Final Report

Study Title

Quantitative ELISA Analysis of poCry1F and PAT Protein Expression Levels, Composition and Efficacy of Hybrid Lines 1360 and 1507 – EU Field Sites

Data Requirements

None

Authors

Cynthia Stauffer

Study Completed On

September 28, 2000

Performing Laboratory

Pioneer Hi-Bred International, Inc.
7300 NW 62nd Ave.
Johnston, Iowa 50131-1004

Study Number

PHI99-005

Sponsor

Pioneer Hi-Bred International Inc.
7250 N.W. 62nd Ave.
Johnston, Iowa 50131-0552

Total number of pages: 39

Study Number:	PHI99-005
Title:	Quantitative ELISA Analysis of poCry1F and PAT Protein Expression Levels, Composition and Efficacy of Hybrid Lines 1360 and 1507 – EU Field Sites
Testing Facility:	Pioneer Hi-Bred International, Inc. 7300 NW 62nd Ave. Johnston, Iowa 50131-1004
Field Sites:	Malagnino (Cremona), Italy Castellucchio (Mantova), Italy Terranova dei Passerini (Lodi), Italy 16240 Longre, France 64300 Bonnut, France 31700 Daux, France
Study Sponsor:	Rod Townsend, Pioneer Hi-Bred Int'l Inc.
Study Director:	Cynthia Stauffer, Pioneer Hi-Bred Int'l Inc.
Contributors:	Analytical Principal Investigator – Jacqueline Rivas Field Principal Investigator (Italy) – Gianni Manuini Field Principal Investigator (Longre, France) – Jean-Jacques Ferron Field Principal Investigator (Bonnut, France) – Jean Lacazedieu Field Principal Investigator (Daux, France) – Eric Perroud
Study Initiation Date:	5/3/99
Records Retention:	All study specific raw data, protocols, final reports and facility records are archived at Pioneer Hi-Bred Int'l Inc.
Specimen Storage:	All retain samples will be stored at Pioneer in Johnston, IA.

I certify that this report accurately represents the results observed during the course of this study.

Report issued by:

Cynthia Stauffer
Cynthia Stauffer, Study Director

28 September, 2000
Date

Rod Townsend
Rod Townsend, Study Sponsor

10-02-00
Date

TABLE OF CONTENTS **PAGE**

INTRODUCTION

A. Background

Maize lines, designated 1360 and 1507 were modified to express the Cry1F protein from *Bacillus thuringiensis* subsp. *aizawai*. This protein confers resistance to the European corn borer (*Ostrinia nubilalis* Hubner) insect pest. Additionally, these lines contain a synthetic *pat* gene, derived from *Streptomyces viridochromogenes,* which encodes phosphinothricin acetyl transferase (PAT). The PAT protein is an enzyme that inactivates the herbicide glufosinate-ammonium and thus makes genetically modified plants that accumulate this protein resistant to the herbicide.

The study initially included maize hybrids derived from Bt maize lines 1360 and 1507. However, after the study was initiated, it was determined that Bt maize line 1360 was not performing appropriately so the test lines containing event 1360 were removed from this study.

B. Purpose

The purpose of the field trials was:

1) To generate leaf, V9 whole plant, pollen, silk, stalk, R1 whole plant, R4 whole plant, grain and senescent whole plant samples from a hybrid derived from maize line 1507. The tissue samples were also collected from a control line designated Mycogen brand hybrid 2722
2) To measure levels of Cry1F and PAT proteins in tissue collected from the test and control line plants.
3) To measure levels of various nutritional composition traits in grain and whole plant tissue samples from both the test and control lines.
4) To collect efficacy data for controlling second generation European corn borer (ECB)

MATERIALS

A. Test substance

The test substances for this study were seed of a 1507 maize hybrid that was capable of expressing the Cry1F and PAT proteins.

Initial characterization of the test substance consisted of documentation of the breeding lineage of the seed. Pedigree information for the hybrid and inbred is proprietary information and is on file with staff breeders at Mycogen Seeds, Huxley, Iowa. Prior to planting, the seed was stored under appropriate conditions to maintain seed viability and vigor (Wych, 1988). Definitive characterization of the test substance occurred during the study – confirmation of glufosinate tolerance in the field and specific ELISA's for the detection and quantification of the Cry1F and PAT proteins were performed.

B. Control substance

The control substance, Mycogen brand hybrid 2722, was seed from a maize line that had not been genetically modified, but that had background genetics representative of the test substance. Pedigree information for Mycogen brand hybrid 2722 is on file with staff breeders at Mycogen Seeds, Huxley, Iowa. Prior to planting, the seed was stored under appropriate conditions to maintain seed viability and vigor (Wych, 1988).

C. Reference substances

The following reference standards were used in this study:

Cry1F protein standard. The Cry1F protein (lot number 082597) was purified from *Pseudomonas fluorescens* (strain MR872) that contained a gene coding truncated Cry1F toxin. Strain MR872 was developed by Mycogen Corp. The purified protein was stored at a concentration of 1.4 mg/ml in a storage buffer containing glycerol at 2-8°C. Characterization of the standard was accomplished by SDS-PAGE and amino acid analysis.

PAT protein standard. The PAT protein (lot number 050195) was purified from *E. coli* strain BL21 (Novagen) encoding the *pat* gene. The *pat* gene was subcloned into Pharmacia's pGEX4T1 expression plasmid (pstock#6484) and transformed into the *E. coli* strain. The purified protein was stored as a 0.89 mg/ml protein solution in 50 mM Tris-HCl at -80°C for long term storage and at 2-8°C for short term storage. Characterization of the standard was accomplished by SDS-PAGE/silver stain, sequencing and amino acid analysis.

The reference substance for the Bradford assay was:

Bio Rad Protein Assay Standard II. (Bio Rad #500-0007 or equivalent). The Protein Assay Standard II was obtained from Bio Rad (lot number 60092A) with a BSA purity of 64.46% (Bio Rad reference). The concentrated protein was diluted in distilled water (as per manufacturer's instructions) to achieve a 1.23 mg/ml protein solution (lot number 60092A-011399). The diluted solution was kept at –10 to -24°C for long-term storage and at 2-8°C for short term storage.

D. Test system

Field Test: The test system for this study was the environment in which the maize plants were grown. The field sites were Malagnino (Cremona), Castellucchio, (Mantova) and Terranova dei Passerini (Lodi) all located in Italy and 16240 Longre, 64300 Bonnut and 31700 Daux all located in France. These sites are located in the major maize growing regions of Italy and France. Each site was identified by a unique 3-character code.

At each site in Italy, there were 3 blocks (block = replicate) that were arranged in a randomized complete block design. All blocks contained plots consisting of 4 rows per entry. The entries were maize line 1507 sprayed with glufosinate ammonium herbicide (1507s), maize line 1507 not sprayed with glufosinate ammonium (1507lp) and Mycogen brand hybrid 2722.

At each site in France, there were 6 blocks that were arranged in a randomized complete block design. Block 1 contained plots consisting of 3 rows per entry, blocks 2-6 contained plots consisting of 2 rows per entry. The entries were maize line 1507 not sprayed with glufosinate ammonium and Mycogen brand hybrid 2722.

Field plot maps showing the randomization schemes for each site are shown in Tables 1-6. Each entry was tracked by a specific designation unique to each position in the field.

METHODS

A. Summary of experimental design

The test and control lines were grown at 3 field sites in Italy and 3 field sties in France. Leaf, V9 whole plant, pollen, silk, stalk, R1 whole plant, R4 whole plant, grain and senescent whole plant tissue samples were collected from plants from the test and control lines of the unsprayed entries. Only R4 whole plant and grain were collected from the sprayed entries. The tissues were evaluated for Cry1F and PAT protein levels using specific ELISA methods developed for each protein. Additionally, R4 whole plant and grain samples from the hybrid test and control lines were analyzed for nutrient composition.

B. Field trial

All sites were managed so that the identity and integrity of all samples was maintained. Important crop dates (i.e., planting, pollinations and harvest) are listed in Table 7.

1. Agronomic practices

Agricultural practices for growing the test and control plants were typical for producing maize in the regions chosen for this study. Chemical and fertilizer applications were appropriate for each location and are listed in Tables 8 - 13.

2. Planting

The land at each site went through multiple plowings and cultivations to prepare the soil prior to planting. The test and control lines were planted at a depth of 3 – 7 cm. Between 19 and 23 kernels of each line were planted per row of each plot. Depending upon the site and replication, the plot dimensions ranged from 8.0 m^2 to 16.5 m^2.

The test lines were from early generations and therefore segregating at 1:1 for the presence of the *cry*1F and *pat* genes. At each location, every plant of the unsprayed test entry was leaf-painted with glufosinate ammonium to identify the plants containing the *pat* gene. Plants that were damaged by the herbicide were assumed to lack the *pat* and *cry*1F genes and were rouged from the plots of each test line. At the Longre, France site, the control lines were inadvertently leaf-painted with the herbicide and the damage was so severe that the plants all died.

3. Climate

Weather conditions were documented at each site and are summarized in Tables 14 and 15. Rainfall and supplemental irrigation was sufficient to produce maize typical of the growing area.

4. Sampling

Protein expression. Leaf, pollen, silk and stalk tissues were collected (see Table 16 for dates) from five randomly selected plants from the unsprayed test entries in the first replicate and ears were collected from five randomly selected plants in both the unsprayed and sprayed test entries in the first replicate. Control tissue samples of leaf, pollen, silk and stalk tissue were collected from a single plant in the first replicate and ears were collected from three plants in the first replicate. Whole plant tissue was collected from three plants in the first replicate in both the test entries and control entries, the R4 whole plant samples were collected from both sprayed and unsprayed plots. Leaf tissue was collected when plants were at the V9 growth stage (nine emerged leaves with visible collars). Pollen, silk and stalk tissues were collected when plants were at the R1 growth stage (beginning silk emergence). Whole plant tissue was collected when the crop was at the V9, R1 and R4 (or forage) growth stage (Iowa State University, 1993). Grain was collected when plants were physiologically

mature, corresponding to the time of typical commercial grain harvest. Each sample was uniquely identified with a code that completely identified the line, replicate and sample number.

Each individual leaf, pollen, silk and stalk sample was placed in a labeled resealable plastic bag on ice then either kept frozen at approximately -20°C until shipment on dry ice or shipped immediately on artificial ice to the Pioneer facility in Epuiseau, France where they were lyophilized. After lyophilization the samples were kept at approximately 10°C, then shipped to the analytical principal investigator in Johnston, IA, where they were stored at -80°C until analysis.

All whole plant samples were chopped, dried at approximately 55°C then ground to a fine powder. The samples were kept at approximately 10°C until shipment to Johnston, IA at ambient temperature. The samples were stored at –80°C until analysis.

Nutritional composition. R4 whole plant tissue from three randomly selected plants and grain tissue from three randomly selected plants was collected from all sprayed and unsprayed entries from all replicates at each site. Tissues were processed and stored as described for protein expression. Samples were then forwarded to Woodson-Tenent Laboratories, Inc., Des Moines, Iowa for compositional analysis. A portion of the grain sample was retained for tocopherol analysis at Pioneer. The samples from the first replicate were divided and used for ELISA analysis or nutrient composition analysis.

C. Analysis

Protein expression

Extraction of proteins from maize tissues
The leaf and silk samples were reduced to a fine powder using a paint shaker. Grain samples were ground using a Kleco Ball mill then lyophilized. All processed samples were stored at approximately -80°C until the ELISA analyses were performed. A portion of each lyophilized or dried sample was weighed and then extracted in a buffer solution (PBST) using a proprietary tissue homogenizer. Insoluble material was removed by centrifugation for approximately 10 minutes.

Total extractable protein The total extractable protein (TEP) concentration of the supernatant was determined by the Bradford method (1976) using the microtiter plate application of the Bio-Rad Protein Assay. Bio Rad's Protein Assay Standard II was used as the protein standard. Results from this assay were expressed in µg total protein/ml of extract. Based on these concentrations, the volume of extract for further analyses was determined such that a constant amount of protein was delivered in each well of the plates.

Cry1F ELISA. A direct double antibody sandwich enzyme-linked immunosorbent assay (ELISA) was developed in house to quantify levels of Cry1F protein in genetically modified maize. The method uses a polyclonal rabbit antibody specific to Cry1F protein to capture the protein in the microtiter well. The captured protein is detected by the same polyclonal antibody conjugated to biotin. The binding of the biotinylated antibody to the captured protein was detected by a conjugate of streptavidin-alkaline phosphatase (SA/AP). The enzyme substrate, para-nitrophenyl phosphate (pNPP), was added for the color development. Quantification of Cry1F protein was accomplished by extrapolation (based on sample absorbance (optical density; OD) value) from a Cry1F standard protein concentration curve. The Cry1F ELISA concentrations were expressed in pg/µg total protein.

PAT ELISA. A direct double antibody sandwich enzyme-linked immunosorbent assay (ELISA) was developed in house to quantify levels of PAT protein in genetically modified maize plants. The method uses a polyclonal rabbit antibody specific to the PAT protein to capture the protein in the microtiter well. The captured protein is detected by the same polyclonal antibody conjugated to biotin. The binding of the biotinylated antibody to the

captured protein is detected by a conjugate of SA/AP. The enzyme substrate, pNPP, was added for color development. Quantification of the PAT protein was accomplished by extrapolation (based on sample absorbance (OD) value) from a PAT standard protein concentration curve. The PAT ELISA concentrations were expressed in pg/μg of total protein.

Limits of detection

In all tissues, the samples yielding an interpolated concentration of <10 pg/well (200 pg/ml) for the Cry1F assay and <20 pg/well (400 pg/ml) for the PAT assay were considered below the level of detection (LOD). These values correspond to <10 pg/μg total protein for the Cry1F assay and <20 pg/μg total protein for the PAT assay. The assays were developed using a total protein load of 1μg/well.

Nutrient composition analysis

Whole Plant

Moisture Analysis
Moisture analysis was conducted according to American Oil Chemist's Society (AOCS) method #Ba 2a-38. Moisture is the actual water content and any material that is volatile under the conditions of the test.

Fat Content
Crude fat content was determined by use of the AOCS method #Ba 3-38. This determines the substances extracted by petroleum ether under the conditions of the method.

Protein Content
Crude protein content was determined by use of the AOCS method #Ba 4d-90. Mixtures of true proteins, composed of amino acids, and non-protein nitrogen make up the crude protein content. Protein is determined by measuring the amount of nitrogen and multiplying by 6.25. The general term "Protein" refers to crude protein that includes both available and unavailable crude protein.

Ash Analysis
Ash analysis was conducted according to AOCS method #Ba 5a-49. This method determines as ash the residue remaining after incineration under the conditions of this test.

Fiber Content
Acid Detergent Fiber (ADF) and Neutral Detergent Fiber (NDF) content was determined by use of the ANKOM$^{200/220}$ method by Woodson-Tenent Laboratories Inc.

Carbohydrates
Carbohydrates are calculated as the percentage of dry weight =100% - % protein - % fat - % ash. Fiber (ADF and NDF) is included in the carbohydrates.

Grain
Moisture, crude fat, crude protein, ash, fiber and carbohydrate content were analyzed as described for whole plant tissue samples.

Fatty Acid Composition (% relative)
The percent relative fatty acid composition was determined using the AOCS method #Ce 1e-91.

Amino Acid Profile
The amino acid profile was determined using the method by the Association of Official Analytical Chemists (AOAC) 1990 15th Ed 982.30 D,E,F.

Minerals: Ca, P, Cu, Fe, Mg, Mn, K, Zn.

The calcium levels were determined by AOAC (1990 15th Ed.) method 968.08 with modifications as performed by Woodson-Tenent Laboratories.

Phosphorous was determined by AOAC method 965.17.

Potassium, Copper, Iron, Magnesium, Manganese and Zinc were determined using established AOAC methods as performed by Woodson-Tenent Laboratories.

Vitamins: B1, B2, E, and folic acid
Vitamin B1 - AOAC 942.23 with fluorometric quantitation.
Vitamin B2 - AOAC 970.65 with fluorometric quantitation.
Vitamin E - AOAC 971.30 with HPLC quantitiation.
Folic Acid - AOAC 992.05.

Phytic Acid
Phytic acid levels were measured by methods described in Analytical Biochemistry (1977) 77:536-539.

Trypsin Inhibitor
Trypsin inhibitor levels were measured by AOCS method Ba 12-75.

The following analysis was conducted by the Analytical Biochemistry Laboratory (Jan Hazebroek) at Pioneer.

Tocopherols
Tocopherol levels were conducted according to AOAC method 971.30.

D. Control of Bias

The test and control lines were planted in a randomized manner at the field sites. Samples were obtained non-systematically within the plot. The grain samples removed were representative of the entire ear. The lyophilized tissues were ground to a fine powder and mixed before extraction to minimize tissue bias. Plant tissue matrix was added to analytical reference standards where appropriate to control for matrix effects.

E. Data reduction and statistical analyses

Protein expression

All samples were analyzed in specific ELISA's for Cry1F and PAT. Duplicate wells were used for each sample. Sample interpolated values were only used from standard curves which passed the stated quality control criteria.

Absorbance readings from the ELISA's and TEP determinations were recorded using the Bio-Rad Model 3550 plate reader. Data was transferred to a JMP file where mean pg/µg TEP and standard deviation calculations were made for each. If the ELISA value was below the limit of detection (LOD) then a 0 replaced the value and was used in calculating the mean.

The dilution performed to load a constant amount of protein to each PAT-ELISA or Cry1F-ELISA plate well was determined by the following equations:

Volume of extract (µl) = $\frac{\text{amount of total protein/well (µg/well) X final volume (µl)}}{\text{volume/well (µl/well) X total protein concentration (mg/ml)}}$

Volume of PBST (µl) = final volume (µl) – volume of extract (µl)

For the ELISA calculation the absorbance reading from the plate reader was converted into units of pg/well (= x)

Concentration of Cry1F extract $c = \frac{x}{\text{volume of well (µl/well)}}$ in pg/µl

Titer of Cry1F of extract $t = \frac{c}{\text{concentration of protein loaded (µg/50µl)}}$ in pg/µg total protein

A polynomial regression (fit to the second degree) was used to determine the equation for the curve. The regression equation was applied using Bio-Rad software as follows:

$$y = ax^2 + bx + c$$

Solving the above equation for x,

$$x = \frac{\left(-b + \sqrt{b^2 - 4a\,(c - y)}\right)}{2a}$$

Where, x = Cry1F (or PAT) protein concentration, pg/well
y = Absorbance Reading

Therefore, $\text{Cry1F Protein (pg/well)} = \frac{\left(-b + \sqrt{b^2 - 4a\,(c - \text{Absorbance Reading})}\right)}{2a}$

The mean concentration of the duplicate wells was calculated as follows :

$$\text{Mean Cry1F (pg/well)} = \frac{\text{Cry1F well 1} + \text{Cry1F well 2}}{2}$$

The adjusted Mean Results in pg/µg TEP was determined as follows :

Adjusted Mean Cry1F (pg/µg TEP) = Mean Cry1F (pg/well) ÷ Concentration of protein (µg TEP/well)

For example, using the data from maize leaf sample BON-15Ip-1-1-L that was diluted to 0.5 µg TEP/well, pipetted in well A4 (duplicate sample in well B4), and had an absorbance reading of 0.743.

$$\text{Adjusted Mean Cry1F (pg/µg TEP)} = \frac{\text{Mean Cry1F (pg/well)}}{\text{Concentration (µg TEP/well)}}$$

$$= \frac{126.43}{0.5}$$

$$= 252.85$$

The mean values were determined for each tissue as stated above.

Calculations for curve criteria:

$$\text{Predicted pg} = \frac{\left(-b + \sqrt{b^2 - 4a\,(c - \text{Absorbance Reading})}\right)}{2a} \qquad 1\mu g/50\mu l$$

$$\%\,\text{error} = \left(\left|\frac{\text{Predpg} - \text{pg}}{\text{pg}}\right|\right) * 100$$

Nutrient compositional analysis

The experiment was arranged in a randomized complete block design. There were three or six blocks per location, depending on the country. Country was not taken into consideration. There were three entries (treatments), 1507Ip (unsprayed), 1507s (sprayed with glufosinate-ammonium), and 2722 (control).

A mixed model analysis of variance was used to analyze all response variables. The following model was used with the random effects in italics:

Response = TRT *LOC Rep(LOC) LOC*TRT*

Where TRT, LOC and Rep refer to treatment, location and replicate, respectively. Treatment was the only fixed effect in the model.

The analyses were performed by Miliken Associates, Inc. using SAS® PROC MIXED with means and differences generated using the ESTI<ATE statement.

RESULTS AND DISCUSSION

A. Field Trial

The Cry1F maize was grown under conditions representative of the major maize-growing regions of both France and Italy. Samples of test and control hybrids were collected, identified, shipped and stored in a manner to preserve line identity and sample integrity.

B. Protein expression in maize tissue samples

1. **Total protein levels in maize tissues.** The tissue extracts were found to have a total protein concentration ranging from 0.19 to 2.72 mg/ml. The concentration result of each extract was used to calculate the dilution volumes for preparing ELISA plates. Initially, all extracts were diluted to a constant concentration – serial dilutions were then performed to obtain the desired concentrations. No further discussion of the total protein levels will be made in this report.

2. **Cry1F protein levels in leaf, V9 whole plant, pollen, silk, stalk, R1 whole plant, R4 whole plant, grain, and senescent whole plant samples.** Table 17 summarizes the levels of Cry1F protein in the tissue samples. The values represent means across all sites.

   For the test lines there were a total of 20 leaf samples analyzed, five samples from BON, LON and DAU, four samples from MAL, a single sample from TER, and no samples from CAS. A single control sample was analyzed from BON, DAU, MAL, TER. The missing samples were destroyed due to mold problems. The range in Cry1F expression levels across all sites for the test substance leaf samples was from 193.2 to 651.4 pg/µg TEP. The Cry1F expression was below LOD in all leaf samples from the control line.

Three whole plants at the V9 stage of development were pooled at each location to make a single sample for each test entry. Three whole plants of the control line were also pooled at every location except LON, the control plants at the LON location were destroyed by glufosinate-ammonium application. The range of Cry1F expression in the test line ranged from 409.6 to 1526.6 pg/µg TEP. The Cry1F expression was below the LOD in all V9 whole plant control samples.

For pollen, silk and stalk samples there were a total of 30 samples (five from each location) for each of the test lines for each tissue analyzed. For pollen, the level of Cry1F expression ranged from 141.9 to 630.8 pg/µg TEP. The range of Cry1F expression levels in silk was 61.1 to 265.3 pg/µg TEP. And for stalk, the level of Cry1F expression ranged from 417.9 to 917.7 pg/µg TEP. The Cry1F expression levels of all three tissues in the control line were below the LOD.

Three whole plants at the R1 stage of development were pooled at each location to make a single sample for each test entry. Three whole plants of the control line were also pooled at every location except LON, the control plants at the LON location were destroyed by glufosinate-ammonium application. The samples from the BON site were destroyed before analysis due to mold problems. The range of Cry1 expression in the R1 test line ranged from 323.4 to 1206.4 pg/µg TEP. The Cry1F expression was below LOD in all R1 whole plant control samples.

Three whole plants at the R4 stage of development were pooled at MAL, CAS and TER to make a single sample for each test entry (both sprayed and unsprayed) and control entry. Three unsprayed R4 whole plants at LON were pooled to make a single sample for the test and control entries. A storm at DAU prevented the collection of R4 whole plant samples. The test and control samples from BON were moldy upon arrival in Johnston, so they were not analyzed. The range of Cry1F expression in the R4 test line samples ranged from 874.4 to 1576.1 pg/µg TEP in the unsprayed lines and from 556.7 to 575.8 in the sprayed lines. The Cry1F expression was below the LOD in all R4 whole plant control samples.

For the unsprayed test lines there were a total of 20 grain samples analyzed; samples from BON and LON were moldy upon receipt in Johnston and therefore not analyzed. There were a total of 15 grain samples analyzed from the sprayed test lines. Twelve control line grain samples were analyzed, the samples from BON and LON were not analyzed. The level of Cry1F expression levels in the test line grain samples ranged from 44.8 to 135.3 pg/µg TEP in the unsprayed lines and from 57.4 to 131.8 in the sprayed lines. The Cry1F expression was below the LOD in all control grain samples.

The sample for senescent whole plants at each location consisted of three plants pooled together for both the test and control lines. The samples from BON and LON were not analyzed due to mold problems. The range of Cry1F expression levels in the test line senescent whole plants ranged from 171.2 – 219.5 pg/µg TEP. The Cry1F expression in the control line senescent whole plant samples was below the LOD.

3. **PAT protein levels in hybrid leaf, pollen, silk, stalk, whole plant, grain, and senescent whole plant samples.** Table 18 summarizes the levels of PAT protein in the tissue samples. The values represent means across all sites.

   There were 20 test line and control line leaf samples analyzed for PAT expression. The range in PAT expression levels for the test substance leaf samples was from below LOD to 136.8 pg/µg total protein. The PAT expression level was below the level of detection for all control line leaf samples.

For both test and control lines a total of 30 pollen samples, 30 silk samples, 30 stalk, 20 unsprayed grain samples and 15 sprayed samples were analyzed. The PAT expression in all (test and control) pollen, silk, stalk and grain samples was below the level of detection.

The R1, R4 and R9 whole plant samples were collected as described for Cry1F expression. The mean PAT expression was below the level of detection for all test and control samples.

The sample for senescent whole plants at each location also consisted of three plants pooled together, so a total of 4 samples were analyzed for each of the test and control lines. The PAT expression was below the level of detection for all test and control samples.

Since the data are reported in pg/μg TEP and the protein levels vary between tissues it is not possible to make any comparisons between expression levels for the different tissues.

C. Composition analyses

The conclusions in this report are primarily derived from a comparison of nutrient levels in the test line to levels in the literature, based on standard approaches in food safety assessment. A report from food experts (IFBC, 1990) states that "In evaluating a genetically modified food, a comparison with its traditional counterpart will be necessary in order to determine whether the significant nutrients in the new food as consumed fall within the range typical of the product. If the new product is found to have essential nutrients in the same range as its traditional counterpart, no further nutritional evaluation of the product would be required." The term "traditional counterpart" is defined as the comparable traditional foods and ingredients. The report states that the general classes of inherent constituents that should be evaluated include key nutrients, naturally occurring toxicants, and constituents that affect the processing of food; however, an exhaustive analytical comparison is not necessary in most cases. This concept, known as substantial equivalence, has been embodied in regulatory policies such as the US Food and Drug Administration policy on new plant varieties (FDA, 1992).

The statistical analysis included means for the test lines, *B.t.* Cry1F maize line 1507 not sprayed with herbicide, Bt Cry1F maize line 1507 sprayed with glufosinate-ammonium (the test lines), the control line, and a comparison of means.

The forage samples were all dried to between 7 and 14 % moisture before processing. The grain was dried to between 9 and 12 % moisture before shelling. Woodson-Tenent determined the exact moisture content for each sample so the results could be reported on a dry weight basis.

1. Forage analysis

Proximate analysis of maize forage

An analysis of the protein, fat, acid detergent fiber (ADF), neutral detergent fiber (NDF), carbohydrate and ash levels of forage in the test lines and the control hybrid was conducted and the results are shown in Table 19. The levels of protein, fat, carbohydrates, and ash in the test lines and the control line were all within the reported literature ranges for maize forage. The published literature reports means for forage ADF and NDF of 30% and 51%, respectively; however, no range of values is available. The ADF and NDF levels in the test lines and the control were similar to the means reported in the literature. Spraying with glufosinate-ammonium did not have an effect on the proximate levels.

2. Grain analysis

Proximate analysis of maize grain
An analysis of the protein, fat, acid detergent fiber (ADF), neutral detergent fiber (NDF), carbohydrate and ash content of grain from the test lines and the control hybrid was conducted and the results are shown in Table 20. With the exception of ADF, the levels of fat, NDF, carbohydrates, and ash were all within the literature range for maize grain for the test lines and the control. The protein level of 1507 sprayed (12.04) was slightly above the published range of 6.0 – 12.04, but not statistically significantly different than 1507 unsprayed. Levels of ADF in the test lines and the control line were all slightly lower than the published range. However, there was no statistically significant difference in ADF levels between the test and control lines, indicating that the slightly depressed levels in the test lines was not due to the presence of the transgenes or spraying with glufosinate-ammonium.

Mineral analysis of maize grain
Levels of nine minerals (calcium, phosphorous, copper, iron, magnesium, manganese, potassium, sodium, and zinc) were analyzed in the test lines and the control hybrid (Table 21). The mineral levels in the test lines and control lines were within the literature range for maize grain.

Fatty acid composition of maize grain
Grain from the test lines and the control hybrid was analyzed for the five major fatty acids in maize: palmitic (C16:0), stearic (C18:0), oleic (C18:1), linoleic (C18:2), and linolenic (C18:3) (Table 22). The levels of the five fatty acids were all within the literature range for maize grain in the test and control lines.

Amino acid analysis of maize grain
Both the essential and non-essential amino acids were analyzed in grain from test lines and the control hybrid (Table 23). With the exception of threonine and isoleucine, levels of all other essential amino acids were within the range published in the literature or within the range determined by the analysis of 22 commercial Pioneer® Brand hybrids. Levels for threonine in maize line 1507unsprayed and sprayed (0.41 %) were slightly above the published range of 0.29 - 0.39 %. Levels of isoleucine in maize line 1507 unsprayed (0.41 %) and sprayed (0.40 %) were also slightly above the published range of 0.26 – 0.40 %. Levels for several of the non-essential amino acids were slightly above the expected range for both test lines. The minor increases in levels of the above mentioned amino acids were not considered to be nutritionally significant.

Vitamin analysis of maize grain
Grain from the test lines and the control hybrid was analyzed for vitamin content (Table 24). Vitamin B1, vitamin B2, total tocopherols and folic acid levels were determined and levels of vitamins B1 and B2 were found to be within the published range for maize grain. There is no typical range available for folic acid in maize grain, although an average value of 0.3 ppm is reported (Watson, 1987). Levels of folic acid in the test lines and the control line were not significantly different. There was no statistically significant difference in total tocopherols between the test and control lines, but they were all lower than the literature ranges. Tocopherols are rapidly degraded and the storage times and conditions of these samples may have resulted in an overall loss of tocopherol.

Secondary metabolites and anti-nutrients
Grain from the test lines and the control hybrid was analyzed for secondary metabolites and two potential anti-nutrients (Table 25). There were no statistically significant differences between the test lines and the control line for the levels of inositol, p-coumaric acid and ferulic acid. Levels of furfural were below the level of quantitation (0.500 mg/100 g) for the test lines and the control line. There were no literature ranges available except for raffinose. Levels of this metabolite were within the range published in the

literature and there were no significant differences between the test lines and the control line.

Maize grain typically contains low concentrations of the anti- nutrient phytic acid (Cheryan, 1980). Phytic acid levels in the test lines and the control were not significantly different. Maize usually contains only low levels of trypsin inhibitor (Watson, 1987; Del Valle, 1983). As expected, trypsin inhibitor levels in the test lines and the control line were below the limit of quantitation for the enzyme assay that was used in this analysis. This confirms that no unusually high levels of trypsin inhibitor are present in maize line 1507 and that spraying with glufosinate ammonium does not affect the levels of trypsin inhibitors or phytic acid.

CONCLUSIONS

Tissue samples collected from the genetically modified maize hybrid lines and the control hybrid grown in the field site locations were representative of commercially grown maize. Therefore, data collected on protein expression levels in the genetically modified maize hybrid lines were representative of the levels expected in the commercial crop of these maize lines.

Expression of the Cry1F protein was found at measurable levels in all test substance tissues sampled.

Expression of the PAT protein was only found at measurable levels in the leaf tissue samples of the test substances.

The analysis of nutrient composition of forage from maize line 1507 showed that it is comparable to forage from commercial maize hybrids and spraying with glufosinate-ammonium does not have an effect on the nutrient composition of maize forage.

The analysis of nutrient composition of grain from maize line 1507 showed that it is comparable to grain from commercial maize hybrids and spraying with glufosinate-ammonium does not have an effect on the nutrient composition of maize grain.

REFERENCES

Bradford, M.M. 1976. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein Dye-Binding. *Anal. Biochem.* 72: 248-254.

Cheryan, M. 1980. Phytic acid and interactions in food systems. In *CRC Critical Review in Food Science and Nutrition*. pp. 297-335.

Del Valle, F.R., Pico, M.L., Camacho, J.L., Bourges, H. 1983. Effect of processing parameters on trypsin inhibitor and lectin contents of tortillas from whole raw corn-soybean mixtures. *J. Food Science* 48: 246-252.

Iowa Gold Catalog, 1993. Iowa Department of Agriculture and Land Stewardship. 1993 Catalog.

Iowa State University. 1993. How a Corn Plant Grows. ISU Cooperative Extension Service Special Publication 48.

Watson, S.A. 1982. Corn: Amazing Maize. General Properties. pp. 3-29. *In:* CRC Handbook of Processing and Utilization in Agriculture, Vol. II: Part 1 Plant Products. I.A. Wolff (ed.). CRC Press, Inc., Florida.

Watson, S.A. 1987. Structure and Composition. pp. 53-82. *In:* Corn: Chemistry and Technology, S.A. Watson and P.E. Ransted (eds.). American Association of Cereal Chemists, Inc., Minnesota.

Wych, R. D. Production of Hybrid Seed Corn. 1988. pg 603. In *Corn and Corn Improvement*, 3rd ed.; G.F. Sprage and J.W. Dudley, (eds). American Society of Agronomy, Inc. Wisconsin.

Table 1. Field Plot Map: Malagnino, Italy (MAL)

		Plot 1	Plot 2	Plot 3	Plot 4	Plot 5	
	Border 3394	Border 3394	Border 3394	Border 3394	Border 3394	Border 3394	Border 3394
Range 3	Border Control Hybrid 2722	Hybrid 1360 (leafpainted)	Hybrid 1360 (sprayed)	Control Hybrid 2722	Hybrid 1507 (leafpainted)	Hybrid 1507 (sprayed)	Border Control Hybrid 2722
Range 2	Border Control Hybrid 2722	Control Hybrid 2722	Hybrid 1360 (sprayed)	Hybrid 1360 (leafpainted)	Hybrid 1507 (sprayed)	Hybrid 1507 (leafpainted)	Border Control Hybrid 2722
Range 1	Border Control Hybrid 2722	Hybrid 1507 (sprayed)	Hybrid 1507 (leafpainted)	Hybrid 1360 (leafpainted)	Hybrid 1360 (sprayed)	Control Hybrid 2722	Border Control Hybrid 2722
	Border 3394	Border 3394	Border 3394	Border 3394	Border 3394	Border 3394	Border 3394

Border: 1 range both ends (3394) and 2 rows control hybrid 2722 on each side

Table 2. Field Plot Map: Castellucchio, Italy (CAS)

		Plot 1	Plot 2	Plot 3	Plot 4	Plot 5	
	Border 3394	Border 3394	Border 3394	Border 3394	Border 3394	Border 3394	Border 3394
Range 3	Border Control Hybrid 2722	Control Hybrid 2722	Hybrid 1360 (leafpainted)	Hybrid 1360 (sprayed)	Hybrid 1507 (leafpainted)	Hybrid 1507 (sprayed)	Border Control Hybrid 2722
Range 2	Border Control Hybrid 2722	Hybrid 1507 (leafpainted)	Hybrid 1507 (sprayed)	Control Hybrid 2722	Hybrid 1360 (leafpainted)	Hybrid 1360 (sprayed)	Border Control Hybrid 2722
Range 1	Border Control Hybrid 2722	Hybrid 1360 (leafpainted)	Hybrid 1360 (sprayed)	Hybrid 1507 (leafpainted)	Hybrid 1507 (sprayed)	Control Hybrid 2722	Border Control Hybrid 2722
	Border 3394	Border 3394	Border 3394	Border 3394	Border 3394	Border 3394	Border 3394

Border: 1 range both ends (3394) and 1 row control hybrid 2722 on each side

Table 3. Field Plot Map: Terranova dei Passerini, Italy (TER)

		Plot 1	Plot 2	Plot 3	Plot 4	Plot 5	
	Border 3394	Border 3394	Border 3394	Border 3394	Border 3394	Border 3394	Border 3394
Range 3	Border Control Hybrid 2722	Hybrid 1507 (sprayed)	Hybrid 1507 (leafpainted)	Hybrid 1360 (leafpainted)	Hybrid 1360 (sprayed)	Control Hybrid 2722	Border Control Hybrid 2722
Range 2	Border Control Hybrid 2722	Hybrid 1507 (leafpainted)	Hybrid 1507 (sprayed)	Control Hybrid 2722	Hybrid 1360 (leafpainted)	Hybrid 1360 (sprayed)	Border Control Hybrid 2722
Range 1	Border Control Hybrid 2722	Control Hybrid 2722	Hybrid 1507 (leafpainted)	Hybrid 1507 (sprayed)	Hybrid 1360 (sprayed)	Hybrid 1360 (leafpainted)	Border Control Hybrid 2722
	Border 3394	Border 3394	Border 3394	Border 3394	Border 3394	Border 3394	Border 3394

Border: 1 range both ends (3394) and 2 rows control hybrid 2722 on each side

Table 4. Field Plot Map: Bonnut, France (BON)

		Plot 1	Plot 2	Plot 3	Plot 4	Plot 5	Plot 6	
	Border 3394	Border 3394	Border 3394	Border 3394	Border 3394	Border 3394	Border 3394	Border 3394
Range 3	Border 3394	Hybrid 1360 (leafpainted)	Control Hybrid 2722	Hybrid 1507 (leafpainted)	Control Hybrid 2722	Hybrid 1360 (leafpainted)	Hybrid 1507 (leafpainted)	Border 3394
Range 2	Border 3394	Hybrid 1507 (leafpainted)	Hybrid 1360 (leafpainted)	Control Hybrid 2722	Hybrid 1507 (leafpainted)	Control Hybrid 2722	Hybrid 1360 (leafpainted)	Border 3394
Range 1	Border 3394	Control Hybrid 2722	Hybrid 1507 (leafpainted)	Hybrid 1360 (leafpainted)	Hybrid 1360 (leafpainted)	Hybrid 1507 (leafpainted)	Control Hybrid 2722	Border 3394
	Border 3394	Border 3394	Border 3394	Border 3394	Border 3394	Border 3394	Border 3394	Border 3394

Border: 1 range both ends (3394) and 2 rows (3394) on each side

Table 5. Field Plot Map: Longre, France (LON)

		Plot 1	Plot 2	Plot 3	Plot 4	Plot 5	Plot 6	
	Border 3394	Border 3394	Border 3394	Border 3394	Border 3394	Border 3394	Border 3394	Border 3394
Range 3	Border 3394	Hybrid 1360 (leafpainted)	Hybrid 1507 (leafpainted)	Control Hybrid 2722	Hybrid 1360 (leafpainted)	Hybrid 1507 (leafpainted)	Control Hybrid 2722	Border 3394
Range 2	Border 3394	Control Hybrid 2722	Hybrid 1360 (leafpainted)	Hybrid 1507 (leafpainted)	Control Hybrid 2722	Hybrid 1360 (leafpainted)	Hybrid 1507 (leafpainted)	Border 3394
Range 1	Border 3394	Hybrid 1507 (leafpainted)	Control Hybrid 2722	Hybrid 1360 (leafpainted)	Hybrid 1507 (leafpainted)	Control Hybrid 2722	Hybrid 1360 (leafpainted)	Border 3394
	Border 3394	Border 3394	Border 3394	Border 3394	Border 3394	Border 3394	Border 3394	Border 3394

Border: 1 range both ends (3394) and 2 rows (3394) on each side

Table 6. Field Plot Map: Daux, Fance (DAU)

		Plot 1	Plot 2	Plot 3	Plot 4	Plot 5	Plot 6	
	Border 3394	Border 3394	Border 3394	Border 3394	Border 3394	Border 3394	Border 3394	Border 3394
Range 3	Border 3394	Hybrid 1360 (leafpainted)	Hybrid 1507 (leafpainted)	Control Hybrid 2722	Hybrid 1360 (leafpainted)	Hybrid 1507 (leafpainted)	Control Hybrid 2722	Border 3394
Range 2	Border 3394	Control Hybrid 2722	Hybrid 1360 (leafpainted)	Hybrid 1507 (leafpainted)	Control Hybrid 2722	Hybrid 1360 (leafpainted)	Hybrid 1507 (leafpainted)	Border 3394
Range 1	Border 3394	Hybrid 1507 (leafpainted)	Control Hybrid 2722	Hybrid 1360 (leafpainted)	Hybrid 1507 (leafpainted)	Control Hybrid 2722	Hybrid 1360 (leafpainted)	Border 3394
	Border 3394	Border 3394	Border 3394	Border 3394	Border 3394	Border 3394	Border 3394	Border 3394

Border: 1 range both ends (3394) and 2 rows (3394) on each side

Table 7. Important Crop Dates for the 1999 Field Trials Conducted in Italy and France.

Field Site Site Code	Planting	Pollination	Harvest
Malagnino, Italy MAL	5/14/99	7/14/99 – 7/22/99	10/15/99
Castellucchio, Italy CAS	5/14/99	7/8/99 – 7/14/99	10/18/99
Terranova, Italy TER	5/18/99	7/15/99 – 7/23/99	11/2/99
Bonnut, France BON	5/27/99	8/7/99 – 8/13/99	11/17/99
Longre, France LON	5/26/99	7/27/99 – 8/10/99	11/10/99
Daux, France DAU	5/12/99	8/10/99*	after 10/25/99*

* Actual dates were not recorded for these activities; these dates were derived from the sampling dates for pollen and grain.

Table 8. Chemical and Fertilizer Applications for the 1999 Field Trials Conducted in Malagnino, Italy.

Field Site Site Code	Date	Product	Rate (kg ai/ha)
Malagnino, Italy MAL	5/12/99	N-P-K	0-0-180
	5/12/99	N-P-K	92-0-0
	5/15/99	metolachlor + terbuthylazine	1.29 + 0.64
	5/15/99	isoxaflutole	0.05
	5/14/99	carbofuran + isofenphos	10
	6/8/99	N-P-K	184-0-0

Table 9. Chemical and Fertilizer Applications for the 1999 Field Trials Conducted in Castellucchio, Italy.

Field Site Site Code	Date	Product	Rate (kg ai/ha)
Castellucchio, Italy CAS	5/12/99	N-P-K	32-96-96
	5/12/99	N-P-K	138-0-0
	5/15/99	glyphosate	2
	5/15/99	terbuthylazine 37	1
	5/14/99	carbofuran + isofenphos	10
	5/24/99	N-P-K	138-0-0

Table 10. Chemical and Fertilizer Applications for the 1999 Field Trials Conducted in Terranova, Italy.

Field Site Site Code	Date	Product	Rate (kg ai/ha)
Terranova, Italy TER	5/13/99	N-P-K	48-144-144
	5/13/99	N-P-K	135-0-0
	6/16/99	N-P-K	135-0-0
	5/19/99	metolachlor terbuthylazine	1.93 + 0.97
	5/18/99	carbofuran + isofenphos	10
	6/16/99	N-P-K	135-0-0

Table 11. Chemical and Fertilizer Applications for the 1999 Field Trials Conducted in Bonnut, France.

Field Site Site Code	Date	Product	Rate (kg ai/ha)
Bonnut, France BON	4/6/99	N-P-K	0-80-150
	5/30/99	alachlor	1.68
	5/30/99	atrazine	1
	5/27/99	chlormephos	0.3
	7/3/99	N-P-K	270-0-0

Table 12. Chemical and Fertilizer Applications for the 1999 Field Trials Conducted in Longre, France.

Field Site Site Code	Date	Product	Rate (kg ai/ha)
Longre, France LON	4/25/99	N-P-K	0-54-138
	5/10/99	dimethenamid	1.44
	4/25/99	atrazine	0.5
	6/5/99	N-P-K	150-0-0

Table 13. Chemical and Fertilizer Applications for the 1999 Field Trials Conducted in Daux, France.

Field Site Site Code	Date *	Product	Rate (kg ai/ha)
Daux, France DAU	March	N-P-K	0-100-100
	planting	carbofuran	0.6
	V3-V4 stage	N-P-K	40-0-0
	after planting	alachlore	1.92
	after planting	isoxaflutol + aclonifen	0.0525 + 0.35
	V4 stage	N-P-K	90-0-0
	V8 stage	N-P-K	90-0-0

* exact dates not recorded

Table 14. Summary of Rainfall and Temperatures for the 1999 Growing Season in Italy.

Maximum temperature: monthly mean of maximum temperatures
Minimum temperature: monthly mean of minimum temperatures
Total Rainfall: total monthly rainfall

Month	Parameter	MAL	TER
May*	Maximum temperature - °C	25	25
	Minimum temperature - °C	14	14
	Average temperature - °C	20	20
	Total Rainfall – mm	33.60	3.80
June	Maximum temperature - °C	28	27
	Minimum temperature - °C	14	16
	Average temperature - °C	21	21
	Total Rainfall – mm	67.60	22.60
July	Maximum temperature - °C	31	30
	Minimum temperature - °C	17	18
	Average temperature - °C	24	24
	Total Rainfall – mm	22.40	35.21
August	Maximum temperature - °C	30	30
	Minimum temperature - °C	17	18
	Average temperature - °C	23	24
	Total Rainfall – mm	79.50	88.80
September	Maximum temperature - °C	26	25
	Minimum temperature - °C	15	16
	Average temperature - °C	20	21
	Total Rainfall – mm	59.70	150.90
October**	Maximum temperature - °C	22	23
	Minimum temperature - °C	9	10
	Average temperature - °C	16	16
	Total Rainfall – mm	0	24.60

* From the time of planting
** Until the last sample was collected

Weather data was not available for the Castellucchio location.

Table 15. Summary of Rainfall and Temperatures for the 1999 Growing Season in France.

Maximum temperature: monthly mean of maximum temperatures
Minimum temperature: monthly mean of minimum temperatures
Total Rainfall: total monthly rainfall

Month	Parameter	BON	DAU	LON
May*	Maximum temperature - °C	29	22	23
	Minimum temperature - °C	16	13	11
	Average temperature - °C	23	18	17
	Total Rainfall – mm	19.70	60.60	58.50
June	Maximum temperature - °C	25	25	24
	Minimum temperature - °C	14	14	11
	Average temperature - °C	20	20	18
	Total Rainfall – mm	42.40	14.60	37.50
July	Maximum temperature - °C	28	29	28
	Minimum temperature - °C	16	17	14
	Average temperature - °C	22	23	21
	Total Rainfall – mm	66.00	23.40	16.50
August	Maximum temperature - °C	28	29	27
	Minimum temperature - °C	16	18	14
	Average temperature - °C	22	23	20
	Total Rainfall – mm	112.70	38.60	84.50
September	Maximum temperature - °C	27	27	24
	Minimum temperature - °C	14	15	13
	Average temperature - °C	20	21	18
	Total Rainfall – mm	153.80	54.00	168.00
October	Maximum temperature - °C	NA†	20	18
	Minimum temperature - °C	NA	10	9
	Average temperature - °C	NA	15	14
	Total Rainfall – mm	NA	34.20	60.00
November**	Maximum temperature - °C	NA	11	NA
	Minimum temperature - °C	NA	4	NA
	Average temperature - °C	NA	7	NA
	Total Rainfall – mm	NA	71.80	NA

* From the time of planting
** Until the last sample was collected
† Not Available

Table 16. Summary of Tissue Sampling Dates for the 1999 Field Trials.

Field Site Site Code	Malagnino, Italy MAL	Castellucchio, Italy CAS	Terranova, Italy TER	Bonnut, France BON	Longre, France LON	Daux, France DAU
Leaf	6/25/99	6/21/99	6/29/99	7/12/99	7/21/99	7/1/99
V9 Whole Plant	6/25/99	6/21/99	6/29/99	7/12/99	7/21/99	6/28/99
Pollen	7/17/99	7/12/99	7/19/99	8/11/99	8/2/99	8/10/99
Silk	7/17/99	7/12/99	7/19/99	8/13/99 8/11/99	8/4/99	8/10/99
Stalk	7/15/99	7/13/99	7/19/99	8/14/99	8/4/99	8/10/99
R1 Whole Plant	7/16/99	7/13/99	7/21/99	8/14/99	8/4/99	8/5/99
R4 Whole Plant	8/23/99	8/6/99	8/23/99	9/24/99	9/8/99 9/7/99 9/6/99 9/3/99	NR*
Grain	9/22/99	9/21/99	10/7/99	11/8/99	10/5/99	10/25/99
Senescent Whole Plant	10/10/99	9/30/99	10/14/99	11/8/99	11/9/99	NR

* R4 whole plant samples were not collected due to storm damage.

Table 17. Summary of Cry1F protein levels measured in tissue collected from Bt maize hybrid line 1507 during the 1999 growing season in France and Italy

Tissue/Line	Mean pg/µg TEP*	Std Deviation	Min/Max Range pg/µg TEP	Number of Samples**
Leaf				
1507 lp***	348.0	160.9	193.2 - 651.4	20/ 0<LOD†
V9 Whole Plant				
1507 lp	743.7	394.2	409.6 - 1526.6	6/ 0<LOD
Pollen				
1507 lp	190.5	84.4	141.9 - 630.8	30/ 0<LOD
Silk				
1507 lp	133.0	58.1	61.1 - 265.3	30/ 0<LOD
Stalk				
1507 lp	630.8	141.6	417.9 - 917.7	30/ 0<LOD
R1 Whole Plant				
1507 lp	671.9	348.2	323.4 - 1206.4	5/ 0<LOD
R4 Whole Plant				
1507 lp	1073.1	338.2	874.4 - 1576.1	4/ 0<LOD
1507 s‡	569.4	11.0	556.7 - 575.8	3/ 0<LOD
Grain				
1507 lp	96.4	25.9	44.8 – 135.3	20/ 0 <LOD
1507 s	90.3	21.8	57.4 - 131.8	15/ 0 <LOD
Whole Plant Senescent				
1507 lp	198.9	21.4	171.2 – 219.5	4/ 0<LOD

* TEP = Total extractable protein.
** Total number of samples used in the calculations, and number of samples with values below the LOD.
*** lp = leaf painted with glufosinate-ammonium.
† <LOD= below the empirical limit of detection. [10pg/µg TEP]
‡ s = sprayed with glufosinate-ammonium.

Table 18. Summary of PAT protein levels measured in tissue collected from Bt maize hybrid line 1507 during the 1999 growing season in France and Italy

Tissue/Line	Mean pg/µg TEP*	Std Deviation	Min/Max pg/µg TEP	Number of Samples**
Leaf				
1507 lp***	42.0	32.3	<LOD† - 136.8	20/ 3<LOD
V9 Whole Plant				
1507 lp	<LOD	15.5	<LOD - 38.0	6/ 5<LOD
Pollen				
1507 lp	0	NA#	NA	30/ 30<LOD
Silk				
1507 lp	0	NA	NA	30/ 30<LOD
Stalk				
1507 lp	0	NA	NA	30/ 30<LOD
R1 Whole Plant				
1507 lp	0	NA	NA	5/ 5<LOD
Whole Plant Forage				
1507 lp	0	NA	NA	4/ 4<LOD
1507 s‡	0	NA	NA	3/ 3<LOD
Grain				
1507 lp	0	NA	NA	20/ 20 <LOD
1507 s	0	NA	NA	15/ 15 <LOD
Whole Plant Senescent				
1507 lp	0	NA	NA	4/ 4<LOD

* TEP = Total extractable protein.
** Total number of samples used in the calculations, and number of samples with values below the LOD.
*** <LOD= below the empirical limit of detection. [20 pg/µg TEP].
† lp = leaf painted with glufosinate-ammonium.
‡ s = sprayed with glufosinate-ammonium.
# NA = not applicable.

TABLE 19. PROXIMATE ANALYSIS – FORAGE

Proximate Analysis of Forage				
Response Variable	**1507lp (unsprayed)**	**1507s (sprayed)**	**2722 (control)**	**Range of Values in Literature***
Fat %	2.48[a] ± 0.30	2.42[a] ± 0.30	2.48[a] ± .030	0.7 - 6.7
Protein %	8.72 [a] ± 0.29	9.27[a] ± 0.31	8.87[a] ± 0.29	3.5 - 15.9
ADF %	28.07[a] ± 1.55	28.46[a] ± 1.58	28.68[a] ± 1.54	30**
NDF %	50.62[a] ± 1.93	50.15[a] ± 2.03	50.83[a] ± 1.96	51**
Carbohydrates %***	84.25[a] ± 0.63	83.50[a] ± 0.65	84.00[a] ± 1.31	66.9 – 94.5
Ash %	4.56[a] ± 0.28	4.81[a] ± 0.29	4.63[a] ± 0.28	1.3 - 10.5

Estimated mean values (across all sites) ±SE followed by different letters are significantly different ($\alpha = 0.05$). Data presented as % of dry weight.

* Watson, 1982
** Watson, 1982 reports an average value for ADF of 30% and NDF of 51%.
*** Carbohydrates are calculated as the percentage of dry weight =100% - % protein - % fat - % ash. Fiber (ADF and NDF) is included in the carbohydrates.

TABLE 20. PROXIMATE ANALYSIS – GRAIN

Proximate Analysis of Grain				
Response Variable	**1507Ip (unsprayed)**	**1507s (sprayed)**	**2722 (control)**	**Range of Values in Literature***
Fat %	4.21[a] ± 0.12	4.41[a] ± 0.14	4.41[a] ± 0.12	3.1 - 5.7*
Protein %	11.73[a] ± 0.24	12.04[a] ± 0.28	10.98[b] ± 0.24	6.0 – 12*
ADF %	2.37[a] ± 0.17	2.52[a] ± 0.18	2.29[a] ± 0.17	3.0 – 4.3**
NDF %	10.16[a] ± 0.30	10.54[a] ± 0.35	10.13[a] ± 0.30	8.3 – 11.9*
Carbohydrates % ***	82.46[ab] ± 0.57	81.97[b] ± 0.25	83.00[a] ± 0.28	63.3 – 89.7**
Ash %	1.60[a] ± 0.04	1.67[a] ± 0.05	1.56[a] ± 0.04	1.1 - 3.9**

Estimated mean values (across all sites) ±SE followed by different letters are significantly different ($\alpha = 0.05$). Data presented as % of dry weight.

* Watson, 1987.
** Watson, 1982.
*** Carbohydrates are calculated as the percentage of dry weight =100% - % protein - % fat - % ash. Fiber (ADF and NDF) is included in the carbohydrates.

TABLE 21. MINERAL COMPOSITION – GRAIN

Mineral Analyses of Grain				
Response Variable	**1507lp (unsprayed)**	**1507s (sprayed)**	**2722 (control)**	**Range of Values in Literature****
Calcium %	0.008[a] ±0.001	0.007[a] ± 0.001	0.007[a] ± 0.001	0.01 - 0.10 0.002 - 0.011***
Phosphorous %	0.33[ab] ± 0.008	0.34[a] ± 0.010	0.31[b] ± 0.008	0.26 - 0.75
Copper ppm	1.88[a] ± 0.52	1.53[a] ± 0.57	1.35[a] ± 0.52	0.9 – 10
Iron %	0.0021[ab] ± 0.00005	0.0021[a] ± 0.00006	0.0019[b] ± 0.00005	0.0001 – 0.01
Magnesium %	0.114[a] ± 0.0036	0.117[a] ± 0.0041	0.106[a] ± 0.0036	0.09 - 1.0
Manganese %	0.0008[a] ± 0.00004	0.0008[a] ± 0.00050	0.0008[a] ± 0.00004	0.00007 – 0.0054
Potassium %	0.416[a] ± 0.016	0.417[a] ± 0.012	0.380[b] ± 0.016	0.32 – 0.72
Sodium* %	0.0015[a] ± 0.0	0.0015[a] ± 0.0	0.0015[a] ± 0.0	0.0 – 0.15
Zinc %	0.0018[a] ± 0.0001	0.0017[a] ± 0.0001	0.0019[a] ± 0.0001	0.0012 – 0.0030

Estimated mean values (across all sites) ±SE followed by different letters are significantly different ($\alpha = 0.05$). Data presented as % of dry weight.

* All values were below the level of quantitation for sodium of 0.01%
** Watson, 1982.
*** Data from analysis of 22 commercial Pioneer® Brand Hybrids.

TABLE 22. FATTY ACID COMPOSITION – GRAIN

Fatty Acid Analyses of Grain				
Response Variable	**1507lp (unsprayed)**	**1507s (sprayed)**	**2722 (control)**	**Range of Values in Literature***
Palmitic %	9.87[a] ± 0.072	9.85[a] ± 0.081	9.83[a] ± 0.072	7 – 19
Stearic %	2.09[a] ± 0.065	2.03[a] ± 0.067	2.11[a] ± 0.065	1 – 3
Oleic %	33.06[a] ± 0.50	33.21[a] ± 0.52	32.63[a] ± 0.50	20 – 46
Linoleic %	51.38[a] ± 0.79	51.42[a] ± 0.82	51.90[a] ± 0.79	35 – 70
Linolenic %	1.16[a] ± 0.03	1.17[a] ± 0.03	1.17[a] ± 0.03	0.8 – 2

Estimated mean values (across all sites) ±SE followed by different letters are significantly different ($\alpha = 0.05$). Data presented as % of total fat.

* Watson, 1982.

TABLE 23. AMINO ACID COMPOSITION – GRAIN

Amino Acid Analyses of Grain				
Essential Amino Acids				
Response Variable	**1507lp (unsprayed)**	**1507s (sprayed)**	**2722 (control)**	**Range of Values in Literature**
Glycine	0.41[a] ± 0.0090	0.42[a] ± 0.0102	0.38[b]± 0.0090	0.26 - 0.47* 0.24 - 0.41**
Threonine	0.41[a]± 0.0080	0.41[a] ± 0.0094	0.37[b] ± 0.0080	0.29 - 0.39* 0.21 - 0.37**
Valine	0.51[a] ± 0.0106	0.52[a] ± 0.0125	0.47[b] ± 0.0106	0.21 - 0.52* 0.25 - 0.67**
Isoleucine	0.41[a] ±0.0098	0.41[a] ± 0.0116	0.36[a] ± 0.0098	0.26 - 0.40* 0.19 - 0.39**
Leucine	1.38[a] ± 0.03	1.41[a] ± 0.04	1.23[b] ± 0.04	0.78 -1.52* 0.43 - 1.35**
Phenylalanine	0.55[a] ± 0.018	0.56[a] ± 0.014	0.49[b] ± 0.012	0.29 - 0.57* 0.04 - 0.54**
Histidine	0.31[a] ± 0.0065	0.32[a] ± 0.0076	0.29[b] ± 0.0065	0.20 - 0.28* 0.21 - 0.32**
Lysine	0.32[a] ± 0.008	0.33[a] ± 0.009	0.31[a] ± 0.008	0.20 - 0.38* 0.19 - 0.36**
Arginine	0.47[a] ± 0.012	0.48[a] ± 0.014	0.44[a] ± 0.012	0.29 - 0.59* 0.28 - 0.55**

Table 23. Amino acid composition - Grain (continued)

Response Variable	1507lp (unsprayed)	1507s (sprayed)	2722 (control)	Range of Values in Literature
Cysteine	0.22[a] ± 0.004	0.23[a] ± 0.005	0.22[a] ± 0.004	0.12 - 0.16* 0.13 - 0.27**
Methionine	0.20[a] ± 0.0035	0.21[a] ± 0.0041	0.20[a] ± 0.0035	0.10 - 0.21* 0.12 - 0.26**
Tryptophan	0.10[a] ± 0.0035	0.10[a] ± 0.0037	0.09[a] ± 0.0035	0.05 - 0.12* 0.05 - 0.10**
Non-essential Amino Acids				
Serine	0.55[a] ± 0.012	0.56[a] ± 0.014	0.50[b] ± 0.012	0.42 - 0.55* 0.25 - 0.46**
Alanine	0.83[a] ± 0.018	0.85[a] ± 0.022	0.74[b] ± 0.018	0.64 - 0.99* 0.37 - 0.81**
Glutamic Acid	2.12[a] ± 0.050	2.18[a] ± 0.060	1.90[b] ± 0.050	1.24 - 1.96* 0.89 - 2.02**
Proline	1.00[a] ± 0.0212	1.04[a] ± 0.0258	0.92[b] ± 0.0217	0.66 - 1.03* 0.43 - 1.01**
Aspartic Acid	0.79[a] ± 0.0157	0.81[a] ± 0.0186	0.71[b] ± 0.0157	0.58 – 0.72* 0.37 - 0.80**
Tyrosine	0.21[a] ± 0.0048	0.21[a] ± 0.0057	0.19[b] ± 0.0048	0.29 - 0.47* 0.17 - 0.31***

Estimated mean values (across all sites) ±SE followed by different letters are significantly different ($\alpha = 0.05$). Data presented as % of dry weight.

* Watson, 1982.
** Data from analysis of 22 commercial Pioneer® Brand Hybrids.
*** Iowa Gold Catalog, 1994.

TABLE 24. VITAMIN COMPOSITION – GRAIN

Vitamin Analyses of Grain				
Response Variable	**1507lp (unsprayed)**	**1507s (sprayed)**	**2722 (control)**	**Range of Values in Literature***
Thiamine Hydrochloride (B1) ppm	3.502[a] ± 0.184	3.874[a] ± 0.208	3.818[a] ± 0.184	3.0 – 8.6
Riboflavin (B2) ppm	1.208[b] ± 0.037	1.199[b] ± 0.045	1.314[a] ± 0.037	0.25 – 5.6
Folic Acid ppm	0.158[a] ± 0.005	0.161[a] ± 0.007	0.154[a] ± 0.005	0.3**
Total tocopherols ppm	28.51[a] ± 1.15	29.30[a] ± 1.40	29.24[a] ± 1.15	42 - 87

Estimated mean values (across all sites) ±SE followed by different letters are significantly different ($\alpha = 0.05$). Data presented as parts per million (ppm) on a dry weight basis.

* Watson, 1982
** Watson, 1987 reports an average value for folic acid in grain as 0.3 mg/kg.

TABLE 25. SECONDARY METABOLITES AND ANTI-NUTRIENTS– GRAIN.

Secondary Metabolites in Grain				
Response Variable	**1507Ip (unsprayed)**	**1507s (sprayed)**	**2722 (control)**	**Range of Values in Literature***
Inositol mg/100 g	45.75 [a] ± 3.17	48.303 [a] ± 3.73	45.43 [a] ± 3.17	NA
Raffinose %	0.099 [a] ± 0.019	0.089 [a] ± 0.021	0.107 [a] ± 0.019	0.08 – 0.30
P-Coumaric Acid mg/100 g	20.34 [a] ± 2.65	20.98 [a] ± 2.99	16.83 [a] ± 2.65	NA
Furfural mg/100g	0**	0**	0**	NA
Ferulic Acid mg/100 g	273.85 [a] ± 6.84	284.51 [a] ± 8.21	260.54 [a] ± 6.84	NA
Anti-nutrient Analyses of Grain				
Phytic acid %	1.03 [a] ± 0.022	0.98 [a] ± 0.027	0.969 [a] ± 0.022	0.7 – 1.0
Trypsin Inhibitor TIU/g	0***	0***	0***	NA

Estimated mean values (across all sites) ±SE followed by different letters are significantly different (α = 0.05). Data presented as percentage (%) or mg/100 g on a dry weight basis. Trypsin inhibitor is expressed in units of trypsin inhibitor enzyme activity per gram on a dry basis.

* Watson, 1982.
** Below level of quantitation for furfural of 0.500 mg/100 g.
*** Below level of quantitation for trypsin inhibitor of 2000 TIU/g.
NA Ranges are not available in the published literature.