SUBSTANTIAL EQUIVALENCE ASSESSMENT OF TRANSGENIC CORN PLANTS

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ABSTRACT

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Transgenic crops are regulated until they are judged to be as safe as non-transgenic plants. To grow them without permits, they must attain regulatory approval. . A lot of data are needed to show "substantial equivalence" and safety . This work deals with corn lines that contain cellulase genes. We hypothesize that no difference exists between Genetically Engineered (GE) and non-Genetically Engineered (non-GE) corn seeds at the morphological, protein, and DNA levels, except for the protein expressed specifically from the corresponding gene. This research will address concerns in commercializing GE crops, such as those containing a gene for either an exo- or endo-cellulase. Study objectives are: 1) examine seed morphological features. 2) Evaluate protein profiles and compare them the GE with non-GE control. 3) Examine corn line DNA to determine quality of insert. The outcome of this project will provide substantial information that will support regulatory approval for cellulase corn.

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LIST OF ABBREVIATIONS

1-D SDS-PAGE: One-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis

2-D SDS-PAGE: Two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis

^{32}P: Phosphorous isotope 32

BCIP: Bromo-4-chloro-3'-indolyphosphate p-toluidine salt

BCC: CBH I-expressing line -cell wall targeted protein

BCF: E1-expressing lines –endoplasmatic reticulum (ER) targeted protein

BCH: E1-expressing lines -vacuole targeted protein

BSA: Bovine serum albumin

B. subtilis: *Bacillus subtilis*

Bt: Bacillus thuringiensis Cry3Bb1 protein

CBH I: Cellobiohydrolase I

CP4 EPSPS: 5-enolpyruvylshikimate-3-phosphate synthase isolated from *Agrobacterium* sp. strain CP4

CRW: Corn rootworm

CTAB: Cetyltrimethylammonium bromide

DNA: Deoxyribonucleic acid

DTT: Dithiothreitol

E1: beta-1,4-endoglucanse

E. coli K-12: *Escherichia coli K-12* strain

EDTA: Ethylenediaminetetraacetic acid

ELISA: Enzyme-linked immunosorbent assay

GE: Genetically engineered

GOI: Gene of interest

JECFA: Joint FAO/WHO Expert Committee on Food Additives

kDa: kilo Dalton

MOPS: 3-(N-morpholino) propanesulfonic acid

NaOAc or **$NaC_2H_3O_2$**: Sodium acetate

NBT: Nitro-blue tetrazolium chloride

OECD: Organization for economic co-operation and development

PAT: Phosphinothricin-acetyltransferance

PBS: Phosphate buffered saline

PCR: *Polymerase chain reaction*

pI: Isoelectric point

RCF: Relative centrifugal force

RT: room temperature

SE: substantial equivalence

TAE: Tris base, acetic acid and Ethylenediaminetetraacetic acid

TBE: Tris Borate Ethylenediaminetetraacetic acid

TBS: Tris Buffered Saline

Tris-HCL: *Tris-Hydrochloride*

Tris-HEPES-SDS: Tris 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid sodium dodecyl sulfate

I. INTRODUCTION

The U.S. government has regulations governing any genetically engineered (GE) plant that is released into the environment and/or commercialized. Compliance with these regulations must be achieved before GE crops or products can be grown and consumed freely. Because growing a GE-crop under containment is expensive and tedious, regulatory approval is often sought. However, regulatory approval is also expensive and tedious. But once approval status is obtained, profits to crop developers are enabled and safety to the public is ensured.

Small businesses often cannot afford either process described above because a large amount of data is needed to show that the transgenic crop is not harmful (regulatory requirement of safety and substantial equivalence, SE). Thus, a new model is being developed to aid data collection. This model incorporates computer data mining to obtain background information needed for the regulatory approval process, as illustrated in Figure 1, and compares this information to data obtained in the lab and the field. The ultimate goal of this work is to ease the regulatory barriers to commercialization with three parallel lines of investigation to address regulatory approval of GE crops. First, data-mining of existing published literature to understand what is normal for a non-GE plant or crop, second, gather laboratory and field data from a non-GE crop to confirm the data mined from published literature, and third assess GE plant characteristics determined

from laboratory and field data and compare the data to normal standards to determine safety and SE. To integrate with this model, laboratory data must be collected from GE and non-GE maize seeds/plants and compared to determine if mined data (provided by computer scientists) coincide with laboratory-collected data. The overall results will provide evidence to support substantial equivalence and safety.

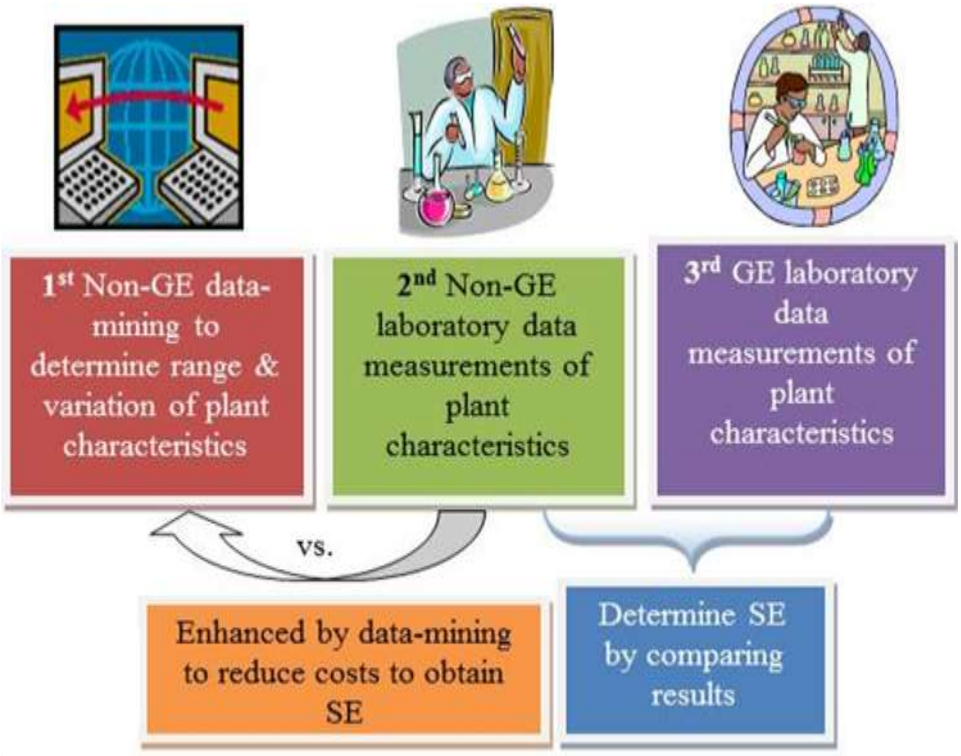


Figure 1. Original Plan of the three way comparison this project wants to achieve.

Unfortunately, the data mining program was not finished and we only performed laboratory data comparisons among the GE and non-GE corn plants in terms of seed morphology and protein quality and quantity.

II. LITERATURE REVIEW

Maize Production Improvement

Throughout history, the human race has been producing food through the growth of different crops worldwide. In the early years, plants were farmed without any alteration; therefore many acres had to be planted to produce the desired yield. Currently, that same number of acres can produce up to four-times more than what was produced in 1944. In the 1960's the maize hybrids that were introduced exhibited "heterosis" that improved yield (*Fraley et al. 2009*). Introduction of better cultivation practices also enriched yield (*http://www.actionbioscience.org/biotech/borlaug.html*).

Maize (*Zea mays, L.*) is a major food crop around the world and must have high yields to meet consumption demands. The hybrids introduced in corn production were at first "double crosses", crosses of two inbred parents at the same time as another two, to enhance hybrid vigor (improved offspring with higher yields). Then the "single cross" process (a cross of two parents with the desired characteristics to give an improved plant with higher yields) became a standard in the U.S. A. (*Fraley et al. 2009*).

The need for maize crop improvement in the 1990's stimulated the development and application of a new technology to plant production, biotechnology. Biotechnology facilitates the introduction of desired traits into commercial maize and other crops and is now used worldwide. In maize these traits allow, for example, plant growth under drought stress, better nitrogen usage, plants that manufacture their own insecticide, and

improvement in the quality of grains for animal feed, biofuels and food (*Fraley et al. 2009*). These advances have improved maize production to the extent that now it is more sustainable in terms of land, fertilizer and water use. For example, yields have increased by 50% while fertilizer rates have been held constant. Also, biotechnology has provided detailed information about the maize crop that will allow new findings in molecular breeding and biotechnology (*Fraley et al. 2009*).

Input Traits and Output Traits

Input traits are genes that improve crop production. These include herbicide, insect and disease resistance traits that replace chemical use (*Castle et al., 2006).* Transgenic crops that possess these traits were adopted quickly in agricultural production and usage has been increasing. For example, "global adoption rates for input traits have grown 10% or more each year since 1996" (*Castle et al., 2006*).

The most widely adopted input trait has been herbicide resistance. In corn for instance, glyphosate-resistance combined or "stacked" with insect resistance traits is increasing rapidly. Insect resistance traits by themselves, such as the *Bacillus thuringiensis* (*Bt*) genes in corn, have reduced the use of insecticides significantly. Moreover, disease resistance traits, such as virus resistance, have made a big impact in the papaya industry since the introduction of the ringspot virus resistant papaya in 1997 (*Castle, et al., 2006*).

In contrast to input traits, output traits are the product of genes put into plants that do not affect production directly. These traits include nutritional improvements as well as bio-production of industrial products and pharmaceuticals. Bio-production as an output trait is becoming quite common in research and will likely yield new products in the near

future. Several studies in over-expression of proteins in corn for different uses, such as pharmaceuticals, vaccines and industrial enzymes have been published (*Fischer et al., 2004; Hood et al. 2004; Howard and Hood, 2005*). Plant bio-products are free of human pathogens, are produced at low cost, and the products can be delivered to the consumer directly and safely. Using corn to produce a non-food product "will provide to the public with direct benefits they can easily recognize, such as orally delivered health products, environmentally friendly solutions to caustic chemicals, and an alternative supply of ethanol" (*Hood and Howard, 2009*). Nevertheless, the safety of the crop for a non-food application must be ensured.

Commercialization of Transgenic Products

Transgenic plants are one of several technologies to fulfill societal needs for food, feed, fuel and fiber. However, the governmental regulatory system is a barrier that slows down the commercialization of these products and the development of new ones because the process is tedious and very expensive. To meet the regulatory requirements, and commercialize a product can cost $20-30 million per product per event *(Bradford et al., 2005*). A new paradigm is needed to navigate the regulatory approval process to allow the benefits of biotechnology to be available to society.

The goal for many crop developers is to achieve transgenic crop regulatory approval by collecting relevant data that determine "substantial equivalence" and safety with the non-transgenic crop and achieve an approved status cost-effectively. However, developers of transgenic crops have a heavy burden to fulfill these requirements before commercialization, whereas varieties produced through conventional breeding that have similar new traits are exempt from those requirements *(Bradford et al., 2005*).

Substantial Equivalence (SE)

SE refers to the global standard that measures similarities between GE foods or crops based on health and nutritional characteristics with their non-transgenic (conventional) counterpart. Non-substantially equivalent products can still be safe, but they must be tested in more aspects to be marketed (*www.whybiotech.com*). Demonstrating SE status is an important objective for success with a GE product, because marketing under regulated status is expensive and therefore profits may be limited.

This project focuses on determining substantial equivalence between three genetically engineered corn lines and a non-genetically engineered control. The maize plants were transformed with cellobiohydrolase I (CBH1; *Shoemaker et al., 1983*) and beta-1,4-endoglucanse (E1; *Mohagheghi et al. 1986*; *Nieves et al., 1995*) genes that specifically produce the cellulases in the corn embryo (Hood *et al.*, 2007). The three transgenic lines were compared to their near-isogenic controls to determine if differences were detectable between them, and thus determine if the three transgenic lines are substantially equivalent to the control, apart from the specific gene inserted.

Studies have shown that "it is possible to develop GE lines which are substantially equivalent to conventional varieties" in wheat *(Shewry et al., 2007).* In order to determine substantial equivalence, Shewry *et al.* (*2007*) applied various technologies such as: genomics, transcriptomics, proteomics, metabolomics and functional properties, and compared the two crops. In each case, more variation was observed among multiple varieties of control plants than between the GE and non-GE plants, suggesting that the engineered trait is not responsible for the greatest variation. The present study on GE

corn focuses primarily in the use of morphology, proteomics and DNA hybridization to determine what changes, if any, are observed in transgenic maize containing cellulases.

Proteins as a model to determine SE

Seed proteins are a major nutritional part of plants. Ten percent of the grain comprises protein. Three groups of proteins are common: storage proteins, structural and metabolic proteins, and protective proteins *(Shewry and Halford, 2002).* Storage proteins are typically 50-70% of the total proteins in seed and thus the most abundant *(Holding and Larkins, 2009).* Therefore, proteins, and more specifically storage proteins, are one good indicator of substantial equivalence.

Cereal seed storage proteins are produced and accumulated through the secretory pathway. Maize storage protein fractions consist of prolamins, albumins, and globulins. Maize prolamins are called zeins, and are the more abundant protein class *(Shewry and Halford, 2002).* Because of their abundance in corn seed and their majority location in the endosperm, in contrast with the GE lines that have an extra protein (cellulase enzyme) that is located in the embryo, these storage proteins should be a good indicator of whether the addition of the transgene-encoded protein has an effect. Therefore they have been chosen for analysis of GE and non-GE lines, as a model to measure SE.

Origin of transgenic lines

Many corn lines were transformed with two genes at a biotechnology company that utilized corn as a cellulase producing system (Hood et al., 2007). They developed these corn lines to produce enzymes (cellulases) that would degrade cellulose for future ethanol production. The genes selected to transform these lines were two cellulases called E1 and CBH I, enzymes that showed good synergistic activity on lignocellulosic

substrates, were thermostable at 45 ^{o}C and had an optimum compatible pH of 5 (Baker et al., 1998). These genes originate from the bacterium *Acidothermus cellulolyticus (E1)* and the fungus *Trichoderma reesei (CBH I).* The company created many different constructs that contained these genes targeted to different subcellular locations and possessed a selectable marker PAT ((phosphinothricin-acetyltransferase). Of all the lines produced, three were chosen based on their percent of TSP (Total soluble protein) in seed for further breeding and analysis. The lines were: BCF (ER targeted-E1), BCH (vacuole-targeted E1) and BCC (cell wall-targeted CBH I). These lines were utilized in a backcross program to obtain better agronomic performance of the GE lines. After their backcrossing with the parent lines (LH244 and LH283) was complete (6 generations), the transgenic parent lines were crossed to generate a high-yielding hybrid. These hybrids were the lines we utilized to perform the substantial equivalence assessment experiments shown in this work (Hood *et al.*, 2007)

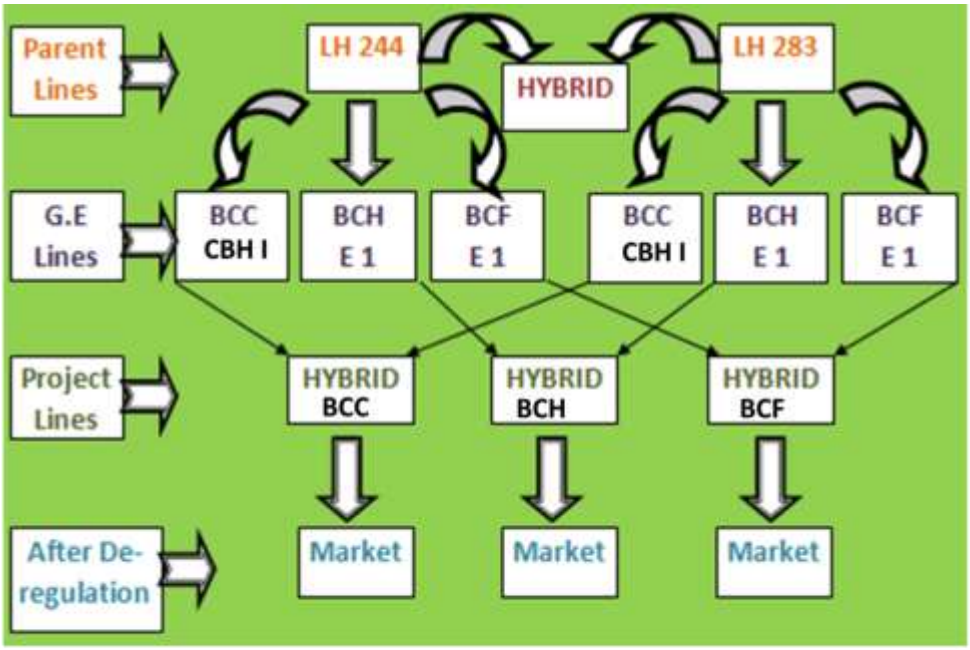


Figure 2. Corn lines used in the project, their origin and desired outcome after de-regulation. Each hybrid was grown in two fields; Maroa And Philo (IL). Planted in 2009.

III. MATERIALS & METHODS

Seed Morphology

Morphology of maize seeds was determined as follows. Seed weight, size, color, shape, and number of germinated seeds were analyzed. The seeds were harvested from two fields planted in 2009 in Maroa, IL and Philo, IL in randomized complete block designs. Four lines from each field were analyzed for a total of eight lines sampled including the controls.

Seed weight: Pools of ten seeds per line were measured with a digital scale, repeated five times. The ten seeds were weighed and recorded, followed by the next ten seeds until five repetitions were made. The same process was repeated for each line.

Seed size, color and shape were analyzed by photography and comparison analysis. A ten representative seeds sample from each transgenic line and controls was selected providing representative batches of shape, size, and color. Photographs were taken with no flash against a black surface and with a ruler next to the seeds. Each photograph contained the control line and one transgenic line, providing a direct comparison. These photographs were analyzed by thirty randomly chosen individuals to ensure that the observed similarities or differences were or were not significant.

The number of germinated seeds was first determined in petri dishes to observe closely how and when germination occurred and also to measure root length over time. Thirty seeds per line were randomly chosen, surface sterilized with 20% Clorox for 20

minutes and ten seeds placed in each petri dish over a wet filter paper and covered with a second filter paper. The plates were kept in growth chambers at 25 ^{o}C. Seeds were observed every day until at least 90% were germinated, which happened between 2 to 5 days. The number of germinated seeds per day was recorded. Once they germinated, the root length was measured and recorded. After five days in petri dishes the germinated seeds were potted and kept in the greenhouse to observe emergence of the shoot.

After 7 days, transgenic plants were identified by leaf painting with 2% active ingredient Liberty^{TM} herbicide the second oldest leaf. The GE lines have also a gene that has resistance to Liberty^{TM} herbicide, moPAT (*maize optimized phosphinothricin-acetyltransferase* gene), that allowed us to select the GE lines. After two days, resistant plants were identified by their dark green color at the application site and four of them were kept in the greenhouse to produce more seeds. The remaining transgenic plants were harvested for further studies.

Protein profiles

Proteins were extracted from transgenic and non-transgenic corn seeds with multiple buffers and the extracts compared. A series of 1-dimensional (1-D) and 2-dimensional (2-D) polyacrylamide gels were run, and Western blots conducted. Protein extractions were done in (A) phosphate buffered saline pH 7 (PBS), (B) 70% ethanol (primarily for zein storage proteins), and (C) sodium acetate, pH 5 (NaOAc), the first and the last were preferentially used to extract the GE proteins (CBH I and E1). Western blots were performed with the protein extracts from PBS and NaOAc buffers, which contained the GE protein of interest (described below).

1-D gels were used to separate the complex mixture of proteins and to investigate composition. They were performed for all lines in three repetitions. These gels were stained with coomassie blue & silver nitrate (Pierce silver stain kit) to identify the protein profile.

2-D gel electrophoresis was used for separation of the complex protein mixture of sodium acetate extracted proteins by the independent parameters of isoelectric point (pI) and molecular weight, utilizing a Proteome IEP unit from Bio-Rad and then running an SDS- polyacrylamide gel for each sample. The polyacrylamide gels were stained with Silver Nitrate to identify the protein profile.

Phosphate buffered saline (PBS, 50mM pH 7) (*Hood et al., 1997*) extraction was performed utilizing 10ml of PBS per 1g of ground corn seed that was pulverized in liquid nitrogen. The slurry was centrifuged at 805 RCF for 15 minutes. The supernatant was recovered and the Nano-drop (ND-1000 spectrometer) used to obtain protein concentration. These samples were size separated on 1-D gels (PIERCE Precast Polyacrylamide Gels 12%) with 1X Tris-HEPES-SDS (20ug protein per well per corn line).

Western blot analysis was also performed on these extracts as previously described (*Hood et al., 2007*). Inmunoblots were blocked for 1h at room temperature (RT) with 3% Bovine Serum Albumin (BSA) in Tris Buffered Saline (TBS, 1X). The blot was incubated overnight in primary antibody (anti-E1 and CBH I 1:2000 dilution in TBS) at 4^{o}C and in secondary antibody (goat anti-rabbit alkaline phosphatase conjugated, diluted 1:5000 in TBS) also at 4^{o}C overnight. The blot was then developed by the alkaline phosphatase detection reaction using nitro-blue tetrazolium chloride (NBT) and -

bromo-4-chloro-3'-indolyphosphate p-toluidine salt (BCIP) substrates (33µl and 66µl of the substrates in 10ml of alkaline phosphatase reaction buffer respectively).

An ethanol extraction was performed with 70% ethanol (v/v), 5% 2-mercaptoethanol (v/v) and 0.5% $NaC_2H_3O_2$ buffer (w/v) in a ratio of 50mg corn flour per 250µl of extraction buffer. Samples were vortexed for 10 sec each six times, then shaken at 65ºC for one hour, and centrifuged at 4ºC for 5 min at 1600 RCF. The supernatant was recovered and protein concentration was measured using the Nano-drop. These extracts were size separated on 1-D gels (PIERCE Precast Polyacrylamide gradient Gels 12%) with 1X MOPS buffer (5ug per well per corn line). The gels were stained with coomassie blue and silver stain.

Sodium acetate (50mM pH 5) (*Hood et al., 2007*) extraction was performed utilizing 10ml of $NaC_2H_3O_2$ per 1g of ground corn seed that was pulverized in liquid nitrogen. The slurry was centrifuged at 453 RCF for 15 min. The supernatant was recovered and the Nano-drop (ND-1000 spectrometer) used to obtain protein concentration. Extracted proteins were size separated on 1-D gels (PIERCE Precast Polyacrylamide Gels 12%) with 1X Tris-HEPES-SDS buffer (20ug per well per corn line).

Western blot analysis was also performed on these sodium acetate extracts following a standard protocol (*Hood et al., 2007*). Immunoblots were blocked for 1h at RT, then the blot was incubated overnight in primary antibody at 4ºC and the secondary conjugated antibody was also incubated at 4ºC overnight. The blot was then developed by the alkaline phosphatase detection reaction.

These samples were also used for the 2-D gel electrophoresis analysis. Isoelectric focusing was done on 11-cm-long IPG strips (Bio-Rad) with a non-linear pH gradient of 3-10 in a Proteome IEP unit from Bio-Rad. The strips were rehydrated in 200µl of rehydration buffer for 14h at 20V active rehydration. After rehydration, focusing was done for 9hs total (30 min, 1.30h, 7h -11cm pre-programed run). Prior to the second dimension, on SDS-PAGE, the IPG strips were equilibrated at RT for 15min in 50mM Tris-HCL, pH 8.8, 6M urea, 30% (v/v) glycerol, 2% SDS and 1% DTT, then another 15 min with 1% (v/v) 2-mercaptoethanol (instead of DTT). SDS-PAGE on Bio-Rad Criterion Precast Polyacrylamide 12% Gels was performed in a Bio-Rad Criterion cell system. The gels were run at 150V for 1h in 1x MOPS buffer at RT with a Precision plus Protein Dual Color Standard. Gels were stained with silver nitrate, or were used to perform a western blot as described above.

DNA analysis

At ten days post planting, transgenic plant selections were made by leaf painting with 2% (active ingredient) Liberty™ herbicide. Herbicide was applied with a cotton swab to the second oldest leaf. After two days resistant plants were identified, harvested and frozen (-80^{o}C), to extract DNA and perform Southern blot analysis (*Southern, 1975*). DNA was extracted from corn leaves using a cetyltrimethylammonium bromide (CTAB)-based buffer (*http://gmo-crl.jrc.it/detectionmethods/MON-Art47-dnaextraction.pdf*). Twenty micrograms of each DNA sample were digested with restriction enzyme (RE) *Kpn*I [New England Bio-labs (Ipswich, MA, USA), high concentration]. DNA fragments were separated on a 1% agarose gel with 1X Tris Borate EDTA (TBE) buffer, and capillary transferred to nitrocellulose. Hybridization was performed in Church buffer

(*Church and Gilbert, 1985*) (6% SDS, 5% bovine serum albumin (BSA), 1 mM EDTA, 0.5 M phosphate buffer) with ^{32}P nick translation labeled probe. DNA to be labeled was PCR amplified from the transformation plasmids containing the cellulase coding region, and included only the cellulase gene sequences. The PCR products were run in a 0.8% agarose gel in 1X Tris base, acetic acid and Ethylenediaminetetraacetic acid (TAE) buffer for 1.5h at 110V and then eluted with Qiagen gel extraction kit. Reconstructions of several copies per cell were generated from linearized plasmid and added to the blots to estimate the copy number. Blots were hybridized for 24 h at 65 °C, washed in the Church buffer system (6% SDS, 0.5 M phosphate buffer) five times and exposed to Kodak Bio-Max XAR film for three days at -80^{o}C.

IV. RESULTS

Corn plants were grown in the summer of 2009 in randomized field blocks in two locations in Maroa and Philo (central Illinois). Three transgenic lines (*Hood et al., 2007*) including a CBH I-expressing line (BCC-cell wall targeted protein) and two E1-expressing lines (BCF-ER targeted protein and BCH-vacuole targeted protein) were grown beside a near-isogenic control hybrid. Seed was harvested and compared for various characteristics including morphology, protein content and DNA content.

Morphology Studies

Morphological studies of maize seeds were conducted to determine whether substantial equivalence could be observed among all corn lines (GE and non-GE) analyzed.

Seed germination, weight, and root length: Figure 3A shows the average of seed germination measurements from five separate pools of ten seeds at each time point. Differences among the seed lots were not significant at the 95% confidence level**.** Figure 3B shows the average root growth from three separate pools of ten seeds at a time, measured starting after germination (48 h) and every following 24 h period up to 120 h total. The GE lines demonstrated no statistical difference between each of them and the control. Figure 3C shows average seed weight of five separate pools of ten seeds measured once for each transgenic line and control from each field location. Although batch to batch variations were observed, the overall differences among the averages per

line (BCF, BCC, BCH and control) were not significant at the 95% confidence level shown by the statistical analysis performed on the averages (Appendix II).

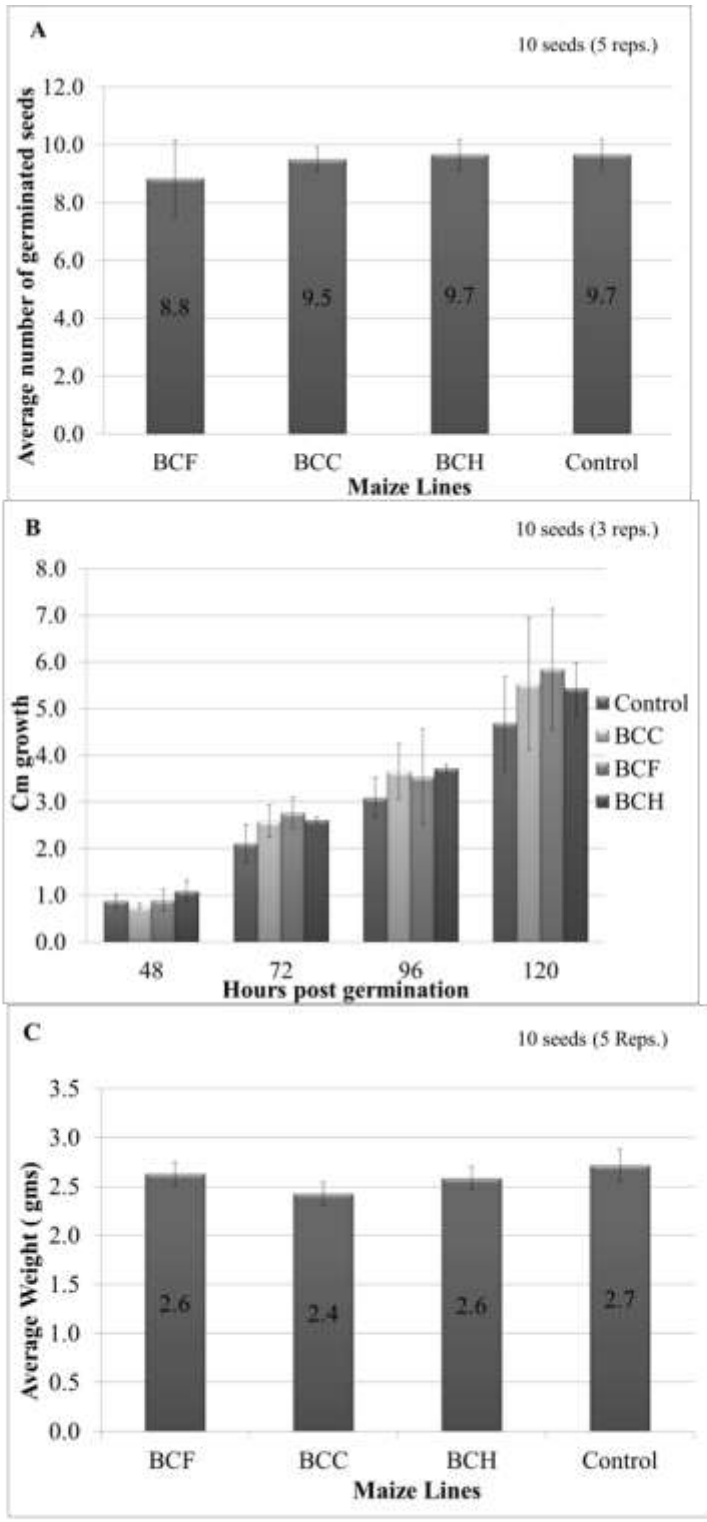
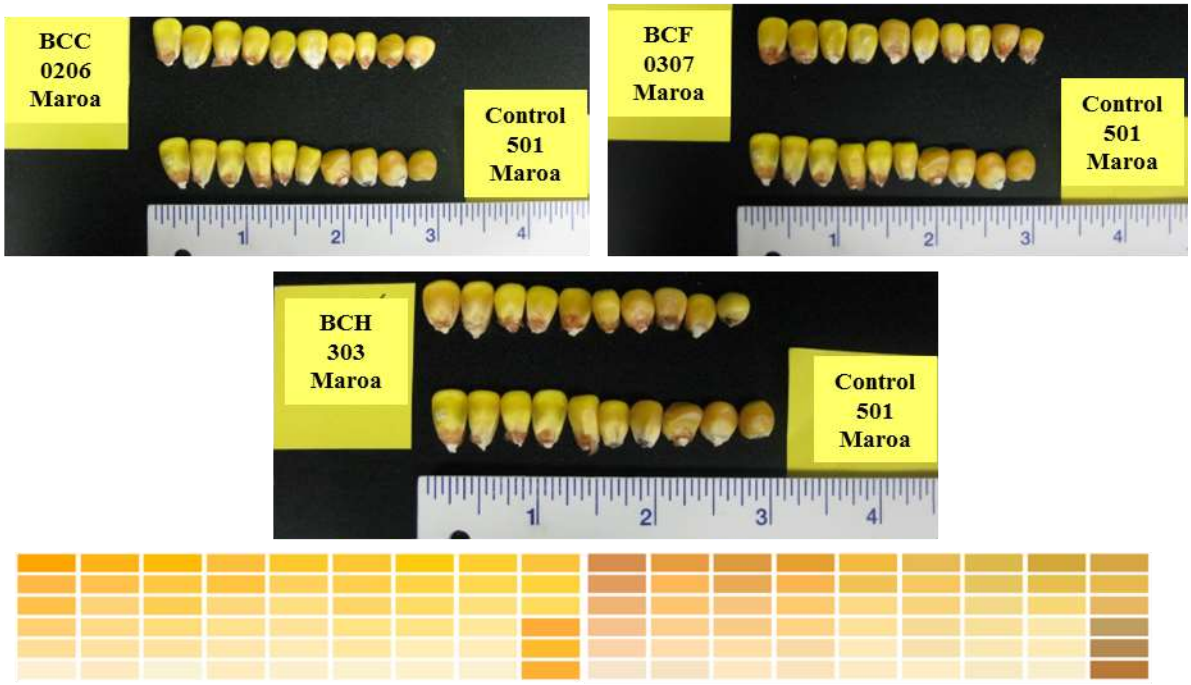


Figure 3. Corn seed morphology studies from two fields in Illinois (Maroa and Philo) from summer 2009. **A**: Average number of germinated seeds (5 batches of 10 each) from the three transgenic lines (BCC, BCF and BCH) and the untransformed control. **B**: Average root length of germinated seeds from BCC, BCF and BCH lines and the isogenic untransformed control line, measured in cm from 48 to 120 hours after germination. **C**. Average weight of seed harvested from each of the transgenic lines and the isogenic untransformed control line. All averages are from both fields combined.

Seed size, color and shape: Figure 4A and B shows seed size, color and shape of the three GE lines, BCF, BCC, and BCH as well as the control line (non-GE) from each field. Photographs of a representative sample of seeds were taken, directly comparing each line to its near-isogenic control. These photographs were shown to a random sampling of 30 individuals to identify any relevant differences. No differences between transgenic and control lines were observed for the four lines by any of the 30 individuals. Some differences can be observed in the Philo field and the comparison could be re-done to determine if these were due to environmental or transgenic factors.

A



B

Figure 4: Size, color and shape of seed samples visually scored by 30 individuals for consistency. A representative amount of seeds from each transgenic line from each field was randomly chosen and directly compared to an equal representative sample size of the isogenic control line. **A**: Maroa, **B**: Philo.

Protein Analyses

Seed proteins were extracted from transgenic and non-transgenic plants with phosphate buffered saline (PBS), 70% ethanol, or sodium acetate (NaOAc). A series of 1-dimensional (1-D) and 2-dimensional (2-D) polyacrylamide gels were used to analyze these extracts, and Western blots conducted to detect the cellulase proteins.

1-D gel electrophoresis: Figure 5 shows the surveyed extracts from seed purchased from two commercial sources and their protein profiles were quite variable. Thus, the minor differences we observed among transgenics and controls were determined to be not significant. CBH I protein has a calculated molecular weight of 52.5 kDa while E1 has a catalytic domain which corresponds to a calculated molecular weight of 40 kDa. At these calculated molecular weights, no band corresponding to CBH I or E1 was visible on the stained gels even though they represent a substantial amount of the soluble protein. However, they were highly detectable on Western blots (Fig. 6B). Figure 6A shows a 1-D polyacrylamide gel of the corn proteins extracted in PBS buffer. Although minor differences can be seen, these 1-D gels show no large differences in protein content among the lines tested, either transgenic or control.

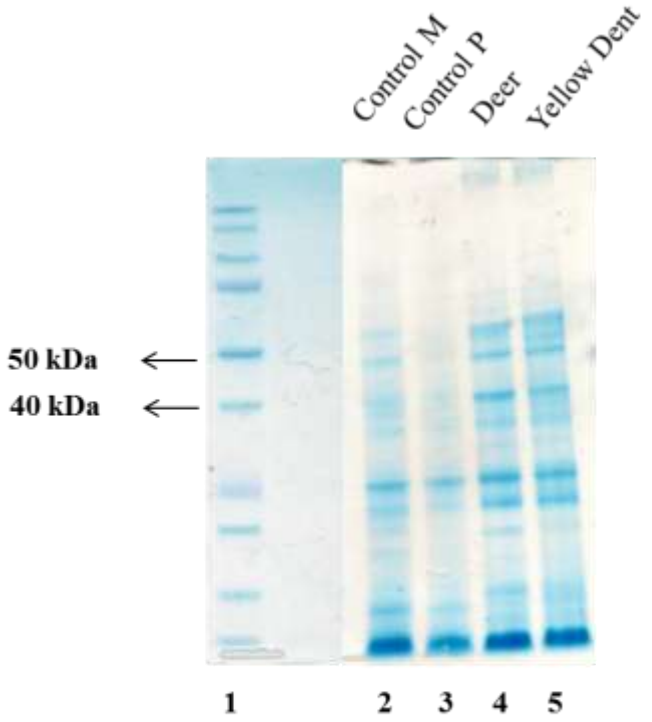


Figure 5. SDS-PAGE analysis of different corn seed varieties extracted in PBS from. In lane 1 is the Precision plus Protein Dual Color Standard. Similarly lane 2: Maroa field control. Lane 3: Philo field control. Lane 4: Deer corn variety. Lane 5: Yellow Dent corn variety.

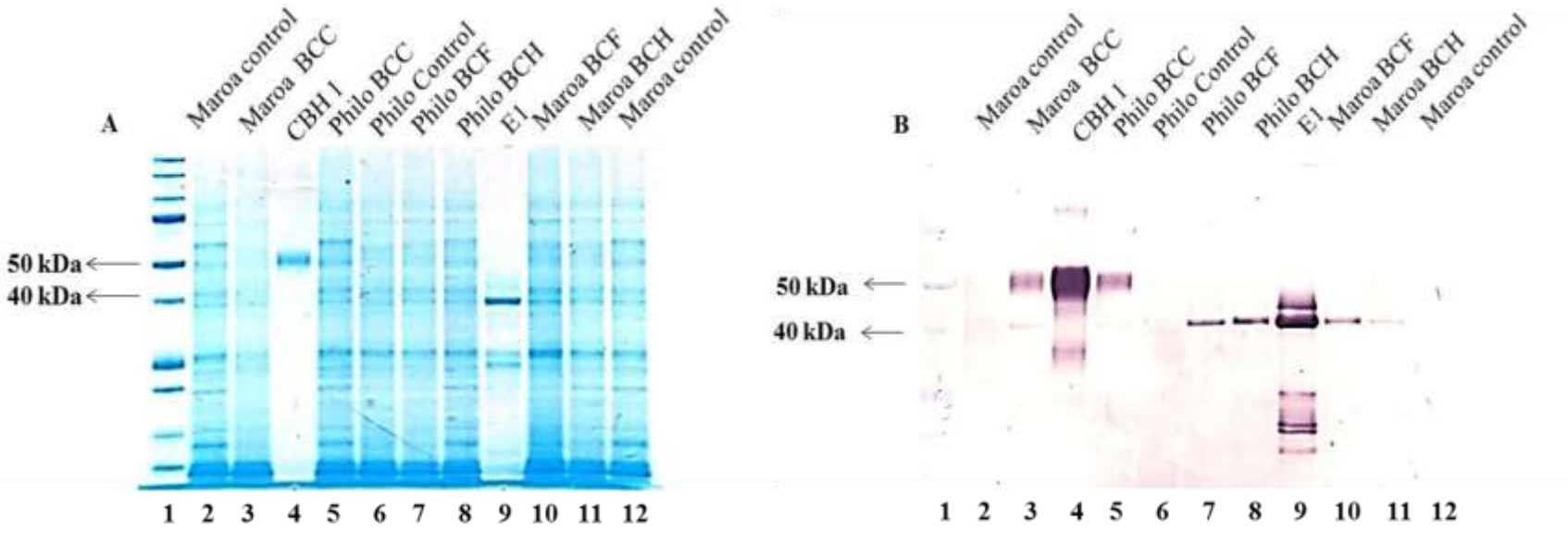


Figure 6. A- SDS-PAGE analysis of corn proteins extracted in PBS buffer. **B**- Western blot analysis of an equivalent SDS-PAGE gel. Lanes 1 contain the Precision plus Protein Dual Color Standard (Bio-Rad). Lanes 4 and 9: purified proteins of interest (CBH I and E1 respectively) as positive controls. Lanes 2, 6 and 12: negative untransformed corn controls (from Maroa and Philo fields). Lanes 3 and 5: Cellobiohydrolase I (CBH I) lines from Maroa and Philo fields, respectively. Lanes 7, 8, 10 and 11: E1 cellulase lines, two from Philo and two from Maroa fields from left to right, respectively.

Figure 7A shows a 1-D polyacrylamide gel of seed proteins extracted in sodium acetate (NaOAc), pH=5. Proteins extracted with this general extraction buffer do not show any major differences among transgenic lines and controls, nor between fields. CBH I and E1 were not visible on the stained gels even though they represent a substantial amount of the soluble protein. However, our proteins of interest were highly detectable on Western blots (Fig. 7B). This confirms the presence of the proteins of interest in the GE lines and their absence in the non-GE lines. Figure 8 shows zeins extracted from ground seed in 70% ethanol and size separated on a 1-D polyacrylamide gel. As seen in the gel, all the extracts, whether from controls or transgenic lines, look similar to each other, although a few minor differences are apparent. In addition, small differences were detected in samples harvested from different fields.

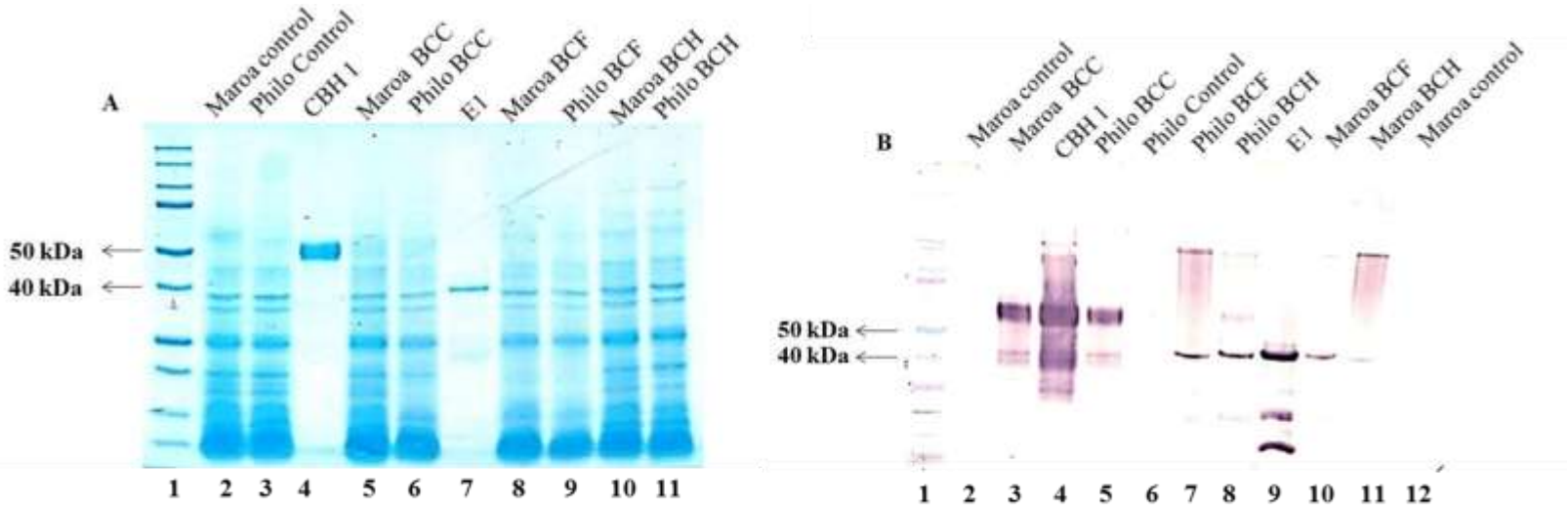


Figure 7. A- SDS-PAGE analysis of corn proteins extracted in NaOAc buffer. Lanes 4 and 5: purified proteins of interest (CBH I and E1 respectively). Lines 2 and 3: negative untransformed corn controls (from Maroa and Philo fields). Lanes 5 and 6: Cellobiohydrolase I (CBH I) lines from Maroa and Philo fields (BCC), respectively. Lanes 8, 9, 10 and 11: E1 cellulases lines, two from Philo and two from Maroa fields (2 BCF lines and 2 BCH) from left to right , respectively. **B-** Western blot analysis of similar SDS-PAGE gel. Lanes 1 contain the Precision plus Protein Dual Color Standard (Bio-Rad). Lanes 4 and 9: purified protein of interest (CBH I and E1 respectively) as positive controls. Lines 2, 6 and 12: negative untransformed corn controls (from Maroa and Philo fields). Lanes 3 and 5: CBH I lines from Maroa and Philo fields (BCC), respectively. Lanes 7, 8, 10 and 11: E1 cellulases lines, 2 from Philo (BCF and BCH, respectively) and 2 from Maroa (BCF and BCH, respectively)

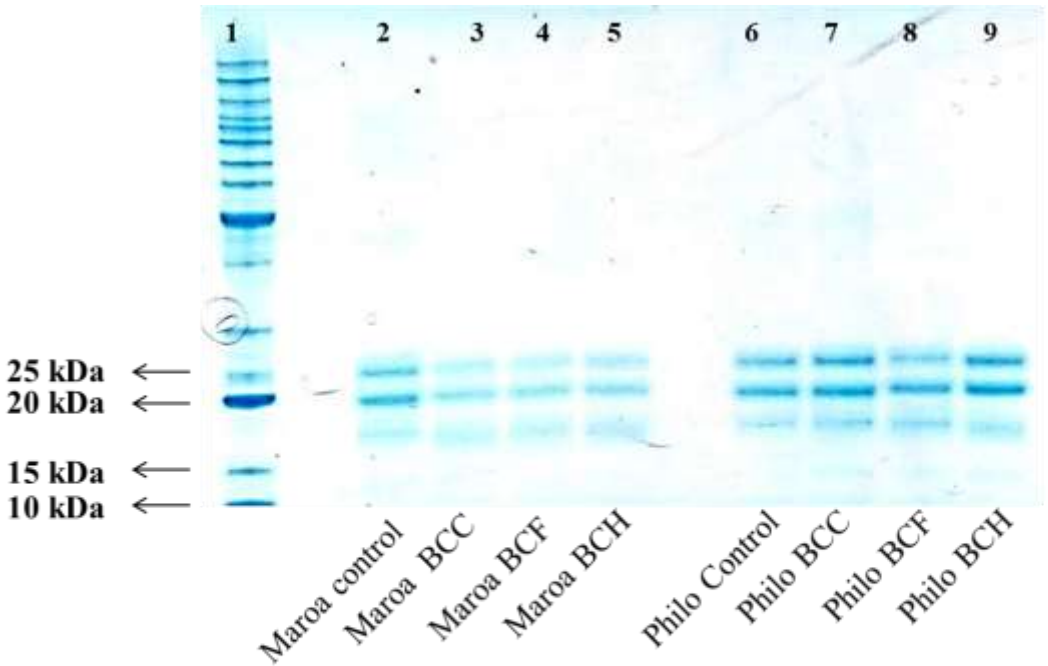


Figure 8. SDS-PAGE analysis of corn seed Zeins extracted in 70% ethanol. Lane 1 contains the Precision plus Protein Dual Color Standard. Lanes 2 and 6: non-GE lines from the respective field. Lanes 3, 4 and 5: lines BCC, BCF and BCH from the Maroa field, respectively. Similarly lanes 7, 8 and 9 contain the same lines harvested from the Philo field, respectively.

2-D gel electrophoresis: Proteins extracted in NaOAc buffer from seed harvested from the Philo field were analyzed in a 2-D SDS-PAGE system. These protein profiles (Figures 9, 10 and 11) show differences compared to their control, indicated by squares. In Figure 9, two protein spots at 50kDa and at 4 to 5 pH range are observed, that likely represent CBH I protein. This observation was then confirmed by a Western blot analysis (Fig. 12B). CBH I protein has a calculated molecular weight of 52.5 kDa and a pI of 5.2. In both Figures 12A and 12B CBH I is visible. Other variable proteins closer to pH10 were also observed. E1 cellulase lines (Fig. 10 B and 11B) look somewhat different than the control (Fig. 10A and 11A). This difference is partially due to the presence of the E1 protein, as well as other proteins distributed throughout the gel. E1 presence was confirmed by a Western analysis of the two E1 lines (BCF Fig. 13B, and BCH Fig. 14B). In these images a protein spot at 40kDa and approximately pH 5 is observed, and represents E1 protein. E1 protein catalytic subunit has a calculated molecular weight of 40 kDa and a pI of 5.

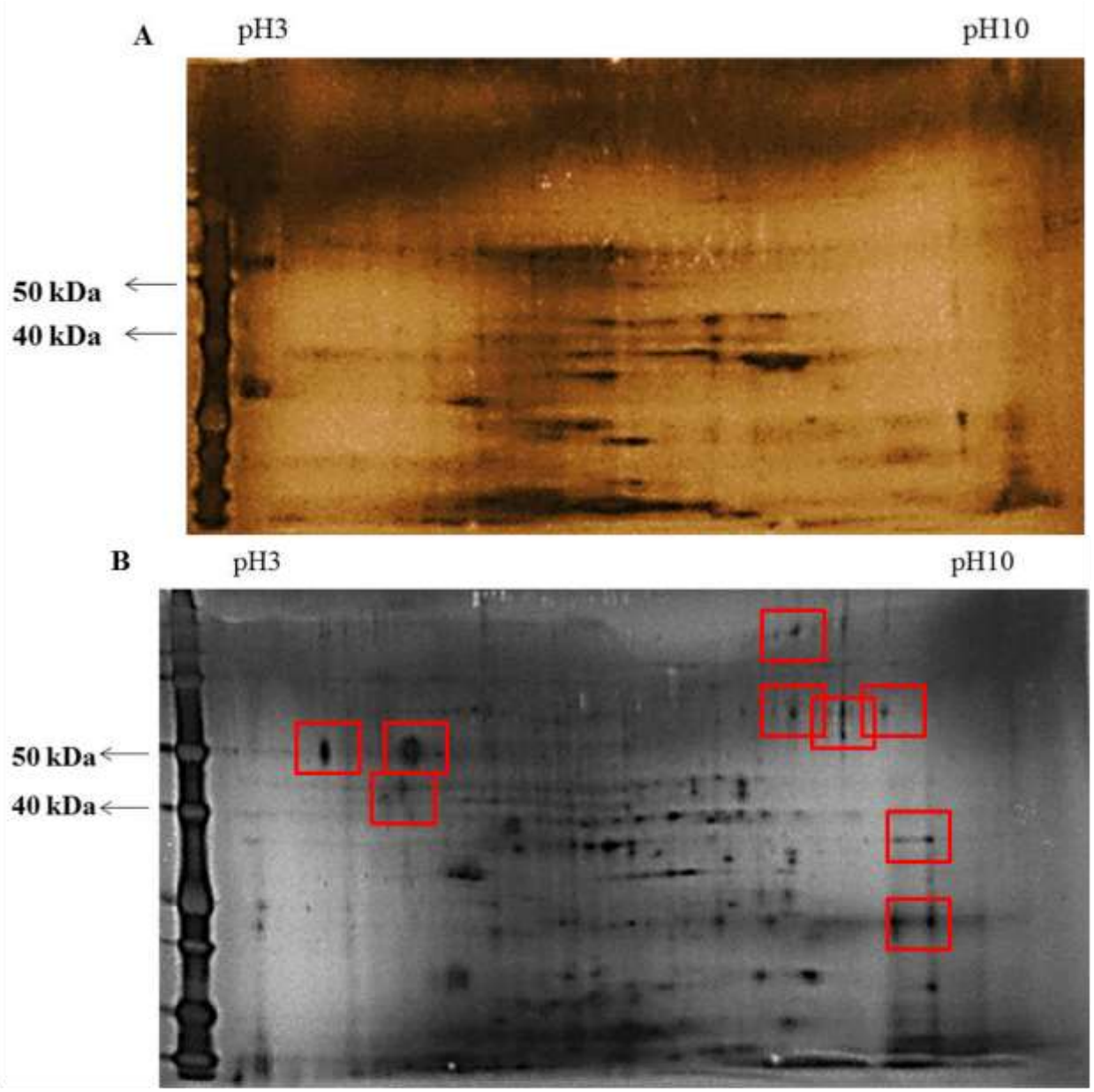


Figure 9- A Two-dimensional SDS-PAGE analysis of the Philo field control corn line extracted in NaOAc (100ug TSP), stained with silver nitrate. The pH gradient ranged from 3 to 10. **B-** Two-dimensional SDS-PAGE analysis of GE corn line BCC from the Philo field (CBH I line) extracted in NaOAc (100ug TSP). These gels were stained with silver stain.

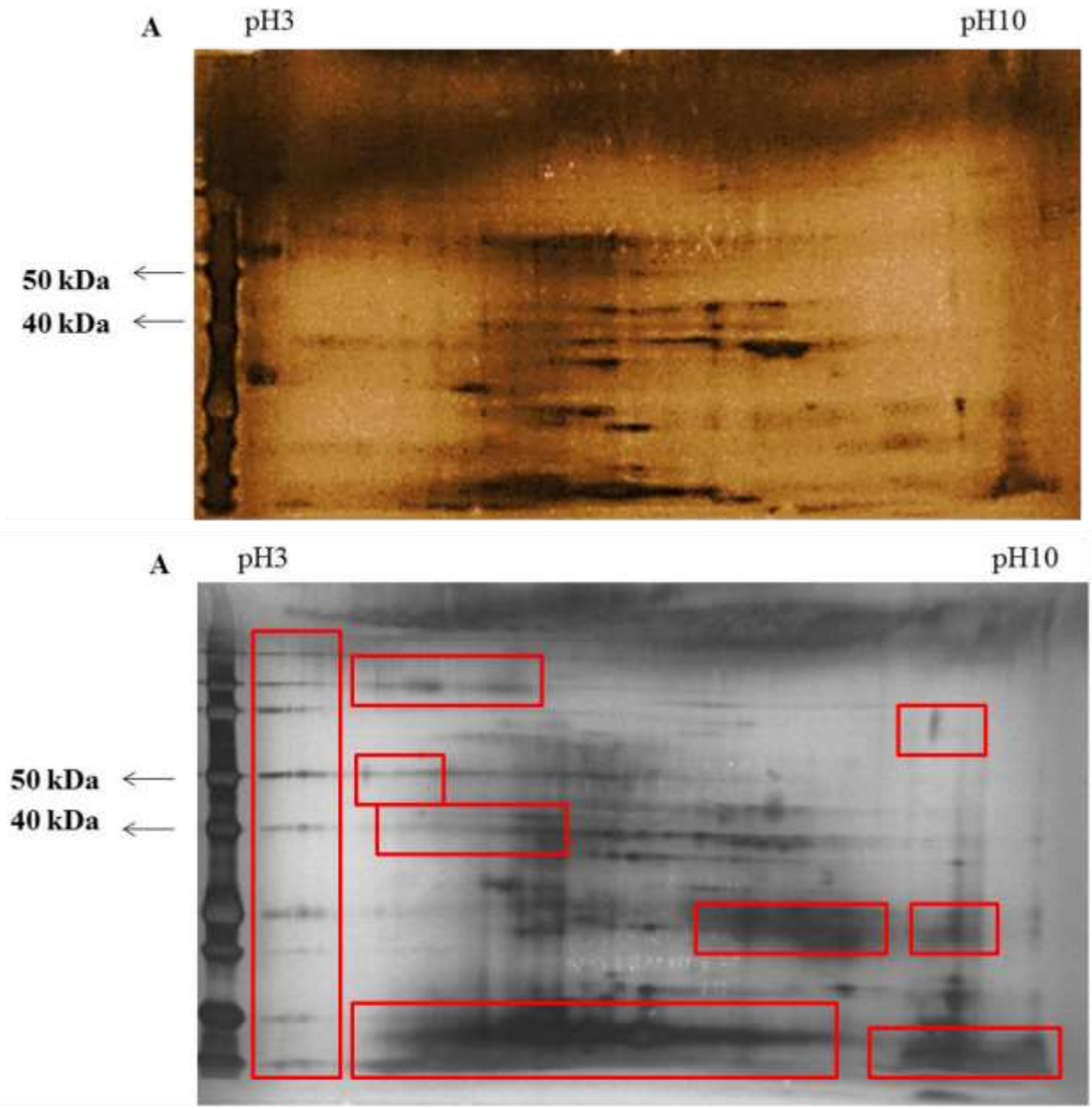


Figure 10- A Two-dimensional SDS-PAGE analysis of the Philo field control corn line extracted in NaOAc (100ug TSP), stained with silver nitrate. The pH gradient ranged from 3 to 10. **B**- Two-dimensional SDS-PAGE analysis of GE corn line BCF from the Philo field (E1 line) extracted in NaOAc (100ug TSP). These gels were stained with silver stain.

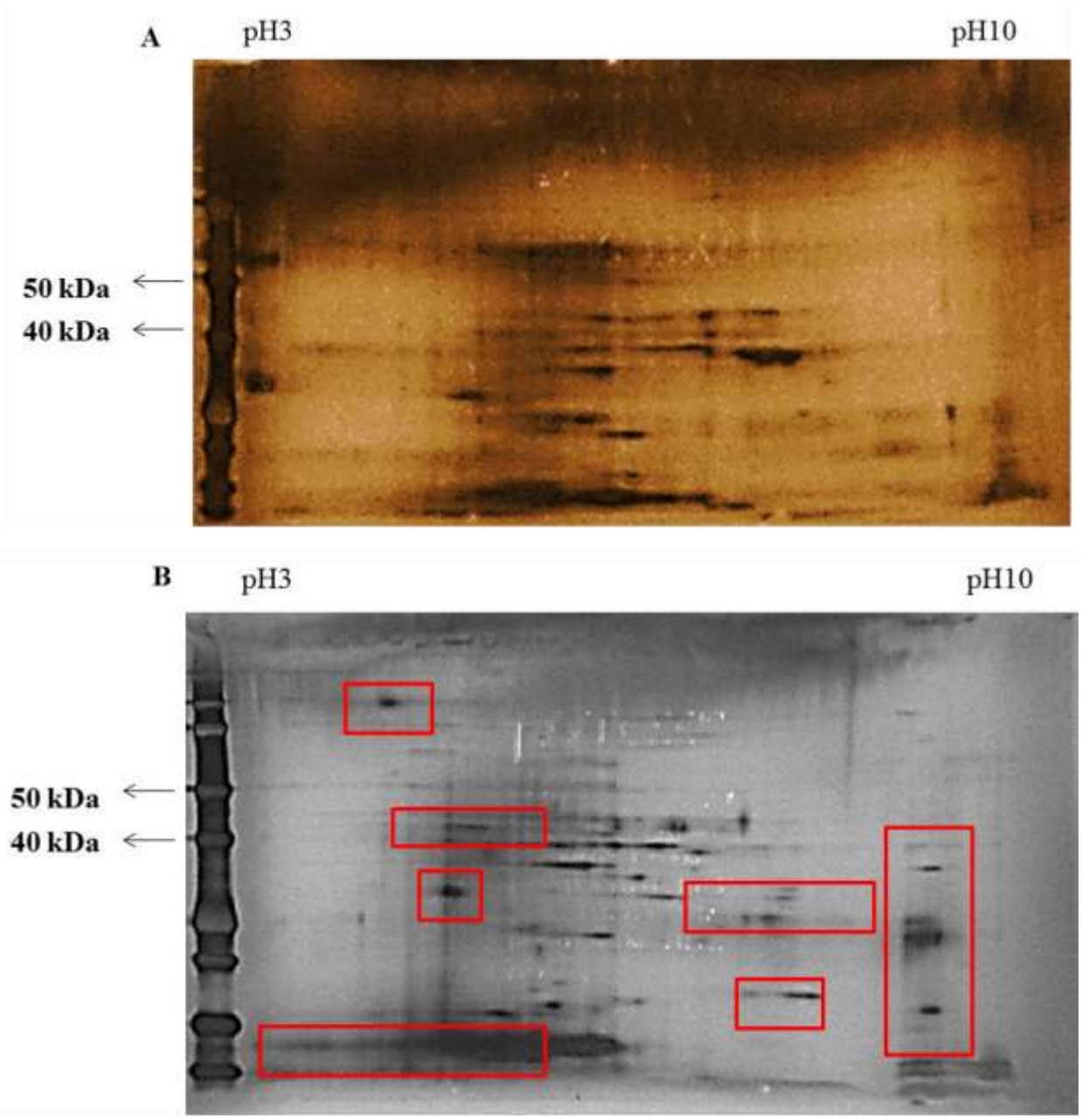


Figure 11- A Two-dimensional SDS-PAGE analysis of the Philo field control corn line extracted in NaOAc (100ug TSP), stained with silver nitrate. The pH gradient ranged from 3 to 10. **B-** Two-dimensional SDS-PAGE analysis of GE corn line BCH from the Philo field (E1 line) extracted in NaOAc (100ug TSP). These gels were stained with silver stain.

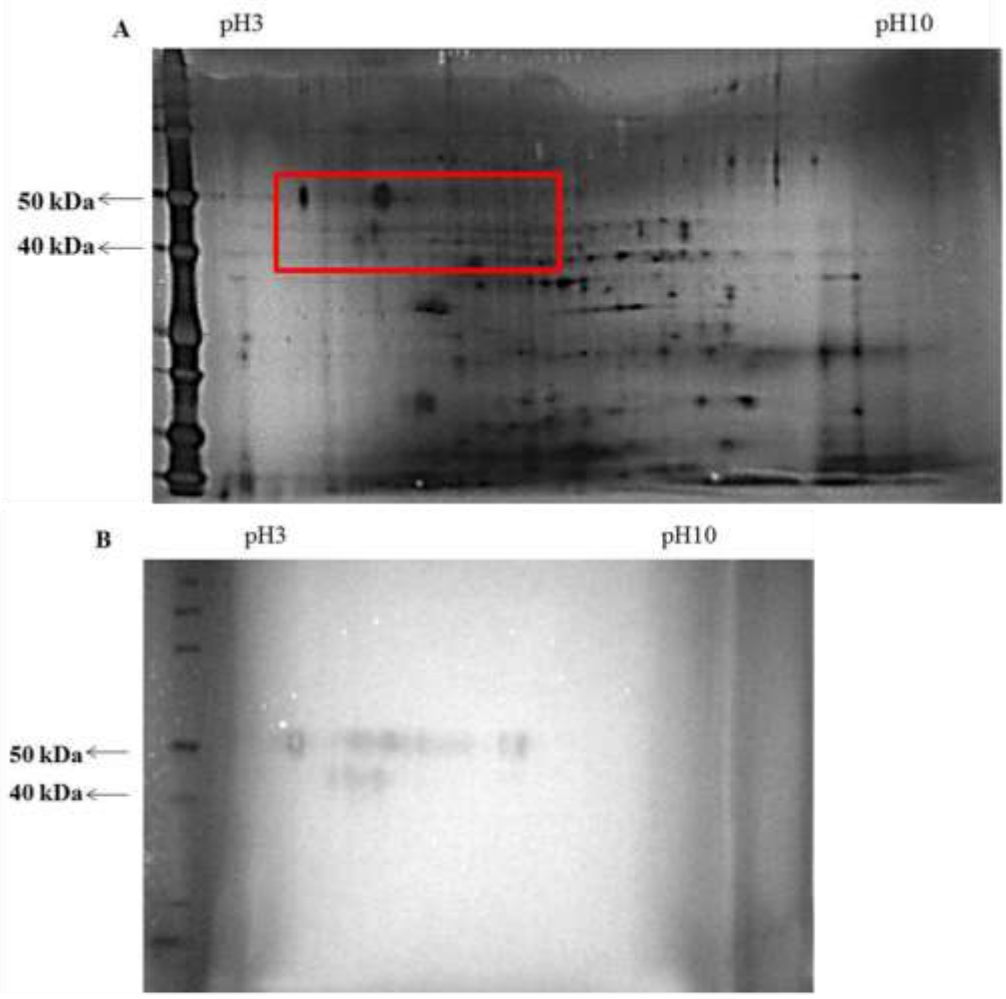


Figure 12. A- Two-dimensional SDS-PAGE analysis of GE corn line BCC from Philo field (CBH I) extracted in NaOAc (100ug TSP). This gel was silver stained. **B-** Western blot of Two-dimensional SDS-PAGE analysis of GE corn line BCC from Philo field (CBH I line) extracted in NaOAc (150ug TSP)

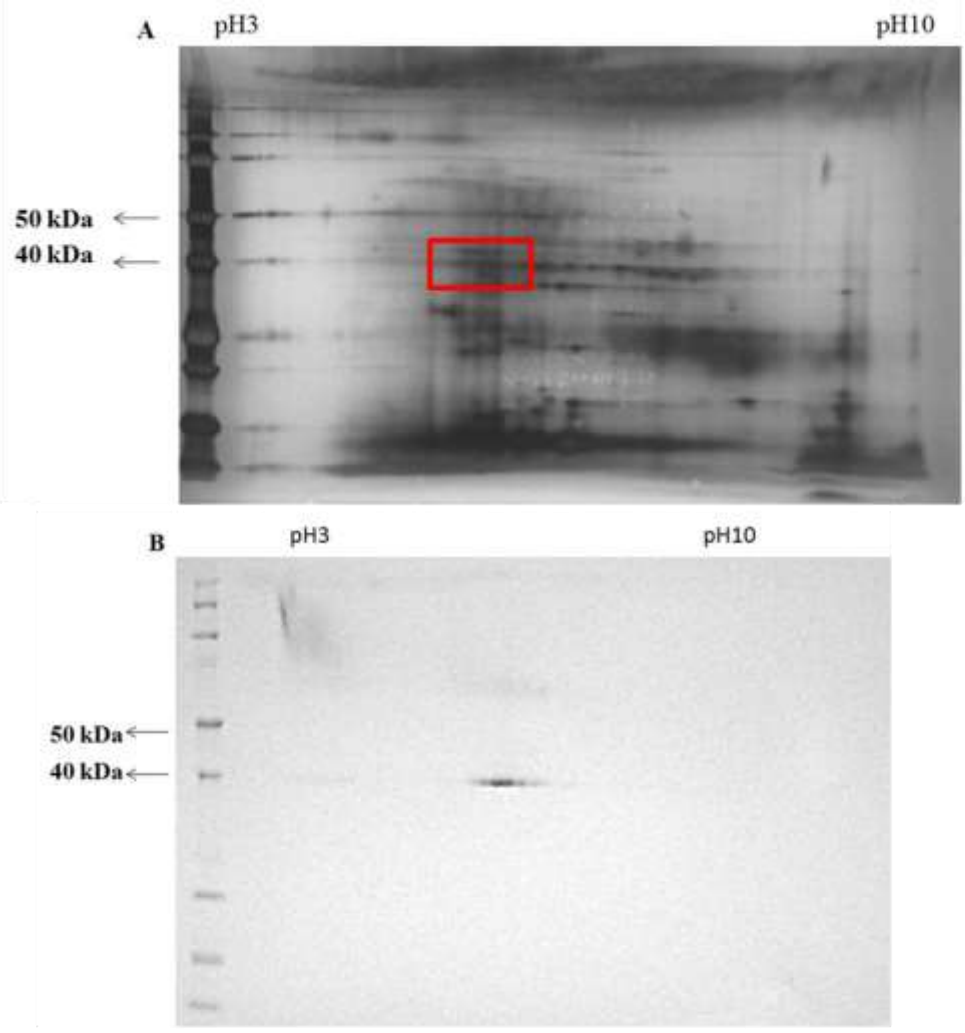


Figure 13. **A-** Two-dimensional SDS-PAGE analysis of GE corn line BCF from Philo field (E1 line) extracted in NaOAc (100ug TSP). This gel was silver stained. **B-** Western blot of a two-dimensional SDS-PAGE analysis of GE corn line BCF from Philo field (E1 line) extracted in NaOAc (100ug TSP).

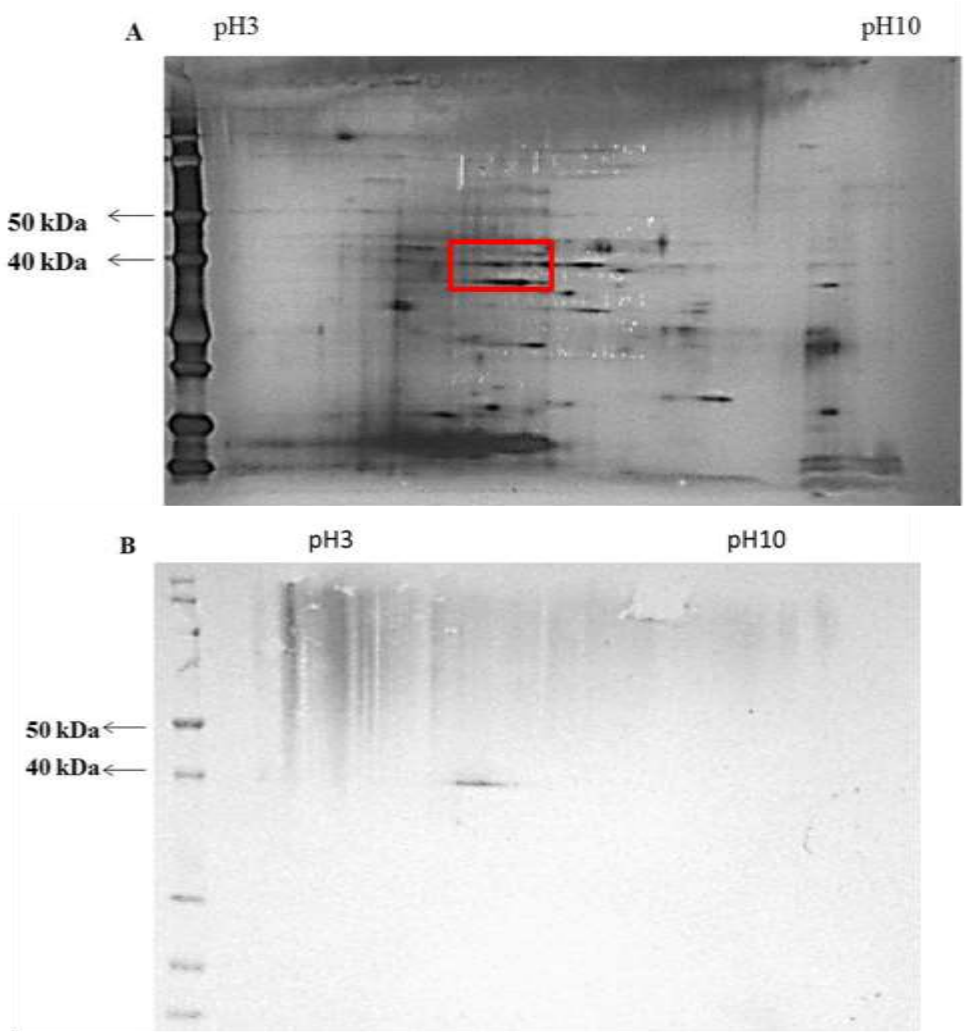


Figure 14. **A-** Two-dimensional SDS-PAGE analysis of GE corn line BCH from Philo field (E1 line) extracted in NaOAc (100ug TSP). This gel was silver stained. **B-** Western blot of a two-dimensional SDS-PAGE analysis of GE corn line BCH from Philo field (E1 line) extracted in NaOAc (100ug TSP).

DNA Analyses

DNA was extracted from corn leaves using a cetyltrimethylammonium bromide (CTAB)-based buffer, and Southern blot hybridization was performed for each set of lines. The positive controls used were CBH I, E1 and spectinomycin genes. Although DNA blot hybridization analysis does not establish substantial equivalence, it is an essential part of the data package that is submitted to the USDA in order to complete the characterization of the transgenic lines.

DNA blot hybridization analyses (*Southern 1975*) were performed to confirm that the two genes of interest (CBH I and E1) are present in the transgenic lines, to determine how many copies per GE line were present and to demonstrate that the control line did not hybridize to the probe (Figure 15A and B). A third probe was utilized to confirm that only the gene of interest and the selectable marker were introduced in the transgenic lines without any additional vector sequences (e.g., the *spectinomycin* resistance gene). The results obtained with the *spectinomycin* gene showed no hybridization in the transgenic lines (Figures 16A and B). The background bands seen in the transgenic lines are also seen in the controls and correspond to repetitive DNA bands visible on the stained gel (data not shown).

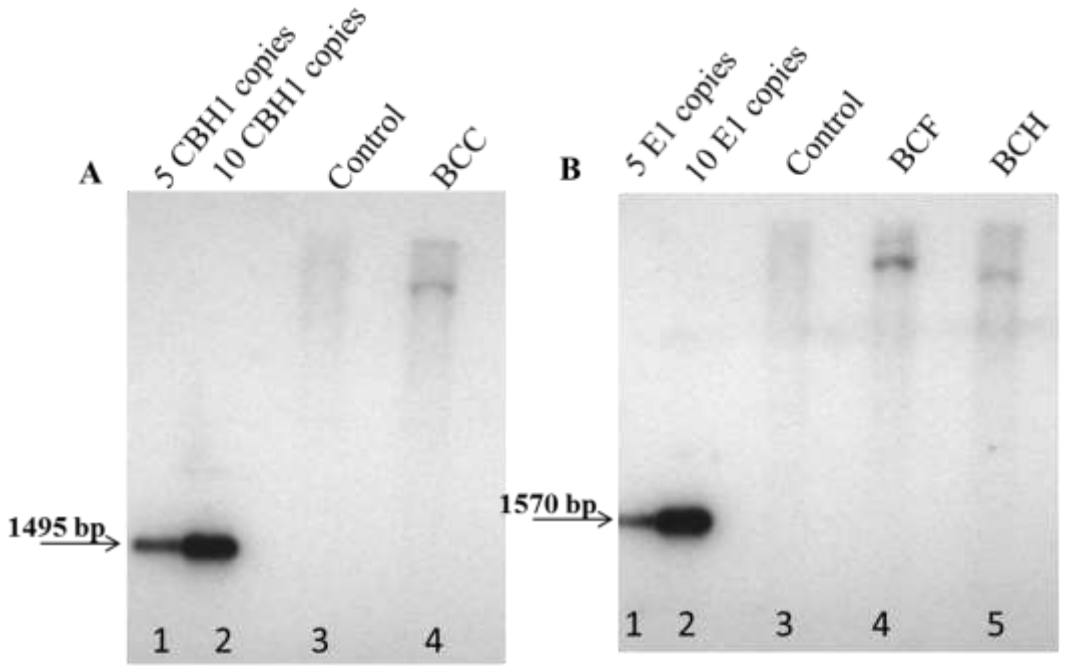


Figure 15. **A**- Southern blot of *Cellobiohydrolase I* (CBH I). Lanes 1 and 2: 5 and 10 copies of *CBH I* gene as a positive control; Lane 3: negative untransformed corn control; Lane 4: BCC line of Maroa and Philo fields. **B**- Southern blot *E1* cellulase. Lanes 1 and 2: 5 and 10 copies of *E1* gene as a positive control; Lane 3: negative untransformed corn control; Lane 4: BCC line of Maroa and Philo fields; Lanes 4 and 5: BCF and BCH lines (from Maroa and Philo fields), respectively. Each lane contains 10 µg of Kpn1 digested genomic corn DNA. Both blots were probed with ^{32}P-labeled CBH I and E1, respectively.

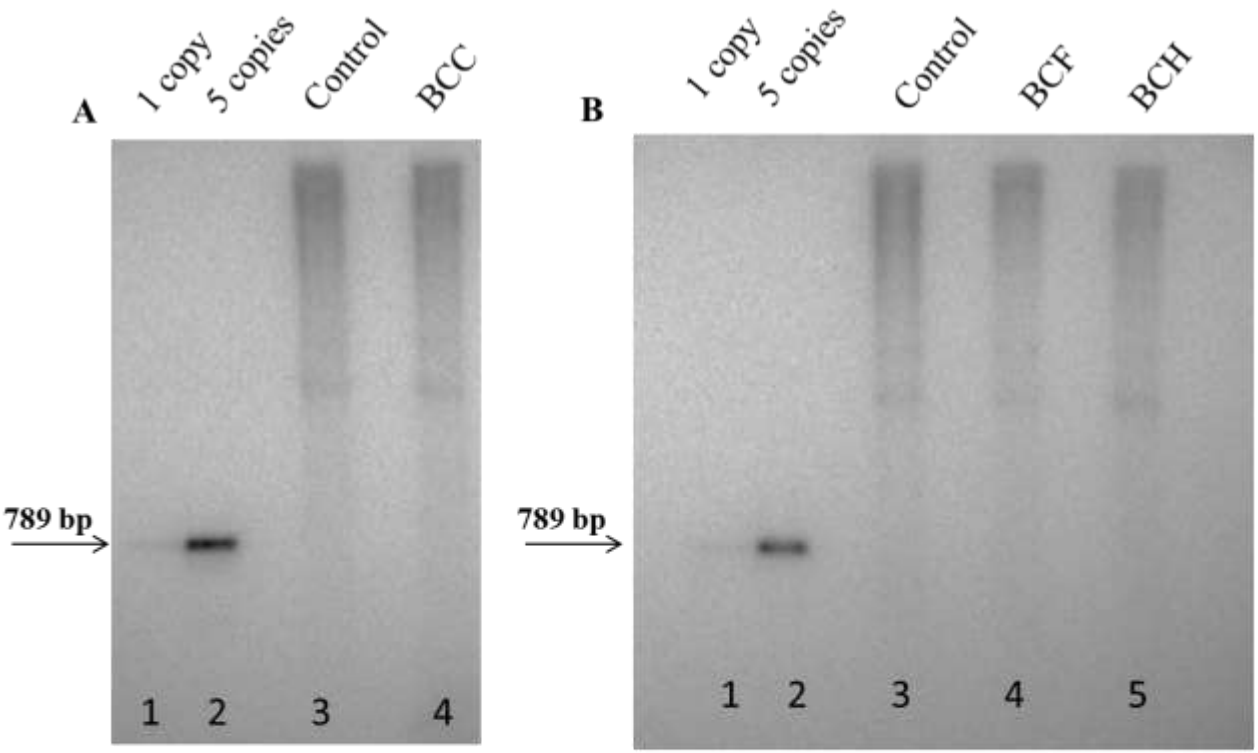


Figure 16. Southern blot analyses of CBH I and E1 corn lines probed with a ^{32}P-labeled *spectinomycin* gene. Each lane contains 10 µg of Kpn1 digested genomic DNA isolated from corn leaves. **A:** Control and BCC CBH I lines; **B:** Control, BCF and BCH E1 lines. Lanes 1 and 2: 1 and 5 copies of the *spectinomycin* gene as a positive control. Lanes 3: negative untransformed corn control. All corn DNA samples are from Maroa and Philo fields combined.

V. DISCUSSION

A set of experiments was performed to support substantial equivalence of cellulase-expressing transgenic corn lines and their near-isogenic control. Experiments included morphology, protein and DNA analyses. Morphological studies consisted of measuring corn seed weight, germination percent and root length, as well as shape, color and size of kernels. Protein analyses were performed using three extraction buffers, phosphate-buffered saline, sodium acetate and ethanol (PBS, NaOAc and 70% ethanol) and 1-dimensional SDS-PAGE and Western blots (PBS and NaOAc extracts), as well as 2-dimensional SDS-PAGE and Western blots (NaOAc extracts). Lastly, DNA was analyzed by Southern blot hybridization with three ^{32}P labeled probes, two for the GOI (Gene of Interest; CBH I and E1) and a third for the *spectinomycin* gene to identify potential presence of a non-T-DNA portion of the transformation plasmid. These data analyses were performed to contribute to the regulatory approval of CBH I and E1 cellulases in transgenic maize for biomass conversion. This was a first step to standardize and simplify the data collection required for a regulatory approval status.

Achieving regulatory approval for biotechnology crops is a known process. Companies like Syngenta, Monsanto, and DuPont/Pioneer have created GE crop varieties, and their regulatory team had to produce the entire intense load of analyses to

achieve regulatory approval of their crops (*Ravinder et al., 2004 and Tuttle et al., 2007*). They have successfully obtained this status for a number of traits, particularly input traits.

In our DNA and morphology experiments, we found no differences among transgenic and control plants, other than those that were intended. Plant DNAs hybridized with their respective ^{32}P labeled probes in the GE lines. The backbone plasmid gene, spectinomycin, did not hybridize (as desired), and morphological observations did not show any statistical differences as desired as well. Similarly, Syngenta developed an alpha-amylase enzyme in corn grain (endosperm) for future use in the dry-grind fuel ethanol process in the US (*Tuttle et al., 2007*). This product would replace amylase addition from microbial production processes. After several years of data collection, regulatory approval was obtained February 15, 2011. Under the identification and characterization of the introduced gene data set, Syngenta demonstrated that the alpha-amylase gene (amy797E, event 3272) was inserted as a single copy and that the transgenic event does not contain any parts of the transforming plasmid other than those intended to be put into the corn plants. They utilized Southern blot hybridization as part of their experimental design to demonstrate this fact. Additionally they showed that no vector sequence was introduced by hybridization that showed a negative result. They also conducted protein analyses utilizing ELISA (*enzyme-linked immunosorbent assay*) methods. The alpha-amylase protein was highly detectable in maize kernels. Furthermore, they tested germination rates, comparing both GE and non-GE plants, and observed that there were no statistical differences at the 95% confidence level.

Similarly Monsanto has engineered a corn crop with improved agronomic traits--resistance to Roundup herbicide, 5-enolpyruvylshikimate-3-phosphate synthase isolated

from *Agrobacterium* sp. strain CP4 (CP4 EPSPS protein, http://cera-gmc.org/docs/cera_ publications/pub_01_2010.pdf), with an extra introduced modified protein (*Bacillus thuringiensis* Cry3Bb1, http://www.epa.gov/oppbppd1/biopesticides/ingredients/ factsheets/factsheet_006484.htm) that selectively controls corn rootworm (CRW) species. These traits provide a broad-spectrum of weed control with minimal crop injury by utilizing Roundup herbicide, and additionally provide protection against CRW. The Monsanto petition to obtain non-regulated status was approved after they showed substantial equivalence, in addition to other safety characteristics, between the GE crop and the non-GE control.

We determined no significant differences in the protein extracts of the GE and non-GE corn lines by 1-D SDS-PAGE and some distinct differences in 2-D SDS-PAGE. Our proteins of interest were detected and confirmed by Western blots. Southern blot hybridizations demonstrated presence of only one copy of the intended genes. Likewise, Monsanto performed a set of Southern blot hybridizations and demonstrated that there was only one copy of the Cry3Bb1 gene and the CP4 EPSPS gene per GE line and that no bacterial plasmid DNA was present (*Ravinder et al., 2004*). They also performed protein analyses to characterize CP4 EPSPS and Cry3Bb1 proteins by SDS-PAGE and Western blots, to determine their molecular weight and their binding to specific antibodies, respectively. These protein analyses demonstrated the right size and proper antigenicity of the proteins introduced into the corn lines. Similarly we determined no significant differences in the protein extracts of the GE and non-GE corn lines by 1-D SDS-PAGE and some distinct differences in 2-D SDS-PAGE. Our proteins of interest were detected

and confirmed by Western blots. Southern blot hybridizations demonstrated presence of only one copy of the intended genes.

The concept of substantial equivalence (SE) as described by the Organization for Economic Co-operation and Development (*OECD, 1993*) for microbial recombinant enzyme preparations can be applied if three conditions are met. The enzymes have similar functions and intended uses; the microbial strains are from safe species with a safe history of food usage; and the purification and manufacturing processes are substantially equivalent. However there is no agreement yet on the criteria to determine substantial equivalence of each of these parameters and therefore safety evaluations are made to determine that there are low to no risks on the enzyme preparations. Having said this and having made evaluations on chymosin and alpha-amylase production through methods other than the conventional process previously used, SE was determined under the concept described above.

In the case of *E. coli* K-12 producing chymosin for cheese manufacture, the organism and method utilized for production were totally different from their traditional source, animal-derived rennet. Bacterial-derived impurities and characteristics of the preparations were different as well, and therefore the Food and Drug Administration had to assess whether the enzymatic activity was equal and if the impurities affected the safe use of it. Nevertheless, although their preparation was different, their function and safety were assessed as substantially equivalent.

In the case of alpha-amylase obtained from recombinant *B. subtilis*, the enzyme product was analyzed and no toxins or undesirable products were found by the Joint FAO/WHO Expert Committee on Food Additives (JECFA). Therefore, JECFA

established the enzyme production system as substantially equivalent and safe for its intended use to control for chocolate syrup viscosity (*www.OECD.org, http://www.oecd.org/dataoecd/37/18/41036698.pdf*).

In the present study, the substantial equivalence definition used was that "the composition of the GE [product] is within the range of variation shown by conventionally bred cultivars when grown under similar conditions" (*Shewry et al, 2007*). Currently, the data set required to achieve deregulated status is quite extensive. These data include: **1)** A description of the biology of the non-modified recipient plant and information necessary to identify the recipient plant. **2)** Relevant experimental data (Genomics, transcriptomics, proteomics, metabolomics and/or functional properties) and publications. **3)** A detailed description of the differences in genotype between the regulated article and the non-modified recipient organism. **4)** A detailed description of the phenotype of the regulated article, including but not limited to: Plant pest risk characteristics, disease and pest susceptibilities, expression of the gene product, new enzymes produced or changes to plant metabolism, weediness of the regulated article, impact on the weediness of any other plant with which it can interbreed, agricultural or cultivation practices, effects of the regulated article on non-target organisms, indirect plant pest effects on other agricultural products, transfer of genetic information to organisms with which it cannot interbreed, and any other relevant information. **5)** Field test reports for all trials conducted under permit or notification procedures. Field test reports shall include the APHIS reference number, methods of observation, resulting data, and analysis regarding all deleterious effects on plants, non-target organisms, or the environment (*www.aphis.usda.gov, http://www.aphis.usda.gov/brs/pdf/7cfr340.pdf*). This current study

was the first step to build the data package required for determining substantial equivalence and thus regulatory approval of cellulase corn. We only focused on the altered portions of the corn lines, the corn kernels (embryos, more specifically) where the proteins of interest are present. Shewry *et al*. (2007) demonstrated no differences between GE and non-GE wheat and therefore these were considered to be substantially equivalent under the concept of SE being variations accepted within the range that they appear in the non-GE crop grown under the same conditions. This result was partially obtained in our experiments. All the morphological and 1-D SDS-PAGE protein analyses showed SE. The lines had very insignificant differences that fell within the range expected (95% confidence level) between the GE and non-GE cellulase corn lines.

VI. CONCLUSION

Utilizing standard methods to analyze morphology, protein and DNA, the obtained results showed no significant differences at the 95% confidence level in terms of seed morphology and no observed significant differences at the protein level other than the intended difference among the GE corn lines containing cellulase and their isogenic control, thus substantial equivalence was demonstrated for morphology and 1-D SDS-PAGE analyses. 2-D SDS-PAGE studies showed differences that suggest that more experiments are needed to demonstrate if those differences fall within non-GE corn protein variation. DNA analyses confirmed the existence of the GOI which characterized the lines.

The differences found in the 2-D SDS PAGE analysis could be attributed to the fact that the hybrid lines are not finished yet, as they still contain some heterozygosity, due to their T6 backcross stage.

USDA-APHIS regulations are designed to protect human and environmental health and many products have been approved and no approved product has been shown to be harmful. We predict that will be the case for these cellulase corn lines as well.

Remaining studies for completion of a non-regulated status include: more 2-D SDS-PAGE, more proteomics, metabolomics, functional properties, allergenic and feeding studies as well as environmental interactions.

VII. SUMMARY

Corn is grown worldwide and utilized for human food, animal feed and for raw material in industrial products. Ethanol production from corn starch has grown a lot in the US over the past 25 years and improved corn crops are being genetically engineered to improve this production even more. Regulatory approval is quite extensive in material required and expensive in scope to move these crops into the market. We wanted to show with standard methods a set of experiments to demonstrate SE within the cellulase corn lines (for future ethanol production) and their isogenic control. These experiments were chosen from those within the Monsanto and Syngenta petition approvals.

We demonstrated at a morphological, protein and DNA level that the corn plants containing cellulases were substantially equivalent to their isogenic control.

Morphological studies consisted of measuring corn seed weight, germination rate and root length, as well as shape, color and size of corn kernels.

Protein analyses were performed using three extraction buffers (PBS, NaOAc and 70% ethanol) by 1-dimensional SDS-PAGE and Western blots (PBS and NaOAc extracts) and 2-dimensional SDS-PAGE (NaOAc extracts).

Lastly, DNA was analyzed by Southern blot hybridization using three separate ^{32}P labeled probes, two for the GOI (CBH I and E1) to confirm presence of our gene of interest and a third for the *spectinomycin* gene to confirm that no sequence of the transformation plasmid backbone was present.

With all these analyses we conclude that GE cellulase corn lines show substantial equivalence to the isogenic non-GE control line at the morphological level, also show SE to the non-GE line at the 1-D SDS-PAGE protein analysis level and that the DNA insert is single copy with no unwanted vector sequences.

APPENDICES

Appendix I: Seed morphology raw data

Seed germination

Control	BCC	BCF	BCH	Lines	Field
10	10	10	10	Rep 1 # seeds	Maroa
9	10	7	9	Rep 2 # seeds	
9	9	10	10	Rep 3 # seeds	
10	10	10	10	Rep 4# seeds	Philo
10	10	8	10	Rep 5 # seeds	
10	10	8	9	Rep 6 # seeds	
0.516398	0.40824829	1.32916	0.516398	Std. Dev.	

Line	Average	Stdv
BCF	8.8	1.329
BCC	9.5	0.408
BCH	9.7	0.516
Control	9.7	0.516

Seed root length

Field/Hour	Line	48H	72H	96H	120H
Philo	Control*	0.77	1.82	2.79	3.98
	BCC*	0.72	2.34	3.23	4.53
	BCF*	1.06	2.53	2.82	4.92
	BCH*	1.25	2.65	3.78	5.03
Maroa	Control**	0.98	2.39	3.39	5.39
	BCC**	0.80	2.83	4.07	6.54
	BCF**	0.73	3.00	4.26	6.77
	BCH**	0.92	2.56	3.65	5.83

Averages

Line/Hour	48	72	96	120
Control	0.88	2.11	3.09	4.69
BCC	0.76	2.59	3.65	5.54
BCF	0.90	2.77	3.54	5.85
BCH	1.09	2.61	3.72	5.43

Standard deviations

Line/Hour	48	72	96	120
Control	0.15	0.40	0.42	1.00
BCC	0.06	0.35	0.59	1.42
BCF	0.23	0.33	1.02	1.31
BCH	0.23	0.06	0.09	0.57

Seed weight

Weight (g)	Lines							
Rep	BCF 101	BCF 202	BCC 503	BCC 304	BCH 403	BCH 303	Control 402	Control 501
1	2.58	2.54	2.67	2.46	2.98	2.40	2.74	2.79
2	3.00	2.67	2.35	2.57	2.70	2.37	2.87	2.58
3	2.59	2.56	2.34	2.42	2.52	2.38	2.81	2.62
4	2.51	2.59	2.50	2.45	2.79	2.26	3.05	2.77
5	2.71	2.52	2.30	2.19	3.05	2.41	2.31	2.60
Avg	2.68	2.58	2.43	2.42	2.81	2.36	2.76	2.67
Std Dev	0.19	0.06	0.15	0.14	0.21	0.06	0.27	0.10

Weight (g)	Lines			
Rep	BCF	BCC	BCH	Control
1	2.56	2.57	2.69	2.77
2	2.84	2.46	2.54	2.73
3	2.58	2.38	2.45	2.72
4	2.55	2.48	2.53	2.91
5	2.62	2.25	2.73	2.46
Avg	2.6	2.4	2.6	2.7
Stdv	0.12	0.12	0.12	0.16

Seed appearance

Maroa Field										
Characteristic/ Person #	1	2	3	4	5	6	7	8	9	10
Color	N	N	N	N	N	N	N	N	N	N
Size	N	N	N	N	N	N	N	N	N	N
Shape	N	N	N	N	N	N	N	N	N	N
Characteristic/ Person #	11	12	13	14	15	16	17	18	19	20
Color	N	N	N	N	N	N	N	N	N	N
Size	N	N	N	N	N	N	N	N	N	N
Shape	N	N	N	N	N	N	N	N	N	N
Characteristic/ Person #	21	22	23	24	25	26	27	28	29	30
Color	N	N	N	N	N	N	N	N	N	N
Size	N	N	N	N	N	N	N	N	N	N
Shape	N	N	N	N	N	N	N	N	N	N
Over all major differences?		Yes (Y)	No (N)							

Philo Field										
Characteristic/ Person #	1	2	3	4	5	6	7	8	9	10
Color	N	N	N	N	N	N	N	N	N	N
Size	N	N	N	N	N	N	N	N	N	N
Shape	N	N	N	N	N	N	N	N	N	N
Characteristic/ Person #	11	12	13	14	15	16	17	18	19	20
Color	N	N	N	N	N	N	N	N	N	N
Size	N	N	N	N	N	N	N	N	N	N
Shape	N	N	N	N	N	N	N	N	N	N
Characteristic/ Person #	21	22	23	24	25	26	27	28	29	30
Color	N	N	N	N	N	N	N	N	N	N
Size	N	N	N	N	N	N	N	N	N	N
Shape	N	N	N	N	N	N	N	N	N	N
Over all major differences?		Yes (Y)	No (N)							

Appendix II: Seed morphology statistics

Seed germination

Unpaired t test results

P value and statistical significance:

The two-tailed P value equals 0.5490

By conventional criteria; this difference is considered to be not statistically significant.

Confidence interval:

The mean of Control Germ minus BCC Germ equals -0.17

95% confidence interval of this difference: From -0.77 to 0.43

Intermediate values used in calculations:

t = 0.6202

df = 10

standard error of difference = 0.269

Review your data:

Group	*Control Germ*	*BCC Germ*
Mean	9.67	9.83
SD	0.52	0.41
SEM	0.21	0.17
N	6	6

P value and statistical significance:

The two-tailed P value equals 0.1828

By conventional criteria; this difference is considered to be not statistically significant.

Confidence interval:

The mean of Control Germ minus BCF Germ equals 0.83

95% confidence interval of this difference: From -0.46 to 2.13

Intermediate values used in calculations:

t = 1.4315

df = 10

standard error of difference = 0.582

Review your data:

Group	*Control Germ*	*BCF Germ*
Mean	9.67	8.83
SD	0.52	1.33
SEM	0.21	0.54
N	6	6

P value and statistical significance:

The two-tailed P value equals 1.0000

By conventional criteria; this difference is considered to be not statistically significant.

Confidence interval:

The mean of Control Germ minus BCH Germ equals 0.00

95% confidence interval of this difference: From -0.66 to 0.66

Intermediate values used in calculations:

t = 0.0000

df = 10

standard error of difference = 0.298

Review your data:

Group	*Control Germ*	*BCH Germ*
Mean	9.67	9.67
SD	0.52	0.52
SEM	0.21	0.21
N	6	6

Seed root length

Unpaired t test results

P value and statistical significance:

The two-tailed P value equals 0.7420

By conventional criteria; this difference is considered to be not statistically significant.

Confidence interval:

The mean of Control Root minus BCC Root equals -0.4425

95% confidence interval of this difference: From -3.5824 to 2.6974

Intermediate values used in calculations:

t = 0.3448

df = 6

standard error of difference = 1.283

Review your data:

Group	*Control Root*	*BCC Root*
Mean	2.6925	3.1350
SD	1.6096	1.9989
SEM	0.8048	0.9995
N	4	4

P value and statistical significance:

The two-tailed P value equals 0.6757

By conventional criteria; this difference is considered to be not statistically significant.

Confidence interval:

The mean of Control Root minus BCF Root equals -0.5725

95% confidence interval of this difference: From -3.7604 to 2.6154

Intermediate values used in calculations:

t = 0.4394

df = 6

standard error of difference = 1.303

Review your data:

Group	*Control Root*	*BCF Root*
Mean	2.6925	3.2650
SD	1.6096	2.0491
SEM	0.8048	1.0245
N	4	4

P value and statistical significance:

The two-tailed P value equals 0.6844

By conventional criteria; this difference is considered to be not statistically significant.

Confidence interval:

The mean of Control Root minus BCH Root equals -0.5200

95% confidence interval of this difference: From -3.5014 to 2.4614

Intermediate values used in calculations:

t = 0.4268

df = 6

standard error of difference = 1.218

Review your data:

Group	*Control Root*	*BCH Root*
Mean	2.6925	3.2125
SD	1.6096	1.8297
SEM	0.8048	0.9148
N	4	4

Seed weight

Unpaired t test results

P value and statistical significance:

The two-tailed P value equals 0.3600

By conventional criteria; this difference is considered to be not statistically significant.

Confidence interval:

The mean of BCF Weight minus Control Weight equals -0.0880

95% confidence interval of this difference: From -0.2970 to 0.1210

Intermediate values used in calculations:

t = 0.9710

df = 8

standard error of difference = 0.091

Review your data:

Group	*BCF Weight*	*Control Weight*
Mean	2.6300	2.7180
SD	0.1204	0.1630
SEM	0.0539	0.0729
N	5	5

P value and statistical significance:

The two-tailed P value equals 0.0126

By conventional criteria; this difference is considered to be statistically significant.

Confidence interval:

The mean of BCC Weight minus Control Weight equals -0.2900

95% confidence interval of this difference: From -0.4989 to -0.0811

Intermediate values used in calculations:

t = 3.2010

df = 8

standard error of difference = 0.091

Review your data:

Group	*BCC Weight*	*Control Weight*
Mean	2.4280	2.7180
SD	0.1203	0.1630
SEM	0.0538	0.0729
N	5	5

P value and statistical significance:

The two-tailed P value equals 0.1861

By conventional criteria; this difference is considered to be not statistically significant.

Confidence interval:

The mean of BCH Weight minus Control Weight equals -0.1300

95% confidence interval of this difference: From -0.3373 to 0.0773

Intermediate values used in calculations:

t = 1.4464

df = 8

standard error of difference = 0.090

Review your data:

Group	*BCH Weight*	*Control Weight*
Mean	2.5880	2.7180
SD	0.1176	0.1630
SEM	0.0526	0.0729
N	5	5

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