# Response of diazotrophic microbial community to nitrogen input and glyphosate application in soils cropped to soybean

by

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**Abstract**

Diazotrophic bacteria play a critical role in biological N fixation by converting atmospheric nitrogen to ammonium that can be used by plants. About 80% of biological N fixation can be attributed to diazotrophs in symbiosis with legumes. Soybean (*Glycine max* L. Merr.), a leguminous food crop, is the second largest crop (by value) in the U.S., with 93% of the planted soybean being genetically modified to tolerate the herbicide glyphosate. Inclusion of leguminous crops in crop production systems reduces the need for nitrogenous fertilizer and thus reduces cost, possible N pollution and fossil fuel consumption. Many factors affect symbiotic N fixation. This work examined the effects of two important factors—N input and glyphosate application—on the soil diazotrophic community and N fixation associated with soybean.

The first study assessed the response of the diazotrophic community to N input in a century-old crop rotation experiment, the Cullars Rotation, located in Auburn, Alabama. The field experiment consists of a three-year rotation of cotton, corn, wheat, and soybean with differing agronomic inputs. Soil samples were collected in June and October 2008 and February 2009 for six experimental treatments at two depths (0-5 cm and 5-15 cm). The abundance and diversity of the *nifH* gene encoding dinitrogenase reductase in diazotrophic bacteria were determined using the quantitative polymerase chain reaction (qPCR) and PCR amplification coupled with denaturing gel gradient electrophoresis (PCR-DGGE), respectively. The *nifH* gene copy numbers ranged from $6.1 \times 10^5$ to $2.3 \times 10^7$ copies/g soil. The lowest gene copy number was found in the treatment without lime application. In general, the *nifH* gene abundance was

highest in February and lowest in October. More *nifH* genes were found in surface soil than subsurface soil. DGGE banding patterns showed that diazotrophic community structure tended to vary with soil pH, soil organic carbon content, seasonal change, and inorganic N fertilization.

The second study focused on the effect of glyphosate on diazotrophs associated with soybean since there is concern that the application of glyphosate may negatively affect N fixation in glyphosate-resistant (GR) soybean. Greenhouse and field experiments were conducted using isogenic conventional and GR soybean cultivars. Glyphosate at 1.5 times the field use rate was applied on the GR cultivar: none, once, or twice during the study period; the conventional cultivar did not receive any herbicide and served as a control. The plants were harvested two days after each glyphosate treatment. The data from the greenhouse experiment show that glyphosate treated GR soybean had lower chlorophyll content, root mass, nodule mass, total plant nitrogen, and nitrogenase activity compared with the conventional cultivar, especially for the second harvest (V5 to V6 stage). However, these results were not consistently supported under field conditions because of high environmental variability in the field. *In vitro* growth experiments using rhizobial isolates also showed that glyphosate inhibited pure cultures of rhizobia to different extents. Most of the parameters measured in the greenhouse and field experiments showed no significant differences between the conventional cultivar and the GR cultivar without glyphosate application. The *nifH* gene abundance in the soybean rhizosphere soil was not affected by either glyphosate application or soybean cultivar. In addition, although the glyphosate resistant gene, *CP4 EPSPS* gene, was detected by qPCR in the rhizosphere soil, its abundance did not vary with different glyphosate applications.

Taken together, the diversity of the diazotrophic community was influenced by soil pH, soil organic carbon content, soil depth, nitrogen input, and seasonal change; liming appeared to

be the most important factor affecting the *nifH* gene abundance. Multiple glyphosate applications at high rates may reduce symbiotic N fixation in GR soybean, especially when soybean is under certain environmental stress.

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## List of Abbreviations

| | |
|---|---|
| AMAP | Aminomethylphosphonic acid |
| ARA | Acetylene Reduction Assay |
| ARISA | Automated Ribosomal Intergenic Spacer Analysis |
| DGGE | Denaturant Gradient Gel Electrophoresis |
| GC/FID | Gas Chromatography/ Flame Ionization Detector |
| GR soybean | Glyphosate Resistant Soybean |
| LOD | Limit of Detection |
| LLOQ | Lower limit of Quantification |
| MPN | Most Probable Number |
| PCR | Polymerase Chain Reaction |
| PLFA | Phospholipid Fatty Acid |
| qPCR | Quantitative Polymerase Chain Reaction |
| RFLP | Restriction Fragment Length Polymorphism |
| T-RFLP | Terminal-Restriction Fragment Length Polymorphism |

# Chapter I. Introduction and Literature Review

## Introduction

Biological nitrogen fixation by diazotrophic bacteria is one of the most important processes in natural and agricultural systems. Diazotrophs have been found in diverse habitats such as lakes, oceans, estuaries, marshes, microbial mats, sediment, soil, plants, and invertebrates (Zehr et al., 2003). Almost 80% of biological nitrogen fixation can be attributed to diazotrophs in symbiosis with legume plants. Many studies on biological nitrogen fixation in different ecosystems begin by focusing on the diazotrophic community. The diazotrophic community is generally studied using the *nifH* gene that encodes the iron protein subunit of the nitrogenase complex. The *nifH* gene can provide tangible information on nitrogen fixation potential, including both diversity and the abundance of nitrogen fixing bacteria. Many environmental factors affect the diversity and abundance of the diazotrophic community in the soil, such as the climate, agricultural management, and the characteristics of the soil.

Soybean (*Glycine max* L. Merr.), a leguminous food crop, is the second largest crop in the U.S. agricultural production, 93% of which is genetically modified to tolerate the herbicide, glyphosate. Glyphosate, the active ingredient in Roundup®, is currently one of the most widely used herbicides in agriculture (Duke et al., 2003). From 1995 to 2002, glyphosate use in soybean and cotton crops increased from 2.5 to 30 million kg/yr and from 700,000 to 3,870,000 kg/yr in the United States alone, respectively (USDA, 2004). However, glyphosate can be easily translocated from shoots to roots of glyphosate resistant crops and released into the rhizosphere

where nitrogen-fixing bacteria might be affected or inhibited. Glyphosate inhibits 5-enolpyruvyl shikimate-3-phosphate synthase (EPSPS), which is involved in the shikimate pathway of plants and some microorganisms. Glyphosate-resistant crops confer resistance to glyphosate via the insertion of a transgene (*CP4 EPSPS*) from *Agrobacterium* sp. strain CP4, which encodes a glyphosate-insensitive version of EPSPS (Duke et al., 2003). The adoption of glyphosate-resistant crops has brought about noticeable improvements in production efficiency, and hence economic benefits, as glyphosate usage and glyphosate-resistant crop acreage increase. Meanwhile, other aspects of this technology have also drawn attention as a result of its potential adverse impact on the environmental ecosystem.

Culture-independent methods could provide a more complete picture on which to base estimates of microbial community diversity and structure compared with traditional culture-dependent methods. Denaturing gradient gel electrophoresis (DGGE) has become a commonly common technique in molecular biology to study the characterization of population structure and dynamics in the field of environmental microbiology. It is particularly effective for evaluating microbial diversity, community composition and shifts. In addition, real time PCR, a very efficient molecular biological technique, is being widely used to understand and assess the absolute and relative abundance of genes, the dynamics of functional genes, and community composition in natural ecosystems. Acetylene reduction assays are another good option for measuring nitrogenase activity.

## Research Objectives

The overall objective of this work was to determine the response of the diazotrophic community to N input and glyphosate treatment. The specific objectives were to:

1 ) Examine the effect of nitrogen input on the abundance and diversity of the diazotrophic community in a long-term crop rotation system;

2) Investigate the impact of glyphosate on the characteristics of glyphosate-resistant soybean by:

   a) Assessing the impact of glyphosate application on symbiotic nitrogen fixation and the fate of glyphosate resistant genes associated with GR soybean; and

   b) Determining the effect of glyphosate on the growth of diazotrophic bacteria.

## Literature Review

### A. Biological nitrogen fixation

#### a) Overview

Biological nitrogen fixation involves the process of enzymatic reduction of nitrogen to ammonia. It is unique to prokaryotes, including bacteria and archaea. Eukaryotic organisms are only able to obtain fixed nitrogen through their symbiotic interactions with nitrogen fixing prokaryotes. Biological nitrogen fixation is a key point for understanding the nitrogen cycle, assessing ecosystem functions, and predicting the responses of future ecosystems to global environmental change (Cleveland et al., 1999). Estimates of the total annual terrestrial inputs of nitrogen through biological nitrogen fixation range from 139 million to 175 million tonnes, with symbiotic associations growing in arable land accounting for 25 to 30% (Burns and Hardy, 1975; Paul, 1988). Among bacteria, anaerobes, aerobes, cyanobacteria and bacteria possess the nitrogen fixing ability (Young, 1992). There are two kinds of biological nitrogen fixers, namely free-living (non-symbiotic) bacteria such as *Cyanobacteria, Azotobacter*, and *Clostridium*, and symbiotic bacteria such as *Rhizobium, Bradyrizhobium*, and *Spirillum.*

Symbiotic nitrogen fixation associated with soybean can provide from 65 to over 160 kg fixed nitrogen/ha (Klubek et al., 1988), accounting for almost 40-70% of the plants nitrogen requirement. Symbiotic nitrogen fixation is very important for the yield of crops when soil nitrogen is low. The major symbiotic biological nitrogen fixation is achieved by root-nodule symbiosis, which receives carbon from the plant and in return provides fixed nitrogen. Legumes play an important role in the ecosystem because they convert nitrogen from atmospheric nitrogen to fixed forms. Symbiotic nitrogen fixation by legumes and root-nodule bacteria contributes at least 70 million metric tons per year into terrestrial ecosystems (Brockwell et al., 1995). Free-living nitrogen fixation can also produce critical nitrogen inputs to terrestrial ecosystems in the absence of symbiotic nitrogen fixing plants (Reed et al., 2011). Free living diazotrophs might fix 0-60 kg of nitrogen $ha^{-1}year^{-1}$ and have become an important source of nitrogen when lower proportions of nitrogen are provided in an available form (Cleveland et al., 1999; Orr et al., 2011).

**b) Diazotrophic communities in the soil**

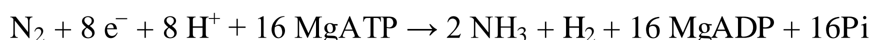
Soil diazotrophs are the main source of the nitrogen input in primary production ecosystems (Poly et al., 2001a). Biological nitrogen fixation during nitrogen cycling in natural ecosystems is carried out by diazotrophic bacteria. Most heterotrophic diazotrophs in soil are prokaryotes (Zehr et al., 2003) including *α-, β-, γ-, δ- Proteobacteria, Firmicutes and Archaea* (Wakelin et al., 2010). About 80% of the biological nitrogen fixation is provided by diazotrophs in symbiosis with legumes. Diazotrophs are widely distributed and have high diversity in bacterial or archael taxa. Only a small proportion of species are able to fix nitrogen and these have been identified as diazotrophs, which include about 87 species in 2 genera of archaea, 38 genera of bacteria, and 20 genera of cyanobacteria (Dixon and Wheeler, 1986; Sprent and

Sprent, 1990). All diazotrophs contain nitrogenase, and in order to achieve biological nitrogen fixation they must utilize different strategies to cope with varying concentrations of oxygen, which is lethal to the nitrogenase (Izquierdo and Nusslein, 2006).

### c) Mechanism of biological nitrogen fixation

#### i) Nitrogenase complex

Most biological nitrogen fixation carried out by the diazotrophic community is catalyzed by the nitrogenase complex, which is primarily composed of the Fe protein (dinitrogenase reductase) and the MoFe protein (dinitrogenase). Some of the diazotrophic microorganisms carry alternative vanadium and/or iron-only nitrogenase. The homodimeric Fe protein with twofold symmetry (MW ≈ 60,000) is encoded by the *nifH* gene. The *nifD* and *nifK* genes specify the α and β subunits of heterotetrameric MoFe protein (MW ≈ 220,000), including iron-molybdenum cofactor (FeMo-co) and P-cluster (Fischer, 1994; Dixon and Kahn, 2004). The nitrogenase complex functions as follows: In each nitrogen fixation cycle, the Fe protein hydrolyzes ATP and uses the required energy to provide electrons to the MoFe protein, which subsequently binds a dinitrogen molecule and reduces it to ammonium. The general nitrogen reduction reaction catalyzed by nitrogenase under optimal conditions is typically presented as follows:



$$N_2 + 8\,e^- + 8\,H^+ + 16\,MgATP \rightarrow 2\,NH_3 + H_2 + 16\,MgADP + 16Pi$$

The nitrogenase enzymes are irreversibly inactivated by oxygen, and the process of nitrogen fixation uses a large amount of energy (Postgate, 1982). Nitrogenase activity is usually measured by an acetylene reduction assay and $^{15}N$ isotopic method (Hardy et al., 1968).

#### ii) Characteristics of the *nifH* gene

The diazotrophic communities are generally studied using molecular-based tools, as in the PCR-based detection of the *nifH* gene. The amino acid sequence of the Fe protein is very

similar and is conserved among nitrogen fixing organisms (Ben-Porath and Zehr, 1994). The sequence divergences obtained from the *nifH* gene, which is the oldest existing functional gene in the history of gene evolution, has been reported to be consistent with the phylogeny inferred from *16S rRNA* gene sequences (Hennecke et al., 1985; Ben-Porath and Zehr, 1994; Rosado et al., 1998). The conserved characteristics could be partly explained by horizontal gene transfer, the horizontal *nifH* gene transfer came from the pioneering phylogeny studies has been reported (Normand et al., 1992). The diazotrophic community is often studied by using the *nifH* gene that encodes the iron protein subunit of nitrogenase. Both Poly et al. (2001b) and Zehr et al. (2003) demonstrated that the *nifH* gene can be used as a genetic marker to study the diversity, activity, and abundance of a diazotrophic community. Today, the *nifH* gene is the most thoroughly studied among all genes in the *nif* operon, with an extensive collection of sequences obtained from both cultured and uncultivated organisms from many different environments (Izquierdo and Nusslein, 2006).

**d) Factors that affect biological nitrogen fixation**

Microbial community composition can shift due to soil disturbance, soil amendment, tillage, irrigation, fertilization, and the composition of the plant community (Buckley and Schmidt, 2001). Specifically, symbiotic nitrogen fixing bacteria are highly sensitive to perturbation. Environmental factors such as soil texture (clay or sandy) and aggregate size, salinity, oxygen, temperature, soil moisture, soil pH, and carbon or nitrogen availability must all be considered (Roper and Smith, 1991; Hungria and Vargas, 2000; Poly et al., 2001a; Hsu and Buckley, 2009). For instance, high salt concentrations may have a detrimental effect on nitrogen fixation because they retard the initiation or growth of nodules (Berstein and Ogata, 1966). Nitrogen fixation is also affected by low soil moisture due to the resulting reduction in the

nitrogenase activity of soybean nodules (Albrecht et al., 1984). High root temperature has been shown to strongly affect bacterial infection and nitrogen fixation in several legume species (Michiels et al., 1994). Since biological nitrogen fixation is favored by neutral or slightly acid soil conditions, soil acidity is a severe problem for nitrogen fixation because the legume nodules fail to form and some rhizobia become inactive. In addition, soil salinity and acidity usually lead to problems with nutrient deficiency or mineral toxicity during nitrogen fixation (Zahran, 1999).

### e) The influence of environmental factors on the diazotrophic community

#### i) The influence of environmental factors on community diversity

Diversity encompasses richness and evenness in terms of community structure. Richness represents the number of unique operational taxonomic units (OTUs) in a given community, while evenness describes the distribution of individuals within a given community. Determining the diversity of a diazotrophic community can help us understand the effect of environmental factors on nitrogen fixing bacteria.

The diversity of nitrogen-fixing bacteria in aquatic environments has been well studied in recent years. However, in terrestrial environments many uncertain factors remain to be addressed because of the many complicating factors. In general, the community structure of soil microorganisms depends on a series of environmental factors (moisture, plant growth, soil texture, temperature) as well as human activities (agricultural practices). Soil carbon is closely related to the diazotrophic ecology. The input of carbon in soil is one reason for changes in the diversity and abundance of nitrogen-fixing bacteria (Wakelin et al., 2010). In addition, physicochemical properties, rather than the geographic distance between soils, affect *nifH* diversity. Poly et al. (2001a) also showed that the structure of the *nifH* gene is not associated with geographical location, although the relationship between the *nifH* structure and

physicochemical properties could not be explained. The long-term management of balanced fertilizer inputs has no obvious effect on the bacterial communities based on an analysis of the *nifH* gene (Ogilvie et al., 2008), while other studies have supported fertilization as a major factor influencing the *nifH* diversity (Tan et al., 2003).

Microbial diversity can be measured using techniques such as traditional plate counting and direct counting (Bakken, 1997; Johnsen et al., 2001), molecular-based methods (Izquierdo and Nusslein, 2006; Coelho et al., 2009), and fatty acid analysis (Frostegård and Bååth, 1996; Bååth and Anderson, 2003). However, the culture dependent method is not a good choice for an analysis of diversity, because only 1-10 % of the overall bacteria in soil can be recovered by plate counting.

**ii) The influence of environmental factors**

The diazotrophic communities associated with biological nitrogen fixation have been studied using quantitative PCR to estimate the response of nitrogen fixing microorganisms to land use and management, nitrogen fertilizer, cultivars, soil pH, carbon/nitrogen source, soil organic matter, soil horizon, and seasonal change (Colloff et al., 2008; Coelho et al., 2009; Hayden et al., 2010; Levy-Booth and Winder, 2010; Jung et al., 2012).

Diazotrophs are sensitive to acidity in the soil, so soil pH can be used as a predictor of the diversity and composition of microorganisms in soil (Fierer and Jackson, 2006; Lauber et al., 2009). Hayden et al. (2010) indicated that less *nifH* gene was observed in the soil at lower pH, with the greatest abundance of *nifH* being found in neutral to slightly alkaline soil. Similar results were also reported by Deluca et al. (1996). Suitable lime application has been shown to increase the abundance of diazotrophic communities (Wakelin et al., 2009), which again suggests that a reduction in diazotrophic populations was caused by the low soil pH level.

Nitrogen fertilizer as an effective agricultural input is considered to have an impact on the diazotrophs in the soil. Rather unexpectedly, nitrogen fertilizer has no consistent influence on the abundance of diazotrophs in soil (Coelho et al., 2009; Orr et al., 2011).

Many studies have indicated that the abundance of diazotrophic communities is influenced by season. An early study revealed a seasonal fluctuation of microbial populations in the soil (Higashida and Takao, 1985), while later work found that the concentration of cultured nitrogen fixing bacteria showed significant seasonal fluctuations, with the highest numbers in winter and the lowest in summer (Mergel et al., 2001). Orr et al. (2012) also indicated that the number of nitrogen fixing bacteria could change from season to season. Many researchers agree that there is an increase in the nitrogen fixing bacteria population when temperatures are low, such as in the winter (Hiltbold et al., 1985). However, this may not be the whole story; Jung et al. (2012) observed a reduction of *nifH* gene abundance in the winter. It is possible the abundance of the diazotrophic community is influenced by a number of complex integrated factors, including season and other environmental conditions. In short, estimates of the diazotrophic community must take a wide range of environmental factors into consideration.

## B. Glyphosate (Roundup®)

### a) The history of glyphosate

Glyphosate is a non-selective, systemic herbicide that is used to control a broad spectrum of weeds. Glyphosate molecule was first synthesized by Henri Martin working at a small Swiss pharmaceutical company (Cilag) in 1950. However, it was not tested and utilized as a herbicide until John E. Franz, an organic chemist at Monsanto, discovered its herbicidal effect in May 1970 (Franz et al., 1997). Glyphosate was tested in the greenhouse in July of that year and then brought to the market as Roundup ® herbicide by the Monsanto Company (St. Louis, MO) in

1971 (Baird et al., 1971). The acid form of glyphosate is not typically an effective herbicide. Commercial formulations of glyphosate in the market are available as the isopropylamine salt, the trimethylsulfonium salt, the diammonium salt, etc. Glyphosate was originally produced exclusively by Monsanto and was formulated as the isopropylamine salt, entering the marketplace in 1974 as a post-emergence, nonselective herbicide (Duke, 1996). Many other brands came out after the patent expired. Later, improved products formulated with surfactants such as Roundup Ultra® entered the marketplace. As glyphosate application increased, concerns regarding the potential effect of glyphosate on soil characteristics and the structural composition and abundance of microorganisms began to be raised.

**b) The mode of action and mechanism of glyphosate used for weed control**

Glyphosate (*N-phosphonomethylglycine*) is a white, odorless, crystalline amino acid with an empirical formula of $C_3H_8NO_5P$ and a molecular weight of 169.1. Its solubility in water at 25℃ is 1.157 wt %. Commercial herbicide products are generally synthesized as more soluble monoanionic salts. The unique biological properties of glyphosate on plants include: (1) broad-spectrum, postemergence control on most of annual and perennial plants and (2) rapid translocation from foliage to the roots, shoots, rhizome, and apical tissues of treated plants. Glyphosate inhibits the enzyme 5-enolpyruvylshikimic acide-3-phosphate synthase (EPSPS) of the shikimate pathway in plants, which is necessary for the synthesis of aromatic amino acids (phenylalanine, tyrosine, and tryptophan) (Franz et al., 1997). The EPSPS of all higher plants appears to be inhibited by glyphosate. To date, glyphosate is the only known EPSPS inhibitor; no analogs or alternative chemical classes that target EPSPS have yet been discovered (Duke and Powles, 2008). Glyphosate can control a wide range of weeds, including grasses, sedges, and broadleaf weeds. It can lead to several metabolic disturbances, including interrupting protein

production, secondary product biosynthesis, and a general metabolic disruption of the phenylpropanoid pathway after reducing the biosynthesis aromatic amino acids (Franz et al., 1997; Duke et al., 2003). The target EPSPS is found only in plants and certain microorganisms. Animals do not possess the EPSPS enzyme.

**c) Uptake and translocation of glyphosate**

Under most circumstances, glyphosate enters plants through their foliage and is transported by diffusion across the cuticle. Many factors can affect the efficiency of this process, including temperature, chemical formulation, concentration, the thickness of the cuticle and epicuticular wax. Glyphosate penetrates the cuticle through the hydrophilic pathway and rapidly enters into symplasts under favorable conditions. Several processes might affect glyphosate uptake, including reduced stomatal aperture, reduced transpiration, increased water potential, and reduced carbon dioxide fixation although respiration and membrane integrity are unaffected (Grossbard and Atkinson, 1985).

Glyphosate can be translocated throughout the plant via the phloem and then transported to the meristems of shoots, roots, soybean grain and nodules, and other metabolic sinks after absorption by the foliage. About 80% of glyphosate absorbed after foliar applications can be translocated into the shoot, apex, and root (Feng et al., 2003). Glyphosate is not readily degraded in soybean and will concentrate in the young roots and developing or mature nodules (Kearney and Kaufman, 1975). Preferential transport may lead to downward movement of highly-absorbed glyphosate in the soil. Many factors will influence this preferential transport, including soil structure such as biopores and burrows, rainfall intensity, and time of application.

Leaching studies from a tiled field have indicated that glyphosate can be transported to deeper soil layers in combination with high rainfall intensity shortly after glyphosate application,

although strong adsorption and rapid degradation are possible (Vereecken, 2005). Glyphosate can enter surface waters through three major routes, including direct application to aquatic vegetation, binding to soil, and drifting from treated area to areas close to water (Monsanto, 2003). However, runoff from fields treated with recommended rates of glyphosate should not create a serious environmental hazard (Edwards et al., 1980). There are two main factors that determine whether glyphosate leaches through soil to groundwater or moves into surface water via runoff: the rate of degradation in the soil and the tendency of glyphosate to bind to soil. The rate of glyphosate degradation in water is generally slow because fewer microorganisms exist in water than in most soil.

**d) Fate of glyphosate in soil**

Glyphosate that enters the soil can be rapidly and tightly bound by adsorption to soil constituents such as clay minerals, organic materials, and humic substances. It can be transported and finally degraded by different mechanisms. Many factors can influence glyphosate bioavailability in soil, including the mechanism of adsorption, the duration of glyphosate in contact with the soil, physicochemical properties of the sorbate and the sorbent, and the characteristics of microorganisms (Haney et al., 2000).

**i) Adsorption and desorption of glyphosate in soil**

Adsorption of glyphosate is usually an equilibrium process and so is reversible. Glyphosate is bound to soil by its phosphonate moiety. The mobility and leachability of glyphosate in the soil is determined by its adsorption characteristics. Strong adsorption will make adsorbed glyphosate immobile, but weak adsorption can promote its leachability. Glyphosate is generally considered to be almost immobile on the basis of its adsorption properties (Sprankle et al., 1975). The adsorption of glyphosate in soil is affected by pH, phosphate level, and soil type

(Gimsing et al., 2004). Generally, the phosphonic moiety is considered to control the adsorption by hydrogen bonding through complexation. The mobility of glyphosate can increase as the phosphate levels increase. Since there is strong competition between phosphate and glyphosate for adsorption sites, with phosphate being preferred, the bioavailability of glyphosate in the soil may be affected (Vereecken, 2005). The adsorption of glyphosate to the soil occurs rapidly within the first hour and increases slowly thereafter (Sprankle et al., 1975). Hance (2006) indicated that glyphosate adsorption is correlated to the unoccupied phosphate sorption capacity rather than the total phosphate adsorption capacity of the soil.

Glyphosate is adsorbed onto soils by binding with variable-charge surface sites, including aluminum and iron oxides and aluminum silicates. Furthermore, it is strongly bonded to specific sorption sites by phosphonates, which group together to form complexes, as with phosphate adsorption. The bioavailability of glyphosate in soil therefore depends on soil characteristics such as its mineral contents and types, pH, phosphate content, temperature, and also possibly the amount of soil organic matter. Shushkova et al. (2010) indicated that the efficiency of biodegradation and bioavailability might also depend on favorable conditions for the reproduction of introduced microorganisms, plowing, humidification, and the introduction of organic additives.

**ii) Degradation of glyphosate in soil**

The degradation of glyphosate can be achieved by chemical, photochemical, and biological processes. Photochemical decomposition due to ultraviolet light and sunlight have been reported to stimulate glyphosate degradation, while chemical degradation includes both hydrolytic and oxidative processes. However, in most cases photodecomposition and chemical degradation contribute little to the decomposition of glyphosate in soil. The predominant

degradation mechanism for glyphosate in the soil is via various microorganisms, including bacteria, actinomycetes, fungi, and unidentified microorganisms. Of these, bacteria appear to play the most active role in glyphosate degradation, and not all microorganisms in the soil are capable of degrading glyphosate. Glyphosate can be readily metabolized by some soil microorganisms, which convert it into nutrients that plants can later utilize (Franz et al., 1997). The half-life of glyphosate in soil ranges from 3 to 130 days (Schuette, 1998). The rate of glyphosate degradation can vary based on soil type, microbial activity, and other soil-related factors.

Glyphosate can be rapidly and completely degraded by soil microorganisms to produce water, carbon dioxide and phosphate. Some bacteria can utilize glyphosate as a phosphorus source. There are two principal pathways of glyphosate degradation by microbial mechanisms. Aminomethylphosphonic acid (AMPA) is one of common intermediates during the glyphosate degradation. The first biological degradation pathway, the AMPA pathway, cleaves glyphosate molecule yielding AMPA and glyoxylate (Rueppel et al., 1977). The second is the C-P lyase pathway, with cleavage of the carbon–phosphorus bond yielding phosphate and sarcosine (Pollegioni et al., 2011). *Pseudomonas* is capable of degrading glyphosate via the sarcosine pathway. AMAP can generally be detected more frequently than sarcosine in the soil. The rate of glyphosate degradation depends on soil characteristics and the microbial activity in the soil. Soil pH, aeration and organic matter content may exert an effect on the rate of degradation of glyphosate. Shushkova et al. (2012) indicated that the degradation of glyphosate was most effective at pH 6.0-7.0 and aeration at 10-60 % of air saturation with suitable sources of carbon and nitrogen.

The degradation of glyphosate can take place under both aerobic and anaerobic conditions and is a cometabolic process. The initial mineralization of glyphosate in soil is rapid, followed by a gradual decrease in the rate caused by the rate-limiting desorption of glyphosate from soil particle surfaces. The degradation of glyphosate in soil is inversely correlated with the adsorption of glyphosate in soil, because strong adsorption could lead to lower bioavailability (Franz et al., 1997). Additional phosphate may have a positive effect, no effect or even a negative effect on the degradation of glyphosate. Stalker et al. (1985) indicated that the addition of phosphate can stimulate glyphosate degradation in some soils. The addition of phosphate can promote desorption of glyphosate, increase its bioavailability, and finally accelerate the degradation, although glyphosate already absorbed on specific sites will be little affected. Furthermore, the degradation rate of glyphosate will significantly drop as a result of the addition of iron or aluminum oxides, which can bind with glyphosate.

The influence of temperature on glyphosate degradation has also attracted attention. The degradation rate of glyphosate increases with temperature, and the long term effect of changes in the moisture level in the soil due to factors such as global warming will also have an impact (Borgaard and Gimsing, 2008). The half-life of glyphosate in the soil can therefore range from several days to months, depending on conditions (Monsanto, 2005). Giesy et al. (2000) indicated the average half-life of glyphosate to be 32 days based on 47 agricultural and forestry study.

**e) Toxicity of glyphosate**

In general, glyphosate is considered to be an environmentally benign herbicide. It has a very low level of toxicity in mammals, birds, and fish and glyphosate and its decomposition products are not harmful to either soil or water microorganisms (Franz et al., 1997). Druart et al. (2011) indicated there was a low effect due to glyphosate at relevant concentrations in soil, but

the detection of residues such as AMPA suggests there may be a potential risk. Glyphosate has a relatively short environmental half-life due to effective microbial degradation in the soil (Feng et al., 1990) and will not result in atmospheric contamination because it is not volatile. There is very little glyphosate movement in either soil or groundwater owing to its tight adsorption by soil. However, glyphosate can move down into groundwater due to the presence of preferential flow. At commercial use rates, glyphosate should have little or no effect on non-target organisms (Duke and Powles, 2008). Many studies have indicated that the risk of glyphosate leaching to groundwater is minor, but it cannot be excluded completely. Both the United States and Europe have observed a low occurrence of glyphosate in groundwater. Therefore, it is unlikely that glyphosate will affect water quality and aquatic organisms at the recommended concentration rates.

In addition to the effect of glyphosate on water and groundwater quality, one of the most important effects of glyphosate is that the soil microbial activity could be affected. The presence of glyphosate in soil may lead to changes in the microbial population and activity in soil. Glyphosate can either stimulate or inhibit soil microorganisms based on the soil characteristics and glyphosate application rates (Carlisle and Trevors, 1986). The presence of glyphosate in soil has been linked to temporary increases in the number of bacteria and overall microbial activity in soil (Wardle and Parkinson, 1990a; 1990b). Haney et al. (2000) found glyphosate significantly stimulated soil microbial activity but did not affect soil microbial biomass over short time periods. Glyphosate in the soil could be directly and rapidly degraded by soil microorganisms without adversely affecting the microbial activity over longer times, even at high concentrations of glyphosate.

**f) The advantages and disadvantages of glyphosate**

There are several reasons for the success of glyphosate application since its commercial introduction in 1974. Firstly, it is not only a highly effective broad spectrum herbicide, but also has an excellent toxicological and environmental profile. Secondly, glyphosate is taken up rapidly through plant surfaces and translocates well to growing points. Thirdly, its limited degradation and slow mode of action are also important factors. Glyphosate is the only herbicide that targets 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) that has no competing herbicide analogs (Duke and Powles, 2008). Weed management by glyphosate offers significant environmental and other benefits over the technologies that it replaces. Furthermore, glyphosate has a relatively short soil half-life, and does not move easily to contaminate ground water (Duke et al., 2003). The advantage enjoyed by glyphosate in the market place is also related to its economic benefits, production efficiency, and facilitation of conservation tillage (Dill, 2005).

However, the advantages of glyphosate and glyphosate-resistant crop application are accompanied by the development of herbicide resistant weeds. The tillage and herbicide regimes are the two most important selective pressures on the weed community in an agroecosystem. Increased selection pressures such as frequent herbicide application in herbicide-resistant crop systems can lead to weed population composition shifts, so that the natural herbicide resistance of the particular weed species to the herbicide or the evolution of herbicide resistance within the weed population will occur (Heard et al., 2003). The advent of herbicide-resistant weeds does not begin with herbicide resistant crops but evolves with repeated use of herbicides. Numerous weed species have evolved resistance, with 291 resistant biotypes reported in 2004 (Heap, 2004). Weed resistance may become a problem after repeated use of a single herbicide over several years. The most common and economical recommendation for managing herbicide-resistant

weeds is tank mixing herbicides with different modes of action, by which the usage and utility of glyphosate can be reduced and preserved (Diggle et al., 2003; Culpepper, 2006). In addition, glyphosate also affects glyphosate-resistant crops by decreasing their chlorophyll content (Pline et al., 1999), increasing the sensitivity of plants to various soil-borne pathogens in the root exudates caused by increased growth of selected fungal populations in soil (Kremer et al., 2005), and inhibiting nodular metabolisms (De Maria et al., 2006).

**C. Glyphosate resistance**

**a) The development of glyphosate-resistant genes**

Herbicide-resistant crops resistant to herbicides have been created either by transgene technology or by selection in cell or tissue culture for mutations that confer resistance (Duke, 1996). Three basic strategies have been assessed and used to introduce glyphosate-resistance into crop species: over-expression of the sensitive target enzyme, detoxification of glyphosate molecule, and expression of an insensitive form of the target enzyme (Dill, 2005). Three transgenes are used for glyphosate-resistance: the *CP4 EPSPS* gene encodes a glyphosate resistant form of EPSPS from *Agrobacterium*; a gene from *Ochrobactrum anthropi* encodes glyphosate oxidase (GOX), a glyphosate-degrading enzyme, and is used with the *CP4 EPSPS* gene to degrade glyphosate (Padgette et al., 1996); and the third glyphosate-resistant EPSPS transgene is obtained from maize produced by mutagenesis (Dill, 2005). Many commercial glyphosate-resistant products on the market today contain the first of these, the bacterial *CP4 EPSPS* gene from the *Agrobacterium* sp. strain CP4. In addition, several other naturally occurring glyphosate-tolerant microorganisms have been identified, including the *Achromobacter* sp. strain LBAA and *Pseudomonas* sp. strain PG2982 (Barry et al., 1997). Since the *Agrobacterium* sp. strain CP4, isolated from a waste-fed column at a glyphosate production

facility, has very favorable glyphosate resistance kinetic parameters, namely high glyphosate resistance, it has generally been chosen for the production of transgenic glyphosate resistant crops (Funke et al., 2006).

**b) Glyphosate resistant crops**

The development of glyphosate-resistant crops has been pursued since the early 1980s (Padgette et al., 1995) and glyphosate tolerance is now the most widely planted transgenic crop trait. Glyphosate-resistant crops are developed by splicing the glyphosate resistant *CP4 EPSPS* gene into plants in order to allow plants to survive glyphosate applications. The use of genetically engineered crops has expanded rapidly in the past few years, increasing to over 40 million hectares worldwide by 2000 (Pretty, 2001). Six glyphosate resistant agronomic crops have been deregulated in the USA, namely soybean, cotton, canola, maize, sugarbeet, and alfalfa. Over 90% of the soybean and 70% of the cotton planted in the United States are now glyphosate-resistant. In 2006, 10 million farmers in 22 countries planted more than 100 million hectares with glyphosate-resistant crops and the area devoted to genetically modified crops increased more than 60-fold from 1996 to 2007, becoming one of the most quickly adopted farming technologies in modern history (Nebraska, 2007).

**i) Development of glyphosate-resistant crops by inserting *EPSPS* gene**

Glyphosate-resistant (GR) soybean was engineered by the stable integration of a transgene from *Agrobacterium* species that encode the herbicide insensitive enzyme, 5-enolpyruvylshikimic acide-3-phosphate synthase (EPSPS) in the shikimate pathway (Padgette et al., 1995). In 1983, the scientists at Monsanto and Washington University isolated a common soil bacterium, *Agrobacterium tumefaciens* strain CP4, which is highly tolerant to glyphosate

(Watrud et al., 2004). They successfully inserted the *CP4 EPSPS* gene into the plant genome to achieve glyphosate resistant crops after three years.

### ii) Characteristics and mechanism of the *EPSPS* gene

5-Enolpyruvylshikimate-3-phosphate synthase (EPSP synthase) is a chloroplast-localized enzyme of the shikimate pathway in plants. It is present in all plants, bacteria and fungi, but not in animals. In plants, EPSPS is localized in the chloroplasts or plastids (Della-Cioppa et al., 1986). The EPSPS encoded by the *EPSPS* gene is the only cellular target for glyphosate (Steinrucken and Amrhein, 1984). The inhibition of the EPSPS by glyphosate leads to the failed conversion of phosphoenolpyruvic acid (PEP) and 3-phosphoshikimic acid to 5-enolpyruvyl-3-phosphoshikimic acid, high levels of shikimate accumulation, and the inhibition of biosynthesis of plant hormones and vitamins. Two necessary elements for genetically modified glyphosate resistant crops are a resistant target enzyme and sufficient expression of the enzyme in the transgenic plant. Many studies have searched for EPSPS enzymes or variations with high glyphosate resistance from native and mutagenized microbial and plant sources (Ruff et al., 1991; Barry et al., 1997)

The naturally occurring EPSPS enzyme from *Agrobacterium* sp. strain CP4 was identified as CP4 EPSPS, possessing high glyphosate tolerance with favorable glyphosate resistant kinetic parameters (Barry et al., 1997; Padgette et al., 1995). CP4 EPSPS allows glyphosate resistant crops to overcome the inhibition in the shikimate pathway by preventing aromatic amino acids starvation and deregulation of the metabolic pathway. *CP4 EPSPS* genes coding for Class II EPSPS enzymes that have been isolated and cloned from the *Agrobacterium tumefaciens* sp. strain CP4 can be fused with chloroplast transit peptides in plants to produce glyphosate-resistant plants (Padgette et al., 1995).

### iii) Development of glyphosate resistant crops: Roundup Ready® Soybean

More than 50% of the world's soybean crops are now genetically modified. In the USA, glyphosate resistant soybean application increased from 2% in 1996 to 93% in 2010 (USDA, 2010). The Roundup Ready® resistant soybean from Monsanto is one of the most important genetically modified soybean crops worldwide. As a transgenic plant, glyphosate resistant soybean represents a revolutionary breakthrough in the weed control field. Glyphosate resistant soybean is created by incorporating a gene encoding a glyphosate resistant enzyme (CP4 EPSPS), which acts in the shikimate pathways regulating the production of phenolic compounds and lignin synthesis. A glyphosate resistant soybean line, 40-3-2, is achieved via the expression of bacterial EPSPS from *Agrobacterium* sp. strain CP4. The molecular characteristics of glyphosate resistant soybean line 40-3-2 contain a portion of the cauliflower mosaic virus 35S promoter (P-E35S), the *Petunia hybrid* EPSPS chloroplast transit peptide (CTP), the *CP4 EPSPS* gene, and a portion of the 3' nontranslated region of the nopaline synthase gene (NOS3 ') terminator (Padgette et al., 1995). The Prichard RR soybean used in this study possesses a single gene for resistance to glyphosate derived from a backcross programming initiated by the University of Georgia in May 1996, to transfer glyphosate tolerance from a glyphosate resistant donor line to 'Prichard' (Boerma et al., 2001).

### iv) Safety of glyphosate-resistant crops

Several studies have reported the presence of recombinant DNA from genetically modified crops in the food chain (Vaitilingom et al., 1999) and genetically modified crops fed to livestock (Duggan et al., 2000), as well as in the air (Losey et al., 1999) and in agricultural soils (Lerat et al., 2005). One of the potentially most serious environmental risks of genetically modified crops is gene transfer from genetically modified crops to bacteria in soil, animal,

human and other wild relatives (Ellstrand et al., 1999). Perhaps of most interest for the public is the potential for transgenes to transfer novel traits that have never appeared before, leading to transformed populations. In the UK, the possibility of horizontal gene transfer from genetically modified oil seed rape to weeds could lead to the weeds also developing a resistance to herbicides (Cresswell et al., 2008). In addition, viruses or bacteria may incorporate transgenes into their genomes to produce undesirable traits with high fitness. In order to detect genetically modified organisms in genetically modified crops, analytical methods such as PCR amplification have therefore been developed (Meyer, 1999).

**c) The advantages and disadvantages of glyphosate resistant crops in agriculture**

The generally accepted advantages of glyphosate resistant crops are that they offer a flexible, easy and effective management system for farmers. The cost of weed control has plummeted since glyphosate resistant crops were introduced. The adoption of glyphosate resistant crops is also associated with some statistically significant reductions in aggregated pesticide use (Heimlich et al., 2000). The American Soybean Association states that glyphosate-resistant soybean crops protect the environment through changes in tillage practices and herbicide application, and improved weed control (Teasdale, 2003). One of the important environmental benefits of glyphosate resistant crops is that they promote reduced, minimum, or no tillage agriculture, thus preventing the loss and erosion of top soil often caused by tillage. The incorporation of glyphosate application and no-tillage has become an important weed management practice (Duke, 2005). Furthermore, there is no evidence that herbicide resistant crops have a significant direct effect on soil nutrient transformations in field environments (Motavalli et al., 2004).

As glyphosate usage and glyphosate resistant crop acreage have increased, the challenges of sustainability of this technology have also become evident and problems with herbicide resistance are increasingly being identified. Two selective pressures among others on a weed community in the agroecosystem are the tillage and herbicide regimes. The increase usage of glyphosate resistant crops results in the greater use of and reliance on herbicides, so that naturally herbicide resistant weed species will tend to increase and replace those species controlled by glyphosate. Composition shifts in the weed population and the evolution of herbicide resistance may thus occur due to increased selection pressure (Heard et al., 2003). In addition to the appearance of herbicide resistance, the movement of transgenes from glyphosate resistant crops into natural populations is another concern for environmentalists. Furthermore, it is possible that the diversity, function and distribution of microbial communities might be influenced by the widespread use of glyphosate resistant crops in the future.

**D. The influence of glyphosate on glyphosate-resistant crop systems**

**a) Effect of glyphosate on the growth of glyphosate-resistant crops**

The adoption of glyphosate resistant soybean has been rapid in the United States. Almost 93% of the country's soybean acreage was planted with glyphosate resistant soybean in 2010 (USDA, 2010). Although the soybean is resistant to glyphosate, the application of glyphosate could still have an adverse effect under certain conditions. The potential for yield suppression in a glyphosate resistant crop is a concern for both producers and seed companies. In general, there have been no significant yield reductions reported in either the vegetative or reproductive stages of the crop since the introduction of glyphosate resistant crops (Delannay et al., 1995; Reddy et al., 2000). Some studies have even suggested that glyphosate-resistant crops with applications of glyphosate have the same or higher yields compared with conventional preemergence or

postemergence herbicide applications (Nelson and Renner, 1999). However, yield suppression of glyphosate soybean might occur under stress conditions (King et al., 2001; Elmore et al., 2001). The physiological effects on glyphosate resistant soybean caused by glyphosate applications are not clearly understood (Reddy and Zablotowicz, 2003). Reddy et al. (2000) found glyphosate treatments at 1.12 kg/ha had little or no effect on the chlorophyll content and dry weight of the shoots and roots of glyphosate resistant soybean, although treatments with glyphosate at the higher rate of 2.24 kg/ha had the potential to cause soybean injury. Lee (1981) found some inhibition of chlorophyll synthesis by glyphosate. In addition, the effect of glyphosate on nitrogen fixation and the accumulation of glyphosate in glyphosate resistant crops have been observed (King et al., 2001). Nitrogen fixation in glyphosate resistant soybean is slightly affected at the label use rate, but is noticeably reduced above label use rates. The greatest reduction of nitrogen fixation in glyphosate resistant soybean was observed under soil moisture stress following glyphosate application (Zablotowicz and Reddy, 2007). The effect of glyphosate on nodulation and plant growth has also been studied (Reddy et al., 2000; King et al., 2001). There is no significant effect of glyphosate on the mineral content of glyphosate-resistant soybean at recommended rates for weed management in the field (Duke et al., 2012a). In summary, the effects of glyphosate on glyphosate resistant soybean depend on the time of glyphosate treatment, the concentration of glyphosate, the number of glyphosate applications, glyphosate formulation, and the plant cultivar (Duke et al., 2012b).

**b) Effect of glyphosate on the diazotrophic community associated with glyphosate-resistant crops**

The introduction of glyphosate resistant soybean seems to have an impact on its associated diazotrophic community due to the translocation, metabolic sink, and exudation of

glyphosate after glyphosate application (Moorman, 1989; Duke, 1996; Reddy and Zablotowicz, 2003; Kremer et al., 2005). Symbiotic nitrogen fixation associated with soybean can be affected by a herbicide because of its potentially negative effects on both rhizobial symbionts and host plants (Moorman, 1989). The effect of glyphosate on the diazotrophic community may pose a potential risk for glyphosate resistant soybean cropping systems. Since glyphosate can interfere with the symbiotic relationship of the nitrogen-fixing symbiont in soybean root nodules because some of nitrogen fixing bacteria have no insensitive EPSPS, biological nitrogen fixation in soybean might be affected. Various studies have found that glyphosate can indeed inhibit the growth of *Bradyrhizobium japonicum*, which does not contain resistant EPSPS (Jaworski, 1972; Moorman et al., 1992; Zablotowicz and Reddy, 2004). There is a differential growth inhibition caused by glyphosate in different strains of *Bradyrhizobium japonicum* (Hernandez et al., 1999; Zablotowicz and Reddy, 2004). Moorman et al. (1992) also reported that the repeated application of glyphosate could affect *Bradyrhizobium japonicum*. The adverse effects on nodulation and nitrogen fixation of glyphosate resistant soybean can be partly explained by the effect of glyphosate on *Bradyrhizobium japonicum.* In addition, glyphosate could physiologically influence the nodulation potential of some *Rhizobium* (Eberbach and Douglas, 1989). Therefore, the effect of glyphosate on the diazotrophic community needs to be considered in the glyphosate resistant soybean production systems.

**c) Other changes in soil microbial communities associated with glyphosate degradation**

Since glyphosate and its degradation products such as AMPA can be utilized by microbial communities as nutrient sources in the soil and rhizosphere, the application of glyphosate may also influence the soil microbial activity in both the short and long terms (Araújo

et al., 2003; Kremer et al., 2005). Herbicides can affect root growth and morphology, so rhizosphere microbial activity can be affected by root exudation or chemical composition. Glyphosate applications can increase the soil microbial biomass, respiration, and carbon and nitrogen mineralization (Haney et al., 2000; 2002). In addition, there is an increased fungal population observed under glyphosate treatment, which can further adversely influence the plant growth and biological processes in the soil and rhizosphere. Heavy root colonization and specific plant diseases could be caused by glyphosate applications (Kremer et al., 2005). The primary soilborne fungi colonizing susceptible plants after applications of glyphosate are *Fusarium* and *Phytophthora* (Johal and Huber, 2009).

**E. Horizontal gene transfer**

**a) Principle of horizontal gene transfer**

Horizontal gene transfer is an evolutionary phenomenon based on the flow of genes between species (Koonin et al., 2001). Gene transfer in plants carried out by pollen has been reported (Watrud et al., 2004). Except for pollen hybridization, the possibility and barriers for gene transfer from plants to bacteria depend on the genetic mechanisms that affect transformation frequency under natural conditions. The main barriers preventing gene transfer from plants to bacteria may be the lack of sequence homology and non-competent recipient cells (Nielsen et al., 1997). Three major bacterial gene transfer processes for naturally occurring DNA transfer include natural genetic transformation, transduction by bacterial viruses, and conjugation by bacterial plasmids. In the case of gene transfer from plants to bacteria, the uptake of existing plant DNA in the environment is expected in natural transformations (Lorenz and Wackernagel, 1994). Widmer et al. (2008) reported that transgenic plant DNA remained PCR-detectable for more than 130 days. It is thus possible for soil to become a potential reservoir of plant DNA

released by transgenic plants. Nevertheless, there are several key steps required in order to achieve this transformation under natural conditions, including the release of DNA molecules into the environment, the presence of genetically adapted bacterial genotypes, efficient DNA uptake, and successful chromosomal integration (Smith et al., 1981).

**b) *EPSPS* gene transfer in glyphosate-resistant cropping systems**

A number of glyphosate resistant crops have been developed since the early 1980s (Gasser and Fraley, 1989). Glyphosate-resistant crops remain unaffected by the continuing introduction of the glyphosate resistant EPSPS enzyme (Padgette et al., 1995) and examples of recombinant gene transfer between glyphosate resistant plants and wild plants have been found in the field. In addition, competent bacteria can integrate the foreign genetic material into their own genomes. The detection and quantification of recombinant DNA from glyphosate resistant soybean has focused on the junction between the chloroplast transit peptide element (CTP4 for RR soybean) and the *CP4 EPSPS* gene. Many studies have detected RR soybean by using other target sites (Table 1.1). Small amounts of the *CP4 EPSPS* gene can be found in soil samples of glyphosate resistant corn and soybean (Lerat et al., 2005). Previous studies have shown that recombinant DNA from residues of decaying plants can persist in the soil for several months (Lerat et al., 2007). Levy-Booth et al. (2008) found that *CP4 EPSPS* genes entering the soil from decomposing soybean leaf biomass can be detected for at least 30 days. The persistence of recombinant DNA in soil depends on many factors, such as the type of plant, soil type, soil moisture content, temperature, activity of soil organisms, and climatic conditions. Recombinant genes from glyphosate resistant soybean have been found in the surface soil during the growing season, but transgenes in the soil follow a cycle that differs among different crops. The numbers of transgenes differ between the growing season and the following spring and recombinant DNA

in the soil surface can be quickly degraded over the winter (Lerat et al., 2007). Recombinant *CP4 EPSPS* genes introduced into the soil from glyphosate resistant crops are already considered an environmental risk due to the possibility of horizontal gene transfer to soil microorganisms. Since microbial community structure and function may be changed by gene transfer in the soil, the crop system and agricultural management could also be indirectly affected by gene transfer.

**F. Fundamentals of denaturing gradient gel electrophoresis and quantitative PCR**

Our understanding and knowledge of the natural microbial communities in the soil are limited because of the limited numbers of microorganisms that can be isolated from natural ecosystems through culturing techniques. Culture-independent analysis makes the direct extraction of DNA from environmental samples possible. Denaturing gradient gel electrophoresis (DGGE) has already been widely used to study the structural diversity of microbial communities using a culture-independent method. A DGGE profile reflects the evenness and richness of populations in environmental samples, allowing PCR products of the same length but of different sequence compositions to be separated in gradient gels according to the melting behavior of the DNA (Heuer et al., 2001). The double stranded fragments cannot separate until reaching conditions where the lower temperature melting domains start to melt. At the same time, the branching DNA fragments are mobilized in the gel. To prevent the complete melting of the double stranded DNA fragments, a GC-clamp (GC rich sequence 40-45 bases in length) is attached to the 5’ end of the one of the primers (Heuer and Smalla, 1997). In DGGE, the double-stranded DNA is separated in a linearly increasing gradient of formamide and urea. DGGE can detect single base changes in DNA fragments so it is an effective way of screening for new species, mutations and polymorphisms (Myers et al., 1987). Although DGGE can be used as a good genetic tool to generate fingerprints of bacterial communities, several limitations

need to be considered. The detection level of DGGE can vary for a number of reasons, including different numbers of rRNA operons, mismatches to the primer, similar electrophoretic mobilities, and its ineffectiveness for PCR products above 500 bp (Heuer et al., 2001).

The difference between real-time quantitative PCR (qPCR) and conventional PCR is the amount of PCR product at each cycle that can be monitored using fluorescent reporter molecules in real time PCR. Rather than recording the amount of target accumulated after a fixed number of cycles, real- time PCR measures the point in time during cycling when the first PCR products appear. In real-time PCR, the increase in fluorescent signal is directly proportional to the number of PCR products generated in the exponential phase of the reaction. The intensity of the reporter dye emission is monitored. The cycle number is referred to as the threshold cycle (Ct). The plot of fluorescence against cycle number is then generated by the real-time PCR instrument. Real-time PCR assays are highly reproducible and can discriminate between twofold differences in target numbers (Heid et al., 1996; Bustin, 2005). Absolute quantification is achieved by establishing a standard curve based on known amounts of the target template, thus allowing the determination of the concentration of the unknown samples. For relative quantifications, the changes of gene expression in different samples are measured based on an external standard or a reference sample (Wong and Medrano, 2005). In addition, there are several well-established probe-based techniques such as the hybridization TaqMan probes and Hairpin Probes of molecular beacons. Real time PCR is now has become a common, sensitive, fast, and effective method for measuring the gene expression and abundance that is widely used in the analysis of functional gene abundance in various environments.

In summary, the diversity and abundance of diazotrophic communities in the soil are affected by several environmental factors, including climate, soil pH, the input of nitrogen

fertilizer, the carbon source, and so on. Since diazotrophs are indicators of overall soil ecological health, with great diversity and wide distribution across the ecosystem, it is vital to understand those factors that might lead to changes in the diazotrophic community. Both benefits and potential risks caused by increased usage of glyphosate resistant crops and glyphosate applications have already become apparent. Specifically, the glyphosate resistant soybean system and associated microorganisms in the soil deserve closer scrutiny from researchers.

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Table 1. 1. Primers for detection of RR soybean by PCR/qPCR

| Target sequence (gene) | Source | Amplicon length (bp) | Method |
|---|---|---|---|
| CaMV 35S promoter/Petunia CTP | Van Hoef et al., 1998 | 352<br>156 | PCR |
| CaMV 35S promoter/EPSPS gene | Hurst et al., 1999 | 320 | PCR |
| CaMV 35S promoter/Petunia CTP | DMIF-GEN, 1999 | 110 | PCR |
| CaMV 35S promoter/Petunia CTP | Wurz and Willmund, 1997 | 172 | PCR |
| P-35S/CP4 EPSPS<br>P-35S/CTP- EPSPS | Meyer and Jaccaud, 1997 | 447<br>169 | PCR |
| P-35S/CP4 EPSPS,<br>CTP-EPSPS/ CP4 EPSPS | Köppel et al., 1997 | 509<br>180 | PCR |
| P-35S, nos 3', CP4 EPSPS/nos 3' | Van et al., 1997 | 260 | PCR |
| CP4 EPSPS /nos 3' | Hörtner, 1997 | 259 | PCR |
| CTP/CP4 EPSPS | Lerat et al., 2005 | 92 | qPCR |
| CaMV 35S promoter/CP4 EPSPS | Battistini and Noli, 2009 | 254 | qPCR |

## Chapter II. Abundance and diversity of diazotrophic community in a long-term crop rotation system


### Abstract

Diazotrophic bacteria in soil are key members of microbial communities that convert atmospheric nitrogen to biologically available ammonium. The objective of this study was to assess the abundance and diversity of diazotrophic bacteria in a long-term crop rotation system. The field experiment, Cullars Rotation established in 1911, consists of a three-year rotation of soybean, cotton, corn, and wheat and various soil amendment levels. Soil samples were collected in June and October 2008 and February 2009 for six experimental treatments at two depths (0-5 cm and 5-15 cm). The abundance and diversity of the *nifH* gene was determined by quantitative polymerase chain reaction and denaturing gradient gel electrophoresis (DGGE). The *nifH* gene copy numbers ranged from $6.1 \times 10^5$ to $2.3 \times 10^7$ copies/g soil. The lowest gene copy number was found in the treatment without lime application. There was a significant interaction between sampling month and soil depth. In general, the *nifH* gene abundance was the highest in February and lowest in October. More *nifH* genes were found in surface soil than subsurface soil. DGGE banding patterns showed that soil pH, seasonal change, and inorganic N fertilization all affected the diazotrophic community. These results indicate that liming, season, soil depth, and N fertilization are important factors affecting the abundance and diversity of diazotrophic microorganisms in this century-old crop rotation system.

## Introduction

Biological nitrogen fixation carried out by the diazotrophic community is the natural process for the reduction of atmospheric nitrogen gas to biologically available ammonium. It contributes 100-290 Tg nitrogen per year to the terrestrial ecosystem (Cleveland et al., 1999). Several environmental factors are known to influence nitrogen fixation in soil, including soil moisture (Rajaramamohan-Rao, 1976; Sangakkara et al., 1996), oxygen (James and Crawford, 1998; Kondo and Yasuda, 2003), pH (Limmer and Drake, 1996; Mensah et al., 2006; Nelson and Mele, 2006), carbon source (Rajaramamohan-Rao, 1976; Keeling et al., 1998), nitrogen availability (McAuliffe et al., 1958; Keeling et al., 1998) and trace elements (Iswaran and Sundara Rao, 1960). Soil diazotrophs are the main source of nitrogen input in primary-production ecosystems (Poly et al., 2001b). Diazotrophs share a common operon in which the *nifH* gene encodes for nitrogenase reductase (Fe protein subunit) of the nitrogenase complex. The *nifH* gene is highly conserved through evolution and has been widely studied (Young, 1992; Poly et al., 2001b; Zehr et al., 2003). The diazotrophic community is most often studied using *nifH* as a marker gene to determine the abundance of potential diazotrophic microorganisms in natural environments (Levy-Booth and Winder, 2010). Nitrogen-fixing bacteria are known to be sensitive to perturbation (Doran et al., 1997). The abundance of the *nifH* gene seems to be affected by environmental conditions. The *nifH* gene has been quantified using quantitative PCR (qPCR) to determine the response of nitrogen-fixing microorganisms to land use and management, nitrogen fertilizer, crop cultivar, soil pH, carbon/nitrogen source, soil organic matter, soil horizon, and seasonal change (Colloff et al., 2008; Coelho et al., 2009; Hayden et al., 2010; Levy-Booth and Winder, 2010; Jaejoon et al., 2012).

Agricultural ecosystems provide a good opportunity to study the functional significance of soil microbial communities in a less complex setting than that found in natural ecosystems (Hsu and Buckley, 2009). Long-term agricultural experiments are thus particularly useful for the study of microbial communities and how they respond to changes in agricultural management (Buckley and Schmidt, 2001). The Cullars Rotation experiment, located on a Marvyn loamy sand soil in Alabama's Coastal Plain physiographic region, was designed primarily to study the long-term effect of soil fertilization in a three-year crop rotation (Mitchell et al., 2005). Hiltbold et al. (1985) found that seasonal variations in *Rhizobium japonicum* numbers were observed in the long-term Cullars Rotation using the most probable number (MPN) method. Today, qPCR is widely used for estimating the potential or expression of nitrogen cycling activities in soils.

Microbial community structure and functional potential are important for determining the relationship between microorganisms and the ecosystem they live in (McGuire and Treseder, 2010). In recent years, many studies have addressed the contribution of the diazotrophic community response to the environment by focusing on the diversity of the *nifH* gene (Tan et al., 2003). Currently, nucleic acid based methods for the analysis of microbial community structure include DGGE (Orr et al., 2011), restriction fragment length polymorphism (RFLP) (Poly et al., 2001a), terminal-restriction fragment length polymorphism (T-RFLP) (Ohkuma et al., 1999), automated ribosomal intergenic spacer analysis (ARISA) (Gros et al., 2006), soil metagenomics (Rondon et al., 2000), and high throughput sequencing (Moisander et al., 2006). Diazotrophic community diversity has been shown to respond to various environmental conditions in studies utilizing DGGE to detect the *nifH* gene (Wakelin et al., 2007; Wartiainen et al., 2008; Coelho et al., 2009; Wakelin et al., 2009; Orr et al., 2011; Shu et al., 2012). In this study, we examined the

abundance and diversity of the diazotrophic community in the century-old Cullars Rotation experiment.

## Materials and Methods

### Field experiment and soil sampling

The Cullars Rotation experiment, established in 1911, is the oldest continuous soil fertility experiment in the southern United States and is located in Auburn, Alabama. The soil type is Marvyn loamy sand (fine loamy, siliceous, thermic, typic hapludults). A detailed description of the Cullars Rotation experiment can be found in Mitchell et al. (2005).

Soil samples were collected in June and October in 2008 and February in 2009. Samples were taken from two depths (0-5 cm and 5-15 cm) for six experimental treatments: no nitrogen fertilizer with winter legume, no nitrogen without winter legume, no input, complete fertilizer (N, P, K) with winter legume, complete fertilizer without winter legume, and no lime application with complete fertilization and winter legume (Table 2.1). The experiment was arranged in an ordered block design replicated three times, with one replicate for each 3-year crop rotation sequence. Six cores of soil were randomly collected from each plot using tube samplers and mixed to form one composite sample per plot. Field moist soil was sieved (4 mm) and stored at -20°C before extraction of soil DNA. Soil chemical properties are shown in Table 2.2.

### Soil DNA extraction

Total DNA was extracted from 10 g of soil using a MoBio Power Max Soil® DNA isolation kit (MoBio laboratories, Solana Beach, CA, USA) according to the manufacturer's instructions, and quantified spectrophotometrically (NanoDrop®ND-1000, Thermo Scientific, Wilmington, DE, USA). Soil DNA was stored at -80° C until further analysis.

**Quantitative real-time PCR analysis of the *nifH* gene**

The abundance of *nifH* genes was measured using Applied Biosystems StepOne$^{TM}$ Real-Time PCR system (ABI, Carlsbad, CA, USA) with fluorescent dye SsoAdvanced SYBR-Green Supermix (Bio-Rad laboratories, Hercules, CA, USA). The degenerate oligonucleotide primers used in qPCR to amplify the *nifH* gene were PolF and PolR (Poly et al, 2001a) (Table 2.3), corresponding to a 360 bp region between sequencing positions 115 and 476 (referring to the *Azotobacter vinelandii nifH* coding sequence [M20568]). Two replicates of each sample were used for detection. Quantitative PCR of *nifH* was performed in a 20 µl reaction mixture containing: 5 µl of template DNA (~3-15 ng/µl), 10 µl of SsoAdvanced SYBR Green Supermix (2×), 0.6 µl of the each primer stock solution (10 µM), and nuclease-free water. The initial denaturation step was at 95°C for 10 min prior to reaction step, followed by 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. Melting curve analyses was performed at 95°C for 15 s, 60°C for 30 s, and 95°C for 15 s. The limit of detection (LOD) of 20 copies per reaction and the lower limit of quantification (LLOQ) of 60 copies per reaction for *nifH* gene were determined according to procedures described by Ermer and Miller (2005) and Shrivastava and Gupta (2013).

A standard was prepared for PCR in order to determine the absolute quantities of the *nifH* gene present in soil. *nifH* genes were amplified by conventional PCR with the PolF and PolR primer set. The PCR product was extracted from agarose gel, purified with Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) and cloned into a TOPO-TA plasmid vector (Invitrogen Corporation, USA) in *Escherichia coli.* Plasmid DNA was extracted from *E. coli* colonies with inserts using a QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA, USA). Plasmid DNA was assessed for quantity and quality using a NanoDrop®ND-1000

spectrophotometer and sequenced. A 10-fold dilution series of plasmid DNA was prepared, ranging from $10^2$ to $10^7$ copies. The *nifH* copies were calculated from a standard constructed by plotting plasmid DNA concentrations versus quantification cycles. The $C_q$ values of the no-template control in this study were above 38 ± 3. The range of the slopes and intercepts of the standard curves were from -3.28 to -3.43 and from 33.81 to 39.87, respectively. The standard curves produced were linear ($R^2 > 0.99$) and the efficiency of the reactions was around 99%.

**DGGE of *nifH* gene**

The *nifH* gene was amplified by a nested PCR-DGGE method as described by Wartiainen et al. (2008). The first PCR was carried out with PolF and PolR primers (Poly et al., 2001a) generating a product of 370 bp. The second PCR was performed with the forward primer PolFI and the reverse primer AQER-GC30 containing a GC clamp (Table 2.3; Wartiainen et al., 2008), yielding a 368 bp fragment. The 25 µl reaction mixture included 5 µl of template DNA (~3-15 ng /µl), 12.5 µl of GoTaq Colorless Master Mix (2×), 2.5 µl of the each primer stock solution (5 µM), 25 µg of bovine serum albumin and nuclease-free water. The thermocycling conditions were as follows: 15 min at 95°C, 30 cycles of 60 s at 94°C, 60 s at 55°C and 60 s at 72°C, and a final 10 min at 72°C. For the second PCR, the same PCR mixture and thermocycling conditions were used, but 2 µl of the first PCR products were used as a template. The PCR products were screened using agarose gel in order to confirm the presence of products before running DGGE. DGGE was performed on a Dcode system (Biorad, Hercules, CA, USA) using 1mm thick acrylamide-bisacrylamide (37.5:1) according to the manufacturer's instructions. The products were separated on an 8% (w/v) acrylamide-bisacrylamide gel with a 40-65% denaturing gradient in 1× TAE (40 mM Tris-HCl, 20 mM acetic acid, 1 mM EDTA at pH 8.3) at a constant temperature of 60°C and 70 V for 16 hours. The gels were stained for 15 min in 1× SYBR Green

I Nucleic Acid Stain (Lonza Rockland Inc., Rockland, ME, USA), visualized under a UV transilluminator and recorded using a Kodak Gel Logic 200 Imaging System (Eastman Kodak Co., Rochester, NY, USA) for further analysis. To normalize for differences among the different gels, a DGGE reference was prepared using a rhizobial isolate. Its PCR product covering the size range of samples was used in the reference lanes on each gel. In addition, each gel included eighteen samples from each sampling time at one depth and three reference lanes. Later on, to control gel to gel variability, some of samples from different sampling times were run on the one gel.

The Shannon-Wiener diversity index was used to determine the diversity of diazotrophic community and reflect species richness and evenness (Shannon, 1948). The index was calculated by the following equation:

$$\text{Shannon-Wiener index (H')} = -\sum_{i=1}^{s}(P_i)(\ln P_i)$$

where $P_i$ is the ratio of the specific band intensity to the total intensity of all bands and s is the number of species in the sample.

**Statistical analysis**

Gene copy numbers per gram of dry soil were used as the input data for the SAS software package (SAS® system for Windows version 9.2, SAS Institute Inc., Cary, NC). Three blocks served as the random effect in the statistical analysis. The month, depth and treatment were the fixed effects. All variables were checked for normal distribution and transformed when necessary. The Glimmix Procedure was used to provide an appropriate model to estimate the effects of each variable and interaction between variables on the abundance of *nifH*. Type III Tests of fixed effects of the model from the Glimmix Procedure indicated statistically significant differences at P-values less than 0.05. Least square means (LS-mean) calculated from the model

represented the trend of abundance in different months, depths and treatments. The obtained DGGE patterns were subsequently normalized and analyzed with BioNumerics software (Version 5.0, Applied Math, Kortrijk, Belgium). Normalized intensity values and positions of the detected bands in all lanes were used for cluster analysis and statistical analysis after conversion, normalization, and background subtraction. The dendrogram was constructed using the method of unweighted pair groups with mathematical averages (UPGMA), with similarity expressed by the Pearson Product-moment correlation coefficient. Principal components analysis (PCA) was used to determine the distribution of fingerprint patterns by sampling months and treatments.

## Results

### Effect of treatments on *nifH* gene abundance

The *nifH* was detected in all the soil samples collected from the six experimental treatments in the Cullars Rotation during the sampling period. The results showed that the *nifH* gene copy number ranged from $6.1 \times 10^5$ to $2.3 \times 10^7$ copies per gram of soil. There were no significant interactions among treatment, sampling month and soil depth. The quantification of *nifH* did reveal significant treatment effects ($P = 0.0271$) (Table 2.4). The means of *nifH* abundance ranged from $1.4 \times 10^6$ to $1.5 \times 10^7$ gene copies per gram of soil, with the lowest being in subsurface soil without lime application in June 2008 and the highest in surface soil of the NPK fertilizer with winter legume treatment in February 2009 (Fig. 2.2). In general, the lowest gene copy numbers were found in the treatments without lime application (Fig. 2.2). The comparison of least square means of the natural log transformed *nifH* gene copies per gram of soil indicated a significant difference between the no lime treatment (plot 8) and the no nitrogen without legume treatment (plot B) (Fig. 2.1a). No nitrogen input for nearly 100 years (plot C) did

not appear to affect the abundance of *nifH* genes; nor did the type of nitrogen (legume or inorganic nitrogen) (Fig. 2.1 and 2.2).

**Effect of sampling time on *nifH* gene abundance**

The abundance of *nifH* genes was significantly affected by sampling time (P < 0.0001) (Table 2.4). In the surface soil (0-5 cm), the abundance ranged from $8.9\times10^{5}$ to $1.4\times10^{7}$ *nifH* gene copies per gram of soil in June and from $2.0\times10^{6}$ to $1.1\times10^{7}$ *nifH* gene copies per gram of soil in October. The range of *nifH* gene abundance in February was from $2.1\times10^{6}$ to $2.3\times10^{7}$ copies per gram of soil. In the subsurface soil (5-15 cm), the range of *nifH* gene abundance was from $6.1\times10^{5}$ to $7.9\times10^{6}$ in June and from $8.4\times10^{5}$ to $9.4\times10^{6}$ in October. In February, the *nifH* gene abundance ranged from $2.9\times10^{6}$ to $1.4\times10^{7}$. In general, *nifH* gene copies were at their highest in February and lowest in October (Fig. 2.2). The full set of data across the three sampling times showed a trend of increasing *nifH* copy numbers from June 2008 to February 2009 (Fig. 2.2). The *nifH* gene numbers were significantly different among February, June and October (Fig. 2.1b).

**Effect of soil depth on *nifH* gene abundance**

The abundance of *nifH* genes was significantly affected by soil depth (Table 2.4). Higher *nifH* gene abundance was found in the surface soil (0-5 cm) than subsurface soil (5-15 cm) in all treatments except for the no N with legume treatment in October (Fig. 2.2). The *nifH* gene copies in the surface soil (0-5 cm) were 1.2 times and 1.6 times higher than in the subsurface soil (5-15 cm) in October 2008 and February 2009, respectively. In June 2008, the number of gene copies in the upper layer was 2.5 times higher than in the subsurface soil. Moreover, the quantification of the *nifH* gene at two sampling depths across three sampling times revealed that there was a significant interaction between sampling time and soil depth (P = 0.0007) (Table 2.4).

**DGGE analysis of the *nifH* gene**

In the DGGE gel, each band represents at least one species. The band numbers showed that there were no significant differences among the six treatments in the surface soil. However, a significantly lower number of bands were detected in the no lime treatment (8 ± 2.5) than in the no input treatment (13 ± 2.7) in the subsurface soil (Table 2.5). Lower band numbers were observed in the no lime treatment at both depths across three sampling months, except for the subsurface soil in October 2008. The results of Shannon-Wiener index revealed that there was a significantly lower diversity in the no input surface soil (0-5 cm) (2.94 ± 0.12) than the no N with winter legume (3.23 ± 0.22) and NPK fertilizer without winter legume (3.22 ± 0.15) soil samples, at $P < 0.05$ (Table 2.6). In the subsurface soil (5-15 cm), the Shannon-Wiener index for the diversity in the no input treatment (2.88 ± 0.32) was significantly lower than for the no N with winter legume (3.50 ± 0.04) and NPK fertilizer with winter legume (3.35 ± 0.08) treatments. No big differences were observed among the other five treatments in this study.

Principal components analysis of the DGGE banding patterns was performed for each sampling month (Fig. 2.4). The first two principal components (PC) explained 58% of the total sample variation for the June samples, 62% for October, and 52% for February. Data points for the no lime and no input treatments formed separate clusters (Fig. 2.4a). Although the data points for the no N with and without winter legume treatments were intermixed, those for the NPK fertilizer with and without winter legume were separated into two clusters. In October, data points for all treatments formed individual clusters (Fig. 2.4b), but in February only the data points from the no lime and no N without winter legume treatments formed distinct clusters (Fig. 2.4c). Although the soil pH values were similar in the no input and no lime treatments, the data points from these two treatments did not overlap. PCA was also performed to determine the

effect of season on diazotrophic organisms. Fig. 2.4 shows a clear seasonal effect, consistent with the results of the qPCR analysis.

## Discussion

In this study, given the importance of nitrogen fixation for nitrogen cycling, we determined the abundance and diversity of diazotrophic communities in a long-term crop rotation system using real-time PCR and DGGE. Our findings suggested that there was no significant effect of nitrogen input on the abundance of nitrogen-fixing bacteria throughout this study. No suppression of the *nifH* gene copies under the condition with additional inorganic nitrogen across the season was observed. There are two major reasons that could account for this observation. First, low nitrogen levels in soils are favorable for free-living diazotrophs, resulting in increased numbers in the soil (Orr et al., 2011). Many previous studies have indicated a tight coupling between a low number of diazotrophs and a high nitrogen level because of the competitive suppression of non-diazotrophs (Cejudo and Paneque, 1986; Kolb and Martin, 1988; DeLuca et al., 1996; Coelho et al., 2009; Wu et al., 2009; Meng et al., 2012), but the nitrogen level in the long-term crop rotation experiment might not be a major limiting factor for the abundance of the nitrogen-fixing bacteria compared with many other factors, such as the temperature and moisture of the soil, and the presence of organic substrates and mineral nutrients that could also be limiting the development of diazotrophs.

Alternatively, the lack of fluctuations in the abundance of *nifH* genes could be interpreted by r/k selection theory. In this theory, the numbers of organisms typically remain very constant and close to the maximum under favorable conditions with unlimited resources. Consequently, we considered it possible that the long-term crop rotation experiment across a hundred years had

created a relatively stable condition, where only minimal competition between populations could occur because these populations had developed the capacity to maintain ecological function in the environment. The lack of significant changes in the *nifH* gene abundance when inorganic nitrogen and legume were added in this study demonstrated that diazotrophs had already achieved the ideal psychological characteristics to adapt to their habitat and their populations had already arrived at a maximum, stable level. Therefore, any change in nitrogen level caused by either adding inorganic nitrogen or from legume growth was not sufficient to destablize the diazotroph population in the long term rotation experiment. Ogilvie et al. (2008) found that nitrogen-fixing bacteria were stable and relatively non-responsive to long-term management of balanced nitrogen fertilizer inputs. The same conclusion is also supported by the previous findings of Bagwell and Lovell (2000) and Orr et al. (2011).

In contrast to the effect of nitrogen fertilizer on the abundance of *nifH* genes, some shifts in the diazotrophic community structure were associated with inorganic nitrogen fertilizer applications for each sampling month in the Cullars Rotation experiment. The consistent effect of inorganic N input on the diversity of the bacterial community was indicated across three months. In theory, the populations of k selected species exhibit more complex adaption, so that it is reasonable for them to develop to maximize biodiversity in order to adapt to a heterogeneous habitat in a complicated environment. Different species of diazotrophs have different sensitivities and responses to nitrogen application. Coelho et al. (2008) suggested that the use of nitrogen fertilizer is an important factor that can influence nitrogen-fixing community structures. Nitrogen fertilizer could induce a shift in the microbial community during microbial life-history strategies (Fierer et al., 2012). Shu et al. (2012) suggested that soil nitrogen content is important for nitrogen fixing bacterial communities among soil management treatments. However, Poly et al.

(2001b) argued that the amounts of nitrogen were not correlated to *nifH* gene pool differentiation. Different types of available nitrogen in soil sometimes had no influence on the structure of the potential nitrogen fixing community (Wartiainen et al., 2008).

In our study, the number of bands and the Shannon diversity index among treatments were examined. The lowest diversity in the diazotrophic community was observed in the no input treatment, corresponding to the lowest Shannon diversity index compared with the other treatments. These results suggest that there was less richness and evenness in the diazotrophic community in the no input soil, which is in agreement with the result for soil organic carbon found in this study. The significant relationship between the diversity of the diazotrophic community and the soil organic carbon was observed at both soil depths; the no input treatment had the lowest amount of soil organic carbon compared to the other treatments in the study. Microorganisms may have a positive relationship with the organic carbon when the carbon content is lower than 17.6 mg $g^{-1}$ (Yan et al., 2000). In our no input soil, the organic carbon was low (4.8 mg $g^{-1}$), thus limiting the growth of microorganisms in the soil. The addition of fertilizer could stimulate soil biological activity by providing more organic materials such as N, K, and P to the microorganisms (Haynes and Naidu, 1998). In our study, the surface soil of the no input treatment exhibited lower total nitrogen content, which could be another limitation affecting the diversity of the diazotrophic community.

The analysis of the effect of sampling time on the abundance of diazotrophs generally revealed lower numbers of *nifH* genes during the summer (June), then increasing into the winter (February). This was in accordance with the results reported for soil populations of *Rhizobium japonicum* in the same Cullars Rotation system using the Most Probable Number (MPN) determination method (Hiltbold et al., 1985). Mergel et al. (2001b) found the concentration of

cultured nitrogen-fixing bacteria in forest soils in Norway showed seasonal fluctuations, being at their lowest in summer and highest in early spring, fall and winter. More *nifH* genes at gene level in spring than autumn have also been observed by other researchers (Yeager et al., 2012). Eckford et al. (2002) suggested that free-living diazotrophs may be active and change seasonally. The seasons are known to have an effect on the nitrogen-fixing bacteria activity (Nohrstedt, 1982). Temperature and moisture are two of the most important factors affecting nitrogen-fixing bacteria activity and diversity in the soil across the seasons. In our study, a greater abundance of diazotrophic bacteria in February was observed compared with the other two months. This peak abundance in February may be caused by the high moisture content and low temperature in soil during the winter, which is good for the development of nitrogen-fixing bacteria. In general, the microbial activity decreases as the soil becomes drier. The microbial numbers and activities of microbes could be limited by water availability, which can affect the physiological performance (Harris, 1981; Kieft et al., 1993).

Lower temperatures will also slow the decomposition of crop residues so that the remaining residues from incomplete decomposition, including organic matter and organic intermediate products, can accumulate and thus support the development of nitrogen-fixing bacteria in the soil (Okon et al., 1976; Hegazi et al., 1979). The decomposition of crop residues during the winter fallow period can also enhance the activity of diazotrophic bacteria and may facilitate the decomposition of organic matter to produce carbon sources (Wakelin et al., 2007). Hegazi et al. (1986) found that maize stubble contributes large amounts of carbon to the soil, so that the abundance of free-living nitrogen-fixing bacteria increases significantly. The long-term crop rotation at the Cullars Rotation site might improve the soil structure, moisture content, and soil organic content, which could support the growth of diazotrophs in soil across its hundred

year duration. The evaporation rate of water in soil becomes slower and crop residue decomposition is limited in winter so that it is reasonable that the accumulation of crop residues in the long-term crop rotation and the presence of adequate moisture might support the development of nitrogen-fixing bacteria.

Anaerobic conditions greatly enhance the activities of nitrogen fixing bacteria compared with aerobic conditions (Limmer and Drake, 1996). Rajaramamohan-Rao (1976) suggested that greater nitrogen fixation under flooded conditions may result from the optimum moisture levels present that favor the development of the nitrogen-fixing bacteria. During the summer in Alabama, non-ideal soil conditions with low moisture levels and high temperatures are not optimum for the development of nitrogen-fixing bacteria. Dry soil conditions and high temperatures are likely to influence the activities of microorganisms in the soil by limiting their enzyme activity or the physiological activity of microbes to reduce the abundance of *nifH* gene copies, as observed in June.

PCA plots obtained from DGGE patterns of the *nifH* gene indicated that seasonal changes may be responsible for the variable structure of the diazotrophic community in a long-term crop rotation system. The effect of seasonal changes on the diazotrophic community structure was also reported by Gamble et al. (2010), while Wartiainen et al. (2008) found that the active diazotrophic community varied strongly with sampling time. The diversity of the nitrogen-fixing bacteria across nine months in the present study may be due to the fact that plants at different seasons have different plant exudates, organic matter availability, and oxygen diffusion into the soil because of changes in soil temperature and moisture.

The soil temperature, moisture content, and long-term crop rotation would also affect soil pH, another important factor affecting the microorganism composition and function in soil. Soil

pH can be used as a predictor of soil microbial community composition and diversity (Fierer and Jackson, 2006; Lauber et al., 2009). Soil acidity can affect infection, nodulation and nitrogen fixation by disrupting the survival of rhizobia in soil. Low or high pH can reduce populations of *Rhizobium*. The optimal pH range for the growth of *Rhizobium* is pH 6-7. Most soil microbes thrive in this pH range due to the high availability of nutrients. The soil pH could increase 4 to 6 after liming in the Cullars Rotation. Lower amounts of *nifH* gene copies were found in the soil without lime compared with lime application across three sampling months, probably because the acidity affected the survival and growth of nitrogen-fixing bacteria. Low copy numbers of the *nifH* gene have been observed in strongly acidic surface soil in North-East Victoria and South-West Victoria (Hayden et al., 2010). The population and activity of free-living, $N_2$-fixing bacteria was somewhat reduced by low soil pH levels (DeLuca et al., 1996).Wakelin et al. (2009) demonstrated that liming application increased the abundance of the *nifH* gene and affected the bacterial community structure. The abundance of diazotrophic bacteria was highly correlated with soil pH, with the greatest number of *nifH* genes being observed in neutral pH. Ivarson (1977) reported that lime applications can remove some microbial-inhibiting substances such as phenolic compounds and also tend to increase the humification. In the present study, the long-term crop rotation experiment led to low soil pH when no lime was applied. Under such low soil pH conditions, not only was the availability of most nutrients limited, but some of the enzymes that support the survival and development of nitrogen-fixing bacteria could also be inhibited.

In addition to the effect of pH on the abundance of nitrogen-fixing bacteria, pH was strongly correlated with variations in composition, size and structure of $N_2$-fixing communities (Pereira e Silva et al., 2011). The long-term effects of liming may increase both soil organic matter content and soil biological activity (Haynes and Naidu, 1998). Nelson and Mele (2006)

also found that additions of lime caused significant changes in *nifH*-containing rhizobacteria. Soil pH was found to be a significant factor in determining community structures by Orr et al. (2012). The inhibitory effect of low pH might be indirect because of the decreased availability of soil nutrients at low pH, which affects the activity of nitrogen-fixing bacteria (Limmer and Drake, 1996). Soil pH can be viewed as an integrating factor that drives the variance in bacterial community composition because it is often directly or indirectly related to other soil characteristics. In addition, pH can directly impose a physiological constraint on the soil bacterial community (Lauber et al., 2009). Bannert et al. (2011) observed changes in the abundance and diversity of nitrogen fixing microorganisms depending on the chemical and physical properties of soils. Higher numbers of cultural bacteria and greater microbial activity, along with changes in the bacterial community composition due to liming have also been reported (Bååth and Arnebrant, 1994). In the Cullars Rotation experiment, we found there was a shift in the pH response of the diazotrophic microbial community in the soil, suggesting the altered soil pH could lead to stress on the structure of the diazotrophic community during the long-term crop rotation.

It is generally accepted that microbial activity will decrease with soil depth (Speir et al., 1984; Higashida and Takao, 1985). The abundance of the *nifH* gene can be used as an indicator of potential diazotrophic activity. In this study, the diazotrophic numbers (as determined by gene abundance) were significantly higher in the surface soil than in the subsurface soil. The higher *nifH* gene abundance observed in the surface soil could be explained by the greater amounts of organic and inorganic materials released from plants, which would provide more nutrients and energy source for the diazotrophic microorganisms. In addition, the lower depth soil had limited oxygen content compared with the upper layer, where the microorganisms could consume more

oxygen. Speir et al. (1984) suggested there could be some influence of depth on soil chemical and biochemical properties, including lower moisture, organic carbon, total nitrogen content, and some enzyme activity, as the soil depth increased. Soil organic matter, carbon content, and nitrogen content were all found to be significantly related to nitrogen fixing gene abundance (Levy-Booth and Winder, 2010). Higher numbers of diazotrophs are generally detected in the upper (5 cm) soil layer, decreasing with increasing soil depth (Kloos et al., 1998; Mergel et al., 2001a; 2001b). Although we found the soil depth to be a strong driver of the *nifH* gene abundance in soil, no effect of depth on diazotrophic community structure was observed in this study. It is possible that variations in the structure of the diazotrophic community depend on several environmental factors, such as agricultural management and soil characteristics. A lack of differences in the structures of *nifH* genes produced in the two soil layers was also reported by Widmer et al. (1999) and Culman et al. (2010) found that soil depth was a major driver of community structure. Although the diazotrophic community was affected by a complex interaction between soil depth, management history and the site, the soil depth had less influence on the community structure of diazotrophic bacteria than for general bacterial communities.

It is clearly necessary to take into account all the factors that might affect the diversity and numbers of diazotrophs. Our findings suggested that the season, soil pH, and soil depth had an overarching influence on the *nifH* gene abundance. In addition to the season and soil pH, the nitrogen input also had a substantial effect on the diversity of diazotrophs in the long-term Cullars Rotation experiment.

All methodologies have limitations; PCR-DGGE and qPCR used in this study are no exception. Nested PCR- DGGE used in this study might have a potential to underestimate the *nifH* gene diversity. Firstly, PCR is likely to amplify the dominant members of community; This

effect may be magnified after two rounds of PCR. Secondly, a single band in a DGGE gel might represent more than one species because of multiple operons or the same electrophoresis mobility between two species. In addition, the potentially horizontal *nifH* gene transfer carried out by plasmid could also affect *nifH* phylogeny. Besides the underestimation of diversity caused by preferential amplification of PCR reaction, other biases of detecting *nifH* gene abundance also exist. One reason is that detection of the *nifH* gene abundance does not allow the separation of *nifH* genes expressed and gene not expressed. The other is that *nifH* genes maybe present on multiple plasmids instead of chromosome.

## Conclusions

The abundance and diversity of the diazotrophic community were determined in a long-term crop rotation system using real-time PCR and DGGE. The results of this study indicate that season and soil pH influenced both the *nifH* gene abundance and diversity. The effect of nitrogen fertilizer on the diversity of the diazotrophs was observed across three months, but no significant difference in *nifH* gene abundance was shown. In summary, the long-term Cullars Rotation experiment provides a relatively stable and heterogeneous habitat for the development of the diazotrophic community. A further step would be to isolate and identify diazotrophs related to specific seasons, soil pH, nitrogen fertilization and other soil management regimes.

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Table 2.1. Treatments sampled from Cullars Rotation

| Plot number | Treatment | Fertilization |
| --- | --- | --- |
| A | No N + legume | Legume, P, K, Lime |
| B | No N + no legume | P, K, Lime |
| C | No input | None |
| 1 | NPK + no legume | N, P, K, Lime |
| 3 | NPK + legume | Legume, N, P, K, Lime |
| 8 | No lime | Legume, N, P, K |

Table 2.2. Soil chemical properties averaged across the sampling period (Mathew 2012)

| Treatment | Soil pH $_{water}$ 1:1 Soil/water* | | Soil organic carbon mg $g^{-1}$ soil* | | Soil total nitrogen content (%)* | |
|---|---|---|---|---|---|---|
| | 0-5 cm | 5-15 cm | 0-5 cm | 5-15 cm | 0-5 cm | 5-15 cm |
| No N + legume | 6.1 | 5.4 | 9.6 | 5.1 | 0.06 | 0.02 |
| No N + no legume | 6.2 | 5.7 | 8.3 | 4.1 | 0.05 | 0.02 |
| No input | 4.8 | 4.4 | 4.8 | 4.0 | 0.04 | 0.02 |
| NPK + legume | 5.8 | 5.7 | 7.7 | 4.3 | 0.04 | 0.02 |
| NPK + no legume | 6.0 | 5.6 | 9.6 | 5.2 | 0.04 | 0.02 |
| No lime | 4.2 | 3.9 | 9.2 | 5.6 | 0.02 | 0.01 |
| $LSD_{(0.05)}$ | 0.7 | 0.6 | 1.4 | 0.8 | 0.01 | 0.01 |

**Significant at $P \leq 0.001$, *Significant at $P \leq 0.05$.

Table 2.3. Oligonucleotide primers used for *nifH* gene this study

| Primer | Sequence (5'→3') | Reference |
|---|---|---|
| PolF | TGC GAY CCS AAR GCB GAC TC | Poly et al. (2001a) |
| PolR | ATS GCC ATC ATY TCR CCG GA | Poly et al. (2001a) |
| PolFI | TGC GAI CCS AAI GCI GAC TC | Wartiainen et al. (2008) |
| AQER-GC30 | CGC CCG CCG CGC CCC GCG CCC GGC CCG CCC GAC GAT GTA GAT | Wartiainen et al. (2008) |
| Modified bases: I = Inosine, Y = CT, S = CG, R = AG, B = GCT | | |

Table 2.4. Analysis of variance for the *nifH* gene abundance

| Source of variation | *F* - test probability[a] |
|---|---|
| Treatment | **0.0271** |
| Depth | **<.0001** |
| Month | **<.0001** |
| Depth × Treatment | 0.6095 |
| Month × Treatment | 0.6161 |
| Month × Depth | **0.0007** |
| Month × Depth × Treatment | 0.2632 |

[a] Type III Tests of Fixed Effects indicated statistically significant differences at $P < 0.05$. Boldface is used for a significance (P) of $< 0.05$.

Table 2.5. Number of bands detected on DGGE gels

| Plot No. | Treatment | Shannon diversity index | | | Average across month |
|---|---|---|---|---|---|
| | | June 2008 | October 2008 | February 2009 | |
| *Depth 0-5 cm* | | | | | |
| A | No N + legume | 3.00 | 3.45 | 3.24 | 3.23 ± 0.22 |
| B | No N + no legume | 3.09 | 3.08 | 3.13 | 3.10 ± 0.03 |
| C | No input | 3.05 | 2.81 | 2.97 | 2.94 ± 0.12 |
| 1 | NPK + no legume | 3.27 | 3.34 | 3.05 | 3.22 ± 0.15 |
| 3 | NPK + legume | 3.11 | 3.29 | 3.10 | 3.16 ± 0.11 |
| 8 | No lime | 3.13 | 3.19 | 3.13 | 3.15 ± 0.04 |
| $LSD_{0.05}$ | | | | | 0.232 |
| *Depth 5-15 cm* | | | | | |
| A | No N + legume | 3.52 | 3.52 | 3.46 | 3.50 ± 0.04 |
| B | No N + no legume | 3.16 | 2.87 | 3.51 | 3.18 ± 0.32 |
| C | No input | 2.94 | 3.16 | 2.53 | 2.88 ± 0.32 |
| 1 | NPK + no legume | 3.15 | 3.08 | 2.87 | 3.03 ± 0.14 |
| 3 | NPK + legume | 3.30 | 3.31 | 3.44 | 3.35 ± 0.08 |
| 8 | No lime | 2.95 | 3.54 | 3.27 | 3.26 ± 0.30 |
| $LSD_{0.05}$ | | | | | 0.413 |

Significant at $P \leq 0.05$ by LSD test

Table 2.6. Shannon diversity indices

| Plot No. | Treatment | No. of bands | | | Average across month |
|---|---|---|---|---|---|
| | | June 2008 | October 2008 | February 2009 | |
| *Depth 0-5 cm* | | | | | |
| A | No N + legume | 12 | 15 | 8 | 12 ± 3.5 |
| B | No N + no legume | 10 | 11 | 11 | 11 ± 0.6 |
| C | No input | 10 | 11 | 10 | 10 ± 0.6 |
| 1 | NPK + no legume | 8 | 15 | 11 | 12 ± 3.5 |
| 3 | NPK + legume | 11 | 9 | 10 | 10 ± 1.0 |
| 8 | No lime | 10 | 9 | 8 | 9 ± 1.0 |
| $LSD_{0.05}$ | | | | | 3.85 |
| *Depth 5-15 cm* | | | | | |
| A | No N + legume | 10 | 10 | 11 | 10 ± 0.3 |
| B | No N + no legume | 11 | 12 | 14 | 12 ± 1.3 |
| C | No input | 11 | 16 | 11 | 13 ± 2.7 |
| 1 | NPK + no legume | 14 | 17 | 14 | 15 ± 1.8 |
| 3 | NPK + legume | 10 | 7 | 14 | 10 ± 3.2 |
| 8 | No lime | 8 | 11 | 6 | 8 ± 2.5 |
| $LSD_{0.05}$ | | | | | 3.90 |

Significant at $P \leq 0.05$ by LSD test

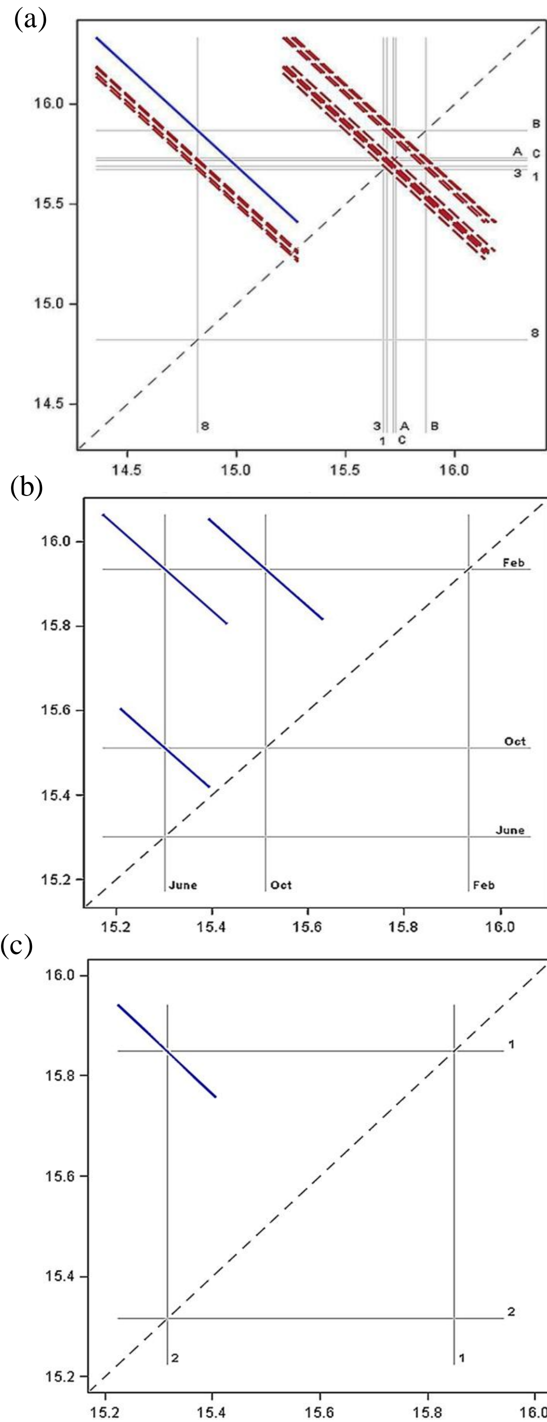


Fig. 2.1. Comparison of least square means of natural log of *nifH* gene copies per gram of soil. Both x and y axes represented the least square means of gene copies. (a) Differences among six treatments represented by plot numbers as described in Table 1; (b) Differences among three months (June 2008, October 2008, and February2009); (c) Differences between two depths (1: 0-5 cm; 2: 5-15 cm). Dashed lines indicate no significant difference; Solid lines indicate significant differences. Statistically significant differences were at P < 0.05.

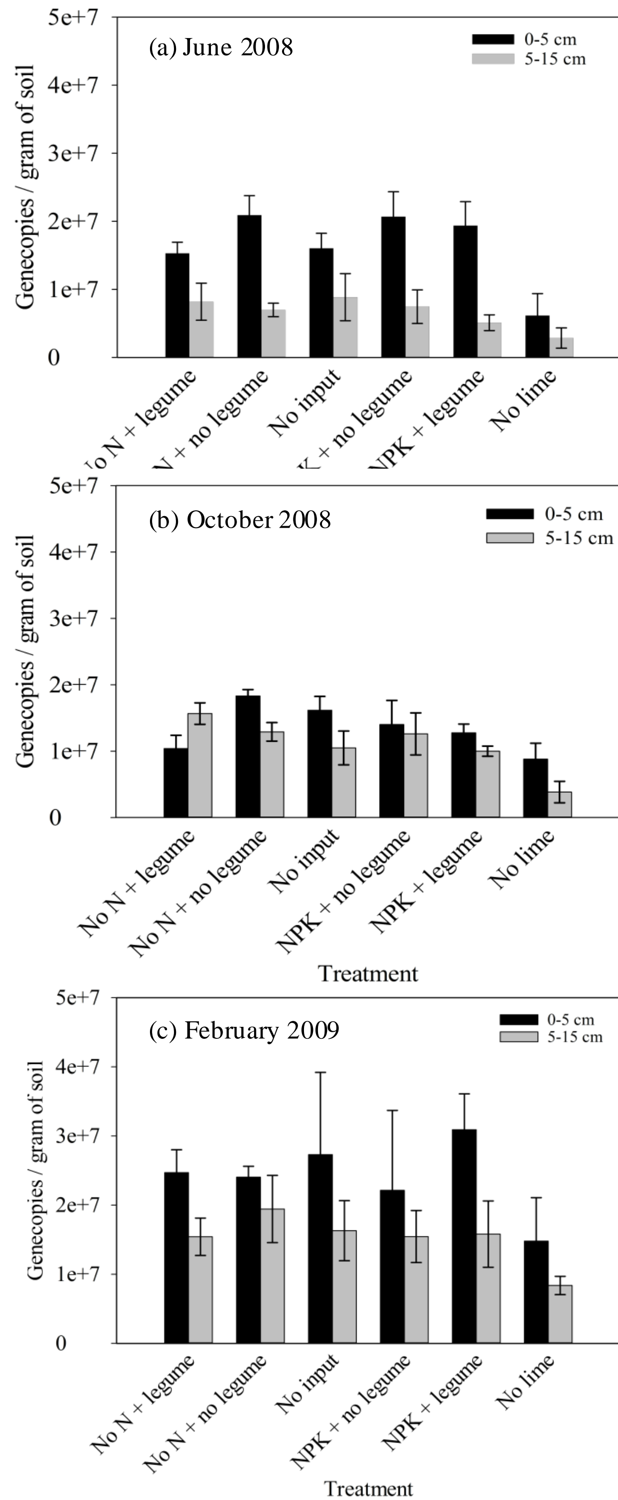


Fig. 2.2. Abundance of *nifH* gene in three months with six treatments at two depths expressed as gene copies per gram of dry soil (Mean ± Standard deviation): (a) June 2008; (b) October 2008; (c) February 2009.

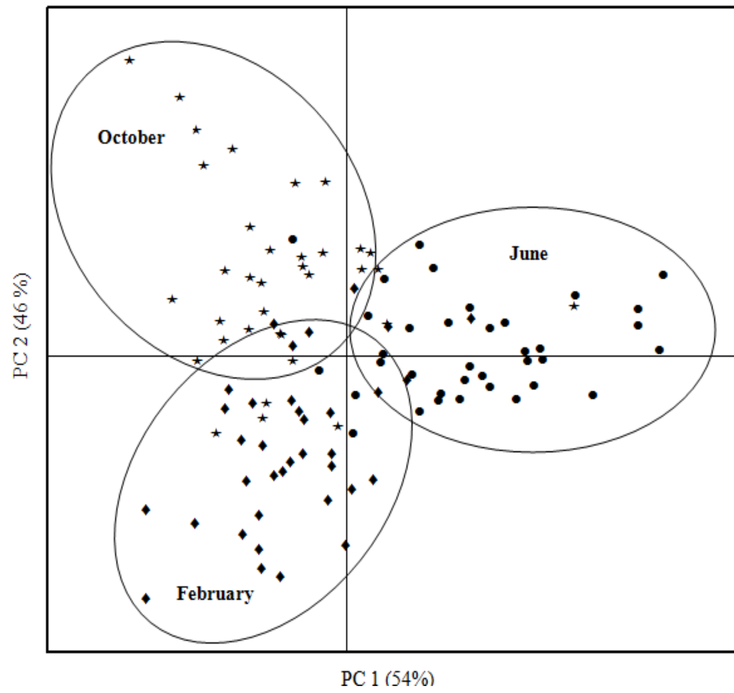


Fig. 2.3. The PCA plot of *nifH* DGGE profiles obtained from soil samples across three months.

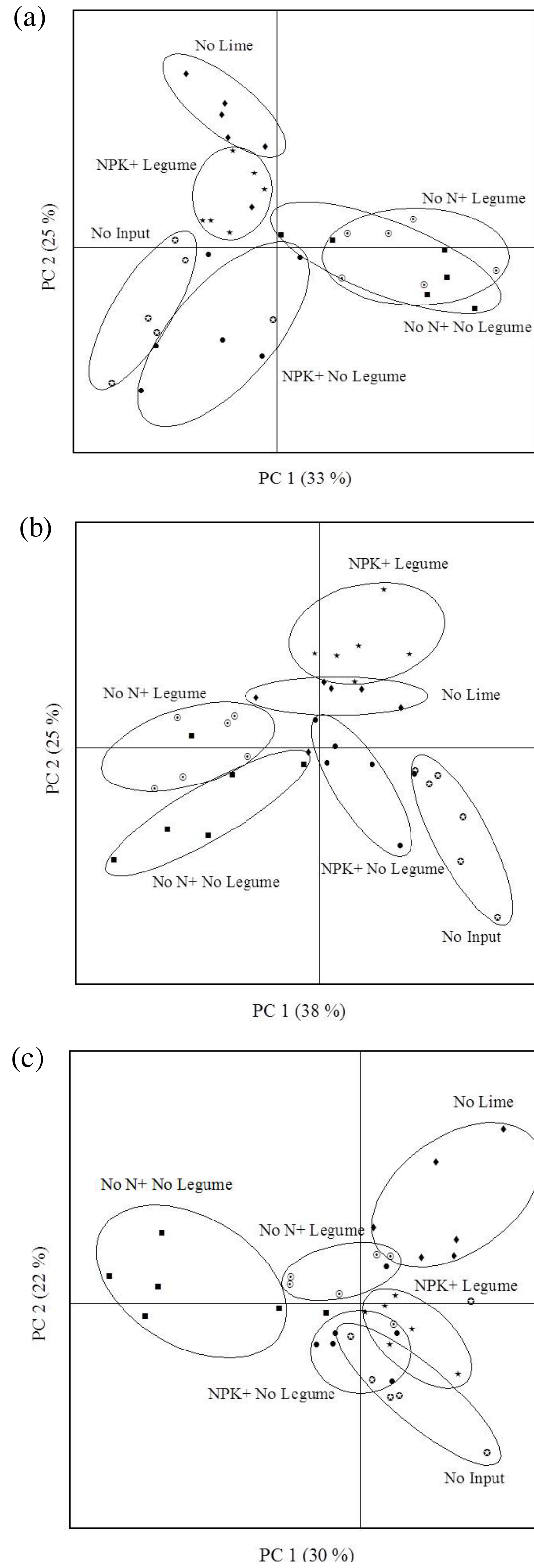


Fig. 2.4. PCA plots of *nifH* DGGE profiles by month: (a) June 2008; (b) October 2008; and (c) February 2009.

## Chapter III. Effect of glyphosate on glyphosate resistant soybean: symbiotic nitrogen fixation and the transgenetic *CP4 EPSPS* gene abundance in the rhizosphere

### Abstract

Most of the soybeans (*Glycine max* L. Merr.) now grown in the U.S. are the Roundup Ready® cultivar, genetically modified to be resistant to glyphosate herbicide. Concerns have been expressed that the application of glyphosate may negatively affect plant growth and nitrogen fixation in glyphosate resistant (GR) soybean. In this study, greenhouse and field experiments were conducted to examine the impacts of single and sequential glyphosate application on the growth and nitrogen fixation activity in GR soybean. GR soybean received zero, one, or two times foliar applications of glyphosate during the study period; the conventional cultivar did not receive any herbicide and served as a control. Plants were harvested two days after each glyphosate application. In addition to soybean growth parameters, the nitrogenase activity of root nodules and *nifH* gene abundance in the rhizosphere soil were determined using the acetylene reduction assay and quantitative polymerase chain reactions (qPCR), respectively. In the greenhouse experiment, glyphosate treated soybean had lower chlorophyll content, root mass, nodule mass, total plant nitrogen, and nitrogenase activity than the conventional cultivar, especially for the second harvest (V5 to V6 stage). However, no significant differences were observed in the field. For most of the parameters measured in this study, no significant differences were observed between the conventional cultivar and resistant cultivar without glyphosate application in both greenhouse and field experiments. The *nifH* gene

abundance in the rhizosphere of GR soybean was not affected by glyphosate application in either experiment. *In vitro* growth experiments using rhizobial isolates also showed that glyphosate inhibited pure cultures of rhizobia to different extents. The presence of the *CP4 EPSPS* gene in the soil was detected using qPCR. Although *CP4 EPSPS* gene transfer from the GR soybean to the soil was observed, the rate of glyphosate application had no effect on gene transfer.

## Introduction

Genetically modified glyphosate resistant crops have become an important tool in crop production practice and weed management. Glyphosate resistant soybean is engineered by the insertion of a transgene (*CP4 EPSPS*) from the *Agrobacterium* sp. strain CP4, which encodes a glyphosate-insensitive version of EPSPS (Duke et al., 2003b). Glyphosate, the active ingredient of Roundup®, is one of the most widely used herbicides in agriculture (Araújo et al., 2003). It inhibits the biosynthesis of aromatic amino acids (phenylalanine, tyrosine, and tryptophan) and leads to several metabolic disturbances, including the interruption of protein production, secondary product biosynthesis, and a general metabolic disruption of the phenylpropanoid pathway after reducing the biosynthesis of aromatic amino acids (Franz et al., 1997; Duke et al., 2003a).

The glyphosate resistant soybean is supposed to be resistant to glyphosate, but glyphosate application might still cause the soybean some injury, such as a reduction in its chlorophyll content under certain conditions (Reddy et al., 2000; Zobiole et al., 2010a; 2011). Glyphosate could also decrease the biomass of root/shoot/nodule and nodule numbers (King et al., 2001; Reddy and Zablotowicz, 2003; Kremer and Means, 2009; Zobiole et al., 2010d). Previous studies have indicated that the effect of glyphosate on the nitrogen fixation activity of soybean is not consistent (Zablotowicz and Reddy, 2007). The nitrogen content of glyphosate resistant soybean has been shown to be affected by glyphosate under different glyphosate rates, the growth stage of the soybean, and the soybean cultivar itself (King et al., 2001; Reddy and Zablotowicz, 2003).

Although many studies have shown the growth of glyphosate resistant soybean may be affected by glyphosate application, the adverse physiological effects on glyphosate resistant soybean treated by glyphosate are not fully understood (Reddy and Zablotowicz , 2003). One of

the most important impacts of glyphosate on the soybean plants concerns its biological nitrogen fixation. Glyphosate directly affects the rhizobial symbiont of soybean via the accumulation of hydroxybenzoic acids within the bacterial symbiont (Moorman, 1989; Moorman et al., 1992). Zablotowicz and Reddy (2004) concluded that glyphosate could inhibit the nodulation and nodule leghemoglobin content of glyphosate resistant soybean based on both greenhouse and field experiments and the nitrogenase activity of *Bradyrhizobium japonicum* could be affected. There is a potential risk of reducing the nitrogen fixation of glyphosate resistant soybean by the application of glyphosate, affecting its symbiont microorganism under natural conditions. Glyphosate can be transported to metabolic sinks such as plant roots, from which it is eventually released into the rhizosphere (Coupland and Caseley, 1979). Mijangos et al. (2009) suggested that microbial activity and diversity in the rhizosphere of glyphosate treated plants could be changed by glyphosate translocation and release from roots. Glyphosate has diverse effects on the ecology and biology of rhizosphere microorganisms when it is released into the rhizosphere of glyphosate resistant crops (Kremer and Means, 2009).

In the US alone, Roundup Ready® soybean was planted on about 74.3 million acres in 2010 (USDA-NASS, 2010). As the usage of glyphosate resistant crops increase, however, concerns have been raised regarding the potential glyphosate resistant gene transfer from plants to indigenous bacteria in the soil. The transgenes carried by transgenic plants can be released into the surrounding environment by roots, plant decomposition, or by pollen dispersal (Pontiroli et al., 2007). Saxena et al. (1999) reported that *Bt* toxin possessing the *cry1Ab* gene was released into the rhizosphere soil in root exudates from *Bt* corn. Glyphosate resistant crops could release transgenic DNA through their roots into the soil (Dunfield and Germida, 2004), which indicates

the possibility that indigenous bacteria in the soil might take up plant DNA containing the glyphosate resistant gene and incorporate the DNA into their genomes (Nielsen et al., 1997).

The *nifH* gene encodes for nitrogenase reductase (Fe protein subunit) of nitrogenase. Soil diazotrophs containing the *nifH* gene are the main source of nitrogen input in primary-production ecosystems except for symbiotic nitrogen fixing bacteria (Poly et al., 2001b). Many studies have suggested that the abundance of the *nifH* gene directly or indirectly depends on environmental conditions such as land use and management, nitrogen fertilizer, cultivar, soil pH, carbon/nitrogen source, soil organic matter, soil horizon, and seasonal change (Colloff et al., 2008; Coelho et al., 2009; Hayden et al., 2010; Levy-Booth and Winder, 2010; Jung et al., 2012). However, there has been no reported assessment of the effect of glyphosate on *nifH* gene abundance in the rhizosphere of glyphosate resistant soybean.

In this study, we assessed the effect of glyphosate application on the physiological characteristics of glyphosate resistant soybean. The responses of *nifH* gene and *CP4 EPSPS* gene copies to glyphosate in the rhizosphere soil of glyphosate resistant soybean were investigated. Given that the rhizobium associated with soybean is important for the growth of glyphosate resistant soybean, an experiment was set up to look into the effect of glyphosate on the growth of rhizobia.

## Materials and Methods

The Compass sandy loam soil collected from the E.V. Smith Research Center, Milstead, AL, was used in the greenhouse experiments. The soil pH is 6.8, which is appropriate for soybean. Soil test results showed that no additional P and K were needed and concentrations of

cations important for soybean growth were: Ca, 454; K, 140; Mg, 102; Fe, 5; Mo, < 0.1; Ni, <0.1 ppm.

**Greenhouse Experiment 1: The effect of glyphosate on glyphosate resistant soybean**

**Soil preparation and growth conditions**

Soil samples were collected and fumigated using Basamid, with the active ingredient being dazoment (1.2 gram/gallon), before planting. After about 10 days of fumigation, the soil was air dried for another 10 days and sieved to pass a 4 mm sieve. Greenhouse temperatures were maintained at 27 ± 1°C during the day and 24 ± 1°C at night using an evaporative cooling system. Natural daylight was supplemented with sodium vapor lamps to provide a total of 18 hours of illumination. Pots were rotated periodically to reduce variability of light exposure.

**Seed and glyphosate treatments**

Soybean cultivars 'Prichard RR' were obtained from the University of Georgia Foundation, Inc. Seeds were disinfected by immersing in 0.1% sodium hypochlorite (2% Chlorox bleach) for 2 minutes, washed several times using sterile distilled water, and air dried in a Class I biosafety cabinet. The sterile seeds were planted in plastic pots with an approximate soil volume of 3 L. Four seeds were sown at 3 cm depth in each pot. Each gram of seed was inoculated with approximately 1.5 g of Rhizo-stick® inoculant, containing $10^8$ viable *Bradyrhizobium japonicum* per gram of inoculant (Becker Underwood Inc., Ames, IA, USA) at planting. Soil was saturated with water after planting. At about 10 days after planting, soybean plants were thinned to one plant per pot. Plants were provided with water as needed.

Glyphosate (Roundup Ultra® Herbicide, Monsanto, St. Louis, Mo, USA) was applied at 1.68 kg a.e. $ha^{-1}$ using an indoor spray chamber equipped with an air-pressurized system at a volume of 30 gallons per acre at 32 psi using a single Teejet 8002 E flat fan nozzle. Each

application was as a postemergence over-the-top spray to plants. Three herbicide treatments consisted of a single application, a sequential application, and a control without herbicide, with four replications of each.

**Harvest descriptions**

Plants were harvested twice, at two days and at 14 days after the second glyphosate application (Table 3.1). Plants were watered 3 days before harvesting.

**Chlorophyll content** The chlorophyll content was measured using a SPAD meter immediately before harvesting. Three leaflets from the youngest fully developed trifoliolate leaf were measured from each plant. Three SPAD meter readings were taken from each leaflet and averaged.

**Separation of roots from soil** The whole plant with soil was removed from each pot. The shoot was cut at the cotyledon node with scissors and placed in a plastic bag. The root was recovered from the soil by gently shaking to remove loosely adhering soil and put into 150 mL sterile distilled water. Both root and shoot were placed in containers and transported to the lab on ice.

**Acetylene Reduction Assay**

Nitrogen fixation activity or nitrogenase activity in the soybean nodules was measured by an acetylene reduction assay. Acetylene ($C_2H_2$) can be reduced to ethylene ($C_2H_4$) by nitrogenase. The conversion of $C_2H_2$ to $C_2H_4$ was quantified by gas chromatography with a flame-ionization detector (GC-FID). Roots with nodules intact were washed using sterile water, dried by blotting, and incubated in 920 mL Mason jars. The jar lids were fitted with a 15 mm rubber stopper. Forty six milliliters of air (5% of total volume) were removed from each jar and replaced with an equal volume of acetylene. After 30 minutes of incubation at 20 ±1°C, the gas

sample was collected using a BD double-ended needle and a vacutainer (Becton Dickinson & Company, NJ, US) after equilibration for 5 min. An HP 5890 Series II (Agilent Technologies, Santa Clara, CA, USA) gas chromatograph equipped with a splitless inlet and a FID was used for gas analyses. A volume of 5 µL of gas sample was injected manually into a 60 m length × 0.32 mm diameter GS-GasPro column (Agilent Technologies, Santa Clara, CA, USA). The oven temperature was held constant at 50°C and helium gas was used as the carrier gas at a flow rate of 10 ml/min. Both injector and detector temperatures were set at 250°C. Two distinct peaks were observed, with $C_2H_4$ at about 2.05 min and $C_2H_2$ at about 2.85 min. Standard curves for ethylene and acetylene were prepared using serial dilutions of pure gas for each analysis and used to determine the acetylene reduction activity of the unknown samples.

**Biomass and total N**

After the acetylene reduction assay, the number of nodules and the fresh weights of shoots, roots, and nodules were recorded. All were oven dried at 60°C for 48 hours and the dry weights determined. The oven dried plant sections, including shoots and roots, were ground to pass a 1 mm sieve and total N content was determined by dry combustion.

**Rhizosphere soil sampling**

The soil attached to the roots that had previously been placed in sterile distilled water was sonicated in a FS28 sonicator (Fisher Scientific, Pittsburgh, PA, USA) for 6 min to remove rhizosphere and rhizoplane microorganisms. The suspension was then centrifuged at 10,000 rpm for 10 min at 4°C to collect the rhizosphere soil samples and the moisture content of each soil sample determined. Soil samples were stored at 4°C overnight prior to soil DNA extraction.

**Soil DNA extraction**

Total DNA was extracted from 1 g of moist rhizosphere soil using the MoBio Power Soil® DNA isolation kit (MoBio laboratories, Solana Beach, CA, USA) according to the manufacturer's instructions except that two replicates of each sample were loaded onto the spin filter membrane. The extracted DNA was quantified spectrophotometrically (NanoDrop®ND-1000, Thermo Scientific, Wilmington, DE, USA). The soil DNA was stored at -80° C until further analysis.

**Quantitative Real-Time PCR amplification for the *CP4 EPSPS* gene**

The detection and quantification of recombinant DNA of the glyphosate resistant soybean depended on the amplification of the junction between the chloroplast transit peptide element and the *CP4 EPSPS* gene (Lerat et al., 2005). Real-time PCR amplifications were performed using Applied Biosystems StepOne™ Real-Time PCR system (ABI, Carlsbad, CA, USA) with fluorescent dye SsoAdvanced SYBR Green Supermix (Bio-Rad laboratories, Hercules, CA, USA). The primer sets included the forward primer (CTP4-5) 5'-ATCAGTGGCTACAGCCTGCAT-3' and the reverse primer (CTP4-12) 5'-GAATGCGGACGG TTCCGGAAAG-3'corresponding to a 92 bp region (Lerat et al., 2005). Quantitative PCR of *CP4 EPSPS* gene was performed in a 20 µl reaction mixture containing: 5 µl template DNA (~3-15 ng /µl), 10 µl of SsoAdvanced SYBR Green Supermix (2×), 0.6 µl of the each primer (10 µM), and nuclease-free water. The initial denaturation step was at 98°C for 2 min prior to reaction step, followed by 40 cycles of 98°C for 5 s, 55°C for 30 s, and 72°C for 30 s. Melting curve analysis was performed as follows: 95°C for 15 s, 60°C for 30 s, and 95°C for 15 s. The range of the slopes and intercepts of the standard curves were from -3.33 to -3.34 and from 36.30 to 39.27, respectively. The efficiency of the reaction was around 99% and the $R^2$

value was better than 0.99. The limit of detection (LOD) of 8 copies per reaction and the lower limit of quantification (LLOQ) of 25 copies per reaction for *CP4 EPSPS* gene were determined according to the procedures described by Ermer and Miller (2005) and Shrivastava and Gupta (2013). The LLOQ corresponds to $3 \times 10^4$ copies/g of soil.

**Quantitative Real-Time PCR of the *nifH* Gene**

The abundance of *nifH* genes in soil was measured using an Applied Biosystems StepOne$^{TM}$ Real-Time PCR system. The degenerate oligonucleotide primers used in qPCR to amplify the *nifH* gene were the forward primer (PolF) 5’-TGCGAYCCSAARGCBGACTC-3’and the reverse primer (PolR) 5’- ATSGCCATCATYTCRCCGGA-3’corresponding to a 360 bp region between sequencing positions 115 and 476 (referring to the *Azotobacter vinelandii nifH* coding sequence[M20568]) (Poly et al, 2001a). The qPCR reaction mixture (20 µl) contained 5 µl of template extracted DNA (~3-15 ng /µl), 10 µl of SsoAdvanced SYBR Green Supermix (2×), 0.6 µl of the each primer (10 µM), and nuclease-free water. The initial denaturation step was at 95°C for 10 min prior to the reaction step, followed by 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. Melting curve analysis was performed as follows: 95°C for 15 s, 60°C for 30 s, and 95°C for 15 s. The range of the slopes and intercepts of the standard curves were from -3.27 to -3.40 and from 30.20 to 39.49, respectively. The efficiency of the reaction was around 99% and the $R^2$ value was better than 0.99. The limit of detection (LOD) of 20 copies per reaction and the lower limit of quantification (LLOQ) of 60 copies per reaction for *nifH* gene were determined according to the procedures described by Ermer and Miller (2005) and Shrivastava and Gupta (2013).

**Greenhouse Experiment 2**

Soil collected from the field was used directly without fumigation in this experiment. About 3300 grams of soil were packed into plastic pots (13 cm in diameter and 24 cm in height, Stuewe & Son Inc., OR, USA) with an approximate soil volume of 2.54 L to obtain a bulk density of about 1.3 g/cm$^3$. Three disinfected seeds of isogenic soybean cultivars 'Prichard Conventional' or 'Prichard RR' were planted in each pot. Each gram of seed was inoculated with approximately 1.5 g of Rhizo-stick® inoculants (containing $10^8$ viable *Bradyrhizobium japonicum* per gram) at planting. About 10 days after planting, the soybean plants were thinned to one plant per pot. Plants were watered as needed and each pot received 400 ml of tap water after emergence based on the water holding capacity of the soil. Growth conditions were the same as in Experiment 1. The herbicide treatments on GR soybean consisted of none, single application, and sequential application with four replications (Table 3.2). The conventional cultivar served as a control and did not receive any herbicide. Plants were harvested two days after the first and second glyphosate applications (Table 3.2). The growth parameter measurements were the same as described in Greenhouse Experiment 1 except for the following. The rhizosphere soil was sampled by gently shaking off the soil that attached to the roots. The roots were then put into wide-mouth glass jars containing 150 mL sterile distilled water and placed on a platform shaker at 150 rpm for 15 min at room temperature. The rhizosphere soil was collected by centrifugation of the root washings at 10,000 rpm for 10 min at 4°C. Total DNA was extracted from 0.2 g of moist rhizosphere soil using the MoBio Power Soil® DNA isolation kit following the manufacturer's instructions except for the first step: an additional incubation of bead tubes at 65°C for 10 min followed by 2 min of bead beating to limit DNA shearing (Lauber et al., 2010). A molecular beacon specifically designed for the CTP/EPSPS junction was used in

this study (Lerat et al., 2005). Quantitative real time PCR of the *CP4 EPSPS* gene in this experiment contained 5 µl template extracted DNA (~5-15 ng /µl), 10 µl of SsoFast Probe (2×) (Bio-Rad laboratories), 1 µl of each primer (7 µM), 1 µl of molecular beacon (5 µM) Fam-5'-CGCGATCATTTGCGGGCGGTTGCGGGCGATCGCG-3'-Dabcyl, 1 µg/µl Bovine Serum Albumin (Sigma-Aldrich, Saint Louis, MD, USA), and nuclease-free water in a 20 µl reaction mixture. The initial denaturation step was 3 min at 95°C prior to the reaction step, followed by 40 cycles of 95°C for 10 s and 60°C for 20 s. The range of the slopes and intercepts of the standard curves were from -3.23 to -3.35 and from 39.92 to 40.92, respectively. The efficiency of the reaction was around 99% and the $R^2$ value was better than 0.99. The limit of detection (LOD) of 4 copies per reaction and the lower limit of quantification (LLOQ) of 11 copies per reaction for *CP4 EPSPS* gene were determined. The LLOQ corresponds to $1 \times 10^3$ copies/g soil.

**Greenhouse Experiment 3**

The glyphosate application and harvesting times are shown in Table 3.2. All measurements were the same as described in Greenhouse Experiment 2 except for the acetylene reduction assay. In this experiment, the nodules were removed, dried in air for 20 min and then used for incubation. After 60 minutes incubation at 20 ± 1°C, the gas sample was collected by using a BD double-ended needle and a vacutainer after equilibration for 10 min. The carrier flow rate used for the GC/FID was 5 ml/min.

**Field Experiment**

Field experiments were carried out in 2013 at the E.V. Smith Research Center, Milstead, AL, USA, on a Compass sandy loam soil. The experiment was a randomized design with four replications. The plot size was 6 m × 11 m and row spacing was 0.9 m, providing four rows per plot.

Isogenic conventional and glyphosate-resistant Prichard soybean cultivars inoculated with Rhizo-stick® inoculants were planted on May 22, 2013. Glyphosate was applied at 1.68 kg a.e. $ha^{-1}$ 21 and 35 days after planting. Soybean plants were harvested two days after each glyphosate application. The third harvest was conducted 14 days after the second glyphosate application. The GR soybean received foliar applications of glyphosate zero, one, or two times during the study period. Weeds in the conventional soybean treatment were controlled by conventional herbicides. The experiment was managed according to the Alabama Agricultural Experiment Station recommendations.

**Effect of glyphosate on the growth of rhizobia**

*Bradyrhizobium japonicum* USDA 110 was obtained from the Agricultural Research Service Culture Collection of the USDA. The other two rhizobia were isolated from inoculants of Rhizo-Stick® and nodules of GR soybean. The isolation of rhizobia from the nodules was based on the previous methods (Somasegaran and Hoben, 1994). The bacteria were multiplied on yeast mannitol broth agar (YEM) plates for further use. All inocula for this experiment were prepared by inoculating the cultures into an AG broth medium. The cultures were incubated for 1 to 5 days at room temperature on a rotating shaker at 144 rpm. Late log phase cultures were centrifuged at 10,000 × rpm for 20 min, washed twice in sterile physiological saline (0.85% NaCl, w/v), and resuspended in the sterile physiological saline for further use.

Technical grade (purity ≥ 96%) glyphosate (*N*-(phosphonomethyl)glycine) was used to prepare 5 mM and 0.05 mM glyphosate stock solutions in sterilized distilled water, which were then filter-sterilized using 0.22 μm pore size filters. Each culture was inoculated into 10 mL AG media containing five glyphosate concentrations (0.0004, 0.004, 0.04, 0.4 and 1 mM) in triplicate (Table S1). Meantime, three test tubes without glyphosate served as controls. The initial

concentration was $10^5$ colony-forming units CFU/mL broth medium for *Bradyrhizobium japonicum* USDA 110, $10^6$ CFU/mL for the Rhizo-Stick isolate, and $10^5$ CFU/mL broth medium for the GR soybean isolate. Cultures were incubated in the dark for 2 to 5 days at room temperature on a reciprocal shaker at 144 oscillations per minute. The bacterial growth was monitored by measuring optical density (OD) at 560 nm periodically.

**Statistical analysis**

For the greenhouse experiments, the data were analyzed using the Glimmix procedure (SAS® system for Windows version 9.2, SAS Institute Inc., Cary, NC) for a randomized block design with four replicates. Statistical significant differences among treatments, harvesting time, and interaction of harvesting time and treatment were at $P = 0.05$. Gene copies were natural log transformed prior to data analysis.

## Results

**Greenhouse Experiment 1**

Statistical analyses showed that there was no significant effect of harvesting time and interaction of treatments and harvesting time on chlorophyll content, the growth of soybean, or *CP4 EPSPS* gene abundance at $P < 0.05$. Chlorophyll content (SPAD units) was significantly different between sequential glyphosate applied plants and non-treated plants. The lowest and highest chlorophyll contents were observed in sequential glyphosate applied plants and non-treated plants, respectively (Table 3.4). The chlorotic symptoms (yellow) observed may have been caused by chlorophyll damage. Although chlorophyll content was lower in the single glyphosate treated plants compared with the non-treated soybean plants, the effect of a single glyphosate application on the chlorophyll content of the soybean was not as significant as the

sequential application. The sequential glyphosate application had a significant effect on both the shoot and root mass of the soybean, with the dry mass of both shoot and root reduced compared with non-treated soybean. The single glyphosate application had a significant influence only on the root mass. However, glyphosate application frequency did not affect the shoot and root mass or the nodule mass and number (Table 3.4). Only sequential glyphosate treatment had a significant effect on the dry mass per nodule in comparison with non-treated plants (Fig. S3a). The nitrogen fixation activity of the GR soybean was determined by acetylene reduction assay after the second harvest. There was no significant difference observed among the treatments ($P < 0.05$). However, the non-treated glyphosate resistant soybean had the highest nitrogen fixation activity numerically compared to the treated plants (Fig. 3.1a). A significant interaction between *nifH* gene abundance and harvesting time was observed at $P < 0.05$. Furthermore, the *nifH* gene abundance was affected more by the sequential glyphosate application than either the single or no glyphosate application in the second harvest (Fig. 3.5a). No significant effects of glyphosate applications on the *CP4 EPSPS* gene abundance were observed (Fig. 3.6a).

**Greenhouse Experiment 2**

A single glyphosate application at 1.68 kg a.e./ha significantly affected the dry mass of nodule and mass per nodule in Harvest 1. The nodule dry mass of GR soybean treated with a single glyphosate application was decreased by 40% and 39% compared with the conventional soybean and non-treated GR soybean, respectively (Table 3.5). The single glyphosate application also led to 39% and 40% decreases in the mass per nodule of the GR soybean compared with the conventional soybean and non-treated GR soybean, respectively (Table 3.5). However, the dry mass of root and shoot, nodule numbers, and chlorophyll content was not significantly influenced by glyphosate application or soybean cultivar. No significant differences were

observed in the nitrogen fixation activities among the treatments (Fig. 3.1b). The total nitrogen content was not significantly influenced by a single glyphosate application ($P < 0.05$), but the percentage of total nitrogen content (Fig. 3.2a) and the C/N ratio (Fig. 3.3a) were affected by glyphosate application.

For Harvest 2 of Greenhouse Experiment 2, both single and sequential applications had significant effects on the dry mass of root and nodule, chlorophyll content, and nodule numbers compared with conventional soybean. However, no significant difference was observed between glyphosate treated plants and non-treated plants except for their chlorophyll contents (Table 3.5), which decreased 9%, 15%, and 12% in non-treated GR soybean, single glyphosate treated GR soybean, and sequential glyphosate treated GR soybean, respectively, compared with the conventional soybean (Table 3.5). The root dry mass was also significantly reduced by glyphosate relative to the conventional soybean. However, no effect of glyphosate on shoot dry mass was found. The non-treated GR soybean had 15% less dry nodule mass than the conventional soybean. Single and sequential glyphosate applications reduced the dry weight of nodules by 36% and 32%, respectively, in comparison to conventional soybean. The nodule numbers for conventional soybean were 24% higher than for non-treated GR soybean, and the nodule numbers of single and sequential glyphosate applied plants were 33% and 45% lower, respectively, relative to the conventional soybean (Table 3.5). The acetylene reduction assay showed that conventional soybean also had higher nitrogen fixation activity than GR soybean and there was a decreasing trend as the rate of glyphosate application increased (Fig. 3.1c). Significant effects of both glyphosate treatments on the total nitrogen content were observed (Fig. 3.2b). However, the percentage of nitrogen content in shoots was not affected by glyphosate application. Only the single glyphosate application had a significant effect on the percentage of

nitrogen content in the soybean roots. The C/N ratio was not affected by glyphosate application, but there was a difference observed between conventional soybean and non-treated GR soybean (Fig. 3.3b).

The abundance of *nifH* gene copies in the rhizosphere was not affected by the glyphosate applications and no relationship between the *nifH* gene abundance and the rate of glyphosate application was found (Fig. 3.5b and 3.5c). Some transfer of the *CP4 EPSPS* gene from GR soybean to rhizosphere soil was detected by real time PCR amplification in both harvests of Greenhouse Experiment 2, although it was not affected by the rate of glyphosate application (Fig. 3.6b and 3.6c). The abundance of the *CP4 EPSPS* gene found in the rhizosphere soil of the conventional cultivar was similar to that in unplanted soil, which served as a background control. Based on the concentration $2 \times 10^5$ ng/mL of plasmid DNA inserted by *CP4 EPSPS* gene was corresponding to $1\times 10^{11}$ gene copies, the background soil *CP4 EPSPS* gene concentration was around $7 \times 10^{-5}$ ng/ml in this study.

**Greenhouse Experiment 3**

Glyphosate applications in both harvests of Greenhouse Experiment 3 caused a decrease in the chlorophyll content of the GR soybean ($P < 0.05$) (Table 3.6). Differences were also observed in the chlorophyll contents between single glyphosate treated plants and sequential glyphosate treated plants in Harvest 2. For Harvest 1, the root/shoot/nodule mass and nodule number of soybean were not affected regardless of glyphosate application and soybean cultivar. For Harvest 2, the root mass of the GR soybean was lower than that of the conventional soybean, but there was no difference for the two rates of glyphosate application (Table 3.6). The single and sequential glyphosate applications did have an effect on the shoot mass and nodule mass of the GR soybean. The nodule number was not significantly affected by glyphosate application and

soybean cultivar in Harvest 2. Numerical reductions in the chlorophyll content, root/shoot/nodule mass, and nodule number of glyphosate treated soybean were observed compared with conventional soybean and non-treated soybean except for the nodule number in Harvest 1. Significantly different responses of the nitrogenase activity of GR soybean to the glyphosate applications at both rates were observed compared with conventional soybean and non-treated soybean in the second harvest ($P < 0.05$), but the rate of glyphosate had no effect (Fig. 3.1d and 3.1e). There were no differences in the nitrogenase activities of conventional soybean and non-treated GR soybean. For Harvest 1, the single glyphosate application had no effect on the total nitrogen content of the soybean. However, for Harvest 2, both rates of glyphosate applications caused reductions in the total nitrogen contents of the GR soybean compared with the conventional soybean and the non-treated GR soybean ($P < 0.05$) (Fig. 3.2c and 3.2d). The percentages of nitrogen contents in the roots and shoots of the soybean were not affected by either glyphosate application or soybean cultivar in both harvests, except for the effect of a single glyphosate application on the percentage of root nitrogen content in Harvest 2 (Fig. 3.4d; 3.4e; 3.4f; and 3.4g). There was no significant different in the C/N ratio observed in this experiment (Fig. 3.3c and 3.3d).

In the third greenhouse experiment, the abundance of *nifH* gene copies in the rhizosphere was not affected by either glyphosate application or soybean cultivar (Fig. 3.5d and 3.5e). The transfer of *CP4 EPSPS* gene from GR soybean plants to the soil was again detected, but the GR soybean had the same response to different rates of glyphosate applications in spite of the increasing trend (Fig. 3.6d and 3.6e). The abundance of the *CP4 EPSPS* gene found in the rhizosphere soil of the conventional cultivar was also similar to the background level in the soil.

### Field experiment

For Harvest 1 of the Field Experiment, the single application of glyphosate caused a decrease in the root mass of the GR soybean compared with the conventional soybean ($P < 0.05$) (Table 3.7). However, no influence of the glyphosate applications and soybean cultivar on other parameters measured, such as chlorophyll content, shoot mass, nodule mass, and nodule number was observed (Table 3.7). For Harvest 2, no significant differences were observed (Table 3.7). For Harvest 3, only chlorophyll content was lower in soybean with a single glyphosate application than conventional soybean (Table 3.7). There were no consistent numerical reductions in chlorophyll content, root/shoot/nodule mass, and nodule number across the three runs under the field conditions. The nitrogenase activity was not significantly affected by either glyphosate application or soybean cultivar in any of the three harvests (Fig. 3.7). Effect of glyphosate application on total nitrogen content of soybean was not observed (Fig. 3.7).

Under the field conditions, no differences in the responses of soybean or the *nifH* gene abundance to the glyphosate application and soybean cultivar were found (Fig. 3.8). However, consistent results for *CP4 EPSPS* gene transfer were obtained in both greenhouse and field experiments (Fig. 3.9).

### Effect of glyphosate on the growth of rhizobia

All three strains were affected by glyphosate treatment to different extents. Bacterial growth of all three strains was not inhibited completely, even at the highest concentrations of up to 1 mM (Fig. 3.10). The growth of rhizobia isolated from the Rhizo-stick® inoculums was reduced at all five glyphosate concentrations. There was a gradual decline of growth as concentrations of glyphosate increased from 0.0004 mM to 1 mM (Fig. 3.10a). Glyphosate at all concentrations except for 0.0004 mM reduced the growth of rhizobia isolated from nodules of

glyphosate resistant soybean (Fig. 3.10b). *Bradyrhziobium japonicum* USDA 110 was only affected by 0.4 mM and 1 mM glyphosate (Fig. 3.10c). Glyphosate concentrations of 0.0004 mM, 0.004 mM, and 0.04 mM did not exhibit any significant adverse effect on the growth of *Bradyrhziobium japonicum* USDA 110.

## Discussion

In this study, we used a glyphosate rate of 1.68 kg a.e./ha, which is higher than the recommended rate of 0.7 lb a.e./ac for soybean before harvest. The results of our greenhouse study indicated glyphosate treated soybean had a lower chlorophyll content, root mass, nodule mass, total plant nitrogen, and nitrogenase activity than the conventional genotype, especially for the late harvest (V5 to V6 stage). Previous researchers have indicated that some glyphosate resistant soybean cultivars can sustain injury from glyphosate application, although glyphosate resistant soybean is supposed to be resistant to glyphosate (Reddy et al., 2000; King et al., 2001). In the greenhouse experiment, the glyphosate applications at two rates had significant effects on the chlorophyll content compared with conventional soybean in all three experiments, except for Harvest 1 of Experiment 2. However, a numerical reduction in the chlorophyll content of glyphosate treated GR soybean relative to conventional soybean and non-treated GR soybean was observed throughout the greenhouse experiments. We did not find significantly different chlorophyll content between a single glyphosate application and sequential glyphosate applications in greenhouse experiments, except for Harvest 2 of Experiment 3. These results are in agreement with the study reported by Zobiole et al. (2010a), who also found the chlorophyll content to be lower in glyphosate treated plants compared with non-treated plants, but the difference between single and sequential glyphosate application was not significant. A numerical

reduction in the chlorophyll content of glyphosate treated soybean was observed with increasing glyphosate rate across all three greenhouse experiments except for one harvest in our study, which is consistent with the results reported by others (Pline et al., 1999; Zobiole et al., 2011), although it is not always the case that glyphosate application affects the chlorophyll content of soybean (Reddy et al., 2000; Reddy and Zablotowicz, 2003). In contrast to greenhouse experiments, this consistent response of chlorophyll content to the glyphosate application was not observed in the field experiment. However, in general, the chlorophyll content of the soybean in the field was higher than that of the soybean in the greenhouse in this study. The temperature may be an important factor here: the average temperature of 25°C in the greenhouse was higher than the average temperature of 23°C in the field. In our study, the highest and lowest temperatures were 36°C and 18 °C in the greenhouse, respectively, while in the field, they were 31°C and 13°C. Pline et al. (1999) indicated that glyphosate resistant soybean grown at higher temperatures had higher chlorophyll loss than those grown at lower temperatures. This may be due to an increased translocation of glyphosate to new meristematic areas and the secondary effects of glyphosate. Zobiole et al. (2010a) also suggested that higher temperatures in the greenhouse than in the field could contribute to lower chlorophyll contents. In addition to the different temperatures between greenhouse and field, the temperature across the three greenhouse experiments was not always consistent. Experiments 1 and 3 were conducted during the summer and so had higher temperatures than experiment 2, which was conducted during the winter. It is therefore reasonable that the average chlorophyll contents in greenhouse experiments 1 and 3 should be lower than in experiment 2.

Glyphosate at the recommended label rates does not inhibit the root growth of GR soybean (Bott et al., 2008). However, some reduction in the root mass of GR soybean has been

reported under greenhouse conditions (King et al., 2001). It is possible that the root mass can be affected by glyphosate application because the photosynthesis, water use efficiency, and micronutrients accumulations of soybean plant could all be decreased by glyphosate (Zobiole et al., 2010d). In the current study, the glyphosate application significantly reduced the root mass of soybean in the greenhouse experiments except for Harvest 1 in Experiments 2 and 3. This result is consistent with a previous report that indicated that the root mass was reduced by single and sequential glyphosate applications at the four-leaf and five-leaf stages (Zobiole et al., 2010a). As glyphosate rates increased, the root mass was reduced. Reddy et al. (2000) also found that sequential glyphosate applications could injure root growth. The reduction of root mass could affect soybean plant by limiting the availability of nutrients and water from the soil and reducing the number of beneficial microorganisms attached to the soybean roots, including symbiotic nitrogen fixing bacteria. However, Zobiole et al. (2010e) found no differences in the root mass of soybean between single and sequential glyphosate applications. Under field conditions, we did not observe any effect of glyphosate application on the root mass of the soybean, possibly because the variability in the field was considerably higher than in the greenhouse with regard to soil conditions, weather, and other environmental factors. There was also no guarantee of extracting the complete root system when we sampled in the field. Reddy and Zablotowicz (2003) indicated that the root mass of GR soybean was unaffected by glyphosate regardless of the number of applications and formulation in the field. Zobiole et al. (2010a; 2010b) also suggested that the presence of glyphosate resistant genes had no effect on the root mass compared to conventional soybean. In our study, we found no effect of the soybean cultivar on the root mass. In contrast to our results, a greater reduction in the root mass of soybean due to glyphosate at the early growth stage (V2 stage) was observed than at the late stage (V6 stage)

was observed by Zobiole (2010c). This could be because this soybean cultivar had a better capacity for recovery from stress later after glyphosate application under contain conditions.

The nodule mass can be used simply as an indicator of biological nitrogen fixation in soybean. King et al. (2001) indicated that applying glyphosate at 1.68 kg a.e. $ha^{-1}$ had no consistent effect on the nodule mass and Bellaloui et al. (2008) also found that there was no effect of glyphosate application on the nodule mass. However, our data suggest that glyphosate application led to a reduction in the nodule mass in all three greenhouse experiments, and most of these experiments also showed a significant reduction in the nodule mass of glyphosate treated soybean compared with conventional soybean. This impact of glyphosate application on the nodule mass of GR soybean is supported by many other studies (Reddy and Zablotowicz, 2003; Zobiole et al., 2010a; 2010d). Zobiole et al. (2011) suggested that the reduction in the nodule mass was related either to the toxicity of glyphosate or its intermediates to nodule formation or to decreased Ni, which is important for biological nitrogen fixation. In our greenhouse study, we did not find that increased glyphosate rates significantly reduced the nodule mass of GR soybean, which is in agreement with the findings of Zobiole et al. (2010a). In addition, we found the GR soybean always had a lower nodule mass compared with conventional soybean. Kremer et al. (2009) also found that nodulation was consistently lower in glyphosate resistant soybean compared with conventional soybean. However, the effects of glyphosate application and soybean cultivar on the nodule mass of soybean were not observed in the field experiment in our study, probably because of the complex environmental conditions.

Effects of glyphosate on shoot mass and nodule number did not show a consistent trend in our greenhouse experiments. The inconsistent effect of glyphosate application on the shoot mass of soybean has also been reported by other researchers (Reddy et al., 2000; 2003; 2004;

King et al., 2001; Bellaloui et al., 2008; Zobiole et al., 2010a; 2010d; 2011). Under field conditions, our results indicated that glyphosate application and soybean cultivar had no effect on the shoot mass and nodule number of soybean.

Glyphosate has a minimal effect on the nitrogen content of glyphosate resistant soybean, although some chlorosis has been observed (Bellaloui et al., 2006). The label use rates of glyphosate in a glyphosate resistant soybean have minimal effects on leaf nitrogen (Zablotowicz and Reddy, 2007). The GR soybean treated by glyphosate had a significantly lower total nitrogen content compared with conventional soybean at the second harvest in our greenhouse experiments. However, there was no significant difference in the total nitrogen content observed between the two rates of glyphosate application. Most of our results suggested that a numerical reduction in the total nitrogen content in the treated GR soybean was observed compared to the conventional soybean and non-treated GR soybean in the greenhouse experiments. The soybean cultivar had no effect on the total nitrogen content in this study. King et al. (2001) showed that the total nitrogen content and shoot/root nitrogen content of GR soybean were reduced by glyphosate application. The soybean with glyphosate applications had a lower level of total nitrogen in their shoots compared to non-treated plants (Reddy et al., 2000). Nevertheless, Reddy and Zablotowicz (2003) indicated that glyphosate resistant soybean at the fifth trifolioliate stage with glyphosate application had a higher nitrogen content than non-treated controls because of the recovery of the stressed soybean after glyphosate treatments.

The soybean root nodule is an organelle specialized for biological nitrogen fixation. The nitrogenase activity of root nodules was measured by acetylene reduction assay in this study. Our results indicated that the glyphosate treated GR soybean had lower nitrogenase activity compared with conventional soybean in the greenhouse experiments. The conventional soybean had the

highest nitrogenase activity. A significant reduction in the nitrogenase activity of GR soybean as a result of the glyphosate applications was observed at the second harvest. The soybean at the later growth stage had higher nitrogenase activity than at the early growth stage. This result was in accord with the total nitrogen content; the higher nitrogen content could be generally related to the higher nitrogenase activity of the root nodules. However, the increased glyphosate frequency had no effect on the nitrogenase activity of the soybean. Bellaloui et al. (2006) indicated that glyphosate had negative effects on nitrogen fixation, significantly decreasing nitrogenase activity. The reason for this transient decrease in nitrogenase activity in glyphosate resistant soybean could be that glyphosate has a direct effect on the soybean nitrogen fixing symbiont and an indirect effect on the host plant (Moorman, 1989). Some strains of soybean rhizobial symbionts, *Bradyrhizobium japonicum,* possess a glyphosate sensitive enzyme so that glyphosate could interrupt aromatic amino acid synthesis and disturb the energy drain in the organisms (Fischer et al., 1986). In contrast to the results of our greenhouse experiments, there was no effect of glyphosate application on the nitrogenase activity of root nodules in the field experiment. An inconsistent effect of glyphosate on acetylene reduction activity was also observed by Zablotowicz and Reddy (2004; 2007). King et al. (2001) indicated that the non-treated plants had greater nitrogenase activity compared with the glyphosate treated soybean. However, glyphosate treated soybean had the highest nitrogenase activity one week later and there was no significant effect of glyphosate on the nitrogenase activity observed the next year. An assessment of the response of nitrogenase activity to glyphosate application conducted at the USDA Southern Weed Science Research Unit farm in 2002 found a large variance in the nitrogenase activity among plots. Under our experimental conditions, no initial inhibition of nitrogenase activity by glyphosate was observed, but after the second glyphosate application the

nitrogenase activity of GR soybean dropped below that of the conventional soybean. This may be because glyphosate was translocated from the leaves to the nodules and accumulated, affecting the enzyme content and leading to metabolic disturbance. Our results suggest that the effect of glyphosate on nitrogenase activity probably depends on the rate of glyphosate application and the growth stage of the soybean.

Since the introduction of genetically modified crops, the assessment of the potential environmental impact of genetically modified crops has been ongoing. Many previous studies have indicated that transgenic plants could release transgenes into the surrounding environment via their roots, plant decomposition, or by pollen dispersal (Ellstrand, 1988; Widmer et al., 1996; 1997; Eastham and Sweet, 2002; Meier and Wackernagel, 2003; Watrud et al., 2004; Lerat et al., 2005; Pontiroli et al., 2007). Widmer et al. (1996) reported that genetically modified crops could lead to the release of trangenes into the soil, raising concerns regarding the potential transfer of glyphosate resistant genes carried by glyphosate resistant crops to the soil and its indigenous bacteria. de Vries et al. (2003) found that plant roots can release DNA, either as free molecules or within plant tissue material into the soil during plant growth. They identified recombinant DNA from transgenic potato plants in the rhizosphere soil. In our study, the *CP4 EPSPS* gene, a marker gene encoding the EPSPS enzyme, was detected and quantified in the rhizosphere soil of glyphosate resistant soybean. The transfer of *CP4 EPSPS* genes from GR soybean to the surrounding soil was detected in both greenhouse and field experiments. This indicates that there may be some glyphosate resistant genes transfer from glyphosate resistant plants to rhizosphere soil via releases from roots. Transgenes released from root cells, tissues, and root exudates have also been detected by other studies (Dunfield and Germida, 2004; Pontiroli et al., 2007). *Bt* toxin containing an inserted truncated *cry1Ab* gene was released into the rhizosphere soil in root

exudates from *Bt* corn, along with other proteins normally present in root exudates (Saxena et al. 1999; Saxena and Stotzky, 2000), and genetically modified *Bt* corn could release the *Bt* toxin from its root throughout the entire growth of a crop. The glyphosate resistant gene from GR soybean has been detected in the leachate water (Gulden et al., 2005). Lerat et al. (2007) reported that recombinant DNA from glyphosate resistant soybean and corn was detected in the field over a full growing season although no differences were observed between herbicide treatments. Our data also showed that the frequency of glyphosate application had no significant effect on the *CP4 EPSPS* gene abundance. Duke et al. (2003b) demonstrated that the CP4 EPSPS was either not inhibited or minimally inhibited by glyphosate treatment.

In our study, the *CP4 EPSPS* gene was detected in the rhizosphere of the conventional soybean cultivar in both greenhouse and field experiments. However, the levels found in the rhizosphere were similar to those in unplanted soils used in the greenhouse and soil collected outside the field plots. Fate of this transgene in the soil environment should be investigated further.

Since the transgenes of plants could be detected in the soil, the ecological consequences must be carefully considered once genetically modified genes enter the soil, based on our results. One of the most important reasons for this is the subsequent uptake in soil by indigenous bacteria, which could develop the natural ability to accept novel genetic elements by natural transformation (Lorenz and Wackernagel, 1994). Several researchers have attempted to evaluate the natural transformation of plant DNA to native soil microorganisms under field conditions, but evidence has yet been found. Widmer et al. (1997) indicated the possible transfer frequency of transgenes from plants to microorganisms in the soil might be very low. The horizontal gene transfer from plants to bacteria under field conditions was not supported (Gebhard and Smalla,

1999). There was no transfer of the *CP4 EPSPS* gene from glyphosate resistant soybean to root associated *B. japonicum* by natural transformation under either field or laboratory conditions (Isaza, 2009; Isaza et al., 2011). In general, there are barriers to horizontal gene transfer from plants to bacteria in soil, including spatial and temporal localization of available DNA and competent bacterial cells, gene transfer, establishment and expression of plant DNA, and selection of bacterial transformants (Nielsen et al., 1998). Several studies have suggested some bacteria take up transgenes from plants under certain condition (Ceccherini et al., 2003). The natural transformation of bacteria in soil is known to be stimulated by compounds exuded from plants, including carbon sources and inorganic salts (Nielsen and van Elsas, 2001). Kay et al. (2002) concluded that gene transfers could take place between transgenic plants and bacteria under natural conditions, at least without certain natural barriers. The natural indigenous microorganisms in soil could take up and incorporate the transgenes carried by genetically modified plants into their genomes if homologous sequences exist in the recipient cell (de Vries et al., 2001).

We found no reports in the literature of the effect of glyphosate on the *nifH* gene. In our study, the qPCR data indicated that the glyphosate application and soybean cultivar had no impact on the abundance of the *nifH* gene in either the greenhouse or the field study. One possible interpretation of this finding is that diazotrophic bacteria, including free-living nitrogen fixing bacteria, associative nitrogen fixing bacteria, and some dead nitrogen fixing bacteria, were detected during the determination of the abundance of the *nifH* gene. Detection of the *nifH* gene abundance does not allow the separation of *nifH* genes associated with live and dead cells. At present, we need to pay more attention to the effect of glyphosate on the diazotrophic community associated with soybean, so it is necessary to first study the effect of glyphosate on the symbiotic

nitrogen fixing bacteria in the soybean nodule before moving on to consider all the nitrogen fixing bacteria in the soil. In addition, future research could focus on improving the method used for determining the nitrogenase gene based on our results. Measuring the abundance of the *nifH* gene by qPCR, as in this study, can only tell us about the existence of *nifH* genes, but the presence of *nifH* genes does not always mean that diazotrophic bacteria could actually express the active nitrogenase enzyme, which could be regulated at the transcriptional level (Masepohl and Klipp, 1996). It is vital to know whether diazotrophic bacteria are actively fixing nitrogen in the natural environment when determining the impact of glyphosate on the diazotrophic microbial community. In addition to the acetylene reduction assay used for detecting the nitrogenase activity in this study, reverse transcription PCR (RT-PCR) could be used to detect the actively expressing *nifH* genes by determining the gene expression (Zani et al., 2000). It would also be interesting to identify a genetic marker or a method, which is more suitable for detecting symbiotic nitrogen fixing associated with soybean in the environment.

The growth of rhizobial strains in symbiosis with GR soybean was evaluated in the present study. All three rhizobial strains were inhibited by glyphosate at 1 mM. The rhizobia isolated from the Rhizo-Stick inoculant and the GR soybean was more sensitive to glyphosate than the *B. japonicum* USDA 110. Our results suggest that rhizobial strains respond to glyphosate differently. Many previous studies have also indicated that glyphosate has different effects on the growth of rhizobia (Jaworski, 1972; Moorman, 1986; Moorman et al., 1992; De Maria et al., 2006). Hernandez et al. (1999) suggested that the sensitivity of *B. japonicum* to glyphosate was an interstrain characteristic. Moorman (1986) indicated that glyphosate used at recommended rates was not sufficiently toxic to the survival of *B. japonicum* populations in soil. *B. japonicum* could be affected by repeated applications of glyphosate to glyphosate resistant

soybean. After glyphosate application at 1.12 kg/ha, an average of 1 mg/kg of glyphosate was expected in the surface 7 cm of soil. In addition, the concentration of glyphosate accumulated in the bulk root tissue was approximately from 0.06 to 0.3 mM after a single application of 0.5 kg/ha (Honegger et al., 1986). In our study, glyphosate at 1.68 kg a.e./ha was applied, which was expected to accumulate more than 0.06 - 0.3 mM in the root of the glyphosate resistant soybean. McWhorter et al. (1980) indicated that higher rates or repeated glyphosate applications could lead to much greater glyphosate accumulations in metabolic sinks such as root nodules. Reddy and Zablotowicz (2003) concluded that glyphosate can be detected in the nodules of glyphosate resistant soybean regardless of the number of applications and formulations, with the concentration of glyphosate ranging from 39 to 147 ng $g^{-1}$ nodule dry weight. It is not certain that to what extent the glyphosate rate used in our study affected the rhizobial symbiosis in GR soybean because we did not determine the concentration of glyphosate in the soybean nodules. Nevertheless, the potential risk of glyphosate on the growth of rhizobia exists.

## Conclusions

The results presented in this chapter demonstrate that the negative effects of glyphosate application on the chlorophyll content, nitrogenase activity, and growth of GR soybean can be observed in the greenhouse. Under these controlled conditions, the GR soybean was more sensitive to glyphosate application. In contrast, the field experiments experienced a more complicated environment with greater variability in the growing conditions, so the effect of the glyphosate application was less apparent and failed to conclusively show a negative impact on the GR soybean. No significant differences were observed between the conventional cultivar and resistant cultivar without glyphosate application in this study. The transfer of *CP4 EPSPS* genes

from GR soybean to the soil was detected consistently throughout the study, so the incorporation of transgenes by indigenous bacteria and other plants in the soil is a serious concern. In addition, the results of the effect of glyphosate on the rhizobial growth suggested that the inhibition of rhizobia by glyphosate should be considered when using glyphosate for weed control. The detection of the *nifH* gene, which is important for biological nitrogen fixation, indicated that glyphosate application had no effect on the existence of the *nifH* gene in the environment. It is not surprising that we failed to observe any differences in the *nifH* gene copies between glyphosate applications over this short- term experiment. However, future experiments utilizing more specific and effective approaches such as reverse transcription-PCR should be used to investigate diazotrophic bacteria actively expressing the *nifH* gene in the soil.

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Table 3.1. Glyphosate treatment and harvest timings for Greenhouse Experiment 1

| Treatment | Rate (kg a.e./ha) | Application timing | | Harvest timing （DAP） | |
|---|---|---|---|---|---|
| | | DAP[a] | Growth stage | 1st | 2nd |
| None | 0 | - | - | 36 | 48 |
| Single | 1.68 | 20 | V3 | 36 | 48 |
| Sequential | 1.68+1.68 | 34 | V5-V6 | 36 | 48 |

[a] DAP, days after planting.

Table 3.2. Glyphosate treatment and harvest timings for Greenhouse Experiments 2 and 3

| Treatment | Rate (kg a.e./ha) | Application timing | | Harvest timing (DAP) | |
|---|---|---|---|---|---|
| | | DAP[a] | Growth stage | 1st | 2nd |
| *Experiment 2* | | | | | |
| Conv[b] | 0 | - | - | 21 | 31 |
| None | 0 | - | - | 21 | 31 |
| Single | 1.68 | 19 | V2-V3 | 21 | 31 |
| Sequential | 1.68 + 1.68 | 29 | V5 | - | 31 |
| *Experiment 3* | | | | | |
| Conv | 0 | - | - | 24 | 32 |
| None | 0 | - | - | 24 | 32 |
| Single | 1.68 | 22 | V3-V4 | 24 | 32 |
| Sequential | 1.68 + 1.68 | 30 | V5-V6 | - | 32 |

[a] DAP, days after planting.

[b] Conv, conventional soybean as a control

Table 3.3. Glyphosate treatment and harvest timings for the Field Experiment

| Treatment | Rate | Application timing | | Harvest timing (DAP) | | |
|---|---|---|---|---|---|---|
| | (kg a.e./ha) | DAP[a] | Growth stage | 1st | 2nd | 3rd |
| Conv[b] | 0 | - | - | 23 | 37 | 49 |
| None | 0 | - | - | 23 | 37 | 49 |
| Single | 1.68 | 21 | V2 | 23 | 37 | 49 |
| Sequential | 1.68 + 1.68 | 35 | V5 | - | 37 | 49 |

[a] DAP, days after planting.

[b] Conv, conventional soybean as a control

Table 3.4. Effect of glyphosate application on chlorophyll content and growth of glyphosate resistant soybean in Greenhouse Experiment

| Treatment | Dwt_Root (g/plant) | Dwt_Shoot (g/plant) | Chlorophyll (SPAD unit) | Nodule No./plant | Nodule mass (mg/plant) | Mass/nodule (mg) |
|---|---|---|---|---|---|---|
| None | 1.37 a | 4.68 a | 24.2 a | 53 a | 140.00 a | 2.58 a |
| Single[1] | 1.12 b | 4.18 ab | 22.5 ab | 50 a | 136.25 a | 2.86 ab |
| Sequential[2] | 1.05 b | 3.91 b | 18.0 b | 42 a | 161.25 a | 3.90 b |
| SE[3] | 0.07 | 0.22 | 1.92 | 7.32 | 27.08 | 0.33 |

[1]Single: glyphosate applied foliarly at 1.68 kg a.e./ha 20 days after planting.
[2]Sequential: glyphosate applied foliarly at 1.68 kg a.e./ha 20 and 34 days after planting.
[3]SE: Standard error
[*] LS-mean within a column followed by the different letters signifies a significant difference at $P < 0.05$

Table 3.5. Effect of glyphosate application on chlorophyll content and growth of glyphosate resistant soybean in Greenhouse Experiment 2

| Treatment | Dwt_Root (g/plant) | Dwt_Shoot (g/plant) | Chlorophyll (SPAD unit) | Nodule No./plant | Nodule mass (mg/plant) | Mass/nodule (mg) |
|---|---|---|---|---|---|---|
| *Harvest 1* | | | | | | |
| Conv [1] | 0.14 a | 0.44 a | 18.01 a | 13 a | 29.38 a | 2.37 a |
| None [2] | 0.17 a | 0.52 a | 19.52 a | 13 a | 29.18 a | 2.43 a |
| Single [3] | 0.14 a | 0.43 a | 17.78 a | 12 a | 17.68 b | 1.45 b |
| SE | 0.02 | 0.04 | 0.85 | 1.30 | 2.33 | 0.28 |
| *Harvest 2* | | | | | | |
| Conv | 0.52 a | 1.56 a | 26.27 a | 51 a | 169.2 a | 3.45 a |
| None | 0.47 ab | 1.53 a | 23.78 b | 39 ab | 143.4 ab | 3.69 a |
| Single | 0.33 b | 1.22 a | 22.27 b | 34 b | 108.2 b | 3.51 a |
| Sequential [4] | 0.33 b | 1.19 a | 23.05 b | 28 b | 113.7 b | 4.26 a |
| SE[5] | 0.05 | 0.09 | 0.59 | 5.33 | 12.10 | 0.49 |

[1]Conv: conventional soybean
[2]None: glyphosate resistant soybean without glyphosate application
[3]Single: glyphosate applied foliarly at 1.68 kg a.e./ha 19 days after planting.
[4]Sequential: glyphosate applied foliarly at 1.68 kg a.e./ha 19 and 29 days after planting.
[5]SE: Standard error
[*] LS-means within a column followed by the different letters signifies a significant difference at $P < 0.05$.

Table 3.6. Effect of glyphosate application on chlorophyll content and growth of glyphosate resistant soybean in Greenhouse Experiment 3

| Treatment | Dwt_Root (g/plant) | Dwt_Shoot (g/plant) | Chlorophyll (SPAD unit) | Nodule No./plant | Nodule mass (mg/plant) | Mass/nodule (mg) |
|---|---|---|---|---|---|---|
| *Harvest 1* | | | | | | |
| Conv [1] | 0.42 a | 1.51 a | 17.32 a | 45 a | 122.90 a | 2.75 a |
| None [2] | 0.41 a | 1.53 a | 17.31 a | 50 a | 116.10 a | 2.36 a |
| Single [3] | 0.40 a | 1.49 a | 14.93 b | 52 a | 111.10 a | 2.13 a |
| SE | 0.02 | 0.07 | 0.47 | 3.38 | 6.35 | 0.15 |
| *Harvest 2* | | | | | | |
| Conv | 1.25 a | 3.98 a | 18.21 a | 159 a | 462.6 a | 2.93 a |
| None | 1.04 b | 3.54 a | 16.97 a | 140 a | 390.7 a | 2.95 a |
| Single | 0.81 c | 3.04 b | 13.66 b | 131 a | 351.5 a | 2.75 a |
| Sequential [4] | 0.88 bc | 3.23 a | 9.86 c | 112 a | 329.5 b | 2.96 a |
| SE[5] | 0.04 | 0.18 | 0.77 | 14.75 | 27.70 | 0.23 |

[1]Conv: conventional soybean
[2]None: glyphosate resistant soybean without glyphosate application
[3]Single: glyphosate applied foliarly at 1.68 kg a.e./ha 22 days after planting.
[4]Sequential: glyphosate applied foliarly at 1.68 kg a.e./ha 22 and 30 days after planting.
[5]SE: Standard error
[*] LS-means within a column followed by the different letters signifies a significant difference at $P < 0.05$

Table 3.7. Effect of glyphosate application on chlorophyll content and growth of glyphosate resistant soybean in the Field Experiment

| Treatment | Dwt_Root (g/plant) | Dwt_Shoot (g/plant) | Chlorophyll (SPAD unit) | Nodule No./plant | Nodule mass (mg/plant) | Mass/nodule (mg) |
|---|---|---|---|---|---|---|
| *Harvest 1* | | | | | | |
| Conv [1] | 0.19 a | 0.73 a | 23.06 a | 28 a | 43.80 a | 1.60 a |
| None [2] | 0.18 a | 0.71 ab | 23.90 a | 36 a | 46.88 a | 1.33 a |
| Single [3] | 0.19 a | 0.62 b | 21.27 a | 32 a | 48.44 a | 1.53 a |
| SE | 0.02 | 0.06 | 1.08 | 4.93 | 6.48 | 0.11 |
| *Harvest 2* | | | | | | |
| Conv | 0.46 a | 2.58 a | 25.16 a | 52 a | 127.6 a | 2.44 a |
| None | 0.55 a | 2.68 a | 27.00 a | 62 a | 131.7 a | 2.17 a |
| Single | 0.47 a | 2.19 a | 25.19 a | 60 a | 138.7 a | 2.34 a |
| Sequential [4] | 0.44 a | 2.52 a | 25.52 a | 52 a | 119.1 a | 2.57 a |
| SE[5] | 0.05 | 0.22 | 1.11 | 10.48 | 18.87 | 0.26 |
| *Harvest 3* | | | | | | |
| Conv | 0.95 a | 7.32 a | 29.25 a | 69 a | 216.4 a | 3.21 a |
| None | 0.86 a | 6.62 a | 26.91 ab | 80 a | 230.5 a | 2.89 a |
| Single | 0.93 a | 6.57 a | 26.35 bc | 87 a | 250.0 ab | 2.88 a |
| Sequential | 0.72 a | 5.29 a | 26.60 ab | 70 a | 163.1 ac | 2.40 a |
| SE | 0.12 | 0.69 | 0.74 | 7.35 | 17.15 | 0.21 |

[1]Conv: conventional soybean
[2]None: glyphosate resistant soybean without glyphosate application
[3]Single: glyphosate applied foliarly at 1.68 kg a.e./ha 21 days after planting.
[4]Sequential: glyphosate applied foliarly at 1.68 kg a.e./ha 21 and 35 days after planting.
[5]SE: Standard error
[*] LS-means within a column followed by the different letters signify a significant difference at $P < 0.05$.

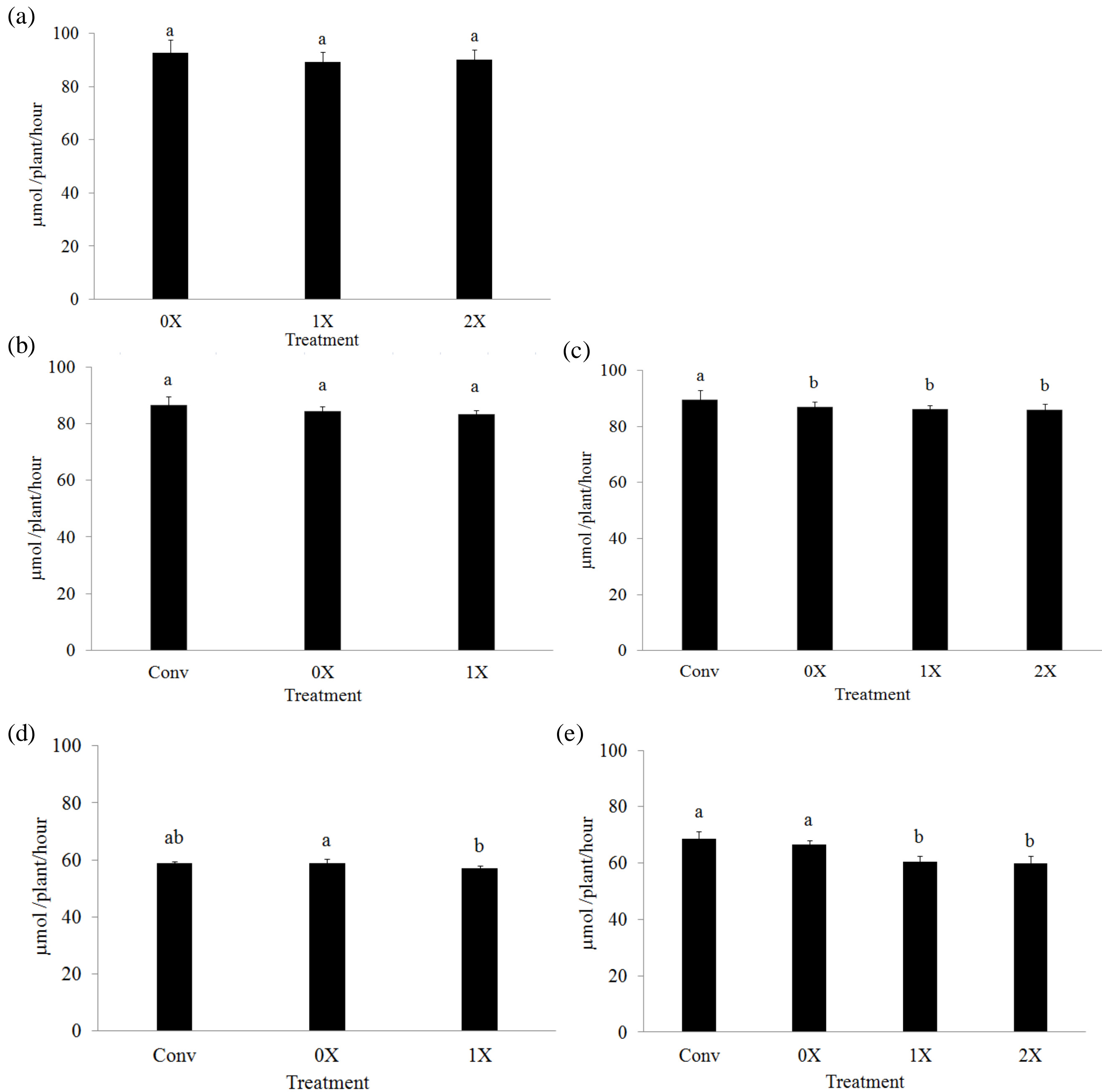


Fig. 3.1. Effect of glyphosate on the nitrogen fixation activity determined by ARA. Data are represented by LS-means (± standard deviation). (a) Greenhouse Experiment 1; (b) and (c) Harvests 1 and 2 in Greenhouse Experiment 2; (d) and (e) Harvests 1 and 2 in Greenhouse Experiment 3.

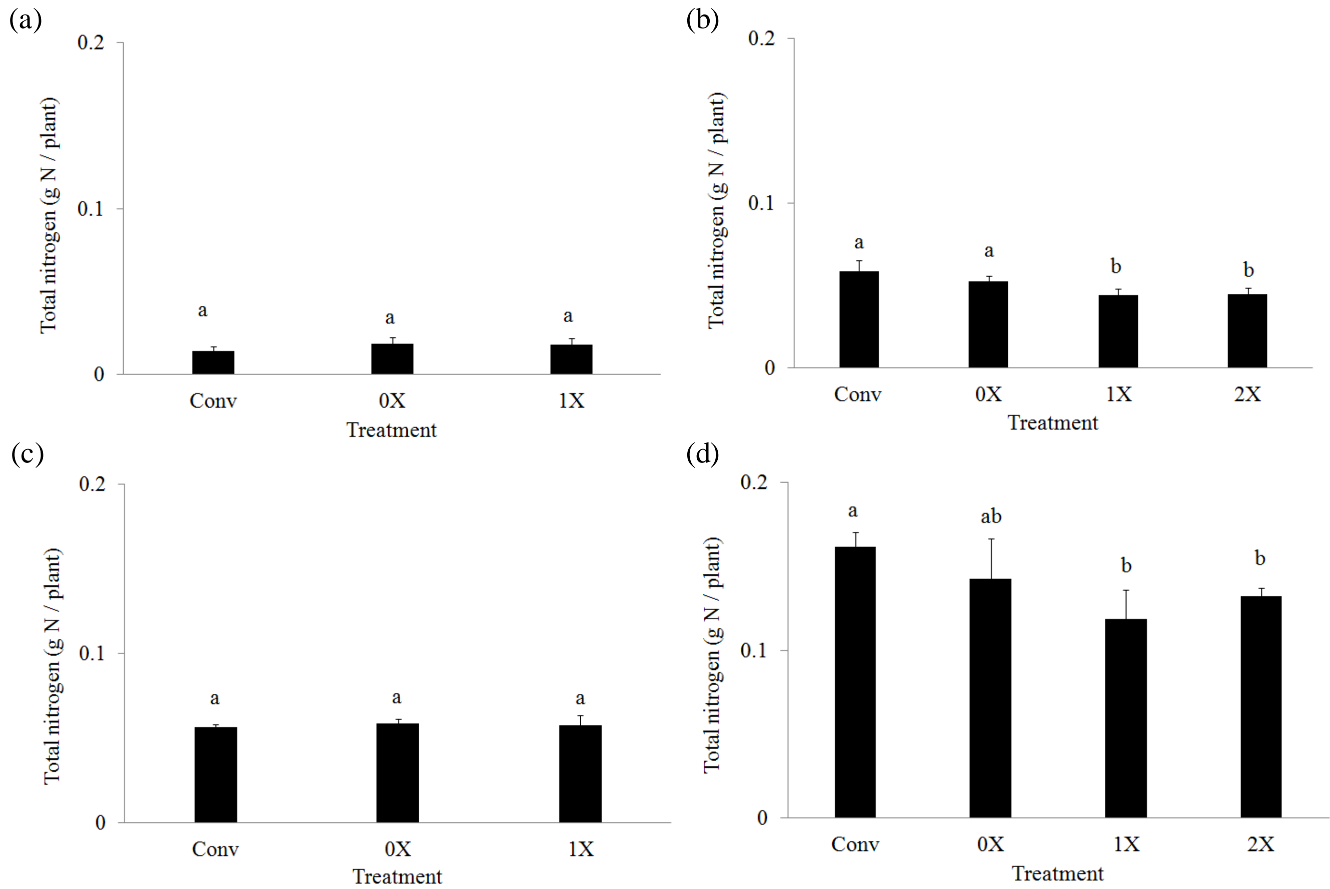


Fig. 3.2. Effect of glyphosate on total nitrogen content of soybean. Data are represented by LS-means (± standard deviation). (a) and (b) are for Harvests 1 and 2 in Greenhouse Experiment 2; (c) and (d) are Harvests 1 and 2 in Greenhouse Experiment 3.

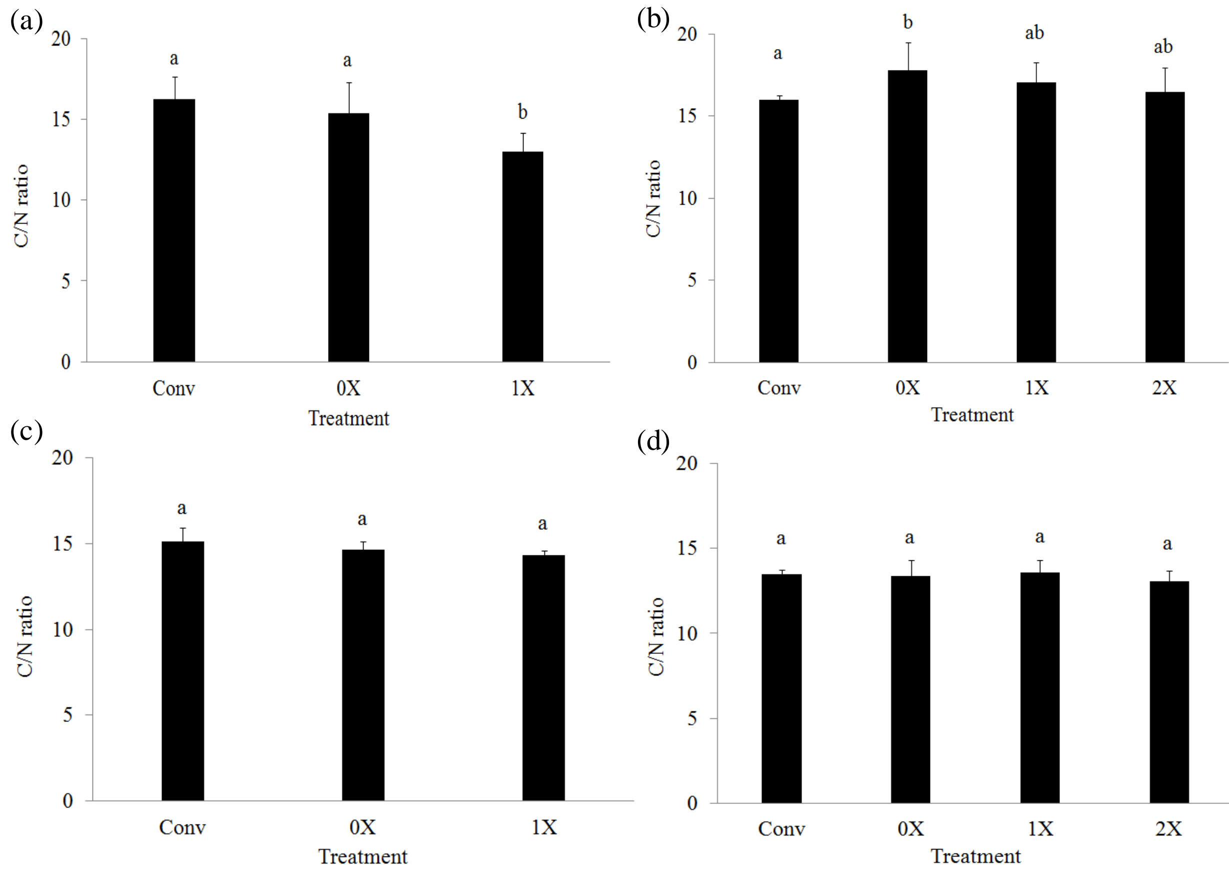


Fig. 3.3. Effect of glyphosate on C/N ratio of soybean. Data are represented by LS-means (± standard deviation). (a) and (b) are for Harvests 1 and 2 in Greenhouse Experiment 2; (c) and (d) are for Harvests 1 and 2 in Greenhouse Experiment 3.

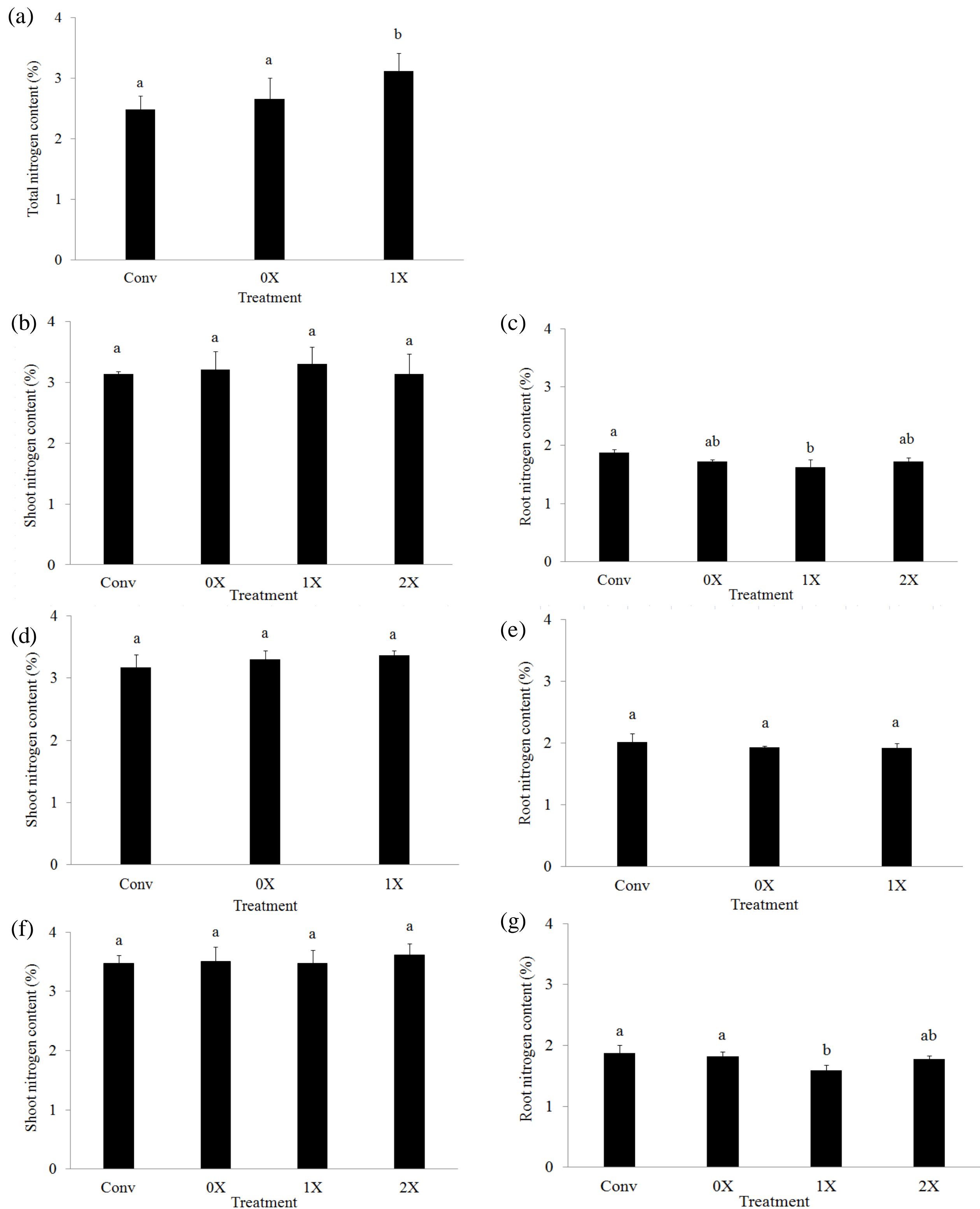


Fig. 3.4. Effect of glyphosate on the nitrogen contents of soybean. Data are represented by LS-means (± standard deviation). (a) Harvest 1 in Greenhouse Experiment 2; (b) and (c) Harvest 2 in Greenhouse Experiment 2; (d) and (e) Harvests 1 in Greenhouse Experiment 3; (f) and (g) Harvests 2 in Greenhouse Experiment 3.

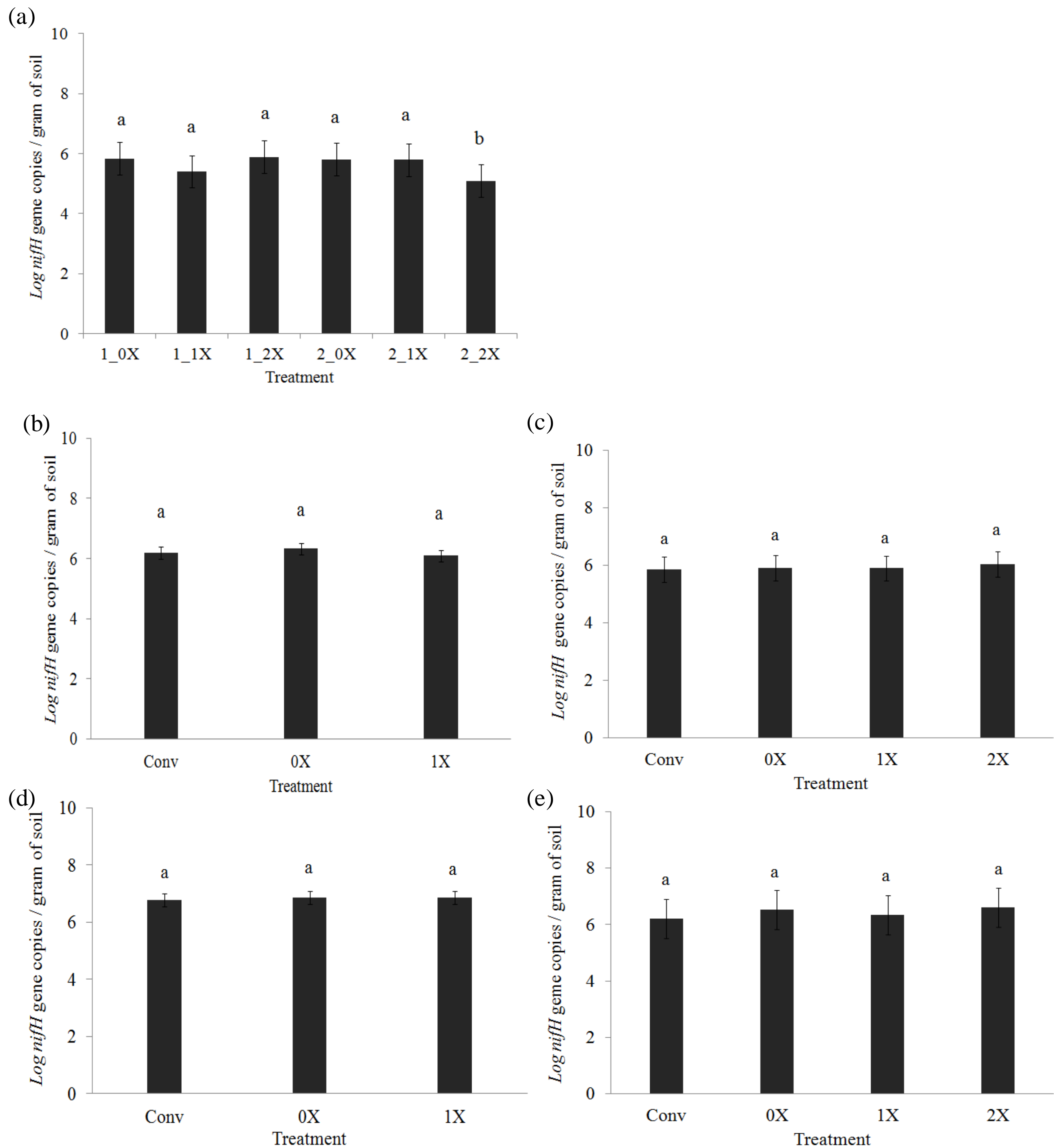


Fig. 3.5. *nifH* gene copies in rhizosphere soil samples of Prichard soybean under different glyphosate treatments. Data are represented by LS-means (with 95% confidence interval) of log base 10 of gene copies per gram of soil. (a) Greenhouse Experiment 1 (m_nX: m, sampling time; n, glyphosate treatment); (b) and (c) Harvests 1 and 2 in Greenhouse Experiment 2; (d) and (e) Harvests 1 and 2 in Greenhouse Experiment 3.

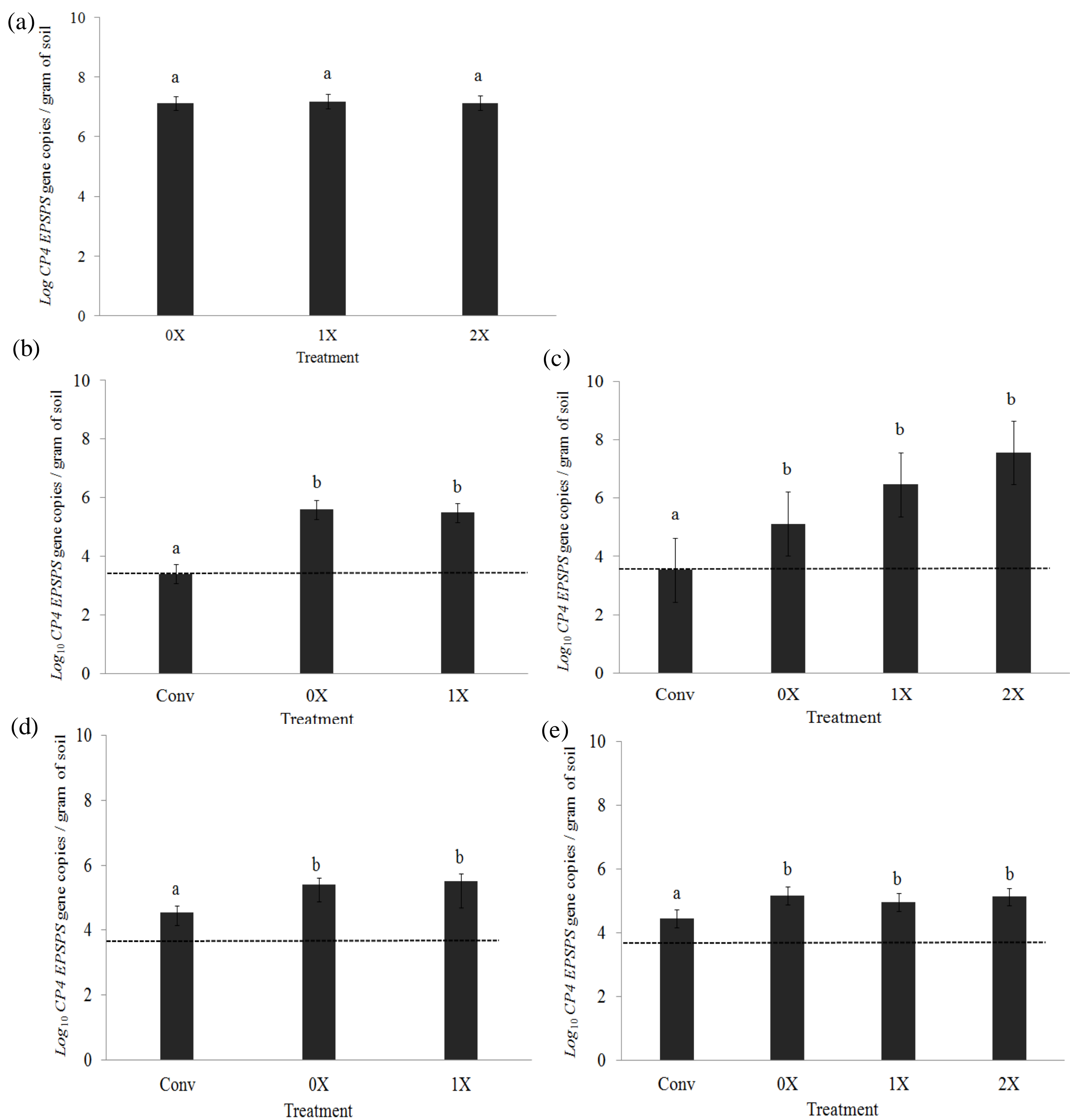


Fig. 3.6. *CP4 EPSPS* gene copies in rhizosphere soil samples of Prichard soybean under different glyphosate treatments. Data are represented by LS-means (with 95% confidence interval) of log base 10 of gene copies per gram of soil. (a) Greenhouse Experiment 1; (b) and (c) Harvests 1 and 2 in Greenhouse Experiment 2; (d) and (e) Harvests1 and 2 in Greenhouse Experiment 3. Dash line was background in bulk soil.

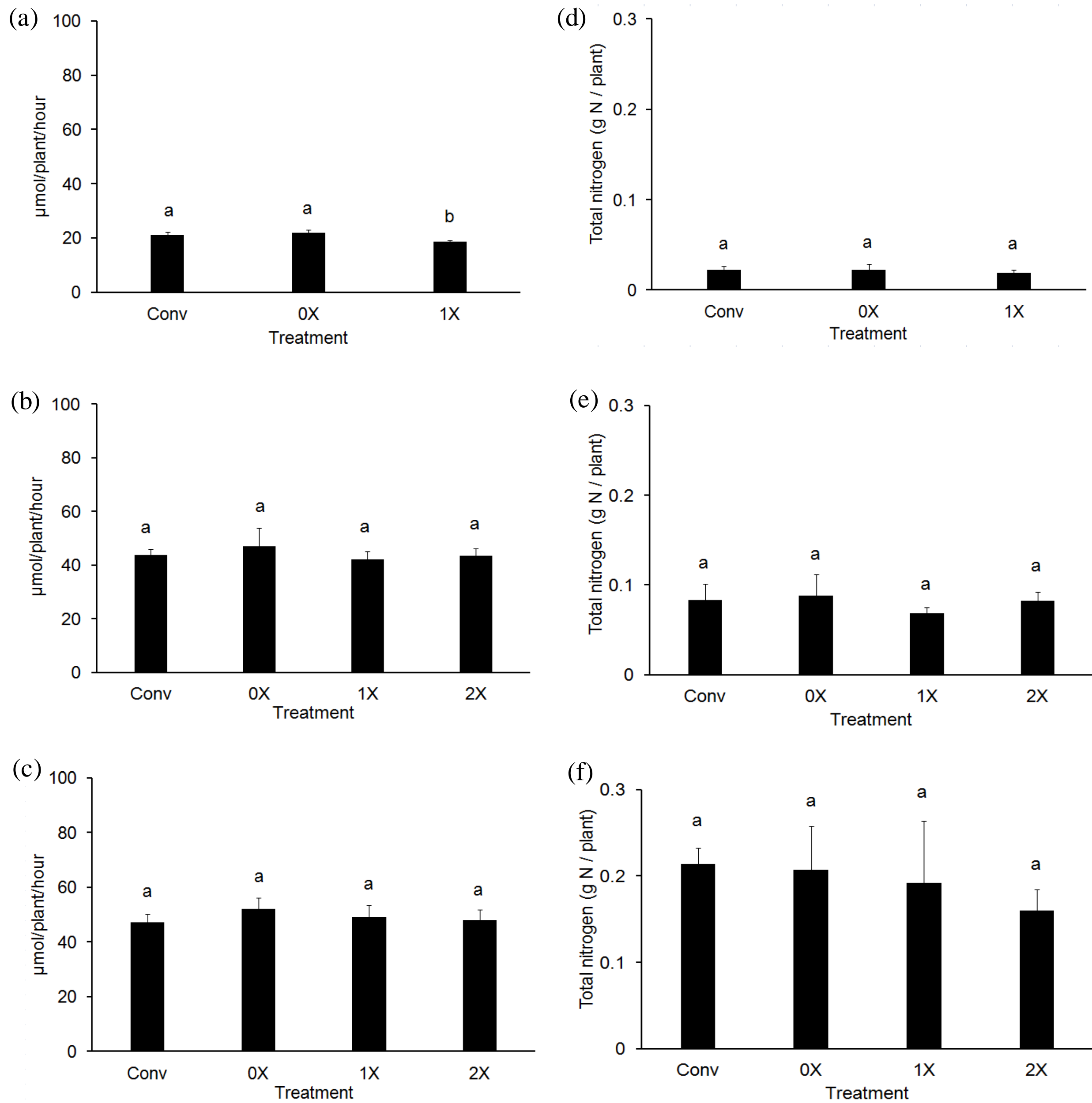


Fig. 3.7. Effect of glyphosate on the nitrogen fixation activity (a-c) and total nitrogen content (d-f) in the field experiment. Data are represented by LS-means (± standard deviation). (a and d) The first harvest; (b and e) the second harvest; and (c and f) the third harvest.

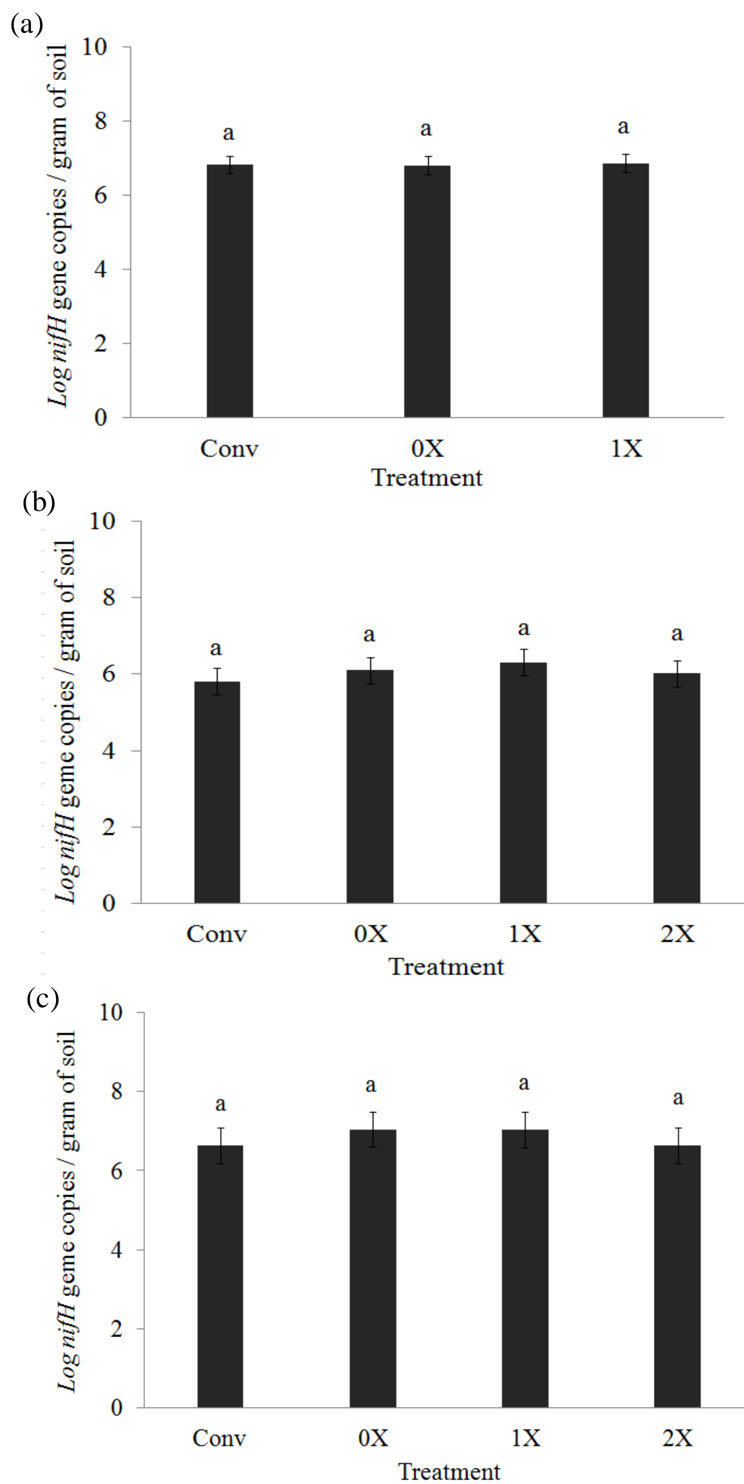


Fig. 3.8. *nifH* gene copies in rhizosphere soil samples of Prichard soybean under different glyphosate treatments in the field experiment. Data are represented by LS-means (with 95% confidence interval) of log base 10 of gene copies per gram of soil. (a) The first harvest; (b) the second harvest; and (c) the third harvest.

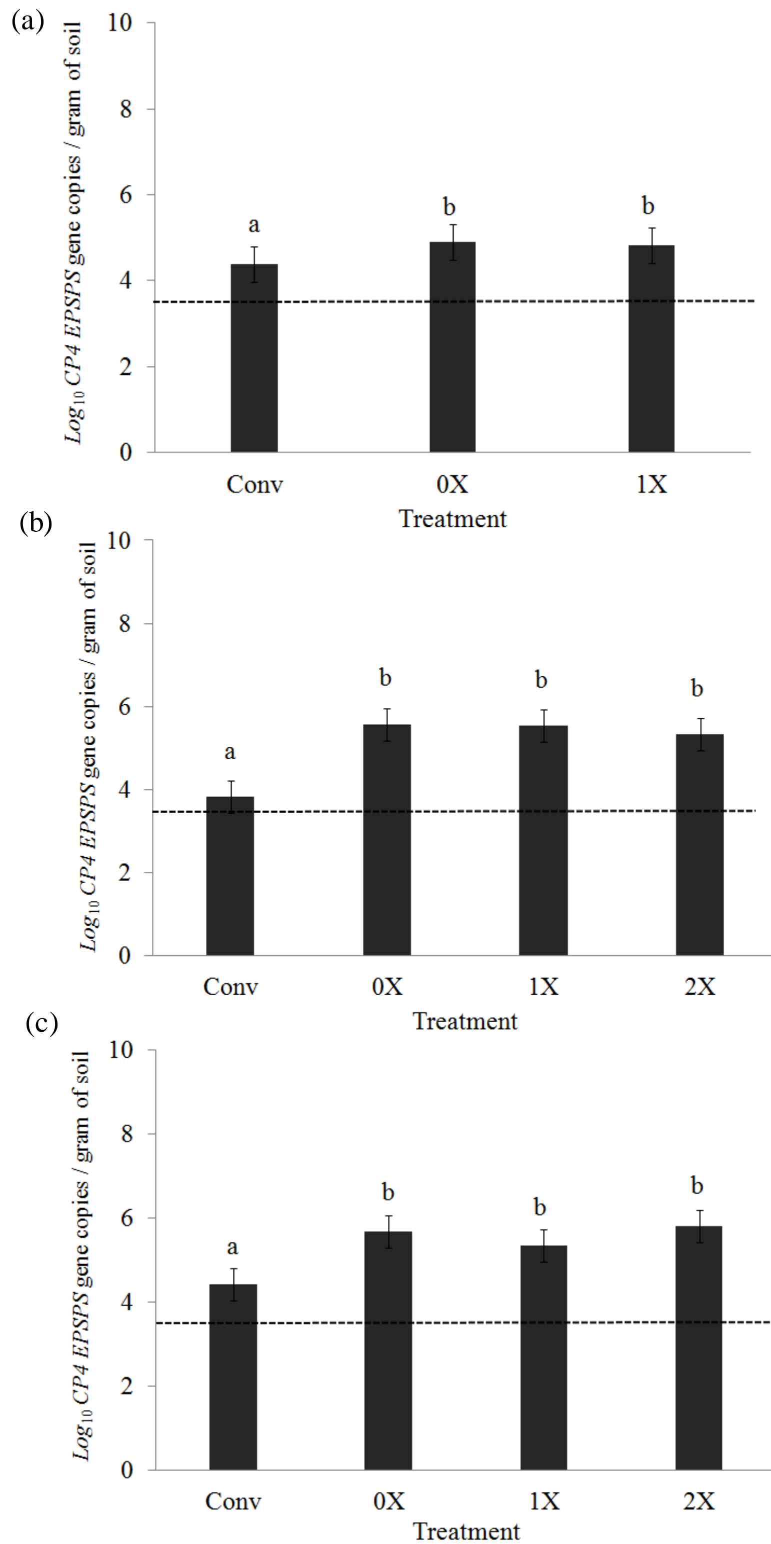


Fig. 3.9. *CP4 EPSPS* gene copies in rhizosphere soil samples of Prichard soybean under different glyphosate treatments in the field experiment. Data are represented by LS-means (with 95% confidence interval) of log base 10 of gene copies per gram of soil. (a) The first harvest; (b) the second harvest; and (c) the third harvest. Dash line was background in bulk soil.

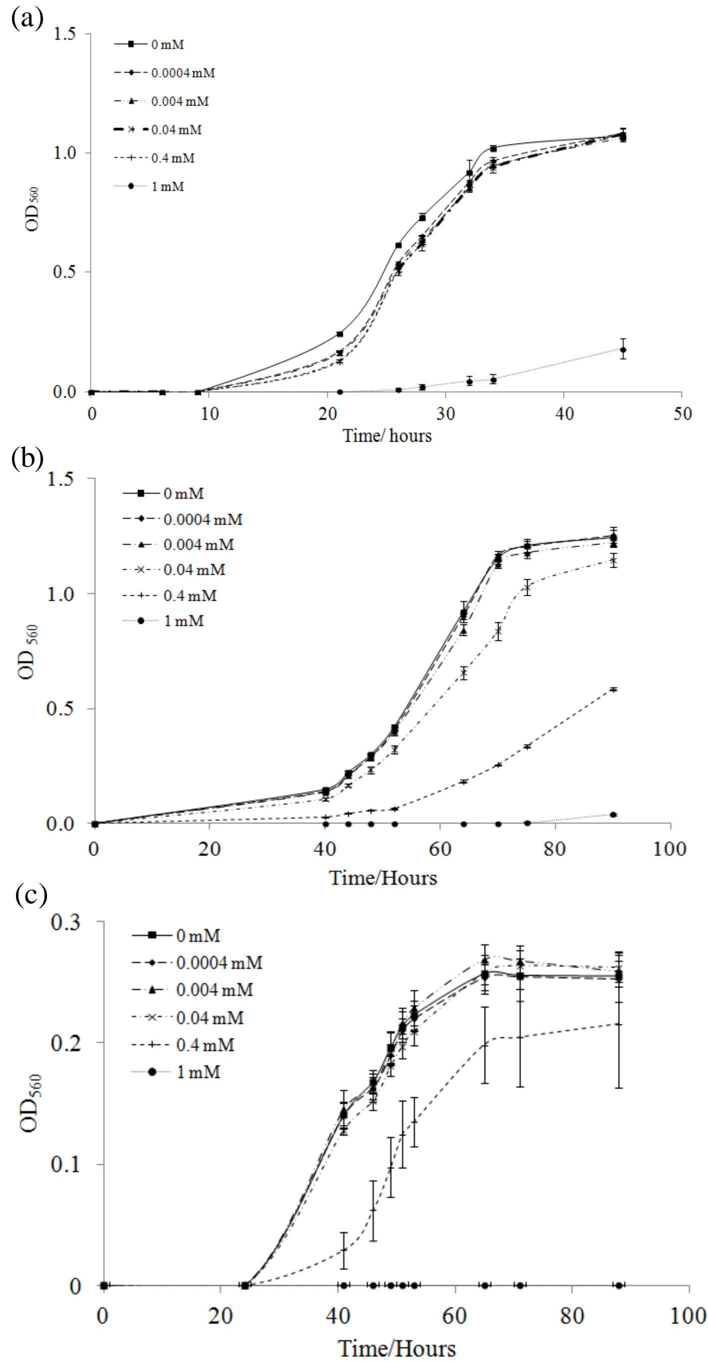


Fig. 3.10. Effect of glyphosate at different concentrations on growth of rhizobia. (a) *Bradyrhizobium* isolated from Rhizo-Stick®; (b) *Bradyrhizobium* isolated from nodules of glyphosate resistant soybean; (c) *Bradyrhizobium japonicum* USDA 110.

## Chapter IV. Conclusions and Future Work

The diazotrophic microbial community is a key part of the biological nitrogen fixation process. In this study, the effects of environmental factors such as season, soil characteristics, and nitrogen input on the diversity and abundance of the diazotrophic bacteria community were assessed in a century-old crop rotation system using DGGE and qPCR. The abundance of diazotrophic bacteria were affected by soil pH, season, and soil depth. The types of nitrogen input did not affect the *nifH* gene copy numbers during the sampling period. Shifts in the structure of the diazotrophic community depend on soil pH, season, and nitrogen fertilization in long-term crop rotation systems. In addition, glyphosate applications have negative effect on the growth and nitrogen fixing activity of GR soybean to a certain extent. The presence of transgenes from GR soybean was detected in the rhizosphere soil. As the usages of GR soybean and glyphosate continue to increase, this gives rise to concerns about their potential negative effect on ecosystems.

Given that the diversity of the diazotrophic community was affected by the soil pH, nitrogen input, and season, further research should be conducted to identify the phylogenetic characteristics of diazotrophic bacteria under different conditions using more effective and advanced technologies such as next generation sequencing. For example, it would be interesting to investigate specific diazotrophic species under the conditions such as high nitrogen levels and lower soil pH in order to explore the relationship between the microorganisms and their ecological functions. In addition, the *nifH* gene expression should be examined instead of simply

the gene abundance in future research. In terms of the effect of glyphosate on the rhizobia, it would be useful to detect the concentration of glyphosate that accumulates in the soybean nodules so that the effect of glyphosate concentrations on the rhizobia symbiosis within nodules could be assessed.

## Appendices

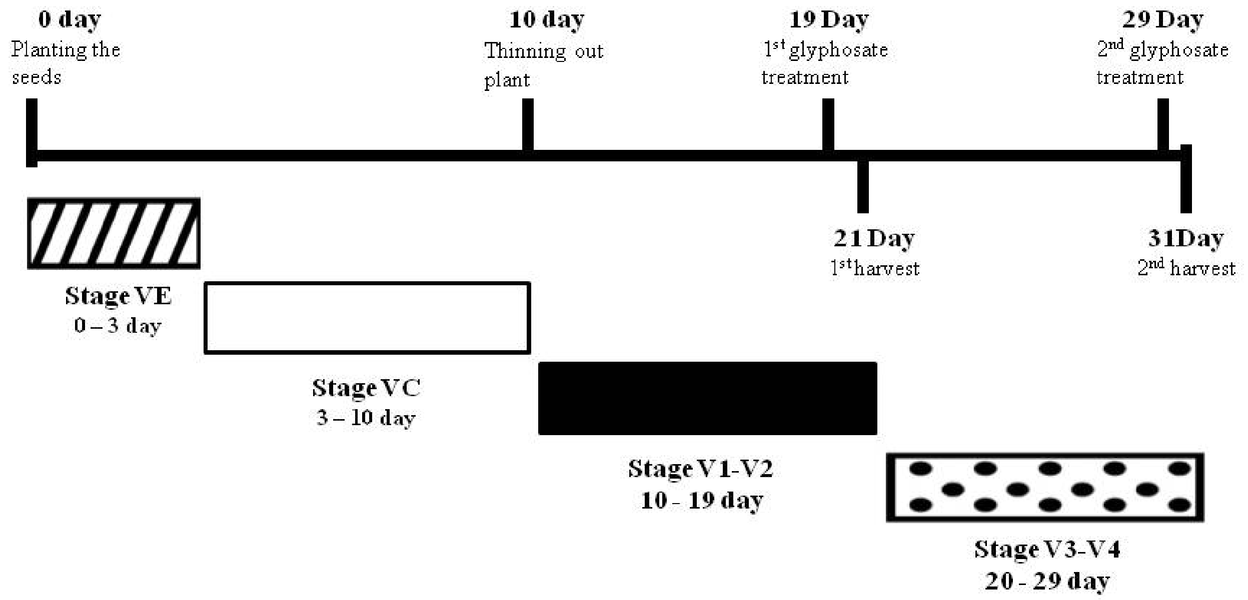


Fig. S1. Planting and harvest timeline.

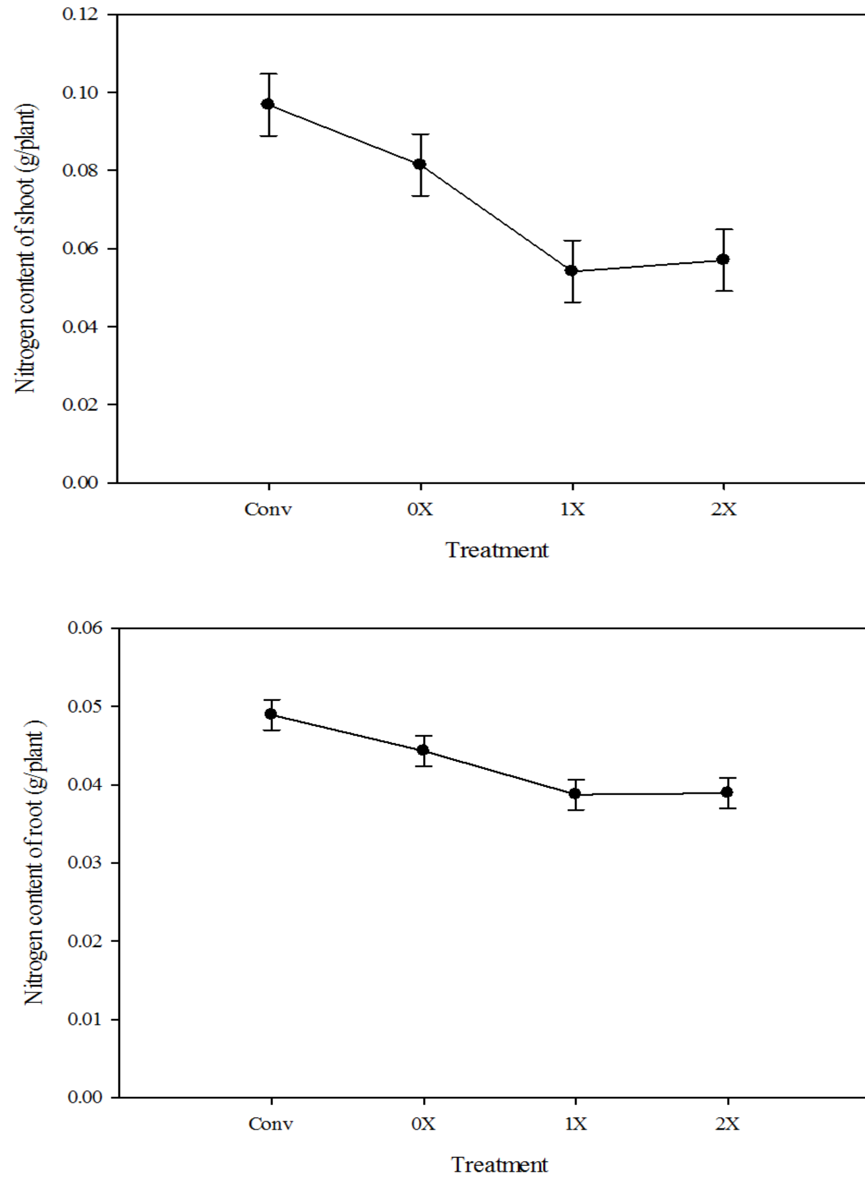


Fig. S2. Effect of glyphosate on nitrogen contents of soybean roots and shoots from Harvest 2 in Greenhouse Experiment 2. Data are represented by LS-means (± standard error).

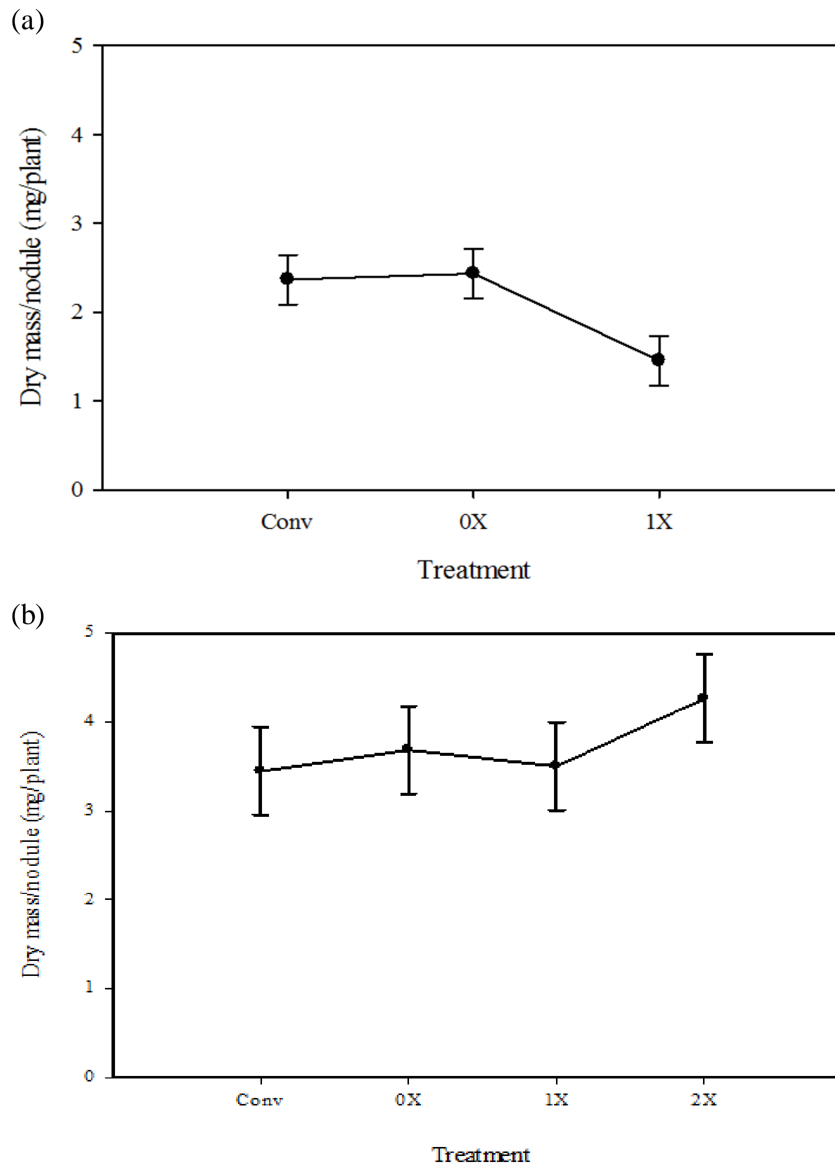


Fig. S3. Effect of glyphosate on dry mass per nodule. Data are represented by LS-means (± standard error). (a) Harvest 1 in Greenhouse Experiment 2; (b) Harvest 2 in Greenhouse Experiment 2.

CACATAAAACCCCAAGTTCCTAAATCTTCAAGTTTTCTTGTTTTTGGATCTAAAAAACTGAAAAATTCAG

CAAATTCTATGTTGGTTTTGAAAAAAGATTCAATTTTTATGCAAAAGTTTTGTTCCTTTAGGATTTCAGC

ATCAGTGGCTACAGCCTGCATGC**TTCACGGTGCAAGCAGCCGGCCCGCAACCGCCCGCAAATCCTCTGGC**

**CTTTCCGGAACCGTCCGCATTC**CCGGCGACAAGTCGATCTCCCACCGGTCCTTCATGTTCGGCGGTCTCG

CGAGCGGTGAAACGCGCATCACCGGCCTTCTGGAAGGCGAGGACGTCATCAATACGGGCAAGGCCATGCA

GGCCATGGGCGCCAGGATCCGTAAGGAAGGCGACACCTGGATCATCGATGGCGTCGGCAATGGCGGCCTC

CTGGCGCCTGAGGCGCCGCTCGATTTCGGCAATGCCGCCACGGGCTGCCGGCTGACCATGGGCCTCGTCG

GGGTCTACGATTTCGACAGCACCTTCATCGGCGACGCCTCGCTCACAAAGCGCCCGATGGGCCGCGTGTT

GAACCCGCTGCGCGAAATGGGCGTGCAGGTGAAATCGGAAGACGGTGACCGTCTTCCCGTTACCTTGCGC

GGGCCGAAGACGCCGACGCCGATCACCTACCGCGTGCCGATGGCCTCCGCACAGGTGAAGTCCGCCGTGC

TGCTCGCCGGCCTCAACACGCCCGGCATCACGACGGTCATCGAGCCGATCATGACGCGCGATCATACGGA

AAAGATGCTGCAGGGCTTTGGCGCCAACCTTACCGTCGAGACGGATGCGGACGGCGTGCGCACCATCCGC

CTGGAAGGCCGCGGCAAGCTCACCGGCCAAGTCATCGACGTGCCGGGCGACCCGTCCTCGACGGCCTTCC

CGCTGGTTGCGGCCCTGCTTGTTCCGGGCTCCGACGTCACCATCCTCAACGTGCTGATGAACCCCACCCG

CACCGGCCTCATCCTGACGCTGCAGGAAATGGGCGCCGACATCGAAGTCATCAACCCGCGCCTTGCCGGC

GGCGAAGACGTGGCGGACCTGCGCGTTCGCTCCTCCACGCTGAAGGGCGTCACGGTGCCGGAAGACCGCG

CGCCTTCGATGATCGACGAATATCCGATTCTCGCTGTCGCCGCCGCCTTCGCGGAAGGGGCGACCGTGAT

GAACGGTCTGGAAGAACTCCGCGTCAAGGAAAGCGACCGCCTCTCGGCCGTCGCCAATGGCCTCAAGCTC

AATGGCGTGGATTGCGATGAGGGCGAGACGTCGCTCGTCGTGCGTGGCCGCCCTGACGGCAAGGGGCTCG

GCAACGCCTCGGGCGCCGCCGTCGCCACCCATCTCGATCACCGCATCGCCATGAGCTTCCTCGTCATGGG

CCTCGTGTCGGAAAACCCTGTCACGGTGGACGATGCCACGATGATCGCCACGAGCTTCCCGGAGTTCATG

GACCTGATGGCCGGGCTGGGCGCGAAGATCGAACTCTCCGATACGAAGGCTGCCTGATG

Fig. S4. DNA sequence of the *CTP4-CP4 EPSPS* junction in RR soybean (GenBank: AF464188.1). Arrow indicates the position of primers. The forward primer starts from position 141 and reverse primer from 232. Underlined letters indicate the *CTP* gene from *Petunia. hybrida*. Highlighted letters in bold indicate the qPCR amplicon from *Agrobacterium sp.* CP4.

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TTTAAGGTTTCTGCATCGGTCGCCGCCGCAGAGAAGCCGTCAACGTCGCCGGAGATCGTGTTGGAACCCA
TCAAAGACTTCTCGGGTACCATCACATTGCCAGGGTCCAAGTCTCTGTCCAATCGAATTTTGCTTCTTGC
TGCTCTCTCTGAGGGAACAACTGTTGTAGACAACTTGTTGTATAGTGAGGATATTCATTACATGCTTGGT
GCATTAAGGACCCTTGGACTGCGTGTGGAAGATGACAAAACAACCAAACAAGCAATTGTTGAAGGCTGTG
GGGGATTGTTTCCCACTAGTAAGGAATCTAAAGATGAAATCAATTTATTCCTTGGAAATGCTGGTACTGC
AATGCGTCCTTTGACAGCAGCTGTGGTTGCTGCAGGTGGAAATGCAAGCTACGTACTTGATGGGGTGCCC
CGAATGAGAGAGAGGCCAATTGGGGATTTGGTTGCTGGTCTTAAGCAACTTGGTGCAGATGTTGATTGCT
TTCTTGGCACAAACTGTCCACCTGTTCGTGTAAATGGGAAGGGAGGACTTCCTGGCGGAAAGGTGAAACT
GTCTGGATCAGTTAGCAGTCAATACTTGACTGCTTTGCTTATGGCAGCTCCTTTAGCTCTTGGTGATGTG
GAAATTGAGATTGTTGATAAACTGATTTCTGTTCCATATGTTGAAATGACTCTGAAGTTGATGGAGCGTT
TTGGAGTTTCTGTGGAACACAGTGGTAATTGGGATAGGTTCTTGGTCCATGGAGGTCAAAAGTACAAGTC
TCCTGGCAATGCTTTTGTTGAAGGTGATGCTTCAAGTGCCAGTTATTTACTAGCTGGTGCAGCAATTACT
GGTGGGACTATCACTGTTAATGGCTGTGGCACAAGCAGTTTACAGGGAGATGTAAAATTTGCTGAAGTTC
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GCTGTTGTTGCACTATTTGCTAATGGTCCCACTGCTATAAGAGATGTGGCAAGTTGGAGAGTTAAAGAGA
CTGAGAGGATGATAGCAATCTGCACAGAACTCAGAAAGCTAGGAGCAACAGTTGAAGAAGGTCCTGATTA
CTGTGTGATTACTCCACCTGAGAAATTGAATGTCACAGCTATAGACACATATGATGACCACAGAATGGCC
ATGGCATTCTCTCTTGCTGCTTGTGGGGATGTTCCAGTAACCATCAAGGATCCTGGTTGCACCAGGAAGA
CATTTCCTGACTACTTTGAAGTCCTTGAGAGGTTAACAAAGCACTAA
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Fig. S5. DNA sequence of glyphosate sensitive *EPSPS* gene in soybean (GenBank: XM_003516991.1). Marked letters indicate overlapping sequence compared with the *CP4 EPSPS* gene.

TTTAAGGTTTCTGCCTCCGTCGCCGCCGCCGCAGAGAAGCCTTCGACGGCGCCGGAGATCGTGTTGGAAC
CTATCAAAGACATCTCGGGTACCATCACATTGCCAGGGTCTAAGTCTCTGTCCAATCGAATTTTGCTTCT
TGCTGCTCTCTCTGAGGGAACAACTGTTGTAGACAACTTGCTGTACAGCGAGGATATTCATTACATGCTT
GGTGCATTAAGGACCCTTGGACTGCGTGTGGAAGACGACCAAACAACCAAACAAGCAATTGTGGAAGGCT
GTGGGGGATTGTTTCCCACTATTAAAGAATCTAAAGATGAAATCAATTTATTCCTTGGAAATGCTGGTAC
TGCGATGCGTCCTTTGACAGCAGCTGTAGTTGCTGCAGGTGGAAATGCAAGCTACGTACTTGATGGAGTG
CCCCGAATGAGAGAGAGGCCAATTGGGGATTTGGTTGCTGGTCTTAAGCAGCTCGGTGCAGATGTTGATT
GCTTTCTTGGCACAAACTGTCCACCTGTTCGTGTAAATGGGAAGGGAGGACTTCCTGGCGGAAAGGTGAA
ACTGTCTGGATCAATTAGCAGTCAATACCTAACTGCTTTGCTTATGGCAGCTCCTTTAGCTCTTGGCGAC
GTGGAAATTGAGATTGTTGATAAACTGATTTCTGTTCCATATGTTGAAATGACTCTGAAGTTGATGGAGC
GTTTTGGAGTTTCTGTGGAACACAGTGGTAATTGGGATAAGTTCTTGGTCCATGGAGGTCAAAAGTACAA
GTCTCCTGGCAATGCTTTTGTTGAAGGTGATGCTTCAAGTGCCAGTTACTTCCTAGCTGGTGCAGCAGTT
ACTGGTGGGACTATCACTGTTAATGGCTGTGGCACAAACAGTTTACAGGGAGATGTAAAATTTGCTGAAG
TTCTTGAAAAGATGGGAGCTAAGGTTACATGGTCAGAGAACAGTGTCACCGTTACTGGACCACCACAAGA
TTCTTCTGGTCAAAAAGTCTTGCAAGGCATTGATGTCAATATGAACAAGATGCCAGATGTTGCCATGACT
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AGACTGAGAGGATGATAGCAATCTGCACAGAACTCAGAAAGCTAGGAGCAACAGTTGAAGAAGGTCCTGA
TTACTGTGTGATTACTCCACCTGAGAAATTGAATGTCACAGCTATAGACACATATGATGACCACAGAATG
GCCATGGCATTCTCTCTTGCTGCTTGTGGGGATGTTCCAGTAACCATCAAGGATCCTGGTTGCACCAGGA
AGACATTTCCCGACTACTTTGAAGTCCTTGAGAGGTTCACAAGGCACTAA

Fig. S6. DNA sequence of the glyphosate sensitive *EPSPS* gene in soybean (GenBank: XM_003521809.1). Marked letters indicate overlapping sequence compared with the *CP4 EPSPS* gene.

Table S1. Vegetative stages of a soybean plant (Hanway et al., 1967)

| Abbr | Vegetative stages |
| --- | --- |
| VE | Emergence |
| VC | Cotyledon |
| V1 | First-node |
| V2 | Second-node |
| V3 | Third-node |
| V4 | Fourth-node |

Table S2. Herbicide treatments for the growth of diazotrophic bacteria in AG broth medium

| Herbicide concentration (mM) | Herbicide concentration (mg/L) | Volume (ml/flask) | | | |
|---|---|---|---|---|---|
| | | AG broth medium | Distilled water | Herbicidal solution **(0.05 mM)** | Total |
| 0 | 0 | 8 | 2 | 0 | 10 |
| 0.0004 | 0.07 | 8 | 1.96 | 0.08 | 10 |
| 0.004 | 0.7 | 8 | 1.4 | 0.8 | 10 |
| | | AG broth medium | Distilled water | Herbicidal solution **(5 mM)** | Total |
| 0.04 | 7 | 8 | 1.92 | 0.08 | 10 |
| 0.4 | 70 | 8 | 1.2 | 0.8 | 10 |
| 1 | 169 | 8 | 0 | 2 | 10 |