The potential for bradyrhizobia and phosphate solubilising microorganisms to improve soybean (*Glycine max* (L.) Merr.) production in acid soils in Ethiopia

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Acronyms and abbreviations

AGB	above ground biomass
Al $(H_2PO_4)_3$	aluminum dihydrogen phosphate
ANOVA	analysis of variance
ATP	adenosine tri-phosphate
AZ	Assossa zone
BD	bulk density
$Ca_3(PO_4)_2$	tri-calcium phosphate
$CaHPO_4$	calcium hydrogen phosphate
CCaMK	calcium/calmodulin-dependent kinase
CD	colony diameter
cfu	colony forming unit
CSA	Ethiopian Central Statistics Agency
D	Day
E	effective
EPS	exopolysaccharide
$FePO_4$	iron phosphate
HD	halo zone diameter
HE	highly effective
Its	infection threads

ITS	inter-transcribed spacer
LCO	Lipochitooligosaccharide
LE	low effectiveness
LPS	lipopolysaccharide
LSD	least significant difference
LysM–RLK	lysine motif receptor-like kinase
MPN	most probable number
$\%N_{dfa}$	%N derived from atmosphere
Nod	nodulation gene
Nif	nitrogen fixing gene
NF	nod factor
N_2	Dinitrogen
Nr	reactive nitrogen
NH_3	Ammonia
NA	not applicable
NS	not significant
O_2	Oxygen
PBI	phosphorus buffering index
PGPR	plant growth promoting rhizobacteria
pM	pico molar

PSM	phosphate solubilising microorganism
RCBD	randomised complete block design
SDW	shoot dry weight
SE	south Ethiopia
SNNP	Southern nations and nationalities peoples of Ethiopia
SWE	South west Ethiopia
T	Tonne
TCP	tri-calcium phosphate
TD	total diameter
Tg	tera gram
TSP	triple super phosphate
V	Vanadium
WE	Western Ethiopia
YMA	yeast mannitol agar
Ml	micro-litre

Abstract

Nitrogen (N) and phosphorus (P) are two significant plant growth-limiting elements that are required in relatively large amounts and often are deficient. Both N and P are typically deficient in soils of Sub Saharan Africa. Amelioration of these deficiencies is difficult in Sub Saharan Africa since chemical fertilizers are prohibitively expensive for small holder farmers. Microbial inoculants that enhance the access to N and P could potentially be used to alleviate N and P deficiencies and provide an inexpensive means of providing these nutrients in this region.

In Ethiopia, rhizobial inoculants are currently being promoted to legume growers. Site-specific field experiments have demonstrated yield improvements due to rhizobial inoculants for different legume crops. However, for some of the grain legumes, such as soybean, multi-location demonstrations have shown inconsistent responses to inoculation. The reasons for the variability in the responses to inoculants is, however, not clear.

Around forty percent of the arable land in Ethiopia is of low pH (less than 5.5), which is one of the constraints for successful cultivation of crops. Soil acidity limits N fixation through its detrimental effect on legume growth, the survival of rhizobia, and its influence on the symbiotic interactions. In addition, soil acidity reduces the availability of P to plants, further limiting both plant growth and N fixation. Therefore, this study aimed to identify acid tolerant rhizobial inoculants and phosphate dissolving bacterial inoculants as a means to improve soybean production in acid soils of Ethiopia. In doing so, the presence, effectiveness, acid tolerance and diversity of soybean rhizobial populations resident in Ethiopian soils were investigated. Phosphate solubilising microorganisms were also isolated from soils that grew soybean and their effect on soybean yield was investigated.

Rhizobial strains isolated from nodules of soybean grown on Ethiopian soils were screened *in vitro* for their acid tolerance. The acid tolerant strains were then evaluated for symbiotic effectiveness in a controlled environment. Following this, the most effective acid tolerant strains were evaluated in six field experiments in major soybean growing areas of Ethiopia. Inoculation with a commercial rhizobial strain, or two locally-sourced isolates of rhizobia were used as inoculants resulted in improved soybean yield. The yield increase due to inoculation with the commercial strain was consistent and greater than that of other treatments, while the increase due to these two most effective locally-sourced strains was comparable to, or greater than, application of 46 kg N ha^{-1} in soils, where the resident rhizobial population was $\leq 1.4 \times 10^3$ cfu g^{-1} soil. For soils with high background rhizobial populations, there was no nodulation response to inoculation. At one of the experimental sites (Bako), the percentage of N derived from the atmosphere ($\%N_{dfa}$) was 55% for the commercial strain and 35% for a local isolate, *Bradyrhizobium japonicum* strain ES3. Field validation was observed to be a necessary step in the selection of acid tolerant strains of rhizobia, to increase soybean production in Ethiopia.

Genetic diversity of twenty of the 55 acid tolerant isolates was determined by comparing their 16S-23S internal transcribed spacer sequences. The acid tolerant strains were found to have high symbiotic and phylogenetic diversity, relative to the type strains. The acid tolerant strains were also shown to be phylogenetically distinct from most of the type strains used, as well as from most of the previously isolated Ethiopian soybean strains. However, multilocus sequence analysis of the core and symbiotic genes are required to determine their exact taxonomic position relative to Bradyrhizobium and other related genera.

During acid tolerance screening of rhizobial isolates, P solubilising *Bacillus* spp. and a *Pseudomonas* sp. were isolated from soils that grew soybean. Selected phosphate solubilising strains significantly increased the soluble P relative to controls in liquid cultures containing

Al, Fe and Ca bound P sources. The increase in available P in the culture solution was accompanied by a decrease of up to 1.7 pH units in the culture media. Strains that dissolved the highest amount of P in liquid cultures were selected and tested in six field experiments in Ethiopia, in separate plots adjacent to the N fixation experiments. Field experiments where soybean was inoculated with phosphate solubilising organisms appeared to show trends of yield increases over the controls, but the increases were not statistically significant. As an example, among the inoculant strains, strain EPS1 resulted in an average yield increase of 13.8% over the control that was not supplied with P. Further investigation of this strain in a new inoculant formulation, such as seed co-inoculation with an effective rhizobial inoculant would be worthwhile.

Finally, the presence and abundance of soybean nodulating rhizobia and the N fixing effectiveness of soil rhizobial populations were tested in 55 soils collected from major soybean growing areas of Ethiopia, using the most probable number and whole soil inoculation techniques in a controlled environment. Rhizobial population estimates of the soils ranged from non-detectable to $>1.5\times10^4$ cfu g^{-1} soil and 49% of the soils had rhizobial populations of <300 cfu g^{-1} soil. Soybean plants that received soil suspensions had shoot dry weights ranging between 45 to 142 mg $plant^{-1}$, compared with 60 mg $plant^{-1}$ when un-inoculated and 136 mg $plant^{-1}$ when inoculated with a reference strain (*Bradyrhizobium japonicum* strain CB1809). Comparison of shoot dry weights of plants inoculated with a reference bradyrhizobial strain (CB1809), soil suspensions or control treatments (un-inoculated and not fertilized) showed that among soils with rhizobial populations of >300 cfu g^{-1} soil, 13% contained ineffective rhizobial populations, while 15% contained moderately effective and 72% of soils contained effective rhizobial populations. Soils from southwestern Ethiopia had larger and more effective rhizobial populations while soils from South Ethiopia, West Ethiopia, and Assossa areas mostly contained few and/or ineffective populations.

Therefore, widespread inoculation responses are unlikely in Southwestern Ethiopia, while extension efforts related to inoculation of soybean should be targeted to South Ethiopia, West Ethiopia, and Assossa areas to provide the greatest likely benefit.

The results of this study are relevant to the soybean industry, inoculant companies, and the rhizobial inoculation programs of various governmental and non-governmental institutions in Ethiopia and potentially in other African countries with low soil pH where soybean is grown. Low soil pH and the population density of resident soil rhizobia are shown here to be important factors that have contributed to inconsistent responses to soybean inoculation in Ethiopia. The results showed soybean yield in acid soils can be improved as a result of selection and testing of new isolates from a combination of controlled environment screening and field evaluation. Genetic analysis has indicated effective rhizobia are not specific to a particular taxonomic group, or to a particular location, which indicates that an effective strain may be selected randomly from any region.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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September 2017

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Chapter 1 Introduction

Ethiopia is an agrarian country, where 85% of the population directly participates in agriculture. The occurrence of diverse agro-ecological regions in Ethiopia allows the cultivation of several legume crops, with different adaptations. These legume crops are categorized as cool season legumes (e.g. lentil, faba bean, field pea, chickpea, grass pea, lupin and fenugreek) and mid- to low-altitude legume crops (e.g. haricot bean, mung bean, cowpea, and soybean). In the 2015/2016 cropping season, pulses were grown on 2.1 million hectares of land, accounting for 14.7% of the total cultivated land, with a total production of 3.5 million t (CSA 2017).

Legumes play a significant role in Ethiopian agriculture. The crop legumes (pulses) are important sources of cheap protein for the wider population and are consumed daily. The tree and forage legumes are used as a source of feed for animals and play a crucial role in maintaining soil fertility and preventing soil degradation. Additionally, legume crops are sown as a break crop to prevent disease in cereal crops, and to restore soil fertility when the soil nutrient supply declines due to repeated cultivation and due to low fertiliser inputs. Legume crop production is also used as a means of income generation for small holder farmers, and it is a significant source of income for the country (Atnaf et al. 2015).

Among the crop legumes cultivated in Ethiopia, soybean is gaining popularity among small holder farmers and commercial soybean growers because of its local consumption as food and use in animal feed, and its use in industrial processing as food additives and supplements. It is also becoming an export commodity in the international market, making it a valuable crop for the country (Hailu and Kelemu 2014). Between 2002 and 2012, the cultivation of soybean in Ethiopia increased 10-fold while its production volume increased 21-fold (Hailu and Kelemu 2014).

Despite soybean growing in importance in Ethiopia, its productivity is low at two t ha^{-1}, (Bekabil 2015), being constrained by low soil fertility, limited agricultural inputs and lack of improved varieties (Bekabil 2015; Jaiswal et al. 2016). All these factors slow its wider adoption in the country (Hailu and Kelemu 2014).

Improving the productivity of legume crops such as soybean is critical for improving the livelihoods of the population, the environment, and the economy of the country. Ethiopian soils are generally low in N and P (Haileslassie et al. 2005) while soybean has a high N demand (Herridge, 2002) and P deficiency severely limits N fixation . 1988). The occurrence of acid soils on 40% of the arable land of Ethiopia (Bekabil 2015) further limits crop production in the country. Low soil pH affects the legume crop, the symbiotic rhizobia and the function of the symbiosis in general (Giller 2001). Available P is limited in low pH soils due to precipitation with Al and Fe. In acid soils, P fertilisers have low efficiency due to reaction with these readily available cations, rendering P insoluble (Richardson 2001). In addition, low pH soils may have toxic concentrations of Al and Mn, while they are typically deficient in Ca, Mg, P, Fe and Mo, which further limits the symbiotic potential (Richardson 2001; Indrasumunar et al. 2011). Fertilizers are prohibitively expensive for small holder farmers in Ethiopia and the application of alternative cheap microbial inoculants is currently being pursued to improve soil fertility.

Application of inoculants to improve soybean N fixation and yield has been successful in Ethiopia (Aserse et al. 2012; Jefwa et al. 2014) but not in all growing areas (Aserse et al. 2012). The reasons for inconsistent responses to soybean inoculation are not clear (Aserse et al. 2012) but were proposed to be due to poor adaptation of exotic inoculant strains (Aserse et al. 2012) or competition with soil resident rhizobial populations (Jaiswal et al. 2016). In contrast to the proposal that there are significant populations of soil rhizobia, it is considered that soybean nodulating rhizobia are generally absent from Ethiopian soils, based on limited

observations (Aserse et al. 2012) and taking into consideration that the crop was a recent introduction to Ethiopia (Abate et al. 2012).

1.1. Aims

The purpose of this study was to evaluate the responses of soybean to inoculation with acid tolerant and phosphate solubilising microbes isolated from acid soils of Ethiopia. In addition, soils were sampled in key regions to assess the population densities of soil resident soybean nodulating rhizobia to understand the potential to improve soybean production through inoculation. Selected isolates of soybean rhizobia were collected from key regions to investigate the genetic diversity of rhizobia present in those soils.

In this study we hypothesised that soil acidity and variable populations of rhizobia resident in soils accounted for the variable responses to soybean inoculation observed previously in Ethiopia. Therefore, soybean production in the acidic soils was hypothesised to be improved through the application of acid tolerant rhizobial inoculants and phosphate dissolving microbes. In addition, we hypothesised that soybean rhizobial populations would be of low genetic diversity, due to the limited introduction and a short history of cultivation.

1.2. Thesis structure

The thesis is structured according to the questions listed in Figure 1.1; each question is addressed in the associated chapters. **Chapter 1** provides a general introduction, states the purpose of the research and outlines the structure of the thesis. **Chapter 2** reviews pertinent literature about the theoretical background to the research and methodologies of the experiments. **Chapter 3** describes an investigation into the yield responses of soybean to inoculation with acid-tolerant strains. The genetic diversity of acid-tolerant rhizobial strains are analysed in **Chapter 4. Chapter 5** examines the responses to inoculation of soybean with phosphate solubilizing bacteria. The abundance and effectiveness of soybean nodulating

rhizobial populations in Ethiopian soils is assessed in **Chapter 6**. In the general discussion, **Chapter 7,** the significance of results of the experiments conducted are presented in the context of the literature and potential future research activities are suggested.

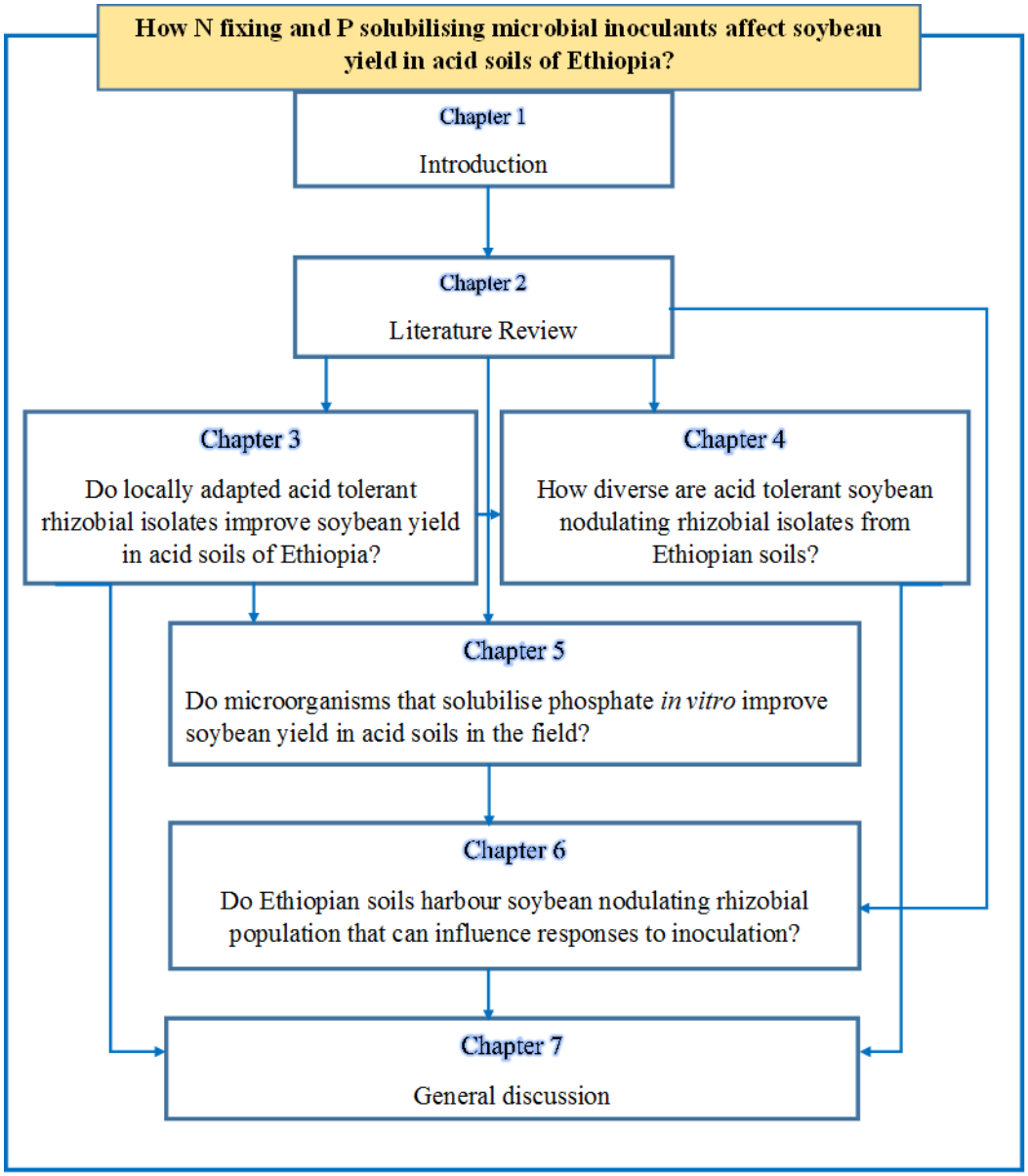

Figure 1.1. Thesis structure

Chapter 2 Literature Review

2.1. Biological N fixation

Nitrogen is one of the most important macronutrients that is required for growth and reproduction of all life forms. It is an essential building block of nucleic acids, amino acids, ATP, chlorophyll and several enzymes needed to maintain physiological processes of living things (Newton 2004). Even though N is abundant in the atmosphere (78%), in its diatomic form (N_2) only members of the bacteria and archaea are able to convert and utilise it. Other living organisms are not equipped with the ability to convert atmospheric N into a form available for plant growth.

The process that converts diatomic N into its reactive forms (Nr) is termed N fixation. N fixation can occur naturally through the action of microorganisms (diazotrophs) and hence is called biological N fixation, or through lightning or through anthropogenic activity, such as the Haber-Bosch process or combustion of fuels and biomass (Vitousek et al. 2013).

Industrial N fixation is an energy demanding process carried out at high temperatures (500-800 °K and pressure (500 atm) (Howard et al. 1996). However, diazotrophs use an enzyme, nitrogenase, to catalyse the conversion of N_2 into a reduced form under specific physiological conditions. The common and widely distributed nitrogenase enzyme, called Mo-nitrogenase, is a metalloprotein having two components, a Fe-protein component and Mo-Fe-protein component (Zhao et al. 2006). The first component is encoded by the *nif*H gene while the latter component is encoded by *nif*D and *nif*K, whereas the coordinated action of other genes is also required for the functioning of the enzyme (Yousuf et al. 2014). Three more genetically distinct and alternative nitrogenase systems have been identified. One has vanadium (V-nitrogenase) while another has Fe (Fe-nitrogenase) instead of Mo. The final one has Mo but contains additional sulphur and can function in the presence of O_2 (Newton 2006;

Zhao et al. 2006; Masson-Boivin et al. 2009). Although the N fixation process is catalysed by the nitrogenase enzyme in diazotrophs, the process of N fixation is energy-intensive requiring the expenditure of 16 moles of ATP to convert one mole of N_2 into two moles of NH_3 (Cheng 2008).

The estimated global N fixation is 413 Tg N yr^{-1} (Vitousek et al. 2013). Biological N fixation is naturally carried out in terrestrial and marine ecosystems through free living, associative and symbiotic diazotrophs (Herridge et al. 2008). A recent review of estimates of such natural N fixation showed the contribution to be 198 Tg N yr^{-1} to the global amount of fixed N, while the contribution of lightning was estimated to be 5 Tg annually (Vitousek et al. 2013). Human cultivation of crops and pastures in agricultural systems fixes 50-70 Tg N annually, of which the main N fixers are the rhizobia in symbiosis with crop legumes and oilseeds that fix 21 Tg N yr^{-1}; the rhizobial symbioses with pasture legumes and fodder legumes fix 12-25 Tg N yr^{-1} (Herridge et al. 2008). Industrial N fixation through the Haber-Bosch process provides 120 Tg N annually and the combustion of fuels and biomass together are estimated to contribute 30 Tg N yr^{-1} to the global Nr pool (Vitousek et al. 2013).

Industrial and biological N fixation are the two most important processes that contribute significantly to the flux of Nr into the terrestrial ecosystem, contributing 120 and 118 Tg N yr^{-1} respectively (Vitousek et al. 2013). Harnessing the efficient utilization of these two major processes is necessary to meet the growing world demand for agricultural produce in a sustainable manner (Herridge et al. 2008). Although industrial N fixation is the major source of fixed N for agricultural production, its economic and environmental impacts are extremely large as discussed below, and efforts are needed to efficiently exploit and enhance the biological N fixation process (Herridge et al. 2008; de Vries et al. 2012).

2.2. Industrial inputs and sustainability

The United Nations estimate that the world's population will reach 8.9 billion people by 2050 (Nelson et al. 2010). This indicates the scale of the challenge to increase agricultural production. In the past half century, the 'Green Revolution' has helped to meet the food requirements of fast-growing populations in many countries (Dyson 1999). More effective use of fertilizers and pesticides, improved germplasm, improved agronomic management, and extended use of irrigation have contributed to the success of the 'Green Revolution', resulting in hunger reduction and improvements in nutrition and have, in addition, limited the rapid conversion of ecosystems into farming land (Tilman 1998; Vance 2001; Adesemoye et al. 2009).

Applications of higher amounts of fertilizers and pesticides in intensive agriculture have resulted in a series of environmental and economic problems in past decades (Cakmak 2002). Erosion and leaching of surface soil, loss of fertility, contamination of surface and ground water bodies, eutrophication, emission of greenhouse gases, loss of diversity in plants, microorganisms and insects, spread of plant diseases and parasites are among the main agronomic and environmental problems (Tilman 1998; Vance 2001; Sing et al. 2011; Kaur et

al. 2013). Currently, claims of nutritional quality and health issues related to the use of fertilizers are being raised (Reganold et al. 1990; Carey Jr 2009; Shi et al. 2010).

On top of their profound environmental impacts, chemical fertilizers are expensive, costing agriculture more than $45 billion per year (Ladha et al. 1997); their unit cost is increasing each year in due to the high energy demand of production and transportation, and they are becoming less available to subsistence farmers in developing countries (Chianu et al. 2010). Production of N and phosphorus fertilizers depends on non-renewable resources and their supply is not sustainable in the long term (Dima et al. 1997; Richardson 2001). Consequently, the common trend of world agriculture in past decades has shifted from a focus on improving genotypes and increasing productivity using intensive inputs, to emphasizing sustainability (Peoples et al. 1995). Sustainable agriculture attempts to effectively use and manage natural resources to meet current human needs, without compromising the ecosystem for future generation use (Lal 2009) while intensive agriculture does not have this as an explicit aim.

2.3. Microbial Inoculants in sustainable agriculture

2.3.1 Microbial inoculants / Biofertilizers definition

The term “Microbial inoculants” is often used interchangeably with “biofertilizers”. However, there is no standard definition for the term ‘‘biofertilizers’’. Among the various definitions given to the term ‘‘biofertilizers’’ (Bashan 1998; Vessey 2003; Bünemann et al. 2006; Ahmad et al. 2008), is a broader definition by Fuentes-Ramirez (2006 p.144) as follows: “a product that contains living organisms that exert direct or indirect beneficial effects on plant growth and crop yield through different mechanisms”. This definition is adhered to in this thesis and the term “microbial inoculant” is used interchangeably with the same definition. In addition, the microorganisms in microbial inoculants are termed plant growth-promoting rhizobacteria (PGPR). PGPR are generally defined as “rhizosphere bacteria that can enhance plant growth

and protect plants from disease and abiotic stresses through a wide variety of mechanisms" (Souza et al. 2015 p.401). However, a recent definition by Mus et al. (2016) excludes nodule forming N fixers (rhizobia and *Frankia*) while applying it to all other organisms that enhance plant growth including associative and free-living N fixers as a subset of PGPR.

Exploitation of microbial technologies is considered as an alternative approach to help overcome the environmental, economic and supply constraints of chemical fertilisers, while ensuring a sustainable increase in production. According to Higa et al. (1994 p.6) ''beneficial and efficient microorganisms applied as soil, plant and environmental inoculants hold the greatest promise for technological advancement in crop production, crop protection, and natural resource conservation''. Hence microbial inoculants are considered as one of the major components of these technologies and there is a growing need to use beneficial microorganisms in the form of microbial inoculants either alone, or in combination with lower rates of chemical fertilizers to improve the nutrient use in low input systems (Vessey 2003; Adesemoye et al. 2009).

2.3.2 Microbial inoculants versus inorganic inputs

Microorganisms in inoculants can provide N to farming systems, mediate the transformation of important nutrients (e.g. P) from insoluble to more soluble forms, and enhance efficient recycling of organic matter (Kaur et al. 2013). In contrast to chemical fertilizers, inoculant production technology is relatively simple and fermenter installation cost is low, and the technology uses renewable natural resources (Chen et al. 2006), hence their cost is affordable to subsistence farmers in developing countries (Chianu et al. 2010). Cost of transportation of inoculants is another advantage over chemical fertilizers, as inoculants are usually produced in small packs that are applied to large areas; this is particularly important for developing countries with limited transport infrastructure (Chianu et al. 2011). On the other hand, special cool storage conditions or handling instructions might need to be provided. Microbial

inoculants are also found to improve the nutritional quality of plant products in comparison to chemical fertilizers, as demonstrated in lettuce (Baslam et al. 2011), *Moringa* (Zayed 2012) and in other organic produce (Worthington 2001; Savita 2007). Hence the scientific endeavour devoted to exploiting this technology is justifiable from environmental, social and economic points of view.

2.3.3 Microbial inoculant types and function

Many species of PGPRs are found in association with plant roots in the rhizosphere and can enhance plant growth via mechanisms including N fixation, phosphate and potassium dissolution, and phytohormone and siderophore production. (Vessey 2003; Velivelli et al. 2014; Gupta et al. 2015; Souza et al. 2015; Ambrosini et al. 2016).

2.4. Biological N fixing organisms

The world terrestrial agricultural ecosystem receives 118 Tg of fixed N annually from biological N fixation (Vitousek et al. 2013). The N fixing microbes, diazotrophs are widely distributed among phylogenetically diverse groups. Often, the only common characteristic among these diazotrophs is having the enzyme nitrogenase (Newton 2006; Yousuf et al. 2014). The *nif*H gene that codes for nitrogenase is usually used to study the phylogeny, diversity, and abundance of diazotrophs (Yousuf et al. 2014), and it is used for the estimation of populations of non-culturable diazotrophs (Van Dommelen et al. 2007). The culturable diazotrophs are grouped into free-living (those living without association), associative (living in close association with plants) and symbiotic N fixers (those that live inside plants forming a specialized structure, the nodule) based on their lifestyle (Newton 2006).

The microorganisms that are found in plant tissue are generally called endophytic microorganisms (endosymbionts) (Mus et al. 2016).

2.5. Rhizobia

Among the microorganisms that fix N, rhizobia are known to be the most efficient diazotrophs (Hassen et al. 2016). The rhizobia association with crop legumes (pulses and oil seed legumes) and pasture legumes fix 21 Tg and 12-25 Tg N, respectively, per annum, while the non-symbiotic N fixers altogether fix <24 Tg N annually into the terrestrial agricultural systems (Herridge et al. 2008).

Rhizobia are gram negative rod-shaped soil bacteria that form nodules on the roots or stems of legumes (Loureiro et al. 1995; Martins et al. 2015) and fix N symbiotically (Willems 2006).

2.5.1 Techniques to study rhizobial diversity

Large diversity in rhizobial populations have been documented (Martínez-Romero et al. 1996). Phenotypic (classical) characteristics of rhizobia including morphology, biochemical, physiological, and serological characteristics of rhizobia, together with host specificity, were used as means to identify, characterise and classify rhizobia (Vandamme et al. 1996; Nick 1998). Accordingly, rhizobial population diversity was observed among strains isolated from a single host species grown in different environments, among strains isolated from different hosts grown in the same environment, and among strains isolated from different hosts in different environments (Pongsilp 2012). Even cultivars of the same host species grown in a given environment have been shown to be nodulated preferentially by different rhizobial populations (Collins et al. 2002). However, the methods used had reproducibility issues and low discriminatory power to clearly distinguish rhizobia from each other and from other soil bacteria. Historically, grouping of legumes/rhizobia based on host specificity (cross inoculation grouping) was abandoned (Wilson 1944) as strains with very wide host-range, such as *Rhizobium* sp. NGR234 which has a host range as large as 120 legume specie were

identified. In addition, legumes like soybean and common bean are found to be infected by more than one genetically different rhizobial strain (Sessitsch et al. 2002).

Studying such a wide diversity of agriculturally important beneficial microorganisms could be difficult when based only on classical characteristics of the rhizobia (Thies et al. 2001). Understanding the diversity and phylogeny of rhizobia is currently more easily obtained using several genetic methods (Thies et al. 2001; Pontes et al. 2007). A list of molecular techniques used in the study of diversity and taxonomy of rhizobia is given in Table 2.1. However, each of the techniques has a drawback as reviewed in Lagos et al. (2015); for example, those techniques used to study bacterial community were able to capture only dominant groups in a community. In identifying rhizobia at strain level, specifically *bradyrhizobia*, sequencing 16s-23s rRNA region gave similar results to DNA-DNA hybridization, which is considered as a gold standard in identifying microorganisms. Hence, sequencing 16s-23s rRNA region is used in identifying strain in this thesis.

Table 2.1. Molecular markers and methods used at different taxonomic levels of resolution in the study of microorganisms.

(Adapted from Thies et al. (2001)).

Level of resolution	**Target**	**Method**	**Reference**
Community DNA	16S rDNA	T-RFLP	Clement et al. (1998)
	16S rDNA	DGGE	Vallaeys et al. (1997)
Genus	16S rDNA	TGGE	Heuer et al. (1999)
	16S rDNA	PCR, sequencing	Ludwig et al. (1998)
	16S rDNA	T-RFLP	Clement et al. (1998)
	23S rDNA	PCR, RFLP	Terefework et al. (1998)
Species	16S rDNA	PCR, ARDRA, RFLP	Vinuesa et al. (1998)
	16S-23S rDNA IGS	PCR, RFLP	Frémont et al. (1999)
Strain	Rep elements	PCR-fingerprinting	Niemann et al. (1999)
	RAPDs	PCR-fingerprinting	Paffetti et al. (1998)
	Directed primers	PCR-fingerprinting	(Hebb et al. 1998)
	GC-rich arbitrary PCR primers	PCR-fingerprinting	González-Andrés et al. (1998)
	16S-23S rRNA,	PCR, sequencing	Willems (2006)
Gene: plasmid	*nif* or *nod* genes	Nested PCR	Widmer et al. (1999)
	*nif*H gene	PCR, RFLP	Chelius et al. (1999)
	*nfe*A gene	PCR	Hartmann et al. (1998)
Gene: chromosome	*dct* or *rec*A genes	Targeted PCR-fingerprinting	Perret et al. (1998)

Level of resolution	Target	Method	Reference
	leghemoglobin genes	FISH	Uchiumi et al. (1998)
Gene: plasmid or chromosome	*gus*A or *lac*I markers	Introduced marker gene technique	Wilson et al. (1999)
All levels	Specific DNA sequences	Subtractive hybridisation	Cooper et al. (1998)
	Core genes	Multilocus sequence analysis (MLSA)	Zeigler (2003), Mousavi et al. (2015)
	homologous genomes	Average nucleotide identity (ANI)	Konstantinidis et al. (2007), Rashid et al. (2015)
	Combination of rhizobial characteristics: Classical (Phenotypic, physiological, biochemical) and genetic characteristics including G+C content, DDH, rRNA sequences, FAME profiles)	Polyphasic taxonomy	O'Hara et al. (2016), Vandamme et al. (1996)
	Whole genome sequence	Genotaxonomy	Ormeno-Orrillo et al. (2015), Shamseldin et al. (2017)

2.5.2 Rhizobia taxonomy

Rhizobial taxonomy has been refined over time as techniques used to characterize rhizobia have improved and new symbiotic legumes have been investigated (Berrada et al. 2014; Shamseldin et al. 2017). All legume-nodulating rhizobial strains were initially categorized under the genus *Rhizobium* consisting of only six species (Somasegaran et al. 1985). Just over a decade ago, rhizobia were categorized under six genera (Sylvia et al. 2005) including *Allorhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium*. After several reshufflings within the groups and addition of new groups (Sylvia et al. 2005; Willems 2006; Rivas et al. 2009; Zhang et al. 2012; Mousavi et al. 2014; Mousavi et al. 2015; Ormeno-Orrillo et al. 2015), rhizobia are currently classified into 238 species in 18 genera and two super clades of α-proteobacteria and β-proteobacteria (Shamseldin et al. 2017).

Only 23% of the nearly 19,300 species of the legume family Fabaceae (Andrews et al. 2017) have been studied for their symbiotic association with rhizobia; identification of new rhizobial groups in the future is highly likely (Berrada et al. 2014).

2.5.3 Nodulation

Both the process of nodule formation and signal transduction between the symbionts have been reviewed intensively (Sprent 2007; McAdam et al. 2017; Demidchik et al. 2018; Diédhiou et al. 2018) and only a brief account of the process is presented. Nodulation of legumes by rhizobia is a result of chemical signalling between the two partners. The host plants initiate nodulation by sending out signalling molecules (flavonoid) (Nelson et al. 2015) during N limiting conditions (Masepohl et al. 2007). The molecular dialog between the partners can be nod factor (NF) dependent or independent (Giraud et al. 2007; Masson-Boivin et al. 2009; Bonaldi et al. 2011; Gourion et al. 2015). In the NF dependent signalling, the

inducer molecules released by the plants can be either flavonoids (including luteolin, methoxychalcone, naringenin, or daidzein) or non-flavonoid inducers (trigonelline or stachydrine) (Long 1996). Once signalling molecules reach the rhizobial cells, they initiate transcription of nod genes (*nod*ABC) to form nod factor (NF), a lipochitooligosaccharide (LCO). The NF is recognised by receptor-like kinases of the epidermal cells (Oldroyd 2013) of the root, and initiates the formation of infection threads (ITs) through which the bacterial cells enter into the cortical cells of the root. While the cortical cells grow into the nodule structure, certain cells inside the growing nodule engulf the rhizobial cells and encircle them with a peribacteriod membrane (Simms et al. 2002), resulting in organelle like structures, symbiosomes (Downie 2014) with differentiated bacteroids inside, ready to fix N. Both the N fixed by the bacteroids and the carbohydrate from the plant can cross the peribacteroid membrane.

In the molecular dialogue between legumes and their rhizobia, there are points of control at different levels of the interaction to ensure correct matching between the groups of host plants and the specific rhizobial species or strains with which the legumes form a symbiosis (specificity) (Simms et al. 2002; Oldroyd 2013; Andrews et al. 2017). The first level of specificity regulation is the secretion of specific flavonoids by the legume (Wang et al. 2012) that will be recognised by only specific species or strains of rhizobia. The specific flavonoids that are recognised by the rhizobial transcription regulator (NodD) trigger the production of NF. NFs differ in their primary structure and in their decorations or functional groups and only a specific type of NF that is recognised by the plant can induce root hair curling, infection thread formation, and nodule organogenesis. This is the second point where specificity is maintained in the host-microsymbiont interaction. Further specificity control by the plant is carried out during the movement of the rhizobia through the infection thread and their release into the cortical cells of the roots (Simms et al. 2002; Wang et al. 2012). The

plants recognise the surface polysaccharides of the bacteria through the release of receptor lectins, in which case the correct rhizobia are allowed to multiply, are released into cortical cells and differentiate (Simms et al. 2002).

During NF-independent signalling, the rhizobial cells enter through cracks of the root or directly through junctions of root epidermal cells (Ardley et al. 2013; Czernic et al. 2015). Nodule organogenesis might be initiated via auto-activation of the legume independent of the rhizobial inducer (Gleason et al. 2006) or due to the accumulation of cytokinin-like compounds released by the rhizobia (Masson-Boivin et al. 2009). However, the exact nature of the initial plant inducers that attracts rhizobia is yet to be determined (Masson-Boivin et al. 2009).

2.6. Factors affecting response to rhizobial inoculants

Improving global N fixation by 10% would result in an economic return close to US$ 1 billion annually (Herridge et al. 2000). One way of improving N fixation in farming systems is through inoculation of legumes with effective N fixing strains (Brockwell et al. 1988; Brockwell et al. 1989; Sessitsch et al. 2002). However, inoculation doesn't always result in increased N fixation in legumes (Thies et al. 1991; Singleton et al. 1992) due to factors related to technology and quality of inoculants (Brockwell et al. 1995), biotic and abiotic factors that interact with both the inoculant strain and the host plant (Hungria et al. 2000; Giller 2001; Sessitsch et al. 2002; Vuong et al. 2017).

2.6.1 Inoculant delivery method

Currently, commercial inoculants are available for seed inoculation, usually to be applied in the form of slurry or suspension, or for soil application (in-furrow application) either in granular or liquid forms (Deaker et al. 2004; Denton et al. 2017). Although each method has its merits and disadvantages (Brockwell et al. 1995; Brockwell et al. 1995; Denton et al. 2007;

Drew et al. 2012; Deaker et al. 2016), their suitability to the environmental conditions, and the crop type needs to be considered (Denton et al. 2017). Novel formulations of carrier material that can maintain high populations of the inoculant strains upon storage and on seeds is a valuable research area, with significant gains to be made (O'Callaghan 2016).

2.6.2 Inoculant quality

Many of the inoculants produced worldwide have been of poor or substandard quality (Brockwell et al. 1995; Lupwayi et al. 2000; Catroux et al. 2001; Bullard et al. 2005) due to low populations of inoculant strains resulting from either contamination, toxic effects of the carrier material or poor storage conditions. A national independent regulatory body is important to ensure that growers receive quality products and to avoid dissemination of contaminants or ineffective strains into the environment (Brockwell et al. 1995; Lupwayi et al. 2000; Deaker et al. 2016)

2.6.3 Competition for nodulation

Competition for nodulation and nodule occupancy between the inoculant strain and the soil resident rhizobial population (which may be ineffective or less effective) is a common challenge for inoculation programmes around the world (Brockwell et al. 1982; Denton et al. 2000; Hungria et al. 2000; Brockwell 2001; Sessitsch et al. 2002; Wielbo 2012).

Clear understanding of the mechanisms of interactions among competing strains in the soil and strains of inoculants for nodulation of the host plant is lacking, however, interventions to manage the competition problem are based on the assumption that successful nodulation is a result of numerical dominance (Thies et al. 1991; Brockwell et al. 1995; Sessitsch et al. 2002). Based on this assumption, a number of conventional and genetic approaches to understanding competition have been used, at least in controlled conditions, as reviewed by Sessitsch et al. (2002). As indicated in Sessitsch et al. (2002), the techniques focus on

increasing competitiveness of the required rhizobia through selection traits that would render competitive advantage over the resident populations or using management practices that would favour the target strain to get numerical advantage for nodulation. Among the mentioned techniques, selection of acid tolerant rhizobia helps to maintain rhizobia population needed for nodulation and nitrogen fixation under acid stressed soils.

2.6.4 Environmental factors affecting inoculation response

The effect of environmental factors on both the symbionts and the N fixing process has been reviewed in some detail previously (Zahran 1999; Slattery et al. 2001; Sadowsky 2005; Andrés et al. 2012; Lebrazi et al. 2014).

The selection and development of stress tolerant strains have contributed to increasing legume production and N fixation in several crops. Examples include acid tolerant strains nodulating common bean (Graham et al. 1982), annual medic (Howieson et al. 1986) and soybean (Hungria et al. 2000). Similarly, high temperature (40 °C) tolerant common bean nodulating rhizobia (Hungria et al. 1993) and salt tolerant soybean rhizobia (Elsheikh et al. 1995) have been reported. Consequently, it is noted that low pH inhibit the signalling between rhizobia and legume partner as indicated by Richardson et al. (1988) and Hungria and Stacey (1997) which critically affects nodulation and nitrogen fixation. When the rhizobia are exposed to low pH, they undergo structural and physiological changes to adapt the new environment as reviewed by Lebrazi and Benbrahim (2014) that ultimately affect the symbiotic nitrogen fixation. Hence, to improve nitrogen fixation under low pH, screening acid tolerant strains would be critical.

2.7. Biological N fixation in soybeans

2.7.1 Soybean use and world production

Soybean is one of the most valuable and versatile crops in the world. It is a cheap source of quality protein for both human consumption and animal feed and is used in the production of numerous products including biodiesel blends, inks, plasticizers, paints and cosmetics (Cahoon 2003; Erickson 2015). Twentynine percent of the vegetable oil in the world market is produced from soybean, and hence it is considered as an important oilseed crop (USDA 2017).

Due to the demand for soybean products, production has increased worldwide by an average of 4.6% annually from 1961 to 2007 (Masuda et al. 2009). Annual production of soybean was 351.74 million metric t in 2016/17 and 312.87 million metric t in the 2015/16 cropping season (USDA 2017). In 2016/17, the world average yield for soybean was 2.92 metric t per hectare, while it was 3.5 for USA and 3.36 for Brazil (USDA 2017). Soybean production was predicted to continue growing at an annual rate of 2.2% and reach 370 million metric t by 2030 (Masuda et al. 2009). However, soybean production is low in Africa, and well below 2 t per hectare particularly in Sub Saharan soybean-growing countries. An example, Uganda's average yield was 0.6 t per hectare in 2016/17 (USDA 2017).

2.7.2 Soybean biological N fixation

Among cultivated annual legume crops, soybean fixes the largest amount of N, up to 450 kg N ha^{-1} (soybean harvested from 67.6 $x10^6$ ha of different countries) (Unkovich et al. 2000). In good field conditions, soybean fixed over 300 kg N ha^{-1} at Washington (Bezdicek et al. 1978; Keyser et al. 1992), hence farming systems that include soybean have a lower requirement for N fertilizer (Sinclair et al. 2014). This high N fixation capacity of soybean is related to its higher physiological N demand as it produces seeds with high N content (Pagano et al. 2015).

Recent estimates showed that soybean contributes up to 10.4 Tg N annually to the world agricultural system (Hungria et al. 2005; Gelfand et al. 2015).

Soybean is estimated to fulfil its 50-60% of its N demand from N fixation. Other estimates from Brazil also showed that it can fulfil up to 80% of its physiological (Alves et al. 2003) demand from N fixation. Application of 50 kg N ha^{-1} either at sowing or at pod filling stage when N fixation is thought to slow down did not affect N fixation and yield of soybean have shown that it has no effect (Zapata et al. 1987; Albareda et al. 2009) and the current recommendation of application of 50 kg N ha^{-1} for North America is questionable (Gelfand et al. 2015). Higher amounts of nitrates are known to affect nodule formation, function and N fixation in soybean (Saito et al. 2014) and application of N to soybean is generally considered unnecessary and harmful to the environment (Gelfand et al. 2015).

2.7.3 Soybean nodulating rhizobia

Several strains of rhizobia forming a symbiotic association with soybean have been reported in *Bradyrhizobium* genera, while few strains are also identified from *Sinorhizobium* and *Mesorhizobium* genera. Based on their growth rate and taxonomic position, slow growers such as *B. japonicum*, *B. ottawaense* and *B. diazoefficiens* (Jordan 1982; Delamuta et al. 2013; Yu et al. 2014), fast growers such as *S. fredii* (Chen et al. 2000; Hungria et al. 2001) and S. *xinjiangense* (Peng et al. 2002) are identified. Species with variable generation time are also reported, such as *M. tianshanese* (Chen et al. 1995). While the taxonomic positions of the above-mentioned species are already determined, distinct species that need further description are reported from different regions of the world (Vinuesa et al. 2008; Appunu et al. 2009; Zhang et al. 2011; Aserse et al. 2012).

2.7.4 Soybean production and N fixation in Ethiopia

Soybean is a recently introduced crop in Ethiopia (Abate et al. 2012). Due to a growing demand for local consumption and for the export market (Hailu et al. 2014) the area of land allocated for its production is expanding quickly, with the total yield of 1600 t in 2002 to 61,000 t in 2014 (Bekabil 2015). However, the productivity of the crop is low in small holder farms (CSA, 2012). Due to the involvement of commercial farmers and technology dissemination efforts, it was only in 2014 that the national average was raised to 2 t per ha in Ethiopia (Bekabil 2015).

Among the factors contributing to low soybean yield in Ethiopia, limited access to improved varieties, inorganic fertilizers, and low soil fertility are the major ones (Atnaf et al. 2015; Jaiswal et al. 2016). In particular, Ethiopian soils are low in N and P due mainly to erosion (Haileslassie et al. 2005) and there are large areas (40%) covered with low pH soils (Schlede 1989). Hence efficient crop production, including soybean, is a major challenge as agricultural inputs are limited.

There is a growing need to use soybean rhizobial inoculants to improve soybean production in Ethiopia. Imported commercial inoculants have shown improved yields in some areas of the country (Solomon et al. 2012; Jefwa et al. 2014); however, the results were inconsistent for different locations and the reasons for this are not clear (Aserse et al. 2012). Lack of adaptation of the imported strains for the local conditions (Aserse et al. 2012) or competition with indigenous rhizobial populations (Jaiswal et al. 2016) were proposed as possible reasons. However, the presence of indigenous soil rhizobia population has not been systematically addressed, and there was even an assumption that soybean nodulating rhizobial populations might be absent from Ethiopian soils as the crop is a recent introduction (Aserse et al. 2012). Therefore due to the growing demand of the crop and the limited fertiliser supply in the

country, utilising rhizobial inoculant more widely and effectively would be beneficial for the soybean industry in the country.

In order to effectively utilise the benefit of N fixation in Ethiopia, assessment of the population distribution of soybean rhizobia in agricultural soils and selection of acid tolerant effective strains are crucial.

2.7.5 Assessment of resident soil population size and effectiveness

Previous experiments indicated that inoculation with compatible rhizobia was necessary to improve nodulation and yield of soybean where the crop is introduced into tropical soils in which the associated rhizobia are naturally absent or low in number (Caldwell and Vest, 1968). In places where sufficient numbers of effective bradyrhizobia are present, soybean achieves satisfactory yields (Bhangoo et al. 1976; Abaidoo et al. 2007).

The population size of effective rhizobia that are resident in a soil is a reliable predictor of the need to inoculate a legume (Brockwell et al. 1988; Thies et al. 1991). Determining the effectiveness of indigenous rhizobial populations can be a lengthy process that typically involves isolation of representative strains and screening their effectiveness in microbiologically controlled environments (Brockwell et al. 1988). Conversely, the use of agronomic trials to determine the need for inoculation is often limited by soil heterogeneity, climatic variability and statistical interpretation (Singleton et al. 1992). Hence, there are limitations to both microbiological and agronomic procedures for determining the need for inoculation.

Assessment of the symbiotic effectiveness of a population of soil rhizobia can be more accurately and quickly performed in the laboratory or greenhouse using the “whole soil inoculation” technique, developed by Brockwell et al. (1988). The whole soil inoculation technique involves inoculating plants that are grown aseptically in pots receiving N-free

nutrient solution with a suspension of the test soil. The whole soil inoculation technique, coupled with determining the number of rhizobia per gram of soil via the MPN technique, provides an assessment of the need for inoculation (Denton et al. 2000).

2.7.6 Screening of acid tolerant rhizobia

Agricultural production in acid soils is mainly constrained by toxic concentrations of H^+, Al, and Mn, and deficiencies in Ca, Mg, P, Fe and Mo (Richardson 2001; Indrasumunar et al. 2011). Thus, legume production in acidic soils is constrained by these acidity related problems affecting the growth of the legume, the persistence and effectiveness of rhizobia, the nodulation process and the function of the symbiosis in general (Date et al. 1979; Howieson et al. 1986).

Amelioration of soil acidity problems by the addition of lime is expensive (Foy et al. 1987). A relatively cheaper approach to tackling the problem is a selection of tolerant crops (Taylor 1991) and/or their associated microorganisms (White 1966). Successful selection of acid and aluminium tolerant strains of soybean nodulating rhizobia from Brazil soils have been reported (Hungria et al. 2000). Similarly, acid tolerant rhizobia for common bean (Vargas et al. 1988) and for *Medicago* (Howieson et al. 1986) were selected from soils and resulted in significant yield increase in acid soils. Many researchers have attempted to identify acid tolerant strains of rhizobia for forage and food legumes using laboratory based protocols (Date et al. 1979; Keyser et al. 1979; Wood et al. 1984; Howieson et al. 1986).

The media developed for acid tolerance screening were mainly liquid and were not efficient for screening large numbers of isolates. Ayanaba et al. (1983) used solid media based on a liquid medium previously devised by Keyser et al. (1979) to manage a large number of strains. Media developed for screening acid tolerance vary either in the concentrations of Al,

Mn, Ca and P (Keyeser et al. 1979; Ayanaba et al. 1983; Richardson et al. 1988; Howieson et al. 1988; Gemell et al. 1993).

The majority of laboratory screening programs have not been translated into the field and some of them that tried to do so failed to repeat the laboratory result in the field soil environment (Howieson et al. 1988; Indrasumunar et al. 2012) as the screening media used were not good simulators of the soil environment. Recently, Indrasumunar et al. (2012) compared previously developed acid tolerance screening media and determined that (Gemell et al. 1993) agar media was suitable for the quick and efficient screening of acid tolerant rhizobia for soybean. While Indrasumunar et al. (2012) validated the survival and multiplication of the laboratory screened acid tolerant strains through estimating populations in sterilized acid soils, their nodulation in acidic soil environments was suggested to be an avenue for future exploration.

2.8. Phosphate solubilising microorganisms

Phosphorus (P) one of the macronutrient required by plants in large quantities and often limits crop production. Despite its abundance in the soil as organic and inorganic forms, P is often found in forms that are not accessible for plants (insoluble) as P precipitates with other inorganic ions or is locked in organic compounds. In acidic soils, P is usually bound with iron (Fe) or Aluminium (Al), while in alkaline soils, P is predominantly associated with calcium (Khan et al. 2009).

Phosphatic fertilizers are often applied to amend the deficiency of P. However, the cost of such fertilizers is continuously increasing as it requires high energy and as the raw materials are in competition with other industries. In addition, the deposits of quality phosphate rocks are finite. P use efficiency is very low and it is estimated that plants can use only up to 25% of

applied P fertilizers. On top of that, the fertilizers can have negative environmental impacts (Richardson 2001; Khan et al. 2009; Vitorino et al. 2012).

Phosphate solubilising microorganisms (PSM) are being considered to alleviate the deficiency of available P in soils, as they have the capability of dissolving the plant inaccessible insoluble P pool in the soil (Richardson 2001; Khan et al. 2009; Vitorino et al. 2012; Saharan and Nehra 2011). A large number of laboratory and greenhouse experiments have shown phosphate solubilisation traits of selected organisms applied either in pure form or in combination with other beneficial bacteria (Cattelan et al 1999; Vikram et al. 2007; Tauran et al. 2010; Viruel et al. 2011).

Most research has focused on the *in vitro* solubilisation properties of PSM either singly or with other microorganisms. Consistent results from field evaluation of phosphate solubilising bacteria have not been widely reported compared to laboratory evaluations (Richardson 2001; Khan et al. 2009; Bashan et al. 2013). It is suggested that laboratory procedures and methods commonly used to select P mobilizing organisms might not be appropriately related to their effect in soil (Richardson 2001).

A common laboratory medium used to isolate PSM contains tri calcium phosphate (TCP). However, isolation of such microorganisms needs to take into consideration the characteristic of the soils on which they will be applied; PSMs isolated from alkaline soils to solubilise TCP may not solubilise $FePO_4$ or $Al(H_2PO_4)_3$ as found in low pH soils (Bashan et al. 2013). Vitorino et al. (2012) showed the ability of such organisms to solubilise $FePO_4$ using Reyes Basal Media while Marra et al. (2012) used GELP media to test the solubilisation of P from inorganic $CaHPO_4$, $Al(H_2PO_4)_3$ and $FePO_4.2H_2O$.

Further limitations in increasing field efficacy of PSMs are a lack of clear understanding of the mechanisms of P solubilisation, environmental factors involved in the process and lack of

knowledge in the development of appropriate carrier materials. Publications showing phosphate solubilisation efficiency of PSM on common laboratory media are increasing. However, Bashan et al. (2013) proposed that such research activities should consider the amount of acid production, the amount of phosphorus solubilised in liquid media and that the activities should include rigorous field testing.

2.9. Summary

Microbial inoculants are attractive from environmental and economical points of view. Application of microbial inoculants will contribute to the wider adoption of soybean as cheap source input for small resource limited small holder farmers in Ethiopia. However, the limited reports on soybean have shown inconsistent responses to inoculation in Ethiopia. Assessing the rhizobia population distribution in soybean growing regions of Ethiopia will assist further inoculation efforts. The majority of soybean growing soils in Ethiopia are acidic. Selection of acid tolerant and locally adapted N fixing strains can potentially improve soybean yield in acid soils. P is also limiting in acid soils, which in turn impacts N fixation, hence P solubilising bacterial inoculation can enhance soybean N fixation in acid soils.

The purpose of this thesis is, therefore, to address the questions:

- Do locally adapted acid tolerant rhizobial isolates improve soybean yield in acid soils of Ethiopia? (Chapter 3)
- Do microorganisms that solubilise phosphate *in vitro* improve soybean yields in field conditions in acid soils of Ethiopia? (Chapter 4)
- Do Ethiopian soils harbour soybean nodulating rhizobial populations that can influence responses to inoculation? (Chapter 5)
- How diverse are acid tolerant soybean nodulating rhizobial isolates from Ethiopian soils? (Chapter 6)

Chapter 3 The potential for rhizobial inoculation to increase soybean grain yields on acid soils in Ethiopia

Chapter three consists of an article published from this thesis, which investigates the yield responses of soybean to inoculation with acid-tolerant strains in acidic soils of Ethiopia. A summary of the manuscript is provided, followed by a statement of authorship and then the published paper.

3.1. Manuscript summary

Based on controlled environment assessment of acid tolerance and symbiotic effectiveness, selected strains and a commercial inoculant were evaluated for their impact on yield of soybean. Acid tolerant commercial inoculant was found to consistently increase yield while two of the four locally selected strains resulted in similar or better yield than the application of 46 kg N ha^{-1} in places where the resident populations were $\leq 1.4 \times 10^3$ cfu g^{-1} soil.

3.1.1 Context

To enhance soybean N fixation in acidic soils, the identification of symbiotically efficient and acid tolerant strains is crucial. Improved N fixation was achieved with such an effort with soybean in Brazil (Hungria et al. 2000), *Medicago* in West Australia (Howieson et al. 1991) and common bean in South America (Sadowsky et al. 1998). The existence of a large expanse of soybean growing land predominantly acid soils (40%) is proposed to contribute to inconsistent soybean response to inoculation in Ethiopia. Investigating the performance of soybean under inoculation in acid soils of Ethiopia will contribute to maximise the benefit of biological N fixation.

3.1.2 Research objective

Evaluating the field efficacy of locally adapted and acid tolerant strains that were screened in controlled environments.

3.1.3 Methods

Local strains and a commercial inoculant were screened for their acid tolerance and symbiotic effectiveness in laboratory and controlled environments. The yield performances of soybean due to inoculation of these strains were evaluated under field conditions, at six areas representing major soybean growing regions of Ethiopia.

3.1.4 Findings

Yield improvements due to inoculation were achieved under field conditions, in places where the soil resident populations were low. Selection of acid tolerance under controlled conditions is useful when supported by field validation.

3.1.5 Implications

Inoculation programmes should give due attention to acid tolerant screening, both in laboratory and field conditions. Concurrently, evaluating the soil resident rhizobia population is critical to predicting the response to inoculation, despite the commonly held assumption that soybean rhizobia are absent in Ethiopian soils, as the crop is a recent introduction.

3.2. Statement of Authorship

Statement of Authorship

Title of Paper	The potential for rhizobial inoculation to increase soybean grain yields on acid soils in Ethiopia.
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Muleta, D., Ryder, M., Denton, M.D., 2017. The potential for rhizobial inoculation to increase soybean grain yields on acid soils in Ethiopia. Soil science and plant nutrition.

Principal Author

Name of Principal Author (Candidate)	Daniel Muleta Fana		
Contribution to the Paper	Contributed in designing the experiment, conducting the experiment and writing the paper.		
Overall percentage (%)	80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	28/8/2017

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Maarten Ryder		
Contribution to the Paper	Contributed in designing the experiment and writing the paper.		
Signature		Date	28/08/2017

Name of Co-Author	Matthew Denton		
Contribution to the Paper	Contributed in designing the experiment and writing the paper.		
Signature		Date	28/08/2017

3.3. Article

The actual article is embedded in the following pages.

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The potential for rhizobial inoculation to increase soybean grain yields on acid soils in Ethiopia

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ABSTRACT

In Ethiopia, inoculation of soybean with rhizobial inoculants is not common practice, but could provide an option to increase grain yields in low nitrogen (N) acidic soils. In these acid soils, the selection of acid tolerant rhizobia is one strategy that may increase the performance of soybean. In this study, rhizobial strains isolated from Ethiopian soils were evaluated for their acid tolerance and symbiotic N fixation efficiency with soybean, in controlled environments. Following this, four isolated rhizobial strains were evaluated in six field experiments in major soybean growing areas of Ethiopia. Inoculation with the commercial strain or with one of two locally sourced isolates, that were developed as inoculants, improved soybean yield. The yield increase due to inoculation with the commercial strain was con-sistent and greater than other treatments, while the increase due to the two locally sourced strains was comparable to, or greater than, application of 46 kg N/ha in soils, where the resident rhizobial population was ≤1.4 × 10^3 cfu/g soil. For soils with high background rhizobial populations, there was no response to inoculation. In one of the experimental sites (Bako), the percentage of N fixed (%Ndfa) was 55 for the commercial strain and 35 for the local strain, ES3. This study demonstrated that field validation is a necessary step in the selection of acid-tolerant strains of rhizobia to increase soybean production for Ethiopia.

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Soybean; rhizobia; acid tolerance; nodulation; symbiotic effectiveness

1. Introduction

Soybean, Glycine max (L.). Merr. has the largest worldwide production of any crop legume (Sinclair et al. 2014) and con-tributes greater symbiotic N fixation than any other crop legume (Herridge et al. 2008). Soybean production is increas-ing in sub-Saharan Africa, driven by its high value for food and feed, and its ability to improve soil fertility (Sinclair et al. 2014). In Ethiopia, soybean is cultivated mainly in the southern and western regions, and production has increased from 1,620 t in 2002 to 61,000 t in 2014 (CSA 2012; Bekabil 2015). Although the average yield of soybean has recently increased in Ethiopia to 2 t/ha, it is still below the world average of 2.35 t/ha (Abate et al. 2012) which may be related to soil constraints and management of the crop.

The cultivation of soybean in sub-Saharan Africa is often affected by low soil fertility, soil acidity, soil salinity, and organic matter depletion (Bromfield and Ayanaba 1980; Abaidoo et al. 2007; Amissah-Arthur and Jagtap 2007; Thuita et al. 2012). In Ethiopia, about 40% of the cultivated land is sufficiently acidic that it reduces crop production (Abdenna et al. 2007). Production of soybean in acid soils can be con-strained by toxic concentrations of H^+, Al, and Mn, and defi-ciencies in Ca, Co, Cu, Mg, P, Fe, and Mo (O'Hara 2001; Indrasumunar et al. 2011). Although soybean yields can be increased in acid soils by application of N fertilizer, this is uneconomical when compared with the costs of inoculation with rhizobia (Ronner et al. 2016), and is unsuitable for small-holder farmers.

Symbiotic N fixation in acidic soils is itself constrained by several acidity-related problems affecting the symbiosis, viz. the persistence of rhizobia, nodule formation, and the function of the symbiosis (Date and Halliday 1979; Howieson and Ewing 1986; Peoples et al. 2009). It was previously demonstrated that a com-mercial strain of Bradyrhizobium japonicum (strain 532c) increased soybean yield in Ethiopia, Kenya, and Nigeria (Jefwa et al. 2014), although the effectiveness of the strain was incon-sistent across Ethiopia, and performed poorly in Bako and Assossa regions (Asrat, personal communication). Hence, despite the potential to improve yield and soil fertility, biological N fixation by soybean in areas with acid soil is greatly constrained. The inconsistent results of inoculation with strain 532c (Aserse et al. 2012), may therefore be related to soil acidity. Improving the symbiosis through amelioration of soil acidity by the addition of lime is expensive (Foy 1988), and a more cost-effective approach has been the selection of acid-tolerant crops (Taylor 1991) com-bined with selection of suitable acid-tolerant microorganisms (White 1966; Howieson and Ewing 1986; Howieson et al. 1988).

In this study, soybean-nodulating rhizobia were isolated from Ethiopian soils and then tested for their acid tolerance. These strains were then evaluated for their symbiotic effec-tiveness in controlled conditions. Four selected isolated strains were then used to produce inoculants, and the ability of the

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inoculants to increase grain yield was investigated in six field experiments in soybean-growing regions of Ethiopia. We hypothesized that the strains isolated would be adapted to acid soils, and that those found to be symbiotically efficient may provide effective inoculants for use by farmers.

2. Material and methods

2.1. Soil sampling

Soils were collected from 154 sites in the major soybean production areas of Ethiopia, including Jimma and Illubabur zones of the Oromia regional state, Southern Ethiopia (from Hawassa to Amaro), Bako area of Oromia, and Assossa area in the Benishangul Gumuz regional state. Soil samples (0–20 cm) were taken from four points in each field and mixed, and used for isolation of rhizobia after storing at 4°C for 20 d.

2.2. Isolation of rhizobia

Soybean cv. Clark, a widely grown cultivar preferred by Ethiopian farmers for its short maturity, was grown (3 seeds/pot) in 1 kg pots containing field soil in a green-house at Holleta Agricultural Research Center in January and February 2014. After 8 weeks of growth, nodules were collected from the roots and rhizobia were isolated from nodules. The strains isolated were maintained on yeast mannitol agar (YMA) slants, as described by Somasegaran and Hoben (1985).

2.3. Assessment of acid tolerance

Cultures of strains from Ethiopian soils and the Australian commercial soybean strain (CB1809) were grown in yeast extract mannitol broth (YMB) medium (Somasegaran and Hoben 1985). After 7 d of growth, 100 µl of culture were streaked on duplicate plates of pH 4.5 medium containing high Mn and Al, and low P and Ca (Gemell et al. 1993). The inoculated plates were incubated at 28°C for 12 d, and the presence or absence of colonies was monitored visually to assess growth on the acidic media.

2.4. Assessment of nodulation and symbiotic effectiveness

River sand was washed with sulfuric acid (38%; 5 L/20 kg sand) to reduce organic matter that could be an N source for the plants (Lupwayi and Haque 1994). The sand was then rinsed with tap water until its pH was neutral. The washed sand was autoclaved and placed in 1 L pots that had been washed, sprayed with 95% alcohol and dried. Before sowing, the soy-bean seeds were surface sterilized with 95% ethanol for 10 s and then with 2.5% sodium hypochlorite for 3 min, followed by rinsing with seven changes of sterile distilled water. Each pot was sown with five seeds, and after emergence the seed-lings were thinned to three per pot before inoculation. Each treatment was applied to three replicate pots.

Reference and isolated strains that grew on acid-stressed media were streaked on standard YMA plates and incubated at 28°C for 7 d. The reference strains used were 532c, a commercial strain used in Ethiopia (Legume Technology Ltd, Notts, UK) and CB1809 isolated from EasyRhiz® freeze-dried inoculant (New Edge Microbials Pty Ltd, Albury, Australia). Single colonies were inoculated into YMB and grown on a shaker incubator at 28°C, 120 rpm for 5 d. The plants were inoculated 7 days after sowing (DAS) with 1 mL of bacterial suspension (10^9 cfu/mL) per plant. The replicates were arranged in a completely randomized design in a greenhouse at Holleta Agricultural Research Centre where the average day and night temperatures were 26°C and 15°C, respectively, with a 12 h photoperiod.

The plants were supplied with N-free nutrient solution twice a week (Broughton and Dilworth 1970). In addition, an N-supplied control treatment received 20 mL of ammonium nitrate solution (5 mM) per pot, once per week. Plants were grown in April and May 2014, and harvested 60 DAS.

2.5. Field experiments

Six soybean inoculation field experiments were conducted at sites with nitisol soils (FAO-Unesco 1974) at Jimma (three sites, one at the Jimma Agricultural Research Center and two on farms in Ababiya and Seifu farms), Bako on a farm and Assossa (two sites, one at the Agricultural Research Center and the other on a farm) from June to November 2014). Details of soils at these sites are provided in Table 1.

Table 1. Location and selected physical, chemical and biological properties of soils at the experimental sites.

	Jimma, Ababiya farm	Jimma, Seifu farm	Jimma, Research Center	Bako	Assossa, Research Center	Assossa, farm
Latitude	0.7°42.770'	0.7°42.633'	0.7°06.668'	9°04.503'	10°03.251'	10°01.237'
Longitude	37°00.461'	37°00.305'	36°07.867'	36°59.620'	34°59.412'	34°45.613'
Altitude (m)	1781	1767	1753	1755	1588	1578
% Clay	64	71	44	61	71	64
%Silt	24	19	12	19	15	10.0
%Sand	12	10	44	27	14	26
pH H_20	4.37	4.26	4.53	4.39	4.81	4.62
P mg/kg	4.92	4.72	2.12	4.94	5.28	8.21
CEC meq/100g	17.19	17.92	19.6	14.55	34.7	31.6
%OC	2.05	1.98	2.07	1.56	2.96	2.61
Ex. Acidity[b]	0.46	0.81	1.74	0.78	0.18	0.42
Total N %	0.16	0.15	0.45	0.1	0.18	0.14
Rhizobia MPN cfu/g soil	250	16	$>10^6$	ND[a]	$1.4x10^3$	$>10^6$

[a]ND: Not detectable with the MPN procedure used. See methods section for further details.
[b]Exchangeable acidity (Van Reeuwijk 2002).

2.6. Inoculant preparation

Based on symbiotic effectiveness in controlled conditions, four Ethiopian strains (designated as ES1 to ES4 for the purpose of this report) with the greatest measured symbiotic effective-ness, plus the commercial strain 532c were prepared as inocu-lants for use in the field. The carrier was filter mud, a by-product of sugar cane processing, that has previously been used as a carrier (Philpotts 1976). The filter mud was ground and passed through a 200 mesh sieve and neutralized by the addition of $CaCO_3$, sealed in polyethylene bags (125 g per bag) and autoclaved. The strains were grown in 25 ml YMB broth in 50 ml flasks. After 5 d of growth, the broths were transferred to 1 L YMB broth and grown for 5 days to achieve 10^9 cfu/ml, before being used to inoculate the carrier. Rhizobial numbers in the final inoculant were determined by plate count.

2.7. Experimental design

Each field experiment consisted of eight treatments with four replicates arranged in individually randomized complete block designs. Treatments included five rhizobial treatments (four isolated strains and a commercial strain, strain 532c), N-supplied treatments with either 46 or 23 kg N/ha, and one control treatment receiving neither rhizobia nor N fertilizer. The Bako experiment had an additional treatment of non-nodulating haricot bean as a reference treatment sown in one plot for each of the four replicate blocks. Phosphorus was applied at a rate of 20 kg P/ha as triple superphosphate, to ensure that P availability did not limit the expression of N fixation. Both P and N fertilizers, where applicable, were applied in rows to a depth of 5 cm and incorporated into the soil in a separate operation before seeding, according to farmer practice. Seeds were inoculated with the carrier mate-rial under shade immediately before sowing. The carriers con-taining the test strains (10 g containing 10^9 cfu/g) were mixed with seeds that were moistened with sugar solution (2 g in 20 ml water, as a sticker) to enhance inoculant contact with the seed. Treatments were applied taking care to avoid cross contamination of the test strains. Seeds were hand sown in rows 60 cm apart and seeds in rows were 5 cm apart from each other; rows were 4 m long and there were 4 rows per plot. Blocks and plots were separated by 1 m wide buffers, to assist in maintaining hygiene and to limit the chance of con-tamination from one plot to another.

2.8. Soil sample collection and analysis

The soils collected from the field were air-dried, ground, passed through a 2-mm sieve. Samples were analyzed for texture using a hydrometer (Gee and Bauder 1986), pH (1:2.5, H_2O), soil phosphorus (Bray II), cation exchange capacity (Chapman 1965), organic carbon (Walkley and Black 1934), and exchangeable acidity (Van Reeuwijk 2002) at Holleta Agricultural Research Soil Laboratory, using the protocols out-lined by Sertsu and Bekele (2000). The most probable number of soybean-nodulating rhizobia per gram of soil was deter-mined for each sample using the methods as outlined by Somasegaran and Hoben (1985).

2.9. Data collection

In the greenhouse experiment, plants were harvested at 60 DAS. The shoots were dried for 48 h at 70°C, weighed and the total shoot N was analyzed in Holleta Agricultural Research Laboratory using the Kjeldahl method (Bremner and Mulvaney 1982). Nodule numbers were recorded for each plant. For the field experiments, nodules were counted on the roots of five plants removed at 80-cm intervals along the outer rows at 60 DAS. At the same time, shoots were harvested and oven dried at 70°C for 48 h and dry weight was recorded. Grain yield and total above-ground biomass (AGB, dried for 48 h at 70°C) including both shoots and grain were recorded during harvest.

2.10. Assessment of N fixation

The ^{15}N natural abundance technique (Unkovich et al. 2008) was used to estimate the N fixation in the treatments at the Bako experimental site. Dried shoot samples were finely ground and analyzed for total N concentration (µg N/g) and ^{15}N com-position using a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20–20 isotope ratio mass spectro-meter (Sercon Ltd., Cheshire, UK). The percentage of soybean N derived from N fixation ($\%N_{dfa}$) was calculated by comparing the ^{15}N in soybean plants ($\delta^{15}N$ legume) with the $\delta^{15}N$ of the non-nodulating haricot bean reference ($\delta^{15}N$ haricot) which was assumed to reflect the $\delta^{15}N$ of the plant-available soil N. A $\delta^{15}N$ value for soybean grown under N-free media entirely reliant upon N fixation for its N (B value; −1.83‰) was used (Unkovich et al. 2008). The following formula was used for calculating $\%N_{dfa}$:

$$\%N_{dfa} = 100\ \delta^{15}N \text{ haricot} \quad \delta^{15}N \text{ legume} = \delta^{15}N \text{haricot} \quad B$$

The shoot N and total shoot N derived from fixation were calculated using the following formulae:

Legume shoot N ¼ ð %N=100Þ x ð legume shoot weightÞ and

Amount shoot N fixed ¼ ð$\%N_{dfa}$=100Þ x ð Legume shoot NÞ

2.11. Statistical analysis

The data from the greenhouse experiment were subjected to analysis of variance (ANOVA) using the General Linear Model procedure of GenStat (VSN International 2014). Means of all the treatments were calculated and the differences were tested and considered significant when $p < 0.05$. The means of the treatments were differentiated using the LSD. The symbiotic effectiveness percentage was calculated by the method described in Purcino et al. (2000) as:

Effectiveness (%) = (Shoot Dry Weight of inoculated plant /Shoot Dry Weight of N-fertilized treatment) × 100.

Based on this effectiveness scale, isolates were considered highly effective (HE) when percentage of effectiveness >80%, effective, (E) between 50 and 80%, and of low effectiveness (LE) between 50 and 35%. Isolates were considered ineffective when the percentage effectiveness was less than 35.

Data collected from each field experiment were combined and subjected to Bartlett's test for homogeneity of variance. Data from each site were also independently subjected to

ANOVA and LSD was used to separate the treatments, using GenStat software (VSN International 2014).

3. Results

3.1. Soil collection, rhizobial isolation, and acid tolerance screening

From the initial 154 soils collected from the major soybean growing regions, 80 strains were isolated from nodules (Fig.1 and Table 2). Of the 80 rhizobial strains, 50 grew on acid-stressed media at pH 4.5 and these strains were further evaluated for symbiotic effectiveness in the greenhouse.

3.2. Evaluation of symbiotic effectiveness under controlled conditions

The evaluation of symbiotic N fixation of soybean in the greenhouse indicated variation in N fixation among the 50 isolates and the two reference strains (Fig. 2). Shoot dry weight (SDW) among treatments varied from 4.13 g/plant and 3.67 g/plant when inoculated with the commercial strains 532c and CB1809, to only 1.37 g/plant in the un-inoculated treatment. The N-supplied treatment weighed 3.67 g/plant and four strains were identified that had higher SDW than the remaining local strains; these are listed as ES1, ES2, ES3, and ES4 (Fig.2). Results for selected strains that were the most effective are shown in Table 3. The shoot N content of the plants ranged from 0.8% (negative control) to 3.3% (strain 532c), while the shoot dry weight ranged from 1.4 g/plant (negative control) to 4.1 g/ plant (strain 532c). The N-fertilized plants had a shoot N content of 2.9%. Seedlings inoculated with the four isolates ES1–ES4 (Table 2) and reference strains had significantly greater shoot N concentrations and shoot dry weights than the negative control and other isolates. No relationship was observed between the shoot dry matter of the plants and their nodule number (R^2 = 0.0061, data not shown). The selected strains were categorized as either effective or highly effective. The four Ethiopian isolates with the greatest symbiotic effectiveness (ES1–ES4) were selected for field evaluation. Reference strain, 532c, was superior to CB1809 in the above three measures and was used in the field as a reference strain.

Table 2. Soil samples collected from major soybean growing areas of Ethiopia and the number of strains isolated from the soils using soybean cv. Clark as a host plant.

Soil collection areas	Soils sampled	Rhizobial strains isolated
Southern Ethiopia	40	14
Jimma and Illubabor zone	40	36
Bako area	43	11
Assossa zone	31	19
Total	154	80

3.3. Rainfall at field sites

The Jimma sites had received less precipitation prior to sowing (June; 144 mm) compared with other sites, but that site received more rainfall over the June–November growing

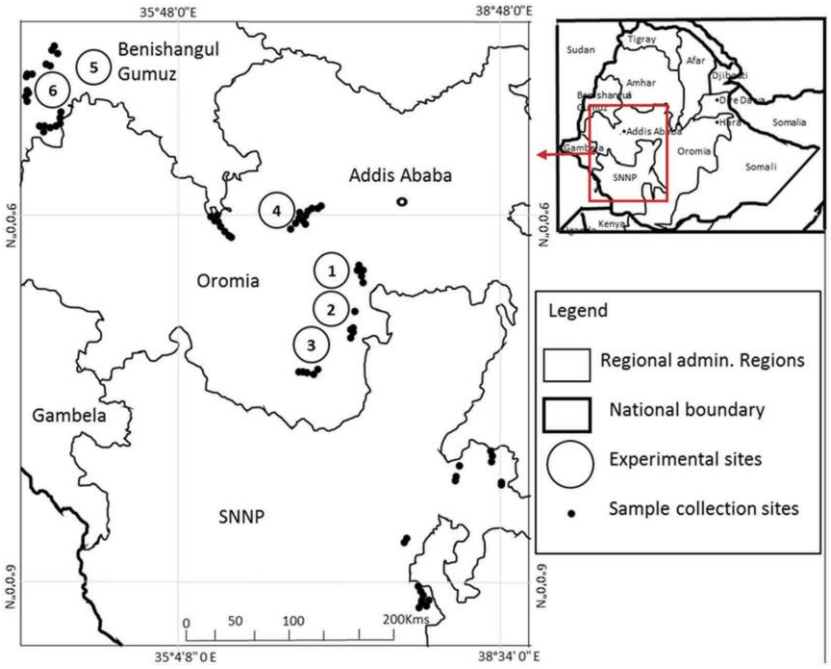

Figure 1. Experimental sites in South Western and West Ethiopia indicated by numbers 1–6. The site numbers indicate: (1) Ababiya farm, (2) Seifu farm, (3) research station in the Jimma area, (4) Bako farm, (5) Assossa research Centre, and (6) Assossa farm.

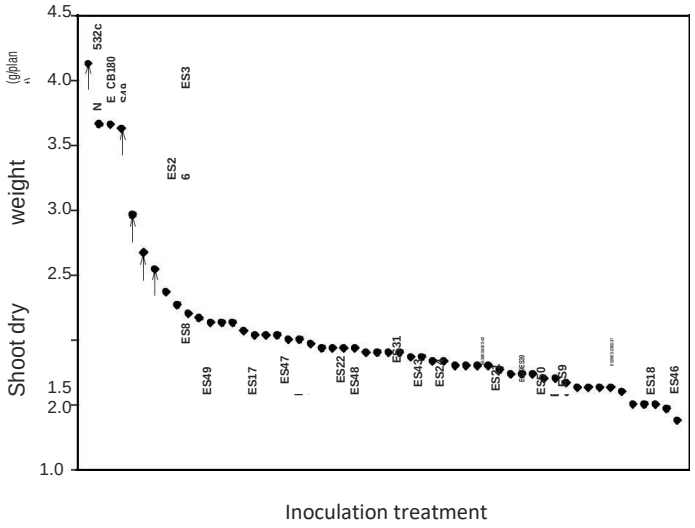

Figure 2. Shoot dry weight responses of soybean to inoculation with one of 50 different rhizobial strains (1–50) and two commercial reference strains, 532c and CB1809, an N-supplied control (N) and a non-inoculated control (Control). LSD = 0.48 at P = 0.05; 3 replicate pots. Arrowed symbols represent strains selected for field experimentation: 532c and four local strains.

Table 3. Effectiveness of selected rhizobial strains inoculated on to soybean grown in N-free conditions in a controlled environment, compared with controls and reference strains.

Treatment	%N	mg N/shoot	% Effectiveness	Rank[a]
Strain 532c	3.3 ± 0.15a	135 ± 7.6a	113	HE
N-fed treatment	2.9 ± 0.03bc	107 ± 1.4b	100	
CB1809	3.0 ± 0.05b	110 ± 1.9b	100	HE
ES4	3.0 ± 0.11ab	110 ± 5.7b	99	HE
ES3	2.8 ± 0.09 cd	82 ± 3.8c	81	HE
ES2	2.6 ± 0.04d	69 ± 2.7d	73	E
ES1	2.5 ± 0.04d	65 ± 30d	69	E
Control	0.8 ± 0.01e	11 ± 0.4e	-	
$LSD_{0.05\%}$	0.24	11.96		

[a]Rank indicates: effective (E) or highly effective (HE).
Means followed by different letters are significantly different at P ≤ 0.05.

season (total 1009 mm) (Fig. 3). The Bako site received more precipitation in the first two months of the growing season (483 mm), but received the least over the rest of the growing season (348 mm). The Assossa sites received the least total precipitation (790 mm), although the amount of rainfall received during the last four months (493 mm) was greater than that at Bako.

3.4. Soil analysis

Soil physical, chemical, and biological analytical results are shown in Table 1. The soils at the experimental sites were either extremely acidic (Sites 1, 2, and 4) or strongly acidic (Sites 3, 5, and 6) with pH (H_2O) ranging between 4.26 and 4.81. The total soil N and P status at the experimental sites were low.

Soybean-nodulating rhizobia were not detected in the Bako soil, while soils at Ababiya farm and Seifu farm contained low-resident soybean-nodulating rhizobial populations (16 and 250 cfu/g soil, respectively) compared with the other sites. The soil at the Assossa Agricultural Research Center site con-tained 1.4×10^3 cfu/g soil while the remaining two sites had abundant background rhizobial populations of $>10^6$ cfu/g soil (Table 1).

3.5. Field experiments

Combined data analysis was initially conducted for all sites to determine if there were overall trends. A combined analysis of soybean yield for the six sites indicated var-iance heterogeneity (Bartlett's test for homogeneity of var-iances, $\chi = 108.4$ with 5 degree of freedom and $p < 0.001$). This indicated the likelihood of high variation among sites due to factors such as soil properties or rainfall (Table 1, Fig. 3). Therefore, general treatment effects could not be compared across sites and results were subsequently ana-lyzed independently for each location.

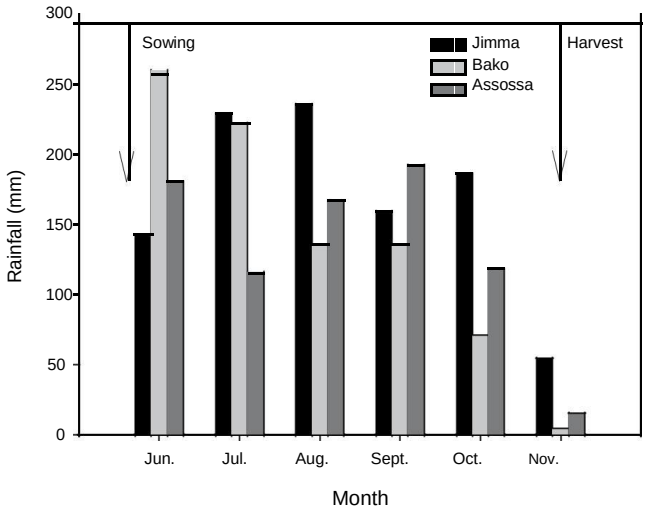

Figure 3. Monthly rainfall distribution across experimental sites during the 2014 growing season. Jimma area represent farms at Ababiya, Seifu, and Jimma research stations, Bako area represents an on-farm site, and Assossa area represents sites at the research station and a farm.

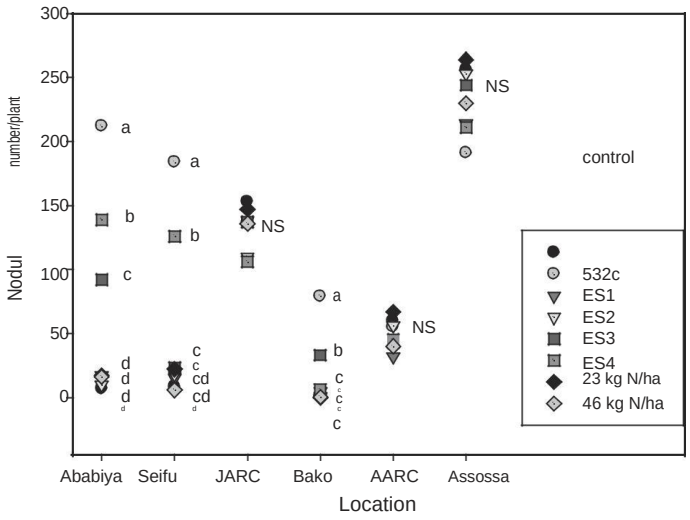

Figure 4. Nodule number per plant of soybean following application of different inoculants and N supply at six experimental sites in major soybean growing areas of Ethiopia, 2014. The means of the treatments for each location is separated by 95% LSD, and means with the same letters are not significantly different.

At Ababiya, inoculation with 532c produced the largest number of nodules per plant, followed by ES4 and ES3, respectively, while the other treatments were not significantly different from the negative control (Fig. 4). All the treatments resulted in higher yield than the control (Fig. 5). Strain 532c produced the highest yield followed by ES3 and ES4 that resulted in a similar yield with appli-cation of 46 kg/ha N. ES1 and ES2 resulted in a yield similar to that obtained with the application of 23 kg/ha N (Fig. 5).

At the Seifu site, inoculation with 532c resulted in significantly greater nodule numbers than the negative con-trol, followed by ES4, whereas other treatments were not different from the negative control in nodule numbers. Inoculation with 532c also resulted in the highest grain yield, followed by ES4, ES3 and application of 46 kg N/ha. Inoculation with ES1, ES2 and application of 23 kg/ha N gave no significant yield increase over the control.

At Bako, inoculation with 532c and ES3 resulted in greater nodule numbers than the other treatments (Fig. 4). The

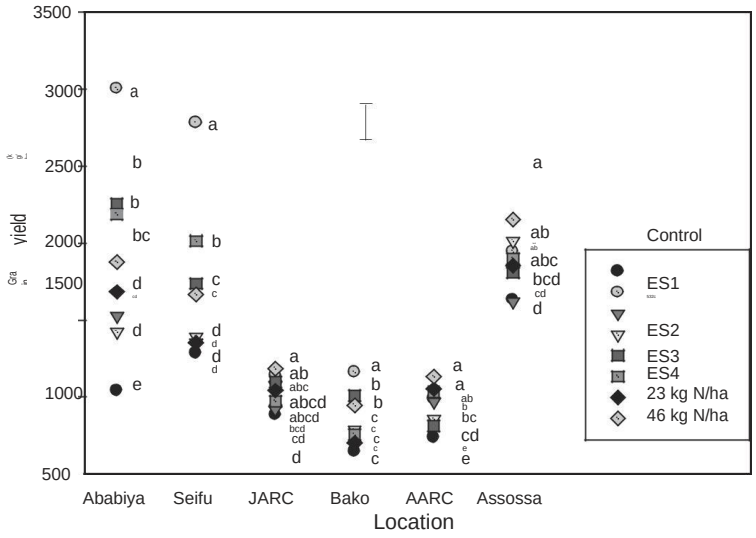

Figure 5. Grain yield of soybean following application of different inoculants and N supply at six experimental sites in major soybean growing areas of Ethiopia in 2014. The means of the treatments for each location is separated by 95% LSD. Means with the same letters are not significantly different.

Table 4. Aboveground biomass including grain at harvest AGB, kg/ha, %N, total shoot N, percentage of shoot N fixed, %Ndfa, and total shoot N fixed by soybean at harvest following application of different inoculants at Bako.

Treatment	AGB kg/ha	%N	Total shoot N kg/ha	$\%N_{dfa}$	Fixed N in shoots kg/ha
Control	1325 ± 206c	4.2 ± 0.4a	50± 5d	0.7± 0.4c	1± 1c
Strain 532c	2693 ± 204a	8.4 ± 2a	226± 26a	55± 7a	126± 26a
ES1	1609 ± 402bc	4.5 ± 0.4a	78± 4d	5± 0.7c	4± 1.5c
ES2	2025 ± 235b	7.6 ± 1.5a	164± 15abc	12± 6c	22± 15bc
ES3	2091 ± 383ab	8 ± 2a	191± 66ab	35± 9b	60± 18b
ES4	1467 ± 154bc	6 ± 1a	97± 4cd	10± 1c	10± 1c
23 kg N/ha	1602 ± 136bc	6 ± 1.3a	82± 22cd	0.08± 0.2c	1± 2c
46 kg N/ha	1778 ± 142bc	7 ± 0.4a	130± 8bcd	0.7± 0.9c	1± 0.1c
$LSD_{0.05}$	643	NS	82	14.2	38

Means in a column followed by different letters are significantly different at P ≤ 0.05.

experiment conducted at Bako had lower grain yields in its negative control compared with the negative control of other sites (13.7–150% decrease compared with Assossa Research Center and Assossa farm (Fig. 5). Yield increased following inoculation with 532c and ES3, but not more than with the application of 46 kg N/ha.

At Bako, the use of a sown reference species allowed ^{15}N natural abundance N fixation measurements to be made on soybean. Total shoot N at harvest ranged from 50 kg/ha (un-inoculated control) to 226 kg/ha (532c) and the three strains (532c, ES2, and ES3) had significantly higher shoot N than the control (Table 4). The percentage of N derived from the atmosphere was greater in plants inoculated with 532c (55%) and ES3 (35%) than for other treatments. Accordingly, the amount of fixed N in the shoots of plants inoculated with 532c and ES3 (126 and 60 kg/ha, respectively) were signifi-cantly greater than the other treatments (Table 4).

At the Jimma Agricultural Research Center experimental site, only strain 532c, ES3 and application of 46 kg/ha N resulted a higher yield over the negative control, while nodule numbers did not differ, and were universally high in all treatments.

At the Assossa Agricultural Research Center site, there were no differences observed among treatments for nodule num-bers (Fig. 4). Treatments other than ES3 had greater grain yield than the negative control. Yield increase resulting from inocu-lation with ES4 was comparable to those obtained for N applications, while strain 532c significantly improved yield over the control but did not achieve the largest yield at this site. Treatments ES1 and ES2, that did not lead to high yields at other sites, produced a higher yield than the control.

At the Assossa farm site, nodule numbers were universally high (Fig.4) and did not differ among treatments. Yields did not show significant difference among inoculant treatments, however, strain 532c and ES2 increased yield significantly relative to the control.

Overall, the responses of soybean to the application of 23 and 46 kg N/ha, and inoculation with strain 532c increased grain yield, relative to the uninoculated control treatments (Fig. 6). The association between yield and nodule number per plant at the six experimental sites was examined by linear regression, which revealed that in three of the six experi-ments (Fig. 7: A, B, D) there were positive correlations

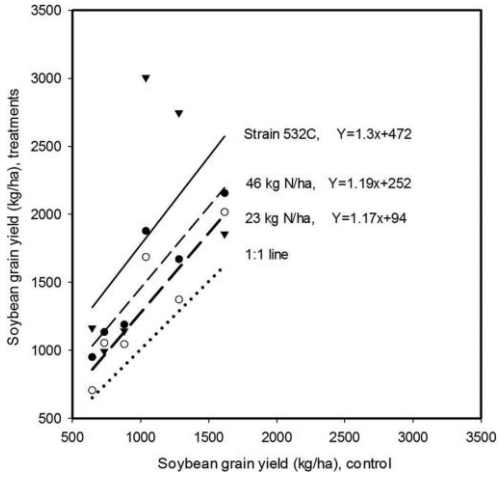

Figure 6. Soybean grain yields following inoculation with commercial strain (closed triangles) or with application of 46 kg N/ha (closed circles), or 23 kg N/ ha (open circles) in comparison with the yield of the untreated control across the six experimental sites. A 1:1 dotted line is shown for comparative purposes.

between nodule number and grain yield, while for the other three sites (Fig. 7, C, E, F) correlations did not exist.

4. Discussion

4.1. Field response to inoculation

We hypothesized that locally adapted strains that were evaluated for acid tolerance and symbiotic effectiveness would provide greater acid tolerance and prove to be robust inoculants across different soybean-growing regions of Ethiopia. In contrast to our expectations, the commercial strain proved to be a more effective inoculant than locally isolated strains used as inoculants, when assessed at six experimental sites. At two of the experimental sites, inoculation with field isolates from Ethiopian soils (ES3 and ES4) resulted in yields similar to, or greater than, the application of 46 kg N/ha, indicating the value that they could provide to soybean crops grown in Ethiopian soils with low mineral N. In con-trast, ES1 and ES2 performed poorly except in the Assossa area, and are unsuited to field application in other areas, potentially due to poor acid tolerance. This study confirmed the importance of field evaluation of isolates, in addition to evaluation in the laboratory, before suitable strains can be identified (Howieson et al. 1988).

Application of 46 kg N/ha improved yield at five of the six sites, indicating the extent of soil N limitation in grain production in Ethiopia (Atnaf et al. 2015). Application of 23 kg N/ha, however, improved yield at only two sites, indicating that the N demand of the soybean crop exceeded this level of N application, which agrees with previous research that demonstrated the high N demand of soybean (Herridge 2002). In Ethiopia, farmers typically use N fertilizer where they have access, rather than inoculating their soybeans. However, fertilizer costs are high for smallholder farmers in developing countries. Soybean requires 120 kg N to produce a tonne of grain (Herridge 2002) and urea was 60 USD per 100 kg in Ethiopia in 2015 (Ayele et al. 2016) so that N sufficient for a 2 t/ha crop would cost at least $144/ha, assuming limited soil mineral N supply and a 100% use efficiency of the fertilizer. In contrast, a commercial inoculant to treat 1 ha of soybean (320 g /80 kg) costs 13.3 USD (Jefwa et al. 2014), while local inoculants (500 g) applied to 1 ha cost less than 7.5 USD in Ethiopia. Thus, in using inoculants instead of N fertilizers, there is a cost savings of $130 USD/ha, based on recent data. It is, however, unlikely that Ethiopian farmers would provide these quantities of N fertilizer to their crops, further highlighting the importance of inoculant use. Similarly, on-farm research on soybean responses to inoculation and P application in Northern Nigeria indicated that 95% of the farmers achieved an economic benefit by using soybean inoculant (Ronner et al. 2016).

Plants inoculated with strain 532c and ES3 fixed 105 and 55 kg N/ha at the Bako experimental site, respectively. Strain 532c fixed 55% N, while ES3 fixed 35% of the N in the crop. Previous estimates of $\%N_{dfa}$ for soybean were 68% in experimental sites and 58% in farmers' fields (Herridge et al. 2008). The lower $\%N_{dfa}$ for some of the strains in the current study might be due to low precipitation during pod-filling in 2014, when N fixation is known to be important (Zapata et al. 1987; Bergersen et al. 1992). N fixation is known to be limited by factors that affect biomass accumulation of the host plant (Giller 2001), including environ-mental and agronomic factors, and cultivar (Herridge et al. 2008).

Two types of responses were observed in the relationship between nodule number per plant and grain yield: three sites had strong and positive associations between nodule numbers and grain yield, and three sites had no associations. Inoculation with a commercial strain in Ethiopia previously improved soybean yield and nodulation in some parts of Ethiopia, although the responses were not universal (Jefwa et al. 2014). The relationships observed in our study could be explained by the background popula-tion densities of soil rhizobia. At sites with strong correla-tions between nodule number and yield, the initial soil rhizobial numbers were low and ranged from non-detect-able to 250 cells/g soil. Sites with weak or negative corre-lations between nodule number and yield, i.e., Jimma and the Assossa sites, had high soil rhizobial populations ran-ging from 1.4×10^3 to $>10^6$ cells/g soil. The inverse relation-ship between yield response to inoculation and resident rhizobial population has been observed previously (Thies et al. 1991; Brockwell et al. 1995; Denton et al. 2000).

4.2. Screening acid-tolerant rhizobia in the laboratory

The sites chosen for field experiments had soil pH (H_2O) as low as 4.26. Rhizobia were screened on agar media of pH 4.5, since low pH reduces rhizobial survival to a greater extent on plates than in soil of the same pH (Appunu and Dhar 2006). This might be due to the presence of microsites in soils with a higher pH, or the association of the microorganisms with cations, anions, or organic molecules in the soil (Howieson et al. 1988; Appunu and Dhar 2006). Growth of the freshly isolated rhizobial strains on acidic agar media ranged from

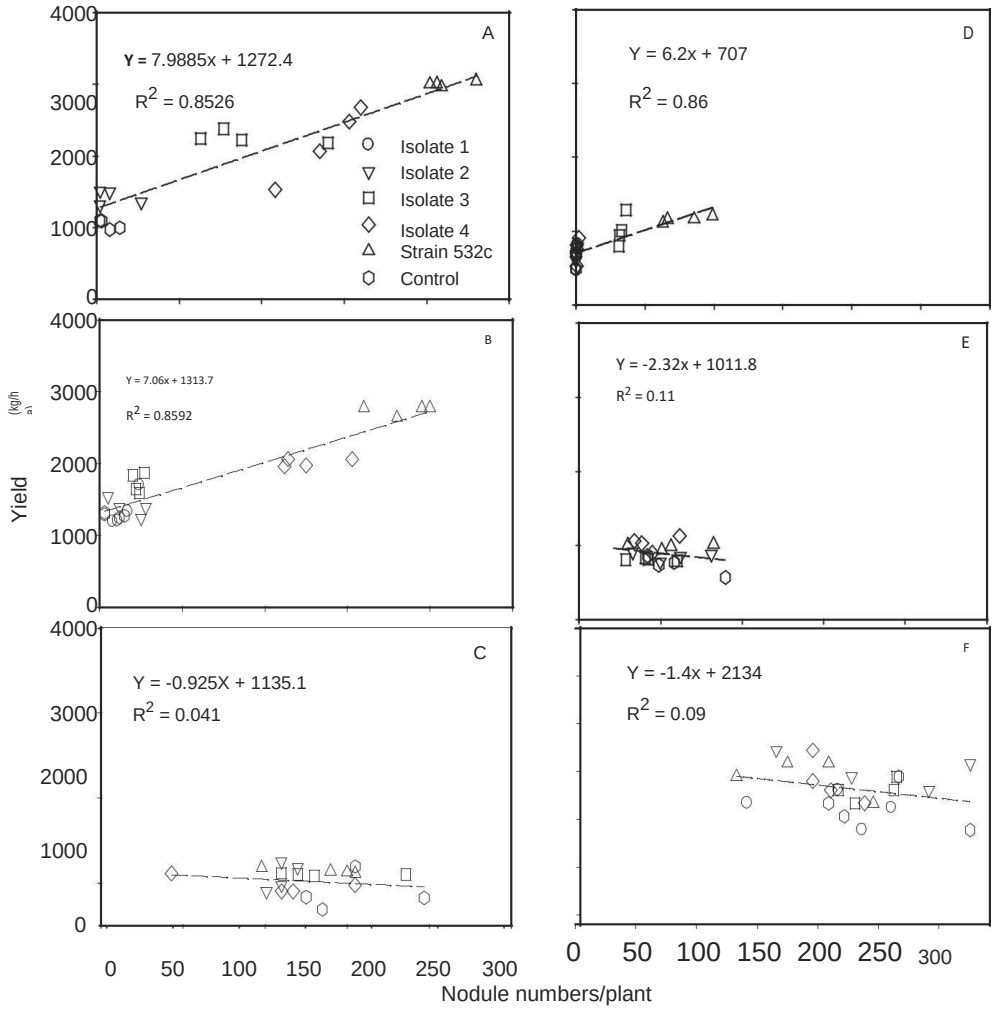

Figure 7. Regression analysis of nodule number per plant and grain yield among different treatments at the six experimental sites. Sites are (A) Jimma, Ababiya farm; (B) Jimma, Seifu farm; (C) Jimma, Agricultural Research Centre; (D) Bako; (E) Assossa, Agricultural Research Centre; (F) Assossa, farm.

none to profuse. Such differences in tolerance to acidity among strains of the same species have been reported pre-viously for various Rhizobium and Bradyrhizobium (Howieson et al. 1988; Graham et al. 1994; Appunu and Dhar 2006). Hence, strains that showed profuse growth on low pH agar media, with high concentrations of Al and Mn and low con-centrations of Ca and P, coupled with high symbiotic poten-tial, were selected for testing effectiveness in the field. The commercial strain, 532c, was also able to grow on the same acid-stressed media. Among the four strains selected, three demonstrated symbiotic effectiveness under field conditions (ES3 and ES4 at Sites 1 and 2; ES3 at Site 4; and ES1 and ES4 at Site 5), supporting the need for a rapid screening of rhizobia for acid tolerance to assist in selection of strains in the field (Indrasumunar et al. 2011).

4.3. Assessment of n fixation effectiveness in the greenhouse

Assessment of N fixation effectiveness in the greenhouse indicated that 40% of the strains that tolerated pH 4.5 on agar media had low effectiveness and 56% were considered effective in fixing N, but only 4% of the strains were found to be highly effective. However, since strains that grew on acid-stressed media were evaluated for N fixation efficiency in the greenhouse in soils of neutral pH, it is possible that the results of the effective strains could have differed, had the green-house screening been performed at an acidic pH. The yield responses to inoculation with the commercial strain 532c in the field were generally better than with the locally sourced strains, although inoculation with ES3 and ES4 were similar in

yield to the commercial strain at one field site each. Assessment of symbiotic effectiveness on acidic media should be considered as an option to identify an acid-tolerant strain that is symbiotically effective, and may assist in developing locally adapted strains, well suited to soil constraints (Alexandre et al. 2009).

5. Conclusion

Soils of Ethiopia are typically low in N status and acidic. Inoculation of soybean with effective rhizobial isolates increased yield, although locally adapted isolates from acidic Ethiopian soils were not as effective as the commercial strain when tested at multiple sites. The use of effective strains was demonstrated to bring economic benefits to resource-limited small holder farmers over the current practice of using N fertilizers. Screening of strains with high acid-tolerance in vitro and symbiotic effectiveness in the greenhouse was a rapid way to identify acid-tolerant strains that may increase grain yield in acid soils, but field testing is a necessary step in demonstrating this value for soybean production.

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References

Abaidoo R, Keyser H, Singleton P, Dashiell K, Sanginga N 2007: Population size, distribution, and symbiotic characteristics of indigenous Bradyrhizobium spp. that nodulate TGx soybean genotypes in Africa. Appl. Soil Ecol., 35, 57–67. 10.1016/j.apsoil.2006.05.006

Abate T, Alene AD, Bergvinson D, Shiferaw B, Silim S, Orr A, Asfaw S 2012: Tropical Grain Legumes in Africa and South Asia: knowledge and Opportunities, ICRISAT, Nairobi, Kenya.

Abdenna D, Negassa C, Tilahun G 2007. Inventory of soil acidity status in crop lands of Central and Western Ethiopia. Utilisation of diversity in land use systems: Sustainable and organic approaches to meet human needs, Tropentag Witzenhausen.

Alexandre A, Brígido C, Laranjo M, Rodrigues S, Oliveira S 2009: Survey of chickpea rhizobia diversity in Portugal reveals the predominance of species distinct from Mesorhizobium ciceri and Mesorhizobium mediter-raneum. Microb. Ecol., 58, 930–941. 10.1007/s00248-009-9536-6

Amissah-Arthur A, Jagtap S 2007: Soybeans in Sub-Saharan Africa: model-ling possibilities and potential. Ier., 9, 44–54. 10.1504/IER.2007.053979 Appunu C, Dhar B 2006: Symbiotic effectiveness of acid-tolerant Bradyrhizobium strains with soybean in low pH soil. Afr. J. Biotechnol., 5, 842–845.

Aserse A, Räsänen LA, Aseffa F, Hailemariam A, Lindström K 2012: Phylogenetically diverse groups of Bradyrhizobium isolated from nodules of Crotalaria spp., Indigofera spp., Erythrina brucei and Glycine max growing in Ethiopia. Mol. Phylogenet. Evol., 65, 595–609. 10.1016/j. ympev.2012.07.008

Atnaf M, Tesfaye K, Kifle D 2015: The importance of legumes in the Ethiopian farming system and overall economy: an overview. Am. J. Exp. Agric., 7, 347–358. 10.9734/AJEA/2015/11253

Ayele GK, Gessess AA, Addisie MB, Tilahun SA, Tebebu TY, Tenessa DB, Langendoen EJ, Nicholson CF, Steenhuis TS 2016: A biophysical and economic assessment of a community based rehabilitated gully in the Ethiopian Highlands. Land Degrad. Dev., 27, 270–280. 10.1002/ ldr.2425

Bekabil UT 2015: Empirical review of production, productivity and market-ability of soya bean in Ethiopia. Ijunesst, 8, 61–66. 10.14257/ijunesst Bergersen FJ, Turner GL, Peoples MB, Gault RR, Morthorpe LJ, Brockwell J 1992: nitrogen fixation during vegetative and reproductive growth of irrigated soybeans in the field: application of 15n methods. Aust. J Agric. Res. 43, 145-153.

Bremner JM, Mulvaney C 1982: Nitrogen total. In Methods of Soil Analysis. Part 2. Chemical and Microbiological Properties, eds. Page AL, Miller RH, Keeney DR, pp. 595–624. Amercan Society of Agronomy, Madison.

Brockwell J, Bottomley PJ, Thies JE 1995: Manipulation of rhizobia micro-flora for improving legume productivity and soil fertility: a critical assessment. Plant Soil., 174, 143–180.

Bromfield E, Ayanaba A 1980: The efficacy of soybean inoculation on acid soil in tropical Africa. Plant Soil., 54, 95–106. 10.1007/BF02182002

Broughton WJ, Dilworth MJ 1970: Plant nutrient solutions. In Methods in Legume-Rhizobium Technology: handbook for Rhizobia, eds. Somasegaran P, Hoben H, pp. 245–249. University of Hawii Press, Maui, Hawii.

Chapman H 1965: Cation-exchange capacity. In Methods of Soil Analysis. Part 2. Chemical and Microbiological Properties, ed. Black CA, et al., pp. 891–901. American Society of Agronomy, Madison, WI.

CSA 2012: Agricultural sample survey 2011-2012. In Annual Bulletin, CSA, Addis Ababa, Ethiopia.

Date RA, Halliday J 1979: Selecting Rhizobium for acid, infertile soils of the tropics. Nature, 277, 62–64. 10.1038/277062a0

Denton MD, Coventry DR, Bellotti WD, Howieson JG 2000: Distribution, abundance and symbiotic effectiveness of Rhizobium leguminosarum bv. trifolii from alkaline pasture soils in South Australia. Aust. J. Exp. Agric., 40, 25–35. 10.1071/EA99035

FAO-Unesco 1974: FAO/UNESCO Soil Map of the World 1: 5,000,000, UNESCO, Paris.

Foy CD 1988: Plant adaptation to acid, aluminum toxic soils. Commun. Soil Sci. Plant Anal., 19, 959–987. 10.1080/00103628809367988

Gee GW, Bauder JW 1986: Particle-size analysis. In Methods of Soil Analysis. Part 1. Physical and Mineralogical Methods, ed. Klute A, pp. 383–411. American Society of Agronomy, Inc. and Soil Science Society of America, Inc., Madison, WI.

Gemell L, Roughley R, Reed M, Hartley EJ 1993: Screening of Rhizobium leguminosarum bv. Trifolii for adaptation to acid and neutral soils using a selective agar medium. Soil Biol. Biochem., 25, 1463–1464. 10.1016/ 0038-0717(93)90063-H

Giller KE 2001: Nitrogen Fixation in Tropical Cropping Systems, CAB International, Wallingford, UK.

Graham PH, Draeger KJ, Ferrey ML, Conroy MJ, Hammer BE, Martinez E, Aarons SR, Quinto C 1994: Acid pH tolerance in strains of Rhizobium and Bradyrhizobium, and initial studies on the basis for acid tolerance of Rhizobium tropici UMR1899. Can. J. Microbiol., 40, 198–207. 10.1139/ m94-033

Herridge D ed 2002: Legume N and Inoculants: global and Vietnamese Perspectives, ACIAR, Brisbane, Australia.

Herridge DF, Peoples MB, Boddey RM 2008: Global inputs of biological nitrogen fixation in agricultural systems. Plant Soil., 311, 1–18. 10.1007/ s11104-008-9668-3

Howieson J, Ewing M 1986: Acid tolerance in the Rhizobium meliloti–Medicago symbiosis. Aust. J. Agric Res., 37, 55–64. 10.1071/AR9860055

Howieson J, Ewing M, D'antuono M 1988: Selection for acid tolerance in Rhizobium meliloti. Plant Soil.,105, 179–188. 10.1007/BF02376781

Indrasumunar A, Dart PJ, Menzies NW 2011: Symbiotic effectiveness of Bradyrhizobium japonicum in acid soils can be predicted from their

sensitivity to acid soil stress factors in acidic agar media. Soil Biol. Biochem., 43, 2046–2052.

Jefwa J, Pypers P, Jemo M, Thuita M, Mutegi E, Laditi M, Faye A, Kavoo A, Munyahali W, Herrmann L 2014: Do commercial biological and chemi-cal products increase crop yields and economic returns under small-holder farmer conditions? In Challenges and Opportunities for Agricultural Intensification of the Humid Highland Systems of sub-Saharan Africa, eds. Vanlauwe B, VanAsten PG. pp. 81–96. Springer International Publishing, Cham, Switzerland.

Lupwayi N, Haque I 1994: Legume-Rhizobium technology manual. In ILCA Environmental Sciences Working Document (ILCA). No. 29, ILCA, Addis Ababa, Ethiopia.

O'Hara G 2001: Nutritional constraints on root nodule bacteria affecting symbiotic nitrogen fixation: a review. Aust. J. Exp. Agric., 41, 417–433. 10.1071/EA00087

Peoples M, Brockwell J, Herridge D, Rochester I, Alves B, Urquiaga S, Boddey R, Dakora F, Bhattarai S, Maskey S 2009: The contributions of nitrogen-fixing crop legumes to the productivity of agricultural systems. Symbiosis, 48, 1–17. 10.1007/BF03179980

Philpotts H 1976: Filter mud as a carrier for Rhizobium inoculants. J. Appl. Bacteriol., 41, 277–281. 10.1111/j.1365-2672.1976.tb00631.x

Purcino H, Sé N, Vr'ana M, Scotti MF, Vargast IMM 2000: Response of Arachis pintoi to inoculation with selected rhizobia strains in Brazilian Cerrado. Pasturas. Tropicales, 25, 26–29.

Ronner E, Franke AC, Vanlauwe B, Dianda M, Edeh E, Ukem B, Bala A, Van Heerwaarden J, Giller KE 2016: Understanding variability in soybean yield and response to P-fertilizer and rhizobium inoculants on farmers' fields in northern Nigeria. Field Crop Res., 186, 133–145. 10.1016/j. fcr.2015.10.023

Sertsu S, Bekele T eds 2000: Procedures for Soil and Plant Analysis, National Soil Reseach Center EARO, Addis Ababa, Ethiopia.

Sinclair TR, Marrou H, Soltani A, Vadez V, Chandolu KC 2014: Soybean production potential in Africa. Glob. Food Sec., 3, 31–40. 10.1016/j. gfs.2013.12.001

Somasegaran P, Hoben H 1985: Methods in Legume-Rhizobium Technology; NIFTAL, MIRCEN, University of Hawaii Press, Maui, Hawaii.

Taylor GJ 1991: Current views of the aluminum stress response: the physiological basis of tolerance. Curr. Top. Plant Biochem. Physiol., 10, 57–93.

Thies JE, Singleton PW, Bohlool BB 1991: Influence of the size of indigenous rhizobial populations on establishment and symbiotic performance of introduced rhizobia on field-grown legumes. Appl. Environ. Microbiol., 57, 19–28.

Thuita M, Pypers P, Herrmann L, Okalebo RJ, Othieno C, Muema E, Lesueur D 2012: Commercial rhizobial inoculants significantly enhance growth and nitrogen fixation of a promiscuous soybean variety in Kenyan Soils. Biol. Fert. Soils, 48, 87–96. 10.1007/s00374-011-0611-z

Unkovich M, Herridge D, Peoples M, Cadisch G, Boddey B, Giller K, Alves B, Chalk P 2008: Measuring Plant-Associated Nitrogen Fixation in Agricultural Systems, Australian Centre for International Agricultural Research (ACIAR), Cannbera, Australia.

Van Reeuwijk L 2002: Procedures for Soil Analysis, International soil reference and information centre (ISRIC), Wageningen, The Netherlands.

VSN International 2014: GenStat for Windows, 17th. VSN International, Hemel Hempstead, UK.

Walkley A, Black IA 1934: An examination of the Degtjareff method for determining soil organic matter, and a proposed modification of the chromic acid titration method. Soil Sci., 37, 29–38. 10.1097/00010694-193401000-00003

White JG 1966: Introduction of lucerne into acid soils. Proceedings of the 28th New Zealand Grasslands Association, Alexandra, New Zealand.

Zapata F, Danso SKA, Hardarson GFried M 1987: Time course of nitrogen fixation in field-grown soybean using nitrogen-15 methodology. Agron. J, 79, 172–176.

Chapter 4 Evaluation of the diversity among acid tolerant soybean rhizobia from key agricultural regions of Ethiopia

4.1. Introduction

Evaluation of the genetic relationships among rhizobial strains provides insights about the structure of their population, and the potential for gene transfer, and adaptation to environmental factors (Pongslip 2012). Such information can assist in understanding the interaction of rhizobial inoculants with the soil background rhizobia and may generate important information that can be used in the development of inoculants and in the prediction of inoculation responses.

The 16S rRNA gene sequences are widely used to study the genetic and evolutionary relationship of organisms. The 16S rRNA gene sequences from a wide range of microbes are deposited in the three primary databases including DDBJ (Japan), GenBank (USA) and European Nucleotide Archive (Europe), along with other biological databases. However, classifying microorganisms using the 16S rRNA sequences is considered imprecise at the species level (Thies et al. 2001; Martens et al. 2008). Among the drawbacks of the technique are the presence of duplicate copies of the 16S rRNA gene in some bacterial genomes (Acinas et al. 2004), the transfer of the gene among diverse microorganisms and within a group of microorganisms through horizontal transfer and genetic recombination (Van Berkum et al. 2006), and conservation of the sequence among members of same group organisms (Van Berkum et al. 2006), especially among *Bradyrhizobium* spp. (Willems et al. 2003). Therefore, there is a need to identify more reliable genetic markers. A previous study showed limitations in the ability of 16S rRNA to discriminate rhizobia below the level of genus (Van Berkum et al. 2003). Currently, multi-locus sequence analysis (MLSA) of protein coding genes (constitutive genes or house-keeping genes and N fixation genes, *nif* genes) (Young et al. 2006) and the sequence analysis of 16S-23S (internal transcribed spacer, ITS) are used to

study rhizobial diversity (Jaiswal et al. 2016). The internal transcribed region of the 16S-23S sequence provides similar results to that of DNA-DNA hybridization in bradyrhizobia strains, except for those isolated from *Aeschynomene* species (Willems et al. 2003). The ITS sequence for strains isolated from *Aeschynomene* species was observed to be much more diverse.

Genetic studies on soybean-nodulating rhizobia from Ethiopia have been conducted using MLSA (Aserse et al. 2012) and ITS (Jaiswal et al. 2016). These studies indicated the existence of diverse bradyrhizobia in Ethiopia. The strains identified in these two studies were grouped within *Bradyrhizobium elkanii* (Aserse et al. 2012; Jaiswal et al. 2016), and *Bradyrhizobium japonicum* (Aserse et al. 2012) in addition to distinct rhizobial strains that need further investigation to confirm their species allocation (Aserse et al. 2012; Jaiswal et al. 2016).

Inconsistent responses of soybean to inoculation with exotic strains have been observed in Ethiopia. Poor adaptation to soil environment of the imported strains (Aserse et al. 2012) and competition with indigenous rhizobial populations (Jaiswal et al. 2016) were proposed as possible reasons for inoculant failures.

Inoculation failure due to low pH is common. Forty percent of the arable land in Ethiopia is acidic (Abdenna et al. 2007), including soybean growing areas, and low pH can affect the inoculant strains, the legume, and the N fixation process. Fifty acid tolerant rhizobial strains were selected *in vitro* and tested for their symbiotic effectiveness in a controlled environment (Chapter 3). The most efficient 4 isolates, together with acid tolerant commercial strain were evaluated under six field sites. Significant inoculation responses were observed when acid tolerant strains were used in soils with low background rhizobia populations (see Chapter 3).

The two previous genetic studies on soybean-nodulating rhizobia from Ethiopian soils (Aserse et al. 2012; Jaiswal et al. 2016) focussed on the genetic diversity and effectiveness of

local isolates. In this current study, our focus was on soybean-nodulating rhizobia that were able to grow *in vitro* at a pH of 4.5. In addition, the strains from the above studies were sampled from two economically important soybean growing areas of Ethiopia, South Ethiopia, and Benishangul Gumuz. The present study provides additional information on the influence of location on diversity, as sampling zones included isolates from SW Ethiopia (particularly Jimma zone and Chewaka area), and Western Ethiopia (Bako and Gute areas), where farmers frequently use soybean as a cash and rotational crop.

This chapter outlines the results from an analysis of the diversity of soybean-nodulating, acid tolerant rhizobia from major soybean growing regions of Ethiopia, using the 16S-23SrRNA ITS sequence analysis with the aims of 1) assessing the genetic diversity of acid tolerant rhizobia in the main growing regions. 2) determining if acid tolerant and effectiveness were related to particular genetic groups, and 3) comparing the taxonomic position of the acid tolerant isolates with previous studies.

4.2. Material and methods

4.2.1 Rhizobia isolates

Twenty rhizobia strains were selected out of fifty strains (Table 4. 1) that had been screened on acidic agar plates (see chapter 3) for sequencing of the ITS. Five isolates were randomly selected from each of the four soybean growing areas of Ethiopia: South Ethiopia, South West (Jimma area and Chewaka area), and West Ethiopia (Bako to Gute area) and Assossa Zone. The strains were re-authenticated in glasshouse and re-isolated from nodules (See chapter 3 for detailed methodologies) before PCR amplification.

4.2.2 PCR amplification

Whole bacterial colony was used as a source of template DNA, as the initial annealing temperature was high enough to lyse the rhizobial cell and release the genomic DNA into the

PCR reaction mixture (Versalovic et al. 1994). The 16S-23S rRNA region was amplified using the primers FGPS1490-72 (5′TGCGGCTGGATCACCTCCT3′) and FGPL132-38 (5′ CCGGGTTTCCCCATTCGG3′) (Bioline, Australia) with a thermal cycler (GeneTouch, Bioer). Polymerase chain reaction (PCR) was carried out in 23-µl reaction volume containing 5 µl (5×) MyFi Reaction Buffer, 1 µl (5 U μl^{-1}) MyFi DNA polymerase (Bioline, Australia), 1 µl (10 pM) of each of the primers and double distilled water. Rhizobial cells were transferred to the appropriate tubes by lightly touching a freshly-grown culture with a sterile pipette tip and swirling in the PCR solution to transfer cells; tubes were briefly centrifuged before the reaction was carried out. The PCR reaction conditions were set for lysis (and initial denaturation) at 95°C for 5 min, followed by 35 cycles of 30 s of denaturation at 95°C, 30 s of annealing at 56 °C, 2 min of extension at 72°C, followed by a final extension for 5 min at 72°C.

4.2.3 PCR confirmation

The amplicons were checked by horizontal gel electrophoresis on 1.5 % agarose gel (Table 4. 1) stained with SYBR safe with 2 kb DNA marker (EASY ladder I, Bioline, Australia) and photographed using a gel documentation system (GeneFlash, Syngene, UK).

4.2.4 DNA purification and sequencing

The amplicons were purified with Isolate II PCR and Gel purification kit (Bioline, Australia) according to the manufacturer's instruction. The purified samples were sequenced with Sanger sequencing technique (Applied Biosystems genetic analysis systems, ThermoFisher Scientific) at AGRF (Adelaide, South Australia).

4.2.5 Phylogenetic analysis

The quality of all sequences was checked using Geneious 8.1.3 software (Kearse et al. 2012). LPSN bacterio.net website was used to get the type strains of *Bradyrhizobium* spp. NCBI

GenBank databases were used to confirm the match of sequences of the test strains to the bradyrhizobia-related species using the BLASTn program. The NCBI website was also used to access the ITS sequences of 30 *Bradyrhizobium* spp. type strains and strains previously isolated from Ethiopian soils (Jaiswal et al. 2016). *Bradyrhizobium* spp. type sequences and the test sequences were aligned with Muscle program and a phylogenetic tree was constructed using MEGA 7.0 software (Tamura et al. 2013) and using the neighbour-joining method algorithm (Saitou et al. 1987) and Kimura-2 parameter model (Kimura 1980) with 1000 bootstraps (Felsenstein 1985). The rate variation among sites was modelled with the gamma distribution (shape parameter=1) (Jaiswal et al. 2016). Similarly, a separate phylogenetic tree was constructed using the ITS sequences of the test strains, type strains and ITS sequences mentioned in Jaiswal et al. (2016) using the same alignment and algorithm used above.

4.2.6 Symbiotic effectiveness of acid tolerant strains

The symbiotic effectiveness of the 50 acid tolerant bradyrhizobial isolates were tested in a controlled environment (See chapter 3, Fig. 2). The symbiotic effectiveness of the 20 isolates was placed alongside their phylogeny to infer if there was a relationship between phylogenetic position and symbiotic effectiveness.

4.3. Results

4.3.1 PCR amplification

Following gel electrophoresis, Single bands between 700 kb and 1000 kb (for ESS27 and ESS22 respectively) were detected for the amplicons of each strain (Table 4.1; Figure 4.1).

4.3.2 Phylogeny of test strains based on the 16S-23S rRNA ITS sequence

A phylogenetic tree was constructed using the neighbour-joining method with the Kimura-2 model based on the sequences of the 21 test strains, 30 type strains, and 2 outgroups. The multiple sequence alignment of the test and type strains with Muscle gave 1,602 bp long

alignment. Based on the 16S-23S ITS phylogeny (Figure 4.2), all the Ethiopian strains were identified as being in the genus *Bradyrhizobium sp.* except a single strain, ES11, which was grouped with *Rhizobium* spp. Similarly, all the strains showed similarity with *Bradyrhizobium* species following a BLAST search.

In the phylogenetic tree, two large clusters (cluster D with 5 isolates and cluster E with 9 isolates), and one small cluster (cluster C with 3 strains) were observed (Figure 4.2). In addition, other isolates were clustered in five independent clusters (A, B, E, and G). The largest cluster (F) contained isolates from all of the four collection sites. However, this cluster did not comprise any of the type strains (reference strains) used for comparison. The first cluster (A), strain ES32 from Bako area of SW Ethiopia grouped with the type strain *B. tropiciagri* and *B. embrapense*, while strain ES29 clustered with *B. subterraneum* type species. Strains ES1, ES21 and ES23 clustered with *B. ottawaense* type strain (C) with 94%, 64% and 70% bootstrap support respectively. The second largest cluster (D) comprises 6 isolates from two of the collection sites (Assossa zone, Bako and SW Ethiopia), and the type strain *B. diazoefficiens.* Isolate ES27 from South Ethiopia (E) grouped with the type strains *B. oligotrophicum* and *B. denitrificans* with a bootstrap support of 58%. Strain ES11 (Cluster G) grouped with the *Rhizobium sp.* type strains rather than the *Bradyrhizobium sp.* type strains.

4.3.3 Phylogeny and symbiotic effectiveness of test strains

The four most efficient strains were distributed into three clusters; ES1 in cluster C with *B.ottawaense*, ES2, and ES3 in cluster D, with *B. diazoefficens* and ES4 in a phylogenetically distinct cluster (cluster D). These three clusters containing the 4 most effective strains also contains strains that had symbiotic effectiveness of <50%, while cluster A and D contain strains with symbiotic effectiveness of >50% and cluster B and E <50% (Figure 4.2).

4.3.4 Phylogenetic comparison of test strains with previously identified strains.

A combined phylogenetic analysis of the acid tolerant strains of the current study with a previous study (Jaiswal et al. 2016) based on their ITS sequence revealed that the two studies isolated strains that were genetically distinct. The single exception was a strain from the current study (ES1 from South Ethiopia) that clustered with the type strain *B. ottawaense.* A large group of isolates from Jaiswal et al. (2016) studies clustered with *B. elkanii* (19 strains) while none of the acid tolerant strains grouped in this group. In contrast, 6 of the strains from current study form cluster with *B. diazoefficiens* and *B .japonicum* (USDA122) while none of the isolates from the previous study clustered in this group. Besides this observed trend, isolates from both studies formed a separate group.

4.4. Discussion

Phylogenetic analysis of the acid tolerant bradyrhizobial strains was carried out using a neighbour-joining algorithm of their 16S-23S ITS region sequence, to investigate their diversity and phylogenetic position within established *Bradyrhizobium* spp. Our results showed that the acid tolerant strains were diverse, scattered through the *Bradyrhizobium* genus. Similarly, the symbiotic effectiveness of strains was distributed among the different clusters, so effectiveness did not appear to be related to the phylogeny. Four of the most effective strains were grouped into three separate clusters, however, these three clusters also contain ineffective members. Moreover, the acid tolerant strains had a separate phylogenetic position from strains previously isolated from Ethiopia.

Genetic analysis of strains isolated from four regions in Ethiopia, combined with *in silico* phylogenetic analysis, identified that all the strains used in this study belong to the *Bradyrhizobium* spp. except for a single strain, ES11. ES11 clustered with the fast growing

Rhizobium sp. Similarly, fast growing soybean nodulating rhizobial strains were isolated previously (Jarvis et al. 1992; Hungria et al. 2001; Peng et al. 2002)

The seven clusters identified from the current ITS phylogeny indicates that isolate positioning is scattered across the *Bradyrhizobium* genus. Only strain ES32 (Cluster A) from Bako area is isolated in the phylogenetic tree, closer to *B. embrapense* and *B. tropiciagri,* both strains that were isolated from tropical pasture legumes (Delamuta et al. 2015). However, ES32 is on a separate branch and hence it is highly probable that this is an unnamed *Bradyrhizobium* sp. Multilocus phylogenetic analysis would be required before assigning this taxon to a particular species (Figueras et al. 2014).

With regard to the individual clusters, ES29 (cluster B) from south Ethiopia is closely grouped with *B. subterraneum* that was originally isolated from ground nut (Grönemeyer et al. 2015), a plant that is grown in some parts of Ethiopia. *Arachis hypogaea* (ground nut) is nodulated by *Bradyrhizobium* sp. (Urtz et al. 1996), however, there is no record whether these two crops are nodulated by similar rhizobia like ES29. Further research on cross inoculation between soybean and ground nut may give important information on the pool of rhizobia nodulating the two crops.

ES1 and ES23 from South Ethiopia, and ES21 from SW Ethiopia, were closely clustered with *B. ottawaense*, where ES1 had shown a symbiotic efficiency of 69% under greenhouse conditions (See chapter 3, Fig 2). Similarly, Jaiswal et al. (2016) reported strains clustered with *B. ottawaense.*

Strains under cluster D closely grouped with a *B. diazoefficiens* type strain. Strains ES2 and ES3 were effective and highly effective respectively, based on the shoot dry matter produced, after inoculation under greenhouse conditions (See chapter 3, Fig 2). Out of the four effective strains selected for field strains (Chapter 3), the clustering of the two efficient and acid

tolerant strains in this group suggests that this group can be targeted for future isolation of symbiotically effective acid tolerant strains. In two previous studies on genetic diversity of soybean nodulating rhizobia from Ethiopian soils (Aserse et al. 2012; Jaiswal et al. 2016), strains closely related to *B. diazoefficiens* were not reported. This might be due to the current study being focused only on strains that are tolerant to acid soil conditions, or that a greater diversity of sites was considered in the current work.

The closest type strain for ES27 (Cluster E) is *B. denitrificans* and *B. oligotrophicum*, with a bootstrap support of 58%. *B. denitrificans* was reported to fix N with the plant *Aeschynomene indica* that is also found in Ethiopia. Further study is required to determine whether *A. indica* and soybean can be nodulated by a common strain, such as ES27.

Strains from cluster F (9 strains from the four growing areas) form separate groups that did not match with either of the type *Bradyrhizobia* spp. used. Such separate groups that do not cluster with type strains have been previously reported from Ethiopia (Aserse et al. 2012; Jaiswal et al. 2016). Similarly, distinct *Bradyrhizobium* spp. are reported from studies in Myanmar, India, Nepal, and Vietnam (Vinuesa et al. 2008), India (Appunu et al. 2009), and China (Zhang et al. 2011) indicating wider geographical distribution and diversity of soybean nodulating rhizobia. This group consists of strain ES4, which showed a symbiotic effectiveness of 99% relative to a N-fed control in greenhouse conditions and was effective when used in field conditions (Chapter 3) showing the potential of this group to enhance soybean production as a locally adapted strain.

Cluster G strain, ES11, did not closely group with any of the *Bradyrhizobium* sp. type strains and tended to be closer to the fast growing *Rhizobium spp.* Fast growing soybean nodulating rhizobia, S. *fredii* (Keyser et al. 1982; Chen et al. 2000; Hungria et al. 2001) and *S. xinjiangense* (Peng et al. 2002) were also identified. These two strains of *Sinorhizobium* sp. clustered closer to *Rhizobium* sp. than ES11 (data not shown), indicating the possibility that

ES11 is an unnamed soybean nodulating rhizobia. The ITS sequence analysis is a powerful tool to discriminate most of the *Bradyrhizobium* spp. and the result from the analysis of this 16S-23S gene is found to be similar to results of DNA-DNA hybridization (Willems et al. 2003). Taking this into account, the current analysis of acid tolerant soybean nodulating rhizobia are diverse, corroborating previous reports (Aserse et al. 2012; Jaiswal et al. 2016).

The symbiotic effectiveness of acid tolerant strains in the *Bradyrhizobium* spp. was not related to phylogeny in any defined pattern, which was in contrast to our expectations, as the four most effective strains (ES1, ES2, ES3, and ES4) were found in three different clusters, while the rest of the clusters displayed a symbiotic effectiveness of either greater or less than 50%. However, as mentioned above, as two of the four efficient isolates were grouped under cluster D, this group should be targeted for the screening of symbiotically effective and acid tolerant strains.

A comparison of ITS sequences of the current acid tolerant strains with those of a previous study (Jaiswal et al. 2016) showed that the current acid tolerant strains are distinct, except for ES1 from South Ethiopia, which clustered with strains grouped with *B. ottawaense* (Figure 4.3). Strains clustering near *B. elkanii* type strain were reported in previous studies (Aserse et al. 2012; Jaiswal et al. 2016). However, our study did not capture strains that clustered together with *B. elkanii*, as they were detected in large numbers in a previously study (Jaiswal et al. 2016). Hence, there is potential that the Ethiopian strains that clustered with B. *elkanii* may be acid sensitive, since they were not captured in the current study.

Generally, the phylogenetic tree showed that the acid tolerant strains nodulating soybean in Ethiopia are scattered across the *Bradyrhizobium* genus. In addition, the acid tolerant strains that clustered together were found in different branches, indicating that they are likely to be different species. In addition, a comparison with previous studies showed that the acid tolerant isolates are distinct from those previously isolated on neutral pH media. Despite our

assumption that acid tolerant strains and/or efficient strains may form a particular cluster, the isolates were found to be scattered across the *Bradyrhizobium* genus and presumably were phylogenetically unrelated. Despite this, isolates clustered with *B. diazoefficiens* could be possible targets for future selection of efficient acid tolerant strains.

Table 4. 1. Strains isolated from major soybean growing areas of Ethiopia, their effectiveness, ITS sequence length and their cluster group

No	Strains	Origin of isolates	Effectiveness	Sequence length (bp)	Cluster	Source
1	ES1	SE*	69	992	C	present study
2	ES2	SWE**	73	921	D	present study
3	ES3	Assossa zone	81	928	D	present study
4	ES4	Bako area	99	920	F	present study
5	ES10	SE	55	884	F	present study
6	ES11	SWE	52	905	G	present study
7	ES13	SWE	56	910	D	present study
8	ES14	SWE	52	908	F	present study
9	ES15	SWE	58	897	F	present study
10	ES21	Bako area	48	902	C	present study
11	ES23	SE	45	913	C	present study
12	ES26	SE	62	710	F	present study
13	ES27	SE	46	878	E	present study
14	ES29	SE	47	888	B	present study
15	ES30	Bako	52	746	F	present study
16	ES32	Bako	53	944	A	present study
17	ES34	Assossa zone	41	747	F	present study
18	ES40	Assossa zone	45	673	D	present study
19	ES42	Assossa zone	49	811	F	present study
20	ES45	Assossa zone	46	811	D	present study
21	ES50	Bako area	47	811	F	present study
22	TUTSFWI-63	SE	NA	578	I	Jaiswal et al. 2016
23	TUTSBAGS-33	SE	NA	598	I	Jaiswal et al. 2016
24	TUTSBAW-117	SE	NA	749	I	Jaiswal et al. 2016
25	TUTSBAGS-98	SE	NA	740	I	Jaiswal et al. 2016
26	TUTSMCL-79	NEW***	NA	726	I	Jaiswal et al. 2016
27	TUTSDGI-51	SE	NA	737	I	Jaiswal et al. 2016
28	TUTSBCK27	SE	NA	735	I	Jaiswal et al. 2016

No	Strains	Origin of isolates	Effectiveness	Sequence length (bp)	Cluster	Source
29	TUTSBCK-30	SE	NA	1102	I	Jaiswal et al. 2016
30	TUTSPWG-81	NWE	NA	855	I	Jaiswal et al. 2016
31	TUTSMCF-75	NWE	NA	556	I	Jaiswal et al. 2016
32	TUTSBWI-25	SE	NA	585	I	Jaiswal et al. 2016
33	TUTSDTGx-49	SE	NA	813	I	Jaiswal et al. 2016
34	TUTSFCF-59	NWE	NA	752	I	Jaiswal et al. 2016
35	TUTSMCL-77	NWE	NA	708	I	Jaiswal et al. 2016
36	TUTSPCK-93	NWE	NA	748	I	Jaiswal et al. 2016
37	TUTSPNV-107	NWE	NA	756	I	Jaiswal et al. 2016
38	TUTSAWI-16	SE	NA	877	I	Jaiswal et al. 2016
39	TUTSBWG-37	SE	NA	879	I	Jaiswal et al. 2016
40	TUTSAAW95-6	SE	NA	793	II	Jaiswal et al. 2016
41	TUTSDCF-44	SE	NA	838	II	Jaiswal et al. 2016
42	TUTSBWI-23	SE	NA	808	II	Jaiswal et al. 2016
43	TUTSBAGS-35	SE	NA	811	II	Jaiswal et al. 2016
44	TUTSBAW95-113	SE	NA	789	II	Jaiswal et al. 2016
45	TUTSBAW04-120	SE	NA	813	II	Jaiswal et al. 2016
46	TUTSAGI-12	SE	NA	615	II	Jaiswal et al. 2016
47	TUTSBAW95-111	SE	NA	867	II	Jaiswal et al. 2016
48	TUTSBCK-28	SE	NA	563	III	Jaiswal et al. 2016
49	TUTSACF-4	SE	NA	797	IV	Jaiswal et al. 2016
50	TUTSACF-2	SE	NA	903	IV	Jaiswal et al. 2016
51	TUTSACF-1	SE	NA	900	IV	Jaiswal et al. 2016

SE*= South Ethiopia, SWE**= South West Ethiopia, and NWE= North West Ethiopia

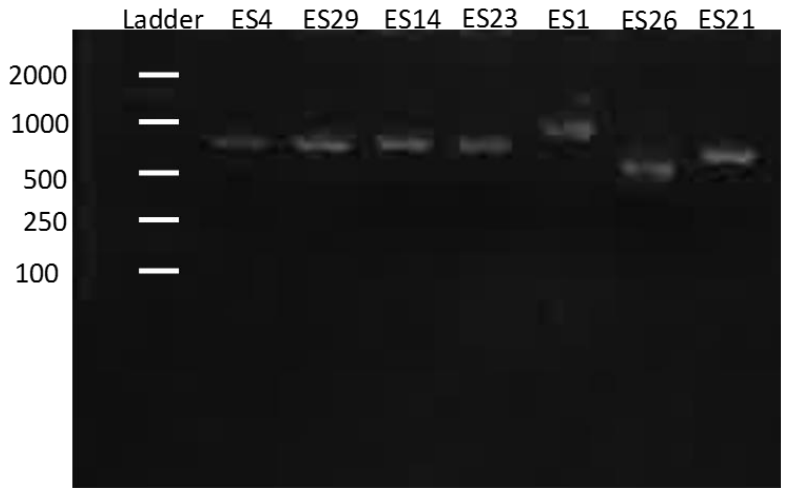

Figure 4.1. ITS sequence of selected strains of soybean nodulating rhizobia from Ethiopia

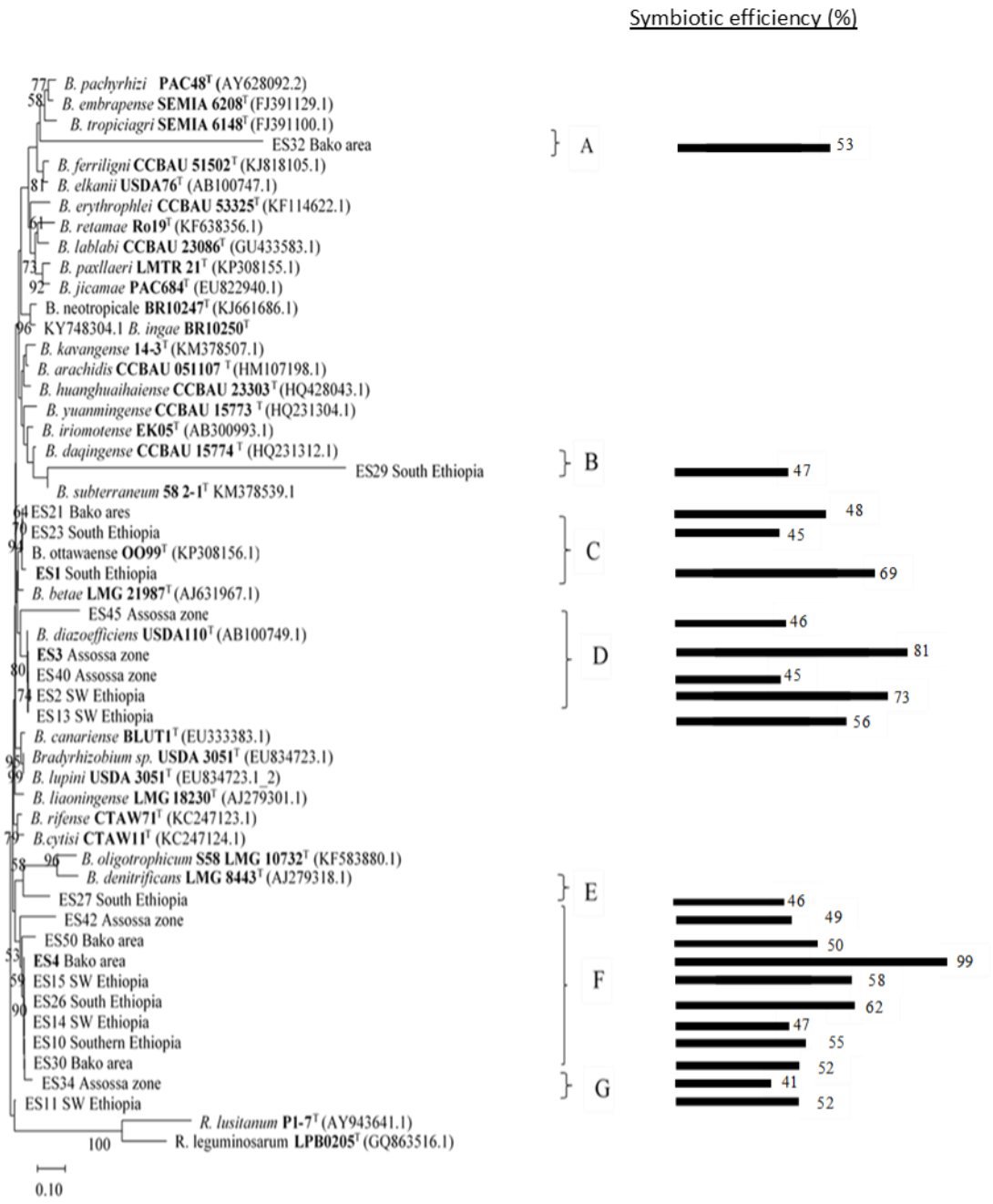

Figure 4.2. Neighbour-joining phylogenetic analysis of the 16S-23S rDNA inter-transcribed spacer (ITS) sequences of acid tolerant soybean nodulating *Bradyrhizobium* spp. from major growing areas of Ethiopia.

The symbiotic effectiveness of strains, determined in Chapter 3, is indicated as bars on the right-side of the Figure.

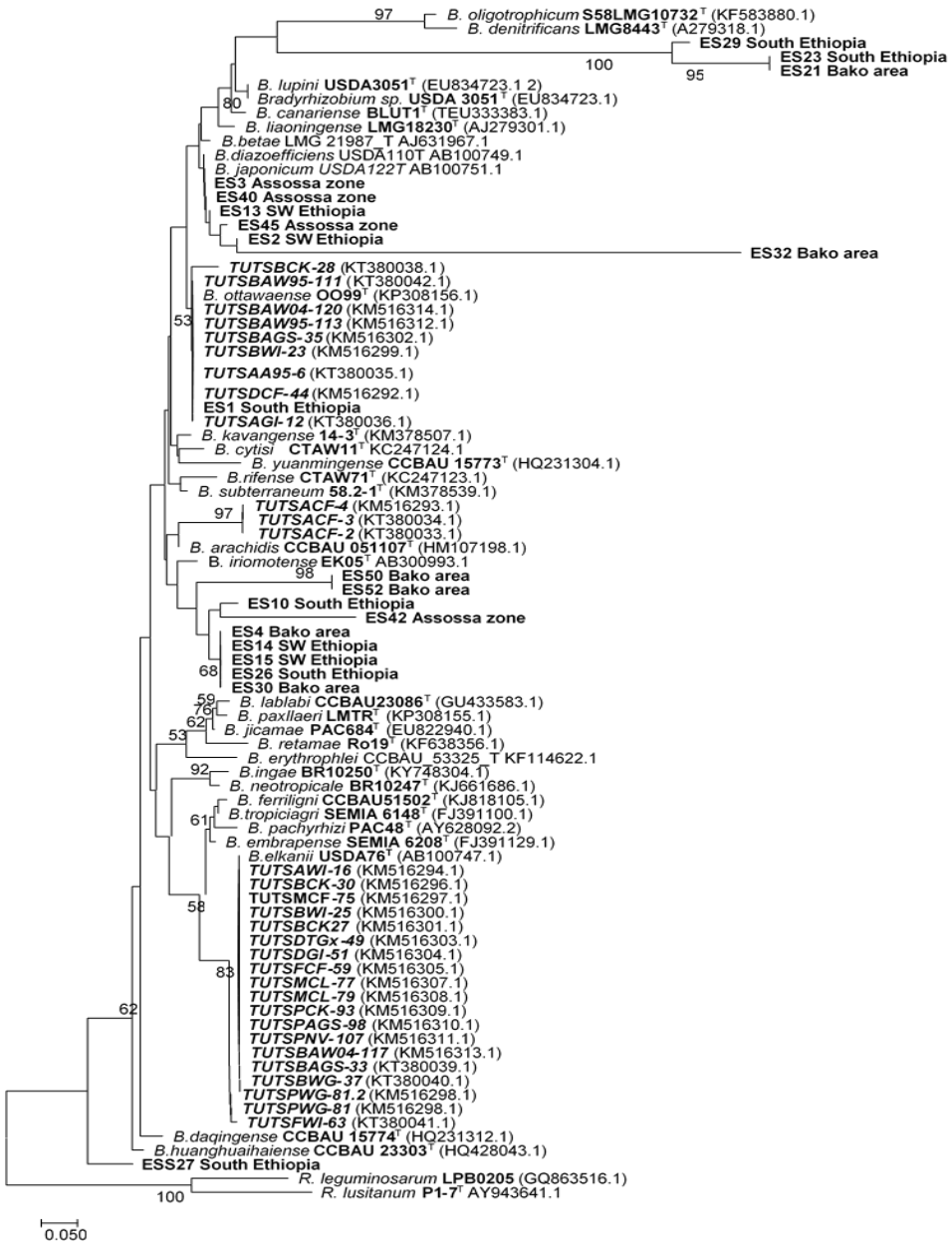

Figure 4.3. Neighbour-joining phylogenetic analysis of the 16S-23S rDNA inter-transcribed spacer (ITS) sequences of acid tolerant *Bradyrhizobium* spp. nodulating soybean from major growing areas of Ethiopia

(bold) compared with isolates from a previous study (Jaiswal et al. 2016) that were isolated from South and North West Ethiopia (bold and italicised).

Chapter 5 Isolation and evaluation of phosphate-solubilising bacteria associated with soybean

5.1. Introduction

Phosphorus (P) is one of the macronutrients required by plants in large quantities. Despite its abundance in the soil in organic and inorganic forms, P is often found in forms that are not accessible (soluble) to plants, as P precipitates with inorganic cations (Richardson 2001) or is locked in organic compounds (Goldstein 1986). In acidic soils, P is usually bound with iron (Fe) or Aluminium (Al); in alkaline soils, P is predominantly associated with calcium (Richardson 2001; Khan et al. 2009).

Phosphatic fertilizers are often applied to amend the deficiency of available soil P. However, the cost of such fertilizers is continually increasing, as they require high energy input and the availability of raw P-containing materials for farming is in competition with other industries. In addition, the deposits of quality phosphate rocks are finite (Neset et al. 2012). P use efficiency is low and it is estimated that plants can use up to only 25% of the applied fertilizers (Rodríguez et al. 1999; Stevenson 1999; Turan et al. 2006). In addition, the fertilizers can have negative environmental impacts through leaching and run off (Richardson 2001; Khan et al. 2009; Vitorino et al. 2012).

Phosphate solubilising microorganisms (PSM) have been considered as options to alleviate a plant deficiency of available P in soils. PSMs have the capability of dissolving the plant-inaccessible P pool in the soil (Richardson 2001; Khan et al. 2007; Saharan 2011; Vitorino et al. 2012). Secretion of low molecular weight organic compounds is thought to be the major mechanism for releasing cation-bound P into soil solution (Richardson 2001). Laboratory and greenhouse experiments have shown that phosphate solubilisation traits of microorganisms

can result in plant growth promotion when plants are under P deficiency (Cattelan et al. 1999; Vikram et al. 2007; Taurian et al. 2010; Viruel et al. 2011).

Research on microbial phosphate dissolution has focused on the P solubilising properties of microorganisms *in vitro* either singly or with other microorganisms. However, field evaluation of their effect has not been widely reported and consistent results have not been obtained (Richardson 2001; Khan et al. 2009; Bashan et al. 2013). Accordingly, this study investigated the potential for P-solubilising microorganisms from Ethiopia to increase the yield of soybean across major soybean growing regions of Ethiopia.

5.2. Materials and methods

5.2.1 Initial in-vitro phosphate dissolution

Five soil samples from four soybean growing areas of Ethiopia (Chapter 3, Figure 1) (South Ethiopia, South Western and Western part of Oromia region and Benishangul Gumuz) were used as sources of phosphate dissolving bacteria (Table 5.1). Serially diluted soils (1g each) were streaked on plates (dilution levels from 10^{-6} to 10^{-8}) containing 5g L^{-1} tricalcium phosphate (TCP) (Pikovskaya 1948), 5 g L^{-1} $AlPO_4$ or 2 g L^{-1} $FePO_4$ on Reyes basal media (Reyes et al. 1999). The plates were incubated at 28°C for 10 d. The isolates that showed a halo zone were spot inoculated into the above three types of agar plates and incubated for 10 d at 28°C. Total diameter (TD) of strains that form halo zone (solubilisation zone), halo zone diameter (HD) and colony diameter (CD) were measured and their phosphate solubilisation index (SI) was determined. The phosphate solubilisation index was calculated according to Kumar et al. (1999) using the formula :

SI= TD/CD, where TD= CD+HD

5.2.2 Determination of phosphate dissolution efficiency in liquid cultures

Three replicates of liquid solutions of the above three media (Ca, Al and Fe as P sources) were prepared and inoculated with 0.2 mL (10^8 cfu mL^{-1}) of each of the bacterial isolates that were pre-grown on 20 mL of trypticase soy broth (those with wider halo diameter when grown on Pikovskayas (PKV) medium). Un-inoculated controls were used for each P-source. After 10 d of growth on shaker at 28ºC and a speed of 120 rpm, the pH of each of the solutions was determined and 1 mL of each culture solution was taken and centrifuged at 14,000 rpm for 5 min. The available P was quantified from the supernatant solution using a spectrophotometer (Jenway, 6300, UK) following Bray-II procedure.

5.2.3 Characterization of phosphate solubilising bacteria using inter transcribed region (ITS) sequence of 16S-23S rRNA.

5.2.3.1 PCR amplification

The 16S-23S rRNA region of the isolates was amplified based on method in a paper (Jaiswal et al. 2016) using the primers FGPS1490-72 (5′TGCGGCTGGATCACCTCCT3′) and FGPL132-38 (5′ CCGGGTTTCCCCATTCGG3′) (Bioline, Australia) with a thermal cycler (GeneTouch, Bioer). Polymerase chain reaction (PCR) was carried out in 23-µl reaction volume containing 5 µl (5×) MyFi Reaction Buffer, 1 µl (5 U $µl^{-1}$) MyFi DNA polymerase (Bioline, Australia), 1 µl (10 pM) of each of the primers and double distilled water. Bacterial cells were transferred to the appropriate tubes by lightly touching a freshly grown culture with a sterile pipette tip and swirling in the PCR solution to transfer cells; tubes were briefly centrifuged before the reaction was carried out. The PCR reaction conditions were set for lysis (and initial denaturation) at 95°C for 5 min, followed by 35 cycles of 30 s of denaturation at 95°C, 30 s of annealing at 56 °C, 2 min of extension at 72°C. The final step involved an extension for 5 min at 72°C.

5.2.3.2 PCR confirmation

The presence of amplicons was checked by horizontal gel electrophoresis on 1.5 % agarose gel stained with SYBR safe with 2 kb DNA marker (EASY ladder I, Bioline, Australia).

5.2.3.3 DNA purification and Sequencing

The amplicons were purified with Isolate II PCR and Gel purification kit (Bioline, Australia) according to the manufacturer's instruction. The purified samples were sequenced with Sanger sequencing (Applied Biosystems genetic analysis systems, ThermoFisher Scientific) at AGRF (Adelaide, South Australia).

5.2.4 Field Experiment on phosphate solubilisation

Three isolates with the highest measured TCP dissolution, based on the liquid dissolution experiment (designated as EPS1, EPS2, and EPS3), were prepared for field inoculation. The carrier material used was filter mud, a by-product of sugar cane processing, which has been used previously to formulate microbial inoculants (Philpotts 1976). The filter mud was ground, passed through a 200 mesh sieve (0.09 mm) and neutralised by addition of $CaCO_3$. The carrier material was sealed in polyethylene bags (125 g per bag) and autoclaved. The isolates (EPS1, EPS2, and EPS3) were grown in 25 mL nutrient broth in 50 mL flasks. After 5 d of growth, the broths were transferred to 1 L Nutrient broth in sterilized 2 L flasks and grown with shaking for 5 d to 10^9 cfu mL^{-1} before being used to aseptically inoculate prepared carriers. Carries sachets of 125 g were inoculated with 45 ml of the nutrient broth containing the bacterial isolates in a laminar flow.

5.2.5 Experimental design

Experiments were established in six farmers' fields side by side to the previous acid tolerance experiment using a randomized complete block design (RCBD) with six treatments replicated four times. Three treatments were the bacterial strains and two treatments were full and half

dose of phosphorus fertilizers (20 kg P ha^{-1} and 10 kg P ha^{-1}) applied as Triple Super Phosphate (TSP), Ca $(H_2PO_4)_2.H_2O$. The negative control treatment received neither isolate nor P fertilizer. Soybean cv. Clark was used for all treatments and seed were inoculated with a commercial N fixing strain (MAR 1495, TSBF-Nairobi) while the phosphate dissolving strains were applied (500 g ha^{-1} at 10^9 cfu g^{-1} carrier) in furrow at 5 cm before seeding at the same depth.

5.2.6 Data collection

The presence and absence of halo zones, the diameters of the halo zones and the diameter of the colony were recorded for phosphate dissolution activity of the microorganisms grown on the three P sources contained in agar plates. The concentration of available P and the pH were determined for the phosphate dissolution experiment. In the field experiments, plants were hand harvested and grain yield was recorded after oven drying for three days at 70 °C.

5.2.7 Statistical analysis

The data from phosphate dissolution in liquid cultures and yield data from field experiments were subjected to analysis of variance (ANOVA) using the General Linear Model Procedure of GenStat (VSN International, 2014) and considered significant when $P<0.05$. Means of all the treatments were calculated and differentiated using LSD test for the experiments that produced quantitative data.

5.3. Results

5.3.1 Initial phosphate dissolution screening

The halo zone indicating phosphate dissolution of the strains were measured on PKV media. Five isolates that form halo were selected (Table 5.1). The solubilisation indices of the five isolates were between 1.5 and 3.3 while their colony diameters were between 2.9 and 3.2 at the 10^{th} d. The five strains changed the colour of the growth media containing both $AlPO_4$ and

$FePO_4$ into yellow, indicating a reduction of pH but there was no clear halo zone formation as seen on $Ca_3(PO_4)_2$.

5.3.2 Phosphate dissolution in liquid culture

In the laboratory experiment, the amount of available P released into solution differed among the bacterial isolates and the P sources used (Table 5.1). The largest available P in the liquid culture was recorded for $Ca_3(PO_4)_2$ (187.4 mg L^{-1}). EPS1 was able to release the highest amount of available P both from $Ca_3(PO_4)_2$ (187.4 mg L^{-1}) and $FePO_4$ (32.4 mg L^{-1}) which were 216 and 73% larger than the P concentration of their respective controls. EPS1 to EPS4 were able to dissolve larger concentrations of P from $AlPO_4$ and the increase in soluble P ranged between 34-86% compared to the un-inoculated control. Among these four isolates, EPS2 released the highest amount of P (12.9 mg L^{-1}) from $AlPO_4$, which was 86% larger than the P concentration compared to the control. EPS3 increased P concentration of $Ca_3(PO_4)_2$ containing solution by 37% compared to the control. The rest of the isolates increased soluble P by 20-73%, except EPS4, which was similar to that of the control.

The increase in P concentration in the solution was also accompanied by a decrease in pH of the media relative to the respective controls, as the correlation coefficients between pH and available P were negatives. The correlation coefficients between pH and available P were -0.8,-0.7 and -.93 for $Ca_3(PO4)_2$, $FePO_4$ and $AlPO_4$ respectively. The highest soluble P concentrations from both $AlPO_4$ and Ca_3 $(PO_4)_2$ were recorded from the lowest numerical pH values, while for $FePO_4$ it was from the third lowest pH (Table 5.1). Both $AlPO_4$ and $FePO_4$ resulted in a maximum pH decrease by 1.7 units while Ca_3 $(PO_4)_2$ was accompanied by a maximum pH decrease of 1.3 pH units (Table 5.1).

5.3.3 ITS sequences of the phosphate solubilising bacteria

The ITS sequences of the five phosphate solubilising bacterial isolates were compared with the database *in silico* and identified based on their similarity with the known strains. Accordingly, the first isolate (EPS1) was found to belong to the *Pseudomonas* genera while the rest of the four isolates (EPS2-EPS5) were from the genera of *Bacillus* (Table 5.1).

5.3.4 Field experiments

For the field experiments, a significant difference in grain yield was not observed in yield among controls, P applications and inoculation treatments except at site 2 (Table 5.2). Even though the differences were non-significant, consistent yield increases compared to negative controls were observed at the individual sites. Yield increase due to the application of the full dose of P (20 kg ha^{-1}) ranged between 5-50% (site 1 and site 6 respectively) for the six experimental sites with a mean increase of 22% while inoculation with EPS1 increased yield between 3.5-54% with a mean yield increase of 13.8%. The lowest yield increase among the treatments was observed for EPS2, ranging from -12.5 to 26.8% with a mean increase of 5.6%. The yield increase with EPS 3 compared to control ranged between -12.5 to 27% with a mean value increase of 13.4%. Yield increase due to the application of a half dose of P over the control ranged from 4 to31.7% with a mean of 10.3% across the six experimental sites.

5.4. Discussion

Inoculation with phosphate solubilising microorganisms can be beneficial in increasing the phosphorus nutrition of plants. Al-P and/or Fe-P in acid soils or Ca-bound P in alkaline soils are released into the plant available P pool of the rhizosphere due to the action of the PSMs (Goldstein 1986). The bacterial isolates in this study increased the concentration of available P in the three tested P sources under laboratory condition compared with the un-inoculated controls. The highest concentrations of P released, compared to the controls were 86, 73 and

216% for Al, Fe and Ca sources, respectively. Plant growth promotion by similar species of this study, *B.subtilis*, *B. velenzesis*, and *P. fluorescens*, were previously reported from acidic soils of coffee growing areas of Ethiopia (Muleta et al. 2009). Strains of *B. safensis* were also found in diverse terrestrial and marine environments and are known for their plant growth promoting properties (Lateef et al. 2015).

The major soybean growing areas of Ethiopia are covered by acid soils (Chapter 3, Table 1). The major P forms in acidic nitisols of Ethiopia are mainly comprised of Fe-P and smaller amounts of Al-P and Ca-P (Piccolo et al. 1986; Mamo et al. 1987). Hence, isolates that have the capacity to dissolve larger amounts of Fe-P together with Al and Ca bound P are important to increase the plant available P content. Because of this, EPS1 that increased the available P from Fe-P by 73% *in vitro* was considered the best candidate, followed by EPS2 and EPS3 although not significantly different from the control under field conditions.

The increase in available P in the liquid solution due to inoculation of the isolates was negatively correlated with pH of the media. Such negative association between pH and P concentration was reported in previous studies (Illmer et al. 1995; Whitelaw et al. 1999; Chen et al. 2006; Yu et al. 2012) and secretion of short carbon chain organic acids was considered as one of the main mechanisms that lead to the dissociation of cations and P (Whitelaw et al. 1999; Richardson 2001). In one study, HPLC analysis of the media in which phosphate dissolving bacteria had grown indicated the secretion of organic acids, including citric acid, gluconic acid, lactic acid, propionic acid, with the most common forms of organic acids being gluconic acid, oxalic acid and citric acid (Richardson 2001).

There is increasing interest in the application of phosphate solubilising microorganisms to improve P nutrition and yield. Promising results have been demonstrated in the field and greenhouse for plant growth promotion through inoculation of these microorganisms on several crops; for example, rice (Estrada et al. 2013), canola (De Freitas et al. 1997), maize

(Hameeda et al. 2008), and sunflower (Ekin 2010). However, it is not uncommon that microorganisms displaying phosphate solubilising traits in a laboratory fail to demonstrate significant plant growth promotion in field conditions (Richardson 2001, Bashan 2013). Further investigation is required on the methodologies of selecting these microorganisms, carrier material formulation for inoculations, and interaction of the organisms with the host crops and the environment (Richardson 2001).

In this study, the isolates demonstrated phosphate solubilising capacity, especially in liquid cultures although inoculated into furrows did not result in significant yield increases compared to control treatments. Even though the differences were not statistically significant, the grain yield of control plants was the lowest or near the lowest among the control treatments across all six field sites. Application of full P resulted in an average of 22% yield increase across the six sites ranging from 5% in site 1 to 49.9% in site 6. Responses to inoculation with the isolates varied due to location. Inoculation with EPS1 led to an average of 13.8% yield increase across locations ranging from 3% to 54%, better than the half application of P. This isolate can be further investigated for possible growth promotion and the effect on plant P content. Lack of significant yield increase of soybean for application of 20 kg P at Assossa, near to site 5 and 6, was also reported previously (Argaw 2011). A lack of P response could be due to the high phosphorus buffering index of the soil at the experimental sites (See table 5S2 in chapter 5) such that the blanket recommendation of 20 kg P ha^{-1} did not show significant difference, since the P might be converted into unavailable forms. As shown in Chapter 5, our soil sample collection sites had phosphorus buffering index (PBI) as high as 558, the average being 280. The blanket recommendation might not consider the high PBI values at sites with low pH. Such un-responsive rates of fertilizer applications could be changed with the ongoing calibration work being conducted by the Ethiopian Institute of Agricultural research intended to develop soil test based fertilizer recommendations for

various crops including soybean. The impact of soil type on these interactions is also important. Higher rates of P application (30 kg P ha^{-1}) were required to increase the production of soybean in a previous study at Alabama in acidic, and Kaolinitic soils with low organic content (Cope 1981). In other circumstances application of 60 kg P ha^{-1} did not show significant soybean yield increases over controls, until 60 kg P ha^{-1} was accompanied with 112 kg K ha^{-1} (Jones et al. 1977). Clearly, the soil phosphorus chemistry is an important factor for understanding the potential for responses to inoculants.

Table 5.1. Taxonomy and colony size of phosphate solubilising test isolates, and concentration of dissolved P, 10 d after inoculation of three P sources with 5 different types of phosphate dissolving bacteria

			Size			**P concentrations and respective P sources**					
			Initial *in vitro* screening			**$AlPO_4$**		**$FePO_4$**		**$Ca_3(PO_4)_2$**	
Test Strains	**Confirmed Genus**	**Taxonomic affiliation, ITS (99% similarity)**	CD	TD	SI	pH*	P(mg L^{-1})	pH	P(mg L^{-1})	pH	P(mg L^{-1})
EPS1	*Pseudomonas*	*P. fluorescens*	2.9	9.5	3.3	5.1	11.3 ± 0.06b	5.4	32.4 ± 0.5a	5.2	187.4 ± 6.8a
EPS2	*Bacillus*	*B. subtilis*	3.2	9.4	2.9	4.2	12.9 ± 0.2a	4.7	24.9± 0.5b	5.8	68.2 ± 0.4c
EPS3	*Bacillus*	*B. safensis*	3.0	8.8	2.9	5.6	9.6 ± 0.3c	4.3	24.9 ± 0.8b	5.7	81 ± 0.6b
EPS4	*Bacillus*	*B. velenzesis*	3.2	6.6	2.1	5.7	9.3 ± 0.2c	5.8	20.7 ± 0.6c	6.2	62.53 ± 1.3cd
EPS5	*Bacillus*	*B. velenzesis***	3.0	4.5	1.5	5.8	8.37 ± 0.6cd	5.9	22.5 ± 0.06d	5.8	62.17 ± 1cd
Control						5.9	6.93 ± 0.2d	6.0	18.7 ± 0.5e	6.5	59.27 ± 0.2d
$LSD_{0.05}$							0.4		1.67		8.8

* Final pH of treatments was similar among the majority of replicates. ** 92% similarity

Table 5.2. Yield response of soybean at harvest for the application of different phosphate dissolving inoculants at six experimental sites

	Grain yield (kg ha^{-1})					
Treatment	Site1 (Ababiya)	Site2 (Seifu)	Site3 (JARC)	Site4 (Bako)	Site5 (AARC)	Site6 (Assossa on farm)
Control	2160a	1389ab	856.0a	757.7a	758.4a	420.5a
EPS1	2299a	1458ab	885.5a	1167.8a	961.9a	451.1a
EPS2	2257a	1215b	758.7a	881.0a	961.9a	463.6a
EPS3	2247a	1215b	941.0a	960.5a	959.1a	498.3a
1/2P	2254a	1458ab	878.5a	790.7a	869.2a	553.9a
P**	2268a	1667a	928.9a	935.5a	938.3a	630.3a

*JARC= Jimma Agricultural Research Centre, AARC=Assossa Agricultural research Centre

** P= 20 kg P ha^{-1}

Chapter 6 Assessment of symbiotic effectiveness and population size of soybean rhizobia in Ethiopian soils to assist appraisal of crop inoculation requirements

Chapter five contains a manuscript submitted for publication from this thesis that investigates the population size and effectiveness of soybean nodulating rhizobia from key soybean growing regions of Ethiopia.

6.1. Manuscript summary

Soils from soybean growing regions of Ethiopia were assessed for the resident rhizobia population density and effectiveness. Variable population density, ranging from 0 to >1.5 × 10^{-4} cfu g^{-1} soil were recorded. Nearly half of the soils had a low population (<300 cfu g^{-1} soil), while the majority (72%) of the other half of soil samples had effective rhizobial population and these effective populations were found mainly in one region, South west Ethiopia. The other three regions had low and ineffective rhizobial population indicating high likelihood of response to inoculation.

6.1.1 Context

The newness of the crop, coupled with anecdotal evidence on the lack of nodulation, suggested that low numbers of soybean rhizobia were expected in soils. In contrast, several previous studies have demonstrated that competition with soil resident populations of rhizobia can result in non-responsive inoculation (Brockwell et al. 1982; Thies et al. 1991b; Denton et al. 2000). Considering these experiences, investigating the soil resident population density and effectiveness of soybean nodulating rhizobia is critical in predicting and planning inoculation programs.

6.1.2 Research objective

Investigating the population size and effectiveness of soybean nodulating rhizobia to assist in predicting soybean nodulation requirements.

6.1.3 Methods

Controlled environment assessment of the soil samples was performed using inoculation of soybean with soil suspensions ("Whole soil inoculation") to determine the effectiveness and the most probable number of the rhizobial population (MPN technique).

6.1.4 Findings

Inoculation response is likely in three out of the four regions studied, while the fourth region (South west Ethiopia) harbours effective strains that can be a further source of locally adapted strains.

6.1.5 Implications

Large areas of soybean growing regions of Ethiopia are likely to benefit from soybean inoculation provided that locally adapted and quality inoculants are distributed for growers.

6.2. Statement of Authorship

Statement of Authorship

Title of Paper	Assessment of the symbiotic effectiveness and population size of soybean rhizobia in Ethiopian soils to assist appraisal of crop inoculation.
Publication Status	☐ Published ☐ Accepted for Publication ☑ Submitted for Publication ☐ Unpublished and Unsubmitted w ork w ritten in manuscript style
Publication Details	Muleta, D., Ryder, M., Denton, M.D., 2017. Assessment of the symbiotic effectiveness and population size of soybean rhizobia in Ethiopian soils to assist appraisal of crop inoculation. Soil science and plant nutrition.

Principal Author

Name of Principal Author (Candidate)	Daniel Muleta Fana		
Contribution to the Paper	Contributed in designing the experiment, conducting the experiment and writing the paper.		
Overall percentage (%)	80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	28/8/2017

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Maarten Ryder		
Contribution to the Paper	Contributed in designing the experiment and writing the paper.		
Signature		Date	28/08/2017

Name of Co-Author	Matthew Denton		
Contribution to the Paper	Contributed in designing the experiment and writing the paper.		
Signature		Date	28/08/2017

6.3. Article, as submitted to Soil Science and Plant Nutrition (excluding references)

Assessment of the symbiotic effectiveness and population size of soybean rhizobia in Ethiopian soils to assist appraisal of crop inoculation requirements

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Running title: inoculant requirements for soybean in Ethiopia

Abstract: Soil rhizobial population density and symbiotic N fixation effectiveness are factors that assist in predicting a legume response to inoculation, but are largely unknown for soybean in Ethiopia. The abundance and symbiotic effectiveness of soybean-nodulating rhizobial populations were assessed in fifty-five soils from major soybean-growing areas of Ethiopia, using the most probable number and whole soil inoculation techniques. Estimated population densities of soybean rhizobia ranged from non-detectable to $>1.5\times10^4$ cfu g^{-1} soil; 49% of the soils had rhizobial populations of <300 cfu g^{-1} soil. Shoot dry weight of plants inoculated with soil suspensions ranged between 45 to 142 mg $plant^{-1}$, compared with 60 mg $plant^{-1}$ when un-inoculated and 136 mg $plant^{-1}$ when inoculated with a reference strain

(*Bradyrhizobium japonicum* strain CB1809). Comparison of shoot dry weights showed that in soils with populations of >300 cfu g^{-1} soil, 13% contained ineffective rhizobial populations, while 15% contained moderate and 72% of soils contained effective rhizobial populations. Soils from southwestern Ethiopia had larger and more effective rhizobial populations; here, widespread crop inoculation responses are unlikely. Responses to inoculation would be very likely in South Ethiopia, West Ethiopia and Assossa areas, as the soils mostly contained few and/or ineffective populations. In these latter regions, extension efforts related to inoculation of soybean should be targeted to provide the greatest likely benefit.

Keywords: most probable number, N fixation, resident rhizobia, soybean, symbiotic effectiveness, whole soil inoculation.

1. Introduction

Soybean is one of the most valuable and versatile crops in the world. It is a cheap source of quality protein for both human consumption and animal feed, and is used in the production of numerous products including biodiesel blends, inks, plasticizers, paints and cosmetics (Cahoon 2003; Erickson 2015). Twentynine percent of the vegetable oil in the world market is produced from soybean, demonstrating its importance as an oilseed crop (USDA 2017).

Soybean makes an important contribution to soil N through symbiotic N fixation. In field conditions, soybean fixes 334 kg N ha^{-1} on average and can fix up to 450kg N ha^{-1} in favourable conditions (Boddey et al. 1984; Keyser et al. 1992; Unkovich et al. 2000), hence farming systems that rely on soybean N fixation have a lower requirement for N fertilizer (Sinclair et al. 2014).

Due to the demand for soybean products, production has increased worldwide by an average of 4.6% annually from 1961 to 2007 (Masuda et al. 2009) with annual production growing from 217.6 million t in 2005-07 to 319.73 million t in the 2014/15 (Masuda et al. 2009; USDA 2016). In 2014/15, the world average yield for soybean was 2.7 metric t per hectare, while it was 3.2 for USA (USDA 2016).

Similarly, soybean production is increasing in Sub-Saharan Africa due to demand for cheap protein and for soil fertility maintenance (Sinclair et al. 2014). As an example, Uganda's soybean production has increased from 158,000 t in 2005 to 213,000 t in 2011 (Murithi et al. 2016). In Ethiopia, soybean production has increased thirty-eight fold from 1,600 t in 2002 to nearly 61,000 t in 2014 (Bekabil 2015). However, soybean productivity is particularly low in Africa, and well below 2 t per hectare in most of these soybean-growing countries in Africa (USDA 2016). In Ethiopia, the yield was below 1 tonne per hectare (CSA 2012) and it is only in the 2014/15 cropping season that the average yield rose to 2 t per hectare (Bekabil 2015).

Soybean N fixation is accomplished through nodulation with rhizobia, *Bradyrhizobium* spp. Absence or low abundance of rhizobia compatible with soybean was reported in soils of nine African countries (Abaidoo et al. 2007), which might explain the lower yields of soybean, in addition to low soil fertility and limited agricultural inputs (Jaiswal et al. 2016).

The population density and effectiveness of rhizobia in a soil have been used as reliable predictors of the need to inoculate legumes (Thies et al. 1991; Furseth et al. 2010). Determining the effectiveness of resident rhizobial populations can be constrained by the perceived need to isolate individual strains of rhizobia to assess effectiveness (Brockwell et al. 1988) or the resources required to conduct agronomic trials (Singleton et al. 1992). A

more effective assessment of the symbiotic effectiveness of a population of soil rhizobia can be made using the whole soil inoculation (WSI) technique (Brockwell et al. 1988), which can provide a rapid assessment of the entire soil population, which is more likely to represent the overall crop response to a genetically or phenotypically diverse population. The WSI method, coupled with the most probable number (MPN) technique, can provide an assessment of the need for inoculation (Denton et al. 2000), as these combined techniques estimate both density and symbiotic effectiveness of the soil population of rhizobia.

Although studies have recently focused on the genetic diversity of soybean rhizobia (Aserse et al. 2012; Jaiswal et al. 2016), information is lacking on the abundance of resident soybean-nodulating rhizobia in Ethiopian soils. The absence of nodules on soybean roots in some fields has been observed (Aserse et al. 2012); however, observations have not been made across all the areas where soybeans are grown. Inoculation responses for soybean have been obtained using local and imported commercial inoculants in field conditions (Bekere 2012; Jefwa et al. 2014; Muleta et al. 2017); however, inoculation has not consistently improved yield, and failures have been observed (Aserse et al. 2012) in areas such as Bako and Assossa. The reasons for poor performance of inoculants is not clear but poor adaptation of rhizobia to low soil pH is one possibility (Aserse et al. 2012). Soil acidity was previously reported to significantly decrease the response of soybean to inoculation in Southwestern Ethiopia (Bekere et al. 2013).

In this study, we assessed the abundance and symbiotic effectiveness of rhizobia in soils sampled from the four major soybean-growing areas of Ethiopia, using the WSI and MPN techniques. Soils were collected from farms and nearby lands, and cropping and inoculation

histories were established. It was hypothesised that low populations and/or low effectiveness of soybean-nodulating rhizobia in Ethiopian soils may limit effective N_2 fixation and the production of soybean. The objectives of this study were therefore to 1) assess the population density of soybean nodulating rhizobia across the main soybean growing areas of Ethiopia, 2) evaluate the effectiveness of the soil resident rhizobial population on soybean yield and 3) provide recommendations on future inoculation practices based on observed soil rhizobial population sizes and symbiotic effectiveness.

2. Materials and Methods

2.1. Site selection and collection of soil samples

Fifty-five soil samples were collected from four major soybean-growing areas of Ethiopia: SNNP (SE, n = 11), Southwestern Oromia (SWE, n = 21), Western Oromia (WE, n = 16) and Assossa Zone of Benishangul Gumuz (AZ, n=7) (Table S1, Figure 5.1). Farmers at most of the collection sites provided at least two years of cropping and inoculation history of the fields. Soil samples (0-20 cm depth) were taken from four points in each field and were mixed to make a composite sample that was stored at 4°C until the experiment was conducted.

2.2. Soil analysis

The soils collected from the field were air-dried, ground, and passed through a 2 mm sieve. All the analysis followed the methods of Rayment et al. (1992) and included total N using a Leco analyser (Method 6B1), pH H_2O (Method 4A1), pH $CaCl_2$ (Method AB1), electrical conductivity (Method 3A1), nitrate N and ammonium N (Method 7C2b), Colwell phosphorus (Method 9B), Bray II P (Method 9E2), organic carbon (Walkley and Black, Method 6A1), phosphorus buffering index (PBI, Method 912c), texture using mid-infrared (Method 6B4b). Analyses indicated that the pH ($CaCl_2$) of the soil samples ranged from 4.0 to 6.8, with a mean of 5.5 (Table S2). The samples were generally low in total N (mean 0.23%), organic carbon (mean 2%), electrical conductivity (median 0.09 units) and P (Bray-I, median 0.1 units). The samples had high PBI (median 279.8) indicating high P fixation potential of the soils. Clay content was generally high, with a maximum of 49.5% (mean 34.4). Soil texture ranged from clays (e.g. soils 29 and 57) to sandy clay loams (e.g. soils 19 and 30) and included clay loam soils (e.g. soil 36) and sandy clay (e.g. soil 1).

2.3. Most probable number determination and whole soil inoculation

The population density of soybean-nodulating rhizobia and N fixation effectiveness of the soil rhizobial population were determined concurrently in one experiment. To determine MPN of the soils (Brockwell 1963; Woomer 1994), six levels of serial dilution of each soil (5^{-1} to 5^{-6}) were prepared and each soil dilution was used to inoculate four pots (each with one plant). Symbiotic effectiveness using the whole soil inoculation technique (Brockwell et al. 1988) was determined from the replication of plants that received the lowest dilution, i.*e*. the highest concentration of the soil suspension (5^{-1}). Soils with <300 cfu g^{-1} soil are considered to be

small populations and may not provide an accurate representation of the effectiveness of the rhizobial population, as this number may limit the expression of symbiotic effectiveness (Denton et al. 2000); those categorised as having >300 cfu g^{-1} soil are considered to be large populations.

Pots were prepared using aseptic techniques (washed with detergent, dried and sprayed with 70% alcohol) and the experiment was conducted in a quarantine-approved facility. Approximately 460 g of pre-washed sand and vermiculite (1:1, vv^{-1}) was added to each 500-ml pot, moistened with McKnight's nutrient solution (McKnight 1949) to 60% of the pore space, and sterilised for 20 min at 121°C. Soybean (*Glycine max* cv. Soy791) seeds were surface sterilised using 95% ethanol for 10s and 3% sodium hypochlorite for 3 min (Somasegaran et al. 1985) followed by rinsing with six changes of sterile water. Seeds were aseptically transferred to pots (4 seeds pot^{-1}) and thinned to one plant per pot 7 d after germination.

Soil suspension and inoculation were carried out aseptically. Soil samples (100 g) were suspended in 400 ml of sterilised saline solution (0.89% NaCl) and mixed on a shaker at 150 rpm for 25 min. Each of the soil suspensions was diluted through a six-step fivefold series (5^{-1} to 5^{-6}), transferring 5 ml of suspension to 20 ml of sterile saline solution at each step (Woomer 1994). For each soil, four pots for each dilution were inoculated with 1 ml of suspension, a total of 1320 pots. A reference strain (CB1809, obtained from the Australian Inoculants Research Group, Gosford, NSW) was grown in yeast extract mannitol broth (Somasegaran et al. 1985) for 7 d and used to inoculate four replicate plants. Negative controls without inoculation or other N addition were also included. After inoculation, the pots were

randomised and the sand surface was covered with sterile 3 mm alkathene beads to reduce water loss and contamination. The plants were grown in a controlled environment room with a 12 h photoperiod with day / night temperatures of 25°C / 15°C, respectively.

2.4. Harvest

Plants were harvested 6 weeks after inoculation. The roots were assessed for the presence or absence of nodules, to determine the MPN and its correlation with other growth parameters. The four plants with the first dilution (5^{-1}) were assessed for symbiotic N fixation effectiveness, relative to the un-inoculated control and the reference strain. During harvest, the nodules were counted, and shoots and roots were oven dried at 70°C for 48 h before being weighed.

2.5. Data analysis

The MPN estimate was determined following the procedure outlined by (Woomer 1994). The lower and upper confidence interval limits ($p = 0.05$) for each estimate were calculated by dividing and multiplying the respective estimate by 3.40 (Woomer 1994).

The shoot dry weight data of the plants used for whole soil inoculation (the first four plants that received the lowest dilution) were taken and subjected to analysis of variance (ANOVA) using GenStat (VSN International 2014). The least significant difference (LSD) was used to separate treatment means at 5% level of significance and mean shoot dry weights of the treatments were compared with the un-inoculated plants and/or plants inoculated with CB1809. An isolate was considered effective if the mean value of the dry matter accumulated in the inoculated plants was not significantly different ($P < 0.05$) from that of the CB1809-

inoculated host, while being significantly different from the un-inoculated control. An isolate was classified as moderately effective if the performance of the inoculated plant was significantly higher than the un-inoculated control but significantly lower than that of the CB1809-inoculated host, and ineffective if the dry matter accumulated was not significantly different from that of the un-inoculated control (Abaidoo et al. 2007).

3. Results

3.1. Rhizobial population density

The density of rhizobial populations in the soils ranged from non-detectable to > 1.5×10^4 cfu g^{-1} soil (antilog $10^{4.18}$). Forty- nine percent of the soils (27 of 55) had a low rhizobial count (<300 cfu g^{-1} soil, Table S1). Soils from SWE, WE and AZ had maximum rhizobial populations of >1.5×10^4 cfu g^{-1} soil, while the maximum density of the rhizobial population for SE was 5.9×10^4 cfu g^{-1} soil. No rhizobia were detected in two of the 16 soil samples from the Bako area (soils 45 and 51) in WE (Table S1).

The highest proportion of soil samples with large rhizobial populations (>300 cfu g^{-1} soil) were typically identified from SWE of the Oromia Regional State, while soil samples from WE of the same Oromia Regional State had the lowest proportion of soil samples with large rhizobial populations (Table S1). Conversely, the highest proportion of soil samples with small rhizobial populations (<300 cfu g^{-1} soil) were found from WE, while the lowest proportion was from SWE.

3.2. Rhizobial population and inoculation history

The rhizobial population density was not significantly correlated with either the years since soybean was last sown ($R^2 = 0.02$) or with the history of inoculation ($R^2 = 0.0004$) (Table S1).

3.3. Symbiotic effectiveness

The symbiotic effectiveness of the soil rhizobial populations was assessed by comparing shoot dry weight of inoculated plants with shoot dry weights of the negative control (without inoculation or N addition) and of CB1809-inoculated plants. Inoculation with the soil samples resulted in shoot dry weights ranging from 45 mg (soil 3 from WE) to 142 mg (soil 26 of SWE), whereas the un-inoculated plants and the plants inoculated with CB1809 had means of 62 and 136 mg, respectively. In 25% of the soils sampled, rhizobial populations were less than half the symbiotic performance of CB1809, while 62% of the surveyed soils had ineffective rhizobial populations. Soils with <300 cfu of rhizobia g^{-1} soil (49% of soil samples) did not increase shoot dry weight above that of the un-inoculated plants. The soil samples with >300 cfu g^{-1} soil (51%) were categorised as having effective (31%), moderately effective (7%) and ineffective (13%) rhizobial populations (compared to CB1809 and the un-inoculated control; Figure 5.2).

Soil samples from the different soybean-growing areas differed in the proportion of effective rhizobia populations that they contained. SWE soils had the highest frequency of effective (62%) and moderately effective (24%) rhizobial populations, relative to CB1809, while none of the soils sampled from AZ harboured effective populations. The proportions of soil samples containing effective rhizobial populations for SE and WE were 2% and 19% respectively (Figure 5.3).

3.4. Associations among measured variables

Rhizobial density (MPN) was positively correlated with shoot dry weight ($R^2 = 0.33$, $P < 0.001$, Figure 5.4). Nodule number per plant ranged between 0 (soils 45 and 51, and the un-inoculated control) and 69 (soil 49), and was positively and significantly correlated with shoot dry weight ($R^2 = 0.38$, $P< 0.01$, data not shown).

3.5. Soil properties correlated with rhizobia population density and effectiveness

There were no meaningful correlations between soil properties and rhizobial population size and effectiveness (Table S2). However, a significant relationship ($R^2 =0.47$) was observed between symbiotic effectiveness and rhizobial population size.

4. Discussion

This is the first study to quantify the soil population densities of soybean-nodulating rhizobia and their symbiotic effectiveness in Ethiopia. The assessment of rhizobial numbers and symbiotic effectiveness is useful in allowing the prediction of responses to inoculation (Brockwell et al. 1988; Ballard et al. 2004; Herridge 2008) and are therefore likely to be important determinants that can improve N_2 fixation, yield, and grain protein level.

In this study, 96% of the soil samples assessed contained rhizobia that were able to form nodules on soybean. The soil samples that formed nodules were taken from fields that grew soybean in rotation with other crops or had grown soybean only once (e.g. soils 9 and 19), or where soybean had not been grown (e.g. soil 36). Two fields from natural forests adjacent to fields were also tested (e.g. soils 44 and 52) and contained rhizobia. The detection of soybean

rhizobia in soils from natural forests and from soils that had never been cultivated suggests that soybean-nodulating rhizobia may be indigenous to some Ethiopian soil or that rhizobia have moved to these regions from neighbouring fields, such as through air travel (Rosselli et al. 2015). We are not aware of the native plant species that might be nodulated by these rhizobia, except for *Indigofera arrecta* and *Crotalaria incana*, which are commonly found in Ethiopia and have been found to nodulate soybean (Aserse et al. 2012). Reports by Aserse et al. (2012) and Jaiswal et al. (2016) identified that native isolates of soybean nodulating *Bradyrhizobium spp*. from Ethiopia formed a distinct cluster from previously described *Bradyrhizobium spp*. type strains in phylogenetic trees. Soybean-nodulating indigenous rhizobia, potentially from native legumes, have similarly been found in other African countries, such as Nigeria (Sanginga et al. 1996), Zimbabwe (Musiyiwa et al. 2005) and Zambia (Javaheri 1996).

The lack of association between rhizobial population density with inoculation history and the years since soybean was last grown was unexpected. In other studies, stronger relationships between paddock crop rotation history and rhizobial populations were observed (Drew et al. 2012). The lack of association is likely to be related to the detection of ineffective rhizobia in large numbers in uncultivated areas. As previously mentioned, Aserse et al. (2012) demonstrated that isolates from *I. arrecta* and *C. incana* were able to form effective nodules on soybean, while other isolates from different *Indigofera* spp., *Crotalaria* spp. and *Erythrina brucei* either formed white ineffective nodules or failed to nodulate soybean. However, such cross inoculation has not been reported, with the exception of this single report. Hence, cross-

inoculation studies that identify efficient soybean nodulating rhizobia could increase the selection pool for elite strains.

The frequency of detection of soybean-nodulating rhizobia in Ethiopian soils was higher than reported for nine other African countries (Abaidoo et al. 2007), where *Bradyrhizobium* spp. nodulating soybean cv. TGx and *Bradyrhizobium japonicum* nodulating soybean cv. Clark were detected in 72% and 37% of the soils, respectively. In the present work, fivefold dilution series were used to detect rhizobial populations as small as 2 cfu g^{-1} soil (Somasegaran et al. 1985), and this sensitivity may have contributed to a higher frequency of rhizobial detection. Additional to the sensitivity of the dilution ratio used, the use of a different cultivar, Soya79, in the current study might have contributed for the higher frequency detection.

Despite the relatively high frequency of detection of rhizobia in the present study, 49% of the soil samples had <300 cfu g^{-1} soil and 42% of soils had <50 cfu g^{-1} soil. A high percentage of low numbers of soybean-nodulating rhizobia (300 cfu g^{-1} soil) was found in soil samples from AZ, WE and SE. In these areas with smaller populations of rhizobia, soybean yields will likely be improved through inoculation (Herridge 2008; Jefwa et al. 2014). Soybean growth has been improved by rhizobial inoculation in soils with low rhizobia population densities in Nigeria (Ronner et al. 2016), Egypt (Youseif et al. 2014) and Kenya (Thuita et al. 2012; Herrmann et al. 2014). A meta-analysis on soybean inoculant effectiveness similarly showed that the largest nodulation due to inoculation occurred in soils with no or low background rhizobial population (Thilakarathna et al. 2017). In such cases, inoculation is a cheap and sustainable alternative to increase soybean yield compared with the supply of inorganic N or

organic inputs, which are expensive for smallholder farmers (Hungria et al. 2013; Ronner et al. 2016).

In contrast to our expectations, large populations of soybean-nodulating rhizobia were detected in 51% of the soil samples in the present study. Ninety-five percent of the samples from SWE fall in this category and 61% of these SWE soil samples contained $>10^4$ cfu g^{-1} soil. The presence of indigenous or naturalised rhizobial populations is a significant factor that can affect the response to inoculation (Thies et al. 1991; Brockwell et al. 1995; Denton et al. 2000; Slattery et al. 2002; Denton et al. 2007; Denton et al. 2013) and soil populations can reduce nodule occupancy by commercial inoculants (Denton et al. 2002). When the background population exceeds 1000 cfu g^{-1} soil, inoculation is not expected to be beneficial (Herridge 2008). The variable numbers of soil rhizobia identified in the present study may account for some inconsistent responses to inoculation that were previously observed with commercial soybean inoculants in Ethiopia (Aserse et al. 2012).

Understanding the need to inoculate legumes, and how this varies geographically, provides useful information regarding the likelihood of responses expected from inoculation. According to our results, inoculation is unlikely to increase yields in many soils in SWE, as the numbers exceed 1000 cfu g^{-1} soil in more than 90% of the samples (Herridge 2008). This would only occur if a significantly more effective and competitive strain is used in high concentration (Hungria et al. 2017), or isolated areas with low population numbers are identified. Recent studies on soybean inoculation rates in Brazilian soils indicated that responses to inoculation can be achieved by increasing the rates of inoculant with a minimum inoculum rate of 1.2×10^6 cfu $seed^{-1}$ (Hungria et al. 2017). Increasing the inoculant rate to 2.4

$\times 10^6$ cfu seed^{-1} also showed increased grain yields in areas with high background rhizobial populations. Ethiopian inoculants are prepared in 125 g sachets having 10^9 cfu g^{-1} carrier material and distributed mainly by governmental research centres and one private company. However, the recommendation is to inoculate this 125 g inoculant on seeds sufficient to cover 0.25 hectare. The recommended seeding rate for cultivar Clark in the Jimma area is 60 kg ha^{-1}, equivalent to 1.1×10^6 cfu seed^{-1}. Therefore, it would be necessary to increase the inoculation rate to increase the likely benefit of inoculation, especially for soils with >300 cfu g^{-1}. Furthermore, some cultivars require higher seeding rates and these would also benefit from an increased inoculant application rate.

Inoculation is more likely to be important in Assossa, as 71% of the samples contained <100 cfu g^{-1} soil, and in WE and SE. Farmers in these regions should be advised to inoculate their soybean crops and appropriate extension messages targeting these regions would be more worthwhile than targeting the SWE region.

With regard to symbiotic N fixation effectiveness, 62% of soil samples from SWE contained effective populations of soybean rhizobia. It is likely that the soils from this region can be a good source of inoculants, as demonstrated by the greenhouse effectiveness test in which 62% of the soils from this region had similar effectiveness to the reference strain. Similar effectiveness of indigenous populations has been detected in other African soils, e.g. in Nigeria (Sanginga et al. 2000) and Zimbabwe (Musiyiwa et al. 2005). An understanding of genetic diversity influences the effectiveness of these populations might be a useful area of research and assist in targeting diverse strains that are highly effective.

5. Conclusions

The soil population of soybean-nodulating rhizobia and their effectiveness varied among fields and areas in Ethiopia, providing both opportunities and challenges to the soybean industry in improving soybean grain yield through inoculation. Among the major growing areas, soil samples from SE, WE and AZ generally contained smaller soybean-nodulating rhizobial populations (76% with <300 cfu g^{-1} soil), providing circumstances where inoculation is likely to improve the growth of soybean. As the abundance and distribution of the soybean nodulating rhizobia in Ethiopia is assessed in this work with improved variety (cv. Soya791), validation of this work with smaller samples is needed if similar result is achieved with the different varieties that are currently being released and also with those varieties commonly used in the different agro-ecologies. Otherwise, similar work may be required based on the widely used cultivar in the respective growing regions. Extension efforts to improve the availability and application rates of inoculants would, therefore, be very worthwhile in these regions. Higher proportions of soil samples from SWE contained large numbers of rhizobia (95% of soils contained >300 cfu g^{-1} soil) with similar effectiveness to CB1809 (86%). Inoculation with rhizobia in the majority of fields in SWE should be considered carefully and studies using high rates of inoculation should be conducted to evaluate the potential for inoculation responses. Although there were limited correlations between soil rhizobial numbers and other soil properties and cropping history, higher populations in the SWE region may provide an opportunity to isolate new, effective strains that are well-adapted to Ethiopian soils.

6. Acknowledgments

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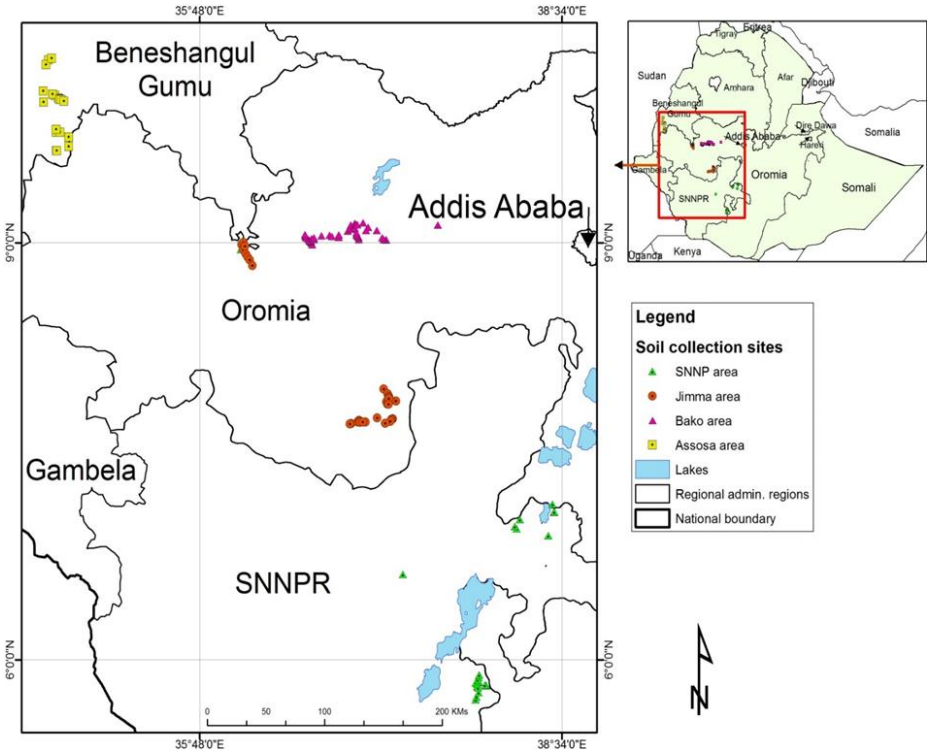

Figure 6.1. Soil collection sites of major soybean growing areas of Ethiopia. Assossa Zone (AZ) is within the Benishangul Gumuz Regional State, Southern Ethiopia (SE) comprises the Southern Nations and Nationalities People (SNNP) Regional State, South West Ethiopia (SWE) represents Jimma and Chewaka areas, and Western Ethiopia (WE) comprises Bako and Gute areas of the Oromia Regional State.

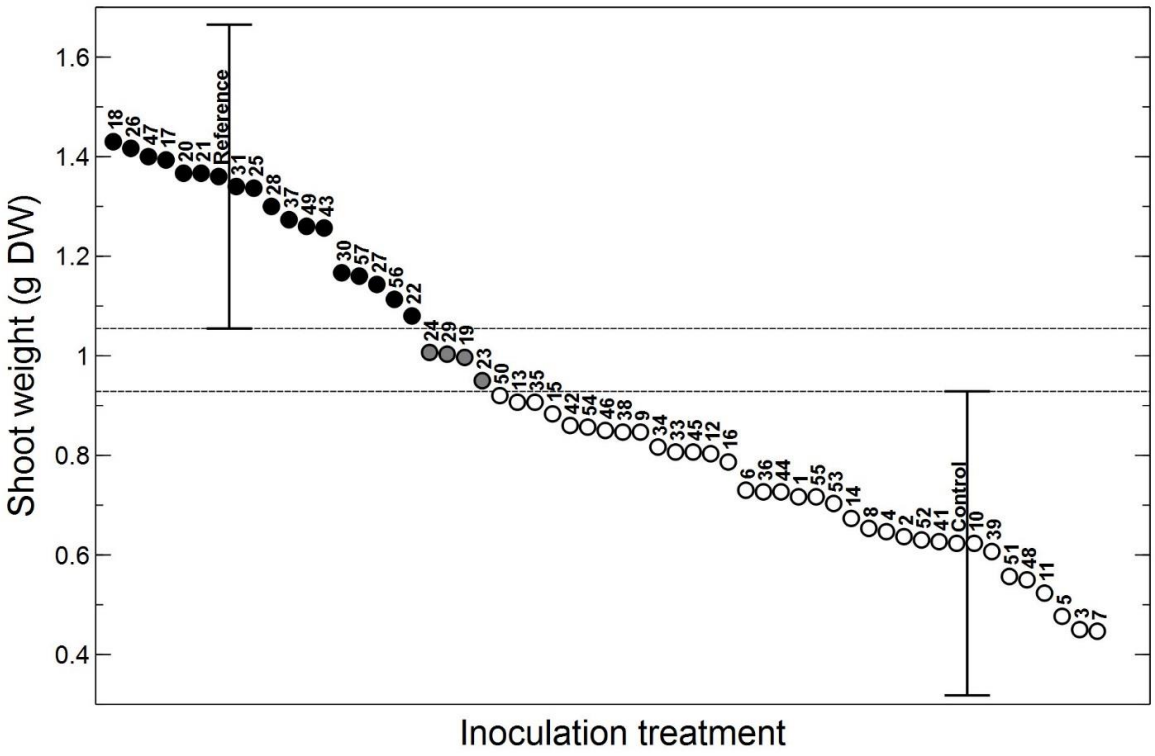

Figure 6.2. Shoot dry weight for plants inoculated with one of 55 soil samples suspensions collected from major soybean growing areas of Ethiopia, compared with a reference strain (CB1809) and an un-inoculated control. The soil inoculants are divided into three groups: those not significantly different from the reference (●), those not significantly different from the control (O) and those significantly different from both the reference and the control (◍). Error bars are the 95% LSD and the dashed lines indicate the boundaries between the three groups.

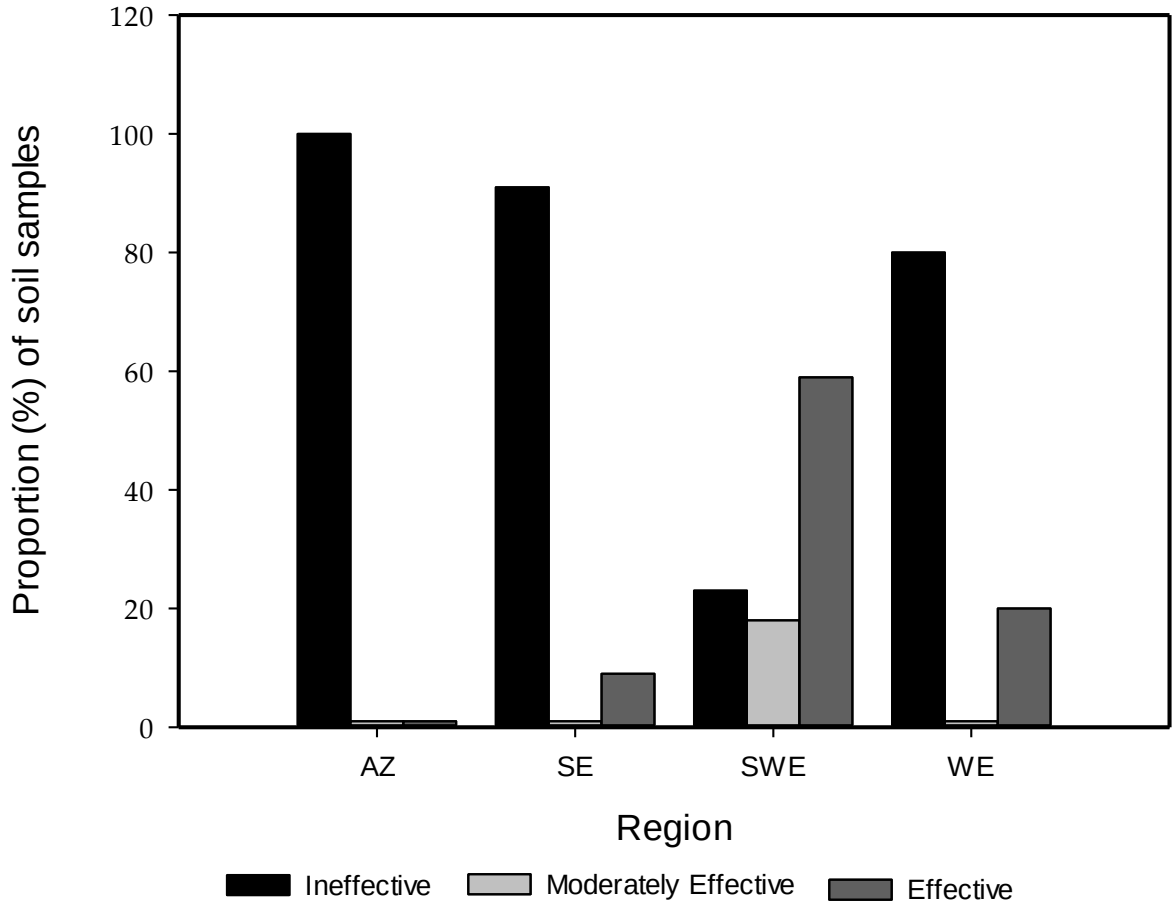

Figure 6.3. Effectiveness of soil rhizobial populations from different areas of Ethiopia where the rhizobia population was >300 cfu g^{-1} soil as determined by the most probable number technique. AZ, Assossa Zone in Benishangul Gumuz Regional State; SE, Southern Ethiopia (SNNP); SWE, Southwestern Ethiopia and WE, Western Ethiopia of Oromia Regional State.

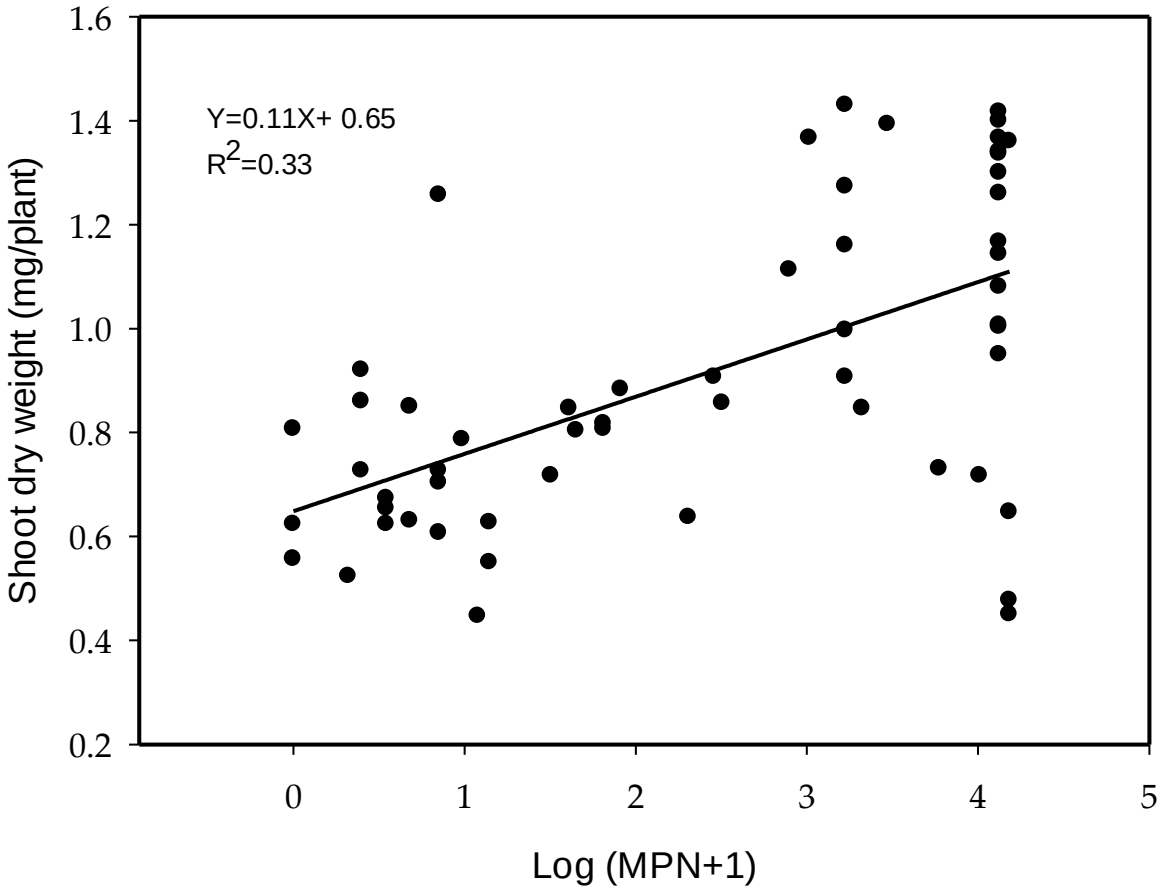

Figure 6.4. Regression line showing the association between rhizobial populations (cfu g^{-1} soil) determined by the most probable number (MPN) technique and shoot dry weight of plants ($P < 0.001$).

Supplementary Materials

Table 6S1. Soil collection areas and regions with cropping, inoculation history and rhizobial populations determined using most probable number (MPN) technique. Oromia Regional State is represented by Southwestern Ethiopia (Jimma, and Chewaka areas), and Western Ethiopia (Bako and Gute areas). Southern Ethiopia represents the Southern Nations and Nationalities People (SNNP) and extends from Hawassa to Amaro areas. Benishangul Gumuz (BG) Regional State is represented by Assossa Zone (AZ).

Number	Soil code	Region	Area	Inoculation history	Years since last soybean	Log (MPN+1)
1	Jimma-03	Oromia	SWE	No	1	4.01
2	SE-32	SNNP	SE	No	4	2.31
3	Bako-03	Oromia	WE	No	6	4.18
4	Assossa-1	BG	AZ	No	1	4.18
5	Jimma-11	Oromia	SWE	No	0.4	4.18
6	SE-39	SNNP	SE	Yes	1	3.77
7	Bako-10	Oromia	WE	No	0.4	1.08
8	Assossa-2	BG	AZ	No	1	0.54
9	Jimma-24	Oromia	SWE	No	0.4	3.32
10	SE-10	SNNP	SE	No	6	0.54
11	Bako-04	Oromia	Oromia	WE	6	0.32
12	Jimma-14	Oromia	SWE	No	4	1.65
13	SE-20	SNNP	SE	No	6	2.46
14	Bako-18	Oromia	WE	No	1	0.54
15	Assossa-3	BG	AZ	No	1	1.91
16	Assossa-4	BG	AZ	No	2	0.99
17	Jimma-40	Oromia	SWE	No	2	3.47
18	Jimma-37	Oromia	SWE	No	∞^{1}	3.23

Number	Soil code	Region	Area	Inoculation history	Years since last soybean	Log (MPN+1)
19	Jimma-08	Oromia	SWE	No	1	3.23
20	Jimma-05	Oromia	SWE	No	2	3.02
21	Jimma-34	Oromia	SWE	No	1	4.12
22	Jimma-27	Oromia	SWE	No	0.4	4.12
23	Jimma-25	Oromia	SWE	Yes	1	4.12
24	Jimma-16	Oromia	SWE	No	∞	4.12
25	Jimma-12	Oromia	SWE	Yes	1	4.12
26	Jimma-01	Oromia	SWE	No	1	4.12
27	Jimma3	Oromia	SWE	No	0.4	4.12
28	Jimma-10	Oromia	SWE	No	1	4.12
29	Jimma-28	Oromia	SWE	No	1	4.12
30	Jimma-09	Oromia	SWE	No	1	4.12
31	Jimma-32	Oromia	SWE	Yes	1	4.12
32	SE-33	SNNP	SE	No	3	1.81
33	SE-26	SNNP	SE	No	3	1.81
34	SE-36	SNNP	SE	No	0.4	3.23
35	SE-05	SNNP	SE	No	∞	0.85
36	SE-38	SNNP	SE	Yes	1	3.23
37	SE-37	SNNP	SE	Yes	1	1.61
38	SE-30	SNNP	SE	No	0.4	0.85
39	Bako-30	Oromia	WE	No	0.4	1.15
40	Bako-36	Oromia	WE	No	0.4	0.4
41	Bako-40	Oromia	WE	No	1	0.85
42	Bako-20	Oromia	WE	No	∞	0.4
43	Bako-07	Oromia	WE	No	4	0

Number	Soil code	Region	Area	Inoculation history	Years since last soybean	Log (MPN+1)
44	Bako-31	Oromia	WE	No	0.4	0.68
45	Bako-25	Oromia	WE	Yes	0.4	4.12
46	Bako-28	Oromia	WE	No	0.4	1.15
47	Bako6	Oromia	WE	No	0.4	4.12
48	Bako-12	Oromia	WE	No	6	0.4
49	Bako-32	Oromia	WE	Yes	0.4	0
50	Bako-22	Oromia	WE	No	1	0.68
51	Assossa-8	BG	AZ	No	4	0.85
52	Assossa-6	BG	AZ	No	1	2.51
53	Assossa-5	BG	AZ	No	3	1.51
54	Jimma-39	BG	AZ	No	1	2.9
55	Jimma-38	BG	AZ	No	1	3.23

∞^1 represents farms where soybean had never been grown

Table 6S2. Soil characteristics measured for 55 soil samples collected from soybean-growing areas of Ethiopia and their linear regression with rhizobial abundance and effectiveness. Mean, median and minimum and maximum values are provided for the measurement of each variable.

Variable	Mean (± s.e.)	Median	Min	Max	Linear regression Log(MPN+1)			Linear regression effectiveness		
					p-value	R^2	slope	p-value	R^2	slope
pH H_2O	6 (± 0.1)	6	5	8	0.1	-	-	0.9	-	-
pH $CaCl_2$	6 (± 0.1)	6	4	7	0.9	-	-	0.9	-	-
Total N%	0.2 (± 0.01)	0.2	0.04	0.4	0.05	0.0	-0.01	0.8	-	-
Nitrate (mg/kg)	10(± 1.1)	7	1	30	0.2	0.0	-	0.1	-	-
Ammonium (mg/kg)	22 (± 1.3)	23	4	35	0.7	-	-	0.5	-	-
C%	2 (± 0.08)	2	1	3.3	0.02	0.1	-0.10	0.6	-	-
Conductivity (ds/m)	0.1 (± 0.01)	0.1	0.03	0.2	0.6	-	-	0.5	-	-
P(Bray-I) (mg/kg)	3 (± 0.7)	1	0.1	24	-	-	-	0.7	-	-
P(Cowell) (mg/kg)	16 (± 1.2)	14	4	44	1.0	-	-	0.9	-	-
K (mg/kg)	449(± 43)	347	98	1097	0.9	-	-	0.1	-	-
PBI	280 (± 21)	309	57	558	0.9	-	-	0.6	-	-
Clay%	34 (± 0.9)	34	25	50	0.2	-	-	0.1	-	-
Silt%	10 (± 0.5)	10	2	19	0.4	-	-	0.3	-	-
Sand%	55 (± 1)	56	36	67	0.07	-	-	0.02	0.0	-2.3
Effectiveness	2	1	1	3	<0.001	.47	0.4			

Statement of Authorship

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Contribution to the Paper	Contributed in designing the experiment, conducting the experiment and writing the paper.		
Overall percentage (%)	80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Chapter 7 General discussion

7.1. Introduction

Nitrogen and phosphorus are the two of the most important plant growth limiting elements, next to sunlight and water (Vance 2001). Both N and P are deficient in the majority of soils in countries in Sub Saharan Africa due to inherent soil infertility, weathering and leaching (Okalebo et al. 2006). Chemical fertilizers are unaffordable for the vast majority of small holder farmers in Africa, particularly Sub Saharan Africa. Application of microbial inoculants can play a vital role in integrated soil fertility management, to alleviate crop N and P deficiencies in the farming systems of African countries (Sanginga et al. 2009).

Rhizobial inoculants are being promoted to farmers in Ethiopia through the Ministry of Agriculture and Rural Development and the Ethiopian Institute of Agricultural Research, in cooperation with different national and international institutes. Site-specific field experiments from the national agricultural research system have shown encouraging yield improvements with the application of rhizobial inoculants for different legume crops in Ethiopia including faba bean (Argaw 2012; Tsegaye et al. 2015), field pea (Belay et al. 2011; Argaw et al. 2017), chick pea (Tena et al. 2016), lentil (Jida et al. 2011; Tena et al. 2016) haricot bean (Argaw et al. 2015), and soybean (Solomon et al. 2012; Argaw 2014; Jefwa et al. 2014). However, multi-location trials of some of the grain legumes, such as soybean, have shown variable results in response to inoculation (Aserse et al. 2012). The reasons for the variability in the responses to inoculants are, however, not clear. Poor adaptation of imported strains to the local soil environment (Aserse et al. 2012) and competition with indigenous rhizobial populations (Jaiswal et al. 2016) were proposed as possible reasons for inoculant failures.

Responses to rhizobial inoculation are affected by various environmental factors including low soil pH, drought, and nutrient deficiency (Zahran 1999; Hungria et al. 2000; Giller 2001; Thilakarathna et al. 2017). Forty percent of the arable land of Ethiopia is covered with low pH soils (Schlede 1989) out of which 27.7 % of the arable land is covered by moderately to weakly acidic soils (pH in KCl of 4.5 -5.5) while 13.2 % are strongly acidic soils (pH in KCl, <4.5), including soils of major soybean growing regions. Hence, soybean N fixation in these areas is expected to be limited as a result of low soil pH (Bekere et al. 2013). Plant growth, rhizobial survival and the interactions between rhizobia and the plant (nodulation and N fixation) are all sensitive to highly acidic soil pH (Ferguson et al. 2013). In low pH soils, P reacts with Al and Fe to form insoluble compounds that are not available for plant uptake, (Richardson 2001) which further limits biological N fixation in low pH soils.

The experiments in this thesis were aimed at increasing the yield of soybean in acid soils of Ethiopia using microbial inoculants as inexpensive alternatives to fertilizers to improve biological N fixation and P availability. To this end, the experiments evaluated 1) yield improvement of soybean through selection and testing of acid tolerant N fixing rhizobial strains in major soybean growing areas of Ethiopia (Chapter 3), 2) the potential for yield improvement of soybean through inoculation of locally isolated P solubilizing bacteria together with N fixing inoculants (Chapter 4), 3) the size and effectiveness of the resident rhizobial populations in soil samples from major growing areas to assist the prediction of responses to inoculation (Chapter 5), and 4) the genetic diversity of the acid tolerant rhizobial isolates in reference to known *Bradyrhizobium* type strains (Chapter 6). The discussion below will highlight some of the new information gathered during this work and how it addresses the overall aims of the thesis.

7.2. Summary of results

7.2.1 The potential for rhizobial inoculation to increase soybean grain yields on acid soils in Ethiopia:

Based on an initial screening for acid tolerance on acidified agar media and another for screening N fixation on soybean grown in a controlled environment, a commercial strain and four local isolates were evaluated for yield improvement in six field experiments in soybean-growing areas of Ethiopia with soil pH (H_2O) ranging from 4.3 to 4.8. The yields in response to application of the commercial strain (532c) were consistently greater than for the other treatments at all of the test sites. Inoculation with newly-selected local strains showed increases relative to the control, with regard to yield and nodule number at field sites, where the resident soil rhizobial populations were $\leq 1.4 \times 10^3$ cfu g^{-1} soil. In the soils with population $\leq 1.4 \times 10^3$ cfu g^{-1} soil, inoculation with local isolates resulted in yields greater than, or comparable to, the application of fertilizer at 46 kg N ha^{-1}. However, in soils with resident rhizobia populations $> 1.4 \times 10^3$ cfu g^{-1} soil, there were no significant nodulation responses to inoculation. Despite the expression of acid tolerance *in vitro*, two of the local isolates didn't perform well relative to the control at five of the six field sites, due potentially to by acid soil related stresses, which indicated the critical importance of field validation during screening for acid tolerance.

7.2.2 Evaluation of the diversity in acid tolerant soybean rhizobia from key agricultural regions of Ethiopia

The genetic diversity of acid tolerant strains was studied using the ITS portion of the 16S-23S region of DNA. Analysis of the ITS region showed that the acid tolerant strains were

distributed within the *Bradyrhizobium* genus and were genetically distinct from the *Bradyrhizobium* type strains and from each other, corroborating previous studies (Aserse et al. 2012; Jaiswal et al. 2016) of soybean-nodulating rhizobia isolated from Ethiopian soils.

Similarly, symbiotic effectiveness was found to be distributed relatively evenly among the different groups, so was not found to occur in particular clusters. The most efficient strains were, however, grouped in three different clusters (Cluster C, D, and F). In addition, on a phylogenetic tree constructed using the neighbour-joining algorithm of the ITS sequences of the 16S-23S region, the acid tolerant strains isolated in the current study occupied different phylogenetic positions (clusters) compared with those of strains previously isolated from soils of Ethiopia (Jaiswal et al. 2016).

7.2.3 Isolation and evaluation of phosphate dissolving bacteria associated with soybean

Strains of *Bacillus spp.* and a *Pseudomonas* spp. were isolated from soils collected from farms that grew soybean and selected for field testing after an *in vitro* screening process. When these strains were grown in liquid culture that contained Al, Fe and Ca phosphates, they significantly increased the available P relative to controls. The increase in soluble P in the culture solution was accompanied by a decrease in pH.

Two strains of *Bacillus* spp. and a strain of *Pseudomonas* sp. were tested in 6 field experiments through furrow application of a filter mud carrier. There appeared to be some soybean yield increases over the controls, but these were not statistically significant. Inoculation with strain EPS1 (a *Pseudomonas* spp.), in particular, resulted in an average yield

increase of 13.8% over the control. There would be merit in testing this strain further with a different inoculation strategy, such as seed inoculation, in concert with rhizobial inoculants. The lack of responses following the addition of P solubilising bacteria in the field experiments were considered to be due to high phosphorus buffering index (PBI) of the soils (PBI up to 558 was identified in Chapter 5). Since the application of P at a rate of 20 kg ha^{-1} did not elicit a yield response, any effects of the inoculant strains were considered to have released less than 20 kg P ha^{-1}. Further investigation of the influence of P levels in different soils in the range of their PBI values and through an understanding of soil P chemistry may help in understanding whether P solubilizing organisms can help to improve soybean nutrition.

7.2.4 Assessment of the symbiotic effectiveness and population size of soybean rhizobia in Ethiopian soils to assist appraisal of crop inoculation requirements

The abundance and symbiotic effectiveness of background populations of soil rhizobia in 55 soils from major soybean growing areas of Ethiopia were analysed in a controlled environment. The population size of the resident soil rhizobia varied from 0 to > 1.5×10^4 cfu g^{-1} soil while 49% of the soils had populations <300 cfu g^{-1} soil.

The highest proportion of soil samples with >300 cfu g^{-1} soil were typically identified from SWE of the Oromia Regional State, while soil samples from WE of the Oromia Regional state had the lowest proportion of soil samples with large rhizobial populations and two of the soil samples were devoid of detectable soybean nodulating rhizobia.

Where the soils contained rhizobial populations >300 cfu g^{-1} soil, the N fixation effectiveness was variable. Among the soil samples assessed, there were populations of rhizobia that were effective (72%), moderately effective (15%) or ineffective (13%). On the other hand, all soils

with < 300 rhizobial cfu g^{-1} soil were ineffective (49% of soils) presumably due to the low numbers of rhizobia. Soils from SWE contained large populations of resident rhizobia with higher proportions of effective rhizobia, and hence the decision whether to inoculate soybean in this region of Ethiopia needs careful attention; increasing the rate of inoculant application might benefit soybean production when soybean is sown into soils with large backgrounds of rhizobia. The soils in SE, WE and AZ contained smaller and/or ineffective soybean nodulating rhizobial populations; here there are greater opportunities to benefit from an inoculation response (Figure 7.1).

7.3. Conclusions and recommendations

Inconsistent responses to inoculation of soybean were previously observed in Ethiopia during efforts to verify and promote the use of commercial soybean inoculants. The reasons for this variable response to inoculation was not clear, as it was thought the soybean-nodulating rhizobia may be absent from the soils (Aserse et al. 2012). This research indicated that low soil pH and resident rhizobial population sizes were two potential factors affecting soybean inoculation responses in major soybean growing areas of Ethiopia. One commercial and two local soybean rhizobial isolates were demonstrated in this research to be effective when soybean was grown in low pH soils, depending on the size of the background soil rhizobial population, while two other selected soybean strains appeared to be more sensitive to acid related factors, and did not increase nodulation in the field relative to controls. Further screening of additional soil samples from the SWE is proposed, to enable the selection of better adapted, acid tolerant and effective N fixing strains, such as those reported in a recent investigation focussed on locally adapted and highly efficient soybean nodulating rhizobial

strains from Mozambique (Chibeba et al. 2017). For further screening works, including poorly acid tolerant strains as a control will give a more clear picture on the performance of acid tolerant strains. The background rhizobial population size in soils from the major soybean growing areas of Ethiopia was variable; soils from SWE contained larger and more effective rhizobia populations. Rhizobial population densities are usually correlated with the number of years since soybean was last grown and /or with inoculation history (Parr et al. 2017), although the results of this study showed no significant correlation with the presence and level of soil resident populations in the majority of soil samples in SWE. In this area, agricultural extension programs should be judicious in promoting the use of rhizobial inoculants. Lack of response to inoculation due to competition from native soil rhizobia might challenge growers' trust for inoculant technology, not only for soybean but for other legumes too. Additional surveys to determine the population sizes of the rhizobia across SWE will help to identify areas that can benefit from inoculation within the region and may assist with an extension to target inoculation programs. Increasing the rate of inoculant application to seed has been shown in other situations to overcome competition for nodulation (Hungria et al. 2017). A study on the response to different inoculation rates in soils with high background rhizobial populations is critical, as failure to nodulate in the presence of background rhizobia population is well-established (Brockwell et al. 1995). In addition, even though high rates of inoculation are found to be effective in soils with background rhizobial populations, careful attention to product quality is required, to ensure that inoculants distributed to growers are up to the required standards, as the numerical abundance of rhizobia is crucial to successful inoculation (Brockwell et al. 1995; Hartley et al. 2005; Denton et al. 2013). With respect to the soybean industry in the remaining areas studied (SE, WE and AZ), a large proportion of

soils from these regions had small rhizobial populations, and hence a dedicated inoculation program with acid tolerant strains in these regions will likely be of sound economic benefit for the growers.

The current study shows that a combination of screening on acidified agar plates and evaluation of N fixation effectiveness in controlled environments is an effective method to select acid tolerant strains for use in acid soils. However, after screening acid tolerance using plate assays, evaluation of their symbiotic effectiveness in low soil pH under controlled environment conditions might be an important additional step to enable selection of strains that can efficiently tolerate acidic soil conditions and fix N. In the process of selecting strains, validating the most tolerant and efficient strains in field conditions is equally important. To clearly show the performance of acid tolerant strains, including poorly acid tolerant strains, either from the plate screenings or from other know source as controls would be of paramount importance. From genetic characterisation of *Bradyrhizobium* strains in chapter 4, it was suggested that *B.elkanii* might be acid sensitive. Including this strain in acid tolerance evaluation, both on plate and field condition will help in proving whether it is really acid sensitive.

The inoculation of P solubilizing bacteria in similar (acidic) field soils indicated some potential to increase the yield of soybean, however, the increases were not significant and the identification of responses was constrained due to the high PBI of the soils, and the inability of the blanket P fertilizer recommendation to increase yield significantly. To clearly show the potential benefit of P solubilizing microorganisms, soils in which there is a response to applied P or the interaction of P with other nutrients need to be identified, as observed for K

and Mg in Kenyan soils (Keino et al. 2015). Efforts will be made to evaluate these P solubilising strains in low PBI soils. Furrow application of N fixing inoculants has been shown to be inferior to seed inoculation (Denton et al. 2017). Similarly, the furrow application of P dissolving bacteria (as done in this study) might not be as effective as seed inoculation. Further work on improved application methods for inoculation of P solubilizing organisms will be important to enable exploitation of these microorganisms.

An analysis of the diversity of the *Bradyrhizobium* spp. isolated in this study revealed that the acid tolerant strains are diverse. The ITS sequence analysis of the 16S-23S rDNA region has been shown to give similar groupings to that produced by analysis of DNA-DNA hybridization in most of the *Bradyrhizobium* species, except for *Aeschynomene* species, which showed variable ITS sequence (Willems et al. 2003). Due to the high discrimination power of the analysis of ITS sequences, the acid tolerant strains were analysed for their diversity using this method. Corroborating previous work, the acid tolerant strains were found to be diverse, interspersed in the *Bradyrhizobium* ITS-based phylogenetic tree, and distinct from the previously identified *Bradyrhizobium* type strains. In addition, the positions of newly isolated soybean rhizobia in the ITS-based phylogenetic tree were found to be different from previously isolated Ethiopian *Bradyrhizobium* strains (Jaiswal et al. 2016). The different position on the phylogenetic tree of these strains isolated in different seasons supports the observation that screening for acid tolerant rhizobia on acidified media enabled the identification of different rhizobial groups of interest among soybean nodulating rhizobial strains from Ethiopia. Conversely, the absence of certain rhizobial groups (e.g. isolates that grouped with *B. elkanii*) among the acid tolerant strains also suggested that screening on acidified media helps in the selection of acid tolerant strains. Some of the strains were closely

grouped with strains isolated from different legumes such as *Aeschynomene indica* and *Arachis hypogaea*. The role of these species in sharing similar rhizobia types with soybean is not clear and we know very little about the existence of other legumes with the same cross inoculation group as soybean. However, similar expectations on the existence of legumes with same inoculation group with soybean were reported in Malawi (Parr et al. 2017). Identification of such legumes will benefit from increasing the pool from which elite strains for soybean can be screened. The acid tolerant strains in this study were found to be diverse but distinct from currently described bradyrhizobial type strains. To locate the taxonomic position of these acid tolerant strains more precisely, multilocus sequence analysis of the core and symbiotic genes is required.

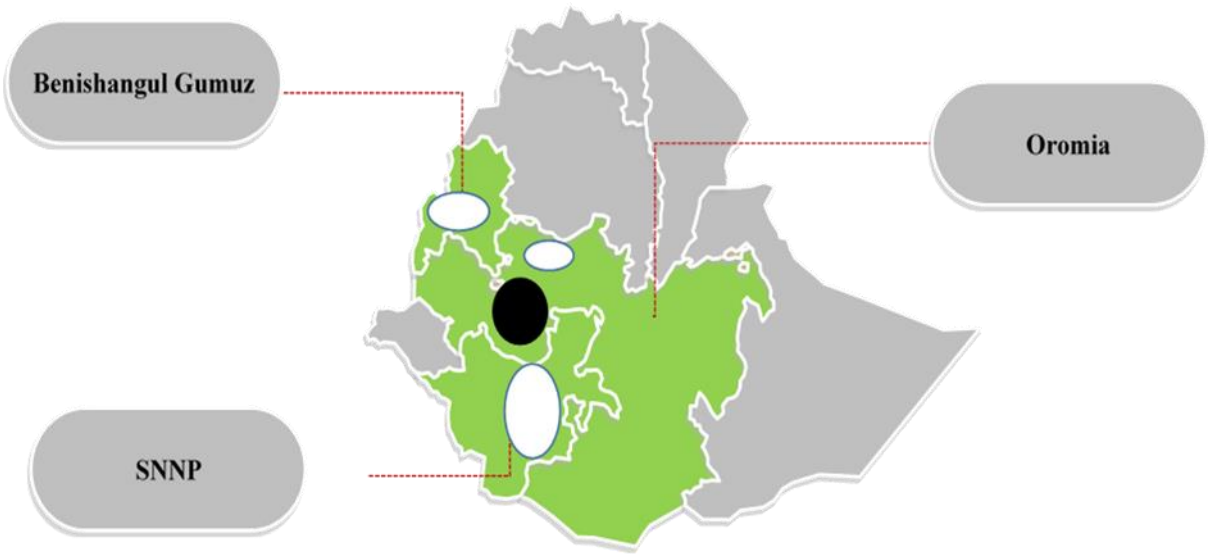

Figure 7.1. Soybean growing regions of Ethiopia (shaded in green) and localised areas that are found to respond to inoculation (in white) and areas that are with a large background rhizobial population (shaded in black). SNNP stands for Southern Nations and Nationality Peoples of Ethiopia.

References

Abaidoo, R, Keyser, H, Singleton, P, Dashiell, K & Sanginga, N 2007, 'Population size, distribution, and symbiotic characteristics of indigenous *Bradyrhizobium* spp. that nodulate TGx soybean genotypes in Africa', *Applied and Environmental Microbiology*, vol. 35, no. 1, pp. 57-67.

Abate, T, Alene AD, Bergvinson D, Shiferaw B, Silim S, Orr A & Asfaw S 2012, *Tropical Grain Legumes in Africa and South Asia: Knowledge and Opportunities.*, ICRISAT, Nairobi, Kenya.

Abdenna, D, Negassa, C & Tilahun, G 2007, 'Inventory of soil acidity status in crop lands of Central and Western Ethiopia', *Utilisation of diversity in land use systems: Sustainable and organic approaches to meet human needs*, Witzenhausen.

Acinas, SG, Marcelino, LA, Klepac-Ceraj, V & Polz, MF 2004, 'Divergence and redundancy of 16S rRNA sequences in genomes with multiple rrn operons', *Journal of bacteriology*, vol. 186, no. 9, pp. 2629-2635.

Adesemoye, AO & Kloepper, JW 2009, 'Plant–microbes interactions in enhanced fertilizer-use efficiency', *Applied microbiology and biotechnology*, vol. 85, no. 1, pp. 1-12.

Ahmad, F, Ahmad, I & Khan, M 2008, 'Screening of free-living rhizospheric bacteria for their multiple plant growth promoting activities', *Microbiological research*, vol. 163, no. 2, pp. 173-181.

Albareda, M, Rodríguez-Navarro, DN & Temprano, FJ 2009, 'Soybean inoculation: Dose, N fertilizer supplementation and rhizobia persistence in soil', *Field Crops Research*, vol. 113, no. 3, pp. 352-356.

Alves, BJ, Boddey, RM & Urquiaga, S 2003, 'The success of BNF in soybean in Brazil', *Plant and Soil*, vol. 252, no. 1, pp. 1-9.

Ambrosini, A, de Souza, R & Passaglia, LM 2016, 'Ecological role of bacterial inoculants and their potential impact on soil microbial diversity', *Plant and Soil*, vol. 400, no. 1-2, pp. 193-207.

Andrés, JA, Rovera, M, Guiñazú, LB, Pastor, NA & Rosas, SB 2012, 'Interactions between legumes and rhizobia under stress conditions', *Bacteria in Agrobiology: Stress Management*, Springer, pp. 77-94.

Andrews, M & Andrews, ME 2017, 'Specificity in legume-rhizobia symbioses', *International journal of molecular sciences*, vol. 18, no. 4, p. 705.

Appunu, C, Sasirekha, N, Prabavathy, VR & Nair, S 2009, 'A significant proportion of indigenous rhizobia from India associated with soybean (*Glycine max* L.) distinctly belong to *Bradyrhizobium* and *Ensifer* genera', *Biology and Fertility of soils*, vol. 46, no. 1, p. 57.

Ardley, JK, Reeve, WG, O'Hara, GW, Yates, RJ, Dilworth, MJ & Howieson, JG 2013, 'Nodule morphology, symbiotic specificity and association with unusual rhizobia are distinguishing features of the genus *Listia* within the southern African crotalarioid clade *Lotononis s.l.*', *Annals of botany*, vol. 112, no. 1, pp. 1-15.

Argaw, A 2011, 'Evaluation of co-inoculation of *Bradyrhizobium japonicum* and Phosphate solubilizing *Pseudomonas spp.* effect on soybean (*Glycine max* L. Merr.) in Assossa Area', *Journal of Agricultural Science and Technology*, vol. 14, no. 1, pp. 213-224.

—— 2012, 'Characterization of symbiotic effectiveness of rhizobia nodulating Faba bean (*Vicia faba* L.) isolated from Central Ethiopia', *Research Journal of Microbiology*, vol. 7, no. 6, p. 280.

—— 2014, 'Symbiotic effectiveness of inoculation with *Bradyrhizobium* isolates on soybean [*Glycine max* (L.) Merrill] genotypes with different maturities', *SpringerPlus*, vol. 3, no. 1, p. 753.

Argaw, A & Akuma, A 2015, '*Rhizobium leguminosarum bv. viciae sp*. inoculation improves the agronomic efficiency of N of common bean (*Phaseolus vulgaris* L.)', *Environmental Systems Research*, vol. 4, no. 1, p. 11.

Argaw, A & Mnalku, A 2017, 'Symbiotic effectiveness of *Rhizobium leguminosarum* bv. *vicieae* isolated from major highland pulses on field pea (*Pisum sativum* L.) in soil with abundant rhizobial population', *Annals of Agrarian Science*.

Aserse, A, Räsänen, LA, Aseffa, F, Hailemariam, A & Lindström, K 2012, 'Phylogenetically diverse groups of *Bradyrhizobium* isolated from nodules of *Crotalaria* spp., *Indigofera* spp., *Erythrina brucei* and *Glycine max* growing in Ethiopia', *Molecular phylogenetics and evolution*, vol. 65, no. 2, pp. 595-609.

Atnaf, M, Tesfaye, K & Kifle, D 2015, 'The importance of legumes in the Ethiopian farming system and overall economy: An overview', *American Journal of Alternative Agriculture*, vol. 7, no. 6, pp. 347-358.

Ayanaba, A, Asanuma, S & Munns, D 1983, 'An Agar Plate Method for Rapid Screening of Rhizobium for Tolerance to Acid-Aluminum Stress 1', *Soil Science Society of America Journal*, vol. 47, no. 2, pp. 256-258.

Ballard, R, Charman, N, McInnes, A & Davidson, J 2004, 'Size, symbiotic effectiveness and genetic diversity of field pea rhizobia (*Rhizobium leguminosarum* bv. *viciae*) populations in South Australian soils', *Soil Biology and Biochemistry*, vol. 36, no. 8, pp. 1347-1355.

Bashan, Y 1998, 'Inoculants of plant growth-promoting bacteria for use in agriculture', *Biotechnology advances*, vol. 16, no. 4, 7, pp. 729-770.

Bashan, Y, Kamnev, AA & de-Bashan, LE 2013, 'A proposal for isolating and testing phosphate-solubilizing bacteria that enhance plant growth', *Biology and Fertility of soils*, pp. 1-2.

Baslam, M, Garmendia, I & Goicoechea, N 2011, 'Arbuscular mycorrhizal fungi (AMF) improved growth and nutritional quality of greenhouse-grown lettuce', *Journal of agricultural and food chemistry*, vol. 59, no. 10, pp. 5504-5515.

Bekabil, UT 2015, 'Empirical review of production, productivity and marketability of soya bean in Ethiopia', *IJUNESST*, vol. 8, no. 1, pp. 61-66.

Bekere, W 2012, 'Liming Effects on Yield and Yield Attributes of Nitrogen Fertilized and *Bradyrhizobia* Inoculated Soybean (*Glycine max L.*) Grown in Acidic soil at Jimma, South Western Ethiopia', *Journal of Biology, Agriculture and Healthcare*, vol. 3, no. 14, p. 201.

Bekere, W, Kebede, T & Dawud, J 2013, 'Growth and nodulation response of soybean (*Glycine max* L.) to lime, *Bradyrhizobium japonicum* and nitrogen fertilizer in acid soil at Melko, South western Ethiopia', *International journal of Soil Science*, vol. 8, no. 1, p. 25.

Belay, Z & Assefa, F 2011, 'Symbiotic and phenotypic diversity of *Rhizobium leguminosarum* bv. *viciae* from Northern Gondar, Ethiopia', *African Journal of Biotechnology*, vol. 10, no. 21, pp. 4372-4379.

Berrada, H & Fikri-Benbrahim, K 2014, 'Taxonomy of the Rhizobia: Current Perspectives', *British Microbiology Research Journal*, vol. 4, pp. 616-639.

Bezdicek, D, Evans, D, Abede, EB & Witters, R 1978, 'Evaluation of Peat and Granular Inoculum for Soybean Yield and N Fixation Under Irrigation 1', *Agronomy journal*, vol. 70, no. 5, pp. 865-868.

Bhangoo, M & Albritton, D 1976, 'Nodulating and non-nodulating Lee soybean isolines response to applied nitrogen', *Agron. J.*, vol. 68, no. 4, pp. 642-645.

Boddey, RM, Chalk, PM, Victoria, RL & Eiichi, M 1984, 'Nitrogen fixation by nodulated soybean under tropical field conditions estimated by the 15 N isotope dilution technique', *Soil Biology and Biochemistry*, vol. 16, no. 6, pp. 583-588.

Bonaldi, K, Gargani, D, Prin, Y, Fardoux, J, Gully, D, Nouwen, N, Goormachtig, S & Giraud, E 2011, 'Nodulation of *Aeschynomene afraspera* and *A. indica* by photosynthetic *Bradyrhizobium* sp. strain ORS285: the nod-dependent versus the nod-independent symbiotic interaction', *Molecular plant-microbe interactions*, vol. 24, no. 11, pp. 1359-1371.

Brockwell, J 1963, 'Accuracy of a plant-infection technique for counting populations of *Rhizobium trifolii*', *Applied microbiology*, vol. 11, no. 5, pp. 377-383.

—— 2001, '*Sinorhizobium meliloti* in Australian soils: population studies of the root-nodule bacteria for species of *Medicago* in soils of the Eyre Peninsula, South Australia', *Australian Journal of Experimental Agriculture*, vol. 41, no. 6, pp. 753-762.

Brockwell, J & Bottomley, PJ 1995, 'Recent advances in inoculant technology and prospects for the future', *Soil Biology and Biochemistry*, vol. 27, no. 4, pp. 683-697.

Brockwell, J, Bottomley, PJ & Thies, JE 1995, 'Manipulation of rhizobia microflora for improving legume productivity and soil fertility: a critical assessment', *Plant and Soil*, pp. 143-180.

Brockwell, J, Gault, R, Herridge, D, Morthorpe, L & Roughley, R 1988, 'Studies on alternative means of legume inoculation: microbiological and agronomic appraisals of commercial procedures for inoculating soybeans with *Bradyrhizobium japonicum*', *Australian Journal of Agricultural Research*, vol. 39, no. 6, pp. 965-972.

Brockwell, J, Gault, R, Zorin, M & Roberts, M 1982, 'Effects of environmental variables on the competition between inoculum strains and naturalized populations of *Rhizobium trifolii* for nodulation of *Trifolium subterraneum* L. and on rhizobia persistence in the soil', *Australian Journal of Agricultural Research*, vol. 33, no. 5, pp. 803-815.

Brockwell, J, Gault, RR, Morthorpe, LJ, Peoples, MB, Turner, GL & Bergersen, FJ 1989, 'Effects of soil nitrogen status and rate of inoculation on the establishment of populations of *Bradyrhizobium japonicum* and on the nodulation of soybeans', *Australian Journal of Agricultural Research*, vol. 40, no. 4, pp. 753-762.

Brockwell, J, Holliday, R & Pilka, A 1988, 'Evaluation of the symbiotic nitrogen-fixing potential of soils by direct microbiological means', *Plant and Soil*, vol. 108, no. 1, pp. 163-170.

Bullard, G, Roughley, R & Pulsford, D 2005, 'The legume inoculant industry and inoculant quality control in Australia: 1953–2003', *Australian Journal of Experimental Agriculture*, vol. 45, no. 3, pp. 127-140.

Bünemann, EK, Schwenke, G & Van Zwieten, L 2006, 'Impact of agricultural inputs on soil organisms—a review', *Soil Research*, vol. 44, no. 4, pp. 379-406.

Cahoon, EB 2003, 'Genetic enhancement of soybean oil for industrial uses: prospects and challenges', *AgBioForum*, vol. 6, pp. 11-13.

Cakmak, I 2002, 'Plant nutrition research: Priorities to meet human needs for food in sustainable ways', *Plant and Soil*, vol. 247, no. 1, pp. 3-24.

Carey Jr, D 2009, 'Guatemala's green revolution: synthetic fertilizer, public health, and economic autonomy in the Mayan highland', *Agricultural history*, pp. 283-322.

Catroux, G, Hartmann, A & Revellin, C 2001, 'Trends in rhizobial inoculant production and use', *Plant and Soil*, vol. 230, no. 1, pp. 21-30.

Cattelan, A, Hartel, P & Fuhrmann, J 1999, 'Screening for plant growth–promoting rhizobacteria to promote early soybean growth', *Soil Science Society of America Journal*, vol. 63, no. 6, pp. 1670-1680.

Chelius, M & Lepo, J 1999, 'Restriction fragment length polymorphism analysis of PCR-amplified nifH sequences from wetland plant rhizosphere communities', *Environmental Technology*, vol. 20, no. 8, pp. 883-889.

Chen, LS, Figueredo, A, Pedrosa, FO & Hungria, M 2000, 'Genetic characterization of soybean rhizobia in Paraguay', *Applied and Environmental Microbiology*, vol. 66, no. 11, pp. 5099-5103.

Chen, W, Wang, E, Wang, S, Li, Y, Chen, X & Li, Y 1995, 'Characteristics of Rhizobium tianshanense sp. nov., a moderately and slowly growing root nodule bacterium isolated from an arid saline environment in Xinjiang, People's Republic of China', *International journal of systematic and evolutionary microbiology*, vol. 45, no. 1, pp. 153-159.

Chen, YP, Rekha, PD, Arun, AB, Shen, FT, Lai, WA & Young, CC 2006, 'Phosphate solubilizing bacteria from subtropical soil and their tricalcium phosphate solubilizing abilities', *Applied soil ecology*, vol. 34, no. 1, pp. 33-41.

Cheng, Q 2008, 'Perspectives in biological nitrogen fixation research', *Journal of integrative plant biology*, vol. 50, no. 7, pp. 786-798.

Chianu, JN, Nkonya, EM, Mairura, F, Chianu, JN & Akinnifesi, F 2010, 'Biological nitrogen fixation and socioeconomic factors for legume production in sub-Saharan Africa: a review', *Agronomy for Sustainable Development*.

—— 2011, 'Biological nitrogen fixation and socioeconomic factors for legume production in sub-Saharan Africa: a review', *Agronomy for Sustainable Development*, vol. 31, no. 1, pp. 139-154.

Chibeba, AM, Kyei-Boahen, S, de Fátima Guimarães, M, Nogueira, MA & Hungria, M 2017, 'Isolation, characterization and selection of indigenous *Bradyrhizobium* strains with outstanding symbiotic performance to increase soybean yields in Mozambique', *Agriculture, ecosystems & environment*, vol. 246, pp. 291-305.

Clement, BG, Kehl, LE, DeBord, KL & Kitts, CL 1998, 'Terminal restriction fragment patterns (TRFPs), a rapid, PCR-based method for the comparison of complex bacterial communities', *Journal of microbiological methods*, vol. 31, no. 3, pp. 135-142.

Collins, M, Thies, J & Abbott, L 2002, 'Diversity and symbiotic effectiveness of *Rhizobium leguminosarum* bv. *trifolii* isolates from pasture soils in south-western Australia', *Soil Research*, vol. 40, no. 8, pp. 1319-1329.

Cooper, J, Bjourson, A, Streit, W & Werner, D 1998, 'Isolation of unique nucleic acid sequences from rhizobia by genomic subtraction: Applications in microbial ecology and symbiotic gene analysis', *Plant and Soil*, vol. 204, no. 1, pp. 47-55.

Cope, J 1981, 'Effects of 50 years of fertilization with phosphorus and potassium on soil test levels and yields at six locations', *Soil Science Society of America Journal*, vol. 45, no. 2, pp. 342-347.

CSA 2012, *Agricultural Sample Survey 2011-2012*, Annual Bulletin, CSA, Addis Ababa, Ethiopia.

Czernic, P, Gully, D, Cartieaux, F, Moulin, L, Guefrachi, I, Patrel, D, Pierre, O, Fardoux, J, Chaintreuil, C & Nguyen, P 2015, 'Convergent evolution of endosymbiont differentiation in dalbergioid and inverted repeat-lacking clade legumes mediated by nodule-specific cysteine-rich peptides', *Plant Physiology*, vol. 169, no. 2, pp. 1254-1265.

Date, RA & Halliday, J 1979, 'Selecting *Rhizobium* for acid, infertile soils of the tropics', *Nature*, vol. 277, no. 5691, pp. 62-64.

De Freitas, J, Banerjee, M & Germida, J 1997, 'Phosphate-solubilizing rhizobacteria enhance the growth and yield but not phosphorus uptake of canola (*Brassica napus* L.)', *Biology and Fertility of soils*, vol. 24, no. 4, pp. 358-364.

de Vries, FT & Bardgett, RD 2012, 'Plant–microbial linkages and ecosystem nitrogen retention: lessons for sustainable agriculture', *Frontiers in Ecology and the Environment*, vol. 10, no. 8, pp. 425-432.

Deaker, R, Hartley, E, Gemell, G, Herridge, DF & Karanja, N 2016, *Inoculant production and quality control*, Working with rhizobia, eds JG Howieson & MJ Dilworth, Australian Center for Internation Agricultural Research, Canberra.

Deaker, R, Roughley, RJ & Kennedy, IR 2004, 'Legume seed inoculation technology—a review', *Soil Biology and Biochemistry*, vol. 36, no. 8, pp. 1275-1288.

Delamuta, JRM, Ribeiro, RA, Ormeño-Orrillo, E, Melo, IS, Martínez-Romero, E & Hungria, M 2013, 'Polyphasic evidence supporting the reclassification of *Bradyrhizobium japonicum group* Ia strains as *Bradyrhizobiumdiazoefficiens* sp. nov', *International journal of systematic and evolutionary microbiology*, vol. 63, no. 9, pp. 3342-3351.

Delamuta, JRM, Ribeiro, RA, Ormeño-Orrillo, E, Parma, MM, Melo, IS, Martínez-Romero, E & Hungria, M 2015, '*Bradyrhizobium tropiciagri sp.* nov. and *Bradyrhizobium embrapense sp*. nov., nitrogen-fixing symbionts of tropical forage legumes', *International journal of systematic and evolutionary microbiology*, vol. 65, no. 12, pp. 4424-4433.

Demidchik, V, Maathuis, F & Voitsekhovskaja, O 2018, 'Unravelling the plant signalling machinery: an update on the cellular and genetic basis of plant signal transduction', *Functional Plant Biology*, vol. 45, no. 2, pp. 1-8.

Denton, M, Coventry, D, Bellotti, W & Howieson, J 2000, 'Distribution, abundance and symbiotic effectiveness of *Rhizobium leguminosarum* bv. *trifolii* from alkaline pasture soils in South Australia', *Australian Journal of Experimental Agricultural Research*, vol. 40, no. 1, pp. 25-35.

Denton, M, Coventry, D, Murphy, P, Howieson, J & Bellotti, W 2002, 'Competition between inoculant and naturalised *Rhizobium leguminosarum* bv. *trifolii* for nodulation of annual clovers in alkaline soils', *Crop and Pasture Science*, vol. 53, no. 9, pp. 1019-1026.

Denton, MD, Hill, CR, Bellotti, WD & Coventry, DR 2007, 'Nodulation of *Medicago truncatula* and *Medicago polymorpha* in two pastures of contrasting soil pH and rhizobial populations', *Applied soil ecology*, vol. 35, no. 2, pp. 441-448.

Denton, MD, Pearce, DJ & Peoples, MB 2013, 'Nitrogen contributions from faba bean (*Vicia faba* L.) reliant on soil rhizobia or inoculation', *Plant and Soil*, vol. 365, no. 1-2, pp. 363-374.

Denton, MD, Phillips, LA, Peoples, MB, Pearce, DJ, Swan, AD, Mele, PM & Brockwell, J 2017, 'Legume inoculant application methods: effects on nodulation patterns, nitrogen fixation, crop growth and yield in narrow-leaf lupin and faba bean', *Plant Soil*, pp. 1-15.

Diédhiou, I & Diouf, D 2018, 'Transcription factors network in root endosymbiosis establishment and development', *World Journal of Microbiology and Biotechnology*, vol. 34, no. 3, p. 37.

Dima, S & Odero, A 1997, 'Organic farming for sustainable agricultural production. A brief theoretical review and preliminary empirical evidence', *Environmental and resource Economics*, vol. 10, no. 2, pp. 177-188.

Downie, JA 2014, 'Legume nodulation', *Current Biology*, vol. 24, no. 5, pp. R184-R190.

Drew, E, Denton, M, Sadras, V & Ballard, R 2012, 'Agronomic and environmental drivers of population size and symbiotic performance of *Rhizobium leguminosarum* bv. *viciae* in Mediterranean-type environments', *Crop and Pasture Science*, vol. 63, no. 5, pp. 467-477.

Drew, E, Herridge, D, Ballard, R, O'Hara, G, Deaker, R, Denton, M, Yates, R, Gemell, G, Hartley, E & Phillips, L 2012, 'Inoculating legumes: a practical guide', *Grains Research and Development Corporation*.

Dyson, T 1999, 'World food trends and prospects to 2025', *Proceedings of the National Academy of Sciences*, vol. 96, no. 11, pp. 5929-5936.

Ekin, Z 2010, 'Performance of phosphate solubilizing bacteria for improving growth and yield of sunflower (*Helianthus annuus* L.) in the presence of phosphorus fertilizer', *African Journal of Biotechnology*, vol. 9, no. 25, pp. 3794-3800.

Elsheikh, E & Wood, M 1995, 'Nodulation and N_2 fixation by soybean inoculated with salt-tolerant rhizobia or salt-sensitive bradyrhizobia in saline soil', *Soil Biology and Biochemistry*, vol. 27, no. 4, pp. 657-661.

Erickson, DR 2015, *Practical handbook of soybean processing and utilization*, AOCS Press, Champaign.

Estrada, GA, Baldani, VLD, de Oliveira, DM, Urquiaga, S & Baldani, JI 2013, 'Selection of phosphate-solubilizing diazotrophic *Herbaspirillum* and *Burkholderia* strains and their effect on rice crop yield and nutrient uptake', *Plant and Soil*, vol. 369, no. 1-2, pp. 115-129.

Felsenstein, J 1985, 'Confidence limits on phylogenies: an approach using the bootstrap', *Evolution*, pp. 783-791.

Ferguson, B, Lin, M-H & Gresshoff, PM 2013, 'Regulation of legume nodulation by acidic growth conditions', *Plant signaling & behavior*, vol. 8, no. 3, p. e23426.

Figueras, MJ, Beaz-Hidalgo, R, Hossain, MJ & Liles, MR 2014, 'Taxonomic affiliation of new genomes should be verified using average nucleotide identity and multilocus phylogenetic analysis', *Genome announcements*, vol. 2, no. 6, pp. e00927-00914.

Foy, CD, Lee, EH & Wilding, SB 1987, 'Differential aluminum tolerances of two barley cultivars related to organic acids in their roots', *Journal of plant nutrition*, vol. 10, no. 9-16, pp. 1089-1101.

Frémont, M, Prin, Y, Chauviere, M, Diem, H, Pwee, K & Tan, T 1999, 'A comparison of *Bradyrhizobium* strains using molecular, cultural and field studies', *Plant Science*, vol. 141, no. 1, pp. 81-91.

Fuentes-Ramirez, LE & Caballero-Mellado, J 2005, 'Bacterial biofertilizers', *PGPR: Biocontrol and biofertilization*, Springer, pp. 143-172.

Furseth, BJ, Conley, SP & Ané, J-M 2010, 'Enumeration of soybean-associated rhizobia with quantitative real-time polymerase chain reaction', *Crop Science*, vol. 50, no. 6, pp. 2591-2596.

Gelfand, I & Robertson, GP 2015, 'A reassessment of the contribution of soybean biological nitrogen fixation to reactive N in the environment', *Biogeochemistry*, vol. 123, no. 1-2, pp. 175-184.

Gemell, L, Roughley, R, Reed, M & Hartley, EJ 1993, 'Screening of *Rhizobium leguminosarum* bv. *Trifolii* for adaptation to acid and neutral soils using a selective agar medium', *Soil Biol. Biochem.*, vol. 25, no. 10, pp. 1463-1464.

Giller, KE 2001, *Nitrogen fixation in tropical cropping systems*, CAB International, Wallingford, UK.

Giraud, E, Moulin, L, Vallenet, D, Barbe, V, Cytryn, E, Avarre, J-C, Jaubert, M, Simon, D, Cartieaux, F & Prin, Y 2007, 'Legumes symbioses: absence of Nod genes in photosynthetic bradyrhizobia', *Science*, vol. 316, no. 5829, pp. 1307-1312.

Gleason, C, Chaudhuri, S, Yang, T, Munoz, A, Poovaiah, B & Oldroyd, GE 2006, 'Nodulation independent of rhizobia induced by a calcium-activated kinase lacking autoinhibition', *Nature*, vol. 441, no. 7097, pp. 1149-1152.

Goldstein, AH 1986, 'Bacterial solubilization of mineral phosphates: historical perspective and future prospects', *American Journal of Alternative Agriculture*, vol. 1, no. 02, pp. 51-57.

González-Andrés, F & Ortiz, JM 1998, 'Biodiversity of rhizobia nodulating *Genista monspessulana* and *Genista linifolia* in Spain', *New Zealand Journal of Agricultural Research*, vol. 41, no. 4, pp. 585-594.

Gourion, B, Berrabah, F, Ratet, P & Stacey, G 2015, '*Rhizobium*–legume symbioses: the crucial role of plant immunity', *Trends in plant science*, vol. 20, no. 3, pp. 186-194.

Graham, P, Viteri, S, Mackie, F, Vargas, A & Palacios, A 1982, 'Variation in acid soil tolerance among strains of *Rhizobium phaseoli*', *Field Crops Research*, vol. 5, pp. 121-128.

Grönemeyer, JL, Chimwamurombe, P & Reinhold-Hurek, B 2015, '*Bradyrhizobium subterraneum* sp. nov., a symbiotic nitrogen-fixing bacterium from root nodules of groundnuts', *International journal of systematic and evolutionary microbiology*, vol. 65, no. 10, pp. 3241-3247.

Gupta, G, Parihar, SS, Ahirwar, NK, Snehi, SK & Singh, V 2015, 'Plant growth promoting rhizobacteria (PGPR): current and future prospects for development of sustainable agriculture', *Journal of Microbial & Biochemical Technology*, vol. 7, no. 2, pp. 096-102.

Haileslassie, A, Priess, J, Veldkamp, E, Teketay, D & Lesschen, JP 2005, 'Assessment of soil nutrient depletion and its spatial variability on smallholders' mixed farming systems in Ethiopia using partial versus full nutrient balances', *Agriculture, ecosystems & environment*, vol. 108, no. 1, pp. 1-16.

Hailu, M & Kelemu, K 2014, 'Trends in Soy Bean Trade in Ethiopia', *Research Journal of Agriculture and Environmental Management.*, vol. 3, no. 9, pp. 477-484.

Hameeda, B, Harini, G, Rupela, O, Wani, S & Reddy, G 2008, 'Growth promotion of maize by phosphate-solubilizing bacteria isolated from composts and macrofauna', *Microbiological research*, vol. 163, no. 2, pp. 234-242.

Hartley, E, Gemell, L, Slattery, J, Howieson, J & Herridge, D 2005, 'Age of peat-based lupin and chickpea inoculants in relation to quality and efficacy', *Australian Journal of Experimental Agriculture*, vol. 45, no. 3, pp. 183-188.

Hartmann, A, Giraud, JJ & Catroux, G 1998, 'Genotypic diversity of *Sinorhizobium* (formerly *Rhizobium*) meliloti strains isolated directly from a soil and from nodules of alfalfa (*Medicago sativa*) grown in the same soil', *FEMS Microbiology Ecology*, vol. 25, no. 2, pp. 107-116.

Hassen, AI, Bopape, F & Sanger, L 2016, 'Microbial inoculants as agents of growth promotion and abiotic stress tolerance in plants', *Microbial inoculants in sustainable agricultural productivity*, Springer, pp. 23-36.

Hebb, M, Richardson, AE, Reid, R & Brockwell, J 1998, 'PCR as an ecological tool to determine the establishment and persistence of *Rhizobium* strains introduced into the field as seed inoculant Diane', *Australian Journal of Agricultural Research*, vol. 49, no. 6, pp. 923-934.

Herridge, D 2008, 'Inoculation technology for legumes', in MJ Dilworth, EK James, JI Sprent & WE Newton (eds), *Nitrogen-fixing leguminous symbioses*, Springer, Dordrecht, The Netherlands, pp. 77-115.

Herridge, D & Rose, I 2000, 'Breeding for enhanced nitrogen fixation in crop legumes', *Field Crops Research*, vol. 65, no. 2, pp. 229-248.

Herridge, DF, Peoples, MB & Boddey, RM 2008, 'Global inputs of biological nitrogen fixation in agricultural systems', *Plant and Soil*, vol. 311, no. 1/2, pp. 1-18.

Herridge, DF, Peoples, MB & Boddey, RM 2008, 'Global inputs of biological nitrogen fixation in agricultural systems', *Plant and Soil*, vol. 311, no. 1-2, pp. 1-18.

Herrmann, L, Chotte, J-L, Thuita, M & Lesueur, D 2014, 'Effects of cropping systems, maize residues application and N fertilization on promiscuous soybean yields and diversity of native rhizobia in Central Kenya', *Pedobiologia*, vol. 57, no. 2, pp. 75-85.

Heuer, H, Hartung, K, Wieland, G, Kramer, I & Smalla, K 1999, 'Polynucleotide probes that target a hypervariable region of 16S rRNA genes to identify bacterial isolates corresponding to bands of community fingerprints', *Applied and Environmental Microbiology*, vol. 65, no. 3, pp. 1045-1049.

Higa, T & Parr, JF 1994, *Beneficial and effective microorganisms for a sustainable agriculture and environment*, vol. 1, International Nature Farming Research Center Atami,, Japan.

Howard, JB & Rees, DC 1996, 'Structural basis of biological nitrogen fixation', *Chemical Reviews*, vol. 96, no. 7, pp. 2965-2982.

Howieson, J & Ewing, M 1986, 'Acid tolerance in the *Rhizobium meliloti–Medicago* symbiosis', *Australian Journal of Agricultural Research*, vol. 37, no. 1, pp. 55-64.

Howieson, J, Ewing, M & D'antuono, M 1988, 'Selection for acid tolerance in *Rhizobium meliloti*', *Plant Soil*, vol. 105, no. 2, pp. 179-188.

Howieson, J, Ewing, M, Thorn, C & Revell, C 1991, 'Increased yield in annual species of *Medicago* grown in acidic soil in response to inoculation with acid tolerant *Rhizobium meliloti*', *Plant-Soil Interactions at Low pH*, Springer, pp. 589-595.

Hungria, M, Araujo, RS, Júnior, S, Barbosa, E & Zilli, JÉ 2017, 'Inoculum Rate Effects on the Soybean Symbiosis in New or Old Fields under Tropical Conditions', *Agronomy journal*, vol. 109, no. 3, pp. 1106-1112.

Hungria, M, Campo, R, Chueire, L, Grange, L & Megias, M 2001, 'Symbiotic effectiveness of fast-growing rhizobial strains isolated from soybean nodules in Brazil', *Biology and Fertility of soils*, vol. 33, no. 5, pp. 387-394.

Hungria, M & Franco, AA 1993, 'Effects of high temperature on nodulation and nitrogen fixation by *Phaseolus vulgaris* L', *Plant and Soil*, vol. 149, no. 1, pp. 95-102.

Hungria, M, Loureiro, M, Mendes, I, Campo, R & Graham, P 2005, 'Inoculant preparation, production and application', *Nitrogen fixation in agriculture, forestry, ecology, and the environment*, Springer, pp. 223-253.

Hungria, M, Nogueira, MA & Araujo, RS 2013, 'Co-inoculation of soybeans and common beans with rhizobia and azospirilla: strategies to improve sustainability', *Biology and Fertility of soils*, vol. 49, no. 7, pp. 791-801.

Hungria, M & Vargas, MA 2000, 'Environmental factors affecting N_2 fixation in grain legumes in the tropics, with an emphasis on Brazil', *Field Crops Research*, vol. 65, no. 2, pp. 151-164.

Illmer, P, Barbato, A & Schinner, F 1995, 'Solubilization of hardly-soluble AlPO 4 with P-solubilizing microorganisms', *Soil Biology and Biochemistry*, vol. 27, no. 3, pp. 265-270.

Indrasumunar, A, Dart, PJ & Menzies, NW 2011, 'Symbiotic effectiveness of *Bradyrhizobium japonicum* in acid soils can be predicted from their sensitivity to acid soil stress factors in acidic agar media', *Soil Biol. Biochem.*, vol. 43, pp. 2046-2052.

Indrasumunar, A, Menzies, NW & Dart, PJ 2012, 'Laboratory prescreening of Bradyrhizobium japonicum for low pH, Al and Mn tolerance can be used to predict their survival in acid soils', *Soil Biology and Biochemistry*, vol. 48, pp. 135-141.

Israel, DW 1987, 'Investigation of the role of phosphorus in symbiotic dinitrogen fixation', *Plant Physiology*, vol. 84, no. 3, pp. 835-840.

Jaiswal, SK, Beyan, SM & Dakora, FD 2016, 'Distribution, diversity and population composition of soybean-nodulating bradyrhizobia from different agro-climatic regions in Ethiopia', *Biology and Fertility of soils*, vol. 52, no. 5, pp. 725-738.

Jarvis, B, Downer, H & Young, J 1992, 'Phylogeny of fast-growing soybean-nodulating rhizobia supports synonymy of *Sinorhizobium* and *Rhizobium* and assignment to *Rhizobium fredii*', *International journal of systematic and evolutionary microbiology*, vol. 42, no. 1, pp. 93-96.

Javaheri, F 1996, 'Naturally nodulating (promiscuous) soybean varieties for smallholder farmers: the Zambian experience', *Soyabeans in Smallholder Cropping Systems of Zimbabwe. SoilFertNet/CIMMYT, Harare, Zimbabwe*, pp. 63-73.

Jefwa, J, Pypers, P, Jemo, M, Thuita, M, Mutegi, E, Laditi, M, Faye, A, Kavoo, A, Munyahali, W & Herrmann, L 2014, 'Do commercial biological and chemical products increase crop yields and economic returns under smallholder farmer conditions?', in B Vanlauwe & PaB VanAsten, G. (eds), *Challenges and opportunities for agricultural intensification of the humid highland systems of sub-Saharan Africa.*, Springer International Publishing, Cham, Switzerland, pp. 81-96.

Jida, M & Assefa, F 2011, 'Phenotypic and plant growth promoting characteristics of *Rhizobium leguminosarum* bv. *viciae* from lentil growing areas of Ethiopia', *African Journal of Microbiology Research*, vol. 5, no. 24, pp. 4133-4142.

Jones, G, Lutz, J & Smith, T 1977, 'Effects of phosphorus and potassium on soybean nodules and seed yield', *Agronomy journal*, vol. 69, no. 6, pp. 1003-1006.

Jordan, D 1982, 'Transfer of *Rhizobium japonicum* Buchanan 1980 to *Bradyrhizobium* gen. nov., a genus of slow-growing, root nodule bacteria from leguminous plants', *International journal of systematic and evolutionary microbiology*, vol. 32, no. 1, pp. 136-139.

Kaur, S, Dhillon, G, Brar, S, Chauhan, V, Chand, R & Verma, M 2013, 'Potential Eco-friendly Soil Microorganisms: Road Towards Green and Sustainable Agriculture', in A Malik, E Grohmann & M Alves (eds), *Management of Microbial Resources in the Environment*, Springer Netherlands, pp. 249-287.

Keino, L, Baijukya, F, Ng'etich, W, Otinga, AN, Okalebo, JR, Njoroge, R & Mukalama, J 2015, 'Nutrients Limiting Soybean (*glycine max* l) Growth in Acrisols and Ferralsols of Western Kenya', *PloS one*, vol. 10, no. 12, p. e0145202.

Keyser, H & Munns, D 1979, 'Effects of calcium, manganese, and aluminum on growth of rhizobia in acid media', *Soil Science Society of America Journal*, vol. 43, no. 3, pp. 500-503.

Keyser, HH, Bohlool, BB, Hu, T & Weber, DF 1982, 'Fast-growing rhizobia isolated from root nodules of soybean', *Science*, vol. 215, no. 4540, pp. 1631-1632.

Keyser, HH & Li, F 1992, 'Potential for increasing biological nitrogen fixation in soybean', *Biological Nitrogen Fixation for Sustainable Agriculture*, Springer, pp. 119-135.

—— 1992, 'Potential for increasing biological nitrogen fixation in soybean', *Plant Soil*, vol. 141, no. 1-2, pp. 119-135.

Khan, MS, Zaidi, A & Wani, PA 2007, 'Role of phosphate-solubilizing microorganisms in sustainable agriculture—a review', *Agronomy for Sustainable Development*, vol. 27, no. 1, pp. 29-43.

—— 2009, 'Role of phosphate solubilizing microorganisms in sustainable agriculture-a review', *Sustainable agriculture*, Springer, pp. 551-570.

Kimura, M 1980, 'A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences', *Journal of molecular evolution*, vol. 16, no. 2, pp. 111-120.

Konstantinidis, KT & Tiedje, JM 2007, 'Prokaryotic taxonomy and phylogeny in the genomic era: advancements and challenges ahead', *Current opinion in microbiology*, vol. 10, no. 5, pp. 504-509.

Kumar, V & Narula, N 1999, 'Solubilization of inorganic phosphates and growth emergence of wheat as affected by *Azotobacter chroococcum* mutants', *Biology and Fertility of soils*, vol. 28, no. 3, pp. 301-305.

Ladha, J, De Bruijn, F & Malik, K 1997, 'Introduction: assessing opportunities for nitrogen fixation in rice-a frontier project', *Plant and Soil*, vol. 194, no. 1-2, pp. 1-10.

Lagos, L, Maruyama, F, Nannipieri, P, Mora, M, Ogram, A & Jorquera, M 2015, 'Current overview on the study of bacteria in the rhizosphere by modern molecular techniques: a mini–review', *Journal of soil science and plant nutrition*, vol. 15, no. 2, pp. 504-523.

Lal, R 2009, 'Laws of sustainable soil management', in E Lichtfouse, M Navarrete, P Debaeke, V Souchère & C Alberola (eds), *Sustainable Agriculture*, Springer, Netherlands, pp. 9-12.

Lateef, A, Adelere, IA & Gueguim-Kana, EB 2015, 'The biology and potential biotechnological applications of Bacillus safensis', *Biologia*, vol. 70, no. 4, pp. 411-419.

Lebrazi, S & Benbrahim, KF 2014, 'Environmental stress conditions affecting the N_2 fixing *Rhizobium*-legume symbiosis and adaptation mechanisms', *African Journal of Microbiology Research*, vol. 8, no. 53, pp. 4053-4061.

Long, SR 1996, '*Rhizobium* symbiosis: nod factors in perspective', *The Plant Cell*, vol. 8, no. 10, p. 1885.

Loureiro, M, James, E, Sprent, J & Franco, A 1995, 'Stem and root nodules on the tropical wetland legume *Aeschynomene fluminensis*', *New Phytologist*, vol. 130, no. 4, pp. 531-544.

Ludwig, W, Amann, R, Martinez-Romero, E, Schönhuber, W, Bauer, S, Neef, A & Schleifer, K-H 1998, 'rRNA based identification and detection systems for rhizobia and other bacteria', *Molecular microbial ecology of the soil*, Springer, pp. 1-19.

Lupwayi, N, Olsen, P, Sande, E, Keyser, H, Collins, M, Singleton, P & Rice, W 2000, 'Inoculant quality and its evaluation', *Field Crops Research*, vol. 65, no. 2, pp. 259-270.

Mamo, T & Haque, I 1987, 'Phosphorus status of some Ethiopian soils', *Plant and Soil*, vol. 102, no. 2, pp. 261-266.

Marra, LM, Soares, CRFS, de Oliveira, SM, Ferreira, PAA, Soares, BL, de Fráguas Carvalho, R, de Lima, JM & de Souza Moreira, FM 2012, 'Biological nitrogen fixation and phosphate solubilization by bacteria isolated from tropical soils', *Plant and Soil*, vol. 357, no. 1-2, pp. 289-307.

Martens, M, Dawyndt, P, Coopman, R, Gillis, M, De Vos, P & Willems, A 2008, 'Advantages of multilocus sequence analysis for taxonomic studies: a case study using 10 housekeeping genes in the genus *Ensifer* (including former *Sinorhizobium*)', *International journal of systematic and evolutionary microbiology*, vol. 58, no. 1, pp. 200-214.

Martínez-Romero, E & Caballero-Mellado, J 1996, '*Rhizobium* phylogenies and bacterial genetic diversity', *Critical Reviews in Plant Sciences*, vol. 15, no. 2, pp. 113-140.

Martins, CM, Borges, WL, Júnior, C, de Souza, J & Rumjanek, NG 2015, 'Rhizobial diversity from stem and root nodules of *Discolobium* and *Aeschynomene*', *Acta Scientiarum. Agronomy*, vol. 37, no. 2, pp. 163-170.

Masepohl, B & Forchhammer, K 2007, 'Regulatory cascades to express nitrogenase', *Biology of the nitrogen cycle. Elsevier, Amsterdam*, pp. 131-145.

Masson-Boivin, C, Giraud, E, Perret, X & Batut, J 2009, 'Establishing nitrogen-fixing symbiosis with legumes: how many *Rhizobium* recipes?', *Trends in microbiology*, vol. 17, no. 10, pp. 458-466.

Masuda, T & Goldsmith, PD 2009, 'World soybean production: area harvested, yield, and long-term projections', *International Food and Agribusiness Management Review*, vol. 12, no. 4, pp. 143-161.

McAdam, EL, Hugill, C, Fort, S, Samian, E, Cottaz, S, Davies, NW, Reid, JB & Foo, E 2017, 'Determining the site of action of strigolactones during nodulation', *Plant Physiology*, p. pp. 00741.02017.

McKnight, T 1949, 'The efficiency of isolates of *Rhizobium* in the cowpea group with proposed additions to this group.', *Queensland journal of agricultural science*, vol. 6, pp. 61-67.

Mousavi, SA, Österman, J, Wahlberg, N, Nesme, X, Lavire, C, Vial, L, Paulin, L, De Lajudie, P & Lindström, K 2014, 'Phylogeny of the *Rhizobium–Allorhizobium–Agrobacterium* clade supports the delineation of *Neorhizobium* gen. nov', *Systematic and applied microbiology*, vol. 37, no. 3, pp. 208-215.

Mousavi, SA, Willems, A, Nesme, X, De Lajudie, P & Lindström, K 2015, 'Revised phylogeny of *Rhizobiaceae*: proposal of the delineation of *Pararhizobium* gen. nov., and 13 new species combinations', *Systematic and applied microbiology*, vol. 38, no. 2, pp. 84-90.

Muleta, D, Assefa, F, Hjort, K, Roos, S & Granhall, U 2009, 'Characterization of Rhizobacteria isolated from Wild Coffea arabica L', *Engineering in Life Sciences*, vol. 9, no. 2, pp. 100-108.

Muleta, D, Ryder, M & Denton, MD 2017, 'The potential for rhizobial inoculation to increase soybean grain yields on acid soils in Ethiopia', *Soil Science and Plant Nutrition*.

Murithi, H, Beed, F, Tukamuhabwa, P, Thomma, B & Joosten, M 2016, 'Soybean production in eastern and southern Africa and threat of yield loss due to soybean rust caused by Phakopsora pachyrhizi', *Plant Pathology*, vol. 65, no. 2, pp. 176-188.

Mus, F, Crook, MB, Garcia, K, Costas, AG, Geddes, BA, Kouri, ED, Paramasivan, P, Ryu, M-H, Oldroyd, GE & Poole, PS 2016, 'Symbiotic nitrogen fixation and the challenges to its extension to nonlegumes', *Applied and Environmental Microbiology*, vol. 82, no. 13, pp. 3698-3710.

Musiyiwa, K, Mpepereki, S & Giller, K 2005, 'Symbiotic effectiveness and host ranges of indigenous rhizobia nodulating promiscuous soyabean varieties in Zimbabwean soils', *Soil Biology and Biochemistry*, vol. 37, no. 6, pp. 1169-1176.

Nelson, GC, Rosegrant, MW, Palazzo, A, Gray, I, Ingersoll, C, Robertson, R, Tokgoz, S, Zhu, T, Sulser, TB & Ringler, C 2010, *Food security, farming, and climate change to 2050: Scenarios, results, policy options*, vol. 172, Intl Food Policy Res Inst.

Nelson, MS & Sadowsky, MJ 2015, 'Secretion systems and signal exchange between nitrogen-fixing rhizobia and legumes', *Frontiers in plant science*, vol. 6.

Neset, TSS & Cordell, D 2012, 'Global phosphorus scarcity: identifying synergies for a sustainable future', *Journal of the Science of Food and Agriculture*, vol. 92, no. 1, pp. 2-6.

Newton, WE 2004, *Nitrogen fixation: origins, applications, and research progress*, eds D Werner & WE Newton, Springer, Netherland.

Newton, WE 2006, 'Physiology, biochemistry and molecular biology of nitrogen fixation', *Biology of the Nitrogen Cycle; Bothe, H., Ferguson, SJ, Newton, WE, Eds*, pp. 109-130.

Nick, G 1998, *Polyphasic taxonomy of rhizobia isolated from tropical tree legumes*, University of Helsinki, Helsinki (Finlandia). Department of Applied Chemistry and Microbiology.

Niemann, S, Dammann-Kalinowski, T, Nagel, A, Pühler, A & Selbitschka, W 1999, 'Genetic basis of enterobacterial repetitive intergenic consensus (ERIC)-PCR fingerprint pattern in *Sinorhizobium meliloti* and identification of *S. meliloti* employing PCR primers derived from an ERIC-PCR fragment', *Archives of microbiology*, vol. 172, no. 1, pp. 22-30.

O'Hara, G, Zilli, J, Poole, P & Hungria, M 2016, 'Taxonomy and physiology of rhizobia', in JG Howieson & MJ Dilworth (eds), *Working with Rhizobia*, Australian Centre for International Agricultural Research, Canberra.

O'Callaghan, M 2016, 'Microbial inoculation of seed for improved crop performance: issues and opportunities', *Applied microbiology and biotechnology*, vol. 100, no. 13, pp. 5729-5746.

Okalebo, J, Othieno, CO, Woomer, PL, Karanja, N, Semoka, J, Bekunda, M, Mugendi, DN, Muasya, R, Bationo, A & Mukhwana, E 2006, 'Available technologies to replenish soil fertility in East Africa', in A Bationo, B Waswa, J Kihara & J Kimetu (eds), *Nutrient Cycling in Agroecosystems*, vol. 76, Springer, Dordrecht, The Netherlands, pp. 153-170.

Oldroyd, GE 2013, 'Speak, friend, and enter: signalling systems that promote beneficial symbiotic associations in plants', *Nature reviews. Microbiology*, vol. 11, no. 4, p. 252.

Ormeno-Orrillo, E, Servín-Garcidueñas, LE, Rogel, MA, González, V, Peralta, H, Mora, J, Martínez-Romero, J & Martínez-Romero, E 2015, 'Taxonomy of rhizobia and agrobacteria from the *Rhizobiaceae* family in light of genomics', *Systematic and applied microbiology*, vol. 38, no. 4, pp. 287-291.

Paffetti, D, Daguin, F, Fancelli, S, Gnocchi, S, Lippi, F, Scotti, C & Bazzicalupo, M 1998, 'Influence of plant genotype on the selection of nodulating *Sinorhizobium meliloti* strains by *Medicago sativa*', *Antonie van Leeuwenhoek*, vol. 73, no. 1, pp. 3-8.

Pagano, MC & Miransari, M 2015, 'The importance of soybean production worldwide', *Abiotic and Biotic Stresses in Soybean Production: Soybean Production*, vol. 1, p. 1.

Parr, M, Griffith, E & Grossman, J 2017, 'Drivers of promiscuous soybean associated rhizobia diversity in un-inoculated soil in Malawi', *Symbiosis*, vol. 71, no. 2, pp. 129-141.

Peng, GX, Tan, ZY, Wang, ET, Reinhold-Hurek, B, Chen, WF & Chen, WX 2002, 'Identification of isolates from soybean nodules in Xinjiang Region as *Sinorhizobium xinjiangense* and genetic differentiation of *S. xinjiangense* from *Sinorhizobium fredii*', *International journal of systematic and evolutionary microbiology*, vol. 52, no. 2, pp. 457-462.

Peoples, MB, Herridge, DF & Ladha, JK 1995, 'Biological nitrogen fixation: An efficient source of nitrogen for sustainable agricultural production?', *Plant and Soil*, vol. 174, no. 1-2, 1995/07/01, pp. 3-28.

Perret, X & Broughton, WJ 1998, 'Rapid identification of *Rhizobium* strains by targeted PCR fingerprinting', *Plant and Soil*, vol. 204, no. 1, pp. 21-34.

Philpotts, H 1976, 'Filter mud as a carrier for Rhizobium inoculants', *Journal of applied microbiology*, vol. 41, no. 2, pp. 277-281.

Piccolo, A & Huluka, G 1986, 'Phosphorus status of some Ethiopian soils', *Tropical agriculture*, vol. 63, no. 2, pp. 137-142.

Pikovskaya, R 1948, 'Mobilization of phosphorus in soil in connection with vital activity of some microbial species', *Mikrobiologiya*, vol. 17, no. 362, p. 370.

Pongsilp, N 2012, 'Phenotypic and genotypic diversity of rhizobia', Bentham Science Publishers, Thailand.

Pongslip, N 2012, *Phenotypic and Genotypic Diversity of Rhizobia*, Bentham Science Publishers, Thailand.

Pontes, DS, Lima-Bittencourt, CI, Chartone-Souza, E & Nascimento, AMA 2007, 'Molecular approaches: advantages and artifacts in assessing bacterial diversity', *Journal of industrial microbiology & biotechnology*, vol. 34, no. 7, pp. 463-473.

Rashid, MH-o, Young, JPW, Everall, I, Clercx, P, Willems, A, Braun, MS & Wink, M 2015, 'Average nucleotide identity of genome sequences supports the description of *Rhizobium lentis* sp. nov., *Rhizobium bangladeshense* sp. nov. and *Rhizobium binae* sp. nov. from lentil (*Lens culinaris*) nodules', *International journal of systematic and evolutionary microbiology*, vol. 65, no. 9, pp. 3037-3045.

Rayment, G & Higginson, FR 1992, *Australian laboratory handbook of soil and water chemical methods*, Inkata Press Pty Ltd, Melbourne.

Reganold, JP, Papendick, RI & Parr, JF 1990, 'Sustainable agriculture', *Scientific American*, vol. 262, no. 6, pp. 112-121.

Reyes, I, Bernier, L, Simard, RR & Antoun, H 1999, 'Effect of nitrogen source on the solubilization of different inorganic phosphates by an isolate of *Penicillium rugulosum* and two UV-induced mutants', *FEMS Microbiology Ecology*, vol. 28, no. 3, pp. 281-290.

Richardson, AE 2001, 'Prospects for using soil microorganisms to improve the acquisition of phosphorus by plants', *Functional Plant Biology* vol. 28, no. 9, pp. 897-906.

Richardson, AE, Simpson, RJ, Djordjevic, MA & Rolfe, BG 1988, 'Expression of nodulation genes in *Rhizobium leguminosarum* biovar *trifolii* is affected by low pH and by Ca and Al ions', *Applied and Environmental Microbiology*, vol. 54, no. 10, pp. 2541-2548.

Rivas, R, García-Fraile, P & Velázquez, E 2009, 'Taxonomy of bacteria nodulating legumes', *Microbiology Insights*, vol. 2, p. 51.

Rodríguez, H & Fraga, R 1999, 'Phosphate solubilizing bacteria and their role in plant growth promotion', *Biotechnology advances*, vol. 17, no. 4, pp. 319-339.

Ronner, E, Franke, AC, Vanlauwe, B, Dianda, M, Edeh, E, Ukem, B, Bala, A, van Heerwaarden, J & Giller, KE 2016, 'Understanding variability in soybean yield and response to P-fertilizer and *rhizobium* inoculants on farmers' fields in northern Nigeria', *Field Crop Res.*, vol. 186, 2/1/, pp. 133-145.

Rosselli, R, Fiamma, M, Deligios, M, Pintus, G, Pellizzaro, G, Canu, A, Duce, P, Squartini, A, Muresu, R & Cappuccinelli, P 2015, 'Microbial immigration across the Mediterranean via airborne dust', *Scientific reports*, vol. 5, no. 16306 (2015).

Sadowsky, MJ 2005, 'Soil stress factors influencing symbiotic nitrogen fixation', *Nitrogen fixation in agriculture, forestry, ecology, and the environment*, Springer, pp. 89-112.

Sadowsky, MJ & Graham, PH 1998, 'Soil biology of the *Rhizobiaceae*', *The Rhizobiaceae*, Springer, pp. 155-172.

Saharan, B 2011, 'Plant growth promoting rhizobacteria: a critical review', *Life Sciences and Medicine Research*, vol. 21, no. 1, p. 30.

Saito, A, Tanabata, S, Tanabata, T, Tajima, S, Ueno, M, Ishikawa, S, Ohtake, N, Sueyoshi, K & Ohyama, T 2014, 'Effect of nitrate on nodule and root growth of soybean (*Glycine max* (L.) Merr.)', *International journal of molecular sciences*, vol. 15, no. 3, pp. 4464-4480.

Saitou, N & Nei, M 1987, 'The neighbor-joining method: a new method for reconstructing phylogenetic trees', *Molecular biology and evolution*, vol. 4, no. 4, pp. 406-425.

Sanginga, N, Abaidoo, R, Dashiell, K, Carsky, R & Okogun, A 1996, 'Persistence and effectiveness of rhizobia nodulating promiscuous soybeans in moist savanna zones of Nigeria', *Applied soil ecology*, vol. 3, no. 3, pp. 215-224.

Sanginga, N, Thottappilly, G & Dashiell, K 2000, 'Effectiveness of rhizobia nodulating recent promiscuous soyabean selections in the moist savanna of Nigeria', *Soil Biology and Biochemistry*, vol. 32, no. 1, 1, pp. 127-133.

Sanginga, N & Woomer, PL 2009, *Integrated soil fertility management in Africa: principles, practices, and developmental process*, Tropical Soil Biology and Fertility Institute of the International Center for Tropical Agriculture, Nairobi, Kenya.

Savita, V 2007, 'Knowledge and adoption of bio-fertilizers in horticultural and other crops in rural communities of Haryana', *Haryana Journal of horticultural Sciences*, vol. 36, no. 1/2, pp. 86-88.

Schlede, H 1989, 'Distribution of acid soils and liming materials in Ethiopia', *Ethiopian Institute of Geological Survey, Ministry of Mines and Energy, Addis Ababa, Ethiopia*.

Sessitsch, A, Howieson, J, Perret, X, Antoun, H & Martinez-Romero, E 2002, 'Advances in *Rhizobium* research', *Critical Reviews in Plant Sciences*, vol. 21, no. 4, pp. 323-378.

Shamseldin, A, Abdelkhalek, A & Sadowsky, MJ 2017, 'Recent changes to the classification of symbiotic, nitrogen-fixing, legume-associating bacteria: a review', *Symbiosis*, vol. 71, no. 2, pp. 91-109.

Shi, WM, Xu, WF, Li, SM, Zhao, XQ & Dong, GQ 2010, 'Responses of two rice cultivars differing in seedling-stage nitrogen use efficiency to growth under low-nitrogen conditions', *Plant and Soil*, vol. 326, no. 1-2, p. 291.

Simms, EL & Taylor, DL 2002, 'Partner choice in nitrogen-fixation mutualisms of legumes and rhizobia', *Integrative and Comparative Biology*, vol. 42, no. 2, pp. 369-380.

Sinclair, TR, Marrou, H, Soltani, A, Vadez, V & Chandolu, KC 2014, 'Soybean production potential in Africa', *Global Food Security*, vol. 3, no. 1, pp. 31-40.

Singleton, P, Bohlool, B, Nakao, P, Lal, R & Sanchez, P 1992, 'Legume response to rhizobial inoculation in the tropics: myths and reality', *Myths and science of soils of the tropics: proceedings SSSA, Las Vegas, Nevada, 17 October 1989.*, pp. 135-155.

Slattery, J, Coventry, D & Slattery, W 2001, 'Rhizobial ecology as affected by the soil environment', *Australian Journal of Experimental Agriculture*, vol. 41, no. 3, pp. 289-298.

Slattery, J & Pearce, D 2002, 'The impact of background rhizobial populations on inoculation response', *Inoculants and Nitrogen Fixation of Legumes in Vietnam. ACIAR Proceedings No. 109e*, pp. 37-45.

Solomon, T, Pant, LM & Angaw, T 2012, 'Effects of inoculation by *Bradyrhizobium japonicum* strains on nodulation, nitrogen fixation, and yield of soybean (*Glycine max* L. Merill) varieties on nitisols of Bako, Western Ethiopia', *ISRN Agronomy*, vol. 2012.

Somasegaran, P & Hoben, H 1985, *Methods in Legume-rhizobium technology; NIFTAL, MIRCEN*, University of Hawaii Press, Maui, Hawaii.

Souza, Rd, Ambrosini, A & Passaglia, LM 2015, 'Plant growth-promoting bacteria as inoculants in agricultural soils', *Genetics and molecular biology*, vol. 38, no. 4, pp. 401-419.

Sprent, JI 2007, 'Evolving ideas of legume evolution and diversity: a taxonomic perspective on the occurrence of nodulation', *New Phytologist*, vol. 174, no. 1, pp. 11-25.

Stevenson, FJ 1999, *Cycles of soils: carbon, nitrogen, phosphorus, sulfur, micronutrients*, 2nd edn, John Wiley & Sons, New York, USA.

Sylvia, DM, Fuhrmann, JJ, Hartel, PG & Zuberer, DA 2005, *Principles and applications of soil microbiology*, Pearson Prentice Hall Upper Saddle River, NJ:.

Tamura, K, Stecher, G, Peterson, D, Filipski, A & Kumar, S 2013, 'MEGA6: molecular evolutionary genetics analysis version 6.0', *Molecular biology and evolution*, vol. 30, no. 12, pp. 2725-2729.

Taurian, T, Anzuay, MS, Angelini, JG, Tonelli, ML, Ludueña, L, Pena, D, Ibáñez, F & Fabra, A 2010, 'Phosphate-solubilizing peanut associated bacteria: screening for plant growth-promoting activities', *Plant and Soil*, vol. 329, no. 1-2, pp. 421-431.

Taylor, GJ 1991, 'Current views of the aluminum stress response: the physiological basis of tolerance', *Curr. Top. Plant Biochem. Physiol.*, vol. 10, pp. 57-93.

Tena, W, Wolde-Meskel, E & Walley, F 2016, 'Response of chickpea (*Cicer arietinum* L.) to inoculation with native and exotic *Mesorhizobium* strains in Southern Ethiopia', *African Journal of Biotechnology*, vol. 15, no. 35, pp. 1920-1929.

—— 2016, 'Symbiotic efficiency of native and exotic *Rhizobium* strains nodulating lentil (*Lens culinaris* Medik.) in soils of Southern Ethiopia', *Agronomy*, vol. 6, no. 1, p. 11.

Terefework, Z, Nick, G, Suomalainen, S, Paulin, L & Lindström, K 1998, 'Phylogeny of *Rhizobium galegae* with respect to other rhizobia and agrobacteria', *International journal of systematic and evolutionary microbiology*, vol. 48, no. 2, pp. 349-356.

Thies, J, Holmes, E & Vachot, A 2001, 'Application of molecular techniques to studies in *Rhizobium* ecology: a review', *Animal Production Science*, vol. 41, no. 3, pp. 299-319.

Thies, JE, Singleton, PW & Bohlool, BB 1991, 'Influence of the size of indigenous rhizobial populations on establishment and symbiotic performance of introduced rhizobia on field-grown legumes', *Applied and Environmental Microbiology*, vol. 57, no. 1, pp. 19-28.

Thilakarathna, MS & Raizada, MN 2017, 'A meta-analysis of the effectiveness of diverse rhizobia inoculants on soybean traits under field conditions', *Soil Biology and Biochemistry*, vol. 105, pp. 177-196.

Thuita, M, Pypers, P, Herrmann, L, Okalebo, RJ, Othieno, C, Muema, E & Lesueur, D 2012, 'Commercial rhizobial inoculants significantly enhance growth and nitrogen fixation of a promiscuous soybean variety in Kenyan Soils', *Biology and Fertility of soils*, vol. 48, no. 1, pp. 87-96.

Tilman, D 1998, 'The greening of the green revolution', *Nature*, vol. 396, no. 6708, 11/19/print, pp. 211-212.

Tsegaye, D, Assefa, F, Gebrekidan, H & Keneni, G 2015, 'Nutritional, eco-physiological and symbiotic characteristics of rhizobia nodulating faba bean (*Vicia faba* L.) collected from acidic soils of Ethiopia', *African Journal of Environmental Science and Technology*, vol. 9, no. 7, pp. 646-654.

Turan, M, Ataoğlu, N & Şahın, F 2006, 'Evaluation of the capacity of phosphate solubilizing bacteria and fungi on different forms of phosphorus in liquid culture', *Journal of Sustainable Agriculture*, vol. 28, no. 3, pp. 99-108.

Uchiumi, T, Kuwashiro, R, Miyamoto, J, Abe, M & Higashi, S 1998, 'Detection of the leghemoglobin gene on two chromosomes of *Phaseolus vulgaris* by in situ PCR linked-fluorescent in situ hybridization (FISH)', *Plant and cell physiology*, vol. 39, no. 7, pp. 790-794.

Unkovich, MJ & Pate, JS 2000, 'An appraisal of recent field measurements of symbiotic N_2 fixation by annual legumes', *Field Crops Research*, vol. 65, no. 2, pp. 211-228.

Urtz, BE & Elkan, GH 1996, 'Genetic diversity among *Bradyrhizobium* isolates that effectively nodulate peanut *(Arachis hypogaea)*', *Canadian Journal of Microbiology*, vol. 42, no. 11, pp. 1121-1130.

USDA 2016, 'World Agricultural Production', no. WAP 11-16, Foriegn Agricultural Service, USA, viewed 10/11/2016, <http://apps.fas.usda.gov/psdonline/circulars/production.pdf>.

—— 2017, *World Agriculture Production*, viewed 30/08/2017, <https://apps.fas.usda.gov/psdonline/circulars/production.pdf>.

USDA, FAS 2017, *Oil seeds: World Markets and Trade*, viewed 30/08/2017, <https://apps.fas.usda.gov/psdonline/circulars/oilseeds.pdf>.

Vallaeys, T, Topp, E, Muyzer, G, Macheret, V, Laguerre, G, Rigaud, A & Soulas, G 1997, 'Evaluation of denaturing gradient gel electrophoresis in the detection of 16S rDNA sequence variation in rhizobia and methanotrophs', *FEMS Microbiology Ecology*, vol. 24, no. 3, pp. 279-285.

Van Berkum, P, Leibold, JM & Eardly, BD 2006, 'Proposal for combining *Bradyrhizobium spp.*(*Aeschynomene indica*) with *Blastobacter denitrificans* and to transfer *Blastobacter denitrificans* (Hirsch and Muller, 1985) to the genus *Bradyrhizobium* as *Bradyrhizobium denitrificans* (comb. nov.)', *Systematic and applied microbiology*, vol. 29, no. 3, pp. 207-215.

Van Berkum, P, Terefework, Z, Paulin, L, Suomalainen, S, Lindström, K & Eardly, BD 2003, 'Discordant phylogenies within the rrn loci of rhizobia', *Journal of bacteriology*, vol. 185, no. 10, pp. 2988-2998.

Van Dommelen, A & Vanderleyden, J 2007, 'Associative nitrogen fixation', *Biology of the Nitrogen Cycle*, pp. 179-191.

Vance, CP 2001, 'Symbiotic nitrogen fixation and phosphorus acquisition. Plant nutrition in a world of declining renewable resources', *Plant Physiology*, vol. 127, no. 2, pp. 390-397.

Vandamme, P, Pot, B, Gillis, M, De Vos, P, Kersters, K & Swings, J 1996, 'Polyphasic taxonomy, a consensus approach to bacterial systematics', *Microbiological reviews*, vol. 60, no. 2, pp. 407-438.

Vargas, AA & Graham, PH 1988, '*Phaseolus vulgaris* cultivar and *Rhizobium* strain variation in acid-pH tolerance and nodulation under acid conditions', *Field Crops Research*, vol. 19, no. 2, pp. 91-101.

Velivelli, SL, Sessitsch, A & Prestwich, BD 2014, 'The role of microbial inoculants in integrated crop management systems', *Potato Research*, vol. 57, no. 3-4, pp. 291-309.

Versalovic, J, Schneider, M, De Bruijn, F & Lupski, JR 1994, 'Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction', *Methods in molecular and cellular biology*, vol. 5, no. 1, pp. 25-40.

Vessey, JK 2003, 'Plant growth promoting rhizobacteria as biofertilizers', *Plant and Soil*, vol. 255, no. 2, 2003/08/01, pp. 571-586.

Vikram, A, Hamzehzarghani, H, Alagawadi, A, Krishnaraj, P & Chandrashekar, B 2007, 'Production of plant growth promoting substances by phosphate solubilizing bacteria isolated from vertisols', *Journal of Plant Science*, vol. 2, no. 3, pp. 326-333.

Vinuesa, P, Rademaker, JL, de Bruijn, FJ & Werner, D 1998, 'Genotypic Characterization of *Bradyrhizobium* Strains Nodulating Endemic Woody Legumes of the Canary Islands by PCR-Restriction Fragment Length Polymorphism Analysis of Genes Encoding 16S rRNA (16S rDNA) and 16S-23S rDNA Intergenic Spacers, Repetitive Extragenic Palindromic PCR Genomic Fingerprinting, and Partial 16S rDNA Sequencing', *Applied and Environmental Microbiology*, vol. 64, no. 6, pp. 2096-2104.

Vinuesa, P, Rojas-Jiménez, K, Contreras-Moreira, B, Mahna, SK, Prasad, BN, Moe, H, Selvaraju, SB, Thierfelder, H & Werner, D 2008, 'Multilocus sequence analysis for assessment of the biogeography and evolutionary genetics of four *Bradyrhizobium* species that nodulate soybeans on the Asiatic continent', *Applied and Environmental Microbiology*, vol. 74, no. 22, pp. 6987-6996.

Viruel, E, Lucca, ME & Siñeriz, F 2011, 'Plant growth promotion traits of phosphobacteria isolated from Puna, Argentina', *Archives of microbiology*, vol. 193, no. 7, pp. 489-496.

Vitorino, LC, Silva, FG, Soares, MA, Souchie, EL, Costa, AC & Lima, WC 2012, 'Solubilization of calcium and iron phosphate and in vitro production of indoleacetic acid by endophytic isolates of *Hyptis marrubioides* Epling (*Lamiaceae*)', *International Research Journal of Biotechnology*, vol. 3, pp. 47-54.

Vitousek, PM, Menge, DN, Reed, SC & Cleveland, CC 2013, 'Biological nitrogen fixation: rates, patterns and ecological controls in terrestrial ecosystems', *Philosophical Transactions of the Royal Society of London B: Biological Sciences*, vol. 368, no. 1621, p. 20130119.

VSN International, 2014, *GenStat for Windows, 17th edn.*, VSN International, Hemel Hempstead, UK.

Vuong, HB, Thrall, PH & Barrett, LG 2017, 'Host species and environmental variation can influence rhizobial community composition', *Journal of Ecology*, vol. 105, no. 2, pp. 540-548.

Wang, D, Yang, S, Tang, F & Zhu, H 2012, 'Symbiosis specificity in the legume–rhizobial mutualism', *Cellular microbiology*, vol. 14, no. 3, pp. 334-342.

White, JG 1966, 'Introduction of lucerne into acid soils', *Crop and Pasture Improvement (Selection and Breeding)*, Alexandra, Newzealand vol. 28, pp. 105-113.

Whitelaw, M, Harden, T & Helyar, K 1999, 'Phosphate solubilisation in solution culture by the soil fungus *Penicillium radicum*', *Soil Biology and Biochemistry*, vol. 31, no. 5, pp. 655-665.

Widmer, F, Shaffer, B, Porteous, L & Seidler, R 1999, 'Analysis of *nif*H gene pool complexity in soil and litter at a Douglas fir forest site in the Oregon Cascade Mountain Range', *Applied and Environmental Microbiology*, vol. 65, no. 2, pp. 374-380.

Wielbo, J 2012, 'Rhizobial communities in symbiosis with legumes: genetic diversity, competition and interactions with host plants', *Open Life Sciences*, vol. 7, no. 3, pp. 363-372.

Willems, A 2006, 'The taxonomy of rhizobia: an overview', *Plant and Soil*, vol. 287, no. 1-2, pp. 3-14.

Willems, A, Munive, A, de Lajudie, P & Gillis, M 2003, 'In most *Bradyrhizobium* groups sequence comparison of 16S-23S rDNA internal transcribed spacer regions corroborates DNA-DNA hybridizations', *Systematic and applied microbiology*, vol. 26, no. 2, pp. 203-210.

Wilson, J 1944, 'Over five hundred reasons for abandoning the cross-inoculation groups of the legumes', *Soil science*, vol. 58, no. 1, pp. 61-70.

Wilson, KJ, Parra, A & Botero, L 1999, 'Application of the GUS marker gene technique to high-throughput screening of rhizobial competition', *Canadian Journal of Microbiology*, vol. 45, no. 8, pp. 678-685.

Wood, M, Cooper, J & Holding, A 1984, 'Soil acidity factors and nodulation ofTrifolium repens', *Plant and Soil*, vol. 78, no. 3, pp. 367-379.

Woomer, PL 1994, 'Most probable number counts', in RW Weaver (ed.), *Methods of Soil Analysis: Part 2—Microbiological and Biochemical Properties*, vol. Soil Sci. Soc. Am. 5, Soil Sci. Soc. Am., Madison, WI, pp. 59-79.

Worthington, V 2001, 'Nutritional quality of organic versus conventional fruits, vegetables, and grains', *The Journal of Alternative & Complementary Medicine*, vol. 7, no. 2, pp. 161-173.

Young, JPW, Crossman, LC, Johnston, AW, Thomson, NR, Ghazoui, ZF, Hull, KH, Wexler, M, Curson, AR, Todd, JD & Poole, PS 2006, 'The genome of *Rhizobium leguminosarum* has recognizable core and accessory components', *Genome biology*, vol. 7, no. 4, p. R34.

Youseif, SH, El-Megeed, FHA, Ageez, A, Mohamed, ZK, Shamseldin, A & Saleh, SA 2014, 'Phenotypic characteristics and genetic diversity of rhizobia nodulating soybean in Egyptian soils', *Eur. J. Soil Biol.*, vol. 60, pp. 34-43.

Yousuf, B, Kumar, R, Mishra, A & Jha, B 2014, 'Differential distribution and abundance of diazotrophic bacterial communities across different soil niches using a gene-targeted clone library approach', *FEMS microbiology letters*, vol. 360, no. 2, pp. 117-125.

Yu, X, Cloutier, S, Tambong, JT & Bromfield, ES 2014, '*Bradyrhizobiumottawaense* sp. nov., a symbiotic nitrogen fixing bacterium from root nodules of soybeans in Canada', *International journal of systematic and evolutionary microbiology*, vol. 64, no. 9, pp. 3202-3207.

Yu, X, Liu, X, Zhu, T-H, Liu, G-H & Mao, C 2012, 'Co-inoculation with phosphate-solubilzing and nitrogen-fixing bacteria on solubilization of rock phosphate and their effect on growth promotion and nutrient uptake by walnut', *European Journal of Soil Biology*, vol. 50, pp. 112-117.

Zahran, HH 1999, '*Rhizobium*-legume symbiosis and nitrogen fixation under severe conditions and in an arid climate', *Microbiology and molecular biology reviews*, vol. 63, no. 4, pp. 968-989.

Zapata, F, Danso, S, Hardarson, G & Fried, M 1987, 'Time course of nitrogen fixation in field-grown soybean using nitrogen-15 methodology', *Agronomy journal*, vol. 79, no. 1, pp. 172-176.

Zayed, MS 2012, 'Improvement of growth and nutritional quality of Moringa oleifera using different biofertilizers', *Annals of Agricultural Sciences*, vol. 57, no. 1, pp. 53-62.

Zeigler, DR 2003, 'Gene sequences useful for predicting relatedness of whole genomes in bacteria', *International journal of systematic and evolutionary microbiology*, vol. 53, no. 6, pp. 1893-1900.

Zhang, YM, Li, Y, Chen, WF, Wang, ET, Tian, CF, Li, QQ, Zhang, YZ, Sui, XH & Chen, WX 2011, 'Biodiversity and biogeography of rhizobia associated with soybean plants grown in the North China Plain', *Applied and Environmental Microbiology*, vol. 77, no. 18, pp. 6331-6342.

Zhang, YM, Tian, CF, Sui, XH, Chen, WF & Chen, WX 2012, 'Robust markers reflecting phylogeny and taxonomy of rhizobia', *PloS one*, vol. 7, no. 9, p. e44936.

Zhao, Y, Bian, SM, Zhou, HN & Huang, JF 2006, 'Diversity of nitrogenase systems in diazotrophs', *Journal of integrative plant biology*, vol. 48, no. 7, pp. 745-755.