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

Diversity, symbiotic and plant growth promoting properties of rhizobia and rhizobacteria of grass pea (*Lathyrus sativus* L.) from central Ethiopia: implication to the selection and use of microbial inoculants in low input agriculture in Ethiopia

A Thesis submitted to the School of Graduate Studies of the Addis Ababa University in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biology (Applied Microbiology)

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April 26, 2018

Addis Ababa, Ethiopia

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

Declaration

This is to certify that the Dissertation prepared by Mussa Adal Mohammed, entitled "**Diversity, symbiotic and plant growth promoting properties of rhizobia and rhizobacteria of grass pea (*Lathyrus sativus* L.) from central Ethiopia: implication to the selection and use of microbial inoculants in low input agriculture in Ethiopia"** and submitted in fulfillment of the Requirements for the Degree of Doctor of Philosophy (Biology: Applied Microbiology) complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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Addis Ababa, Ethiopia

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Approval of dissertation by examining board

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Supervisor_________________________________Signature________________Date_________

____

Chair of Department

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Table of contents

Contents **page**

Acknowledgment…………………………………………………………………………………i
Table of contents……………………………………………………………………………....iii
List of tables…………………………………………………………………………………...ix
List of figures………………………………………………………………………………….xi
List of appendices……………………………………………………………………………..xii
List of Abbreviations…………………………………………………………………………xiii
Abstract…………………………………………………………………………………………xiv
Chapter 1. Introduction………………………………………………………………………1
    1.1 General introduction……………………………………………………………………..1
    1.2 Objectives of the study……………………………………………………………………...7
        1.2.1 General objective ………………………………………………………………………..7
        1.2.2 Specific objectives ………………………………………………………………….……7
    1.3. Literature Review…………………………………………………………………………..8
        1.3.1 Grass pea ………………………………………………………………………………..8
        1.3.2 Definition and taxonomy of rhizobia: Overview ……………………………………10
        1.3.2.1 Rhizobial taxonomy ………………………………………………………………..10
        1.3.2.2 History of rhizobial taxonomy ……………………………………………………..12
        1.3.2.3 Methods of studing rhizobial taxonomy …………………………………………...13
        1.3.2.4 Review on rhizobia nodulating grass pea ………………………………………….14
        1.3.2.5 Rhizobia- legume symbiosis ……………………………………………………….14
        1.3.2.6 Rhizobia- non-legume symbiosis …………………………………………………..15
        1.3.3 Rhizosphere …………………………………………………………………………16
        1.3.4 Plant Growth Promoting Rhizobacteria (PGPR) …………………………………...17
        1.3.5 Rhizobacteria …………………………………………………………………………19
        1.3.6 Some PGPR works in Ethiopia ……………………………………………………19
        1.3.7 Mechanisms of PGPR ………………………………………………………………20

1.3.7.1 Plant growth promoting mechanisms ……………………………………...............20
1.3.7.2 Biocontrolling mechanisms ……………………………………………………..23
1.3.8 Biological Nitrogen Fixation (BNF) …………………………………………………24
1.3.9 Ecological factors affecting BNF ……………………………………………………26
1.3.9.1 Temperature ……………………………………………………………………..27
1.3.9.2 Soil pH ……………………………………………………………………………..28
1.3.9.3 Salinity ……………………………………………………………………………..29
1.3.9.4 Antibiotics and heavy metals ……………………………………………………30
1.3.9.5 Carbohydrate and amino acid utilization ………………………………………..31
1.3.10 Microbial inoculation and co-inoculation ………………………………………..31
Chapter 2. Diversity of ecologically competent and symbiotically effective grass pea rhizobia to enhance nitrogen fixation for grass pea (*Lathyrus sativus* L.) and other cross-inoculating cool season legumes in Ethiopia…………………33
Abstract……………………………………………………………………………………….33
2.1 Introduction……………………………………………………………………………..34
2.2. Materials and Methods…………………………………………………………………36
2.2.1 Sample collection and isolation of rhizobia ……………………………………36
2.2.2 Isolation and purification of rhizobia ……………………………………………37
2.2.3 Cultural characterization ……………………………………………………………40
2.2.3.1 Experimental set up ……………………………………………………………...40
2.2.3.2 Colony characteristics …………………………………………………………...40
2.2.3.3 Growth rate determination (GMT) ……………………………………….......40
2.2.4 Presumptive test for root nodule bacteria …………………………………………41
2.2.4.1 Growth on YEMA-CR ……………………………………………………………..41
2.2.4.2 Gram reaction ……………………………………………………………………....41
2.2.4.3 Growth on Glucose Peptone Agar (GPA) medium ……………………………...41
2.2.5 Authentication of the isolates and preliminary screening of their symbiotic effectiveness on sand experiment ………………………………………………………41
2.2.6 Relative symbiotic effectiveness of the rhizobial isolates ……………………………42

2.2.7 Physiological characterization ……………………………………………………42
2.2.7.1 pH, temperature and salt tolerance ………………………………………….......43
2.2.7.2 Inherent antibiotic resistance (IAR) and heavy metal (HM) of isolates ………......43
2.2.7.3 Carbohydrate and amino acid utilization ……………………………………….43
2.2.8 Numerical analysis and construction of dendrogram ……………………………44
2.2.9 Genetic Characterization ……………………………………………………………44
2.2.9.1 PCR method of amplification …………………………………………………..44
2.2.9.2 Gel electrophoresis and Extraction of PCR products ……………………………...45
2.2.9.3 Sequencing and phylogentic analysis of 16S rRNA and nif-H nigenes …………...45
2.2.10 Data analysis…………….. ………………………………………………………46
2.3 Result and Discussion …………………………………………………………….....46
2.3.1 Isolation and purification of the isolates ………………………………………….46
2.3.2 Presumptive tests of the isolates ……………………………………………………46
2.3.3 Cultural characterization and growth rate of the isolates ……………………………46
2.3.4 Symbiotic effectiveness of the isolates on sand culture ……………………………...49
2.3.5 Pattern of ecological tolerance of isolates …………………………………………53
2.3.5.1 Tolerance to salt, pH and temperature …………………………………………..54
2.3.5.2 Intrinsic antibiotic and heavy metal resistance ………………………………….55
2.3.5.3 Pattern of carbohydrate and amino acid utilization of isolates……………………..56
2.3.6 Numerical analysis…………………………………………………………………58
2.3.7 Molecular characterization…………………………………………………………59
2.3.7.1 Sequencing of 16S rRNA and nif-H genes………………………………………59
2.3.7.2 Phylogentic relations of the Rhizobium strains for 16S rRNA and nif-H genes…...60
Chapter 3. Diversity and plant growth promoting properties of rhizobia and rhizobacteria isolated from the nodules and rhizosphere of Grasspea (*Lathyrus sativus* L.) growing areas in central Ethiopia**…………………………...63**
Abstract…………………………………………………………………………………63
3.1 Introduction……………………………………………………………………………..65
3.2 Materials and Methods…………………………………………………………………67

3.2.1.Selected rhizobial and rhizobacterial isolates ……………………………………..........67
3.2.2 Selection and activation of *Rhizobium* strains ……………………………………68
3.2.3 Sources of rhizosphere bacteria ………………………………………………69
3.2.4 Designation and preservation of purified rhizobacterial isolates ……………………69
3.2.5 Characterization of the isolates………………………...69
3.2.5.1 Gram reaction for rhizobacterial isolates ……………………………………69
3.2.5.2 *In vitro* screening of the isolates for plant growth promotion ……………………70
3.2.5.2.1 Phosphate solubilising characteristics ……………………………………………70
3.2.5.2.2 Indole-acetic-acid (IAA) Production …………………………………………….71
3.2.5.2.3 Nitrogen fixation …………………………………………………………………71
3.2.6 *In vitro* screening of the *Rhizobium* isolates for their biocontrol traits ……………72
3.2.6.1 In vitro Antifungal activity ……………………………………………………...72
3.2.6.2 Chitinase Production ……………………………………………………………72
3.2.6.3 Cellulase activity determination ………………………………………………...73
3.2.6.4 Protease Production ……………………………………………………………..73
3.2.6.5 Cyanide (HCN) Production ……………………………………………………..73
3.2.6.6 Ammonia (NH_3) Production ……………………………………………………74
3.2.7 Growth response of selected rhizobial isolates on potted soil under greenhouse conditions ………………………………………………………………………74
3.2.7.1 Microbial inoculants ……………………………………………………………74
3.2.7.2 Soil analysis and Estimation of rhizobial population (MPN) ……………………74
3.2.7.3 Performance evaluation of selected isolates on potted soil ……………………75
3.2.8 Identification of rhizobacterial isolates using 16S rRNA ……………………………76
3.2.8.1 DNA extraction and amplification using colony PCR ……………………………76
3.2.8.2 Purification of PCR products ……………………………………………………76
3.2.8.3 Sequencing of 16S rRNA genes and phylogentic analysis ……………………76
3.2.9 Data analysis ……………………………………………………………………77
3.3 Result and Discussion…………………………………………………………………..77
3.3.1 Gram reaction for rhizobacterial isolates ………………………………………………77

3.3.2 *In vitro* characterization of bacterial isolates for plant growth promotion……………......77
3.3.2.1 Phosphate solubilization by rhizobial isolates ……………………………………77
3.3.2.2 Phosphate solubilization by rhizobacterial isolates ………………………………..79
3.3.2.3 IAA production by rhizobial isolates ………………………………………………80
3.3.2.4 IAA production by rhizobacterial isolates ………………………………………….82
3.3.2.5 Nitrogen fixation by rhizobacterial isolates ………………………………………..82
3.3.3. Antifungal activity of the isolates…………………………………………………83
3.3.3.1 In vitro antifungal activity of rhizobial isolates ……………………………………83
3.3.3.2 Hydrolytic enzyme production …………………………………………………….85
3.3.3.3 Hydrogen cyanide and ammonia production……………………………………….85
3.3.3.4 Antifungal activity of the rhizobacterial isolates …………………………………..87
3.3.3.4.1 Dual culture antifungal test ………………………………………………………88
3.3.3.4.2 Hydrolytic enzyme production test ………………………………………………88
3.3.3.4.3 Hydrogen cyanide and ammonia production test ………………………………...88
3. 3.4 Growth response of selected rhizobial isolates on potted soil under conditions ……91
3. 3.4. 1 Response of the isolates to the root and shoot growth of grass pea ………………91
3.3.5 Molecular characterization…………………………………………………………95
3.3.5.1 PCR amplification and gel visualization of 16S rRNA and nif-H genes…………...95
3.3.5.2 Extraction of PCR products and sequencing of 16S rRNA gene…………………...96
3.3.5.3 Phylogenetic analysis of 16S rRNA gene…………………………………………..98
Chpater 4. The effect of inoculation and co-inoculation of rhizobia and rhizobacteria on growth and symbiotic effectiveness response of grass pea under greenhouse condition……………………………………………………...101
Abstract…………………………………………………………………………………..101
4.1 Introduction…………………………………………………………………………102
4.2 Materials and Methods……………………………………………………………...104
4.2.1 Sources of inoculant ………………………………………………………..104
4.2.2 Soil analysis and determination of most probable number of rhizobia (MPN……...104
4.2.3 Inoculant preparation………………………………………………………..106

4.2.4 Evaluating the compatibility of the isolates…………………………………………106
4.2.5 Treatments and experimental design ……………………………………………..106
4.2.6 Preparation of the pot soil experiment ……………………………………………..107
4.2.7 Cultivation and harvest ……………………………………………………………..107
4.2.8 Statistical analysis…………………………………………………………………...108
4.3 Result and Discussion……………………………………………………………………108
4.3.1 Compatibility test ……………………………………………………………………108
4.3.2 Evaluation of nodulation and growth parameters under greenhouse conditions……108
4.3.2.1 Effect of inoculation and co-inoculation on nodulation ………………………….108
4.3.2.2 Effect of inoculation and co-inoculation on shoot growth and symbiotic effectiveness ………………………………………………………………………...113
Chapter 5. General conclusion and recommendations …………………………………………118
5.1 General conclusion ……………………………………………………………………………118
5.2. General recommendation ……………………………………………………………………….119
6. References ……………………………………………………………………………………...121
7. Appendices ……………………………………………………………………………………..156

List of tables

Tables Page

Table 2.1. Sampling sites for the collection of soil and nodule samples of grass pea

from central parts of Ethiopia …………………………………………………………...39

Table 2.2 Primers used for PCR amplification of 16S rRNA and nif-H genes ………………...45

Table 2.3 Cultural characteristics of representative isolates of grass pea grown on YEMA…….48

Table 2.4 Authentication of the isolates under greenhouse conditions …………………………50

Table 2.5 Pattern of symbiotic effectiveness of *Rhizobium*species of cross inoculating
hosts of the viciae family ………………………………………………………………..52

Fig. 2. 6 Ecophysiological tolerance and nutrional versatility of highly effective grass pea
isolates grown on YEMA/YEMB medium ……………………………………………...53

Table 2. 7 Pattern of ecophysiological tolerance of *Rhizobium* species of cross inoculating
hosts of the viciae family …………………………………………………………………57

Table 3. 1 Sampling sites for the selected rhizobial and rhizobacterial isolates ………………..68

Table 3. 2 The soil chemical analysis and rhizobial determination using MPN ………………..74

Table 3. 3. The plant growth promoting traits of selected *Rhizobium* strains …………………..78

Table 3. 4 PGP properties of grass pea rhizobacterial isolates under *in vitro* conditions………81

Table 3. 5 Antagonistc in vitro properties of grass pea rhizobia against *Fusarium*
oxysporum …………………………………………………………………………………..84

Table 3. 6 Antagonistc in vitro properties of grass pea rhizobacteria against *Fussarium*
oxysporum …………………………………………………………………………………..87

Table 3. 7 Multiple growth promoting and biocontrolling traits of the rhizobacterial isolates….90

Table 3.8 Performance of selected *Rhizobium* isolates on potted soil under

greenhouse conditions ……………………………………………………………...92

Table 3. 8 16S rRNA gene partial sequencing of selected rhizobacterial isolates of grass
pea …………………………………………………………………………………96

Table 4. 1 Plant growth promotion and biocontrol properties of selected

Rhizobium and rhizobacterial strains ……………………………………………..105

Table 4. 2 The effect of inoculation and co-inoculation with four *Rhizobium* sp. and two *E. casseliflavus/gallinarum* on nodulation and symbiotic properties of wasse variety grown for 60 days under greenhouse conditions …………….........................111

List of figures

Figure Page

Figure 1.1 Root nodulating proteobacteria ……………………………………………………..12

Figure 1.2 The role of nitrogenase enzyme in nitrogen fixation ………………………………..25

Figure 2. 1 Map of the study sites ………………………………………………………….....37

Figure 2.2 Dendrogram highlighting the phenotypic diversity of grass pea rhizobial isolates …58

Figure 2.3 Phylogenetic tree constructed based on 16S rRNA gene sequences…………………61

Figure 2.4 Phylogenetic tree constructed based on nif-H gene sequences………………………62

Figure 3.1 Phylogenetic tree of 16S rRNA gene of selected rhizobacteria ……………………..99

List of appendices

Appendices page

Appendix 1. Correlation matrix of the rhizobia on sand culture ………………………………156
Appendix 2. Correlation matrix of selected rhizobia on potted soil different symbiotic parameters ……………………………………………….156
Appendix 3. Correlation matrix of inoculation and coinoculation of rhizobia and rhizobacteria …………………………………………………157
Appendix 4. Greenhouse authentication of the isolates on sand culture (partial view) ………..157
Appendix. 5. Selected authenticated isolates on sand culture (AAUGR-11, 14, 15, 50)............158
Appendix 6. Nodulation of authenticated isolates (AAUGR-39 & 45) ……………………….159
Appendix 7. PCR amplifications of 16S rRNA and nif-H genes ……………………………..159
Appendix 8. Phosphate solubilization index (PSI) of selected rhizobia ……………………….160
Appendix 9. IAA production by rhizobia ……………………………………………………...160
Appendix 10. HCN and NH_3 production by selected rhizobia ………………………………...161
Appendix 11. Phosphate solubilization index (PSI) and IAA production of rhizobacteria ……161
Appendix 12. Antifungal activity of rhizobacteria …………………………………………….162
Appendix 13. Other biocontrol activity of rhizobacteria ………………………………………163
Appendix-14. Performance of selected rhizobial isolates on potted soil under greenhouse…...163
Appendix 15. Compatibility of selected rhizobia and rhizobacteria …………………………...164
Appendix 16. Greenhouse inoculation and co-inoculation experiments ………………………164

List of Abbreviations

BLAST = Basic local alignment search tool

BNF = Biological nitrogen fixation

BTB = Bromothymol blue

DNA = Deoxyribonucleic acid

gl^{-1}= Gram per litre

IAA = Indole-3 acetic acid

MEGA6 = Molecular evolutionary genetic analysis version 6

MLSA = Multilocus sequence analysis

NCBI = National Center for Biotechnology Information (US)

NDW = Nodule dry weight

NN = Nodule number

NTSYS21 = Numerical taxonomic system version 21

PDA = Potato dextrose agar

PGP = Plant growth promoting

PGPR = Plant growth promoting rhizobacteria

rpm = revolutions per minute

PVK = Pikovskaya medium

RNA = Ribonucleic acid

rRNA = Ribosomal RNA

SDW = Shoot dry weight

SE = Symbiotic effectiveness

SL = Shoot length

TCP = Tri-calcium phosphate

UPGMA = Unweighed pair group method with arithmetic mean

YEMA = Yeast extract mannitol agar

YEMB = Yeast extract mannitol broth

µgml^{-1} = microgram per milliliter

Abstract

Grass pea (*Lathyrus sativus* L.) is widely cultivated for food and feed in some developing countries including Ethiopia. However, its alkaloid content did not attract attention to the research community for its over-exaggerated neuro-lathyrism causing paralysis of limbs making it as one of the most neglected orphan crops in the world. However, the crop is considered as an insurance crop by resource-poor farmers for it produces reliable yields when all other crops fail. The increase in production in several areas in Ethiopia presupposes that it is an important source of protein to the diet of the population and makes it the fifth most important pulse crops in Ethiopia after faba bean, chickpea, field pea and haricot bean in terms of production. This indicates the necessity of looking beyond the prejudices held on the crop for long to expand the frontier of food security and soil fertility against the changing climate in the country. To this end, a study was made to characterize and select rhizobial and rhizobacteria from grass pea from central Ethiopia using standard methods. Isolates were tested for their phenotypic, symbiotic, genetic, *in vitro* stress tolerance, variations in substrate utilization and PGP properties within the context of their taxonomic characteristics and selection of tolerant isolates for field applications. Based on their preliminary symbiotic effectiveness, stress tolerance (pH tolerance, intrinsic antibiotic resistance (IAR)), PGP (phosphate solubilization, indole acetic acid production, etc), and antagonistic properties (suppression of fungal pathogen, etc), four rhizobial and two rhizobacteria isolates were selected and tested for single and co-inoculation trials of the crop on soil culture under greenhouse conditions. The data showed that all but one rhizobium isolates (49 isolates) were authenticated as root nodule bacteria (renodulating the host variety upon re-inoculation) and 86% of the isolates accumulated more than 50% of the shoot dry weight (SDW) (0.273-1.148g/p in relation to N-fetilized control plants indicator of symbiotic effectiveness (SE%) in nitrogen fixation. The inoculated plants also showed variations in nodule number (NN) ranging from 17.7-116/plant, Nodule dry weight (NDW) 0.011g-0.098/plant indicating a seven-fold and ten-fold difference among the isolates. Among the highly symbiotic effective isolates (SDW of >80%), isolates AAUGR- 2, 5, 6, 9, 11, 14, 15, 19, 20, 24, 30 and 50 acquired a wide range of substrate utilization and ecological tolerance under *in vitro*-conditions and the

majority of these isolates showed multiple plant growth promoting properties (MPGP) (4-8) ranging from good potential of phosphate solubilization and suppression of fungal pathogens. The PCR amplification of 16SRNA and nif-H genes showed that the majority of the isolates (87%) showed 99% sequence homology with *Rhizobium* spp. obtained from NCBI gene data base and a few isolates (AAUGR-2, AAUGR-24) were grouped into *Ensifer meliloti* and *Rhizobium leguminosarum* var viceae indicating that grass pea rhizobia were more diverse than the hitherto established cross-nodulation grouping to *Rhizobium leguminosarum* var viceae. All the selected rhizobial isolates were highly significant (P=0.000) in their ability to solubilize TCP with zones of solubilisation (solubilization index; SI) ranging from 1.24- 3.37 cm and the capacity of producing organic acids within the range of 256- 418 μg/ml. The highest amount of IAA (74.69±1.72 μg/ml) was produced by *Rhizobium* species (AAUGR-14) followed by 74.18±1.95 μg/ml produced by AAUGR-30. Regarding the antagonistic properties, all isolates produced catalase and ammonia whereas, a number of isolates were positive for chitinase, protease, cellulase, HCN and *in vitro* fungal inhibition assay. Among a total of 100 isolates of rhizobacteria, 39% were phosphate solubilizers of which 22 isolates that showed SI of greater than 2.4 cm were selected for further screening process for multiple growth promoting properties (MPGP). The 16S rRNA gene sequence analysis of five rhizobacterial isolates, AAUGPR- 38, 53, 73, 91 and 92 showed 94- 99 % identity to different rhizobacterial genera including *Enterobacter, Enterococcus, Kluyvera, pantoea* and *Serratia* type. Under the circumstances most of them displayed 5-8 MPGP of which AAUGPR-53 identified as *Enterococcus* species, *Enterococcus casseliflavus* strain LAHHAB-24 and *Enterococcus gallinarum* strain F1 showed the highest phosphate solubilization index (PSI) and IAA production efficiency of 4.81±0.02 (cm) and 56.55±0.45 (μg/ml), respectively. Seventeen (77.3%) of the isolates showed *in vitro* antifungal inhibition against *Fusarium oxysporum* f. sp. lentis with isolates AAUGPR- 92 and 91 identified as *Enterococcus* species, *Enterococcus casseliflavus* and *Enterococcus gallinarum* exhibiting a percent radial growth inhibition of 73 and 83%, respectively. In general, 46%-100% of the isolates showed variations in their ability to produce chitinase, protease, cellulase, HCN and NH_3 associated with antagonistic properties of the isolates. With regard to the performance of the selected inoculants to inoculation and coinoculation result, *Rhizobium*. sp. 14 performed

best in all single and co-inoculation trials with *E. casseliflavus/gallinarum*- 92. In general, the mixed inoculants; *Rhizobium* sp. 6, *R*. sp. 9, *R*. sp. 11, *R*. sp. 14 and *E. casseliflavus/gallinarum*-53 & 92 displayed the highest performance in all parameters. In all cases, treatments increased nodulation, growth and symbiotic effectiveness over the negative and positive controls. The mixed inoculants can be recommended as good candidates for plant growth improvement trials and validation under field conditions to exploit the crop for its multipurpose use with higher tolerance under extreme environmental factors, higher protein content and high yield under low soil fertility/input in the country.

Keywords/phrases: Co-inoculation, Rhizobacteria, *Rhizobium* spp; *Rhizobium leguminosarum*, 16S rRNA, stress tolerance

Chapter 1. Introduction

1.1 General introduction

Pulse crops are the most widely cultivated grain legumes in Ethiopian farming. They are mostly grown in Amhara and Oromia states accounting for 48 % and 36 % of production in the country, respectively (Samina Zekaria, 2003). The major types of pulse crops include faba bean, field pea, chick pea, haricot bean, grass pea, lentil and lupine. In Amhara region, more than 61% of pulses produced are used for household consumption, about 16% for seed, and 19% for sale and the remaining 4% for animal feed and others (Samina Zekaria, 2003).

Grass pea (*Lathyrus sativus* L.) is a food, feed and fodder crop belonging to the family Leguminosae (= Fabaceae), subfamily Papilionoideae, tribe Vicieae (Campbell and Clayton, 1997*)*. Nowadays, grass pea is grown for stock-feed and human consumption in Asia (Bangladesh, China, India, Nepal and Pakistan), in the Middle East (Iraq, Iran, Afghanistan, Syria and Lebanon), in Northern Africa (Ethiopia, Egypt, Morocco, Algeria and Libya), in Southern Europe (France, Spain and Italy) and some parts of South America (Rachele *et al*., 2012). It is the only cultivated grain legume within the genus *Lathyrus* and showed tolerance to drought as well as water logging, resistance to insects and pests, adaptability to nearly all types of soils and adverse climatic conditions and high seed protein content (Dibyendu, 2009). It is known by many common names, including chickling, vetch, Indian vetch (UK and USA), Khesari or Batura (Indian) and Guaya (Ethiopia) (Jennifer, 2003).

Grass pea seed contains 18.2-34.6% protein, 0.6% fat, 58.2% carbohydrates (about 35% starch) (Muehlbauer and Abebe Tullu, 1997). The authors also reported that it contains essential amino acids (in grams per 16 grams of nitrogen): arginine 7.85, histidine 2.51, leucine 6.57, isoleucine 2.34, tryptophan 0.40, and valine 4.68 (like other cool season food legume, grass pea is deficient in methionine and tryptophan). It is regarded as an orphan (neglected) crop that causes **Lathyrism** or **neurolathyrism** which is a neurological disease of humans and domestic animals. The consumption of large quantities of *Lathyrus* grain containing high concentrations of the glutamate analogue neurotoxin β-oxalyl-L-α,β-diaminopropionic acid (ODAP, also known as β-N-oxalyl-amino-L-alanine, or BOAA) causes paralysis, characterized by lack of strength or inability to move the lower limbs, and may involve pyramidal tracts producing UMN signs (William, 2012).

In Ethiopia there are five different species, *Lathyrus sativus, L. pratensis, L. sphaericus, L. apaca, L adoratus* of which *Lathyrus sativus* is the most important in agriculture (Dawit Tadesse, 1991). It is mostly grown in the highlands of Ethiopia at altitudes ranging from 1700-2220m above sea level with a maximum temperature of 15-20 ^{O}C in an operative range of 5-30 ^{O}C (Asmare Ayalew *et al.*, 2000). It is the fifth most important pulse crop in Ethiopia after faba bean, field pea; chickpea and red haricot bean and occupies 9 % of the total area and 11.03 % of the total production of food legumes in Ethiopia (CSA, 2018). It is a highly popular food and feed legume in the farming system due to its tolerance of drought, flooding and disease and its importance in ameliorating soil fertility. It performs well on heavy black soils which are characterized by water logging.

Grass pea production is mainly concentrated in the North West Zone (58%), the central zone (16.3%), the North East (12%) and the Northern as well as the Southeast (12.9%) regions of Ethiopia (Wuletaw Tadesse and Endashaw Bekele, 2001). However, the major grass pea producing administrative regions of Ethiopia are Shoa, Gojam, Gonder, Tigray, Wollo and Arsi (Adugna Negere and Shelemew Woldemariam, 2001). In the highlands of Ethiopia, major soils suitable for cool season food legumes are predominantly brown and chestnut vertisols (Adugna Negere and Shelemew Woldemariam, 2001). Major soil related constraints to cropping are poor fertility, soil erosion on the slopes, acidity, and low phosphate availability (Angaw Tsigie and Asnakew Woldeab, 1994). Although the severity and incidence ranged between 5-9 % and 5-25%, grass pea is also infected by root rot and wilt diseases (*Fusarium* sp.) (Campell and Clayton, 1997).

Low soil fertility and limited external inputs are the major constraints to low yield of crops in Ethiopia (EARO, 2000). The low soil fertility is mainly due to low level of nitrogen and available phosphorus in the country (Getachew Agegnehu and Rezene Fessehaie, 2006). The chemical based agriculture is facing challenges that include degradation of soil and water resources, increasing pathogen and pest attacks, diminishing biodiversity and increase in environmental pollution which in turn lead to loss of soil fertility, reduction of crop yield and productivity (Gopalakrishana *et al.*, 2014). The excessive use of agro-chemicals (fertilizers and pesticides) is posing serious threats to the environment (Azeem *et al.*, 2009). Developing means of applying biological nitrogen fixation and microbially solubilized phosphorus are among the major mechanisms for both increasing agricultural productivity and reducing the economical and ecological impacts associated with application of chemical fertilizer (Hvistendahl, 2010). Thus, application of rhizobia and other soil beneficial rhizobacteria as an alternative to ameliorate soil fertility has become an important part of integrated soil fertility management and environmentally sustainable way of improving nutrients available for plants (Ponmurugan and Gopi, 2006).

Grass pea also plays an important role as a legume crop in crop rotations, reportedly adding around 67 kg/ha of nitrogen to the soil from symbiosis with *Rhizobium* species in a single season and conferring yield and protein benefits on the subsequent non-legume crop (Jennifer, 2003). Apart from BNF rhizobia have also PGP characters including solubilization of nutrients, production of growth hormones, production of hydrolytic enzymes, siderophore production, HCN production and good colonizer. Such activity directly or indirectly increases the plant growth and productivity.

Besides rhizobia, there has been a growing interest in the search for plant growth promoting rhizosphere bacteria to enhance crop productivity in various parts of the world during the past decade. These root associated bacteria that are competent in the rhizosphere and are able to multiply and colonize plant roots at all stages of plant growth are rhizobacteria (Brahim, 2013). Most of them are known as plant growth promoting rhizobacteria (PGPR) that significantly increase the growth and yield of crops (Valverde *et al.*, 2006) and stimulate plant growth by plethora of mechanisms (Vessey, 2003). The most important PGPR belongs to genera *Azospirillum, Alcaligenes, Arthrobacter, Acinetobacter, Bacillus, Burkholderia, Enterobacter, Erwinia, Flavobacterium, Pseudomonas, Rhizobium* and *Serratia* (Tilak *et al.*, 2005).

The main mechanisms by which PGPR directly contribute to plant growth include phytohormone production such as auxins, cytokinins and gibberellins, enhancing plant nutrition by solubilization of minerals such as phosphorus and iron, production of siderophores and enzymes and lowering ethylene levels (Bhattacharyya and Jha, 2012). The indirect benefit of PGPR that involves bacterial antagonism against phytopathogenic fungi via the production of lytic (fungal cell wall degrading) enzymes such as chitinase, cellulase, glucanase and protease (Pal and Gardener, 2006), antibiotic production (Whipps *et al.*, 2001), hydrogen cyanide (HCN) production (Viosard *et al.*, 1989), competition for nutrients, niche exclusion, induced systemic resistance and antifungal metabolite production (Lugtenberg and Kamilova, 2009).

Three years yield data of grass pea indicated decline from 18.73-11.63 qt/ha (CSA, 2017). Besides agroecological constraints, the crop encounters a range of biotic stresses and abiotic factors which reduce its yield potential by15–25% (Campbell and Clayton, 1997). Some of the constraints can be solved by investigating and producing effective and competitive rhizobia and rhizobacteria which can increase soil fertility that can in turn enhance crop yield. Co-inoculation of legumes with PGPR and rhizobia significantly increased the growth and yield of legume crops (Valverde *et al.*, 2006). Similarly, co-inoculation of rhizobia having one or more growth promoting and biocontrolling properties showed more growth and yield than the application of any one of them (Zahir *et al.*, 2004). The synergistic role between them can increase the competitiveness and efficiency of *Rhizobium* inoculation which consequently result into considerable increase in the growth and yield of crops (Bhattacharyya and Jha, 2012).

Several previous studies showed that nodule and rhizosphere of different legumes harbor different types of rhizobia and rhizobacteria with various PGP properties. Rhizobial isolation and their phenotypic and symbiotic characterization were conducted for various legumes of Ethiopia including faba bean (*Vicia faba* L.) (Abere Minalku *et al.*, 2009; Zerihun Belay and Fassil Assefa, 2011; Solomon Legesse and Fassil Assefa, 2013), lentil (*Lens culinaris*) (Adigo Setargie *et al*., 2015; Anteneh Argaw, 2012; Mulisa Jida and Fassil Assefa, 2011), field pea (*Pisum sativum* L.) (Amha Gebremariam and Fassil Assefa, 2018; Aregu Amsalu *et al*., 2012; Kassa Baye *et al*., 2015), Fenugreek (*Trigonellafoenum-graecum* L.) (Mekasha Tsegaye *et al*., 2015), chickpea (*Cicer aeritinum* L.) (Mulisa Jida *et al*., 2013), Haricot bean (Mulugeta Fentahun *et al*., 2013), Soybean (Driba Temesgen, 2017) and common bean *(Phaseolus Vulgaris L.)* (Getaneh Tesfaye, 2016).

Similarly, rhizobacterial isolation and their PGP and molecular characterization were carried out for different legumes that included lentil (*Lens culinaris)* (Mulisa Jida *et al*., 2015); chickpea (*Cicer aeritinum* L.) (Mulisa Jida *et al*., 2016); common bean (*Phaseolus vulgaris* L.) (Getaneh Tesfaye, 2016), soybean [*Glycine max* (L) (Driba Temesgen, 2017), Coffee (*Coffea arabica* L.) (Diriba Muleta *et al*., 2007/ 2009/2013). However, very limited works were reported focusing on phenotypic and symbiotic characterization of grass pea (Amha Gebremariam and Fassil Assefa, 2017; Mussa Adal, 2009) whereas, no work was reported on grass pea rhizobacteria and their characterization studies from Ethiopia.

Similarly, previous studies focusing on genotypic and phenotypic diversity of *Lathyrus japonicus* rhizobia (Aoki *et al*., 2010), low temperature tolerating *Rhizobium leguminosarum* associated with *Lathyrus* species (Drouin *et al*., 2000), performance of

Rhizobium leguminosarum strains on dry matter production of *Lathyrus sativus* (Barrientos *et al*., 2003), taxonomic classification of *Rhizobium leguminosarum* strains nodulating *Lathyrus japonicas* and *Lathyrus pratensis* using 16S rRNA gene sequencing (Drouin *et al*., 1996) were done. However, there is a dearth of information on symbiotic, phenotypic, PGP and genotypic characterization of rhizobia and rhizobacteria isolated from the nodules and rhizosphere of grass pea (*Lathyrus sativus* L.), respectively.

Therefore, the present study was conducted to assess the potential of grass pea rhizobial and rhizobacterial isolates endowed with multiple plant growth and biocontrolling properties as they can be good candidates for improving the growth and yield of grass pea and its ability to prevent disease causing fungal phytopathogens. This study may contribute to the introduction of PGPR application that can address the abiotic and biotic constraints limiting grass pea yield and other crops in Ethiopia. Moreover, studying the diversity of selected isolates of rhizobia and rhizobacteria at phenotypic and molecular level was another aim of this study.

1.2 Objectives of the study

1.2.1 General objective

The general objective of the study was to evaluate the diversity and plant growth promoting properties of rhizobia and rhizobacteria from grass pea and prepare mixed microbial inoculant for improving grass pea productivity in low input agriculture in Ethiopia.

1.2.2 Specific objectives

The specific objectives of the study were:

- ✓ To isolate native grass pea rhizobia and screen for their effectiveness in symbiotic nitrogen fixation under greenhouse conditions.
- ✓ To isolate and characterize the screened rhizobia for their multiple PGP traits and ecological competitiveness (tolerance to pH, temperature, heavy metals, antibiotics and heterotrophic competence) under laboratory conditions
- ✓ To isolate, screen and evaluate grass pea rhizobacteria for their multiple PGP traits under *in vitro* conditions
- ✓ To evaluate the growth response of grass pea to inoculation and co-inoculation of selected rhizobial and rhizobacterial species under greenhouse conditions.
- ✓ To investigate diversity of some selected grass pea rhizobia and rhizobacteria using phenotypic and genetic methods.

1.3. Literature Review

1.3.1 Grass pea

Pulse crops are important components of agricultural and food systems all over the world. In Ethiopia, pulse crops occupy 12.33% of the total cultivated land and 9.69% of the total production of major crops (CSA, 2017). They are cheap source of protein in the diets of the Ethiopian population. In addition, these crops also play a significant role in export market and enhancing soil fertility. The main pulse crops cultivated in Ethiopia include Faba bean, chickpea, grass pea, Lentil, field pea, cow pea, soybean, common bean, Haricot bean and Lupine. They are essential part of the dietary requirement of most Ethiopians. Of the total area and production of these crops in Ethiopia, Amhara region accounts for about 48% of the area and production in the country followed by Oromia region which accounts for 36% of area and 37% of production (Samina Zekaria, 2003). In Amhara region, more than 61% of pulses produced are used for household consumption, about 16% for seed, and 19% for sale and the remaining 4% for animal feed and others (Samina Zekaria, 2003).

Grass pea (*Lathyrus sativus L.*) is the only cultivated grain legume within the genus *Lathyrus* and showed tolerance to drought as well as water logging, resistance to insects and pests, adaptability to nearly all types of soils and adverse climatic conditions and high seed protein content than other legumes (Hillocks and Maruthi, 2012). Grass pea is grown for stock-feed and human consumption in Asia (Bangladesh, China, India, Nepal and Pakistan), in the Middle East (Iraq, Iran, Afghanistan, Syria and Lebanon), in Northern Africa (Ethiopia, Egypt, Morocco, Algeria and Libya), in Southern Europe (France, Spain and Italy) and some parts of South America (Rachele *et al*., 2012) .

Grass pea is the fifth most important pulse crop in Ethiopia after faba bean, chickpea, field pea and red haricot bean and occupies 10.2 % of the total area and 11.03 % of the total production of food legumes in Ethiopia (CSA, 2017). It performs well on heavy black soils which are characterized by water logging. Its production is mainly concentrated in the North West Zone (58%), the central zone (16.3%), the North East

(12%) and the Northern as well as the Southeast (12.9%) regions of Ethiopia (Wuletaw Tadesse and Endashaw Bekele, 2001). However, the major grass pea producing administrative regions of Ethiopia are Shoa, Gojam, Gonder, Tigray, Wollo and Arsi (Adugna Negare and Shelemew Woldemariam, 2001). It is mostly grown in the highlands of Ethiopia at altitudes ranging from 1700-2220m above sea level with a maximum temperature of 15-20 ^{0}C in an operative range of 5-30 ^{O}C (Asmare Ayalew *et al*., 2000).

Grass pea is a staple food during famines, crop failures and in cases of extreme poverty as well as in times of food crisis. It is a low-input crop mostly planted on marginal land where it sustains on residual moisture (Anteneh Girma *et al*., 2011); thrives with minimal external inputs (Hillocks and Maruthi, 2012) and grows to maturity during the dry season (Das, 2000). These agronomic properties make it the cheapest pulse available during times of drought and food insecurity and often considered as life saver crop or as insurance crop (Lambein *et al*., 2008).

The seed contains protein, fat, carbohydrates (about 35% starch) and also contains glucose, Pentosans, Phytin, lignin, albumin, prolamine globulin, guttllin, several essential amino acids and minerals (Muehlbauer and Abebe Tullu, 1997 and Demelash Hailu *et al.*, 2015). It is a cheap source of protein for human and animal consumption (Campbell and Clayton, 1997 and Urga *et al.*, 2005). The protein content of grass pea seeds collected from 15 major production areas of Ethiopia was 27–32% (Urga *et al*., 2005), which is higher than the average percentage of protein content (21–25%) in other legume seeds (Monsoor and Yusuf, 2002). The yield (kg/ha) obtained from grass pea doubles the yield obtained from cultivating the same area/ha of other legumes (Bashu, 2013). This implied that the crop has a unique inherent potential to cope up the changing harsh argo-climatic conditions and produce better yield than other pulse crops. Although it has multi- purpose agronomic benefits, it is grouped among the orphan or understudied legume crops (Zerihun Tadele, 2007). The yield of the crop from 2014-2016 indicated a decline from 18.73-11.63 qt/ha (CSA, 2017). Besides agroecological constraints, the crop encounters a range of biotic stresses and abiotic factors which reduce its yield potential by15–25% (Campbell and Clayton, 1997).

1.3.2 Definition and taxonomy of rhizobia: an overview

The term ‘’rhizobia’’ in the strictest sense, refers to members of the genus *Rhizobium* but later becomes a repository for all bacteria capable of nodulation and nitrogen fixation with legumes (Willems, 2006; Rivas *et al.*, 2009). Giller (2001) defined rhizobia as ‘’a group of diverse’’ bacterial genera that are able to induce and infect nodules on roots or stems of plants in the family *Leguminosae* or *Fabaceae,* irrespective of their ability to fix nitrogen”. Rhizobia are a group of bacteria that have the capacity to form nodules on legume roots (and occasionally on stems) that can fix atmospheric nitrogen to partially or fully meet the nitrogen requirements of the host plant. To describe bacteria from root nodules (Frank, 1889) proposed name “rhizobia”, and after this proposal all nodule-forming bacteria have been known as rhizobia. It represents the genera *Rhizobium, Bradyrhizobium, Ensifer, Mesorhizobium, Allorhizobium,* and *Azorhizobium*.

The interactions between rhizobia and legume roots result in formation of root nodules, in which rhizobia use energy from the host plant to fix atmospheric N_2 into plant-available forms of nitrogen. Biological nitrogen fixation (BNF: atmospheric nitrogen fixation through different members of prokaryotes, specifically by diazotrophs) contributes approximately 16% of total nitrogen input in crop land (Ollivier *et al.*, 2011). Rhizobia are a major contributor to BNF and the legume-rhizobium symbiosis can fix up to 450 Kg N/ha/year (Unkovich *et al.*, 2000). The amount of nitrogen fixed by a legume crop varies widely because it depends on the legume genotype, rhizobia strain and the soil environment (Lupwayi *et al.*, 2011).

1.3.2.1 Rhizobial taxonomy

Rhizobia are classically defined as symbiotic bacteria capable of eliciting and invading root and stem tissue forming nodules on leguminous plants where they undertake symbiotic nitrogen fixation (Sahgal and Johri, 2003). It is the common name given to a group of small, rod-shaped, Gram-negative bacteria that collectively have the ability to produce nodules on the roots of leguminous plants and belong to the family *Rhizobiaceae*, which are part of the α-proteobacteria. The *Proteobacteria* represents the s00econd largest group of bacteria and consist of a diverse range of organisms including purple phototrophic, nitrifying and enteric bacteria as wellas symbiotic and free-living nitrogen-fixing bacteria (Jordan, 1984).

All *Proteobacteri*a are Gram-negative and motile by means of flagella. Based on analyses of the highly conserved small subunit 16S ribosomal RNA (16S rRNA) gene sequence, the *Proteobacteria* are divided into the five groups α-, β-, γ-, δ- and ε- *Proteobacteria* (Zakhia & de Lajudie, 2001). Nodulation and nitrogen fixation is restricted to the α-*Proteobacteria* and β- *Proteobacteria.* The terms α- and β- rhizobia were proposed to distinguish rhizobia of α- *Proteobacteria* and β- *Proteobacteria* (Chen *et al*., 2003a). The most recent taxonomy of rhizobia consists of 62 species found in 12 genera of nodule-forming diazotrophic bacteria, nine of which are α- rhizobia (A*zorhizobium, Bradyrhizobium, Devosia, Mesorhizobium, Methylobacterium, Ochrobactrum, Phyllobacterium, Rhizobium* and *Sinorhizobium)* and three β- rhizobia (*Burkholderia, Cupriavidus and Herbaspirillum*) (Wolde-Meskel *et al*., 2005; Weir, 2006).

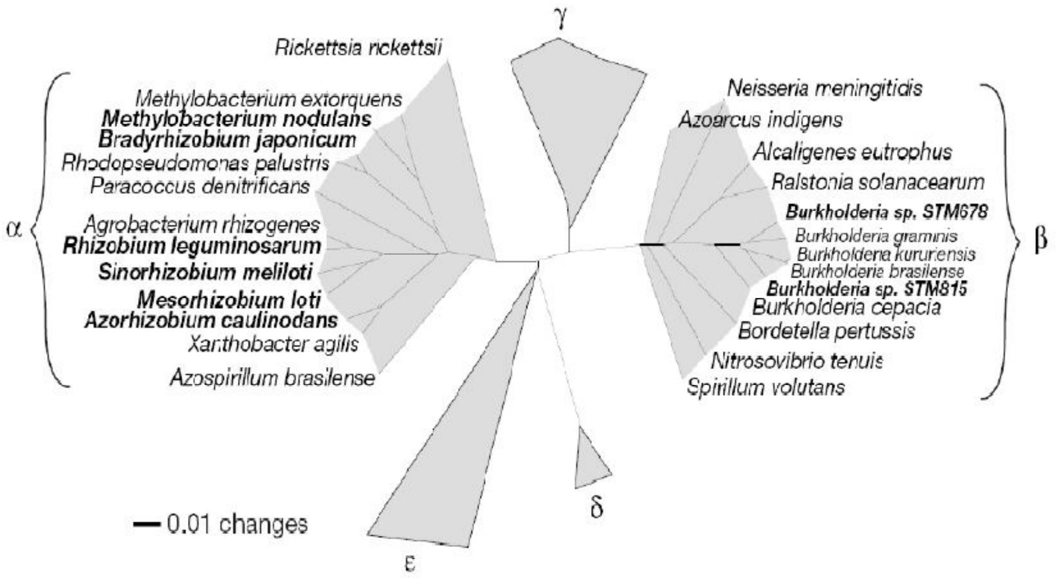

Fig. 1.1 Root nodulating proteobacteria (from Moulin *et al*., 2001).

1.3.2.2 History of rhizobial taxonomy

The taxonomy of root nodule bacteria has changed considerably over the last 20 years. Beijerinck (1888) had for the first time isolated a bacterium from root noduleof legume and named it *Bacillus radicicola*. This was subsequently renamed *Rhizobium* (Frank, 1889). The earliest classification of rhibzobia was based on specificity of symbiotic plant range of bacterial species (Sahgal and Johri, 2003). Fred *et al.* (1932) recognized six species in the genus Rhizobium viz. *R. leguminosarum* (*Lathyrus*, *Lens, Pisum* and *Vicia*), *Bradyrhizobium* (Glycine max), *R. lupini* (Lupinus), *R. Meliloti* (*Melilotus, Medicago, Trigonella*) *R. phaseoli* (*Phaseolus*) and *R. Trfolii* (*Trifolium*) based on their host range and certain morphological and physiological properties.

The majority of rhizobial species belong to five principal genera of α- proteobacteria (*Rhizobium, Sinorhizobium, Mesorhizobium, Bradyrhizobium* and *Azorhizobium*) and research in rhizobia-legume symbioses has focused on species within these genera because many of their primary host plants are important food and fodder crops. However, the list of rhizobial species is continually expanding and now includes: *Methylobacterium nodulans*, a methylotroph that induces the formation of root nodules on various *Crotalaria* spp. (Sy *et al*., 2001); a species of *Devosia* that forms nodules on the tropical aquatic legume *Neptunia natans* (Rivas *et al*., 2009); and several rhizobial species within the genera *Ochrobactrum* (Zurdo-Pineiro *et al*., 2007) and *Phyllobacterium* (Mantelin *et al*., 2006). Moreover, rhizobia have also included the β-proteobacteria, consisting species of *Burkholderia*, *Cupriavidus* (formerly *Ralstonia*) and *Herbaspirillum* (Chen *et al*., 2008). Rhizobial species therefore do not belong to a homogenous clade (fig. 1.1) and phylogenetic analyses indicate that many rhizobia have acquired genes required for symbiosis via multiple horizontal gene transfer events (Giraud *et al*., 2007).

1.3.2.3 Methods of studing rhizobial diversity

Early attempts at classification were based on specificity amongst rhizobia for their respective host plants and led to the concept of crossinoculation groups in which each group of legume host species could only be nodulated by a corresponding group of compatible rhizobial strains, while incompatible strains from another group would be incapable of nodulation. This system provided the basis for initial classification of species in the genus *Rhizobium*, although inconsistencies such as differences in host specificity of strains within species and overlapping nodulation ranges between groups later showed that this system was not practical.

Cross-inoculation was often used together with other differentiation methods for the identification of rhizobial strains. These included cultural characteristics such as type and rate of growth on Congo red yeast mannitol medium, as well as serological and bacteriophage crossreactivity (Somasagaren & Hoben, 1994). Other characterization techniques include substrate utilization, enzyme linked immunosorbent assay (ELISA), antibiotic resistance, plasmid profile analysis, multilocus enzyme electrophoresis and fatty acid analysis (Niemann *et al.,* 1997). A variety of DNA-based methods have emerged for the identification of rhizobia including genomic restriction fragment length polymorphisms (RFLP), random amplified polymorphic DNAs (RAPDs) using random probes, rRNA analysis, DNA-DNA hybridization, Southern blot analysis of nitrogen-fixation (*nif*) and nodulation (*nod*) genes and polyacrylamide gel analysis of total proteins (Thies *et al*., 2001).

The current rhizobial classification procedures involve a polyphasic approach that results in reliable resolution of relationship among microorganisms (Martens *et al.,* 2008). Vinuesa *et al.,* (2005b) determined the adequacy of the combined phylogenetic and population genetic methods for the description of bacterial species, more especially *Bradyrhizobium* species. The move to a polyphasic approach (using several characters) was made to ensure that only valid new species or genera are created. For the description of a new taxa all genotypic (DNA-DNA hybridization, etc.), phenotypic (substrate utilization, etc.) and phylogenetic (rRNA gene sequence analysis, etc.) information should therefore be combined (Martens *et al.,* 2008). As a result rhizobial classifications should be based on phylogenetic and phenotypic data relating to the symbiotic, cultural, and morphological properties of the bacteria.

1.3.2.4 Review on rhizobia nodulating grass pea

Grass pea (*Lathyrus sativus* L.) is one of the viciae group member’s nodulated by *Rhizobium leguminosarum*. However, there is a dearth of information indicating *Rhizobium*- grass pea symbiosis. Barrientos *et al*. (2003) have isolated rhizobial strains from the nodules from grass pea and have evaluated their symbiotic properties under greenhouse conditions. Mahdavi *et al.* (2007) have also studied the effect of temperature on nodulation of grass pea ecotypes. Moreover, Mussa Adal (2009) has studied nodulation of grass pea by rhizobial isolates while studying the phenotypic and symbiotic characterization of grass pea rhizobial isolates.

1.3.2.5 Rhizobia- legume symbiosis

Legumes are the third largest flowering plant family, containing 700 genera and about 20,000 species. It is an extremely diverse plant family with a great number of different characters, and a few characters are common to all species. Nodulation is an important characteristic of majority legumes but it is absent in the earliest divergent lineages of this family. Legume evolved about 65 MYA and nodulation have evolved in legumes after the origin of this family approximately 30 MY (Doyle, 2011) later.

Rhizobia are taxonomically diverse members of the α-subdivision of the proteobacteria and can exist in two states: as a free-living saprophyte in the soil and in a symbiotic relationship with leguminous plants. The latter interaction begins with a specific molecular signal exchange between the legume and the free-living *Rhizobium*. Plant roots secrete many different organic compounds into the soil, some of which allow microorganisms to grow in the rhizosphere and include carbohydrates, amino acids, organic acids, vitamins and phenolic derivatives. In terms of symbiosis, flavonoids are the most important of these compounds, as they trigger the induction of bacterial nodulation (*nod*) genes (Redmond *et al.*, 1986), although oxygen limitation also plays a key role in symbiotic gene expression (Soupène *et al.*, 1995).

The development of this symbiotic relationship is a multistep process finely choreographed by molecular signal exchange between the symbiotic partners (Wang *et al.*, 2012) in which the nodulation process and the establishment of symbiosis may be divided into three continuous processes: i) Recognition *via* molecular signal exchange (molecular dialogue) between the partners ii) invasion or infection phase when the rhizobia enter into root or stem nodules and iii) symbiotic phase when the rhizobia occupying the nodules are transformed into bacteroides and start to fix N2 (Masson-Bovin *et al.*, 2009).

1.3.2.6 Rhizobia- non-legume symbiosis

Most legume species can engage a symbiosis with nitrogen fixing soil bacteria collectively referred to as rhizobia. Rhizobia (species of *Rhizobium*, *Mesorhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Allorhizobium*, and *Sinorhizobium*) naturally infect legumes and form intimate symbiotic relationships with them by responding chemotactically to flavonoid molecules released as signals by the legume host. However, it was reported that some *Rhizobium* strains can form symbiotic relationships with non-legume species such as *Parasponia* initiated by rhizobium-secreted LCOs which is similar to rhizobia- legume association (Graqvit, 2015).The nodulation observed in *Parasponia* by both *Rhizobium* and *Bradyrhizobium* strains provided further encouragement that rhizobial infection and nodule formation in non-legume crops is a possibility in the future and has increased the prospect and search for rhizobial nodulation of non- legume plants such as cereals(Matiru *et al*., 2004).

The possibilities of extending the host range of rhizobia to non-legumes were encouraged by the discoveries that *Rhizobium* forms nodules in *Parasponia* and that *Rhizobium parasponium* RP 501 and *Bradyrhizobium* CP 283 induce nodulation in oilseed rape (Saikia and Jain, 2007). In addition, several attempts have been made to extend nodulation and N_2-fixing ability to other non-legume crops (Stone, 2001). Rhizobia have also been isolated as natural endophytes from roots of other non-legumes species such as rice, cotton, sweet corn, maize, wheat and canola either grown in rotation with legumes or in a mixed cropping system involving symbiotic legumes (Matiru and Dakora, 2004).

1.3.3 Rhizosphere

The term rhizosphere was first defined over a century ago by Lorentz Hiltner and redefined by Pinton as the zone that includes the soil influenced by the root along with the root tissues colonized by microorganisms (Huang *et al.,* 2014). However, for the sake of practical investigation, the rhizosphere is most often defined as the soil adhering to plant roots when they are rigorously shaken, throughout which the rhizosphere effect must be observed to some extent (Kang and Mills, 2004). The parts of plants that are both underground and above ground serve as a niche where microbial activity takes place. The rhizosphere is a biologically active zone of the soil around plant roots where soil-borne microbes including bacteria and fungi reside. The specific region in the underground part of the plant is the site of interaction between plants and soil microorganisms that plays an integral and unique role in ecosystem functions such as decomposing, mineralizing organic matters and releasing as well as transforming inorganic nutrients is termed as the rhizosphere.

The rhizosphere is the soil–plant root interphase and, in practice, consists of the soil adhering to the root besides the loose soil surrounding it (Ngoma *et al.*, 2012). In this environment, the interactions between plant roots, soil, and microbes significantly alter soil physical and chemical properties, which in turn alter the microbial population in the rhizosphere (Huang *et al.*, 2014). The concept rhizosphere is also defined as the volume of soil surrounding a plant root in which very important and intensive interactions are taking place between soil, microorganisms, and plant roots (Marta *et al.*, 2010). The rhizosphere is about 1-5 mm wide, but has no distinct edge and is different of the bulk soil (Lines-Kelly, 2004). This is the narrow region of soil surrounding the root system where the biology and chemistry of the soil are influenced directly by root secretions, the root systems and associated soil microorganisms. There are three distinct zones in the rhizosphere: the endorhizosphere, the rhizoplane, and the ectorhizosphere (Huang *et al.*, 2014).

Bhattacharyya and Jha (2012) reviewed that soil microorganisms including free-living as well as associative and symbiotic rhizobacteria belonging to the genera like *Acinetobacter, Alcaligenes, Arthrobacter, Azospirillum, Azotobacter, Bacillus, Burkholderia, Enterobacter, Erwinia, Flavobacterium, Proteus, Pseudomonas, Rhizobium, Serratia, Xanthomonas* in particular, are the integral parts of rhizosphere biota exhibiting successful rhizosphere colonization.

1.3.4 Plant Growth Promoting Rhizobacteria (PGPR)

The term “plant growth promoting rhizobacteria” (PGPR) is coined to refer to root colonizing bacteria that cause the increase in growth and yield and to differentiate them from other mechanisms found in rhizosphere that don’t colonize roots or enhance plant growth (Gupta *et al*., 2000). According to Vessey (2003) PGPR is the collective name for the various species of soil bacteria that dominates and thrive in the rhizosphere of plants and that may grow in, on or around plant tissues and trigger plant growth by a diverse mechanisms The plant growth promoting rhizobacteria (PGPR), are characterized by the following inherent distinctiveness's: (i) they must be proficient to colonize the root surface (ii) they must survive, multiply and compete with other microbiota at least for the time needed to express their plant growth promotion/protection activities, and (iii) they must promote plant growth (Rajeshwar *et al.*, 2014)**.**

The bacteria that can promote plant growth(PGPB) include those that are free-living, those that form specific symbiotic relationships with plants (e.g., Rhizobia spp. and Frankia spp.), bacterial endophytes that can colonize some or a portion of a plant's interior tissues, and cyanobacteria (formerly called blue-green algae) (Glick, 2012). Bhattacharyya and Jha (2012) reviewed that plant growth-promoting rhizobacteria (PGPR) are the rhizosphere bacteria that can enhance plant growth by a wide variety of mechanisms like phosphate solubilization, siderophore production, biological nitrogen fixation, rhizosphere engineering, production of 1-Aminocyclopropane-1carboxylate deaminase (ACC), quorum sensing (QS) signal interference and inhibition of biofilm formation, phytohormone production, exhibiting antifungal activity, production of volatile organic compounds (VOCs), induction of systemic resistance, promoting beneficial plant-microbe symbioses, interference with pathogen toxin production and others.

PGPR are classified based on their functional activities as (i) biofertilizers (increasing the availability of nutrients to plant), (ii) phytostimulators (plant growth promotion, generally through phytohormones), (iii) rhizoremediators (degrading organic pollutants) and (iv) biopesticides (controlling diseases, mainly by the production of antibiotics and antifungal metabolites (Munnes and Mulugeta (2013). Based on their relationship with host plants, Gopalakrishnan *et al.*, 2014 classified as (1) extracellular PGPR (ePGPR), which exists in the rhizosphere, on the rhizoplane, or in the spaces between cells of the root cortex including *Agrobacterium, Arthrobacter, Azotobacter, Azospirillum, Bacillus, Burkholderia, Caulobacter, Chromobacterium, Erwinia, Flavobacterium, Micrococcous,* *Pseudomonas*, *Serratia* and (2) intracellular PGPR (iPGPR), which exist inside root cells, generally in specialized nodular structures (for example, *Bacillus, Pseudomonas, Azotobacter, Allorhizobium, Azorhizobium, Bradyrhizobium, Mesorhizobium, Sinorhizobium* and *Rhizobium* of the family Rhizobiaceae).

1.3.5 Rhizobacteria

Several microorganisms including protozoa, fungus, viruses and bacteria use soil as an excellent niche. The bacteria that are able to colonize the soil surrounding the plant roots, the rhizosphere making come under the influence of plant roots are termed as rhizobacteria (Kennedy, 2005). Similarly, Pankaj *et al.,* (2012) has defined rhizobacteria as the dominant soil bacteria that live in close vicinity to the root or on its surface and are important for soil health and plant growth. As reviewed by Brahim (2013) rhizobacteria are rhizosphere competent bacteria able to multiply and colonize plant roots at all stages of plant growth, in the presence of a competing microflora where they are in contact with other microorganisms. The same author reported the intent made by numerous researchers with the aim to enlarge this restrictive definition of rhizobacteria as any root-colonizing bacteria and consider endophytic bacteria in symbiotic association: Rhizobia with legumes and the actinomycete Frankia associated with some phanerogams as PGPR genera.

1.3.6 Some PGPR works in Ethiopia

Several researches have been carried out to characterize and select symbiotically effective indigenous rhizobial strains isolated from faba bean (Abere Mnalku *et al*., 2015; Alemayehu Workalemahu, 2009; Ayneabeba Adamu *et al*., 2001; Asfaw Hailemariam and Angaw Tsige, 2006; Dereje Tsegaye *et al*., 2015; Solomon Legesse and Fassil Assefa, 2013; Zerihun Belay and Fassil Assefa, 2011; Anteneh Argaw, 2012), lentil (Adigo Setargie *et al*., 2015; Anteneh Argaw, 2012, Mulisa Jida, 2011), field pea (Kassa Baye *et al*., 2015), chickpea (Mulisa Jida *et al*., 2013), Haricot bean (Habtamu Bekele *et al*., 2013; Mulugeta Fentahun *et al*., 2013) and fenugreek(Mekasha Tsegaye *et al*., 2015). These studies are mainly focused on phenotypic characterization and symbiotic effectiveness. However, researches that focused on plant growth promotion and biocontrolling aspects are meager.

Similarly, some researches that involved plant growth promotion and biocontrol activities of rhizobacteria isolated from chickpea (Mulisa Jida *et al*., 2013), coffee (Diriba Muleta *et al*., 2007/9/13); faba bean (Ahmed Indris *et al*., 2008/9); soybean (Driba Temesgen, 2017), common bean (Getaneh Tesfaye, 2017) and cowpea (Girma Kenassa, 2017). However, preparation of effective and competitive rhizobia and rhizobacteria and their commercialization is not yet reported.

1.3.7 Mechanisms of PGPR

Plant growth promotion is described as increased in plant growth and crop yield that occurred among inoculating seeds or roots with certain specific root- colonizing bacteria (Bashan and Bashan, 2005). PGPR enhance plant growth either directly or indirectly (Glick, 2012).

1.3.7.1 Plant growth promoting mechanisms

Nitrogen is an essential plant nutrient which is most commonly deficient in soils, contributing to reduced agricultural fields throughout the world (Montanez, 2000).The element nitrogen is an essential part of many of the chemical compounds, such as proteins, nucleic acids and other metabolites, which are the basis of all life forms. The atmospheric nitrogen in order to be incorporated into amino acids and subsequently become an integral part of plant tissue, it has to be transformed in to ammonia (NH_3) through nitrogen fixation (Postgate, 1998). Although nitrogen fixation is most common in species of rhizobia, small amount is fixed by a number of free-living bacteria. Rajeshwar (2013) has reviewed symbiotic (*Rhizobium, Mesorhizobium, Bradyrhizobium, Azorhizobium, Allorhizobium* and *Sinorhizobium sp.*) and non-symbiotic (*Azotobacter, Azospirillum, Acetobacter, Diazotrophs, Bacillus* and *Klebsiella sp.*) PGPR that fix nitrogen.

BNF is estimated to contribute 180 X 10^6 mt /year globally, of which 80% comes from symbiotic association and the rest 20% from free-living or associative systems (Tilak *et al.*, 2005). This is by far greater than that of the industrially fixed nitrogen supply which is 65 X 10^6 tons per year. Roughly, half of the 23 million mt of nitrogen consumed as human food sources (grains and live stocks) come from BNF by prokaryotes (Frink *et al.*, 1999). Out of this, rhizobia in root nodules are estimated to carry out about 80% of the world's BNF (Burris and Roberts, 1993). Inoculation with rhizobia has the potential of increasing dry matter yield, N yield, and residual N levels (Vessey, 2003). The application of *Rhizobium* in *Phaseolus vulgaris* L. significantly improved the shoot dry matter yield, number of pods per plant, number of seeds per plant, 100-seed weight, seed yield, total N content and protein yield (Bambara and Ndakidemi, 2010).

Assefa Keneni *et al.* (2010) have reported that inoculation of *Rhizobium* on *Vicia faba* L. increased dry matter yield, nodulation and nodule wet weight in pouch culture. Growth and yield responses to inoculation are dependent upon many factors, but legume species and soil N levels prior to seeding are two important factors (Vessey, 2003). The nodulation and nitrogen fixation experiments carried out on Faba bean rhizobia (Abere Minalku *et al.*, 2009; Assefa Kenneni *et al.*, 2010) and lentil (Adigo Setargie *et al.*, 2012; Mulisa Jida *et al.*, 2011) showed variations in the symbiotic parameters recorded. SNF is dependent on several factors including the host plant genotype, the *Rhizobium* strain and the interaction of these symbionts with the pedoclimatic factors and the environmental conditions (Bordeleau and Prevost, 1994).

Phosphorus, the second most important macro-nutrient required by the plants, next to nitrogen, is reported to be a critical factor of many crop production systems, due to the fact that the limited availability in soluble forms in the soils (Xiao *et al.*, 2011). Phosphorus is a structural component of many co-enzymes, phospho-proteins, phospholipids (Ozanne, 1980) and part of the genetic memory "DNA" of all living things. Phosphate in soil mostly exists in insoluble (bound) forms and the concentration of soluble phosphate in soil solution is very low (400–1,200 mg kg^{-1} of soil) (Rodriguez and Fraga, 1999). Consequently, about 98% soils have inadequate supply of available Phosphorous (Hasan, 1996) and likely to induce deficiency of this mineral. The phosphatic fertilizers that are applied to the soil often become insoluble (more than 70%) and are converted into complexes such as calcium phosphate, aluminum phosphate and iron phosphate in the soil (Mittal *et al.,* 2008). The application of chemical fertilizer is also a costly affair and environmentally undesirable (Reddy *et al*., 2002).

This has created a fertile ground to think to use phosphate solubilizing microorganisms that are environmental-friendly and economically feasible alternative strategies for improving crop production in low or phosphorus deficient soils. The microbial solubilization of soil phosphorus in liquid medium has often been due to the excretion of organic acids including citric, lactic, acetic, malic, tartaric, oxalic, 2-ketogluconic, gluconic and succinic acids (Bolan *et al*., 1996) which can either directly dissolve the mineral phosphate as a result of anion exchange of PO_4^{2-} by acid anion or can chelate both iron and aluminium ions associated with phosphate (Omar, 1998). The most important P solubilizing bacterial genera are *Pseudomonas, Bacillus, Rhizobium, Burkholderia, Achromobacter, Agrobacterium, Microccocus, Aereobacter, Flavobacterium and Erwinia* (Rodriguez and Fraga, 1999).

Symbiotic and non-symbiotic bacteria may promote plant growth directly through production of plant hormones (Vivas *et al.*, 2005) and other PGP activities (Dobbelaere *et al.*, 2003). One of these phytohormones is indole-3-acetic acid (IAA). Indole-3-acetic acid a main auxin in the plants is known to control many important physiological processes of plants such as cell enlargement, cell division, root initiation, growth rate, phototropism and apical dominance (Zaidi *et al.,* 2009). Bacterial IAA increases root surface area and length, and thereby provides the plant greater access to soil nutrients and loosens plant cell walls and as a result facilitates an increasing amount of root exudation that provides additional nutrients to support the growth of rhizosphere bacteria (Glick, 2012).

It is reported that 80% of microorganisms belonging to the genera *Azospirillum*, *Pseudomonas*, *Xanthomonas*, and *Rhizobium* as well as *Alcaligenes faecalis*, *Enterobacter cloacae*, *Acetobacter diazotrophicus* and *Bradyrhizobium japonicum* isolated from the rhizosphere of various crops possess the ability to synthesize and release auxins as secondary metabolites and have been shown to produce auxins which help in stimulating plant growth (Patten and Glick, 1996). Indoleacetic acid production by microbial isolates varies greatly among different species and strains and dictated by the availability of a substrate IAA in the presence of a suitable precursor such as L-tryptophan (Karnwal, 2009).

1.3.7.2 Biocontrolling mechanisms

The indirect mechanisms include limiting pathogen- caused damage that in turn includes local antagonism of soil borne pathogens (Salme, 2003) or induction of systemic resistance against pathogens throughout the entire plant, production of antibiotics, secretion of siderophores, production of HCN, lytic enzymes and successful competition (Gopalakrishnan *et al*., 2014).

Phytopathogenic microorganisms are a major and chronic threat to sustainable agriculture and ecosystem stability worldwide subverts the soil ecology, disrupt environment, degrade soil fertility and consequently show harmful effects on human health, along with contaminating ground water (Gupta *et al.*, 2015). Soil borne diseases affect all crops and encompass the whole spectrum of plant pathogens including fungi, bacteria, and nematodes. Several groups of soil borne fungi attack most of the economically important crops causing infection resulting in huge yield losses (Gohel *et al*. 2006). Generally plant diseases cause 10–20 % loss in production (James, 1981). The usual strategy for the control of phytopathogens is to apply chemical pesticides, but this strategy has led to increased concerns over environmental contamination and has resulted in the so-called pesticide treadmill in which pathogens develop resistance to individual chemical controls over time, needing a constant development of new pesticides (Fernando *et al*., 2006). Moreover, these agrochemicals although vital for controlling the pathogens, they can drastically affect the microbial diversity and functional properties of natural microbial communities of soils, leading thereby to imbalanced agro-ecosystems (Mubeen *et al*., 2008).

The use of antibacterial and antifungal chemicals is deprecated in view of sustainable agricultural practices. Hence, an alternative to chemical control of plant diseases by the use of bacteria able to antagonize phytopathogenic is considered as a more environmentally friendly process. A large number of mechanisms are involved in biocontrol and can involve direct antagonism via production of antibiotics, siderophores, HCN, hydrolytic enzymes (chitinases, cellulases, glucanases proteases, lipases, etc.), or indirect mechanisms in which the biocontrol organisms act as a probiotic by competing with the pathogen for a niche (infection and nutrient sites). For example, the strains including *R. leguminosarum bv. trifolii, R. leguminosarum bv. viciae, R. meliloti, R. trifolii, S. meliloti* and *B. japonicum* have been reported to secrete antibiotics and cell-wall degrading enzymes that can inhibit the phytopathogens (Chandra *et al.,* 2007).

1.3.8 Biological nitrogen fixation

Nitrogen is commonly the most limiting element in agricultural production and or the most expensive to purchase as fertilizer although there is an abundant supply form air (78% air is nitrogen gas, amounting to about 8,000 pounds nitrogen in the air over every area at land). BNF, the process of reducing the triple bond of atmospheric nitrogen (N=N) in to ammonia and other usable forms take place in the presence of an oxygen sensitive enzyme, the nitrogenase. Nitrogenase is a complex – enzyme containing two oxygen sensitive metalloprotein components that are active independently but work synergetically (Leigh, 2002).

Dinitrogenase (Component I), the larger heterotetramer component of nitrogenase with two different metalloclusters has two α- protein subunits and two β-protein subunits, 24 molecules of iron, two molecules of molybdenum and an iron molybdenum cofactor (FeMoCo) (Leigh, 2002). Component I also called the dinitrogenase reductase catalyses the actual conversion of N_2 to ammonia. Similarly, component II is composed of two protein subunits (different from that of component I) and a large number of iron molecules or iron-protein which plays a role in donating electrons to component I (Haward and Rees, 1996).

Each individual α, β- dimmer component containing Fe-Mo-Co and a P-cluster is considered as a functional unit of nitrogen fixation and the Fe-protein is ahomodimmer component containing two MgATP binding sites and a single (4Fe-45) cluster (Peters *et al.*, 1995). Dinitrogenase, a 240-KDa heterotertramer, binds N_2 and holds it while it is being reduced whereas; Dinitrogenase reductase a 64-KDa homodimer provides dinitrogenase with high energy electrons. Following binding, an electron is transferred from the Fe-protein to the Mg Fe-protein with concomitant hydrolysis of both bound ATP molecules to ADP; ultimately, the two components of nitrogenase dissociate (Giller, 2001). Thus, nitrogenase catalyses the conversion of N_2 to NH_3 which is represented as:

$$N_2 + 16\ ATP + 8e + 8H^+ \xrightarrow{Mg^{++},\ nitrodenase} 2NH_4{}^+ + H_2 + 16\ ADP + 16\ pi$$

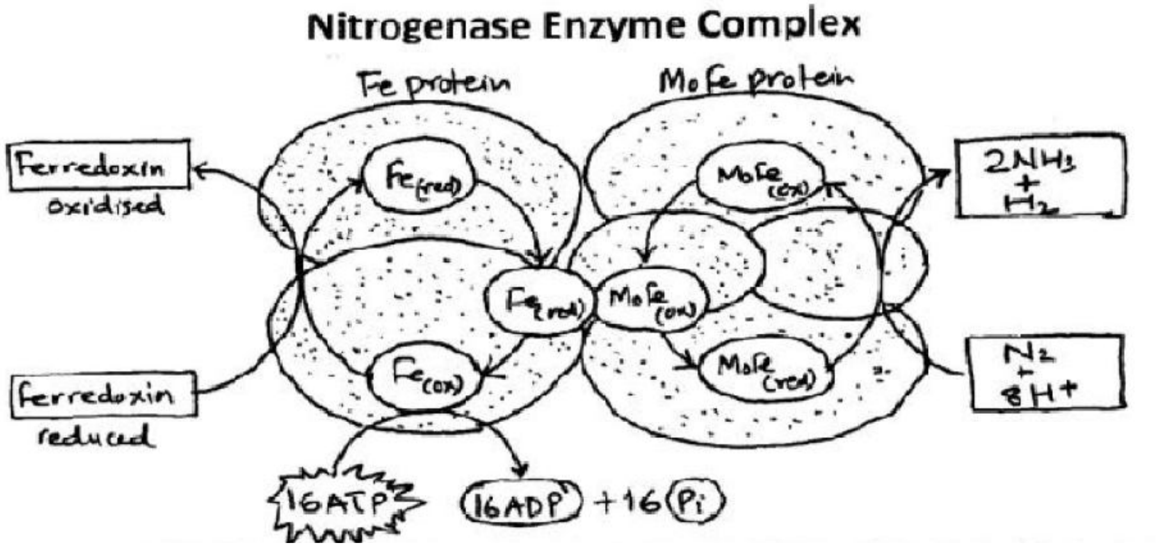

Fig. 1.2 The role of nitrogenase enzyme in nitrogen fixation

For the reaction to be catalyzed, nitrogenase requires anaerobic environment and a source of large amount of energy. BNF is energy intensive, efficient but requires much lower energy than Haber process (Zahran *et al.*, 1992). In symbiotic association, nitrogenase activity is protected form oxygen damage in two ways: one with strong and variable physical barrier to oxygen diffusion into nodule cortex and the second component of oxygen protection is with the presence of leg-hemoglobin within the infected cells. The role of leg-hemoglobin in the nodule is to facilitate oxygen supply for respiration, so that high oxygen tension support bacteroidal oxidative phosophorylation while keeping oxygen sequestered away from nitrogenous enzyme (Shaw, 1983).

1.3.9 Ecological factors affecting BNF

The biological nitrogen fixation (BNF) which is a natural process that consists of the conversion of atmospheric nitrogen into ammonia by the nitrogenase enzyme complex is the result of rhizobium-legume symbiosis that in turn results due to a balance between environmental factors affecting both plant and bacteria. Consequently, the success of the legume infection and nodulation depends on environmental factors and rhizobium survival. BNF is therefore affected by edaphic and climatic factors that include temperature, salinity, pH, antibiotics and heavy metals which interfere with the infection and nodulation process and influence the symbiosis of nitrogen fixation (Kinkema *et al.*, 2006). Environmental factors can influence the ratio between rhizobia in soil and rhizosphere, and can also influence other steps in the nodulation process that includes colonization, attachment, infection and nodule formation resulting in different outcome of competition (Abdullah, 2002).

Bordeleau and Prevost (1994) have also described that BNF can be successful when there is establishment of effective symbiosis which requires the following factors: colonization and survival in soil by rhizobia as saprophytes in competition with other endogenous microbes which is also associated with soil infertility and extremes of temperature, rapid colonization of the rhizosphere prior to root infection and genetic compatibility between host and root nodule bacteria to establish an effective nodule, and a favorable environment to allow maximum fixation. For effective BNF, rhizobia with different PGP and stress relieving traits desired. The response of legumes to various stresses depends on the host plant reaction that in turn can be influenced by the rhizobia and the process of symbiosis (Yang *et al*., 2009).

1.3.9.1 Temperature

Rhizobial species have their own range of optimum temperature although it varies from species to species. High temperatures lead to increased drought intensity, due to enhanced transpirational water loss that can lead to reduction in nodule number, rhizobial growth, rate of colonization and infectious events, and can lead to delay in nodulation or restrict the nodule to the subsurface region (Gopalakrishnan *et al.,* 2014). The optimum temperature for rhizobial growth is 28–31^{0}C, while many of them are unable to grow beyond 38 ^{0}C (Graham, 1992). As reviewed by Gopalakrishnan *et al.* (2014), *R. leguminosarum bv. phaseoli* subjected at 35 and 38 ^{0}C was found to be infective and formed nodules in *P. vulgaris,* but these nodules were found to remain ineffective. Screening of *R. leguminosarum* bv. phaseoli showed that some strains were able to nodulate *Phaseolus vulgaris* at high temperatures (35 and 38°C) but that the nodules formed at high temperatures were ineffective and plants did not accumulate N in shoots (Hungria and Franco, 1993).

The growth of rhizobia isolated from faba bean was shown to grow at low and high temperature at 4^{0}C and 55 ^{0}C (Berrada *et al.,* 2012). The survival of the rhizobia and the exchange of molecular signals between the symbiotic partners can be influenced by temperature (Sadowsky, 2005). Moreover, high temperature can induce an inhibiting effect on bacterial adherence to root hairs, bacteroid differentiation, nodule structure and legume root nodule functioning can be inhibited by high temperature (Zahran, 1999) and accelerates nodule senescence (Hungria and Franco, 1993). Likewise, it was also found that low temperature decrease nodulation and nitrogen fixation (Waughman, 1997). However, the survival of bacteria in soil is more affected by high temperatures than by low temperature because it can be deleterious (Niste *et al.,* 2013).

However, selection of temperature tolerant and adapted host legume and rhizobial strains shock proteins in heat-tolerant bean genotypes and rhizobial strains (Bordeleau and Prevost, 1994). Adaptation of microorganisms to stress is a complex regulatory process, as it involves the use of proteins and lipopolysaccharide (LPS) with the up-regulation of an array of genes. Upon exposing the wild and heat resistant *Rhizobium* sp. to 30 and 43 ^{0}C, changes in the cell surface including extracellular polymeric substances/exo polysaccharides (EPS), LPS and proteins had been demonstrated (Nandal *et al.*, 2005).

1.3.9.2 Soil pH

Soil acidity is a significant problem facing agricultural production in many areas of the world and limits legume productivity (Correa and Barneix, 1997). Acid soil conditions pose problems for the plant, the bacteria and the symbiosis (Giller and Wilson, 1991). Most leguminous plants require a neutral or slightly acidic soil for growth, especially when they depend on symbiotic nitrogen fixation (Bordeleau and Prevost, 1994). Large variations in tolerance to acidity factors are found both within and between Rhizobium species where fast growing rhizobia are generally considered more acid sensitive than Bradyrhizobium, but low pH- tolerant strains exist in many species (Serraj and Adu-Gyamfi, 2004). Thus, this large variation in acid tolerance within species of root nodule bacteria imply genetic basis to low pH and studies of acid - sensitive mutants suggested that a large number of genes and regulatory system could be involved (Glenn *et al.*, 1999).

Bacteria develop mechanisms to resist extremes of pH. Various physiological and biochemical mechanisms of rhizobial adaptation to acidic conditions are reported that include the exclusion and expulsion of H^+, the increase of potassium and glutamate contents in the cytoplasm of stressed cells (Lebranzi and Benbrahim, 2014), the change in the lipopolysaccharides composition, and the accumulation of polyamines/ glutamate concentrations in the cell (Gopalakrishnan *et al.*, 2014 and Lebrazi and Benbrahim, 2013. Watkin *et al.* (2003) reported the ability of acid tolerant *Rhizobium leguminosarum bv.*

trifolii in accumulating higher level of potassium and phosphorous than an acid sensitive strain. The production of acid shock proteins (ASPs) is another common response contributing to this stress tolerance by conferring acid production on the bacteria with no alteration of the cellular pH (John, 1993). Furthermore, several genes, such as *actA, actP, exoR, lpiA, actR, actS* and *phrR* were shown to be essential for rhizobia growth at low pH (Abdel *et al*., 2014).

1.3.9.3 Salinity

Salinity is concentration of dissolved mineral salts comprising cations and anions present in the soil and in water. The principal cations in solution consists of Na^+, Ca^{2+}, Mg^{2+}, and K^+ and anions are Cl^-, SO_4^{2-}, HCO^{3-}, CO_3^{2-}, and NO_3^-. Salinity is one of the major factors threatening agriculture in arid and semi-arid areas and about 40% of the world's land surface can be categorized as having a potential salinity problem (Zahran, 1999). The main cause of salinity is the nutrient imbalance in the soil, which is considered as a constraint influencing the nitrogen fixing symbiosis and the survival of both partners (Mohammad *et al*., 2012).

Salt stress result in growth depression which can be attributed to the accumulation of toxic ions such as sodium and chlorine in plant tissue, where they can disturb enzyme activities (Bordeleau and Prevost, 1994), affect microbial population, plant growth and yield as the result of inhibition of rhizobia-legume symbiosis, nodule initiation and nodule formation (Zahran, 1999), causes nutrient imbalance such as potassium, iron, boron and calcium when nodulated *Pisum sativum* were analyzed (El-hamdaoui *et al*., 2003) and affect the *Rhizobium*- legume symbiosis and nodule formation (Zahran, 1991). They added that the reduction on nitrogen fixing activity by salt stress is usually the result of reduction in respiration of nodules and in cytosolic protein production particularly leg-hemoglobin by nodules. It was reported that rhizobia can generally tolerate a higher level of salinity than the host legume and fast growing rhizobial strains are more salt tolerant than slow growing ones (Serraj and Adu Gyamfi, 2004). Thus, inoculation of legumes with salt-tolerant bacteria could improve the biological nitrogen fixation under salinity stress.

1.3.9.4 Antibiotics and heavy metals

Various antibiotic substances in the soil can be produced either by pathogenic or beneficial bacteria that symbiotically inhabit with the host plant. Thus, soil beneficial bacteria that enhance plant growth and yield through their PGP and biocontrol activities should have mechanisms to tolerate the antibiotics produced. Bacterial resistance to antibiotics as reviewed by Sarma *et al.*, (2010) is achieved through four main strategies: (i) reduction of membrane permeability to antibiotics; (ii) drug inactivation; (iii) rapid efflux of the antibiotic and (iv) mutation of cellular target(s). Biofilm production also has potential contribution in metal and antibiotic tolerance (Thakur *et al.*, 2007).

Heavy metals such as Zn, Cu, Ni and Cr are essential or beneficial micronutrients for plants, animals and microorganisms, whereas others, such as Cd, Hg and Pb have no known biological and/or physiological functions (Perira *et al.*, 2006). The environmental pollution by heavy metals comes from anthropogenic sources such as smelters, mining, power stations and the application of pesticides containing metal, fertilizer and sewage sludge (Munees, 2013). Although many metals are essential, all metals are toxic at higher concentrations, because they cause oxidative stress by formation of free radicals through replacing essential metals in pigments or enzymes disrupting their function (Abou-Shanab *et al.*, 2003).

Soils contaminated with toxic metals can adversely affect both beneficial rhizospheric microbes and plant growth at elevated concentrations (Rajkumar *et al.*, 2006) which can be reduced by applying resistant microorganisms (Wani *et al.*, 2009). Such beneficial bacteria with antibiotic and heavy metal tolerance could be of greater importance both in bioremediation and plant growth promotion. Moreover, the variation in antibiotic resistance shown by bacterial strains could be an indication of useful aspect of antibiotics as complementary tool for characterization and discrimination of rhizobial isolates

(Zahran *et al*., 2012) and is important for their competitiveness in soil (Brockwell *et al*., 1995).

1.3.9.5 Carbohydrate and amino acid utilization

Evaluating the carbohydrate and amino acid utilization patterns of rhizobial strains is essential to determine their nutritional preference for survival advantage in soil (Gauri *et al*., 2011). Variations in utilization pattern among in rhizobia nodulating lentil (Mulisa Jida and Fassil Assefa, 2011; Adigo Setargie *et al*., 2015) and faba bean (Zerihun Belay and Fassil Assefa, 2011; Dereje Tsegaye *et al*., 2015) was observed which indicated variation in rhizobial strain competitiveness capability to metabolize the nutrients as carbon and energy sources (Wielbo, 2012). The variation observed in nutritional utilization of carbohydrates can be used as marker for rhizobial classification (Zabaloy and Gomez, 2005).

1.3.10 Microbial inoculation and co-inoculation

The chemical fertilizers that are currently used to improve soil fertility, crop production and pest control is contaminating deep-water reservoirs; creating significant direct hazards to the rural population, disrupting the local environment and decreasing the quality of products. Moreover, the excessive use of the chemical fertilizers and plant protection chemicals has been affecting the rhizosphere microflora greatly and in place of the beneficial associative bacteria, harmful types are now predominating in the rhizosphere (Verma *et al*., 2009). These negative effects could partially be eliminated by using highly efficient strains of PGPR as one of the most promising biotechnological practices to improve soil fertility, crop production and crop quality with low input of chemical fertilizers, energy and costs. However, not all the rhizobial inoculation gives positive response to nodulation because a variety of biotic or abiotic factors affects nodulation of plants. There were many approaches which tried to overcome this problem. Among them, co-inoculation of rhizobia with proper plant growth promoting rhizobacteria (PGPR) is one of the popular methods. Co-inoculation studies with PGPR and *Rhizobium*/*Bradyrhizobium* spp. have been shown to increase root and shoot biomass, nodule dry matter, nitrogenase activity, N-fixation and grain yield in legumes (Elkoca *et al*., 2008).

The co-inoculation of non-rhizobial plant growth promoting strains to improve the nodulation and N-fixing potential of the inoculated rhizobial strains has received much attention in the recent past. Dual inoculation of *Azotobacter vinelandii* or *Azospirillum lipoferum* with *Rhizobium* strains showed a synergistic effect on nodulation, yield and N uptake in soybean, clover and peanut (Raverker and Konde, 1988). Similarly, co-inoculation of *Pseudomonas* spp. with *Rhizobium* spp. has been reported to enhance nodulation and N- fixation, plant biomass and grain yield in various leguminous crops such as alfalfa (Knight and Langston-Unkefer, 1988) and pea (Bolton *et al*. 1990).

The use of mixed biofertilizers is advocated to get the maximum benefits due to additive and synergistic effect. The role of symbiotic nitrogen fixing bacteria, PGPR and phosphate slolubilising microorganisms in crop productivity is well documented (Hariprasad and Niranjana, 2009). The PGPR play crucial role in soil health and plant growth and have been used in biocontrol of plant pathogens. Co-inoculation studies with PGPR and *Rhizobium*, *Bradyrhizobium* species have shown to increase root and shoot weight, plant vigor, nitrogen fixation and grain yield in various legumes (Yadegari *et al*., 2008). Combined inoculation of *Rhizobium* with *Pseudomonas Striata* or *Bacillus polymyxa* and with *Bacillus megaterium* have shown increased dry matter, grain yield and phosphorus uptake significantly over the uninoculated control in legumes (Elkoca *et al*., 2008). Gross average of seed yield increased from 3000 kg/ha for *Rhizobium* alone to 4693 kg/ha after co-inoculation with *P. Fluorescens* (Yadegari *et al*., 2010).

Chapter 2. Diversity of ecologically competent and symbiotically effective grass pea rhizobia to enhance nitrogen fixation for grass pea (*Lathyrus sativus* L.) and other cross-inoculating cool season legumes in Ethiopia

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Abstract

Grass pea (*Lathyrus sativus* L.) is one of the cool season legume crops widely grown in several countries including Ethiopia. It fixes nitrogen in association with rhizobia that also cross nodulate the vicieae tribe including faba bean (*Vicia faba* L.), field pea (*Pisum sativum* L.), and lentil (*Lens esculenta* L.). Its high protein content and yield potential to enhance soil fertility, and tolerance to extreme environmental factors attract farmers in low input agriculture in Ethiopia. However, it is one of the orphan crops neglected for research due its alkaloid content and lathyrism. In this study, fifty rhizobial isolates from central Ethiopia were isolated and characterized from grass pea nodules using standard methods. The isolates displayed cultural and growth features of the fast growing and acid producing rhizobia. The data showed that all but AAUGR- 47 (98%) of the isolates were authenticated as root nodule bacteria, and 86 % were symbiotically effective accumulating 50-100% of shoot dry matter compared to nitrogen fertilized control plants. The numerical analysis based on 59 phenotypic features grouped the 22 isolates into three distinct clusters at 25 % disimilarity level with a couple of subclusters. The 16S rRNA gene sequence showed that the majority of the isolates (86 %) displayed 99% homology with *Rhizobium* spp., whereas isolates AAUGR-24 and 42 showed 99% identity with *Rhizobium leguminosarum* var viceae. The nifH sequence result of two strains, AAUGR-2 and AAUGR-24 showed 96 and 99% sequence homology with *Ensifer meliloti* and *Rhizobium leguminosarum* type strains indicating the high diversity of grass pea rhizobia other than the specific

Rhizobium leguminosarum var viceae that needs more research using several nod and housekeeiping genes to reveal variability of the endosymbionts. The isolates also showed variations in tolerance to ecological stresses and nutritional versatility. The isolates AAUGR- 2, 5, 6, 9, 11, 14, 15, 19, 20, 24, 30 and 50 performed top on sand culture authentication and were consistently tolerated environmental factors and showed wide range of nutrient versatility. They were thus selected for further PGP and biocontrol assay and BNF evaluation on potted soil under laboratory and greenhouse conditions.

Keywords: Nodulation, heterotrophic competence, PCR, 16S rRNA, taxonomic diversity, stress tolerance

2.1 Introduction

Grass pea *(Lathyrus* spp) is one of the cool-season leguminous crops within the tribe *Viciae* that include the genera *Vicia, Pisum*, and *Lens* that fix inorganic nitrogen with root nodule bacteria known as rhizobia. Previous studies on *Vicia, Pisum, Lathyrus* (grass pea) and Lens (Jordan, 1984; Perret *et al.*, 2000; Rivas *et al.*, 2009) showed the endosymbionts belong to *Rhizobium leguminosarum* biovar *vicieae.* However, recent reports revealed that nodules from *Vicia faba*, *Pisum sativum* and *Lens esculenta* also harbor other groups of root nodule bacteria other than *Rhizobium leguminosarum* biovar *vicieae* (Santillana *et al.*, 2008, Wendwessen Tena *et al.*, 2016). These include *Rhizobium leguminosarum* biovar trifolli that nodulate trifolium spp, *Rhizobium etli* that nodulate *Phaseolus vulgaris* in Ethiopia (Desta Beyene *et al.*, 2004). Recently, Wendwesen Tena *et al.*, 2016) made a polyphasic study on root nodule from lentil, and identified three distinct sub-lineages into *Rhizobium etli* and *Rhizobium leguminosarum*, and *Rhizobium* spp.

Other studies also showed the different levels of symbiotic effectiveness of cross-nodulating rhizobia isolated from one group of host may not necessarily effective on another one indicating diversity and specificity even among the *Rhizobium leguminosarum* var *viceae* populations (Laguerree, 2003; Amha Gebremariam and Fassil

Assefa, 2017). For several years now extensive studies have been under taken on phenotypic and symbiotic properties of the leguminous crops of Viceae tribe in Ethiopia. Some of these studies were focused on Faba bean (Abere Minalku *et al*., 2009; Assefa Keneni *et al*., 2010; Zerihun Belay and Fassil Assefa, 2011; Solomon Legesse and Fassil Assefa, 2013), lentil (Adigo Setargie *et al*., 2015; Mulisa Jida *et al*., 2011;) and field pea (Aregu Asresie *et al*, 2012; Kassa Baye *et al*., 2015), and cross inoculation studies on Viceae tribe (Amha Gebremariam and Fassil Assefa, 2017)

Grass pea (*Latyrus sativus L.*) is one of the widely farmer grown orphan crops which was not given due attention despite the fact it is a source of protein, a break crop (crop rotation/soil amelioration) that is grown on marginal lands and with a residual moisture after the rainy seasons in Ethiopia.The inadvertent neglect of the crop is due to the negative perception of the crop for the seed contains alkaloids associated with lathyrism (Girma Moges *et al.,* 2004).

Although extensive researches have been undertaken on other related pulse crops, little is known about its contribution to soil fertility and plant health by its rhizobia. The limited studies also showed that the root nodulating rhizobia of grass pea were as diverse as other cross nodulating hosts such as faba bean, field pea and lentil (Drouin *et al*., 1996; Mahdavi *et al*., 2007; Aoki *et al*., 2010) contributing to diversity studies on *R leguminosarum* var viceae. The fact that the crop has not been given attention in the scientific community as it was the case with other orphan crops such as chickpea until recently notwithstanding, the exploitation of the fifth most important feed and cover crop in the country can not be realized unless the legume-rhizobium symbiosis and the legume-rhizobacteria association of the crop are studied.

Rhizobial populations are also known to vary in their tolerance to major environmental factors (Wei *et al*., 2008). Consequently, exploiting the enormous potential of symbiotic nitrogen fixation requires the selection and development of efficient inoculant strains compatible to legume host and the target edaphic environment (Bhargava *et al*., 2016). To this end, studying the potential of the isolates for physiological versatility is important to select them for their competitiveness in the complex soil environment (Fitouri *et al.*, 2012). Thus, screening and selecting highly effective and most tolerant isolates with ecological adaptation that can be introduced as microbial inoculants not only for the grass pea crop, but also for the cross-nodulation crops such as faba bean, field pea, and lentil was the main aim of this study.

2.2. Materials and Methods

2.2.1 Sample collection and isolation of rhizobia

Soil and nodules were collected from the rhizosphere and roots of selected standing crops grown from 50 sampling field sites (Fig. 2. 1 and Table 2. 1) from which 35 were from some districts of South Wollo (Jamma, Woreilu, Tenta, Borena, Kalu, Tehuledere and Kutaber), 3 from Oromia zone (Artumafursi) and 12 were from West Shoa (A/ Gindeberet, Jeldu and Dendi). The soil samples were kept in polyethylene plastic bags whereas the nodules were collected in vials containing a desicant (silica gel) covered with 1cm of cotton wool (Somasegaren and Hoben, 1994) and brought to Addis Ababa University.

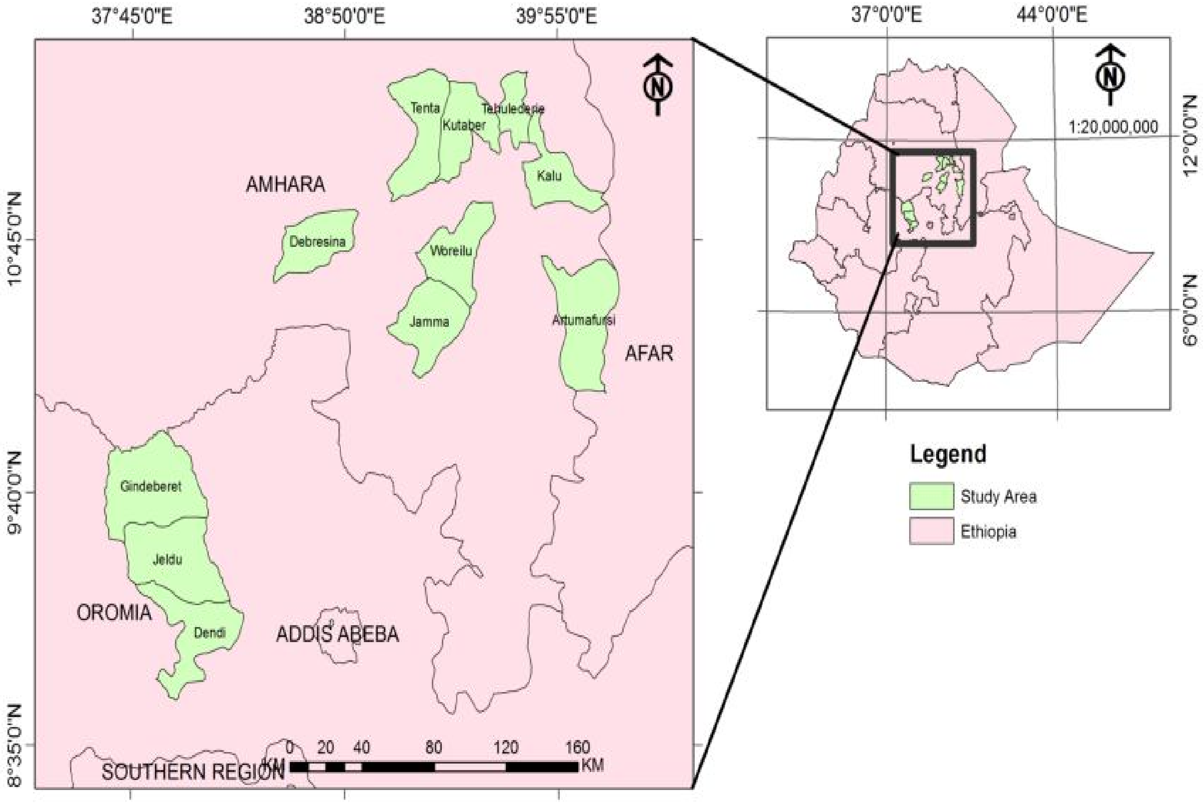

Fig. 2. 1 **Map of the study site**

2.2.2 Isolation and purification of rhizobia

Dehydrated or desiccated root nodules were immersed in sterile distilled water for overnight in labeled sets of petri-dishes (Vincent, 1970) and were then surface sterilized according to Somasegaren and Hoben (1994). They were first subjected to 70% ethanol for 10 seconds and then to 5 % (v/v) solution of sodium hypochlorite (NaOCl) for 4 minutes. The surface sterilized nodules were then rinsed in five changes of sterile distilled water to completely rinse the sterilizing chemicals.

The surface sterilized nodules were transferred into sterile petri-dishes one by one and were crushed with alcohol flamed sterile glass rod in a drop of normal saline solution (0.85% NaCl) inside a laminar air flow hood. A loop-full of suspensions (crushed nodule saps) were streaked across the surface of Yeast Extract Mannitol Agar (YEMA) plates containing 25 ppm of congored (YEMA-CR) and were incubated at 28 ± 2^{0}C for 3-5 days. Single and seemingly different colonies were selected and purified through repeated re-streaking on YEMA-CR and incubated for 3- 5 days. The purified isolates were designated as AAUGR (Addis Ababa University Grasspea Rhizobia) with different numbers representing each strain and kept at -20 ^{0}C for further work.

Table 2. 1 Sampling sites for the collection of soil and nodule samples of grass pea from central parts of Ethiopia

Isolate	Sampling sites			Altitude	Soil pH
	Adm. Zone	District	Kebele		
AAUGR- 1	SW	Borena	Derami	2400.00	6.9
AAUGR -2	SW	Borena	Tewa	2435.00	6.8
AAUGR -3	SW	Borena	D/ Senbo	2507.00	6.7
AAUGR -4	SW	Borena	Dega Dibi	2754.00	7.0
AAUGR -5	SW	Borena	Chefebelo	2563.00	7.1
AAUGR -6	SW	Borena	Hulagosh	2789.00	7.1
AAUGR -7	SW	Tenta	Yamed	2854.00	7.0
AAUGR -8	SW	Tenta	Ababor	3165.00	6.9
AAUGR -9	SW	Tenta	Chelemie	2973.00	6.8
AAUGR -10	SW	Tenta	Ambemariam	2989.00	6.9
AAUGR -11	SW	Tenta	Qul/Amba	2844.00	7.0
AAUGR -12	SW	Woreilu	Degnu	2886.00	6.8
AAUGR -13	SW	Woreilu	Sekela	2778.00	6.9
AAUGR -14	SW	Woreilu	Mariam	2718.00	7.0
AAUGR -15	SW	Woreilu	T/sertsu	2702.00	7.1
AAUGR -16	SW	Woreilu	Geshober	2656.00	7.0
AAUGR -17	SW	Woreilu	Abajale	2684.00	6.9
AAUGR -18	SW	Jamma	Aejerti	2595.00	6.9
AAUGR -19	SW	Jamma	Degolo afaf	2674.00	6.8
AAUGR- 20	SW	Jamma	Debreguracha	2653.00	7.0
AAUGR -21	SW	Jamma	Ketari	2678.00	7.1
AAUGR -22	SW	Kutaber	Hamusit	2687.00	7.0
AAUGR -23	SW	Kutaber	Boru	2652.00	7.1
AAUGR -24	SW	Kalu	Jerjero	1937.00	6.6
AAUGR -25	SW	Kalu	Keteteya	2258.00	6.7
AAUGR -26	SW	Kalu	Adame	2521.00	6.5
AAUGR -27	SW	Kalu	Argeo	2507.00	6.6
AAUGR -28	SW	Kalu	Ancharo	2096.00	6.6
AAUGR -29	SW	Kalu	Ardibo	2438.00	6.5
AAUGR -30	SW	Tehuledere	Gobeya	1984.00	6.8
AAUGR -31	SW	Tehuledere	Jari	1816.00	7.0
AAUGR -32	SW	Tehuledere	Amumo	1937.00	6.9
AAUGR -33	SW	Tehuledere	Hitecha	2054.00	6.9
AAUGR -34	SW	Tehuledere	Bededo	2364.00	6.8
AAUGR -35	SW	Tehuledere	Korkie	2492.00	7.0
AAUGR -36	KS	Artumafursi	Mermashasho	1820.00	6.4
AAUGR -37	KS	Artumafursi	Chireti	1423.00	6.6
AAUGR- 38	KS	Artumafursi	Chireti	1427.00	6.5
AAUGR -39	WS	A/Gindeberet	Kersa	2634.00	6.9
AAUGR -40	WS	A/Gindeberet	Gitre	2653.00	7.0
AAUGR -41	WS	Jedlu	Gojo	2671.00	7.0
AAUGR -42	WS	Jedlu	Tulubultuma	2639.00	6.9
AAUGR -43	WS	Jedlu	Sunko	2661.00	6.8
AAUGR -44	WS	Jedlu	Felo	2669.00	6.8
AAUGR -45	WS	Jedlu	Chilankie	2658.00	7.0
AAUGR- 46	WS	Dendi	Awash Bolto	2650.00	6.7
AAUGR -47	WS	Dendi	Ubdo legabatu	2599.00	6.8
AAUGR -48	WS	Dendi	Ababor	2645.00	6.8
AAUGR -49	WS	Dendi	Awaro kolo	2676.00	6.9
AAUGR -50	WS	Dendi	Tulu meda	2667.00	7.1

SW= South Wollo, KS= Kemissie, WS= West Shewa

2.2.3 Cultural characterization

2.2.3.1 Experimental set up

All tests were conducted in triplicates on YEMA medium inoculated with a loop full culture (~ 10^{6} cells mL^{-1}) and incubated at 28±2 ^{0}C for 3-5 days. For each test, positive control plates were included and rhizobial growth was scored qualitatively by visual inspection as (+) and (-) for positive growth and no growth, respectively.

2.2.3.2 Colony characteristics

The isolates (~ 10^{6} cells mL^{-1}) were inoculated onto YEMA medium to look into colony diameter, texture and rate of growth according to Lupwayi and Haque (1994). They were incubated at 28 ± 2 ^{0}C for 3-5 days. The isolates were inoculated into YEMA- BTB (0.5 % Bromothymol blue) medium and incubated at 28 ± 2 ^{0}C for 3-5 days to detect color change due to acid or alkali production according to Jordan (1984).

2.2.3.3 Growth rate determination (GMT)

Each isolate was inoculated into a 10ml YEM broth (YEMB), vortex-dispersed and shaken on orbital shaker at 120 rev. min^{-1} for 48 hrs at room temperature. Then, half ml of each broth culture (cell suspensions) was inoculated into 50ml sterilized YEM broth in 125ml Erlenmeyer flask and kept on orbital shaker (Gollen hamp, England) at a rev. of 120 min^{-1}. Turbidity was measured by taking samples every 6 hrs and reading optical density (OD 540nm) using spectrophotometry (Jenway, 6405 UV/ VIS spectrophotometer) after calibrating it to zero with sterile un-inoculated YEM broth as a blank. Simultaneously, samples (0.1 ml) were taken serially diluted (10^{-1} – 10^{-10} with sterile distilled water) and dispensed onto sterilized YEMA plates to determine the colony forming units (CFU) (Somasegaren and Hoben, 1994). Mean generation (doubling) time was calculated from the logarithmic phase using the formula:

$\boxed{g = [\log 2\ (t)]/[\log X-\log Xo]}$ where t is time elapsed, Xo is first OD reading and X is second OD reading in logarithmic phase (White, 1995).

2.2.4 Presumptive test for root nodule bacteria

2.2.4.1 Growth on YEMA-CR

In order to distinguish rhizobia from other contaminants, isolates were inoculated and streaked onto YEMA-CR media. They were incubated at 28 ± 2 ^{0}C for 48 hours to detect congo red absorption (Vincet, 1970).

2.2.4.2 Gram reaction

The isolates were tested for their Gram reaction using the KOH method (Buck, 1982). A 24 hour old colony of each rhizobial isolate was picked and mixed with 3 % KOH on clean microscope slide. The mixture was lifted with inoculating loop about 1 cm from the slide to detect the presence of obvious stringiness (viscosity).

2.2.4.3 Growth on Glucose Peptone Agar (GPA) medium

Rhizobial isolates were streaked on glucose peptone agar content of the medium supplemented with bromocrysol purple and incubated at 28 ± 2 ^{0}C for 3-5 days. The presence of growth and pH change of the medium was recorded according to Vincet (1970).

2.2.5 Authentication of the isolates and preliminary screening of their symbiotic effectiveness on sand experiment

All the rhizobial isolates were subjected to authentication test (definitive test) under pot sand culture according to Somasegaren and Hoben (1994). “Wasse” grass pea variety which was obtained from Debrezeit Agricultural Research Centre was surface sterilized using 95% ethanol (briefly) and 3% sodium hypochlorite and rinsed with five changes of sterile water and germinated on water agar (0.75 w/v) for 3 days. Five germinated seeds were transferred into 3kg capacity plastic pots washed with acid (95% H_2SO_4). After 7 days of growth, each seedling was inoculated with 1ml (10^9cells/ml) of 72 hrs YEMB grown culture. The experiment was statistically laid out in a complete random design

(CRD) with three replicates in a greenhouse with a 12hr photoperiod and an average of 28^0C and 15^0C day and night temperature. The treatment included a negative control without treatment with chemical N-fertilizer (KNO_3^-) and inoculation and a positive control treated with 70 mg/l nitrogen (0.05 KNO_3 (w/v)) solution every week. All pots were fertilized with quarter strength of Broughton and Dilworth N-free medium per week and watered every two days.

2.2.6 Relative symbiotic effectiveness of the rhizobial isolates

Sixty days after planting (DAP), plants were uprooted to record nodule number, nodule dry weight and shoot dry weight. The symbiotic effectiveness (SE) of the isolates was calculated using the formula:

$$\%SE = \frac{\text{Inoculated plant SDW}}{\text{N – Fertilized plant SDW}} \times 100 \text{ (Molungoy, 2004)}$$

Where, SDM = shoot dry weight, N= nitrogen, SE= symbiotic effectiveness

The rate of nitrogen fixing effectiveness was evaluated as: Highly effective > 80%, Effective 50-80%, Lowly effective 35-49% and Infective <35%.

2.2.7 Physiological characterization

The highly symbiotically effective (22) isolates were selected for their *in vitro* ecological characteristics; tolerance to pH, temperature, salt, resistant to antibiotics and heavy metals on YEMA medium (Somasegaren and Hoben, 1994); whereas their heterotrophic competence; carbohydrate and amino acid utilization was carried out on basal medium developed by Amarger *et al.*, (1997). All tests were carried out in triplicates inoculating 10^6 cells/ml bacterial cultures and bacterial growth were recorded and compared against their controls.

2.2.7.1 pH, temperature and salt tolerance

Each isolate was grown on YEMA medium to determine their capacity to grow at different pH (4.0, 4.5, 5.0, 8.5, 9.0, 9.5, 10.0); salt concentrations of 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, of NaCl (Bernal and Graham, 2001) and their resistance to different incubation temperature of 4^0C, 10 ^{0}C, 15 ^{0}C, 35 ^{0}C, 40 ^{0}C and 45 ^{0}C as indicated in (Lupwayi and Haque, 1994) and incubated 28±2 ^{0}C for 3-5 days. Growth of bacterial colonies was recorded as an indication of resistance.

2.2.7.2 Inherent antibiotic resistance (IAR) and heavy metal (HM) tolerance of isolates

The inherent antibiotic resistance (IAR) of the rhizobial isolates was determined by inoculating (10^6cells ml^{-1}) on solid YEMA medium containing filter sterilized (0. 22 mm Millipore filters) antibiotics (µg/ml): Ampicillin (30), Chloroamphinicol (40), Erythromycin (30), Nalidixic acid (20), Neomycin (20), Streptomycin (10), and Tetracycline (30). The isolates (10^6 cells/ml) grown for 48 hrs were inoculated to the same medium containing filter-sterilized heavy metals at concentrations (µg/ml): Hg ($HgCl_2$) (5), Kr ($K_2Kr_2O_7$) (50), Mn ($MnCl_2$) (200), Ni ($NiCl_2$) (200), Pb (Pb $(CH_3COO)_2$) (50) and Zn ($ZnCl_2$) (100) culture (Mohamed *et al.*, 2012). All plates were incubated at 28±2 ^{0}C for 3-5 days.

2.2.7.3 Carbohydrate and amino acid utilization

Carbon utilization of isolates was determined on a basal medium containing each of the 12 carbohydrates: starch, cellobiose, dextrin, lactose, sucrose, galactose, maltose, fructose, glucose, arabinose, xylose and sorbitol as 10% (w/v) by reducing the yeast extract to 0.05 gl^{-1} liter following the method of Somasegaran and Hoben (1994). The basal medium contained 1 g of KH_2PO_4; 1 g K_2HPO_4; 0.01 g $FeCl_3.6H_2O$; 0.2 g $MgSO_4.7H_2O$; 0.1 g $CaCl_2$; 15 g agar. Similarly, the ability of isolates to utilize different nitrogen sources was tested on the same basal medium containing each of the 7 amino acids and supplemented with 1g/l of mannitol and lacking ammonium sulphate: L-lysine, L-leucine, L-alanine, Glycine, L-aspargine and L-cystine and L-Diphenylamine (Amarger *et al.*, 1997). The plates were incubated at 28±2 ^{0}C for 3-5 days.

2.2.8 Numerical analysis and construction of dendrogram

The phenotypic variability of the matrix was constructed using 22 isolates and 59 traits; in terms of resistance to temperature, pH, salt, antibiotics, heavy metals and utilizations of carbohydrates and amino acids were used to construct phenogram. Traits were coded 1 for positive and 0 for negative. A computer cluster analysis of the phenotypic variables was carried out using Ward's linkage and Squared Euclidean distance as a measure of dissimilarity coefficient(Ward,1963) and a dendrogram was constructed PAST ver.2.17c (Hammer *et al.*, 2001).

2.2.9 Genetic characterization

Characterization of the isolates at the molecular level was conducted at the laboratory of Institute for Agricultural Biosciences, Oklahoma State University.

2.2.9.1 PCR method of amplification

Fourteen and two rhizobial isolates that showed better performance in greenhouse and stress tolerance tests and for which amplicons were obtained were selected for 16S rRNA and nif-H gene characterization. A small piece of a single colony from 48 hrs rhizobia was taken using sterile pipette tip and added into a 50 µl PCR mix containing phusion DNA polymerase mix. A typical PCR reaction containing 10µl 5x phusion HF buffer, 1µl 10mM dNTPs, 2.5µl 10µM forward primer, 2.5µl 10µM reverse primer, 0.5 µ phusion DNA polymerase, DNA template (single colony) and 33.5 µl Nuclease Free Water was thoroughly mixed by pipetting up and down (Kun *et al.*, 2016) and the PCR for 16S rRNA was run under the following conditions: preheating at 98^{0}C for 30'', 35 cycles of 98^{0}C for 10'', 56 ^{0}C for 30'', 72 ^{0}C for 30'' and a final extension at 72 ^{0}C for 10' (Weisburg *et al.*, 1991) (Table 2. 2). The final volume of PCR reaction for nifH was 50 µl and cycles for amplification were performed as follows: 2 min at 94 ^{0}C; 35 cycles of 30 s at 94 ^{0}C, 30 s at 57 ^{0}C, 2 min at 72 ^{0}C; followed by 5 min at 72 ^{0}C.

Table. 2.2 Primers used for PCR amplification of 16S rRNA and nif-H genes

S/N	Primer	Sequence	Length of PCR product	Reference
1	16S rRNA (8.27F)	5’ AGA GTT TGA TCC TGG CTC AG 3’	1473	(Weisburg *et al*., 1991)
	16S rRNA-R(rD1)	5' ACGGCTACCTTGTTA CGACTT 3'	1473	(Weisburg *et al*., 1991)
2	nif-H- F	5’ TAC GGN AAR GGS GGN ATC GGCAA 3’	780	(Bontempts et al., 2010)
	nif-H- R	5’ AGC ATG TCY TCS AGY TCN TCC A 3’	780	(Bontempts et al., 2010)
3	nif-H- 1	5'-AAGTGCGTGGAGTCCGGTGG-3'	500	(Eardly *et al.*, 2005)
	nif-H- 2	5'-GTTCGGCAAGCATCTGCTCG-3'	500	(Eardly *et al.*, 2005)

2.2.9.2 Gel electrophoresis, extraction and purification of PCR products

A total of 50µl amplified PCR products of each sample was loaded on agarose gel (2%) containing ethidium bromide and run in 1 TAX buffer at 120 volts for 1 hr. Gel purification, visualization and documentation was done using the Gel Logic 200 Imaging System (Sambrook *et al*., 1989). The PCR amplicons of 14 16S rRNA rhizobial genes and 2 nifH genes were selected and extracted from the gel and purified using PCR purification kit (QIAGEN) according to the standard protocol recommended by the manufacturer.

2.2.9.3 Sequencing and phylogentic analysis of 16S rRNA and nif-H genes

The gene fragments were directly sequenced at Noble DNA sequencing division (USA) from the 16S rRNA and nif-H PCR products of both strands using the same primers used in PCR amplification. Sequence results were edited using Bioedit software. The 16S rRNA and nif-H gene sequences were compared to *Rhizobium* group and *Rhizobium leguminosarum* sequences in the public database using Basic Local Alignment Search

Tool (BLAST) on the National Center for Biotechnology Information (NCBI) website (Shayne *et al.,* 2003). They were aligned with highly similar sequences from Genbank with ClustalW and the evolutionary history was inferred using the maximum composite likelihood method (Saitou & Nei, 1987).

2.2.10 Data analysis

The data recoded were analyzed and interpreted using ANOVA. The experimental treatments were compared and contrasted against their control following Duncan's multiple range test (DMRT). The correlation between different greenhouse data was evaluated by Pearson correlation coefficient using SPSS v.20.

2.3 Result and Discussion

2.3.1 Isolation and purification of the isolates

A total of 50 legume nodulating bacteria were isolated from grass pea nodule samples of South Wollo, Kemissie and West Shewa of which, 35 were from South Wollo, 3 from Kemissie and 12 were from West shewa.

2.3.2 Presumptive tests of the isolates

All isolates except AAUGR - 47 were Gram negative bacteria, failed to grow on PGA medium, and absorb congored. Therefore, based on the presumptive tests, 98% of the isolates were authenticated as root nodule bacteria (Vincet, 1970).

2.3.3 Cultural characterization and growth rate of the isolates

The isolates showed variations in colony size in that 7 isolates (14 %) that included AAUGR- 2, 5, 11, 12, 30, 44 and 50 displayed the highest colony size (5.5 mm) whereas 10 (20.4%); AAUGR- 3, 9, 15, 17, 19, 21, 22, 33, 41 and 43 displayed the smallest colony diameter of 2.0 mm. Dereje Tsegaye *et al*. (2015) reported a colony size of less than 3 cm and greater than 5 cm. The isolates exhibited large watery (LW) (45%) and large mucoid (LM) (55%) colony textures, respectively. Solomon Legesse and Fassil

Assefa (2013) reported 81 % and 19 % of faba bean rhizobia that displayed large mucoid and large watery characteristics, respectively. Most of the isolates (86 %) displayed 1.27-3.93 hrs whereas, 7 (14 %) of them showed doubling time above 4 hrs. Zerihun Belay and Fassil Assefa (2011) reported 19 % and 81 % faba bean rhizobia that showed generation time of greater than and less than 4 hrs, respectively. All these colony and growth features accompanied by acid production (yellow on YEMA- BTB) medium were characteristics of fast growing and acid producing rhizobia.

Table. 2. 3 Cultural characteristics of representative isolates of grass pea grown on YEMA at 28±2 ^{0}C **for 3- 5 days**

Isolate	Sampling sites	Colony diameter (mm)	Colony morphology	Growth on YEMA-BTB	Growth on YEMA-CR	PGA-BCP	MGT
AAUGR- 1	Derami	2.5	LW	Yellow	Colourless	NG	1.27
AAUGR -2	Tewa	5.5	LM	Deep yellow	Colourless	NG	2.29
AAUGR -3	D/ Senbo	2.0	LW	Yellow	Colourless	NG	1.89
AAUGR -4	Dega Dibi	3.5	LM	Deep yellow	Colourless	NG	2.19
AAUGR -5	Chefebelo	5.5	LW	Yellow	Colourless	NG	2.35
AAUGR -6	Hulagosh	2.5	LW	Yellow	Colourless	NG	3.16
AAUGR -7	Yamed	3	LM	Yellow	Colourless	NG	4.27
AAUGR -8	Ababor	3	LM	Deep yellow	Colourless	NG	3.81
AAUGR -9	Chelemie	2	LW	Yellow	Colourless	NG	1.64
AAUGR -10	Ambemariam	3.5	LM	Deep yellow	Colourless	NG	3.69
AAUGR- 11	Qul/Amba	5.5	LW	Yellow	Colourless	NG	3.31
AAUGR -12	Degnu	5.5	LW	Yellow	Colourless	NG	2.47
AAUGR -13	Sekela	3.5	LW	Yellow	Colourless	NG	4.12
AAUGR -14	Mariam	2.5	LW	Yellow	Colourless	NG	3.51
AAUGR -15	T/sertsu	2	LW	Yellow	Colourless	NG	3.65
AAUGR -16	Geshober	3	LM	Yellow	Colourless	NG	3.23
AAUGR -17	Abajale	2	LW	Yellow	Colourless	NG	3.91
AAUGR -18	Aejerti	4	LM	Deep yellow	Colourless	NG	3.57
AAUGR -19	Degolo afaf	2	LW	Yellow	Colourless	NG	1.87
AAUGR- 20	Debreguracha	3	LW	Yellow	Colourless	NG	1.98
AAUGR -21	Ketari	2	LW	Yellow	Colourless	NG	3.79
AAUGR -22	Hamusit	2	LW	Yellow	Colourless	NG	2.35
AAUGR -23	Boru	3	LM	Yellow	Colourless	NG	2.89
AAUGR -24	Jerjero	3.5	LW	Deep yellow	Colourless	NG	2.13
AAUGR -25	Keteteya	3.5	LM	Deep yellow	Colourless	NG	1.45
AAUGR -26	Adame	3	LM	Yellow	Colourless	NG	2.93
AAUGR -27	Argeo	3.5	LM	Moderate yellow	Colourless	NG	3.34
AAUGR -28	Ancharo	2.5	LM	Yellow	Colourless	NG	3.24
AAUGR -29	Ardibo	2.5	LM	Yellow	Colourless	NG	2.15
AAUGR -30	Gobeya	5.5	LW	Deep yellow	Colourless	NG	3.01
AAUGR -31	Jari	3	LM	Moderate yellow	Colourless	NG	4.22
AAUGR -32	Amumo	2.5	LM	Yellow	Colourless	NG	2.93
AAUGR -33	Hitecha	2	LW	Moderate yellow	Colourless	NG	1.99
AAUGR -34	Bededo	3.5	LM	Moderate yellow	Colourless	NG	3.85
AAUGR -35	Korkie	4	LM	Yellow	Colourless	NG	4.73
AAUGR -36	Mermashasho	4.5	LM	Yellow	Colourless	NG	2.21
AAUGR -37	Chireti	4	LM	Yellow	Colourless	NG	3.32
AAUGR- 38	Chireti	3.5	LM	Moderate yellow	Colourless	NG	2.49
AAUGR -39	Kersa	3	LM	Yellow	Colourless	NG	3.72
AAUGR -40	Gitre	3.5	LM	Deep yellow	Colourless	NG	2.19
AAUGR -41	Gojo	2	LW	yellow	Colourless	NG	4.33
AAUGR -42	Tulubultuma	3.2	LW	Deep yellow	Colourless	NG	4.05
AAUGR -43	Sunko	2	LM	Yellow	Colourless	NG	1.97
AAUGR -44	Felo	5.5	LW	Moderate yellow	Colourless	NG	2.56
AAUGR -45	Chilankie	4.5	LW	Deep yellow	Colourless	NG	3.87
AAUGR- 46	Awash Bolto	4	LM	Yellow	Colourless	NG	2.83
AAUGR -47	Ubdo legabatu	-	-	-	-	-	-
AAUGR -48	Ababor	4	LM	Deep yellow	Colourless	NG	4.31
AAUGR -49	Awaro kolo	4.5	LM	Deep yellow	Colourless	NG	2.99
AAUGR -50	Tulu meda	5.5	LM	Moderate yellow	Colourless	NG	3.93

SW= South Wollo, KS= Kemissie, WS= West Shewa, -ve= no growth, MGT= mean generation time, PGA= peptone glucose agar, YEMA-BTB= yeast extract mannitol agar- bromothymol blue, YEMA-CR= yeast extract mannitol agar-congo red, Gm= gram negativity reacton, NG= No growth, LM= large mucoid, LW= large watery

2.3.4 Symbiotic effectiveness of the isolates on sand culture under greenhouse condition

Among the rhizobial isolates authenticated under green house conditions, 98 % of them induced nodules on the host plant. Earlier studies on authentication of rhizobia nodulating cross inoculation legumes reported 74 % - 100 % nodule induction (Tables 2.5). Variations in nodulation and growth characters were observed (Table 2. 4). Accordingly, the isolates induced nodules on the host plant with nodule number (NN) ranging between 17 /plant (AAUGR-22) up to 116 /plant (AAUGR -15, *Rhizobium* sp.) and mean nodule dry weight ranging from 0.010g/p (AAUGR- 39) to 0.098 g/p (AAUGR- 15) indicating 7 and 10 times difference between the highest and lowest nodulating isolates, respectively. Other authors that worked on cross inoculation group rhizobia also reported similar variations ranging from 2.5 times to 9 times and 2.5 times and 15 times for nodule number/p and nodule dry weight/p, respectively (Table 2.5). This indicated the existence of variations in rhizobial effectiveness which accounts due to low rhizobial density, incompatibility of the rhizobia, and difference in rhizobial strain and host plant type (Vincet, 1970).

Table 2.4. Authentication of the isolates under greenhouse conditions

Isolate code	Collection sites	NN/p	NDW/p (g)	SDW/p (g)	% SE	Rate
AAUGR 1	SW	49±2 ^{ijk}	0.015±0.000 ^{pqr}	0.477±0.054^{h}	62.5	E
AAUGR 2	SW	71±6.7^{def}	0.049±0.005 ^{ghi}	0.778±0.026^{def}	101.2	HE
AAUGR 3	SW	48.± 5.7.6^{ijkl}	0.039±0.017 ^{ijk}	0.707±0.047^{efg}	92.7	HE
AAUGR 4	SW	28.7 ± 12.7^{nopq}	0.013±0.001 ^{qr}	0.275±0.138^{ijk}	36.0	LE
AAUGR 5	SW	98.3±6.1^{c}	0.062±0.006 ^{ef}	0.898±0.027^{jkl}	117.7	HE
AAUGR 6	SW	103.3±9.7 ^{abc}	0.066±0.008 ^{de}	0.988±0.102^{bc}	129.5	HE
AAUGR 7	SW	50±3.6^{hijk}	0.027±0.005 ^{jklmn}	0.668±0.008^{fg}	87.6	HE
AAUGR 8	SW	50.3±8.1^{hijk}	0.030±0.005 ^{jklmn}	0.714±0.344^{efg}	93.6	HE
AAUGR 9	SW	102±7.0^{bc}	0.074±0.005 ^{cd}	1.005±0.088^{bc}	131.7	HE
AAUGR 10	SW	37.7±9.6 ^{klmno}	0.013±0.001 ^{qr}	0.438±0.092^{hi}	57.4	E
AAUGR 11	SW	105.7±7 ^{abc}	0.082±0.005 ^{bc}	1.013±0.080^{bc}	132.8	HE
AAUGR 12	SW	67±2 ^{defg}	0.035±0.005 ^{ijklmn}	0.785±0.103^{def}	102.9	HE
AAUGR 13	SW	31±2 ^{mnopq}	0.014±0.001 ^{qr}	0.484±0.068^{h}	63.4	E
AAUGR 14	SW	115.3±8.5^{ab}	0.085±0.006 ^{b}	1.060±0.089^{ab}	138.9	HE
AAUGR 15	SW	116±9.5^{a}	0.098±0.002 ^{a}	1.148±0.191^{a}	150.6	HE
AAUGR 16	SW	51±7 ^{hijk}	0.028±0.006 ^{ijk}	0.670±0.058^{fg}	87.8	HE
AAUGR 17	SW	53±8.2 ^{ghij}	0.034±0.012 ^{ijklmn}	0.653±0.050 ^{fg}	82.6	HE
AAUGR 18	SW	28.3±3.1^{nopq}	0.012±0.00^{qr}	0.318±0.024^{ijkl}	41.7	LE
AAUGR 19	SW	60±2.7^{efghi}	0.048±0.006^{ghi}	0.763±0.024^{efg}	100.1	HE
AAUGR 20	SW	80±8.5^{d}	0.056±0.006^{fg}	0.845±0.069^{de}	110.7	HE
AAUGR 21	SW	42.7±7.1^{jklmn}	0.011±0.003 ^{r}	0.366±0.127^{ijkl}	49.3	E
AAUGR 22	SW	17.7±2.1 ^{q}	0.011±0.002 ^{r}	0.306±0.044^{ijkl}	40.1	LE
AAUGR 23	SW	27±3 ^{opq}	0.013±0.001 ^{qr}	0.398±0.038^{hijkl}	52.2	E
AAUGR 24	SW	72.3±8.3 ^{de}	0.051±0.008 ^{gh}	0.763±0.015^{defg}	100.1	HE
AAUGR 25	SW	33. 7±4.5 ^{mnopq}	0.020±0.00 ^{opqr}	0.432±0.202^{hi}	56.6	E
AAUGR 26	SW	64.3±7.2 ^{efgh}	0.041±0.007 ^{hij}	0.728±0.031^{efg}	95.4	HE
AAUGR 27	SW	57.7±21.0 ^{fghij}	0.025±0.017 ^{nop}	0.429±0.074^{hi}	56.2	E
AAUGR 28	SW	20±3.5^{pq}	0.026±0.005 ^{lmno}	0.365±0.103^{ijkl}	49.7	E
AAUGR 29	SW	33.7±6.7 ^{mnopq}	0.019±0.004 ^{opqr}	0.363±0.085^{ikjl}	49.6	E
AAUGR 30	SW	68.3±9.5^{def}	0.041±0.006 ^{hij}	0.764±0.017^{defg}	100.1	HE
AAUGR 31	SW	26±5.3^{opq}	0.024±0.003 ^{nopq}	0.395±0.032 ^{hijkl}	51.8	E
AAUGR 32	SW	28±2.7 ^{nopq}	0.049±0.006 ^{klmno}	0.422±0.056 ^{hij}	55.3	E
AAUGR 33	SW	27.3±4.2 ^{opq}	0.029±0.004 ^{klmno}	0.383±0.049 ^{hijkl}	50.2	E
AAUGR 34	SW	24.7±3.5 ^{opq}	0.029±0.003 ^{klmno}	0.456±0.110 ^{hi}	59.8	E
AAUGR 35	SW	42.7±13.7 ^{jklmn}	0.029±0.006 ^{klmno}	0.377±0.063 ^{hijkl}	49.4	E
AAUGR 36	KS	26.7±4.5 ^{opq}	0.028±0.004 ^{klmno}	0.274±0.030 ^{jkl}	35.9	LE
AAUGR 37	KS	67±4 ^{defg}	0.040±0.006 ^{ijk}	0.680±0.035^{fg}	89.1	HE
AAUGR 38	KS	43.7±3.2 ^{jklm}	0.034±0.002 ^{ijklmn}	0.448±0.021 ^{hi}	58.7	E
AAUGR 39	WS	37.3±8.5 ^{klmno}	0.010±0.012 ^{rs}	0.265±0.044 ^{kl}	34.7	LE
AAUGR 40	WS	51.7±5.0 ^{hijk}	0.028±0.003 ^{klmno}	0.436±0.013 ^{hi}	57.1	E
AAUGR 41	WS	37.3±6 ^{klmno}	0.025±0.010 ^{mnop}	0.411±0.032 ^{hijk}	53.9	E
AAUGR 42	WS	51.5±5.0 ^{hijk}	0.028±0.002 ^{klmno}	0.435±0.013 ^{hi}	80.5	HE
AAUGR 43	WS	45±15.4 ^{jklm}	0.013±0.001 ^{qr}	0.273±0.047 ^{jkl}	35.8	LE
AAUGR 44	WS	43.7±14. ^{jklm}	0.030±0.009 ^{jklmno}	0.371±0.116 ^{hijkl}	49.6	E
AAUGR 45	WS	28±4.4^{nopq}	0.013±0.001 ^{qr}	0.385±0.148 ^{hijkl}	50.5	E
AAUGR 46	WS	61.3±4.7^{efghi}	0.039±0.006 ^{ijk}	0.619±0.064 ^{g}	81.1	HE
AAUGR 48	WS	36.7±5.5^{klmno}	0.013±0.001 ^{qr}	0.315±0.021 ^{ijkl}	41.3	LE
AAUGR 49	WS	31±8.9^{mnopq}	0.030±0.004 ^{jklmno}	0.405±0.079 ^{hijkl}	53.1	E
AAUGR 50	WS	71±7.8^{def}	0.049±0.007 ^{ghi}	0.779±0.027 ^{def}	102.1	HE
+ve		.0000 ^{r}	0.000±0.000 ^{ijk}	0.763±0.011^{defg}	100	-
-ve		.0000 ^{r}	0.000±0.000s	0.237±0.074 l^{lm}	-	-

The means followed by different letters are significantly different at $P < 0.01$ (Duncan test)- SPSS 20.0 version. HE= >80%, E= 50-80%, LE= 35-49%, IE= < 35%, NN= nodule number, NDW= nodule dry weight, SDW= shoot dry weight, %SE= percent symbiotic effectiveness, p=plant

The difference in shoot dry matter accumulated between the highest (1.15g/p) and the least (0.27g/p) inoculated plant by isolate AAUGR-15 (*Rhizobium* sp.) and AAUGR-38, respectively was greater than four folds. Similarly, variation in shoot dry weight was reported from earlier works (Table 2.5). The host plants inoculated with AAUGR (*Rhizobium sp*.- 15, 14, 11, 6) and isolate AAUGR-5 that displayed similar pattern in NN and NDW showed high correlation between nodule dry weight and shoot dry weight (r= 0.864 and 0.817).

Based on the relative shoot dry matter accumulation of the inoculated plants with uninoculated and nitrogen fertilized control (positive control), 22 (45%) of the isolates were highly effective in nitrogen fixation with >80 % of shoot dry matter accumulation whereas, 20 (41 %) of them accumulated 50- 80% of shoot dry matter compared to the nitrogen-fertilized positive control (Table 2. 4). Among the isolates, 13 (27 %) that included AAUGR- 2, 5, 6, 9, 11, 12, 14, 15, 19, 20, 24, 30 and 50 accumulated higher shoot dry weight than the positive control. In general, 86 % of the isolates performed well in their symbiotic effectiveness. Likewise, several authors reported 55 % - 100 % effective and highly effective isolates compared to the positive control (Table 2.5) that may depend on differences in rhizobial strains and plant species (or cultivars) (Neelawan, 2012). The correlation between shoot dry weight and percent symbiotic effectiveness showed a relatively more direct and perfect linear relationship than the other parameters (r= 0.988). According to Mulongoy (2008), shoot dry matter is a good indicator of relative effectiveness and there exists a sound correlation between the nitrogen fixing capacity of legumes and their shoot dry matter accumulation.

Table 2. 5 Pattern of symbiotic effectiveness of *Rhizobium*species of cross inoculating hosts of the viciae family

Researcher	Host plant	% of isolates authenticated	Nodule data/p						Shoot data/plant				
			Minimum NN/p	Maximum NN/p	Difference between max. and min. NN/p	Minimum NDW/plant	Maximu NDW/plant	Difference between max. & min. NDW/p	Minimum SDW/plant	Maximum SDW/plant	Difference between max. & min. SDW/p	% isolates that scored SE above 50%	Max. % SE on sand
Abere Minalku *et al*., 2009	*Vicia faba* L.	100	60	183	3X	0.045	0.125	2.8x	0.061	2.31	38x	55	100.9
Anteneh Argaw (2012)	*Vicia faba* L.	82	60	325.3	5.4x	0.059	0.298	5x	1.233	4.3	3.5x	100	169.8
Solomon Legesse & Fassil Assefa (2014)	*Vicia faba* L.	75	18	91	5x	0.02	0.05	2.5x	0.57	1.5	2.6x	74	115
Zerihun Belay & Fassil Assefa (2011)	*Vicia faba* L.	95	67	168	2.5x	0.04	0.1	2.5x	0.4	2.3	5.8x	81	96
Amha Gebremariam and Fassil Assefa(2017)	*Vicia faba* L.	-	25	118	4.7x	0.006	0.090	15x	2.80	3.17	1.1x	80	96
Amha Gebremariam and Fassil Assefa(2017)	*Pisum sativum* L.	-	25	120	5x	0.007	0.054	7.7	0.54	1.56	2.9x	80	101
Aregu Amsalu *et al*., 2012	*Pisum sativum* L.	96	29	108	3.7x	ND	ND	ND	0.68	3.35	5x	79	133
Amha Gebremariam and Fassil Assefa(2017)	*Lens culinaris*	-	18	55	3x	0.002	0.017	8.5x	0.05	0.1	2x	80	91
Anteneh Argaw (2012)	*Lens culinaris*	74	17	68	4x	0.002	0.013	6.5x	0.079	0.36	4.5x	98.4	275
Mulisa Jida & Fassil Assefa (2011)	*Lens culinaris*	86	21	62	3x	0.004	0.1	25x	0.34	0.73	2.2	87	100
Mekasha Tsegaye *et al*., 2015	*Trigonellafoenum -graecum* L.	88	10	41	4x	0.010	0.027	2.7x	0.046	0.086	2x	88	84
Amha Gebremariam and Fassil Assefa(2017)	*Lathyrus sativus*	-	24	135	5.4x	0.035	0.086	7.7x	0.11	0.21	2x	60	84
Mussa Adal (2009)	*Lathyrus sativus*	93	27	135	9x	0.011	0.086	7.8x	0.066	0.296	4.5x	61	120
This study	*Lathyrus sativus*	98	18	116	6.4x	0.011	0.098	9x	0.264	1.15	4.4x	86	151

ND= not determined, NF =not found

2.3.5 Pattern of ecological tolerance of isolates

All the isolates showed significant difference in their eco-physiological tolerance to pH, salt, temperature, IAR, antibiotic, heavy metals, and nutritional versatility to utilization of carbohydrates and amino acids substrates (Table 2. 6).

Fig. 2. 6 Ecophysiological tolerance and nutrional versatility of highly effective grass pea isolates grown on YEMA/YEMB medium

Isolates	Sampling sites	% utilization		Temperature Stress tolerance			IAR resistance		HM tolerance			
		CHOs	Aas	Temp. (^{0}C)	pH	%Salt	%IAR	Antibiotics	%	HM cpds resisted	Total	Rank
AAUGR -2	Tewa	1	1	1	1	1	1	AM, ER, CL, NA, NE, ST	1	Hg, Cr, Mn, Ni, Pb, Zn	7	1
AAUGR- 3	D/ Senbo	1	2	1	2	3	3	AM, ER, NA, NE	3	Mn, Pb, Zn	15	14
AAUGR -5	Chefebelo	1	1	1	1	1	2	AM, ER, NA, NE, TE	1	Cr, Mn, Ni, Pb, Zn	8	5
AAUGR -6	Hulagosh	1	1	1	1	1	1	AM, ER, CL, NA, NE, ST, TE	1	Cr, Mn, Ni, Pb, Zn	7	1
AAUGR -7	Yamed	2	3	3	3	2	3	ER, NA, NE	3	Mn, Pb	19	21
AAUGR -8	Ababor	3	3	3	3	2	3	ER, NA, NE, TE	3	Mn, Zn	20	22
AAUGR -9	Chelemie	1	1	1	1	1	1	AM, ER, CL, NA, NE, ST, TE	1	Hg, Cr, Mn, Ni, Pb, Zn	7	1
AAUGR- 11	Qul/Amba	1	1	1	1	1	1	AM, ER, CL, NA, NE, TE	1	Cr, Mn, Ni, Pb, Zn	7	1
AAUGR -12	Degnu	2	2	3	3	2	3	ER, CL, NA, NE	3	Mn, Ni	18	20
AAUGR -14	Mariam	1	1	1	2	1	1	AM, ER, CL, NA, NE, TE	1	Cr, Mn, Ni, Pb, Zn	8	5
AAUGR -15	T/sertsu	1	1	1	2	1	1	AM, ER, CL, NA, NE, ST	2	Cr, Mn, Ni, Pb	9	8
AAUGR -16	Geshober	1	2	2	3	3	3	ER, CL, NA, TE	3	Cr, Pb, Zn	17	17
AAUGR -17	Abajale	1	2	2	3	3	3	AM, ER, NA, NE	3	Mn, Zn	17	17
AAUGR -19	D/ afaf	1	1	3	1	3	1	AM, ER, CL, NA, NE, TE	2	Mn, Ni, Pb, Zn	12	10
AAUGR- 20	D/guracha	1	1	2	1	2	2	AM, ER, NA, NE, TE	2	Cr, Mn, Ni, Pb	11	9
AAUGR -24	Jerjero	1	1	1	1	1	1	AM, ER, CL, NA, NE, ST, TE	2	Cr, Mn, Ni, Pb, Zn	8	5
AAUGR -26	Adame	1	2	2	3	2	3	ER, NA, TE	3	Mn, Zn	17	17
AAUGR -30	Gobeya	1	2	1	3	2	1	AM, ER, CL, NA, NE, ST	2	Hg, Mn, Ni, Pb	12	10
AAUGR -37	Chireti	1	3	2	2	2	3	ER, CL, NA	2	Mn, Ni, Pb, Zn	15	14
AAUGR -42	Tulubultuma	1	3	2	2	2	3	ER, CL, NA	2	Mn, Ni, Pb, Zn	15	14
AAUGR- 46	Awash Bolto	1	2	2	3	3	3	CL, NA, NE	1	Hg, Cr, Mn, Pb, Zn	15	14
AAUGR -50	Tulu meda	1	2	2	1	2	2	AM, ER, NA, NE	2	Hg, Cr, Mn, Ni, Pb, Zn	12	10

CHos= carbohydrates, AA= amino acids, IAR= intrinsic antibiotic resistance, HM= heavy metal, cpds= compounds, AM= ampcillin, ER= erythromycin, CL= chloroamphenicol, NA= nalidixic acid, NE= neomycin, ST=stryptomycin, TE= tetracycline; (1=80-100%; 2=60-79%;3=<60%), ($T^o{}_c$ = 1=10-40, 2=15-35, 3=20-40) (salt %= 1=1-7/8, 2= 1-5/6, 3= <5), (%pH= 1= 4-10, 2= 5-9, 3= 6-9). Rank on pattern of resistance, tolerance of ecological stress and substrate utilization. Cumulative=7-10(best); 10-15=intermediate; <16=poor performers.

2.3.5.1 Tolerance to salt, pH and temperature

The potential of the isolates to tolerate a wide range of salt, pH and temperature was determined (Table 2. 6). All isolates displayed growth at 3% salt concentrations whereas; 14% of the isolates were tolerant to 8% NaCl. Likewise, rhizobial isolates from V*icia faba*, *Lens culinaris*, *Pisum sativum* and *Lathyrus sativus* tolerated salt concentrations of 8 % and above (Table 2. 7) indicating that fast growing rhizobia are salt tolerant (Zahran, 1999). Isolates AAUGR- 2, 5, 6, 9, 11 and 24 tolerated all the tested salt concentrations which could be used as a remedy in areas where 40% of the world's land surface is categorized as having potential salinity problems (Zahran, 1999).

All isolates were tolerant to 6- 9 % of pH whereas, 5 (23 %) grew at pH 4% and 9 (40 %) grew on the medium adjusted at pH 10 indicating that the isolates were more tolerant to high pH than acidic pH. Lebrazi and Benbrahim (2014) reported that rhizobia can grow at high pH since alkalinity is less harmful to the survival of rhizobia. Four (18%) isolates that include AAUGR- 2, 6, 9 (*Rhizobium* species) and 24 (*Rhizobium leguminosarum*) tolerated the minimum and maximum pH of 4 and 10. Previous works on pH tolerance of rhizobia of cross inoculation groups and other rhizobia reported percent tolerance greater than the value recorded in this work (Table 2.7). Although all of the isolates grew between 20 and 35 ^{0}C (Table 2. 6), a progressive decline was observed below and above these temperatures of less 20 and above 40 ^{0}C. Seven (32 %) and 2 (9 %) of the isolates were tolerant to wide range of temperature between 10 and 40 ^{0}C that was similar to other rhizobia isolated from different legumes that grew at temperatures between 10 – 45 ^{0}C (Table 2. 7) implying that rhizobia are tolerant to heat ((Michiels *et al.*, 1994) and freezing temperature which is as low as -10 ^{0}C (Cloutier *et al*., 1992).

2.3.5.2 Intrinsic antibiotic and heavy metal resistance

The isolates also showed variations in antibiotic resistance (IAR) and heavy metal resistance (HMR) (Table 2. 6). Many isolates were resistant to nalidixic acid, erythromycin and neomycin but were sensitive to streptomycin and tetracycline. This result was similar to the work of Hewedy *et al.*, (2014) and Assefa Kenenni *et al*., (2010) who reported faba bean rhizobia sensitive to tetracycline at 30 µg/ml concentrations and to the findings of Tolera *et al*., (2015) who reported the sensitivity of all faba bean isolates to tetracycline and chloroamphenicol. Among the isolates; AAUGR- 6, 9, 24, 2, 11, 14, 15, 19 and 30 were tolerant to the majority of the tested antibiotics. This is contrary to the result of Zahran *et al.*, (2012) who reported that streptomycin, tetracycline and nalidxic acid and erythromycin were sensitive and resistant to the tested isolates.The variation in antibiotic resistance is a useful marker for the characterization and discrimination of rhizobial isolates (Zahran *et al*., 2012) and is important for identifying their competitiveness in soil (Brockwell *et al*., 1995).

The isolates were resistant to manganese (96%), lead (82%), and Zn (77%); and fairly resistant to nickel (68%), cromium (55%), but sensitive to mercury (23%), and 40% of the isolates; AAUGR- 9, 50, 5, 6, 11 14, 24, and 46 were highly tolerant to many of the tested heavy metals. Three (14 %) of the isolates (AAUGR- 2, 9 and 50) tolerated all the tested heavy metals. Zerihun Belay and Fassil Assefa (2011) reported 12 % of faba bean rhizobia that tolerated all the tested heavy metals. Isolates AAUGR- 2, 6, 9, 11, 24 and 50 that showed the highest resistance to the tested antibiotics also showed the same pattern to the tested heavy metals which indicated correlation between metal and antibiotic tolerance in bacteria because of the likelihood that resistance genes to both (antibiotics and heavy metals) may be located closely together on the same plasmid in bacteria and are thus more likely to be transferred together in the environment (Elizabeth, 2003).

2.3.5.3 Pattern of carbohydrate and amino acid utilization of isolates

The potential of the isolates to utilize a wide range of carbohydrates and amino acids showed that they catabolized 80- 100 % of carbon and 46 – 100 % amino acid sources (Table 2. 6). With regard to pathern of carbohydrates utilized, all isolates utilized most of the monosaccharides and disaccharides except trehalose (63.6) and Na- citrate (41%) that were utilized by fewer isolates. Isolates AAUGR 2, 9, 11, 12, 24, and 42 utilized all the tested carbohydrates whereas AAUGR- 2, 5, 11 and 24 catabolized all nitrogen sources indicating the versatility of the isolates to utilize the carbon and nitrogen sources. Studies showed that faba bean rhizobia (Zerihun and Fassil, 2011) and lentil rhizobia (Mulisa and Fassil, 2011) utilized 80- 100 % and 69- 100 % carbohydrates and amino acids, respectively. The potential of the isolates to utilize various carbohydrates indicated variation in growth and competitiveness of rhizobia to metabolize the nutrients as carbon and energy sources (Gauri *et al*., 2011; Wielbo, 2012).

The amino acids that were utilized less relatively were leucine (77.3%) and lysine (82 %). Isolates catabolized and used the amino acids as a sole source of nitrogen by 68 % - 86 %. This result was close to Faba bean rhizobia that catabolized 67- 100% of the amino acids (Solomon Legesse and Fassil Assefa, 2013) and lentil rhizobia utilizing 64- 86 % of the amino acids tested as reported by Mulisa Jida and Fassil Assefa (2011). The variation observed in nutritional utilization of carbohydrates and amino acids can be used as marker for rhizobial classification (Zabaloy and Gomez, 2005).

In general, the isolates AAUGR- 2, 5, 6, 9, 11, 14, 15, 19, 20, 24, 30 and 50 that showed top performance on sand culture authentication were consistent in ecophysiological tolerance tests and also showed nutrient versatility through utilizing wide range of carbon and nitrogen sources. Thus, these isolates were selected for further PGP and biocontrol assay.

Table 2. 7 Pattern of ecophysiological tolerance of *Rhizobium* species of cross inoculating hosts of the viciae family

Researcher	Host plant	pH tolerance				Temperature tolerance				Salt tolerance			
		Maximum pH (%)	%of isolates grown	Minimum pH(%)	%of isolates grown	Maximum Temp.(0C)	% of isolates grown	Minimum temp.(0C)	% of isolates grown	Maximum NaCl (%)	% of isolates grown	Minimum salt conc. (%)	% of isolates grown
Alemayehu Workalemahu (2009)	*Vicia faba* L.	8	92	4.5	17	45	8	10	75	2	83	0.5	75
Amha Gebremariam and Fassil Assefa (2017)	*Vicia faba* L.	12	67	4	33	45	33	5	67	10	33	1	100
Solomon Legesse & Fassil Assefa (2013)	*Vicia faba* L.	10	96	4.5	96	45	26	10	33	7	37	0.1	100
Zerihun Belay & Fassil Assefa (2011)	*Vicia faba* L.	9	100	5	6	40	12	5	12	5	6	0.1	53
Amha Gebremariam and Fassil Assefa (2017)	*Lens culinaris*	11	33	4	33	45	33	5	33	9	33	1	100
Mulisa Jida & Fassil Assefa (2011)	*Lens culinaris*	8.5	20	4.5	27	35	37	10	10	1	40	0.8	100
Adigo Setargie *et al*., (2015)	*Lens culinaris*	10	25	5	50	ND	ND	ND	ND	8	12.5	1	100
Amha Gebremariam and Fassil Assefa (2017)	*Pisum sativum*	11	33	4.5	33	40	33	5	33	8	33	1	100
Aregu Asresie *et al*., (2012)	*Pisum sativum*	9.5	21	5	53	38	16	4	21	6	5	0.1	80
Kassa Baye *et al*., (2015)	*Pisum sativum*	9	100	4.5	100	40	100	15	88	0.1	100	0.1	100
Mekasha Tsegaye *et al*., 2015	Fenugreek	9.5	100	4.5	100	40	21	15	50	6	7	0.1	100
Mulugeta Fentahun *et al*., 2013	Haricot bean	11	75	4	33	50	17	35	100	4	17	1	100
Mulisa Jida *et al*., 2012	Chickpea	10	22	4.5	39	40	14	10	22	5	11	0.5	100
Amha Gebremariam and Fassil Assefa (2017)	*Lathyrus sativus*	12	67	4	100	45	67	5	67	10	67	1	100
Mussa Adal (2009)	*Lathyrus sativus*	12	100	4	84	50	25	5	71	13	11	0.5	100
This study	*Lathyrus sativus*	10	46	4	23	40	59	10	46	8	14	1	100

ND=Notdetermined

The pattern of ecophysiological tolerance of grass pea rhizobia of this study as compared to rhizobium species of other cross inoculating legumes and other legumes showed close similarity (Table 2. 7). Regarding salt tolerance, isolates of this study showed similarity with lentil rhizobia whereas, rhizobia of other legumes displayed less tolerance.

2.3.6 Numerical analysis

The numerical analysis conducted based on 59 phenotypic features showed the grouping of the 22 isolates into three distinct clusters at 25 % dissimilarity level (Fig. 2. 2). Cluster I was further divided into two sub-clusters comprising of three isolates in sub-clusters IA (AAUGR- 37, 42 and 16) and four isolates in sub-cluster IB (AAUGR- 3, 19, 17,and 46). Cluster II was also further divided into to two sub-clusters with four isolates consisting of AAUGR- 7, 8, and 26 in sub- cluster IIA and AAUGR-12 as sub-cluster IIB. Cluster III was further classified into three sub-clusters comprising of eleven isolates that included isolates AAUGR- 2, 5, 6, 9, 11, 14, 15, 20, 24, 30 and 50. Clustering decreased below 5% level of dissimilarity indicating their phenotypic diversity. All taken together, the clusters indicated high diversity among grass pea rhizobia.

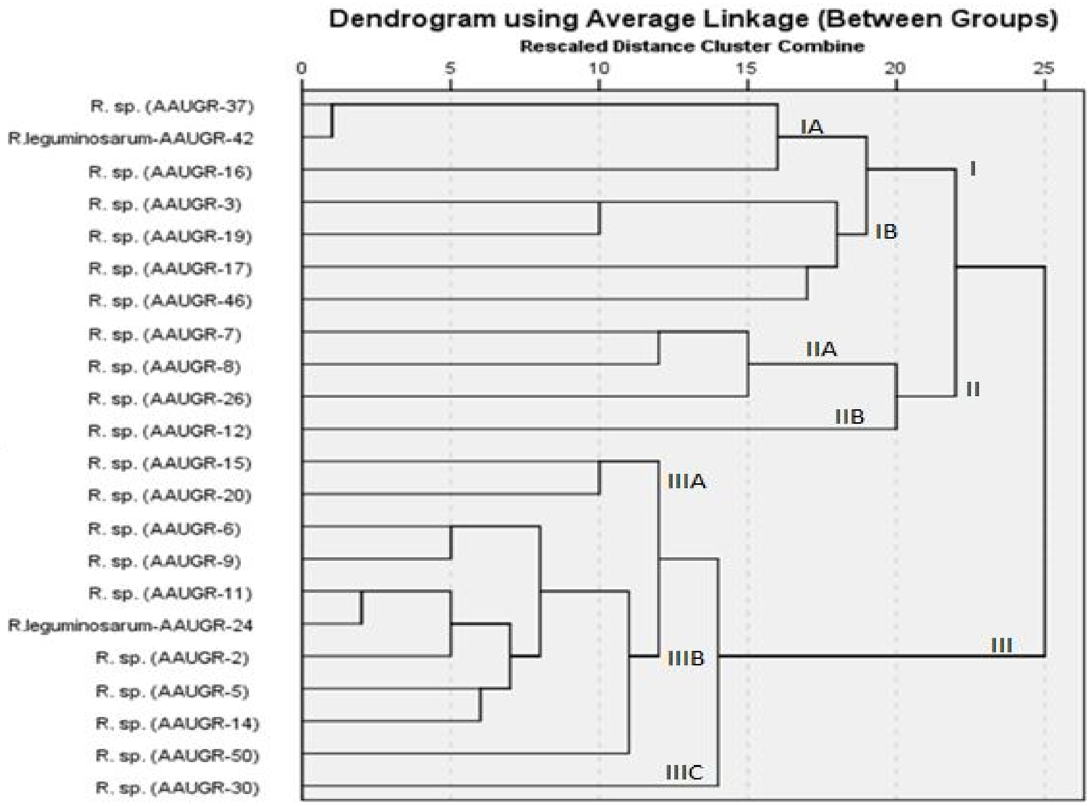

Fig.2.2 Dendrogram highlighting the phenotypic diversity of grass pea rhizobial isolates

2.3.7 Molecular characterization

2.3.7.1 Sequencing of 16S rRNA and nif-H genes

The result of the sequence analysis of 16S rRNA gene indicated that two isolates (14.3%) (AAUGR- 24 & 42) showed 99 % identity with *Rhizobium leguminosarum* bv.viciae strain CAF431 and SWD43-1 with accession numbers FJ405377.1 and KF749034.1, respectively whereas, 12 (85.7 %) isolates (AAUGR- 2, 6, 9, 11, 14, 19, 20, 30, 37, 38, 40 and 49) had 99 % identity with *Rhizobium* species with different accessions. The nif-H gene sequences result of two selected *Rhizobium* spcies (AAUGR- 2 & 24) showed 99 % identity with different strains of *Ensifer meliloti* and 99 % similarity with several strains of *Rhizobium leguminosarum* in different accessions, respectively (Table 2. 9).

The 16S rRNA gene sequencing result showed that out of the 14 rhizobial isolates, 12 (85.7 %) of them were closely related to *Rhizobium* species with 96- 99% identity and two (14.3 %) isolates to *R. leguminosarum* with 99% identity which corroborated with the findings of Berrada *et al*., (2012) who reported the identification of 2 *Rhizobium leguminosarum* and 12 *Rhizobium* species from bean plants with 98- 100 percent identity. Rivas *et al*. (2009) reported that *R. leguminosarum* bv. viciae is the specific microsymbiont of the legumes of the tribe Vicieae, which comprises the genera *Vicia*, *Pisum*, *Lens*, and *Lathyrus*. On the other hand, Aoki *et al*., (2010) reported 97- 100% sequence homology of 16S rRNA gene of rhizobia isolated from *Lathyrus japonicas* nodules with 16S rRNA gene sequences of *Rhizobium* species, *Rhizobium phaseoli*, *Rhizobium pisi*, *Rhizobium multihospitum*, *Rhizobium tropici* and *Rhizobium radiobacter* which coincided with the findings of this study. Moreover, Drouin *et al*., (1996) reported 100% sequence identity of two *Lathyrus japonicas* and *Lathyrus pratensis* 16S rRNA gene with *Rhizobium* species, *Rhizobium etli*, *Rhizobium meliloti* and *Rhizobium leguminosarum* bv.phaseoli. Similarly, Wondosen Tena *et al*., (2017) also reported genotypically diverse rhizobia nodulating lentil *(Lens culinaris* Medik.) implying that the phylogentic positions of grass pea nodulating rhizobia to *Rhizobium* species was not a miracle.

The nifH gene sequence analysis of AAUGR-2 showed 99 % identity with *Ensifer (Sinorhizobium meliloti)* instead of *Rhizobium leguminosarum* which agreed with the result of Belal *et al*., (2013) and Rashid *et al*., (2012) who reported the identification of *Rhizobium etli* from faba bean and *Rhizobium etli* and several *Rhizobium* species from lentil nodules, respectively. The identification of *Rhizobium etli*, *Rhizobium pisi*, *Rhizobium tropici*, *Rhizobium radiobacter*, *Ensifer meliloti* and other several *Rhizobium* species were reported from *Lathyrus japonicas* nodules (Aoki *et al*., 2010) implying that the identification of *Ensifer (Sinorhizobium meliloti)* from grass pea nodule is not a miracle.

2.3.7.2 Phylogentic relations of the *Rhizobium* strains for 16S rRNA and nif-H genes

A phylogenetic tree drawn from the aligned sequences of related *Rhizobium* species deposited in NCBI Genebank database and native rhizobial species showed sequence similarity amongst each other and formed four distinct clusters (Fig. 2. 3). Native isolates of AAUGR- 2, 6, 9, 11, 14, 19, 20, 37, 40 and 49 and their related sequences obtained from the NCBI Genbank database were grouped sub-cluster I with 61 % similarity with each other whereas, AAUGR-30 that showed 100% similarity with all the sequences of isolates of this study and retrived sequences formed separate group. Isolates, AAUGR- 24 displayed 100% similarity to all the three sub-clusters. On otherhand, isolate AAUGR-42 showed 100% similarity to retrived *Rhizobium leguminosarum* sequences. In general, the grouping of the isolates into clusters and subclusters with different percent of identity was an indication of genetic diversity. In general, 86% of the isolates were grouped into *Rhizobium* spp; whereas only AAUGR-24 and AAUGR- 42 were categorized into *R. leguminosarum* var viceae.

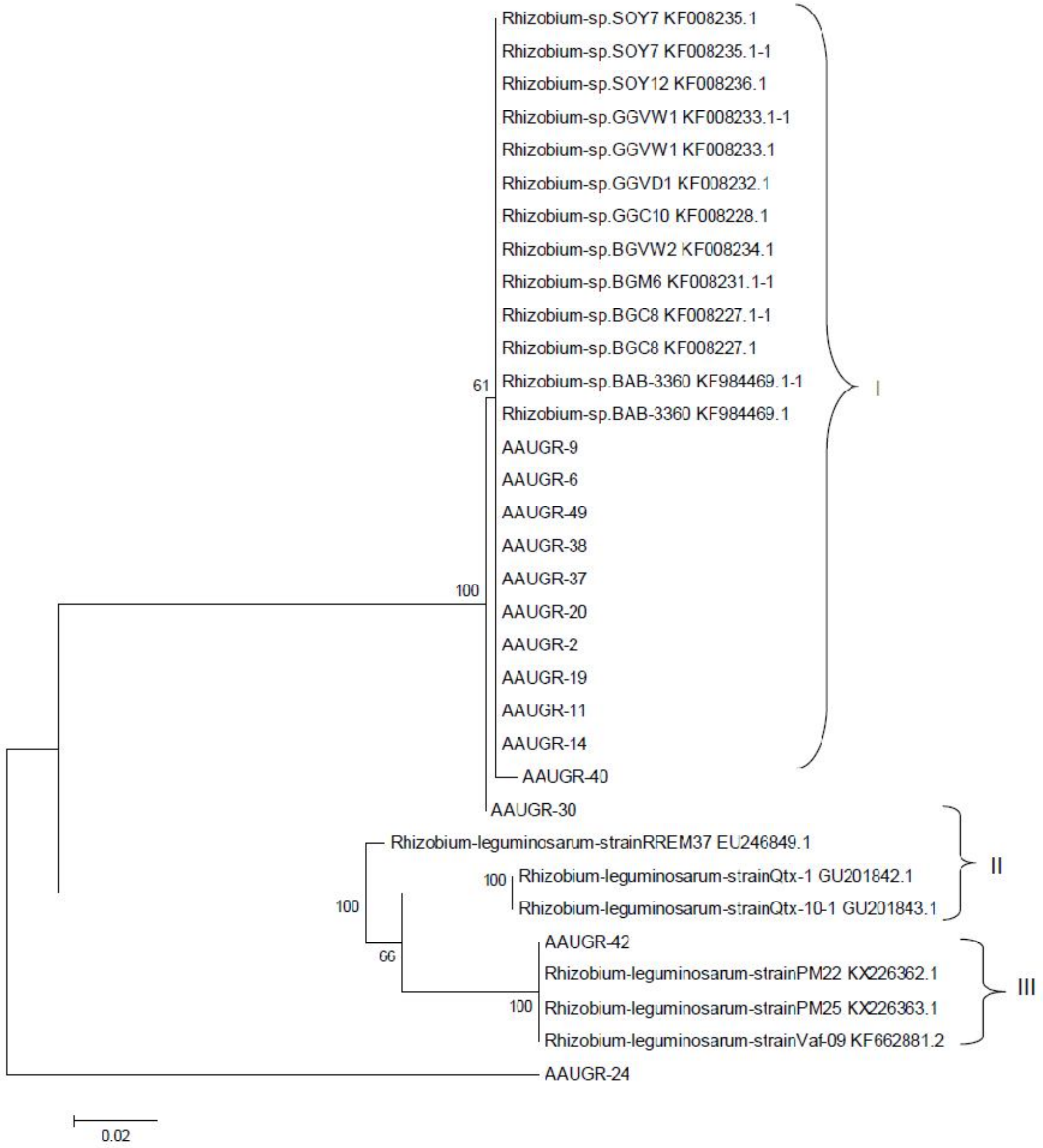

Fig 2. 3 Phylogenetic tree constructed by maximum liklihood method derived from analysis of the 16S rRNA gene sequences at 404 aligned positions (without gaps) of native isolates (designated by AAUGR- with different numbers) and related sequences obtained from NCBI. Scale bar, 0.1 substitutions per nucleotide position. The numbers at the branching points are the percentage of occurrence in 1000 bootstrap values as indicated at the nodes.

The sequences of both native and related strains were grouped into four sub-clusters. AAUGR-2 exhibited 76% similarity with both AAUGR-24 and sub-cluster I whereas, AAUGR-24 showed 64% similarity with sub-cluster I. Similarly, both AAUGR-2 and AAUGR- 24 showed phylogenetic relation with both sub-cluster III and IV at 67 % and 99% similarity.

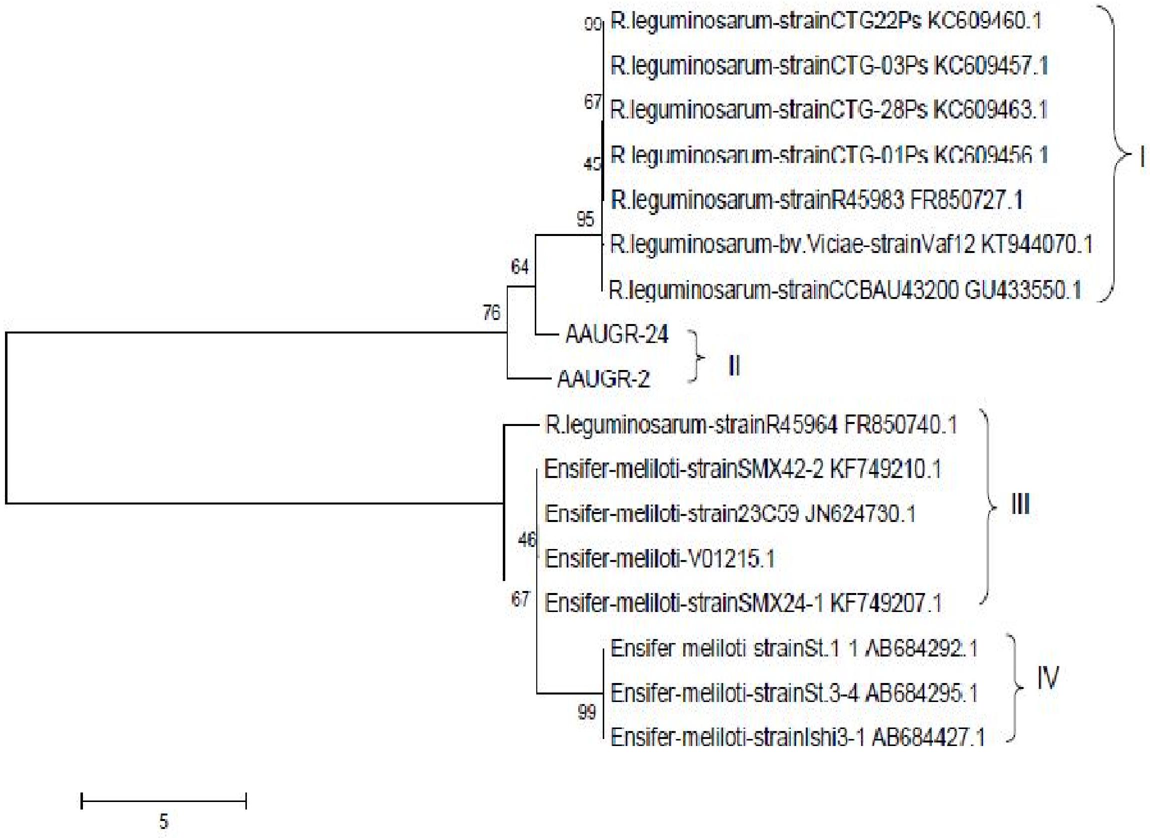

Fig 2. 4 Phylogenetic tree constructed by maximum composite liklihood method derived from analysis of the nif-H gene sequences at 374 aligned positions (without gaps) of native isolates (designated by AAUGR- 2 and AAUGR-24) and related sequences obtained from NCBI. Scale bar, 5 substitutions per nucleotide position. The numbers at the branching points are the percentage of occurrence in 1000 bootstrap values as indicated at the nodes.

Chapter 3. Diversity and plant growth promoting properties of rhizobia and rhizobacteria isolated from the nodules and rhizosphere of Grasspea (*Lathyrus sativus* L.) growing areas in central Ethiopia

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Abstract

Rhizobia and rhizobacteria are root nodule and rhizospheric bacteria that promote plant growth and reduce the effects of phytopathogens by diverse mechanisms. Twelve isolates of rhizobia and twenty two rhizobacteria were screened and characterized for their PGP and biocontrol traits using standard methods. The rhizobial and rhizobacterial isolates solubilized phosphate with PSI ranging from 1.24- 3.37 and 2.0- 4.81 cm, respectively. The phosphate quantification assay of 256.33±26.64- 418.33±6.11 µg/ml and 288.00 ±7.00- 340.67±28.3 µg/ml was produced by rhizobial and rhizobacterial isolates, respectively. AAUGR- 6 (*Rhizobium* species) and AAUGPR-92 (*Enterococcus casseliflavus/gallinarum*) were the highest phosphate solubilizers. The rhizobial isolates produced IAA at a range of 31.06±1.82-74.69±1.72 whereas, rhizobacterial isolates produced 12.01±0.03 - 56.55±0.45 µg/ml ranges of IAA. The isolates that produced the highest IAA were AAUGR- 14 (*Rhizobium* species) and AAUGPR- 53 (*Enterococcus casseliflavus/gallinarum*). Two *Rhizobium* species (AAUGR- 11 & 14) inhibited *Fusarium oxysporum* f. sp. lentis with 35 and 44 % of inhibition, respectively whereas, 16 (72%) of the rhizobacterial isolates were antagonistic to *Fusarium oxysporum* f. sp. lentis with 25- 82 % of inhibition. The potential rhizobacterial antagonist was AAUGPR-92(*Enterococcus casseliflavus/gallinarum*) with 82 % radial growth inhibition. Among the rhizobial isolates, all produced ammonia whereas, 16.7%, 83.3%, 75% and 33.3% of the isolates were positive for chitinase, protease, cellulose and HCN, respectively. Among the rhizobacterial isolates, 68.2%, 91.3%, 45.5%, 72.7% and 100% were positive for chitinase, protease, cellulase, HCN and ammonia production. The isolates AAUGPR-29, 91 and 92 with the latter two identified as *Enterococcus casseliflavus/gallinarum* displayed all the tested biocontrol traits. The 16S rRNA gene sequence showed that the majority of the rhizobial isolates (87%) were grouped into *Rhizobium spp;* whereas isolates AAUGR 24 & 42 were categorized into *Rhizobium leguminosarum var* viceae. The nifH sequence result of two strains, AAUGR-2 and AAUGR-24 showed 96 and 99% sequence homology with *Sinorhizobium meliloti/ Rhizobium meliloti* and *Rhizobium leguminosarum* type strains indicating the high diversity of grass pea rhizobia other than the specific *Rhizobium leguminosarum* var viceae that needs more research using several nod and housekeeping genes to reveal variability of the endosymbionts. The 16S rRNA gene sequence of the isolates AAUGR- 38 and 73 showed homology with different genus with 99 and 94 % identity, respectively whereas, AAUGPR- 53, 91 and 92 showed a maximum sequence similarity of 99% to *Enterococcus* species, *Enterococcus casseliflavus* and *Enterococcus gallinarum* with different accession numbers. *Rhizobium* species AAUGR-6, 9, 11 and 14 accumulated the shoot dry weight greater than the positive control. The overall plant growth and biocontrolling assay revealed that *Rhizobium* species- 6, 9, 11 and 14 and AAUGPR-53 and 92 (*Enterococcus casseliflavus/ gallinarum)* were recommended as potential candidates of microbial inoculants for inoculation and co-inoculation applications under greenhouse and field conditions.

Keywords: Antagonistic, IAA, nifH, phosphate solubilization, PCR, Rhizobia, Rhizobacteria, 16S rRNA

3.1 Introduction

The rhizosphere is a very complex environment with intricate and interdependent interaction amogst microorganisms in the soil (Mukerji *et al.*, 2006). Inoculation of legumes with plant growth promoting rhizobacteria (PGPR) and rhizobia significantly increased the growth and yield of legume crops (Valverde *et al.*, 2006). Rhizobacteria are competent bacteria in the rhizosphere that are able to multiply and colonize plant roots at all stages of plant growth (Brahim, 2013) and stimulate plant growth by plethora of mechanisms (Vessey, 2003). The main mechanisms by which PGPR directly contribute to the plant growth are phytohormone production such as auxins, cytokinins and gibberellins, enhancing plant nutrition by solubilization of minerals such as phosphorus and iron, production of siderophores and enzymes, lowering ethylene levels and induction of systemic resistance (Bhattacharyya and Jha, 2012).

The indirect benefits of PGPR that involve bacterial antagonism against phytopathogens through production of lytic (fungal cell wall degrading) enzymes such as chitinase, cellulase, glucanase and protease. (Pal and Gardener, 2006), antibiotic production (Whipps, 2001), hydrogen cyanide (HCN) production (Viosard *et al.*, 1989), siderophore production (Siddiqui, 2006) and competition for nutrients, niche exclusion, induced systemic resistance and antifungal metabolites production (Lugtenberg and Kamilova, 2009) enhance plant growth and crop yield. A number of bacterial species belonging to genera *Azospirillum*, *Azotobacter, Alcaligenes, Arthrobacter, Acinetobacter, Bacillus, Beijerinckia, Burkholderia, Enterobacter, Erwinia, Flavobacterium, Klebsiella, Microbacterium, Pseudomonas, Rhizobium* and *Serratia* are associated with the plant rhizosphere and are able to exert a beneficial effect on plant growth (Bhattacharyya and Jha, 2012; Glick, 2012*;* Tilak *et al.*, 2005).

Rhizobia besides N_2-fixation are endowed with many characteristics of PGPR including production of phytohormones, siderophores, HCN, solubilization of sparingly soluble organic and inorganic phosphates, and can colonize the roots of many non-legume plants (Antoun and Kloepper, 2001; Rajesh, 2013). Rhizobia have a good potential to be used as biological control agents against some plant pathogens. Buonassisi *et al. (*1986) showed that rhizobia antagonistic to *F. solani* f. sp. phaseoli isolated from commercial snap bean, appeared to have a good potential for controlling fusarium rot. Similarly, rhizobia isolated from chickpea and common bean showed antagonistic inhibitory effect against *Fusarium oxysporum* f.ciceris (Arfaoui *et al*., 2006).

Three years yield data of grass pea (2014- 2016) indicated decline from 18.73-11.63 qt/ha (CSA, 2017). Besides agroecological constraints, the crop encounters a range of biotic stresses and abiotic factors which reduce its yield potential by15–25% (Campbell and Clayton, 1997). Some of the constraints can be solved by investigating and producing effective and competitive rhizobia and rhizobacteria which can increase soil fertility that can in turn enhance crop yield. The application of rhizobia and rhizobacteria is eco-friendly and economically safe which can be applied as key strategies to improve nutrient solubilization and controlling phytopathogens (Ponmurugan and Gopi, 2006). Thus, screening and selecting effective and competitive rhizobia and rhizobacteria conferred with multiple plant growth promoting and biocontrolling traits can have high contributition to growth and yield improvement of grass pea and other crops in Ethiopia.

Several works regarding grass pea have been done on its neurolathyrism (Yang and Zang, 2005), its physiology (Zewdu and Solomon, 2008), agronomic performance (Dereje Tsegaye *et al*., 2005) and its improvement (Hillocks and Maruthi, 2012). Works focusing on genotypic and phenotypic diversity of *Lathyrus japonicus* rhizobia (Aoki *et al*., 2010), low temperature tolerating *Rhizobium leguminosarum* associated with *Lathyrus* species (Drouin *et al*., 2000), performance of *Rhizobium leguminosarum* strains on dry matter production of *Lathyrus sativus* (Barrientos *et al*., 2003), taxonomic classification of *Rhizobium leguminosarum* strains nodulating *Lathyrus japonicas* and *Lathyrus pratensis* using 16S rRNA gene sequencing (Drouin *et al*., 1996) were also done. However, there is a dearth of information on PGP and genotypic characterization of rhizobia and rhizobacteria isolated from the nodules and rhizosphere of grass pea (*Lathyrus sativus* L.), respectively. Therefore, the present study was conducted to assess the potential of rhizobial and rhizobacterial isolates endowed with multiple plant growth and biocontrolling properties as they can be good candidates for improving the growth and yield of grass pea and its ability to prevent disease causing fungal phytopathogens.

3.2 Materials and Methods

3.2. 1. Selected rhizobial and rhizobacterial isolates

The sampling sites of some rhizobial and rhizobacterial isolates that were selected for further plant growth promoting and biocontrol tests were presented (Table 3.1).

Table 3. 1 Sampling sites for the selected rhizobial and rhizobacterial isolates

Isolate	Sampling sites			Altitude	Soil pH
	Adm. Zone	District	Kebele		
AAUGR -2	SW	Borena	Tewa	2435.00	6.8
AAUGR -5	SW	Borena	Chefebelo	2563.00	7.1
AAUGR -6	SW	Borena	Hulagosh	2789.00	7.1
AAUGR -9	SW	Tenta	Chelemie	2973.00	6.8
AAUGR -11	SW	Tenta	Qul/Amba	2844.00	7.0
AAUGR -14	SW	Woreilu	Mariam	2718.00	7.0
AAUGR -15	SW	Woreilu	T/sertsu	2702.00	7.1
AAUGR -19	SW	Jamma	Degolo afaf	2674.00	6.8
AAUGR- 20	SW	Jamma	Debreguracha	2653.00	7.0
AAUGR -24	SW	Kalu	Jerjero	1937.00	6.6
AAUGR -30	SW	Tehuledere	Gobeya	1984.00	6.8
AAUGR -50	WS	A/Gindeberet	Gitre	2653.00	7.0
AAUGPR-2	SW	Borena	Derami	2400.00	6.9
AAUGPR-3	SW	Borena	Tewa	2435.00	6.8
AAUGPR-4	SW	Tenta	Yamed	2854.00	7.0
AAUGPR-7	SW	Woreilu	T/sertsu	2702.00	7.1
AAUGPR-29	SW	Woreilu	Geshober	2656.00	7.0
AAUGPR-36	SW	Jamma	Aejerti	2595.00	6.9
AAUGPR-38	SW	Jamma	Debreguracha	2653.00	7.0
AAUGPR-47	SW	Kalu	Jerjero	1937.00	6.6
AAUGPR-48	SW	Kalu	Keteteya	2258.00	6.7
AAUGPR-49	SW	Tehuledere	Jari	1816.00	7.0
AAUGPR-53	SW	Tehuledere	Amumo	1937.00	6.9
AAUGPR-55	KS	Artumafursi	Mermashasho	1820.00	6.4
AAUGPR-67	KS	Artumafursi	Chireti	1423.00	6.6
AAUGPR-71	WS	A/Gindeberet	Kersa	2634.00	6.9
AAUGPR-72	WS	A/Gindeberet	Gitre	2653.00	7.0
AAUGPR-73	WS	Jedlu	Gojo	2671.00	7.0
AAUGPR-77	WS	Jedlu	Tulubultuma	2639.00	6.9
AAUGPR-89	WS	Jedlu	Sunko	2661.00	6.8
AAUGPR-90	WS	Jedlu	Chilankie	2658.00	7.0
AAUGPR-91	WS	Dendi	Awash Bolto	2650.00	6.7
AAUGPR-92	WS	Dendi	Ubdo legabatu	2599.00	6.8
AAUGPR-98	WS	Dendi	Ababor	2645.00	6.8

SW= South Wollo. KS= Kemissie, WS= West Shoa

3.2.2 Selection and activation of Rhizobium strains

Twelve *Rhizobium* strains that were highly effective in their symbiotic effectiveness and resistant to abiotic factors were selected from the previous works (culture collection) (Table 3.1). The selected strains were activated by inoculating them on YEMA and incubated for 3-5 days at 28± 2 ^{0}C (Somasegaren and Hoben, 1994).

3.2.3 Sources of rhizosphere bacteria

Rhizosphere bacteria were isolated from the roots of grass pea grown in South Wollo, Kemmissie and West Shoa (Fig. 2. 1) using nutrient agar medium supplemented with 100 μgml^{-1} of cycloheximide to suppress fungal growth (Somasagaren and Hobben, 1994). They were purified and preserved at -4^{0}C.

3.2.4 Designation and preservation of purified rhizobacterial isolates

All the selected isolates were re-streaked on a new plate of the same media to obtain pure colonies and incubated at 28 ± 2 ^{0}C for 3-5 days. Ultimately, all the isolates obtained were maintained on their respective agar slants for short term storage at - 4^{0}C. The pure cultures were stored at -20 ^{0}C in eppendorf tubes containing 20% glycerol to be used for further experiments. All the isolates were designated as AAUGPR (Addis Ababa University grasspea plant growth promoting rhizobacteria) with different numbers representing each strain.

3.2.5 Characterization of the isolates

3.2.5.1 Gram reaction for rhizobacterial isolates

The rhizobacterial isolates were tested for determining their Gram reaction type using the KOH method (Buck, 1982). A drop of 3% KOH was placed on a clean microscope slide and a loopfull rhizobacterial isolate was picked, dipped and mixed properly for l minute. The mixture was lifted with inoculating loop about 1 cm from the slide and presence and absence of obvious stringiness (viscosity) were recorded as Gram negative and Gram positive bacteria, respectively.

3.2.5.2 *In vitro* screening of the isolates for plant growth promotion

3.2.5.2.1 Phosphate solubilising characteristics

The rhizobia were screened for inorganic phosphate solubilization according to Nautiyal (1999). A loop full of freshly prepared (48 hrs) bacterial culture was streaked onto Pikovskaya agar medium containing (g l^{-1}): glucose (10), Ca_3 $(PO_4)_2$ (5), $(NH_4)_2SO_4$ (0.5), NaCl (0.2), $MgSO_4.7H_2O$ (0.1), KCl (0.2), yeast extract (0. 2), $MnSO_4.7H_2O$ and $FeSO_4.7H_2O$ (0.0003 each), and agar (15) and incubated at 28 ± 2 °C for 3-5 days. After 5 days, clear halo zone around colonies indicated solubilization of mineral phosphate. Phosphate solubilization index (SI) was calculated based on the formula (Nautiyal, 1999):

Phosphate Solubilization Index= A/B × 100
A= total diameter (colony + halo zone)
B= diameter of colony

Five isolates that showed higher PSI were selected and quantitatively determined in PKV broth media (Hayat *et al.*, 2013). Accordingly, 500µl of 48 hr freshly prepared broth culture of the isolates (10^6 cells/ml) was inoculated into 100ml of PVK medium in 250ml conical flask containing $5gl^{-1}$ insoluble phosphate in the form of tricalcium phosphate (TCP) and placed on shaker at 120 rpm for 5 days at room temperature. The pH of the medium was recorded after 5 days. The culture was harvested by centrifuge at 8500 rpm for 25 minutes and available phosphorus in the supernatant was determined according to Watanabe and Olsen (1965). Optical density was recorded using spectrophotometer (Wagtech International, UK) at 700 nm and concentration of phosphorus solubilized was measured using a standard curve constant from a known concentration of phosphate (μgml^{-1}).

3.2.5.2.2 Indole-acetic-acid (IAA) Production

The freshly prepared cultures of the isolates as before were inoculated into 50 ml of yeast extract mannitol broth (YEMB) containing 0.1% DL- tryptophan in 200 ml flasks and incubated on shaker at room temperature at 180 rpm for 48 hrs. The bacterial cultures were centrifuged at 10,000 rpm for 10 min at 4 °C. Indole-3-acetic acid (IAA) in the supernatant was determined using colorimetric assay (Loper and Schroth, 1986). Accordingly, 2ml of the supernatant was mixed with 4 ml Salkowski reagent and absorbance of the resultant pink color was read after 30 min at 535 nm in UV/Visible Spectrophotometer (Jenway, 6405 Uv/vis spectrophotometer). The IAA was quantitatively determined using a standard curve of known concentrations of pure IAA (HiMedia; 5, 10, 20, 50, 80 and 100 µg mL^{-1}) in un-inoculated L-tryptophan amended YEM broth. Non-inoculated L-tryptophan supplemented YEM broth medium was used as control.

3.2.5.2.3 Nitrogen fixation

The rhizobacterial isolates were tested for their BNF capacity by stabbing in and assessing their capacity to grow and form pellicle in Burk's N-free semi-solid medium containing (g L^{-1} of distilled glucose (10), KH_2PO_4 (0.41), Na_2SO_4 (0.52), $CaCl_2$ (0.2), $MgSO_4.7H_2O$ (0.1), $FeSO_4.7H_2O$ (0.005), $Na_2MoO_4.2H_2$ (0.0025) and agar (18) and incubated at 30 ^{0}C for 5 days (Laskar and Sharma, 2013). Relative sizes of pellicles were recorded to relate to the relative BNF potential of the isolates.

3.2.6 *In vitro* screening of the Rhizobium isolates for their biocontrol traits

3.3.6.1 *In vitro* Antifungal activity

The antagonistic activity of both rhizobia and rhizobacteria was tested using dual culture technique as described by Landa *et a*l., (1997). Loop full of the isolates (10^6 cells ml^{-1}) was equidistantly spotted on the margins of PDA plates amended with sucrose (0.5%) and incubated at 28±2°C for 48 hrs. A 4 mm in diameter of the fungal pathogens from Potato Dextrose Agar (PDA) cultures was placed at the center of the PDA media containing bacterial isolate and incubated 28±2°C for 5 days. Plates containing fungal disc without bacteria was included as control. Fungal growth inhibition by the bacterial isolates was checked by presence of inhibition zone on the dual culture; and radii of the fungi inhibited by bacterial isolates were estimated by measuring the inhibition zone. Finally, percentage of inhibition was calculated as:

$$I = C - T/C * 100 (\text{Landa } et\ al., 1997)$$

Where, I= Percentage inhibition, C= Growth of the pathogen on control plate, T= Growth on treatment plate

3.2.6.2 Chitinase Production

Chitinase production was determined by growing the bacterial isolates on chitin agar medium containing (g L^{-1}) colloidal chitin (4), $MgSO_4.7H_2O$ (0.5), K_2HPO_4 (0.7), KH_2PO_4 (0.3), $FeSO_4.7H_2O$ (0.01), $MnCl_2$ (0.001), NaCl (0.3), yeast extract (0.2) and agar (20) using the method described by (Renwick *et al.*, 1991). The bacterial cells (10^6 ml^{-1}) were spot inoculated on the chitin medium and incubated for 3-5 days at 28±2^0C.

Clear zone formation around colonies was considered as a positive test for chitinase activity.

3.2.6.3 Cellulase activity determination

The isolates (48 hr old, 10^6 ml^{-1}) were spot inoculated on CMC agar medium with yeast extract plates containing (g L^{-1}) $NaNO_3$ (2), K_2HPO_4 (1), $MgSO_4$ (0.5), KCl (0.5), CMC sodium salt (2), peptone (0.2), and agar (17) to evaluate their cellulase activity according to Kasana *et al*. (2008).and incubated for 3-5 days at $28±2^0$C. Clear zone surrounding the colonies was indicative of cellulase production.

3.2.6.4 Protease Production

The same inoculums size (10^6 cells ml^{-1}) 48 hrs grown isolates was spot inoculated on Skim milk agar (Skim milk powder 10g ml^{-1}, agar 15g ml^{-1}) and incubated for 3-5 days at $28±2^0$C. Clear zones around the inoculation spots indicated casein hydrolysis or protease activity (Simbert and Krieg, 1994).

3.2.6. 5 Cyanide (HCN) Production

Isolates (10^6 ml^{-1}) were streaked on nutrient agar slant medium. Filter paper strips dipped in picric acid and 2 % sodium carbonate were inserted in the tubes. The test tubes were incubated at 28 ± 2°C for 3-5 days after sealing them with parafilm (Ahemad and Khan, 2012). HCN production was checked on the basis of changes in colour from yellow to light brown, moderate brown or strong brown of the yellow filter paper strips.

3.2.5.6 Ammonia (NH_3) Production

Each isolate was tested for the production of ammonia in peptone water. Freshly grown bacterial broth cultures (10^6 ml^{-1}) were inoculated in 10 ml nutrient broth and incubated in a rotatory shaker at 28 ± 2°C for 72 hrs. Afterwards 0.5ml of Nessler's reagent was added to each culture. Development of deep yellow to brown color was recorded as a positive test for ammonia production (Cappucino and Sherman, 1992).

3.2.7 Growth response of selected rhizobial isolates on potted soil under greenhouse conditions

3.2.7.1 Microbial inoculants

Rhizobial isolates that included AAUGR- 2, 5, 6, 9, 11, 14, 15, 19, 20, 24, 30 and 50 were selected based on their sand culture performance and stress tolerance potential. Their effect on growth of grass pea was tested on potted soil under greenhouse conditions.

3.2.7.2 Soil analysis and Estimation of rhizobial population (MPN)

The soil brought from Kutabr, one of the sampling sites was chemically analysed and determined for rhizobial population density (Table 3.1).

Table 3. 2 The soil chemical analysis and rhizobial determination using MPN

Parameters	pH	Texture	Organic carbon	Total nitrogen	C:N ration	P (mg kg-1)	MPN(No. of rhizobia g-1 soil
Value	7.1	CL	3.68	0.085	12.53	10.75	2.3×10^1

CL=Clay loam, C:N= carbon nitrogen ratio, MPN= most probable number, P= phosphorus

The pH of the soil was nearly neutral whereas, the organic carbon, total nitrogen and available phosphorus was high, low and moderate, respectively as described by Bruce and Rayment (1982); Charman and Roper (2007) and Holford and Cullis (1985). The result of most probable number of rhizobia (2.3 x 10^1) revealed that though it is not too much, it can induce infection and nodulation.

3.2.7.3 Performance evaluation of selected isolates on potted soil

The highly effective isolates were selected and their symbiotic effectiveness was further determined through pot soil experiment in a greenhouse. The soil was properly mixed, sieved and air–dried. 3 Kg of this soil was distributed to plastic pots. “Wasse” grass pea variety which was obtained from Debrezeit Agricultural Research Centre was surface sterilized using 95% ethanol (briefly) and 3% sodium hypochlorite and rinsed with five changes of sterile water. Five un-germinated seeds were sown in each pot and later thinned down to three after germination for one week. After a week, each seedling was inoculated with 1 ml of each isolate grown for 72 hrs in YEM broth. The experiment was set up in replicates under a greenhouse temperature of 19 ± 1^0C mean minimum and $30+/-1^0$C mean maximum. The pots were arranged in complete random design (CRD) with each block consisting of negative control (without nitrogen and uninoculated) and positive control (uninoculated but with nitrogen). The nitrogen fertilizer (KNO_3) was given at a concentration of 0.5 g / l per week until the plants were harvested. All the pots were watered every two days.

After sixty days of cultivation, the plants were uprooted to record nodule number, nodule dry weight, shoot length and shoot dry weight. The effectiveness of the isolates in accumulating plant shoot dry matter was calculated as described in Molungoy (2004).

3.2.8 Identification of rhizobacterial isolates using 16S rRNA

3.2.8.1 DNA extraction and amplification using colony PCR

Based on their PGP and biocontrol activities, the most effective rhizobacterial isolates were selected for genetic characterization. A loop full of rhizobacterial isolates grown in broth culture for 48 hrs was inoculated by streak plating onto NA and incubated at 28 ± 2 ^{0}C for 48 hrs (Manivannan *et al.*, 2012). A small piece of a single colony from 48 hrs rhizobacterial was taken using a sterile pipette tip and added into a 50 µl PCR mix containing phusion DNA polymerase mix. A typical PCR reaction containing 10µl 5x phusion HF buffer, 1µl 10mM dNTPs, 2.5µl 10µM forward primer (8.27F, 5' AGA GTT TGA TCC TGG CTC AG 3') and reverse primer (R (rD1), 5' AGA GTT TGA TCC TGG CTC AG 3') each with 1473 bps length of PCR products, 0.5 µ phusion DNA polymerase, DNA template (single colony) and 33.5 µl Nuclease Free Water was thoroughly mixed by pipetting up and down (Kuan *et al.*, 2016) and was run under the following conditions: preheating at 98 ^{0}C for 30'', 35 cycles of 98^{0}C for 10'', 56 ^{0}C for 30'', 72 ^{0}C for 30'' and a final extension at 72 ^{0}C for 10' (Kuan *et al.*, 2016; Kumar and Gera, 2013; Gaunt *et al.*, 2001).

3.2.8.2 Purification of PCR products

Among the ten (10) PCR amplified rhizobacterial isolates, the PCR products of five selected isolates were extracted from the gel and purified using PCR purification kit (QIAGEN) according to the standard protocol recommended by the manufacturer.

3.2.8.3 Sequencing of 16S rRNA genes and phylogentic analysis

Five micro-liter of each purified PCR product was used for Sanger sequencing at the Oklahoma State University Core Facility. PCR products of both strands were sequenced using the same primers used in the PCR amplification. Sequence reads were edited using

Bioedit software and compared for homology with sequences of other organisms deposited in the National Center for Biotechnology Information (NCBI) GenBank database using Basic Local Alignment Search Tool (BLAST) to determine identity to known sequences (Shayne *et al.*, 2003). The 16S rRNA gene sequences determined in this study were aligned with highly similar sequences from GenBank using ClustalW and evolutionary history was inferred using the Neighbor- Joining phylogenetic tree (Saitou & Nei, 1987) with bootstrap values derived from 1000 replicates.

3.2.9 Data analysis

The data recoded were analyzed and interpreted using ANOVA. The experimental treatments were compared and contrasted against their control following Duncan's multiple range test (DMRT) (Duncan, 1955). The correlation between different phosphate solubilization, IAA production and greenhouse data was evaluated by Pearson correlation coefficient using SPSS v. 20.

3.3 Result and Discussion

3.3.1 Gram reaction for rhizobacterial isolates

Among the rhizobacterial isolates selected for conducting characterization, the gram reaction test showed that 6 (27.3%) and 16(72.7%) of them were Gram positive and Gram negative, respectively.

3.3.2 *In vitro* characterization of bacterial isolates for plant growth promotion

3.3.2.1 Phosphate solubilization by rhizobial isolates

Isolates were screened for phosphate solubilization and the result showed that 25% of rhizobia were phosphate solubilisers (Table 3. 2).

Table 3. 3. The plant growth promoting traits of selected *Rhizobium* strains

Isolatcode	16SrRNA identification	Collection sites	Soil pH	Phosphate solubilization activity			IAA production activity
				PSI (cm)	µg/ml	pH	µg/ml
AAUGR- 2	R. sp.	SW	6.9	2.05± 0.07 cde	356.64±15.14 cd	5.65±0.16 c	69.10±1.39 b
AAUGR- 5	UN	SW	7.0	2.07± 0.09 cde	359.67±15.14 cd	5.68±0.19 c	70.11±1.39 b
AAUGR- 6	*R.* sp	SW	6.8	3.37± 0.22 a	418.33±6.11 a	4.87±0.08 e	60.96±1.19 c
AAUGR- 9	*R.* sp	SW	6.7	2.98 ±0.14 ab	401.00±15.13 ab	5.22±0.22 d	62.90±1.49 c
AAUGR- 11	*R.* sp	SW	6.9	2.68 ± 0.10 bc	375.00±7.94 bc	5.68 ±0.09 c	60.78±2.34 c
AAUGR- 14	*R.* sp	SW	6.8	2.68 ±0.26 bc	375.67±13.32 bc	5.47±0.08 c	74.69±1.72 a
AAUGR- 15	UN	SW	6.9	1.63 ±0.22 ef	331.67±25.58 de	6.64±0.09 a	20.86 ±2.43 g
AAUGR- 19	*R.* sp	SW	6.6	1.24 ± 0.11 f	278.00±10.54 f	6.71±0.08 a	39.92±1.35 e
AAUGR- 20	*R.* sp	SW	7.0	2.35 ±0.36 cd	353.33±10.97 cd	6.05±0.07 b	50.68±1.36 d
AAUGR- 24	*R. legu.*	SW	6.9	2.32±0.33 cd	351.33±10.87 cd	6.01±0.05 b	50.63±1.32 d
AAUGR- 30	*R.* sp	Sw	6.7	1.99± 0.15 de	256.33±26.64 f	6.85±0.12 a	74.18±1.95 a
AAUGR- 50	UN	WS	6.8	2.35±0.12 bcd	316.67±17.62 e	6.15±0.13 b	31.06±1.82 f

All values are average of three replicates. * Initial pH 7.0, P=0.000, UN= undetermined, R.sp.= *Rhizobium* species, *R.legu.=R. leguminosarum*

All the selected isolates solubilized phosphate with a range of 1.24 – 3.37 cm solubilization index showing a significant variation (P=0.00). Accordingly, among the isolates AAUGR-6 (*Rhizobium* species) displayed the highest solubilization index (3.37 cm) followed by isolate AAUGR-9 (*Rhizobium* species) with a solubilisation index of 2.98 cm. On the contrary, isolate AAUGR-19 (*Rhizobium* species) showed the least solubilisation index of 1.24 cm. Variation in phosphate solubilization with PSI of 1.13-1.18 cm was recorded for lentil rhizobia (Mulisa Jida *et al*., 2011). Hajjam *et al*. (2016) and Girmaye Kenasa *et al*. (2014) also reported PSI of 2.01- 3.18 and 1.25 - 2.10 for faba bean rhizobia, respectively. The variation in the ability of the isolates to form a halo zone was due to the variation in the amount and type of organic acid produced (Tan *et al*., 2014).

The quantitative determination of phosphorous indicated that isolates AAUGR-6 (*Rhizobium* species) and AAUGR-9 (*Rhizobium* species) were effective phosphate solubilizers with the release of 418.3 µg/ml and 401.0 µg/ml phosphate, respectively which was significantly higher than the ones produced by the rest of the rhizobial isolates. The least effective PSB were AAUGR-30 and 50 that released 256.3 and 278 µg/ml phosphate. A release of 359 µg/ml phosphate was reported for faba bean rhizobia (Hajjam *et al.*, 2016). The isolates AAUGR- 6, 9, 11 and 14 solubilized TCP along with decreased pH in broth culture. Strong negative correlation between phosphate solubilization and pH was observed which indicated the acidic condition required for phosphate solubilization. An inverse relationship was observed between pH value of culture medium and soluble phosphate which was an indication of organic acid secretion (Table 3. 2). Singh *et al.* (2014) reported same inverse relationship for rhizobia from chickpea.

3.3.2.2 Phosphate solubilization by rhizobacterial isolates

Out of the total of 99 bacterial colonies collected from the rhizosphere samples of grass pea, 39% were phosphate solubilizers (PSB) of which 22 PSB showed better solubilization indices from tricalcium phosphate (TCP) ranging from PSI 2.41- 4.81 (Table 4.2). Among the isolates, isolate AAUGPR-53 (*Enterococcus casseliflavus* and *Enterococcus gallinarium)* showed the highest solubilization index of 4.81, followed by isolates AAUGPR-90 and AAUGPR-91 (*Enterococcus casseliflavus* and *Enterococcus gallinarium)* with SI of 4.57±0.10 and 4.57±0.02, respectively. The effectiveness of PSB in terms of PSI was better than Mulisa Jida *et al.* (2015; 2016) who reported SI of lentil and chickpea strains that ranged from 1.34 to 2.25cm and 1.44- 3.06 cm, respectively.

Regarding quantitative determination of phosphate solubilization, an inverse relation was observed between phosphate solubilisation and amount of pH measured. The selected isolates solubilized TCP with a solubilization potential of 288-340 μgml^{-1} with significant drop in pH of the culture medium from an initial pH 7.0 - 4.87 showing moderate weak correlation ($r = -0.333$, $p = 0.05$ and $r = -0.358$, $p > 0.05$) between phosphate solubilization and broth pH which indicated the acidic condition required for phosphate solubilization. Similarly, Driba Muleta *et al.*, (2013) reported a solubilization of 140 µgml^{-1} with strong negative correlation ($r = -0.73$, $p < 0.01$) by rhizobactera originated from coffee. However, phosphate solubilizing lentil rhizobacteria identified as *Enterobacter kobei* have produced much higher (674 µg ml^{-1}) than the value of this study (Mulisa Jida *et al.*, 2015). Microorganisms including *Serratia* and *Enterobacter* (Malleswari and Bagyanarayana, 2013) and *Kluyvera* (Hariprasad and Niranjana, 2009), *Pantoea* species, *Enterobacter* species and *Enterobacter cloacae* (Sharon *et al.*, 2016) were reported as phosphate solubiliser in which some of them were identified in this study.

3.3.2.3 IAA production by rhizobial isolates

All PSB isolates produced IAA with isolates AAUGR-14 (*Rhizobium* species) producing the highest IAA (74.69 µg/ml) followed by isolates AAUGR- 30 (*Rhizobium* species) (74.18 µg/ml) and AAUGR-5 (70.11 µg/ml). Isolate AAUGR- 15 (*Rhizobium* species) produced the lowest IAA (20. 86 µg/ml)) which was greater than that produced by field pea rhizobia (Waheed *et al*, 2014) who reported 10.50-14.25µgml^{-1}. Isolates AAUGR-14, 30, 5, 2, 9, 6 and 11 produced the first top consecutive amount of IAA relative to other isolates indicating that IAA production depends on bacterial growth, their metabolic activities and growth medium among other things (Spaenpen *et al.*, 2007). The result of phosphate solubilization and IAA production taken together showed that isolates AAUGR- 14, 9, 11, 6, 5 and 2 were among the best top performing isolates.

Table 3. 4 PGP properties of grass pea rhizobacterial isolates under *in vitro* conditions

S/N.	Iisolate	Collection sites	Phosphate solubilisation			IAA	N-fixation	Gram rxn
			PSI(cm)	Quantitative ((µg mL -1) ± S.E	pH released	(µg mL -1) ± S.E		
1	AAUGPR-2	SW	$3.09\pm_{0.07}^{e}$			$36.40\pm_{0.08}^{e}$	-	-ve
2	AAUGPR-3	SW	$4.12\pm_{0.09}^{c}$			$35.53\pm_{0.06}^{e}$	-	-ve
3	AAUGPR-4	SW	$4.27\pm_{.0.09}^{c}$			$56.58\pm_{0.08}^{a}$	+	-ve
4	AAUGPR-7	SW	$2.95\pm_{0.03}^{ef}$			$36.07\pm_{0.06}^{e}$	-	-ve
5	AAUGPR-29	SW	$3.66\pm_{0.55}^{d}$			$40.85\pm_{0.08}^{dc}$	+	-ve
6	AAUGPR-36	SW	$2.77\pm_{0.02}^{fgh}$			36.50 ± 0.02^{e}	-	-ve
7	AAUGPR-38 (*Pantoa* sp., *Enterobacter* sp.)	SW	$4.53\ \pm_{0.0}^{b}$	$288.00\ \pm_{7.00}^{b}$	6.13^{c}	$49.99\pm_{2.39}^{c}$	+	-ve
8	AAUGPR-47	SW	$2.44\pm_{0.04}^{k}$			$49.80\pm_{0.02}^{c}$	-	-ve
9	AAUGPR-48	SW	$2.63\ \pm_{0.03}^{hij}$			$29.80\pm_{0.05}^{f}$	-	-ve
10	AAUGPR-49	SW	$3.06\pm_{0.07}^{e}$			$31.15\pm_{1.00}^{f}$	-	-ve
11	AAUGPR-53 *(Enterococcus casseliflavus/gallinarum*)	SW	$4.81\pm_{0.02}^{a}$	$337.67\pm_{2.03}^{a}$	5.04^{a}	$56.55\pm_{0.45}^{a}$	+	+ve
12	AAUGPR-55	KS	$2.70\pm_{0.04}^{ghi}$			$17.57\pm_{0.07}^{i}$	-	-ve
13	AAUGPR-67	KS	$2.63\pm_{0.02}^{hij}$			$22.18\pm_{0.08}^{g}$	+	-ve
14	AAUGPR-71	WS	$2.55\pm_{0.06}^{ijk}$			$23.41\pm_{0.03}^{g}$	-	-ve
15	AAUGPR-72	WS	$2.48\ \pm0.07\ ^{jk}$			$29.8\pm_{20.05}^{f}$	-	-ve
16	AAUGPR-73 (*Serratia*, *Enterobacter*, *Kluyvera* sp.)	WS	$2.71\pm_{0.07}^{ghi}$	$289.67\pm_{8.76}^{b}$	6.21^{c}	$12.01\pm_{0.03}^{j}$	+	-ve
17	AAUGPR-77	WS	2.85±0.05 fg			$18.79\pm_{0.12}^{hi}$	-	+ve
18	AAUGPR-89	WS	2.41±0.08 k			$19.7\pm_{50.13}^{h}$	-	-ve
19	AAUGPR-90	WS	4.57±0.10 b			$50.88\pm_{0.88}^{bc}$	+	+ve
20	AAUGPR-91 *(Enterococcus casseliflavus/gallinarum*)	WS	4.57±0.02 b	$309.67\pm_{6.17}^{ab}$	5.23^{b}	$54.87\pm_{0.83}^{a}$	+	+ve
21	AAUGPR-92 *(Enterococcus casseliflavus/gallinarum*)	WS	4.46±0.03 b	$340.67\pm_{28.30}^{a}$	4.87^{a}	$53.89\ \pm_{1.64}^{ab}$	+	+ve
22	AAUGPR-98	WS	$2.94\ \pm_{0.03}$ef			$34.25\pm_{0.27}^{e}$	+	+ve

Phosphate and IAA values are average of three replicates. * Initial pH 7.0,

3.3.2.4 IAA production by rhizobacterial isolates

All isolates produced IAA with variations from 12.01 µg/ml by AAUGPR-73 (*Serratia marcescens* and others) to 56.58 µg/ml by AAUGPR- 53 (*Enterococcus casseliflavus* and *Enterococcus gallinarum* PR-53) (Table 3. 3). The maximum IAA (56.58 µg/ml) was produced by isolate AAUGPR-53 followed by isolate AAUGPR-91(54.87 µg/ml). The least amount of IAA production (12.01 µg/ml) was produced from isolate AAUGPR- 73 that showed 94% identity with different *Serratia* species, *Serratia marcescens, Serratia nematodiphila, Serratia ureilytica, Enterobacter* species, *Enterobacter aerogenes, Kluyvera georgiana, Kluyvera ascorbata, Kluyvera cryocrescens*, and *Kluyvera intermidia.* The amount of IAA produced by grass pea rhizobacteria was lower than the amount of IAA (146 µg/ml) released by soybean rhizobacteria Driba Temesgen (2017). Malleswari and Bagyanarayana (2013) reported 11.6- 60 µg/ml IAA by rhizobacteria from medicinal plant that was in the range of the findings of this study.

3.3.2.5 Nitrogen fixation by rhizobacterial isolates

Among the rhizobacterial isolates grown on Burk's N-free semi-solid medium for their capacity to fix nitrogen on Burk's N-free semi-solid medium, 10 (46 %) were able to form conspicuous pellicles (Table 4.2) indicating their potential of BNF. Among these isolates, 6 of them were PCR amplified and formed distinct band for nif-H gene confirming that they belonged to the free-living nitrogen fixing *Enterobacter* and *Serratia* isolated from the rhizosphere of various crops contributing fixed nitrogen to the associated plants (Yadave *et al*., 2016).

3.3.3 Antifungal activity of the isolates

3.3.3.1 *In vitro* antifungal activity of rhizobial isolates

Among the tested rhizobial isolates, only 2(16.7%) of the isolates, AAUGR 11 and 14 both of which were identified as *Rhizobium* species were antagonistic to the phytopathogen, *Fusarium oxysporum f*. sp. lentis with a mycelial growth inhibition of 44 % and 35 %, respectively. Driba Temesgen (2017) reported that 10% of soybean rhizobia were antagonistic to *Fusarium oxysporum* with 17 and 33 % of fungal growth inhibition. Hoque *et al*. (2015) reported antifungal inhibition by *Rhizobium leguminosarum* with a fungal mycelial growth inhibition percent of 51 - 55 % indicating grass pea rhizobia do possess the potential to be used as a biocontrol agent against soil born root pathogens.

Table 3. 5 Antagonistc in vitro properties of grass pea rhizobia against *Fusarium oxysporum* f. sp. lentis

Isolate code	16S rRNA identification	Sampling Sites	Antagonistic test							No. of combined traits
			Chitinase	Protease	Cellulase	HCN	NH3	Dual culture	PIRG	
AAUGR- 2	*R*. sp.	SW	-	+	+	+	+	-	-	5
AAUGR- 5	UD	SW	-	+	-	-	+	-	-	3
AAUGR- 6	*R*. sp.	SW	-	+	+	-	+	-	-	4
AAUGR- 9	*R*. sp.	SW	+	+	-	+	+	-	-	5
AAUGR- 11	*R*. sp.	SW	-	+	+	+	+	+	44	6
AAUGR- 14	*R*. sp.	SW	+	+	+	-	+	+	35	6
AAUGR- 15	UD	SW	-	-	+	-	+	-	-	3
AAUGR- 19	*R*. sp.	SW	-	+	+	-	+	-	-	4
AAUGR- 20	*R*. sp.	SW	-	+	+	-	+	-	-	4
AAUGR- 24	*R. legum.*	SW	-	+	+	+	+	-	-	5
AAUGR- 30	*R*. sp.	WS	-	+	+	-	+	-	-	4
AAUGR- 50	UD	WS	-	-	-	-	+	-	-	2
Number			2	10	9	4	12	2		
%			16.7	83.3	75	33.3	100	16.7		

PIRG= percent radial growth inhibition, R= *Rhizobium*, sp.= species, UD= undetermined

3.3.3.2 Hydrolytic enzyme production

The capacity of the isolates to produce chitinase, cellulase and protease was determined (Table 3. 4). Accordingly, 2(17 %), 10(83 %) and 9 (75%) of the isolates were capable of producing chitinase, protease and cellulase, respectively. The isolates that showed chitinase property were AAUGR-11 (*Rhizobium* species) and 14 (*Rhizobium* species). Strain AAUGR- 14 (*Rhizobium* species) showed enzymatic activity for all the three enzymes tested. This study showed that 9 (75%) of the isolates were cellulase producers which was close to the result of Hung and Annapurna (2004) and Bhagat *et al.,* (2014) who reported 70 % and 88% of cellulase producing soybean and chickpea rhizobia, respectively. Driba Temesgen (2017) also reported cellulase (62 %) producing soybean rhizobia.

Out of the isolates, 10 (83.3%) showed production of protease by the formation of clear zone which was greater than Bhagat *et al*., (2014) who reported 65 % of protease producing chickpea rhizobial isolates. These proteases producing isolates can contribute to the eradication of some fungal plant pathogens (Ruchi *et al.,* 2012). Many of the isolates produced three enzymes out of the tested enzymes, whereas only one isolate, AAUGR- 14 (*Rhizobium* species) showed the potential for all tested enzymatic activity.

3.3.3.3 Hydrogen cyanide and ammonia production

The ability of the isolates to produce cyanide and ammonia was evaluated (Table 3. 4). Accordingly, 4 (33.3%) of the isolates, AAUGR- 2,9,11 and 14 produced hydrogen cyanide that concided with the result of Kaur *et al*., (2015) who reported 7 (33.3 %) of HCN producing chickpea rhizobial isolates. The rhizobial isolates can be important in assisting grass pea crop improvement in drought affected areas as bacteria possessing this trait can increase plant growth (Glick *et al*., 1995) through inhibiting pathogen growth by producing volatiles that includes antibiotics, hydrolytic and cyanides (Arfoui *et al.*, 2006) and through affecting their respiratory system (Viveros *et al* 2010 and Verma *et al.*, (2012) or inhibiting the electron transport and hence disruption of energy supply to the cells (Reddy, 2013).

Regarding ammonia production, all of the rhizobial isolates were positive for the test. Rajpoot and Panwar (2013) and Singh *et al*., (2013) reported that 66.7 % of ammonia producing rhizobial isolates from *Vagina radiate* and *Pigeon pea*. The production of volatile ammonia by these rhizobial isolates is an implication to control soil borne pathogens (Paulitz *et al*., 2000). It can also be said that inoculation with such NH_3 producing rhizobia may enhance the plant growth as a result of their ability to fix nitrogen (N_2) to ammonia (NH_3) making it an available nutrient for plant growth (Hayat *et al*., 2010).

The overall biocontrolling activities of the isolates showed that only 17 % and 33.3% produced chitinase and HCN, respectively. Similarly, only 2(17 %) of the isolates inhibited the fungal antagonist. In general, all the tested rhizobial isolates produced ammonia whereas, most of them showed moderate activity to protease and cellulase production. Isolates AAUGR- 11 & 14 (*Rhizobium* species) showed diverse features in the mechanism of biocontrol whereas, isolates AAUGR- 50, 5 and 15 displayed low performances in these activities. In this study, the *Rhizobium* isolates from the nodules of grass pea (*Lathyrus sativus* L.) produced antifungal metabolites, cell wall degrading enzymes viz. chitinase, cellulase and protease, hydrogen cyanide and ammonia that can enhance plant growth (Kucuk *et al*., 2013). The *Rhizobium* isolates showed similar pattern with Bhagat *et al.*, (2014) who reported isolates that showed antagonistic activity for antifungal test, chitinase, cellulase, protease, ammonia and hydrogen cyanide production. The variations in producing different hydrolytic enzymes among the isolates were attributed to their individual competencies.

3.3.3.4 Antifungal activity of the rhizobacterial isolates

Table 3. 6 Antagonistc in vitro properties of grass pea rhizobacteria against *Fusarium oxysporum* f. sp. lentis

Isolates	Sampling Sites	Antagonistic properties							No. of combined traits
		Chitinase	Protease	Cellulase	HCN	NH_3	Dual culture	PIRG	
AAUGPR 2	SW	+	-	+	+	+	-	-	5
AAUGPR 3	SW	+	+	-	+	+	-	-	5
AAUGPR 4	SW	+	-	+	+	+	-	-	5
AAUGPR 7	SW	+	+	-	-	+	-	-	4
AAUGPR 29	SW	+	+	+	+	+	+	25	7
AAUGPR 36	SW	-	+	+	+	+	+	30	6
AAUGPR 38	SW	-	+	+	+	+	+	69	6
AAUGPR 47	SW	-	+	-	+	+	+	36	5
AAUGPR 48	SW	-	+	-	+	+	+	40	5
AAUGPR 49	SW	-	+	-	+	+	+	46	5
AAUGPR 53	SW	+	+	+	-	+	+	74	6
AAUGPR 55	KS	+	+	-	-	+	+	44	5
AAUGPR 67	KS	-	+	-	+	+	+	63	5
AAUGPR 71	WS	+	+	-	+	+	+	66	6
AAUGPR 72	WS	-	+	+	+	+	+	51	6
AAUGPR 73	WS	+	+	+	-	+	+	71	6
AAUGPR 77	WS	+	+	-	-	+	-	-	5
AAUGPR 89	WS	+	+	-	++	-	-	-	4
AAUGPR 90	WS	+	+	-	+	+	+	78	6
AAUGPR 91	WS	+	+	+	+	+	+	73	7
AAUGPR 92	WS	+	+	+	+	+	+	83	7
AAUGPR 98	WS	+	+	-	+	+	+	70	6
Total		15	20	10	17	22	16		
%		68.2	90.9	45.5	77.3	100	72.7		

PIRG= percent radial growth inhibition, KS= Kemissie, SW= South Wollo, WS= West Shoa

3.3.3.4.1 Dual culture antifungal test

Among the isolates tested for their antifungal activity, 16 (73 %) inhibited the growth of *Fusarium oxysporum* f. sp. lentis (Table 3. 5). The isolates AAUGPR- 53(74 %), AAUGPR- 90(78 %) and AAUGPR-92(83 %) where the former and the latter isolates belonged to *Enterococcus* species, *Enterococcus casseliflavus* and *Enterococcus gallinarum* species showed the highest fungal mycelial inhibition. Driba Muleta *et al.* (2007) reported fungal radial growth inhibition ranging 40.1- 71.8 % by coffee rhizobacterial isolates against *Fusarium oxysporum*. Similarly, 52% of soybean rhizobacterial strains have showed antifungal activity with an inhibition percent ranging from 19- 82% (Driba Temesgen, 2017) implying that isolates of this study showed better antifungal activity.

3.3.3.4.2 Hydrolytic enzyme production test

Among the tested isolates, 68.2%, 91.3% and 45.5% of them were positive for chitinase, protease and cellulase production, respectively (Table 3. 5). Kavitha *et al*., (2013) reported the production of chitinase (60%) and cellulase (50%) by rhizobacteria isolated from sunflower whereas, 50% protease producing soybean rhizobibacteria is reported by Driba Temesgen *et al*., (2017) indicating that the isolates of this study were good at their biocontrols activities. Protease and chitinase production which are involved in cell wall degradation during antagonism, was reported for various genera of endophytic bacteria including *Enterobacter*, and *Serratia* (Wang *et al*., 2013) and are important strategy to inhibit fungal spore germination, germ tube elongation and lyse hyphal tips (Ordentlich *et al*., 1988), respectively.

3.3.3.4.3 Hydrogen cyanide and ammonia production test

Out of the tested isolates, 17(77 %) of them produced HCN that corroborated with the findings of Kavitha *et al*., (2013) and Prashar *et al*., (2013) who reported 80% of cyanide degrading rhizobacterial isolates from sunflower and tomato, respectively that inhibit pathogenic fungal growth through affecting their respiratory system (Verma *et al.*, 2012) or inhibiting the electron transport and hence disruption of energy supply to the cells (Reddy, 2013). Consequently, these HCN producing isolates could be used as biocontrol agents of *Fusarium* wilt as they do not have adverse effect on plant growth (Goel *et al.*, 2002). All the isolates produced ammonia (NH_3) similar to Geetha *et al*., (2014) who reported the production of ammonia by all rhizobacteria isolated from Green gram. Isolates AAUGPR-29, 72, 91 and 92 were positive for all the biocontrolling mechanisms tested followed by isolates AAUGPR-36, 38, 53, 71, 72, 73, 90 and 98 with 85.7% of efficiency that can thus deserve these isolates to be used as microbial inoculants in disease suppressive soil.

Isolates AAUGPR-29, 91 and 92 were found positive in all the salient features of pathogen inhibition with isolate PR- 29 being the least inhibitor. The isolates that showed the least performance in their biocontrol potential were isolates AAUGPR-7 and 89 with 57.1% of cumulative biocontrol traits, appearing negative in four of the eight tests (Table 3. 5).

Table 3. 7 Multiple plant growth promoting and biocontrolling traits of the rhizobacterial isolates

Isolates	Sampling sites	Phosphate solubilisationn	IAA production	Chitinase	Protease	Cellulase	HCN	NH3	Dual culture	PIRG(%)	Total traits	Efficiency rate(%)
AAUGPR 2	SW	3.09±0.07^{e}	36.40±0.08^{e}	+	-	+	+	+	-	-	7	77.8
AAUGPR 3	SW	4.12±0.09 ^{c}	35.53±0.06^{e}	+	+	-	+	+	-	-	7	77.8
AAUGPR 4	SW	4.27±.0.09 ^{c}	46.58±0.08^{d}	+	-	+	+	+	-	-	7	77.8
AAUGPR 7	SW	2.95±0.03 ^{ef}	36.07±0.06 ^{e}	+	+	-	-	+	-	-	6	66.7
AAUGPR 29	SW	3.66±0.55 ^{d}	40.85±0.08^{dc}	+	+	+	+	+	+	25	9	100
AAUGPR 36	SW	2.77±0.02 ^{fgh}	36.50±0.02^{e}	-	+	+	+	+	+	30	8	88.9
AAUGPR 38	SW	4.53 ±0.01^{b}	49.99±2.39 ^{c}	-	+	+	+	+	+	69	9	88.9
AAUGPR 47	SW	2.44±0.04 ^{d}	49.80±0.02 ^{c}	-	+	-	+	+	+	36	7	77.8
AAUGPR 48	SW	2.63 ±0.03^{hij}	29.80±0.05 ^{f}	-	+	-	+	+	+	40	7	77.8
AAUGPR 49	SW	3.06±0.07 ^{e}	31.15±1.00 ^{f}	-	+	-	+	+	+	46	7	77.8
AAUGPR 53	SW	4.81±0.02 ^{a}	56.55±0.45 ^{a}	+	+	+	-	+	+	74	8	88.9
AAUGPR KS	WS	2.70±0.04^{ghi}	17.57±0.07 ^{i}	+	+	-	-	+	+	44	7	77.8
AAUGPR KS	WS	2.63±0.02 ^{hij}	22.18±0.08 ^{g}	-	+	-	+	+	+	63	7	77.8
AAUGPR 71	WS	2.55±0.06 ^{ijk}	23.41±0.03 ^{g}	+	+	-	+	+	+	66	8	88.9
AAUGPR 72	WS	2.48 ±0.07^{jk}	29.8±20.05 ^{f}	+	+	+	+	+	+	51	9	100
AAUGPR 73	WS	2.71±0.07^{ghi}	12.01±0.03 ^{j}	+	+	+	-	+	+	71	8	88.9
AAUGPR 77	WS	2.85±0.05^{fg}	18.79±0.12 ^{hi}	+	+	-	-	+	+	-	7	77.8
AAUGPR 89	WS	2.41±0.08 ^{k}	19.7±50.13 ^{h}	+	+	-	++	-	-	-	6	66.7
AAUGPR 90	WS	4.57±0.10 ^{b}	50.88±0.88 ^{bc}	+	+	-	+	+	+	78	8	88.9
AAUGPR 91	WS	4.57±0.02 ^{b}	54.87±0.83 ^{a}	+	+	+	+	+	+	73	9	100
AAUGPR 92	WS	4.46±0.03 ^{b}	53.89 ±1.64 ^{ab}	+	+	+	+	+	+	83	9	100
AAUGPR 98	WS	2.94 ±0.03^{ef}	34.25±0.27 ^{e}	+	+	-	+	+	+	70	8	88.9
Total		22	22	15	20	10	17	21	17	16		
%		100	100	68.2	90.9	45.5	77.3	95.5	77.3	72.3		

PIRG= Percent radial growth inhibition

Among the many different modes of action of fungal inhibition, protease, cellulase and chitinase assays, and cyanide and ammonia production were performed as salient features of the same (Table 3. 5). All the tested isolates showed better performance in both plant growth promotion and biocontrol properties tested (Table 3. 6). Four (18.2%) of the isolates that included AAUGPR- 29, 72,91 and 92 were positive in all the tested traits followed by isolates AAUGPR- 36, 38, 53, 71, 73, 90 and 98 that acquired many features of biocontrol and PGP traits. Isolates AAUGPR- 38 (that showed 99 % homology with the genus *Pantoea* and *Enterobacter*), AAUGPR-73 (94 % homology with *Serratia*, *Enterobacter* and *Kluyvera* genus) and AAUGPR- 53, 91 and 92 that both showed 99 % identity with *Enterococcus caseliflavus/gallinarum* displayed top performance in all PGP and biocontrol properties taken together. However, based on the cumulative efficiency of phosphate solubilization, indole acetic acid production and antifungal inhibition, isolates AAUGPR- 53 (SW) and 92 (WS) were selected for co-inoculation trial under greenhouse conditions.

3. 3.4 Growth response of selected rhizobial isolates on potted soil under greenhouse conditions

3. 3.4. 1 Response of the isolates to root and shoot growth of grass pea

Based on the plant growth promotion and biocontrolling test performance conducted and result obtained, out of the 12 isolates 9 potential *Rhizobium* isolates, AAUGR- 2, 5, 6, 9, 11, 14, 20, 24 and 30 were selected for greenhouse test on potted soil. “Wasse” variety was subjected to grow on potted slightly neutral soil (7.1 pH) that was brought from Kutaber, one of the sampling sites in South Wollo.

Table 3. 8 Performance of selected *Rhizobium* isolates on potted soil under greenhouse conditions

Isolate code	16S rRNA identification	NN/p	NDW/p (g)	SL (cm)	SDW/p (g)
AAUGR 2	*R.* sp.	103.0±8.5^{e}	0.074±0.02^{cde}	51.4 ± 3.8^{de}	1.01±0.03^{d}
AAUGR 5	UD	105.0±8.7^{e}	0.076±0.02^{cde}	51.7 ± 4.2^{de}	1.01±0.05^{d}
AAUGR 6	*R.* sp.	120. 7±7.8^{cd}	0.086±0.09^{c}	60.0± 10.4^{cd}	1.34±0.09 c
AAUGR 9	*R.* sp.	132.0±5.6^{c}	0.100±0.05^{b}	65.3± 2.5^{bc}	1.49±0.06^{b}
AAUGR 11	*R.* sp.	144.7±6.7^{b}	0.105±0.07^{b}	70.3 ±2.1^{ab}	1.59±0.06^{ab}
AAUGR 14	*R.* sp.	157.7±12.9^{a}	0.125±0.09^{a}	75.5 ±1.8^{a}	1.65±0.01^{a}
AAUGR 20	*R.* sp.	88.3±3.1^{f}	0.070±0.08^{de}	55.2± 5.1^{d}	0.80±0.05^{e}
AAUGR 24	*R. legum.*	104.0±7.7^{e}	0.076±0.01^{cde}	51.9 ± 4.0^{de}	1.00±0.07^{d}
AAUGR 30	*R.* sp.	78.3±7.0^{f}	0.066±0.02^{e}	59.0 ±5.0^{cd}	0.87±0.11^{e}
+VE	-	12.3±1.5^{g}	0.019±0.0^{f}	51.7 ± 1.5^{de}	1.22±0.11^{d}
-VE	-	21.3±6.1^{g}	0.024±0.02^{f}	43.2±5.8^{e}	0.77±0.06^{f}

Data are means of three replicates ± SE. The means followed by different letters are significantly different at $P < 0.01$ (Duncan test)- SPSS 20.0 version. HE= >80%, E= 50-80%, LE= 35-50%, IE= < 35%, UD= undetermined. NN= nodule number,NDW= nodule dry weight, SDW= shoot dry weight, SL= shoot length.

The symbiotic test conducted on potted soil showed variations in nodule number, nodule dry weight, shoot length and shoot dry weight amongst the inoculated plants (Table 3. 7). Accordingly, the highest nodule number (157 nodules/plant) was obtained from plants inoculated with isolates AAUGR- 14 (*Rhizobium* species) which was followed by 144 nodules/plant from plants treated with isolates AAUGR- 11 (*Rhizobium* species). Similarly, the least nodule number (78 nodules/plant) was recorded from plants inoculated with AAUGR- 30 (*Rhizobium* species). In this study, 101 % difference was recorded between the highest and lowest nodule numbers/plant. However, Abere Minalku *et al*., (2009) and Anteneh Argaw (2012) reported a nodule number difference of

16 % and 127 % from rhizobia inoculated faba bean and lentil plants, respectively implying that isolates of this study showed better symbiotic efficiency. In this study, the positive and negative control plants formed few nodules that were small in size and white in colors from the indigenous rhizobia which may be due to ineffective native rhizobia (Laurette *et al*., 2015). In this study, better nodulation enhancement was observed as a result of *Rhizobium* inoculation that indicated poor status of native rhizobia and better competitive ability of the introduced rhizobial isolates for nodule formation (Fobert *et al*., 1991).

The nodule dry weight of inoculated plant was highly significant amongst rhizobial isolates. The highest nodule dry weight (0.125g/p) was recorded from plants inoculated with isolates AAUGR-14 (*Rhizobium* species) which was followed by a nodule dry weight of 0.105g/p and 0.100g/p recorded from plants inoculated with isolates AAUGR-11 (*Rhizobium* species) and AAUGR-9 (*Rhizobium* species). The least nodule dry weight of 0.066 g/p was obtained from plants inoculated with isolates AAUGR-30 (*Rhizobium* species). All rhizobial inoculated plants accumulated a nodule dry weight greater than the controls. The difference in nodule dry weight between the maximum and minimum accumulated amongst rhizobial treated plants was 89% which was nearly similar with Zerihun Belay and Fassil Assefa (2011) who reported 93 % difference from rhizobia inoculated faba bean plants.

The highest shoot length (76 cm/p) was recorded from plants inoculated with isolate AAUGR- 14 (*Rhizobium* species) that was followed by a shoot length of 70.3 cm/p from plants treated with AAUGR- 11(*Rhizobium* species). Whereas, the shortest shoot length (52 cm/p) was recorded by plants inoculated with isolate AAUGR- 5. Regarding difference in shoot height, 44 % difference was obtained between the most effective and least effective inoculants which was less than the findings of Ayeabeba *et al*., (2001) who reported 85 % shoot height difference between longest and shortest height of rhizobia inoculated faba bean plants. Hoque and Haq (1994) reported that inoculation of seed with *Rhizobium* significantly increased plant height of lentil. It is therefore, possible to deduce rhizobial inoculation significantly increased shoot length of grass pea.

Regarding the shoot dry weight accumulation, all rhizobium inoculated plants accumulated a shoot dry weight greater than the negative control (Table 3. 7). The difference in shoot dry weight accumulated by the least effective and most effective isolate ranged 4- 114 % as compared to the negative control plant. Anteneh (2012) reported 26- 62 % faba bean shoot dry weight difference between the most and least effective inoculant compared to the negative control indicating better nitrogen fixation efficiency of grass pea rhizobia.

The fact that all bacterial treated plants accumulated a shoot dry weight greater than the negative control plants indicated inoculation improved the growth of plants. It is interesting to note that inoculated plants with isolates AAUGR- 6, 9, 11 and 14 which were identified as *Rhizobium* species accumulated shoot dry weight higher than the control plants in the presence of indigious rhizobial population which implied the superiority of the rhizobial inoculants to the native soil rhizobia (Anshu and Gupta, 2014). Anteneh Argaw (2012) reported a higher shoot dry weight from rhizobia inoculated faba bean plants than that of control plants. The isolates AAUGR- 6, 9, 11 and 14 that were identified as *Rhizobium* species were highly effective in fixing nitrogen under pot soil experiment, PGP and biocontrol activities. Consequently, they were selected for coinoculation trial under greenhouse conditions.

3.3.5 Molecular characterization

3.3.5.1 PCR amplification and gel visualization of 16S rRNA and nif-H genes

Out of the total of 22 phenotypically characterized rhizobacterial isolates, the DNA of ten (10) of them was extracted and amplified for the identification of 16S rRNA and nif-H genes of which all and 6 formed distinct bands, respectively. The amplification and gel visualization of 16S rRNA and nif-H genes resulted in the formation of corresponding thick bands at 1470, 780 or 500 bps, respectively. All the PCR amplified rhizobacterial isolates formed a thick band of 16S rRNA gene which was used for sequencing to identify their phylogenetic relationship to other bacteria since it has universal distribution, highly conserved nature, fundamental role in protein synthesis, do not horizontally transfer and its slow rate of evolution which represents an appropriate level of variation between organisms (Clarridge, 2004).

The amplification of the 16S rRNA gene of the selected rhizobacterial isolates used in this study showed a single band of nearly 1.5 kb in size that corresponded to the expected size reported by Bontempts *et al*., (2010) and Berrada *et al*., (2012). Among the isolates of rhizobacteria six of them amplified approximately 500 bp and 780 bp fragment of the nifH gene (Gaunt *et al*., 2001).

3.3.5.2 Extraction of PCR products and sequencing of 16S rRNA gene

Table 3. 9 16S rRNA gene partial sequencing of selected rhizobacterial isolates of grass pea

S/N	I.code	Length	Target organism	Closest gene bank similarity	Accession number	Query Cover	Identit y (%)
1	AAUG PR-38	1406	*Pantoea* species	*Pantoea*-sp.1	KF017289.1	100	99
			Pantoea agglomerans	*Pantoea agglomerans* strain S5-44	KC202283.1	99	99
			Enterobacter species	*Enterbacter ludwigii* strain AR-165	KF843728.1	100	99
			Enterobacter species	*Enterobacter sp. WTNC1*	JQ398852.1	100	99
			Enterobacter species	Enterobacter cloacae strain LY-62	KU867644.1	100	99
			Enterobacter species	Enterobacter kobei strain DSM 13645T	LT547821.1	100	99
2	AAUG PR-53	821	*Enterococcus casseliflavus*	*Enterococcus casseliflavus* strain LAHHAB-24	KX908026.1	100	99
			Enterococcus gallinarum	*Enterococcus gallinarum* strain *F1*	KX890228.1	100	99
			Enterococcus species	*Enterococcus*-sp.687B1-12EGalle	KU644378.1	100	99
3	AAUG PR-73	*506*	*Serratia* species	*Serratia sp. JY-2*	KY432775.1	*99*	*94*
			Serratia marcescens	*Serratia marcescens* strain 6-1	*KY790421.1*	*99*	*94*
			Serratia nematodiphila	*Serratia nematodiphila* strain RCB885	KT261097.1	*99*	*94*
			Serratia ureilytica	*Serratia ureilytica* strain *RCB891*	KT261103.1	*99*	*94*
			Enterobacter species	*Enterobacter sp.* strain *jx-10*	*KY780224.1*	*99*	*94*
			Enterobacter species	*Enterobacter aerogenes-strainBAC006*	*KU161309.1*	*99*	*94*
			Kluyvera species	*Kluyvera ascorbata* strain*RCB318*	*KT260530.1*	*99*	*94*
			Kluyvera species	*Kluyvera cryocrescens* strain*VJ-5*	*KX226333.1*	*99*	*94*
			Kluyvera species	*Kluyvera georgiana* strain*AF1*	*KY438192.1*	*99*	*94*
			Kluyvera species	*Kluyvera intermedia* strain*RCB78*	*KT260290.1*	*99*	*94*
4	AAUG PR-91	*1435*	*Enterococcus* species	*Enterococcus* sp. CL2	KJ124182.1	99	99
			Enterococcus casseliflavus	*Enterococcus casseliflavus* strain ZZUA83	LC119138.1	99	99
			Enterococcus gallinarum	*Enterococcus casseliflavus* strain 99B(BR46)	KF254559.1	99	99
5	AAUG PR-92	1433	*Enterococcus* species	*Enterococcus sp.123PY*	AB675128.1	100	99
			Enterococcus gallinarum	*Enterococcus gallinarum* strain *CCFM8319*	KJ124182.1	99	99
			Enterococcus gallinarum	*Enterococcus gallinarum* strain *FMAC104*	KF060264.1	100	99

The nucleotide sequence analysis of the isolates showed that each isolate shared similar maximum homology with different bacterial isolates (Table 3.8). As the nucleotide sequence analysis of the strains performed confirmed, isolates AAUGPR-38 showed 99%

homology with several *Pantoea* species, *Pantoea agglomeran* strains, *Enterobacter* species, *Enterbacter ludwigii* strains, *Enterobacter cloacae* strains and *Enterobacter kobei* strains for which representatives were presented. Mulisa *et al.*, (2016) reported 99-100 % sequence homology of lentil rhizobacteria with *Enterobacter ludwigii* (JX979114), *Enterobacter kobei* (JX979115), *Enterobacter cloacae* (JX979128) and *Enterobacter kobei* (NR 028993.1) and *Pantoea agglomerans* (AY900169.1) with 99 % identity.

Similarly, the 16S rRNA gene sequence of isolate AAUGPR-73 showed a maximum homology of 94% with several genera and species of organisms in the NCBI Genedata base however, those that showed a repeated identity that included several *Serratia* species, *Serratia marcescens* strains, *Serratia nematodiphila* strains, *Serratia ureilytica* strains, *Enterobacter* species, *Enterobacter aerogenes*, *Kluyvera ascorbata*, *Kluyvera cryocrescens, Kluyvera georgiana* and *Kluyvera intermidia* were presented (Table 3. 8). *Diriba* Muleta *et al*. (2009) reported coffee rhizobacteria that showed 100 % identity with *Serratia marcescens* (ATCC 13880).Whereas, the 16S rRNA gene sequence of the isolates AAUGPR- 53, 91 and 92 showed a maximum sequence similarity of 99% to different *Enterococcus* species, *Enterococcus casseliflavus* strains and *Enterococcus gallinarum* strains with different NCBI accession numbers.

The blast search result of the 16S rRNA gene sequences of the native isolates against sequences of related organisms deposited at the NCBI GenBank database failed to show maximum identity with only one target organisms but with different genus or same genus but different species of organisms which implied that the 16S rRNA gene sequences though widely used as genetic markers for bacterial classification, the findings of this study confirmed that it is not an accurate method of identification at the species level. It was reported that the precise identification and description of closely related bacterial species requires multilocus sequence analysis (MLSA) using different protein-coding genes (Martens *et al*., 2008). The sequences of the native isolates showed 30-99%

similarity with each other and with sequences of related organisms obtained from the NCBI forming four clusters which indicated the existence of molecular diversity.

3.3.5.3 Phylogenetic analysis of 16S rRNA gene

A phylogenetic tree was drawn from the aligned sequences using Mega 6 software (Fig. 3. 1). All the isolates showed 30-99% similarity amongst each other and were found forming four clusters. Native isolates of AAUGPR- 73 and their related sequences obtained from the NCBI Gendata base were grouped in one cluster with 40-63% similarity with each other. Similarly, isolate AAUGPR- 38 and related sequences were grouped in the second cluster with 30-38% similarity. Whereas, two isolates of AAUGPR- 91 and 92 (*Enterococcus caseliflavus/gallinaru*) were grouped in the third cluster with 99% similarity with each other. Likewise, sequences of AAUPGR-53 and related sequences were found in the fourth clustering group with 99% sequence identity among one another. The sequence obtained from the NCBI Gendata base (*R. leguminosarum*) used as an out group was found grouped in the first and second clusters with 78 and 73% of similarity, respectively both with sequences of native isolates and related sequences taken from the NCBI.

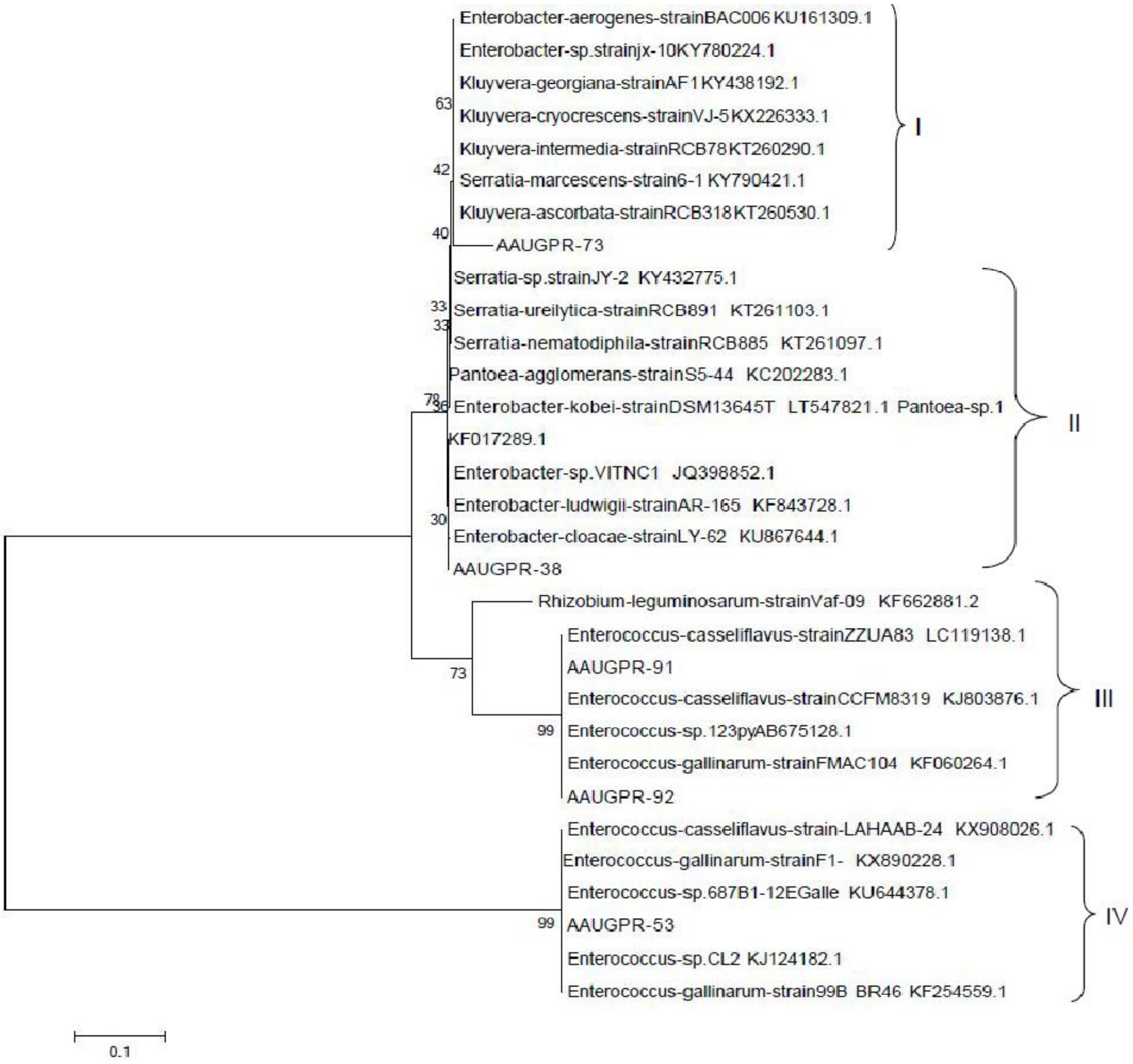

Fig 3.1 Phylogenetic tree constructed by Neighbor-Joining method derived from analysis of the 16S rRNA gene sequences at 496 aligned positions (without gaps) of native isolates (AAUGPR-38, 53, 73, 91, 92) and related sequences obtained from NCBI. The tree was rooted by *Rhizobium leguminosarum* strain Vaf-09 (KF662881.1) as an out group. Scale bar, 0.1 substitutions per nucleotide position (AAUGPR represents test strains in this study). The numbers at the branching points are the percentage of occurrence in 1000 bootstrap values as indicated at the nodes.

The phylogenetic tree (Fig. 3. 1) and 16S rRNA gene sequence analysis of the isolates AAUGPR-53, 91, and 92 showed 99% sequence identity with several *Enterococcus* species, *Enterococcus casseliflavus* strains and *Enterococcus gallinarum* strains whose genome is found at the NCBI Gendatabase where the only selected examples were presented (Table 4.7). Whereas, the 16S rRNA gene sequence analysis of the rhizobacterial isolates represented by AAUPGR-38 and 73 showed 99% and 94% sequence homology, respectively with several different genus and species of microbial organisms whose genome is deposited in the NCBI Gendatabase in which only few were selected and presented (Table 3. 8).

Chpater 4. The effect of inoculation and co-inoculation of rhizobia and rhizobacteria on growth and symbiotic effectiveness response of grass pea under greenhouse condition

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Abstract

A greenhouse experiment was conducted to study the application of inoculation and co-inoculation of *Rhizobium* and rhizobacteria in improving the nodulation, growth and subsequent nitrogen fixation which are important for determining the productivity of legumes. Nodulation and nitrogen fixation can be improved by applying combined inoculation of rhizobia with plant growth promoting rhizobacteria (PGPR). Hence, four rhizobial isolates AAUGR- 6, 9, 11 and 14 (identified as *Rhizobium* species) and two rhizobacteria AAUGPR- 53 and 92 (*Enterococcus casseliflavus/ gallinarum*) that were highly effective and competitive in their symbiotic effectiveness, tolerance to abiotic stresses, plant growth promoting and biocontrol properties under greenhouse and *in vitro* conditions were selected. Their compatibility was checked on dual plate culture. Single inoculations, combined and mixed co-inoculations were conducted and the treated plants were allowed to grow for 60 days. Nodulation and shoot growth associated data were collected after 60 days of cultivation and the result was analyzed. Inoculation and coinoculation enhanced nodulation, growth promotion and nitrogen fixation. The enhancement in accumulating shoot dry weight by inoculation with rhizobia and coinoculation with rhizobia and rhizobacteria was in the range of 8- 47 % over the positive control. Four coinoculation treatments accumulated 2- 23 % shoot dry weight over the most effective inoculation by rhizobia alone (AAUGR- 11). AAUGR- 11 (*Rhizobium* species) performed best in all single inoculation treatments. Likewise, among combined co-inoculation treatments, the highest efficiency in all measured parameters was obtained by plants co-inoculated with AAUGR- 14 (*Rhizobium* species) and AAUGPR- 92 (*Enterococcus casseliflavus/gallinarum*). Similarly, plants coinoculated with all mix scored highest in all measured parameters. All and most treatments increased nodulation, growth and symbiotic effectiveness over the negative and positive controls. In general, from the result recorded, AAUGR- 11 and 14 (*Rhizobium* species) and AAUGPR- 53 and 92 (*Enterococcus casseliflavus/gallinarum*) can be recommended as coinoculant candidates for grass pea growth improvement under field conditions.

Keywords: Grass pea, Nodulation, Plant growth promotion, Rhizobacteria, *Rhizobium*

4.1 Introduction

Grass pea (*Lathyrus sativus* L.) is a crop belonging to the family Leguminosae (= Fabaceae), subfamily Papilionoideae, tribe Vicieae (Campbell and Clayton, 1997*)* and is widely grown in Asia (Bangladesh, China, India, Nepal and Pakistan), in the Middle East (Iraq, Iran, Afghanistan, Syria and Lebanon), in Northern Africa (Ethiopia, Egypt, Morocco, Algeria and Libya), in Southern Europe (France, Spain and Italy) (Rachele *et.al*., 2012) and some parts of South America (Chinnasamy *et .al*., 2005). It is used as food, feed and fodder. The seed contains protein, fat, carbohydrates (about 35% starch) and also contains glucose, Pentosans, Phytin, lignin, albumin, prolamine globulin, guttllin, several essential amino acids and minerals (Demelash Hailu *et al.,* 2015).

It is interesting to note that most of the hitherto legume rhizobia symbiosis research were undertaken on the same cross nodulating *Rhizobium* species on faba bean, field pea, lentil (Zerihun Belay and Fassil Assefa, 2011; Solomon Legesse and Fassil Assefa, 2013; Mulisa Jida *et al.*, 2012). There is a dearth of information on diversity and symbiotic property of grass pea in Ethiopia. This is due to a deliberate neglect or a taboo on the crop because of its alkaloid content (β-oxalyl-L-α, β-diaminopropionic acid, ODAP).

Grass pea is one of the leguminous crops used in crop rotation and soil fertility. It is also grown in residual moisture after the rainy seasons indicating that it is a drought resistant crop. Besides its nutritional benefits, grass pea has an important role as a legume crop in crop rotations, reportedly adding around 67 kgha^{-1} of nitrogen to the soil from symbiosis with *Rhizobium* sp. in a single season conferring yield improvement as a result of nitrogen supply to subsequent non-legume crops (Jennifer, 2003).

The beneficial contribution of extracellular PGPR (ePGPR) including *Agrobacterium, Arthrobacter, Azotobacter, Azospirillum, Bacillus, Burkholderia, Caulobacter, Chromobacterium, Erwinia, Flavobacterium, Micrococcous, Pseudomonas* and *Serratia* (Bhattacharyya and Jha, 2012) and intracellular PGPR (iPGPR) such as *Allorhizobium, Azorhizobium, Bradyrhizobium, Mesorhizobium, Sinorhizobium* and *Rhizobium* (Munees, A. and Mulugeta, kibret, 2013) to plant and soil health has been recently recognized. The co-inoculation of *Bacillus megaterium* SNji in common bean plants with *Rhizobium phaseoli* provided the largest increases in nodule number, nodule dry weight, shoot length and shoot dry weight (Olivera *et al.*, 2011).

In Ethiopia, it is the most neglected crop in terms of research not withstanding the compound to other crops such as faba bean, field pea, lentil and chickpea. Although the crop is rich in all important nutrients, it is the most neglected crop in terms of research in breeding, disease and biological nitrogen fixation. The production of the crop is reduced to its 15- 25% yield potential due to a range of biotic stresses and abiotic factors (Girish *et al*., 2016). The demand for food to the ever-increasing population of the world which is projected to research 8.9 billion by 2050 (Rajeshwar, 2014) necessitate the search for the exploitation of biological resources including the neglected crops such as grass pea. Grass pea (*Lathyrus sativus* L.) is the fifth most important pulse crop in Ethiopia after faba bean, chickpea, red haricot bean and field pea in terms of production area and level of production (CSA, 2017).

The protein content of grass pea seeds collected from 15 major production areas of Ethiopia was 27–32% (Urga *et al*., 2005), which is higher than the average percentage of protein content (21–25%) in other legume seeds (Monsoor and Yusuf, 2002). It is reported that co-inoculation of *Rhizobium* with *Pseudomonas* strains enhanced nodulation and other nitrogen fixation parameters in common bean (*Phaseolus vulgaris*) (Sanchez *et al*., 2014). Co-inoculation of PGPR with rhizobia has shown improvement in nodulation, root and shoot growth, symbiotic effectiveness, plant health and yield to inoculation with *Rhizobia* alone (Rajendran *et al*., 2012). The coinoculation of rhizobia and PGPR on lentil brought a synergistic effect through enhancing nodulation, root length, shoot length and biomass (Zafar-ul-hye *et al*., 2013). The main aim of this work was to evaluate the effect of single, combined and mixed inoculation and co-inoculation with four selected rhizobia and two rhizobacterial isolates up on nodulation, growth and BNF efficiency in grass pea.

4.2 Materials and Methods

4.2.1 Sources of inoculants

The effective rhizobial and rhizobacterial isolates under laboratory and greenhouse conditions were selected and used as sources of inoculants. They were *Rhizobium* (AAUGR-6, 9, 11, 14) and rhizobacteria (AAUGPR-53, 92) (Table 4. 1).

4.2.2 Soil analysis and determination of most probable number of rhizobia (MPN)

The soil pH, total nitrogen, organic carbon and available phosphorus (Sahlemedhin Sertsu and Taye Bekele, 2002) and density of the indigenous rhizobia in the soil used for pot experiment was enumerated using the MPN method (Somasegaren and Hoben, 1994) as determined already (Table 3. 1).

Among all the rhizobial and rhizobacterial isolates, four rhizobial and two rhizobacterial isolates that showed better performance in greenhouse and *in vitro* tests were selected (Table 4. 1).

Table 4. 1 Plant growth promotion and biocontrol properties of selected *Rhizobium* and rhizobacterial strains

Isolate code	16S rRNA identification				PGP traits	Biocontrol properties						
		PSI	IAA	SE % sand culture	% SE soil	Chitinase	Protease	Cellulose	HCN production	NH_3 production	Dual culture	PIRG
AAUGR-6	*Rhizobium* sp.	3.37± 0.22 a	418.33±6.11 a	129.5	121.2 ±19.6b	-	+	+	-	+	-	-
AAUGR-9	*Rhizobium* sp.	2.98 ±0.14 ab	401.00±15.13 ab	131.7	132.7 ±13.2ab	+	+	-	+	+	-	-
AAUGR-11	*Rhizobium* sp.	2.68 ± 0.10 bc	375.00±7.94 bc	132.8	143 ± 11.7a	-	+	+	+	+	+	44
AAUGR-14	*Rhizobium* sp.	2.68 ±0.26 bc	375.67±13.32 bc	138.9	148.3 ±14.6a	+	+	+	-	+	+	35
AAUGPR-53	*E.case/gal.*	4.81±0.02 a	56.55±0.45 a	-	-	+	+	+	-	+	+	74
AAUGPR-92	*E.case/gal.*	4.46±0.03 b	53.89 ±1.64 ab	-	-	+	+	+	+	+	+	83

PIRG= percent radial growth inhibition, E.case/gal= *Enterococcus caseliflavus/gallinarum,* PSI=phosphate solubilization index, IAA= indole acetic acid production, %SE= percent symbiotic effectiveness

4.2.3 Inoculant preparation

The rhizobial and rhizobacterial strains selected from the stock culture (Table 4. 1) were streaked on YEMA and NA media, respectively and incubated at 30± 2 ^{0}C for 3 days. Single colony from each strain was separately inoculated into 150ml (the same broth culture media) in 250 ml Elenmeyer flasks. The flasks were incubated at 30 ^{0}C on orbital shaker ((Gollen hamp, England)) at 120 rpm until the cell concentrations reach 10^8- 10^{10} cfu ml^{-1} (OD_{650} nm = 1.0).

4.2.4 Evaluating the compatibility of the isolates

The compatibility of the PGPR and rhizobial isolates was undertaken using cross streaking method on YEMA medium according to the method of Martins *et al.*, (2004).

4.2.5 Treatments and experimental design

The microbial treatments included six single inoculation and co-inoculation with consortia and two controls. The experiment was arranged in a completely randomized design (CRD) with 17 treatments in triplicates resulting in 51 experimental units. The pots were arranged in complete random design with each block consisting of negative control (without nitrogen and uninoculated) and positive control (uninoculated but with nitrogen). The nitrogen fertilizer (KNO_3) was given at a concentration of 0.5 g / l per week until the plants were harvested. All the pots were watered every two days. The seventeen treatments were:

T1 = AAUGR- 6,

T2 = AAUGR- 9,

T3 = AAUGR- 11,

T4 = AAUGR- 14,

T10 = AAUGR- 9 + AAUGPR- 92

T11 = AAUGR- 11 + AAUGPR- 53

T12 = AAUGR- 11 + AAUGPR- 92

T13 = AAUGR- 14 + AAUGPR- 53

T5 = AAUGPR- 53,

T6 = AAUGPR- 92,

T7 = AAUGR- 6 + AAUGPR- 53,

T8 = AAUGR- 6 + AAUGPR- 92,

T9 = AAUGR- 9 + AAUGPR- 53

T14 = AAUGR- 14 + AAUGPR- 92

T15 = All mix

T16 = non-inoculated and unfertilized control

T17 = non-inoculated but fertilized control

4.2.6 Preparation of the pot soil experiment

The soil was brought from one of the sampling sites, Kutaber in South Wollo. It was properly mixed, sieved (0. 2mm) and air–dried and filled in 3 kg capacity plastic pots. Grass pea "Wasse variety" brought from Holeta research center was surface sterilized using 95% ethanol and 3% hypochlorite and rinsed in five changes of distilled sterilized water (Somasagaren and Hoben, 1994). Five seeds were sown in each pot and later thinned down to three after one week of germination. There after, each seedling was inoculated and co-inoculated with 1 ml of each isolate grown for 72 hrs in YEM broth and NB for rhizobia and rhizobacteria, respectively. The experiment was set up in replicates under a greenhouse conditions with maximum temperature of 30+/-1^{0}C and minimum temperature of 19 ± 1^{0}C.

4.2.7 Cultivation and harvest

After 60 days of planting (DAP), data were collected on nodule number, nodule dry weight, shoot length and shoot dry weight per a single plant (Somasegaran and Hoben, 1985).

4.2.8 Statistical analysis

The data were analyzed and interpreted using ANOVA. The experimental treatment means were compared and contrasted against their control and with each other following

Duncan's multiple range test (DMRT) at significance level of 0.05. The correlation between different greenhouse data was evaluated by Pearson correlation coefficient using SPSS v.20.

4.3 Result and Discussion

4.3.1 Compatibility test

All of the selected rhizobacterial and rhizobial isolates didn't antagonize one another showing no inhibitory effect against one another indicating that they were safe for application.

4.3. 2 Evaluation of nodulation and growth parameters under greenhouse conditions

4.3. 2. 1 Effect of inoculation and co-inoculation on nodulation

The nodulation and shoot growth response of grass pea to inoculation and co-inoculation with four *Rhizobium* sp. and two *Rhizobacterium* (*E. casseliflavus/gallinarum*-53 & 92) was presented (Table 4. 2). The result of inoculation and co-inoculation with *Rhizobium* sp. and rhizobacterial strains showed variations in nodulation data. Regarding nodule number, the highest (199.7± 3.5/p) was recorded by plants co-inoculated with the mixture of all the tested rhizobia and rhizobacteria followed by 165.3/p and 160.7/p from plants co-inoculated with AAUGR-14(*Rhizobium* species) + AAUGPR-92 (*E. casseliflavus/gallinarum*) and AAUGR-14 (*Rhizobium* species) + AAUGPR- 53 (*E. casseliflavus/gallinarum*), respectively.

The data showed plants treated with individual *Rhizobium* sp. AAUGR- 6, 9, 11 and 14 induced nodule numbers between 126 NN/p and 155 NN/p. Whereas, plants co-inoculated with AAUGR- 14 *(Rhizobium,* sp.) + AAUGPR- 92 (*E. casseliflavus/gallinarum*) and mixed inoculants induced maximum nodule number of 165 NN/p and 199 NN/p with an increase of 10 and 44 NN/, respectively showing that co-inoculation induced more nodule number than single inoculation. Among the bacteria treated plants, plants inoculated by AAUGPR- 53 (*E. casseliflavus/gallinarum*) and AAUGPR- 92 (*E. casseliflavus/gallinarum*) scored the least nodule number of 64.0/p and 65.7/p, respectively. Moreover, all the bacteria inoculated and co-inoculated plants showed the greater nodule number than both treatments with no bacteria and the negative control and nitrogen fertilizer supplemented positive control plants.

The result of this study was in close agreement to the findings of Martins (2004) who reported nodule numbers of 140 NN/p and 175 NN/p by common bean plants inoculated with *Rhizobium leg*uminosarum bv. *phaseoli* strain BR10049 and co-inoculated with *Rhizobium leguminosarum* bv. *phaseoli* strain BR10049 and the rhizobacterial isolates ENA 4413 and ENA 4419 with an increase of 35 NN/p, respectively which is an indication of synergistic effect. A minimum nodule number difference of 34 NN/p and 45 NN/p was recorded between plants co-inoculated with all mix and AAUGR-14 (*Rhizobium*. sp.) + AAUGPR-92 (*E. casseliflavus/gallinarum*) and co-inoculated with all mix and inoculated with AAUGR- 14 (*R*. sp.) alone. Similarly, a difference of nodule number of 39 NN/p was recorded by plants co-inoculated with AAUGR- 14 (*Rhizobium* sp.) + AAUGPR-92 (*E. casseliflavus/gallinarum*) and plants inoculated with AAUGR- 6 (*Rhizobium.* sp.) alone. Olivera *et al*. (2011) reported a difference of 52 NN/p between plants co-inoculated with *Rhizobium phaseoli* and *Bacillus megaterium* SNji and *Rhizobium phaseoli* alone.

In this study, nodule number difference of 131 NN/p and 142 NN/p was obtained between plants inoculated with AAUGR- 14 (*Rhizobium*. sp.) alone and positive control and plants coinoculated with AAUGR- 14 (*Rhizobium*. sp.) + AAUGPR-92 (*E. casseliflavus/gallinarum*) and positive control, respectively. In greenhouse co-inoculation experiment conducted on common bean plants, Olivera *et al*., (2011) reported a difference of 27 NN/P and 79 NN/p between plants inoculated with *Rhizobium phaseoli* alone and positive control and plants co-inoculated with *Rhizobium phaseoli* and *Bacillus megaterium* SNji and positive control, respectively. All *Rhizobium* alone and *Rhizobium* and rhizobacterium inoculated and coinoculated plants induced nodule numbers ranging from 66 NN/p – 139 NN/p over the negative control. Ckickpea cv. NIFA 88 plants inoculated and coinoculated with *Rhizobium* Rr2 and *Rhizobium* Rnl + *Enterobacter* B showed a nodule number difference of 36 NN/p and 19 NN/p over the negative control, respectively (Mizra *et al*., 2007).

Table 4. 2 The effect of inoculation and co-inoculation with four *Rhizobium* sp. and two *E. casseliflavus/gallinarum* on nodulation and symbiotic properties of Wasse variety grown for 60 days under greenhouse conditions

Strains	**Date of flowering**	**NN/p**	**ND W (g)/p**	SL (cm)/p	SDW.(g)/p
AAUGR-6 *(R*. sp.)	48	126.0 ± 3.2^{e}	0.015 ± 0.002^{h}	62.3 ± 5.84^{e}	1.19 ± 0.07^{def}
AAUGR-9 (*R*. sp.)	47	133.7 ± 7.4^{de}	0.017 ± 0.001^{gh}	61.7 ± 1.45^{e}	1.21 ± 0.15^{def}
AAUGR-11 *(R*. sp. 11)	46	145.0 ± 2.1^{cd}	0.022 ± 0.003^{g}	$64.3 \pm 1.20d^{e}$	1.32 ± 0.06^{cde}
AAUGR-14 *(*R. sp.)	46	154.7 ± 3.3^{bc}	0.033 ± 0.002^{f}	$65.2 \pm 3.03d^{e}$	1.31 ± 0.02^{cde}
AAUGPR-53 *(E. casseliflavus/gallinarum*)	49	64.0 ± 9.7^{f}	0.012 ± 0.001^{hi}	42.8 ± 2.46^{h}	0.79 ± 0.04^{g}
AAUGPR-92 *(E. casseliflavus/gallinarum*)	50	65.7 ± 5.0^{f}	0.013 ± 0.002^{hi}	45.0 ± 3.12^{gh}	0.80 ± 0.01^{g}
AAUGR-6 *(R*.sp.) + AAUGPR-53 *(E.casseliflavus/gallinarum*)	48	130.3 ± 3.0^{de}	0.033 ± 0.004^{f}	66.0 ± 6.03^{cde}	1.21 ± 0.10^{def}
AAUGR-6 *(R*.sp.) + AAUGPR-92 (*E. casseliflavus/gallinarum*)	47	130.7 ± 5.4^{de}	0.035 ± 0.002^{f}	65.3 ± 5.37^{def}	1.17 ± 0.09^{ef}
AAUGR-9 *(R*. sp.) + AAUGPR-53 (*E. casseliflavus/gallinarum*)	45	136.0 ± 6.1^{de}	0.042 ± 0.002^{e}	67.3 ± 1.42^{cde}	1.31 ± 0.08^{cde}
AAUGR-9 *(R*. sp.) + AAUGPR-92 (*E. casseliflavus/gallinarum)*	45	138.7 ± 7.5^{de}	0.049 ± 0.001^{d}	68.3 ± 2.42^{bcde}	1.24 ± 0.04^{def}
AAUGR-11 (*R*. sp.) + AAUGPR-53 (*E. casseliflavus/gallinarum*)	45	155.7 ± 0.09^{bc}	0.054 ± 0.001^{d}	69.0 ± 0.95^{abcd}	1.39 ± 0.03^{bcd}
AAUGR-11 (*R*. sp.) + AAUGPR-92 (*E. casseliflavus/gallinarum*)	45	154.7 ± 3.0^{bc}	0.061 ± 0.002^{c}	70.1 ± 1.04^{ancd}	1.34 ± 0.02^{cde}
AAUGR-14 (*R*. sp.) + AAUGPR-53 (*E. casseliflavus/gallinarum*)	46	160.7 ± 3.2^{bc}	0.067 ± 0.001^{b}	71.2 ± 0.93^{ab}	1.52 ± 0.03^{abc}
AAUGR-14 (*R*. sp.) + AAUGPR-92 (*E. casseliflavus/gallinarum*)	45	165.3 ± 3.4^{b}	0.070 ± 0.002^{ab}	70.9 ± 0.29^{abc}	1.56 ± 0.02^{ab}
All mix	44	199.7 ± 3.5^{a}	0.089 ± 0.004^{a}	74.6 ± 0.90^{a}	1.62 ± 0.02^{a}
PC	49	23 ± 2.52^{g}	0.008 ± 0.002^{i}	60.3 ± 2.33^{f}	$1.\ 10 \pm 0.12^{fg}$
NC	NF	60.0 ± 2.65^{f}	0.012 ± 0.001^{hi}	41.2 ± 2.13^{h}	0.68 ± 0.04^{g}

Note: NN= nodule number, NDW= nodule dry weight, SL= shoot length, SDW= Shoot dry weight, NF = No flower. Levels not followed by the same letter/letters are significant at $p < 0.05$ ((DMRT test)

Variation in nodule dry weight was observed among bacterial treated and control plants. The number of nodules produced by inoculated plants followed the general pattern of all mix > rhizobia combined with rhizobactria > rhizobia alone and rhizobacteria. Consequently, the highest (0.089g/p) and lowest (0.012 g/p) were recorded by plants inoculated with mixed inoculants and the rhizobacteria AAUGPR- 53 (*E. casseliflavus/gallinarum*), respectively. The maximum nodule dry weight 0.089g/p was accumulated by plants inoculated with all mix followed by 0.070g/p accumulated by plants coinoculated with AAUGR- 14 (*Rhizobium*. sp.) + AAUGPR- 92 (*E. casseliflavus/gallinarum*). Whereas, the least nodule dry weight of 0.008g/p was accumulated by positive control plants which was too less by 0.081 and 0.062 compared to nodule dry weight of plants coinoculated with mixture of inoculants and AAUGR- 14 (*Rhizobium*. sp.) + AAUGPR- 92 (*E. casseliflavus/gallinarum*).

A nodule dry weight difference of 0.037 g/p and 0.059 g/p was recorded between common bean plants co-inoculated with *Rhizobium phaseoli* + *Bacillus megaterium* SNji and inoculated with *Rhizobium phaseoli* alone and plants coinoculated with *Rhizobium phaseoli* + *Bacillus megaterium* SNji and positive control plants (Olivera *et al*., 2011), respectively. Similarly, Martins *et al*., (2004) reported a difference of 0.25g/p by common bean plants coinoculated with fluorescent *Pseudomonas* ENA 4413 and *Rhizobium leguminosarum* bv. *phaseoli* strain BR 10049 and inoculated by *Rhizobium leguminosarum* bv. *phaseoli* strain BR 10049 alone. These previous reports indicated that the inoculants of this study showed better performance. All *Rhizobium* alone and *Rhizobium* and rhizobacteria inoculated plants of this study accumulated a nodule dry weight that ranged 0.003- 0.074g/p over the negative control. Mizra *et al*. (2007) reported nodule dry weight difference of 0.30g/p and 0.20g/p accumulated by chickpea plants coinoculated and inoculated with *Rhizobium* Rnl + *Enterobacter* B and *Rhizobium* Rr2, respectively over the negative control.

4.3. 2. 2 Effect of inoculation and co-inoculation on shoot growth and symbiotic effectiveness

Variation in shoot length and shoot dry weight that were regarded as indicators of grass pea growth response to inoculation and co-inoculation with four *Rhizobium* sp. and two *Rhizobacterium* AAUGPR- 53 & 92 (*E. casseliflavus/gallinarum*) was presented (Table 4. 2). Plants co-inoculated with AAUGR- 11 (*Rhizobium* sp.) + AAUGPR- 53 (*E. casseliflavus/gallinarum*), AAUGR- 11 (*Rhizobium* sp.) + AAUGPR- 92 (*E. casseliflavus/gallinarum*), AAUGR- 14 (*Rhizobium* sp.) + AAUGPR- 53 (*E. casseliflavus/gallinarum*), AAUGR- 14 (*Rhizobium* sp.) + AAUGPR- 92 (*E. casseliflavus/gallinarum*) and all mix containing all tested *Rhizobium* and rhizobacteria showed higher shoot length than other treatments and the controls. Taking the maximum height measured into account across each parameters, height differences of 3.4 cm, 6 cm and 9.4 cm was recorded by plants coinoculated and inoculated between mixed inoculants and AAUGR- 14 (*Rhizobium*. sp.) + AAUGPR- 53 (*E. casseliflavus/gallinarum*), AAUGR- 14 (*Rhizobium* sp.) + AAUGPR- 53 (*E. casseliflavus/gallinarum*) and AAUGR- 14 (*Rhizobium* sp.) and mixed inoculants and AAUGR- 14 (*Rhizobium*. sp.), respectively. Aamir *et al*., (2013) reported a shoot length difference of 15. 38 cm between mug bean (*Vigna radiata* L.) plants coinoculated with PGPR and *Rhizobium* and inoculated with *Rhizobium* compared to control plants.

Furthermore, plants coinoculated with all mix and inoculated with AAUGR- 14 (*Rhizobium*. sp.) + AAUGPR- 53 (*E. casseliflavus/gallinarum*) and AAUGR- 14 (*Rhizobium*. sp.) + AAUGPR- 92 (*E. casseliflavus/gallinarum*) showed a difference of 14.3 cm, 10.9 cm and 10.6 cm, respectively over the positive control. Shoot length difference of 3.5/8.8 cm, 5.4/14.3 cm and 1.9/5.5 cm was obtained from lentil and pea plants coinoculated and inoculated by *Rhizobium leguminosarum* PR1 + *Bacillus thuringeinsis* KR1 and *Rhizobium leguminosarum* PR1, *Rhizobium leguminosarum* PR1 + *Bacillus thuringeinsis* KR1 and positive control and

Rhizobium leguminosarum PR1 and positive control, respectively (Mishra *et al*., 2009) implying that isolates of this study were also good enough to be recommended as microbial inoculants. In this study, all bacterially inoculated and co-inoculated plants but rhizobacterium inoculated plants showed shoot height difference ranging from 50- 81 % compared to the negative control. Shoot height difference of 23 % and 76 % were recorded by chickpea plants inoculated and coinoculated with *Rhizobium* Rr2 and *Rhizobium* Rnl + *Enterobacter* B over the negative control (Mizra *et al*., 2007).

Regarding the shoot dry weight, all of the inoculated and co-inoculated treatments showed significant difference at $P < 0.05$ with the negative control. Whereas, all but two treatments AAUGPR- 53 (*E. casseliflavus/gallinarum*) and AAUGPR- 92 (*E. casseliflavus/gallinarum*) accumulated greater shoot dry weight than the positive control with the highest (1.62± 0.02 g/p) and least (1.17± 0.09 g/p) shoot dry weight accumulated by all mix and AAUGR- 6 (*Rhizobium*. sp.) + AAUGPR-92 (*E. casseliflavus/gallinarum*), respectively.

The highest shoot dry weight (1.62 g/p) was accumulated by plants co-inoculated with all mix followed by 1.56 g/p and 1.52 g/p co-inoculated with AAUGR- 14 (*Rhizobium* sp.) + AAUGPR-92 (*E. casseliflavus/gallinarum*) and AAUGR- 14 (*Rhizobium* sp.) + AAUGPR- 53 (*E. casseliflavus/gallinarum*) which was 47%, 42% and 38% over the positive control, respectively. Similarly, 42% and 20% of shoot dry weight was accumulated by plants coinoculated and inoculated with AAUGR- 14 (*Rhizobium* sp.) + AAUGPR- 53 (E. *casseliflavus/gallinarum*) and AAUGR- 11 (*Rhizobium* sp.) alone over the positive control, respectively. Olivera *et al*. (2011) reported 30% and 20% increment of shoot dry weight by common bean plants co-inoculated with *Rhizobium phaseoli* + *Pseudomonas* sp. LG and *Rhizobium* alone over positive control implying that isolates of this study have the potential to improve grass pea productivity.

Plants coinoculated with combination of rhizobia and rhizobacteria and all mix accumulated shoot dry weight greater than single inoculations by rhizobia and rhizobacteria alone. Plants co-inoculated with all mix accumulated 39% shoot dry weight difference over the least accumulated rhizobium inoculated plant and 47% over positive control which was less than the result of Younesi *et al*. (2013) who reported an increase of 57% shoot dry weight of alfalfa plant co-inoculated with *Rhizobium* + *Pseudomonas* over rhizobia alone and 78% over the positive control. In this study, all *Rhizobium* and *Rhizobium* and rhizobacterium inoculated and coinoculated plants except plants inoculated with rhizobacterium alone showed 75 – 138 % shoot dry weight difference over the negative control. However, Mizra *et al*., (2014) reported 200- 400 % shoot dry weight difference between chickpea plants inoculated and coinoculated with *Rhizobium* Rr2 and *Rhizobium* Rnl + *Enterobacter* B over the negative control.

All inoculated and co-inoculated treatments except AAUGPR- 53 & 92 (*E. casseliflavus/gallinarum*) were relatively highly effective in their symbiotic effectiveness. Eleven bacterial treatments (73.3%) showed a symbiotic effectiveness ranging 106 - 147.3 % greater than the positive control with all treatments scoring greater than the negative control. Plants co-inoculated with all mix showed the highest symbiotic effectiveness (147.3 %) followed by 141.8 % and 138.2 % recorded by plants co-inoculated with AAUGR- 14 (*Rhizobium* sp.) + AAUGPR-92 (*E. casseliflavus/gallinarum*) and AAUGPR- 14 (*Rhizobium* sp.) + AAUGPR- 53 (*E. casseliflavus/gallinarum*), respectively. The co-inoculation of AAUGR- 11 & 14 (*Rhizobim*. sp.) with AAUGPR- 53 & 92 (*E. casseliflavus/gallinarum*) enhanced the accumulation of shoot dry matter greater than the positive control and hence showed higher symbiotic effectiveness. Olivera *et al*. (2011) reported that 80% of the co-inoculated treatments showed higher shoot dry matter accumulation and higher symbiotic efficiency than both the positive control and *Rhizobium* alone indicating that isolates of this study have better BNF efficiency.

Among the inoculated, co-inoculated plants and controls, plants co-inoculated with all mix, co-inoculated with AAUGR- 14 (*Rhizobium*. sp.) + AAUGPR- 53 (*E. casseliflavus/gallinarum*) and AAUGR- 14 (*Rhizobium*. sp.) + AAUGPR- 92 (*E. casseliflavus/gallinarum*) showed the highest and next records in all measured parameters consistently. The result of all the measured parameters indicated that plants inoculated with all mix showed higher record than plants treated with single co-inoculation that in turn was higher than single inoculation by rhizobia alone. In general, all bacterially treated plants showed better improvement in all measured parameters over the negative control whereas, 13 (87 %) of the inoculants showed better improvement in shoot length and shoot dry weight over the positive control.

A positive correlation of r= 0.646- 0.921 among the measured parameters including nodule number, nodule dry weight, shoot length and shoot dry weight for response of grass pea to inoculation and co-inoculation with *Rhizobium* – rhizobacteria was observed. The result of correlation enhanced to suggest that inoculation and co-inoculation played major role in the rate of nitrogen fixation and eventually on healthy growth and yield improvement. All the measured parameters showed positive correlation at P= 0.01. Moderate to strong positive correlation of $r > 0.707$ at $p = 0.01$ was recorded. Chibeba *et al.* (2015) reported a highly significant correlation of ($r = 0.92$, $p < 0.001$) between NN and NDW and ($r = 0.95$, $p < 0.001$) between NDW and SDW. Olivera *et al*. (2011) also reported highly positive correlation between NN and NDW. Chibela *et al*. (2015) demonstrated that NDW is the best variable to evaluate biological N-fixation since it is highly correlated with SDW which was also confirmed in this study.

Inoculation of grass pea with rhizobia and rhizobacteria with single inoculation and combined and mixed co-inoculation resulted in a significant increase in nodulation and shoot growth compared with non-inoculated controls. Mizra *et al*. (2014) reported maximum increase in nodule number, nodule dry weight, plant height and shoot dry weight by chickpea plants coinoculated with *Rhizobium* strain Rnl and *Enterobacter* strain B over all treatments compared. In general, in all measured parameters co-inoculation with all mix showed the highest record followed by AAUGR- 14 (*Rhizobium* sp.) + AAUGPR- 92 (*E. casseliflavus/gallinarum*) and AAUGR- 14 (*Rhizobium*. sp.) + AAUGPR- 53 (*E. casseliflavus/gallinarum*) that in turn showed better result than single co-inoculation by others inoculants and *Rhizobium* alone. Likewise, most and all bacterial treated plants displayed higher performance in all measured parameters over the positive and negative control indicating that inoculation and co-inoculation is highly preferred to the application of the costly and uneco-friendly chemical fertilizer. Similar increased nodulation, growth promotion and BNF efficiency as a result of combined inoculations of rhizobia and rhizobacteria were reported (Prasad *et al*., 2002) which indicated that the combined inoculants were endowed with better traits of plant growth promotion and biocontrolling activities (Zahir *et al*., 2004).

Plants co-inoculated with all mix showed the highest performance in all tested parameters indicating that microbial inoculants when used as composite inoculum exhibited maximum efficiency in growth promotion and the suppression of diseases with the characteristic increase in chlorophyll content, total number of leaves, shoot height and thereby facilitating overall crop yield than when inoculated singly (Bhattacharyya and Jha, 2012). The study suggested that though the performance of the inoculants on the tested parameters was good, selection of effective strains which were more compatible to *Rhizobium* would be necessary for obtaining the meaningful benefits from coinoculation.

Chapter 5. General conclusion and recommendations

5.1 General conclusion

Grass pea is the fifth important leguminous crop in area coverage and production in Ethiopia. Although it is an important source of protein and a break crop to enhance soil fertility, there is realistic information on its agronomic and symbiotic properties. It seems that there is deliberate research neglect on the crop. This is due to the presence of alkaloids in seeds that cause lathyrism. Interestingly, it is a member of a viciae tribe that is cross nodulated by the same rhizobial groups infecting faba bean, field pea and lentil. Nevertheless, there is deliberate research black out on the crop on the phenotypic and symbiotic properties of root nodule rhizobia, not the least the PGP properties of its rhizobacteria, the wild one. However, it is widely used as a multipurpose crop in countries such as India and Ethiopia that deserves attention at least from the soil management point of view in low in put agriculture.

The data showed that grass pea rhizobia were as good as other cross inoculation highland legumes in terms of authentication rate (98 %) and symbiotic effectiveness (86 %). The root nodule bacteria from grass pea were best harbored more diverse group *Rhizobium* species (87 %) compared to the traditionally established *Rhizobium leguminosarum* bv. Viciae. The grass pea rhizobia also displayed tolerance to a wide range of environmental stress (pH 4-10, 8 % NaCl, 10- 40 ^{0}C) that other fast growing rhizobia failed to survive. They are also endowed with ability of phosphate solubilization and other multiple growth promoting properties that have a potential as biocontrol agents. The data also showed a diverse group of PGP rhizobacteria in the rhizosphere displayed several PGP and biocontrol traits.

The 16S rRNA and nifH gene sequencing demonstrated that the root nodule and rhizosphere bacteria belonged to different bacterial genera: *Rhizobium, Ensifer (Sinorhizobium meliloti), Enterobacter, Enterococcus, Kluyvera, Pantoea* and *Serratia*. Most of the rhizobial and rhizobacterial isolates were most potent bacteria with 6- 7 multiple plant growth promoting properties. All taken together, the inoculation and coinoculation of isolates AAUGR- 6, 9, 11, 14 (*Rhizobium* species) with AAUGPR- 53, 92 (*Enterococcus caseliflavus/ gallinarum*) showed the most prolific and effective N-fixing and PGPR potential in pot trial under greenhouse conditions via enhancing nodulation and other growth parameters.

5.2. General recommendation

Based on the findings of this study the here under are recommended:

- The rhizobial isolates AAUGR- 6, 9. 11, 14 (*Rhizobium* species) that showed high potential in their symbiotic effectiveness, stress tolerance potential, PGP and antifungal inbition through diverse mechanisms are highly recommended for field applications under different soil and climatic conditions.

- Similarly, the rhizobacterial isolates AAUGR- 53 & 92 that were identified as *Enterococcus caseliflaus/gallinarum* displayed multiple PGP and biocontrol properties with high radial growth inhibition of *Fusarium oxysporum* f. sp. lentils are recommended for field applications particularly for soil suppression.

- The coinoculation of AAUGR- 11, 14 (*Rhizobium* species) with AAUGPR- 53, 92) and all mix showed a significant result in nodule number, nodule dry weight and shoot dry weight. The potential of these isolates in improving the growth and yield of grass pea should be evaluated under field conditions.

- The 16S rRNA gene sequences of AAUGPR- 38 and 73 showed homology with different genus. Multisequence analysis using housekeeping genes such as *ropB, glnII, recA, atpD* and others needs to be conducted for true taxonomic classification of these isolates.

- The 16S rRNA gene sequence showed that 87 % of the rhizobial isolates nodulating grass pea was *Rhizobium* species which was expected to be *Rhizobium leguminosarum* bv. viciae. Thus, its confirmation needs further molecular characterization using multisequence analysis of nod, nif and other housekeeping genes such as *ropB, glnII, recA, atpD.*

- The 16S rRNA and nifH gene sequence blast search result for *Rhizobium* strain designated by AAUGR-2 was identified as *Rhizobium* species and *Sinorhizobium meliloti,* respectively which is not known as a microsymbiont associated with grass pea and other viciae tribe members. Thus, its confirmation requires repeating authentication at greenhouse and characterization at a molecular level again.

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6. References

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7. Appendices

Appendix 1. Correlation matrix of the rhizobia on sand culture

	NN	NDW (g)	SDW(g)	SE
NN	1			
NDW (g)	.864**	1		
SDW (g)	.813**	.817**	1	
SE %	.800**	.811**	.988**	1

** Correlation is significant at 0.01 level

Appendix 2. Correlation matrix of selected rhizobia on potted soil different symbiotic parameters

Correlations

	NN	NDW	SHL	SDW	SE
NN	1				
NDW	.963**	1			
SHL	.774**	.802**	1		
SDW	.742**	.734**	.897**	1	.
SE	.719**	.732**	.896**	.960**	1

**Correlation is significant at the 0.01 level, BRN= branch number, NN= nodule number; NDW= nodule dry weight(g); SL= shoot length (cm);; SDW= shoot dry weight(g) and SL= shoot length, SE (%)= symbiotic effectiveness efficiency.

Appendix 3 Correlation matrix of inoculation and coinoculation of rhizobia and rhizobacteria

	NN	NDW	BRN	SL	SDW
NN	1				
NDW	.804**	1			
BRN	.721**	.801**	1		
SL	.647**	.776**	.646**	1	
SDW	.744**	.753**	.707**	.921**	1

** Correlation is significant at the 0.01 level, BRN= branch number, NN= nodule number; NDW= nodule dry weight(g); SL= shoot length (cm);; SDW= shoot dry weight(g) and SL= shoot length, SE (%)= symbiotic effectiveness efficiency

Appendix 4. Greenhouse authentication of the isolates on sand culture (partial view)

Appendix 5. Selected authenticated isolates on sand culture (AAUGR-11, 14, 15, 50)

Appendix 6. Nodulation of authenticated isolates (AAUGR- 39 & 45)

Appendix 7. PCR amplification of 16S rRNA and nif-H genes

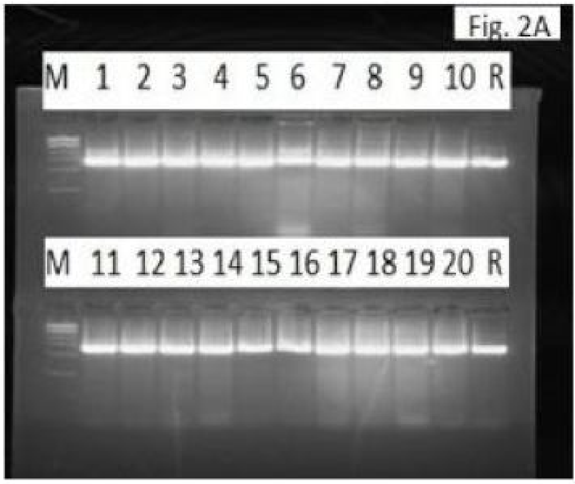


Fig. 2.3A. 16S rRNA gene- M, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, R (top) and

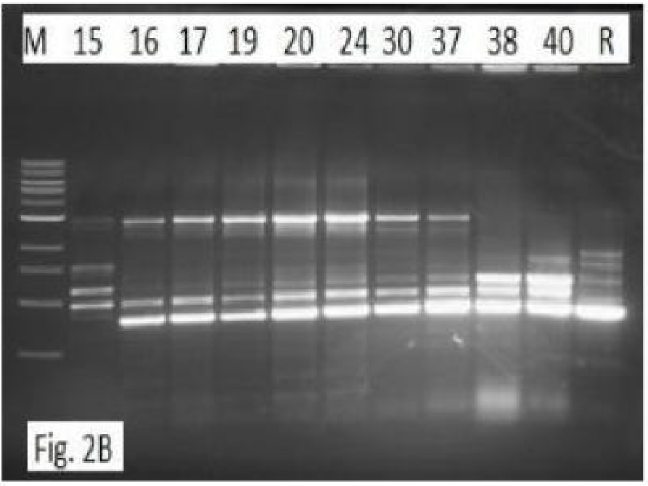


Fig. 2.3B. nif-H-M, 15, 16, 17, 18, 19, 20, 24 30, 37, 38, R

Appendix 8. Phosphate solubilisation index (PSI) of selected rhizobia

M, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, R (bottom)

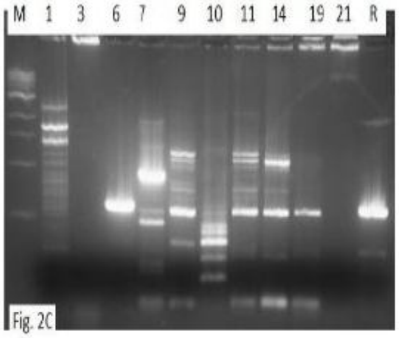
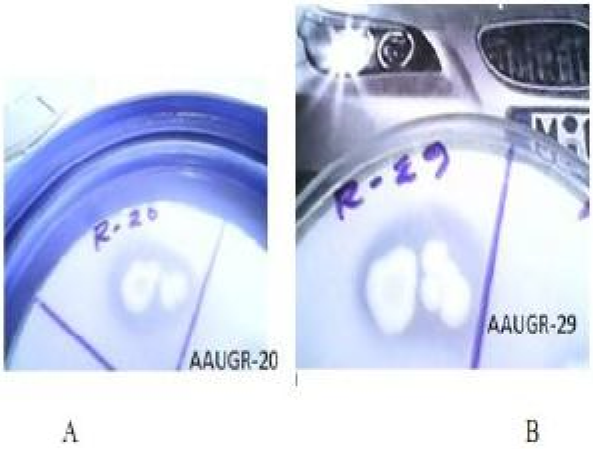
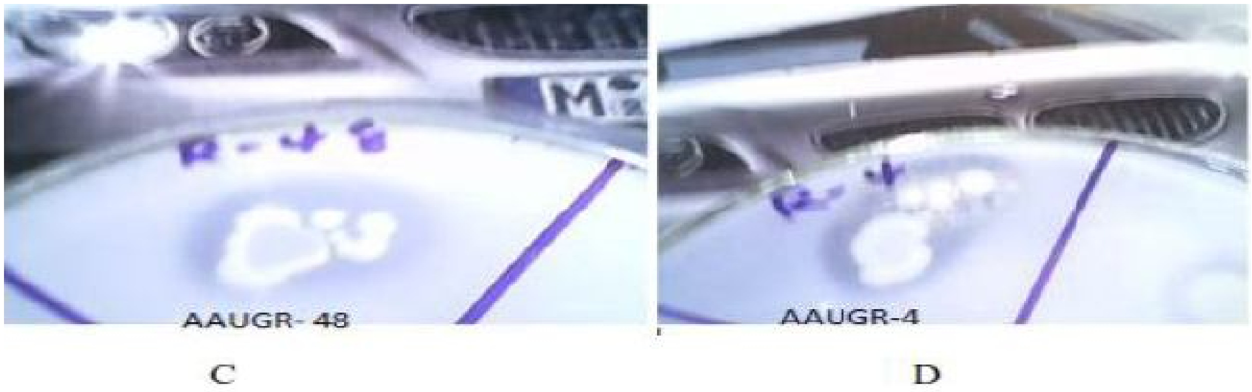


Fig. 2.3C. nif-H-M, 1, 3, 6, 7, 9, 10, 11, 14, 19, 21, R



A

B

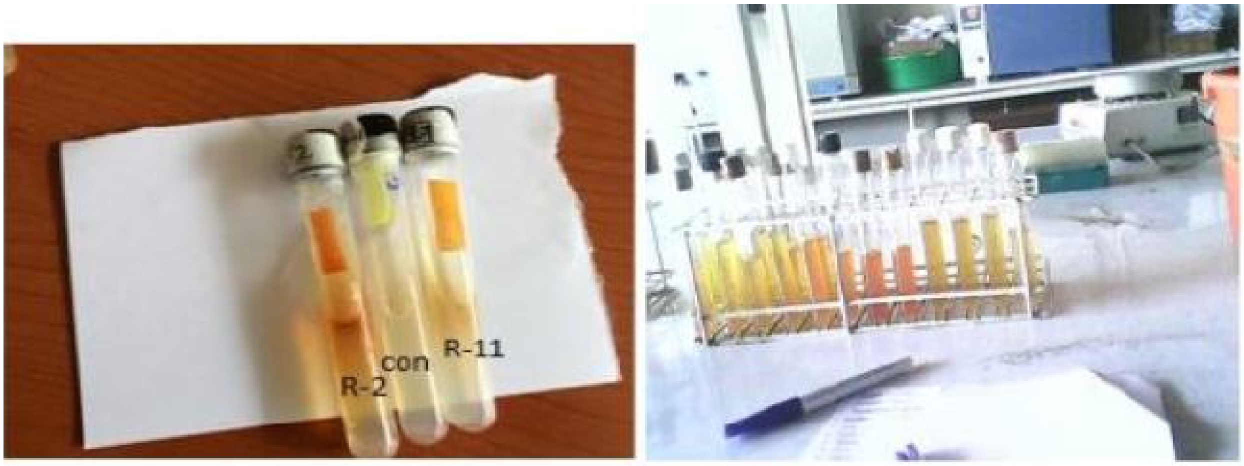


C

D

Appendix 9. IAA production by rhizobia

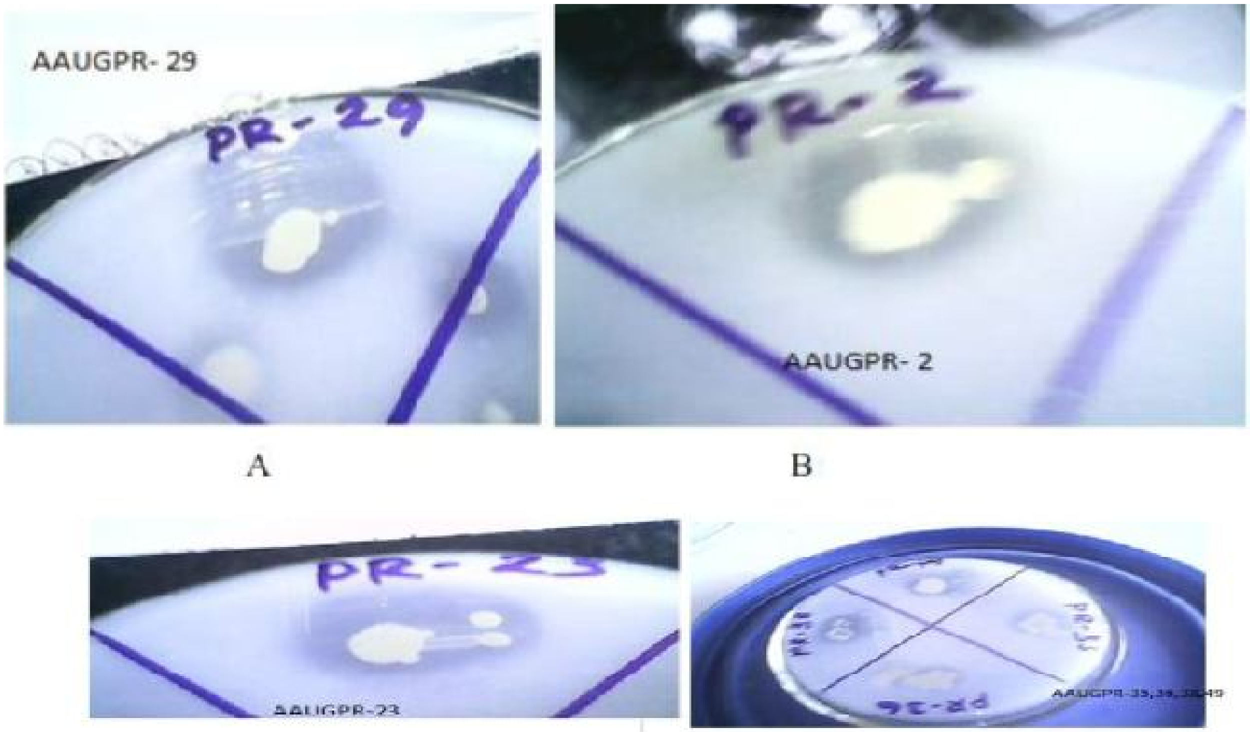
Appendix 10. HCN and NH_3 production by selected rhizobia



HCN production

NH_3 production

Appendix 11. Phosphate solubilisation index (PSI) and IAA production by rhizobacteria



A

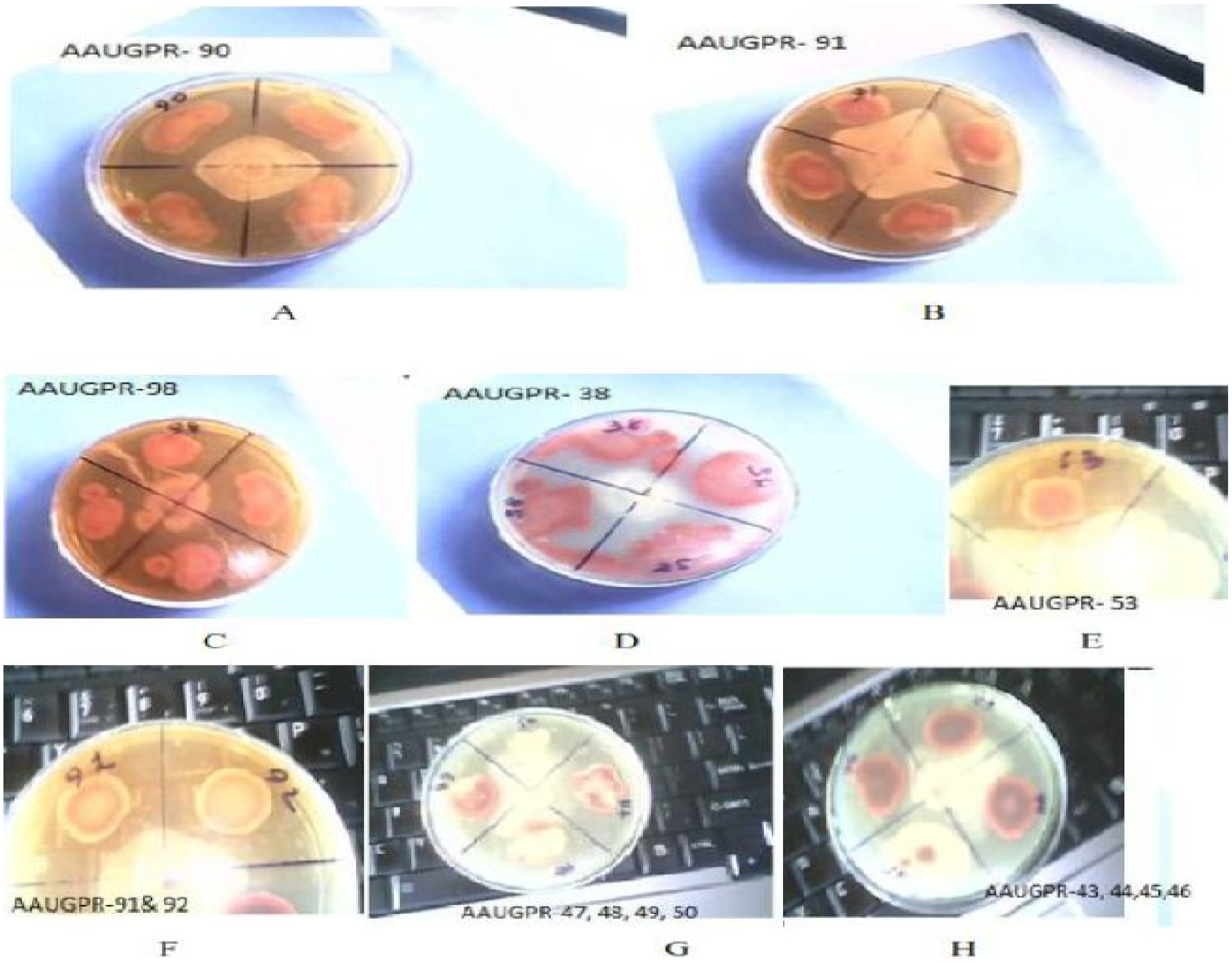
B

C

D

Indole acetic acid production

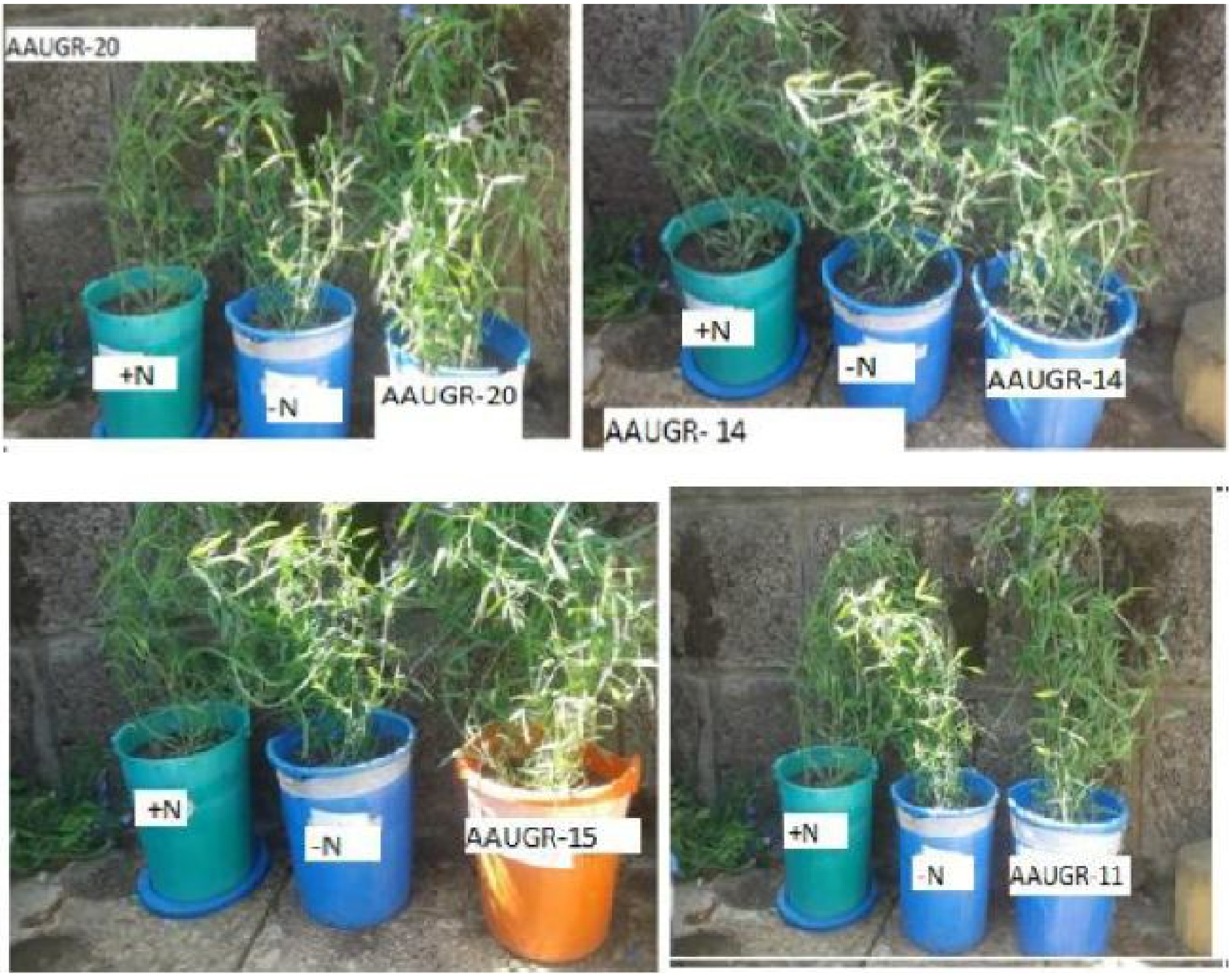
Appendix 12. Antifungal activity of rhizobacteria



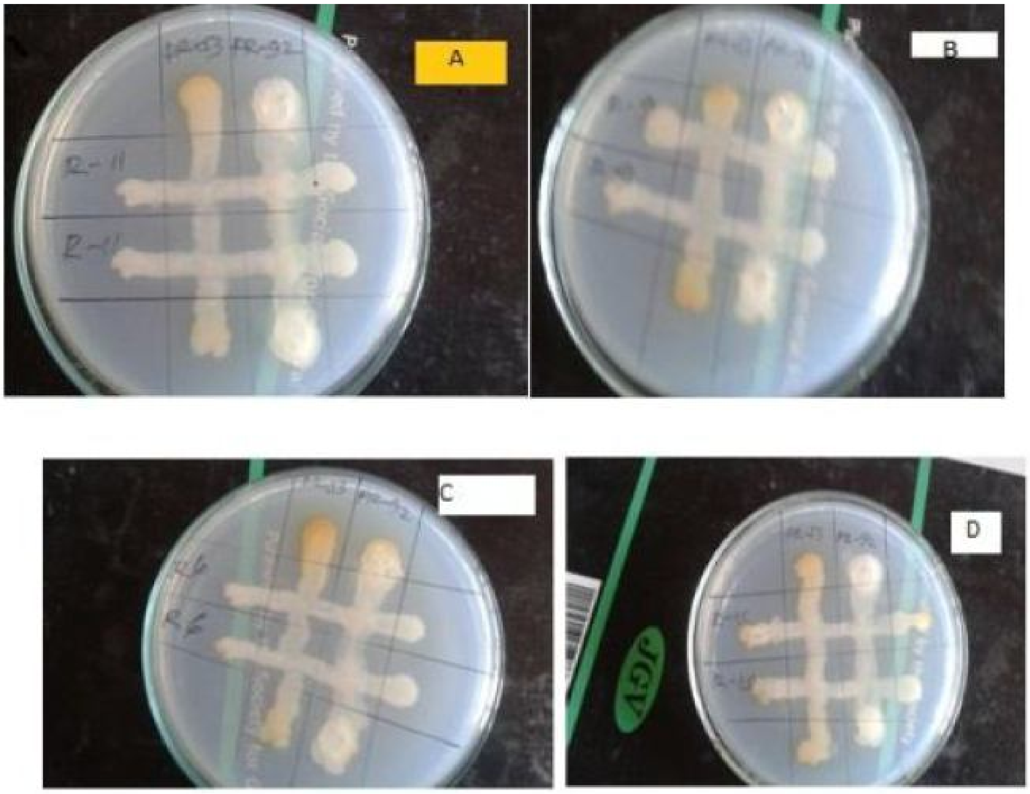
Appendix 13. Other biocontrol activity of rhizobacteria

Cellulase activity HCN production NH_3 production

Appendix 14. Performance of rhizobial isolates on potted soil under greenhouse condition



Appendix 15. Compatibility of selected rhizobia and rhizobacteria



Compatibility test between: A/ R-11 & PR-53, 92, B/ R-9 & PR- 53, 92 C/R6& PR- 53, 92 D/ R-14 & PR- 53, 92

Appendix 16. Greenhouse inoculation and co-inoculation experiment (done twice)

A

B

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