GENETIC DIVERSITY AND EFFECTIVENESS OF ELITE INDIGENOUS NODULATING RHIZOBIA ON SOYBEAN PRODUCTIVITY IN SOUTH KIVU PROVINCE, DEMOCRATIC REPUBLIC OF CONGO

BINTU NABINTU NDUSHA

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UNIVERSITY OF NAIROBI

2021

DECLARATION

This thesis is my original work and has not been submitted for award of a degree in any other university.

……..... Date:18/08/2021……

Bintu Nabintu Ndusha

This thesis has been submitted with our approval as University supervisors.

Supervisors:

Signature …  Date 21/08/2021 ……..

Prof. Shellemiah Okoth Keya

Department of Land Resource Management and Agricultural Technology, University of Nairobi

Signature ……  Date 21/08/2021 ……..

Prof. Richard Onwonga

Department of Land Resource Management and Agricultural Technology, University of Nairobi

Signature ……  Date: 20/08/2021 …….

Dr. Leon Nabahungu

International Institute of Tropical Agriculture, D.R. Congo

Signature…….  Date: 21/08/2021 ……..

Prof. Gustave Mushagalusa Nachigera

Université Evangelique en Afrique, D.R. Congo

DECLARATION OF ORIGINALITY

Name of student: BINTU NABINTU NDUSHA

Registration number: A80/50110/2015

College: COLLEGE OF AGRICULTURE AND VETERENARY SCIENCES (CAVS)

Faculty/School/Institute: FACULTY OF AGRICULTURE

Department: LAND RESOURCE MANAGEMENT AND AGRICULTURAL TECHNOLOGY

Course name: SUSTAINABLE SOIL RESOURCE MANAGEMENT

Title of work: **GENETIC DIVERSITY AND EFFECTIVENESS OF ELITE INDIGENOUS SOYBEAN NODULATING RHIZOBIA ON SOYBEAN PRODUCTIVITY IN SOUTH KIVU PROVINCE, DEMOCRATIC REPUBLIC OF CONGO**

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DEDICATION

I dedicate this work to the entire Ndusha family. A special dedication to my husband Burhama Norbert, for the infinite encouragement and my children Glodie Nshokano, Armel Ashuza, Grace Nabintu, Jordan Koko and David Agisha for their unconditional love.

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LIST OF ABBREVIATIONS AND ACRONYMS

BLAST:	Basic local alignment tool
BNF:	Biological nitrogen fixation
Bp:	Base pair
CIALCA:	Consortium for Improving Agriculture Livelihood in Central Africa
DNA:	Deoxyribonucleic acid
DRC:	Democratic Republic of Congo
DSCRP :	Dossier Strategique pour la croissance et la reduction de la pauvrete.
EDTA:	Ethylenediaminetetraacetic acid
FAO:	Food and Agriculture Organization
FAOSTAT:	The Food and Agricultural Organization corporate statistical database
ICSP:	International Committee on Systematics of Procaryotes
IITA:	International Institute of Tropical Agriculture
LSD:	Least significant difference
MGT:	Mean generation time
MINAGRI:	Ministry of Agriculture
MLSA:	Multilocus sequence analysis
N:	Nitrogen
N_2 Africa:	Nitrogen Africa
N_2:	Nitrogen gas
PCR:	Polymerase chain reaction

RecA:	Recombinase A gene
RFLP:	Restriction fragment length polymorphism
rRNA:	Ribosomal ribonucleic acid
SDW:	Shoot dry weight
SNR:	Soybean-nodulating rhizobia

ABSTRACT

Soybean (*Glycine max L. Merr.*) is an important crop in South Kivu Province of the Democratic Republic of the Congo (DRC), but its productivity has been limited by poor soil fertility. Inoculation of rhizobium is touted as an effective and sustainable way to improve soil fertility and soybean productivity. Despite the introduction of soybean nodulating rhizobia (SNR) through inoculants in South Kivu, biological nitrogen fixation and soybean yield have not increased to the desired level. This study was therefore conducted in South Kivu province to assess the genetic diversity and the effectiveness of indigenous rhizobia in enhancing biological nitrogen fixation and soybean productivity in South Kivu. The genetic diversity was assessed based on *16S rRNA*, *recA, glnII-2* and *glnII-12* genes. Full genomes of 24 selected SNR were obtained on Miseq, libraries prepared using Nextera xt protocols and compared with the published genomes of the commercial strain *Bradyrhizobium japonicum* USDA 110, accession CP011360. Greenhouse and field experiments were conducted to determine the effectiveness of selected indigenous SNR on soybean's BNF and yield enhancement. Completely Randomized Design and Randomized Complete Block design were employed in the greenhouse and field experiment respectively. A survey was conducted to assess the perception and determinants of rhizobium inoculants'adoption among soybean smallholder farmers. The *16S rRNA* phylogeny showed 70 indigenous rhizobia in two major clusters while two housekeeping genes (*recA* and *glnII*) based phylogeny divided them into three clusters showing a high diversity. Six indigenous rhizobia strains and *B. japonicum* USDA 110 strain clustered together with high bootstrap values (84%) suggesting a high degree of relatedness. Genome features of 24 indigenous rhizobia were determined and varied significantly. The genome size was 8.383 Mb ±0.762 Mb in length with an average Guanine-Cytosine (GC) content of 62%. The chromosomes comprised a mean of 8063±975 genes, 7992±978 potential protein-coding genes, 1.2±0.43 set of rRNA genes and 57±9.8 tRNA genes. Based on genome size, the number of protein-coding genes, C-G content and tRNA, six indigenous rhizobia showed high similarity (mean genetic distance=0.04) with the commercial strains USDA110. The best inoculation treatments across all experiments were the indigenous strains NAC46 and NAC17 which improved nodulation equally or better than the commercial strain USDA 110. In the field NAC46 and NAC17 increased soybean grain yield from 2.4 t ha^{-1} to 3.3 t ha^{-1} and 3.4tha^{-1} respectively; indicating an increase of 68.7% and 70.8% respectively, over the commercial strain

USDA110. The survey results indicated that smallholder farmers perceive rhizobium inoculants as an affordable means (58%) of improving soybeans productivity and strongly agreed that they could use rhizobium inoculants if available in the market (73%), to enhance soybean's biological nitrogen fixation and productivity. However, the adoption of soybean inoculants was very low (23.9%) and was highly influenced ($P<0.01$) by the farmer's location, gender, type of education, the awareness of nodules, the household income and inoculant perception. This study concludes that indigenous rhizobia have higher potential for increasing soybeans yields and BNF in the South Kivu province of DRC. These indigenous SNR could be used for inoculant production for the region.

CHAPTER ONE: GENERAL INTRODUCTION

1.1. Background

The world population in 2014 was estimated at 7.2 billion (Gerland *et al.,* 2014). In the next 2050, this population is projected to double (Cleland, 2013) and this situation will be aggravated in Sub Saharan Africa (Thomas and Zuberi, 2012). Feeding the African population will be the major challenge and if tremendous transformations are not made to establish food security in the near future, this challenge will be irreversible. Enhancing yield of major cash crops, source of extra income for many households, by intensification using efficient and adaptable technologies will be crucial instead of increasing croplands (Sanginga and Woomer, 2009). One of the potential pathways for agriculture intensification is the incorporation of legumes in cropping systems. They can fix nitrogen from air; thus, improve the fertility status of soils, which are mostly depleted in Sub Saharan Africa (Vanlauwe *et al*., 2015). Legumes are also important in supplying rich protein for the population and combat malnutrition which is high in Sub Saharan Africa (Gibson and Ferguson, 2008).

Amongst legumes, soybean [*Glycine max* (L.) Merr.] is a crop of global importance cultivated for its higher protein and edible oil (Hungria et al., 2005). Soybean is fourth among the top traded commodities after wheat, rice and corn (Hartman et al., 2011). This crop has been promoted in South Kivu province (Eastern Democratic Republic of Congo), since 1985 by the humanitarian organization and United Nations agencies such as Food and Agriculture Organization (FAO), to address the issues of malnutrition induced human diseases following the political strife of 1985 (Kismul et al., 2015). Therefore, soybean cultivation has increased mainly as result of its utilization as medicinal food in public schools and hospitals to prevent and cure the wasting effects of malnutrition (Bisimwa et al., 2012; Kismul et al., 2015). Soybean is also used in household to improve nutrition status (Pypers et al., 2011; de Jager et al., 2019) and in livestock especially in poultry production and aquaculture (Khojely et al., 2018). Consequently, soybean acreage has increased from 13,310 ha to 55,863 ha (4.197 folds) in between 1990 and 2018 with total

production of 12,070 t and 25,772 t, respectively (FAO, 2018). These observations indicate a decline in soybean productivity between 1990 (0.90 t/ha) and 2018 (0.51 t ha^{-1}).

Besides high nutritive value, soybean fixes nitrogen from the atmosphere by forming a symbiotic association with a group of bacteria (rhizobia) through a process known as biological nitrogen fixation (BNF) (Collino et al., 2015). Nitrogen fertilizers, for instance, are the most important inputs to maximize crop production (Salvagiotti et al., 2008). It should be noted that the soils of South Kivu have been depleted due to continuous cropping without soil replenishment as a consequence of population pressure (Bashagaluke et al., 2015). The Exploitation of BNF in legume crops such as soybean through rhizobia is an economical, renewable and environmentally friendly source of nitrogen that increases nitrogen availability to crops (Salvagiotti et al., 2008). Moreover, nitrogen derived from BNF is readily available to plants and less vulnerable to leaching, denitrification and volatilization losses (Olivares et al., 2013).

Two main approaches have been pursued by researchers from international organizations to improve soybean' yield and BNF. First, promiscuous soybean cultivars were developed to form nodules freely with native rhizobia (Tefera, 2011). Second, inoculation with highly effective rhizobia strains has been disseminated (van Heerwaarden et al., 2018). These developed varieties were widely disseminated all over Africa (Sanginga and Okogun, 2003) but it was observed that native rhizobia were not always effective in enhancing BNF and productivity (Thuita et al., 2012). Therefore, Biofix Legume inoculant containing rhizobia strain, *Bradyrhizobium japonicum* USDA110 was introduced in South Kivu by N2 Africa project since 2010 (www.n2africa.org) and distributed among farmers by agricultural extensions services and humanitarian organization (van Heerwaarden et al., 2018).

From trials and farmer's fields results, the commercial inoculant increased legume yield from 500 to 1343 kg ha^{-1} (van Heerwaarden et al., 2018), but still not to the desired levels (Ulzen et al., 2016) as the potential soybean yield is 3000 kg ha^{-1} (Salvagiotti et al., 2008; Zanon et al., 2016). That low improvement was attributed to the effect of environmental and edaphic conditions on the introduced commercial strains in addition to the failure to overcome the competition barriers caused by native rhizobia (van Heerwaarden et al., 2018).

Many studies in Africa have reported the presence of effective rhizobia strains among indigenous rhizobia populations (Musiyiwa et al., 2005; de Almeida Ribeiro et al., 2015; Chibeba et al., 2017). In addition, indigenous rhizobia strains were reported to be more persistent and well adapted; consequently, more competitive compare to commercial strains (Fening and Danso, 2002). Therefore, this study will contribute to the identification of highly effective strains among indigenous populations.

1.2 Statement of the problem

Despite the importance of soybean in improving food security, nutrition and soil fertility, in addition to crop promotion, the yield in South Kivu remains low. Only 0.5t ha^{-1} of grain yield was reported in DRC by FAO (2018), while the potential grain yield of soybean is 3t ha^{-1} (Salvagiotti *et al*., 2008). This yield is also low compared to neighboring countries with the same climatic conditions such as Uganda (1.2t ha^{-1}) and Tanzania (1.5t ha^{-1}) (FAO, 2018). Declining soil fertility coupled with low utilization of mineral fertilizers that are exorbitant and not affordable to poor farmers is the major factor contributing to low soybean productivity in South Kivu (Pypers et al., 2011; Minagri, 2016; Walangululu *et al.,* 2010).

Despite the potential of the introduced strains through commercial inoculants to increase soybean's yield in South Kivu, the increment is below the anticipated productivity (van Heerwaarden et al., 2018). The indigenous rhizobia population in South Kivu soils remains uncharacterized and unclassified (Ndusha, 2014). Besides being recognized as a high humid forest zone characterized by high vegetation diversity and highlands (Potapov et al., 2012), limited information is available on indigenous SNR diversity in the South Kivu province of DRC. The rhizobia systematic has been revised in recent years with the addition of several new genera and species, there is certainly much more to discover (Berrada and Fikri-Benbrahim, 2014; De Lajudie et al., 2019).

Although, there are many studies on the selection of highly effective rhizobia among indigenous populations (O'Hara et al., 2002; Chibeba et al., 2017; Chibeba et al., 2018), they are time consuming and hence there is a need for an efficient and rapid selection approach in South Kivu where soils lack nitrogen. Concerning soybean, there is also limited information on the effectiveness of indigenous rhizobia strains on BNF and soybeans yield under different soil

conditions prevailing in South Kivu (Sanginga et al., 2016). Besides, there is a paucity of information on perception and rhizobium inoculants adoption's determinants among soybean farmers of South Kivu. Therefore, this study was conducted to assess the genetic diversity and effectiveness of indigenous rhizobia in enhancing biological nitrogen fixation and soybean productivity.

1.3 Justification

Soybean [*Glycine max* (L.) Merr.] is a benefit crop that could alleviate malnutrition, improve soil fertility, increase farmers's income and consequently increased food security in South Kivu, a region that is characterized by high malnutrition rate due to repetitive wars. The characterization of indigenous soybean-nodulating rhizobia in South Kivu could allow understanding their similarity and particularity compare to those of other countries and thus facilitate selection of elite strains. The genome comparison of indigenous rhizobia would allow the identification of genetic components associated with high nitrogen fixation ability in order to orient a selection program.

Testing indigenous strains in contrasting soil conditions could permit the identification of the indigenous strains that are adaptive, competitive and highly effective in improving soybean's BNF and productivity. These strains could replace the commercial strains that are expensive and less adapted to local condictions. Assessing the perception and determinants toward adoption of rhizobium inoculants would inform on action for increasing the use of inoculants products. The use of indigenous rhizobia strains as inoculants is a cheap and environment friendly alternative to enhance soybean yields where farmers cannot mostly afford the expensive N mineral fertilizers. Use of these strains on varieties adapted to local conditions will enhance soybean production and BNF. Improving soybean production and BNF in different soil conditions has beneficial effect on food and nutrition security (FNS) and on the environment.

1.4. Objectives

1.4.1. Overall objective

This study's overall objective was to assess the genetic diversity and effectiveness of indigenous rhizobia in enhancing soybean productivity and Biological Nitrogen Fixation for improved food and nutrition security in South Kivu, Eastern DRC.

1.4.2. Specific objectives

(i) To assess the genetic diversity of indigenous soybean-nodulating rhizobia strains isolated from South Kivu soils,

(ii)To assess the genomic characteristics of selected indigenous soybean-nodulating rhizobia and genetic components associated with high nitrogen fixing ability in indigenous rhizobia,

(iii)To determine the effectiveness of elite indigenous soybean-nodulating rhizobia in enhancing soybean BNF and yield in different soil conditions,

(iv)To assess the adoption' determinants and perception of rhizobium inoculants among soybean smallholder farmers of South Kivu.

1.5. Hypotheses

(i) Indigenous soybean nodulating rhizobia are not highly diverse and not vary considerably in different types of land uses;

(ii) Considerable variations in genomic features do not exist among indigenous rhizobia;

(iii) The effectiveness of indigenous rhizobia in yield and BNF improvement do not vary significantly under different soil conditions.

(iv) Demographic factors do not have greater influence in the adoption of rhizobium inoculants among smallholder farmers of Southern Kivu province.

CHAPTER TWO: LITTERATURE REVIEW

2.1. Soybean crop

2.1.1. Classification and origin

Soybean is an annual crop that belongs to the order *Fabales*, the family *Fabaceae,* the subfamily *Faboidae* and the genus glycine (Ghosh *et al.,* 2013). According to the legend, Soybean was domesticated for the first time in China, especially in North East of China around 1700–1100 B.C. (Han et al., 2016). In Africa, soybean was introduced in the early 19th century through missionary, trade and colonialism (Abuli, 2016). Egypt was the first country in Africa to cultivate soybean in 1858, followed by Tunisia (1873) and Algeria in 1880 (Shurtleff and Aoyagi, 2009). IITA started soybean breeding for improvement around 1974, and in 1970 some National Agricultural Research Services (NARS) started promotion of soybean production and inclusion in population diets (Tefera, 2011).

In DRC, soybean was first introduced by colonialists in 1908 (Shurtleff and Aoyagi, 2009). The cultivation became intense in some of the provinces like South Kivu, only in 1985, when due to civil conflict in rural areas, there appeared a high malnutrition rate among children and women and malnutrition induced diseases called Kwashiorkor. Nutrition specialists recommended soybeans to deal with these diseases (DSCRP, 2011; Kismul et al., 2015).

In the last decades, important investments and researches on soybean have been carried out in Africa in general and in the South Kivu province of DRC. This is in partly because of its high nutritive value which can improve nutrition and food security but also because of the aspect of Biological Nitrogen fixation (BNF), which is a cheaper source of nitrogen for low income farmers and environmentally friendly (Chianu et al., 2009).

The recent researches on soybean in South Kivu were conducted by N2 Africa program of IITA (Sanginga *et al*., 2016). N2 Africa is a large research program aimed at putting Nitrogen to work for smallholder farmers. The objectives of N2 Africa are to increase average legume grain yield,

promote BNF and improve smallholder farmers income (Turner *et al*., 2011). The program brings together a team of scientists, students, inoculants manufacturers and legume breeders.

In South Kivu, research was conducted on the performance of introduced improved soybean varieties (CIALCA 2010) and the commercial inoculants' effectiveness in improving soybean yields (Sanginga et al., 2016; van Heerwaarden et al., 2018). There is limited information on the nature, abundance and the diversity of the indigenous population because introduced commercial strain through inoculant did not improve yields and BNF at desired levels (van Heerwaarden et al., 2018). This failure of commercial strains was attributed to the less adaptive ability and failure to overcome competitiveness barriers opposed by indigenous rhizobia (Gyogluu et al., 2016). There is a need to characterize these indigenous populations of rhizobia and identify adapted and highly effective strains for inoculants production to enhance soybean BNF and productivity. This study contributes to identifying adapted local rhizobia for enhancing soybean Biological Nitrogen Fixation and productivity in South Kivu.

2.1.2. Importance and uses of soybeans

The soybean is called by some authors the "miracle crop" or "meat for the poor" (Chianu et al., 2011; Khojely et al., 2018). Currently, there exist a high number and many types of soybean-based products (Shurtleff and Aoyagi, 2009). It is mainly pressed to extract oil and the remaining is utilized to produce soybean meal (Ali, 2010). Soybean is mainly used to produce animal feeds; 98% of soybean production is utilized as animal and fish feeds and only 2% is consumed directly by humans as food (Hartmann *et al.,* 2011).

Soybean is used in the food processing industry to produce numerous food products such as margarine, soymilk, infant formula, tofu, fried foods, flower, baked goods, snack bars, noodles and many others (Shurtleff and Aoyagi, 2009). Consumption of soybean products increased significantly when the link between animal fat and cardiovascular diseases was established (Raghuvanshi and Bisht, 2010). Soybean also has many therapeutic qualities such as very low content of fatty acid and high antioxidants content namely vitamins C, K, D, folic acid, B complex and nicotinic (Ghosh et al., 2013). The high isoflavones content, with inhibitory effect on cancer cells, was also reported (Qiu and Chang, 2009).

In South Kivu soybean is considered as a cash crop. In this region, women and youth contribute economically in their households, for example by paying school fees or livestock purchasing by growing soybean (CIALCA, 2010). Apart from using soybean to bring in extra income, women use it to improve their children's diets. From a survey conducted in South Kivu, soybean is mostly cultivated and sold to processing units that produce livestock feeds, soyflour, infant formula, soymilk and recently okara, soy bread, and others (Sanginga *et al*., 2016).

2.1.3. Soybean' production trend and yield

The world production and yield of soybean is increasing continuously (Figure 1) and is estimated to be respectively 312 metric tons and 2.9t ha^{-1} (USDA, 2017). The USA occupies the position of soybeans main producer in the world (117.21 metric tons with 3.5 t ha^{-1}) followed by Brazil and Argentina. Other important producers include China, South Korea, and India (USDA, 2017). African soybean producing countries are South Africa (1.3 metric tons of production and 2.9 tons per hectare of yield) followed by Nigeria, with average production of 0.68 metric tons. Zambia and Uganda take the third and the fourth places (Ude et al., 2003; USDA, 2017).

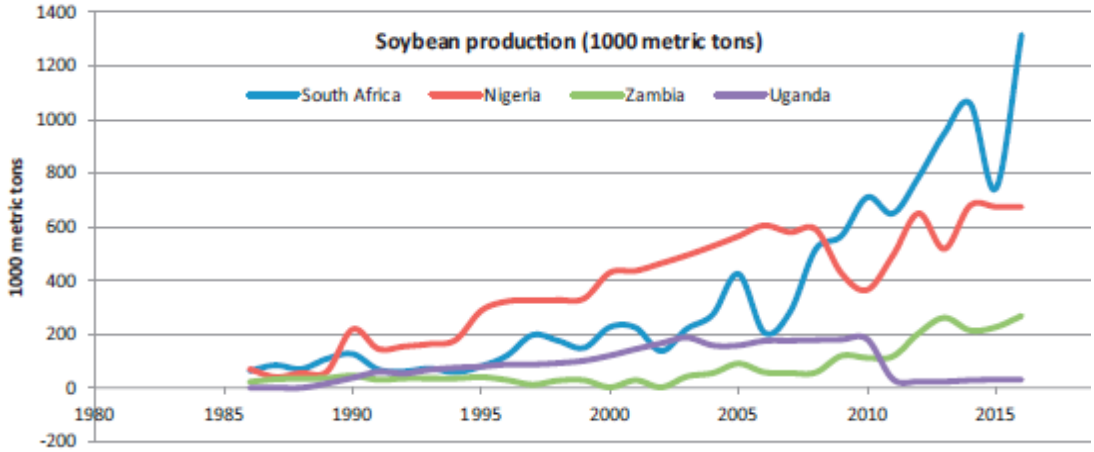

Figure 1: African Soybean production trends source: Khojely et al., 2018

In South Kivu, soybean production and cultivated area have increased these last decades as result of its promotion and high market demand, as demonstrated in Table 1. The improved production is the result of the increase of cultivated area rather than enhanced yield (MINAGRI, 2016). The

noted yield variations between the USA, Brazil and DRC are attributed to high potential cultivars adapted to climatic conditions and the application of the best techniques of soil fertility management (Singh, 2010). Soybean growth periods ranged between 90 and 120 days and required the range of 470-700mm of water and cool temperatures (Dogan et al., 2007). The agro-ecological conditions for South Kivu province of DRC are characterized by 900-1800mm, high solar radiation, cool temperatures and a growing period extended up to 325 days (Pypers et al., 2011). This province has conditions permitting good soybean growth.

International research institutes such as IITA, national research institutions, and universities in Africa released up to 195 soybean varieties suited to African conditions between 1970 and 2011 (Khojely et al., 2018). These soybeans cultivars have specific adaptation traits suited to African conditions; the main traits include high yields, tolerance to drought and high temperatures and, insensitivity to short day length, better disease resistance and susceptibility to nodulation with indigenous rhizobia strains (Sanginga and Okogun, 2003). Although these varieties were highly adopted in Africa (Chianu et al., 2011), the reported yields are still low due to declining soil fertility coupled with unconventional agronomic practices rather than variety adaptation to agroecological conditions (Ronner et al., 2016). Even the so-called promiscuous varieties yielded low and these low yields were attributed to the fact that most of indigenous rhizobia present in tropical soils are ineffective (Fenning and Danso, 2002).

Therefore, the inoculation technique was introduced in Africa and in South Kivu precisely in 2010 (Sanginga et al., 2016). Since, some studies reported the high performance of these inoculants (Thuita et al., 2012; Chibeba et al., 2018) on soybean's BNF and grain yield improvement while other studies did not find any significant improvement (Sanginga and Okogun, 2003). These differences were attributed to the genetic and effectiveness variability among these exotic commercial strains (Ulzen et al., 2016) on one hand and on the other hand to the failure of introduced strains to face the competitiveness barriers opposed by indigenous populations. Also, indigenous rhizobia strains were reported to be of high persistence with adaptive ability to in-situ conditions and thus higher competitivity ability at the expense of exotic strains (Fenning and Danso, 2002).

Table 1: South Kivu annual soybean cultivated area, production and yield

Year	Area (ha)	Production (tons)	Yield (Kg ha^{-1})
2001	8906	4781	537
2002	3744	2270	606
2003	4309	2791	648
2004	3897	1979	508
2005	4131	2251	545
2006	22904	3627	557
2007	8368	4562	545
2008	11123	5957	536
2009	11391	6389	561
2010	6652	4657	700
2011	12895	7163	555
2012	12965	7324	565
2013	13801	7432	539
2014	14048	7374	525

Source: MINAGRI, 2016.

2.1.4. Constraints to soybean production and productivity improvement strategies

Numerous biotic and abiotic factors constrain soybean production. The main biotic factors constraining soybean production include pathogens, pests and weeds. Abiotic factors include weather-related constraints (such as drought, flooding and frost), soil nutrient availability, salinity, and response to photoperiod (Hartman et al., 2011). In Africa the main abiotic factors constraining soybean production include poor soil fertility, poor nodulation and poor seed quality (Murithi et al., 2016). In South Kivu province of DRC, these abiotic factors include declining soil fertility accentuated by low access and use of fertilizers, lack of improved varieties and poor agronomic practices (CIALCA, 2010; Pypers et al., 2011).

From many authors, these constraints may be addressed with the use of high yielding soybean cultivars (Thao et al., 2002; Kumaga and Ofori, 2004; Gicharu et al., 2013), use of adapted fertilizers (Okereke et al., 2001; Tien et al., 2002; Kamanga et al., 2010; Solomon et al., 2012) and application of best management such as right time planting and right spacing (De bruin, 2007).

Soybean's local and international research efforts have been allocated to breeding and spreading improved soybean cultivars (Tufa et al., 2019). For instance, between the years 1970 and 2011, about 197 improved soybean varieties were introduced in Africa (Khojely et al., 2018). The qualities claimed for these new varieties include higher-yielding, stress tolerance and the capacity to nodulate effectively with native rhizobia (Tufa et al., 2019). This dissemination has been done by organizations such as N2Africa, the Syngenta Foundation for Sustainable Agriculture (SFSA), the African Agricultural Technology Foundation (AATF) and the Tropical Legume project of IITA (Chigeza et al., 2017; Santos, 2019).

However, many authors demonstrated that these varieties can perfom well only in combinations with best agricultural practices such as right planting dates (Salmerón et al., 2016), optimum plants population (Ren et al., 2016), grain inoculation with rhizobia (Ronner et al., 2016; Chibeba et al., 2017; Thuita et al., 2018) and phosphorus application (Thioub et al., 2019; He et al., 2019). These agricultural practices combined with the use of high yielding soybean cultivars were reported to increase yield to maximum (Van Vugt et al., 2017). Moreover, very high soybean yield has been reached in many countries without fertilizers, only with the contribution of Biological Nitrogen Fixation (BNF) (Hungria et al., 2015). The state of utilization of inoculation techniques for soybean improvement in the South Kivu province of DRC has not yet been assessed. Inoculation techniques using adaptive and competitive indigenous rhizobia in improving BNF and soybean productivity have not yet been assessed under different South Kivu agro ecological conditions (CIALCA, 2010). This study, therefore, evaluated the effectiveness of inoculation techniques using indigenous rhizobia.

2.2. Importance of Biological Nitrogen Fixation in soybean production

2.2.1. Importance of Biological Nitrogen Fixation

Nitrogen is the main constituent of all living organisms and consequently the most nutrient limiting their growth. Although 78% of the atmosphere is composed of nitrogen (N_2), this form is inaccessible to most organisms, and consequently, it needs to be converted into usable forms such as NH_3, NH_4^+, and NO_3^- (Bellenger et al., 2020). Biological nitrogen fixation (BNF) is a process by which bacteria (mostly rhizobia) enter into a symbiotic relationship with legumes resulting in nodulation where the inert form of N (N_2) is converted into ammonia (Vitousek et al., 2013). The reduction of N_2 into ammonia is achieved through the following equation:

$N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16P_i$ (Dixon and Kahn, 2004).

BNF is important because it reduces the application of N mineral fertilizers in agroecosystems. It is an inexpensive option for farmers because its cost is low than the N-fertilizers. Silva and Uchida (2000) demonstrated that the cost of N due to the application of rhizobia-based inoculants is only $3/ha and is equivalent to fertilizer N that cost $87. Besides, inoculants are also environmentally friendly as it prevents the contamination of water resources by excess fertilizers from leaching and runoff (Vitousek et al., 2013). The amount of Nitrogen fixed through BNF was estimated at around 16.4 Tg per annum (Herridge et al., 2008). Soybean has been rated as the most important legume that contributes to BNF with up to 450 kg $Nha^{-1}year^{-1}$(Hungria and Mendes, 2015). The effect of soybean variety, the rhizobium strain nodulating soybean, environment, agronomic practices and their interactions determine the success of BNF in soybeans (Giller et al., 2013).

Better yields have been achieved by some countries namely Brazil in 2003 (3 t ha^{-1}) and their success is totally attributed to the use of high yielding soybean varieties in the conditions that favor high BNF and continuous attention paid to the selection of highly effective rhizobian strains adapted to the used varieties (Alves et al*.,* 2003). Some African countries also, have achieved higher yield with the use of high effective indigenous strains as inoculants combined with better soybean variety; for example, yield of 1.5 t ha^{-1} was reported in Kenyan soils (Waswa et al., 2014). In South Kivu, only a few studies were conducted on soybean and BNF; namely isolation and

testing of indigenous rhizobium strains in controlled conditions (Ndusha, 2014). There is still many missing information in Nitrogen fixation in South Kivu (Turner et al., 2011).

2.2.2. Soybean nodulating rhizobia diversity

Rhizobia refer to the group of bacteria that can establish symbiosis with legumes and form root nodules where the nitrogen fixation occurs (Zakhia and de Lajudie, 2001; Peix et al., 2015). These bacteria are, in general gram-negative, prokaryotic, heterotrophs and belong to the alpha-proteobacteria group, and recently, some species were discovered in the group of beta-proteobacteria (Rivas et al., 2009).

The taxonomy of rhizobia has changed considerably from the discovery in 1888 by Beijerinck to date (De Lajudie et al., 2019). At the origin, this taxonomy comprised of only one genus (*Rhizobium*), but now many other species representing several genera were discovered. For example, in 2001, six more genera were reported; in 2003, twelve more genera were reported; in 2014, fourteen genera and in 2017, sixteen genera (De Lajudie et al., 2019). The evolution of rhizobia taxonomy was summarized by Shamseldin et al., (2017). This taxonomy is in permanent evolution (Ahmad *et al*., 2008) due essentially to the continuous development of accurate molecular methods of microorganisms' identification (Shamseldin et al., 2017).

According to De Lajudie et al., (2019), the current taxonomy describes all known rhizobia as belonging to the group of alphaproteobacteria and few belong to the group of beta-proteobacteria. Those belonging to the alphaproteobacteria include the families of *Rhizobiacea, Ensifer (syn. Sinorhizobium), Allorhizobiacea, Pararhizobiacea, Neorhizobiacea, Shinella, Phyllobacteriaceae (Mesorhizobium, Aminobacter, Phyllobacterium), Brucellaceae (Ochrobactrum), Methylobacteriaceae (Methylobacterium, Microvirga), Bradyrhizobiaceae (Bradyrhizobium), Xanthobacteraceae (Azorhizobium)* and *Hyphomicrobiaceae (Devosia)*. Those belonging to the beta proteobacteria include the family of *Burkholderiaceae (Paraburkholderia, Cupriavidus, Trinickia*).

Many authors reported that most of the rhizobia nodulating soybean belong to the *Bradyrhizobium* genus (Wasike et al., 2009; Shiro et al., 2013; Gyogluu et al., 2018). However, other authors

demonstrated that soybean is also nodulated by strains representing other genera, for example Wu et al. (2011) who detected a *Sinorhizobium* in soybean's nodules and Biate et al., (2014) who reported the nodulation of soybean by other genera such as *Rhizobium, Mesorhizobium* and *Sinorhizobium*. There is no available data on the identity and genetic diversity of indigenous rhizobia strains present in the Democratic Republic of Congo, a forest region, especially in South Kivu province. In addition to those mentioned above, there is a need to continue discovering of new strains of indigenous rhizobia because this can contribute to improved soybean BNF and productivity, particularly in South Kivu where high malnutrition rates were noted (Pypers et al., 2011). Rhizobia identification and diversity studies were performed only in few African countries (Thilakarathna et al., 2019).

2.2.3. Methods to study rhizobia characteristics and diversity

There are many methods to characterize rhizobia and assess their genetic diversity between different microorganisms' species. The first characteristic of rhizobia is that the nodule isolate must produce a nodule on a legume (Tindall et al., 2010). Traditional methods are based on characteristics such as growth rate, appearance on a specific media, morphology of the colony, and resistance to antibiotics (Graham et al., 1991; Somasegaran and Hoben 1994).

Based on their growth characteristics on yeast mannitol medium, rhizobia were described as fast growing (2 to 3days) and slow growing (7 days and above). Therefore, rhizobia could be classified as fast growers in the genus of *Rhizobium*, while, the slow growers (from 7days) and alkaline producers were classified as *Bradyrhizobium* (Valerie and Sharon, 1999). However, these methods are not accurate and discriminative. Another method of rhizobia characterization is the serology test, which was used to classify different rhizobia in serogroups (Date, 2001). This was performed by generating antisera to specific strains and use the sera, to generate a serological scheme. This method has many limitations because one serogroup can be identified by three different DNA and new standards were defined for new strains rhizobia identification and characterizations (De Lajudie et al., 2019).

The International Committee on Systematics of Prokaryotes (ICSP) proposed standards for rhizobia identification and characterization and are essentially based on molecular methods. These

standards are explained by de Lajudie et al. (2019). In recent years, molecular methods have been used to detect differences within and between strains of rhizobia (Ampomah and Huss-Dannel, 2016; Delestre et al., 2015).

The study of bacterial species, including rhizobia is performed by sequencing the *16S rRNA* genes (Mwenda et al., 2018). Ribosomal ribonucleic acid (rRNA) including the *16S rRNA* are among highly conserved genes that identify microorganisms to the genus level but may fail to differentiate closely related strains (De Lajudie et al., 2019). Recent findings demonstrated that the *16S rRNA* based phylogeny is not informative enough to compare two close species of rhizobia (Ampomah and Dhuss-Dannel, 2016). For example, Mwenda et al., (2018), in their study on Genetic characterization of Phaseolus *vulgaris*-nodulating rhizobia in Kenya used the *16S rRNA* phylogeny and the phylogeny based on housekeeping genes (*atpD and recA*); they found 3 and 6 clades respectively with *16S rRNA* and housekeeping genes suggesting that the use of both phylogenies is more consistent. Therefore, the sequencing method using different genes like the *atpD, RecA, nodA* can be more accurate for genetic characterization of rhizobia because it distinguishes close strains in the same species (Rashid *et al.,* 2015; Saidi *et al.,* 2014).

As mentioned previously, there exist studies on indigenous identification in many African countries like Zimbabwe, Ethiopia, Kenya, Zambia et., based on the *16S rRNA* and housekeeping genes. Still nothing is known about the genetic characteristics of indigenous soybean-nodulating rhizobia in South Kivu (Turner et al., 2016). Indigenous rhizobia strains were isolated from South Kivu soils and were characterized only by their ability to nodulate soybean but any genetic identification, which identifies strains at species and strains levels was not performed (Ndusha, 2014).

2.2.4. Genetic component associated with high nitrogen fixation in rhizobia

The symbiotic capacity, nitrogen-fixing ability, environmental adaptation, survival and competitiveness in rhizobia are all under the control of genes (Garg and Chandel, 2011). Symbiotic nitrogen fixation is a complex process involving several genes'expression (Menna and Hungria, 2011). These genes are called nitrogen fixation genes; they include genes responsible of nodules formation (*nod* genes), genes in charge of nitrogenase formation (*nif* genes) and function (*fix* genes) and are located in the portion of genome called symbiotic island and plasmid (Young et al., 2006).

The symbiotic nitrogen fixation ability is restricted to some strains therefore sequencing the whole genome now helps to identify strains with high potential for nitrogen (Amadou et al., 2008).

The genome refers to an ensemble of genes within species and includes both coding and non-coding DNA, and comprises core and accessory genomes (Young et al., 2006). The genomic features of rhizobia refer to the characteristics like genome size, number of orthologous genes, the percentage of protein-coding genes, cluster of orthologous genes (COG), number of RNA according genes, number of replicons, chromosome size, size of the symbiotic Island, the presence and number of nitrogen fixation genes (Han et al., 2009; Li et al., 2011; Reeve et al., 2010). By the knowledge of the genome, all characteristics of strains can be identified (Sablok et al., 2017). It is possible to identify rhizobia strains with a high potential of nitrogen fixation by careful genome analysis (Weidner et al., 2003). For example, Dos Santos et al., (2012), in their study on nitrogen fixation assessment, could predict nitrogen fixation based on a careful comparison of nitrogen fixation genes within a genome. Furthermore, they proposed a new criterion for the prediction of Nitrogen fixation by analyzing a bacteria genome. Gonzalez et al. (2006) have also concluded that the large size of the rhizobia genome may be related to high adaptative ability.

The availability of a higher number of genomes in the Genbank facilitates a better understanding of the process of nitrogen fixation for its better improvement (Tian et al., 2012). To date, only a few whole genomes of soybean–nodulating rhizobia has been sequenced and still new recoveries are being made about these organisms (Thilakarathna and Raizada, 2017). Giraud et al (2007), for example, discovered three *Bradyrhizobium* species that lack the *nod* genes though they effectively nodulate soybeans suggesting that these species have different symbiosis strategies with the host. The availability of many genomes of rhizobia will help understand important mechanisms such as the survival ability and competitiveness for nodules occupation of rhizobia, which are still major obstacles in the success of rhizobial inoculants (Tian et al., 2012). To date, no other studies have sequenced the whole genome of indigenous soybean-nodulating rhizobia in the soils of the Democratic Republic of Congo (Turner et al., 2016). The current study compared 24 soybean-nodulating rhizobia from DRC soils based on genomics features to document their potential of improving BNF and soybean productivity.

2.2.5. Effectiveness of rhizobia in BNF and legume yield improvement

There exist differences between rhizobia in their ability to fix nitrogen although the process of BNF is similar (Dixon and Kahn, 2004). Based on this criterion, rhizobia were grouped into four categories (non-infective, infective but ineffective, partially effective and effective). An effective strain is defined as a strain that produces nodules where nitrogen fixation occurs (Howieson et al., 2005). In addition to having higher N fixation rates, this effective strain must have a higher competitiveness ability for nodules occupation than the indigenous population of rhizobia (Batista et al., 2015). The less competitiveness of introduced strains is among the principal reasons for non-response to inoculation when exotic rhizobia are introduced in a new soil highly concentrated in indigenous rhizobia population (Estrella et al., 2009). A good strain must be highly competitive and highly effective at the same time.

The effectiveness of rhizobia is complex and depends on the legume host, the rhizobia strain, the environment, the agronomic management and their interaction. Giller et al. (2013) expressed this effectiveness by the following equation: (GL × GR) × E × M where GL means legume germplasm; GR means rhizobia strain; E means environment, and M means agronomic management.

Each rhizobia strain prefers to nodulate a specific group of legumes and vice versa (Wang et al., 2012). This phenomenon is referred to as degrees of specificity, which is controlled by the exchange of compatible signaling molecules between the host (legume) and the symbiont (rhizobia) (Hassen et al., 2020). In the host legume, the difference in nitrogen fixation occur depending on the growth potential and the length of the vegetative stage while in the symbiont (rhizobia), this difference depends in their survival ability and ability to utilize different types of carbon (Nelson and Sadowski, 2015). Many studies have demonstrated the different responses to inoculation of soybean cultivars. Rurangwa et al., (2018) in their study on the benefit of rhizobial inoculation, Phosphorus fertilization, and manure application on soybean and common bean, found that the benefit of the two legumes to inoculation vary greatly and observed that soybean benefit more from inoculation compare to the common bean. Equally, some strains have been demonstrated to be superior compare to others in symbiotic nitrogen fixation (Dwivedi et al., 2015).

Environmental factors also affect legume growth, rhizobia survival and consequently the effectiveness of symbiotic nitrogen fixation (Graham and Vance, 2003). The environmental factors affecting the effectiveness of nitrogen fixation include nutrient deficiency (Dwivedi et al., 2015), salinity (Faghire et al., 2011; Garg and Chandel, 2011), unfavorable pH (Nohwar et al., 2019), drought (Staudinger et al., 2019), extreme temperatures (Santachiara et al., 2019) and diseases (Joshi et al., 2014). The essential mineral nutrients required for legume effective symbiotic nitrogen fixation are those required for plant growth (C, H, O, N, P, K, S, Ca, Mg, Fe, Zn, Mn, Cu, B, Mo, Cl, Ni, and Co) (Dwivedi et al., 2015). Each essential nutrient plays specific physiological and biochemical roles and is critical for optimum nitrogen fixation (Weisany et al., 2013). However, many studies demonstrated that phosphorous is particularly critical for nitrogen fixation (Ronner et al., 2016). Under field condition, the population of indigenous rhizobia also highly influence the effectiveness of nitrogen fixation (Bogino et al., 2011). A successful rhizobial inoculant must out-compete less effective bacterial strains native to the soil (Lindstrom et al., 2010).

In Africa many studies have been conducted on the effectiveness of indigenous rhizobia strains in BNF and yield improvement of soybean in different soil conditions. For example, Gyogluu et al., (2016) assessed the symbiotic response of soybeans to inoculation by different *Bradyrhizobium japonicum* strains at three experimental sites in Mozambique. They found that response varies depending on different sites and suggested that there are specific effects of sites on nodulation and dry matter improvement by rhizobia. In South Kivu, indigenous rhizobia strains were tested in controlled environment for nodulation ability with soybean and for increasing biomass (Ndusha, 2014). The effectiveness of these strains in improving soybean'BNF and productivity in different soil conditions was not assessed. Therefore, this study determined the effectiveness in N fixation of selected highly effective indigenous rhizobia in different soils conditions of South Kivu province.

2.2.6. Rhizobia based fertilizers: inoculants

Rhizobia bacteria are naturally present in soils (Aserse et al., 2012) but often native rhizobia are ineffective to nodulate a legume (Gyogluu et al., 2018) or present in low concentration to sustain an optimum BNF (Ogola et al., 2020). Therefore, rhizobial inoculation is always needed to sustain BNF and higher legumes yields. Rhizobial inoculation was defined as a technique of introducing a high population of effective rhizobia to the legume rhizosphere (Deaker et al., 2004). It can be applied directly to the soil or coated on seeds (Date, 2001). *Rhizobium* inoculation has been described as a cheap and effective source of fertilizers for legumes yields enhancement (Ndakidemi et al., 2006).

There have been contradicting results on the effectiveness of inoculants products among African farmers; numerous authors have reported a yield increase of legumes while others did not observe a significant increase in grain legumes. For example, Chibeba et al. (2017) reported a yield increase of 5% over the non-inoculated plots and Van Heerwarden et al., (2018) who reported a yield increase from 1.2 to 1.3 tons per hectare of inoculated plots over the non-inoculated plots across African countries. These differences in the performance were attributed to the genetic, effectiveness, and competitiveness differences in these commercial strains and the environmental conditions, including soil physico-chemical condition and the concentration of native population of rhizobia in soils. In Nigeria for example Sanginga and Okogun (2003) did not observe any difference in soybean grain yield in inoculated treatments while Mpepereki et al. (2000) reported high significant increase in soybean grain yield in Zimbabwe with rhizobia inoculated treatments. Futhermore, Thuita et al., (2012) documented the benefit of soybeans rhizobial inoculation in Kenya.

Rhizobial inoculation technique is a popular practice for soybean cultivation in America, Australia, and Europe with many documented success stories (Martins et al., 2003; Yates et al., 2005; and Albareda et al., 2009). However, in spite of these success stories and its reported accessible cost (Thuita et al., 2012), its adoption among SSA farmers was reported to be very low (Mutuma et al., 2014). The factors affecting the wide utilization of these products in SSA include poor quality of

products (Van Heerwarden et al., 2018), lack of awareness as well as the inaccessibility of inoculants products (Chianu et al., 2011).

While Woomer et al., (1997) and Chianu et al., (2011) identified limited farmers' awareness and inoculants unavailability as an important constraint to its the adoption, there is no information on adoption and demographic factors in rhizobium inoculant adoption among smallholder soybeans farmers in South Kivu province of D.R Congo. In addition, the adoption and profitability of the inoculants product were assessed in other African countries (Getachew, 2016; Mutuma et al., 2014; Nekesah, 2017; Ulzen et al., 2016) however limited information is available in South Kivu. Furthermore, previously conducted studies did not assess the perception of smallholder farmers of the inoculants products since the adoption largely depends on perceptions (Ojiako et al., 2007). Therefore, this study assessed the demographic factors and perceptions that are likely to influence the adoption of rhizobium inoculants among soybean smallholder farmers of South Kivu province.

2.3. Research gaps

Several knowledge gaps exist on indigenous soybean-nodulating rhizobia in South Kivu soils. These gaps include lack of knowledge on nature, abundance and diversity of indigenous soybean-nodulating rhizobia in different types of land uses in South Kivu soils. There is also a lack of knowledge of these native SNR'genomics. Furthermore, indigenous rhizobia from South Kivu were isolated but their performance in BNF enhancement and soybean productivity improvement under diverse soil conditions was not assessed. Finally, there is a lack of knowledge on rhizobium inoculants' perception and factors influencing the adoption of these products in South Kivu province of Democratic Republic of Congo.

CHAPTER THREE: GENETIC CHARACTERIZATION OF SOYBEAN (*GLYCINE MAX (L.) MERR.)* NODULATING RHIZOBIA FROM SOUTH KIVU PROVINCE OF THE EASTERN DEMOCRATIC REPUBLIC OF CONGO

3.1. Abstract

Soybean (*Glycine max (L.) Merr.*) is an important crop in South Kivu province of the Democratic Republic of the Congo (DRC), but its productivity has been affected by poor soil fertility. Inoculation with rhizobia is an effective and sustainable way to improve soil fertility and soybean productivity. One hundred and seven rhizobia strains were isolated from nodules of 213 legume plants collected from cultivated fields and grasslands of South Kivu. These rhizobia were inoculated to soybean variety SB24. Plants were examined for nodulation and shoot biomass production along with a commercial rhizobium strain, *Bradyrhizobium japonicum* USDA 110. The effectiveness index (EI) was determined for each rhizobium strain. Of the 107 isolated strains sequenced for *16S rRNA, recA, glnII-2* and *glnII-12* genes, informative sequences were obtained for 70 strains. Sequences were analysed using bioinformatics tools and clustering was performed in MEGA7 software. Nodulation assay revealed significant benefits ($P<0.01$) among four indigenous strains (NAC14, NAC40, NAC42 and NAC74) for nodule production and 16 strains for dry shoot biomass. The EI of 17 indigenous strains were higher (≥2) compared to the commercial strains. The *16S rRNA* sequence-based taxonomy showed a high genetic diversity of rhizobia in South Kivu with a high frequency of *Bradyrhizobium* (20%), *Kosakonia* (20%), *Enterobacter* (14%), and *Rhizobium* (10%). In contrast, less frequent rhizobia were *Bacillus*, *Beinjerinckia*, *Burkolderia*, *Microvirga*, *Cupriviadus*, *Mesorhizobium* and *Agrobacterium*. The rhizobia diversity was higher in grasslands than in cultivated fields. The *16S rRNA* phylogeny showed 70 native strains into two major clusters and multiple sub-clusters while two housekeeping genes (*recA* and *glnII*) based phylogeny divided them into three clusters. Six native rhizobia strains and commercial strain clustered together with high bootstrap support (>80%), indicating a close genetic relationship. We suggest further studies on these indigenous rhizobia strains as possible candidates for improving soil fertility and crop productivity in South Kivu province.

Keywords: *Bradyrhizobium diazoefficiens* USDA 110, Biological nitrogen fixation, Genetic diversity, Native rhizobia, Soybean.

3.2.Introduction

Soybean (*Glycine max L.Merr.*) is a crop of global importance cultivated for protein and edible oil (Hungria et al., 2005). Soybean is the fourth top traded commodities after wheat (*Triticum aestivum*), rice (*Oryza sativum*) and corn (*Zea mays*) (Hartman et al., 2011). Its cultivation was promoted in Democratic Republic of the Congo (DRC) since 1990 to address the issues of malnutrition induced human diseases following the political strife of 1985 (Shurtleff and Aoyagi, 2010; Kismul et al., 2015). Consequently, soybean acreage has increased from 13,310 ha to 55,863 ha (4.197 folds) in between 1990 and 2018 with total production of 12,070 t and 25,772 t, respectively (FAO, 2018). These observations indicate a decline in soybean productivity between 1990 (0.90 t/ha) and 2018 (0.51 t/ha). Besides its high nutritive value, soybean is capable of fixing atmospheric nitrogen by forming symbiotic association with a group of bacteria (rhizobia), through a process called biological nitrogen fixation (BNF) (Collino et al., 2015).

Despite the importance of soybean in improving food and nutrition security (FNS) and soil fertility, the reported yield in DRC is among the lowest in the world (FAO, 2018). This low yield is attributed to the poor soil fertility and limited use of mineral fertilizers that are expensive and not affordable to poor farmers (Pypers et al., 2011). Therefore, an accessible, affordable and sustainable approach of improving soil fertility is imperative. Nitrogen fertilizers, for instance, are the most important inputs to maximize agricultural production (Salvagiotti et al., 2008). Exploitation of BNF in legume crops (through rhizobia) is an economical, renewable and environmentally friendly source of nitrogen that increase nitrogen availability to agricultural crops (Deaker, 2004; Garg and Geetanjali, 2007; Salvagiotti et al., 2008; Zou et al., 2016). Moreover, nitrogen derived from BNF is readily available to plants and less vulnerable to leaching, denitrification and volatilization losses (Peoples et al., 1995).

Rhizobium refers to a group of bacteria that fixes atmospheric nitrogen in symbiosis with a compatible legume. They are also known as diazotrophic bacteria or legumes nodulating bacteria (Zakhia and de Lajudie, 2001; Menna and Hungria, 2011). The majority of these bacteria belong to the genera *Azorhizobium, Bradyrhizobium, Rhizobium, Mesorhizobium, Ensifer and Paraburkholderia* (Zakhia and de Lajudie, 2001; Zakhia et al., 2004). Recently, rhizobia were

reported from other genera such as *Allorhizobium, Aminobacter, Devosia, Methylobacterium, Microvirga, Ochrobactrum, Phyllobacterium, Shinella and Cupriavidus* (Shamseldin et al., 2017). The presence of rhizobia in soils depends on the presence of the host, climate and soil conditions including soil pH and micro-nutrient concentration (Pádua Oliveira et al., 2017).

Soybean is well appreciated for high BNF ability. Combined use of compatible rhizobium and appropriate agronomic practices can supply up to 85% of soybean nitrogen needs (Alves et al., 2003; Hungria et al., 2006). Many studies have reported soybean nodulation exclusively by rhizobia from genus *Bradyrhizobium* (Delamuta et al., 2013; Chibeba et al., 2017). In comparison, other studies have reported nodulation of soybean by other rhizobia genera (Tian et al., 2012). For example, Ramirez et al. (2018) found that *Burkholderia* and *Paraburkholderia* are the predominant soybean rhizobial genera in Venezuelan soils. The success of soybean nodulation and BNF are determined by microsymbiont genotypes (Zou et al., 2016). Therefore, it is imperative to use adapted rhizobial inoculants to enhance BNF and subsequently the soybean yield (Ronner et al., 2016).

Environmental conditions play a major role *in-situ* microorganism diversity (Zou et al., 2016). Therefore, knowledge of *in-situ* microbial diversity is important for the exploration and better use of microbes in agriculture. This has inspired several studies on native rhizobia diversity in Africa (e.g. Kenya, Mozambique, and South Africa). These countries' agro ecosystem represent the arid and moist Savannah zones (Wasike et al., 2009; Naamala et al., 2016; Chibeba et al., 2017). However, to our best knowledge, such information is not available for high humid forest zone characterized by a high vegetation diversity and highlands (Potapov et al., 2012) like DRC's South Kivu province. Since rhizobia taxonomy has gone through continuous changes in recent years with the addition of several new genera and species, there is certainly much more to discover (Berrada and Fikri-Benbrahim, 2014) about native rhizobium diversity in South Kivu.

Presently, there are several techniques available to assess the rhizobia diversity. These techniques include: (i) whole genome sequencing - it allows identification of species with specific characteristics (Sablok et al., 2017) but this method is time, resources and expertise demanding. (ii) The *rRNA* genes are commonly used in estimation of evolutionary history, taxonomic

assignment and diversity of individual organisms (Janda and Abbott, 2007; Caporaso et al., 2011; Eigen et al., 1985; Kuntzel et al., 1981; Woese, 1987). Therefore, *16S rRNA* phylogeny is commonly used in microbial diversity studies (Janda and Abbott, 2007) despite challenges in differentiating closely related species (Ampomah and Huss-Danell, 2016). However, sequences of housekeeping genes have been extremely useful in distinguishing closely related species (Zhang et al., 2012). Housekeeping genes that are commonly used in rhizobia diversity analysis are *recA*, *glnII* and *atpD* (Berrada and Fikri-Benbrahim, 2014). Some authors have reported significant recombination signal in the concatenated *recA-glnII-atpD* genes sequences (Zhang et al., 2012). Therefore, I used *16S rRNA, recA* and *glnII* gene sequences for establishing genetic diversity of native soybean nodulating rhizobium (SNR) population in DRC's South Kivu province.

3.3.Materials and methods

3.3.1. Source of rhizobia Strains

This study was carried out in DRC's South Kivu province situated between 1°36' - 5° South and 26°49'- 29°20' East with estimated surface of 69,130 km^2 (Pypers et al., 2011). It is recognized as a high humid forest zone depicted by high vegetations diversity (Potapov et al., 2012), highlands and soils that are mostly infertile, Humic Ferralsols and Dystric or Humic Nitisols (Van Engelen et al., 2006). This region has a tropical climate with annual rainfall averaged 1500 mm and average temperature of 18°C (Nash and Endfield, 2002).

3.3.2. Plant Samples, Collection of nodules and Rhizobium Isolation

A total of 213 plant samples comprising cultivated and non-cultivated legumes, obtained from N2 Africa project of IITA, were sampled from cultivated fields and grasslands of Uvira, Kalehe and Walungu villages of DRC's South Kivu province. These villages are situated in major soybean growing zones of South Kivu and represent diverse agroclimatic conditions. Plant samples were identified to the species level (where possible) using the botanical key (Boughey et al., 1968). All cultivated fields used for plant sample collection, were selected randomly and had no previous record of rhizobium inoculation. Plants were uprooted carefully not to detach and damage secondary roots since nodules may be present on both lateral and tap roots.

For rhizobium isolation, minimum of four pink nodules were collected from each plant sample and stored in airtight vials containing silica gel (Mwenda et al., 2018). Nodules were surface desinfected following procedure established by Somasegaran and Hoben, (1994) and crashed on Yeast Mannitol Agar (YMA). Isolates were streaked on YMA multiple times to obtain single cell culture. Working cultures were maintained at 4°C on YMA and stock cultures were maintained at -80°C in 25% glycerol (v/v) for long term storage (Somasegaran and Hoben, 1994) and assigned collection number following the procedure adopted by N2Africa Rhizobiology Laboratory (Ndusha, 2014).

3.3.3 Soybean Nodulation Test

A greenhouse experiment was conducted to test rhizobium strains for nodulation ability on soybean variety SB24 at IITA - Kalambo Station in DRC. The promiscuous soybean SB24, the most cultivated soybean variety in South Kivu, was used as test crop (Walangululu et al., 2014; Mwenda et al., 2018). Soybean seeds were surface disinfected in ethanol (95%) and $HgCl_2$ (0.2%) for 5 min and 3 min, respectively, and washed 7 times in autoclaved distilled water. Seeds were softened by immersion in autoclaved water for 3 hrs. Four seeds, later thinned to three, were planted on sterile sand (pH=7) in three liters capacity PVC pots and inoculated with rhizobia following established procedures (Somasegaran and Hoben, 1994)**.** The experiment was laid out as a completely randomized design with four replicates. Watering was done using nitrogen-free nutrient solution (Broughton and Dillworth, 1970). Plants were harvested at early flowering (R3 stage), and the presence/absence of nodules, nodules numbers, and dry shoot weight were recorded after oven-drying at 70°C for 48 hours (Nash and Endfield, 2002). The EI (Effectiveness Index) was calculated for each strain (Chibeba et al., 2017), where,

$$\text{EI} = \frac{\text{shoot dry weight from indigenous strain}}{\text{shoot dry weight from } \textit{Bradyrhizobium japonicum} \text{ USDA 110 strain}}$$

Based on EI value, indigenous SNR were classified into two groups: effective group (strains with EI ≥ *B. diazoefficiens* USDA110) and non-effective group (strains with EI value less than *B. diazoefficiens* USDA 110 strain). Rhizobia were re-isolated from SB24 nodules and authenticated as indigenous SNR. The data from nodulation were submitted to ANOVA test using GenStat

software version 16. The Least Significant Difference (LSD) test was used to separate treatment means.

3.3.4. DNA extraction and quality check

DNA was obtained by lysis of seven days old single colony cultures. DNA extraction was done through the DNA extraction kit, QIAamp DNA Mini Kit (Qiagen) (Ghimire et al., 2010) following the manufacturer' instructions. The DNA quality control was performed on 0.8% agarose Tris Acetate EDTA (TAE) buffer and visualized under UV light using the GelDoc-It2 imager. The nanodrop and Qubit analysis were performed to determine DNA concentrations.

3.3.5. Amplification of *16S rRNA* regions, *recA, glnII-2* and *glnII-12* genes

The polymerase chain reaction (PCR) was performed on 20µl reaction composed as follow: 10µl of mastermix (Bioneer premix), 7.4µl of Milli-Q water (molecular grade), 0.4µl of each primer (forward and reverse) and 2µl of DNA template (concentrated at 20ng/µl). The PCR was performed on the Mastercycler (Eppendorf) nexus GSx7 in three steps consisting of initial denaturation at 94°C for five minutes, final extension at 72°C for ten minutes, and customized intermediate PCR cycles specific for each set of primer (Table 2). The quality of amplified product was checked in 1.5% agarose gel electrophoresis stained with gel red and read on GelDoc-It2 imager.

Table 2: Target genes, primer, primer sequences and customized PCR conditions

Target gene	Primer	Sequence	PCR conditions	Reference
16S rRNA	27F 1492I	AGAGTTTGATCMTGGCTCAG GGTTACCTTGTTACGACTT	35x (2min 94°C 90s 56.4°C 45s 72°C)	(Janda and Abbott, 2007)
glnII-2	glnII-1F *glnII-2*R	AACGCAGATCAAGGAATTCG ATGCCCGAGCCGTTCCAGTC	35x (2min 94°C 90s 62°C 45s 72°C)	(Tan et al., 2012)
glnII-12	glnII12F glnII689R	YAAGTTCGAGTACATYTGGC TGCATGCCSGAGCCGTTC	35x (2min 94°C 90s 65.3°C 45s 72°C)	(Tan et al., 2012)
recA	recA6F recA504R	CGKCTSGTAGAGGAYAAATC TTGCGCAGCGCCTGGCTCAT	35x (2min 94°C 90s 60°C 45s 72°C)	(Tan et al., 2012)

3.3.6. DNA purification and sequencing

Amplified PCR product was purified using QIAquick PCR Purification Kit according to manufacturer's instruction. The DNA sequencing was performed by Macrogen Europe using Sanger Sequencing platform.

3.3.7. *16S rRNA*, *recA* and *gnlII* Sequences and Construction of Phylogenetic Trees

Sequences were received in three formats: ab1, pdf and html format. The ab1 format was loaded in CLC Genomics Main Workbench version 7 and using the tool Trim, low-quality parts of sequences were removed (Kumar et al., 2012). The assembling of forward and reverse sequences was performed using the same software to obtain consensus sequences (Kumar et al., 2012; Gascuel, 2006). Consensus sequence files were exported from CLC as FASTA files and loaded in MEGA version 7.0 software for alignment using Clustal W (Kim et al., 2013). Sequences were manually corrected in MEGA and best fit models of data analysis were selected based on BIC values determined using same program. Consensus sequences were submitted to BLASTn program in NCBI genebank (www.ncbi.nlm.nih.gov/blast) for authentication and identification of test strains (Deaker, 2004; Garg et al., 2011; Yang, 2017; Martens et al., 2008).

3.3.8. Data analysis

Clean and authenticated sequences were deposited in NCBI genebank and published with accession numbers MK872302 to MK872366 and MK905508 to MK905512. Phylogeny trees were constructed in MEGA 7 software where clustering was performed using Maximum Likelihood and Neighbour Joining methods. To reduce errors from data, all gaps were exluded in the sequences alignments. To support clusters, bootstrap analysis was performed using 1000 replicates (Garg et al., 2011; Yang, 2017). Pairwise analysis and genetic diversity were performed in MEGA7 software (Parvathy et al., 2018).

3.4.Results

3.4.1. Nodulation and biomass yield of indigenous soybean-nodulating rhizobia

One hundred and seven rhizobium strains, viable on plates, isolated from different legume species from South Kivu province were subjected to nodulation test along with a commercial rhizobium strain *B. diazoefficiens* USDA 110. All but two indigenous strains (NAC05 and NAC08) and the commercial strain produced nodules on soybean variety SB24 (Table 3). These nodulating strains differed significantly for nodule production ($p<0.001$) with mean number of nodules in between 0.25 and 30 per plant (Table 3). Similarly, the shoot biomass production ranged from 0.679 to 11.281g per plant and this difference was highly significant ($p<0.001$) (Table 3).

Five indigenous strains (NAC14, NAC40, NAC42, NAC66 and NAC74) produced higher number of nodules compared to the commercial strain USDA 110 ($p<0.001$). Sixteen indigenous rhizobium strains were superior to the commercial strain for shoot biomass production, and the best performing strain (NAC67) yielded 2.6 folds higher shoot biomass than the commercial strain USDA110 (Table 3). A total of 17 indigenous rhizobium strains had effectiveness index (EI) between 1.166 and 3.604 confirming them as effective strains. Two rhizobium strains, NAC40 and NAC42 outperformed the commercial strain in terms of nodule number and shoot biomass production with EI value of 2.166 and 2.364, respectively (Table 3). Interestingly, 15 effective rhizobium strains had the effectiveness index ≥ 2.

Table 3: Nodulation status (+/-), nodule numbers, dry shoots biomass (g plant -1) and effectiveness index of native rhizobia and a commercial rhizobium strain

Rhizobia strain	Nodulation (+/-)	Nodules numbers	Shoot dry weight (g/plant)	Effectiveness Index
NAC01	+	2.50	1.654	0.528
NAC02	+	0.50	1.363	0.435
NAC03	+	0.25	1.389	0.443
NAC04	+	16.25	2.078	0.663
NAC05	-	0.00	1.554	0.367
NAC06	+	0.50	1.435	0.458
NAC07	+	1.50	1.237	0.395
NAC08	-	0.00	0.679	0.196
NAC09	+	1.25	1.141	0.373
NAC10	+	19.00	8.059	2.574
NAC11	+	5.25	1.916	0.612
NAC12	+	0.50	0.693	0.221
NAC13	+	1.00	1.437	0.459
NAC14	+	24.50	2.970	0.948
NAC15	+	7.00	1.626	0.432
NAC17	+	18.75	2.222	0.709
NAC18	+	1.00	1.253	0.400
NAC19	+	19.00	9.036	2.886
NAC20	+	16.50	1.072	0.342
NAC21	+	12.75	0.739	0.207
NAC22	+	17.75	9.351	2.987
NAC23	+	19.50	7.554	2.413
NAC24	+	0.50	1.409	0.450
NAC25	+	6.50	1.181	0.377
NAC26	+	5.00	1.503	0.480
NAC27	+	1.00	1.434	0.458
NAC28	+	1.25	2.018	0.644
NAC29	+	7.00	1.882	0.601
NAC30	+	22.00	3.349	0.921
NAC31	+	1.00	1.483	0.473
NAC32	+	9.50	2.688	0.858
NAC33	+	0.50	1.491	0.476
NAC34	+	7.50	1.518	0.484

Rhizobia strain	Nodulation (+/-)	Nodules numbers	Shoot dry weight (g/plant)	Effectiveness Index
NAC35	+	17.00	1.606	0.507
NAC36	+	5.00	2.156	0.688
NAC37	+	15.75	6.420	2.051
NAC38	+	17.00	9.238	2.951
NAC39	+	10.25	1.614	0.515
NAC40	+	30.00	8.377	2.166
NAC41	+	4.25	1.397	0.446
NAC42	+	27.50	7.401	2.364
NAC43	+	3.25	1.286	0.410
NAC44	+	7.00	1.278	0.408
NAC45	+	21.00	9.290	2.968
NAC46	+	10.50	1.663	0.531
NAC47	+	19.75	7.394	2.362
NAC48	+	1.75	1.658	0.529
NAC49	+	9.75	2.015	0.643
NAC50	+	16.00	8.957	2.861
NAC51	+	0.50	1.675	0.535
NAC52	+	0.75	1.357	0.433
NAC53	+	0.75	1.713	0.547
NAC54	+	0.50	1.467	0.468
NAC111	+	14.00	4.193	1.166
NAC55	+	0.75	1.691	0.540
NAC56	+	19.00	9.770	3.121
NAC57	+	3.75	2.164	0.691
NAC58	+	1.75	1.377	0.439
NAC59	+	18.00	3.779	1.207
NAC60	+	0.50	1.751	0.559
NAC61	+	11.50	3.120	0.996
NAC62	+	2.50	1.648	0.367
NAC63	+	2.25	1.486	0.474
NAC64	+	3.00	1.649	0.526
NAC65	+	6.00	1.728	0.552
NAC66	+	24.00	8.572	2.738
NAC67	+	20.00	11.281	3.604
NAC68	+	1.00	2.162	0.448
NAC69	+	0.50	1.361	0.434
NAC70	+	16.00	2.955	0.738

Rhizobia strain	Nodulation (+/-)	Nodules numbers	Shoot dry weight (g/plant)	Effectiveness Index
NAC71	+	0.50	0.794	0.253
NAC72	+	8.00	1.730	0.552
NAC73	+	21.75	2.645	0.845
NAC74	+	25.75	1.702	0.543
NAC75	+	14.75	9.898	2.464
NAC76	+	15.00	1.559	0.498
NAC77	+	7.50	1.511	0.435
NAC78	+	2.25	1.243	0.397
NAC79	+	3.50	1.756	0.561
NAC80	+	0.75	1.510	0.391
NAC81	+	1.25	1.619	0.436
NAC82	+	5.00	1.855	0.592
NAC84	+	4.25	1.269	0.405
NAC85	+	13.25	2.065	0.610
NAC86	+	12.75	2.362	0.696
NAC87	+	10.00	1.972	0.565
NAC88	+	12.25	2.151	0.687
NAC89	+	1.25	0.703	0.224
NAC91	+	2.00	1.959	0.625
NAC92	+	8.75	2.126	0.496
NAC93	+	3.25	0.967	0.243
NAC94	+	0.50	1.384	0.442
NAC95	+	4.00	1.522	0.486
NAC96	+	2.75	1.660	0.530
NAC97	+	5.75	1.996	0.521
NAC98	+	0.75	1.312	0.380
NAC99	+	1.00	1.524	0.422
NAC100	+	20.50	1.675	0.460
NAC101	+	17.75	2.495	0.595
NAC102	+	14.75	2.146	0.734
NAC103	+	16.67	2.961	0.752
NAC104	+	10.75	1.752	0.465
NAC105	+	2.25	1.558	0.360
NAC107	+	1.75	1.710	0.446
NAC108	+	2.00	1.620	0.444
NAC109	+	19.75	3.371	0.867
NAC110	+	0.50	0.693	0.200
USDA110	**+**	**15.75**	**3.130**	**1.000**

Rhizobia strain	Nodulation (+/-)	Nodules numbers	Shoot dry weight (g/plant)	Effectiveness Index
N+	**-**	**0.00**	**3.389**	**1.082**
N-	**-**	**0.00**	**0.798**	**0.254**
P value	NA	< 0.001	< 0.001	-
CV (%)	NA	8.8	12.2	-
LSD	NA	6.707	0.899	-

3.4.2. *16S rRNA* isolates and gene sequences of indigenous soybean-nodulating rhizobia

Sequencing of 107 indigenous soybean nodulating rhizobium (SNR) strains from South Kivu province of DR Congo (Table 1) for *16S rRNA* gene made it possible to establish molecular identity of only 70 SNR (Table 4). Information on rhizobia strain, host plant species, origin, land use types, rRNA sequence length and similarity, molecular identity and GenBank accession numbers for matching sequences and query sequences are presented in Table 4. The sequence length of rhizobium strains ranged between 1268 to 1440 bp, and sequences similarity was ≥ 97% for the most strains.

Bradyrhizobium, *Kosakonia*, *Enterobacter* and *Rhizobium* were the most frequently isolated rhizobium strains in South Kivu Province representing 20%, 20%, 14% and 10% of total population. The less frequent genera were *Bacillus*, *Beinjerinckia*, *Burkolderia*, *Microvirga*, *Cupriviadus*, *Mesorhizobium* and *Agrobacterium*. The rhizobia diversity was higher in grassland (8 reported genera) compared to cultivated fields (5 reported genera) (Figure 2). *Bradyrhizobium* (45%) and *Kosakonia* (29%) were frequently isolated rhizobia from cultivated fields and grasslands, respectively (Figure 2, 3).

Table 4: Rhizobia strains, host plants species, location, types of land uses, *16S rRNA* sequences characteristics, molecular identity of rhizobium, and GenBank accession numbers for matching sequences and query sequences

Strain	Host plant	location	Land use	*16S rRNA* sequence		Molecular identity	Accession numbers	
				Length (bp)	Similarity (%)		Matching Sequence	Query Sequence
NAC01	*Tephrosia sp.*	Uvira	Grassland	1440	98.2	*Enterobacter tabaci*	JN210900.1	MK872302
NAC02	*Rhyncosia sp.*	Uvira	Grassland	1407	98.1	*Enterobacter asburiae*	KF747680.1	MK872310
NAC03	*Crotalaria. incana*	Uvira	Grassland	1412	99.6	*Stenotrophomonas bentonitica*	JQ359091.1	MK872320
NAC04	*Desmodium sp.*	Walungu	Grassland	1392	100.0	*Paraburkholderia caledonia*	HF674686.1	MK905508
NAC06	*Glycine max*	Walungu	Grassland	1410	99.6	*Stenotrophomonasrhizophila*	KJ361468.1	MK872344
NAC07	*Tephrosia vogelii*	Katana	Cultivated field	1320	100.0	*Beijerinckia fluminensis*	MF443190.1	MK872351
NAC10	*Trifolium sp.*	Uvira	Grassland	1409	89.5	*Brevibacillus formosis*	LC005608.1	MK872303
NAC11	*Trifolium sp.*	Uvira	Grassland	1344	99.4	*Rhizobium jaguaris*	KM192231.1	MK872304
NAC12	*Trifolium sp.*	Uvira	Grassland	1408	98.9	*Klebsiella variicola*	KY887765.1	MK872305
NAC13	*Vigna sp.*	Uvira	Grassland	1412	98.9	*Bacillus aerius*	MG937680.1	MK872306
NAC14	*Arachis hypogea*	Uvira	Cultivated field	1402	98.5	*Kosakonia oryzae*	CP015113.1	MK872307
NAC17	*Glycine max*	Uvira	Cultivated field	1349	98.3	*Rhizobium multihospitium*	MF944248.1	MK872308
NAC19	*Glycine max*	Uvira	Cultivated field	1352	92.2	*Bradyrhizobium japonicum*	KF995085.1	MK872309
NAC20	*Crotalaria sp.*	Uvira	Cultivated field	1415	98.1	*Enterobacter tabaci*	CP017087.1	MK872311
NAC22	*Glycine max*	Uvira	Cultivated field	1359	99.4	*Bradyrhizobium japonicum*	MF944235.1	MK872312
NAC23	*Glycine max*	Uvira	Grassland	1409	99.1	*Enterobacter tabaci*	CP017087.1	MK872313
NAC24	*Tephrosia sp.*	Uvira	Grassland	1416	98.9	*Bacillus pumilus*	KY072775.1	MK872314
NAC25	*Phaseolus vulgaris*	Uvira	Cultivated field	1409	98.8	*Kosakonia oryzae*	CP015113.1	MK872315
NAC26	*Phaseolus vulgaris*	Uvira	Cultivated field	1347	98.8	*Rhizobium miluonense*	MG786749.1	MK872316
NAC27	*Glycine max*	Uvira	Cultivated field	1339	98.7	*Bradyrhizobium elkanii*	GU552898.1	MK872317
NAC28	*Glycine max*	Uvira	Cultivated field	1355	98.7	*Bradyrhizobium huanghuaihaiense*	KY426358.1	MK872318
NAC29	*Tephrosia sp.*	Uvira	Grassland	1412	98.7	*Kosanica oryzae*	CP015113.1	MK872319
NAC31	*Tephrosia sp.*	Uvira	Grassland	1402	99.6	*Brevibacillus formosus*	KY368167.1	MK872321

Strain	Host plant	location	Land use	*16S rRNA* sequence		Molecular identity	Accession numbers	
				Length (bp)	Similarity (%)		Matching Sequence	Query Sequence
NAC32	*Phaseolus vulgaris*	Uvira	Cultivated field	1356	99.5	*Bradyrhizobium huanghuaihaiense*	KY426358.1	MK872322
NAC33	*Rhyncosia sp.*	Uvira	grassland	1411	99.5	*Enterobacter tabaci*	JN210900.1	MK872323
NAC34	*Tephrosia sp.*	Uvira	Grassland	1405	99.5	*Paenibacillus sonchi*	GU328693.1	MK872324
NAC36	*Sesbania sesban*	Walungu	Grassland	1407	99.5	*Kosakonia oryzae*	CP015113.1	MK872325
NAC37	*Sesbania sesban*	Walungu	Grassland	1409	99.9	*Enterobacter tabaci*	JN210900.1	MK872326
NAC38	*Calliandra sp.*	Walungu	Grassland	1399	99.9	*Cupriavidus plantarum*	HQ438088.1	MK872327
NAC39	*Calliandra sp.*	Walungu	Cultivated field	1356	100.0	*Bradyrhizobium elkanii*	JQ689186.1	MK872328
NAC42	*Mimosa pudica*	Walungu	Grassland	1412	99.9	*Kosakonia oryzae*	CP015113.1	MK872329
NAC43	*Vigna sp.*	Walungu	Grassland	1411	99.9	*Kosakonia oryzae*	CP015113.1	MK872330
NAC44	*Vigna sp.*	Walungu	Grassland	1407	99.9	*Enterobacter tabaci*	JN210900.1	MK872331
NAC45	*Phaseolus vulgaris*	Walungu	Cultivated field	1268	99.9	*Bradyrhizobium japonicum*	KY794773.1	MK872332
NAC46	*Phaseolus vulgaris*	Walungu	Grassland	1410	99.9	*Pantoea aglomerans*	KY660470.1	MK872333
NAC47	*Glycine max*	Walungu	Grassland	1355	99.9	*Bradyrhizobium huanguanensis*	KY426358.1	MK872334
NAC48	*Glycine max*	Walungu	Grassland	1411	100.0	*Kosakonia oryzae*	CP015113.1	MK872335
NAC50	*Phaseolus vulgaris*	Walungu	Grassland	1395	100.0	*Burkholderia caledonia*	MG846104.1	MK905509
NAC51	*Phaseolus vulgaris*	Walungu	Grassland	1404	100.0	*Kosakonia oryzae*	CP015113.1	MK872336
NAC52	*Desmodium sp.*	Walungu	Cultivated field	1408	100.0	*Enterobacter tabaci*	JN210900.1	MK872337
NAC53	*Glycine max*	Walungu	Cultivated field	1348	100.0	*Rhizobium lupine*	KY587906.1	MK872338
NAC54	*Glycine max*	Walungu	Cultivated field	1346	99.9	*Beijerinckia fluminensis*	KM894194.1	MK872339
NAC55	*Glycine max*	Walungu	Cultivated field	1307	99.6	*Rhizobium lupine*	KY000637.1	MK872340
NAC56	*Phaseolus vulgaris*	Walungu	Cultivated field	1357	99.6	*Bradyrhizobium diazoefficiens*	KU862338.1	MK872341
NAC57	*Glycine max*	Walungu	Grassland	1355	99.6	*Novosphingobium bardani*	JF716064.1	MK905510
NAC58	*Glycine max*	Walungu	Grassland	1400	99.6	*Bacillus subterraneus*	KY202699.1	MK872342
NAC59	*Phaseolus vulgaris*	Walungu	Cultivated field	1352	99.6	*Rhizobium pusense*	LC368035.1	MK872343

Strain	Host plant	location	Land use	*16S rRNA* sequence		Molecular identity	Accession numbers	
				Length (bp)	Similarity (%)		Matching Sequence	Query Sequence
NAC60	*Glycine max*	Katana	Cultivated field	1343	99.9	*Bradyrhizobium elkanii*	KY660605.1	MK872345
NAC61	*Glycine max*	Katana	Cultivated field	1353	99.9	*Bradyrhizobium japonicum*	EU010398.1	MK872346
NAC63	*Glycine max*	Katana	Cultivated field	1354	99.9	*Bradyrhizobium huanguanensis*	JF266645.1	MK872347
NAC64	*Rhyncozia sp.*	Katana	Grassland	1402	99.8	*Pantoea aglomerans*	KT280494.1	MK905511
NAC65	*Phaseolus vulgaris*	Katana	Grassland	1292	100.0	*Pseudomonas hubiscus*	MG905320.1	MK872348
NAC66	*Arachis hypogeae*	Katana	Cultivated field	1358	100.0	*Bradyrhizobium huanguanensis*	JF266657.1	MK872349
NAC67	*Sesbania sesban*	Katana	Grassland	1410	100.0	*Pantoea aglomerans*	DQ068848.1	MK905512
NAC69	*Rhyncosia hirta*	Katana	Grassland	1402	100.0	*Kosakonia oryzae*	CP015113.1	MK872350
NAC71	*Indigofera repens*	Katana	Cultivated field	1354	97.2	*Mesorhizobium acaciae*	KF891405.1	MK872352
NAC72	*Glycine wightii*	Katana	Cultivated field	1362	97.2	*Microvirga lotonidis*	AY725254.1	MK872353
NAC73	*Arachis hypogeae*	Katana	Grassland	1409	97.2	*Klebsiella pneumoniae*	LT671911.1	MK872354
NAC74	*Cassia mimosoides*	Katana	Grassland	1409	97.1	*Enterobacter tabaci*	MG754444.1	MK872355
NAC76	*Rhyncosia hirta*	Katana	Grassland	1412	97.1	*Kosakonia oryzae*	CP015113.1	MK872356
NAC78	*Cassia mimosoides*	Katana	Cultivated field	1353	97.7	*Bradyrhizobium japonicum*	EU010398.1	MK872357
NAC79	*Phaseolus vulgaris*	Katana	Cultivated field	1421	97.6	*Paenibacillus taidurgensis*	KF475860.1	MK872358
NAC82	*Phaseolus vulgaris*	Katana	Grassland	1412	96.9	*Kosanica oryzae*	CP015113.1	MK872359
NAC83	*Pisum sativum*	Katana	Grassland	1420	96.9	*Bacillus thuringensis*	KT720292.1	MK872360
NAC84	*Arachis hypogeae*	Katana	Grassland	1410	96.9	*Kosakonia oryzae*	CP015113.1	MK872361
NAC88	*Glycine max*	Katana	Grassland	1411	96.9	*Kosakonia oryzae*	CP015113.1	MK872362
NAC89	*Arachis monticola*	Katana	Cultivated field	1362	96.8	*Microvirga lotonidis*	AY725254.1	MK872363
NAC91	*Glycine max*	Katana	Grassland	1407	96.8	*Kosakonia oryzae*	CP015113.1	MK872364
NAC94	*Phaseolus vulgaris*	Katana	Cultivated field	1350	96.7	*Rhizobium indigofera*	KY587901.1	MK872365
NAC96	*Rhyncosia sp.*	Katana	Grassland	1411	97.6	*Enterobacter ludungwe*	CP017181.1	MK872366

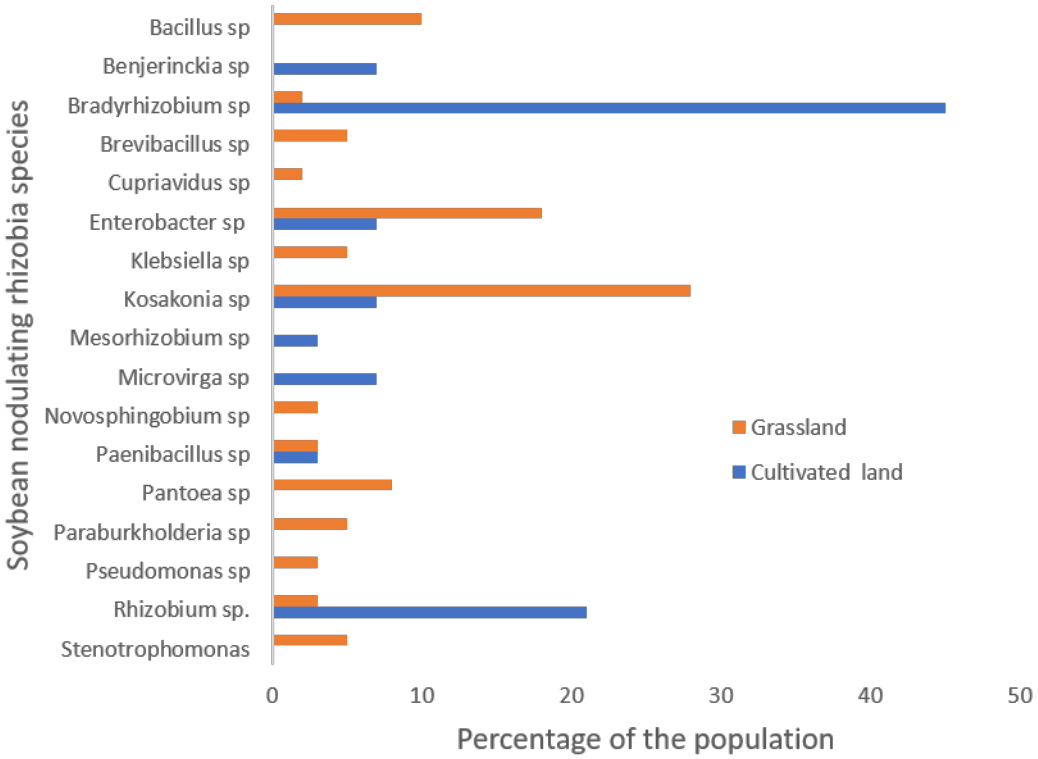

Figure 2 : Soybean nodulating rhizobia genera in cultivated land and grassland of South Kivu, Democratic Republic of the Congo

3.4.3. *16S rRNA* Gene Phylogeny

The *16S rRNA* phylogeny of 70 SNR from South Kivu province and a commercial strain divided them in two major clusters (Figure 3). The first cluster was composed of 43 rhizobia strains further divided into three clades while the second cluster was composed of 27 rhizobia strains divided further into two clades. Of 43 strains, 30 were present in clade I, five in clade II and eight in clade III. The second cluster was formed by 26 strains where only one strain was present in clade IV and 25 in clade V (Figure 3). Most strains from grassland were grouped in cluster 1 which represented eight genera - *Kosakonia, Klebsiella, Enterobacter, Pantoea, Stenophomonas, Pseudomonas, Cupriavidus* and *Paraburkholderia* exhibiting a high diversity (Figure 3). Most strains from cultivated field were also grouped together in cluster 2 and they represented five genera - *Novosphingobium, Rhizobium, Beijerinckia, Mesorhizobium* and *Bradyrhizobium* (Figure 3). Based on *16S rRNA* phylogeny, five SNR strains from South Kivu province (NAC19, NAC28, NAC32, NAC47 and NAC55) grouped together with the commercial strain *Bradyrhizobium diazoefficiens* USDA 110 and had a high bootstrap support of over 80%.

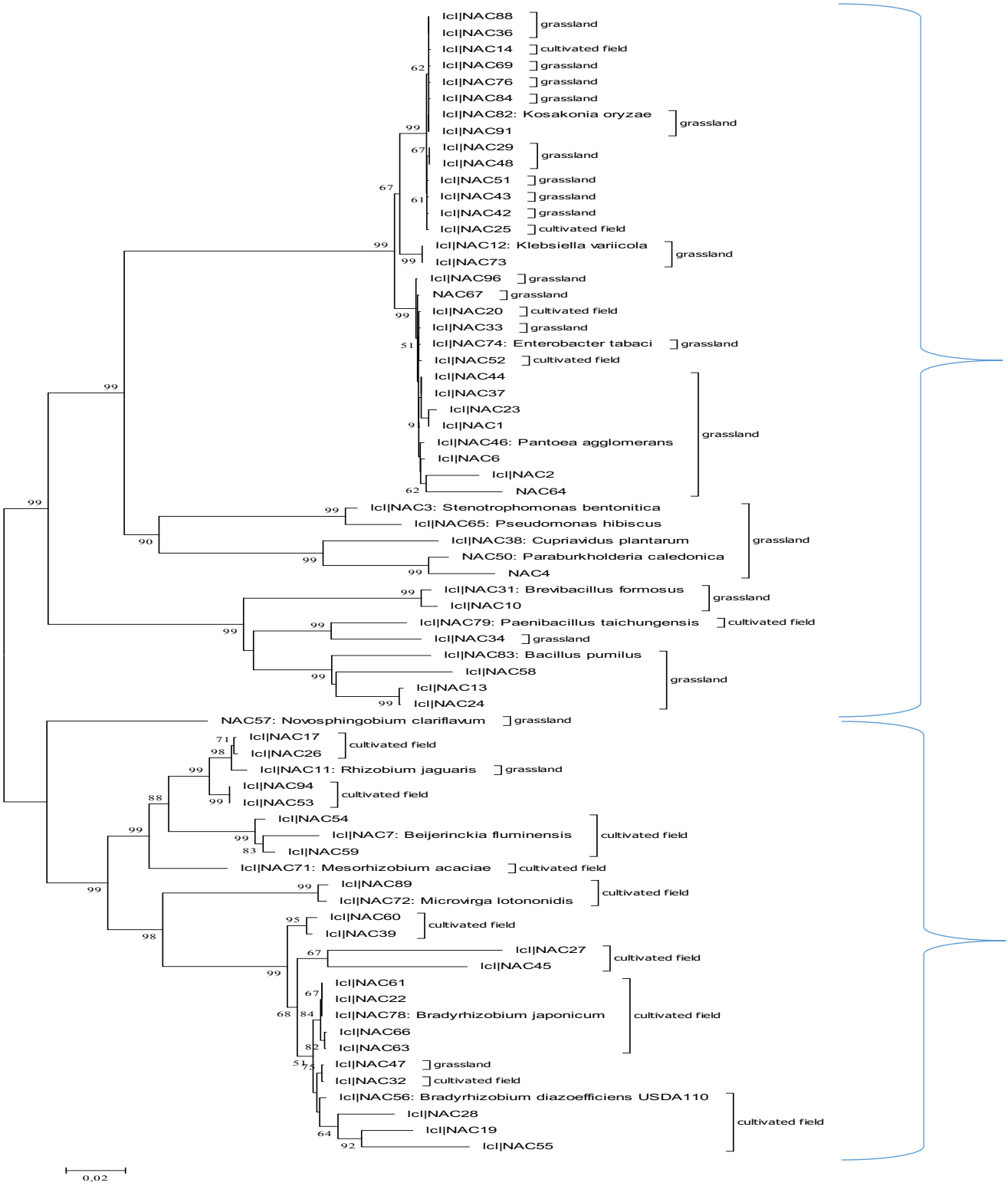

Figure 3: Phylogenetic relationships among indigenous SNR isolates based on *16S rRNA* gene. The evolutionary history was inferred using the Neighbour-Joining method and Tamura-3.

3.4.4. Housekeeping genes phylogenies

Three housekeeping genes (*glnII-2*, *glnII-12* and *recA*) were successfully amplified for 41 randomly selected strains from *16S rRNA* clusters. The analysis of the single sequence *glnII-2* gene gave three distinct clusters mostly composed of *Bradyrhizobium* and *Rhizobium* genera (Figure 4). Of the 41 sequences, 25 strains belonged to cluster I, eight strains each to cluster II and III. Strains from cluster I belonged to genus *Bradyrhizobium* and strains from cluster II and III were *Rhizobium*. Most strains from cultivated fields clustered in the *Bradyrhizobium* group while the strains from grassland were found in all clusters affirming higher rhizobia diversity in grassland (Figure 4). Six strains of *Bradyrhizobium* group were closely related to the commercial strain *Bradyrhizobium diazoefficiens* USDA 110 with 100% bootstrap value suggesting that they may have same genetic features as the commercial rhizobia strain.

The phylogeny based on *recA* gene gave also three well defined clusters (Figure 5). Based on *recA* phylogeny, all strains belonged to the *Rhizobium* and *Agrobacterium* (www.ncbi.nlm.nih.gov). The analysis of concatenated sequences of three housekeeping genes (*glnII-2*, *glnII-12* and *recA*) provided further discrimination of the rhizobia strains (Figure 6). With this classification, strains were divided into three clusters, the second cluster subdivided into clades showing high diversity.

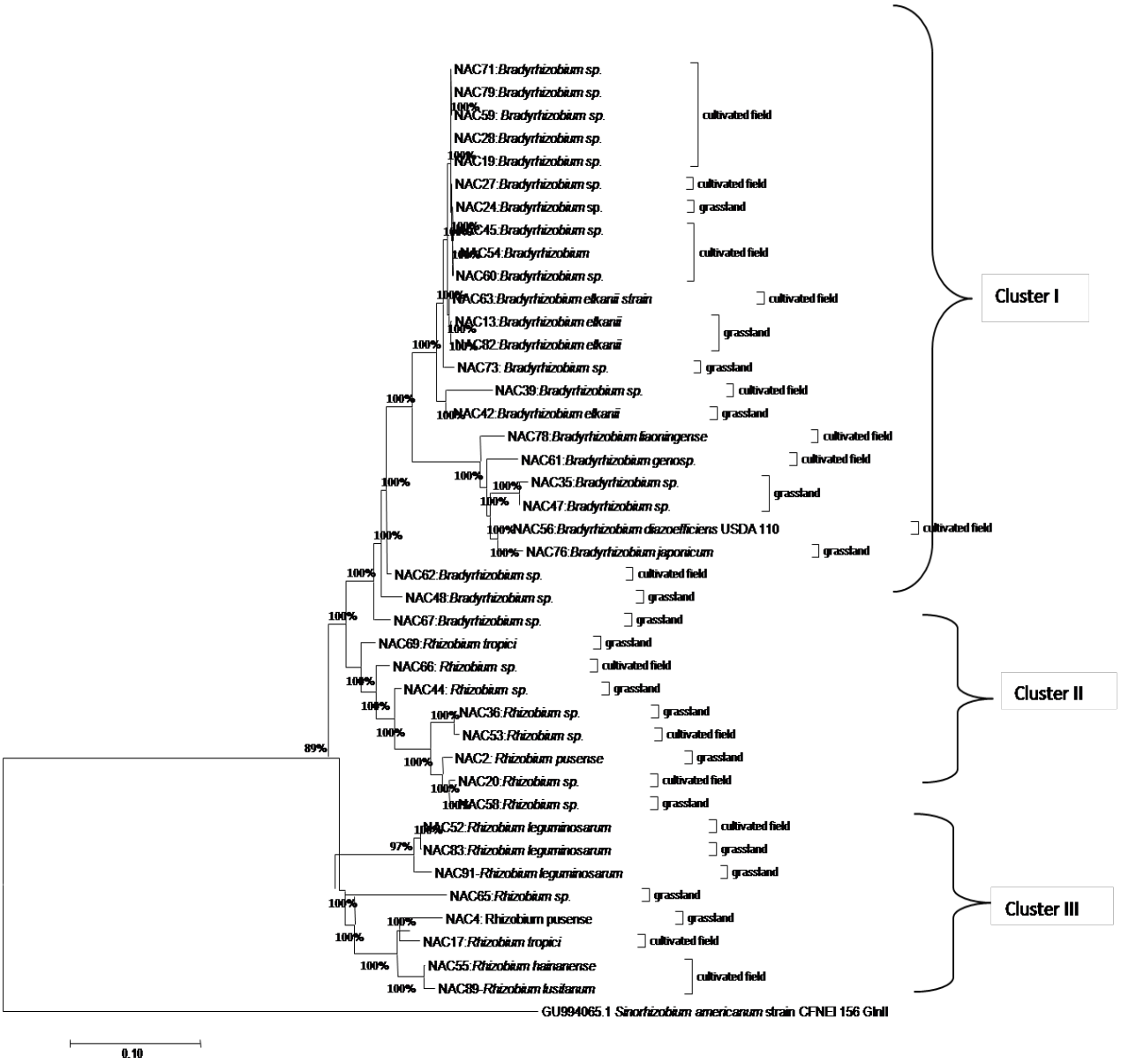

Figure 4: Tree constructed using the glnII gene sequence. The tree was built using Neighbour Joining method based on the Tamura Nei model with a gamma distribution. The species Sinorhizobium americanum was used as outgroup.

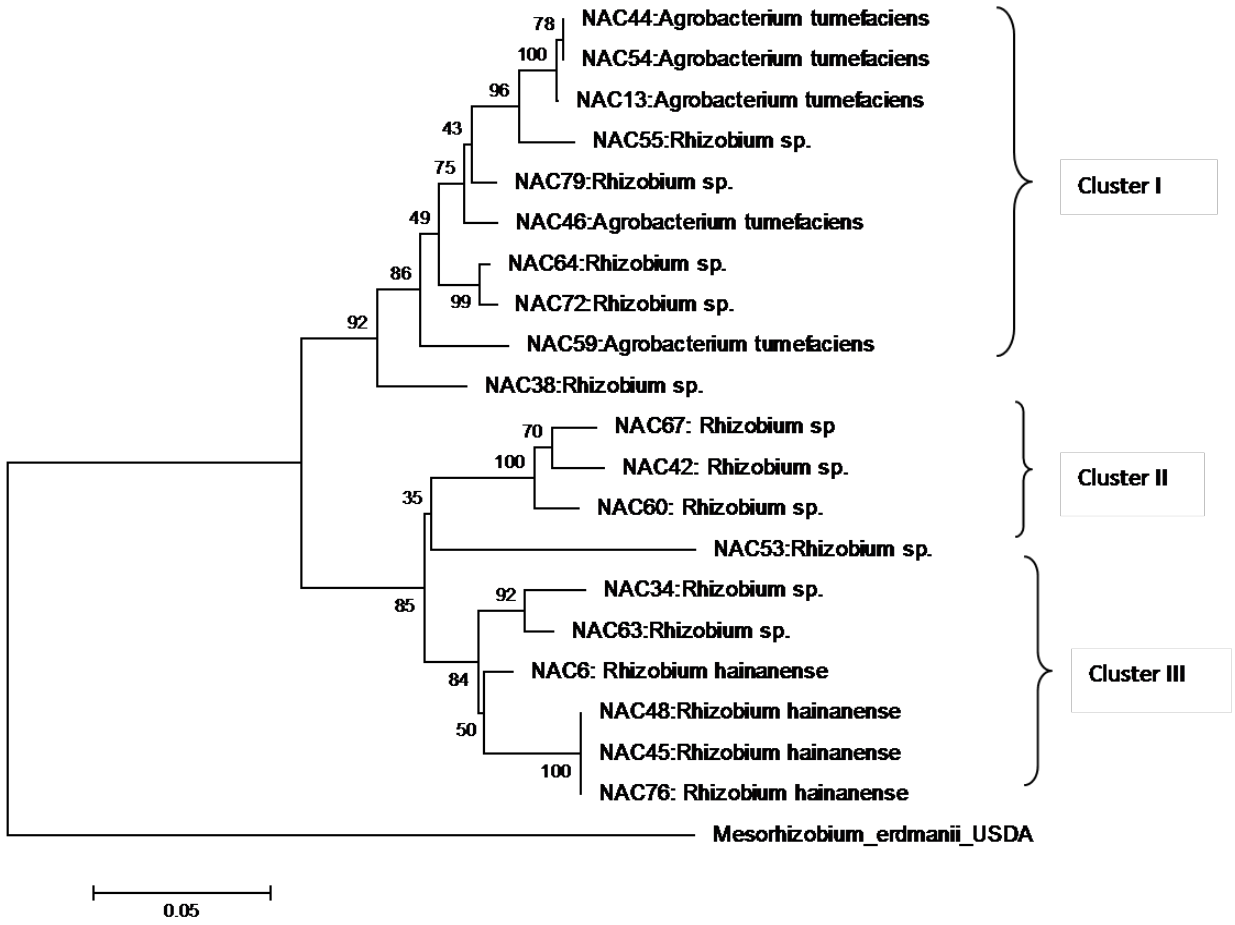

Figure 5: Phylogenetic tree constructed using the recA gene sequences using the Neighbor-Joining method and the Tamura 3-parameter model. The specie Mesorhizobium erdmanii was used as out-group.

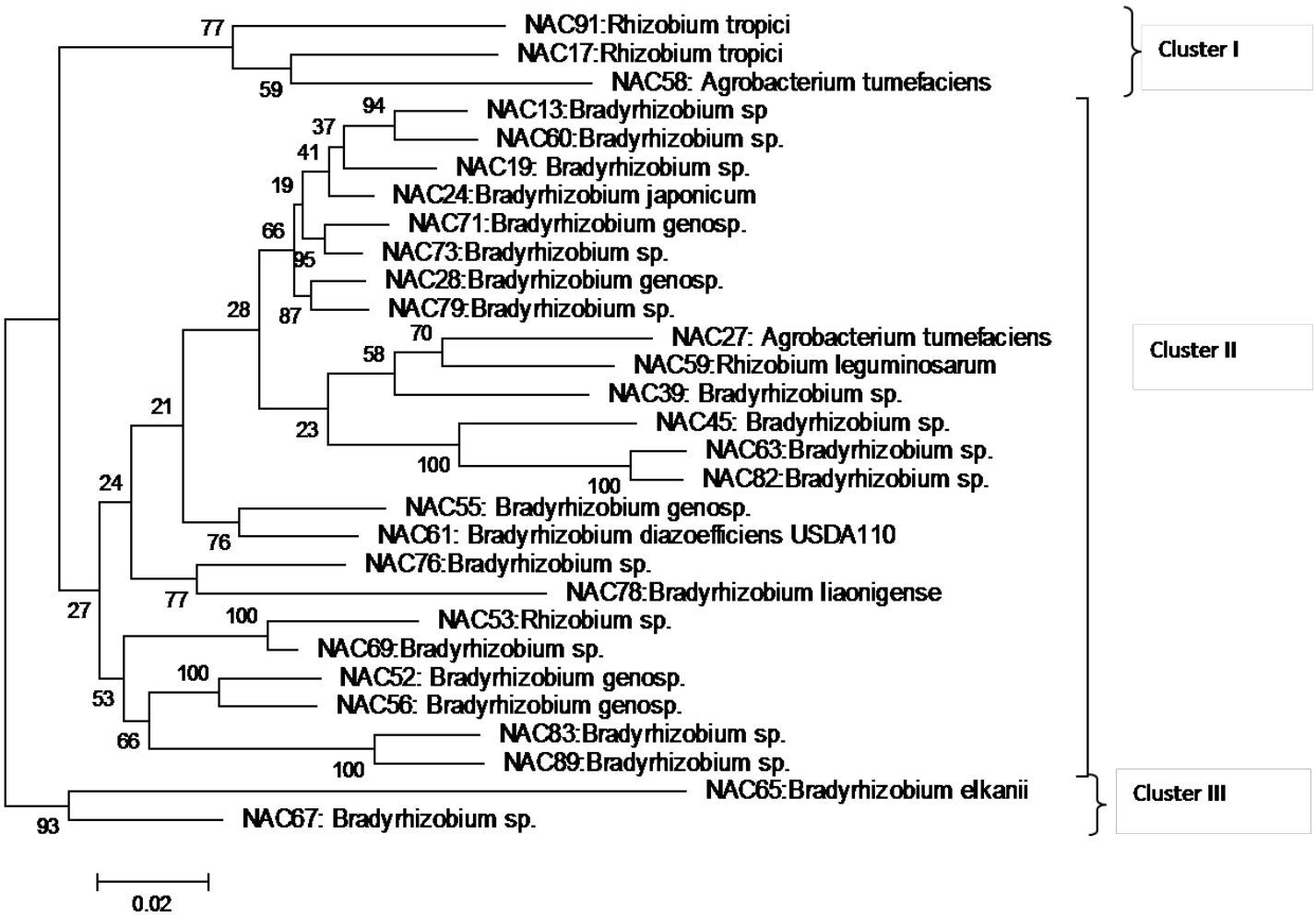

Figure 6: The phylogeny tree constructed using concatenated genes *glnII-2*, *glnII-12* and recA. Evolutionary history was inferred by using the Maximum Likelihood method based on the General Time Reversible model.

3.5. Discussion

3.5.1. Indigenous rhizobia nodulation and biomass improvement

Difference in nodule production ability, nodule numbers and shoot biomass production have been attributed to genetic differences among these indigenous rhizobium strains and host-rhizobium interaction. These results agree with other studies conducted in Africa that have reported great variation in symbiotic effectiveness among the indigenous rhizobium strains (Alves et al., 2003;

Chibeba et al., 2017). This study also confirmed the ability of most indigenous rhizobium strains to establish effective symbiosis with soybean. Furthermore, some indigenous SNR (15 strains of 107 strains) from South Kivu province performed better than commercial strain (*B. japonicum* USDA 110) for shoot biomass production as reported in other studies (Ndusha, 2014; Chibeba et al., 2018). This may be due to the fact that indigenous rhizobia, in addition of being highly effective, they adapt very well to the environment conditions. This finding agrees with Chibeba et al., (2018) who observed that highly effective commercial strains fail to overcome the competition barrier for nodules occupancy imposed by indigenous rhizobia. Expectedly, the majority of these performing strains belong to the *Bradyrhizobium* genus and clustered together with the commercial strain *Bradyrhizobium Japonicum* USDA110 (cluster 2) in the *16S rRNA* phylogeny (Figure 2). However, these results disagree with a study (Alves et al., 2018) that reported high effectiveness of commercial rhizobium strain (*B. japonicum* USDA 110) than indigenous rhizobia.

3.5.2. Identity of indigenous soybean nodulating rhizobia from South Kivu soils

This study revealed that, apart from the two commonly reported rhizobia genera in soybeans nodules (i.e. *Bradyrhizobium* and *Rhizobium*) we recovered other nodulating and free living bacterial genera - *Kosakonia*, *Enterobacter, Pantoea, Brevibacillus, Klebsiella, Bacillus, Beinjerinckia, Mesorhizobium*, *Burkolderia*, *Microvirga*, *Cupriviadus* and *Agrobacterium* (Figure 1) -that successfully formed nodules in soybean. The high rhizobia diversity detected in this study could be explained by: 1) use of promiscuous soybean variety (SB24) as trapping plant, 2) the diversity in legume species used in rhizobia isolation, 3) collection of plant samples from the fields with diverse land use patterns.

Some legume species can be nodulated by different bacterial genera while other are really restrictive. Legumes that can nodulate with a wide range of bacteria are named promiscuous legumes (Franche et al., 2008). Soybean was considered as a specific legume until the breeding program at IITA, Nigeria, developed and disseminated soybean cultivars (TgX or Tropical glycine cross), with ability to form effective nodules with native rhizobia strains (Tefera, 2011). In this study we found the nodulation of soybean variety used (promiscuous soybean SB24) by a wide range of nodulating rhizobia ever reported before. This finding is in agreement with Musiyiwa et

al., (2005) and Sanginga and Okogun, (2003) who found that the nodulation of promiscuous soybean varieties is greater in African soils.

For successful collection of rhizobia to overcome poor nitrogen fixation of existing rhizobia strains or to select well adapted strains to the environment, the collection and isolation of strains must be performed from a broad range of rhizobium. Therefore, Howieson et al., (2016) suggested that rhizobia strains should be isolated from a large pool of nodules (different regions and different plant species). Isolating rhizobia from one legume species limit the chance to get high diversity. Alberton et al., (2005) conducted a study on the sampling effects on the genetic diversity of rhizobia associated with legumes, they suggested that sources of rhizobia must be diversified for collection of a larger spectrum of bacteria. In addition, the soils and environmental conditions influence the in-situ diversity of rhizobia. Many authors stated that rhizobia strains are present in soils where the compatible host have been grown recently. Bizaro et al., (2011) in their study on soybean bradyrhizobia population genetic variability under different soil management found higher bradyrhizobia diversity from fields under no-tillage.

3.5.3. Diversity of indigenous soybean nodulating rhizobia based on *16S rRNA* phylogeny

High diversity of indigenous soybean-nodulating rhizobia in South Kivu was reported; in addition to the most reported genera *Bradyrhizobium* (Adhikari et al., 2012; Shiro et al., 2013; Yan et al., 2014)*, Rhizobium* (Hungria et al., 2006)*, Sinorhizobium (Ensifer)* (Wu et al., 2011; Yan et al., 2014) and *Mesorhizobium* (Biate et al., 2014), other bacteria genera were found. These include kosakonia*, Klebsiella, Enterobacter, Bacillus, Pseudomonas, Pantoea, Agrobacterium, Burkholderia, Microvirga* and *Cupriavidus*. The presence of these bacteria in soybeans nodules may be explained by continuous exchange of nitrogen fixation genes among bacteria by the events of lateral genes transfer (Menna and Hungria, 2011; Okubo et al., 2016). These finding has practical implication on the choice of genes to be used in nodulating bacteria diversity studies. These findings are in agreement with many other authors on the presence of non-rhizobial strains in legumes nodules apart the rhizobia group. Franche et al., (2009) found that these bacteria use diverse strategies to enter in plant roots. Kawaka et al., (2018) found the effective nodulation of *P. vulgaris* with the genera of *Pantoea, Klebsiella, Enterobacter and Bacillus* in Kenyan soils. Lu et al., (2017) also

observed the presence of non-rhizobial bacteria such as *Bacillus, Lactococcus* and *Klebsiella* in nodules of *Dalbergia odorifera*.

A rich and high rhizobia diversity was detected in grassland compared to cultivated field. *Bradyrhizobium* and *Rhizobium* genera were two commonly isolated genera from cultivated fields while a quite number of other genera e.g. *Kosakonia*, *Bacillus, Beinjerinckia, Mesorhizobium*, *Burkolderia*, *Microvirga*, *Cupriviadus* and *Agrobacterium* were exclusively isolated from the grassland agroecosystem. It can be explained that the intense presence of a compatible leguminous crop tends to decrease the diversity and number of the *Rhizobium* population in situ. These results agree with previous study conducted by NgoNkot et al., (2008) on groundnut (*Arachis hypogea*) who observed that rhizobia diversity is lower where its host (legume) was cultivated for subsequent seasons. Many studies have suggested that the introduction of a leguminous plant promotes the selection of rhizobium (Tamimi et al., 2004). These findings will have practical implications on the choice of sources of rhizobia strains for increasing BNF in soybean and improving soil fertility.

Many studies reported that soybean nodulate essentially by the rhizobia of *Bradyrhizobium* genus (Wasike et al., 2009; Chibeba et al., 2017). In contrast, in this study, it was observed that, in addition to *Bradyrhizobium* genus, soybean was nodulated by other genera. This can be supported by the fact that these rhizobia strains were derived from different legume species grown in two distinct land use patterns. These findings agree with those of Zou et al., (2016) who reported nodulation of soybean by other genera than *Bradyrhizobium*. Kawaka et al., (2018) also found that, apart from rhizobia, other bacteria were present in legumes nodules. These findings, however, differ from two previous studies that examined genetic diversity of rhizobia nodulating soybeans in Mozambique and in Kenya (Chibeba et al., 2017; Wasike et al., 2009). In both studies, rhizobia strains were collected essentially from cultivated field and all indigenous rhizobia were *Bradyrhizobium*.

This study detected high nucleotide identity (99.2 to 100%) between six indigenous SNR and the commercial strain, *Bradyrhizobium japonicum* USDA 110 (CP011360.1). The mentioned strain is an outstanding soybean symbiont used as commercial inoculants worldwide (Chibeba et al., 2018). A high genetic relatedness of South Kivu's indigenous strains with the commercial strain

USDA110 suggests that there exist rhizobia strains among indigenous population in Africa that are potentially good for nitrogen fixation and enhanced soybean productivity. It is also noted that some reports have suggested a possibility for tropical origin of *Bradyrhizobium diazoefficiens* USDA 110 strain (Delamuta et al., 2013).

3.5.4. Diversity of indigenous soybean-nodulating rhizobia based on housekeeping genes glnII and recA phylogenies

The *16S rRNA* phylogeny produced only two clusters while the three housekeeping genes and concatenated housekeeping genes produced three clusters. The lower number of clusters in *16S rRNA* phylogeny may be explained by the fact that *16S rRNA* is a conserved region for the big group of bacteria while housekeeping genes is conserved for smaller taxa (Zhang et al., 2012). This observation corroborates previous reports showing that the *16S rRNA* gene sequences alone is not sufficient to discriminate between the strains (de Almeida Ribeiro et al., 2015). The phylogenetic analysis of the housekeeping genes showed a clear differentiation between clusters and all tested isolates belonged to closely related bacterial genera.

3.6. Conclusion

Indigenous strains of SNR in South Kivu soils were genetically diverse. This study reported higher SNR diversity in grassland ecosystem compared to cultivated fields suggesting that in bioprospecting targeting the exploration of grasslands offers good probability for succcess. The high levels of similarity between some indigenous rhizobia strains (NAC19, NAC28, NAC32, NAC47 and NAC55) and a Commercial strain of *B. diazoefficiens* USDA110 suggest that there exist indigenous SNR in South Kivu soils with high potential for BNF. Furthermore, the higher EI of indigenous strains NAC56, NAC67, NAC75, NAC50, NAC 45, NAC47, NAC 22, NAC23 and NAC10 suggest that these strains are performant in improving soybean BNF. We suggest further studies on genetic characterization of SNR using full genome sequencing approach and evaluation of these five indigenous SNR for BNF effectiveness. The cultivated fields especially those under cereal crops and legume for extended period would benefit from SNR inoculation through enhanced BNF and increased crop yields.

CHAPTER FOUR: GENOMES COMPARISON FOR RAPID IDENTIFICATION OF ELITE INDIGENOUS SOYBEAN NODULATING RHIZOBIA

4.1. Abstract

The use of indigenous rhizobia as inoculants is very important in improving soybeans productivity and BNF in South Kivu province of Democratic Republic of Congp (DRC). The selection method to date of effective rhizobia nodulating legumes among indigenous population to be included in inoculants formula is time consuming. We sequenced 24 genomes of indigenous Soybean nodulating rhizobia (SNR) isolated from soybean's root nodules grown in South Kivu province of DRC in order to identify rapidly the highly effective indigenous rhizobia strains. Full genomes of 24 indigenous rhizobia were obtained on Miseq, libraries prepared using Nextera xt protocols and compared with genome of the commercial strain *Bradyrhizobium japonicum* USDA 110 (accession number CP011360.1). Indigenous SNR and commercial strain were compared based on their genomic features, the presence of nitrogen fixation genes and phylogenetic distance. Out of 24 samples, 14 high quality genomes of indigenous SNR were obtained, of 8.383 Mb ±0.762 bp mean size with mean GC content of 62%. These SNR belonged mostly to *Bradyrhizobium* (64%) genus and few to *Rhizobium, Microvirga and Kosakonia* genera. The chromosomes comprised a mean of 8063±975 genes, 7992±978 potential protein-coding genes, 1.2±0.43 set of rRNA genes and 57±9.8 tRNA genes. Based on genome size, number of protein-coding genes and phylogenetic comparison, six strains (NAC53, NAC46, NAC22, NAC76, NAC37, NAC17, NAC28 and NAC42) were very close to the commercial strains USDA110 (mean genetic distance=0.004) and could be thus considered as candidate elite strains.

Key words: genomics, indigenous rhizobia, rhizobia selection, South Kivu.

4.2. Introduction

The Rhizobium-legume symbiosis, characterized by the formation of root nodules, is the most important bacteria–plant interaction (Hirsch et al., 2001). It is an important process in sustainable agriculture, as this symbiotic association is able to enhance soil nitrogen status and legume productivity (Alves et al., 2003). The symbiosis involving soybean is the most exploited in the world; it produces as much as 300 kg of N ha^{-1} in addition to the release, in the soil, of 20–30 kg N ha^{-1} per year (Hungria et al., 2013).

Soybean is an important legume considered as "meat for the poor" (Hartman et al., 2011) that provide high protein and edible oil. This crop was introduced first in 1908 in Democratic `Republic of Congo by missionaries and promoted in 1990 as a medicinal food to prevent and cure malnutrition-induced diseases (Khojely et al., 2018). Consequently, its cultivation is expanding rapidly and this crop has become one of cash crops (Barhebwa et al., 2015). In addition, soybean fixes nitrogen from atmosphere through Biological Nitrogen Fixation (BNF) process (Collino et al., 2015).

For higher yield achievement, soybean must accumulate important amount of nitrogen in grain through high photosynthesis rates provided by BNF (Thuita et al., 2018). Successful BNF depends on both good legume genotype and dominating nodule occupancy with highly and adapted efficient rhizobia strains (Alves et al., 2003; Checcucci et al., 2017). A soybean-breeding programme by International Institute of Tropical Agriculture (IITA) has led to the development of “promiscuous” soybeans. These cultivars have capacity to form root nodules with indigenous rhizobia, thus alleviating the need to inoculate with commercial rhizobia strains (Osunde et al., 2003). Despite this success, recent studies demonstrated yields responses to inoculation in these promiscuous varieties and hence, suggested the importance of inoculation of these soybean cultivars (Ronner et al., 2016). In addition to that indigenous rhizobia are not usually effective and thus the success of these varieties relies on the selection of highly effective rhizobia among indigenous populations, isolates them and applies them as inoculants (O’Hara et al., 2002).

There exist numerous studies on selection of highly effective rhizobia among indigenous populations (O'Hara et al., 2002). The empirical approach of selecting highly effective and competitive rhizobia strains consists on native rhizobia strains collection, isolation and authentication, isolates screening against reference strains for symbiotic effectiveness, competitiveness for nodules occupancy testing and isolates performance testing under varied field conditions (Yates et al., 2005). Each step eliminates the worst performing isolates for further consideration. This selection method is time consuming thus there is need of an adequate and rapid selection approach especially in Sub-Saharan Africa where most soils are depleted of important nutrient and most of farmers cannot afford mineral fertilizers.

More recently, numerous studies have demonstrated the effectiveness of genomic approaches on detection of genetic component associated with nodules formation in rhizobia, nitrogenase regulation, competitiveness for nodules occupation and other processes involved in BNF (Amadou et al., 2008; Checcucci et al., 2017). In addition, several studies have defined bacterial genes responsible for root-nodule formation, host specificity and nitrogen fixation, (Laguerre et al., 2003; MacLean et al., 2007). These genomics approaches have been used to describe the role of the entire bacterial genomes in the symbiotic nitrogen fixation process (Bailly et al., 2011). In the field of microbiology, bacteria like rhizobia represent ideal candidates for this new development because it is easy to sequence the genomes of bacteria and analyse its composition (Galibert, 2001). For rhizobia species genomes sequenced to date, the genomes size and composition differ considerably (Galibert, 2001; Amadou et al., 2008; Bailly et al., 2011; Delestre et al., 2015). Some species have demonstrated higher ability in nitrogen fixation compared to others; for example, the *Bradyrhizobium* strain USDA110 which has demonstrated higher ability in nitrogen fixation and consequently is beeing used in commercial inoculants (Kaneko et al., 2002; Kumar et al., 2013; Shah and Subramaniam, 2018). In this study, 24 indigenous soybean nodulating rhizobia isolated from South Kivu soils were sequenced and analyzed in order to determine their genomic features, select indigenous rhizobia with high potential of Nitrogen fixation and identify genetic components associated with high N and high productivity in these rhizobia.

4.3. Materials and methods

4.3.1. Extraction and preparation of genomic DNA

Rhizobia cultures were obtained from N2 Africa project of International Institute of Tropical Agriculture. Genomic DNA from the isolates was obtained by lysis of single colonies streaked out on Yeast Mannitol (YMA) medium for 7 days (Ampomah and Huss-Danell, 2016). DNA was extracted using Qiagen Plant Mini kit following the manufacturer's instructions (Qiagen, Hilden, Germany) (Ghimire et al., 2010; Di Bella et al., 2013). The DNA quality check was performed on 0.8% agarose-buffer TAE and read on UV light using GelDoc-It2 imager (Batista et al., 2017). The concentration of DNA was measured using Qubit High Sensitivity (Batista et al., 2017).

4.3.2. Libraries preparation and sequencing

In brief, a mean of 450 base-pair libraries preparation was done by the Nextera™ XT Library Prep Kit following the manufacturer'instructions (Illumina, San Diego) (Ring et al., 2017). Quality controls were performed on Tape Station controller (Agilent Technologies) and lectures performed using 2200 Tape Station analysis software A.01.04 (Dong et al., 2019). Genomes sequencing was conducted at the Bioscience Eastern and Central Africa of International Livestock Research institute (BecA-ILRI), Nairobi Kenya. Reads were generated on an Illumina MiSeq instrument, using 2 × 150 bp paired end (PE) library with an average insert size of 480 bp (Sugawara et al., 2013).

4.3.3. Analysis of Sequences

Raw reads obtained from MiSeq sequencer, were analyzed for quality using fastqc software (Leggett et al., 2013). Low quality reads were removed by Trimomatric and loaded in CLC main Workench version 7 for denovo assembling (Li et al., 2010). Assembled sequences were exported as fasta and assembled first in contigs and then in scaffolds using SSPACE Basic software version 2.0. and Unicycler version 0.4.7. Scaffolds were mapped to reference genome of *Bradyrhizobium diazoefficiens* USDA110 (accession number CP011360.1) obtained from NCBI databank (whole genome shotgun sections) (Sablok et al., 2017). Scaffolds were further improved using CLC software version 7 and are vailable at hpc.ilri.cgiar.org.

4.3.4. Genome annotation

Improved scaffolds were submitted to Blastn program available in NCBI genebank (www.ncbi.nlm.nih.gov/blast) for strains identification and were used for gene prediction and annotation using Prokka bacteria annotation tool which uses Prodigal. To increase the robustness of the annotation the references genomes *Bradyrhizobium diazoefficiens* USDA 110 and *Rhizobium sullae* were used (Sablok et al., 2017).

4.3.5. Data analysis

Descriptive statistics were performed in XLstat version 2014. Genomes were compared to the commercial strain based on genome size, total number of genes, number of proteins-coding genes, C-G content and number of nitrogen fixation genes. The rhizobia phylogeny and genetic distance among studied indigenous rhizobia strains and commercial strain were computed using pairwise analysis in MEGA6 software. The clustering was performed by using the Maximum likelihood method and the Tamura-Nei model using the same software. The bootstrap value of 1000 replicated was applied to support the clusters and the indigenous rhizobia were compared to closely related sequences using BLASTn in NCBI (99% identity and 98% coverage).

4.4. Results

4.4.1. Genomes'sequences quality

Twenty-four genomes of indigenous soybean-nodulating rhizobia were sequenced and compared to the genome of commercial rhizobia *Bradyrhizobium diazoefficiens* available in the NCBI genebank, accession number CP011360.1. From 24 genomes sequences, 14 yielded higher quality genomes (figures of quality scores for all samples are presented in appendix1) and were considered for this study. The Figures 7 and 8 present the per base quality scores of the sample NAC1 and NAC22 respectively. The quality was scored using 0-38 scale, the acceptable quality scores starting from 20 to 38. From these figures, most of sequences were of high quality (Figures 7 and 8).

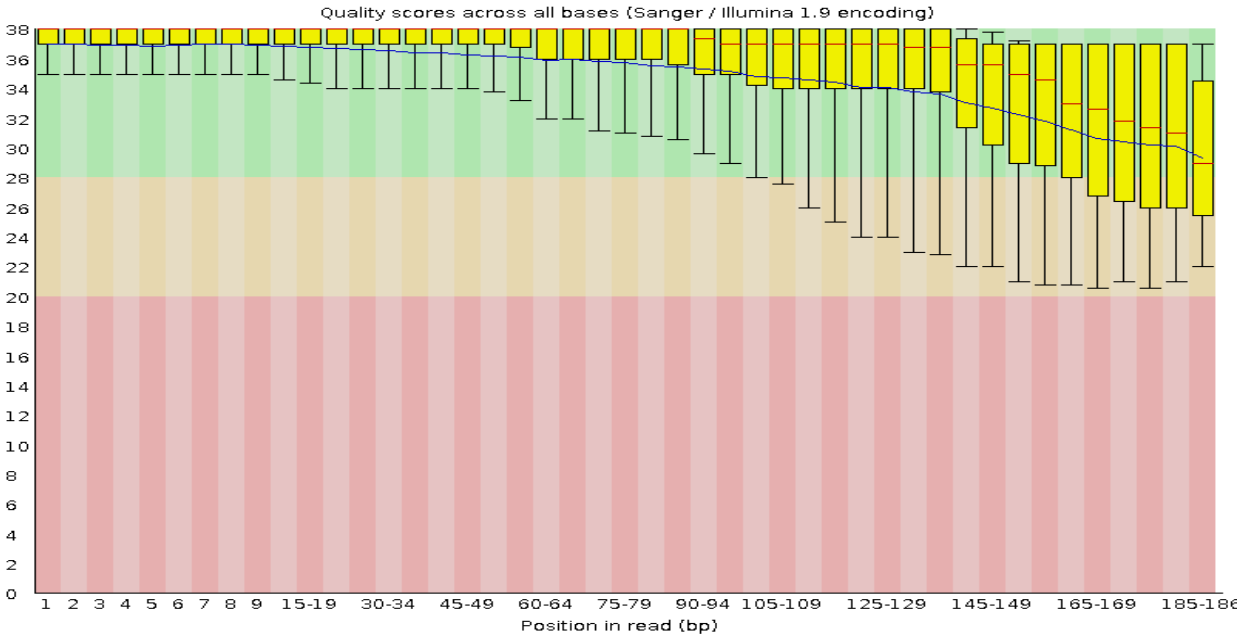

Figure 7: sequences quality score of sample NAC1

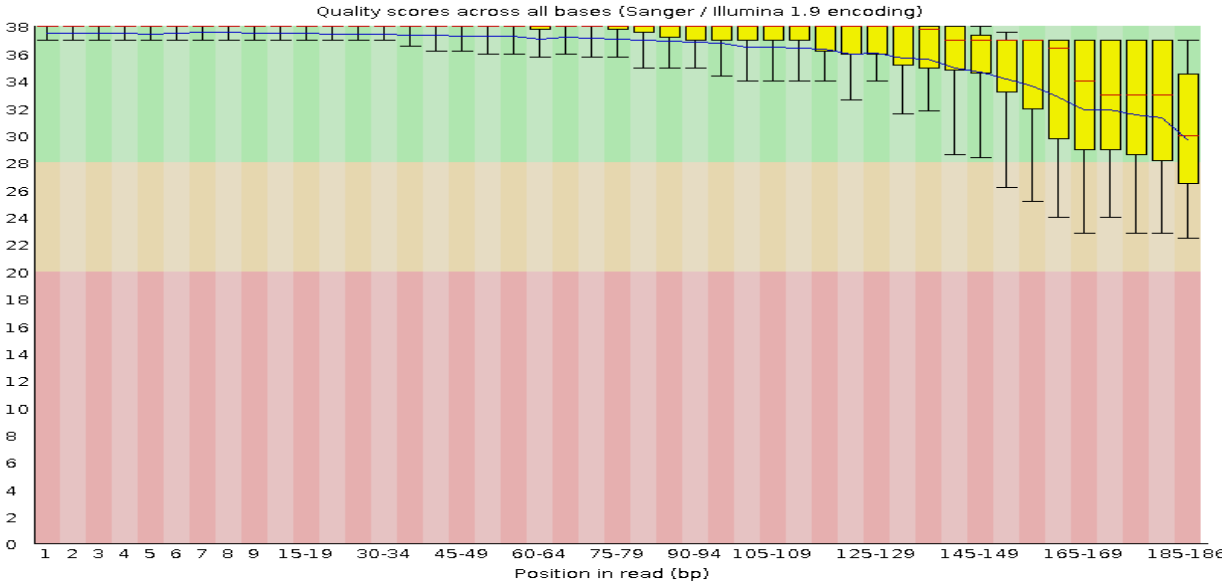

Figure 8: sequences quality score of sample NAC22

4.4.2. General genomic features

Draft genomes of indigenous rhizobia strains nodulating soybeans belonged to five genera of alpha-proteobacteria namely *Bradyrhizobium* (62%), *Rhizobium* (14%)*, Microvirga* (14%), *Kosakonia* (7%) and *Agrobacterium* (7%) (table 5). *Bradyrhizobium* was the most represented genus nodulating soybean in South Kivu soils (Table 5). However, this study found that, in addition to the mentioned genus, soybean is nodulated by other genera like *Rhizobium*, *Agrobacterium*, *Microvirga* and *Kosakonia*. From these results, eight strains (NAC53, NAC46, NAC22, NAC76, NAC37, NAC17, NAC28 and NAC42) belong to the same genus (identity index range 98-100%) as the commercial strain USDA110.

Genomes size varied considerably (CV=14.6%) among indigenous strains and ranged from 5.669Mb and 9.963Mb with the mean genomes estimated at 8.383 Mb in size (Table 5). The *Bradyrhizobium* genus holds the largest genomes while *Microvirga* genus holds the smallest. From this study, seven indigenous strains (NAC1, NAC22, NAC76, NAC37, NAC17, NAC28 and NAC42) hold a genome size closer to the commercial strain USDA110 (≥9Mb). The mean GC content was 62% while the mean number of 8063 genes was identified and 99 % of these were protein coding (Table 5). The indigenous rhizobia genomes also comprised of either one or two sets of transfer-messenger RNA (tmRNA) and a mean of 57 transfer RNA (tRNA).

Table 5: summary of genomics features

Strain	Identity(NCBI)	genome size(Mb)	G-C content(%)	number of genes	Protein-coding genes	tmRNA	tRNA
NAC1	*Agrobacterium sp.*	9.247	58.04	8747	8667	2	78
NAC53	*Bradyrhizobium diazoefficiens*	7.722	60.53	7376	7325	1	50
NAC46	*Bradyrhizobium diazoefficiens*	8.327	63.12	8200	8148	1	51
NAC22	*Bradyrhizobium elkani*	9.082	63.58	8567	8516	1	50
NAC76	*Bradyrhizobium japonicum*	9.963	63.47	9656	9597	1	58
NAC37	*Bradyrhizobium ottawaense*	9.567	63.68	9031	8973	1	57
NAC17	*Bradyrhizobium ottawaense*	9.312	63.27	9224	9174	1	49
NAC28	*Bradyrhizobium sp.*	9.469	63.90	9015	8962	1	52
NAC42	*Bradyrhizobium sp.*	9.024	63.95	8893	8840	1	52
NAC69	*Kosakonia oryzae*	5.640	54.25	5390	5314	1	75
NAC71	*Microvirga ossetica*	6.973	62.27	6830	6757	2	71
NAC72	*Microvirga ossetica*	7.019	62.28	6887	6847	2	71
NAC11	*Rhizobium jaguaris*	7.557	59.48	7170	7119	1	50
NAC94	*Rhizobium leguminosarum*	7.740	60.54	7390	7337	1	52
USDA110	*Bradyrhizobium diaazoefficiens* (Kaneko et al., 2002)	9.110	64.10	8571	8317	1	50
Min		5.640	54.25	5390	5314	1	49
Max		9.963	64.10	9656	9597	2	78
Mean		8.383	61.76	8063	7992	1,20	57.73
CV (%)		14.61	4.49	14.51	14.61	34.50	18.05

4.4.3. Comparative genomics

The number of *nod*, *fix* and *nif* genes recorded in indigenous soybean-nodulating rhizobia and soybean-nodulating commercial strain USDA110 are presented in Table 6. The *nif* genes number recorded in indigenous strains varied from 0 to 2 with the indigenous strain NAC94 holding the higher number (2) of *nif* genes (2). In contrast, three indigenous strains (NAC1, NAC22 and NAC76) hold no *nif* genes. Concerning *fix* genes, their number varied from 2 to 11 with the higher number of these genes recorded with the indigenous strains NAC17 (11) followed by the strains NAC76 and NAC37 (10). Besides, *nod* genes number varied from 4 to 11 with the higher number recorded with indigenous strain NAC22 (11) followed by NAC78 and NAC72 (10).

Table 6: Number of nif, nod and fix genes

Strain	*nif*	*Nod*	*fix*
NAC1	0	5	2
NAC53	1	8	7
NAC46	1	7	4
NAC22	0	11	5
NAC76	0	10	10
NAC37	1	7	10
NAC17	1	9	11
NAC28	1	8	6
NAC42	1	8	6
NAC69	1	4	2
NAC71	1	9	7
NAC72	1	10	7
NAC11	2	7	6
NAC94	1	8	7
Min	0	4	2
Max	2	11	11
Mean	0.85	7.92	7,57
CV	62.36	23.96	41.14

In addition, indigenous and commercial strains were compared based on their *16S rRNA* region approximating 1400bp. Based on this phylogeny tree (Figure 9), indigenous soybean-nodulating rhizobia and the commercial strain USDA110 were partitioned into two main clusters (Figure 9). The first cluster comprised of twelve strains and was separated with the second cluster (only two strains) by 100% bootstrap value. From this classification, six indigenous strains (NAC28, NAC42, NAC46, NAC76, NAC37 and NAC17) clustered together with the commercial strain USDA110 (98% bootstrap value).

The genetic distance between indigenous strains and the commercial strainUSDA 110 is presented Table 7. The genetic distance ranged from 0 to 0.364 with the recorded mean genetic distance of 0.156. The indigenous strain NAC46 had the lowest distance (around 0) to the commercial strain USDA 110. In addition, four more indigenous strains had lower genetic distances to the commercial strain (genetic distance range from 0.006 and 0.007) namely NAC76, NAC42, NAC37 and NAC17.

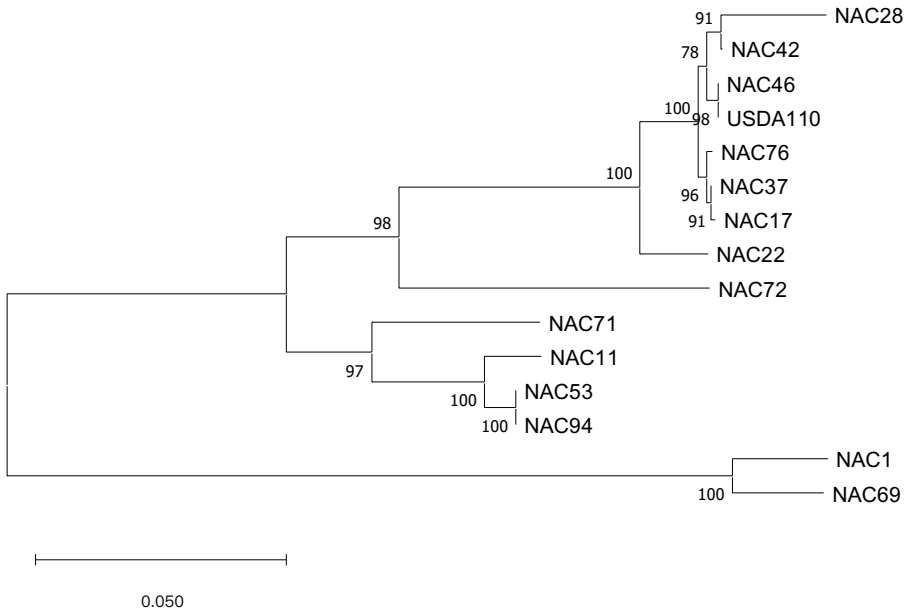

Figure 9: Phylogeny tree based on 16SrRNA constructed using Tamura-Nei model and Maximum Likelihood method. Bootstrap values are shown next to the branches.

Table 7: Genetic distances between indigenous rhizobia strains and commercial strain

Strains	NAC11	NAC53	NAC76	NAC28	NAC42	NAC22	NAC37	NAC17	NAC46	USDA110	NAC1	NAC69	NAC94	NAC71	NAC72
NAC11															
NAC53	0.018														
NAC76	0.140	0.132													
NAC28	0.166	0.157	0.029												
NAC42	0.138	0.130	0.008	0.021											
NAC22	0.140	0.140	0.027	0.051	0.031										
NAC37	0.140	0.132	0.002	0.029	0.007	0.026									
NAC17	0.141	0.133	0.003	0.029	0.008	0.027	0.001								
NAC46	0.139	0.131	0.007	0.025	0.006	0.031	0.006	0.007							
USDA110	0.139	0.131	0.007	0.025	0.006	0.031	0.006	0.007	0.000						
NAC1	0.301	0.290	0.333	0.363	0.332	0.320	0.333	0.333	0.336	0.336					
NAC69	0.293	0.278	0.330	0.364	0.326	0.336	0.330	0.330	0.333	0.333	0.037				
NAC94	0.018	0.000	0.132	0.157	0.130	0.140	0.132	0.133	0.131	0.131	0.290	0.278			
NAC71	0.067	0.065	0.142	0.165	0.140	0.124	0.145	0.146	0.145	0.145	0.292	0.297	0.065		
NAC72	0.138	0.136	0.126	0.149	0.120	0.127	0.127	0.128	0.125	0.125	0.333	0.332	0.136	0.129	

4.5. Discussion

4.5.1. Genomes'sequences quality

In this study 24 genomes were sequenced and from them, only 14 yielded higher quality. This rate can be explained by the sequencing platform used. Quality control of sequences is very important for meaningful analysis. In a study conducted by Degnan and Ochman (2012), up to 85% of sequences were removed because they did not meet the treshhold accuracy. The most reported sequences removal rate on MiSeq sequencing platform, due to lower quality range from 40-70% (Degnan and Ochman, 2012).

4.5.2. Genomes features

Draft genomes of indigenous rhizobia strains nodulating soybeans belonged to five genera of alpha-proteobacteria (table 5). *Bradyrhizobium* genus was the most represented because this genus is the most reported compatible host of soybean. These findings are in agreement with many authors who found that soybean is nodulated mostly by *Bradyrhizobium* genus in tropical soils (Wasike et al., 2009; Li et al., 2011; Chibeba et al., 2017; Gyogluu et al., 2018). This study found that in addition to the mentioned genus, soybean is nodulated by other genera like *Rhizobium*, *Agrobacterium*, *Microvirga* and *Kosakonia*. Higher diversity was found because, strains were isolated from different environments (cultivated fields and grassland) and on diverse legumes plants. Youseif et al. (2014) also observed the nodulation of soybean by two more genera in addition to the *Bradyrhizobium* group namely *Rhizobium* and *Ensifer*. From these results, eight strains (NAC53, NAC46, NAC22, NAC76, NAC37, NAC17, NAC28 and NAC42) out of fourteen were similar (98-100%) to the commercial strain USDA110.

Genomes size varied considerably among indigenous strains (Table 1). These variations in genome size are attributed to different adaptation mechanisms to changing environment conditions (Geddes et al., 2007). This size is consistent with other findings. For example, Bromfield et al., (2019) and Kaneko et al. (2011) who found the *Bradyrhizobium* genome size of 7.04Mb and 9.207Mb respectively. The *Bradyrhizobium* genus holds the largest genomes while *Microvirga* genus holds the smallest. Black et al. (2012), Kaneko et al. (2000) and Giraud et al. (2007) also found larger

size of genome in *Bradyrhizobium* genus. From this study, seven indigenous strains (NAC1, NAC22, NAC76, NAC37, NAC17, NAC28 and NAC42) hold a genome size closer to the commercial strain USDA110 showing their close relatedness and suggesting the potential higher nitrogen fixation ability.

The mean GC content was 62% which is similar to related strains reported such as *Rhizobium leguminosarum* strain TA1 (60.9%) (Sablok et al., 2017) and *Bradyrhizobium diazoefficiens* strain USDA 110 (64%) (Kaneko et al., 2002). In total, a mean number of 8063 genes was identified and 99 % of these were protein coding (Table 1). The number of genes recorded by this study is in range of that found by other authors (Kaneko et al., 2011) and suggesting that the phenomenon of insertion and deletion are not very important. These findings show that indigenous soybean-nodulating rhizobia are well adapted to their environment. This is in agreement with findings of many researchers suggesting that 80 to 90% of the bacteria genome is formed by protein-coding DNA (Bobay and Ochman, 2017). The indigenous rhizobia genomes also comprised of either one or two sets of transfer-messenger RNA (tmRNA) and a mean of 57 transfer RNA (tRNA). This number of tmRNA and tRNA was also found by Kaneko et al. (2011) and Kaneko et al. (2002) and suggest that there is no significant modification of indigenous rhizobia genomes.

4.5.3. Comparative genomic

The comparative genomics of indigenous soybean-nodulating rhizobia and commercial strains USDA 110 was achieved in three steps: 1) nitrogen fixation genes were quantified and compared, 2) the phylogenetic analysis of 16S region was performed to analyze their genetic relatedness and 3) the genetic distance between strains was determined by pairwise analysis.

Three types of genes are involved in nitrogen fixation namely *nod*, *nif* and *fix* genes. *Nod* genes are responsible of nodules organogenesis while *nif* genes and *fix* genes are responsible for nitrogenase synthesis and activity respectively (Geddes et al., 2020; Menna and Hungria, 2011). The *nif* genes number recorded in indigenous strains varied from 0 to 2 with the indigenous strain NAC94 holding the highest number of *nif* genes. In contrast, three indigenous strains (NAC1, NAC22 and NAC76) hold no *nif* genes suggesting that the nitrogen fixation might not be possible and thus, these strains could not be considered in a selection program. This finding is consistent with Okazaki et al. (2015)

who reported in maximum 2 *nif* genes in their studied genomes. In addition, De Meyer et al. (2016) also did not detect any *fix* genes in six of genomes under their investigation although these strains were reported to form nodules and fix nitrogen with their host.

Concerning *fix* genes, their number varied from 2 to 11. Meyer et al., (2016) also found a total number of 13 *fix* genes in rhizobia genomes under their study. The presence of the full set of nitrogen fixation genes is equivalent to higher capacity of both nodulation and nitrogen fixation. Many authors sustain that the presence of nitrogen fixation is essential for nodules formation and consequentlty for nitrogen fixation. For example, Okazaki et al., (2015) and Andrews et al., (2018) found *Bradyrhizobium* sp. DOA9 strain of particular biological interest because it possesses divergent *nod* genes compared with other bradyrhizobia and consequently a broader host range.

In this study, 11 soybean-nodulating rhizobia strains (NAC53, NAC46, NAC37, NAC17, NAC28, NAC42, NAC69, NAC71, NAC72, NAC11, NAC94) out of 14 (Table 5) possess the full set of the nitrogen fixation and could be considered for a program of selection of effective rhizobia to be included in the inoculants commercial. Others studies, have also demonstrated the nodulation and nitrogen fixation in some strains despite the absence of nitrogen fixation genes. This suggest that some strains have alternative mechanisms to perform the nodulation and nitrogen fixation process (Menna and Hungria, 2011). Therefore, the selection of effective rhizobia based on genomic comparative must be validated by the nodulation test on hosts.

In addition, indigenous and commercial strains were compared based on their *16S rRNA* region approximating 1400bp. This size is the fragment size acceptable for bacterial identification of this target gene (Kawaka et al., 2018; Mwenda et al., 2018). From this classification, six indigenous strains clustered together with the commercial strain USDA110 (98% bootstrap value) suggesting that they may have similar genomic features and thus could be considered as candidate elite strains for testing in order to be included in inoculants formulation. Many studies suggested that indigenous rhizobia from tropical soils are not very different from commercial strains (Chibeba et al., 2017; Chibeba et al., 2018). This finding agrees with past studies demonstrating that indigenous rhizobia are similar to commercial rhizobia in legume grain yield improvement (Tena et al.,2016; Abou-shanab et al., 2019) and in genetic similarity (Kawaka et al., 2018; Mwenda et al., 2018).

Pairwise analysis was performed to quantify the genetic distance between indigenous strains and the commercial strain USDA 110 (Table 3). The indigenous strain NAC46 had the lowest distance (around 0) to the commercial strain USDA 110 suggesting that this strain isolated from South Kivu soils could be same as the commercial strain. This may be introduced through inoculation using commercial inoculant disseminated in Democratic Republic of Congo by the N2 Africa project (Van Heerwarden et al., 2018). The genetic distance close to 0 has been attributed to a common ancestry and a very low rate of recombination among rhizobia genomes (Hellwig et al., 2020). This finding suggests that there exist effective indigenous rhizobia in tropical soils that have the same ancestry as the commercial strains. The implication is that instead of introducing exotic strains for BNF and productivity improvement of legumes, it is preferable to isolate effective strains locally and use them for inoculant production as they are already adapted to environmental conditions.

4.6. Conclusion

This study has demonstrated the existence of indigenous soybean-nodulating rhizobia in South Kivu soils that have same genomics characteristics as the commercial rhizobia USDA110 namely NAC46, NAC76, NAC42, NAC37 and NAC17. These indigenous rhizobia strains exhibited comparable nitrogen fixation characteristics comparable to the commercial strain USDA110. I suggest further investigations and testing of these indigenous rhizobia under different environmental conditions to confirm their nitrogen fixation superiority. Comparative genomics can be considered a time saving method for rapid selection of effective rhizobia to be included in commercial formulation but must always be coupled with field testing.

CHAPTER FIVE: EFFECTIVENESS OF ELITE INDIGENOUS RHIZOBIA STRAIN IN ENHANCING BIOLOGICAL NITROGEN FIXATION AND SOYBEAN YIELDS UNDER DIFFERENT SOILS CONDITIONS

5.1. Abstract

Soybean is an important crop in the Democratic Republic of Congo, a country faced with high malnutrition level. However, its production has been limited by poor soil fertility. Commercial rhizobia strains introduced in 2010 failed to adapt in local soil conditions. In this study, six selected indigenous rhizobia strains were tested toward enhancing soybean productivity compared to two commercial strains USDA110 and SEMIA5019. The study was conducted in the greenhouse and in the station field of International Institute of Tropical Agriculture (IITA), Kalambo, D.R. Congo during 2016/2017 cropping season. The treatments included: (1) N-, control without inoculation and N-fertilizer; (2) N+, non-inoculated control with 80 kg ha^{-1} of nitrogen; (3)plots inoculated with commercial strain *Bradyrhizobium diazoefficiens* USDA110; (4) plots inoculated with commercial strain *B. elkanii* SEMIA5019; (5) plots inoculated with local strains NAC17; (6) NAC22; (7) NAC37, (8) NAC42 (9) NAC 46 and (10) NAC76. Greenhouse and field experiments were laid out as completely randomized design and Randomized Complete Block Design respectively. In the greenhouse nodulation and biomasse production varied significantly among treatments ($P<0.01$). The best inoculation treatments across all experiments were the indigenous strains NAC46 and NAC17 which nodulated twice compared to the commercial strain USDA 110. In the field NAC46 and NAC17 increased soybean grain yield from 2.4 t ha^{-1} to 3.3 t ha^{-1} and 3.4t ha^{-1}; translating into an increase of 68.7% and 70.8% respectively, over the commercial strain USDA110. The results demonstrated that indigenous rhizobia NAC46 and NAC17 would thus be the solution to enhanced BNF and Soybean yields in South Kivu.

Key words: inoculation, elite rhizobia, local rhizobia; soil fertility, USDA110.

5.2. Introduction

Soybean, *Glycine max* (L.) Merryl is an important crop worldwide and is increasingly becoming important and popular in South Kivu due to its potential to curb high malnutrition (Hartman et al., 2011). Soybean was introduced in Africa from Asia in the 19th century (Khojely et al., 2018) to address the need for cropping systems diversification dominated by maize (Giller et al., 2011a). In these systems, soybeans enhance soil fertility through Biological Nitrogen Fixation process (Collino et al., 2015). At the same time, this crop provides smallholders farmers the opportunity to increase their households' income while fighting malnutrition issues because of its important nutritional value in terms of protein, amino acid and micronutrient (Arslanoglu, 2011; Xu et al., 2015).

Since the last decades, in South Kivu province of DRC, where the economy depends largely on agriculture (Jeníček and Grófová, 2016; Maass et al., 2012), there is an increase of soybean demand due to the presence of market created by the development of livestock (Rudel et al., 2015) and industry of soybean processing (Bisimwa et al., 2012). The most common soybean-based formula consumed in South Kivu province includes soy infant formula (Bahwere et al., 2016; Owino et al., 2011), soymilk, soy oil, soybean flowers, soybean biscuits, soybean spices, soybean meat, soybean bread and cakes and soybean waste industry used as animal feed (Shurtleff and Aoyagi, 2009). This crop is essentially cultivated by smallholders' farmers and maintained by women to improve nutrition and generate income for their households, and by youth to pay costs of their education (CIALCA, 2010).

In South Kivu, farmers generally plant legumes, including the soybean without adding mineral fertilizers because they are not available, unaffordable and are less economic (Vanlauwe et al., 2010; Pypers et al., 2011; Lambrecht et al., 2016). Therefore, soils have been depleted due to a continuous cropping without soil replenishment as consequence of population pressure (Bashagaluke et al., 2015). In the case of South Kivu, soybean yields depend upon N fixation by native rhizobia that are not always effective (Ojo et al., 2015) thus obtaining low yields, estimated at 0.5 t ha^{-1} (FAO, 2018).

Inoculation of soybean with appropriate, highly effective, adapted and compatible rhizobia has been stated as the most economic (Chianu et al., 2011), productive (Saturno et al., 2017) and environment friendly (Collino et al., 2015) approach to improve crop yield. Two main pathways have been pursued by international research organizations to improve soybean yield: first, promiscuous soybean cultivars were developed to nodulate freely with native rhizobia (Tefera, 2011); second, inoculation with highly effective rhizobia strains has been promoted (van Heerwaarden et al., 2018). In that line, commercial inoculants, Biofix Legume inoculants, containing *Bradyrhizobium diazoefficiens* USDA110 strain, was introduced among South Kivu farmers by N_2 Africa program since 2010 (www.n2africa.org) and disseminated among farmers by agricultural extensions services and humanitarian organization.

From trials and farmer's fields results, the commercial inoculants increased legume yield from 500 kg ha^{-1} to 1343 kg ha^{-1} (van Heerwaarden et al., 2018), but still not at desired levels in certain farms and with no increase in other farmers, while the potential soybean yield is 3000 kg ha^{-1} (Salvagiotti et al., 2008; Zanon et al., 2016). That low improvement was attributed to the effect of environmental and edaphic conditions on the introduced commercial strains in addition to the failure to overcome the competition barriers opposed by native rhizobia (van Heerwaarden et al., 2018).

Many studies in Africa have reported the presence of effective rhizobia strains among indigenous rhizobia populations (Musiyiwa et al., 2005; de Almeida Ribeiro et al., 2015; Chibeba et al., 2017). In addition, indigenous rhizobia have been described by many studies as more persistent, well adapted to local conditions and therefore more competitive for nodules occupancy compared to introduced exotic strains (Fening and Danso, 2002). From our results on genetic characterization of soybean-nodulating rhizobia in South Kivu, some indigenous rhizobia clustered together with the commercial strains USDA110 (bootstrap value: 99%) showing possible relatedness of indigenous strains with this commercial strain. There is need to test these indigenous strains and identify highly effective indigenous strains suitable for South Kivu environment and edaphic conditions. Six indigenous rhizobia strains were tested for their ability to improve soybean nodulation and yield in order to propose indigenous strains with potential to be included in soybean inoculants.

5.3. Materials and methods

5.3.1. Study area

The current study was conducted in South Kivu province, Eastern Democratic Republic of Congo, in the greenhouse and station field of International Institute of Tropical Agriculture (IITA), Kalambo station. South Kivu is located in Eastern DRC between 1°36' - 5° South and 26°49'-29°20' East and the surface is estimated to be 69,130 km^2 with 3.8 million people of population with the estimated density of 91 people per km^2 (Pypers et al., 2011). It is recognized as a high humid forest zone depicted by high vegetation diversity (Potapov et al., 2012) and highlands. Soils are mostly Dystric, Humic Nitisols and Humic Ferralsols (van Engelen et al., 2006; Eswaran et al., 1997). This region has a tropical climate, the Aw3 type according to Koppen classification with an average annual rainfall of 1500 mm and mean temperature of 18 °C (Nash and Endfield, 2002).

The site is characterized by smallholders farming systems that have an average acreage ranging between 0.5 and 1 ha (Pypers et al., 2011). The main cultivated crops include cassava, common beans, maize and banana (Maass et al., 2012). Soybean crop was promoted since 1990 to fight high malnutrition rate induced by repetitive wars (Kismul et al., 2015) and since then its cultivation is increasing (FAO, 2018). The current study was conducted using three sites soils namely Walungu, Kalehe and Murhesa soils. These were selected regarding the fact that they are soybean production zones and different soils conditions. Walungu is characterized by ferralsols while Kalehe and Murhesa are characterized by Humic soils (Van Engelen et al., 2006).

5.3.2. Soil sampling and analysis

Selected field had no history of neither soybean cultivation nor rhizobia inoculation. Two weeks before sowing, twenty composite soil samples were collected from 0–20 cm depth along the field diagonal (Carter and Gregorich, 2008). Soil samples were pretreated (dried and sieved through a 2mm perforation size) prior for analysis. Total Soil Organic Carbon was analyzed using the modified Walkley-Black (Okalebo et al., 2002**),** total nitrogen and available phosphorus analysis were performed, respectively by the Kjeldahl and Olsen methods **(**Anderson and Ingram, 1993**).** Exchangeable potassium, Magnesium and Calcium were determined by Mehlich 3 method

(Okalebo et al., 2002). Soil pH was determined using the 1:10 water method and measured by the seven compact, S210 Metler Toledo pH meter, after 60 min of agitation. The population of native rhizobia was determined by the plant infection technique (Somasegaran and Hoben, 1994).

Table 8: Greenhouse and field soils characteristics

Sampling site	pH	SOC	N	P	K	Ca	Mg	Rhizobia population.
Walungu	5.45	3.32	0.21	7.70	245	1061	302	$2x10^2$
Kalehe	6.89	3.24	0.19	19.14	205	6980	258	$5x10^3$
Murhesa	7.9	3.33	0.21	22.95	455	3230	537	$1x10^3$

P: extractable P in mg/kg; K: exchangeable K in mg/kg; Ca: exchangeable Ca in mg/kg; Mg: exchangeable Mg in mg/kg; rhiz. pop.: rhizobia population in number of cells per gram of soils.

5.3.3. Rhizobia isolates identification

Six indigenous rhizobia to be considered for testing in the greenhouse and field experiments were selected based on their high genetic similarity with the commercial strain `USDA110. For similarity checking, DNA was obtained as explained in the section 3.3.4., amplified as described in section 3.3.5. and sequenced as described in section 3.3.6. The *16S rRNA* sequences were analysed in MEGA7 to check their similarity with the commercial strain USDA 110 and SEMIA available in the NCBI Genebank.

5.3.4. Rhizobia culture and inoculant preparation for seeds inoculation

Indigenous rhizobia strains isolates were inoculated on YMB (Yeast Extract Mannitol broth) (Somasegaran and Hoben, 1994), incubated at 25 ^{0}C until the concentration attained 10^9cells ml^{-1}. Inoculants were prepared from indigenous rhizobia cultures using sterilized peat as carrier material, incubated for two weeks and inoculated at the rate of 10 g per kg of seed with 20% sugar-water (w/v) used as adhesive following the two-step inoculation method (Woomer et al., 2011).

5.3.5. Trial management and experimental design

Two experiments were carried out to compare six selected indigenous strains, based on the high genetic similarity with the commercial strain, with two commercial strains USDA 110 and SEMIA5019: 1) testing for effectiveness carried in potted field soils in the greenhouse using two tdifferent sites soils (ferralsols and humic soils) (Table 1) and 2) on station field testing.

5.3.6. Indigenous rhizobia testing under controlled conditions

Greenhouse experiment was conducted in the IITA Kalambo station's greenhouse, temperature in the greenhouse varied from 22 to 38 ^{0}C. Two site soils: from Walungu and Kalehe villages, were used as substrate in 3 liters pot containers. The two villages were selected because they are all soybean production zones and their soils conditions are different (Table1). Sterilized 3 liters capacity PVC pots were filled with soil substrate (2.5kg) and covered with a sterile plastic plate to avoid contamination. Soybean seeds were surface sterilized using the described procedure (Somasegaran and Hoben, 1994), pre-germinated in agar plates; and 3 seeds per pot were sewed, thinned to 2 after emergence for appropriate spacing. Seeds were inoculated with 1 ml of broth concentrated at 10^9 cells ml^{-1} pre-cultured (described in section 2.3).

For the mineral N control, urea was applied at a rate of 80 kg ha^{-1} (Pypers et al., 2011). Watering was done regularly at the frequency of 3 times per week adjusted according to plant needs. After 7 weeks, at early flowering, plants were harvested; nodules counted, weighted and shoot weight determined by weighing after oven dried at 70 ^{o}C for 48 h. A Completely Randomized Design consisting of 10 treatments including 6 indigenous rhizobia and 2 commercial strains (SEMIA5019 and USDA110), and non-inoculated control with (N+) and without mineral N (N-), with 3 replicates was established. Soybean variety SB24 was used as the test crop, selected for their high adoption among farmers (Walangululu et al., 2014).

5.3.7. Indigenous rhizobia testing under field condition

A field experiment was conducted at the field station of IITA Kalambo located in Murhesa during 2016-2017 long rains (September to January). Soils characteristics of field were determined (Table1). Six indigenous rhizobia strains from South Kivu were compared with two commercial strains USDA110 and SEMIA5019 on promiscuous soybean variety SB24. The experiment was laid out as Randomized Complete Block design with 3 replicates.

The treatments included: (1) N-, control without inoculation and N-fertilizer; (2) N+, non-inoculated control with 80 kg of N ha^{-1}; and inoculated with (3) commercial strain *Bradyrhizobium diazoefficiens* USDA110; (4) commercial strain *B. elkanii* SEMIA5019; (5) local strains *B. japonicum* NAC17; (6) NAC22; (7) NAC37, (8) NAC42 (9) NAC 46 and (10) NAC78. Each plot measured 6 m x 4 m, seeds were planted at 5cm intervals and 45cm spacing between rows equivalent to 1066666 plants per hectare density. To avoid cross contamination, plots were separated by four non-inoculated lines. Legume inoculants were prepared from isolates as described in the section 2.3.

The trial management was done according to known farmer's practice; weeding as per need before the canopy closure. The intensity of green color in leaves was measured at different growth stages using a chlorophyll Meter (Dey et al., 2016). Plants were assessed for nodulation at flowering stage, seven weeks after planting. Plants were uprooted carefully, roots rinsed, nodules counted, dried for 48h and 70 ^{0}C at oven and dry weight recorded. Soybean grains were harvested at maturity (4 months), dried and dry weight recorded.

5.3.8. Data analysis

Data were subjected to analysis of variance (ANOVA) using the software R version 3.5.1. When differences between treatments were detected, Tukey test was used to compare means at $p > 0.05$ level of significance. Relative effectiveness (RE) was determined by dividing the shoot dry weight of treatment over that of the N+ treatment, in the same block (Chibeba et al., 2018).

5.4. Results

5.4.1. Phylogenetic relationship between indigenous strains and commercial strains USDA110

The tested isolates and commercial strain USDA 110 clustered together with bootstrap value of over 90% (Fig.10).

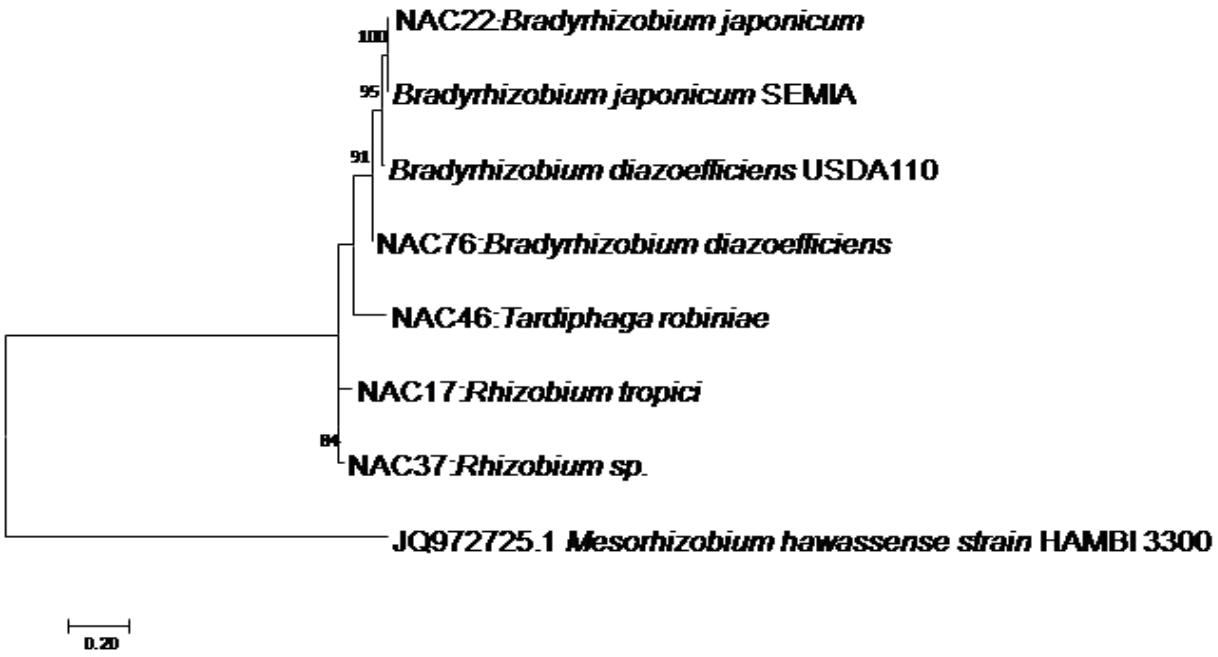

Figure 10: Phylogenetic relationship between indigenous strains and commercial strains USDA110 and SEMIA using the Maximum Likelihood method based on the Tamura 3-parameter model. *Mesorhizobium hawassense* was used as out-group.

5.4.2. Nodulation and shoot dry weight of indigenous rhizobia recorded in the greenhouse

Nodules number reported in the greenhouse varied significantly among strains (P=0.0001) and between the two soils types (P=0.0203). The recorded nodules numbers varied from 3 nodules to 21 nodules per plant in Walungu soil while it varied between 1 to 36 nodules per plant in Kalehe soil (Table 7). The highest nodules number in Walungu was recorded with the treatments inoculated by the indigenous strain NAC46 and NAC76 (±21 nodules per plant), followed by

commercial strains (USDA110 and SEMIA 5019) and indigenous strains (NAC22, NAC17 and NAC37). The lowest number of nodules in Walungu site soils was recorded in the treatment without inoculation but with mineral N fertilizer (N+), where recorded nodules numbers averaged only 3 nodules per plant (Table 2). The highest nodules number in Kalehe soil was recorded with the same indigenous strains NAC46 and the control (N-) (average 34 nodules per plant), followed by NAC76 (21 nodules) (Table 2). The lowest number of nodules per plant was also reported in the treatment N+, where the nodule number averaged only 1.

The nodules dry weight (DW) recorded in the greenhouse experiment also varied between site soils and among strains (P=0.0001) (Table 8). The highest nodules dry weight was recorded in Kalehe soil (average 0.373 g per plant DW) while the lowest nodules weight was recorded in Walungu soil (average 0.284 g per plant DW). The inoculation with indigenous strains NAC46 produced the highest nodules weight followed by NAC37 and NAC76 in Walungu soil (Table 7). The lowest nodules weight was recorded with N+ control for both soils.

Shoot dry weight variation (P=0.0012) was recorded only in the Kalehe soils while in Walungu no difference was recorded among the inoculated strains. In Kalehe soils, the highest shoot weight was recorded by N+ control (9.6 g plant DW), followed by indigenous strain NAC46, and by the commercial strain SEMIA5019. The lowest shoot dry weight in Kalehe soils was recorded by the indigenous strain NAC37 (5.9 g plant DW) (Table 9).

Table 9: Nodule number, nodule dry weight and shoot dry weight recorded in the greenhouse by the effects of different rhizobia strains

	Nodule number		Nodule weight(g)		Shoot dry weight(g)	
Treatment	Walungu	Kalehe	Walungu	Kalehe	Walungu	Kalehe
NAC46	21.0a	36.0a	0.66a	0.88a	8.00a	9.06ab
NAC76	20.0a	21.3b	0.30bc	0.26ab	6.63a	6.30bc
NAC22	11.0b	10.6cd	0.18bc	0.18c	6.23a	6.50bc
NAC17	10.0b	9.0cde	0.17bc	0.16c	6.10a	6.36bc
NAC37	9.3b	17.0bc	0.46ab	0.62ab	5.66a	5.90c
NAC42	8.0bc	7.0de	0.40abc	0.20c	5.80a	6.50bc
USDA110	12.6b	9.0cde	0.21bc	0.24c	6.10a	6.53bc
SEMIA5019	10.6b	8.3cde	0.15bc	0.20c	6.23a	7.33abc
N-	7.0bc	33.0a	0.20bc	0.60b	5.06a	6.30bc
N+	3.0c	1.3e	0.09c	0.15c	7.10a	9.60a
P-value	< 0.0001	< 0.0001	0.0001	< 0.0001	0.6439	0.0012

Means followed by the same letter are not statistically different (P<0.001).

5.4.3. Nodule number, nodule dry weight, shoot dry weight, leaf greenness, plant height and crop yield recorded in the field study

In the field, all treatments nodulated but their number varied considerably across treatments (P< 0.0001). The nodules number varied from 30 to 69 nodules per plant. Even the non-inoculated and not fertilized (N- and N+) control plants nodulated abundantly (average 40 nodules per plant). The highest nodule number was recorded by the treatments of both indigenous and commercial strains, which did not differ among them, except for NAC42, with a lowest number of nodules (Table 10).

Nodules dry weight did not vary with the treatments but shoot dry weight significantly varied among rhizobia strains (P< 0.0001). The highest biomass was recorded with the indigenous strain NAC17 (8.0 g plant DW), followed by NAC46. These shoot dry weights were even higher than the commercial strains USDA110 and SEMIA5019. The lowest shoot dry weight was recorded by the treatment N- control (5.8 g plant DW) (Table 10).

The plant leaf greenness also varied among treatments (P< 0.0001). The highest intensity of green color measured on leaves was recorded by the indigenous strains NAC17 and NAC46 (Table 3). The lowest green color intensity was recorded by the control N- and the indigenous strain NAC76 (about 55) (Table 10).

Plant height also varied significantly across treatments (P< 0.0001). The highest plant height was recorded by the indigenous rhizobia strain NAC17 (64cm). The commercial strain SEMIA5019 took the third place while USDA 110 took the fourth position. The lowest treatment in terms of plant height was the treatment NAC46 (55cm) (Table 10).

Finally, grain yield also varied across treatments (P< 0.0001) (Table 10). Yield improvement was recorded by the indigenous strains NAC17 and NAC46 that yielded 1.4-fold than the commercial strain USDA110 and 1.6-fold than the control with nitrogen (N+). The lowest grain yield was recorded by the treatment N-, followed by N+ (Table 10).

Table 10: Nodule number (NN), nodule dry weight (NDW), shoot dry weight (SDW), leaf greenness (LG), plant height (PH) and crop yield recorded in the field by the effects of different rhizobia strains

Treatment	NN	NDW(g)	SDW(g)	LG	PH(cm)	Yield (kg/ha)
NAC17	69.0a	1.40a	7.97a	43.83a	64.4a	3397a
NAC46	65.0a	1.10a	7.60ab	47.57a	55.2d	3409a
NAC76	65.0a	0.83a	6.04ef	34.71d	60.2abc	2342ef
NAC37	62.6a	0.93a	7.15bc	37.90cd	62.8ab	2924bc
NAC22	59.6ab	0.96a	6.43de	40.08c	62.2ab	2720cde
NAC42	46.6c	0.42a	6.46de	41.27bc	58.4cd	3148ab
USDA110	64.6a	3.19a	6.73cd	37.90cd	58.9bcd	2416de
SEMIA5019	62.0a	0.98a	6.64cd	39.32c	61.1abc	2768bcd
N-	50.6bc	0.38a	5.75f	35.55d	58.4cd	1543g
N+	30.0d	0.20a	6.63cd	39.72c	58.5cd	2012f
P value	< 0.0001	0.3426	< 0.0001	< 0.0001	< 0.0001	< 0.0001

Means followed by the same letter are not statistically different (P<0.001).

5.4.4. Relative effectiveness of indigenous rhizobia strains compared to the reference strains

In the greenhouse, the effectiveness index of all tested isolates did not exceed the reference treatment N+ but the indigenous rhizobia NAC46 had a relative index higher than the commercial strains USDA110 and SEMIA 5019 (Figure 9). However, in the field, the relative effective index by the 3 indigenous rhizobia strains (NAC17, NAC46 and NAC37) exceeded both commercial strains and control with N (N+) (Figure 10).

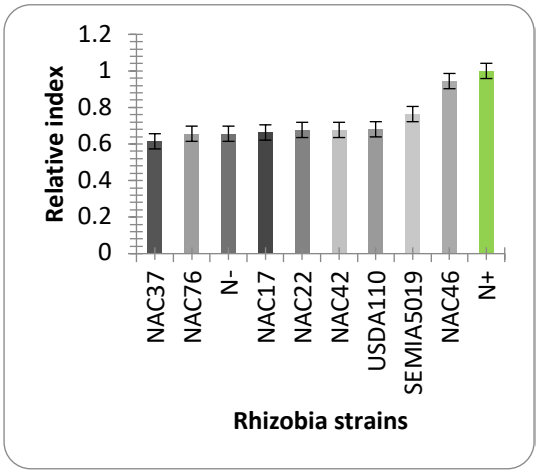

Figure 11: Relative index in the greenhouse.

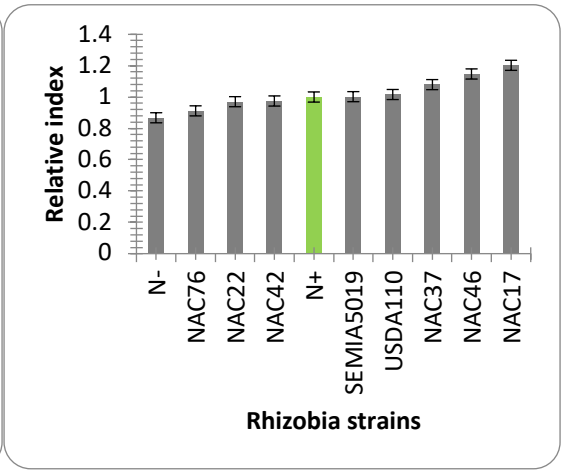

Figure 12: Relative index in the field.

5.5. Discussion

5.5.1. Genetic similarity between selected indigenous rhizobia and commercial strains

The six tested indigenous rhizobia clustered together with the commercial strain USDA110 supported by high bootstrap value (91%). This strain is an outstanding soybean symbiont used as commercial inoculants worldwide (Chibeba et al., 2018). The high genetic relatedness of this strain with indigenous strains suggests that there exist rhizobia strains among indigenous population potentially good for nitrogen fixation and enhanced soybean productivity. In addition, some reports have suggested a possibility for tropical origin of this *Bradyrhizobium diazoefficiens* USDA 110 strain (Delamuta et al., 2013).

5.5.2. Effectiveness of indigenous strains under controlled environment

Many parameters have been used to identify a rhizobia strain with superior nitrogen fixation ability and legume productivity improvement. The most important of them include the estimate of the amount of nitrogen fixed through BNF by ^{15}N natural abundance method (Pacheco et al., 2020), nodulation assessessment, shoot weight, the accumulation of nitrogen in leaves (Cardoso et al., 2012), ureide technique (Kanu and Dakora, 2012) and grain yield assessment (Gresta et al., 2019). The ^{15}N natural abundance method was reported as the most accurate method of BNF measurent (Chalk et al., 2016) but it is very expensive. Therefore, the correlated parameters have been proposed for BNF assessment and identification of highly effective rhizobia strain. A positive correlation was demonstrated between the amounts of N fixed throught BNF and nodulation, plant shoot weight and grain yield (Cordova et al., 2019).

This study revealed significant differences in nodulation among treatments in the two soils, in the greenhouse experiment, suggesting that there is need to inoculate the soils of the two sites (Leggett et al., 2017). The important factors that limit the expression of inoculation response include: i) failure of the introduced strain to establish nodules due to competition from native rhizobia (Ulzen et al., 2016) and ii) failure of the introduced strain to adapt to the new environment (Koskey et al., 2017). These differences may be explained by the low abundance or less effectiveness of native rhizobia population in the Walungu and Kalehe soils (Table 6). Sanginga and Okogun (2003) and Osunde et al. (2003) stated that responses to inoculation are more likely to occur when the population of native rhizobia is less than 10^3 cells per gram of soil or when the native rhizobia are less effective. Koskey et al (2017) in their study on efficiency of native rhizobia in N fixation improvement and yield increase of climbing beans in contrasting environments of Kenya observed that the yield improvement and nodulation of introduced rhizobia strains depended largely on the population size of viable native rhizobia in the soil.

This study revealed significant differences in nodulation between the two soils; higher nodules number was produced in Kalehe soils (Table 7). This is because of the differences in soils conditions (Table 6). Nodulation depends on a number of soils factors, especially the soil pH

(Lapinskas, 2007), P availability and the indigenous rhizobia abundance and effectiveness (Sanginga et al., 2000; Slattery et al., 2004). Kalehe soil had better conditions for growth and survival of rhizobia bacteria namely the neutral pH and higher P content compared to Walungu soil (Table 6). This result agrees with other authors, for instance Gyogluu et al., (2016) who studied the symbiotic effectiveness of soybeans to inoculation by different *Bradyrhizobium japonicum* strains at 3 experimental sites in Mozambique. They found response variation depending on different sites and suggested that there are specific effects of sites on nodulation and dry matter improvement by rhizobia. This result is also in agreement with Boucho et al., (2019) who reported that the response to inoculation is highly affected by soils conditions and for their case phosphorus availability in the soil promote nodulation and biomass. Therefore, this study suggests the improvement of soils conditions prior to inoculation such as liming where soil pH is low and phosphorus application at optimum level.

The inoculation of soybean increased shoot dry weight (biomass) only in Kalehe soils while any change was observed in Walungu soils. The nonresponse of soybean to inoculation in Walungu soils can be ascribed to unfavourable soils conditions such as low pH and low P levels, which limited the process of nitrogen fixation despite the presence of nodules (Table 6). This is in agreement Collino et al., (2015) who reported that the BNF intensity depends on the crops, soils and meterological factor. This study also revealed differences among strains in both nodulation and shoot dry weight improvement which is primarily due to their genetic differences (Chibeba et al., 2017, 2018). Many studies detected a consistent difference in nitrogen fixation effectiveness among studied rhizobia strains (Abaidoo et al., 2007; Chibeba et al., 2017, 2018) and consequently this study further contributes to the evidence that effective rhizobia do occur in African soils.

5.5.3. Effectiveness of indigenous strains under field conditions

In the field, nodules were observed on all treatments including the controls non-inoculated and without nitrogen (N-); the N- control produced even higher number of nodules compared to the inoculated plots. This finding could be attributed to the presence in soils of native rhizobia strains highly competitive. Many studies have demonstrated that infective rhizobia occur naturally in soils but mostly they are less efficient (Abaidoo et al., 2007; Gyogluu et al., 2016; Jaiswal and Dakora,

2019; Wongphatcharachai et al., 2015). These less efficient strains are even more competitive compared to the inoculants strains and occupy a significant higher number of the nodules, affecting the impact of the introduced commercial strain on improving BNF (Batista et al., 2015). Irisarri et al. (2019) also reported higher nodules occupation by native rhizobia compared to introduced strains. This has important practical implication for agriculture to the effect that highly effective rhizobia for inoculant production must be selected among native population and be provided in higher concentration through inoculants. Furthermore, the host plant shows preference on native rhizobia compared to exotic strains (Osunde et al., 2003).

Only the N+ control produced very few nodules. This could be attributted to the fact that the presence of mineral nitrogen inhibits the biological nitrogen fixation by inhibiting the nodules formation and nitrogenase activity (Kaschuk et al., 2016). This finding agrees with Ulzen et al., (2016) who reported very few nodules with the application of 100 kg of nitrogen. In addition, many authors have stated that N doses as low as 20kg of N ha^{-1} highly decrease legume nodulation and BNF, with no yield benefits (Hungria et al., 2005, 2006; Saturno et al., 2017).

The differences in nodulation among tested strains could be attributed to the fact that natives or naturalized rhizobia were less effective in one hand and on the other hand introduced strains were highly effective (Gyogluu et al., 2016). These findings agree with Osunde's et al., (2003) who tested the nodulation of two soybean promiscuous varieties by introduced elite rhizobia and indigenous rhizobia and found that the introduced rhizobia through inoculants were less competitive but highly effective compared to native rhizobia. The differences in nodulation may be attribute to their genetic makeup expressed through symbiotic efficiency, competitiveness for nodule occupancy, compatibility with the host plant and adaptive ability to soil stress conditions (Mwenda et al., 2018). The indigenous rhizobia NAC17 and NAC46 produced higher number of nodules compared to the commercial strain USDA110 suggesting that these strains had higher symbiotic efficiency and higher adaptation ability to local conditions compared to the commercial strains.

The increase in leaves green color noted with the native strains compared to the controls may be attributed to the fact that nitrogen is the principal component of chlorophyl that confers green colors

to the plants. Inoculation improves nitrogen content in leaves and thus promotes the formation of chlorophyll, which is also important for photosynthesis (Sinclair, 2004; Hakeem et al., 2012). The same results were observed by Abaidoo et al., (2007) who classified rhizobia strains investigated into four groups based on green color intensity. The less effective group comprised isolates that recorded lower green color intensity on leaves of soybean genotypes.

In the field, the significant differences of shoot dry weight were observed among treatments as result of enhanced nodulation. Nitrogen is the component responsible for vegetative development; it has been demonstrated that 80% of the N accumulation is attributed to biological nitrogen fixation by rhizobia (Hungria et al., 2006). The N- control produced a higher number of nodules but lower shoot dry weight. This is a proof of the native rhizobia being less effective (Osunde et al., 2003). The same observation has been made by Chibeba et al., (2018) who observed that an appreciable proportion of rhizobia population in Mozambican soils was composed of ineffective rhizobia. The N+ control produced high shoot dry weight; this was expected because mineral N is absorbed by the plant at early stages compared to the fixed N and thus improved vegetative formation (Saturno et al., 2017). This finding agrees with that of Kinugasa et al., (2012) who found higher biomass production with mineral N treatment but which did not always result in grain yield increase.

This study demonstrated significant differences in yields among treatments. There was yield improvement with inoculated plots compared to the plots where N had been applied and N-control. The indigenous strains NAC46 and NAC17 increased yields by 1.7 and 1.6 fold respectively compared to the N+ control, 2.2 folds compared to the N-control and 1.4 and 1.3 fold compared to the commercial strain USDA110. These yield gains are within the 3.2–14.5% interval of inoculation yield benefit reported in Brazil (Hungria et al., 2006) and in Mozambique (Chibeba et al., 2018). This study has further confirmed other authors' findings (Chibeba et al., 2017, 2018; Hungria et al., 2005, 2006) that BNF is the most efficient way of improving soybean productivity.

The best inoculation treatments across all experiments (greenhouse and field experiment) were the indigenous strains NAC46 and NAC17. These indigenous strains were among the best in nodulation, biomass improvement across the two experiments and importantly in grain yields

enhancement. These strains nodulated and improved shoot weight equally or better than the commercial strain USDA 110. In the field NAC46 and NAC17 increased soybean grain yield from 2.4 t ha^{-1} to 3.3 t ha^{-1} and 3.4tha^{-1}. These strains are confirmed as well adapted elite candidate rhizobia inoculant strains for improved BNF and soybean productivity in South Kivu province of D.R. Congo.

5.6. Conclusion

This study assessed the effectiveness of indigenous rhizobia in improving BNF and soybean productivity. The inoculation with indigenous rhizobia improved the nodulation, the biomass production and grain yield across all the experiments. The best strains across all the experiments are NAC17 and NAC46 with average yields gain 60-70% over commercial strains and controls suggesting that these strains hold the best potential as commercial inoculants in South Kivu soils conditions. USDA 110 and SEMIA 5019 are also effective but it is preferable to use adapted and competitive native strains of rhizobia. Therefore, the native strains are likely to adapt well not only in South Kivu, but also in other countries with similar agro-climatic conditions.

CHAPTER SIX: DEMOGRAPHIC FACTORS AND PERCEPTION OF SMALLHOLDER FARMERS TOWARDS ADOPTION OF RHIZOBIUM INOCULANTS FOR SOYBEAN IN SOUTH KIVU

6.1. Abstract

The rhizobium inoculants have been practiced in soybean production for over a century all over the world but in Africa this technology is relatively new. Since 2010, Biofix rhizobium inoculants have been disseminated in Eastern Democratic Republic of Congo (DRC) by Nitrogen 2 Africa (N2AFRICA) program of CIAT and later of IITA but the perception and factors towards its adoption remain unknown. Therefore, the perception and adoption'deteminants of rhizobium inoculants were assessed among 193 soybeans smallholder's farmers of South Kivu province of DRC. The information collected in September 2018 included farms and farmers' socio-economic characteristics such as farmers education, group memberships, the knowledge of nodules etc. The perception about inoculants effectiveness, accessibility and affordability was measured using 5-point Likert-type scale. Multivariate probit model was used to assess the factors that influenced the adoption. Results indicated that the adoption of rhizobium inoculants was very low in South Kivu (21%) and was highly influenced ($P<0.01$) by gender of the household head, farmer's location, education type of household head, the knowledge of nodulation and the household income. The farmers'perception of inoculants also highly influenced its adoption ($P<0.01$). Moreover, rhizobium inoculant was strongly perceived by farmers as an affordable nitrogen source for enhancing soybeans productivity but hardly available in the market. More effort is needed to strengthen farmers's education about BNF in order to improve inoculants adoption.

Key words: adoption, perception, smallholder's farmers, Biofix, Soybeans, N2 Africa, rhizobium inoculants

6.2. Introduction

The Democratic Republic of Congo (DRC) is among the largest countries in Africa and offers enormous potential for increased agricultural productivity (Lecoutere et al., 2009). Currently DRC's agricultural production is among the lowest in Africa and in the world (FAO, 2018) due to declining soil fertility (Pypers et al., 2011), and aggravated by lack of specific information on soil management and sustainability at farm level (Bashagaluke et al., 2015). The majority of crops, cereals and legumes, are cultivated without application of fertilizers and consequently produce low yields (Lambrecht et al., 2016).

Soybean is one of the most emerging crops (Hartman et al., 2011) cultivated by smallholder farmers in South Kivu province of Democratic Republic of Congo for cash and diverse uses (Bisimwa et al., 2012). This crop has been promoted since 1985 by humanitarian organization and United Nations agencies such as FAO, to address the issues of malnutrition induced human diseases following the political strife of 1985 (Kismul et al., 2015). Since, its cultivation has increased as result of its utilization in hospital and nutrition centers for prevention and malnutrition-induced diseases treatment in public schools and hospitals (Bisimwa et al., 2012; Kismul et al., 2015), in household to improve nutrition status (Pypers et al., 2011; de Jager et al., 2019) and in livestock especially in poultry production and aquaculture (Khojely et al., 2018).

Despite its importance, the potential productivity of this crop is limited by poor soil fertility and low accessibility to mineral fertilizers by poor-resources farmers (Pypers et al., 2011; Khojely et al., 2018). Consequently, reported yields are very low (0.51 t/ha) (FAO, 2018) compared to reported potential yield of 3t/ha (Salvagiotti et al., 2008). The application of organic fertilizers to alleviate this problem is constrained by their very limited availability leading to a very low utilisation per unit area (Lambrecht et al., 2016). The mineral fertilizers are also very expensive to farmers; Odame (1992) estimated that a farmer must sell 5kg of common beans or 10 kg of maize grain to purchase only 1 kg of nitrogen and phosphorus mineral fertilizers.

Fortunately, soybean is able to fix its own nitrogen from the atmosphere in symbiosis with rhizobia bacteria through the Biological Nitrogen Fixation process (Dakora and Keya, 1997; Hungria et al., 2005; Giller et al., 2011; Collino et al., 2015). This crop is estimated to fix up to 80% of its nitrogen

needs and thus alleviate the need of applying mineral fertilizers that are neither available nor affordable by smallholder's farmers (Chianu et al., 2011).

Many soils contain *Rhizobia,* but often in small populations and they are less effective and mostly non-compatible to soybeans (Abaidoo et al., 2007). To sustain higher legumes yields the inoculation with a highly effective and competitive Rhizobia strain in high quality formulations is required. Two approaches were used by international organization to address the problem of soybean's low yields in Africa: breeding for development of soybean cultivars that can nodulate freely with native rhizobia population (Mpepereki et al., 2000; Tefera, 2011) and introduction of inoculants containing highly effective rhizobia strains (van Heerwaarden et al., 2018).

Consequently, rhizobium inoculants (biofertilizers) were introduced among smallholder's farmers of South Kivu by the N2 Africa program of International Institute of Tropical Agriculture(IITA) and partners' organizations, first in 2010 (Chianu et al., 2011, Van Heerwaaden et al., 2018). The introduced commercial formula was the BIOFIX®. This inoculant is produced in Kenya and was initiated as part of the Microbial Resources Centre Network (MIRCEN) established by the University of Nairobi (Mutuma et al., 2014; Chianu et al., 2011). This product is licensed and marketed by MEA Limited, which started its production in 2010 (Ampadu-Boakye et al., 2017). Yield increase was observed in soybean inoculated by this inoculant in many countries (Waswa et al., 2014; Ulzen et al., 2016; Van Heerwaarden et al., 2018; Thilakarathna et al., 2019).

BIOFIX® for soybean contains the *Bradyrhizobium japonicum* strain USDA 110, a widely used industry product concentrated at $>10^9$ Rhizobia g^{-1} in an organic carrier material (Ulzen et al., 2016). This is one of the main legume inoculants commercially available in East Africa and is steadily being promoted among farmer groups of many countries assisted by many organizations such as N2 Africa (Chianu et al., 2011; Karanja et al., 1998; Wafulah, 2013; Farrow et al., 2016).

While Woomer et al., (1997) and Odame (1997) identified limited farmers awareness and inoculants unavailability as an important constraint to the adoption of inoculants, adoption and profitability of the inoculants product assessed in other countries (Getachew, 2016; Mutuma et al., 2014; Nekesah, 2017; Ulzen et al., 2016); there is no information on demographic factors in rhizobium inoculant adoption among smallholder soybeans farmers in South Kivu province of D.R

Congo. Moreover, previous conducted studies did not assess the perception of smallholder's farmers of the inoculants products since the adoption largely depend on perceptions (Negatu and Parikh, 1999; Ojiako et al., 2007). Therefore, the objective of this study was to assess the demographic factors and perceptions that are likely to influence the adoption of rhizobium inoculants among soybean smallholders' farmers of South Kivu.

6.3. Methodology

6.3.1. Study area

The current study was conducted in South Kivu province of DRC, targeting three villages namely Lurhala, Kalehe and Kamanyola where N2 Africa program of IITA has disseminated BNF technologies including rhizobium inoculants (Chianu et al., 2011). South Kivu province is located in the Eastern part of Democratic Republic of Congo between 1° 36' and 5° South latitude and 26° 47' and 29° 20' East longitude (Pypers et al., 2011). The province of South Kivu has an area of 69,130 Km^2 and its population is currently estimated at 3,500,000 peoples with an average density of 50.6 inhabitants per km^2 (DSCRP, 2011).

The area is recognized as a high humid forest zone depicted by high vegetation diversity (Potapov et al., 2012) and characterized by highlands (van Engelen et al., 2006). This region has a tropical climate with average annual rainfall of 1500 mm and average temperature of 18°C (Nash and Endfield, 2002). The main activity in the region is agriculture with most cultivated crops including banana, cassava, beans and traditional livestock comprised of cattle, sheep, goats, chicken and pigs (Maass et al., 2012).

6.3.2. Sampling and data collection

The survey was conducted in two stages; a pre-survey (conducted from 23rd to 25th June 2018) was done in consultation with N2 Africa country coordinator and Field specialist to determine the villages where inoculant product was promoted. From this stage, three villages were purposively selected namely Lurhala, Kalehe and Kamanyola and the sampling frame determined.

At the second stage, two lists of soybeans farmers; (i) participating and (ii) not participating in the N2 Africa program was drawn in each village with the help of the farmer's group contact person and N2 Africa program field technician. Lastly, a random equal number (100 respondent per group) of farmers were drawn from the two lists to participate in the survey conducted from 1st to 30th September 2018. A total number of 66 per village was considered. From this process 200 farmers were selected but only 193 respondents were considered as they met the requirements of the survey. The true sample size was determined as guided by Murongo et al., (2018).

Data were collected through personal interviews, using pretested questionnaires. Information collected for demographic factors in inoculants adoption included farmers' characteristics (gender, education, household size and management, etc.), farm characteristics (farm size, number of cultivated land, etc.), institutional factors (group membership, credit access etc.) and capital endowment. Concerning perception of inoculants product, farmers were asked about their perception on the importance of soybean, the effectiveness of rhizobium inoculant in improving soybeans productivity, its availability, its accessibility and affordability. The market prices were used to estimate the cost of farm inputs and value of outputs in order to compute the gross margin.

6.3.3. Analytical framework

Technology adoption can be modeled using a utility maximization problem (Sidibé, 2005). A farmer will only adopt a new technology, for example an improved crop variety or fertilizer, when the utility he derives from this technology (U_n) is greater than the utility of a traditional technology he had been using (U_t) (Mercer, 2004). The utility obtained from an innovation is considered as a vector of several factors ranging from farm observed attributes to perceived innovation attributes (X_i) through institutional factors (e.g., distance to the market, membership to farmers' organizations), farmer characteristics (e.g., gender of the farmer, age) and a disturbance term with mean zero (Sattler and Nagel, 2010). Perceived technology characteristics, or perceived varietal attributes under crop technology adoption, are also function of subjective and/or objective characteristics of the technology itself, but also farm and farmer-specific characteristics (Mariano et al., 2012). Thus, a given farmer, in the adoption process, will always consider the benefits and

losses (both economic and social) of the new technology and eventually chooses the technology (T) that promises higher utility compared to the old one.

Suppose an individual household's utility of adopting a new technology, controlled by a vector of social, economic and physical factors (X), expressed by $U_n(X)$, and the utility of remaining with the traditional technology (in other words the utility of adopting the traditional technology), also designated by $U_t(X)$, then the utility models associated with adoption of the old and new technologies can be apprehended through a linear relationship:

$$\boldsymbol{U_n(X) = \theta_n' X + E_n} \quad \textbf{(1)}$$

$$\boldsymbol{U_t(X) = \theta_t' X + E_t} \quad \textbf{(2)}$$

Where $\theta_n' X$, $\theta_t' X$ and E_n, E_t are response coefficients and disturbances associated with adoption of the new and old technologies respectively.

Under the adoption framework, the state of mind of the farmer is not observable but can only be seen through outcome of a decision-making process and this allows the classification of farmers into two groups: adopters and non-adopters. The adoption process can thus be modelled using a latent variable (Horrace and Oaxaca, 2006) denoted by (y^*). In our case, it measures the difference between the utility derived from the new technology and that of the old technology $[U_n(X) - U_t(X)]$. This variable can take both positive and negative values depending on whether the utility of the new technology outweigh that of the old technology and vice versa. So, in the real world, the outcome variable (Y)will be assigned the value of 1 if the farmer adopted or is susceptible to adopt the inovation and 0 in the opposite case. Mathematically, this probability can be expressed as follows:

$$P(Y = 1) = P(U_n > U_t)$$
$$= P(\theta_n' X + E_n > \theta_t' X + E_t)$$
$$= P[X(\theta_n - \theta_t) > E_t - E_n]$$
$$= P[X\theta > E]$$
$$= \boldsymbol{F(X\theta)} \textbf{ (3)}$$

Where P is the probability function, $\theta = (\theta_n - \theta_t)$, a vector of unknown parameters to be estimated and which can be interpreted as net influence of explanatory variables on technology adoption; $E = (E_t - E_n)$ a random disturbance term; and $F(XB)$ the cumulative distribution function of F evaluated at XB.

The parameters of such model can be estimated using maximum likelihood technique due to the non-linearity nature of the model (probabilistic model). Several empirical models can be used to map the relationship between the dependent variable and the independent variables. These include the Linear Probability Model (LPM) (Horrace and Oaxaca, 2006), logit and probit models (Briz and Ward, 2009). One of the major flaws of the LPM model comes from its estimation technique. It uses ordinary least squares (OLS) to estimate parameters of a binary-outcome variable. The predicted probability for such model may also go beyond 1 or below 0, violating basic principles of probability (Horrace and Oaxaca, 2006). This has made the model less used in studying technology adoption in empirical studies. Therefore, Logit and Probit are suitable for the current situation but the choice between them has always been subject to several controversies. However, the results provided by both models are similar and they can be used interchangeably (Zamasiya et al., 2014). In the current study, we used a probit model to identify determinants of adoption of rhizobium inoculants among soybean farmers. Farmers 'perception towards rhizobium inoculants was measured using 5-point Likert-type scale (Preston and Colman, 2000; Bagheri, 2010; Li, 2013).

6.4. Results

6.4.1. General characteristics of soybean farmers

The mean age of soybean farmers of South Kivu was 47 years with the standard deviation of 14.6 (Table 1) with most of farmers being within productive age (more than 46 years old). Of the interviewed farmers, 68.7% were men while 31.2% were women. The education type among soybeans farmers was mostly formal education with a mean of 5years spent to school. Most of the interviewed farmers had a mean of 26years of experience in growing soybean and most practised religion was Catholicism (72.9%) followed by Protestantism (23.9%).

The principal source of income in the study area was the sale of agricultural products (88.4%) followed by small trade of articles of first use in households such us soaps, body lotions etc. (6.8%). The household income was controlled mostly by both husband and wife (45%) followed by the husband alone (23%) and was in the range of 50-100 US dollars. Only few households (28%) received a mean credit of 128 USD and many households (68.7%) were members of farmer's group. The mean number of fields allocated to soybean was 2 fields per household with mean area under soybean crop of 0.46 ha.

Table 11: general characteristics of soybean farmers

Factor	Category	N	Percent
Mean age	<18	1	0.5
	18-25	24	7.2
	26-35	36	18.7
	36-45	27	27.6
	>46	89	45.8
gender of the farmer	Male	125	68.7
	Female	67	31.2
Type of education	Formal	139	72.4
	non formal	14	7.3
	Any	38	19.8
	Other	1	0.5
Religion	catholicism	140	72.9
	Jehova witness	2	1.0
	Protestantism	46	24.0
	Adventist	3	1.6
	Traditional	1	0.5
household management	Husband is the head	42	22.1
	wife is the head	45	23.7
	Conjoints	87	45.8
	another person	16	8.5
montly income interval	<30$	27	17.3
	30-50$	41	26.3
	50 -100$	46	29.5
	100 -200$	33	21.2
	200 -300	8	5.1
	>500	1	0.6
Source of income	sale of agric.products	169	88.5
	small trade	13	6.8
	Employees	2	1.0
	Other	5	2.6

6.4.2. Comparative characteristics of soybean inoculants users and non-users

The number of soybean inoculants users was 41 against 152 of non-users (Table 2) showing an adoption rate of 21%. The users of soybean inoculants had more access to credit than non-users (P<0.01), they were more involved in groups and had stayed longer in groups than non-users (P<0.01). In addition, many of them were beneficiary of development or humanitarian projects (P<0.01). In the other hand, users of soybean inoculants had more awareness on roots nodules roles (P<0.01) and were in contact with organization promoting inoculants (P<0.01).

Table 12: characteristics of soybean inoculants users versus non-users

Variables	Overall sample	users	Non-users	Mean/Prop.diff.
Age	46	46	46	-0.28
Gender		0.425	0.51	0.093
Education level	5.86	5.57	5.97	0.40
Experience in agriculture	26.48	25.75	26.74	0.995
Household head	7.81	8.4	7.6	-0.8
Household workers	3.16	3.38	3.07	-0.31
Credit access	0.3	0.5	0.22	-0.27***
Credit amount	11478.4	17640	6549.12	-11090.88
Group membership	0.73	1	0.63	-0.36***
Duration in farmer's group	7.09	9.87	5.67	-4.21***
Project beneficiary	0.52	0.87	0.39	-0.48***
Number of cultivated land	2.72	2.97	2.60	-0.36
Knowledge of root nodules	0.50	0.75	0.401	-0.35***
Contact with inoculant promotors	0.47	0.81	0.30	-0.51***
Total number of farmers (N)	193	41	152	

*Note: *** and ** are significant at 1% and 5% respectively*

6.4.3. Determinants of inoculants adoption

The location, gender, education, knowledge of root nodules, household income and perception of rhizobium inoculant were significant in explaining adoption of rhizobium inoculants in the study areas (Table 3). Farmers located in Lurhala were more likely to adopt Rhizobium inoculants than those located in Kamanyola.

Table 13: Factors affecting adoption of rhizobium inoculants

Variables	Adoption coefficients	Probability	Marginal effects	Probability
Gender	-1.049**	0.013	-0.167**	0.011
	(0.423)		(0.0654)	
Age	-0.0268	0.129	-0.00425	0.126
	(0.0177)		(0.00278)	
Type of education	-0.841*	0.051	-0.134**	0.049
	(0.431)		(0.0679)	
litteracy	1.224	0.131	0.194	0.132
	(0.812)		(0.129)	
Farming experience	-0.00137	0.932	-0.000218	0.932
	(0.0161)		(0.00255)	
Religion	-0.125	0.764	-0.0198	0.764
	(0.419)		(0.0659)	
Household size	0.0791	0.127	0.0126	0.123
	(0.0518)		(0.00813)	
Credit access	0.262	0.521	0.0415	0.525
	(0.407)		(0.0653)	
Membership to farmer organization	0.519	0.207	0.0824	0.200
	(0.411)		(0.0643)	
knowledge of roots nodules	3.011***	0.000	0.478***	0.000
	(0.529)		(0.0653)	
Contact with extension services	0.627	0.130	0.0995	0.122
	(0.414)		(0.0643)	
Income variables				
30-50	1.119**	0.043	0.170**	0.041
	(0.554)		(0.0830)	
50$ -100$	1.341**	0.014	0.207***	0.008
	(0.548)		(0.0783)	
100-200	0.947*	0.099	0.142*	0.092
	(0.575)		(0.0844)	
200-300	0.310	0.750	0.0433	0.754
	(0.971)		(0.138)	
Location variables				
Kamanyola	-0.902	0.441	-0.125	0.353
	(1.169)		(0.134)	
Lurhala	1.722***	0.000	0.279***	0.000
	(0.396)		(0.0564)	
Perception variables				

Variables	Adoption coefficients	Probability	Marginal effects	Probability
Affordable price	-1.604***	0.000	-0.255***	0.000
	(0.459)		(0.0680)	
Inoc. accessibility	-0.519	0.412	-0.0824	0.411
	(0.633)		(0.100)	
Inoc.effictiveness	1.307***	0.007	0.207***	0.004
	(0.483)		(0.0720)	
Availability at sale points	0.901*	0.057	0.143*	0.053
	(0.474)		(0.0738)	
Inoc not important for soy	0.461	0.283	0.0732	0.286
	(0.430)		(0.0686)	
Soybean importance	-0.0152	0.972	-0.00242	0.972
	(0.426)		(0.0677)	
Constant	-2.276**	0.037		
	(1.093)			
Wald chi2(24)	81.39			
Prob>Chi2	0.0000			
Pseudo R2	0.5678			
Observations	140		140	

In bracket robust standards, ***P<0.001, **P<0.05, *P<0.1

Gender of the household head had unexpectedly a negative effect on the adoption of rhizobium inoculants fertilizer meaning that when a household is men headed, he is not likely to adopt the rhizobium inoculant. The type of education, also, unexpectedly negatively affects the use of inoculants meaning that farmers with informal education were more likely to adopt inoculants fertilizers compared to those with formal education. The awareness of roots nodules positively affected the adoption of inoculants. Household income positively and significantly (P<0.01) affected the adoption of soybean inoculants. Furthermore, farmers' perceptions of rhizobium inoculants also played a key role in adoption of the latter.

6.4.4. Farmers' perception of rhizobium inoculants adoption

Inoculants users strongly agreed (65%) that soybean is an important crop, strongly agree that inoculant improves soybean's yield (50%) and agreed that rhizobium inoculant is available at sale points. Inoculants users were not sure (21% agree, 21% moderate agreed and 21% disagreed) on the importance of rhizobium inoculants for soybeans production (Figure 13). However, inoculants users strongly agreed that inoculants price is affordable with the majority of farmers' users of inoculants stating that the sales points of inoculants are inaccessible (Figure 13).

Concerning inoculants non-users; they also strongly agreed that soybean is an important crop, moderately agreed that inoculation can promote soybeans production, moderately agreed or disagreed (37%, 37% respectively) on inoculants availability (Figure 14). In addition, they agreed that inoculant is not important for soybean's production and disagreed on inoculants' easy access. However, most of them strongly agreed that inoculants 'price is affordable (Figure 14).

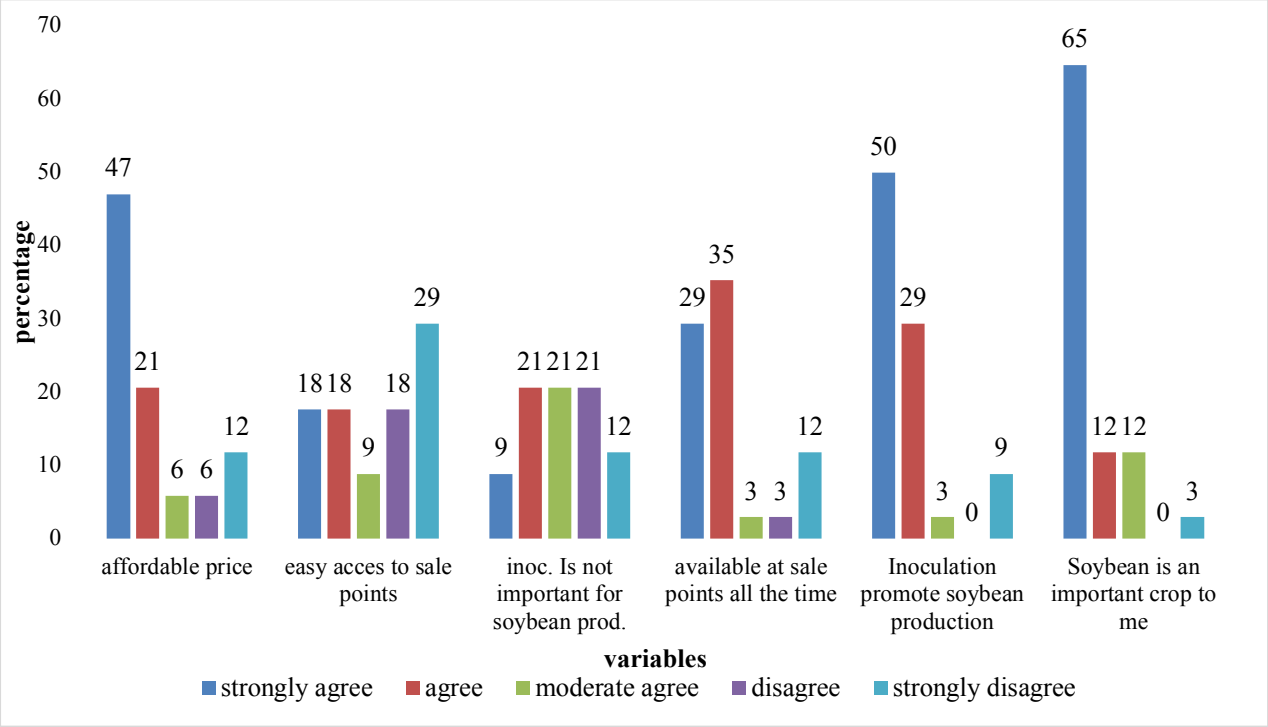

Figure 13: Inoculants perception by users

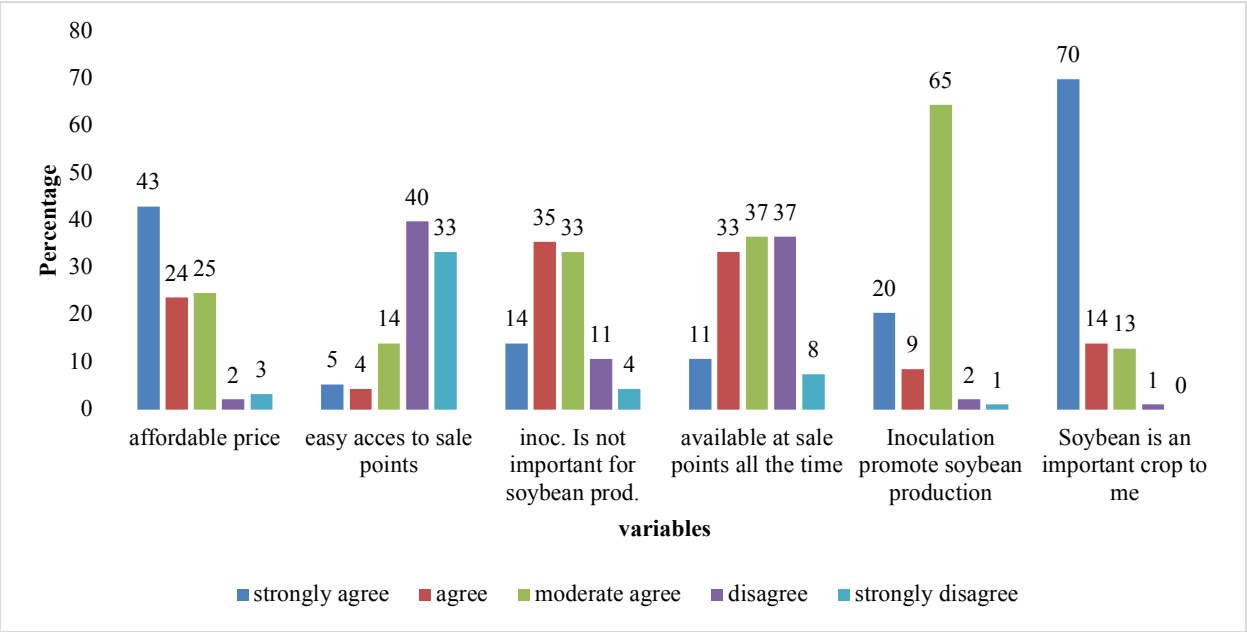

Figure 14: Inoculant perception by non-users

6.5. Discussion

6.6.1. Characteristics of smallholder's soybean farmers

The mean age of soybean farmers of South Kivu was 47 years showing a low involvement of the youths in soybean production, consequently highlighting the need to encourage youth involvement for reduced youth enemployment that is estimated to be severe in Africa (ILO, 2013). The interest of farmers within productive age on soybean could be attributed to the fact that soybean is a crop cultivated for both nutrition security and cash income generation of the household; which is an elders' concern in one hand. On the other hand, this can be explained by the fact that young ones are not interested in performing various agriculture related activities. This result is consistent with Zamasiya et al., (2014) and Ojiako et al., (2007) who found that most soybean farmers are within productive age (43-50years).

Of the interviewed farmers, the majority were men; this could be due to the fact this crop is becoming a cash crop in South Kivu due to the increasing market opportunity; men are mostly interested in market-oriented crops (Carr, 2008). This is a very interesting fact, especially for

soybean, because it was demonstrated that in Africa, men have more access to the land than anyone in the household (Carr, 2008). These findings are in discordance with Mutuma' et al., (2014) who found that in Kenya women are more involved in soybeans culture than men. This discordance is noted probably because the market opportunity for soybean in Kenya is not that considerable compared to D.R. Congo, where soybean crop is used like medicinal food to cure malnutrition diseases (Bisimwa et al., 2012).

This study revealed that the education type among soybeans farmers was mostly unformal education. The low education status is explained by the less access to education in rural area due to poverty. A study conducted by Mariano et al., (2012) also reported a low education status of farmers in Philippine and the same study demonstrated the negative influence of this low education on adoption of modern rice technologies. Most of the interviewed farmers had a mean of 26years of experience in growing soybean. This indicated that farmers have quite good experience in growing soybean suggesting that they have more knowledge and skills to improve soybean production. Futhermore, it was demonstrated that long experience in growing a certain crop has a positive impact on the adoption of new technologies related to this particular crop (Paustian and Theuvsen, 2017).

6.6.2. Determinants of rhizobium inoculants adoption

The location, gender, education, knowledge of root nodules, household income and perception of rhizobium inoculant were significant in explaining adoption of rhizobium inoculants in the study area. Farmers located in Lurhala were more likely to adopt Rhizobium inoculants than those located in Kamanyola. This was expected and may be due to the fact that soil conditions vary among these villages. Lurhala for instance, is characterized by highlands and less fertile soils compared to Kamanyola which is a plain with moderate fertility soils (Pypers et al., 2010). The higher inoculant adoption in Lurhala could be attributed to the lower soil fertility status and need of an affordable source of fertilizer for soybean. This finding agrees with Mutuma' et al., (2014) to the effect that farmers in Bondo were more likely to use inoculants than Mumias and Bungoma because of low soil fertility status.

Gender of the household head had unexpectedly a negative effect on the adoption of rhizobium inoculants fertilizer meaning that when a household is men headed, it is not likely to adopt the rhizobium inoculant. The higher adoption of women may be due to the fact that women have higher accessibility to products compared to men and considering that women can even get price reduction when purchasing. This observation is in contradiction of the finding of Nekesah (2017) who found that male farmers are more likely to adopt inoculants fertilizers because they can leverage on their equity capital with which to purchase external farm inputs than women. Our study findings were in discordance perhaps because at the beginning of the project, inoculants were distributed for free by organization promoting it and thereafter they remained cheap and accessible to farmers (Ampadu-Boakye et al., 2017). However, these results agreed with Zamasiya et al., (2014) who reported that a female-headed household is likely to adopt new technologies related to legumes because legumes are usually considered as female crop oriented to improve household nutrition status (Carr, 2008).

The education's type, also, unexpectedly negatively affects the use of inoculants meaning that farmers with informal education were more likely to adopt inoculants fertilizers than to those with formal education. This may be attributed to the fact that farmers who went through informal education undertook technical studies including short trainings in agricultural techniques organized by extension workers in rural areas. These findings are consistent with numerous authors (Mignouna et al., 2011; Namara et al., 2013; Sumane et al., 2017). They stated that informal knowledge and learning is a valuable resource that can reorient modern agriculture towards more sustainable and resilient paths of development because this type of learning adress the knowledge and learning needs of farmers.

The awareness of roots nodules positively affected the adoption of inoculants. This was expected because being aware of the existence of root nodules in leguminous plants, knowing their role in nitrogen fixation and perceiving that the nodules are enhanced by inoculant use, increase the decision of using inoculants. This was also observed by Mutuma et al., (2014) and Nekessah (2017). The adoption was also positively affected by the household income. This is because when farmers are getting more income from farm crop, they take a risk and responsibility about a new technology. Duressa (2015) also reported that farmers' income has significant and positive effect

on adoption of technologies. Households with relatively higher level of income are more likely to purchase or exchange improved technologies.

Moreover, farmers' perceptions of rhizobium inoculants also played a key role in adoption of the latter. Perceiving that inoculant's price is affordable by farmers negatively affected its adoption meaning that cost is not the most important factor for adoption of inoculant. This might be due to the fact that when a technology is perceived to be affordable, its efficacy is questionable. These results are consistent with Sattler and Nagel (2008) in the study factors affecting farmers' acceptance of conservation measures in Germany; costs were not the most important factor for adopting conservation measures. Other factors, like effectiveness, associated risks, or time and effort necessary to implement a certain measure were more important.

Perceiving that rhizobium inoculant is effective and available at sale points was more important for its adoption in our study area. This is due to the fact that the inoculation technology has been subjected to intense promotion among farmers under Integrated Soil Fertility Management (ISFM) as one of soil fertility replenishment technologies that are suitable for different types of resource-poor farm households (sanginga and Woomer, 2007). These findings are in agreement with Farrow's et al., (2016) who reviewed the litteratures on factors affecting inoculants adoptions. He mentioned that the commonly mentioned factors affecting the adoption of inoculants as one of legume technologies, were the relevance of the technology (such as suitability for the agro-ecological zone), followed by the effectiveness and availability of the technology.

Diagnostic tests through Wald statistic showed that the model was globally significant, justifying the use of the selected covariates to predict the response variable. As for the reported pseudo R2, its value of 0.56 indicated that the retained variables (the location, gender, education, knowledge of root nodules, household income and perception of rhizobium inoculants etc.) are useful in predicting adoption of rhizobium inoculants (Table 3). To measure goodness-of-fit, other statistical tests such as the Hosmer-Lemeshow test was performed. Results ($\chi^2 = 4.94, P - value = 0.7635$) showing that the used Probit model fitted well the data. As for multicollinearity test, Variance Inflation Factors (VIF) reported figures less than 5 for most of the variables; this implies low level of multicollinearity among variables. Robust standard errors were used to control the

problem of heteroskedasticity and possible sample selection-bias in the data. At last, a link test was performed for model specification and possible omitted-variables problem. The result of the test indicated that the model was well specified and is not affected by any omitted-variable problem.

6.6.3. Rhizobium inoculants perceptions

Inoculants users and non-user farmers strongly agreed that soybean is crop of high importance. This is explained by the fact that soybeans, in South Kivu, are being used in households for malnutrition fighting and for cash income generation due to the presence of markets. This is in accordance with many authors who stated that soybean is becoming an important and popular crop in Sub-Saharan Africa (Hartman et al., 2010; Murithi et al., 2016; Khojely et al., 2017). This importance is explained by for its roles in food and nutrition security (Rossi et al., 2005; Owino et al., 2011; Bahwere et al., 2016), in cash income generation (Bangsund et al., 1999), in animal nutrition (Huang et al., 2014; Yuan et al., 2016) and in in soil fertility improvement (Sanginga, 2003; Miransari et al., 2013). This suggest that effort should be done to promote the productivity of this crop.

Inoculants users strongly agreed that inoculation promotes soybean production whereas the non-users only agreed moderately. This may be explained by the higher contact of inoculants users with organizations promoting inoculants and their long duration in farmers groups, as demonstrated in section 3.2. This facilitates their easy access to information and evaluation of new technologies. The low agreement of non-users on the effectiveness of inoculants may be explained by their less education on inoculation but also by the fact that the response of soybeans to inoculation is variable and depends on many factors namely the population size and effectiveness of indigenous rhizobia together with environmental factors such as water stress (Serraj et al., 1999), high temperature (Michiels et al.,1994), soil acidity and salinity (Delgado et al., 1994) and nutrient deficiencies (Cassman et al., 1981). Marufu et al., (1995) observed that farmers' education on BNF and inoculation is among major determinants for the adoption of inoculants. Organizations promoting inoculants and extension services should determine the need to inoculate a certain area before the implementation of demonstration trials for good perception and high adoption of the product.

Concerning the inoculants availability at sale points, inoculants users agreed that this product is available in the market while in non-users'group, the same number of farmers either agreed moderately or disagreed. This shows a moderate availability of inoculants which may be owing to the fact that this product was produced under a project by limited number of technicians who could produce only limited quantity of inoculants (Ampadu-Boakye et al., 2017). A study on farmers' inoculants adoption conducted in Zimbabwe demonstrated also a less availability of inoculants (Bala, 2008). According to this study, the less availability was explained by the complexity of inoculants production' techniques. These findings are in agreements with other studies that demonstrated a very low access to inoculants as major constraint to its adoption (Odame, 1997; Woomer et al., 1997; Kipkoech et al., 2007).

The two groups (users and non-users) strongly agreed that the price of inoculants is affordable. This is in agreements of other studies (Mutuma et al., 2014; Nekesah et al., 2017). For example, Chianu et al., (2011) demonstrated that a 100g-packet of inoculant sufficient to inoculate 15g of seeds and enough to plant one acre costs only 1.2 US dollars while inorganic nitrogen fertilizer in form of Calcium Ammonium Nitrate needed for the same size of plot costs 34 US dollars. This shows that rhizobium inoculant was cheaper 28 times compared to inorganic N fertilizer and should be promoted among smallholders farmers.

6.6. Conclusion

The objective of this study was to assess the perception and determinants of rhizobium inoculants among soybean smallholder's farmers of South Kivu. The adoption of rhizobium inoculants in South Kivu is affected positively by the knowledge of roots'nodules, the inoculants perceptions and farmers'income. This adoption is negatively affected by the gender and the type of education. However, farmers perceive rhizobium inoculant as an affordable source of nitrogen for soybean but less accessible. Much effort is needed in strengthening extension services in order to ensure advanced farmers 'education about rhizobium inoculation technology. Local private firms and agro-dealers involvement is important for access and sustainance of the product.

CHAPTER SEVEN: GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS.

7.1. General discussion

This study had four key objectives. The first objective was to determine the genetic diversity of indigenous soybean-nodulating rhizobia (SNR) in South Kivu soils. This diversity has not been determined before in South Kivu despite a previous study that has demonstrated that rhizobia exist in South Kivu soils (Ndusha, 2014). The second was to assess the genomic features of indigenous SNR for a rapid identification of candidate elite strains among the highly diverse strains identifyied in the first study. This would serve as a quick approach of identification of highly effective strains to be used in inoculants production as the traditional approach was shown to be time consuming (O'Hara et al., 2002). Whereas the two objectives were very important, the study would not be complete without identifying highly effective indigenous rhizobia strains among local populations. These strains would serve as alternatives to replace the commercial strain USDA 110 that has demonstrated a poor adaptation to DRC soils (van Heerwarden et al., 2018). Therefore, the third objective was to assess selected indigenous rhizobia strains for their ability to fix nitrogen and improve soybean productivity under different soil conditions. The fourth objective was to assess the perception and adoption'determinants of rhizobium inoculants among smallholders' farmers.

Analyses of the *16S rRNA, recA* and *glnII* genes of 70 indigenous soybean-nodulating rhizobia (SNR) strains from cultivated and grassland of South Kivu province of DRC revealed that indigenous strains were highly diverse. These SNR belong to several genera; *Bradyrhizobium* (20%), *Kosakonia* (20%), *Enterobacter* (13%), *Rhizobium* (10%) and a few belonged to *Bacillus*, *Beinjerinckia*, *Burkolderia*, *Microvirga*, *Cupriviadus*, *Mesorhizobium* and *Agrobacterium* (Table 3). Many of indigenous SNR were *Bradyrhizobium* and six of them were very close to the commercial strains *Bradyrhizobium japonicum* USDA 110, a commercial strain that was introduced in this region (Van Heerwarden et al., 2018).

Most of genetic diversity studies are guided by three major hypotheses (Anyango et al., 1995; Mwenda et al., 2018). The first suggest that the strains were introduced with the crop on seeds and therefore they originate from the seeds' provenance or crop growing zones. The second suggest that the strains were introduced through commercial inoculants. The third suggest that the strains are indigenous to local soils and belong to either rhizobial species that do not

traditionally nodulate the crop but possess symbiotic genes corresponding to symbiovars known to nodulate the particular crop, either undescribed rhizobial species that carry very host range symbiotic genes that allow successful nodulation with many types of legumes.

In this study, results that support the tenability of the second and third hypotheses were obtained and could not verify the first hypothesis because the region of soybean seeds provenance was not investigated. In support of hypothesis 2, many of indigenous SNR were *Bradyrhizobium* very close to the commercial strains *Bradyrhizobium japonicum* USDA 110, a commercial strain that was introduced in this region (Van Heerwarden et al., 2018). Moreover, this result revealed that six indigenous SNR clustered together with the commercial strain supported by high bootstrap value (90%). This finding agrees findings of other many authors stipulating that *Bradyrhizobium* is the most common host of soybean in Africa (Grönemeyer et al., 2014; Jaiswal et al., 2016; Naamala et al., 2016; Chibeba et al., 2017; Gyogluu et al., 2018; Mohammed et al., 2019). On the other side nodules were sampled in fields with no history of inoculation, supporting that those indigenous strains have same ancestral with the commercial strain. Some studies on rhizobia have established that some exotic strains called naturalized strains undergo genetic changes and may acquire superior competitive abilities (Chibeba et al., 2018).

The identification of the other group of strains (*Kosakonia, Enterobacter*, *Bacillus*, *Beinjerinckia*, *Burkolderia*, *Microvirga*, *Cupriviadus*, *Mesorhizobium* and *Agrobacterium*) in the soybean nodules support the third hypothesis that stipulated that these indigenous bacteria possess symbiotic genes that allow successful nodulation with soybean. These strains are indigenous to South Kivu soils and either possess host range of symbiotic genes that allow them nodulate with a wide range of legumes (Denison, 2019) or belong to the genera that normally nodulate soybean and that are undescribed to date. These indigenous non-rhizobial strains have acquired the nitrogen fixation genes by the phenomenon of horizontal genes transfer (transfer of genes to a non-parent) as explained by Andrews et al., (2018). They stated that "the transfer of symbiosis genes to bacteria adapted to local soil conditions can allow these bacteria to become rhizobial symbionts of previously incompatible legumes growing in these soils".

These results corroborate Kawaka et al., (2018) who found that root nodules of *P. vulgaris* can be occupied by a diverse group of bacteria namely *Bacillus, Enterobacter, Klebsiella and Pantoea*. Lu et al. (2017), in their study co-existence of rhizobia and diverse non rhizobial bacteria in the rhizosphere and nodules of *Dalbergia odorifera* seedling inoculated with

Bradyrhizobium elkanii, Rhizobium multihospitium and *Burkholderia pyrrocinia*-like strains. They also found the abundance in nodules, of non-rhizobial bacteria such as *Bacillus, Lactococcus and Pseudomons.* However, many other authors consider as contaminants any nodule isolates lacking typical rhizobia characteristics (Simon et al., 2014). The implication of these findings is that effective rhizobia to be used in a certain environment should be selected from indigenous population. The inoculation by exotic rhizobia is necessary only the first season of legume cultivation, after then, the highly effective and competitive rhizobia must be selected among indigenous population.

Indigenous SNR diversity in cultivated land and grassland were compare and it was reported that grassland maintained a higher diversity of rhizobia. Only five species were identified in cultivated fields while about 8 species were discovered in grassland. The presence of diverse legumes crops favors the rhizobial abundance and diversity (Yan et al., 2014). Similarly, NgoNkot et al., (2008) stated that the presence of a compatible legume decreases the diversity and the abundance of the *Rhizobium* population in situ. Of what consequence is this finding? For bioprospecting to identify and collect effective rhizobia to be included in inoculants formulation, the grassland environment must be targeted. In addition, this study shows clearly the importance of frequent inoculation of fields where soybean is cultivated in monocrop and for subsequent seasons.

From these results, high nucleotide identity (99.2 to 100%) was detected between six indigenous SNR and the commercial strain, *Bradyrhizobium diazoefficiens* USDA 110 (CP011360.1), an outstanding soybean symbiont used as commercial inoculants worldwide (Chibeba et al., 2018). A high genetic relatedness of South Kivu's indigenous strains with the commercial strain suggests that there exist highly effective rhizobia strains among indigenous population. There is need to test those strains for nitrogen fixation in diverse environments.

Twenty four indigenous rhizobia strains were selected from the genetic study along all the clusters for genomic study. From the genomes comparison, fourteen high quality draft genomes of indigenous soybean-nodulating rhizobia that can contribute to ecological and physiological microbial studies were obtained. The results from this chapter indicated that sequences genomes belong mostly to the *Bradyrhizobium* and few to the genera of *Rhizobium, Agrobacterium, Kosakonia and Microvirga*. *Bradyrhizobium* is the most common genus nodulating soybeans (Tian et al., 2012) and are present in African soils (Chibeba et al., 2017). However, it was found that, in addition to the common symbiont of soybean, other bacteria like *Rhizobium,*

Agrobacterium, Kosakonia and Microvirga in nodules of soybean suggesting that these indigenous species share symbiotic genes with soybean host (Amadou et al., 2008) or have obtained these genes by lateral transfer (Tartaglia et al., 2019).

Genomes features varied considerably among indigenous soybean-nodulating rhizobia (SNR) suggesting that these strains are highly diverse. This result is consistent with earlier studies and shows that we uncovered almost the totality of genomes of studied organisms (Gonzalez et al., 2006; Kaneko et al., 2011; Wibberg et al., 2011; Mohd Suhaimi et al., 2014; Li et al., 2020). The *Bradyrhizobium* genus hold a higher genome size (9.058Mb±0.720) suggesting that it has a wide range of metabolic capacities (Amadou et al., 2008). Number of genes and G C content obtained from this study were in agreement with earlier studies (Kaneko et al., 2011; Mohd Suhaimi et al., 2014).

The important finding of this study is that 11 indigenous strains out of 14 hold the full set of nitrogen fixation genes while three lack some genes. This suggest that strains with the full set of genes could be considered for a selection program as candidate elite strains. This is in agreement with studies that found that some rhizobia strains possess divergent nitrogen fixation and consequently broader host range (Okazaki et al., 2015; Andrews et al., 2018). The phylogeny and pairwise analysis revealed that six strains (NAC76, NAC42, NAC22, NAC17, NAC46 and NAC28) were highly similar to the commercial strain USDA110 and may be considered as candidate elite strains for South Kivu province of DRC. Many studies identified indigenous strains genetically similar to commercial strains and equally or higher effective in nitrogen fixation compare to the commercial rhizobia. For example, Mwenda et al., (2018) found indigenous isolates with high genetic similarity with commercial strains. Another study conducted by Muthini et al., (2013) found indigenous rhizobia that are more effective in improving beans yields than commercial rhizobia. This method, the comparative genomics can be considered a time saving method for selection of effective rhizobia to be included in commercial formulation.

Using data on genetic study and comparative genetic, six strains (NAC46, NAC17, NAC22, NAC42, NAC76 and NAC37) that showed higher similarity with the commercial strains USDA 110 were selected and evaluated under differents soils conditions in the greenhouse and field condition on the variety SB24 of soybean. From the results, indigenous strains were found to be variable in their ability to nodulate and increase grain yield in diverse conditions which is primarily due to their genetic differences. Other studies conducted in Africa have reported

consistent variation in symbiotic effectiveness among indigenous rhizobia strains (Abaidoo et al., 2007; Chibeba et al., 2017, 2018) and consequently this study further contributes to the evidence that effective rhizobia do occur in African soils.

The best inoculation treatments across all experiments were the indigenous strains NAC46 and NAC17 which nodulated equally or better than the commercial strain USDA 110. In the field NAC46 and NAC17 increased soybean grain yield from 2.4 t ha^{-1} to 3.3 t ha^{-1} and 3.4tha^{-1}; indicating the increase of 68.7% and 70.8% respectively over the commercial strain USDA110. These strains are the leading inoculants elite strains for soybean in South Kivu. These strains will need to be assessed for genetic stability and persistence in inoculants as some author demonstrated that there is risk of loss of ability to nodulate over a period of time (Sachs et al., 2010).

There was significant difference in nodulation between the two sites soils (Table 2). This is because of the differences in soils conditions (Table 1). This finding is consistent with earlier study; for example, Boucho et al., (2019) and Collino et al., (2015) who found that the response to inoculation is highly affected by soils conditions and for their case phosphorus availability in the soil promote nodulation and biomass. Therefore, this study suggests soils amendment prior to inoculation.

The results for the study on rhizobium inoculants'perceptions and adoption determinants revealed that the adoption of inoculant is positively influenced by the location, the knowledge of root nodules, household income and perception of rhizobium inoculant. Location influenced the adoption because some soils need the inoculation compared to others. Many authors stated that innovations are likely to be adopted if they exhibited good results on site and are easy to be applied (Chianu et al., 2011). These finding suggest that before dissseminating the inoculation technology there is need to assess the need to inoculate these soils and the adaptability of the introduced strains in the soils.

Gender of the household head negatively influenced the adoption. There are evidences now that women and man, in agriculture, do not adopt new technology equally (Tanellari et al., 2014; Rola-Rubzen et al., 2020). In this study it was found that women are likely to adopt inoculant than men; these findings are consistent with Murage et al., (2015) who stated that women are bound to benefit from new technology because they are the most vulnerable of farmers. This study suggests gender balance should seriously be considered when disseminated rhizobia

inoculants technology. This study also showed that the knowledge of roots nodules and its roles on legumes also highly influenced the adoption of incoculants. Most of farmers are not aware about BNF (Chianu et al., 2011); thus, there is need to educate farmers on Biological nitrogen fixation for higher adoption. Household income positively influenced the adoption of inoculants because there is always cost for new technology and only farmers with income can invest more in agriculture. Finally, the perception of inoculants as effective and available on market influenced positively its adoption. Therefore, farmer's education about BNF is crucial in its adoption.

7.2. General conclusion

In this study, the genetic diversity of indigenous soybean-nodulating rhizobia (SNR) was firstly analysed and it was concluded that these SNR are genetically diverse and agreed with other authors who consider sub-Saharan Africa as a centre of high rhizobium biodiversity. This diversity is higher in grassland compared to cultivated field suggesting targeted exploration of grassland is necessary for bioprospecting. Six indigenous SNR strains (NAC19, NAC28, NAC32, NAC47 and NAC55) and the commercial strain of *B. diazoefficiens* USDA110 are highly similar suggesting that there exist indigenous SNR in South Kivu soils with high potential for BNF.

Secondly, fourteen genomes of indigenous SNR were compared and it was found that there exist indigenous soybean-nodulating rhizobia in South Kivu soils that have same genomics characteristics as the commercial rhizobia USDA110. These indigenous rhizobia exhibited comparable ability for nitrogen fixation characteristics compared to the commercial strain USDA110. These selected elite isolates based on genomic comparative should be subjected to further investigations under different environmental conditions to confirm their nitrogen fixation superiority. Comparative genomics can be considered a time saving method for selection of effective rhizobia to be included in commercial formulation but must be validated by a field testing.

Thirdly, six selected indigenous SNR were tested for symbiotic effectiveness and compared to the commercial strains USDA110 and SEMIA5019 and it was found that the best strains across all the experiments are indigenous SNR NAC17 and NAC46 with average yields gain 60-70% over commercial strains and controls. These results suggest that these indigenous strains hold the best potential as commercial inoculants in South Kivu soils conditions. USDA 110 and

SEMIA 5019 are also effective but it is preferable to use adapted and competitive strains. Therefore, the native strains are likely to adapt well not only in South Kivu, but also in other countries with similar agro-climatic conditions.

At last, the rhizobium inoculants' perception and adoption determinants among smallholder's farmers in South Kivu province were assessed. This study reported that the adoption is influenced by the gender of household head, the type of education, the awareness of nodules roles on legumes and the household income. Moreover, farmers perceive rhizobium inoculant as an affordable source of Nitrogen for soybean but less accessible. Much effort is needed in extension services strengthening to ensure advanced farmers 'education about inoculation and rhizobium inoculant promotion. Local private firms and agro-dealer's implication is important for more availability and accessibility of the product.

7.3. Recommendations

From this study's results, the use of indigenous strains NAC46 and NAC 17 are recommended for improved biological nitrogen fixation and soybean productivity as they are adapted to local conditions and exhibite comparable nitrogen fixation ability to the commercial strain USDA 110. The use of comparative genomics as a quick method of identifying highly effective rhizobia strains is also recommended. However, these indigenous selected elite isolates should be subjected to further investigations under different environmental conditions to assess their stability. For increasing the adoption of rhizobium inoculants as an affordable mean of increasing soybean productivity among smallholders' farmers, the strengthening of extension service to ensure advanced farmers 'education about inoculation is recommended. The local private firms and agro-dealer's implication for more availability and accessibility of inoculants products is recommended.

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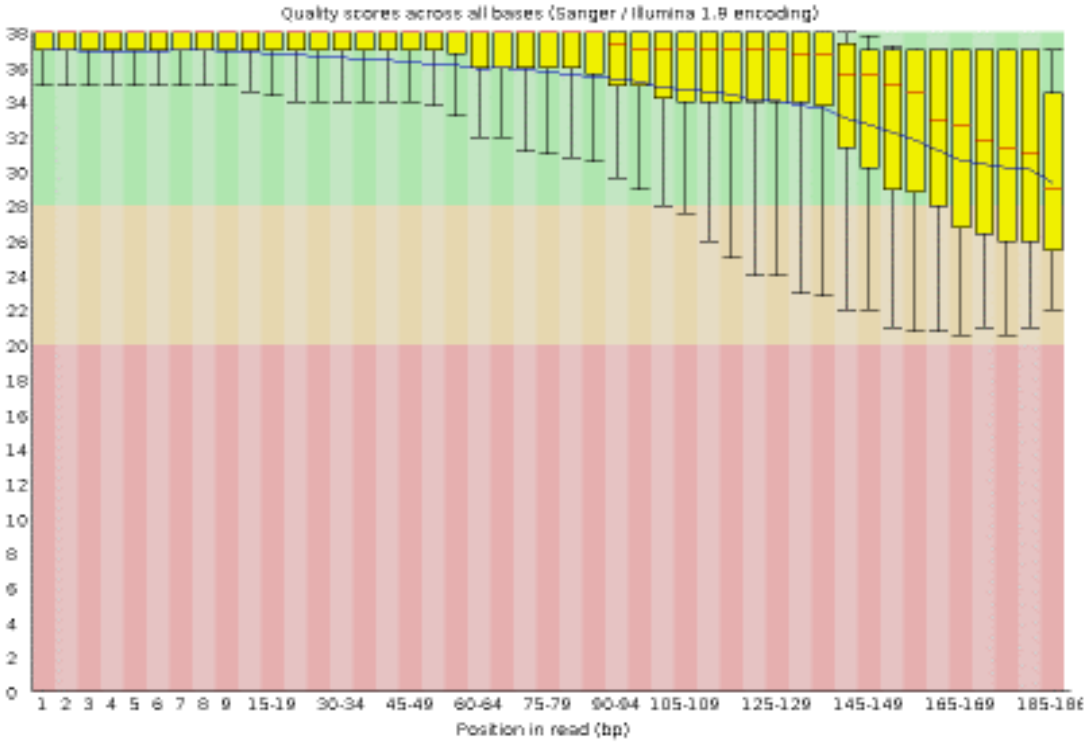
APPENDICES

Appendix 1: Genomes sequences quality scores. The report of quality was generated by FastQC software



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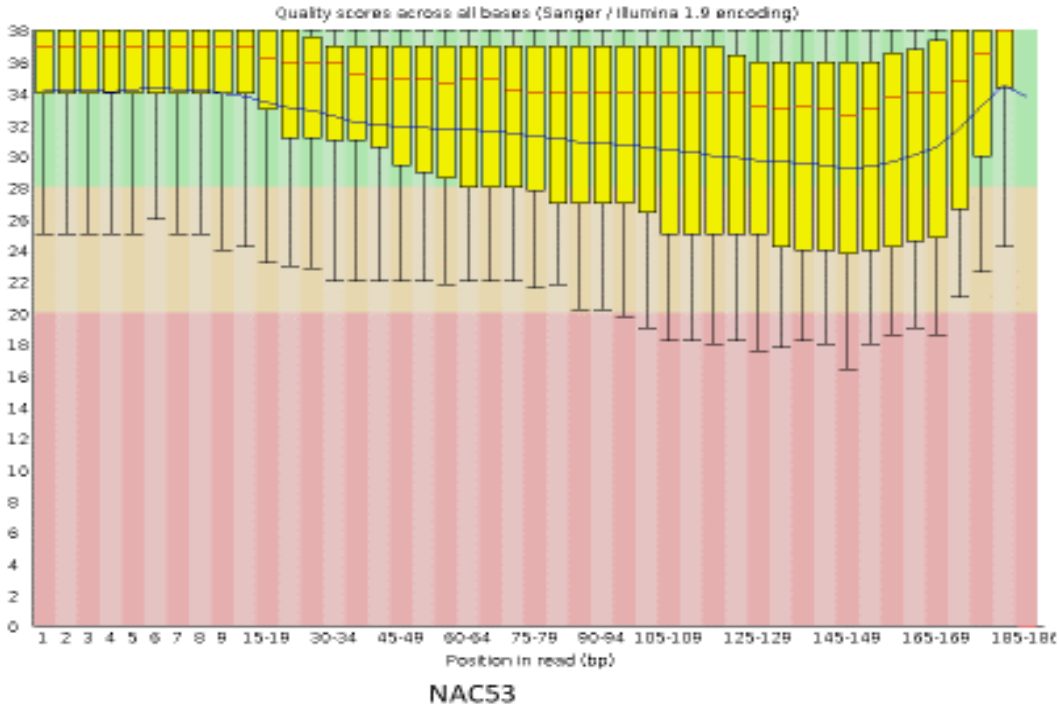

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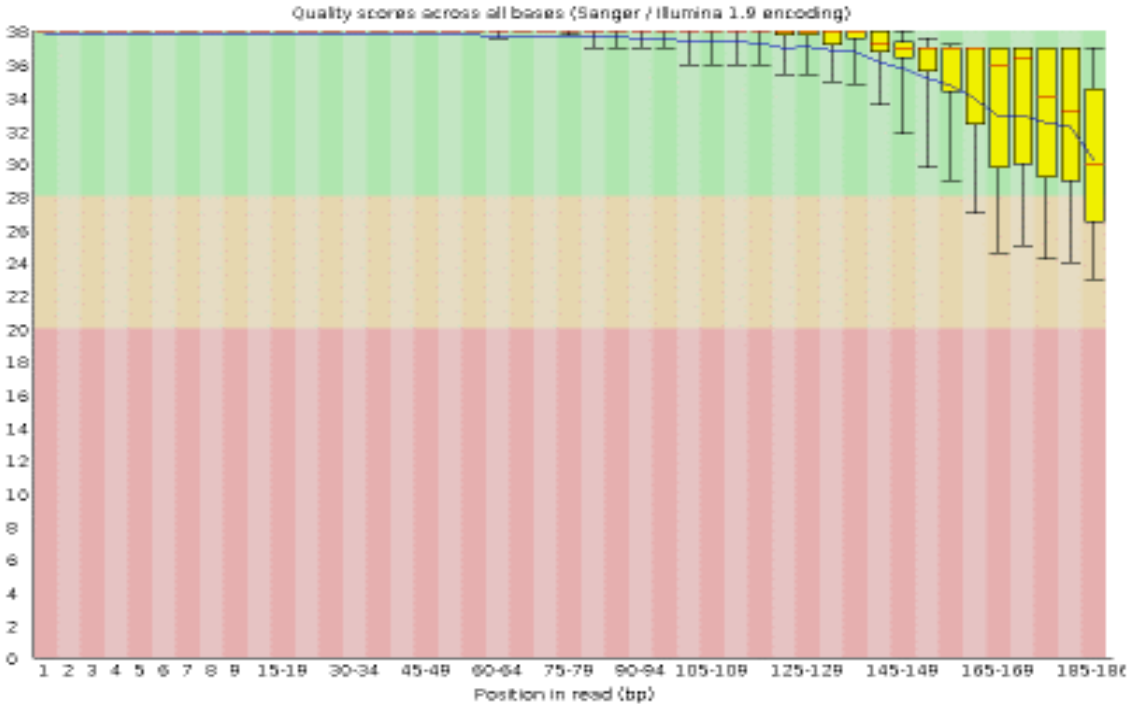

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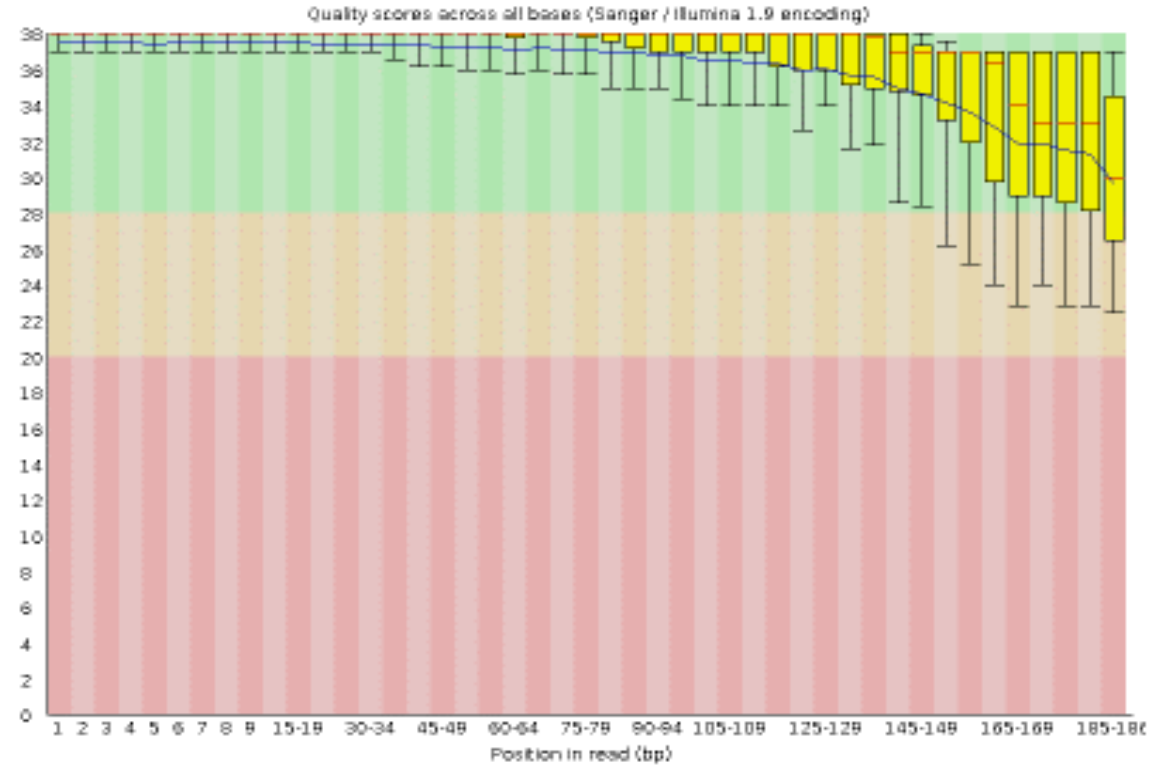

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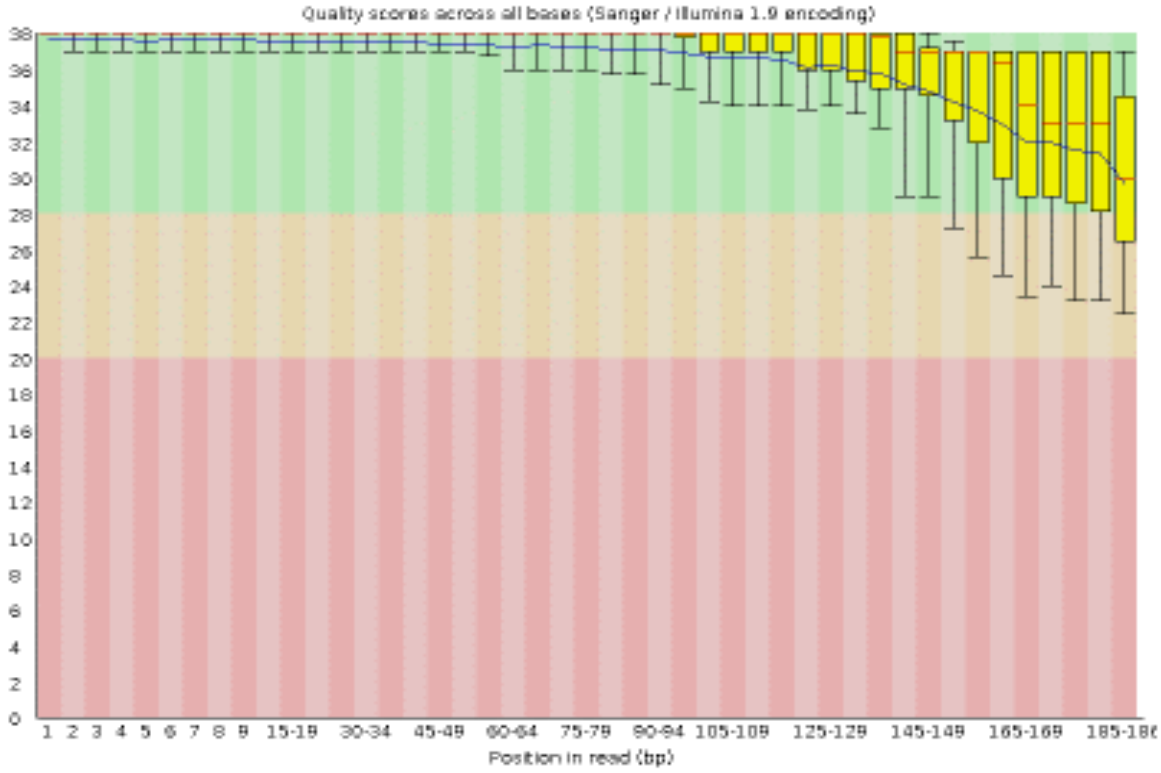

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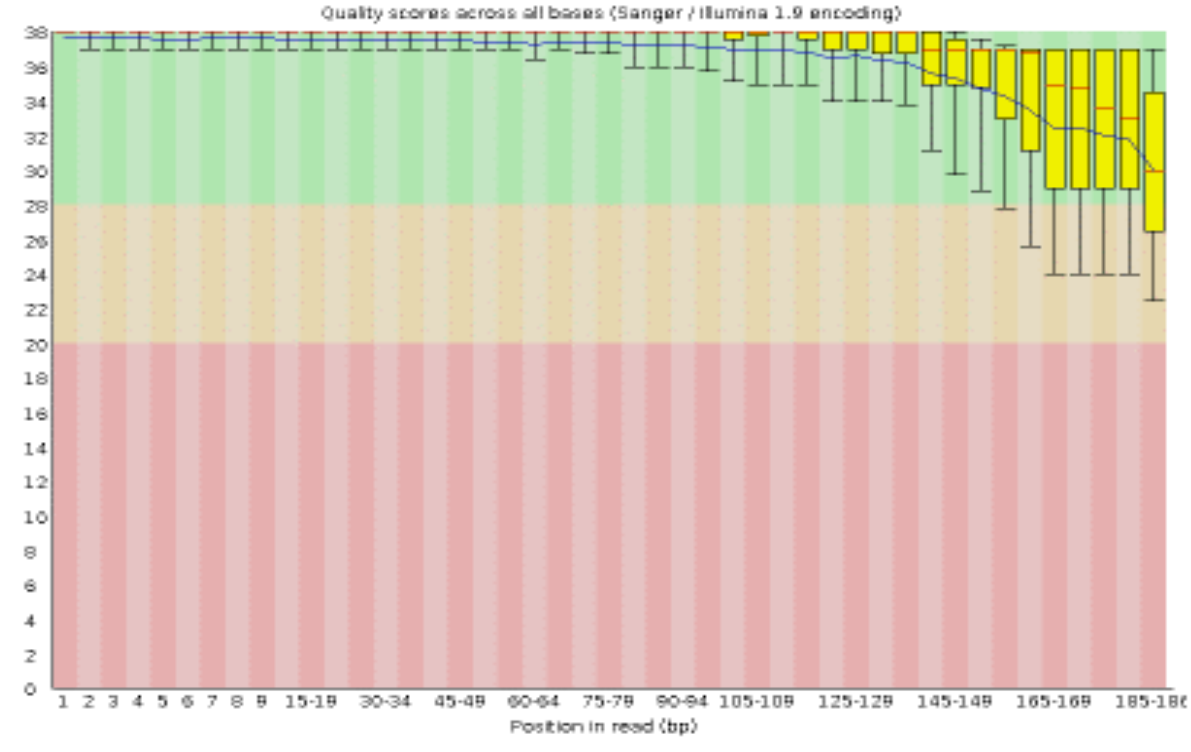

NAC63

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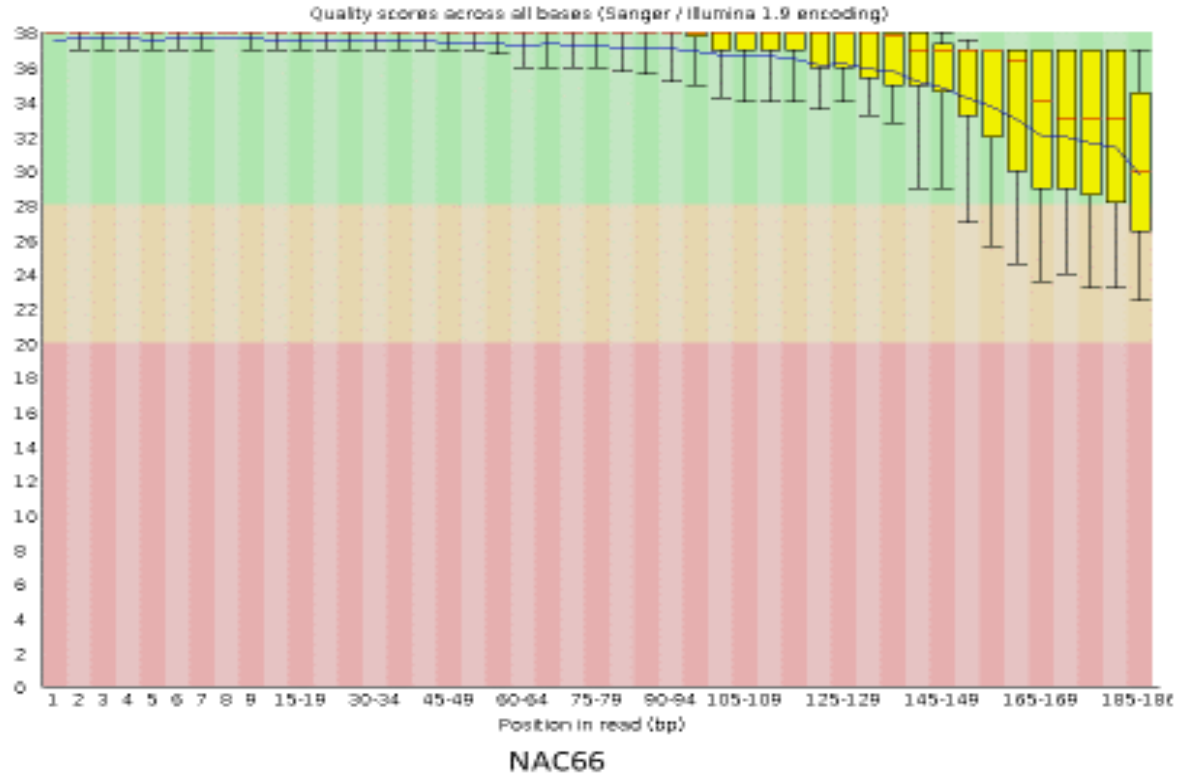

Appendix 2: Table of analysis of variance for nodules number for indigenous rhizobia testing of chapter three

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
repetition stratum	3	184.85	61.62	4.36	
repetition.variety stratum					
Variety	1	153.08	153.08	10.84	0.046
Residual	3	42.38	14.13	0.62	
repetition.variety.strains stratum					
Strains	112	54418.43	485.88	21.38	<.001
variety.strains	112	2378.17	21.23	0.93	0.667
Residual	672	15268.27	22.72		
Total	903	72445.18			

Appendix 3: Table of Analysis of Variance of testing indigenous rhizobia in the green house

Parameter	factor	F values	Df	PR
Nodules number	strain	41.488	9	<0.001
	location	18.160	1	<0.001
	strainxlocation	0.886	9	<0.001
Nodules weight	Strain	22.251	9	<0.001
	location	5.836	1	0.0203
	strainxlocation	0.478	1	0.0029
Shoot weights	strain	3.346	9	0.0038
	location	4.777	1	0.0347
	strainxlocation	0.526	9	0.8465

Appendix 4: Table of Analysis of Variance of field testing of indigenous rhizobia

Parameter	factor	F values	Df	PR
Nodules number	strain	74.292	9	<0.001
Nodules weight	Strain	1.119	9	0.343
Shoot weight	strain	34.230	9	<0.001
Plantgreenness 01	strain	61.11	9	<0.001
Plant greenness o2	strain	29.69	8	<0.001
Plant height 01	strain	7.480	9	<0.001
Plant height 02	strain	10.06	9	<0.001
Plant height 03	strain	68.66	9	<0.001
Grain yield	strain	57.87	9	<0.001

Appendix 5: questionnaire used to assess the adoption and perception of rhizobium inoculants among smallholders soybean's farmers

0. IDENTIFICATION DE L'ENQUETE ET PRESENTATION DE L'ENQUETEUR

Coordonnées géographiques du ménage

Altitude : b. Latitude : c. Longitude

1. Nom du répondant……..Age……Sexe…… ……..
2. Village…….. Localité…… Territoire…….
3. Nom de l'enquêteur…….Date de l'interview…….

SECTION A: CARACTERISATION SOCIOECONOMIQUE DU MENAGE

4. S'il vous plait, donnez les informations suivantes sur le chef du ménage.

Genre *[Code: 1 = Male, 2 = Femelle]*	
Age (Années)	
Type de scolarité? (Formelle, Formation professionnelle, autre)	
Nombre d'années d'études	
Peux lire et écrire. *1 = Oui, 0 = Non*	
Expérience dans l'agriculture (Années)	
Religion *(**Code** : **1**= Catholique, **2** = Islam, **3** = témoins de Jéhovah, **4** = Adventiste, **5**= Protestante, **6**= Religion Traditionnelle Africaine, 7= Autres*	

5. Quel est nombre de membre du ménage [____] nombre de ceux qui ont moins de 18 ans [____], entre 18 et 45 [____] plus de 45ans [____]

6. Quel est le nombre total des membres du ménage de sexe masculin [____] sexe féminin [____]

7. Quel est le nombre des membres du ménage qui travaillent dans le champ au sein du ménagé [____]

8. En commençant par le plus important, choisissez les trois sources de revenu les plus importantes pour le ménage [____], [____] et [____]

Code *: 1= ventre de la production agricole 2= vente des produit de l'élevage y compris les animaux 3= petit commerce 4= emploi formel 5= autres à préciser*

9. Qui contrôle le revenu du ménage. [_____]

Code *: 1 = homme du ménage 2 = Femme dans le ménage 3= Conjoints (hommes et femmes) 4= personne étrangère au ménage 5= autre à spécifier*

10. Selon vous quelle l'intervalle de revenu moyenne mensuel de votre ménage [____]

Code *: 1 = moins de 30$ 2= entre 30 et 50$ 3 = entre 50 et 100$ 4= entre 100 et 200$ 5= entre 200 et 300 6=plus de 500*

11. Avez-vous reçu un crédit au cours de ces … dernières années ? [____] et quel est la taille du crédit [____]

- Si oui quelle est la source du crédit [____] Pour quel motif [____] et qui emprunte [____]
- Si non pourquoi ? [____]

Motifs d'emprunt	Qui emprunte	Source du crédit
1= Investissement non agricole business	1= Homme du ménage	**1 =** Banque
3 = Financement élevage	3= Conjoints (homme & femme)	**3 =** ONG/EMF
2 = Financement agricole	2= Femme dans le ménage	**2=**Epargne communautaire/Coopérative
4 = Frais médicaux	4= Personne étrangère au ménage	**4 =** Commerçant
5 = Frais de scolarité	5= Autre (spécifier):	**5 =** Groupe d'entraide
6 = Amélioration/maintenance maison		**6 =** Ami/proche
7 = Autres (spécifier)_____		**7 =** Autres

12. Si vous n'avez pas emprunté, quelles ont été les raisons du non emprunt (*énumérez les raisons par ordre d'importance*) [__] [__] [__] [__] [__] (**code**)

Code *: **Raisons du non emprunt : 1** = N'a pas besoin de crédit (fonds propres suffisants), **2** = Pas d'endroit où emprunter, **3** = N'a pas la capacité de rembourser, **4** = Il est difficile d'avoir un crédit, **5** = Peur d'emprunter, **6 =** taux d'intérêt, 7= Echéance de remboursement, 8= Autre (à préciser)* _____

13. Quels sont les outils de production que le ménage dispose et utilise dans l'exploitation ? [____]

Code *: 1= Outils aratoires 2= recours à la motorisation 3= les deux*

14. Etes-vous membre d'une association ? *1=Oui, 2=Non* [____]

15. Si oui, quel type d'association [____]

Code : *1= Association d'agriculteurs; 2= Platform; 3= Association Culturelle; 4= Association Politique; 5= Association Religieuse; 6= ONG; 7= Autres (spécifier)*

16. Depuis combien d'années êtes-vous membre ? [____]

17. Etes-vous bénéficiaire d'un projet de développement ou humanitaire ? [____]

Code *: 1=N2 Africa (IITA) 2=Autre (A préciser)*

SECTION B: INFORMATIONS GENERALES SUR L'EXPLOITATION

18. Combien de champs (parcelles) possédez-vous au total ? [____] *1 = Oui 2= Non*
19. Donnez les informations sur vos différentes parcelles dans le tableau ici-bas :

Numéro de la parcelle	Nom de la parcelle (Lieu où elle se trouve)	Culture principale dans la parcelle (***Code 1)***	Taille de la parcelle en Ha	Appréciation de la pente (**Code 2)**	Appréciation de la fertilité (**Code 3)**	Niveau d'érosion (**Code 4**)	Technique de lutte antiérosive (**Code 5**)	Distance de la maison (Minutes)	Propriété de la parcelle (**Code 6)**

Code 1 ***Cultures: 1****= **Soja**, **2 =** Sorgho, **3 =** Manioc, **4 =** Haricot volubile, **5 =** Pomme de terre, **6 =** Patate douce, **7 =** Taro, **9=** Banane, **10 =** Riz, **11 =** Canne à sucre, **12** = Igname, **13** = Thé, **14 =** Café, **15 =** Maïs, **16 =** Choux**, 17 =** Tomate**,** 18=haricot nain, 19 = Autres*

Code 2 ***Appréciation de la pente :*** *1 = faible 2 = moyenne 3 = forte*

Code 3 ***Appréciation fertilité :*** *1 = Très bonne qualité 2 = Bonne qualité 3 = Satisfaisant 4 = mauvaise qualité5 = Je ne sais pas*

Code 4 ***Niveau d'érosion :*** *0=Absente 1 = très sévère 2 = sévère3 = moyen4 = Aucun5 = Ne sais pas*

Code 5 ***Technique de lutte antiérosive*** *: 1 = haie vive 2 = fosse d'infiltration 3 = billon 4 = autre à préciser*

Code 6 ***Propriété de la parcelle :*** *1 = Location temporaire, 2= don, 3 =location de longue durée, 4 = droits d'accès communautaire, 5 = autres (à préciser)*

20. Donner l'ordre d'importance des principales cultures que vous exploitez dans vos différents champs.
    Première culture [___], 2ème culture [___] 3ème culture [___] 4ème culture [___] 5ème culture [___] 6ème culture [___]

21. Faites-vous de l'association des cultures ? [___] 1 = oui 2 = non

22. Si oui, quels sont les différentes cultures souvent associées? (utiliser les codes des cultures pour compléter les différents modes d'associations utilisés)
    - [___] X [___] X [___] X [___]
    - [___] X [___] X [___] X [___]

- [___] X [___] X [___] X [___]

23. Ces associations sont-elles identiques à celles de la saison passée ? [___] 1 = oui 2 = non
24. Si non quelle étaient les associations de la saison passée ?
    - [___] X [___] X [___] X [___]
    - [___] X [___] X [___] X [___]
    - [___] X [___] X [___] X [___]

SECTION B: INFORMATIONS GENERALES SUR L'EXPLOITATION DE SOJA

25. S'il vous plait, donnez les informations suivantes concernant les champs de Soja

Numéro de la parcelle	Variétés pratiqués	Type de variété **(Code 1)**	Depuis combien d'années utilisez-vous cette variété?	Source de la variété **(Code 2)**	Motivation du choix des variétés **(Code 3)**	Taille de la parcelle (Ha)	Culture associée (si en association) (**Code 4**)	Proportion de superficie occupée par le soja dans l'association (%)	Type de main d'œuvre **(Code 5)**	Cout de la main d'œuvre en FC (de la préparation du terrain à la récolte)	Quantité récoltée Saison A	Quantité récolté saison B	Proportion de la production vendue (%)	Unité de vente (**Code 6**)	Prix de vente unitaire (**en FC**)

Code 1 ***Type de variété:*** *1 = Améliorée 2 = Locale 3 = Je ne sais pas*
Code 2 ***Source de la variété :*** *1= Achat ; 2=Récolte précédente ; 3=Association de producteur; 4= Voisin ; 5= ONG ; 6=Centre de recherche ; 7= Autres*
Code 3 ***Motivation du choix*** *: 1=cycle court ; 2=production élevée ; 3=résistance aux maladies ; 4=adaptation aux conditions édapho-climatique)*

Code 4 ***Cultures:*** ***1****= **Soja**, **2** = Sorgho, **3** = Manioc, **4** = Haricot volubile, **5** = Pomme de terre, **6** = Patate douce, **7** = Taro, **9**= Banane, **10** = Riz, **11** = Canne à sucre, **12** = Igname, **13** = Thé, **14** = Café, **15** = Maïs, **16** = Choux**, **17** = Tomate**, 18=haricot nain, 19 = Autres*

Code 5 ***Type de Main d'œuvre:*** *1 = Familiale 2 = Salariée 3 = Les deux (Familiale et salariée)*

Code 6 ***Unité de vente*** *: 1=kg, 2= Sac, 3=Tonne, 4=Panier, 5=Autre, (Spécifier)*

Application et couts d'intrants dans la <u>production du soja</u> au cours de la dernière saison. Veuillez faire en sorte que les numéros des parcelles concordent avec ceux dans les tableaux précédents.

26. Complétez les informations requises dans le tableau ici-bas s'il vous plaît

Numéro de la parcelle	Quantité de semences utilisées issues de sa propre réserve (en kgs)	Quantité de semences achetées (en kgs)	Coût total, **(en FC)**	Type de fertilisant biologique utilisé (**code**1).	Quantité de fertilisant utilisée 1 (en kg)	Coût total, **(en FC)**	Type de fertilisant minérale utilisé (**code1**).	Quantité de fertilisant 2 (kg)	Coût total, **(en FC)**	Type d' intrants minerale2 (**code2**)	Quantité d' intrants organiques utilisée (équiv. secs) (en kg)	Quantité de pesticides (kg/litres)	Cout pesticides **(en FC)**	Quantité d' herbicides (kg/litres)	Cout herbicide **(en FC)**	Pratiques ESSENT. (au moins 3, séparées par une virgule) **code3**	Coût - main d' œuvre embauchée **(en FC)**	Coût - main d' œuvre embauchée (labour)	Coût - main d' œuvre embauchée (sarclage) FC	Mode de semis **(Code4)**	Utilis. de résidus de cultures après la récolte **(code5)**

<u>Code 1 fertilisants</u>: *0= Sans fertilisant; 1=Phosphate dibasique d'ammonium (DAP); 2=UREE; 3=NPK; 4=Nitrate d'ammonium avec calcium (CAN); 5= Phosphate d'ammonium (MAP); 6=Triple Super phosphate (TSP); 7=Super phosphate (SSP); 8=**Inoculum**, 9=Autres (Spécifiez) _____*

<u>Code 2</u> intrants organiques: *1= Résidus de cultures; 2= Fumier d'origine animale; 3= Composte; 4 =Jachère naturelle; 5=Jachère améliorée; 6 =Cultures-abris légumineuses; 7 = Transfer de la biomasse; 8=Agroforesterie; 9=Déchets ménagers, 10= Autres (spécifiez)_____*

<u>Code</u> *3 Pratiques agricoles essentielles (HORMIS la préparation des terres et le désherbage): 1=Semis en sillons, 2=Distançage de plantes, 3= Paillage, 4= Démariage/ Ebourgeonnage, 5=Elagage, 6=Labour minimum / Semis direct, 7= Autres (spécifiez)*

<u>Code 4</u> Mode de semis /plantation *: 1=en ligne, 2=en vrac, 3=en quinconce*

<u>Code 5</u> Utilisation de résidus de récoltes : *1=Laissées dans le champ pour se décomposer; 2= Laissées dans le champ pour nourrir les animaux, 3=Enlevées du champ pour nourrir les animaux ailleurs; 4= Enlevées du champ et mélangées à la composte; 5= Vendues; 5= Enlevées du champ pour servir de combustible, 6=Autre (spécifiez)*

27. Quel est le mode d'application ? [____] *1 = en poquet 2 = en ligne (sillon de semis) 3 = à la volée 4=en couronne*
28. Quelle est la source du fumier utilisé [____] *1 = exploitation propre 2 = acheté 3 = autre à préciser*
29. Quelle est la destination de vos récoltes [____] *1=Autoconsommation 2=vente 3=transformation 4=semence*
30. Qui s'occupent de cette culture [____] *1=hommes 2=femmes 3=enfants 4=tous*
31. Quelles sont les principales contraintes de cette culture [____] *1=maladies et ravageurs 2=fertilité du sol 3=manque des variétés améliorés et intrants 4=autres.*

SECTION C: CONNAISSANCE ET UTILISATION DE L'INOCULUM

32. Connaissez-vous le fertilisant appelé inoculum ? [_____] *1= oui 2 = non*
33. D'où avez-vous appris son existence ?
34. Utilisez-vous ce fertilisant ? [_____] *1= oui 2 = non*
35. Si non pourquoi ne le faites-vous pas ?_____
36. Si on envisagerait introduire ce fertilisant dans votre milieu l'achèteriez-vous ? [_____] *1= oui 2 = non*
37. Si vous utilisez l'inoculum quelle est le nom du produit que vous utilisez ?
38. D'où vous procurez-vous ce produit ?
39. Quelle est votre appréciation de ce produit ?
40. Quand avez-vous commencé à utiliser l'inoculum ? (Année)
41. Comment avez-vous obtenu ce produit pour la première fois ?
42. Connaissez-vous les nodules ? [_____] *1= oui 2 = non*
43. Connaissez-vous le rapport entre nodule et production du Soja ? quelle est le rapport ?
44. Avez-vous été en contact avec une organisation vulgarisant ce produit ? [_____] *1= oui 2 = non.* Laquelle ?
45. Avez-vous reçu une formation sur son utilisation ? [_____] 1= oui 2 = non
46. S'il vous plait, donner un ordre d'importance aux différentes assertions suivantes au sujet de l'utilisation de l'inoculum.

Assertions	Importance (**code**)
1. Je peux acheter l'inoculum si son prix est acceptable	
2. J'ai accès au point de vente de l'inoculum	
3. Le rendement de Soja cultivé par moi est satisfaisant même sans utilisation de	
4. Je peux acheter facilement l'inoculum en cas de besoin n'importe quand	
5. L'inoculation améliore le rendement de Soja	

6. La culture de Soja est très importante pour moi	

<u>Code</u> Importance: 1 *= Fortement d'accord,* ***2*** *= D'accord tout de même,* ***3*** *= N'est ni pour ou contre, 4= Désapprouve tout de même,* ***5*** *= Désapprouve fortement*

MERCI BEAUCOUP