ASSESSING THE EFFECTIVENESS OF NATIVE RHIZOBIA AS POTENTIAL STRAINS FOR LOCAL INOCULANT PRODUCTION FOR ENHANCED COWPEA AND GROUNDNUT YIELDS IN NORTHERN GHANA

BY

OPHELIA OSEI

OCTOBER, 2018

KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY,

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SCHOOL OF GRADUATE STUDIES

COLLEGE OF AGRICULTURE AND NATURAL RESOURCES

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BY

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(BSc Agriculture (Hons); MSc Soil Science)

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DOCTOR OF PHILOSOPHY

IN

SOIL SCIENCE

OCTOBER, 2018

DECLARATION

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any degree or diploma at Kwame Nkrumah University of Science and Technology, Kumasi or any other educational institute, except where due acknowledgement is made in the thesis.

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ABSTRACT

Efficient exploration of the legume-rhizobium symbiosis via inoculation with rhizobia is constrained by the limited knowledge on the fate of introduced strains under field conditions and the unavailability of effective native strains for use as local inoculants. This research therefore sought to address these challenges in four studies: i) nodule occupancy determination ii) identification of effective native isolates iii) symbiotic performance evaluation of elite native isolates under field conditions and iv) assessment of the persistence of elite isolates following field inoculation.

Significant differences ($p < 0.05$) in shoot biomass of cowpea following inoculation was observed. The differences were explained via nodule occupancy studies by designing specific primers for the test strain *Bradyrhizobium pachyrhizi* BR 3262 (a recommended cowpea strain in Brazil) using the comparative genomics approach. Out of eleven specific primer pairs designed, the primers 2645 and 2736 were observed to be highly sensitive and reliably detected the target strains in nodules extracts of gnotobiotic system and potted soil grown cowpea. These two primers are thus novel tools for determining the fate of BR 3262 in field inoculation studies. The approach for designing specific primers in this study should be applicable to other bacteria/rhizobium strains whose genomic sequences are available.

Bioprospecting for nodules of groundnut and cowpea cultivated in farmers' fields without inoculation was done followed by isolation of rhizobium in the laboratory. Authentication and symbiotic effectiveness evaluation of the isolated rhizobium strains led to the selection of seven effective isolates. The nitrogen accumulated via BNF of the selected effective isolates was significantly higher ($p < 0.05$) compared to the –N control treatment. Treatment with isolate KNUST 1002 resulted in a total N accumulation that was comparable to the reference strain 32H1 on groundnut (BR 1 variety) grown in potted soils. Genetic characterization of the seven effective isolates revealed them as diverse with isolates KNUST 1003 and KNUST 1007 belonging to the *Rhizobium tropici* speices. The five remaining isolates were identified to belong to the *Bradyrhizobium yuanmingense* species.

The two most effective isolates were evaluated in the field alongside a positive (with nitrogen) and negative (without nitrogen or inoculation) control on cowpea (Songotra variety) and groundnut (Chinese variety) in multi-locational trials in the Northern region

of Ghana using the randomized complete block design. On the average, cowpea grain yields produced by the inoculated treatments were significantly ($p< 0.05$) larger than the –N treatment. Isolate KNUST 1002 produced the best average yield (1056 kg ha^{-1}) which was significantly different from the other treatments. Groundnut yields on the other hand were significantly larger with isolate KNUST 1006 (1234 kg ha^{-1}). The chemical properties of soils in the study locations generally showed low soil fertility. The different soil types significantly influenced the performance of treatments across the study locations. Twenty-six and 23% of the variability in grain yields of cowpea and groundnut respectively, was explained by the soil type and treatment interaction. These results imply that effective native isolates in this study can improve grain yields of cowpea and groundnut based on location specific recommendations.

The persistence of effective isolates following previous inoculation on cowpea and groundnut was assessed in the 2017 cropping season on ten of the sites considered in 2016. Nodulation in both previously inoculated and re-inoculated plots were significantly higher than the un-inoculated plots (+N and –N). Nodule dry mass recorded for isolate KNUST 1006 in previously inoculated plots did not differ significantly from that of re-inoculated plots on both target host. However, the nodule dry mass recorded for isolate KNUST 1002 on cowpea in previously inoculated plots was significantly lower than in re-inoculated plots. Increases in grain yield of groundnut were significantly larger for treatment with isolate KNUST 1006 than the other treatments for previously inoculated and re-inoculated plots. Isolate KNUST 1002 on cowpea produced significantly higher yields than the other treatments in previously inoculated and re-inoculated plots. The type of legume host in each case influenced the performance of the isolates. There were no significant differences in the yields produced by either of the isolates in previously inoculated and re-inoculated plots. These results indicate that effective native isolates persisted and effectively nodulated the target legumes obviating the need for re-inoculation in subsequent cropping season.

The outcomes of these studies have important implications for the use of effective isolates from this study as inoculants to improve cowpea and groundnut yields particularly in Northern Ghana.

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CHAPTER ONE

1.0 INTRODUCTION

Legumes form a major part of smallholder farmers' cropping systems due to their high nutritional and economic benefits (Arnoldi *et al.*, 2015). Both cowpea and groundnut serve as a cheap source of protein to the poor (Ennin *et al.*, 2004) and can potentially be used to alleviate malnutrition problems. They also contribute immensely to soil fertility and have the potential and ability to meet most of their nitrogen requirement through biological nitrogen fixation (BNF) (Naab *et al.*, 2009; Yakubu *et al.*, 2010). The association between legumes and compatible rhizobia, which leads to BNF, is a natural phenomenon that has been utilized to improve agricultural productivity (Thies *et al.*, 2001). The process has also been identified as an environmentally friendly and economically viable source (Herridge *et al.*, 2008) through which legume yields can be improved in tropical farming systems (Yakubu *et al.*, 2010; Abaidoo *et al.*, 2013). Despite these benefits, yields of grain legumes are still just a fraction of their maximum potential. These low yields have been attributed to the low fertility status of soils particularly in the savannah agro-ecological zones of Ghana (Buri *et al.*, 2010).

Grain legumes can meet their N requirements by accessing atmospheric N_2 via a symbiotic association with compatible micro-symbionts (rhizobia) which results in effective nodulation and nitrogen fixation (McInnes *et al.*, 2004). Most soils of the sub-Saharan Africa region are characterized with large populations of cowpea miscellany rhizobia that cause nodulation in tropical legumes (Allen and Allen, 1939). The presence of such large populations of indigenous rhizobia led to the assumption that nodulation of tropical legumes by indigenous rhizobia was adequate (Zahran, 1999). However, members of the cowpea miscellany group were found to vary in their effectiveness with a large proportion being ineffective (Thies *et al.*, 1991a; Mpepereki *et al.*, 1996; Fening and Danso, 2002; Herridge *et al.*, 2002). Large numbers of ineffective indigenous rhizobia adversely affect nodulation and subsequently nitrogen fixation of grain legumes. This is evident from the low grain yields observed when these legumes are cultivated without inoculation or fertilization.

The inoculation of legumes with superior exotic strains coupled with improved agronomic practices such as application of basal P serves as means to improve legume yields by increasing the rhizosphere populations of effective rhizobia for enhanced nodulation and yield responses (Ronner *et al.*, 2016; Ulzen *et al.*, 2016). This technique has also led to improvements in soil nutrients levels for succeeding crops (GRDC, 2013). Boddey *et al.* (2016) reported an increase of between 39 and 57% in grain yields of cowpea in Ghana following inoculation and up to 30% in some parts of Brazil (Martins *et al.*, 2003; Soares *et al.*, 2006). Similarly, yields of groundnut have been improved following inoculation with rhizobia and phosphorus application in Nigeria (Yakubu *et al.*, 2010). These findings run contrary to the widely held notion that promiscuous legume species such as cowpea and groundnut do not require inoculation.

Meanwhile, realization of the full benefits of legume-rhizobia symbiosis remains a challenge in lots of environments. Thies *et al.* (2001) mentioned in a review that, the agronomic value from symbiosis was yet to be fully exploited due generally to the failure of introduced strains to outcompete native rhizobia in nodule occupancy among other factors. Thus, an introduced strain highly competitive in nodule occupancy is advantageous and is more likely to increase grain yields of legumes. The authors also indicated that knowledge of the fate of introduced strains is vital in order to improve their performance. Generally, paucity of information is available on the fate of introduced elite strains, for example, BR 3262 and BR 3267 from Brazil, used in previous inoculation studies in Ghana due to methodological challenges.

The methods that were employed in the past to determine the fate of introduced strains were not without limitations. For instance, serological methods, which are examples of traditional techniques used in the past, were limited by target specificity (Mpepereki and Wollum II, 1991). Cross-reaction of target strain's antisera with closely related strains in the soil has been a problem associated with the use of polyclonal antibodies (Johnsson *et al.*, 1998). The natural occurrence of antibiotic resistant markers that were used to monitor selected strains in soil rhizobia populations also limits the discrimination power of this tool (Bushby, 1981). Other methods such as protein profiling and multi-locus enzyme

electrophoresis are respectively sophisticated and limited in use on a large scale (Thies *et al.*, 2001). Currently, advances in molecular methods have provided an easy means to obtain information about all members of a particular community and to discriminate between them. In addition, most of the molecular based methods do not require prior culturing of strains before analysis, which makes them advantageous over serological methods (Thies *et al.*, 2001). However, most of these methods are sophisticated, require specialised equipment, skilled technicians and involve high costs and therefore not easily accessible to rhizobiologists in developing countries. Fortunately, advances in DNA sequencing, which is one of the molecular methods have facilitated the acquisition of (almost) complete sequences of strains of interest and the performance of comparative genomics. This has led to the development of more specific primers and gene probes for studies in rhizobium ecology (Thies *et al.*, 2001). Stets *et al.* (2015) successfully used this DNA sequencing and comparative genomics approach to design strain-specific primers for quantification of *A. brasilense* FP2 in wheat roots. The authors concluded that, the designed specific primers could effectively monitor the population of plant-growth promoting bacteria (PGPB) in inoculated plants and that the approach used for the primer design was possible for any microorganism whose complete genomic sequence is available. It would therefore be possible to assess the fate of elite strain (e.g. BR 3262) used in inoculation studies in Ghana by employing this methodology since its complete genomic sequence is available.

On the other hand, imported inoculants are often beyond the reach of most smallholder farmers, particularly in Ghana due to restricted access, availability or both. Conversely, moderate to highly effective rhizobia strains have been found to be well represented in soils, which can equally improve nodulation and N_2 fixation (Sattar *et al.*, 1995; Bogino *et al.*, 2006). In addition, Sattar *et al.* (1995) reported that strains isolated from a particular region are most effective for a given crop in the same region. Such strains must be selected and identified for local inoculant production since effective performance of exotic strains is not always guaranteed. The strain selection process involves bioprospecting for nodule isolates and subsequently testing these isolates for their symbiotic and saprophytic potentials (Somasegaran and Hoben, 2012). Prior to these tests, authentication of isolates

as true rhizobia is carried out under sterile conditions, which sometimes results in the acquisition of rhizobia strains of the same genotype (Thies *et al.*, 2001). In order to prevent a culture collection containing strain reiteration, molecular characterization of the isolates is necessary. Genetic fingerprinting of rhizobia leads to identification and selection of isolates with unique abilities, like nitrogen fixation (Laguerre *et al.*, 1996; Niemann *et al.*, 1999). In addition, competitiveness and persistence are important traits considered during a strain selection process. Elite strains with the ability to occupy a greater proportion of nodules under field conditions are essential for effective N_2 fixation (Triplett, 1990). A persistent strain on the other hand possess the ability to establish in soils in the absence of the host legume, which may obviate the need for re-inoculation in subsequent seasons (Zengeni *et al.*, 2006). However, unlike exotic strains, native rhizobia with abilities to effectively nodulate grain legumes have received very little research attention in the context of Ghanaian agriculture, and as such, limited information is available on their genetic diversity, symbiotic potential and saprophytic competence. In effect, no local strain has been recommended for grain legume cultivation in Ghana. To exploit the full benefits of the legume-rhizobia symbiosis, there is the need to identify elite native rhizobia strains and determine their performance in comparison with introduced strains. This research sought to answer the following questions:

i. will the use of available DNA sequences and comparative genomics information help develop strain-specific primers for assessing nodule occupancy of inoculated cowpea (using the elite strain *Bradyrhizobium pachyrhizi* BR 3262 as the test strain)?

ii. will molecular characterization lead to the identification of effective isolates from native rhizobia population and establishment of their phylogenetic position?

iii. can the selected isolates effectively nodulate with and improve yields of target legumes under field conditions?

iv. can the selected effective isolates persist in soil and enhance grain yields in the subsequent cropping season?

The main objective of this research was to identify elite cowpea and groundnut rhizobia strains to improve grain yields in smallholder farms in Northern Ghana.

The specific objectives were to:

i. develop strain specific PCR primers for determining the nodule occupancy of *Bradyrhizobium pachyrhizi* strain BR 3262 inoculated on cowpea, ii. identify the diversity within effective native rhizobia nodulating groundnut and cowpea,

iii. evaluate the symbiotic performance of effective isolates on groundnut and cowpea grown under varying environmental conditions,

iv. assess the persistence of elite isolates and its effects on grain yields of cowpea and groundnut in subsequent planting seasons.

1.1 Significance of the study

This study will lead to i) the development of strain specific primers that can be used to determine the fate of introduced exotic strains to improve their performance, ii) the identification and establishment of the phylogeny of effective native isolates for improved N_2 fixation efficiency in cowpea and groundnut locally, iii) the characterization of native isolates in terms of their ability to enhance grain yields of cowpea and groundnut under field conditions and (iv) establishment of the ability of the effective isolates to persist in soils in the absence of the target legumes thus aiding in the decision of whether or not inoculation in subsequent seasons is necessary. Generally, the outcomes of this research will lead to enhanced nodulation and BNF, which will subsequently translate into improved cowpea and groundnut yields and livelihoods of smallholder farmers in Northern Ghana.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Functions of grain legumes in cropping systems

Grain legumes are of great importance in modern cropping systems characterized by the use of high external inputs (e.g. fertilizers and agrochemicals) and a decreasing crop diversity for environmental safety and socioeconomics (Plaza-Bonilla *et al.*, 2017). In Africa, grain legumes such as cowpea and groundnut occupy over 12 million and 14 million hectares (ha) of land respectively. In Ghana however, cowpea occupies approximately 200,000 ha while the area for groundnut is nearly 340, 000 ha (FAOSTAT, 2016). Peoples *et al.* (2009) grouped the agronomic benefits of planting legume as precrops into nitrogen effect components and break crop effect components. The nitrogen effect components benefits are realized when N is made available to crops via BNF. For example, Osunde *et al.* (2003) reported that intercropping with soybean resulted in 40% N accumulation compared to sole cropping. Chu *et al.* (2004) observed a 29-37% and 47% increase in rice and peanut respectively. In most cases, the nitrogen benefits to subsequent crops are more evident under low N conditions (Preissel *et al.*, 2015). For example, quantified yield benefits for legume-cereal sequence compared to cereal-cereal sequence were in the range of 40-50% for low N levels and 10-17% for high N levels in Australia (Angus *et al.*, 1991). The BNF derived from legumes has a positive effect on quality and yields of succeeding crops (Luce *et al.*, 2015; Preissel *et al.*, 2015). For instance, yield benefits obtained in a legume-wheat cropping system was on the average 30% more compared to a wheat-wheat cropping system in Australia (Angus *et al.*, 2015). Yusuf *et al.* (2009) reported a 46% increase in maize yield when cultivated subsequent to soybean than maize and natural fallow. Studies on cowpea in rotation to or intercropped with maize in Zimbabwe also revealed 88% increase in yield of maize (Chibudu, 1998). Several researchers (e.g. Peoples and Craswell, 1992; Chalk, 1998; Kirkegaard *et al.*, 2008; Stagnari *et al.*, 2017) have also reported on yield benefits of legumes to subsequent crops. Break crop effect component on the other hand is viewed as the non-legume specific benefits. Examples of such benefits include reduction in pest and disease incidence,

improvement in soil fertility and soil conservation (Robson *et al.*, 2002; Hernanz *et al.*, 2009; Shen *et al.*, 2011; Angus *et al.*, 2015). The pest and disease associated with legumes differ from those of cereals, as such, incorporating legumes into farming systems particularly cereal-based farming results in the control of pest and diseases (Zander *et al.*, 2016). Legumes also provide soil cover, which is advantageous in the control of weeds and erosion (Odendo *et al.*, 2011; Seymour *et al.*, 2012). When legumes are intercropped with cereals, a competition for nitrates arises which stimulates N_2 fixation (Hinsinger *et al.*, 2011) subsequently increasing the soil N. Some grain legumes also possess the ability to mobilize fixed P in soils through the secretion of organic acids from their roots (Hocking, 2001). In a study by Li *et al.* (2014) on maize/faba bean intercropping, 23.9 and 49% increases in P and grain yield were observed over time (4 years) in P – deficient calcareous soils. Hydrogen gas produced as by-product of BNF influences the composition of microbial population in soils, particularly favouring the development of plant growth promoting bacteria (Angus *et al.*, 2015). In terms of soil conservation, intercropping legumes with cereals have resulted in runoff and soil loss reduction of 2030% and 50% respectively (Dwivedi *et al.*, 2015). There have also been reports on improvement in soil water retention in legumes fields compared to cereals (Miller *et al.*, 2003; Angus *et al.*, 2015; Gan *et al.*, 2017; Stagnari *et al.*, 2017). For sustainable agriculture, incorporating legumes into farming systems is a plausible option, which necessitates measures to improve their cultivation and yields. Furthermore, the benefits obtained from grain legumes have been either under estimated or overestimated and as such, studies are needed to accurately quantify the contributions of these legumes in cropping systems.

2.2 The legume-rhizobium symbiosis: what is it?

The symbiotic interaction between legumes and rhizobia begins with the exchange of molecular signals (like Nod factors) between the host plant and compatible rhizobia, which leads to nodule organogenesis (Oldroyd and Downie, 2008). This is referred to as the infection process (Dupont *et al.*, 2012). Following the exchange of signal molecules, the rhizobia are attached to the root hairs. Root hair deformation and branching then begins, providing an entry point into the legume host for the rhizobia. This is followed by

the curling of root hairs around the rhizobia forming a structure referred to as the shepherd's crook. An infection site is formed within the shepherd's crook from where rhizobia gain access to the internal tissues of plants through tubular structures known as infection threads. Rhizobia grow and multiply within the infection thread through cell division. Concomitant with rhizobia invasion of plant roots, Nod factors also stimulate root cortex cells dedifferentiation and division to form nodule primordia which then differentiates into nodules (Oldroyd *et al.*, 2011). The growing infection thread then penetrates the primordial cells releasing rhizobia into plant in the form of bacteriods (Timmers, 2008; Ivanov *et al.*, 2010). These bacteriods are surrounded by peribacteriod membrane that forms a symbiosome(s). In the symbiosome, bacteria differentiates into nitrogen fixing bacteriods (Jones *et al.*, 2007). Matured nodules actively make nitrogen available to plants via fixation until they enter senescence at a later growth stage (Dupont *et al.*, 2012). Nevertheless, not all nodules are occupied by rhizobia that can effectively fix nitrogen for plant use. Some rhizobia strains just enter into association with legumes by depending on the plant-derived carbon at the expense of fixing nitrogen (Kiers and Denison, 2008; Heath and Tiffin, 2009). Such strains are referred to as ineffective, result in poor yields, and reduced nutritional quality (Hafeez *et al.*, 2001). According to Perret *et al*. (2000), legumes vary in their ability to enter into symbiosis with rhizobia and vice versa with some legumes having the capability of nodulating with a wide range of rhizobia species. This is referred to as promiscuity. Some grain legumes considered promiscuous include cowpea (*Vigna unguiculata* Walp L.), common bean (*Phaseolus vulgaris* L.) and groundnut (*Arachis hypogaea* L.) (Perret *et al*., 2000; Alwi *et al*., 1989). Thus in order to enhance legume-rhizobia symbiosis there is the need for closer matching of effective rhizobia strains within the native population with improved varieties of legumes (Howieson *et al*., 2000).

2.3 Significance of the legume-rhizobia symbiosis

The symbiotic relationship between root-nodule bacteria and legumes results in the reduction of the inert atmospheric nitrogen into plant usable forms. Knowledge in, and scientific demonstrations of, the potential of this unique symbiotic association of legumes and their micro-symbionts to enrich the soil with nutrients date back to the 19th century

(Zhang *et al.*, 1999). The N derived from symbiotic association between legumes and their micro-symbionts reduces the need for chemical nitrogen fertilizers in farming systems and the potential for N contamination of water resources as well as providing grains with high nutritional value in the case of pulses (Hardarson and Atkins, 2003). The process of N fixation in legumes also serves as an economic means of reducing environmental pollution associated with chemical fertilizer usage (Silva and Uchida, 2000). For instance, legumes such as cowpea in association with rhizobia has been reported to fix up to 240 kg/ha N and can make 60 – 70 kg/ha N available to crops cultivated in rotation with them (Aikins and Afuakwa, 2008). Groundnut on the other hand has been estimated to fix 65 - 100 kg/ha N per year (Yakubu *et al.*, 2010). According to Vance *et al.* (2000), grain legumes alone contribute 33% of dietary protein requirements of humans and for the chicken industries with soybean and peanut being the major contributors of this nutrient. Furthermore, over

35% of the world's processed vegetable oil is obtained from soybean and peanut (Graham and Vance, 2003).In the presence of compatible rhizobia legumes are able to fix 20 million tonnes of nitrogen per year (Herridge *et al.*, 2008). Despite the enormous benefits derived from legume-rhizobium symbiosis, reported yields for these crops particularly in SSA are just a fraction of their maximum potential (Abaidoo *et al.*, 2013). For instance, the average yield of cowpea and groundnut in some major growing areas in the northern regions of Ghana is estimated at <1.0 t ha^{-1}, which is far below the potential yield of about 4.0 t ha^{-1} and 3.5 t ha^{-1} for cowpea and groundnut respectively (Mensah, 2014). These low yields have been attributed to the use of low yielding varieties coupled with poor soil fertility and high cost of inputs such as fertilizers (Kolawole, 2012). This therefore necessitates the search for a cost effective and environmentally friendly approach such as the use of inoculant technology and improved legume varieties to save the situation (Catroux *et al.*, 2001; Deaker *et al.*, 2004; Hungria and Mendes, 2015).

2.4 Factors affecting legume-rhizobium symbiosis

Effective legume-rhizobium symbioses usually expressed as the amount of N fixed is constrained by some environmental factors including temperature (Niste *et al.*, 2013), moisture stress (Tate, 1995) and also soil nutrient deficiencies such as N, P and

micronutrients (Sanginga *et al.*, 1995). The physiological state of the host plant also strongly influences the symbiotic association. All these factors have been shown to either directly or indirectly affect symbiotic N_2 fixation by impacting the population size of rhizobia, their diversity and survival as well as the nodule formation process. For example, soil moisture is important in the function of nitrogenase and nodulation (Danso *et al.*, 1992). A decrease in soil moisture due to drought thus reduces the number of rhizobia in soils leading to poor nodulation and subsequently N_2 fixation (Sellstedt *et al.*, 1993; Zahran, 1999). High soil temperatures in the tropics and subtropics also affects rhizobia growth and survival (Michiels *et al.*, 1994). The sensitive nature of root hair infection and early nodule development to soil temperature can impede or delay onset of nodulation (Junior *et al.*, 2005). According to Bordeleau and Prévost (1994) high temperatures can also result in decay and senescence of soybean nodules. On the other hand, deficiency in soil N has a positive effect on N_2 fixation since amount of N derived via symbiotic association under such condition is high provided the level of other nutrients and soil moisture are adequate (Unkovich *et al.*, 2008). Inadequate levels of available phosphorus in soils also affects the legume-rhizobium due to the substantial need of P as an energy source in the nodulation and N_2 fixation processes (Giller, 2001). Such low levels of P may be induced by low or high soil pH (Chen, 2006). Favorable soil and environmental conditions are thus required in order to enhance the growth of both legumes and rhizobium and the association between them.

2.5 Limitations of some of the approaches employed to increase legume yields and the need for rhizobium inoculation

Mineral fertilizers served as sources of nutrients for improving yields of crops until increases in their prices and extensive use raised environmental safety concerns, which led to a shift in interest towards low external input systems in the late 80s. The low external input systems mainly focused on biological management of soil fertility (Sanginga *et al.*, 2003). The Balance Nutrient Management (BNM) technology was also tested with the aim to counteract N and P depletion in countries like Togo, Nigeria and Benin. The technology involved a combination of organic and inorganic nutrient sources, which resulted in a 50% savings in the cost of fertilizer N (Vanlauwe *et al.*, 2001). However, the

quality and quantity of organic materials required posse a challenge to their use in improving both soil fertility and crop yields. Transportation as well as handling cost of this resource further exacerbates the problem (Palm *et al.*, 1997; Ayoola, 2006). The International Institute of Tropical Agriculture (IITA) in the early 1990s also developed improved varieties of grain legumes such as promiscuous soybean and cowpea through their breeding programs (Pulver *et al*., 1985). These promiscuous varieties were characterised as highly efficient in N_2 fixation and high yielding as well. Such characteristics of improved grain legume varieties made them an important resource in improving soil fertility and yields of resource poor farmers. Unfortunately, the ability to maintain the viability of seeds of the improved varieties remained a challenge. Furthermore, the promiscuous nature of the improved varieties, which made them able to nodulate freely with native rhizobia in soils, could not meet the full nitrogen needs of plants at all times due to ineffective symbiosis (Sanginga *et al.*, 2003). Under such conditions, inoculation with highly effective rhizobia strains is required for effective nodulation, N_2 fixation and optimum yields (Catroux *et al.*, 2001; Deaker *et al.*, 2004; Abaidoo *et al.*, 2007). The practice of inoculation with superior rhizobia strains particularly on soybean dates back to the 1950s in Zimbabwe and South Africa (Bala, 2011). An important index for determining the need for inoculation is the cropping history of a particular site (Herridge *et al.*, 2002; Peoples *et al.*, 2009). In tropical areas where legumes like cowpea and groundnut are considered as traditional crops (i.e. cultivated for centuries), inoculation with rhizobia may not be necessary (Thompson *et al.*, 1991). This is because continuous cultivation of legumes in soils results in the attraction of free-living rhizobia for nodule formation (Vlassak *et al.*, 1996; Ojo and Fagade, 2002). However, majority of the free-living rhizobia are ineffective leading to less effective symbiosis (Herridge *et al.*, 2002). Conversely, inoculation of cowpea and groundnut with superior strains until recently was rare and impossible in most developing countries including Ghana owing to scarcity or unavailability of inoculants stemming for the lack of inoculant production facilities. Several studies on the response of promiscuous legumes to inoculation have been carried out which showed positive results (Boddey *et al.*, 2016; Masso *et al.*, 2016; Ronner *et al.*, 2016; Ulzen *et al.*, 2016). Nevertheless, superior exotic strains may be limited in saprophytic capacity and sometimes outcompeted by the less

effective native strains due to their minimal adaptation to prevailing environmental conditions (Sessitsch *et al.*, 2002; Shamseldin and Werner, 2004). For improved inoculation response by tropical legumes, Nkot *et al.* (2008) suggested the use of indigenous rhizobia as inoculants. Furthermore, strains selected from a particular region have been identified as the most effective for a given crop in the same region (Sattar *et al.*, 1995). However, little is known about the diversity and symbiotic potential of indigenous rhizobia at different locations particularly in the context of Ghanaian agriculture (Fening *et al.*, 2002; Fening *et al.*, 2004; Ampomah *et al.*, 2008; Osei *et al.*, 2018). There is therefore the need for a holistic approach to improve inoculation response and yields of legumes through isolation and selection of effective indigenous strains for use in local inoculant production.

2.6 Selection of indigenous rhizobia for use as inoculants

Until recently, many developing countries including Ghana did not have inoculant production facilities. Accessibility of inoculants by farmers in these regions therefore remained a challenge, which made farmers resort to the use of promiscuous legumes in Zimbabwe (Mpepereki *et al.*, 2000; Musiyiwa *et al.*, 2005). Other farmers in developing countries also successfully depended on indigenous rhizobia in their natural state (Lindström *et al.*, 2010). Meanwhile, the diverse nature of indigenous rhizobia remains a challenge to their use in the natural state. As such, some studies were carried out to understand the diversity within native rhizobia that nodulate cultivated legumes in Africa (Mpepereki *et al.*, 1996; Fening *et al.*, 2004; Abaidoo *et al.*, 2007). In addition, Fening and Danso (2002) studied the variability in symbiotic effectiveness of native rhizobia in Ghanaian soils and concluded that they comprised strains that were highly effective, moderately effective and ineffective. Thies *et al.* (1991a) have indicated that an increase in the population of symbiotically effective indigenous rhizobia will give indigenous strains competitive advantage over introduced strains due to the characteristic ability of adaptation to the prevailing environment by the former.

In order to increase the population of effective indigenous strains, the selection programme must be geared towards testing of indigenous isolates for their symbiotic and

saprophytic characteristics (Somasegaran and Hoben, 2012). In addition, the capacity of selected strains to perform well under diverse field conditions should be considered. The process of rhizobia strain selection involves several steps. First is the preparation of a collection of nodule isolates; nodule isolates may be obtained from field-grown plants or from plants inoculated with soils from the target area. Another means is to obtain isolates from other researchers (Lupwayi *et al.*, 2000; Somasegaran and Hoben, 2012). The second step in the selection process involves pre-screening of isolates obtained from nodule samples to ensure that they are truly rhizobia. This process is commonly referred to as authentication. Isolates that induce nodule formation on the target host are re-isolated, purified and stored for further studies (Lupwayi *et al.*, 2000). In order to prevent storing the same genotype of isolates in a culture collection (i.e. strain reiteration), isolates should be selected from diverse locations (Howieson *et al.*, 1995). In addition, PCRfingerprinting may be used to pre-select isolates of different genotypes prior to greenhouse and field screenings (Riffkin, 1999). This approach leads to the identification of strains with unique abilities in N_2 fixation and saves time and resources. Evaluation of symbiotic properties of the isolates is the next step and this is carried out under controlled conditions (e.g. in the greenhouse). In these evaluations, seed(lings) of the target legume are cultivated under aseptic conditions using sterile assemblies such as test tubes and Leonard jars. Broth cultures of the test isolates are used to inoculate the seed(lings) and plants response to inoculation are compared with un-inoculated controls (Date, 2000). Field confirmation of the final selection of isolates with superior symbiotic characteristics from the previous experiment is the last step in the strain selection process. Field conditions for testing isolates should be similar to that of the target location for which strains are being selected. This experiment is then repeated under several other environments to confirm elite strains' performance (O'Hara *et al.*, 2002).

Exploiting indigenous rhizobia as a source of inoculant has proven to be a successful means of enhancing legume-rhizobia symbiosis and subsequently yields in lots of environments (Aguilar *et al.*, 2001; Drevon *et al.*, 2001; Fening and Danso, 2002; Sessitsch *et al.*, 2002; Nkot *et al.*, 2008). Bogino *et al.* (2006) reported significant nodulation and N_2 fixation with subsequent yield improvement in groundnut inoculated

with native rhizobia. These successes have led to the recommendation of indigenous rhizobia as inoculants. As an example, strain PRF81 isolated from Brazilian soils improved yields of common bean and has been recommended as a commercial inoculant strain (Hungria *et al.*, 2000; Mostasso *et al.*, 2002). The success reports following the use of indigenous rhizobia elsewhere and by Fening and Danso (2002) and Ampomah *et al.* (2008) in Ghana emphasize the need to search for such elite strains for use as inoculants.

2.6.1 Characterising native rhizobia for their symbiotic and saprophytic competence

For an isolate to qualify as an inoculant strain, it should have the ability to form highly effective nodules with the intended host and subsequently increase N_2 fixation under a wide range of field conditions (Date, 2000). An isolate that is able to fix N_2 equivalent to, or even higher than, the nitrogen-treated plants or the reference strain (with known N_2 fixing abilities) is considered effective (Yates *et al.*, 2016). However, nitrogen fixation capacity of strains has been reported to vary, ranging from those that fix little or no nitrogen to those that fix substantial amounts of nitrogen (Terpolilli *et al.*, 2008). This implies that, the legume-rhizobium symbioses may not always lead to optimum nitrogen fixation. It thus becomes necessary to critically assess the effectiveness of a strain during strain selection before it is finally recommended for use as inoculant.

Competitiveness with ineffective native strains and persistence in the absence of a host plant are also important traits required of an elite strain (Date, 2000). The ability of a given strain to nodulate in the presence of other strains of the same species is referred to as nodulation competitiveness (Triplett, 1990). Native rhizobia have been identified to occupy greater proportion of nodules (Rupela and Sudarshana, 1990) as such; elite strains with superior N_2 fixing abilities must be selected under natural conditions in competition with these native populations (Rengel, 2002). Strains with high competitive ability in comparison with native rhizobia are as critical as the effectiveness of symbiotic nitrogen fixation (Triplett, 1990). Some factors have been indicated to influence the competitiveness of strains and these include soil and genetic traits of host and rhizobia symbionts (Rengel, 2002). Other qualitative traits such as rhizobia mobility enables the

rhizobia to reach and nodulate lateral roots in additions to the crown ones thus enhancing competitiveness (McLoughlin *et al.*, 1990).

The capacity of rhizobia strains to establish in soils in the absence of the host legume is usually referred to as persistence (saprophytic competence) (Dowling and Broughton, 1986). Persistence is an important characteristic considered during selection of superior rhizobia strains. A persistent inoculant strain may obviate the need for re-inoculation (Zengeni *et al.*, 2006). In the absence of the host plant, rhizobia are able to survive saprophytically in soils particularly those with adequate nutrients (Sanginga *et al.*, 1996). Soil organic matter serves as a source of energy (in the form of C and N) for bacteria in the absence of the host (O'Hara, 2001).

Identification of native strains with the characteristics above is crucial for enhanced nodulation, N_2 fixation and subsequently improved adaptation of the inoculant technology.

2.6.2 Methods used in establishing the identity and diversity of rhizobia strains

Earlier classification of these bacteria were based on their ability to nodulate particular legumes, a concept known as cross inoculation (Somasegaran and Hoben, 1985). This concept was however disputed because some rhizobial strains have the capability of infecting a wide range of legumes (Pueppke and Broughton, 1999; Giller, 2001) and vice versa (Perret *et al.*, 2000). Later, Schleifer (2009) in classifying bacteria in general including rhizobia, considered phenotypic properties such as physiology, morphology and biochemical markers. However, the emergence of molecular techniques, particularly gene sequencing turned over the phase of bacterial taxonomy through the development of phylogenies that were used to infer genetic relatedness. This was further supported by the recommendation of International Subcommittee for the Taxonomy of *Rhizobium* and *Agrobacterium* that a combination of approaches be adopted when describing new genera and species of root-and stem-nodulating bacteria (Graham *et al.*, 1991). This combined approach, also referred to as polyphasic taxonomy, is an integration of phenotypic, genotypic and phylogenetic data (Vandamme *et al.*, 1996). Several authors have employed these methods of classifying rhizobia associated with grain legumes which has resulted in

identification and assignment of strains into various genus and species (Van Rossum *et al.*, 1995; Gillette and Elkan, 1996; Laguerre *et al.*, 2001; Abaidoo *et al.*, 2002; Fening *et al.*, 2004; Taurian *et al.*, 2006; Steenkamp *et al.*, 2008; Ibáñez *et al.*, 2009; Muñoz *et al.*, 2011). Characterising rhizobia using these approaches has revealed rhizobia diversity to be larger than originally anticipated (Sawada *et al.*, 2003; Willems, 2006; Lindström *et al.*, 2010). The importance of studying rhizobia taxonomy is realised in the economic value and the valorization of biodiversity of a large number of microorganisms and this can also serve as a means to improve productivity in agriculture through biotechnological manipulation (Azevedo *et al.*, 2015).

2.6.2.1 Phenotypic classification

The phenotypic classification approach is based on properties such as morphology, physiology and chemotaxonomy and has been used as a routine identification method (Schleifer, 2009). According to Bochner (2008), phenotypic description of rhizobium strains not only allows for their classification but also provides important information about the roles and adaptation of these strains to the natural environment. Vandamme *et al.* (1996) reported the determination of bacterial morphology (such as cellular shape, presence of endospores, flagella, gram staining) and colony characteristics as the conventional basics for formal description of bacterial taxa phenotypically. Generally, rhizobia have been reported as gram-negative bacteria with typical translucent, viscid, slimy growth on yeast mannitol agar media. Individual colonies are usually dump-shaped with elevated features and entire margins (Somasegaran and Hoben, 2012). Analysis on growth of bacteria at different temperatures, pH levels and salt concentrations has also been used as a means to characterize rhizobia (Rosselló-Mora and Amann, 2001). Somasegaran and Hoben (2012) grouped rhizobia into fast and slow growers based on their ability to produce acidic or alkaline substrates respectively on YMA with bromothymol blue as indicator. The bacteria normally thrive under a temperature of between 25 and30 ^{o}C and a pH of between 6 and 7. Other sources of phenotypic data include gene expression and protein analysis and function (Vandamme *et al.*, 1996). The phenotypic classification method however, provides only limited information on the phylogenetic relationship of bacteria (Schleifer, 2009).

2.6.2.2 Genotypic classification

Description of bacterial taxonomy using the genotypic method of classification generally employs methods such as DNA base ratio determination, DNA-DNA hybridization (Schleifer, 2009), restriction-fragment-length polymorphism (RFLP), pulsed-field gel electrophoresis and PCR-finger printing (Stackebrandt *et al.*, 2002). The DNA base ratio involves the determination of percentage in moles of guanosine plus cytosine of the bases. Following this determination, organisms are characterized as belonging to different genus when they have less than 90 mol% similarity. On the other hand, species should share greater than 95 mol% similarity (Schleifer, 2009). Nonetheless, bacteria with similar DNA base ratios are not always likely to belong to the same phylogeny (Rosselló-Mora and Amann, 2001). The indirect measure of sequence similarity between two entire genomes, referred to as DNA-DNA hybridization, has also been used to delineate species. Strains that share 70% or more DNA-DNA relatedness are regarded as belonging to the same species (Stackebrandt and Goebel, 1994). This method of classifying bacteria however, is limited as it (i) gives only a rough measurement of average genetic relationship, (ii) requires a lot of time and energy, (iii) can only distinguish between closely related species or subspecies (> 90% genome similarity), (iv) is also impossible to develop additional database (Schleifer, 2009). Despite the limitations associated with this method, it has been proposed as a reference standard (Stackebrandt *et al.*, 2002) until a better alternative is identified (Schleifer, 2009). These genotypic classification methods are however, difficult to adapt in developing countries such as Ghana, owing to infrastructural and financial constraints.

2.6.2.3 Phylogenetic classification

The advent of molecular protocols for sequencing bacterial genes has led to the reconstruction of valid phylogenies (Woese, 1987). For instance, phylogenetic inference based on comparative analysis of 16S rRNA gene revealed rhizobia to be diffused within the Alpha- and Beta-proteobacteria and also within taxa that do not constitute legume micro-symbionts (Sawada *et al.*, 2003; Masson-Boivin *et al.*, 2009). This method has led to the description of 98 species in 13 rhizobial genera

(http://www.rhizobia.co.nz/taxonomy/rhizobia). Most of these species are classified within the class alpha-proteobacteria with genera including *Bradyrhizobium*, *Rhizobium*, *Mesorhizobium* and *Ensifer* (Garrity *et al.*, 2004). Some other strains characterized with this class are the *Devosia* (Rivas *et al.*, 2002), *Methylobacterium* (Sy *et al.*, 2001), *Ochrobactrum* (Trujillo *et al.*, 2005) and *Shinella* (Lin *et al.*, 2008). The degree of relatedness between several microorganisms can be revealed through taxonomy. For example, Woese (1987) reported that phylogenetic relatedness of bacteria could be inferred through the sequencing of 16S or 23S rRNA genes. Several authors have also explained that, for strains to be considered as different species, less than 97% 16S rRNA similarity should be found as species with greater than or equal to 70% DNA similarity usually have a sequence similarity of greater than 97% (Stackebrandt and Goebel, 1994; Vandamme *et al.*, 1996; Gevers *et al.*, 2005). Although the 16S rRNA gene has been used widely for classification of bacteria – rhizobia (Garrity and Holt, 2001), it is limited in its ability to mark the differences within closely related species (Wang and MartínezRomero, 2000). It has also been reported that species of *Bradyrhizobium* share a high level of sequence similarity for the 16S rRNA gene (Willems, 2006) hence restricting the sole use of this gene as a determinant of bacterial taxonomy. Regardless of the limitation surrounding the use of the 16S rRNA gene (Young and Haukka, 1996); there is extensive publication and availability of sequence data for this gene in databases for the comparison of new isolates with already described species leading to the establishment of phylogenetic relationships (Sessitsch *et al.*, 2002). An example of such databases is the National Center for Biotechnology Information (NCBI), (U.S. National Library of medicine, Bethesda, USA). Analysis of the intergenic sequences of ribosomal genes (ITS region) was suggested as an approach to improve species identification (Sessitsch *et al.*, 1997; Vinuesa *et al.*, 1998; Willems *et al.*, 2001; Willems *et al.*, 2003). The region is highly variable in length and sequence among closely related strains (Gürtler and Stanisich, 1996), thus can provide additional information for establishing the relationship between and within species (Goncalves and Rosato, 2002). Maiden (2006) and Stackebrandt *et al.* (2002) proposed the use of several housekeeping genes in the phylogenetic analysis of a taxon. These housekeeping genes that act to maintain basic cell functions (Stackebrandt *et al.*, 2002) have a higher degree of sequence divergence

compared with the rRNA gene thereby providing a greater resolution of taxonomy at the sub species or species level (Palys *et al.*, 2000; Martens *et al.*, 2008). Among the widely used housekeeping genes in phylogenetic studies of bacteria including rhizobia are *recA, gyrB, rpoB, atpD, dnaK, glnA,* GSI and GSII (Stępkowski *et al.*, 2003; Vinuesa *et al.*, 2005a; Martens *et al.*, 2008). Multilocus sequence analysis (MLSA) of these genes and Multilocus sequence typing (MLST) have been used as reliable means for the assessment of genetic relatedness of species (Martens *et al.*, 2008). MLSA involves the comparison of several housekeeping genes and thus serves as a buffer against the problem of alteration in the recombination of one of the loci (Schleifer, 2009). An ad hoc committee for the re-evaluation of species came to a consensus that, to obtain sufficient information for species delineation, at least 5 genes of diverse chromosomal loci be considered (Stackebrandt *et al.*, 2002). Gevers *et al.* (2005) proposed that MLSA could be used to replace DNA-DNA hybridization in the classification of prokaryotes. In addition to the housekeeping genes (also known as core genes), accessory genes which constitute 28% of an average bacterial genome (Lapierre and Gogarten, 2009) are also considered in rhizobia description (Silva *et al.*, 2005). These genes are located on plasmids in fast- and some middle-growing species and in symbiotic islands in middle and slow growers (Barnett *et al.*, 2001). Examples of some accessory genes considered include *nodA, nodC, nodD* and *nifH* (Laguerre *et al.*, 2001; Stępkowski *et al.*, 2007; Estrella *et al.*, 2009). Though symbiotic genes usually reveal host range (Perret *et al.*, 2000) and biogeography (Wei *et al.*, 2009) of rhizobia, they are not useful in taxonomic classification due to their ability to move from plasmids to islands (Nakatsukasa *et al.*, 2008), bacteria to plants (Broothaerts *et al.*, 2005) and among bacteria (Rogel *et al.*, 2001).

The phylogenetic method of classifying bacteria is increasingly improving taxonomic studies due to the use of DNA sequencing approaches. The method is relatively rapid, low cost and can be repeated by almost any research group at specialized sequencing centers. In addition, database of gene sequences and softwares to compare these genes are readily available (Zeigler, 2003) and can be utilized to assess the diversity, and establish the identity of Ghanaian native rhizobia at the strain level.

Of all the works that have been carried out to identify and characterize elite native rhizobia as inoculants for grain legumes, very few reported on strains in Ghanaian soils. For instance, Fening *et al.* (2004) and Fening and Danso (2002) studied the variability in cowpea *Bradyrhizobium* strains in Ghanaian soils in relation to their genome and symbiotic effectiveness. The authors concluded that the strains were widely diverse with N_2 fixing capacities comparable to reference strains and N treated controls. However, they suggested that native isolates were a potential source of inoculants strains. In addition, most of the works on groundnut nodulating strains reported were carried out elsewhere (Torres-J *et al.*, 2014; Grönemeyer *et al.*, 2015) with little information on such strains in Ghana (Ampomah *et al.*, 2008). It thus becomes imperative that native strains that can effectively nodulate with groundnut in Ghanaian soils in addition to cowpea strains be identified, characterized and recommended for local inoculant production.

2.7 The successes and failures of rhizobium inoculant technology

Rhizobium inoculants are formulations containing rhizobium strains in an easy-to-use carrier material, which is either organic, inorganic or synthesized from defined molecules. Inoculating legumes seeds is probably the oldest agro biotechnological application (Lindström *et al.*, 2010) and dates back to ancient times when productive soils were transferred from one site to another to enhance crop productivity (Fred *et al.*, 1932).
Artificial inoculation using pure cultures of rhizobium (in the form of inoculants) is the method now being employed (Bala, 2011). The formulation (rhizobium inoculant) is such that sufficient quantities of the effective strains are maintained alive in the carrier to ensure that large proportions of nodules are occupied by the inoculant strain upon inoculation. Rhizobium inoculants have therefore been available on the market for more than 100 years, mostly in developed countries (Giller, 2008).

The increased use of this technology is evident in the large number of tonnes of soybean inoculated annually in USA, Brazil and Argentina (Bala, 2011). The legume inoculation practice has led to many success stories in several countries (Giller, 2001; Martins *et al.*, 2003; Yates *et al.*, 2004; Albareda *et al.*, 2009). In Ghana, studies by Boddey *et al.* (2016) revealed increases in cowpea grain yields by 39-57% following inoculation. Ulzen *et al.*

(2016) and Asei *et al.* (2015) also reported increases in soybean and cowpea grain yields based on their inoculation studies using Legumefix and Biofix commercial inoculants. Grain yield increases of above 2000 kg ha^{-1} were also reported with soybean inoculation in Zimbabwe (Mpepereki *et al.*, 2000). Studies by Bambara and Ndakidemi (2010) on *Phaseolus vulgaris* inoculation in South Africa, demonstrated significant increases in the measured grain yield parameters. The FAO of the United Nations and International Atomic Energy Agency (IAEA) also reported that inoculation with suitable rhizobium strains during sowing is a good agronomic practice, which maximizes legume yields (Hardarson and Atkins, 2003). Conversely, some rhizobia inoculation studies did not yield positive responses. For example, inoculation of cowpea in Zambia resulted in insignificant responses (Mweetwa *et al.*, 2014). Another inoculation studies on cowpea, conducted by Mathu *et al.* (2012) showed no significant effect on nodulation, biomass yield and shoot N content. Okogun and Sanginga (2003) reported no significant improvement in grain yields of soybean following inoculation. Several authors in various countries have also reported inconsistent results following inoculation with rhizobia (Awonaike *et al.*, 1990; Javaheri, 1994; de Freitas *et al.*, 2012; Thuita *et al.*, 2012).

These inconsistent responses have been attributed to several factors including the inability of introduced strains to out-compete the native rhizobia due to their less adaptation to the prevailing environment by the former (Vlassak *et al.*, 1997). Native rhizobia population in tropical soils is large with a greater proportion being ineffective (Mpepereki *et al.*, 1996; Fening and Danso, 2002; Herridge *et al.*, 2002). However, these strains possess a high level of saprophytic competence as they are well adapted to the prevailing environment (Zengeni *et al.*, 2006), a characteristic that poses a challenge to the successful use of inoculants. High predictability of nodule occupancy by introduced rhizobia thus becomes a necessary tool for a more judicious use of inoculants (Thies *et al.*, 2001; RodríguezNavarro *et al.*, 2011).

2.7.1 Techniques for monitoring introduced strains in-vitro and in the soil environment

The techniques for studying the characteristics and performance of individual strains introduced into the natural environment in the past were based on immunology, antibiotic resistance, protein profiling and multi-locus enzyme electrophoresis. Nowadays, molecular techniques have been relied upon and developed to an extent where many of the shortcoming in using previous methods are addressed (Thies *et al.,* 2001). Immunological methods such as cell agglutination was used to identify rhizobia in starter or fermentor broth. A cross-reaction between a suitable rhizobia and corresponding antibodies results in the formation of macro or microscopically visible clumps. Agglutination between the cells is thus an indication that the cells in the culture are either similar to or the same as the cells used to prepare the antiserum (Lupwayi *et al.,* 2000). Olsen *et al.* (1998) also used the syringe filter enzyme immunoassay method to identify and enumerate rhizobia strains in broth. Other immunological methods include the enzyme-linked immunosorbent assay which was used by Mpepereki and Wollum II (1991) to determine soybean nodule occupants, the spot blot (Ayanaba *et al.*, 1986) and the indirect fluorescent antibody identification method (Lupwayi *et al.,* 2000). The examples of immunological techniques mentioned are however limited in a number of ways. For instance, some authors have reported the natural occurrence and loss of antibiotic resistant markers in soils, cross-reactivity of polyclonal antibodies with target strains among others (Bushby, 1981; Mpepereki and Wollum II, 1991). Multi-locus enzyme electrophoresis was considered as a standard method for assessing rhizobia diversity and relatedness (Martínez-Romero and Caballero-Mellado, 1996) due to its simplicity and robustness (Richardson *et al.*, 1986). This method, is however, challenged by the inability to use it when the sample size is large (Thies *et al.*, 2001). On the other hand, polyacrylamide gel electrophoresis of total cell proteins (SDS-PAGE), a protein profiling method has also been successfully used to screen large numbers of strains revealing their taxonomic relationships and identities (Moreira *et al.*, 1993). Nevertheless, most protein profiling methods are laborious. Generally, most of the methods described above require prior culturing of strains, a process that may bias results towards only those bacteria that remain cultivable after nodule formation. Fortunately, other molecular based

methods have provided a window through which strain assessment can be done with ease. Examples of such molecular methods employed to assess the outcome of competitions between strains are: i) polymerase chain reaction (PCR) using primers designed to target repetitive sequences such as the enterobacterial repetitive intergenic consensus (ERIC) (Niemann *et al.*, 1999), ii) arbitrary primers to produce randomly amplified polymorphic DNA (RAPD) (Harrison *et al.*, 1992), iii) repetitive extragenic palindromic and interspersed repetitive DNA (BOX) sequences (Versalovic *et al.*, 1991; Versalovic *et al.*, 1994), iv) electrophoresis of nucleic acids, such as denaturation and temperature gradient gel electrophoresis (Sheffield *et al.*, 1989; Wartell *et al.*, 1998) and v) DNA sequencing. Most of these molecular based methods are very useful but quite sophisticated and expensive (Tas *et al.*, 1995; Gillings and Holley, 1997; González-Andrés and Ortiz, 1998; Van Berkum and Fuhrmann, 2000). The DNA sequencing method has however contributed immensely to rhizobial ecological studies. The contributions include the possibility of targeting genes sequences of strains of interest for use in the development of specific primers and gene probes (Thies *et al.*, 2001). Advances in this method have also led to the sequencing of (near) complete genome of most rhizobia strains enabling comparative genomic studies. This approach has been used successfully by some authors to design strain specific primers for the quantification of *A. brasiliense* FP2 in wheat roots (Stets *et al.*, 2015).

To this end, the DNA sequencing and comparative genomics approach appears to offer a means through which specific primers could be designed to monitor the fate strains introduced into soils.

2.8 Summary of literature review

The benefits of incorporating legumes into cropping systems make them an important resource for sustainable agriculture. The legume–rhizobium symbiosis process contributes huge amounts of nitrogen to smallholder farming systems. The process is an environmentally friendly and economically viable means to replenish nitrogen in soils hence attracting immense research attention. Nevertheless, yields of legumes are still low due to low soil fertility status, erratic environmental conditions and the non-use of external inputs particularly by legume farmers. This thus necessitating the need for less costly but sustainable measures to improve yields in less privileged countries such as Ghana. The rhizobium inoculant technology is one of the approaches that has been used to enhance yields of legumes in lots of other environments. However, improved yields following inoculation has always not been the case due to the less competitive nature of exotic strains used in most inoculants. In order to improve the situation, measures that can help monitor the fate of these exotic strains are required. DNA-based methods for monitoring nodule occupancy of strains have provided a window through which the performance of strains can be assessed which will subsequently lead to improvement in nodulation, nitrogen fixation and yields of legumes. On the other hand, isolation and characterization of native rhizobia for use as inoculants have shown positive results. This approach has been used successfully in several countries, which led to the identification of highly effective strains, some of which are currently being used as inoculants in their countries of origin and elsewhere. Meanwhile the native rhizobia population is yet to be explored for use as inoculants in the context of Ghanaian agriculture. Fortunately, some authors in this region have stimulated the discussion on the potential of using these strains for inoculant production.

This current study thus seeks to evaluate nodule occupancy of introduced strain (BR 3262) by designing and using strain-specific primers based on DNA sequencing and comparative genomics and also characterize and recommend effective native strains for inoculant production.

CHAPTER THREE

3.0 GENERAL MATERIALS AND METHODS

In this chapter, procedures used in primer design, genes amplification and laboratory protocols for soil analyses have been elaborated.

3.1 Selection of strain and procedure for specific primers design

The *Bradyrhizobium* strain BR 3262, is one of the elite strains that has been used to improve cowpea grain yields in Brazil. This strain was imported to Ghana to evaluate its potential to improve yield of cowpea in the search for good inoculant strain. Design of specific primers pairs were thus targeted at this *Bradyrhizobium* strain BR 3262 in order to monitor its performance in the field.

The draft genomic sequence (about 8000000 base pairs) of the *Bradyrhizobium* strain BR 3262 was downloaded from the GenBank (www.ncbi.nlm.nih.gov). The genome sequence was fragmented into non-overlapping sequences using Perl script software (SimõesAraújo *et al.*, 2016) resulting in 16000 fragments with each having 500 base pairs. The number of fragments were reduced by selecting those specific to the target strain using BLAST with an e value cut off of e^{-20}. A locally installed BLAST server set was used to compare the fragmented genome of BR 3262 with the genome of its closest type strain *Bradyrhizobium pachyrhizi* PAC 48^T. This analysis resulted in the selection of 2006 fragments that were specific to the target strain. A further manual analysis based on default parameters of the nucleotide collection (nr/nt) database using BLAST (Altschul *et al.*, 1997) was carried out which resulted in the final selection of 55 fragments that were complementary with the target strain, but not with any other strains. A total of 11 primer pairs located at different annealing sites of the strain's chromosome were designed from the 55 strain-specific fragements using the Primer 3 plus (Untergasser *et al.*, 2012) and Oligo Explorer v1.1.0 (http://www.softpedia.com/get/Science-CAD/Oligo-Explorer.shtml) softwares. The specifications on the parameters for primers design were: (i) product size of between 80 and 150 bps and 18–22 bps in length, (ii) melting

temperature difference of <2 °C and (iii) absence of predicted primer dimer formation, hairpin loops and duplexes. Genomic content of the designed primer pairs was visualized on the annotated genome sequence of *Bradyrhizobium pachyrhizi* strain BR 3262 using the Artemis software (Rutherford *et al.*, 2000).

3.2 Amplification of genes

Prior to amplification of genes, DNA was extracted from pure cultures of the seven isolates (KNUST 1001 – 1007) using the Wizard™ Genomic DNA Isolation kit (Promega). About 50 ng of total extracted DNA for each isolate was used for the amplification of selected genes.

3.2.1 16S rRNA gene and ITS region amplification

Conventional PCR was carried out using primers 27f/1492r (Lane, 1991) and ITSreubf/r for the amplification of 16Sr RNA gene and ITS region respectively. The PCR reaction mix was prepared with Flexi GoTaq kit (Promega). Fifty microlitres reaction mix was prepared for each gene. The mix for 16S rRNA gene contained 1 µL DNA (50 ng), 10 µL Taq DNA polymerase buffer (1×), 3 µL $MgCl_2$ (1.5 mM), 1 µL dNTPs (200 µM of each dNTP), 1 µL of each primer (10 pmol) and 0.2 µL Taq (1 U) DNA polymerase and 32.8 µL PCR water. The volume of reagents used for preparing the mix for ITS region was the same as that of the 16S rRNA gene except for the primers and Taq DNA polymerase, which were 0.5 µL (of each primer) and 0.3 µL respectively. The cycling conditions for the 16S rRNA gene programme included an initial denaturation at 96 °C for 5 min, followed by 39 cycles consisting of denaturation at 95 °C for 1 min, annealing at 60 °C for 45 s, extension at 72 °C for 45 s and final elongation for 5 min at 72 °C. The conditions for ITS region differed slightly as follows: 3 min initial denaturation at 94 °C, 30 cycles of denaturation at 94 °C for 45 s, annealing at 55 °C for 1 min, extension at 72°C for 2 mins and finally, elongation at 72 °C for 7 min.

3.2.2 Amplification of housekeeping genes

For identifying the diversity within native rhizobia population considered in this study, the *recA, gln II, gyrB*, and *rpoB* genes were amplified. The primers used for amplification of these genes were 41f/640r for *RecA*, 12f/689r for glnII, 343f/1043r for *gyrB* and rpoBfJ/rJ (Martens *et al.*, 2008; Menna *et al.*, 2009). Reaction mix of 50 µL volume was prepared for each gene and comprised 1 µL DNA (50 ng), 10 µL Taq DNA polymerase buffer (1×), 3.0 µL $MgCl_2$ (1.5 mM), 1.25 µL dNTPs (200 µM of each dNTP), 1.25 µL of each primer (10 pmol) and 0.5 µL 0.3 µL Taq (1 U) DNA polymerase and 31.95 µL PCR water. A thermocycler programme with two phases (i.e. a touch down PCR) was used. The programme included a first phase with 5 min initial denaturation at 95 °C, followed by 14 cycles consisting of denaturation at 95 °C for 1 min, annealing at 62 °C (with a decrease of 0.3 °C for every repeat) for 1 min and extension at 72 °C for 2 min. The second phase followed generic PCR amplification with 18 cycles using the final annealing temperature reached in the first phase (58 ^{o}C) and an ultimate elongation for 10 min at 72 °C.

3.2.3 Amplification of symbiotic genes

The NodCfor540/NodCrev1160 (Sarita *et al.*, 2005) and PolF/PolR (Poly *et al.*, 2001) primers were used in a PCR assay to amplify *nodC* and *nifH* genes for the test isolates. The assay for both genes was carried out in a 25 µL volume of reaction mix but had different thermocycler programmes. For the *nodC* gene, the 25 µL volume mix contained 4 µL DNA (50 ng), 5 µL Taq DNA polymerase buffer (1×), 2.5 µL MgCl2 (1.5 mM), 0.63

µL dNTPs (200 µM of each dNTP), 2 µL (10 pmol) of each primer and 0.25 µL Taq (1 U) DNA polymerase and 8.63 µL PCR water. The thermocycler programme for this mix was a touch down PCR including an initial denaturation at 95 °C for 5 min, followed by 3 cycles consisting of denaturation at 94 °C for 2 min, annealing at 58 °C for 2 min and extension at 72 °C for 1 min. This was followed by another 30 cycles of generic PCR amplification but with an annealing temperature of 56 ^{o}C and a final elongation for 5 min

at 72 °C. The reaction mix for *nifH* also contained 1 uL DNA (50 ng), 5 µL Taq DNA polymerase buffer (1×), 1.5 µL MgCl2 (1.5 mM), 0.6 µL dNTPs (200 µM of each dNTP), 1 µL of each primer (10 pmol) and 0.5 µL Taq (1 U) DNA polymerase and 14 µL PCR water. Cycling conditions for *nifH* PCR reaction included an initial denaturation at 94 °C for 5 min, followed by 35 cycles consisting of denaturation at 94 °C for 45 s, annealing at 57 °C for 30 s, extension at 72 °C for 30 s and final elongation for 1 min at 72 °C.

3.3 Soil sampling and preparation

Eight core soil samples from a depth of 0 - 15 cm were taken from each experimental site (29 m X 23 m) using an augur. The sampling was done following a W design. Soil samples were bulked to form a composite per site. Part of the composite samples for each site was air dried and sieved through a 2 mm wire mesh for determination of soil properties in the Soil Chemistry and Microbiology laboratories of KNUST.

3.3.1 Determination of soil physico-chemical characteristics

3.3.1.1 The procedures for laboratory soil analyses

The soils were characterized prior to the establishment and application of treatments at the various experimental sites. Characteristics considered included soil texture, pH, organic C, total N, available P and exchangeable cations.

3.3.1.2 Soil texture

Soil texture was determined by the hydrometer method described by Bouyoucos (1962). This method relies on the principle of dispersion and sedimentation. A fifty-gram portion of air-dried soil was weighted into a 250 mL beaker. Fifty milliletres of calgon solution and 10 ml of deionized water were added followed by vigorous stirring for 1 min. The suspension was then left to stand for 30 min after which it was transferred to a mixer for 15 min at a medium speed. The content of the beaker after the mixing was transferred into a sedimentation cylinder and the volume topped up to 1 L with deionized water. In the sedimentation cylinder, the suspension was carefully mixed by using a plunger in several

vertical movements for 1 to 2 min. The cylinder was then set on a flat surface with a soil hydrometer placed into the suspension. After 40 s, the first hydrometer and temperature readings were taken. A second hydrometer and temperature reading was taken after the suspension was left to stand for a further 3 hrs. Hydrometer and temperature readings were taken in duplicates and their average used for calculation as shown below. The textural class of the soils were obtained from the textural triangle.

$$\% \text{ Sand} = 100 - [H_1 + 0.2 (T_1 - 20) - 2] \times 2$$

$$\% \text{ Clay} = [H_2 + 0.2 (T_2 - 20) - 2] \times 2$$

$$\% \text{ Silt} = 100 - (\% \text{ Sand} + \% \text{ Clay})$$

where:

H_1 = average of first two hydrometer readings at 40 s
T_1 = average of first two temperature readings at 40 s
H_2 = average of second hydrometer readings at 3 h
T_2 = average of second temperature readings at 3 h

3.3.1.3 Soil pH

The electrometric method was used to determine pH of soils. A 1:2.5 soil to distilled water ratio (w/v) was prepared by adding 10 g of soil to 25 mL of distilled water in a 50 mL beaker. The mixture was stirred vigorously for 20 min and allowed to settle for 30 min. The Jenway 3510 pH meter (England) was calibrated and pH of soils read by inserting the electrode into the partly settled suspension.

3.3.1.4 Soil organic carbon

The Walkley and Black procedure by Nelson and Sommers (1982) which is a modification of the wet oxidation method was employed in the determination of organic carbon contents in soil samples. The procedure involved a wet combustion of organic matter using a mixture of potassium dichromate ($K_2Cr_2O_7$) solution and sulphuric acid. One gram of each soil sample was weighed into a 500 mL Erlenmeyer flask, followed by the addition

of 10 mL of 1 *N* $K_2Cr_2O_7$ solution and a gentle swirling to disperse the soil. Twenty milliliters of concentrated sulphuric acid was then added to the suspension, swirled gently and allowed to stand for 30 min on an asbestos sheet. After cooling, 250 mL of distilled water and 10 mL of orthophosphoric acid were added. Finally, 1 mL of diphenylamine indicator was added and the resulting mixture was titrated with 1 *M* $FeSO_4$ until a colour change of dark blue, and then the end point colour, green. The titre value was then recorded. A blank was included which was treated the same way as the soil samples except that no soil was added.

Percentage organic carbon (% OC) was calculated as follows:

$$\% \text{ OC} = \frac{M \times (V_1 - V_2) \times 0.39 \times \text{mcf}}{g}$$

where:

M = molarity of $FeSO_4$ (from blank titration)

V_1 = volume of $FeSO_4$ required for blank titration

V_2 = volume of $FeSO_4$ required for sample titration

g = mass of soil in grams

$0.39 = 3 \times 10^{-3} \times 1.33 \times 100$; i.e. milli-equivalent weight of C in grams x compensation for incomplete combustion of organic matter in the procedure.

3.3.1.5 Total nitrogen

Percent total N in soil samples was determined using the Kjeldahl method (Bremner and Mulvaney, 1982). This is a two-step method for estimating N, which involves initial digestion of soil samples to convert organic N to ammonium – N and then the determination of this ammonia in the digested sample.

Two grams of air-dried soil were weighed into a 500 mL long-necked Kjeldahl flask and 10 mL-distilled water was added. The flask and its content was allowed to stand for 10 minutes to moisten. A spatula full of Kjeldahl catalyst and 30 mL concentrated sulphuric acid were added and digested for about 1 – 2 h until a colourless or light green solution

was obtained. The flask was allowed to cool after which the resulting digest solution was decanted into a 100 mL volumetric flask and made to volume with distilled water. Ten milliliters aliquot of the digest from soil samples and a blank were separately pipetted into the Kjeldahl distillation apparatus and 20 mL of 40% NaOH was added to each. The mixture was distilled and collected over 4% boric acid and 3 drops of mixed indicator. The collected distillates were titrated with 0.1 *N* HCl until a colour change (blue to pink and then pink flashes) was observed.

% total N was calculated as follows:

14 g of N contained in one equivalent weight of NH_3

$$\text{Weight of N in the soil} = \frac{14 \times (A - B) \times N}{1000}$$

Considering the dilution and aliquot taken for distillation,

$$\text{Weight of soil used} = \frac{10 \text{ g} \times 10 \text{ mL}}{1.0 \text{ g} \quad 1000 \text{ mL}}$$

Thus the % of nitrogen in the soil sample;

$$\% \text{ Total N} = \frac{14 \times (A - B) \times N \times 1000}{1000 \times 1}$$

where:

A = volume of standard HCl used for sample titration (mL)

B = volume of standard HCl used in blank titration (mL)

N = Normality of standard HCl (0.1)

3.3.1.6 Available phosphorus

Soil available phosphorus was extracted using the Bray's No. 1 extracting solution (0.03 *M* NH_4F and 0.025 *M* HCl) as reported by Bray and Kurtz (1945). Using the blue ammonium molybdate method with ascorbic acid as the reducing agent, phosphorus in the extract was measured using the spectrophotometer.

Five grams of each soil sample were weighed into centrifuge tubes and 15 mL Bray 1 solution added. The mixture was shaken for 5 min on a mechanical shaker and allowed to stand for 2 min. The suspension was then centrifuged at 3000 rpm for 5 min. A 2 mL aliquot from the suspension and a blank together with 10 mL of distilled water were pipetted into a clean centrifuge tube and mixed well. Two milliliters each of colour reagent and ascorbic acid were added to the mixture and mixed thoroughly. The solution was allowed to stand for 15 min for maximum colour development after which the absorbance was measured on a spectronic 21 D Spectrophotometer at a wavelength of 660 nm at medium sensitivity.

A standard series of 0, 1.2, 2.4, 3.6, 4.8 and 6.0 mg P/L was prepared by pipetting respectively 0,10, 20, 30, 40 and 50 ml of 12.0 mg P/L into 100 mL volumetric flask and made to volume with distilled water.

Calculation:

$$P\ (\text{mg/kg soil}) = \frac{(A - B) \times 35 \times 15 \times mcf}{W}$$

where:

A = mg/L P in sample extract B = mg/L P in blank mcf = moisture correcting factor 35 = mL extracting solution

15 = mL final sample solution

W = sample weight in grams

3.3.1.7 Determination of exchangeable bases

Exchangeable bases (Na^+, K^+, Ca^{2+} and Mg^{2+}) in soils were extracted using the method described by Black (1965) with 1.0 *M* ammonium acetate extract.

Ten grams of soil were weighed into a leaching tube and leached with a 250 mL of buffered 1.0 *M* ammonium acetate (NH_4OAc) solution at pH 7. For combined

determination of exchangeable calcium and a magnesium, a 25 mL portion of the extract was transferred into a conical flask and the volume made to 50 mL. To this, 1 mL hydroxylamine hydrochloride, 1 mL of 2.0% potassium ferrocyanide, 1 mL of 2.0% potassium cyanide, 10 mL ethanolamine buffer and 0.2 mL Eriochrome Black T solution were added. The solution was then titrated with 0.01 *M* EDTA (ethylene diamine tetracetic acid) until a pure turquoise blue colour was obtained. To determine calcium only, 20 mL

0.01 *M* EDTA in the presence of 25 mL 1.0 *M* ammonium acetate solution was added to provide a standard blue colour after titration. titre values were recorded and the concentration of calcium + magnesium was calculated using the equation below;

$$Ca + Mg(cmol(+)/kg) = \frac{0.01 \times (V_1 - V_2) \times 1000}{0.1 \times m}$$

where:

V_1 = volume of 0.01 *M* EDTA used in sample titration (mL)

V_2 = volume of 0.01 *M* EDTA used in blank titration (mL) m

= mass of air-dried soil used

0.01 *M* = concentration of EDTA

Flame photometry was used to determine potassium (K) and sodium (Na) in the leachate. Standard series of K and Na were prepared by diluting both 1000 mg/L K and Na solutions, respectively to 100 mg/L. This was done by measuring 25 mg portion of each solution into a 250 mL volumetric flask and made up to the mark with distilled water. From the 100 mg/L standard solutions, portions of 0, 5, 10, 15, 20 mL were put into separate volumetric flasks. This was followed by the addition of 100 mL of 1.0 *M* NH_4OAc solution to each flask. Distilled water was used to top up to the mark to obtain standard series of 0, 2.5, 5.0, 7.5 and 10 mg/L were obtained for K and Na. Potassium and sodium were measured directly in the leachate by flame photometry at wavelengths of 766.5 and 589.0 nm respectively.

Calculation:

$$(A - B) \times 250 \times mcf$$

Exchangeable K ($cmol_{(+)}$/kg soil) =

$$\frac{(A - B) \times 250 \times mcf}{10 \times 39.1 \times W}$$

Exchangeable Na ($cmol_{(+)}$/kg soil) =

$$\frac{(A - B) \times 250 \times mcf}{10 \times 23 \times W}$$

where:

A = concentration of K or Na in diluted sample (mg/L)

B = concentration of K or Na in blank sample (mg/L)

W = weight (g) of air-dried sample
mcf = moisture correcting factor

3.3.2 Enumeration of soil rhizobia population

Rhizobium population in soil of the various sites were estimated using the most probable number method (MPN) described by Vincent (1970). Cowpea (*Vigna unguiculata* Walp L.) was used as the trap host. Uniform seeds of good viability were surface sterilized with 95% ethanol for 10 s and 3% (v/v) hydrogen peroxide solution for 3 min. Sterilized seeds were then rinsed in seven changes of sterile distilled water (Somasegaran and Hoben, 2012). The seeds were pre-germinated in Petri-dishes lined with moistened filter paper and incubated at 28 ºC. Seeds with equal radicle length were selected and asceptically transferred into plastic growth pouches (Mega International, USA). Prior to filling with seeds, growth pouches were filled with N – free nutrient solution (Broughton and Dilworth, 1970). The setup was arranged on wooden racks and kept in the greenhouse under controlled conditions. Five – fold serial dilution of soils was prepared by weighing one hundred grams of soil into a test tube containing 400 mL of sterile distilled water. This resulted in 5^{-1} soil to water solution and was mixed thoroughly using a vortex mixer. Series of soil dilutions from 5^{-2} to 5^{-6} were prepared by pipetting 5 mL of the initial solution (5^{-1}) into 20 mL of sterile distilled water. Soil dilutions were used to inoculate a week old cowpea seedlings. Four plants were inoculated per dilution level. The setup was monitored for 28 days after which nodulation was assessed using the MPNES software (Woomer *et al.*, 1990).

4.0 CHAPTER FOUR

4.1 PCR assay for direct specific detection of *Bradyrhizobium* elite strain BR 3262 in root nodule extracts of soil-grown cowpea

Abstract

Successful inoculation of legume crops with rhizobia depends on dominating nodule occupancy with highly efficient strains. The aim of this study was to develop a rapid and reliable conventional PCR methodology to specifically detect an elite *Bradyrhizobium* strain in root nodule extracts from soil-grown cowpea plants. The draft genome sequence of *Bradyrhizobium pachyrhizi* BR 3262 was compared to the closely related strain PAC 48^T. BR 3262-specific regions were selected to design specific primer pairs, which were tested with respect to PCR amplification specificity and efficiency on extracted DNA, bacterial cells and root nodules from cowpea plants grown under gnotobiotic conditions and in soil. Eleven designed primer pairs were specific for BR 3262 amplification and two of them (pairs 2645 and 2736) were highly sensitive and selected for further analyses. Experiments with gnotobiotic and soil-grown plants showed that both primer pairs were suitable to reliably determine nodule occupancy and confirmed the competitiveness of strain BR 3262 in soil. Primer pairs 2645 and 2736 are novel tools to accompany the fate of strain BR 3262 in inoculation experiments of cowpea in soil. This strategy should be applicable to other rhizobium/legume symbioses in the field.

Keywords: *Bradyrhizobium*; strain specific PCR assay; real time PCR; nodule occupancy; inoculation; *Vigna unguiculata*

4.1.1 Introduction

Cowpea (*Vigna unguiculata* Walp. L.) is the most important food legume in terms of area cultivated in Ghana (https://csirsavannah.wordpress.com). Unfortunately, the demand for this legume is above its production (Al-Hassan and Diao, 2007). Cowpea can obtain 200

kg N ha^{-1} via biological nitrogen fixation (BNF) through symbiotic association with rhizobia mainly from the genus *Bradyrhizobium* (Giller, 2001). Despite this contribution, yields of cowpea have often been reported to be low and inconsistent (Deaker *et al.*, 2004). However, farmers rarely apply nitrogen fertilizer or rhizobium inoculants to this crop as the assumption reigns that cowpea is able to effectively nodulate with native soil rhizobia to meet its N needs (Ndakidemi *et al.*, 2006). Nevertheless, microbial products such as inoculants have been reported to substantially influence the productivity of specific crops such as soybean (Giller, 2001). Recently, inoculation studies on cowpea in Ghana using some recommended Brazilian strains increased grain yields between 39 and 57%, challenging the widely held belief that inoculation of this crop is not necessary (Boddey *et al.*, 2016). Nevertheless, some contradicting results have also been reported following the use of inoculants in some African countries (Sanginga *et al.*, 1997; Mpepereki *et al.*, 2000; Thuita *et al.*, 2012). Thus in order to demonstrate the benefits of inoculation and to make this technology attractive to smallholder farmers, important factors such as poor inoculant quality, poor survival of the introduced bacteria on seeds, roots or in soil (Khalid *et al.*, 2004) must be considered.

Determination of nodule occupancy has been reported to be an important approach to evaluate the competitiveness and quality of rhizobium inoculant strains (Dowling and Broughton, 1986). Consequently, most methods for assessing the competitiveness and contribution of exotic strains to grain yields following inoculation remain a major challenge partly because existing approaches to measure competition between introduced and native strains are not specific enough while others are sophisticated, time consuming and/or require advanced/expensive technologies.

Traditionally, serological methods have been widely used to study nodule occupancy in legume nodules, especially soybean (Yokoyama *et al.*, 1999). However, problems related to target specificity may occur, for example when different polyclonal antibodies recognize a single nodule occupant (Moawad and Schmidt, 1987; Mpepereki and Wollum II, 1991). Intrinsic or induced antibiotic resistance markers employed to monitor selected strains in the field (Josey *et al.*, 1979; Bushby, 1981; Turco *et al.*, 1986) were also shown to be limited by the natural occurrence of such markers in rhizobium populations (Bushby,

1981) and also due to loss of natural resistance in the environment. DNA-based methods have revealed limitations of serology-based methods and provided a window through which strain detection in a highly specific manner could be achieved. For example, a comparative study of rhizobia applying serology and amplified fragment length polymorphism (AFLP) showed that strains from the same serogroup might be genetically distinct (Van Berkum and Fuhrmann, 2000). Therefore, DNA-based identification methods need to be explored for the strain-specific detection of nodule bacteria. In this sense, multilocus sequence analysis has been used to study *Bradyrhizobium* genotypes occupying nodules of field-grown uninoculated soybean plants (Van Berkum *et al.*, 2012). To validate the occupation of nodules by the inoculated strain, PCR using primers of target repetitive sequences including repetitive extragenic palindromic (REP) sequences (Versalovic *et al.*, 1991), enterobacterial repetitive intergenic consensus (ERIC) sequences (Niemann *et al.*, 1999), and the interspersed repetitive DNA (BOX) sequences (Versalovic *et al.*, 1994) have been used to generate PCR–fingerprints for characterizing rhizobial isolates at the strain level (Thies *et al.*, 1999). Harrison *et al.* (1992) also designed arbitrary primers to produce randomly amplified polymorphic DNA (RAPD) fragments, which have often been used in rhizobium studies for strain discrimination. However, the use of target repetitive sequences and arbitrary primers for rhizobial isolate characterization and strain discrimination, respectively, are not specific enough due to their ability to provide fingerprints for any specific target genome and variation in fingerprint patterns based on a subtle difference in PCR conditions (Gillings and Holley, 1997; González-Andrés and Ortiz, 1998). In addition, these techniques generally require the isolation and purification of strains from nodules, which is laborious, and time consuming. The rapid advances in sequencing technology and bioinformatics have facilitated obtaining (almost) complete genome sequences of bacteria and performing comparative genomics. As an example, a recent study reported on the quantification of *A. brasilense* FP2 in wheat roots using a qPCR protocol with strain-specific primers (Stets *et al.*, 2015).

The present study describes the development of a strain-specific PCR assay for the detection of the Brazilian elite strain *B. pachyrhizi* BR 3262 in the extracts of nodules

from soil grown inoculated cowpea plants. The methodology described in this paper can be performed in a relatively simple molecular biology laboratory, which is a great advantage for application in developing countries such as Ghana.

4.1.2 Materials and Methods

4.1.2.1 Selection and design of strain-specific primer pairs

Draft genomic sequences of *B. pachyrhizi* strain BR 3262, *B. pachyrhizi* PAC48^T, *B. elkanii* USDA 76^T, *B. paxllaeri* DSM 18454^T, *B. tropiciagri* CNPSo 1112^T, *B. neotropicale* BR 10297^T and *B. japonicum* USDA 6^T were downloaded from the genbank (www.ncbi.nlm.nih.gov). Accession numbers and some general characteristics of these sequences can be found in Appendix 1. The phylogenetic relatedness of *B. pachyrhizi* BR 3262 to the type strain *B. pachyrhizi* PAC 48 ^{T} and other *Bradyrhizobium* species and strains was inferred by analysis of the partial nucleotide sequence (375 basepairs) of the recombinase A gene (*recA*). Analyses were performed using MEGA 7, applying the maximum likelihood method and the Tamura 3-parameter model to infer evolutionary history (Kumar *et al.*, 2016). Average nucleotide identity analyses among the different *Bradyrhizobium* species and strains were performed using the downloaded draft genome sequences and the OrthoANI software (Lee *et al.*, 2016).

In order to identify strain-specific DNA sequences, the BR 3262 genome was fragmented *in silico* in 500 basepair non-overlapping fragments using Perl Script software (SimõesAraújo *et al.*, 2016). The genome of strain PAC48^T, which was the closest bacterial strain with an available sequenced genome, was used as reference and automated BLAST search analyses were performed using a locally installed BLAST server comparing the fragmented sequence of BR 3262 to the PAC48^T genome, with an e-value of e^{-20} as cutoff level. Selected BR 3262-specific 500 bp fragments were subjected to manual BLASTn analyses (Altschul *et al.*, 1997) at NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) between December 2015 and January 2016 using default parameters of the nucleotide collection (nr/nt) database and the fragments without any hits were selected. These fragments were used to design primers using the

Primer 3 plus (Untergasser *et al.*, 2012) and Oligo Explorer v1.1.0 (http://www.softpedia.com/get/Science-CAD/Oligo-Explorer.shtml) softwares. Efforts were made to design primer pairs based on the following characteristics: (i) product size of between 80–150 bps and 18–22 bps in length (Table 4.1.1), (ii) melting temperature difference of <2^{o}C, (iii) absence of predicted primer dimer formation, hairpin loops and duplexes. Primer synthesis was done by Alpha DNA Company, Canada. In order to map the designed primers annealing sites on the BR 3262 genome, the 116 contigs were re-annotated using the Rapid Annotation of Microbial Genomes using Subsystems Technology (RAST) (Overbeek *et al.*, 2014) and the sites of interest were localized using the Artemis software (Rutherford *et al.*, 2000). The primer pairs were tested to verify their specificity *in vitro* using the DNA of target (BR 3262) and several additional *Bradyrhizobium* strains as shown in Table 4.1.2.

Table 4.1.1. Characteristics of designed primers

Primer pair ID	Primer sequence	Orientation	GenBank accession number of target contig	Amplicon size (bp)
793	CTACTTACGACGCTCCTCTG	F	LJYE01000005	86
	AGTCTGCAATGATGTCGAAC	R		
1052	CGGATCAACGTAGAGATGAG	F	LJYE01000010	100
	TTTGCTCTCACCAAGACTTC	R		
2614	ATGCTTACGAGGATTGGAC	F	LJYE01000021	125
	CCACTTCGTTTCGATGAC	R		
2631	ATGATCTTGAAGTGCGACTG	F	LJYE01000021	113
	CCAGGAAGTTGAGTTGGTC	R		
2645	TAGAGGGCTGCTATCATGTC	F	LJYE01000021	140
	GAGATGATTACCGCAATGAG	R		
2736	TGGCTCTTCTCTTGACTAGG	F	LJYE01000022	94
	TACATCCTCCGATGTGACTC	R		
2754	AGAACCACTTCGCTATTCAC	F	LJYE01000025	80
	ATTGTGCTCATTCCAGAAAC	R		
9801	AGCTATCATAGACGGATTGC	F	LJYE01000081	133
	AGTATAGTTGGCCGCTTATC	R		
10273	ACCTCGATACAGGCAAATAG	F	LJYE01000084	120

	TCAGATGGAAGGTCAATTTC	R		
17852	TGTCATTATCTACGCAAACG	F	LJYE01000113	143
	GACATCGCTATTTCCGTAAG	R		
18665	TGATCCAGCTTTATCCTGTC	F	LJYE01000116	129
	ATTGAACTTCTATGCCAAGC	R		

[a]F: Forward primer; R: Reverse primer

4.1.2.2 Bacteria strains and culture conditions

Ten closely related *Bradyrhizobium* strains were obtained from the Johanna Döbereiner Biological Resources Center at Embrapa Agrobiologia and grown on YMA plates (Fred and Waksman, 1928). The BR 3262 strain was originally isolated from the Embrapa Agrobiologia Experimental Station, Seropédica, Rio de Janeiro, Brazil as isolate E7-6 (Zilli *et al.*, 1999). All liquid cultures used in this study were prepared by inoculating pure single colonies in TY liquid medium (Somasegaran and Hoben, 1994b). Liquid cultures were incubated in an orbital incubator at 28 °C and 150 rpm until the mid-exponential growth phase with an optical density at 600 nm ($O.D._{600}$) of approximately 0.8, as determined by spectrophotometry.

4.1.2.3 Molecular techniques

DNA was extracted from bacterial cells cultivated to mid-logaritmic phase in liquid TY medium using the Wizard™ Genomic DNA Isolation kit (Promega). For PCR reactions, the Flexi GoTaq kit (Promega) was used and 25 µL reaction mixtures contained DNA (50 ng), Taq DNA polymerase buffer (1x), $MgCl_2$ (1.5 mM), 200 µM of each dNTP, 10 pmol of each primer and 1 U Taq DNA polymerase. Primers used are given in Table 1 and as a positive control, the 16S rRNA gene was amplified with primers E786F (5’ GATTAGATACCCTGGTAG 3’) and E1115R (5’ AGGGTTGCGCTCGTTG 3’) (Baker *et al.*, 2003). In the case of colony PCR, or PCR with nodule extracts, 2 µL of cell suspensions/extracts were used as template. The thermocycler programme included a 10 min initial denaturation at 95 ºC, followed by 30-35 cycles consisting of denaturation at 95 ºC for 15s, annealing at 60 ºC for 1 min, extension at 72 ºC for 30 s and final elongation

of 10 min at 70 °C modified from (Stets *et al.*, 2015). All PCR analyses mentioned in this paper unless otherwise stated were carried out according to this method.

PCR products were analysed by electrophoresis on 2% agarose gels prepared with 1 x TAE buffer during two hours at 90 V. The low mass DNA ladder (Invitrogen) was used as a molecular size marker. Gels were stained using ethidium bromide and visualized on an ultraviolet transilluminator.

PCR amplicons were submitted to sequencing reactions using the BigDye® Terminator v3.1 Cycle Sequencing Kit (ThermoFisher). 16S rRNA gene amplicons were sequenced using primer E786F and BR 3262-specific amplicons 2645 and 2736 were sequenced using the primers 2645F and 2736F, respectively (Table 4.1.1). Sequencing reaction products were submitted to post-reaction clean up and analysed using a ABI 3500 Genetic Analyser (Thermo Fisher).

Real-time PCR reactions were carried out in 96-well optical plates on a 7500 Fast Real Time PCR system (Applied Biosystems) using QuantiTect® SYBR® Green PCR kit (QIAGEN) in a total volume of 15 µL. Nodule extracts were diluted five times using ultrapure water and then used as reaction template. Each reaction mixture included 7.5 µL QuantiTect® SYBR, 1 µL of 2645 F and R primers (10 µM), 2 µL of DNA or nodule extract and ultrapure water. The reaction conditions for amplification were 95 °C for 15 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min. To determine the specificity of amplification, analysis of the product-melting curve was performed after the last cycle of each amplification. For each biological sample three technical replicates were performed. Absolute quantification was carried out using qPCR standard curves constructed with serial dilutions (10, 1, 0.1, 0.01, 0.001 and 0.0001 ng per reaction) of *B. pachyrhizi* BR 3262 genomic DNA using the same conditions describe above. The Ct (threshold cycle) values for these reactions were used to calculate the standard curve. Because the targetsequence of primer pair 2645 occurs as a single copy on the BR 3262 genome, the number of target-sequence copies was considered equal to the number of BR 3262 cells.

4.1.2.4 Validation and sensitivity of primer pairs

The efficiency of primer pairs was tested using different cell densities following serial dilution of a turbid broth culture of BR 3262. Ten-fold serial dilutions were prepared in sterile distilled water. Using the drop plate method, 20 µL of each dilution level was plated on YMA in order to estimate the cell number based on colony forming units (CFUs) for each of the dilution levels. Aliquots of 2 µL of each cell dilution levels were used as template for PCR analyses as described above.

4.1.2.5 Gnotobiotic system for studying cowpea nodulation by BR 3262

Clean and uniform cowpea seeds (BRS Guariba) were surface-sterilized for 30 s using 70% ethanol, followed by 2.5% sodium hypochloride for 3 min (Somasegaran and Hoben, 1994b). The seeds were then rinsed in several changes of sterile distilled water and left to dry for 10 min. The sterilized seeds were placed on 1% water agar and incubated at 28 °C for 3 d until germination. Seedlings with uniform radicle length (2 cm) were transplanted into 350 mL glass bottles containing sterilized nitrogen–free nutrient solution (Norris and Mannetje, 1964).

For the gnotobiotic experiment, seedlings were inoculated with 1 mL cultures of BR 3262 or PAC 48^T 24 h after transplanting. All procedures were carried out under axenic conditions. The experiment which consisted of a total of three treatments (inoculation with BR 3262 or PAC 48^T and a negative control without inoculation) and three replicates per treatment was set up in a growth room with 12 h photoperiod provided by light emitting diode (LED) illumination at 27 °C and a humidity of 60%. Nodules were sampled and processed for PCR analysis 28 DAP.

4.1.2.6 Preparation and use of nodule suspension

Nodules from inoculated plants in the gnotobiotic system were superficially sterilized in 70% ethanol for 30 s followed by 3 min in 2.5% sodium hypochloride. Nodules from soilgrown plants were also treated with 70% ethanol for 30 s and then sterilized in 2.5% sodium hypochloride for 5 to 7 min. Surface sterility was confirmed by placing surfacesterilized nodules briefly on YMA plates and verifying the absence of bacterial

growth after incubation. Each nodule was individually crushed in a sterile microtube with 100 µL of sterile distilled water using heat-sterilized forceps. The resulting suspensions were vortexed and left to stand for about 30 min. Aliquots of 2 µL of each suspension were used as template in PCR analyses. Aliquots of the suspensions were also plated and strains inhabiting the nodules were re-isolated and submitted to colony-PCR.

4.1.2.7 Estimation of rhizobium cell numbers in soil

The enumeration of rhizobia population in soils was carried out using the Most Probable Number (MPN) count method (Somasegaran and Hoben, 1994a). Uniform, clean cowpea seeds were surface sterilized as in section 4.1.2.5. Six levels of five-fold dilutions of experimental soil were prepared using sterilized saline solution and 1 mL aliquot of each dilution was used to inoculate cowpea seedling with four replicates. Nodulation was assessed at 28 days after planting (DAP) based on the presence or absence of root nodules. The population of rhizobia was estimated using the MPNES software (Woomer *et al.*, 1990).

4.1.2.8 Competitiveness of BR 3262 in the presence of native rhizobium population

A pot experiment using soil from pasture fields of Embrapa Agrobiologia Research Station, Seropédica, State of Rio de Janeiro, Brazil, was setup under greenhouse conditions. The experiment was a 3x3 factorial plus one control without soil dilution and inoculation, arranged in a randomized complete block design (RCBD) with three replicates. The factors included three levels of soil concentrations (100% undiluted soil, 10% and 1% soil diluted with sterile river sand); and three inoculation levels ($1x10^9$, $1x10^7$ and $1x10^5$ CFU $seed^{-1}$) in addition to a negative control treatment without any soil dilution or inoculation. The different soil concentrations were obtained by mixing soil with sterilized river sand in plastic bags at the various proportions. BR 3262 liquid cultures were centrifuged to concentrate cells into a pellet. Pelleted cells were suspended in 0.85% saline solution from which 10-fold serial dilutions were prepared to obtain different inoculant densities, which were determined by serial dilution and plating followed by colony (CFU) counting.

Aliquots of 100 µL, containing the indicated cell (CFU) numbers, were used to inoculate surface sterilized cowpea seeds at planting. The experiment was monitored for 28 days and shoot dry mass assessed after oven drying at 65°C for 72 h. At least five nodules per experimental unit were selected and prepared for PCR analysis. Extracts from surfacesterilized fresh (for two replicates) and dried and rehydrated nodules (third replicate) were used in PCR analyses. In order to obtain extracts from dried nodules, freshly collected nodules were stored on desiccated silica gel for 10 days and rehydrated for 1 h before sterilization. As a proof of concept, aliquots of the nodule suspensions used for PCR analyses were plated and resultant bacterial colonies were used for a second PCR analysis in order to confirm the results of the nodule extract PCR assay.

4.1.2.9 Statistical analysis

All measured data were subjected to analyses of variance using SISVAR (Ferreira, 2008). Where overall probability was significant ($p<0.05$), means were separated using Scott Knott at 5 % probability.

4.1.3 Results

4.1.3.1 Sensitivity of strain-specific primer pairs

Phylogenetic analysis of the *recA* gene (Fig 4.1.1) and OrthoANI analyses (Appendix 1) confirmed a high level of similarity between *B. pachyrhizi* strain BR 3262 and the type strain PAC48^T. The draft genomes of these two strains were therefore compared in order to identify BR 3262-specific sequences, which resulted in the selection of 2006 potentially specific fragments. Finally, 55 BR 3262-specific 500 bp fragments were selected and 11 specific primer pairs were designed based on the nucleotide sequence of these fragments. Analyses of the genomic context of the primer annealing sites revealed that four primer pairs were located in intergenic regions; six were located in regions with predicted hypothetical protein functions and one in an RNA-splicing ligase protein RtcB (Appendix 2).

PCR analyses with genomic DNA of BR 3262, the closely related strain PAC48^T, and other *Bradyrhizobium* type strains with available genome sequences were applied to

verify the specificity of the designed primer pairs. These analyses showed that all the designed primers produced amplicons of the expected size only for BR 3262 DNA and not for any other strain whereas a general 16S rRNA primer pair produced amplicons for all the strains (Table 4.1.2). When PCR analyses were conducted with cell suspensions instead of purified DNA of the same strains, the same result was obtained (data not shown).

For the direct strain-specific detection of rhizobia in nodule extracts by PCR, the amplification sensitivity of the primers is an important trait. Therefore, the minimal number of BR 3262 cells that could be detected by the 11 strain-specific primer pairs was determined. All primer pairs were effective in amplifying DNA from $3.3x10^5$ cells. Two of the primer pairs showed outstanding sensitivity; primer pair 2736 giving a positive reaction at $3.3x10^3$ cells whereas primer pair 2645 detected down to $3.3x10^2$ cells (Table 4.1.3). These sensitivity levels are more than sufficient to detect rhizobia in a typical cowpea nodule. Blast analysis with the BR 3262 *recA* gene indicated that several

Bradyrhizobioum strains, such as SEMIA 6152, SEMIA 6160 and SEMIA 6099 could be more similar to BR 3262 than PAC 48^T (Fig. 4.1.1). Therefore, primer pairs 2645 and 2736 were tested in PCR reactions using DNA of these strains as template. Although the 16S rRNA gene of these strains could be amplified, no amplicons were obtained with the specific primers, thus confirming the high level of their specificity towards BR 3262 DNA (Table 4.1.2).

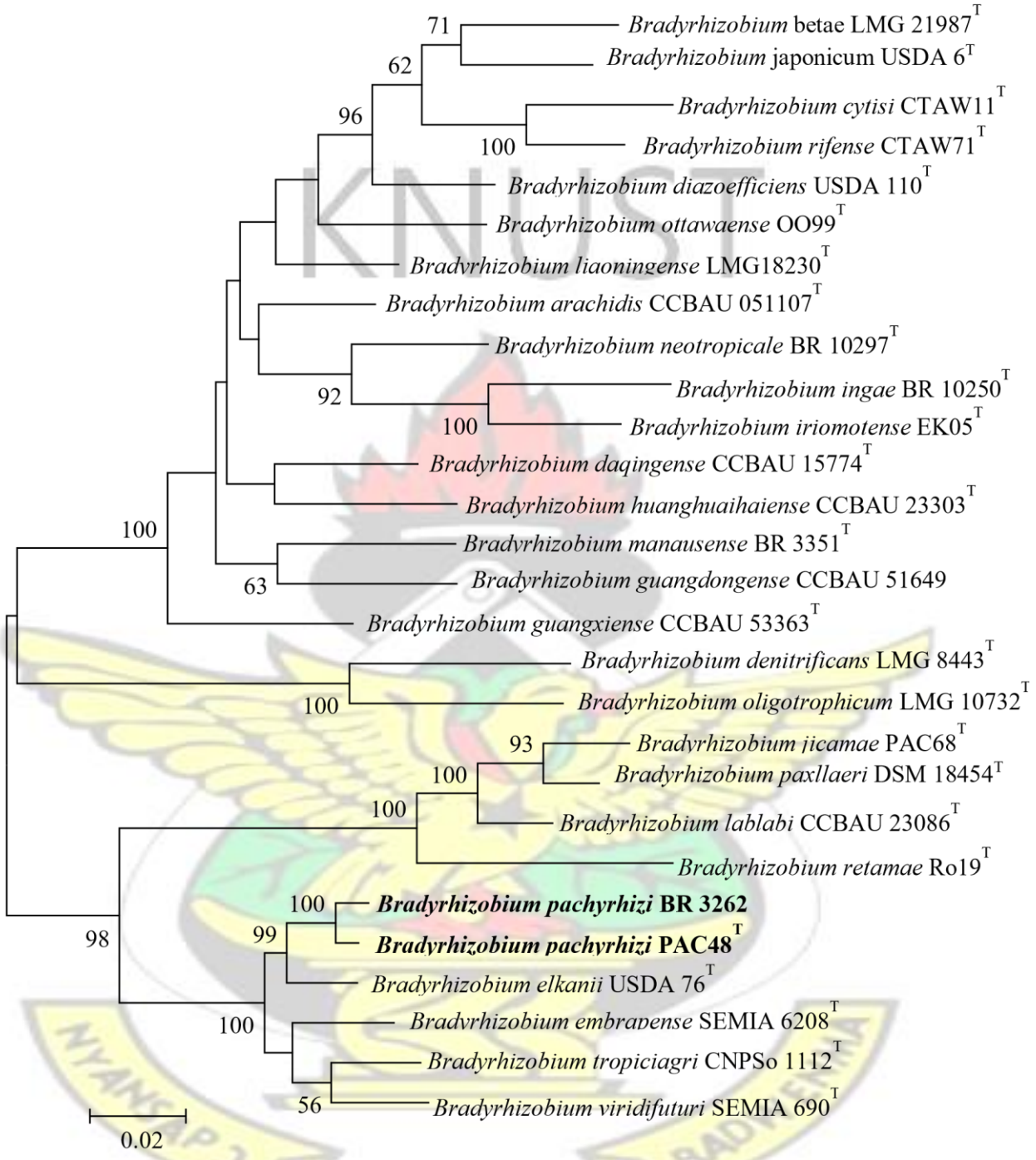


Figure 4.1.1. Molecular phylogenetic analysis of strain *B. pachyrhizi* strains BR 3262 and PAC48^T (in bold) and other *Bradyrhizobium* strains based on partial recA gene sequences

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura 3parameter model (Tamura, 1992). Bootstrap values are shown when the represented relationships were observed in at least 50% of 500 pseudoreplicates. The scale bar represents 0.02 nucleotides substitutions per site. There was a total of 375 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

Table 4.1.2. Specificity of primer pairs in amplifying target DNA

Bradyrhizobium strain	OrthoANI % with BR 3262	Primer pair											
		793	1052	2614	2631	2645	2736	2754	9801	10273	17852	18665	16S rRNA
B. pachyrhizi BR 3262	100	+	+	+	+	+	+	+	+	+	+	+	+
B. pachyrhizi PAC 48^{T}	95.18												+
B. elkanii USDA 76 ^{T}	94.62												+
B. paxllaeri DSM 18454^{T}	81.18												+
B. tropiciagri CNPSo 1112 ^{T}	89.72												+
B. neotropicale BR 10297 ^{T}	80.47												+
B. japonicum USDA 6 ^{T}	80.63												+
Bradyrhizobium sp. SEMIA 6152	n.d.	n.d.	n.d.	n.d.	n.d.			n.d.	n.d.	n.d.	n.d.	n.d.	+
Bradyrhizobium sp. SEMIA 6160	n.d.	n.d.	n.d.	n.d.	n.d.			n.d.	n.d.	n.d.	n.d.	n.d.	+
Bradyrhizobium sp. SEMIA 6099	n.d.	n.d.	n.d.	n.d.	n.d.			n.d.	n.d.	n.d.	n.d.	n.d.	+

+: positive PCR reaction, -: negative PCR reaction; n.d.: not determined.

Table 4.1.3. Efficiency of primer pairs in target amplification from different numbers of BR 3262 cells

CFU per reaction	PCR	Primer pair										
		79 3	105 2	261 4	263 1	264 5	273 6	275 4	980 1	102 73	178 52	186 65
$3.3x10^5$		+	+	+	+	+	+	+	+	+	+	+
$3.3x10^4$		+	+	+	+	+	+	+	+	+	+	+
$3.3x10^3$						+	+					
$3.3x10^2$						+						
33												
3.3												
0.33												

+: positive PCR reaction, -: negative PCR reaction

4.1.3.2 Specificity of primers in detecting DNA of target strain BR 3262 in nodules of plants grown under gnotobiotic conditions

Since the aim of this study was to detect the BR 3262 strain directly in nodule extracts, gnotobiotically grown cowpea plants were inoculated with strain BR 3262 or the closely related strain PAC48^T. Results for nodule extract PCR analysis for sole inoculation of BR 3262 and PAC 48^T are shown in Fig. 4.1.2. Amplicons were observed for nodules from plants inoculated with BR 3262. On the contrary, nodules from plants which received PAC 48^T did not give amplification with any of the primer pairs. In all cases, the blank (without template) did not give any amplification indicating that the specific primer pairs are suitable for amplifying BR 3262 DNA directly from nodule suspension.

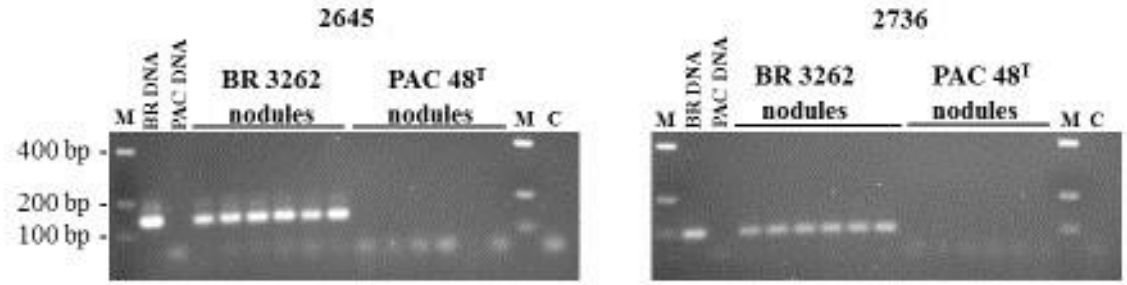


Figure 4.1.2. Specific PCR detection of strain BR 3262 in macerated nodules from gnotobiotic cowpea plants

Primer pairs 2645 (left) and 2736 (right) were used to amplify BR 3262 DNA directly from macerated surface sterilized nodules. As controls, reactions were performed with DNA from BR 3262 (BR DNA) or PAC 48^T (PAC DNA) or without any template (C). A low mass DNA ladder was used as size reference (M).

4.1.3.3 Competitiveness of target strain BR 3262 in the presence of native rhizobia

In native soils, a high diversity of known and yet uncharacterized rhizobia are present. Among these, there may be bacteria that are genetically more similar to the targeted BR 3262 strain than the tested *Bradyrhizobium* type strains. Therefore, to validate the specificity of the primer pairs 2645 and 2736 towards strain BR 3262, a test in a more natural setting with microbial competition by means of a greenhouse inoculation experiment with cowpea plants grown in native soil was done. This study sought to establish the bases of inoculation response by simulating different soil conditions (in terms of rhizobium population) and inoculant quality (in terms of cell numbers). The experimental soil had a rhizobium population of 1.3×10^4 rhizobia g^{-1} soil as estimated by the MPN method. At the end of the experiment, it was found that soil dilution level, inoculant quality, as well as their interaction, promoted significant differences ($p<0.05$) in shoot dry mass 28 DAP (Table 4.1.4). Interaction between soil concentration and inoculation level significantly influenced shoot dry weight at 1% soil concentration and $1x10^7$ and $1x10^5$ inoculation levels. At 100% soil concentration, the highest shoot dry mass was produced when plants were inoculated with $1x10^7$ CFU $seed^{-1}$, which was significantly more ($p< 0.05$) than the dry mass produced after inoculation with $1x10^5$ and $1x10^3$ CFU $seed^{-1}$ under the same soil conditions. Interaction between inoculation level and soil concentration also resulted in significant differences in shoot dry mass with the $1x10^7$-

inoculation level producing the highest dry weight in 100% soil concentration. Shoot dry mass observed for the interaction between 100% soil concentration and $1x10^3$ inoculation level was 1.49 g pot^{-1} and was significantly less ($p < 0.05$) than the mass produced for the other inoculation levels (Table 4.1.4).

Table 4.1.4. Effect of soil concentration and inoculant cell number on dry shoot biomass yield

Soil concentration (%)	Number of ted BR 326 2 cells $seed^{-1}$) inocula (CFU			
	$1x10^3$	$1x10^5$	$1x10^7$	Mean
1	1.65 b B	2.31 a A	2.51 a C	2.16 B
10	2.24 b A	2.65 a A	2.85 a B	2.58 A
100	1.49 c B	2.54 b A	3.57 a A	2.53 A
Mean	1.79 c	2.50 b	2.98 a	

Values are means of two plants per pot in g. Different lowercase letters in rows (inoculation levels) and upper case letters in columns (soil concentrations) indicate significant differences ($p < 0.05$) between treatments by Scott-Knott test.

PCR analyses with primers 2645 and 2736 were performed with extracts from surfacesterilized nodules from cowpea plants grown in different soil dilution levels after inoculation with different doses of BR 3262 and the PCR-results for one replicate with 100% soil are shown (Fig. 4.1.3). Surface-sterility of the analyzed nodules (five per inoculant dosage per replicate) was confirmed after placing them briefly on YMA plates and verifying the absence of bacterial growth after incubation. Clearly positive reactions were observed after PCR with nodule extracts (Fig. 4.1.3a). In the example shown, all five nodules of plants inoculated with $1x10^7$ CFUs were positive and after inoculation with $1x10^5$ and $1x10^3$ CFUs, four and no nodules were positive, respectively with primer pairs 2645 and 2736. The amplicons obtained with both strain-specific primer pairs were submitted to sequencing, which confirmed the specific amplification of the BR 3262 target sequence (data not shown). When plants were inoculated with $1x10^3$ CFUs, very faint

PCR products could be observed for primer pair 2645 (Fig. 4.1.3a). PCR analyses were repeated using isolated rhizobia from the same nodules, in order to confirm the presence/absence of strain BR 3262 (Fig. 4.1.3b). These analyses produced similar results as observed in Fig 4.1.3a, but differently, the faint PCR-products in the 'negative' samples were absent. This shows that these 'negative' nodules did not contain the BR 3262 strain. It was thus hypothesized that these faint PCR products were as a result of traces of DNA of strain BR 3262 present on the nodule-surface and which apparently were sufficient to produce a PCR product, demonstrating the high sensitivity of the PCR reaction. Substantiating this hypothesis, uninoculated plants grown in soil never gave any amplification with the strain-specific primers, whereas the 16S rRNA PCR was positive for all nodules (Fig. 4.1.3c). Sequencing of 16S rRNA PCR products confirmed that they represented *Bradyrhizobium* strains in all cases (data not shown). This therefore demonstrated that native *Bradyrhizobium* strains from the soil were occupying the nodules (Fig. 4.1.3c).

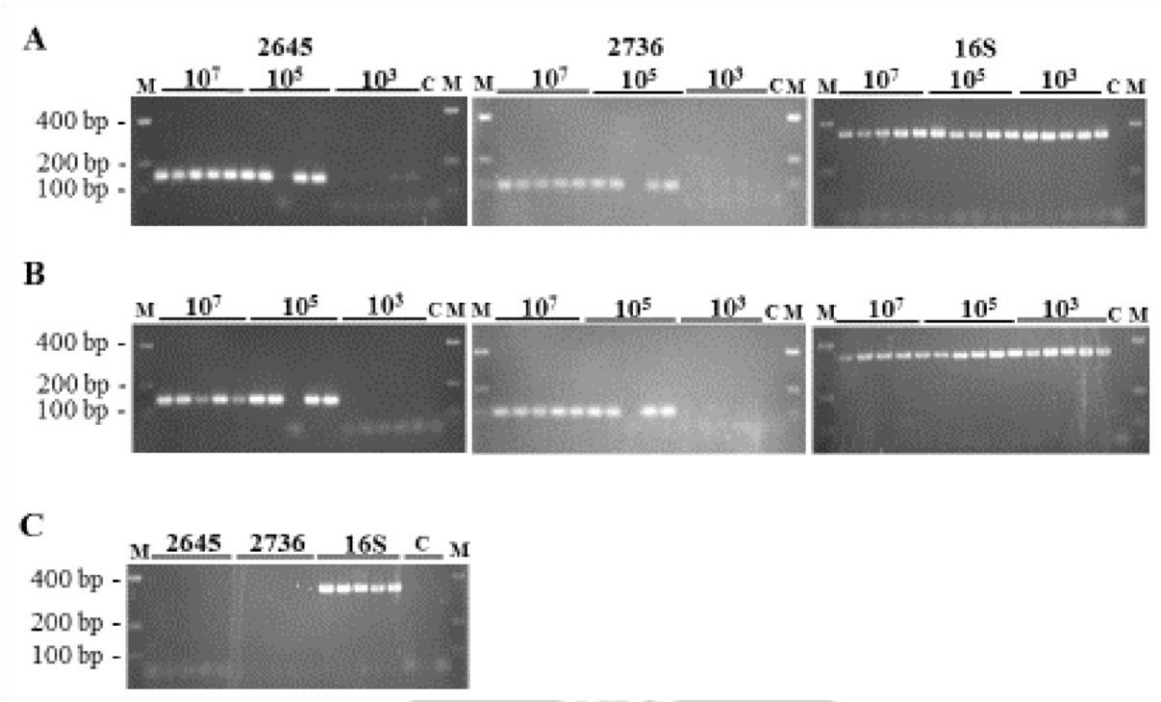


Figure 4.1.3. Specific PCR detection of strain BR 3262 in macerated nodules from inoculated cowpea plants grown on 100% soil

Primer pairs 2645 (left), 2736 (center) and E786F/E1115R (right, 16S rRNA control) were used to amplify BR 3262 DNA directly from macerated surface-sterilized nodules (a) or using reisolated rhizobia from the same nodules (b). Nodule suspension from un-inoculated plants were tested as well (c). The number of inoculated BR 3262 cells per plant in CFU seed^{-1} is indicated (10^3, 10^5, and 10^7). Low mass DNA ladder (M). Negative control without template (C).

A real-time PCR experiment was performed to obtain more information about the faint PCR products observed in Fig. 4.1.3a. For this, a standard curve was elaborated using serially diluted BR 3262 genomic DNA and the function describing the relationship between Ct and the log (copy number) for the qPCR assays was: $Ct = -3.5951x + 34.616$, $R^2 = 0.9936$. The experiment showed that the extracts from BR 3262-occupied nodules contained approximately 10^3 times more DNA from the target strains than the nodules that were occupied by native soil rhizobia and the number of copies in the occupied nodules indicated the presence of approximately 10^6-10^7 BR 3262 bacteroids per nodule (Fig. 4.1.4). This observation corroborates the hypothesis that the weak PCR products were a result of surface contamination of nodules with BR 3262 DNA.

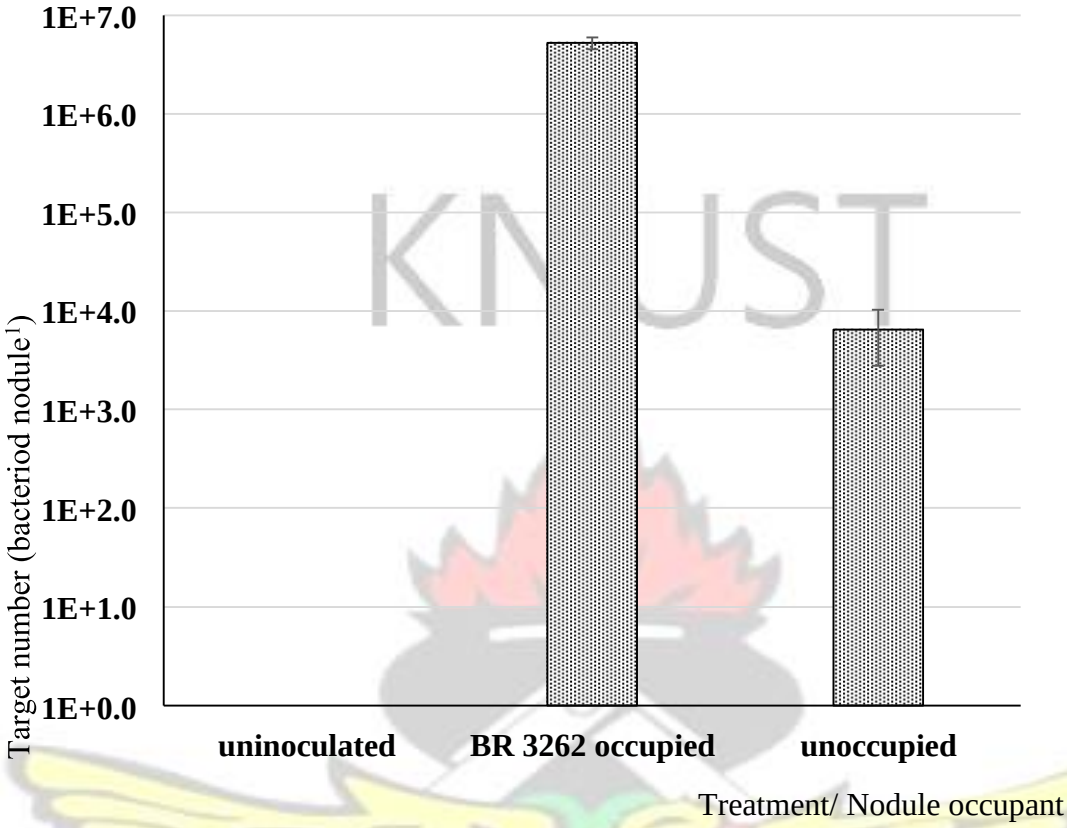


Figure 4.1.4. Number of BR 3262 target sequence copies detected in soil-grown cowpea nodules

Analyses of the PCR results from all soil concentrations and three replicates showed nodule occupancy levels between 100% and 7%, and, as expected, there was a clear tendency towards higher occupancy levels by BR 3262 at lower soil concentrations and at higher inoculant densities (Table 4.1.5). It is a common practice in rhizobiology to dehydrate and store nodules on silica gel at the time of sampling in the field, so that the tissue can be stored at room temperature for several days to weeks, maintaining viability of the rhizobia. An analysis was carried out to test the possibility to specifically detect BR 3262 in silica gel-dehydrated nodules. Dessicated nodules from one of the three replicates (blocks) of the greenhouse experiment gave similar results as were obtained when fresh nodules were used, confirming the feasibility of the methodology for dehydrated nodules.

Table 4.1.5. Effect of soil concentration and inoculant cell number on % nodule occupancy

Soil concentration (%)	Number of inoculated BR 3262 cells (CFU)			
	$1x10^3$	$1x10^5$	$1x10^7$	Mean
1	67 a A	78 a A	100 a A	82 A
10	13 c B	53 b A	87 a A	51 B
100	7 b B	67 a A	93 a A	56 B
Means	29 c	66 b	93 a	

Values are means of % nodule occupancy for three replicates. Different lowercase letters within a row (inoculation levels) and upper case letters within a column (soil concentrations) indicate significant differences ($p < 0.05$) between treatments by Scott-Knott test.

4.1.4 Discussion

Many tropical legumes have been considered promiscuous nodulators that can form effective symbiotic interactions with native rhizobia from the soils where these crops are cultivated thereby dispensing the need for inoculation. Nevertheless, experiences in the Brazilian semi-arid region and more recently in Africa have shown that, inoculation for example with the *B. pachyrhizi* elite strain BR 3262 contributed to significant grain yield increases (de Almeida *et al.*, 2010; Costa *et al.*, 2011; de Freitas *et al.*, 2012; Fernandes Jr *et al.*, 2012; Ferreira *et al.*, 2013; de Alcantara *et al.*, 2014; Boddey *et al.*, 2016).

When evaluating the efficacy of rhizobium inoculants for legume crops, one essential requirement is the effective colonization of the root nodules by the introduced strain. In majority of cases, this has been done using immunological techniques (Lupwayi *et al.*, 2000) which have the advantage of permitting the detection of bacteria directly in nodule extracts without the need of bacterial cultivation, for example by applying the enzymelinked immunosorbent assay (ELISA) methodology. A drawback of immonulogical methods may be the occurrence of cross-reactivity of polyclonal antibodies with bacterial strains other than the target strain. For example, it has been observed that genetically different strains may pertain to the same serogroup (Van Berkum and Fuhrmann, 2000). Therefore, different strains may be indistinguishable based on immunology.

The advancement in molecular microbiology has revealed a huge microbial diversity among natural rhizobia and other bacteria, which can explain cross-reactivity issues and lack of specificity of immunological methods (Thies *et al.*, 2001). This study was thus based on the hypothesis that the use of molecular genetics information from rhizobia and comparative genomics could be a valuable strategy to develop more sensitive and specific assays based on PCR. Strain-specific PCR-based methods have been previously developed for plant growth promoting bacteria of the genus *Azospirillum* (Couillerot *et al.*, 2010; Stets *et al.*, 2015). In these cases, the authors applied qPCR, which allowed for the quantification of these *Azospillum* bacteria, which inhabit rhizospheres and roots of plants and are not bound to forming symbiotic plant structures, such as nodules. In the case of

legumes, however, root nodules tend to be occupied by one or few strains of rhizobia and as such, the simple confirmation of presence or absence suffices to evaluate inoculation success, thus qPCR would be unnecessary to evaluate nodule occupancy. Conventional PCR, which is nowadays a routine molecular biology technique that can be performed in any basically equipped molecular biology laboratory, is a big advantage for the application of this technique for many laboratories in developing countries.

In this study, the draft genome sequences of cowpea elite strain BR 3262 and the closelyrelated PAC48^{T} of the species *B. pachyrhizi* were compared in order to identify BR 3262specific sequences to develop a strain-specific PCR assay. Based on BR 3262-specific sequences, 11 primer pairs were randomly designed for BR 3262-specific regions. Sequence analyses showed that four of these regions were intergenic and six encoded hypothetical protein functions thus without any experimentally confirmed role. Such regions have no clear biological function, therefore suffer less selective pressure, and tend to show larger sequence variability than functional genes. This is in accordance with the localization of ten out of 11 (91%) strain-specific primers in regions with no clear biological function while only 6-14% of bacterial genomes is non-coding (Rogozin *et al.*, 2002).

All the 11 primer pairs developed for the BR 3262-specific regions specifically amplified BR 3262 DNA but did not produce amplicons with DNA or cells of closely or more distantly related *Bradyrhizobium* strains. The use of different cell concentrations of BR 3262 showed variations in the efficiency of the primers. Primer pair 2645 was highly sensitive amplifying its target region at cell numbers down to 330 CFUs. When using nodule extracts, in some cases it was even necessary to dilute the nodule extract to obtain reliable and repeatable results with this primer pair. Primer pair 2736 could amplify its target from 3300 CFUs of the target strain. These results show that these primers were highly sensitive in detecting the target strain even at cell numbers far lower than usually found in infected nodules (Somasegaran and Hoben, 2012).

Amplification of the target strain directly from nodule extracts of plants solely inoculated with BR 3262 proved that the selected primers were specific and efficient in their

amplification of the target even with nodule extract as the template irrespective of the fact that the concentrations of rhizobia in the extracts may have differed. Kremer and Peterson (1982) also used nodule extracts as the template in an antibiotic typing of rhizobia experiment, which led to the testing of variable numbers of rhizobia due to variation in cell concentrations of the extracts. They therefore concluded that, to obtain a standard inoculum size for antibiotic testing, isolation of rhizobia from nodules was critical. In the present study, no PCR products were observed for the nodules of PAC 48^{T}-inoculated plants.

In contrast to gnotobiotically-grown plants, tropical soils naturally habour a native population of rhizobia capable of nodulating cowpea. To confirm the validity of the PCR methodology, a competitiveness experiment was performed in which different inoculant densities were tested on substrates with different concentrations of native rhizobia. In this study, the BR 3262 specific primers could not amplify DNA from surface-disinfected nodules of non-inoculated plants grown in a soil that contained approximately 1.3×10^{4} rhizobia g^{-1} soil. This showed that the strain-specific primers could not detect the native soil rhizobia, even though the target strain BR 3262 was originally isolated from soil from within 1 km of the soil used in our experiment (Zilli *et al.*, 1999). As would be expected, there was a clear tendency towards higher levels of occupancy by BR 3262 when the inoculant density was increased from 10^{3} to 10^{7} CFU $seed^{-1}$. When the native rhizobium population was lower (that is, in highly diluted soil) a higher percentage of nodule occupancy by BR 3262 was also observed. Even in non-diluted soil with a high concentration of native rhizobia, the strain BR 3262 was able to occupy more than 50% of the nodules after inoculation with $1x10^{5}$ CFUs, thereby confirming previously demonstrated performance of strain BR 3262 (Zilli *et al.*, 2009; de Alcantara *et al.*, 2014). In this study, all the PCR reactions were repeated on *Bradyrhizobium* cultures obtained after re-isolations from nodule and in all cases, these experiments confirmed PCR experiments performed directly on nodule extracts.

In order to evaluate the effect of the inoculation with BR 3262, apart from nodule occupancy, dry matter accumulation in cowpea shoots was also determined as a measure of BNF efficiency (Somasegaran and Hoben, 1994b). There was a clear positive response

after inoculation with increasing inoculant doses of BR 3262 even when undiluted soil was used. This thus confirms the positive effect of the BR 3262 strain after nodule occupancy and, in line with previous results, which showed increased BNF after inoculation (de Alcantara *et al.*, 2014). Even though the native rhizobia population was estimated to be high in the experimental soil, these were probably less competitive and less effective (Brockwell *et al.*, 1995). However, the low shoot dry mass and nodule occupancy by BR 3262 with undiluted soil inoculated with 10^3 CFU $seed^{-1}$ confirmed that inoculant quality, especially bacterial cell numbers, is an important factor that affects inoculation response (Khalid *et al.*, 2004). The success of rhizobial inoculation is often limited by several factors among which are the number of infective cells applied and the presence of competitive indigenous rhizobia (Bogino *et al.*, 2008).

Very faint PCR amplicons could be observed after using primer pair 2645 on nodules of plants inoculated with low doses of BR 3262, whereas this strain was not present inside these nodules. These faint PCR products were probably a result of BR 3262 DNA traces present on the plant surface, because they were only observed on inoculated plants and never appeared in nodules from un-inoculated plants. Nevertheless, these false positive reactions could be easily distinguished from true positives. Additionally, real-time PCR analysis using primer pair 2645 showed that the BR 3262-occupied nodules contained 1000 times more BR 3262 DNA than the false positives.

4.1.5 Conclusion

The approach described in this chapter should be applicable for the design of primers for other bacterial/rhizobial strains whose genome sequences are available. Of course, the use of this approach in other laboratories and on other plant-microbe systems may require slight adaptations, for example with respect to PCR conditions or the procedure of preparation of nodule suspension. The described conventional PCR method has the advantage of being applicable in molecular biology laboratory with basic equipment. This new approach will be used to monitor field inoculation studies of cowpea in Ghana and Brazil.

4.2 Bacteria related to *Bradyrhizobium yuanmingense* from Ghana are effective groundnut micro-symbionts

Abstract

The identification of locally adapted rhizobia for effective inoculation of grain legumes in Africa's semiarid regions is strategic for developing and optimizing cheap nitrogen fixation technologies for smallholder farmers. This study was aimed at selecting and characterising effective native rhizobia from Ghanaian soils for groundnut (*Arachis hypogaea* L.) inoculation. From surface-disinfected root nodules of cowpea and groundnut plants grown on farmers' fields, 150 bacterial isolates were obtained, 30 of which were eventually found to nodulate groundnut plants. After testing the symbiotic potential of these isolates on groundnut on sterilized substrate, seven of them, designated as KNUST 1001-1007, were evaluated in an open field pot experiment using ^{15}N-labelled soil. Although ^{15}N dilution analyses did not indicate differences among treatments in the proportion of nitrogen (N) derived from the atmosphere (%Ndfa), all seven strains increased total N derived from N_2 fixation by groundnut plants relative to the noninoculated control. Inoculation with KNUST 1002 led to total N accumulation as high as that of the groundnut reference strain 32H1. Genetic characterisation of the isolates by sequence analysis of 16S rRNA gene, 16S – 23S rRNA intergenic transcribed spacer (ITS) and *nodC* gene revealed that isolates KNUST 1003 and 1007 were related to *Rhizobium tropici*, a common bean symbiont. The other five isolates, including KNUST 1002 belonged to the *Bradyrhizobium* genus, being closely related to *Bradyrhizobium yuanmingense*. Therefore, this study revealed novel native Ghanaian rhizobia isolates with potential for the development of groundnut inoculants.

Keywords: Native isolates, Biological nitrogen fixation, Genetic diversity, *Arachis hypogaea* L.

4.2.1 Introduction

Groundnut (*Arachis hypogaea* L.) is a multipurpose grain legume, which is considered a nutritious component in diets and a source of income for smallholder farmers in developing countries (Carlberg, 2012). In Ghana, about 94% of the groundnut production is concentrated in the northern region, which is considered as one of West Africa's main groundnut production areas (Tsigbey *et al.*, 2003). In terms of symbiotic nitrogen fixation, groundnut has been found to form effective association with both fast and slow growing 'rhizobia' of the *Rhizobium* and *Bradyrhizobium* genera, respectively (Taurian *et al.*, 2002). Among the *Bradyrhizobium* strains identified to nodulate groundnut are: *Bradyrhizobium arachidis, Bradyrhizobium japonicum, Bradyrhizobium elkanii, Bradyrhizobium lablabi, Bradyrhizobium yuanmingense and Bradyrhizobium iriomotense* (Taurian *et al.*, 2006; El-Akhal *et al.*, 2008; Chang *et al.*, 2011; Muñoz *et al.*, 2011; Wang *et al.*, 2013). Other species that nodulate groundnut include *Rhizobium gardinii* and *Rhizobium tropici* (Taurian *et al.*, 2006). Despite the nitrogen-fixing ability of groundnut, yields are often below their maximum potential (Nutsugah *et al.*, 2007). These low yields have been partially attributed to low inherent soil fertility and nutrient deficiencies in N and P which mostly limit productivity of this crop (Maheswar and Sathiyavani, 2012; Mohamed and Abdalla, 2013).

Options such as mineral nitrogen application and or rhizobium inoculation have been considered as means to supply legumes with N (Mweetwa *et al.*, 2014). Apart from the possible adverse environmental consequences of excessive mineral nitrogen application (Trindade *et al.*, 2001; Flechard *et al.*, 2007), farmers are unable to exploit this option due to financial constraints. Thus, the more feasible alternative is the use of rhizobium inoculants. The practice of inoculation with highly effective rhizobium strains has been identified, among other factors, as an essential means to promote biological nitrogen fixation (BNF) with subsequent increases in grain yields (Unkovich and Pate, 2000). The usefulness of this BNF process is made evident when legumes depending on atmospheric N_2 produce increased yields in soils in which non-legume crops would require a substantial amount of mineral nitrogen. In addition, inoculation of groundnut has led to considerable

increases in nodulation, growth and productivity (Sajid *et al.*, 2010; Sharma *et al.*, 2011; Mohamed and Abdalla, 2013). Despite the potential benefits of inoculation, farmers rarely apply inoculants to groundnut because of the consensus that the association between groundnut and native soil rhizobia is usually adequate. Another factor that could contribute to the limited use of inoculants by farmers is the low awareness of the higher economic returns from the use of inoculants relative to mineral nitrogen (Ndakidemi *et al.*, 2006). Limited availability of groundnut inoculants (i.e. of exotic origin) and lack of local strains for inoculating the crop particularly in Ghana further exacerbate the limited use of inoculants.

To improve inoculation response of tropical legumes, Nkot *et al.* (2008) suggested the use of indigenous rhizobia as inoculants. For example, improvement in nodulation and N_2 fixation was reported when groundnut was inoculated with native rhizobia (Bogino *et al.*, 2008). Shishido and Pepper (1990) and Sattar *et al.* (1995) suggested that strains isolated from a particular region are the most effective for a given crop in that same region. In addition, rhizobia that are moderately to highly effective have been found to be well represented among the native population (Herridge *et al.*, 2008) and could serve as a source of elite strains for local inoculant production. This emphasizes the need to identify elite isolates adapted to the prevailing environmental conditions for improved BNF. In selecting rhizobia strains for use as inoculants, the characteristics competitiveness in nodule formation and effectiveness in nitrogen fixation are considered (Stephens and Rask, 2000).

Conversely, the symbiotic potential and genetic diversity of groundnut-nodulating rhizobia is yet to be investigated, particularly in the context of Ghanaian agriculture. Previous reports based on the analyses of 16S rRNA and RFLP revealed a large diversity within cowpea- and soybean-nodulating strains only at the genus level (Abaidoo *et al.*, 2000; Fening *et al.*, 2004). Therefore, it is imperative to assess the diversity within the native rhizobium populations that nodulate groundnut and to estimate their contribution to N_2 fixation in grain legumes. Therefore, the aim of this study was to characterise rhizobia capable of nodulating groundnut using molecular tools and to identify elite strains for groundnut inoculation. To this end, symbiotic potential and phenotypic tests in addition to sequence analyses of 16S rRNA gene, 16S – 23S rRNA intergenic transcribed spacer (ITS)

region and symbiotic genes *nodC* and *nifH* were carried out to reveal the diversity within groundnut nodulating rhizobium and identify elite strains for improved inoculation response.

4.2.2 Materials and Methods

4.2.2.1 Recovery and authentication of Rhizobium isolates

Groundnut and cowpea nodules were collected from farmers' fields across the three regions in northern Ghana at the flowering stage and sampling points were located using a GPS (Figure. 4.2.1). Recovered nodules were kept on desiccated silica gel and transported to the microbiology laboratory, KNUST in Kumasi, Ghana, for isolation. Dried nodules were rehydrated in sterile distilled water overnight. After rehydration, whole nodules were surface sterilised using 95% ethanol for 10 s and transferred into a 3% hydrogen peroxide solution for 3 min. The nodules were then rinsed in seven changes of sterilised distilled water to remove the remaining hydrogen peroxide as described by Somasegaran and Hoben (1994a). Sterilised nodules were carefully crushed onto YMA (yeast mannitol agar) plates (Fred and Waksman, 1928) under aseptic conditions using heat-sterilised forceps. The resulting plates were incubated at 28^{o}C and monitored for 10 days. Bacterial colonies were repeatedly streaked on YMA medium to obtain pure cultures.

To authenticate isolates as true rhizobia, a nodulation test was carried out under aseptic and controlled conditions using cowpea (*Vigna unguiculata* L. Walp, cv. Asontem) as the test host. Cowpea was selected for this initial screening because of its highly promiscuous nodulation pattern and for being easily cultivable in growth pouches. Cowpea seeds were prepared, pre-germinated on moist sterile tissue paper in Petri dishes, and incubated at 28°C for three days. Seedlings with equal radicle length (2 cm) were selected and aseptically transferred into plastic growth pouches (Mega International, USA) containing N-free plant nutrient solution (Broughton and Dilworth, 1970). After seeding, the growth pouches were arranged on a wooden rack and placed in the greenhouse at KNUST, Kumasi, Ghana. A week after transplanting, broth cultures of each of the isolates were used to inoculate the cowpea seedlings. At 28 days after inoculation, the seedlings were

assessed for nodulation and isolates that induced nodule formation on the test host were considered as true rhizobia. Where no nodules were observed, the isolate was not subjected to further studies. Isolates confirmed as true rhizobia were maintained on agar slants and in 25% (w/v) glycerol (at -20^{o}C) for short term and long term (-80^{o}C) storages, respectively.

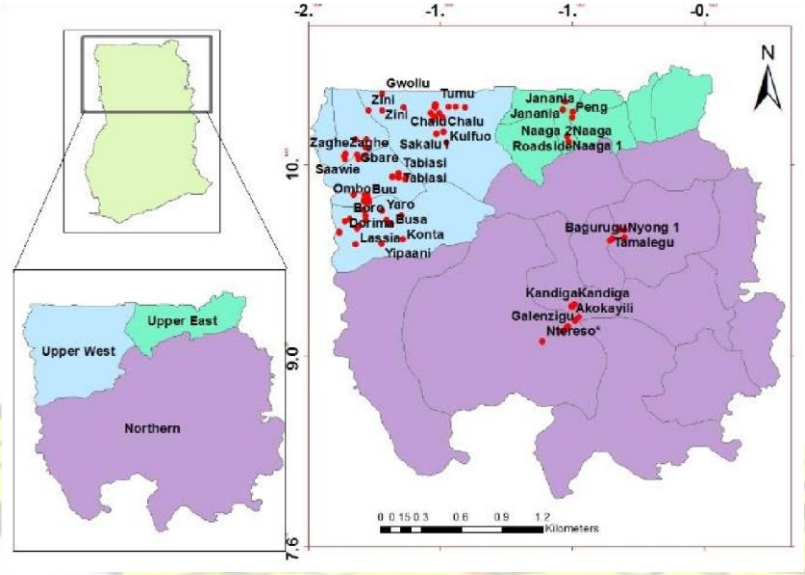


Figure 4.2.1. GPS locations of nodule sampling sites in Northern Ghana

4.2.2.2 Symbiotic potential of native isolates on groundnut in sterilized river sand in Ghana

The sixty-five isolates, that were considered true rhizobia based on the authentication test on cowpea, were evaluated for their symbiotic potential together with recommended/commercial strains namely; *Bradyrhizobium diazoefficiens* USDA 110 (soybean strain from Florida, USA) (Delamuta *et al.*, 2013), and two Brazilian elitestrains; *Bradyrhizobium pachyrhizi* strain BR 3262 and *Bradyrhizobium yuanmingense* strain BR 3267 (Leite *et al.*, 2017). USDA 110 is a strain widely used in commercial inoculants for soybean in Africa and in characterising newly cultured isolates.

The groundnut variety 'Chinese' (an early maturing variety preferred by most farmers in Ghana) was used. For the first experiment, four-litre capacity pots were filled with 3 kg of

sterilised river sand and arranged in the greenhouse at KNUST, Kumasi, Ghana. Prior to filling the pots, the sand was sterilised in an autoclave at 121^{o}C for 1 h (Lupwayi and Haque, 1994). Broughton and Dilworth (Broughton and Dilworth, 1970) N-free nutrient solution was used to irrigate the plants weekly. The strains were classified by a symbiotic effectiveness index (SEI) that was calculated from the shoot dry matter (SDM) of the groundnut plants inoculated with a specific isolate divided by the SDM of groundnut plants inoculated with the reference strain BR 3267, expressed as a percentage (Yates *et al.*, 2016). Both trials were arranged in a randomized complete block design (RCBD) with four replicates.

4.2.2.3 Nitrogen fixation contribution of isolates on groundnut in ^{15}N labelled soil in Brazil

The second experiment was conducted in pots in the open field at Embrapa Agrobiologia, Seropédica, Brazil. The planting medium used was soil classified as an Alfisol (US Soil Taxonomy Classification) obtained from Piracicaba, São Paulo State, Brazil, with a history of ^{15}N enrichment since the 1980s through the application of ^{15}N labelled organic matter (Tsai, Siu Mui, CENA, Piraicicaba, personal communication). The soil had high clay content and thus mixed with 50% sand to improve drainage. Prior to the experiment, the chemical properties of the soil were analysed using the methods of Souza and Nogueira (2005): pH in H_2O, 5.3; exchangeable Al, Ca and Mg: 0.04, 0.96 and 0.18 $cmol_{(+)}$ kg^{-1}, respectively; and P and K were 16.2 and 21.7 mg kg^{-1}, respectively. Seven effective isolates identified from the first experiment alongside the reference strains BR 3267 and three other effective/recommended groundnut strains, *Bradyrhizobium* sp. strain BR 10254 (Torres-J *et al.*, 2014), *Bradyrhizobium* sp. strain 32H1 (Urtz and Elkan, 1996) and SEMIA 6144 (Menna *et al.*, 2009) were used as treatments. Also included were three nonN_2-fixing reference plants: non-nodulating (NN) soybean (*Glycine max*), NN common bean (*Phaseolus vulgaris*) and sorghum (*Sorghum bicolor*, cv. BR 305). Each experimental unit consisted of five-litre capacity pot filled with 4 kg of soil, amended with 732 mg P_2O_5, 241 mg K_2O and the specific treatment. Clean tap water was used to irrigate the experiment

every week. In both experiments, two un-inoculated controls; (i) with nitrogen (70 ppm in the form of 0.05% KNO_3 for the first experiment and 100 mg N in the form of NH_4NO_3 for the second experiment) and (ii) without nitrogen (-N) were included.

4.2.2.4 Bacterial culture and experimental management

Broth cultures of each of the isolates and reference strains used in this study were prepared by inoculating a loop-full of pure culture in yeast mannitol broth (YMB). The cultures were then incubated in an orbital incubator at 125 rpm and 28 ^oC until the late logarithmic growth phrase where an $O.D._{600\ nm}$ of 1.0 was achieved. Groundnut seeds were surfaced sterilized with 95% ethanol for 30 s and 3% hydrogen peroxide solution for 3 min followed by several rinses in sterilised distilled water (Somasegaran and Hoben, 1994a). Five seeds were planted per pot with the help of a pair of sterile forceps and thinned to two plants one week after planting. One mL culture of each of the test isolates or reference strain was used to inoculate the seeds at planting. Unless otherwise stated, all bacteria culture, seed preparation and planting in this study followed the procedure outlined in this section.

4.2.2.5 Data collection

The plants were harvested 45 days after planting for the experiments conducted in sand and soil/sand mixture (i.e. in Ghana and Brazil, respectively). Groundnut shoot was separated from the root at the soil surface level. The nodulated roots and detached nodules were collected and stored in polythene bags. Samples were transported to the microbiology laboratory, KNUST in Ghana and Embrapa-Agrobiologia in Brazil respectively, for processing. Nodules were separated from roots by gently washing the root system under running tap water to remove all debris and adhering sand or soil after which the nodules were detached, counted and oven dried together with shoots at 65 °C for 72 h to estimate dry biomass.

For the second experiment, dried plant shoots were ground to fine powder using a roller miller similar to that described by Arnold and Schepers (2004). Total N contents of all plant samples and seeds were analysed using the semi-micro Kjeldahl procedure as

described by Urquiaga *et al.* (1992). The ^{15}N enrichment of aliquots of sub-samples containing between 35 and 70 µg of N were weighed into tin capsules and determined using an automated continuous-flow isotope-ratio mass spectrometer consisting of a Costech EA Model ECS 4010 automatic C and N analyzer coupled to a Thermo Delta V Advantage mass spectrometer (Costech Analytical, Valencia, CA, USA). Since the ^{15}N dilution technique was used for this study, the ^{15}N enrichment of the N derived from the soil was estimated by discounting the N derived from seed and its excess ^{15}N content using the formula proposed by Boddey *et al.* (1995):

^{15}N enhrichment (SC) ={(plant N □ at.%xs plant) - (seed N □ Ps □%xs seed)}/(plant N - seed N) where SC indicates corrected for seed N, at. % xs is the atom% ^{15}N excess and Ps is the proportion of the seed N that was assimilated by the plant tissue. Ps was assumed to be 50% since the correction was done for shoot tissue only (Okito *et al.*, 2004).

For the proportion of N derived from the air (% Ndfa) via BNF, the equation of Chalk (1985) was used:

%Ndfa =100 □ { 1 - (at.% ^{15}Nxs of legume /at.% ^{15}Nxs of reference)} where at. % ^{15}Nxs is the atom% ^{15}N excess of the shoot tissue of the plants. As three different non-N_2-fixing reference crops were included, individual as well as combined estimates of the % Ndfa for each isolate could be calculated using both the total N difference method and ^{15}N isotope dilution method.

4.2.2.6 Statistical Analysis

Data measured for the two experiments were subjected to analysis of variance using SISVAR (Ferreira, 2008). Where overall probability was significant ($p < 0.05$), means were separated using Scott Knott at 5% probability.

4.2.2.7 Morpho - cultural characteristics of effective isolates

Characterisation of effective isolates was carried out when at least three isolated colonies were observed after streaking on YMA with bromothymol blue as pH indicator.

Characteristics analysed were pH reaction of culture medium, number of days to form colonies, colony elevation and form and mucus production. Bacterial cultures were given identification numbers (Table 4.2.1) and deposited in the culture collections of Johanna Döbereiner Biological Resources Center (CRB-JD), Embrapa Agrobiologia, Brazil and the KNUST Microbiology Laboratory, Kumasi, Ghana.

4.2.2.8 DNA extraction, PCR amplification and gene sequencing

Bacterial genomic DNA was extracted using the Wizard genomic DNA purification kit (Promega, USA). Extracted DNA was sujected to amplification of 16S rRNA gene (Lane, 1991) and the intergenic transcribed spacer (ITS) region between the 16S and 23S rRNA genes (Cardinale *et al.*, 2004). Additionally, the symbiotic gene *nodC* (Sarita *et al.*, 2005) and the nitrogenase reductase gene *nifH* (Poly *et al.*, 2001) were amplified. For each of the genes amplified, conditions for the PCR specified by the cited references were employed. PCR amplicons were subjected to bi-directional sequencing using the BigDye® Terminator v3.1 Cycle Sequencing Kit (ThermoFisher). Sequencing reaction products were subjected to post-reaction clean up and analysed using an ABI 3500 Genetic Analyzer (Thermo Fisher). Quality control and sequence assembly were performed using BioNumerics 7 (Applied Maths, Belgium).

4.2.2.9 Phylogenetic analyses

Multiple nucleotide sequence alignments were generated using CLUSTAL W (Thompson *et al.*, 1994) and phylogenetically analysed using MEGA7 software (Kumar *et al.*, 2016). Concatenated sequence analyses of the 16S rRNA and the ITS region for the isolates in this study as well as type-strains of recognized *Bradyrhizobium* species were aligned and trimmed to the same length. Aligned sequences of the different genes were then concatenated using the Seaview programme (Galtier *et al.*, 1996). The maximum likelihood reconstruction method was used in calculating the phylogenetic trees of individual and concatenated genes. The most suitable models for generating phylogenetic trees of individual and concatenated genes were determined for each alignment using the integrated model selection tool of MEGA7. The strength of the phylogenetic tree

topologies was evaluated using the bootstrap method by applying 500 pseudo replicates (Felsenstein, 1985). DNA sequences obtained for the various isolates after sequencing have been deposited in the GenBank database under the accession numbers in Table 4.2.1.

Table 4.2.1. GenBank accession numbers of sequences obtained in this study

Bradyrhizobium isolates[a]		GenBank accession number			
KNUST ID	CRB-JD ID	16S rRNA	ITS	nodC	nifH
KNUST 1001	BR 10839	KY229769	MF108830	KY040459	KY040464
KNUST 1002	BR 10840	KY229770	MF108831	KY040460	KY040465
KNUST 1004	BR 10824	KY229772	MF108832	KY040461	KY040466
KNUST 1005	BR 10841	KY229773	MF108833	KY040462	KY040467
KNUST 1006	BR 10842	KY229774	MF108834	KY040463	KY040468
Rhizobium isolates					
KNUST 1003	BR 10837	KY229771			
KNUST 1007	BR 10838	KY229775			

[a]KNUST ID = Kwame Nkrumah University Technology culture collection identification, CRB-JD = Johanna Döbereiner Biological Resource Center culture collection identification.

4.2.3 Results

4.2.3.1 Symbiotic potential of rhizobial isolates on groundnut in sterilized river sand in Ghana

A total of 65 bacterial isolates were authenticated after inducing root nodules when inoculated individually on cowpea plants grown in sterile growth pouches in a greenhouse. These 65 authenticated strains were then tested for their performance by inoculating them on groundnut grown in pots with sterilized sand medium. The negative control (groundnut plants without inoculation or nitrogen fertilizer) did not form any nodules and showed nitrogen deficiency symptoms. Among the 65 authenticated rhizobia, only 30 induced nodulation in groundnut, demonstrating a difference in micro-symbiont specificity between groundnut and cowpea. Significant treatment effects on nodule number were observed following analysis of variance with three of the isolates producing statistically more ($p < 0.05$) nodules than all the reference strains (Table 4.2.2). Isolate KNUST 1006 produced nodule numbers similar to that of the reference strain BR 3267. With the exception of KNUST 1002, all the isolates that induced increased nodule numbers also resulted in increased nodule dry weights that were significantly ($p < 0.05$) higher than that observed for the reference strain, BR 3267. Isolate KNUST 1007 also caused a significant increase in nodule dry weight. Treatment with isolates KNUST 1001 and 1002 caused the greatest increase ($p < 0.05$) in shoot dry weight (Table 4.2.2). Symbiotic effectiveness of isolates also varied significantly ($p < 0.05$) among the isolates with isolates KNUST 1001 and 1002 performing better than the reference strain BR 3267 (Table 4.2.2). Twelve of the isolates had significantly lower symbiotic effectiveness indices (SEI) than all the reference strains. The lowest SEI was recorded for the control treatment without nitrogen and isolate KNUST 1020. Profiling the symbiotic effectiveness of isolates placed 23% into the effective group (i.e. SEI >75%). The remaining isolates were considered as partially effective (Table 4.2.2).

Table 4.2.2. Nodulation and shoot dry weight of inoculated groundnut and symbiotic effectiveness of isolates in sterilized river sand

Isolate/strain	Nodule dry	Shoot dry	Symbiotic

FIELD ID	KNUST ID	Source	Nodule number	weight (mg pot^{-1})	weight (g pot^{-1})	effectiveness index (%)	
2NAG 52b1	KNUST 1001†	Konta	137.3 a	150.0 c	7.04 a	132.95 a	
2NAG 53e	KNUST 1002†	Yipaani	105.0 b	100.0 d	7.20 a	136.00 a	
2NAG 9d	KNUST 1003†	Punyoro kb	99.3 b	290.3 a	4.94 c	93.34 c	
2NAG 8a	KNUST 1004†	Kandiga 2	24.0 e	70.0 e	3.89 e	73.56 e	
2NAG 75b	KNUST 1005†	Akuokayili	18.0 e	44.0 f	4.23 d	79.98 d	
2NAG 08e	KNUST 1006†	Kandiga 2	84.7c	183.3 b	5.84 b	110.48 b	
2NAG 87c	KNUST 1007†	Boro	12.3 f	180.0 b	5.02 c	94.86 c	
2NAG 01e	KNUST 1008	Tamale	37.3 d	47.7 f	2.34 h	44.23 h	
2NAG 08d	KNUST 1009	Kandiga 2	10.3 f	45.7 f	3.25 f	61.35 f	
2NAG 09b	KNUST 1010	Punyoro kb	5.0 f	24.7 f	2.54 h	47.95 h	
2NAG 11d	KNUST 1011	Kandiga	6.3 f	34.0 f	2.93 g	55.33 g	
2NAG 11f	KNUST 1012	Kandiga	13.3 f	23.0 f	2.39 h	45.19 h	
2NAG 11g	KNUST 1013	Kandiga	15.3 f	86.3 d	3.88 e	73.32 e	
2NAG 13e	KNUST 1014	Naaga	7.0 f	24.7 f	2.41 h	45.62 h	
2NAG 19d	KNUST 1015	Akuokayili 1	9.3 f	24.3 f	2.36 h	44.53 h	
2NAG 20a	KNUST 1016	Pishigu	6.3 f	28.7 f	2.45 h	46.23 h	
2NAG 70g	KNUST 1017	Kuncheni	5.3 f	28.3 f	3.17 f	59.96 f	
2NAG 71b	KNUST 1018	Zaguo deryiri	5.0 f	36.3 f	3.73 e	70.36 e	
2NAG 72a	KNUST 1019	Zaguo deryiri	13.0 f	46.0 f	3.70 e	69.99 e	
2NAG 73e	KNUST 1020	Gbare	6.7 f	44.0 f	2.01 i	37.91 i	
2NAG 75b	KNUST 1021	Saawie	12.3 f	46.0 f	3.79 e	71.65 e	
2NAG 80d	KNUST 1022	Varimpere	12.0 f	21.3 f	3.13 f	59.13 f	
2NAG 81b	KNUST 1023	Varimpere	8.3 f	57.7 e	2.65 h	50.03 h	
2NAG 84e	KNUST 1024	Chiatanga	8.7 f	19.7 f	2.48 h	46.86 h	
2NAG 85c	KNUST 1025	Dorima	7.0 f	34.3 f	2.57 h	48.59 h	
2NAG 87a	KNUST 1026	Boro	9.3 f	56.0 e	3.32 f	62.75 f	
2NAG 87d	KNUST 1027	Boro	12.3 f	40.7 f	3.47 f	65.66 f	

2NAG 92b	KNUST 1028	Tabiasi 1	5.3 f	19.0 f	2.94 g	55.49 g
2NAG 93e	KNUST 1029	Tabiasi 2	15.3 f	41.3 f	2.84 g	53.59 g
2NAG 97a	KNUST 1030	Kpalga	22.7e	130.0 c	4.18 d	78.90 d
	Non-Inoculated		-	-	1.81 i	34.21 i
	Reference strains					
	USDA 110	USA	10.0 f	31.7 f	3.39 f	64.11 f
	BR 3262	Brazil	21.3 e	60.3 e	3.54 f	66.84 f
	BR 3267	Brazil	80.7 c	78.3 d	5.29 c	100.00 c
CV (%)			27.93	23.35	7.17	7.34

Means in the same column followed by the same letter are not significantly different at P <0.05 (Scott Knott Test).
2NAG = Phase 2N2Africa Ghana, † Isolates selected for second experiment.

Principal component analysis gave a better understanding of the symbiotic potential of the isolates and their biogeographic distribution across sampling sites. Two principal components explained 94.5% of the variation in symbiotic potential of the test isolates and three reference strains. The first principal component explained 84.1% of the variation, which was dominated by shoot dry weight. The second component explained 10.5% of the variation with nodule dry weight being the main contributing variable (Appendix 3). Shoot dry weight and symbiotic effectiveness index pointed towards the same direction and were close to each other demonstrating a correlation between these two variables (Fig. 4.2.2). Isolates that clustered in the direction of the shoot dry weight and symbiotic effectiveness variables, together with the reference strain BR 3267, recorded high values. On the other hand, isolates that clustered on the opposite side (i.e. to the left) produced lower values for the variables considered. A large proportion of the sampling sites harboured strains with SEI of between 25 – 75% and clustered on the left side.

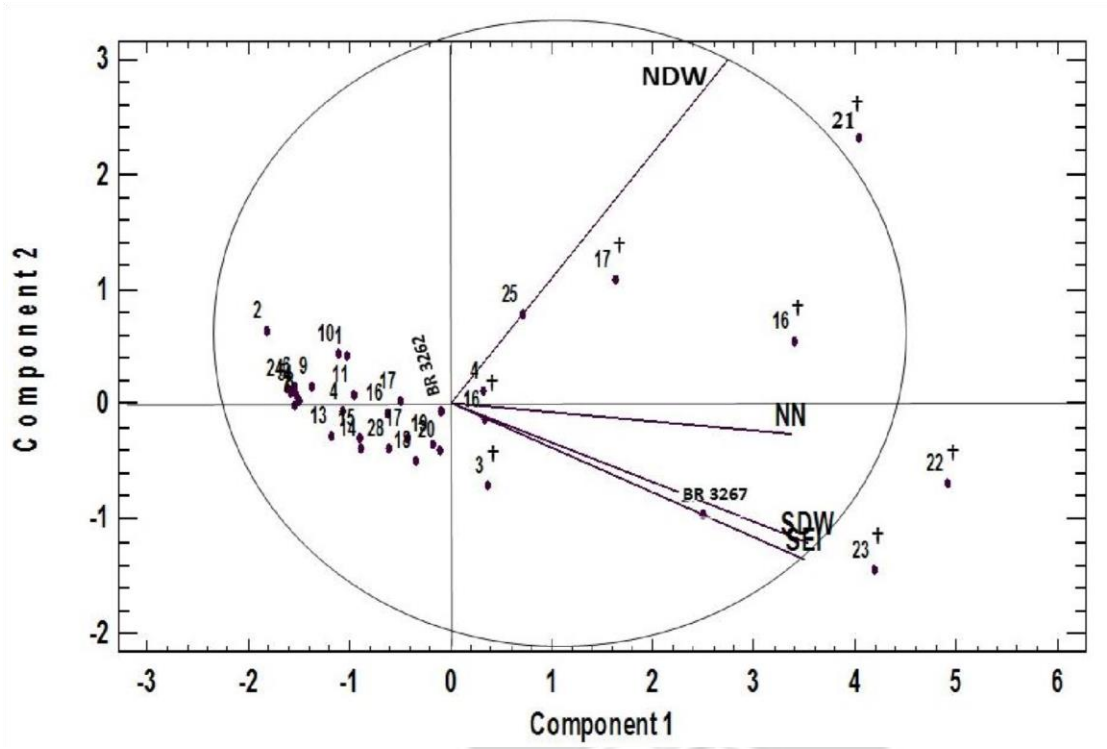


Figure 4.2.2. Principal component analysis for the relationships among sampling sites as sources of effective or ineffective isolates

Reference strains *Bradyrhizobium yuanmingense* strain BR 3267 and *Bradyrhizobium pachyrhizi* strain BR 3262 represent sources of good perfoming strains. Numbers represent sampling sites: 1- Tamale; 2- Gbare; 3- Akokayilli; 4- Kandiga; 5- Naaga; 6- Pishegu; 7- Chiatanga; 8- Punyoro; 9- Dorima; 10- Varimpere; 11- Tabiasi; 12- Kandiga; 13- Tabiasi4 14- Varimpere 2; 15- Kuncheni; 16- Kandiga 2; 17- Boro; 18- Zaguo daryiri; 19- Zaguo daryiri; 20- Saawie; 21- Punyoro kb; 22- Konta; 23- Yipaani; 24- Tabiasi 2; 25- Kpalga. † Isolates selected for second experiment. SDW: shoot dry weight, NN: nodule number, NDW: nodule dry weight, SEI: symbiotic effectiveness index.

4.2.3.2 Nitrogen fixation potential of selected isolates on groundnut grown in soil in Brazil

Seven best-performing isolates from the first experiment were selected for a second test using ^{15}N labelled soil as the growth medium. All the selected isolates, except KNUST 1004, were ranked effective (with SEI > 75%) in the previous experiment. Although isolate KNUST 1030 had SEI > 75% in the first experiment, it was not selected because re-isolation after the first experiment failed, thus the next best performing isolate from the partially effective group (i.e. KNUST 1004 with SEI = 73.6%) was selected. Significant ($p < 0.05$) differences were observed among the isolates in terms of nodulation, shoot dry weight and nitrogen accumulated in the shoot. Nodulation in all the treatments was significantly ($p < 0.05$) higher than the un-inoculated control (-N). The non-nodulating

(NN) soybean and common bean on the other hand did not show any signs of nodulation. The symbiotic association between three of the isolates and the test host resulted in increased nodule dry weight comparable to the control treatment inoculated with the 32H1 reference strain. Isolates that induced high nodule dry weights did not necessarily produce higher nodule numbers and vice versa (Table 4.2.3).

Table 4.2.3. Nodulation and shoot dry weight of inoculated groundnut and reference plants grown in ^{15}N labelled soil

Isolate/strain/reference plant	Nodule number	Nodule dry weight	Shoot dry weight
		kg ha^{-1}	
KNUST 1001	341.0 e	146.6 a	2672.5 c
KNUST 1002	411.5 d	147.5 a	2867.5 b
KNUST 1003	564.8 a	132.9 a	2745.0 c
KNUST 1004	337.5 e	146.5 a	2556.3 d
KNUST 1005	456.3 c	114.8 b	2331.3 e
KNUST 1006	495.8 c	120.4 b	2687.5 c
KNUST 1007	488.3c	120.1 b	2596.3 d
NON-INOCULATED	416.0 d	87.0 c	1677.5 g
BR 10254	523.0 b	147.8 a	2340.0 e
BR 3267	499.5 c	126.4 b	2565.0 d
32H1	498.3 c	151.3 a	3146.3 a
SEMIA 6144	476.3 c	137.1 a	2193.8 f
NN common bean			466.3 i
NN Soybean			712.5 h
Sorghum			300.0 j
CV (%)	5.9	8.6	2.9

Means in the same column followed by the same letter are not significantly different at $P < 0.05$ (Scott Knott Test). NN: non-nodulating.

Among the reference strains used, 32H1 was the most effective in terms of shoot dry weight while isolate KNUST 1002 produced the highest ($p < 0.05$) shoot dry weight among

the test isolates. Considering the effect of inoculation on shoot N accumulation, isolate KNUST 1002 promoted a significant ($p < 0.05$) increase in N accumulation of groundnut shoot when compared to all the other test isolates (Fig. 4.2.3). The performance of isolate KNUST 1002 was not significantly different from that of the reference strain 32H1. Generally, the shoot dry weight and N accumulation of inoculated groundnut plants were superior to the reference plants (Table 4.2.3 and Figure 4.2.3).

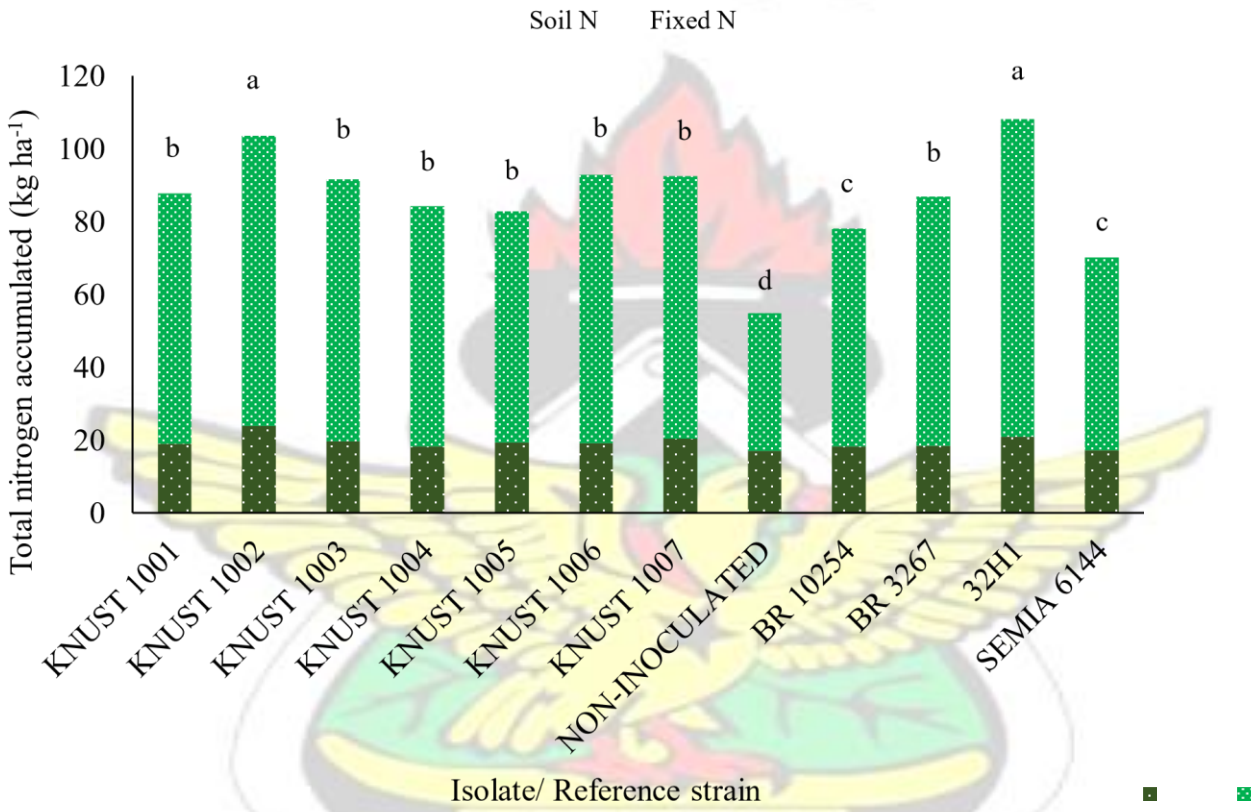


Figure 4.2.3. Total nitrogen accumulated and estimates of N derived from the air. Bars followed by the same letter are not significantly different at P <0.05 (Scott Knott Test).

In general, the ^{15}N enrichment values of inoculated plants were lower than all the reference plants. The ^{15}N enrichment data showed that there were very small amounts of N derived from the soil. The level of ^{15}N enrichment suggests that, for the N-rich legume seeds of NN common bean (9.3 mg N seed^{-1}) and NN soybean (13.0 mg N seed^{-1}), the large differences in seed N content (which were not enriched with ^{15}N) were responsible for much of the isotope dilution. For this reason, only the sorghum plant (seed N content 0.62 mg N seed^{-1}) was used as a reference for estimating the ^{15}N enrichment of the N derived

from the soil with the assumption that 50 % of the shoot N was derived from the seed. The values for the percentage of nitrogen derived from the atmosphere (% Ndfa) ranged from 88 to 93 % with no significant ($p < 0.05$) difference between treatments (Appendix 4). However, there were large and significant differences between the values for the total N derived from BNF (Fig. 4.2.3). Isolate KNUST 1002 fixed more N than any other strain except the reference strain 32H1. Most of the other strains isolated in Ghana accumulated N for the atmosphere (N fixed) that was statistically similar to that of the reference strain BR 3267. The lowest amount of N fixed was recorded for the non-inoculated treatment.

4.2.3.3 Morpho - cultural and genetic characterisation of effective isolates

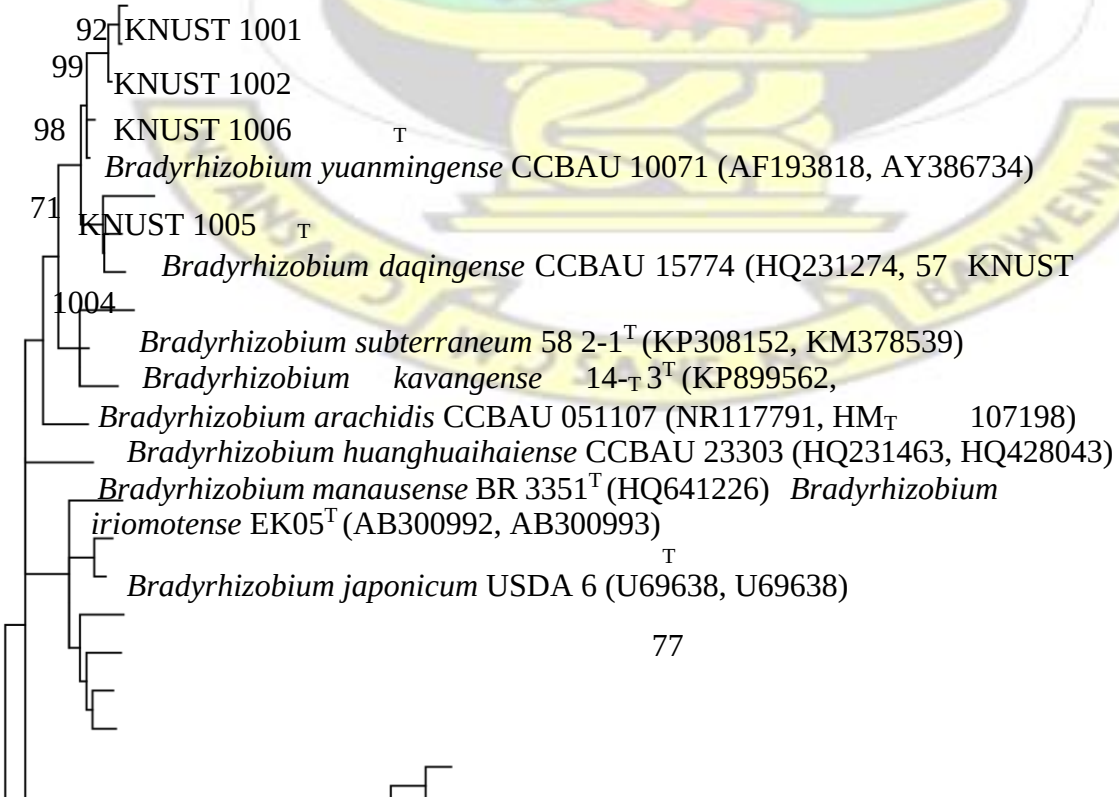
The seven isolates selected as most effective on groundnut grown on sterilized sand were characterised based on morpho-cultural and molecular genetic characteristics. Five isolates (KNUST 1001, 1002, 1004, 1005 and 1006) formed isolated colonies within six to seven days, alkalinized the culture medium and formed opaque-white colonies; traits that are consistent with the genus *Bradyrhizobium*. Isolates KNUST 1003 and KNUST 1007 acidified the culture medium, forming circular and elevated colonies. These fastgrowing isolates, the colonies of which formed within three days, produced abundant mucus that was shiny in appearance while slow-growing isolates produced colonies with reduced mucus and were more opaque in appearance (Table 4.2.4).

Table 4.2.4. Morpho-cultural characteristics of effective isolates

Isolates	Days to single colony(C) appearance	pH reaction	Colony morphology	Colony transparency/Colour	Mucus produced
KNUST 1001	6	Alkaline	Circular	Opaque/ White	Less
KNUST 1002	7	Alkaline	Circular	Opaque/ White	Less
KNUST 1003	3	Acidic	Circular	Translucent/ Yellow	More
KNUST 1004	7	Alkaline	Irregular	Opaque/ White	Less
KNUST 1005	7	Alkaline	Circular	Opaque/ White	Less
KNUST 1006	7	Alkaline	Circular	Opaque/ White	Less
KNUST 1007	3	Acidic	Circular	Translucent/ Yellow	More

The phylogeny of the selected strains was studied by analysing the near-complete sequence of their 16S rRNA gene and ITS sequences. Basic Local Alignment Search Tool (BLAST) analysis of the 16S rRNA sequences confirmed that isolate KNUST 1001, 1002, 1004, 1005 and 1006 belonged to the genus *Bradyrhizobium*. The fast-growing isolates KNUST 1003 and 1007 were highly similar to members of the genus *Rhizobium* (Appendix 5a and b).

Within the genus *Bradyrhizobium*, 16S rRNA sequences are too conserved to permit for a more detailed phylogenetic classification at the species level. The ITS sequence between the 16S and 23S rRNA genes can be used to improve this phylogenetic resolution. Therefore, in this study, phylogenetic analysis was performed on the concatenated sequences of the 16S rRNA gene (1243 nt) and the ITS sequence (1027 nt) giving a total of 2270 nt. In this analysis, the five *Bradyrhizobium* isolates from this study clustered together on the same branch with close relation to *B. yuanmingense* CCBAU 10071^{T}, *Bradyrhizobium daqingense* CCBAU 15774^{T} and *Bradyrhizobium subterraneum* 48 2-1^{T} with bootstrap support of at least 71% and with nucleotide sequence similarity values between 99.1 and 99.3% (Fig. 4.2.4, Appendix 10). Generally, the concatenated analyses of 16S rRNA gene and ITS region revealed a clearer relationship between clustering of *Bradyrhizobium* strains or isolates compared to their individual analyses (Appendix 5a and 7).



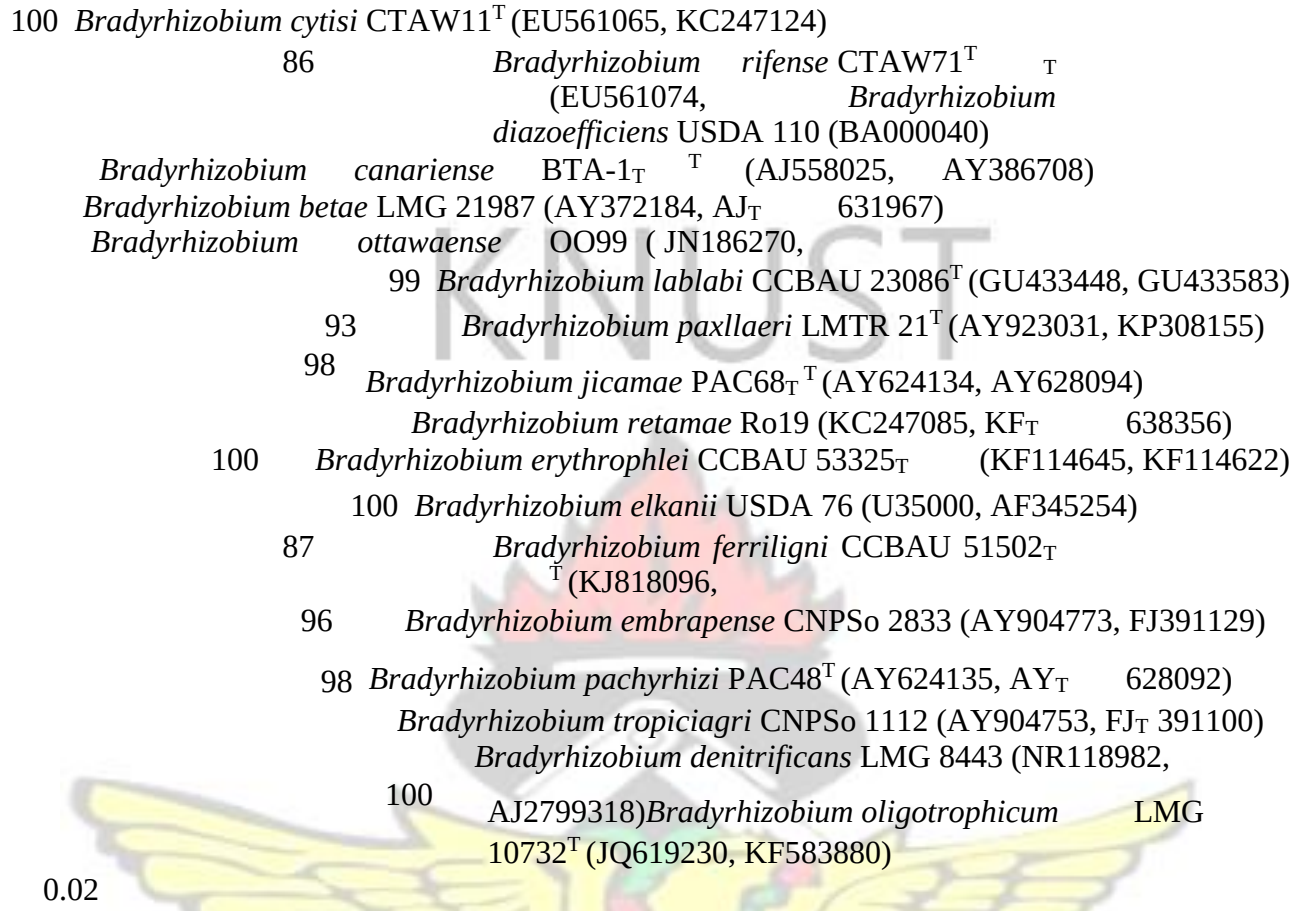


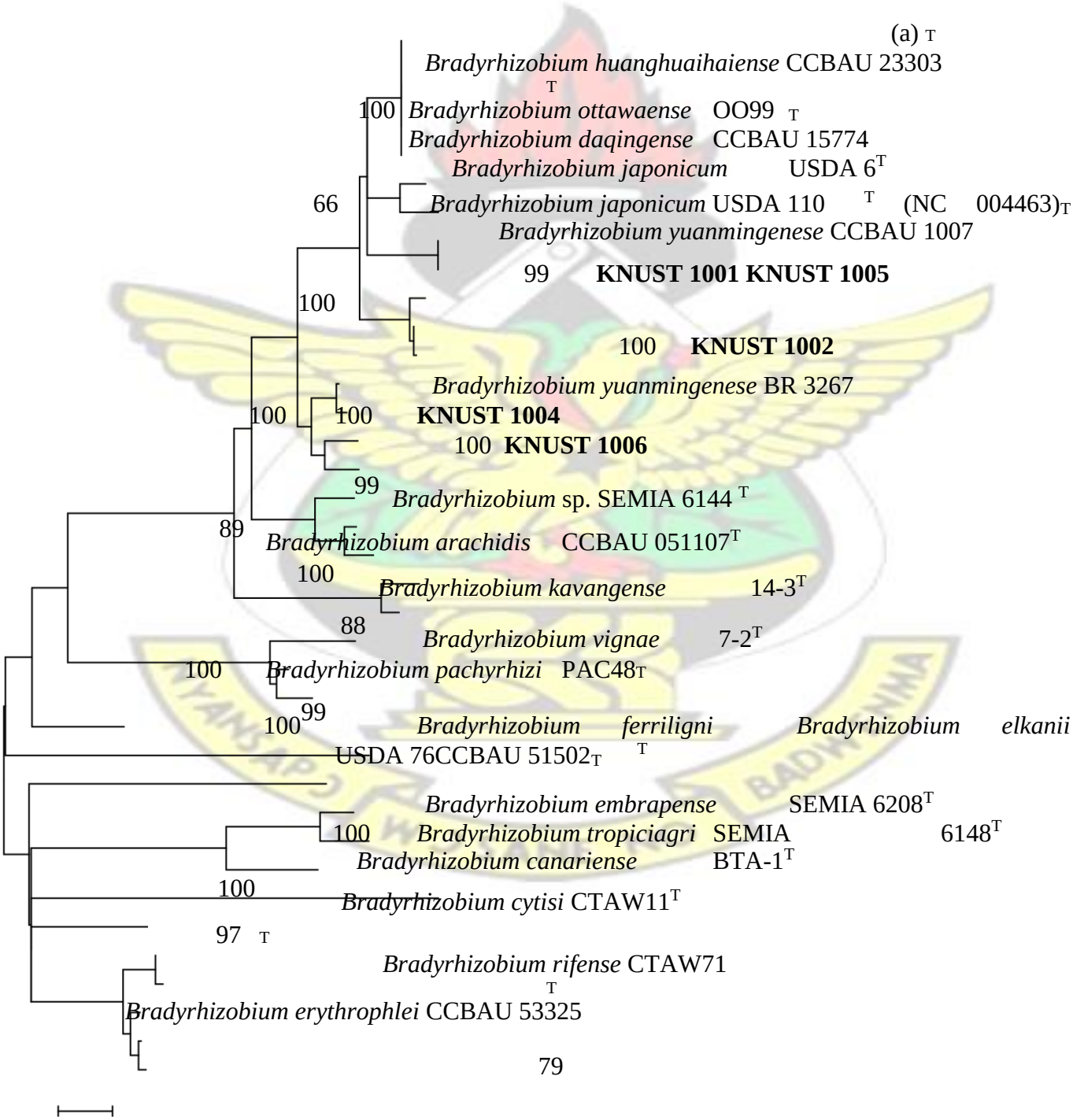
Figure 4.2.4. Unrooted maximum likelihood phylogenetic tree based on concatenated 16S rRNA gene and ITS sequences showing relationships among isolates and typestrains (^{T}) of the genus *Bradyrhizobium*

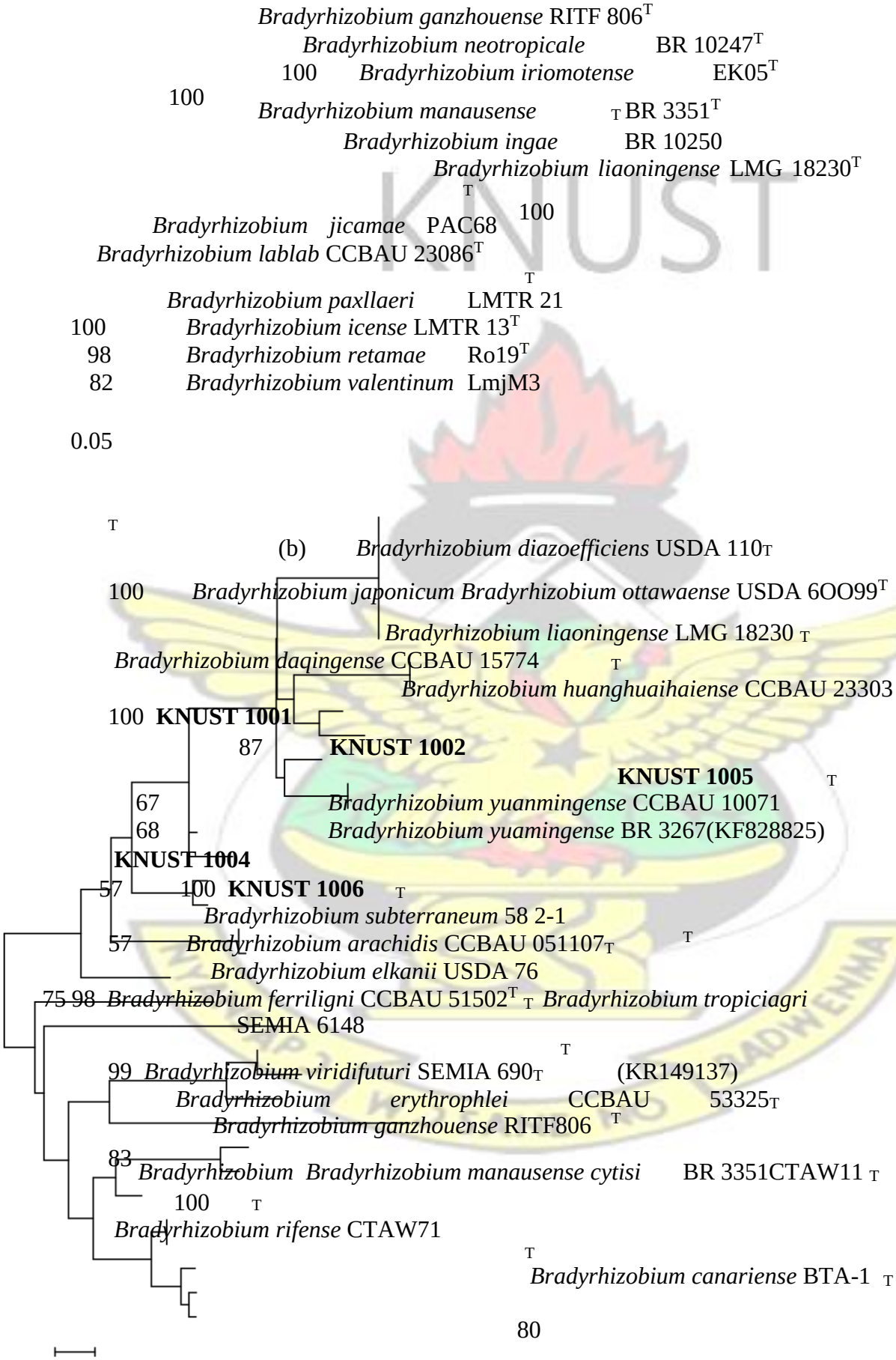
Bootstrap values were inferred from 500 replicates and are indicated at the tree nodes when ≥50%. GenBank accession numbers are provided in parentheses. The bar represents two estimated substitutions per 100 nucleotide positions.

4.2.3.4 Analyses of symbiotic genes

The phylogenetic relationship of the *nodC* gene from the novel *Bradyrhizobium* isolates in relation to validly described species was studied. The *nodC* phylogenetic analyses placed two of the isolates, KNUST 1004 and 1006, on a branch together with *B. yuanmingense* BR 3267^T with 100% bootstrap support (Fig. 4.2.5a). The isolates KNUST 1001, 1002 and 1005 were together on a branch with their sequences most closely related to *B. yuanmingense* CCBAU 1007^T, *Bradyrhizobium ottawaense* OO99^T, *Bradyrhizobium japonicum* USDA 6^T, *Bradyrhizobium huanghuaihaiense* CCBAU 23303^T and *Bradyrhizobium denitrificans* LMG 8443^T, with 65% bootstrap support. In agreement with

the *nodC* phylogeny, phylogenetic analyses of the *nifH* gene also placed the isolate KNUST 1005 in a branch together with *B. yuanmingense* CCBAU 10071^{T} followed by the inclusion of isolates KNUST 1001 and 1002. Isolates KNUST 1004 and 1006 also shared close relation to *B. yuanmingense* BR 3267^{T} (Fig. 4.2.5b). Therefore, in general, the *nodC* and *nifH* phylogenies were congruent with the phylogeny estimated based on the concatenated 16S rRNA and ITS sequences.




Bradyrhizobium ganzhouense RITF 806T
Bradyrhizobium neotropicale BR 10247T
100 *Bradyrhizobium iriomotense* EK05T
100
Bradyrhizobium manausense T BR 3351T
Bradyrhizobium ingae BR 10250
Bradyrhizobium liaoningense LMG 18230T
T
100
Bradyrhizobium jicamae PAC68
Bradyrhizobium lablab CCBAU 23086T
T
Bradyrhizobium paxllaeri LMTR 21
100 *Bradyrhizobium icense* LMTR 13T
98 *Bradyrhizobium retamae* Ro19T
82 *Bradyrhizobium valentinum* LmjM3
0.05
T
(b) *Bradyrhizobium diazoefficiens* USDA 110T
100 *Bradyrhizobium japonicum Bradyrhizobium ottawaense* USDA 6OO99T T
Bradyrhizobium liaoningense LMG 18230 T
Bradyrhizobium daqingense CCBAU 15774 T
Bradyrhizobium huanghuaihaiense CCBAU 23303
100 **KNUST 1001**
87 **KNUST 1002**
KNUST 1005 T
67 *Bradyrhizobium yuanmingense* CCBAU 10071
68 *Bradyrhizobium yuamingense* BR 3267(KF828825)
KNUST 1004
57 100 **KNUST 1006** T
Bradyrhizobium subterraneum 58 2-1
57 *Bradyrhizobium arachidis* CCBAU 051107T T
Bradyrhizobium elkanii USDA 76
75 98 *Bradyrhizobium ferriligni* CCBAU 51502T T *Bradyrhizobium tropiciagri*
SEMIA 6148
T
99 *Bradyrhizobium viridifuturi* SEMIA 690T (KR149137)
Bradyrhizobium erythrophlei CCBAU 53325T
Bradyrhizobium ganzhouense RITF806 T
83
Bradyrhizobium Bradyrhizobium manausense cytisi BR 3351CTAW11 T
100 T
Bradyrhizobium rifense CTAW71
T
Bradyrhizobium canariense BTA-1 T
80

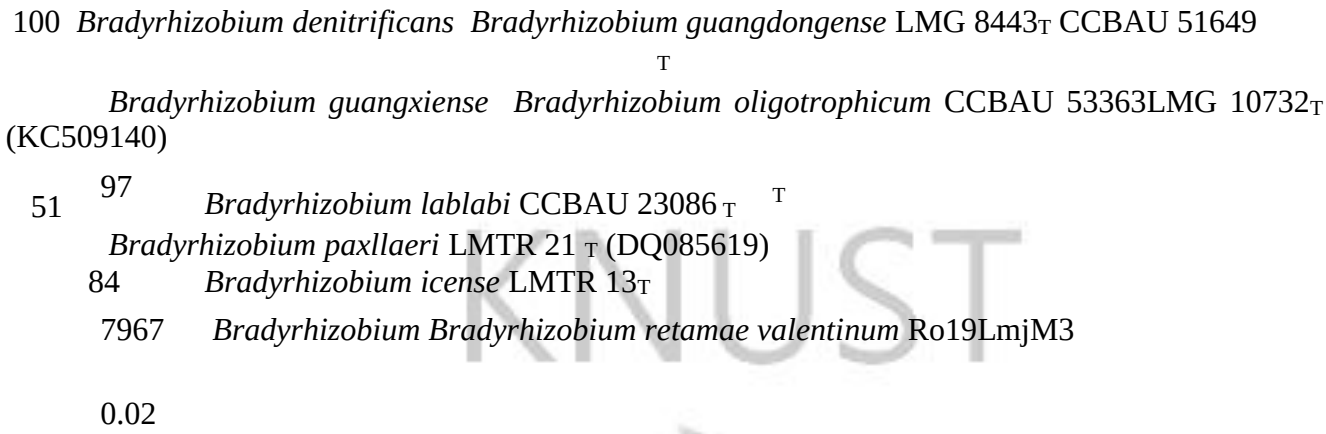


Figure 4.2.5. Unrooted maximum likelihood phylogenetic tree based on *nodC* (a) and *nifH* (b) genes showing relationships among isolates and type-strains (^{T}) of the genus *Bradyrhizobium* Bootstrap values were inferred from 500 replicates and are indicated at the tree nodes when ≥50%. GenBank accession numbers are provided in the parenthesis. The bar represents five or two estimated substitutions per 100 nucleotide positions.

4.2.4 Discussion

4.2.4.1 Authentication and symbiotic potential of isolates

In tropical soils, there is an enormous diversity of rhizobia with different nodulation capacities, which forms a natural reserve of germplasm for the selection of strains with desired characteristics (Dilworth *et al.*, 2001). When assessing the relationship between rhizobia and their host, infectivity and symbiotic effectiveness are the two essential features commonly considered (Brockwell, 1998). The symbioses between legumes and rhizobium must be effective for enhanced BNF and subsequent yield improvement to be realized. In this study, a preliminary screening for authentic rhizobia was performed using cowpea in growth pouches because this species is easier to grow under such conditions than the target species groundnut. The variation in numbers of infective isolates between cowpea and groundnut reflects differences in host-range among bacteria and host plant species and also confirms earlier studies that report that groundnut has a more restrictive nodulation pattern than cowpea (Thies *et al.*, 1991a). Nevertheless, the inoculation experiments described herein demonstrate that it was possible to obtain several effective rhizobia for groundnut inoculation, notably *Bradyrhizobium* sp. KNUST 1002, which caused increments in nodulation, shoot dry weight and amount of N fixed in both

experiments. The effectiveness of the symbiotic association between the infective isolates and their host in this study revealed varying effectiveness classes with some test isolates resulting in significantly higher SEI than the reference strains used. Useful variations in characteristics required in inoculant strains such as symbiotic effectiveness have also been observed within the natural pool of soil rhizobia (O'Hara *et al.*, 2002). Nitrogen fixation efficiency has been found to be diverse, ranging from symbiotic interactions leading to little or no nitrogen fixation, to those that obtain nitrogen in levels equivalent to or even greater than plants treated with mineral N (Terpolilli *et al.*, 2008). Similar results have been reported in other studies carried out to evaluate rhizobium cultures of various tropical legumes for their symbiotic capacity (Florentino *et al.*, 2010; Marra *et al.*, 2012). Additionally, the uneven distribution of effective isolates demonstrated in the principal component analysis (Supplementary Fig. S2) highlights a wide variation in terms of geographic distribution and symbiotic performance (Abaidoo *et al.*, 2007). In order to overcome sub-optimal N_2 fixation, the need arises to acquire rhizobium with high N_2 fixing ability that are also well adapted to the prevailing environment (Yates *et al.*, 2016). Estimation of the contribution of strains to nitrogen fixation has been based on methods such as N_2 balance, N_2 difference, ^{15}N natural abundance, ureide analyses, acetylene reductase assay, hydrogen evolution and ^{15}N isotope dilution method (Unkovich *et al.*, 2008). The ^{15}N isotope dilution method was employed in this study to quantify the amount of N_2 fixed for the selected effective isolates (experiment 2). Three reference plants were included in this experiment to estimate % Ndfa, because of the difficulty in directly determining which reference crop would accumulate N with the same ^{15}N enrichment as the legume crop. Boddey *et al.* (1995) thus recommended that several reference plants should be utilized to produce individual estimates of BNF contribution with the range of these estimates considered as an index of their accuracy. The lower ^{15}N enrichment recorded by inoculated plants implies that they received contributions of unlabeled N through BNF. On the other hand, the estimates of ^{15}N enrichments of the NN legume reference plants were extremely high and which could be due to their large seed N contents. Naegle *et al.* (2005) indicated that the response of seedlings' (of soybean) to available N is strongly related to available seed N resources. Since the seed N content of the NN reference plants were so high, and the availability of labelled soil N was so low, the results suggest that the N derived from the seed was considerably higher than 50%. The estimates

^{15}N enrichments for these two NN legume crops of approximately 0.19 to 0.20 atom % excess were far higher than that of the small-seeded sorghum and as such, it is thought impossible that these NN legumes obtained only half of their N from seed reserves. Therefore, to avoid over estimation of BNF, the proportion of N derived from the atmosphere was estimated using sorghum as the reference crop.

The insignificant differences observed for the various treatments in terms of the ^{15}N enrichment was not surprising since all the isolates selected for this experiment were potentially effective. The un-inoculated treatment resulted in the proportion of N derived from the atmosphere that was similar to all other treatments, but the amount of BNF contributed by the former was significantly lower. The results also show that, the proportion of plant accumulated N derived from BNF by inoculated plants ranged between 88 and 93%. The differences between the strains was apparent only in the total accumulated N and the total N derived from BNF. The *Bradyrhizobium* strain 32H1 was the best and most consistent reference strain in all parameters measured supporting the claim that it is an effective strain on groundnut (Urtz and Elkan, 1996). All isolates except KNUST 1005 performed similar to the reference strain BR 3267. The results from this study indicate the presence of indigenous rhizobia strains with highly effective symbiotic capacities that can be used as inoculants.

4.2.4.2 Genetic characterisation of effective isolates

Morpho-cultural characterisation of isolates used in this study indicated that they belong to the genera *Bradyrhizobium* and *Rhizobium* and this was confirmed by BLAST analysis of 16S rRNA gene sequences. Groundnut has been found previously to form associations with strains from the *Rhizobium* genus in addition to *Bradyrhizobium* symbionts (Van Rossum *et al.*, 1995; Urtz and Elkan, 1996; Yang *et al.*, 2005). Five out of seven isolates in this study belong to the *Bradyrhizobium* genus affirming the observation by several authors that bacteria associated with peanut are predominantly *Bradyrhizobium* (Van Rossum *et al.*, 1995; Zhang *et al.*, 1999; Yang *et al.*, 2005). The classification of novel species has been based on a polyphasic approach, which considers phenotypic and genetic characteristics (Vandamme *et al.*, 1996). This approach employs the sequencing of 16S rRNA gene as the backbone of genetic classification (Garrity and Holt, 2001). However, this gene has been found to be limited in delineating the diversity within *Bradyrhizobium* at the species level (Wang and Martínez-Romero, 2000) corroborating the findings in this

study where diversity within *Bradyrhizobium* isolates was not clearly defined with this gene. The *Rhizobium* isolates characterised in this study showed close relation to the *Rhizobium tropici* group (Dall'Agnol *et al.*, 2013). Strains belonging to this group are characterised with broad-host-range, high tolerance to environmental stress and genetic stability (Hungria *et al.*, 2000; Hungria *et al.*, 2003). This observation is interesting since the isolates in this study were obtained from areas with harsh environmental conditions. The analysis of concatenated 16S rRNA and ITS regions in this study revealed that the isolates shared more than 95.5% similarity to the *B. yuanmingense* strain CCBAU 1007^{T}; implying that these isolates belong to this species (Willems *et al.*, 2001). Willems *et al.* (2003) reported that the ITS gene sequencing and DNA-DNA hybridization shared a high correlation such that a sequence similarity of more than 95.5% shared by strains indicates that they belong to the same genospecies and have more than 60% DNA-DNA hybridization.

Symbiotic genes, on the other hand, have been found useful in the determination of host range, nodulation capacity and symbiovars between rhizobia and legumes (Rogel *et al.*, 2011). The observation from the analyses of symbiotic genes of isolates in this study revealed that some areas in northern Ghana harbour strains related to *B. yuanmingense* in addition to the already identified geographical origins of this species (So *et al.*, 1994; Vinuesa *et al.*, 2005b; Ormeno-Orrillo *et al.*, 2006; Gu *et al.*, 2007; Steenkamp *et al.*, 2008; Leite *et al.*, 2017). This suggests that strains of this species may be widely distributed in nature. Although *nifH* phylogeny is characterised by lateral gene transfer related to the host (Vinuesa *et al.*, 2005a), isolates that effectively nodulated groundnut in this study did not necessarily show any close relation with typical groundnut symbionts such as *Bradyrhizobium arachidis* (Wang *et al.*, 2013), *Bradyrhizobium subterraneum* (Grönemeyer *et al.*, 2015) and *Bradyrhizobium vignae* (Grönemeyer *et al.*, 2016). This observation may be because effective isolates used in this study were originally obtained from nodules of cowpea plants. To this end, *B. yuanmingense* has been confirmed to be an important micro-symbiont of groundnut as reported previously in other studies (Gu *et al.*, 2007; Steenkamp *et al.*, 2008; Leite *et al.*, 2017). Strains of the *B. yuanmingense* species have been identified to possess superior nitrogen fixation abilities due to the presence of efficient nitrogen fixing genes (Sarr *et al.*, 2009).

4.2.5 Conclusion

Increased nodulation, biomass production and N accumulation in soil-grown groundnut were achieved after inoculating with native rhizobium strains of northern Ghana. Among the isolates tested, KNUST 1002 was highly effective, performing similar to the groundnut reference strain 32H1. Apart from two *Rhizobium* isolates (KNUST 1003 and 1007); all the strains selected in this study were closely related to *B. yuanmingense*, confirming this species as a major micro-symbiont of groundnut.

4.3 Can native *Bradyrhizobium* isolates from Ghana enhance grain yields of fieldgrown cowpea and groundnut?

Abstract

Two elite *Bradyrhizobium* isolates from Ghanaian soils have previously been identified as potential inoculant strains based on their symbiotic potential under controlled conditions. However, their field performance in improving yields of cultivated legumes is yet to be established. This study was designed to evaluate the symbiotic potential of elite *Bradyrhizobium* isolates in improving cowpea and groundnut yields in multi-location trails. The experiment was set up in 26 locations in the Northern region of Ghana. Four treatments were considered at each location: two elite isolates (KNUST 1002 and KNUST 1006), a positive (+N) control and an absolute control (without nitrogen or inoculation) arranged in randomized complete block design with four replications. Grain yields for both cowpea and groundnut varied significantly at the different locations for the treatments based on the results of the analysis of variance. Analysis of the contribution of each factor (i.e. treatment and location) to the observed variation revealed that location contributed significantly higher proportion of variation in the data compared to the treatments for both crops. The treatment view of the GGE biplot analysis revealed isolate KNUST 1002 to be the best strain for cowpea, producing consistently higher average grain yields (1056 kg ha^{-1}) than the other treatments. Inoculation with isolate KNUST 1006 resulted in relatively consistent higher average grain yields for groundnut (1234 kg ha^{-1}) than isolate KNUST 1002. The location evaluation view of the biplot showed that most of the locations were suitable for assessing the effectiveness of the elite isolates for use as inoculants. The

which-won-where biplot analysis made it possible to identify the best treatment for each location based on the grouping of the study locations into megaenvironments. The results from this study revealed that isolate KNUST 1006 and KNUST 1002 improved yield of groundnut and cowpea, respectively and can thus, be recommended for local inoculant production. Furthermore, better yield responses can be achieved based on location-specific recommendation and use of identified elite isolates from this study.

Keywords: Native isolates, multiple location trials, GGE biplot, site-specific response.

4.3.1 Introduction

Cowpea and groundnut form a vital part of the traditional cropping system particularly in the northern part of Ghana due to their rich protein contents (Ajeigbe *et al.*, 2012). Other advantages of these crops are their contribution to soil fertility improvement and assistance in reducing the incidence of pest and diseases (Graham and Vance, 2000). Until recently, legumes were cultivated as secondary crops to cereals by most smallholder farmers and as such, received little or no fertilizer (Naab *et al.*, 2009). Farmers nowadays cultivate these legumes particularly cowpea and groundnut on a large scale as cash crops but still without the application of adequate quantities of fertilizer. It is also evident that, yields obtained are just a fraction of their maximum potential. Although legumes are characterized by the ability to partially or fully meet their nitrogen (N) needs through the biological nitrogen fixation (BNF) (Hungria and Kaschuk, 2014), the process is often limited by nutrient deficiencies in soils particularly phosphorus (P). Such nutrient deficiencies in soils are predominant in the legume growing areas of the savanna zones of Ghana. For the past decade, several approaches have been recommended as means to improve BNF and subsequently yields of legumes. Among these approaches include the use of high yielding varieties (Okogun *et al.*, 2005; Buruchara *et al.*, 2011), phosphorus (P)-based fertilizers (Kamara *et al.*, 2007; Kamanga *et al.*, 2010; Kolawole, 2012), rhizobia inoculants (Sanginga *et al.*, 2000; Osunde *et al.*, 2003; Thuita *et al.*, 2012) or a combination of these approaches (Ndakidemi *et al.*, 2006; Ronner *et al.*, 2016). The International Institute of Tropical Agriculture (IITA) bred promiscuous legumes particularly soybeans with the aim of improving BNF (Vanlauwe and Giller, 2006). Consequently, smallholder farmers in Zimbabwe and South Africa for instance, preferred these promiscuous varieties to the high yielding ones due to limited access to inoculants (Mpepereki *et al.*, 2000; Musiyiwa *et al.*,

2005). However, the performance of promiscuous soybeans in terms of nodulation and N_2 fixation has been erratic due to the diverse nature of native rhizobia present in soils (Mpepereki *et al.*, 2000; Sanginga, 2003). In effect, it was concluded that, to obtain a promiscuous soybean variety that nodulates in all environments was impossible. Hence, the need for rhizobium inoculation remains paramount.

Recent studies that employed the use of rhizobia inoculants in combination with phosphorus fertilizers revealed clearly that this approach results in increased yields of legumes (Ronner *et al.*, 2016; Ulzen *et al.*, 2016; Kyei-Boahen *et al.*, 2017). However, the use of rhizobia inoculants by smallholder farmers in Ghana is very low owing to limited access. Until recently, there were no facilities in the region to produce and distribute inoculants, as such all the inoculants available on the market were imported. This made it impossible to meet farmers' demands at the right time. In addition, the quality of imported inoculants are variable (Brockwell and Bottomley, 1995; Singleton *et al.*, 1997) which have resulted in inconsistent responses. Native rhizobia on the other hand also pose a competition barrier against introduced strains precluding inoculation responses in some cases (McInnes and Haq, 2007).

For the reason that all strains used in inoculant formulations originate from indigenous pools, isolation and characterization to identify elite local strains, will offer an opportunity to improve BNF. Studies that focused on the characterization of indigenous rhizobia have revealed them as important sources of inoculant strains that are able to improve nodulation and N_2 fixation in grain legumes (Fening and Danso, 2002; Bogino *et al.*, 2006; Ampomah *et al.*, 2008; Grönemeyer *et al.*, 2014). Furthermore, effective native strains when identified will serve as an import source of local inoculants that will better enhance BNF and subsequently yields of target legumes in the local region compared to exotic strains. Native strains with better performance will help address in part, the skepticism associated with the use of exotic inoculant strains.

This study thus aimed to assess the symbiotic potential of elite native strains to improve groundnut and cowpea yields in multiple environments. Identification of elite strain(s) that can effectively nodulate with target legumes will serve as resources for inoculant production and use by farmers.

4.3.2 Materials and Methods

4.3.2.1 General description of study locations

Trials were conducted during the 2016 cropping season in two districts in the Northern region of Ghana. The region falls within the Guinea savannah agro ecological zone with a unimodal rainfall pattern and an annual rainfall distribution of 1000 – 1200 mm. Mean temperature for the area ranges from 26 to 30 ºC with slight variations within a year. Twenty-six locations with no known history of recent legume cultivation and inoculation were selected for the field trials. The geographical positions and soil types identified across the study locations are presented in Table 4.3.1

Table 4.3.1. Description and GPS coordinates of study locations

District	Community	ID	Latitude	Longitude	Alt(m)	Soil type	Planting date
Cowpea							
Savelugu	Sankpem	L1	N09º38'56.5"	W000º45'03.5"	155	Acrisols	22/ 07/16
Savelugu	JegunKukuo	L2	N09º39'11.0"	W000º41'51.9"	129	Acrisols	05/ 08/16
Karaga	Karaga	L3	N09º56'36.4"	W000º26'20.3"	184	Lixisols	01/ 08/16
Karaga	Kunang	L4	N09º54'57.4"	W000º37'44.5"	147	Plinthosols	29/07/16
Karaga	Nangunkpang 1	L5	N09º58'58.6"	W000º25'55.4"	161	Lixisols	28/07/16
Karaga	Nangunkpang 2	L6	N09º59'40.7"	W000º26'24.6"	160	Lixisols	24/ 07/16
Karaga	Nangunkpang 3	L7	N09º59'15.6"	W000º26'23.4"	153	Lixisols	25/ 07/16
Karaga	NangunNayilli	L8	N09º57'15.9"	W000º27'19.1"	183	Planosols	28/ 07/16
Savelugu	Nanton-Kurugu	L9	N09º37'18.3"	W000º44'00.1"	140	Planosols	22/ 07/16
Savelugu	Nyeko	L10	N09º40'50.4"	W000º39'57.3"	184	Acrisols	07/ 08/16
Savelugu	Nyeko Kukusoli	L11	N09º41'42.1"	W000º39'03.3"	175	Lixisols	07/ 08/16
Karaga	Pishegu	L12	N09º57'14.2"	W000º37'36.4"	158	Plinthosols	21/ 07/16
Groundnut							

Savelugu	Nanton-Kurugu	L1 N09°37'00.8" W000°44'53.0"	149 Acrisols	14/ 07/16
Savelugu	Zoggu	L2 N09°40'47.4" W000°40'56.6"	178 Acrisols	15/ 07/16
Karaga	Karaga	L3 N09°56'36.4" W000°26'20.3"	184 Lixisols	01/ 08/16
Karaga	NangunNayilli	L4 N09°57'15.9" W000°27'19.1"	183 Planosols	02/ 08/16
Savelugu	Sankpem	L5 N09°38'56.5" W000°45'03.5"	155 Acrisols	12/ 07/16
Savelugu	Nyeko D	L6 N09°41'36.3" W000°39'07.3"	187 Lixisols	07/ 08/16
Savelugu	Nyeko B	L7 N09°42'52.4" W000°40'13.3"	169 Planosols	15/ 07/16
Karaga	Nangunkpang 3	L8 N09°59'15.6" W000°26'23.4"	153 Acrisols	26/ 08/16
Karaga	Nangunkpang 1	L9 N09°58'46.6" W000°25'51.3"	167 Planosols	23/ 08/16
Karaga	Nangunkpang 2	L10 N09°58'58.6" W000°25'55.4"	161 Plinthosol	25/ 08/16
Karaga	Pishegu	L11 N09°58'19.0" W000°39'41.7"	161 Plinthosols	08/ 07/16
Savelugu	Batanyilli	L12 N09°37'37.7" W000°43'30.7"	122 Acrisols	12/ 07/16
Karaga	Kunang	L13 N09°56'22.1" W000°37'22.9"	153 Planosols	16/ 07/16
Karaga	Kunang 2	L14 N09°55'26.6" W000°37'28.8"	151 Plinthosols	14/ 07/16

4.3.2.2 Physico-chemical analyses of soils from the study locations

Prior to establishment of trials, eight soil core samples were taken and thoroughly mixed to form a composite for each of the fields. A portion of the composite sample for each field was air-dried, sieved through a 2 mm wire mesh and kept for physico-chemical analyses. The hydrometer method by Bouyoucos (1962) was used to determine soil texture. Soil pH was measured in a 1: 2.5 (w/v) soil: water suspension with a pH meter (Jenway 3510, England), organic carbon and total N were determined by the modified Walkley and Black method described by Nelson and Sommers (1982) and the Kjeldhal method by Bremner and Mulvaney (1982), respectively. Available phosphorus was extracted using the Bray's No 1 solution (Bray and Kurtz, 1945). Exchangeable cations were extracted using the procedure by Black (1965).

4.3.2.3 Most probable number estimate of indigenous soil rhizobia

Rhizobia population in each composite soil sample was estimated using the most probable number (MPN) method described by Vincent (1970) with a cowpea variety (Asontem) as the trap host. Cowpea seeds of equal radicle length, which were pre-germinated under aseptic conditions, were transferred into growth pouches (Mega International, USA). Growth pouches with healthy seedlings were selected and inoculated with serial dilutions of the soil sample a week after transplanting. The soil dilutions were prepared using the six steps, five-fold serial dilution approach. One-milliliter aliquot of each dilution was used to inoculate the seedlings with four replicates. The setup was monitored for 28 days after which the most probable number of rhizobia in each soil was assessed using the MPNES software (Woomer *et al.*, 1990).

4.3.2.4 Preparation of planting materials

For the groundnut trials, Chinese groundnut variety was used while the cowpea variety (Songotra) was used for the cowpea experiments. Two elite isolates (KNUST 1002 and 1006) identified from preliminary controlled experiments based on their symbiotic effectiveness (Osei *et al.*, 2018) were processed into inoculant formulations. The isolates were cultured in yeast mannitol broth (YMB) until the late logarithm phase and used to inoculate gamma-radiated peat. Fifty milliliters of each culture was introduced into 50 g peat aseptically and sealed. The inoculated peat was gently massaged for even distribution of inoculum and incubated at 28 ^{o}C for 2 weeks to cure and reach a cell concentration of at least 10^{8} CFU g peat^{-1} (Somasegaran and Hoben, 2012).

4.3.2.5 Field preparation and imposition of treatments

Experimental fields were ploughed, harrowed and laid out in a randomized complete block design with four replications. The treatments were: i) KNUST 1002, ii) KNUST 1006, iii) +N (i.e. un- inoculated with applied N in the form of urea) and iv) –N (i.e. without ininoculation or applied N). Each plot measured 5 m x 5 m spaced by 1 m alleys between blocks and treatments. The inoculants were seed applied and planted at a spacing of 50 cm x 20 cm for both cowpea and groundnut. Prior to planting, seeds were coated with gum Arabic solution, followed by 5 g of either of the inoculant strains. The seeds were mixed

gently and air-dried for 30 minutes before planting. Each treatment received a basal application of 30 kg P ha^{-1}. One hundred kg N ha^{-1} in the form of urea was applied as the +N treatment and was split during the application; 20 kg N ha^{-1} at two weeks after planting and the remaining 80 kg N ha^{-1} at 50 % flowering (R_2 growth state).

4.3.2.6 Harvesting and data collection

Ten plants in each plot were randomly selected at R_2 growth stage (Fehr *et al.*, 1971) for nodulation assessment. This was done by carefully digging around and uprooting the plants using a spade. Plant roots were then washed carefully using clean water to remove all adhering soil and debris after which nodules were detached and counted. The nodules were oven dried at 65 ^oC for 72 h for dry weight determination. Pods of both groundnut and cowpea were harvested at full maturity (R_8 stage) (Fehr *et al.*, 1971). In the case of cowpea, pods were dried to constant weight, threshed and winnowed for grain yield determination. Grain yield of groundnut was determined unshelled.

4.3.2.7 Statistical analyses

Since two different legume hosts (groundnut and cowpea) were used, separate analyses were done for each crop. The data from all the locations were pooled together and subjected to analysis of variance (ANOVA) using SISVAR (Ferreira, 2008). The ANOVA model included treatment, location and their interaction. Significant mean differences were separated using Scott Knott method at 5 % probability. Treatment by location interaction was further evaluated using the GGE biplot function in GEA-R (Genotype x Environment Analysis with R for windows) software version 4.0 (Pacheco *et al.*, 2015). The analysis considered mean yields of the treatment tested at the 26 locations (i.e. 12 for cowpea and 14 for groundnut). The GGE biplot shows the first two principal component (PC) derived from subjecting location-centred yield data to singular value decomposition (SVD). Through SVD, treatment by location (T x L) matrix of mean yield were approximated as the product of treatment and location matrices. This was displayed as T x L means with T points for treatment and L points for locations (Yan *et al.*, 2000). In order to compare the performance of the various treatments and the response at the different locations, the genotype-focused and environment focused scaling respectively were used. Symmetric scaling was used to visualize the which-won-where pattern of the multi-locational yield

data (Yan, 2002). A linear mixed model regression using R software version 3.3.2 (R Core Team, 2017) was performed to explain the variability in yields based on the interaction between soil type and treatments.

4.3.3 Results

4.3.3.1 Soil chemical properties

The soils from all the study locations were slightly acidic with pH ranging from 5.5 to 6.8. Soil organic carbon and total N contents of the study locations ranged from 0.03% to 1.8% and 0.03 to 0.14% respectively on average and were considered very low. The available phosphorus values averaged 5.8 mg kg^{-1} (5.3 – 6.2 mg kg^{-1}) and 5.7 mg kg^{-1} (5.3 – 5.9 mg kg^{-1}) for locations under the Karaga and Savelugu districts, respectively and were considered low. Native rhizobia population of soils from the study locations averaged 57 and 126 rhizobia cells g^{-1} of soil for Karaga and Savelugu districts, respectively. In general, the fertility status of the soils considered were low (Table 4.3.2) based on the description of Landon (2014).

Table 4.3.2. Average soil chemical properties at study locations

	Karaga (n = 15)		Savelugu (n = 11)	
Parameter	Mean	Range	Mean	Range
pH (1:2.5) (H_2O)	6.1 ± 0.08* a	5.5 – 6.8	6.0 ± 0.04 a	5.5 – 6.8
Organic carbon (%)	0.7 ± 0.08a	0.2 – 1.8	0.5 ± 0.03 b	0.03 – 0.8
Total N (%)	0.08 ± 0.004a	0.05 – 0.12	0.07 ± 0.006 a	0.03 – 0.14
Available P (mg kg^{-1})	5.8 ± 0.06a	5.3 – 6.2	5.7± 0.03 a	5.3 – 5.9
Exchangeable K ($cmol_{(+)}$ kg^{-1})	0.10 ± 0.006a	0.08 – 0.2	0.10 ± 0.003 a	0.08 – 0.2
Exchangeable Ca ($cmol_{(+)}$ kg^{-1})	4.8 ± 0.14a	2.6 – 7.0	4.0 ± 0.12 b	2.4 – 5.8
Exchangeable Mg ($cmol_{(+)}$ kg^{-1})	1.3 ± 0.08b	0.56 – 2.6	1.6 ± 0.10 a	0.4 – 2.6
Exchangeable Na ($cmol_{(+)}$ kg^{-1})	0.04 ± 0.004b	0.02 – 0.08	0.07 ± 0.03 a	0.007 –0.08
IRP[a] cells g of $soil^{-1}$	75(26.2 – 217.6)†	11 - 218	126(43.7 – 363.4)	24 - 273

Different lowercase letters within a row indicate significant differences ($p < 0.05$) by Scott-Knott test. IRP; Indigenous rhizobia population [a] Most probable number method. [*] Standard error of mean.[†] Values in parentheses are confidence intervals.

4.3.3.2 Rainfall distribution during the cropping period

The daily amounts of rainfall received at the study sites are presented in Appendix 9. The amount of rainfall a day before or at planting varied at the study locations for both cowpea and groundnut. For the cowpea study locations, the rainfall amount at planting was 9 mm or more for only four out of the 12 sites considered. At the time of planting of groundnut, the amount of rainfall recorded was 11 mm or more for four out of the 14 study locations.

4.3.3.3 Response of target legumes to treatments

In Tables 4.3.3a and b, analysis of variance of data from this study revealed that different treatments produced the highest grain yields (referred to as crossover TLI) at the various locations (i.e. significant treatment by location interaction, TLI prevailed). For instance, on cowpea, isolates KNUST 1002 and KNUST 1006 produced significantly highest yields at L11 and L3 respectively, (Table 4.3.3a). Groundnut yields also followed a similar trend with isolate KNUST 1002 producing significantly higher yield at L5 while KNUST 1006 produced significantly higher yield at L13 (Table 4.3.3b). Nevertheless, some treatments produced the best yields across a number of locations (referred to as non-cross over TLI) for both cowpea and groundnut. Isolate KNUST 1002 produced the largest cowpea yields at locations 1, 2 and 4 whereas isolate KNUST 1006 produced highest yields at L6 and L7. Treatment with +N resulted in the largest cowpea yields at L5 and L9 (Table 4.3.3a). On groundnut, inoculation with isolate KNUST 1002 resulted in significantly highest yields at L3 and L10 while isolate KNUST 1006 produced the largest yields at location L1, L8 and L12 (Table 4.3.3b).

Table 4.3.3a. Grain yield response of cowpea to treatments at the different study locations

	Grain yield (kg ha^{-1})				
Location/Treatment	KNUST 1002	KNUST 1006	N+	N	Mean
L1	1213^{cA}	1125^{cA}	1053^{dA}	821^{bB}	1053^{c}
L2	1330^{bA}	1005^{cB}	760^{eC}	693^{bC}	947^{d}

L3	1553^{aB}	1720^{aA}	1305^{bC}	493^{cD}	1268^{b}
L4	1130^{cA}	1100^{cA}	950^{dB}	835^{bB}	1004^{d}
L5	515^{eB}	438^{eB}	665^{eA}	175^{eC}	448^{g}
L6	465^{eB}	805^{dA}	425^{fB}	325^{dB}	505^{f}
L7	685^{dA}	803^{dA}	675^{eA}	325^{dB}	622^{e}
L8	503^{eA}	410^{eA}	500^{fA}	200^{eB}	403^{g}
L9	1080^{cB}	838^{dC}	1227^{cA}	740^{bC}	971^{d}
L10	1453^{aA}	1280^{bB}	1375^{bA}	1173^{aB}	1320^{b}
L11	1563^{aA}	1250^{bB}	1535^{aA}	1229^{aB}	1394^{a}
L12	1180^{cA}	1060^{cB}	1210^{cA}	760^{bC}	1053^{c}
Mean	1056^{A}	986^{B}	973^{B}	647^{C}	
CV (%)		10.1			

Means followed by different uppercase letters within columns (treatment) and lowercase letters within rows (locations) are significantly different ($p< 0.05$) based on Scott-Knott test. Details of study locations are shown in Table 1.

Table 4.3.3b. Grain yield response of groundnut to treatments at the different study locations

Location/Treatment	KNUST1002	KNUST1006	N+	N	Mean
L1	1000^{fB}	1455^{bA}	1075^{dB}	1000^{dB}	1133^{e}
L2	1213^{eA}	1289^{cA}	1294^{cA}	960^{dB}	1189^{e}
L3	595^{gA}	590^{eA}	580^{eA}	305^{fB}	518^{h}
L4	515^{gB}	495^{eB}	670^{eA}	410^{fB}	523^{h}
L5	2175^{aA}	1829^{aB}	1809^{aB}	1665^{aC}	1870^{a}
L6	1050^{fB}	1355^{bA}	1295^{cA}	1385^{bA}	1271^{d}
L7	1220^{eA}	1165^{cA}	1330^{cA}	1210^{cA}	1231^{d}
L8	1295^{eA}	1305^{cA}	1135^{dB}	930^{dC}	1166^{e}
L9	585^{gA}	590^{eA}	630^{eA}	605^{eA}	603^{g}
L10	1575^{cA}	1370^{bB}	1390^{bB}	1510^{bA}	1461^{c}
L11	930^{fA}	775^{dB}	1023^{dA}	880^{dB}	902^{f}
L12	1420^{dB}	1874^{aA}	1510^{bB}	1470^{bB}	1569^{b}
L13	1860^{bA}	1935^{aA}	1508^{bB}	1105^{cC}	1602^{b}
L14	1230^{eA}	1245^{cA}	1110^{dA}	1165^{cA}	1188^{e}

Mean	1190[B]	1234[A]	1168[B]	1043[C]
CV (%)		**18.7**		

Means followed by different uppercase letters within columns (treatment) and lowercase letters within rows (locations) are significantly different ($p < 0.05$) based on Scott-Knott test. Details of study locations are shown in Table 1.

The general contribution of treatment (T), location (L) and their interaction (TL) are presented in Table 4.3.4. Considering the relative contribution of each source to the total variation (T + L + TL), it was observed that location was the most important source of variation in cowpea and groundnut yields. For cowpea, location accounted for 70.9% of the variation in grain yield. Treatment (T) and its interaction with location (TL) accounted for 16.3% and 12.8%, respectively. The data for groundnut on the other hand showed that, location contributed 87.7% of the variation observed while the contribution of T and TL were 2.5% and 9.8%, respectively.

Table 4.3.4. Combined ANOVA for grain yield at the study locations for two cropping seasons

Legume host	Source	df	SS	Explained (%)
Cowpea	Treatment (T)	3	4794892*	16.3
	Location (L)	11	20822792*	70.9
	T x L	33	37629556*	12.8
	Residuals	144	1225188	
Groundnut				
	Treatment (T)	3	981812*	2.5
	Location (L)	13	34582158*	87.7
	T x L	39	3872460*	9.8
	Residuals	168	2219295	

* Significant at $p < 0.05$

4.3.3.4 Identifying the best treatment in each location

The observed crossover and non-crossover TLI suggest that the study locations may be divided into groups called mega-environments. Mega-environment is explained as a group of locations that consistently share the best set of treatment(s). The which-won-where function of GGE biplot was used to delineate mega-environments in this study. For the cowpea locations, four mega-environments were identified. The +N treatment produced high yields in the locations within the first mega-environment. Locations L1, L8, L10 and L12 formed the second mega-environment with isolate KNUST 1002 producing high yields (Figure 4.3.1). In the third mega-environment, isolate KNUST 1006 produced high yields at L2, L3, L4, L6 and L7. Similarly, in the fourth mega-environment the –N treatment produced high yields but no location was found in this sector. This indicates that the all the locations responded to treatments with either inoculation or N fertilization.

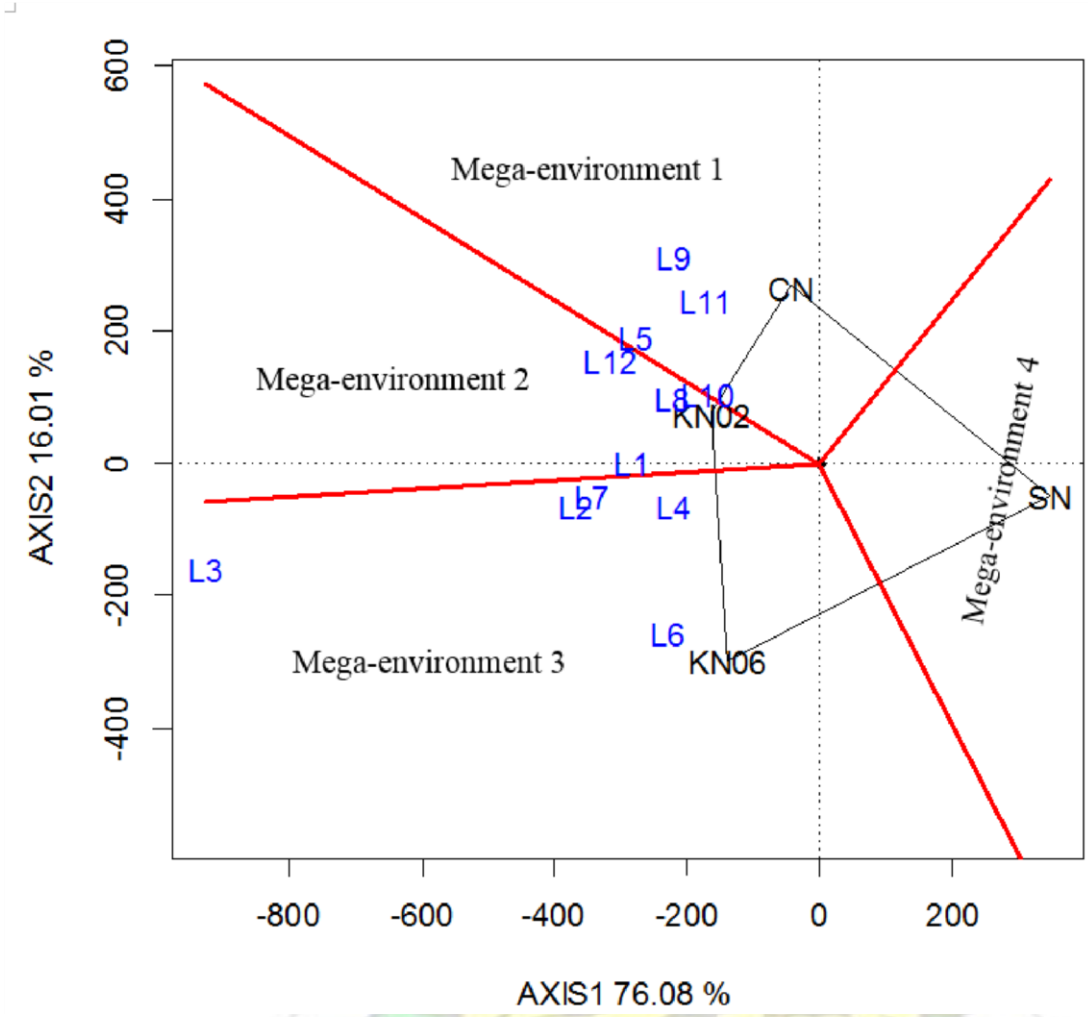


Figure 4.3.1. GGE biplot of the which-won-where pattern showing which treatment was the best at which location for cowpea

KN02, KN06, CN and SN represent isolate KNUST 1002, KNUST 1006, positive nitrogen and absolute controls, respectively and L1-L12 represent study locations 1 to 12, respectively. Details of study locations are shown in Table 1.

The lines that divide the biplot into sectors for the groundnut data delineated three megaenvironments. The first mega-environment comprised six locations with isolate KNUST 1006 producing the highest yield (i.e. the treatment at the polygon vertex) for these locations. The second mega-environment comprised only L6 with the negative control treatment recording the highest yield at this location. Seven locations fell in the third mega-environment with isolate KNUST 1002 producing higher yields, followed by the +N control treatment (Figure 4.3.2).

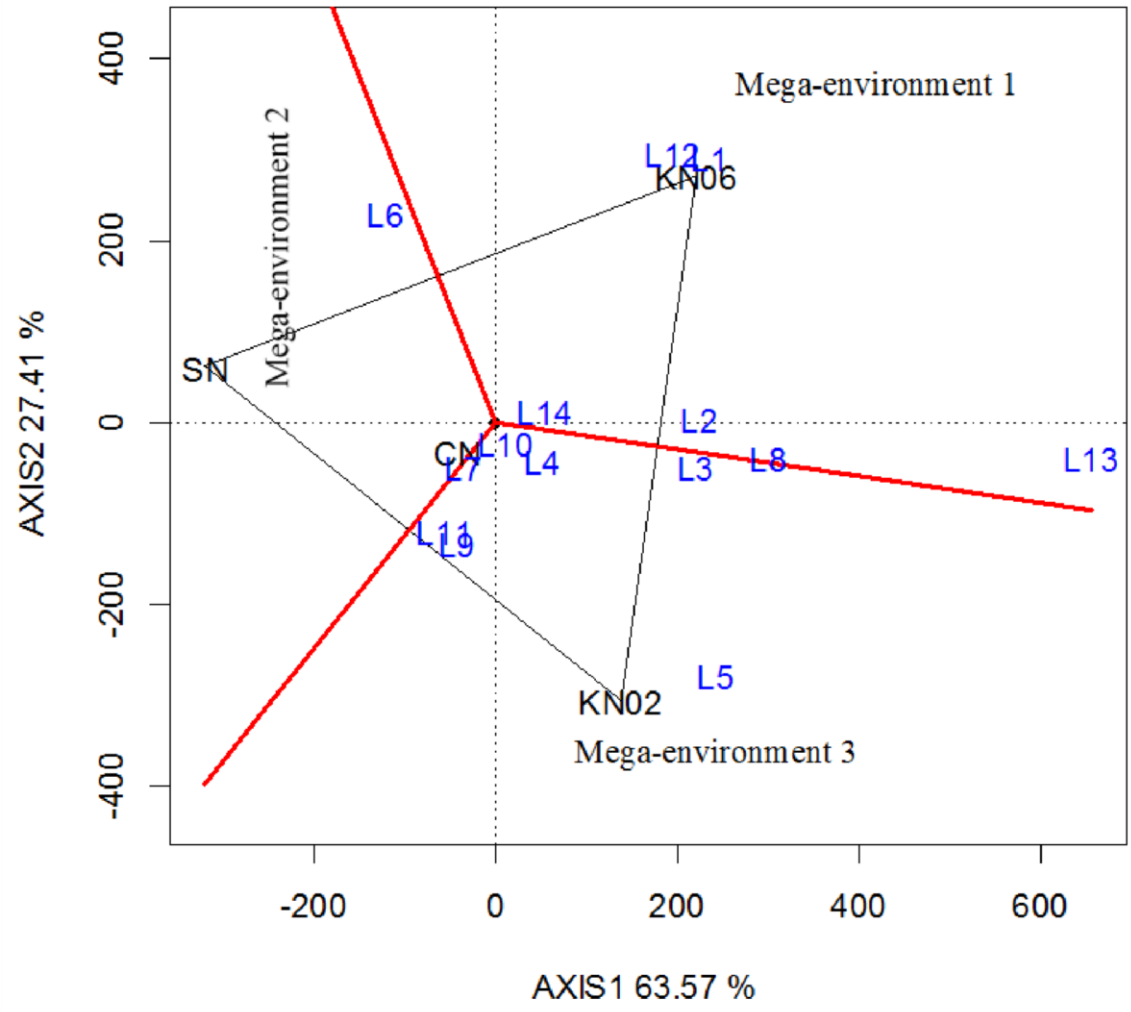


Figure 4.3.2. GGE biplot of the which-won-where pattern showing which treatment was the best at which location for groundnut.

KN02, KN06, CN and SN represent isolate KNUST 1002, KNUST 1006, positive nitrogen and absolute controls, respectively and L1-L14 represent study locations 1 to 14, respectively. Details of study locations are shown in Table 1.

4.3.3.5 Mean performance and consistency of treatments across study locations on cowpea and groundnut (Treatment evaluation)

The first two principal components (PC) explained 92.1% of the variation in grain yield for cowpea (Figure 4.3.3). The average scores of the two PCs of all locations is considered as the average location score. Thus, based on the average environment coordination

(AEC), the yield performance and consistency of treatments were evaluated. An average environment axis (AEA) which serves as an abscissa of the AEC was drawn, which pointed to the direction of the treatment with the highest main effect. Greater treatment by location interaction (TLI), reduced consistency and treatments with below average means were identified using the AEC ordinate constructed perpendicular to the AEA. To illustrate this, above average mean grain yields for cowpea were observed for isolate KNUST 1002, followed by KNUST 1006 and +N control. The absolute control plots produced below average grain yields. Isolate KNUST 1002 and the absolute control treatment had short projections to the AEC ordinate (Figure 4.3.3). This indicates a high consistency in performance of treatments across the study locations.

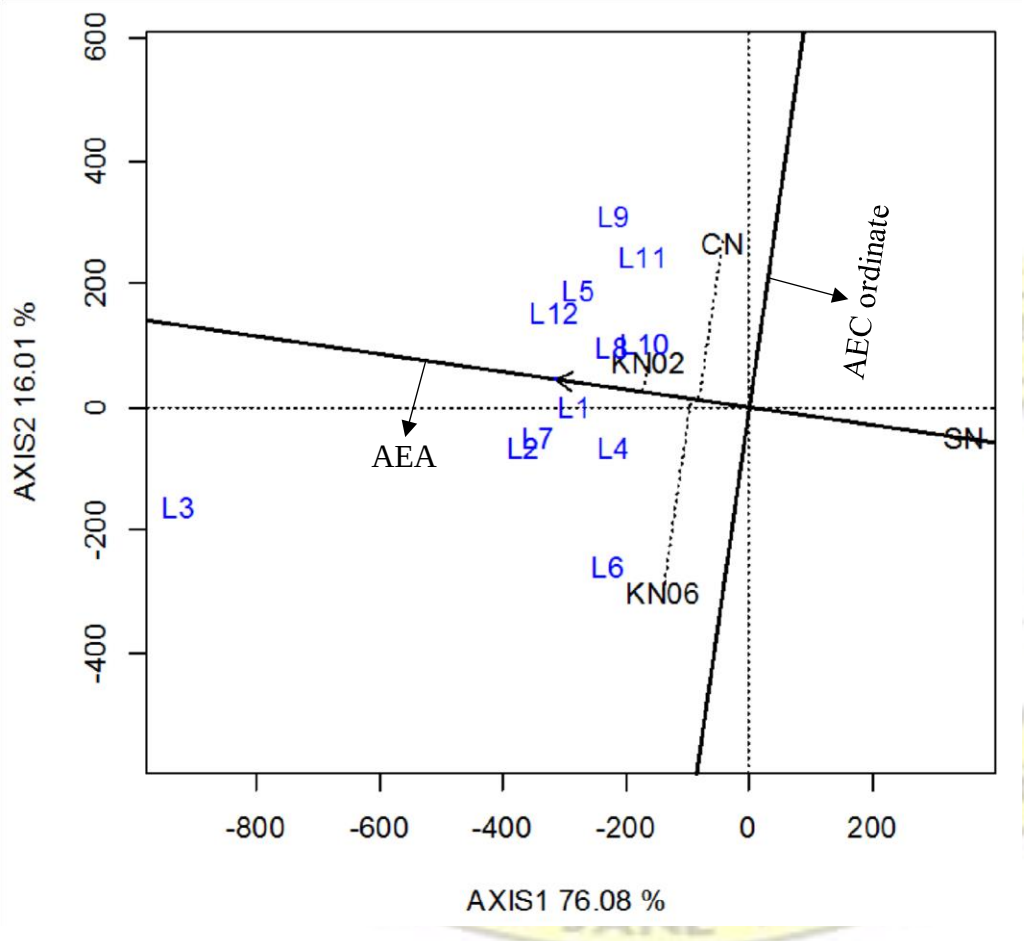


Figure 4.3.3. GGE biplot based on the treatment-focused evaluation of the variability in cowpea yields

KN02, KN06, CN and SN represent isolate KNUST 1002, KNUST 1006, positive nitrogen and absolute controls, respectively and L1-L12 represent study locations 1 to 12, respectively. Details of study locations are shown in Table 1.

To identify the best treatment, all the four treatments considered in this study were compared to an ideal treatment (i.e. a treatment that produced the highest yields across all environments) (Figure 4.3.4). Such an ideal treatment is defined as having the greatest vector length (i.e. high yield) on the AEA and the shortest projection on the AEC ordinate (i.e. zero or reduced TLI). Concentric circles drawn to visualize the distance between each treatment and the ideal treatment showed that isolate KNUST 1002 was closer to the ideal treatment (i.e. at the centre of the concentric circles) than all the other treatments for cowpea (Figure 4.3.4)

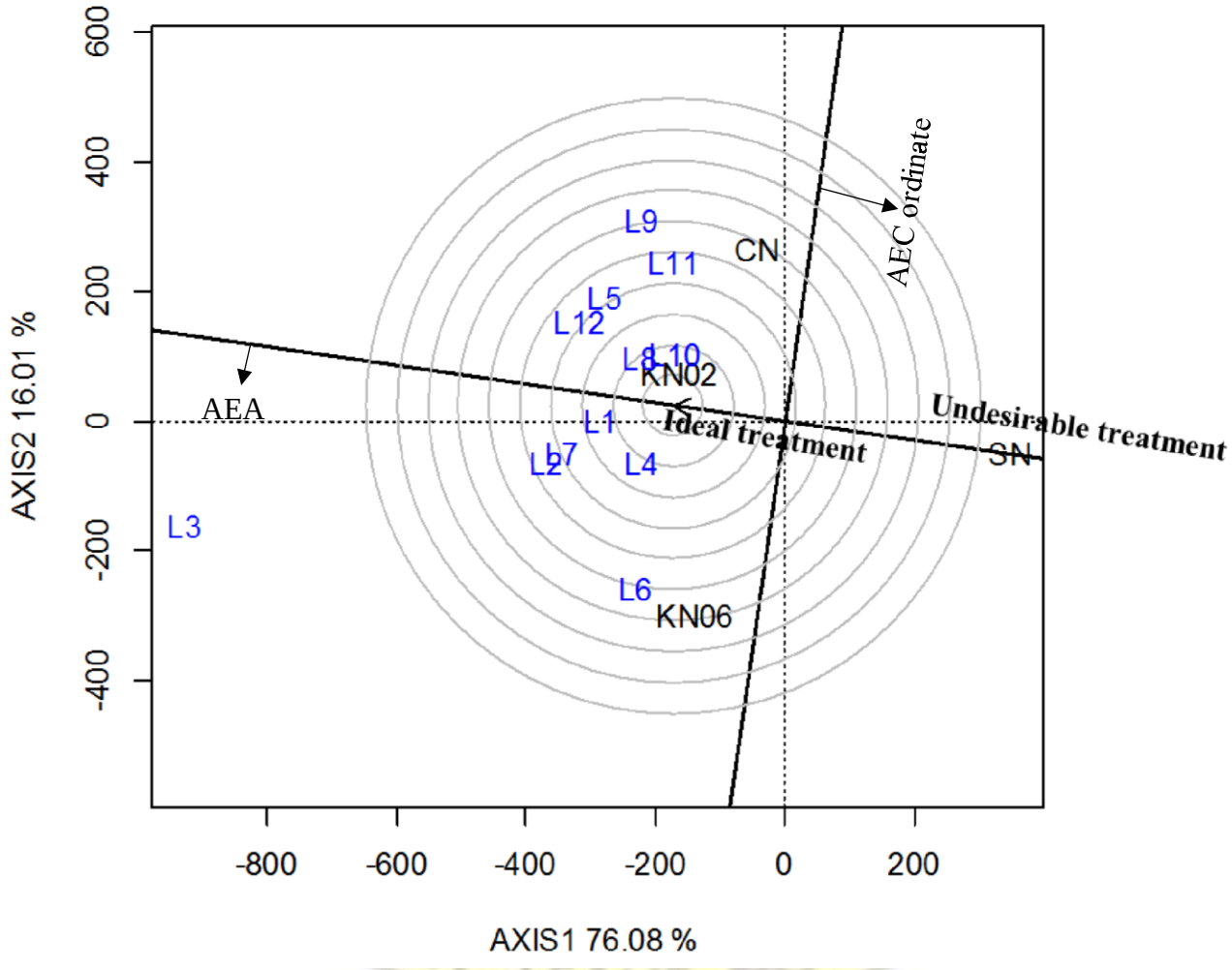


Figure 4.3.4. GGE biplot based on treatment-focused evaluation for comparing treatment performance on cowpea to an ideal treatment

KN02, KN06, CN and SN represent isolate KNUST 1002, KNUST 1006, positive nitrogen and absolute controls, respectively and L1-L12 represent study locations 1 to 12, respectively. Details of study locations are shown in Table 1.

The treatment-focused view for the groundnut data showed that inoculation with isolates KNUST 1006 and KNUST 1002 resulted in above average groundnut yields while the positive and negative controls produced below average yields. Based on the projection of mean yields of each treatment on the AEC ordinate, the positive and negative control treatments produced consistent yields across the study locations (indicated by the short projections) (Figure 4.3.5). The inoculated treatments on the other hand, had longer projections on the AEC ordinate with isolate KNUST 1002 producing relatively inconsistent yields (indicated by a longer projection). With reference to an ideal treatment, isolate KNUST 1006 was identified as a better treatment compared with the others since it was relatively closer to the centre of the concentric circles (ideal treatment) (Figure 4.3.6).

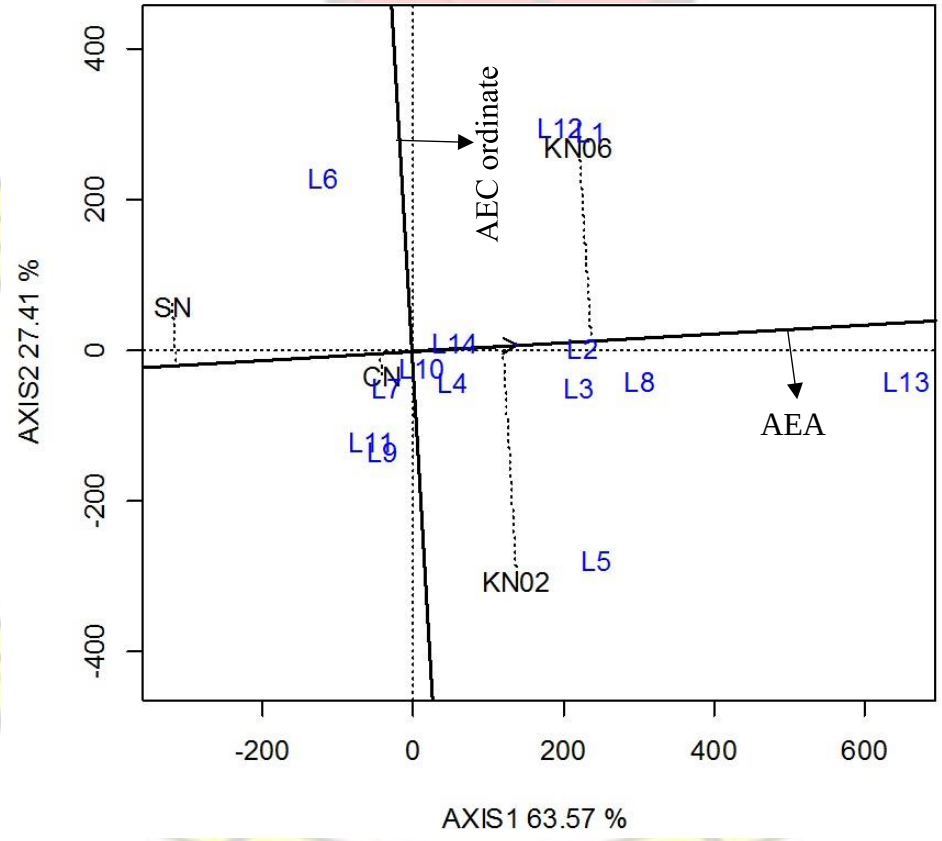


Figure 4.3.5. GGE biplot based on the treatment-focused evaluation of the variability in groundnut yields.

KN02, KN06, CN and SN represent isolate KNUST 1002, KNUST 1006, positive nitrogen and absolute controls, respectively and L1-L14 represent study locations 1 to 14, respectively. Details of study locations are shown in Table 1.

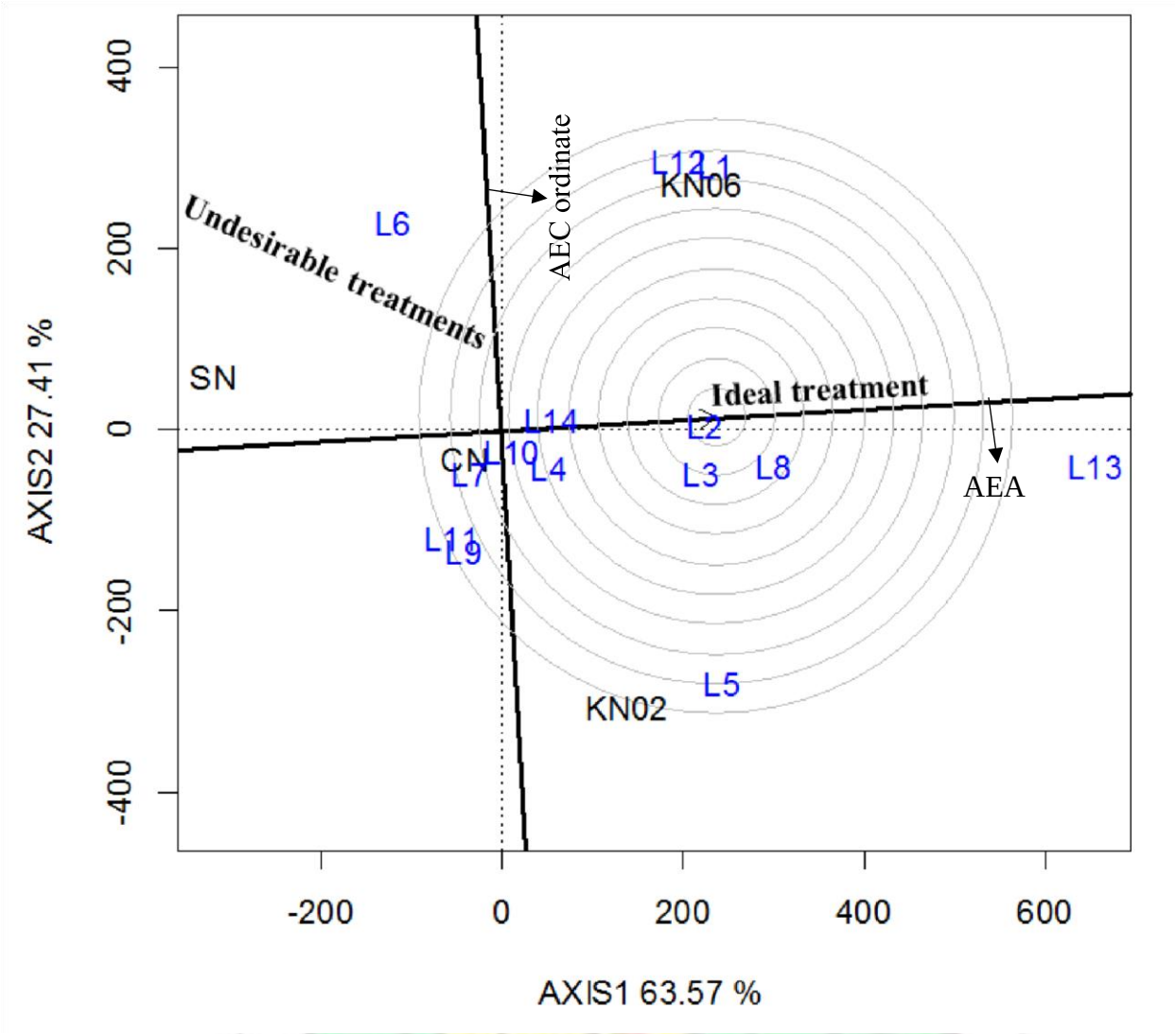


Figure 4.3.6. GGE biplot based on treatment-focused evaluation for comparing treatment performance on groundnut to an ideal treatment

KN02, KN06, CN and SN represent isolate KNUST 1002, KNUST 1006, positive nitrogen and absolute controls, respectively and L1-L14 represent study locations 1 to 14, respectively. Details of study locations are shown in Table 1.

4.3.3.6 Identifying favorable locations for selected potential inoculant strains for cowpea and groundnut (Location evaluation)

The first PC in the location-focused view of the GGE biplot drawn using the cowpea data had only negative scores indicating that the grain yield differences for each treatment was proportional across locations (i.e. non-crossover TLI) . Scores for the PC2 axis on the other

hand were both positive and negative. This means that the significant yield differences between the treatments occurred across locations on this axis (i.e. crossover TLI). The location view biplot also shows the interrelationship among the study locations. With the exception of L6, L9 and L11, all the locations were positively correlated, having acute angles between their vectors (Figure 4.3.7).

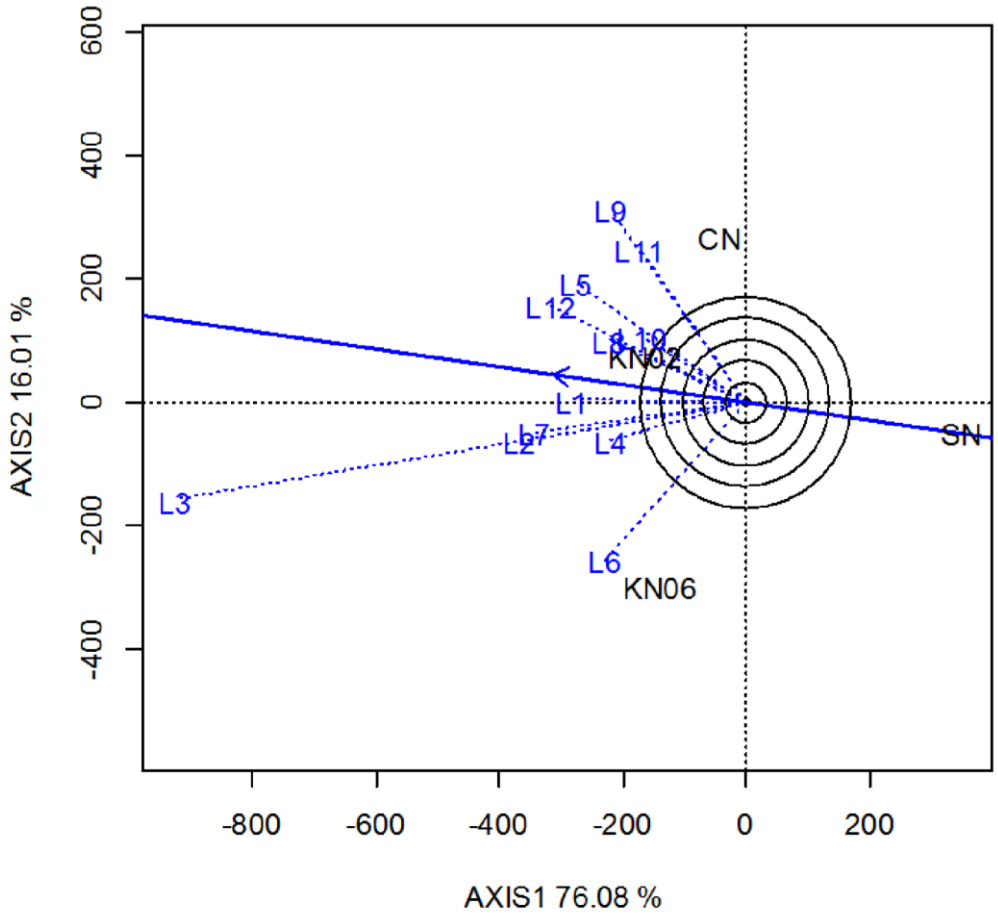


Figure 4.3.7. GGE biplot based on location-focused evaluation of the cowpea study locations

KN02, KN06, CN and SN represent isolate KNUST 1002, KNUST 1006, positive nitrogen and absolute controls, respectively and L1-L12 represent study locations 1 to 12, respectively. Details of study locations are shown in Table 1.

The association between locations was also shown in Table 4.3.5a. The highest correlation coefficient was between L3 and L7 and was as depicted in Figure 4.3.7. Forty out of the 66 correlations were found to be significant. To identify the most favorable location(s) for selecting the best strain, mean yields of each of the study locations were compared to an ideal location (i.e. a location with the ability to distinguish between the best and the worse treatments and representative of the average environment). The results in Figure 4.3.8

shows that L3 was the most favorable location (located at the centre of the concentric circles) followed by L2, L7 and L12. Treatment performance varied significantly at all the favorable locations (Table 4.3.3)

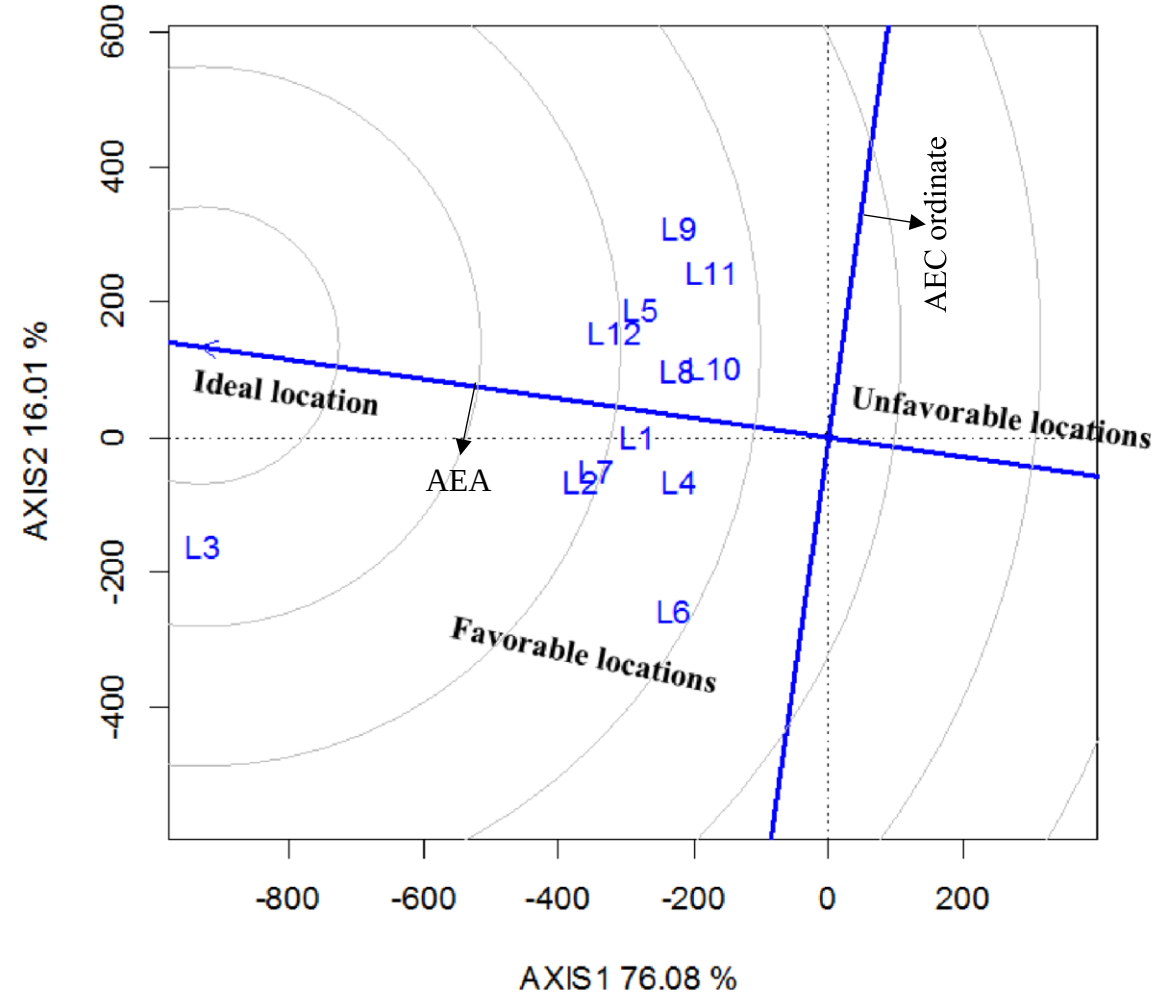


Figure 4.3.8. GGE biplot based on location-focused evaluation for comparing study locations to an ideal location

L1-L12 represent study locations 1 to 12. Details of study locations are shown in Table 1.

Table 4.3.5a. Correlation coefficient among cowpea study locations

Location	L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	L11	L12
L1	1.00											
L2	0.75*	1.00										
L3	0.88*	0.64*	1.00									
L4	0.66*	0.69*	0.63*	1.00								
L5	0.67*	0.31	0.65*	0.40	1.00							
L6	0.50*	0.32	0.74*	0.45	0.16	1.00						
L7	0.79*	0.49*	0.91*	0.52*	0.67*	0.73*	1.00					
L8	0.73*	0.48*	0.74*	0.38	0.77*	0.33	0.77*	1.00				
L9	0.50*	0.22	0.38	0.18	0.86*	-0.16	0.48	0.75*	1.00			
L10	0.67*	0.48*	0.55*	0.33	0.62*	0.030	0.38	0.61*	0.50*	1.00		
L11	0.58*	0.40	0.38	0.34	0.75*	-0.25	0.31	0.72*	0.78*	0.73*	1.00	
L12	0.66*	0.37	0.61*	0.36	0.68*	0.31	0.61*	0.85*	0.57*	0.57*	0.69*	1.00

* indicate significance at $p< 0.05$, Figures without asterisk are not significant at $p< 0.05$. L1-L12 represent study locations 1 to 12. Details of study locations are shown in Table 1.

For groundnut, the location vector view showed that PC1 and PC2 had both positive and negative scores. This indicates significant yield differences across locations (i.e. crossover TLI) (Figure 4.3.9). The figure also shows a succinct summary of the interrelationship between the 14 groundnut study locations. The angle between the vectors of two locations gives the correlation coefficient between them. Based on this explanation, majority of the locations were considered to be positively correlated. Table 4.4.5b shows the correlation coefficients among the study locations. It was observed that, L1 significantly correlated with L11 and L12, while L2 correlated significantly with L3 and L13. Furthermore, L3 correlated significantly with L4, L8 and L13. The highest coefficient was found between L3 and L13. However, Figure 4.3.9 showed that L13 closely correlated with L2 and L8. Ranking the locations with reference to an ideal location revealed L13 to be the most favorable location (located at the centre of the concentric circles), followed by L1, L2, L3, L5, L8 and L12 (Figure 4.3.10). Significant differences between treatments were clearly seen at the favorable locations (Table 4.3.3).

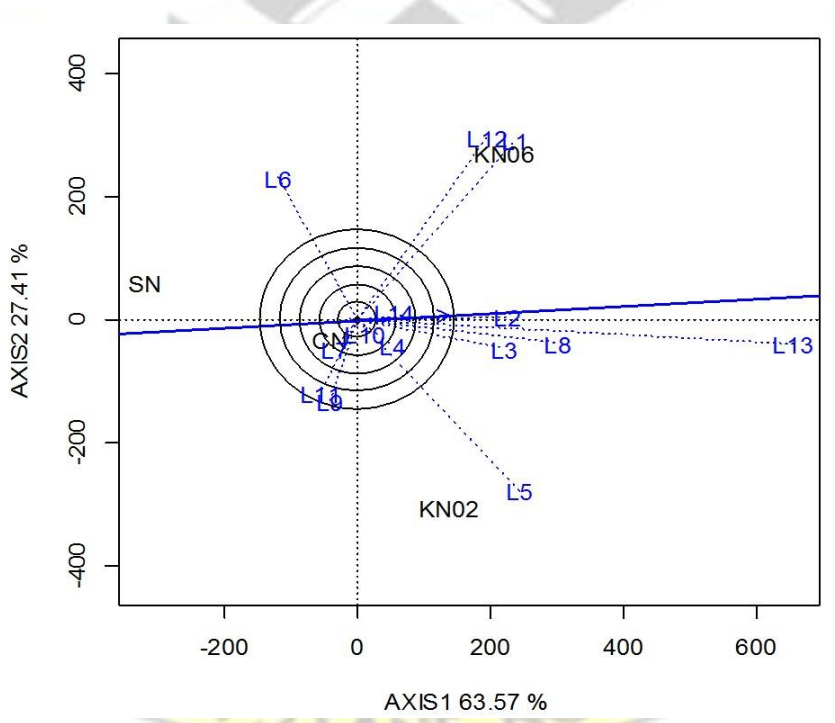


Figure 4.3.9. GGE biplot based on location-focused evaluation of the cowpea study locations.

KN02, KN06, CN and SN represent isolate KNUST 1002, KNUST 1006, positive nitrogen and absolute controls, respectively and L1-L12 represent study locations 1 to 12 respectively. Details of study locations are shown in Table 1.

Table 4.3.5b. Correlation coefficient among groundnut study locations

Location	L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	L11	L12	L13	L14
L1	1.00													
L2	0.31	1.00												
L3	0.32	0.55*	1.00											
L4	-0.13	0.47	0.56*	1.00										
L5	-0.09	0.32	0.45	0.12	1.00									
L6	0.35	-0.26	-0.38	-0.23	-0.59*	1.00								
L7	-0.28	-0.02	0.17	0.55*	-0.24	-0.19	1.00							
L8	0.44	0.46	0.75*	0.22	0.41	-0.45	0.14	1.00						
L9	-0.37	-0.38	-0.11	-0.14	0.24	-0.33	0.28	-0.13	1.00					
L10	0.12	-0.05	-0.07	0.36	-0.18	0.15	0.22	-0.02	0.11	1.00				
L11	-0.63*	0.10	0.18	0.66*	0.12	-0.38	0.49*	-0.24	0.26	0.16	1.00			
L12	0.73*	0.39	0.26	-0.14	-0.15	0.42	-0.33	0.26	-0.36	-0.22	-0.44	1.00		
L13	0.45	0.53	0.82	0.22	0.51*	-0.32	-0.06	0.76*	-0.01	-0.14	-0.26	0.38	1.00	
L14	0.28	0.34*	0.17*	-0.33	0.22	-0.22	-0.53*	0.22	0.08	-0.15	-0.28	0.34	0.36	1.00

* indicate significance at $p < 0.05$, Figures without asterisk are not significant at $p < 0.05$. L1-L14 represent study locations 1 to 14. Details of study locations are shown in Table 1.

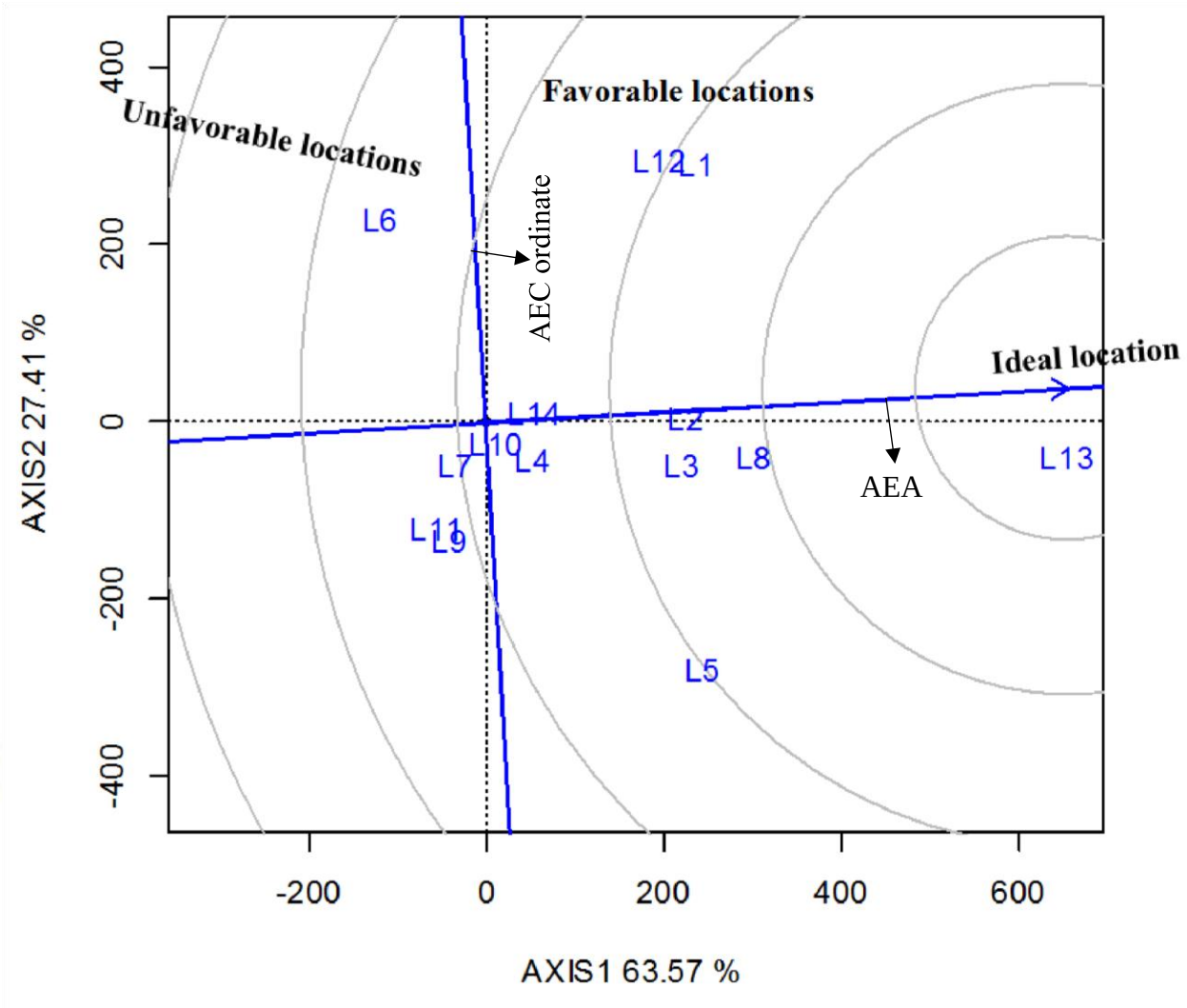


Figure 4.3.10. GGE biplot based on location-focused evaluation for comparing study locations to an ideal location

L1-L14 represent study locations 1 to 14. Details of study locations are shown in Table 1.

4.3.3.7 Variability in yield responses to treatments at the various location

The variability in grain yields observed at the different study locations was explained by constructing a linear mixed model based on the interaction between treatment and the identified soil types. For cowpea, the model could be used to predict (i.e. p = 3.452e-09) only 26% of the observed yield variation. For instance, inoculation of cowpea with either of the test isolates on Acrisols resulted in a significant positive effect on grain yield. Significantly, low yield was observed on Lixisols for the treatment without inoculation or fertilization (i.e. -N treatment). For groundnut, 22% of the variation in yields was

significantly explained by the linear model equation. The yields of groundnut on the different soil types following treatment application showed a similar pattern as that observed in cowpea. The application of treatments on Acrisols resulted in significant positive effect on groundnut yields. (Table 4.3.6).

Table 4.3.6. Explanatory variables for the variability in cowpea and groundnut yields at the different study locations

Coefficients	Estimate	Standard error	t value	Pr (>|t|)
Cowpea				
(Intercept)	797.50	121.46	6.57	5.61e-10***
Soil type_Acrisols:+N	264.67	156.81	1.69	0.093
Soi ltype_Lixisols:+N	123.50	143.72	0.86	0.39
Soil type_Planosols:+N	66.00	171.78	0.38	0.70
Soil type_Plinthosols:+N	282.50	171.78	1.65	0.10
Soil type_Acrisols: KNUST 1002	534.17	156.81	3.41	0.00082***
Soil type_Lixisols: KNUST 1002	158.50	143.72	1.10	0.27
Soil type_Planosols: KNUST 1002	-6.25	171.78	-0.036	0.97
Soil type_Plinthosols: KNUST 1002	357.50	171.78	2.081	0.039*
Soil type_Acrisols: KNUST 1006	339.17	156.81	2.16	0.032*
Soil type_Lixisols: KNUST 1006	205.40	143.72	1.43	0.155
Soil type_Planosols: KNUST 1006	173.75	171.78	-1.011	0.31
Soil type_Plinthosols: KNUST 1006	282.50	171.78	1.65	0.10
Soil type_Acrisols: -N	97.83	156.81	0.62	0.53
Soil type_Lixisols: -N	-288.30	143.72	-2.01	0.046*
Soil type_Planosols: -N	-327.50	171.78	-1.91	0.058
Adjusted R-squared: 0.26 F-statistic: 5.55 on 15 and 176 DF *p-value*: 3.45e-09				
Groundnut				
(Intercept)	883.33	109.70	8.052	6.20e-14***
Soil type_Acrisols: +N	538.67	145.12	3.71	0.00026***
Soil type_Lixisols: +N	120.00	155.14	0.774	0.44
Soil type_Planosols:+N	341.04	145.12	2.350	0.020*
Soil type_Plinthosols: +N	37.50	155.14	0.24	0.81
Soil type_Acrisols: KNUST 1002	568.54	145.12	3.92	0.00012***
Soil type_Lixisols: KNUST 1002	96.67	155.14	0.62	0.53
Soil type_Planosols: KNUST 1002	409.17	145.12	2.82	0.0053**
Soil type_Plinthosols: KNUST 1002	31.67	155.14	0.20	0.84
Soil type_Acrisols: KNUST 1006	728.04	145.12	5.017	1.12e-06***
Soil type_Lixisols: KNUST 1006	200.00	155.14	1.289	0.198769
Soil type_Planosols: KNUST 1006	357.92	145.12	2.47	0.014*
Soil type_Plinthosols: KNUST 1006	-13.33	155.14	-0.086	0.93
Soil type_Acrisols:-N	390.42	145.12	2.69	0.0077**
Soil type_Lixisols:-N	-10.00	155.14	-0.064	0.95

Soil type_Planosols:-N	175.42	145.12	1.209	0.228118
Adjusted R-squared: 0.23 F-statistic: 5.36 on 15 and 208 DF *p-value*: 4.33e-09				

Significant levels: *p< 0.05, **p< 0.01, and ***p< 0.001

4.3.4 Discussion

Efficient exploration of BNF in order to enhance grain yields of legumes can be achieved via the use of effective native rhizobia as inoculants. Currently, no local strain has been recommended for inoculant production and use by cowpea and groundnut farmers in Ghana despite various research efforts made to address this issue (Fening and Danso, 2002; Ampomah *et al.*, 2008; Osei, 2015) as has been the case in some countries (Hungria *et al.*, 2000; Mostasso *et al.*, 2002). An important characteristic considered during the selection of elite rhizobia strains is the preference for the capacity to perform well under a wide range of field conditions (Rodríguez-Navarro *et al.*, 2011). However, variability in strain performance do occur, leading to variable yield responses. The significant yield differences between treatments observed at the various locations is not uncommon and may be attributed to inherent variations in the isolates nitrogen fixing abilities and in the fertility status of soils of the study locations. This result is in agreement with reports from the Nif-TAL group that inoculation responses could be highly variable and site specific (Singleton *et al.*, 1992; Date, 2000). Thus, for a better assessment of the site specific performance of elite native isolates at the different study locations, GGE biplot analysis was explored. Yan *et al.* (2007) identified genotype (G) (herein referred to as treatments) and genotype x environment (GE) interaction as the most relevant source of variation in a genotype and test environments evaluation. GGE biplot analysis has been used effectively in plant breeding to detect the interaction patterns between plant genotypes and the study environments and to delineate mega-environments among study sites (Yan and Tinker, 2006; Yan *et al.*, 2007; Rakshit *et al.*, 2012). However, this approach is yet to be employed in the analysis of multi-locational (ML) data from rhizobiology trials. The significance of this approach is in the fact that it reveals the differences in relative performance of treatments over locations, which is not possible by just considering mean yields based on ANOVA alone (Samonte *et al.*, 2005). In this study, the analyses of GEI between treatments across different locations revealed that location contributed 70.9 – 87.7% of the variation in the data for both legume hosts. The contribution of treatment (T) (2.5 - 16.3)

and its interaction with location (T x L) (9.8 – 12.8) on the other hand were lesser. These results agree with the report of Gauch and Zobel (1997); Kaya *et al.* (2006) and

Sabaghnia *et al.* (2008) who found that in multi-environment trial (MET) data, environment could account for over 80% of the total variation. The highly significant effect of location indicates large differences in the performance of a given treatment across the various study locations. However, some treatments produced the highest yields in two or more locations, which indicate non-cross over treatment by location interactions (TLI). Non-cross over TLI is an indication that the performance of treatments was consistent across some location with significant differences only in the magnitude of response. Rhizobial isolates that show non-cross over type of TLI are desirable since they can be recommended for inoculation in a wide range of environment. For general adaptation of genotypes in a genotype selection process, plant breeders recommend genotypes with noncross over GEI (Matus-Cadiz *et al.*, 2003). In this study, the differences in the magnitude of response can be attributed to the variability in populations and effectiveness of native soil rhizobia and the weather conditions at the various locations. For instance, the variability in amount of rainfall before or at planting, and planting and weeding dates can also influence the performance of introduced strains and the efficient use of applied N (+N treatment), which was the case in this study. Legumes particularly cowpea have been reported to grow well in soils with sufficient moisture (i.e. 15 mm and above) (Ajeigbe *et al.*, 2010). Since factors that affect the host also affects the micro-symbiont (Rhizobia) (Thies *et al.*, 1995), insufficient (below 15mm) amount of rainfall received at some of the locations affected the growth of both cowpea and groundnut resulting in the differences in yield responses to treatments (Tittonell *et al.*, 2008; Subedi and Ma, 2009). In addition, the linear regression model, which showed that interaction between soil type and treatment significantly explained 26% and 22% of the overall variability in grain yield of the target legumes, agrees with the finding of Falconnier *et al.* (2016). These authors opined that the soil type and its influence on soil nutrients affects crop response to treatments. For instance, differences in organic carbon content in the soils at the study locations may have resulted in difference in the metabolic activities of the test isolates thus influencing their competitiveness (Wielbo *et al.*, 2007), hence BNF and yields. Argaw (2017) reported similar results of variation in nodulation and plant N accumulation at different locations in Eastern Ethiopia following inoculation. The author in this case attributed the observed variation to environmental factors such as soil and weather conditions prevailing during the period of the experiment. Given that, the linear model could be used to predict only 26% and 22% of the variation in cowpea and groundnut yields respectively, there may be additional factors that influenced the variation in yields, which were not considered in this study. For instance, host plant identity, which influences competitiveness of strains (Wielbo *et al.*, 2012), may have played a significant role in the observed variation. For GGE biplot analysis to be used effectively in the interpretation of variability in multilocation trial data, the first two principal components (PC) and the combined (G + GL) effect must explain more than 60% and 10% of the (G + GL) variability in the data set, respectively (Yang *et al.*, 2009; Yan *et al.*, 2010). These conditions were met for the data in this study. From the GGE biplot for treatment evaluation, inoculation with isolate KNUST 1002 produced yields that were consistently higher than the overall average grain yields of cowpea. On the contrary, yield performance of this same isolate on groundnut was inconsistent and less than the overall average. Isolate KNUST 1006 on the other hand, produced higher yields on groundnut compared with cowpea though relatively inconsistent. These results therefore suggest that isolate KNUST 1002 was stable on cowpea while KNUST 1006 was stable on groundnut. A treatment is referred to as highly stable when it performs consistently better or worse in a measured parameter (Yan and Tinker, 2006). The consistently

higher average yields produced with isolate KNUST 1002 and KNUST 1006 on cowpea and groundnut, respectively suggest that these isolates could be recommended for inoculating the target legumes. Boddey *et al.* (2016); Ulzen *et al.* (2016); Martins *et al.* (2003) and Bogino *et al.* (2006) have also reported positive responses following inoculation of cowpea and groundnut but none of these considered the stability of the performance of the strains. The difference in performance of the test isolates on the different legume host may be attributed to differences in the compatibility between the host plant and rhizobia strain. Several authors have reported similar observations of variability in rhizobial strains performance on different legume genotypes (Tirichine *et al.*, 2000; Simsek *et al.*, 2007; Schumpp and Deakin, 2010).

In the GGE biplot analysis for location evaluation, a cosine of the angle between two vectors is an indication of their correlation (Yan and Tinker, 2006). Most of the locations considered in this study were found to be significantly correlated. Such significant correlations suggest that selection of the best treatment can be practical across locations

(Kaya *et al.*, 2006). For instance, the treatment that produced high cowpea yields at location L3 may also produce high yields at locations L1, L2, L4 and L7 (Yan and Tinker, 2006; Yan *et al.*, 2007; Rakshit *et al.*, 2012). Thus, for optimum use of scarce resources in selecting effective strains, a subgroup from locations that are closely related can be considered for further studies. Another important criterion considered during the selection of elite strains for inoculant production is the assessment of the need to inoculate the same field repeatedly. According to Herridge *et al.* (2002) inoculation is likely to increase yields under soil conditions such as where compatible native rhizobia are absent, where the population of compatible native rhizobia is too small to induce effective nodulation and where native rhizobia are ineffective in nitrogen fixation. Although the population of native rhizobia in soils of the study locations were on the average, relatively high (75 – 126 cell g soil-^{1}), grain yields at the identified favorable locations were improved following inoculation with test isolates. This observation gives an indication that the prevailing soil conditions at the identified suitable locations were probably similar to those outlined by Herridge *et al.* (2002). To this end, the most effective isolate could be selected based on the results from the suitable locations.

The study locations which were partitioned into mega-environments based on the whichwon-where view of the GGE biplot (Figures 4.3.1 and 4.3.2) with either the test isolates or the +N treatment producing the highest yield in each of the mega-environments implies that there was a need for inoculation or mineral N fertilizer in the study locations. However, in the mega-environment 2 in Figure 4.3.2 the negative control treatment (-N) produced the highest yield at L6. This observation may be attributed to the native rhizobia population of this location which exceeded the threshold of 50 cell g soil^{-1} reported by Thies *et al.* (1991b) thus precluding inoculation responses. A mega-environment is a term used to refer to a group of locations that consistently share the best set of treatment. Grouping of study locations into mega-environments serves as a basis for selecting specifically adapted treatments (test isolates) for each of the mega-environment. The

advantage of using GGE biplot lies in the ease of identifying the best strain and most favorable environments and also serves as a prerequisite for meaningful recommendation of treatments (Yan *et al.*, 2000) which would be difficult to determine using mean tables alone (Rakshit *et al.*, 2012).

4.3.5 Conclusion

Isolate KNUST 1002 produced consistently high yields when inoculated on cowpea while KNUST 1006 produced high yields on groundnut. The grouping of locations into megaenvironments, with different treatments producing high average yields in each megaenvironment suggests that location-specific recommendation and use of identified elite isolates can improve yields of target legumes.

4.4 Introduced effective native *Bradyrhizobium* improves grain yields of cowpea and groundnut and obviate the need for re-inoculation

Abstract

Bradyrhizobium isolates from soils in Ghana have been identified as candidates for local inoculant production. However, their ability to persist in soils to obviate the need for reinoculation is unknown. This study sought to evaluate the effectiveness and need for reinoculation of two previously selected native elite strains for field-grown cowpea and groundnut in Ghana. Ten sites previously inoculated with native elite isolates (KNUST 1002 and KNUST 1006) in 2016 were considered for this experiment (in 2017). Plots that were inoculated in the first cropping season (2016) were divided into two with one-half receiving re-inoculation with the same isolate from the previous year and the other half left un-inoculated in the second season (2017). This resulted in a split plot design with previous inoculation as the main plot factor and re-inoculation as the subplot factor plus two controls (i.e. plus nitrogen (+N) and without nitrogen or inoculation (-N)). The six resulting treatments were replicated four times. Estimation of rhizobium population prior to planting in 2017 showed that numbers of rhizobia in inoculated plots had increased considerably compared to the population prior to planting in 2016. On average, significant differences in grain yields were observed for the treatments on both legume hosts. Grain yield increases were significantly high with isolates KNUST 1006 and KNUST 1002 on groundnut and cowpea, respectively in both previously inoculated and re-inoculated plots. The yield increases in both target legumes on previously inoculated plots and reinoculated plots did not differ significantly ($p < 0.05$). Spatial variability in yields between locations was influenced by the difference in some measured parameters including soil type, most probably number of rhizobia and cumulative rainfall. The study revealed that inoculation with effective native isolates obviates the need for annual re-inoculation of the target legumes. Furthermore, better yield responses can be obtained when inoculation with effective isolates is targeted at locations with suitable soil and environmental conditions.

4.4.1 Introduction

Incorporating legumes like cowpea and groundnut benefits farming systems in diverse ways including the acquisition of renewable N through biological nitrogen fixation (BNF) (Aikins and Afuakwa, 2008; Yakubu *et al.*, 2010; Kermah *et al.*, 2018). Legumes are also

very important as sources of human and animals protein, vegetable oil and feed for livestock (Vance *et al.*, 2000; Graham and Vance, 2003). These have resulted in aggravated interest in the use of both native and exotic rhizobium strains as inoculants for enhanced exploration of the associated benefits particularly BNF. For example, several studies using imported rhizobium inoculants on crops like soybean and cowpea in Ghana reported improved yield responses (Asei *et al.*, 2015; Boddey *et al.*, 2016; Ulzen *et al.*, 2016). Nonetheless, the inoculant formulations of exotic origin have not always yielded the desired results (Brockwell *et al.*, 1995; Okogun and Sanginga, 2003; Chemining'wa *et al.*, 2007) partly due to their poor competitiveness against native rhizobia and limited adaptation to local environments (Streeter, 1994; Vlassak *et al.*, 1997). Such limited abilities of some exotic strains have stimulated the search for native elite strains as alternatives for effective nodulation and N_2 fixation (Fening and Danso, 2002; Bogino *et al.*, 2006; Ampomah *et al.*, 2008; Grönemeyer *et al.*, 2014). Nevertheless, it is not enough for an elite strain to form effective symbiosis; it must also be highly competitive in nodule occupancy (Triplett, 1990) and persistent in soils in the absence of the legume host (Date, 2000). A persistent strain may obviate the need for re-inoculation in subsequent season(s) (Zengeni *et al.*, 2006).

Unfortunately, there is paucity of information on the availability of elite native strains with such traits in Ghana even though several studies have been conducted in other countries that reported the persistence of strains in the field (Abaidoo *et al.*, 1990; Woomer *et al.*, 1992; Vlassak *et al.*, 1996). Recent studies have alluded to the potential of local biological resources to perform as good candidates for inoculant production. For example, Osei (2015), Adjei (2017) and Wilson (2017) studied the competitiveness and/or persistence of some native strains for soybean, cowpea and groundnut in Ghana under greenhouse conditions. It has however been argued that results from works conducted under controlled conditions may not reflect the actual performance of strains field situation (Pitkäjärvi *et al.*, 2003). The need therefore arises to evaluate the saprophytic competence of elite native isolates under field conditions.

Native rhizobia have the ability to persist in soils between growing seasons in the absence of the host due to their adaptation to prevailing environmental conditions. For instance, Leite *et al.* (2017) observed that the *Bradyrhizobium yuanmingense* strain BR 3267 had a

high tolerance to temperature, pH and NaCl characteristics that predict their adaptation to conditions of tropical soils. A recent study by Osei *et al.* (2018) identified some effective native isolates from soils of Ghana to be closely related to this *Bradyrhizobium yuanmingense* species. However, the saprophytic competence and competitiveness abilities of these isolates under field conditions are yet to be established. The significance of this research is realised in the fact that grain legumes like cowpea and groundnut are of high agricultural and nutritional value especially to resource poor farmers.

This study thus sought to evaluate the effectiveness and need for re-inoculation of two previously selected native elite strains for field-grown cowpea and groundnut in Ghana. The outcome of this study will serve as a basis for making decisions on whether or not to re-inoculate in subsequent seasons with such strains introduced as commercial inoculants.

4.4.2 Materials and Methods

4.4.2.1 Experimental site and design

In order to study the persistence of introduced native isolates after a year of inoculation, an extended study was carried out in 2017 on fields previously inoculated in 2016 (Section 4.3.2). It must be noted that not all the locations considered in 2016 were available for the current study because most of the farmers were of the notion that the fertility status of their lands had been improved by the 2016 treatments and hence wanted to benefit from this improvement by planting cereal crops. The current study was thus conducted in only ten available sites (5 previously planted to cowpea and the other 5 planted to groundnut). The sites were located in the Guinea Savannah agro ecological zone characterized by a unimodal rainfall pattern and a rainfall distribution of 1000 - 1200 mm per year. Physicochemical properties of the soils at the experimental sites were analysed and averages for all the sites were determined. The average soil pH (1:2.5) was 6.1, exchangeable Ca, Mg, K and Na were 5.0, 1.2, 0.10 and 0.05 $cmol_{(+)}$ kg^{-1} respectively following the method of Black (1965). Total nitrogen (Bremner and Mulvaney,1982), organic carbon (Nelson and Sommers, 1982); and available phosphorus (Olsen and Sommers, 1982) were 0.08%, 0.54% and 5.7 mg kg^{-1} P respectively. The soil type at the study locations in Kunang and Pishegu (two sites at each location) were identified as

Plinthosols while the soils at Nyeko 2, Nangun-Nayilli and Karaga were classified as Lixisols. The third soil type at Zoggu and NantonKurugu was identified as Acrisols.

In 2016, four treatments were considered and arranged in a randomized complete block design with and four replications. In 2017, the field layout for 2016 was maintained but each of the previously (2016) inoculated plots was divided into two. For each plot, the soil was loosened with a hoe. The hoe was sterilized with 70% alcohol intermittently to prevent cross contamination. Seeds planted in one-half of the plot were re-inoculated with the same treatment (isolate) from the previous year while those for the second half were sown without inoculation. This resulted in a split plot design plus two controls (i.e. the nitrogen treated plants and the un-inoculated and unfertilized treatment). The main plot factor was the bacterial treatment from the previous season and the sub plot factor was the re-inoculation (R) or no re-inoculation treatments. The control plots (i.e. with nitrogen and without nitrogen or inoculation) were also divided into two. The nitrogen treated plots in 2016 were re-fertilized in the current season.

4.4.2.2 Field preparation and soil analysis

The soils at each experimental site were loosened manually using hoes. This was done on treatment basis to prevent transferring the isolates applied in the previous season from one plot to another. The alleys between plots were raised to form ridges in order to reduce cross contamination of strains. Each plot measured 2 m x 5 m with 1 m alleys between plots and blocks. Necessary precautions were taken during field preparation, soil sampling, sowing, fertilization, weeding and harvesting to avoid cross contamination. Five core samples of soil were taken from each plot representing a particular treatment. Twenty (20) core samples for each treatment were bulked and mixed thoroughly to form a composite sample. Sub samples of each composite were taken to the laboratory for enumeration of rhizobia population as described by Vincent (1970).

4.4.2.3 Seed inoculation, planting and trial management

Peat based inoculants for each isolate (KNUST 1002 and 1006) were prepared by inoculating gamma radiated peat (50 g bag^{-1}) with 50 mL broth cultures of each isolate. Broth cultures were prepared by incubating yeast mannitol broth inoculated with pure

cultures of each isolate in an orbital shaker until the late logarithm phase. The number of viable cells in each peat-based inoculant was estimated to be 6.12 x 10^8 and 6.50 x 10^8 CFU g $peat^{-1}$ respectively for KNUST 1002 and KNUST 1006. One kilogram of seeds (cowpea or groundnut) was inoculated with 5 g of inoculant for each isolate. The inoculation was done using the two-step method in which Gum Arabic solution was applied as sticker followed by the application of the inoculant. Both inoculated and uninoculated seeds were planted at a rate of two seeds per hill and a spacing of 50 cm x 20 cm. Chinese (groundnut) and Padi-tuya (cowpea) varieties were used as test hosts. Each treatment received a basal application of 30 kg P ha^{-1} in the form of triple super phosphate (TSP). The nitrogen treated plots in the previous year were re-fertilized with 100 kg N ha^1 in the form of urea in the current study. The urea was split applied; 20 kg N ha^{-1} at two weeks after planting and the remaining 80 kg N ha^{-1} at 50 % flowering (R_2 growth state) for both cowpea (Fehr *et al.*, 1971) and groundnut (Boote, 1982).

4.4.2.4 Enumeration of rhizobia population

Numbers of rhizobia in soils and inoculants were estimated using MPN method by Vincent (1970) and the surface drop plate method by Miles *et al.* (1938), respectively. Cowpea seeds used as the trap host for the MPN counts were sterilized (Somasegaran and Hoben, 2012) and pre-germinated on moistened tissue at 28 ^oC. Pre-germinated seeds were transferred into plastic growth pouches (Mega International, USA) filled with N-free nutrient solution (Broughton and Dilworth, 1970). After a week of transplanting, seedlings were inoculated with five-fold serial dilutions of soils with each dilution level replicated four times. The presence or absence of nodules was assessed 28 days after inoculation and the MPNES software (Woomer *et al.*, 1990) was used to assign the rhizobium population in soils. For the plate counts, broth cultures of each isolate at the late logarithm phase were serially diluted using the 10-fold dilution steps. Twenty microliters of three high dilution levels (10^{-5}, 10^{-6} and 10^{-7}) were deposited onto yeast mannitol agar (YMA) plates and allowed to dry. The plates were incubated at 28 ^oC for 8 days after which colonies were counted using the colony counter and cells numbers estimated as colony forming units per milliliter (CFU ml^{-1}).

4.4.2.5 Data collection and harvesting

At the R_2 growth stage (50% flowering), 10 plants from each treatment were sampled by carefully cutting the shoots at about 5 cm above soil level. The roots of plants were carefully dug out, collected into sealable plastic bags and transported to the laboratory. The roots were washed carefully under running tap water to remove adhering soil and debris. Nodules were then detached and oven dried at 65 ^{o}C for 72 h in order to estimate their dry masses. At full maturity (R_8) (Fehr *et al.*, 1971), grain yields for cowpea and pod yields for groundnut were estimated after drying to a constant weight.

4.4.2.6 Statistical Analysis

Prior to the analyses, data were checked for normal distribution using residual plots in JMP® Pro 13.0.0 (SAS Institute Inc., Cary, NC, 1989-2007). Preliminary analysis of the data revealed a significant interaction for the previously inoculation and re-inoculation subplots (i.e. treatments in 2017); hence, separate models were fitted for the previously inoculated and re-inoculated subplots. A linear mixed model was fitted to the data on the measured growth and yield parameters using the fit model platform based on the Restricted Maximum Likelihood (REML) method in JMP®. In the mixed model, treatment was considered a fixed factor and location as random factor. Treatment means for groundnut and cowpea data were separated by lsmeans with Tukey HSD and student's t test, respectively. Orthogonal contrast analysis was used for direct comparison of pairs of treatments that were not significantly different in the main analysis. T-test was used to compare the performance of test isolates across previously inoculated and re-inoculated plots. GG plot in R software was used to determine the distribution of responses to individual treatments in the various locations (R core team, 2017).

4.4.3 Results

4.4.3.1 Rhizobia population estimates

Prior to planting in the 2016 cropping season, the population of rhizobia in soils ranged between $\log_{10}$ 1.1 to $\log_{10}$ 2.2 cells g soil^{-1}. However, by the second planting season (i.e. after the land had been left fallow), population of rhizobium in the control and treated plots in almost all the study locations had doubled and in some cases tripled ($\log_{10}$ 1.6 to $\log_{10}$

2.5 cell g soil^{-1}). The increases in population following inoculation in the previous season ranged between 1.1 to 1.9 folds for isolate KNUST 1002 and 1.2 to 1.9 for isolate KNUST 1006 (Figure 4.4.1).

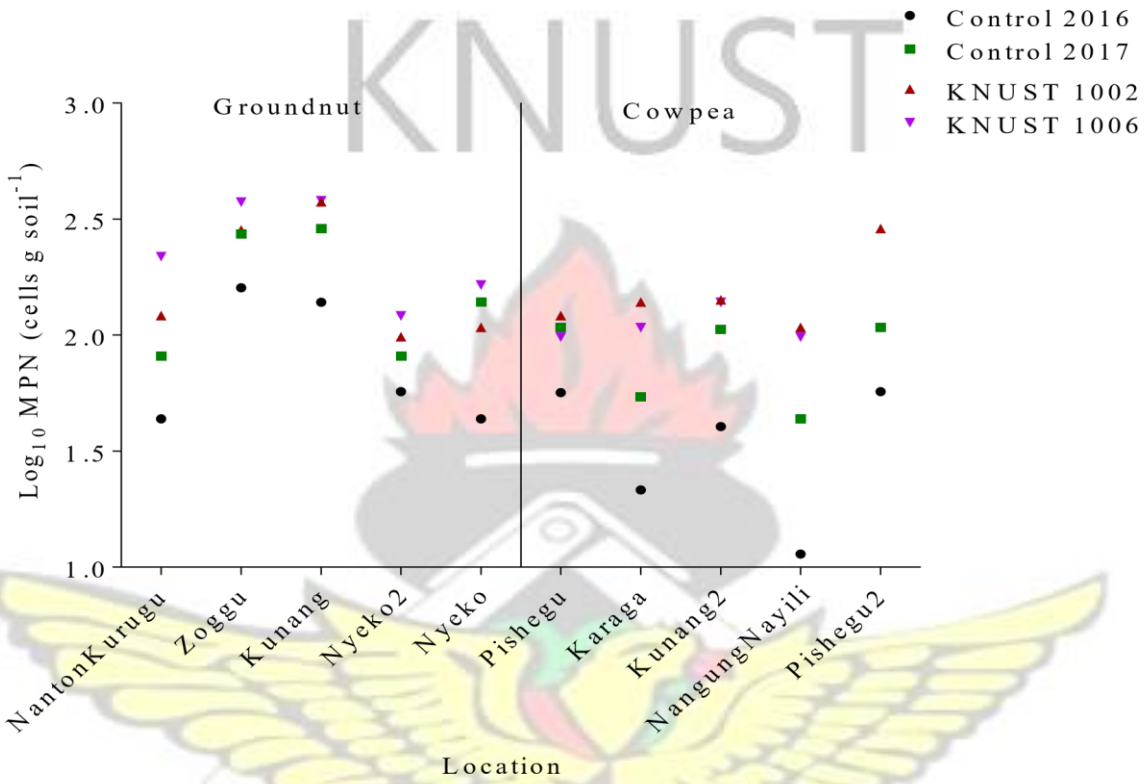


Figure 4.4.1. Variation in rhizobia population for previously inoculated and un-inoculated plots. Control 2016 = MPN of rhizobium prior to planting in 2016 cropping season, Control 2017 = MPN of rhizobium prior to planting in 2017 cropping season.

4.4.3.2 Nodulation response of target legumes to inoculation with test isolates

Nodule dry mass of groundnut

The application of treatments in the previous season (2016) resulted in significant (p = 0.0001) variations in nodule dry mass following re-introduction of the host in 2017. The nodule dry mass (NDM) in plots previously inoculated with the test isolates were higher than those of the un-inoculated plots (i.e. –N and +N control plots). Re-application of treatments in the second season (2017) caused significantly (p = 0.0001) higher nodule dry mass in plots inoculated with isolate KNUST 1006 compared to the other treatments. The nodule dry mass produced with isolate KNUST 1002 was not statistically different from that of the +N control treatment (Figure 4.4.2a). However, a direct comparison between the two treatments based on the contrast analysis revealed that NDM of isolate KNUST

1002 was significantly (p = 0.02) higher than that of the +N control (Table 4.4.1). There were no significant differences in NDM following previous inoculation in 2016 and re-inoculation in 2017 with both isolates. The NDM obtained for plots inoculated with isolate KNUST 1002 (Mean (M) = 681 mg 10 plants^{-1}, Standard deviation (SD) = 208) was not significantly different from that of re-inoculated plots with the same isolate (M = 686 mg 10 plants^{-1}, SD = 219, t = 0.08, p = 0.936). Similarly, the NDM recorded for plots previously inoculated with isolate KNUST 1006 (M = 811, SD = 196) did not differ significantly from that of re-inoculated KNUST 1006 plots (M = 848 mg 10 plants^{-1}, SD = 274, t = 0.48, p = 0.63) (Appendix 9).

Nodule dry mass of cowpea

Nodulation response of cowpea was such that, previous application of treatments had a significant (p = 0.0124) effect on nodule dry mass with the highest being produced by isolate KNUST 1002. The contrast analysis did not show any significant (p = 0.41) difference between the nodule dry mass produced with isolates KNUST 1006 and KNUST 1002 (Table 4.4.1). Following re-inoculation in 2017, significant (p = 0.0001) differences in nodule dry mass were observed between the plots treated with isolates KNUST 1002 and KNUST 1006 (Figure 4.4.2b). Comparing the performance of isolates in both previously inoculated and re-inoculated plots revealed that NDM for isolate KNUST 1002 in the former plots were significantly lower (M = 93 mg 10 plants^{-1}, SD = 43.6) than those in the latter plots (M = 160 mg 10 plants^{-1}, SD = 97.4, t = 2.78, p = 0.010) (Appendix 9). Conversely, NDM of plots previously inoculated with isolate KNUST 1006 (M = 86 mg 10 plants^{-1}, SD = 56.0) did not vary significantly from that of re-inoculated plots with the same isolate (M = 117 mg 10 plants^{-1}, SD = 74.8, t = 1.46, p = 0.15) (Appendix 9).

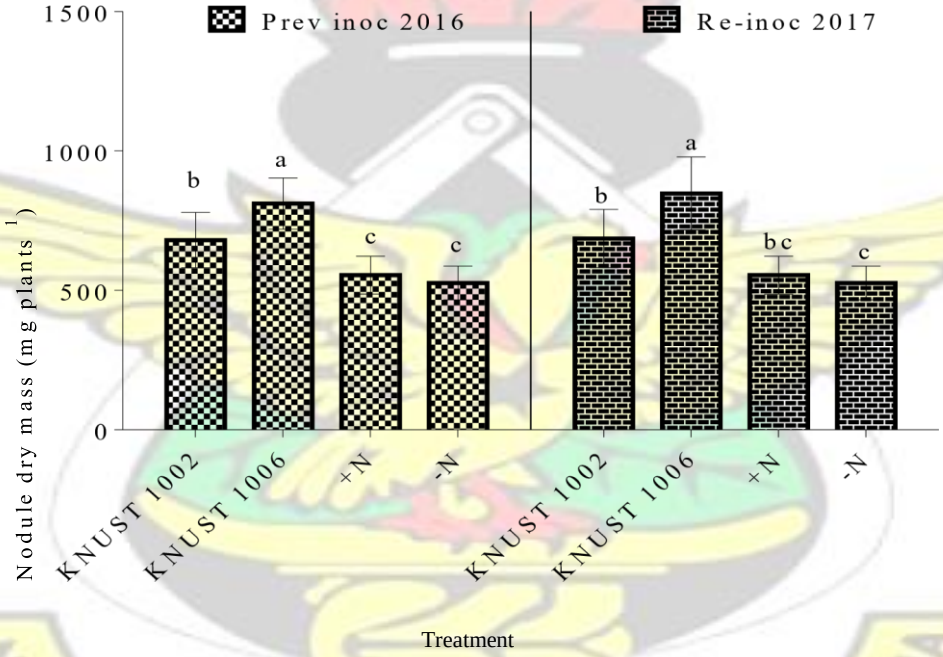
4.4.3.3 Grain yield response of target legumes to inoculation with test isolates

Groundnut yields

Grain yields of groundnut (unshelled) were significantly (p = 0.0001) affected by inoculation with test isolates. In previously inoculated plots, significantly higher yields were observed for treatment with isolate KNUST 1006 compared to the other treatments. Grain yield observed following previous application of isolate KNUST 1002 was however, not statistically different from that of the +N control treatment. The contrast analysis on

the other hand revealed that yields produced with isolate KNUST 1002 was significantly ($p = 0.02$) higher than that of the -N control treatment (Table 4.4.2). Reinoculation with test isolates resulted in significant ($p = 0.0012$) yield differences and followed a similar pattern as the previously inoculated plots (Figure 4.4.3a). The highest yield was observed for treatment with isolate KNUST 1006. Comparing the performance of treatments in previously inoculated to re-inoculated plots revealed that both isolates KNUST 1002 and KNUST 1006 induced grain yields that were not significantly different in each case (Appendix 10).

(a)



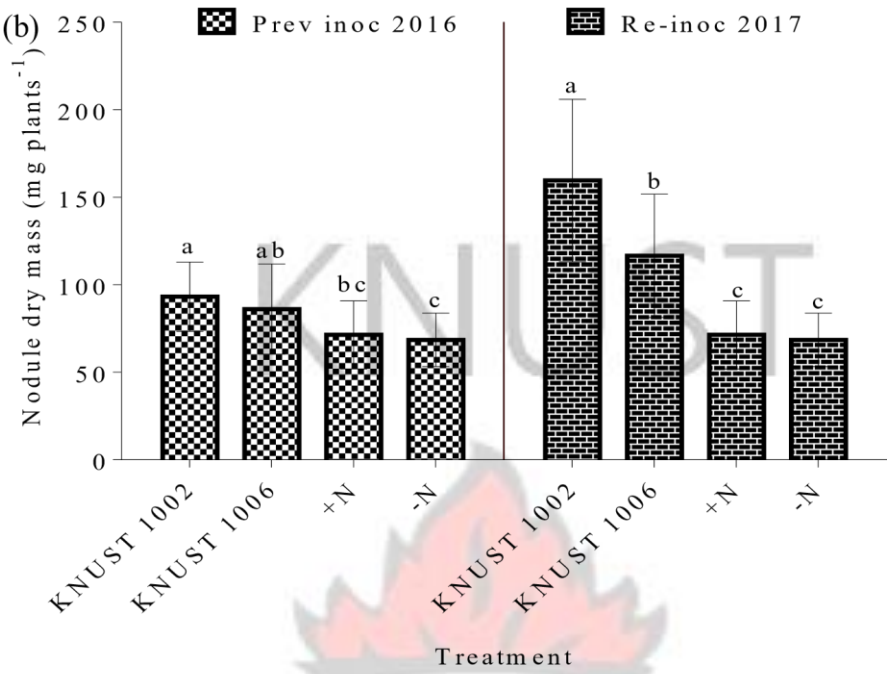


Figure 4.4.2. Nodule dry mass of groundnut (a) and cowpea (b) plants previously inoculated in 2016 and re-inoculated in 2017

Values are means of 10 plants per plot. Bars denote standard error of means. Letters compare means within a year and bars with similar letters are not significantly ($p < 0.05$). Prev inoc 2016; Previously inoculated in 2016, Re-inoc 2017; Re-inoculated in 2017

Table 4.4.1. Orthogonal contrast probabilities of treatments for nodule dry mass responses

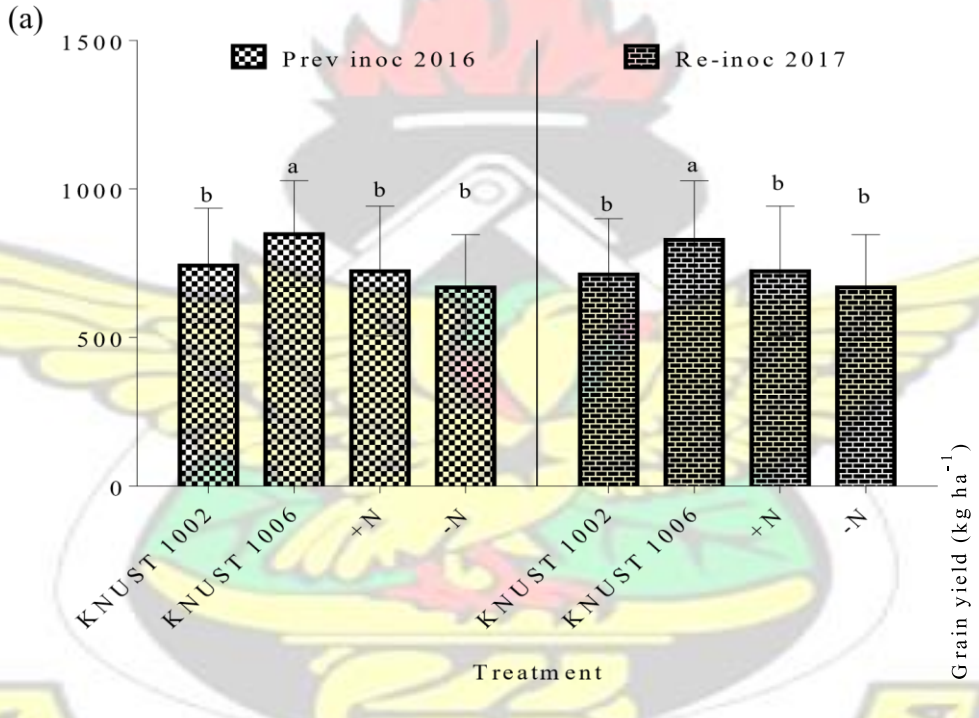
Contrast	Nodule dry mass			
	Prev inoc 2016		Re-inoc 2017	
	Groundnut	Cowpea	Groundnut	Cowpea
Overall Fpr	<0.001	0.012	<0.001	<0.001
KNU02 vr KNU06	-	0.41	-	-
KNU02 vr +N	-	-	0.02	-
KNU02 vr -N	-	-	-	-
KNU06 vr +N	-	0.085	-	-
KNU06 vr -N	-	-	-	-
+N vr -N	0.44	0.73	0.61	0.81

Prev inoc; Previously inoculated, Re-inoc; Re-inoculated

Cowpea yields

The yields of cowpea from previous inoculation were such that significantly ($p = 0.0125$) higher yields were obtained in plots that were inoculated with isolate KNUST 1002. The

performance of isolate KNUST 1002 was not statistically different from that of the +N control treatment. Re-inoculation with test isolates in 2017 caused significant ($p = 0.0221$) differences in yields of cowpea with the highest of 965 kg ha^{-1} recorded for isolate KNUST 1002. The +N control treatment produced yield that was not significantly different from the yield of isolate KNUST 1002 (Figure 4.4.3b). There was no significant difference in grain yields between the plots previously inoculated with isolate KNUST 1002 and the reinoculated plots with the same isolates. A similar performance of no significant difference in grain yield in previously inoculated and re-inoculated plots was observed for the treatment with isolate KNUST 1006 (Appendix 10).



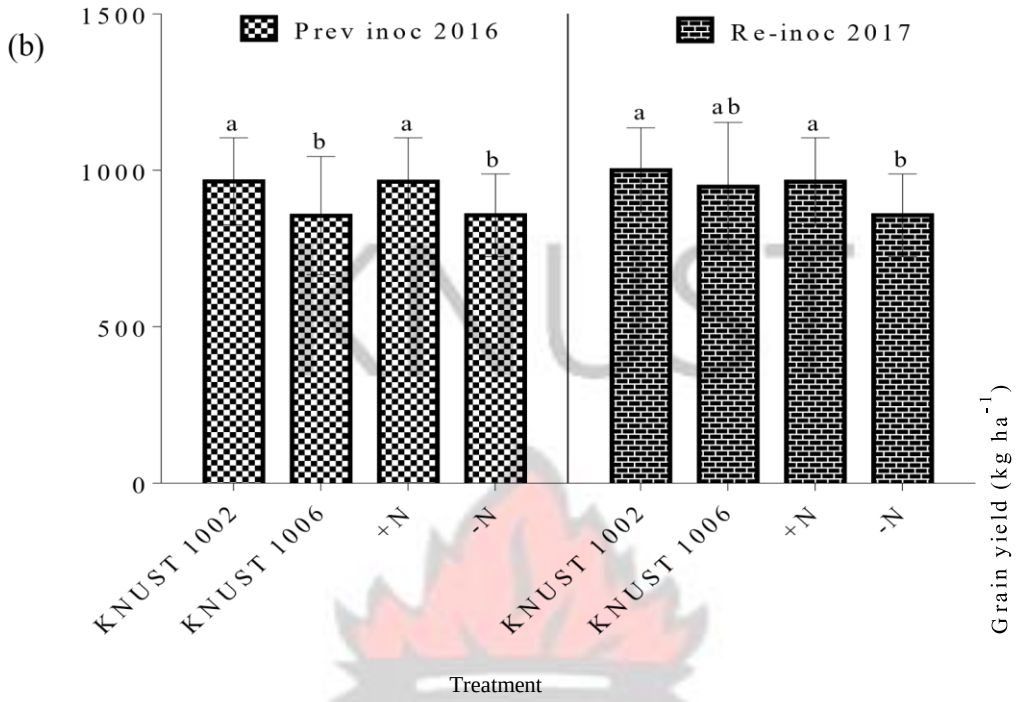


Figure 4.4.3. Grain yield responses of groundnut (a) and cowpea (b) in previously inoculated and re-inoculated plots

Values are means of 10 plants per plot. Bars denote standard error of means. Letters compare means within a year and bars with similar letters are not significantly ($p < 0.05$) different. Prev ino 2016; Previously inoculated in 2016, Re-ino 2017; Re-inoculated in 2017

Table 4.4.2. Orthogonal contrast probabilities of treatments for grain yield responses

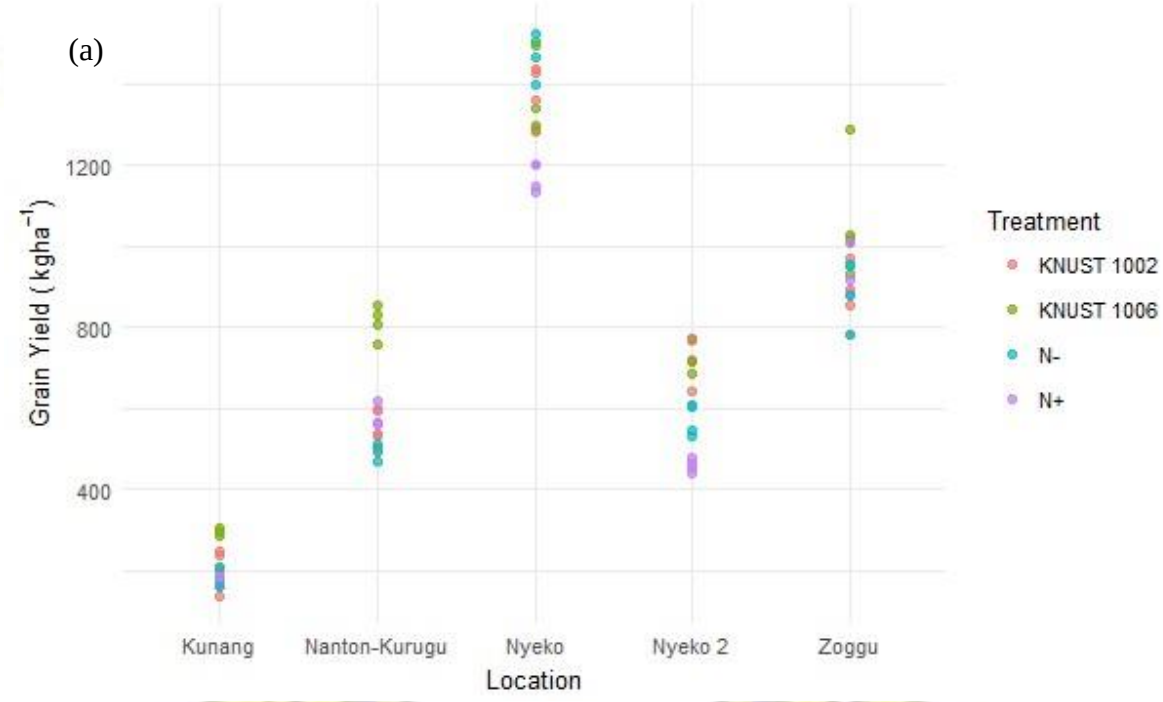
Contrast	Grain yield			
	Prev inoc 2016		Re-inoc 2017	
	Groundnut	Cowpea	Groundnut	Cowpea
Overall Fpr	< 0.001	0.013	0.001	0.022
KNU02 vr KNU06	-	-	-	0.26
KNU02 vr +N	0.53	0.98	0.77	0.44
KNU02 vr -N	0.02	-	0.27	-
KNU06 vr +N	-	-	-	0.73
KNU06 vr -N	-	0.98	-	0.056
+N vr -N	0.07	-	0.17	-

KN02 = KNUST 1002, KN06 = KNUST 1006, +N = positive nitrogen control and –N = absolute control without nitrogen or inoculation.

4.4.3.4 Variability in grain yield response to treatments in two cropping seasons

Variations in grain yield were observed within and between the study locations for the treatments (Figure 4.4.4). For instance, at the groundnut trial sites, yields in previously inoculated plots ranged between 138 kg ha^{-1} to 1494 kg ha^{-1}. With the exception of Nyeko, grain yields for the –N control plots were low ranging between 168 kg ha^{-1} to 954 kg ha^{1}. Isolate KNUST 1006 treated plots produced higher yields compared to the other treatments at three out of the five study locations (Figure 4.4.4a). In re-inoculated plots, a similar trend of treatment performance was observed for all the locations except Nyeko 2 where re-inoculation with isolate KNUST 1006 produced the lowest grain yield (Figure 4.4.4b).

(a)



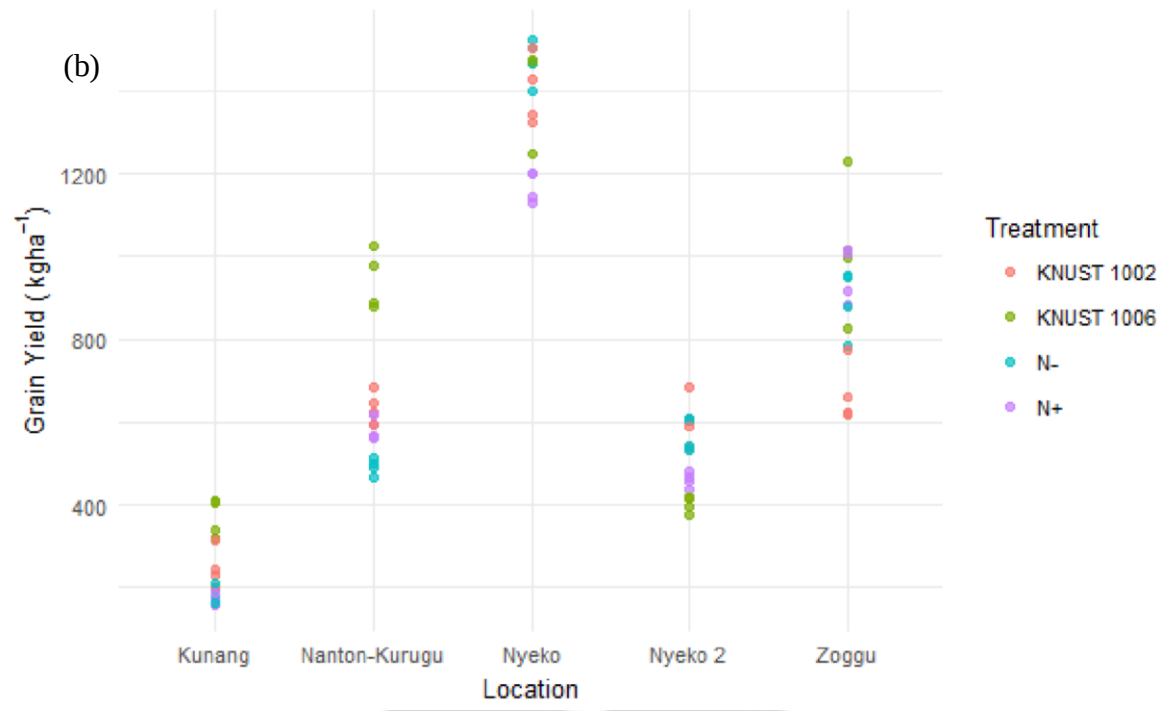


Figure 4.4.4. GG plot of the variability in groundnut yield responses to treatments at the different locations

(a) Previously inoculated and (b) Re-inoculated plots.

Variable yield responses in cowpea were also observed at the cowpea study locations with plots previously inoculated with isolate KNUST 1006 producing poor responses at Karaga, Nangun-Nayilli and Pishegu. Isolate KNUST 1002 treated plots in 2016 resulted in grain yield responses that were 800 kg ha^{-1} or more at all the locations except NangunNayilli. In general, yield responses at Pishegu and Pishegu 2 were higher than those of the other locations (Figure 4.4.5a). Re-inoculation of cowpea in 2017 also caused variable yield responses at the study locations with yields ranging between 300 to 1700 kg ha^{-1}. Contrary to the yields observed in previously inoculated plots, higher yields (900 kg ha^{-1} or more) were obtained following re-inoculation with isolate KNUST 1006 at three (Kunang, Pishegu and Pishegu 2) out of the five study locations (Figure 4.4.5b).

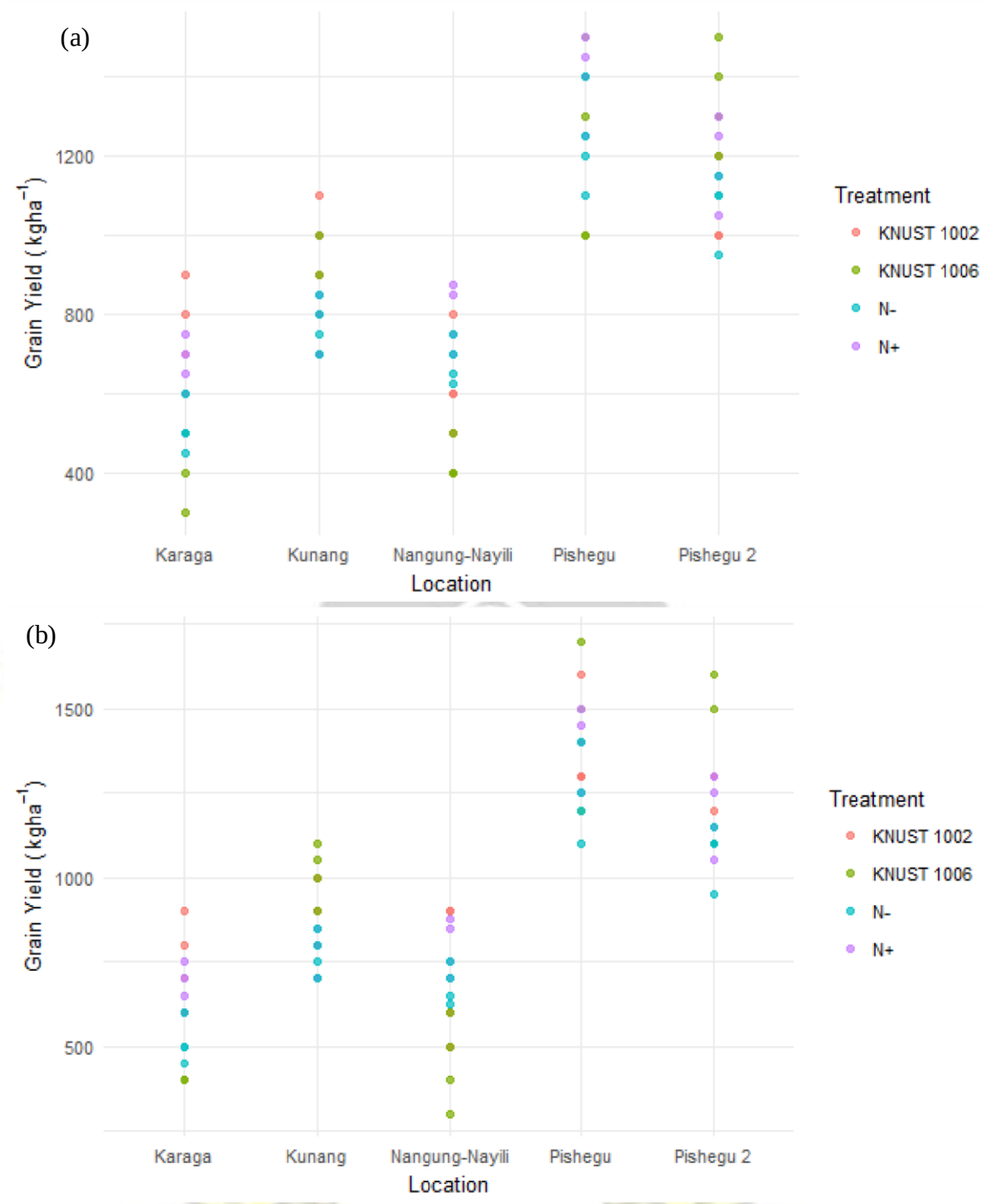


Figure 4.4.5. GG plot of the variability in cowpea yield responses to treatments at the different study locations

(a) Previously inoculated and (b) Re-inoculated plots.

4.4.3.5 Linear regression between grain yield and soil variables

Multiple linear regression was done using grain yield as the dependent variable against some soil and environmental factors (independent variable) to understand the variability in the yield responses observed (Table 4.4.3). Overall, the regression model showed that soil type, MPN and cumulative rainfall factors explained 98% and 92% of the variability observed in grain yields for previously inoculated and re-inoculated plots of groundnut, respectively. The resident rhizobia MPN (i.e. rhizobia that persisted after previous inoculation or due to introduction of legume host in control plots) had a significant positive effect on grain yield in both previously inoculated and re-inoculated plots of groundnut. Lixisols had a significant positive effect on groundnut yields in both previously inoculated and re-inoculated plots.

Table 4.4.3. Multiple linear regression explaining the variability in grain yields of previously and re-inoculated groundnut plots at the various locations

Coefficients				
	Estimate	Standard error	t value	Pr (>|t|)
Prev inoc				
(Intercept)	-132.43	1303.00	-0.102	0.92
$MPNLog_{10}$	714.87	162.43	4.40	0.0017**
Cumulative rainfall	-1.95	2.65	-0.74	0.48
Soil type_Lixisols	713.37	77.022	9.26	6.75e-06***
Soil type_Planosols	81.94	94.69	0.87	0.41
Soil type_Plinthosols	-376.90	509.003	-0.740	0.48
Adjusted R-squared: 0.98 F-statistic: 122.6 on 5 and 9 DF *p-value*: 5.35e-08				
Re-inoc				
(Intercept)	-2607.66	2443.63	-1.067	0.31
$MPNLog_{10}$	781.56	304.62	2.57	0.030*
Cumulative rainfall	4.23	4.97	0.85	0.42

Soil type_Lixisols	849.60	144.45	5.88	0.00023***
Soil type_Planosols	52.67	177.58	0.30	0.77
Soil type_Plinthosols	-1454.53	954.59	-1.52	0.16
Adjusted R-squared: 0.92 F-statistic: 34.93 on 5 and 9 DF *p-value*: 1.24e-05				

Significant levels: *p< 0.05, **p< 0.01, and ***p< 0.001, Prev inoc; Previously inoculated, Re-inoc; Reinoculated.

In cowpea, the model explained 63 and 61% of the variability in the grain yields of previously and re-inoculated plots respectively. Resident rhizobia population (MPN) had a negative effect on yields in previously inoculated plots but a positive effect on reinoculated plots. However, these effects were not significant. Plinthosols had a significant positive effect on cowpea grain yields in both previously inoculated and re-inoculated plots (Table 4.4.4).

Table 4.4.4. Multiple linear regression explaining the variability in grain yields of previously and re-inoculated cowpea plots at the various study locations

Coefficients				
	Estimate	Standard error	t value	Pr (>|t|)
Prev inoc				
(Intercept)	-757.37	2218.11	-0.34	0.74
$MPNLog_{10}$	-100.11	332.13	-0.30	0.77
Cumulative rainfall	7.42	10.59	0.70	0.50
Soil type_Plinthosols	567.21	121.49	4.67	0.00068***
Adjusted R-squared: 0.63 F-statistic: 9.053 on 3 and 11 DF *p-value*: 0.003				
Re-inoc				
(Intercept)	-957.49	2394.57	-0.40	0.70

$MPNLog_{10}$	46.01	358.56	0.13	0.90
Cumulative rainfall	7.18	11.44	0.63	0.54
Soil type_Plinthosols	556.26	131.16	4.24	0.0014**
Adjusted R-squared: 0.61				
F-statistic: 8.19 on 3 and 11 DF *p-value*: 0.004				

Significant levels: *p< 0.05, **p< 0.01, and ***p< 0.001, Prev inoc; Previously inoculated, Re-inoc; Reinoculated. The other soil types were not included in this regression because they had weak correlations with the dependent variable

4.4.4 Discussion

The persistence of a strain in the absence of the legume host is an important trait which influences inoculation responses and hence worth considering during the selection of inoculant strains because of the higher potential to persist in their source environments. The adaptation of native strains to local environments make them advantageous over exotic strains as inoculants. This study thus hypothesized that; native isolates from Ghana will persist in soils and obviate the need for annual re-inoculation. Results from the estimates of rhizobia populations in previously inoculated plots showed considerably increases over the control plots. This indicates that introduced isolates in this study multiplied in numbers in the presence of the host and subsequently persisted during the dry season. This could be due to their compatibility with legume hosts and adaptation to local conditions. Isolates KNUST 1002 and KNUST 1006 were identified to be closely related with the *Bradyrhizobium yuanmingense* species by Osei *et al.* (2018). Strains belonging to this species have been characterized as well adapted to tropical soil conditions (Leite *et al.*, 2017). Recently, Ulzen *et al.* (2018) confirmed the saprophytic abilities of the *Bradyrhizobium yuanmingense* strain BR 3267 in some locations in the northern part of Ghana. There was also a modest increase in the population of rhizobia in un-inoculated plots prior to the 2017 cropping season, which was not surprising since the study locations did not have any history of legume cultivation prior to planting in 2016. This observation could have been influenced by the presence of the legume host in the previous cropping season (i.e. 2016). The introduction of legumes in an area results in the attraction of free-

living soil rhizobia for nodule formation, which are subsequently, released into soils via nodule senescence (Brockwell *et al.*, 1995; Vlassak *et al.*, 1996; Ojo and Fagade, 2002). The non-significant difference in NDM observed between previously inoculated and re-inoculated plots in this study suggests that the isolates persisted in soils after a year of introduction and effectively nodulated with host legumes particularly groundnut in the subsequent cropping season. Conversely, the significantly low NDM recorded for previously inoculated cowpea plots with isolate KNUST 1002 implies that although persistent, the ability of this isolate to re-infect a large number of nodules might have been limited. According to Lupwayi *et al.* (1997), it is possible for a strain to persist but fail to re-infect plants. In this case, re-introduction of these isolates in the subsequent season may have led to an increase in the number of infective cells hence the observed increases in nodule dry mass (Deaker *et al.*, 2004). Increases in grain yields following inoculation with test isolates in both previously inoculated and re-inoculated plots over the control may be attributed to effective nodulation and optimum N_2 fixation. Nodule dry mass has been used as an indicator of symbiotic effectiveness as such higher nodule dry mass may result in high grain yields (Graham *et al.*, 2004). Thuita *et al.* (2018) have also reported increases in grain yield as a result of increased nodulation and high levels of nodule occupancy by introduced strains in Kenya. The observation that isolate KNUST 1002 produced significantly low NDM in previously inoculated cowpea plots but recorded yields that were comparable to those of re-inoculated plots is interesting. This gives an indication that although the ability of this isolate to re-infect large numbers of nodules might have been limited, it did not affect its effectiveness in N_2 fixation (Lupwayi *et al.*, 1997). However, there is the need to confirm the number of nodules formed by test isolates via nodule occupancy studies, which was not possible in this study due to limited availability of equipment.

Spatial variability in soil and environmental factors such as MPN, soil type and cumulative rainfall contributed to the variation in yield responses observed at the different locations in this study. In general, between 60 to 98% of the variability in grain yield was explained by the measured variables. The observation agrees with the reports by Bielders and Gérard (2015) and Ronner *et al.* (2016) that environment and management factors contribute reasonably to variability of millet and soybean yields, respectively. For instance, the effect of Plinthosols on cowpea was positive and significant but negative and non-significant on

groundnut. This implies that the Plinthosols might have favored cowpea growth and yield in this study. Plinthosols are generally characterized as less fertile, having drainage deficiencies, reduced water storage capacity and an underlying hard pan, which limits rooting volume (IUSS, 2015). However, the effective root zone of cowpea, which is about 120 cm deep (Hudson, 1975), coupled with its tap root system, which enhances water uptake, and increases plant water status under drought conditions is a plausible explanation for the observed responses (Timsina *et al.*, 1994). The significant positive response to treatments on Lixisols particularly for groundnut is not surprising since recurrent inputs of fertilizers (in this case inoculation) are a precondition for continuous cultivation on this soil type (IUSS, 2015). The results of this study provide empirical evidence that inoculating with effective elite isolates can obviate the need for reinoculation in subsequent seasons.

4.4.5 Conclusion

Inoculation with isolates KNUST 1002 and KNUST 1006 led to effective nodulation and subsequently enhanced grain yields of cowpea and groundnut after a year, thus reinoculation may not be necessary in the subsequent season.

CHAPTER FIVE

5.0 GENERAL DISCUSSION

Legumes like cowpea and groundnut form an integral part of cropping systems in Ghana. However, their yields are only a fraction of their maximum potential (Abaidoo *et al.*, 2013). The rhizobium inoculant technology is one of the approaches that is increasingly gaining aggravated interest as a means to improve yields of grain legumes (Bala, 2011). The technology serves as an environmentally friendly and economically viable means of incorperating sustainable inputs of nitrogen into agricultural soils (Lindström *et al.*, 2010) which subsequently results in increased yields. In order to harness the full benefits of this sustainable nitrogen inputs via BNF, both exotic and native rhizobia strains with high effectiveness have been introduced into soils through inoculation (Fening and Danso, 2002; Deaker *et al.*, 2004; Bogino *et al.*, 2006; Ampomah *et al.*, 2008; Grönemeyer *et al.*, 2014; Asei *et al.*, 2015; Boddey *et al.*, 2016; Ulzen *et al.*, 2016).

5.1 PCR assay for direct specific detection of *Bradyrhizobium* elite strain BR 3262 in root nodule extracts of soil-grown cowpea

Knowledge of the fate of introduced exotic strains is very critical for improving their performance and subsequently yields of legumes. In this current study, identification of gene sequences specific to the target strain based on comparative genomics was successful and resulted in the design of strain specific primer pairs for monitoring the presence and fate of this strain. Results from this study thus supports the conclusion by Stets *et al.* (2015) that comparative genomics for designing strain specific primers is theoritically possible for any organism whose whole genomic sequence is available. Strain-specific primers designed in this study were effective in detecting the target strain BR 3262 using a conventional PCR assay. The ability of primer 2645 to detect the target strain in bacteria cultures with cell concentrations as low as 3.3×10^2 indicates high sensitivity. This level of sensitivity is high enough to detect the target strain in a typical cowpea nodule (Somasegaran and Hoben, 2012). Effective and competitive strains when used as inoculants provide beneficial results such as increased yields in the absence of all other limiting factors. This is corroborated by the findings of this study where cowpea dry matter was increased following inoculation with 10^7 CFU seed^{-1} in soils with high native rhizobia

(i.e. 100% soil). Where adequate numbers of effective or compatible soil rhizobia are abscent in soils, inoculation with highly effective rhizobia becomes necessary (Catroux *et al.*, 2001; Abaidoo *et al.*, 2007). The effective performance of BR 3262 in this study affirms the previous findings of de Alcantara *et al.* (2014) and Zilli *et al.* (2009). Effectiveness of the target strain (BR 3262) in increasing shoot dry weight of cowpea was also supported by the high nodule occupancy observed in the treatments with this strain over the control treatment (without inoculation or fertilization). Contrary to nodules of inoculated plants, nodules from control plants gave no amplification following PCR. This indicates that the designed primer pairs were highly specific in the detection of the target strains even when nodule extracts were used. Enhanced nodulation and increase in shoot dry weight can thus be viewed as physical demonstration of the efficacy of target strains in dorminating nodule occupancy.

The primer pairs designed in this study will be a useful tool in determining the performance of BR 3262 in field studies hence serving as a basis for explanation of inoculation responses of plants to this strain. In addition, the approach can be used to develop such specific primers for other strains of interest whose whole genomic sequences are available. Finally, in developing countries such as Ghana, where infrastructure for molecular works are limited, the conventional PCR method described in this study is suitable in any basically equiped molecular biology laboratory.

5.2 Bacteria related to *Bradyrhizobium yuanmingense* from Ghana are effective groundnut micro-symbionts

Genetic, phenotypic and ecological characterisation of rhizobia during strain selection is an important criteria which leads to the attainment of information that contributes to our understanding of the legume-rhizobia symbosis (García-Fraile *et al.*, 2007). The current study revealed that native isolates obtained from soils in northern Ghana are variable in their phenotypic, genetic and N_2 fixing characteristics when groundnut was used as the test host. For instance, the N accumulated via BNF following the use of native isolates varied with some isolates resulting in N accumulation comparable to recommended reference strains (32H1 and BR 3267). This observation affirms that native rhizobia that can effectively nodulate with legumes are present amongst the native population (Herridge *et*

al., 2008) and can thus be exploited as a means to enhance BNF and to substitute the use of exotic strains. The phenotypic characteristics which revealed the isolates to belong to both fast and slow growing rhizobia was in agreement with the results from the phylogenetic analysis of the 16S rRNA gene. Analysis of this gene clustered five of the isolates in the same group with some already described bradyrhizobium while the remaining two showed close relation to rhizobium species. Groundnut has been identified to form effective symbiosis with both bradyrhizobium and rhizobium species (Taurian *et al.*, 2002) coroborating the results of this study. The concatenated analyses of 16S rRNA gene and ITS region revealed a well defined phylogenetic relationship between the already described strains and the isolates than did their individual analysis. It has been established that the 16S rRNA gene is limited in its ability to delineate the diversity within *Bradyrhizobium* species (Wang and Martínez-Romero, 2000). The clustering of isolates with already described species base on symbiotic genes analyses were in agreement with the result of concatenated 16S rRNA gene and ITS region. The observation that isolates in this study shared a close relation with *Bradyrhizobium yuanmingenese* is interesting since this species have been confirmed as an important microsymbiont of cultivated legumes like groundnut and cowpea (Steenkamp *et al.*, 2008; Leite *et al.*, 2017). Effective isolates that have been identified in this study thus serve as an important genetic resource for further field testing and recommendation as local inoculant strains.

5.3 Can native *Bradyrhizobium* isolates from Ghana enhance grain yields of fieldgrown cowpea and groundnut?

Field testing of elite native strains is an important step in selecting effective rhizobium for use in inoculant production. However, limited studies have been conducted along this line of research particularly in the context of Ghanaian agriculture. In effect, no local strain(s) has been recommended yet for inoculant production in the country. This study sought to assess the symbiotic potential of identified effective isolates in improving groundnut and cowpea yields under field conditions. Results from this study revealed site specific performance of test isolates on both groundnut and cowpea. This was marked by the significant ($p < 0.0001$) differences in grain yields within and between locations. The study thus provides additional evidence that inoculation responses could be highly variable and site specific. However, some non-cross over treatment by locations interaction (TLI) were

observed which implied that there was consistency in the performance of some treatments with significant difference only in the magnitude of response. Rhizobium isolates that show non-cross over type of TLI are desirable since they can be recommended for inoculation in a wide range of environment (Matus-Cadiz *et al.*, 2003). Considering the extent to which each isolate improved grain yields of the target legumes, isolate KNUST 1002 produced consistently high yields across the cowpea study locations while isolate KNUST 1006 produced better yields on groundnut. There are different levels of compatibility between host plant and rhizobia; and this may have resulted in the difference in performance of isolates on the different hosts (Schumpp and Deakin, 2010).

Identification of the best strain and most suitable location in this study based on the whichwon-where biplot has an important implication for the recommendation and use of isolates in this study, which will lead to optimum inoculation response. Effective native *Bradyrhizobium* isolates in this study improved grain yields of target legumes and represent a potential resource for local inoculant production.

5.4 Introduced effective native *Bradyrhizobium* improves grain yields of legumes and obviate the need for re-inoculation

The persistence of introduced strains is an important trait, which obviates the need for annual re-inoculation. A knowledge of the ability of identified native strains to persist after a year of introduction will serve as a resource for increasing yields of cultivated legumes in a sustainable manner in smallholder farms. This study demonstrated a marked increase in grain yields of groundnut and cowpea cultivated on plots previously inoculated with effective native isolates. Inoculation with effective strains has an important implication in increasing grain yield responses of tropical legumes (Giller, 2001). The increases in yields were stimulated by effective nodulation and subsequently N_2 fixation of introduced isolates. The effective persistence of introduced native isolates is therefore viewed as a plausible explanation for the enhanced yield of target legumes. The results from this study suggests that, re-inoculation with effective native isolates after a year of introduction may not be necessary. However, the performance of test isolates varied within and between the locations considered in this study. The within location variance can be attributed to differential responses of isolates to edaphic conditions due to intrinsic characteristics (Sanging *et al.*, 1996). Soil and environmental factors also contributed between 60 to 90%

of the observed variation, which agrees with the results of Bielders and Gérard (2015) and Ronner *et al.* (2016). This observation implies that, the use of effective native isolates should be beneficial when targeted at the locations where increased yield responses were obtained as well as other areas with similar soil and environmental conditions. Furthermore, the non-significant yield responses following previous inoculation with effective native isolates is an indication that re-inoculation in subsequent season may not be necessary.

CHAPTER SIX

6.0 GENERAL CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

The objectives and results from this study made it possible for the following general conclusions to be drawn:

i. *Bradyrhizobium pachyrhizi* strain BR 3262 is an effective cowpea microsymbiont, which increased shoot biomass. The positive response to inoculation was adequately explained by the determination of nodule occupants. The methodology employed in this study and the primers designed will serve as important resources for designing primers for other important bacteria and for monitoring the fate of BR 3262 strain in field inoculation studies.

ii. Symbiotic and molecular characterization of native rhizobium strains from northern Ghana revealed them as important sources of local inoculant strains. The effective isolates identified in this study have high potential in increasing nodulation, shoot biomass and N accumulation in groundnut under controlled conditions. Furthermore, the close association between *Bradyrhizobium* isolates from this study and *Bradyrhizobium yuanmingense* strain confirms the former as important micro-symbionts of groundnut.

iii. Field inoculation with elite native isolates increased grain yields of cowpea and groundnut as a result of the effective symbiotic association established between the host legumes and introduced strains. The results obtained indicated that isolate KNUST 1002 was the most effective on cowpea while isolate KNUST 1006 was the most effective on groundnut. Inoculation with effective native isolates from this study is thus a feasible option for enhancing cowpea and groundnut production in the study areas.

iv. Previous inoculation with test isolates improved nodule dry mass and grain yields of cowpea and groundnut in the subsequent cropping season. Nodulation and yield increased in previously inoculated plots that were comparable to re-inoculated plots in the subsequent cropping season indicate that the isolates were persistent

and hence enhanced N_2 fixation leading to increased grain yields. The results provide empirical evidence that re-inoculation in subsequent seasons may not be necessary when effective native isolates in this study are inoculated on cowpea and groundnut.

6.2 Recommendations

In order to improve grain yields of the target legumes in a cost effective and environmentally manner, the following recommendations are made:

i. isolate KNUST 1002 and KNUST 1006 should be adopted for local inoculant production for farmers in the study locations,

ii. the competitiveness of effective isolates from this study should be established via nodule occupancy studies which was not possible in the current study due to limited availability of equipment and

iii. strain-specific primers should be designed for the elite isolates identified using the comparative genomics method in order to effectively monitor their nodulation and persistence ability.

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APPENDICES

Appendix 1. General characteristics of (draft) genome sequences used in this study

Bradyrhizobium strain	GenBank accession number	Number of contigs	Genome size (Mbp)	%GC
B. pachyrhizi BR 3262	LJY00000000.1	116	9.97	63.7
B. pachyrhizi PAC 48^{T}	LFIQ00000000.1	124	8.71	63.7

B. elkanii USDA 76 ^{T}	ARAG00000000.1	25	9.48	63.7
B. paxllaeri DSM 18454^{T}	MAXB00000000.1	146	8.29	62.5
B. tropiciagri CNPSo 1112 ^{T}	LFLZ00000000.1	189	9.77	63.5
B. neotropicale BR 10297 ^{T}	LSEF00000000.1	125	8.68	63.6
B. japonicum USDA 6 ^{T}	AXAV00000000.1	1	9.21	63.7

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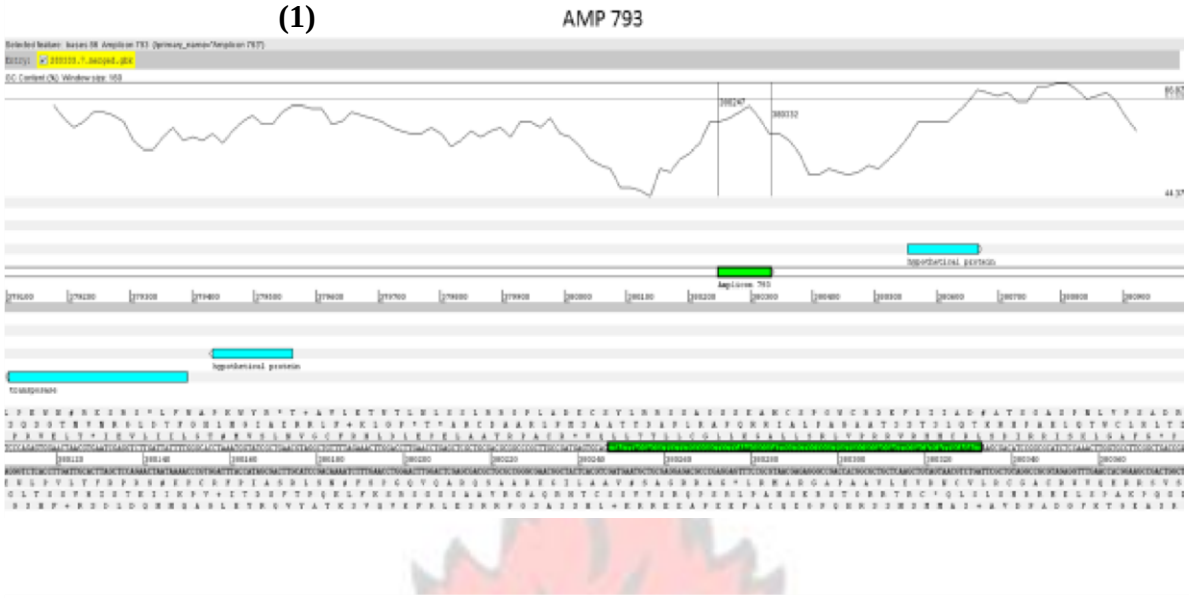

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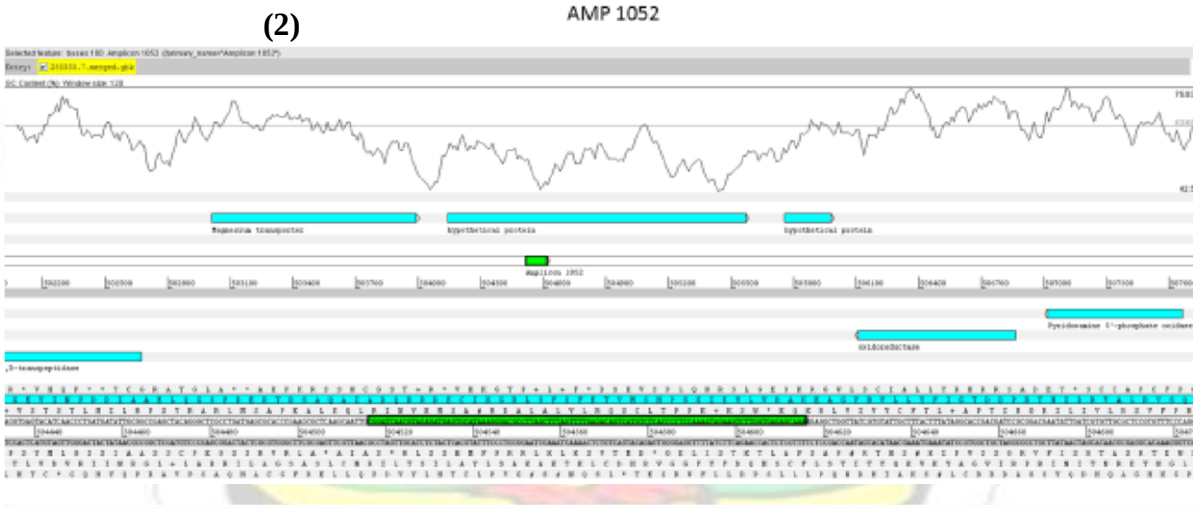

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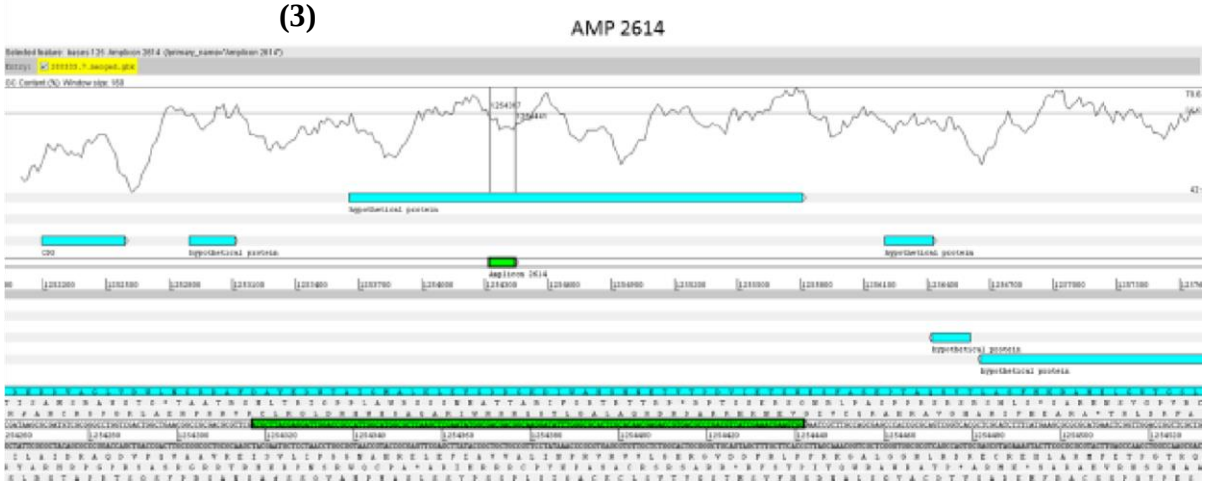

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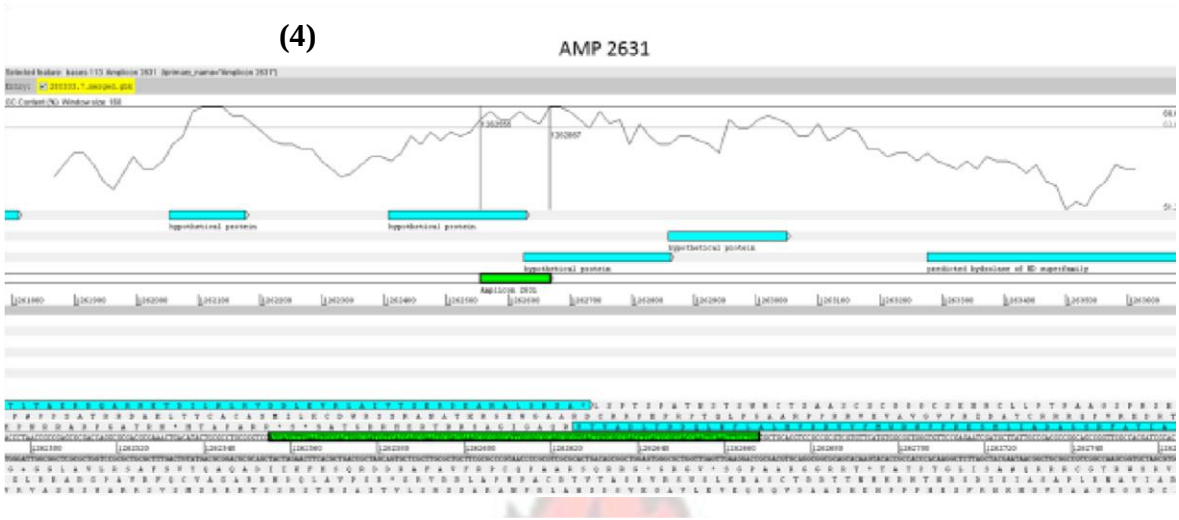

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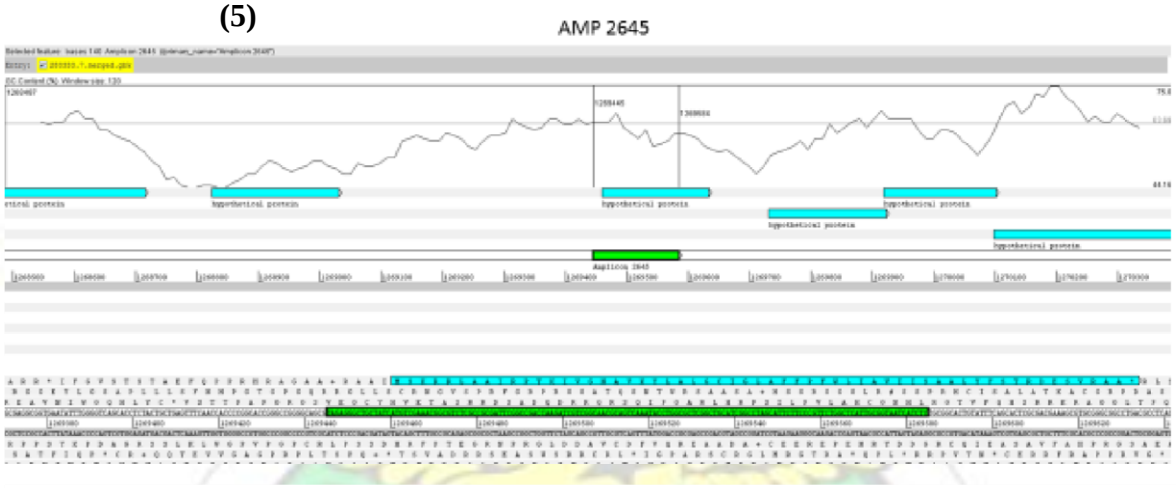

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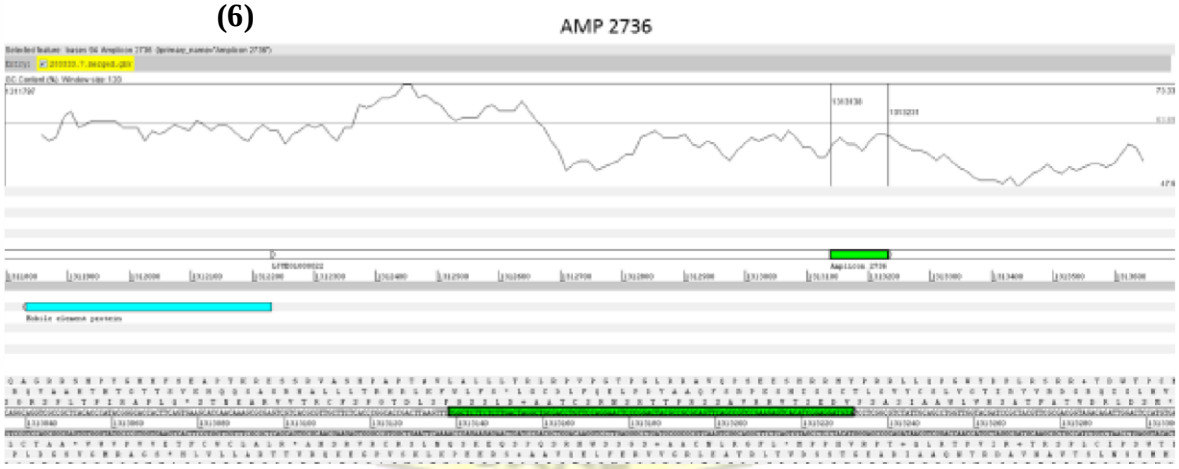

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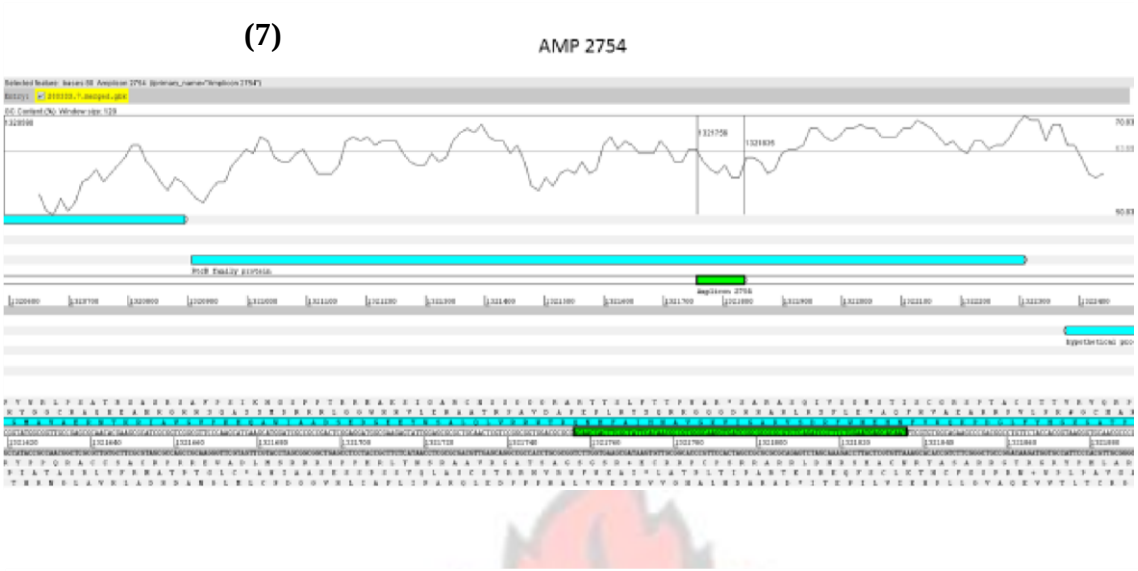

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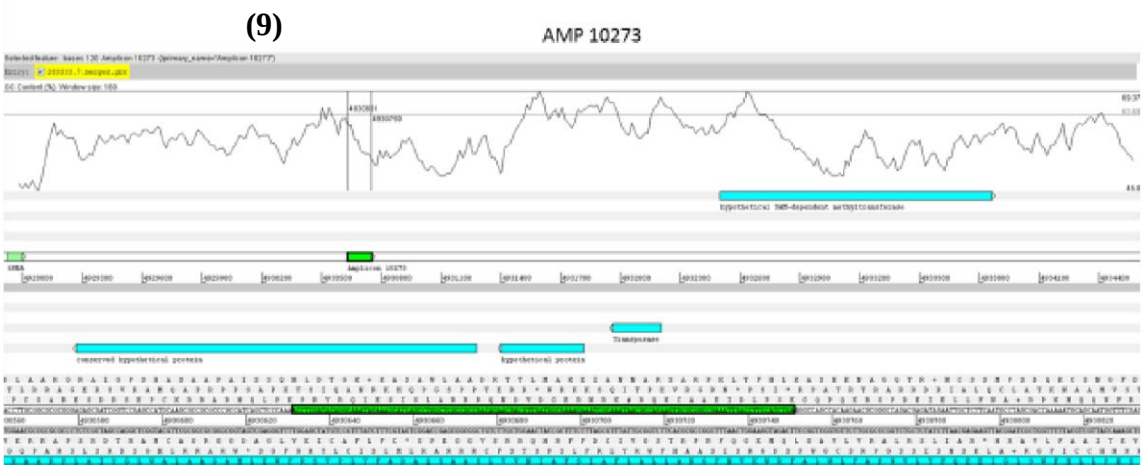

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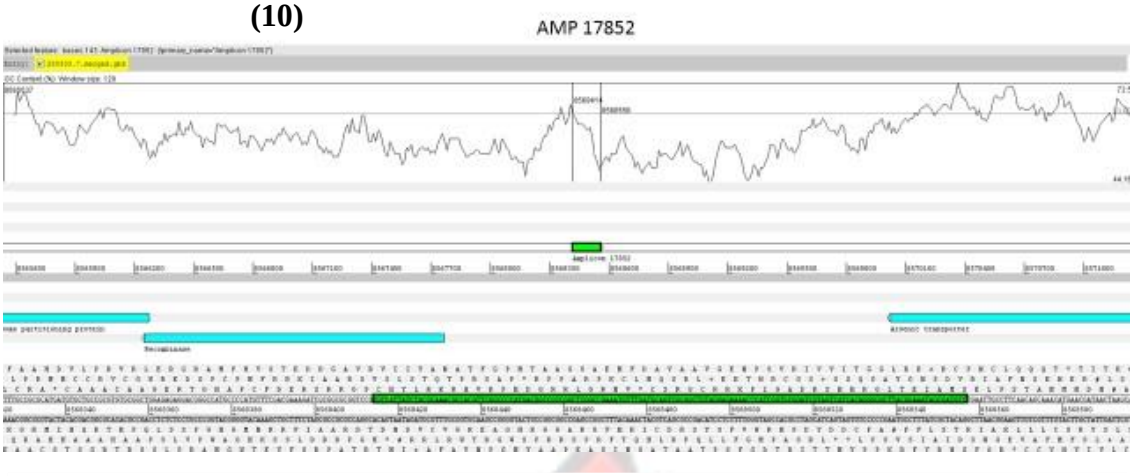

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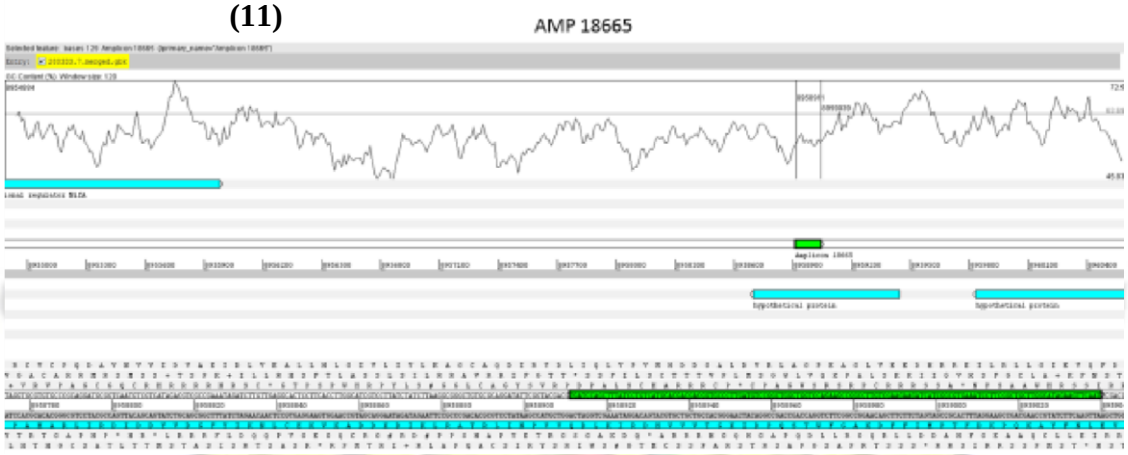


Appendix 2. Genomic representation of specific primer pair sequences (1 – 11) on the chromosome of the target strain the BR 3262.

Amplicons are represented by green boxes. Light blue arrows represent annotated protein functions. AMP numbers represent primer pair IDs as show in table 4.1.1

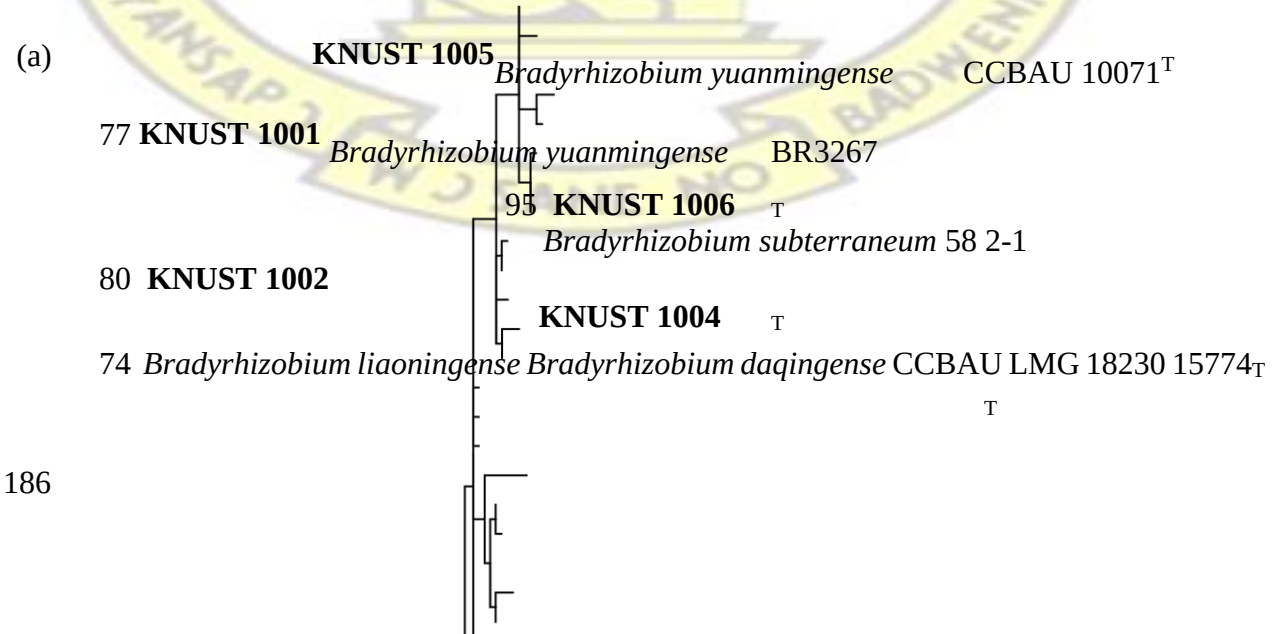
Appendix 3. Principal component table

	Eigen Value	Percent Variance	of Cumulative Percentage
1	3.36	**84.1**	84.05
2	0.42	**10.5**	**94.5**
3	0.20	5.052	99.6
4	0.016	0.421	100.0

Appendix 4. ^{15}N enrichment and proportion of nitrogen derived from air (%Ndfa) of groundnut plants inoculated with test isolates/reference strains and non-N_2 fixing reference plants

Isolate/strain/reference plant	Atom % ^{15}N excess	% Ndfa calculated using sorghum as the non-N_2- fixing reference plant
KNUST 1001	0.00592 c	91.8 a
KNUST 1002	0.00872 c	87.8 a
KNUST 1003	0.00613 c	91.4 a
KNUST 1004	0.00550 c	92.3 a
KNUST 1005	0.00664 c	90.8 a
KNUST 1006	0.00551 c	92.3 a
KNUST 1007	0.00661 c	90.7 a
Non-inoculated	0.00692 c	90.2 a
BR 10254	0.00594 c	91.7 a
BR 3267	0.00536 c	92.5 a
32H1	0.00601 c	91.5 a
SEMIA 6144	0.00542 c	92.4 a
Sorghum	0.07125 b	
NN Soybean	0.18750 a	
NN Common bean	0.20574 a	
CV (%)	48.8	

Means in the same column followed by the same letter are not significantly different at $p< 0.05$ (Scott Knott Test).



Bradyrhizobium japonicum USDA 6$_T$

Bradyrhizobium canariense BTA-$_T$1

Bradyrhizobium ottawaense OO99$_T$

Bradyrhizobium betae Bradyrhizobium diazoefficiens LMG 21987USDA 110 ^{T} $_T$

Bradyrhizobium guangxiense CCBAU 53363$_T$

63 *Bradyrhizobium vignae* 7-2 $_T$

67 *Bradyrhizobium guangdongense* CCBAU 51649$_T$

Bradyrhizobium manausense BR 3351

Bradyrhizobium ganzhouense RITF806$_T$ ^{T}

Bradyrhizobium cytisi CTAW11 $_T$

94 51 *Bradyrhizobium rifense* *Bradyrhizobium ingae* CTAW71BR 10250$_T$ $_T$

Bradyrhizobium iriomotense *Bradyrhizobium kavangense* EK0514-3 $_T$ $_T$

Bradyrhizobium arachidis CCBAU 051107$_T$

62 *Bradyrhizobium* sp. BR 10254 $_T$

Bradyrhizobium japonicum SEMIA 6144

^{T}

Bradyrhizobium huanghuaihaiense CCBAU 23303$_T$

98 *Bradyrhizobium denitrificans* *Bradyrhizobium oligotrophicum* $_T$

LMG 8443LMG 10732 $_T$

Bradyrhizobium retamae *Bradyrhizobium neotropicale* Ro19 BR 10247$_{TT}$

Bradyrhizobium erythrophlei CCBAU 53325$_T$

99 55 *Bradyrhizobium embrapense* *Bradyrhizobium jicamae*

PAC68SEMIA 6208 $_T$ $_T$

Bradyrhizobium viridifuturi SEMIA 690

60 *Bradyrhizobium icense* *Bradyrhizobium valentinum* LMTR 13LmjM3$_T$ $_T$

Bradyrhizobium paxllaeri LMTR 21 $_T$

Bradyrhizobium lablabi CCBAU 23086 $_T$

Bradyrhizobium ferriligni CCBAU 51502

Bradyrhizobium pachyrhizi BR 3262$_T$

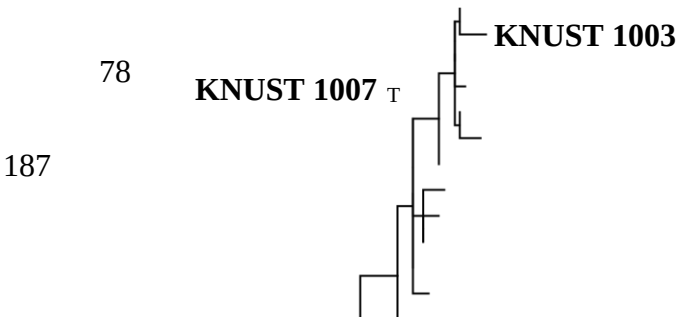
72 *Bradyrhizobium pachyrhizi* PAC48$_T$

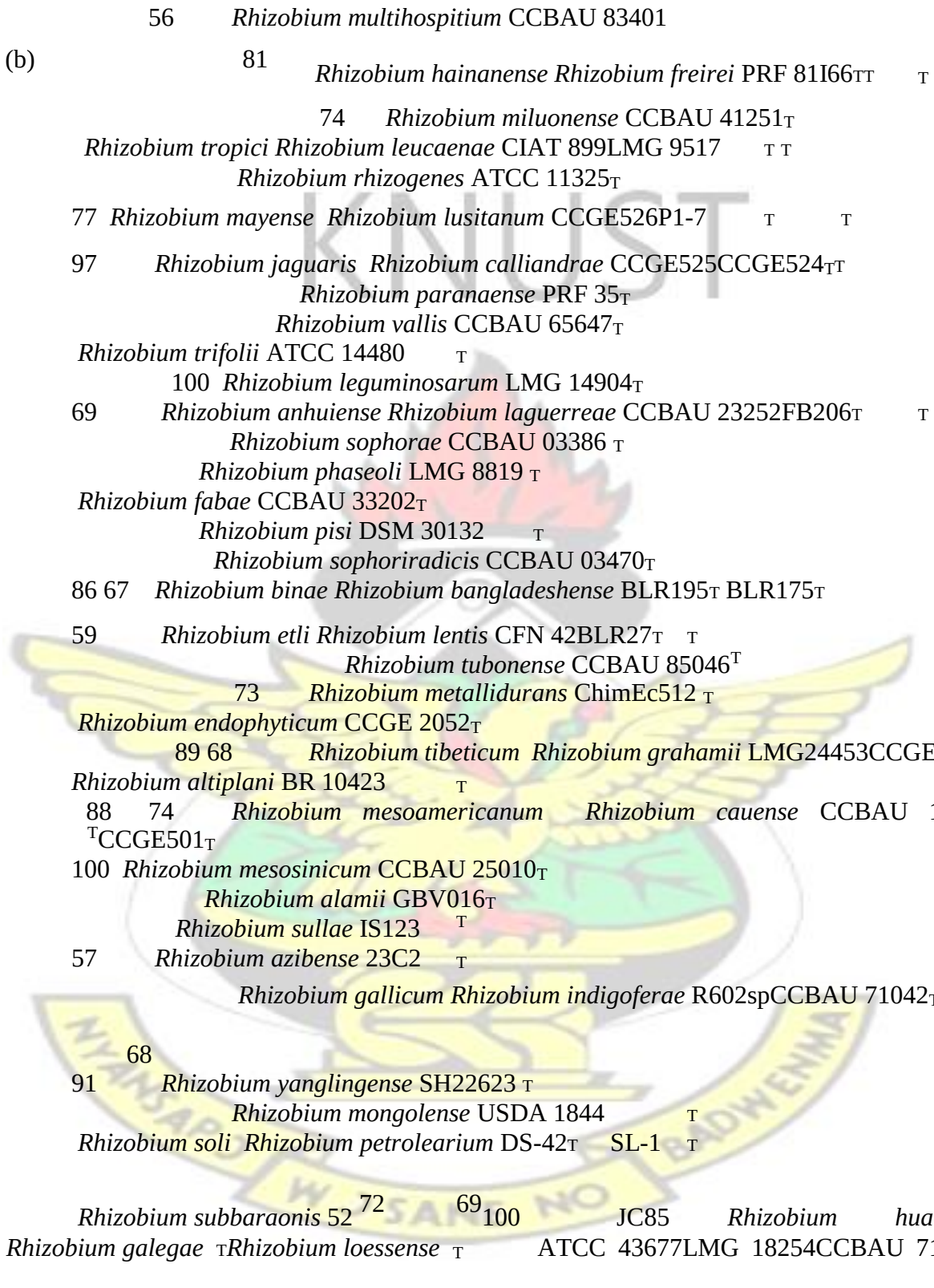
Bradyrhizobium elkanii USDA 76 ^{T}

Bradyrhizobium tropiciagri SEMIA 6148$_T$

Microvirga vignae BR3299

0.02




56 Rhizobium multihospitium CCBAU 83401
(b) 81
Rhizobium hainanense Rhizobium freirei PRF 81I66TT T
74 Rhizobium miluonense CCBAU 41251T
Rhizobium tropici Rhizobium leucaenae CIAT 899LMG 9517 T T
Rhizobium rhizogenes ATCC 11325T
77 Rhizobium mayense Rhizobium lusitanum CCGE526P1-7 T T
97 Rhizobium jaguaris Rhizobium calliandrae CCGE525CCGE524TT
Rhizobium paranaense PRF 35T
Rhizobium vallis CCBAU 65647T
Rhizobium trifolii ATCC 14480 T
100 Rhizobium leguminosarum LMG 14904T
69 Rhizobium anhuiense Rhizobium laguerreae CCBAU 23252FB206T T
Rhizobium sophorae CCBAU 03386 T
Rhizobium phaseoli LMG 8819 T
Rhizobium fabae CCBAU 33202T
Rhizobium pisi DSM 30132 T
Rhizobium sophoriradicis CCBAU 03470T
86 67 Rhizobium binae Rhizobium bangladeshense BLR195T BLR175T
59 Rhizobium etli Rhizobium lentis CFN 42BLR27T T
Rhizobium tubonense CCBAU 85046T
73 Rhizobium metallidurans ChimEc512 T
Rhizobium endophyticum CCGE 2052T
89 68 Rhizobium tibeticum Rhizobium grahamii LMG24453CCGE 502T T
Rhizobium altiplani BR 10423 T
88 74 Rhizobium mesoamericanum Rhizobium cauense CCBAU 101002 TCCGE501T
100 Rhizobium mesosinicum CCBAU 25010T
Rhizobium alamii GBV016T
Rhizobium sullae IS123 T
57 Rhizobium azibense 23C2 T
Rhizobium gallicum Rhizobium indigoferae R602spCCBAU 71042T T
68
91 Rhizobium yanglingense SH22623 T
Rhizobium mongolense USDA 1844 T
Rhizobium soli Rhizobium petrolearium DS-42T SL-1 T
Rhizobium subbaraonis 52 72 69100 JC85 Rhizobium huautlense
Rhizobium galegae TRhizobium loessense T ATCC 43677LMG 18254CCBAU 7190BTT
Rhizobium fredii Rhizobium giardinii LMG 6217 H152T
67 Rhizobium loti ATCC 33669T

0.01

Appendix 5. Maximum Likelihood phylogenetic tree based on 16S rRNA gene showing relationships between isolates and type species (^{T}) of the genera *Bradyrhizobium* (a) and *Rhizobium* (b).

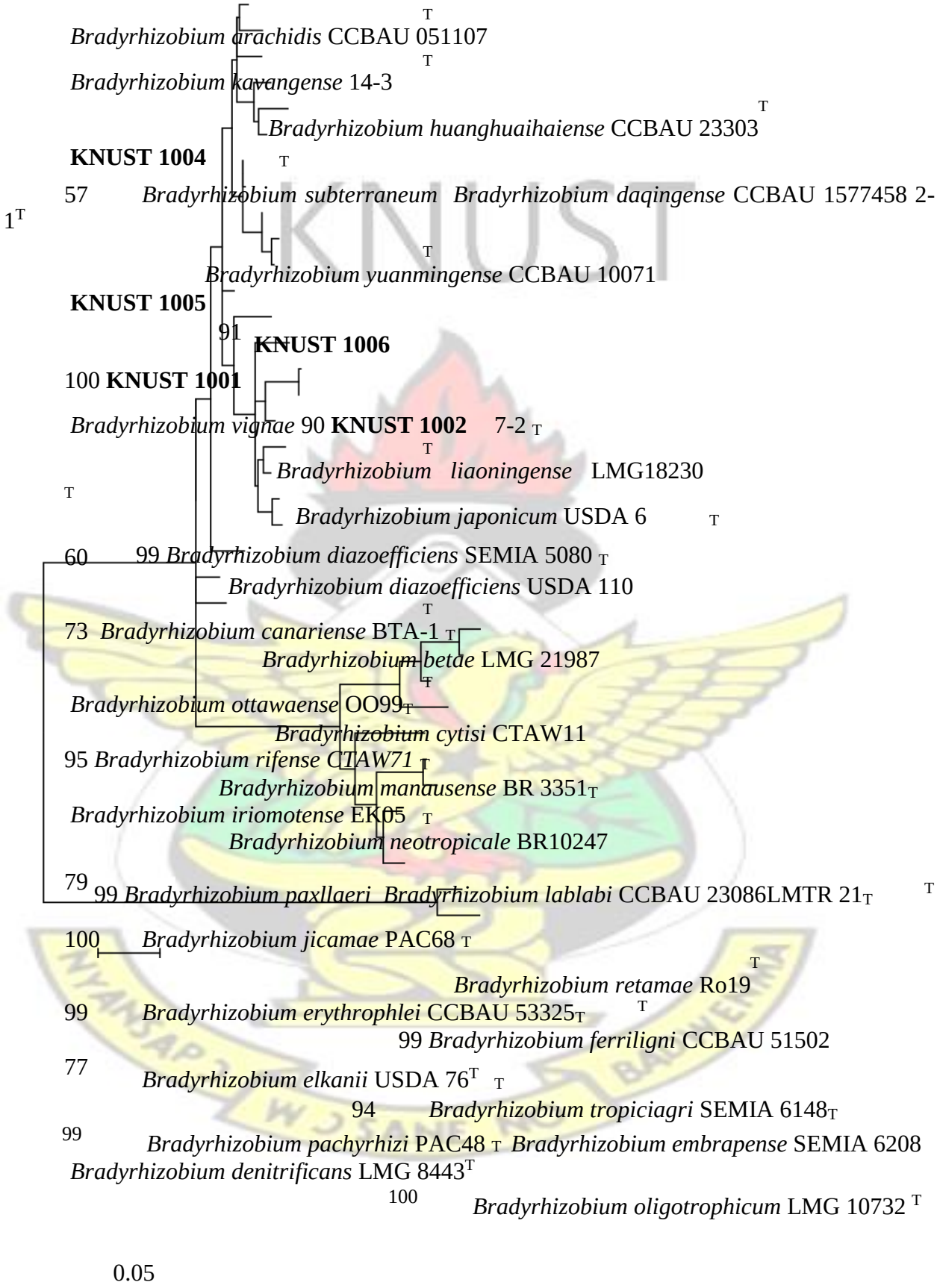
The phylogenetic tree was built based on the Tamura 3-parameter model. Bootstrap values were inferred from 500 replicates and are indicated at the tree nodes when ≥ 50%. The bar represents two or one estimated substitutions per 100 nucleotide positions.

Appendix 6. Percentage nucleotide similarity at different genetic loci between already described *Bradyrhizobium* strains and isolates

Strains/ genes	**16S rRNA**	**ITS**	***nodC***	***nifH***	**ITS**	**16S rRNA,**
Bradyrhizobium yuanmingense CCBAU 10071 ^{T}						
KNUST 1001	99.75%	97.52%	89.74%	92.54%		99.05%
KNUST 1002	99.59%	97.88%	89.74%	92.54%		99.16%
KNUST 1004	99.59%	97.89%	90.28%	92.19%		99.16%
KNUST 1005	99.75%	99.83%	94.33%	96.62%		99.78%
KNUST 1006	99.43%	98.59%	90.03%	92.19%		99.33%
Bradyrhizobium yuanmingense BR3267	99.26%	-	89.25%	93.12%		-
Bradyrhizobium japonicum SEMIA 6144 ^{T}	98.60%	-	86.29%	-		-
Bradyrhizobium sp. BR 10254 ^{T}	98.68%	-	-	-		-
Bradyrhizobium arachidis CCBAU 051107 ^{T}	98.85%	98.24%	85.43%	91.88%		98.66%
Bradyrhizobium viridifuturi SEMIA 690 ^{T}	96.99%	-	-	88.53%		-
Bradyrhizobium embrapense SEMIA 6208 ^{T}	96.99%	86.61%	78.34%	-		94.15%
Bradyrhizobium subterraneum 58 2-1 ^{T}	99.51%	97.17%	-	93.09%		98.82%
Bradyrhizobium betae LMG 21987 ^{T}	99.01%	94.58%	-	-		97.58%
Bradyrhizobium canariense BTA-1 ^{T}	99.10%	95.69%	65.71%	83.85%		97.97%
Bradyrhizobium cytisi CTAW11 ^{T}	99.01%	94.94%	68.72%	83.32%		97.75%
Bradyrhizobium daqingense CCBAU 15774 ^{T}	99.43%	96.07%	91.57%	91.40%		98.32%
Bradyrhizobium denitrificans LMG 8443 ^{T}	98.18%	78.59%	-	85.01%		92.44%
Bradyrhizobium elkanii USDA 76 ^{T}	96.73%	88.28%	80.49%	89.17%		94.45%
Bradyrhizobium erythrophlei CCBAU 53325 ^{T}	96.99%	89.04%	71.20%	88.04%		94.87%
Bradyrhizobium ferriligni CCBAU 51502 ^{T}	94.89%	86.82%	81.03%	89.63%		92.67%
Bradyrhizobium ganzhouense RITF806 ^{T}	99.18%	-	63.98%	85.38%		-
Bradyrhizobium guangdongense CCBAU 51649 ^{T}	99.26%	-	-	82.43%		-
Bradyrhizobium guangxiense CCBAU 53363 ^{T}	99.18%	-	-	85.58%		-
Bradyrhizobium huanghuaihaiense CCBAU 23303 ^{T}	98.68%	97.17%	91.57%	91.40%		98.15%
Bradyrhizobium icense LMTR 13 ^{T}	96.90%	-	68.99%	85.71%		-

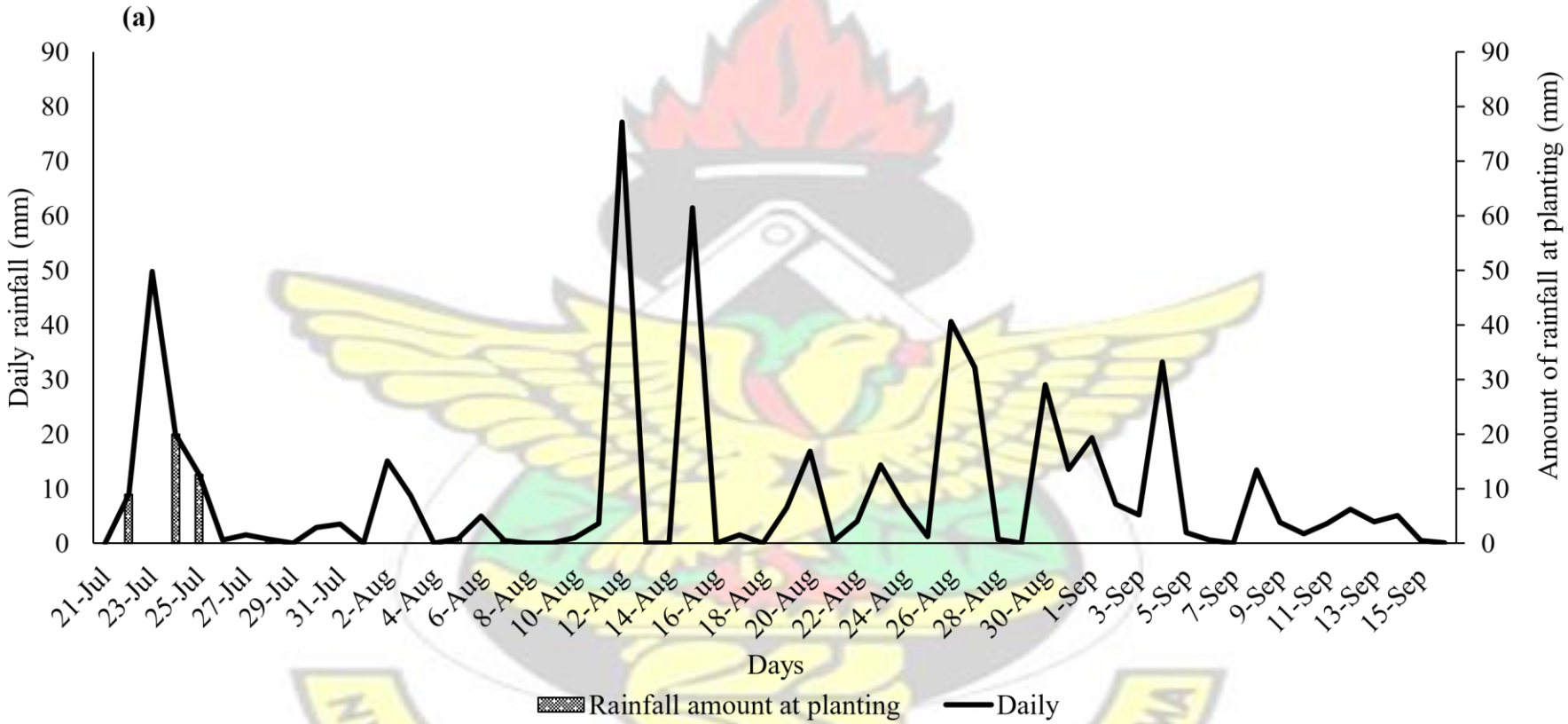
Bradyrhizobium ingae BR 10250 ^{T}	98.35%	-	63.82%	-	-
Bradyrhizobium iriomotense EK05 ^{T}	98.35%	95.51%	63.34%	-	97.46%
Bradyrhizobium japonicum USDA 6 ^{T}	99.43%	95.34%	91.57%	91.40%	98.09%
Bradyrhizobium jicamae PAC 68 ^{T}	96.65%	88.04%	64.54%	-	94.33%
Bradyrhizobium kavangense 14-3 ^{T}	98.93%	97.34%	85.72%	-	98.43%
Bradyrhizobium lablabi CCBAU 23086 ^{T}	96.82%	87.25%	69.66%	85.73%	94.21%
Bradyrhizobium liaoningense LMG 18230 ^{T}	99.51%	95.51%	59.52%	91.40%	-
Bradyrhizobium manausense BR 3351 ^{T}	99.18%	96.25%	63.77%	81.61%	98.26%
Bradyrhizobium oligotrophicum LMG 10732 ^{T}	98.43%	76.86%	-	84.87%	92.25%
Bradyrhizobium ottawaense OO 99 ^{T}	99.34%	95.69%	91.57%	91.40%	98.15%
Bradyrhizobium pachyrhizi PAC 48 ^{T}	96.82%	87.43%	81.35%	-	94.27%
Bradyrhizobium paxllaeri LMTR 21 ^{T}	96.90%	87.01%	69.63%	85.73%	94.21%
Bradyrhizobium retamae Ro 19 ^{T}	97.24%	86.76%	68.56%	86.14%	94.38%
Bradyrhizobium rifense CTAW 71 ^{T}	99.10%	94.74%	67.53%	83.38%	97.69%
Bradyrhizobium valentinum LmjM3	96.82%	-	68.85%	86.21%	-
Bradyrhizobium vignae 7-2 ^{T}	99.01%	97.88%	85.70%	-	-
Bradyrhizobium tropiciagri SEMIA 6148 ^{T}	96.21%	87.38%	80.58%	88.08%	93.84%
Bradyrhizobium diazoefficiens USDA 110 ^{T}	99.01%	94.20%	91.57%	91.40%	97.52%
Bradyrhizobium neotropicale BR 10247 ^{T}	96.39%	95.34%	62.87%	-	-
Microvirga vignae BR 3299^{T}	90.47%	-	-	-	-

The last column shows values for concatenated sequences of two loci. Similarity values were determined using the Mega 7 software. -: data not available

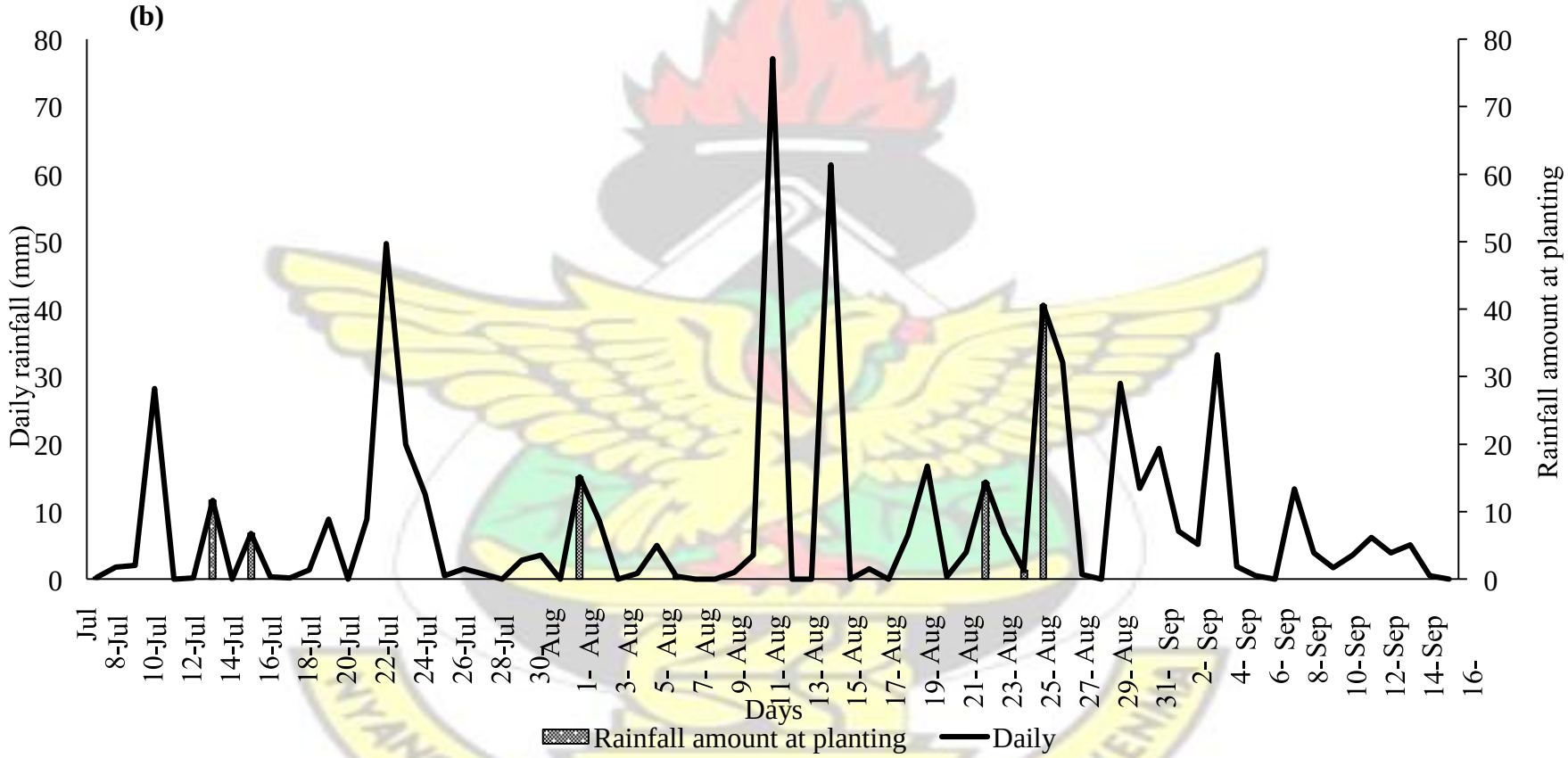

Bradyrhizobium arachidis CCBAU 051107
Bradyrhizobium kavangense 14-3
Bradyrhizobium huanghuaihaiense CCBAU 23303
KNUST 1004
57 Bradyrhizobium subterraneum Bradyrhizobium daqingense CCBAU 1577458 2-1
Bradyrhizobium yuanmingense CCBAU 10071
KNUST 1005
91 KNUST 1006
100 KNUST 1001
Bradyrhizobium vignae 90 KNUST 1002 7-2
Bradyrhizobium liaoningense LMG18230
Bradyrhizobium japonicum USDA 6
60 99 Bradyrhizobium diazoefficiens SEMIA 5080
Bradyrhizobium diazoefficiens USDA 110
73 Bradyrhizobium canariense BTA-1
Bradyrhizobium betae LMG 21987
Bradyrhizobium ottawaense OO99
Bradyrhizobium cytisi CTAW11
95 Bradyrhizobium rifense CTAW71
Bradyrhizobium manausense BR 3351
Bradyrhizobium iriomotense EK05
Bradyrhizobium neotropicale BR10247
79
99 Bradyrhizobium paxllaeri Bradyrhizobium lablabi CCBAU 23086LMTR 21
100 Bradyrhizobium jicamae PAC68
Bradyrhizobium retamae Ro19
99 Bradyrhizobium erythrophlei CCBAU 53325
99 Bradyrhizobium ferriligni CCBAU 51502
77 Bradyrhizobium elkanii USDA 76
94 Bradyrhizobium tropiciagri SEMIA 6148
99 Bradyrhizobium pachyrhizi PAC48 Bradyrhizobium embrapense SEMIA 6208
Bradyrhizobium denitrificans LMG 8443
100 Bradyrhizobium oligotrophicum LMG 10732
0.05

Appendix 7. Maximum likelihood phylogenetic tree based on ITS region showing relationships between isolates, type species (^{T}) and reference strains of the genus *Bradyrhizobium*.

The phylogenetic tree was built based on the Kimura 2-parameter model. Bootstrap values were inferred from 500 replicates and are indicated at the tree nodes when ≥50%. The bar represents five or two estimated substitutions per 100 nucleotide positions.



Planting day for each experimental site: 21st July = Pishegu, 22nd July = NantonKurugu/Sampkem, 24th July, Nangunkpang 2, 25th July = Nangunkpang 3, 28th July = NangunNayilli/Nangunkpang, 29th July = Kunang, 1st August = Karaga, 5th August = Jegunkukuo, 7th August = Nyeko kukusoli/Nyeko



Planting day for each experimental site: 8th July = Pishegu, 12th July = Sampkem/Bantanyilli, 14th July = NantoKurugu/Kunang 2, 15th July = Zoggu/Nyeko B, 16th July = Kunang, 1st August = Karaga, 2nd August = NangunNayilli, 7th August = Nyeko D, 23rd August = Nangunkpang 1, 24th August = Nangunkpang 2, 26th August = Nangunkpang 3

Appendix 8. Rainfall distribution during 2016 cropping season for cowpea (a) and groundnut (b)

Appendix 9. T-test on nodule dry mass between previously inoculated and re-inoculated plots

Host legume	Treatment	Mean NDM (mg 10 plants^{-1})	SD	*t-value*	*Pvalue*	95% CI
Groundnut	KNUST 1002 Prev inoc	681	208	0.08	0.94	-131.3, 142.3
	KNUST 1002 Re-inoc	686	219			
	KNUST 1006 Prev inoc	811	196	0.48	0.63	-116.7, 189.3
	KNUST 1006 Re-inoc	848	274			
Cowpea	KNUST 1002 Prev inoc	93	44	2.78	0.010	17.3, 115.5
	KNUST 1002 Re-inoc	160	97			
	KNUST 1006 Prev inoc	86	56	1.46	0.15	-12.0, 72.9
	KNUST 1006 Re-inoc	117	75			

Prev inoc = Previously inoculated, Re-inoc = Re-inoculated, NDM = Nodule dry mass, SD = Standard deviation, CI = Confidence Interval

Appendix 10. T-test on grain yield between previously inoculated and re-inoculated plots

Host legume	Treatment	Mean Grain yield (kg ha^{-1})	SD	*t-value*	*Pvalue*	95% CI
Groundnut	KNUST 1002 Prev inoc	742	401	0.24	0.81	-223, 283
	KNUST 1002 Re-inoc	712	390			
	KNUST 1006 Prev inoc	848	377	0.15	0.89	-235, 273
	KNUST 1006 Re-inoc	829	416			
Cowpea	KNUST 1002 Prev inoc	835	347	0.05	0.96	-216, 226
	KNUST 1002 Re-inoc	830	342			
	KNUST 1006 Prev inoc	668	347	0.94	0.35	-132, 362
	KNUST 1006 Re-inoc	783	420			

Prev inoc = Previously inoculated, Re-inoc = Re-inoculated, NDM = Nodule dry mass, SD = Standard deviation, CI = Confidence Interval