Improving the legume-rhizobium symbiosis in Zimbabwean Agriculture: A study of rhizobia diversity & symbiotic potential focussed on soybean root nodule bacteria

by

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DECLARATION

I declare that this thesis is an original report of my research, while a doctoral student at Murdoch University, Western Australia. Due references have been provided on all supporting literature and sources. The work has not been submitted for the award of any other degree.

Mazvita Sheila Chiduwa

Abstract

Legumes are important components for both smallholder and commercial agriculture in Zimbabwe in relation to food and income security and improvement of soil fertility through a symbiotic association with rhizobia. The efficiency of biological nitrogen fixation is largely unknown in most situations in Zimbabwe. While rhizobia inoculant is available for many legumes, only soybean is consistently inoculated. Native soybean rhizobia have not been genetically characterized or taxonomically identified.

The inoculation response of cowpea, groundnut, lablab, sunn hemp, pigeon pea and soybean was investigated under Zimbabwean field conditions, together with effects on a subsequent maize crop. Separately, soybean microsymbionts were obtained from soils with known inoculation histories from nine smallholder and three commercial farms to isolate naturalized inoculant strains and native rhizobia. Isolates were genetically characterized using partial *recA* gene sequences. Phylogenetic analysis of representative isolates was undertaken using *recA*, *glnII*, and 16S rDNA sequences. Symbiotic genes *nifH* and *nodC* were analysed. Isolates were screened for nitrogen fixation efficiency and the two best fixers per species were tested for compatibility with three soybean varieties under glasshouse conditions. The best isolate of each species was tested across different field sites in Zimbabwe.

Inoculation generally increased grain yield, shoot biomass and nitrogen accumulation. Maize biomass was higher when succeeding inoculated legumes than when succeeding uninoculated legumes. Partial *recA* gene sequencing grouped the isolates into four species: *Bradyrhizobium diazoefficiens* (13%), *B. japonicum* (21%), *B. elkanii* (61%) and *B. ottawaense* (5%). *B. ottawaense* had the widest host range across 13 legumes, followed by *B. elkanii, B. diazoefficiens* and *B. japonicum*.

Phylogenetic analyses were consistent with vertical transmission of core genes and horizontal transfer of symbiotic genes. Based on symbiotic performance and edaphic competence, strains *B. japonicum* NAZ554 and NAZ710 and *B. diazoefficiens* NAZ629 were identified as potential elite inoculant strains for soybean in Zimbabwe.

Dedication

This thesis is dedicated to the smallholder farmers of Africa. May the treasure of symbiotic nitrogen fixation and good agronomic practices broadly yield for them a sustainable farming experience, towards eradicating food and nutrition insecurity and poverty.

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Table of contents

List of Tables

List of Figures

CHAPTER 1

Introduction and Literature Review

1.1 Biological Nitrogen Fixation

Nitrogen (N) is an essential crop macronutrient, a component of proteins, chlorophyll and nucleic acids. While N_2 is abundant in the atmosphere, making up around 78% of the volume of air, the N atoms in the dinitrogen (N_2) molecule are tightly bound, such that N is ironically often the most limiting element in crop production (Graham and Vance, 2000, Smil, 1999, Valentine et al., 2018). However, this atmospheric N_2 may be converted to biologically available forms via the Haber-Bosch process, lightning and symbiotic nitrogen fixation (SNF).

The Haber Bosch process is an energy-intensive industrial process for combining atmospheric N with hydrogen gas to form ammonia, which is then used for the production of inorganic nitrogen fertilizer. Therefore, while energy prices are high, inorganic nitrogen fertilizer is expensive. In addition, use of inorganic nitrogen fertilizer is associated with environmental damage due to acidification of soils, contamination of drinking water, greenhouse gas emissions and eutrophication from leaching and surface runoff (Golding and Dong, 2010, Vance, 2001). As such, the use of alternative options for nitrogen nutrition for crop production is a global goal (Mus et al., 2016).

This can occur via SNF, where atmospheric nitrogen is converted to ammonia by the enzyme nitrogenase (Unkovich et al., 2008). Nitrogenase is only found in microorganisms – bacteria and archaea. The SNF process, like the Haber-Bosch process, is also energetically expensive. It requires 16 ATP molecules for every N_2 gas molecule to generate two molecules of ammonia, as summarized in the following equation:

$$N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16P_i.$$

Globally, SNF has been estimated to generate between 88 Tg N $year^{-1}$ (Davies-Barnard and Friedlingstein, 2020) and 175 Tg N $year^{-1}$ (Herridge et al., 2008). It occurs in free-living, associative or symbiotic mode. In free-living nitrogen fixation, as seen for example in cyanobacteria (Zehr, 2011), the micro-organism fixes nitrogen for its own use and there is no

controlled exchange of carbon (C) or N between plant and microorganisms. In associative nitrogen fixation, the microorganisms are in close association with plants. They proliferate in the root zone due to nutrients released by the plant and the exchange of nutrients is mutual. Examples include grass-*Azospirillum* associations (Steenhoudt and Vanderleyden, 2000). In symbiotic association, the microorganism becomes established inside the plant, provided with nutrients and energy by the plant. A novel organ, the nodule, develops in which nitrogen fixation occurs, for use by the plant. Examples include *Frankia*-actinorhizal symbioses and legume-rhizobia symbioses (Poole et al., 2007). Commonly observed ranges of free-living, associative and symbiotic nitrogen fixation are less than 5 to 30; 5 to 65; and 10 – 250 kg N/ha per year, respectively. Hence, symbiotic nitrogen fixation (SNF) contributes the most nitrogen to the biologically fixed nitrogen economy (Unkovich et al., 2008).

1.2.1 Legume-Rhizobia Symbiotic Nitrogen Fixation

The legume-rhizobia symbiosis is the most commonly used biological fertilizer system in agricultural lands and is also important for natural ecosystems; it accounts for 20% of the nitrogen fixed annually worldwide (Dresler-Nurmi et al., 2009). It is found in many agriculturally important grain and fodder crops such as soybean (*Glycine max*), lucerne/ alfalfa (*Medicago sativa*), clovers (*Trifolium* spp.) and vetches (*Vicia* spp.). Nitrogen derived from SNF in agricultural systems alone has been estimated at 33-46 Tg N $year^{-1}$ (Herridge et al., 2008).

The legume-rhizobia symbiosis is an intimate relationship, where the microsymbiont becomes established within the macrosymbiont cells in new structures called nodules (Long, 1996). The core basis of the symbiosis is that the microsymbiont provides fixed nitrogen to the plant, which in return supplies fixed carbon needs to the microsymbiont (Mus et al., 2016). The relationship is generally specific, guided by a molecular dialogue between the legume and the rhizobia (Perret et al., 2000).

Deliberate efforts have been made to exploit legume symbiotic nitrogen fixation in agricultural systems for sustainable economic gain (Hungria et al., 2006). The following sections present a review of relevant information on legumes and rhizobia towards understanding the potential benefits of nodulated legumes in agriculture, the need to inoculate and rhizobia inoculant strain selection.

1.3 Legumes

Legumes are thought to have originated between 75 and 100 million year ago (Doyle, 2019), on either side of the Tethys seaway and have since diversified and spread into various ecosystems, ranging from rainforest to hyper-arid; alpine and arctic regions to equatorial tropics (Sprent, 2007, Doyle, 2019). Their morphology is highly diverse, ranging from the small, cosmopolitan herbaceous clovers to woody climbers such as *Wisteria* as well as rainforest trees (LPWG, 2017, Doyle, 2019).

Legumes are flowering plants that belong to the monophyletic Fabaceae or Leguminosae family within the Rosid I Clade (Sprent, 2007). With about 18000 species, the Leguminosae is the third-largest plant family after the Asteraceae (with 24000 species) and the Orchidaceae (with about 20000 species) (Doyle, 2019). Legumes are typically distinguished by their familiar fruit set in a pod, characteristic floral structures and, for a majority of them, capacity for SNF (Graham and Vance, 2003, Doyle, 2019).

Based on the conspicuous floral structures, the Leguminosae were traditionally organized into three subfamilies, viz Papilionoideae, Mimosoideae and Caesalpinioideae, although Caesalpinioideae was known to be paraphyletic (Sprent, 2007). Recent re-classification based on the plastid *matK* gene from nearly all genera of the family and about 20% of all known species in the family defined the Leguminosae as comprising 503 genera distributed in 6 subfamilies viz Caesalpinioideae (148 genera), Cercidoideae (12 genera), Detariodeae (84

genera), Dialioideae (17 genera), Duparquetioideae (1 genus), and Faboideae (Papilionoideae) (Figure 1) (LPWG, 2017).

Highlights of the new classification are that the Caesalpinioideae encompasses the group formerly known as Mimosoideae. Nodulation has not been reported in the four subfamilies Cercidoideae, Detariodeae, Dialioideae and Duparquetioideae. Nodules are variably present and indeterminate in the Caesalpinioideae; and usually present in the Faboideae, suggesting that nodulation evolved separately on multiple occasions in the two subfamilies (LPWG, 2017, Sprent et al., 2017). However, the latest research suggests that nodulation evolved once and was subsequently lost in some taxa due to selection pressures, as supported by results of phylogenetic analysis (Griesmann et al., 2018, Van Velzen et al., 2019).

Within the subfamilies, species are organized into genera, then into tribes. In the largest subfamily, the Faboideae (Papilionoideae), the largest clade is marked by a 50 kb inversion of the chloroplast genome and includes the *Papilionoideae* (*Millettioid/Phaseoloid*) clade to which soybean, cowpeas (*Vigna unguiculata*), lablab (*Lablab purpureus*) and pigeon pea (*Cajanus cajan*) belong; the tribe *Dalbergieae*, to which groundnut (*Arachis hypogaea*) belongs; and the tribe Crotalarieae, to which sunn hemp (*Crotalaria juncea*) belongs (Figure 1.1).

The Leguminosae is the second most important family for food to humans, after the Gramineae/Poaceae (Graham and Vance, 2003, Baumann et al., 2000). Legumes are high in protein, critical for human growth and development. Grain legumes contribute 33% of dietary protein nitrogen needs of humans and this figure can double under subsistence farm conditions (Graham and Vance, 2003). They are also important for sustainable agriculture where they are incorporated into rotation sequences to break pest and disease cycles in

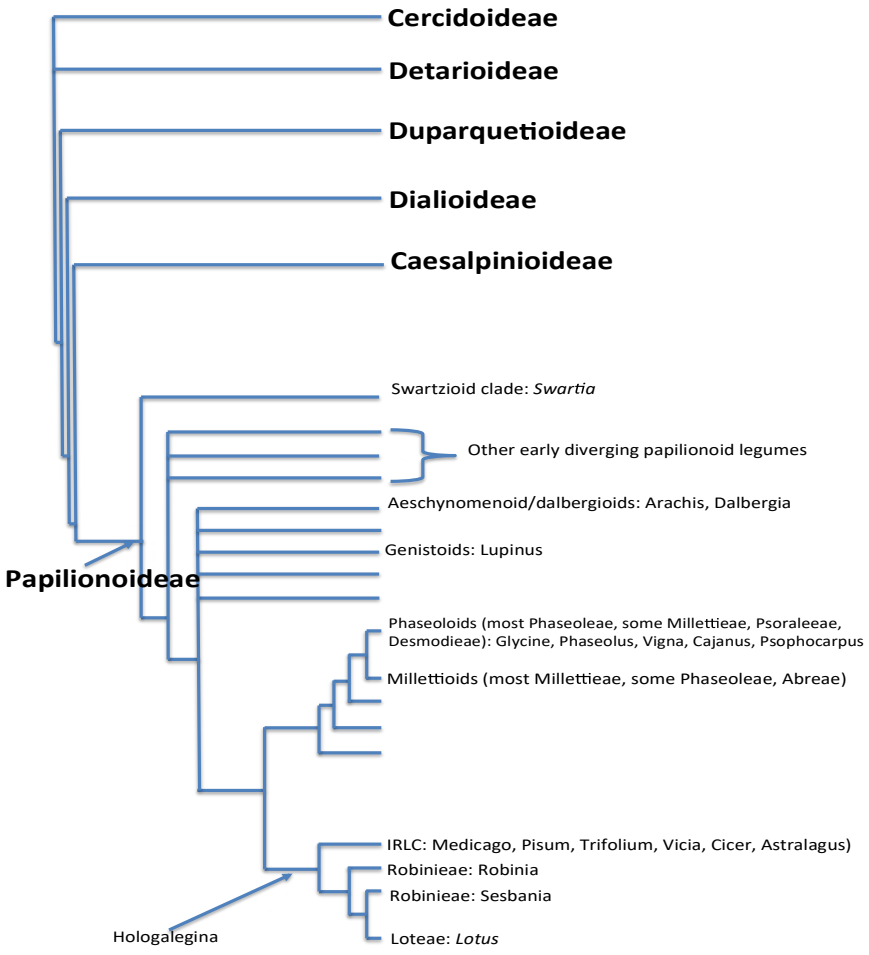


Figure 1.1 Phylogeny of the Leguminosae, showing all six subfamilies (in bold) and major lineages of the Papilionoideae. Figure adapted from LPWG, (2017) and Doyle and Luckow, (2003)

cereal-based cropping systems (Giller et al., 2011). Many subsistence-farming models depend on legume-derived N fixed in association with rhizobia (Mapfumo et al., 2005, Nezomba et al., 2008). World production trends and the share of developing countries in global legume production is on an upward trend, with global production of soybean of about 350 Mt annually, and an overall average yield of 2.8 t/ha achieved (FAOSTAT, 2020). Tropical legumes

important for dietary protein, edible oils and livestock feed include soybean, groundnuts, cowpeas and common beans (*Phaseolus vulgaris*) (Ojiewo et al., 2019), while temperate/Mediterranean food legumes include *Cicer*, *Vicia*, *Pisum* and *Lens* spp. (Maxted et al., 2012). Tropical pasture legumes include lablab and pigeon pea (Giller et al., 2019, Murungweni et al., 2004, Mapfumo et al., 2000), while temperate pasture legumes include clovers (*Trifolium* spp.), lucerne (*Medicago sativa*), *Lotus* spp. and *Ornithopus* spp. (Frame et al., 1998). Sunn hemp has been widely used as a green manure for soil improvement due to its superior capacity to fix N (Nezomba et al., 2008). Tropical legumes are generally believed to be more promiscuous in their rhizobial associations than their temperate counterparts, and able to rely on nodulation with abundant indigenous rhizobia to satisfy their N requirements (Singleton et al., 1992).

Legumes have been recommended for soil improvement since 37 BC, although the mechanisms of soil improvement were not then understood (Fred et al., 1932, Graham and Vance, 2003). For more than 100 years, it has been known that many legumes perform nitrogen fixation in association with rhizobia (Beijerinck, 1888).

This study is focused on the symbiotic associations between rhizobia and the tropical agricultural legumes soybean, cowpea, groundnut, lablab, pigeon pea and sunn hemp, because of their perceived potential for improving the fortunes of smallholder farmers by improving soil N, leading to food and income security.

1.4 Rhizobia

Rhizobia are Gram-negative, motile, non-sporulating and phylogenetically diverse proteobacteria that infect legumes (and the nonlegume *Parasponia*), leading to the formation of root or stem nodules in which they differentiate into bacteroids and fix N (Op Den Camp et al., 2012, Masson-Boivin et al., 2009). While rhizobia were first discovered as soil bacteria, they are now known also to be aquatic, epiphytic and endophytic (Yates et al., 2011).

1.4.1. Rhizobia classification and taxonomy

After their initial discovery, rhizobia were all classified as *Rhizobium leguminosarum* (Schneider, 1892, Frank, 1889), to mean the bacteria that live in association with legumes. Differences between rhizobia became apparent with selective nodulation of legumes, leading to the cross-inoculation theory and methods of classifying rhizobia. There was an early subdivision of the genus into six species on the basis of their host association, establishing *R. trifolii*, *R. japonicum*, *R. phaseoli*, *R. meliloti* and *R. lupini* in addition to the initial *R. leguminosarum* (Fred, Baldwin & McCoy, 1932).

The 1960s saw several new methods of analysis developed, and the development of technologies to classify different rhizobial taxa. Methods included antibody based techniques such as serology; physiological based methods such as intrinsic antibiotic resistance (IAR); patterns of utilization of carbon and nitrogen sources; and molecular composition such as G+C content (Johnson and Means, 1963, Dudman and Brockwell, 1968, Means and Johnson, 1968, Schmidt et al., 1968). In this era, rhizobia were also described based on the growth rate, as slow or fast growers (Allen & Allen, 1950). Fifty years after the initial naming of six species within the *Rhizobium* genus, a new genus, *Bradyrhizobium*, was named in 1982. It contained only one species *B. japonicum*, based on cultural and physiological analysis, most notably the slow growth rate (Jordan, 1982).

In the 1980s, bacterial taxonomy was revolutionized by the arrival of molecular techniques, and gene sequencing in particular. With this came phylogenies that reflected the genomic relationships of bacteria (Woese, 1987). Comparisons of DNA relatedness, which included DNA-DNA hybridization and rRNA gene sequencing, defined relatedness and diversity within the rhizobia with higher resolution, and more precision than phenotypic measures (Woese, 1987). The gold standard for defining bacterial species was set by DNA-DNA hybridization with a species boundary threshold at 70% (Barco et al., 2020). By 1991,

there were two more rhizobial genera described, *Azorhizobium* and *Sinorhizobium* (later reclassified as *Ensifer*) and it was recognized that there was a further need to clarify the taxonomic position of several unnamed groups of strains within the Rhizobiaceae (Graham et al., 1991, Willems, 2006, Chen et al., 1988, Dreyfus et al., 1988).

At the turn of the century, a discovery was made that extended rhizobial taxonomy beyond the *Alphaproteobacteria*, to *Betaproteobacteria* within the *Burkholderiaceae* (Moulin et al., 2001). *Burkholderiaceae* is now known to host rhizobia within the genera *Paraburkholderia*, *Cupriavidus* and *Trinickia* (De Lajudie et al., 2019). In addition, rhizobia were described from Alphaproteobacterial genera that were previously not defined as rhizobia, including genera within the families *Methylobacteriaceae (Methylobacterium* and *Microvirga)* (Sy et al., 2001, Ardley et al., 2012), *Brucellaceae* (*Ochrobactrum*) (Trujillo et al., 2005), *Hyphomicrobiaceae* (*Devosia*) (Rivas et al., 2002), *Blastobacter* (Van Berkum and Eardly, 2002), *Phyllobacteriaceae* and *Xanthobacteraceae* (De Lajudie et al., 2019). Clearly, the rhizobia are a paraphyletic assemblage of bacterial lineages.

1.4.1.1. Gene-based classification of rhizobia

Genomic characterization is the currently preferred primary method of bacterial classification (De Lajudie et al., 2019). Bacterial genomes consist of core genes, which are shared with related taxa, and accessory genes, which are sporadic in their distribution in related taxa (Young et al., 2006). Within the core genes are housekeeping genes, which are required for the essential functions of the cell. Examples of housekeeping genes include *glnII, recA, atpD* and *dnaK*. They are protein-coding genes that support core functions of the strains, and are found on the chromosome. They are unlike accessory genes that a cell can otherwise function without, but whose presence confers specific advantages, such as nitrogen fixation. Core genes are conserved, like the 16S rRNA gene, and their sequence differences are useful for phylogenetic investigation (Gevers et al., 2005, Wernegreen and Riley, 1999).

The 16S rRNA gene is found in all bacteria and is characterized by a very slow rate of change. Therefore it is ideal for identification of rhizobia and phylogenetic analysis (Young and Haukka, 1996). Large databases, including the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) and the Joint Genome Institute (https://img.jgi.doe.gov), maintain gene sequences for comparison with new sequences. In phylogenetics, scientists seek to reconstruct the evolution of strains using genetic loci acquired from ancestors of the strain. In the bradyrhizobia, however, the sequence of the 16S rRNA gene is highly conserved, making the delineation of different species difficult (Vinuesa et al., 2008a).

A combination of the 16S rRNA gene sequences, along with that of other housekeeping genes, is the standard of molecular systematics and phylogenetics of rhizobia (Vinuesa, 2005). Integrating several phylogenetic parameters is termed **m**ulti**l**ocus **s**equence **a**nalysis (MLSA). It improves phylogenetic resolution, giving a robust classification/identity of rhizobia (Gevers et al., 2005, Menna, 2009, Vinuesa, 2010). For example, in a study of 169 bradyrhizobial isolates, phylogenetic analysis of 16S rRNA gene sequences could only resolve two broad groups for strains that were subsequently aligned to nine different species by multi-locus sequence analysis (MLSA) of the *glnII, recA, atpD* and *dnaK* housekeeping genes (Menna, 2009). Table 1 shows examples of gene combinations used for phylogenetic analysis.

Table 1.1 Examples of housekeeping genes used for phylogenetic analysis

Housekeeping genes combination	**Reference**
atpD, dnaK, glnII, recA,	(Menna, 2009, Stępkowski et al., 2005)
atpD, glnII, recA, rpoB	(Vinuesa et al., 2008b)
glnII, recA, rpoB	(Aserse et al., 2012a)
dnaK, glnII, gyrB, recA, rpoB	(Zilli et al., 2014)

A consequence of the higher resolution of analysis using genetic tools, combined with an increase in studies describing rhizobia isolated from diverse agricultural and non-agricultural legumes has been that the number of validly described species within the rhizobia has rapidly expanded in the last two decades. There are now more than 200 species of rhizobia recognized in 18 genera within the *Alpha-* and *Betaproteobacteria* (De Lajudie et al., 2019). In comparison to the one recognized *Bradyrhizobium* species within the genus in 1982, this genus now houses 53 distinct species (as of June 2020) and is estimated to house many more based on genomes found in public libraries (Ormeno-Orrillo and Martinez-Romero, 2019).

With increasing advances in research and technology, it is now becoming possible to compare full genome sequences of rhizobial strains (Aserse et al., 2017, Bromfield et al., 2020, Bromfield et al., 2019). Many rhizobia genomes, including several bradyrhizobia, have been fully sequenced, providing a future platform for genetic comparisons to explain physiological differences (Lee et al., 2008, Kaneko et al., 2000, Kaneko et al., 2011, Bromfield et al., 2020, Bromfield et al., 2019).

1.4.2 Infection, nodule initiation and symbiotic genes in rhizobia

Symbiotic genes are accessory genes that confer the ability for nodulation and nitrogen fixation. In general, these processes are controlled by a molecular dialogue between the rhizobia and the legume, leading to the formation of nodules (Long, 2001). Compatibility of

symbiotic partners is determined by their ability to recognize molecules elicited from their partners (Perret et al., 2000). The infection process begins with host plant secreted flavonoids (Kosslak et al., 1987) being perceived by the rhizobium transcriptional regulator *nodD* (Begum et al., 2001). *NodD* then induces the common *nodABC* genes to produce lipochito-oligosaccharide (LCO) nod factors (Debellé et al., 2001, Perret et al., 2000). The structure of the nod factors has been determined to consist of a backbone of four or five glucosamine residues that are N-acylated at the non-reducing end (Debellé et al., 2001). The LCO backbone is decorated by various moieties encoded by other *nod*, *nol* and *noe* genes (Perret et al., 1999, Pueppke and Broughton, 1999, Stacey et al., 1994). When the nod factors are recognized by the legume's LysM receptor kinases, curling and deformation of the root hair begins, creating a shepherd's crook shape enveloping the rhizobia. An infection thread forms, to deliver the rhizobia, which continues to divide, into the plant and meristematic nodule primordium (Baev et al., 1992).

Legumes are infected by rhizobia in three alternate infection processes, namely root hair infection, crack entry and epidermal infection (Boogerd and Van Rossum, 1997). Root hair infection is an elaborate, relatively common and well-studied mode of infection, described above. It occurs with most temperate legumes, including *Vicia*, *Trifolium*, *Pisum* and *Medicago* species, producing indeterminate nodules; and with subtropical legumes including soybean, cowpea, lablab and pigeon pea, producing determinate nodules (Boogerd and Van Rossum, 1997).

The crack entry mode of infection occurs in the Dalbergioid clade legumes, including groundnut, and *Aeschynomene*, among others (Boogerd and Van Rossum, 1997, Bonaldi et al., 2011). In this case, rhizobia enter the host through cracks in the epidermis, such as those caused by adventitious roots (Caetano-Anollés and Bauer, 1988, Giraud et al., 2007)

The third mode of infection is the epidermal mode of infection. In this mode, the rhizobia

enter the host at the boundary of adjacent epidermal cells which has been reported in *Mimosa scabrella* and lupin (De Faria et al., 1988, Van Rhijn and Vanderleyden, 1995, González-Sama et al., 2004). Here, rhizobia penetrate the walls and proliferate intercellularly. When successful, the rhizobia are released into the cells of the nodule primordium, resulting in nodulation (Van Rhijn and Vanderleyden, 1995).

Within the nodule, the bacteria differentiate into bacteroids and begin to fix atmospheric nitrogen into ammonia (Caetano-Anollés and Bauer, 1988). Differentiation of rhizobia into bacteroids involves a new, plant-derived membrane called a symbiosome enveloping the bacteroid (Vasse et al., 1990).

Nodules may be determinate or indeterminate. Determinate nodules arise from hemispherical meristems. They begin formation from the outer cortex and are spherical. Indeterminate nodules grow from the inner cortical cells, leading to continuous apical meristematic growth that results in cylindrical nodules (Boogerd and Van Rossum, 1997). Corby (1989) described five types of legume nodules, and these appear to be related to the classification of the legumes. However, the nodule structure has been shown to vary within the same legume taxonomic group (Doyle, 1994, Sprent, 2009).

1.4.2.3 Horizontal/Lateral gene transfer of symbiotic genes

Within the rhizobial genome, symbiotic genes may be wholly located on the chromosome or symbiotic islands or Integrative and Conjugative Elements (ICE's) or separately on plasmids or chromids (Haskett et al., 2016, Fricke et al., 2009, Perret et al., 1999, Sadowsky and Bohlool, 1983). In the bradyrhizobia, symbiotic genes are found on symbiotic islands on the chromosome (Kaneko et al., 2002, Kaneko et al., 2011). Symbiotic genes have been demonstrated to be transferrable between strains of the same species, but also between more distantly related organisms (Sullivan et al., 1995, Bailly et al., 2007), by a process termed horizontal or lateral gene transfer (HGT). Symbiotic genes are often subject to HGT, which

has been shown to be prevalent in rhizobial evolution (Sullivan et al., 1995, Ramsay et al., 2006, Haskett et al., 2017).

Horizontal gene transfer of symbiosis genes within the bradyrhizobia has been reported under field conditions (Barcellos et al., 2007, Batista et al., 2007). The symbiosis genes of bradyrhizobia are carried on symbiosis islands (Kaneko et al., 2011, Nguyen et al., 2018, Kaneko et al., 2002). In *Mesorhizobium*, horizontal transfer of symbiosis genes contained within an ICE under laboratory conditions is well researched (Haskett et al., 2016, Ramsay et al., 2009, Sullivan and Ronson, 1998, Sullivan et al., 1995). Horizontal gene transfer of symbiotic genes in bradyrhizobia has been reported under laboratory conditions, but recipient strains subsequently lost the genes (Minamisawa et al., 2002).

Horizontal gene transfer allows the evolution of strains that are able to nodulate with a new legume host. A free-living non-fixing bacterium becomes a symbiont with the acquisition of a symbiosis island (Sullivan and Ronson, 1998). The resultant capacity for nitrogen fixation is highly variable (Nandasena et al., 2006). For the loss or gain of horizontally transferred genes to be sustained, the new genetic status must confer some advantage that promotes survival or prevents demise against a selection pressure (Ochman and Moran, 2001). In this case, the ability to nodulate a legume host can provide a selective advantage where that host is present. Transfer of symbiosis genes from one rhizobial host to another can extend the legume symbionts that the recipient rhizobia strain can infect (Remigi et al., 2016).

Due to their mobile nature, symbiotic genes should not be used in phylogenetic analysis. Otherwise, they may show phylogenies that are discordant with that demonstrated by the core genes. Instead, they can give an indication of the host association. For example, the symbiotic genes of *R. leguminosarum* biovar *viciae* nodulating pea will differ from *R. leguminosarum* biovar *trifolii* nodulating clover (Chirak et al., 2016).

1.4.3 Bradyrhizobia phylogeny and biogeography

Bradyrhizobium is the largest and most diverse genus of the rhizobia. Bradyrhizobia are a common symbiont of tropical legumes and are of particular interest in this study due to their association with the target legumes. It is estimated that there may be more than 800 species of bradyrhizobia in nature (Ormeno-Orrillo and Martinez-Romero, 2019). Strains from the *Bradyrhizobium* genus collectively nodulate the widest range of legumes of all the rhizobia as well as the nonlegume *Parasponia* (Sprent et al., 2017, Avontuur et al., 2019, Ormeno-Orrillo and Martinez-Romero, 2019). The genomes of bradyrhizobial strains are large, up to 9 million base pairs (9MB), consistent with the evident genetic capacity for the diversity of metabolic and physiological functions that allow their wide occurrence in geography and ecology (Ormeno-Orrillo and Martinez-Romero, 2019, Kaneko et al., 2011, Avontuur et al., 2019). Rhizobia occurrence is responsive to geography, climate and ecology, as these factors define the boundaries within which the individual bacterial species survive (Vinuesa et al., 2008a, Parker, 2015). This diversity points to possibilities of bradyrhizobia being the ancestral symbionts of legumes (Parker, 2015). Individual bradyrhizobia species also nodulate widely, as is the case with *B. yuanmingense* that can nodulate *Lespedeza* spp. in Northern China, *Phaseolus lunatus* in Peru, *Indigofera hirsuta* in Mexico, soybean in Asia and *Vigna* species in southern Africa and subtropical China (Vinuesa et al., 2008a).

Bradyrhizobia have been isolated from cool temperate environments as well as hot, arid biomes (Menna, 2009, Aserse et al., 2012a, Yu et al., 2014). They have been isolated from Namibia, the extreme opposite of the geographic and ecological spectrum from Canada, and under both agricultural and non-agricultural conditions (Bünger et al., 2018).

Of note is a subset of the bradyrhizobia that are photosynthetic; and have been shown to infect and nodulate legumes of the genus *Aeschynomene* using a Nod-factor independent infection process (Giraud et al., 2007).

The very widely globally grown and economically important soybean was the first major crop legume from which the slow-growing rhizobia (*Bradyrhizobium*) were isolated from several different locations. Soybean alone is known to be nodulated by the species *B. diazoefficiens*, *B. elkanii*, *B. japonicum*, *B. ottawaense* and *B. liaoningense* (Yu et al., 2014, Delamuta et al., 2013, Kuykendall et al., 1992, Jordan, 1982, Yao et al., 2002, Xu et al., 1995). The type strain for the species *Bradyrhizobium japonicum* is USDA 6, which was originally isolated in Japan in 1929 (Kaneko et al., 2011). The type strain for *B. diazoefficiens*, which for a long time was known as *B. japonicum,* was isolated in Florida in 1957 (Kaneko et al., 2002, Delamuta et al., 2013). *B. liaoningense* was described in China, and *B. elkanii* occurs widely in Africa (Chibeba et al., 2017, Xu et al., 1995).

1.5 Biogeography of nodulated legumes and rhizobia in Africa

Legumes are the most common plant family encountered among native flora in Africa (Sprent et al., 2009). The mimosoid genera *Vachellia* and *Senegalia* (formerly *Acacia* (Kyalangalilwa et al., 2013)), are the most extensive across the continent, important for honey, gum production, timber and fuel (Sprent, 2005).

Zimbabwe has a rich diversity of native legumes, as illustrated by the report of 550 wild indigenous legume species in Rhodesia (now Zimbabwe, Zambia and Malawi combined), spanning 19 tribes distributed in the three legume sub-families (Corby, 1971). A more recent and more localized Zimbabwean study reported more than 36 species from 11 genera, including *Crotalaria*, *Tephrosia* and *Indigofera* (Mapfumo et al., 2005). These legumes, including, *Crotalaria laburnifolia*, *C. ochroleuca*, *Eriosema ellipticum*, *Indigofera astragalina*, *Rothia hursuta*, *Tephrosia purpurea*, and *Zornia glochidiata* are considered weeds on agricultural land. As such, they are not well studied. However, they are well adapted to their native conditions and have been shown to establish well where conventional exotic legumes have failed (Mapfumo et al., 2005, Nezomba et al., 2008) and therefore represent a resource for the

potential development of new legumes of economic importance and more extensive use or soil improvement for the small-holder farmers. Interestingly, new legumes are still being discovered, such as Zhombwe (*Neorautanenia brachypus* (Harms) C.A.Sm), important for livestock feed and medication (Murungweni et al., 2012). More research into legume in Zimbabwe will reveal as yet unknown potential for sustainable legume-based crop systems.

Legumes are also important in farming systems around Africa. They are traditionally intercropped with cereals in an irregular fashion (Grönemeyer et al., 2015a). From the Phaseoleae tribe, and genus *Vigna*, are important local subsistence crops Bambara roundnut (*Vigna subterranea*) and cowpea, whose centre of origin is West Africa. They are drought tolerant through their deep roots, reduced leaf sizes and thickened cuticles.

Some introduced exotic legumes have found a niche in agriculture because of their economic importance. Soybean, groundnuts and common bean are introduced legumes of great significance, contributing to diets by providing oil and protein (Aserse et al., 2012b), and in the case of soybean, a cash crop for both large and smallholder farmers.

A wide diversity of rhizobia accompanies the vast diversity of legumes across Africa. The soils harbor rhizobia with which these native legumes are compatible. In general, a wide diversity of strains has been reported, with some displaying narrow host ranges while others showed wider host ranges (Mpepereki et al., 1996a). Rhizobia from both alpha (*Azorhizobium*, *Bradyrhizobium*, *Ensifer*, *Mesorhizobium* and *Rhizobium*) and beta-proteo-rhizobial (*Paraburkholderia*) lineages were recovered in a study with 65 Fynbos legumes in the Cape Floristic region, dominated by *Mesorhizobia* and *Paraburkholderia* strains (Lemaire et al., 2015). Rhizobia isolates of the *Rhizobium* and *Ensifer* genera have also been reported (Sprent et al., 2009). Many studies also report the slow growing rhizobia, most likely belonging to the *Bradyrhizobium* species (Musiyiwa et al., 2005a, Mpepereki et al., 1996b, Mpepereki and Makonese, 1995) (Abaidoo et al., 2007, Pule-Meulenberg, 2014, Musiyiwa et al., 2005a) while

others have specifically identified bradyrhizobial isolates from African legumes by molecular means (Grönemeyer et al., 2015a, Aserse et al., 2012a, Chibeba et al., 2017, Jaiswal and Dakora, 2019, Puozaa et al., 2017). There is still a need for molecular studies that can elucidate the species designations of the diverse Zimbabwean and African indigenous rhizobia, as knowledge of these indigenous rhizobia is still scant.

1.6. Legumes in Zimbabwean agriculture

Zimbabwean farming is broadly split into smallholder and commercial farm production. In smallholder agriculture, farmers are subsistence-oriented, and crop production is dominated by the staple food crop, maize. Legumes are low priority crops, traditionally allowed on the more fertile fields only as companions to maize, in rotation or intercrop systems (Jeranyama et al., 2000). Limited use of external yield and soil improvement options drives production systems into low output.

Agriculture research and extension approaches are applying legume-based interventions to reverse the negative trend in soil productivity (Mapfumo et al., 2005). Conservation agriculture programs are designed to promote the use of legumes as cover crops, to take advantage of the many benefits of legumes (Mhlanga et al., 2015). Conservation agriculture is a form of farming where soil disturbance is minimized and mulching is promoted, including use of retained crop residues. This is to conserve both the soil and moisture for sustained higher yields. Legumes are incorporated because they fix nitrogen and can contribute to high quality crop residues into the systems (Mhlanga et al., 2015). Legumes also break pest, disease and weed cycles that arise from parasitic agents that thrive on cereal mono-crops (Mpepereki and Mudyazhezha, 2009). The comprehensive soil cover of legumes helps with weed control; provides high protein content that is important for family diets and livestock feed; while the capacity for SNF can improve soil N content and reduce nitrogen fertilizer input costs (Mpepereki et al., 2000). For legumes to be effective in improving agriculture production

systems, they must fix nitrogen and generate large amounts of high quality biomass (Nezomba et al., 2008). Legume-maize rotations are therefore recommended as the most viable option for sustainable agriculture for smallholders (Chikowo et al., 2004).

The legumes traditionally incorporated in the smallholder farming systems, albeit as lesser crops and therefore on smaller, less fertile land areas, include cowpea, groundnut, and Bambara nut (Sumberg, 2002). Legumes of African origin such as cowpea and Bambara nut (Sprent et al., 2009) are well adapted to Zimbabwean farm conditions. They are expected to nodulate widely with slow-growing rhizobia in Zimbabwean soils. Cowpea and Bambara nut are important sources of dietary protein in Zimbabwe. Cowpea is the second most popular legume crop for smallholder farmers. It is drought tolerant (Tindwa and Semu, 2018) making it ideal for smallholder farmers who generally do not irrigate.

Groundnut, though exotic, originating from South America, was an early introduction by traders. It has naturalized in Zimbabwean farming systems and is the most common legume grown by subsistence farmers. It is rich in protein (25%) and oil (50%), important for subsistence food security for farm families. Groundnuts are used in various commercial products and therefore hold potential as cash crops. However, it is a typical “women’s crop”, given low priority by household decision-makers.

Some more recently introduced legumes, such as pigeon pea, and sunn hemp, both originating in India, and lablab originating in Asia or Africa, are produced in even more limited amounts for their grain and biomass yield. Their agronomy is generally not optimized (Mapfumo et al., 1998, Mhlanga et al., 2015). Pigeon pea is often incorporated in tropical agroecosystems, to fulfil food security, soil improvement and fodder functions. In Zimbabwe, it has also found application in conservation agriculture programs where it has been reported to contribute significant nitrogen to the cropping system (Mhlanga et al., 2015).

Sunn hemp produces high biomass and fixed nitrogen; contributing to nitrogen fertilizer

replacement in subsequent maize. This crop can generate more than 3 tha^{-1} in biomass in a maize-sunn hemp relay intercrop (Jeranyama et al., 2000). It is also tolerant to moisture stress and contributes to weed reduction in maize-sunn hemp green manure cover crop rotation treatments (Mhlanga et al., 2015).

Lablab is a diverse underutilized crop, which potentially originated from Africa and therefore may have more indigenous rhizobia that are compatible with it. It is very drought tolerant, and adapted to acid soils with low levels of available phosphorus (Maass et al., 2010, Abdel-Wahab et al., 2002).

Despite the potential held by these legumes individually and collectively, they are faced with very similar challenges. The market for improved grain legume and pasture seeds is not well developed in Zimbabwe (Mapiye et al., 2006). As opposed to purchasing certified seed, farmers depend on passing on seed within communities. Where the practice is not well starndardised and a quality assurance system is lacking, this leads to build up of pests and diseases and a dilution in improved genetics. As a result, there is a large discrepancy between the yield projections from research and that obtained by the farmers (Matikiti et al., 2012). A demonstration of the potential of these crops, under research conditions and farm conditions, may contribute to the realization of their potential by smallholder farmers. Only soybean seems to have a different status from the other legumes, as outlined below. And yet, there is even room for the yields achieved in soybean production to improve.

1.7. Soybean

Soybean, was domesticated 3000 years ago (Graham and Vance, 2003) from the wild soybean *Glycine soja* in China (Carter Jr et al., 2004). Recent molecular investigation narrows the origin of soybean to the Liaoning province of China (Wang and Li, 2014). From natural variation, the Chinese farmers selected the traits of choice, including thicker stems to support erect plants bearing larger seeds (Piper, 1943, Wang and Li, 2014, Carter Jr et al., 2004).

Soybean consists of high levels of both oils (ca. 20%) and protein (ca. 40%). Soybean oil has many uses, comprising food, pharmaceutical and industrial uses, including biodiesel. After oil extraction, the remaining soy cake is useful for dietary protein and fibre, as well as animal feeds. It provides eight essential amino acids for human nutrition.

Soybean is now grown in the Americas, Europe, Asia and Africa, and is the single highest contributor to world oilseed production. Plant breeders continue to develop new soybean varieties that yield high under different local conditions. According to FAO statistics, the United States was the highest producer of soybean, as of 2018, producing over 123 million metric tonnes of the commodity, followed by Brazil at just over 117 million metric tonnes (FAOSTAT, 2020).

It is possible to produce soybean entirely on SNF-derived N, without the use of inorganic nitrogen fertilizer. This translates to substantial economic benefits. Soybean is estimated to fix 8.7 Tg N in 2019 (FAOSTAT, 2020), with a value of US$6.1 billion (assuming urea cost at US$260/tonne and 20% of applied urea-N lost through volatilization, denitrification and leaching. Globally, soybean was estimated to fix 24 Tg N in 2019 (DF Herridge, pers comm). The domestication of N-fixing legumes and their rhizobia can certainly be harnessed to support sustainable agriculture production.

1.7.1. History of Soybean in Zimbabwe

Soybean, with prostrate growth habit, was introduced to Zimbabwe in 1906 and received enthusiastically by large-scale commercial farmers. The high protein grain was favoured for combining with maize for livestock feed, or haymaking when cut at pod filling stage. Grain was sold to Lever Brothers Company in South African for oil extraction. However, inconsistent yields led to an abandonment of the crop in 1915 (Shurtleff and Aoyagi, 2009). Production resumed with new varieties in 1924, but even the best varieties were characterized by loss of leaves, uneven ripening and pod shattering.

An intensive soybean-breeding program led by the Crop Breeding Institute of the National Agriculture Research System, the predecessor of the current Department of Research and Specialist Services, began in the 1950s, to generate varieties suited to the Zimbabwean environment, starting with an extensive evaluation of imported soybean varieties, many of which were obtained from South Africa. Although the programs did not specifically direct efforts to nitrogen fixation efficiency, there was a collaboration with the country's inoculant production facility for the provision of rhizobial inoculants in breeding programs. To date, significant improvements have been achieved with respect to yield, resistance to diseases and quality of genotypes (Tichagwa et al., 2004).

Soybean is the one legume with well-developed seed breeding programs in different organizations in Zimbabwe. The country's largest seed company joined in the breeding of soybean early, and now several other companies are also involved in seed production. Soybean was a commercial sector crop, produced as a component of a two crop per year rotation, with irrigated wheat in the winter and rain-fed soybean in the summer. More recently, several initiatives have sought to introduce a more diverse farming system in the smallholder communities, most notably the Soyabean Promotion Task Force (SPTF) hosted by the University of Zimbabwe and the Soil Productivity Research Laboratory (SPRL), seeking to arrest soil degradation, diversify and improve diets, and give smallholders opportunities for income generation (Marufu et al., 1995, Whingwiri, 1996). The country's producer price of soybean is attractive for farmers and demand remains high.

High soybean yields attained with the support of research and extension personnel, and viable producer prices made soybean attractive to the men, rescuing this legume from the "women's crop" status in the rural communities. However, in comparison to potential, soybean yields in smallholder communities remain low, with averages of 0.4t/ha reported, against a potential 5t/ha on research plots. Soybean is now produced in all eight rural provinces of the

country, and is most suited to natural regions IIa, IIb, and III (Figure 1.2).

1.8. Occurrence of indigenous soybean rhizobia in Zimbabwe

Soybean root nodule bacteria have been reported in various places around Zimbabwe in varying numbers and efficiencies. Early studies of soybean production and research were carried out with rhizobial inoculants, and inoculation responses were reported with cultivar Hernon 237 in 1952/3 (Mpepereki et al., 2000). In 1977, Hernon 147 nodulated effectively in a farmer's field in Zambia, with no history of inoculation (Mpepereki et al., 2000). However, spontaneous nodulation was also reported as early as 1964/5 (Corby, 1965). At the time, the national research system served what are now Zimbabwe, Zambia and Malawi together, as Rhodesia then. The promiscuous soybean cultivar Hernon 147 nodulated effectively in uninoculated plots, and did not respond to inoculation at research stations Mount Makulu and Magoye in Zambia; and at Henderson, Kadoma and Chimanimani in Zimbabwe, suggesting that plants were nodulated with indigenous rhizobia. The Zimbabwean sites fall in natural regions IIa, III and I, respectively (Figure 1.2). However, at Save valley, which is in natural region IV, free nodulation was ineffective and there was a positive response to inoculation (Corby, 1965). Free nodulation refers to nodulation with native rhizobia, achieved without inoculation. Soil moisture decreases and ambient temperature increases from natural region I to V, implying that indigenous soybean rhizobia populations respond to rainfall amounts across the country. It has also been suggested that populations respond to soil organic carbon with populations increasing and decreasing with the parameters (Davis and Mpepereki, 1995, Zengeni et al., 2003, Zengeni et al., 2006, Mpepereki and Makonese, 1998).

Zimbabwe appears to be dominated by slow growers among soybean rhizobia (Musiyiwa et al., 2005a, Mpepereki and Makonese, 1995). These are most likely bradyrhizobial strains, but some fast growers have also been found in Zimbabwean soils (Mpepereki and Makonese, 1998, Davis and Mpepereki, 1995). More than 50% of the fast

growers were found to tolerate high temperature, beyond 40ºC. This was linked to exopolysaccharide production, as opposed to altitude, annual temperature or soil properties (Davis and Mpepereki, 1995).

The rhizobia in all previous studies of native soybean rhizobia in Zimbabwe have not been described to species level. Still, the phenotypic characterizations suggest wide diversity, in line with the diverse legume life in Zimbabwe. Some soybean root nodule bacteria also nodulated cowpea, siratro, pigeon pea and sunn hemp, but failed to nodulate common bean, groundnut and sesbania (*Sesbania sesban)* (Musiyiwa et al., 2005b, Mpepereki and Makonese, 1995). Indigenous rhizobia with high capacity for nitrogen fixation with soybean have been obtained, and there are potential candidates for use as inoculant strains (Mpepereki and Makonese, 1995, Musiyiwa et al., 2005b).

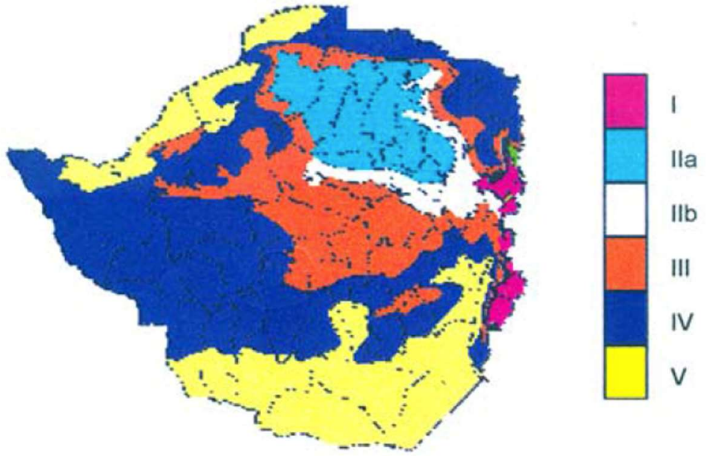


Figure 1.2 A map of natural regions in Zimbabwe. Natural regions are designated I - V, based on decreasing amount of rainfall received.

1.9. Use of rhizobia inoculants with legumes

Rhizobial inoculants are amendments for crop production that carry rhizobial cells for specified crops, in order to facilitate nodulation and optimum SNF. Inoculation ensures nodulation and improves nitrogen fixation where soils may carry rhizobia that are incompatible with the cultivated crop or populations of compatible rhizobia are too small for economic yields. In Zimbabwe, there are more than ten crops for which rhizobia inoculants are made,

including groundnut, cowpea, pigeon pea, lablab, sunn hemp and others. However, farmers often do not take up the technology, and therefore lose out on the potential benefits of using the inoculants.

Cowpea is promiscuously nodulating (Woliy et al., 2019), but inoculation with selected efficient strains can improve SNF (Sena et al., 2020). A recent study at one location in Zimbabwe recorded remarkable yield improvements with cowpea at elevated inoculation rates (Kanonge-Mafaune et al., 2018). This suggests that beyond establishing the need to inoculate and the potential of the crops under inoculation, there will be other research questions that will be defined.

Groundnut may be considered slightly less promiscuous than cowpea (Woliy et al., 2019), and in Zimbabwe, it is generally believed that the promiscuous nodulation is sufficient to generate economic yields. One study found at least 46% and up to 85% effective nodulation of cowpea and Bambara groundnuts with slow-growing rhizobia isolated from 13 legumes in Zimbabwean soils (Mpepereki et al., 1996a). These were groundnut (cv. Natal Common); pigeon pea, sunn hemp, rattlepod (*Crotalaria ochraleuca*), soybean (cv. SCS-1), silky indigo (*Indigofera astragalina*), true indigo (*Indigofera tinctoria*), peas (*Pisum sativum*), common beans (cv. Carioca), siratro (*Macroptilium atropurpureum*), Egyptian riverhemp (*Sesbania sesban*), cowpea (cv. Local Mixed) and Bambara groundnuts. The nodulation of groundnut and soybean in the same study was much lower, at 12% and 13% respectively, pointing to a need for wide inoculant use. Only limited amounts of groundnut rhizobia inoculant are sold from the inoculant factory. This poor uptake of the technology may be due to lack of sufficient promotion of the rhizobia inoculant use other than soybean rhizobia inoculant. Elsewhere, groundnut has been reported to respond to inoculation, including improvements in nodulation, nitrogen fixation and grain yields (Gericó et al., 2019, Jain et al., 2020).

Pigeon pea and sunn hemp have been reported to nodulate freely but to a limited extent

in Zimbabwean soils (Mpepereki et al., 1996a). Pigeon pea crop responses to inoculation have been recorded (Araujo et al., 2020). Inoculation will assist the crop to realize yield potential. There are currently no studies into the effectiveness of inoculating sunn hemp crop in Zimbabwe, which could potentially improve the benefits on the cropping systems.

While lablab is promiscuously nodulating, there is no documented evidence of the need or lack of need to inoculate in Zimbabwe. There occur strains that are competitive under different on-farm conditions with the potential to improve yields significantly. Lablab is known to be nodulated by *B. namibiense* (Grönemeyer et al., 2017). There are no known reports of noduation by lablab in the country.

The effectiveness of nodulation and nitrogen fixation, particularly with inoculation, of legumes other than soybean in Zimbabwe, have not been documented sufficiently. Rhizobia inoculation is an effective way to improve nodulation and nitrogen fixation. Still, there are no reports of any studies into the response to inoculation in Zimbabwe with most of these legumes. While cross inoculation suggests that some specific crops can be inoculated with strains that have been isolated from the same crops, as is the case for cowpea, *Crotalaria* and pigeon pea (Araujo et al., 2020), the effectiveness of the symbioses may vary (Woliy et al., 2019). A good starting point is to establish the need for inoculation for the individual crops, and the response to current inoculant strains. Isolation and selection of new rhizobia inoculation strains of indigenous origin potentially discovers strains that are well adapted to native abiotic factors such as high temperatures, and saline and acidic soils, while fixing high levels of nitrogen (Sena et al., 2020). Research studies generate empirical evidence to inform and guide farmers.

1.9.1 Nitrogen fixation efficiency of different rhizobia strains

Different rhizobia strains have different capacity to fix nitrogen with a given legume. Therefore, it is important to screen isolates and select elite strains. Candidates must be screened for effectiveness in N fixation, edaphic adaptation and performance in situ, which includes

competitiveness (Howieson et al., 2000, Hardarson et al., 1984). Ideally, strains are screened first under controlled conditions such as glasshouse conditions before those that are shortlisted can be assessed under field conditions. Under field conditions, the shortlisted strains must perform in a range of soil physical and chemical properties such as low and high soil pH as well as agro-ecological conditions, which are important for rhizobia survival (Howieson et al., 2000, Hungria and Vargas, 2000).

Soil chemical properties such as pH and nutrient levels must be optimized for efficiency of SNF. Soil phosphorus is the second most limiting nutrient for crop production (Vance, 2001) and has been found often to limit SNF and nodulation. Nitrogen fixation and crop yields generally increase in response to phosphorus application (Ulzen et al., 2018a, Smith, 1992).

Host range is also crucial in screening rhizobia strains. Broad host range isolates are important where the capacity to produce many different isolates in commercial formulations is limited (Howieson et al., 2000). It is also important across different cultivars of the same crop where the market for the crop has many cultivars.

Nitrogen fixation efficiency may be evaluated directly or indirectly. Direct methods include stable N isotope dilution technique that can be used to separate the nitrogen derived from the atmosphere from that derived from the soil and fertilizer (Hardarson et al., 1984). Acetylene reduction is yet another direct method of analysis. Indirect methods compare the biomass or the nitrogen accumulated by inoculated legumes with that by uninoculated ones.

1.9.2. Use of rhizobia inoculants with soybean in Zimbabwe

Rhizobia inoculants formed a cornerstone of soybean production in Zimbabwe since the 1960s. The widely studied superior nitrogen fixation strain *Bradyrhizobium diazoefficiens* USDA 110^{T} (= MAR1491) was obtained from the USDA Culture Collection in Maryland in 1950 (and re-imported in 1980) to the government inoculant production facility at Marondera, 70 km outside the capital city, Harare, towards the North East (Mary Ryder, pers. comm). It is

the established strain of choice for soybean because of its superior capacity for nitrogen fixation. Rhizobia inoculants have been widely used and promoted for soybean production in Zimbabwe. Soybean rhizobia sales make up more than 90% of sales at Soil Productivity Research Laboratory, the sole producer of rhizobia inoculant in Zimbabwe for a long time.

Commercial production for soybean inoculants began in 1967. Strains were stored and maintained on agar slants (Sandmann, 1969). From 1995, they were backed up by lyophilization (Marufu et al., 1995). Farmers were supplied with pure inoculum in returnable laboratory flat bottles from the rhizobia inoculant factory since the 1960s. Efforts to provide inoculant to farmers in a solid formulation found that the nearest source of peat for a solid carrier was South Africa. The logistics for importation were too cumbersome and the opportunity was abandoned. Since 1981 (to date, September 2020), bagasse, a solid by-product of sugar cane production, is used as the inoculant carrier.

In the 1980s, elsewhere on the continent, the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria had begun a breeding program for promiscuous soybean to take advantage of SNF but without depending on rhizobia inoculant that was not available in most places on the continent. This was around the time that soybean popularization for smallholder communities was starting. Because of the lack of refrigerators in smallholder communities in Zimbabwe, it was also assumed that promiscuous cultivars would be appropriate for smallholder farmers in the country. Direct comparison under field conditions revealed a higher propensity of promiscuous soybean to freely nodulate and for the more specific soybean cultivars to respond to inoculation (Davis and Mpepereki, 1995, Kasasa et al., 1998).

However, because of the long history of soybean breeding and soybean production on commercial farms in Zimbabwe, only specifically nodulating, high yielding soybean cultivars were available on the market for farmers to buy (Zengeni et al., 2006, Kasasa et al., 1999). In

addition, the symbiotic performance of indigenous soybean rhizobia was quite erratic, such that nodulation and yields were inconsistent. Also, the promiscuous cultivars responded to rhizobial inoculation (Javaheri et al., 1994). The only sensible thing to do was to promote rhizobia inoculant along with the introduction of high yielding soybean production in the smallholder communities.

The introduction of rhizobia inoculant to the smallholder communities was followed up by research to adapt scientific findings to the smallholder farmer settings. For example, while fridges were placed at different centres around the country to keep the cold chain up to the selling points, close to the smallholder farmers, smallholder farmers were advised to store their sachets of rhizobia in granaries or on the floor to keep them cool.

The yield of soybean crops under smallholder conditions have been known to more than double in inoculated plots (Zengeni et al., 2006). However, there is a need to update recommendation rates to reflect the differences in edaphic and agro-climatic conditions around the country. In one study, soybean continued to give an inoculation response on sandy soils when inoculant was added at up to ten times the rate recommended by the factory (Chirinda et al., 2003).

Currently, rhizobia inoculants from the inoculant factory are distributed through the national agriculture extension service, Agriculture Technical and Extension Services (AGRITEX), which has an extensive network in the country. Only strategic districts, with interest in soybean production, stock the product in time for farmers to access them to establish crops for the season. Gaps in yields still persist as smallholder farmers apply limited to no fertility amendments and use manual operations while large-scale farmers are mechanized, and use fertilizers, inoculants and herbicides (Svubure et al., 2010).

Nitrogen fixation efficiency in the field is a function of the genetics of both the host legume and the microsymbiont, and also agricultural management practices and environment.

The availability of compatible rhizobia in the soil is not a guarantee for efficient nitrogen fixation. Competition from other rhizobia and suboptimal agro-ecological conditions that include unfavourable soil pH, temperature and low soil moisture content are the common challenges agronomists must address. The elite rhizobia inoculant strain appears not to persist in the soils in Zimbabwe (Zengeni et al., 2003). Competition from indigenous rhizobia cannot be ruled out. It is important to screen indigenous rhizobia for rhizobia inoculant strain candidates that are superior to the current elite USDA 110^{T}, because indigenous rhizobia would persist more than the exotic strain and superiority in nitrogen fixation is an improvement.

1.10. Challenges to greater use of legumes in Zimbabwean agriculture

Legume-rhizobia associations naturally cycle large amounts of N, converting inert atmospheric N into biologically usable forms. Legumes are a significant component of Zimbabwean farming systems, important for their contributions to dietary protein, livestock feed, and income generation and soil improvement. They can be exploited in agronomy for economic gains and environmentally friendly provision of nitrogen fertility for soils. Different legumes have different capacities for SNF. This must be tested. Choices of legumes to incorporate into farming systems must reflect farmers' needs and must be well adapted to allow superior performance. The easiest way to exploit symbiotic nitrogen to improve the fertility of soils would be to grow crops and make use of indigenous rhizobia populations. However, because legume-rhizobia associations are specific, resident native rhizobia may not be appropriate for the legume crop grown. There is a need to evaluate free nodulation of legumes and compare the response to inoculation with elite strains of rhizobia that have been determined to be highly effective for nitrogen fixation with the specific legumes. Inoculation is an effective way to improve nodulation and nitrogen fixation where resident rhizobia are incompatible with the legume grown. However, inoculation is not without its own challenges, particularly the soybean rhizobia inoculant in Zimbabwe, already reported to lack saprophytic competence.

Rhizobia inoculants sometimes face competition from indigenous rhizobia that are well adapted to the native conditions. These native rhizobia potentially make improved rhizobia inoculants in the event that they fix higher amounts of nitrogen than the inoculant strain.

Legume-rhizobia technology is attractive to smallholder farmers due to the more affordable cost in comparison to inorganic fertilizers. With this background, it is important to look at the potential of a range of legumes to fix N and produce biomass with native Zimbabwean rhizobia without the need for inoculation. This must be compared to the inoculated legumes, to weigh the value of inoculation.

1.11. Aims of the thesis

Accordingly, the aims of this thesis are to:

i) Investigate the feasibility of free-nodulation and the inoculation response in different Zimbabwean soils of cowpea, groundnut, soybean, lablab, pigeon pea and sunn hemp, and evaluate the N-fixation effectiveness of these symbioses.

ii) Describe the diversity and determine the phylogeny of soybean root nodule bacteria in Zimbabwean soils where the elite inoculant MAR1491 has been inoculated five years prior to the dates of sampling.

iii) Investigate the symbiotic efficiency of soybean root nodule bacteria isolated in (ii) above, and verify compatibility with a range of commercial soybean cultivars and performance in a range of soil and climatic conditions.

CHAPTER 2

Effect of inoculation with rhizobia of six grain and pasture legumes on biomass yields and residual benefits on rotation maize in Zimbabwe

2.1 Introduction

Nitrogen fertilizer is one of the critical limiting nutrients for crop production in Zimbabwe, where smallholder agriculture is dominated by maize production (Nyoka et al., 2004, Chigariro, 2004). Application of inorganic fertilizers is often below recommended rates due to prices that are beyond the reach of the majority of farmers and/ or erratic distribution systems. Other organic options that include manure are limited in quality and quantity due to the small numbers of livestock owned by the farmers (Mapfumo and Giller, 2001). Legumes have emerged as the most potent option for improving nitrogen fertility in smallholder farmers, supported by a modest amount of fertilizers (Mapfumo et al., 2005). Legumes can form nitrogen-fixing symbiotic relationships with soil microorganisms called rhizobia, in a process of symbiotic nitrogen fixation (SNF) (Wang et al., 2012). Incorporating legumes with high rates of SNF into agricultural systems SNF can save farmers and governments money by eliminating the need to purchase nitrogen fertilizers (Hungria and Mendes, 2015a). Residual benefits can be extended to other crops in the system by using legumes as rotation, intercrops, green manure, ley crops, or various other options (Kasasa et al., 1999). In addition to nitrogen fixation, legumes are important for the provision of dietary protein, high protein livestock feed, and reducing pest and disease pressures as rotation crops.

The increasing area under legumes and improving legume yields across Africa will improve SNF contributions (Ulzen et al., 2018a). However, the legumes vary widely in their adaptation to different environments, their uses for the farmers and their capacity for nitrogen fixation (Daryanto et al., 2015). The choice of legume must be a well-informed, deliberate decision. Legumes may be categorized as food security (Foti et al., 2020), livestock feed (Chigariro, 2004), soil improvement (Nezomba et al., 2008) and cash crop (Giller et al., 2011). In Zimbabwe, groundnut and cowpea are two of the most widely produced crops for food security. Pigeon pea and lablab are crops with potential for both human and livestock consumption while

lablab along with sunn hemp can be promoted for soil improvement. Soybean is the most economically valued legume in Zimbabwe, with well-developed input and output markets. In fact, only soybean has readily available improved seed on the market whereas farmers pass on seed to each other for the other crops.

Efficiency in SNF will depend on the genetic capacity of the crop and cultivar, and the availability of appropriate rhizobia in sufficient numbers. Soils often lack the rhizobia required by legumes for optimum SNF. Addition of the appropriate rhizobia strains at planting by inoculation can improve nodulation and nitrogen fixation (Ulzen et al., 2018b, Thuita et al., 2012, Lindström et al., 2010). Inoculation has been reported to result in higher grain yields and higher nitrogen fixation by legumes (Ulzen et al., 2018a, Samago et al., 2018, Tauro et al., 2011). Over 40% increase in nodule numbers and more than 10% increase in grain yield has been recorded relative to uninoculated controls of cowpea and soybean (Ulzen et al., 2016).

However, inoculation response may be compromised by competition from resident indigenous rhizobia that are well-adapted to their native conditions (Thies et al., 1991, Bogino et al., 2008, Zdor and Pueppke, 1990). Sub-Saharan Africa hosts a wide range of rhizobia, with only a limited number of these studied (Aserse et al., 2012b, Gronemeyer and Reinhold-Hurek, 2018, Aserse et al., 2012a). The diverse indigenous soil populations of rhizobia that compete with introduced rhizobia strains may have an exceptional adaptation to local conditions such as extreme temperatures (Mpepereki et al., 1996b, Gronemeyer and Reinhold-Hurek, 2018, Grönemeyer et al., 2014a). This is particularly important as climate extremes become more common, as shown by an analysis of Zimbabwe's historical climate data which demonstrates increasing temperatures and rainfall variability, in line with climate change predictions (Moyo et al., 2012). Other abiotic conditions that introduced strains must contend with include extremes of soil pH, and low soil fertility and moisture, which all affect the establishment, maintenance and function of the symbiosis (Valentine et al., 2018, Igiehon and Babalola, 2018,

Brear et al., 2013, Zahran, 1999). Because of their adaptation, indigenous rhizobia present a reservoir for selection of new rhizobia inoculant strains (Chibeba et al., 2017, Grönemeyer et al., 2014a). Native rhizobia can be screened for selection of potential strains for nitrogen fixation (Chibeba et al., 2017, Koskey et al., 2017, Howieson et al., 2011) particularly where inoculant strains in culture collections are not satisfactory for nitrogen fixation.

The present study investigates SNF with six legumes, namely soybean, cowpea, groundnut, lablab, pigeon pea and sunn hemp. Of these crops, only soybean has been widely studied for its nitrogen fixation under inoculation. Information on biomass yield, nitrogen fixation and benefits for rotation maize is not readily available, yet is essential to guide farmers to make the best use of legume-rhizobia technology. The right crop must be selected for the farming system, and the right approach to providing the microsymbiont will support a sustainable legume-integrated system.

To provide this information, this study was designed to investigate the symbiotic potential of six legumes groundnut, cowpea, pigeon pea, sunn hemp, lablab and soybean. The objectives were to (i) assess the SNF potential of the six legumes with native rhizobia *in situ* over a range of sites, (ii) evaluate and compare the inoculation response of six legumes with respect to biomass production (iii) compare the residual effect of inoculation with repeat sowing of legumes on the same land, and (iv) compare rotation effects on the staple crop maize. This data will inform research priorities and guide agriculture practitioners towards more effective use of the legume-rhizobia symbiosis in farming systems.

2.2 Materials and methods

A field experiment was carried out in the first season, to compare legume growth with and without inoculation. In the second season, one half of each plot was rotated with maize to evaluate residual effects while the legume was resown in the other half. In the third season,

three new sites were used to evaluate the occurrence of rhizobia for the legumes in the farming systems.

2.2.1 Hosts and bacterial strains

Seeds for cowpea, groundnut and soybean were commercial cultivars produced by the Crop Breeding Institute of the Division of Crops Research, Harare, Zimbabwe (Table 2.1). Lablab, pigeon pea and sunn hemp were obtained from Grasslands Research Institute of the Division of Livestock Research, Marondera, Zimbabwe (Table 2.1).

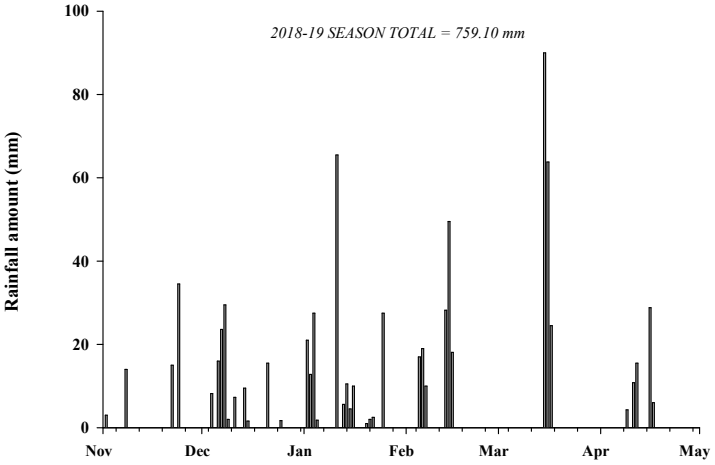
All strains used are recommended ones for the respective legumes, obtained from the Soil Productivity Research Laboratory of the Division of Specialist Services, Marondera, Zimbabwe. Strains were cultured in yeast extract mannitol agar, and the broth was generated from single colonies in yeast extract mannitol broth. The broth was inoculated into steam-sterilized sachets of bagasse, incubated to achieve 10^9 CFU per gram.

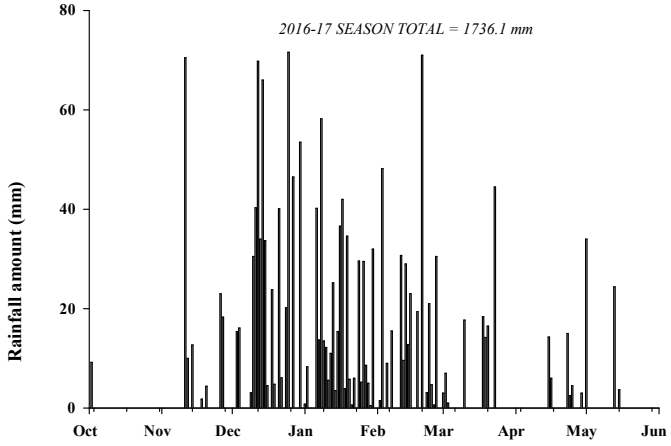
Table 2.1 Summary of legumes and rhizobia inoculant strains used in the study

Legume	Centre of origin	Subfamily	Tribe	Seed source for study	Main use in Zimbabwe	Reference	Cultivar	Rhizobia inoculant strain
Cowpea	Sub-Saharan Africa	Papilionoideae	Phaseoleae	CBI	Food	(Chidebe et al., 2018)	CBC2	MAR1510
Groundnut	South America	Papilionoideae	Dalbergieae	CBI	Food	(Jaiswal et al., 2017)	Illanda	MAR411
Lablab	Sub-Saharan Africa	Papilionoideae	Phaseoleae	GRS	Forage	(Murungweni et al., 2004)	Highworth	MAR411
Pigeon pea	India	Papilionoideae	Phaseoleae	GRS	Forage	(Mapfumo et al., 1999)	Unknown	MAR472
Soybean	China	Papilionoideae	Phaseoleae	CBI	Oil expression, food	(Tian et al., 2012)	Mhofu	MAR1491
Sunn hemp	India	Papilionoideae	Crotalarieae	GRS	Green manure	(Schomberg et al., 2007)	Unknown	MAR1510

2.2.2 Study sites

This study was carried out at Grasslands Research Station (GRS), Marondera, about 70 km northeast of Harare, Zimbabwe (18°10.916′ S, 031°29.870′ E), elevation 1648 masl during the rainfall seasons 1 (2015-16) and 2 (2016-17) (Figure 2.1); while for season 3 (2018-2019), three sites were used (Figure 2.1; Figure 2.2). These were at GRS, at Makoholi Research station (MRS), Masvingo, about 280 km South-East of Harare (19º50'S; 30º47'E) elevation 1204 masl and at Panmure Experiment Station (PES) elevation 881 masl, Shamva, about 95 km northeast of Harare, Zimbabwe (17°10′0″S, 31°40′0″E). Grasslands Research Station, PES and MRS are in agro-ecological regions (AER) IIa, IIb and IV, respectively. They receive mean annual rainfall of 900 mm at GRS, between 700 and 900 mm at PES and 650 mm at MRS. While traditionally rainfall is described to fall between November and March, rain fell between October and June during the course of the study. Soil characterization is listed in Table 2.2. Mean maximum temperatures are 24°C at GRS, 28°C at MRS and 30°C at PES.


2018-19 SEASON TOTAL = 759.10 mm
Rainfall amount (mm)
100
80
60
40
20
0
Nov
Dec
Jan
Feb
Mar
Apr
May


2016-17 SEASON TOTAL = 1736.1 mm
Rainfall amount (mm)
80
60
40
20
0
Oct
Nov
Dec
Jan
Feb
Mar
Apr
May
Jun

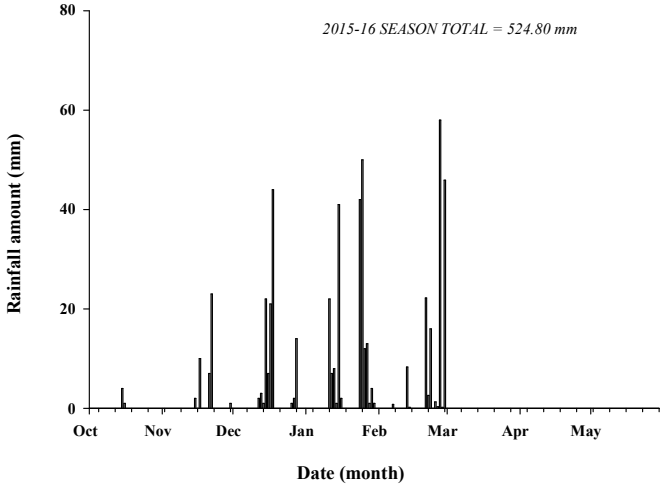

2015-16 SEASON TOTAL = 524.80 mm
Rainfall amount (mm)
80
60
40
20
0
Oct
Nov
Dec
Jan
Feb
Mar
Apr
May
Date (month)

Figure 2.1 Rainfall amount and distribution at Grasslands Research Station, Marondera, Zimbabwe, during the course of the study

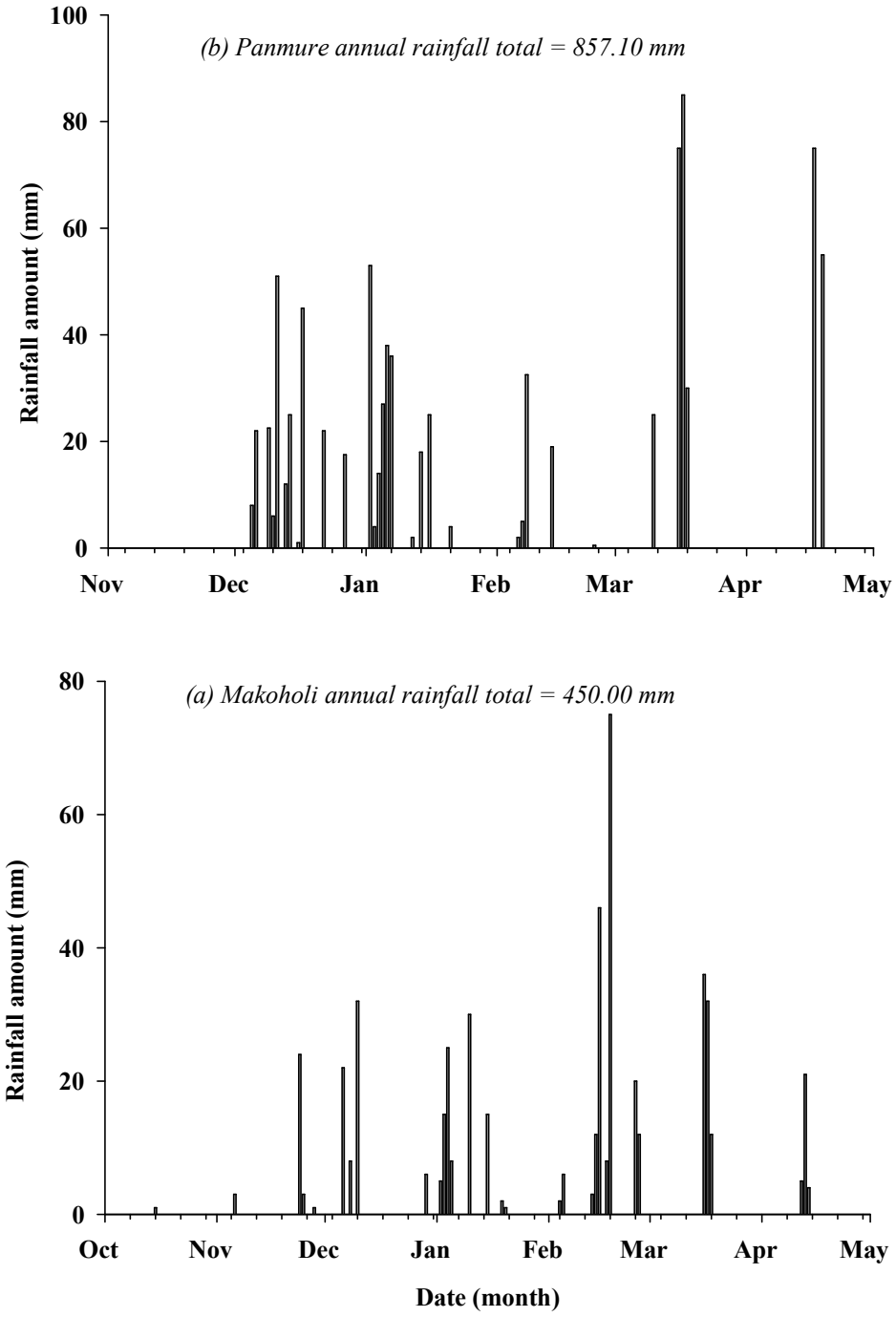


Figure 2.2 Rainfall amount and distribution during the 2018-19 season at (a) Makoholi Research Station and (b) Panmure Experiment Station in Zimbabwe.

All experiments were carried out at sites that had not previously been planted to the same legumes. However, as all sites are located on research or experiment stations, there is a history of inoculated soybean at least within the farm. The third season experiment at GRS was sited 2km away from the first to reduce possibilities of contamination from the previous inoculation.

Field sites were ploughed and disced using tractor drawn implements except in the second season at GRS where the site was ox-ploughed to avoid moving soil out of original plots. All treatment plots received fertilizer Compound D (7 N - 14 P_2O_5 - 7 K_2O) at 300 kg ha^{-1} applied by broadcasting, and incorporated using handheld hoes before plants were established.

Table 2.2 Soil characterization for the different sites used in the study in seasons 2015/16; 2016/17 and 2018/19

						Exchangeable cations meq per 100 g		
Site	NR	Texture	pH (CaCl)	Mineral N ppm	Available P (resin extract) ppm	Potassium	Calcium	Magnesium
GRS 1	IIa	mgS	4.4	18	49	0.14	0.89	0.85
GRS	IIa	mgS	4.5	25	13	0.18	0.93	0.68
PES	IIb	mgSCL	5.9	9	85	0.39	2.31	1.21
MRS	IV	mgS	4.3	21	17	0.05	0.45	0.38

The inoculation treatment was carried out in season 2015/16 at GRS 1. The legumes were re-established in the 2016/17 season on half of the plots and the other half planted to maize. In 2018/19 season, the legumes were planted without inoculation at GRS, PES and MRS.
Abbreviations for soil texture: m = medium; g=grained; S=sand/sandy; C=clay; L=loam

2.2.3 Experimental description

2.2.2 Season 1: 2015/2016

Field experiments of cowpea, groundnut, lablab, pigeon pea, soybean and sunn hemp were established under inoculation and no inoculation treatments in December 2015 at GRS only. The trial was a Randomised Complete Block Design (RCBD) with plot sizes of 3.5 m by 5 m, blocked by a slope. Uninoculated plots were established first. Seeds for the inoculated treatments were inoculated with a slurry of 1% sugar solution and bagasse-based inoculant at 10^{-9} rhizobial cells per gram. The rhizobia inoculant strains used for the legumes are listed in Table 2.1. Seed inoculation was undertaken in the shade and the seeds were immediately planted and covered with moist soil. Each treatment was replicated four times. Plots were kept weed-free using a hoe and maintained under rain-fed conditions.

Biomass production of the legumes was determined at mid-flowering eight weeks after planting (8WAP) by a destructive sampling of plants from a net plot measuring 1.0 m × 1.0 m. All plants within the net plot were cut at the first internode point and packaged in khaki paper bags. Biomass was determined after drying the aboveground plant parts at 75ºC in an oven for 48 hrs. After drying, the samples were ground and analyzed for Total N using the micro-Kjeldahl technique (Anderson and Ingram, 1989). Nitrogen (N) accumulation (kg ha^{-1}) was determined as a product of %N content and biomass productivity. Grain yield (t ha^{-1}) and 100 seed weight (g) were determined at physiological maturity after 12% moisture correction of the grain.

2.2.3 Season 2: 2016/2017

In the subsequent season, the experimental plots from season 1 were split into two. In one half of the plot, the legume from season 1 was re-established, without inoculation, to evaluate the nitrogen fixation capacity of the legumes on residual rhizobia populations from inoculation in the preceding year and the N fixation capacity of a legume following a legume. In the second half of the plot, maize crop was established to evaluate the residual nitrogen effect on maize following an inoculated or non-inoculated legume crop. Biomass production was determined at 8WAP by a destructive sampling of three plants each from the middle three rows of the plot for both the maize and the legume options. Determination of biomass and total nitrogen accumulated was carried out as in season 1.

2.2.4 Season 3: 2018/2019

In the third season (2018 -19), the availability of indigenous root nodule bacteria at three sites, GRS, MRS and PES was evaluated. Plot sizes, layout and establishment were adopted from the initial experiment, but with no inoculation in all treatments. Legumes were maintained for eight weeks, until mid-flowering for all plots and then harvested for nodules. To harvest, plots were drenched with water and allowed to soak for two hours, after which ten random plants were excavated from the middle three rows of the plot. The plants were cut off at the first node and the plant tops discarded. The nodules were counted and recorded. Nodule colors for three random nodules were checked and recorded. The nodules were cut off the root with about 2 mm of root on either side, and their fresh weights were recorded. The dry weights were determined after drying in silica gel for two weeks.

2.2.5 Data analysis

The data generated for biomass accumulated and N by legumes in seasons 1 and 2; grain yield by legumes in season 1, 100 seed weight of legumes in season 1 and nodule numbers of legumes without inoculation in season 3 were subjected to analysis of variance in SPSS version 22 (IBM Corp, released 2013) and means were subsequently separated using least significant differences (LSD).

2.3 Results

Soils at the research stations varied (Table 2.2) but were dominated by medium-grained sands at GRS and MRS, while PES had a medium grained sandy clay loam. The soils were generally acidic, ranging from 4.3 to 4.5 while PES had a less acidic soil of pH 5.9. The soil at PES was generally more fertile, with the highest levels of potassium and phosphorus of the four sites, as well as exchangeable cations.

2.3.1 Nodulation of legumes across different natural regions during the 2018/19 season

At GRS, average nodule counts ranged from zero for groundnut, to 93 for cowpea at GRS; 23 for groundnut to 104 for cowpea at MRS; and from 11 for cowpea to 72 for soybean at PES (Table 2.3). The nodulation was highly variable across and within the legumes and sites. There were no significant differences between the nodule numbers nor the nodule weights (Table 2.4) recorded on the legumes. Nodulation was only evaluated in season 3.

Table 2.3 Number of nodules recovered on the roots of six uninoculated legumes cowpea, groundnut, lablab, pigeon pea, soybean and sunn hemp established at three sites- Grasslands Research Station (GRS), Makoholi Research Station (MRS) and Panmure Experiment Station in the 2018/2019 agriculture season

	Site		
Legume	GRS	MRS	PES
Cowpea	93	104	11
Groundnut	0	23	22
Lablab	43	29	21
Pigeon pea	37	22	48
Soybean	22	31	72
Sunn hemp	65	50	37
lsd (p<0.05) CROP	30.11		

Table 2.4 Average nodule weight (g) of six uninoculated legumes cowpea, groundnut, lablab, pigeon pea, soybean and sunn hemp established at three sites Grasslands Research Station (GRS), Makoholi Research Station (MRS) and Panmure Experiment Station in 2018/2019 agriculture season

	Site (soil pH)		
Legume	GRS (pH 4.5)	MRS (pH 4.3)	PES (pH 5.9)
Cowpea	3.8 g	7.1 g	8.2 g
Groundnut	0 g	3.9 g	2.2 g
Lablab	13.7 g	8.6 g	60.9 g
Pigeon pea	23.0 g	7.9 g	8.4 g
Soybean	48.5 g	5.3 g	5.0 g
Sunn hemp	4.4 g	20.0 g	43.6 g
lsd (p<0.05) CROP	6.63		

2.3.2 Biomass accumulated by legumes over two seasons

Uninoculated legumes yielded low biomass from 1.21 t ha^{-1} for groundnut to 2.14 t ha^{-1} for lablab (Figure 2.3a) in season 1, 2015/2016. The highest biomass accumulated by legumes without inoculation was by lablab. All crops responded positively to inoculation ($p<0.001$) with soybean giving the highest increase in biomass after inoculation, at 225% biomass higher than the respective uninoculated treatment at 8 WAP. Inoculated sunn hemp (4.2 tha^{-1}), lablab (4.11 tha^{-1}) and soybean (3.93 tha^{-1}) had

the greatest amounts of biomass accumulated at 8 WAP, with no difference between them ($P<0.05$). This was followed by inoculated cowpea, which itself was not different from inoculated groundnut, inoculated pigeon pea and uninoculated lablab ($P<0.05$). Biomass generated by uninoculated lablab was similar to that generated by inoculated groundnut, pigeon pea and cowpea. The lowest biomass was generated by uninoculated groundnut.

In the second season, 2016/2017, while there was no inoculation, there were significant differences with respect to biomass due to the inoculant treatment ($p<0.001$) of the previous season, and the different crops ($p = 0.021$). The biomass accumulated by legumes from the uninoculated treatments ranged from 1.6 t ha^{-1} to 2.2 t ha^{-1} while that from the previously inoculated plots ranged from 3.09 t ha^{-1} to 4.17 t ha^{-1}. Under the previously inoculated treatment, lablab and sunn hemp again provided the greatest biomass accumulation with no difference between them (4.17 t ha^{-1} and 4.0 t ha^{-1}, respectively ($P<0.05$), Figure 2.3b). Sunn hemp biomass was not different from that of soybean while pigeon pea had the lowest biomass, which was not different from groundnut and cowpea ($P<0.05$). The legumes from the plots that were not inoculated in either season also generated greater biomass in the second season than the first, except for cowpea and sunn hemp.

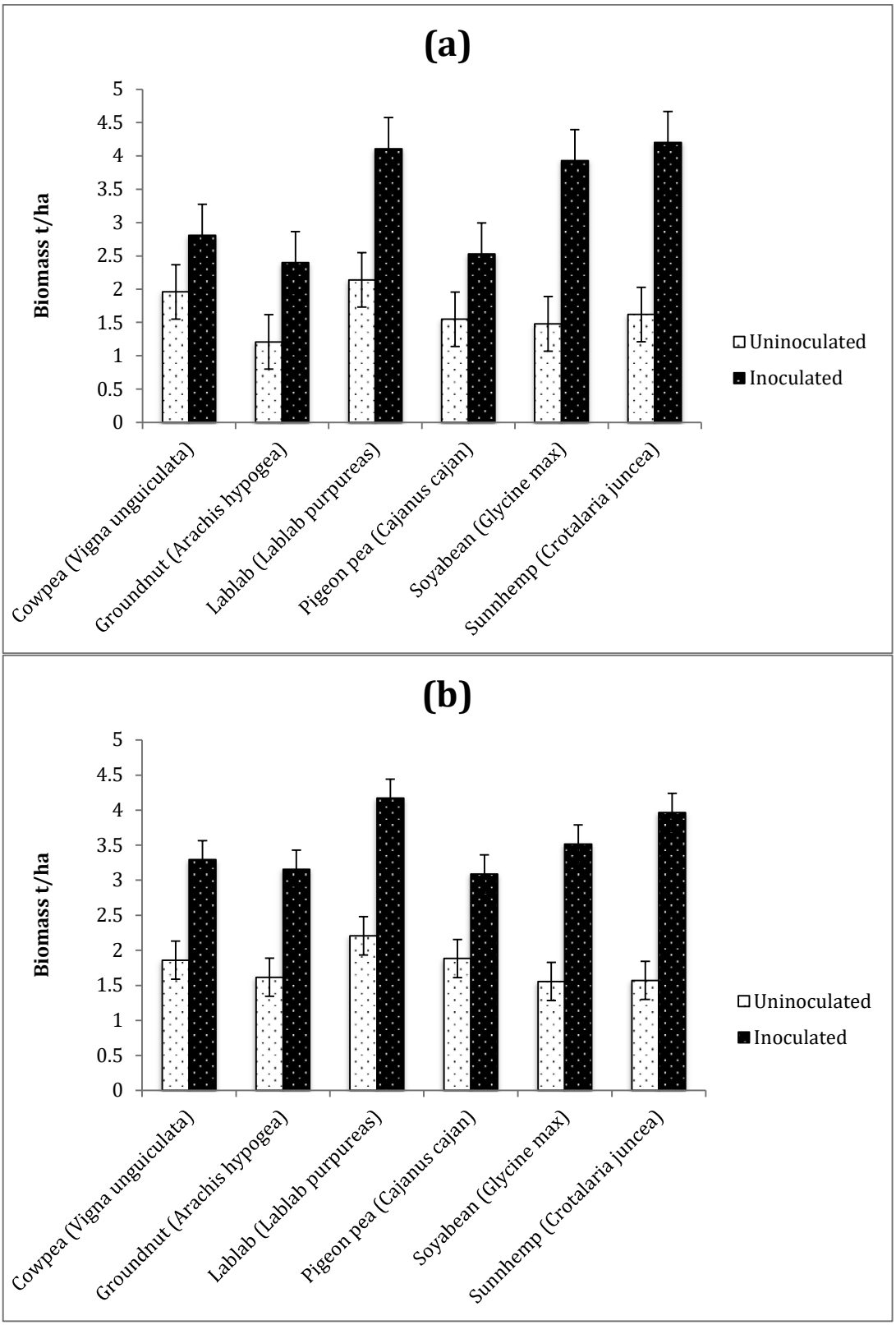


Figure 2.3 Biomass production of inoculated and uninoculated legumes at 8 WAP during (a) 2015/16 and (b) 2016/17 seasons at GRS. Inoculation was only carried out in 2015/16 season. Error bars represent LSD ($P<0.05$)

2.3.3 Biomass accumulated by maize

Biomass of maize accumulated following inoculated legumes was significantly higher than that of maize following uninoculated legumes ($p = 0.007$) (Figure 2.4). Maize after uninoculated legumes generated 496 kg ha^{-1} after groundnut and 988 kg ha^{-1} after lablab while that after inoculated legumes generated from 1.04 t ha^{-1} after groundnut to 1.38 t ha^{-1} after sunn hemp. Of the maize after uninoculated legumes, the biomass of maize after uninoculated lablab was the highest; and it was comparable to the lowest of the maize biomass after inoculated legumes - groundnut.

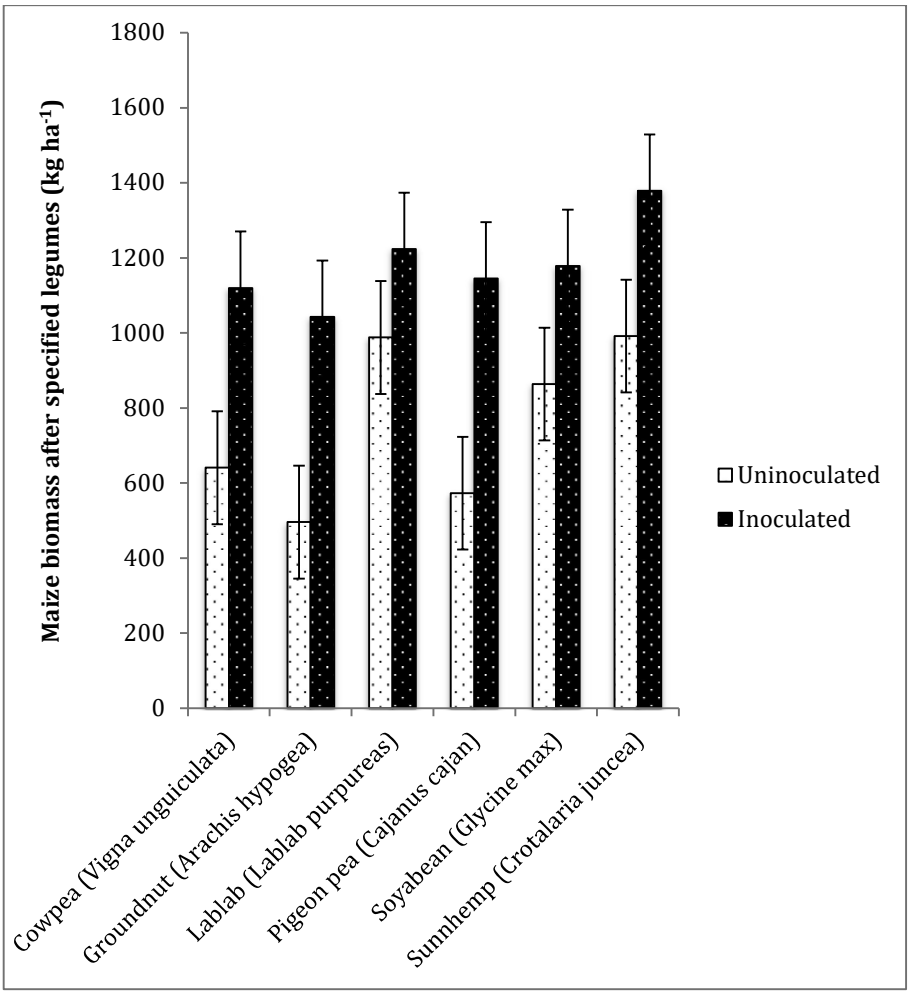


Figure 2.4 Maize biomass at GRS in 2016/17 season following different legumes grown with and without inoculation during 2015/16 season

2.3.4 Legume N accumulation over two seasons

In season 1, 2015/2016, the N accumulation was higher ($P<0.001$) where legumes were inoculated than where they were uninoculated. Legumes differed in their N accumulation ($p < 0.01$); groundnut and soybean had the lowest N accumulation of the uninoculated legumes, at 28 kg ha^{-1} and 30 kg ha^{-1} (Fig. 2.5a), respectively. Inoculating groundnut resulted in a 58% increase in N accumulation. Sunn hemp had the highest N accumulation (105 kg ha^{-1}) of all legumes when inoculated. Lablab and soybean N accumulation were not different from each other when inoculated ($P<0.05$).

Legume N accumulation under the uninoculated treatment in the second season ranged from 28 kg ha^{-1} to 59 kg ha^{-1} (Figure 2.5). There was a positive inoculation response for all legumes ($p < 0.001$) with respect to N accumulation with inoculated legumes yielding from 31 kg ha^{-1} to 105 kg ha^{-1}. The greatest N accumulation increase was generated following inoculation of sunn hemp while the lowest was generated by the uninoculated groundnut.

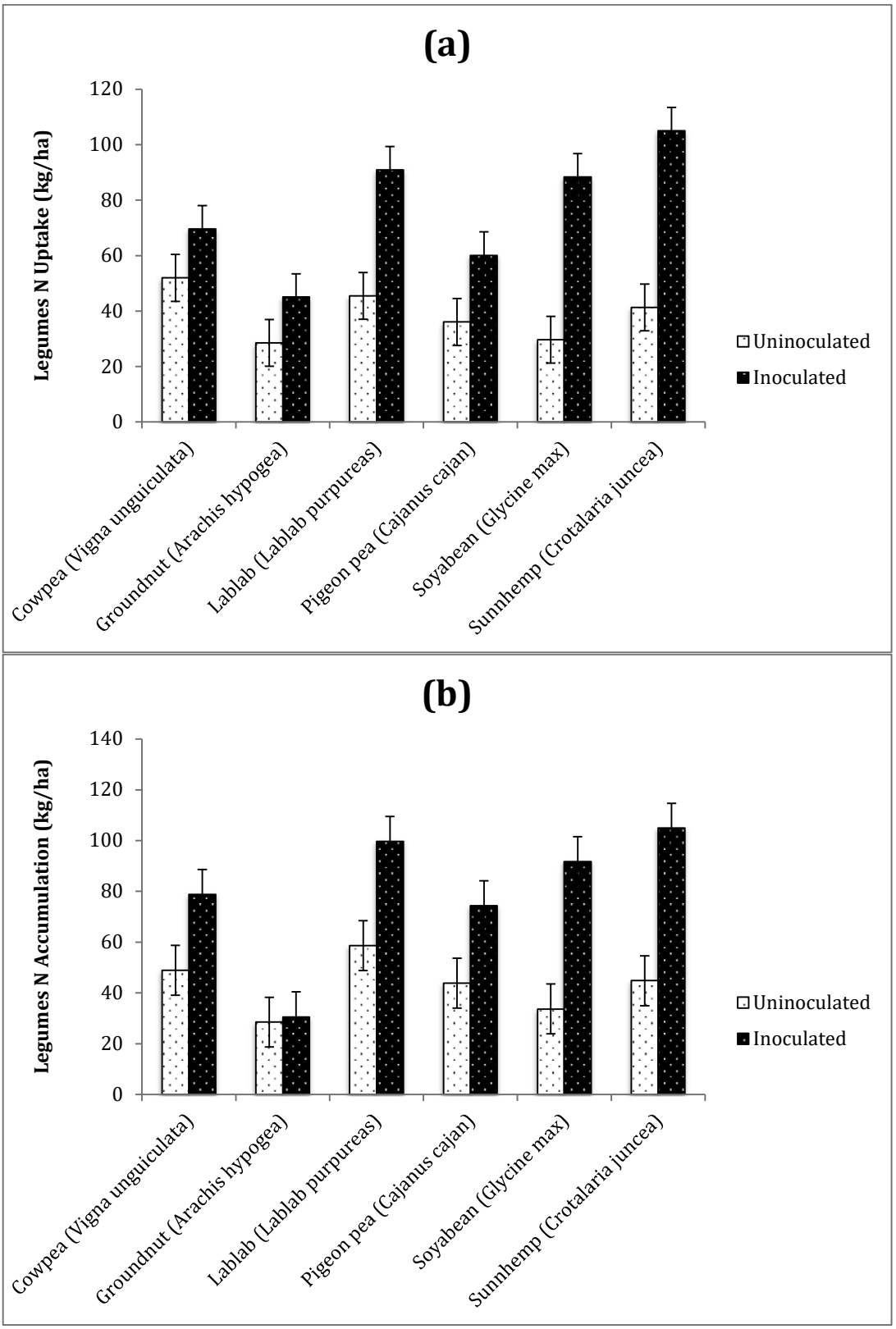


Figure 2.5 N accumulation of legumes at GRS with and without inoculation at 8 WAP during (a) 2015/16 and (b) 2016/17 seasons. In 2016/17 season, legumes were re-established without any inoculation .

2.3.5 Grain yield during season 1 (2015/16) season

The grain yield for the crops ranged from 0.71 t ha^{-1} for lablab to 1.73 t ha^{-1} for groundnut (Figure 2.6) under the uninoculated treatment. All crops registered a positive inoculation response for the grain yield, except for groundnut and pigeon pea that had no difference in grain yield between the two treatments ($p < 0.01$). Under the inoculated treatment, pigeon pea produced the lowest amount of grain, but this was not different from that of lablab at 1.02 and 1.07 t ha^{-1}, respectively ($P>0.05$). The highest grain yield was produced by soybean at 3.45 t ha^{-1}, which was 118 % greater than the respective uninoculated treatment. Grain yield was only recorded in season 1.

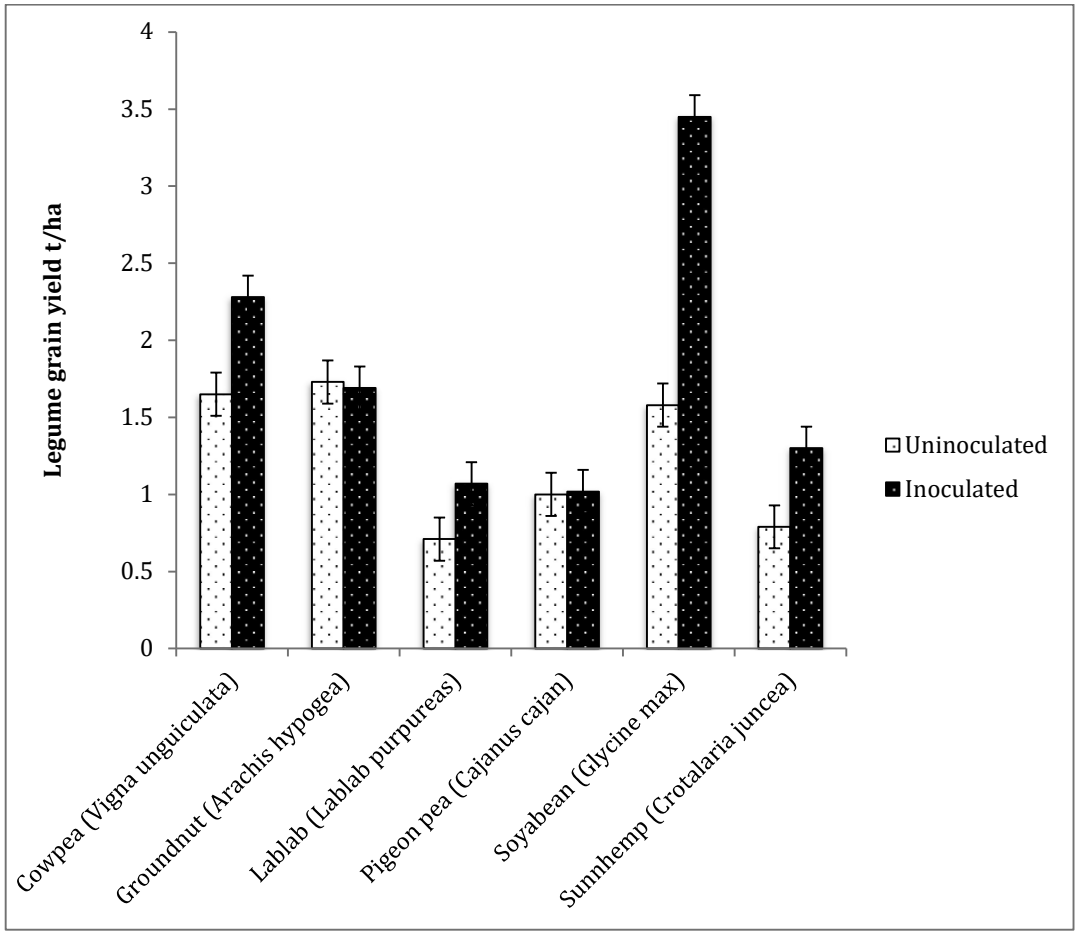


Figure 2.6 Legume grain yield at GRS year 1 during 2015/16 season

2.3.6 100 seed weight

The 100 seed weights of the legumes were not significantly different ($p < 0.01$) between inoculated and uninoculated legumes with the exception of soybean (Table 2.5). The 100 seed weight of the legumes was only recorded in season 1.

Table 2.5 100 seed weight of inoculated and uninoculated legumes

	100 SEED WEIGHT (g)	
CROP TYPE	INOCULATED (I)	UNINOCULATED (U)
Cowpea	13.82	13.1
Groundnut	57.3	56.38
Lablab	19.7	19.5
Pigeon pea	14.3	14.3
Soybean	18.25	16.78
Sunn hemp	4.38	4.3
LSD ($p<0.05$) TREATMENT (I/U)	0.88	
LSD ($p<0.05$) CROP	1.53	

2.4 Discussion

This chapter reports on the added value of using rhizobia inoculants, the variable contributions of legumes to maize-based rotations, and the need and potential for developing local adapted rhizobia inoculants.

2.4.1 Legume biomass, N accumulation, grain yields

The supply of appropriate rhizobia via inoculant to promote nodulation and nitrogen fixation generally supported higher biomass (Figure 2.3), N accumulation (Figure 2.5) and legume grain yields (Figure 2.6) for the legumes. Supplying rhizobia traditionally overcomes (i) low population numbers of the appropriate rhizobia in the soil (Evans et al., 1993) and (ii) compatibility failures between legume and

microsymbiont combinations in free nodulation scenarios (Thies et al., 1991). (Okogun et al., 2005) reported increased biomass production, N fixation, N and P uptake and grain yield following inoculation of soybean in Nigeria. Furthermore, inoculation with elite rhizobia strains commonly improves biomass, yields, and total nitrogen content even with crops traditionally considered to be promiscuous (Leite et al., 2018, Kyei-Boahen et al., 2017, Ulzen et al., 2016). All other legumes were nodulated by resident rhizobia in the current experiments, except for groundnut at GRS (Table 2.3). At GRS, where production parameters were measured, these legumes required inoculation to achieve maximum production. However, the usefulness of rhizobia is sometimes obscured by a failure to employ correct and effective ways of inoculation (Botha et al., 2004). As such, education on the benefits and the proper methods of inoculation is still required in smallholder communities, as the data indicate resident rhizobia are widespread in the study area, and not necessarily efficient at N fixation.

2.4.2 Rotational benefits

Improved productivity with inoculated legumes promoted higher maize biomass (Figure 2.4) in the plots succeeding these treatments, compared to those without inoculation. Maize biomass is a good indicator of potential yields (Mtambanengwe et al., 2007). Legume biomass is a high-quality source of soil organic matter which improves the efficiency of nutrient cycling and inorganic additions to soils (Kumwenda et al., 1996). Nitrogen accumulation of legumes is important because C: N ratio directly affects N mineralization. The simple linear relationship between legume N and maize biomass can be described as $y = 8.588\ x + 4.66.1$ ($r2 = 0.62$, $P<0.01$), where y is maize biomass and x is legume N (both in kg/ha) (Figure 2.4). Translation of inoculation benefits, including the increase of soil nitrogen accrued by legumes and benefiting the subsequent maize has been reported previously (Kasasa et

al., 1999, Zingore and Giller, 2012). It is for this reason that sustainable farming systems models recommend incorporation of legumes (Rusinamhodzi et al., 2006, Ojiem et al., 2014, Rusinamhodzi et al., 2012, Chikowo et al., 2007).

2.4.3 Promotion of rhizobia populations in soils

The presence of a legume in soil itself promotes the populations of nodulating rhizobia (Zengeni et al., 2006, Ulzen et al., 2018b). While populations fall in the dry season, they are reactivated by improved soil moisture when it rains, and more-so when the crop is repeated (Zengeni et al., 2003). High soil moisture may also contribute to the higher productivity. These reasons explain the maintenance of higher productivity of the inoculated plots in the second season where there was no re-inoculation, compared to the twice-non-inoculated plots. While re-inoculation may not be critical in the second season (Ulzen et al., 2018b), it is often advisable to re-inoculate as insurance for maximum nodulation and nitrogen fixation (Mendes et al., 2004, Hungria and Mendes, 2015a). It is considered a small price to pay relative to the potential loss of biomass and grain yield. Re-inoculation also protects against the development of ineffective background rhizobia, promoted by the presence of the legume in the field, as was the case with Biserrula ineffective rhizobia strains in Australia (Nandasena et al., 2007). However, the data from GRS suggest that with further attention to selection of strains that are both well adapted to the soil, and to the target legumes, the cost of re-inoculation every year might be avoided, without a high risk of compromised production. This may well be an appropriate research target to lower the cost of production of legumes for small holder farmers.

2.4.4 Appropriate indigenous rhizobia and development of new adapted inoculant strains

All six crops were nodulated in at least one location in the multi-site experiments without inoculation in the final season of the present study, with high

variability across sites. Lablab originated in sub-Saharan Africa, (Maass et al., 2010) and this may explain the good nodulation reported with lablab in this study.

The rainfall amounts across the sites, in different natural regions, were very variable, with MRS recording the lowest rainfall as expected (Figures 2.1 and 2.2). Despite the promiscuity of groundnut, and high rainfall amounts at GRS (Figure 2.1), groundnut was not nodulated at the GRS location tested (Table 2.1), with similar pH to the MRS site (Table 2.2) where nodulation was recorded. Only Panmure had ideal soil pH for nodulation. It is likely that groundnut or any other legume in the same cross-inoculation group has not grown in this location previously and hence compatible rhizobia are lacking. Soybean, usually considered a specifically nodulating crop, nodulated quite widely, as has been the case in other Zimbabwean studies (Musiyiwa et al., 2005b).

Characterization of the indigenous rhizobia responsible for all the nodulation outcomes will contribute to the body of knowledge on African root nodule bacteria. Their evaluation for usefulness as inoculant strains is a high priority considering the urgency for low-cost soil improvement (Musiyiwa et al., 2005b). The findings of (Grönemeyer et al., 2014b) indicated quite specific ecological adaptations of rhizobia, with evidence of adaptation to acidic soils, subhumid ecology, arid ecology and high temperatures. The background rhizobia detected in this study are a resource for potential development of inoculants, as they are adapted to their native locations.

2.4.5 General discussion

Individual crops vary in their genetic/inherent capacity for biomass generation, nitrogen fixation, grain yield and other characteristics (Giller, 2001). Crops with high biomass yields have the potential for application in green manure/animal feed. This leads to improvement of soil fertility for better maize and whole system farming.

2.5 Conclusions

Results from our study show the benefit of growing a legume consecutively on the same land. Inoculated legumes were superior to uninoculated legumes in biomass and N accumulation in the first year and even without inoculation in the second year. Groundnut, pigeon pea and cowpea still require further research to develop and select cultivar and rhizobia strains for higher SNF benefits. Resident rhizobia that nodulated these legumes in uninoculated treatments present a resource for evaluation of well-adapted strains, however there are concerns about their N fixation efficiency given that yields in uninoculated treatments were less than in inoculated treatments and that maize biomass was higher when following inoculated legumes than following uninoculated legumes. Until further data is acquired, annual inoculation to repeat the remarkable performance of the first year legumes is recommended, although the potential for relying on inoculant survival through seasons is acknowledged.

CHAPTER 3

Genetic diversity of soybean root nodule bacteria in Zimbabwe

3.1 Introduction

Soybean, an exotic crop introduced to Zimbabwe in 1906 (Shurtleff and Aoyagi, 2009), is an important cash crop for both smallholder and commercial farming in the agriculture-led economy of Zimbabwe (Giller et al., 2011). In Zimbabwe, smallholder subsistence farms are typically managed with minimal external inputs of machinery, seeds, fertilizers and chemicals due to resource constraints. In contrast, commercial farms maximize yields with high external inputs, following research recommendations for pH and soil fertility management (Mpepereki et al., 2000). As a result, the two farming systems may be expected to differ in their fertility profiles, responses to different agricultural practices and effects on biological/biotic parameters of the farming systems. Soybean production is mostly carried out in agro-ecological region (AER) 2, the second-highest crop production potential region of Zimbabwe's five regions, which receives between 750 and 1000 mm of rainfall annually (Vincent et al., 1960), in a unimodal rainy season that begins in November and ends in March (Mugandani et al., 2012). Because they span from November of one year to March of the next, rainfall seasons are therefore defined by year 1/year 2. Dominant soils in this region are Cambisols, Luvisols and Arenosols with pH ranges of 4 to 5 (FAO, 2006).

The N requirements for soybean production may be met by either inorganic N fertilizer or effective exploitation of symbiotic N fixation (SNF) via the legume-rhizobia symbiosis. Inorganic nitrogen fertilizer is more expensive and maybe beyond the reach of smallholder farmers, making rhizobia even more critical for soybean production in the country. In its native homeland of China, soybean is nodulated and fixes nitrogen with root nodule bacteria (RNB), collectively known as rhizobia, belonging to either the genus *Bradyrhizobium* or *Ensifer*, depending on soil and environmental factors (Han et al., 2009).

At the time of introduction of soybean to Zimbabwe, rhizobia compatible with soybean were not expected to be present in the soils, and it was recommended that introduction of

soybean be accompanied by inoculation with compatible rhizobia strains (Sprl, 1958). Soybean crops responded positively to inoculation, as demonstrated by improved figures for nodulation, nitrogen fixation, growth and grain yields. However, nodulation was also observed in the uninoculated experimental plots, confirming the presence of compatible, indigenous rhizobia (Sandmann, 1970). Several studies have described the diversity of indigenous soybean nodulating rhizobia with respect to phenotype and physiology (Musiyiwa et al., 2005a, Davis and Mpepereki, 1995, Mpepereki et al., 1996b). This is an important resource to understand because it represents a potential source of competition against introduced elite rhizobia inoculants; and at the same time a source for new elite adapted rhizobia inoculant strains.

The taxonomy and phylogeny of the Zimbabwean root nodule bacteria is as yet unknown, a research gap that limits (i) the country's ability to improve the management and application of soybean inoculant programs, as well as (ii) contribute to the rapidly growing research repository on rhizobia taxonomy and phylogeny and their link to the effective exploitation of soybean SNF.

While Zimbabwean indigenous rhizobia populations can nodulate soybean, nodulation is inconsistent and the nitrogen fixation efficiency highly variable (Musiyiwa et al., 2005a, Musiyiwa et al., 2005b, Zengeni and Giller, 2007), necessitating the continued use of rhizobia inoculant. As such, inoculation remains a conventional and important element of Zimbabwe's soybean production under both commercial and smallholder agriculture (Mpepereki et al., 2000, Giller et al., 2011). The current recommended inoculant strain used in Zimbabwe, MAR1491, is highly efficient in N fixation with soybean (Delamuta et al., 2013). There have been reports of poor survival of this strain in Zimbabwean soils, however, the extent is unknown because the studies did not characterize these rhizobia beyond their ability to form nodules with soybean and did not identify them according to molecular methods (Zengeni et

al., 2003, Zengeni et al., 2006). Persistence and stability of inoculant strains after introduction into soils is critical for sustained agriculture performance (Brunel et al., 1988).

The present study was designed to determine the diversity of soybean root nodule bacteria in Zimbabwean soils and to characterize them using a polyphasic approach. Soils that had last been inoculated five years prior to this study were selected in order to assess the persistence of the inoculant strain. Samples were taken from both commercial and smallholder farms. Species designation of isolates was determined using molecular means, and the phylogeny determined. Nodulation capacity across a range of legumes is described.

3.2 Materials and Methods

3.2.1 Site selection and sampling

Nodules and soil samples were collected in March 2012 just before the end of the 2011/2012 agriculture season from sites that were last inoculated at least five years prior to sampling. The rhizobia inoculant that had been used at all sites was prepared in bagasse (the waste material from sugar cane after sugar extraction), carrying 10^9 CFU per g, and applied at 100 g per ha of soybean. Samples were collected from AER 2 at 15 sampling sites that included 12 smallholder and three commercial farms, in different districts of the provinces Harare, Mashonaland East, Mashonaland West and Mashonaland Central (Figure 3.1). Site characterization is detailed in Table 3.1.

Nodules for rhizobia isolation were obtained differently from smallholder farmer fields and commercial farms. Random nodule samples from a transect of each smallholder farmer's field were obtained directly from mature plants at R6 stage of development. Nodules were maintained under cool conditions, washed and transferred to silica gel vials until they were used for RNB isolation (Howieson and Dilworth, 2016).

Soil samples from commercial soybean farms were used to isolate rhizobia via host-trapping under glasshouse conditions. At each site, ten soil subsamples were taken to create a

composite sample, which was then used for trapping. Two composite samples were taken at each location, but only one was used from HRS and PRF, while both samples were used from RARS where soil types were contrasting. Samples were stored at 4^{o} C for three months and then transferred to Murdoch University (Perth, Western Australia), where trapping was carried out under glasshouse conditions in three-litre pots according to the methods of Howieson and colleagues (Howieson and Dilworth, 2016). Briefly, pots were ¾ filled with a sterile sand mix, then holes were made in four positions around the pot and 20 ml of soil from the soybean commercial farms was placed into each hole, two centimetres below the surface. Soybean seeds were sorted for uniform size, surface sterilized, pre-germinated then planted above the trap soils, before being covered with the sterile sand mix. Different varieties of soybean, viz Bimha, PAN 981), Squire and Status, were sown to improve the diversity of rhizobia isolated. For each pot, two plants were removed after two weeks, to maintain two even-sized, healthy plants. Triplicate sets of pots were set up per treatment and maintained for six weeks, and irrigated with nitrogen-free nutrient solution (Howieson and Dilworth, 2016). Whole plants were then harvested at flowering and nodules recovered, followed by immediate isolation of the rhizobia or storage in silica gel vials.

3.2.2 Isolation of root nodule bacteria and authentication

Root nodule bacteria (RNB) isolation was carried out under aseptic techniques in the laboratory, using nodules from the smallholder farmers' fields and from the commercial farms. Nodules were surface-sterilized (Howieson and Dilworth, 2016) then transferred to an empty, sterile Petri dish and crushed in a drop of sterile water. The suspension was streaked onto half lupin agar (½LA) plates [1.25 gL^{-1} Yeast extract; 26.7 mM D-mannitol; 27.7 mM D-glucose; 3.24 mM $MgSO_4.7H_2O$; 1.71 mM NaCl; 180 µM $FeSO_4.7H_2O$; 100 mM K_2HPO_4; 100 mM KH_2PO_4; 11.6 µM $Na_2B_4O_7$; 9.1 µM $MnSO_4.4H_2O$; 760 nM $ZnSO_4.7H_2O$; 320 nM $CuSO_4.5H_2O$; 520 nM $Na_2MoO_4.2H_2O$; 0.15% agar (Howieson et al., 1988)] using dilution

streaking (Somasegaran and Hoben, 1994). Plates were incubated at 28ºC and monitored for 14 days. Putative RNB were selected based on colony appearance and growth rate and these were then dilution streaked to achieve single colonies on yeast extract mannitol agar (YMA) [0.5gL^{-1} Yeast extract; 26.7 mM D-mannitol; 0.81 mM $MgSO_4.7H_2O$; 1.71 mM NaCl; 312.5 mM K_2HPO_4; 0.15% agar (Howieson and Dilworth, 2016)]. Single colonies were incubated then authenticated by inoculation onto soybean cultivar Bimha growing in pots under axenic conditions. Nodulation, leaf color and general plant vigor were observed. Authenticated isolates (i.e. those that nodulated soybean) were used for subsequent work.

Pure cultures of authenticated isolates were maintained on YMA slants at 4º C for routine use, and stored at -80º C as glycerol stocks containing 15% (v/v) glycerol in 0.89% (w/v) saline for long-term storage. Strains were deposited in the Murdoch University WSM Culture Collection, Perth, Western Australia and the Grasslands MARCulture Collection, Marondera Zimbabwe.

3.2.3 Morphological characteristics

Isolates were plated on YMA and incubated at 28ºC for seven days for characterization and comparison. Colony morphology for single colonies was observed under the light microscope and isolates were grouped by similarity of morphology according to shape, color and size. Isolates were also observed for pH reaction on YMA plates supplemented with bromothymol blue (BTB) at a concentration of 25 ppm (Howieson and Dilworth, 2016).

3.2.4 PCR amplification and sequencing

Four-day-old broth cultures obtained by inoculation with single colonies were centrifuged for 2 minutes at 3500 rpm to concentrate whole cells. The supernatant was discarded. The pellet was resuspended in fresh sterile saline solution and the process repeated for a total of three centrifugations. The final pellet was resuspended in with PCR grade water

standardized to an optical density (OD_{600}) of 6.0. Cell suspensions were maintained in -20ºC (Gerding et al., 2013).

Initially, partial amplification and sequencing of the genes **16S rRNA**, *recA*, *rpoB*, *glnII* and nifH were undertaken for 20 individuals using whole cells for DNA templates (Gerding et al., 2013) with primers detailed in Table 3.2.

As preliminary results indicated that *Bradyrhizobium recA* partial gene sequences were the most phylogenetically informative, amplification and partial sequencing of the *recA* gene was performed on all 137 authenticated isolates, using previously described primers and protocols (Vinuesa et al., 2005). The sequence reads were edited manually and assembled with Geneious software version 7 (Biomatters Ltd, NZ). Isolates were grouped with the closest related bacterial species identified using BLASTN on the National Centre for Biotechnology Information Genbank database (Altschul et al., 1990).

Further PCR amplification and sequencing work was carried out with representatives of the *recA* gene phylogenetic lineages obtained. Nearly full-length amplification and sequencing for the 16S rDNA was achieved with universal 16S rDNA primers and two internal primers (Weisburg et al., 1991, Yanagi and Yamasato, 1993). Partial amplification and sequencing of the *recA* and *glnII* housekeeping gene sequences, as well as symbiotic genes *nifH* and *nodC* were determined using previously described protocols (Table 3.2) (Vinuesa et al., 2005, Laguerre et al., 2001).

3.2.5 Phylogenetic analyses

The assembled gene sequences were imported into MEGA version 7 along with the gene sequences of other *Bradyrhizobium* type strains. For phylogenetic reconstruction, gene sequences were aligned using the MUSCLE program within MEGA. Neighbor-joining (NJ) (Saitou and Nei, 1987) and maximum likelihood (ML) (Tamura et al., 2011, Felsenstein, 1981) phylogenetic trees of the aligned and trimmed sequences were constructed using the best-fit

models determined within MEGA 7 for each analysis. The NJ trees were bootstrapped by 500 replications and the ML trees by 1000 replications.

3.2.6 Evaluation of host range

Single representatives of the *recA* gene phylogenetic lineages obtained in section 2.5. viz *B. diazoefficiens*, *B. elkanii*, *B. japonicum* and *B. ottawaense* were tested for their ability to nodulate and fix nitrogen with 13 legume species namely sandhill wattle (*Acacia ligulata*), Adzuki bean (*Vigna angularis*), cowpea, mung bean (*Vigna radiata*), pigeon pea, *sunn hemp, common bean,* lupin *(Lupinus angustifolius*), peas, faba bean (*Vicia faba*), groundnut, seradella *(Ornithopus compressus*) and their original trap-host soybean. An uninoculated control was included, and the experiment was performed under glasshouse conditions as previously described (Da Silva et al., 2014, Howieson et al., 2013), using a 4-replicate, split-plot design (Howieson and Dilworth, 2016, Howieson et al., 2011). Nodules were dissected and observed for internal colors (Da Silva et al., 2014).

3.2.7 Antibiotic resistance

Two representatives, each of the *recA* gene phylogenetic lineages obtained in section 2.5. were tested for their intrinsic antibiotic resistance on YMA agar supplemented with filter-sterilized solutions of five antibiotics at three concentrations. Triplicate plates were prepared for chloramphenicol (10, 20, 40 μg ml^{-1}), kanamycin (10, 20, 30 μg ml^{-1}), rifampicin (5, 10, 20 μg ml^{-1}), tetracycline (10, 20, 30 μg ml^{-1}) and streptomycin (50, 75, 100 μg ml^{-1}). Representatives of the rhizobia clades identified by *recA* sequences were inoculated into 3 ml of YMA broth and grown for three days at 28 ºC and 200 rpm to the exponential growth phase. Aliquots (3 μl) were then drop-plated onto the Petri dishes containing antibiotics and subsequent growth observed for seven days at 28 ºC (Yates et al., 2004).

3.3 Results

3.3.1 Recovery of rhizobia from Zimbabwean soils

Two hundred and fifteen nodule isolates were obtained from the smallholder farmers' fields and soybean breeding facilities in Zimbabwe. Authentication yielded 11 to 33 RNB per district, with a total of 137 RNB (Table 3.3). The authenticated RNB isolates varied in the effectiveness of N fixation on soybean, as evidenced by the leaf color of the nodulated plants, which ranged from pale yellow to rich green.

3.3.2 Colony Morphology and Growth Characteristics

All authenticated isolates were slow-growing, with colonies visible after five to seven days of incubation on YMA at 28ºC. All isolates were Gram-negative rods and alkalinized BTB-containing YMA, causing blue coloration around colonies. In general, colonies were circular and dome-shaped with an entire margin. The isolates differed in colony size, from small colonies of about 1 mm to large colonies of about 4 mm, after seven days of growth. Most isolates produced copious amounts of mucus. The isolates formed seven different colony morphology groups (Table 3.4).

3.3.3 Classification of all isolates based on *recA* gene sequence

Preliminary genetic analysis of all 137 isolates, using a sequenced 339 base-long internal portion of the *recA* gene, resolved the isolates into four distinct clades, with closest identities to *Bradyrhizobium diazoefficiens, B. elkanii, B. japonicum* and *B. ottawaense* type strains (Figure 3. 2). The four separate *recA* clades consisted of 83 *B. elkanii*-like isolates that were split into two smaller sub-clades of 48 and 35 isolates; 29 *B. japonicum*-like isolates that were split into three groups of 17, 8 and four isolates; 18 *B. diazoefficiens*-like isolates, which all had identical *recA* partial gene sequences; and seven *B. ottawaense*-like isolates, also with identical *recA* partial gene sequences (Figure 3.2). The four *B. japonicum* isolates NAZ553,

NAZ554, NAZ535 and NAZ755 were identical and had 99.41% sequence identity with *B. japonicum* USDA6^T *recA* gene; the eight strains NAZ543, NAZ706, NAZ708, NAZ710, NAZ711, NAZ715, NAZ728 and NAZ712 were identical and had 100% sequence similarity to type strain USDA6^T *recA* gene. The remaining 17 *B. japonicum*-like strains had a 99.71% sequence similarity with the type strain's *recA* gene. In the *B. elkanii* group, the smaller clade of 36 consisted of isolates whose sequences were identical and had 99.71% similarity with the type strain USDA76^T *recA* gene. The larger clade of 48 isolates had 100% sequence similarity with the type strain USDA76^T *recA* gene. All isolates and their sites of origin are shown in Table 3.5.

All *B. diazoefficiens* isolates were recovered from smallholder farm sites (nodules collected *in-situ*), and none were recovered from the research facilities (host trapping). One isolate was recovered from Chinhoyi and the rest from Marondera. The most widely distributed species was *B. elkanii,* which was recovered from all districts under both smallholder and research sites. *B. elkanii* was most abundant with totals of 36 and 46 respectively, under research and smallholder sites. The second most widely distributed was *B. japonicum* which was found everywhere except for Bindura, and with an aggregate 11 strains under smallholder conditions and 16 under commercial farm conditions. Isolates of *B. ottawaense* origin were only found in a total of three sites; Pannar research facility, Rattray Arnold research facility and Chinhoyi communal areas.

3.3.4 Phylogeny based on 16S rDNA, *recA* and *glnII* genes

Sequence analysis based on the core genes 16S rDNA, *recA* and *glnII,* allowed phylogenetic analysis of isolates within the *Bradyrhizobium* genus. Nearly full-length sequences (1342 to 1422 bp long) of the 16S rRNA gene of representative isolates were generated and deposited in NCBI (Table 3.6). Each sequence presented 100% sequence similarity to that of its respective type strain, *B. diazoefficiens*, *B. japonicum*, *B. elkanii* or *B.*

ottawaense. Phylogenetic reconstruction (Figure 3.3) placed the *B. elkanii* strains NAZ584 and NAZ521 within the *B. elkanii* superclade, while the *B. diazoefficiens, B. japonicum* and *B. ottawaense* isolates fell within the *B. japonicum* superclade, consistent with the findings of other researchers (Zilli et al., 2014, Da Silva et al., 2014, Yu et al., 2014, Vinuesa, 2005). Phylogenetic reconstruction based on 375 bp of *recA* and 520 bp of *glnII* genes (Figures 3.4 and 3.5) gave similar topologies, with higher bootstrap support in the *glnII* phylogenetic tree (Figure 3.5) than in the *recA* phylogenetic tree (Figure 3.4). In general, the topologies of the 16S rRNA, *recA* and *glnII* gene phylogenetic trees were congruent.

3.3.5 Analysis of the symbiosis genes

The symbiosis gene topologies differed from those generated from 16S rRNA, *recA* and *glnII*. There were still two large clades within the *Bradyrhizobium* genus; however, all our strains fell in one clade. The 648 bp long *nifH* gene sequence was identical for the Zimbabwean isolates affiliated with *B. diazoefficiens*, *B. japonicum* and *B. ottawaense,* along with *nifH* of the type strains for these three species and that of the type strains of *B. lupini*, *B. liaoningense*, and *B. huanghuaihaiense* (Figure 3.6). The three were all placed together in the phylogenetic tree, at a bootstrap value of 99% along with *B. daqingense,* while the Zimbabwean *B. elkanii* isolates and the *B. elkanii* type strain fell into a separate cluster. Similarly, over the 441 bases of the *nodC* sequences, the species *B. diazoefficiens* and *B. japonicum* were identical (Figure 3.7). Similar to the *nifH* gene tree topology, but in contrast to the housekeeping genes, the *B. elkanii* strains were found in the same cluster but further from the rest.

3.3.6 Phenotypic analyses

3.3.6.1 Evaluation of host range

The *B. japonicum* isolate (NAZ505) failed to elicit nodules on either *sunn hemp*, mung bean or common bean (Table 3.7). The *B. ottawaense* isolate (NAZ732) formed nodules with the three legumes sunn hemp, mung bean and common bean, but only those with sunn hemp and mung bean were effective. The *B. elkanii* (NAZ661) isolate formed an effective symbiosis with sunn hemp, non-fixing nodules with common bean and failed to nodulate mung bean. The *B. diazoefficiens* isolate (NAZ641) failed to form nodules with sunn hemp and common bean and only formed ineffective nodules on mung bean. Sunn hemp nodules were coralloid, while those of sandhill wattle were elongate and the rest were globose. All four species nodulated and fixed nitrogen with sandhill wattle, adzuki bean, pigeon pea, cowpeaand the control, soybean. None formed nodules with lupin, peas, faba bean, groundnut and seradella.

3.3.6.2 Antibiotic resistance

When challenged by the antibiotics chloramphenicol, tetracycline, kanamycin, rifampicin and streptomycin, growth patterns varied from no growth to good growth and varied even within the same species (Table 3.8). Isolates of *B. diazoefficiens* origin had the highest sensitivity to antibiotics. Both isolates NAZ634 and NAZ692 did not grow under kanamycin and streptomycin at the lowest concentrations tested. Isolates belonging to *B. ottawaense* species also completely failed to grow under streptomycin. The *B. elkanii*-like isolates displayed the greatest tolerance to antibiotics, with both NAZ501 and NAZ578 growing under all antibiotic test conditions. Chloramphenicol at 10 ug/ul was the most tolerated but sensitivity was higher at double and triple the concentration. Tetracycline was the least tolerated antibiotic.

3.4 Discussion

All isolates in this study showed characteristics consistent with the phenotypic designation of bradyrhizobia as being Gram-negative, slow-growing bacteria that produce an alkaline reaction with mannitol as carbon source (Mpepereki et al., 1996b, Chen et al., 2000) and supports previous characterization of Zimbabwean isolates as diverse bradyrhizobia (Musiyiwa et al., 2005a). However, classification of rhizobia based on colony morphology and phenotype appears inconsistent and therefore ineffectual for specific taxonomic determination. All the *B. diazoefficiens*, *B. ottawaense* and some of the *B. japonicum* isolates were similar in colony morphology (Table 3.4). The elite *B. diazoefficiens* could not be distinguished from other species according to colony morphology.

Wide diversity has been reported for soybean root nodule bacteria in Zimbabwe previously, but without the species designation (Musiyiwa et al., 2005a, Davis and Mpepereki, 1995, Mpepereki and Makonese, 1995). Partial sequencing of housekeeping genes allowed us to determine the taxonomic position of all our isolates. Sequencing of *recA* gene has been found to produce accurate results in determining the taxonomic position of rhizobia to species level (De Meyer et al., 2018, Kämpfer and Glaeser, 2012, Glaeser and Kämpfer, 2015). Partial sequencing of the *recA* gene confimed that all strains belong to *Bradyrhizobium*, and revealed a diverse population of rhizobia from Zimbabwean soils, across at least four *Bradyrhizobium* species, namely, *B. diazoefficiens, B. elkanii, B. japonicum* and *B. ottawaense* that are able to nodulate with soybean. These species have all been shown to nodulate with soybean previously (Jordan, 1982, Yu et al., 2014, Kuykendall et al., 1992, Delamuta et al., 2013). This is the first study to detail the species designation of soybean root nodule bacteria in Zimbabwe. Other studies across Africa have also indicated the presence of effective *Bradyrhizobium* microymbionts among genera nodulating soybean (Aserse et al., 2012a, Abaidoo et al., 2007, Chibeba et al., 2017).

The 16S rDNA and two housekeeping and two symbiosis loci were used to estimate the phylogenetic signal of the Zimbabwean soybean root nodule lineages determined in this study using the Maximum likelihood approach. Representative isolates of the four species of Zimbabwean bradyrhizobia able to nodulate soybean were selected for the phylogenetic and phenotypic characterization. Multi-locus sequence analysis approach has been proven useful in determining taxonomy and phylogeny, particularly for the bradyrhizobia where the 16S rDNA sequences alone often do not carry sufficient polymorphisms for this determination (Vinuesa et al., 2008a, Menna, 2009). There was congruence between the 16S rDNA gene phylogenies and the housekeeping gene phylogenies (Gaunt and Gaunt, 2001). *B. diazoefficiens*, *B. japonicum* and *B. ottawaense*, belong to one *Bradyrhizobium* superclade while the *B. elkanii* belongs to the *B. elkanii* superclade (Vinuesa et al., 2008a).

While the *nodC* and *nifH* gene phylogenies were congruent to each other, they displayed a different phylogeny from that of the core genes, suggesting monophyly for *B. diazoefficiens*, *B. japonicum* and *B. ottawaense*, separate from the *B. elkanii.* This is again consistent with *Bradyrhizobium* comprising two superclades, defined as the *B. elkanii* superclade and the *B. japonicum* superclade (Vinuesa et al., 2008a, Yu et al., 2014). These findings support the assertion that core genes within *Bradyrhizobium* have been transmitted via vertical descent while the *sym* loci were later acquired by horizontal gene transfer (HGT) (Vinuesa et al., 2005, Moulin et al., 2004). These results confirm that the *B. elkanii* in Zimbabwe have not acquired symbiosis genes by HGT from the introduced inoculant strain. The possibility that *B. japonicum* and *B ottawaense* in Zimbabwean soils have obtained their symbiosis genes from the *B. diazoefficiens* inoculant strain, MAR1491, cannot be ruled out. This description of Zimbabwean soybean root nodule bacteria, to this detail, is important in revealing the biogeography of these species and contributes to the discussion about historical, environmental and ecological influences on the species distributions (Vinuesa et al., 2008a).

The species *B. ottawaense* has been isolated from soybean growing in Canada, where soils and climate contrast with Zimbabwean conditions (Yu et al., 2014). It has also been recovered in Brazil and Ethiopia, which have soil and climatic conditions that are more similar to Zimbabwean conditions (Menna, 2009, Aserse et al., 2012a). This species demonstrates an interesting biogeographic adaptation by transcending soil and climate categories and would be a worthwhile candidate for studying biogeographic adaptation.

Results showed only 13% recovery of *B. diazoefficiens,* to which the rhizobia inoculant strain MAR1491 belongs, despite years of continual inoculation (Table 3.4; Table 3.5). In contrast, there was higher recovery of *B. elkanii* at 61%, and *B. japonicum,*at 21% of the total number of isolates recovered in this study (Table 3.4; Table 3.5). Low recovery of any rhizobia at all in subsequent years after inoculation has been reported following inoculation with MAR1491 in Zimbabwean soils (Zengeni et al., 2003, Zengeni et al., 2006). Other introduced legume-rhizobia symbioses have also reported similar low % recovery of inoculant strain (Gerding et al., 2013, Ham et al., 1971) while others suggest that re-inoculation of soybean in the following year after inoculation is unnecessary (Brunel et al., 1988, Ulzen et al., 2018b). The failure of an inoculant strain to persist in a field may be due to biotic and abiotic challenges in the local environment or failure to enter nodules due to competition from indigenous rhizobia (Thies et al., 1991, Thies et al., 1992, Zengeni et al., 2006).

In contrast, native rhizobia are expected to be well adapted to the native conditions; which, in Africa, often include high temperatures, soil acidity and drought (Zahran, 1999). In the present study, the results suggest that native *B. elkanii* are competitive against the other species. Interestingly, *B. diazoefficiens* was not recovered at all from commercial farm sites in our study. While MAR1491 is associated with significant improvements in crop yield (Chirinda et al., 2003, Kasasa et al., 1999), the failure to persist in the soils means farmers may have to contend with frequent inoculation; or research must supply new candidates with

improved survival rates in Zimbabwean soils. It is important to note that different sampling approaches for rhizobia in soils, such as directly from field, potted soil, or Leonard jars at different soil dilutions affect the composition of the population recovered (Alberton et al., 2006). Therefore, only limited inferences can be made concerning how the rhizobia populations differ between smallholder farmers' fields and commercial farmers fields. In addition, there is the possibility that the field at Chikonyora farm, Marondera, was inoculated in the year of sampling due to the elevated recovery of the *B. diazoefficiens*. Verbal agreement suggested that there had not been inoculation recently, but the results suggest otherwise. A long-term study with interval monitoring of the inoculant and indigenous rhizobia dynamics would provide some useful information.

Nodulation (*nod* genes) and nitrogen fixation (*nif* genes) genes are responsible for the molecular signaling in the rhizobia to determine matching of symbiotic partners and facilitating nitrogen fixation. They can be acquired by HGT, thereby changing the host range of the bacterial species. The capacity of the different indigenous strains in Zimbabwe to nodulate soybean could arise from three options. These strains could carry a suite of symbiosis genes that naturally give them access to soybean because many rhizobia nodulate a wide range of hosts and many legumes are nodulated by a wide range of rhizobia (Laguerre et al., 2001). Rhizobia may have been brought on soybean seed by accident, as the case with lupins and serradella strains in Australia (Stepkowski, 2005). A third alternative is the acquisition of symbiosis genes carried by the inoculant strain MAR1491 by HGT in the soils, as has happened in the cerrados of Brazil (Barcellos et al., 2007). This is particularly plausible for the *B. japonicum* and *B. ottawaense* strains that share 100% sequence similarity of the sections of symbiosis genes evaluated, with the inoculant strain.

The *B. elkanii* isolates were revealed to have higher intrinsic antibiotic resistance while *B. diazoefficiens* had the least. Soil antibiotics are secreted by a range of soil microorganisms,

including the rhizobia themselves, as an antagonist strategy against closely related strains and species (Sessitsch et al., 2002), and antibiotic resistance, a selection pressure over many seasons (Brockman and Bezdicek, 1989), allows strains and species to persist and thrive under these conditions (Naamala et al., 2016). Intrinsic antibiotic resistance has been linked to survival of strains in soil environments (Xavier et al., 1998, Brockman and Bezdicek, 1989). It is curious whether this is linked to dominance of the *B. elkanii* in the soils in our study. Further studies on competitiveness and adaptation to environmental conditions, particularly for strains earmarked for inoculant use, including tolerance to temperature, salt and pH, are warranted.

Africa has a wide diversity of legumes (Mapfumo et al., 2005, Sprent et al., 2009), and to go with that a wide diversity of indigenous rhizobia, compatible with the legumes, including bradyrhizobia that nodulate soybean (Abaidoo et al., 2007, Aserse et al., 2012a, Botha et al., 2004, Musiyiwa et al., 2005a, Musiyiwa et al., 2005b), lablab (Grönemeyer et al., 2017), cowpea (Ndungu et al., 2018, Mpepereki and Makonese, 1995, Mpepereki et al., 1996a) and many others (Doignon-Bourcier et al., 1999). Many of these legumes are promiscuous., including cowpea and vachellia. Common bean nodulates widely but only effective with a narrow range. All strains tested in our present study formed N fixing nodules with cowpea, which is known for promiscuity and wide diversity and occurrence in Africa (Sprent et al., 2010). They also nodulated sandhill wattle, native to Australia, and cowpea and pigeon pea, whose centers of origin, like soybean, are in Asia. Adzuki bean has been reported to noduate with soybean rhizobia (Stajković et al., 2010). Many rhizobia nodulate more than one legume (Musiyiwa et al., 2005b, Mpepereki and Makonese, 1995, Mpepereki et al., 1996a). The nodulation pattern of *B. ottawaense* with cowpea and common bean were similar to those found by other workers (Yu et al., 2014). The unique combinations of legume-host relationships, as seen in Table 3.6, are defined by the *nod* genes borne by each rhizobia species or strain (Carlson et al., 1993, López-Lara et al., 1996, Lohrke et al., 1998). These *nod* genes are transmitted

vertically but may also be acquired by HGT, such that strains acquire the ability to nodulate a legume that their ancestor did not nodulate and a legume acquires new symbionts in a new environment (Barcellos et al., 2007, Batista et al., 2007).

3.5 Conclusions

This study revealed the diversity of RNB compatible with soybean in Zimbabwean soils. There are at least four *Bradyrhizobium* species that nodulate soybean in Zimbabwean soils. The study was based on one agro-ecological zone and it is possible that a different diversity occurs in the other agro-ecologies.

Limited persistence of the elite rhizobia inoculant strain MAR1491 was confirmed. More research is essential to understand the factors causing this and to find indigenous strains with high nitrogen fixation effectiveness. The species *B. elkanii* dominates soybean nodulating root nodule bacteria populations, suggesting that it is well adapted to Zimbabwean conditions. The isolates from this study should be evaluated for candidacy as inoculant strains, with ecological and manufacturing studies, as well as a broader evaluation of their capacity for nitrogen fixation with the new cultivars of soybean emerging from breeding programs, with the objective of finding a Zimbabwean *B. elkanii* strain that is high N fixing, and able to persist in soils. The diversity of African bradyrhizobia is a rich resource, which has yet been poorly characterised and considering the challenges of soil fertility and the poverty of smallholder farmers, holds great potential for obtaining improved inoculant strains and thus improving livelihoods.

Table 3.1 Site characterization for sites where isolates were obtained

Province, District	Site	Texture	Clay%	Silt%	pH (0.01M $CaCl_2$)	available P resin extract ppm	exchangeable K (meq/100g)	Total CEC (meq/100g)
Mash East, Marondera	Ngundu	cSaL	13	9	5.16	54	0.26	5.9
Mash East, Marondera	Sigauke	mSaL	14	9	7.19	35	0.29	2.9
Mash East, Marondera	Mapingire	cSaL	14	19	5.29	27	0.61	9.3
Mash East, Marondera	Chikonyora		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Mash Central, Bindura	Kwaramba	mSaL	9	9	4.99	44	0.39	6.5
Mash Central, Bindura	Paraziva	n.d.	n.d.	n.d.	0.93	63	0.56	6.5
Mash Central, Bindura	Guvamombe	n.d.	n.d.	n.d.	4.76	7	0.54	
Mash Central, Bindura	Maponga	n.d.	n.d.	n.d.	6.44	43	0.41	9.5
Mash West, Chinhoyi	Chivenda	CL	27	36	5.82	21	0.55	11.6
Mash West, Chinhoyi	Mwenyendiani	CL	33	27	5.16	12	0.52	12.3
Mash West, Chinhoyi	Razawu	fSaCL	31	22	6.39	11	0.56	6.2
Mash West, Chinhoyi	Muchineripi	CL	28	32	5.37	11	0.23	9
Mash East, Goromonzi	RARS II	fSaCL	32	19	5.68	63	0.34	12.1
Mash East, Goromonzi	RARS I	C	43	23	5.35	33	0.22	9.1
Mash East, Goromonzi	PRF	C	42	25	5.25		0.35	9.1
Harare, Harare	HRS	fSaCL	31	22	6.2		0.28	6.1

Table 3.2 Primer details for gene amplification of isolates in the study

Gene	Forward primer	Reverse primer	Forward primer sequence	Reverse primer sequence	Reference
16S rRNA	fD1	rD1	5′AGAGTTTGATCCTGGCTCAG -3′	5′ -AAGGAGGTGATCCAGCC -3′	(Weisburg et al., 1991)
16S rRNA	420f	920r	5'-GATGAAGGCCTTAGGGTTGT-3'	5'-CCCCGTCAATTCCTTTGAGT-3'	(Yanagi and Yamasato, 1993)
recA	recA41F	recA640 R	5′-TTCGGCAAGGGMTCGRTSATG -3′	5′ -ACATSACRCCGATCTTCATGC -3′	(Vinuesa et al., 2005)
glnII	glnII 12F	glnII 689R	5′-YAA GCT CGA GTA CAT YTG GCT-3′	5′-TGC ATG CCS GAG CCG TTC CA-3′	(Vinuesa et al., 2005)
nifH	nifH40f	nifH817R	5′-GGNATCGGCAAGTCSACSAC-3′	5′-TCRAMCAGCATGTCCTCSAGCTC-3′	(Vinuesa et al., 2005)
nodC	nodCF	nodCI	5′ -AYGTHGTYGAYGACGGTTC -3′	5′ - CGYGACAGCCANTCKCTATTG-3′	(Laguerre et al., 2001)

Table 3.3 Summary of isolation and authentication of bacteria from soybean breeding facilities and smallholder farmer field sites all previously inoculated at least five years prior to sampling in the present study.

	Soybean breeding facilities				Smallholder farmers' fields				
SITE	PRF	RARS	HRS	TOTAL	M'NDERA	BINDURA	CHINHOYI	TOTAL	SUM
TOTAL	27	46	39	112	40	26	37	103	215
AUTHENTICATED	24	29	25	78	33	11	15	59	137
% AUTHENTICATED	89	63	64	70	83	42	41	57	64

Table 3.4 A summary of the colony morphology of the RNB isolated in this study.

Colony type	SPECIES* BD	BJ	BE	BO	Total	Colony diameter	Representatives for preliminary MLSA	ADDITIONAL NOTES
1	18	12		7	37	2-4 mm	BdMAR1491, BjNAZ554, BdNAZ692, BjNAZ728	Circular, dome shaped
2		15			15	1-4 mm	BjNAZ750	Circular, dome shaped
3		1	7		8	1 mm	BeNAZ584, BeNAZ589, BjNAZ694	Circular, dome shaped
6		1			1	1 mm		Circular, raised dome shape

							BeNAZ599, BeNAZ600, BeNAZ683, BeNAZ689, BeNAZ747	Watery, mucilaginous
8			52		52	2-4 mm		
							BeNAZ506, BeNAZ567, BeNAZ727	Circular, dome shaped, elastic
9			14		14	2-4 mm		
							BeNAZ521, BeNAZ587, BeNAZ588, BeNAZ719	Circular, dome shaped, elastic, firm
10			10		10	1-4 mm		
Total	18	29	83	7	137			
Percent of total	13	21	61	5	100			

*In general, colonies were circular and dome shaped with an entire margin. All isolates were Gram-negative and alkalinized BTB. Strains are disaggregated by their species designations viz *B. diazoefficiens* (BD), *B. elkanii (*BE), *B. japonicum* (BJ) and *B. ottawaense* (BO).

Table 3.5 Proposed taxonomic position of isolates recovered in this study, as revealed by polymorphisms in partial recA analysis. The isolates are listed per their sites of origin.

Province	**District**	**Site Name**	**Commercial (C) smallholder (S)**	**Species Identity**	**Isolate identities**	**Total # isolates**
Harare	Harare	HRS	C	*B. elkanii*	NAZ582, NAZ583, NAZ584, NAZ586, NAZ587, NAZ588, NAZ589, NAZ592, NAZ597, NAZ598, NAZ599, NAZ600, NAZ601, NAZ603, NAZ604, NAZ605, NAZ609, NAZ614, NAZ615, NAZ616, NAZ619, NAZ623, NAZ624	23
				B. japonicum	NAZ606, NAZ607	2
Total						**25**
Mash East	Goromonzi	PRF	C	*B. elkanii*	NAZ501, NAZ502, NAZ503, NAZ504, NAZ506, NAZ508, NAZ510, NAZ511, NAZ512, NAZ514, NAZ515, NAZ516, NAZ517, NAZ520, NAZ521, NAZ523, NAZ524, NAZ526, NAZ529, NAZ530, NAZ531	21
				B. japonicum	NAZ505, NAZ527	2
				B. ottawaense	NAZ519	1
Total						**24**
Mash East	Goromonzi	RARS 1	C	*B. elkanii*	NAZ533, NAZ534, NAZ538, NAZ539, NAZ541, NAZ545, NAZ548, NAZ549, NAZ551, NAZ555, NAZ557	11
				B. japonicum	NAZ532, NAZ535, NAZ536, NAZ543, NAZ553, NAZ554, NAZ556	7
		RARS 2	C	*B. elkanii*	NAZ561, NAZ567, NAZ568, NAZ571, NAZ574, NAZ576, NAZ577, NAZ578, NAZ581	9
				B. ottawaense	NAZ572, NAZ575	2
Total						**29**
Mash East	Marondera	Chikonyora	S	*B. diazoefficiens*	NAZ625, NAZ626, NAZ627, NAZ628, NAZ629, NAZ630, NAZ632, NAZ633, NAZ634, NAZ635, NAZ636, NAZ637, NAZ638, NAZ639, NAZ640, NAZ641, NAZ643	17

		Mapingire	S	*B. elkanii*	NAZ719, NAZ720, NAZ721, NAZ723, NAZ725	5
				B. japonicum	NAZ718	1
		Sigauke	S	*B. elkanii*	NAZ667, NAZ747	2
				B. japonicum	NAZ666, NAZ750, NAZ751, NAZ753, NAZ753	5
		Ngundu		*B. elkanii*	NAZ661	1
			S	*B. japonicum*	NAZ755, NAZ756	2
Marondera Total Isolates						**33**
Mash Central	Bindura	Kwaramba	S	*B. elkanii*	NAZ683, NAZ685, NAZ688, NAZ689	4
				B. japonicum	NAZ684	1
Mash Central	Bindura	Paraziva	S	*B. japonicum*	NAZ706, NAZ708, NAZ710, NAZ711, NAZ712, NAZ715	6
Bindura Total Isolates						**11**
Mash West	Chinhoyi	Muchineripi	S	*B. elkanii*	NAZ727, NAZ729, NAZ730	3
				B. japonicum	NAZ728	1
				B. ottawaense	NAZ732	1
Mash West	Chinhoyi	Chivende	S	*B. ottawaense*	NAZ757, NAZ758, NAZ760	3
Mash West	Chinhoyi	Razawu	S	*B. diazoefficiens*	NAZ692	1
				B. elkanii	NAZ693, NAZ695, NAZ700, NAZ702	4
				B. japonicum	NAZ694, NAZ701	2
Chinhoyi Total Isolates						**15**

Footnote: Isolates have been designated with NAZ and a number, starting at NAZ501. The letters NAZ are derived from N2Africa – Zimbabwe (The project on which this work was carried out – and the country from which isolates were obtained); and the number was assigned in the order of isolation.

Table 3.6: Strain and accession numbers of sequences generated in this study

Species	**Strain**	**16S rDNA**	***rec A***	***glnII***	***nifH***	***nodC***
B. diazoefficiens	NAZ634	MK480225	MK496319			
	NAZ692	MK480226	MK496318	MK496311	MK496328	MK496333
B. elkanii	NAZ521	MK480227	MK496320		MK496325	MK496331
	NAZ584	MK480228	MK496321	MK496312	MK496327	MK496332
B. japonicum	NAZ505	MK480229	MK496316			
	NAZ606	MK480230	MK496317	MK496310		
	NAZ728				MK496329	MK496335
	NAZ750				MK496330	MK496334
B. ottawaense	NAZ519	MK424234	MK496322		MK496324	
	NAZ572	MK424235	MK496323	MK396315	MK496326	

Table 3.7: Host range/ specificity of Bradyrhizobium inoculant species recovered in this study, across 13 different legumes. Each species that was isolated is represented by one isolate. B. japonicum NAZ505, B. ottawaense NAZ732, B. diazoefficiens NAZ641 and B. elkanii NAZ661.

Common name	*B. diazoefficiens* NAZ641	*B. elkanii* NAZ661	*B. japonicum* NAZ505	*B. ottawaense* NAZ732	Nodule type	Legume clade
Soybean	E	E	E	E	Globose	Phaseoleae
Acacia	E	E	E	E	Elongate	Acacieae
Adzuki bean	E	E	E	E	Globose	Phaseoleae
Pigeon pea	E	E	E	E	Globose	Phaseoleae
Cowpea	E	E	E	E	Globose	Phaseoleae
Sunn hemp	N	E	N	E	Coralloid	Crotalarieae
Mung bean	I	N	N	E	Globose	Phaseoleae
Common bean	N	I	N	I	Globose	Phaseoleae
Lupin	N	N	N	N	-	Genisteae
Pea	N	N	N	N	-	Viciae
Faba bean	N	N	N	N	-	Viciae
Groundnut	N	N	N	N	-	Dalbergieae
Seradella	N	N	N	N	-	Loteae
TOTAL E	5	6	5	7		

TOTAL I	1	1	0	1
TOTAL N	7	6	8	5

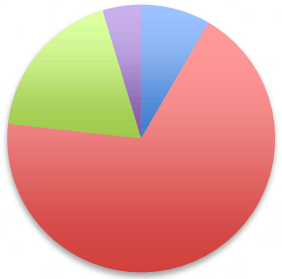
N denotes failure to nodulate (Nod-); E denotes effective nodulation (Fix+) and I denotes ineffective nodulation (Nod+Fix-). Isolates were only tested for effectiveness of nitrogen fixation but not ranked for the efficiency of nitrogen fixation.

Table 3.8 Antibiotic resistance of 9 RNB – 2 isolates from each of the four species and the commercial inoculant strain MAR1491

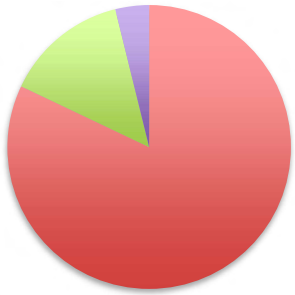
Species		Level of growth exhibited by the isolates under named antibiotic conditions														
	ANTIBIOTIC	Chloramphenicol (ug/ml)			Tetracycline (ug/ml)			Kanamycin (ug/ml)			Rifampicin (ug/ml)			Streptomycin (ug/ml)		
	Conc.	10	20	40	10	20	40	10	20	30	5	10	20	50	75	100
B. diazoefficiens	NAZ634	1	0	0	1	0	0	0	0	0	1	1	1	0	0	0
B. diazoefficiens	NAZ692	1	0	0	1	0	0	0	0	0	3	2	2	0	0	0
B. diazoefficiens	MAR1491	1	0	0	1	0	0	1	0	0	3	2	2	0	0	0
B. elkanii	NAZ501	4	3	2	4	4	3	3	3	3	3	3	3	3	3	3
B. elkanii	NAZ578	4	3	2	4	4	3	4	3	3	4	4	3	4	3	2
B. japonicum	NAZ505	2	0	0	0	0	0	1	0	0	0	0	0	0	0	0
B. japonicum	NAZ701	4	3	2	3	3	2	2	3	3	2	3	3	3	2	2
B. ottawaense	NAZ519	4	0	0	0	0	0	3	3	3	2	1	1	0	0	0
B. ottawaense	NAZ732	3	2	2	2	0	0	2	3	2	3	3	2	0	0	0

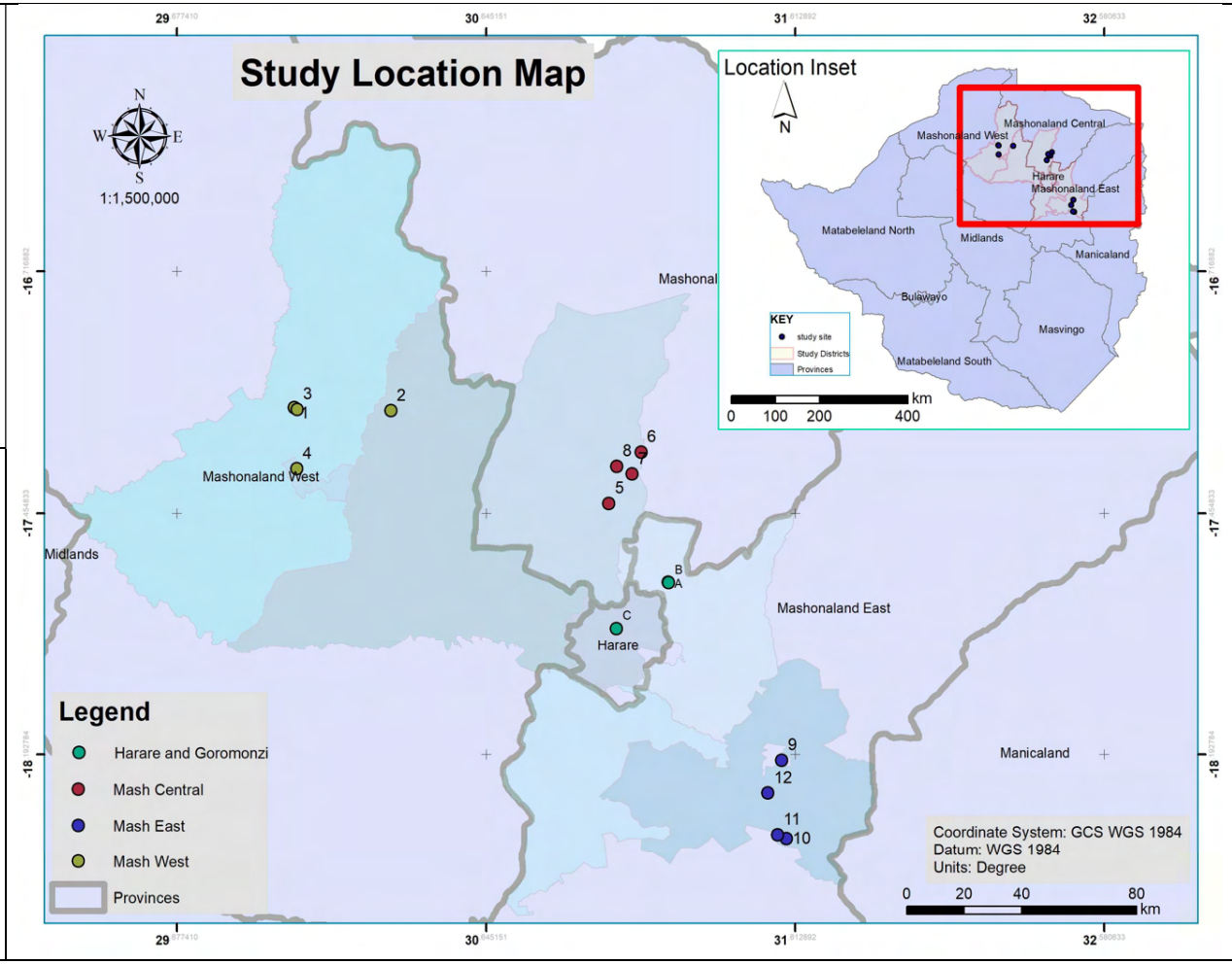
The numbers 0, 1, 2, 3 and 4 denote no growth, limited growth, fair growth, normal growth and luxurious growth. Values were generated from three replicates.

Global Population Distribution



Research Stations A, B and C





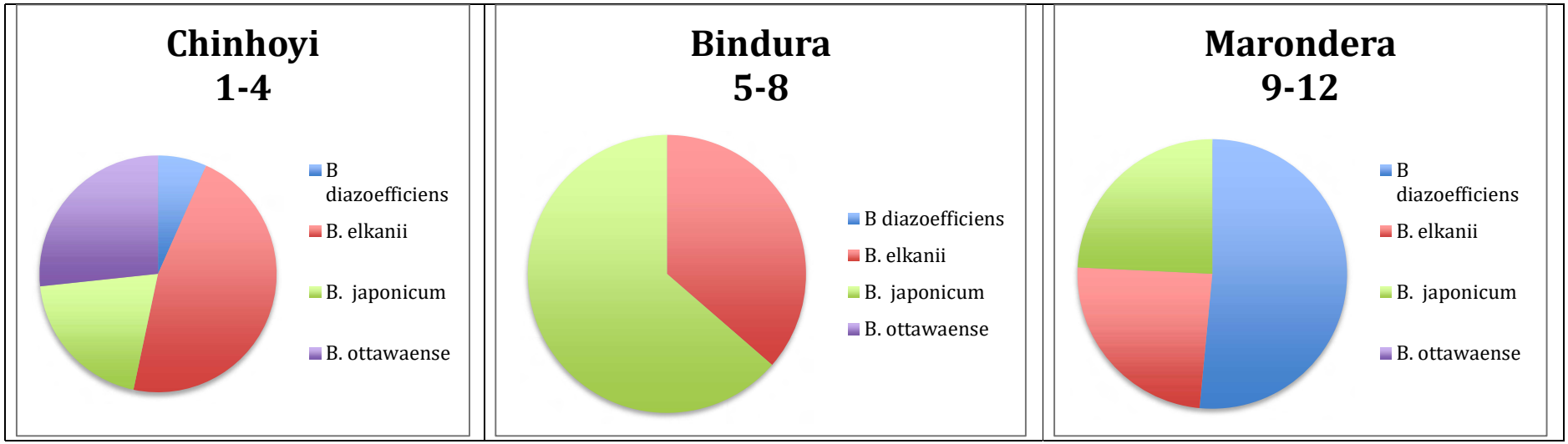


Figure 3.1 Map of Zimbabwe showing sampling sites and distribution of rhizobia population recovered as shown on pie charts. Sampling sites are marked by dots. They are found in four administrative provinces, namely Harare, Mashonaland Central, Mashonaland East and Mashonaland West. Within the provinces, districts where the samples were obtained are delineated with light boundaries. Smallholder farm sites are marked by a number from 1 to 12 with four in each of three provinces and the commercial farm sites are marked by letters A, B and C.

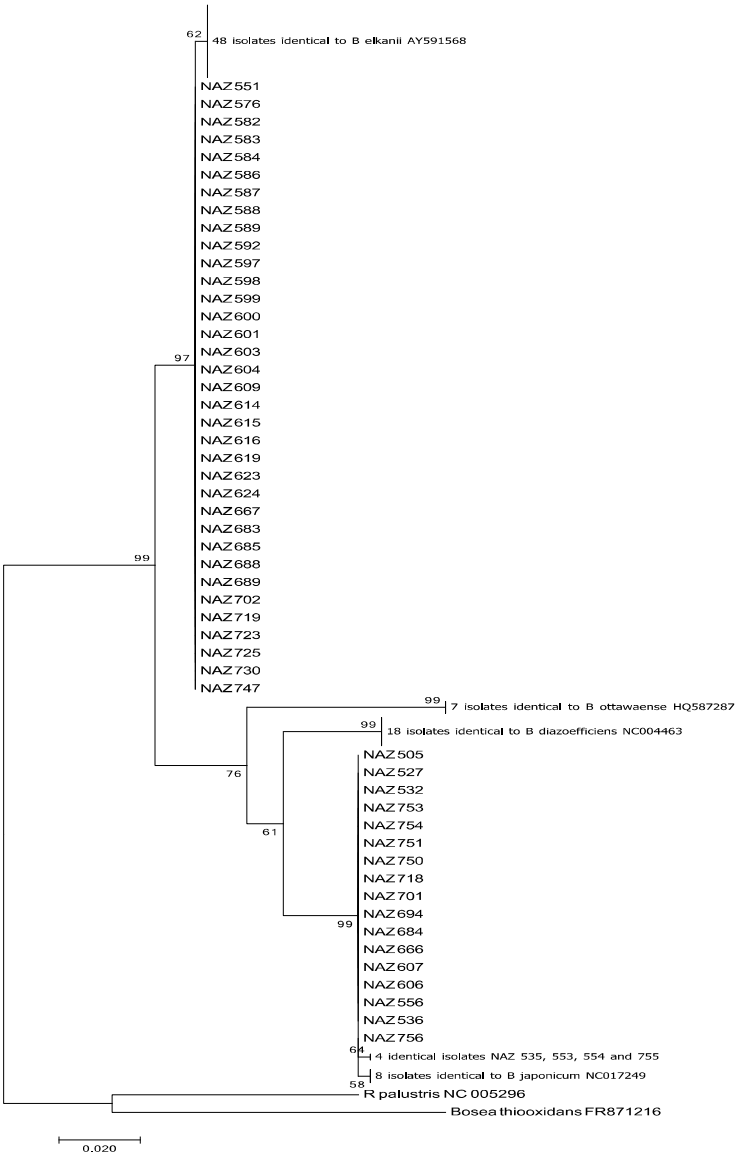


Figure 3.2 The genetic diversity of 137 soybean RNB isolated from Zimbabwean soils according to phylogeny of a 339 base-long intragenic *recA* sequence. The image is a maximum likelihood phylogenetic tree generated by MEGA 7. Isolates in the study had at least 97% similarity to one of four *Bradyrhizobium* type strains, *B. diazoefficiens* USDA 110^{T}, *B. elkanii* USDA 76^{T}, *B. japonicum* USDA 6^{T} and *B. ottawaense* OO99^{T}. The GenBank accession numbers are given next to the type strains. Node labels are bootstrap values expressed as percentages of 1000 replications at node labels. The tree is compressed for some identical sequences to fit it on the page.

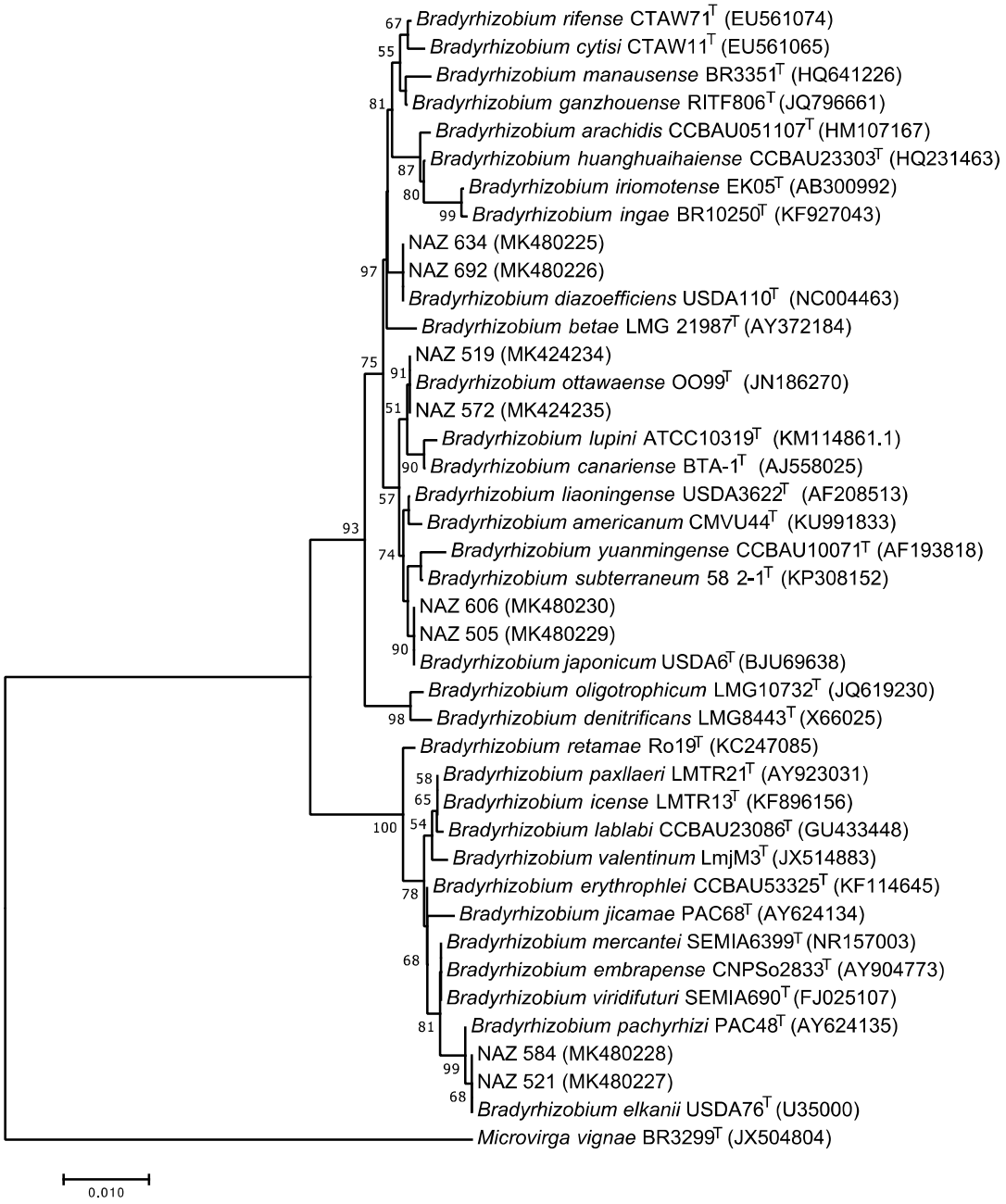


Figure 3.3 Neighbor joining phylogeny for RNB from Zimbabwean soils isolated in this study, inferred from a 1329 base-long internal portion of the 16S rRNA gene. Two representative isolates each were included, along with type strains of recognized *Bradyrhizobium* species. Phylogenetic analysis was performed using MEGA7, with Kimura 2-parameter model of substitutions for the nucleotides and gamma distributed rates among sites. Numbers are derived as a percentage of bootstrap support from 500 replications. Bootstrap support above 50% is shown.

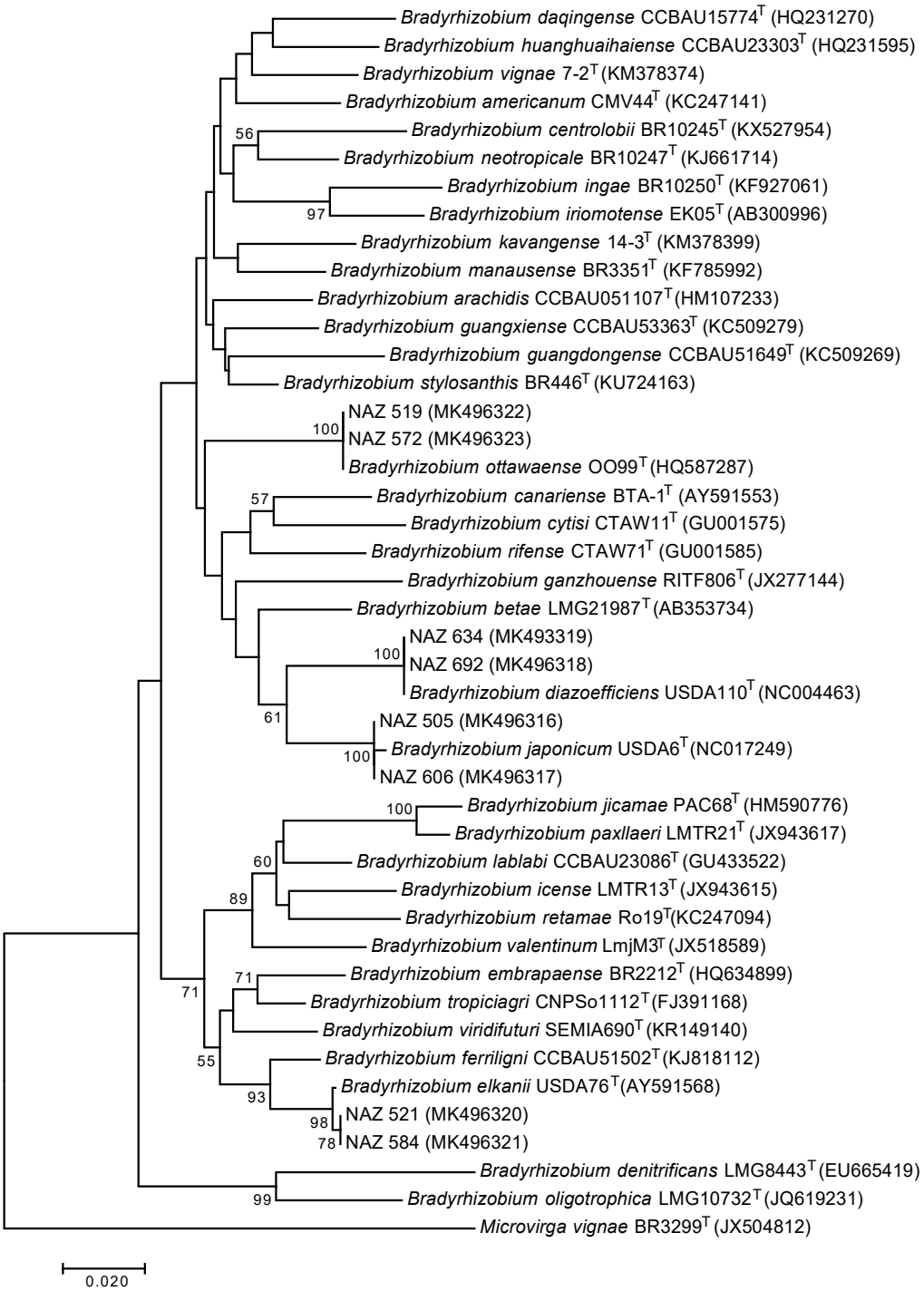


Figure 3.4 Phylogenetic gene tree of the relationship of Zimbabwean soybean RNB to *Bradyrhizobium* type strains inferred from a 375 base long internal portion of the *recA* gene. Study isolates are represented by two isolates per clade detected in this study. Analysis is based on MEGA7 neighbor joining phylogenetic analysis with 500 bootstrap replications. Bootstrap support above 50% is shown.

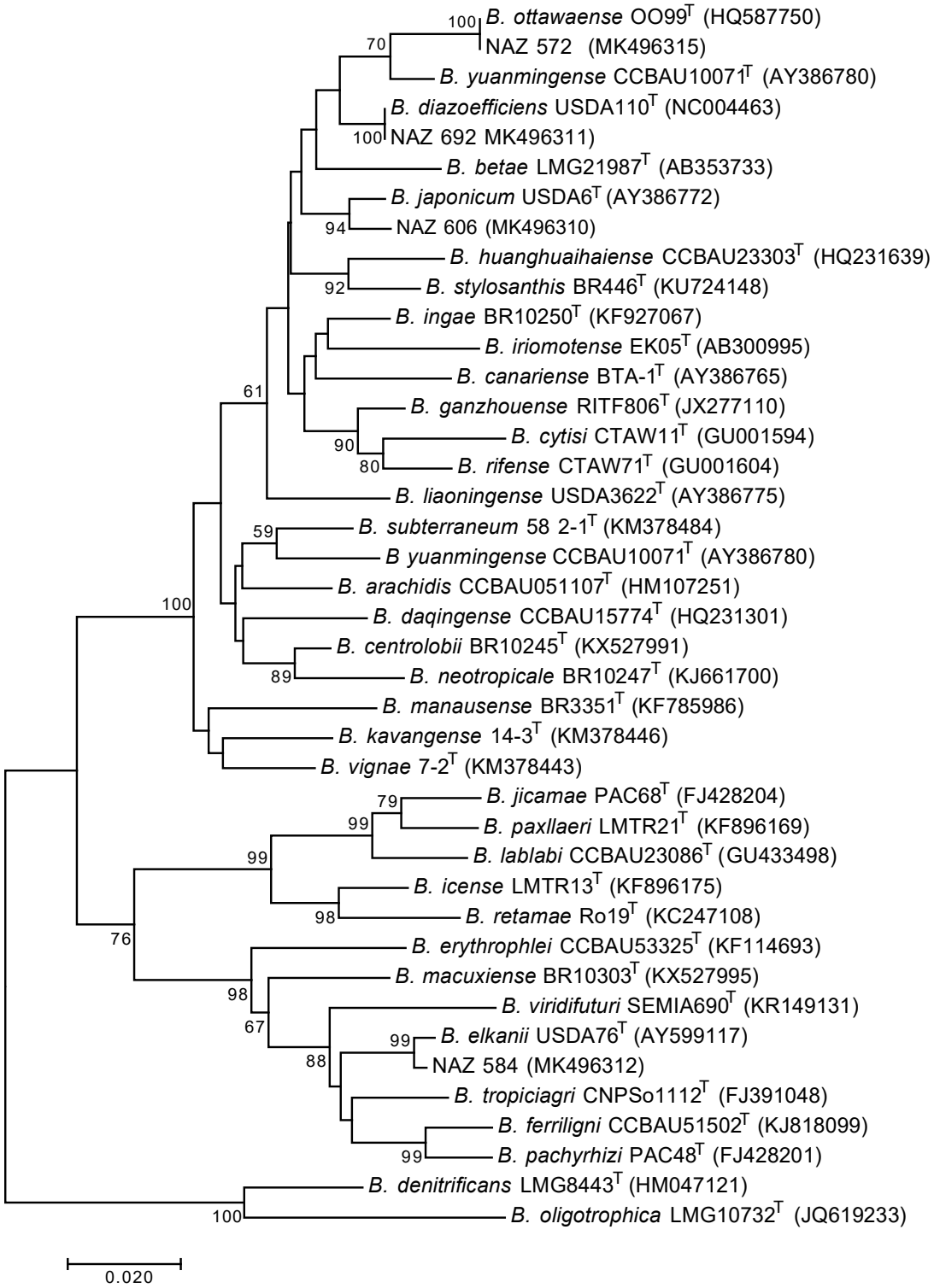


Figure 3.5 Phylogenetic gene tree based on 520 base long internal portion of *glnII* gene of isolates representing the four *Bradyrhizobium* clades detected in this study with the type strains of recognized *Bradyrhizobium* species. Analysis is based on MEGA7 neighbor joining analysis with 500 bootstrap replications. Bootstrap support above 50% is shown as a percentage.

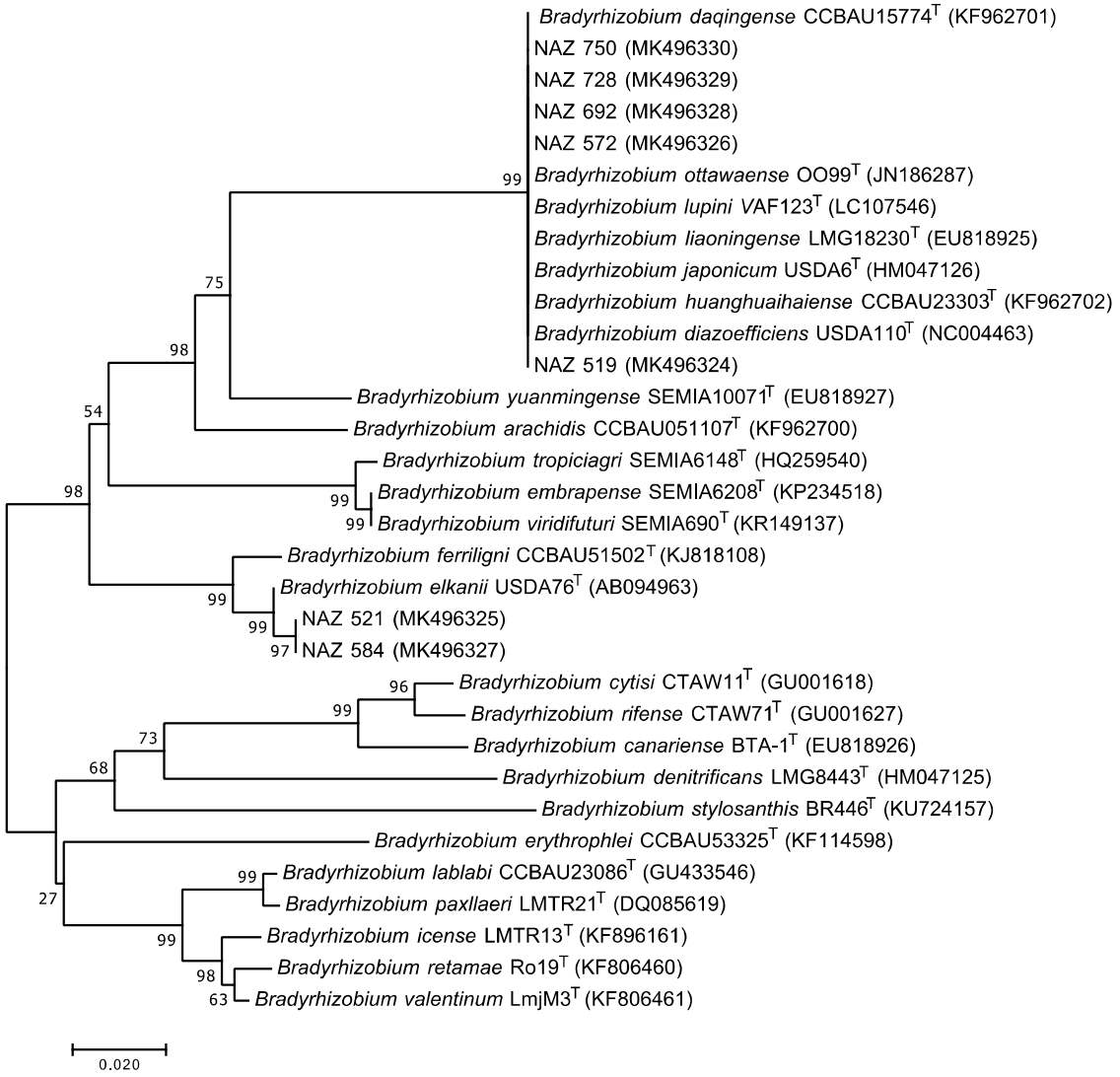


Figure 3.6 Phylogenetic gene tree of isolates based on partial *nifH* genes of Zimbabwean soybean RNB with named *Bradyrhizobium* species. The four species are all represented by two species, except *B. diazoefficiens*, which is represented by one. Analysis is a neighbor-joining tree based on 500 bootstrap replications in MEGA7, with bootstrap support values above 50% shown at the nodes, as percentages.

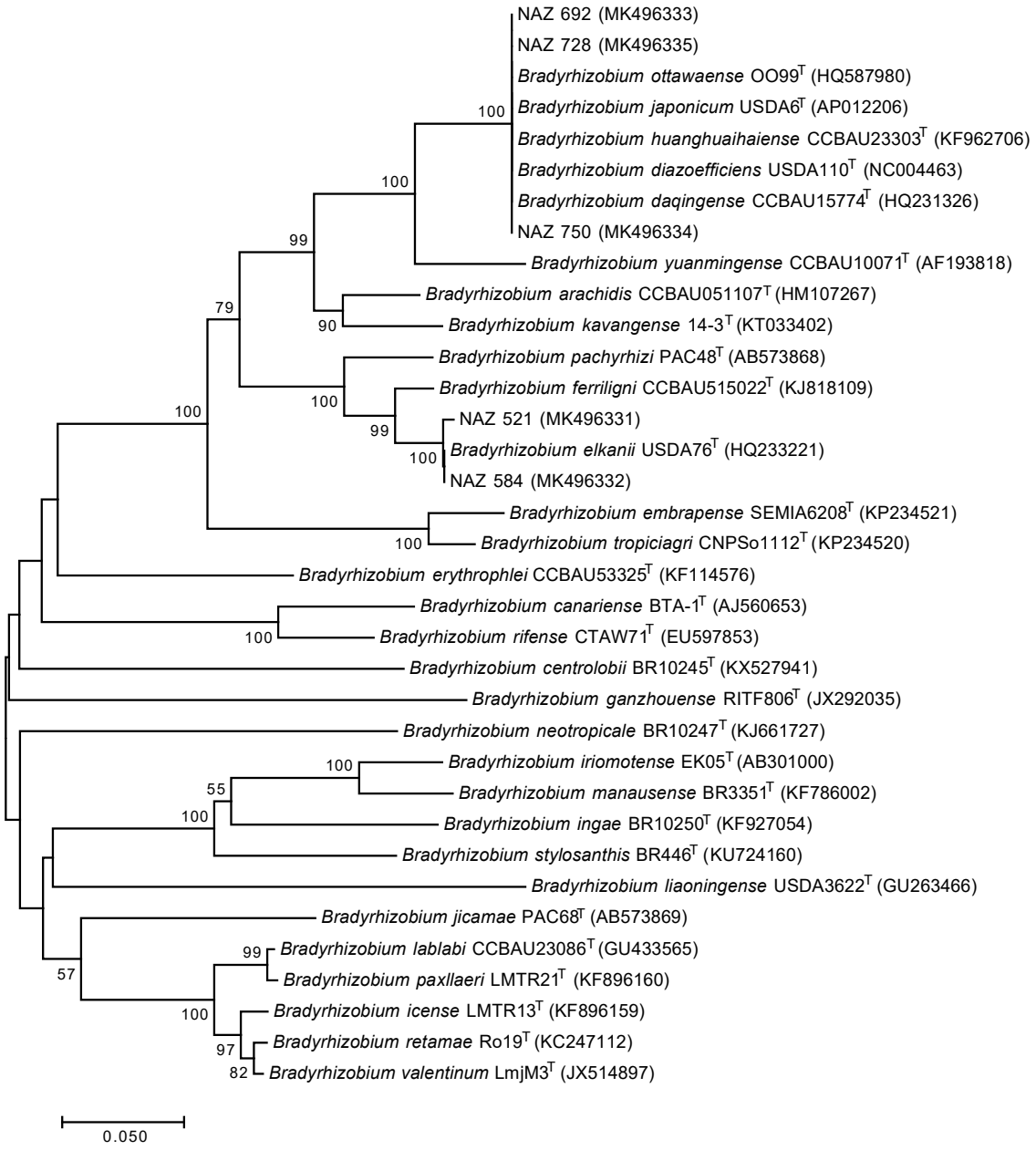


Figure 3.7Phylogenetic gene tree of isolates based on partial *nodC* genes of Zimbabwean soybean RNB *B. diazoefficiens, B. japonicum and B. elkanii* isolates. The tree is an unrooted neighbor joining tree based on 441 base long internal portion of *nodC* executed in MEGA7. Bootstrap values are shown at the nodes, as percentages of 500 replications.

CHAPTER 4

Selecting improved soybean rhizobia inoculant strains from indigenous and naturalized soil populations in Zimbabwe

4.1 Introduction

Soybean is the most important legume worldwide according to land under cultivation and global trade (Alves et al., 2003, Pagano and Miransari, 2016). It is a major crop in Zimbabwe, despite being introduced only in the last century (Shurtleff and Aoyagi, 2009). Production is widespread in different parts of the country (Giller et al., 2011). Indigenous rhizobia compatible with soybean have been reported in Zimbabwean soils (Chiduwa, 2021; Musiyiwa, 2000, Musiyiwa et al., 2005a, Mpepereki and Makonese, 1995). However, the natural distribution of soybean rhizobia in Zimbabwean soils is highly variable (Chiduwa, 2021; Musiyiwa et al., 2005b, Zengeni and Giller, 2007), necessitating inoculation (Lindström et al., 2010).

Inoculated soybean production as a package is well promoted as a strategy for sustainable improvement of productivity on both smallholder and commercial farms in Zimbabwe (Giller et al., 2011). The capacity to fulfil a large proportion of its nitrogen requirement through SNF is important for Zimbabwean smallholder farmers who are mainly constrained for agricultural inputs (Mpepereki et al., 2000). The use of SNF is a practical way for resource-constrained farmers to supply nitrogen nutrition to their farming systems.

While the elite inoculant strain MAR1491 has high nitrogen fixation capacity, it has limited saprophytic competence in Zimbabwean soils (Zengeni et al., 2006, Zengeni et al., 2003) (results, Chapter 3). There is a need for highly efficient, saprophytically competent rhizobia strains to improve the sustainability of rhizobia inoculant use. High efficiency in SNF of strains will generate higher yields and saprophytic competence will accrue to farmer savings from reducing the frequency of inoculation, especially before distribution networks are improved.

A possible solution to this two-fold challenge is to screen isolates from sites where indigenous rhizobia occur and elite strains have been introduced. Elsewhere, screening re-isolates of soybean inoculant strains has been effective in identifying strains with improved N-fixation capacity compared to progenitor strains (Torres et al., 2012). This present study details a series of experiments with soybean root nodule bacteria isolated from soils that were previously inoculated with MAR1491 at least five years prior to sampling for the study. With the overall objective being to select elite candidates for use as inoculant strains, the study was organised to screen a population of indigenous and naturalised soybean rhizobia, obtained in Chapter 3, for (i) high nitrogen fixation capacity, (ii) effectiveness across a range of soybean cultivars and (iii) adaptation to a range of edaphic and climatic conditions.

4.2 Materials and methods

4.2.1 Rhizobia germplasm

This study utilised 137 authenticated soybean root nodule bacteria isolated from Zimbabwean soils, last inoculated with MAR1491 at least five years prior (Chapter 3). Rhizobia were isolated from nodules from smallholder farmers or by trapping from soils collected from soybean breeding facilities. All isolates were authenticated on soybean cultivar Bimha (Howieson et al., 2016). *Bradyrhizobium diazoefficiens* strain MAR1491, the current inoculant strain in Zimbabwe, was the inoculant control in all experiments. Isolates are listed in Table 4.1.

Table 4.1 Description of rhizobia isolates used in the study, their species designations and the experiments in which they were used

Inoculant strain	*Bradyrhizobium* species	Experiment
NAZ599	*B. elkanii*	1, 2 and 3
NAZ629	*B. diazoefficiens*	1, 2 and 3
NAZ710	*B. japonicum*	1, 2 and 3
NAZ758	*B. ottawaense*	1, 2 and 3
NAZ510	*B. elkanii*	1 and 3
NAZ554	*B. japonicum*	1 and 3
NAZ760	*B. ottawaense*	1 and 3
NAZ626	*B. diazoefficiens*	1 and 3

4.2.2. Legume germplasm

Soybean germplasm was sourced from Zimbabwe (Seed-co Pvt. Ltd, Pannar Seeds, Crop Breeding Institute) and Australia (Leichhardt) (Table 4.2).

Table 4.2 Description of legume germplasm used in the study.

Legume	Cultivar	Source	Experiment
Soybean	Leichardt	Australia	Experiment 1
Soybean	Mhofu	CBI, Zimbabwe	Experiment 2
Soybean	Status	Seed-Co Ltd. Zimbabwe	Experiment 2
Soybean	PAN 1867	Pannar Seeds, Zimbabwe	Experiment 2
Soybean	Solitaire	Seed-Co Ltd. Zimbabwe	Experiment 3

4.2.3. Site selection and soil characterization

Experiments 1 and 2 were carried out under glasshouse conditions at Murdoch University, Western Australia. Field testing of rhizobia isolates (Experiment 3) was carried out at three sites in Agroecological region 2 in three districts of Zimbabwe, at smallholder farms in Gotora (18º8'24''S, 31º11'24''E), 1527 m.a.s.l.; Mswaka (18º7'12''S; 31º14.'23.9''E), 1602 m.a.s.l; and Grasslands Research Station (18º6'0''S, 31º17'23.99 E), 1674 m.a.s.l. in agriculture season 2016/2017. All sites receive between 750 and 1000 mm rainfall annually. Gotora has medium grained loam sands; Mswaka and Grasslands Research Station (GRS) have coarse sandy loams. Site characterization is detailed in Table 4.3.

Table 4.3 Chemical and physical properties of 20 cm topsoil at the site of field experiments in Zimbabwe

	Physical						Chemical					
Farm Site	Soil Texture	Clay	Silt	Fine Sand	Medium Sand	Coarse Sand	pH (0.01 M $CaCl_2$)	P (ppm)	K (meq/ 100g)	% C	Na (meq/ 100g)	CEC (meq/100g)
Gotora	mLS	5	4	28	44	19	5.36	4	0.11	0.57	0.1	6.3
Mswaka	cSaL	12	14	10	28	36	5.19	12	0.18	0.71	0.06	3
GRS	cSaL	14	5	14	27	40	4.9	34	0.08	0.39	0.06	5.8

Soil physical texture was determined using the hydrometer method; pH was determined using the $CaCl_2$ method; exchangeable cations were determined by AAS after extracting with acidified ammonium solution; % carbon was determined by the Walkley Black method

4.2.4. Isolate screening for nitrogen fixation: Experiment 1

A total of 137 isolates were screened for nitrogen fixation, as determined by plant dry mass after eight weeks of growth, under glasshouse conditions. Because the isolates could not be simultaneously accommodated in the glasshouse, isolates were evaluated in one of three separate glasshouse experiments. Each experiment consisted of 3 replicates of inoculation treatments, a control inoculated with MAR1491, a non-inoculated control and nitrogen (KNO_3)-fed control.

Even-sized seeds of the Australian cultivar Leichardt were selected and surface-sterilized (Somasegaran and Hoben, 1994). Three-litre pots were filled with sand, steam sterilized, then washed off any nitrates with sterile, boiling water before being allowed to drain overnight to field capacity. Four planting holes were made aseptically then the pre-germinated seed was planted and inoculated with 1 ml of a single rhizobium isolate. The inoculum was prepared by washing cells off a 7-day old petri dish with sterile one-percent sugar solution. Pot assemblies were covered with plastic film and arranged in a completely randomized design. After plants emerged, the plastic film was removed, a sterile watering tube placed in the center of the pot, then the sand surface covered with a layer of sterilized propylene beads to prevent contamination between treatments. Plants were maintained with nitrogen-free nutrient solution (Howieson and Dilworth, 2016). After eight weeks of growth in the glasshouse, plants were carefully cut at the first node, shoots dried at 60^{o}C for 72 hours then weighed. Shoot weight was used to evaluate nitrogen fixation (Howieson et al., 2011).

Data were expressed as a percentage of the value obtained for the control strain MAR1491 inoculated treatment in the respective experiment and then the three experiments were combined collectively for analysis in SPSS. A two-way analysis of variance was carried out on mean shoot dry weights with respect to individual isolates.

In addition, data was aggregated by species, and two-way analysis of variance was carried out on the species, with the inoculated control, and the other controls each treated separately. Treatment means were separated using the LSD.

4.2.5. Rhizobia preference for soybean cultivar: Experiment 2

The isolates that generated the highest plant dry matter for each of the four bacterial species in experiment 1, viz NAZ629 (*B. diazoefficiens*), NAZ599 (*B. elkanii*), NAZ710 (*B. japonicum*) and NAZ758 (*B. ottawaense*), were evaluated under glasshouse conditions for nitrogen fixation with the three Zimbabwean soybean cultivars: Mhofu from the Crop Breeding Institute, PAN 1867 from Pannar Seeds and Status from Seed-co. Each isolate x strain combination was replicated six times in a randomised complete block design (RCBD) experiment set up and maintained as in 'Experiment 1: Isolate screening' (Howieson et al., 2011). The experiment was harvested by cutting shoots at the first node, drying at 60^{o}C for 72 hours then weighing. Treatment means for the data generated was evaluated by two way analysis of variance in SPSS and means separated by LSD.

4.2.6. Field evaluation of four rhizobia species as inocula: Experiment 3

The best two isolates per species from Experiment 1, to include strains confirmed to fix nitrogen across three different soybean cultivars in Experiment 2, were evaluated under field conditions at three sites in Zimbabwe, and compared to MAR1491, as inoculant for the most widely grown soybean cultivar in the country, Solitaire. Experimental sites were ploughed and fertilized with 300 kg ha^{-1} compound D (N-P-K = 7:14:7) fertilizer. Experiments were set up in January 2017. Plots were pegged to measure 5 m long and 3.5 m wide at Mswaka and Gotora; and 5m long by 4.5 m wide at GRS. Plant populations were set according to standard farm practice of 45 cm apart with intra-row spacing at 5 cm. At eight weeks after planting, six

representative plants were carefully uprooted from the middle 2 m of the middle two rows. Nodule numbers for all six plants were counted, and the average numbers of nodules per plant were recorded. Plant matter was dried at 60^{o}C for 48 hours and plant biomass was recorded. The same plant matter was used for evaluating total nitrogen accumulated by the Kjeldahl method. Nitrogen content of the grain from net plots, harvested at physiological maturity, was also evaluated using the Kjeldahl method. The experimental design was a completely randomized block design with three replicates at each site. Treatment means were evaluated by two way analysis of variance in SPSS and separated using LSD.

4.3. Results

4.3.1. Isolate screening for nitrogen fixation: Experiment 1

The mean plant dry weight of the 137 isolates evaluated under glasshouse conditions ranged from 49.56% to 103% of that generated by plants inoculated with the elite MAR1491 (Figures 4.1a-e). The variability was high both within and between rhizobial species. Ten isolates generated biomass of more than 90% of MAR1491, drawn from all four species, with seven of these isolates belonging to *B. elkanii*. The isolates that generated the highest biomass belonged to *B. diazoefficiens* and *B. japonicum* ($P<0.05$), namely NAZ629 and NAZ710, respectively. Strains NAZ599 (*B. elkanii*) and NAZ758 (*B. ottawaense*) were the most effective of their species. These strains, NAZ629, NAZ710, NAZ 599 and NAZ758 were isolated from diverse sites, Chikonyora farm of Marondera, Paraziva farm of Bindura, HRS of Harare and Chivende farm of Chinhoyi, respectively (chapter 3). Most isolates elicited a positive inoculation response and only *B. elkanii* had isolates with N fixation comparable to the uninoculated control. In general, the bulk of the isolates ranged in N fixation potential

from 70 to 90% of that of MAR1491, for all species. A limited number of isolates were found in the lower and in the exceptionally high ranges.

When aggregated by species, the four rhizobia species yielded less biomass than the N-fed control and the inoculated control. Of the four rhizobia species, *B. japonicum* generated the highest mean shoot dry weight, followed by the *B. elkanii*, while the *B. diazoefficiens* and the *B. ottawaense* were not significantly different from each other and were only superior to the uninoculated control ($p<0.05$). Each species was represented by at least one isolate in the lowest and the highest 10% of the strains. Isolates of *B. elkanii* were spread across the range of N fixation capacity isolates and dominated both the highest and lowest 10% at 50% of the treatments. More than half the *B. diazoefficiens* isolates generated dry weights in the bottom 50% of the range.

Figure 4.1: Experiment 1 Dry weights of plants, soybean cultivar Leichardt under glasshouse conditions, inoculated with the 137 rhizobium isolates recovered in the study, presented as a percentage of that of those inoculated with the control strain MAR1491. Means presented are averages of three replicates for each of (1a) 42 isolates of *B. elkanii*, (1b) 41 isolates of *B. elkanii*, (1c) 29 isolates of *B. japonicum*, (1d) 18 isolates of *B. diazoefficiens*, and (1e) 7 isolates of *B. ottawaense*. The black line separates the elite strains (>80% dry weight of the MAR1491 dry weight).

Fig. 1a

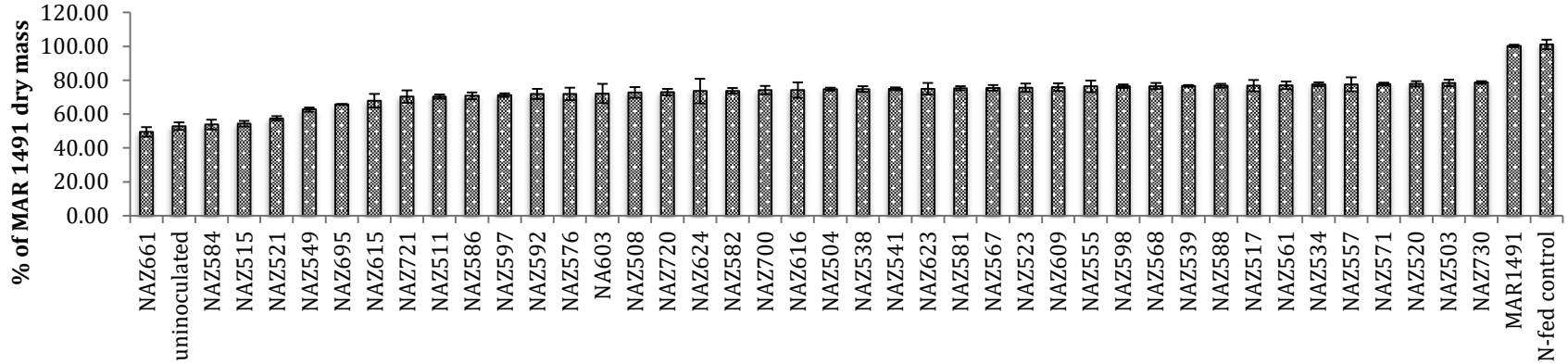

B. elkanii (i)
% of MAR 1491 dry mass
120.00
100.00
80.00
60.00
40.00
20.00
0.00
NAZ661
uninoculated
NAZ584
NAZ515
NAZ521
NAZ549
NAZ695
NAZ615
NAZ721
NAZ511
NAZ586
NAZ597
NAZ592
NAZ576
NA603
NAZ508
NAZ720
NAZ624
NAZ582
NAZ700
NAZ616
NAZ504
NAZ538
NAZ541
NAZ623
NAZ581
NAZ567
NAZ523
NAZ609
NAZ555
NAZ598
NAZ568
NAZ539
NAZ588
NAZ517
NAZ561
NAZ534
NAZ557
NAZ571
NAZ520
NAZ503
NAZ730
MAR1491
N-fed control

Fig. 1b

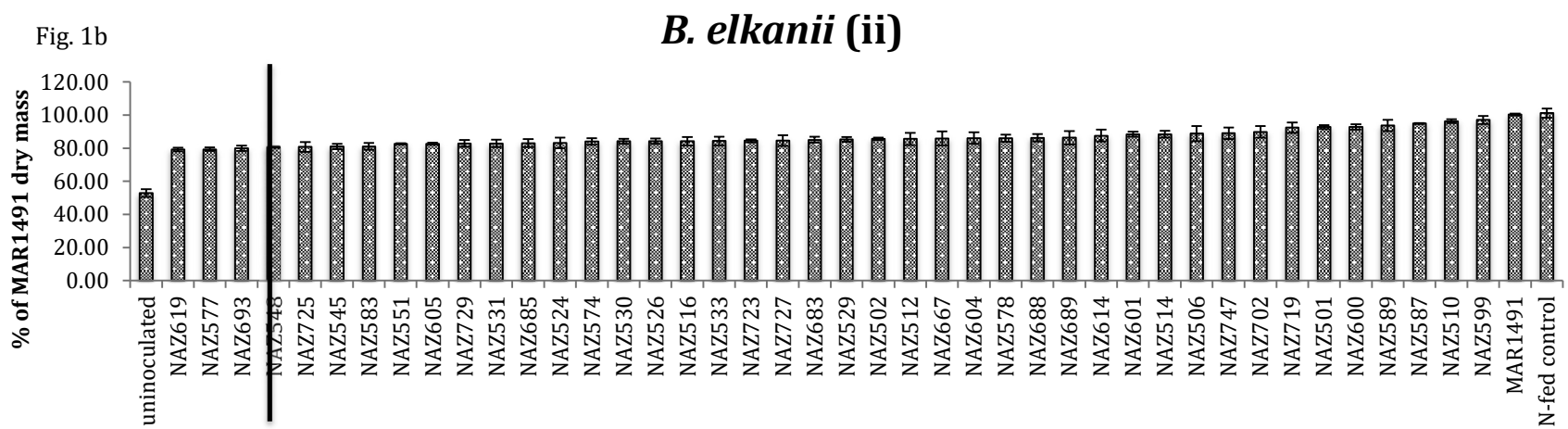

B. elkanii (ii)
% of MAR1491 dry mass
120.00
100.00
80.00
60.00
40.00
20.00
0.00
uninoculated
NAZ619
NAZ577
NAZ693
NAZ548
NAZ725
NAZ545
NAZ583
NAZ551
NAZ605
NAZ729
NAZ531
NAZ685
NAZ524
NAZ574
NAZ530
NAZ526
NAZ516
NAZ533
NAZ723
NAZ727
NAZ683
NAZ529
NAZ502
NAZ512
NAZ667
NAZ604
NAZ578
NAZ688
NAZ689
NAZ614
NAZ601
NAZ514
NAZ506
NAZ747
NAZ702
NAZ719
NAZ501
NAZ600
NAZ589
NAZ587
NAZ510
NAZ599
MAR1491
N-fed control

Fig. 1c

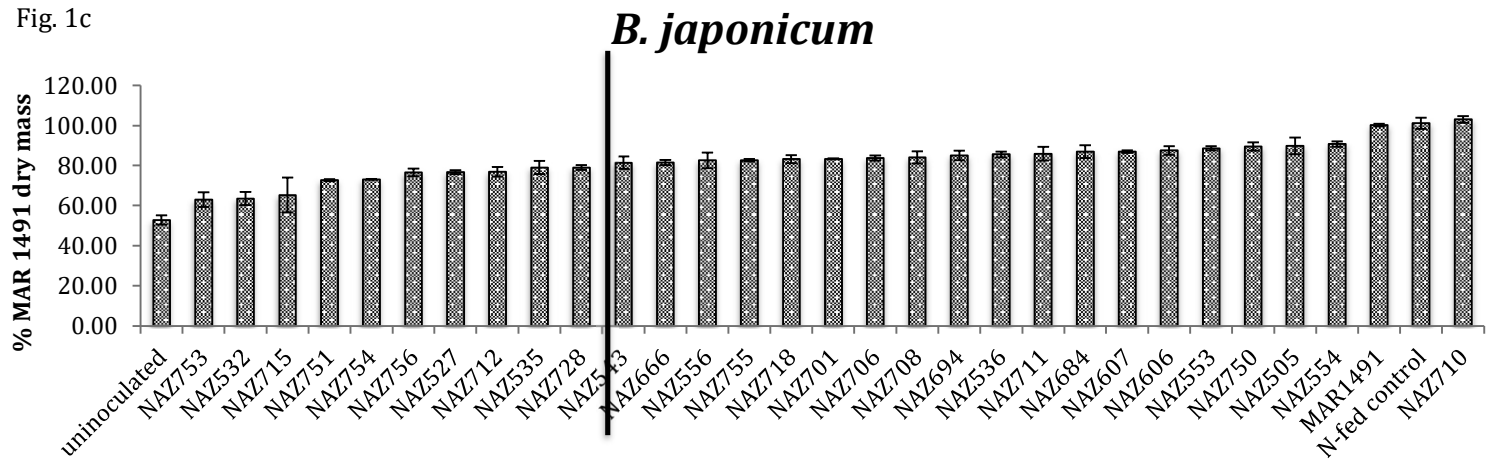


Fig. 1d

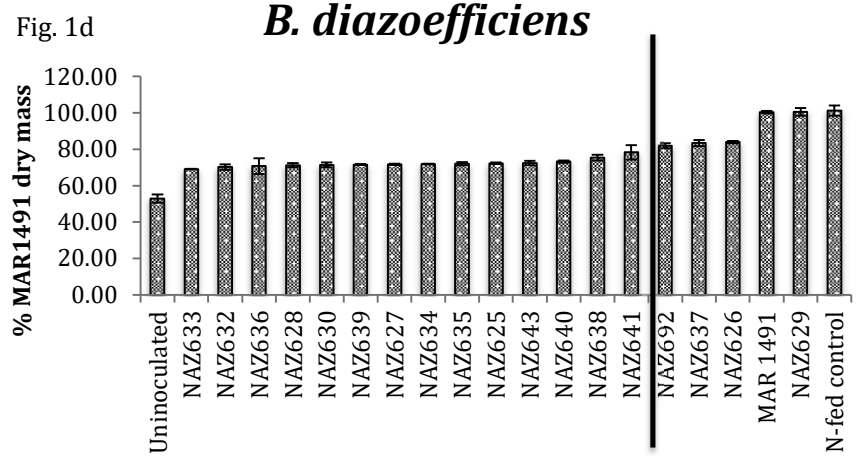
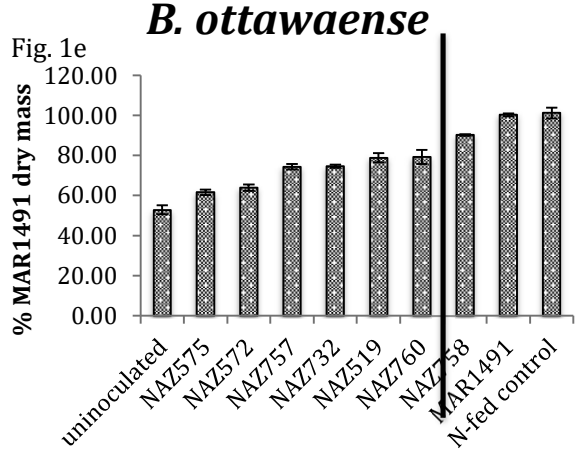


Fig. 1e



4.3.2. Rhizobia preference for soybean cultivar: Experiment 2

All three cultivars responded positively ($p<0.05$) to inoculation with the best isolate from each of the four species of bradyrhizobia. There were interaction effects between isolates and cultivars. Rhizobia strain NAZ710 generated the greatest mean inoculation response overall ($p<0.05$), compared to other inoculation treatments, when inoculated on cv. Mhofu. The strain performed similarly or better than the elite inoculant strain MAR1491 with all three cultivars. Isolate NAZ629 inoculated on cv. Mhofu and cv. Status performed similarly or superior to MAR1491 while performance with PAN1867 was inferior to the inoculant strain (Figure 4.2). Strains NAZ599 and NAZ758 performed similarly or superior to the inoculant strain with PAN1867 and Status but worse than the inoculant strain with Mhofu. When uninoculated, cv. Mhofu generated higher shoot dry weight than cv. Status and cv. PAN1867, whose shoot dry weights were not significantly different from each other. In all inoculation treatments, cv. Mhofu generated the greatest shoot dry weight than cv. Status, except with NAZ710, where they are not significantly different ($p<0.05$).

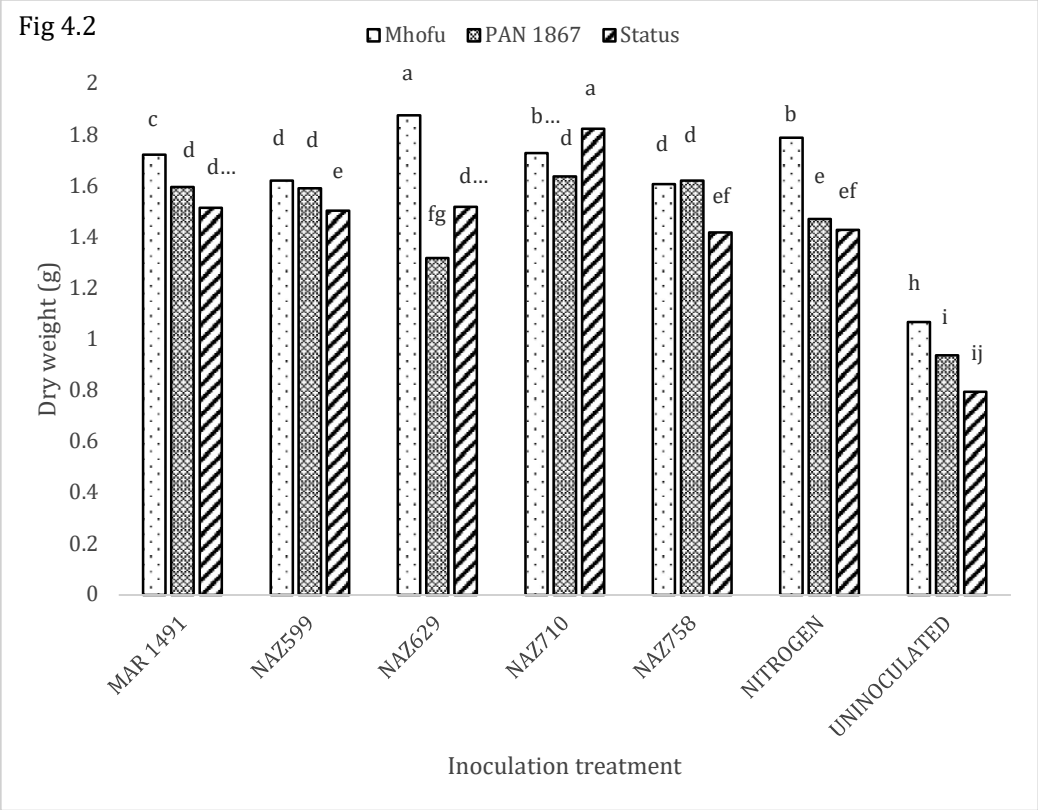


Figure 4.2 Experiment 2 Dry weights of plants of soybean cultivars Mhofu, PAN 1867 and Status, inoculated with the best isolate for each of the four species recovered, with respect to N fixation potential. Each species is represented by the best strain as such: MAR1491 = Inoculated control; NAZ599 = *B. elkanii*; NAZ629 = *B. diazoefficiens*; NAZ710 = *B. japonicum* and NAZ758 = *B. ottawaense*.

4.3.3. Field evaluation of four rhizobia species as inocula: Experiment 3

In the multi-site field experiment, the best two inoculant strains of each of the four species were inoculated with the most commonly planted soybean cultivar in the country, Solitaire. MAR1491 generated the lowest nodule numbers of all the strains, including the uninoculated treatment, at Gotora and GRS ($p<0.05$) (Figure 4.3a). Strain MAR1491 (16), NAZ626 (28) and NAZ629 (42) are all *B. diazoefficiens* strains and they elicited low nodule numbers in comparison to the other species (Figure 4.3a). The

greatest number of nodules was achieved by inoculation with NAZ599, followed by NAZ510, both *B. elkanii* isolates. Nodule numbers by *B. japonicum* isolates NAZ554 and NAZ710 differed at all sites with the latter being lower than the former ($P<0.05$). Nodulation by the *B. ottawaense* isolates NAZ760 and NAZ758 was in the middle of the range but with the latter lower than the former. In general, the highest nodulation was at Gotora, followed by Mswaka and GRS had the least ($P<0.05$).

At Gotora site, the greatest biomass was generated by NAZ629, followed by NAZ710 then NAZ554 and NAZ758 (Figure 4.3b). At Mswaka, there was no significant difference in the biomass yields of plots inoculated with MAR1491, NAZ710, NAZ554 and NAZ629. The lowest biomass accumulation in general, was achieved at GRS ($P < 0.05$) (Figure 4.3b). However, NAZ 554 performed well at GRS and superior to performance at Mswaka. The rest of the strains performed poorly at GRS. NAZ554 was followed by NAZ710 and then NAZ629 ($P < 0.05$). Overall, strain NAZ554 generated the mean highest biomass at the three sites, followed by NAZ629 then NAZ710, ahead of MAR1491, the standard inoculant strain ($P < 0.05$).

There were interaction effects of the strains and the sites on the N content of shoots at eight weeks and grain at physiological maturity (Figure 4.3c). At Gotora, the highest plant shoot N content was generated by NAZ629, which was not significantly different from NAZ510; while NAZ710 was highest at Mswaka, similar to NAZ629. The greatest plant shoot N content of all sites was generated by NAZ710 and NAZ629 at GRS and this was not significantly different from NAZ554 ($p<0,05$). The lowest plant shoot N content of all sites was generated by NAZ599 at Mswaka ($p<0.05$). The N content in the grain at physiological maturity was highest at Gotora and lowest at Mswaka (Figure 4.4).

Gotora site had a pH of 5.36, Mswaka had a pH of 5.19 and GRS had pH 4.9 (Table 4.3).

Fig 4.3a

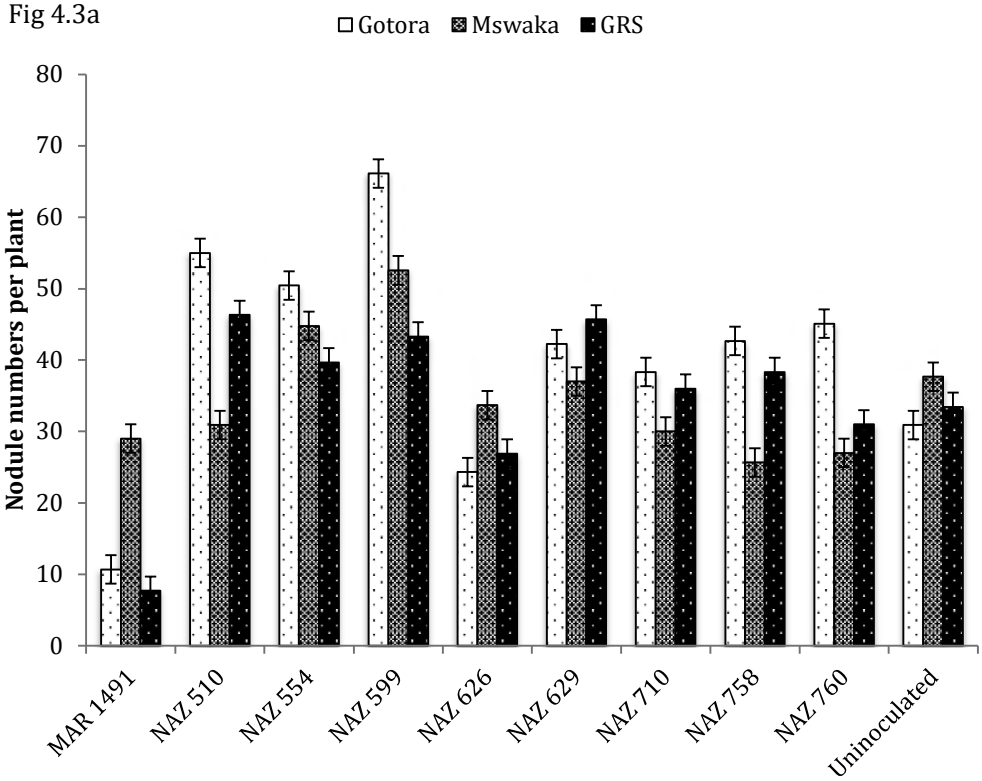
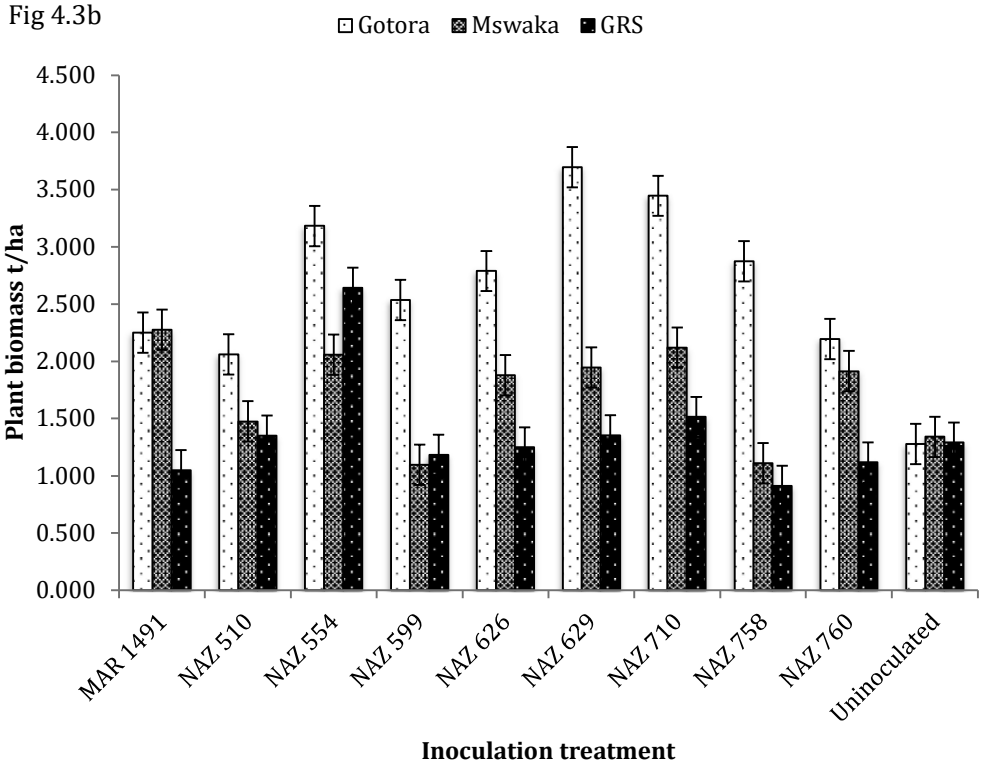


Fig 4.3b



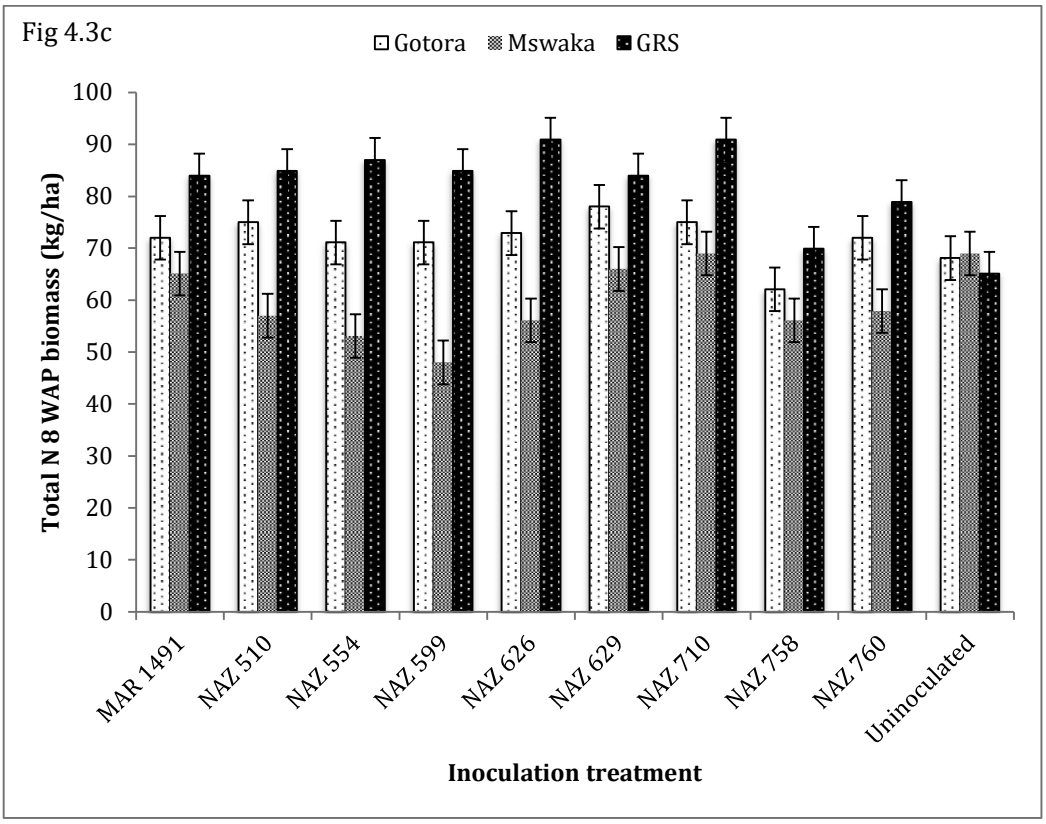


Figure 4.3a Experiment 3 Number of nodules observed on; (Figure 4.3b) Plant biomass generated and (Figure 4.3c) Total N generated by soybean plants, cultivar Solitaire, at eight weeks after planting under field conditions at three field sites, in Zimbabwe with strains *B. diazoefficiens* NAZ626 and NAZ629; *B. elkanii* NAZ510 and NAZ599; *B. japonicum* NAZ554 and NAZ710; and *B. ottawaense* NAZ758 and NAZ760.

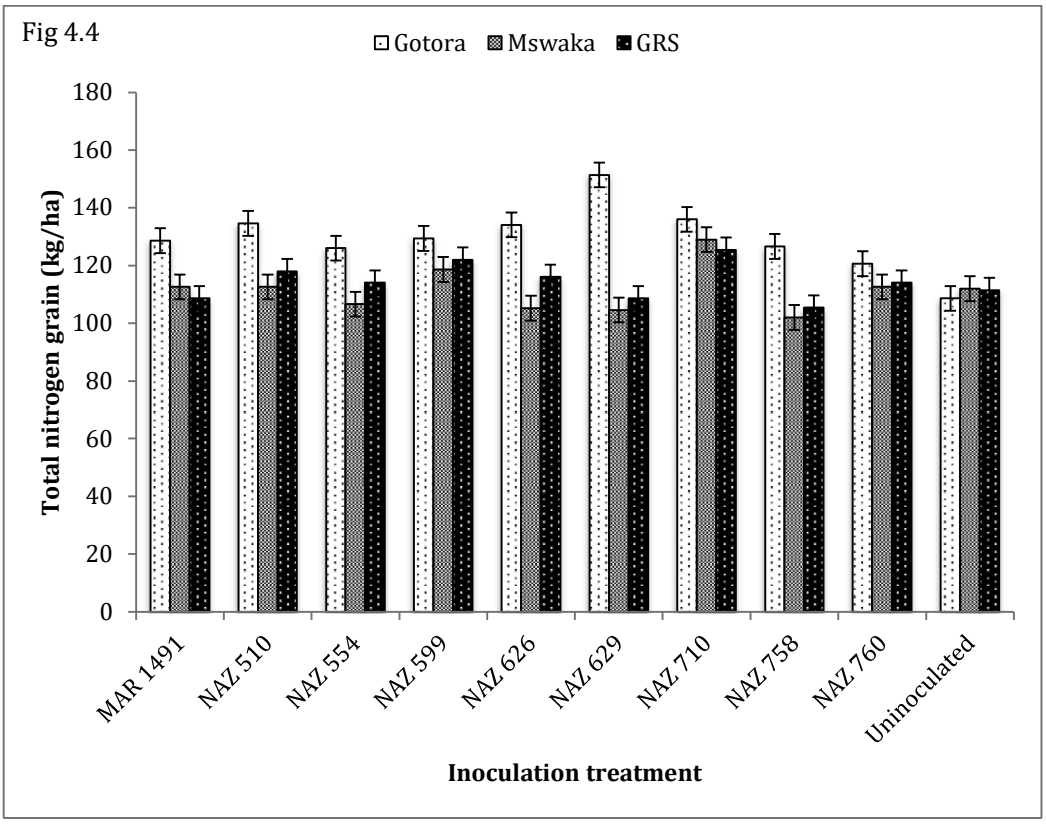


Figure 4.4 Experiment 3 Total N generated in grain at physiological maturity of cv. Solitaire, under field conditions at three field sites in Zimbabwe. Seeds were inoculated with the best two isolates for each of the four species isolated in this study, as such *B. diazoefficiens* NAZ626 and NAZ629; *B. elkanii* NAZ510 and NAZ599; *B. japonicum* NAZ554 and NAZ710; and *B. ottawaense* NAZ758 and NAZ760.

4.4. Discussion

This study firstly compared biomass accumulation of soybean inoculated with 137 Zimbabwean soybean isolates, with biomass obtained from inoculation with the commercial strain MAR1491 and uninoculated and N-fed controls. Improved rhizobia inoculant strain selection by screening from native and adapted re-isolates has been proven to work as a strategy to obtain superior rhizobia strains for use as inoculants (Chibeba et al., 2017, Torres et al., 2012). Notably, the 137 Zimbabwean soybean isolates were predominantly strains of *B. elkanii*, suggesting superior adaptation and

survival or competitiveness in attaining nodule occupancy in Zim soils. The initial screening generated a normally distributed performance for shoot dry weight with only two isolates *B. japonicum* NAZ 710 and *B. diazoefficiens* NAZ 629 surpassing the current inoculant stain MAR1491 (Figure 4.1a - e). This underscores the need to select superior rhizobia inoculant strains that can infect soybean and fix high amounts of nitrogen as native rhizobia are known for competing with inoculant strains but fixing poorly (Gerding et al., 2013, Torres et al., 2012).

Rhizobia can contribute to soybean production by symbiotically supplying a significant amount of the crop's nitrogen requirements (Albareda et al., 2009, Musiyiwa et al., 2005b). The residual value of this N to soil fertility depends upon the harvest index of soybean in any given year, and the amount of N exported in the grain. Several studies have revealed that the benefit of SNF in farming systems is not dominantly due to residual soil N provided by SNF *per se*, as there is often lower soil N after grain harvest than before crop establishment (Patra et al., 2012). Others have demonstrated that inclusion of the below-ground N additions brings the N balance to neutral (Salvagiotti et al., 2008). It is clear that a well-matched rhizobia-soybean combination benefits from effective SNF and saves on fertilizer costs.

There are several soybean cultivars on the market in Zimbabwe, and it is important for commercial rhizobia strains to be highly effective with as wide a range of cultivars on the market as possible. Several studies reported interaction effects between soybean cultivars and rhizobia strains (Appunu et al., 2008, Megueni et al., 2006, Shutsrirung et al., 2002). This may be due to the genetic capacity of the cultivar for nitrogen fixation, or incompatibility between the soybean cultivar and the rhizobia strains (Caetano-Anollés and Bauer, 1988). (Heron and Pueppke, 1987) showed cultivar strain interactions that included inhibition of nodulation; as well as cohabiting

within nodules by some rhizobia, which can be disadvantageous for biomass accumulation for the macrosymbiont. (Musiyiwa et al., 2005b) found that the promiscuous cultivar Magoye formed nodules in 80 % of soils tested, while Hernon 147 formed them in 50 % and the specific cultivar Roan formed nodules only in 25 % of test soils.

The best single strains for each of the four rhizobia species determined in experiment 1 were compared with the commercial inoculant MAR1491 for compatibility with different soybean cultivars. Positive inoculation response in all cultivars (Fig. 4.2) with overall superior performance by NAZ 710 performed the best, followed by NAZ 629 showed the two strains to be stable high-performance strains. Strains NAZ 599 and NAZ 758 performed within the range of the commercial inoculant. Cultivar Mhofu exceeded the other cultivars in dry matter accumulation, while PAN 1867 and Status were not significantly different (Figure 4.2). This emphasizes the presence of other factors that the rhizobia strains must interact within the environment. Inclusion of the current rhizobia inoculant strain in the soybean cultivar breeding programs may have contributed to compatibility with the control.

Evaluation of inoculant performance under field conditions is critical when screening strains of potential agricultural application. Introduced rhizobia have often been challenged by competition from indigenous rhizobia (Thies et al., 1992, Thies et al., 1991) and the soil and environment also offer additional challenges for the rhizobia (Hungria and Vargas, 2000, Elias and Herridge, 2015). *B. elkanii* strains in the present study exhibited superior nodulation to strains of other species. *B. diazoefficiens* strains, including the standard strain MAR1491, exhibited lower numbers of nodulation in comparison to the other strains. However, higher nodulation does not always result in higher SNF benefits (Terpolilli et al., 2008). Therefore, the recommended inoculant

strains are not the *B. elkanii* strains with the high nodulation but the *B. japonicum* and *B. diazoefficiens* strains with superior nitrogen fixation.

It is interesting to compare these results for Zimbabwean soybean isolates with those obtained for potential Brazilian soybean inoculant strains. Brazilian soils were originally devoid of soybean rhizobia and elite strains were introduced from other countries. Subsequent strain selection with extensive field testing (Hungria et al., 2005) based on isolates recovered from soils in Brazil, including where the exotic strains have been introduced by inoculation (Santos et al., 1999a), has led to the development of elite inoculant strains that are well adapted to local conditions. Of the four strains used in Brazilian inoculants, two are *B. elkanii*, and local screening of more *B. elkanii* strains in Zimbabwe may reveal competitive high nitrogen-fixing isolates. In soils where indigenous and exotic rhizobia interact *in situ*, horizontal gene transfer may lead to the development of strains that have the survival and persistence capacity and ability to nodulate of the indigenous strains, with the nitrogen fixation of the elite, exotic strains (Barcellos et al., 2007, Batista et al., 2007). Such strains make ideal inoculant strains. There is no information on genetic transfer between soybean *bradyrhizobia* in African soils. Variation in field results may be attributable to a cultivar's effectiveness in accessing essential crop nutrients in the dynamic soil conditions (Fatima et al., 2006). It is important to continue to investigate the stability of these strains over several seasons and sites.

By the low recovery numbers of *B. diazoefficiens*, our studies confirmed the lack of persistence of the rhizobia inoculant strain MAR1491. Nodulation under field conditions revealed the complexity of challenges that are not found under controlled glasshouse conditions. For this reason, it was critical to validate findings from glasshouse experiments from Western Australia to the field conditions in Zimbabwe,

particularly with the best isolates. The rhizobia, the crop and the symbiosis all experience an array of biotic and abiotic stress factors under field conditions including low soil fertility and the deficiency of essential and enhancer crop nutrients, drought, high temperatures and low pH (Rawat et al., 2008, Hungria and Vargas, 2000). Rhizobia strains vary in their response to the different stress factors and screening the potential candidates under those conditions allows for the selection of strains that are adapted to the environmental conditions (Appunu and Dhar, 2006). Soybean has been reported to fix 15-450 kg N/ha/year (Smil, 1999), suggesting that the soybean rhizobia symbiosis in the study farming system can be further optimized. Soils must be conditioned to optimum soil pH of 5.5 to 6.5 for optimum performance of the soybean-rhizobia symbiosis.

In view of the positive gains in yields and SNF linked with annual inoculation (Hungria and Mendes, 2015b), and the low cost of rhizobia inoculation, it is prudent to pursue annual inoculation in order to maintain high nodulation and high nitrogen fixation and yields. Making use of SNF can save money otherwise committed to N fertilizers, enhance environmental health by reducing the total N fertilizer used in the farming system which eventually pollutes groundwater and contributes to greenhouse emissions (Hungria and Mendes, 2015b).

4.5. Conclusions

Strains NAZ 710 and NAZ 629 are recommended for use ahead of MAR1491 for inoculant production because these strains displayed high N fixation capacity, across a range of soybean cultivars and a range of edaphic and climatic conditions. Annual inoculation is recommended, along with testing of more isolates for survival

and persistence together with higher nitrogen fixation capacity. Further testing of NAZ 710 and NAZ 629 may also yield new insights into how they work.

CHAPTER 5

General discussion

5.1 Recap of aims/ introduction

Legumes involved in efficient symbiotic nitrogen fixation (SNF) hold the answer to many challenges of smallholder farmers. As well as providing dietary protein and high protein livestock feed, nodulated legumes can increase soil N content (Mpepereki et al., 2002). This is a cheaper and more sustainable route to improve the fertility of soils in comparison to the use of chemical fertilizers, especially for smallholder farmers, who typically have limited capacity to invest in external inputs (Nezomba et al., 2008). The increased soil N content can improve yields of crops grown in rotation, such as maize. Surpluses from the improved yields can be sold for income security. Additionally, legumes in rotation can break pest and disease cycles that are associated with the continuous cultivation of mono-crops (Giller et al., 2011).

A significant proportion of Zimbabwean smallholder farmers are resource-constrained, such that adopting an efficient legume-rhizobia combination on their farm has the potential to make a significant contribution to the farm's overall N budget and sustainable farming, without requiring considerable monetary investment. Smallholder-farming systems are characterized by suboptimal levels of SNF. As such, the maximum benefits that legumes can provide to food, nutrition and income security, as well as the development of sustainable agriculture, have not yet been realized in Zimbabwe.

The overall aim of the present study was to increase the benefits of SNF for smallholder farmers in Zimbabwe. Legumes that take part in SNF are many and they vary in their capacity for SNF. Some legumes, such as groundnut and cowpea, are commonly grown in Zimbabwe for subsistence. Other legumes, such as pigeon pea, sunn hemp and lablab, may have the potential for several uses around the farm. The information on nodulation and N fixation of these legumes are limited. Many studies have focused on soybean and its symbiosis (Kasasa et al., 1999, Musiyiwa, 2000, Davis

and Mpepereki, 1995). While well studied and promoted, the soybean-rhizobia symbiosis has its own challenges relating to saprophytic competence of its inoculant strain (Zengeni et al., 2003, Zengeni et al., 2006).

The present study investigated the effectiveness and the potential of N fixation of six legume-rhizobia symbioses in Zimbabwean agriculture and then pursued the soybean microsymbionts to greater detail. The three specific objectives of the study were to (i) evaluate six legumes for their potential for high rates of nitrogen fixation, (ii) determine the survival of the elite soybean inoculant strain *B. diazoefficiens* MAR1491 in Zimbabwean soils and conduct molecular characterization of rhizobial strains that nodulate soybean in Zimbabwe, and (iii) evaluate the potential of Zimbabwean soybean nodulating strains for N fixation effectiveness under glasshouse and field conditions, with the aim of developing an improved rhizobial inoculant for soybean.

5.2 Evaluating legumes for effective SNF and determining the need to inoculate

The efficiency of SNF depends upon several factors that can be shown by the equation

$$(G_L \times G_R) \times E \times M,$$

where G_L refers to legume genotype, G_R refers to rhizobium genotype, E refers to the environment, which encompasses both climate and soils and M refers to the agronomic management, which includes fertilizer application and weed and pest control (Schilt-Van Ettekoven et al., 2017). The appropriate rhizobial species must be available in sufficiently large numbers in the soil or on the seed for legumes to nodulate and have high rates of SNF. Soil rhizobia populations are dynamic and diverse, and depend on biotic and abiotic factors (Abaidoo et al., 2007, Mcinnes et al., 2004). Nodulation of the host legume by less effective but saprophytically competent rhizobial strains will

result in decreases in N fixation, thus affecting crop production and yield (Thuita et al., 2012, Musiyiwa et al., 2005b).

Crops select the subset of soil rhizobia that they will nodulate with, based on the molecular dialogue with the microsymbionts. In chapter 2, six legumes were tested over two seasons for their capacity to fix N with and without inoculation in Zimbabwean farming conditions. Inoculation was performed in season 1 only. The tested legumes included soybean, groundnut, cowpea, lablab, pigeon pea and sunn hemp. The residual benefits that accrued to a subsequent maize crop in season 2 were also evaluated.

In season 1, the greatest amount of biomass without inoculation was generated by lablab, while groundnut generated the least. All legumes had a positive response to inoculation, shown by increases in biomass, nitrogen content of plant tissue and grain yield. Under the inoculation treatment, sunn hemp generated the greatest biomass, but the amount of biomass was not significantly different from that of lablab and soybean. Although groundnut responded to inoculation, the amount of biomass it generated was still low in comparison to that of the other legumes. The benefits of inoculation persisted into the second season. Biomass of legumes in the plots that were inoculated in the first year was higher than from the plots that were not inoculated in the first year. Maize biomass was highest after lablab in the uninoculated treatment and after sunn hemp after inoculated legumes. However, our data told us little about how much shoot biomass would be produced by each species in the course of its growth and how much N would be fixed. To do this, sampling would need to be done for each species, likely at different times, just prior to physiological maturity when pods are full of beans and leaves are starting to turn yellow.

High-biomass-producing legumes have been shown to improve soil N contents (Wortmann et al., 2000). Early biomass accumulation is also important for weed suppression, thereby reducing the labour required for keeping farmland weed-free (Cheminingwa et al., 2007). Therefore, based on our results, sunn hemp and lablab are good candidates for use in Zimbabwean farming systems. Lablab is additionally suitable for livestock feed (Nyoka et al., 2004).

Tropical legumes have been expected to engage in effective SNF without the need for inoculation, as it has been assumed that there are abundant strains of effective rhizobia in tropical soils (Singleton et al., 1992). Groundnut failed to nodulate at GRS while cowpea recorded an average of only 11 nodules at PES in the third season. This re-iterates that the presence of compatible native rhizobia cannot be guaranteed. Some studies have shown that planting even the model tropical legume, cowpea, into soils that lack desired rhizobia results in varied nodulation and/or inefficient N fixation, affecting crop productivity; and positive inoculation responses have been recorded (Kanonge-Mafaune et al., 2018, Ulzen et al., 2016). Although only soybean is traditionally inoculated in Zimbabwe, all legumes in our study responded positively to inoculation. This study has shown that legumes in Zimbabwean farming systems can benefit from inoculation with effective rhizobia strains. However, the large variability means that it is important for farmers to make an informed choice of legume and decision to inoculate or not, to satisfy their requirements for soil improvement.

The soils in the present study, like many soils in smallholder communities in Zimbabwe, are low pH soils. Although one site had a pH of 5.9, the remaining three sites had a pH of 4.5, 4.3 and 4.2. While bradyrhizobia are more adapted to acid soils that to alkaline soils, soil pH levels of 5.5 to 7 are more ideal. This may explain the low rhizobia numbers leading to low nodulation. Soil conditioning for optimum pH to the

recommended pH 5.5 ($CaCl_2$) would benefit not only the rhizobia populations and subsequent SNF, but also the performance of most other crops grown by smallholder farmers, the staple crop, maize, included. Future research studies must include both soil pH correction as well as screening soil rhizobia for strains that are tolerant to low pH for overall system performance.

Strain selection programs must be accompanied by cultivar improvement programs. SNF in Zimbabwean farming systems is also compromised by the lack of improved cultivars. Farmers dependence on passing seed fromother farmers, rather than using improved cultivars, perpetuates low yields. Crop breeders and rhizobiologists must work together to develop legume cultivars and rhizobia inoculant strain combinations for high SNF, which will result in higher yields. A rather extreme suggestion has been that research pursues the development of soybean cultivars that nodulate exclusively with the desired elite inoculant strain (Minamisawa and Mitsui, 2000). The model for the optimization of soybean production in Brazil over several decades has resulted in high yielding soybean cultivars that depend on competitive, high N fixing rhizobia inoculant strains (Alves et al., 2003).

Soybean is considered a strategic crop in Zimbabwean agriculture, suitable for both commercial and smallholder production (Giller, 2008). Initial work introducing soybean to smallholder communities sought to promote promiscuous soybean cultivars (Mpepereki et al., 2000). However, rhizobia inoculant use has demonstrated its potential to improve yields, even with promiscuous soybean cultivars (Mpepereki and Pompi, 2003, Giller et al., 2011, Kasasa et al., 1999). Subsequently, specific, high yielding soybean cultivars have been promoted for production along with the use of rhizobia inoculant. This is unlike the case with cowpea and groundnut, which are popularly grown without many external inputs, nor rhizobia inoculant.

Therefore, soybean is the crop of choice that can readily be deployed to harness the benefits of SNF. There are several cultivars on the market, rhizobia inoculant use in association with soybean production is widely known and the inoculated crop generates good yields. Also, the crop has well-developed input and output markets, with attractive profits for farmers who engage in its production.

5.3 The diversity of Zimbabwean soybean root nodule bacteria

Soybean is an important cash crop in Zimbabwe. Unlike subsistence legumes, improved cultivars of soybean are available on the market and cultivation is accompanied by inoculation with the elite strain, MAR1491 (*B. diazoefficiens* USDA110^{T}), originally isolated from an effective soybean nodule in Florida, USA (Kaneko et al., 2002, Delamuta et al., 2013). Previous studies have suggested that this inoculant strain survives poorly in Zimbabwean soils (Zengeni et al., 2006, Zengeni et al., 2003). At the same time, soybean grown in Zimbabwe has also been reported to nodulate with slow-growing native strains. While the native strains have not been molecularly characterized, they are likely to be bradyrhizobia based on phenotypic characterization (Davis and Mpepereki, 1995, Musiyiwa et al., 2005a). This study sought to genetically characterize the rhizobia populations in soils with a history of rhizobia inoculation with the elite soybean strains.

For the first time, molecular characterization has shown that Zimbabwean soils host diverse populations of slow-growing soybean rhizobia, reported in chapter 3. This study demonstrated that sequencing of the *recA* gene is effective for identification of *Bradyrhizobium* species. The isolates recovered were identified as strains of *B. diazoefficiens, B. elkanii*, *B. japonicum* and *B. ottawaense. B. elkanii* was the dominant nodule occupant, comprising 61% of the total nodule isolates. The remaining isolates consisted of *B. japonicum* (21%), *B. diazoefficiens* (13%) and *B. ottawaense* (5%). The

B. elkanii and *B. japonicum* strains were broadly distributed, with the occurrence at 10 sites each, of the total 13 sites and with *B. elkanii* found in larger numbers. The results definitively show that the inoculant strain MAR1491 does not persist in the Zimbabwean farming system soils. Species *B. diazoefficiens*, to which the inoculant strain MAR1491 belongs, only made up 13% of rhizobia recovered. Strains of *B. ottawaense* were only found at four sites, in two districts. Although only a few isolates of *B. ottawaense* were recovered, they do not appear to be associated with any particular soil type or biogeography. *B. diazoefficiens* dominated numbers at two sites and was the only species isolated from Chikonyora farm in Marondera district. This strongly suggests that the site was inoculated more recently than five years previously and more likely in the season of sampling. The overall result demonstrates that MAR1491 does not persist well in Zimbabwean soils. If MAR1491 continues to be used as the inoculant strain for soybean, it is recommended that it is applied annually.

Previous reports have shown that Zimbabwean soybean root nodule bacteria are slow-growing (Davis and Mpepereki, 1995, Musiyiwa et al., 2005a) suggesting that they belong to the *Bradyrhizobium* genus. Soybean has been reported to nodulate with bradyrhizobia elsewhere in African including Mozambique (Chibeba et al., 2017), South Africa (Botha et al., 2004), Kenya (Maingi et al., 2006) Ethiopia (Aserse et al., 2012a). This diversity includes some yet unnamed species. A previous study has shown that 75 % of the strains nodulating soybean in Mozambique were bradyrhizobia while 25 % was made up of *Agrobacterium*/*Rhizobium* (Chibeba et al., 2017) . Bradyrhizobial strains have also been reported to nodulate widely in Africa including cowpea, groundnut, lablab, Bambara nut and some traditional legumes (Botha et al., 2004, Jaiswal and Dakora, 2019, Grönemeyer et al., 2017, Grönemeyer et al., 2015a, Grönemeyer et al., 2016, Grönemeyer et al., 2015b).

The *nodC* and *nifH* sequences of strains of *B. diazoefficiens*, *B. japonicum* and *B. ottawaense* showed 100% identity among themselves. Isolates tested were similar to those of the elite rhizobia inoculant strain *B. diazoefficiens* MAR1491. This suggests that they may harbour the same symbiotic island. The closest related *nodC* gene is harboured by strains of *B. huanghuaihaiense* and *B. daqingense*, isolated in China and also nodulating soybean (Wang et al., 2013, Zhang et al., 2012). The *B. elkanii* strains carried a different set of those symbiosis genes.

The wide occurrence and dominance of *B. elkanii* around the sites in Zimbabwe suggest that the strains are widely adapted to various edaphic and climatic conditions. This species may be widespread in Zimbabwean soils due to association with some native legumes. *B. elkanii* is most likely the dominant indigenous rhizobia species capable of nodulating soybean in Zimbabwean soils, while *B. japonicum* and *B. ottawaense* are found only in small numbers. *B. diazoefficiens* most likely does not occur naturally in Zimbabwean soils.

Similar to native rhizobia, failure of rhizobia inoculant strain to persist in a given soil may be a result of lack of adaptation to the prevailing biotic and/or abiotic conditions. Factors such as pH and soil nutrients (for example, phosphorus) can and should be addressed to optimize SNF. While rhizobia inoculation improves nodulation, N fixation and yields, the response is higher when phosphorus fertilizer is also applied. Soil pH and nutrient deficiencies could potentially be the reason for the limited persistence of inoculant strain MAR1491. Future research should test local strains for their adaptation to local soil pH condition, even as efforts to improve soil pH are also important.

5.4 Prospecting for new Zimbabwean soybean rhizobia inoculant strains with both saprophytic competence and effective N fixation

It is a useful property for rhizobia inoculant strains to be well-adapted to edaphic and climatic conditions present in the agricultural systems where they are being used. This supports rhizobia persistence in the soil, and thereby reduces the requirement for annual inoculation and consequently reduces farmers' costs. However, exotic elite inoculant strains can be poorly adapted to the local soil conditions and may not be competitive against the native rhizobia that are well-adapted.

There are a number of potential sources of strains that have both saprophytic competence and are highly efficient in N fixation. Horizontal gene exchanges between native and elite exotic strains have been shown to occur (Barcellos et al., 2007), and can result in new strains that have superior saprophytic competence as well as improved efficiency in N fixation (Batista et al., 2007, Chibeba et al., 2017). Horizontal gene transfer of symbiotic genes in bradyrhizobia has been reported under laboratory conditions, but recipient strains subsequently lost the genes (Minamisawa et al., 2002). Additionally, new elite strains have been postulated to be recovered from soybean nodules in croplands due to adaptation to harsh conditions by the exotic inoculant strains. Thirdly, there may be a potential pool of rhizobia strains that have been introduced with the exotic crop, soybean, but not properly recorded (Santos et al., 1999b). Finally, there may be some native Zimbabwean rhizobia that are highly effective on soybean. Therefore, the strategy used in the present study to identify potential inoculant strains that were also saprophytically competent, as shown by their ability to persist in the soils, and then screen them for N fixation efficiency. The hypothesis was that putative inoculant strains with saprophytic competence and efficient N fixation would be most readily obtained from soils with a history of

inoculation but where there was also a large population of native and/ or naturalized rhizobia.

The sites selected for bio-prospecting in this study had a history of inoculation with the elite MAR1491 and had last been inoculated five years prior to sampling. The time period between the last inoculation and sampling was to allow potential horizontal gene transfer (HGT) to occur between indigenous and elite exotic strains, as has been previously demonstrated with *Mesorhizobium* strains (Nandasena et al., 2007). The soybean rhizobial isolates recovered from these sites (chapter 3) were then evaluated for their potential to be developed as superior inoculant strains for soybean cultivars in Zimbabwe.

The isolates were firstly evaluated by measuring biomass accumulated by the inoculated soybean cultivar, Leichardt, under glasshouse conditions. From the glasshouse screening, the best-performing strains of the four species (*B. diazoefficiens*, *B. elkanii*, *B. japonicum* and *B. ottawaense*) were then tested for compatibility with three soybean cultivars commonly grown in Zimbabwe (Mhofu, Status and PAN1867) in a subsequent glasshouse trial. Finally, multi-site field-testing was conducted with each of the two best isolates per rhizobial species inoculated onto soybean cultivar Solitaire. This is the ultimate test for a potential inoculant strain, because it provides an assessment of N fixing performance under the local biotic and abiotic conditions that a strain must contend with. This approach of screening germplasm to develop elite inoculant strains has been followed with success in various Australian farming systems (O'hara et al., 2002, Howieson et al., 2011).

The results of the glasshouse trials showed that the isolates identified in chapter 3 vary greatly in their N fixation ability as measured by their biomass accumulation. This ranged from 49 to 103% in comparison to that accumulated by plants inoculated

with the elite inoculant strain MAR1491. Variability was high both within and between species, and no single species could be singled out for superior performance. Although *B. elkanii* strains made up 61% of the isolates, the best performance was by strains of *B. diazoefficiens* and *B. japonicum*, namely NAZ629 and NAZ710, respectively. The same strains NAZ710 performed the best with the three soybean cultivars under glasshouse conditions, followed by NAZ629. The other two strains belonging to *B. ottawaense* and *B. elkanii* performed similarly or worse than MAR1491. Nodulation under field conditions was highly variable and *B. elkanii* strain NAZ599 elicited the highest nodule numbers across all sites. However, the overall best strain for biomass accumulation was NAZ554> NAZ629 >NAZ710. Total N accumulation was highest at Gotora and lowest at GRS sites.

This confirms that this bio-prospecting method is a successful strategy for obtaining potential improved inoculant strains. The three strains NAZ554, NAZ629, and NAZ710 can be recommended for development as Zimbabwean soybean inoculants. The strains that showed the superior capacity for biomass accumulation of inoculated plants were of different genetic identity and geographic origin, to underscore the importance of screening a wide range of candidates (Chibeba et al., 2017). Improved inoculant strains have been recovered successfully after screening populations at least five years after the initial inoculation (Santos et al., 1999b, Batista et al., 2007, Melchiorre et al., 2011). However, the evolution of strains in the field does not always result in strains that are more efficient in N fixation (Nandasena et al., 2007).

Although *B. elkanii* was the predominant strain isolated from nodules, none of the *B. elkanii* isolates was highly effective for N fixation on soybean. It is most likely that the *B. elkanii* strains are the local indigenous rhizobia (chapter 3). This agrees with the premise that background native rhizobia are often competitive enough or in large

enough numbers to exclude elite strains in nodulation, despite that their N fixation is suboptimal (Thies et al., 1991). Elsewhere, in Mozambique and Brazil, strains of *B. elkanii* designation are used as rhizobia inoculants (Chibeba et al., 2017, Menna et al., 2006). Perhaps further searches for new rhizobia inoculants may yield *B. elkanii* strains, which have the superior saprophytic competence. It appears that *B. ottawaense* is not as effective for N fixation as other soybean rhizobia species. *B. diazoefficiens* strains have been shown to be highly effective in N fixation with soybean over a range of biogeographic sites (Brazil, Australia, Canada, China).

Beyond high N fixation, and competitiveness for nodule occupancy, rhizobia inoculant strains must display other properties that can be tested under controlled environments. Strains must be amenable to laboratory multiplication for inoculant production (Deaker et al., 2016, O'hara et al., 2002). They must be compatible with as wide a range of target crop cultivars as possible. Results from the present study showed that the test strains had compatibility with the five test soybean cultivars, Leichardt, Mhofu, PAN1867, Status and Solitaire (chapters 3 and 4).

Strain performance under field conditions is confronted by many biophysical conditions. In the tropics, this often comes with challenges of high temperature, low soil pH, low soil fertility and extended drought periods, leading to low soil moisture. Rhizobia strains vary in their capacity to tolerate a range of stresses (Asanuma and Ayanaba, 1990, Ozawa et al., 1999, Appunu and Dhar, 2006). Soil acidity is critical because it affects other factors, such as modification of the availability of many nutrients (Asanuma and Ayanaba, 1990). The *B. japonicum* strain NAZ554 performed the best of the eight test strains and the elite standard strain MAR1491. Soil pH was lowest at GRS of the test sites and the other strains performed poorly. Since the performance of MAR554 exceeded that of the other strains, overall, and performed very

well at the low pH site, it is the best-recommended strain for progression as a new rhizobia inoculant strain.

The present study suggests that it is possible to screen for tolerance to specific soil challenges, such as low pH. The potential of NAZ554, along with NAZ710 and NAZ629 for use as new inoculant strains should be investigated by wider on-farm trials that include low pH soils. However, because soybean grow poorly in low pH soils (Fageria and Baligar, 1999), it may be better to ameliorate soils for optimum pH along with bio-prospecting for saprophytically competent rhizobial strains.

Conclusions

This study suggests that legumes are not being used to their full potential in Zimbabwean farming systems. This study has identified that there is a need to educate farmers more on the benefits of high biomass legumes. There is also a need for the development of improved cultivars of the legumes cowpea, groundnut and pigeon pea to improve yields and SNF. The benefits of rhizobia inoculation were demonstrated in this study and farmers should be educated about these, along with improving access to the rhizobia products.

This study has demonstrated for the first time that soybean in Zimbabwe is nodulated by taxonomically diverse species of Bradyrhizobium (*B. diazoefficiens*, *B. elkanii, B. japonicum* and *B. ottawaense*). *B. elkanii* strains were the predominant nodule occupants. However, these were less effective in N fixation than the *B. japonicum* and *B. diazoefficiens* strains.

As a result of screening *Bradyrhizobium* isolates from soybean, three isolates have been selected as potential improved inoculant strains that outperformed MAR1491 in field trials. However, these trials were conducted at sites with comparably

low pH soils and there may be a need to ameliorate these soils to achieve optimum yields.

5.5 Future directions

There is a need for more research that explores the options for farmers to integrate more legumes into their farming systems. Breeding programs for higher-yielding legumes with higher SNF capacity must be prioritized. Future work must focus on the need to identify rhizobia inoculant and crop cultivar combinations for groundnut, cowpea and pigeon pea that work well in Zimbabwean conditions. New methods for identification of elite and competitive rhizobia strains such as matrix-assisted laser desorption/ionization- time of flight (MALDI-TOF) should be adopted (Ziegler et al., 2015). One approach would be to trial elite strains that may be sourced from other culture collections. This would be followed up by testing them for suitability in Zimbabwean conditions, in a similar pattern to the studies reported in this thesis for soybean.

Conducting the trials from this thesis with several legumes at one site, focusing on legumes and rhizobia inoculants should be carried out on-farm to demonstrate the potential of legumes and give farmers a visual from which to choose what fits their farming system best.

There is a need for more research to support the inoculant industry with more rhizobia strains that are saprophytically competent, competitive and efficient in nitrogen fixation. For soybean, the strains discovered in this thesis should be progressed for commercial production. This must be preceded by studies on the suitability of the strains for commercial inoculant production and shelf life with the Zimbabwean carrier materials.

The option for strains that are tolerant of otherwise stressful edaphic conditions, including low soil pH and low soil fertility will increase the extent of land that can be put under SNF. Research must include the molecular basis of survival and tolerance of low pH conditions. However, for immediate benefit to the farmers, it may be more productive to ameliorate soil pH and soil fertility to improve the performance of both the soybean crop and the rhizobia.

Field-testing of any agronomic technologies is critical. The challenges to maximizing SNF in the field are diverse. They reduce the efficiency of SNF by constraining the rhizobia, the crop and the symbiosis. There is also a need to address these challenges, such as soil pH and nutrition, in order to improve overall system performance.

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