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Effects of Glyphosate on Soybean Nutrition, Endophytic Colonization by *Cercospora* cf. *flagellaris* and Development of *Cercospora* Leaf Blight

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EFFECTS OF GLYPHOSATE ON SOYBEAN NUTRITION, ENDOPHYTIC
COLONIZATION BY *CERCOSPORA* CF. *FLAGELLARIS* AND
DEVELOPMENT OF *CERCOSPORA* LEAF BLIGHT

A Thesis
Submitted to the Graduate School Faculty of the
Louisiana State University and
Agricultural and Mechanical College
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by

Teddy Garcia Aroca
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ABSTRACT

Glyphosate (Roundup®, Monsanto, Inc., St. Louis, MO) is the most widely used herbicide in the world because of its broad spectrum and its efficacy in controlling annual broadleaf weeds and undesired grasses. The effect of glyphosate on mineral nutrition and plant diseases has been an important topic during the past decade, because of its controversial effects on plant mineral nutrition. In order to test the hypothesis that glyphosate affects soybean mineral nutrition, and therefore predisposes soybean to *Cercospora* leaf blight, six glyphosate-resistant (GR) soybean varieties were either not treated or treated with glyphosate in field experiments. Plants were then evaluated for leaf concentrations of 13 nutrients, foliar disease symptoms, and biomass of *Cercospora cf. flagellaris* as assessed with real-time PCR assays. Experiments were conducted at three locations in Louisiana. Three sets of soybean GR varieties were used in the experiments, six each year at each location, for a total of 18 varieties. These varieties corresponded to maturity groups III, early and late IV, and V. Control plots received no glyphosate application on the soybean foliage. Disease assessments were performed at R6 growth stage at one location using predefined scales for purple symptoms in 2014 and 2016. No symptoms of CLB were observed at any other location during the three-year period of the study. Differences were detected in nutrient uptake among research stations and years, and there were variations in fungal biomass across varieties. The effects of glyphosate on leaf concentrations of Al, Fe, Mn, N, Na, and K were location-dependent. Glyphosate enhanced uptake of Zn in all experiments. Real-time PCR analyses of the CTB6 gene of *C. cf. flagellaris* consistently detected higher fungal biomass in glyphosate-treated samples compared to controls, indicating that glyphosate affected colonization of host plants during the latent period of infection. Enhanced uptake of Zn, which is a key part of a transcriptional activator (CTB8) in the cercosporin biosynthetic pathway, could be associated with higher concentrations of *C. cf. flagellaris* DNA in glyphosate-treated leaves. However, CLB purple leaf symptom severity was significantly lower in glyphosate-treated plots compared to controls in 2014 and 2016. Therefore, colonization by *C. cf. flagellaris* was not associated with disease severity. These results suggest glyphosate may affect colonization by *C. cf. flagellaris* only while the pathogen is in its endophytic stage of development.

1. INTRODUCTION

Glyphosate (Roundup, Monsanto, Inc., St. Louis, MO) has been the most used herbicide in the world since its introduction in the 1970's by Monsanto (Duke & Powles, 2008). Even though glyphosate is applied to control weeds, the material may also affect soil properties, soil chemistry and availability of nutrients, some specific microorganisms, and the host plant (Altman & Campbell, 1977). Many plant diseases have been associated with glyphosate in previous research, but results arguing both inhibition and synergistic properties of glyphosate for some specific pathogens have been reported, including increases of root rot, target spot, sudden death syndrome, and white mold in soybean (*Glycine max* (L) Merr.) (Johal and Huber, 2009), as well as growth inhibition of *Macrophomina phaseolina*, causal agent of charcoal rot of soybean, in *in vitro* assays (Mengistu, *et al.*, 2013). For other organisms, such as *Cercospora beticola*, causal agent of sugar beet leaf spot, glyphosate had shown no fungicidal effect in field tests (Kanh & Bradley, 2013).

One of the reasons why glyphosate has been associated with plant diseases is because of the findings of Johal & Huber (2009) regarding the effects of glyphosate on micronutrient uptake, which linked glyphosate to reduced levels of certain nutrients in soybean. This could ultimately predispose plants to infection (Johal & Huber, 2009). Application of sub-lethal doses of glyphosate to non-glyphosate resistant soybean was the main factor causing a reduction in leaf and seed concentrations of Ca, Mn, Mg and Fe, suggesting that glyphosate interferes with uptake and translocation of these elements by binding to them and therefore inhibiting their mobilization into the plant (Cakmak, *et al.*, 2009). Similar results were shown in GR soybean by Zobiolo, *et al* (2012) regarding nutrient uptake. Furthermore, Eker *et al* (2006), using sub-lethal doses of glyphosate, showed that glyphosate drift suppressed leaf uptake of Mn and Fe in sunflower 48-72 hours after application. On the other hand, Duke *et al* (2012a) and Duke *et al* (2012b), in an extensive literature review of the effect of glyphosate in GR crops (soybean, cotton, and corn), as well as experimenting in greenhouse and field plots, showed no significant differences between glyphosate-treated plants and controls in tissue concentrations of Ca, Mg, Mn, Zn, Fe, Cu, Sr, Ba, Al, Cd, Cr, Co, or Ni. These findings created controversy concerning whether or not glyphosate should be applied to crops.

In this research project, we tested the hypothesis that glyphosate may have important effects on soybean mineral nutrition and therefore may impact soybean diseases in Louisiana. We focused our efforts on Cercospora leaf blight, caused by *Cercospora cf. flagellaris*. We tested this hypothesis by applying glyphosate to the foliage of six glyphosate-resistant (GR) soybean varieties to compare nutrient uptake and disease development between glyphosate-treated plants and their respective controls (no foliar application of glyphosate). We also determined the direct effect of glyphosate on the CLB causal agent, *C. cf. flagellaris* (Albu *et al.*, 2016), formerly known as *C. kikuchii* (Matsumoto & Tomoyasu, 1925), by measuring DNA concentration/amplification in glyphosate-treated and control leaf samples.

1.1 Cercospora Leaf Blight (CLB)

Cercospora leaf blight is a major disease of soybean (*Glycine max* (L) Merr.) in many states in the USA. Cercospora leaf blight is caused by *Cercospora cf. flagellaris* (Albu, *et al.*, 2016), formerly known as *C. kikuchii* (T. Matsumoto & Tomoy.) M.W. Gardner. Cercospora leaf blight may be linked to purple seed stain of soybean. However, it can also infect leaves, stems, and pods. Upper leaves exposed to the sun can show a light purple appearance when infected by *C. cf. flagellaris* (Sinclair & Backman, 1989). A reddish, purple symptom highlighted with a bronzing appearance also can be seen in affected leaves. When infection is severe, necrosis and chlorosis can develop from these lesions and defoliation may occur, although this can be mistaken for early senescence (Sinclair & Backman, 1989). As reported by Walters (1978), symptoms of CLB are observed at the beginning and throughout seed set (R5-R7) under field conditions. *Cercospora cf. flagellaris* is known to produce cercosporin, a red pigmented phytotoxin (Kuyama & Tamura, 1957).

Cercospora cf. flagellaris can now be targeted with specific primers and probe developed by Chanda *et al* (2014) for real-time PCR assays. These primers were developed based on the CTB6 region of the *C. kikuchii* genome from the same isolates that later were shown to be *C. cf. flagellaris* by Albu *et al* (2016). Amplification of this region was used to detect trace amounts of fungal DNA in plant tissue or cultures, and, using Chanda's equation, to convert cycle threshold (CT) value to relative DNA concentrations of *C. cf. flagellaris*.

1.2 History of Glyphosate

Glyphosate [N-(phosphonomethyl) glycine] was first synthesized in 1950 by Henri Martin in a small Swiss pharmaceutical company (Cilag) (Franz *et al*, 1997; Duke & Powles, 2008). It was not until 1970 that John E. Franz of Monsanto Co. synthesized and tested the molecule for herbicidal use, and the compound was patented as a herbicide. The broad-spectrum herbicide was rapidly accepted by farmers because of its high efficacy. In fact, two decades later, glyphosate was considered the “first billion dollar product” of the pesticide industry (Cox, 1998). However, since the day of its introduction, a problem emerged with glyphosate because the herbicide also killed crops. This brought about a need to avoid killing desired plants with applications of glyphosate, so glyphosate could only be used against undesired plants (Franz *et al*, 1997).

1.3 Molecular Basis for Glyphosate Resistant (GR) Varieties

Before we can understand the molecular basis of glyphosate resistance, we need to look at the mode of action of glyphosate in susceptible plants. In such plants (non-GR), glyphosate binds to the 5-enolpyruvylshikimate-3-phosphate enzyme (EPSP synthase), inhibiting the penultimate step of the shikimate acid pathway, where shikimate is converted to chorismate (Amrhein, Schab, & Steinrücken, 1980; Tzin & Galili, 2010; Haslam, 1974; Steinrücken & Amrhein, 1980). Three aromatic amino acids, phenylalanine (Phe), tyrosine (Tyr) and tryptophan (Trp), are produced at the end of this pathway when glyphosate is not present. When glyphosate is present in the cytoplasm, it inhibits the production of these three amino acids by blocking the EPSP synthase recognition site. Plants use these amino acids, or primary metabolites, to build proteins and other basic processes, including production of some compounds involved in plant defense such as flavonoids (Palo & Robbins, 1991). These amino acids also play an important role in the food chain since they are essential for animals, but only plants can produce them in the shikimate acid pathway. To obtain plants that were able to avoid dying by amino acid starvation caused by glyphosate, a GR plant was engineered with the ability to produce previously mentioned aromatic amino acids through the shikimate acid pathway even when glyphosate is present in the cytoplasm or the chloroplast. One way to obtain GR plants is by mutagenesis of EPSP synthase (Cao, et al., 2012). An EPSP mutant (Pro101 to Ser) of *Salmonella typhimurium* activated glyphosate resistance in transgenic tobacco plants (Comai *et*

al., 1985). In soybean, the changes in the genome to obtain glyphosate resistance were made to obtain an EPSP synthase with high affinity for Phe, Tyr, and Trp but low affinity for glyphosate. A mutant with an alanine (Ala) residue at position 100 that leads to CP4 EPSP (obtained from *Agrobacterium* sp. strain CP4) being glyphosate-insensitive, was identified, even though most bacterial and plant EPSP enzymes contain a strictly bound glycine (Gly) residue at that position (Funke, *et al.*, 2006; Cao, *et al.*, 2012). This led to GR soybeans using *Agrobacterium* sp. strain CP4.

1.4 Effects of Glyphosate on Plant Diseases

The initial controversy of glyphosate associated with plant diseases started long ago and has recently increased because of several studies conducted in the past decade (Duke *et al.*, 2012a). Johal & Huber (2009) linked glyphosate to several plant diseases through an extensive literature review. In soybean, glyphosate was linked to increases in *Fusarium virguliforme*, causal agent of sudden death syndrome (SDS), as well as *Corynespora asiicola*, the pathogen causing target spot (Huber, Cheng & Winsor, 2005). These diseases were reported to increase in incidence because of weed control programs based on glyphosate (Johal & Huber, 2009; Keen, *et al.*, 1982). However, Kandel *et al.* (2015) found no significant effects of glyphosate on SDS. No effects on glyphosate on charcoal rot, caused by *Macrophomina phaseolina* where found in field studies (Mengistu *et al.*, 2013). In other cases, glyphosate has shown suppressing effects on certain diseases in soybean. Applications of glyphosate inhibited soybean rust, caused by *Phakopsora pachyrhizi* in glyphosate resistant soybeans (Feng *et al.*, 2005). These results have led investigators to think that the effects of glyphosate on plant nutrition and diseases are dependent on many factors, since mixed results are found across crops and microorganisms (Duke, 2012).

One of the arguments used to support the hypothesis that glyphosate increased the incidence of diseases was the fact that glyphosate is a strong chelator. When the molecule was first isolated in the 1950's, the only use known for glyphosate was as a chelator until its herbicidal properties were discovered in the 1970's (Bromilow, *et al.*, 1993). This means that, when applied to the foliage, glyphosate readily translocates to the roots where it can immobilize most cations that are in close contact with the rhizosphere or even throughout the plant, especially in meristems where most micronutrients are needed to activate enzymes and other

essential physiological activities of the plant (Pline, *et al.*, 200; Schuette, 1998). Therefore, glyphosate can potentially reduce Mn, Fe and other metals making them unavailable for plants and microorganisms (Johal & Huber, 2009; Thompson & Huber, 2007). However, these effects of glyphosate that have caused controversy in the last decade are based only on reports of reduction of Fe, Mn, and other nutrients by glyphosate applied to non-GR soybeans, in a greenhouse test, using 0.06% and 1.2% of the recommended application rate (Cakmak, *et al.*, 2009); as well as research performed in other crops (Ozturk, *et al.*, 2008). Previous research in our laboratory showed that these minor elements play an important role in the disease cycle (i.e., high levels of Fe suppressed severity of CLB, and high levels of Mn increased severity) (Feng *et al.*, 2005; Ward, *et al.*, 2015; Silva, *et al.*, 2014). However, no effect of glyphosate affecting *C. cf. flagellaris* has been reported. The possibility of glyphosate binding to soil particles and chelating metals also exists, but the extent at which this could have an effect in the host plant, causing secondary effects on some diseases of soybean and other hosts by predisposing plants to infections that depend on the nutritional status of the plant, is still unknown.

1.5 Plant Nutrition and Plant Diseases

Plant nutrition can play an important role in plant diseases. If certain nutrients are deficient, plants are predisposed to some diseases by modifying resistance or susceptibility (Huber, 1980; Datnoff *et al.*, 2007). Among many nutrients, micronutrients are needed to activate most plant physiological processes (Barker & Pilbean, 2007; Datnoff *et al.* 2007; Marschner, 2012). Some pathogens, such as species of *Gaeumannomyces*, *Magnaporthe*, *Phymatotricum*, *Corynespora* and *Streptomyces* utilize Mn oxidation to reduce defense mechanisms in plants involving the shikimate acid pathway (Thompson & Huber, 2007; Schulze, *et al.*, 1995; Melgar, *et al.*, 1998; Huber, *et al.*, 2000). Two elements often mentioned in literature are Fe and Mn, to which glyphosate is associated to cause detrimental effects in plant uptake (Cakmak, 2009; Duke, 2012; Johal & Huber, 2009; Huber, 2005). Recent field studies with CLB demonstrated that Fe and Mn play important roles in the disease cycle. Brandt™ EDTA-Fe formulations applied to soybean foliage showed higher CLB purple symptom severity in field experiments. Formulations of Brandt™ Manni-plex Fe applied to soybean foliage showed lower CLB purple symptom severity. Both EDTA-Fe and Manni-plex Fe reduced blight symptoms of CLB (Silva, *et al.*, 2014; Silva, 2014). High concentration of Mn *in planta* increased CLB severity in petioles

and exacerbated symptoms. Moreover, *in vitro* tests showed that Fe, Mn and Zn were effective in inhibiting mycelial growth of the CLB causal agent (Ward, *et al.*, 2015; Ward, 2015). Based on these findings and conclusions, we would expect glyphosate to play an indirect role in the CLB life cycle by limiting the concentration of Fe and Mn in soybean leaves.

2. MATERIALS AND METHODS

2.1 Field Test of Six Glyphosate Resistant Soybean Varieties

In order to test the hypothesis of glyphosate affects micronutrient content in soybean, resulting in impacts on disease severity and biomass of *C. cf. flagellaris*, six soybean GR varieties were tested in field experiments where plants were either treated or not treated with glyphosate. Disease assessments were performed in 2014 and 2016. No assessments were made in 2015 because of low disease severity. Samples were collected in all locations each year that the experiment was conducted (2014, 2015, and 2016) to determine nutrient concentrations through tissue analysis. Real-time PCR was conducted for all samples collected to determine the biomass of *C. cf. flagellaris* in glyphosate-treated and control samples. This study was conducted at three locations in Louisiana: Ben Hur Research Station in Baton Rouge (BHRS) in 2014, Dean Lee Research Station in Alexandria (DLRS) in 2014, 2015 and 2016, and Macon Ridge Research Station (MRRS), in Winnsboro during 2015 (Table 1). No fertilizers were applied to any of the plots used in these research stations for this test. Standard management practices for insects were followed at each research station.

Table 1. List of research stations and years when the varieties were planted. Sampling dates and growth stages at which samples were collected for later analyses. Soybean growth stages were estimated based on Fehr, *et al.*, (1971). Asterisk (*) indicates the year in which each set of varieties presented in Table 2 were planted.

Code	Research Station/Year	Sampling Date	Growth Stage	Varieties*
DLRS2014	Dean Lee/2014	8/30/2014	R5-R6	2014
BHRS2014	Ben Hur/2014	8/29/2014	R3, R6	2014
DLRS2015	Dean Lee/ 2015	9/3/2015	R6	2015
MRRS2015	Macon Ridge/2015	10/8/2015	R6	2014
BHRS2015	Ben Hur/2015	9/24/2015	R6	2015
DLRS2016	Dean Lee/2016	8/25/2016	R6	2016

2.1.1 Spatial Configuration of Field Experiments

Six soybean GR varieties belonging to maturity groups III to V were planted at three research stations in Louisiana from 2014 to 2016 (Table 2). Maturity groups were assigned as described by Cregan & Hartwig (1984) and Zhang, *et al.* (2007). The experiment was repeated in

2015 and 2016 with different varieties each year, but the same number of maturity groups. One block with four replicates of each variety was planted for the Roundup® (50.2% glyphosate) treatment. A control treatment was planted consisting of one block with four replicates of each variety, in a randomized block design (RBD). The plots were composed of two rows (20 ft long and row spacing of 30” at BHRS, 38” at DLRS, and 40” at MRRS) per experimental unit with no borders in between. Each experimental unit was composed of a variety (plot) either sprayed (glyphosate-treated) or not sprayed (control). Glyphosate was applied at label rates and times indicated below (Section 2.2).

All of the varieties used in this experiment were planted as part of a statewide research project named “Official Variety Trials (OVT)” conducted by Dr. Ronald Levy. Yield was measured for each of the varieties planted at DLRS as well as other stations and published in the LSUAgcenter Soybean Variety Yields and Production Practices annual report (Levy, *et al.*, 2016). Some varieties could not be replanted in successive years because of regulations regarding seed storage as well as loss of viability. For this reason, a different set of varieties had to be used each year (Table 2). The same set of varieties was planted in 2014 at DLRS and BHRS. These varieties also were planted in 2015 at MRRS in order to have a complete replication of the varieties planted in 2014. The seed of the varieties that were planted in 2015 at MRRS was kept at -4 °C in the off season (from 2014 to 2015). However, different varieties had to be planted at DLRS and BHRS in 2015 for the reasons mentioned above. The test was repeated in 2016 only at DLRS with a new set of varieties (Table 2). Terral REV 47R53 was the only variety that was planted during all three years of the study using new seed each year. Planting dates differed at the each location (Table 1) to increase the probabilities of CLB development. For example, the field was planted early at DLRS (May 14th) and late at BHRS (July 2nd) in 2014.

Table 2. Soybean varieties planted at Dean Lee, Ben Hur, and Macon Ridge Research Stations from 2014 to 2016. Maturity groups were assigned as described by Cregan & Hartwig (1984) and Zhang, *et al.* (2007). An asterisk (*) indicates that the variety also was planted in another station/year of the study.

Maturity Group	Varieties/Year		
	2014	2015	2016
Late III and IV	DynaGro 39RY43*	TerralREV 38R10	Asgrow 3931
	Pioneer 94Y82	TerralREV 39A35	TerralREV 47R53*
	TerralREV 47R53*	DynaGro 39RY43*	Armor 48R70
V	TerralREV 52A94	TerralREV 47R53*	Pioneer P54T94R
	DynaGro S56RY84	Armor 55-R22	TerralREV 56R63*
	Asgrow 5332	TerralREV 56R63*	DynaGro 32Y55

2.2 Application of Glyphosate

Roundup® (50.2% glyphosate [isopropylamine salt]) was applied at a rate of 22 fl oz/acre (0.65 L/acre) to the foliage of plots labeled as glyphosate-treated using a backpack sprayer calibrated to deliver 20 gal/A (75.7 L/A). Two applications were made in 2014 at BHRS (R1 and early R3) and one at DLRS at R1. The R3 application at BHRS in 2014 was an off-label application, which was needed because of high weed pressure. However, we did not exceed the maximum allowed per season (44 fl oz/A in soybean). The other half of the experiment was left non-treated for all six varieties with four replicates each. Mechanical control of weeds, using hoes, was performed on both sides of the field (glyphosate-treated and control) three times in 2014 at DLRS and once at BHRS using a tractor outfitted with a cultivator. Mechanical control of weeds was not needed in 2015 and 2016. Roundup® was applied to grasses between rows of the non-treated block at R1 at all research stations in 2015 and DLRS in 2016 using a hooded backpack sprayer at a rate of 22 fl oz/A. This application method (hooded backpack sprayer) allowed us to avoid contact of glyphosate with soybean foliage in the control plots.

2.3 Sampling and Storage of Samples for Real-time PCR (qPCR) and Leaf Tissue Analysis

Leaf samples were collected at all locations from all varieties each year. Two sets of samples, one for analysis of tissue concentrations of 13 nutrients and one for qPCR for *C. cf. flagellaris*, were collected at each plot for all varieties. Samples for analysis of tissue concentrations of nutrients were from collected in paper bags (Duro Bulwark, Novolex, Hartsville, SC). Each sample consisted of 20 leaflets from the third node from the top that randomly collected from the glyphosate-treated and nontreated (control) plots at R6 growth stage at all locations. Samples for qPCR were kept in plastic bags (Zip-Loc, SC Johnson, East Haddam, CT) and dry ice during transportation back to the lab. For long-term storage, samples were kept in a -80 °C freezer (Baxter, Deerfield, IL). For short-term storage, samples were kept at -20 °C in a household freezer (Avantco, Kuala Lumpur, Malaysia).

2.4 Tissue Analyses for Nutrients

2.4.1 Washing, Desiccating, and Grinding of Leaves

Leaflets were put in paper bags during sampling and immediately brought to the lab in boxes or ice chests if the samples were being carried for a long distance (i.e. from DLRS or MRRS to our lab) for washing using a solution of 0.1 M HCL and 0.05 M Liquinox (sodium dodecylbenzenesulfonate). Leaves were then dried at room temperature (21-23 °C) and transferred to new paper bags. These bags were then put in an oven (Fisher Scientific, Hampton, NH) to be dried at 60 °C for 3-4 days. Leaves were ground in a coffee grinder (Intertek, London, United Kingdom) under a laminar flow hood until a powder was obtained. The coffee grinder was cleaned between samples with compressed air to avoid cross-contamination. Samples were transferred to small envelopes and sent for tissue analysis of 13 elements using inductively coupled plasma mass spectrometry (ICP-mass spectrometry) at the LSU AgCenter Soil Testing and Plant Analysis Lab.

2.4.2 Spectrophotometry for Macro and Micronutrients

In order to measure the concentrations of elements in plant tissue, glyphosate-treated and control samples were analyzed following the plant tissue digestion for multi-element ICP protocol (nitric acid- hydrogen peroxide method) (Hansen, et al., 2009). Tissue was digested as

follows: 1) 0.5 g was wrapped in a Kimwipe (Kimberly-Clark, Irving, TX) for each sample then placed in digestion tubes consisting of disposable standard glass tubes (Wheaton 358607 16 X 125mm Tube, 15-415 Cap). Note: for every 36 samples, two blanks (negative control/water) and two reference (positive control) samples were weighed; 2) 5 mL of HNO₃ were added to the tubes to ensure elimination of plant tissue on the walls of the digestion tube. The digestion block was heated to 152-155 °C, and 50 minutes later the digestion block tray was removed from the digestion block; 3) each sample was mixed for about 5 seconds using a Vortex™ mixer prior placing the tubes in the digestion block tray; 4) the tray was returned to the digestion block for 5 minutes to initiate vigorous boiling; 5) the tray was then removed from the digestion block and tubes were removed and allowed to cool for 10 minutes; 6) 3mL of H₂O₂ were then added to each tube, covered with a small glass funnel, and placed on the digestion block for 2 hours and 45 minutes; 7) after digestion, samples were removed from the block and allowed to cool to room temperature; 8) once cooled, samples were stirred using the Vortex then transferred to a 15 mL centrifuge tube. The solution was brought to 12.5 mL total volume with distilled water; and 9) samples were filtered through Whatman No. 1 filter paper (Sigma-Aldrich, St. Louis, MO) using a Fisherbrand porcelain funnel (Fisher Scientific, Pittsburgh, PA), poured into ICP tubes (Polypropylene Autosampler Tubes) and covered with Parafilm™ prior to analysis. Samples were analyzed for concentrations of 13 elements: Al, B, Ca, Cu, Fe, Mg, Mn, N, P, K, Na, S, and Zn.

2.5 Real-time PCR for *Cercospora cf. flagellaris*

2.5.1 Sample Processing for DNA Extraction

Frozen samples from the 2014, 2015 and 2016 growing seasons were ground to a powder using mortars and pestles and liquid nitrogen to keep them frozen during the process. After obtaining a powder, samples were stored at -80 °C in scintillation vials until DNA extraction.

2.5.2 Plant Genomic Total DNA Extraction

Sigma's GenElute™ Plant Genomic DNA Purification Miniprep Kit (Sigma–Aldrich, St. Louis, MO) was used to obtain total DNA from the soybean leaf powder. The initial amount of powder used to extract DNA was 0.1-0.2 grams, as specified in the kit. We made some minor

modifications to the suggested protocol to obtain more DNA: 1) the temperature used in the dry bath was changed from 65 to 70 °C; 2) on the third step, the centrifuge time was extended to 10 minutes at a speed of 14,000X g; and 3) in the last step of DNA extraction only 50 µL of Elute solution was used.

2.5.3 Measuring DNA Concentration and Dilutions

In order to measure DNA concentrations in the 50 µL of eluted DNA extracted from the soybean leaflets, a drop (~2 µL) of the eluted DNA was put in a Nanodrop Spectrophotometer ND-1000 (NanoDrop Technologies Inc, Wilmington, DE). The purity ratio used for a concentration to be accepted was the standard for this type of spectrophotometer (1.7-1.9). The reports containing DNA concentration in ng/µL were saved to be used later for calculations for making dilutions. Tubes containing this initial concentration of DNA were kept at -20 °C and labeled as working stocks. This procedure provided the amount of DNA in µL needed in 100 µL of total solution to obtain a concentration of 10 ng/µL. Dilutions were made for each sample diluting the initial concentration to obtain 100 µL of 10 ng/µL, which was the same concentration of the *C. cf. flagellaris* standards that we used as positive controls in our qPCR plates. The new diluted samples were properly labeled as templates to be used in qPCR.

2.5.4 Real-time PCR Plate Setup

The protocol for detection of *C. kikuchii*, developed by Chanda *et al* (2014), was used to perform qPCR analyses to quantify DNA of the CLB causal agent, *C. cf. flagellaris*. The primers and probe used for this part of the study were: CKCTB6-2F: 5'-CACCATGCTAGATGTGACGACA-3' as the forward primer, CKCTB6-2R: 5'-GGTCCTGGAGGCAGCCA-3' as the reverse primer, and CKCTB6-PRB: 5'-FAMCTCGTCGCACAGTCCCGCTTCG-TAMRA-3' as the fluorescent probe. These primers and probe are specific for the CTB6 gene present in the *C. cf. flagellaris* genome. Isolates used by Chanda *et al* (2014) to develop these primers and probe (CKCTB6-2R, CKCTB6-2F and CKCTB6-PRB) also were used by Albu *et al* (2016) to demonstrate that *C. cf. flagellaris* is the most common pathogen causing CLB in Louisiana. We also tested our primers with isolates used by Albu *et al* (2016) to verify amplification (Supplementary Figure 1).

A premix solution was prepared using the Taqman Universal Master Mix (12.5 μL), Nucleoside Free Water (NFW) (8.9 μL), forward primer CKCTB6-2F (1 μL), reverse primer CKCTB6-2R (1 μL) and fluorescent probe CKCTB6-PRB (0.6 μL) per technical replication. Three technical replications were performed per biological sample. Once the premix solution was prepared, 24 μL were added to each well of a 96-well reaction plate, and 1 μL of template obtained from dilutions of total DNA for the glyphosate-treated and nontreated samples was used as the source of DNA to be amplified resulting in a total solution of 25 μL /well. The plate was setup following a previously designed spreadsheet in order to maintain a record of samples. After the template DNA was added to the plate, it was processed for measuring fluorescence of the probe (CKCTB6-PRB) using a qPCR detection system.

2.5.5 DNA Amplification and Quantification by qPCR

A 96-well MicroAmp[®] Optical Reaction Plate (Applied Biosystems-Thermo Fisher, Foster City, CA) containing the templates diluted in the master mix, plus a positive control (*C. cf. flagellaris* 10ng/ μL) and a negative control (NFW labeled as NTC on the plate), and covered with MicroAmp 8 cap-strips (Applied Biosystems-Thermo Fisher, Foster City, CA) was centrifuged for 3 min at 4000X g in a 96-well plate centrifuge (Eppendorf [5810R], Hamburg, Germany) for a 3-minute spin cycle at 4000X g. The plate was then transferred to an ABI PRISM[®] 7000 Sequence Detection System (Applied Biosystems-Thermo Fisher, Foster City, CA) where 40 temperature cycles were performed by the qPCR thermocycler, and fluorescence (Delta Rn) was measured after each temperature cycle. The temperature pattern for each cycle in the ABI PRISM[®] 7000 comprised three stages: 1) 50 °C for 2 minutes; 2) 95 °C for 10 minutes; and 3) divided in two steps: 95 °C for 15 seconds for step 1 and 60 °C for 1 minute for step 2. The last stage was repeated 40 times.

DNA concentration was estimated using the cycle threshold (Ct) value, which reflects the cycle number at which the fluorescence (Delta Rn) generated within a reaction crosses the threshold (statistically significant point above calculated baseline), usually at the linear phase of amplification (calculated automatically by ABI 7000 software). Ct-values were transformed to DNA concentration in picograms per nanogram of total DNA using a linear regression developed by Chanda *et al* (2014).

2.6 Disease Assessments

Disease assessments for foliar symptoms of CLB were performed at DLRS in 2014 and 2016. These assessments relied on two different rating scales for CLB foliar symptoms (Figures 1 and 2) that were developed by Silva (2014 and unpublished, respectively) using a disease symptom image analysis system, ASSESS 2.0 (APS Press, St. Paul, MN) with field-collected symptomatic leaves. The values in the scales used for CLB (Figures 1 and 2) represent disease severity on soybean leaves for two common symptoms associated with CLB. The purple symptom of CLB (Figure 1A) ranged from 0% disease severity, which is a healthy leaf, to >20% disease severity representing the highest value of CLB purple leaf severity for the scale used in 2014. The majority of severities did not exceed 15%. The blight symptom of CLB is shown in lower row of the scale in Figure 1B, and the values range from 0% (healthy) to 20%. These ratings were done in 2014 at the R6 growth stage. Ratings also were repeated at the same time of the season the following years (2015 and 2016). However, symptoms of CLB were not observed in 2015 at any of the research stations where the experiment was repeated. Both symptoms of CLB (blight and purple) were observed in 2016 at DLRS, and a different scale was used to estimate disease severity (Figure 2), which also was created in ASSESS 2.0, as well as our previous scale. This rating scale used a wider range of symptomatic leaves, which provided more accurate estimates of severity for both purple and blight symptoms.

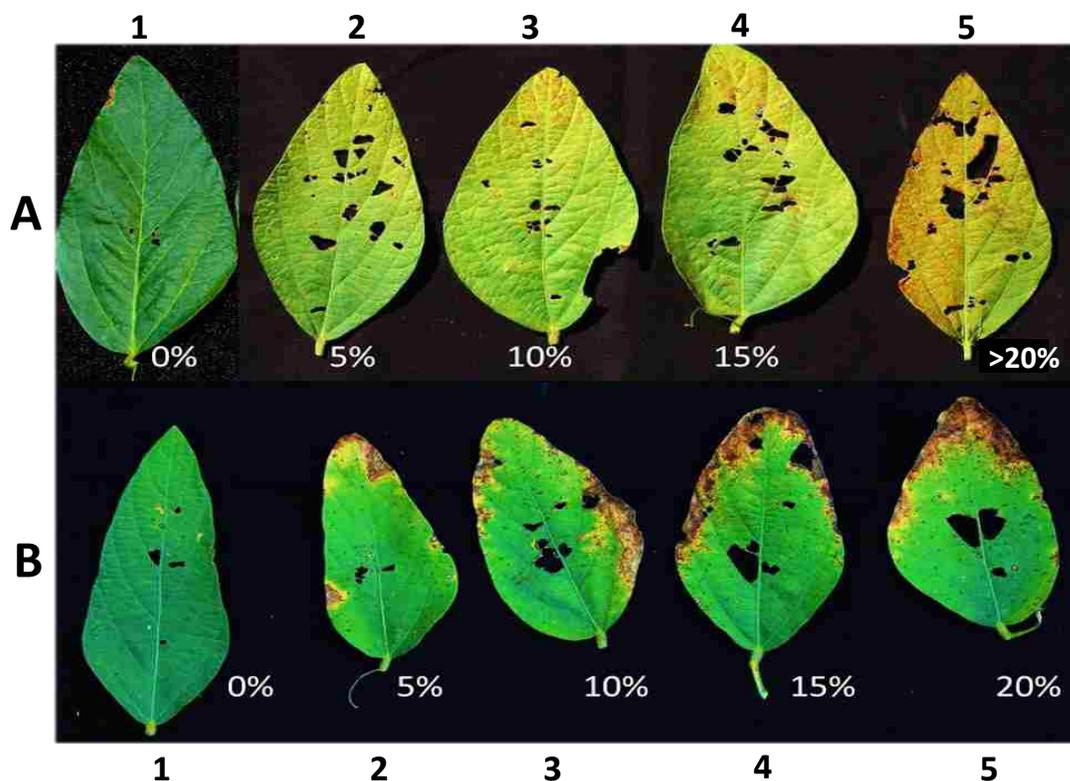


Figure 1. Scale used to assess purple and blight symptoms of *Cercospora* leaf blight of soybean at the R6 growth stage at the Dean Lee Research Station in 2014. A) Purple symptom percentages estimated by ASSESS 2.0. B) Blight symptom percentages estimated by ASSESS 2.0. Modified from Silva (2014).

Assessment of CLB was conducted at DLRS in 2014 in a single-blind fashion where evaluators knew only the glyphosate-treatment (whether the plots being evaluated were treated or nontreated). The values assigned by ASSESS 2.0 were A) CLB purple symptom values (1-5) were 1=0% (healthy), 2=5%, 3=10%, 4=15% and 5=>20%. B) CLB blight symptom values were assigned as follow: 1=0% (healthy), 2=5%, 3=10%, 4=15%, and 5=20% (Figure 1). To evaluate glyphosate-treated and control plots based on this scale, and the entire plot was assigned a value based on the number of leaves affected by the disease. If more than one evaluator was in the same field, an average between the two evaluators was obtained for each plot. In case of widely differing values assigned to a certain plot, both evaluators went back to that plot and discussed the values until reaching a consensus. Raw data collected in 2014 were converted to percentages prior to analysis. This procedure was repeated at DLRS in 2015 at R6 growth stage using the new scale (Figure 2).

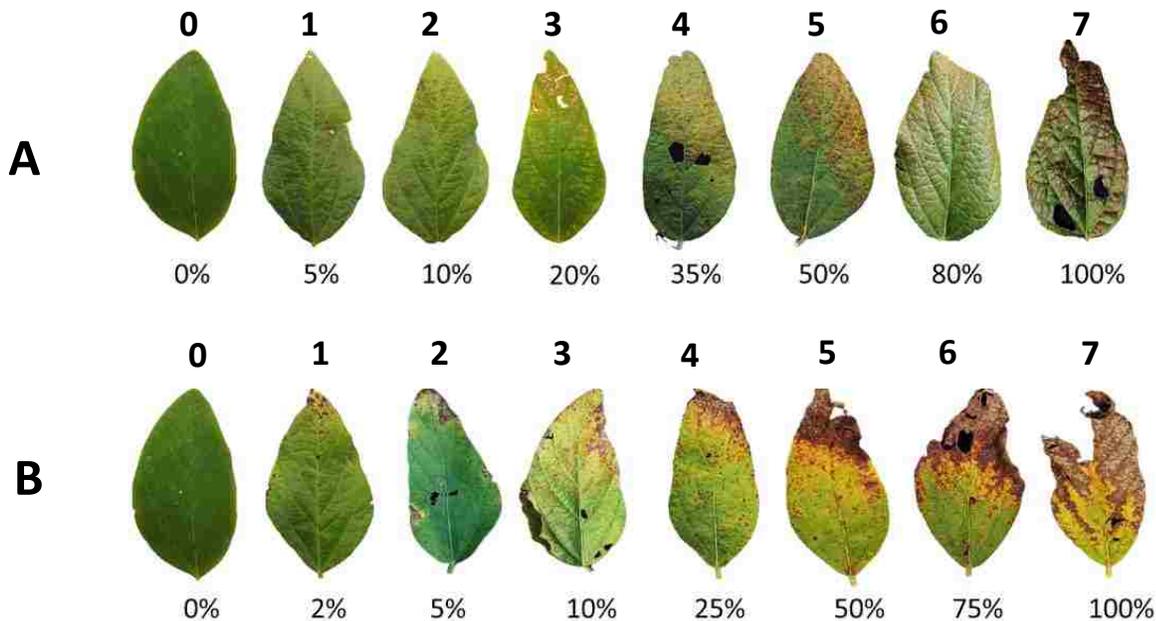


Figure 2. Scale used to assess purple and blight symptoms of *Cercospora* leaf blight of soybean at the R6 growth stage at the Dean Lee Research Station in 2016. A) Purple symptom percentages were estimated by ASSESS 2.0. B) Blight symptom percentages also were estimated with ASSESS 2.0. Developed by Silva (unpublished).

In 2016, assessments of CLB were conducted at DLRS using the revised, expanded scale above (Figure 2). The values assigned by ASSESS 2.0 were on a 0-7 scale as follows: A) CLB purple symptom values (0-7): 0=0% (healthy), 1=5%, 2=10%, 3=20%, 4=35%, 5=50%, 6=80%, and 7=100%; B) CLB blight symptom values were assigned as follows: 0=0% (healthy), 1=2%, 2=5%, 3=10%, 4=25%, 5=50%, 6=75%, and 7=100%.

2.7 Analysis of DATA

Datasets for concentrations of nutrients and DNA amplification were created using all the information for each data point (i.e. year, location, variety, nutrient, treatment, and percentage of each nutrient or DNA concentration for qPCR data). Datasets were then input into SAS 9.4 through the LSU virtual lab application and analyzed using PROC UNIVARIATE to test normality and PROC GLM for interactions across our sources of variation (random effects: year, location, and nutrient; fixed effects: treatment and variety) and PROC MIXED for significant differences ($P \leq 0.05$) between glyphosate-treated and control across our model. Field ratings for CLB purple leaf symptoms were transformed to percentages based on the scales described in

Figures 1 and 2 and input in SAS 9.4 for analysis using PROC UNIVARIATE to test for normality, PROC GLM for interactions across sources of variation (random: year and location; fixed: treatment and variety) and PROC MIXED for significant differences ($P \leq 0.05$) between glyphosate-treated and control across varieties

Major flooding affected our field at BHRS in 2015 and 2016 (heavy rain in June). Even though samples were collected in 2015 at this location, only a maximum of three replications for five varieties could be recovered because of low stand and poor germination of soybeans, which resulted in unbalanced data that could decrease our statistical power in PROC GLM test for interactions. For this reason, data from BHRS collected in 2015 were dropped from our analyses.

3. RESULTS

3.1 Leaf Tissue Analysis for Concentrations of 13 Nutrients

Significant differences were detected in the field experiment main random effects: location, year and nutrient, as well as the fixed effects treatment and variety. However, these differences were influenced by interactions across the sources of variation. Results from SAS PROC GLM regarding interactions across sources of variation included in the class statement for each data point (i.e. year, location, variety, nutrient, and treatment) showed several significant two-way and three-way interactions (Table 3). Two-way interactions were detected for treatment*location, location*year, treatment*variety, nutrient*treatment, location*nutrient, year*nutrient, and variety*nutrient (Table 3). Three-way interactions for location*nutrient*treatment, location*year*nutrient, variety*nutrient*treatment, and location*variety*nutrient were detected (Table 3). These interactions indicated data had to be kept separated by year, location, nutrient, and variety for analyses with PROC MIXED for treatment effects.

Table 3. SAS output from PROC GLM for fixed and random effects, and interactions affecting nutrient concentrations. Effects and interactions were considered significant when $P \leq 0.05$.

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Treatment	1	0.142728	0.142728	7.64	0.0057
Location	2	0.374943	0.187472	10.04	<.0001
Location*Treatment	2	0.224143	0.112072	6.00	0.0025
Year	2	1.096403	0.548202	29.38	<.0001
Year*Treatment	2	0.022675	0.011337	0.61	0.5449
Location*Year	1	0.221851	0.221851	11.88	0.0008
Location*Year*Treatment	1	0.015836	0.015836	0.85	0.3572
Variety	15	0.809918	0.053995	2.89	0.0002
Variety*Treatment	15	0.538158	0.035877	1.92	0.0174
Location*Variety	7	0.175118	0.025017	1.34	0.2271
Location*Variety*Treatment	7	0.048671	0.006953	0.37	0.9188
Year*Variety	1	0.045792	0.045792	2.45	0.1175
Year*Variety*Treatment	1	0.014606	0.014606	0.78	0.3765
Location*Year*Variety	0	0.000000	.	.	.
Location*Year*Variety*Treatment	0	0.000000	.	.	.
Nutrient	12	3135.296619	261.274718	13893.5	<.0001
Nutrient*Treatment	12	1.331919	0.110993	5.94	<.0001
Location*Nutrient	24	41.069500	1.711229	91.65	<.0001
Location*Nutrient*Treatment	24	1.433734	0.059739	3.20	<.0001
Year*Nutrient	24	19.678462	0.828186	44.38	<.0001
Year*Nutrient*Treatment	24	0.340108	0.014171	0.76	0.7918
Location*Year*Nutrient	12	3.053422	0.254452	13.63	<.0001
Location*Year*Nutrient*Treatment	12	0.082466	0.006872	0.28	0.9825
Variety*Nutrient	180	30.784105	0.171023	9.16	<.0001
Variety*Nutrient*Treatment	180	4.176569	0.023203	1.24	0.0184
Location*Variety*Nutrient	84	3.900103	0.046430	2.49	<.0001
Location*Variety*Nutrient*Treatment	84	0.562768	0.006700	0.36	1.0000
Year*Variety*Nutrient	12	1.102941	0.091912	4.92	<.0001
Year*Variety*Nutrient*Treatment	12	0.194883	0.016240	0.87	0.5777
Location*Year*Variety*Nutrient	0	0.000000	.	.	.
Location*Year*Variety*Nutrient*Treatment	0	0.000000	.	.	.

3.1.1 Results from Ben Hur Research Station in 2014

Results from soybean varieties analyzed for macro and micronutrient content in leaf tissue showed significantly higher concentrations of Mn, N, and Zn in samples collected at BHRS in 2014 from six GR varieties. Concentrations of Mn were significantly higher in glyphosate-treated samples in three out of six varieties (Asgrow 5332, Terral REV 47R53, and Terral REV 52A94) in 2014 (Figure 3). The concentrations of N also were significantly higher in glyphosate-treated samples for four out of six varieties analyzed in 2014 at BHRS (DynaGro 39RY43, TerralREV 47R53, TerralREV 52A94, and Asgrow 5332) (Figure 4). Concentrations of Zn were found in significantly higher amount in glyphosate-treated samples for five out of six varieties at this location in 2014 (DynaGro 39RY43, Pioneer 94Y82, TerralREV 47R53, DynaGro S56RY84, and Asgrow 5332) (Figure 5).

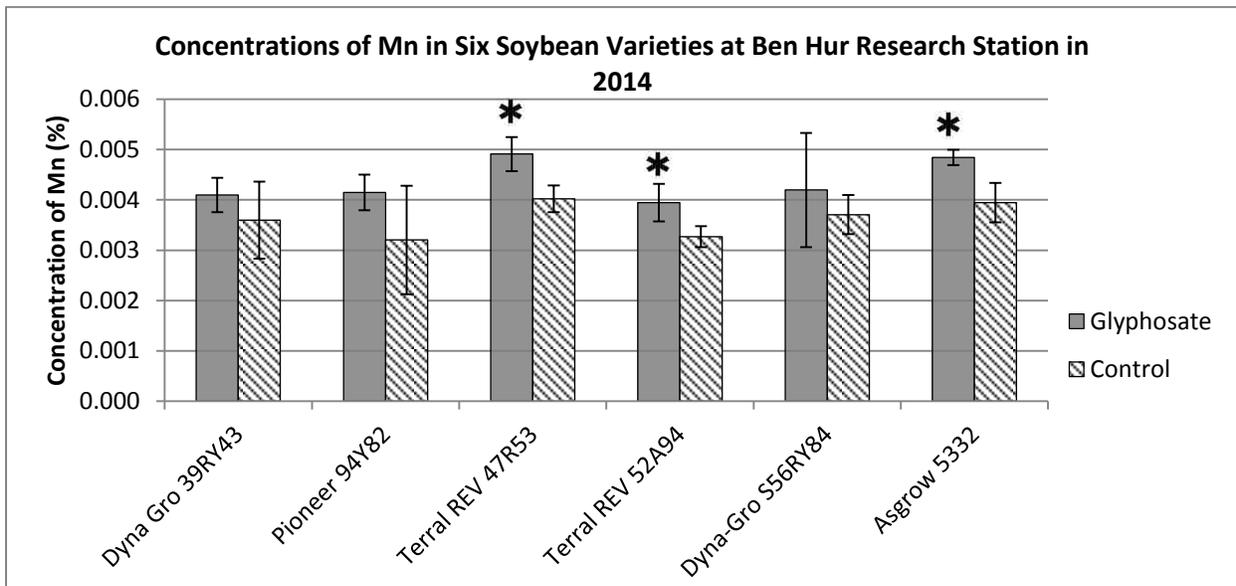


Figure 3. Concentrations of Mn in six soybean varieties for glyphosate-treated and control samples collected at the Ben Hur Research Station in 2014. Bars represent means for each variety (n=4) and error bars represent standard error. Statistical differences ($P \leq 0.05$) between glyphosate-treated and control are marked with an asterisk (*).

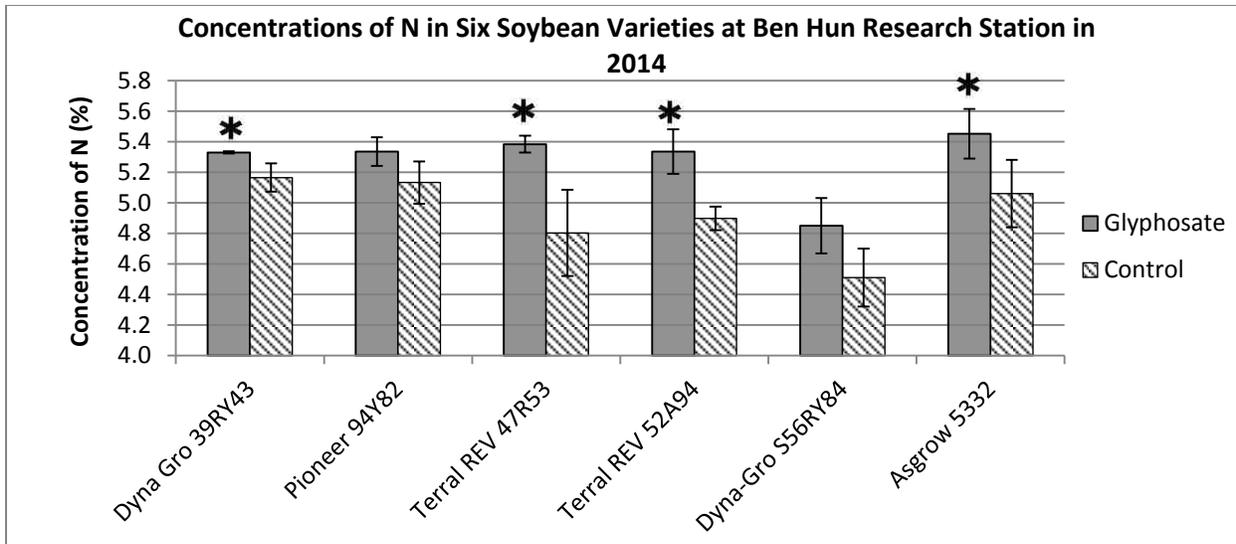


Figure 4. Concentrations of N in six soybean varieties for glyphosate-treated and control samples collected at the Ben Hur Research Station in 2014. Bars represent means (n=4), and error bars represent standard error. Statistical differences ($P \leq 0.05$) between glyphosate-treated and control are marked with an asterisk (*).

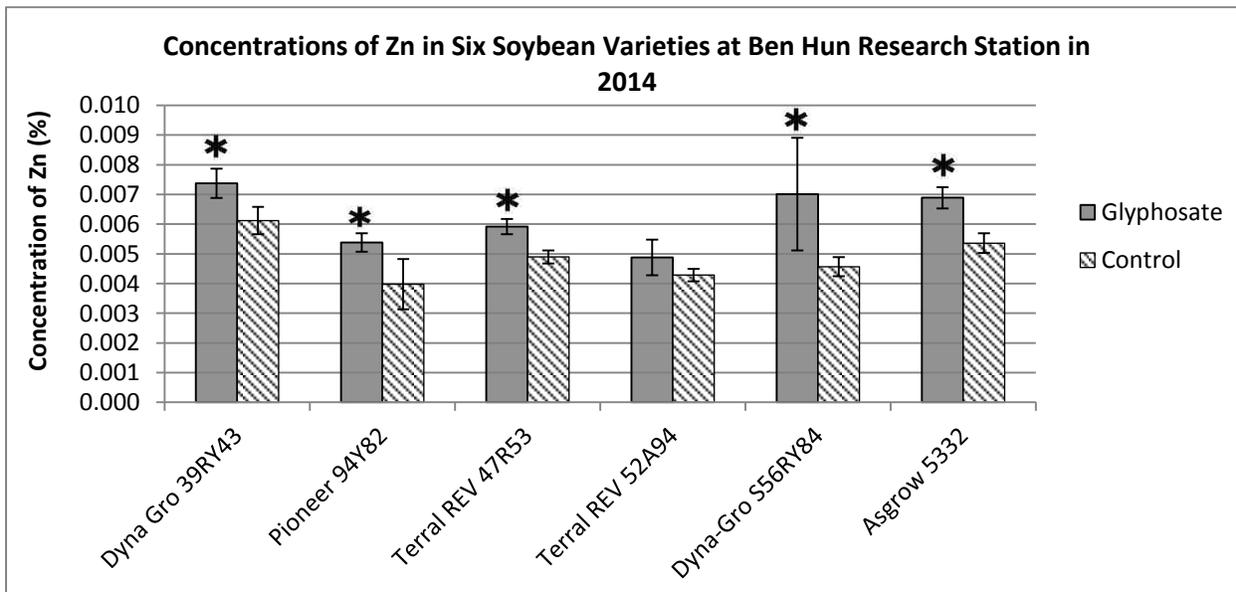


Figure 5. Concentrations of Zn for six soybean varieties in glyphosate-treated and control samples collected at the Bun Hur Research Station in 2014. Bars represent means for each variety (n=4), and error bars represent standard errors. Statistically significant differences ($P \leq 0.05$) are marked with an asterisk (*).

Sodium was found in significantly higher concentration in nontreated plants from all varieties tested at this research station in 2014 (Figure 6). No significant differences ($P \leq 0.05$) were observed in the rest of nutrients analyzed (Al, B, Ca, Cu, Fe, Mg, P, K, and S).

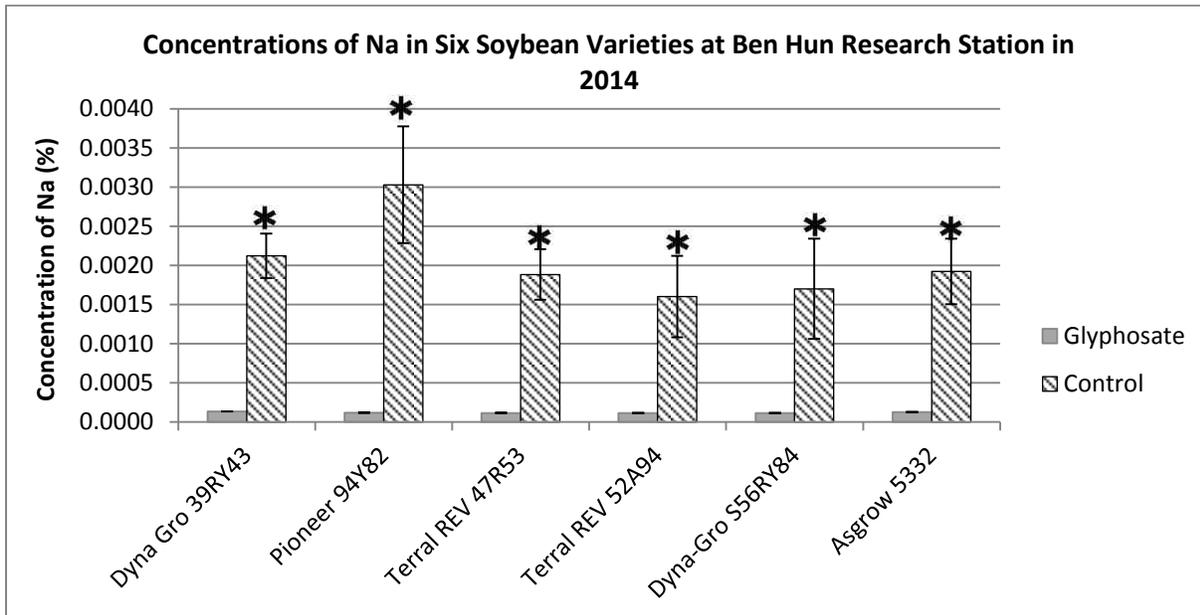


Figure 6. Concentrations of Na for six soybean varieties in glyphosate-treated and control samples collected at the Ben Hur Research Station in 2014. Bars represent means for each variety (n=4), and error bars represent standard error. Statistically significant differences ($P \leq 0.05$) are marked with an asterisk (*).

3.1.2 Results from Macon Ridge Research Station in 2015

Results from MRRS in 2015 showed significantly higher concentrations of Mn, Fe, Al, Na, and Zn for glyphosate-treated samples across six varieties. Concentration of Mn was significantly higher in all varieties tested at MRRS in 2015 (DynaGro 39RY43, Pioneer94Y82, TerralREV 47R53, TerralREV 52A94, DynaGro S56RY84, and Asgrow 5332)(Figure 7). The concentration of Fe was significantly higher in samples treated with glyphosate for four out of six varieties in 2015 at MRRS (DynaGro 39RY43, TerralREV 47R53, TerralREV 52A94, and DynaGro S56RY84) (Figure 8). Aluminum also was found in higher concentration in glyphosate treated samples at this research station in 2015. Five out of six varieties showed significantly higher concentration of Al (DynaGro 39RY43, TerralREV 52A94, DynaGro S56RY84, and Asgrow 5332) (Figure 9).

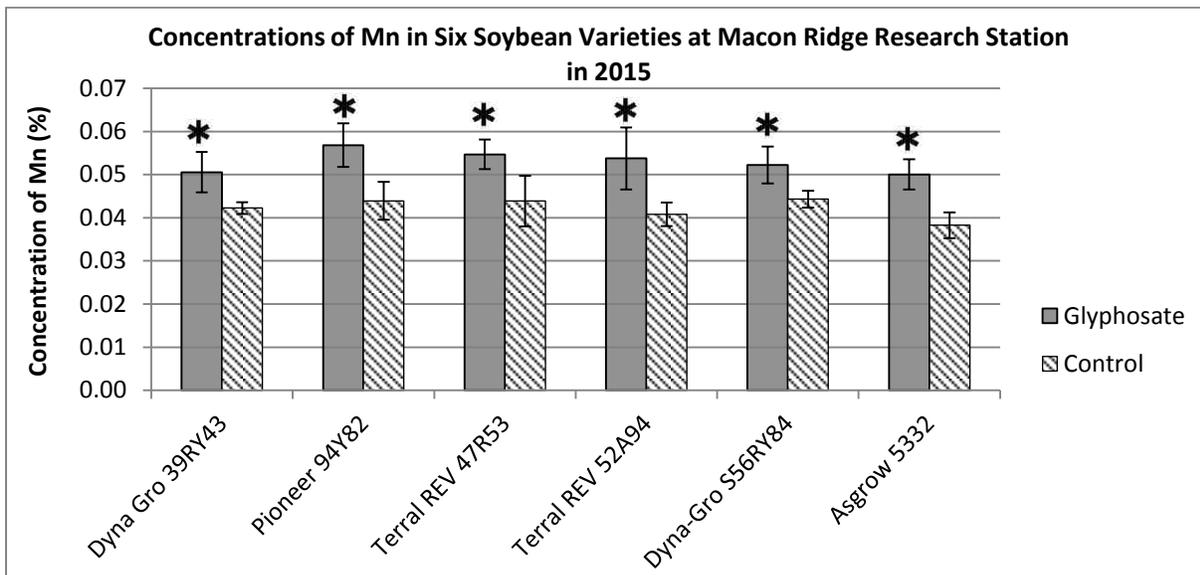


Figure 7. Concentrations of Mn for six soybean varieties in glyphosate-treated and control samples collected at the Macon Ridge Research Station in 2015. Bars represent means across variety (n=4) and error bars represent standard error. Statistically significant differences ($P \leq 0.05$) are marked with an asterisk (*).

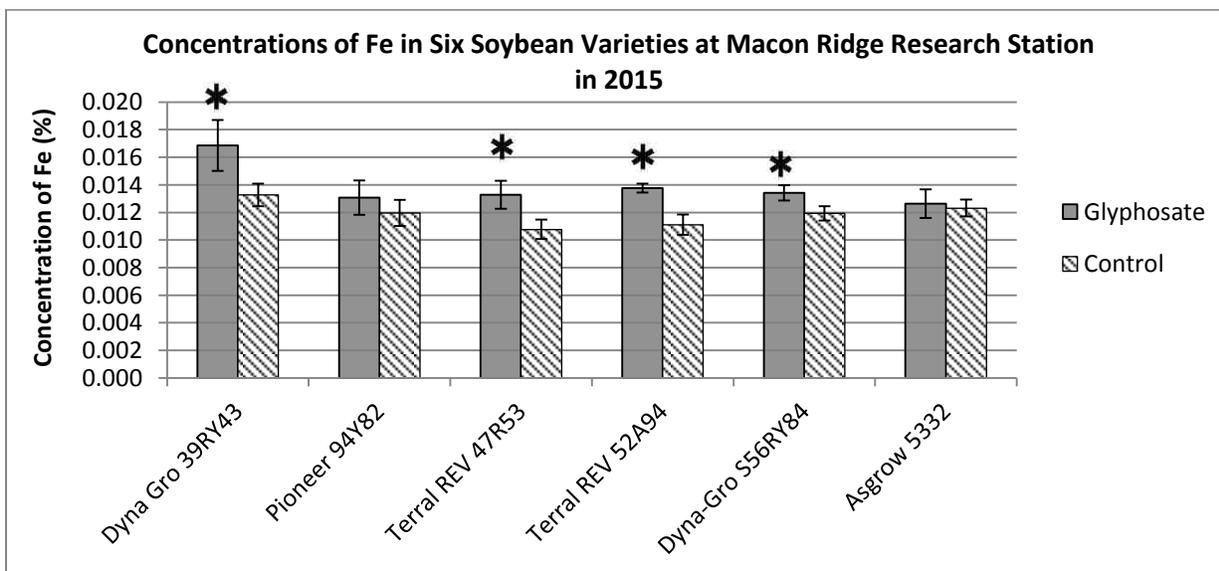


Figure 8. Concentrations of Fe for six soybean varieties in glyphosate-treated and control samples collected at the Macon Ridge Research Station in 2015. Bars represent means across variety (n=4) and error bars represent standard error. Statistically significant differences ($P \leq 0.05$) are marked with an asterisk (*).

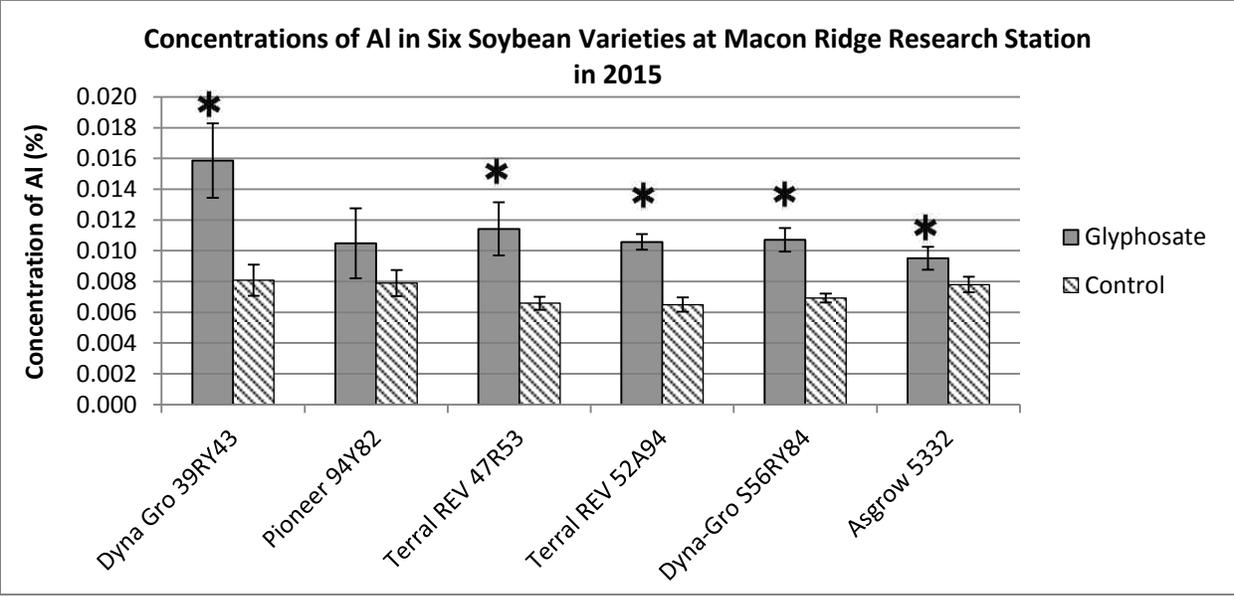


Figure 9. Concentrations of Al for six soybean varieties in glyphosate-treated and control samples collected at the Macon Ridge Research Station in 2015. Bars represent means for each variety (n=4) and error bars represent standard error. Statistically significant differences ($P \leq 0.05$) are marked with an asterisk (*).

Sodium was found in significantly higher concentration in glyphosate-treated samples collected from five out of six varieties tested at MRRS in 2015 (DynaGro 39RY43, Pioneer94Y82, TerrallREV 47R53, DynaGro S56RY84, and Asgrow 5332) (Figure 10). The concentration of Zn also was found in higher concentration in glyphosate treated samples at this location in 2015 in four out of six varieties tested (DynaGro 39RY43, TerralREV 47R53, TerralREV 52A94, and Asgrow 5332) (Figure 11).

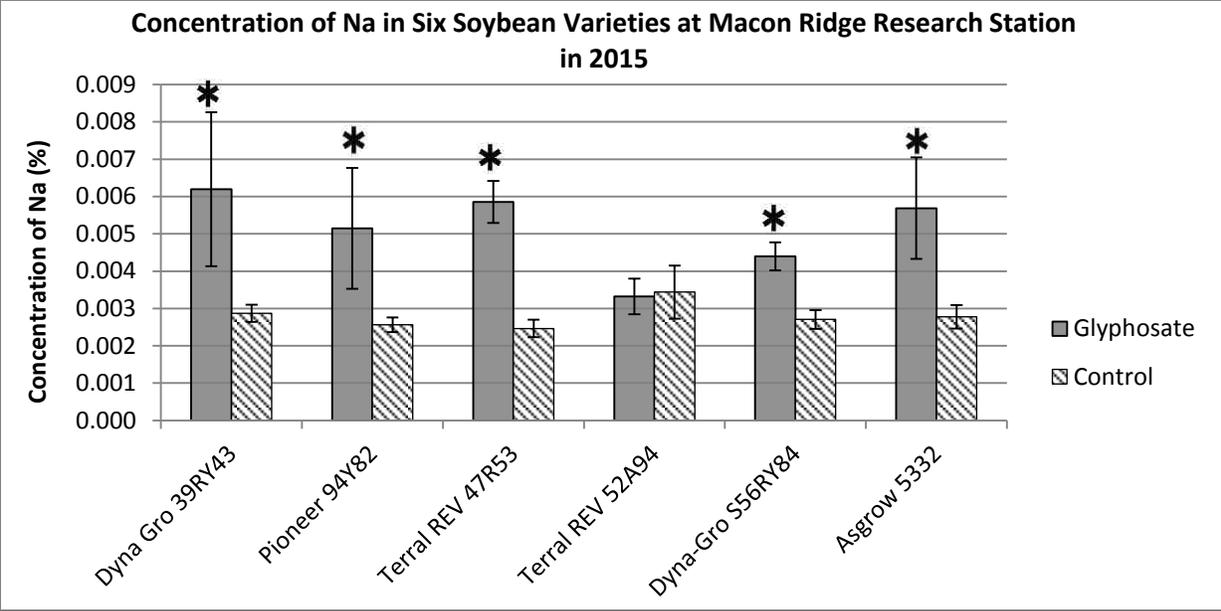


Figure 10. Concentrations of Na for six soybean varieties in glyphosate-treated and control samples collected at the Macon Ridge Research Station in 2015. Bars represent means for each variety (n=4) and error bars represent standard error. Statistically significant differences ($P \leq 0.05$) are marked with an asterisk (*).

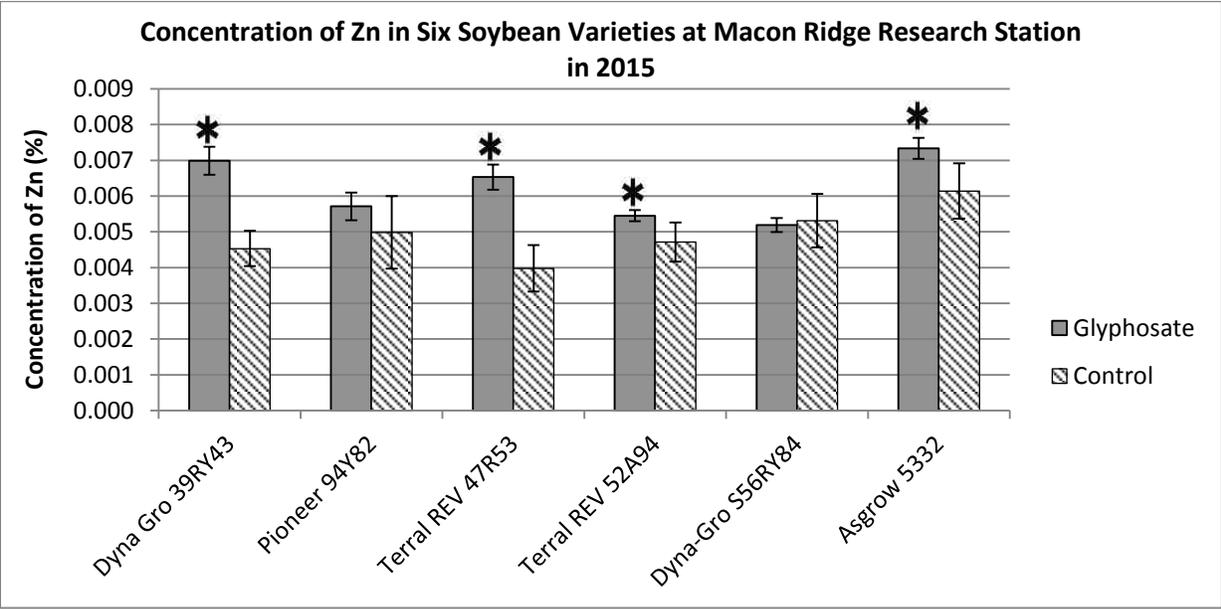


Figure 11. Concentrations of Zn for six soybean varieties in glyphosate-treated and control samples collected at the Macon Ridge Research Station in 2015. Bars represent means for each variety (n=4) and error bars represent standard error. Statistically significant differences ($P \leq 0.05$) are marked with an asterisk (*).

Concentrations of N and K were significantly lower for glyphosate-treated samples at MRRS in 2015. Nitrogen was found in significantly lower concentration in glyphosate-treated samples from three out of six varieties (TerralREV 52A94, DynaGro S56RY84, and Asgrow 5332) (Figure 12). Potassium was found in significantly lower concentration in glyphosate-treated samples from all varieties tested in 2015 at MRRS (DynaGro 39RY43, Pioneer94Y82, TerralREV 47R53, TerralREV 52A94, DynaGro S56RY84, and Asgrow 5332) (Figure 13). No significant differences were observed for all other nutrients at MRRS in 2015.

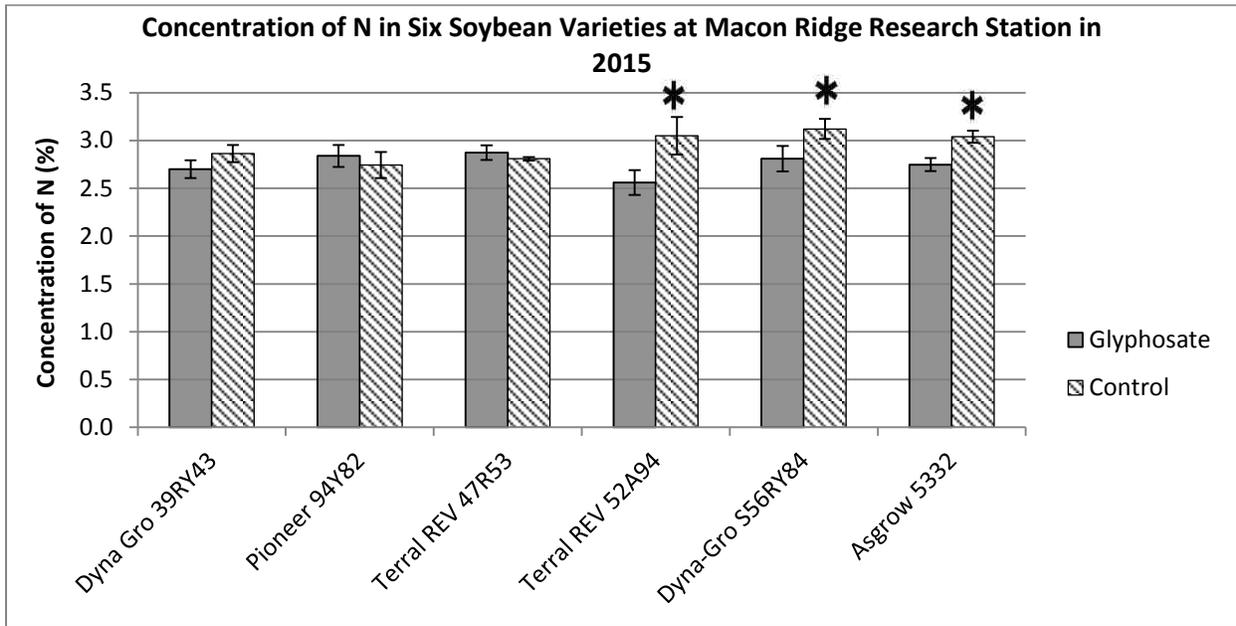


Figure 12. Concentrations of N for six soybean varieties in glyphosate-treated and control samples collected at the Macon Ridge Research Station in 2015. Bars represent means for each variety (n=4) and error bars represent standard error. Statistically significant differences ($P \leq 0.05$) are marked with an asterisk (*).

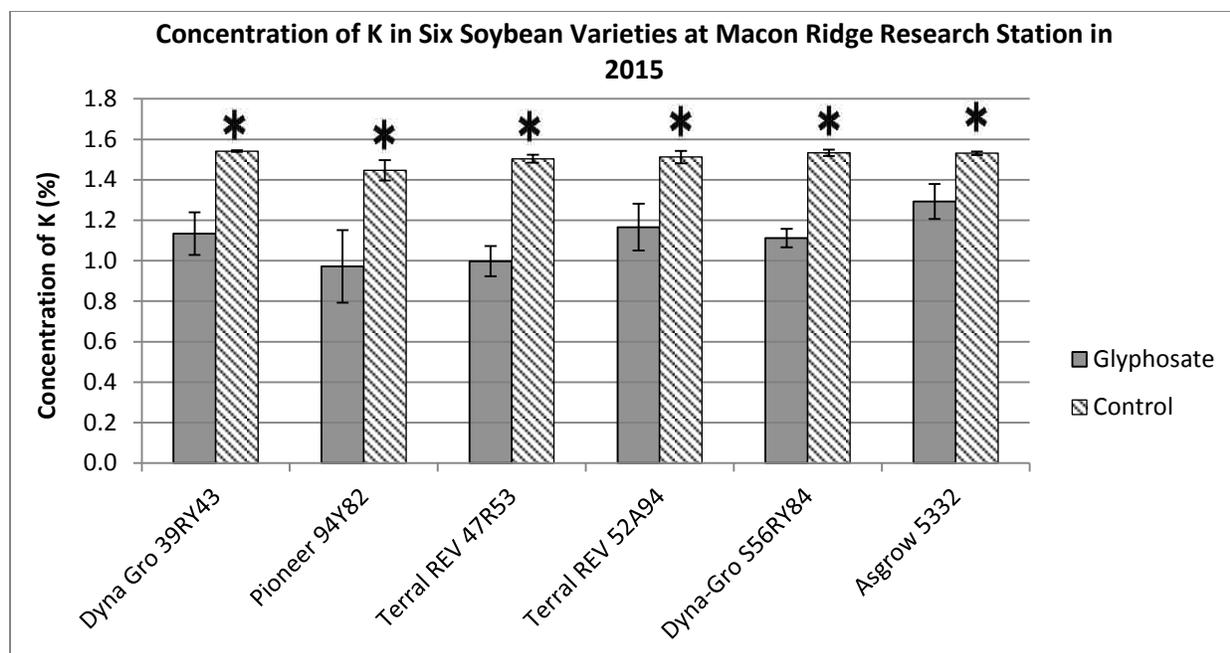


Figure 13. Concentrations of K for six soybean varieties in glyphosate-treated and control samples collected at the Macon Ridge Research Station in 2015. Bars represent means for each variety (n=4) and error bars represent standard error. Statistically significant differences ($P \leq 0.05$) are marked with an asterisk (*).

3.1.3 Results from Dean Lee Research Station from 2014 to 2016

Differences in concentrations of nutrients were detected between glyphosate treated and nontreated plants in samples collected at DLRS in 2014, 2015 and 2016, using different varieties each year (Table 2). Results from samples collected in 2014 at DLRS showed significantly higher concentration of Zn in glyphosate-treated samples for four out of six varieties (DynaGro 39RY43, Pioneer94Y82, DynaGro S56RY84, and Asgrow 5332) (Figure 14). Nitrogen and potassium were found in significantly lower concentrations in glyphosate-treated samples at this location in 2014. The concentrations of N were significantly lower in glyphosate-treated samples from all varieties tested at DLRS in 2014 (DynaGro 39RY43, Pioneer94Y82, TerralREV 47R53, TerralREV 52A94, DynaGro S56RY84, and Asgrow 5332) (Figure 15). Potassium was found in lower concentration in glyphosate-treated samples from five out of six varieties tested at DLRS in 2014 (Pioneer94Y82, TerralREV 47R53, TerralREV 52A94, DynaGro S56RY84, and Asgrow 5332) (Figure 16).

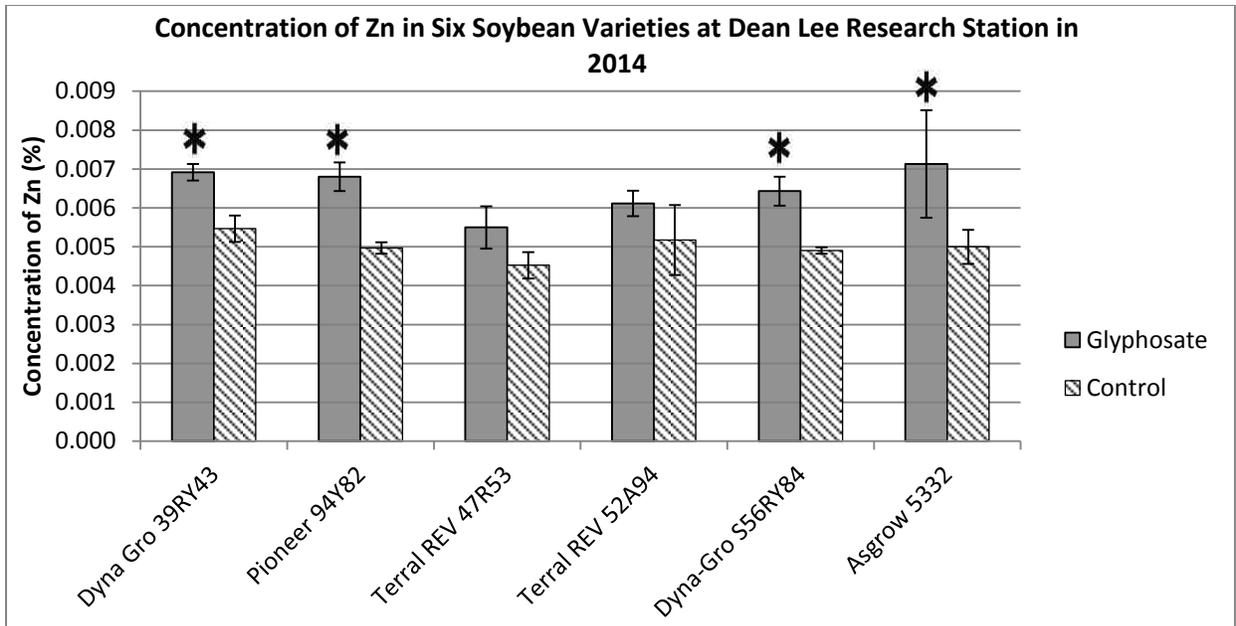


Figure 14. Concentrations of Zn for six soybean varieties in glyphosate-treated and control samples collected at the Dean Lee Research Station in 2014. Bars represent means for each variety (n=4) and error bars represent standard error. Statistically significant differences ($P \leq 0.05$) are marked with an asterisk (*).

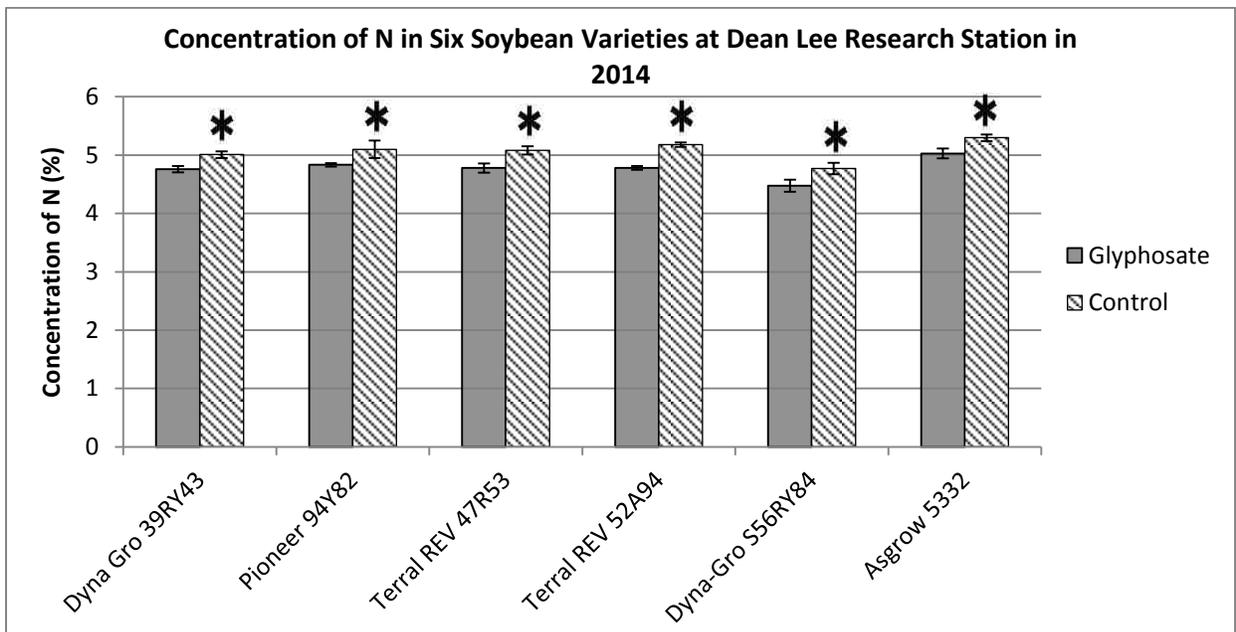


Figure 15. Concentrations of N for six soybean varieties in glyphosate-treated and control samples collected at the Dean Lee Research Station in 2014. Bars represent means for each variety (n=4) and error bars represent standard error. Statistically significant differences ($P \leq 0.05$) are marked with an asterisk (*).

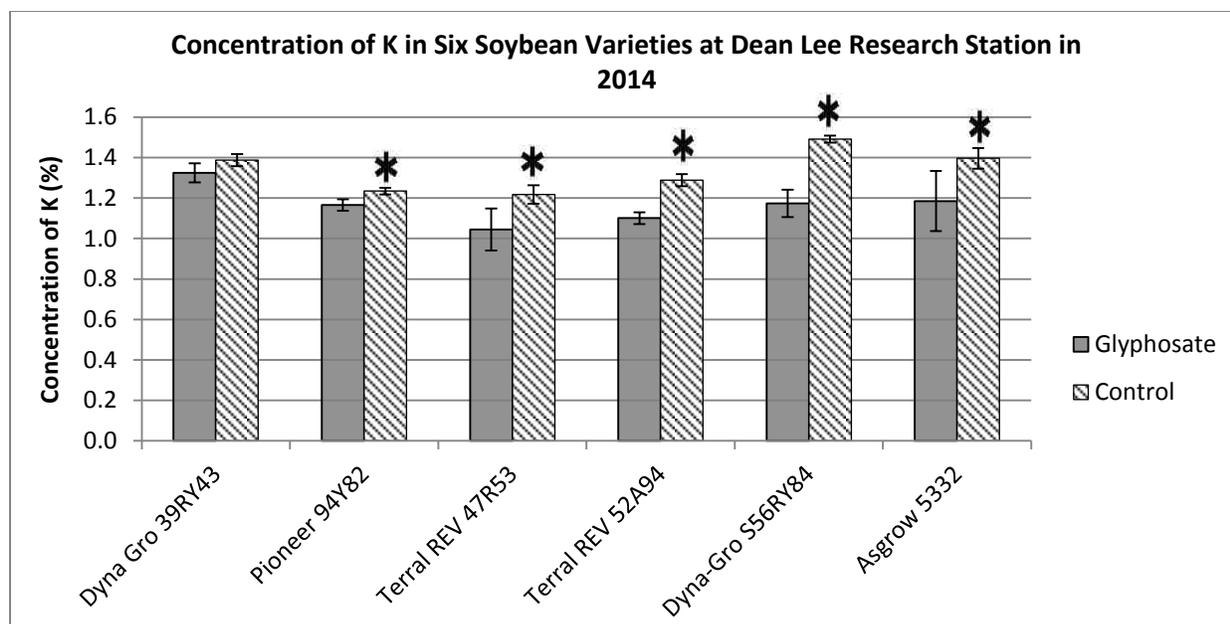


Figure 16. Concentrations of K for six soybean varieties in glyphosate-treated and control samples collected at the Dean Lee Research Station in 2014. Bars represent means for each variety (n=4) and error bars represent standard error. Statistically significant differences ($P \leq 0.05$) are marked with an asterisk (*).

In 2015, Zn was found in significantly higher concentrations in glyphosate-treated samples collected at DLRS. Zinc was found in higher concentration in four out of six varieties tested at DLRS in 2015 (TerralREV 38-R10, TerralREV 47R53, Armor 55-R22, and TerralREV 56R63) (Figure 17). Iron was detected in significantly lower concentration in glyphosate-treated samples collected at DLRS in 2015 for two out of six varieties tested (TerralREV 39A35, TerralREV 47R53) (Figure 18). No significant differences were detected between glyphosate-treated and control plants for all other nutrients analyzed in 2015 at DLRS.

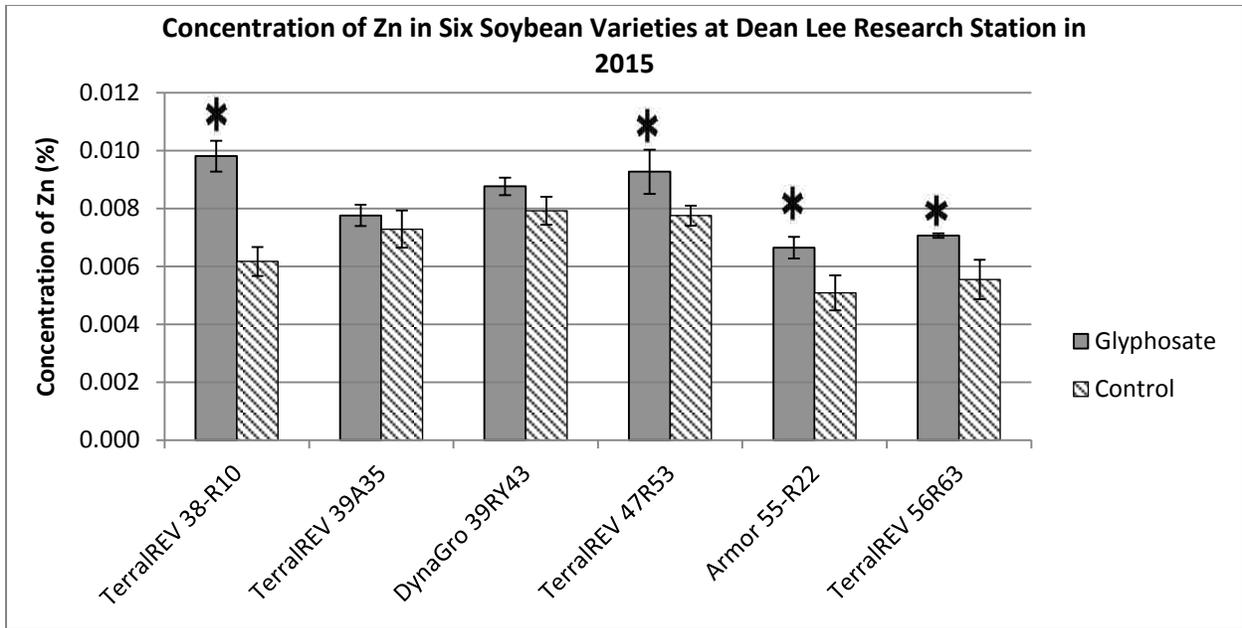


Figure 17. Concentrations of Zn for six soybean varieties in glyphosate-treated and control samples collected at the Dean Lee Research Station in 2015. Bars represent means for each variety (n=4) and error bars represent standard error. Statistically significant differences ($P \leq 0.05$) are marked with an asterisk (*).

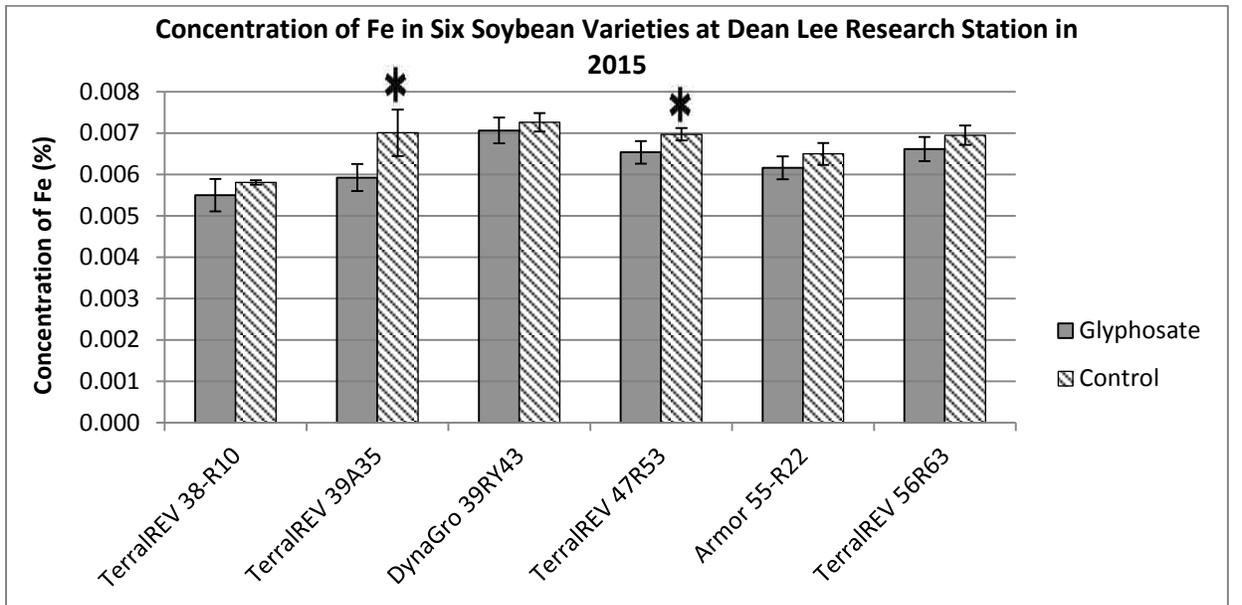


Figure 18. Concentrations of Fe for six soybean varieties in glyphosate-treated and control samples collected at the Dean Lee Research Station in 2015. Bars represent means for each variety (n=4) and error bars represent standard error. Statistically significant differences ($P \leq 0.05$) are marked with an asterisk (*).

Similar results were obtained in 2016 at DLRS. The concentration of Zn and Mn was significantly higher in glyphosate-treated plants. Zinc was detected in higher concentrations in glyphosate-treated plants in samples collected from all six varieties tested in 2016 (Asgrow 3931 TerralREV 47R53, Armor 48R70, Pioneer P54T94R, TerralREV 56R63, and DynaGro 32Y55) (Figure 19). Manganese also was detected in significantly higher concentration in glyphosate-treated plants at this research station for four out of six varieties tested (Asgrow 3931 TerralREV 47R53, Armor 48R70, and Pioneer P54T94R) (Figure 20). One variety (TerralREV 56R63) showed significantly lower concentration of Mn in glyphosate-treated plants at DLRS in 2016 (Figure 20).

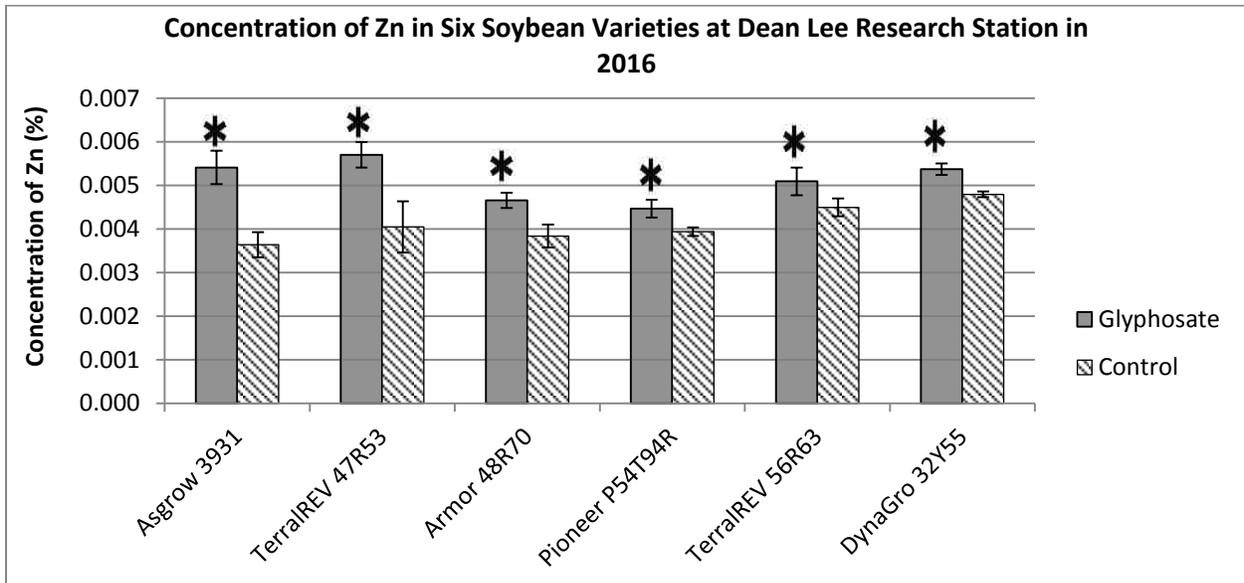


Figure 19. Concentrations of Zn for six varieties with four replications each (n=4) in samples collected at the Dean Lee Research Station in 2016. Bars represent means for each variety and error bars represent standard error. Statistically significant differences ($P \leq 0.05$) are marked with an asterisk (*).

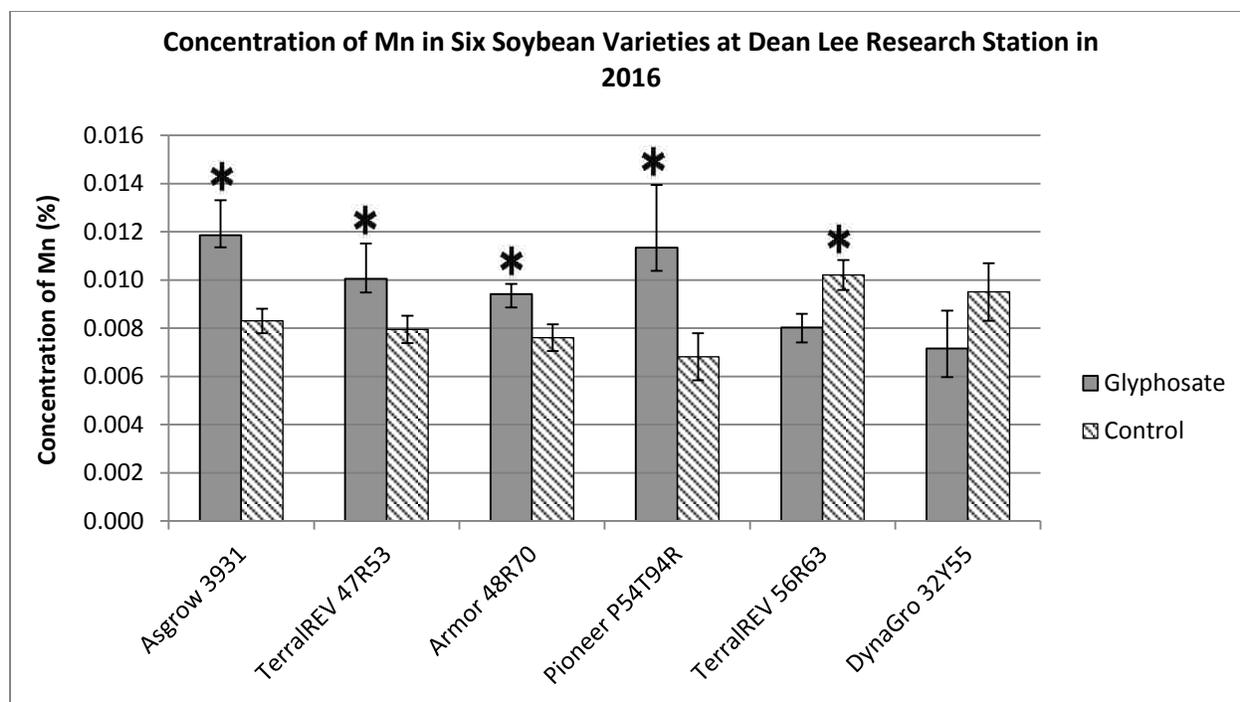


Figure 20. Concentrations of Mn for six soybean varieties in glyphosate-treated and control samples collected at the Dean Lee Research Station in 2016. Bars represent means for each variety (n=4) and error bars represent standard error. Statistically significant differences ($P \leq 0.05$) are marked with an asterisk (*).

Nitrogen and potassium were again found in lower concentration in glyphosate-treated plants at DLRS in 2016. The concentration of N was significantly lower in glyphosate-treated samples for three out of six varieties tested at this research station in 2016 (Armor 48R70, Pioneer P54T94R, and DynaGro 32Y55) (Figure 21). The concentration of K was significantly lower in glyphosate-treated plants for four out of six varieties tested at DLRS in 2016 (Asgrow 3931, Armor 48R70, Pioneer P54T94R, and DynaGro 32Y55) (Figure 22). No significant differences between glyphosate-treated and control plants were observed in 2016 among all other nutrients analyzed.

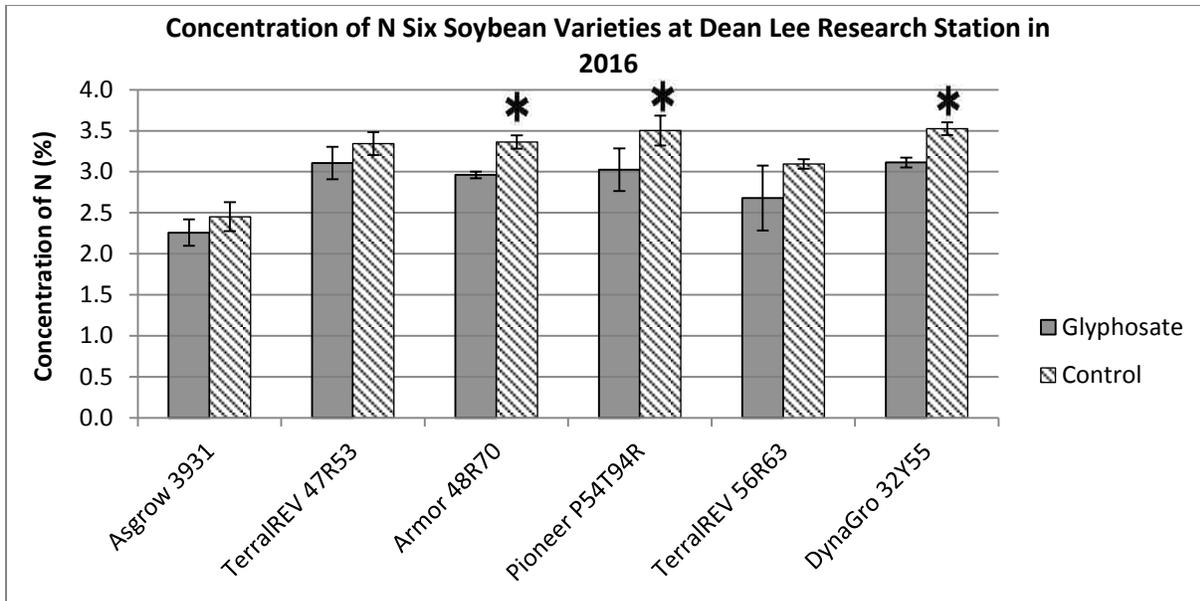


Figure 21. Concentrations of N for six soybean varieties in glyphosate-treated and control samples collected at the Dean Lee Research Station in 2016. Bars represent means for each variety (n=4) and error bars represent standard error. Statistically significant differences ($P \leq 0.05$) are marked with an asterisk (*).

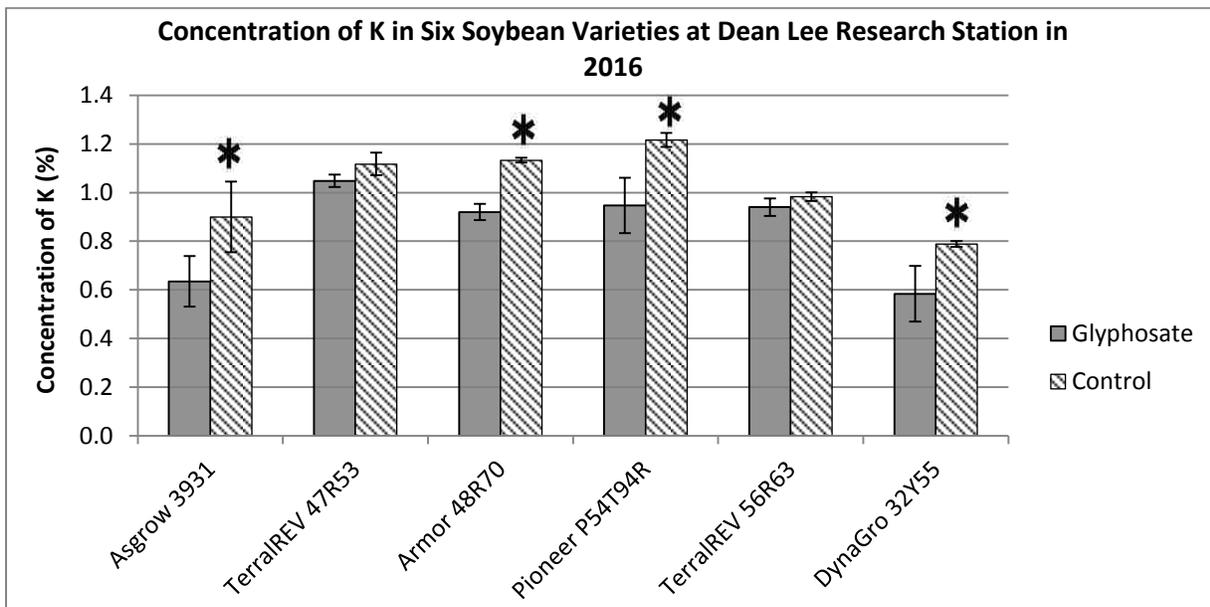


Figure 22. Concentrations of K for six soybean varieties in glyphosate-treated and control samples collected at the Dean Lee Research Station in 2016. Bars represent means for each variety (n=4) and error bars represent standard error. Statistically significant differences ($P \leq 0.05$) are marked with an asterisk (*).

3.2 Relative DNA Concentrations of *Cercospora cf. flagellaris*

Expression of *C. cf. flagellaris* CTB6 gene was estimated through real-time PCR in leaflets collected at all research stations during the three-year period that the experiment was conducted. Differences were apparent in most assays between control and glyphosate-treated samples with more *C. cf. flagellaris* DNA detected in samples from treated plants. An example of a typical amplification plot in the ABI 7000 is shown in Figure 23.

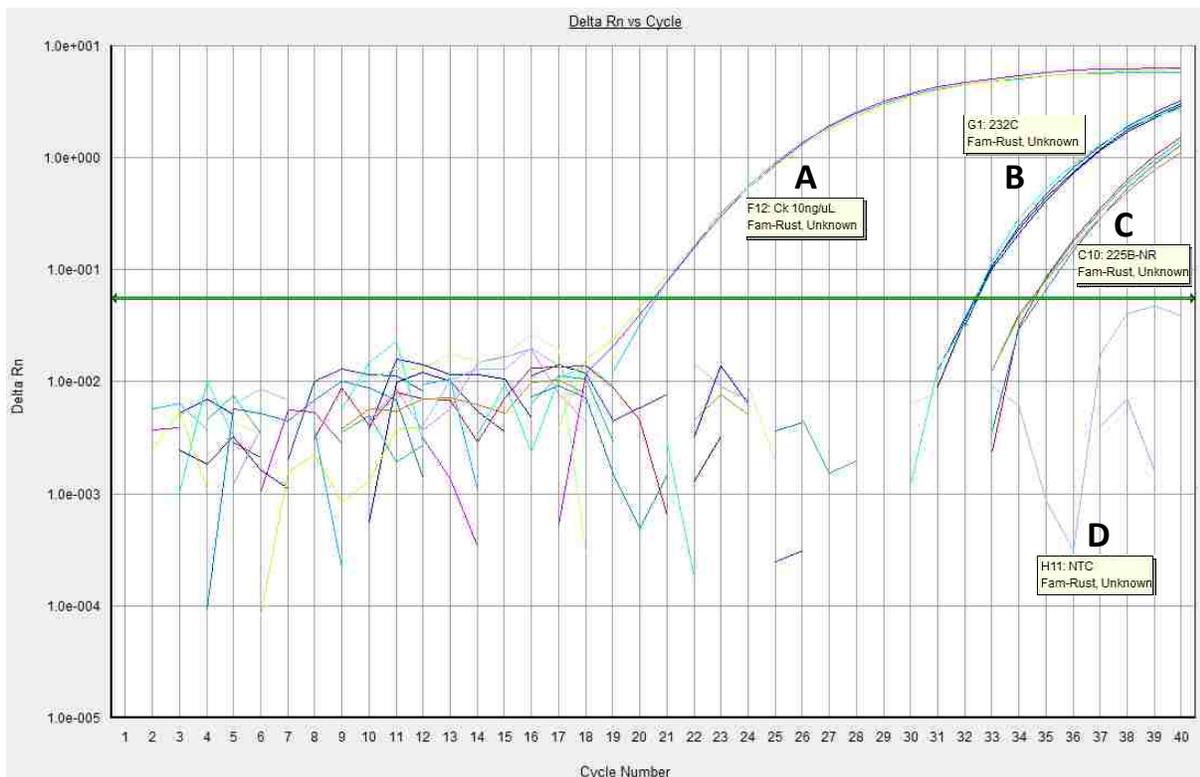


Figure 23. Typical qPCR amplification plot for glyphosate-treated vs control samples. Y-axis represents Delta Rn (fluorescence value of an experimental reaction minus the Rn value of the baseline signal generated by ABI 7000). X-axis represents the temperature cycle number (40 cycles total). A) Positive control, *Cercospora cf. flagellaris* diluted to 10 ng/ μ L. B) Glyphosate-treated sample (diluted to 10 ng/ μ L of total DNA). C) Control sample (NR=no-roundup) from the same variety as B. D) Negative control or nontreated cell (NTC), which was 1 μ L of Nucleoside Free Water (NFW). Green line represents threshold at which Delta Rn reached the linear phase of amplification for all samples.

Results from PROC GLM in SAS for all sources of variation included in the class statement for year, location, variety, and treatment showed significant differences across the main fixed effects treatment ($P=0.0198$) and variety ($P<0.0001$), as well as random effects for

location ($P < 0.0001$) and year ($P < 0.0001$). However, these differences were influenced by two-way and three-way interactions across sources of variation (Table 4). Two-way interactions ($P \leq 0.05$) between variety*treatment and year*treatment were detected. Three-way interactions ($P \leq 0.05$) between year*variety*treatment were detected (Table 4). These results indicated the data had to be kept separated in ANOVA using PROC MIXED.

Table 4. SAS output from PROC GLM for fixed and random effects, and interactions affecting concentrations of *Cercospora cf. flagellaris* DNA. Effects and interactions were considered significant when $P \leq 0.05$.

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Treatment	1	55820.0015	55820.0015	5.53	0.0198
Variety	13	775227.5529	59632.8887	5.91	<.0001
Variety*Treatment	13	619486.2154	47652.7858	4.72	<.0001
Location	2	364227.0254	182113.5127	18.05	<.0001
Location*Treatment	2	19697.9729	9848.9865	0.98	0.3788
Location*Variety	10	70147.5592	7014.7559	0.70	0.7283
Location*Variety*Treatment	10	44072.7736	4407.2774	0.44	0.9270
Year	2	610258.2472	305129.1236	30.23	<.0001
Year*Treatment	2	118628.2106	59314.1053	5.88	0.0034
Year*Variety	2	35213.6403	17606.8201	1.74	0.1777
Year*Variety*Treatment	2	468301.3911	234150.6956	23.20	<.0001
Location*Year	0	0.0000			
Location*Year*Treatment	0	0.0000			
Location*Year*Variety	0	0.0000			
Location*Year*Variety*Treatment	0	0.0000			

In 2014, DNA concentrations of *C. cf. flagellaris* were significantly higher for glyphosate-treated samples from four varieties (DynaGro 39RY43, Pioneer 94Y82, Terral REV 52A94, and Asgrow 5332) planted at DLRS (Figure 24).

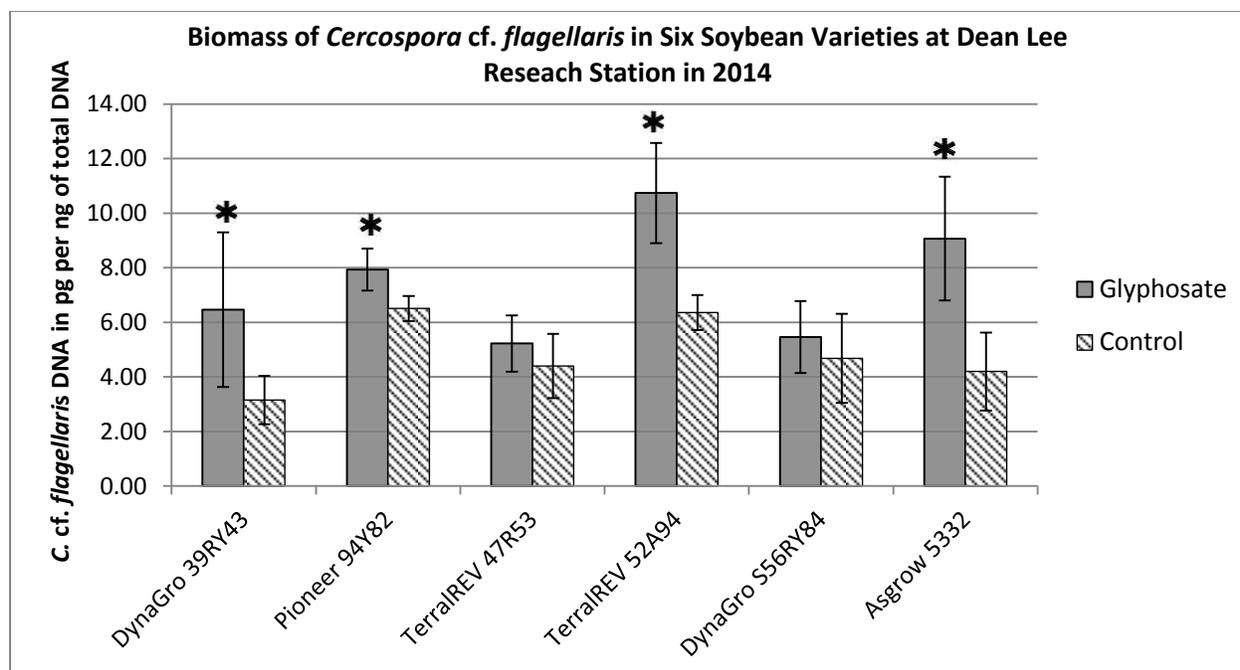


Figure 24. Biomass of *Cercospora cf. flagellaris* for glyphosate-treated and control samples from six soybean varieties collected at the Dean Lee research station in 2014. Bars represent mean DNA concentrations of *C. cf. flagellaris* in picograms per nanogram of total DNA. Error bars represent standard error calculated among four biological replications and three technical replications during qPCR procedure. Significant differences ($P \leq 0.05$) between glyphosate-treated and control are marked with an asterisk (*).

Results from qPCR analyses for samples collected at BHRS in 2014 showed significantly higher amounts of *C. cf. flagellaris* DNA for three out of six varieties (DynaGro 39RY43, Terral REV 47R53, and Asgrow 5332) treated with glyphosate, which indicated that higher amplifications of the CTB6 gene of *C. cf. flagellaris* occurred in those varieties when treated with glyphosate (Figure 25). DynaGro 39R43 and Asgrow 5332 had higher amounts of *C. cf. flagellaris* at both locations.

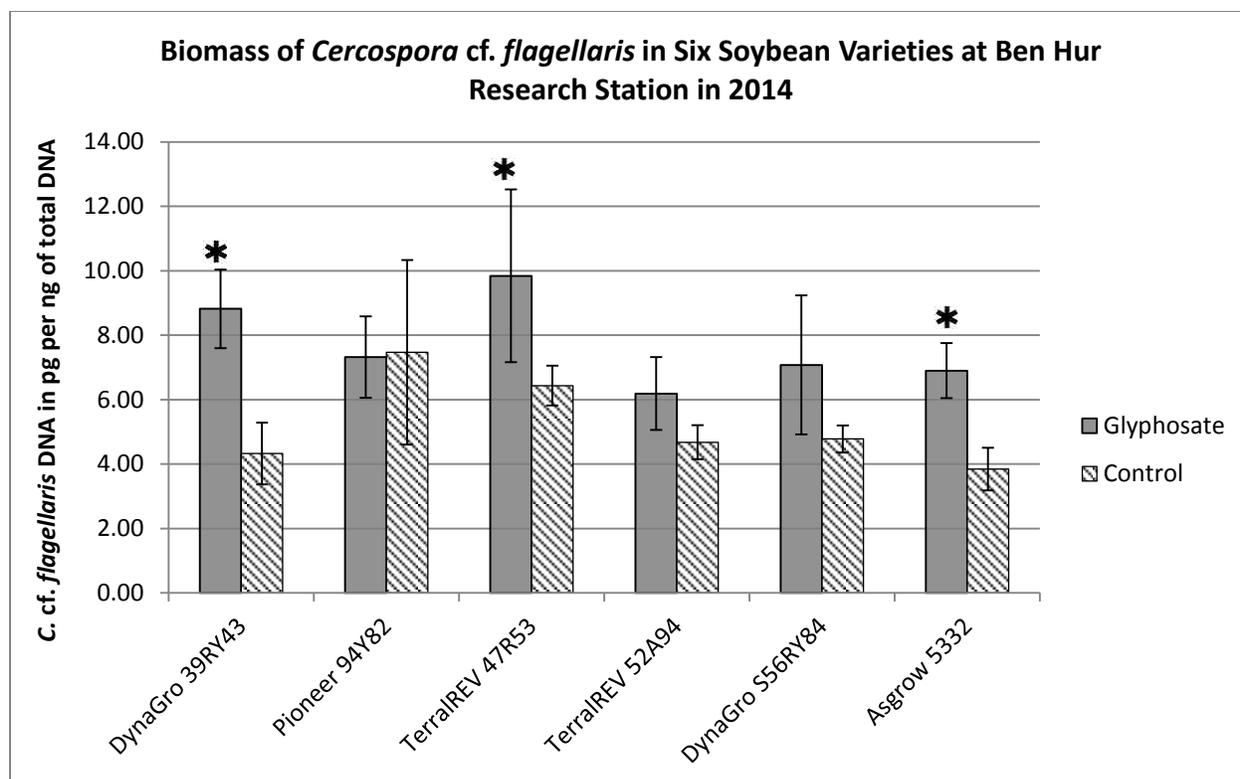


Figure 25. Biomass of *Cercospora cf. flagellaris* for glyphosate-treated and control samples from six soybean varieties collected at Ben Hur Research Station in 2014. Bars represent mean DNA concentrations of *C. cf. flagellaris* in picograms per nano grams of total DNA. Error bars represent standard error calculated among four biological replications and three technical replications during qPCR procedures. Significant differences ($P \leq 0.05$) between glyphosate-treated and control are marked with an asterisk (*).

Samples collected at DLRS in 2015 showed significantly higher amplification of *C. cf. flagellaris* in glyphosate-treated samples in four out of six varieties tested. The varieties that showed significantly higher concentrations of *C. cf. flagellaris* in glyphosate-treated samples were TerralREV 38-R10, TerralREV 39A35, DynaGro 39RY43, and TerralREV 47R53 (Figure 26). TerralREV 56R63, showed a significantly lower concentration of *C. cf. flagellaris* in glyphosate-treated samples (Figure 26). Data obtained from the ABI 7000 also suggested that this location-year (DLRS 2015) had the highest values of DNA concentrations of *C. cf. flagellaris* compared to all research stations and years, ranging from 36.02 pg of *C. cf. flagellaris* DNA per ng of total DNA (control of TerralREV 39A35) to 624.10 pg of *C. cf. flagellaris* DNA per ng of total DNA (control of TerralREV 56R63).

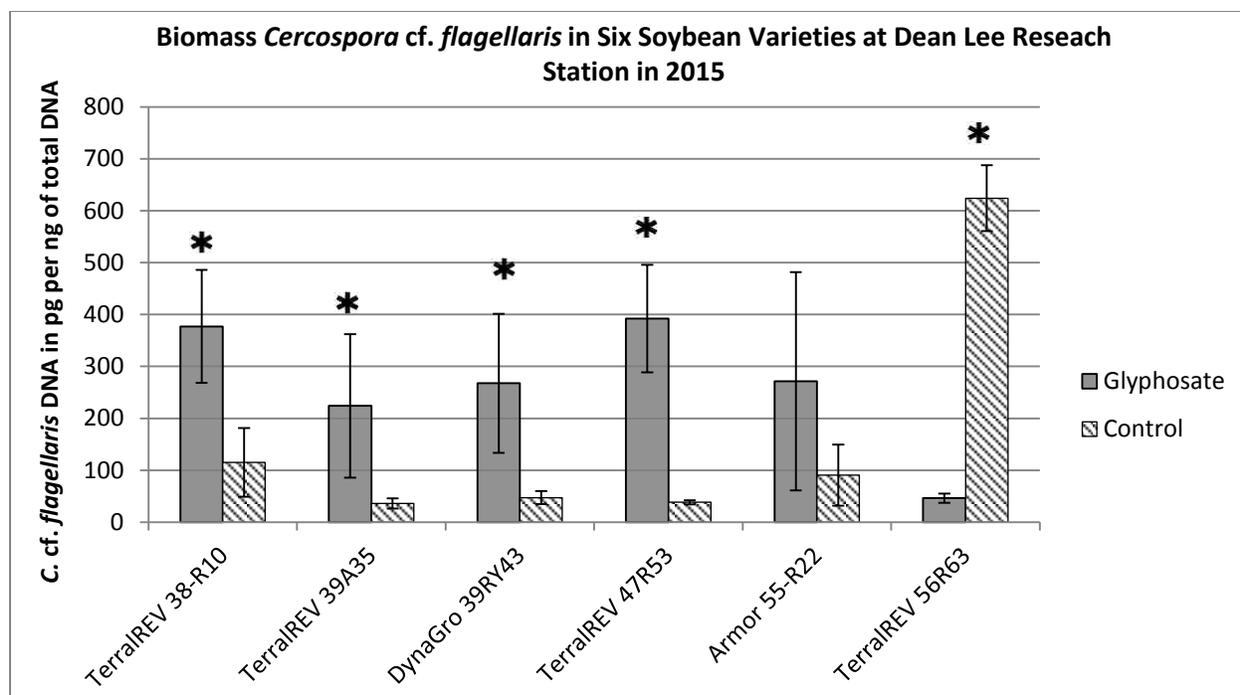


Figure 26. Biomass of *Cercospora cf. flagellaris* for glyphosate-treated and control samples from six soybean varieties collected at the Dean Lee Research Station in 2015. Bars represent mean DNA concentrations of *C. cf. flagellaris* in picograms per nanogram of total DNA. Error bars represent standard error calculated for four biological replications and three technical replications during the qPCR procedure. Significant differences ($P \leq 0.05$) between glyphosate-treated and control samples are marked with an asterisk (*).

Three out of six varieties planted at MRRS in 2015 showed significantly higher concentration of *C. cf. flagellaris* DNA in 2015: Pioneer 94Y82, TerralREV 47R53, and Asgrow 5332 (Figure 27).

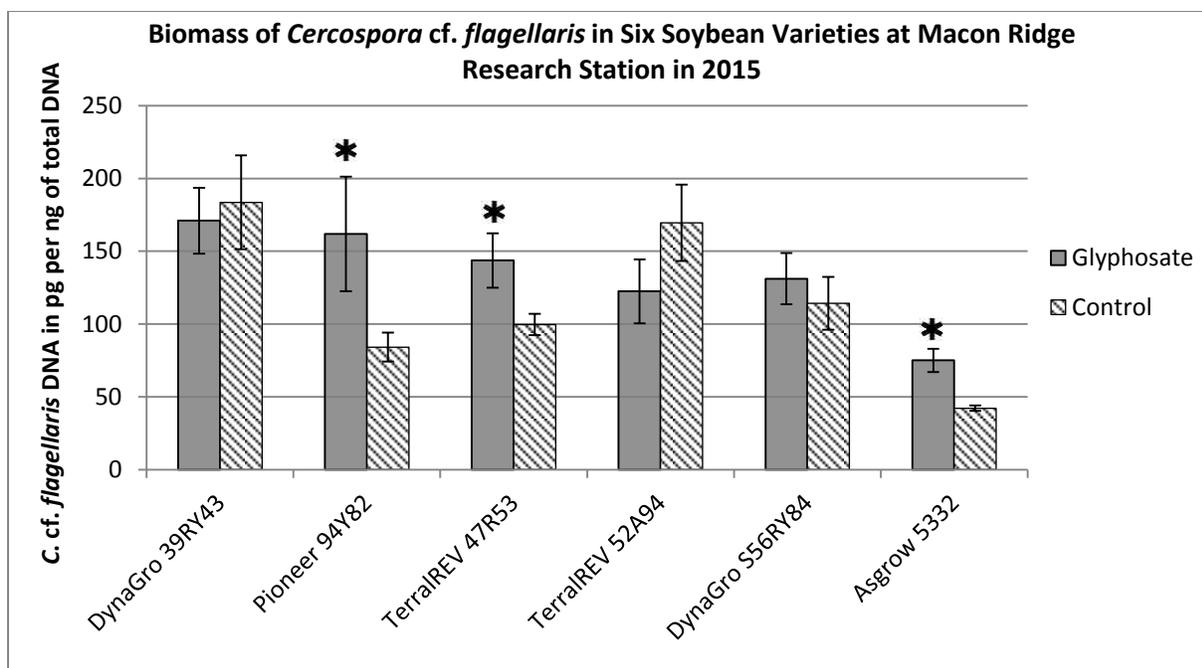


Figure 27. Biomass of *Cercospora cf. flagellaris* for glyphosate-treated and control samples from six soybean varieties collected at the Macon Ridge Research Station in 2015. Bars represent mean DNA concentrations of *C. cf. flagellaris* in picograms per nanogram of total DNA. Error bars represent standard error calculated for four biological replications and three technical replications during the qPCR procedure. Significant differences ($P \leq 0.05$) between glyphosate-treated and control samples are marked with an asterisk (*).

In 2016, five out of six varieties planted at DLRS showed significantly higher concentrations of *C. cf. flagellaris* DNA in glyphosate-treated samples. The varieties that showed significantly higher DNA of the fungus at this research station were TerralREV 38R10, Asgrow 3931, Armor 48R70, TerralREV 56R63, and DynaGro 32Y55 (Figure 28).

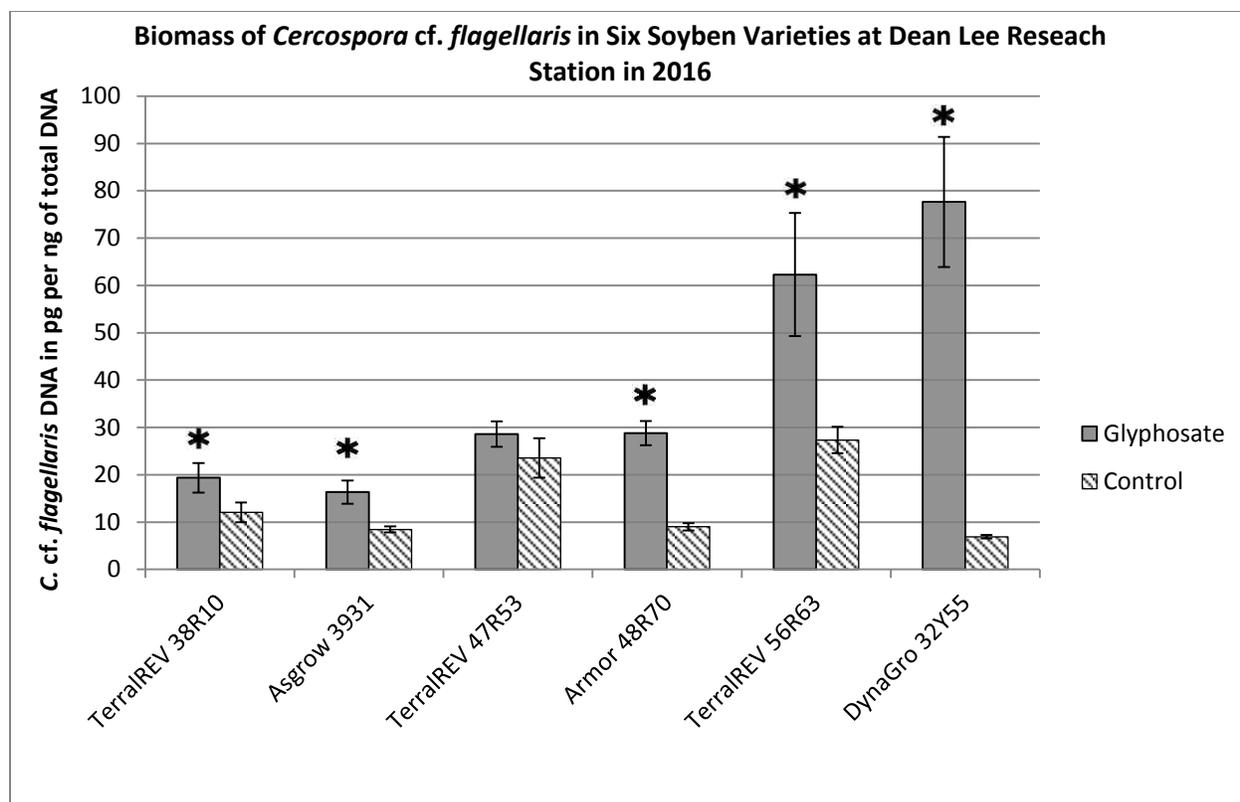


Figure 28. Biomass of *Cercospora cf. flagellaris* for glyphosate and control samples from six soybean varieties collected at the Dean Lee Research Station in 2016. Bars represent mean DNA concentrations of *C. cf. flagellaris* in picograms per nanogram of total DNA. Error bars represent standard error calculated among for four biological replications and three technical replications during the qPCR procedure. Significant differences ($P \leq 0.05$) between glyphosate-treated and control are marked with an asterisk (*).

3.3 Disease Assessment of *Cercospora* Leaf Blight Purple Symptom in 2014 and 2016

Results obtained with PROC GLM showed significant differences across the main fixed effects treatment ($P = 0.0014$) and variety ($P \leq 0.0001$). This analysis did not show significant differences across the random effect year. Location effect was not included in the model because purple symptom of CLB was observed twice at the same location (DLRS in 2014 and 2016). Based on these results, varieties were kept separated in our analyses with PROC MIXED for differences between glyphosate-treated and controls.

Purple leaf symptoms of CLB were observed at DLRS in 2014 and 2016. CLB blight symptoms were observed only in 2016 at DLRS (not shown because no significant differences

were observed between glyphosate-treated and control). No symptoms of either CLB blight or purple leaf were observed in 2015. Overall, glyphosate-treated plots showed lower severities of CLB purple symptoms in 2014. However, only three out of six varieties showed significantly lower severities of CLB purple symptom in glyphosate-treated plots ($P \leq 0.05$) (Figure 29).

The varieties that showed statistically significant differences ($P \leq 0.05$) with higher disease severity in control plots were Dyna-GroS56RY84, Terral REV52A94, and Pioneer 94Y82 (Figure 29). Asgrow 5332 did not show CLB purple leaf symptoms in either glyphosate-treated or control plots in 2014 at DLRS.

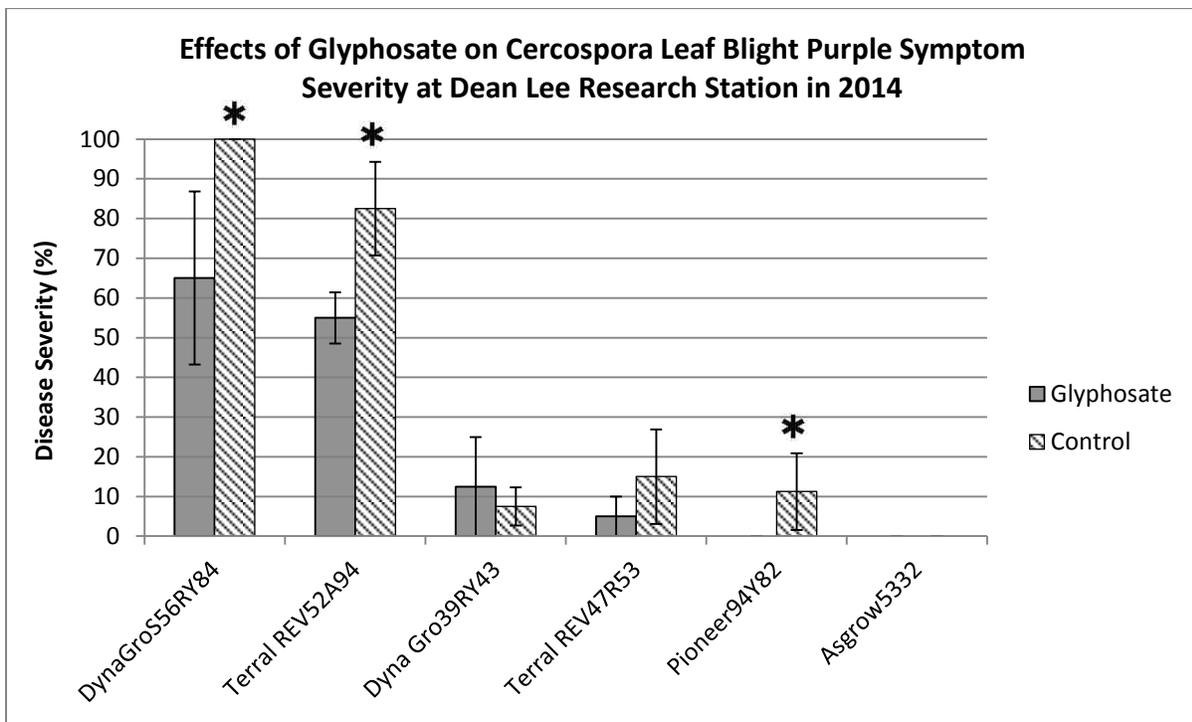


Figure 29. Disease assessment for the Cercospora leaf blight purple symptom at the Dean Lee Research Station in 2014. Bars represent means across four replications for each variety, and error bars represent standard error. Statistical differences ($P \leq 0.05$) between glyphosate-treated and control are marked with an asterisk (*).

In 2016, purple symptoms of CLB were observed at DLRS. Disease symptoms were assessed in six varieties in each half of the field (treated and non-treated). CLB purple leaf symptoms were numerically lower in glyphosate-treated plots in five out of six varieties. However, only two out of six varieties showed significantly lower purple leaf severity, Pioneer

P54T94R and TerraREV 56R63 in glyphosate-treated plots. Asgrow 3931 did not show any purple leaf symptoms at either treated or nontreated plots (Figure 30). The scale used in 2016 (Figure 2) was enhanced using ASSESS2.0 to obtain more accurate estimates of disease severity above 20%. However, only Pioneer P54T94 and DynaGro 32Y55 showed percentages higher than 20% at DLRS in 2016 (Figure 30).

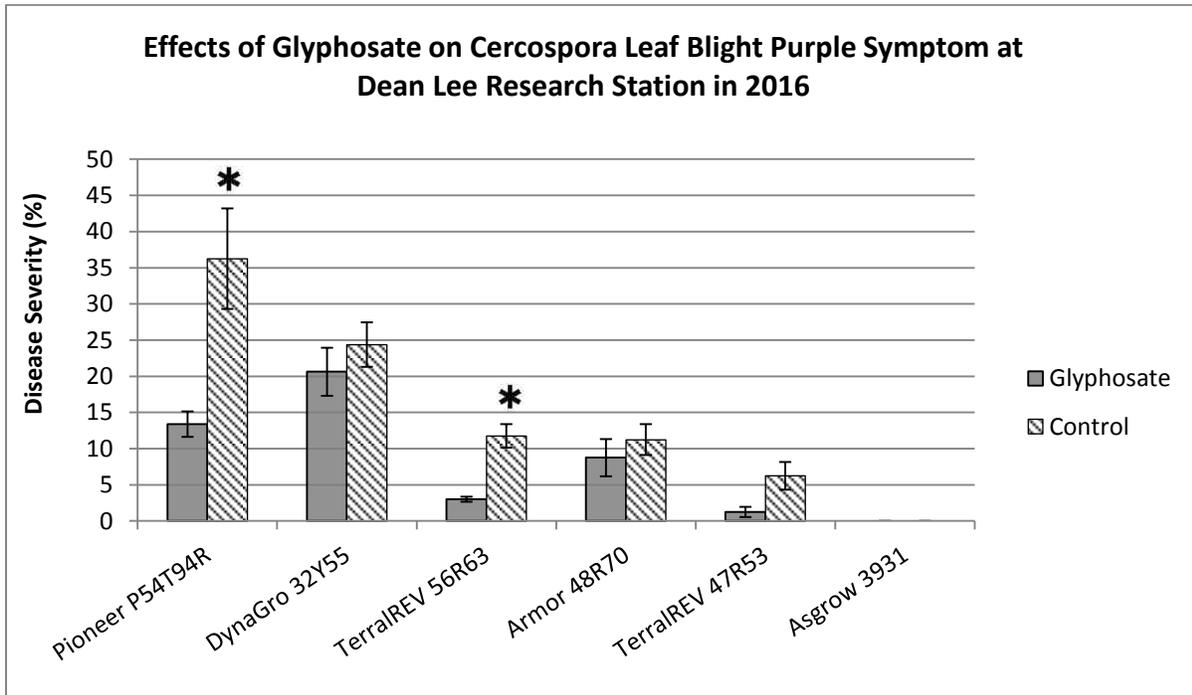


Figure 30. Disease assessment for the Cercospora leaf blight purple symptom at the Dean Lee Research Station in 2016. Bars represent mean disease severity across four replications for each variety, and error bars represent standard error. Statistical differences ($P \leq 0.05$) between glyphosate-treated and control are marked with an asterisk (*).

4. DISCUSSION

4.1 Effects of Glyphosate on Nutrient Uptake

Glyphosate can affect nutrient availability and uptake in plants and potentially affect host plant resistance and disease severity. There has been concern that disease severity could be enhanced in transgenic soybean for glyphosate resistance (Buiatti, *et al.* 2013; Cerdeira & Duke, 2006; Williams, *et al.* 2000). Therefore, a field study was conducted to evaluate the effects of glyphosate application to GR soybeans on nutrient uptake and the development of an important disease, *Cercospora* leaf blight. The results indicated that glyphosate does affect nutrient uptake, fungal development, and disease severity.

Glyphosate affected concentrations of some nutrients in GR soybeans, including key nutrients involved in plant resistance responses. However, the effects were not consistent across locations and years. Overall nutrient results from tissue analyses were not meaningful because of the high variation observed among research stations and years of the study. Concentrations of some nutrients were significantly higher for glyphosate-treated plants at some locations, whereas concentrations of the same nutrient in plants at another research station showed significantly lower concentrations. Nitrogen, for example, was found in significantly higher concentration in glyphosate-treated plants collected at BHRS in 2014 but significantly lower concentrations for glyphosate-treated at DLRS in 2014 and MRRS in 2015. Similar variations were observed in the concentrations of Al, Fe, K, Mn, and Na, which were found in significantly higher, no significant differences, or significantly lower concentrations in glyphosate-treated plants at different locations.

Variability in the uptake of most nutrients was not observed across varieties within experiments. Rather, tissue analyses detected variability across locations and years. These results suggested that glyphosate affects nutrient uptake similarly in GR soybean varieties but more so in some varieties than others. However, this process is dependent upon edaphic conditions.

The results regarding uptake of Fe and Mn, which are involved in CLB life cycle, disagreed with those of Johal & Huber (2009), who argued that glyphosate has a detrimental

effect in the uptake of these elements. Iron was found in lower concentrations in glyphosate-treated plants at DLRS in 2015, but only two out of six varieties showed significantly lower concentration. However, the concentration of Fe was higher in glyphosate-treated plants at MRRS in 2015 and four out of six varieties showed significantly higher concentration. The concentration of Mn was higher in glyphosate-treated plants from three out of six varieties at BHRS in 2014, six out of six varieties at MRRS in 2015, and four out of six varieties at DLRS in 2016. The concentration of Mn was lower in glyphosate-treated plants from one of six varieties tested at DLRS in 2016. Mn did not show significant differences between glyphosate-treated and control plants at DLRS in 2014 and 2015. The results indicate that the uptake of Fe and Mn is location-dependent as it is for the other nutrients mentioned above. However, when Mn was affected, it was found in higher concentrations in glyphosate-treated plants.

Routine soil nutrient analyses showed variability across research stations in soil concentration of some nutrients (Supplementary Table 1). However, no nutrient was found to occur at levels regarded as deficient. Although, natural variations in the soil profile could have influenced leaf concentration of nutrients. In general, soil pH did not show high variability across fields (with some exceptions).

Zinc was the only nutrient that was always found in significantly higher concentrations in glyphosate-treated plants at all research stations and years of study, when there was a significant treatment effect. The results with Zn did not support those of Moreira *et al* (2016), who found that tissue concentrations of Zn were lower in glyphosate-treated soybean leaves. This greenhouse study used different varieties, and the soil type probably differed compared to this study.

The higher uptake of Zn when glyphosate was applied has been studied in detail by Li, *et al.* (2013), and their findings help to explain the current study results. They showed that the synergistic activity of glyphosate and Zn was dependent on soil pH. The highest uptake of Zn in the presence of 1.0 mM glyphosate occurred when gamma-alumina was at pH=5.5 (lowest at pH 8.0). The average pH for each of the study soils was: BHRS in 2014, pH=5.8 (moderately acid); DLRS in 2014, pH=6.6 (slightly acid); DLRS in 2015, pH=5.9 (moderately acid); MRRS,

pH=4.8 (very strongly acid); and DLRS in 2016, pH=6.5 (slightly acid). The acidic soil conditions provide an explanation for the increased concentration of Zn in treated plants.

The variability in tissue concentrations of nutrients among research stations and years of study could have been caused by many factors, including glyphosate application timing and variable edaphic and environmental conditions. Glyphosate was applied twice during the season at most research stations (except for BHRS in 2014), one application prior to planting and one application at R1 (one extra application at BHRS in 2014 because of high weed pressure) at a rate of 0.65 L/acre. However, plants were collected at the R5-R6 stage of development of soybeans at each research station every year. Plants were evaluated at that time to investigate nutrient concentrations and biomass of *C. cf. flagellaris* at the time when CLB symptoms are usually observed in soybean foliage (R5-R6) (Hartman, *et al.* 2015). For most varieties planted in Louisiana, the time period between R1 (beginning bloom) and R6 (full seed) would be approximately 50-60 days (Mueller, *et al.* 2016; Casteel, 2016; Pedersen, 2016).

This gap between application and sampling would have resulted in glyphosate degradation in the soil. Glyphosate degradation depends on the type of soil and environmental conditions, varying from 4 to 189 days, with an average of 49 days (Grundmann, *et al.*, 2008; Laitinen, *et al.*, 2006; Capri & Vicari, 2010; Williams, Kroes, & Munro, 2000). A report by Monsanto states that the half-life of glyphosate in soil is 32 days, but this does not mean that glyphosate will be gone in 64 days, since it can be detected after 3-4 half-lives (Backgrounder, 2005). Glyphosate is readily translocated to roots after it is applied to the foliage of GR soybeans, and it may degrade in soil in a relatively short time (around 49 days) depending on environmental conditions; therefore, it should not have a large direct effect on the foliage (Franz, *et al.* 1997; Grundmann, *et al.*, 2008; Laitinen, *et al.* 2006; Capri & Vicari, 2010; Williams, *et al.* 2000).

Another source of variation could be the time of sampling in different varieties. Samples were collected at a time when most of the varieties had reached R5-R6. However, since three maturity groups were included in the study (III, IV, and V), some varieties reached R6 earlier than others (varieties of maturity group III would reach R6 earlier, even though planted at the

same time as IV and V). Including different varieties and maturity groups in the study increased chances of observing symptoms of CLB by planting several varieties with no prior knowledge of susceptibility to disease, since CLB symptoms are hard to predict from season to season and across varieties (some varieties might or might not show CLB symptoms from season to season).

4.2 Effects of Glyphosate on DNA of *Cercospora cf. flagellaris* and Disease Symptoms

DNA concentrations of *C. cf. flagellaris* were significantly higher in leaves of glyphosate-treated plants at all locations (with some minor exceptions) indicating higher biomass of the pathogen in leaves. Variability in amplification of DNA occurred among varieties as would be expected because of variability in levels of susceptibility to the fungus for each variety. However, the pattern for increased fungal biomass in glyphosate treated plants was similar across varieties with the exception of one variety in one experiment at one location.

The portion of the genome associated with cercosporin biosynthesis in *C. cf. flagellaris* was amplified (CTB6 gene), and higher concentrations of Zn in soybean leaves could enhance toxin production. Zinc is part of a major transcriptional activator identified as Zn (II) Cys6 domain in the CTB8 gene cluster that regulates the cercosporin biosynthetic pathway in *Cercospora nicotianae* (Chen, *et al.* 2007; Newman & Townsend, 2016; Campbell, *et al.* 2008). However, increased Zn levels in treated plants and increased fungal biomass were not associated with increased symptom expression. The effect of glyphosate on cercosporin production in *Cercospora*-infected GR soybean needs to be evaluated.

The highest DNA amplification values were found in samples collected at DLRS in 2015 in glyphosate-treated plots, which also showed the lowest pH and highest Zn concentrations in leaves (90-112ppm). However, symptoms of CLB were not detected at this site in 2015. The lowest values of relative DNA concentrations of *C. cf. flagellaris* were obtained from samples collected in glyphosate-treated plots at DLRS in 2014. Purple symptoms of CLB were observed in these plots, and three of six varieties treated with glyphosate showed significantly lower severities of the CLB purple symptom. At DLRS in 2016, there were higher DNA concentrations but lower disease severities for glyphosate-treated plants compared to 2014. In 2016, three of six varieties again showed lower disease severity in treated plants.

The disease assessment results demonstrated that glyphosate reduced CLB purple symptom severity in some varieties during the only two seasons that disease symptoms occurred (2014 and 2016 at DLRS). These results failed to support a link between biomass of the fungus and disease severity found by Chanda *et al* (2014). The results were similar to the reported reduced severity for soybean rust in glyphosate treated plants (Feng, *et al.* 2005).

The residual activity of glyphosate in soil and soybean foliage may not be sufficient to have a direct effect on the development of symptoms of CLB. Instead, glyphosate has been suggested to affect disease development by its effects on nutrient uptake and disease resistance. If cercosporin production depends on Zn concentration, then glyphosate could indirectly influence the development of CLB symptoms by enhancing the conditions for toxin production. However, disease severity was reduced rather than increased following glyphosate application while fungal biomass was increased in the leaves. The reasons for these contradictory results are unclear, but the study results did not produce evidence that glyphosate will increase CLB severity in GR soybean.

Cercospora leaf blight is a complex disease. *Cercospora* will infect soybean and develop endophytically (Cai & Schneider, 2008; Costa-Silva, *et al.* 2010; Douanla-Meli, *et al.* 2013; Soares, *et al.* 2015), and disease expression is erratic. The results suggest that glyphosate enhances endophytic colonization in GR soybean. However, some effects on nutrient uptake separately affect the CLB resistance response and symptom development.

4.3 Overall Conclusions

Glyphosate affected nutrient uptake in GR soybean. Differences in nutrient uptake between glyphosate treated and nontreated plants were similar across GR soybean genotypes but variable by location and year.

Glyphosate application consistently increased fungal biomass in treated GR soybean plants across varieties and locations. However, the purple symptom of CLB was unrelated to the concentration of DNA found in leaves in contrast to earlier research (Chanda, *et al.* 2014) that

associated higher concentrations of DNA of *C. cf. flagellaris* with more severe CLB blight symptoms. The application of glyphosate to GR soybean increased endophytic colonization by the fungus, but treatment either decreased or had no effect on CLB severity.

Glyphosate significantly affected concentrations of Fe and Mn, two minor elements involved in the CLB disease cycle, but not in a consistent manner. Glyphosate consistently increased Zn uptake, and the increased availability of Zn could have a direct effect on the replication of CTB8 and cercosporin production. However, disease severity was decreased in treated plants rather than increased.

The overall results suggest glyphosate has complex effects on the uptake of different nutrients involved in plant resistance, fungal colonization, and CLB development in GR soybean that are dependent on edaphic and environmental conditions.

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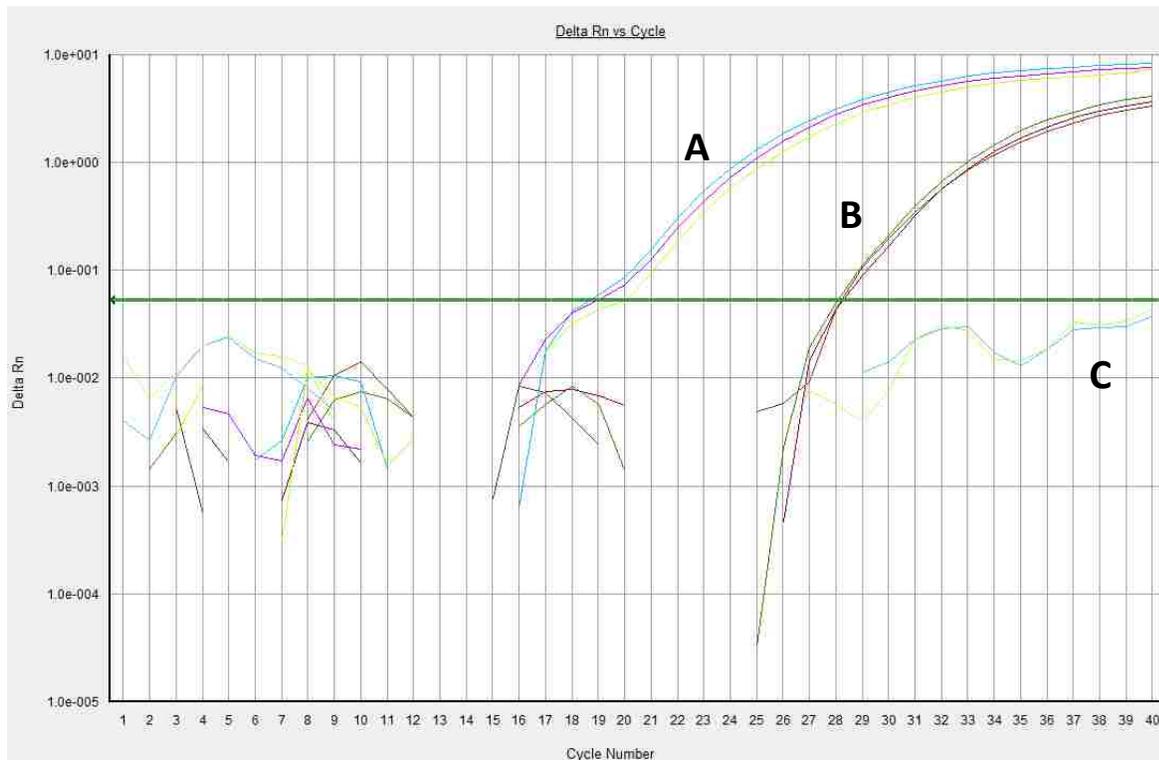
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6. APPENDIX



Supplementary Figure 1. Amplification of Albu *et al* (2016) isolate SA1019 (B) diluted at 0.1 ng/ μ L compared to Chanda *et al* (2014) isolate DLL 6013-1B (A) diluted at 10 ng/ μ L with primers and probe developed using the latter isolate. Negative control (C) was the premix used in all other wells (iTaq/Taqman DNA polymerase, CKCTB6-2R/2F primers and CKCTB6-PRB, and nucleoside free water) and 1.0 μ L nucleoside free water instead of template DNA.

Supplementary Table 1. Results from routine soil analyses at each location and year of the study. Values represent average nutrient concentrations in soil at the time of sampling (midseason). Data provided by Dr. Brenda Tubana.

Nutrient	Ben Hur Research Station			Dean Lee Research Station			Macon Ridge Research Station		
	2014	2015	2016	2014	2015	2016	2014	2015	2016
Al	0.06443	-	-	0.06752	0.06738	0.06338	-	0.06098	-
B	0.00016	-	-	0.00047	0.00047	0.00005	-	0.00009	-
Ca	0.19490	-	-	0.19482	0.19409	0.42967	-	0.11244	-
Cu	0.00019	-	-	0.00019	0.00019	0.00030	-	0.00014	-
Fe	0.04905	-	-	0.05087	0.05059	0.04195	-	0.04770	-
Mg	0.03357	-	-	0.03377	0.03362	0.03129	-	0.01106	-
Mn	0.00647	-	-	0.00654	0.00650	0.00663	-	0.04204	-
N	0.14821	-	-	0.15063	0.15055	0.11946	-	0.13906	-
P	0.00713	-	-	0.00712	0.00705	0.00574	-	0.00194	-
K	0.01465	-	-	0.01552	0.01552	0.02096	-	0.00774	-
Na	0.00638	-	-	0.00550	0.00542	0.00480	-	0.00201	-
Zn	0.00018	-	-	0.00017	0.00017	0.00021	-	0.00016	-

VITA

Teddy Garcia Aroca was born in El Paraiso, Honduras. He went to the local high school named “Instituto Tecnico Alejandro Flores” from 2003 to 2007. He started his bachelor’s degree in Agronomy at “Universidad Nacional de Agricultura (UNA)” in Catacamas, Honduras in January, 2008. He interrupted his bachelor’s degree at UNA to participate in a European program for exchange of international students named “Erasmus Mundus” in which he traveled to Europe and spent one year at the “University of the Basque Country/Universidad del Pais Vasco” in Vitoria-Gasteiz, Spain; and earned 60 ETCs in Food Science. Teddy also did his undergraduate research project at University of Georgia in 2012. After that he went back to Honduras and graduated of his B.S. in Agronomy. In 2013, he came to LSU and joined Dr. Raymond Schneider’s lab as an intern. During this time he learned many laboratory and field techniques working with Dr. Ashok Chanda in the development of a real-time PCR protocol for *Cercospora kikuchii*. He became a graduate student under Dr. Raymond Schneider in 2014 and started working in a research project to determine the effects of glyphosate on soybean nutrition and foliar diseases. He anticipates graduating with his master’s degree in December 2016. He plans to continue with his Ph.D. at LSU in 2017.