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Ph.D. Dissertation

"Biodiversity of symbiotic nitrogen fixing rhizobia isolated from arable and virgin soils from Huambo province, Angola"

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Abstract

"Biodiversity of symbiotic nitrogen-fixing rhizobia isolated from arable and virgin soils from Huambo province, Angola"

The aim of the study was the evaluation of the presence of symbiotic BNF rhizobia in different soils in central Angola. Soil samples were collected from natural forest, fallow, and arable land. A total of 221 isolates of potential BNF rhizobia was isolated from the nodules of two promiscuous species used as trap plants (*Phaseolus vulgaris* L., *Vigna angularis* (Willd.) Ohwi & H. Ohashi) and directly from the soil. Twenty-one isolates stimulated the growth of the common bean but did not induce nodules. Most plant growth-promoting bacteria were *nifH* positive. Seventeen of them produced IAA. The most active IAA producers were identified as *Enterobacter huaxiensis*. The majority of PGPB was identified as *Burkholderia diffusa* and *Beijerinckia fluminensis*. Also, *Herbaspirillum huttiense* and *Rhizobium pusense* were identified among PGPB. Seventy-six isolates with phenotypic characteristics of rhizobia were selected. Among them, 22 isolates were selected as effective nitrogen fixation rhizobia. The majority of selected strains were isolated from acidic soils with pH 4.2 - 5.2. The biodiversity of BNF rhizobia in the tested soils was limited. Most of the strains isolated from the root nodule of the adzuki bean were phylogenetically similar to *Paraburkholderia kirstenboschensis*, to *Burkholderia diffusa*, or to *Rhizobium miluonense*. Most isolates from nodules of the common bean were identified as *Rhizobium aegyptiacum/R. bangladeshense/R. binae* group, or as *R. miluonense*. The most efficient in pot experiment and under field condition were strains HCC321, HBA15a, and HLo8 identified as *R. aegyptiacum/R. bangladeshense/R. binae.* Under the field condition at Gongoinga (Huambo, Angola) inoculation of seed with the strain HCC321 improved the grain yield of the common bean by 755% in comparison to the uninoculated plants grown on unfertilized plots. Moreover, the application of this strain in combination with NPK fertilization increased the yield by 57.2% in comparison to the yield on plots with NPK fertilization, only. Two strains, HEC1 and HC4 related to genus *Paraburkholderia,* are probably new species because they are not clustered with the any of recognized species.

Keywords: BNF rhizobia, PGPB, *P. vulgaris, V. angularis,* trapping method

Streszczenie

“Biodiversity of symbiotic nitrogen-fixing rhizobia isolated from arable and virgin soils from Huambo province, Angola”.

Celem pracy była ocena występowania symbiotycznych bakterii brodawkowych w różnych glebach w środkowej Angoli. Próbki gleb pobrano z naturalnego lasu, z ugoru i z gruntów ornych. Z brodawek dwóch gatunków roślin pułapkowych (*Phaseolus vulgaris* L., *Vigna angularis* (Willd.) Ohwi & H. Ohashi) i bezpośrednio z gleby pozyskano łącznie 221 izolatów potencjalnych rizobiów. Dwadzieścia jeden z tych izolatów stymulowało wzrost fasoli zwyczajnej, ale nie indukowało tworzenia brodawek. Większość tych PGPB zidentyfikowano jako *Burkholderia diffusa* i *Beijerinckia fluminensis*. Ponadto stwierdzono obecność gatunków *Herbaspirillum huttiense* i *Rhizobium puesense*. Większość tych bakterii wykazywała obecność genu *nifH*, 17 z nich produkowało IAA. Najbardziej aktywnymi producentami IAA były szczepy zidentyfikowane jako *Enterobacter huaxiensis*. Spośród 76 izolatów o fenotypowych cechach bakterii brodawkowych, wyselekcjonowano 22 izolaty wiążące azot w symbiozie z fasolą zwyczajną. Większość wybranych szczepów pochodziła z kwaśnych gleb o pH 4,2 - 5,2. W badanych glebach bioróżnorodność symbiotycznych rizobiów była niewielka. Większość szczepów izolowanych z brodawek korzeniowych fasoli adzuki była filogenetycznie podobnych do gatunków *Paraburkholderia kirstenboschensis*, *Burkholderia diffusa* lub *Rhizobium miluonense*. Podczas gdy izolaty uzyskane z brodawek fasoli zwyczajnej zostały zidentyfikowane jako *R. aegyptiacum/R. bangladeshense/R. binae* lub *R. miluonense*. W doświadczeniach laboratoryjnych i w warunkach polowych najbardziej efektywne były szczepy HCC321, HBA15a i HLo8, które zidentyfikowano jako *Rhizobium aegyptiacum/R. bangladeshense/R. binae*. W doświadczeniu polowym w Gongoinga (Huambo, Angola) inokulacja nasion fasoli zwyczajnej szczepem HCC321 poprawiła plon ziarna o 755%, w porównaniu z niezaszczepionymi roślinami rosnącymi na poletkach bez nawożenia. Zastosowanie tego szczepu w połączeniu z nawożeniem NPK zwiększyło plon o 57,2%. Dwa szczepy z rodzaju *Paraburkholderia*, HEC1 i HC4, są prawdopodobnie nowymi gatunkami, ponieważ nie były filogenetycznie podobne do żadnych uznanych gatunków z tego rodzaju.

Słowa kluczowe: BNF rhizobia, PGPB, *P. vulgaris, V. angularis,* metoda pułapkowa

ACRONYMS AND ABBREVIATIONS

ACB	–	African Centre for Biodiversity
ADF	–	African Development Found
AfDB	–	African Development Bank
ATP	–	Adenosine triphosphate
BNF	–	Biological nitrogen fixation
CIAT	–	International Center for Tropical Agriculture
EPA	–	Environmental Protection Agency
FAO	–	Food and Agriculture Organization of the United Nations
IAA	–	Indole -3-acetic acid
ICRISAT	–	International Crop Research Institute for the Semi-Arid Tropics
IITA	–	International Institute of Tropical Agriculture
INE	–	Instituto Nacional de Estatística
LPWG	–	Legume Phylogeny Working Group
MINADERP	–	Ministério da Agricultura e do Desenvolvimento Rural e Pesca
MLEE	–	Multilocus Enzyme Electrophoresis
MLSA	–	Multilocus Sequencing Analysis
NAN	–	Number of active nodules
NEPAD	–	New Partnership for Africa's Development
NN	–	Nodule number
NPK	–	Nitrogen Phosphorus and Potassium fertilizer
PCR	–	Policycle Reaction
PGPB	–	Plant Growth Promoting Bacteria
RAP	–	Regional Agricultural Policy
RDM	–	Root dry mass
SADC	–	Southern African Development Community
SDM	–	Shoot dry mass
SOLAW	–	State of the World's Land and Water Resources for Food and Agriculture
SSA	–	Sub Saharan Africa
UAN	–	Universidade Agostinho Neto
UN	–	United Nations
USA	–	United States of America
USAID	–	United States Agency for International Development

1. INTRODUCTION

The *Fabaceae* (Lindl.) family that provide 25-35% of the worldwide protein intake (Vance, 1998), subdivided into three subfamilies, *Mimosoideae, Ceasalpinioideae,* and *Papilionoideae*, is one of the largest families of plants with about 643 genera, grouped into 40 tribes growing worldwide and their socio-economic importance is second after the *Poaceae.* There are in genus *Phaseolus* thousand species described, however, only a few are cultivated (Martínez-Romero, 2003; Broughton *et al.,* 2003) and among them, the species *Phaseolus vulgaris* L. (common bean) is the most important species. One of the unique characteristics of members of the *Fabaceae* is their capacity to the formation of root nodules, which were observed as early as the 16th century and by the 19th century was considered of diagnostic value for taxonomic identification (Gunn *et al.,* 1992). Around the world common bean (*P. vulgaris* L.) is the third crop, in the *Fabaceae* family, supplanted only by soybean (*Glycine max* L. Merry) and peanut (*Arachis hypogaea* L.). The major producers of dry beans are India, Brazil, Mexico, and Myanmar as well as in Africa are Tanzania, Uganda, and Kenya (Broughton *et al.,* 2003). Common bean is a staple food nourishing more than 100 million people in sub-Saharan Africa (SSA) countries (Aserse *et al.,* 2012) as well as the primary source of dietary protein providing over 65% of the protein, and 35% of the caloric intake (Wortmann *et al.,* 1998; Dwivedi *et al.,* 2015). It is characterized by the low glycemic index (GI), which is rich in fiber, vitamins, miners, and very low content of saturated fat as well as cholesterol (Wortmann *et al.,* 1998; Broughton *et al.,* 2003). Consumption of common bean *per capita* was estimated from 14 kg, up to more than 66 kg *per* year in SSA countries (Shellie-Dessert and Bliss, 1991; Spilsbury *et al*., 2004; Buruchara, 2007). Solely in SSA that comprise about 25% of the global production, common beans are cultivated on more than 3.5 million hectares by more than 95 % of farmers (CIAT, 2004). Mainly managed by women and cultivated in fields with low fertility (Katungi *et al.,* 2010; Woomer *et al*., 2014).

However, despite the great social-economic importance of the common bean and their direct impact on the diet of people, the attention on this crop has been ignored, not only by governments, policymakers but also by researchers. In general grain yield in Africa remains among the lowest in the world with an average of 550 kg ha^{-1} for maize, 350 kg ha^{-1} for millet, 390 kg ha^{-1} for groundnut, and 220 kg ha^{-1} for beans (ADF, 2005). Among several factors limiting productivity, the low availability of nitrogen is the main one (Global Soil Partnership, 2013). In addition the low yield of common bean in SSA in comparison with high yield

potential, that can be even more than 5,000 kg ha^{-1} under experimental conditions (Graham and Ranallin, 1997; CIAT, 2004), is the result of lack proper crop management as well as lack of knowledge that common bean could be a factor improving nitrogen availability. Nitrogen is the essential element for agriculture crops and due to the relatively high price of nitrogen fertilizers for farmers in SSA is rarely applied in this region (Vieira *et al.,* 2010). For example, the cereals yield about 7 Mg ha^{-1} requires the application up to 300 kg N ha^{-1} (Bockman *et al.,* 1990). Therefore, to supply this amount of nitrogen, agriculture practices use synthetic nitrogen fertilizers (Elkoca *et al.,* 2007), which became more available due to the industrial implementation of the process known as Haber-Bosch synthesis of ammonia, which converts into ammonia atmospheric nitrogen (Smil, 1999). Since the Green Revolution that took place (1959 – 1960) the massive use of synthetic fertilizers, mostly nitrogen, improved worldwide crop yield (Global Soil Partnership, 2013). However, the industrial production, transport, and application of 1 kg of synthetic nitrogen as a fertilizer require 45 MJ, which is equivalent to 0.7 m^3 of natural gas or 1 L of diesel oil (Kendall *et al.,* 2015). Since that time the amount of synthetic nitrogen applied to crops has risen dramatically mainly in Europe and North America. In the last forty years, the application of synthetic nitrogen increased from 12 Tg $year^{-1}$ to 104 Tg $year^{-1}$, hence the agricultural productivity has been enabled by the growth of crop yields that has been accompanied by increasingly intensive use of land (FAO, 2003; McAlister *et al.,* 2012). The higher yields require higher fertilizer application rates and this trend is expected to continue for the next 30 years (Ramírez and Worrell, 2005; Walker *et al.,* 2011). However, farmers in SSA that comprise over 10% of the global population, they have applied actually an annual average of 8 to 9 kg ha^{-1} of fertilizer, mainly concentrated on main crops like maize (*Zea mays* L.) and cassava (*Manihot esculenta* Crantz.) (Morris *et al.,* 2007; Gilbert, 2012). Niazi (2004) noticed that despite an increase in agricultural productivity as a result of the Green Revolution, applying a considerable amount of synthetic nitrogen, however hunger, malnutrition, poverty, not only persist but have increased considerably in developing countries. FAO (2004) reported that agriculture in the twenty-first century is facing unprecedented challenges, on one side dramatic increase in population and on other decreasing food productivity and the problem of improving productivity to feed the population became very important (Barea *et al.,* 2017). The Green Revolution left behind at least 750 million people that live below the extreme poverty line and trapped in subsistence agriculture (FAO, 2004). Projections suggest that over 25% of the population in Africa may

remain in extreme poverty by 2030 (FAO *et al.,* 2017; UN, 2018). FAO (1984) and later Bohlool *et al.* (1992) reported that the malnutrition and high level of poverty in developing countries are strongly related to the lack of possibilities to import fertilizers, mainly nitrogen, which are neither available nor affordable for smallholder farmers due to high prices. Analyzing this scenario, Morris *et al.* (2007) concluded that Africa has not yet experienced its Green Revolution, as well as according to projections by 2030, SSA will be ongoing out of the Green Revolution agenda. In addition, Africa is facing over the past two decades not only for low yield but also with decline agriculture productivity as a result of vicious poverty cycle among decrease soil fertility, soil erosions, droughts, acidification, salinity, poor agricultural practices and lack of proper technology (Odendo *et al*., 2004; Bationo *et al.,* 2004; Morris *et al.,* 2007; Katungi *et al.,* 2010; Gilbert, 2012; Shin *et al.,* 2016; ACB, 2016). The study of over some 200 million ha of arable soils in 13 countries in SSA region presented by Global Soil Partnership (2013) showed that average soil depletion of NPK was on the level of 660 kg N ha^{-1}, 75 kg P ha^{-1} and 450 kg K ha^{-1}, while replenished is only about 18% of losses. This causes significant problems in maintaining the fertility of tropical soils (Morris *et al.,* 2007). For nitrogen as an example, whereas 4.4 million Mg is lost per year, only 0.8 million Mg is applied and farmers in Africa use only an average of 9 kg ha^{-1} of fertilizer compared to more than 87 kg ha^{-1} of fertilizer applied in developed countries (Bationo *et al.,* 2004; Global Soil Partnership, 2013; Huising, 2013; Woomer *et al*., 2014). According to the forecast for the 2030 year published by Bruinsma *et al.* (2003) for sub-Saharan Africa, the main challenge for the agriculture sector in this region will be satisfying demand for food, that due to low doses of applied fertilizers (less than 10 kg ha^{-1}) and high rate of volatilization from synthetic nitrogen fertilizers strongly limit the possibilities of increase of crop productivity. The African agriculture productivity in general still remains extremely low, and estimation indicates that the continent spent more than 18 billion USD annually for food importing (Bationo *et al.,* 2004). All these factors and others lead to poor agriculture productivity, poverty, and food insecurity (Igbozurike, 1971; Janzen, 1973).

Several researchers pointed out that one of the strategies to overcome these challenges could be improving the production of leguminous plants, the systematic effective integration of legumes crops into the farming system, which effectively symbiosis with nitrogen-fixing bacteria (Bohlool *et al.,* 1992; Amede and Kirkby, 2004). The implementation of legumes crops into agriculture systems as a soil cover will reduce soil erosion, maintain and improve

soil physical properties, increase soil organic matter and cation exchange capacity, increase microbial activity and reduce soil temperature (Abayomi *et al.,* 2001) and weed suppression as well (Versteeg *et al*., 1998). Therefore, one of the sustainable ways that we can embrace to face futures challenges of SSA is Biological Nitrogen Fixation (BNF) process, which actually is gaining more attention of researchers not only to improve crop productivity but also to improve soil fertility and health through interactions of plant roots and soil microorganisms (Lugtenberg *et al*., 2002; Oliveira *et al.,* 2002; Buruchara *et al*., 2011). Successful use of BNF can be an environmentally friendly and effective alternative to synthetic nitrogen fertilizers on the medium- and long-term solution for the African population (Vance and Graham, 1995; FAO, 2004; Sanfo and Gérard, 2012). The application of high doses or mismanagement of nitrogen fertilizers contributes significantly to the volatilization of nitrogen oxides into the atmosphere (Vance, 1998). In extreme cases, up to 75% of the applied nutrients are lost in the environment (Sutton *et al.,* 2013). United States Environmental Protection Agency (EPA) as well as the international organization the State of the World's Land and Water Resources for Food and Agriculture (SOLAW) predicted that due to the increase of application of synthetic nitrogen the global N_2O emissions will increase from 35% to 60% by 2030 (FAO, 2011). Therefore, considering the social-economic importance of the common bean in SSA, Katungi *et al.* (2010) suggested that this crop can be used as a strategic crop to hold the fight against hunger, increasing farmers' income and improving soil fertility in Sub Saharan Africa.

1.1. Environmental conditions of agricultural production in Sub-Saharan Africa.

The soil type in Africa varies considerably between regions of production. Luvisols (alfisols) dominate in Eastern and Southern Africa whereas histic soils, eutric soil, dystric nitosols dominate in Eastern Africa and humic, orthic, and rhodic ferralsols (oxisols) dominate in Southern Africa (Allen *et al.,* 1989). The soil pH is mostly between 5.0 and 6.0, but in Eastern Africa and in Southern Africa the soil pH is below or equal to 5.0 on 23 - 20% of the production area. The temperature in Eastern and Southern Africa ranges from 15 to 24°C and the precipitation range from 500 to 2,000 mm, generally characterized as bimodal distribution (White *et al.,* 1992; Wortmann *et al*., 1998). Therefore, the rainfall is very irregular, varying substantially from place to place and from year to year as well, particularly in the drier regions (Bunting, 1961). However, in the majority of regions where the common bean has cultivated the mean of precipitation during the season varies from 400 mm, that is about the

minimum rainfall required, to 800 mm (Allen *et al.,* 1989). The seasonal length, from sowing to harvest, varies from about 70 days in drier lowlands to 150 days or more in humid highlands, although obviously, seasonal length depends also on latitude, cultivars' vegetation period, and growth habit of the cultivar (Allen *et al.,* 1989). Crop system is variable, for example, at high altitudes, between 2,000 and 2,300 m a.s.l., generally predominates monoculture, nevertheless, intercropping is more often and complex characterized by associations with more than two crops species. In Rwanda and Burundi, for example, the most common associations are bananas, maize, and sweet potatoes, while in the north of D.R of Congo, the intercropping practiced with maize, bananas, and coffee (Allen *et al.,* 1989).

1.2. Conditions for agricultural production in Angola.

Angola is the third-largest country in sub-Saharan Africa located between parallels 4º22' and 18º02'S and longitudes 11º41' and 24º05'E, extending over an area of 1,246,700 km^2. The population is estimated for 25.8 million, with a 2.7% growth rate and 37.4% of people live actually in the rural zone and more than 70% of the population depends upon agriculture and livestock (ADF, 2005; INE, 2014). Angola is considered one of the potentially richest countries in SSA in terms of agriculture. The potentially arable land is estimated for more than 35 million hectares that correspond about 28.1% of the total land and about 30 million ha is considered as virgin land while the remaining 5 million ha approximately is the land that has been previously cleared and cultivated. However, 2.5 million ha are currently cultivated, only (ADF, 2005; Cihlář, 2010; SADC, 2011;). The hydrography and the renewable surface water availability is estimated at almost 184 BCM $year^{-1}$, including relatively smaller lakes as well as greater resources of groundwater that can be found in almost all provinces and constitute major sources of water supply (ADF, 2005; NEPAD, 2005; SADC, 2011). Renewable groundwater resources have been estimated at 72 BCM $year^{-1}$, at depths 10-30 m in the Central Plateau, 5-30 m in the coastal zone, and below 200 m in the semi-arid areas of the Southern and Southern-east zone. The aforementioned resources potentially can be used for irrigation about 6.7 million ha. However, currently, little more than 65,000 ha is irrigated, only (NEPAD, 2005). The prevailing climate conditions are variables from northern to south along the coast and western-central plateau. Taking into consideration the Köppen climate classification, Angola is characterized by tropical savannah (Aw) in the north; temperate dry winter and hot summer (Cwa) in north-east; temperate dry winter and warm summer (Cwb) in

center-south; arid steppe and hot (BSh) in the south-east; and arid desert and hot (BWh) in the south-west region (Peel *et al.,* 2007). The temperature is moderate whose annual mean is about 22 ºC and characterized by two seasons, rainy and dry season (Ngolo *et al.,* 2018). Mean annual rainfall decreases towards the southern and increases with increasing altitude, with the highest annual rainfall are 1,700 mm in the northern-east region and the lowest, about 100 mm in the southern-west region (Beernaert, 1997; Carvalho *et al.,* 2016). Soils mainly are classified as Lixisols, Acrisols (ultisols); Ferralsols (oxisols); Arenosols, and Fluvisols (Figure 2) (Missão de Pedologia de Angola, 1961; Preetz *et al.,* 2009). Maize (*Zea mays* L.), cassava (*Manihot esculenta* Crantz.), common bean (*Phaseolus vulgaris* L.), sweet potato (*Ipomoea batatas* (L.) Lam.), groundnut (*Arachis hypogaea* L.), and sorghum (*Sorghum spp.*), are mainly cultivated (SADC, 2011). Until the mid-fifties of the last century, Angola was self-sufficient in term of food crops production, however, due to the civil war that affected the country (1975-2002), farmers became dependent on food aid and Angola has been compelled to become chronically dependent on imports of agricultural products to meet its food requirements (AfDB, 2017). The agriculture sector still is the principal economic activity for the Angolan household. The common bean is one of the most important crops, occupying about 17.3% of the total area used of agriculture annually, with average productivity about 277,000 Mg per year, while domestic consumption is about 468,000 Mg per year (AfDB, 2017) and to fulfill domestic consumption common bean has to be imported. The common bean is considered a strategic crop in the national program for the guarantee of food security and nutrition as a principal source of protein (Jones, 1999; Fonseca *et al.,* 2002; Manuel, 2013). Despite the great importance of the common bean, the yield remains very low (MINADERP, 2010; Manuel, 2013; FAOSTAT, 2017). The high price of fertilizers, low doses of applied fertilizers (average 8.8 kg ha^{-1}), lack of skills, poor research, and ineffective land management systems drive to low agricultural productivity as well as increase hunger and poverty (FAO, 1984; AfDB, 2017).

1.3. Common bean (*Phaseolus vulgaris* L.): Origin, domestication, and evolution.

Over a period of at least 7,000 to 8,000 years, the common bean has evolved from a wild form growing in the highlands of Mesoamerica and Andes to one of the major cultivated food leguminous worldwide sown in a broad range of environments and cropping systems as well (Gepts and Debouck, 1991). However, the exact single local origin of the cultivated common

bean has not certainly been known. The evolution process of wild common bean displays evidence for multiple origins in Mesoamerica (Gentry, 1968) and in South America (Berglund-Brucher and Brucher, 1976). Such evidence contradicts the old hypothesis that the common bean originated from Asia (Kaplan, 1967). The wild common bean was identified in Guatemala (Middle America) by McBryde (1947) and further description provided by Miranda-Colin (1967) and Gentry (1968). Moreover, Berglund-Brucher and Brucher (1976) identified wild populations of *P. vulgaris* growing sporadically from western central Mexico through central and southern America, along with the eastern Andean slopes into northwestern Argentina. These populations later became sources of primary genes pools and centers of diversification (Kaplan and Kaplan, 1988). Two primary genes pools of wild common beans were identified, one originated from the north of Mexico to Colombia (known as Mesoamerican gene pool) and another one from southern Peru to northwestern Argentina (known as Andean gene pool) (Gepts *et al.,* 1986; Gepts, 1988; Koenig and Gepts, 1989; Singh *et al.,* 1991; Angioi *et al.,* 2009; Kwak and Gepts, 2009; Bitocchi *et al.,* 2012). Additionally, Kami *et al*. (1995) described wild common beans lines from Ecuador and northern Peru that are intermediate between the two major gene pools. The comprehensive genetic analysis presented by Tohme *et al.* (1996) reveals the presence at least four wild common bean gene pools: (1) Mesoamerica (Mexico and Central America); (2) Colombia; (3) western Ecuador and northern Peru; and (4) the southern Andes. However, the gene pool of cultivated bean originates mainly from the Mesoamerican pool and Andean pool (Debouck, 1999; Broughton *et al.,* 2003; Beebe *et al.,* 2013).

Domestication was a complex long-term process when a wild common bean plant became a crop. This process changed several morphological and physiological features, such as differences in growth habit, seed dormancy, photoperiod sensitivity, shape, color, and size of the plant, pods, and seeds as well the dissemination mechanisms (Bellucci *et al.,* 2014). All of these structural and functional modifications make cultivated plants different from their wild types, and confer better adaptation to different agro-ecosystems (Gepts and Papa, 2002). One of the consequences of the domestication process is the reduction of genetic diversity due to a founder effect (Glémin and Bataillon, 2009). It was shown that the domestication process of the common bean in Mesoamerica induced a severe reduction (about 72%) in genetic diversity while in the Andes only about 27% loss of diversity was confirmed (Bellucci *et al.,* 2014). The expansion and the pathways of distribution of the common bean out of the

domestication centers were very complex, including the breakdown of the spatial isolation between Mesoamerican and Andean gene pools that leads to increasing the potential for their hybridization and introgression. Also, the cultivation of common bean under variable agro-ecological conditions increased opportunities for both natural and human-mediated selection (Bellucci *et al.,* 2014). Common bean was probably introduced into Africa via the Iberian Peninsula through western Europe during the Portuguese trades and colonial period in the sixteenth century and was established as a food crop before the colonial era (Greenway, 1945; Kaplan, 1965; Gepts and Debouck, 1991; Wortmann *et al*., 1998). According to status and expansion as well of common bean, several continents and countries have been proposed as the secondary centers of diversification for *P. vulgaris*, e.g. Europe (Santalla *et al.,* 2002; Angioi *et al.,* 2010), Brazil (Burle *et al*., 2010), central-eastern and southern Africa (Martin and Adams, 1987; Asfaw *et al.,* 2009; Blair *et al*., 2010) and China (Zhang *et al*., 2008).

1.3.1. Classification, morphology, and conditions of growth of the common bean.

The general classification of common bean is, as follows: Kingdom, *Plantae*; Division, *Magnoliophyta;* Class, *Magnoliopsida;* Order, *Fabales;* Family, *Fabaceae* (*Leguminosae*)*;* Sub-family, *Papilionoideae* (*Faboideae*); Tribe, *Phaseoleae;* Sub-tribe, *Phaseolinae;* Genus, *Phaseolus,* and Specie, *Phaseolus vulgaris* L. According to LPWG (2013), the *Fabaceae* family is one of the most successful lineages of flowering plants with 650–700 genus and 18,000 up to 20,000 species, divided into three subfamilies: *Caesalpinioideae*, *Mimosoideae*, and *Papilionoideae* (*Faboideae*) (Isley and Polhill, 1980). Therefore, *Papilionoideae* is by far the largest sub-family with 476 genera and about 14,000 species (Lewis and Schrire, 2003)*,* within the tribe, *Phaseoleae* contain about 84 genus and 1,500 species, consequently the sub-tribe *Phaseolinae,* (Kloz and Kolozova, 1974) and genera *Phaseolus,* with approximately 55 species (Kass and Wink, 1997). However, only five, out of fifty-five, such as common bean (*Phaseolus vulgaris* L.), scarlet runner bean (*P. coccineus* L.), tepary bean (*P. acutifolius* A. Gray), lima bean (*P. lunatus* L.), and year bean (*P. polyanthus* Greenman) are currently cultivated (Martínez-Romero, 2003; Broughton *et al.,* 2003). From centers of diversification, common bean cultivars were independently disseminated worldwide (Gepts and Debouck, 1991; Broughton *et al.,* 2003) giving rise to two major cultivar groups: cultivar "S" *phaseolin* from Mesoamerican gene pool gave rise to small-seeded cultivars group, while cultivar "T" *phaseolin* from Andean gene pool gave rise to large-seeded cultivars group (Singh and

Gutierrez, 1984; Gepts and Bliss, 1985; Gepts and Debouck, 1991). Therefore, Mesoamerican cultivars, became predominant in South America and in the southwestern of the United States, whereas Andean cultivars became predominant in Africa, Europe, and the northeastern of the United States (Kaplan, 1965; Gepts and Debouck, 1991; Graham and Ranalli, 1997). The germplasm of common bean was clustering also according to seed size: such as small (less than 25 g per 100 seeds); medium (between 25 and 40 g per 100 seeds) and large (over 40 g per 100 seeds) (Hidalgo, 1988). Moreover, based on grows habit, common bean genotypes were clustering into four categories: type I, determinate bush upright; type II, indeterminate bush upright, upright habit, with an erect stem and branches, and often without a guide; type III, indeterminate bush prostrate, a bush habit with weak and prostrate stem and numerous branches; having a short or long guide and with variable ability to climb, and type IV, the indeterminate climber is supported on a suitable tutor, with a weak, long and twisted stem and reduced branching (Kelly and Adams, 1987; Kelly *et al*., 1998; Broughton *et al.,* 2003).

The morphology of wild common bean is generally a slender, much-branched climber. It is an annual or, rarely, a short-lived perennial, climbing vines, flowering, and fruiting the first year, developing from an elongate or fibrous root system profusely nodulated (Gentry, 1968). The stems are angulate, and occasionally can be corky or lignescent with 3-4 mm. The leaves have triangular to lanceolate stipules, spreading or reflexed. The petioles are stout, rounded, with 2.5, 8, or 16 cm long if smallest, medium or largest respectively; rachises 5.5 cm long. The leaflets are membranaceous, ovate to sub-rhombic-lanceolate, 3.2-9.0 cm long, 3-7 cm wide; acute at the tip, round to truncate at the base; upper and lower surfaces sparsely to densely covered with strigillose pubescence and interspersed hooked hairs (Duke, 1981; Delgado-Salinas *et al.,* 1988; Debouck, 1991; Freytag and Debouck, 1996). The inflorescence is a pseudoraceme which is up to 7 cm long, with 2-10 bi-florous nodes. The bracteoles are ovate to lanceolate, rarely orbiculate, 3.5-6.0 mm long, 2.5-4.0 mm wide, not completely adnate to the calyx in fresh material, usually persistent. The flowers are small, 13-18 mm long, normally pink, pale purple, or white (fading to yellow) in color. The gynoecium has an ovary containing 7 to 10 ovules; the styles are bearded introrsely and with an introrse stigma. The pods are straight to slightly falcate, up to 8.3 mm long, and 1.0 cm wide and weigh approximately 0.46g. The valves are thin, papyraceous, beige, or green, often red-pigmented in fresh material and purple in dry ones, bulging slightly over the 8 to 10 seeds. The seeds are oblong, reniform, or trapezoidal in shape. The testa may be greenish, beige, yellow, pale, or

dark brown, grey, black, or pinto usually striped or mottled with black (Delgado-Salinas *et al.,* 1988). The wild common bean was found growing mostly on igneous soils in sloping, moist, and well-drained areas from 500 to 1,900 m a.s.l., but especially between 1,500 to 1,900 m a.s.l. Annual rainfall ranges from 550 to 1,000 mm (Delgado-Salinas *et al.*, 1988) the growth is severely limited with less than 200 mm or more than 710 mm during the growing season, and have optimum growth between 363 and 450 mm (Beebe *et al.,* 2011) with the annual mean temperature from 16 to 25°C (Laing *et al*., 1984; Delgado-Salinas *et al.,* 1988; White *et al.,* 1992). Cultivated common bean, in general, expire with temperatures below 0ºC, reduce significantly growth below 13.6ºC or above 25.6ºC, therefore, the optimum growth temperature is between 17.5ºC and 23.1ºC (Wortmann *et al.,* 1998). Seeds for germination require 5-7 days at 12.5ºC (Kooistra, 1971; Austin and Maclean, 1972). Flowering is usually initiated 28-42 days after planting, but amongst climbing varieties grown at high elevation, can be significantly later. The flowering of determinate cultivars occurs over a very short period of time (usually 5-6 days), while indeterminate cultivars produce additional nodes after initial flowering, with flower formation thereby extended to 15-30 days. Seed filling periods may extend from 23 days in the case of the determinate cultivars to nearly 50 days in indeterminate and climbing varieties. Physiological maturity may occur only 60-65 days after planting amongst those early varieties used in areas where the growing season is very short or extend to 200 days after planting amongst climbing varieties used in cooler upland elevations (Graham and Ranalli, 1997).

1.3.2. Cultivars and potential yield of common bean.

There is a greater diversity of common bean varieties worldwide with the different potential of productivity. The following varieties are the most often cultivated in Angola; Branco Grado a small white, Sachinongue an elongated medium red, Sondeyombua a small red, Calembe, and Chumbo medium-sized cream-seeded varieties as well as Manteiga a large cream and yellow. These cultivars are very similar to the South American varieties known as Manteca, Jalo, or Mexican Canarios; Catarino and Maravilla, similar to the Cranberry types, and Ervilha, a medium-sized yellow bean, respectively. Nearly all Angolans varieties are bush beans and often are cultivated in association with maize or cassava as well as in monoculture (UAN, 1987). Generally, farmers produce, storage, and conserve their own seeds for every sowing season. Their experiences and skills enable them to keep biodiversity and resilient

seeds. The potential productivity of common bean is very high, for example, Fanjul *et al*. (1982) reported the yield of common bean at the level of 8 Mg ha^{-1} and Izquierdo (1991) reported a yield over 6 Mg ha^{-1}. They concluded that to achieve such production all conditions for crop growth should be secured. They reported that whether four independent factors, planting density, water management, fertilizer applications, and soil compaction, were at the level of 95% of their optimum, it resulted in a 19% yield reduction from the potential of 6 Mg ha^{-1} to 4.9 Mg ha^{-1}. There are several factors that contribute to achieving high yield like agricultural practices, climate conditions, selection of cultivars, fertilization, etc. These factors strongly affect the yields of common bean harvested in different regions. Several reports show that in developed countries like Ireland, Netherlands and the USA in which seed inoculation is common practice an average yield of the dry bean was in the range from 6,069 kg ha^{-1} to 1,965 kg ha^{-1}, while in Angola the average yield was 487 kg ha^{-1}, only (Broughton *et al.,* 2003; Katungi *et al.,* 2009; FAOSTAT, 2019). This comparison indicates that improving the agricultural practice in Angola, including the implementation of proper inoculation of seeds, can significantly increase the productivity of the common bean.

1.4. Biological nitrogen fixation.

The role of BNF prokaryotic microbes, also called diazotrophs, has been the subject of studies for over 120 years (Ormeno-Orrillo *et al.,* 2012) and in soils, worldwide huge genetic biodiversity could be exploited (Saravanakumar, 2012). It is noticed that symbiotic bacteria in the area of approximately 250 million hectares of legumes worldwide fix about 90 Tg of nitrogen per year (Vance, 1998). BNF microbes can supply up to 90% of nitrogen plant's needs, promoting plant development, high crop yields, increases soil tilth, environmental stability and support the productivity of farmers with low income (Vance, 1998; FAO, 2004; Kawaka *et al.,* 2014; Pohajda *et al.,* 2016). BNF is a fairly rapid natural process by which diazotrophs bacteria convert atmospheric nitrogen (N_2) into ammonium (NH_3) biologically active forms (Smil, 2002; Galloway *et al*., 2004). This conversion takes place through enzymatic action using energy from the hydrolysis of 16 molecules of ATP (Equation 1) (Kaminski *et al.,* 1998 Pankievicz *et al.,* 2015; Pohajda *et al.,* 2016). However, restricted microorganism members of the domains Archaea and Bacteria are able to perform BNF processes (Peter *et al.,* 1995; Dixon and Kahn, 2004; Galloway *et al*., 2004; Boody and De Vore, 2006; Herder *et al.,* 2010). The BNF process that involves several bacterial genera,

collectively known as Rhizobia, is the most important and the main stabilizers of the nitrogen cycle in the tropics and subtropics (Smil, 2002; Lindstrom *et al.,* 2010; Kapembwa *et al.,* 2016). Assimilated nitrogen is embedded mostly in the amino acids. After plants and heterotrophs organisms die, the decomposition (ammonification) moves nitrogen from dead biomass to NH_3, which is again oxidized. Finally, denitrification returns nitrogen from NO_3 via NO_2 to atmospheric N_2. Globally was estimated that the terrestrial flux of nitrogen from BNF has been calculated to range from 139-170 x 10^6 Mg per year, clearly greater in comparison to nitrogen inputs from synthetic fertilizer (65 x 10^6 Mg per year) (Burns and Hardy, 1975; Paul, 1988; Ishizuka, 1992; Cleveland *et al.,* 1999).

Different authors estimated that N_2 fixation through rhizobia symbiosis is in the range 40-500 kg N ha^{-1} *per* year, in the range 27-150 kg N ha^{-1} per year by symbiotic Actinomycetes, in the range 5-8 kg N ha^{-1} per year by blue-green algae, by free-living bacteria, in the range 1-10 kg ha^{-1} per year, and in the range 3-150 kg N ha^{-1} per year by associate bacteria (Bothe *et al*., 1983; Boddey *et al.,* 1995; Cleveland *et al.,* 1999; Bottomley and Myrold, 2015). Symbiotic nitrogen fixation systems have become well understood and exploited as an effective means of raising the nitrogen status of the soils, providing nitrogen for crops and pastures (Vincent, 1984). When the plants are growing in symbiosis it is presumed that it will satisfy all, or at least main part, of nitrogen requirements from atmospheric N_2, and the surplus nitrogen fixed will subsequently accrue in the soil and benefit other crops (Peoples and Craswell, 1992; Peoples *et al.,* 1995). This makes BNF one of the most 'environmentally friendly' approaches to obtaining nitrogen into agroecosystems (Jensen and Hauggaard, 2003).

The specific enzyme that responds to nitrogen fixation is known as nitrogenase. Nitrogenase is a complex enzyme consisting of two components: Component I, FeMo-protein, or dinitrogenase is a heterotetramer containing 4 (Fe_4S_4) clusters covalently bound to the MoFe protein bridging the α- and β-subunits and a cofactor with Fe and Mo, which is the catalytic site for N_2 reduction. Component II, *NifH* is a dinitrogenase reductase also designated Fe-protein (Hurek *et al.,* 2002; Shin *et al.,* 2016; Skorupska *et al.,* 2017), homodimer contains a single Fe_4S_4 cluster, *NifD* and *NifK* are α and β subunits of dinitrogenase, respectively (Kaminski *et al.,* 1998; Dixon and Kahn, 2004).

Equation 1. $N_2 + 16\ MgATP + 8e^- + 8\ H^+ \rightarrow 2\ NH_3 + 16\ MgADP + 16Pi + H_2$

A second, major, feature of nitrogenase is its oxygen sensitivity, mainly contributed by the Fe-protein component. Nitrogenase requires a strict anaerobic environment for activity and, as

a consequence, rhizobia need to protect their enzyme from oxygen (Giller and Wilson 1991; Hill 1992). Partners, plants, and bacteroid contribute to solving the oxygen paradox. The low O_2 concentration in the infected nodule cells (5-30 nM) is maintained by an oxygen diffusion barrier, by the buffering capacity of leghaemoglobin and by bacteroid respiration (Kaminski *et al.* 1998). Therefore, to be an effective symbiosis requires also the expression of *nod* (or *nol*) genes (Laguerre *et al.,* 2001). The bacterial nodulation (*nod*) genes encode the synthesis of "Nod factors" and stimulate the plants to produce symbiotic nodules signaling pathway promoting root invasion by the rhizobia and the formation of nodules C, after bacterial establish and nodule formation, initiates the expression of *nif* genes, responsible for their ability to fix atmospheric nitrogen (Laguerre *et al.,* 2001; Tan, 2014). According to Bohlool *et al.* (1992), the full utilization of benefit BNF systems can be realized only through analysis and resolution of major constraints to their optimal performance in the field, and the implementation on farmer's level. For long-term sustainability of agricultural systems must rely on the use and management efficiency of internal resources focused on BNF.

1.4.1. Symbiotic rhizobia.

Soil bacteria capable of inducing the formation of nodules on root systems of legumes were discovered at the end of the nineteenth century when it was reported for the first time that atmospheric nitrogen was being assimilated through the root nodules of legume plants. In 1888, Beijerinck isolated bacteria from root nodules and concluded that those bacteria were responsible for atmospheric nitrogen fixation. Therefore he named these bacteria *Bacillus radicicola* (Willems, 2006; Velázquez *et al.,* 2017). Later on, it was renamed as *Rhizobium leguminosarum* (Frank, 1889). In the beginning, the criteria used for classification of rhizobial in different species was based on the legume host they nodulated such as *Rhizobium phaseoli* nodulating *Phaseolus*, *R. trifolii* nodulating *Trifolium, R. meliloti* nodulating *Melilotus* (Dangeard, 1926), *Rhizobium japonicum* nodulating *Glycine* (Buchanan, 1926), and *Rhizobium lupini* nodulating *Lupinus* (Eckhardt *et al.,* 1930) all were included in the validation lists of Skerman *et al.* (1980). *R. japonicum* was reclassified into the genus *Bradyrhizobium* as the first species with the name *Bradyrhizobium japonicum* (Jordan, 1982) and later the genus *Phyllobacterium* was isolated and reported by Knösel (1984). The aforementioned papers changed the classification of *Rhizobiaceae* with three genera;

Rhizobium, Bradyrhizobium, and *Phyllobacterium* as well the species *R. trifolii* and *R. phaseoli* were reclassified as *R. leguminosarum* (Jordan, 1984).

The most relevant change for bacterial taxonomy was proposed based on 16S rRNA gene sequences (Woese and Fox, 1977). Rhizobia were placed within the alpha subdivision of *Proteobacteria* (Woese *et al*., 1984). Since then, the 16S ribosomal gene became an essential tool for bacterial classification and identification (Velázquez *et al.,* 2017). From, 1991 onwards, the 16S rRNA gene sequences were included in all descriptions or reclassifications of the different taxa within family *Rhizobiaceae*, and the reclassification of *R. fredii* into genus *Sinorhizobium* was confirmed by the analysis of this gene (Jarvis *et al*., 1992) as well as *Rhizobium tropici* was the first species described using partial sequencing of the 16S rRNA gene (Martínez-Romero *et al.,* 1991). Only in the 1980s when species *R. fredii* as fast-growing rhizobia was discovered nodulating soybean the status of rhizobia biodiversity become more interesting for researchers and led to the reclassification of *R. fredii* into a new genus named *Sinorhizobium* (Chen *et al.,* 1988). Moreover, from that time, the number of phenotypic characteristics included in the description of new taxa was higher, as occurred in the case of *R. galegae* isolated from Galega nodules (Lindström, 1989) and *R. huakuii* isolated from *Astragalus* nodules (Chen *et al.,* 1991). Based on phenotypic characteristics and rRNA-DNA hybridization, were described new genus named *Azorhizobium* with type species *Azorhizobium caulinodans* isolated from stem nodules of *Sesbania rostrata* (Dreyfus *et al*., 1988). The studies have proceeded and in the 1990s several new rhizobial species were described (Velázquez *et al.,* 2017). De Lajudie *et al.* (1992) described the new genus *Allorhizobium* with a single species named *Allorhizobium undicola* isolated in Senegal from nodules of *Neptunia natans.* Other species were changed from the old to the newly described genera e.g. the species *R. meliloti*, was reclassified into the new genus *Sinorhizobium,* and became *Sinorhizobium meliloti* (de Lajudie *et al.,* 1994). A new genus *Mesorhizobium* with an intermediate growth rate between *Rhizobium* and *Bradyrhizobium* was proposed by Jarvis *et al.* (1997). The species *R. ciceri, R. huakuii, R. loti, R. mediterraneum,* and *R. tianshanense* were reclassified from the genus *Rhizobium* to the new genus *Mesorhizobium* as *M. ciceri, M. huakuii, M. loti, M. mediterraneum,* and *M. tianshanense.* Young *et al*. (2001) based on comparative 16S rRNA gene sequences reclassified the genera *Agrobacterium* and *Allorhizobium* into genus *Rhizobium*, published in the International Journal of Systematic and Evolutionary Microbiology (IJSEM). However, many researchers did not recognize the

reclassification of the genus *Agrobacterium* into the genus *Rhizobium* (Farrand *et al*., 2003; Mousavi *et al.,* 2014). Furthermore, the reclassification of the genus *Sinorhizobium* into genus *Ensifer* (Casida, 1982) was performed according to the decision of the Judicial Commission of the International Committee on Systematics of Prokaryotes (2008).

Another important contribution to the taxonomy of rhizobia was the sequencing of several housekeeping genes summarized by Velázquez *et al.* (2017). Two of these genes, *recA,* and *atpD* are used nowadays very often in rhizobia taxonomy (Gaunt *et al*., 2001). Other genes such as *glnII*, *rpoB*, *dnaK* or *gyrB,* and multilocus sequences analysis (MLSA) has actually used for the description of new genera, new species, used also for reclassification of genera, and the replacement of old genera which were not been earlier recognized in *Rhizobiaceae* (Velázquez *et al.,* 2017). For example, the new genera *Neorhizobium* (Mousavi *et al.,* 2014) and *Pararhizobium* have been described, and the old genera *Agrobacterium* and *Allorhizobium* have been reclassified (Mousavi *et al.,* 2015). Also, as the separate species *R. phaseoli* (Ramírez-Bahena *et al*., 2008) and the reclassification of the *R. lupini* into the genus *Bradyrhizobium* as *Bradyrhizobium lupini* (Peix *et al.,* 2015) were confirmed. Furthermore, the complete genome sequence is currently available for the type strains of several old and recent rhizobial species and has already been used to describe new rhizobial species isolated from lentils, such as *R. lentis*, *R. binae* and *R. bangladeshense* (Rashid *et al*., 2015), and from soybean, such as *Ensifer glycinis* (Yan *et al*., 2016).

Conversely, classic rhizobial genera now also contain many species isolated from other sources than *Fabaceae* (Velázquez *et al.,* 2017). Some of them coming from non-rhizobial genera, such as *Blastobacter denitrificans* and *Agromonas oligotrophica* that were transferred to genus *Bradyrhizobium* (van Berkum *et al*., 2006; Ramírez-Bahena *et al.,* 2013) and *Blastobacter aggregatus* that was first transferred to genus *Rhizobium* (Kaur *et al*., 2011) and later to the new genus *Pararhizobium* (Mousavi *et al.,* 2015). Nevertheless, the most relevant advancement in the present century was the discovery that bacteria which do not belong to classic rhizobial genera are able to induce nodules in legumes. Most of these bacteria belong to genera from the alpha subdivision of Proteobacteria, while some of them belong to the beta subdivision of Proteobacteria (Velázquez *et al*., 2010; Peix *et al*., 2015a). The first beta Proteobacteria isolated from legume nodules were included into the genera, *Burkholderia* and *Ralstonia*, able to nodulate *Mimosa* (Moulin *et al.,* 2001; Chen *et al.,* 2003a), but they are currently included in the genera *Paraburkholderia* (Sawana *et al.,* 2014) and *Cupriavidus*

(Vandamme and Coenye 2004). Although some studies have shown that in some American and Asian countries *Mimosa* is preferably nodulated by alpha Proteobacteria (Gehlot *et al.,* 2013; Bontemps *et al.,* 2016).

According to the summary (Velázquez *et al*., 2017) of rhizobia classification, more than one hundred species of symbiotic nitrogen-fixing bacteria were recognized recently. Majority of them belong to subclass α-Proteobacteria the old genus *Allorhizobium, Azorhizobium, Bradyrhizobium, Mesorhizobium, Rhizobium,* and *Sinorhizobium*; the new genus, *Aminobacter*, *Devosia*, *Methylobacterium, Microvirga*, *Neorhizobium*, *Ochrobactrum*, *Phyllobacterium* and *Shinella* (Sy *et al.,* 2001; Latif *et al.,* 2013; Mousavi *et al.,* 2014)*;* on the subclass β-Proteobacteria: *Burkholderia, Paraburkholderia* (formerly *Burkholderia*) and *Cupriavidus* (Velázquez *et al.* 2017); and the subclass γ-Proteobacteria have also been found to be involved in nodulation (Benhizia *et al*., 2004; Peix *et al.,* 2015a; Suneja *et al.,* 2016). These species and genus are phylogenetically separate from each other based on 16S rDNA sequences. Native bacteria nodulating common bean were studied in Mesoamerica and South America. Strains nodulating common bean originating from Mexico and South America were identified as a heterogeneous complex strongly differentiated phylogenetic lineages (Piñero *et al.,* 1988). One of the lineages was subsequently classified as *R. tropici* (Martínez-Romero *et al.,* 1991) with the identification of two subgroups *R. tropici* type A and *R. tropici type* B (Willems and Collins, 1993). Moreover, Eardly *et al.* (1992) described two species as *R. leguminosarum bv. phaseoli* type I and type II. Segovia *et al.* (1993) proposed the species *Rhizobium etli* as a separate species based on its difference in the 16S ribosomal RNA (rRNA) gene compared with *R. leguminosarum*. The results showed clear evidence that *R. etli* was distinguishable genetically and phenotypically different (Segovia *et al.,* 1993). Full-length of small subunit ribosomal RNA (SSU rRNA) sequencing and DNA-DNA reassociation analyses have confined the independent species status of *R. etli* (van Berkum *et al.,* 1996; Tamimi and Young, 2004; Junier *et al.,* 2014). Two new species *Rhizobium gallicum* and *Rhizobium giardinii* were identified in France by Amarger *et al.* (1997) and consequently, authors proposed the subdivision of each species into two biovars: *R. gallicum bv. gallicum* and *R. gallicum bv. phaseoli*; and *R. giardinii bv. giardinii* as well as *R. giardinii bv. phaseoli.*

However, knowledge gaps still exist in the genetic diversity and biogeographic distribution of rhizobia, where they have not been intensively studied so far like in Sub-Saharan Africa ecozones. For example, several new isolates of rhizobia described recently isolated from

common bean (Mwenda, 2017) as well as from many indigenous legumes in Sub-Saharan Africa (Chidebe *et al.,* 2017) are probably new species.

1.4.2. Taxonomy of rhizobia nodulating common bean in Sub-Saharan Africa.

Rhizobia nodulating common bean isolated in the east and south Africa were taxonomically related to *R. tropici* (Anyango *et al.,* 1995; Dagutat and Steyn, 1995). While most of the indigenous strains isolated in central Africa and in western Africa were closely related to *R. tropici*, *R. tropici* type B, and *R. etli* (Tjahjoleksono, 1993; Diouf *et al.,* 2000). Additionally, strains of *R. gallicum* were also identified in soils of central Africa nodulating common bean (Mhamdi *et al.,* 1999; Diouf *et al.,* 2000; Beyene *et al.,* 2004; Faghire *et al.,* 2012). Aserse *et al.* (2012), using MLSA observed that most frequent rhizobia nodulating common bean in soils of Ethiopia were closely related to *R. phaseoli, R. etli,* and the novel group of *Rhizobium* spp. While few strains considered sporadic symbionts of common bean were closely related to *R. leucaenae* and *R. giardinii*. Therefore, they observed that several strains in the concatenated tree showed 99–100% partial 16S rRNA sequences similarity to *R. etli* CFU 42^T as major species in the *R. leguminosarum* complex. Authors noted that most of the isolated rhizobia nodulating common bean had very similar *nifH* gene sequences. Aserse *et al.* (2012) gave support to Segovia *et al.* (1993) reporting that species *R. phaseoli,* also known as *R. leguminosarum bv. phaseoli,* first identified in Europe, might have arisen from the addition by horizontal transfer of the symbiotic plasmid from *Rhizobium sp*. type I to *R. leguminosarum* chromosome. Common bean in Western, Eastern, and Southern Africa, according to Diouf *et al.* (2000) is naturally nodulating by the same species of rhizobia as that nodulating common bean at its site of origin. Concluding that rhizobia nodulating common bean are cosmopolitan, and was co-introduced with common bean seeds. This hypothesis was also supported by Aserse *et al.* (2012) reporting that native rhizobia may have acquired the molecular characteristics of *R. phaseoli* from the introduced *R. etli bv. phaseoli*.

Discrepancy argument with Diouf *et al.* (2000) and Aserse *et al.* (2012) was reported by Beyene *et al.* (2004). According to Beyene *et al.* (2004) based on MLEE and 16S rDNA sequences similarities, rhizobia nodulating common bean in Ethiopia was related to *R. leguminosarum* or to *R. etli.* Therefore they reported that it is unlikely that rhizobia nodulating common bean originating from Americas or Europe and extensively colonized soils of Ethiopia because Beyene *et al.* (2004) did not identify *R. tropici, R. gallicum* and *R. giardinii*

that occur very often in common bean symbiotic rhizobia species on soils of Americas or Europe. According to Beyene *et al.* (2004), it is not completely clear how *R. leguminosarum* in Ethiopian soils acquired the genetic information for nodulation of common bean, nor it is apparent how *R. leguminosarum* gained the 16S rRNA gene sequence characteristic of *R. etli*. Anyango *et al.* (1995) observed that indigenous rhizobia nodulating common bean from regions of acidic soil stress (pH 4.5) were predominantly close to *R. tropici*, while the majority of regions with pH 6.8 showed species phenotypically similar to *R. etli.* However, Odee *et al.* (2002) have done relevant research in Kenya concerning the genetic diversity and symbiotic status of 41 indigenous bacteria isolated from root nodules of 13 trap host trees and herbaceous *Fabaceae* plants. Authors found BNF rhizobia with 99 – 100% similarities *B. elkanii, B. japonicum, Mesorhizobium sp., R. leguminosarum, R. tropici IIB,* and *Sinorhizobium sp.* Otherwise, in discrepancy with Anyango *et al.* (1995) findings, they observed that indigenous rhizobia related to *R. tropici* were isolated from range soil pH conditions including alkaline. Studies have revealed that there are in Kenya a greater number of unknown rhizobia diversity that form an effective symbiotic relationship with the common bean. Examination of 185 isolates from common bean done by Mwenda (2017) indicated that 65% of isolates effectively nodulating common bean were putative novel taxa where others 45% isolates were identified as *R. sophoriradicis, R. phaseoli, R. leucaenae, R. paranaense,* and *R. etli*. The putative novel taxa had *recA* sequences with less than 96.6% identity to known *Rhizobium* strains. Results that illustrate the high level of promiscuity of common bean were described by Wekesa *et al.* (2017). Among 24 isolates from nodules of common bean cultivated on western Kenya, only three were closely related to *Rhizobium* sp. The remaining isolates were related to diverse genera representing *Acinetobacter calcoaceticus*, *Delftia sp., Enterobacter* sp., *Klebsiella pneumoniae,* and *Providencia* sp. Authors reported that the wide phylogenetic diversity of rhizobia isolated from a relatively small number of leguminous species in the SSA region is an indicator as an important center of rhizobia biodiversity (Odee *et al.,* 2002; Wolde-meskel *et al.,* 2005).

Indigenous rhizobia isolated from root nodule of common bean and runner bean cultivated in South Africa was assessed by Lindeque (2006) demonstrating that the majority of 18 isolates from common bean were similar to *R. leguminosarum, R. tropici* and others isolates were identified as *R. etli*, *R. leguminosarum bv. phaseoli* or *R. lusitanum*. The author observed that 50 % of isolated strains from nodules of runner bean were similar to *Burkholderia unamae*

and others were identified as *B. tropicalis, R. leguminosarum,* and *R. tropici* while the remaining isolates were not able to be assigned to a specific genus or species. Among the aforementioned species, *R. tropici* is recognized as opportunistic BNF rhizobacteria having a broad range of hosts including common bean (Hernández-Lucas *et al.,* 1995). Strains of this species showed a wide spectrum of symbiotic efficiency with common bean (Oliveira *et al.,* 1998; Mostosso *et al.,* 2002). Several studies revealed that *R. tropici* strains are more successfully nodulating common bean in acid soils (Vargas and Graham, 1989; Graham *et al.,* 1994; Ribeiro *et al.,* 2012), and had been isolated from acid soils in France (Amarger *et al.,* 1997), in the east and south Africa (Anyango *et al.,* 1995) as well as in tropical environmental (Segovia *et al.* 1993). Moreover, strains of this species are more tolerant to high temperature (Martínez-Romero *et al.,* 1991; Hungria *et al.,* 1993), are well adapted to sandy soils (Acosta-Duran and Martínez-Romero, 2002), and are tolerant to high concentrations of salts in soils (Priefer *et al.,* 2001).

1.4.3. Common bean-rhizobia symbiotic interaction.

Common bean (*Phaseolus vulgaris*) is frequently considered as a poor nitrogen fixer, and the *Phaseolus-Rhizobium* symbiosis has frequently been identified as an unreliable symbiotic system (Pena-Cabriales *et al*., 1993; Epping *et al.,* 1994). Peoples *et al.* (2009) analyzed the global average shoot nitrogen fixed by several legumes in farmer´s fields their findings illustrated that nitrogen fixed by *P. vulgaris* was 15 kg N ha^{-1}; soybean about 137 kg N ha^{-1}; pea 108 kg N ha^{-1}; lentil 71 kg N ha^{-1}; cowpea 20 kg N ha^{-1}; pigeon pea 59 kg N ha^{-1}, and groundnut 103 kg N ha^{-1} (Peoples *et al*., 2009). Several other reports similarly have indicated the common bean as the most inefficient nitrogen-fixing in comparison to other legume species (Graham, 1981; Bliss, 1993; Hardarson, 1993; Herridge and Danso, 1995). The host genotype, the rhizobia strain, and the interaction between the inoculation method as well as environmental conditions may all be contributing to this poor fixation (Chaverra and Graham, 1992; George and Singleton 1992; Pena-Cabriales *et al*., 1993; Hardarson *et al.,* 1993; Buttery *et al*., 1997; Nleya *et al.,* 1999; Bressers, 2014). However, Saito and Ruschel (1978) have indicated that, once these factors are corrected, the naturally established symbiotic interaction in common bean fields occurs hence increasing the rates of nitrogen fixation. Similarly, Westermann and Kolar (1978) reported that it is possible to increase the symbiotic nitrogen fixation and seed yields of the common bean by isolating and recombining lines with

high nitrogen fixation capabilities, as well as improving the rhizobia strain-host symbiosis. Piha and Munns, (1987a, 1987b) showed that there are certain varieties of common bean that can satisfy their nitrogen needs exclusively through nitrogen fixation when inoculated with suitable rhizobia strain. Also, several studies in SSA have shown possibilities of improving the rhizobia-common bean interaction and efficacy of nitrogen fixation (Saito, 1982; Hungria *et al*., 2000; Mostasso *et al.,* 2002; Tamimi, 2002). Inoculation of common bean with selected strains of *Rhizobium etli*, *R. tropici,* or *R. leguminosarum bv. phaseoli* (Michiels *et al*., 1998), with a consortium of native rhizobia (Ouma *et al.,* 2016), with native rhizobia, a strain named ELM3 (Koskey *et al.,* 2017), with *R. tropici* CIAT 899, and with several indigenous strains, related to *R. fabae, R. pisi, R. phaseoli R. etli,* or *R. suphoriradicis* (Mwenda, 2017) showed higher effectiveness of BNF and increase of grain yield in comparison with uninoculated plants.

According to Broughton *et al.* (2003), the insufficient nitrogen fixation of common bean symbiotic rhizobia often derives from poor root infection and nodule development. Root systems are responses to interaction with symbiotic soil organisms, uptake water, and nutrients (Hetrick, 1991; Feddes and Raats, 2004; Parniske, 2008). The common bean root architecture is characterized by the main root and abundance of adventitious roots, together with enabling the plant to explore a larger volume of soil (Lynch and Ho, 2005; Lynch, 2007; Miguel *et al*., 2013). However, the root system varies according to the growth habit of common bean varieties: The type I varieties have generally a shallow root system with abundant adventitious roots; type II, exhibits a dominant deep root system with few adventitious roots, while type III has a highly branched root system and is superficial. One of the most important structures in the root system is root hairs. Root hairs are subcellular protrusions of root epidermal cells, usually are found on 1-2 cm from the root tip, responsible for the acquisition of immobile nutrients (Peterson and Farquhar, 1996; Jungk, 2001), dispersion of exudates throughout the rhizosphere (Hinsinger, 2001; Ryan *et al.,* 2001), and beyond others process that allows the effectiveness of symbiotic interaction process.

The symbiotic interaction starts with a dialogue between partners, when the host plant root exudates, such diverse classes of flavonoids that induce *nod* genes in rhizobia, including chalcones, flavanones, flavones, flavonols, isoflavonoids, coumestans and anthocyanidin (Dazzo *et al.,* 1988) are released into the rhizosphere. Rhizobia in response produce lipo-chitin oligosaccharides, known as *Nod* factors, that afterward induce plants to various

responses such as root hair deformation, infection threads, cortical cell division, pseudo nodule and nodule formation (Long, 1989; Lerouge *et al.,* 1990; Bergman *et al.,* 1991; Gottfert *et al.,* 1992; Dakora *et al.,* 1993; Cárdenas *et al.,* 2000; Perret *et al.,* 2000; Cooper, 2004; Geurts *et al.,* 2005; Sánchez-López *et al.,* 2011). *Nod* factors induce responses in three different root tissues of the host; epidermis, cortex, and pericycle. Rhizobia enters into the root hair through the infection thread and the underlying cortical cells (van Brussel *et al.,* 1992). The infection thread filled with proliferating rhizobia grows from the root hair towards the nodule primordium localized in the inner cortex (Bakhuizen, 1988). Inoculation induces root hairs to curl shortly after emergence, thereby trapping rhizobia at the cell junction, from which the infection thread initiates at the root hair base (Hadri *et al.,* 1998). Root hair deformation and curling are the first morphological changes induced by rhizobia and they are preceded by a rapid change of cytoplasmic streaming. (Hadri and Bisseling, 1998). The rhizobia into the cytoplasm colonize the central tissue due to rapid cell division producing a new structure called Bacteroides leading to the development of the nodules (Brewin *et al.,* 1992; Cermola *et al.,* 2000; Emons and Mulder, 2000).

1.4.4. Promiscuity symbiotic interaction.

One of the interesting characteristics of the common bean is its ability to establish symbiotic interaction and fix nitrogen with different species of rhizobia. This characteristic is known as promiscuity due to the fact that some of the flavonoids produced by common bean roots have been found to be the inducers of *nod* genes in the different rhizobia species allowing them to nodulating plants (Broughton *et al*., 2000; Perret *et al.,* 2000), and several *Rhizobium* species have multiples copies of *nod* genes (Davis and Johnston, 1990; Girard *et al*., 1991; van Rhijn *et al*., 1994: Martínez-Romero, 2003). According to Martínez-Romero (2003), the poor inoculation responses hence the low rate of atmospheric nitrogen fixation is directly correlated to the promiscuity behaviors of common bean. Similarly, Vlassak *et al.* (1997) reported that symbiotic promiscuity is one of the characteristics that can be a disadvantage for the host, due to the fact that it increases the number of potential competitors to inoculants, leading to a high incidence of inoculation failure. Michiels *et al*. (1998) observed that common bean was able to recognize *Nod* factors with different chain lengths from 10 different rhizobia species (*R. etli, R. tropici, S. fredii, M. loti, R. leguminosarum bv. trifolii*, *R. leguminosarum* bv. *viciae, B. japonicum, S. meliloti*, and *Rhizobium* sp. NGR234 and GRH2),

however, authors notice that 70% of the strains that induce nodules on common bean are unable to form nitrogen-fixing nodules. However, according to Pacovsky *et al*. (1984) even if the common bean is promiscuous, plants seem to have some degree of preference for certain rhizobia and Aguilar *et al*. (2001) reported that in nature common bean have some selectivity for *R. etli* bv. *phaseoli.* Therefore, the strategy to improve the rate of nitrogen fixation in the common bean would be the interaction of common bean varieties with high capacity to a symbiotic relationship in combination with *Rhizobium* strains with superior capacities to fix nitrogen and compete with native strains (Martínez-Romero, 2003).

1.4.5. Commercial inoculants and factors affecting biological nitrogen fixation.

Rhizobia may be applied to soils as commercial inoculants, through seeds, aerial plants, or soils inoculations. Commercial inoculants are frequently used with a significant positive impact on agronomic systems. According to Somasegaran and Hoben (1994) generally, high-quality inoculants applied to seed, achieve inoculation rates from $2.5x10^3$ to $1x10^6$ rhizobia $seed^{-1}$ corresponding to an application rate about $8x10^{10}$ rhizobia ha^{-1} in common bean field (Brockwell *et al.,* 1995). Therefore, Sanginga *et al*. (1996) and Houngnandan *et al*. (2000) reported that there a positive effect of commercial inoculants when in the field the indigenous rhizobia are absent, ineffective or less than 5 cells g^{-1} soil and also Ulzen *et al*. (2016) noted the positive effect of commercial inoculants if the number of native rhizobia was < 10 cells g^{-1} soil and probably ineffective. This factor was reported by several researchers (Mead *et al.,* 1985; Thies *et al.,* 1991*,* Namkeleja *et al.,* 2016). However, to maintain high populations, up to 10^2 cells g^{-1} of the soil of very effective introduced rhizobia in the soil for at least 3 years, the soil might have high clay content (> 20 %) and high organic carbon content (> 1 %) (Zengeni *et al.,* 2006). The growth and survival of the introduced microorganisms depend on the physiological adaptation in the introduced cells, such as adaptation to nutrient-limited conditions and/or other physical-chemical conditions, efficient utilization of root-released compounds or specific interactions with plants (van Overbeek and van Elsas, 1997; Devliegher *et al*., 1995). However, in the soil, the survival of the inoculated bacteria largely depends on the availability of an empty niche, helping them withstand competition with the often better-adapted native microflora (Rekha *et al*., 2007).

1.5. Factors affecting biological nitrogen fixation.

There are several factors that contribute to effective symbiotic interaction between inoculant and host plants. Among them, in literature, the number of viable indigenous rhizobia in soil, soil acidity, salinity, amount of available nutrients as well as the temperature and water stress as factors affecting the biological nitrogen fixation of symbiotic rhizobia were discussed.

1.5.1. Soil biotic and abiotic factors affecting biological nitrogen fixation.

One of the most often discussed factors is the number of indigenous rhizobia previously present in the soil (Pohajda *et al.,* 2016). The number of indigenous rhizobia in the soils can range from 0 up to 10^7 cells g^{-1} soil (Singleton and Tavares, 1986; Triplett *et al.,* 1993; Brockwell *et al.,* 1995; Rougbley *et al.,* 1995). According to Evans *et al.* (1996) commercial inoculants rarely infect the host in soils that contain more than 10^2 indigenous rhizobia g^{-1} of soil. Similarly, several researchers observed reduction and failure of nodule formation on fields with more than 10 indigenous rhizobia g^{-1} of soil (Singleton and Tavares, 1986; Thies *et al.,* 1991; Sanginga *et al.,* 1996). While Fening and Danso (2002) observed high effective symbiotic interaction of indigenous rhizobia at $1.3x10^3$ cells g^{-1} of rhizobia. Similar results were found by Tena *et al.* (2016) in Ethiopia.

Agricultural practices are important factors that strongly affect the soil acidity, soil salinity, and the availability of the nutrients. Both alkaline and acidic soils have a direct effect on rhizobial growth, survival, and effective symbiotic interaction (Abd-Alla *et al.,* 2014). The increasing soil acidity noticeable reduce the symbiotic BNF process, reduce survival and growth of rhizobia, reduce attachment of rhizobia to root hair and infection what reduce plant growth (Carter *et al.,* 1995; Sadowsky and Graham, 1998; Hungria and Vargas, 2000) and grain yields (Martínez-Romero, 2003). Weak or no nodulation was recorded when the pH of the soil was below 5.8 (Diouf *et al.,* 2007; Agbenyega, 2015). Moreover, as the pH of soils decreased, the isoflavonoids secretion was reduced (Richardson *et al*., 1989; Hungria and Stacey, 1997) and the profile of secreted nod factors was noticeably different even by *R. tropici* strain CIAT 899, which is tolerant of acidic conditions (Morón *et al.,* 2005). The level of soil acidity generally results in the dominance of one rhizobia species only. In neutral or alkaline soils, the symbiotic interaction of common bean was dominated by *R. etli* (Piñero *et al.,* 1988*;* Souza *et al.,* 1994) while *R. tropici* generally dominates under acid soil conditions (Vargas and Graham, 1988; Frey and Blum, 1994). The majority of indigenous rhizobia

isolated from acid soils (pH about 4.5) were related to *R. tropici* what was described in Brazil (Vargas and Denardin, 1992) and also in Kenya (Anyango *et al.,* 1995). The most effective rhizobia-legumes interaction was observed in East Africa on soils with a pH of about 6.5 (Argaw, 2012; Alemu, 2016). The varied optimum values of soil pH were reported for the growth of different species of rhizobia; Chen *et al.* (1993) reported that mutants of *R. leguminosarum* have been able to grow at pH 4.5, Foster (2000) observed that *S. meliloti* grew up well at pH 5.5, while Fujihara and Yoneyama (1993) reported that *S. fredii* can grow well between pH 4 – 9.5. The major problem in alkaline soils is the reduction of nutrient availability. However, whether the BNF process is facing alkalinity stress, generally, delays the growth of rhizobia as well as the establishment of effective nitrogen-fixing symbiosis (Abd-Alla *et al.,* 2014). Although *R. leguminosarum bv. trifolii* has been reported to colonize soil, induce nodule formation as well as effectively fix nitrogen even at soil pH 11.5 (Zahran, 1999).

The soil acidity strongly influences nutrients status, which are essential elements for the growth of bacteria or plants, which can cause a reduction in the nodule numbers, nodule size, and in the amount of nitrogen fixed (Peoples and Herridge, 1990; Giller and Wilson, 1991). Rhizobia are very sensitive to the application of nitrogen fertilizer. A nodule formation and nitrogen fixation are inhibited due to increases in the levels of application of synthetic nitrogen in the soil (Streeter, 1988; Barbulova *et al*., 2007). Phosphorus deficiency affects strongly the growth of plants dependent on BNF, reducing the number of effective nodules and nodule development (Cassman *et al*., 1981; Sa and Israel, 1991). Potassium stimulates the infection and nitrogen fixation process. The symptoms of potassium deficiency appear on the surfaces of the lower leaves in the form of white spots on their edges and progressing toward the top as the severity of the deficiency increases (Maheshwari *et al.,* 2012). Sulfur deficiency causes a reduction of nodule number, size, considerable declines in the rate of nitrogen fixations, and protein synthesis (Munns, 1977; Maheshwari *et al.,* 2012). Under Zinc deficiency, carbon fixation activity is completely inhibited and decline in photosynthetic activity (Marschner, 1995; Romheld and Marschner, 1991). Calcium is an important factor regulating rhizobia attachment to the root hair of leguminous plants (Caetano-Anolles *et al*., 1989), increasing the *nod* gene induction and expression activities of plants (Richardson *et al*., 1988). Other nutrients such as manganese, magnesium, and aluminum are known to affect nodulation and nitrogen fixation as well (Brady *et al*., 1990).

Salinity stress is also an important factor limiting the productivity of leguminous crops. Almost 40% of the world's land surface is affected by salinity problems (Vriezen *et al.,* 2007). Inhibition of plant growth by salinity is mostly due to the toxic level of sodium chloride (NaCl), decreasing the ability of the root system to control the entry of ions to the shoot (Hajibagheri *et al.,* 1989; Abd-Alla *et al.,* 2014). According to Zahran (1999), Legume-Rhizobia symbioses are more sensitive to salinity stress than rhizobia alone. Salt stress inhibits the initial steps of rhizobia-legume symbioses (Zahran *et al.,* 1997), causing changes in root hair morphology and decreases curling root hairs (Abd-Alla *et al.,* 2014), affect the expression of *nod* genes, decreasing in lipochitooligosaccharide (Dardanelli *et al.,* 2008; Penttinen *et al.,* 2013), reduction the number and weight of nodule per plant (Abd-Alla, 1992). Salinity induces also the formation of inactive and deformed nodules, degradation of the peribacteroid membrane (Bolaños *et al.,* 2003).

Strains vary in their tolerance to salinity. According to Elsheikh (1998), fast-growing rhizobia are generally considered to be more tolerant of saline stress than slow-growing rhizobia and strains isolated from saline soils are typically more tolerant Hua *et al*. (1982). However, Zahran (1999) observed several fast-growing rhizobia that isolated from saline soils were salt sensitive. The ability of rhizobia to adapt to fluctuations in the osmolarity of their surroundings is fundamental for their survival (Abd-Alla *et al.,* 2014).

1.5.2. The environmental factors affecting biological nitrogen fixation.

The influence of natural factors like the temperature in connection with water stresses strongly affected the BNF efficacy of symbiotic rhizobia, especially in SSA. The soil temperature and water deficiency play an important role in the exchange of molecular signals between rhizobia and their host. Many soil microorganisms have the capacity to survive at relatively low soil temperatures, therefore, their growth rates and metabolic activities decline below optimal temperatures (Sadowsky and Graham, 1998). It is noticeable that low temperature generally inhibits the biosynthesis and rhizosecretion of signal and signal exchange between the two symbiotic partners (Abd-Alla, 2011). Low temperature also inhibits the induction of bacterial nodulation genes (Zhang *et al.,* 1995). Additionally, the root zone temperatures below 17.5 °C cause reduction and delay nodule formation. Trinick (1982) previously reported that even with rhizobia that tolerate low (4°C) soil temperature, the growth rate decreases considerably. Moreover, high temperatures above 40 ºC near the

surface, can affect the survival and persistence of rhizobia and the interaction with legume hosts (Karanja and Wood, 1988; Andrés *et al.,* 2012), decrease nodulation (Hungria and Vargas, 2000). Hungria and Franco (1993) observed a decline in nitrogenase activity at a temperature above 28°C. Similarly, Aranjuelo *et al*. (2007) observed a drastic reduction of nitrogenase activity, nitrogen, and CO_2 fixation rates as a result of high temperature. The optimum temperature for nodule formation and rhizobia growth is between 25 and 33 °C (Pankhurst and Sprent, 1976; Zhang *et al*., 1995). Rhizobia nodulating common bean can survive up to 47 °C, (Karanja and Wood, 1988). However, *R. leguminosarum bv. phaseoli* were able to nodulate common bean at temperatures, not higher than 35 - 38°C (Hungria and Franco, 1993). Therefore, nodule formation and fix nitrogen are optimal between 25 and 30°C and are hampered by root temperatures between 30 and 33°C (Piha and Munnus, 1987). The high temperatures of soil resulted in South Tropical and Southern Africa ecozones. Generally, the phase of root infection by BNF rhizobia is the most sensitive to water stress and high temperature (Singleton and Bohlool, 1984). Once nodules have formed, the sensitivity of the symbiotic process decreases (Purwantari *et al*., 1995). It was reported that water deficiency during vegetative growth decreases the activity of nitrogenase, decreasing respiratory activity followed by decreases in nodule surface area (Weisz *et al.,* 1985; Albrecht *et al.,* 1994; Zahran, 1999; Gerosa-Ramos *et al.,* 2003), affect the deposition of exudates into the rhizosphere (Drew, 1990), decrease the growth of nodule and accelerate nodule senescence (Abd-Alla *et al.,* 2014). With increasing water deficiency, the plant biomass production and nitrogen fixation decrease (Pimratch *et al.,* 2008) as well as decrease the transport of fixed nitrogen out of the nodule (Serraj *et al.,* 1999; Andrés *et al.,* 2012). BNF in the stage of nodule development and function is also sensitive to excess of water (Abd-Alla *et al.,* 2014). The diffusion of O_2 within the nodules is in part regulated by a physical barrier located in nodular parenchymal cells (Andrés *et al.,* 2012). Under excess water conditions, the diffusion resistance increases by identifying a lack of O_2 inside the nodule, leading to inhibiting the nitrogenase activity (Day and Copeland, 1991). The ability of rhizobia to survive underwater stress depends on the capacity of bacteria to cope with radiation stresses, reactive oxygen, salts and solutes, and temperature extremes without being killed (Potts, 1994; Billi and Potts, 2002; Ramos *et al.,* 2001).

1.6. Methods of determination of rhizobial diversity.

Interest in the diversity of soil rhizobia has increased in recent years with the development of improved techniques for strain characterization and grouping, and the recommendation for polyphasic taxonomy of Rhizobia (Graham *et al.,* 1991). The current taxonomy of rhizobia reveals their wide diversity at the genus, species, and intraspecies levels (Laguerre *et al.,* 2001). Many studies have described the diversity of bacteria. The use of PCR techniques for selectively amplifying sequences of partial bacteria diversity related to different phylogenetic origins is nowadays widespread (Zézé *et al.,* 2001; Sarita *et al.,* 2005). The first study using the nod gene as a target for a PCR approach was published by Zézé *et al.* (2001). A rapid method to screen rhizobial diversity at any location to check any aspect related to the rhizobial community is the upcoming interest (Sarita *et al.,* 2005). They have developed this method to characterize the soil rhizobia diversity using nod genes as the genes responsible for initiating the symbiosis process and found in all nodulating rhizobia. PCR primers that match nod gene sequences can be used, with soil bacterial DNA as a template, to amplify sequences that must come from rhizobia, and the specific origin of these sequences can be identified by comparing them with the extensive database of published nod gene sequences (Sarita *et al.,* 2005). On the other hand, Ueda *et al.* (1995) analyzed rhizobia diversity using *nif*H gene sequences after PCR amplification of mixed organism DNA. While Laguerre *et al.* (2001) analyzed the diversity of rhizobia based on PCR-amplified 16S-rDNA analyses of *nod*C and *nif*H fragments. *nod*C sequence is relatively long, which enabled the PCR amplification of large DNA fragments a priori to ensure maximum specificity of RFLP fingerprints and maximum robustness of phylogeny inferred from nucleotide sequences. Using the *nif*H gene as a nitrogen fixation marker (Laguerre *et al.,* 2001) as well as a *nod*C gene marker was also discussed by Kamst *et al.* (1999) and Perret *et al.* (2000) for determination of biodiversity of rhizobia nodulating common bean.

Phenotypic characterization of isolated strains in the soil can be used also for the determination of rhizobia diversity, however, these methods exclude several bacteria that cannot be isolated and cultured in laboratory media (Torsvik *et al.,* 1990). To estimate the diversity of the bacterial community more effectively, according to Torsvik *et al.* (1990) can be using the soil DNA heterogeneity by thermal denaturation and reassociation. Also, Amann *et al.* (1995) reported that the determination of soil bacteria diversity by DNA-DNA

re-association studies of samples directly extracted from soil using PCR, 16S rRNA gene fragments is more effective.

1.7. Problem statement and justification.

The presented above summary of the present status of knowledge related to BNF rhizobia biodiversity, occurrence, and its efficacy in soils of the SSA region noticeably indicated that soils in Angola were not practically investigated recently. Also, the review focused on the biodiversity of BNF rhizobia in Sub-saharan Africa notes a noticeable gap in the knowledge about the presence and biodiversity of BNF in central Angola (Bongo and Pietr, 2019). The increasing food insecurity in Angola is attributed to several factors mainly to declining agricultural productivity caused by soil infertility, diseases, the high price of input for farmers, and lack of knowledge (ADF, 2005). In Angola, production of common beans is mainly carried out by smallholder farmers who, in most cases, have difficulty getting access to input, mainly, fertilizers and high yielding certified common bean seeds varieties in order to optimize their production. The effort to meet high food demand by increasing population, farmers generally intensify the use of land for crop production, a factor which has contributed to the decline in soil fertility along with soil acidity challenge. The use of inorganic fertilizers, mainly nitrogen is limited due to the high cost; therefore common beans are cultivated without the application of fertilizers. In addition, the lack of the complete absence of commercial inoculant, as well as the lack of knowledge of BNF technology use altogether, leads to actual low yield that common bean has attained in Angola.

1.8. The aim of the study.

As it was mentioned by several researchers the effective use of BNF rhizobia in the agriculture system can be very useful as one of the tools to face the actual and future challenges of agriculture productivity and minimizing adverse effects on the environment. Improvement of common bean yield through BNF in Angola will contribute, in the short term, to poverty reduction, enhance food security and generate income for smallholder farmers as well as in medium- and long-term agriculture sustainability. The characterization of rhizobia diversity in soils of central Angola will help in selecting more effective rhizobia strains capable of improving common bean yield in Angola. Therefore, I decided to study for the first time in Angola the diversity of rhizobia in different soil of Huambo province and

identify the effective indigenous rhizobia nodulating common bean. Effective strains of native rhizobia with higher nodulation and symbiotic nitrogen fixation efficiencies can be used to produce inoculant as bio-fertilizer that might be available to smallholder farmers in Angola. The use of native rhizobia inoculants will also reduce the reliance on nitrogen fertilizers.

Therefore I hypothesize that there are indigenous rhizobia species in the soils of Huambo, that are able to establish an effective symbiotic relationship with *Phaseolus vulgaris* and improve common bean yield through the BNF process. Moreover, I hypothesize that the use of two different promiscuous species as trapping plants will be more effective for the isolation of a greater variety of BNF rhizobia. For this purpose, I decided to compare the isolation effectiveness of BNF rhizobia directly from the soil on semi-selected medium to trapping technique based on promiscuous common bean which has a long-lasted history of cultivation in Angola and to trapping technique based on adzuki bean which is not yet cultivated in central Angola.

General objective

Evaluation of the usefulness of different methods of isolation of BNF rhizobia and characterization of the diversity of indigenous rhizobia isolated from different soils in the Huambo province of Angola.

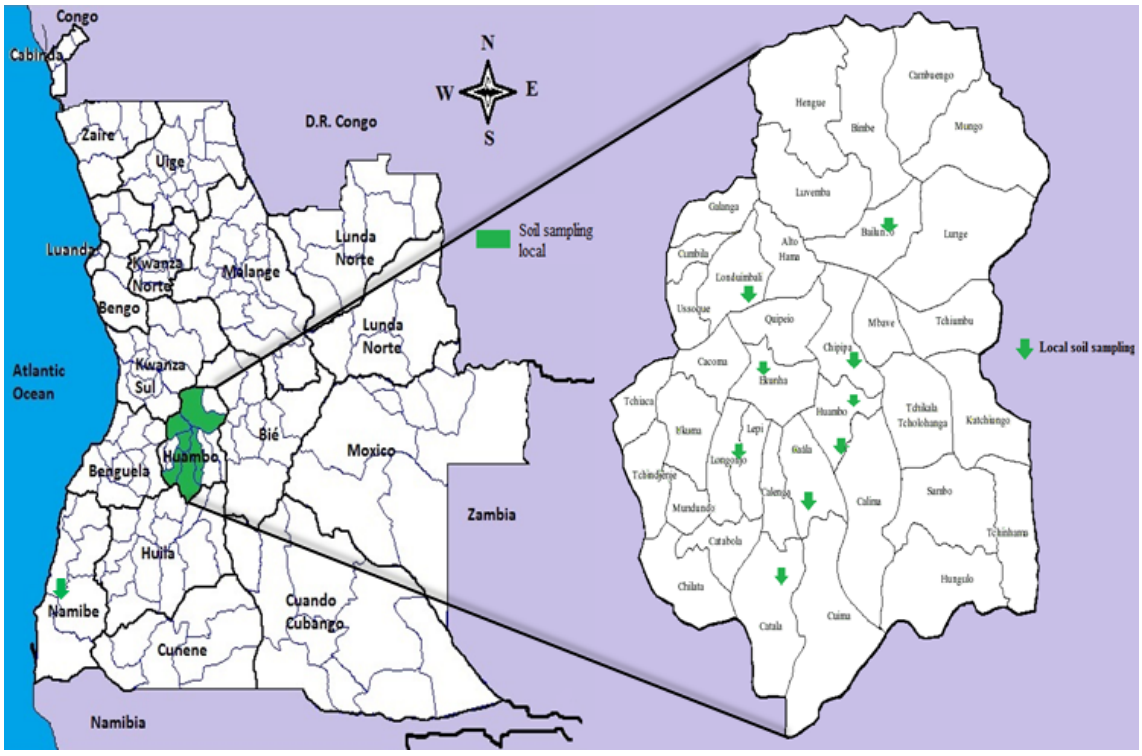
Specific objective

Determination of efficiency of different indigenous rhizobia in nodulating and nitrogen-fixing in symbiosis with the common bean.
Selection of effective indigenous rhizobia suitable for inoculation of common bean.

2. MATERIALS AND METHODS

2.1. Collection the soil samples and descriptions of the sampling sites.

The soil samples used for isolation of indigenous rhizobia were collected from natural forest, fallow, and cultivated fields at different regions of Huambo province, and one soil sample was collected from the desert of Namibe province (Map 1).



Map 1. Geographic distribution of sampling sites of soils in Angola.

The description of sampling sites is summarized in table 1. The soil samples were collected from four points at 15 - 20 cm depth on each site using the soil auger (Photo 1).

To avoid cross-contamination between sampling sites, the auger was washed with alcohol (95%). The soil samples from each site were thoroughly mixed to form an average sample of about 1 kilogram. The pebbles and other visible plant debris were removed and soil was sieved through 2 mm sieve. Finally, a subsample of 300 g of each soil was packed into sterile bags and stored at +4ºC. Each soil sample was then divided for the physical-chemical analyses and for rhizobia isolation research that was done in Wroclaw University of Environmental and Life Science, Poland.

Table 1. Description of tested soil samples.

Place	Geographic Coordinate		Soil Type [a,c]	Texture [a,b,c]	Clay (%)[a]
	Latitude (S)	Longitude (E)			
Arable land					
Bailundo	12°11'12.0"	015°53'13.0"	RWF	FC	12.00
Chipipa	12°34'38.4"	015°44'33.7"	OWF, B	FC	ND
Elande	13°03'58.2"	015°22'24.3"	YWF, B-G	C-S	8.45
Gongoinga	12°51'45.7"	015°43'50.2"	OWF, B-G	FC	ND
Chianga	12°44'21.2"	015°49'35.8"	O	C-S	53.3
Natural forest					
Cabinda	13°07'13.3"	015°19'29.9"	YWF	F-C-S	ND
Chilela	12°33'40.9"	015°24'08.4"	WF, B-G	F-C-Y	ND
Fallow					
Alto Hama	12°20'45.5"	015°37'15.7"	RWF	SL	11.05
Desert					
Namibe	15°40'17.7"	012°05'32.8"	C X-R	Co	1.25

Legend: [a]Missão de Pedologia de Angola, (1961), [b] Beernaert (1997).
Soil type - C X-R: Chromopsammic xero-regosols; OWF, B: Orange weakly ferrallitic, brown; OWF, B-G: Orange weakly ferrallitic, brown grayish; O: Oxisols; RWF: Red weakly ferrallitics; WF, B-G: Weakly ferrallitic, brown-grizzly; YWF: yellow weakly ferrallitic; YWF, B-G: Yellow weakly ferrallitic, brown grizzly.
Texture - C-S: Clay-sandy; Co: Coarse; FC: frank clayey; F-C-S: Frank-clayey-sandy; SL: Sandy loam; ND: not determined.

Photo 1. Collection of the soil samples.

2.2. The physical-chemical analyses of soil samples.

The analysis of the physical-chemical properties of tested soils was done at the laboratory of the Department of Plant Nutrition of Wroclaw University of Environmental and Life Sciences, Poland. The soil pH was determined in 1:2.5 soil:1 M KCl suspensions using a digital pH meter CP 505 (Elemetron, Poland). The overall sulfur content was determined by the Butters-Chenery method (Butters and Chenery, 1959). The content of organic carbon was

determined using the Tiurin method (Tiurin, 1935), the total nitrogen content was determined using the Kjeldahl method (Kjeldahl, 1883; Bremner and Mulvaney, 1982). The content of phosphorus and soluble potassium was analyzed using the DL method (Egner and Riehm, 1955), while the content of the soluble magnesium was determined by Schactschabel method (Schachtschabel, 1954). The content of soluble micronutrients and heavy metals in soil samples, such as manganese, iron, copper, zinc, nickel, cadmium, lead, and chromium were determined according to the recommendation of the Rinkis method (Rinkis, 1972).

2.3. Media and solutions used in the study.

The following media and solutions for isolation, cultivation, and phenotypic characterization were used.

YEMA - yeast extract mannitol agar according to Vincent (1970). The composition of the medium; 0.5 g K_2HPO_4, 0.2 g $MgSO_4x7H_2O$, 0.1 g NaCl, 10.0g Mannitol, 0.5 g $CaCO_3$, 0.5 g Yeast Extract, 15.0 g Bacto Agar, 1000.0 mL distilled water. The pH was adjusted to 6.8 before adding agar and autoclaving at 121ºC for 15 minutes.

YEMA-CR. The YEMA medium supplemented with 10.0 mL of 1% (w/v) filter sterile sterilized solution of Congo red (CR) (Somasegaran and Hoben, 1994).

YEMA-BTB - YEMA medium supplemented with 5.0 mL of 0,5% (w/v) filter sterilized solution of Bromothymol blue (BTB) (Somasegaran and Hoben, 1994).

YEMB - Yeast extract mannitol broth medium (Somasegaran and Hoben, 1994). The composition of the medium; 10.0 g Mannitol, 0.5 g K_2HPO_4, 0.2 g $MgSO_4.7H_2O$, 0.1 g NaCl, 0.5 g Yeast extract, 1000.0 mL distilled water. The pH was adjusted to 6.8 before adding agar and autoclaving.

S1 medium (Gould *et al.,* 1985) a selective medium for fluorescent pseudomonas. The composition of the medium; 10.0 g saccharose, 10.0 mL glycerol, 5.0 g casamino acid, 1.0 g $MgSO_4x7H_2O$, 1.0 g $NaHCO_3$, 2.3 g K_2HPO_4, 1.2 g sodium lauroyl sarcosine (SLS), 16.0 g Difco Bacto Agar, 1000.0 mL distilled water. The pH was adjusted to 7.2 before adding agar and autoclaving. After the autoclaving filter-sterilized solution of 20.0 mg trimethoprim in 5 mL of ethanol was added.

D1 medium according to Kado and Heskett (1970) for agrobacterium determination. The composition of the medium; 15.0 g mannitol, 5. 0 g $NaNO_3$, 6.0 g LiCl, 20.0 mg $Ca(NO_3)_2.4H_2O$, 2.0 g K_2HPO_4, 0.2 g $MgSO_4x7H_2O$, 0.1 Bromothymol blue (BTB), 15.0 g

Difco Bacto Agar, distilled water 1000 mL. The pH was adjusted to 7.2 before adding agar and autoclaving.

YMAA - yeast mannitol antibiotic agar a semi-selective medium for rhizobia (Graham, 1969). The composition of the medium; 1000.0 mL of distilled water, 5.0 g mannitol, 5.0 g lactose, 0.5 g K_2HPO_4, 0.2 g NaCl, 0.2 g $CaCl_2.2H_2O$, 0.1 g $MgSO_4.7H_2O$, 0.1 g $FeCl_3.6H_2O$, 0.5 yeast extract, 20.0 g Difco Bacto Agar, distilled water 1000 mL. The pH was adjusted to 7.0 before adding agar and autoclaving. After the autoclaving solutions of 200.0 mg cycloheximide, 100.0 mg pentachloronitrobenzene, 25 mg sodium benzylpenicillin, 10.0 mg chloromycetin, 25 mg sulfathiazole, 2.5 mg neomycin, and 2.5 mL of 1% solution of Congo red were added.

YEMA-slants. Yeast extract mannitol agar slants for storage of isolated rhizobia. The composition of the medium; 0.66 g K_2HPO_4, 0.34 g KH_2PO_4, 0.20 g $MgSO_4$, 0.10 g NaCl, 0.10 g $CaCl_2$, 0.4 g yeast extract, 5.0 g $CaCO_3$, 20.0 mg $FeSO_4$, 20.0 mg $MnSO_4$, 10.0 g mannitol, 12.0 g agar, 1000.0 mL distilled water. The pH was adjusted to 6.9 – 7.1 before adding agar and autoclaving.

TY - Tryptone yeast medium (Somasegaran and Hoben, 1994). The composition of the medium; 5.0 g Tryptone, 0.3 Yeast extract, 0.87 g $CaCl_2$, 1000 mL distilled water. The pH was adjusted to pH 6.8 before autoclaving.

Nitrogen-free nutrient solution (Broughton and Dilworth, 1971). The composition of the medium: 0.1g $CaCl_2$, 0.12 g $MgSO_4.7H_2O$, 0.1 g KH_2PO_4, 0.15 g $Na_2HPO_4.2H_2O$, 0.005 g ferric citrate, 0.1 mL trace element, 1000 mL distilled water.

Trace elements solution (Broughton and Dilworth, 1971; Kawaka *et al.,* 2014). The composition of the solution; 2.86 g H_3BO_3, 2.03 g $MnSO_4.7H_2O$, 0.22 g $ZnSO_4.7H_2O$, 0.08 g $CuSO_4.5H_2O$, 0.14 g $NaMoO_2.2H_2O$, 1000 mL distilled water. The pH was adjusted to pH 6.8 before autoclaving.

PBS - Phosphate buffered saline (Wall, 2003). The composition of the buffer; 1.2 g Na_2HPO_4, 0.18 g NaH_2PO_4, and 8.5 g NaCl, 1000 mL distilled water. The pH was adjusted to pH 7.6 before autoclaving.

Salkowski. The composition of Salkowski reagent: 20 mL H_2O, 43.9 mL H_2SO_4 96%, and after cooling 2 g $FeCL_3$ x $6H_2O$ supplement with H_2O to 100 mL (Gordon and Weber, 1951).

Saline solution. The solution of 9.0 g NaCl in 1000 mL distilled water.

Deep frozen solution. The solution of 10 g of skim milk (Difco Ltd.) in 100 mL distilled

water. This solution was also used for the lyophilization of selected isolates for long storage. All media and solutions were sterilized by autoclaving for 15 min at 121 ºC unless otherwise indicated. All chemicals used in this study were provided by Sigma-Aldrich Sp. z o.o. (Poznań, Poland) unless otherwise indicated.

2.4. Cultivars of common bean (*Phaseolus vulgaris* L.) used in the study.

The following cultivars of common bean were used in this study;

- "CATIOLO". An Angolan early bush cultivar with small white seeds (~250 g per 1000 seeds) grown for dry grain (UAN, 1987),
- "MANTEIGA". An Angolan early bush cultivar with medium-sized cream seeds (~400 g per 1000 seeds) grown for dry grain (UAN, 1987),
- "SONDEYOMBUA". An Angolan early bush cultivar with meddle and red seed (~350 g per 1000 seeds) grown for dry grain (UAN, 1987),
- "IGOŁOMSKA". A polish very early dwarf cultivar with medium-sized white seeds (~420 g per 1000 seeds) grown for dry grain.
- "BASTA". An early dwarf snap bean cultivar with small seeds (~160 g per 1000 seeds) and yellow pods usually grown for fresh pod consumption (Łabuda, 2012).

Additionally, for comparison the adzuki bean (*Vigna angularis* (Willd.) Ohwi & H. Ohashi), a species which is not cultivated in Angola, a bush cultivar with very small red seeds (~50 g per 1000 seeds) grown for dry grain was used as a trap plant. This species will be named in the text as "ADZUKI". The adzuki bean is a typical tropical legume and it is also known as a promiscuous host (Kimura *et al.,* 2004).

2.5. Isolation of rhizobia.

The indigenous rhizobia were isolated from collected soil samples by plate method as well as by trapping method using two promiscuous species, common bean, and adzuki bean. The semi-selective medium YMAA was used for enumeration and isolation of putative rhizobia directly from tested soil samples. For this purpose, 10 grams of tested soils were suspended in 90 mL of sterile saline solution and 1 mL of soil suspensions was streaked directly onto Petri plates containing YMAA. The inoculated plates were incubated at 28ºC for 3 – 10 days in the dark (Graham, 1969; Somasegaran and Hoben, 1994). Bacterial colonies characteristic for rhizobia appearance were enumerated and selected for purifications and further analysis.

The BASTA cultivar of common bean and ADZUKI cultivar of adzuki bean as trapping plants were used according to the methodology recommended by Somasegaran and Hoben (1994). Seeds were previously surface sterilized using ethanol 90% for 5 min followed by 2.5% of sodium hypochlorite solution for 3 min and rinsed several times with sterile distilled water. After seeds were put aseptically on sterilized wet filter paper in Petri dishes for 48h in dark to allow the pre-germination of the seeds (Cárdenas *et al.,* 1995; Sánchez-López *et al.,* 2011). Four seedlings were transferred aseptically into experimental plastic pots containing 450 grams of silica sand previously washed and autoclaved (15 min at 121 ºC). Each seedling in the pot was inoculated with 1 mL of a suspension of tested soil prepared by shaking for 5 min. 10 g of soil in 90 mL of sterile saline solution. After inoculation, 30 mL of the sterile nitrogen-free nutrient solution was added per pot. The pot experiment was laid out in a completely randomized design with soil types in four replicates for each soil as well as one treatment without soil inoculation as the control. The plants were grown in a phytotron set up with 24.5ºC temperature and 12/12 hour days/night. Pots were regularly watered alternatively with 30 mL of nitrogen-free nutrient solution and sterile distilled water as required. Forty-five days after emergence, plants were harvested, washed up very carefully the root with tap water. The nodules that showed pink-red coloration were selected and removed from the roots as recommended by Somasegaran and Hoben (1994). The pink-red color is an indicator of the presence of active nitrogen-fixing bacteria (Ceccatto *et al.,* 1998). The nodules were washed in sterile distilled water and surface sterilized with 90% ethanol for 3 min, followed by immersed in 2.5% sodium hypochlorite solution for 3 min and rinsed in seven changes sterile distilled water (Somasegaran and Hoben, 1994). Under aseptic conditions, the sterile nodules were crushed, in a sterile Petri dish containing a drop of 0.9% of the saline solution, and a loopful of resulting crushed nodule suspension was streaked on the surface of the petri dish containing yeast extract mannitol agar (YEMA). The plates were incubated in the dark at 28ºC for 3 – 10 days (Vincent, 1970; Somasegaran and Hoben, 1994; Muthini *et al*., 2014).

2.6. Selection of putative rhizobia.

The selection of putative rhizobia from isolated bacteria from soil and nodules was carried out according to the procedure described by Odee *et al.* (1997). The visible single colonies were subcultured by streaking on solid YEMA-CR, on the S1 medium (Gould *et al.,* 1985), and on the D1 medium (Kado and Heskett, 1970). Selected strains were stained using standard

Gram's procedure (Somasegaran and Hoben, 1994). The isolates that showed typical rhizobia colonies on the YEMA-CR medium (photo 2), which were Gram-negative and did not grow on S1 and on the D1 medium were considered as putative rhizobia (Tekele, 2015). The selected putative pure isolates were labeled according to the local sampling place for further analyzes. Isolates were preserved on YEMA slants and stored at + 4°C. The viability of preserved isolates was checked by inoculation in the YEMB medium.

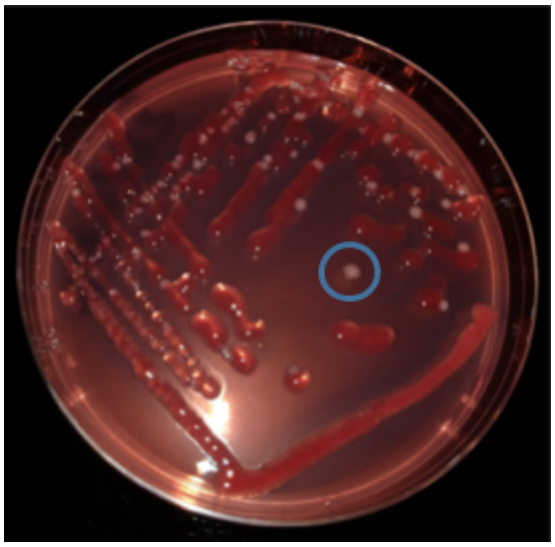


Photo 2. The typical characteristics of rhizobia colonies on the YEMA-CR medium.

2.7. Phenotypic characterization of isolated bacteria.

The colony morphology of tested isolates was examined on YEMA after incubation at 28ºC for 7 days (Sinclair and Eaglesham, 1984; Somasegaran and Hoben, 1994). The following parameters were described:

- The colony color (watery translucent, white, white translucent, yellow, and milky);
- The colony shape (conical, dome, raised, and flattened);
- The colony appearance (dry and wet);
- The colony size (small less than 3 mm diameter, medium 3-5 mm diameter, and large more than 5 mm diameter).

Moreover, tested isolates were cultivated on the YEMA-BTB medium at 28ºC for 7 days. The time of appearance of the colonies as well as their abilities to change the pH of the medium was checked daily. Based on this study isolates were described as:

- Fast growers and acid producers (colony appear on 1 to 3 days and change the growth medium from green to yellow);
- Slow growers and alkaline producers (colonies appear for 4 days or more and change the growth medium from green to blue) (Vincent, 1970).

2.8. Primary screening of isolated bacteria.

The pot experiment was set up as primary screening of the ability or no of the putative pure isolates to nodulate common bean cv. Basta. For this purpose, there were selected 158 isolates, based on the phenotypic characteristic that showed typical rhizobia colonies, according to the procedure described above in section 2.6. The experimental plastic pots contain 450 grams of autoclaved quartz. Seeds before inoculation were previously surface sterilized using the procedure described above in section 2.5. The pot experiment with inoculated seeds was done in duplicates and the results annotated as yes, whether the isolates induce the active nodule formation or no, if the isolates did not induce nodulation. The condition of growth is described above in section 2.5. Additionally, the shoot dry mass was determined. On each shelf in the phytotron, the control pot was randomly added and the standard deviation of SDM was calculated.

2.9. Genetic characterization of isolated strains.

Bacterial cultures for total genomic DNA extraction were grown in YEMA and a single colony of each bacterial strain was collected by using a plastic disposable tip. The extraction of the DNA of the ninety-three pure isolated strains was carried out by following the procedure of the GenElute™ Bacterial Genomic DNA Kit according to the manufacturer's instructions (SIGMA-ALDRICH 2012). DNA concentration was determined using the BioPhotometer Eppendorf. The list of primers used in the study is presented in Table 2.

Table 2. The primers used for the determination of the presence of specific genes using PCR methods and sequencing.

Gene	Name	Sequence 5' - 3'	Reference
*Nif*H [A]	IGK3	GCIWTHTAYGGIAARGGIGGIATHGGIAA	Gaby and Buckley, 2012
	DVV	ATIGCRAAICCICCRCAIACIACRTC	
NodC [B]	NodCfor540	TGATYGAYATGGARTAYTGGCT	Sarita *et al.*, 2005
	NodCrev	CGYGACARCCARTCGCTRTTG	
16S rRNA [A]	27F	AGAGTTTGATCMTGGCTCAG	Eden *et al.,* 1991
	1492R	TICAGCATTGTICCATIGG	Eden *et al.,* 1991

Abbreviations: Y = C or T, R = A or G, M = A or C.
[A] - Primers were provided by Genomed SA (Warsaw, Poland)
[B] - Primers were provided by Syngen Biotech Sp. z o. o. (Wrocław, Poland).

The *nodC* gene (620 bp) was amplified using primers NodCfor540 and NodCrev described above (Table 2). The conditions of the *nodC* PCR were optimized with a total volume of 20 µL, containing 4.0 µL of PCR master mix Blend Master Mix RTL (Solis Blodyne), 3.0 µL of fresh DNA template, and 2.0 µL of primers, 2.0 µL of dimethyl sulphoxide (DMSO) and 9.0

µL of ultra-pure PCR grade water. PCR thermal cycler adjusted to the following program: initial denaturation for 15 min at 95ºC, 35 cycles of denaturation for 1 min at 95ºC, annealing for 30 sec. at 50ºC, the extension for 1 min. at 72ºC, and final extension for 10 min at 72ºC. 10 µL of the PCR products were loaded onto horizontal gel electrophoresis and 10 µL of Marker DNA M50-3000 also was loaded onto each gel as a molecular weight marker. The agarose gel was prepared with: 2.25 g of agarose, 135.0 mL of ultra-pure water, 15.0 mL of buffer, and stained with 7.5 µL of ethidium bromide. The gel electrophoresis was run at 97V for approximately 150 minutes then the DNA bands were visualized under UV trans-illumination and photographed (Sarita *et al.,* 2005). The *nodC* gene was chosen considering that it is a common nod gene essential for nodulation in all rhizobial species investigated so far and it is also a determinant of host range (Perret *et al.,* 2000; Laguerre *et al.,* 2001).

The amplification of *nifH* using the primers IGK3 and reverse DVV (400 bp) described above (Table 2) and PCR thermal cycler was adjusted to the following program: initial denaturation for 15 min. at 95ºC, 35 cycles of denaturation for 1 min. at 94ºC, annealing for 1 min. at 58ºC, the extension for 1 min. at 72ºC, and the final extension for 10 min. at 72ºC. To visualize, 10 µL of the PCR products were loaded onto horizontal gel electrophoresis and 10 µL of Marker DNA M50-3000 provided by Blirt S.A. (2013) also was loaded onto each gel as a molecular weight marker. The gel was prepared with: 2.25 g of agarose, 135.0 mL of ultra-pure water, 15.0 mL of buffer, and stained with 7.5 µL of ethidium bromide. The gel electrophoresis was run at 97 V for approximately 150 minutes then the bands were visualized under UV trans-illumination and photographed (Gaby and Buckley, 2012).

2.10. Authentication of isolated putative rhizobia.

The authentication of isolated putative rhizobia was done using the strains that were selected at the primary screening. Isolates that stimulate the growth of the common bean cv. Basta on the pot experiment, at the primary screening, but did not induce the nodule formation were tested for the ability of indole-3-acetic acid (IAA) production. For this objective, different concentrations of tryptophan were used to evaluate the production of IAA of the seventeen isolated strains in triplicate. Strains were grown in 50 mL TY-broth medium in 100 mL Erlenmeyer flasks supplemented with the filter-sterilized solution of L-tryptophan was added to final concentration 10.0 μg mL^{-1}. The flasks were inoculated with 100 μL of bacterial cell suspension adjusted to an optical density of 0.45 measured at 540 nm by spectrophotometer and incubated at 20ºC for 72 h on a rotary shaker (160 rpm). After incubation, cells were removed from stationary phase cultures by centrifugation at for 5 min, and auxin was detected in 1 mL of supernatant using Salkowski reagent as described by Ali and Hasnain (2007). Color development was first visible at the highest IAA concentration within minutes and intensity continued to increase for a period of 30 min. Hence optical density was measured spectrophotometrically at a wavelength of 530 nm.

The experiment for authentication was carried out using 19 isolated bacteria (HA2a, HA8, HBA11, HBA15, HCA10, HC4, HCC321, HEC1, HLD1, HLE11, HLE22, HKK321, HLE131, HLo8, HNG33, HNG13, HSL1, HSL13, and HSL13a) out of thirty-seven strains that gave positive *nifH* and *nodC* band, along with one reference strain (F17) from the Institute of Soil Science and Plant Cultivation (PIB-IUNG) at Puławy, Poland. Following aseptic laboratory procedure to analyze their ability to form nodules on common bean and determination of their effectiveness, all strains were grown in YEMB for five days on a rotary shaker (160 rpm, at 26ºC) and cell density in the cultures was estimated by measuring optical density (OD) using a spectrophotometer at 540 nm. The common bean cultivar "BASTA" was used. The seeds sterilization, pre-germination, and plantation were carried out as described above (section 2.3.3). The seedlings were inoculated on time of planting with 1 mL YEMB culture approximately (10^{-8} to 10^{-9} cells mL^{-1}) of each isolate (Boakye *et al.,* 2016). The pot experiment was carried out following the standard procedures described by Somasegaran and Hoben (1994) on a completely randomized design with 20 treatments along with 1 nitrogen supplied treatment as positive control and one treatment without bacteria inoculation and without mineral nitrogen fertilizer as the negative control. The mineral nitrogen treatment was

supplied with 50 mL of sterile KNO_3 solution at ten, twenty, and thirty days after the emergency (Argaw, 2012). Each treatment was done with four replications, in total 88 experimental units. The plants were grown in a phytotron set up with 27ºC temperature and 12/12 hour days/night, for forty days regularly watering alternatively with 30 mL of nitrogen-free nutrient solution and sterile distilled water as required until the harvest.

Plant harvesting was carried out 40 days after emergence. The appearance of the plants, the color of the leaves, and the nodulation status of roots as well as the internal color of nodules was checked. The greenness of plants was qualitatively scored by comparing the plant color with the color of the uninoculated plants, as the negative control (without inoculation and without mineral nitrogen), and the positive controls with nitrogen fertilization. The shoots were then separated from the roots by cutting, the roots and washed using tap water and root nodules were separated and counted for each plant. The nodulated and non-nodulated plants were recorded. Nodule number per plant was scored as absent nodules (zero nodules), rare (<5 nodules), few (5 - 10 nodules), moderate (11 - 20 nodules), abundant (21 - 50 nodules) and super nodulated (> 50), while internal color was analyzed by cutting the nodule and scored as white, green, pink or red (Bala *et al.,* 2011). The nodules number (NN), the number of active nodules (NAN), the nodules dry mass (NDM), the shoot dry mass (SDM), root dry mass (RDM), and shoot dry mass, root dry mass ratio was recorded. Nodule, shoot, and root dry weights were recorded after drying for 24 hours at +70°C. Green plants and the presence of a single active nodule (pink nodule) on the roots was an indication and confirmation that the isolated bacteria are effective nitrogen fixation bacteria in the common bean. Ineffective nitrogen fixation bacteria were considered when plants looked yellowish and without or had only inactive (white) nodules (Aserse *et al.,* 2012ab; Morad, 2013).

The relative effectiveness (RE) of isolates was determined using the following equation described by Gibson (1987).

$$RE = (Xi / Xr)\, x\, 100$$

Where, Xi is the mean of the shoot dry mass of the inoculated plants and Xr is the mean of the shoot dry mass of the reference strain (F17). For data analysis, shoot dry mass (SDM) of inoculated plants were expressed as a percentage of the mean weight of the SDM of reference strain and the resulting data compared across isolated rhizobia treatment. The putative rhizobia that induced weights ≥ 80%, 79% - 50%, 49 - 20%, and < 20% of the reference were classified as effective, partially effective, poorly effective, and ineffective respectively

(Fening, 1999; Mwenda, 2017). The percentage of symbiotic efficiency was calculated by dividing the shoot dry mass of inoculated plants by shoot dry mass of plants supplemented with nitrogen as described by (Gibson, 1987; Mwenda *et al.*, 2011).

$$SE = (Xi / Xn)\, x\, 100$$

Where, *Xi* is the mean of the shoot dry mass of the inoculated plants and *Xn* is the mean of the shoot dry mass of the plants supplemented with nitrogen. Moreover, the total Nitrogen content in harvested shoots from pot experiments was determined using the Kjeldahl procedure that involves digestion and distillation (Kjeldahl, 1883; Bremner and Mulvaney, 1982).

2.11. The evaluation of the symbiotic interaction with four common bean cultivars.

The experiment was performed to evaluate the capability of the selected potentially effective isolates with high and moderate symbiotic efficiency (HA2a, HA8, HC4, HKK321, HLD1, HLE22, HLE131, HLo8, and HSL1) to nodulate four common bean cultivars. Two common bean cultivars from Angola ("CATIOLO" and "MANTEIGA") and two cultivars ("IGOŁOMSKA" and "BASTA") from Poland. The experiment was laid out randomized complete block design (RCBD) with 36 treatments (nine strains x four cultivars) and replicated four times; all the procedure was done as described above (section 2.10). Plant harvesting was carried out 40 days after emergence. The appearance of the plants, the color of the leaves, and the nodulation status of roots as well as the internal color of nodules was checked. The greenness of plants was qualitatively scored as well as the nodules number (NN), the nodules dry mass (NDM), the shoot dry mass (SDM), the root dry mass (RDM), and shoot dry mass, root dry mass ratio was determined as was described above (section 2.8.).

2.12. Sequencing of the 16S rRNA gene of the isolated bacteria.

The bacteria isolates HA2a, HA8, HA2b, HA8b, HBA11, HBA15, HBA15a, HC4, HCA10, HCC32, HCC31, HCC321, HCK23, HEC1, HCP8, HKK213, HKK213a, HKK321, HLD1, HLD211, HLD311, HLE11, HLE131, HLE22, HLE111a, HLo2, HLo8, HLo11, HLo23, HNG13, HNG33, HNG33a, HNG331, HNG332, HSL1, HSL13, and HSL13a selected for sequence analysis of the partial 16S rRNA gene. The total DNA was isolated using GenElute™ Bacterial Genomic DNA Kit following the manufacturer's instructions (SIGMA-ALDRICH 2012). The sequencing of the 16S rRNA gene of bacterial isolates was conducted with primers 27F and 1492R in a commercial laboratory (Genomed S.A., Warsaw, Poland). Partial DNA sequences were aligned using the Clustal V method of the DNAStar

software package (DNAStar Lasergene, Inc., Madison, USA). A BLASTn search of the National Center for Biotechnology Information Database was used to compare the gene sequences of the isolates tested with those available online.

2.13. Field experiment.

The field experiment was carried out on the Experimental field at Gongoinga of University José Eduardo dos Santos from August to December 2019. The Experimental field at Gongoinga is located at latitudes (S) 12°51'45.7" and longitudes (E) 015°43'50.2". The soil is Orange weakly ferrallitic brown grayish (Missão de Pedologia de Angola, 1961; Beernaert, 1997). The experimental plot was on fallow for 3 years, to allow the good sowing was thoroughly plowed and leveled mechanically using the tractor, plow and harrow, after which the layout was done and sowing manually using the hoe. The weather conditions during the experiment are present in Table 3. Soil samples for physic-chemical determination were taken from field experiments as described above (section 2.1 and 2.2) and analyzed at the Department of Plant Nutrition of the Wroclaw University of Environmental and Life Sciences.

The plate count technique was used to determine the presence of native and viable rhizobia cells in the soil at the Experimental field at Gongoinga and cultures were grown at the YEMA-CR medium. For this purpose, soil samples were taken, as was described above (section 2.1) before planting from each plot and were analyzed in the laboratory of the Faculty of Agrarian Science of the University José Eduardo dos Santos.

The inoculant was prepared with sterile peat as a carrier of bacteria in the Laboratory of Plant Protection of the Wroclaw University of Environmental and Live Science in Poland. Peat-based inoculants were prepared with strains that showed the highest effectiveness and symbiotic efficiency at pots experiment. The following rhizobia strains were used for field study; HA2a ($112x10^{-10}$ UFC g^{-1}) isolated from a nodule of adzuki bean, HLo8 ($8.7x10^{-10}$ UFC g^{-1}) isolated directly from soil of the arable land at Bailundo, HCC321 ($21x10^{-10}$ UFC g^{-1}), and HBA15a ($96x10^{-10}$ UFC g^{-1}) isolated from a nodule of common bean. The inoculant was added to the plastic bag containing the seeds and swirled gently until seeds were uniformly covered with inoculant and allowed to air dry for 30 min to enable the inoculant to stick well enough onto the surface of the seeds before planting. The dose of the inoculant was done at the recommended rate (10 g per kilogram of seed) (Abdula, 2013). The uninoculated seeds

were sown before the inoculated ones to avoid contamination. All was done early in the morning to avoid its exposure to direct sun rays that might affect the quality of the inoculant as the recommendation of Abdula (2013). The certified seeds of common bean cultivar "Sondeyombua" were used for the field experiment and were supplied by the Institute of Agrarian Research (IIA) in Chianga Huambo.

The fields were laid out using a randomized complete block design (RCBD) replicated three times. Each main plot had nine subplots with 6 m^2 (3 x 2 m) as experimental units, the inter-row spacing of 50 cm and intra-row plant spacing of 7 cm, and 3 seeds per hole sowing at a depth of 3 - 5 cm. Each subplot consisted of 4 rows and the outer two rows were considered as border rows as well as each plot was separated by a distance of 1 m and 1.5 m between the blocks to enable easy management and data collection. All treatments were done with three repetitions as subplots, in total 45 subplots, according to scheme 1 presented below, and were completely randomly set-up on the field as it is shown on Scheme 2.

Scheme 1. The scheme of the field experiment.

Fertilization	Seed treatment				
	Control	HA2a	HLo8	HBA15a	HCC321
None	3 rep.	3 rep.	3 rep.	3 rep.	3 rep.
NPK (N 12, P 24, K 12 kg ha^{-1})	3 rep.	3 rep.	3 rep.	3 rep.	3 rep.
NPK (N 12, P 24, K 12 kg ha^{-1}) + Urea (N 21.8 kg ha^{-1})	3 rep.	3 rep.	3 rep.	3 rep.	3 rep.

The mineral fertilizer NPK (12:24:12) at a rate of 100 kg ha^{-1} was applied before sowing as the starter fertilizer for a common bean as was suggested by Daba and Haile (2002). This fertilizer is commonly used by Angolan farmers. After 20 days urea at the rate 21.8 N kg ha^{-1} was added on some plots as listed above.

Initially, the field was irrigated twice per week to keep soil moisture content close to field capacity, due to the fact that the experiment started at the beginning of the rainy season there was no precipitation yet (Table 3). Weeding control was done twice manually using hoe in the third and sixth week after germination to ensure that the plots were kept weed-free throughout the growing season. Each weeding operation was completed on the same day for all plots. The management practices were applied equally to all plots and the crops were grown for 110 days.

Scheme 2. The layout of the field experiment.

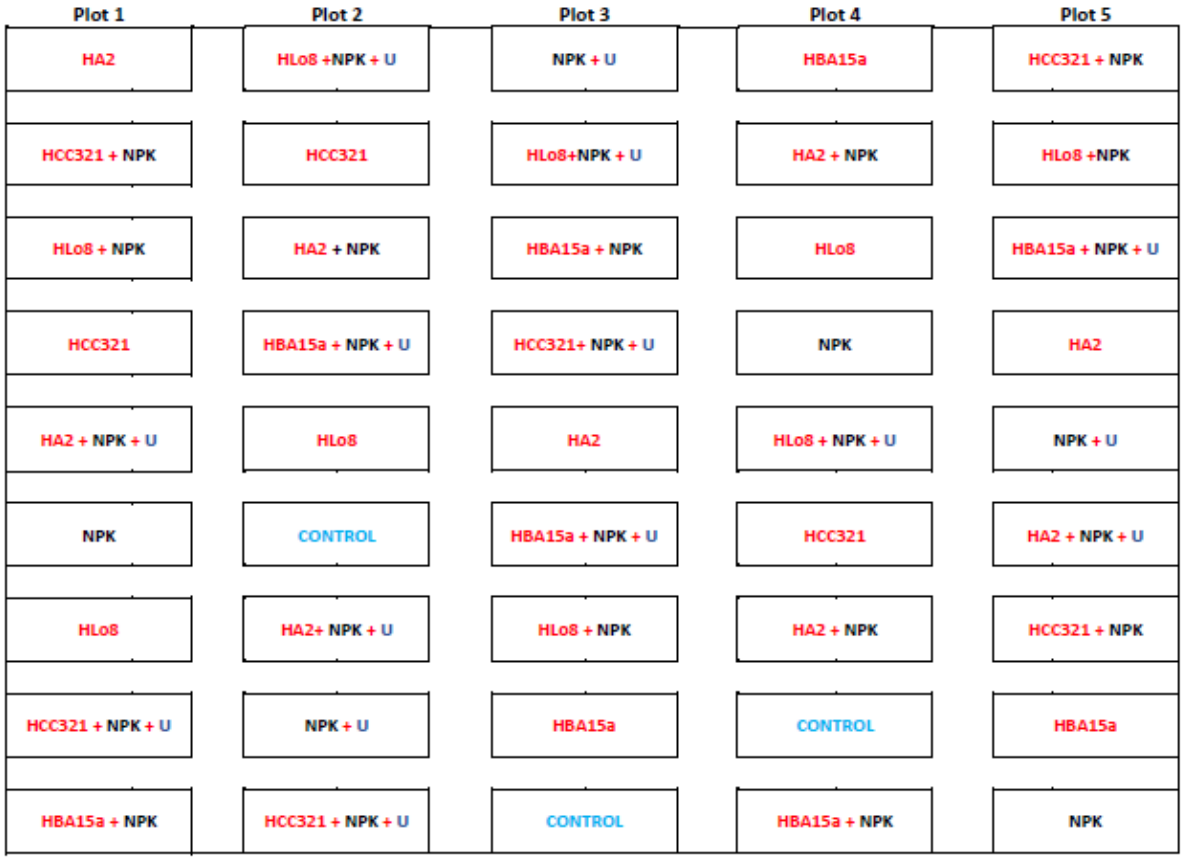


Table 3. Weather conditions during the field experiment.

	Air temperature (ºC)		Precipitation (mm)		Humidity (%)	
Month	2014 - 2018	2019	2014 - 2018	2019	2014 - 2018	2019
August	19.58	19.1	0.36	0	41.58	41.5
September	21.46	21.6	51.36	2.2	53.24	48
October	21.22	21.3	80.76	78.2	70.38	72.7
November	19.8	20.1	192.9	120.6	83	83
December	19.48	19.8	179.8	108.6	86.67	82.5

Source: Data from sasscalweathernet.org at the Experimental Field Gongoinga of the University José Eduardo dos Santos.

2.14. Observations and data collection.

The appearance of plants was evaluated and qualitatively scored by comparing the color (greenness) as well as the vigorousness of the treated plants and control (without inoculation and without mineral nitrogen). The nodulation determination was conducted 60 days after germination. Nine (9) plants were selected from the two inner rows of each repetition and

gently uprooted. The root was washed with tap water to remove the adhering soil. The number of nodules (NN) per plant was counted and the values averaged to give the number of nodules per plant and scoring according to a protocol developed by Bala *et al*. (2010) where plants with nodule number of 0, <5, 5-10, 11-20, 21-50, and >50 were given the categorical values 0, 1, 2, 3, 4 and 5 respectively (absent nodule, rare nodules, few nodules, moderate nodules, abundant nodules, and super nodulated root). In addition, the nodules were severed from the roots, oven-dried at 70°C for 48 hours, and their dry mass was recorded. At the harvest time, the shoots were separated from the root, and SDM and RDM were determined as was described above (section 2.9.).

At harvest time, parameters of yields from each plot such as number of pods per plant (NPP), number of grain per plant (NG), the mass of thousand seeds (MTS), the number of seeds per pod (NSP), and the grain yield (GY) were recorded. Nine plants were randomly selected from the interior rows, counted the number of pods per plant, and left to sun-dry for three days and then threshed separately for each plot after the seeds were separated from debris or husks. The grain yield per square meter was determined by threshing the harvested plants from the harvested area square meter central of each plot.

2.15. Data analyses.

The data generated were analyzed using software R i386 (3.6.3 version for Windows). The data were subjected to analysis of variance (ANOVA) and means were compared using Tukey's HSD test at $p = 0.05$. The correlation analysis was used to determine the relationship and magnitude of the association between the studied parameters.

3. RESULTS

3.1. Physical-chemical characteristics of tested soils.

For the purpose of the study of the occurrence of rhizobia nodulating common bean in arable and natural soils samples were collected at different locations of Huambo and Namibe provinces of Angola. Samples of soils were collected from five arable fields (Bailundo, Chianga, Chipipa, Elande, and Gongoinga), four from the natural forest soils (Cabinda, and Chilela), one from the fallow soil (Alto Hama), and one from desert soil (Namibe). The physicochemical characteristics of tested soil samples are presented in tables 4 and 5. The measurement of pH of the studied soils indicated that, except for the alkaline desert soil from Namibie, all other soils were acidic. The soil samples from the majority of arable fields and natural forests were strongly acidic, pH < 4.5. While the soil from fallow at Alto Hama with dominant growth of Welw plant (*Brachystegia tamarindoides* Benth.) was moderately acidic. The evaluation of physicochemical parameters of tested soils was done based on laboratory methods of soil and plant analysis manual of Okalebo *et al.* (2002) and the agronomic soil evaluation was done according to African soil categorization. The total nitrogen content as well as of organic carbon content was very low in the majority of studied arable and forest soils and varied from 0.031% to 0.073% of N and from 0.044% to 0.098% of C. A moderate level of total nitrogen content (0.114% to 0.141% N) and a moderate level of organic carbon (2.01% to 2.44% of C) were found in soil samples from the arable fields at Chianga and Elande and in soil from the forest at Cabinda. While desert soil showed the lowest level of the organic carbon but nitrogen content was in the range of most other soils. The highest soil C/N ratios above 17 were found in arable soil at Elande and in forest soil at Cabinda. Except for the desert soil, all other soils exhibited a C/N ratio above 11.

The content of available phosphorus was very low in all soils, except in soil from forest soil at Mande was found at medium level. The content of available potassium in the soils from most tested sites were low or very low. Only, the content of available potassium was very high in soil from the forest at Gandavila (530 mg kg^{-1}) and in soil from the arable field at Chianga (303 mg kg^{-1}) and was medium is soil at Gongoinga (151 mg kg^{-1}).

Table 4. The physical-chemical properties of tested soils.

Soils from	pH_{KCl}	Total N	Organic S	N/S	Organic carbon	C/N	Available P	Available K	Available Mg
		(g kg^{-1} soil)			(g kg^{-1} soil)		(mg kg^{-1} soil)		
Arable land									
Bailundo	4.30	0.52	0.28	2.00	7.26	13.90	9	32	33
Chipipa	4.40	0.58	0.24	2.50	8.91	15.30	9	72	47
Elande	4.30	1.14	0.13	8.90	20.10	17.70	10	50	15
Gongoinga	4.40	0.63	0.20	3.10	9.81	15.60	9	151	55
Chianga	5.20	1.41	0.63	2.30	20.20	14.30	15	303	120
Natural forest									
Cabinda	4.20	1.41	0.13	10.60	24.40	17.30	10	46	71
Chilela	4.50	0.73	0.13	5.80	8.27	11.40	10	10	50
Fallow									
Alto Hama	5.80	0.31	0.11	2.80	4.44	14.00	10	48	28
Desert									
Namibe	8.00	0.73	1.25	0.10	1.59	9.80	10	37	308

Table 5. The content of micronutrients and heavy metals in tested soils.

Soils from	Micronutrients				Heavy metals			
	Mn	Fe	Cu	Zn	Ni	Cd	Pb	Cr
	mg kg^{-1} soil							
Arable land								
Bailundo	50.30	307.0	3.65	3.14	0.58	0.020	2.27	0.75
Chipipa	39.20	320.0	2.47	3.60	0.91	0.025	3.26	0.95
Elande	7.43	889.0	2.03	4.04	0.75	0.040	3.61	0.40
Gongoinga	70.70	308.0	4.22	3.91	2.46	0.030	2.97	3.35
Chianga	292.00	860.0	4.07	9.04	1.09	0.075	6.69	0.75
Natural forest								
Cabinda	96.20	527.0	2.97	3.70	1.55	0.040	2.33	0.55
Chilela	51.80	195.0	1.87	3.62	0.78	0.040	5.10	0.85
Fallow								
Alto Hama	131.00	166.0	2.90	14.00	1.19	0.025	2.23	1.80
Desert								
Namibe	68.40	546.0	3.44	5.41	5.28	0.665	7.50	4.15

The content of available magnesium was found to be very high in the desert soil (308 mg kg^{-1}) and was at medium level in soil from the arable field at Chianga with (120 mg kg^{-1}). The remaining region showed low and very low content of plant available magnesium. The content of available manganese in most of the tested soils was low. The very low content of Mn ions was found in soil from arable land at Elande (7.43 mg kg^{-1}). The very high content of manganese ions was determined in soil from the arable field at Chianga (292 mg kg^{-1}) and from fallow soil at Alto Hama (131 mg kg^{-1}), only. The highest values of iron ions content

were both from the soil of arable land of Elande with 889 mg kg^{-1} soil and Chianga with 860 mg kg^{-1}, while the lowest was from fallow with 166 mg kg^{-1}. The content of copper and zinc ions were very low and noticeable differences among regions were not observed. Only in the soil from fallow at Alto Hama the zinc ions content was noticeably higher in comparison with other locations. The content of nickel, lead, and chromium ions were found at a very low level at all tested soils except the soil from the desert that showed moderate nickel and lead contents, while the high level of chromium was found. The content of plant-available chromium strongly correlated with the soluble Ni content (r = 0.820). The high level of soluble chromium can be toxic for plants and microbes. Tested soils were characterized with a noticeable correlation of acidity with Mn ions (r = 0.646) and Zn ions (r = 0.953). Moreover, the total content of nitrogen correlated strongly with soil organic matter (r = 0.974), magnesium (r = 0.646), and iron (r = 0.844) content as well as with cadmium (r = 0.779). Summarizing the results of the agronomic evaluation of soils used for the study it was noticeable that arable soils as well as natural soils, except desert soil, were acidic and contained low amounts of organic carbon as well as low amount of plant-available macro- and micronutrients

3.2. Isolation of putative rhizobia from different soils of Angola.

3.2.1. Isolation of putative rhizobia directly from tested soils.

Isolation of putative rhizobia was done by a serial dilution method of tested soils on the YMAA medium. After 10 days of incubation at 28°C, the number of CFU of bacteria showing the typical appearances of rhizobia was enumerated and isolated for further study. They were later purified on the YEMA-CR medium and YEMA-BTB medium. The results of the enumeration of CFU of rhizobia-like are presented in table 6. The highest numbers of CFU were found in soils from the arable land of Elande and Chianga. These soils were characterized by the higher content of organic carbon, plant-available phosphorus, and magnesium. But any viable cell was found in the soil from the desert, which was alkaline and contained a low amount of organic carbon and nutrients. Moreover, the high level of soluble chromium in an alkaline environment could be toxic for Gram-negative bacteria. The highest number of morphologically different rhizobia-like bacteria were selected from nodules of common bean inoculated with the soil's suspensions from most arable fields as well as from natural forest. A noticeable lower number of morphologically different rhizobia-like bacteria

were selected from nodules of adzuki bean plants. The lowest number of rhizobia-like bacteria was isolated from fallow soil at Alto Hama independently from the method of isolation. The number of isolated rhizobia-like bacteria from nodules was noticeably related to the number of CFU determined by plate count.

Table 6. Enumeration of colony-forming units of rhizobia-like bacteria in tested soils on the YMAA medium and the number of selected isolates.

Soil samples	Viable cells (CFU g^{-1})	Number of selected isolates from		
		soil	nodules of common bean	nodules of adzuki bean
		Arable land		
Bailundo	8.0×10^2	4	5	4
Chipipa	5.0×10^2	5	15	8
Elande	$25{,}7 \times 10^2$	13	16	12
Gongoinga	15.0×10^2	8	13	6
Chianga	23.0×10^2	7	19	13
		Natural forest		
Cabinda	6.4×10^2	5	15	13
Chilela	19.0×10^2	3	14	4
		Fallow		
Alto Hama	6.0×10^2	6	8	5
		Desert		
Namibe	N.O*	N.O*	N.O*	N.O*
Total		**52**	**105**	**65**

*Not observed

The results of this study showed a very low number of rhizobia-like bacteria in tested soils. Moreover, the comparison of physicochemical parameters of tested soil with the enumerated number of CFU noticeable indicated that the most important factor influencing their presence in tested soils was the N/S ratio and the content of organic carbon (Table 7).

Table 7. Correlations factors of the physicochemical parameters of tested soils and CFU of rhizobia-like bacteria, nodulation of common bean, and of adzuki bean.

	Viable cells (CFU g^{-1})	Number of nodules per plant	
		Common bean	Adzuki bean
pH	-0.401	0.358	-0.503
N	0.575	0.288	0.323
OS	-0.379	**0.887*****	-0.406
N/S	**0.789****	-0.486	0.295
OC	**0.632***	0.168	0.443
N/C	0.324	-0.099	0.269
P.	0.238	-0.191	0.137
K	-0.303	0.194	0.264
Mg	-0.213	**0.833****	-0.470
Mn	0.029	**0.774****	0.345
Fe	0.104	0.255	0.237
Cu	-0.447	0.620	0.098
Zn	-0.291	0.253	0.036
Ni	-0.297	0.203	-0.533
Cd	-0.309	0.550	-0.553
Pb	-0.163	0.501	-0.067
Cr	-0.482	0.208	-0.433

Computed correlation used Pearson-method with listwise-deletion.

Codes: '**' significant at $p < 0.01$, '*', significant at $p < 0.05$.

3.2.2. Isolation of putative rhizobia using trapping plants.

The common bean and adzuki bean were used as trap plants. After inoculation with suspensions of tested soil samples, both species showed significantly different nodulation patterns (Table 8 and 9). Most of the arable and natural soils showed the ability to induce nodulation of common bean cv. BASTA and the significantly highest number of nodules, 42 per plant, was observed in plants inoculated with soil from arable land at Chianga. Similarly, inoculation of adzuki bean cv. ADZUKI with this soil resulted in the highest number of nodules, 10.75 per plant, in comparison with other soils. Any nodules were found after the inoculation of common bean and adzuki bean with soil from the desert. Moreover, the inoculation of adzuki bean with the soil from arable land at Chipipa did not induce nodulation and the number of nodules on common bean was also lowest in comparison with other soil samples. The different types of nodules were observed on root systems such as pink-red nodules (active nodules), white nodules (inactive nodules) that were generally small in size, and green nodules as well (Photo 3). Significant differences in the number of active nodules (NAN) per plant between species and among tested soils used for inoculation were observed.

The highest values of NAN were observed on the roots of both trapping plants inoculated with soil from arable land at Chianga. Similarly, significant differences in nodule dry mass (NDM) and shoot dry mass (SDM) between plant species were found. The cultivar BASTA of common bean inoculated with soils from arable land at Chianga and from the forest at Cabinda showed the highest values NDM. However, the highest value of SDM of cultivar BASTA was determined after inoculating with soil from the forest at Chilela, while the highest SDM of the ADZUKI was observed after inoculating with soils from arable lands at Chianga and Elande.

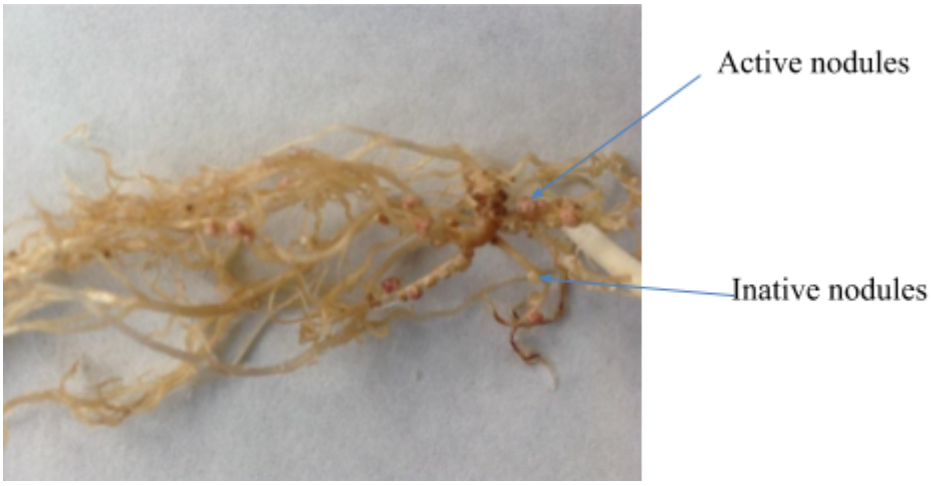


Photo 3. Common bean cultivar "BASTA" root nodules. Type of nodules with different colors and sizes.

Table 8. The effect of soil inoculation on common bean cultivar Basta growth.

Soil from	NN	NAN	NDM (g)	SDM (g)
		Arable land		
Bailundo	28.25c*	17.50d	0.003b	0.17b
Chipipa	9.00f	6.50g	0.002c	0.12c
Elande	19.00e	12.50f	0.003b	0.18ab
Gongoinga	24.25d	15.50e	0.003b	0.19ab
Chianga	40.75a	29.25a	0.005a	0.19ab
		Forest		
Cabinda	38.75a	26.00b	0.005a	0.18ab
Chilela	34.50b	22.75c	0.004ab	0.21a
		Fallow		
Alto Hama	22.00d	15.00e	0.004ab	0.19ab
		Desert		
Namibe	0.00g	0.00h	0.000d	0.11c
Control	0.00g	0.00h	0.000d	0.11c

**Values in the colons followed by the same letters are not significantly different at p = 0.001 using the Tukey HSD test.*

Table 9. The effect of soil inoculation on adzuki bean growth.

Soil from	NN	NAN	NDM (g)	SDM (g)	SNC (%)
		Arable land			
Bailundo	7.25b	5.75cd	0.0018bc	0.16a	3.56ab
Chipipa	0.00d	0.00f	0.0000d	0.11de	3.05c
Elande	8.50b	7.25ab	0.0018bc	0.17a	3.74a
Gongoinga	8.00b	6.00bc	0.0033a	0.12de	3.39b
Chianga	10.75a	8.50a	0.0033a	0.17a	3.37b
		Forest			
Cabinda	5.25c	4.25e	0.0019bc	0.12de	3.058
Chilela	8.50b	7.00bc	0.0023c	0.12de	2.83d
		Fallow			
Alto Hama	5.00c	4.50de	0.0013c	0.13b	3.14c
		Desert			
Namibe	0.00d	0.00f	0.000d	0.10e	3.04c
Control	0.00d	0.00f	0.000d	0.11de	2.66d

**Values in the colons followed by the same letters are not significantly different at p = 0.001 using the Tukey HSD test.*

The significant difference was found regarding the content of nitrogen in the above-ground parts. The plants inoculated with the soil from the arable fields at Elande and Bailundo showed the highest nitrogen content, 3.74% N, and 3.56% N, respectively. And both of them outperform significantly all others. Only in the case of plants inoculated with soils from Chipipa the content of nitrogen (3.05% N) was not higher than in control plants (2.65% N), which indicated that the BNF process was ineffective. The calculated correlation coefficients between determined parameters revealed the significant strong correlations values between NAN and NDM, NDM, and SDM in the case of common bean (Table 10) as well as between SDM and nitrogen content in the case of adzuki bean (Table 11).

Table 10. Correlations factors of the biometric parameters of cv. BASTA

	NN	NAN	NDM	SDM
NN				
NAN	0.988^{***}			
NDM	0.890^{***}	0.875^{***}		
SDM	0.847^{***}	0.846^{***}	0.813^{***}	

Computed correlation used Pearson-method with listwise-deletion.

Codes: '***' Significant at p < 0.001.

Table 11. Correlations factors of the biometric parameters of adzuki bean.

	NN	NAN	NDM	SDM
NN				
NAN	0.989***			
NDM	0.890***	0.860***		
SDM	0.676***	0.699***	0.454**	
SNC	0.591***	0.584***	0.468**	0.738***

Computed correlation used Pearson-method with listwise-deletion.

Codes: '***' significant at $p < 0.001$, '**', significant at $p < 0.01$.

The results of the above studies showed similar effectiveness of common bean and adzuki bean as a trapping plant for isolation of putative rhizobia. Moreover, the nodulation potential of common bean, but not of adzuki bean, was correlated with the content of organic sulfur, with the content of magnesium, and manganese (Table 7). A similar number of putative rhizobia isolates originated from nodules of common bean and adzuki bean but very small originated directly from soil indicating that the use of promiscuous trapping plants for isolation of rhizobia was the more fruitful method. Also, it was noticeable that the occurrence of diverse putative rhizobia able to nodulate overseas promiscuous species like common bean and adzuki in tested arable Angolans soils at Huambo province are scarce like in natural soils.

3.3. Selection and purification of putative rhizobia.

In total, two hundred and twenty-one potential rhizobia-like isolates were selected. Among them, one hundred and seventy were originated from nodules of trapping plants and fifty-one were isolated from soils on the semi-selective medium YMAA. The highest number of isolates (42 isolates) were selected from arable land at Chianga and the lowest number of bacteria was isolated from fallow soil at Alto Hama, while from the desert soil any bacterial colony on YMAA was observed. The higher number of rhizobia-like bacteria were isolated from nodules of common bean cv. Basta and from nodules of adzuki bean inoculated with suspensions of the tested arable soils than directly from soils. All isolates were identified as Gram-negative rods. However, based on further screening at different media, 30 isolates grown on D1 medium specific for *Agrobacterium*, 12 isolates grown on Gould medium specific for fluorescent pseudomonas and absorbed Congo Red on YEMA-CR medium were excluded from further study.

One hundred fifty-eight pure isolates were used for primary screening of the nodulation activity in a pot experiment. The results of the primary screening revealed that sixty-five isolates did not induce nodule formation and did not improve the plant's growth. Sixteen tested isolates (HNG331, HNG332, HLE321, HLo23, HLE111a, HCC31, HKK213, HCC32, HCK23, HLD221a, HMA3, HLD211, HBA151, HLo2, HLo11, and HLo7) did not induce nodule formation but noticeable stimulated plant growth. Among them, eight were isolated from the forest, eight were isolated from arable lands, and one plant growth-promoting bacteria was isolated from fallow soil at Alto Hama. Any PGPB from soil collected at Chianga was found. Their application as a seed inoculant resulted in the development of more than 45% higher shoot dry mass in comparison to the uninoculated control plants in pots with sterilized quartz sand (Table 12). Moreover, the yield of SDM in comparison to reference *Rhizobium leguminosarum* strain F17 and to effective putative rhizobia isolates HLE11 and HCC321 were not lower.

3.3.1. Determination of indole-3-acetic acid production.

Mentioned above 17 isolates were used for further analysis of the ability of indole-3-acetic acid (IAA) production (Table 12) and the presence of the *nifH* gene (Table 12, Table 15). After incubation with L-tryptophan, 13 isolates were found to produce significantly more of IAA than was determined in sterile medium and in the supernatants of the reference strains (Table 12). It was found a significant correlation ($r = 0.657$; $p < 0.05$) between SDM and potential IAA productivity. Most active IAA producers were isolated from the nodules of common bean or from adzuki bean induced by the soil from the forest at Cabinda and from the forest at Chilela, respectively. Among 17 isolates stimulating the development of the common bean in pot experiment most of them after amplification of DNA with specific primers for the *nifH* revealed the presence of a specific band at 400 bp.

This suggests that bacteria inhabiting nodules producing phytohormones e.g. IAA and fix nitrogen can stimulate the growth of legumes independently from the plant growth-promoting effect of BNF rhizobia, which supply plants with nitrogen due to symbiosis.

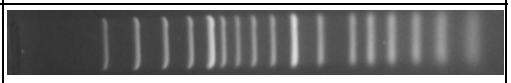
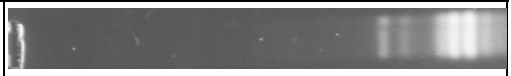
Table 12. The effect of non-nodulating bacteria on common bean development and their ability to IAA production in the LY-broth medium with tryptophan.

Inoculants *	**Source**	**Active nodules**	**SDM (g plant^{-1})**	***nifH***	**IAA (µg mL^{-1})**
HLD221a	Common bean	No	0.35	+	1.48 a
HLE111a	Adzuki bean	No	0.39	-	1.04 b
HLE321	Adzuki bean	No	0.27	+	0.77 c
HLo2	Soil	No	0.32	+	0.65 d
HCC31	Adzuki bean	No	0.33	-	0.65 d
HNG331	Common bean	No	0.31	+	0.61 de
HMA3	Common bean	No	0.24	-	0.56 ef
HLo11	Soil	No	0.30	+	0.57 efg
HLD211	Common bean	No	0.18	-	0.57 efgh
HLo7	Soil	No	0.18	-	0.53 fghi
HKK213	Common bean	No	0.22	+	0.53 ghij
HCC31	Common bean	No	0.18	+	0.50 ijk
HNG332	Common bean	No	0.31	-	0.49 ijkl
HCC32	Adzuki bean	No	0.20	+	0.48 jkl
HCK23	Common bean	No	0.19	+	0.48 kl
HLo23	Adzuki bean	No	0.20	+	0.45 lm
HNG33a	Common bean	No	0.21	+	N.D.
HKK312a	Common bean	No	0.14	+	N.D.
	Reference treatments				
HLE11	Common bean	Yes	0.12	+	0.43 lm
HCC321	Common bean	Yes	0.17	+	0.44 lm
R. leguminosarum F17	Reference	Yes	0.13	+	0.45 lm
Uninoculated	Control	No	0.09±0.014	X	X
Control medium	X	X	X	X	0.42 m

**Values in the colons followed by the same letters are not significantly different at p = 0.001 using the Tukey HSD test.*

** Name codes of isolates: HCA or HMA - soil from Cabinda; HA, HLE or HEC - soil from Chilela, HLo or HBA - soil from Bailundo; HCK, HCP or HKK - soil from Chipipa; HCC or HCH - soil from Chianga; HNG, HGO or HGA - soils Gongoinga; HAH - soil from Alto Hama; HLD or HSL - soil from Elande.*

Table 13. The results of the amplification of DNA of plant-growth promoting bacteria with specific *nifH* primers.

Tested samples	***nifH* 400 bp**
Size Marker	
F17*	
HLD221a	
HMA3	
HLD211	
HLo23	
HNG331	
HNG332	
HKK213	
HLo11	
HLE111a	
HLE321	
HCK23	
HLo2	
HLo4	

***Reference strain R. leguminosarum F17*

The remaining 76 isolates of potential-rhizobia were selected as putative rhizobia and were used for further study. None of the putative rhizobia was isolated from the desert soil and the absence of rhizobia in this environment was also confirmed by nodulation test with common bean and adzuki bean. The lowest number of putative rhizobia was isolated from fallow soil at Alto Hama as well as the nodulation potential of this soil was weak. The results of primary screening of the nodulation activity in a pot experiment, of phenotypic characteristics on YEMA-CR and on YEMA-BTB media, and of identification of *nifH* and *nodC* genes of 76

isolated putative rhizobia and of 2 reference strains of *Rhizobium leguminosarum* are presented in Tables 14, 16, and 18.

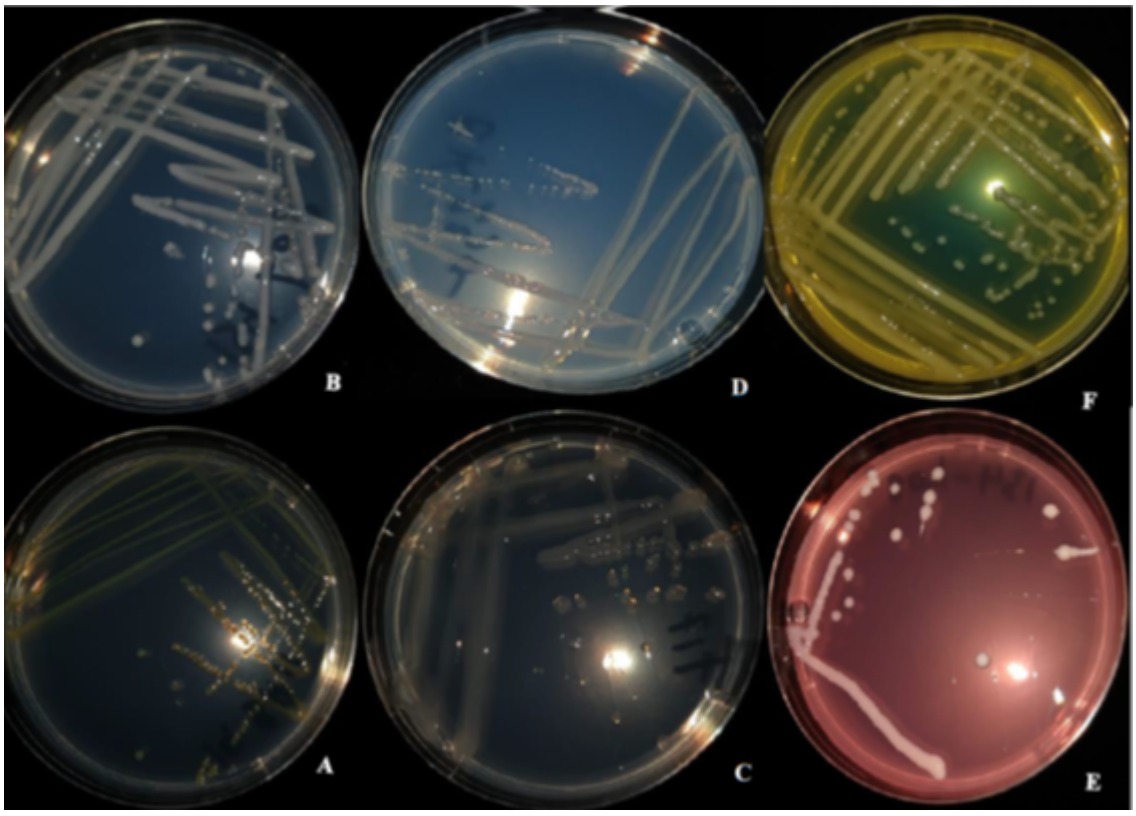


Photo 4. Morphological characteristics of isolated bacteria: A - yellow colonies, B - white colonies, C - white translucent colonies, D - white milk colonies, E - not absorb the dye, F - the acidic reaction of rhizobia isolates on YEMA-BTB.

The characteristic of 19 putative rhizobia isolated directly from tested soils on the YMAA medium is summarized in Table 14. The results showed some diversity among tested isolates. All isolates and reference strains were identified as Gram-negative rods and they did not absorb or weakly absorb Congo Red when incubated in the dark on YEMA-CR medium (e.g. Photo 5E). The growth rate and ability to acidify the YEMA-BTB medium showed that all tested putative rhizobia strains and reference strains are fast-growing and acid producers (e.g. Photo 5F). All tested putative rhizobia strains grown as a white wet (e.g. Photo 5B) or dry colony but all reference strains grown as white translucent wet colonies (e.g. Photo 5C). The shape of the colonies was noticeably diverse and most of them (11 isolates) showed raised shape when the 7 tested strains like the reference strains were flattened.

Table 14. Characteristics of putative rhizobia isolated directly from different soil samples on YMAA medium and of reference strains of *R. leguminosarum* F10 and F17,

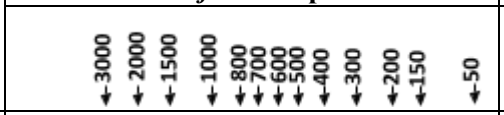
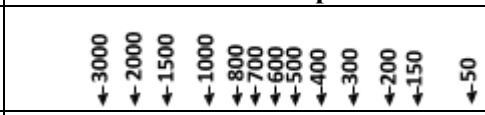
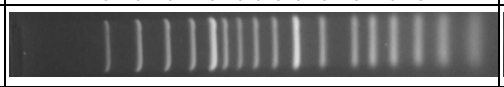
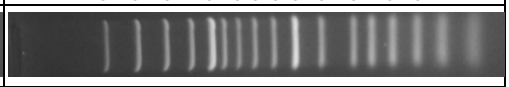
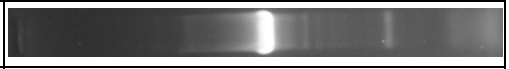
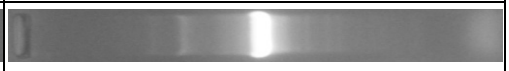
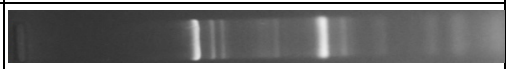
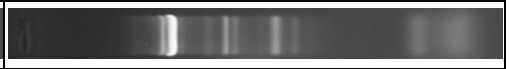
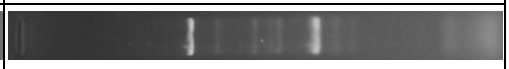
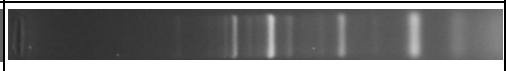
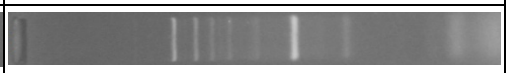
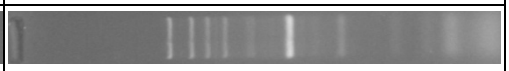
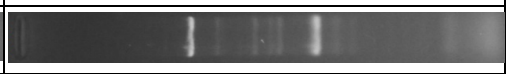
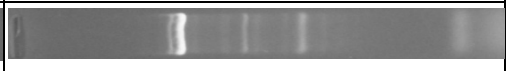
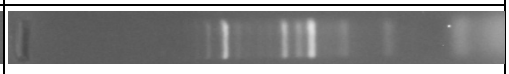
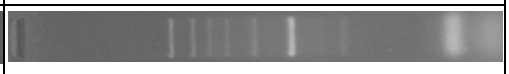
Isolates	YEMA - BTB		YEMA - CR	YEMA medium		NAN / SDM (g plant^{-1})	Amplification	
	Growth rate	Acid release		Colony shape	Colony color / appearance		*nodC*	*nifH*
Isolated strains								
HCH1	FG	Ac. P	N.A	Conical	W / Dry	No / 0.13	+	-
HCH3	FG	Ac. P	N.A	Flattened	W / Wet	No / 0.16	-	+
HCH10a	FG	Ac. P	W.A	Raised	W / Dry	No / 0.10	-	+
HLo2	FG	Ac. P	N.A	Raised	W / Dry	No / 0.13	-	+
HLo3	FG	Ac. P	N.A	Raised	W / Dry	No / 0.11	-	+
HLo5	FG	Ac. P	N.A	Raised	W / Dry	No / 0.10	+	+
HLo8	FG	Ac. P	N.A	Raised	W / Dry	Yes / 0.20	+	+
HLo9	FG	Ac. P	N.A	Raised	W / Dry	No / 0.12	-	+
HLo11	FG	Ac. P	N.A	Raised	W / Dry	No / 0.11	+	+
HLo15	FG	Ac. P	N.A	Raised	W / Dry	No / 0.11	+	+
HLo18	FG	Ac. P	N.A	Conical	W / Dry	No / 0.09	-	+
HLo22	FG	Ac. P	N.A	Raised	W / Dry	No / 0.08	-	+
HLo23	FG	Ac. P	N.A	Raised	W / Dry	No / 0.11	+	+
HLo32	FG	Ac. P	N.A	Raised	W / Dry	No / 0.10	+	+
HGA8	FG	Ac. P	N.A	Flattened	W / Wet	No / 0.12	-	+
HGA9	FG	Ac. P	N.A	Flattened	W / Wet	No / 0.12	-	+
HGO6	FG	Ac. P	N.A	Flattened	W / Wet	No / 0.09	-	+
HGO8	FG	Ac. P	N.A	Flattened	W / Wet	No / 0.09	-	+
HMA1	FG	Ac. P	N.A	Flattened	W / Wet	No / 0.07	-	+
Reference strains *R. leguminosarum*								
F10	FG	Ac. P	W.A	Flattened	W. Tr. / Wet	No / 0.09	+	+
F17	FG	Ac. P	W.A	Flattened	W. Tr. / Wet	Yes / 0.13	+	+
Uninoculated								
Control	X	X	X	X	X	No/0.09±0.014	-	-

FG – fast-growing, Ac.P – acid producer, N.A – not absorbs CR dye, W.A – weakly absorb CR dye, W.- white colony, Y -yellow colony, W Tr. - white translucent colony, N.D. - not determined
** Name codes of isolates: HCA or HMA - soil from Cabinda; HA, HLE or HEC - soil from Chilela, HLo or HBA - soil from Bailundo; HCK, HCP or HKK - soil from Chipipa; HCC or HCH - soil from Chianga; HNG, HGO or HGA - soils Gongoinga; HAH - soil from Alto Hama; HLD or HSL - soil from Elande.*

The evaluation of the nodulation potential in the primary pot experiment revealed that only one HLo8 isolate originated from Bailundo soil induced active nodules and SDM was noticeable greater than of control plants and similar to SDM inoculated with reference strain F17. The amplification of DNA of the reference strain of *Rhizobium leguminosarum* F17 with specific primers for *nod*C and *nifH* genes gave positive bands 620 bp and 400 bp, respectively. Among tested putative rhizobia isolates 18, except the HCH1 isolate from

Chianga soil, revealed the presence of *nifH* genes while the presence of *nodC* gene was found only in the case of 7 isolates, including HLo8 isolates isolated from arable land at Bailundo. This isolate HLo8 was selected for further study. However, four isolates that did not show active nodule on the common bean and give positive *nodC* and *nifH* genes, I can presume that these isolates can be rhizobia nodulating another specific *Fabaceae* species.

Table 15. Amplification of the *nif*H and *nod*C genes of the putative rhizobia isolated from different soil samples from Huambo on YMAA medium and of the reference strain of *R. leguminosarum* F17.

Tested samples	***nifH* 400 bp**	***nodC* 620 bp**
		
Size Marker		
F17*		
HLo8		
HLo11		
HLD221a		
HLo2		
HGO8		
HGA9		
HLo32		
HLo23		
HLo9		
HLo18		
HGA8		
HLo3		
HGO6		
HLo5		
HLo22		

*****Reference strain R. leguminosarum F.17.*

The majority of isolates (11) that did not give positive *nodC* were originated from arable land with a history of soybean inoculation, or common bean cultivation. Only one isolates HMA1 with the negative *nodC* gene originated from the forest at Cabinda. It was noticeable that none of the isolates selected directly from the soil present phenotypic characteristics as reference strains. The aforementioned results indicate that isolation of effective rhizobia directly from soils is not sufficiently effective, principally for rhizobia biodiversity identification. Most isolates selected on the YMAA medium from tested soils are able to fix nitrogen but form inactive nodules even some of them are carrying *nodC* genes.

The characteristic of 27 putative rhizobia isolated from nodules of adzuki bean inoculated with soil suspensions of tested soils is summarized in Table 16. The results showed bigger phenotypic diversity among them in comparison with isolates selected directly from soils with the YMAA medium. All isolates and reference strains were identified as Gram-negative rods and they did not absorb or weakly absorb Congo Red when incubated in the dark on YEMA-CR medium (e.g. Photo 5E). The growth rate and ability to acidify the YEMA-BTB medium showed that 24 among them and reference strains are fast-growing and acid producers (e.g. Photo 5F). Five isolates (HEC21a, HEC23, HEC24, HEC27, and HMA7) from the nodules of adzuki bean alkalized YEMA-BTB medium and were slow-growing. They were excluded from further study. Most of the tested putative rhizobia grown as a white wet (e.g. Photo 5B) or dry colony. Only colonies of eight isolates had an appearance as a white translucent wet colony like reference strains (e.g. Photo 5C). The shape of the colonies was noticeably diverse and most of them were flattened like the reference strains and also raised and conical shape was observed. It was noticeable that 6 isolates selected from the nodules of adzuki bean present phenotypic characteristics like reference strains. The evaluation of the nodulation potential in the primary pot experiment revealed that 7 isolates (HC4, HNG33, HCA10, HEC1, HSL1 HA2a, HA8) induced active nodules and SDM was noticeable greater than of control plants and similar to SDM inoculated with reference strain F17 (Table 16). The result of the amplification of DNA of the isolates from adzuki bean and reference strain of *Rhizobium leguminosarum* with specific primers for *nodC* and *nifH* genes is presented in Table 17. The majority of tested putative rhizobia isolates, including all inducing active nodules, revealed the presence of the *nifH* gene. The amplification of DNA of two isolates HLE11a and HLE12a from the arable land at Chilela did not show a specific band for the *nifH* gene.

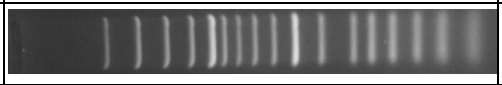
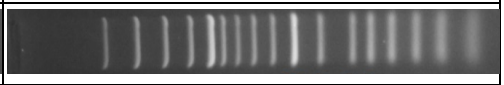
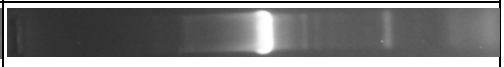
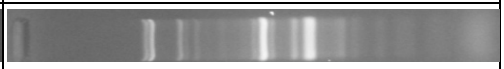
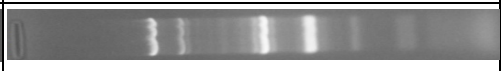
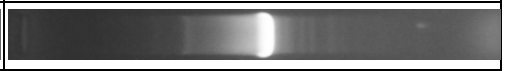
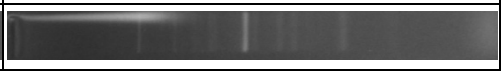
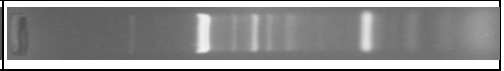
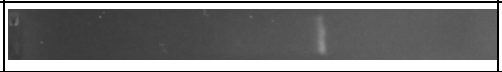
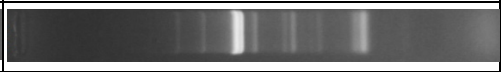
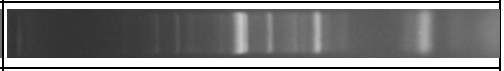
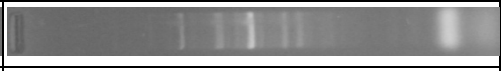
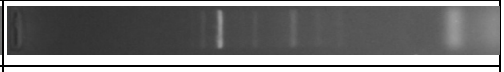
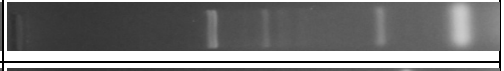
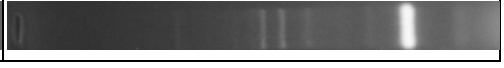
Table 16. Characteristics of putative rhizobia isolated from nodules of *V. angularis* inoculated with soil samples from Huambo and of reference strains of *R. leguminosarum* F10 and F17.

Isolates	YEMA - BTB		YEMA - CR	YEMA medium		NAN / SDM (g plant^{-1})	Amplification	
	Growth rate	Acid release		Colony shape	Colony color / appearance		*nodC*	*nifH*
Isolated strains								
HMA2	FG	Ac. P	W.A	Conical	W / Dry	No / 0.03	-	+
HMA7	SG	ALR	N.A	Raised	W / Dry	No / 0.09	-	+
HLD311	FG	Ac. P	W.A	Flattened	W. Tr. / Wet	Yes / 0.08	+	+
HC4	FG	Ac. P	N.A	Raised	W / Dry	Yes / 0.17	+	+
HNG33	FG	Ac. P	W.A	Flattened	W. Tr. / Wet	Yes / 0.24	+	+
HCA4a	FG	Ac. P	N.A	Conical	W / Dry	Yes / 0.11	+	+
HCA4	FG	Ac. P	N.A	Conical	W / Dry	No / 0.07	-	+
HCA6	FG	Ac. P	N.A	Conical	W / Dry	No / 0.06	-	+
HCA7	FG	Ac. P	N.A	Conical	W / Dry	No / 0.14	-	+
HCA10	FG	Ac. P	N.A	Raised	W / Wet	Yes / 0.30	+	+
HEC1	FG	Ac. P	N.A	Raised	W / Dry	Yes / 0.14	+	+
HEC1a	FG	Ac. P	N.A	Raised	W / Dry	No / N.D.	-	+
HEC21a	SG	ALR	N.A	Flattened	W / Wet	No / 0.12	-	+
HEC23	SG	ALR	N.A	Flattened	W / Wet	No / 0.08	-	+
HEC24	SG	ALR	N.A	Flattened	W / Wet	No / 0.14	-	+
HEC27	SG	ALR	N.A	Flattened	W / Wet	No / 0.08	-	+
HLE11a	FG	Ac. P	W.A	Flattened	W. Tr. / Wet	No / 0.07	+	-
HLE12a	FG	Ac. P	W.A	Flattened	W. Tr. / Wet	No / 0.04	+	-
HLE13	FG	Ac. P	W.A	Raised	W. Tr. / Dry	No / 0.11	-	+
HSL1	FG	Ac. P	W.A	Raised	W. Tr. / Wet	Yes / 0.13	+	+
HA1	FG	Ac. P	W.A	Flattened	W. Tr. / Wet	No / 0.06	-	+
HA2a	FG	Ac. P	W.A	Flattened	W. Tr. / Wet	Yes / 0.16	+	+
HA8	FG	Ac. P	W.A	Flattened	W. Tr. / Wet	Yes / 0.16	+	+
HA4	FG	Ac. P	W.A	Flattened	W / Dry	No / 0.12	-	+
HA5	FG	Ac. P	W.A	Flattened	W / Wet	No / 0.08	-	+
HA7	FG	Ac. P	W.A	Flattened	W / Wet	No / 0.11	-	+
HA9	FG	Ac. P	W.A	Flattened	W / Wet	No / 0.06	-	+
Reference strains *R. leguminosarum*								
F10	FG	Ac. P	W.A	Flattened	W. Tr. / Wet	No / 0.09	+	+
F17	FG	Ac. P	W.A	Flattened	W. Tr. / Wet	Yes / 0.13	+	+
Uninoculated								
Control	X	X	X	X	X	X / 0.09±0.014	-	-

FG – fast-growing, Ac.P – acid producer, N.A – not absorbs CR dye, W.A – weakly absorb CR dye, W.- white colony, Y -yellow colony, W Tr. - white translucent colony, N.D. - not determined

** Name codes of isolates as above in Table 16.*

Table 17. Amplification of the *nif*H and *nod*C genes of the strains isolated from nodules of *V. angularis* inoculated with soil samples from Huambo and of the reference strain of *R. leguminosarum* F17.

Tested samples	nifH 400 bp	nodC 620 bp
	3000 2000 1500 1000 800 700 600 500 400 300 200 150 50	3000 2000 1500 1000 800 700 600 500 400 300 200 150 50
Size Marker		
F17*		
HA2a		
HA8		
HLD311		
HSL1		
HC4		
HA9		
HA5		
HCA4a		
HCA6		
HEC1		
HNG33		
HLE11a		
HA1		
HA7		
HEC24		
HCA4		
HCA10		

***** Reference strain R. leguminosarum F17*

The presence of the *nodC* gene was found in the case of 14 isolates. In the case of isolates from root nodules of adzuki bean that did not give positive *nodC,* the majority of them (9) were from the forest but the remaining five strains were from arable land at Elande with no story of common bean cultivation. The isolate HLD311 from arable land at Elande revealed

the presence of *nifH* and *nodC* genes, however, it did not improve the growth of common bean in primary screening at pot experiment so they were not used for authentication. Also, the isolate HCA4a from the forest at Cabinda showed the presence of *nifH* and *nodC* genes but did not significantly improve the growth in comparison to control. I presume that those isolates that showed positive *nifH* and *nodC* but did not improve the growth can be an ineffective isolate. All those isolates along with isolates that gave positive *nodC* only (HLE11a, HLE12a) as well as those that gave positive *nifH* only, were excluded for further study of authentication. The remaining 7 isolates were selected for further study.

The characteristic of 30 putative rhizobia isolated from nodules of common bean cv. Basta inoculated with soil suspensions of tested soils is summarized in Table 18. The results showed also higher phenotypic diversity among them in comparison with isolates selected directly from soils on the YMAA medium. All isolates and reference strains were identified as Gram-negative rods and they did not absorb or weakly absorb Congo Red when incubated in the dark on YEMA-CR medium (e.g. Photo 5E). As well as all showed fast-growing and acid producers (e.g. Photo 5F) as reference strains when incubated in dark at the YEMA-BTB medium. The shape of the colonies was noticeably diverse and most of them (12 isolates) showed flattened shape like the reference strains, and only one putative rhizobium showed conical shape. Growing on the YEMA medium, seventeen isolates showed white translucent colonies and wet (e.g. Photo 5C), nine were white (e.g. Photo 5B), two isolates were white milk (e.g. Photo 5D) and only one showed pale yellow (e.g. Photo 5A). Phenotypically the putative rhizobia isolated from the root nodule of the common bean showed higher diversity in comparison to isolates from adzuki bean as well as in comparison to isolates originated directly from the soil. However, only two isolates, HSL13 and HSL13a, both from the forest soil at Elande were phenotypically like the reference *R. leguminosarum* strains.

The nodulation potential in the primary pot experiment revealed that twelve isolates (HBA11, HBA15, HBA15a, HCC321, HKK321, HLD1, HLE11, HLE22, HLE131, HNG13, HSL13, HSL13a) induced active nodules and SDM was noticeable greater than control plants and similar or higher than SDM inoculated with reference strain F17 (Table 18).

The results of the amplification of DNA of the isolates from the common bean and 2 reference strains of *Rhizobium leguminosarum* with specific primers for *nod*C and *nifH* genes are presented in Table 19. The majority of tested putative rhizobia revealed the presence of *nifH* genes, only in the case of two isolates HCK2 and HEC20 both from arable land at the

presence of the *nifH* gene was not confirmed. While thirteen isolates the presence of the *nodC* gene was also not found and most of them were from arable land with a history of common bean cultivation or inoculation. Among the isolates that gave positive *nodC* and *nifH* genes, the isolates HAH2 from fallow soils at Alto Hama and HBA 12 from arable land at Bailundo showed ineffective with the manifestation of the lowest value of SDM in comparison to the control. These isolates along with HCK2 and HEC20 isolates that did not give positive *nifH* as well as the isolates with negative *nodC* were excluded for further analysis of authentication. The remaining isolates mentioned above that originated from root nodules of common, which gave positive results on the primary experiment as well as were confirmed the presence of the *nifH* and *nodC* was used for authentication. Most of them were from arable land, and only two isolates were from the forest at Elande. In addition, it is noteworthy that most of the strains that revealed to be effective on primary screening consequently with positive *nifH* and *nodC* were originated from arable land with the history of common bean cultivation or seed inoculation. So I presume that most of those isolates presented in the soils were introduced or by the seed of legume plants or by the inoculation process in the past. Moreover, the annual cultivation of the common bean and soybean in the study regions has influenced positively in the maintenance and survival of nitrogen fix bacteria. The isolate HCP8, from arable land at Chipipa revealed the presence of *nifH* and *nodC* genes, however, it did not improve the growth of common bean in pot experiments so it was not used for authentication.

The relatively high number of isolates with different phenotypic characteristics (Table 18) that were found among the isolates originating from nodules of common bean is the evidence of the higher promiscuity of the common bean in comparison to adzuki bean.

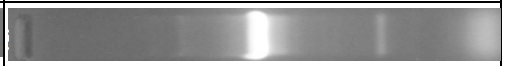
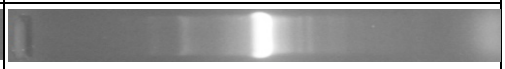
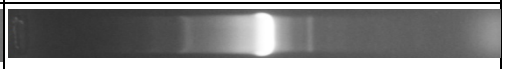
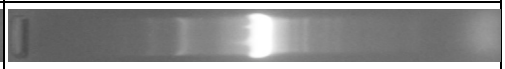
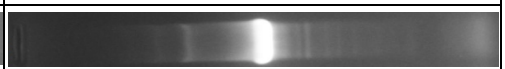
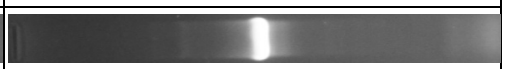
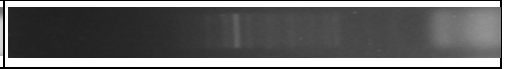
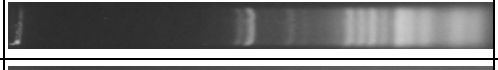
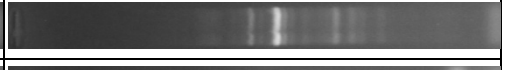
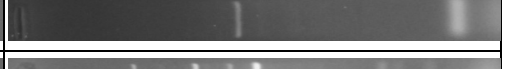
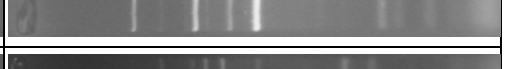
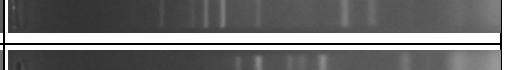
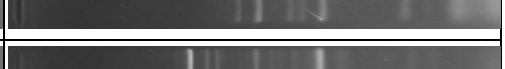
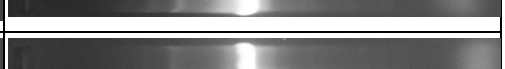
Table 18. Characteristics of putative rhizobia isolated from nodules of *P. vulgaris* inoculated with soil samples from Huambo and reference strains of *R. leguminosarum* F10 and F17.

Isolates	YEMA - BTB		YEMA - CR	YEMA medium		NAN / SDM (g plant^{-1})	Amplification	
	Growth rate	Acid release		Colony shape	Colony color / appearance		*nodC*	*nifH*
Isolated strains								
HBA2	FG	Ac. P	W.A	Raised	W / Dry	N.D. / N.D.	-	+
HBA11	FG	Ac. P	W.A	Flattened	Y / Wet	Yes / 0.18	+	+
HBA15	FG	Ac. P	W.A	Raised	W. Tr. / Wet	Yes / 0.15.	+	+
HBA 12	FG	Ac. P	W.A	Raised	W. Tr. / Wet	Yes / 0.04	+	+
HBA15a	FG	Ac. P	W.A	Raised	W. Tr. / Wet	Yes / 0.15	+	+
HCC321	FG	Ac. P	W.A	Raised	W. Tr. / Wet	Yes / 0.17	+	+
HCC322	FG	Ac. P	W.A	Raised	W. Tr. / Wet	No / 0.09	-	+
HCH52	FG	Ac. P	W.A	Raised	W. Tr. / Wet	Yes / 0.10	-	+
HCK2	FG	Ac. P	W.A	Raised	W. Tr. / Wet	Yes / 0.09	+	-
HCK211	FG	Ac. P	W.A	Raised	W. Mi. / Wet	No / N.D.	-	+
HKK321	FG	Ac. P	W.A	Raised	W. Mi. / Wet	Yes / 0.15	+	+
HEC20	FG	Ac. P	W.A	Raised	W / Dry	No / N.D.	+	-
HNG13	FG	Ac. P	W.A	Flattened	W. Tr. / Wet	Yes / 0.12	+	+
HCP8	FG	Ac. P	N.A	Flattened	W / Wet	N.D / N.D.	+	+
HCP2	FG	Ac. P	N.A	Raised	W / Wet	No / 0.09	+	-
HLD1	FG	Ac. P	N.A	Raised	W. Tr. / Wet	Yes / 0.33	+	+
HLD12	FG	Ac. P	W.A	Raised	W. Tr. / Wet	Yes / 0.12	-	+
HCP10	FG	Ac. P	W.A	Raised	W / Wet	No / 0.10	+	+
HLE11	FG	Ac. P	N.A	Raised	W / Wet	Yes / 0.12	+	+
HLE22	FG	Ac. P	W.A	Raised	W. Tr. / Dry	Yes / 0.49	+	+
HLE131	FG	Ac. P	W.A	Raised	W. Tr. / Wet	Yes / 0.12	+	+
HSL12	FG	Ac. P	W.A	Flattened	W. Tr. / Wet	No / 0.12	-	+
HSL13	FG	Ac. P	W.A	Flattened	W. Tr. / Wet	Yes / 0.12	+	+
HSL13a	FG	Ac. P	N.A	Flattened	W. Tr. / Wet	Yes / 0.15	+	+
HAH1a	FG	Ac. P	N.A	Flattened	W. Tr. / Wet	No / N.D.	-	+
HAH2	FG	Ac. P	N.A	Flattened	W / Dry	No / 0.06	+	+
HAH3	FG	Ac. P	N.A	Flattened	W / Wet	No / 0.09	-	+
HAH6	FG	Ac. P	N.A	Flattened	W / Wet	No / 0.10	-	+
HAH9	FG	Ac. P	N.A	Flattened	W / Wet	No / 0.08	-	+
Reference strains *R. leguminosarum*								
F10	FG	Ac. P	W.A	Flattened	W. Tr. / Wet	No / 0.09	+	+
F17	FG	Ac. P	W.A	Flattened	W. Tr. / Wet	Yes / 0.13	+	+
Uninoculated								
Control	X	X	X	X	X	X / 0.09±0.014	-	-

FG – fast-growing, Ac.P – acid producer, N.A – not absorbs CR dye, W.A – weakly absorb CR dye, W.- white colony, Y -yellow colony, W. Tr. - white translucent colony, N.D. - not determined

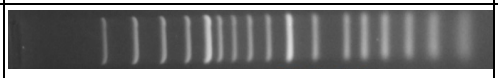
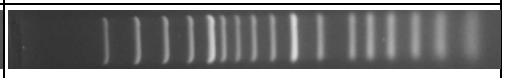
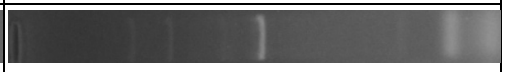
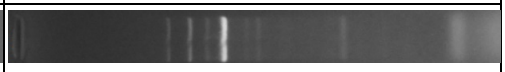
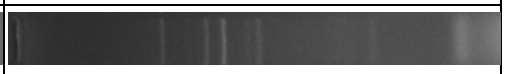
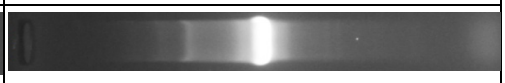
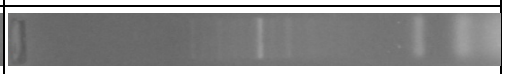
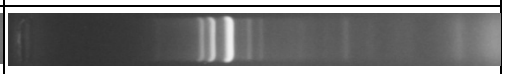
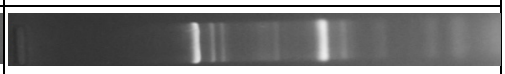
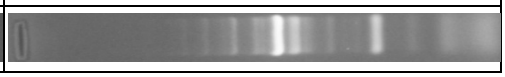
** Name codes of isolates as above in Table 16.*

Table 19**.** Amplification of the *nif*H and *nod*C genes of the strains isolated from nodules of *P. vulgaris* inoculated with soil samples from Huambo and of the reference strain of *R. leguminosarum* F17.

Tested samples	***nifH* 400 bp**	***nodC* 620 bp**
	3000 2000 1500 1000 800 700 600 500 400 300 200 150 50	3000 2000 1500 1000 800 700 600 500 400 300 200 150 50
Size Marker		
F17*		
HCP8		
HBA15a		
HBA15		
HBA 12		
HCC321		
HLE131		
HSL13		
HNG13		
HCP9		
HBA2		
HCH52		
HMA5		
HSL13a		
HCH4		
HLE321		
HBA11		
HCC31		
HKK321		
HEC20		
HAH1a		
HAH9		

*****Reference strain R. leguminosarum F17*

Table 19. Continued

Tested samples	*nifH* 400 bp	*nodC* 620 bp
	←3000 ←2000 ←1500 ←1000 ←800 ←700 ←600 ←500 ←400 ←300 ←200 ←150 ←50	←3000 ←2000 ←1500 ←1000 ←800 ←700 ←600 ←500 ←400 ←300 ←200 ←150 ←50
Size Marker		
HCK211		
HAH2		
HCK2		
HLD1		
HBA12		
HLD12		
HEC5		
HSL12		
HLE11		
HLE22		
HCH41		
HLD212		
HLD211a		
HCP2		

**Reference strain R. leguminosarum F17*

3.4. Authentication of isolated rhizobia.

The nineteen previously selected putative rhizobia were used for authentication and assess their effectiveness with one common bean cultivar BASTA in a pot experiment. Among them, eleven isolates from nodules of common bean (HBA15a, HLE11, HCC321, HLD1, HBA11, HLE22, HNG13, HSL13, HSL13a HKK321, HLE131), seven isolates from adzuki bean nodules (HA2a, HA8, HCA10, HC4, HEC1, HNG33, HSL1), and HLo8 isolate from soil were applied for inoculation of disinfected seeds of common bean cultivar BASTA.

3.4.1. Plant growth and appearance.

The evaluation of plant development was done after 45 days of growth. Inoculation of a common bean with all tested 19 isolates resulted in a noticeable response on plant growth parameters presented below (Photo 5).

Photo 5. Differences in plant growth and leaves color as the response of the inoculation. C - control, N - nitrogen fertilization, F17 - treatment with reference *R. leguminosarum* strain F17, BA11 - treatment with isolated putative rhizobia HBA11.

As it is exemplified in photo 5 application of most effective isolates and of reference strain F17 resulted in visible better growth of common bean in comparison with nitrogen fertilization as well as with the noticeable greenish color of the leaves in comparison to yellowish leaves of control plants.

3.4.2. Effect on root nodulation.

Nodulation of roots of all inoculated common bean cv. BASTA plants were observed after the application of the mentioned above 19 selected putative rhizobia. The results of the nodulation study are presented in Table 20. The performance of the root nodulations was related to the isolates. As it is exemplified on photo 6 with the application of most effective isolate HA2a resulted in the presence of nodules along with the all root system mostly on the main root and few nodules were observed on lateral or secondary roots.

The inoculation with less effective isolates e.g. HLE11 induced only a few nodules on the root system of the plant (e.g. Photo 6). The root systems of control non-inoculated plants and nitrogen fertilized plants were not nodulated (e.g. Photo. 6). All tested strains were found to be effective nodulating bacteria. The evaluation of common bean development is summarized

in Table 20. The average number of nodules and the number of active nodules per plant differed significantly among inoculation treatment (Table 20).

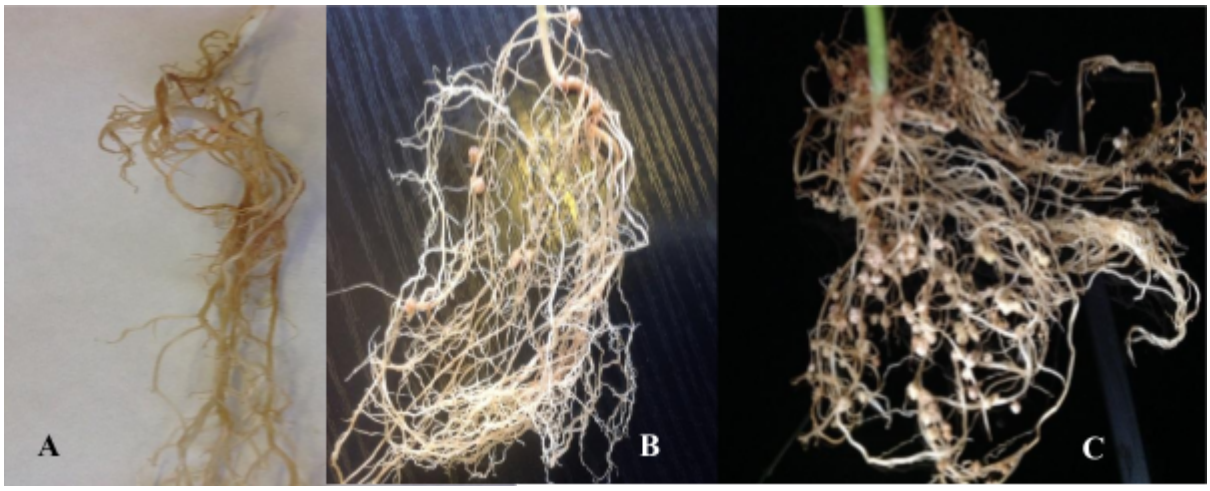


Photo 6: The differences between the three root systems.
A - weak root system of the un-inoculated plants without any nodules,
B - root system with few nodules after inoculation with moderate effective isolate HLE11.
C - vigorous and abundant nodules on the root system after inoculation with HA2a.

The plants inoculated with an HA8 isolate produced 173.0 nodules per plant and it was significantly the highest number among all tested putative rhizobia. This isolate originated from adzuki bean nodules induced by soil suspension from Chilela. Also isolates originated from adzuki bean nodules (HA2a) and from nodules of common bean (HBA11, HSL13, HSL13a) induced a similar number of nodules like reference strain F17. Whereas the lowest number of nodules as well as of active nodules were observed on plants inoculated with isolate HLE11 from common bean, originated from the soil at Chilela and HSL1 from nodules of adzuki bean originated from soils at Elande. The significantly highest nodule dry mass was determined on plants inoculated with the isolate HSL13a which also induced one of the highest numbers of active nodules per plant. This isolate originated from the common bean nodules induced by the soil from Elande. But the significantly lowest NDM per plant, in comparison with the others inoculants, was determined on the plants inoculated with isolate HLE11. This isolate originated from common bean nodules induced by soil suspension from Chilela. This isolate also induced the lowest value of NAN ($p < 0.001$) (Table 20).

3.4.3. Effect on the shoots and roots dry mass.

The effect of inoculation of common bean with 19 selected putative rhizobia in comparison to nitrogen fertilization as well as to unfertilized and uninoculated control plants is presented in Table 14. The significant differences among treatments were found at $p < 0.001$. The value of

the SDM of control plants was the lowest one. The treatment with nitrogen fertilization yields higher SDM per plant, however without a significant difference in comparison to SDM of plants inoculated with HCC321 and HSL13a. Both isolates originated from the nodules of common bean induced by the soil from Chianga and Elande, respectively. Particularly in the case of isolate HSL13a, I presume that it can be native nitrogen fixing bacteria, due to the fact that it was isolated from the natural forest with no history inoculation or agriculture practices. In addition, these isolates induced SDM similar reference strain *R. leguminosarum* (Table14).

The effect of the 19 selected putative rhizobia on root dry mass per plant was significantly different and varied from 0.08 to 0.24 g plant^{-1} (Table 20). The significantly highest value of RDM per plant similarly like SDM per plant was determined after treatment with isolate HCC321 selected from common bean nodules induced by the soil from Chianga. This isolate also induced the highest SDM as was mentioned above. The RDM after application of isolate HCC321 was about 84% higher than uninoculated plants. Plants inoculated with isolate HCC321 did not show significant differences of RDM in comparison with plants supplied with nitrogen fertilization. It was not observed significant differences in the RDM values of the most inoculant plants in comparison with control. However, the lowest RDM was observed after the application of isolate HEC1 originated from the adzuki bean nodules induced by the soil from Chilela. This isolate also induced one of the lowest numbers of active nodules per plant. The SDM/RDM ratio was observed in the range from 2.58 to 3.92 and the differences among tested treatments were not significant in most cases. Among tested strains, the lowest SDM/RDM ratio was found also after inoculation with HEC1 (Table 20). The calculated correlation coefficients between determined parameters revealed the significant correlation values between NAN and NDM, NDM, and SDM as well as between SDM and RDM (Table 21). These results illustrated that the use of the most effective isolates can improve the biomass yield of the common bean. Most of the effective isolates originated from arable land and two of them HSL13 and HSL13a originated from the natural forest.

Table 20. The effect of inoculation with selected putative rhizobia of *P. vulgaris* cv. BASTA on nodule number, number of active nodules, nodule dry mass, shoot dry mass, root dry mass, shoot dry mass, and the ratio of the shoot - root dry mass.

Strains	NN (plant^{-1})	NAN (plant^{-1})	NDM (g plant^{-1})	SDM (g plant^{-1})	RDM (g plant^{-1})	SDM/RDM (g plant^{-1})
HA2a	133.5 b	89.75 bcd	0.056 abc	0.52 cde	0.19 bcd	2.67 ab
HA8	173.0 a	148.00 a	0.055 abc	0.38 efg	0.14 def	2.99 ab
HBA11	129.5 bc	101.00 b	0.063 a	0.45 defg	0.20 abc	2.58 b
HBA15a	86.2 def	41.00 ghi	0.047 bcd	0.48 def	0.21 abc	2.88 ab
HCC321	96.7 de	72.00 def	0.057 abc	0.66 ab	0.24 a	2.85 ab
HC4	94.5 def	81.25 bcd	0.042 cd	0.34 fg	0.09 f	3.09 ab
HCA10	53.7 hi	33.00 ij	0.020 ef	0.36 fg	0.12 ef	3.28 ab
HEC1	68.5 fgh	43.75 ghi	0.035 de	0.34 fg	0.08 f	3.92 a
F17*	129.0 bc	100.75 bc	0.046 bcd	0.57 abc	0.15 cde	3.55 ab
HKK321	83.0 efg	65.50 efg	0.050 bcd	0.47 def	0.17 bcde	2.80 ab
HLD1	98.7 de	75.25 cde	0.045 bcd	0.35 fg	0.13 def	3.01 ab
HLE11	21.2 jk	14.25 j	0.011 f	0.41 efg	0.15 cde	2.88 ab
HLE22	88.0 def	70.50 def	0.045 bcd	0.37 gf	0.16 cde	2.79 ab
HLE131	59.0 ghi	39.00 ij	0.042 cd	0.47 def	0.17 bcde	3.61 ab
HLo8	93.5 def	54.50 ghi	0.048 bcd	0.56 bcd	0.19 bcd	3.01 ab
HNG13	98.75 de	71.75 def	0.040 cd	0.39 efg	0.12 df	3.24 ab
HNG33	105.50 cde	60.00 efg	0.050 bcd	0.58 abc	0.21 abc	2.75 ab
HSL1	34.50 ij	14.00 j	0.018 ef	0.33 fg	0.09 f	3.54 ab
HSL13	111.50 bcd	74.25 def	0.059 ab	0.57 abc	0.16 cde	3.70 ab
HSL13a	130.00 bc	90.00 bcd	0.064 a	0.64 ab	0.19 bcd	2.87 ab
N Fert.	0.00 k	0.00 k	0.000 g	0.76 a	0.22 ab	3.56 ab
Control	0.00 k	0.00 k	0.000 g	0.29 g	0.13 def	2.80 ab

*Results represent means ± standard deviation, * Reference strain, NN - Nodule number, NAN – Number of the active nodule, NDM – Nodule dry mass, SDM – Shoot dry mass, RDM – Root dry mass, SDM/RDM – Shoot dry mass and root dry mass ratio, N. Fert. – Nitrogen fertilization, Control – pots without nitrogen fertilization and without bacteria inoculation. Values in the colons followed by the same letters are not significantly different at p = 0.05 using the Tukey HSD test.*

Table 21. Correlations variables of the tested parameters evaluated after the inoculation of common bean cv. Basta with 19 putative rhizobia.

	NN	*NAN*	*NDM*	*SDM*	*RDM*
NAN	0.930***				
NDM	0.812***	0.735***			
SDM	0.423	0.291	0.546**		
RDM	0.294	0.175	0.394	0.613***	

Computed correlation used Pearson-method with listwise-deletion.

*Codes: '***' significant at p < 0.001, '**', significant at p < 0.01.*

3.4.4. Relative effectiveness and symbiotic efficiency.

Relative effectiveness (RE) and the symbiotic efficiency (SE) of 19 selected putative rhizobia are presented in table 22. The SDM of inoculated plants with selected putative rhizobia as well as of plants inoculated with the reference *R. leguminosarum* strain F17 was determined after 45 days of growth. Based on the comparison of SDM of common bean plants inoculated with tested rhizobia with SDM of plants inoculated with reference strain F17, the putative rhizobia were classified as effective, partially effective, poorly effective, and ineffective respectively. The six isolates (HBA15a, HCC321, HKK321, HLE131, HSL13, HSL13a) originated from nodules of common bean, two isolates (HA2a and HNG33) isolated from adzuki bean, and one (HLo8) isolated directly from the soil on YMAA medium were found to be relatively effective in comparison to references strain. The mentioned above isolates showed higher or similar NAN and SDM in comparison to reference strains as was described above (section 3.4.4) (Table 20). The remaining 10 isolates were partially effective.

Moreover, the symbiotic efficiency was calculated by comparison of the SDM of inoculated plants with SDM of plants supplemented with nitrogen fertilization. The results of the evaluation of SE indicated a noticeable relation to the RE. The same isolates which showed high RE had SE above 50% efficacy of nitrogen fertilization like reference *R. leguminosarum* strain F17. The highest SE (86.8%) was observed after the application of the isolate HCC321 which also showed the highest RE among isolates originated from the common bean nodules.

Table 22. Symbiotic effectiveness and symbiotic efficiency of selected 19 putative rhizobia used for the inoculation of P. vulgaris cv. BASTA.

Isolates	SDM.pl^{-2} (g)	Relative Effectiveness (%)	Symbiotic Efficiency (%)
HA2a	0.52 ± 0.023*	91.2	68.4
HA8	0.38 ± 0.070	66.7	50.0
HBA11	0.45 ± 0.030	78.9	59.2
HBA15a	0.48 ± 0.095	84.2	63.2
HCC321	0.66 ± 0.097	115.8	86.8
HC4	0.34 ± 0.049	59.7	44.7
HC10	0.36 ± 0.068	63.2	47.4
HEC1	0.34 ± 0.028	59.6	44.7
F17**	0.57 ± 0.061	100.0	75.0
HKK321	0.47 ± 0.044	82.5	61.8
HLD1	0.35 ± 0.050	61.4	46.1
HLE11	0.41 ± 0.078	71.9	54.0
HLE22	0.37 ± 0.044	64.9	48.7
HLE131	0.47 ± 0.083	82.5	61.8
HLo8	0.56 ± 0.118	98.9	73.7
HNG13	0.39 ± 0.059	68.4	51.3
HNG33	0.58 ± 0.073	101.8	76.3
HSL1	0.33 ± 0.050	57.9	43.4
HSL13	0.57 ± 0.118	100.0	75.0
HSL13a	0.64 ± 0.117	112.4	84.2
Nitr. Fert.	0.76 ± 0.064	133.3	100.0
Control	0.29 ± 0.029	50.9	38.2

**mean± standard deviation, **Reference strain R. leguminosarum F17, SDM – Shoot dry mass.*

The calculated correlation coefficients between determined parameters revealed significant correlations between NAN and NDM, NDM, and SDM for tested rhizobia independently of their origin (Table 23). However, the most efficient BNF isolates HCC321 (SE = 86.8 %) and HLS13a (SE = 84.2%) originated from common bean nodules were more efficient than the best BNF rhizobia HA2a (SE = 68.4 %) and HA8 (SE = 50.0%) originated from adzuki bean nodules. Also, the isolate HLo8 from the soil was efficient BNF rhizobium with SE = 73.7% in relation to nitrogen fertilization.

3.5. Effect of putative rhizobia on different common bean cultivars.

It is noticeable the differences in effectiveness and efficiency among the isolates (Table 15). For the possibilities of using the isolates according to their effectiveness on a host cultivar

plant, the isolates from the common bean, adzuki bean as well as from the soil, were selected for the inoculation of four common bean cultivars on pot experiment trials. Common bean cultivars originating from Angola (CATIOLO, MANTEIGA) and from Poland (BASTA, IGOŁOMSKA) were tested. Nine isolates, among them two with high symbiotic efficiency (HKK321, HLE131) and moderate symbiotic efficiency (HLD1, HLE22) previously isolates from common bean, high symbiotic efficiency (HA2a, HA8), and moderate symbiotic efficiency (HC4, HSL1) isolates from adzuki bean and the isolates with high symbiotic efficiency (HLo8) isolated from soil were tested.

3.5.1. The effect on nodulation.

The results of the NN, NAN, and NDM per plant are presented in figure 1, figure 2, and figure 3 respectively. The NN, NAN, and NDM per plant showed significant differences among isolates within cultivar ($p < 0.001$) and significant differences among cultivars. The isolates HA2a and HA8 both originated from arable land at Chipipa, showed the highest number of nodules (Figure 1), active nodules (Figure 2), and nodule dry mass per plant (Figure 3) in comparison with other tested isolates independently of the cultivar. The isolates HLo8 showed similar NN in comparison with isolates HA2a and HA8 on cultivar CATIOLO and MANTEIGA (Figure 1). However, the highest NAN on the cultivar CATIOLO (Figure 2). These isolates, on previous experiments of authentication (section 3.4.2) also showed the highest NN and NAN per plant (Table 14). The isolates HLo8 induced high values of NN and NAN on the cultivars CATIOLO, IGOŁOMSKA, and MANTEIGA but a low value of the NDM was observed. This result confirmed earlier observations with cv. Basta which showed also that nodules induced by isolate HLo8 were mostly small. The lowest value of NN, NAN, and NDM was noted at plants inoculated with isolates HLE22, HLE131, HLD1, HC4, and HSL1 independently of the cultivar (Figure 1, Figure 2, Figure 3**)**. These results indicate that these isolates, primarily isolated from the nodules of the adzuki bean, were also found as effective inoculants of four different common bean cultivars. This experiment with four cultivars confirmed the results of the previous experiment (section 3.4) with one cultivar, only.

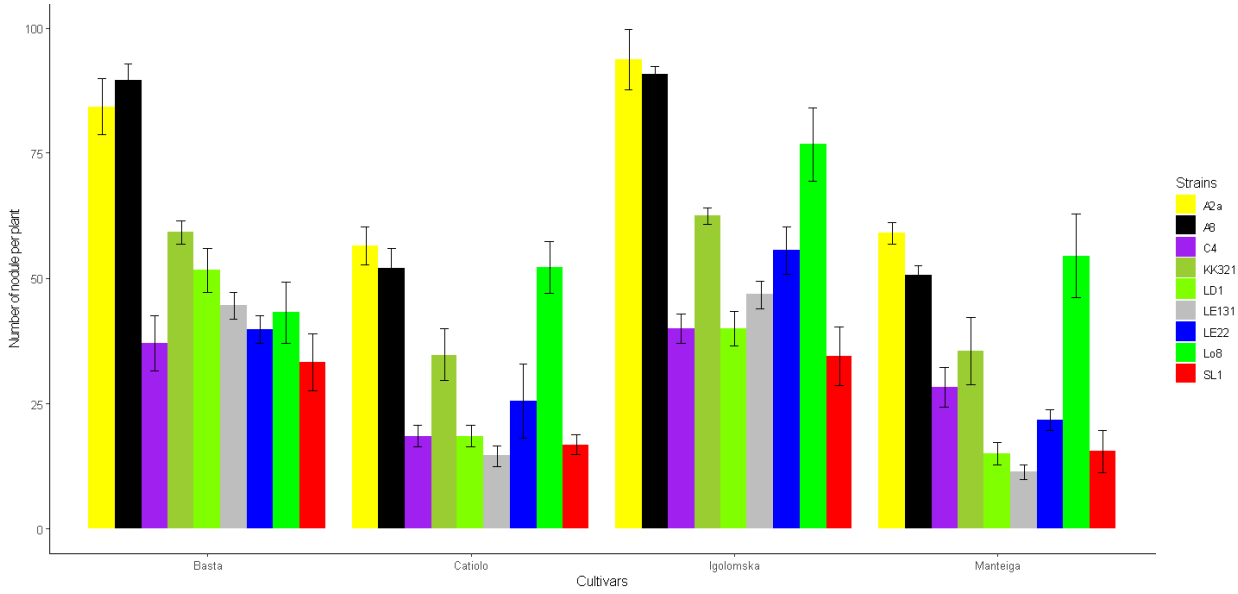


Figure 1. The effect of tested isolates on nodule number at four common bean cultivars

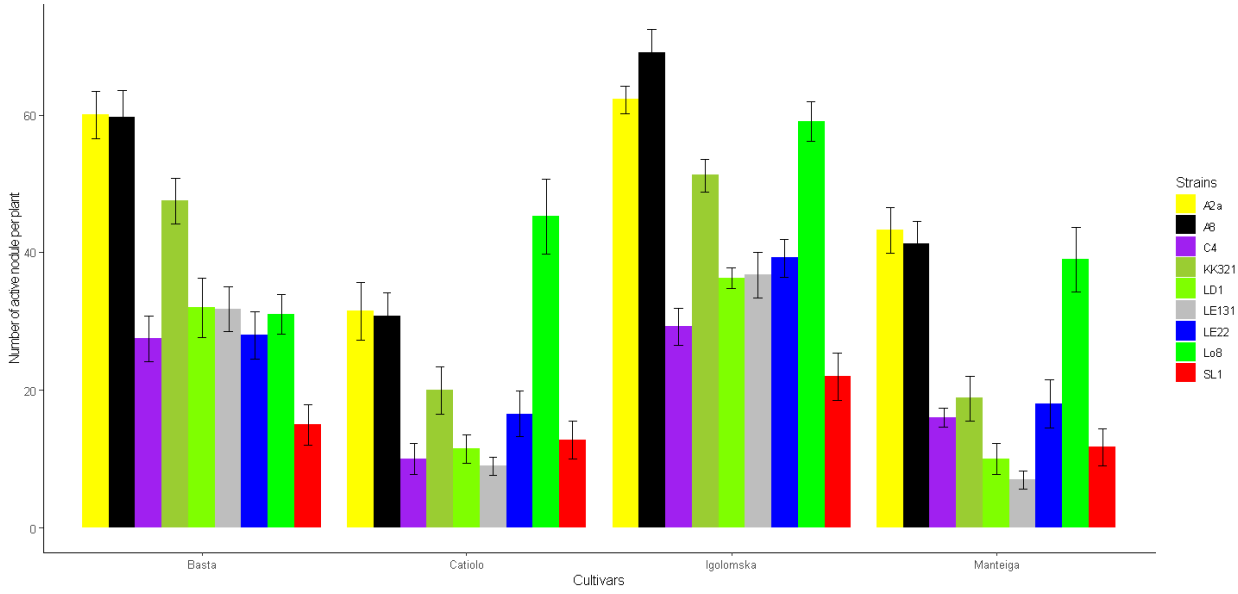


Figure 2. The effect of tested isolates on active nodules at four common bean cultivars.

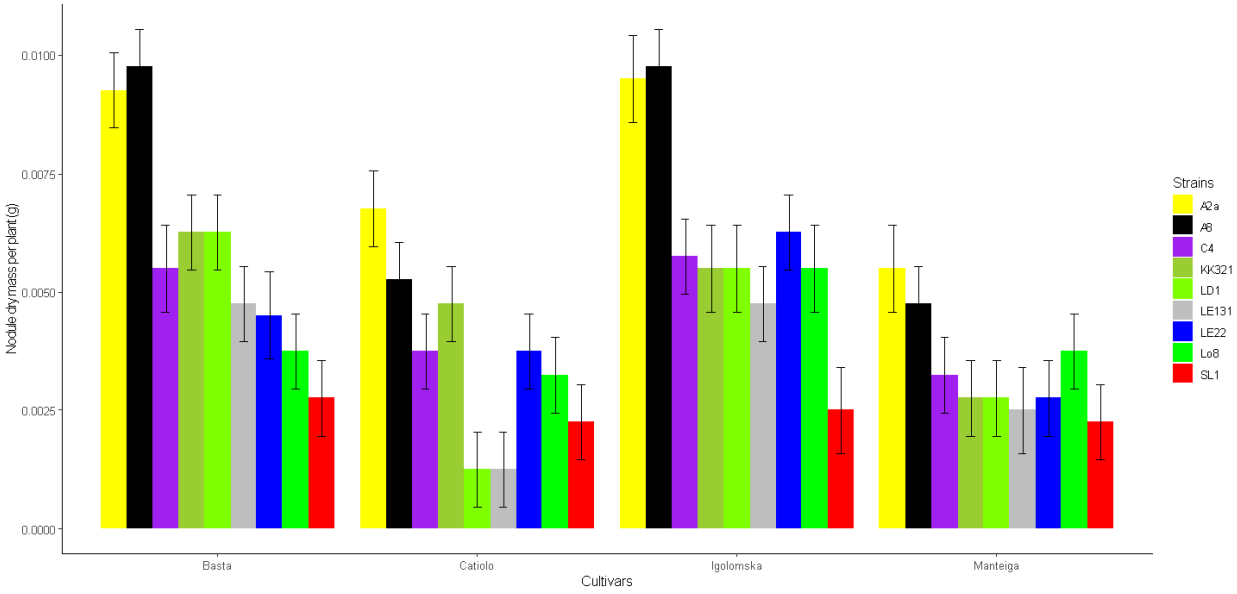


Figure 3. The effect of tested isolates on nodule dry mass at four common bean cultivars.

3.5.2. The effect on the shoots and roots dry mass.

The significant differences in SDM were observed among isolates within cultivar as well as the significant interaction between isolates and cultivar was found (Figure 4). The application of HA2a and HA8 isolates from the adzuki bean showed the highest SDM in comparison to the other isolates on cultivars BASTA and IGOŁOMSKA. The isolate HLo8 isolated from soil induced higher or similar SDM in comparison with isolates HA2a and HA8 on cultivar CATIOLO and MANTEIGA (Figure 4). The lowest SDM of plants was determined after inoculation by HSL1 isolated from the adzuki bean.

The result of the effect of selected isolates on the RDM per plant showed also significant differences among isolates within cultivars (Figure 5). The isolates HA2a and HA8 isolates from adzuki bean showed the highest SDM in comparison to the others isolate on cultivars BASTA and IGOŁOMSKA. The high value of SDM was observed after inoculating with isolate HLo8 on cultivar CATIOLO without significant difference in comparison with isolates HA2a and HA8 as well as with significant differences in cultivar MANTEIGA (Figure 5). The calculated correlation coefficients confirm the previous findings described above, that the high effective isolated nitrogen fixation bacteria, showed higher effectiveness independently of the cultivar as well as the primary origin of the isolates (Table 23).

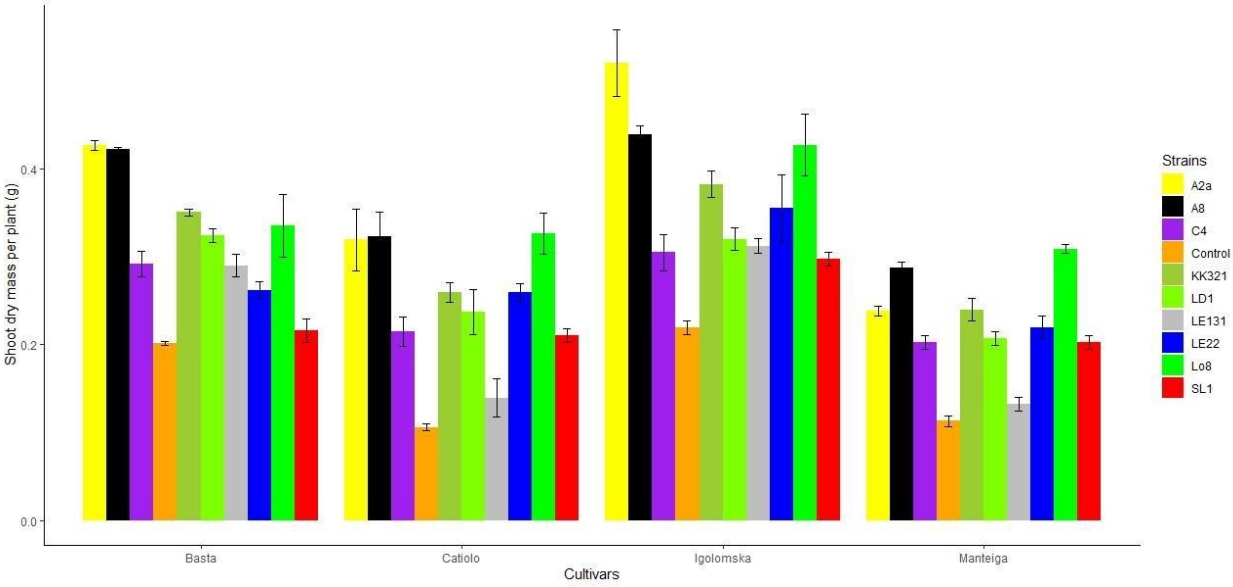


Figure 4. The effect of tested isolates on shoots dry mass at four common bean cultivars.

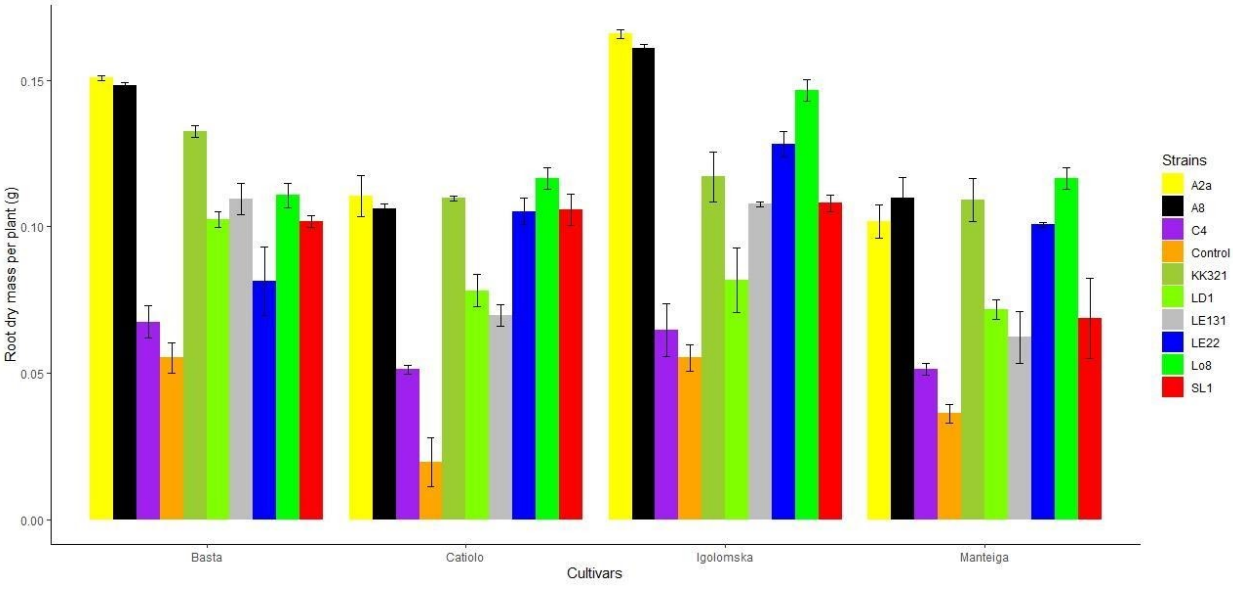


Figure 5. The effect of tested isolates on root dry mass at four common bean cultivars.

Table 23. Correlations factors of the biometric parameters of four *P. vulgaris* cultivars.

	NN	*NAN*	*NDM*	*SDM*	*RDM*
NAN	0.971***				
NDM	0.912***	0.879***			
SDM	0.915***	0.910***	0.852***		
RDM	0.880***	0.857***	0.761***	0.858***	

Computed correlation used Pearson-method with listwise-deletion.
*Codes: '***' significant at p < 0.001*

It was evident that the isolation of effective rhizobia directly from soils is not an efficient method for rhizobia isolation principally to assess biodiversity. I presume that the use of a trap plant is the better way of determining rhizobia biodiversity. The use of both common bean and adzuki bean for isolation revealed to be a greater advantage for the possibility of the isolated wide range of putative rhizobia. Most of the isolates from the root nodule of adzuki bean revealed also effective nitrogen-fixing bacteria nodulating common bean.

The authentication the efficacy and effectiveness of the putative rhizobia previously identified with common bean cv. BASTA was performed in a pot experiment using four different common bean cultivars. Most of the isolates, which showed high symbiotic efficiency, regardless of whether they were isolated from common bean, adzuki bean, or directly from the soil, also showed similar effectiveness after their use for inoculation of different cultivars. I presume that the use of one cultivar for authentication and selection of the effective isolates for field study is sufficient and faithful. The use of more than one cultivar results in time consumption and waste of money. Moreover, it is important that it was a possible selection of effective putative rhizobia nodulating common bean from different acid soils of the Huambo province. These results led me to select, among effective putative rhizobia, two isolates, HCC321 and HBA15a from common bean nodules, one isolate HA2a from adzuki bean nodule, and on isolate HLo8 originated from the soil, for further study under field condition in Angola.

3.6. Field experiment.

The field experiment was set-up at Gongoinga (Huambo province, Angola) with the common bean cultivar SONDEYOMBUA. Seeds were inoculated with isolates that showed the highest effectiveness and symbiotic efficiency previously in pot experiment. For this purpose, two putative rhizobia (HBA15a, HCC321) isolated from the nodule of common bean, one putative rhizobium (HA2a) isolated from the nodule of adzuki bean, and one (HLo8) isolated directly from the soil were used.

The climatic parameters during the vegetation period are summarized in Table 3. During the vegetation periods, the mean daily air temperature, as well as the humidity, were relatively similar in comparison to the average value of the previous four years. The average of the precipitation from August to October was lowest in comparison to the same period of the previous four years. Due to any precipitation in August and extremely low precipitation in September and the beginning of October, it was necessary to irrigate the experimental plots twice per week at approximately 10 - 15 mm from August to 10th October.

3.6.1. The effect of tested treatments on plant growth and nodulation.

The estimation of the number of putative indigenous rhizobia on the soil at the experimental field was done before sowing using the plate count technique on the YEMA-CR medium. The number of putative indigenous rhizobia was found at the level of 30 CFU g^{-1} of soil. The response of the plants to *Rhizobium* inoculation and fertilization treatments showed significantly different effects at all variables measured. Treatments inoculated only with isolate HBA15a (e.g. Photo 7 A), HCC321 (e.g. Photo 7 B), and HLo8 (e.g. Photo 7 C) showed good growth, good vigorous, and green color of the leaves (e.g. Photo 7 A), similar to plants treated with NPK, and urea fertilization (e.g. Photo 7 D). This result under field condition confirms the previous findings from the pot experiment as was described above in section 3.4.1. The positive synergic effect between inoculation and fertilization was observed due to the fact that treatment with the combination of inoculation and fertilization showed the best development of the plants. The control plants (e.g. Photo 7 E) showed a weak, little, and yellowish color of their leaves. In addition, plants inoculated with the isolate HA2a alone (Photo 7 E) were not so robust as the plants inoculated with isolates HBA15a, HCC321, and HLo8. However, it outperformed the control plants.

Photo 7. Differences in plant growth and on the color of leaves among treatments as the response of the inoculation under field conditions.

A – Plants inoculated with strain HBA15a, B – plants supplied with NPK and urea, C – Plants inoculated with strain HCC321, D – Plants inoculated with strain HLo8, E – Plants control (without inoculant and without fertilization), F – plants inoculated with strain HA2a.

The nodulation was determined sixty days after germination and the number of nodules per plant was determined by selecting nine plants from the two inner rows of each repetition and gently uprooted and washed. The NN per plant showed a significant difference among tested treatments (Table 24). The significatively highest NN (64.0), was found on roots of plants inoculated with the isolate HCC321 in the combination with NPK fertilization. The value of

NN was 3.4-times higher in comparison with plants supplied with NPK fertilization alone, and 6.2-times higher in comparison with control plants. The NN of plants treated with isolates HCC321 alone was 5-times higher than the control plants. Similarly, plants inoculated with isolates HBA15a and supplemented with NPK and urea fertilization showed 53.2 nodules per plant, and it was a 5-times higher number per plant in comparison to plant control. The lowest NN was observed on roots of plants inoculated with isolates HA2a in combination with NPK and additional with urea. However, when the plants were inoculated with HA2a alone showed a 3-times higher number of nodules per plant in comparison with control plants. These findings confirm the effectiveness of nodulation of the common bean by these isolates observed previously at the pot experiment described above (section 3.4.1). Independently whether the isolates were isolated from the soil or from nodules of the adzuki bean or of the common bean, the number of nodules observed under field condition showed the effectiveness of these strains for common bean inoculation under field conditions in Huambo province.

Table 24. The effect of tested treatments on the number of nodules per plant in the field experiment with *P. vulgaris* cv. SONDEYOMBUA.

Seed treatment	**Soil fertilization**			
	None	**NPK (N 12, P 24, K 12)**	**NPK (N 12, P 24, K 12) + Urea (N 21.8)**	**Average**
Control	10.4 g	18.8 f	19.3 b	16.2
HA2a	30.7 g	24.3 e	9.0 g	21.3
HLo8	34.3 cd	16.4 fg	41.6 de	30.8
HBa15a	35.7 cd	28.1 de	53.2 b	39.0
HCC321	52.2 b	64.0 a	30.8 de	40.0
Average	32.7 A	30.3 B	30.8 B	X

**values followed by the same small letters in the column and values of averages followed by the same capital letters are not significantly different according to Tukey HSD test ($p < 0.001$).*

I presume that the use of NPK fertilizer simultaneously with seed inoculation on the time of sowing influenced negatively the earlier stage of rhizobia infection and nodule formation. The isolates HA2a originally from the adzuki bean revealed more sensitivity to fertilization which resulted in decreasing the NN from 30.7 nodules per plant to 9 nodules when NPK and urea were applied (Table 24). In addition, the isolate HA2a and another effective putative rhizobium isolated originally from adzuki bean are an important step to implement the production of Adzuki bean in Angola.

The result of the determination of NDM per plant is presented in Table 25. The result shows a significant difference ($p < 0.001$) among the treatments. The highest NDM was found after seed treatment with rhizobium HBA15a (0.51 g plant^{-1}) alone in comparison to all other treatments. Similarly, the NDM of the plants treated only with isolates HCC321, HLo8, and HA2a showed significantly higher values in comparison to uninoculated plants. All plots supplied with NPK and urea fertilization showed the lowest NDM in comparison to plots with inoculation alone, except the plots treated with HCC321 in combination with NPK that showed a relatively high value of NDM. As was mentioned above the application of NPK fertilizer influenced negatively the nodulation. The lowest NDM was found after seed treatment with strain HA2a in combination with NPK and urea. These results led me to presume that the increase of available nitrogen in the soil by application of NPK fertilizer inhibited the effectiveness of tested isolates, and the HA2a isolate was most sensitive.

Table 25. The effect of tested treatments on the dry mass of nodules per plant in the field experiment with *P. vulgaris* cv. SONDEYOMBUA

Seed treatment	**Soil fertilization**			
	None	**NPK (N 12, P 24, K 12)**	**NPK (N 12, P 24, K 12) + Urea (N 21.8)**	**Average**
Control	0.03 h*	0.06 g	0.06 g	0.05
HA2a	0.31 e	0.31 e	0.02 h	0.21
HLo8	0.38 c	0.21 f	0.32 de	0.30
HBa15a	0.51 a	0.32 e	0.43 b	0.45
HCC321	0.35 d	0.44 b	0.24 f	0.34
Average	0.32 A	0.27 B	0.23 C	X

**values followed by the same small letters in the column and values of averages followed by the same capital letters are not significantly different according to Tukey HSD test ($p < 0.001$).*

The result of the determination of shoot dry mass per plant is presented in Table 26. The response of the plants to treatments showed significant differences ($p < 0.001$) among them similarly as was found for others biometric plant parameters. The highest value of SDM (35 g plant^{-1}) was found after the seed inoculation with isolate HBA15a in combination with NPK fertilization similarly like the highest value of NDM was found after seed treatment with this rhizobium. The SDM induced by HBA15a in combination with NPK was 27.1% higher than plants fertilized with NPK alone and 23.3% higher than plants fertilized with NPK and supplied with urea. These results show the synergetic effect between BNF rhizobia and fertilization. The use of selected putative rhizobia alone to improve the growth of tested

cultivar of common bean was clearly evident. The isolate HCC321 which was one of the most effective at pot experiments also under field conditions improved SDM by 78.7% in comparison with control plants. Other highly effective selected rhizobia, HLo8 and HBA15a, increase SDM in comparison to control plants by 71.2 and 67.1%, respectively. The effect of isolate HA2a was not significantly different in comparison to the control plants (Table 26).

Table 26. The effect of tested treatments on the shoot dry mass per plant in the field experiment with *P. vulgaris* cv. SONDEYOMBUA.

Seed treatment	**Soil fertilization**			
	None	**NPK (N 12, P 24, K 12)**	**NPK (N 12, P 24, K 12) + Urea (N 21.8)**	**Average**
Control	5.1 g	26.7 bcd	27.3 c	19.7
HA2a	6.7 g	11.9 f	17.5 e	12.0
HLo8	17.7 e	17.6 e	29.6 b	21.6
HBa15a	15.5 e	35.6 a	25.6 cd	22.2
HCC321	23.9 d	29.3 b	27.9 bc	27.0
Average	13.8 C	22.2 B	25.6 A	X

**values followed by the same small letters in the column and values of averages followed by the same capital letters are not significantly different according to Tukey HSD test ($p < 0.001$).*

The results of the determination of the RDM is presented in Table 27. A significant difference ($p < 0.001$) among treatment was observed. Plants inoculated with isolates HBA15a in combination with NPK give the highest value (3.25 g) of RDM per plant similarly like was found for SDM what was described above. The value of RDM of plants inoculated with this isolate corresponded to a 49.5% increase of RDM in comparison to plants fertilized with NPK alone and to 30.5% increase of RDM compared to plants supplied with NPK and urea. It is also noticeable that two other isolates HLo8, and HCC321, stimulated the RDM in control soil as well as on plots fertilized with NPK and urea similarly like HBA15a. There was no significant difference among treatments of bacteria inoculation in the combination with NPK and urea fertilization. Among the treatments with bacteria inoculation, the lowest RDM of plants was observed on plants inoculated with HA2a alone, without significant difference in comparison to control plants (Table 27). The lowest RDM of plants determined after application of this isolate is noticeable similar to the effect of this isolate on NN, NDM, and SDM, which also were the lowest one in comparison with three other isolates. These results also show the positive effect of selected isolates on the growth of the root system, as was described above in the pot experiment.

Table 27. The effect of tested treatments on the roots dry mass per plant in the field experiment with *P. vulgaris cv.* SONDEYOMBUA.

Seed treatment	Soil fertilization			
	None	NPK (N 12, P 24, K 12)	NPK (N 12, P 24, K 12) + Urea (N 21.8)	Average
Control	1.11 e	1.64 d	2.26 c	1.67
HA2a	1.17 e	1.69 d	2.29 c	1.72
HLo8	1.76 d	1.76 b	2.67 b	2.04
HBa15a	1.74 d	3.25 a	2.69 b	2.75
HCC321	1.65 d	2.37 c	2.66 b	2.13
Average	1.49 B	2.14 AB	2.55 A	X

**values followed by the same small letters in the column and values of averages followed by the same capital letters are not significantly different according to Tukey HSD test ($p < 0.001$).*

Table 28. The effect of tested treatments on SDM/RDM ratio in the field experiment with *P. vulgaris* cv. SONDEYOMBUA.

Seed treatment	Soil fertilization			
	None	NPK (N 12, P 24, K 12)	NPK (N 12, P 24, K 12) + Urea (N 21.8)	Average
Control	4.94 i	16.05 a	12.08 c	11.0
HA2a	5.36 i	7.15 h	7.77 g	6,8
HLo8	10.09 e	6.94 h	11.06 d	9.4
HBa15a	8.90 f	10.97 n	9.85 e	9.9
HCC321	14.23 b	12.05 c	11.13 d	9.3
Average	8.7 C	19.6 A	10.6 BC	X

**values followed by the same small letters in the column and values of averages followed by the same capital letters are not significantly different according to Tukey HSD test ($p < 0.001$).*

3.6.2. The effect of tested treatments on yield parameters.

The effect of tested treatments on the yield parameters (the number of pods per plant, the mass of 1,000 seeds, the final crop per square meter) was determined after the harvest. All these parameters were significantly different among treatments. The number of pods per plant (NPP) like the NN, NDM, RDM, and SDM was also significantly different among treatments ($p < 0.001$) (Table 29). The number of pods per plant is one of the important parameters which affect the final yield. The positive effect of tested isolates on the number of pods per plant was found as was expected based on the results of symbiotic effectiveness and efficiency of these isolates observed in the pot experiment as well as based on their effect on NDM, SDM, and RDM as was described above. Plants inoculated with isolates HLo8,

HCC321, and HBA15a on plots fertilized with NPK and urea, produced 33.9, 29.0, and 25.2% respectively higher NPP in comparison to plants with NPK and urea fertilization, only. Moreover, these isolates on plots fertilized with NPK improved the NPP in the range from 37.5 to 107.5% and on plots with NPK with urea improved the NPP in the range from 24.7 to 108.05% in comparison to unfertilized plots. Plants inoculated with isolates HCC321, HLo8, and HBA15a alone showed, respectively, 71.8, 56.3, and 54% NPP higher than on control plants. The isolate HA2a showed lower NPP in comparison with three other tested isolates but it was still 39.5% higher than on control plants. These results confirm the results of previous pot experiments with four different cultivars of *P. vulgaris* used for the evaluation of the efficacy of the selected putative rhizobia to fix atmospheric nitrogen.

Table 29. The effect of tested treatments on the number of pods per plant in the field experiment with *P. vulgaris* cv. SONDEYOMBUA.

Seed treatment	**Soil fertilization**			
	None	**NPK (N 12, P 24, K 12)**	**NPK (N 12, P 24, K 12) + Urea (N 21.8)**	**Average**
Control	4.9 g	17.4 d	15.4 d	12.6
HA2a	8.1 f	19.6 c	19.6 c	15.8
HLo8	11.2 e	15.4 d	23.3 a	16.6
HBa15a	10.7 e	22.2 ab	20.6 bc	17.8
HCC321	17.4 d	21.0 bc	21.7 ab	20.0
Average	10.5 B	19.1 A	20.1 A	X

******values followed by the same small letters in the column and values of averages followed by the same capital letters are not significantly different according to Tukey HSD test ($p < 0.001$).*

The effect of seed treatment and of soil fertilization on the dry mass of 1,000 seeds (MTS) and of the final yield of seeds of the common bean is presented in Table 30 and Table 31, respectively. A significant effect of tested treatments on the MTS and yield of harvested seeds was found. The harvested seeds of common bean inoculated with strain HBA15a in combination with NPK showed 377.9 g which was 12.5% higher than seeds collected from plants fertilized with NPK alone and was 15.9% higher than harvested on the plots fertilized with NPK and urea. Similarly, the high MTS was determined after the harvest of plants inoculated with the isolate HA2a in combination with NPK and urea which was more than 13% higher than collected from uninoculated plants (Table 30).

Table 30. The effect of tested treatments on the mass of 1,000 seeds in the field experiment with *P. vulgaris* cv. SONDEYOMBUA.

Seed treatment	Soil fertilization			
	None	**NPK (N 12, P 24, K 12)**	**NPK (N 12, P 24, K 12) + Urea (N 21.8)**	**Average**
Control	308.7 g	330.5 ef	317.8 fg	319.0
HA2a	307.8 g	340.2 de	369.4 ab	339.1
HLo8	340.7 de	341.3 de	358.8 bc	346.9
HBa15a	320.8 fg	377.9 a	348.0 cd	348.9
HCC321	322.9 f	340.7 de	338.3 de	338.3
Average	320.2 B	346.1 A	346.5 A	X

**values followed by the same small letters in the column and values of averages followed by the same capital letters are not significantly different according to Tukey HSD test ($p < 0.001$).*

The observed significant different effect of tested treatments on grain yield per square meter was a result of the multiplier effect of NPP and MTS. The final yield of the harvested grain of the common bean is presented in Table 31. The isolates HCC321 and HLo8 in combination with NPK and urea showed the highest common bean yield, 414.8 and 412.4 g m^{-2} that correspond to 57.2% and 56.3% increase of yield in comparison to plants from plots fertilized with NPK and urea, only. Plants inoculated with HBA15a and additional fertilized with NPK yielded 333.9 g m^{-2}, which is 14.3% higher than plots fertilized with NPK alone. Among tested isolates, the lowest values of yield (94.5 g m^{-2}) were observed on plots with plants inoculated with HA2a alone. However, this strain applied for seed inoculation significantly improved the grain yield by 303.9% higher than harvested from control plots. Under field conditions in Huambo province of Angola, I observed that application of the most effective strains alone, such as HCC321, HBA15a, and HLo8 resulted with an increase of the yield of the harvested grain of *P. vulgaris* at the level of 854.9, 605.5, and 506.8% in comparison to control plants, respectively.

Table 31. The effect of tested treatments on the grain yield per square meter in the field experiment with *P. vulgaris* cv. SONDEYOMBUA.

Seed treatment	Soil fertilization			
	None	**NPK (N 12, P 24, K 12)**	**NPK (N 12, P 24, K 12) + Urea (N 21.8)**	**Average**
Control	31.1 l	236.5 g	263.9 f	177.2
HA2a	94.5 k	225.5 h	283.4 d	201.1
HLo8	157.6 j	263.5 f	412.4 a	277.6
HBa15a	188.3 i	333.4 c	374.6 b	298.8
HCC321	265.9 ef	275.0 de	414.8 a	318.6
Average	147.6 C	266.8 B	349.8 A	X

**values followed by the same small letters in the column and values of averages followed by the same capital letters are not significantly different according to Tukey HSD test ($p < 0.001$).*

Table 32. Correlation factors of measured parameters *P. vulgaris* cv. SONDEYOMBUA in the field experiment.

	NN	*NDM*	*SDM*	*RDM*	*SDM.RDM*	*NPP*	*GY.m^{-2}*
NDM	0.771***						
SDM	0.389***	0.150					
RDM	0.188*	0.158	0.767***				
SDM.RDM	0.372***	0.063	0.769***	0.265**			
NPP	0.332***	0.150	0.772***	0.761***	0.505***		
GY.m^{-2}	0.324***	0.156	0.717***	0.693***	0.451***	0.917***	0.475***

Computed correlation used Pearson-method with listwise-deletion

*Codes: '***' significant at $p < 0.001$, '**', significant at $p < 0.01$.*

The strong correlation (r = 0.717) between SDM and the yield, NPP, and other measured parameters (Table 32) under field conditions strongly support the effectiveness of isolates and their effects on the common bean described on pot experiment. The high correlation effect between SDM and the yield could be also explained as the result of the synergetic effect of inoculation fertilization. The yield of the common bean showed also a direct and positive relationship with the number of nodules, indicating the effectiveness of tested isolates and the importance of nodulation to improve the common bean yield. As was described above at pot experiment Shoot dry mass is one of the most faithful parameters to estimate the yield.

Summarizing the results of field experiments at Gongoinga, independently of the origin of the isolates, whether it was isolated directly from the soil or from nodules using common bean or adzuki bean as a trap plant, selected rhizobia revealed effective nitrogen fix under field conditions in central Angola. I assume that all isolates tested under field conditions, as well

as, majority of previously isolated bacteria can be acidic tolerant putative rhizobia. Because, for example, the isolates HBA15a and HLo8, as well as HA2a were originated from acidic soils (pH 4.3) at Bailundo and Elande, and isolate HCC321 from arable land at Chianga with pH 5.2. The results of this study focussed on the BNF rhizobia in arable and virgin soils of Huambo province clearly showed that in acid soils this group of bacteria is scarce. Moreover, the selection of BNF bacteria able to induce nodulation of two different promiscuous legume species also resulted in a limited number of rhizobia, and among them, only a few were found to be potentially high effective strains useful for agricultural practices and inoculant production.

3.7. Identification of isolates and phylogenetic analysis based on the sequencing of the 16S rDNA gene.

The 16S rDNA of 17 isolates of PGPB (section 3.3.1.) and 22 selected rhizobia strains (section 3.4.) were sequenced using the primers 27F and 1492R. The identification and phylogenetic analysis were done for mentioned above isolates and for two reference strains from collection of PIB-IUNG Puław, Poland *R. leguminosarum* F10 and F17.

The majority of PGPB were isolated from the nodules of the common bean or of adzuki bean inoculated with soils from arable land. The sequence and phylogenetic analysis revealed that most of them were clustered in *Burkholderia* and *Beijerinckia* genus (Table 33, Figure 9 and 11). Five isolates, HKK312a, HNG33a, HBA151, HKK213 and HCC31, were phylogenetically very similar to each other (Figure 11) and were in 100% similar to *Beijerinckia fluminensis* UQM 1685 as well as to *Agrobacterium tumefaciens* IAM12048. Four isolates HLo2, HLo11, HCC32, and HNG332 were phylogenetically very similar to each other (Figure 9). These isolates were closely related to *Burkholderia diffusa* strain R-15930 and to *Burkholderia puraguae* strain CAMPA with 99.7 - 99.8% of similarity. Two the most active IAA producers HLD221a and HLE111a were 99.8% similar to *Enterobacter huaxiensis* KHED8. Moreover, 3 other isolates were clustered with the Enterobacter genus. The isolate HLD211 was closely related to *E. wuhouensis* WCHE 120002 with 99.5% of similarity, the isolate HA8a was closely related to *E. ludwigii* EN-119 but the isolate HA8a was not closely related to none of recognized species of this genus (Figure 10). Among PGPB single isolates were closely related to *Herbaspirillum huttiense, Rhizobium pusense* or *Paraburkholderia caribiensis.*

Table 33. The results of the identification of selected PGPB isolates based on the sequencing of the 16S rDNA gene.

Selected isolates	Origin of the soil sample	Closer relative from the NCBI database	
		Name of species	Accession number Similarity (%)
HLD221a	Elande**	*Enterobacter huaxian is* KHED8	QZCT00000000 (99,8%)
HLE111a	Chilela**	*Enterobacter huaxian is* KHED8	QZCT00000000 (99,8%)
HLE321	Chilela*	N.D.	-
HLo2	Bailund***	*Burkholderia diffusa* R-15930	AM747629 (99,8%)
HCC31	Bailundo*	*Beijerinckia fluminensis* UQM 1685	EU401907 (100%)
HNG331	Gongoinga**	*Herbaspirillum huttiense* ATCC 14670	AB021366 (99,85%)
HMA3	Mande*	N.D.	-
HLo11	Bailundo***	*Burkholderia diffusa* R-15930	AM747629 (99,8%)
		Burkholderia puraguae CAMPA 1040	NBYX01000000 (99,8%)
HLD211	Elande**	*Enterobacter wuhouensis* WCHE 120002	SJOK00000000 (99,5%)
HLo7	Bailundo***	N.D.	-
HKK213	Chipipa*	*Beijerinckia fluminensis* UQM 1685	EU401907 (100%)
		Agrobacterium tumefaciens IAM12048	D12784 (100%)
HBA151	Bailundo**	*Beijerinckia fluminensis* UQM 1685	EU401907 (100%)
HNG332	Gongoinga**	*Burkholderia diffusa* R-15930	AM747629 (99,8%)
		Burkholderia puraguae CAMPA 1040	NBYX01000000 (99,8%)
HCC32	Chianga*	*Burkholderia diffusa* R-15930	AM747629 (99,7%)
HCK23	Chipipa*	*Rhizobium pusense* NRCPB10	KP142169 (99,4%)
		Agrobacterium salinitolerans YIC 5082	FJ969841 (99,4%)
HLo23	Bailundo***	*Paraburkholderia caribiensis* CIP10684	
HKK321a	Chipipa	*Beijerinckia fluminensis* UQM 1685	EU401907 (100%)
HNG33a	Bailundo	*Beijerinckia fluminensis* UQM 1685	EU401907 (100%)

*isolated from nodules of *P. vulgaris,* ** isolated from nodules of *V. angularis,* *** isolated directly from soil,

Most of the strains that showed an effective symbiotic relationship with the common bean cultivar BASTA and positive *nodC* and *nifH* were phylogenetically related to the *Rhizobium* genus (Table 34). Among them six strains (HBA15a, HLE11, HLE22, HLS13, HLS13a, HSL1) were clustered together and also with *Rhizobium miluonense* CCBAU 41251 with 99.6 - 100% identity. Isolate HLD1 was closely related to the mentioned strains however with 99.0% of similarity to *R. miluonense* CCBAU 41251 (Figure 6). The isolate HBA11 was phylogenetically different than described above cluster with *R. miluonense*. This strain was noticeable closely related to *Rhizobium tropici* NBRC 15247, *Rhizobium hainanense* I66, and

Rhizobium multihospitium CCBAU 83401 however with 99.0% of similarity, only. Other six active rhizobia (HCC321, HBA15, HLE131, HLo8, HLD311, HCP8) were clustered together and also with *R. aegyptiacum* 1010, *R. bangladesh tense* BLR175, and *R. binae* BLR195 with 100% of identity (Figure 6). This cluster was significantly phylogenetically different than described above groups. The reference strains F10 and F17 were most closely related to strain USAD2370 of *R. leguminosarum bv. vicia* and also clustered with *Rhizobium acidisoli* FH13, and *Rhizobium anhuiense* CCBA U23252 with 99.7 - 100% similarity. None of the isolated native rhizobia from Huambo soils was clustered together with reference strains (Figure 6). Additionally, as a member of *Rhizobium* genus were phylogenetically related one low effective isolate HNG13 and PGPB isolate HCK23 (Figure 11). They were very closely related and were similar to *R. pusense* strain NRCPB 10 with 99.4% identity. Also isolate HCA10 selected as active nodule inducers was found to be closely related to *Paraburkholderia kirstenbosch nsis* Kb15 with 99.1% of similarity. It was noticeable some differentiation between the biodiversity of BNF rhizobia related to their origin. Most of the strains isolated from the root nodule of the adzuki bean were phylogenetically similar to *Paraburkholderia kirstenboschensis*, to *Burkholderia diffusa*, or to *Rhizobium miluonense*. However, most isolates from nodules of the common bean were identified as *Rhizobium aegyptiacum/R. bangladeshense/R. binae,* or as *R. miluonense*. Also is noticeable that all strains identified as *R. miluonense* were isolated from forest virgin soils. Noteworthy is the fact that two strains HC4 and HEC1 were phylogenetically related to the *Paraburkholderia* genus. These strains are partly similar to each other. These strains were noticeably grouped out of clusters of recognized *Paraburkholderia* species. I assume that it can be a novel unidentified species. The identified as *Rhizobium miluonense* HLS13, *Paraburkholderia kirstenbosch nsis* HCA10, *Paraburkholderia sp.* HC4, and *Paraburkholderia sp.* HEC1 isolated from virgin soils originating from natural forests at Chillela and Cabinda of Huambo suggest that these species can be native to Huambo.

Table 34. The results of the identification of selected rhizobia based on the sequencing of 16S rDNA gene.

Selected isolates	Origin of the soil sample	Closer relative from the NCBI database	
		Name of species	Accession number Similarity (%)
Isolates originated from nodules of *P. vulgaris*			
HCC321	Chianga	*Rhizobium aegyptiacum* 1010	NR113739.1 (100%)
		Rhizobium bangladesh tense BLR175	JN648931 (100%)
		Rhizobium. binae BLR 195	JN648932 (100%)
HLD1	Elande	*Rhizobium miluonense* CCBAU 41251	EF061096 (98.0%)
HNG33	Bailundo	*Rhizobium sp.*	-
HBA15	Bailundo	*Rhizobium aegyptiacum* 1010	NR113739.1 (100%)
		Rhizobium bangladeshense BLR175	JN648931 (100%)
		Rhizobium. binae BLR 195	JN648932 (100%)
HBA15a	Bailundo	*Rhizobium miluonense* CCBAU 41251	EF061096 (100%)
HKK321	Chipipa	*Rhizobium sp.,*	-
HLE11	Chilela	*Rhizobium miluonense* CCBAU 41251	EF061096 (99.9%)
HLE22	Chilela	*Rhizobium miluonense* CCBAU 41251	EF061096 (99.6%)
HLS13	Elande	*Rhizobium miluonense* CCBAU 41251	EF061096 (99.6%)
HLS13a	Elande	*Rhizobium miluonense* CCBAU 41251	EF061096 (99.8%)
HNG13	Gongoinga	*Rhizobium pusense* NRCPB10	FJ969841 (99.4%)
		Agrobacterium salinitolerans YIC 5082	KP142169 (99.4%)
HLE131	Chilela	*Rhizobium aegyptiacum* 1010	NR113739.1 (100%)
		Rhizobium bangladeshense BLR175	JN648931 (100%)
		Rhizobium. binae BLR 195	JN648932 (100%)
HCP8	Chipipa	*Rhizobium aegyptiacum* 1010	NR113739.1 (99.9%)
		Rhizobium bangladeshense BLR175	JN648931 (99.9%)
		Rhizobium. binae BLR 195	JN648932 (99.9%)

Table 34. Continuation

Selected isolates	Origin of the soil sample	Closer relative from the NCBI database	
		Name of species	Accession number Similarity (%)
Isolates originated from nodules of *V. angularis*			
HA2a	Chilela	*Rhizobium sp.*	-
HA8	Chilela	*Rhizobium sp.*	-
HEC1	Chilela	*Paraburkholderia sp.*	-
HBA11	Bailundo	*Rhizobium hainanense* I66	U71078 (99.0%)
		Rhizobium multihospitium CCBAU 83401	EF035074 (99.0%)
		Rhizobium tropici NBRC 15247	NR113739.1 (99.0%)
HSL1	Elande	*Rhizobium miluonense* CCBAU 41251	EF061096 (99.8%)
HCA10	Cabinda	*Paraburkholderia kirstenboschensis* Kb15	HF674707 (99.1%)
HC4	Cabinda	*Paraburkholderia sp.*	-
HLD311	Elande	*Rhizobium aegyptiacum* 1010	NR113739.1 (100%)
		Rhizobium bangladeshense BLR175	JN648931 (100%)
		Rhizobium. binae BLR 195	JN648932 (100%)
Isolates originated directly from soil samples			
HLo8	Bailundo	*Rhizobium aegyptiacum* 1010	NR113739.1 (100%)
		Rhizobium bangladeshense BLR175	JN648931 (100%)
		Rhizobium. binae BLR 195	JN648932 (100%)
Reference strains			
F10*	Poland	*Rhizobium acidisoli* FH13	KJ921033 (99.7%)
		Rhizobium anhuiense CCBAU 23252	KF111890 (99.7%)
		Rhizobium indigoferae NBRC 100398	NR_113895 (99.7%)
		Rhizobium laguerreae FB206	JN558651 (99.7%)
		Rhizobium leguminosarum LMG 14904	AF345271 (99.7%)
F17*	Poland	*Rhizobium acidisoli* FH13	KJ921033 (100%)
		Rhizobium anhuiense CCBAU 23252	KF111890 (100%)
		Rhizobium indigoferae NBRC 100398	NR_113895 (100%)
		Rhizobium laguerreae FB206	JN558651 (100%)
		Rhizobium leguminosarum LMG 14904	AF345271 (100%)

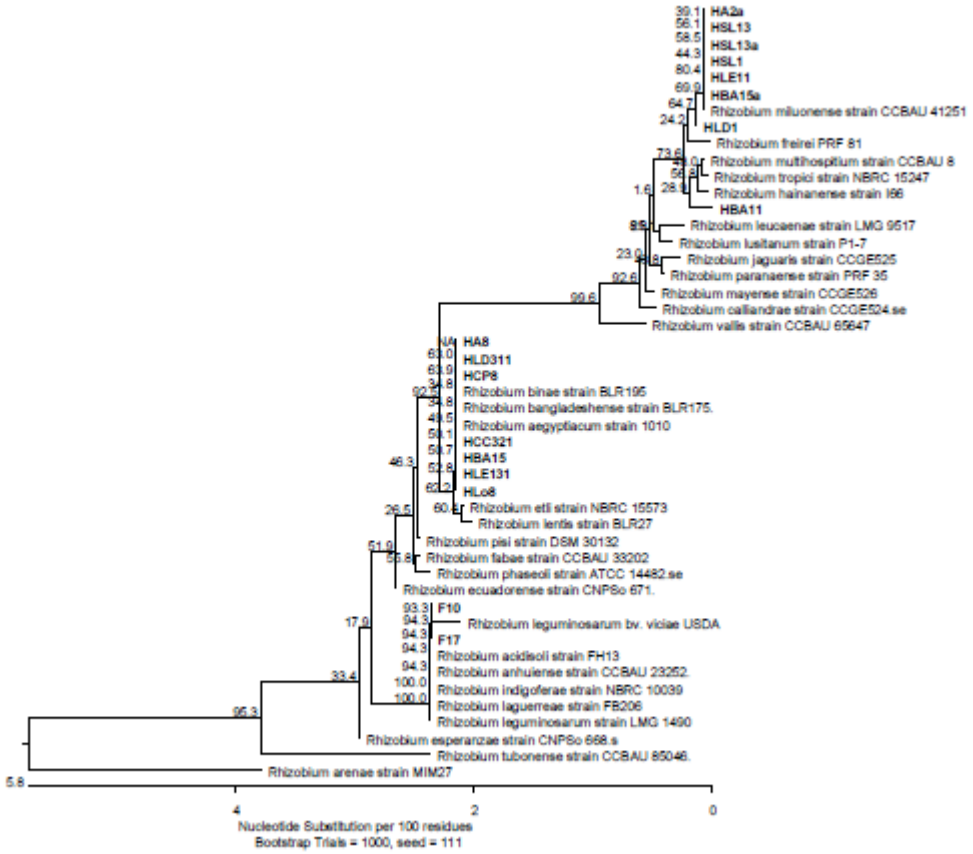


Figure 6. Phylogenetic tree based on analysis of the partial 16S rRNA gene sequences, showing the relationships among recognized species of the genus *Rhizobium*. The tree was constructed by the Clustal V method of the DNAStar the neighbor-joining method using a software package (DNAStar Lasergene, Inc., Madison, USA). Bootstrap values (Trials = 1000) are indicated at the branching points.

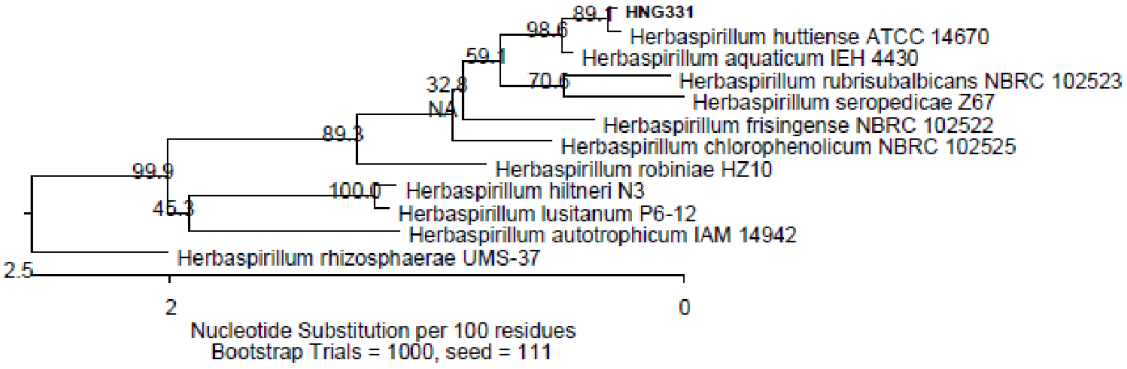


Figure 7. Phylogenetic tree based on analysis of the partial 16S rRNA gene sequences, showing the relationships among recognized species of the genus *Herbaspirillum*. The tree was constructed by the Clustal V method of the DNAStar the neighbor-joining method using a software package (DNAStar Lasergene, Inc., Madison, USA). Bootstrap values (Trials = 1000) are indicated at the branching points.

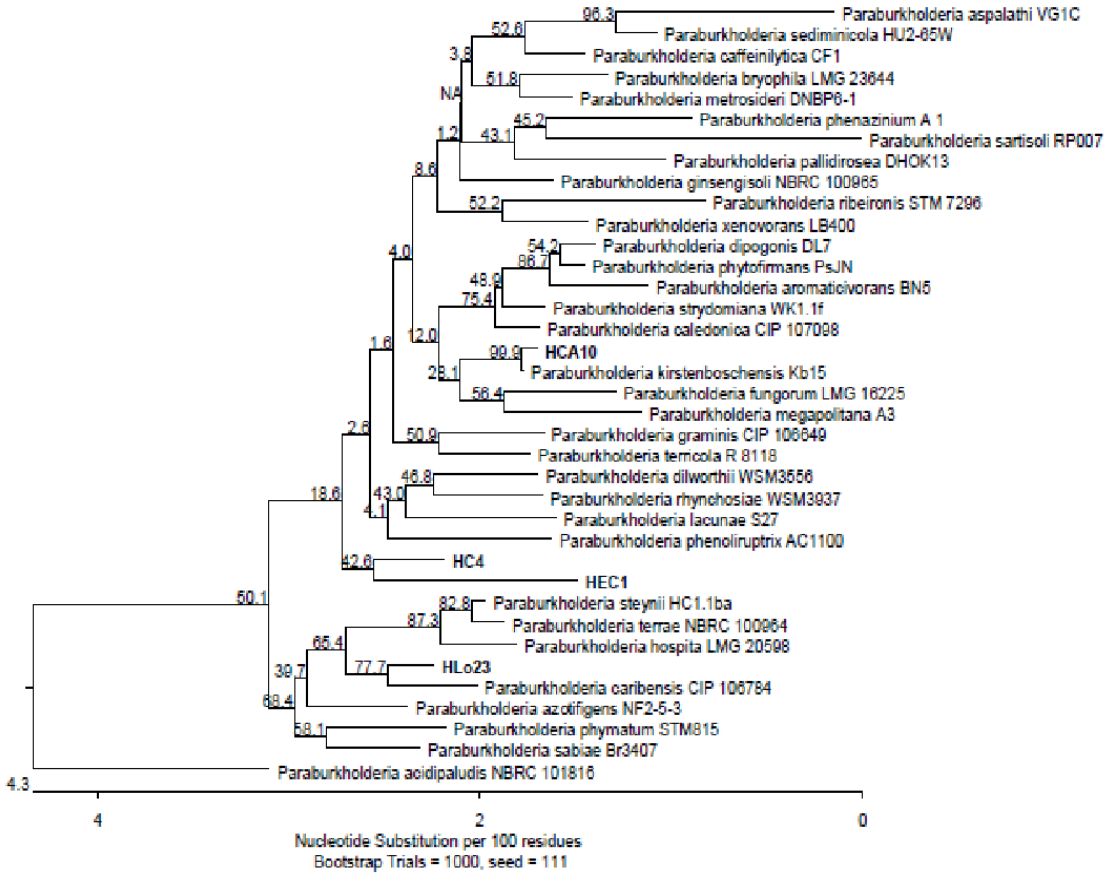


Figure 8. Phylogenetic tree based on analysis of the partial 16S rRNA gene sequences, showing the relationships among recognized species of the genus *Paraburkholderia*. The tree was constructed by the Clustal V method of the DNAStar the neighbor-joining method using a software package (DNAStar Lasergene, Inc., Madison, USA). Bootstrap values (Trials = 1000) are indicated at the branching points.

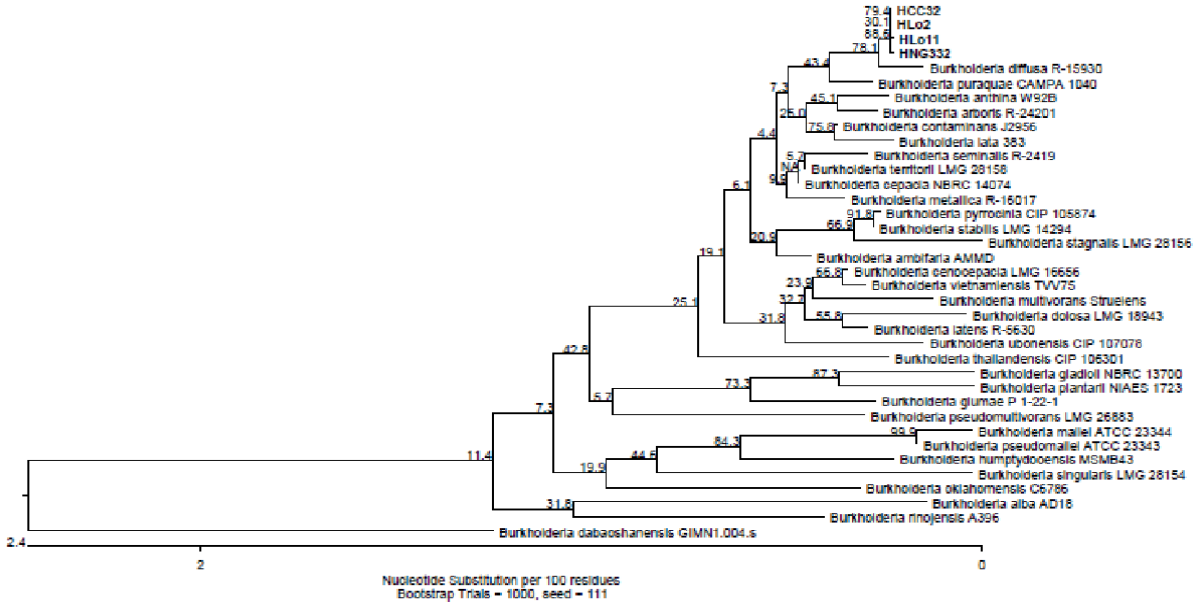


Figure 9. Phylogenetic tree based on analysis of the partial 16S rRNA gene sequences, showing the relationships among recognized species of the genus *Burkholderia.* The tree was constructed by the Clustal V method of the DNAStar the neighbor-joining method using a software package (DNAStar Lasergene, Inc., Madison, USA). Bootstrap values (Trials = 1000) are indicated at the branching points.

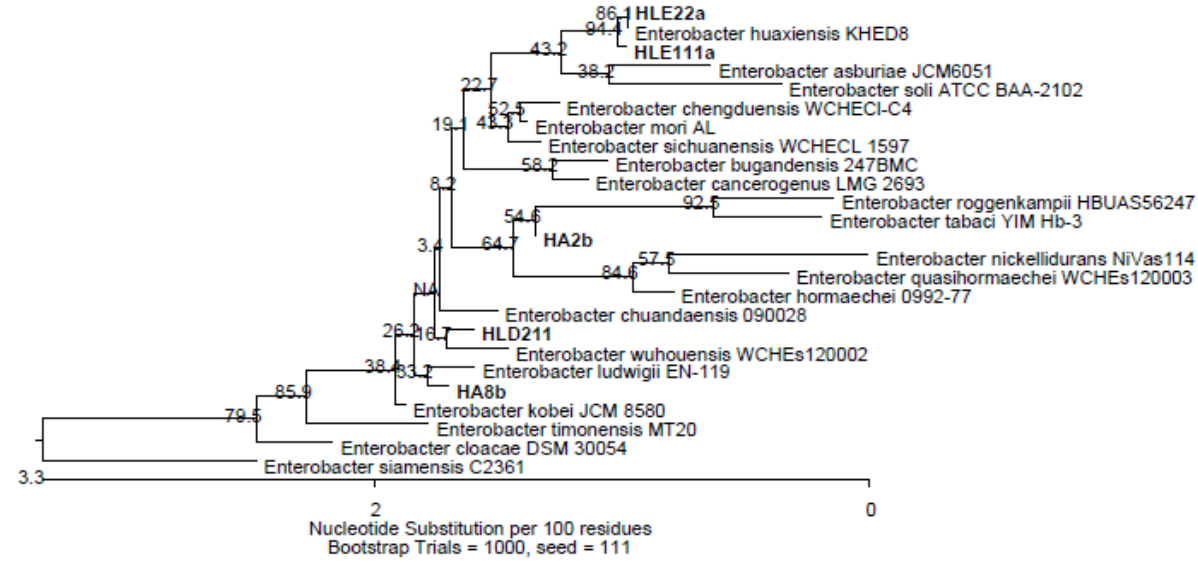


Figure 10. Phylogenetic tree based on analysis of the partial 16S rRNA gene sequences, showing the relationships among recognized species of the genus *Enterobacter.* The tree was constructed by the Clustal V method of the DNAStar the neighbor-joining method using a software package (DNAStar Lasergene, Inc., Madison, USA). Bootstrap values (Trials = 1000) are indicated at the branching points.

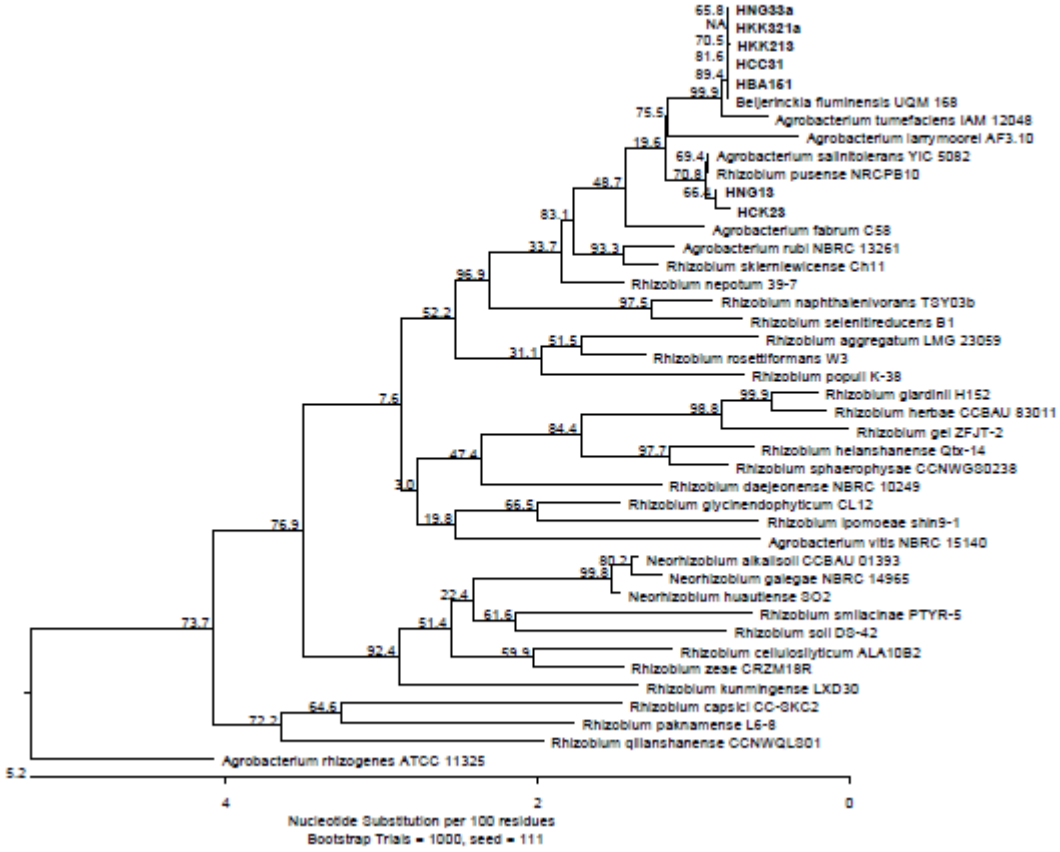


Figure 11. Phylogenetic tree based on analysis of the partial 16S rRNA gene sequences, showing the relationships among recognized species of the genus *Beijerinckia.* The tree was constructed by the Clustal V method of the DNAStar the neighbor-joining method using a software package (DNAStar Lasergene, Inc., Madison, USA). Bootstrap values (Trials = 1000) are indicated at the branching points.

4. DISCUSSION

For isolation of a wide range of BNF rhizobia from different soils at Huambo province and from the desert soil at Namide province, I used the common bean (*P. vulgaris* L.) and the adzuki bean (*V. angularis* (Willd.) Ohwi & H. Ohashi) as trapping plants because they are well known promiscuous species (Perret *et al.,* 2000; Broughton *et al.,* 2000; Kimura *et al.,* 2004). Adzuki bean is an overseas promiscuous specimen, which is not grown in Angola. The aim of its use as a trapping plant was the isolation of another BNF rhizobia not able to nodulate common bean. Additionally, I used it for isolation of BNF rhizobia directly from the same soils, a semi-selective YMAA medium (Graham, 1969). The study on this magnitude was the pioneer ones in Angola. The only available study related to the characterization of indigenous rhizobia in Angola was performed in the Chitembo area in the Okavango River region by Grönemeyer *et al.* (2013; 2014). They isolated BNF rhizobia from nodules of cowpea (*V. unguiculata*), Bambara groundnut (*V. subterranea*), peanut (*Arachis hypogaea*), hyacinth bean (*Lablab purpureus*), and common bean (*Phaseolus vulgaris*), cultivated by smallholder farms in this region. The majority of indigenous rhizobia identified by Grönemeyer *et al.* (2014) were related to the *Bradyrhizobium* genus, only six were identified as *Rhizobium sp.* nodulating common bean. A review focused on the biodiversity of BNF rhizobia in Sub-saharan Africa also notes a noticeable gap in the knowledge about the presence of fast-growing BNF rhizobia in Angola (Bongo and Pietr, 2019).

The rhizobia-like bacteria were not found in the desert soils. The noticeable number of rhizobia-like bacteria were isolated from arable land, forest, and follow soils in Huambo. The main factor which influences the presence of rhizobia like bacteria was the physicochemical parameters of the soils. The soil from the desert was found to have a high concentration of Pb, Cd, and Ni and it gave the support for the assumption that these heavy metals have influenced negatively the development of the plants and the presence of the like-rhizobia bacteria on this region. Seed inoculated with the soil suspension from the desert did not induce nodules on promiscuous species as well as incubating on the YMAA medium none bacteria grew. These findings are supported by a study done by Bondarenko *et al.* (2010) who reported that a high concentration of Zn, Cd, and Ni inhibited the growth of rhizobia and other microorganisms. Also, corroborates with Ahmad *et al.* (2012) who reported that the soil with a high content of heavy metal becomes uninhabitable for microbial communities or unsuitable for crop

production. Similarly, Rother *et al.* (1983) observed a reduction in growth and symbiosis due to cadmium, lead, and zinc.

The soil properties significantly influence the presence of indigenous bacteria in the soils, consequently on the rhizobia diversity. The results of this study revealed a strong correlation between the number of nodules per plant and the CFU of rhizobia-like bacteria. Moreover, these biotic parameters correlated with soil properties such as the content of organic sulfur, the content of soil organic carbon, N/S ratio, the content of available Mg, and Mn. The study was done by Zephania *et al*. (2015) in northern Tanzania reported that the rhizobia number in the soil was influenced by the content of phosphorus and magnesium. Also, Kawaka *et al.* (2014) in the west of Kenya observed a high number of rhizobial in soil with low Al and Cu. Moreover, Benson *et al.* (2015) reported that soil acidity and P levels influenced the relative dominance or absence of the different groups at any particular site. They observed that the low value of CFU of rhizobia-like bacteria, as well as the small number of nodules per plant, was noticeably correlated with the soil acidity. According to Jordan (1984), the optimum soil acidity for BNF rhizobia occurrence is considered to be between 6.0 and 7.0, and relatively few rhizobia remaining viable at pH less than 5.0 (Graham *et al*., 1994). Lately, Diouf et al. (2007) in Senegal and Agbenyega (2015) in Ghana noted the limited number and no nodules on the roots of *Acacia seyal* (Del.) and on the roots of African yam bean (*Sphenostylis stenocarpa* Sonder) grown in acidic soils with pH in the range 5.8 - 5.4, respectively.

Active nodules on the roots of used promiscuous plants common bean and adzuki bean were evidence of the presence of the active BNF rhizobia in the tested soils. Except for the soil from the desert, all tested soil samples showed the existence of active indigenous BNF bacteria with the ability of symbiotic interaction with both trapping species. Most of them can be natural to Huambo soils, precisely those bacteria that were isolated from the virgin lands. These results are supported by several earlier studies that reported that native BNF rhizobia can be isolated from different SSA soils without a history of growing *Fabaceae* plants or deliberate inoculation (Zephania *et al*., 2015; Kawaka *et al.,* 2014; Chemining'wa *et al*., 2013; Mwangi *et al.,* 2011; Mwenda *et al.,* 2011; Anyango *et al.,* 1995).

Taking into consideration that the majority of the arable land in Huambo is an acidic, fact that I found active nodules on both promiscuous plants, common bean, and adzuki bean, indicated the potential resources of active BNF rhizobia in virgin and arable soils. That is the evidence

of the presence of native rhizobia, with the ability to tolerate soil acidity and the ability to effectively nodulate legumes plants. what opens the opportunity for future production of the inoculant based on soil acidity tolerant rhizobia. My result is corroborated with Hungria and Vargas (2000) and Abd-Alla *et al.* (2014) who reported that acidic soils can be used as a source for isolation of BNF rhizobia that tolerate acidic soil environments and may improve the acid tolerance of the legume through efficient symbiotic nitrogen fixation under acidic conditions. Also, other studies reported the occurrence of BNF rhizobia in acidic soils (Muleta *et al.,* 2017; Foster, 2000; Chen *et al*., 1993). According to Waswa *et al*. (2014), the native rhizobia that showed high symbiotic effectiveness is a suitable source for inoculant production. In addition, the native strains are more useful for inoculant production due to their competitiveness in nodule infection and the good adaptation to the local agro-climatic conditions (Meghvansi *et al*., 2010; Tena *et al.,* 2016; Ulzen, 2013;).

Besides the isolation of BNF rhizobia, I found several bacteria that did not induce nodule formation, however, they were able to increase SDM by 45% in comparison to no-inoculated plants common bean. This led to presume that there are plant-growth-promoting bacteria. This was confirmed by analysis of IAA production that showed a positive correlation between IAA production and SDM. My presumptuousness was supported by Grönemeyer *et al.* (2013) and Fierro-Coronado *et al.* (2014) who reported that in nodules and in the rhizosphere of different legumes occurring bacteria which stimulated the growth due to production of IAA. Most PGPB that I identified in my study belong to *Burkholderia* and *Beijerinckia* genus, however, the most active IAA producers were closely related to *Enterobacter huaxiensis.*

The low number of isolated bacteria, and the phenotypic characteristics that were found among isolates in this study, indicate that the rhizobia diversity in Huambo regions is narrow. Several isolated bacteria were found in soils without a history of inoculation and common bean cultivation. On the other hand, the majority of isolates originated from arable land at Elande and Chianga, both soils have a history of common bean and soybean cultivation. This result suggests that the survival and occurrence of BNF rhizobia communities in soils are supported by the host plant often cultivated. I presume that the majority of putative rhizobia present in soil with common bean cultivation or history of seed inoculation were introduced in these regions from abroad. This is supported by the findings reported by Parr *et al*. (2013), indicating that the diversity of rhizobia is higher in areas where the host plant is cultivated,

while there is very low biodiversity in natural virgin lands. Also, the review paper of Bongo and Pietr (2019) concluded that in the published papers indicated there is low biodiversity of BNF rhizobia of *P. vulgaris* in the SSA region.

Moreover, the use of the YMAA medium to evaluate the diversity of rhizobia revealed it as an inefficient method, fundamentally in soils with no history of legume cultivation or seed inoculation. This was proved based on the fact that only a few putative rhizobia were isolated directly from the soil as well as the majority of them were originated from soils at Elande and Chianga with history of legume cultivations**.**

All strains isolated from nodules of common bean were acid producers and fast-growers. It is in agreement with several studies reported that the fast-growers and acid producers rhizobia are typical characteristics of rhizobia nodulating common bean. Several papers also described similar results. Among others, Kawaka *et al*., (2014), Muthini *et al.* (2014), as well as Koskey et al. (2018, 2017) in Kenya and in Lake Victoria Basin, found that all isolates from nodules of the common bean were fast growers and acid producers. Also, Torres-Gutiérrez *et al*. (2017) in Ecuador found only fast growers and acid producers rhizobia nodulating common bean. Besides fast growers, all slow growers and alkali producers that were found were isolated from the adzuki bean. This result is corroborated with the previous study noted by Han *et al.* (2009) in China and Delić *et al.* (2010) in Serbia, who reported that the adzuki bean is nodulated by slow growers rhizobia as typical characteristics of microsymbiont *Bradyrhizobium spp*.

The results of authentication support that all 19 selected putative rhizobia used for reinfecting host plant common bean are potential nitrogen fixation bacterial with a diverse level of symbiotic effectiveness and efficiency. Independently whether it was primarily isolated from adzuki bean, common bean, or directly from the soil. All selected isolates were able to induce the nodulation on the common bean with the high number of active nodules, promoting the growth of plants, as well as increased the SDM. Several reports described that the pinkish-red color of the inner of the nodule is good indicators of the existence of effective symbiotic rhizobia and nodulation efficacy is a suitable indicator of the presence of active BNF rhizobia in the soil, as well as the yield of shoot dry mass, is correlated with nodule mass (e.g. Tajima *et al*., 2007; Delic *et al.,* 2010; Mungai and Karubiu, 2010; Woomer *et al*., 2011; Patra *et al.*, 2012; Mhango 2015; Navabi, 2015; Namkeleja 2017). So it makes an appropriate proxy for

assessing the efficiency of the symbiosis and useful parameters to measure the nitrogen fixation potential of rhizobia as well as SDM is one of the best ways to measure the symbiotic effectiveness between rhizobia and legumes. The high correlation found in this study between NN and SDM as well as between SDM and NAN supports the effectiveness of selected isolates as nitrogen BNF rhizobia. Similarly, the positive correlation of these parameters was reported by several other researchers (Otieno *et al.,* 2009; Kawaka *et al*., 2014; Agoyi *et al.,* 2016; Ouma *et al.,* 2016; Rahim *et al.,* 2016; Koskey *et al*., 2017). In addition, Mungai and Karubiu (2010), observed that under field condition commercial inoculant Biofix and USDA 9030 performed poorly and did not show the correlation between the nodulation and the SDM due to competition with the high numbers of indigenous rhizobia present in the soil, as well as low adaptation to the local environment and others factors.

The majority of the native isolates described in this study on authentication assay revealed highly effective or moderately effective for common bean independently of the source of isolation (common bean, adzuki bean or directly from the soil) some of them showed higher symbiotic effectiveness than the European reference strain *R. leguminosarum* strain F17. A similar result was reported by Mungai and Karubiu (2010) in Kenya that native rhizobia nodulating common beans had higher symbiotic effectiveness compared to the commercial overseas inoculants Biofix and USDA 9030. Kawaka *et al.* (2014) in Kenya observed that all native isolates significantly increase in RDM, nitrogen concentration, and symbiotic efficiency in comparison to commercial CIAT 899. Similar results were reported by Koskey *et al.* (2017) in Eastern Kenya who observed that native rhizobia showed higher symbiotic efficiencies compared to the commercial Biofix.

Based on authentication comparative research of the effectiveness of selected strains of BNF rhizobia used to inoculate four different varieties of common bean I found that using one cultivar for selection active strains is faithful. Reactions of tested cultivars were similar after the application of the same isolates in comparison to control plants. My findings are further supported by Koskey *et al.* (2017) who reported that the symbiotic effectiveness of native isolated strains was no significant difference between the two common bean cultivars tested. As was described most of the identified rhizobia form effective symbiotic interaction with the common bean independently if were primarily isolated from common bean, adzuki bean, or directly from the soil.

The majority of isolated strains that showed effective symbiotic interaction with common bean were identified as *Rhizobium miluonense* or as a group *R. aegytiacum/R. bangladeshense/R. binae.* The result showed that these BNF rhizobia dominate in the soils of Huambo and probably tolerate the acidity of soils and high temperatures. The presence of different BNF rhizobia species, which are able to resist similar stress conditions was also reported from several SSA countries (e.g. Mwenda, 2017; Dall Agnol *et al.* 2017; Chibebe *et al.,* 2017; Shamseldin *et al.,* 2016; Onyango *et al.* 2015; Aserse *et al.* 2012; Odee *et al.* 2002). The results reported by Aserse *et al.* (2012) from Ethiopia, by Zinga *et al.* (2017) from Mozambique and South Africa, and by Attar *et al.* (2019) from Morocco who isolated BNF strains from the root nodules of common bean grown in acidic soils closely related to *Rhizobium miluonense* and/or to *R. leucaenae* within the *R. tropici* group according to phylogenetic analysis. The strains identified as *R. miluonense* were also isolated from nodules *Vigna unguiculata* by Chibebe *et al.* (2017) in Mozambique. Similarly, Ferreira *et al.* (2012) observed in the Amazonian region and Ribeiro *et al.* (2015) in Peru and Ecuador, that the strains tolerate low soil pH were also closely related to *Rhizobium miluonense*, *R. tropici,* and *R. multihospitium*. Mentioned above papers corroborate with my findings. The identified in my study 6 strains were closely related to *Rhizobium miluonense* CCBAU 41251. This strain was firstly isolated from the nodules of the *Lespedeza chinensis* (G.Don) as the original host (Gu *et al.,* 2007; 2008). However, I assume that these strains phylogenetically closely related to the *Rhizobium miluonense* CCBAU 41251 represent probably a species/subspecies also native in the region of Huambo due to the fact that it was isolated from virgin soils as well as similar strains were found under similar environmental conditions most SSA countries.

Six strains identified as *R. aegytiacum, R. bangladeshense,* and *R. binae* were originated from arable land with soils pH 4.3 - 5.2. Four strains HCC321, HBA15, HLE131, and HCP8 were isolated from nodules of common bean, one strain HLD311 was isolated from the nodule of adzuki bean, and the strain HLo8 was isolated directly from the soil. Mwenda *et al.* (2018) in Ethiopia also found strains nodulating common bean which clustered together with *Rhizobium aegytiacum, R. bangladeshense,* and *R. binae*. The strain *R. aegyptiacum* 1010, was primarily isolated from nodules of *Trifolium alexandrinum* (L.) in Egypt (Shamseldin *et al.* 2016). Another study was done by Chidebe *et al.* (2017) who isolated BNF rhizobia from nodules of *Vigna unguiculata* in Mozambique closely related to *Rhizobium binae*. *R. bangladeshense* and *R. binae* were primarily isolated from the nodules of *Lens culinari*s in Bangladesh by Rashid

et al. (2015). Lentils are the oldest pulse crop known, and among the earliest crops domesticated more than thirteen thousand years ago in the Old World. The lentil is indigenous to Western and Central Asia. Described above results, as well as my findings, suggest that these species are typical BNF rhizobia inhabiting soils in a warmer climate and probably were introduced from Asia by seeds thousands of years ago and remaining in soils.

Interesting was the confirmation of the presence of strains related to the *Paraburkholderia* genus, and all of them were originated from virgin sites or with no history of legume cultivation. Moreover, these *Paraburkholderia* strains revealed effective symbiotic rhizobia nodulating the common bean. Except for the strain closely related to *Paraburkholderia caribiensis* CIP 106484 that was isolated directly from the soil, others were isolated from the nodule of adzuki bean. One was closely related to *Paraburkholderia kirstenboschensis* Kb15 and two *Paraburkholderia sp.* were not clustered with any recognized species. The strain Kb15 of *Paraburkholderia sp.* was found originally in the root nodules of *Hypocalyptus* spp. in South Africa (Steenkamp *et al.,* 2015). Lately, it was reclassified as *Paraburkholderia kirstenboschensis* by Dobritsa and Samadpour (2016). Previously Gyaneshwar *et al.* (2011) reported that strains of *Paraburkholderia spp.* were found to nodulate several *Fabaceae* species including common bean in different regions. The ability of the *Paraburkholderia sp.* nodulating common bean was also reported by Dall'Agnol *et al.* (2016; 2017) in Brasil. The two unidentified *Paraburkholderia* strains originated from the natural forest at Cabinda and Chilela. I presume that these *Paraburkholderia* species can be native in subtropical SSA regions.

One of the isolated strains HBA11, isolated from the nodule of common bean, was closely related to *R. hainanense, R. tropici,* or *R. multihospitium.* These species were also isolated from the root nodule of *Vigna unguiculata* in Mozambique by Chibebe *et al.* (2017). The species identified as *R. hainanens*e was isolated firstly by Chen *et al.* (1997) from nodules of different legumes grown in China which are native for Asia and Africa. Described above results, as well as my findings, suggest that these species are typical native BNF rhizobia inhabiting soils in a warmer climate.

This was the first time to identify these rhizobia species effectively nodulating common bean from the soils of Huambo. A study was done by Grönemeyer *et al.* (2014), however, authors

focused on strains related to the *Bradyrhizobium* genus, nodulated other pulses only isolated strains identified as *Rhizobium sp.* were identified as the nodulating common bean.

Under field conditions, the *R. miluonense* strain HBA15a, *R. aegytiacum/R. bangladeshense/ R. binae* strains HCC321 and strain HLo8 showed high symbiotic effectiveness with the common bean. Plants inoculated with these strains showed a significantly high value of the NN, NDM SDM, and RDM in comparison to control plots. I strongly believe that the low number of bacteria in soil, at the level about 30 CFU g^{-1} of soil and the very low nitrogen content in the field, can be the factors that influenced positively on the symbiotic interaction. This result is supported by the previous findings reported by Asei *et al* (2015) and Agbenyega (2015) in Ghana who observed positive symbiotic interaction due to the absence of competition between the inoculant and native bacteria in the soil. Other studies reported by Rahim *et al.* (2016) in Pakistan and Hultman, (2018) in Swedish observe high symbiotic interaction with common bean when the rhizobia community in the soil is low. In addition, Mathenge *et al*. (2016) in Kenya and Wilson (2017) in Ghana reported that the effectiveness of symbiotic interaction of BNF rhizobia tends to be higher when the mineral nitrogen in the soil is low. Under field conditions, I observed that the use of inoculation in combination with fertilization resulted in a significant increase of SDM and RDM as the results of the synergistic effect. However, the nodule number was reduced on the plots with additional application of urea at the rate of 21.8 N kg ha^{-1}. These findings are consistent with Rebeschini *et al.* (2014) and Argaw and Akuma (2015) in Ethiopia who concluded that regardless of inoculation treatments, the NN and NDM decreased with increasing rates of nitrogen fertilization above 20 kg N ha^{-1}. The high level of nitrogen fertilization could totally inhibit legume-rhizobia symbiosis as was exemplified by Abdelmalik *et al.* (2015) in Sudan. They observed that nodule formation was completely inhibited in groundnut when nitrogen fertilization in the form of KNO_3 at the rate of 100 kg N ha^{-1} was added.

The yield of *P. vulgaris* inoculated with *R. aegyptiacum* strain HCC321, *R. aegyptiacum* strain HLo8, and *R. miluonense* HBA15a alone resulted with an increase of the yield at the level of 854.9, 506.8%, and 605.5 in comparison to control uninoculated plants, respectively. Moreover, the synergetic effect was observed between inoculants and fertilizer. After seeds treated with mentioned above selected strains of *R. aegyptiacum, R. miluonense,* and *Rhizobium sp.* in combination with NPK fertilization with additional urea, the yield was 14.3 -

57.2% higher in comparison to uninoculated plants on plots with the same level of NPK and urea fertilization. Among tested strains, *R. aegyptiacum* HCC321 and *R. miluonense* HBA15a were performed better on plots with additional fertilization under field conditions than strain HLo8. These findings indicated that the use of effective strain *R. aegyptiacum* HCC32 and strain *R. miluonense* HBA15a as seed inoculants for the common bean is sufficient enough to improve the yield either with or without additional fertilization.

However, my results are not in line with Arrow and Akuma (2015) who observed in Ethiopia that additional application of nitrogen fertilization in the range from 20 kg to 100 kg N ha^{-1} in the combination with inoculants did not significantly change grain yield of the common bean in comparison with nitrogen fertilization applied alone. Also, the results reported by Akter *et al.* (2013) from Kenya showed that the common bean inoculated with native rhizobia strains in combination with nitrogen fertilization up to 100 kg N ha^{-1} did not significantly affect the yield compared with control. Nevertheless, my results are consistent with a report of Wilson (2017) from Ghana who observed a significant increase in yield of groundnut due to inoculation with effective rhizobia in comparison to the control. In another study, Kyei-Boahen *et al* (2017) observed that inoculant together with phosphates increased grain yield by 56% compared with that for the control plants and applying phosphates alone increased grain yield by 30% compared to the control. Muleta *et al* (2017) reported that the isolates from Ethiopian soils resulted in yields similar to, or greater than, the application of 46 kg N ha^{-1}. Elkoca *et al* (2010) and Beshir *et al* (2015) observed that inoculation with different native *Rhizobium* strains increased common bean seed yields in the range from 6.8% to 18% in comparison to the control under field conditions.

In conclusion, I observed a predominance of distinct genotypes closely related to *Rhizobium miluonense, Rhizobium aegyptiacum, Paraburkholderia kirstenboschensis,* and *Parabulkholderia sp.* that was to date found in sub-Saharan Africa and other tropical regions. These results imply that the indigenous rhizobia are highly adapted to exclusive environmental conditions and should thus be preferred for implementation for the technology of inoculant in Angola, as one of the sustainable and rapid ways to improve the common bean production. Moreover, it could be helpful for the expansion of common bean production to the farms in the areas where this crop has not been cultivated yet.

5. CONCLUSIONS

1. The use of common bean and adzuki bean as the trap plants was more efficient to access the rhizobia diversity than isolated directly from the soil by semi selective medium.
2. The biodiversity of the BNF rhizobia nodulating the common bean in the studied regions of Huambo is low.
3. The use of one cultivar for authentication is a faithful method to identify the effectiveness and efficiency of the rhizobia.
4. Several isolates from the nodules of common bean and adzuki bean were Plant growth Promoting Bacteria for common bean.
5. The PGPB isolates were found in the phylogenetic tree closely related to *Burkholderia diffusa, Beijerinckia fluminensis*. *Herbaspirillum huttiense, Enterobacter ludwigii, Enterobacter wuhouensis*, and *Rhizobium puesense*.
6. The native rhizobia nodulating common bean that dominate in the regions of Huambo were closely related to *R. miluonense* or *R. aegyptiacum/R. bangladeshense/R. binae/ R. miluonense* group.
7. The strains isolated from the nodule of adzuki bean were closely related to *Paraburkholderia kirstenboschensis*, *Paraburkholderia caribiensis*.
8. Two isolated strains of the genus *Parabulkholderia* were not phylogenetic similar to any recognized species.
9. Seeds treated with *R. miluonense* strain HBA15a, *R. aegyptiacum* strains HCC321 and HLo8 alone improved common bean in the range 407 - 755%, in comparison to control plants.
10. Inoculation with strains HBA15a, HCC321, and HLo8 in combination with NPK fertilization with additional urea, increase yield in the range 14.3 - 57.2% higher in comparison to plants with the same level of NPK and urea fertilization.
11. The most effective rhizobia can be a potential candidate for the production of inoculants for common bean in Angola.

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