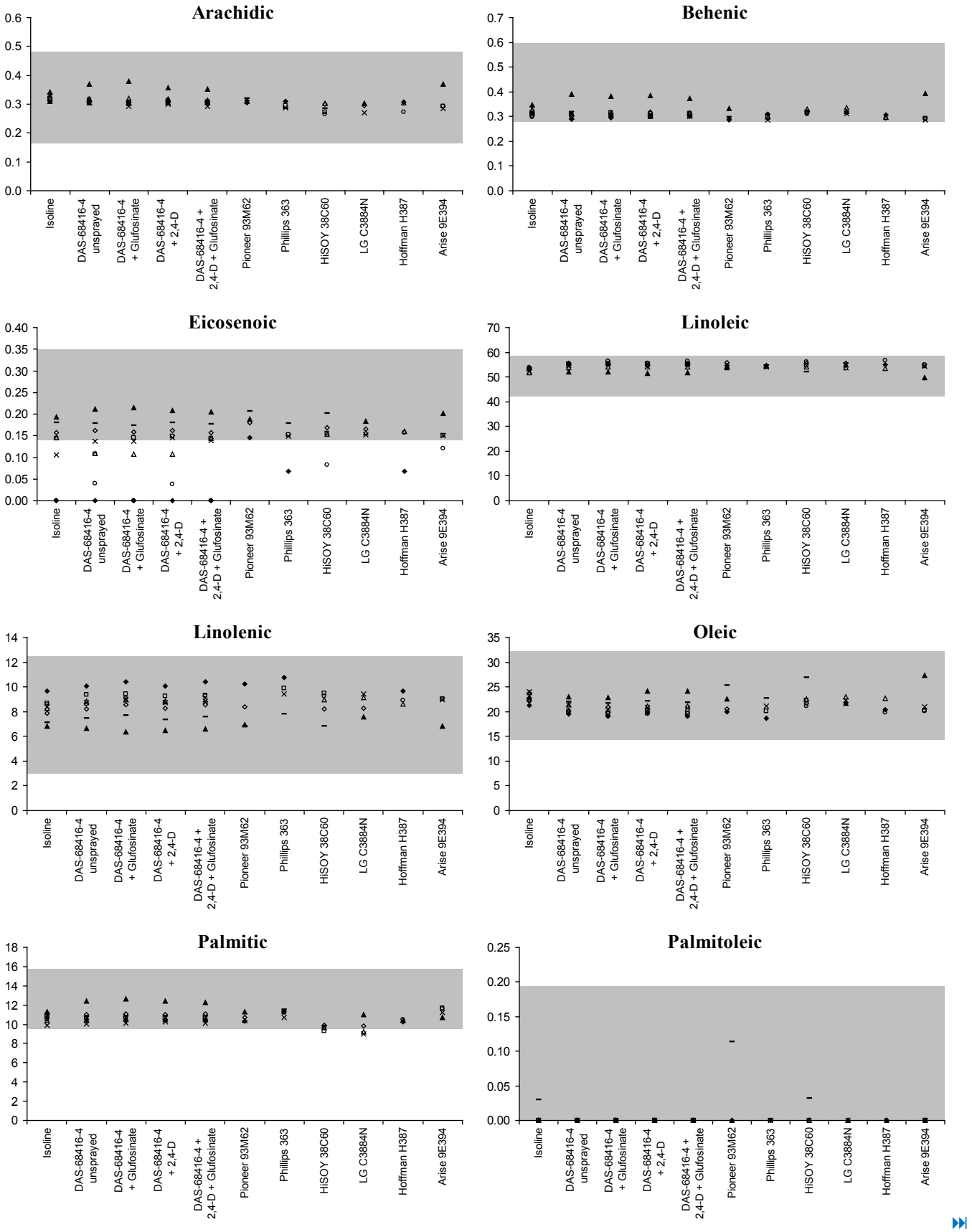


Figure 5: Non-essential amino acid levels in non-transgenic control soybean seed, unsprayed DAS-68416-4 (AAD-12), 2,4-D-treated DAS-68416-4, glufosinate-treated DAS-68416-4, 2,4-D + glufosinate-treated DAS-68416-4, Pioneer 93M62, Phillips 363, HiSOY 38C60, LG C3884N, Hoffman H387, and Arise 9E394 seed. The shaded area spans the published values for each analyte, and levels are expressed as a percentage of dry weight. Symbols in the plots are as follows: ▲, Lonoke (AR); ×, Richland (IA); ◊, Carlyle (IL); □, Wyoming (IL); ○, Rockville (IN); Δ, La Plata (MO); –, Dudley (MO); ♦, York (NE).

with 2,4-D (37.8% dw) compared with the protein level in the non-transgenic entry (38.7% dw). Again, the protein levels for all transgenic entries were typical of non-transgenic soybean grain (Figure 2). Of the minerals analyzed in grain, only calcium levels in the DAS-68416-4 entry not sprayed with 2,4-D or glufosinate (312 mg/100g dw) significantly differed from the non-transgenic near-isogenic control (281 mg/100g dw). Higher calcium levels in soybean grain do not raise any safety issues, and the effect of the weather stress at the Arkansas site (hail damage) overshadowed any potential effect of transgenesis or herbicide treatment (Figure 3). The average levels of four amino acids (alanine, arginine, glutamic acid, and proline) were significantly lower in the DAS-68416-4 entry treated with glufosinate (1.73, 2.83, 6.38, and 1.97% dw, respectively) compared with the non-transgenic near-isogenic entry (1.76, 2.93, 6.63, and 2.03% dw, respectively), but in all cases, levels were typical of those observed for the commercial non-transgenic entries in the study (Figure 4 and Figure 5).

Average oleic fatty acid levels were significantly lower for the DAS-68416-4 entries (20.5 – 21.0% of total fatty acids) compared with the non-transgenic near-isogenic entry (22.9% of total fatty acids), and


Arachidic
Behenic
Eicosenoic
Linoleic
Linolenic
Oleic
Palmitic
Palmitoleic
Isoline
DAS-68416-4 unsprayed
DAS-68416-4 + Glufosinate
DAS-68416-4 + 2,4-D
DAS-68416-4 + 2,4-D + Glufosinate
Pioneer 93M62
Phillips 363
HiSOY 38C60
LG C3884N
Hoffman H387
Arise 9E394

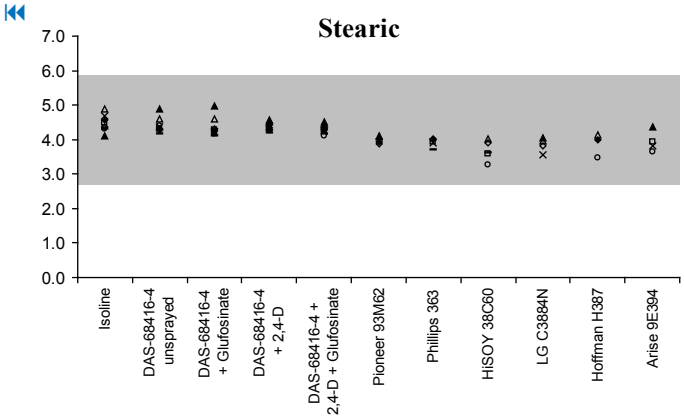
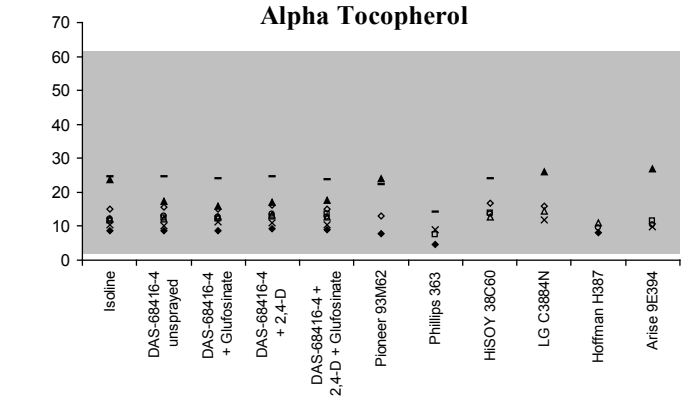
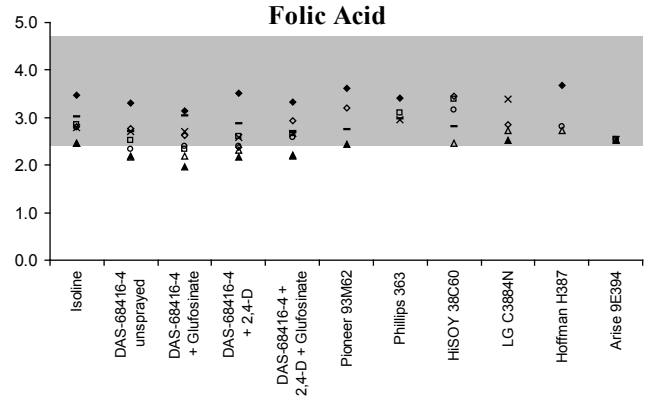
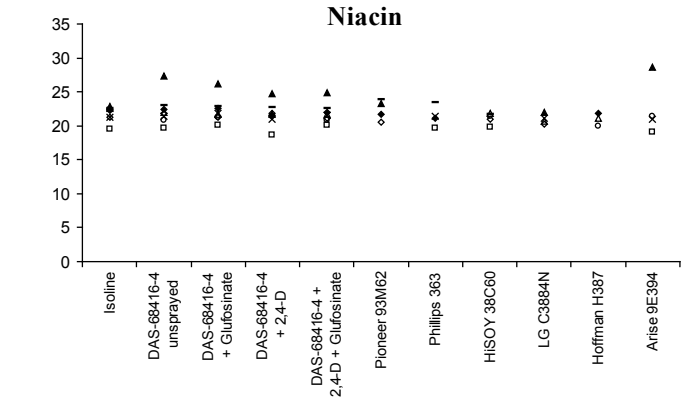
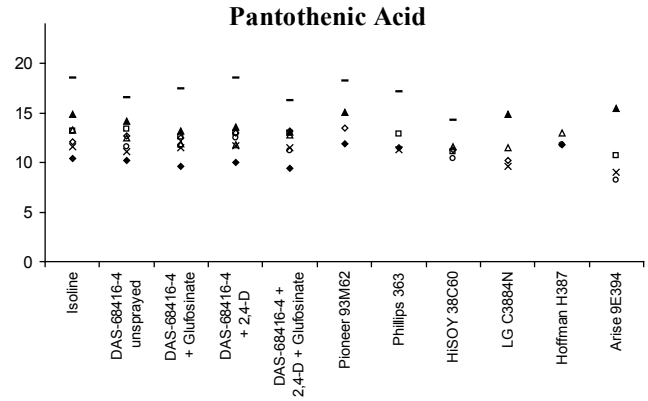


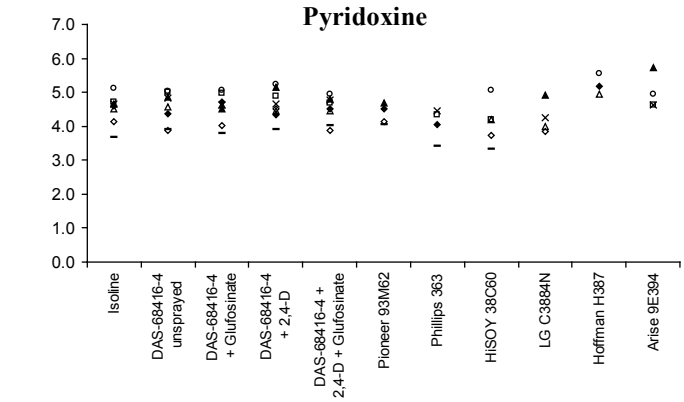
Figure 6: Fatty acid levels in non-transgenic control soybean seed, unsprayed DAS-68416-4 (AAD-12), 2,4-D-treated DAS-68416-4, glufosinate-treated DAS-68416-4, 2,4-D + glufosinate-treated DAS-68416-4, Pioneer 93M62, Phillips 363, HiSOY 38C60, LG C3884N, Hoffman H387, and Arise 9E394 seed. The shaded area spans the published values for each analyte, and levels are expressed as a percentage of total fatty acids. Symbols in the plots are as follows: ▲, Lonoke (AR); ×, Richland (IA); ◊, Carlyle (IL); □, Wyoming (IL); ○, Rockville (IN); △, La Plata (MO); –, Dudley (MO); ♦, York (NE).

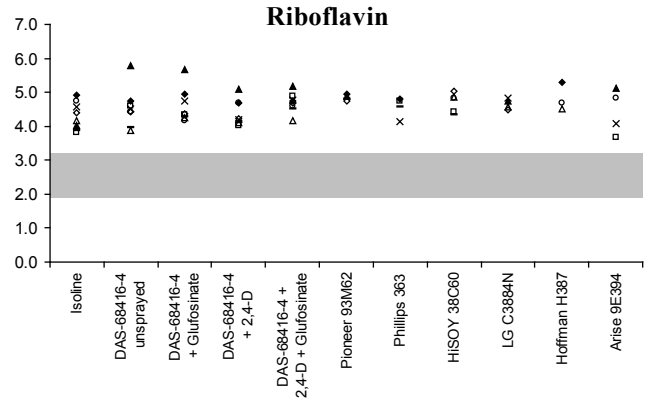












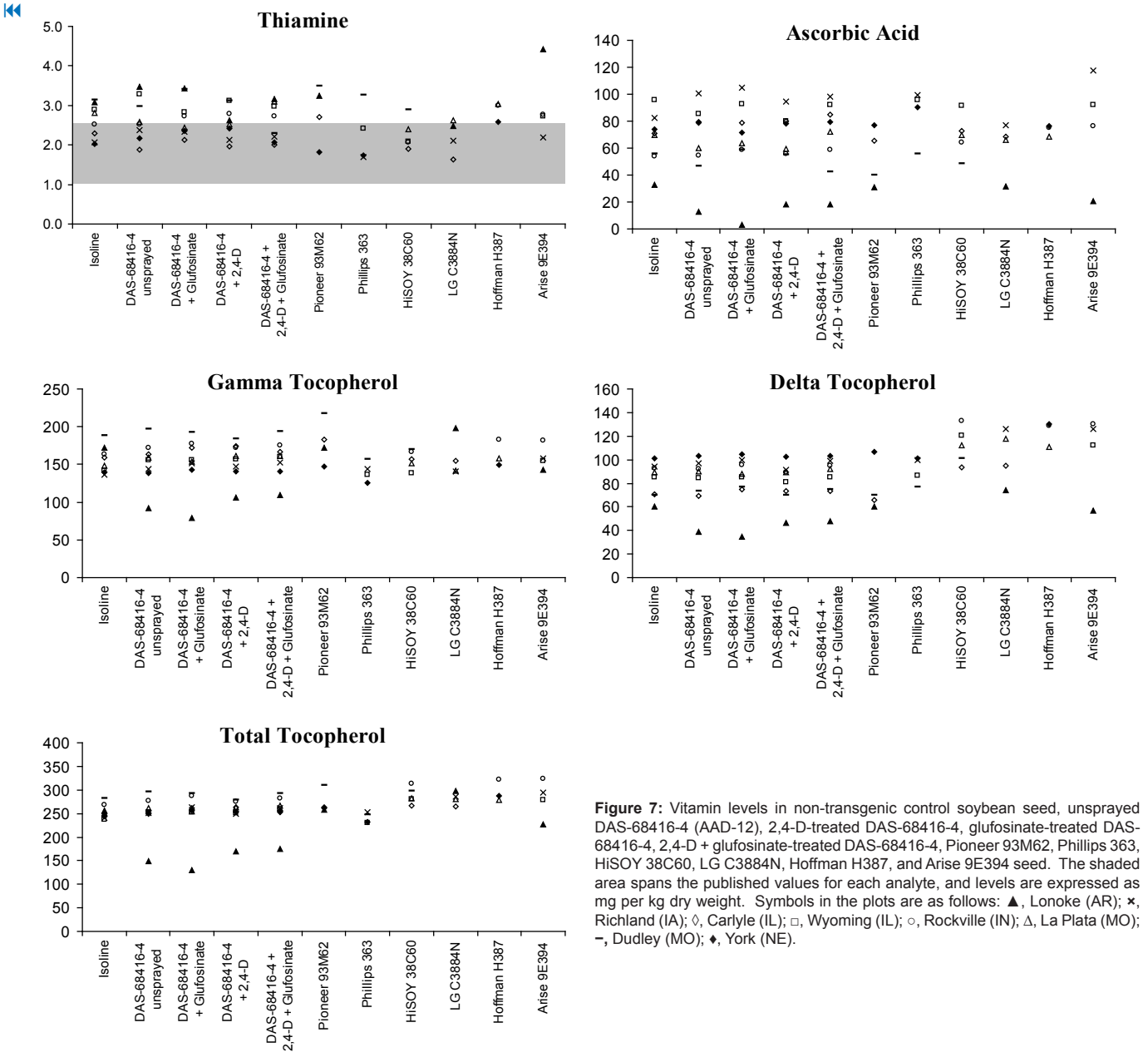
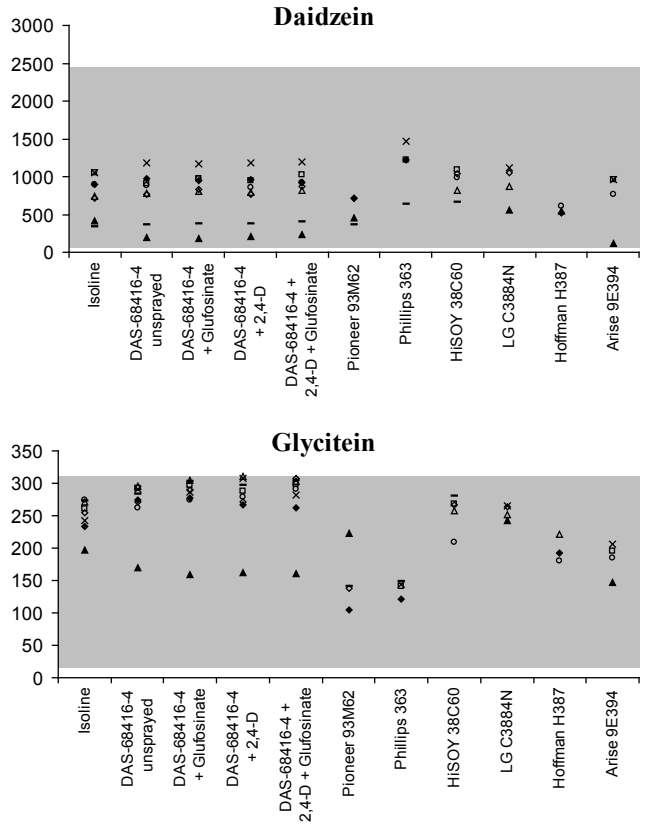


Figure 7: Vitamin levels in non-transgenic control soybean seed, unsprayed DAS-68416-4 (AAD-12), 2,4-D-treated DAS-68416-4, glufosinate-treated DAS-68416-4, 2,4-D + glufosinate-treated DAS-68416-4, Pioneer 93M62, Phillips 363, HiSOY 38C60, LG C3884N, Hoffman H387, and Arise 9E394 seed. The shaded area spans the published values for each analyte, and levels are expressed as mg per kg dry weight. Symbols in the plots are as follows: ▲, Lonoke (AR); ×, Richland (IA); ◊, Carlyle (IL); □, Wyoming (IL); ○, Rockville (IN); Δ, La Plata (MO); –, Dudley (MO); ♦, York (NE).

linoleic and linolenic levels for the DAS-68416-4 entries (54.6 – 54.8 and 8.46 – 8.69% of total fatty acids, respectively) were significantly higher compared with the non-transgenic near-isogenic entry (53.0 and 8.17% of total fatty acids, respectively). However, levels of these fatty acid levels were all typical of non-transgenic soybean grain (Figure 6). Average folic acid levels for three of the four DAS-68416-4 entries (2.55 – 2.60 mg/kg dw; excluding the entry treated with both herbicides) were significantly lower compared with the level for the non-transgenic near-isogenic entry (2.83 mg/kg dw), but were again typical of non-transgenic soybean grain although some site means were below literature ranges (Figure 7). Low folic acid levels were most evident at the Arkansas site where severe hail damage occurred. Total glycitein levels were found to be statistically higher for the DAS-68416-4 entries treated with 2,4-D and with both 2,4-D and glufosinate (273.7 and 276.3 µg/g dw, respectively) compared with the non-transgenic near-isogenic entry (250.5 µg/g dw), but fell within the literature range for non-transgenic soybean grain (Figure 8). Soybean isoflavones have significant health benefits so a moderate increase in glycitein, if seen across soybean varieties, would be beneficial [66]. No significant differences between the transgenic entries and the non-transgenic near-isogenic entry were seen for antinutrients (Figure 9).

The number of small differences between the transgenic and non-transgenic near-isogenic entries that were detected as significant by the statistical analysis demonstrates the high power of the experimental design and analysis. The small magnitude of these differences, their questionable impact on safety, and the similarity of the composition of DAS-68416-4 to non-transgenic soybean lines indicates the substantial equivalence between DAS-68416-4 soybean and non-transgenic



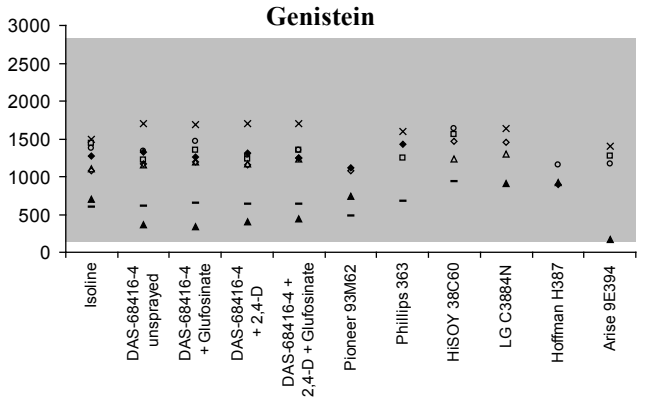
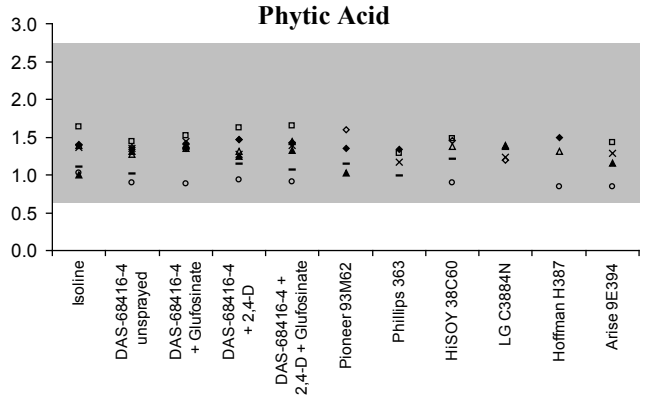
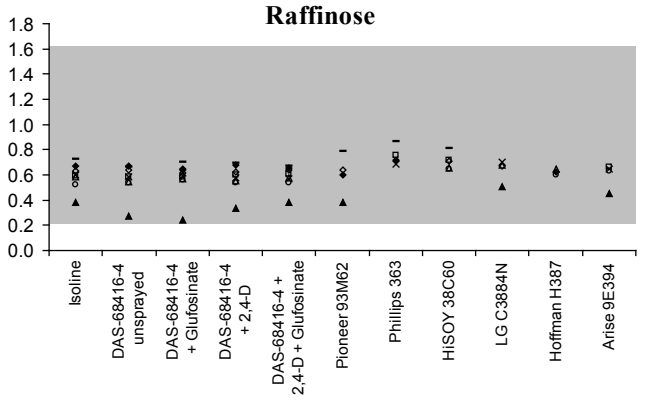
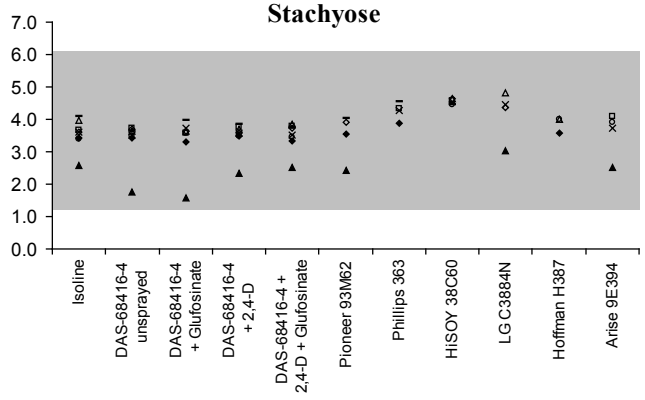
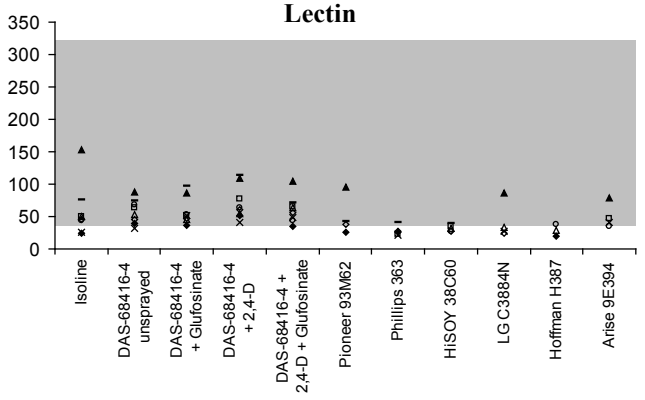


Figure 8: Isoflavone levels in non-transgenic control soybean seed, unsprayed DAS-68416-4 (AAD-12), 2,4-D-treated DAS-68416-4, glufosinate-treated DAS-68416-4, 2,4-D + glufosinate-treated DAS-68416-4, Pioneer 93M62, Phillips 363, HiSOY 38C60, LG C3884N, Hoffman H387, and Arise 9E394 seed. The shaded area spans the published values for each analyte, and levels are expressed as mcg per g dry weight. Symbols in the plots are as follows: ▲, Lonoke (AR); ×, Richland (IA); ◊, Carlyle (IL); □, Wyoming (IL); ○, Rockville (IN); Δ, La Plata (MO); **–**, Dudley (MO); ♦, York (NE).









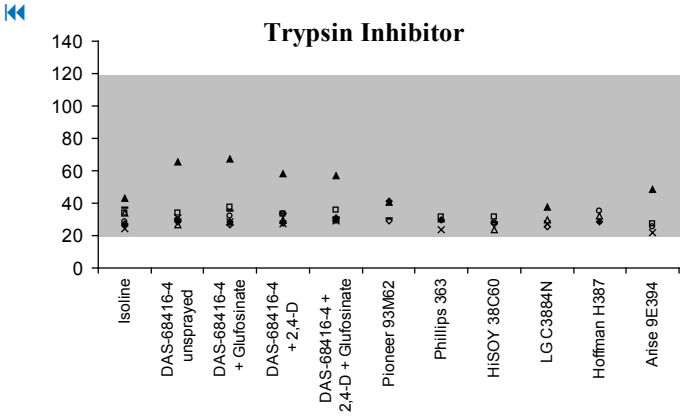


Figure 9: Anti-nutrient levels in non-transgenic control soybean seed, unsprayed DAS-68416-4 (AAD-12), 2,4-D-treated DAS-68416-4, glufosinate-treated DAS-68416-4, 2,4-D + glufosinate-treated DAS-68416-4, Pioneer 93M62, Phillips 363, HiSOY 38C60, LG C3884N, Hoffman H387, and Arise 9E394 seed. The shaded area spans the published values for each analyte, and levels are expressed as percentage of dry weight (Phytic Acid, Raffinose, and Stachyose), hemagglutinating units per mg protein dry weight (Lectin), and trypsin inhibitor units per mg dry weight (Trypsin Inhibitor). Symbols in the plots are as follows: ▲, Lonoke (AR); ×, Richland (IA); ◊, Carlyle (IL); □, Wyoming (IL); ○, Rockville (IN); Δ, La Plata (MO); **–,** Dudley (MO); ♦, York (NE).

soybean, and confirms the compositional safety of this transgenic event. This study adds to the growing literature supporting the expectation that unintended compositional changes due to transgenesis pale in comparison to those imparted during traditional breeding and those caused by environmental variability [2,3]. Considering the long history of safety associated with traditional breeding, the value of conducting extensive and non-targeted compositional studies with transgenic crops to look for unintended effects does not seem warranted. However, as transgenic traits are developed that are designed to interact with endogenous genes or metabolic cascades, hypothesis-driven composition studies may be useful for informing the dietary risk assessment.

Conflict of Interest

RAH, AMP, & MDL are employees of Dow AgroSciences LLC, a wholly owned subsidiary of The Dow Chemical Company, which develops transgenic crops and produces insecticides, herbicides, and fungicides for agricultural applications and residential pest control. JS is employed by Covance Laboratories, Inc. which performed the compositional analysis under contract from Dow AgroSciences LLC.

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