ABSTRACT

PARR, MARY. Promiscuous Soybean: Impacts on Rhizobia Diversity and Smallholder Malawian Agriculture. (Under the direction of Julie Grossman and Jot Smyth).

Legumes associate with soil-dwelling nitrogen fixing rhizobia bacteria, and through this relationship can maintain and increase soil fertility while improving human nutrition, an important function in low-resourced nations such as Malawi. Increasing legume production requires the presence of appropriate rhizobia partners. In recent years soybean (Glycine max) in Malawi has increased as an economically and nutritionally important crop, however without inoculation or soybean production history, significant soybean-nodulating-rhizobia populations are unlikely to exist in most soils. Tropical glycine cross (TGx) soybean varieties, bred to nodulate with resident rhizobia populations, form nodules with a wider range of rhizobia than standard varieties, but little is known about the community of TGx soybean-nodulating rhizobia The purpose of this project is to reveal the environmental drivers of TGx soybean-nodulating-rhizobia diversity in un-inoculated soils, and use this information to improve nodulation and nitrogen-fixation of soybean grown on resource-limited farms. Soils were collected from 39 small-holder farms in the Ekwendeni region of northern Malawi from fields with a history of soybean production (11 soils), non-soybean legume production (8 soils), non-legume crop production (12 soils) and non-cultivated areas (8 soils). Soils were analyzed for Mehlich 3 P, Ca, Mg, K and Fe, particle size distribution and total organic matter (OM) content. Extracted soybean-nodulation rhizobia populations were fingerprinted using the BOX A1R primer in a rep-PCR and

diversity indexes calculated from resulting fingerprints. Unique isolates from each cluster were selected for multi-locus sequencing of atpD, recA, glnII and nifH genes to determine phylogeny and symbiotic ecotypes comprising the rhizobia community. Soils varied in extractable P from 2 – 112 mg kg-1 soil, OM from 0 – 7.5% and clay content from 4% to 44%, however, these variables were independent of past cropping history. Soils from fields with a history of soybean production resulted in a greater number of nodules per plant than those cultivated fields without a history of soybean (15 and 16 nodules per plant as opposed to 8 and 10), fields with legume or native history had greater number of nodules per plant than non-legume cultivated soils. Genomic fingerprinting of rhizobia isolates resulted in 32 clusters with at least 70% fingerprint similarity. Soil history also had a significant impact on isolate distribution across clusters with native soils having the most unique fingerprint cluster distribution. Other soil factors affecting community structure include clay content, organic matter, Mg, K, Fe and P content. Sequence analysis indicate that isolates represent both Bradyrhizobium and Rhizobium genera, with the majority of strains belonging to B. elkanii, two isolates to B. canariense and one isolate similar to B. japonicum. Among isolates belonging to Rhizobium genera, 7 strains were related to R. tropici, one to R. etli, and two isolates to unknown Rhizobium species. Sequence analysis of nifH gene indicates horizontal transfer of from cowpea (Vigna unguiculata) nodulating Bradyrhizobium to other Bradyrhizobium and Rhizobium species. Three isolates had nifH regions similar to Glycine nodulating species. Understanding community structure

will lead to improved plant-microbe interactions resulting in greater legume BNF and increased soil N status.

Promiscuous Soybean: Impacts on Rhizobia Diversity and smallholder Malawian Agriculture

by
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DEDICATION

To my husband, who embodies the best of the Warm Heart of Africa.

BIOGRAPHY

Mary Parr was born and raised in the town of Lakeview Oregon. She earned her undergraduate degree in French Language and Literature at Fairfield University in Connecticut in 2004. After completing her bachelor's degree, she spent 2 years in the Jesuit Volunteer Corp, including a year as a community organizer in Hartford CT followed by two years as a GED teacher at an alternative to incarceration center in New York, NY. Mary started graduate school in the Soil Science Department at North Carolina State University in 2008 and earned her M.S. in 2010. Mary was awarded an NSF Graduate Research Fellowship and began doctoral studies at NCSU in 2010 which allowed her the opportunity to work in Malawi for approximately a year where she met and later married her husband, Bon Phiri.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1: Introduction: Challenges to Improving Soybean Nitrogen Fixation in Malawi

Overview

Over the next 30 years, Sub-Saharan Africa is expected to experience the greatest percent population increase in the world (UN Department of Economic and Social Affairs, 2004). Ensuring global food security over the coming decades will require a concerted effort to improve agricultural yields in this region, which currently experiences the lowest average grain yields in the world. Across Sub-Saharan Africa, maize yields for the past decade averaged less than 2 Mg ha^{-1} compared to a 3 – 10 Mg ha^{-1} for other continents (FAO STAT 2013). Low yields are driven by persistent nitrogen deficits afflicting smallholder farmers who repeatedly remove more nitrogen in harvested crops than they are able to replenish through fertilizer or organic N inputs (Vitousek et al., 2009). The southern African country of Malawi epitomizes this challenge. The most densely populated country on the continent, Malawi struggles to feed 15 million people on 9 million hectares of arable land. Decreasing yields, drought, and widespread famine in 2004 and 2005 prompted the government to adopt a national fertilizer subsidy that has allowed farmers to increase yields from < 1 Mg ha^{-1} to nearly 2 Mg ha^{-1} in 2006 (Sanchez et al., 2009). However, poorly resourced farmers still struggle to maintain adequate yields through fertilizers alone.

A critical soil-borne bacteria collectively known as *rhizobia* plays an essential role in sustaining global agriculture production, particularly the production of grain legume crops such as soybean (*Glycine max* L.), by increasing the plant's access to nitrogen (N),

a necessary nutrient. Through a symbiotic relationship, rhizobia infect root hairs of leguminous plants, forming organs called nodules. Inside nodules, rhizobia are able to reduce atmospheric nitrogen (N_2), an otherwise plant-unavailable form of nitrogen, into ammonium (NH_4) a form plants are able use. This process, known as biological nitrogen fixation (BNF), accounts for between 50 – 70 Tg of annual N inputs in agricultural systems with 17Tg coming from soybean production (Herridge et al., 2008). Biological nitrogen fixation is particularly important in low input farming systems in regions where farmers have less access to costly inputs such as nitrogen fertilizer (Sanginga, 2003). Rhizobia are naturally occurring in most soils; however the symbiotic relationships are governed by complex signaling processes such that a particular host legume can only form symbiotic relationships with a subset of rhizobia (Graham, 2009; Laguerre et al., 2003; Vinuesa et al., 2005c; Young and Haukka, 1996). Improving growth and production of nitrogen-fixing-legume crops in smallholder farming systems will lead to enhanced soil nutrient status and contribute increased agricultural yields and improved food security in these systems. Doing this will require an improved understanding of soil rhizobia ecology.

Understanding the population biology of native rhizobia is critical for improving agricultural production in low-input and sustainable farming systems. **The purpose of this dissertation is to reveal the environmental drivers of diversity in naturally occurring soybean-nodulating-rhizobia in Malawian soils, to determine the phylogenetic and symbiotic community able to form nodules with TGx soybean,**

and to determine the factors contributing to reduced soybean yields in the Ekwendeni region. This study conducted a bio-geographical study of native rhizobia populations across gradients in various soil factors in the Ekwendeni watershed in northern Malawi, a smallholder farming region with where farmers have a strong interest in soybean production and limited access to inoculants. This study was coupled with multivariate analysis of environmental factors contributing to this diversity, as well as diagnostic work identifying other potential constraints to improving soybean yield. Many previous studies have attempted to describe community structure and diversity in native populations(Appunu et al., 2011; Giongo et al., 2008; Grossman et al., 2011; MingZhou et al., 2005; Mothapo et al., 2013; Nkot et al., 2008; Rincon et al., 2008; Stepkowski et al., 2007; SuFang et al., 2011),but few studies have attempted to correlate diversity and community structure with background environmental factors that drive diversity (Li et al., 2011; Ormeno-Orrillo et al., 2012).

Cropping Systems and Soybean production in Malawi

Food security is a perennial concern in Malawi. Through the cultivation of grain legumes, farmers are able to increase soil nitrogen further through the process of biological nitrogen fixation (BNF). Grain legume cultivation also diversifies the diet and provides a higher protein yield (Mhango et al., 2013; Snapp et al., 2010). An extensive study comparing cropping systems over the past decade in Malawi has shown that legume diversified systems not only provide equivalent maize yields to fertilized monoculture maize, but provide increased protein due to the additional legume grain,

improved N use efficiency, soil protection, and more favorable value cost ratios for farmers (Snapp et al., 2010). Legume intercropping, especially for the poorly resourced farmers, has been shown to be the least risky method to improve maize yields because input cost is low. This is especially true for long-duration legume crops such as pigeon pea combined with a small amount of fertilizer (Kamanga et al., 2010a).

Legumes traditionally grown in Malawi include groundnut (*Arachis hypogaea*), Bambara ground nut (*Vigna subterranea*), common bean (*Phaseolus vulgaris*). pigeon pea (*Cajanus cajun*) has historically been grown in Malawi, and in the past 20 years, farmers have shown an increased interest in and adoption as an intercrop for maize and other short- season legumes, prized for its versatility, ability to improve soil fertility, and tasty grain (Bezner Kerr et al., 2007; Snapp and Silim, 2002; Snapp et al., 2003). Soybean (G*lycine max*) is a crop that has only recently garnered interest in Malawi, however because of its nutritional value, this interest has been strong (Kerr et al., 2007). In the Ekwendeni region of Malawi, soybean has been promoted by the local hospital as a nutritional supplement for infants and small children. Interest in soybean and other legume cultivation has increased dramatically following the formation in 2000 of the organization Soils, Food, and Healthy Communities (SFHC) associated with Ekwendeni Hospital ((Bezner Kerr et al., 2007). This organization was formed to address the problem of child malnutrition associated with poor diets and low soil fertility and has been successful in increasing farmer interest in legume cultivation, particularly soybeans, and improving food security in the region (Bezner Kerr et al.,

2011). Surveys in the Ekwendeni region have found that soybean adoption has increased rapidly over the past 12 years, from 3% to over 90% of farmers growing soybean. An ongoing research and development project funded by the McKnight Foundation in collaboration with SFHC and Bunda College of Agriculture entitled Best Bets Legumes is working to identify farmer preferred legume species and varieties and new models for legume cultivation. Farmers associated with this project are greatly interested in soybean production. Farmers cite nutritional value for children as their main interest in the crop (Mhango et al., 2013). Unfortunately, soybean grain yields in Malawi remain low, < 1 Mg ha^{-1} (Mhango et al., 2008), compared to other tropical regions which produce >4 Mg ha^{-1} soybean (Hamawaki et al., 2010). A likely cause is low numbers of native rhizobia capable of nodulating soybean, yet this has not yet been assessed via appropriate research. Farmers in the region rarely practice inoculation (*personal communication* SFHC 2011), and preliminary data from an initial field trial conducted in 2010 show very low numbers of soil rhizobia capable of nodulating soybean, and inconsistent improvements when inoculants were used with non-promiscuous varieties.

Biological N Fixation in Soybean:

Soybean has tremendous capacity for nitrogen fixation, and has thus been heavily promoted as a crop in low N settings. Estimates for Brazil suggest that soybean acquire up to 250 kg N ha, approximately 80% of the total tissue N through BNF (Alves et al.,

2003). In order for a legume crops to effectively add nitrogen to a cropping system, they must have appropriate rhizobia partners. While native rhizobia populations exist in most soils, it is difficult to predict whether there will be significant numbers capable of nodulating a particular host legume. The capacity for nitrogen fixation in differing rhizobia sub-species or *strains* varies (Albareda et al., 2008). The issue is further complicated by the fact that many host legumes can be nodulated by multiple types of rhizobia, and many rhizobia types are capable of nodulating multiple host plant species (Young and Haukka, 1996). In commercial agriculture, farmers will apply purchased inoculants, which tend to contain strains of rhizobia that have been shown to effectively nodulate a legume crop and fix adequate amounts of nitrogen in controlled settings (Materon and Hagedorn, 1982; Thies et al., 1991). The amount of N accumulated in soybeans has been shown to correlate with inoculation rate of appropriate rhizobia ((Bergersen et al., 1989).

However, in many regions, access to inoculants is limited, and farmers rely solely on native rhizobia populations for legume nodulation and BNF. If the population of soil rhizobia is low, or if the existing population is unable to form nodules on a particular crop, little to no nitrogen fixation will take place. On the other hand, a large diverse rhizobia population is likely to include rhizobia capable of forming nodules on a wide range of legumes (Kahindi et al., 1997), and inoculant performance can vary according to competitiveness of indigenous populations and the encountered soil environment, with different strains of rhizobia demonstrating differential competitiveness in their

ability to form nodules as well in their ability to survive in various soil environments (Bogino et al., 2008; Thies et al., 1991). If the native population is less efficient at N fixation than the inoculant, this can reduce overall N fixation and cause reduced growth and yield of the crop (Thies et al., 1992). Understanding background diversity of rhizobia populations can provide for better use of inoculant strains, as well as improved nitrogen fixation of legumes.

TGx Soybean

Soybean expansion generally requires the use of rhizobia inoculants because in areas where soybean is a non-native species, such as in South America and Africa, native populations of rhizobia capable of nodulating soybean are low. In resource poor countries such as Malawi with limited infrastructure, distribution and access to refrigeration for inoculant storage is poor, thus widespread inoculation is not feasible. In response to this challenge, the International Institute for Tropical Agriculture, (IITA) began developing "promiscuous" varieties of soybean capable of forming nodules with a wider range of rhizobia already present in African soils, thus eliminating the need for inoculation(Kueneman et al., 1984). The resulting soybean varieties – denoted as "TGx" (Tropical Glycine Cross) – form significantly more nodules in un-inoculated native soils than traditional varieties, and rarely respond to inoculation (Kasasa et al., 1998).

Agronomic studies of promiscuous soybean in the past decade has shown that despite relatively abundant nodulation, these varieties only acquire approximately 50 % of their N through BNF and are routinely unable to meet yield potentials due to N

limitations (Sanginga et al., 2002). Research by IITA in Nigeria has shown that most promiscuous varieties obtain a maximum of 75 kg N ha^{-1} from BNF, less than half of total plant N, resulting in a net loss of N after harvest of soybean grain (Okogun and Sanginga, 2003; Osunde et al., 2003). Attempts to increase BNF through inoculation have had inconsistent results in terms of biomass N or yield responses. In some attempts, competition from native rhizobia prevented inoculant strains from occupying nodules (Pule-Meulenberg et al., 2011), while in other cases, inoculant strains did form nodules, but N fixation capacity remained unchanged(Okogun and Sanginga, 2003). One particular variety, TGx 1740-2F, released in Malawi as "Tikolore" (meaning "We will harvest much"), been shown to have high BNF potential, be moderately promiscuous, and yield adequately without inoculation yet respond to favorably to inoculation by increasing N fixation and yield (Pule-Meulenberg et al., 2011; Thuita et al., 2012).

Evidence suggests that low phosphorous availability might be the greatest limitation to increasing BNF by soybean in most African soils. Research on promiscuous soybeans in Cameroon shows that nodule and shoot dry matter, grain yield and N uptake all increase significantly with P fertilization and that there was a positive linear correlation between P and N uptake (Jemo et al., 2010). Research in Malawi has shown that while a significant yield increase from P fertilization is not obtained for maize(Phiri et al., 2010), legume grain yield does increase with the use of P fertilizers, suggesting that P may be a limiting factor for legumes(Kamanga et al., 2010b). Research in Kenya assessing diversity of rhizobia nodulating promiscuous soybeans under P and lime amendments

showed that both P and lime improved nodulation and that rhizobia strains differed between treatments, suggesting that soil amendments may have an impact on the ability of some rhizobia to form nodules(Wasike et al., 2009). Thus, poor yields and BNF of soybean are likely limited by soil P status as well as the native rhizobia community.

Rhizobia evolutionary ecology

The understanding of rhizobia evolutionary ecology has been, until recently, complicated by the confounding of classification based on phylogenetic descent, with classification based on symbiotic partners. Historically, six species of rhizobia, in one genus, *Rhizobium,* were recognized, and classification was based primarily on the host plant on which rhizobia were able to form nodules, known as the "cross inoculation" concept. Over time this was abandoned due to wide overlap in host range, and in 1982, the first proposed classification was made based on physiological properties, with the proposal of the genera *Bradyrhizobium* representing slow growing acid intolerant bacteria (Jordan, 1982; Young and Haukka, 1996). However, Jordan (1982) still suggests naming conventions within *Bradyrhizobium* to fall along host ranges with *B. japonicum* representing soybean nodulating bacteria. Recently however, DNA sequencing has made it possible to make distinctions based on gene structure and descent, allowing for more accurate speciation, and proposed phylogenies are continually being revised. For example, an early review in 1996 of phylogenies based on 16s sequences recognized seventeen species in 4 genera (Young and Haukka, 1996),

while a more recent review by Graham (2009) indicates more than 50 species of nodule forming bacteria in 12 genera including both α- and β-proteobacteria (Graham, 2009). In recent years, speciation has become dependent on more than 16s sequences, with constitutive genes *atpD*, *glnII* and *recA* becoming increasingly important in understanding phylogeny (Appunu et al., 2011; SuFang et al., 2011; Vinuesa et al., 2005b). Recent work by several researchers, has demonstrated how symbiotic ecotypes (biovarieties) can exist across multiple rhizobia species and genera, as symbiotic regions of rhizobia genomes have been shown to be mobile, conferring nodulation ability of the same host across multiple rhizobia species (Barcellos et al., 2007; Batista et al., 2007; Hungria et al., 2006; Silva et al., 2005; Vinuesa et al., 2005d).

Diversity and evolution of bacterial communities relating to soybean cultivation

Cropping history and past inoculation can have a significant impact on rhizobial diversity in a soil. In a study of peanut nodulating rhizobia in Cameroon, the highest diversity was found in sites with no history of peanut cultivation, suggesting that simply the introduction of a legume is capable of selecting for field presence of particular rhizobia taxa(Mothapo, 2011; Nkot et al., 2008). However, in Brazilian soils after 18 years of cropping where only 4 strains of bacteria had been introduced, genetic diversity of soybean rhizobia (*Bradyrhizobium japonicum*) was found to be much greater than these 4 strains. As these soils had previously had no native rhizobia capable of nodulating soybean, this suggests rapid evolution in and adaptation to the

harsh Cerrado oxisols (Loureiro et al., 2007). Five to ten years after expansion of soybean growing area in the Northern great plain region of the US and Canada, diversity of soybean-nodulating-rhizobia was found to be great, identifying over 103 different ERIC-PCR profiles generated from 105 total strains analyzed. Shannon diversity index (SDI) did not correlate to soil or climate factors but did correlate with geo-political boundaries (Canada vs. US) – suggesting that this could be a result of differences in fertilizer use and crop rotation between nations(Farooq and Vessey, 2009).

Very little is known about native soil rhizobia in Malawi, especially about those nodulating soybeans. While evidence suggests that the rhizobia community that typically nodulate promiscuous varieties are less efficient in terms of BNF than those found in commercial inoculants, it is still unknown whether this can be improved. A vast amount of mutation and potential horizontal gene transfer can happen when inoculants are introduced into harsh tropical soil environments (Barcellos et al., 2007; Hungria et al., 2006). Studies in Brazil have shown that prior to soybean expansion in the 1960's soils were devoid of rhizobia capable of nodulating soybean (Loureiro et al., 2007), and inoculants used by farmers were primarily from the slow growing species *Bradyrizobia japonicum* and *B. elkanii*). However, by the 2000's, isolates from soybean nodules were shown to be both slow and fast growing and classified phylogenetically as *Rhizobia tropici*, *Rhizobia sp*., *Agrobacterium sp*., as well as fast growing *B. japonicum* and *B. elkanii*., indicating that either inoculants strains underwent mutations, allowing them to speed up their lifecycle, or horizontal gene transfer allowed native strains that

previously were incapable of nodulating soybean to become capable, or both(Hungria et al., 2006).

Characterizing Rhizobia Diversity

Methods for detecting diversity in rhizobial communities are extensive. Molecular methods include gene sequencing, gene amplification by PCR and characterization using restriction fragment length polymorphism (RFLP) as well as repetitive element PCR to create strain specific "fingerprints". Repetitive element PCR using the BOX A1R primer (BOX PCR) is a robust method of detecting diversity between strains of rhizobia within a species. While an excellent tool for tracking rhizobial strains that have been introduced to soils, it is unsuited for determining speciation/taxinomical groupings, showing no correlation with host legumes, and not correlating well sequencing techniques (Binde et al., 2009; Menna et al., 2009). Sequencing of the 16S rRNA portion of the rhizobia genome is useful in highlighting diversity in tropical diazotrophs and can contribute to understanding of phylogenetic relationships between strains. A polyphasic approach combining 16SrRNA and BOX PCR has proven powerful in detecting both diversity and species relationships between strains (Menna et al., 2009). However, these techniques give phylogenetic information, but do not distinguish between functional genes responsible for legume symbiosis. Other researchers have found that multi-locus sequencing of neutral housekeeping genes other than the 16S region, especially *atpD* (ATP synthase protein D) , *glnII* (glutamine synthase protein 2),

and *recA* (DNA recombinase protein) give well resolved phylogenetic trees to the species level, as well as giving evidence for re-combination within lineages(Vinuesa et al., 2005a; Vinuesa et al., 2008). Inferring symbiotic function diversity is possible through sequence analysis of symbiotic genes particularly n*if* genes (responsible for nitrogenase protein complex involved in biological nitrogen fixation) and *nod* genes (responsible for nod-factors involved in plant signaling prior to nodule formation). Previous studies have revealed that these genes, located in a region referred to as the symbiotic island are prone to lateral transfer across lineages and species, giving information regarding microbial ecotype but not descendancy (Vinuesa et al., 2005a). Using both neutral housekeeping genes and symbiotic genes, can reveal a more complete understanding of community phylogeny and function than either one alone.

Summary

Improving agricultural production in Malawi will require increasing soil N inputs through legume BNF. In the Ekwendeni region, legume integration in cropping systems has increased in recent years, and farmers are particularly interested in improving soybean performance. Economic and infrastructure limitations in Ekwendeni prevent farmers from using inoculants when growing legumes, promiscuous TGx soybean varieties are a promising technology for increasing soybean BNF in this region. Additionally, promiscuous varieties of soybean are known to rely exclusively on native strains for nodulation and BNF. Native soil rhizobia become increasingly important resources in soil fertility management. However, native soil rhizobia diversity is known

to vary widely. Rhizobia diversity will affect the success of TGx soybean nitrogen fixation. The purpose of this study is to explore the diversity of native soil rhizobia across the landscape in the Ekwendeni region of Malawi and correlate this diversity with biotic and abiotic soil factors including nutrient availability, soil texture, soil organic matter content, land use history, and climatic factors.

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CHAPTER 2: Drivers of Rhizobia Diversity in Un-inoculated Soil in Malawi

Introduction

Low soil nitrogen (N), poor infrastructure and population pressure are major barriers to improving agricultural yields across sub-Saharan Africa. Heavy reliance on maize (*Zea maize)* without adequate fertility inputs continues to reduce soil N resources (Vitousek et al., 2009). Malawi, a country in the southern region of Africa is typical of nations in this region, where 16 million people struggle to produce adequate food on 9 million hectares of arable land. Multiple studies have indicated that increasing biological nitrogen fixation (BNF) by integrating leguminous crops into agricultural systems in Malawi and other countries may lead to improved soil N levels as well as yield increases beyond what is possible with fertilizer alone (Akinnifesi et al., 2009; Bezner Kerr et al., 2007; Mwato et al., 1999; Snapp et al., 2010). Through a symbiotic relationship with soil bacteria collectively known as rhizobia, legume plants, form nodules on plant roots and are able to reduce atmospheric nitrogen (N_2), into biologically active NH_4 for plant uptake. Among the potential legume crops that Malawian farmers are interested in adopting, soybean (*Glycine max*) has the greatest potential for nitrogen contribution though BNF of major legume crops, with a global average 176 kg N ha^{-1} $year^{-1}$ (Herridge et al., 2008), as well as high nutritional value important for human nutrition (Bezner Kerr et al., 2007; Mhango, 2011).

Soybean expansion generally requires the use of rhizobia inoculants, usually in the form a peat-based powder, because in areas where soybean is a non-native species,

such as in South America and Africa, native populations of nodulating-soybean-rhizobia are low, however inoculation is difficult in smallholder African agriculture due to infrastructure limitations. In response to this challenge, the International Institute for Tropical Agriculture, (IITA) has developed "promiscuous" varieties of soybean capable of forming more nodules in un-inoculated soils than traditional varieties (Kasasa et al., 1998; Kueneman et al., 1984). Despite relatively abundant nodulation, these "TGx" (Tropical Glycine Cross) varieties only acquire approximately 50 % of their N through BNF and are routinely unable to meet yield potentials due to N limitations (Okogun and Sanginga, 2003; Osunde et al., 2003; Sanginga et al., 2002). For most varieties of TGx soybean, attempts to increase BNF through inoculation have not resulted in biomass N or yield responses, likely due to competition from native rhizobia preventing inoculant strains from occupying nodules (Kasasa et al., 1998), while in other cases, inoculant strains did form nodules, but N fixation capacity remained unchanged (Okogun and Sanginga, 2003). A new variety of TGx soybean, TGx 1740-2F, released in Malawi under the name "Tikolore," has been shown to have improved BNF over older standard varieties both with and without inoculation (Thuita et al., 2012).

It has long been understood that rhizobia strains differ in their nitrogen fixation efficiency and subsequently affect the nitrogen accumulation of the host legume (Boonkerd et al., 1978). In an agricultural system where a host legume relies on the resident soil rhizobia population to provide rhizobia symbionts, the nitrogen fixation efficiency can vary substantially between soils as a result of population differences. For

this reason, understanding the drivers of rhizobia population size, diversity and community structure can have a major impact on agricultural production.

Cropping history and past inoculation can have a significant impact on rhizobia diversity in a soil. The number of nodule-forming isolates usually increases the longer a soil is exposed to a particular host-legume crop (Elkins et al., 1976; Mothapo et al., 2013; Nkot et al., 2008). Additionally, the use of commercial inoculants can lead to an increase in diversity through the introduction of novel strains and genetic material (Loureiro et al., 2007). Crop management, including the use of fertilizers, pesticides and herbicides has also been shown to impact rhizobia diversity, with differences observed along geo-political boundaries due to differences in cropping history and cultural practices between regions (Farooq and Vessey, 2009) as well as between specific agricultural management practices and systems, such as conventional and organic (Grossman et al., 2011). However, little is known about how similar management factors might impact small-holder farms where inoculation has not been practiced previously, and where the host-legume is already capable of forming nodules with a very wide variety of natural rhizobia strains present in the soil. There is some evidence to suggest that other soil factors such as soil pH, clay and nutrient content might influence size and diversity of rhizobia populations. Total soil surface area determined by clay, organic matter and soil carbon impacts total microbial diversity of a soil and protects microbes against management induced changes to a soil (Neumann et al., 2013). Soil pH is known to affect total microbial diversity as well as diversity of

rhizobia (Fierer and Jackson, 2006; Li et al., 2011). Among individual species and strains of rhizobia, optimum pH and acid tolerance varies, both for saprophytic survival as well as nodulation ability (Appunu and Dhar, 2006; O'Hara et al., 1989). Nutrients such as calcium (Ca) and phosphorous (P) are also known to affect the ability of rhizobia to form nodules and fix nitrogen (Indrasumunar et al., 2012a; Israel, 1987).

Given the known effects of legume cropping history, clay content, soil C pools, pH, Ca and P on rhizobia survival and microbial diversity, we hypothesize that cropping history, soil organic matter content and particle size distribution will affect rhizobia diversity and community structure. The objectives of this study are to 1) assess how cropping history affects the ability of promiscuous soybean to form nodules with natural soil rhizobia populations 2) determine how physical, biological and chemical soil properties affects rhizobia community diversity, and 3) determine drivers of community structure in natural rhizobia populations on smallholder Malawian farms.

Methods

Site identification and soil sampling

In collaboration with the Ekwendeni Mission Hospital Soils Food and Healthy Communities (SFHC) we identified and sampled soil from 39 fields on smallholder farms. All farmers were participants in SFHC activities. We obtained land use history for the previous five years through farmer interviews; all soils were classified as "Soya" – having a history of soybean cultivation, "Legume" – having a history of legume cultivation other than soybean, "Non-legume" – having a history of cultivated crops that were not legumes, usually maize, tobacco and cassava, or "Native" – uncultivated areas,

either woodlands or grasslands (Table 1). Following the interview, soil samples were collected using a 5 cm diameter soil probe to a depth of 8 inches. In each field, a single core was taken every three meters along a diagonal transect with a total of 15 to 20 cores taken per field. In fields where the soil had been ridged by farmers, samples were taken from ridge centers. All cores were pooled and mixed in a plastic bucket, and then a sub-sample collected in a plastic bag. Samples were transported back to Bunda College, where they were divided and one half stored moist at 4ºC for rhizobia trapping, the other air dried and sieved through 2mm sieve for physical and chemical analysis.

Soil Analysis

Particle Size Analysis

Particle size analysis was conducted at the Bunda College Soil Science laboratory the hydrometer method of particle size analysis as described previously . Hydrometer and temperature measurements were taken at 5 minutes and 2 hours corresponding to sand and silt settling times. Blank measurements of Na-hexametaphosphate were taken throughout the procedure, and all measurements were corrected to a temperature of 25C.

Mehlich-3 Nutrient availability

Available phosphorous was measured at Bunda College using a Mehlich-3 extractant. (Mehlich, 1984) Samples were shaken with the extractant for 5 minutes, allowed to settle for 5 minutes and filtered through cotton wool. Phosphate in the extract was measured colorometrically using a spectrophotometer at 660 Nm. Mehlich 3 extractable P data was used along with clay content to determine a phosphorous

index (Pi) for each soil using Numass software, where Pi is the ratio of actual Mehlich 3 extractable P to the optimal P level for crop growth as determined by the buffering capacity of the clay fraction. A Pi less than 1.0 indicates a P deficiency in the soil, whereas a Pi greater than 1.0 indicates excess P.

To analyze cations including Magnesium (Mg), Calcium (Ca), Iron (Fe), and Potassium (K), soils were brought to North Carolina State University (NCSU) where a second Mehlich 3 extraction was performed. Cations in the extract were measured using Flame Atomic Adsorption Spectroscopy with an air-acetylene flame on a Fisher Scientific F.S ICE 3300 AA. Solutions were diluted with lithium-lanthenum when measuring Mg, Ca and K to prevent ionization.

Soil pH was measured in DI water in a 1:1 solution. Soil slurries were allowed to equilibrate for one hour before analysis.

Organic Matter

Organic matter was measured using the loss on ignition (LOI) method at Bunda College. Oven dried samples were weighed, heated to 550ºC for 5 hours and cooled to room temperature in a desiccator and weighed again. Labile organic matter was determined using the Permanganate Oxidizeable Carbon (PoxC) method as described in (Culman et al., 2012).

Rhizobia trapping

Rhizobia were extracted from soil using the host-plant trap method described previously (Mothapo et al., 2013). Briefly, seeds of soybean variety TGx 1740-2F were sterilized by immersing in 3% NaClO for 4 minutes and then rinsing 5 times in sterile

water. Two seeds each were planted in a locally available 300 ml clay pot filled with sterilized river sand. For each soil, a slurry was prepared of 10 g soil in 40 ml 0.85% NaCl, and then shaken vigorously. Seven days after planting, pots were thinned to one seedling and inoculated with 1 ml of the appropriate soil slurry. Three replicates, each containing one plant for each soil type, were included, as well as two negative controls and a positive inoculated control using the locally produced soybean inoculant. Soybean plants were arranged in a complete randomized block design in the IITA screenhouse at the Chitedze research station and watered daily with a sterilized dilute N-free plant nutrient solution. After seven weeks, shoot biomass, and nodule counts were taken for all plants.

High mortality of soybean plants due to low water holding capacity of the sand, poor germination, and the uncontrolled environment in the IITA screenhouse necessitated repeating the rhizobia trapping experiment in a controlled environment at North Carolina State University (NCSU) with the following modifications. Modified Leonard jars made of Magenta GA-7 Plant Culture Boxes (Magenta LLC, Chicago IL), containing a 1:1 mixture of sterile vermiculite and sand were used as growth containers (Tlusty et al., 2004). Five surface-sterilized seeds were directly planted into each experimental unit and were thinned to one plant after seven days. Plants were grown indoors under full-spectrum florescent lights with 12 hours light per day for seven weeks. Plants were bottom watered with a sterile dilute N-free nutrient solution (Broughton and Dilworth, 1971). At harvest, total nodule number and mass, and shoot

and root biomass were measured. Shoot biomass was ground and analyzed for total N and C content in the Environmental and Agricultural Testing Services lab at NCSU.

Rhizobia Characterization

Nodules from both the Chitedze and NCSU grown soybean plants were extracted for rhizobia isolates. Plant roots were gently rinsed, and 20 nodules collected from each soil treatment. If fewer than 20 nodules were present across all plants for a treatment, all nodules were collected. Nodules were sterilized by immersing in 3% NaClO for 3 minutes followed by rinsing 5 times in sterile deionized water, then crushed and cultured on yeast mannitol agar (YMA) plates (Vincent, 1970). Colonies began to appear between 3 and 10 days after culturing. A single round, smooth-edged, glossy colony from each nodule was further isolation and purified. Where colonies were very small, a dissecting microscope was used to confirm morphology. In some instances, a single nodule produced colonies with two distinct growth rates and both were morphologically similar to rhizobia. In these cases, both types of colonies were selected and cultured separately. Pure colonies were isolated and stored on BYMA slants, and in glycerol for further analysis

Cell Preparation and Genomic fingerprinting

Pure isolates were grown on liquid tryptone yeast (TY) broth in 1.5 ml micro-centrifuge tubes on a rotary shaker at 150 rpm for 3 to 7 days at room temperature. Cells were pelleted using a centrifuge at 1000 rcf for 5 min, then TY broth poured off and the pellet washed with 1M NaCl, re-pelleted and re-suspended in sterile water. The resulting cell suspensions were used as template for PCR reactions. Genomic

fingerprinting was conducted using the repetitive extragenic primer BOX A1R (Versalovic et al., 1994), in a 25 µl reaction as described by Mothapo et. al (2013). Resulting products were visualized after gel electrophoresis in a 25 cm gel containing 3% agarose and ethidium bromide run for 5 minutes at 300 V followed by 18 hours at 80 volts, and imaged using UV light. A 10 kb ladder was run in three lanes of each gel to estimate fragment size.

Statistical Analysis

Statistical analysis was performed using SAS software (SAS Institiute, Cary NC). Correlations between history and other soil characteristics were tested using proc GLM. Results of the soybean trapping experiment were analyzed using the GLM procedure with history as the main factor and field nested within history. Nodule weight, nodule mass and biomass accumulation were tested as response variables. Means separations were conducted using the pdiff option in the Proc GLM procedure with alpha at 0.05.

Gel images were analyzed using Bionumerics GelCompar II software version 6.1 (Applied Mathematics, Belgium). Cluster analysis was conducted using UPGMA (Unweighted Pair Group Method with Arithmetic Mean) and the cosine parameter. Strains were grouped into clusters using the resulting dendrogram, with each cluster comprised of at least 3 strains with greater than 70% similarity. The soil origin of strains in each cluster was recorded so that strain distribution from each soil could be further analyzed. Band matching was performed in order to conduct a principle component analysis (PCA) on banding pattern. In order to determine soil history effect on overall diversity, Shannon diversity index was calculated for each soil.

To determine the effect of management history on rhizobia strain distribution, we performed a discriminant analysis in SAS using the STEPDISC procedure for model selection. This was followed by a discriminate analysis using the selected model to analyze clusters using the variables selected by PROC STEPDISC. This analysis included cross-validation to help determine the predictive power of the discriminant analysis. In order to determine how chemical and physical soil properties influenced rhizobia strain distribution, logistic regressions were then run on each cluster, modeling the log-odds of membership in each cluster as a function of the soil characteristics using the LOGISTIC procedure in SAS.

Results

Soil Characteristics

Soils collected were from an approximately even distribution of cultivation histories, with 12 soils classified as Soya, 9 classified as Legume, 11 as Non-legume and 8 as Native (Table 2-1). Clay content in soils ranged from 0 to 44%, with native soils having greater clay content than cultivated soils ($p < 0.05$). Phosphorous content ranged from 2 to 112 mg P kg^{-1} soil, with phosphorous index (Pi) levels ranging from 0.21 to 6.18, with 19 indices falling below 1.0, and 20 being 1.0 or greater. Extractable P was lower ($p<0.05$) in native soils than in other cultivation histories. Calcium levels ranged from zero to 982 mg kg^{-1} Mg levels from 18 to 248 Mg mg kg^{-1}, K levels from to 27 - 260 mg kg^{-1}, and Mehlich 3 extractable Fe from 21 to 137 mg kg^{-1}. Cultivation history had no effect on Ca or Mg levels.

Rhizobia community

Total nodule number per plant was affected by field history, with soils having a history of soybean production producing more nodules per plant (16 nodules) than other histories, followed by native (10 nodules), legume (8 nodules) and then non-legume (6 nodules ; Figure 1). Soya soils had the greatest numbr of nodules; Native soils resulted in a greater number of nodules than non-legume soils, but legume and non-legume were not different ($p > 0.05$). These trends were similar for nodule mass and shoot N content (Figure 1), with the exception that nodule mass was approximately equal for Soya and Native treatments, but native was greater than Legume and Non-legume treatments where Soya was only greater than Non-legume treatments ($p>0.05$), indicating that nodules from Native treatments were overall similar in mass than those from Soya treatments. Shoot N content followed a similar trend as nodule mass, with Native, Soya and legume treatments all having similar masses (18 – 20 mg N plant) with non-legume having less than Native and Soya, but similar to legume, (, >15 mg N plant^{-1}, $p>0.05$).

Fingerprinting

A total of 691 rhizobia strains were fingerprinted using rep-BOX-PCR. Cluster analysis in GelComparII revealed 32 distinct clusters having at least a 70% banding pattern similarity, ranging in size from 3 to 120 strains (Figure 2). Of these, 2 clusters contained strains from only one cultivation history – cluster 1 represents strains only from Native soils and cluster 23 only contains strains from Soya fields, both being relatively small with 12 and 3 strains, respectively, in each. Five clusters contain soils

with 2 histories, 10 clusters represent 3 histories and 15 of the clusters represent all four histories (Figure 3). Cluster 30 had the greatest number of strains overall as well as from fields with Native, Legume and Non-legume histories. However, within fields with a Soya history, clusters 10, 24 and 13 contained the greatest number of strains.

The first 3 principal components (PC) accounted for 16.75%, 9.94% and 6.92% of variation in banding patterns (Figure 2-3). In the plot of PC1 vs PC2, samples from cluster 30 can be seen clustering together on the right side of graph A of Figure 3. From this concentration, the remaining banding patterns fan out to the left. In the dispersion it can be seen that strains from Soya and Native fields disperse farther than those from legume and non-legume fields which remain relatively close to the main cluster. In the plot of PC2 vs PC3 (graph B) this dispersion is composed of three major axes with the lower left axes dominated by strains from fields with a Soya history.

Community Composition

Discriminate analysis of the fingerprint strains indicated that Native soils have a distinctly different community composition than the cultivated soils. Model selection using the STEPDISC procedure indicated that clusters 1, 15, 18, 26, 28 and, 30 were significant in being able to predict field history based on the genotypes that were isolated from a particular soil (Table 3). Of these, Cluster 1 was entirely composed of rhizobia originating from Native soils. Cluster 30 was the largest cluster identified, including nearly 20% of all strains, mostly from fields that did not have a Soya history; Cluster 30 contained 23% of all Native strains, 28% of all non-legume strains and 17 % of all legumes strains; however it only comprised 6% of strains from fields with

soybean history. In general, stains from Soya fields had a more even cluster distribution with the largest group, cluster 25, comprising only 13% of the strains and cluster 10 comprising 12% of the strains. Using the DISCRIM procedure and this model to predict field history, all soils classified as Native were classified correctly, however, only two of the four soils the model predicted to be Legume and 6 of the 12 predicted to be Non-Legume were correctly identified. Nine of the 17 soils classified as Soya were correctly classified indicating a success rage of greater than half (Table 4). This indicates that Native soil rhizobia communities capable of forming nodules with promiscuous soybean are distinctly different in their composition than rhizobia from cultivated fields. Overall diversity as calculated by Shannon diversity index was not affected by soil history.

Drivers associated with rhizobia diversity

Logistic analysis of each cluster indicated that soil organic matter, total carbon, PoxC, clay content, phosphorous, calcium, magnesium, potassium iron and soil pH had effects on the probability of specific clusters appearing in a given soil. Significant log-odds ratio estimates have been plotted in Figure 2-5. For example, the odds of strain membership in cluster 10 decreased with an increase in soil carbon, but increased slightly with an increase Mg. Clusters that were most influenced by soil chemical and physical properties were clusters 6, 10, 12, 14, 17, 19 21 and 26. Soil OM, Ca and clay content had the greatest significance in that they affected the odds of strain membership in four clusters each. Organic matter (OM) predicted the presence of clutsers 6, 14, 19 and 21; Ca predicted clusters 12, 21, 26 and 32 and Clay content had

an impact on membership in clusters 4, 6, 14 and 21. Total carbon was a significant predictor of presence of clusters 10 and 26 and PoxC was a predictor of clusters 12, 26 and 28. Phosphorous was a predictor of clusters 12 and 21, extractable Mg had a significant effect on the odds of membership in clusters 10, 17 and 19, and extractable K had an effect on the odds of membership in clusters 10, 19 and 21, extractable Fe had an effect on cluster 10, and pH was a predictor of clusters 12, 24 and 18.

Discussion

The goal of this study was to determine the drivers of rhizobia diversity and community structure in order to better understand how this may affect biological nitrogen fixation in promiscuous soybean on smallholder farms. We found that cropping history, organic matter and nutrient content were the biggest drivers of rhizobia community structure.

Overall, a higher OM and clay content and lower phosphorous was found in Native soils than in Soya, Legume or Non-legume cropped soils. Previous studies have found that over long histories of cultivation, notable soil quality decreases are observed, particularly in soil OM, water stable aggregation, and water holding capacity in African soils (Moebius-Clune et al., 2011). This may also explain the reduced clay content in cultivated soils compared to Native soils, as reductions in water stable aggregates may encourage increased erosion and loss of clay particles. Increased soil P is explained by historical fertilizer applications, particularly the use of 23-21-0 fertilizer on tobacco and maize crops in the sampled regions.

Field history effects on rhizobia population and community composition

As expected, soil with a history of host crop cultivation resulted in greater nodule number, most likely as a result of past host-rhizobia interaction leading to increased rhizobia population size, as seen in previous studies (Mothapo et al., 2013; Nkot et al., 2008; Thies et al., 1991). Additionally, Native, uncultivated soil was able to produce an equal number of nodules on promiscuous soybean as soils with a history of legume cultivation. While this result was unexpected, it is likely a result of the diverse wild legume community comprising the Native *Miombo* woodland vegetation in the area. Miombo woodlands include an abundance of legume species from the families *Papilionaceae* and *Mimosaceae* (Mwase et al., 2007) which are likely sources of the Native rhizobia population found in these soils. In this study we defined a cropping history of "Legume" to indicate any history of any legume cultivation, including grain crop legumes such as groundnut (*Arachis hypogaea* L.), common bean (*Phaseolus vulgarus*), pigeon pea (*Cajunus cajun*) and cowpea (*Vigna unguiculata*) as well as semi-perennial legume green manures including fish bean (*Tephrosia vogelii* Hook) and gliricidia (*Gliricidia sepium*). As a result, the rhizobia community in two different legume fields may be affected by different species hosts being present in the rotation. Since both Legume and Native soils have a history of not having soybeans in the rotation, there may be parallel influences of host presence affecting the rhizobia community leading to their similarities in nodulation capacity and rhizobia effectiveness. A similar study investigating the effect of land use on *Bradyrhizobium* communities found an overall loss in species diversity when forest was converted to

cropland, and that Shannon diversity index correlated with *Bradyrhizobium*-specific legume density of a particular land use system (Ormeno-Orrillo et al., 2012). Other research has indicated that in semi-arid regions a close positive correlation exists between Native legume-nodulating-rhizobia populations and legume-tree hosts in that areas with the presence of legume trees results in a greater population of Native legume-nodulating rhizobia (Sene et al., 2013). This would explain the high nodule count we found in Native soils, as well as the lower nodule counts and nodule mass of non-legume cropped soils (Figure 1). Despite this, our sampled fields with a history of legume cultivation had a genetically distinct rhizobia community composition when compared to Native fields, indicating that some aspect of cultivation other than host presence is causing the difference in community structure.

Discriminate analysis of cluster distribution and cropping history revealed that Native soils had a distinct clustering profile from other cropping systems. A history of soybean production resulted in a shift away from the dominant fingerprint strain type (cluster 30), towards a more even strain distribution, with particular increases in cluster 24 and cluster 10. These results are in contrast to a study of peanut nodulating rhizobia in Cameroon where a history of peanut cultivation decreased rhizobia diversity resulted in a single strain dominating a soil as a result of selection of a particular Rhizobium taxa by the peanut host legume (Nkot et al., 2008). However, a study of hairy vetch (*Vicia villosa)* nodulating rhizobia showed that a history of hairy vetch cultivation was the single most important driver of increasing rhizobia diversity

(Mothapo et al., 2013). As a relatively recent introduction into the cropping systems of northern Malawi, soybean does seem to be selecting for a different community of rhizobia than previous legume crops or wild legumes selected for, as evidenced by the shifts in community composition seen in Figure 4 , however as TGx soybean are known to form nodules with a diversity of Rhizobium taxa, that chances of continued cultivation of soybean to result in a single taxa dominating the soil are low. Furthermore, whereas previous studies considering cropping systems effects on rhizobia diversity were conducted in production agriculture settings with access to inoculants, TGx soybean was developed for cultivation by small-holder farmers for whom growing multiple legume crops is important for food security (Bezner Kerr et al., 2007; Kueneman et al., 1984). In the Ekwendeni region, soybean is grown in rotation with a variety of crops including maize, common bean, pigeon pea and peanut. In addition, some farmers purposefully maintain leguminous trees such as gliricidia and *Faidherbia albida* as additional fertility sources (Saka et al., 1994). The combination of multiple legume hosts and the promiscuous nature of TGx soybean suggests cultivation of TGx soybean may shift the population structure through selection of a variety of Native rhizobia taxa, but will not reduce overall diversity.

Organic matter as a driver of soil rhizobia diversity

Soil organic carbon has been shown to be one of the primary drivers of overall microbial community size, diversity and structure (Sul et al., 2013), as well as influences on the soil rhizobia population. In rangelands, soil organic carbon between 2

and 3.5% corresponded to greater soil rhizobia population size, where above and below this range, the population began to decline (Swanepoel et al., 2011). In our experiment, OM was found to be the most important soil factor other than cropping history driving rhizobia community structure. In particular, soil OM was found to be positively associated with the presence of rhizobia strains from clusters 14 and 19 and negatively associated with clusters 6 and 30. Soil OM is important for the saprophytic survival of rhizobia when they are not in the presence of a host legume (Rinaudi et al., 2006). It is also associated with improved water holding capacity and aggregation, both of which are associated with supporting microbial activity (Lagomarsino et al., 2012; Sul et al., 2013).

Extractable cations Ca, Mg and K affected rhizobia clustering

Other than soil organic matter and cropping history, soil Ca, Mg and K, and to a lesser extent Fe content, were influential in defining community structure, with Ca predicting the presence of cluster 12, 21, 26 and 32, Mg influencing the presence of clusters 10, 17 and 19, K influencing 10, 19, and 21, and Fe influencing cluster 10. While these results were unexpected, there is evidence from other studies that extractable cations can be important drivers of rhizobia community structure. Biogeography of *Bradyrhizobium* species associated with soybean in China has been shown to be affected by pH, P and K, with pH and phosphorous being the biggest predictors of species distribution, and OM only slightly predictive (Li et al., 2011). Phosphorus in particular is an important element in the nitrogen fixation process, with specific roles in nodule initiation, growth and function (Israel, 1987) . While few studies

have investigated the role of Mg and Ca on rhizobia diversity, there is evidence that rhizobia respond differently to the toxic effects of high Mg salt concentration (Elsheikh and Wood, 1990), and more recent studies indicate that Mg and Ca affect the ability of rhizobia to form protective biofilms in the soil which allow them to survive in the absence of host legumes (Rinaudi et al., 2006).

Implications for Smallholder Production

In the Ekwendeni region of Malawi, soybean has been promoted locally as a nutritional supplement for infants and small children. Interest in soybean and other legume cultivation has increased dramatically following the formation in 2000 of the organization Soils, Food, and Healthy Communities (SFHC) associated with Ekwendeni Hospital (Bezner Kerr et al., 2007). This organization was formed to address the problem of child malnutrition associated with poor diets and low soil fertility and has been successful in increasing farmer interest in legume cultivation, particularly soybeans, and improving food security in the region (Bezner Kerr et al., 2011). Unfortunately, soybean grain yields in Malawi remain low, < 1 Mg ha^{-1} (Mhango et al., 2008), compared to other tropical regions which produce >4 Mg ha^{-1} soybean (Hamawaki et al., 2010). Yet, surveys in the Ekwendeni region have found that soybean adoption has increased rapidly. In 2000, less than 50 farmers in the region grew soybean, but by 2004, nearly 1000 farmers were growing soybean (Bezner Kerr et al., 2007), and general farmer interest in legumes has continued to grow (Mhango et al., 2013). Farmers cited nutritional value for children as their main interest in the crop

(Mhango, 2011); however the residues remaining after soybean harvest were also be a valuable resource for improving soil fertility (Snapp et al., 2010). Findings of this study suggest that where farmers are planting soybean for the first time, they are likely to achieve greater nodule count and nitrogen fixation choosing a field where they have grown other legumes previously, or a field that is newly cleared.

Further improvements to smallholder soybean production could potentially be found by selecting strains for survival in local soil conditions as well as for high N-fixation capacity. For example, in regions where soil pH and aluminum content adversely affects inoculant effectiveness subsequent nitrogen fixation, selection of acid tolerant rhizobia strains for use in inoculants has proved effective (Indrasumunar et al., 2012b; Situmorang et al., 2009). In Malawi a barrier to consistent inoculant effectiveness is likely due to widely varying soil properties (Lowole, 2001; Snapp, 1998) which would likely prevent a single strain from persisting across soil types. Creating rhizobia inoculants that consist of multiple strains with complementary environmental requirements could result improved soybean n-fixation without requiring yearly application of inoculant.

Conclusion

Overall we found that cropping history was the biggest driver of promiscuous-soybean-nodulating-rhizobia population and community structure. Soils that had a history of soybean cultivation resulted in increased nodule number on soybean plants compared to all other field histories. Native fields and fields with a history of legume cultivation demonstrated increased nodulation and nitrogen accumulation compared to

those with a non-legume history, suggesting that other legume hosts may influence the population of rhizobia capable of forming nodules with promiscuous soybean TGx. This has implications for crop management in that soybean cultivation in fields where other legume have been growing in the past, particularly soybean will likely result in increased BNF and yields for farmers where only non-legume crops have been planted, including planting of soybean in uncultivated or Native fields. These results may suggest that increasing legume integration could result in improved legume crop performance over time if the rhizobia community that nodulates other important legume crops such as common bean and peanut behave in the same manner as the soybean-nodulating rhizobia community examined here . We found other influences on rhizobia community structure to be soil organic matter content, clay content, Mg, K, Ca and Fe content, suggesting that these elements are important to the saprophytic competence of different rhizobia strains. .By understanding rhizobia diversity and community dynamics we can begin to improve BNF and legume crop performance on small-holder farms. Future research into understanding the speciation and cross-inoculation potential of rhizobia partners of promiscuous soybean would help to further understand how host diversity and rhizobia diversity interact.

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Tables

Table 2-1: Physical and Chemical Properties of Soil Samples

Soil Number	History	Clay (%)	OM (%)	Carbon (%)	PoxC*	pH	P*	Pi§	Ca *	Mg *	K *	Fe*
DRD 1	Legume	4	0	0.45	149	6.05	40	1.57	303	80	72	61
DRD 2	Legume	12	2.77	0.22	285	5.77	29	1.4	434	117	143	50
DRD 11	Legume	12	0.15	0.00	5	5.76	14	0.69	235	69	106	48
DRD 14	Legume	8	1.32	0.29	115	7.58	24	1.04	794	112	102	58
DRD 16	Legume	14	2.01	0.13	92	5.58	7	0.35	146	61	89	28
DRD 21	Legume	18	2.31	0.47	115	5.18	25	1.5	98	44	107	34
DRD 25	Legume	14	1.97	0.18	139	5.48	25	1.3	118	52	97	29
DRD 27	Legume	8	1.32	0.49	22	6.37	25	1.08	197	68	102	28
DRD 35	Legume	16	2.68	0.45	5	5.77	8	0.44	111	55	193	39
Mean:		***12 b***	***1.61 b***	***0.30***	***103***	***5.95***	***22***	***1***	***271***	***73***	***112***	***42***
DRD 8	Native	20	1.62	0.25	163	5.57	7	0.48	165	109	171	38
DRD 15	Native	36	4.81	0.22	259	6.17	3	0.47	404	248	260	26
DRD 30	Native	16	1.78	0.30	22	5.79	50	2.74	490	142	207	137
DRD 33	Native	4	1.2	0.11	0	6.83	5	0.21	42	18	27	27
DRD 34	Native	8	1.56	0.32	7	6.79	91	3.89	440	61	68	67
DRD 36	Native	18	3.23	0.16	3	6.69	14	0.82	589	109	98	60
DRD 38	Native	44	7.57	0.55	21	5.33	2	0.22	982	209	51	27
DRD 39	Native	40	6.59	0.41	22	5.57	3	0.46	887	177	131	32
Mean:		***23 a***	***3.55 a***	***0.29***	***62***	***6.09***	***22***	***1***	***500***	***134***	***127***	***52***

Table 2-1 (cont.) Physical and Chemical Properties of Soil Samples

Soil Number	History	Clay (%)	OM (%)	Carbon (%)	PoxC*	pH	P*	Pi§	Ca *	Mg *	K *	Fe*
DRD 3	non-legume	10	2.31	0.32	281	5.68	37	1.68	521	103	108	70
DRD 5	non-legume	12	0.97	0.26	249	5.86	21	1	509	116	167	43
DRD 6	non-legume	16	2.15	0.59	208	5.62	112	6.18	431	83	93	48
DRD 9	non-legume	26	2.64	0.22	291	5.52	25	2.11	430	140	190	35
DRD 13	non-legume	10	2.42	0.19	236	5.98	28	1.29	269	94	102	53
DRD 17	non-legume	10	1.73	0.19	129	5.67	23	1.04	188	86	117	34
DRD 19	non-legume	28	3.9	0.72	180	5.92	10	0.93	340	219	246	36
DRD 22	non-legume	8	1.68	0.21	157	6.46	88	3.76	362	75	128	62
DRD 23	non-legume	16	1.79	0.19	147	5.31	21	1.15	139	78	143	40
DRD 28	non-legume	8	1.44	0.23	21	6.36	14	0.6	169	55	93	21
DRD 29	non-legume	8	1.77	0.35	16	6.66	14	0.61	271	97	115	44
	Mean:	***14 b***	***2.07 b***	***0.32***	***174***	***5.91***	***36***	***2***	***330***	***104***	***137***	***44***
DRD 4	Soya	10	1.9	0.21	206	5.70	15	0.68	0	35	62	45
DRD 7	Soya	20	1.98	0.20	200	5.70	14	0.88	213	99	159	47
DRD 10	Soya	28	2.33	0.20	173	5.04	11	1	198	76	108	34
DRD 12	Soya	8	0	0.18	76	7.34	67	2.9	467	57	83	54
DRD 18	Soya	16	1.63	0.39	119	6.19	8	0.63	263	78	89	32
DRD 20	Soya	14	1.88	0.22	48	6.20	24	1.23	236	67	127	31
DRD 24	Soya	18	2.21	0.24	133	7.23	44	2.58	449	121	173	29
DRD 26	Soya	12	1.57	0.24	115	6.09	17	0.81	249	83	120	32
DRD 31	Soya	24	3.21	0.14	5	5.40	2	0.15	133	96	77	36
DRD 32	Soya	26	3.77	0.16	10	6.11	3	0.21	220	157	158	36
DRD 37	Soya	24	4.73	0.22	15	5.13	4	0.29	635	169	138	47
	Mean:	***18 ab***	***2.29 b***	***0.22***	***100***	***6.01***	***19***	***1***	***278***	***94***	***118***	***38***

Mean values with a different letter are significantly different from each other within a column and as impacted by soil history.

Table 2-2: Rhizobia fingerprint clusters selected through Stepdisc model selection procedure used in Discriminate model

Step	Cluster	Partial R-Square	F Value	Pr > F	Pr < Lambda	Pr > ASCC
1	C1	0.2962	4.91	0.0060	0.0060	0.0060
2	C30	0.2195	3.19	0.0360	0.0019	0.0025
3	C15	0.2756	4.18	0.0129	0.0002	0.0011
4	C26	0.1801	2.34	0.0917	0.0002	0.0006
5	C28	0.1805	2.28	0.0993	0.0001	0.0007
6	C18	0.2546	3.42	0.0299	<.0001	0.0007

Table 2-3: Discriminate analysis of rhizobia cluster distribution between soil histories. Where soils are classified correctly into their appropriate history, they possess a distinct community composition.

Classified As:	Actual Field History				Total	% correctly classified
	Legume	Native	Non-Legume	Soya		
Legume	25	0	8	9	10	60
Native	0	75	0	0	15	100
Non-Legume	37.5	25	50	9	30	41
Soya	37.5	0	42	82	43	51

Figures

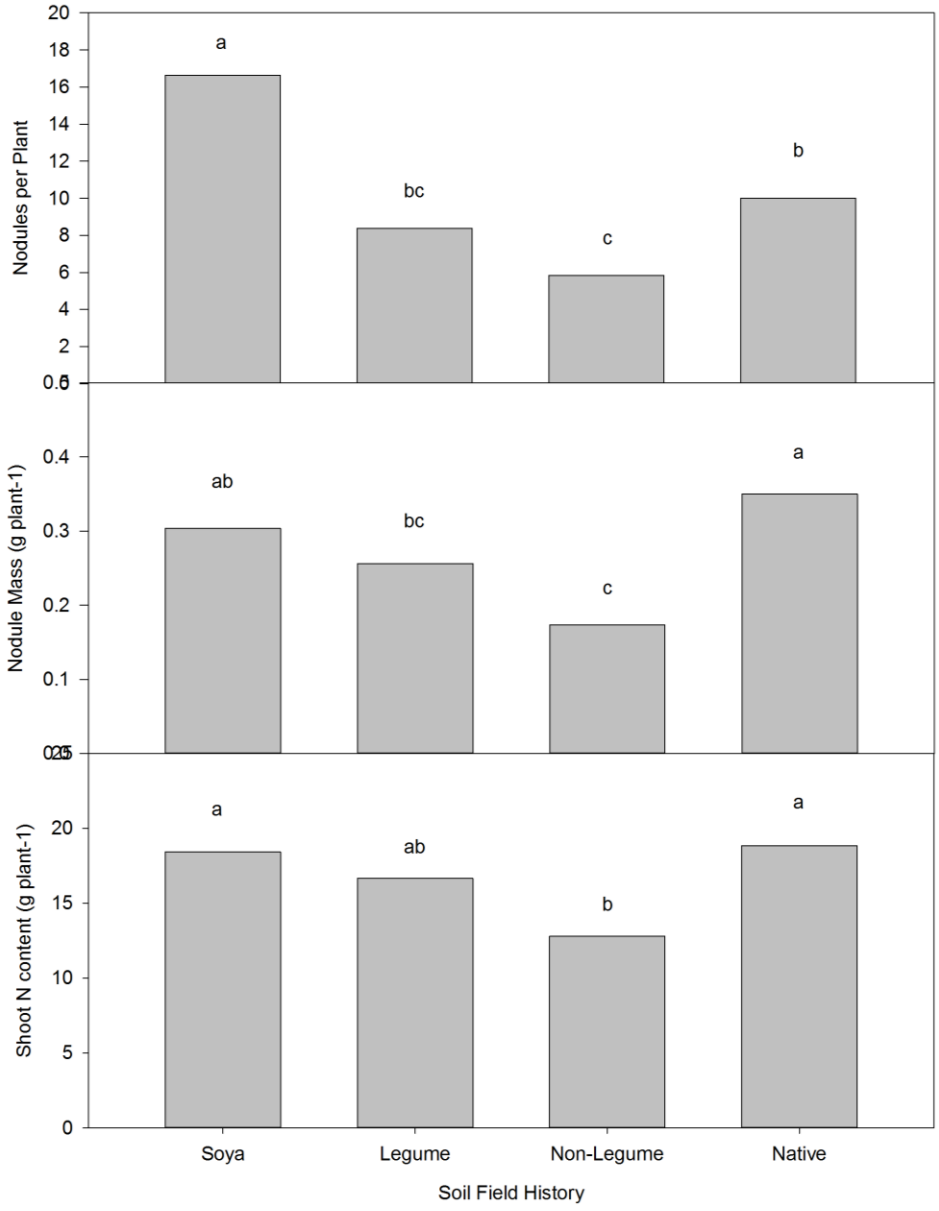

Figure 2-1: Nodule number, mass and shoot N content of soybean plants relative to origin of soil inoculant. Variable with the same letter are not significantly different at α<0.5

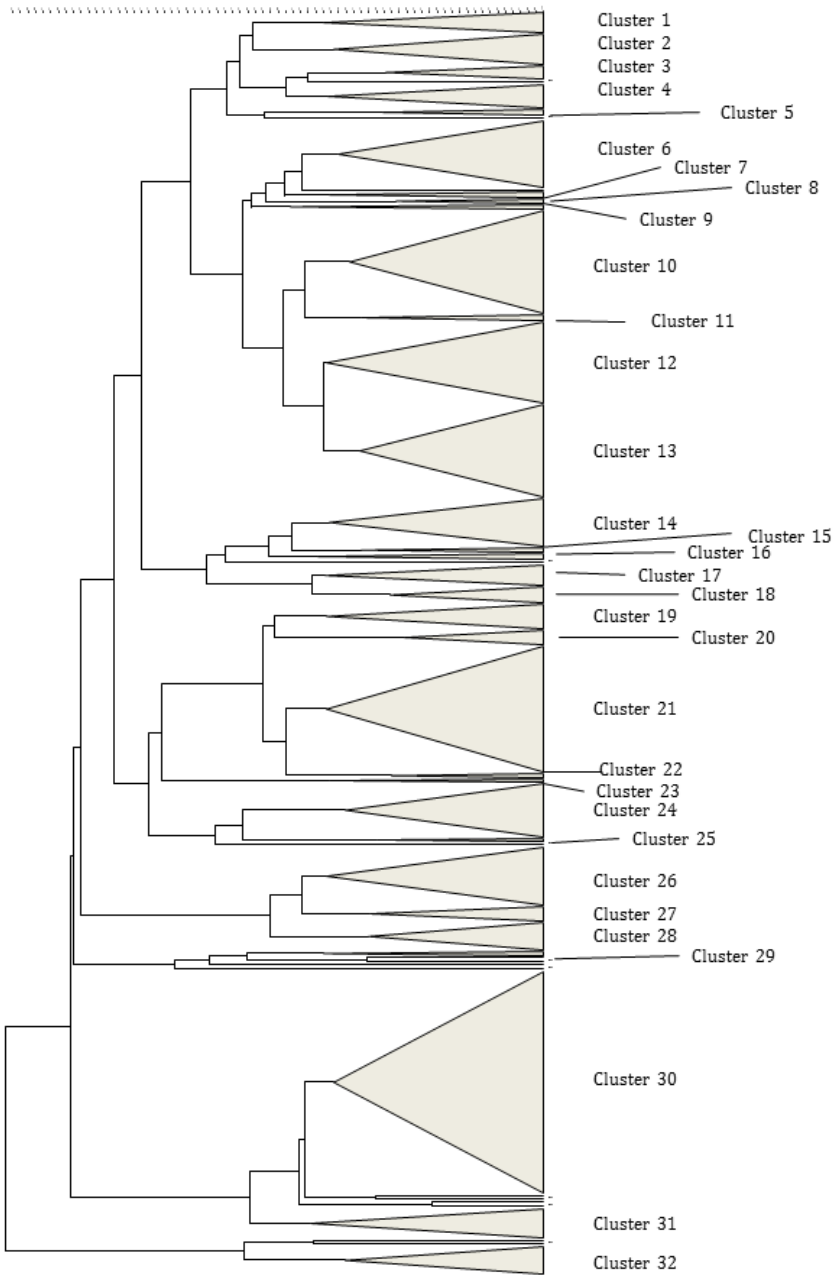

Figure 2-2: Cluster analysis of BOX-PCR fingerprint data. constructed using COSINE function in GelComparII software

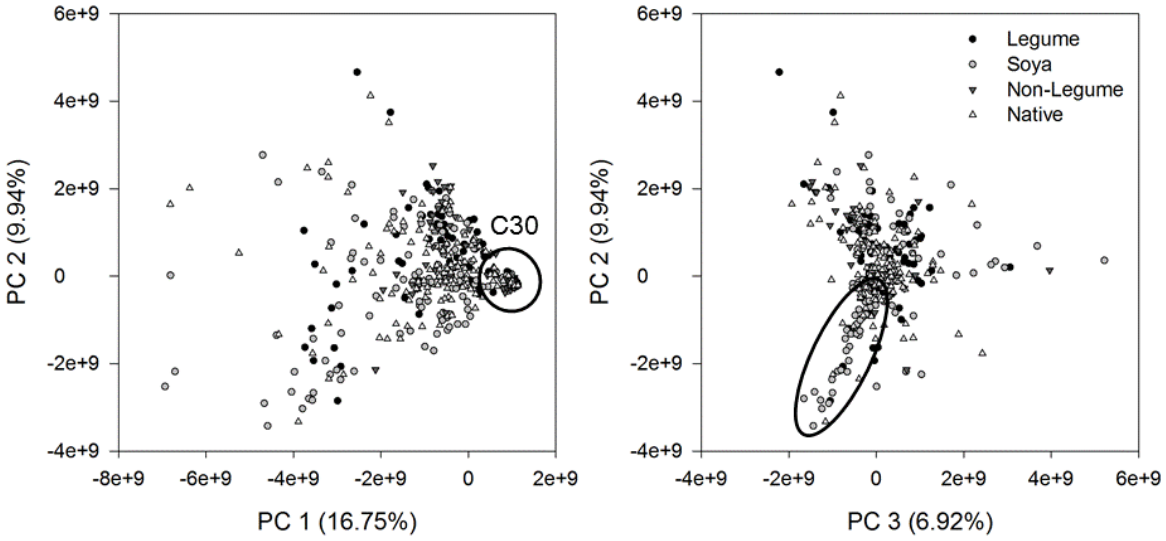

Figure 2-3: Principle component analysis BOX-PCR fingerprints bands.

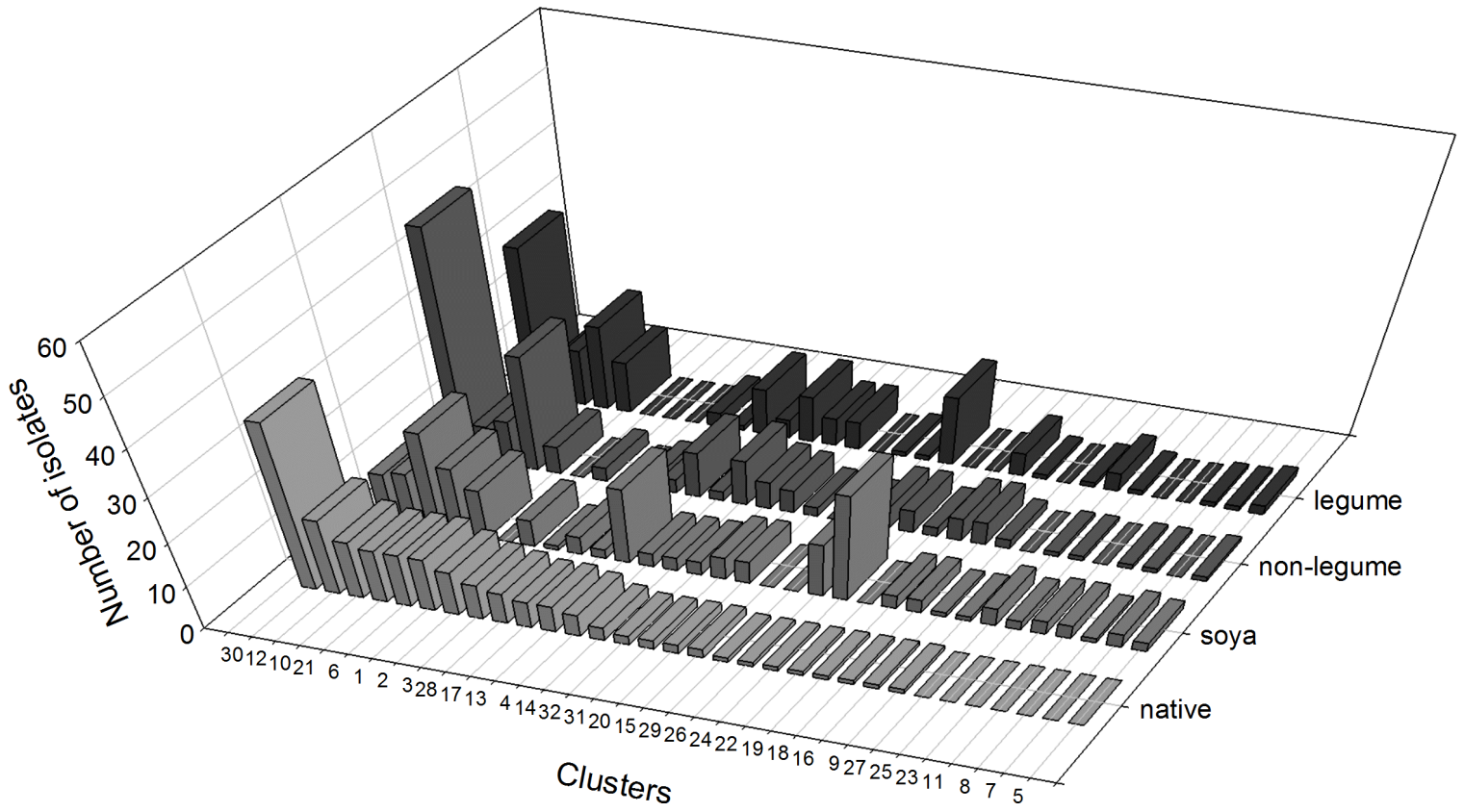

Figure 2-4: Isolate distribution from fingerprint clusters relative to field cultivation history.

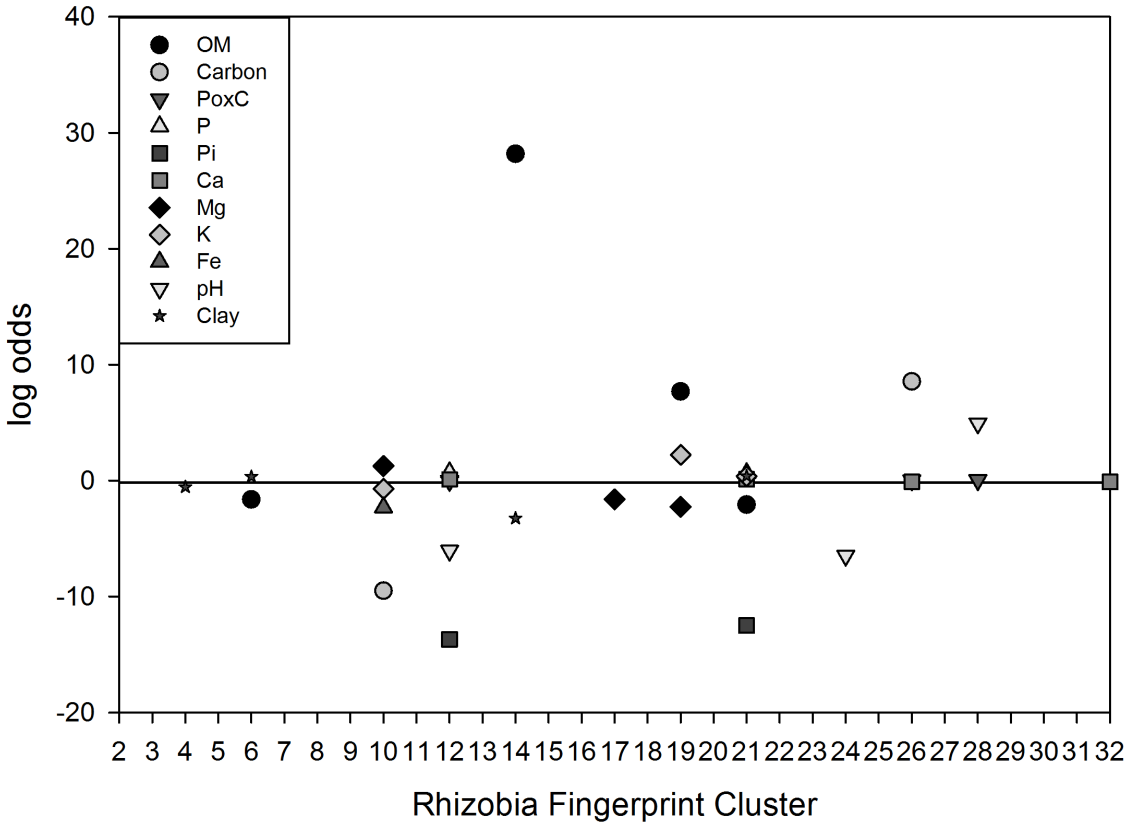

Figure 2-5: Odds ratio of soil characteristics predicting distribution of rhizobia fingerprint clusters . Symbols present on the figure indicate the log of the odds ratio of soil factor that predict the presence of indicated cluster at a significance level of $\alpha < 0.10$.

CHAPTER 3: Phylogeny and Diversity of Rhizobia Forming Nodules With Promiscuous TGx Soybean In Malawi

Introduction

The soil-borne bacteria collectively known as *rhizobia* play an essential role in sustaining global agriculture production, particularly the production of grain legume crops such as soybean (*Glycine max* L.), by increasing the plant's access to nitrogen (N), a necessary nutrient. Through the process of symbiotic biological nitrogen fixation (BNF), rhizobia form nodules on legume plant roots wherein they reduce otherwise plant un-available atmospheric nitrogen (N_2), into ammonium (NH_4) a form plants are able use. Through the cultivation of grain legumes, farmers are able to increase soil nitrogen through utilization of BNF.

Nitrogen inputs through legume BNF is particularly important in such countries as Malawi where low soil nitrogen and limited access to fertilizers has led to reduced crop yields and food insecurity. An extensive study comparing cropping systems over the past decade in Malawi has shown that legume diversified systems not only provide equivalent maize yields to fertilized monoculture maize, but also increase protein production, improve N use efficiency and soil protection, and deliver more favorable value cost ratios for farmers (Snapp et al., 2010). Successful BNF requires host plant legumes as well as specific soil rhizobia. Soybean (*Glycine max*) has one of the highest N fixation potentials of legume crops appropriate for growth in Malawi (Herridge et al., 2008). Not traditionally grown in Malawi, soybean has recently garnered interest because of its nutritional value and potential for soil fertility improvement (Bezner Kerr

et al., 2007; Mhango, 2011). In the Ekwendeni region of Malawi, soybean has been promoted by the local hospital as a nutritional supplement for infants and small children. Interest in soybean and other legume cultivation has increased dramatically following the formation in 2000 of Soils, Food, and Healthy Communities (SFHC) organization associated with Ekwendeni Hospital (Bezner Kerr et al., 2007).

Standard improved soybean varieties are known to form nodules with few species of *Bradyrhizobium*, particularly *B. japonicum* and *B. elkanii.* In fields where soybean has been grown on a regular basis, naturalized populations of these species may remain high enough for adequate nodule formation that no additional N fertilization for maximum growth is required. However where soybean is a novel species, it requires inoculation with appropriate rhizobia when planted. Due to challenges such as weak infrastructure, lack of local development non-existent inoculant production facilities, and low farmer awareness, attempts to distribute inoculant to smallholder farmers in Africa have repeatedly failed (Chianu et al., 2011).

To address the challenge of potentially low soybean-nodulating-rhizobia population sizes in African soils, the International Institute of Tropical Agriculture (IITA) developed the Tropical Glycine Cross (TGx) soybean lines (Kueneman et al., 1984). TGx soybeans have been shown to form a greater number of nodules in un-inoculated soils than standard varieties and are therefore known to be "promiscuous" (Kasasa et al., 1998; Sanginga et al., 1997). Despite widespread dispersal of TGx varieties, research regarding the rhizobia community able to form nodules with TGx soybean is still

limited. Molecular fingerprinting of the IGS region of rhizobia symbionts of TGx soybean, reveal a wide variation in the symbiotic communities of different varieties (Pule-Meulenberg et al., 2011; Wasike et al., 2009). Additional evidence suggests that native rhizobia forming nodules with TGx soybean are not necessarily efficient at N-fixation (Sanginga et al., 1997), and that inoculation may still be required for maximum yield (Thuita et al., 2012; Wasike et al., 2009). However by nature of host promiscuity, inoculant strains don't always compete well for nodule occupancy so that inoculation success depends on the background community of indigenous rhizobia (Okogun and Sanginga, 2003; Pule-Meulenberg et al., 2011).

Soybean variety TGx 1740-2F, released in Malawi under the name "Tikolore" has been shown to have high BNF potential, is moderately promiscuous, and yield adequately without inoculation yet responds favorably to inoculation by increasing N fixation and yield (Pule-Meulenberg et al., 2011; Thuita et al., 2012). Tikolore was released in Malawi as an official variety in early 2011, and promises to be a popular variety among farmers in the Ekwendeni region (*Personal Communication*, SFHC personnel 2011). However little is known about the phylogeny or speciation of the rhizobia that form nodules with this variety.

In recent years, rhizobia speciation and phylogeny has moved toward a multi-locus sequencing approach. Through this approach, neutral housekeeping, or constitutive, genes, are used to determine phylogenetic relationships between isolates. Previous work has used genes responsible for production of ATP Synthase (*atpD)*, glutamine

synthase (*glnII)* and DNA recombinase protein A (*recA)* to determine phylogeny of bacteria, resulting in well resolved phylogenetic tree as well as providing evidence for re-combination within lineages (Vinuesa et al., 2005c; Vinuesa et al., 2008). In addition to analyzing housekeeping genes, analysis of symbiotic genes including *nif* and *nod* genes (responsible for nitrogenase protein complex and nod-factor formation, respectively) can explain variation in symbiotic function or "ecotype" between strains, including-host relationships and nitrogen fixation capacity (Vinuesa et al., 2005a). Recent work, has revealed that symbiotic regions of rhizobia genomes are mobile, conferring nodulation ability of a single plant host across multiple rhizobia species (Barcellos et al., 2007; Batista et al., 2007; Silva et al., 2005). As a result, one ecotype can exist across multiple rhizobia species and genera, and one rhizobia species, may contain multiple ecotypes.

The overall goal of our research is to better understand the microbial community present in smallholder Malawian farm soils to improve BNF of soybean. Specific objectives include determination of the phylogeny and speciation of rhizobia capable of forming nodules with TGx 1740-2F soybean in Malawi, multi-locus sequence analysis of neutral housekeeping genes, and determination of rhizobia ecotype relationships with promiscuous soybean through nifH gene analysis.

Methods

Rhizobia Isolation and cell preparation

A collection of 55 rhizobia isolates were chosen from the NCSU Rhizobia Library for multi-locus sequence analysis. All strains originated from 39 smallholder farms in the Ekwendeni region in Northern Malawi, from fields representing 4 different cultivation histories and were isolated from soil samples using soybean variety TGx 1740-2F as a 'trap' plant asdescribed in the previous chapter. Strains were screened using genomic fingerprinting via repetitive element PCR with the BOX A1R primer, resulting in 32 rhizobia clusters of 70% similarity in fingerprint pattern. Between one and thee strains from each cluster were chosen for further analysis in this experiment. Additionally, reference strains listed in Table 2 were obtained from USDA (personal correspondence, Patrick Elia). All strains were grown on 750 µl Tryptone Yeast broth (TY) or Modified Arabinose Gluconate broth (MAG) for 5-7 days in a 1.5 ml micro-centrifuge tubes. Cells were pelleted in a centrifuge, media removed and cells re-suspended in 100 µl sterile water and stored at -20C for further analysis.

Amplification of Target Sequences

Partial fragments of genes *recA*, *glnII*, *atpD* and *nifH* were amplified using polymerase chain reaction (PCR). DNA oligos used as primers for these reactions are listed in table 3. The *recA* gene was amplified in a 15µl reaction volume consisting of 7.5 µl of 2x Econo Taq MasterMix (Lucigen), 200 nM of primers recA 41F and recA

640R, and 1 µl frozen cell suspension. Reaction cycle consisted of 94ºC for 5 min, 35 cycles of 94ºfor 30 s, 59ºC for 30s and 72ºC for 45 s, followed by final annealing at 72ºC for 5 min. The DNA concentration of the PCR product was estimated by comparing the ethidium bromide signal under UV light to a known standard. If less than the optimum amount of DNA for sequencing was present, 1 µl of product was used as a template in a 30 µl PCR reaction, with two times the reaction mixture to re-amplify the gene. All PCR products were treated with exosap PCR clean-up before sequencing at the NCSU genomic services lab (GSL). The *atpD* gene was amplified using the same reaction as above with an annealing temperature of 64ºC. For some strains, amplification of the *atpD* gene resulted in a weak signal of the PCR product even after multiple attempts. Where this happened 40 µl of one PCR reaction were run on an agarose gel and the resulting band cut from the gel and the DNA purified using the EZNA v-spin gel extraction kit from Omega (Omega Bio-Tek Inc.). The *glnII* sequence was amplified with a reaction using the primer glnII-12F and glnII-689R with an annealing temperature of 56ºC (Table 3). If less than the optimum amount of DNA was produced in the first reaction, 1 µl of product was used in a second reaction using primers GS1 and GS 2 with similar parameters as the first reaction. To amplify the *nifH* gene, we used a nested PCR reaction in order to reduce non-specific amplification (Gaby and Buckley, 2012). The first reaction used primers nifH-F and nifH-I. Reaction mixture and parameters were similar to those for *recA*. The nested reaction used the primers nifH B-112 and nifH CDHP 723R at 0.25uM concentration, 15 µl 2x EconoTaq and1 µl of

the first PCR product in a total volume of 30 µl. Final PCR products were treated with exosap PCR clean-up before sequencing as above. All PCR products were sequenced in both the forward and reverse primers. The resulting two sequences were aligned using BLAST Align program (NCBI). Any sequence discrepancies or gaps were corrected in reference to their chromatograms.

Sequence Analysis and alignment

For each gene, evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011). Phylogenetic reconstruction and evolutionary history were conducted using Maximum likelihood method based on the Tamura-Nei model with the bootstrap method as a test of phylogeny (Tamura and Nei, 1993). The tree with the highest log likelihood is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. All positions containing gaps and missing data were eliminated.

Results

Phylogeny based on *recA*

The phylogenetic tree of the *recA, glnII* and *atpD* were similar, resulting in 2 main groups one consisting of *Bradyrhizobium sp.* (bootstrap value (BV) 98), and the other of *Rhizobium* species. Among those who fell in the genus *Bradyrhizobium,* most were

closely related to *B. elkanii*, as can be seen in the upper branch of the tree shown in Figure 1.

In the phylogeny of the *recA* gene, six small sub-groups form a polyphyletic group of strains related to *B. elkanii*. One isolate, NCSU 2401 had a *recA* sequence related to *B. japonicum*, seen in figure 1 in a cluster with *B. japonicum* strains USDA 6 and USDA 110. In addition to the strains related to *B. japonicum* and *B. elkanii*, two clusters were related to *Bradyrhizobium sp.*

Of the remaining isolates, all fell within the genus *Rhizobium*. Strain NCSU 2363 was closely related to *R. etli*, as seen in the cluster containing *R. etli* strain USDA 9032. Two strains, NCSU 2642 and 2658 formed a group with a unique *recA* sequence of which the closest sequence was *Rhizobium grahamii* CCGE 502, a strain from Mexico known to form nodules with *Dalea leporina* L., a tropical vine. The *recA* sequences of several strains on the lower branch of the tree were closely related to *Rhizobium tropici* (BV 32).

Phylogeny based on glnII

Phylogeny based on *glnII* (Figure 2) was similar to that of *recA*. however the population exhibited less variation in the *glnII* gene than in the *recA* gene. As with *recA,* the large cluster identified as *B. elkanii* (BV 99) was present, the within species variation of this gene is very low with over 30 strains exhibiting a nearly identical sequence. As with *recA* phylogeny, the *glnII* gene of strain NCSU 2401 fell within the cluster of B*. japonicum* (BV 99) and two strains NCSU 2477 and 2509 clustered together

with *Bradyrhizobium* sp. SE05 (BV 100) . Eight strains, NCSU 2453, 2459, 2609, 2076, 2099, 1953, 2200 and 1943, form a distinct clade in the lower part of the phylogenetic tree which includes two type strains of *R. tropici* (BV 49). Strain NCSU 2363 forms a cluster with a type strains of R. etli (HBR5). Strains NCSU 2642 and NCSU 2658 fall within a unique space within the *Rhizobium* genera, where the closest related reference strain is *R. pusense*.

Phylogeny based on atpD

Phylogeny of *atpD* sequences resulted in a similar three as with *recA* and *glnII* (Figure 3). The same large group of closely related strains found in *recA* and *glnII* phylogenies also appears in this tree; however the closest related strain was *Bradyrhizobium sp.* SEMIA 6145 (BV 85), a strain from BrazilBrazil that is in the cowpea nodulating group of *Bradyrhizobium* rather than *B. elkanii*. *Bradyrhizobium elkanii* formed a polyphyletic group with *B. japonicum* and *Bradyrhizobium sp.* Only four of our analyzed strains showing genetic similarity of this region to *B. elkanii*. *Rhizobium* species also formed a polyphyletic groups with four strains grouping with *R. tropici* and *Rhizobium* sp. and one strain, NCSU 2363 grouping with *R. leguminosarum*. One strain NCSU 2642 grouped with *Sinorhizobium fredii* (BV 67). Despite repeated attempts, we were unable to amplify the *atpD* sequence from strains NCSU 1953, NCSU 2509, NCSU 2477, and NCSU 2401. For other housekeeping genes, NCSU 2509 and 2477 were closely related to a suspected *B. canariense* strain and NCSU 2401 was most related to *B. japonicum.*

Speciation based on constitutive genomic elements

Because of recombination and shifting of the atpD sequence between lineages, phylogeny based on multiple genes was only conducted with *recA* and *glnII*. Phylogeny of concatenated sequences of *recA* and *glnII* show the same segregation of strains with those related to *Bradyrhizobium* forming a large clade distinct from those related to *Rhizobium.* (Figure 4). Within the *Bradyrhizobium* group, the majority of strains formed a monophyletic cluster with *B. elkanii.* Strain NCSU 2401 again clusters with *B. japonicum*, and NCSU 2477 and NCSU 2509 form a group with *Bradyrhizobium* sp. SE03. Within the group that clustered with *Rhizobium*, two strains, NCSU 2658 and NCSU 2642 fell outside of clusters containing known type strains, potentially forming a unique species of *Rhizobium*. Several strains, as seen in individual gene trees, formed a group with *R. tropici*.

Ecotype of isolates based on nifH

The phylogeny of nifH sequences differed considerably from those of the housekeeping genes. Aside from NCSU 2363, all analyzed strains matched nifH sequences from the *Bradyrhizobium* genus. A large group of similar strains clustered with *B. elkanii* and *Bradyrhizobium sp.*, however many of these strains had housekeeping sequences different from each other. This large group has a nifH sequence matching reference strains originating from nodules of wild legumes such as *Sophora alopecuriodes* (sophora root), *Vicia hirsute* (tiny vetch), and *Aeschynomene* (jointvetch) as well as crop legumes including V*igna unguiculata* (cowpea) and

Phaseolus lunatus (lima bean). Three strains, NCSU 2401, NCSU 2690 and NCSU 2135 were similar to strains originating from the nodules of *Glycine max*, and seven strains were similar to the nodule occupants of *Aeschynomene*.

Discussion

Phylogeny of rhizobia forming nodules with promiscuous soybean

We found a wide variety of both *Rhizobium* and *Bradyrhizobium* species in the nodules of promiscuous soybean isolated from fields in Malawi, including isolates related to *B. elkanii, B. japonicum, Bradyrhizobium sp*., *R. etli, R. tropici, and Rhizobium sp.* Among the strains clustering with *Bradyrhizobium*, the majority were most closely related *to B. elkanii* with only one strain, NCSU 2401, related to *B. japonicum.* In many studies, *B. japonicum* is one of the most common species to be isolated from soybean nodules, and we expected to see a large group of *B. japonicum* isolates. Strains NCSU 2477 and NCSU 2509 clustered closely with *Bradyrhizobium sp.* SE03, which is likely a strain of *B. canariense* (Stepkowski et al., 2007). *B. canariense* is an acid-tolerant lineage of *Bradyrhizobium* that was found to form nodules with *Genisteae* and *Loteae* species, but not *Glycine* species (Vinuesa et al., 2005b). The presence of these strains in nodules of TGx soybean contributes to the evidence of the promiscuity of these soybean lines.

Among isolates that grouped with *Rhizobium*, eight are most likely strains of *R. tropici*. A fast growing strain that has appeared in nodules of soybean in BrazilBrazil (Hungria et al., 2006). One strain NCSU 2363 is most likely a strain of *R. etli*, and two

strains NCSU 2658 and NCSU 2642, fall within the *Rhizobium* genera, but do not consistently group with any known species. None of the unknown isolates grouped with *Sinorhizobium* despite that fact that *Sinorhizobium* is known to form nodules with soybean.

Horizontal gene transfer evidenced by incongruous phylogenies.

In comparing the phylogenies of *recA*, *glnII* and *atpD* with that of *nifH*, we see possible evidence of horizontal gene transfer of the *nifH* region. While the phylogeny of constitutive genes resulted in similar patterns for our test strains, *nifH* phylogenetic analysis resulted in a unique tree, with more sequences aligning with *Bradyrhizobium* species than with *Rhizobium*. Specifically, in constitutive phylogenies, strains NCSU 2099, NCSU 1953, NCSU 2200, NCSU 2453, and NCSU 2459 form a clade, or single branch of an ancestor and its descendants, closely related to *Rhizobium tropici*. However the *nifH* sequence of one of these, NCSU 2459, grouped with *B. elkanii,* while NCSU 2099, 1953, 2200 and 2453 grouped with *B. denitrificans and Bradyrhizobium sp.* Similarly, constitutive genes of NCSU 2642 are most related to a strain of *Rhizobium sp.,* but the *nifH* gene is most closely related to *B. elkanii*. In *Bradyrhizobium japonicum*, the nifH gene is located in the symbiotic island which includes several genes involved in plant signaling and nodule formation (Kaneko et al., 2002). Symbiosis islands have been identified previously as transmittable DNA segments of approximately 500 kb containing genes critical for nodule formation and nitrogen fixation (Sullivan and Ronson, 1998). In *Rhizobium leguminosarum*, the symbiotic island makes up part of

plasmid pRL10 (Young et al., 2006). As a "transmittable" segment, there have been numerous phylogenetic studies suggesting that these symbiotic regions are susceptible to horizontal gene transfer both within and between species and genera (Barcellos et al., 2007; Menna and Hungria, 2011; Silva et al., 2005). The similarity of nifH genes in phylogenetically dissimilar isolates that we found suggests a fairly recent transfer event from *Bradyrhizobium* into *Rhizobium* species. Since *nifH* and nodulation genes are both part of the symbiotic island and usually transferred together, this likely responsible for their ability to form nodules with soybean.

Horizontal gene transfer has been observed in harsh tropical soil environments when strains not native to a soil are introduced through inoculation (Barcellos et al., 2007; Hungria et al., 2006). Studies in BrazilBrazil have shown that prior to soybean expansion in the 1960's soils were devoid of rhizobia capable of nodulating soybean (Loureiro et al., 2007), and inoculants used by farmers were primarily from the slow growing *Bradyrhizobium japonicum* and *B. elkanii* species. However, by the year 2000, isolates from soybean nodules were shown to be both slow and fast growing and classified phylogenetically as *R. tropici*, *Rhizobia sp*., *Agrobacterium sp*., as well as fast growing *B. japonicum* and *B. elkanii*, indicating that horizontal gene transfer allowed native strains that previously were incapable of nodulating soybean to become capable, and that mutation in inoculant strains resulted in fast growing strains of *Bradyrhizobium* (Hungria et al., 2006). The Malawian soils in this study had never been subject to inoculation, therefore the most probable source of the *nifH* sequence are

Bradyrhizobium sp. capable of nodulating wild legumes and/or common crops such as *Vigna unguiculata.*

TGx soybean has a diverse symbiotic community

The *nifH* sequence phylogeny not only suggests horizontal gene transfer, but also that the origin of the symbiotic region in our isolated rhizobia strains is more similar to the symbionts of wild legumes than to soybean. The largest clade on the nifH phylogeny contained reference sequences of *Bradyrhizobium sp.* originating from diverse hosts including *Sophora alopecuriodes* (CCBAU 83335), Vicia *hirsuta* (CCBAU 83502) and *Vigna unguiculata* (CCBAU 51012 and TUXTLAS-17 Pop 321). These reference strains were isolated from legumes in China (CCBAU strains) and Mexico (TUXTLAS strain). The TUXTLAS-17 strain has a *nodZ* sequence belonging to *nodZ* group III (López-López et al., 2013), which is a lineage of *Vigna* nodulating *Bradyrhizobium* genes with historical origins in Africa (Steenkamp et al., 2008). Vigna unguiculata, cowpea, is a commonly grown throughout Malawi as an important food source and cowpea nodulating rhizobia are likely plentiful in the soil. Only three of the isolated strains, NCSU 2135, NCSU 2690 and NCSU 2401 had a nifH gene region similar to *Glycine max*-nodulating isolates (USDA 122 and SEMIA 5027). Additionally, the phylogeny of the *nifH* sequence NCSU 2363 indicates that this strain is most likely related to *R. leguminosarum*, a species that is not usually known to form nodules with soybean. Combined, our data suggests that the TGx soybean variety assessed in this study does indeed form nodules with a wider symbiotic group than non-promiscuous

varieties of soybean, one that is likely similar to the symbiotic group of cowpea and *Aeschynome indica*, a common green manure used throughout Africa.

Limited *B. japonicum* presence impediment to enhanced BNF

In the Ekwendeni region where these strains originated, soybean grain yields remain low, < 1 Mg ha^{-1} (Mhango et al., 2008), compared to other tropical regions which produce >4 Mg ha^{-1} soybean (Hamawaki et al., 2010). A proposed cause of these low yields is low numbers of native rhizobia capable of nodulating soybean (Chilimba et al., 2001). When growing standard non-promiscuous soybean lines, an adequate soybean-nodulating-rhizobia population is critical to maintain yields. Only one of the strains analyzed from this region was related to *B. japonicum* and three strains had *nifH* sequences similar to those from a non-promiscuous soybean nodulating rhizobia (*B. japonicum* USDA 122 and *B. elkanii* SEMIA 5027), indicating that there are likely a limited number of bacteria in these soils capable of forming nodules on traditional non-promiscuous soybean. Previous studies in Africa have shown that TGx 1740-2F responds to inoculation with *B. japonicum* strains with increased BNF, nodule count and plant nitrogen, indicating that despite the ability to form nodules with local *Bradyrhizobium* strains, total BNF is enhanced when *B. japonicum* is present (Pule-Meulenberg et al., 2011; Thuita et al., 2012). The limited presence of *B. japonicum* may explain why farmers in this region have observed low nodulation on traditional soybean varieties. We do not know the N-fixation capacity of the strains studied here, but learning this would be important because if the large group of strains clustering

with *B. elkanii* and *Bradyrhizobium sp.* exhibit reduced N-fixation capacity, this would present a formidable barrier to improving N-fixation capacity of soybean through inoculation (Okogun and Sanginga, 2003).

Implications for soybean production and improving BNF of promiscuous soybean,

We demonstrate here that TGx 1740-2F soybean has the ability to form nodules with a wide variety of *Bradyrhizobium* and *Rhizobium* species representing a range of ecotypes much wider than that of traditional soybean. Of the strains analyzed here, only 11 were isolated from fields with a history of soybean cultivation, all of which took place in the past 10 years, 11 strains were from fields with a history of crop legume cultivation other than soybean, 13 were from native, uncropped fields and 17 were from fields cropped with non-legumes. While it is known that a history of soybean cultivation will increase the number of soybean-nodulating rhizobia present in a soil, sometimes for as long as 8 years after the last cultivation (Elkins et al., 1976), this study suggests that cultivation of TGx soybean could possibly also affect the symbiotic community of other agronomically important host legumes, specifically, *Phaseolus vulgaris* and *Vigna unguiculata*. This is especially important as a rhizobia strain that forms effective nodules on one host may form ineffective nodules on a different host (Odee, 1989). Horizontal gene transfer of nifH sequences from *Bradyrhizobium* to *Rhizobium* species suggests that transfer between local lineages of rhizobia has contributed to the diversity of the symbiotic community of TGx soybean to include rhizobia beyond the *Bradyrhizobium* group.

Conclusion

Promiscuous soybean is able to form nodules with a wide diversity of indigenous *Rhizobium* and *Bradyrhizobium* strains in Malawian soils. Most of these strains are closely related to *B. elkanii, Bradyrhizobium sp*. and *R. tropici,* with only one isolate identified as *B. japonicum*. Horizontal gene transfer of symbiotic genes from *Bradyrhizobium* into *Rhizobium* species may account for the wide host range observed here. Applied research regarding N-fixation capacity of local strains will serve to further improve N fixation on smallholder farms leading to improved soil quality and food security.

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Tables

Table 3-1: Strain and Accession Numbers included in investigation

Strain	Area	Field history	cluster	atpD	recA	glnII	nifH
NCSU 2192	Chilida	legume	5	-	KJ535959	KJ536012	KJ546599
NCSU 2265	Chilida	legume	6	KJ535916	KJ535968	KJ536021	KJ546608
NCSU 2438	Kaluhoro	legume	10	KJ535928	KJ535980	KJ536032	KJ546619
NCSU 2631	Chilida	legume	19	KJ535942	KJ535995	KJ536046	KJ546633
NCSU 2322	Luhomero	legume	21	KJ535918	KJ535970	KJ536023	KJ546610
NCSU 2446	Kaluhoro	legume	21	KJ535929	KJ535981	KJ536033	KJ546620
NCSU 2099	Bwabwa	legume	25	KJ535903	KJ535954	KJ536007	KJ546594
NCSU 2632	Chilida	legume	26	KJ535943	KJ535996	KJ536047	KJ546634
NCSU 1943	Luhomero	legume	27	KJ535898	KJ535948	KJ536001	KJ546588
NCSU 1953	Luhomero	legume	27	-	KJ535949	KJ536002	KJ546589
NCSU 2320	Luhomero	legume	30	KJ535917	KJ535969	KJ536022	KJ546609
NCSU 1933	Luhomero	legume	32	KJ535897	KJ535947	KJ536000	KJ546587
NCSU 2609	Zombwe	native	1	KJ535939	KJ535993	KJ536045	KJ546632
NCSU 2248	Bwabwa	native	4	KJ535915	KJ535967	KJ536020	KJ546607
NCSU 2207	Luhomero	native	9	KJ535911	KJ535963	KJ536016	KJ546603
NCSU 2028	Zombwe	native	10	KJ535899	KJ535950	KJ536003	KJ546590
NCSU 2209	Luhomero	native	12	KJ535912	KJ535964	KJ536017	KJ546604
NCSU 2459	Enyezini	native	13	KJ535931	KJ535983	KJ536035	KJ546622
NCSU 2185	Bwabwa	native	15	KJ535907	KJ535958	KJ536011	KJ546598
NCSU 2642	Chilida	native	16	KJ535944	KJ535997	KJ536048	KJ546635
NCSU 2039	Zombwe	native	28	KJ535900	KJ535951	KJ536004	KJ546591
NCSU 2211	Luhomero	native	31	KJ535913	KJ535965	KJ536018	KJ546605

Table 3-1 (cont.): Strain and Accession Numbers included in investigation

Strain	Area	Field history	cluster	atpD	recA	glnII	nifH
NCSU 2369	Luhomero	non-legume	8	KJ535923	KJ535975	KJ536028	KJ546615
NCSU 2333	Luhomero	non-legume	10	KJ535919	KJ535971	KJ536024	KJ546611
NCSU 2477	Zombwe	non-legume	13	-	KJ535986	KJ536038	KJ546625
NCSU 2583	Chisangano	non-legume	14	KJ535938	KJ535992	KJ536044	KJ546631
NCSU 2476	Zombwe	non-legume	16	KJ535933	KJ535985	KJ536037	KJ546624
NCSU 2473	Zombwe	non-legume	17	KJ535932	KJ535984	KJ536036	KJ546623
NCSU 2363	Luhomero	non-legume	18	KJ535922	KJ535974	KJ536027	KJ546614
NCSU 2112	Baula	non-legume	20	KJ535904	KJ535955	KJ536008	KJ546595
NCSU 2513	Baula	non-legume	21	KJ535934	KJ535988	KJ536040	KJ546627
NCSU 2204	Enkongolweni	non-legume	22	KJ535910	KJ535962	KJ536015	KJ546602
NCSU 2509	Baula	non-legume	24	-	KJ535987	KJ536039	KJ546626
NCSU 2200	Chisangano	non-legume	25	-	KJ535961	KJ536014	KJ546601
NCSU 2401	Enyezini	non-legume	29	-	KJ535977	KJ536030	KJ546617
NCSU 2199	Chisangano	non-legume	30	KJ535909	KJ535960	KJ536013	KJ546600
NCSU 2335	Luhomero	non-legume	30	KJ535920	KJ535972	KJ536025	KJ546612
NCSU 2384	Luhomero	non-legume	30	KJ535925	KJ535976	KJ536029	KJ546616
NCSU 2576	Chisangano	non-legume	30	KJ535936	KJ535990	KJ536042	KJ546629
NCSU 2579	Chisangano	non-legume	30	KJ535937	KJ535991	KJ536043	KJ546630
NCSU 2536	Enkongolweni	non-legume	32	KJ535935	KJ535989	KJ536041	KJ546628
NCSU 2453	Enyezini	soya	3	KJ535930	KJ535982	KJ536034	KJ546621
NCSU 2658	Chilida	soya	4	KJ535945	KJ535998	KJ536049	KJ546636
NCSU 2358	Luhomero	soya	7	KJ535921	KJ535973	KJ536026	KJ546613
NCSU 2223	Baula	soya	11	KJ535914	KJ535966	KJ536019	KJ546606
NCSU 2135	Enkongolweni	soya	13	KJ535906	KJ535957	KJ536010	KJ546597
NCSU 2404	Enyezini	soya	23	KJ535926	KJ535978	KJ536031	-
NCSU 2690	Chisangano	soya	24	KJ535946	KJ535999	KJ536050	KJ546637
NCSU 2076	Enkongolweni	soya	25	-	KJ535953	KJ536006	KJ546593
NCSU 2114	Enkongolweni	soya	26	KJ535905	KJ535956	KJ536009	KJ546596
NCSU 2061	Enkongolweni	soya	32	KJ535901	KJ535952	KJ536005	KJ546592

Table 3-2: USDA Reference strains and species included in investigation.

Strain	Species
USDA 6	*B. japonicum*
USDA 76	*B. elkanii*
USDA 110	*B. japonicum*
USDA 191	*S. fredii*
USDA 201	*S. fredii*
USDA 205	*S. fredii*
USDA 207	*S. fredii*
USDA 257	*S. fredii*
USDA 2370	*R. leguminosarum*
USDA 2145	*R. leguminosarum*
USDA 3384	*Bradyrhizobium sp.*
USDA 4893	*S. Saheli*
USDA 9032	*R. etli*
USDA 9039	*R. tropici*

Table 3-3: Oligos used as primers in sequencing reactions

Target Gene	Primer Name	Sequence	Reference
recA	recA 41F	TTCGGCAAGGGMTCGRTSATG	(Vinuesa et al., 2005a)
	recA 640R	ACATSACRCCGATCTTCATGC	
glnII	glnII 12F	YAAGCTCGAGTACATYTGGCT	(Vinuesa et al., 2005a)
	glnII 689R	TGCATGCCSGAGCCGTTCCA	
	GSII-1	AACGCAGATCAAGGAATTCG	(Turner and Young, 2000)
	GSII-2	ATGCCCGAGCCGTTCCAGTC	
atpD	atpD 255F	GCTSGGCCGCATCMTSAACGTC	(Vinuesa et al., 2005a)
	atpD 782R	GCCGACACTTCMGAACCNGCCTG	
	atpD 273 F	SCTGGGSCGYATCMTGAACGT	(Gaunt et al., 2001)
	atpD 771 R	GCCGACACTTCCGAACCNGCCTG	
nifH	nifH-F	TACGGNAARGGSGGNATCGGCAA	(Laguerre et al., 2001)
	nifH-I	AGCATGTCYTCSAGYTCNTCCA	
	nifH B1-112	GGC TGC GAT CCC AAG GCT GA	(Noar and Buckley, 2009)
	nifH CDHP-723R	GATGTTCGCGCGGCACGAADTRNATSA	

Figures

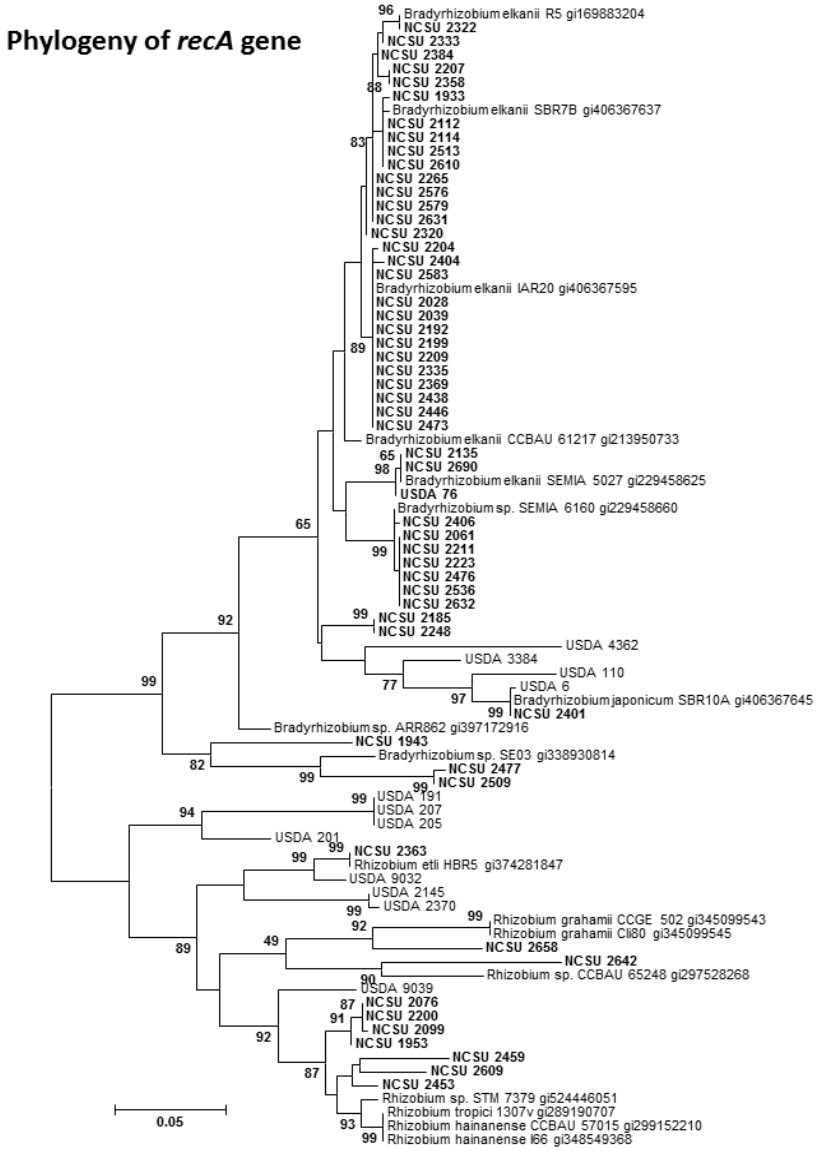

Figure 3-1: Phylogeny of Rec A Gene.

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei,1993). The tree with the highest log likelihood (-3964.9968) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 90 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 402 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al. 2011)

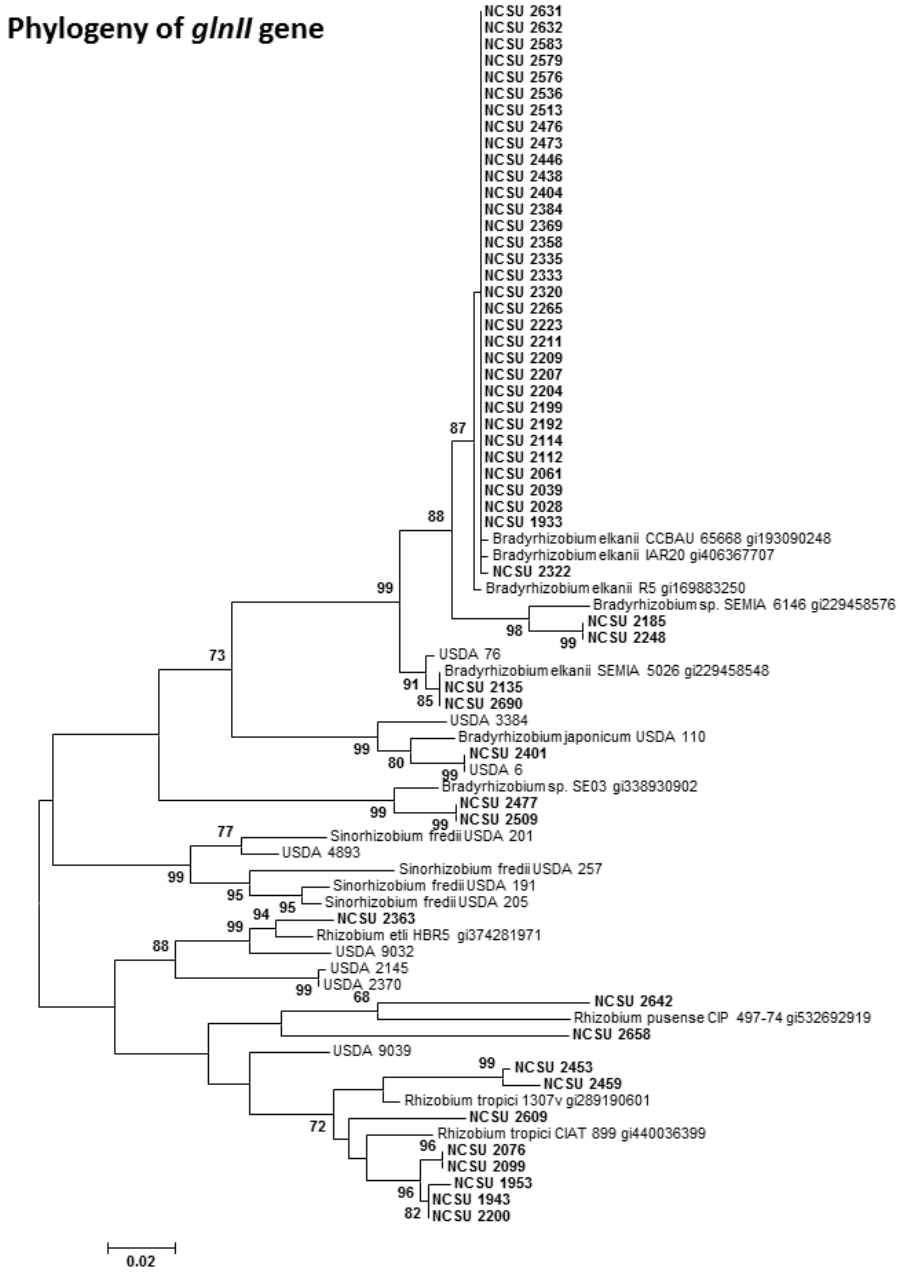

Figure 3-2:. Phylogeny of glnII gene.

Molecular Phylogenetic analysis by Maximum Likelihood method The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model [1]. The tree with the highest log likelihood (-3631.4837) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 80 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 454 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 [2].

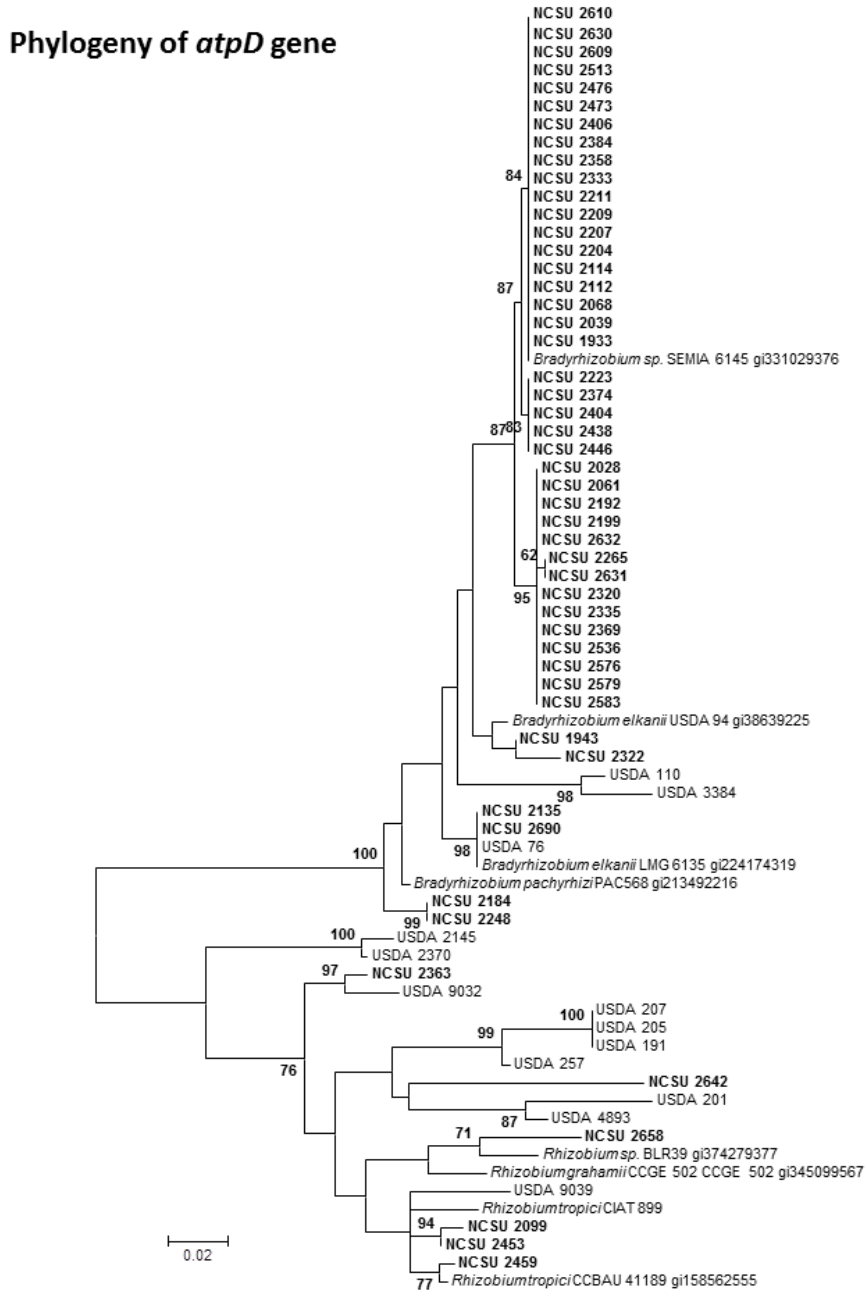

Figure 3-3: Phylogeny of atpD gene

. Molecular Phylogenetic analysis by Maximum Likelihood method . The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model [1]. The tree with the highest log likelihood (-2496.5033) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 71 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 400 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 [2].

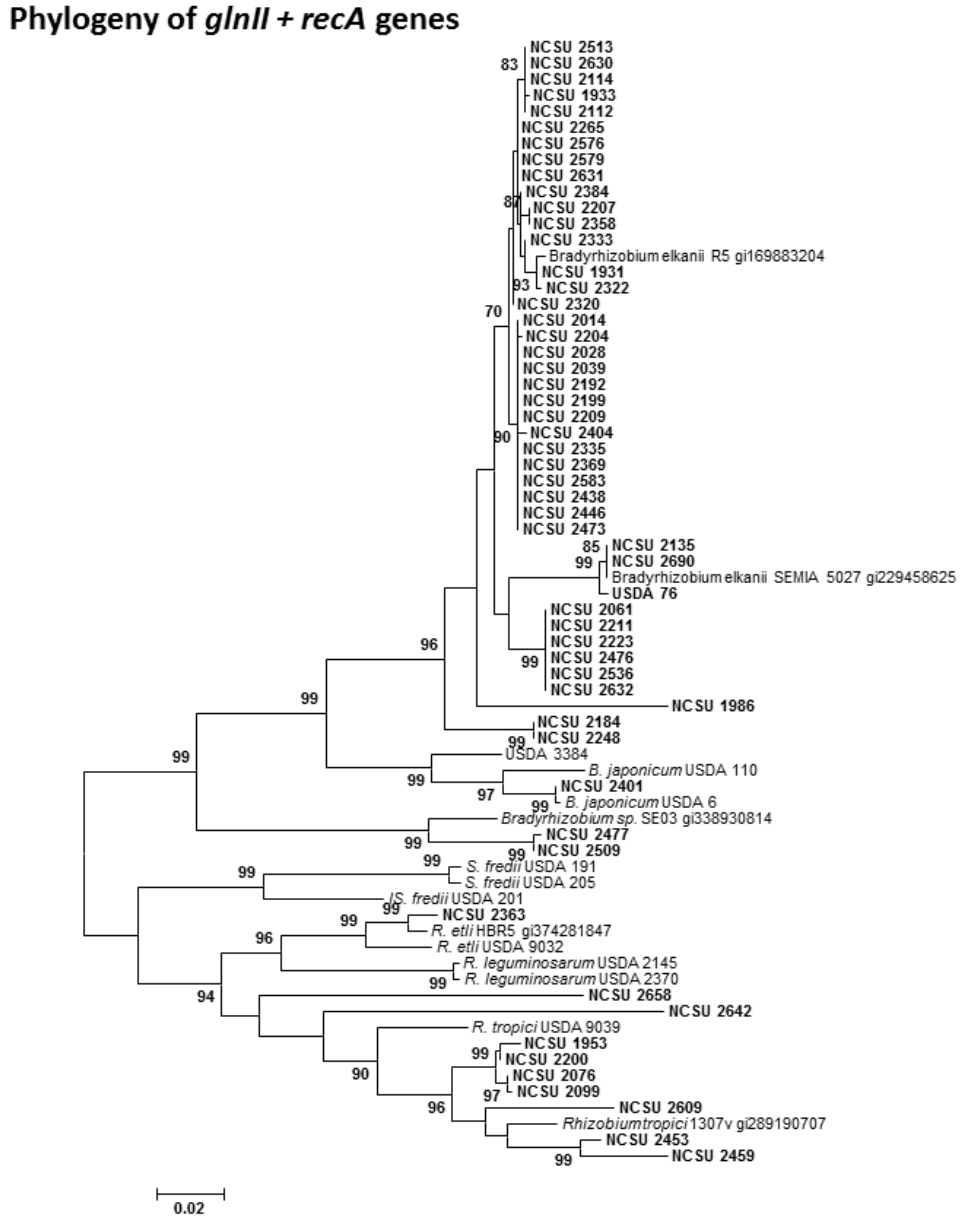

Figure 3-4: Phylogeny of glnII + recA genes.

Molecular Phylogenetic analysis by Maximum Likelihood method :The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model [1]. The tree with the highest log likelihood (-6632.8266) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 70 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 850 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 [2

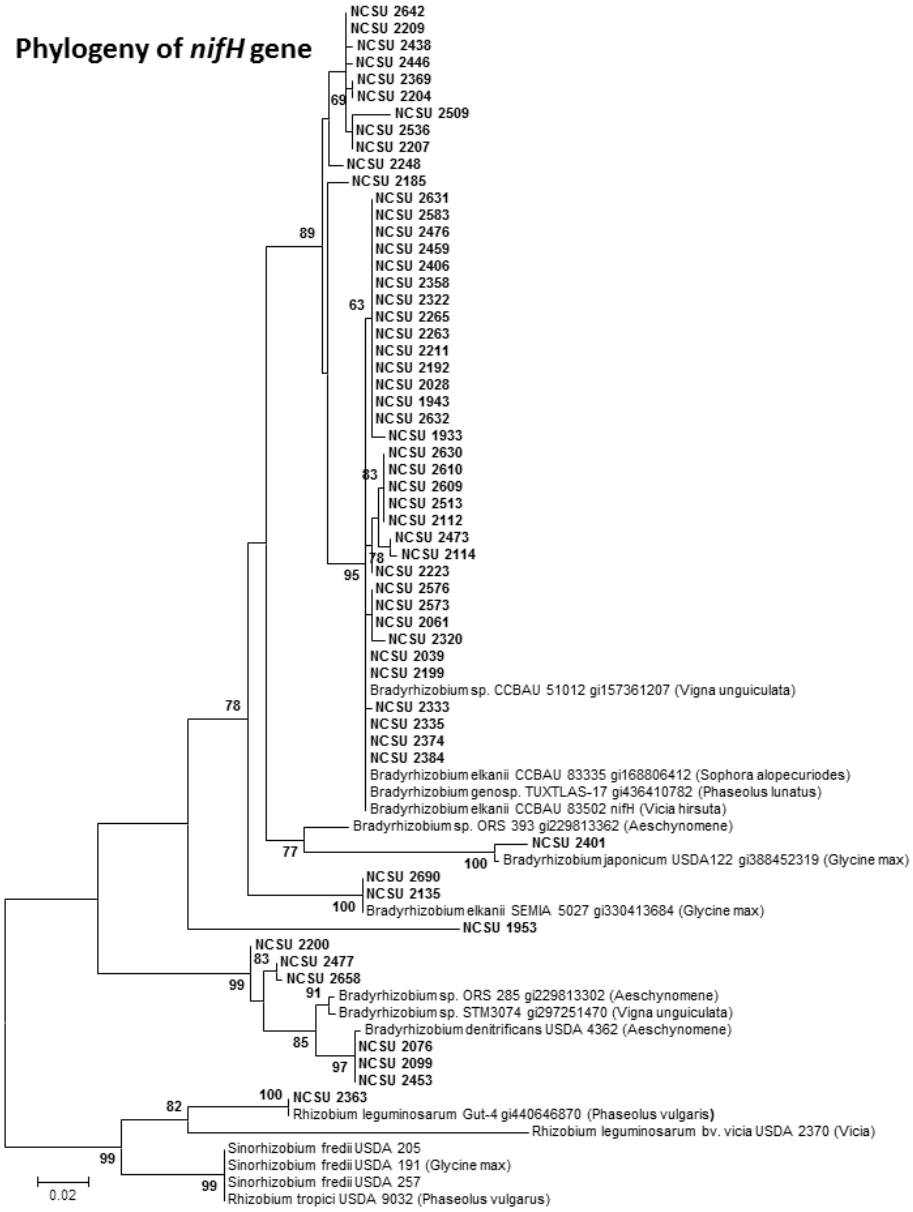

Figure 3-5 : Phylogeny of nifH gene

. Molecular Phylogenetic analysis by Maximum Likelihood method The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model [1]. The tree with the highest log likelihood (-2223.7946) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 76 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 389 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 [2]

CHAPTER 4: Constraints to Improving Soybean Production on Smallholder Farms in Malawi: Implications for Future Research

Introduction

Achieving food security in Africa will require increasing yields on smallholder farms through improved soil fertility. Continuous cultivation of high N-use crops such as maize, without nutrient supplementation through fertilizer inputs has resulted in reduced yields in response to very low soil nitrogen (N) throughout the continent (Sanchez et al., 2009; Vitousek et al., 2009). Integrating legume crops such as soybean (G*lycine max*) into cropping systems can improve soil fertility through symbiotic legume biological N fixation (BNF) with rhizobia bacteria. Long-term legume integration in small-holder agricultural systems is associated with improved protein output, greater soil cover, greater soil N and organic matter content, and improved yields of non-legume grain crops (Snapp et al., 2010).

Malawi, a country in the southeastern region of Africa, epitomizes the challenges of improving agricultural production in Africa. Soybean has recently garnered increased interest in Malawi because of its nutritional value (Bezner Kerr et al., 2007). In the Ekwendeni region of Malawi, soybean has been promoted by the local hospital as a nutritional supplement for infants and young children. Interest in soybean and other legume cultivation has increased dramatically following the formation in 2000 of the organization Soils, Food, and Healthy Communities (SFHC) associated with Ekwendeni Hospital (Bezner Kerr et al., 2007). Unfortunately, soybean grain yields in Malawi

remain low, < 1 Mg ha^{-1} (Mhango et al., 2008), compared to other -tropical regions which often produce > 4 Mg ha^{-1} soybean (Hamawaki et al., 2010).

Improving soybean yields on smallholder farms remains a challenge. Due to limited fertilizer access and the perceived ability of legumes to fix N, farmers usually reserve fertilizer application for maize crops and rarely fertilize legumes (Bezner Kerr et al., 2007; Mhango et al., 2013). Phosphorous is a critical nutrient in the BNF process, and is often the most limiting nutrient for legume production (Israel, 1987). Previous work in the Ekwendeni region of Malawi has shown that legume crops are typically grown on fields with P status below the critical level, and that many legume crops would likely benefit from P fertilizer (Mhango et al., 2013). Studies in other regions of Malawi have shown a positive response by maize and soybean grain to P and sulfur (S) application (Mhango et al., 2008). Other than N and P, little work has been done to determine possible limitations of essential nutrients such as K, Ca, Mg, and Fe tocrop growth in Malawi. For example, Fe in particular is essential to leghemoglobin production – an essential component of the rhizobia N fixation process (Ragland and Theil, 1993).

While not a direct contributor to plant growth and production, soil organic matter (OM) is often used as an indicator of soil quality (Fortuna et al., 2008; Moebius-Clune et al., 2011), and has been shown to correlate with crop yields (Aase and Pikul, 1995; Lucas and Weil, 2012). Organic matter is known to have a major impact on various soil quality indicators including infiltration, water holding capacity, microbial biomass and nutrient cycling (Lagomarsino et al., 2011; Lagomarsino et al., 2012; Meek et al., 1982).

Crop production in the Ekwendeni region is rain-fed. The rainy season in Malawi begins in November and continues through early April with a brief period of reduced rainfall in February. However, both inter-annual and spatial variation of rainfall is high, particularly toward the end of the rainy season, in March and April (Nicholson et al., 2014). For this reason, planting time has been shown to be critical for maize production (MacColl, 1990), but little formal research has been done on soybean or other legume crops in Malawi to determine the impact of planting time. It is known that even mild water stress can have negative impacts on nodule formation and BNF (Serraj and Sinclair, 1998; Sinclair et al., 2000).

Previous studies on soybean growth in Africa have shown that low numbers of soil rhizobia are present (Chilimba et al., 2001; Thuita et al., 2012). Such reduced rhizobia population sizes can be remedied through the use of rhizobia inoculants at planting, but these are generally unavailable to smallholder farmers. Preliminary studies in the Ekwendeni region of Malawi showed wide variation in nodule counts between farms (*unpublished data* Snapp, 2010), which may be limiting BNF and soybean growth in the region.

Agronomic studies on soybean have been conducted but research trials do not often reflect practices of smallholder farmers (Kamanga et al., 2010; Mhango et al., 2008). In order to improve soybean production, information regarding production drivers and limitations to yield on smallholder farmers' fields are needed. In this study, we used the farmer-network structure of the Soils Food and Healthy Communities organization to

conduct a region wide-survey on actively growing soybean fields ranging from high producing fields to those with moderate to low production. Since farmers in this region face land limitations, evidence that soybean production will result in high yields is needed in order to encourage sowing of soybean and not maize, as is traditionally planted in this region. While some farmers have success with the new crop, others experience crop failure and are discouraged from continued soybean cultivation (personal communication, SFHC staff). The purpose of this study was to determine variations in soil quality, rhizobia population size, and agronomic practices between fields where farmers determined the soybean crop to be a success and fields where farmers determined the crop to perform poorly. Additionally, we sought to identify constraints to improving soybean yield on smallholder farms in Malawi with the goal of developing guidelines for smallholder farmers to improve the chances of a successful crop and identification of knowledge gaps for further agronomic research. We hypothesized that late planting date, low soybean-nodulating rhizobia population sizes and/or soil nutrient limitations are responsible for low performance of soybean fields.

Methods

Selection of fields

This study was conducted in collaboration with the Soils Food and Healthy Communities Farmer research groups associated with the Ekwendeni Hospital in Ekwendeni, Malawi. Through focus group meetings, farmer groups identified soybean

fields in their communities that were performing well (HIGH fields) and fields that were low performing (LOW fields). Based on farmer interest and availability, a sub-sample of both HIGH and LOW fields were selected for in-depth analysis.

Field sampling and field histories

Farmer interviews, soil, above ground biomass and root samples were taken on 18 farms in late February 2012. At each field, oral interviews were conducted with the farmer through translators from SFHC. Farmers identified planting date, variety, rhizobia inoculation history fertilizer inputs utilized, soybean planting history, and qualitative observations regarding the success or failure of their soybean field.

Soybean and soil sampling

Soil samples were taken using a 2 cm diameter soil probe. A 20 cm core was sampled from every 3rd ridge in a diagonal transect across the field. Soil was collected in a plastic bucket, thoroughly mixed, and a subsample collected in a plastic bag. The collection bucket and soil probe were cleaned with water between each field. At each field a ½ meter length of row was sampled from a randomly chosen part of the field. Stems were cut at the soil surface and placed in a large paper sample bag. Using a hoe, roots were also excavated from the ridge, attempting to collect all viable roots, and collected in a plastic bag. Root nodules were counted and separated from the root mass immediately following biomass harvest. Because the study area was far from a drying oven, biomass samples were allowed to air-dry at approximately 25ºC to 30ºC for 2 to 3 days until they could be transported to the Chitedze research station near Lilongwe and

then dried at 70˚C. Oven dried samples were then weighed for final dry weight. Dry biomass was ground to pass through a 1mm mesh and submitted to the Environmental and Agricultural Testing Services Lab (NCSU, Raleigh NC) where it was analyzed for elemental carbon and nitrogen using a Perkin Elmer 2400 CHNS/O Elemental Analyzer.

Soil Analysis

Soils were air-dried and sieved to pass a 2mm mesh. A Mehlich 3 extraction was conducted on dried soil samples as described previously (Mehlich, 1984). Dissolved orthophosphate in Mehlich extracts was analyzed colorimetrically using the ascorbic acid method (Yuen and Pollard, 1955) on QuikChem 2000 flow injection autoanalyzer (Lachat Instruments, Loveland CO). Extracts were also analyzed for magnesium (Mg), calcium (Ca), iron (Fe), and potassium (K) using flame atomic adsorption spectroscopy with an air-acetylene flame on a Fisher Scientific F.S ICE 3300 AA. Solutions were diluted with lithium-lanthenum when measuring Mg, Ca and K to prevent ionization. Soil pH was measured at a 1:1 soil slurry in distilled water. Soil slurries were allowed to equilibrate for 1 hour before measurements were taken.

Three measurements of soil carbon were made that have been shown to represent three distinct pools of soil carbon (Beedy et al., 2010; Culman et al., 2012; Szava-Kovats, 2009). Total soil organic matter was measured using the loss on ignition method (OM_{LOI}). A 10 g sample of dry soil was ignited in a muffle furnace at a low temperature of 400 C for 16 hours to reduce the loss of structural water from kaolinite clays that can occur at temperatures above 450˚C (Ball, 1964; Rhodes et al., 1981). For total C

measurements, dry soil was pulverized in a ball grinder and analyzed for elemental C and N using a Perkin Elmer 2400 CHNS/O Elemental Analyzer Environmental and Agricultural Testing Services Lab (NCSU, Raleigh NC). Permanganate oxidizeable organic carbon (PoxC) was measured as previously described (Culman et al., 2012). Absorption values of final supernatant was measured on a Biotek Synergy HT Microplate Reader in triplicate with 200 µl of $KMnO_4$ solution per well.

DNA extraction and qPCR

To determine how soil rhizobia population may affect nodule count and biomass yield, DNA was extracted from 0.5 g of soil using FASTDNA Spin Kit for Soil (MP Biomedicals). Due to high humic acid contamination, samples were further treated using the POWERCLEAN DNA Clean-up kit (MoBio). Final elution of DNA was conducted in 50 µl Tris pH 8.0. Total DNA was quantified using a Thermo Scientific NanoDrop™ ND‑2000 Spectrophotometer.

Quantitative PCR was performed in 96-well plates using a BioRad CFX Connect Real-Time system. To estimate total bacterial population we amplified a 180 bp segment of the 16s gene using primers Eub338 and EUB518. To estimate soybean nodulating rhizobia population, we amplified a 228 bp segment of the *nodZ* gene using primers nodZA-F and nodZA-R (Furseth et al., 2010). To amplify the 16s gene, a 10 µl reaction containing 5 µl SSO advantage SYBR Green Master-mix, 0.1 µl each of 20 µM primer, and 0.5 µl DNA template in a 10x dilution. Reaction parameters consisted of 2 min denaturation at 98ºC, followed by 40 cycles of 10 s at 98ºC, 30 s at 55º C and 30 s at

72ºC. Analysis was conducted in triplicate for each sample and each of six standards ranging from 3 x 10^9, to 3 x 10^5, copies per reaction. Standards were created using the respective primers in a reaction using pure culture Bradyrhizobium japonicum USDA 110 cells. The resulting fragment was visualized using gel electrophoresis and the band was cut and extracted from the agarose gel. The lengths of each fragment and a mass of 650 g mol^{-1} bp^{-1} were used to calculate total copies per reaction. For the nodZ fragment, the reaction mixture was similar to that of the 16s reaction, except that it included 0.25 µl of each primer and 2 µl of full strength soil DNA extract template. In order to improve resolution of low copy numbers of the nodZ gene, six cycles of touchdown PCR preceded normal amplification. For the touchdown step, annealing temperature started at 63ºC for the first cycle and dropped 1C each following cycle until reaching 58ºC, after which cycling proceeded as with 16s with the exception of the annealing temperature, which remained at 58ºC. Standards for the *nodZ* gene included six 10-fold dilutions ranging from 2 x 10^6 to 2 x 10^1 copies of the *nodZ* gene. Total starting gene copies were determined for both 16s and nodZ using standard curve data and normalized to gene copies per gram soil.

Statistical analysis

Data were analyzed using SAS software (SAS Institute, Cary NC). Farmer ratings were converted to binary data (1 for HIGH and 0 for low) and regressed against biomass and biomass N. Soil and growth parameters most influencing biomass production were determined using step-wise regression in proc REG. Input variables

included history of soybean production, nodule count, OM_{LOI}, PoxC, total carbon, soil pH, P, Ca, Mg, Fe, K, planting date, and copy numbers of *nodZ* and 16s genes. Significant parameters were confirmed by re-running the resulting model in proc REG. In addition to the step-wise analysis, correlation between nodZ copy number, in-field nodule count, and biomass N was determined using proc REG.

Results

A total of 18 farms were sampled in the Ekwendeni region, with eight sampled fields rated HIGH by the community and 10 rated "low". Total above ground biomass correlated with farmer ratings, with mean soybean biomass production of 2,127±294 kg ha^{-1} for HIGH fields and 324±83 kg ha^{-1} for " low" fields. Biomass and biomass N were predicted by planting date, OM_{LOI}, K, PoxC, and history of soybean production. (Table 4-3).

Farmer interviews

Interview results revealed that no farmers were practicing inoculation or adding external inputs to their soybean crops. Planting dates ranged from December 7th for early planting to January 15th for late plantings, only five fields had history of soybean cultivation, with 14 farmers never planting soybean in sampled fields (Table 1). Fields were split evenly between variety with 9 fields containing a local, unnamed variety and 9 fields containing an improved Seed-Co variety available through the government fertilizer subsidy. Among the LOW fields, farmer qualitative observations regarding the

reasons they believe the crop failed included insect and disease pests as well as drought.

Biomass and biomass N correlates with planting date

At the time of sampling, most soybean crops were at full podding to beginning seed stage (R4-R5). Total above ground soybean biomass ranged from a high of 3,560 kg ha^{-1} to a low of 50 kg ha^{-1}. Tissue nitrogen content ranged similarly from a high of 92 kg N ha^{-1} to a low of 1.6 kg N ha^{-1}. Significant predictors of both soybean biomass and biomass N included planting date, K content, OM_{LOI}, PoxC, and soybean planting history (Table 3). Parameter estimates indicate that both K and PoxC are positively correlated with Biomass and biomass N, OM_{LOI} was negatively correlated with biomass and biomass N, the negative correlation of soybean planting history indicates that fields where soybean had previously been grown had lower biomass and biomass N than fields new to soybean. Planting date indicated that later planted crops had lower biomass than earlier planted crops.

Soil organic matter and PoxC

The majority of soil OM_{LOI} values ranged from 0.9 % to 2.8% with one field, SQ8, containing 9% OM_{LOI} (Table 2). Total carbon values ranged from 0.15% to 0.73% with the exception of SQ8 which had a total carbon value of 2.04%. The majority of PoxC values ranged from 15.5 to 213 mg C Kg^{-1} soil with SQ 8 containing 608 mg C Kg^{-1} soil (Table 2). The field SQ8 was located in a "Dambo", which is a low flood plain near a river that regularly receives alluvial sediments and organic deposits. . Taking into

account planting date, planting history and K, OM_{LOI} and PoxC had a significant impact on total biomass accumulation by sampling time but total C did not (Table 3).

Soil nutrient analysis

Mehlich 3 extractable P ranged from 5 to 72 mg P kg^{-1} soil (Table 2). Extractable Ca ranged from 72 to 1880 mg kg^{-1} soil (Table 2). Extractable Mg ranged from 41 to 366 mg kg^{-1} soil, Fe from 36 to 119 mg kg^{-1} soil, and K from 182 to 2757 mg kg^{-1} soil (Table 2). Field SQ8 ranked highest in all nutrient extractions except soil P. Soil pH values ranged from 5.78 to 7.60. Mg content correlated with OM_{LOI} ($p<0.001$). However, only soil K was a significant predictor of total biomass or biomass N (Table 3).

Rhizobia population, nodulation and qPCR

Nodule counts ranged from zero nodules on plants in 7 of the fields tested to over 200 nodules per 50 cm of row in field SQ 17. Ten fields had less than 10 nodules per 50 cm of row, three fields had between 10 and 50 nodules per 50 cm of row and 5 fields had greater than 100 nodules per 50 cm of row. Of the fields that had less than 10 nodules, two were from HIGH fields and 8 were from LOW fields. Two of the fields that had less than 50 nodules were rated LOW and one was rated HIGH. All of the fields with more than 100 nodules were rated HIGH. Nodule count did not correlate with biomass or biomass N.

Soil DNA extraction yielded between 13.1 and 19.6 ng DNA/µl in a total volume of 50 µl. The efficiency of the 16s qPCR reaction was 77.2%, and the standard curved returned a slope of -4.01. Quantitative PCR on the 16s gene was used as a control to

quantify the prokaryotic community in the soil. Copy number of 16s genes present, ranged between 1.0 x 10^7 and 1.9 x 10^7 for all samples except SQ8 which had a high of 4.9 x 10^7 copies g^{-1} soil. When analyzing the complete data set, 16s copy number correlated to PoxC ($p < 0.0001$) (Figure 4-1). If the SQ8 soil data was left out as an outlier, there was not a significant correlation.

The *nodZ* amplification had an efficiency of 95.43%. Our standard curve returned a slope of -3.437 and an r^2 value of 0.998. From the 10-fold standard dilutions, we determined a lower threshold on detectability of *nodZ* using qPCR at 20 copies per reaction, correlating to approximately 1000 gene copies per gram of soil. Six soils had detectable numbers of copies of *nodZ* ranging from 5400 for field SQ9 to 92,600 for field SQ17 copies per gram soil. The signals of the remaining samples were lower than the lowest detectable standard of 20 copies per reaction and have therefore been designated as > 1000 copies g^{-1} soil. The correlation between nodZ copy number and field nodule counts was not significant (Figure 3).

Discussion

In this study we surveyed soybean fields planted by smallholder farmers that varied in their acceptability for farmers in terms of total biomass production by the end of February. We found that the most significant predictors of total biomass production were planting date, soil K levels, soil PoxC, OM_{LOI} and previous history of soybean planting.

Potassium limitations

The only soil mineral nutrient that was found to have a significant correlation with soybean biomass yields was K. It is often hypothesized that in Malawi, the most limiting nutrient for increasing growth of legume crops will be phosphorous (Mhango et al., 2013), but results of fertilizer studies indicate a mixed response, with P fertilizers resulting in increased yields in other regions of Malawi (Kamanga et al., 2010; Mhango et al., 2008). Farmers who apply fertilizer usually do so through the national fertilizer input subsidy program, which provides vouchers for a 50kg bag of 23:21:0 +4S basal fertilizer and a 50kg bag of urea, neither of which contains K (Dorward and Chirwa, 2011) . Potassium deficiency in legumes is known to result in reduced nodule number and size (Divito and Sadras, 2014). Pot studies have shown that increasing K in legumes can increased shoot N and shoot dry weight, as well as relative water content under water stress (Abd-Alla and Abdel Wahab, 1995; Abdelhamid et al., 2011). Higher soil K levels may help soybean cope with variable rain fall and reduce damage during dry spells.

Planting date and water stress

Planting date had a significant effect on total biomass and biomass nitrogen, with early planted soybean having greater biomass than late planted crops. Planting date interactions with maize performance has been investigated in Malawi, concluding that maize planted later than 4 weeks following the start of the rainy season show reduced yield (MacColl, 1990). The March-April portion of the rainy season contributes about

20 – 25% of the total annual rainfall, however inter-annual variability is considerably greater in March – April than it is from December to February (Nicholson et al., 2014). This means that crops planted in January will experience a shorter growing season, more intermittent rainfall, and more drought periods than crops planted in December, although little data is available to understand how this affects soybean yields.

Lending support to the hypothesis that poor performance of late planted soybean was related to soil moisture is the finding that field SQ8, was rated HIGH, despite being planted on the late date of January 15th. This field was located in a "dambo" – a floodplain near a river identified as poorly drained soil, with high organic matter content and alluvial deposits. The position of dambos allows them to be cultivated even in the dry season due to their proximity to the water table. The high biomass production of SQ8 by late February suggests biomass was not limited by growing days, demonstrating that even a field planted as late as January 15 can accumulate adequate biomass if it has access to consistent moisture. However, all other fields planted after December 20th were rated LOW and had a mean biomass production of 350 kg ha^{-1} whereas crops planted before December 20th had a mean biomass of 2000 kg ha^{-1}. Farmers whose late-planted fields performed poorly indicated that the soybeans had germinated well, and then began to die. Others cited "bugs eating the roots" or dry spells immediately following germination in early or mid-January. Previous surveys of farmers in the Ekwendeni region indicated that the farmers are interested learning how planting date may affect soybean yield (Mhango et al., 2013), and further investigation

of optimizing planting date would help to improve overall performance of soybean crops across the region.

Impact of OM on Biomass

Soil organic matter measurements are often used as an indicator of soil quality (Fortuna et al., 2008; Moebius-Clune et al., 2011). Our results showed that PoxC was positively correlated with total biomass, and that OM_{LOI} had a negative correlation. Permanganate oxidizeable carbon is a pool of highly labile carbon that is sensitive to management and an early indicator of C change in soil and closely related to moderately decomposed particulate organic carbon pools, and small fraction particulate organic matter (Culman et al., 2012). The positive correlation between PoxC and biomass yield in our study is possibly related to management practices such as residue incorporation and tillage. Previous studies have also seen a positive relationship between labile carbon pools and and plant biomass yields. For example, in the United States, greater PoxC values corresponded to greater rye cover crop biomass yields (Lucas and Weil, 2012), and soil organic carbon was found to have a moderately positive correlation with yields on soybean crops (Anthony et al., 2012). A study in India found that for every Mg C ha^{-1} increase in the root zone, there was 0.145 and 0.059 Mg ha^{-1} increase in grain yield of soybean and safflower, respectively (Srinivasarao et al., 2012). This suggests that encouraging soil management factors to farmers that increases labile OM such as residue incorporation and green manure use, may lead to increased crop yields overall. OM_{LOI} is a measurement subject to contamination from charcoal as well as

structural water from clay particles and may not accurately reflect the active organic fraction of carbon in the soil.

Rhizobia population size, nodulation and qPCR enumeration

We expected soil rhizobia counts and *nodZ* copy numbers in this study to be high due to the fact that soil samples were taken from actively growing soybean fields (Elkins et al., 1976; Furseth et al., 2010). While some fields with high nodule counts also had high *nodZ* copy number, no significant correlation was identified between nodule number and *nodZ* presence (Figure 3).

The traditional method for determining field population size of rhizobia is the most probable number (MPN) technique which relies on soil dilutions and probabilities of nodule formation to estimate cell count, but this method is known to produce unreliable data for soybean in tropical soils (Woomer et al., 1988). Quantitative PCR was proposed to offer a faster, higher-throughput alternative and was previously found to correlate well with MPN data for soybean nodulating rhizobia in the United States (Furseth et al., 2010). However Furseth et al. did not use standards of known starting quantities with which to compare their unknowns. Our reference standard data suggest that qPCR may be unreliable in fields where population sizes are low such as those analyzed here, which may be a barrier to wider adoption of this method for estimating cell count in low rhizobia soils. In this study, 20 *nodZ* copies per PCR reaction correlated to approximately 1000 copies gram^{-1} soil. A similar threshold for nodD genes was found for *Rhizobium leguminosarum* (Boonen et al., 2010). From MPN

experiments, 1000 cells gram^{-1} soil is more than adequate to produce nodules on a target host (Thies et al., 1991). However as qPCR is a relatively new technology for determining rhizobia population size, there is little data on how *nodZ* copy number correlates to MPN cell counts. Estimation of population size of *Rhizobium leguminosarum* using qPCR found that estimations in non-inoculated soils were high due to inclusion of non-viable cells (Boonen et al., 2010).

Considering the additional data collected from these fields, total number of soil rhizobia was likely not the only factor influencing nodule formation. In a field setting, high numbers of rhizobia may exist, but nodule formation could be hindered by high soil N (Latimore et al., 1977), for example, or disease and drought (Serraj and Sinclair, 1998). Field SQ 8, the dambo soil, had total soil N of 0.17%N compared to 0.02 – 0.04% for all other soils (Table 2), which is likely a hindrance to nodulation (Latimore et al., 1977). Farmer observations of drought in field SQ 13 and pest problems in SQ 16 and 18 potentially explain the low nodule counts for these fields.

Other possible reasons for lack of correlation between *nodZ* copy number and nodule counts in the field may relate to the variety of soybean planted in this study. The gene nodZ is specific to soybean-nodulating rhizobia. The primer pair used in this study was developed to amplify only soybean specific rhizobia, and no other rhizobia types. Many surveyed farmers planted local varieties of soybean, the seed of which has been multiplied over many years based on varieties such as the promiscuous type soybean "Magoye" as part of the SFHC seed bank program (SFHC staff, *personal*

communication, 2012). Previous research has shown that promiscuous soybean forms nodules with a wide variety of rhizobia, many of which that cannot form nodules with specific soybean varieties (Pule-Meulenberg et al., 2011). If this is the case, amplification of the *nodZ* gene alone may not predict total populations capable of forming nodules with promiscuous soybean.

Implications for improving smallholder soybean production

Improving soybean and legume production on smallholder farms in Malawi is critical in order to enhance overall food security. Limited fertilizer use over decades has depleted soil N, and led to maize yields averaging less than 1 Mg ha^{-1} in 2005, compared to a global average of 5 Mg ha^{-1}, and improvements through a national fertilizer program only increased to approximately 2 Mg ha^{-1}. At the same time, children commonly experience stunting due to protein and micronutrient deficiencies (National Statistical Office and UNICEF, 2008). In the Ekwendeni region, SFHC was formed to address the problem of child malnutrition associated with poor diets and low soil fertility and the promotion program has been successful in increasing farmer interest in legume cultivation, particularly soybeans, and improving food security in the region (Bezner Kerr et al., 2011). Since the formation of SFHC, soybean adoption has increased rapidly, currently, over 90% of farmers associated with SFHC grow soybean (Bezner Kerr et al., 2007), with farmers citing nutritional value for children as their main interest in the crop (Mhango, 2011). The results of this study indicate that

strategies such as timely planting, increasing soil labile organic matter, and increasing soil K content may lead to further improvements of soybean yields.

Conclusions

In this study surveying production drivers of farmer-planted and managed soybean yield we found that planting date was the greatest predictor of crop success. Nearly half of the fields were planted after January 1, indicating that yield improvement might be realized simply by encouraging farmers to plant soybean crops earlier. In addition, we found that, PoxC and soil K content had a positive impact on soybean biomass production, and OM_{LOI} and previous soybean planting had a negative correlation with soybean crop success. Further research is needed on how local soil rhizobia populations impact N fixation by soybean crop and whether inoculation with rhizobia will improve biological N fixation. Additional research on disease and pest incidence in soybean crops in Malawi and how planting date and soil K content affects the drought susceptibility will also be important. Guidelines that help farmers grow more successful soybean crops will ultimately lead to improved soil fertility and livelihoods in Malawi.

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Tables

Table 4-1: Planting and Cultivation information for surveyed fields. HIGH and LOW ratings are based on farmer observations.

	Total Fields Identified	
	HIGH	LOW
Total Fields	8	10
History of Soybean		
Yes	2	3
No	6	7
Variety Planted		
Local	3	6
Seed-Co	5	4
Used Inoculant	0	0
Planting Date		
Early (Before 20 December)	7	1
Mid (Before 10 January)	0	8
Late (After 10 January)	1	1

Table 4-2: Soil Chemical Properties of Soybean fields surveyed in the Ekwendeni region of Malawi

	OM_{LOI} (%)	Total Carbon (%)	PoxC (mg kg^{-1})	pH	Total N (%)	P (mg kg^{-1})	Ca (mg kg^{-1})	Mg (mg kg^{-1})	Fe (mg kg^{-1})	K (mg kg^{-1})
SQ1	2.29	0.33	143.4	7.60	0.03	50.8	621	76	59	614
SQ2	2.26	0.18	135.6	7.00	0.02	10.8	355	70	42	802
SQ3	1.96	0.21	124.0	6.06	0.02	11.6	100	48	32	466
SQ4	2.24	0.35	160.8	7.44	0.02	72.1	709	82	57	497
SQ5	0.94	0.26	15.5	6.19	0.02	30.7	141	23	37	215
SQ6	1.64	0.17	116.2	6.78	<0.02	10.3	342	74	46	523
SQ7	2.20	0.40	114.3	6.99	0.02	56.5	569	115	50	1018
SQ8	9.03	2.04	608.2	6.60	0.17	34.3	1880	366	119	2757
SQ9	2.12	0.18	213.1	6.34	0.02	21.7	455	64	63	368
SQ10	1.41	0.22	104.6	6.03	0.02	11.1	103	22	41	182
SQ11	2.05	0.51	98.8	7.27	0.04	54.1	393	75	50	476
SQ12	2.18	0.22	118.2	6.28	0.02	33.2	177	81	55	361
SQ13	1.81	0.16	46.5	6.07	<0.02	12.1	90	43	35	425
SQ14	1.28	0.15	27.2	6.68	<0.02	27.9	369	50	54	324
SQ15	2.36	0.73	93.0	6.15	0.03	5.6	185	92	36	629
SQ16	1.82	0.56	62.0	5.96	0.03	11.5	124	54	35	398
SQ17	2.82	0.23	162.7	6.36	0.02	16.4	273	86	41	715
SQ18	1.78	0.21	44.6	5.78	0.02	12.9	72	41	36	370

Table 4-3: Model Parameters as determined by stepwise regression for the total soybean biomass and biomass nitrogen

	Total Biomass		Biomass Nitrogen	
Parameter	**Estimate**	**P-value**	**Estimate**	**P-value**
Intercept	806676	0.0004	22048	0.0005
Previous Soybean	-873.6	0.0050	-19.2	0.0180
OM_{LOI}	-1960	0.0007	-44.6	0.0030
PoxC	17.1	0.0002	0.47	0.0003
K	2.7	0.0057	0.05	0.0478
Planting Date	-42.4	0.0005	-1.16	0.0005

Figures

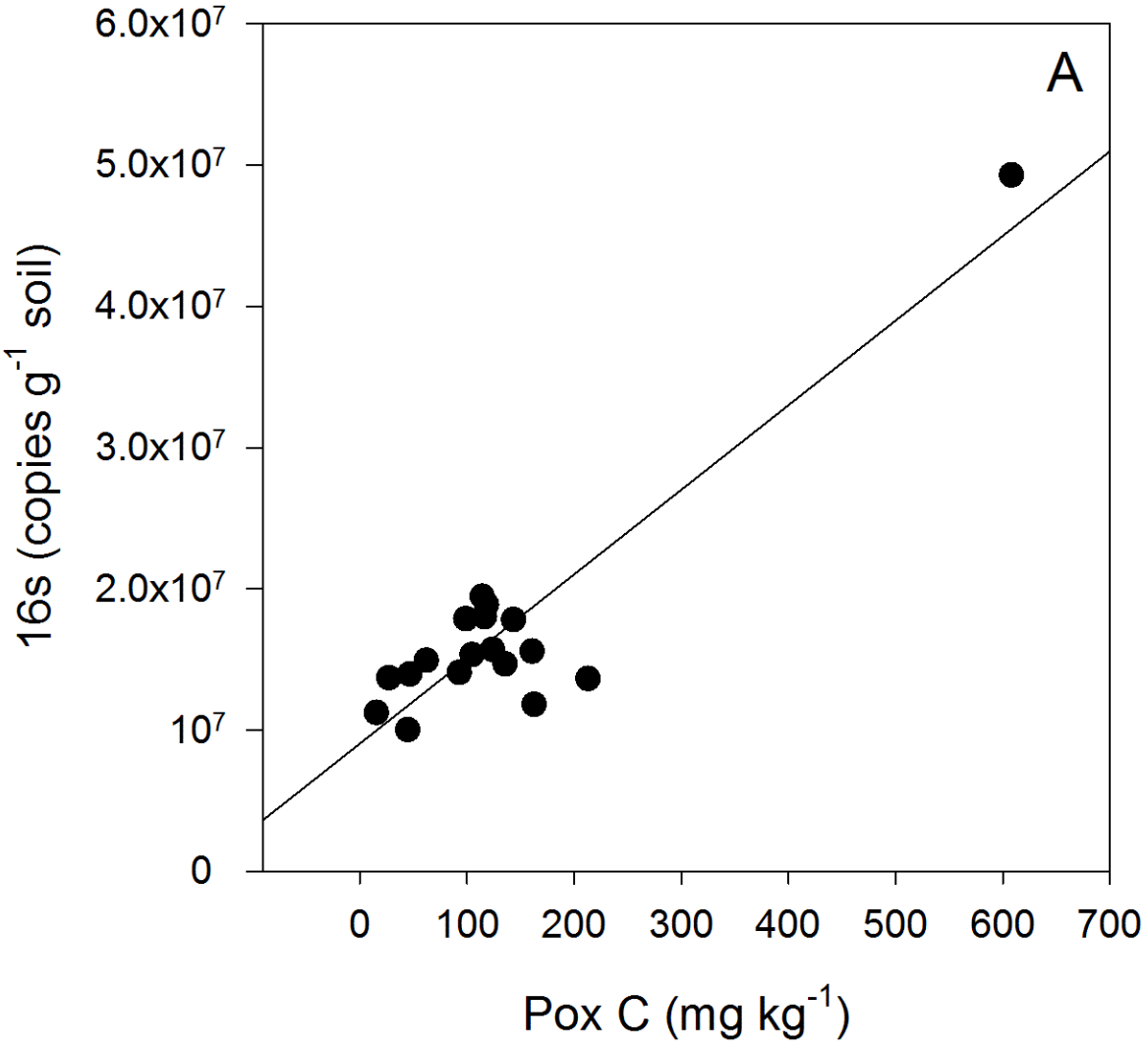

Figure 4-1: PoxC correlates to 16s copy number when accounting for all 18 fields (A) p<0.0001 , when sq8 is excluded from the analysis, this correlation is no longer significant (B) p=0.3724.

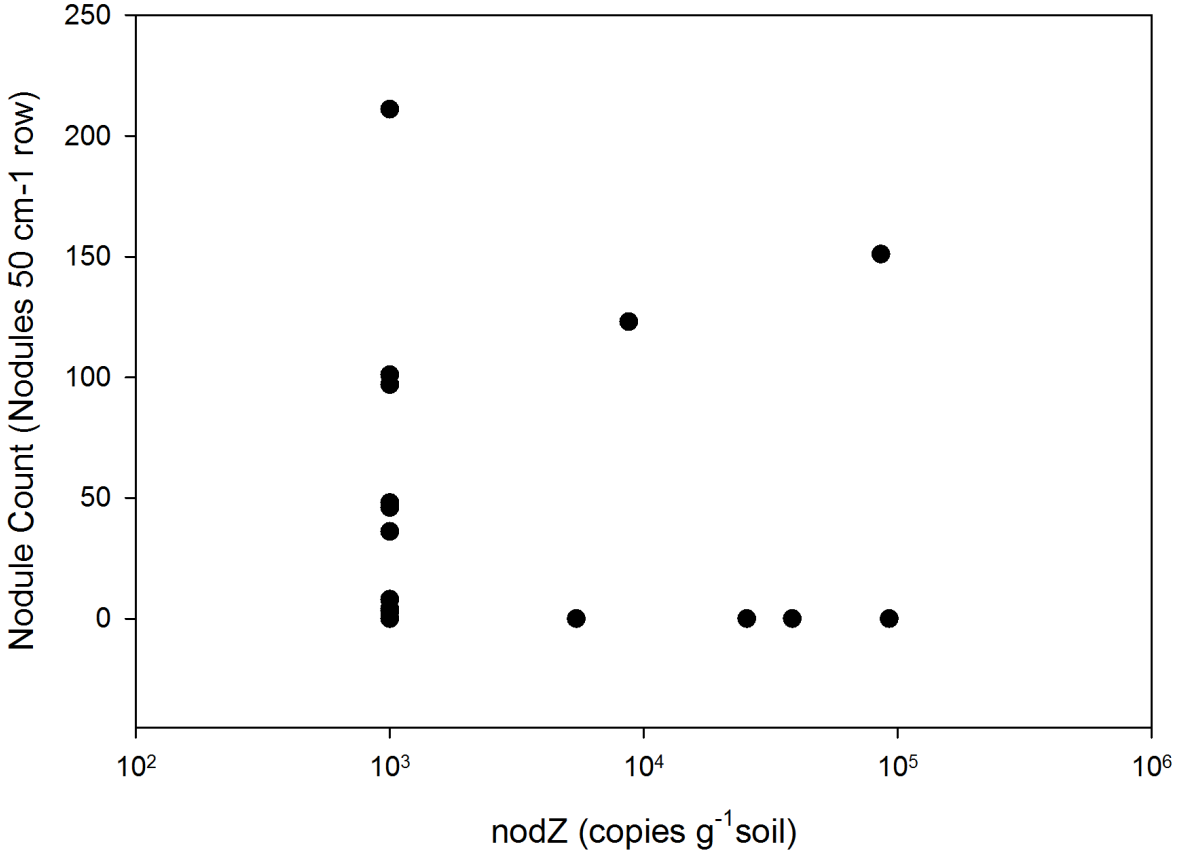

Figure 4-2: Nodule count per field measured on 50 cm of row as related to nodZ copy number in soil, measured using quantitative Polymerase Chain Reaction (qPCR). No correlation was found to exist between the soybean-nodulating-rhizobia specific gene *nodZ* and in-field nodule counts on soybean roots.