**ADDIS ABABA UNIVERSITY**
**SCHOOL OF GRADUATE STUDIES**
**DEPARTMENT OF MICROBIAL, CELLULAR AND MOLECULAR BIOLOGY**

# Genomic diversity, plant growth promoting properties and symbiotic effectiveness of *Mesorhizobum* spp. nodulating chickpea (*Cicer arietinum L.*) on acidic soils of Ethiopia: Implication for inoculant development to enhance production under low inputs Agriculture

**By**

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***A Dissertation submitted to the School of Graduate Studies of Addis Ababa University, in Partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Biology (Applied Microbiology)***

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**July 9, 2020**
**Addis Ababa, Ethiopia**

**Declaration**

I, the undersigned declare that this Dissertation I hereby submit for the Degree of Doctor of Philosophy (PhD) in Biology (Applied Microbiology) to the School of Graduate Studies of Addis Ababa University is my own independent work and has not been submitted to any other institution any where for the award of any academic degree, diploma or certificate. Any material obtained from other sources is duly acknowledged in the Dissertation.

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**Date**: July 9, 2020

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## Abstract

*Nitrogen and phosphorus deficiencies are the two most limiting factors in crop production. Although chickpea fixes nitrogen in association with diverse Mesorhizobium species, effectiveness in the amount of nitrogen fixed depends upon the inherent character and other factors of the endosymbionts. The nitrogen-fixing process requires more phosphrous and phosphorus deficiency aggravated under acidic soils because of sorption. Thus, selection of acid tolerant rhizobia with effective nitrogen fixation and phosphate solubilizing activity is essential in order to improve phosphorus and nitrogen fertility to enhance the growth and production of the crop in acidic soil. This study was initiated to collect chickpea rhizobia from acidic soils of central, western, southern and northern parts of Ethiopia and determine their genomic diversity, and further screen for low pH tolerance, solubilization of insoluble phosphate on tri-calcium phosphate medium and their potential to ecological adaptations and symbiotic effectiveness with host varieties on sand culture at low pH and acidic soil culture under greenhouse conditions. The genomic diversity was studied using illumina sequencing a whole-genome sequence (WGS) approach. The results showed that 81 Mesorhizobium strains were obtained and estimated with average genome sizes 7Mbp, average depth of 143 X coverage. Annotation of the assembled genome predicted an average of 6725 protein-coding genes (CDSs). Genome completeness showed that 81 strains are near complete (>99% complete). Concatenation of 400 universal phylophlan marker genes, present in all genomes (strains) allowed*

*detailed phylogenetic analysis, from which eight well-supported phylogenetic groups were identified. Phylogenetic reconstruction based on the symbiosis-related (nod C and nif H) genes were generally different to those shown by the phylophlan marker genes. The pattern of genomic diversity indicates the existence of multiple, broadly distributed phylotypes, with no relationship between geographic and genetic distance ($r=0.10$, $p<0.01$). The result indicated that 62 strains grew well at low pH 5; and 47 (76%) of them were phosphate solubilizers. The strains showed wide diversity in their substrate utilization and tolerance to salinity, high temperatures, $Mn^{2+}$ and $Al^{3+}$ toxicity, heavy metals and antibiotics. Symbiotic characterization also showed a wide diversity among these mesorhizobial strains. Moreover, most of the phosphate solublizing strains had multiple growth promoting characteristics (production of indole acetic acid, hydrogen cyanide, siderophore, ACC diaminase and inhibition of Fusarium oxysporum f.sp.ciceris growth) under in vitro conditions. Under field conditions, the mesorhizobial inoculants (a.117L2, a.71 and a.15star) showed significant ($P<0.01$) improvement in biological nitrogen fixation, growth, yield, nitrogen and phosphorus uptakes of chickpea compared to the commercially available local reference strain Cp41. The indigenous mesorhizobial strain a.117L2 was superior inoculant for almost all the tested parameters. Generally, the present study indicated that Ethiopian acidic soils contain symbiotically effective, phosphate solublizing, chickpea nodulating Mesorhizobium spp. endowed with different plant growth promoting attributes which are diverse in their genomic and taxonomic identities. Therefore, there is a potential advantage using these mesorhizobial inoculants to enhance chickpea production in acidic soils by improving phosphorus and nitrogen*

*fertility; and provide that further field trials recommended over several seasons and sites in Ethiopia.*

**Keywords:** Inoculants, Whole-genome sequence, Yields,

## Acknowledgements

First, I would like to heartily thank my supervisor Dr. Fassil Assefa for his unreserved guidance, help, and supervision, encouragements beginning from the very inception of this study to completion; useful suggestions and constructive comments on the writing up of the paper.

I am very much grateful to my co-superviser Dr. Kassahun Tesfaye for his wonderful guidance, suggestions and providing all the facilities beginning from sample collection, laboratory, and field activities throughout my study.

I would also like to express my deepest appreciations to Prof. Douglas R. Cook, from the University of California Davis (UCDavis), USA, for his unrelenting support and giving me the opportunity to undertake my study in his genomics laboratory with state-of- the-art facilities and full technical support from his research group. My appreciation and special thanks also extend to Dr. Breandan K. Riely for kindly assisting me in all laboratory activities during my stay in Davis, USA, Noelia Carrasquilla-Garcia for her wonderful support during library development and other activities; and Dr. Alex Greenlon for his support on bioinformatic analysis. I would like to thank all the staff members of the Professor Cook laboratory group for sharing their valuable experiences.

I am grateful to the Department of Microbial, Cellular and Molecular Biology, Addis Ababa University, for providing me the admission to pursue the PhD study. I would like acknowledge University of Gondar for sponsorship. I acknowledge the USAID through

PEER Program Cycle-4, administered by the U.S. National Academies of Sciences, Engineering, and Medicine (NASEM) grant for the financial support.

I am grateful to the staff members of the Bureau of Agriculture, development agents, and farmers from Holleta, Arsi, Bale, Asosa, Wollega, Sodo Gurage, Illu, Tulu Bolo and Wollo Woreds (districts) who kindly spared their time and participated during nodule and soil samples collection. My thanks also go to Debere Zeit Agricultural Research Center, EIAR for providing chickpea seed varieties, greenhouse and necessary materials during greenhouse experiments, especially to Mr. Nigusse Girma, Dr.Yifru Abera, Mr. Eresi Megersa, Mrs. Zehara Mohammed, Mr. Dagnachew Bekele, Mrs. Serkalem Tesfaye, Mr. Mohammed Yimam and Mrs. Alem Menda who helped me during the experiments. I am grateful to Mr. Wibshet Ayano for his help and all other relevant assistance beginning from planting to harvesting in greenhouse experiment.

I am also thankful to Holleta Center, Oromia Agicultural Bureau for providing land and technical assistance for the field work. The help of Mr. Addisu Ejeta and Mrs. Abebech Wordofa of Holeta agricultural Bureau for land preparation and harvesting during field trials is highly appreciated. I also acknowledge Mr. Semu Tuffer and his brother Abule Tuffer, farmer in Sodo (Endebyuo) woreda for providing land, unreserved help and materials support for the field experiment.

My love and heartfelt gratitude goes to my wonderful families for their unreserved encouragement and support through out the study period.

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## Lists of Acronyms and Abbreviations

| | |
|---|---|
| ACC deaminase | 1- aminocyclopropane-1-carboxylic acid |
| ANI | Average Nucleotide Identities |
| ASPs | Acid shock proteins |
| ATPase | Adenosine triphosphatase |
| BMY | Biomass yield |
| BTB | Bromothymol Blue |
| BNF | Biological nitrogen fixation |
| CSA | Central Statistical Authority |
| CDS | Protein-coding sequences |
| CEC | Cation-exchange capacity |
| CV | Coffiecent of varation |
| DAP | Day after emergence |
| DM | Dry Matter |
| DZARC | Debere Zeit Agricultural Research Center |
| EMBL | European Molecular Biology Laboratory |
| EPS | Exopolysaccharides |
| FAO | Food and Agriculture Organization of the United Nation |
| FAOSTAT | FAO Statistics |
| GC | Guanine-cytosine content |
| GHI | Grain harvesting index |
| GPS | Global Positioning System |
| GY | Grain Yield |
| HARC | Holleta Agricultural Research Center |
| HR | heavy metals resistance |
| HSW | Hundred Seed Weight |
| IAR | Intrinsic antibiotics resistance |
| LSD | Least Significant Differences |
| MGT | Mean generation time |
| ML | Maximum Likelyhood |

| | |
|---|---|
| MPN | Most probable number |
| NCBI | National Center for Biotechnology Information |
| Ndf | Nitrogen derived from fixation |
| NDW | Nodule dry weight |
| N50 | The shortest contig at 50% of the total genome |
| NN | Nodule number |
| NPP | Number of pods per plant |
| PGP | Plant growth promoting |
| PGPR | Plant growth promoting rhizobacteria |
| PSM | Phosphate solublizing mesorhizobia |
| RCBD | Randomized Complete Block Design |
| SAS | Stastical Analysis Software |
| SDW | Shoot Dry weight |
| SE | Symbiotic effectivenes |
| SEM | Standard error mean |
| WGS | Whole Genome Sequence |
| YEMA | Yeast Extract Manitol Agar |

**The following papers are prepared / submitted for publication**

1. Greenlon, A., Chang, P. L., Mohammed, Z., **Muleta, A**., and Carrasquilla-Garcia, N., Kim D., Nguyen H.P., Suryawanshi V., Krieg C.P., Yadav S.K., Patel J.S., Mukherjee A., Udupa S., Benjelloun I., Thami-Alami I., Patil B., Singh S., Sarma B.K., Eric J. B. von Wettberg, Yasin M., Kahraman A., Bukun B., Assefa F., Tesfaye K., Fikre A. and Cook D.R. (2019). Global-level population genomics reveals differential effects of geography and phylogeny on horizontal gene transfer in soil bacteria. *Proc. Natl. Acad. Sci. U.S.A.,* **116**: 15200–15209.

2. Atsede Muleta, Alex Greenlon, Kassahun Tesfaye, Fassil Assefa, Douglas R. Cook. Genomic diversity and distribution of *Mesorhizobium* nodulating chickpea (*Cicer arietinum L*.) from low pH soils of Ethiopia. **Prepared for submission.**

3. Atsede Muleta, Kassahun Tesfaye, Douglas R. Cook, Fassil Assefa. Screening for ecologically competent and symbiotically effective chickpea nodulating *Mesorhizobium* spp. isolated from acidic soils of Ethiopia. **Prepared for submission.**

4. Atsede Muleta, Kassahun Tesfaye, Tekle Haimanot Haile Selassie, Douglas R. Cook, Fassil Assefa. Phosphate solubilization and multiple plant growth promoting properties of *Mesorhizobium* species nodulating chickpea from acidic soils of Ethiopia. **Submiited.**

5. Atsede Muleta, Kassahun Tesfaye, Douglas R. Cook, Fassil Assefa. Evaluation of symbiotically effective indigenous *Mesorhizobium* inoculants on growth and productivity of chickpea in acidic soil under greenhouse and field conditions. **Prepared for submission.**

# CHAPTER 1

## General Introduction

### 1. 1. Background

Chickpea (*Cicer arietinum L.*) is mainly cultivated on residual moisture as food and feed crop in the cool, dry season of the semi-arid tropics (ICRISAT, 2013). The diverse agro-climatic conditions in Ethiopia make it very suitable for growing chickpeas in the highlands and semi-arid regions of the country (Gemechu Keneni *et al.,* 2012). Chickpea serves as a source of protein and cash crop to the rural community and parts of the urban population (Tadesse Getachew, 2019).

It is integrated into low input agriculture to improve soil fertility due to its ability to fix atmospheric nitrogen in association with root nodule bacteria belonging to the genus *Mesorhizobium* (Nour *et al*., 1995). According to Werner (2005), chickpea is capable of fixing 90-180 kg N $ha^{-1}$ in one cropping season depending on cultivar, bacterial strain, and environmental factors. There are different biotic and a biotic factors that undermine the full realization of biological nitrogen fixation (BNF). The absence of specific and effective rhizobia in the soil (Aslam *et al*., 2010), and soil acidity are some of the constraints affecting biological nitrogen fixation (Jaiswal *et al.*, 2018).

Soil acidity is one of the environmental factors that limit plant production, because it is associated with increased Aluminum (Al) and Manganese (Mn) toxicity and limit Calcium (Ca) and Phosphorus (P) uptake by plants (Hungria and Vargas, 2000). Jaiswal *et al.* (2018)

reported that phosphorus deficiency and Aluminium (Al) toxicity in acid soils have major effects on legume plant growth and function on symbiotic nitrogen fixation.

It is reported that low pH soil hinders legume production more than any other crops (Graham, 1992), for it affects the performance of nitrogen fixation in one of the following ways; growth of the host plant, the survival of rhizobia in the soil, affecting the exchange of molecular signals between rhizobia and their hosts, thus reducing nodule growth and nodule function (Hungria and Vargas, 2000). However, legumes and their rhizobia exhibit varied responses to acidity (Guo *et al*, 2009).

The negative effect of soil acidity on legume growth can be partly alleviated through selection of tolerant host varieties and endosymbionts that perform better on acid prone soils (Graham, 1992; Rodrigues *et al*., 2006; Brigido *et al.,* 2007). According to Brigido *et al.,* (2007), chickpea is one of the successful legume-rhizobia associations adapted to perform well under acidic conditions, and acid tolerant *Mesorhizobium* strains isolated from chickpea had higher symbiotic effectiveness under greenhouse condition on acidic soils of Portugal compared to the reference strain.

Next, to nitrogen, phosphorus is also the major limiting nutrient for plant production. This is because phosphorus affects legume growth, nodule formation, and development (Alikhani *et al.,* 2006; Nasr Esfahani *et al.,* 2016). Nasr Esfahani *et al.* (2016) have shown that the beneficial effect of P on nitrogen fixation in chickpea and phosphorus is also essential for the growth and functioning of root nodules.

It has been recently established that rhizobia, apart from fixing inorganic nitrogen, improve plant nutrient by mobilizing inorganic and organic P, indicating that they have a dual ability of nitrogen fixation and phosphate solubilization of different crop legumes (Piex *et al.,* 2001; Alikhani *et al.,* 2006; Hmissi Imen *et al*., 2015; Dereje Tsegaye *et al*, 2015). Other studies also showed that these rhizobia have other plant growth promoting (PGP) properties to improve legume production (Piex *et al.,* 2001; Glick, 2012). This indicates that phosphate solubilizing, and other plant growth promoting and nitrogen-fixing *Rhizobium* increase nitrogen and phosphorus status in leguminous crops to enhance plant health and soil fertility in agricultural soils.

In Ethiopia, soil acidity is becoming a serious problem in different highland soils and particularly in the western, central, southern and northwestern parts of the country (Abdenna Deressa *et al*., 2007). It is estimated that about 43% of the total cultivated land area of Ethiopia is affected by soil acidity (Getachew Agegnehu *et al*., 2019). Legume production is seriously affected by soil acidity because of its effect on the host, the endosymbiont and the symbiosis (Graham, 1992; Hungria and Vargas, 2000). This necessitates the search for technologies to correct acid soils, improve nutrient use efficiency and increase crop production on acidic soils by biotechnological applications using microorganisms.

For the last few years, attempts have been made to screen acid tolerant rhizobia to improve production of faba bean under greenhouse conditions (Girmaye Kensa *et al.*, 2011), and field condition (Dereje Tsegaye *et al*., 2015), and soybean under field conditions (Daniel Muleta *et al*., 2017) in Ethiopia. Under the circumstances, the selected acid-tolerant strains improved production of the legume crops over the un-inoculated control plants.

Although studies were made to understand the role of indigenous chickpea rhizobia to enhance growth and nitrogen fixation of the host in slightly acidic and neutral soils (Mulisa Jida and Fassil Assefa, 2012; Daniel Muleta and Fassil Assefa, 2015; Wondwosen Tena *et al.*, 2017; Tassew Sirage and Fassil Assefa, 2018) and proved that Ethiopian soils contain symbiotically effective *Mesorhizobium* nodulating chickpea, there was a dearth of information on the genetic and symbiotic diversity of chickpea *Mesorhizobium* from acidic soils in the country. This study was therefore, initiated to investigate the genetic, ecological, plant growth promoting (PGP) and symbiotic characteristics of chickpea *Mesorhizobium* isolated from acidic soils of Ethiopia.

## 1.2. Objectives of the Study

### 1.2.1. General objective

The general objective of the study was genomic diversity, ecological and plant growth promoting properties and symbiotic effectiveness of chickpea *Mesorhizobium* isolated from acidic soils of Ethiopia using standard methods to select potential elite inoculants to enhance chickpea production under low inputs sustainable agriculture.

### 1.2.2. Specific objectives

The specific objectives of the study were;

- ✓ To isolate and determine the diversity of chickpea *Mesorhizobium* spp. from the different acidic soils of Ethiopia using genetic methods
- ✓ To screen the *Mesorhizobium* isolates for their ecological competitiveness and preliminary effectiveness in symbiotic nitrogen fixation on different chickpea varieties under greenhouse conditions
- ✓ To evaluate the phosphate-solubilizing and other plant growth promoting properties (PGP) of chickpea *Mesorhizobium* under *in vitro* conditions
- ✓ To test the selected strains of *Mesorhizobium* inoculants on host varieties on acidic soils under field conditions

## 1.3. Literature review

### 1.3.1. Chickpea production in Ethiopia

Chickpea *Cicer arietinum* (*L.*) is one of the major food legume crops grown in the tropics, subtropics and temperate regions of the world (FAOSTAT, 2019). It was first produced in the Middle East about 7, 000 years ago, and at present, it is grown world wide as the third most important legume crop next to haricot bean and soybean (Namvar and Sharifi, 2011). The most important chickpea producing countries are India, Turkey, Pakistan, Iran, Mexico, Australia, Ethiopia, Myanmar, Canada and United State of America (FAOSTAT, 2019).

Ethiopia is considered as the center of chickpea diversity (Gemechu Keneni *et al.*, 2012) and is mainly grown in the central, northern and eastern highland areas of the country with an altitude of 1500-2700 m.a.s.l., with an annual rainfall of 500 - 2000 mm and the average temperature of 10–25°C. Under these conditions, the crop takes 120 - 150 days to mature. It prefers well-drained, sandy, sandy loam and black soils with pH 6-7. The farmers essentially plant chickpea on residual moisture after the end of the main rainy season, usually in late August to October (Legesse Dadi *et al.*, 2005).

Generally, there are two types of chickpea produced globally and known as *desi* and *kabuli*. The *desi* type is characterized by small seeds with angular appearance, sharp edges and varying colors but usually light brown. On the other hand, the *kabuli* type produces large round seeds of white or pale cream or yellow color. Although these two chickpea types are currently produced in Ethiopia, the *desi* type chickpea is traditionally and widely grown in the country. The production of *kabuli* type is limited to few pockets, primarily in eastern Shewa zone and produces higher yield under ideal conditions and is more sensitive to environmental stresses with lower yield (Lijalem Korbu *et al*., 2016).

Ethiopia is the leading producer, consumer, and exporter of chickpea and contributes to 4.5% of the global and more than 60% of Africa's chickpea market (FAOSTAT, 2019). In Ethiopia, chickpea cultivation has increased by 55% from about 163,067 ha in 2003 to about 239,786 ha in 2014, while total production reached 499,426 tons, with average productivity of 2.06 t/ha during 2018 (CSA, 2018). Though this growth is impressive, average chickpea productivity in Ethiopia is as low as 2.06 t/ha which is still below the potential production of 6 t/ha (FAOSTAT, 2019). This yield gap between the average and

potential yield of chickpea could be due to different environmental constraints mainly soil acidity, pest and diseases.

The two major producing Amhara and Oromia regions cover more than 90% of the entire chickpea area and constitute about 92% of the total chickpea production in the country. The top 9 chickpea producing zones (north Gonder, south Gonder, north Shewa, east Gojam, south Wello, north Wello, west Gojam, (Gonder Zuria) belong to the Amhara region. In the Oromia region, the major producing zones are in west Shewa, east Shewa and north Shewa (Legesse Dadi *et al.,* 2005).

ICRISAT, (2013) have also projected the hitherto unknown areas of western highlands, Central Rift Valley, western lowlands, southern lowlands, Afar and Somali lowlands of the country have the potential for the extension of chickpea production with supplemental inputs and irrigation. The authors also suggested that there is a great potential to expand its production to the humid and warm regions of Benishangul and Gambella regions provided that Ascocayta blight resistant varieties are available.

Chickpea is grown in rotation with major cereals such as tef (*Erograstic tef*), wheat (*Triticum sp.*), and barley (*Horduem vulgare*) in a traditional low input agricultural system that suffers from soil nutrient depletion (Gemechu Keneni *et al.,* 2012). This is due to its capacity of biological nitrogen fixation in association with root nodule bacteria belonging to the genus *Mesorhizobium* (Nour *et al*., 1995), contributing directly to grain protein and reducing the need for N fertilizer for subsequent crops and has great potential for enhancing soil fertility.

### 1.3.2. Soil acidity in Ethiopia

In Ethiopia, 84 million hectares of land is suitable for agriculture, of which 43% of the area is characterized by acid soils (Getachew Agegnehu *et al*., 2019). The authors estimated that 28.1% of the soil was moderate to weak acids, and 14.5% was strong to moderately acidic with a pH less than 5.0. Most of the strongly acidic soils are found in western and southwestern, central highlands, high rainfall areas of northwestern parts of the country. Soil acidity increases in western and eastern Wollega zones with the large proportions of exchangeable acidity due to exchangeable $Al^{3+}$ while in west Shewa zone it was due to exchangeable $H^{+}$. The acidity problem in east and west Wollega zone of Oromia region is critical (Abdenna Deressa *et al*., 2007). On the contrary, moderately acidic soils are distributed through much of the rest of the country.

Plant nutrients such as phosphorous, calcium, magnesium, and molybdenum become deficient and elements such as aluminium and manganese become toxic to plant growth under low pH. Aluminum and manganese toxicity in acidic soil affects the host plant, its rhizobia and their symbiotic interaction (Hungria and Vargas, 2000). However, aluminum toxicity is the most common plant symptom in acidic soils, causing root stunting and decreases plant productivity (Jaiswal *et al.,* 2018).

It is established that most leguminous plants require a neutral or slightly acid soil pH for normal growth and nodulation. Apart from that the process of nitrogen fixation aggravates acidification of the soil because of the extrusion of hydrogen ions (Graham *et al.,* 1994).

### 1.3.3. Management options for soil acidity problems

Acid soils can be ameliorated with lime to make them highly productive and sustainable. The primary aim of liming is the neutralization of exchangeable hydrogen, aluminum and increasing the degree of base saturation. Therefore, liming acid soil improves the soil environment for plants and microorganisms by raising its pH and increasing availability of essential nutrients (Ca, P, and Mo) for plants and decreasing the solubility of toxic elements Al and Mn (Graham, 1992; Guo *et al*., 2009). However, liming in many areas is not always economically feasible because of the high application rates needed and high transportation cost (Foy, 1988).

Therefore, the selection of acid and high aluminum tolerant strains of *Rhizobium*, as well as tolerant legume species /variety to acid pH is another method that can supplement liming to improve production (Howieson *et al.*, 1988; Taylor, 1991; Graham, 1992). Studies on different legumes showed that selection of tolerant legume varieties and/ or endosymbionts help to reclaim the productivity abandoned acidic soil (Carter *et al.*, 1994; Zahran, 1999; Guo *et al*., 2009). Thus, plant breeding or biotechnology can improve the resistance of plants and microbes to acidity. Jaiswal *et al.* (2018) have reported the existence of genes for Al tolerance in plants and bacteria such as rhizobia that could produce organic acids that chelate and reduce the toxic effect in the rhizosphere of plants.

### 1.3.4. Biological Nitrogen Fixation

Biological nitrogen fixation (BNF) is the process that changes inert Nitrogen to biologically useful $NH_4$ (ammonium). This process evolved between the interaction of soil bacteria (rhizobia) and plants via the formation of nodules. In early stages of the symbiosis, a complex molecular dialogue takes place, involving Nod factors that are synthesized by the bacterium and flavonoids released by legume plant roots, so that both symbiotic partners can recognize each other and initiate nodulation (Hungria and Vargas, 2000). Then, bacteria proliferate within root nodule cells and differentiate into a nitrogen-fixing form of cells called bacteroids, which can fix atmospheric nitrogen. In exchange, rhizobia take advantage of carbon substrates derived from the plants.

Biological nitrogen fixation (BNF) accounts for about 65% of N currently used in nature (Bhattacharyya and Jha, 2012). The recent estimation is that up to 139 to 170 × $10^6$ tons of nitrogen is fixed in the terrestrial ecosystem, where more than 70% is fixed by the symbiotic association of the root nodule bacteria generally known as rhizobia with leguminous plants (De Bruijn, 2015). It is, thus, an environmentally friendly and ideal for sustainable agriculture. In low input agriculture, legumes play a crucial role in agricultural systems in reducing the need for N fertilizer for successive crops.

Chickpea is one of the leguminous crops integrated into low input agricultural system due to its capacity to fix inorganic nitrogen. It can restore and maintain soil fertility (Ben Romdhane *et al.*, 2008; Wondwosen Tena *et al.*, 2017). Werner (2005), reported that

chickpea annually produces up to 90-180 kg N $ha^{-1}$ depending on cultivar, bacterial strain, and environmental factors.

Most leguminous plants grow best on neutral or slightly acidic soil, especially when they depend on symbiotic nitrogen fixation (Graham *et al.,* 1994). Acidity affects the effectiveness of nitrogen fixation in one of the following ways; it affects the growth of the host plant, survival of rhizobia in the soil, the infection process, nodule growth and nodule function (Graham, 1992; Hungria and Vargas, 2000). Low soil pH adversely affects several stages during the development of symbiosis, including the exchange of molecular signals (nodulation factors) between the legume and the microsymbiont. For instance, acid-sensitive isolates have low *nod C* gene products, while acid tolerant host produces more flavonoids (Watkin *et al.,* 2003).

Moreover, in acid soils, Al toxicity and phosphorus deficiency is the limiting factor for biological nitrogen fixation. Due to excess of Al the root systems of legumes are poorly developed, form little fine branching roots, lowered soil rhizobial population, and suppression of nitrogen metabolism involving nitrate reduction, nitrite reduction, and nitrogenase activity and ultimately impairing the N fixation process (Jaiswal *et al.,* 2018). Phosphorus deficiency in legumes inhibits root system development and flowering, impart the dark purple color of leaves, stunted growth of the plant, inhibit symbiotic performance and function (Nasr Esfahani *et al.,* 2016).

The productivity of leguminous crops in acidic soil can be improved by selection of the legume and rhizobia that are able to tolerate low pH stress (Graham, 1992). Hence,

screening of rhizobia for assessment of symbiotic effectiveness on acidic media in the presence of toxic metals such as aluminum has been proved to be useful for identifying an acid-tolerant isolate (Ayanaba *et al*., 1983).

### 1.3.5. Mechanism of low pH tolerance and their control in *Rhizobium*

Legumes and some strains of rhizobia exhibit varied responses to acidity. Some rhizobial species can tolerate acidity better than others, and tolerance may vary among strains within species. Although the optimum pH for rhizobial growth is considered to be between 6 and 7 (Chen *et al.,* 2005), some strains of *Mesorhizobium loti* showed a high degree of acid tolerance in laboratory media, being able to grow at pH values as low as 4.0 (Jarvis *et al.*, 1997) and relatively few *Mesorhizobia* stains can withstand and survive even as low as pH 3 (Brigido *et al.,* 2007).

Acid tolerant rhizobia have developed various physiological and biochemical adaptative mechanisms to acidic conditions. These include maintaining intracellular pH by decreasing membrane permeability, internal buffering, amelioration of external pH, and prevention of metal ions, respectively (Graham, 1994; Tiwari *et al.,* 1996a). Furthermore, the exclusion and expulsion of protons $H^+$, the increase of potassium and glutamate contents in the cytoplasm of stressed cells (Watkin *et al.,* 2003), the change in the lipopolysaccharides composition (Ferguson *et al.,* 2005), the accumulation of polyamines and modification in membrane lipids (Fujihara and Yoneyama, 1993), production of exopolysaccharides (EPS) (Cunningham and Munns, 1984) and glutathione may have a protective role in acid resistance. The production of acid shock proteins (ASPs) is another

common response contributing to stress tolerance by conferring acid protection on the bacteria with no alteration of the cellular pH (Foster, 2000)**.** Moreover, some genes, such as *actA, actP, exoR, lpiA, actR, actS,* and *phrR*, are essential for rhizobial growth at low pH (Abd-Alla *et al.*, 2014).

The production of acid shock proteins (ASPs) contributes to acid tolerance for the *Rhizobium* (Glenn and Dilworth, 1994). There are two main types of ASPs: chaperones and proteases. Chaperones are proteins that either bind to other proteins, preventing them from misfolding and even repairing misfolded ones under stress (Foster, 2000). In order to bring about an acid shock response, the bacteria and/or root nodule must have some form of sensing mechanism. These are generally made up of two components: a sensor and a regulator. For example, *S. meliloti*; the genes *actR* and *actS* encode for the regulator and sensor, respectively (Tiwari *et al.*, 1996b). *ActS* is the membrane-bound product of *actS*, which act on detection of external acidity. ActR then goes on to activate the transcription of other acid response genes via phosphorylation within the bacterium. However, some genes are constitutively expressed that function under stress conditions; e.g. *actA* in *S. meliloti*, the membrane-bound product of *actA* is basic and responsible for maintaining internal pH at around 7 when the external pH drops below 6.5 (Tiwari *et al.*, 1996a).

Research on *S. meliloti* has shown that calcium can also play a key role in acid tolerance. It has been shown that some tolerance mechanisms can function under greater stress (i.e. increasing acidity) on the addition of increasing amounts of calcium. In a similar way, glutathione has been shown to be involved in acid tolerance (as well as other stresses) in

*Rhizobium tropici* (Riccillo *et al.,* 2000). Potassium and phosphorus are also known to increase in concentration in *R. leguminosarum* cells exposed to acid stress (Watkin *et al.,* 2003).

It has been reported that some inducible systems (ABC transporters system) raise the internal pH of the *Rhizobium* in order to counter any intruding acidic molecule or protonated species (Abd-Alla *et al.,* 2014; Fujihara and Yoneyama, 1993). The ABC transporters system; can transport a wide variety of substrates including amino acids, sugars, inorganic ions, polysaccharides and peptides; and this transport mechanisms either move acidic molecules out of the cell or import basic ones (Abd-Alla *et al.,* 2014). This process is only usually successful if the difference between internal and external pH is approximately 1 pH unit.

It was also suggested that rhizobia producing more exopolysaccharides (EPS) are more tolerant under acidic conditions than Rhizobia that can only produce smaller amounts (Cunningham and Munns, 1984). Under the circumstances, slow-growing *Rhizobium* species are typically more acid tolerant than fast-growing ones.

The change in the lipopolysaccharides composition plays an adaptive mechanism to extreme acidity, which mediates bacterial interaction with the environment. It is composed of three covalently-linked domains: lipid A, which is embedded in the outer membrane, the central oligosaccharide core and the O polysaccharide or O antigen (OAg), which is exposed to the bacterial surface (Lerouge and Vanderleyden, 2001). It has been reported

that, under moderate acid conditions, the lipid A domain of LPS is modified by the addition of polar groups that vary between species. The addition of glucose residues to the OAg changes its conformation making it more compact and short (Ferguson *et al.,* 2005).

### **1.3.6. Genus** *Mesorhizobium* and their genome

The genus *Mesorhizobium* is the major group of root nodule bacteria (rhizobia) that include the specific species nodulate chickpea. It also nodulates very diverse temperate legume hosts such as *Biserrula pelecinus, Astragalus* spp*., Amorpha fruticosa, Caragana* spp. and *Lotus* spp. *Chamaecrista,* a caesalpinoid legume, which is nodulated by *M. plurifarium* strains (Sprent, 2007). These root nodule bacteria are mobile, aerobic, gram-negative, non-spore forming rods, characterized by a growth rate intermediate between the fast and slow growing groups (Nour *et al*., 1994; Chen *et al.*, 2005).

The Genus *Mesorhizobium* is among the least studied rhizobia representing an excellent model to study the evolution of symbiosis, due to its wide geographical dispersion, variety of host ranges, diverse symbiosis genes organization and an increasing number of available genomes (Wang *et al*., 2014; Haskett *et al.,* 2016; Wang *et al*., 2018). Currently, it consists of 29 described species that were originally isolated from the root nodules of 15 different legumes (Andrews and Andrews, 2017).

Current knowledge on genome evolution in *Mesorhizobium* achieved through comparative genomics of fully sequenced genomes, showing the rapid evolution of *Mesorhizobia*. Complete sequence for *Mesorhizobium* species is available that provides the basis for even

more profound understanding of the relationship between different nitrogen-fixing symbionts (Laranjo *et al.,* 2014).

Rhizobia genomes are composed of the core and accessory elements (Young *et al*., 2006). The core genome is mostly chromosomal and contains essential genes including housekeeping genes, which are important for basic cell functions. These genes are highly stable; and can provide a robust and consistent physiology-based classification in mesorhizobia (Laranjo *et al.,* 2014). In contrast, the accessory genome includes several groups of a specific gene for instance symbiosis genes which are responsible for special ecological niche adaptation, reflecting physiological diversity

The symbiosis genes are located in chromosomal symbiosis Islands (SI) or only rarely in plasmids and thus are most prone to horizontal transfer (Kloesges *et al.,* 2011). The occurrence of horizontal gene transfer is more associated with the degree of relatedness of the bacteria and among those species sharing the same habitat. The transfer of symbiotic genes could result in the rapid evolution of diverse force for genetic diversity and symbiotic evolution in any case in chickpea rhizobia. The comparison of the phylogeny of the symbiosis genes with that of the core genes is important to understand the evolution of mesorhizobia and the contribution of horizontal gene transfer to rhizobial symbiotic diversity (Laranjo *et al.,* 2012; Greenlon *et al*., 2019).

### 1.3.7. Specificity in mesorhizobia chickpea symbiosis

The symbiotic association is usually specific, when a legume group is nodulated by specific rhizobial strains. For example, chickpea rhizobia are highly host specific to nodulating only ciceri species due to the production of specific Nod factor (Broughton and Perret, 1999). The common Nod genes (*nod* ABC) are responsible for encoding the core structure of the Nod factors, and these genes are found in all rhizobia, their sequences can still vary among rhizobial species or strains that determine the production of specific Nod factors, which in turn, determine symbiotic specificity (Gough and Cullimore, 2011).

The *nod* C gene sequence is one of the components of host-specific nodulation and encodes for Nacetyl-glucosaminyltransferase which is used to understand the symbiotic specificity of rhizobia and their evolutionary adaptation to a specific legume host. All chickpea nodulating *Mesorhizobum* isolates sampled across different countries harbor very similar *nod C* gene sequences specific to chickpea nodulation (Rivas *et al*., 2007; Zhang *et al*., 2012; Wondowesen Tena *et al*., 2017). Study from New Zeland also showed that a specific symbiosis between New Zeland native *Sophara* spp and *Mesorhizobium* spp can be determined by the specific sequences of *nod C* genes **(**Tan *et al*., 2015).

### 1.3.8. Molecular approaches for the identification of *Mesorhizobium*

The classification of prokaryotes has undergone many changes, since the last two decades. This activity has been increased by the introduction of new and more trust-worthy techniques designed to characterize rhizobia. However, the polyphasic combination of

phenotypic, chemotaxonomic properties and genotypic properties can give a reliable method of classification (Kampfer and Glaeser, 2012).

A variety of approaches had been employed for the genetic characterization of root nodule bacteria that include the genomic GC ratios, DNA-DNA hybridization, DNA profiling methods using PCR such as random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), enterobacterial repetitive intergeneric consensus (ERIC) (Nour *et al*., 1994; 1995; Rai *et al*., 2012). BOX element using ribosomal genes (16S, 16S-23S inter generic spacer - IGS) are routinely used to identify and differentiate chickpea nodulating *Mesorhizobium* spp (Suneja *et al.,* 2016). Although PCR-based techniques are rapid, highly sensitive and specific, they might suffer from robustness, carry-over contamination of amplicons, and failure of PCR amplification and may end up in false positives (Kalle *et al.,* 2012).

Sequence analysis of the 16S rRNA gene has been used as a regular protocol for bacterial taxonomic study. However, they show high similarity between species with the *Mesorhizobium* genus, as the previously described *M. metallidurans* and *M. gobiense* (Laranjo *et al.,* 2014). The largest limitation of using the 16S rRNA gene in rhizobia taxonomy is its low discrimination level for closely related species. This has been overcome using multilocus sequence analysis (MLSA) that involves sequencing of several different protein-coding genes or housekeeping genes such as *atpD* (ATP synthase F1, subunit), *dnaJ* (Dna chaperone), *glnA* (glutamine synthetase I), *gyrB* (DNA gyrase subunit) and *recA* (recombinase A) (Brigido *et al.*, 2007; Suneja *et al*., 2016; Zhang *et al*.,

2017; Wondwosen Tena *et al*., 2017). Apart from that symbiotic genes like nod genes (*nod D, nod C*), nif genes (*nif H*), have also been used in combination with 16S rRNA to study the evolutionary relationship among the *Mesorhizobium* spp. (Rivas *et al*., 2007; Alexandre *et al.*,2009; Laranjo *et al.,* 2012; Zhang *et al*., 2012). However, identification using these short DNA sequence specific marker gene can be localized anywhere in the genome (Segata *et al*., 2013). In addition, sequence information from certain regions of the genome can be misleading or inappropriate for inferring accurate phylogenetic relationships among the taxonomy and phylogeny of *Mesorhizobium* species (Suneja *et al*., 2016). Thus, the whole genome sequence analysis provides the solution to the problem associated with the above typing methods (Petersen *et al.,* 2017). Unlike targeted sequencing of specific regions of the genome, whole genome sequencing analysis provides a comprehensive view of the entire genome. Whole genome data give access to complete structural genetic information, including nucleotide variants, gene content, sequence similarity at the genome level and structural variation (Zhang *et al.*, 2011). As a result, whole genome sequences can be used to determine average nucleotide identities (ANI) within the same taxonomic family; robust means for comparing genetic relatedness among strain and make complete taxonomic inferences (Goris *et al.*, 2007; Varghese *et al.,* 2015).

Whole genome data availability provides the gene content-based approaches and provides evidence for diversity pattern in microbial populations. The phylogenetic approaches, for instance, PhyloPhlAn generates high-resolution microbial phylogenies by identifying 400 marker genes and building a phylogenetic tree from the subsequences of these proteins (Segata *et al.*, 2013).

### 1.3.9. Diversity and biography of chickpea *Mesorhizobium*

The diversity of chickpea nodulating *Mesorhizobium* is reported from different geographical locations across different countries. *Mesorhizobium ciceri* (Nour *et al*., 1994) and *Mesorhizobium mediterraneum* (Nour *et al*., 1995) were originally described as a specific chickpea nodulating species in the Mediterranean countries of Asia and Australia; *Mesorhizobium amorphae* and *Mesorhizobium tianshanense* in Spain and Portugal (Rivas *et al*., 2007); *Mesorhizobium huakuii* in Portugal (Alexandre *et al*.,2009), *Mesorhizobium opportunistum* in Portugal (Larinjo *et al*., 2012) and *Mesorhizobium muleiense* in China (Zhang *et al*., 2012, 2017); *Mesorhizobium loti* in Morocco, Portugal, and India (Maatallah *et al*., 2002; Brígido *et al*., 2007; Rai *et al*.,2012).

Studies also showed that chickpea rhizobia can have specific geographic distribution. For example, *M. ciceri* and *M. mediterraneum* are common in the Mediterranian and India, whereas *M. muleiense* were isolated only from northwest China (Zhang *et al*., 2017). Wondowesen Tena *et al*., (2017) have also showed the existence of diverse chickpea rhizobia populations in Ethiopia and identified new chickpea symbionts which were closely related to *Mesorhizobium abyssinicae* and *Mesorhizobium shonense* and undefined *Mesorhizobium* groups.

The diversity and geographic distribution of rhizobial communities may depend on their adaptation to the diverse ecosystem, mainly related to soil conditions (Fierer and Jackson, 2006; Greenlon *et al.,* 2019). Alexandre *et al.* (2009) have showed an association between the distributions of mesorhizobial species with soil pH. Consequently, in southern

Portugal, *M. mediterraneum* and *M. tempratum* isolates were obtained from higher pH soils, while *M. ciceri* and *M. amorphae* were isolated from neutral to acidic soils, and *M. muleiense* was the dominant species identified from the alkaline soils of China (Zhang *et al.*, 2017).

Other studies also showed the type of soil, temperature, moisture, pH, nutrient content can affect the distribution and diversity of the rhizobial population associated with common bean (Wang *et al*., 2016), indicating that environmental factors are important ecological diverse for species diversity of locally adapted rhizobia.

### 1.3.10. Plant growth promoting activities of *Mesorhizobium* spp.

Previous works showed that rhizobia promote plant growth through mechanisms other than nitrogen fixation (Piex *et al.*, 2001; Alikhani *et al.*, 2006). These include phosphate solubilization, production of growth phytohormones such as IAA, gibberellins and cytokinins, and other metabolites such as siderophores and HCN. They also exhibit antagonistic effects against many plant pathogenic fungi (Glick, 2012) and their 1-aminocyclopropane-1-carboxylic acid (ACC deaminase activity) promotes plant growth through lowering of plant ethylene levels (Shaharoona *et al.*, 2006).

All these features are known as plant growth promoting properties (PGP) that enhance plant growth that can massively proliferate root hair production and thus enhance the root's absorptive capacity and nutrient uptake in both legume and non-legume crops (Grichko and Glick, 2001). Peix *et al.* (2001) reported that *Mesorhizobium* acts as plant

growth promoting rhizobacteria (PGPR) that can expand the root architecture of the crop enable them to accumulate more nitrogen, phosphorus, and other nutrients than control plants.

#### 1.3.10.1. Phosphate solubilization

Phosphorus (P) is one of the major plant growth-limiting nutrients and least mobile element in plant and soil that constraints crop production (Khan *et al.,* 2007). It can severely limit plant growth and biological nitrogen fixation in legumes, where both the plants and their symbiotic bacteria require the element for nodule formation, development, and function (Nasr Esfahani *et al.,* 2016).

Liu *et al.* (2014) have reported only 0.1% of the total amount P in the soil is available to plant and the rest became unavailable due to its fixation in the soil. The unavailability of phosphorous in acidic soil is more severe due to precipitation of soluble phosphates with highly reactive cations such as $Al^{3+}$ and $Fe^{3+}$ and $Ca^{2+}$ in calcareous or normal soils.

There is strong evidence that some soil bacteria are capable of mineralizing organic phosphorous and solubilizing inorganic phosphates so as to make it available to plants. According to Jilani *et al.,* (2007), phosphate solubilizing bacteria (PSB) could reduce P fertilizer application by 50% without any significant reduction of crop yield and it infers that PSB inoculants/biofertilizers hold great prospects for sustaining crop production with optimized P fertilization.

Some of the important PSB are grouped into the genera *Pseudomonas*, *Bacillus*, *Rhizobium,* and *Enterobacter*. Chickpea root nodule bacteria of the genus *Mesorhizobium* spp. are the most effective solubilizers *in vitro* (Verma *et al.,* 2013). The main advantage of using rhizobia as a phosphate solubilizing microorganisms is their beneficial nutritional effect resulting both from phosphate mobilization and nitrogen fixation (Piex *et al*., 2001). The fungal genera of *Penicillium* and *Aspergillus* fungi are also powerful phosphorus solubilizers.

#### 1.3.10.1.1. Mechanisms involved in inorganic phosphate solubilization

The major mechanism of mineral phosphate solubilization activity by microbes is associated with the secretion of organic acids such as gluconic, citric, acetic, malic, succinic and oxalic acid (Khan *et al*., 2007; Vyas and Gulati, 2009). The efficacy of phosphate solublizing microorganism depends on the amount and type of organic acids produced (Khan *et al*., 2007). Citric acid is the most effective in releasing phosphate from inorganic sources, and gluconic acid is the most frequent agent in Gram negative bacteria by direct oxidation of mineral phosphate solubilization of glucose to gluconic acid (Alori *et al*., 2017). These organic acids chelate the cations of Ca, Fe and Al bound to phosphates through their hydroxyl and carboxyl groups and the latter being converted to soluble forms. However, it is unknown whether production of one or multiple organic acids is a bacterial species-specific character or is regulated by the substrate availability (Vyas and Gulati, 2009). The excretion of these organic acids is accompanied by a drop in pH that results in the acidification of the microbial cells and the surroundings resulting in the release of P ions (Halder and Chakrabarty, 1993).

An alternative mechanism to organic acid production for solubilization of mineral phosphates is the release of $H^+$ to the outer surface in exchange for cation uptake or with the help of $H^+$ translocation ATPase (Rodríguez and Fraga, 1999). It was also reported that the assimilation of $NH4^+$ within microbial cells is accompanied by the release of protons and this results in the solubilization of phosphorus without the production of any organic acids.

Other mechanisms of mineral phosphate solubilization by microorganisms are the production of inorganic acids (such as sulphuric, nitric, and carbonic acids) and the production of chelating substances. However, the contribution of inorganic acids as chelating agents to the release of phosphorus in soil is less than that of the organic acids (Alori *et al*., 2017).

**1.3.10.2. Indole acetic acid (IAA) production**

Indole acetic acid (IAA) is one of the common phytohormones and is generally considered to be the most important auxin in plants (Verma *et al.*, 2013). Auxins have been implicated with siginal exchange between microorganisms and plant leading to stimulation of cell division, initiation of lateral and adventitious roots. Synthesis via the intermediates indole-3-acetamide or indole-3-pyruvate is widespread among IAA-producing bacteria (Mandal *et al*., 2007).

Rhizobia have the capacity to synthesize IAA *in vitro* in the presence of physiological precursors, mainly tryptophan (Mulissa Jida and Fassil Assefa, 2012). Most *Rhizobium* species produce IAA which is also necessary for nodule formation (Ahemad and Khan, 2012). Inoculation of IAA producing *Rhizobium* in the crop is used to stimulate seed germination, accelerate root growth, modify the architecture of the root system, and increase the root biomass (Verma *et al.*, 2013). This is considered as a direct mechanism to increase the growth and yield of plants. Production of IAA by *Mesorhizobium* sp. isolated from chickpea rhizosphere and root nodules has been reported (Wani and Khan, 2013; Verma *et al.*, 2013).

### 1.3.10.3. Hydrogen cyanide (HCN) production

Over the past few years, the significance of volatile compounds such as cyanide as mediators of bacterium-plant interactions has become increasingly evident (Ahemad and Khan, 2012). Cyanide is a secondary metabolite usually produced at the end of the exponential phase, when the oxygen concentration and cell density is reduced (Blumer and Hass, 2000). It can be produced directly from glycine and from cyanogenic glycosides both of which are found in root exudates. Since glycine is a common root exudate of many plants, it is likely to be available in the rhizosphere as a precursor of HCN Hydrogen cyanide (HCN) has been shown to break seed dormancy in various species and to be involved in induced resistance against viruses (Oracz *et al*., 2009).

About 50% of rhizobacteria are known to produce cyanide, the most important of which are found in the genera *Pseudomonas*, *Chromobacterium*, and *Rhizobium.* *Rhizobium* protects growing plants from pathogen attack by directly killing parasites by producing

HCN (Ahemad and Khan, 2012). *Mesorhizobium* sp. produces HCN in response to contact with pathogenic fungi and inhibits the fungus (Wani and Khan (2013).

### 1.3.10.4. Siderophore production

Iron is necessary for the process of nitrogen fixation as it is a major component of the nitrogenase enzyme complex. Iron deficiency in the soil results in poor nodulation and affects chickpea productivity (Rai *et al.,* 2012). Siderophores are ferric iron specific ligands with high affinity for an iron that is taken into cells via specific membrane receptors (Lutenberg and Kamilova 2009). They may directly stimulate the biosynthesis of other antimicrobial compounds by increasing the availability of these minerals to the bacteria.

They are involved in the suppression of *Fusarium oxysporum* because they sequester the limited supply of iron (III) in the rhizosphere; thereby limiting its availability to pathogens and ultimately suppress their growth (Gupta *et al*., 2000). Ahmad *et al.* (2008) have showed that *Mesorhizobium* sp. inhibits pathogens by producing siderophores that may indirectly influence plant growth. Thus, *Mesorhizobia* possess the specific system for iron chelation to regulate transport of Fe (III) uptake in chickpea (Rai *et al.,* 2012).

### 1.3.10.5. Antagonism against root pathogens

Plant growth promoting microorganisms have also the capacity to suppress root fungal pathogens such as *Fusarium oxysporum f.sp.ciceri, Fusarium solani, Rhizoctonia bataticola, Sclerotium rolfsii, and Rhizoctonia solani* (Khan, 2012). They can be

administered to control fungal root rot as part of integerated pest management (IPM) together with less fungicides and selection of resistant cultivars (Arfaoui *et al.,* 2006).

The application of rhizobia could aid in the management of fusarium wilt of chickpea, for they have various traits (like toxic and/or and stimulatory metabolites, IAA, exopolysaccharides, antimicrobial and bacteriocin-like substances) (Glick, 2012). The suppression of the growth of *Fusarium oxysporum* f. sp. *ciceris in vitro* by *Rhizobium* is further enhanced by the production of antifungal secondary metabolites, cell wall degrading enzymes, etc. that could synergistically act to suppress the pathogen and enable them to serve as biological control agents (Glick, 2012; Kucuk, 2013).

**1.3.10.6. Ethylene levels (1-aminocyclopropane-1-carboxylic acid (ACC deaminase)**

Ethylene is also known as “stress” hormone and its accelerated production is associated with both biotic and abiotic stress. When plants are confronted with conditions that affect their ability to survive, the same mechanism that produces ethylene for normal development functions and produce “stress” ethylene that is synthesized as a consequence of stressful conditions such as heavy metals, flooding, drought and high salt (Grichko and Glick, 2001).

Some soil microorganisms contain an enzyme ACC deaminase that hydrolyzes ACC into ammonia and α-ketobutyrate instead of ethylene to reduce it (Shaharoona *et al.,* 2006). The authors showed that decreased levels of ACC result in lower levels of endogenous ethylene, which eliminate the potential inhibitory effects of stress-induced higher ethylene

concentrations. The prevalence of ACC deaminase genes in rhizobia has been studied primarily in *Mesorhizobium loti* (Duan *et al.,* 2008).

The uptake and cleavage of ACC by ACC-deaminase containing mesorhizobia decreases the amount of ACC, as well as ethylene, in the roots, thereby acting as a sink for ACC. Inoculation with *Mesorhizobium* sp. containing ACC-deaminase increased the growth of the inoculated chickpea plants primarily through regulation of ethylene synthesis in the inoculated roots under gnotobiotic conditions (Shaharoona *et al.,* 2006). Plants treated with *Mesorhizobium* sp. possessing ACC-deaminase activity showed improve nodulation ability and promote the growth of chickpea by alleviating the stress induced by ethylene on chickpea (Duan *et al.,* 2008).

### 1.3.11. Use of *Mesorhizobium* as inoculants in chickpea crop production

The use of *Rhizobium* inoculants in legume seeds is the oldest agrobiotechnological application (Lindstrom *et al.,* 2010). Nowadays, it is widely used in various parts of the world. They are inexpensive, environmentally friendly, and easy to use with no side effects in most cases. The main purpose of legume seed inoculation is to promote BNF in order to provide nitrogenous compounds to the crop. This increases crop yield on one hand and decreases the use of chemical fertilizers on the other hand, with the consequent reduction of environmental pollution. Commercial rhizobia inoculants can be purchased by farmers in solid, liquid or freeze-dried formulations. Solid peat-based inoculants are commonly used and applied to the seed or directly in the soil (Laranjo *et al*., 2014).

The inoculants must be competitive with the indigenous populations for nodule occupancy and effective in nitrogen fixation. Inoculant strains should be selected for individual cultivars of a given host legume, and also preferably for the particular soil and environmental conditions. Another fundamental characteristic is their persistence in soil over time at the recommended inoculation rates, where adequate inoculants strain often dominates in nodulation (Somasegaran and Hoben, 1994). Thus, inoculation of the legume crop with effective and competitive strains of rhizobia is one way of improving $N_2$ fixation.

Studies also indicated that co-inoculation of legumes with rhizobia and PGPR is even more effective for improving nodulation and growth of legume (Garcia *et al.,* 2004). Kaur *et al*. (2015) have verified that co-inoculation of *Mesorhizobium* sp. with PGPR improved grain N and P content by 61.1% and 11.4%, respectively. Field trials in Turkey showed that co inoculation of rhizobia with phosphorus solubilizing bacteria such as *Bacillus subtilis* or *Bacillus megaterium* could enhanced the availability of both nitrogen and phosphorus fertilizers in chickpea crops (Elkoca *et al*., 2008). A study in India proved the positive effects of co-inoculation of *Mesorhizobium* sp. and *Pseudomonas aeruginosa* that increased 32% in grain yield of chickpea, compared to the uninoculated control under field conditions (Verma *et al*., 2013).

Further, field trial in Ethiopia demonstrated that the indigenous *Mesorhizobium* strain Cp41 improved grain yield (66%) and seed protein (20%) compared to the reference strain (Wondwosen Tena *et al*., 2016), Assefa Funga *et al*. (2016) have also reported that inoculation of chickpea with *Mesorhizobium* ICRE-025 increased shoot nitrogen yield in

the range of 13.0 – 31.34%. Endalkachew Wolde-meskel *et al*. (2018) showed inoculation of chickpea with rhizobia over number of test sites was increased grain yield by 10%, while the combined application of *Rhizobium* inoculated and phosphorus fertilized resulted in 38%.

### 1.3.12. Status of Nitrogen fixation research under acidic soils

A study in South Africa showed the positive effects of *Mesorhizobium* inoculation in slightly acidic soil that increased 7.9% grain yield of chickpea compared to the uninoculated control under field conditions (Ogola, 2015). Alexandre *et al.* (2009) have demonstrated that the acid tolerant mesorhizobia strain isolated from chickpea displayed higher symbiotic effectiveness >75% under an acidic condition in Portugal compared to the reference strain. Similarly, Brigido *et al*. (2007) have showed that *Mesorhizobium* spp isolated from Portuguese soils were able to grow at acid pH and increased shoot dry matter in the range of 20-56% of chickpea crop over the uninoculated control in acidic soil.

Hmissi Imen *et al*. (2015) have reported that compatible phosphate solubilizing *Mesorhizobium* strain S27 leads to increased shoot N and P status by 90% in chickpea crops under phosphorus deficient acidic soil. It is, therefore, important to understand the behavior of *Mesorhizobium* in acidic soils for successful nodulation, development of the N fixing symbiosis, and ultimately crop yield under the circumstances.

In Ethiopia, several research activities have been carried out on isolation, identification and symbiotically effective indigenous mesorhizobial strains of chickpea from different

soils (Mulissa Jida and Fassil Assefa, 2012; Daniel Muleta and Fassil Assefa, 2015; Wondwosen Tena *et al*., 2017; Tassew Sirage and Fassil Assefa, 2018). However, only a few studies were carried out in nitrogen fixation on acidic soils of Ethiopia in faba bean (Girmaye Kenasa *et al*, 2014; Dereje Tsegaye, 2015), haricot bean (Belaineh Mekonnen, 2009) and soyabean (Daniel Muleta *et al*., 2017). However, little has been known on the study of symbiotically effective chickpea rhizobia under acidic conditions.

The exploitation of chickpea mesorhizobia for improving chickpea productivity requires, among others factors, the accurate identification and knowledge of the genomic diversity of *Mesorhizobium* species. The only genetic diversity analysis report of *Mesorhizobium* nodulating chickpea in some parts of Ethiopia was by Wondwosen Tena *et al.* (2017). To this effect, the genetic diversity of the genus *Mesorhizobium* in acidic soil has not been well investigated in Ethiopia. Therefore, the genomic diversity study of chickpea *Mesorhizobium* species collected from different acidic soil regions of Ethiopia will help in understanding diversity patterns in the country to apply biotechnolgical tools for crop improvement in acidic soils. Under the circumstances, mesorhizobia can improve tolerance to acid stress conditions and identifying the existence of genes for low pH or Al tolerance of mesorhizobia that could reduce the toxic effect in the rhizosphere of the crop.

# CHAPTER 2

## Genomic diversity and distribution of *Mesorhizobium* nodulating chickpea (*Cicer arietinum L*.) from low pH soils of Ethiopia

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### Abstract

*Chickpea is the most important grain legume, with particular importance in the world. It fixes nitrogen in symbiosis with diverse Mesorhizobium species and its productivity is limited in acidic soil. However, there is the fact that the diversity and distribution of mesorhizobia communities may depend on their adaptation to soil conditions. Therefore, the collection of rhizobial symbionts under locally prevailing acid stresses is one of the methods employed to improve legume production. To this end, studies were initiated to isolate and investigate the diversity pattern and their taxonomic identities of chickpea nodulating Mesorhizobium species from moderately acidic and acidic soils of Ethiopia. A total of 96 chickpea rhizobia were isolated from root nodules of chickpea collected from different areas in central, western, northern and southern low pH soils of the country. A whole-genome sequence (WGS) approach was used to sequence 96 chickpea rhizobia. Considering a representative set of the best-sequenced 81 genomes, the average depth of sequence was 143X coverage; with estimated average genome sizes 7Mbp. Annotation of the assembled genome predicts an average of 6725 protein-coding genes (CDSs). Genome completeness showed that 81 strains are near complete (>99% complete). Concatenation of 400 universal phylophlan marker genes, present in all 81 strains genomes allowed detailed phylogenetic analysis, from which 8 well-supported phylogenetic groups were identified. Phylogenetic reconstruction based on the symbiosis-related (nod C and nif H)*

*genes were generally different to those shown by the phylophlan marker genes, suggesting that Mesorhizobium strains acquired symbiotic genes transfer originally from co-evolving chickpea symbionts. The data showed that the two major genomic groups; M. genospecies (2D, 2E) (M. plurifarium) and M. genospecies (1C, 1D) (M. loti) were widely distributed in almost all the sites. The other six major genospecies groups were isolated from some sites. In general, more diverse genospecies (at least 4 different groups) were distributed in west Shewa, south west Shewa, Asosa, Bale, and south wollow compared to fewer species identified from west Wollega. The geographic pattern of genomic diversity indicates the existence of multiple, broadly distributed phylotypes, with no relationship between geographic and genetic distance ($r=0.10$, $p<0.01$) that may be associated with a limited variability among distant strains and the studied strains probably have originated from the same genetic background. The results generally showed that Ethiopian low pH soils harbor highly diverse and distinct chickpea nodulating symbionts. Thus, selecting elite strains among this phylogenetically diverse mesorhizobia for better symbiotic nitrogen fixation would be expected to improve chickpea production for smallholder farmers.*

**Keywords**: Phylogenetic, Symbiotic genes, Whole-genome sequence (WGS)

## 2.1. Introduction

Chickpea (*Cicer arietinum L*.) is the third most important food legume world-wide, next to haricot bean and soybean (Namvar and Sharifi, 2011). The crop is widely grown in Turkey, India, Pakistan, Iran, Mexico, Australia, Myanmar, Canada, the United States and Ethiopia (FAOSTAT, 2019). Ethiopia is considered as one of the secondary centers and diversity of chickpea. The country's diverse agro-climatic conditions also favor the cultivation of the crop, especially in the highlands and semi-arid regions (Gemechu Keneni *et al.*, 2012). It is an ideal crop to integrate into low inputs agriculture for it improves soil fertility for subsequent crops by reducing fertilizer costs since it fixes atmospheric nitrogen in symbiotic association with diverse species mainly under the genus *Mesorhizobium*.

Earlier it was thought that chickpea was specifically nodulated by *Mesorhizobium ciceri* (Nour *et al*., 1994) and *Mesorhizobium mediterraneum* (Nour *et al*., 1995) for they were originally described from the Mediterranean countries of Asia and Australia. Later, more diverse groups were identified from the root nodules of chickpea from different parts of the world. This was simply because of the advent of various molecular techniques employed that include; random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), fingerprinting using ribosomal genes (16S, 16S-23S intergeneric spacer-IGS), and multilocus sequencing analysis (MLSA) of several different housekeeping genes (Brigido *et al.*, 2007; Rai *et al*., 2012; Suneja *et al*., 2016; Zhang *et al*., 2017; Wondwosen Tena *et al*., 2017). Apart from that, symbiotic genes like *nod* genes (*nod* D, *nod* C, *nod* A), *nif* genes (*nif H*) have been successfully used to determine the

phylogenetic relationships and species identification of root nodule bacteria (Rivas *et al*., 2007; Alexandre *et al*.,2009; Laranjo *et al.,* 2012; Zhang *et al*., 2012).

This revealed other new species and genomic groups were identified from chickpea such as *M. amorphae* and *M. tianshanense* (Rivas *et al*., 2007; Alexandre *et al*., 2009), *M. huakuii* (Laranjo *et al*., 2008), *M. loti* (Brigido *et al.,* 2007; Rai *et al*., 2012), *M. muleiense* (Zhang *et al*., 2012, 2017), *M. opportunistum* (Laranjo *et al.,* 2012). Studies also showed that chickpea rhizobia can have specific geographic distribution; *Mesorhizobium amorphae* and *Mesorhizobium tianshanense* in Spain and Portugal (Rivas *et al*., 2007); *Mesorhizobium huakuii* in Portugal (Alexandre *et al.,* 2009), *Mesorhizobium opportunistum* in Portugal (Laranjo *et al*., 2012) and *Mesorhizobium muleiense* in China (Zhang *et al*., 2012, 2017); *Mesorhizobium loti* in Morocco, Portugal, and India (Maâtallah *et al*., 2002; Brígido *et al*., 2007; Rai *et al*., 2012).

However, sequence information from certain regions of the genome can be misleading or inappropriate for inferring accurate phylogenetic relationships among the taxonomy and phylogeny of *Mesorhizobium* species (Suneja *et al*., 2016). Studies showed that the whole genome sequence analysis provides the solution to the problem associated with the above typing methods (Petersen *et al.,* 2017). The whole-genome sequencing (WGS) delivers maximum phylogenetic power; besides to providing a comprehensive view of the entire genome (Zhang *et al*., 2011). Alternatively, it can be used to determine average nucleotide identities (ANI) within the same taxonomic family; robust means for comparing genetic relatedness among strain and make taxonomic inferences (Goris *et al*., 2007; Varghese *et al.*, 2015).

Ethiopian soils contain symbiotically effective *Mesorhizobium* nodulating chickpea (Mulissa Jida and Fassil Assefa, 2012; Daniel Muleta and Fassil Assefa, 2015; Wondwosen Tena *et al*., 2017, Tassew Sirage and Fassil Assefa, 2018). Most of these works were limited to phenotypic and symbiotic diversity except, a study on the genetic characterization of chickpea rhizobia from central and southern Ethiopia (Wondwosen Tena et *al*., 2017). This shows that there is a dearth of information on the diversity and symbiotic effectiveness of *Mesorhizobium* spp nodulating chickpea under low pH soil conditions.

Given that the diversity and geographic distribution of mesorhizobia communities may depend on their adaptation to the diverse ecosystem, mainly to soil conditions (Fierer and Jackson, 2006; Greenlon *et al.*, 2019), and distributions of different mesorhizobial species is associated with soil pH in Portugal (Brigado et al, 2007; Alexandre *et al.* 2009), China (Zhang *et al.*, 2017), it may well be that diversity of *Mesorhizobium* may be different under low pH soil in Ethiopia. The objective of this study was, therefore, to look into the genomic diversity of chickpea *Mesorhizobium* spp. isolated from low pH soils of central, western, northern and southern parts of Ethiopia using whole genome sequencing. Understanding diversity patterns in the country may help in the identification of mesorhizobia populations having the ability to perform well in acid soils in order to develop inoculant technology to fully realize chickpea productivity.

## 2.2. Materials and Methods

### 2.2.1. Collection of root nodules

Chickpea root nodules were collected from acidic soils of central, western, southern and northern parts of Ethiopia from October - January, 2015/2016 (Figure 2.1). The sampling sites were selected based on soil acidity report by Abdenna Deressa (2007). A total of 79 farmers' field sites were randomly selected to collect nodules from standing two chickpea plants from each site. The nodules collected from each site were com-posited and collected in vials containing silica gel. The soil samples from the rhizosphere of the respective plants were also collected in plastic bags for further soil pH analysis. The GPS coordination, collection sites and cropping systems were recorded and tabulated in (Appendix 4).

### 2.2.2. Rhizobia isolation

The rhizobia were isolated from root nodules using standard protocols of Somasegaran and Hoben, (1994). Thus, nodules were treated with 70% (v/v) ethanol and 4% (v/v) sodium hypochlorite solutions for 10 seconds and 2 minutes, respectively, rinsed with five change of sterile distilled water, subsequently crushed and streaked onto yeast-extract mannitol agar plates (YEMA) medium containing ($gL^{-1}$) $KH_2PO_4$ (0.5), $MgSO_4.H_2O$ (0.2), NaCl (0.1), yeast extract (0.2), mannitol (10) and agar (15). Plates were incubated at 28ºC for 5 to 7 days, and single colonies were sub-cultured, on YEMA plates and pure colonies were stored on YEMA slants at $4^oC$ and 20% (*v*/*v*) glycerol at -20°C for further tests.

Each isolate was then authenticated as root nodule bacteria by inoculating them into the host (Natoli variety) in pot culture under greenhouse conditions using standard methods of Howieson and Dilworth (2016). The true rhizobia were grown on YEMA plates to evaluate their colony size (mean diameter of five colonies, shape and margin, texture (elastic or buttery), appearance (shiny/translucent or opaque). The isolates were also inoculated on YEMA containing 25 g/ml bromothymol blue (YEMA- BTB) to detect color change due to acid or alkali production and the growth rate of the isolates was assessed according to White (1995).

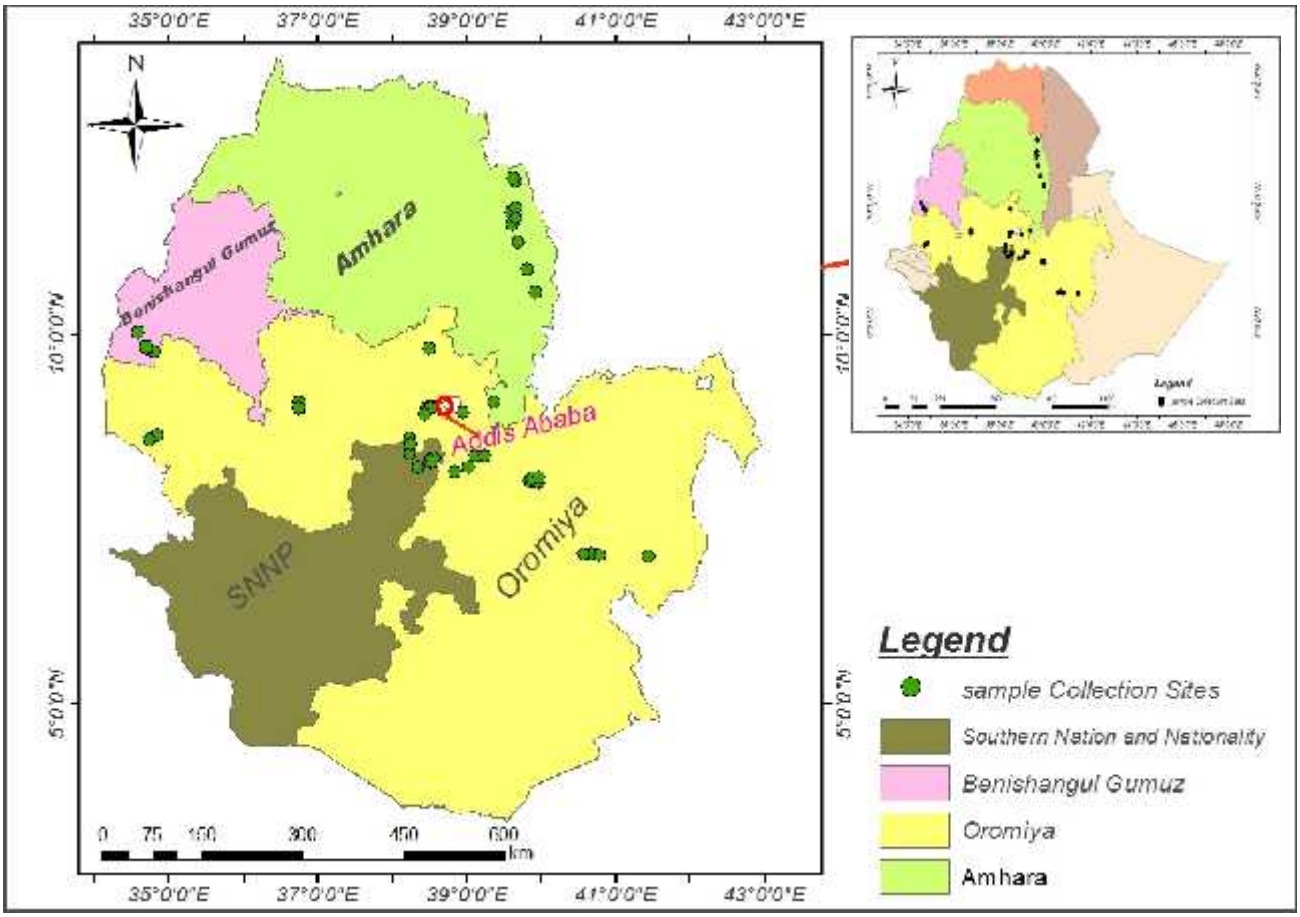


Figure 2.1. Map of chickpea nodules and soil samples collection areas/sites

### 2.2.3. Genomic DNA Extraction

DNA of 96 isolates was extracted from exponential growth phase broth cultures using Qiagen DNeasy®Blood and Tissue Kit (Qiagen Ltd., CA, USA) following the corresponding protocol for Gram-negative bacteria. DNA quantification and purity were determined using Nanodrop Ó 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, USA) and Picogreen (Tecan SPECTRA Fluor plus Microplate Reader, CA, USA) and the samples were resolved on an agarose gel to assess DNA integrity.

### 2.2.4. Library development and whole-genome sequencing

Genome libraries were prepared using the QIAseq FX DNA library kit (96) Handbook, (2015) (Qiagen, CA, USA) following instructions from the manufacturer. This involved aliquot of 17.5 µl normalized DNA of each sample was fragmented and Illumina PE adapters ligated to both ends of the DNA library fragments. A final fragment size selection and clean-up step were performed with AMPure XP beads (Beckman Coulter, Brea, CA) to minimize the presence of low molecular weight, dimerized primers, and then DNA library was amplified with PCR. Fragment size distribution, quantity, and quality of the final library were assessed by capillary electrophoresis on a High Sensitivity DNA chip in a 2100 Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Libraries were normalized to a uniform concentration using Qubit ® 2.0 fluorometry (Invitrogen, CA, USA) and pooled them together, and sequenced using Illumina HiSeq 4000 platform (Illumina, San Diego, CA, USA) at University of California Davis, Genome Center DNA Core Facility USA.

### 2.2.5. Genome assembly and annotation

All Illumina libraries were demultiplexed using Cassava software (Illumina Inc, version 1.7); quality assessment and filtering of sequenced data were accomplished using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc) at a threshold of 0.001 (Phred score of 30). Raw Illumina reads were trimmed and filtered using Trimmomatic version 0.36 (Bolger *et al*., 2014) to remove adaptors, low-quality sequence reads and overlapping sequences not supported by as paired ends. Trimmed reads were assembled into contigs using SPAdes version 3.10.1 (Bankevich *et al.,* 2012). Resulting contigs were aligned using BLAST version 2.2.30+ (Camacho *et al.,* 2009) to reference genomes and verified by CheckM version 1.0.7 (Parks *et al.,* 2015) to check the completeness and contamination. Reads that comprised *Mesorhizobium*-mapped contigs were then assembled using SPAdes version 3.10.1 (Bankevich *et al.,* 2012). The genome statistics such as genome coverage, GC content, *N*50, lengths of contigs and scaffolds are summarized in (Appendix 5) for each strain. Genes were predicted from contigs and annotated *de novo* in draft Illumina assemblies using the Prokka pipeline version 1.13 (Seemann, 2014).

### 2.2.6. Phylogenetic analysis

Phylogenetic placement of *Mesorhizobium* strains was performed using a multilocus protein sequencing phylogeny. Accordingly, ortholog genes from a set of assemblies, along with all assemblies retrieved from the NCBI in the bacterial family phyllobacteriaceae; alignment and concatenation of 400 ubiquitous phylogenetically informative protein sequences were performed in PhyloPhlAn program version 0.99

(Segata *et al*., 2013). A phylogenetic tree was hence constructed using maximum likelihood (ML) methodology on the basis of protein-coding sequences (CDS) of 400 conserved single-copy, universal marker genes, present in all 81 strains. The confidence levels of the phylogenetic trees are evaluated as bootstrap values based on 1,000 replications. The phylogenetic tree was then plotted using iTOL version 4 (Letunic and Bork, 2019). Symbiotic gene phylogenies were inferred with RaxML version 8 (Stamatakis, 2014).

For the sub-species demarcation patterns, pairwise average nucleotide distance on genes from the phylophlan marker set calculated using the JGI's gANI calculator version 1 (Varghese *et al.,* 2015). and clustered genomes into groups using mothur version 1.39.5 (Schloss *et al.,* 2009). To further determine genetic relationships among the strains, the principal coordinate analysis (PCoA) was performed with GenAlEx 6.5 (Peakall and Smouse, 2006) software.

### 2.2.7. Biogeographic analysis

The Mantel test was used to determine population-by-geographic distance relationships of isolates by regressing pairwise average nucleotide distance (calculated as nucleotide dissimilarity distance in concatenated alignments of the 400 phylophlan marker genes) against pair wise spatial distance (latitude and longitude) using GenAlEx 6.5 (Peakall and Smouse, 2006) software.

## 2.3. Results and Discussion

### 2.3.1. Mesorhizobial isolation

Out of 158 nodule samples collected from low pH soil sampling sites widely distributed from 1915-2595 a.s.l, from which 81 rhizobial isolates were recovered and authenticated as chickpea rhizobia (Table 2.1). The relative distribution of the isolates showed that 24 isolates were from western, 40 isolates from central, 5 isolates from southern and 12 from northern parts of Ethiopia. The fact that bacterial isolates were collected from nodules without any history of inoculation may suggest that the chickpea genotype was promiscuously infected by the presence of different groups of indigenous rhizobia adapted to the local environments as previously reported (Mulissa Jida and Fassil Assefa, 2012; Daniel Muleta and Fassil Assefa, 2015; Wondwosen Tena *et al*. 2017; Wubayehu Gebremedhin *et al*., 2018; Tassew Siraj and Fassil Assefa, 2018).

### 2.3.2. Cultural characteristics of chickpea rhizobia

The colony diameter of the isolates was in the range of 1.2 to 4.5 mm in diameter (average 3.6) (Table 2.1). Most isolates formed a medium colony diameter of 2-3 mm to large colony types of >3mm on YEMA after 5 days of incubation, which was similar to the findings obtained by Mulissa Jida and Fassil Assefa (2012).

Most of the isolates 72 (89%) were moderately slow growers with a mean generation time (MGT) 4.1-5.4 h, whereas 9 (11%) of the isolates were slow growers with MGT 5.5-6.2 h). Nour *et al.* (1994, 1995) have defined the specific chickpea symbionts such as *Mesorhizobium ciceri* and *Mesorhizobium mediterraneum* as moderately slow-growing

rhizobia. Mulissa Jida and Fassil Assefa, (2012) have also reported that 89% of the chickpea *Mesorhizobia* species isolated from Ethiopia were moderately slow growers with a mean generation time 4.1-5.4 h. However, the Ethiopian isolates were not growing as slowly as those from Morocco and India with a generation time of up to 9 h (Maâtallah *et al*., 2002; Rai *et al.*, 2012).

All the isolates formed the colony with a circular shape, entire margin, white to watery, translucent to opaque features, and most of the isolates produced copious amounts of mucus. The isolates also changed the color of the green BTB-YMA medium to moderately yellow and deep yellowish irrespective of their generation time (Appendix 4) indicating that the isolates were acid producers (Jordan, 1984).

Table 2.1. Distribution of chickpea *Mesorhizobium* species from different sampling sites of central, western, southern and northern parts of Ethiopia

| Sampling region | Altitude | Number of isolates | Cultural characteristics | | |
|---|---|---|---|---|---|
| | | | Range of colony diameter | Range of generation time (MGT) | Texture |
| **Central Ethiopia** | **1915-2595** | **40** | **1.2-4.5** | **4 - 6.2** | **LWM, LWhM, LWhLM, MWhM, MWhLM, MWM, SWhM, SWLM** |
| **Western Ethiopia** | **1399-2016** | **24** | **2.1-4.5** | **4.1-5.5** | **LWM,MWM, LWhM, MWhLM** |
| **Northern Ethiopia** | **1416-2128** | **12** | **2.6-4.5** | **4.1-5.1** | **LWM, LWhLM, MWhM, LWhM** |
| **Southern Ethiopia** | **1974-2086** | **5** | **2.7 - 4** | **5.1-5.5** | **LWM, LWLM, MWhM** |
| **Total** | | **81** | | | |

LWhM: Large, white, mucoid; SWhM: Small, white, mucoid; LWM: Large, watery, mucoid; LWhLM: Large, white, less mucoid; LWLM: Large watery, less mucoid; MWM: Medium, watery, mucoid; MWhM: Medium, white, mucoid; MWhLM: Large, white, less mucoid; SWLM: Small watery less mucoid; (L :> 3 mm) (M: 2-3mm) (S :< 2 mm).

### 2.3.3. Genome Sequencing and Analysis

Illumina sequencing of 81 strains genomes yielded 7,349,737 base pairs long with an average GC content of 63.2%. The *N*50 values are averaged as 282,018 bp for all 81 genome assemblies and resulted in the generation of 2,078 average contigs and 2,071 average scaffolds (Appendix 5). On average that the 81 strains were sequenced to ~143 fold coverage. The quality and completeness of the draft genome assemblies showed, 81 isolates sequenced were near-complete (>99% complete), indicating a very high level of completeness associated with the coverage of *Mesorhizobium* isolated from low pH soils in Ethiopia.

Comparable genome size was obtained compared to the other studies sequenced *Mesorhizobium opportunistum* type strain WSM2075 genomes (Nandasena *et al.* 2009), *Mesorhizobium huakuii* 7653R genomes (Wang *et al*., 2014) and four other mesorhizobial genomes,including *Mesorhizobium australicum* WSM2073, *Mesorhizobium loti* R7A (Wang *et al*., 2014), *Mesorhizobium ciceri* CC1192 genomes (Haskett *et al.*, 2016*), Mesorhizobium amorphae* CCNWGS0123 genomes (Wang *et al*., 2018) (Table 2.2), indicating the draft assemblies from genomic libraries of each *Mesorhizobum* strains were suitable for analysis of their genome content and taxonomic assignment.

The genome contained an average of 67245 protein-coding genes (CDSs), 56 coding regions of tRNAs (range from 45 to 66), and five rRNAs gene operons (range from 2 to 8) were predicted on the genome. The numbers and types of tRNAs coding regions and

rRNAs gene operons had essentially identical numbers in most cases (Appendix 5), indicating the numbers and types of rRNAs and tRNA of all genomes predicted using the same strategy, which was comparable to that of the earlier report of Wang *et al*. (2014) *Mesorhizobium huakuii* 7653R, *Mesorhizobium australicum* WSM2073, *Mesorhizobium loti* R7A, *Mesorhizobium ciceri* strain CC1192 genomes (Haskett *et al.,* 2016*)* and *Mesorhizobium amorphae* CCNWGS0123 genomes (Wang *et al*., 2018) have essentially matching numbers and types of rRNAs and tRNAs.

Table 2.2. The genome size of genomic groups of this study and previously sequenced *Mesorhizobium* strains

| Strain name | Genome Size | tRNA | rRNA | CDS | Reference |
|---|---|---|---|---|---|
| *Mesorhizobium huakuii* 7653R | 6,881,673 | 51 | 5 | 5,459 | Wang *et al*., 2014 |
| *Mesorhizobium loti* R7A | 6,529,530 | 75 | 2 | 6,323 | Wang *et al*., 2014 |
| *Mesorhizobium opportunistum* WSM2075 | 6,884.944 | 53 | 6 | 4,778 | Nandasena *et al.,* 2009 |
| *Mesorhizobium ciceri* CC1192 | 6,993,628 | 54 | 5 | 6,642 | Haskett *et al.,* 2016 |
| *Mesorhizobium amorphae* CCNWGS0123 | 7,343,952 | 63 | 5 | 7,136 | Wang et *al*., 2018 |
| *Mesorhizobium australicum* WSM2073 | 6,200,534 | 53 | 6 | 4,466 | Wang *et al*., 2014 |

### 2.3.4. Phylogenetic analysis of protein coding gene

The phylogenetic sequence analysis based on concatenation of 400 conserved single-copy, universal marker genes revealed that all the isolates belonged to the *Mesorhizobium* genus with diverse species categories. The isolates were clustered into eight distinct phylogenetic groups with bootstrapping indicated that each branch corresponding to the clad that was well supported (above 67%) (Figure 2.2). Each group being differentiated again into small member sub-populations containing 1 to 30 isolates. Naming and

genospecies groups were further supported by average nucleotide identity values (ANI) from the associated study of Greenlon *et al*. (2019) who showed that 95% ANI as the lower boundary of phylophlan marker genes for investigation with their close relatives and with each other, confirming these groups represent species categories.

Thus, 30 of the isolates were categorized into genospecies 2E,2D showing 95% ANI with *Mesorhizobium plurifarium* STM8774 followed by genospecies 1D,1C containing 16 isolates that were closer to the type strain *Mesorhizobium loti strain* UFLA 01-766, with an average nucleotide identity (ANI) of 95%. Genospecies 8A comprising 15 isolates sharing 95% ANI value that was closer to the type strain *Mesorhizobium australicum* WSM2073. Ten isolates were clustered into genospecies 4B together with *Mesorhizobium amorphae* CCNWGS0123-pacbio at 94% of ANI. The other five isolates were grouped into genospecies 3A corresponding to show 93.3% ANI with unrecognized reference strain, while three isolates were clustered into genospecies 11A with *Mesorhizobium opportunistum* WSM2075 sharing 93% ANI. Genospecies 10A and 5C containing 1 isolate, was closer to the type strain *Mesorhizobium* sp. LSJC280B00 (92% ANI) and with a previously described microsymbiont of chickpea *Mesorhizobium ciceri* ca182 (91.4% ANI), respectively.

Several studies indicated that concatenation of multilocus protein analysis phylogeny provides a better resolution in defining species within a genus (Martens *et al*. 2008; Mohamad *et al*., 2017; Wondwosen Tena *et al*., 2017). Varghese *et al*., (2015) have also demonstrated from a whole genome comparison study that the ANI analyses could surpass

the precision in species delineation. Martens *et al*. (2008), in a study involving seven concatenated genes, inferred an ANI value of 97.3% as cut off point for species delineation in genus *Ensifer*. The phylogenetic analysis of the concatenated 400 genes studied here, a more reliable determination of the phylogenetic positions of our test strains. Accordingly, the isolates were grouped into eight distinct genospecies. The concatenated tree, while reliable and more robust with higher bootstrap values, further highlighted the potential of phylophlan marker genes to delineate species in *Mesorhizobium*.

The phylophlan marker genes sequencing data showed that strains are closely related to symbionts of chickpea, and a few of them are previously unrecognized in chickpea. The dominant genospecies group was related to *Mesorhizobium plurifarium* (Genospecies 2E, 2D) (37%), followed by *M. loti* (Genospecies 1D, 1C) (19.8%) group (Table 2.3). Correspondingly, higher affinity of chickpea nodulating rhizobia with *M. plurifarium* was reported from Indian soil (Prakash *et al*., 2007; Greenlon *et al*., 2019), which was originally isolated from *Acacia* species (de Lajudie *et al*., 1998). *Mesorhizobium loti* strains was found to be a symbionts of legumes such as *Lotus* species *(*Kaneko *et al*., 2000), but they have also identified as chickpea symbionts in Morocco (Maâtallah *et al*., 2002), Portugal (Brígido *et al*, 2007; Laranjo *et al*., 2008) and India (Rai *et al*., 2012). Likewise, in this study strains revealed to *M. australicum* (Genospecies 8A), *M. amorphae* (Genospecies 4B), *M. opportunistum* (Genospecies 11A) accounted 18.5%, 12.3 % and 3.7% of the chickpea isolates respectively (Table 2.3), the strains related to *M. australicum* and *M. opportunistum* were originally isolated from legume tree from

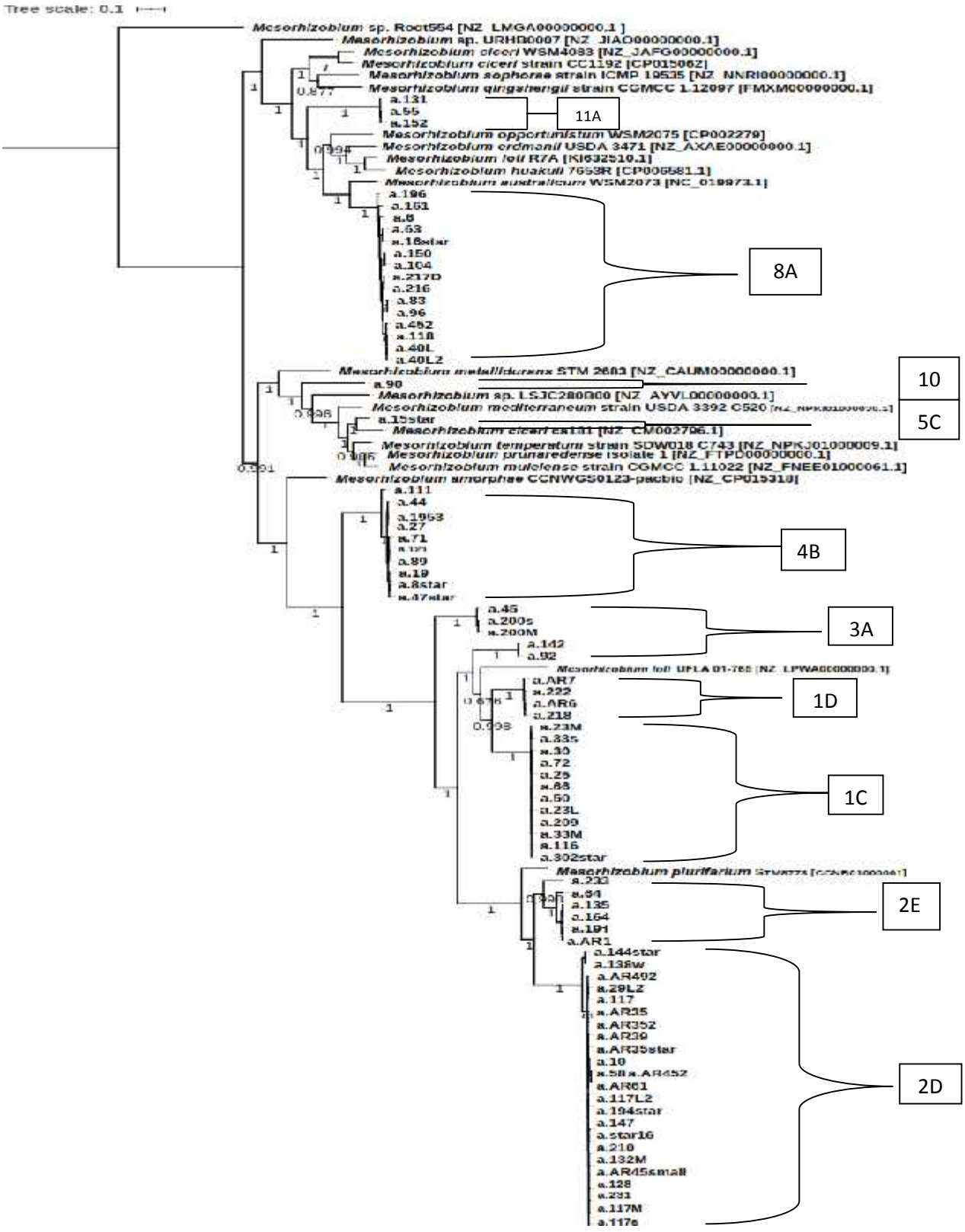


Figure 2.2. Maximum likelihood (ML) tree constructed from 400 conserved universal phylophlan marker genes based for 81*Mesorhizobium* strains along with 21 previously sequenced and publicly available *Mesorhizobium* reference strains, constructed at bootstrap values based on 1,000 replications. Genospecies grouping based on associated study of Greenlon et al. (2019).

*Biserrula pelecinus* L. in Australia (Nandasena *et al*., 2009). The strains that are closely related to *M. australicum* have not been identified as chickpea symbionts, and were suggested as putative new chickpea rhizobia symbionts that require further studies. While *M. opportunistum* was identified as chickpea symbiovar in Portugal (Laranjo *et al*., 2012), and *M. amorphae* strains were first described as a symbionts of leguminous shurub-*Amorpha fruticosa*, but both are also common chickpea nodulating symbionts in Spain and Portugal (Rivas *et al*., 2007; Alexandre *et al.*,2009).

The result generally showed that five genospecies groups belonging to *M. plurifarium* (Genospecies 2E, 2D), *M. loti* (Genospecies 1D, 1C), (Genospecies 8A), *M. amorphae* (Genospecies 4B) and Genospecies 3A were represented by more than 93% of the isolates. The remaining 6% of the isolates were grouped in to *M. opportunistum* (Genospecies 11A), *Mesorhizobium* sp. LSJC280B00 (Genospecies 10A) and *M. ciceri* (Genospecies 5C).

In general, the protein-coding gene sequence data showed that highly diverse and distinct chickpea nodulating *Mesorhizobium* strains existed in the study areas of Ethiopian low pH soils. Interestingly, the common chickpea symbiont, *M.ciceri* (Genospecies 5C) was lower (1.2%) in its population than *M. plurifarium* (Genospecies 2E, 2D), *M. loti* (Genospecies 1D, 1C) and *M. australicum* (Genospecies 8A) contributing to 75% of the isolates. The lower occurrence of *M. ciceri* and the absence of *M. mediterraneum* strains in this study are distinct given that native chickpea's cognate rhizobial partners in its center of origin in the Mediterranean regions (Nour *et al*., 1994; Nour *et al*., 1995; Maatallah *et al*., 2002).

Wondowesen Tena *et al*., (2017) have showed the pattern of chickpea rhizobial species distribution in central and southern parts of Ethiopia, suggesting that ecological diversity and cultivation of the different local chickpea landraces are the most possible driving forces for emergence of diverse chickpea rhizobia, as a result of symbiotic gene transfer originally from the natural chickpea symbionts strains (Rivas *et al*., 2007; Laranjo *et al*., 2008).

Table 2.3. Abundance of each genomic groups of chickpea nodulating *Mesorhizobium* isolates

| *M.*Genospecies | No. of isolates | Abundance (%) | Affiliated reference strains | ANI % |
|---|---|---|---|---|
| 11A | 3 | 3.7 | *Mesorhizobium opportunistum* WSM2075 | 93% |
| 8A | 15 | 18.5 | *Mesorhizobium australicum* WSM2073 | 95% |
| 10A | 1 | 1.2 | *Mesorhizobium* sp. LSJC280B00 | 92% |
| 5C | 1 | 1.2 | *Mesorhizobium ciceri* ca182 | 91.5% |
| 4B | 10 | 12.3 | *Mesorhizobium amorphae* CCNWGS0123-pacbio | 94% |
| 3A | 5 | 6.1 | - | 93.3% |
| 1C,1D | 16 | 19.8 | *Mesorhizobium loti* strain UFLA 01-766 | 95% |
| 2E,2D | 30 | 37 | *Mesorhizobium plurifarium* STM8773 | 95% |

%=abundance of each genomic groups, presented by the ratio of number of the strains in the genomic group per total number of the strains.

### 2.3.5. Phylogenetic analysis based on *nod* C and *nif* H gene

The sequence analysis of *nod* C gene of 79 strains and *nif* H of 80 strains showed their close relation with the important chickpea symbionts, except one strain (a.15star) (Genospecies 5C) on *nif* H clade together with previously unrecognized chickpea symbiont, *Mesorhizobium prunaredense* isolate 1 strain (Figures 2.3, 2.4). The phylogenetic trees of both *nod* C and *nif* H classified the strains into 4 and 5 clades, respectively and each branch corresponding to the clad was supported by bootstrap value (above 65%) (Figure 2.3, 2.4). Consequently, the majority of the strains (i.e 70 (89)%

on *nod* C) were clustered together with *M. ciceri* CC1192, while 6 of the strains were grouped together in a separate clade along with reference strain *M. muleiense* CGMCC1.11022. The 2 strains were clustered with previously described symbionts of chickpea, *M. ciceri* ca181 and 1 strain (a.15 star) (Genospecies 5C) in a separate clade. Similarly, in the *nif* H phylogenetic tree, 28 test strains were clustered with *M. mediterraneum* strain (USDA 3392 C520) and 24 of the strains in a separate clade. Sixteen and 11 of the strains were grouped in a separate clade closer to *M.ciceri* (CC1192) and *M. muleiense* (CGMCC1.11022) strains, respectively. While only 1 strain (a.15star) clade together with *Mesorhizobium prunaredense* isolate 1 strain.

The symbiotic gene analysis showed that the *nod* C and *nif* H phylogeneies of the strains were different from their phylophlan marker genes phylogeny, suggesting the different evolutionary history of the core and symbiosis related genes of the different species group strains. These *nod* C and *nif* H phylogenies of the newly identified *M. australicum* (Genospecies 8A) and (Genospecies 3A) with reference strains (*M. ciceri* CC1192 and *M. muleiense* CGMCC1.11022; *M. mediterraneum* strain USDA3392C520, *M. ciceri* ca181 and *M. muleiense* CGMCC1.110220), respectively may indicate these strains as new chickpea symbionts receiving their symbiotic gene resembling with these reference strains. Interestingly, (a.15star) strain in the *nif* H phylogenetic tree, suggested as new that receiving their symbiotic gene resembling with *Mesorhizobium prunaredense* isolate 1 strain, which was originally isolated from *Anthyllis vulneraria* in France (Mohamad *et al*., 2017) and requires further studies.

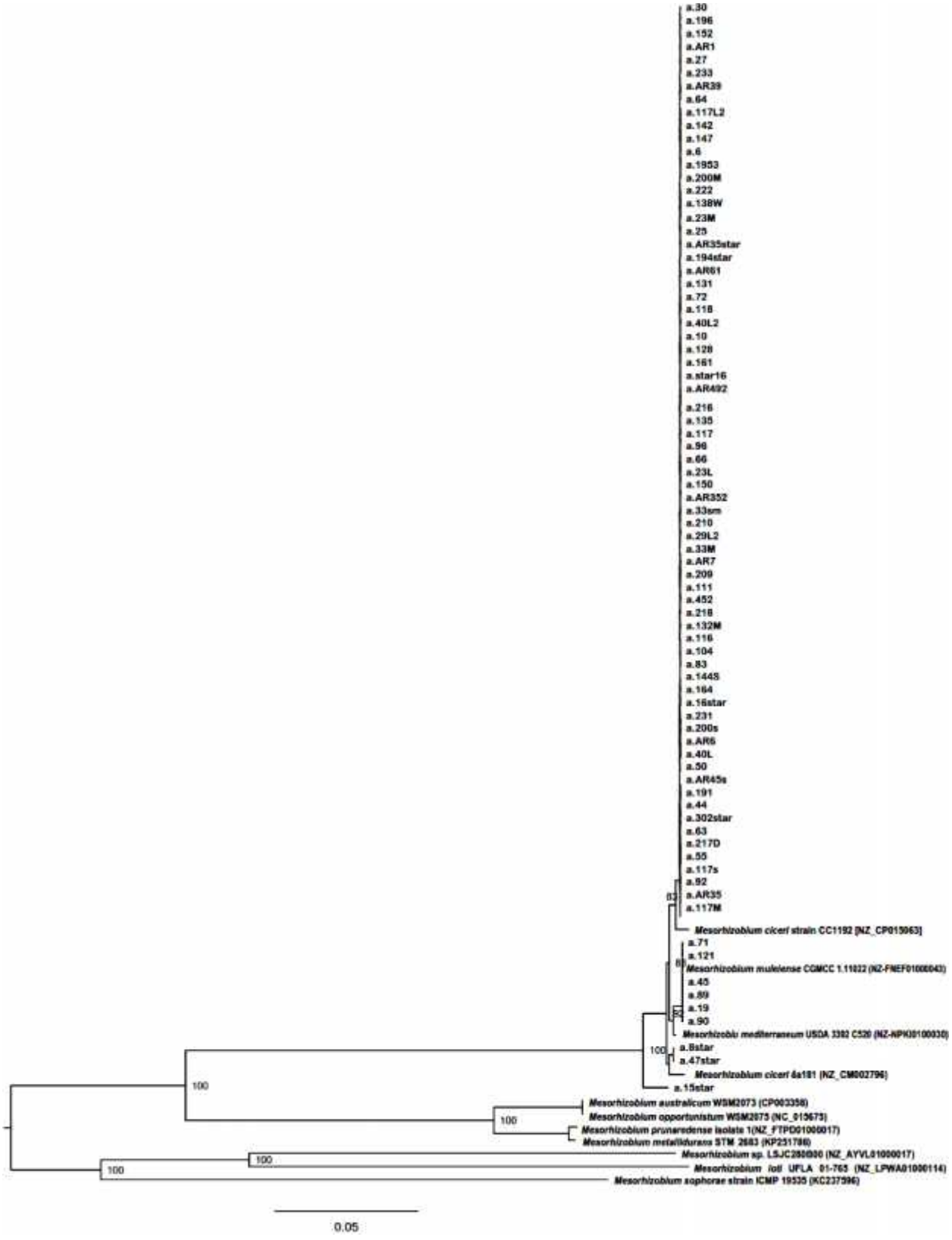


Figure 2.3. Maximum likelihood (ML) tree phylogenetic trees based on sequences of symbiotisis related gene *nod* C gene showing the relationships among the different chickpea isolates and reference strains of the defined *Mesorhizobium* species. Bootstrap values over 83% (based on 1000 replications) are shown at each node. Bars indicate the percentage of estimated nucleotide substitiution.

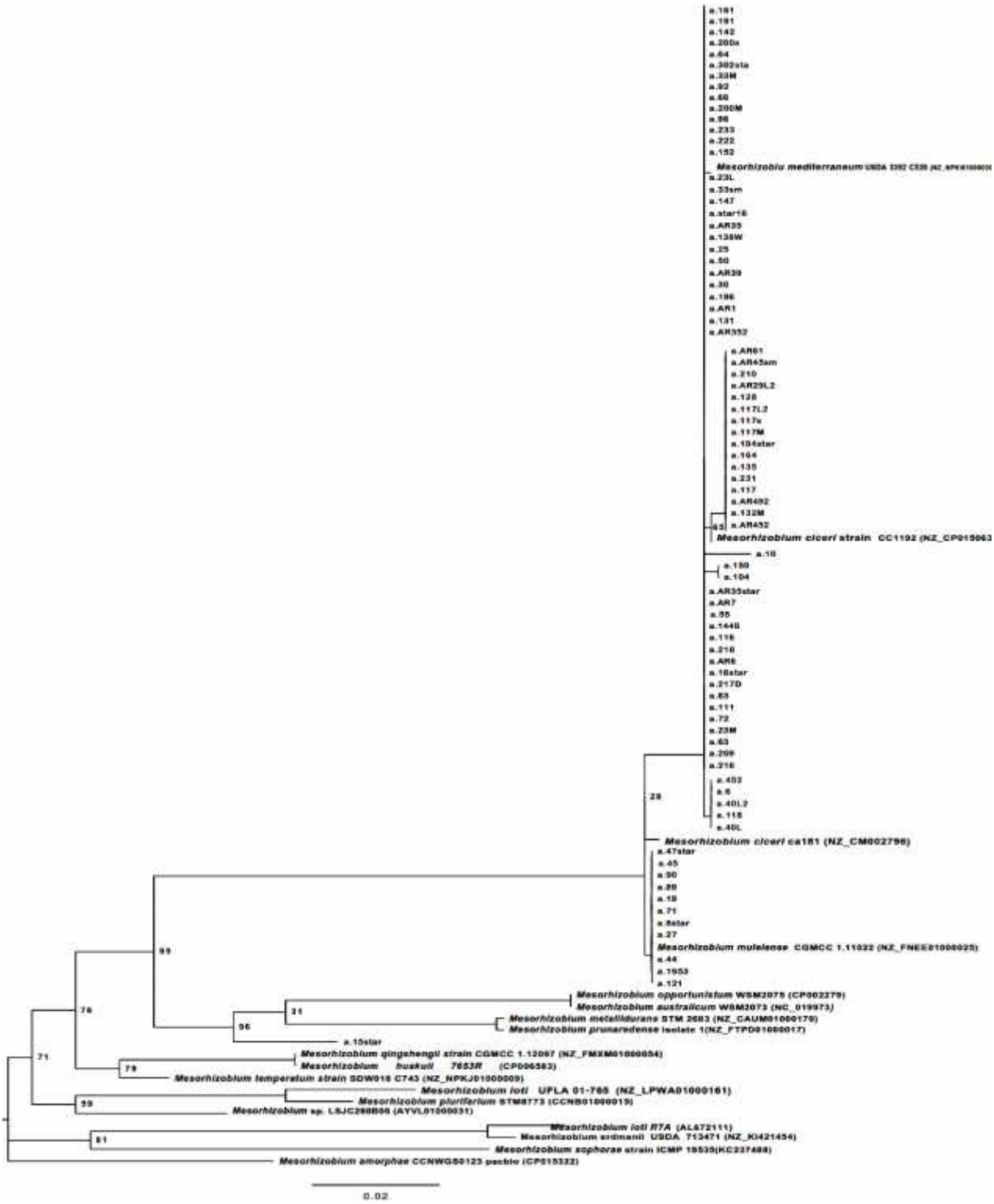


Figure 2.4. Maximum likelihood (ML) tree phylogenetic trees based on sequences of symbiotisis related gene *nif* H gene showing the relationships among the different chickpea isolates and reference strains of the defined *Mesorhizobium* species. Bootstrap values over 65% (based on 1000 replications) are shown at each node. Bars indicate the percentage of estimated nucleotide substitiution.

This study also confirmed as chickpea nodulated by strains of several species of *Mesorhizobium* harboring nearly identical nodulation genes (Rivas *et al*., 2007; Laranjo *et al.,* 2008). In concurrence with this, the study indicated that most genospecies (89%) that nodulate chickpea shared common *nod* C genes, suggesting recognition of similar *nod* genes by chickpea. This study has contributed to the knowledge that mesorhizobia with different chromosomal backgrounds may carry similar symbiosis genes, explaining how a restrictive host for nodulation such as chickpea, is nodulated by several different *Mesorhizobium* species.

Altogether, the relative phylogenetic analysis revealed a high diversity of chickpea nodulating mesorhizobia in the study areas. The symbiotic gene could have brought the evolution of diverse chickpea rhizobial linkages and spreading of nodulation ability among bacteria adapted to local environmental conditions (Rivas *et al*., 2007; Laranjo *et al*., 2008; Zhang *et al*., 2017; Greenlon *et al*., 2019). This might be the case that these isolates of diverse *Mesorhizobium* species groups are able to nodulate chickpea.

### 2.3.6. Distribution of chickpea *Mesorhizobium*

The geographic distribution of chickpea-nodulating isolates in the study area is presented in Table 2.4. Thus, more diverse genospecies groups (at least four different groups) with different proportions were distributed in Bale, west Shewa, south west Shewa, Asossa and south Wollo compared to fewer genospecies identified from west Wollega (Table 2.4). The dominant genospecies, *M*. Genospecies (2E, 2D) (*M. plurifarium*) and *M*. Genospecies (1C,1D) (*M. loti*) were distributed in almost all sampling sites. While *M*.

Genospecies (5C) (*M. ciceri*) and *M.* Genospecies (10A) (*M.* sp. LSJC280B00) were limited to only Arsi and west Shewa, respectively, whereas *M.*Genospecies (11A) (*M. opportunistum*) were isolated from Gurage, north and south Wollo.

The relative abundance of species groups showed that the composition of chickpea mesorhizobia varied in the sampling sites (Table 2.4). In all geographic regions, except Arsi, *M*. Genospecies (2E, 2D) (*M. plurifarium*) group strains were dominant in the range 12.5 % (S/W/Shewa and south Wollo) to 72.7% (Asossa). The *M*. Genospecies (1C,1D) (*M. loti*) strains showed that abundance in south Wollo (50%), west Shewa (27.8%), east Wollega and north Wollo (25%), Gurage and west Wollega (20%), Bale (16.7%) and Asosa (9.1%). The *M*. Genospecies (8A) (*M. australicum*) strains were more abundant in Arsi (62.5%) and west Shewa (22.2%) than the other regions. *M*. Genospecies (4B) (*M. amorphae*) was the most abundant group in shouth west Shewa (37.5%), followed by *M*. Genospecies (3A) strains (25%). Perhaps the recognized new chickpea nodulating genomic groups (*M*. Genospecies 8A and *M*. Genospecies 3A) in this study indicated the occurrence of these strains in the different parts of the country.

The dominance of *M*. Genospecies (2E, 2D) (*M. plurifarium*) and evolution of new distinct genospecies in some regions of Ethiopia could be due to acquisition of the symbiotic gene from co-culturing natural chickpea symbionts and strengthens the assumption that chickpea hosts search certain affiliation with native microsymbiots, well adapted to the local environments (Wondwosen Tena *et al*., 2017; Zhang *et al*., 2017).

Table 2.4. Distribution of chickpea nodulating *Mesorhizobium* genospecies groups in the sampling regions

| **Genospecies group** | **Arsi** | **Bale** | **West Shewa** | **South west Shewa** | **Gurage** | **East Wollega** | **West Wollega** | **Asossa** | **North Wollo** | **South Wollow** | **Total** |
|---|---|---|---|---|---|---|---|---|---|---|---|
| M.Genospecies (2E,2D) *(M.plurifarium)* | - | **1 (16.7%)** | **5 (27.8%)** | **1 (12.5%)** | **3 (60%)** | **5 (62.5%)** | **4 (80%)** | **8 (72.7% )** | **2 (50%)** | **1 (12.5%)** | **30 (37%)** |
| M.Genospecies (4B) *(M.amorphae)* | **2 (25%)** | **1 (16.7%)** | **3 (16.7%)** | **3 (37.5%)** | - | - | - | **1 (9.1%)** | - | - | **10 (12.3%)** |
| M.Genospecies (8A) *(M.australicum)* | **5 62.5%)** | **2 (33.3%)** | **4 (22.2%)** | **2 (25%)** | - | - | - | **1 (9.1%)** | - | **1 (12.5%)** | **15 (18.5%)** |
| M.Genospecies (3A) | - | **1 (16.7%)** | - | **2 (25%)** | - | **1 (12.5%)** | - | - | - | **1 (12.5%)** | **5 (6.1%)** |
| M.Genospecies (1C,1D) ( *M. loti)* | - | **1 (16.7%)** | **5 (27.8%)** | - | **1 (20%)** | **2 (25%)** | **1 (20%)** | **1 (9.1%)** | **1 (25%)** | **4 (50%)** | **16 (19.8%)** |
| M.Genospecies (11A) *(M. opportunistum)* | - | - | - | - | **1 (20%)** | - | - | - | **1 (25%)** | **1 (12.5%)** | **3 (3.7)** |
| M.Genospecies (5C) *(M.ciceri)* | **1 (12.5%)** | - | - | - | - | - | - | - | - | - | **1 (1.2%)** |
| M.Genospecies (10A) *(M.* sp. LSJC280B00) | - | - | **1 (5.6%)** | - | - | - | - | - | - | - | **1 (1.2%)** |
| | **8** | **6** | **18** | **8** | **5** | **8** | **5** | **11** | **4** | **8** | **81** |

%=abundance of each genomic groups of the sampling regions, presented by the ratio of number of the strains in the genomic group per total number of the strains in the region.

In general, the sampling sites in west Shewa, south west Shewa, Asossa, Bale and south Wollo, seems to harbor more diverse populations of chickpea rhizobia as recently reported from different localities of Portugal (Alexandre *et al*., 2009), Ethiopia, India, Turkey and Morocco (Greenlon *et al*., 2019).

### 2.3.7. Principal coordinates analysis on geographic distribution of strains

The principal coordinate's analysis (PCA) indicated the genetic relationships among *Mesorhizobium* strains (Figure 2.5). Thus, 81 indigenous *Mesorhizobium* strains showed a similar pattern as a cluster analysis of the concatenated protein-coding gene. The first and second principal coordinates explained 63.2% and 27.5 % of the variation within strains, respectively, 90% of the variation within the sequence data represented reasonable coverage of the total variation.

This analysis shows a low level of genetic differentiation among indigenous *Mesorhizobium* isolates, showing a stronger, yet similar, pattern of grouping compared to a protein-coding gene analysis. However, the grouping pattern was irrespective of the isolate's geographic locations (Table 2.5), and this is indicative of the possibility of a commonly linked gene within and between *Mesorhizobium* strains. A study by Koskey *et al*. (2018) on PCoA analysis of rhizobia isolates collected from the different agro ecological zone that did not correspond to the geographical locations in Eastern Kenya and linked the genetic homogeneity of the isolates to the ribosomal gene recombination within and between rhizobia strains. Wang *et al*. (2016) also reported that biodiversity and biogeography of rhizobia associated with common beans in Shaanxi province, China,

linked lateral gene transfer of symbiotic genes among different strains of nitrogen-fixing bacteria.

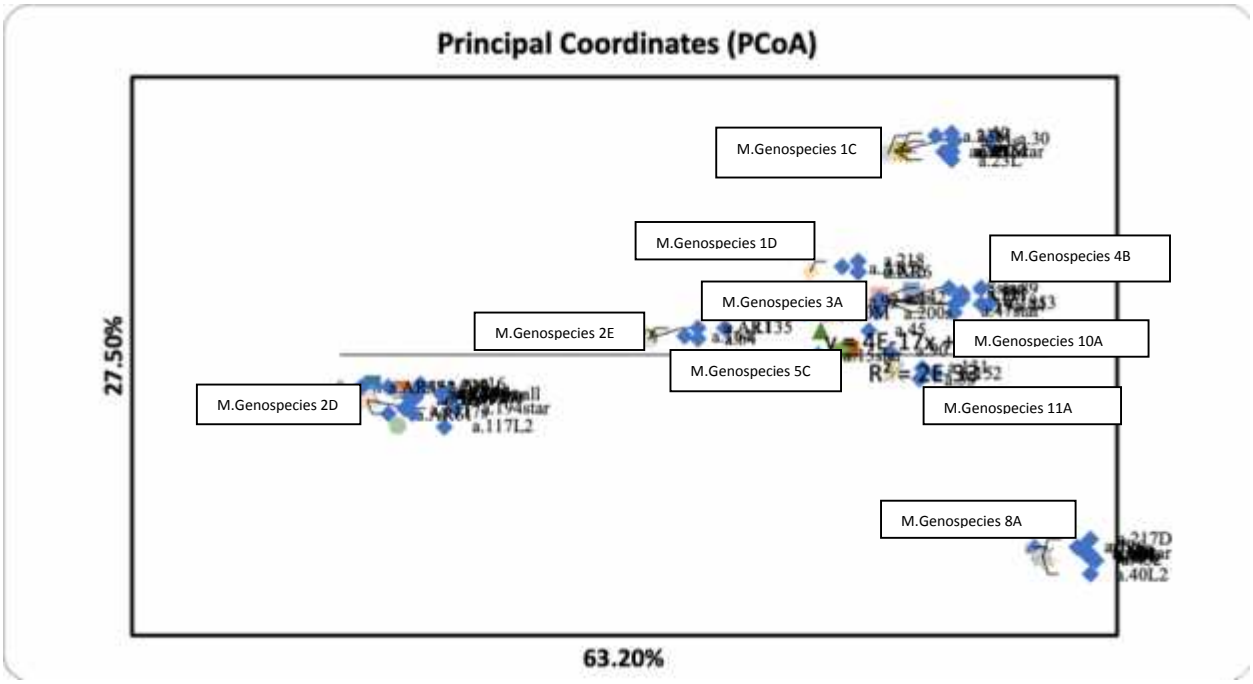


Figure 2.5: Genetic relationships among 81 local *Mesorhizobium* strains on the basis of principal coordinate analysis

### 2.3.8. Relationship between phylogeny and geography

The location of genetic groups plotted according to the sampled site reveals most phylogenetic groups are distributed across the study area (Figure 2.6). The mantel test between pair-wise genetic distance and geographic distance based on coordinates (latitude and longitude) (km) showed the absence of correlation to geographical distribution ($r$ = 0.10, $p<0.01$).

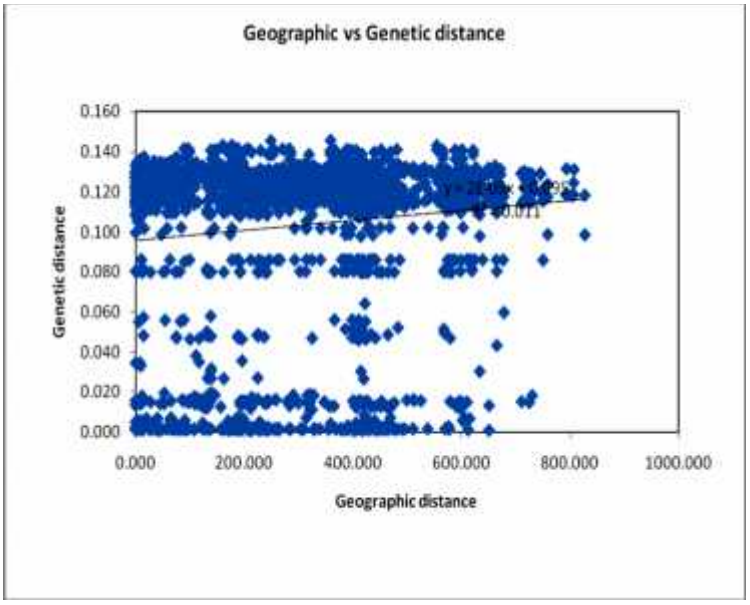


Figure 2.6. Correlation between genetic distance and geographic distance (latitude and longitude) (km) of 81 local *Mesorhizobium* strains

The geographic pattern of genomic diversity indicated the existence of multiple, broadly distributed phylotypes, with the limited relationship between geographic and genetic distance. The geographic distribution of genomic groups indicates most genotypes are distributed across the study area, but with frequent local sub-clusters of related strains. The broad distribution and local clustering of multiple phylotypes across studied locations could be attributed to a combination of local landraces and unique agro-ecology coupled with soil condition.

Thus, the genomic diversity across the studied locations is very low and this may be associated with a limited variability among distant strains and the studied strains probably were originated from the same genetic background (Ismail *et al*., 2013) combined with geographic movement of phylogenetic groups of *Mesorhizobium* strains among locations by means of human activities (e.g. soils, seeds) as a means of limited diversity of strains

across different localities. It also reflects that the nature of Ethiopian agricultural system greatly relies on local crop varieties, where inoculums use is absent thereby restricting the introduction of new genotypes of the *Mesorhizobium* from abroad.

In general, this study contributes to the understanding of the genomic and geographic diversity of chickpea *Mesorhizobium* populations within low pH soils in the country and offers useful insights in developing *Mesorhizobium* inoculants in the country for sustainable agriculture in these localities.

### 2.4. Conclusion and recommendations

In conclusion, acidic soils in Ethiopia harbored more diverse groups of *Mesorhizobium* species and a few of which were not previously known to nodulate chickpea. Phylogenetic analysis revealed eight genospecies were identified across the sampling sites; of which more diverse groups (four genospecies each) belong to Bale, west Shewa, south west Shewa, Asossa and south Wollo than fewer isolates recovered from west Wollega region. *M. plurifarium* (*M*.genospecies 2D, 2E) and *M. loti* (*M*.genospecies 1C, 1D) were widely distributed in almost all sites. The results indicated that the most genospecies (89%) shared common symbiosis genes (*nod* C).

The data also showed that the geographic pattern of genomic diversity indicated the existence of multiple, broadly distributed phylotypes, with no relationship between geographic and genetic distance that may be associated with a limited variability among distant strains and the studied strains probably have originated from the same genetic background. A closer examination of the *Mesorhizobium* strains, for example using a

more detailed examination of the effectiveness of nitrogen fixation or analysis of ecologically competence, and taxonomic patterns can contribute to future biofertilizer selection of the endosymbionts.

To this end, there is a need for further characterization of the genetic diversity amongst isolates by constructing of a pan and accessory genomes from this WGS data and investigation of the roles of both accessory and highly variable common genes concerning niche adaptation. Further examination of the *Mesorhizobium* isolates using, RNA-based approaches, such as RNA-Seq study of chickpea-*Mesorhizobium* interaction, identification of low pH or Al toxicity tolerance associated genes is necessary. This helps to identify the genes that are potentially involved in mechanism of low pH tolerance and their control and map these genes to develop an acid-tolerant specific marker which is critical to improve the productivity of chickpea in acidic region of Ethiopia.

**Data availability**

The Genome sequence assembly for the whole genome sequence projects has been deposited at EMBL under an umbrella BioProject PRJNA453501 with accession numbers PRJNA453501.

# CHAPTER 3

## Screening for ecologically competent and symbiotically effective chickpea nodulating *Mesorhizobium* spp. isolated from acidic soils of Ethiopia

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## Abstract

*Nitrogen fixations are limited in acidic soil due to the sensitivity of legume, rhizobia and the symbiosis to low pH. However, legumes and their rhizobia show different responses to soil acidity. Therefore, it is important to pre-screen mesorhizobial isolates that are symbiotically effective and ecologically competent to their hosts under in vitro low pH conditions. To this end, a total of 81 genetically diverse indigenous Mesorhizobium* spp. *were screened for low pH tolerance and their potential to ecological adaptations under in vitro conditions and their symbiotic effectiveness on two chickpea varieties under greenhouse conditions. The results indicated that 62 strains grew well at low pH 5. The data showed that selected strains within species displayed marked differences in their eco-physiological characteristics such as utilization of different carbon and nitrogen sources, pattern of tolerance to salinity, temperature, $Mn^{2+}$ and $Al^{3+}$ toxicity, heavy metals and inherent resistance to antibiotics. They also displayed significant ($p<0.01$) differences in their nodulation features (nodule number, nodule dry weight) and yield characters (shoot dry weight) on Natoli and DZ-ck-2011s-2-0042 chickpea varieties. Based on their symbiotic effectiveness (SE), all Mesorhizobium strains were highly effective (HE) and effective (E) on DZ-ck-2011s-2-0042, but only 24% and 48% of these strains were highly effective (HE) and effective (E) on Natoli variety, respectively. However, Mesorhizobium strains,* a.55 (*M.genospecies 11A), a.AR7* (*M. genospecies 1D),*

*a.66* (*M. genospecies 1C), a.152* (*M. genospecies 11 A), a.AR1* (*M. genospecies 2E) and a.222* (*M. genospecies 1D) strains were lowly effective on Natoli variety. Five strains, namely a.15star* (*M. genospecies 5C), a.117L2* (*M. genospecies 2D), a.71* (*M. genospecies 4B), a.40L2* (*M. genospecies 8A) and a.200M* (*M. genospecies 3A) showed the best performance on both varieties (SE values 80-100%), even out-performed over the commercially available local strain Cp41 and tolerance to different in vitro ecological conditions. Generally, the present study indicates that Ethiopian acidic soils harbored symbiotically effective, ecologically competent and phosphate solublizing Mesorhizobium species. Thus, these strains may potentially improve chickpea production as inoculant in acidic soil provided that they are validated under different field conditions.*

**Keywords:** Eco-physiological, DZ-ck-2011 s-2-0042 varity, Natoli varity, Symbiotic effectiveness

### 3.1. Introduction

Soil acidity is one of the most important factors that affect nitrogen fixation and the production of leguminous crops, because it increases Aluminum (Al) and Manganese (Mn) toxicity and hampers Calcium (Ca) and Phosphorus (P) uptake by plant (Hungria and Vargas, 2000). Jaiswal *et al.* (2018) have reported that phosphorus deficiency and Aluminium (Al) toxicity in acid soils severely affect growth and symbiotic nitrogen fixation in legumes. These stresses limits the persistence and survival of rhizobia strains in the soil (Graham, 1992), and affect the exchange of molecular signals between rhizobia and their hosts, thus reducing nodulation (Hungria and Vargas, 2000). However, legumes and their specific endosymbiontic rhizobia exhibit varied responses to acidity and effective symbiosis on the host under acidic stress depends upon the strain and legume variety (Guo *et al.*, 2009). Thus, legume production can be improved with the selection of acid-tolerant varieties, effective and competitive strains of rhizobia and liming in acidic soil (Graham, 1992).

Chickpea (*Cicer arietinum* L.) is one of the most important leguminous crops nodulated by specific group of rhizobia under the genus *Mesorhizobium*, to fix atmospheric nitrogen and improve soil fertility. It is estimated that chickpea fixes inorganic nitrogen with suitable rhizobial partner to utilizable form to plants to the tune of 90-180 kg/ha/yr (Werner, 2005); depending upon the host variety, symbiotic and ecological competence of *Mesorhizobium* strains (Aslam *et al.*, 2010; Zhang *et al.*, 2017). Inoculation of chickpea with ecologically fit mesorhizobial strains improved nodulation, growth and yield components of chickpea varieties under adverse conditions (Ben Romdhane *et al.*,

2008), which is partly measured by their *in vitro* ability to utilize different carbon and nitrogen substrates, their inherent resistance to different antibiotics and tolerance to environmental factors such as acidity (pH), temperature and salinity (salt) (Laranjo and Olivera, 2011; Zhang *et al.*, 2017).

According to Brigido *et al.,* (2007), chickpea is one of the successful leguminous crops better adapted to grow and fix nitrogen in acidic soils. Some strains such as *Mesorhizobium loti* have shown a high degree of acid tolerance in laboratory media, being able to grow at pH values as low as 3.0 (Jarvis *et al*., 1997; Brigido *et al*., 2007). Hmissi Imen *et al*. (2015) have demonstrated that *Mesorhizobium* can have a dual purpose of effective symbiotic association for nitrogen fixation and phosphate solubilization to enhance chickpea production in acidic soils. *Mesorhizobia* isolates having nitrogen fixing as well as high P solubilizing capability have great value for sustainable yield enhancement (Peix *et al*., 2001).

Ethiopia is the major chickpea producing country in Africa (FAOSTAT, 2019). However, it is estimated that about 43% of the total cultivated land area is affected by soil acidity (Getachew Agegnehu *et al*., 2019). It has been reported that soil acidity mainly in the central and western parts of the country limits chickpea and other legume crops production (Abdenna Deressa *et al*., 2007; Biru Alemu and Dagnachew Lule, 2018). Under the circumstances, nitrogen and phosphorus are deficient in most highland acidic soils of Ethiopia.

In Ethiopia, several studies were undertaken on eco-physiological and symbiotic properties of chickpea nodulating rhizobia (Mulissa Jida and Fassil Assefa, 2012; Daniel Muleta and Fassil Assefa, 2015; Wondwosen Tena *et al*., 2017; Wubayehu Gebre Medhin *et al*, 2018; Tassew Sirage and Fassil Assefa, 2018). Most of these studies identified (5-10%) chickpea rhizobia isolates combined tolerance to different *in vitro* stress conditions and nutritional versatility with symbiotic effectiveness comparable to Nitrogen fertilized control plants. Other studies also showed effective symbiosis and P solubilization by rhizobia nodulating faba bean (Girmaye Kenasa *et al.*, 2014; Dereje Tsegaye, 2015), and soybean (Daniel Muleta *et al*., 2017) from acidic soils. However, there is still a dearth of information on the pattern of ecological competitiveness, symbiotic effectiveness and phosphate solublization of the different taxonomic groups of *Mesorhizobium* spp from acidic soils of Ethiopia. Therefore, this study was initiated to screen low pH tolerant, phosphate solublizing, hetrotrophically competent and symbiotically effective strains from eight indigenous *Mesorhizobium* species under *in vitro* laboratory and greenhouse conditions.

## 3.2. Materials and Methods

### 3.2.1. Sources of *Mesorhizobium* isolates

The study included 81 chickpea nodulating *Mesorhizobial* strains belonging to eight different *Mesorhizobium* spp. which were retrieved from root nodules of chickpea grown on acidic soils from central, western, southern and northern parts of Ethiopia (Table 3.5). They were isolated on Yeast Extract Mannitol Agar (YEMA) medium, and genetically

identified into the genus *Mesorhizobium* spp and deposited in culture collections at Addis Ababa University and University of California Davis, USA.

### 3.2.2. Screening for low pH tolerance

All the strains were screened for acid tolerance on medium consists of 300 µM MgSO4.7$H_2O$, 300 µM $CaCl_2$, 100 µM Fe EDTA. 10 µM KCI, 1µM $MnCl_2$.4$H_2O$, 0.4 µM $ZnSO_4$.7$H_2O$, 0.1 µM $CuCl_2$.2$H_2O$, 0.02 µM $Na_2MoO_4$.2$H_2O$, 0.02 µM $Co(N0_3)_2$.6$H_2O$, 500 µM $KH_2P0_4$,500 µM $K_2HPO_4$, arabinose (5.0 g), galactose (5.0 g), (1.1 g) Na glutamate, biotin (0.1 mg), thiamine (1.0 mg), (0.005%) bromothymol blue, (15g) agar, and 1L distilled water and adjusted to pH 5 containing high Mn (1.0 mM) and Al (50 µM), and low P (5 µM) and Ca (50 µm) (Gemell *et al.,* 1993).

### 3.2.3. Eco-physiological and nutritional characteristics of selected Mesorhizobial strains

All tests, except carbon and nitrogen utilization, were carried out on YEMA plates by inoculating with 10 µl of inoculums suspension ($10^9$ cfu/ml) and incubated at 28°C for 5 days against uninoculated control unless stated otherwise. All tests were carried out in triplicates and results were recorded visually as ‘’+’’ for growth and ‘’- ‘’ absence of growth.

### 3.2.3.1. Salt, pH and temperature tolerance

Salt and low pH tolerance was determined on YEMA plates containing 1 to 5% (w/v) NaCl concentrations and the medium adjusted to pH (4 and 4.5), respectively (Amarger *et al*., 1997). Temperature tolerance was evaluated by inoculating them on YEMA plates under incubation temperatures (4, 10, 15, 20, 37, 40 and 45 $^{0}$C) (Maatallah *et al.*, 2002).

### 3.2.3.2. Intrinsic antibiotic and heavy metal resistance

The intrinsic antibiotic and heavy metal resistance of strains were determined on solid YEMA medium containing filter sterilized antibiotics or heavy metals. The stock solutions of the antibiotics or heavy metals were sterilized using (0.22 µm Millipore) membrane filters and added to YEMA. The antibiotics used were (µg. $mL^{-1}$): ampicillin (5 and 10), chloramphenicol (5 and 10), erythromycin (10 and 20), nalidixic acid (5 and 10), streptomycin (40 and 80), rifampicin (5, 10), neomycin (5 and 10) and tetracycline (5 and10). Similarly, the heavy metals used were $CoCl_2$ (25,100), $CuCl_2$.2H2O (50, 100), $NiSO_4$ (50,100), and $ZnCl_2$ (50,100), $K_2Cr_2O_7$ (50, 100) (Howieson and Dilworth, 2016). Acidity related $Al^{3+}$ and $Mn^{2+}$ tolerance was tested at two different Al concentrations $KAl(SO4)_3$ (50 µM and 100 µM) and two Mn concentrations $MnCl_2$ (75 µM and 100 µM) using Keyser and Munns (KM) agar medium under acidic conditions at pH 5 (Ayanaba *et al*.,1983).

### 3.2.3.3. Nutritional versatility of strains on different carbon and nitrogen substrates

Different carbohydrates were added as described by Amarger *et al.* (1997) at a final concentration of $1gL^{-1}$ of the basal medium containing (gl/1): $K_2HPO_4$, 1; $KH_2PO_4$, 1; $FeCl_3.6H_2O$, 0.01, $NH_4SO_4$, $1NaCl_2$, 0.1; $MgSO_4.7H_2O$, 0.2; and agar, 15. The following filter sterilized (0.22 µm millipore) heat liable carbon sources; Citric acid, D-sorbitol, D-glucose, D-galactose, xylose, trehalose were added after autoclaving; and heat stable α-lactose, D-fructose, glycerol, α-cellulose, sucrose, and maltose were autoclaved with the basal medium. Filter sterilized L-tryptophan, methionine, L-tyrosine, leucine, riboflavin; DL-β-phenylalanine, L-arginine, glutamic acid, L-lysine, L-serine, glycine, and thiamine were used as a sole nitrogen source to a final concentration of 0.5 $gL^{-1}$ on the basal medium containing ($gL^{-1}$): $K_2HPO_4$, 1; $KH_2PO_4$, 1; $FeCl_3$. $6H_2O$, 0.01; $MgSO_4.7H_2O$, 0.2; $CaCl_2$, 0.1; $NH_4(SO_4)_2$, 1; and agar, 15; from which $(NH_4)_2(SO_4)$ was omitted and mannitol was added after autoclaving (Amarger *et al.*, 1997). All plates were incubated at 28°C for 5 days.

### 3.2.4. Symbiotic characterization mesorhizobia strains

The experiment was carried out at Debre Zeit Agricultural Research Center (DZARC), Debre Zeit, Ethiopia. The study was undertaken in a pot experiment using a sterile sand culture under greenhouse condition (Howieson and Dilworth, 2016). Each *Mesorhizobium* strain was grown on YEMB and incubated at 28°C for 5 days. Seeds of chickpea cultivars called 'Natoli and DZ-ck-2011 s-2-0042' were surface sterilized with 4% sodium hypochlorite for 3 min, then rinsed with five changes of sterile distilled water and allowed to germinate on water agar 1.5 % w/v at $25^0$C for three days.

The germinated seeds were planted in alcohol swabbed plastic pots (3 kg capacity) containing washed and autoclaved sterilized river sand. Five seeds were planted $pot^{-1}$, individually flooded with 1 ml of the culture suspension ($10^{-9}$ cfu/ml) and thinned down to three plants $pot^{-1}$ after 5 days of emergence (DAE). The experiment was laid out with three replications for each treatment using randomized complete design with 12 h photoperiod, day temperature ($28\pm2^0$C) and night temperature ($17\pm3^0$C), by including uninoculated but nitrogen-fertilized (1% w/v $KNO_3$) pots as positive (TN) control and uninoculated non- nitrogen-fertilized (T0) pots as negative controls. The pots were irrigated with nitrogen-free plant growth nutrient solution CRS (Center for *Rhizobium* Studies, Australia) (Howieson and Dilworth, 2016) once a week and with sterile distilled water every three days, respectively. The pH of the nutrient solution was adjusted to pH 5.

Plants were harvested after 8 weeks of planting to record the number of nodules $plant^{-1}$ (NN), nodule dry mass (NDW); shoot dry weight (SDW). The percent symbiotic effectiveness (SE) was calculated according to (Purcino *et al.,* 2000). The percent symbiotic effectiveness of the isolates was expressed as a percentage of the shoot dry biomass of each treatment compared with the shoot dry biomass of the positive control (with N). Finally, symbiotic effectiveness was rated as highly effective (HE) when the percentage of effectiveness >80%, effective, (E) between 50 and 80%, and of low effectiveness (LE) between 35 and 50%. Strains were considered ineffective when the percentage effectiveness was less than 35%. The data were analyzed by one-way analysis of variance (ANOVA) using the general linear model procedure of the SAS software

package (SAS/STAT; version 9.3) and mean values were separated according to Duncan's multiple range test at p = 0.05 (SAS Institute, 2012).

## 3.3. Results and Discussion

### 3.3.1. Screening of mesorhizobia strains for low pH tolerance and phosphate solubilization

Chickpea nodulating *Mesorhizobium* strains were screened based on low pH tolerance and 62 (76%) selected strains showed growth on low pH 5 medium (Appendix 6), indicating the presence of low pH tolerant indigenous strains. Other studies also showed the presence of low-pH tolerant chickpea rhizobia in Portugal (Brigido *et al.,* 2007) and Morocco (Maatallah *et al.,* 2002). The data showed that the growth of isolated strains varied ranging from none to profuse; indicating the strains of a given species varies in their pH tolerance. Such differences in tolerance to acidity among strains have been reported previously for various *Rhizobium* (Ayanaba *et al*., 1983; Asanuma and Ayanaba, 1990; Daniel Muleta *et al*. 2017), indicated that *Rhizobium* strains that survived in the acid soil cannot grow on a nutrient medium with a pH as low as that of the soil from which the strains were isolated. According to Icgen *et al*. (2002), chickpea rhizobia strains displayed a tendency to neutralize the pH of the medium when grown freely in media adjusted to different low pH values, and this might account for the success in acidic conditions. Overall, the present work shows that *in vitro* evaluation of strain growth under pH stress may also be a useful method for finding rhizobial isolates adapted to different soil pH.

### 3.3.2. Eco-physiological characteristics of selected *Mesorhizobium* strains

The mesorhizobial strains were prescreened for tolerance to pH 5, of which 26 strains were selected for further analysis. The strains were tested for *in vitro* tolerance at lower pH, 9 and 25 strains were grown at pH 4 and 4.5, respectively (Table 3.1). Studies also indicated that *Mesorhizobium loti* grown in medium at pH values as low as 4 (Jarvis *et al*., 1997) and other chickpea mesorhizobia isolates are able to grow at strongly acidic pH 3 (Brigido *et al.,* 2007). The result indicated that tolerance to acidity is more strain specific than species specific (Amarger *et al*., 1997). Interestingly, a.15star (*M*.genospecies 5C) showed growth at pH 5 but did not grow at pH (4, 4.5) and thus might be considered a moderate acidophile. Similarly, Brigido *et al.,* 2007; Laranjo and Olivera, 2011) have shown the isolates that belong to the group of *M. ciceri* with preference for tolerance at pH 5, which suggest that a species-related tolerance to acidity. The data did show a negative correlation (r=-0.09,-0.26, p=0.05) between origin of isolation soil pH and pH of isolation medium which is contrary to reports that show a positive correlation between chickpea rhizobia tolerance to different pH values and the origin-soil pH (Rodrigues *et al.,* 2006; Brigido *et al.,* 2007; Alexandre *et al*., 2009). According to Brigado *et al.* (2013), adaptation to acid pH by chickpea rhizobia is due to the presence of chaperones genes in their cells which are important for survival during acid stress.

Table 3.1. Eco-physiological characteristics of selected chickpea nodulating mesorhizobia strains grown YEMA medium and incubated for 5-7 days.

| Mesorhizobia strains | Genospecies name | Close associated type strains | Low pH tolerance | | Temperature tolerance | | | | | | NaCl % tolerance | | | | | Soil pH of the isolation site |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | pH 4 | pH 4.5 | 10°C | 15°C | 20°C | 37°C | 40°C | 45°C | 1% | 2% | 3% | 4% | 5% | |
| **a.AR1** | *M.*genospecies 2E | *M. plurifarium* STM8773 | - | + | - | - | + | + | + | + | + | + | + | + | + | 5.9 |
| **a.64** | ,, | | - | + | + | + | + | + | + | + | + | + | - | - | - | 5.3 |
| **a.AR452** | M.genospecies 2D | | - | + | - | - | + | + | + | - | + | + | - | - | - | 5 |
| **a.138W** | ,, | | + | + | - | - | + | + | + | + | + | + | + | - | - | 5.1 |
| **a.144 S** | ,, | | - | + | - | - | + | + | + | + | + | + | - | - | - | 4.7 |
| **a.117L2** | ,, | | + | + | + | + | + | + | + | + | + | + | + | - | - | 4.9 |
| **a.35star** | ,, | | + | + | - | - | + | + | - | - | + | + | - | - | - | 4.8 |
| **a.AR7** | *M.*genospecies 1D | *M.loti strain* UFLA01-766 | - | + | - | + | + | + | + | + | + | + | + | - | - | 5.3 |
| **a.222** | ,, | | - | + | + | + | + | + | + | + | + | + | - | - | - | 5.2 |
| **a.302star** | *M.*genospecies 1C | | - | + | - | + | + | + | + | - | + | + | + | + | + | 5.4 |
| **a.66** | ,, | | - | + | - | + | + | + | + | - | + | + | + | + | + | 5.9 |
| **a.200M** | *M.*genospecies 3A | | - | + | + | + | + | + | + | + | + | + | - | - | - | 4.4 |
| **a.200s** | ,, | | - | + | - | - | + | + | + | - | + | + | + | - | - | 5.2 |
| **a.71** | *M.*genospecies 4B | *M.amorphae* CCNWGSO123-pacbio | - | + | - | - | + | + | + | + | + | + | - | - | - | 5.1 |
| **a.89** | ,, | | - | + | - | - | + | + | + | - | + | - | - | - | - | 5.1 |
| **a.111** | ,, | | - | + | + | + | + | + | + | - | + | - | - | - | - | 5.5 |
| **a.8star** | ,, | | - | + | - | - | + | + | + | + | + | + | - | - | - | 5.2 |
| **a.40L2** | *M.*genospecies 8A | *M. australicum* WSM2073 | + | + | - | + | + | + | + | - | + | + | + | - | - | 5.9 |
| **a.16star** | ,, | | - | + | - | + | + | + | + | + | + | + | + | - | - | 5 |
| **a.104** | ,, | | - | + | - | + | + | + | + | - | + | + | + | + | - | 5.3 |
| **a.161** | ,, | | + | + | - | - | + | + | + | - | + | + | + | + | + | 5.7 |
| **a.45 2** | ,, | | + | + | - | - | + | + | + | - | + | + | + | + | + | 5 |
| **a.152** | *M.*genospecies 11A | *M.opportunistum* WSM2075 | + | + | - | - | + | + | + | - | + | + | + | + | - | 5.2 |
| **a.55** | ,, | | + | + | - | - | + | + | + | - | + | + | + | + | + | 5.1 |
| **a.15star** | *M.*genospecies 5C | *Mesorhizobium ciceri ca*181 | - | - | + | + | + | + | + | - | + | + | + | + | + | 5.7 |
| **a.90** | *M.*genospecies10A | *M. sp.*LSJC280BOO | + | + | - | + | + | + | + | - | + | + | + | + | - | 4.9 |
| Total | | | 9 (35%) | 25 (96%) | 6 (23%) | 13 (50%) | 26 (100%) | 26 (100%) | 25 (96%) | 11 (42%) | 26 (100%) | 24 (92%) | 16 (62%) | 10 (39%) | 7 (27%) | |

+: the presence of growth, -: the absence of growth

All the strains were able to grow between 20 and 37°C and showed variations below and above these values, where 6(23%) strains were resistant to 10°C; whereas 11 strains (42.3%) were able to grow at 45°C (Table 3.1), which is relatively different from temperature sensitive chickpea rhizobia (40-45°C) isolated from Ethiopia (Wubayehu Gebremedhin *et al.,* 2018) and China (Zhang *et al*., 2017). Conversely, studies from Turkey (Kucuk and Kivanc, 2008), and Morocco (Maatallah *et al.,* 2002) showed chickpea rhizobia were able to grow at high temperature (>$40^0$C), which could be related to local adaptation. *Mesorhizobium* a.117L2 (*M.*genospecies 2D) and a.200M (*M.*genospecies 3A) strains showed wide range of temperature tolerance (10- $45^0$C) than the other strains (Table 3.1). Similarly, Laranjo and Olivera, (2011) and Rai *et al*, (2012) have shown pattern of tolerance to high temprature by *Mesorhizobium plurifarium* and *Mesorhizobium loti* strains, respectively. According to Rodrigues *et al.,* (2006), adaptation of tolerance to high temperature by rhizobia strains in chickpea rhizobia is due to overproduction of a set of proteins, termed heat shock proteins (HSPs), which are important for survival during stress conditions.

Chickpea rhizobia strains displayed high diversity in their salt tolerance (Table 3.1). All strains were tolerant to 1% NaCl, but 24 (92%) of the strains survived at 2% NaCl, and fewer isolates 7 (27%) were able to grow on YEMA medium containing 5% NaCl. This result is comparable to previous finding in Ethiopia that indicates a wide range of variation in salt tolerance to 1-5% (w/v) NaCl concentration (Wondwosen Tena *et al*., 2017). The most tolerant strains were a.AR1 (M.genospecies 2E), a.30s (M.genospecies 1C), a.66 (M.genospecies 1C), a.161 (M.genospecies 8A), a.452 (M.genospecies 8A),

a.55 (M.genospecies 11A) and a.15star (M.genospecies 5C) that were able to grow at 5% NaCl. Studies also showed that *Mesorhizobium ciceri*s, *Mesorhizobium plurifarium* and *Mesorhizobium loti* strains were tolerant to high NaCl concentration (Laranjo and Oliveira, 2011; Rai *et al*., 2012). According to Zahran (1999) salt tolerant rhizobia are endowed with the capacity to tolerate osmotic stress that is mainly associated with their capacity to accumulate low molecular weight organic solutes in their cells.

### 3.3.3. Acidity-$Al^{3+}$/$Mn^{2+}$ tolerance

The strains showed variable responses to $Al^{3+}$ and $Mn^{2+}$ toxicity which is often associated with acidic soils (Table 3.2). Thus, 7(27 %) and 10 (38%) of the *Mesorhizobium* strains displayed tolerance to $Al^{3+}$ and $Mn^{2+}$ toxicity at a concentration of 100 µM /ml at pH 5, respectively. The data showed that strains a.117L2 (*M*.genospecies 2D), a.64 (*M*.genospecies 2E), a.152 (M.genospecies 11A), a.55 (*M*.genospecies 11A), a.200M (*M*.genospecies 3A) and a.71 (*M*.genospecies 4B) were the most tolerant strains that grew well at all tested concentration of $Al^{3+}$ and $Mn^{2+}$. The current result also confirmed the earlier report of Ayanaba *et al*. (1983) who stated that, there is relationship between acid-Al sensitivity of isolates with their colony texture, as large-mucoid rhizobial colonies were more resistance than dry-pinpoint colonies. Previous studies in Ethiopia (Mulissa Jida and Fassil Assefa, 2012) and in Morocoo (Maatallah *et al.,* 2002) also showed relatively same pattern of resistance to $Mn^{2+}$, and sensitivity to $Al^{3+}$ in chickpea rhizobia isolates. According to Jaiswal *et al*. (2018), $Al^{3+}$ toxicity and acidity itself is probably more important limiters of rhizobial growth than $Mn^{2+}$ toxicity in acid soils.

Table 3.2. Soil acidity related metal ($Al^{3+}$ and $Mn^{2+}$ toxicity) tolerance at pH 5 of selected chickpea nodulating mesorhizobia strains

| **Mesorhizobia strains** | **Genospecies name** | **Close associated type strains** | **Soil acidity related metal (Al and Mn toxicity tolerance at pH 5** | |
|---|---|---|---|---|
| | | | **$Al^{3+}$** | **$Mn^{2+}$** |
| | | | **100 μM /ml** | **100 μM /ml** |
| **a.AR1** | *M*.genospecies 2E | *M. plurifarium* STM8773 | + | - |
| **a.64** | ,, | | + | + |
| **a.AR452** | *M*.genospecies 2D | | - | - |
| **a.138W** | ,, | | - | - |
| **a.144 s** | ,, | | - | + |
| **a.117L2** | ,, | | + | + |
| **a.35star** | ,, | | - | - |
| **a.AR7** | *M*.genospecies 1D | *M.loti strain* UFLA01-766 | - | - |
| **a.222** | ,, | | - | - |
| **a.302star** | *M*.genospecies 1C | | - | - |
| **a.66** | ,, | | - | - |
| **a.200M** | *M*.genospecies 3A | | + | + |
| **a.200s** | ,, | | - | - |
| **a.71** | *M*.genospeces 4B | *M.amorphae* CCNWGSO123-pacbio | + | + |
| **a.89** | ,, | | - | - |
| **a.111** | ,, | | - | - |
| **a.8star** | ,, | | - | - |
| **a.40L2** | *M*.genospecies 8A | *M. australicum* WSM2073 | - | + |
| **a.16star** | ,, | | - | - |
| **a.104** | ,, | | - | + |
| **a.161** | ,, | | - | - |
| **a.45 2** | ,, | | - | - |
| **a.152** | *M*.genospecies 11A | *M.opportunistum*WSM2075 | + | + |
| **a.55** | ,, | | + | + |
| **a.15star** | *M*.genospecies 5C | Mesorhizobium_ciceri_ca181 | - | - |
| **a.90** | *M*.genospecies 10A | *M. sp.*LSJC280BOO | - | + |
| Total | | | 7(27%) | 10 (38.5%) |

+: the presence of growth, -: the absence of growth

### 3.3.4. Intrinsic antibiotics (IAR) and heavy metals resistance (HR)

The *Mesorhizobium* strains showed variations in inherent antibiotic resistance in that almost all the strains were resistant to nalidixic acid, kanamycin, ampicillin, chloramphenicol and erythromycin with the exception of *Mesorhizobium* a.71

(*M*.genospeces 4B) and a.89 (*M*.genospeces 4B) that were sensitive to erythromycin (Table 3.3). Three *Mesorhizobium* strains, a.71 (*M*.genospeces 4B), a.55 (*M*.genospeces 11A) and a. AR7 (*M*.genospeces 1D) were resistant to streptomycin and a. AR7 strain from *M*.genospeces 1D group were resistant to tetracycline. *Mesorhizobium* a.15star (*M*.genospeces 5C) and a.AR7 (*M*.genospeces 1D) showed multiple antibiotic resistances by growing on media containing 7-8 of the antibiotics tested (Table 3.3). This is similar to a previous report that showed chickpea rhizobia such as *M. ciceri* strains tolerant to 70-80%) tested antibiotics (Tassew sirage and Fassil Assefa, 2018). Other studies in Ethiopia also showed that chickpea rhizobia were relatively resistant to nalidixic acid and erythromycin; and sensitive to streptomycin and tetracycline (Wondwosen Tena *et al*., 2017). On the contrary, chickpea rhizobia from Morocoo (Maatallah *et al*., 2002) and Turkey (Kucuk and Kivanc, 2008) were sensitive to ampicillin, chloramphenicol and kanamycin, indicating large variability in antibiotic resistance of chickpea endosymbionts.

The mesorhizobial strains showed different response to heavy metals (Table 3.3). Accordingly, all strains failed to grow on Co and Ni (data not shown), whereas most strains (65-96%) were tolerant to aluminium, zinc, manganese, copper and chromium. The data showed that 12 (46) % of the strains showed a wide spectrum of heavy metal resistance growing on five of the heavy metals tested. The study in Ethiopia also showed that chickpea rhizobia were resistant to Mn, Al, and Zn (Daniel Muleta and Fassil Assefa, 2015). A study from Morocco also showed 20-60% of the chickpea rhizobia were resistant to Al, Zn, and Cu (Maatallah *et al*., 2002). This generally indicates that there is

Table 3.3. Intrinsic Antibiotic resistance (IAR), Heavy metals tolerance of selected chickpea nodulating mesorhizobia strains

| Mesorhizobia strains | Genospecies name | Close associated type strains | Intrinsic antibiotics resistance (IAR) | Heavy metals resistance (HR) |
|---|---|---|---|---|
| **a.AR1** | *M*.genospecies 2E | *M. plurifarium* STM8773 | Rif, Kan, Amp, Chl, Er, Nal | Al, Cu, Zn, Cr |
| **a.64** | ,, | | Neo, Kan, Amp, Chl, Er, Nal | Al, Mn, Cu, Zn |
| **a.AR452** | *M*.genospecies 2D | | Kan, Amp, Chl, Er,Nal | Al, Mn, Zn, Cr |
| **a.138W** | ,, | | Kan, Amp, Chl, Er,Nal | Al, Mn, Cu, Zn, Cr |
| **a.144s** | ,, | | Kan, Amp, Chl, Er, Nal | Al, Mn, Cu, Zn, Cr |
| **a.117L2** | ,, | | Neo, Kan, Amp, Chl, Er, Nal | Al, Mn, Cu, Zn, Cr |
| **a.35star** | ,, | | Kan, Amp, Chl, Er, Nal | Al, Mn, Cu, Zn, Cr |
| **a.AR7** | *M*.genospecies 1D | *M.loti strain* UFLA01-766 | Stre, Tetr, Rif, Kan, Amp, Chl, Er, Nal | Al, Mn, Cu, Zn, Cr |
| **a.222** | ,, | | Kan, Amp, Chl, Er, Nal | Al, Mn |
| **a.302star** | *M*.genospecies 1C | | Kan, Amp, Chl, Er, Nal | Al, Mn, Cu, Zn, Cr |
| **a.66** | ,, | | Rif, Kan, Amp, Chl, Er, Nal | Al, Mn, Cu, Zn, Cr |
| **a.200M** | *M*.genospecies 3A | | Neo, Kan, Amp, Chl, Er, Nal | Al, Mn, Zn |
| **a.200s** | ,, | | Kan, Amp, Chl, Er, Nal | Al, Mn, Zn, Cr |
| **a.71** | *M*.genospeces 4B | *M.amorphae* CCNWGSO123-pacbio | Stre, Neo, Kan, Amp, Chl, Nal | Al, Mn |
| **a.89** | ,, | | Rif, Neo, Kan, Amp, Chl, Nal | Al, Mn, Cu, Zn, Cr |
| **a.111** | ,, | | Neo, Kan, Amp, Chl, Er, Nal | Al, Mn, Cu, Zn, Cr |
| **a.8star** | ,, | | Kan, Amp, Chl, Er,Nal | Mn |
| **a.40L2** | *M*.genospecies 8A | | Rif, Kan, Amp, Chl, Er, Nal | Al, Mn, Cu, Zn, Cr |
| **a.16star** | ,, | *M. australicum* WSM2073 | Rif, Kan, Amp, Chl, Er, Nal | Al, Cu, Zn, Cr |
| **a.104** | ,, | | Neo, Kan, Amp, Chl, Er, Nal | Al, Mn, Cu, Zn |
| **a.161** | ,, | | Rif, Kan, Amp, Chl, Er, Nal | Al, Cu, Zn, Cr |
| **a.45 2** | ,, | | Rif, Kan, Amp, Chl, Er, Nal | Al, Cu, Zn |
| **a.152** | *M*.genospecies 11A | *M.opportunistum*WSM2075 | Neo, Kan, Amp, Chl, Er, Nal | Al, Mn, Cu, Zn, Cr |
| **a.55** | ,, | | Stre, Kan, Amp, Chl, Er, Nal | Al, Mn, Zn, |
| **a.15star** | *M*.genospecies 5C | *Mesorhizobium ciceri* ca181 | Rif, Neo,Kan, Amp, Chl, Er, Nal | Al, Mn, Cu, Zn, Cr |
| **a.90** | *M*.genospecies 10A | *M. sp*.LSJC280BOO | Kan, Amp, Chl, Er, Nal | Al, Mn,Cu, Zn |

Am: ampicillin; Chl: chloramphenicol, Kan: kanamycin, Nal: nalidixic acid, Rif: rifampicin, Neo: neomycin, Str: streptomycin, Tetr: tetracycline; Er: Erythromycin,

inherent antibiotic and heavy metal resistance by strains based upon the origin of isolation and the type of chemicals they had been exposed to in the soil.

### 3.3.5. Pattern of carbon and nitrogen source utilization

Most of the chickpea Mesorhizobia strains were able to utilize many of the carbon substrates tested and failed to grow on citrate (Table 3.4). *Mesorhizobium* a.15star (*M.*genospecies 5C) and a.AR452 (*M.*genospecies 2D ) strains were the most versatile of all the strains that were able to grow on more than 90% of the carbon sources; whereas *Mesorhizobium* strains a.71, a.89 and a.8star (*M.* genospecies 4B) group limited to grow on 50% of the tested carbon substrates. This result is concurrent with findings of Tassew Sirag and Fassil Assefa (2018) that showed most *M. plurifarium* and *M. ciceri* strains from Ethiopia were versatile in carbohydrate utilization than the other groups.

Chickpea rhizobia strains also exhibited diversity in utilizing different amino acids and vitamins as sole N-sources (Table 3.4). All strains were grown on all the tested N-sources whereas fewer strains failed to grow on L-lysine and glycine. Unlike that of carbon sources, 13 (50%) of the strains utilized more than 90% of the nitrogen substrates, where as a.8star (*M.* genospecies 4B) strain utilized only 33% of the tested N sources substrates, indicating that most *Mesorhizobium* strains were more versatile in N utilization than C utilization. Other studies in Ethiopia also showed the same pattern of carbon and nitrogen utilization of chickpea rhizobia (Mulissa Jida and Fassil Assefa, 2012; Wondwosen Tena *et al*. 2017). On the contrary, some *M. ciceri* and *M. plurifarium* strains utilized citrate and lysine unlike to the present finding (Tassew sirage and Fassil Assefa, 2018).

Table 3.4. Carbon and nitrogen utilization pattern of selected chickpea nodulating mesorhizobia strains

| Close associated type strains | | *M. plurifarium* STM8773 | | | | | | *M.loti strain* UFLA01-766 | | | | | *M.amorphae* CCNWGSO123-pacbio | | | | *M. australicum* WSM2073 | | | | | *M.opportunistum* WSM2075 | | *M.cicceri* ca181 | *M. sp.*LS JC280 BOO | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *M.*genospecies name | | 2E | | 2D | | | | 1D | 1C | | 1D | 3A | 4B | | | | 8A | | | | | 11A | | 5C | 10A | |
| Mesorhizobia strains | | **a.AR1** | **a.64** | **a.AR452** | **a.138W** | **a.144 s** | **a.117L2** | **a.35star** | **a.AR7** | **a.302star** | **a.66** | **a.222** | **a.200M** | **a.200s** | **a.71** | **a.89** | **a.111** | **a.8star** | **a.40L2** | **a.16star** | **a.104** | **a.161** | **a.45 2** | **a.152** | **a.55** | **a.15star** | **a.90** | Total |
| **Carbon Source utilization** | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| **Carbohydrate utilization** | **Glucose** | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | **Galactose** | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | **Glycerol** | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | **Lactose** | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| | **D- Fructose** | | | | | | | | | | | | | | | | | | | | | | | | | | 100 |
| | **Maltose** | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | **Sucrose** | + | + | + | + | + | + | + | + | + | + | + | + | + | - | - | + | - | + | + | + | + | + | + | + | + | + | 88.5 |
| | **D - Sorbital** | + | + | + | + | + | + | + | - | - | + | + | + | + | - | - | - | - | + | + | + | + | + | + | + | + | - | 77 |
| | **D - Xylose** | + | - | + | - | + | + | + | + | - | + | + | + | + | - | - | - | - | + | + | + | + | - | + | + | + | + | 69 |
| | **Trehalose** | - | - | + | - | + | - | + | - | - | - | + | - | + | - | - | - | - | + | + | + | - | - | - | - | + | - | 34.6 |
| | **Citric Acid** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 0 |
| | **Cellulose** | - | - | + | - | - | - | - | - | + | - | - | - | - | - | - | - | - | - | - | - | + | + | - | - | + | - | 19 |
| | **Total** | 75 | 66.6 | 91.6 | 66.6 | 83.3 | 75 | 83.3 | 66.6 | 66.6 | 75 | 83.3 | 75 | 83.3 | 50 | 50 | 58.3 | 50 | 83.3 | 83.3 | 83.3 | 83.3 | 75 | 75 | 75 | 91.7 | 66.6 | |
| **Nitrogen Source utilization** | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | **L - Arganine** | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | **L - Serine** | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | 100 |
| | **L - Tryptophan** | | | | | | | | | | | | | | | | | | | | | | | | | | |
| | **Glycine** | - | - | - | + | + | - | - | - | - | + | - | - | - | - | + | - | - | + | + | + | + | - | - | + | + | - | 38.5 |
| | **L - Lysine** | + | - | + | - | - | - | + | + | - | - | - | - | - | + | - | - | - | + | + | + | + | + | + | - | - | - | 42.3 |
| | **L - Leucine** | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - | - | + | + | + | + | + | + | + | + | + | 92.3 |
| | **DLβ-Phynylalanine** | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - | 96.2 |
| | **Gulutamic acid** | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - | - | - | - | - | - | + | - | - | + | - | 65.4 |
| | **L - Tyrosine** | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - | - | + | + | + | + | + | + | + | + | + | 92.2 |
| | **Methionine** | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | - | + | + | + | + | + | + | + | + | + | 96.2 |
| | **Thiamine** | + | + | + | + | + | + | + | + | - | + | + | + | + | + | + | - | - | + | + | + | + | - | + | + | + | + | 84.6 |
| | **Riboflavine** | + | - | + | + | + | + | + | + | + | + | + | - | + | + | + | - | - | + | + | + | + | - | + | + | + | - | 76.9 |
| | **Total** | 91.6 | 75 | 91.6 | 91.6 | 91.6 | 83.3 | 91.6 | 91.6 | 75 | 91.6 | 83.3 | 75 | 83.3 | 91.6 | 91.6 | 41.6 | 33.3 | 91.6 | 91.6 | 91.6 | 91.6 | 75 | 83.3 | 83.3 | 91.6 | 66.6 | |

+: the presence of growth, -: the absence of growth

This implies that chickpea rhizobia display large variability in carbon and nitrogen substrate utilization that may be related to differences in strains/genotype or local adaptations (Nour *et al.,* 1994; Maatallah *et al.,* 2002).

### 3.3.6. Symbiotic effectiveness of *Mesorhizobium* strains

The greenhouse trial of selected strains on a sand culture under low pH 5 showed considerable variations among the tested mesorhizobial strains in shoot dry mass, nodule number, nodule dry mass per plant and symbiotic effectiveness (SE) compared to the control ($p \leq 0.01$) (Table 3.5). The inoculated strains induced nodules on both chickpea varieties ranging from 10 NN/plant with *Mesorhizobium* a.AR1 (*M.* genospecies 2E) to 98 NN/plant with *Mesorhizobium* a.64 (*M.* genospecies 2E) (mean= 61 NN/plant) on Natoli variety; and from 12.5 NN/plant for *Mesorhizobium* a.90 (*M.* genospecies 10A) to 85 NN/plant with *Mesorhizobium* a.89 (*M.*genospecies 4B) (mean= 38 NN/plant) on DZ-ck-2011 s-2-0042 variety.

The nodule dry mass was within the range of 49 mg/ plant and 119 mg/ plant (mean=80 mg/ plant) with Natoli variety, and 57 to 102 mg/plant (mean=77 mg/plant) on DZ-ck-2011 s-2-0042 host variety, respectively (Table 3.5). The strains also induced the accumulation of shoot dry weight ranging from 0.63 g/plant to 1.53 g/plant (mean= 0.98 g/plant) on Natoli variety; and 0.66 g/plant to 1.22 g/plant (mean= 0.85 g/plant) on DZ-ck-2011 s-2-0042 variety (Table 3.5).

This study indicated that the number of nodule and dry mass under low pH condition was much lower than recorded in other studies which agreed with reports from Angelini *et al*., (2005) and Girmaye Kenasa *et al.* (2014). The average nodule dry mass obtained from both varieties were also much lower than 120 mg/per plant reported by Mulissa Jida and Fassil Assefa, (2012), 200 mg/plant by Wondwosen Tena *et al*. (2017) and 212 mg/per plant from chickpea which was reported by Brigido (2007) from moderately acidophilic mesorhizobia. The low nodule dry mass could also be attributed to direct pH stress on the plant, limiting nutrient uptake and subsequent dry matter accumulation (Zahran, 1999).

On the basis of relative shoot dry matter accumulation in reference to N fixing and control plants, 5 (24%) strains were highly effective (HE) and 10 (48%) strains were effective (E) on both varieties. However, all the *Mesorhizobium* strains were highly effective (HE) and effective (E) on DZ-ck-2011s-2-0042 variety, but only 24% and 48 % of these strains were highly effective (HE) and effective (E), respectively on Natoli variety; and *Mesorhizobium* strains, a.55 (M.genospecies 11A), a.AR7 (M.genospecies 1D), a.66 (M.genospecies 1C), a.152 (M.genospecies 11 A), a.AR1 (M.genospecies 2E) and a.222 (M.genospecies 1D) strains were lowly effective on Natoli variety, indicating variations in their symbiotic compatibility and interaction between rhizobia and the host genotypes (Ben Romedhane *et al*., 2008; Gemuchu Keneni *et al*., 2012). In all cases, the Natoli variety showed higher nodule number per plant, nodule dry weight, shoot dry weight, but not higher values in symbiotic effectiveness than the DZ-2012-CK-2011s-2-0042 variety. Biru Alemu and Dagnachew Lule, (2018) have indicated that chickpea genotypes showed differential response to acidic pH condition.

Table 3.5. Symbiotic characteristics of chickpea nodulating mesorhizobia strains at pH 5.

| Mesorhizobia strains | Genospecies name | Close associated type strains | Shoot dry weight plant$^{-1}$ (g) | | Symbiotic effectiveness (SE %) | | Number of nodules plant$^{-1}$ (NN/plant) | | Nodule dry weight plant$^{-1}$ (mg) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Natoli | DZ-2012-CK-2011s-2-0042 | Natoli | DZ-2012-CK-2011s-2-0042 | Natoli | DZ-2012-CK-2011s-2-0042 | Natoli | DZ-2012-CK-2011s-2-0042 |
| **a.15star** | M.genospecies 5C | *Mesorhizobium ciceri* ca181 | 1.53±0.319$^{b}$ | 1.21±0.025$^{ab}$ | **96 HE** | **94 HE** | 58±0.278$^{l}$ | 36±0.591$^{h}$ | 89±11.342$^{fe-g}$ | 90±3.22$^{cb-d}$ |
| **a. 90** | M.genospecies 10A | *M. sp.*LSJC280BOO | 0.90±0.419$^{e}$ | 0.93±0.034$^{c}$ | **58 E** | **73 E** | 56±0.867$^{n}$ | 29±1.212$^{k}$ | 102±12.58$^{bdc-e}$ | 82±3.37$^{f}$ |
| **a. 55** | M.genospecies 11A | *M.opportunistum*WSM2075 | 0.80±0.441$^{f}$ | 0.69±0.035$^{e}$ | **40 LE** | **60 E** | 62±0.912$^{k}$ | 45±1.272$^{f}$ | 89±13.23$^{fe-g}$ | 91±3.551$^{cb}$ |
| **a. 152** | ,, | | 0.70±0.051$^{g}$ | 0.82±0.052$^{d}$ | **45 LE** | **61 E** | 74±1.059$^{i}$ | 45±1.478$^{f}$ | 70±15.35$^{j-k}$ | 77±4.127$^{g}$ |
| **a. AR452** | M.genospecies 2D | | 0.90±0.455$^{e}$ | 0.97±0.036$^{c}$ | **57 E** | **73 E** | 55±0.941$^{o}$ | 39±1.317$^{g}$ | 66±13.66$^{k}$ | 101±3.664$^{a}$ |
| **a.AR1** | M.genospecies 2E | | 0.63±0.513$^{h}$ | 0.67±0.043$^{f}$ | **48 LE** | **54 E** | 10±1.057$^{s}$ | 17.7±1.474$^{n}$ | 71±15.33$^{j-k}$ | 68±4.113$^{h}$ |
| **a.64** | ,, | *M. plurifarium* STM8773 | 1.26±0.505$^{d}$ | 0.82±0.043$^{d}$ | **78 E** | **66 E** | 98±1.045$^{a}$ | 36±1.464$^{h}$ | 119±15.14$^{a}$ | 102±4.059$^{a}$ |
| **a.117L2** | M.genospecies 2D | | 1.53±0.466$^{b}$ | 1.00±0.037$^{b}$ | **96 HE** | **81 HE** | 89±0.963$^{b}$ | 37±1.342$^{h}$ | 109±13.96$^{ba-c}$ | 99±3.752$^{ab}$ |
| **a.144s** | ,, | | 0.80±0.506$^{f}$ | 0.66±0.041$^{f}$ | **51 E** | **52 E** | 82±1.025$^{e}$ | 21.3±1.472$^{m}$ | 86±14.78$^{fih-g}$ | 67±4.095$^{h}$ |
| **a.138w** | ,, | | 0.80±0.514$^{f}$ | 0.67±0.048$^{f}$ | **51 E** | **54 E** | 52±1.055$^{p}$ | 12.7±1.473$^{o}$ | 49±15.30$^{l}$ | 57±4.104$^{i}$ |
| **a.71** | M.genospeces 4B | *M.amorphae* CCNWGSO123-pacbio | 1.50±0.474$^{b}$ | 1.22±0.037$^{a}$ | **95 HE** | **96 HE** | 80±0.979$^{f}$ | 57±1.366$^{dc}$ | 87±14.20$^{fih-g}$ | 82±3.812$^{f}$ |
| **a.89** | ,, | | 1.30±0.480$^{d}$ | 0.71±0.038$^{e}$ | **79 E** | **57 E** | 48±0.992$^{q}$ | 85±1.387$^{a}$ | 72±14.39$^{j-k}$ | 76±3.869$^{g}$ |
| **a.AR7** | M.genospecies 1D | | 0.70±0.503$^{g}$ | 0.72±0.042$^{e}$ | **45 LE** | **60 E** | 74±1.040$^{i}$ | 33±1.456$^{i}$ | 104±15.08$^{bd-c}$ | 87±4.037$^{de}$ |
| **a.222** | ,, | *M.loti strain* UFLA01-766 | 0.70±0.501$^{g}$ | 0.73±0.045$^{e}$ | **42 LE** | **59 E** | 78±1.047$^{g}$ | 52±1.469$^{e}$ | 88±15.18$^{feh-g}$ | 86±4.061$^{e}$ |
| **a.302star** | M.genospecies 1C | | 0.93±0.485$^{e}$ | 0.73±0.038$^{e}$ | **59 E** | **59 E** | 58±1.003$^{m}$ | 31±1.398$^{j}$ | 83±14.54$^{jih-g}$ | 89±3.915$^{ce-d}$ |
| **a.66** | ,, | | 0.80±0.480$^{f}$ | 0.81±0.039$^{d}$ | **48 LE** | **65 E** | 75±1.012$^{h}$ | 56±1.412$^{d}$ | 70±14.67$^{j-k}$ | 82±3.915$^{f}$ |
| **a.200M** | M.genospecies 3A | | 1.40±0.499$^{c}$ | 1.20±0.038$^{ba}$ | **86 HE** | **82 HE** | 83±1.025$^{d}$ | 39±1.439$^{g}$ | 116±14.95$^{ba}$ | 100±3.991$^{a}$ |
| **a.200s** | ,, | | 0.90±0.501$^{e}$ | 0.81±0.040$^{d}$ | **56 E** | **67 E** | 84±1.031$^{c}$ | 58±1.448$^{c}$ | 100±15.02$^{fed-c}$ | 92±4.012$^{cb}$ |
| **a.16star** | M.genospecies 8A | *M. australicum* WSM2073 | 0.80±0.493$^{f}$ | 0.83±0.037$^{d}$ | **51 E** | **67 E** | 71±1.050$^{j}$ | 39±1.424$^{g}$ | 91±15.23$^{fed-g}$ | 77±3.941$^{g}$ |
| **a.40L2** | ,, | | 1.40±0.496$^{c}$ | 1.00±0.039$^{b}$ | **86 HE** | **80 HE** | 82±1.019$^{e}$ | 73±1.436$^{b}$ | 109±14.87$^{ba-c}$ | 93±3.974$^{b}$ |
| **a.104** | ,, | | 0.90±0.508$^{e}$ | 0.84±0.046$^{d}$ | **55 E** | **68 E** | 46±1.052$^{r}$ | 37±1.461$^{h}$ | 74±15.38$^{jih-k}$ | 92±4.082$^{cb}$ |
| CP41 | Reference strain | *M. abyssinica* | 0.60±0.542$^{h}$ | 0.70±0.051$^{e}$ | 37 **LE** | 55 **E** | 58±1.060$^{m}$ | 25±1.481$^{l}$ | 75±15.38$^{jih-k}$ | 69±4.128$^{h}$ |
| Nitrogen | | | 1.60±0.611$^{a}$ | 1.24±0.041$^{a}$ | 100 | 100 | | | | |
| Control | | | 0.20±0.630$^{i}$ | 0.30±0.040$^{g}$ | 13 | 23 | | | | |
| CV | | | 2.59 | 2.44 | 1.31 | 1.27 | 0.861 | 1.95 | 9.57 | 2.66 |
| Mean | | | 0.98 | 0.85 | 63.3 | 66.9 | 61.3 | 37.7 | 80 | 77 |
| Significance | | | *** | *** | | | *** | *** | *** | *** |

*Values are mean ± standard error of 3 replicates. Mean values followed by the same letters in each Column and treatment showed no significant difference by Duncan's multiple range test ($p$ = 0.05). Control: without chemical and biological fertilizers, N: with the optimum amount of nitrogen fertilizer.A CV (%): percent coefficient of variability, Significant: probability level. *** indicates a significant difference at a probability level of 0.001

In general, combined evaluation of ecological competitiveness (*in vitro*) and symbiotic effectiveness data showed that five strains namely; a.117L2 (*M.* genospecies 2D), a.15star (*M.* genospecies 5C), a.71 (*M.*genospecies 4B), a.40L2 (*M.* genospecies 8A) and a.200M (*M.* genospecies 3A) performed better than the other strains, and even out-performed over the commercially available local strain Cp41 on both plant varieties at pH 5 under greenhouse conditions. This is concurrent with a report that *M. ciceri* strains from Portugal were effective strains with (SE) > 75% (Alexandre *et al.*, 2009), whereas *M ciceri and M plurifarium* strains from Ethiopia were highly effective on both varieties (Tassew Sirage and Fassil Assefa, 2018).

In this experiment, the most highly effective strains were obtained from pH 5 condition compared to other investigator reports on acidic soil in Ethiopia. Girmaye Kenasa *et al.* (2014) have revealed that rhizobial isolates of faba bean collected from acidic soils of Wollega, western Ethiopia were effective on faba bean, whereas, Daniel Muleta *et al*. (2017) have demonstrated that rhizobia strains of soybean collected from acidic soils of Ethiopia were effective from which, only 4% of the soyabean strains were found to be highly effective. It is likely that symbiotic performance of the strains is significantly dependent on pH condition. Thus, this result underlines the importance for a local screening of symbiotically effective and ecologically competent mesorhizobia isolates from acidic soil to enhance grain yield of the chickpea crop in acidic soil.

### 3.4. Conclusion

In the present study it can be concluded that Ethiopian acidic soils harbored symbiotically effective chickpea nodulating *Mesorhizobium spp.* These strains were also ecologically competent and heterotrophic versatile in carbon and nitrogen utilization, with a wide range of *in vitro* tolerance to various stress conditions, including low pH, $Mn^{2+}$ and $Al^{3+}$ toxicity, salinity, high temperature, heavy metals and antibiotics indicating their potential to effectively nodulate and fix nitrogen under field conditions.

The strains showed variations in their symbiotic effectiveness in nitrogen fixation on the two host varieties showing better performance on DZ-ck-2011s-2-0042 variety. Five strains: a.15star *(M.* genospecies 5C), a.117L2 (*M.* genospecies 2D), a.71 (*M.*genospecies 4B) and a.40L2 (*M.* genospecies 8A), and a.200M (*M.* genospecies 3A) were relatively superior in their symbiotic performance, compatibility with both chickpea varieties, and can be recommended as prospective commercial inoculants provided they can be tested in field trials in acidic soils.

# CHAPTER 4

## Phosphate solubilization and multiple plant growth promoting properties of *Mesorhizobium species* nodulating chickpea from acidic soils of Ethiopia

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## Abstract

*Phosphorus (P) is the second most important nutrient limiting plant growth and productivity of chickpea. Phosphorus deficiency is aggravated in acidic soils because of acid-mediated phosphorus fixation. Mesorhizobium species have a dual function of releasing phosphorus from fixed inorganic phosphate and fixing nitrogen (N) to alleviate the problems of nitrogen and phosphrous nutrition. Thus, the main purpose of this study was to screen and select strains from seven Mesorhizobium spp. for efficient phosphate solubilizing and other plant growth promoting properties. From a total of 62 Mesorhizobium strains, 47(76%) strains formed clear zones with average solublization indiex of 1.9-2.7 on Pikovskaya's agar plate. The selected strains also released soluble phosphorus (125-150 P ($\mu g ml^{-1}$) from tri-calcium phosphate and low level of phosphate i.e, 15.4 µg/ml and 14.5 µg/ml from inorganic ferrous and aluminum phosphates respectively released in a liquid medium after 4 days of incubation. The release of soluble phosphates was significantly ($p<0.001$) correlated with a drop in pH of the growth medium. Moreover, screening for multiple plant growth promoting attributes showed that 40%, 28%, 26%, 21% and 38% of the strains were capable of producing indole-3- acetic acid, hydrogen cyanide, siderophores, ACC diaminase and antagonism against Fusarium oxysporum f.sp.ciceris under invitro conditions. All taken together, the acidic soils harbor numerous and more diverse phosphate solubilizing and plant growth*

*promoting Mesorhizobium spp. However, greenhouse and field conditions should be conducted within the context of improving chickpea production in Ethiopia.*

**Keywords**: ACC deaminase activity, hydrogen cyanide, indole-3- acetic acid, siderophore

## 4.1. Introduction

Phosphorus (P) is the second important plant nutrient after nitrogen, limiting plant growth and productivity of crops. Though, soils may have large reserve of total phosphorus, the amount of phosphorus available to plants is usually small due to pH mediated phosphorus fixation (sorption) or precipitation of soluble phosphates with highly reactive cations such as $Al^{3+}$ and $Fe^{3+}$, and $Ca^{2+}$ in calcareous and acidic soils (Liu *et al.,* 2014). Acidity not only affects the availability of phosphorus, but also induces soil acidity with multiple nutrient disorders, including H, Al, Fe and Mn toxicities of coupled with Ca, Mg, P and Mo deficiencies. This is particularly severe for legumes for it affects the host legume, the endosymbiont and the process of biological nitrogen fixation (Nasr Esfahani *et al.,* 2016).

Microorganisms enhance phosphorus availability to plants by mineralizing organic P in soil and solubilizing phosphate from precipitated phosphates from low and high pH soil or from rock phosphates. The most important phosphate Solubilizing Bacteria (PSB) are; *Bacillus, Pseudomonas,* Rhizobia and *Enterobacter* (Verma *et al.,* 2013). These bacteria convert the insoluble (fixed) phosphates in to available forms to plants by acidification, chelation, exchange reaction and production of organic acid (Rodriguez and Fraga, 1999; Glick, 2012). Jilani *et al.,* (2007) have estimated application of phosphate solubilizing bacteria could reduce P fertilizer application by 50% with significant increase in crop yield.

These microorganisms also acquire one or more multiple plant growth promoting (PGP) characteristics. These include production of phytohormones such as indole acetic acid (IAA) and suppression of different soil borne pathogens, ACC deaminase activity,

production of siderophores and hydrogen cyanide (HCN), etc (Glick, 2012). Several reports demonstrated that inoculation of phosphate solublizing rhizobacteria with multiple plant growth promoting activities significantly improved plant growth and yield (Peix *et al.*, 2001; Yadav *et al.*, 2010).

Rhizobia are one of the soil bacteria that are endowed with both nitrogen fixation with legumes and P solubilization (Alkhani *et al.*, 2006) and other plant growth promoting (PGP) properties (Verma *et al.*, 2013). Several studies showed that *Rhizobium leguminosarum* biovar *viceae* and *Mesorizobium* spp. are most effective in both N2 fixation and P solubilization (Alkhani *et al.*, 2006; Hmissi Imen *et al.*, 2015; Dereje Tsegaye, 2015). It has also been documented that phosphate solubilizing *Mesorhizobium* sp. isolated from chickpea also showed HCN production (Wani and Khan, 2013) and a strong inhibitory effect against other fungal pathogens such as *Ascocyhta rabiei* (Kucuk and Kivanc, 2008) and *Rhizoctonia solani* (Hemissi *et al.*, 2011) and *Fusarium oxysporum* (Mulissa Jidaa and Fassil Assefa, 2012) under *in vitro* conditions.

Although chickpea rhizobia collected from mildly acidic to neutral soils (pH 6.0-7.9 average 6.7) of northern Ethiopia showed solubilization of inorganic phosphate and other growth promoting properties (Mulissa Jidaa and Fassil Assefa, 2012), the work did not show the relationship between the population of phosphate solubilizing chickpea rhizobia and highly acidic soil environments. However, other studies indicated 40% haricot bean rhizobia isolated from acidic soils of Jimma, western Ethiopia (Belaineh Mekonnen, 2009), and 34% rhizobia nodulating faba bean from acidic soils of central and southern highland soils of Ethiopia (Dereje Tsegaye, 2015) were capable of solublizing inorganic

phosphate. Thus, we hypothesized that acidic environments harbor more phosphate solubilizing mesorhizobia to overcome the problem of P deficiency mediated through P sorption.

Apart from that, understanding the dual function of chickpea rhizobia in N fixation and P solublization from acidic soils is important to improve P nutrition, nodulation and N fixing symbiosis. According to Hara and Oliviera (2005) *Rhizobium sp.* isolated from acidic soils of Amazonas Brazil fix N, solubilize insoluble phosphates, and increase the availability of P in the productive systems. Therefore, this study was initiated to look into the pattern of solubilization and growth promoting properties of chickpea rhizobia from the hither to unexplored acidic soils of Ethiopia.

## 4.2. Materials and Methods

### 4.2.1. Source of *Mesorhizobium* isolates

*Mesorhizobium* isolates were retrieved from roots nodule of chickpeas, collected from acidic soils in central, western, southern and northern parts of Ethiopia. They were isolated on Yeast Extract Mannitol Agar (YEMA) medium, phenotypically and genetically identified as genus *Mesorhizobium* spp. (Greenlon *et al*., 2019) and deposited in culture collection centers of the University of California Davis (for further classification into species) and Department of Microbial, Cellular and Molecular Biology, Addis Ababa University.

For all the tests, the mesorhizobial strains were grown to exponential phase in YEMB on a shaker (Thermo Fisher Scientific MaxQ shakers SHKA2000-1CE, USA) at $28^0$C with 200 rpm for 5-7 days unless stated otherwise. The suspension was centrifuged in sterile plastic tubes (10 ml) at 5000 rpm (Thermo Scientific™ Heraeus™ Pico™ 21 Micro-centrifuge) for 10 min. The pellets were re-suspended in normal saline (0.85% w/v of NaCl) solution to give a final concentration of $10^9$ cfu/ml using viable plate count method and optical density measurement by spectrophotometer (Thermo Scientific, Nanodrop© 2000 UV-Vis Spectrophotometer, USA) at 600nm.

### 4.2.2. Determination of phosphate solubilization ability of the *Mesorhizobium* strain

#### 4.2.2.1. Phosphate solubilization in solid medium

Phosphate solubilization was screened according to Edi-Premono *et al.* (1996). One mL of active mesorhizobia culture (about $10^9$cfu/ml cells) was spot inoculated on Pikovaskaya medium (tri-calcium phosphate) (g/l of glucose 10, yeast extract 0.5, $Ca_3$ (PO4)2 (5.0), $(NH4)_2SO_4$ (0.5), NaCl (0.2), KCl (0.2), MgSO4.7H2O (0.1), MnSO4.2H2O (0.002), FeSO4.7H2O (0.002), and agar (15). The plates were incubated at $28^0$C for 7 days. The strains that formed clear halo around their colonies were identified as phosphate solubilizers and the ability of strains to solubilizing the insoluble phosphate was measured to calculate the solublization index (SI) using the following formula

$$SI = \frac{\text{Colony diameter + Halo zone diameter}}{\text{Colony diameter}}$$

#### 4.2.2.2. Phosphate solubilization in liquid medium

Based on the high solublization indices (SI > 2.6) on the tri-calcium phosphate medium, nine strains were selected for quantitative estimation of P solubilization efficiency in the liquid medium. Thus, 1ml of actively grown (about $10^6$ cfu/ml cells) culture from each strain was transferred into 100 ml of Pikovaskaya broth dispensed in 250 ml flasks supplemented separately with Tri-calcium phosphate $Ca_3(PO4)2$ (TCP), Aluminum phosphate ($AlPO_4$) (AP) and Ferrous phosphate ($FePO_4$) (FP) as a sole phosphorus source. All flasks were incubated at $28^0$C temperature on incubator shaker (Thermo Fisher Scientific MaxQ shakers SHKA2000-1CE, USA) at 120 rpm for 4 days.

Ten ml of subsamples were withdrawn from each treatment on day 0 and 4, and analyzed for pH changes and soluble P analysis. The samples were centrifuged (Thermo Scientific™ Heraeus™ Pico™ 21 Microcentrifuge) at 15,000 rpm for 30 minutes and the clear supernatant was used for phosphate analysis using Jackson method (Selvi *et al.*, 2011). Each filtrate was mixed with 10 ml of chloromolibidic acid to detect color formation whose intensity was read at 600 nm by using spectrophotometer (Thermo Scientific, Nanodrop© 2000 UV-Vis Spectrophotometer, USA). The amount of solubilized phosphate was detected from the standard curve constructed from known concentrations of $KH_2PO_4$ (Sigma-Aldrich®, USA). The pH change of the medium was recorded from pH digital meter (Fisher scientific acument pH meter AB150, Singapore).

### 4.2.3. Plant Growth promoting properties of *Mesorhizobium* strains

#### 4.2.3.1. IAA production

IAA production was detected according to Acuna *et al*. (2011). Each strain was grown to exponential phase, inoculated on LB medium supplemented with 5 mM L-tryptophan, and incubated at $28^0$C on incubator shaker (Thermo Fisher Scientific MaxQ shakers SHKA2000-1CE, USA) at 200 rpm for 72 hr. The culture was centrifuged at 15,000 rpm (Thermo Scientific™ Heraeus™ Pico™ 21 Microcentrifuge) for 1 min from which 2ml of the supernatant was mixed with 4 ml of Salkowaski reagent (150 mL concentrated $H_2SO_4$, 250 mL distilled water, 7.5 mL 0.5 M $FeCl_3$ $6H_2O$), and left for 20 min in darkness at room temperature to observe development of a pink-red color and absorbance was measured at 530 nm using a spectrophotometer (Thermo Scientific, Nanodrop© 2000 UV-Vis Spectrophotometer, USA). The amount of IAA was quantified against standard graph constructed from known concentration of IAA.

#### 4.2.3.2. Screening for in vitro antagonistic activity against Fungal Pathogens

The antagonistic activity of the strains was tested against a test organism; *Fusarium oxysporum*f.sp.*ciceris* of chickpea pathogen (obtained from University of California, Davis, USA which was isolated from Ethiopia) using the dual culture technique as described by Landa *et al*. (1997). Ten µl of the inoculums of each isolate was equidistantly placed on the margins of YEMA plates amended with sucrose (0.5%) and incubated at 28°C for 24 hrs. A 5-mm agar disc from 7 days old PDA grown pathogen culture was placed at the center of the YEMA-Sucrose plate and incubated at 28°C for 5-7 days. Percent growth inhibition of the pathogen was measured and calculated using the

formula.

% Inhibition= (R _ r)/ R×100

Where, R is the radius of the fungal colony opposite to the antagonist and r, is the radius of the fungal colony towards the antagonist.

#### 4.2.3.3. HCN production

The strains were separately streaked on Tryptic Soya medium supplemented with glycine (4.4 g/liter) to detect their ability to produce hydrogen cyanide according to the method of Bakker and Schipper, (1987). The inner side of the upper lid of the petridish was covered with Whatman filter paper no. 1 soaked in 2% sodium carbonate and 0.5% picric acid solution. Plates were sealed with parafilm and incubated at $28^0$C for 5 – 7 days. A change in color from yellow to orange brown on the filter paper indicated cyanide production.

#### 4.2.3.4. ACC deaminase (1- aminocyclopropane-1-carboxylic acid) activity

Production of ACC deaminase by the strains was detected as described by Penrose and Glick (2003). The strains were grown in TY broth medium containing ($gL^{-1}$) tryptone (10), yeast extract (5) and NaCl (10) (supplemented with 20 µg/ ml tetracycline) at 28°C for 72 hrs. The cells were harvested by centrifugation (Thermo Scientific™ Heraeus™ Pico™ 21 Microcentrifuge) at 8000 *g* for 10 min and washed twice with 0.1 M Tris–HCl (pH 7.5) and re-suspended in 1 ml of 0.1 M Tris-HCl (pH 7.5). They were spot inoculated on Petri plates containing modified Dworkin and Foster (DF) minimal salt medium containing ($gL^{-1}$) Na2HPO4.$H_2O$ (6), $KH_2PO_4$ (3), NaCl (0.5), $NH_4Cl$ (1), Agar (15) and

after sterilization 1M $MgSO_4$ (1ml), 0.1M $CaCl_2$ (1ml) and 10 % (w/v) (10ml) added. Plates containing only DF without ACC served as negative control and $(NH4)_2SO_4$ (0.2% w/v) was included as positive control. The plates were incubated at 28$^oC$ for 5 -7 days.

#### 4.2.3.5. Siderophore production

The mesorhizobial strains were assayed for siderophore activity on the Chrome Azurol S Agar medium (Sigma, Ltd.) as described by Schwyn and Neilands (1987). Ten microliter of actively grown mesorhizobia strain (about $10^6$cfu/ml cells) was spot inoculated on Chrome Azurol S Agar plates and incubated at 28 °C for 5-7 days to detect color change of the medium from blue to orange around the colonies.

### 4.2.4. Data analysis

Data were analyzed with the General Linear Models of SAS software package (SAS/STAT; version 9.3). The total variation among the strains, P sources and incubation time compared to the controls using LSD at p-value 0.05 (SAS, 2012). The correlation between some parameters was checked by using linear regression analysis.

## 4.3. Results and Discussion

### 4.3.1. Distribution of phosphate solublizing *Mesorhizobium* spp. nodulating chickpea from acidic soils of Ethiopia

In the present study, we screened 62 mesorhizobial strains of which (47) 76% were capable of solubilizing insoluble inorganic phosphate sources (tri-calcium phosphate) with solubilization index (SI) ranging from 1.3 to 3.1 (Table 4.1) of which *Mesorhizobium* a.138w (*M*. genospecies 2D) showed the largest solubilization index (SI =3.1), followed by *Mesorhizobium* a.117L2 (*M*.genospecies 2D) and a.71 (*M*.genospecies 4B) (SI = 3.0) and the least obtained from *Mesorhizobium* a.200s (*M*.genospecies 3A). Most of the sampling sites harbored 70-100% of phosphate solubilizing mesorhizobia, except the Arsi site that was limited to contain 50% phosphate solubilizing mesorhizobia (PSM) showing variation in phosphate solubilizing population among the different sites. The solublization efficiency of the strains (SI 1.3-3.1) on the solid medium was within the intermediate index category (2.0<SI<4.0) recommended by Berraquero *et al*. (1976). Variability in SI on agar plate is due to the type, amount, and diffusion rates of diverse organic acids secreted by rhizobia to solubilize inorganic phosphates (Yadav *et al*., 2010).

The relative abundance of phosphate solubilizing mesorhizobia (PSM) (76%) was much higher than the 30-42% *Mesorhizobium* spp (with SI 0.5 to 2.4) collected from mildly acidic to neutral soil pH (6.0-7.9) of northern and central parts of Ethiopia (Mulissa Jida and Fassil Assefa, 2012; Daniel Muleta and Fassil Assefa, 2015; Tassew Siraj and Fassil Assefa, 2018). Although the population was much lower than the present study, other

phosphate solubilizing rhizobia from faba bean showed the same pattern of phosphate solubilizing (26%-34%) on acidic soils of western and southern Ethiopa (Girmaye Kenasa *et al*., 2014; Dereje Tsegaye, 2015), compared to fewer phosphate solubilizing rhizobia isolated from the same legume form slightly basic soils of northern Ethiopia (Zerihun Belay and Fassil Assefa**,** 2011). It appears that rhizobia in acidic soils acquire more phosphate solubilizing activity to circumvent P deficiency due to acid-mediated P fixation. According to Peix *et al.,* (2001), chickpea rhizobia, are the best phosphate solubilizing of all root nodule bacteria on plates, better mobilize phosphate from TCP and significantly increase growth of the host plant.

The most dominant phosphate solubilizing group was *M*. genospecies 2D, 2E that constituted 43% of the strains (20 strains) collected from six out of the 10 geographic areas (60%) followed by *M*. genospecies 1C,1D containing 8 strains (17%) of the phosphate solubilizing distributed in many geographic regions as *M*.genospecies 2D, 2E. *M*. genospecies 8A and *M*. genospecies 4B contributed to 8 and 6 strains, respectively and their distribution was limited to fewer (30%) sampling sites (Table 4. 2). However, the existence of 5 species out of 11 strains) in western Shewa, and 3 species from 4 strains in Gurage sampling areas showed high diversity of phosphate solubilizing *Mesorhizobium* spp within a limited area. Although a relatively good number of *Mesorhizobium* strains were collected from Assosa sampling site, they were classified into 3 species and dominated by *M*. genospecies 2D, 2E.

Table 4.1. Distribution of phosphate solublizing mesorhizobial strains from acidic soils of the different sampling site of Ethiopia.

| Isolation area | Number of Phosphate solublizing *Mesorhizobium* strains | Total isolates screened | Percentage PSR (%) | Mean PS (SI) | Mean Soil pH |
|---|---|---|---|---|---|
| **Arisi** | **4** | **8** | **50** | **2.4** | **5.3** |
| **Asosa** | **9** | **11** | **82** | **2.0** | **5.1** |
| **Bale** | **1** | **1** | **100** | **2.7** | **5.5** |
| **East Wollega** | **6** | **8** | **75** | **2.2** | **4.7** |
| **Gurage** | **4** | **5** | **80** | **1.9** | **5.7** |
| **North Wollo** | **2** | **2** | **100** | **2.4** | **5.5** |
| **S/W/Shewa** | **2** | **2** | **100** | **2.1** | **5.5** |
| **South Wollo** | **4** | **5** | **80** | **2.2** | **5.4** |
| **West Shewa** | **11** | **15** | **73** | **2.2** | **5.3** |
| **West Wollega** | **4** | **5** | **80** | **2.4** | **5.0** |
| | **47** | **62** | **76%** | | |

Table 4.2. Distribution of the different mesorhizobial species isolated from chickpea nodules along the different acidic soil sampling sites

| Mesorhizobia strains | Genospecies name | Close associated type strains | Isolation site | Altitude (masl) of isolation site | Latitude | Longitude | Soil pH of isolation site | Phosphate solublization activity (S.I.) |
|---|---|---|---|---|---|---|---|---|
| a.161 | M. genospecies 8A | *M. australicum* WSM2073 | Arisi | 2334 | 8.002722 N | 39.91611 E | 5.7 | 1.7 |
| a.104 | ,, | | ,, | 2338 | 8.064417 N | 39.96378 E | 5.3 | 2.3 |
| a.16star | ,, | | ,, | 2325 | 8.000917 N | 39.93356 E | 5.0 | 2.7 |
| a.217D | ,, | | West shewa | 2209 | 8.942449 N | 38.44527 E | 5.7 | 1.9 |
| a.118 | ,, | | ,, | 2259 | 9.008362 N | 38.4611 E | 5.1 | 1.8 |
| a.40L2 | ,, | | S/W/Shewa | 2248 | 8.585578 N | 38.24065 E | 5.9 | 2.5 |
| $a.45_2$ | ,, | | ,, | 2014 | 8.021667 N | 38.09672 E | 5.0 | 1.7 |
| a.150 | ,, | | Asosa | 1448 | 9.850112 N | 34.68784 E | 4.6 | 2.5 |
| a.147 | M genospecies 2D | *M.plurifarium* STM8773 | Gurage | 2003 | 8.337343 N | 38.53053 E | 5.7 | 1.8 |
| a.210 | ,, | | ,, | 2086 | 8.266207 N | 38.50748 E | 5.9 | 1.5 |
| a.231 | ,, | | West shewa | 2264 | 9.003837 N | 38.51457 E | 5.4 | 2.3 |
| a.58 | ,, | | ,, | 2239 | 9.813588 N | 38.4897 E | 5.1 | 2.3 |
| a.144s | ,, | | West wellega | 1905 | 8.646222 N | 34.84814 E | 4.7 | 2.8 |
| a.29L2 | ,, | | ,, | 2016 | 8.577056 N | 34.73936 E | 5.3 | 1.5 |
| a.AR352 | ,, | | East wellega | 1633 | 9.087178 N | 36.7432 E | 4.8 | 2.2 |
| a.AR45s | ,, | | ,, | 1552 | 9.011324 N | 36.74068 E | 4.7 | 2.0 |
| a.AR452 | ,, | | ,, | 1582 | 9.002881 N | 36.74009 E | 5.0 | 2.3 |
| a.AR35star | ,, | | ,, | 1552 | 9.011324 N | 36.74068 E | 4.8 | 2.7 |
| a.138w | ,, | | North wollo | 1416 | 12.08462 N | 39.6654 E | 5.1 | 3.1 |
| a.117M | ,, | | Asosa | 1406 | 9.7659292 N | 34.80069 E | 5.1 | 1.7 |
| a.AR49 | ,, | | ,, | 1406 | 9.7659292 N | 34.80069 E | 5.2 | 1.8 |
| a.star16 | ,, | | ,, | 1399 | 9.763652 N | 34.79209 E | 5.5 | 2.1 |
| a.117L2 | ,, | | ,, | 1399 | 9.763652 N | 34.79209 E | 4.9 | 3.0 |
| a. 117s | ,, | | ,, | 1567 | 10.03125 N | 34.57175 E | 5.2 | 1.7 |
| a.117 | ,, | | ,, | 1428 | 9.831095 N | 34.69295 E | 5.3 | 1.7 |
| a.AR1 | M genospecies 2E | | West shewa | 2237 | 8.995119 N | 38.49062 E | 5.9 | 1.8 |
| a.64 | ,, | | West wellega | 2016 | 8.577056 N | 34.73936 E | 5.3 | 2.9 |
| a.135 | ,, | | Asosa | 1486 | 9.818222 N | 34.70081 E | 5.4 | 2.0 |
| a.8star | M. genospecies 4B | *M.amorphae* CCNWGSO123-pacbio | Arisi | 2326 | 7.985000 N | 39.97147 E | 5.2 | 2.9 |
| $a.195_3$ | ,, | | Asosa | 1428 | 9.831095 N | 34.69295 E | 4.9 | 1.8 |
| a.111 | ,, | | Bale | 2014 | 7.021806 N | 40.69303 E | 5.5 | 2.7 |
| a.71 | ,, | | West shewa | 2231 | 8.054306 N | 39.87258 E | 5.1 | 3.0 |
| a.27 | ,, | | ,, | 2278 | 8.995119 N | 38.49062 E | 5.6 | 1.9 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| a.89 | ,, | | ,, | 2254 | 9.023472 N | 38.51169 E | 5.1 | 2.5 |
| a.209 | M. genospecies 1C | *M.loti strain* UFLA01-766 | Gurage | 2045 | 8.327911 N | 38.56285 E | 5.8 | 1.9 |
| a.30$_2$star | ,, | | West shewa | 2259 | 9.008362 N | 38.4611 E | 5.4 | 2.5 |
| a.33M | ,, | | ,, | 2239 | 9.813588 N | 38.4897 E | 5.3 | 2.1 |
| a.25 | ,, | | East wellega | 1633 | 9.087178 N | 36.7432 E | 4.7 | 2.1 |
| a.66 | ,, | | South wollo | 1461 | 10.57622 N | 39.92128 E | 5.9 | 2.5 |
| a.23L | ,, | | ,, | 1485 | 10.8901 N | 39.80967 E | 5.4 | 2.2 |
| a.116 | ,, | | North wollo | 1847 | 11.75072 N | 39.61308 E | 5.9 | 1.7 |
| a.222 | M. genospecies 1D | | West wellega | 2016 | 8.577056 N | 34.73936 E | 5.2 | 2.5 |
| a.200M | M. genospecies 3A | | East wellega | 1582 | 9.002881 N | 36.74009 E | 4.4 | 1.9 |
| a.200s | ,, | | ,, | 1676 | 11.49973 N | 39.61389 E | 5.2 | 1.3 |
| a.152 | M genospecies 11A | *M.opportunistum*WS M2075 | Gurage | 1974 | 8.287641 N | 38.53556 E | 5.2 | 2.2 |
| a.55 | ,, | | South wollo | 1752 | 11.71503 N | 39.6526 E | 5.1 | 2.0 |
| a.90 | M genospecies 10A | *M. sp.*LSJC280BOO | West shewa | 2219 | 8.386444 N | 38.22611 E | 4.9 | 2.5 |

A recent study on the distribution of *Mesorhizobium* spp in northern Ethiopia, showed that the three major genomic groups; *M. plurifarium, M. amorphae* and *M. ciceri* were widely distributed in all sites, and some sampling sites such as Shewa, Godar, and Gojam seem to harbor more diverse populations of chickpea rhizobia (Tassew Siraj and Fassil Assefa, 2018). Some studies from Portugal (Alexandre *et al.*, 2009) and China (Zhang *et al*., 2017) also showed *Mesorhizobium* species are diverse along different localities and geographic lines which are associated with different soil and climatic factors.

### 4.3.2. Solubilization efficiency by phosphate solublizing mesorhizobial strains in liquid culture

The different mesorhizobial strains released the highest amount of P from tri-calcium phosphate ranging from 125 – 150 µg/P/ml with average value of 134.3 µg P/ml upon 4 days of incubation (Table 4.3). However, they released low level of phosphorous i.e 15.4 µg P/ml and 14.5 µg P /ml from inorganic ferrous and aluminum phosphates, respectively because of strong bonding between the phosphate and the cations that hinders the release of more phosphorus to the medium (Marra *et al*., 2012).

In general, the strains showed slight, but not significant (p=0.05) differences in the amount of P they released from tri-calcium phosphate except, *M*.genospecies 2D, 2E. However, the strains did show significant (p= 0.05) (difference on the average P release from the inorganic iron and aluminium phosphates. Among the strains *Mesorhizobium* a.64 (*M*.genospecies 2E), a.117L2 (*M*.genospecies 2D) and a.71 (*M.* genospecies 4B) performed relatively better in solubilization of calcium phosphate, iron phosphate and aluminum phosphate, respectively. The *Mesorhizobium* a.117L2 (M genospecies 2D) and

a.71 (*M*.genospecies 4B) were consistent in their effectiveness in the solubilization of the inorganic phosphate sources. Other studies showed that *Mesorhizobium* spp are efficient inorganic P solubilizers amongst root nodule bacteria (Peix *et al*., 2001; Rivas *et al*., 2006).

In most cases, there was a steady decrease in the medium pH dropping up to pH 3.1 showing inverse correlation with the amount of phosphate released from tri-calcium phosphate ($r^2$= -0.38, p=0.05), ferrous phosphate ($r^2$ = -0.86, p=0.05) indicating that organic acids were released during the process of phosphate solubilization. Similar studies showed a steady decline in pH upon solublization of tri-calcium phosphate, aluminum phosphate and ferrous phosphate due to production of organic acid and release of protons to the locked P from inorganic P sources (Lin *et al.*, 2006).

The solublization of the different P sources in the liquid medium was more or less similar to the results on cowpea nodulating bacteria under similar incubation period (Marra *et al*., 2012). Although *Mesorhizobium* a.64 (*M*. genospecies 4E) a.117L2 (*M*. genospecies 2D) and a.71 (*M*. genospecies 4B) displayed lower SI than other strains on the solid medium, they performed better in the liquid medium. The low SI is attributed to the resistance of the agar medium to diffuse some organic acid types (Yadav *et al*., 2010).

Table 4.3. Solubilization of $Ca_3$ ($PO_4$), $FePO_4$ and $AlPO_4$ phosphate and pH changes of the culture filtrates in the liquid medium inoculated with Phosphate solublizing *Mesorhizobium* strains after 4th days of incubation

| Mesorhizobia strains | Genospecies species name | Close associated type strains | $Ca_3(PO_4)_2$ | | $(FePO_4)$ | | $(AlPO_4)$ | |
|---|---|---|---|---|---|---|---|---|
| | | | pH | P(µgml$^{-1}$) | pH | P(µgml$^{-1}$) | pH | P(µgml$^{-1}$) |
| **a.64** | M genospecies 2E | | **5.3$^{g}$** | **150$^{a}$** | **4.8$^{c}$** | **14$^{d}$** | **3.9$^{g}$** | **17$^{c}$** |
| **a.AR35 star** | M genospecies 2D | | **5.5$^{e}$** | **134$^{c}$** | **4.7$^{d}$** | **17$^{c}$** | **6.5$^{e}$** | **15$^{d}$** |
| **a.117L2** | ,, | *M. plurifarium* STM8773 | **5.4$^{f}$** | **139$^{b}$** | **4.6$^{e}$** | **25$^{a}$** | **3.4$^{h}$** | **19$^{b}$** |
| **a.144s** | ,, | | **5.7$^{d}$** | **130$^{d}$** | **4.7$^{d}$** | **14$^{d}$** | **6.4$^{f}$** | **17.8$^{c}$** |
| **a.138W** | ,, | | **5.8$^{c}$** | **125$^{e}$** | **4.8$^{c}$** | **12$^{e}$** | **6.8$^{b}$** | **9.8$^{f}$** |
| **a.111** | M. genospecies 4B | | **5.6$^{d}$** | **125$^{e}$** | **4.9$^{b}$** | **12$^{e}$** | **6.7$^{c}$** | **10$^{f}$** |
| **a.71** | ,, | *M.amorphae* CCNWGSO123- | **5.9$^{b}$** | **149$^{ab}$** | **4.7$^{d}$** | **21$^{b}$** | **3.1$^{i}$** | **22$^{a}$** |
| **a.8star** | ,, | pacbio | **5.8$^{c}$** | **119$^{f}$** | **4.9$^{b}$** | **10$^{f}$** | **7.0$^{a}$** | **8.5$^{g}$** |
| **a.16star** | M. genospecies 8A | *M. australicum* WSM2073 | **5.2$^{h}$** | **138$^{b}$** | **4.9$^{b}$** | **13$^{d}$** | **6.6$^{d}$** | **11$^{e}$** |
| **Control** | | | **6.9** | **1.53** | **6.7** | **1.12** | **6.8** | **0.71** |
| **Mean** | | | **5.6** | **134.3** | **4.8** | **15.4** | **5.6** | **14.5** |
| **SEM** | | | **0.005** | **0.48** | **0.005** | **0.53** | **0.017** | **0.462** |
| **CV%** | | | **0.072** | **0.71** | **0.085** | **6.62** | **0.272** | **5.41** |
| **LSD(p=0.05)** | | | **0.007** | **0.713** | **0.0075** | **0.791** | **0.0256** | **0.69** |

This shows that the plate method is reliable only for a preliminary screening of phosphate solubilizing microorganisms that necessitates a definitive test in a liquid culture to authenticate effectiveness in P solubilization (Nautiyal, 1999; Rodriguez and Fraga, 1999).

Although several studies showed an inverse relationship between the release of P and decline in pH of the medium with phosphate solublizing rhizobia (Rodriguez and Fraga, 1999; Alikhani *et al.*, 2006), other authors did not indicate the same from solubilization of P from aluminum phosphate (Marra *et al*., 2012). The discrepancy between SI on solid media and P release without a decline of pH in a liquid culture may indicate the existence of mechanisms of P solubilization other than organic acid production (Rodriguez and Fraga, 1999; Alori *et al*., 2017).

Given that certain strains of root nodule bacteria can solubilize both organic and inorganic phosphates and simultaneously fix nitrogen, the relatively best inorganic P solublizing *Mesorhizobium* a.71 (*M*. genospecies 4B), a.117L2 (*M*. genospecies 2D) and a.64 (*M*. genospecies 4E) can be considered as prospective inocula provided they are proved effective in nitrogen fixation.

### 4.3.3. Multiple PGP characteristics of chickpea *Mesorhizobium*

Eighteen of the 21 mesorhizobial strains (38%) showed antagonistic activity against *Fusarium oxysporum* f.sp.*ciceris* in dual culture assay with mycelial growth inhibition of 9.2% - 57% (Tabel 4.3). Likewise, shows that 28% of the strains were positive for HCN

production and 12 (26%) produced siderophore both of which are directly and indirectly important in disease suppression. Apart from disease suppression, Raychaudhuri *et al*. (2005) have reported that siderophore over producing *Mesorhizobium ciceri* BICC 651 promoted growth and fixed 25% more nitrogen per gram of nodule and caused more than 30% increase in dry weight of plant shoots.

Although 40% of the *Mesorhizobium* strains were implicated with fusarium suppression, only eight (30%) of the strains from *M*. genospecies 2D, 2E, *M*. genospecies 4B, *M*. genospecies 8A, *M*. genospecies 10A, *M*. genospecies 11A combined both HCN and siderophore production. The other strains may have acquired other mechanisms to suppress fusarium and root pathogens such as production of bacteriocin (Gupta *et al*. 2000), detoxifying virulent factors (Compant *et al*. 2005), pathogenic signal interference (Lutenberg and Kamilova, 2009) and production of different cellulolytic, chitinolytic and protease enzymes associated with suppression of the pathogen (Glick, 2012).

With regard to hormone production, 19(40%) of the *Mesorhizobium* strains were capable of producing growth hormone (IAA) ranging from 6.8 µg/ml up to 16.13 µg/ml (Table 4.4). This was much higher than the 28% chickpea rhizobia endowed with production of indole-3-acetic acid (IAA) collected from different parts of Ethiopia (Mulissa Jida and Fassil Assefa, 2012), and the amount of 22.07 µg/100 ml tryptophan produced by *Mesorhizobium* spp (Verma *et al.,* 2013). The large variation in IAA production by different chickpea rhizobia is attributed to the different metabolic adaptation they acquired through the evolutionary process (Leinhos and Vacek, 1994).

The study also revealed that ten *Mesorhizobium* strains (21 %) acquired ACC deaminase activity that can regulate ethylene levels and enhance plant resistance to drought and pathogens (Grichko and Glick, 2001). Nascimento *et al*. (2016) have also indicated that rhizobia that express this enzyme decrease deleterious ethylene levels that affect the nodulation process and increased symbiotic potential. The data showed that mesorhizobia represent more abundant ACC deaminase producing root nodule bacteria than 11% of the rhizobia strains, mainly *Rhizobium leguminosarum*, *Rhizobium gallicum* and *Rhizobium loti* collected from Saskatchewan, Canada (Duan *et al*., 2008). Earlier Ma *et al*. (2003) reported that five out of 13 (38%) tested *Rhizobium* spp displayed ACC deaminase activity (Table 4.4).

In summary, the phosphate solubilizing mesorhizobial isolates showed variation in multiple (1-5) plant growth promoting properties of which *Mesorhizobium* a.71 (*M*. genospecies 4B), a.64 (*M*. genospecies 2E), a.117L2 (*M*. genospecies 2D), and a.40L2 (*M*. genospecies 8A) displayed all the plant growth promoting properties followed by many other strains (up to 30%) that acquired any four multiple growth promoting properties (Table 4.4).

Table.4.4. Multiple PGP characteristics of phosphate solublizing *Mesorhizobium* isolated from chickpea nodule from acidic soils of Ethiopia

| **Mesorhizobia strains** | **Genospecies name** | **Closely associated type strains** | **IAA (µg ml) ± SE** | **HCN production** | **ACC deaminase** | **FOC inhibition (%)± SE** | **Siderophore production** | **Number of PGP** |
|---|---|---|---|---|---|---|---|---|
| **a.104** | M. genospecies 8A | *M. australicum* WSM2073 | **11.33±0.323$^{e}$** | + | + | **20.0±1.347$^{i}$** | - | 4 |
| **a.16star** | ,, | | **16.13±0.279$^{a}$** | + | + | **33.3±1.241$^{e}$** | - | 4 |
| **a.40L2** | ,, | | **10.93±0.292$^{f}$** | + | + | **18.4±1.248$^{j}$** | + | 5 |
| **a.117L2** | M. genospecies 2D | *M. plurifarium* STM8773 | **10.50±0.321$^{f}$** | + | + | **29.0±1.313$^{g}$** | + | 5 |
| **a.138w** | ,, | | **9.4±0.327$^{g}$** | + | - | **55.3±1.362$^{b}$** | + | 4 |
| **a.144 s** | ,, | | - | + | + | **28.6±1.371$^{h}$** | + | 4 |
| **a.AR352** | ,, | | **13.5±0.324$^{c}$** | - | - | **48.6±1.341$^{d}$** | - | 2 |
| **a.AR35star** | ,, | | **10.3±0.342$^{f}$** | - | - | **9.2±1.298$^{l}$** | - | 2 |
| **a.AR452** | ,, | | **11.8±0.341$^{e}$** | + | + | **11.1±1.362$^{k}$** | - | 4 |
| **a.64** | M. genospecies 2E | | **10.8±0.327$^{f}$** | + | + | **30.3±1.338$^{f}$** | + | 5 |
| **a.152** | M. genospecies 11A | *M. opportunistum*WSM2075 | **12.7±0.291$^{d}$** | + | - | - | + | 3 |
| **a.55** | ,, | | **14.4±0.306$^{b}$** | + | - | - | + | 3 |
| **a.200M** | M. genospecies 3A | | **11.87±0.318$^{e}$** | - | - | **57.13±1.36$^{a}$** | + | 3 |
| **a.25** | M. genospecies 1C | *M. loti strain* UFLA01-766 | **11.03±0.309$^{e}$** | - | - | **20.0±1.298$^{i}$** | - | 2 |
| **a.302star** | ,, | | **9.3±0.318$^{g}$** | + | - | **28.6±1.38$^{1}$** | + | 4 |
| **a.66** | ,, | | **6.8±0.307$^{h}$** | - | - | **30.0±1.271$^{f}$** | - | 2 |
| **a.71** | M. genospecies 4B | *M. amorphae* CCNWGSO123-pacbio | **9.4±0.321$^{g}$** | + | + | **50.0±1.311$^{c}$** | + | 5 |
| **a.89** | ,, | | **13.7±0.331$^{c}$** | - | - | **20.0±1.318$^{i}$** | - | 2 |
| **a.8star** | ,, | | - | + | - | **48.0±1.321$^{d}$** | + | 3 |
| **a.111** | ,, | | **13.4±0.341$^{c}$** | - | + | - | - | 2 |
| **a.90** | M. genospecies 10A | *M. sp.*LSJC280BOO | **12.2±0.335$^{d}$** | - | + | **57.36±1.35$^{a}$** | + | 4 |

+: positive for the test, -: negative for the test; FOC, *Fusarium oxysporumf.sp.ciceris*. Means within the column under IAA production and FOC inhibition, having a common letter do not differ significantly at (p = 0.05).

Thus, it can be projected that the different mesorhizobia with multiple PGP characteristics would increase the yield of chickpea and non-legume crops grown in rotation or mixed cropping with it (Alikhani and Yakhchali, 2009; Hemissi *et al.*, 2011, Mulissa Jida and Fassil Assefa, 2012). Nevertheless, it needs further investigations to evaluate their performance under glasshouse and field conditions.

### 4.4. Conclusion and recommendation

This study showed that 76% of chickpea mesorhizobia collected from acidic soils of mainly central and western Ethiopia were capable of solubilizing inorganic phosphorus. They are dominated by *M*. genospecies 2D, 2E, *M*. genospecies 8A and *M*. genospecies 1C, 1D and represented more than 60% phosphate solubilizing *Mesorhizobium* spp are widely distributed in the sampling regions. This is the highest number of phosphate solubilizing root nodule bacteria from all leguminous crops studied so far indicating that acidic soils harbor more phosphate solubilizing rhizobia than mildly acidic and neutral soils. The selected strains solubilized more phosphorus from tri-calcium phosphate than they did from iron and aluminium phosphates. Most of the strains have multiple growth promoting properties that are associated suppression of plant pathogens. The *Mesorhizobium* strains are endowed with the presence of ACC diaminase which was rarely reported elsewhere. All taken together, *Mesorhizobium* a.71 (*M*.genospecies 4B), a.64 (*M*.genospecies 2E), a.117L2, and a.40L2 (*M*. genospecies 8A) displayed most plant growth promoting properties that can be further studied to evaluate their potential as inoculants or biostimulants under greenhouse and field conditions.

# CHAPTER 5

## Evaluation of symbiotically effective indigenous *Mesorhizobium* inoculants on growth and productivity of chickpea in acidic soils under greenhouse and field conditions

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## Abstract

*Inoculation of chickpea with mesorhizobial inoculants is not common practice in Ethiopia, but this could provide an option to increase grain yields in low nitrogen (N) and phosphorus (P) acidic soils. Therefore, the selection of effective acid tolerant mesorhizobia is one strategy to boost the productivity of chickpea in acidic soils. In this study, genetically identified effective indigenous Mesorhizobium species were screened for N fixation efficiency with host varieties on acidic soil culture in controlled environment. Following this, three mesorhizobial strains were selected and evaluated in two field experiments in central (Holleta) and southern (Sodo) acidic soil of Ethiopia to determine their performance on the growth and yield of two cultivars of chickpeas (Natoli and DZ-2012-CK-2011s-2-0042). The inoculated strains showed a pronounced effect on growth, grain yield and biological nitrogen fixation of chickpea compared to the commercially available local strain Cp41 (reference strain) and uninoculated control. The indigenous mesorhizobial strain a.117L2 was superior inoculant for almost all parameters showed maximum nodule dry weight (220 and 261) mg/plant, shoot dry weight (6.3 and 7.3) g/plant, number of pods (49 and 51)per/plant, 100 seed weight (21)gm, grain yield (2265 and 2506.7) kg $ha^{-1}$, nitrogen uptake (91.6 and 101) kg $ha^{-1}$, phosphorus uptake (13.7 and 14.6) kg $ha^{-1}$ and the amount of nitrogen derived from*

*fixation Ndfa% (50.2 and 53.6) at Holleta and Sodo field experiments, respectively. Thus, there is a potential advantage using these mesorhizobial inoculants to improve chickpea production in acidic soils after further field trials over several seasons and sites in Ethiopia.*

**Keywords**: nodulation, yields

## 5.1. Introduction

Ethiopia is the top producer of chickpea in Africa (FAOSTAT, 2019). The crop is mainly cultivated in the central, northern and eastern highland areas of the country with an altitude of 1500 and 2700 m.a.s.l., and annual rainfall of 500-2000 mm (Legesse Dadi *et al.*, 2005). It is a good source of protein for the majority of the population and is one of the major export commodities with significant export market option amongst the field crops (Tadesse Getachew, 2019).

Chickpea is an important component of low input agriculture due to its ability to fix atmospheric nitrogen in symbiosis with root nodule bacteria belonging to the genus *Mesorhizobium* (Nour *et al*., 1995) contributing directly to grain protein and reducing the need for N fertilizer for successive crops. Hence, farmers commonly rotate chickpea with cereal crops to improve production and soil fertility.

Soil acidity is one of the important environmental constraints that affect nitrogen fixation and production of leguminous crops. It increases aluminum and manganese toxicity and aggravate calcium and phosphorus deficiency mediated by phosphorus sorption under low and high soil pH (Hungria and Vargas, 2000). However, nitrogen fixation and production in legumes can be enhanced on acidic soil through application of lime that neutralizes toxicity effect at $H^+$, $Al^{3+}$ and $Mn2^+$ in the soil and raises the pH of the soil conducive for rhizobia-legume symbiosis (Graham, 1992; Guo *et al*., 2009).

Improving the symbiosis through amelioration of soil acidity by the addition of lime is expensive (Foy, 1988), and a more cost-effective approach has been the selection of acid-tolerant crops (Taylor, 1991) combined with selection of suitable acid-tolerant microorganisms (Howieson *et al.,* 1988; Graham, 1992). Studies on different legumes showed that selection of tolerant legume varieties and/ or endosymbionts help to reclaim the productivity of abandoned acidic soils (Carter *et al.,* 1994; Zahran, 1999; Guo *et al*., 2009). Other reports showed that *Rhizobium* sp. isolated from acidic soils of Amazonas Brazil fixed N, solubilized insoluble phosphates, and increased the availability of P in the productive systems (Hara and Oliviera, 2005).

Several studies in the past showed that rhizobial strains show differences among chickpea rhizobial strains in their ability to grow and establish symbiosis under acidic conditions (Rai, 1991; Brigido *et al.,* 2007). Brigido *et al*. (2007) have demonstrated that chickpea rhizobia isolated from acidic soils of Portugual reclaimed and enhanced the growth of host on the acid affected soils and developed highly effective inoculants for chickpea in acid soils. Hmissi Imen *et al*., (2015) have shown that acid-mediated P deficiency in acidic soils could be alleviated by inoculating phosphate solubilizing mesorhizobia isolates.

Soil acidity limits production of crops mainly, in the central, western, southern and northern parts of Ethiopia (Biru Alemu and Dagnachew Lule, 2018), and it is associated with low available phosprous in the soil because of pH-mediated phosprous fixation. According to FAOSTAT, (2019), the average national yield of the crop is about 2 tons ha

$^{-1}$ which is very low compared to the average yield of 6.0 tons ha$^{-1}$ on experimental stations, this is due to different environmental constraints; the most important of which are soil acidity, pest and diseases.

Dereje Tesegaye, 2015) has from Ethiopia also reported that acidic soils harbored symbiotically effective phosphate solubilizing rhizobia nodulating faba bean and showed the potential of such rhizobia can improve production in acidic soils under low input agriculture. Similarly, Daniel Muleta *et al*. (2017) have reported that the potential of rhizobial inoculation increased soybean grain yields on acid soils in Ethiopia.

There is still a dearth of information on the potential of rhizobial inoculation with a dual property of effective nitrogen fixation and phosphate solubilization, to improve nitrogen and phosprous nutrients, thereby enhancing growth and production of chickpea in acidic soils of Ethiopia. Therefore, the objective of this study was to evaluate the performance of genetically identified and symbiotically effective indigenous *Mesorhizobium* species on growth and productivity of two chickpea varieties on acidic soils at Holleta and Sodo (Endebyou) in the central and southern highlands of Ethiopia.

## 5.2. Material and Methods

### 5.2.1. Sources of Microbial inoculants and chickpea cultivars

Five *Mesorhizobium* strains, namely; a.15star (*M.*genospecies 5C), a.117L2 (*M.*genospecies 2D), a.71(*M.*genospecies 4B), a.40L2 (*M.*genospecies 8A) and a.200M (*M.*genospecies 3A) were selected based on their ecological tolerance (*in vitro* laboratory

conditions (i.e) ability to catabolize different carbon and nitrogen substrates, resistance to various eco physiological stresses, and multiple plant growth promoting characteristics including, phosphate solubilization) (chapter 3 and 4). The five strains were evaluated on their symbiotic performance on acidic soils under greenhouse conditions of which, three strains a. 15star (*M.*genospecies 5C), a.117L2 (*M.*genospecies 2D), a.71 (*M.*genospecies 4B) were selected for field studies. The commercially available local strain Cp41 (obtained from Menagesha Biotech Industry PLC) was included as a reference chickpea *Mesorhizobium.*

Two chickpea varieties: Natoli and DZ-2012-CK-2011s-2-0042 (obtained from DZARC) were selected for they are good yielding varieties and tolerant to acidic soil (Biru Alemu and Danachew Lule, 2018). A reference crop (Barely, HB 1307), obtained from the Ethiopian Institute of Agriculture Research, Holleta Research Center, was also included as a non N- fixing reference crop in order to estimate the amount of Nitrogen derived from biological nitrogen fixation (% Ndf).

### 5.2.2. Screening of symbiotic effectiveness on acidic soils in pot experiment under greenhouse conditions

Acidic soils were collected from two sites; agricultural fields from Holleta (west Shewa Zone) and farm lands from Arjo (east Wollega Zone), central and western Ethiopia with soil pH of 4.78 and pH 4.43, respectively. They were sieved to less than 2 mm, and filled in 3 kg capacity of surface sterilized (70% ethanol) pots and fertilized once with fertilizer in g $pot^{-1}$: 0.75 $KH_2PO_4$, 0.5 KCl, 0.075 $MgSO_4.7H_2O$, 0.07 $ZnSO_4.7H_2O$, 0.00214 $(NH_4)_6Mo_7O_{24}.H_2O$ and 0.333 Urea was added as a nitrogen source for N-control pots

recommended by Somasegaran and Hoben, (1994). Healthy seeds of the two varieties were surface sterilized with 4% sodium hypochlorite for 2 to 3 min according to Howieson and Dilworth, (2016), and germinated on 1.5% (w/v) water-agar plates. The selected strains were inoculated into yeast extract mannitol broth (YEMB) and incubated at 30$^{o}$C for 5-7 days. Each seedling was flooded with 1 ml of $10^{9}$ CFU $ml^{-1}$ of the culture suspension, and five inoculated seedlings were planted on each pot and later tinned down to three seedlings per pot. N (urea) fertilized and un-inoculated (non-fertilized) pots were included as positive and negative controls, respectively.

The experiment was set as a Complete Randomized Design (CRD) with three replications. The pots were watered every three days to 75% field capacity. After sixty days of growth, the whole plants were carefully uprooted to determine number of nodules, nodule dry weight, shoot dry matter and total N content (Somasegaran and Hoben, 1994). Dried shoot samples were finely ground to determine nitrogen content in (%) using the Kjeldahl method (Sahlemedhin Sertsu and Taye Bekele, 2000).

### 5.2.3. Field experiments

Field experiments were conducted at two different sites that were characterized as acidic soil: Holleta Agricultural Research office FTC (Bekeka) field site and Sodo (Endebyou) model farmer's field during 2018/2019 cropping seasons (September - January). The Holleta field site was located at 09$^{o}$ 06' 28.676" N, 038$^{o}$ 28'01.361''E and at 2527 meters above sea level (representing central highlands of Ethiopia) in west Shewa Zone. It has a bimodal rainfall distribution with mean annual rainfall between 834 and 1300 mm. It has

a warm sub-humid climate with minimum, maximum and average temperatures of 6.3, 27.1 and 16.7°C, respectively.

The on-farm site at Sodo (Endebyou) was located at 08$^{o}$ 20’ 05.894” N, 038$^{o}$ 28’ 14.270’’E and at 2681 meters above sea level (representing southern highlands of Ethiopia) in Gurage Zone. Sodo (Endebyou) was characterized by a bimodal pattern of rainfall with the mean annual rainfall between 801 and 1200mm. It has a warm humid climate with the minimum; maximum and average temperatures of 10, 25 and 17.5°C, respectively. All climatic data of each field site were obtained from their respective research centers recorded (2014 - 2018).

#### 5.2.3.1. Soil properties and enumeration of indigenous soil rhizobia from field sites

Composite soil samples were collected from each experimental site at the depth of 20 cm at 13 even intervals in a “W” pattern throughout the field (Endalkachew Wolde-Meskel *et al*., 2018). The samples were weighed, air-dried, ground to pass through a 2-mm sieve before analysis. Physico-chemical properties of the soils were analyzed following standard laboratory procedures at the laboratory of soil and water analysis, Horticoop Ethiopia (Horticulture) PLC), Debre Zeit, Ethiopia (Table 5.1).

The population density (population) of root nodule bacteria of the two field soils was determined using the most-probable-number (MPN) plant infection method according to (Howieson and Dilworth, 2016) during the 2018 crop season which was determined at greenhouse of Applied Microbiology, Addis Ababa University. The soils were diluted

appropriately and inoculated on the host grown in surface sterilized pots under greenhouse conditions. The MPN was calculated from the number of units testing positive for each dilution to determine the factor (f). This factor (f) found in MPN tables then multiplied by the lowest dilution before all units were negative for nodulation.

Number of rhizobia population per gram of soils = f × d

Where: f = factor from MPN tables, d = lowest dilution before all units were negative

Table 5.1. Location and selected physicochemical properties of soils at the experimental sites

| **Parameters** | **Holleta** | **Sodo (Endebyuo)** |
|---|---|---|
| Latitude | 09$^{o}$ 06’ 28.676” N | 08$^{o}$ 20’ 05.894” N |
| Longitude | 038$^{o}$ 28’ 01.361’’E | 038$^{o}$ 28’ 14.270’’E |
| Altitude (m.a.s) | 2527 | 2681 |
| pH (1:2:5 $H_2O$) | 4.78 | 5.1 |
| pH (1:2:5 KCl) | 4.08 | 4.72 |
| Exchangeable acidity (meg/100g soil) | 1.83 | 0.06 |
| Exchangeable $Al^{+3}$ | 0.89 | 0.01 |
| Total Nitrogen[b] | 0.12 | 0.15 |
| Available phosphorus mg/kg(ppm)[c] | 4.8 | 9.98 |
| Organic Carbon (OC) (%)[a] | 2.20 | 2.27 |
| C/N | 18.3 | 15.1 |
| CEC cmol (+) kg soil $^{-1}$ [d] | 15.3 | 24.60 |
| K+ cmol (+) kg soil [d] | 1.54 | 1.78 |
| $Ca^{2+}$ cmol (+) kg soil [d] | 10.53 | 21.8 |
| $Mg^{2+}$ cmol (+) kg soil [d] | 2.8 | 3.7 |
| $Na^{+}$ cmol (+) kg soil [d] | 0.35 | 0.38 |
| Fe mg/kg(ppm) [e] | 8.9 | 10.3 |
| Mn mg/kg(ppm) [e] | 42.1 | 54.4 |
| Cu mg/kg(ppm) [e] | 1.71 | 3.22 |
| Zn mg/kg(ppm) [e] | 1.58 | 2.45 |
| Soil texture class [f] | Clay | Clay |
| Sand | 14% | 23.3% |
| clay | 60% | 50% |
| Silt | 26% | 26.8% |

Methods: a= walklay and black; b=Kjeldahl; c= Bray P1; d= Ammonium acetate; e= Mehlich-3; f=Bouyoucos Hydrometer; ppm=parts per million

### 5.2.3.2. Experimental design and land preparation

The field layout for each site was prepared and each treatment was assigned randomly to experimental units within a block. The size of each plot was 4 m by 2.6 (10.4 $m^2$). The spacing between blocks and plots was 1.5 m and 1m, respectively. Thus, each treatment plot consisted of four rows of which, the outer two rows were used as border, leaving twenty centimeters of land between the external rows and the edge of each plot.

All field experimental sites were well prepared, and fertilized according to the standard agricultural practices (Somasegaran and Hoben, 1994). The experiment consisted of 8 treatments with a factorial combination of two chickpea varieties (Natoli and DZ-ck-2011 s-2-0042) with four levels of rhizobia inoculations a.15star (*M.*genospecies 5C), a.117L2 (*M.*genospecies 2D), a.71 (*M.*genospecies 4B), and Cp41), and a control (non-inoculated). Treatments were laid down in a randomized complete block design (RCBD) with three replications making a total number of 24 plots. All plots received a starter dose of P at a rate of 46 kg $P_2O_5$ as TSP (100 kg TSP) recommended by conventional farmers' and fertilizer recommendation level using row methods of application at planting time (Taye Belachew and Asfaw Hailemariam, 2010).

### 5.2.3.3. Inoculum preparation and seed inoculation

Each mesorhizobial strain was grown on yeast extract mannitol broth to late exponential phase (5-7 days). One liter culture of each mesorhizobial inoculant was adjusted to $10^9$cfu/ml and thoroughly mixed with a kg of sterile lignite powder (obtained from National soil of Ethiopia) according to Catroux *et al*. (2001) and cured at 28$^o$C for 2 weeks. The seeds were coated with lignite-based inoculants at the rate of 7 ml/kg of seeds

as recommended by Taye Belachew and Asfaw Hailemariam, (2010) using 5% sucrose as sticker. Coated seeds were spread on a clean plastic sheet under tree-shade to maintain the viability of microbial cells. Two seeds were planted per hole at a spacing of 40 cm between rows and 10 cm between plants and in each row 40-45 seeds were planted. All plots were isolated with ridges to minimize the movement of bacteria from one plot to another. Thinning was done a week after emergence to allow one plant per hole.

**5.2.3.4. Data collection**

Plants were sampled at two different stages; 60 days after planting and at physiological maturity. Sixty days after planting (60 DAP), five plants were randomly taken and uprooted from central rows of each plot at mid-flowering stage and washed to retrieve intact nodules from roots. The number of nodules was counted as the mean number of nodules per plant. The mean nodule dry weight and shoot dry weight per plant was determined by oven drying the samples to constant weight at $70^0$C for 48 hrs.

At physiological maturity 150 day after planting (DAP), five plants were randomly taken from the central two rows of each plot to determine number of pods per plant (representing average count of number of pods per five plants), hundred seed weight in gram, biomass yield (above-ground biomass weight) and grain weight in kg $ha^{-1}$, grain harvest index was also calculated as the ratio of grain yield to biological yield.

#### 5.2.3.5. Determination of N and P uptake

At physiological maturity, five non-border plants were harvested and partitioned into grain and straw. The grain and straw samples were separately oven dried at 70ºC to a constant weight, ground to pass a 1 mm sieve and saved to analyze grain and straw N and P contents. Total N was quantitatively determined by Kjeldahl procedure (Sahlemedhin Sertsu and Taye Bekele, 2000); whereas phosphorus was determined using vanadium phosphomolybdate method (Motsara and Roy, 2008). N uptake and P uptake in the grain and straw was determined after multiplying their respective N and P contents with their respective yields (Taye Belachew and Asfaw Hailemariam, 2010).

#### 5.2.3.6. Measurement of biological nitrogen fixation

$N_2$ fixation (nitrogen derived from fixation) was determined by measuring the total amount of N in the legume crop and nitrogen content in a non-fixing reference crop (Beck *et al.,* 1993). The amount of $N_2$ fixed was calculated by subtracting the N yield of the reference crop from the N yield of legume as follows;

The quantity (Q) of N derived from $N_2$ fixation was calculated as: Q= (Legume DM x %N x 100) - (Reference crop DM x %N x100).

### 5.2.4. Data analyses

The data were analyzed using a mixed linear model procedure of SAS 9.3 (SAS Institute, 2012) after checking the compliance of the data with the assumptions of the statistical test. Mean separation test was done using LSD at p-value 0.05. The correlation between some parameters was checked by using Pearson correlation analysis.

## 5.3. Results and Discussion

### 5.3.1. Symbiotic effectiveness on acidic soil culture under greenhouse conditions

The greenhouse trial in pot culture showed all the strains increased the number of nodules per plant, nodule dry weight, shoot dry weight and total nitrogen content of the two chickpea varieties over the uninoculated control plants (Table 5.2). Thus, the inoculants induced nodules on the host plants ranging from 8 NN/plant (a.200M) to 68 NN/plant (a.117L2) with mean nodule number of 49 NN/plant from Natoli variety at Holleta soil; and 14 NN/plant at Arjo from DZ-ck-2011 s-2-004 variety (Table 5.2).

The strains also showed the same pattern in nodule dry weight with mean NDW of 148 mg/plant on Natoli variety compared to mean NDW of 93 mg/plant from DZ-ck-2011 s-2-004 variety at Holleta farm soil; compared to mean nodule dry mass of 50mg/plant and 45 mg/plant from the two varieties at Arjo soil, respectively. This shows that the inoculants induced more than half times larger nodules and nodule dry weight from Natoli variety at Holleta soil, without showing significant ($p = 0.05$) difference on the two nodulation parameters between the two varieties on Arjo soil (Table 5.2). The difference may be due to the relatively high acidic soil of Arjo soil and variation in soil nutrient availability between the two soils that influenced the number and dry weight of the nodules. It appears that the pH-sensitive stage in nodulation occurs early in the infection process and that *Rhizobium* attachment to root hairs is one of the stages affected by acidic conditions in soils (Vargas and Graham, 1988). Vassileva *et al*. (1997) have also shown that low pH soil decreased number of nodules and nodule dry weights in *Rhizobium* - common bean association.

The inoculants showed significant (p = 0.05) variation in shoot dry weight with mean shoot dry weight of 2.2 gm/plant from Natoli variety at Holleta soil. However, the strains performed less with DZ-ck-2011 s-2-004 variety with mean shoot dry weight of 1.6 g/plant (Table 5.2). With regard to Arjo soil, inoculation increased shoot dry mass with mean shoot dry weight of 1.6 g/plant on Natoli variety and mean shoot dry weight of 1.2 g/plant with DZ-ck-2011 s-2-004 variety.

The mesorhizobia strains induced more than half times (59%) more nodule number and nodule dry weight between the two varieties at Holleta soil. However, the mean shoot dry mass of the Natoli variety was only 38% higher than DZ-ck-2011s-2-004 variety. The shoot dry matter of the Natoli variety was 33% higher than the DZ-ck-2011 s-2-004variety at site irrespective of their difference in the two nodulation parameters, indicating that inoculated plants at Arjo soil increased in shoot dry weight despite nodulation inhibition, for they received nitrogen from the soil. Brigido *et al*. (2007) have obtained a higher shoot dry weight when acid tolerant rhizobia strains were inoculated in chickpea in acidic soil.

The data also showed inter-strain difference in the nodulation and growth parameters in that a.15star (*M.*genospecies 5C) and a.117L2 (*M.*genospecies 2D) strains performed the best on the two varieties at their respective sites; whereas the others showed slight, but not significant (p =0.05) differences depending upon host variety. This can be attributed to variations in their symbiotic compatibility between mesorhizobial strains and host genotype (Ben Romedhane *et al*., 2009; Gemechu Keneni *et al*., 2012), nutrient status

Table 5.2. Symbiotic effectiveness of Mesorhizobia nodulating chickpea on Holleta and Arjo acidic soil under greenhouse condition

| Mesiorhizobial strains | NN/plant | | NDW/ mg/plant | | SDW/ gm/plant | | TN(%) | |
|---|---|---|---|---|---|---|---|---|
| | Natoli | DZ-ck-2011 s-2-0042 | Natoli | DZ-ck-2011 s-2-0042 | Natoli | DZ-ck-2011 s-2-0042 | Natoli | DZ-ck-2011 s-2-0042 |
| **HOLLETA** | | | | | | | | |
| a.15star (*M.*genospecies 5C) | $51^{b}$ | $38^{a}$ | $154^{b}$ | $90^{c}$ | $2.6^{b}$ | $1.8^{b}$ | $2.21^{c}$ | $2.36^{a}$ |
| a.117L2 (*M.*genospecies 2D) | $68^{a}$ | $38^{a}$ | $168^{a}$ | $137^{a}$ | $2.8^{a}$ | $2.0^{a}$ | $2.39^{b}$ | $2.16^{cb}$ |
| a.40L2 (*M.*genospecies 8A) | $34^{d}$ | $24^{c}$ | $146^{c}$ | $52^{de}$ | $2.2^{d}$ | $1.6^{c}$ | $2.20^{c}$ | $2.05^{c}$ |
| a.71 (*M.*genospecies 4B) | $50^{bc}$ | $36^{b}$ | $153^{b}$ | $127^{b}$ | $2.4^{c}$ | $1.9^{a}$ | $2.63^{a}$ | $2.19^{b}$ |
| a.200M (*M.*genospecies 3A) | $43^{c}$ | $19^{d}$ | $118^{d}$ | $57^{d}$ | $2.0^{e}$ | $1.4^{d}$ | $2.32^{bc}$ | $1.69^{de}$ |
| +Control | | | | | $2.4^{c}$ | $1.8^{b}$ | $1.81^{d}$ | $1.72^{d}$ |
| - Control | | | | | $0.9^{f}$ | $0.6^{e}$ | $0.12^{e}$ | $0.10^{e}$ |
| Mean | 49 | 31 | 148 | 93 | 2.2 | 1.6 | 1.95 | 1.75 |
| SEM | 10.6 | | 30.04 | | 1.62 | | 0.07 | |
| CV (%) | 37.1 | | 34.90 | | 8.85 | | 3.75 | |
| **ARJO** | | | | | | | | |
| a.15star (*M.*genospecies 5C) | $16^{b}$ | $13^{b}$ | $58^{b}$ | $51^{b}$ | $1.8^{c}$ | $1.3^{c}$ | $2.09^{b}$ | $1.46^{c}$ |
| a.117L2 (*M.*genospecies 2D) | $24^{a}$ | $28^{a}$ | $75.5^{a}$ | $72.5^{a}$ | $1.9^{b}$ | $1.3^{c}$ | $2.17^{a}$ | $2.02^{b}$ |
| a.40L2 (*M.*genospecies 8A) | $11^{c}$ | $11^{c}$ | $40^{d}$ | $28^{d}$ | $1.5^{d}$ | $1.2^{d}$ | $1.77^{e}$ | $1.13^{d}$ |
| a.71 (*M.*genospecies 4B) | $25^{a}$ | $11^{c}$ | $45.5^{c}$ | $47.5^{c}$ | $1.9^{b}$ | $1.4^{b}$ | $2.17^{a}$ | $2.10^{a}$ |
| a.200M (*M.*genospecies 3A) | $11^{c}$ | $8.0^{d}$ | $32^{e}$ | $24.5^{e}$ | $1.2^{e}$ | $1.1^{e}$ | $1.99^{c}$ | $1.02^{e}$ |
| +Control | | | | | $2.3^{a}$ | $1.7^{a}$ | $1.81^{d}$ | $1.15^{d}$ |
| - Control | | | | | $0.8^{f}$ | $0.6^{f}$ | $0.67^{f}$ | $0.28^{f}$ |
| Mean | 17 | 14 | 50 | 45 | 1.6 | 1.2 | 1.81 | 1.31 |
| SEM | 7.90 | | 35.73 | | 0.02 | | 0.06 | |
| CV (%) | 24.10 | | 17.63 | | 8.62 | | 15.74 | |

NN= nodule number plant$^{-1}$, NDW= nodule dry weight (mg), SDW= shoot dry weight (g), TN%= shoot total nitrogen percent. SEM= Standard error of mean, CV= coefficient of variation, Values with in the column having different letter are significantly different at p=0.05.

and pH of soils, performance of host genotypes and mesorhizobia on both soil type (Zahran, 1999).

In all cases, the Natoli variety yielded more shoot dry matter (30%) than the DZ-ck-2011s-2-004 variety irrespective of inoculation and nitrogen fertilization. Both varieties also showed similar pattern of shoot dry matter increase (33% - 38%) on Holleta acidic soil compared to Arjo acidic soil (Table 5.2), indicating that the relatively high acidic soil of Arjo site that inhibited nodulation and growth parameters of the host plant.

The inoculated plants showed variations with total nitrogen (TN %) depending on individual inoculants on both sites (Table 5.2). Although, the different inoculants did not show significant difference in TN% at Holleta site, they showed a significant ($p = 0.05$) difference (38%) between the good performing Natoli variety and the low performing DZ-ck-2011 s-2-0042 variety on Arjo site indicating that the latter was more sensitive to high acid soils than the Natoli variety. The response of plant N concentration is related to N content available in the soil and the abundant populations of rhizobia with high symbiotic efficiency (Oliveira *et al*., 2017).

In general, the data showed the two chickpea strains namely a.117L2 and a.15star performed better on nodulation and growth parameters of the two host varieties, whereas a.71, which was one of the low performing strain in shoot dry mass, accumulated more total nitrogen (TN %) than the other strains at both Arjo and Holleta sites.

### 5.3.2. Field experiment

#### 5.3.2.1. The rhizobial population of experimental sites

The data showed that Sodo (Endebyuo) soil harbored indigenous rhizobial population of 0.92 x $10^{1}$ $g^{-1,}$ but no rhizobium was detected from Holleta soil. This may be associated with a slightly higher soil acidity observed at Holleta than Sodo (Endebyuo) (Table 5.1), and/or the Holleta soil may not have a history of growing chickpea for a long time. Different works showed that increasing soil acidity affects survival and persistence of the *Rhizobium* which in turn decreases the size of the rhizobial population (Graham, 1992; Andrade *et al*., 2002). According to Thies *et al*. (1991), the populations of rhizobia at both sites were within the low minimum number (less than 100 cells g of $soil^{-1}$). Catroux *et al*., (2001) have argued that if a legume with narrow specificity is not cultivated in a region, it is likely that compatible and effective native rhizobia may be lacking in these soils.

#### 5.3.2.2. Soil physicochemical analysis

Soil physical and chemical analytical results are shown in Table 5.1. The pH value of soils of the experimental sites for both Holleta and Sodo (Endebyuo) were 4.78 and 5.1 which were classified under strongly acidic and moderately acidic soils, respectively as per the pH rating category suggested by Jones, (2003). Thus, the pH values were not within the optimum range of pH (6.0 and 8.2) for chickpea production (Horneck *et al*., 2011) and it was not favorable for the infective strains of rhizobia to nodulate the host variety (Rodrigues *et al.,* 2006). However, Brigido *et al*. (2007) have showed that

chickpea rhizobia isolated from Portuguese reclaim and grow the host on the pH 5 (acidic soils).

As per suggested by Hazelton and Murphy (2007) the total Nitrogen content (TN) of the soil was 0.12% and 0.15% for Holleta and Sodo (Endebyuo), respectively were very low. The available phosphorus (AP) of Holleta soil was 4.8 parts per million (ppm); whereas that of Sodo (Endebyuo) soil was 9.98 ppm falling in a low range (< 20 parts per million (ppm) for Bray P1 test) rating by Horneck *et al* (2011) and the favorable phosphate between (20- 40 ppm) depending on soil properties and crop grown according to Horneck *et al*. (2011). Saxena and Rewari (1991) have found that (20 and 40 ppm) phosphate improved the growth and nodulation of chickpea (*C. arietinum L.*).

**5.3.2.3. Effect of mesorhizobia inoculation on nodulation performance of chickpea**

*Mesiorhizobium* inoculation significantly increased number of nodules per plant, nodule dry weight and shoot dry weight compared to uninoculated plants. Nodulation was not observed on uninoculated treatments (Table 5.3, 5.4), indicating the absence/inefficient number of compatible chickpea rhizobia existed in the experimental sites.

The data showed that inoculants induced mean nodule number of 9 NN/plant with DZ-ck-2011 s-2-004 variety and 11 NN/plant with Natoli variety at Holleta. The highest number of nodules (19 NN/plant) was recorded from varieties inoculated with *Mesorhizobium* strain (a.117L2); whereas the least number of nodules (9 NN/plant) was recorded from plants inoculated with reference strain (Cp41) (Table 5.3). Similarly, *Mesorhizobium* strain a.117L2 induced the highest nodule number (20 NN/plant)

compared to 17 NN/plant produced by (a.71 and a.15 star) strains and 10 NN/plant by (Cp41) at Sodo site. However, the two varieties produced 12-13 nodules per plant without showing significant ($p > 0.05$) difference between them on Sodo site (Table 5.4).

The inoculated plants showed variations on nodule dry weight depending on field sites and variety. Thus, the soil of Sodo showed mean NDW of 261mg/plant with a.117L2 strain compared to 153 mg/plant with reference strain (Cp41) at Holleta (Table 5.3, 5.4). The Natoli variety showed mean nodule dry weight of 222 mg/plant and 156 mg/plant; whereas the mean nodule dry weight of DZ-ck-2011 s-2-0042 variety was 162 mg/plant and 138 mg/plant, on acidic soils of Sodo and Holleta, respectively, showing 42% and 17% difference depending on the site. This showed that the relatively high acidic soil of Holleta sampling site inhibited nodule dry weight of the host plant. Although the plants induced less number of nodules in the field than the pot greenhouse culture on Holleta soil, they displayed higher nodule dry weight ranging from 174-220 mg/plant than the pot culture (52-168 mg/plant) (Table 5.2), indicating that larger nodules were formed under field conditions within the same planting date.

The inoculants also showed significant variation in shoot dry weight ranging from 6.1 gm/plant (Cp41) to 7.3 g/plant (a.117L2) with mean shoot dry weight of 6.0 g/plant on DZ-ck-2011 s-2-004 variety and 6.7 g/plant with Natoli variety at Sodo (Table 5.4). However, they performed less on Holleta with 4.5 - 6.3 g/plant on two varieties with mean shoot dry weight of 4.9 and 5.2 g/plant (Table 5.3).

The low shoot dry mass could also be associated with relatively high acidic soil of Holleta that limited dry matter accumulation of the host plant (Zahran, 1999; Hungria and Vargas, 2000). Studies on different legumes also obtained a lower shoot dry weight in high acidic soil with rhizobia inoculation (Carter *et al.*, 1994; Vassileva *et al*., 1997).

Table 5.3. Effect of mesorhizobial inoculation on nodule number (NN), nodule dry weight (NDW) and shoots dry weight (SDW) at 50% flowering stage of chickpea varieties on acidic soils at Holleta

| **Variety** | **Mesorhizobia inoculants** | **NN / plant** | **NDW mg/plant** | **SDW g/plant** |
|---|---|---|---|---|
| | | **.....Mesorhizobia inoculants.....** | | |
| | Un inoculated control | ND | ND | 3.6$^{d}$ |
| | CP41 | 9.0$^{b}$ | 152.5$^{c}$ | 4.5$^{c}$ |
| | a.15star (*M.*genospecies 5C) | 12$^{b}$ | 186.2$^{b}$ | 5.2$^{bc}$ |
| | a.117L2 (*M.*genospecies 2D) | 19$^{a}$ | 220.0$^{a}$ | 6.3$^{a}$ |
| | a.71 (*M.*genospecies 4B) | 17$^{a}$ | 173.8$^{cb}$ | 5.6$^{b}$ |
| | | **.....Variety.....** | | |
| Natoli | | 11$^{a}$ | 155.5$^{a}$ | 5.2$^{a}$ |
| DZ-ck-2011 s-2-0042 | | 9$^{b}$ | 137.5$^{b}$ | 4.9$^{a}$ |
| **P-value (< 0.05)** | | | | |
| | Inoculants | ** | ** | ** |
| | Varity | ** | ** | NS |
| | I*V | NS | NS | NS |
| | CV% | 29.5 | 14.1 | 12.8 |

* = Significant (P <0.05), ** = highly significant (P <0.01), and NS=non-significant (P>0.05), Not detected, V= Variety, I= Mesorhizobial inoculants. Means within the column having the same letter are not significantly different.

Table 5.4. Effect of Mesorhizobial inoculation on nodule number (NN), nodule dry weight (NDW) and shoots dry weight (SDW) at 50% flowering stage of chickpea varieties on acidic soils at Sodo

| Variety | Mesorhizobia inoculants | NN / plant | NDW mg/plant | SDW g/plant |
|---|---|---|---|---|
| | **.....Mesorhizobia inoculants.....** | | | |
| | Un inoculated control | ND | ND | $5.1^{d}$ |
| | CP41 | $10^{c}$ | $190.0^{c}$ | $6.1^{c}$ |
| | a.15star (*M.*genospecies 5C) | $17^{b}$ | $243.8^{b}$ | $6.8^{b}$ |
| | a.117L2 (*M.*genospecies 2D) | $20^{a}$ | $261.3^{a}$ | $7.3^{a}$ |
| | a.71 (*M.*genospecies 4B) | $17^{b}$ | $242.5^{b}$ | $6.6^{b}$ |
| | **.....Variety.....** | | | |
| Natoli | | $13^{a}$ | $221.9^{a}$ | $6.7^{a}$ |
| DZ-ck-2011 s-2-0042 | | $12^{a}$ | $162.0^{b}$ | $6.0^{b}$ |
| **P-value (< 0.05)** | | | | |
| | Inoculants | ** | ** | ** |
| | Varity | ** | ** | NS |
| | I*V | NS | ** | * |
| | CV% | 30.8 | 14.8 | 7.3 |

* = Significant (P = <0.05), ** = highly significant (P = <0.01), and NS=non-significant (P> 0.05), ND= Not detected, V= Variety, I= Mesorhizobial inoculants. Means with in the column having the same letter are not significantly different.

Inoculation with strain (a.117L2) resulted in significantly greater number of shoot dry weight 6.3 g plant$^{-1}$ and 7.3 g plant$^{-1}$ than 4.5 g plant$^{-1}$ and 6.1 g plant$^{-1}$ recorded from chickpea varieties inoculated with reference strain (Cp41). The inoculants increased shoot dry mass by 75% and 43 % and 25% and 20% over the uninoculated control at Holleta and Sodo field sites, respectively (Table 5.3,5.4), indicating that inoculated plants at Holleta soil increased in shoot dry weight, for they received nitrogen from the soil.

Comparable result was reported from the work of Brigido *et al*. (2007), who showed inoculation with mesorhizobia gave higher shoot dry matter (20-56%) of chickpea crop over the uninoculated control in acidic soil.

Each inoculant also showed significant difference in shoot dry weight depending on soil types and varieties. Consequently, Natoli variety accumulated 29% more shoot dry weight at Sodo site than Holleta site and the mean shoot dry weight of Natoli variety was 11.6% higher than DZ-ck-2011s-2-004 variety, indicating the DZ-ck-2011s-2-004 variety was more sensitive to high acid soils than the Natoli variety.

The data also showed significant difference in nodulation between varieties at Holleta and nodule dry weight and growth parameter at Sodo field experiments. In all cases, the Natoli variety showed higher values than the DZ-2012-CK-2011s-2-0042 variety. This difference might be due to their symbiotic compatibility, agronomic performance of the host genotypes (Gemechu Keneni *et al.*, 2012), nutrient status and pH of soils (Zahran, 1999). Birru Alemu and Dagnachew Lule, (2018) have also indicated that chickpea genotype (Natoli and DZ-2012-CK-2011s-2-0042) showed a different response to acidic soils.

The data in general, showed that indigenous low pH tolerance mesorhizobial strains (a.117L2, a.71 and a.15star) isolated from Ethiopians acidic soils are better adapted to low pH soil environment and symbiotically out-performed more than the commercially available local reference strain (Cp41). *Mesorhizobium* strain a.117L2 performed better

in nodulation and growth parameter, followed by a.71 and a.15star compared to the uninoculated control plants on acidic soils.

#### 5.3.2.4. Effects of mesorhizobia inoculation on yield and yield related characters of chickpea

The mesorhizobia treated chickpea plants significantly ($p< 0.05$) affected the number of pods plant$^{-1}$ (NP), hundred seed weight (HSW), grain yield (GY), and biomass yield (BMY) over the uninoculated control plants at both field sites (Table 5.5, 5.6). However, they did not show significant difference ($p>0.05$) between varieties for grain harvest index (GHI) at both experimental sites.

Plants inoculated with strain a.117L2 induced the higher number of pods plant $^{-1}$ (NP) (51 NP/plant) than 34 NP/plant recorded from chickpea varieties inoculated with Cp41 reference strain compared to the uninoculated control plants at Sodo and Holleta acidic soils, respectively (Table 5.5, 5.6). The inoculants showed the same pattern in hundred seed weight on both field sites, whereas the least number (18 g/plant) was recorded from plants inoculated with strains (a.15star, a.71 and Cp41) at Holleta (Table 5.5). The effects of rhizobia inoculation on number of pod plant$^{-1}$ and hundred seed weight in acidic soil were also reported on other studies in soyabean (Workeneh Bekere, 2013), fababean (Dereje Tsegaye, 2015) and cowpea (Farias *et al*., 2016).

The inoculated plant showed significant difference in hundred seed weight on the two varieties with mean of 24 g with Natoli variety at Sodo compared to mean of 14 g with

DZ-ck-2011 s-2-004 variety at Holleta. This shows that the inoculants induced more than half times more hundred seed weight on Natoli variety, but did not show significant difference on both varieties between two field sites, indicating that Natoli variety higher seed weight than DZ-ck-2011 s-2-004 variety.

The inoculated plants showed variations in grain yield (GY) and biomass yield (BMY) depending on variety with their respective soil types (Table 5.5, 5.6). Thus, the Natoli and DZ-ck-2011 s-2-0042 varieties showed mean grain yield of 2345.5kg $ha^{-1}$and 1866.5 kg $ha^{-1}$ at Holleta; and 2392.3kg $ha^{-1}$ and 2103.6 kg $ha^{-1}$ at Sodo respectively, showing 26% and 14% difference between varieties at Holleta and Sodo field sites, respectively. In terms, of biomass yield (BMY), they also showed difference (32% and 21%) between the good performed Natoli variety and low performed DZ-ck-2011 s-2-0042 variety at Holleta and Sodo field sites, respectively (Table 5.3, 5.4), indicating the DZ-ck-2011 s-2-0042 variety was more sensitive to acid soils than the Natoli variety.

Although, the inoculants did not show significant difference on biomass yield (BMY) and grain yield depending on individual variety between the two soil types, they increased the grain yield over the uninoculated control. The highest grain yield (GY) (2265 and 2506.7) kg $ha^{-1}$ were obtained from varieties inoculated with strain (a.117L2), whereas the least yield (2001.6 and 2185.9) kg $ha^{-1}$ was obtained from plants inoculated with reference strain (Cp41), showing an increase of (22 and 31) % and (8 and 14 %) over the uninoculated control at Holleta and Sodo field sites, respectively (Table 5.5, 5.6). In this

study grain yield increment higher than the report of Ogola, (2015) that obtained 7.9% from South Africa with rhizobia inoculants in a clayed and slightly acidic soil.

In most cases, the value of all yield parameters at Sodo was better than Holleta. This might be due to less soil acidity and the better nutrient availability at Sodo than Holleta from tested soil sample analysis (Table 5.1). Varietal differences were significant for all yield parameters and were higher on Natoli than DZ-ck-2011 s-2-0042 varieties.

In general, indigenous *Mesorhizobium* strain a.117L2 was highly effective for the grain yield and yield components, followed by a.71 and a.15star compared to the commercially available local reference strain (Cp41) and un-inoculated control.

Table 5.5. Effect of mesorhizobia inoculation on yield components and yield of chickpea varieties at Holleta field site

| Variety | Mesorhizobia inoculants | NP ($plt^{-1}$) | HSW (g) | BMY (kg/ ha) | GY (kg/ ha) | GHI |
|---|---|---|---|---|---|---|
| | **.....Mesorhizobia inoculants.....** | | | | | |
| | Un inoculated control | $33^{c}$ | $16^{c}$ | $5289.5^{e}$ | $1849.7^{d}$ | $0.34^{c}$ |
| | CP41 | $34^{c}$ | $18^{bc}$ | $5874.7^{d}$ | $2001.6^{c}$ | $0.35^{b}$ |
| | a.15star (*M.*genospecies 5C) | $44^{b}$ | $18^{b}$ | $6182.8^{c}$ | $2115.8^{bc}$ | $0.35^{b}$ |
| | a.117L2 (*M.*genospecies 2D) | $49^{a}$ | $21^{a}$ | $6615.1^{a}$ | $2265.0^{a}$ | $0.36^{a}$ |
| | a.71 (*M.*genospecies 4B) | $47^{ba}$ | $18^{b}$ | $6235.1^{b}$ | $2144.7^{b}$ | $0.36^{a}$ |
| | **.....Variety.....** | | | | | |
| **Natoli** | | $43^{a}$ | $22^{a}$ | $6584.1^{a}$ | $2345.5^{a}$ | $0.36^{a}$ |
| **DZ-ck-2011 s-2-0042** | | $39^{b}$ | $14^{b}$ | $4994.7^{b}$ | $1866.5^{b}$ | $0.36^{a}$ |
| **P-value (< 0.05)** | | | | | | |
| | Inoculants | ** | ** | ** | ** | ** |
| | Varity | * | ** | ** | ** | NS |
| | I*V | NS | NS | NS | NS | NS |
| | CV% | 10.0 | 8.7 | 14.1 | 12.3 | 1.5 |

* = Significant (P = <0.05), ** = highly significant (P = <0.01), and NS=non-significant (P> 0.05), V= Variety, I= Mesorhizobial inoculants. Means with in the column having the same letter are not significantly different

Table 5.6. Effect of mesorhizobia inoculation on yield components and yield of chickpea varieties at Sodo field site

| Variety | Mesorhizobia inoculants | NP ($plt^{-1}$) | HSW (g) | BMY (Kg/ ha) | GY (Kg/ ha) | GHI |
|---|---|---|---|---|---|---|
| | **.....Mesorhizobia inoculants.....** | | | | | |
| | Un inoculated control | $32^{d}$ | $16^{c}$ | $5621.2^{e}$ | $1909.1^{d}$ | $0.34^{d}$ |
| | CP41 | $41^{c}$ | $19^{b}$ | $6419.6^{d}$ | $2185.9^{c}$ | $0.34^{c}$ |
| | a.15star (*M.*genospecies 5C) | $46^{bc}$ | $19^{b}$ | $6499.7^{c}$ | $2297.5^{bc}$ | $0.35^{b}$ |
| | a.117L2 (*M.*genospecies 2D) | $51^{a}$ | $21^{a}$ | $7025.9^{a}$ | $2506.7^{a}$ | $0.36^{a}$ |
| | a.71 (*M.*genospecies 4B) | $47^{b}$ | $19^{b}$ | $6637.2^{b}$ | $2340.5^{b}$ | $0.35^{b}$ |
| | **.....Variety.....** | | | | | |
| **Natoli** | | $46^{a}$ | $24^{a}$ | $7054.9^{a}$ | $2392.3^{a}$ | $0.34^{a}$ |
| **DZ-ck-2011 s-2-0042** | | $42^{b}$ | $14^{b}$ | $5826.5^{b}$ | $2103.6^{b}$ | $0.36^{b}$ |
| **P-value (< 0.05)** | | | | | | |
| | Inoculants | ** | ** | ** | ** | ** |
| | Varity | ** | ** | * | ** | ** |
| | I*V | NS | NS | NS | NS | ** |
| | CV% | 8.3 | 8.9 | 9.4 | 9.7 | 0.8 |

* = Significant (P = <0.05), ** = highly significant (P = <0.01), and NS=non-significant (P> 0.05), V= Variety, I= Mesorhizobial inoculants. Means with in the column having the same letter are not significantly different

### 5.3.2.5. Nitrogen and phosphorus uptake and nitrogen derived from fixation by *Mesorhizobium* inoculation

Inoculation significantly improved nitrogen and phosphorus uptakes and symbiotic biological nitrogen fixation efficiency compared to the reference strain and uninoculated control on both field sites (Table 5.7, 5.8).

At the Holleta site, inoculation with *Mesorhizobium* strain (a.117L2) improved total nitrogen uptake of 91.6 kg/ha, followed by a.71 , a.15star and Cp41 with 85.5 kg $ha^{-1}$ , 82.3 kg $ha^{-1}$ and 67.9 kg $ha^{-1}$ , showing increases by 48%, 38% , 33% and 10% compared to the uninoculated control plants. Similarly, the highest maximum nitrogen

uptake (101.1 kg/ha) was recorded from *Mesorhizobium* strain a.117L2 followed by 98.3 kg/ha ,92.6 kg/ha and 83.6 kg/ha nitrogen accumulated by strains a.71 , a.15 star and reference strain (Cp41) with increases by 51%, 47% , 38 % and 25%, respectively over uninoculated control at Sodo site. Other study also showed that nitrogen uptake of the legume crops increase (15% - 23%) as a result of inoculation of rhizobia under acidic conditions in faba bean (Dereje Tesegaye, 2015).

Each inoculant showed significant difference on the amount of total nitrogen between the two soil types. This shows that the inoculants accumulate more total nitrogen (10-23%) at Sodo soil than Holleta, but did not show significant difference in total phosphorus uptake and the amount of N derived from fixation between the two soil types.

In addition, the inoculants did not show significant difference between varieties in terms of nitrogen uptakes, phosphorus uptakes and N derived from fixation on both field sites, indicating the two chickpea varieties responded similarly to the inoculant strains, despite different from other yield parameters, for they received from the soil by nitrogen turn over and solubilization of local phosphate by these strains.

The inoculants increased total phosphorus uptake compared to uninoculated control, the highest phosphorus uptake by the plants (13.69 and 14.6 kg $ha^{-1}$) was recorded with *Mesorhizobium* strain (a.117L2) followed by those treated with strain a.71 (13.12 and 14 kg $ha^{-1}$), a.15star (11.18 and 12.3 kg $ha^{-1}$) and Cp41 (11.9 and 12.4 kg $ha^{-1)}$ over uninoculated control plants at Holleta and Sodo field experiments, respectively (Table 5.7, 5.8). According to Hmissi Imen *et al*. (2015), inoculation with mesorhizobia strain

increased phosphorus uptake due to the ability of strains to solublize precipitated phosphorus there by leading to increase phosphorus uptake in chickpea plants.

Plants inoculated with strain a.117L2, a.71 and a.15star derived 50.2 and 53.6%, 38 and 48.4% and 35.9 and 40.2%, more nitrogen than the reference strain Cp41 at Holleta and Sodo field experiments, respectively (Table 5.7, 5.8). Dereje Tesegaye, (2015) demonstrated nitrogen derived from fixation rates could be markedly increased by highly efficient, competitive, and persistent strains of *Rhizobium* nodulating faba bean in acidic soils. The best criteria for *Rhizobium* used as bio-fertilizer is that it must be highly effective in nitrogen fixing ability forming symbiotic association with the host plants (Catroux *et al*., 2001).

Table 5.7. Effect of mesorhizobial inoculation on N, P uptakes and BNF of chickpea varieties at Holleta field

| **Variety** | **Mesorhizobia inoculants** | **Nitrogen uptake (kg/ha)** | **Phosphorus uptake (kg/ha)** | **%Ndfa** |
|---|---|---|---|---|
| | | **..... Mesorhizobia inoculants.....** | | |
| | Un inoculated control | $62.0^{d}$ | $9.43^{e}$ | - |
| | CP41 | $67.9^{c}$ | $11.9^{c}$ | $33.4^{cd}$ |
| | a.15star (*M.*genospecies 5C) | $82.3^{bc}$ | $11.18^{d}$ | $35.9^{c}$ |
| | a.117L2 (*M.*genospecies 2D) | $91.6^{a}$ | $13.69^{a}$ | $50.2^{a}$ |
| | a.71 (*M.*genospecies 4B) | $85.5^{b}$ | $13.12^{b}$ | $38.0^{b}$ |
| | | **.....Variety.....** | | |
| **Natoli** | | $79.2^{a}$ | $11.79^{a}$ | $39.8^{a}$ |
| **DZ-ck-2011 s-2-0042** | | $76.6^{a}$ | $10.69^{b}$ | $38.9^{a}$ |
| **P-value (< 0.05)** | | | | |
| | Inoculants | ** | ** | ** |
| | Varity | NS | NS | NS |
| | I*V | NS | NS | ** |
| | CV% | 13 | 8.3 | 9.3 |

* = Significant (P = <0.05), ** = highly significant (P = <0.01), and NS=non-significant (P> 0.05), V= Variety, I= Mesorhizobial inoculants. Means with in the column having the same letter are not significantly different

Table 5.8. Effect of mesorhizobial inoculation on N, P uptakes and BNF of chickpea varieties at Sodo field

| Variety | Mesorhizobia inoculants | Nitrogen uptake (kg/ha) | Phosphorus uptake (kg/ha) | %Ndfa |
|---|---|---|---|---|
| | | **..... Mesorhizobia inoculants.....** | | |
| | Un inoculated control | 66.9[d] | 10.0[c] | - |
| | CP41 | 83.6[c] | 12.4[b] | 37.8[d] |
| | a.15star (*M.*genospecies 5C) | 92.7[b] | 12.3[b] | 40.2[c] |
| | a.117L2 (*M.*genospecies 2D) | 101.0[a] | 14.6[a] | 53.6[a] |
| | a.71 (*M.*genospecies 4B) | 98.3[ab] | 14.0[a] | 48.4[b] |
| | | **.....Variety.....** | | |
| **Natoli** | | 89.5[a] | 12.7[a] | 36.6[a] |
| **DZ-ck-2011 s-2-0042** | | 87.5[a] | 12.6[a] | 35.4[a] |
| **P-value (< 0.05)** | | | | |
| | Inoculants | ** | ** | ** |
| | Varity | NS | NS | NS |
| | I*V | NS | NS | ** |
| | CV% | 13.7 | 6.8 | 10.1 |

* = Significant (P = <0.05), ** = highly significant (P = <0.01), and NS=non-significant (P> 0.05), V= Variety, I= Mesorhizobial inoculants. Means with in the column having the same letter are not significantly different

In general, indigenous *Mesorhizobium* strain a.117L2 was the best performing inoculant for all of the studied parameters, followed by a.71 and a.15star, respectively, compared to the uninoculated control. Other reports (Evans, 2005; Workeneh Bekere *et al.,* 2013; Farias *et al*., 2016; Daniel Muleta *et al.,* 2017, Bello *et al.,* 2018) demonstrated that indigenous rhizobia inoculations tested in acidic soil environment improve growth, seed yield, nitrogen fixation and also nutrient uptake of legumes. Therefore, these results indicate that the indigenous chickpea nodulating rhizobial strains used in this study are better adapted to the soil environment and survived in adequate numbers as compared to commercially available local strain Cp41.

The types of responses were observed on correlation test results indicated a significant ($p<0.05$) or highly significant ($p<0.01$) positive correlation among most parameters (Table 5.9). Grain yield showed a highly significant ($p< 0.01$) positive correlation with shoot dry matter (r=0.995), hundred seed weight (r=0.998), biological yield (r=0.999), and N derived from fixation (r=0.999). There was also a highly significant ($p< 0.01$) positive correlation between N derived from fixation with shoot dry matter (r=0.997), hundred seed weight (r=0.999), biological yield (r=0.999), grain yield (r=0.999). Similarly, at Sodo field experiment grain yield showed a highly significant ($p< 0.01$) positive correlation with a number of pods per plant (r=0.998), biological yield (r=0.998). There was also a highly significant ($p< 0.01$) positive correlation between N derived from fixation with total nitrogen (r=0.997) and total phosphorus (r=0.990).

Table 5.9. Correlations among different variables at Holleta and Sodo fields

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | HOLETA FIELD | | | | | | |
| | **NN** | **NDW** | **SDW** | **NPP** | **HSW** | **BMY** | **GY** | **GHI** | **TN** | **TP** | **%Ndfa** |
| **NN** | 1 | | | | | | | | | | |
| **NDW** | 0.528 | 1 | | | | | | | | | |
| **SDW** | 0.767 | 0.950 | 1 | | | | | | | | |
| **NPP** | 0.990** | 0.549 | 0.783 | 1 | | | | | | | |
| **HSW** | 0.789 | 0.938* | 0.999** | 0.804* | 1 | | | | | | |
| **BMY** | 0.801* | 0.931* | 0.998** | 0.816* | 0.999** | 1 | | | | | |
| **GY** | 0.827* | 0.914* | 0.995** | 0.841* | 0.998** | 0.999** | 1 | | | | |
| **GHI** | 0.957* | 0.258 | 0.548 | 0.949* | 0.577 | 0.593 | 0.628 | 1 | | | |
| **TN** | 0.960* | 0.745 | 0.916* | 0.967* | 0.930* | 0.937* | 0.952* | 0.837* | 1 | | |
| **TP** | 0.997** | 0.461 | 0.716 | 0.995** | 0.740 | 0.753 | 0.782 | 0.976* | 0.936* | 1 | |
| **%Ndfa** | 0.816* | 0.922* | 0.997** | 0.831* | 0.999** | 0.999** | 0.999** | 0.613 | 0.946* | 0.770 | 1 |
| | | | | | SODO FIELD | | | | | | |
| | **NN** | **NDW** | **SDW** | **NPP** | **HSW** | **BMY** | **GY** | **GHI** | **TN** | **TP** | **%Ndfa** |
| **NN** | **1** | | | | | | | | | | |
| **NDW** | 0.990** | 1 | | | | | | | | | |
| **SDW** | 0.998** | 0.999** | 1 | | | | | | | | |
| **NPP** | 0.975* | 0.968* | 0.958* | 1 | | | | | | | |
| **HSW** | 0.993** | 0.989* | 0.982* | 0.995** | 1 | | | | | | |
| **BMY** | 0.974* | 0.967* | 0.956* | 1.990** | 0.994** | 1 | | | | | |
| **GY** | 0.959* | 0.950* | 0.937* | 0.998** | 0.986* | 0.998** | 1 | | | | |
| **GHI** | 0.999** | 0.998** | 0.995** | 0.982* | 0.996** | 0.981* | 0.968* | 1 | | | |
| **TN** | 0.722 | 0.701 | 0.672 | 0.857* | 0.799 | 0.860* | 0.888* | 0.744 | 1 | | |
| **TP** | 0.678 | 0.656 | 0.626 | 0.824* | 0.761 | 0.827* | 0.859* | 0.702 | 0.998** | 1 | |
| **%Ndfa** | 0.775 | 0.756 | 0.729 | 0.895* | 0.844* | 0.898* | 0.922* | 0.795 | 0.997* | 0.990** | 1 |

*=significant correlation, **=highly significant correlation, NN=nodule number, NDW=nodule dry weight, SDW=shoot dry weight, NPP= number of pods per plant,, HSW= hundred of seed weight, BMY= Biomass yield, GY= Grain yield, GHI=Grain harvest index, TP= Total phosphorus uptake, TN= Total nitrogen uptake, %Ndfa = Nitrogen derived from fixation.

### 5.3.3. Conclusion and recommendation

In conclusion, Holleta and Sodo field soils did not harbor compatible resident mesorhizobia for nodulating chickpea; and inoculation of the chickpea plants with mesorhizobial strain a.117L2 (*M*. genospecies 2D), a.71 (*M*. genospecies 4B), and a.15star (*M*. genospecies 5C) improved biological nitrogen fixation, growth and yield of chickpea compared to reference strain Cp41 and uninoculated. *Mesorhizobium* strain a.117L2 (*M*. genospecies 2D) proved superior in almost all parameters such as grain yield, yield component, nodulation, N and P uptake and amount of N fixed to the other inoculants. The Natoli variety better performed in many parameters than the DZ-ck-2011 s-2-0042 variety. Therefore, there is a potential of using these mesorhizobial inoculants to improve chickpea production in acidic soils after further field trials over several seasons and sites in Ethiopia.

# CHAPTER 6

## 6.1. General Conclusion and Recommendation

### 6.1.1. Conclusion

Acidic soils in Ethiopia harbored more diverse groups of *Mesorhizobium* species and a few of them previously not known to nodulate chickpea, of which more diverse groups (four genospecies each) belong to Bale, west Shewa, southwest Shewa, Asossa and south Wollo than fewer isolates recovered from west Wollega region. Most genospecies (89%) shared common symbiosis genes of *nod* C. However, the grouping pattern was irrespective of the isolates geographic locations. The geographic pattern of genomic diversity indicates no relationship between geographic and genetic distance.

Screening of *Mesorhizobium* spp. for low pH tolerance and phosphate solublization showed that 62 (77 %) of strains were tolerate and 47 (76 %) of strains solublized insoluble tri-calcium phosphate. They were found to have diverse in their heterotrophic versatility in carbon and nitrogen utilization, with a wide range of *in vitro* tolerance to various stress conditions, including low pH, salinity, high temperature, $Mn^{2+}$ and $Al^{3+}$toxicity, heavy metals, antibiotics, indicting their potential to ecological tolerance under field conditions.

The strains showed considerable variation in their symbiotic effectiveness on the two host varieties. Five strains, namely: a.15star *(M.* genospecies 5C), a.117L2 (*M.* genospecies 2D), a.71 (*M.* genospecies 4B) and a.40L2 (*M.* genospecies 8A), and a.200M (*M.* genospecies 3A) was found to be relatively superior in their symbiotic performance.

Further evaluation on N fixation efficiency in a pot experiment on acidic soil of Holleta and Arjo resulted significant improvement on nodulation, growth parameters and accumulation of total nitrogen. The relatively best strains a.15star (*M.* genospecies 5C), a.117L2 (*M.* genospecies 2D) and a.71 (*M.* genospecies 4B) were considered as prospective commercial inoculants for chickpea grown in acidic soils, having low population and in effective indigenous rhizobia, provided that are tested and proved the same under field different conditions.

Relatively large number of phosphate solublizing *Mesorhizobium* spp. was obtained from studied acidic soils of Ethiopia. The selected strains were able to release significant amount of soluble P in liquid medium from different insoluble phosphates sources such as tri-calcium phosphate $Ca_3$ $(PO4)_2$, iron phosphate ($FePO_4$) and aluminum phosphate ($AlPO_4$). Most of these strains also have multiple plant growth promoting traits. The relatively best phosphate solublizing strains (a.117L2 (*M.* genospecies 2D) and a.71 (*M.* genospecies 4B) that displayed all plant growth promoting properties tested can be considered as prospective inoculants in acidic soils, provided that tested under field conditions due to the potentially high importance for the phosphorus nutrition in acidic soil areas.

In the field condition, mesorhizobial strains a.117L2 (*M.* genospecies 2D), a.71 (*M.* genospecies 4B), and a.15star (*M.* genospecies 5C) improved growth, grain yield, biological nitrogen fixations, nitrogen and phosphorus uptakes of chickpea compared to the nationally used commercial reference strain Cp41 and uninoculated plant. Inoculation with mesorhizobial strains a.117L2 (*M.* genospecies 2D) was superior inoculant for

almost all parameters.

Finally, the result of this study will provide useful information on inoculants production and use of such inoculants having multiple performances improve plant growth, the productivity of chickpea in acidic soils by improving phosphorus and nitrogen fertility.

### 6.1. 2 Recommendations

- ✓ Further taxonomic studies should be performed on the potentally novel groups of strains.

- ✓ Further works have to be done on the type of organic acids secreted during inorganic phosphates solublization by *Mesorhizobium* strains.

- ✓ Since the study was limited to few traits, the strain should also be assessed further for additional indices of *in vitro* PGP characteristics such as production of gibberellins and cytokinins, lumichromes etc.

- ✓ Further investigation should require for PGP activity of *Mesorhizobium* strains with host varities in acidic soil under green house expiriments.

- ✓ It is also suggested that mesorhizobial strains a.117L2 (*M*. genospecies 2D), a.71 (*M.* genospecies 4B), and a.15star (*M.* genospecies 5C) having multiple performances should be used as commercial inoculants for chickpea production in acidic soil of Ethiopia after it is tested (validated) at different agro-ecological conditions.

- ✓ Further characterization of the genetic diversity amongst isolates by the construction of a pan and accessory genomes from this WGS data and investigation of the roles of both accessory and highly variable common genes with respect to niche adaptation.

- ✓ Further examination of the *Mesorhizobium* isolates by identifing low pH or Al toxicity tolerance associated genes that are potentially involved in mechanism of low pH tolerance and their control, and map these genes to develop an acid-tolerant specific marker which is critical to improve the productivity of chickpea in acidic region of Ethiopia.

## 7. Referances


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## 8. Appendices

Appendix 1. Composition of Yeast Extract Mannitol Agar (YEMA) (Somasegaran and Hoben, 1994)

Mannitol (10g), K2HPO4 (0.5g), MgSO4.7H2O (0.2g), NaCl (0.1g), Yeast extracts (1g), Agar (15g), Distilled water (1000ml)

Appendix 2. Composition of CRS plant growth nutrient solution e.g for plants grown in sand (Howieson and Dilworth, 2016)

| Stock solution | Quantity (g/l) |
|---|---|
| 1. $MgSO4.7H_2O$ | 12.3 |
| 2. $KH_2PO_4$ | 6.8 |
| 3. $K_2SO4$ | 17.5 |
| 4. Fe-EDTA | 2.5 |
| 5. trace element solution (store at 4°C) | |
| $H_3BO_3$ | 0.464 |
| $Na_2MoO_4.2H_2O$ | 0.018 |
| ZnSO4.7H2O | 0.539 |
| $MnSO4.4H_2O$ | 0.042 |
| CoSO4.7H2O | 0.141 |
| CuSO4.5H2O | 0.125 |
| 6. CaSO4 agitated solution | 2.04 |
| +N control | 5 mL weekly of 10 g/L KNO3 |

**Note:** The advantage of this solution is that it provides adequate calcium with low chloride content and chelated iron. Make five stock solutions, the fifth being for trace elements, and a sixth for calcium.

Appendix 3. Composition of Keyser and Munns basal medium (Ayanaba *et al*., 1983)

300 µM $MgSO4.7H_2O$, 300 µM $CaCl_2$, 100 µM Fe EDTA. 10 µM KCI, 1µM $MnCl_2.4H_2O$, 0.4 µM $ZnSO_4.7H_2O$, 0.1 µM $CuCl_2.2H_2O$, 0.02 µM $Na_2MoO_4.2H_2O$, 0.02 µM $Co(N0_3)_2.6H_2O$, 500 µM $KH_2P0_4$,500 µM $K_2HPO_4$, arabinose (5.0g), galactose (5.0g), 1.1g Na glutamate, biotin (0.1 mg), thiamine (1.0 mg), 0.005% bromothymol blue, 15g agar, and 1L distilled water

Appendix 4. Chickpea Mesorhizobia isolates, the origin of isolation, colony and growth characteristics

| Isolates | Geographic origin of isolates | | | | Altitude (masl) of the isolation site | Latitude | Longitude | SoilpH of the isolation site | Colony morphology | | Growth Rate (MGT) (hrs) | Presumptive test |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Region | Zone | District/town | Location | | | | | Colony size (mm) | Colony Characteristics | | YEMA BTB |
| **a.161** | Oromiya | Arisi | Xiyo | Hella Zenbaba | 2334 | 8.002722N | 39.91611E | 5.7 | 4.1 | LWM | 4.6 | Yellow |
| **a.96** | Oromiya | Bale | Goro | Addis Alema | 2078 | 7.033333 | 40.63417 | 6 | 4.3 | LWM | 4.1 | Yellow |
| **a.152** | SNNP | Gurage | Sodo/Buee | Doajen | 1974 | 8.287641 | 38.53556 | 5.2 | 4 | LWM | 5.1 | M. Yellow |
| **a.63** | Amahra | South wollo | Kemse | Hula tukye | 1463 | 10.56847 | 39.92715 | 6.1 | 4.5 | LWM | 4.8 | Yellow |
| **a.50** | Amahra | South wollo | Ambasel | Abajubane/02 | 1710 | 11.49553 | 39.61255 | 4.6 | 4.2 | LWhLM | 5 | M. Yellow |
| **a.231** | Oromiya | West shewa | Holeta | Berfeta 1 ffa | 2264 | 9.003837 | 38.51457 | 5.4 | 3.7 | LWM | 4.7 | M. Yellow |
| **a.71** | Oromiya | West shewa | Holeta | Geresu seda | 2231 | 8.054306 | 39.87258 | 5.1 | 2.3 | MWM | 4.9 | M. Yellow |
| **a.209** | SNNP | Gurage | Sodo/Buee | Kessay | 2045 | 8.327911 | 38.56285 | 5.8 | 3.5 | LWLM | 5.4 | M. Yellow |
| **a.131** | Amahra | North wollo | Raya kobo | 02/HG6 | 1486 | 12.12385 | 39.62732 | 6.2 | 4.2 | LWM | 4.8 | M. Yellow |
| **a.64** | Oromiya | Westwellega | Dembidolo | Mata | 2016 | 8.577056 | 34.73936 | 5.3 | 4.1 | LWM | 4.6 | Yellow |
| **a.90** | Oromiya | West shewa | Holeta | Geresu | 2219 | 8.386444 | 38.22611 | 4.9 | 2.9 | MWM | 5.9 | M. Yellow |
| **a.23L** | Amahra | South wollo | Kombolcha | Dawa Chiffa/014 | 1485 | 10.8901 | 39.80967 | 5.4 | 3.5 | LWhLM | 4.9 | M. Yellow |
| **a.150** | Benishangul | Asosa | Bambasi | 46 mender | 1448 | 9.850112 | 34.68784 | 4.6 | 4 | LWM | 4.1 | Yellow |
| **a. 83** | Oromiya | Bale | Goro | Chefa mana | 2112 | 7.040833 | 4.560556 | 6 | 4.1 | LWM | 4.7 | M. Yellow |
| **a.92** | Oromiya | Bale | Goro | Giner | 1915 | 7.000472 | 41.43944 | 6.1 | 3.1 | LWhLM | 5.2 | M. Yellow |
| **a.200M** | Oromiya | East wellega | Sire | Oligelte/ mechare | 1582 | 9.002881 | 36.74009 | 4.4 | 3.5 | LWM | 4.9 | M. Yellow |
| **a.147** | SNNP | Gurage | Sodo/firshi | Medhin | 2003 | 8.337343 | 38.53053 | 5.7 | 3.2 | LWM | 5.1 | M. Yellow |
| **a.135** | Benishangul | Asosa | Bambasi | Sonko | 1486 | 9.818222 | 34.70081 | 5.4 | 4.1 | LWM | 4.8 | M. Yellow |
| **a.116** | Amahra | North wollo | Srinka Ag. | Srinka Ag. | 1847 | 11.75072 | 39.61308 | 5.9 | 4 | LWM | 4.1 | Yellow |
| **a.117M** | Benishangul | Asosa | Bambasi | Dabus/3 | 1406 | 9.765929 | 34.80069 | 5.1 | 3.2 | LWM | 4.4 | Yellow |
| **a.142** | Oromiya | S/W/Shewa | Seden sodo | Muden lbayo | 2371 | 8.534798 | 38.25476 | 6.1 | 2.7 | MWhM | 5.6 | M. Yellow |
| **a.195$_3$** | Benishangul | Asosa | Bambasi | 49 mender | 1428 | 9.831095 | 34.69295 | 4.9 | 3.4 | LWM | 5.1 | M. Yellow |
| **a.58** | Oromiya | West shewa | Holeta | Wegdi | 2239 | 9.813588 | 38.4897 | 5.1 | 4.1 | LWhLM | 4.4 | Yellow |
| **a.AR49** | Benishangul | Asosa | Bambasi | Dabus/3 | 1406 | 9.765929 | 34.80069 | 5.2 | 3.8 | LWM | 4.7 | M. Yellow |
| **a.40L2** | Oromiya | S/W/Shewa | Tullu bollo | Becho | 2248 | 8.585578 | 38.24065 | 5.9 | 4.3 | LWhM | 5.3 | M. Yellow |
| **a.104** | Oromiya | Arisi | Shirka | Goro | 2338 | 8.064417 | 39.96378 | 5.3 | 4.2 | LWM | 5.2 | M. Yellow |
| **a.star16** | Benishangul | Asosa | Bambasi | Dabus/03 | 1399 | 9.763652 | 34.79209 | 5.5 | 4.1 | LWM | 4.5 | Yellow |
| **a.216** | Oromiya | West shewa | Holeta | Wajitu Harbo | 2236 | 9.013583 | 38.46469 | 5 | 4.5 | LWhM | 4.5 | Yellow |
| **a.23M** | Amahra | South wollo | Tehule Dere | Hittecha | 2128 | 11.25089 | 39.68306 | 6.1 | 2.6 | MWhM | 5.1 | M. Yellow |
| **a.AR352** | Oromiya | East wellega | Sibu sire | Oligelte | 1633 | 9.087178 | 36.7432 | 4.8 | 3.5 | LWhM | 5.5 | M. Yellow |
| **a.233** | Oromiya | West shewa | Holeta | Geba kemisa/Hurifa | 2077 | 8.917968 | 38.43249 | 5.2 | 3.7 | LWM | 4.4 | Yellow |
| **a.27** | Oromiya | West shewa | Holeta | Geresu seda | 2278 | 8.995119 | 38.49062 | 5.6 | 4.1 | LWM | 4 | Yellow |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **a.55** | Amahra | South wollo | Kuter 8 | Menentera | 1752 | 11.71503 | 39.6526 | 5.1 | 3.8 | LWhM | 4.1 | Yellow |
| **a.30$_2$star** | Oromiya | West shewa | Holeta | Wajitu | 2259 | 9.008362 | 38.4611 | 5.4 | 2.1 | MWhLM | 5.2 | M. Yellow |
| **a.AR39** | Oromiya | West shewa | Holeta | Tuluwato dalecha | 2225 | 8.95242 | 38.95242 | 6 | 2.5 | LWM | 4.9 | M. Yellow |
| **a.66** | Amahra | South wollo | Kemse | Artuma fursa | 1461 | 10.57622 | 39.92128 | 5.9 | 3.6 | LWhLM | 4.7 | M. Yellow |
| **a.47star** | Oromiya | S/W/Shewa | Illu | Tullu mengura | 2064 | 8.826222 | 38.42889 | 6.1 | 3.3 | LWhLM | 5.5 | M. Yellow |
| **a.8star** | Oromiya | Arisi | Shirka | Hella terta | 2326 | 7.985 | 39.97147 | 5.2 | 1.5 | SWLM | 5.4 | M. Yellow |
| **a.AR45s** | Oromiya | East wellega | Sibu sire | Mechare | 1552 | 9.011324 | 36.74068 | 4.7 | 3.7 | LWM | 4.4 | M. Yellow |
| **a.AR452** | Oromiya | East wellega | Sire | Oligelte/ mechare | 1582 | 9.002881 | 36.74009 | 5 | 4.2 | LWM | 5.5 | M. Yellow |
| **a.138w** | Amahra | North wollo | Raya kobo | 07/ Aware | 1416 | 12.08462 | 39.6654 | 5.1 | 4.5 | LWM | 4.5 | Yellow |
| **a.200s** | Amahra | South wollo | Ambasel | Denka meda | 1676 | 11.49973 | 39.61389 | 5.2 | 4 | LWhM | 4.1 | Yellow |
| **a.117L2** | Benishangul | Asosa | Bambasi | Dabus/03 | 1399 | 9.763652 | 34.79209 | 4.9 | 4.1 | LWM | 5.2 | M. Yellow |
| **a.117star** | Benishangul | Asosa | Asosa | Airport/ Amba 12 | 1567 | 10.03125 | 34.57175 | 5.2 | 2.1 | MWM | 5 | Yellow |
| **a.89** | Oromiya | West shewa | Holeta | Berfeta 1 ffa | 2254 | 9.023472 | 38.51169 | 5.1 | 3.4 | LWM | 4.4 | Yellow |
| **a.121** | Oromiya | S/W/Shewa | Illu | Tullu mengura | 2025 | 8.201394 | 38.3445 | 6.1 | 4.5 | LWhLM | 4.8 | M. Yellow |
| **a.194star** | Oromiya | Bale | Goro | Meliyu | 1923 | 7.033667 | 41.24083 | 6.2 | 3.3 | LWM | 5.2 | M. Yellow |
| **a.45 2** | Oromiya | S/W/Shewa | Illu | Tullu mengura | 2014 | 8.021667 | 38.09672 | 5 | 4.4 | LWhM | 4.9 | M. Yellow |
| **a.111** | Oromiya | Bale | Goro | Chefa mana | 2014 | 7.021806 | 40.69303 | 5.5 | 4 | LWhLM | 4.5 | Yellow |
| **a.33M** | Oromiya | West shewa | Holeta | Wegdi | 2239 | 9.813588 | 38.4897 | 5.3 | 3.8 | LWhLM | 4.8 | M. Yellow |
| **a.15star** | Oromiya | Arisi | Shirka | Jelko | 2362 | 8.016972 | 39.85008 | 5.7 | 1.2 | SWhM | 6.2 | M. Yellow |
| **a.210** | SNNP | Gurage | Sodo/kella | Sankaber | 2086 | 8.266207 | 38.50748 | 5.9 | 3.4 | LWM | 5.5 | M. Yellow |
| **a.217D** | Oromiya | West shewa | Holeta | Geba kemisa | 2209 | 8.942449 | 38.44527 | 5.7 | 4.5 | LWhLM | 5.2 | M. Yellow |
| **a.16star** | Oromiya | Arisi | Shirka | Gelbeha | 2325 | 8.000917 | 39.93356 | 5 | 4.3 | LWM | 4.4 | M. Yellow |
| **a.144s** | Oromiya | Westwellega | Dembidolo | Hawa galan | 1905 | 8.646222 | 34.84814 | 4.7 | 4.2 | LWM | 5.2 | M. Yellow |
| **a.44** | Oromiya | S/W/Shewa | Seden sodo | Tole Bale Kesi | 2356 | 8.513059 | 38.25488 | 6.1 | 3.8 | LWhLM | 5.5 | M. Yellow |
| **a.AR35star** | Oromiya | East wellega | Sibu sire | Mechare | 1552 | 9.011324 | 36.74068 | 4.8 | 3.7 | LWM | 4.7 | M. Yellow |
| **a.25** | Oromiya | East wellega | Sibu sire | Oligelte/chne | 1633 | 9.087178 | 36.7432 | 4.7 | 2.2 | MWhLM | 4.1 | Yellow |
| **a.117** | Benishangul | Asosa | Bambasi | 49 mender | 1428 | 9.831095 | 34.69295 | 5.3 | 3.1 | LWM | 5.3 | M. Yellow |
| **a.40L** | Oromiya | West shewa | Holeta | Geba kemisa | 2094 | 8.927031 | 38.43924 | 6.1 | 3.2 | LWLM | 5.4 | M. Yellow |
| **a.118** | Oromiya | West shewa | Holeta | Wajitu | 2259 | 9.008362 | 38.4611 | 5.1 | 2.5 | MWhLM | 4.8 | M. Yellow |
| **a.191** | Amahra | North wollo | Raya kobo | 07/Aware HG14 | 1416 | 12.08462 | 39.6654 | 6.2 | 3.4 | LWhLM | 4.4 | Yellow |
| **a.218** | Oromiya | West shewa | Holeta | Geba kemisa/Hurifa | 2084 | 8.921155 | 38.43337 | 6.1 | 4 | LWM | 5.1 | M. Yellow |
| **a.164** | Amahra | South wollo | Haburu | 15/tachsekela | 1585 | 11.58056 | 39.66279 | 6.2 | 3.2 | LWM | 5.1 | M. Yellow |
| **a.29L2** | Oromiya | Westwellega | Dembidolo | Mata | 2016 | 8.577056 | 34.73936 | 5.3 | 4.1 | LWM | 5.5 | M. Yellow |
| **a.AR1** | Oromiya | West shewa | Holeta | Geresu seda | 2237 | 8.995119 | 38.49062 | 5.9 | 3.7 | LWM | 5.2 | M. Yellow |
| **a.128** | Oromiya | S/W/Shewa | Illu | Tullu mengura | 2014 | 8.021667 | 38.09672 | 6.2 | 4.4 | LWhLM | 4.3 | M. Yellow |
| **a.10** | Benishangul | Asosa | Bambasi | Sonko | 1486 | 9.818222 | 34.70081 | 5.5 | 3.4 | LWhM | 4.9 | M. Yellow |
| **a.132M** | Oromiya | Westwellega | Dembidolo | Hawa galan | 1905 | 8.646222 | 34.84814 | 5.4 | 4.2 | LWM | 5.2 | M. Yellow |
| **a.19** | Oromiya | Arisi | Shirka | Burkutu | 2304 | 7.968111 | 39.87139 | 5.8 | 3.7 | LWhM | 5 | M. Yellow |
| **a.196** | Oromiya | Arisi | Xiyo | Burkitu Alkasa | 2595 | 8.355500 | 39.23403 | 5.6 | 4.1 | LWhLM | 4.5 | M. Yellow |
| **a.222** | Oromiya | Westwellega | Dembidolo | Mata | 2016 | 8.577056 | 34.73936 | 5.2 | 4.5 | LWhM | 4.8 | M. Yellow |
| **a.30** | Oromiya | West shewa | Holeta | Geresu | 2219 | 8.386444 | 38.22611 | 5.7 | 2.3 | MWM | 5.3 | M. Yellow |
| **a.33s** | Oromiya | West shewa | Holeta | Wajitu | 2259 | 9.008362 | 38.4611 | 5.6 | 2.5 | MWM | 5.1 | M. Yellow |
| **a45** | Oromiya | S/W/Shewa | Seden sodo | Urago Kelecha | 2243 | 8.574302 | 38.24453 | 6 | 2.8 | MWhM | 5 | M. Yellow |
| **a.6** | Oromiya | Arisi | Xiyo | Tullu cheba | 2344 | 8.386861 | 39.11511 | 5.8 | 3.7 | LWhM | 4.9 | M. Yellow |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **a.72** | Oromiya | Bale | Goro | Meliyu | 1996 | 7.026806 | 40.68081 | 6.2 | 2.4 | MWhM | 5.2 | M. Yellow |
| **a.AR35** | Oromiya | East wellega | Sibu sire | Mechare | 1552 | 9.011324 | 36.74068 | 5.2 | 3.8 | LWM | 4.8 | M. Yellow |
| **a.AR6** | Oromiya | East wellega | Sire | Oligelte/ mechare | 1582 | 9.002881 | 36.74009 | 5.4 | 3.7 | LWhM | 4.6 | M. Yellow |
| **a.AR61** | SNNP | Gurage | Sodo/Buee | Wollasa | 2051 | 8.337031 | 38.55248 | 5.8 | 2.7 | MWhM | 5.1 | M. Yellow |
| **a.AR7** | Benishangul | Asosa | Asosa | Airport/ Amba 12 | 1567 | 10.03125 | 34.57175 | 5.3 | 3 | MWM | 5.4 | M. Yellow |

LWhM: Large, white, mucoid; SWhM: Small, white, mucoid; LWM: Large, watery, mucoid; LWhLM: Large, white, less mucoid; LWLM: Large watery, less mucoid; MWM: Medium, watery, mucoid; MWhM: Medium, white, mucoid; MWhLM: Large,white, less mucoid; SWLM: Small watery less mucoid; (L :> 3 mm) (M: 2-3mm) (S :< 2 mm), M. Yellow: Medium Yellow.

## Appendix 5. Genome characteristics of chickpea *Mesorhizobium* isolates

| Isolates | Contigs | Scaffolds | Genome size | Longest scaffold | N50 | % GC | CDS | tmRNA | tRNA | rRNA | EC coverage | Completeness |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **a.10** | 1218 | 1214 | 6759804 | 931757 | 321678 | 63.32 | 6355 | 1 | 54 | 6 | 162.7 | 98.89 |
| **a.104** | 2159 | 2152 | 7638698 | 954234 | 345955 | 62.94 | 6945 | 1 | 54 | 6 | 124.45 | 99.51 |
| **a.111** | 1495 | 1488 | 7652926 | 836974 | 288025 | 63.56 | 7105 | 1 | 57 | 3 | 169.96 | 99.92 |
| **a.116** | 1411 | 1405 | 7102005 | 432202 | 176258 | 63.48 | 6580 | 1 | 57 | 6 | 94.78 | 99.51 |
| **a.117L2** | 9428 | 9424 | 10903099 | 603076 | 114143 | 62.96 | 10274 | 1 | 66 | 6 | 95.84 | 98.89 |
| **a.117** | 1098 | 1094 | 7060236 | 858225 | 277634 | 63.14 | 6672 | 1 | 55 | 6 | 144.82 | 98.89 |
| **a.117M** | 1214 | 1210 | 7110462 | 735245 | 313247 | 63.16 | 6699 | 1 | 55 | 6 | 96.46 | 98.89 |
| **a.117s** | 1064 | 1062 | 7063849 | 735371 | 313249 | 63.14 | 6671 | 1 | 56 | 6 | 99.63 | 98.89 |
| **a.118** | 4510 | 4502 | 6938118 | 921370 | 284583 | 63.08 | 6550 | 1 | 45 | 3 | 25.73 | 100 |
| **a.121** | 1787 | 1782 | 7231555 | 875935 | 311710 | 63.74 | 6582 | 1 | 56 | 3 | 164.53 | 99.92 |
| **a.131** | 2281 | 2268 | 7697153 | 921336 | 277717 | 62.77 | 7000 | 1 | 56 | 6 | 141.03 | 99.51 |
| **a.132M** | 1416 | 1413 | 7029886 | 643976 | 319580 | 63.03 | 6561 | 1 | 60 | 7 | 129.82 | 98.89 |
| **a.135** | 2846 | 2834 | 8494907 | 1168222 | 366960 | 63.24 | 7700 | 1 | 56 | 3 | 119.71 | 99.51 |
| **a.138w** | 820 | 814 | 6769988 | 1456957 | 291302 | 63.21 | 6447 | 1 | 55 | 6 | 123.29 | 99.3 |
| **a.142** | 6320 | 6312 | 8657323 | 1295220 | 214201 | 63.03 | 7715 | 1 | 56 | 6 | 96.76 | 99.92 |
| **a.144s** | 1585 | 1578 | 7092673 | 854267 | 291141 | 63.18 | 6675 | 1 | 57 | 6 | 179.63 | 99.3 |
| **a.147** | 6000 | 5997 | 8413508 | 820636 | 241030 | 63.29 | 7234 | 1 | 61 | 6 | 124.06 | 98.89 |
| **a.150** | 2059 | 2052 | 7588744 | 953260 | 287429 | 62.71 | 6894 | 1 | 55 | 6 | 99.46 | 99.51 |
| **a.152** | 1532 | 1521 | 7191009 | 921214 | 264798 | 62.31 | 6588 | 1 | 57 | 7 | 167.1 | 99.51 |
| **a.15star** | 2059 | 2050 | 7144746 | 1150303 | 244695 | 61.98 | 6561 | 1 | 62 | 3 | 136.82 | 100 |
| **a.161** | 1302 | 1293 | 7487981 | 970906 | 255131 | 62.66 | 6949 | 1 | 53 | 6 | 108.58 | 99.51 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **a.164** | 1866 | 1857 | 8196813 | 1167021 | 455904 | 63.23 | 7549 | 1 | 57 | 3 | 133.84 | 99.51 |
| **a.16star** | 2299 | 2289 | 7582416 | 1324465 | 289843 | 62.92 | 6934 | 1 | 54 | 6 | 128.85 | 99.51 |
| **a.19** | 2546 | 2541 | 7149635 | 1015545 | 225276 | 63.84 | 6453 | 1 | 55 | 3 | 113.86 | 99.92 |
| **a.191** | 1384 | 1373 | 7211676 | 2209271 | 578338 | 63.3 | 4929 | 1 | 61 | 2 | 88.81 | 100 |
| **a.194star** | 1195 | 1191 | 7098566 | 751741 | 324693 | 63.18 | 6657 | 1 | 56 | 6 | 163.5 | 98.89 |
| **a.195** | 1937 | 1932 | 7471725 | 1329680 | 302980 | 63.68 | 6835 | 1 | 55 | 3 | 101.48 | 99.92 |
| **a.196**[3] | 3029 | 3018 | 7951155 | 820254 | 288135 | 60.77 | 6906 | 1 | 64 | 8 | 198.47 | 99.51 |
| **a.200M** | 2834 | 2830 | 7069259 | 949056 | 287270 | 63.12 | 6233 | 1 | 46 | 3 | 151.44 | 99.51 |
| **a.200s** | 1551 | 1547 | 6722008 | 948866 | 325169 | 63.15 | 6144 | 1 | 46 | 3 | 152.54 | 99.51 |
| **a.209** | 1997 | 1990 | 7249888 | 452674 | 176258 | 63.41 | 6620 | 1 | 58 | 6 | 189.19 | 99.51 |
| **a.210** | 2502 | 2500 | 7469203 | 635591 | 243205 | 63.19 | 6828 | 1 | 58 | 6 | 137.59 | 98.89 |
| **a.216** | 1679 | 1670 | 7270584 | 1666279 | 348787 | 62.84 | 6621 | 1 | 55 | 6 | 144.36 | 99.1 |
| **a.217D** | 2218 | 2205 | 7422653 | 1770260 | 348787 | 62.8 | 6692 | 1 | 54 | 6 | 167.17 | 99.1 |
| **a.218** | 2089 | 2082 | 7187408 | 941172 | 280180 | 63.54 | 6583 | 1 | 56 | 4 | 106.68 | 99.51 |
| **a.222** | 1629 | 1619 | 6979253 | 941349 | 274288 | 63.52 | 6417 | 1 | 55 | 3 | 168.42 | 99.51 |
| **a.231** | 1074 | 1071 | 7034693 | 743436 | 319580 | 63.07 | 6633 | 1 | 59 | 6 | 168.78 | 98.89 |
| **a.233** | 3699 | 3695 | 7186340 | 625835 | 321184 | 63.5 | 5822 | 1 | 45 | 2 | 96.06 | 100 |
| **a.23L** | 2727 | 2721 | 7468895 | 436429 | 196254 | 63.58 | 6745 | 1 | 62 | 6 | 175.93 | 99.51 |
| **a.23M** | 2156 | 2152 | 7274695 | 432201 | 179273 | 63.14 | 6605 | 1 | 60 | 3 | 150.98 | 99.51 |
| **a.25** | 1850 | 1843 | 7208685 | 436429 | 152605 | 63.44 | 6607 | 1 | 60 | 6 | 144.1 | 99.51 |
| **a.27** | 2108 | 2103 | 7484525 | 757574 | 250710 | 63.65 | 6764 | 1 | 55 | 3 | 132.65 | 99.92 |
| **a.30** | 1179 | 1172 | 7026760 | 426394 | 220555 | 63.46 | 6541 | 1 | 58 | 6 | 124.23 | 99.51 |
| **a.302star** | 1611 | 1605 | 7165423 | 426395 | 176258 | 63.5 | 6637 | 1 | 57 | 6 | 135.44 | 99.51 |
| **a.33M** | 1851 | 1848 | 7212807 | 413513 | 161359 | 63.48 | 6653 | 1 | 54 | 3 | 130.58 | 99.51 |
| **a.33s** | 1872 | 1866 | 7210912 | 627973 | 176258 | 63.49 | 6643 | 1 | 57 | 6 | 148.19 | 99.51 |
| **a.40L** | 1794 | 1785 | 7380344 | 1122257 | 315358 | 62.84 | 6746 | 1 | 53 | 6 | 141.2 | 99.51 |
| **a.40L2** | 1453 | 1445 | 7300060 | 585478 | 271974 | 62.82 | 6703 | 1 | 54 | 6 | 149.7 | 99.51 |
| **a.44** | 1732 | 1726 | 7383120 | 757577 | 302980 | 63.6 | 6738 | 1 | 55 | 3 | 177.39 | 99.92 |
| **a.45** | 3169 | 3164 | 7065542 | 1341504 | 285394 | 63.13 | 6279 | 1 | 47 | 3 | 165.97 | 99.51 |
| **a.452** | 1613 | 1603 | 7273178 | 585478 | 278781 | 62.75 | 6685 | 1 | 53 | 6 | 127.73 | 99.51 |
| **a.47star** | 1239 | 1236 | 7131275 | 726842 | 228164 | 63.83 | 6624 | 1 | 55 | 3 | 134.91 | 99.92 |
| **a.50** | 2429 | 2425 | 7367225 | 554300 | 220555 | 63.37 | 6673 | 1 | 57 | 6 | 177.44 | 99.51 |
| **a.55** | 1153 | 1141 | 7085503 | 921229 | 283306 | 62.89 | 6562 | 1 | 55 | 6 | 122.5 | 99.51 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **a.58** | 1454 | 1451 | 6822915 | 947591 | 324756 | 63.35 | 6392 | 1 | 55 | 6 | 125.33 | 98.89 |
| **a.6** | 1943 | 1933 | 7310798 | 759030 | 349269 | 62.98 | 6673 | 1 | 53 | 7 | 106.06 | 99.51 |
| **a.63** | 1967 | 1957 | 7465318 | 1324465 | 289844 | 62.59 | 6806 | 1 | 55 | 7 | 146.31 | 99.51 |
| **a.64** | 3934 | 3925 | 8165179 | 677664 | 221917 | 63.3 | 7267 | 1 | 55 | 6 | 303.18 | 99.51 |
| **a.66** | 1068 | 1061 | 6990836 | 554267 | 199154 | 63.51 | 6542 | 1 | 57 | 6 | 148.73 | 99.51 |
| **a.71** | 1803 | 1800 | 7226236 | 702235 | 311711 | 63.63 | 6536 | 1 | 60 | 3 | 233.65 | 99.92 |
| **a.72** | 3071 | 3066 | 7557658 | 426395 | 176258 | 63.63 | 6783 | 1 | 63 | 6 | 169.42 | 99.51 |
| **a.83** | 3516 | 3511 | 8029333 | 1054677 | 393751 | 62.91 | 7093 | 1 | 50 | 3 | 149.67 | 99.51 |
| **a.89** | 1646 | 1642 | 7204654 | 667198 | 224722 | 63.49 | 6552 | 1 | 59 | 3 | 158.09 | 99.92 |
| **a.8star** | 1494 | 1492 | 7195098 | 795642 | 266541 | 63.53 | 6654 | 1 | 58 | 3 | 135.87 | 99.92 |
| **a.90** | 2792 | 2777 | 7727715 | 533487 | 142846 | 62.18 | 7119 | 1 | 53 | 3 | 206.64 | 99.51 |
| **a.92** | 2664 | 2653 | 7369142 | 1296248 | 254722 | 62.31 | 6675 | 1 | 58 | 6 | 159.08 | 99.92 |
| **a.96** | 1304 | 1299 | 7373741 | 1035724 | 449143 | 62.79 | 6776 | 1 | 49 | 3 | 130.94 | 99.51 |
| **a.AR1** | 1270 | 1261 | 7515467 | 1201246 | 568373 | 63.28 | 6909 | 1 | 57 | 3 | 151.3 | 99.51 |
| **a.29L2** | 1644 | 1642 | 6841901 | 735635 | 73984 | 63.2 | 6546 | 1 | 52 | 3 | 87.98 | 100 |
| **a.AR35** | 1746 | 1741 | 7157533 | 821161 | 390740 | 63.23 | 6632 | 1 | 56 | 6 | 207.96 | 98.89 |
| **a.AR352** | 1636 | 1632 | 7082887 | 1686719 | 324756 | 63.26 | 6600 | 1 | 52 | 3 | 172.8 | 98.89 |
| **a.AR35star** | 1470 | 1466 | 7511547 | 820730 | 319583 | 62.99 | 7139 | 1 | 62 | 6 | 153.69 | 98.89 |
| **a.AR39** | 885 | 880 | 6887721 | 1289331 | 321678 | 63.26 | 6510 | 1 | 55 | 6 | 143.36 | 98.89 |
| **a.AR452** | 1080 | 1076 | 6747552 | 541928 | 319580 | 63.36 | 6391 | 1 | 56 | 6 | 140.68 | 98.89 |
| **a. AR45s** | 947 | 943 | 7041894 | 743303 | 352673 | 63.16 | 6678 | 1 | 57 | 6 | 166.87 | 98.89 |
| **a. AR49** | 1208 | 1205 | 7044692 | 1182039 | 324607 | 63.2 | 6588 | 1 | 57 | 6 | 123.99 | 98.89 |
| **a.AR6** | 1705 | 1695 | 6999282 | 941349 | 274288 | 63.56 | 6428 | 1 | 57 | 3 | 167.51 | 99.51 |
| **a.AR61** | 1242 | 1238 | 7268500 | 743303 | 214140 | 63.12 | 6952 | 1 | 61 | 6 | 161.04 | 98.89 |
| **a.star16** | 1265 | 1261 | 7052204 | 821164 | 307960 | 63.15 | 6553 | 1 | 56 | 6 | 101.76 | 98.89 |
| **a.AR7** | 2384 | 2376 | 7068262 | 940869 | 274288 | 63.47 | 6381 | 1 | 56 | 3 | 165.17 | 99.51 |
| **Average** | 2078 | 2071 | 7349737 | 889102 | 282018 | 63 | 6725 | 1 | 56 | 5 | 143 | 99 |

Appendix 6. *Mesorhizobium* strains able to tolerate at low (pH 5) and solublizing insoluble tri-calcium phosphate on sold medium isolated from acidic soil of Ethiopia

| *Mesorhizobium* strains | Soil pH of isolation site | Phosphate solublization activity (S.I.) |
|---|---|---|
| a.161 | 5.7 | 1.7 |
| a.104 | 5.3 | 2.3 |
| a.16star | 5 | 2.7 |
| a.217D | 5.7 | 1.9 |
| a.118 | 5.1 | 1.8 |
| a.40L2 | 5.9 | 2.5 |
| $a.45_2$ | 5 | 1.7 |
| a.150 | 4.6 | 2.5 |
| a.147 | 5.7 | 1.8 |
| a.210 | 5.9 | 1.5 |
| a.231 | 5.4 | 2.3 |
| a.58 | 5.1 | 2.3 |
| a.144s | 4.7 | 2.8 |
| a.29L2 | 5.3 | 1.5 |
| a.AR352 | 4.8 | 2.2 |
| a.AR45s | 4.7 | 2 |
| a.AR452 | 5 | 2.3 |
| a.AR35s | 4.8 | 2.7 |
| a.138w | 5.1 | 3.1 |
| a.117M | 5.1 | 1.7 |
| a.AR49 | 5.2 | 1.8 |
| a.star16 | 5.5 | 2.1 |
| a.117L2 | 4.9 | 3 |
| a. 117s | 5.2 | 1.7 |
| a.117 | 5.3 | 1.7 |
| a.AR1 | 5.9 | 1.8 |
| a.64 | 5.3 | 2.9 |
| a.135 | 5.4 | 2 |
| a.8star | 5.2 | 2.9 |
| $a.195_3$ | 4.9 | 1.8 |
| a.111 | 5.5 | 2.7 |
| a.71 | 5.1 | 3 |
| a.27 | 5.6 | 1.9 |
| a.89 | 5.1 | 2.5 |
| a.209 | 5.8 | 1.9 |
| $a.30_2$star | 5.4 | 2.5 |
| a.33M | 5.3 | 2.1 |
| a.25 | 4.7 | 2.1 |
| a.66 | 5.9 | 2.5 |
| a.23L | 5.4 | 2.2 |
| a.116 | 5.9 | 1.7 |
| a.222 | 5.2 | 2.5 |
| a.200M | 4.4 | 1.9 |
| a.200s | 5.2 | 1.3 |

| | | |
|---|---|---|
| a.152 | 5.2 | 2.2 |
| a.55 | 5.1 | 2 |
| a.90 | 4.9 | 2.5 |
| a.15star | 5.7 | - |
| a.AR7 | 5.3 | - |
| a.AR39 | 6 | - |
| a.47s | 6.1 | - |
| a.40L | 6.1 | - |
| a.233 | 5.2 | - |
| a.164 | 6.2 | - |
| a.131 | 6.2 | - |
| a.83 | 6 | - |
| a.63 | 6.1 | - |
| a.50 | 4.6 | - |
| a.44 | 6.1 | - |
| a.23M | 6.1 | - |
| a.216 | 5 | - |
| a.194star | 6.2 | - |

-, no solublization

Appendix 7. Eco-physiological characteristics, Intrinsic Antibiotic resistance (IAR), Heavy metals tolerance, Carbon and nitrogen utilization pattern of chickpea nodulating mesorhizobia strains

| Mesorhizobia strains | Low pH tolerance | | Temprature tolerance | NaCl % tolerance | Intrinsic antibiotics resistance (IAR) | Carbohydrate utilization | | Heavy metals resistance |
|---|---|---|---|---|---|---|---|---|
| | pH 4 | pH 4.5 | | | | C-sources% utilized | N-sources% utilized | |
| a.150 | - | - | 20-40 | 2 | Kan, Amp, Chl, Nal | 41.6 | 50 | - |
| a.216 | - | - | 20- 37 | 2 | Amp, Chl, Er, Nal | 75 | 66.6 | Cr |
| a. 217D | - | - | 20 - 37 | 2 | Amp, Chl, Er, Nal | 41.6 | 58.3 | Cu |
| a.40L | - | - | 20 -37 | 3 | Amp, Chl, Er, Nal | 66.6 | 50 | Cu, Cr |
| a. 131 | - | - | 20 - 40 | 3 | Amp, Chl, Er, Nal | 66.6 | 50 | Zn |
| a. 233 | - | - | 20 - 37 | 2 | Amp, Chl, Er, Nal | 66.6 | 66.6 | Zn, Cr |
| a. 33M | - | - | 20 - 40 | 2 | Amp, Chl, Er, Nal | 41.6 | 75 | Zn |
| a. 23M | - | - | 20 -37 | 2 | Amp, Chl, Nal | 75 | 50 | - |
| a.50 | - | - | 20 - 37 | 1 | Amp, Chl, Er,Nal | 33.3 | 75 | Cr |
| a.209 | - | - | 20 - 40 | 1 | Amp, Chl, Er, Nal | 66.6 | 66.6 | Cu |
| a. 23L | - | - | 20 -37 | 2 | Amp, Chl, Er,Nal | 58.3 | 50 | Zn, Cr |
| a.25 | - | - | 20 - 40 | 1 | Amp, Chl, Er,Nal | 41.6 | 66.6 | - |
| a. 116 | - | - | 20 -37 | 2 | Amp, Chl, Er,Nal | 66.6 | 50 | - |
| a.195 3 | - | - | 20 - 37 | 1 | Neo, Amp, Chl, Nal | 33.3 | 75 | Zn |
| a. 27 | - | - | 20 - 37 | 2 | Amp, Chl, Er,Nal | 41.6 | 50 | Cu, |
| a. 47star | - | - | 20 - 37 | 2 | Amp, Chl, Er, Nal | 58.3 | 66.6 | Zn |
| a. 44 | - | - | 20 - 40 | 2 | Amp, Chl, Er, Nal | 41.6 | 58.3 | Cr |
| a. 231 | - | - | 20 -37 | 1 | Amp, Chl, Er Nal | 33.3 | 50 | - |
| a. 58 | - | - | 20 - 40 | 2 | Amp, Chl, Er,Nal | 33.3 | 66.6 | - |
| a. AR49 2 | - | - | 20 -37 | 3 | Amp, Chl, Er,Nal | 66.6 | 50 | - |
| a. AR45 s | - | - | 20 -40 | 1 | Amp, Chl, Er,Nal | 66.6 | 66.6 | Cu |
| a. 147 | - | - | 20- 40 | 1 | Amp, Chl, Er,Nal | 33.3 | 50 | Zn |
| a.117M | - | - | 20- 37 | 2 | Amp, Chl, Er,Nal | 75 | 66.6 | Mn |
| a.star16 | - | - | 20 -37 | 1 | Amp, Chl, Er, Nal | 75 | 50 | Cr |
| a.117s | - | - | 20 - 37 | 2 | Amp, Chl, Er, Nal | 66.6 | 66.6 | Cu, Zn |
| a.194star | - | - | 20 - 40 | 2 | Amp, Chl, Er, Nal | 66.6 | 50 | Cu, Cr |
| a.117 | - | - | 20 - 40 | 1 | Amp, Chl, Er, Nal | 33.3 | 75 | Cr |
| a.AR352 | - | - | 20 -37 | 2 | Amp, Chl, Er, Nal | 41 | 66.6 | Cu, Zn, Cr |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| a.AR39 | - | - | 20 - 37 | 5 | Amp, Chl, Er, Nal | 50 | 58.3 | - |
| a.135 | - | - | 20 - 37 | 1 | Amp, Chl, Er, Nal | 41.6 | 66.6 | - |
| a.142 | - | - | 20 - 40 | 4 | Kan, Amp, Chl, Er, Nal | 58.3 | 58.3 | - |
| a.118 | - | - | 20 - 37 | 4 | Amp, Chl, Er, Nal | 66.6 | 50 | Zn, Cr |
| a.218 | - | - | 20 - 40 | 2 | Kan, Amp, Chl, Er, Nal | 58.3 | 75 | - |
| a.164 | - | - | 20 - 37 | 2 | Amp, Chl, Er, Nal | 41.6 | 50 | Cu, Zn |
| a.29L2 | - | - | 20 - 37 | 4 | Amp, Chl, Er, Nal | 66.6 | 75 | Cu, Cr |
| a.128 | - | - | 20 - 40 | 3 | Amp, Chl, Er, Nal | 66.6 | 50 | Cu, Cr |

Appendix 8. Rating some soil properties

**A.** Rating for soil pH (Jones, 2003)

| pH | Rating |
|---|---|
| <5 | Strongly acidic |
| 5.5 to 5.1 | Moderately acdic |
| 5.6 to 6 | Slightly acidic |
| 6 to 7 | Neutral |

**B.** Rating for soil OM, total N, and available P

| OC (%)* | Total N (%) ** | available P (mg $kg^{-1}$)*** | Rating |
|---|---|---|---|
| <0.40 | ----- | ----- | Exteremely low |
| 0.40-0.60 | <0.05 | ----- | Very low |
| 0.60-1.00 | 0.05-0.15 | <20 | Low |
| 1.00-1.80 | 0.15-0.25 | 20-40 | Moderate |
| 1.80-3.00 | 0.25-0.50 | 40-100 | High |
| >3.00 | >0.5 | >100 | Very High |
| ---- | ----- | ---- | Extremely high |

Source: Landon, (2014) *, Hazelton and Murphy, (2007) **, Horneck *et al*. (2011) ****

Appendix 9. Some sample on nodule collection and rhizobia isolation

Chickpea root nodule collection

Rhizobia isolation

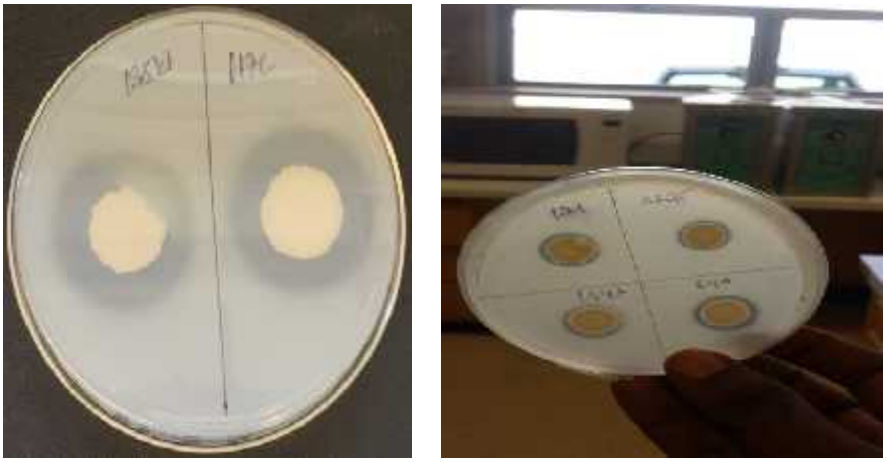
Appendix 10. Some sample of green house expiriments on sand and soil cultures




Green house expiriment on sand culture at DZARC, Debrezite

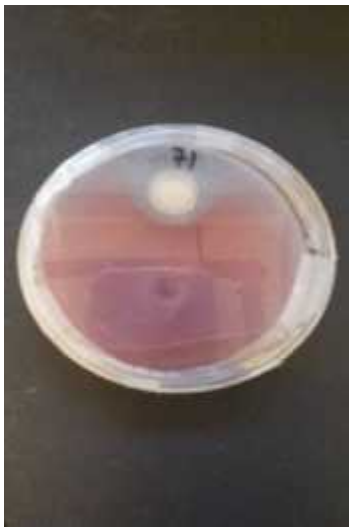

Green house expiriment on soil culture at Addis Ababa University

Appendix 11. Some sample *in vitro* plant growth promoting traits

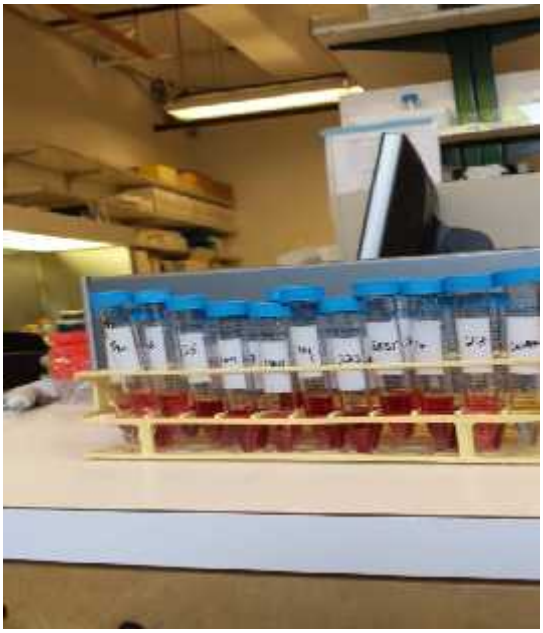


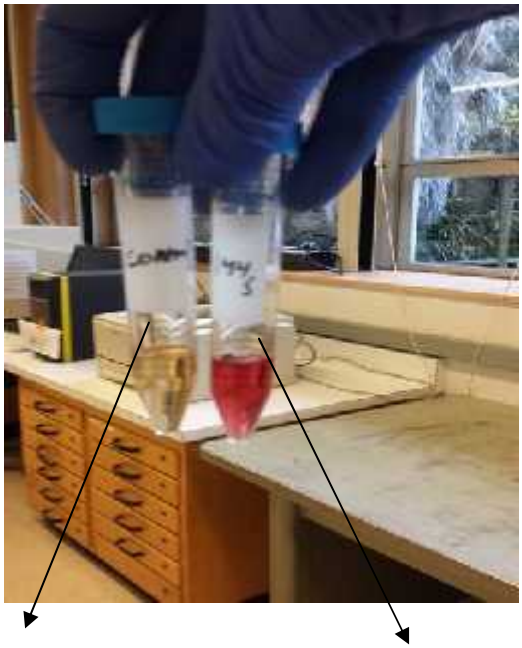
Phosphate solublization on tri-calcium phosphate solid medium

Phosphate solublization on Tri-calcium phosphate, aluminum phosphate and ferrous phosphate in liquid medium



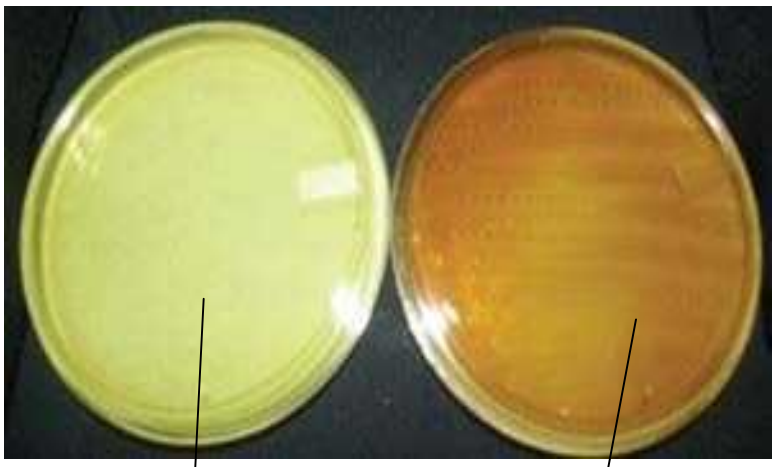
In vitro mycelia growth inhibition activity of *Mesorhizobium* against *Fusarium oxysporium ciceri*

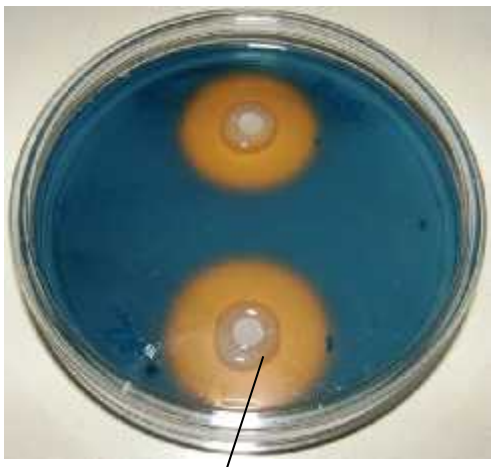




Negative control Positive for IAA production

IAA production from *Mesorhizobium* isolates





Positive for sidrophore production

Negative control Positive for HCN production Siderophore production from a.117L2

Hydrogen cyanide production from a.117L2

Appendix 12. Some sample of field expiriments

Land preparation

Lignite (Carrier) based *Mesorhizobium* Inoculants

Preparation of seed with *Mesorhizobium* strain


Planting


Field experiment at Sodo (Endebyo) and Holleta


Root nodule at 50% of flowering stage

Harvesting at Sodo (Endebyuo)