

SUMMARY

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for public release after registration)

STUDY TITLE (INTERIM REPORT)

Quantitative ELISA Analysis of poCry1F and PAT Protein Expression Levels in Hybrid and Inbred Lines of  
TC1507 and an Inbred Line of TC1360

DATA REQUIREMENTS

None

AUTHOR

[REDACTED]

STUDY COMPLETED ON

February 27, 2001

PERFORMING LABORATORY

Pioneer Hi-Bred International, Inc.  
7100 NW 62<sup>nd</sup> Ave.  
Johnston, Iowa 50131-1000

LABORATORY STUDY ID

PHI99-001

TOTAL NUMBER OF PAGES

2

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The purpose of this study was:

- 1) to generate leaf, whole plant (forage and senescent), silk, pollen, stalk, and grain tissue samples from hybrid and inbred lines of 1507 and non-transformed control lines, and
- 2) to measure levels of Cry1F and PAT proteins in tissues collected in test and control line plants.

The test system for this study consisted of four field sites located in the major maize growing region of the United States. Cry1F and PAT protein levels were measured in each of the samples using specific ELISA (Enzyme Linked ImmunoSorbent Assay) methods.

The conclusions from this study were:

- Expression of the Cry1F protein was found at measurable levels in all test substance tissues sampled. The results are similar to the expression levels found in other *B.t.* Cry1F maize hybrid lines (Stauffer and Rivas, 1999) where it was observed that levels of Cry1F protein expression were highest in whole plant and stalk tissues and lower in leaf, pollen and grain tissues. Cry1F protein expression was lowest in silk tissue in both studies.
- Expression of the PAT protein was only found at measurable levels in leaf tissue samples of the hybrid test lines. These results are comparable to the PAT protein expression levels observed in a *B.t.* Cry1F maize line 1507 hybrid (Stauffer and Rivas, 1999).



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STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

No claim of confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA Section 10 (d)(1)(A)(B), or (C).\*

Company: Dow AgroSciences LLC

Company Agent: [REDACTED]

Title: Regulatory Manager

Signature: [REDACTED]

Date: 2/23/01

\*In the United States, the above statement supersedes all other statements of confidentiality that may occur elsewhere in this report.

THIS DATA MAY BE CONSIDERED CONFIDENTIAL IN COUNTRIES OUTSIDE THE UNITED STATES.

## STATEMENT OF COMPLIANCE WITH GOOD LABORATORY PRACTICE STANDARDS

Title: Quantitative ELISA Analysis of poCry1F and PAT Protein Expression Levels in Hybrid and Inbred Lines of TC1507 and an Inbred Line of TC1360

Study Initiation Date: 05/03/1999 Study Completion Date: 02/27/01

Experimental Start Date: 05/3/1999 Experiment Termination Date: 06/27/00

This report represents data generated after the effective date of the EPA FIFRA Good Laboratory Practice Standards: United States Environmental Protection Agency, Title 40 Code of Federal Regulations Part 160, FEDERAL REGISTER, August 17, 1989. Organisation for Economic Co-Operation and Development, ISBN 92-64-12367-9, Paris 1982

For the purposes of this interim report, all aspects of this study were conducted in accordance with the requirements for Good Laboratory Practice Standards, 40 CFR 160 with the following exceptions:

- There were instances of data having been recorded in pencil, as well as late data entries, data not being initialed and/or dated at the time of entry, and where data corrections were improperly made.  
Impact on study: Minimal - All data were saved and reasonably complete for reconstruction purposes.
- Not all equipment maintenance was recorded according to GLP Standards requirements.  
Impact on study: Minimal - records were reasonably complete.
- A formally approved standard operating procedure (SOP) was not in place for the planter used at the two Johnston field sites.  
Impact on study: Minimal - description and operating procedure for planter was available.
- Weather data at several field sites were not collected or were not collected according to GLP Standards requirements.  
Impact on study: Minimal - weather data is considered incidental.
- Some aspects of the study, i.e. drying and grinding of forage specimens, cannot be totally reconstructed from the raw data.  
Impact on study: Deemed to be minimal.
- Characterization and stability of the reference substances used in this study were not conducted under GLP Standards.  
Impact: on study: Minimal - reference standards were of known and documented purity or strength.  
Stability was verified through consistency of reference standard response in the ELISA procedure over the duration of the study.

None of these departures from the GLP Standards were deemed to have compromised the overall integrity of the study.

02-26-01

Date

Sponsor

Pioneer Hi-Bred International, Inc.

2/23/01

Date

Submitter

Dow AgroSciences LLC

2/27/2001

Date

Study Director

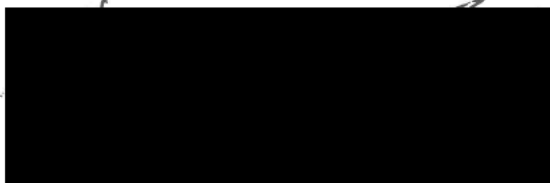
Pioneer Hi-Bred International, Inc.

## QUALITY ASSURANCE STATEMENT

Study PHI99-001 was inspected/audited by the Pioneer Hi-Bred International, Inc. quality assurance unit (QAU) for compliance with EPA's Good Laboratory Practice Standards (40 CFR Part 160). All findings were reported to the study director and study management. The phase inspected/audited, the dates of each inspection or audit, and the dates that findings and recommendations were reported are summarized as follows:

Phase Inspected/Audited	Inspection/Audit Date	Date Reported*
Protocol review	April 19 - 23, 1999	April 26, 1999
In-progress - Planting, AD1, AD2	May 10, 1999	May 14, 1999
In-progress - Planting, NO	May 12, 1999	May 27, 1999
In-progress - Specimen collection, NO	July 19 and 21, 1999	July 23, 1999
In-progress - Pollination & tassel bagging, WN	July 22, 1999	July 30, 1999
In-progress - Specimen collection, NO	Sept 20, 1999	Sept 24, 1999
In-progress - Specimen collection, AD1, AD2	Sept 21, 1999	Sept 27, 1999
Data audit - Field notebook, NO	Nov 19, 1999	Nov 23, 1999
In-progress - ELISA analysis	March 15, 2000	March 15, 2000
Data audit - Field notebook, WN	Jan 26, 27 and 28, 2000	Jan 28, 2000
Data audit - Field notebooks, AD1, AD2	Jan 26, 27 and 28, 2000	Mar 8, 2000
Data audit - ELISA Laboratory	May 11, 12, 19 and 23, 2000	June 1, 2000
Draft final report review	August 21, 22 and 24, 2000	August 24, 2000

\* Date reported to study director and study management



Quality Assurance Representative  
QUALITY ASSOCIATES, INC.

Date

2/22/01

REPORT APPROVAL

**Study Number:** PHI99-001

**Title:** Quantitative ELISA Analysis of poCry1F and PAT Protein Expression Levels in Hybrid and Inbred Lines of TC1507 and an Inbred Line of TC 1360

**Testing Facility:** Pioneer Hi-Bred International, Inc.  
7300 NW 62<sup>nd</sup> Ave.  
Johnston, Iowa 50131-1004

**Field Sites:** Johnston, Iowa – Site Code AD1  
Johnston, Iowa – Site Code AD2  
Noblesville, Indiana - Site Code NO  
Windfall, Indiana – Site Code WN

**Study Sponsor:** [REDACTED], Pioneer Hi-Bred International, Inc.

**Study Director:** [REDACTED], Pioneer Hi-Bred International, Inc.

**Contributors:** Analytical Principal Investigator – [REDACTED], Pioneer Hi-Bred International, Inc.  
Principal Analyst – [REDACTED], Pioneer Hi-Bred International, Inc.  
Field Principal Investigators – [REDACTED], Heartland Technologies, Inc. (NO)  
[REDACTED], Pioneer Hi-Bred International, Inc. (WN)  
[REDACTED], Pioneer Hi-Bred International, Inc. (AD2)  
[REDACTED], Pioneer Hi-Bred International, Inc. (AD1)  
Contributing Scientist – [REDACTED] (forage sample processing), Pioneer Hi-Bred International, Inc., Polk City Livestock Nutrition Center

**Study Initiation Date:** May 3, 1999

**Records Retention:** All study specific raw data, protocols, final reports and facility records will be maintained at the Pioneer Hi-Bred International, Inc. archive facility.

**Test, Control and Reference Substance Storage:** All retain samples will be stored at Pioneer Hi-Bred International, Inc. in Johnston, IA.

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

Report issued by:

[REDACTED]

2/27/2001  
Date

02-26-01  
Date

TABLE OF CONTENTS	PAGE
CERTIFICATION OF NO DATA CONFIDENTIALITY.....	2
STATEMENT OF COMPLIANCE .....	3
QUALITY ASSURANCE STATEMENT .....	4
REPORT APPROVAL .....	5
TABLE OF CONTENTS .....	6
SUMMARY .....	7
I. INTRODUCTION .....	8
II. MATERIALS .....	8
III. METHODS .....	9
IV. RESULTS AND CONCLUSIONS .....	14
V. REFERENCES .....	15
TABLES	
Table 1. Field plot map: Johnston, IA (AD1) .....	16
Table 2. Field plot map: Johnston, IA (AD2) .....	17
Table 3. Field plot map: Noblesville, IN (NO) .....	18
Table 4. Field plot map: Windfall, IN (WN) .....	19
Table 5. Important crop dates for the field trials .....	20
Table 6. Maintenance pesticide and fertilizer applications for the field trial conducted in Johnston, IA (AD1) .....	21
Table 7. Maintenance pesticide and fertilizer applications for the field trial conducted in Johnston, IA (AD2) .....	22
Table 8. Maintenance pesticide and fertilizer applications for the field trial conducted in Noblesville, IN .....	23
Table 9. Maintenance pesticide and fertilizer applications for the field trial conducted in Windfall, IN. ....	24
Table 10. Summary of temperatures and rainfall for the 1999 growing season .....	25
Table 11. Summary of Tissue Sampling Dates for the 1998 Field Trials .....	26
Table 12. Levels of Cry1F protein measured in tissues collected from maize hybrid line 1507 .....	27
Table 13. Levels of Cry1F protein measured in tissues collected from maize inbred line 1507 .....	27
Table 14. Levels of PAT protein measured in tissues collected from maize hybrid line 1507 .....	28
Table 15. Levels of PAT protein measured in tissues collected from maize inbred line 1507 .....	28
APPENDIX I. PROTOCOL AMENDMENTS AND DEVIATIONS .....	29
APPENDIX II. REFERENCES .....	43

## SUMMARY

Maize line 1507 was 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, this line also contained a synthetic *pat* gene, derived from *Streptomyces viridochromogenes*, which encodes phosphinothricin acetyl transferase (PAT). The PAT protein is an enzyme that inactivates herbicides with glufosinate-ammonium as the active ingredient and thus makes genetically modified plants that accumulate this protein tolerant to the herbicides.

The purpose of this study was:

- 3) to generate leaf, whole plant (forage and senescent), silk, pollen, stalk, and grain tissue samples from hybrid and inbred lines of 1507 and non-transformed control lines, and
- 4) to measure levels of Cry1F and PAT proteins in tissues collected in test and control line plants.

The test system for this study consisted of four field sites located in the major maize growing region of the United States. Cry1F and PAT protein levels were measured in each of the samples using specific ELISA (Enzyme Linked ImmunoSorbent Assay) methods.

The conclusions from this study were:

- Expression of the Cry1F protein was found at measurable levels in all test substance tissues sampled. The results are similar to the expression levels found in other *B.t.* Cry1F maize hybrid lines (Stauffer and Rivas, 1999) where it was observed that levels of Cry1F protein expression were highest in whole plant and stalk tissues and lower in leaf, pollen and grain tissues. Cry1F protein expression was lowest in silk tissue in both studies.
- Expression of the PAT protein was only found at measurable levels in leaf tissue samples of the hybrid test lines. These results are comparable to the PAT protein expression levels observed in a *B.t.* Cry1F maize line 1507 hybrid (Stauffer and Rivas, 1999).

## I. INTRODUCTION

### A. Background

Maize lines, designated 1360 and 1507, were genetically modified to express the Cry1F protein from *Bacillus thuringiensis* subsp. *aizawai*. Cry1F protein confers resistance to the European corn borer (*Ostrinia nubilalis* Hubner) insect pest. These lines also contain a synthetic *pat* gene, derived from *Streptomyces viridochromogenes*, which encodes phosphinothricin acetyl transferase (PAT). The PAT protein is an enzyme that inactivates herbicides containing glufosinate-ammonium as the active ingredient. Genetically modified plants that accumulate PAT protein are tolerant to the herbicide. After study initiation, it was determined that line 1360 was not exhibiting the expected agronomic performance and it was removed from the study.

The transgenic hybrid and inbred lines (test substance lines) used in this study were obtained from seed produced during the process of backcrossing the original transformant with elite inbred genetics. The nontransgenic hybrid and inbred control lines possessed background genetics similar to the respective test substance lines, but did not express the Cry1F or PAT proteins.

### B. Purpose

The purpose of this study was to generate data on the expression levels of Cry1F and PAT proteins leaf, silk, stalk, pollen, grain, and whole plant (forage and senescent) tissues from hybrid and inbred test and control substance lines.

## II. MATERIALS

### A. Test substances

The test substances for this study were seeds of a hybrid and an inbred line of event 1507 that were capable of expressing the Cry1F and PAT proteins. The seed source for the test substances was the Dow Mycogen breeding nursery in Puerto Rico. Details regarding the source of the test substances and other necessary supporting information were recorded in the raw data. The test substance seed was segregating for the *cry1F* and *pat* genes.

Initial characterization of the test substances consisted of documentation of the breeding lineage of the seed. Pedigree information for the 1507 hybrid and inbred lines is proprietary information and is on file with staff breeders at Pioneer Hi-Bred International, Inc., Johnston, Iowa. Prior to planting, the seed was stored under appropriate conditions to maintain seed viability and vigor (Wych, 1988). Further characterization of the test substances occurred during the study via confirmation of glufosinate-ammonium tolerance in the field and detection and quantification of the Cry1F and PAT proteins by ELISA. The test substance seed was considered stable for an extended period of time (at least 5 years) under cold room storage conditions.

### B. Control substances

The control lines for this study were comprised of plants from hybrid and inbred lines that did not contain the *cry1F* and *pat* genes. The control lines possessed background genetics similar to the test substance lines. Pedigree information for the control lines is on file with staff breeders at Pioneer Hi-Bred International, Inc., Johnston, Iowa. Prior to planting, the seed was stored under appropriate conditions to maintain seed viability and vigor (Wych, 1988). The control substance seed was considered stable for an extended period of time (at least 5 years) under cold room storage conditions appropriate for seed.



### C. Reference substances

The reference substances for the analytical portion of this study were purified Cry1F and PAT proteins used as standards in the ELISA analysis and bovine serum albumin (BSA) protein as the standard for total protein analysis.

#### Reference substances for ELISA analysis

**Cry1F protein standard.** Cry1F protein standard (Lot #082597; 1.4 mg/mL) was provided by Mycogen Seeds and was purified from *Pseudomonas fluorescens* (strain MR872). Characterization was by SDS-PAGE and amino acid analysis. No expiration date was provided. The solution was stored at 2 - 8°C (long term storage and short term storage).

**PAT protein standard.** PAT protein standard (Lot #050195; 0.89 mg/mL) was purified from *E. coli* strain BL21 encoding the *pat* gene. Characterization was by SDS-PAGE/silver stain, sequencing and amino acid analysis. The solution was stored long-term at -78 to -85°C and short-term at 2-8°C.

#### Reference Substance for the Bradford Assay

**Bio Rad Protein Assay Standard II.** (Bio Rad #500-0007 or equivalent, Lot #64995A, expiration date of 1/12/2005). The Protein Assay Standard II was obtained from Bio Rad with a BSA purity of 64.46% (Bio Rad reference). The concentrated protein was diluted in distilled water. The diluted solution is kept at -10 to -24°C for long term storage (shelf life is one year per manufacturer) and at 2-8°C for short term storage (shelf life is 60 days per manufacturer).

### D. Test system

The test and control substances were planted and grown at several locations within the midwestern maize growing region of the U.S. The fields at these sites were considered the test system.

<u>Field Sites</u>	<u>County</u>	<u>Site Code</u>	<u>Field Principal Investigator</u>
Johnston, Iowa	Polk	AD1	B. Swanson
Johnston, Iowa	Polk	AD2	B. Pauli
Noblesville, Indiana	Hamilton	NO	K. Kiser
Windfall, Indiana	Tipton	WN	K. Freeman

The test system locations represented areas where the maize lines would be suitable commercial products.

## III. METHODS

### A. Summary of experimental design

The test substance and control substance lines were grown at four field sites in the United States. Each field trial (i.e. experiment) was arranged in a randomized complete block design (for field plot maps see Tables 1-4). Replicate 1 contained two hybrid entries (test substance hybrid line 1507 and the control hybrid). Replicate 2 contained three inbred entries (test substance inbred line 1507, the control inbred, and the test substance inbred line 1360, which was later removed from the study by protocol amendment). All replicates were divided into plots consisting of 2 rows each with one plot per hybrid and one plot per inbred entry. Plots containing hybrid and inbred line 1507 were planted at a seeding rate of 40 to 60 plants per plot. Leaf, whole plant (forage and senescent), pollen, silk, stalk, and grain tissue samples were collected from plants from the test and control lines. The samples were processed and then evaluated for Cry1F and PAT protein expression using ELISA methods specific for each protein.

## B. Field trials

All sites were managed so that the identity and integrity of all collected tissue samples were maintained. Important crop dates (i.e. seed receipt, planting, pollination and trial termination) are listed in Table 5.

### 1. Planting

The field at each site went through multiple plowings and cultivations to prepare the soil prior to planting. Plots containing 1507 lines were planted at a seeding rate of 40 to 60 plants per plot. Each row was approximately 17 and 1/2 feet in length with two rows per plot. Within a row, seeds were placed 5 to 12 inches apart. All rows were approximately 30 inches apart. Tables 1-4 show the field plot maps used during planting.

### 2. Agronomic practices

Agricultural practices for growing the test and control plants were typical for producing maize in the regions chosen for this study. Maintenance pesticide and fertilizer applications were applied as appropriate for each location; application dates and amounts are summarized for each location in Tables 6-9.

### 3. Glufosinate Application

Because the test substance lines were segregating for the *pat* and *cry1F* genes, it was necessary to identify positive plants that were expressing the PAT and Cry1F proteins. Since the two genes are genetically linked, plants that are tolerant to glufosinate-ammonium herbicide are also considered to be expressing the Cry1F protein. Therefore, the test substance lines were treated with glufosinate-ammonium herbicide by leaf painting plants at approximately the V4 to V5 stage of development. Plants that were damaged by the herbicide were assumed to lack the *pat* and *cry1F* genes and were removed from the plots.

### 4. Weather

Precipitation and air temperature were monitored and documented at each site and are summarized in Table 10. Rainfall was sufficient to produce maize typical of the growing area.

### 5. Sampling

All samples were uniquely identified by project number (PHI99-001) and samples codes that described the origin of a sample by the site (AD1, AD2, NO, WN); line (hybrid line 1507 or inbred line 1507; control hybrid or inbred); sample # (1 to 5); and tissue type (leaf, pollen, silk, stalk, whole plant forage, grain, whole plant senescent). Table 11 lists the sampling dates for all tissue samples collected.

**Leaf:** For hybrid and inbred line 1507, leaves from five positive plants per replicate were collected at the V9 stage of development. For each of the control lines, a leaf from one plant per replicate was collected at the V9 stage of development. The samples were labeled, shipped on ice by overnight carrier to the analytical principal investigator (PI) and stored at -80°C before and after lyophilization.

**Pollen:** For hybrid and inbred line 1507, pollen from five positive plants per replicate was collected at the R1 (silking) stage of development. For each of the control lines, pollen from one plant per replicate was collected at the same stage of development. Samples were labeled, shipped and stored as described for leaf sampling.

**Silk:** For hybrid and inbred line 1507, the silks from five positive plants per replicate were collected at the R1 (silking) stage of development. For each of the control lines, the silks from one plant per replicate were collected at the same stage of development. Samples were labeled, shipped and stored as described for leaf sampling.

**Stalk:** For hybrid and inbred line 1507, the stalk from five positive plants per replicate was collected at the R1 (silking) stage of development. For each of the control lines, the stalk from one plant per replicate was collected at the same stage of development. Samples were labeled, shipped and stored as described for leaf sampling.

**Grain:** For inbred and hybrid line 1507, ears from five positive plants per replicate were collected at the R6 stage of development (physiological maturity). For each of the control lines, one ear per replicate was collected at the R6 stage (physiological maturity). After drying, the ears were individually shelled and then placed in separate labeled bags. Samples were shipped and stored as described for leaf sampling. Upon receipt in Johnston, samples were ground to a meal. The ground samples were lyophilized, then stored at approximately -80°C until analysis.

**Whole plant (forage):** For inbred and hybrid line 1507, the whole plant at the R4 stage (early dough) was collected for three positive plants, pooled into one sample and labeled. For each of the control lines, three whole plant samples were collected at the R4 stage (early dough), pooled into one sample and labeled. The whole plant samples were shipped, on ice, to the AD1 Field PI in Johnston (B. Swanson) where they were chopped into one to two inch pieces and placed into labeled Ziploc plastic bags. Samples were then forwarded to the Pioneer Livestock Nutrition Center in Polk City, IA and stored frozen until further processing. The chopped samples were dried at 62°C for 24-48 hr in a convection oven and then ground to a fine powder. Afterwards, they were shipped frozen to the Analytical PI in Johnston, IA, and stored at -80°C until analysis.

**Whole plant (senescent):** For inbred and hybrid line 1507, the whole plant at senescence was collected for three positive plants, pooled into one sample and labeled. For each of the control lines, three whole plant samples were collected at senescence, pooled into one sample and labeled. Samples were handled and processed as described above for whole plant forage.

#### C. Extraction of proteins from maize tissues

For all tissue types, previously weighed samples were removed from a -80°C freezer and allowed to gradually equilibrate to ambient temperature (20 - 25 °C) and then extracted in 600 µl PBST (phosphate buffered saline + Tween) buffered solution using a proprietary tissue homogenizer. Insoluble material was removed by centrifugation for 10 min and the supernatant was transferred to a clean microtube for determining total extractable protein and subsequent ELISA analysis.

#### D. Analytical methods

**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. Results from this assay were expressed in µg total protein/ml of extract. Based on these concentrations, the volume of extract for further immunoassay analyses was determined such that a constant amount of protein was delivered in each well of the plates.

**Cry1F ELISA.** A direct double antibody ELISA was used to quantify levels of Cry1F protein in genetically modified maize. The method used a polyclonal rabbit antibody specific to Cry1F protein to capture the protein in antibody-coated microtiter plate wells. The captured protein was detected by the same polyclonal antibody which had been conjugated to biotin. The binding of the biotinylated antibody to the captured protein was detected by an enzyme conjugate of streptavidin-alkaline phosphatase (SA/AP). The alkaline phosphatase enzyme substrate, para-nitrophenyl phosphate (pNPP), was added for color development. Quantification of the 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 extractable protein (TEP).

Samples designated as negative or <LOD were derived from an interpolated concentration of <10 pg/µg TEP for the Cry1F assay. The 10 pg/µg TEP standard concentration was the empirical limit of detection (LOD) for the Cry1F ELISA.

**PAT ELISA.** A direct double antibody sandwich ELISA was used to quantify levels of the PAT protein in genetically modified maize plants. The method used a polyclonal rabbit antibody specific to the PAT protein to capture the protein in antibody-coated microtiter plate wells. The captured protein was detected by the same polyclonal antibody which had been conjugated to biotin. The binding of the biotinylated PAT antibody to the captured protein was detected by an enzyme conjugate of streptavidin-alkaline phosphatase (SA/AP). The alkaline phosphatase 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/ $\mu$ g of TEP.

Samples designated as negative or <LOD were derived from an interpolated concentration of <20 pg/ $\mu$ g TEP for the PAT assay. The 20 pg/ $\mu$ g TEP standard concentration was the empirical limit of detection for the PAT ELISA.

#### E. Control of bias

The test substances were planted in randomly assigned locations within each of the two replicates at each test site. The entire test area was uniformly maintained using practices typical of maize production for each growing area.

#### F. Data reduction

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

Absorbance readings from the ELISA analyses and TEP determinations were recorded using the Bio-Rad Model 3550 plate reader. Data were transferred to a JMP software file (SAS Institute) for mean pg/ $\mu$ g TEP and standard deviation calculations. 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 associated standard deviation was also calculated for this mean.

#### Calculations:

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:

$$\text{Volume of extract } (\mu\text{l}) = \frac{\text{amount of total protein/well } (\mu\text{g/well}) \times \text{final volume } (\mu\text{l})}{\text{volume/well } (\mu\text{l/well}) \times \text{total protein concentration } (\mu\text{g}/\mu\text{l})}$$

$$\text{Volume of PBST } (\mu\text{l}) = \text{final volume} - \text{volume of extract } (\mu\text{l})$$

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

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

$$\text{Titer of Cry1F extract } t = \frac{c}{\text{concentration of protein loaded } (\mu\text{g}/50\mu\text{l})} \quad \text{in pg}/\mu\text{g total protein}$$

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

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

The adjusted mean in pg/ $\mu$ g TEP was determined as follows:

$$\text{Adjusted mean Cry1F/PAT (pg}/\mu\text{g TEP)} = \frac{\text{mean Cry1F/PAT (pg/well)}}{\text{concentration of protein } (\mu\text{g TEP/well})}$$

## Protocol amendments and deviations

Protocol amendments

1. The protocol was amended to be consistent with Pioneer Standard Operating Procedure PHIGLP-09B, which stipulates that every row in each plot should be marked with a stake or flag.
2. The protocol stated that five ears were to be collected from each test substance line during grain specimen collection. However, based on guidance from EPA officials, it was decided that whole plants collected at senescence should include the ears. In order have enough samples to accommodate this need, the Study Director instructed the field Principal Investigators to prioritize sample collection for whole plants at forage (R4) and grain (R6). If necessary, the Principal Investigators were instructed to collect only two forage whole plant samples (instead of three) and three grain samples (instead of five).
3. The protocol was amended to remove the entries designated "hybrid line 1360" and "inbred line 1360" from this study and to not conduct any analysis of samples from this entry. This amendment was based on a decision by Pioneer Research and Product Development to no longer pursue commercialization of this event.

None of the protocol amendments that occurred during the study were deemed to have had an impact on the results of the protein expression analyses conducted in the study.

Protocol deviations

1. The inbred control plants at location AD1 (Johnston, IA) were inadvertently leaf painted with Liberty herbicide, resulting in abnormal plant development. It was decided that the inbred control samples from the nearby AD2 location could be used, if necessary, as control specimens in the ELISA analysis.  
*Impact:* The proximity of the AD1 and AD2 sites at the research station in Johnston allowed the use of the inbred controls at AD2 as the environmental conditions and agronomic practices were very similar at the two locations.
2. At the Windfall, IN test location, the planting data were not initialed in the field notebook at the time of entry.  
*Impact:* This data recording error is included in the GLP compliance statement for this report.
3. Not all specimens collected at R1 could be stored at  $-80^{\circ}\text{C}$  by the Laboratory Principal Investigator because of lack of freezer space. The Principal Investigator and Study Director agreed that stalk tissue specimens could be stored in the  $-10^{\circ}\text{C}$  walk-in freezer maintained by the Pioneer Hi-Bred International, Inc. Livestock Nutrition Center.  
*Impact:* The study director took account of the change in storage conditions for stalk samples in analyzing the results of the protein expression ELISA data for this final report.
4. Two leaf samples, designated NO-CHpo-1-L and NO-CIpo-1-L, could not be located in freezer storage by the Laboratory Principal Investigator. These samples were logged in as received from the NO location on 6-29-99. The samples may have been accidentally discarded. The missing samples are the leaf controls for hybrid and inbred test specimens, respectively.  
*Impact:* The Study Director directed the Laboratory Principal Investigator to use leaf control specimens from the other three locations as these can serve as an adequate control for ELISA analysis.
5. At the Windfall, IN test location, the seeding rate had to be reduced to 30 and 17 plants per plot for the 1507 and 1360 lines, respectively, due to limited seed quantities.  
*Impact:* None. All required tissue specimens were collected.
6. At all test locations, it cannot be reconstructed from the available raw data whether the border rows were planted exactly as described in the study protocol.  
*Impact:* None. Two different borders were utilized, either a hybrid or inbred of similar genetic background. The difference between a hybrid border and inbred border on protein expression is expected to be minimal.
7. Not all specimens collected were shipped on ice to the Laboratory Principal Investigator. At the R1 sampling timepoint the high outdoor temperatures caused the ice pack to melt during shipping. At the R4 sampling timepoint the whole plant samples were not shipped on ice packs.  
*Impact:* None. An analysis of the protein expression results from this study demonstrate that expression levels were similar to the expression levels found in a 1999 study with *B.t.* Cry1F maize hybrid line 1507. (Stauffer and Rivas, 1999). In both studies it was observed that levels of the Cry1F protein were high in whole plant samples and very similar in leaf and grain tissues. PAT protein expression levels in leaf were also similar.



8. Whole plant senescent samples from one location were incorrectly labeled as 'WPS' instead of 'SE' as stated in the study protocol. This was observed during QA review of the study data for the final report.  
*Impact:* None.
9. Labels were for sample collection in July 1999 were printed with site AD instead of NO.  
*Impact:* None. The Laboratory Principal Investigator was informed of the labeling mistake prior to receipt of samples in the laboratory.

#### G. Other Circumstances that may have affected the quality of the data

Grain from a single inbred control sample tested positive for Cry1F. The low level of Cry1F protein in the control sample could have been a result of contamination that occurred during sampling or grinding of the grain. None of the other control samples from either hybrid or inbred tested positive for Cry1F protein. This isolated finding is not considered to have had an impact on the outcome of the study.

### IV. RESULTS AND CONCLUSIONS

#### A. Field Trial

The test and control lines were grown under environmental conditions representative of the major maize growing region of the United States. Tissue samples of the test and control hybrids and the test and control inbreds were collected, identified, shipped and stored in a manner to preserve line identity and sample integrity. Therefore, data collected from the protein expression analysis of the 1507 hybrids can be considered representative of the Cry1F and PAT expression levels in *B.t.* Cry1F maize commercial hybrids.

#### B. Protein expression analyses

Results of the protein expression analysis include the mean, standard deviation, minimum, and maximum values. These values are summarized in Tables 12 to 15.

**Cry1F ELISA.** Table 12 summarizes the Cry1F protein concentration measured in tissues collected from maize hybrid line 1507. Cry1F expression was highest in stalk tissue (1450 pg/μg TEP; mean) and lowest in silk tissue (37.4 pg/μg TEP; mean). These levels of Cry1F protein expression are typical of Cry1F maize lines that exhibit effective control of European corn borer and Cry1F susceptible insect pests of maize. The results are similar to the expression levels found in other *B.t.* Cry1F maize hybrid lines (Stauffer and Rivas, 1999) where it was observed that levels of Cry1F protein expression were highest in whole plant and stalk tissues and lower in leaf, pollen and grain tissues. Cry1F protein expression was lowest in silk tissue in both studies. These results also provide supporting evidence that the change in freezer storage conditions for stalk samples, along with the difficulties in shipping samples on ice, did not impact the results of the study (see protocol deviation #3 and #7 in Section III).

Table 13 summarizes the levels of Cry1F protein measured in tissues collected from maize inbred line 1507. As for the 1507 hybrid, expression was highest in stalk tissue (1770 pg/μg TEP; mean) and lowest in silk tissue (53.5 pg/μg TEP; mean). The relative order of expression levels across tissues was the same as for maize hybrid line 1507 with the exception that the concentration of the Cry1F protein in grain was slightly higher than its concentration in pollen.

The Cry1F protein was detected by ELISA in grain from one of the control inbreds (sample NO-CIpo-1-G). The level of Cry1F protein in that sample (14.2 pg/μg TEP, i.e., just above the limit of detection) was, however, very low when compared to the mean Cry1F protein levels in grain from the test hybrid (116 pg/μg TEP) and inbred (231 pg/μg TEP). The low level of Cry1F protein in the control sample could have been a result of contamination that occurred during sampling or grinding of the grain. None of the other control samples from either hybrid or inbred tested positive for Cry1F protein.

**PAT ELISA.** Table 14 summarizes the levels of PAT protein measured in tissues collected from maize hybrid line 1507. PAT protein was only expressed at measurable levels in leaf tissue and ranged from <LOD – 54.8 pg/μg TEP. In all other tissues, the levels of PAT protein were below the limit of detection (20 pg/μg TEP). The levels of PAT protein expression in maize leaves of the test hybrid were sufficient to confer tolerance to glufosinate-ammonium herbicide.

Table 15 summarizes the levels of PAT protein measured in tissues collected from maize inbred line 1507. The PAT protein was only expressed at measurable levels in leaf tissue and ranged from <LOD– 95.2 pg/μg TEP. In all other tissues, the levels of PAT protein were below the limit of detection (20 pg/μg TEP).

No measurable PAT expression was observed in any samples from the control hybrids or inbreds.

## V. 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.

Stauffer, C. and J. Rivas. 1999. Quantitative ELISA Analysis of Cry1F and PAT Expression Levels in and Compositional Analysis of Maize Inbred and Hybrid Lines 1362 and 1507. MRID #45020104. An unpublished technical report (98-09-RA-NGLP-012) from Pioneer Hi-Bred Intl., Inc.

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

Table 1. Field plot map: Johnston, IA (AD1)

	Range 2	Range 3
Row 2	Hybrid Control	Inbred Line 1507
Row 3	Hybrid Line 1507	Inbred Line 1360 <sup>a</sup>
Row 4	Hybrid Border	Inbred Control

<sup>a</sup> Row 3, Range 3 contained Inbred Line 1360, which was removed from the study by protocol amendment.



Table 2. Field plot map: Johnston, IA (AD2)

	Range 3	Range 4
Row 2	Hybrid Line 1507	Inbred Line 1360 <sup>a</sup>
Row 3	Control Hybrid	Inbred Line 1507
Row 4	Hybrid Border	Inbred Control

<sup>a</sup> Row 2, Range 4 contained Inbred Line 1360, which was removed from the study by protocol amendment.

Table 3. Field plot map: Noblesville, IN (NO)

	Range 2
Row 2	Inbred Line 1360 <sup>a</sup>
Row 3	Control Inbred
Row 4	Inbred Line 1507
Row 5	Inbred Border
Row 6	Hybrid Border
Row 7	Hybrid Line 1507
Row 8	Control Hybrid

<sup>a</sup> Row 2, Range 2 contained Inbred Line 1360, which was removed from the study by protocol amendment.

Table 4. Field plot map: Windfall, IN (WN)

	Range 2	Range 3
Row 2	Control Hybrid	Inbred Line 1507
Row 3	Hybrid Line 1507	Control Inbred
Row 4	Hybrid Border	Inbred Line 1360 <sup>a</sup>

<sup>a</sup> Row 4 , Range 3 contained Inbred Line 1360, which was removed from the study by protocol amendment.

Table 5. Important crop dates for the field trials

Field Site Site Code	Seed Receipt	Planting	Pollination	Trial Termination
Johnston, IA AD1	5/10/99	5/10/99	7/12/99 – 7/27/99	10/15/99
Johnston, IA AD2	5/10/99	5/10/99	7/14/99 – 7/22/99	11/15/99
Noblesville, IN NO	5/11/99	5/12/99	7/13/99 – 7/23/99	10/21/99
Windfall, IN WN	5/11/99	5/21/99	7/20/99 – 7/26/99	10/6/99

Table 6. Maintenance pesticide and fertilizer applications for the field trial conducted in Johnston, IA (AD1)

Date	Product	Rate/Acre
1998 soybeans	Trifluralin 36.35% Flumetsulam 2.67%	2.25 pt/A
4/20/99	Atrazine 32% Metolachlor 26.1%	1.7 qt/A
4/20/99	Metolachlor 82.4%	0.4 pt/A
11/12/98	N-P-K 11-52-0	288 lb/A
11/13/98	N-P-K 0-0-61	197 lb/A
3/29/99	N-P-K 32-0-0	160 lb/A

A = Acre

Table 7. Maintenance pesticide and fertilizer applications for the field trial conducted in Johnston, IA (AD2)

Date	Product	Rate/Acre
1998 soybeans	Trifluralin 36.35% Flumetsulam 2.67%	2.25 pints/A
4/20/99	Atrazine 32% Metolachlor 26.1%	1.7 qt/A
4/20/99	Metolachlor 82.4%	0.4 pt/A
11/12/98	N-P-K 11-52-0	96 lb/A
11/13/98	N-P-K 0-0-61	147 lb/A
3/29/99	N-P-K 32-0-0	120 lb/A

A = Acre

Table 8. Maintenance pesticide and fertilizer applications for the field trial conducted in Noblesville, IN

Date	Product	Rate/Acre
1998 soybeans	Glyphosate: 4 lbs/gal	Unknown
5/10/99	Atrazine 90%	1.5 lbs/A
5/10/99	Metolachlor: 7.8 lbs/gal	1.95 lbs/A
5/12/99	Terbufos 20% (Counter)	6 oz product/1000 ft rows
5/27/99	Permethrin (Pounce 3.2 EC)	4 oz product/A
5/5/99	N-P-K 12-12-12	833 lb/A
5/5/99	N-P-K 46-0-0	110 lb/A

A = Acre

Table 9. Maintenance pesticide and fertilizer applications for the field trial conducted in Windfall, IN

Date	Product	Rate, A/Acre
1998 soybeans	Glyphosate 41%	1 qt/A (Two applications)
5/21/99	Tebupirimifos 2% Cyfluthrin 0.1%	6.7 oz/1000 ft row
5/21/99	Acetochlor 24.8% Atrazine 16.6%	3.3 qt/A
5/21/99	Cyanazine 67.5% Atrazine 21.4%	2 lb/A
6/22/99	Bentazon 44%	2 pts/A
6/22/99	Atrazine 85.5%	0.5 lbs/A
5/21/99	N-P-K 28-0-0	126 lbs/A
6/6/99	N-P-K 28-0-0	612 lbs/A

A = Acre



**Table 10. Summary of temperatures and rainfall for the 1999 growing season.** Total rainfall amounts are presented where available. The study file contains records on daily rainfall amounts for each location.

Month	Parameter	AD1	AD2	NO	WN
May	Maximum temperature - °F	85	85	87	90
	Minimum temperature - °F	42	42	39	42
	Total Rainfall – inches	4.34	4.34	2.13	3.06
June	Maximum temperature - °F	93	93	94	95
	Minimum temperature - °F	44	44	45	44
	Total Rainfall – inches	4.79	4.79	5.34	2.44
July	Maximum temperature - °F	104	104	98	97
	Minimum temperature - °F	54	54	58	50
	Total Rainfall – inches	4.46	4.46	1.59	1.98
August	Maximum temperature - °F	95	95	90	88
	Minimum temperature - °F	49	49	42	47
	Total Rainfall – inches	6.49	6.49	1.12	1.96
September	Maximum temperature - °F	93	93	96	94
	Minimum temperature - °F	31	31	34	32
	Total Rainfall – inches	2.74	2.74	1.18	1.25
October (Through 10/27/99)	Maximum temperature - °F	87	87	Not available	81
	Minimum temperature - °F	25	25		30
	Total Rainfall – inches	1.50	1.50		2.05

Table 11. Summary of tissue sampling dates for the 1998 field trials

Field Site Site Code	Leaf (V0)	Pollen (R1)	Silk (R1)	Stalk (R1)	Whole Plant (R4)	Grain (R6)	Senescent Whole Plant
Johnston, IA AD1	6/28/99	7/19/99 – 7/25/99	7/19/99 – 7/22/99	7/19/99 – 7/22/99	8/13/99 8/16/99	9/21/99 9/24/99	10/5/99
Johnston, IA AD2	6/28/99	7/16/99 – 7/23/99	7/18 – 7/20/99	7/18 – 7/20/99	8/13/99 8/16/99	9/21/99 9/24/99	10/5/99
Noblesville, IN NO	6/28/99	7/21/99 7/26/99	7/21/99	7/21/99	8/17/99	9/20/99	9/28/99
Windfall, IN WN	6/28/99	7/21/99 7/26/99	7/21/99 7/26/99	7/27/99	8/18/99 8/23/99	10/2/99	10/5/99

Table 12. Levels of Cry1F protein measured in tissues collected from maize hybrid line 1507.

Tissue	Mean (pg/ $\mu$ g TEP <sup>a</sup> )	Standard Deviation	Min/Max Range (pg/ $\mu$ g TEP)
Leaf (V9)	138	27.9	99.8 – 179
Pollen (R1)	126	9.79	103 – 145
Silk (R1)	37.4	12.2	13.8 – 67.9
Stalk (R1)	1450	326	1020 - 2160
Grain (R6)	116	20.3	86.6 – 168
Whole Plant (Forage: R4)	628	308	379 – 910
Whole Plant (Senescence)	328	105	217 – 442

a - TEP; total extractable protein.

Table 13. Levels of Cry1F protein measured in tissues collected from maize inbred line 1507.

Tissue	Mean (pg/ $\mu$ g TEP <sup>a</sup> )	Standard Deviation	Min/Max Range (pg/ $\mu$ g TEP)
Leaf (V9)	235	79.6	115 – 417
Pollen (R1)	220	64.8	170 – 361
Silk (R1)	53.8	20.8	33.6 – 113
Stalk (R1)	1770	452	1110 – 2750
Grain (R6)	231	93.0	<LOD <sup>b</sup> – 361
Whole Plant (Forage: R4)	1110	264	932 – 1500
Whole Plant (Senescence)	574	86.2	469 - 673

a - TEP; total extractable protein.

b - <LOD; below the limit of detection (10 pg/ $\mu$ g TEP).

Table 14. Levels of PAT protein measured in tissues collected from maize hybrid line 1507.

Tissue	Mean (pg/ $\mu$ g TEP <sup>a</sup> )	Standard Deviation	Min/Max Range (pg/ $\mu$ g TEP)
Leaf (V9)	<LOD <sup>b</sup>	NA	<LOD – 54.8
Pollen (R1)	<LOD	NA <sup>c</sup>	<LOD
Silk (R1)	<LOD	NA	<LOD
Stalk (R1)	<LOD	NA	<LOD
Grain (R6)	<LOD	NA	<LOD
Whole Plant (Forage: R4)	<LOD	NA	<LOD
Whole Plant (Senescence)	<LOD	NA	<LOD

a - TEP; total extractable protein.

b - <LOD; below the limit of detection (20 pg/ $\mu$ g TEP).

c - NA; not applicable

Table 15. Levels of PAT protein measured in tissues collected from maize inbred line 1507.

Tissue	Mean (pg/ $\mu$ g TEP <sup>a</sup> )	Standard Deviation	Min/Max Range (pg/ $\mu$ g TEP)
Leaf (V9)	38.2	31.5	<LOD – 95.2
Pollen (R1)	<LOD <sup>b</sup>	NA <sup>c</sup>	<LOD
Silk (R1)	<LOD	NA	<LOD
Stalk (R1)	<LOD	NA	<LOD
Grain (R6)	<LOD	NA	<LOD
Whole Plant (Forage: R4)	<LOD	NA	<LOD
Whole Plant (Senescence)	<LOD	NA	<LOD

a - TEP; total extractable protein.

b - <LOD; below the limit of detection (20 pg/ $\mu$ g TEP).

c - NA; not applicable

**APPENDIX I. PROTOCOL AMENDMENTS AND DEVIATIONS**

**EXACT COPY**

**AMENDMENT TO PROTOCOL: AMENDMENT 1**

Study Number: ~~PHIGLP-001~~ PH199-001 (TYPO) [REDACTED] 6/17/99

Study Title: Quantitative ELISA Analysis of poCryIF and PAT Protein Expression Levels in Hybrid and Inbred Lines of TC1507 and an Inbred Line of TC1360

Study Director: [REDACTED]

**Background Information for Protocol Amendment: (Why is the amendment necessary?)**  
PH199-001 12 6/17/99

The protocol for Study Number ~~PHIGLP-001~~ states in Section 4.3 that: "Following emergence of the plants, every 5 rows in the field should be marked clearly with a stake(s) exhibiting the range-row ID for the event(s) planted in those rows." SOP PHIGLP-09B states that every row should be marked with a stake or flag. Therefore, this protocol will be amended to be consistent with SOP PHIGLP-09B.

**Details of Amendment: (Description of amended procedures.)**

Section 4.3 of this protocol is amended to read:

"Every row in the field should be marked clearly within two weeks of planting with a stake exhibiting the range-row ID for the entry."

**Predicted Effects on Study Outcome: (Will the data generation be changed? Will the acceptance criteria be different?)**

No impact on the study is expected as a result of this protocol amendment.

**APPROVALS:**

Study Director

Study Sponsor

5/28/99  
Date

5-28-99  
Date

**EXACT COPY****AMENDMENT TO PROTOCOL: AMENDMENT 2**Study Number: ~~PHIGLP-001~~ PHI99-001 (ENTRY CORRECTION) - 11/24/99  
11-24-99Study Title: Quantitative ELISA Analysis of poCv1F and PAT Protein Expression Levels in Hybrid and Inbred Lines of TC1507 and an Inbred Line of TC1360Study Director: [REDACTED]**Background Information for Protocol Amendment: (Why is the amendment necessary?)**

The protocol for Study Number PHIGLP-001 states that five ears should be collected from each test substance line during grain specimen collection. However, at a meeting with EPA officials in August 1999 it was learned that the agency preferred that whole plants collected at senescence also include the ears (see email message [REDACTED] 8/24/99). In order to obtain enough samples to accommodate this need, the Study Director instructed the field PI's to prioritize sample collection for whole plants at forage and grain. 2. If necessary, the PI's were instructed to collect only two forage whole plant samples (instead of three) and three grain samples (instead of five).

A protocol amendment was issued in July 1999 to address this issue for studies PHI99-003 and PHI99-004. By an oversight, a similar amendment was not issued for this study (PHI99-001). Therefore, by this amendment, PHI99-001 is amended to describe the need for the change in sampling procedures.

**Details of Amendment: (Description of amended procedures.)**

If necessary, the PI's were instructed to collect only two forage whole plant samples (instead of three) and three grain samples (instead of five).

**Predicted Effects on Study Outcome: (Will the data generation be changed? Will the acceptance criteria be different?)**

Since entries that advance in product development will be involved in further regulatory studies of this type there will be additional opportunities to obtain this data if necessary. Therefore, no effect is predicted on the outcome of this study.

ELISA analysis determination of protein levels in some tissues may be based on fewer sample numbers than stated in the protocol. However, the sampling procedures in the protocol were designed to collect more samples per entry than needed in order to accommodate situations where a lower number of positive plants that expected is available for sampling. However, for most entries the estimate of protein levels will be based at a minimum on two plants per entry, which should give a reasonable estimate of protein expression *in planta* across all four locations.

EXACT COPY

APPROVALS

Study Director

Date

11/18/99

Study Sponsor

Date

11/19/99



**EXACT COPY****AMENDMENT TO PROTOCOL: AMENDMENT 3**Study Number: PHIGLP-001Study Title: Quantitative ELISA Analysis of poCry1F and PAT Protein Expression Levels in Hybrid and Inbred Lines of TC1507 and an Inbred Line of TC1360Study Director: [REDACTED]**Background Information for Protocol Amendment: (Why is the amendment necessary?)**

Research and Product Development has decided that transformation event TC1360 is no longer in product development. Therefore, the entry designated "1360 inbred" will be removed from this study and no ELISA analyses will be performed with samples from this entry.

**Details of Amendment: (Description of amended procedures.)**

The entry designated "1360 inbred" will be removed from this study and no ELISA analyses will be performed with samples from this entry. All laboratory and field documentation related to the use of TC1360 will be retained for preparation of the final report.

**Predicted Effects on Study Outcome: (Will the data generation be changed? Will the acceptance criteria be different?)**

No effect is predicted on the outcome of this study.

APPROVALS:

Study Director




Date

Study Sponsor

Date


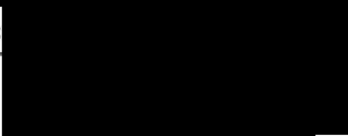
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PIONEER HI-BRED INTERNATIONAL	
Protocol Deviation Record	
Project No.: <u>PHI99-001</u>	Protocol Deviation No.: <u>1</u>
Test Site(s): <u>AD1</u>	
Nature of Deviation	
Date(s) of Occurrence: <u>6/14/99</u>	
Description: Due to oversight, leaves of both test substance lines and control lines were leaf painted with Liberty herbicide. The protocol states that only leaves of the test substance lines were to be leaf painted.	
Recorded by: 	<u>6/22/99</u>
To be completed by Study Director	
Significant Change? Yes <input type="checkbox"/> No <input checked="" type="checkbox"/> : <u>AD2 (TYPO) L2 7/21/00</u>	
Impact on Study The inbred control plants are not developing normally as a result of the leaf painting with Liberty herbicide. If an insufficient amount of inbred controls is available at the AD1 location in Johnston, Iowa then the inbred control samples from the nearby <u>AD2</u> location will be utilized as ELISA controls. The proximity of the AD1 and AD2 sites at the research station in Johnston should allow the use of the inbred controls at AD2 as the environmental conditions and agronomic practices are very similar at the two locations. Therefore, use of the AD2 control inbred plants should not alter ELISA results for this study. The ELISA data from leaf samples at AD1 and AD2 will be reviewed at the time of the final report and assessed for its validity.	
Other Action(s) Taken: None	
Study Directors Signature: 	<u>7/1/99</u>
Management Signature (if necessary): 	<u>2-26-01</u>

<b>PIONEER HI-BRED INTERNATIONAL</b>	
<b>Protocol Deviation Record</b>	
Project No.: <u>PHI99-001</u>	
Deviation No.: <u>X 2</u> <sup>LZ</sup> <u>8/11/99</u>	Test Site: <u>Windfall, IN</u>
<b>Nature of Deviation:</b>	
Protocol Section: <u>10.2</u>	Date(s) of Occurrence: <u>5/21/99</u>
Description: Planting data were not initialed at time of entry.	
Recorded by: Keith Freeman (signed)	<div style="background-color: black; width: 150px; height: 30px; display: inline-block;"></div>
<i>To be completed by Study Director</i>	
Impact on study: Minimal impact.	
Other Action Taken: This data recording deviation will also be included in the GLP Compliance statement in the final report.	
Study Director's Signature:	<div style="background-color: black; width: 200px; height: 30px; display: inline-block;"></div>
Sponsor's Signature:	<div style="background-color: black; width: 150px; height: 30px; display: inline-block;"></div>

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PIONEER HI-BRED INTERNATIONAL	
Protocol Deviation Record	
Project No.: <u>PHI99-001</u>	Protocol Deviation No.: <u>3</u>
Test Site(s): <u>AD1, AD2, NO, WN</u>	
Nature of Deviation	
Date(s) of Occurrence: <u>July 9 to duration of study</u>	
Description: Not all specimens collected at R1 could be stored at - 80 C by the Laboratory Principal Investigator (Jacque Rivas) in the protein lab because of lack of freezer space. The PI and Study Director agreed that stalk tissue specimens should be stored in a walk-in freezer by Mark Donahue at the Pioneer Hi-Bred Intl. Inc. Livestock Nutrition Center (LNC) after processing. The walk-in freezer at the LNC is maintained at - 10°C.	
Recorded by: <u>Jacque Rivas</u>	
<i>To be completed by Study Director</i>	
Significant Change? Yes <input type="checkbox"/> No <input checked="" type="checkbox"/> :	
Impact on Study The study director will take into account the change in storage conditions for stalk samples upon review of the protein expression ELISA data and preparation of the final report for this study. Expression levels that are relatively low or below the limit of detection may be due to storage at - 10 C rather than - 80 C. The study director will also attempt to locate available data on the comparative stability of the Cry1F protein at these two temperatures.	
Other Action(s) Taken: <u>None</u>	
Study Directors Signature: 	<u>9/10/99</u>
Management Signature (if necessary): 	<u>2/26/01</u>


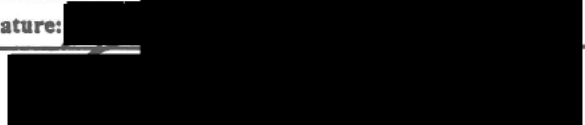
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PIONEER HI-BRED INTERNATIONAL	
Protocol Deviation Record	
Project No.: <u>PHI99-001</u>	Protocol Deviation No.: <u>4</u>
Test Site(s): <u>NO</u>	
Nature of Deviation	
Date(s) of Occurrence: <u>2/24/2000</u>	
Description: Leaf samples NO-CHpo-1-L and NO-CIpo-1-L were missing from the rest of the PHI99-001 leaf samples. The samples are believed to have been received since they were looged in on 6-29-99. The samples may have been accidentally discarded.	
Recorded by: Patrick Garcia	<u>3-1-00</u>
To be completed by Study Director	
Significant Change? Yes <input type="checkbox"/> No <input checked="" type="checkbox"/> :	
Impact on Study. The samples missing are the leaf controls for the hybrid (NO-CHpo-1-L) and inbred (and NO-CIpo-1-L) lines for the Noblesville location of this study. The Study Director has consulted with the Laboratory Principal Investigator and determined that the leaf control hybrid and inbred from the three other locations will serve as an adequate negative control for ELISA analysis of test substance samples.	
Other Action(s) Taken: None	
Study Directors Signature:	<u>3/1/00</u>
Management Signature (if necessary):	<u>2/26/01</u>

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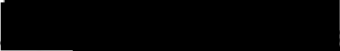


PIONEER HI-BRED INTERNATIONAL	
Protocol Deviation Record	
Project No.: <u>PHI99-001</u>	
Deviation No.: <u>5 SE 6-16-2000</u>	Test Site: <u>Windfall, IN</u>
Nature of Deviation	
Protocol Section: <u>5.1</u>	Date(s) of Occurrence: <u>5/21/99</u>
Description: Instead of the prescribed seeding rates of 40 - 50 plants/plot for 1507 line and 30 - 36 plants/plot for the 1360 line, due to limited seed quantities, these rates had to be reduced to 30 and 17 plants per plot for the 1507 and 1360 lines, respectively.	
Recorded by: Lawrence Zeph	Date:
To be completed by Study Director	
Impact on study: None expected. All required tissue specimens were collected..	
Other Action Taken: None	
Study Director Signature	Date: <u>6/13/00</u>
Sponsor Signature:	Date: <u>06-14-00</u>

EXACT COPY

PIONEER HI-BRED INTERNATIONAL	
Protocol Deviation Record	
Project No.: <u>PHI99-001</u>	
Deviation No.: <u>6 DE 6-16-2000</u>	Test Site: AD1, AD2, WL, <sup>(a)</sup> <del>PR</del> , NO <u>    </u>
Nature of Deviation:	
Protocol Section: <u>5.2</u>	Date(s) of Occurrence: <u>Various (see planting data)</u>
It cannot be reconstructed from the available raw data if the border rows were correctly planted or not as per protocol requirements. This deviation addresses all instances where borders did not meet protocol requirements.	
Recorded by: <u>Lawrence Zeph</u>	Date: <u>    </u>
To be completed by Study Director	
Impact on study: <u>None expected..</u>	
Other Action Taken: <u>None</u>	
Study Director Signature: 	Date: <u>6/13/00</u>
Sponsor Signature: 	Date: <u>06-16-00</u>


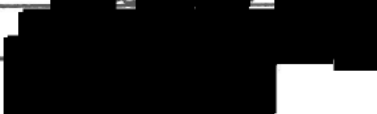
(a) Not applicable DE 6-16-2000

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PIONEER HI-BRED INTERNATIONAL	
Protocol Deviation Record	
Project No.: <u>PHI99-001</u>	Protocol Deviation No.: <u>7</u>
Test Site(s): <u>AD1, AD2, NO, WN</u>	
Nature of Deviation	
Date(s) of Occurrence: Duration of study	
Description: Not all specimens collected were shipped on ice to the Laboratory Principal Investigator (Jacque Rivas) in the protein lab. At the R1 sampling timepoint the high outdoor temperatures caused the ice pack to melt during shipping. At the R4 sampling timepoint the whole plant samples were not shipped on ice packs.	
Recorded by: Larry Zeph	
To be completed by Study Director	
Significant Change? Yes <input type="checkbox"/> No <input checked="" type="checkbox"/> :	
Impact on Study: The study director will take into account the shipping conditions when reviewing the ELISA data for the affected specimens. This protocol deviation will be addressed in the final report for this study. Expression levels that are relatively low or below the limit of detection may be due to temperatures during shipping.	
Other Action(s) Taken: None	
Study Directors Signature: 	2/21/2001
Management Signature (if necessary): 	2/25/01
	



EXACT COPY

PIONEER HI-BRED INTERNATIONAL	
Protocol Deviation Record	
Project No.: <u>PHI99-001</u>	
Deviation No.: <u>8</u>	Test Site: <u>AD1, AD2, WN</u>
Nature of Deviation	
Protocol Section: <u>6.0</u>	Date(s) of Occurrence: <u>7-7-00</u>
Description: Whole plant senescent samples from were incorrectly labeled as 'WPS' instead of 'SE' as stated in the Protocol. This was observed during QA review of the study data for the final report.	
Recorded by: <u>Bonnita Taggart</u>	Date: <u>7-7-00</u>
<i>To be completed by Study Director</i>	
Impact on study: This deviation had no impact on the study. The mislabeling of samples did not cause any errors during the analysis as WPS has often been used to denote whole plant senescent samples in other study protocols.	
Other Action Taken: <u>None.</u>	
Study Director Signature: 	Date: <u>2/21/2001</u>
Sponsor Signature: 	Date: <u>2-25-01</u>

EXACT COPY

## PIONEER HI-BRED INTERNATIONAL

## Protocol Deviation Record

Project No.: PHI99-001Deviation No.: 9Test Site: Protein Analysis Laboratory

## Nature of Deviation

Protocol Section: 6.0Date(s) of Occurrence: July 1999

## Description:

Labels were printed with site AD instead of NO.

Recorded by: Larry Zeph*To be completed by Study Director*

## Impact on study:

This deviation had no impact on the study. The Laboratory Principal Investigator was informed of the labeling mistake prior to receipt of samples in the laboratory.

Other Action Taken: None.Study Director Signature: 2/21/2001Sponsor Signature: 2/26/01.

**APPENDIX II. REFERENCES**

NOTICE: THIS MATERIAL MAY  
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LAW (Title 17 U.S. Code)

## A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding

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Received September 11, 1975; accepted January 29, 1976

A protein determination method which involves the binding of Coomassie Brilliant Blue G-250 to protein is described. The binding of the dye to protein causes a shift in the absorption maximum of the dye from 465 to 595 nm, and it is the increase in absorption at 595 nm which is monitored. This assay is very reproducible and rapid with the dye binding process virtually complete in approximately 2 min with good color stability for 1 hr. There is little or no interference from cations such as sodium or potassium nor from carbohydrates such as sucrose. A small amount of color is developed in the presence of strongly alkaline buffering agents, but the assay may be run accurately by the use of proper buffer controls. The only components found to give excessive interfering color in the assay are relatively large amounts of detergents such as sodium dodecyl sulfate, Triton X-100, and commercial glassware detergents. Interference by small amounts of detergent may be eliminated by the use of proper controls.

Laboratory practice in protein purification often requires a rapid and sensitive method for the quantitation of protein. Methods presently available partially fulfill the requirement for this type of quantitation. The standard Lowry procedure (1) is subject to interference by compounds such as potassium ion (2), magnesium ion (3), EDTA (4), Tris (3), thiol reagents (2), and carbohydrates (5). The relatively insensitive biuret reaction (6) is subject to interference by Tris (7), ammonia (8), and glycerol (9). Even the modified procedure for eliminating problems with the Lowry and biuret assays (10,11) present problems since more complications and time are involved in the modified procedures. The dye binding techniques in the literature are for the most part insensitive assays involving the binding of Orange G to protein (12-16). The exception to this rule is the Amidoschwarz 10-B binding assay (17). This procedure, too, has its drawbacks since the precipitation of the protein by trichloroacetic acid followed by filtration on Millipore membranes is required.

The protein assay herein described eliminates most of the problems involved in the procedures described above, and is easily utilized for

## PROTEIN ASSAY BY DYE BINDING

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processing large numbers of samples, as well as adaptable to automation. It is based on the observation that Coomassie Brilliant Blue G-250 exists in two different color forms, red and blue (18). The red form is converted to the blue form upon binding of the dye to protein (18). The protein-dye complex has a high extinction coefficient thus leading to great sensitivity in measurement of the protein. The binding of the dye to protein is a very rapid process (approximately 2 min), and the protein-dye complex remains dispersed in solution for a relatively long time (approximately 1 hr), thus making the procedure very rapid and yet not requiring critical timing for the assay.

## MATERIALS AND METHODS

**Reagents.** Coomassie Brilliant Blue G-250 was obtained from Sigma, and used as supplied. 2-Mercaptoethanol was obtained from Sigma. Triton X-100 was obtained from Schwartz/Mann. Sodium dodecyl sulfate was obtained from BDH Chemicals Ltd., Poole, England. Hemosol was obtained from Scientific Products. All other reagents were of analytical grade or the best grade available.

**Protein preparation.** Bovine serum albumin (2× crystallized), chymotrypsinogen A, and cytochrome *c* (horse heart) were obtained from Schwartz/Mann. Hemoglobin and human serum albumin were obtained from Nutritional Biochemicals Corporation. Protein solutions were prepared in 0.15 M NaCl. Concentrations were determined for bovine serum albumin, human serum albumin, chymotrypsinogen A, and cytochrome *c* spectrophotometrically in a Bausch and Lomb Spectronic 200 uv spectrophotometer based on  $\epsilon_{280}^{1\%} = 6.6$  (19,20), 5.3 (19,21), 20 (19,22) and 17.1 (23,24) respectively. Hemoglobin solutions were prepared gravimetrically.

**Preparation of protein reagent.** Coomassie Brilliant Blue G-250 (100 mg) was dissolved in 50 ml 95% ethanol. To this solution 100 ml 85% (w/v) phosphoric acid was added. The resulting solution was diluted to a final volume of 1 liter. Final concentrations in the reagent were 0.01% (w/v) Coomassie Brilliant Blue G-250, 4.7% (w/v) ethanol, and 8.5% (w/v) phosphoric acid.

**Protein assay (standard method).** Protein solution containing 10 to 100  $\mu$ g protein in a volume up to 0.1 ml was pipetted into 12 × 100 mm test tubes. The volume in the test tube was adjusted to 0.1 ml with appropriate buffer. Five milliliters of protein reagent was added to the test tube and the contents mixed either by inversion or vortexing. The absorbance at 595 nm was measured after 2 min and before 1 hr in 3 ml cuvettes against a reagent blank prepared from 0.1 ml of the appropriate buffer and 5 ml of protein reagent. The weight of protein was plotted against the corresponding absorbance resulting in a standard curve used to determine the protein in unknown samples.

*Microprotein assay.* Protein solution containing 1 to 10  $\mu\text{g}$  protein in a volume up to 0.1 ml was pipetted into 12  $\times$  100 mm test tubes. The volume of the test tubes was adjusted to 0.1 ml with the appropriate buffer. One milliliter of protein reagent was added to the test tube and the contents mixed as in the standard method. Absorbance at 595 nm was measured as in the standard method except in 1 ml cuvettes against a reagent blank prepared from 0.1 ml of the appropriate buffer and 1 ml of protein reagent. Standard curves were prepared and used as in the standard method.

## RESULTS

*Reproducibility, sensitivity, and linearity of the assay.* Triplicate standard assays of bovine serum albumin as a standard result in a highly reproducible response pattern. Statistical analysis gives a standard deviation of 1.2% of mean value for the assay. There is extreme sensitivity in the assay with 25  $\mu\text{g}$  sample giving an absorbance change of 0.275 OD units. This corresponds to 5  $\mu\text{g}$  protein/ml in the final assay volume. There is a slight nonlinearity in the response pattern. The source of the nonlinearity is in the reagent itself since there is an overlap in the spectrum of the two different color forms of the dye. The background value for the reagent is continually decreasing as more dye is bound to protein. This presents no real problem since the degree of curvature is only slight. If the assay is run with a set of standards and unknowns measured against the response curve of the standards instead of calculated by Beer's Law, there is no difficulty in obtaining satisfactory results.

*Accuracy of the assay.* Figure 1 shows the results of various proteins assayed in the system as to individual responses. There is a scattering of points around the line drawn in the graph. The scattering is believed to be a multifaceted function composed of difficulties in determining the exact amount of protein present in a given sample due to variation of extinction coefficients in the literature, the methods used to determine the exact amount of protein used in measuring extinction coefficients, and some degree of variation in the efficiency of dye binding to various proteins. Figure 2 shows the response pattern obtained from Lowry (1) assays of the same proteins. The degree of scatter in protein response to Lowry (1) assay is similar to that shown for the dye-binding assay presented here. The sensitivity of the Lowry (1) method is an absorbance of 0.110 OD units for the 25  $\mu\text{g}$  standard corresponding to 8  $\mu\text{g}$  protein/ml of final assay volume. By calculation, then, the dye binding assay is approximately four times more sensitive than the Lowry (1) assay. The degree of scatter around the Lowry (1) assay plot also points to the difficulty in establishing a quantitative value for a protein in standard solutions.

*Stability of the protein-dye complex color.* Figure 3 shows the rate of

## PROTEIN ASSAY BY DYE BINDING

251

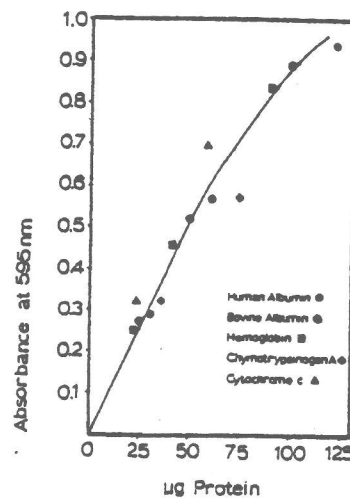


FIG. 1. Protein dye binding response pattern for various proteins.

formation of protein-dye complex in the assay system and the stability of the color complex. The absorbance was monitored at 7.5 sec intervals for 2 min and then at 1 min intervals for a period of 1 hr. As seen from the graph, the color development is essentially complete at 2 min, and remains stable plus or minus 4% for a period of 1 hr. Since the protein-dye complex has a tendency to aggregate with time, there is a decrease in color after this period of time simply by the physical removal of the protein-dye complex from solution. If very precise determinations are required, investigators should take precaution to read the absorbance of

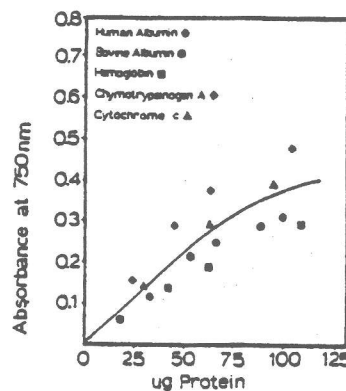


FIG. 2. Lowry (I) response pattern for various proteins.

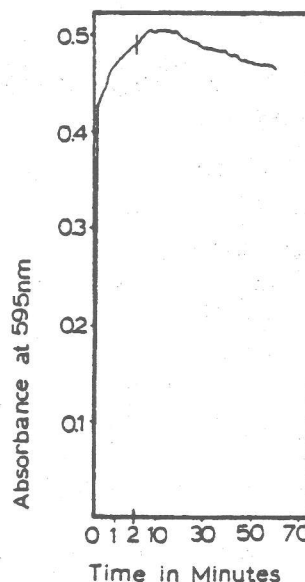


FIG. 3. Protein-dye complex formation rate and color stability.

samples during one of the flatter portions of the color stability curve between 5 and 20 min after reagent addition. This still gives ample time to read a relatively large number of samples.

**Microassay system sensitivity.** When bovine serum albumin is used as the standard in the micro assay system the degree of nonlinearity is similar to that found in the standard assay. There is a loss in protein-dye complex response as compared with the standard assay, i.e., 5  $\mu$ g protein/ml gives an absorbance change of 0.1 vs 0.27 in the standard assay. Perhaps this results from increased dilution of the protein reagent.

**Interference by nonprotein components.** As indicated earlier, there is some interference in the assay system by strongly alkaline buffering agents. This may be overcome by running the appropriate buffer controls and subtracting the value for the control either mathematically or spectrophotometrically. A wide spectrum of components was tested for effects on the protein dye binding assay (Table 1). A lack of effect on the assay by magnesium chloride, potassium chloride, sodium chloride, ethanol, and ammonium sulfate was observed. The small effects due to Tris, acetic acid, 2-mercaptoethanol, sucrose, glycerol, EDTA, and trace quantities of the detergents, Triton X-100, sodium dodecyl sulfate, and Hemosol, can be easily eliminated by running the proper buffer control with the assay. However, the presence of large quantities of the detergents present abnormalities too great to overcome.



## PROTEIN ASSAY BY DYE BINDING

253

TABLE I

EFFECT OF VARIOUS LABORATORY REAGENTS ON COOMASSIE BRILLIANT  
BLUE-G-250-PROTEIN COMPLEX ASSAY\*

Substance	Change in OD 595	( $\mu$ g) Equivalent BSA
1 M KCl	0.000	0.00
5 M NaCl	0.000	0.00
1 M $MgCl_2$	0.000	0.00
2 M Tris	0.026	2.34
0.1 M EDTA	0.004	0.36
1 M $(NH_4)_2SO_4$	0.000	0.00
99% Glycerol	0.012	1.08
1 M 2-Mercaptoethanol	0.004	0.36
1 M Sucrose	0.013	1.17
95% Ethanol	0.000	0.00
Acetone	0.069	6.21
5% Phenol	0.046	4.14
0.1% Triton X-100	0.013	1.17
1% Triton X-100	0.590	53.10
0.1% Sodium dodecyl sulfate	0.011	0.99
1% Sodium dodecyl sulfate	0.495	44.55
0.1% Hemosol	0.004	0.36
1% Hemosol	0.108	9.72

\*The above values were obtained when 0.1 ml of each substance was assayed in the standard assay.

A difficulty observed in performing the assay is the tendency of the protein-dye complex in solution to bind to cuvettes. This results in a blue colored cuvette. The amount of binding is negligible as far as assay readings are concerned, i.e., less than 1% error, as indicated by the standard deviation of triplicate assays in the reproducibility section. The blueness of the cuvettes after assay does present problems in other uses of the cuvettes so the following directions for cleaning the blue complex from cuvettes is included:

Method 1: Rinse cuvettes with concentrated glassware detergent, followed by water and acetone. (Gives immediate removal.)

Method 2: Soak cuvettes in 0.1 M HCL. (Removes complex in a few hours.)

The binding of the protein-dye complex has been observed only with quartz cuvettes and may be eliminated by using either glass or plastic cuvettes.

## ACKNOWLEDGMENTS

This research was supported by Contract No. 1-HD-9-2103 with the Center for Population Research, NICHD and by Ford Foundation Grant No. 680-0805A.

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Study Number  
98-09-RA-NGLP-012

Page 1 of 85

**Final Report**

**Study Title**

**Quantitative ELISA Analysis of Cry1F and PAT Expression Levels in and Compositional  
Analysis of Maize Inbred and Hybrid Lines 1362 and 1507**

**Data Requirements**

**Not Published**

**Authors**

[REDACTED]

**Study Completed On**

**December 1, 1999**

**Performing Laboratory**

**Pioneer Hi-Bred International, Inc.  
7300 NW 62<sup>nd</sup> Ave.  
Johnston, Iowa 50131-1004**

**Study Number**

**98-09-RA-NGLP-012**

**Sponsor**

**Pioneer Hi-Bred International Inc.  
7250 N.W. 62<sup>nd</sup> Ave.  
Johnston, Iowa 50131-0552**

**Total number of pages: 85**

Study Number  
98-09-RA-NGLP-012

Page 2 of 85

## CERTIFICATION OF NO DATA CONFIDENTIALITY

No claim of confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA Section 10(d)(1)(A), (B), or (C). These data are the property of Mycogen Seeds, and as such are considered to be confidential for all purposes other than compliance with FIFRA Section 10. Submission of these data in compliance with FIFRA does not constitute a waiver of any right to confidentiality that may exist under another statute or in another country.

Company: Mycogen Seeds c/o Dow AgroSciences LLC

Company Agent: [REDACTED]

Title: Registration Manager

[REDACTED]

Signature

12/3/99

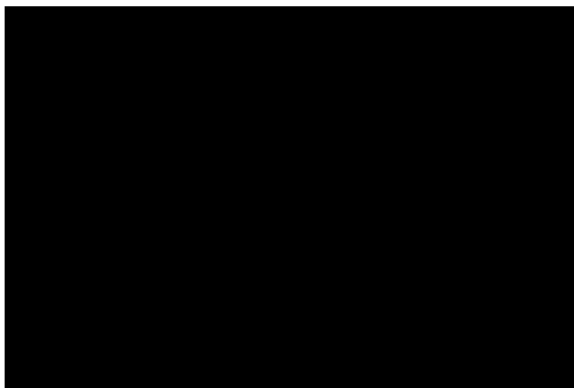
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Study Number  
98-09-RA-NGLP-012

Page 3 of 85

## STATEMENT OF COMPLIANCE

The field and compositional analysis portion of this study was not designed to be conducted in strict adherence with United States EPA FIFRA Good Laboratory Practice Standards (40 CFR Part 160), however, documentation of all critical data and quality control measures were used to ensure the integrity of the study results. Quality control measures included, but were not limited to, maintenance of test substance chain of custody; separation of test and control substances during planting and sampling; and documentation of critical data. Additionally, all raw data was reviewed for accuracy and completeness by the QAU. The ELISA analyses were conducted according to the United States EPA FIFRA Good Laboratory Practice Standards (40 CFR Part 160).



12-3-99

Date

12-2-99

Date

12/1/99

Date

Study Number  
98-09-RA-NGLP-012

Page 4 of 85

## QUALITY ASSURANCE STATEMENT

The following phases of this study were inspected/audited:

Phase Audited	Audit Date	Date Reported
Data audit - Field	10/4/99	10/4/99
Data audit - laboratory	10/4,5/99	10/5/99
Draft report	11/8,9/99	11/9/99

  
Quality Assurance Representative  
  
QUALITY ASSOCIATES, INC.11/29/99  
Date

Study Number  
98-09-RA-NGLP-012

Page 5 of 85

**Study Number:** 98-09-RA-NGLP-012

**Title:** Quantitative ELISA Analysis of Cry1F and PAT Expression Levels in and Compositional Analysis of Maize MPS Inbred and hybrid Lines 1362 and 1507.

**Testing Facility:** Pioneer Hi-Bred International, Inc.  
7300 NW 62<sup>nd</sup> Ave.  
Johnston, Iowa 50131-1004

**Field Sites:** Nancagua, Chile  
Graneros, Chile  
Villuco, Chile  
Buin, Chile

**Study Sponsor:** Rod Townsend, Pioneer Hi-Bred Int'l Inc.

**Study Director:** Steve Ritchie, Pioneer Hi-Bred Int'l Inc. 10/12/98 - 10/19/99  
Cynthia Stauffer, Pioneer Hi-Bred Int'l Inc. 10/19/99 - 12/1/99

**Contributors:** Analytical Principal Investigator - Jacqueline Rivas  
Field Principal Investigator - Ennio Innocenti  
Contributing Scientists - Miguel Ibáñez, Gonzalo Prado

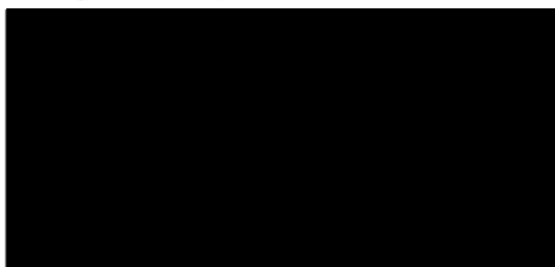
**Study Initiation Date:** 10/12/98

**Records Retention:** All study specific raw data, protocols, final reports and facility records are archived initially at Pioneer Hi-Bred Int'l Inc. and will be retained permanently at QAI in Columbia, MD.

**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:



12/1/99  
Date

12-2-99  
Date

Study Number  
98-09-RA-NGLP-012

Page 6 of 85

TABLE OF CONTENTS	PAGE
Certification of No Data Confidentiality .....	2
Statement of Compliance .....	3
Quality Assurance Statement .....	4
Signatures of Approval .....	5
Table of Contents .....	6
Abstract .....	7
Introduction .....	11
Materials .....	11
Methods .....	12
Results and Discussion .....	16
Conclusions .....	19
References .....	20
Tables	
1. Field Plot Map: Buin, Chile (BUI) .....	21
2. Field Plot Map: Viluco, Chile (VIL) .....	22
3. Field Plot Map: Graneros, Chile (GRA) .....	23
4. Field Plot Map: Nancagua (NAN) .....	24
5. Important Crop Dates for the 1998/99 Field Trials Conducted in Chile .....	25
6. Chemical and Fertilizer Applications for the 1998/99 Field Trials Conducted in Buin, Chile .....	26
7. Chemical and Fertilizer Applications for the 1998/99 Field Trials Conducted in Viluco, Chile .....	27
8. Chemical and Fertilizer Applications for the 1998/99 Field Trials Conducted in Graneros, Chile. ....	28
9. Chemical and Fertilizer Applications for the 1998/99 Field Trials Conducted in Nancagua, Chile. ....	29
10. Summary of glufosinate herbicide segregation. ....	30
11. Summary of Rainfall and Temperatures for 1998/99 Growing Season. ....	31
12. Summary of Tissue Sampling Dates for the 1998 Field Trials. ....	32
13. Summary of Cry1F protein levels measured in tissue collected from maize inbred and hybrid line 1507 during the 1998-1999 growing season in Chile. ....	33
14. Summary of PAT protein levels measured in tissue collected from maize inbred and hybrid line 1507 during the 1998-1999 growing season in Chile. ....	34
Appendix 1. Copies of References Cited .....	35



Study Number  
98-09-RA-NGLP-012

Page 7 of 85

#### ABSTRACT

Maize lines, designated 1362 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. These lines also 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 and thus makes genetically modified plants that accumulate this protein tolerant to the herbicide.

After the study initiation, it was determined that line 1362 was not segregating appropriately so it was removed from the study.

The purpose of the field trials was:

- 1) to generate leaf, pollen, silk, stalk, whole plant, grain and senescent whole plant tissue samples from a MPS (Mycogen Plant Sciences - now known as Mycogen Seeds) hybrid derived from maize line 1507, a MPS inbred derived from maize line 1507 and control lines designated Hybrid A<sub>4</sub> and Inbred A<sub>4</sub>.
- 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. The composition analysis phase of this study will not be presented in this report.

The test system for this study consisted of four field sites located in the major maize growing regions of Chile. The sites are between 10 and 125 Km south of Santiago. The test system represents locations equivalent to regions in the United States where the maize lines would be suitable commercial products, therefore the measurements of Cry1F and Pat expression should be equivalent to what can be expected in the United States.

At each site, leaf, pollen, silk, stalk and grain samples were taken from five discrete plants. The whole plant and senescent whole plant samples consisted of three plants pooled together. Cry1F and PAT protein levels were measured in each of the samples using specific ELISAs developed for each protein.

The results of the assays are summarized below.

Study Number  
98-09-RA-NGLP-012

Page 8 of 85

Summary of Cry1F protein levels measured in tissue collected from maize inbred and hybrid line 1507 during the 1998-1999 growing season in Chile.

Tissue	Mean <sup>1</sup> Cry1F (pg/μg total protein)	Standard Deviation	Min/Max Range
<b>Leaf</b>			
1507 Inbred	169.5	35.4	79.3 – 209.4
1507 Hybrid	110.9	27.2	56.6 – 148.9
<b>Pollen</b>			
1507 Inbred	207.5	10.6	186.3 – 231.1
1507 Hybrid	135.5	13.5	113.4 – 168.2
<b>Silk</b>			
1507 Inbred	58.9	14.9	36.2 – 89.8
1507 Hybrid	50.3	16.5	26.8 – 79.8
<b>Stalk</b>			
1507 Inbred	637.8	101.8	480.5 – 849.0
1507 Hybrid	550.0	104.0	355.9 – 737.4
<b>Whole plant</b>			
1507 Inbred	1357.8	72.3	1283.5 – 1428.0
1507 Hybrid	1063.8	361.7	803.2 – 1572.7
<b>Grain</b>			
1507 Inbred	112.2	20.6	66.5 – 141.5
1507 Hybrid	89.8	23.3	71.2 – 114.8
<b>Senescent whole plant</b>			
1507 Inbred	677.5	215.5	470.5 – 968.3
1507 Hybrid	714.3	95.5	622.2 – 845.3

<sup>1</sup> Values are means across all four sites from mean values calculated from the analysis of five samples per site for leaf, pollen, silk, stalk, grain and one sample per site for both whole plant samples.

Study Number  
98-09-RA-NGLP-012

Page 9 of 85

Summary of PAT protein levels measured in tissue collected from maize inbred and hybrid line 1507 during the 1998-1999 growing season in Chile.

Tissue	Mean <sup>1</sup> PAT (pg/ $\mu$ g total protein)	Standard Deviation	Min/Max Range
<b>Leaf</b>			
1507 Inbred	21.4	22.8	<LOD <sup>2</sup> - 58.2
1507 Hybrid	<LOD	NA <sup>3</sup>	<LOD - 40.8
<b>Pollen</b>			
1507 Inbred	<LOD	NA	<LOD
1507 Hybrid	<LOD	NA	<LOD
<b>Silk</b>			
1507 Inbred	<LOD	NA	<LOD
1507 Hybrid	<LOD	NA	<LOD
<b>Stalk</b>			
1507 Inbred	<LOD	NA	<LOD
1507 Hybrid	<LOD	NA	<LOD
<b>Whole plant</b>			
1507 Inbred	<LOD	NA	<LOD
1507 Hybrid	<LOD	NA	<LOD
<b>Grain</b>			
1507 Inbred	<LOD	NA	<LOD
1507 Hybrid	<LOD	NA	<LOD
<b>Senescent whole plant</b>			
1507 Inbred	<LOD	NA	<LOD
1507 Hybrid	<LOD	NA	<LOD

1 Values are means across all four sites from mean values calculated from the analysis of five samples per site for leaf, pollen, silk, stalk, grain and one sample per site for both whole plant samples.

2 <LOD = below the limit of detection, LOD = 20 pg/ $\mu$ g total protein

3 NA = not applicable.

Study Number  
98-09-RA-NGLP-012

Page 10 of 85

Since the data are reported in pg/ $\mu$ g total protein and the total protein levels vary so greatly between tissues it is impossible to make any comparisons between expression levels of the different tissues.

The Cry1F and PAT expression levels were below the limit of detection for all control substance samples.

The conclusions from this study are:

- 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 samples.

Study Number  
98-09-RA-NGLP-012

Page 11 of 85

## INTRODUCTION

### A. Background

Maize lines, designated 1362 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 and thus makes genetically modified plants that accumulate this protein resistant to the herbicide.

The study initially included maize hybrid and inbred lines 1362 and 1507, both the hybrid and inbred lines were early generations in the breeding program, and it was expected that 50% of the plants would express the PAT protein and would therefore survive application of glufosinate herbicide. However, less than 10% of the plants containing event 1362 survived the herbicide application and the number of plants remaining in both the inbred and hybrid lines was insufficient for protein analysis, so the test lines containing event 1362 were removed from this study.

### B. Purpose

The purpose of the field trials was:

- 1) to generate leaf, pollen, silk, stalk, whole plant, grain and senescent whole plant samples from a MPS (Mycogen Plant Sciences – now known as Mycogen Seeds) hybrid and inbred derived from maize line 1507 and control lines designated Hybrid A<sub>M</sub> and Inbred A<sub>M</sub>.
- 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. The composition analysis phase of this study will not be presented in this report.

## MATERIALS

### A. Test substance

The test substances for this study were seed of 1507 maize inbred and hybrid lines that are 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 substances, Hybrid A<sub>M</sub> and Inbred A<sub>M</sub> were seed from maize lines that had not been genetically modified, but that had background genetics representative of the test substances. Pedigree information for Hybrid A<sub>M</sub> and Inbred A<sub>M</sub> lines 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).

Study Number  
98-09-RA-NGLP-012

Page 12 of 85

### C. Reference substance

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 Buin, Viluco, Graneros and Nancagua, Chile all located between 10 and 125 Km south of Santiago. These sites are located in the major maize growing regions of Chile and are equivalent to regions in the United States where the maize lines would be suitable commercial products, therefore the measurements of Cry1F and Pat expression should be equivalent to what can be expected in the United States. Each site was identified by a unique 2-3-character code.

At each site, there were 6 blocks (block = replicate) that were arranged in a randomized complete block design with nesting. Block 1 consisted of 3-row plots for each entry and contained all test and control lines. Blocks 2-6 were only for composition analysis so are not described here. Each line was tracked by a range/row designation. Field plot maps showing randomization schemes are shown in Tables 1-4.

## METHODS

### A. Summary of experimental design

The test and control lines were grown at 4 field sites in Chile. Leaf, pollen, silk, stalk, whole plant, grain and senescent whole plant tissue samples were collected from plants from the hybrid and inbred test and control lines. The tissues were evaluated for Cry1F and PAT protein levels using specific ELISA methods developed for each protein.

Study Number 98-09-RA-NGLP-012
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Page 13 of 85
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## B. Field trial

All sites were managed so that the identity and integrity of all samples was maintained. Important crop dates (i.e. seed receipt, planting, pollinations and harvest) are listed in Table 5.

### 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. Chemical and fertilizer application dates and amounts are summarized in Tables 6-9.

### 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 5 - 7 cm. Between 19 and 23 kernels of each line were planted per row of each plot. The row length at the Buin and Viluco sites was approximately 3.75 meters and the row length at the Graneros and Nancagua sites was approximately 3.55 meters. Rows at all sites were approximately 0.75 meters apart.

The test lines were segregating for the Cry1F and PAT genes. All test lines were sprayed with glufosinate herbicide using a hand spray bottle at approximately the V5-V6 stage of development. Results from the herbicide spraying are summarized in Table 10. In Viluco, the control lines were inadvertently sprayed with glufosinate instead of the test lines. All the plants in the control plots died; the test lines that had not been sprayed were then sprayed. Plants that were damaged by the herbicide were assumed to lack the *pat* and *cry1F* genes and were rogued from the plots of each test line.

### 3. Climate

Weather conditions were documented at each site and are summarized in Table 11. Rainfall and supplemental irrigation was sufficient to produce maize typical of the growing area.

### 4. Sampling

Sampling dates are summarized in Table 12.

**Leaf sampling** When the plants were at approximately the V9 stage of development the youngest whorl leaf was collected from 5 plants per hybrid test and control line and inbred test and control line at each site. These plants were identified in the field, and the pollen, silk, and stalk tissue samples were collected from the same 5 plants. Each individual sample was placed in a labeled resealable plastic bag on ice and sent to the Pioneer facility near Buin. At the Pioneer facility the samples were kept frozen at approximately -20°C until they were lyophilized. After lyophilization the samples were kept frozen, and then shipped at ambient temperature to the analytical principal investigator. They were stored at -80°C until analysis.

**Pollen sampling** Pollen was collected from each of 5 plants per hybrid test and control lines at the R1 (50% pollen shed) growth stage at each site. The pollen was collected in a tassel bag (either with or without the tassel included), and then individual tassel bags were placed inside a labeled resealable bag on ice and sent to the Pioneer facility near Buin. At the Pioneer facility the samples were kept frozen at approximately -20°C until they were lyophilized. After lyophilization the samples were kept frozen, then shipped at ambient temperature to the analytical principal investigator. Samples were stored at -80°C until analysis.

Study Number  
98-09-RA-NGLP-012

Page 14 of 85

**Silk sampling** Silks were collected from each of 5 plants per hybrid test and control lines at the R1 growth stage. The silks were placed in individual shoot bags, then placed in individual labeled resealable plastic bags on ice and sent to the Pioneer facility near Buin. At the Pioneer facility the samples were kept frozen at approximately -20°C until they were lyophilized. After lyophilization the samples were kept frozen, then shipped at ambient temperature to the analytical principal investigator. Samples were stored at -80°C until analysis.

**Stalk/Pith sampling** A 12 inch section of stalk/pith sample was collected from 5 plants per hybrid test and control lines at the R1-R2 growth stage at each site. Each individual sample was placed in labeled resealable bags on ice and sent to the Pioneer facility near Buin. At the Pioneer facility the samples were kept frozen at approximately -20°C until they were chopped and lyophilized. After lyophilization the samples were kept frozen, then shipped at ambient temperature to the analytical principal investigator. The samples were then shipped to the Pioneer Livestock Nutrition Center at Polk City, IA for grinding. After grinding, samples were returned frozen to the analytical principal investigator where they were stored at -80°C until analysis.

**Whole plant sampling** Three self-pollinated whole plants were collected from each hybrid and inbred test and control plot and chopped using an experimental silage chopper and frozen at the Pioneer facility near Buin. They were then dried for 2 days at approximately 62°C and kept at room temperature until shipment to Johnston, IA. They were then sent to the Pioneer Livestock Nutrition Center in Polk City, IA and stored frozen until ground to a fine powder. They were returned frozen to Johnston, IA, and stored at -80°C until analysis.

**Grain sampling** Grain samples were harvested by hand under normal harvest conditions (after physiological maturity). Ears from 5 self-pollinated plants were collected from each inbred and hybrid test and control plots. After drying, the ears were individually shelled and then placed in separate labeled bags. The grain samples were shipped to the analytical principal investigator at ambient temperature. The grain samples were stored at ambient temperature until they were ground. The ground samples were lyophilized, then stored at approximately -80°C until analysis.

**Senescent whole plant sampling** Three self-pollinated whole plants were collected from each inbred and hybrid test and control plot at senescence. The whole plants were chopped using an experimental silage chopper and frozen at the Pioneer facility near Buin. They were then dried for 2 days at approximately 62°C and kept at room temperature until shipment to Johnston, IA. They were then sent to the Pioneer Livestock Nutrition Center in Polk City, IA and stored frozen until ground to a fine powder. They were returned frozen to Johnston, IA, and stored at -80°C until analysis.

#### C. 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.

#### D. Analytical methods

**Total soluble protein.** Total soluble protein in the maize tissue supernatants was measured by the method of Bradford (1976) using the microtiter plate application of the Bio-Rad Protein Assay. Bovine serum albumin (Sigma, St. Louis, MO) 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.



Study Number  
98-09-RA-NGLP-012

Page 15 of 85

**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/ $\mu$ g 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 streptavidin-alkaline phosphatase (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/ $\mu$ g of total protein.

#### E. 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.

#### D. Data reduction and statistical analyses

On the ELISA reaction plate, duplicate wells were used for each sample and each sample total protein concentration was also assayed in duplicate. Absorbance readings from the ELISAs and total soluble protein determinations were recorded using the Bio-Rad Model 3550 plate reader. If the coefficient of variation (CV) of duplicate wells was equal to or less than 15% the concentration values of the two wells was averaged, however, if the CV was greater than 15% the sample was reanalyzed. The data were transferred to a JMP file where mean pg/ $\mu$ g and standard deviation calculations were performed for each tissue sample across locations.

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:

$$\text{Volume of extract } (\mu\text{l}) = \frac{\text{amount of total protein/well } (\mu\text{g/well}) \times \text{final volume } (\mu\text{l})}{\text{volume/well } (\mu\text{l/well}) \times \text{total protein concentration (mg/ml)}}$$

$$\text{Volume of PBST } (\mu\text{l}) = \text{final volume } (\mu\text{l}) - \text{volume of extract } (\mu\text{l})$$

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

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

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

Study Number  
98-09-RA-NGLP-012

Page 16 of 85

An example of an ELISA calculation using a pollen sample from this study is as follows: The average OD from a leaf sample (ID NAN-5071-1-1-P) was 1.444, the plate reader converted this into 226.55 pg/well.

$$\text{Concentration of Cry1F extract} = \frac{226.55 \text{ pg/well}}{50 \mu\text{l/well}} = 4.53 \text{ pg}/\mu\text{l}$$

$$\text{Titer of Cry1F of extract} = \frac{4.53 \text{ pg}/\mu\text{l}}{1 \mu\text{g}/50 \mu\text{l}} = 226.55 \text{ pg}/\mu\text{g total protein}$$

#### E. Protocol deviations

1. The glufosinate herbicide was inadvertently applied to the control entries at the Viluco site, but was not applied to the test entries. The test entries were sprayed with the herbicide after the error was discovered.

### RESULTS AND DISCUSSION

#### A. Field Trial

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

#### B. Protein expression in maize tissue samples

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/ $\mu\text{g}$  total protein for the Cry1F assay and <20 pg/ $\mu\text{g}$  total protein for the PAT assay. The assays were developed using a total protein load of 1  $\mu\text{g}$ /well. Increasing the protein load per well actually decreases the LOD, however, since the assay was developed using a specific absorbance at the low end of the standard curve that corresponds to the 10 or 20 pg/ $\mu\text{g}$  total protein the LOD was not lowered for this study. Decreasing the protein load however would raise the LOD and the specific absorbance would still be within the acceptable range on the standard curve, so the LOD was raised if the protein load was less than 1  $\mu\text{g}$ /well.

1. **Total protein levels in maize tissues.** The tissue extracts were found to have a total protein concentration ranging from 186 to 3070  $\mu\text{g}/\text{ml}$ . 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 hybrid leaf, pollen, silk, stalk, whole plant, grain, and senescent whole plant samples.** Table 13 summarizes the levels of Cry1F protein in the tissue samples. The values represent means across all sites.

The Cry1F expression level was below the limit of detection in one test line sample from leaf, pollen, silk, stalk and whole plant tissue samples collected from the Buin location and a single leaf sample from Viluco. The chain-of-custody forms indicated that the correct samples were collected. However, since glufosinate application, rather than Cry1F ELISA, was used to identify the plants containing the transgenes, it is possible that not all of the plants that

survived herbicide application contained the *cry1F* gene. Therefore PCR analysis was conducted to determine whether these tissue sample did actually contain the *cry1F* gene. Leaf tissue, pollen and whole plant samples from Buin were very clearly negative for the *cry1F* gene. Buin stalk sample results were equivocal and weight of evidence points to negative results. The PCR reaction for the silk tissue sample from Buin failed, but based on the results from the leaf, pollen and stalk tissue samples that were collected from the same plant in Buin, it was accepted that it was negative for the *cry1F* gene as well. The PCR reaction for the leaf tissue sample from Viluco also failed, but based on the results from the tissue samples from Buin, it was considered negative for the *cry1F* gene. The Cry1F expression values from these samples will not be considered in this report.

For both the test and control lines there were a total of 20 leaf samples analyzed, five samples from each of 4 locations. The Cry1F expression was below the limit of detection in all leaf samples from the control lines. The range in Cry1F expression levels across all sites for the test substance leaf samples was from 56.6 to 148.9 pg/ $\mu$ g total protein.

For pollen and silk samples there were a total of 20 samples for each of the test and control lines for each tissue analyzed. For pollen, the level of Cry1F expression ranged from 113.4 to 168.2 pg/ $\mu$ g total protein. The range of Cry1F expression levels in silk was 26.8 to 79.8 pg/ $\mu$ g total protein. The Cry1F expression levels of both tissues in the control lines were below the level of detection.

There were a total of 20 stalk samples for each of the test and control lines. The range of Cry1F expression levels in the test line stalk samples was 355.9 to 737.4 pg/ $\mu$ g total protein. The Cry1F expression was below the level of detection for all control line samples.

Three whole plants were pooled at each location to make a single sample for each location. The Cry1F expression was below the level of detection for all control line samples. The range of Cry1F expression levels in the test line whole-plant samples ranged from 803.2 to 1572.7 pg/ $\mu$ g total protein.

A total of 20 grain samples were analyzed for each of the test and control lines. The Cry1F expression was below the LOD in all control line grain samples with a single exception. The Cry1F expression level from a control line grain sample from Buin was 86.4 pg/ $\mu$ g total protein. Additionally, a single test line grain sample from Buin was below the LOD for both Cry1F and PAT expression. Based on expression levels, it would appear that the identification for these two samples was reversed. Also, the test line sample was analyzed by PCR to test for the presence of the *cry1F* gene. The PCR reaction was negative for the *cry1F* gene in the test line sample providing further evidence that the test line sample was misidentified, so that value will not be considered. The level of Cry1F expression levels in the test line grain samples ranged from 71.2 to 114.8 pg/ $\mu$ g total protein.

The sample for senescent whole plants at each location consisted of three plants pooled together, so a total of 4 samples were analyzed for both the test and control lines. The range of Cry1F expression levels in the test line senescent whole plants ranged from 622.2 – 845.3 pg/ $\mu$ g total protein. 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 14 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 40.8 pg/ $\mu$ g total protein. The PAT expression level was below the level of detection for all control line leaf samples.

Study Number  
98-09-RA-NGLP-012

Page 18 of 35

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

Three whole plants of each test and control line were pooled at each location to make a single sample for each location. The 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.

4. **Cry1F protein levels in inbred leaf, grain, and senescent whole plant samples.** Table 13 summarizes the levels of Cry1F protein in tissue samples. The Cry1 F expression values were averaged across all sites.

A total of 20 inbred test and control leaf samples were analyzed for Cry1 expression. The Cry1F expression was below the LOD in all of the control lines. The level of expression for the test lines ranged from 79.3 to 209.4 pg/μg total protein.

For pollen, silk and stalk samples there were a total of 20 samples for each of the inbred test and control lines for each tissue analyzed. For pollen, the level of Cry1F expression ranged from 186.3 to 231.1 pg/μg total protein. The range of Cry1F expression levels in silk was 36.2 to 89.8 pg/μg total protein. For stalk, the level of Cry1F expression ranged from 480.5 to 849.0. The Cry1F expression levels in pollen, silk and stalk tissues in the control lines were below the level of detection.

Three whole plants were pooled at each location to make a single sample for each location. The Cry1F expression was below the LOD for all inbred control line samples. The range of Cry1F expression levels in the test line whole-plant samples ranged from 1283.5 to 1428.0 pg/μg total protein.

A total of 20 inbred test and control line grain samples were analyzed for Cry1F expression. The level of Cry1F expression in the test line ranged from 66.5 to 141.5 pg/μg total protein. The Cry1F expression in the control lines was below the level of detection for all samples.

The sample for senescent whole plants at each location consisted of three plants pooled together, so a total of 4 samples were analyzed for both the test and control lines. The level of Cry1F expression in the test line senescent whole plants ranged from 470.5 to 968.3 pg/μg total protein. The Cry1F expression in the control lines was below the level of detection for all samples.

5. **PAT protein levels in inbred leaf, grain, and senescent whole plant samples.** Table 14 summarizes the levels of Cry1F protein in tissue samples. The values were averaged across all sites.

A total of 20 inbred test and control line leaf samples were analyzed for PAT expression. The level of PAT expression in the test line ranged from below the LOD to 58.2 pg/μg total protein. Of the 20 samples, the PAT expression was below the level of detection in 10 of the test samples. The level of PAT expression was below the level of detection in all of the control line leaf samples.

Study Number  
98-09-RA-NGLP-012

Page 19 of 85

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

Three whole plants of each inbred test and control line were pooled at each location to make a single sample for each location. The 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 inbred 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/ $\mu$ g total protein and the protein levels vary so greatly between tissues it is impossible to make any comparisons between expression levels of the different tissues.

#### CONCLUSIONS

Tissue samples collected from the genetically modified maize hybrid and inbred lines and the control hybrid and inbred line 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.

Study Number  
98-09-RA-NGLP-012

Page 20 of 85

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Study Number  
98-09-RA-NGLP-012

Page 21 of 85

Table 1. Field Plot Map: Buin, Chile (BUI)

	Range 2
Row 5	(Block1)
Row 6	Control Inbred A <sub>M</sub>
Row 7	Control Inbred A <sub>M</sub>
Row 8	1507 Inbred
Row 9	1507 Inbred
Row 10	1507 Inbred
Row 11	1362 Inbred
Row 12	1362 Inbred
Row 13	1362 Inbred
Row 14	1362 Hybrid
Row 15	1362 Hybrid
Row 16	1362 Hybrid
Row 17	Control Hybrid A <sub>M</sub>
Row 18	Control Hybrid A <sub>M</sub>
Row 19	Control Hybrid A <sub>M</sub>
Row 20	1507 Hybrid
Row 21	1507 Hybrid
Row 22	1507 Hybrid
Row 23	filler
Row 24	filler

Study Number  
98-09-RA-NGLP-012

Page 22 of 85

Table 2. Field Plot Map: Viliuco, Chile (VIL)

	Range 2
Row 5	(Block 1) 1362 Inbred
Row 6	1362 Inbred
Row 7	1362 Inbred
Row 8	Control Inbred A <sub>M</sub>
Row 9	Control Inbred A <sub>M</sub>
Row 10	Control Inbred A <sub>M</sub>
Row 11	1507 Inbred
Row 12	1507 Inbred
Row 13	1507 Inbred
Row 14	Control Hybrid A <sub>M</sub>
Row 15	Control Hybrid A <sub>M</sub>
Row 16	Control Hybrid A <sub>M</sub>
Row 17	1507 Hybrid
Row 18	1507 Hybrid
Row 19	1507 Hybrid
Row 20	1362 Hybrid
Row 21	1362 Hybrid
Row 22	1362 Hybrid
Row 23	filler
Row 24	filler



Study Number  
98-09-RA-NGLP-012

Page 23 of 35

Table 3. Field Plot Map: Graneros, Chile (GRA)

	Range 2
Row 5	(Block 1)
	1362 Inbred
Row 6	1362 Inbred
Row 7	1362 Inbred
Row 8	Control Inbred A <sub>M</sub>
Row 9	Control Inbred A <sub>M</sub>
Row 10	Control Inbred A <sub>M</sub>
Row 11	1507 Inbred
Row 12	1507 Inbred
Row 13	1507 Inbred
Row 14	1362 Hybrid
Row 15	1362 Hybrid
Row 16	1362 Hybrid
Row 17	1507 Hybrid
Row 18	1507 Hybrid
Row 19	1507 Hybrid
Row 20	Control Hybrid A <sub>M</sub>
Row 21	Control Hybrid A <sub>M</sub>
Row 22	Control Hybrid A <sub>M</sub>
Row 23	filler
Row 24	filler

Study Number  
98-09-RA-NGLP-012

Page 24 of 85

Table 4. Field Plot Map: Nancagua (NAN)

	Range 2
Row 5	(Block 1)
	1362 Inbred
Row 6	1362 Inbred
Row 7	1362 Inbred
Row 8	Control Inbred A <sub>M</sub>
Row 9	Control Inbred A <sub>M</sub>
Row 10	Control Inbred A <sub>M</sub>
Row 11	1507 Inbred
Row 12	1507 Inbred
Row 13	1507 Inbred
Row 14	1362 Hybrid
Row 15	1362 Hybrid
Row 16	1362 Hybrid
Row 17	Control Hybrid A <sub>M</sub>
Row 18	Control Hybrid A <sub>M</sub>
Row 19	Control Hybrid A <sub>M</sub>
Row 20	1507 Hybrid
Row 21	1507 Hybrid
Row 22	1507 Hybrid
Row 23	filler
Row 24	filler

Study Number  
98-09-RA-NGLP-012

Page 25 of 85

Table 5. Important Crop Dates for the 1998/99 Field Trials Conducted in Chile.

Field Site Site Code	Seed Receipt	Planting	Pollination	Harvest
Buin, Chile BUI	prior to 10/28/98	11/4/98	1/19/99 – 2/3/99	4/23/99
Viluco, Chile VIL	prior to 10/28/98	10/30/98	1/11/99 – 1/21/99	4/26/99
Graneros, Chile GRA	prior to 10/28/98	12/2/98	2/3/99 – 2/11/99	5/12/99
Nancagua, Chile NAN	prior to 10/28/98	12/2/98	2/3/99 – 2/10/99	5/12/99

Study Number  
98-09-RA-NGLP-012

Page 26 of 85

Table 6. Chemical and Fertilizer Applications for the 1998/99 Field Trials Conducted in Buin, Chile.

Field Site Site Code	Date	Product	Rate (kg ai/ha)
Buin, Chile BUI	11/3/98	cyanazine	1.5
	11/3/98	chlorpyrifos	2.9
	11/3/98	metolachlor	1.9
	11/4/98	N-P-K	112-95-81
	11/4/98	carbofuran	1.0
	11/4/98	flutriafol + carbofuran	0.05 + 0.60
	11/20, 26/98	chlorpyrifos + cypermethrin	0.5 + 0.05
	12/14/98	N-P-K	180-0-0
	1/9, 20/99	lambda-cyhalothrin	0.01

Study Number  
98-09-RA-NGLP-012

Page 27 of 85

Table 7. Chemical and Fertilizer Applications for the 1998/99 Field Trials Conducted in Viluco, Chile.

Field Site Site Code	Date	Product	Rate (kg ai/ha)
Viluco, Chile VIL	10/29/98	cyanazine	1.5
	10/29/98	chlorpyrifos	2.9
	10/29/98	metolachlor	1.9
	10/30/98	N-P-K	112-95-81
	10/30/98	carbofuran	1.0
	10/30/98	flutriafol + carbofuran	0.05 + 0.60
	11/12, 18, 25/98	chlorpyrifos + cypermethrin	0.5 + 0.05
	11/19/98	bentazon	1.4
	12/7/98	N-P-K	184-0-0
	1/5, 13, 21, 29/99	lambda-cyhalothrin	0.01
	2/23/99	dicofof	0.9
	3/1/99	dicofof	0.7

Study Number  
98-09-RA-NGLP-012

Page 23 of 85

Table 8. Chemical and Fertilizer Applications for the 1998/99 Field Trials Conducted in Graneros, Chile.

Field Site Site Code	Date	Product	Rate (kg ai/ha)
Graneros, Chile GRA	12/2/98	chlorpyrifos	3.4
	12/2/98	atrazine	1.5
	12/2/98	acetochlor	1.9
	12/2/98	N-P-K	91-55-36
	12/12, 19, 30/98	chlorpyrifos	1.4
	12/27/98	N-P-K	138-0-0
	2/26/99	dicofol	1.0
	2/26/99	lambda-cyhalothrin	0.01

Study Number  
98-09-RA-NGLP-012

Page 29 of 85

Table 9. Chemical and Fertilizer Applications for the 1998/99 Field Trials Conducted in Nancagua, Chile.

Field Site Site Code	Date	Product	Rate (kg a/ha)
Nancagua, Chile NAN	12/1/98	chlorpyrifos	2.8
	12/1/98	acetochlor	2.3
	12/1/98	atrazine	1.7
	12/2/98	chlorpyrifos	1
	12/2/98	N-P-K	91-55-36
	12/12, 18, 30/98	chlorpyrifos	1.4
	12/31/98	N-P-K	138-0-0
	2/27/99	dicofol	0.7
	2/27/99	lambda-cyhalothrin	0.01

Study Number  
98-09-RA-NGLP-012

Page 30 of 85

Table 10. Summary of glufosinate herbicide segregation.

entry	Buln		Vitico		Graneros		Nancagua	
	plants emerged	surviving plants	plants emerged	surviving plants	plants emerged	surviving plants	plants emerged	surviving plants
1362I	54-57	4	54-57	6	54	6	49	5
1507I	54-57	29	54-57	21	53	25	54	29
1362H	54-57	2	54-57	5	48	7	51	7
1507H	54-57	35	54-57	37	49	35	53	37



Study Number  
98-09-RA-NGLP-012

Page 31 of 85

Table 11. Summary of Rainfall and Temperatures for 1998/99 Growing Season.

Month	Parameter	BUI	VIL	GRA	NAN
November	Maximum temperature - °F	88	92	not available	not available
	Minimum temperature - °F	39	39		
	Average temperature - °F	62	64		
	Total Rainfall - inches <sup>1</sup>	0.0	0.0		
December	Maximum temperature - °F	97	97	93	99
	Minimum temperature - °F	45	45	44	44
	Average temperature - °F	68	69	68	71
	Total Rainfall - inches <sup>1</sup>	0.0	0.0	not available	not available
January	Maximum temperature - °F	94	93	91	100
	Minimum temperature - °F	45	45	43	44
	Average temperature - °F	67	67	68	71
	Total Rainfall - inches <sup>1</sup>	0.0	0.0	not available	not available
February	Maximum temperature - °F	93	93	93	97
	Minimum temperature - °F	44	45	42	44
	Average temperature - °F	68	68	68	71
	Total Rainfall - inches <sup>1</sup>	0.0	0.0	not available	not available
March	Maximum temperature - °F	88	87	90	91
	Minimum temperature - °F	40	42	37	38
	Average temperature - °F	63	64	64	68
	Total Rainfall - inches <sup>1</sup>	5.8	5.8	not available	not available
April	Maximum temperature - °F	83	83	not available	not available
	Minimum temperature - °F	32	35		
	Average temperature - °F	58	58		
	Total Rainfall - inches <sup>1</sup>	0.06	0.06		

1 Additional irrigation applied in-furrow as needed.

Study Number  
98-09-RA-NGLP-012

Page 32 of 85

Table 12. Summary of Tissue Sampling Dates for the 1998 Field Trials.

Field Site Site Code	V9 Leaf	Pollen	Silk	Stalk	Whole Plant	Grain	Senescent Whole Plant
Buin, Chile BUI	12/23/98	1/25/ - 2/3/99	1/20 - 2/6/99	1/20 - 2/6/99	3/11, 12/99	4/23/99	4/5/99
Viluco, Chile VIL	12/18/98	1/12 - 21/99	1/12 - 19/99	1/12 - 21/99	3/1 - 2/99	4/26/99	4/12/99
Graneros, Chile GRA	1/12/99	2/4 - 9/99	2/4 - 10/99	2/4 - 10/99	4/6/99	5/12/99	4/28/99
Nancagua, Chile NAN	1/12/99	2/4 - 8/99	2/4 - 8/99	2/4 - 8/99	3/31/99	5/12/99	4/29/99

Study Number  
98-09-RA-NGLP-012

Page 33 of 85

**Table 13. Summary of Cry1F protein levels measured in tissue collected from maize inbred and hybrid line 1507 during the 1998-1999 growing season in Chile.**

Tissue	Mean <sup>1</sup> Cry1F (pg/ $\mu$ g total protein)	Standard Deviation	Min/Max Range
<b>Leaf</b>			
1507 Inbred	169.5	35.4	79.3 – 209.4
1507 Hybrid	110.9	27.2	56.6 – 148.9
<b>Pollen</b>			
1507 Inbred	207.5	10.6	186.3 – 231.1
1507 Hybrid	135.5	13.5	113.4 – 168.2
<b>Silk</b>			
1507 Inbred	58.9	14.9	36.2 – 89.8
1507 Hybrid	50.3	16.5	26.8 – 79.8
<b>Stalk</b>			
1507 Inbred	637.8	101.8	480.5 – 849.0
1507 Hybrid	550.0	104.0	355.9 – 737.4
<b>Whole plant</b>			
1507 Inbred	1357.8	72.3	1283.5 – 1428.0
1507 Hybrid	1063.8	361.7	803.2 – 1572.7
<b>Grain</b>			
1507 Inbred	112.2	20.6	66.5 – 141.5
1507 Hybrid	89.8	23.3	71.2 – 114.8
<b>Senescent whole plant</b>			
1507 Inbred	677.5	215.5	470.5 – 968.3
1507 Hybrid	714.3	95.5	622.2 – 845.3

<sup>1</sup> Values are means across all four sites from mean values calculated from the analysis of five samples per site for leaf, pollen, silk, stalk, grain and one sample per site for both whole plant samples.

Study Number  
98-09-RA-NGLP-012

Page 34 of 85

Table 14. Summary of PAT protein levels measured in tissue collected from maize inbred and hybrid line 1507 during the 1998-1999 growing season in Chile.

Tissue	Mean <sup>1</sup> PAT (pg/ $\mu$ g total protein)	Standard Deviation	Min/Max Range
<b>Leaf</b>			
1507 Inbred	21.4	22.8	<LOD <sup>2</sup> - 58.2
1507 Hybrid	<LOD	NA <sup>3</sup>	<LOD - 40.8
<b>Pollen</b>			
1507 Inbred	<LOD	NA	<LOD
1507 Hybrid	<LOD	NA	<LOD
<b>Silk</b>			
1507 Inbred	<LOD	NA	<LOD
1507 Hybrid	<LOD	NA	<LOD
<b>Stalk</b>			
1507 Inbred	<LOD	NA	<LOD
1507 Hybrid	<LOD	NA	<LOD
<b>Whole plant</b>			
1507 Inbred	<LOD	NA	<LOD
1507 Hybrid	<LOD	NA	<LOD
<b>Grain</b>			
1507 Inbred	<LOD	NA	<LOD
1507 Hybrid	<LOD	NA	<LOD
<b>Senescent whole plant</b>			
1507 Inbred	<LOD	NA	<LOD
1507 Hybrid	<LOD	NA	<LOD

1 Values are means across all four sites from mean values calculated from the analysis of five samples per site for leaf, pollen, silk, stalk, grain and one sample per site for both whole plant samples.

2 <LOD = below the limit of detection, LOD = <20 pg/ $\mu$ g total protein

3 NA = not applicable.

## 9

## Production of Hybrid Seed Corn

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The objective of this chapter is to present a current, state-of-the-art description of seed corn (*Zea mays* L.) production as it is commercially practiced in the late 1980s. The chapter draws heavily on the treatment by Craig (1977), but will highlight changes in the industry and in technology since that chapter was written.

## 9-1 HISTORICAL PERSPECTIVE

The first commercial hybrids were produced and sold in the early 1920s. From that modest beginning our present-day sophisticated hybrid seed corn industry has developed. Crabb (1947) described the first production of hybrid seed corn in Iowa:

"The first contract ever drawn for the production of seed for hybrid corn gave [George] Kurtzweil the exclusive right for all time to produce the Copper Cross hybrid, a contract which, although it hasn't been exercised for a good many years, is still one of Kurtzweil's most prized possessions."

"[Henry A.] Wallace said he had foundation inbred material to plant a one-acre seed plot, and the decision was made to produce the first commercial hybrid seed corn ever grown in Iowa . . . Wallace turned the seed over to Kurtzweil [in 1923]. The old East Leaming inbred was used for the seed parent, and the meager supply of the Bloody Butcher line was used as the pollinator parent. Only by very sparse and careful planting was Kurtzweil able to plant the plot that measured almost one acre on a small farm owned by Kurtzweil's father, Mathias Kurtzweil, at Altoona, just east of Des Moines . . ."

"The first detasseling of commercial hybrid seed corn in Iowa was done entirely by a woman, Ruth Kurtzweil, a sister of George . . . From the time the first tassels of the parent plants began to appear on the Leaming inbred, Miss Kurtzweil went up and down, pulling them out. Few fields of hybrid seed corn since have been detasseled with such care and interest. Now that producing hybrid seed corn has become such a tremendous enterprise, Miss Kurtzweil delights in calling her friends' attention to the fact that she once detasseled all the hybrid seed corn production fields in the State of Iowa."

"Copper Cross earned another distinction in 1924 when it became the first hybrid developed in the corn belt to be purchased by farmers of Iowa and elsewhere. Approximately fifteen bushels—all that was available of Cop-

per Cross seed—was sold in the spring of 1924 at the price of \$1.00 a pound, or at the rate of \$56.00 a bushel."

The first hybrids to be developed were adapted primarily to the central Corn Belt; these were accepted slowly, and by 1933 approximately 1% of the Corn Belt corn acreage was planted with hybrid seed (Airy, 1955). Because of the superior performance of hybrids in the severe droughts of 1934 and 1936, farmers rapidly began accepting, and then demanding, hybrid seed.

The rapid acceptance by U.S. farmers of hybrid corn varieties in the 1930s and 1940s provided the basis on which many firms and individuals established themselves in a new and fast-growing industry. Prior to that time, only a few firms had been engaged in the hybrid seed corn business. Development of new hybrids adapted to virtually every corn-growing area of the USA and Canada helped to establish profitable corn production on hundreds of thousands of hectares outside the Corn Belt, where profitable corn production had previously been impossible on a commercial scale.

## 9-2 SIZE OF THE INDUSTRY

Since 1900, the area in the USA planted to corn has varied from a high of 47 million ha (116 million acres) in 1917 to a low of 24.4 million ha (60.2 million acres) in 1983 [the year of the federal government's payment-in-kind (PIK) program]. Excluding 1983, the area planted to corn between 1975 and 1985 averaged 33.4 million ha (82.5 million acres).

Planting rates vary considerably according to soil fertility levels, rainfall and irrigation availability, planting date, intended use (e.g., grain vs. silage), local custom, and finally, adaptation of specific hybrids to high plant populations. Assuming that a seed corn unit of 80 000 kernels will plant 1.4 ha (3.4 acres), it is estimated that a minimum of 24.5 million units of hybrid seed were required to plant the 1985 U.S. crop. At an estimated average retail price of \$65/unit, the domestic hybrid seed corn industry has grown to a gross annual sales volume of \$1.59 billion. Sales in other countries add to the total market.

## 9-3 TYPES OF HYBRIDS

The first hybrids produced and sold commercially were almost exclusively double crosses. However, several factors contributed to a significant transition from double crosses to single crosses within the U.S. Corn Belt, starting in the late 1950s and continuing through the 1980s. The transition to single crosses occurred because: (i) single crosses out-yielded double crosses; (ii) a few companies led the way and others joined them to be competitive; (iii) farmers began to demand single crosses; and

## PRODUCTION OF HYBRID SEED CORN

567

(iv) improved agronomic practices and development by corn breeders of inbreds with higher per se yields made the production of seed of single crosses economically feasible. Single cross hybrids now comprise approximately 90% of the hybrid seed sold in North America (USA and Canada).

Modified single crosses, which are produced using related-line single crosses for either the female or male parent, were used extensively in hybrid seed corn production in the 1960s and early 1970s. Three-way crosses (single cross female parent and inbred male parent) are also a factor in the industry. Today, modified single crosses and three-way crosses account for about 10% of the North American market. While double-cross hybrids were once a significant factor in the market, their importance has decreased over the past two decades. It is estimated that they now comprise <1% of the total U.S. and Canadian market.

## 9-4 SEED CORN COMPANIES "

Many firms have become involved in the production and sale of hybrid seed corn. Small, privately owned companies may produce and distribute only a few thousand units of seed. Operations of this size usually depend on inbred and hybrid development and research conducted by public institutions, or on that conducted by private firms that produce and sell parent seed (foundation seed) stocks. Smaller companies usually purchase foundation seed, produce their supplies of hybrid seed, and then sell it directly to farmers in their local areas.

Large companies usually carry on their own research and development programs, produce their own foundation seed stocks, produce the commercial seed, and distribute it through their own sales organizations. The majority of hybrid seed corn is sold by the various companies to farmer dealers, who, in turn, sell it to farmer customers. It is customary in the industry to deliver to dealers on a consignment basis, and to accept as "returns" seed that remains unsold by the dealer at the end of the planting season. Alternatively, the "sales agent" approach is used, with the seed remaining the property of the company until it is sold to the farmer customer (see section 9-12.2). In some geographical areas, sales are made by the seed company to jobbers or distributors who seek their own retail dealer outlets. This practice is more common in areas of relatively low sales volume.

Hybrid seed corn was at first sold in 25-kg (1-bu) packages. In the early 1960s, there began a trend to package and distribute in 23-kg (50-lb) packages. Later in that decade, the practice of packaging by kernel count became popular. At the present time, most seed corn is sold in units of 80 000 kernels.

The industry has seen both the attrition in the number of companies and great variation in the relative growth rates of individual companies. The many relatively small operations within the industry have a collective

market share of 36%, while the industry's seven largest companies have in total an estimated 64% share of the U.S. hybrid seed corn market (J. Ansorge, 1987, personal communication).

## 9-5 PRODUCTION OF PARENT SEED STOCKS

Large quantities of parent seed stocks (foundation seed) are required annually to plant the several hundred thousand hectares of commercial hybrid seed corn production. Most larger companies have parent seed or foundation seed departments responsible for the production and inventory of inbred and single cross parents needed for commercial seed production. In recent years, many seed companies have devoted increasing attention to developing more effective techniques and procedures to assure adequate supplies of high-quality, genetically pure stocks.

Seed corn companies must forecast future commercial seed sales and seed production plans to ensure availability of adequate parent seed supplies. Since most seed companies produce and sell many different hybrids, the number and supply of different parent seed strains that must be maintained for commercial seed production requirements is often quite large.

### 9-5.1 Foundation Seed Stock Increase

Foundation seed stock increase involves the maintenance and increase of inbred lines and single cross parent seed used to produce commercial hybrids. Inbreds are the basic foundation seed used in hybrid seed corn production. Inbreds must therefore be maintained and increased under rigid control to ensure satisfactory final product performance. Although procedures employed may vary among organizations, at least three important steps are usually taken: (i) establishing and maintaining a supply of breeder seed; (ii) increasing inbred seed; and (iii) producing related-line and/or unrelated-line single cross parent seed.

Breeder seed is usually derived from bulked, self-pollinated seed at the  $F_3$  to  $F_{10}$  generation of inbreeding. The breeder has the responsibility of ensuring that the inbred is homozygous, uniform for plant type, and adequately represents the genetic constitution of the inbred. All inbred increases are made from this base population of breeder seed. Some companies have established separate programs to maintain supplies of breeder seed. Increases are produced in well-isolated blocks by natural random sib mating. In turn, this initial inbred increase is used to plant subsequent inbred seed increases and production of single-cross parents.

Both types of increase are made under stringent isolation. Procedures and standards developed by certification agencies (Hutchcroft, 1957; Cowan, 1972) indicate the importance of minimum isolation distances. Commercial companies certify all foundation seed that will be exported. Much of the parent seed for domestic use is not certified, but guidelines



## PRODUCTION OF HYBRID SEED CORN

569

developed over the years generally exceed, or at least equal, those of certification agencies.

Foundation seed fields are planned with isolation of 201 m (660 ft) as the base distance from other corn. Early studies by Jones and Brooks (1950, 1952) showed that: (i) the greatest contamination occurs in the 50 to 75 m (165-248 ft) nearest contaminating corn; (ii) pollen from border rows dilutes contamination; (iii) natural barriers may reduce contamination; (iv) an abundant supply of male corn pollen at the right time reduces contamination; (v) the direction of a field from contaminating pollen influences the amount of contamination; and (vi) "depth of field" in the direction of contamination source is important. Certification requirements in most states allow for substitution of additional male border rows for some portion of the 201-m isolation distance, but neither natural barriers nor time isolation are allowed to substitute for the required distances.

## 9-5.2 Procedures and Techniques

Generally, the equipment and procedures used in planting, detasseling, harvesting, drying, and conditioning of parent seed increases are similar to those used in commercial hybrid seed production. Some steps are applied more rigorously to ensure maximum genetic purity.

Variability among individual plants within the inbred population will sometimes occur. These off-types must be identified and removed (rogued) to avoid perpetuation of this variability from generation to generation. Careful plant removal (rogueing) must be practiced throughout the growing season to eliminate individual plants that exhibit phenotypes varying from the accepted phenotype of the inbred. As much rogueing as possible should occur prior to pollination to eliminate outcrossing resulting from pollen supplied by undesirable plants. Parent seed is usually harvested on the ear, allowing further selection (i.e., removal of off-type ears) to be practiced on the sorting table prior to drying and shelling (see section 9-9).

## 9-5.3 Quality Control

Rigid requirements must be used to maintain genetic purity at maximum levels. Genetic purity of parent seed not only helps ensure pure commercial hybrid seed but also reduces cost associated with rogueing commercial seed production fields and ear sorting at harvest. Parent seed is usually sized just as commercial seed corn. When genetic impurities occur, particularly those caused by outcrosses, they are often concentrated in specific kernel sizes, especially the large round kernels. As a result, certain kernel sizes within a specific lot may have unacceptably high levels of impurities, while other sizes in the same lot are acceptable. Careful selection of kernel sizes with the highest genetic purity can lead to improved purity of commercial seed corn.

Until relatively recently, the conventional approach to monitoring purity has been field "growouts". Growouts of a shelled corn composite of each seed lot from summer increase are often planted during the subsequent winter season to estimate genetic purity prior to use. In many cases more extensive growouts, sampling each kernel size in the lot, are conducted in the following summer growing season to obtain additional, more precise estimates of purity by kernel size. The accuracy of field growouts depends on: (i) securing a representative sample of the entire seed lot; (ii) a clean, uniform field to minimize volunteer corn and variation in plant height; (iii) favorable growing conditions; and (iv) knowledgeable personnel who are familiar with all the parental and hybrid plant phenotypes to score or "read" the growout.

Growouts are usually made in an area where the crop can be grown in the fall and winter months. Florida, Hawaii, Argentina, and Chile are most often used by U.S. seedsmen. Despite the use of winter growouts at these locations, it is difficult to obtain results early enough to make data-driven decisions on specific seed lots before the seed must be conditioned, bagged, and distributed. This is one of the factors that has led to increased reliance on electrophoresis results by some companies.

Starch gel electrophoresis (Cardy et al., 1980; Smith and Weissinger, 1984) is a recently developed technology that provides an additional means of purity analysis. The advantages of this technique include precision, rapidity relative to field growouts, absence of environmental influence on expression of genetically controlled characters, and the potential to make purity checks on developing embryonic samples collected prior to harvest (Smith and Wych, 1986). The disadvantages of this technique are the initial costs of the specialized equipment required and the cost of laboratory operation, or the relatively high cost per sample charged by commercial laboratories, if this alternative is chosen. Smith and Wych (1986) determined that the costs per seed lot for estimating percentage of female selfs by electrophoresis vs. growouts were approximately equivalent if both procedures were done in-house. However, if analysis for outcrosses is also conducted, the costs for electrophoresis may be somewhat higher than for estimation of selfs, only, due to the need to prepare and stain more gel slices. The urgency of the need for the purity information must be balanced against the comparative costs.

Seedsmen need to be aware that both growouts and electrophoresis provide useful information only to the extent that the samples are representative of a seed lot and adequate in size. The number and size of samples must be sufficient to provide an adequate measure of the variation within the seed lot being sampled.

Both growouts and electrophoresis can be used to identify accidental mechanical mixtures or mislabeling of foundation seed, which may occur at any point in production, conditioning, and inventory. For this application, electrophoresis has the same advantages and disadvantages mentioned above.

## PRODUCTION OF HYBRID SEED CORN

571

Another part of quality control of parent seed is germination. Various methods of measuring seed germination are employed and are generally performed on all usable kernel sizes of each seed lot. These methods are described in more detail later in the chapter (see section 9-11).

## 9-5.4 Storage and Inventory Control

Large inventories of parent seed are required. Accurate records of inventory supplies, genetic purity, and germination are maintained, since parent seed production is typically planned to provide an inventory adequate for 2 to 4 yr. Controlled environment storage facilities are used to maintain viability and quality (see section 9-12 for greater detail).

## 9-6 PRODUCTION OF COMMERCIAL HYBRID SEED

Agronomic practices in seed production fields are in general the same as those used to grow a commercial corn crop. However, there are some additional requirements unique to seed production. Acreages are determined on the basis of projected sales, utilizing yield levels based on breeders' research, production research, and past experience. The transition to the use of inbreds as female parents to produce single-cross hybrids has increased the need for planning, sound scientific and technical knowledge, and production technology to ensure economic success. Cultural practices used in production fields are planned to minimize risks while maximizing yield and seed quality.

## 9-6.1 Selection of Production Areas and Contract Growers

Successful commercial hybrid seed corn production begins with selection of a growing area and contract growers. Innovative farmers in cooperation with the seed companies have helped the seed corn industry grow into a sound business. Most seed corn production acreage is located in the Corn Belt. Expansion of the Corn Belt and increased technical knowledge have created opportunities for seed companies to expand in search of specific seed production areas. Production areas are chosen to provide such necessary factors as growing degree days, day length, lack of extreme temperatures, and specific farming practices, such as irrigation capability. By matching specific inbred needs to growing area characteristics, risk can be minimized and seed yields per female acre maximized.

Selecting growers within a production area is another important step. Generally, someone at the local production plant level will select growers who are among the most progressive and innovative corn growers in the area. The grower's location within the isolation block (see section 9-6.4) is also a factor. Seed companies attempt to select farms with high productivity indices and suitable soils that have been maintained in a state

of high fertility with good weed control. Tillage and cultural practices must be in line with approved hybrid corn production practices. Good soil structure and tillage are important in order to avoid the adverse effects of poor drainage and crusting on inbred stands. Since approximately 90 to 95% of the seed acreage will be devoted to inbred or related-line single cross parents, seed corn growers who will give special attention to management of insect and disease pests, weed competition, and fertility are needed.

Contract growers must be willing to cooperate with seed companies to alter their cultural practices and/or timing, rate, and kind of herbicides, insecticides, or fungicides. Equipment modifications are often necessary. In some areas, the seed company furnishes equipment on a lease or rental basis to the growers. In other cases, growers may cooperate in the purchase and sharing of various specialized pieces of equipment such as unit planters and detasseling and harvesting equipment.

### 9-6.2 Contracts

With the advent of single-cross hybrid seed production, contract growers were not content to assume the greater risks associated with inbred parents. As a result, base guarantees are made that involve payment, up to a predesignated yield level, for complete failure of the seed crop. Incentive payments are often based on published futures or cash market prices at a specified time and place. In some instances, contracts are based on government based yield calculations or locally measured commercial hybrid corn yield checks.

Usually, the type of contract used, base guarantees, and multiplier factors are based upon anticipated yield levels and degree of difficulty encountered in the production of each individual hybrid. Factors such as fertility, herbicide or insecticide costs, seedbed preparation for split-date plantings, volunteer removal, and harvesting are items for consideration within the contract. There are considerable variations in contracts among companies.

### 9-6.3 Management of the Production Area

The management staff at a production plant are responsible for all aspects of production. They are assisted by trained supervisory help, especially during planting, detasseling, and harvesting periods. An area of 4000 to 6000 ha (10 000-15 000 acres) of seed production responsibility is fairly typical for a production plant manager and his/her staff. Within this acreage, regional (or area) supervisors are charged with responsibility for 400 to 800 ha (1000-2000 acres). During detasseling, additional supervisory help may be employed as crew foremen, field foremen, and inspectors. The demand for additional supervisors during the summer months offers opportunities to utilize agriculture and science teachers.

## PRODUCTION OF HYBRID SEED CORN

principals, and other professionally trained personnel during their school vacation periods.

Communication is essential for production plant managers and area supervisors to be effective in the management of their respective areas and for coordination of activities. Particularly during detasseling, it is critical that communication be available so that people and equipment can be effectively used and moved to high priority areas as needed. Many seed companies use a combination of mobile telephones, Citizen's Band radios, and FM two-way radios.

## 9-6.4 Isolation of Seed Fields

Isolation is intended to assure that the hybrid cross is produced with a high degree of genetic purity. It has often been said by seedsmen that the best isolation is a perfect nick, that is, when a pollen parent starts shedding just before the female parent's silks start to emerge beyond the husk or tip of the ear shoot. In addition, plant management personnel must work with each individual contract grower to establish the location and boundaries of each seed field to conform with isolation distance requirements. Preference is also given to crop rotation of corn following soybean [*Glycine max* (L.) Merr.] (rather than following corn), because volunteer corn problems are avoided and seed corn yield is higher.

Minimum standards for isolation of seed corn production fields have been established (Anonymous, 1971) for the USA and Canada; nevertheless, some variation exists among states. When zero or one male border row is present, minimum distances ranging from 125 to 201 m (410-660 ft) are typically required between the female parent of the hybrid being produced and any other corn of the same seed color, maturity, or endosperm type. Isolation distance of 201 m is required where possible contaminant corn may have different kernel color or endosperm type. Additional distance is sometimes employed where contaminating corn may be of decidedly different pollen shedding ability (such as tropical hybrids), and where wind velocities may be high, such as in production areas near large bodies of water.

Minimum isolation distance requirements can be modified by: (i) additional border rows (Fig. 9-1); (ii) size of field and production block; and (iii) adequate natural barriers and differential flowering dates (in some states). Jones and Brooks (1952) found that natural barriers are not as effective as border rows of corn. Differential flowering times are effective in isolation if silks of female parents are not receptive when pollen from other than the male parent is present.

To optimize genetic purity, a timely nick between receptive silks on the female parent and pollen shed by the male parent is required. Differential planting dates of seed parents are often required to achieve this. Abundant amounts of male pollen are also beneficial, and some companies utilize high population density in the male parent rows to increase pollen load.

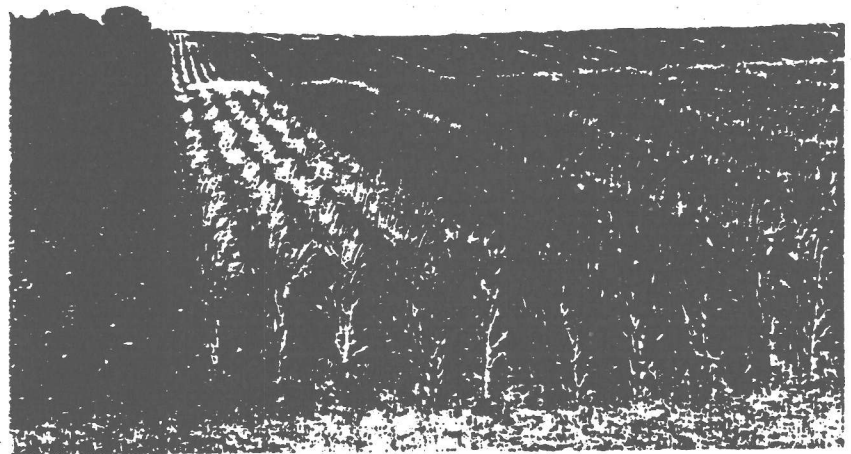


Fig. 9-1. A commercial hybrid seed production field showing additional male parent border rows for the purpose of providing pollen saturation adequate to ensure genetic purity of the hybrid seed. The strip of soybeans on the left provides the required isolation distance.

## 9-7 PLANTING THE SEED FIELD

### 9-7.1 Planting Date

The minimum soil temperature for growth of corn is generally regarded as 10 °C (50 °F). Most agronomists would also agree that the optimum time for planting corn is as soon as the soil temperature at the 5-cm depth reaches that temperature for a relatively sustained period of time. Soil moisture and potential for compaction must also be taken into account. Numerous studies indicating the advantages of early planting upon yield were reviewed by Craig (1977). More recent work is summarized by Hicks and Wright (1987) and Johnson and Mulvaney (1980).

### 9-7.2 Fertility

In general, inbreds have poorer rooting ability than hybrids, and may therefore be more vulnerable to nutrient deficiencies and imbalances. In the past, it has been the tendency of contract growers to overfertilize to protect against possible fertility deficiencies, while at the same time striving for a balanced fertility program. Decreases in commodity prices (upon which contract payments are based) and hence economic pressures on seed growers, as well as growing concern about groundwater contamination, suggest the need for a closer examination of fertilizer recommendations. Contract growers are encouraged to use soil tests regularly and to apply nutrients only as necessary to maintain fertility levels.



## PRODUCTION OF HYBRID SEED CORN

575

## 9-7.3 Herbicides, Insecticides, and Fungicides

Control of weeds, insects, and diseases within the seed field have become an integral and necessary part of seed production. Since inbreds and related-line parents are less competitive than hybrid corn with broad-leaf weeds and grasses, seed growers rely heavily on herbicides for effective weed control. Production personnel work with the grower to develop a weed control program that takes into account specific weed problems, crop rotation, soil type and organic matter, equipment, and the specific parents involved with the hybrid to be produced.

Insecticides for the control of above and below ground insects are generally a must. Most companies have formulated programs that protect against insect damage to stands, the growing plant, and the female parent ear. In recent years, some seed companies have begun to rely heavily on IPM (integrated pest management) principles and scouting of seed fields to determine if and when insecticide application is justified. Selection of the insecticide to use will depend upon the specific insect to be controlled, efficacy of alternative insecticides, the level of infestation, the development stage of the seed crop, safety considerations, and the reentry period.

Fungicides have also become a regular part of the production program for protection of the more susceptible parent lines to damaging fungal diseases. Genetic resistance to disease is preferred, but chemical protection is often needed when resistance is not adequate in the parent line. Spray programs have been effective in reducing damage from foliar disease on the more susceptible lines. Monitoring the crop for disease development is beneficial in making timely applications of chemicals. Fungicides are widely used as seed treatments to give protection against seed and seedling diseases (Shurtleff, 1980).

## 9-7.4 Plant Density

Plant density within the seed field is planned to produce maximum yields of high purity seed of saleable kernel size. Upper limits may be imposed by the particular germ plasm being used, the average rainfall pattern or irrigation availability in the production area, and local labor supply for detasseling. Many investigators have studied plant density effects on yield of hybrid corn (Craig, 1977; Johnson and Mulvaney, 1980). Fewer published studies of inbred response to plant density are available. Some seed companies conduct plant density trials with the female parents they are using in seed production. They evaluate the yield and kernel sizeout responses of those inbreds to increasing plant density. Plant densities in current use in seed fields typically range from 54 000 to 64 000 plants per ha (22 000-26 000 plants per acre) for inbred female parents, and often exceed that level for male parents, especially with inbred males that shed a limited amount of pollen.

### 9-7.5 Planting Patterns

Common planting patterns in seed production fields today include 4:1 (four rows of female parent to one row of male parent) (Fig. 9-2), 4:2, 4:1:4:2, 6:2, and solid female with interplanted male. In the first three patterns, the female parent is never more than two rows from the male parent. One-half of the female parent rows are adjacent to a male parent in the 4:1 and 4:2 patterns, and two-thirds of the female parent rows are adjacent to a male parent in the 4:1:2:1 pattern. These contrast with the formerly conventional 6:2 pattern that was commonly used for production of double-cross hybrids. The 6:2 pattern is still used to produce some single crosses, but its use is generally restricted to male parents that shed an abundant supply of pollen.

Occasionally solid planting of the female parent in 96.5 to 101.6 cm (38-40 in.) rows is utilized with either every other or every fourth between-row space being interplanted with the male parent. This accomplishes two purposes: (i) full utilization of land area for female parent production; and (ii) placement of the male parent closer to the female parent rows. In stress environments, interplanting may lead to yield and quality problems. Solid plantings are typically limited to female parents not so aggressive as to overshadow the male parent and thereby delay pollen shed, and to reasonably short male delays. Also, it is advisable to restrict this practice to male parents of sufficient stalk and root strength to avoid stalk and root lodging, which would make it difficult to detassel the female parent or remove the male parent as soon as pollination is complete.

It is a practice of many seed companies to destroy the male parent by cutting or running it down (if it is brittle enough to break) after pollination is complete. Competition with the developing female parent for nutrients or available soil moisture should, in theory, be minimized and increased kernel size and/or seed yield may result. Production research conducted by the author's company has shown mixed results on kernel size and yield responses. Characteristics of the female and male parents involved and soil moisture availability after pollination are important factors contributing to the response observed. Destroying the male parent at this stage prevents grain formation in the male rows and eliminates the risk of seed contamination at harvest.

### 9-7.6 Parent Delay Techniques

Shoultz (1985) summarized the results of a recent survey of the seed corn industry's use of various parent delay techniques. Split-date planting of parents, the planting of the female and male parents on different dates, is used so that the two parents "nick", or reach the flowering stage concurrently (Fig. 9-3). This has been and continues to be the most popular method of making large alterations in flowering date, so that parents of differing maturities are brought together for a timely nick.



PRODUCTION OF HYBRID SEED CORN

577

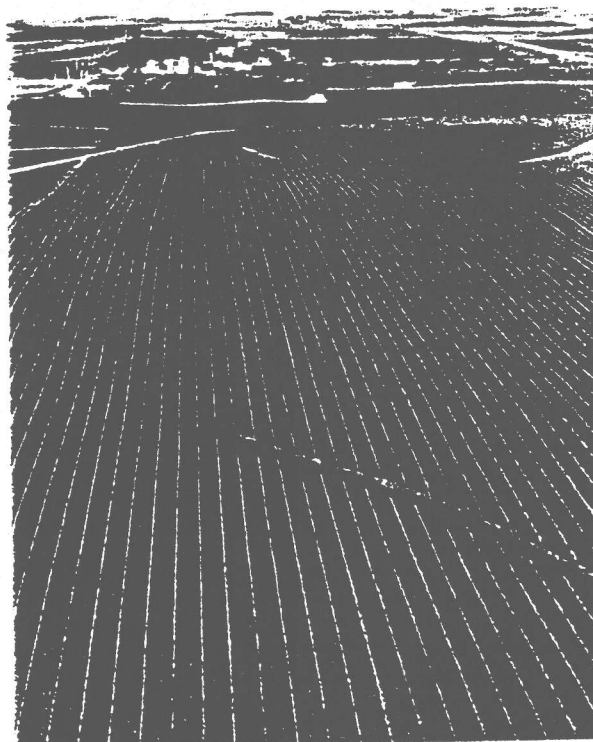


Fig. 9-2. (Top) A commercial hybrid seed production field planted in a 4:1 row pattern, with four detasseled rows (female) and one pollinator row (male). (Bottom) Aerial view of a commercial hybrid seed production field planted in a 4:1 row pattern, shown after detasseling. Note the border male rows on the left side of the field.



Fig. 9-3. Split-date planting of parents in a 6:2 row pattern. The female rows have emerged while the male rows are just being planted.

Split-date plantings are made on the basis of some combination of days, growth stages, and/or heat units accumulated from the time the first parent was planted (Shoultz, 1985). Most success has been realized by a combination of heat units and growth stage coupled with experience and good judgment. Male parents are often planted with a double delay to extend the pollen shedding period. Plantings are timed so that peak pollen shed coincides with maximum female parent silk exposure.

Other methods in common use for obtaining small adjustments to pollen shed are: (i) variable fertilizer rates; (ii) variable planting depths between parents; and (iii) clipping (Cloninger et al., 1974) or flaming (Fowler, 1967) to retard development. These techniques can provide from 1 to 4 d delay in flowering, or extend the duration of flowering by as many as 2 to 4 d (Shoultz, 1985). Clipping and flaming are rarely used to delay the female parent because both techniques typically result in reduced seed yield. Clipping has been used effectively to save a crop when weather conditions have prevented planting the delayed parent when the early parent has already been planted. This has been particularly important when it is too risky to replant the early parent because too few heat units are left in the season or when a seed shortage exists for one or both parents.

### 9-8 POLLEN CONTROL

Pollen control refers to the practices employed to ensure hybridization by forced cross pollination between the female and male parents.

## PRODUCTION OF HYBRID SEED CORN

579

Pollen control in the hybrid seed corn production field is extremely critical. Various methods of pollen control in seed fields have been utilized or investigated. These were aimed primarily at reducing the cost or easing the difficulty at this critical time period, while still maintaining the desired genetic purity. The two most commonly used methods today are detasseling and cytoplasmic male sterility. Craig (1977) described two other methods, genic male sterility and chemical pollen control, which are not currently in widespread use.

## 9-8.1 Detasseling the Seed Field

Detasseling currently represents the most widely used method of pollen control. Detasseling involves the physical removal of the tassel from the female plant, either as a manual operation or in combination with mechanical devices. To ensure that each seed field meets the necessary quality (genetic purity) standards, tassels from the female parent rows must be removed before they shed pollen and/or before silks emerge on the ear shoots of the female parent. This is an expensive operation, currently costing the seed company from \$250 to \$320 per female hectare (\$100 to \$130 per female acre) for an average female parent. Increasing wage rates and deteriorating population demographics (labor supply and its distribution) are two factors that will continue to pose challenges to the industry.

Genetic purity of intended crosses is dependent on compliance with standards established by company management and certifying agencies (Anonymous, 1971). When the female parent has 5% receptive silks (silks emerged and turgid) the following standards, as established by certifying agencies, are employed: (i) the female parent is limited to 1% shedding tassels at any one inspection and to a total of 2% shedding tassels for three inspections at different dates; and (ii) off-types in the male not over 0.2% at any inspection. Some seed companies have established more rigorous standards: e.g., not over 0.5% female shedders allowed at any one inspection and not over 0.1% male off-types per inspection. Tassels are counted as shedding when more than 5 cm (2 in.) of the central spike and/or side branches have emerged and have shedding anthers.

Major seed corn companies hire and train seed field inspectors to observe pollen control operations, report irregularities, and assist in interpreting rules for the detasseling/rogueing supervisors. These inspectors may be assigned as many as 810 to 1620 gross ha (2000 to 4000 gross acres). Before detasseling begins, they establish compliance with isolation requirements and check for volunteer corn and/or off-type plants in both female and male parent rows. When detasseling starts, the inspectors check fields to be sure that female parent tassels and off-types in male rows are properly removed. The objective is to keep those who are responsible for achieving genetic purity (plant management, area foremen, and detasseling contractors) informed, and to prevent any violation of

standards. If isolation, detasseling, or rogue removal standards are not met, inspectors report the details to plant management, so that a decision regarding corrective measures can be made.

### 9-8.2 Manual or Hand Detasseling

Each year thousands of workers, usually teenage youth, are employed by seed companies to perform the hand detasseling operation. This activity may last only 1 week, but may continue up to 5 weeks or more depending upon the volume of production and spread in female parent maturities planted within a seed production area. Several factors influence the magnitude and complexity of this job:

1. Tassels must be removed from all female plants in a timely manner, as previously discussed.
2. When weather conditions favor rapid corn growth, fields must be covered daily; this requires 7-d workweeks, rain or shine.
3. Some female parent plant types are easier to detassel than others.
4. Female parents whose tassels begin shedding pollen before fully emerging from the upper leaves, or which silk at about the same time as pollen shed occurs, create difficult detasseling supervision, management, and purity problems.
5. Weather conditions can greatly aid or complicate the detasseling season. A heavy rain or windstorm can lodge and tangle the female parent just as tassels emerge, making walking or driving through the field more difficult. Extreme heat can affect both the efficiency of detassellers and the emergence of silks and tassels.

Detassellers are usually organized into crews ranging from 6 up to 40 or 50 workers. The crew supervisor is responsible for recruiting, transporting, training, managing, and controlling the detassellers in his crew. With larger crews, the supervisor will have one or more assistants (sometimes called checkers) to help in crew training and managing the job to be done in the field. There will customarily be one supervisor or checker for each 6 to 10 crew members. It is important that each crew member be trained in proper detasseling technique, to minimize leaf damage and to ensure an effective detasseling job in each female parent row. The crew supervisor is also responsible for the safety and comfort of the workers while in the field.

For more effective and efficient labor utilization, detasseling carts or personnel carriers (Fig. 9-4) are frequently used, especially for detasseling taller-growing inbreds or single-cross female parents. These carts are motorized high-clearance machines equipped with platforms upon which the workers stand as they remove the tassels. The machines move slowly through the field, enabling the detassellers to look down into the plant canopy and remove the tassels more effectively and easily than if they were on foot. Usually 12 detassellers will work from each machine; for maximum effectiveness it is therefore important that all detassellers on



Fig. 9-4. A high clearance personnel carrier transporting detassellers through a seed production field. This view depicts the "second pass" through this field: during this trip, late-emerging tassels are removed.

each machine be equally skilled. It is often difficult or impossible to use these machines immediately after heavy rain or windstorms, and the detassellers must then proceed on foot.

Some seed companies employ contract detassellers for at least a part of their seed production. With this method, the contractors agree to detassel specified field areas for an established fee paid to them by the seed company. The contractors may work their own hours as long as they meet the company's established standards for timely removal of tassels. If they fail to do so, the seed company reserves the right to bring in a crew or mechanical detassellers to remove the potential problem-causing tassels before they shed pollen, and to deduct this expense from the contractor's payment. Contractors typically provide transportation for themselves and any other detassellers they employ, as well as the necessary supervision. This method often permits people employed in other jobs to earn extra income during their free time.

### 9-8.3 Mechanical Detasseling

Craig (1977) summarized several factors that led to increased use of mechanical detassellers in the early 1970s. Availability and cost of labor for manual detasseling continue to concern seed companies, and work to improve equipment for mechanical detasseling is ongoing.

Mechanical detassellers (Fig. 9-5) are self-propelled, high-clearance machines capable of operating even in extremely muddy fields. They fall into two basic types:

1. Cutters—a rotating cutter blade or knife cuts or shreds the top of

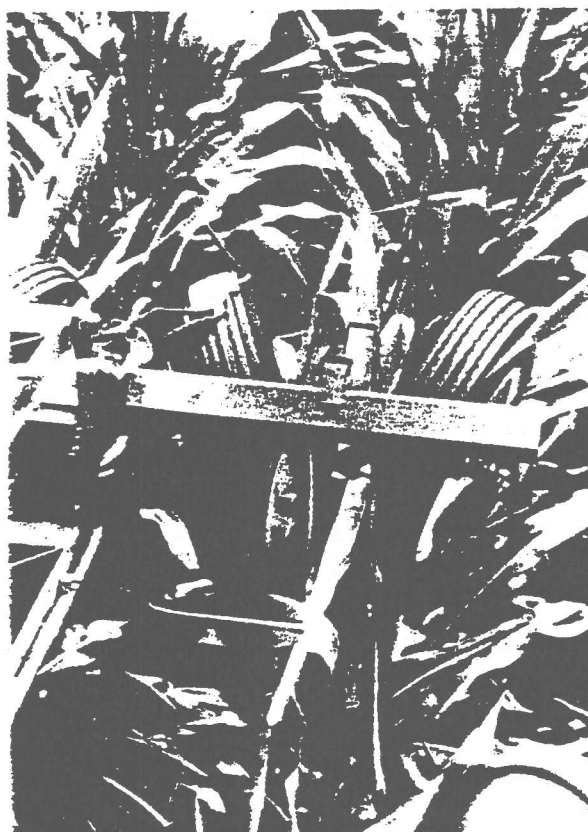


Fig. 9-5. (Top) A "wheel puller" machine used for mechanical detasseling of the female rows in a seed production field. (Bottom) Close-up view of the wheels in action.



## PRODUCTION OF HYBRID SEED CORN

the corn plant, including the tassel: the blades operate at various planes, from horizontal to vertical, and are adjustable in height.

2. Pullers—usually two counter-rotating wheels or rollers, adjustable in height, grasp the tassel and upper leaves and pull them upward in a manner approximating a hand detasseling operation.

The efficiency of mechanical detassellers is affected by many variables in the seed field, such as female parent morphology (leaf and tassel orientation), uniformity of female parent plant height and development, and skill of the operator. Mechanical detasseling produces best results when it is done in a uniform seed field in which the tassel is well exerted ahead of pollen shedding. As conditions become less favorable, percentage of tassels removed per pass will decrease and leaf damage will increase. The typical objective in the use of mechanical detassellers is to delay the operation as long as possible before silk emergence, to permit maximum exertion of tassels, enabling their removal with minimum leaf damage. However, this delay increases the risk of leaving "sprigs" (partial tassels) or "hangers" in the leaf canopy. Hangers, as tassels that become lodged in the leaf canopy are sometimes called, are capable of shedding pollen for 2 to 3 d after removal (D. Langer and P. Downes, 1982, unpublished data). Hangers can lead to increased levels of female selfs, and are one of the chief objections that some companies have to mechanical detasseling machines. In all cases, some hand labor is required to move hangers to the ground and to pull entire tassels or sprigs remaining on missed, late, or short plants, or on tillers.

With most female parents, the combination of mechanical and hand detasseling will result in a cost savings when compared with hand detasseling alone. Current detasseling costs range from \$198 to \$247 per ha (\$80-\$100 per female acre) with a combination of mechanical and hand detasseling, compared to \$296 to \$321 per ha (\$120 to \$130 per female acre) for all hand detasseling (W. Beck, 1987, personal communication).

Cost savings attained through mechanical detasseling may be offset by seed yield reductions if the operation is not carefully managed to minimize leaf damage (Craig, 1977, unpublished data). To decide whether or not to use mechanical detasseling, seed companies and plant management consider variable production costs, especially current detasseling wages, and available labor supply, and weigh them against characteristics of the female parents involved and the size of the detasseling operation they face.

#### 9-8.4 Effect of Detasseling on Seed Yield

The resumption in 1971 of detasseling as the primary method for pollen control (see section 9-8.5) renewed interest in the effect of detasseling on yield. The effect of detasseling on seed yield was considered more critical then, since inbred females had become involved due to the transitional adoption of single-cross hybrids. The development of various

types of mechanical detasseling machines also added a new dimension. A discussion of the effect of detasseling and leaf removal on seed yields of the female parents of double-cross hybrids may be found in Craig (1977).

Published work on leaf removal with inbred lines indicates that the yield response is generally similar to that of single crosses. In theory, removal of only the tassel could result in a yield increase, due to decreased shading of upper leaves and reduced competition for photosynthate and nutrients between the ear and the tassel. Hunter et al. (1973) removed the tassel, only, from 10 inbreds and observed an average increase in yield of 6.9%. As more leaves were removed with the tassel, however, greater yield reductions typically occurred. When one, two, and three leaves were removed with the tassel, yield reductions averaged 1.5, 4.9, and 13.5% relative to the yield where the tassel alone was removed. Cantrell and Geadelmann (1981) removed the tassel with two leaves. They observed yield reductions ranging from 9 to 13% across four early maturity inbreds, with an average of 11.6% yield reduction. Several other workers have reported differences among inbreds in sensitivity to yield reduction following varying amounts of defoliation (Cantrell and Geadelmann, 1981; Hunter et al., 1973; Vasilas and Seif, 1985b).

It is common to observe greater yield reductions after mechanical detasseling than after hand detasseling. Studies conducted by seed companies with mechanical detasseling machines have shown varying results. Craig (1977) cited unpublished research in which the yield of mechanically detasseled plots was from 2 to 40% less than that of hand detasseled treatments, depending upon the inbred involved and the number of mechanical cuttings. Unpublished research by C. Carter and R. York (1979, unpublished data) compared hand detasseling with wheel pullers on inbred females. These workers observed yield reductions ranging from 2 to 46% depending on the inbred line, number of wheel pulls, and timing of the wheel pulling operation.

Measurements have been made on the yield components affected by detasseling, to determine which are primarily responsible for reduced yield (Hunter et al., 1973; Pucaric and Gotlin, 1979; Vasilas and Seif, 1985a; Craig, 1977). The variables involved in these studies included the following: (i) the time of cutting or tassel removal in relation to plant development; (ii) the climatic conditions prior to, during, and after tassel removal; (iii) morphological differences among genotypes; (iv) type of detasseling machine; (v) the number of times cut or pulled; and (vi) the skill and attention of the machine operator. Although kernel number has most often been the major contributing factor, results have varied due to the differences in severity and timing of treatments employed. These complexities mean that precise statements regarding the effect of detasseling treatments on yield components cannot be made.



## PRODUCTION OF HYBRID SEED CORN

## 9-8.5 Cytoplasmic Male Sterility

For about two decades prior to the epidemic of southern corn leaf blight that swept the USA in 1970, the conversion of inbred parents to Texas cytoplasmic male sterility (*cms*-T) replaced detasseling as the predominant form of pollen control (Craig, 1977; Ullstrup, 1972). Though other male sterile cytoplasms were available, the T source (Rogers and Edwardson, 1952) proved to be the most satisfactory, because more inbreds were completely sterilized by T cytoplasm and genetic fertility restoration was more easily accomplished in this cytoplasm.

After the 1970 epidemic, the realization that the nearly complete conversion to T cytoplasm increased the vulnerability of the corn crop (NAS, 1972; Ullstrup, 1972) prompted a retreat from the extensive use of *cms* as a substitute for detasseling. In addition to T cytoplasm, many other male-sterile cytoplasms had been identified (Beckett, 1971; Duvick, 1965); of these, the C and S cytoplasms were the best known (Duvick, 1972). Since the use of *cms* was still a cost-competitive and satisfactory technique for hybrid seed production, C and S cytoplasms became important again in the late 1970s and early 1980s.

The American Seed Trade Association (ASTA) recently conducted a survey of the type of cytoplasm used in the production of seed corn to be sold in the USA. Based on number of units of expected sales for 1987, 66.1% of the seed corn was produced using 100% normal (N) cytoplasm, 22.1% involved production with *cms*-C cytoplasm (1.9% involving 100% *cms*-C and restorers, and 20.2% involving blends with N cytoplasm), and 11.5% involved *cms*-S cytoplasm (0.4% using *cms*-S and restorers, and 11.1% involving blends with N cytoplasm) (W. T. Schapaugh, 1987, letter to member companies responding to ASTA Corn Cytoplasm Survey).

There are two major ways in which *cms* has been used to facilitate the crossing of two inbreds. In the first case, detasseling is eliminated through the use of a female parent for which the *cms* conversion is completely male sterile. No detasseling is required. The other case involves combination of C or S cytoplasms in certain genetic backgrounds that result in only partial male sterility. In this situation, anther exertion is delayed 1 to 10 d (Duvick, 1965) and usually commences after the tassel is fully extended above the leaves. At this point, mechanical detasseling can be accomplished with minimum leaf removal.

Consider the production of the single cross,  $A \times B$ . If inbred A is nonrestorer genotype (*rf/rf*) that has been put into a male sterile cytoplasm by backcrossing, one can plant blocks of *cms* female A alternating with blocks of inbred B (the male) and produce completely cross-pollinated seed on inbred A without detasseling. If inbred B is also a non-restorer genotype, the hybrid plants in a field of commercial corn would also be pollen sterile; if inbred B carries dominant restorer genes (*Rf/Rf*), however, the hybrid (*Rf/rf*) will shed pollen.

Since restored hybrids do not always shed adequate pollen (Duvick,

1959), the use of the restorer system introduces some risk for both the farmer and the seed company. Consequently, most single cross production is likely to involve a nonrestored genotype, in which from 25 to 50% of fertile hybrid seed, produced by detasseling, is blended with 50 to 75% of seed of the same hybrid produced by the cms method. This blending results in 25 to 50% of the hybrid plants in the farmer's field that will shed pollen normally.

Various methods of blending to assure complete mixing are practiced. One method is to flank the pollinator with alternating blocks of cms and normal (fertile) cytoplasm female. Another method increases the scale by planting alternating quarters of the field in sterile or normal female. Harvesting entails making one trip across the field in the cms female and a return trip through the normal female. By the time the ear corn reaches the conditioning plant, the ears of the two cytoplasm are thoroughly mixed.

#### 9-8.6 Other Types of Sterility

Craig (1977) described in some detail the "Patterson method", which employs genic male sterility in the production of hybrid seed corn. This method is not widely used today, however, since the conversion of inbreds is complicated, time consuming, and expensive. Furthermore, additional expenses, in the form of foundation seed production inventory and quality control, are required.

Use of chemically induced male sterility in commercial hybrid seed corn production is an idea that has received considerable attention (Craig, 1977). Despite substantial research and development effort by several agricultural chemical companies, there is essentially no recent published work on this subject for corn. Likewise, a dependable and affordable commercial application of chemical hybridizing agents for seed corn production has not yet been discovered and/or developed (S. L. Kaplan, 1987, personal communication). To date, the major stumbling blocks have been either insufficient sterility percentage (Bollinger et al., 1978) or associated female barrenness (A. J. Cavalieri, 1987, personal communication).

#### 9-9 HARVESTING THE SEED CROP

Harvest of the hybrid seed corn crop is, by necessity, closely coordinated with the operations of conditioning facilities at the production plant. The following discussion describes the operations generally used at a typical large production plant (Fig. 9-6). There are variations among companies and among locations within a single company. All operations from husking to distribution may be done at one location. Alternatively, harvesting, sorting, drying, and shelling may be accomplished at a plant

## PRODUCTION OF HYBRID SEED CORN

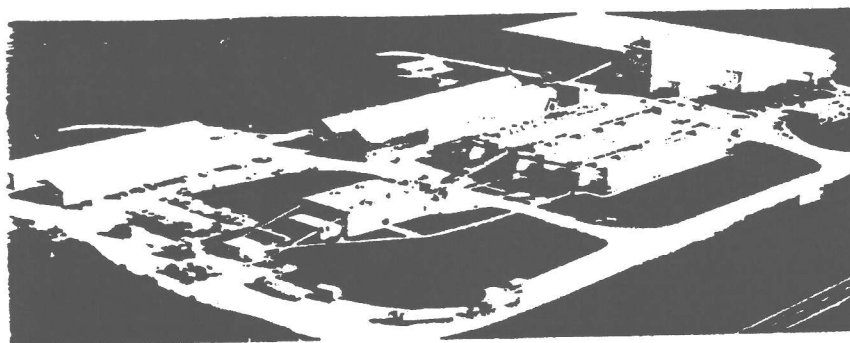


Fig. 9-6. A modern hybrid seed corn production plant located in Iowa, with facilities for all steps in conditioning, bagged seed storage in year-round controlled atmosphere conditions, and distribution of seed.

near the growing location, while the sizing, cleaning, bagging, storage, and distribution are done at other more centralized locations.

### 9-9.1 Maturity and Seed Quality

Harvest of the seed crop usually begins just before the developing kernels approach physiological maturity, the stage at which the kernels have reached their maximum dry matter accumulation. The moisture percentage at which kernels of inbred corn reach physiological maturity varies with genotype and environment, and ranges from 30 to 38% (A. J. Cavalieri, 1987, unpublished data; Knittle and Burris, 1976). Generally, harvest will begin when the moisture level of the seed is between 30 and 38%. The target moisture level depends upon factors such as the female parent, environment, weather forecasts, production volume and, finally, production plant capacity.

For planning purposes, projected harvest dates are typically estimated by monitoring kernel moisture percentage and heat unit (or growing degree day) accumulation, in combination with "black layer" formation (Daynard, 1969, 1972; Daynard and Duncan, 1969) and/or progression of the "milk line" (Afuakwa and Crookston, 1984). Genetic variation for field drying rates has been observed among inbreds (Carter and Poneleit, 1973; A. J. Cavalieri, 1986, unpublished data), and may be taken into account.

Timely harvest of the seed crop provides the seed company several advantages, including minimization of: (i) risk of freeze injury; (ii) field losses from mechanical pickers; (iii) risk of harvest delays due to adverse weather conditions; and (iv) quality deterioration due to insect damage, ear molds, stalk rots, and other diseases. Each of these factors contributes to the quality of the seed crop by reduction of physical damage, preservation of physiological vigor, and enhancement of appearance.

The adverse effect of freeze damage upon seed germination is a major risk to seed corn companies (Airy, 1955; Burris and Knittle, 1985). Ross-

man (1949) concluded that the amount of damage by freezing depended on temperature, duration of exposure, moisture of seed, genotype, husk protection, stage of development, and rate of drying after freezing. Studies reported by Neal (1961) indicated that injury to germination from freeze damage is directly related to kernel moisture as well as intensity and duration of exposure. The higher the moisture, the greater the effect on germination at all levels of freeze treatments.

### 9-9.2 Field Operations

The contract grower is responsible for harvesting the seed crop and delivering it to the production plant. Most mechanical harvesters used today are self-propelled ear corn pickers with three, four, or six-row heads (Fig. 9-7). Reduction in mechanical damage from the harvesters is accomplished by removing pegs from the husking rolls and properly adjusting the husking beds. Since harvest rate is ultimately determined by dryer capacity, a well-coordinated schedule directed by the production plant management is necessary to keep field harvest operations moving smoothly and dryers at full capacity.

### 9-9.3 Plant Operations

The seed is weighed as it is delivered to the plant. As the load is being dumped for movement into the husking/sorting building, the ear corn is sampled to accurately represent the grower's production of each hybrid. If maturity or moisture varies within a grower's fields, more than one sample is secured. These samples are used for measurement of grain moisture percentage and the cob and husk percentage. These data are used to calculate weight of No. 2 shelled corn delivered, which is the basis of payment to the contract grower.

While final husking is still done in the field by some companies, more than 90% of the seed corn produced in the USA is transported to the plant before it is husked a final time, sorted, and then moved to the dryers (Stanfield, 1986). In a typical husking/sorting building, ear corn is conveyed from the green corn receiving area (Fig. 9-8) to storage bins above the husking bed. The action of the husking bed removes the majority of the remaining husks before the ear corn passes over the sorting table (Fig. 9-9). In some operations, a return conveyor takes unhusked ears back over the husking bed. Usually, from four to six workers per table sort the ear corn as it passes over the table. These workers remove any diseased, off-type, or off-color ears from the seed. After sorting, the seed is conveyed to the dryers.

## 9-10 SEED CORN CONDITIONING

Conditioning of seed corn is the series of activities that begins with drying the ear corn and ends with the seed being bagged. The primary

PRODUCTION OF HYBRID SEED CORN

589

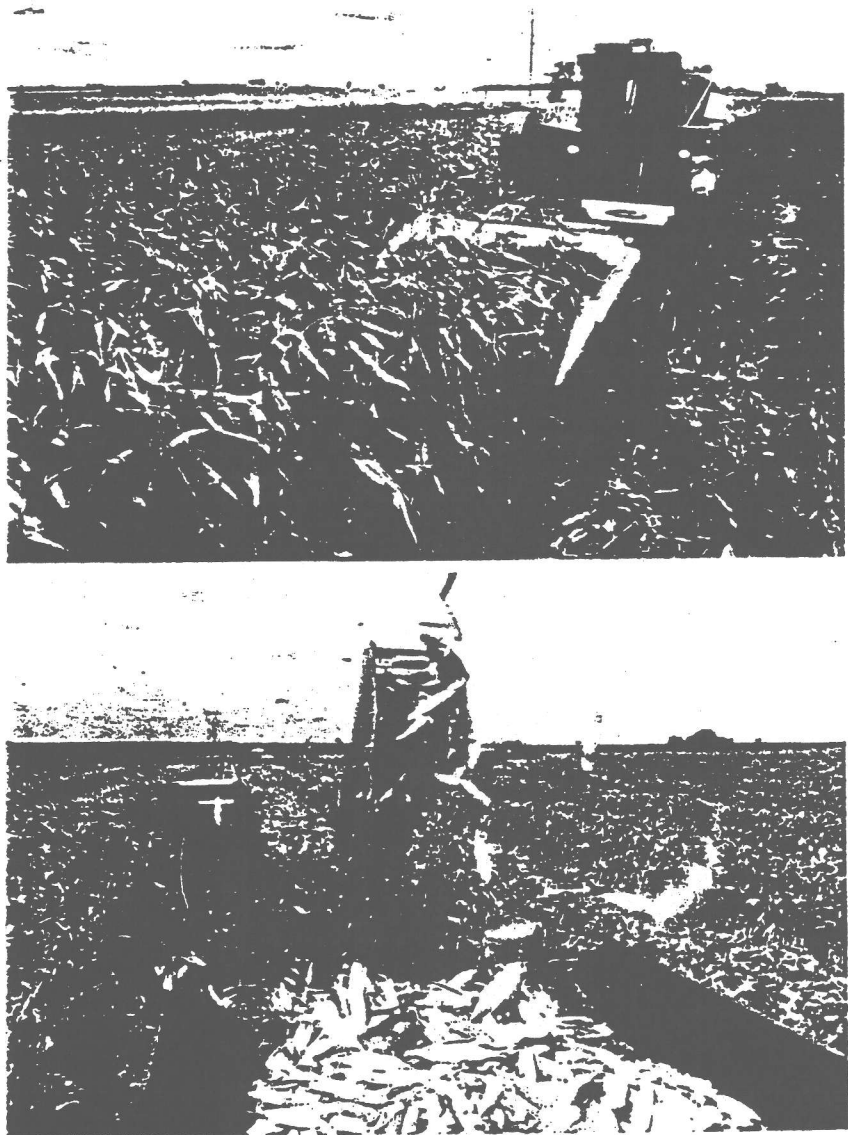


Fig. 9-7. (Top) Mechanically harvesting ear corn from the female rows using a three-row ear corn picker. (Bottom) The ear corn is elevated into a trailing wagon.

steps involved are drying, shelling, cleaning, sizing, treating, and bagging. These activities are accomplished in plants of all sizes and descriptions (Fig. 9-6). Although varying in engineering, all conditioning plants are designed and built to efficiently handle quantities of seed ranging from a few thousand to several million kilograms per year.

Certain objectives must constantly be addressed during the various



Fig. 9-8. Unloading seed corn at the green corn receiving area at a production plant. The ear corn is conveyed from this area to the husking and sorting building.



Fig. 9-9. The sorting operation in progress. Workers remove undesirable ears before the ear corn is conveyed to the dryer. The conveyor above the sorting table returns unhusked ears to the husking bed.

procedures of conditioning. First, the seed must be handled in a manner that minimizes mechanical damage. Any breaks that occur in the seed coat have a direct and detrimental effect on germinability and vigor of the seedling plant (Knake et al., 1986). The extent to which germinability



## PRODUCTION OF HYBRID SEED CORN

and/or vigor are reduced depends on both the severity and location of the mechanical damage (Wortman and Rinke, 1951; Wright, 1948). The effects of seed coat damage can be only partially offset by the application of fungicidal seed treatment (Knake et al., 1986; Tatum and Zuber, 1943).

A second objective of conditioning is to achieve maximum plantability within the limitations imposed by the sizing equipment. The objectives of sizing seed are to achieve uniformity of appearance and to maximize uniformity of kernel size and/or shape so that seeds drop accurately with either plate-type or plateless planters. The contemporary corn farmer, who is optimizing soil fertility, selecting hybrids best adapted to specific population densities, and planting for most efficient production, cannot tolerate significant fluctuations in stand.

Finally, the production plant manager must carefully maintain conditioning schedules to ensure the orderly movement of seed lots through distribution channels to meet delivery and planting schedules.

## 9-10.1 Drying the Seed

Seed corn dryers (Fig. 9-10) vary considerably throughout the industry. The majority of the systems utilize a squirrel-cage or axial-vane fan system to draw fresh air through a burner and force the heated air through bins filled with ear corn. Most burners today are fired with natural, butane, or propane gas.

Drying temperatures vary from 35 to 46 °C (95-115 °F). The entire

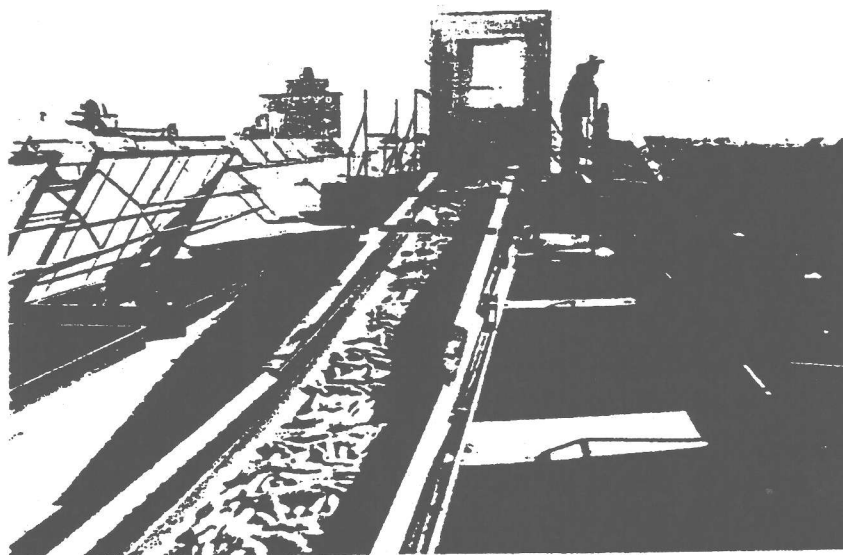


Fig. 9-10. Top view of an ear corn dryer at a seed corn conditioning facility. The doors are opened during bin filling, closed during the first half of the drying cycle, and then opened again to exhaust moisture-laden air during the last half of the drying cycle.

drying procedure is closely monitored since higher temperatures are detrimental to seed quality. Temperatures in the dryer building are measured at the point of contact with the seed. Temperatures above 45 °C (113 °F) have been reported as injurious to seed viability (Navratil and Burris, 1984; Craig, 1977), and high moisture seed is best dried at temperatures of 35 to 40 °C (95-105 °F) (Navratil and Burris, 1984; R. F. Baker, N. M. Frey, and R. D. Wych, unpublished data). It is also known that the higher the initial moisture content, the more susceptible the seed is to germination damage, and that genotypes vary in dryer sensitivity. Air temperatures of 46 °C (115 °F) may be used after moisture content of the seed has decreased to 20% or less, to complete the drying with minimal risk of germination injury. The seed corn is dried to 12 to 13% moisture, at which time it is conveyed to the sheller.

The moisture level to which seed corn is dried and held in storage also is critical. Cal and Obendorf (1972) showed that imbibition of low moisture (6%) corn kernels at temperatures of 5 °C (41 °F) resulted in malformed and delayed seedling growth. Sensitivity to imbibitional chilling was reduced when the initial kernel moisture was 13 or 16%.

### 9-10.2 Shelling the Seed

Shellers used for seed corn are designed so that when operated at low speeds, the seed is more or less rubbed from the cob. As the sheller speed increases, the action becomes increasingly pounding, kernel damage drastically increases, and germination declines (Airy, 1955). Therefore, it is desirable that all contact edges of the moving sheller parts be well smoothed to reduce damage. Experience has shown that with adequate horsepower, shellers can be operated at lower speeds and kernel damage is reduced by keeping the sheller full at all times.

### 9-10.3 Conveying the Seed

Since prevention of kernel damage is one objective of conditioning seed, it follows that each step should be evaluated to determine the amount of damage chargeable to that procedure. When studies such as this were done, some of the conveying equipment formerly used was found to be directly responsible for much of the mechanical damage (Craig, 1977). Grain augers crack and scuff seed; chain conveyors crush and crack kernels; and single tube elevator legs allow seed to be caught under belts and behind cups. Perhaps the greatest single source of damage is the dropping of seed from elevated conveyors into steel bottom bins or onto concrete floors.

Today, elevator legs are the double tube type and are equipped with either all plastic or plastic-lipped buckets. Chain conveyors are seldom used anymore, and augers have been replaced by belted conveyors. Breakage from impact on bin bottoms has been nearly eliminated by using grain spirals or ladders (Fig. 9-11) to lower seed gently with minimal



## PRODUCTION OF HYBRID SEED CORN

593



Fig. 9-11. Bulk seed in storage awaiting further conditioning and bagging. The spiral chute permits filling the bin while subjecting the seed to minimal mechanical injury.

impact. Whenever the fall distances are greater than 2 to 3 m, such equipment should be considered. Lateral conveying of seed is done either on rubber belting or with vibrating trough conveyors.

#### 9-10.4 Cleaning the Seed

Seed corn from the sheller contains varying amounts of foreign material, consisting of bits of cob, husk, silk, pieces of kernels and, on occasion, insect larvae brought in from the field with the ear corn. Storage properties, plantability, and appearance of seed are greatly enhanced by removal of such debris. If not removed, the debris encourages storage insect problems and the development of hot spots when seed is stored for long periods. Aeration and cooling are improved by removing such foreign material, and less storage space is required.

Two types of machines are used in the first steps of cleaning seed, air screen machines and scalperators. With air screen machines, shelled corn is delivered to sloping shaker screens that remove wide, extra large kernels and cob pieces (over a 24/64; see section 9-10.5 for a description of screen size nomenclature) and narrow tip kernels (through a 15/64 or a 16/64). The seed then passes through a blast of air that lifts fines, small cob pieces, and dust which have escaped the screening action. With scalperators, shelled corn is fed onto a rotating wire mesh reel. Kernels and small cob fragments pass through the mesh, thus separating them from the larger cob pieces. An air chamber is then utilized to separate fines.

which are routed to a dust collector. Any grain removed is disposed of as feed or market corn.

When necessary, insects are controlled with an application of a slurry formulation of an appropriate insecticide to the shelled corn stream being conveyed into bulk storage bins. This is especially critical when there may be a delay between shelling and movement of bagged seed to cold storage following sizing, treating, and bagging.

In many operations, the shelled corn is cleaned again as the first step in the sizing towers. The air screen machines or scalperators (with a smaller mesh) are fed at a slower rate to achieve more extensive removal of fines and foreign material. A thorough cleaning prior to sizing is essential to: (i) reduce the amount of dust; (ii) ensure smooth rapid flow through the sizers; and (iii) permit cleaners (aspirators or gravity separators) at the end of the sizing system to operate more efficiently.

## 9-10.5 Sizing the Seed

### 9-10.5.1 Sizing

*Sizing* as used in the seed industry means separating kernels into uniform lots of sizes based on width, thickness, and length. The historic term *grading* carries a connotation of quality measurement that does not apply to the procedure. To determine screens to use in sizing, some seed companies secure a 760 to 1270 kg (30-50 bu) composite sample for each lot. These are run through a sample sizer to determine percent by size, kernel counts, plantability, and germination.

During sizing, seeds are passed through round hole cylinders in a descending series of screen sizes, which are measured in 1/128th in. (commonly referenced as one-half 64th of an inch). Screens are selected to separate the seed into large, medium, and small kernel sizes, and "tips" (narrow seeds for discard). In some systems, a divider cylinder is used to split the seed into "overs" and "throughs". The overs are separated with a larger screen into large and medium portions. A smaller screen divides the throughs into medium (combined with medium from the other separation) and small kernels. Another smaller round hole screen then removes the tips. Following this, slot screens from 12/64 to 14/64 are used to separate large kernels into large flat (LF) and large round (LR), medium kernels into MF and MR, and small kernels into SF and SR. Figure 9-12 illustrates this sequence. In contrast, some systems separate rounds and flats with slotted screens first. Then the rounds and flats are divided by round hole cylinders into large, medium, and small sizes. The goal of both systems of kernel size separations is to achieve seed with acceptable appearance and plantability.

Kernel sizes may be length sized if the range in length would be unacceptable to customers. Length sizers, called uniflows, remove shorter kernels, utilizing a revolving indented cylinder. Shorter kernels ride up higher on the inside of the cylinder before falling out of the indentations

## PRODUCTION OF HYBRID SEED CORN

595

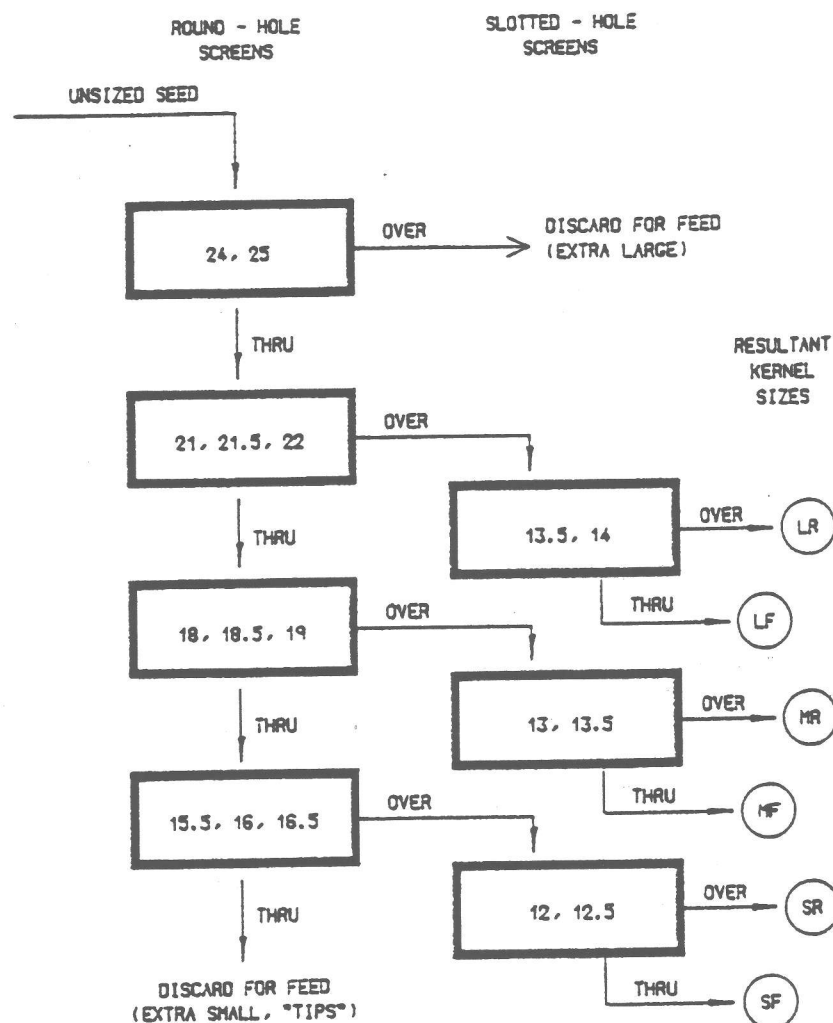


Fig. 9-12. A diagrammatic outline of typical screen sizes used for sizing seed corn, using round-hole screens first and then dividing each portion (large, medium, and small) into flat and round kernels with slotted-hole screens. Screen sizes are given in 64ths of an inch.

and are captured by an adjustable tilt trough through the center of the cylinder. Uniflows may be used to remove long kernels in seed of three-way crosses and modified single crosses. Length sizing is much less common with seed from inbreds, which characteristically have rather short kernels. Length sizing generally has little effect on plantability if the shortest kernels are longer than one-half of the plate cell length.

Separation tolerances of  $\frac{3}{64}$  in. for width and thickness and  $\frac{4}{64}$  in. for length have been proposed (Bateman, 1972; McKee, 1963). The industry, however, commonly accepts a  $\frac{4}{64}$  in. range for kernel width

(round hole) and up to an 8/64 in. range for length with long kernalled seeds. Bateman (1972) suggested plantability (number of kernels dropped) tolerance with plate planters of 3% under-drop to 5% over-drop. This matches well with industry standards, but under-drop must be carefully monitored to be certain that sorting does not occur. Changing round hole screens by one-half 64th of an inch is usually sufficient to greatly improve plantabilities which are heavy or light.

Since the first "plateless" planter (John Deere<sup>®</sup> 'Max-Emerge'<sup>®</sup>) was introduced in the early 1970s, sizing methods have been undergoing change. This change has been accelerated as other equipment manufacturers have also introduced plateless planters. Each of these so-called plateless planters satisfactorily plant seed without prior separation of flat from round sizes. Consequently, most seed corn firms now offer plateless sizes (flats and rounds undivided or blended together), usually sized (and designated) further as large, medium, or small. Since variation in kernel length is no problem for these planters, the short kernels removed in length sizing are often blended into plateless sizes. Current estimates indicate that 85% or more of the corn in some areas is planted with plateless planters. Consequently, up to 60% of the sales of some companies is in plateless kernel sizes.

#### 9-10.5.2 Cleaning Sized Seed

Removal of damaged and diseased kernels, which are usually lighter in weight than sound kernels, is accomplished through the use of aspirators or specific gravity separators. Aspirators have high capacity, but the separation is not as fine as with gravity separators. Consequently, aspirators tend to discard more desirable kernels without as thorough removal of imperfect seeds. Operation of gravity separators requires more skill, because adjustments must be made not only on the feed rate and air flow, but also on the tilt and pitch of the gravity deck and on shaker speed. Quality counts before and after cleaners are vital to satisfactory operation.

Conditioning of seed corn often has little effect on germination but appearance is greatly improved. Improved equipment and a higher comprehension of seed quality by discriminating customers has resulted in excellently conditioned seed being sold in the marketplace today.

#### 9-10.6 Planter Plate Selection

Practically all seed corn companies offer planter plate suggestions for customer convenience. The process of making planter plate checks not only identifies the plates that should be suggested to the customer, but also serves as a check on the accuracy of sizing. If the check planter will not drop the seed accurately, adjustments are made on the sizing equipment to improve accuracy. Most plate selection is done on actual planter test stands provided in recent years by the DICKEY-john<sup>®</sup> Corp. Planter

## PRODUCTION OF HYBRID SEED CORN

stands are often electronically monitored, which makes this step faster and eliminates human error in counting drop accuracy. Planting suggestions are also made for plateless planters.

## 9-10.7 Treating the Seed

Most seed companies apply a fungicide or a combination fungicide/insecticide to the seed before bagging. The purpose of treating conditioned seed is to protect against seedling diseases and to give short-term protection against storage insects. Properly treating seed may also help offset vulnerability to disease caused by mechanical damage (Knake et al., 1986; Wortman and Rinke, 1951; Wright, 1948).

A typical dosage of the fungicide captan [*cis-N*-((trichloromethyl)thio)-4cyclohexene-1,2-dicarboximide] is about 0.6 g a. i. per kilogram of seed (600 ppm; 0.54 oz/bu). In areas where head smut disease is prevalent, Vitavax<sup>®</sup> or Vitavax<sup>®</sup>/thiram [bis(dimethylthio-carbamoyl)disulfide] fungicides are utilized in place of captan. A purplish or reddish dye is usually added to the fungicidal slurry to impart a distinctive color to the seed corn. This enables easy identification that treatment has been added. The treatment is spray metered on the seed in a rolling cylinder drum. A smooth uniform application is desired. During extremely cold weather, methanol may be added to prevent spotty treatment due to crystal formation.

Until recently, malathion (*O,O*-dimethyl phosphorodithioate of diethyl mercaptosuccinate) and methoxychlor [2,2-bis(*p*-methoxyphenyl)-1,1,1-trichloroethane] were the insecticides most commonly included in the treating mix. Use of these chemicals is now declining, due to efficacy and cost considerations, stricter governmental regulations, and the introduction of newer chemistries that are effective at lower application rates. Insecticide and fungicide use will continue to be a dynamic reflection of new chemistry, cooperative research between the chemical companies and the seed corn industry, and the regulation climate imposed by the Environmental Protection Agency and the Food and Drug Administration.

## 9-10.8 Bagging the Seed

Most seed corn today is packaged by automatic or semi-automatic equipment (Fig. 9-13). A specified amount of seed is weighed, the bag is hung and filled, a tag is sewn on, and the bag is coded. Only one or two operators are needed to monitor this operation and keep it running smoothly. Nearly all seed corn in the USA is packaged in multi-ply bags, most of which contain a moisture barrier of free polyethylene or a polyethylene-coated sheet to protect against external water. A crinkled outer ply with a nonslip coating improves handling and stacking ability.

A relatively new innovation is the replacement of sewing by the use of heat-sealed bags. The advantages of heat sealing are to: (i) make entry

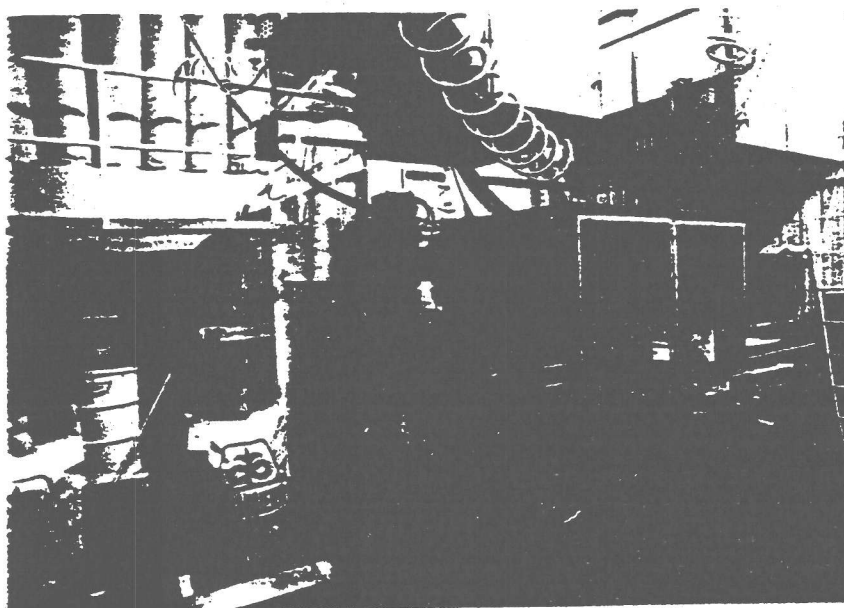


Fig. 9-13. A typical bagging line in which the seed is weighed and dropped into the bag. Information such as bag weight, kernel size, and seed lot code is printed on the bag, and a preprinted tag is attached as the bag is sewn closed while traveling on a belt conveyor. The bags are subsequently stacked on pallets.

into the bag by moisture and insects more difficult; (ii) eliminate problems caused by malfunctioning sewing heads; and (iii) save the cost of sewing thread. Pressure sensitive labels which are preprinted by computers can be used in place of sewn in tags.

Package size has moved from the bushel bag (25 kg, or 56 lb) to units weighing 23 kg (50 lb) and, in recent years, to units with 80 000 kernels per bag. A problem with bagging all units with 80 000 kernels is that different kernel sizes neither weigh the same nor require the same volume. Bag weights may vary from 13 to 32 kg (30-70 lb) and a variety of bag sizes is necessary so that all bags will be full regardless of kernel size. In addition, palletizing bagged seed becomes difficult because of the different bag sizes. A partial solution to these problems has been achieved by some companies who have resumed varying kernel count per unit by kernel size, so that bag weight is near 22 kg (48 lb).

An important final step in conditioning is collecting and saving a representative sample of each seed lot. Trickle samplers are often used, but care must be exercised to ensure that the sampling tube draws from all the flow. Otherwise, stratification in the flow may cause the accumulation of a nonrepresentative sample. The sample thus obtained is used by quality assurance for germination and purity tests, verification of kernel counts, and checks for damaged kernels and inert material. State and federal laws require certain information to be printed on each bag or on a tag or label affixed to each bag. Requirements vary from state to



## PRODUCTION OF HYBRID SEED CORN

599

state, although attempts have been made to standardize these requirements.

## 9-11 QUALITY ASSURANCE

During the past 10 to 15 years, many changes have taken place in the techniques, equipment, and inbred parents that affect seed corn quality. Because of higher yield goals and increased production costs, farmers demand and, in general, receive high-quality seed. It is the goal of seedsmen to supply adequate quantities of a product that retains the genetic gains incorporated into a given hybrid by the corn breeder. To help realize these goals, most companies maintain a quality assurance program to monitor all phases of seed production.

The quality assurance program should have well-defined procedures and standards that are understood by all levels of management. Data should be collected according to rules and procedures outlined in *Rules of Testing Seeds* (Anonymous, 1981), the *AOSCA Certification Handbook* (Anonymous, 1971), and the *International Rules of Seed Testing* (Anonymous, 1985). Often more strict and precise rules and procedures have been adopted by company management.

Isolation standards and tests for genetic purity were described earlier in the chapter as they pertained to production of parent seed stocks and commercial hybrid seed corn (see p. 568-571, 573). The rest of this section deals with standards for specialty corn and quality assurance procedures that are conducted during and after conditioning.

## 9-11.1 Specialty Corn

Seed production and quality control procedures for maintaining genetic purity of white, waxy, high-lysine, and high-amylose corn are somewhat stricter than those outlined above for yellow dent corn. These specialty corns differ from normal yellow dent by being homozygous for recessive alleles at one or more critical loci. Thus, contamination arising from any foreign pollen would mask the expression of the desired trait.

Production standards are more demanding for specialty corn. Any expression of xenia in the kernels must be removed in order to meet genetic purity standards. Most waxy and high-amylose corn is marketed with <3% normal endosperm. See Bear (1975) for a discussion of purity requirements for these specialty corns.

Isolation standards to minimize contamination in specialty seed corn have been set at 201 m (660 ft) plus four border rows when the field size is 4 ha (10 acres) or less. The distance can be decreased as field size and/or the number of border rows increases. However, many commercial companies maintain the 201 m, and some require as much as 402 m (1320 ft), as a minimum isolation distance.

When contamination does occur, such as yellow kernels in white corn,

procedures have been established to remove the obviously impure seeds. The original method was to pick out the yellow kernels at the sorting tables before drying the ears. Although electronic devices sensitive to minute color changes can be used, the equipment is expensive and its capacity is limited, so few companies use color sorters. With waxy corn, a special technique to identify dent contamination is used (Jugenheimer, 1958). An iodine solution applied to exposed endosperm starch causes the waxy kernels to stain reddish brown, while normal-starch kernels stain blue-black. The iodine stain converts a chemical property to a color test that is ultimately equivalent to the detection of yellow kernels in the white seed.

### 9-11.2 Physical Quality

Physical quality is measured by the amount and kind of damaged kernels present and by the viability and vigor of the seed. These traits affect field emergence, establishment and uniformity, and ultimately yield (Knake et al., 1986; Wortman and Rinke, 1951; Wright, 1948; see Craig, 1977 for additional, older references).

Seed corn is said to be in its best physical condition when it has attained physiological maturity and is still in the field on the ear of the female parent plants. Many activities affecting physical quality are performed from the time the seed is harvested until it is planted by the customer. Seed left standing in the field after physiological maturity is subjected to conditions that lower quality, such as cold temperature, disease, and insect damage. Seed corn is subjected to mechanical damage during harvest, drying, shelling, cleaning, sizing, treating, and bagging. Isolating and identifying the source, nature, and extent of physical quality problems, and recommendation of preventive or corrective procedures, are responsibilities of quality assurance.

### 9-11.3 Sampling

Seed quality is measured by testing a representative sample of a lot or prescribed quantity of seed. Samples taken at all steps through the conditioning sequence are vital to the monitoring of seed quality. This is especially true if freezing temperatures have occurred in the field when moisture percentage of kernels exceeds the low temperature, measured in Fahrenheit degrees.

Samples should be large enough to enable running the desired test and a retest, if necessary. However, securing a new sample is usually preferable to retesting the original sample. Between the time the samples are collected and the quality tests are conducted, samples must be stored under conditions preserving the original quality as nearly as possible. Standard procedures for taking representative samples, and the necessary equipment needed, have been described (Anonymous, 1940, 1981; Justice, 1972).



## PRODUCTION OF HYBRID SEED CORN

## 9-11.4 Purity Analysis

Purity analysis for seed corn is done more for genetic purity than for determining percent of pure seed, as is practiced for quality control in small grains, soybean, and forage grasses. Beyond the endosperm purity and color checks described earlier for waxy and white hybrids, electrophoretic tests may be run to check for presence of selfs, outcrosses, off-types, or rogues. Although these tests are time consuming and expensive, they are quite accurate if the sample is representative and the isozymic patterns of the parents of the hybrid have been defined (Smith and Weisinger, 1984; Smith and Wych, 1986).

Maximum tolerances for genetic impurities have not been established by state or federal seed laws or seed certification agencies. Therefore in-house standards are employed by those seed companies that routinely conduct purity analyses. For single-cross hybrids, the presence of more than 5 to 6% selfs, or a combination of selfs and other off-types totaling 6 to 8%, are commonly used criteria for decisions to not offer a seed lot for sale. Growouts, however, might disclose that from 2 to 3% off-types would be neither detectable nor objectionable to customers, if the maturity, kernel color, and stature were similar to the hybrid. Alternatively, even 0.1% of taller off-type plants might generate considerable negative feedback from customers.

## 9-11.5 Germination

The germination test is the most frequently used procedure to measure product quality. Standard methods and guidelines for germination testing of seeds (Anonymous, 1981) and the necessary equipment and procedures (Justice, 1972) have been established. Most of the larger companies have established their own seed testing laboratories, but state, university, or private laboratories are also utilized.

Once a system has been established, accurate and consistent evaluation of the seedlings in the test is of greatest importance. The data collected constitute the germination percentage that is printed on the seed bag (usually on a sew-on tag), as required by both state and federal seed laws.

Standard germination tests are conducted under nearly ideal conditions; since field conditions seldom approximate the ideal, other tests have been developed to measure seed deterioration or vigor. Seed vigor is defined by the Association of Official Seed Analysts (AOSA) as "those properties which determine the potential for rapid, uniform emergence and development of normal seedlings under a wide range of field conditions" (Anonymous, 1983). Differences in vigor between two lots of seed that are otherwise genetically alike may not be obvious from percent germination in the standard warm test (Delouche, 1973; Pollock and Roos, 1972).

#### 9-11.5.1 Cold Test

Most seed companies use some type of cold test as a routine test for vigor. The most commonly used procedures have been reviewed elsewhere (Anonymous, 1983; Martin and O'Neil, 1987; McDonald, 1975). Craig (1977) stated that cold test results indicated the ability of seed to emerge when soil conditions are cold and wet and may reflect the amount of mechanical damage a seed lot has undergone. Currently, however, uniform procedures and agreement on the value of the cold test as a measurement of quality do not exist in the seed industry. Burris and Navratil (1979) discussed the variability inherent in various cold test procedures and the consequent lack of comparability of results from one laboratory to another. Cold testing using one established technique within each seed company may nevertheless be useful for judging seed lots as to suitability for sale following carryover storage conditions, early freezes, etc.

#### 9-11.5.2 Tetrazolium Test

This biochemical test with 2,3,5-triphenyltetrazolium chloride quickly identifies seed viability. Enzymatic activity in living tissue turns the colorless tetrazolium red; loss of enzymatic activity in dead tissue leaves the tetrazolium colorless. The tetrazolium test is further used to evaluate internal seed injury, insect injury, frost damage, and viability of dormant seed (Bennett and Loomis, 1949; Goodsell, 1948; Moore, 1958; Porter et al., 1947).

#### 9-11.5.3 Accelerated Aging Test

In the accelerated aging test (sometimes called the *rapid aging test*), seeds are stressed prior to the germination test (Anonymous, 1983). The conditions suggested by the AOSA for seed corn are to expose the seed to nearly 100% relative humidity at 42 °C (108 °F) for 96 h. The percentage survival is an index for longevity of seed viability in storage and a good measure of seed vigor.

#### 9-11.5.4 Electrical Conductivity Test

Electrical conductivity of steep water (seed leachate) has been studied as an index of seed quality, but has not yet received widespread use because of procedural variability.

### 9-12 STORAGE AND DISTRIBUTION

#### 9-12.1 Storage

The basic requirements for seed storage space are that it be dry, free of rodents and grain storage insects, and capable of being held within

## PRODUCTION OF HYBRID SEED CORN

certain temperature limits (Airy, 1955). Seed is at its highest quality level at physiological maturity and can only deteriorate from that point onward. The goal of seed storage is to maintain physiological quality throughout the storage period by minimizing deterioration. The best storage conditions can only maintain quality.

Storage for both bulk and bagged seed is necessary (Fig. 9-14). In some companies, facilities have been designed to take advantage of the same space for both. As the bulk seed is moved out for conditioning, the bagged seed can be moved into the vacated storage areas. At most facilities, however, the total volume of seed that must be stored exceeds bulk storage capacity; therefore, some seed must be bagged and moved to warehouse storage (often cold storage) to allow the completion of conditioning. Stored bagged seed is normally palletized and moved with forklift trucks (Fig. 9-14).

Storage of seed corn, either bulk or bagged, for prolonged periods at temperatures above 10 °C (50 °F) leads to deterioration of seed quality. For this reason, bulk seed should be cooled after the drying and shelling steps. This is usually accomplished by aeration of bulk bins with ambient air. Further, surplus bagged seed should be stored during the summer at or below 10 °C and at relative humidity levels between 45 to 55% to maintain the desired moisture content and seed quality. Early work (Airy, 1955; Sayre, 1948) indicated the influence of reduced temperature and moisture level on the maintenance of quality and longevity of stored



Fig. 9-14. Bagged seed corn in storage on pallets in preparation for distribution to sales representatives. Some seed companies now use a "stretch wrap" machine that automatically surrounds the pallet of seed corn with a protective plastic wrapping prior to storage and/or shipment. Forklift trucks are used to move pallets around the warehouse.

seed. Specifications for equipment and buildings for controlled atmosphere storage and its effects on stored seed have been reported (Beck, 1969; Dahlberg, 1967; Stanfield, 1971, 1972).

Hybrid seed corn companies must produce seed supplies over and above that which market forecasts indicate will be sold in the subsequent sales year. Some carryover seed, as this unsold seed is called, is desired as insurance against unpredictable consequences of weather, government programs, etc. Seed companies normally anticipate growing approximately 30 to 40% more seed than estimated sales requirements, to fill supply channels and to hedge against possible reduced yields from production acreage. A recent example illustrates how this practice benefits North American agriculture. The 1983 seed corn production acreage in the USA was substantially lower than in previous years, in anticipation of the impact of the federal government's PIK program on 1983 seed sales and anticipated carryover. However, drought reduced 1983 seed field yields severely in many production areas. Seed supplies for the 1984 planting season would have been critically short had it not been for adequate quantities of high-quality carryover seed inventoried in controlled-atmosphere warehouses.

#### 9-12.2 Sales and Distribution

There are basically two common methods of sales and distribution of seed corn from the production plant or warehouse to the customer. The method used most widely throughout the Corn Belt involves farmers serving as farmer-dealers (or sales representatives or sales agents). The second method, involving seed distributors and/or dealers, is more common where corn acreage is less concentrated.

As the name implies, the farmer-dealer or sales representative is usually a farmer who is also a part-time salesman. This method of distribution depends on the local sales representative to: (i) call on his neighboring farmers to solicit their business; (ii) write the order; (iii) receive and store the seed until delivery; (iv) arrange for pick up or delivery; (v) complete the sale and collect the account; (vi) arrange for return of unsold seed to the production plant or warehouse; and (vii) provide service to the customer as needed. The sales representative is usually under the supervision of full-time company employees, commonly referred to as district sales managers. In the author's company, the sales representative never takes actual ownership of the seed corn.

In contrast, a seed distributor or a seed dealer purchases the seed corn from the seed company, and in turn sells the seed to other dealers and/or to farmer customers. Seed may be sold by the seed company directly to a seed dealer, bypassing the seed distributor. Alternatively, seed may be sold to a distributor who in turn sells it to one or more seed dealers. The dealer is often employed in related agricultural endeavors, such as fertilizer or chemicals, other seeds, livestock feeds, farm supply

## PRODUCTION OF HYBRID SEED CORN

605

stores, or elevator operators. This method of operation is especially adapted to areas where sales volumes are not great enough to justify the farm-to-farm calls of the local salesman, but are compatible with the existing business of the store or elevator.

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