Addis Ababa University

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

This is to certify that the thesis prepared by Getaneh Tesfaye, entitled**: Evaluation of Rhizobia and Phosphate Solublizing Rhizobacteria for Enhancing Growth and Yield in Phaseolus Vulgaris L. via Co-Inoculation in Greenhouse and Field"** and submitted in fulfillment of the requirements for the Degree of Doctor of Philosophy (Applied Microbiology) complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

Supervisor: **Fassil Assefa (Associate Prof.)**

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Evaluation of Rhizobia and Phosphate Solublizing Rhizobacteria for Enhancing Growth and Yield in *Phaseolus Vulgaris* L. via Co-Inoculation in Greenhouse and Field

A thesis Presented to the School of Graduate Studies of Addis Ababa University in Partial Fulfilment of the Requirements for the PhD in biology (Applied Microbiology stream).

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Approval of dissertation by examining board

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Chair of Department

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Abstract

The symbiotic nitrogen fixing effectiveness of common bean nodulating rhizobia and phosphate solubilizing abilities of rhizospheric bacteria collected from the major growing areas of Ethiopia were evaluated. Soil samples were randomly collected from farmers' fields. Isolation of rhizobia was conducted using plant infection method in greenhouse whereas the phosphate solubilizing rhizobacteria (PSR) were isolated from the soils adhering to the root surfaces of common bean. Authentication, phenotypic characterization and symbiotic efficiency of 76 rhizobial isolates were determined following standard methods. PSR were screened for solubilization efficiency on solid and liquid media containing different phosphate sources. All the PSR and rhizobia were tested for other plant growth promoting (PGP) traits. Single- and co-inoculation trials with rhizobial and phosphobacterial isolates were conducted under greenhouse and field trials. Analysis of 16S rRNA gene sequences was performed to identify the top two symbiotically effective rhizobial isolates and all the PSR. The rhizobial isolates, except CBR039 and CBR151, were able to reinfect their host plant. The rhizobia displayed diversity in their phenotypic characteristics and formed four clusters with reference strains that include *R.phaseoli*, *R.etli*, *R. gardiani* and *R. tropici*. The two rhizobial isolates, CBR052 and CBR141, with high symbiotic efficiency were identified as *Rhizobium etli* and *Rhizobium* sp., respectively. Fifty six PSR were isolated in this study and identified into 10 genera that include *Bacillus*, *Arthrobacter*, *Brevibacterium*, *Lycinibacillus*, *Enterobacter*, *Acetinobacter*, P*seudomonas*, *Klebsiella*, *Devosia*, and *Chryseobacterium*. The phosphobacteria released as much as 312.7 µg/ml soluble phosphate from tri-calcium phosphate (TCP). As high as 53.2µg/ml IAA production and 51.3% *Fusarium* inhibition was recorded in this experiment. Inoculation of common bean with selected phosphobacterial isolates in greenhouse significantly ($p<0.05$) improved nodulation, nitrogen fixation and other plant growth parameters. *Rhizobium*-PSR co-inoculations significantly

($P<0.05$) increased the nodulation, shoot dry weight, shoot nitrogen, and grain yield compared to both single *Rhizobium* inoculation and the uninoculated control plants in each of the three varieties. The effect of co-inoculation with CBR052 + PGPRC27 on yield and other growth parameters was the highest on all the varieties. The 1436.35 kg/ha yield recorded in the non-inoculated control was increased to 2118.42 kg/ha (47.5%) due to CBR052 single inoculation and this was further raised to 2694.32 kg/ha (87.6%) by the combined inoculation of CBR052 + PGPRC27 on Ibaddo variety at Melkassa site. There was also significant ($P< 0.05$) variability in yield and other plant development responses among the studied common bean varieties as result of inoculation and co-inoculation. Ibadoo was found to be the best-responding variety for the applied bacterial treatments. These findings could therefore reinforce the understanding on interactions between rhizobia, PGPR and the host plant varieties so that as high as 95% more crop yield in common bean could be achieved through co-inoculation of selected strains.

Keywords*:* Common bean, Co-inoculation, Phosphate solubilising rhizobacteria, Rhizobium

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List of Abbreviations

ANI = Average nucleotide identity

BLAST = Basic local alignment search tool

BNF = Biological nitrogen fixation

ERP= Egyptian rock phosphate

IAA = Indole-3 acetic acid

ISFM= Integrated soil fertility management

MEGA5= Molecular evolutionary genetic analysis version 5

MLSA= Multilocus sequence analysis

NTSYS21 = Numerical taxonomic system version 21

PGPR = Plant growth promoting rhizobacteria

PKV= Pikovskaya medium

PSR = Phosphate solubilizing rhizobacteria

SNF = Symbiotic nitrogen fixation

TCP= Tri-calcium phosphate

UPGMA = Unweighed pair group method with arithmetic mean

Chapter 1 Introduction

1.1 General Introduction

Common bean (*Phaseolus vulgaris* L.) is an important legume crop for human nutrition providing 15% of the protein and 30% of the caloric requirement to the world's population, and represents 50% of the grain legumes consumed worldwide (McConnell *et al*., 2010). The annual crop production worldwide exceeds 23 million metric tons (MT), of which 7 million MT are produced in Latin America and Africa (FAOSTAT, 2013). In Ethiopia, the land devoted to common bean production is 359, 235 ha with a total production of 0.42 million tons and average yield of 1.2 t/ha (CSA report, 2015). Common bean is grown throughout Ethiopia and the major production area of the crop is the Rift Valley region (Ferris and Kaganzi, 2008). Most of the production comes from farmers who don't have enough inputs such as improved varieties, fertilizers, pesticides, etc (Setegn, 1997) and because of these, the average national yield (1.2 t/ha) (CSA report, 2015) is very low compared to its yield potential of 4 t/ha (Beebe *et al.,* 2013).

Low soil fertility, smallholder farming and limited access to external inputs are among the major constraints to the low yield of common bean in Ethiopia (IEAR, 2000). The low soil fertility is mainly associated with deficiencies in nitrogen (Allen *et al*., 1997) and low level of available phosphate in most soils in the country (Getachew Agegnehu and Rezene Fessehaie, 2006). While chemically synthesized fertilizers will necessarily be a part of the various options deployed by agricultural and soil scientists throughout the world, exploiting biological nitrogen fixation and microbially solubilized P are among the major steps for both increasing agricultural yield and reducing economical concerns linked to chemical fertilizer use (Hvistendahl, 2010). Therefore, application of root nodule bacteria and other plant growth promoting rhizobacteria as

a means to amend soil fertility has become an important part of integrated soil fertility management and environmentally sustainable way of improving nutrients availability for plants (Peix *et al.* 2001b; Ponmurugan and Gopi, 2006).

Common bean is known to form symbiosis with a wide range of nitrogen fixing root nodule bacteria that include *Rhizobium leguminosarum sv. phaseoli* (Jordan, 1984)*, Rhizobium tropici* (Martínez-Romero *et al.,* 1991)*, Rhizobium etli* (Segovia *et al.*, 1993), *Rhizobium giardinii* and *Rhizobium gallicum* (Amarger *et al.*, 1997). The crop is usually considered as poor nitrogen fixer and that is mainly due to the nodulation promiscuity of the host plant (Michiels *et al*., 1998) and its association with highly competitive but poorly effective indigenous population of rhizobia in soil (Graham, 1981; Giller and Cadisch, 1995; Hungria and Vargas 2000). However, increases in nodulation, nitrogen fixation and grain yield have been observed when the plant was inoculated with effective rhizobial strains (Hungria *et al.* 2000, 2003; Asadi Rahmani *et al*., 2005; Mnasri *et al.,* 2007).

Apart from the root nodule rhizobia, there has been a growing interest in the search for plant growth promoting rhizosphere bacteria to enhance common bean productivity in various parts of the world during the past decade. These root associated bacteria are known as plant growth promoting rhizobacteria – PGPR (Glick, 1995) and acquire one or more plant growth enhancing characteristics. PGPR can improve plant growth and health by various direct and indirect mechanisms (Glick 1995; Gupta *et al.* 2000). The direct mechanisms of plant growth promoting include solubilization of fixed mineral nutrients such as phosphates and production of phytohormones such as IAA (Gupta *et al.* 2000). Indirect mechanism encompasses control of

soil borne pathogens by producing antimicrobial metabolites such as hydrogen cyanide and secretion of lytic enzymes such as chitinase, cellulase, and protease (Whipps, 2001).

The positive effects of plant root associated bacteria in the production system of various crops were reported from different parts of the world (Pan *et al*., 1999; Gray and Smith, 2005; Pérez-Monta˜no *et al*., 2014). Likewise, in Ethiopia, there are reports on the isolation and characterization of PGPR from rhizosphere of coffee (*Coffea arabica*), sorghum (*Sorghum bicolour*), tef (*Erograstis tef*), lentil (*Lens culnaris*) and chickpea (*Cicer arietinum*) testing their potential as a biofertilizer and biocontrol agents (Ahmed Idris *et al*., 2008; Ahmed Idris *et al*., 2009; Diriba Muleta *et al*., 2009; Delelegn Woyessa and Fassil Assefa, 2011, Diriba Muleta *et al*., 2013; Mulisa jida, 2013). However, little efforts have been made in screening, selecting and evaluating PGPR isolates from the rhizosphere of common bean in Ethiopia.

Furthermore, co-inoculation studies with PGPR and rhizobia showed improved nodulation and N fixation in legume plants (Li and Alexander 1988; Vessey and Buss 2002; Silva *et al.,* 2006; Figueiredo *et al*., 2008, Egamberdieva *et al*., 2010). Accordingly, increased plant growth and yield in common bean were reported elsewhere in the world following combined inoculation of PGPR and efficient rhizobia (Remans *et al*., 2008; Yadegari and Rahmani, 2010; Samavat *et al.,* 2012, Sánchez, *et al*., 2014). Even though, few studies were made on common bean in relation to phylogenetic relationship of rhizobia endosymbionts (Berkum *et al.,* 1996; Aserse *et al*., 2012) and its symbiotic effectiveness in the greenhouse condition (Alemayehu Workalemahu and Fassil Assefa, 2007; Anteneh Argaw, 2007) in Ethiopia, there is still a need for an extensive country wide screening of rhizobia and PGPR from common bean to fully utilize their impact on

the production of the crop both at greenhouse and field conditions. Therefore, this study was proposed with the intention of isolation and characterization of rhizobia from the root nodules and phosphate solubilizing rhizobacteria from the rhizosphere of common bean and selecting effective isolates to be used as biofertilizeres in the low input farming systems of Ethiopia.

1.2 Literature review

1.2.1 Legumes

1.2.1.1 General overview

Plants commonly known as legumes belong to the plant family *Leguminosae* or *Fabaceae* which contains approximately 19,400 species within 700 genera that are distributed in the world (Wojciechowski *et al.,* 2004). It is the third largest family of the flowering plants (Gepts *et al.,* 2005). Legumes vary in habit as annual and perennial herbs, shrubs, trees, and vines/lianas and they are found in all continents, except Antarctica (Doyle *et al*., 2003). Legumes are dominant components of most of the vegetation types throughout temperate and tropical regions and are particularly diverse in tropical forests and temperate shrublands with a seasonally dry or arid climate. Several genera have either their major centres of diversity in Africa or are confined to certain regions of Africa (Sprent *et al*., 2010).

The family *Leguminosae* is divided into three subfamilies, namely, the *Caesalpinioideae, Mimosoideae* and *Papilionoideae* (Gepts *et al.,* 2005). Papilionoideae constitute 65% of the legumes and include the grain legumes and some species of trees, shrubs, herbs, and climbers distributed from the tropics to the arctic. The Mimosoideae are the smallest subfamily, comprising 10% of the legumes, mainly trees and shrubs and often found in the dry areas of the tropics/subtropics. The third subfamily, the *Caesalpinioideae* comprises 25% of the

Leguminosae and mainly constitutes trees growing in the moist tropics (Sprent *et al.*, 2010). At least four genera of the family (*Acacia, Astragalus, Crotalaria* and *Indigofera*) contained 500 or more species whereas at least 40 of the other genera consisted of 100 species or more. At the other extreme, nearly 500 genera are small, either monospecific or containing up to 10 species (Wojciechowski *et al.,* 2004).

Legumes probably evolved some 60 million years ago in the early Tertiary period (Lavin *et al*., 2005; Sprent and James, 2007) and their evolutionary success is largely attributed to their ability to enter into nitrogen fixing symbiosis with rhizobia. Several lines of evidence suggest that, at about 55 million years ago, nodulation in legumes was evolved with a major peak in atmospheric carbondioxide, temperature and humidity (Sprent and James, 2007). As the process of nitrogen fixation consumes a significant amount of the total carbon fixed by the host plant, one driving force could have been an excess of carbon dioxide coupled with a deficit in combined nitrogen. It is established that all legumes do not necessarly nodulate. Until recently, approximately 20% of the total legume species have been examined for nodulation, representing all the three subfamilies (Sprent, 2007). Accordingly, nodulation is known to be rare in *Caesalpinioideae* (23%), common in *Mimosoideae* (90%) and very common in *Papilionoideae* (97%) a sequence thought to be consistent with the order in which the three subfamilies evolved (de Faria *et al*., 1989).

1.2.1.2 The role of legumes in agriculture

Leguminosae are second only to *poaceae* (grasses) in terms of agricultural and economic importance (Wojciechowski *et al.,* 2004). Their agricultural importance has been recognized

since the beginning of domestication of *Lens esculenta* (lentils) in Iran dating to 9500 to 8000 years ago and their use as a food source during the prehistory of North and South America (beans more than 3000 years ago) (Graham and Vance, 2003). The ancient Romans practiced crop rotation with legumes to enhance soil fertility.

A large number of domesticated species are harvested as crops for food, feed, oil, fibre, fuel, green manure, timber, medicines, chemicals, and horticultural use. Grain legumes in general contribute to 33% of dietary protein needs of humans, while the two grain legumes; soybeans (*Glycine max*) and peanut (*Arachis hypogeae*) alone provide more than 35% of the world's processed vegetable oil and a rich source of dietary proteins for poultry and pork industries (Graham and Vance, 2003). Furthermore, their ability to fix nitrogen with rhizobia contributes to rapid growth of the legume crops in marginal soils (Giller, 2001). The restoration and maintenance of soil fertility through crop legumes has been basic and critical to most low-input cropping systems, particularly those in the tropics where many soils are poor in soil fertility (Sprent, 2010).

The incorporation of legumes in their cropping systems allows farmers to grow crops with minimal inputs of N-fertilizer. Since part of their N-requirement is met by nitrogen fixation, legumes utilize less amount of the available soil N than cereals, thereby conserving inorganic N for the intercrop or following crop. Non-legume crops grown in companion or in rotation with legumes usually reduce fertilizer N-requirement, which has both economic and environmental benefits. The fixed nitrogen is recycled when legume crop residues decompose and benefit the

non-legume crops grown in rotation or mixed plantation and residues also improve soil structure by enhancing the formation and maintenance of soil aggregates (Lupwayi *et al*., 2011).

Grain legumes occupy about 20 million hectares of arable land in Africa. The major crops include cowpea (*Vigna unguiculata* L.), which is grown on about 11 million hectares of land mostly in West Africa, and common bean (*Phaseolus vulgaris* L.), which covers about 5 million hectares mostly in eastern and southern Africa (Lupwayi *et al*., 2011). In Ethiopia, legume crops constitute 11.5% of the total cultivated land and 9.6% of the total production of major crops (CSA, 2015). They make up the cheap source of protein in the diets of the Ethiopian population. Furthermore, the roles of these crops in contributing to the country's export market as well as improving soil fertility are of paramount importance. Common bean is among the major crop legumes grown widely in the country.

1.2.1.3 Common bean

Common bean (*Phaseolus vulgaris* L.) is one of the five cultivated species of the genus *Phaseolus* and third in importance in terms of its global harvested area coverage after soybean and peanut (Broughton *et al.,* 2003). Common bean is native to the Americas having two primary centers of origin in the Mesoamerican and Andean regions (Blair *et al.,* 2006) and is distributed to the rest of the world starting in the early 16th (Gepts, 1990). The major common bean producing countries are Brazil and Mexico followed by the United States, Canada, Argentina and China. The crop is also important in Central America, the Andean region of South America, and Eastern and Southern Africa (Katungi *et al.*, 2009) where beans are grown both for subsistence agriculture and for regional markets. Much of the world's bean production is on small farms

ranging from 1-10 ha in size. Nutritionally, beans are often called the “poor man’s meat” for they are inexpensive protein source and are rich in minerals (especially iron and zinc) and vitamins (Beebe *et al.,* 2000).

Common bean is characterized by multiple commercial seed types or horticultural classes based on seed color with white, yellow, cream, brown, pink, red, purple, black and mottled, pinto or striped types popular in different regions of the world (Voysest, 1994). There are two types of common beans; namely the short season bush beans and long-season climbing beans (Voysest, 1994). Bush beans mature in 65 days and are grown for two seasons in tropical regions. These crops are often intercropped, or used as a relay crop and planted at the end of the season to take advantage of residual moisture in the soil (Katungi *et al.,* 2009).

Common bean is widely cultivated in Eastern and Southern Africa. However, approximately 80 percent of African bean is produced in 10 countries. Kenya is the leading producer followed by Uganda and Tanzania. The region contributes 3.3 % of the exports, with Ethiopia as the main contributor accounting for an average of 0.92% of the world exports, followed by Uganda (0.49 %) and Tanzania 0.35 % (Katungi *et al*., 2009).

Ethiopia is the ninth common bean producing country in Africa. It is often grown as a cash crop by small-scale farmers with little input and used as a food legume mainly in rural parts of the country (Habtu Assefa, 1994). It is grown between 1200-2000 masl mean maximum and mean minimum temperature of less than 30^0C and greater than 10^0C respectively, and seasonal rainfall of 350-700 mm (Ohlander, 1977). Although it is cultivated in many parts of the country, most of

the production is in semi-arid areas of east and the central Rift Valley, the mid altitude of south and south-west, and the sub-humid climate of the west (Habtu Assefa, 1996). The area coverage of common bean production in Ethiopia is 359, 235 ha with a total production of 0.42 million tons and average yield of 1.2 t/ha (CSA, 2015).

1.2.2 Biological Nitrogen Fixation

Nitrogen is one of the most limiting nutrients of plant production in the terrestrial ecosystem. The ability to convert molecular dinitrogen into useable forms of ammonia (NH_3) is known as biological nitrogen fixation (BNF) and it is an exclusive trait of some diazotrophic bacteria (Franche *et al.,* 2009). It is one of the most important biochemical processes next to photosynthesis, in nearly all ecosystems (Unkovich *et al*., 2008).

The diazotrophs are grouped into free-living, associative and symbiotic nitrogen fixers (Dresler-Nurmi *et al.,* 2007). Free-living diazotrophs fix nitrogen in free-living state as in the case of *Azotobacter*, whereas associative N fixers such as *Azospirillum* colonize the root surface or endophytic tissues of various plant species (Rothballer *et al*., 2009). Symbiotic diazotrophs fix nitrogen within the intracellular space of the host plant forming a novel organ called nodule.

Symbiotic nitrogen fixation (SNF) is undertaken by legume plants in association with nodule forming bacteria collectively called rhizobia (Sprent, 2007). This symbiosis is the most important mechanism for sustainable input of nitrogen into agroecosystems and accounts for about half of the total BNF (Herridge *et al*., 2008). This is because, rhizobia are strategically best situated to meet the high energy demand of nitrogen fixation since they benefit from plant photosynthate.

Sugar is supplied to the nodule in the form of sucrose, which is eventually shunted to the bacteroides in the form of dicarboxylic acids (mainly succinate) after intermediary metabolism in the nodule cortex (White *et al.,* 2007). In the intact nodule, fixed nitrogen is exported to the plant cells in the form of amides (Aspargine) or ureides (Prell and Pool, 2006).

The development of this symbiotic relationship is a multistep process finely choreographed by molecular signal exchange between the symbiotic partners (Wang *et al.,* 2012). For convenience, the nodulation process and the establishment of symbiosis may be divided into three continuous processes (Masson-Bovin *et al.,* 2009). These are: 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 N_2.

1.2.2.1 Biological nitrogen fixation in common bean

Common bean obtains a significant portion of its N requirement when it is nodulated with effective and compatible rhizobial endosymbiont. However, the crop is one of the low nitrogen fixers compared to the other grain legumes (Table 1.1) (Silva and Uchida, 2000). It is estimated that common bean fix 40-70 kg N ha^{-1} y^{-1}, 40 to 50% of the total N found in bean plants at maturity is derived from BNF (Silva and Uchida, 2000).

Table1.1 Estimates of the amount of nitrogen fixed by various legumes (FAO, 1984) cited from Silva and Uchida (2000)

Plant	**Scientific name**	**Nitrogen fixed (kg N/ha/yr)**
Horse bean	*Vicia faba*	45–552
Pigeon pea	*Cajanus cajan*	168–280
Cowpea	*Vigna unguiculata*	73–354
Mung bean	*Vigna mungo*	63–342
Soybean	*Glycine max*	60–168
Chickpea	*Cicer arietinum*	103
Lentil	*Lens esculenta*	88–114
Peanut	*Arachis hypogaea*	72–124
Pea	*Pisum sativum*	55–77
Common bean	*Phaseolus vulgaris*	40–70
Leucaena	*Leucaena leucocephala*	74–584
Alfalfa	*Medicago sativa*	229–290
Clover	*Trifolium* spp.	128–207

The crop is usually considered as a poor nitrogen fixer mainly due to the nodulation promiscuity of the host plant (Michiels *et al*., 1998) and association with highly competitive but poorly effective indigenous population of rhizobia in soil (Graham, 1981; Giller and Cadisch, 1995; Hungria and Vargas, 2000). Another factor for the poor SNF may be the sensitivity of the symbiosis to environmental stresses, mainly low P availability in the soil (Kaschuk *et al*., 2006). It is established that P deficiency generally affect BNF legumes than other species because symbiotic nitrogen fixation requires more P than does plant growth (Olivera *et al*., 2004).

Variation in common bean germplasm was also reported to affect symbiotic nitrogen fixation. Hardarson *et al*. (1983) compared 20 bean genotypes for SNF and reported symbiotic nitrogen fixation differences between 35-70% Ndfa. Afterwards, other researchers also recognized genotypic variability for SNF in this species (Bliss, 1993; Devi *et al*., 2013).

Several researches also showed that improvements in nodulation, nitrogen fixation and grain yield can be obtained by inoculation of selected common bean varieties with efficient strains of rhizobia (Hungria *et al*., 2000; Asadi Rahmani *et al*., 2005; Mnasri *et al*., 2007; Asadi Rahmani *et al*., 2011) as well as coinoculation with selected rhizobia and PGPR (Yadagari *et al.,* 2010; Stajkovi *et al*., 2011; Sanchez *et al.,* 2014).

In Ethiopia, attempts were made to evlauate the diversity and symbiotic effect of indigenous rhizobia isolates collected from different parts of the country (Alemayehu Werkalemahu and Fassil Assefa, 2007). The Ethiopian studies showed that isolates from rift vally (Alemayehu Werkalemahu and Fassil Assefa, 2007) and Tigray (Alemayehu Werkalemahu, 2009) were ineffective whereas four isolates from Sodo and Konso were effective with an increase in shoot dry matter yield to the tune of 50-72% compared to uninoculated control plants under greenhouse condition. Another study conducted on collections from Eastern Ethiopia (Anteneh Argaw, 2007) revealed that two isolates from eastern Showa and western Hararghe were effective with increased shoot dry weight yield of 118% and 140% in varieties Ayenew and Awash Melka over the non inoculated control respectively.

1.2.3 Environmental stresses to symbiotic nitrogen fixation

1.2.3.1 Temperature stress

Temperature is one of the limiting factors for symbiotic nitrogen fixation (SNF) by legumes, not only in semiarid and arid regions, but also in tropical areas. It adversely affects effectiveness of rhizobia and reduces host legume growth and development (Hungria and Kaschuk, 2014). High soil temperatures decrease rhizobia populations and SNF in legumes, as result of a delay in

nodulation or its restriction to the subsurface regions of the soil profile (Graham, 1992). The critical temperature for SNF varies (from 30 to 42^0C) and is a function of the legume and rhizobia strain. Graham (1992) indicated that for most rhizobial strains, 28 to 31^0C is the optimum temperature range of the soil with a threshold of 37°C, as many strains are not able to grow after this temperature.

Inhibitory effects of high temperature were reported in bean plants where a significant decline in nodule size is observed due to high temperature, i.e. smaller nodules are formed at 35°C with a concomitant reduction in nitrogenase activity (Piha and Munns, 1987) and transfer of nodulated plants from a daily temperature of 26 to 35°C significantly decreased symbiotic nitrogen fixation (Hernandez-Armenta *et al.* 1989).

However, selection of temperature tolerant and adapted host legume (Hungria and Franco, 1993) and rhizobial strains (Graham, 1992) could maintain effective SNF as result of producing heat shock proteins in heat-tolerant bean genotypes and rhizobial strains (Graham, 1992; Michiels *et al.,* 1994).

1.2.3.2 Salinity stress

Soil salinity is also a major restriction for SNF and growth in legumes (Serraj and Adu Gyamfi, 2004; Munns, 2005). However, there is genetic diversity for salt tolerance amongest the host and the endophyte to sustain effective BNF. In different legumes some genotypes have been detected with high SNF under salinity stress. Tajini *et al.* (2012) reported SNF tolerance in two common bean genotypes that could maintain adequate leaf area and numerous active nodules under salt stress.

So far, many *Rhizobial* strains have been introduced to improve yielding ability of N fixing legumes under salinity stress (Sharma *et al*., 2013). However, reports showed that the legume-rhizobia symbioses and nodule formation in legumes are more sensitive to salt stress than free-living rhizobia (El-Shinnawi, 1989; Zahran, 1991). Therefore, inoculation of salt-tolerant bacteria into tolerant bean vatieties could improve the SNF under salinity stress.

1.2.3.3 Soil pH stress

Most leguminous plants require a neutral or slightly acidic soil for growth, especially in SNF-dependent environments (Bordeleau and Prevost, 1994). Soil acidity could affect both rhizobia and the plant host. Specially soil pH lower than 5, is a significant factor contributing to the decline in nodulation, even if a viable rhizobial population is present in the field (Graham, 1992). This auther showed that nodulation appears to be more sensitive to low pH than is plant growth. Low pH reduces SNF by limiting the population of rhizobia in the soil and reducing nodulation (Graham *et al*., 1982; Ibekwe *et al*., 1997). Although, both legumes and rhizobia are equally sensitive to the soil pH, there are differences depending upon varities and strains. However, highly tolerant rhizobial strains, usually, but not always, perform better in low pH soil conditions in the field (Graham *et al*., 1994). Slow growing bacteria, *Bradyrhizobium* strains, are generally more acid tolerant than fast growing except some fast-growing rhizobia strains from *R. loti* and *R. tropici* (Graham *et al*., 1994).

Rhizobia are known to present different mechanisms of tolerance such as increasing the cytoplasmic potassium and glutamate levels in response to soil acidity in *R.leguminosarum sv. phaseoli* (Aarons and Graham, 1991), proton exclusion and extrusion in *Rhizobium leguminosarum sv. trifolii* (Chen *et al*., 1993), accumulation of cellular polyamines in *Rhizobium*

fredii (Fujihara and Yoneyama, 1993), and an acid tolerant composition and structure of the outer cell membrane in *Rhizobium tropici* (Graham *et al*., 1994).

Caetano-Anolles *et al*. (1989) and Vergas and Graham (1988) indicated that the sensitive period of nodualtion to pH is an early stage of nodulation process, when the rhizobia are attached to the root hairs. Soil acidity could be the main reason for nodulation failure as it impacts the survival and persistence of rhizobia (Graham *et al*., 1982). There are also some indirect effects of acidity on SNF. Acidic soils, with pH <5.0, induce sensitivities to heavy metals such as aluminum (Al) that inhibits legume nodulation by increasing the availability of these toxic metals (Bordeleau and Prevost, 1994).

1.2.3.4 Macronutrients stress

1.2.3.4.1 Nitrogen

Symbiotic nitrogen fixation in legumes is hampered by the presence of available forms of inorganic N such as nitrate (NO_3). Although there are a few reports of positive effects of low nitrate concentrations (less than 5 mM) on N_2 fixation in legume species such as soybean (Gulden and Vessey, 1997), nitrogen fertilization at recommended rates of 40-60 kg N ha^{-1} negatively affects nodulation and suppress SNF in common bean (Graham, 1981). Increases in available N down-regulates the nif gene expression inhibiting nitrogenase activity (Merrick, 1993). Decreased nitrogen fixation is also the effect of reduced bacteroid respiration, caused by increased resistance to O_2 diffusion in the nodule cortex (Minchin, 1997). In the presence of nitrate, both the energy cost of nitrogen fixation and the gas diffusion resistance increase, concomitantly the efficiency of bacteroid respiration decreases (Arrese-Igor *et al*., 1997; Chamber-Pérez *et al*., 1997,)

The direct influence of nitrate to affect the gas diffusion resistance is that nitrate concentration could cause formation of a local pH gradient or electrochemical gradient due to differences in the concentration of nitrate or in their reduction rate between cell layers. This could affect gas permeability of cortex cell layer, and consequently to cause conditions in which nitrogenase activity was limited by deficiency of $NAD(P)H_2$ and ATP. Lowered bacteroid respiration activity, caused by an increase in the resistance to O_2 diffusion could result in significant ATP deprivation (Chamber-Pérez *et al*., 1997, Lucinski, *et al*. 2002).

The indirect inhibitory effect of nitrate on nitrogenase activity could also be caused by limiting supply of carbohydrates to root nodules. When a large part of the host plant mass is involved in nitrate assimilation, the burden of the demand for reductive power and carbon frameworks is shifted from nodules to other organs. Therefore, intense nitrate metabolism in roots and leaves might lead to decreased supply of carbohydrates to nodules. Consequently, it lowers the energy state and this inhibits the nitrogenase activity and resuls in reduced nitrogen fixation (Arrese-Igor *et al*., 1997, Lucinski, *et al*. 2002).

1.2.3.4.2 Phosphorus

Phosphorus (P) is an essential element in various molecular and biochemical plant processes, including construction of high energy compounds such as ATP and NADPH for energy acquisition, storage and consumption (Epstein and Bloom, 2005). SNF is an expensive process in terms of energy consumption and the availability of P determines the efficacy of any energy-generating metabolic process (Plaxton, 2004). SNF is, therefore, a high P demanding process and any P deficiency can suppress legume SNF (Schulze *et al*., 1999). P deficiency also causes a

severe limitation on nodulation (Leidil and Rodriguez-Navarro, 2000). The P content of nodules is usually greater than the roots and shoots, particularly in environments with P shortages (Adu-Gyamfi *et al*., 1985). These indicate that nodules are stronger sinks for P than are the other parts of plant (Hart, 1989) and P shortage can have a bigger impact on nodulation than on plant growth (Saxena and Rewari, 1991).

P is one of the most deficient nutrients for cultivation of common bean in resource-poor farming of Sub-Saharan Africa (SSA) (Beebe *et al*., 2011). Soils are considered deficient in P if the soil plant-available P content is less than 40 mg kg $^{-1}$ P determined by Bray-1 method (Mourice & Tryphone, 2012). Characteristically, the soils are deficient in P after being subjected to prolonged degradation by erosion and repeated removal in crop harvest without replacement of the removed P (Henao & Baanante, 2006). It is estimated that there is a loss of 9.2 kg P ha $^{-1}$ year $^{-1}$ in East African cultivated soils (Henao and Baanante, 2006) and 6.6 kg ha $^{-1}$ yr $^{-1}$ of P is depleted in Rwanda, Ethiopia and Kenya due to P removal by crops and erosion (Nziguheba, 2007). Furthermore, these soils are acidic and possess high P-fixing capacities (Nziguheba, 2007). Different studies showed that, 65 to 80% of the bean producing areas in Sub Saharan Africa are deficient in plant-available P (Wortman *et al*., 2004; Lunze *et al*., 2007) reducing common bean yield by over 60% (Acosta-Díaz *et al.,* 2009).

1.2.4 Defining phylogenetic positions of rhizobia and PGPR isolates

1.2.4.1 Systematics of rhizobia

The etymology of ‘rhizobia’ stems from two Greek words: Riza = roots and Bios = life (Lindstrom *et al*., 2006). Originally, the term refers only to the genus *Rhizobium* but later becomes a repository for all bacteria capable of nodulation and nitrogen fixation with legumes

(Willems, 2006; and 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". This definition excludes, bacteria that are closely related phenotypicaly or genetically to nodulating strains but cannot induce nodulation by themselves.

Traditionally, rhizobia were classified based on cross-nodulation groupings (Saxena *et al.,* 2005). Cross-nodulation groups are groups of plants which are nodulated by similar rhizobia. Accordingly, six species were recognized comprising of *Rhizobium leguminosarum* (*Lathyrus, Pisum, Vicia, Lens*), *R. trifolii* (*Trifolium*), *R. phaseoli* (*Phaseolus*), *R. meliloti* (*Melilotus*, *Medicago, Trigonella*); *R. japonicum* (*Glycine max*) and *R. lupini* (*Lupinus*), in addition to an undefined "cowpea miscellany" group.

Over reliance on symbiotic properties and cross inoculation had stalled the relative progress of rhizobial taxonomy for some years compared to that of other bacteria (Rivas *et al.,* 2009). For example, the 'cowpea miscellany' group was put up as a repository to hold unclassified rhizobia (by definition they nodulate cowpea in addition to their original legume host). However, this group eventually contained rhizobia from the majority of all nodulated legumes (Broughton, 2003). Moreover, the discovery of genes encoding symbiotic traits (*nod*, *nif*) that often reside on unstable, mobile genetic elements (Symbiotic islands and megaplasmids) reinforeced the unreliability of symbiotic traits as taxonomic criteria (Rivas *et al*., 2009). Gradually, the principle of 'cross-nodulation group' became less acceptable as a major taxonomic indicator.

With the advent of molecular methods in the last decades, the taxonomy of rhizobia has been more and more streamlined with prokaryotic systematics (Sahgal and Johri, 2006). However, the nodulation of legumes is still retained as a secondary criterion in species description. Genes responsible for symbiotic properties are considered part of the accessory genome, which are subject to horizontal gene transfer and recombination (Lindstrom *et al*., 2006). The rhizobial accessory genome is related to lifestyle and can determine different ecological niches (legume host) on similar chromosomal backgrounds (Young *et al*., 2006). Conversely, similar symbiotic genotype can be found in divergent genomes (Silva *et al*., 2005).

The exclusion of symbiotic property from species description means that, rhizobia remain polyphyletic, distributed in distantly related lineages of alpha and beta subdivision of the phylum *Proteobacteria* (Dresler Nurmi *et al*., 2007). According to Weir, (2012) a total of 98 rhizobial species belonging to 13 genera have been recognized in the current taxonomy. The complete list of valid species of rhizobia is recorded in the List of Prokaryotic Names with Standing in Nomenclature that is curated online by Dr Euzeby (http://www.bacterio.cict.fr). The majority of rhizobial genera belong to the family *Rhizobiacea*; however several other families are also known to contain rhizobial genera. These are *Bradyrhizobiaceae*, *Brucellaceae*, *Hyphomicrobiaceae*, *Methylobacteriaceae*, and *Phyllobacteriaceae* in the alpha Proteobacteria subdivision; and *Burkholderiaceae* and *Oxalobacteriaceae* in the Beta proteobacteria sub division (Dresler Nurmi *et al*., 2007).

1.2.4.2 The taxonomy of common bean rhizobia

Until the mid 1980's, the rhizobia that nodulate common bean, *Phaseolus vulgaris*, were placed in a single species, *Rhizobium leguminosarum* (Jordan, 1984). This species was subdivided into three symbiovars, largely on the basis of specificity for host plant infection and nodulation. Strains recovered from nodules of *Phaseolus* species were assigned to *R. leguminosarum sv. phaseoli* and it was noted that this symbiovar was more distinct than the other two (Jordan, 1984). *Rhizobium leguminosarum sv. phaseoli* is commonly found in Europe, and it was also reported to be present in Colombia, Tunisia and Ethiopia (Eardly *et al*., 1995, Herrera-Cervera *et al*., 1999, Mhamdi *et al*., 1999, Mhamdi *et al*, 2002, Aserse *et al*., 2012).

Members of *R. leguminosarum sv. phaseoli* were recognized to be a heterogeneous complex of strongly differentiated phylogenetic lineages (Pinero *et al.,* 1988). Based on molecular genetic analysis, two symbiotypes of *R. phaseoli* have been recognized. Symbiotype I contains the majority of the strains and is characterized by the presence of multiple copies of the nitrogenase structural genes, having a limited host range for nodulation (Martinez *et al*., 1985), and the production of melanin-like pigment (Martinez *et al*., 1988). Whereas, Symbiotype II is characterized by having a single copy of the nif genes, with an extended host range that includes *Leucaena* species (Martinez *et al.,* 1985), and without pigmentation (Martinez *et al*., 1987).

Subsequently, characterization of symbiotype II based on partial sequencing of the 16S rRNA gene, DNA-DNA hybrid-ization, and ribosomal DNA (rDNA) organization led to the creation of a new species, *R. tropici* (Martinez-Romero *et al*., 1991). In addition, two subgroups, subgroups

IIA and IIB, were described under this species to accommodate the relatively low level of DNA-DNA homology (36%) and differences in phenotypic and genotypic characteristics and differences in their specific megaplasmids(Geniaux, 1995).

Rhizobium tropici seems to be native to tropical regions (Marti´nez-Romero *et al*., 1991) and characterized by their tolerance to acidity (Graham *et al*., 1994) and high temperatures (Marti´nez-Romero *et al*., 1991; Hungria *et al.*, 1993), and nodulating *Leucaena* sp. (Marti´nez-Romero *et al*., 1991; Hungria *et al*., 1993). Furthermore, *R. tropici* has greater genetic stability in maintaining symbiotic properties under stressful conditions than the other bean *Rhizobium* species (Marti´nez-Romero *et al*., 1991; Segovia *et al*., 1993). *Rhizobium tropici* is distributed in acid soils of South America (Martı´nez-Romero *et al*., 1991) in France (Amarger *et al*., 1994), Kenya (Anyango *et al.*, 1995), and Gambia and Senegal (Diouf *et al*., 2000).

Later, *Rhizobium etli* was proposed as a species separate from *R. leguminosarum sv. phaseoli* type I strains based on results of variation in chromosomal markers determined by multilocus gel electrophoresis and differences in nucleotide sequence of the 16S rRNA (Segovia *et al*., 1993). *Rhizobium etli sv. phaseoli* is the predominant *P. vulgaris*-nodulating species in Mexico, Colombia, and Argentina (Segovia *et al*., 1993; Eardly *et al*., 1995; Aguilar *et al*., 1998). *R. etli sv. phaseoli* is also found in regions where common bean has been introduced such as Spain (Herrera-Cervera *et al*., 1999), France (Laguerre *et al*., 1993), Austria (Sessitsch *et al*., 1997), Senegal (Diouf *et al*., 2000), Tunisia (Mhamdi *et al.,* 1999, Mhamdi *et al.,* 2002) and Ethiopia (Desta beyene *et al*., 2004; Aserse *et al*., 2012).

Apart from *R. leguminosarum sv. phaseoli*, *R. tropici*, and *R. etli*, classification of common bean rhiozobia further revealed two new species, namely *R. giardinii* from European, Tunisian and Ethiopian soils (Amarger *et al*., 1997, Herrera-Cervera *et al*., 1999, Mhamdi *et al*., 2002, Aserse *et al*., 2012) and *R. gallicum* from soils of Europe (Amarger *et al*., 1997, Herrera-Cervera *et al*., 1999) and Tunisia (Mhamdi *et al*., 1999; Mhamdi et al., 2002), and Mexico (strain FL27) (Sessitsch *et al*., 1997).

1.2.4.3 Phenotypic Clustering and Numerical Taxonomy

Old prokaryotic classification schemes relied heavily upon morphological and physiological characteristics. Subsequent and contemporary schemes have introduced evolutionary information extracted from DNA, RNA and protein sequences by using methods that measure genetic relatedness (Gevers *et al.*, 2006). However, the cut-offs used for species delineation by all the molecular approaches have been calibrated to match species groupings previously determined by phenotypic clustering (Gevers *et al*., 2006).

Today, polyphasic approach, which involves the use of wide range of techniques reveal pheonotypic and genotypic diversity of taxa and forms the basis for prokaryotic systematics (Rosselo-Mora and Amann, 2001). While genomic data alone are sufficient to allocate taxa in a phylogenetic tree and very helpful in drawing the major borderlines in classification systems, the consistancy of phenotypic and genomic characters is required to generate useful classification systems.

Phenotypes of prokaryotic organisms include features such as morphology, physiology and growth conditions of the organisms (Vandamme *et al*., 1996). Phenotypic characteristerization is directly linked to the use of pure cultures, and the laboratory capabilities to cultivate the organisms and analyze their properties. The discontinuous distribution of specific chemical constituents of the cell among strains is used to classify and identify prokaryotes. Phenotypic tests constitute the basis for the formal description of taxa, from species and subspecies up to genus and family (Gevers *et al*., 2006). Individually, many of the phenotypic characteristics are insufficient as parameters for genetic relatedness, yet as a whole, they provide descriptive information enabling us to recognize taxa (Rosselo-Mora and Amann, 2001).

Phenotypic data can mostly be compared phenetically, that means through cluster analysis and numerical taxonomy (Rosselo-Mora and Amann, 2001). The methods require the conversion of information on taxonomic entities into numerical quantities. These comparisons produce results that reflect the degree of similarity of the units under analysis. Phenetic principles state that maximum information: i.e. all possible characters should be studied and equally weighted for the strains, and taxa should be defined on the basis of overall similarity according to the results of the analyses (Rosselo-Mora and Amann, 2001).

Numerical taxonomy has supported the development of stable prokaryotic classifications, especially the determination of homogeneous groups that can be equated with species (Rosselo-Mora and Amann, 2001). Moreover, numerical taxonomy has stimulated several areas of growth, including numerical phylogenetics, molecular taxonomy, morphometrics and numerical identification (Sneath, 1995). Even though numerical taxonomy does not assume phylogenetic

relationships, it is obvious that close correspondence of a large number of phenotypic characteristics has something to say about genetic connections (Rosselo-Mora and Amann, 2001).

However the limitations based on the phenotype is that the whole information of a prokaryotic genome is never expressed, for gene expression is directly related to the environmental conditions (e.g. growth conditions in the laboratory). Therefore, it is usually necessary to grow cultures under carefully standardized growth regimes before comparative phenotypic work can be undertaken (Rosselo-Mora and Amann, 2001).

1.2.4.4 The Molecular approaches in the identification of rhizobia and PGPR

The classification of prokaryotes has undergone many changes, since the last two decades. This activity has been fuelled by the introduction of new and more trust-worthy techniques designed to evaluate the possible similarities and differences. Among these are, DNA: DNA hybridization (DDH), DNA-RNA hybridization, sequencing or restriction fragment length polymorphism (RFLP), analysis of PCR amplified 16S, 23S or 16S-23S intergenic spacer region of rRNA genes (Dresler Nurmi *et al.,* 2007).

Comparison of DNA sequences of 16S rRNA gene is one of the most popular approach in terms of bacterial identification and can be obtained from both culturable and nonculturable bacteria, and sequence data is archival, which can be accessed online (Rosselo-Mora and Amann, 2001). Strains exhibiting more than 70% DDH (or which have more than 94% average nucleotide identity over all shared genes) have been shown to be extremely similar in their 16S rRNA gene sequences (Konstantinidis and Tiedje, 2005). However, the converse is not necessarily true;

strains that have almost identical 16S rRNA sequences may not be closely related generally, whether this is judged by DDH (Rossello-ora & Amann, 2001) or the average nucleotide identity (ANI) between all the shared genes (Konstantinidis and Tiedje, 2005). Two strains with 16S rRNA sequences that are less than 97% identical are therefore assigned with high confidence to different species, but DDH is still required to establish whether strains that have 97% or more 16S rRNA similarity should or should not be placed in the same species (Vandamme *et al*., 1996; Roselo-Mora and Amann, 2001).

The degree of relatedness between organisms above the species level can be measured with high resolution using the 16S rRNA gene sequencing and comparison analysis. However, as more sequence information becomes available, it is evident that the resolving power of 16S rRNA sequences is limited when closely related organisms are being inspected (Vinuesa *et al*., 2005). Species definition using rRNA gene sequences is problematic as this slowly evolving (more conserved) molecule lacks the required level of resolution to distinguish similar species or to address the question of whether species exist and can be clearly resolved (Rosselo-Mora and Amann, 2001). Relationships inferred from 16S rRNA genes may also be distorted by recombination among similar species, which further complicates their use in species definition (Boucher *et al.*, 2004).

In order to assign bacteria to species level, the sequences of a number of protein coding genes have been used (Gevers *et al*., 2006; Staley, 2006). These have the advantage over 16S rRNA of evolving more rapidly and thus provide an increased ability to resolve species within a genus. However, the use of a single gene has major drawbacks as there may be too few informative

nucleotide sites to resolve very similar species and homologous recombination (HR) among similar species that may distort the true relationships between species. The deficiencies of using a single gene to resolve similar species can be overcome by the use of multiple gene sequences or multilocus sequence analysis (MLSA). This approach provides more informative nucleotide sites and also buffers against the distorting effects of recombination at one of the loci (Hanage *et al.*, 2006; Staley, 2006).

1.2.5 Plant growth-promoting bacteria (PGPB)

1.2.5.1 PGPB in the rhizosphere

Soil contains different types of microorganisms, of which bacteria are the most diverse and abundant (Van der Heijden *et al*., 2008). They have relatively rapid growth rate and metabolic versatility to utilize a large number of substrates as either carbon or nitrogen source. While many bacteria are bound to and within soil aggregates, many more live in interactions with roots of plants in the rhizosphere (Hawkes *et al.,* 2007). The rhizosphere is the volume of soil that immediately surrounds and is under the influence of plant roots; usually inclusive of the root surface or rhizoplane along with the thin layer of adherent soil (Dessaux *et al.,* 2009). It is a hot spot of plant-microbe interactions.

The influence of the rhizosphere is such that, more abundant bacteria grow in the immediate vicinity of plant roots, i.e., the rhizosphere, than those found in the bulk soil away from the root. This is because, the nutrient rich plant root exudates and rhizodeposits in the rhizosphere supports higher bacterial density than the bulk soil (Lugtenberg and Kamilova, 2009). In general, microbial association in the rhizosphere may be in the form of symbiotic, associative or parasitic

(pathogenic) relationship depending on the type of microorganism, soil nutrient status and soil environment (Soto *et al*., 2009).

Plants interaction with bacteria and other microbes in the rhizosphere ensures adequate nutrient uptake. In general, the beneficial free-living rhizobacteria that actively colonize plant roots and promote plant growth are termed as plant growth promoting rhizobacteria (PGPR) and are found in association with various plants (Lugtenberg and Kamilova, 2009). They are competent bacteria that actively colonize plant roots and proliferate within all available niche within the plant root at all stages of the plant development in the presence of competing natural microflora (Antoun and Kloepper, 2001).

Although the original definition of PGPR excludes nitrogen fixing root nodule endosymbiosis by rhizobia and frankia, rhizobia may act as PGPR when they interact with non-legume plant species (Saharan and Nehara, 2011). Rhizobia like some PGPR strains may also exist endophytically within the intercellular spaces of various plant tissues locally or systemically (Dodd *et al*., 2010). PGPR colonizes the rhizosphere of many plant species and confer beneficial effects, such as increased growth and yield, reduced susceptibility to phytopathogenes and other biotic and abiotic stresses (Yang *et al.*, 2008).

1.2.5.2 Mechanisms of action of PGPR

PGPR has been the subject of intensive study worldwide and several reviews exist on the mechanisms where more details can be found (Compant *et al*., 2010; Ahemad and Khan, 2011; Saharan and Nehra, 2011). The PGPR strains facilitate plant growth either directly or indirectly (Glick *et al.,* 1999). The direct growth promoting activity of PGPR includes, solubilizaiton of

phosphorous (Khan *et al.,* 2006; Richardson and Simpson, 2011), sequestering of iron by production of siderophores, production of phytohormones such as auxins, cytokinins; gibberellins and lowering of ethylene concentration (Wani *et al.,* 2008; Dodd *et al*., 2010). The indirect mechanisms of plant growth promotion include suppression of phytopathogenes through antibiotic production, depletion of iron from the rhizosphere, synthesis of antifungal metabolites, production of fungal cell wall lysing enzymes, competition for sites on roots and induction of systemic resistance (Compant *et al*., 2005).

From numerous studies, it has become apparent that a particular bacterium may affect plant growth and development using any one or more of the mechanisms. Moreover, since many PGPR strains possess several traits that enable them to facilitate plant growth, a bacterium may utilize different traits at various stages during the life cycle of the plant (Glick *et al.,* 1999). For example, following seed germination, a PGPR strain may lower ethylene concentration in the plant thereby decreasing the ethylene inhibition of seedling root growth. Once the seedling has depleted the resources that are contained within the seed, a PGPR strain may help to provide the plant with sufficient amount of iron and phosphorous from the soil. During the early plant development, plant growth-promoting bacteria may stimulate cell division by providing appropriate levels of auxins, cytokinins or gibberellins and help them tolerate a variety of environmental stress such as drought and flooding by lowering the detrimental level of stress ethylene that can be produced as a result of these stresses (Glick *et al.,* 1999).

1.2.5.2.1 Phosphate solubilization

Phosphorus is the most important element in the nutrition of plants, next to nitrogen (N). It plays an important role in virtually all major metabolic processes in plant including biological nitrogen

fixation in legumes (Saber *et al*., 2005). Although P is abundant in soils in both inorganic and organic forms, it is a major limiting factor for plant growth as it is in an unavailable form for root uptake. Inorganic P occurs in soil mostly in insoluble mineral complexes; some due to fixation, either in the form of iron/aluminium phosphate in acidic soils or in the form of calcium phosphate in neutral to alkaline soils (Sharma *et al.,* 2013) and cannot be absorbed by plants (Rengel and Marschner, 2005).

A major characteristic of P biogeochemistry is that only 1% of the total soil P (400 – 4,000 kg P/ ha in the top 30 cm) is incorporated into living plant biomass during each growing season (10-30 kg P/ha), reflecting its low availability for plant uptake (Quiquampoix and Mousain, 2005). According to Zhou *et al*., (1992), only 0.1% of the total P exists in a soluble form available for plant uptake. However, it has been suggested that the accumulated P in agricultural soils would be sufficient to sustain maximum crop yields worldwide for about 100 years if it were available (Khan *et al*., 2009a).

The use of microbial inoculants possessing P-solubilizing activities in agriculture is considered an economical and environmental-friendly approach to make use of the plant unavailable form of phosphorus in the soil.The insoluble mineral phosphates in the soil are dissociated into plant available P forms through different mechanisms used by P solubilizing rhizobacteria (PSR). PSR are able to release organic acids that 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). Many of the PSR bring down the pH of the medium either by extruding H^+ (Illmer and Schinner, 1995) or by secreting organic acids such as citric, lactic, acetic,

malic, tartaric, oxalic, 2-ketogluconic, gluconic and succinic acids (Bolan *et al*., 1996). Among the bacterial genera with this capacity are *Pseudomonas*, *Bacillus, Rhizobium, Burkholderia, Achromobacter, Agrobacterium, Microccocus, Aereobacter, Flavobacterium, Erwinia, Chryseobacterium, Phyllobacterium, Enterobacter,* and *Klebsiella (*Rodríguez and Fraga, 1999; *Sharma et al*., 2013).

1.2.5.2.2 Indoleacetic Acid (IAA)

Diverse PGPR species produce the naturally occurring auxins, indole-3-acetic acid (IAA) as a direct mechanism to promote plant growth (Spaepen *et al*., 2007). IAA synthesized by bacteria may be involved at different levels in plant-bacterial interactions. In particular, plant growth promotion and root nodulation are both affected by IAA. Bacterial IAA increases root surface area and length, and thereby provides the plant has greater access to soil nutrients. In addition, bacterial IAA 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). Most *Rhizobium* strains that have been examined have been found to produce IAA and several studies have suggested that increases in auxin levels in the host plant are necessary for nodule formation (Mathesius *et al*., 1998).

Bacterial isolates from the rhizosphere of various crop plants displayed a greater potential to biosynthesize and release IAA as secondary metabolites using the ample substrate in the root exudates and rhizodeposit (Costacurta and Vanderleyden, 1995; Lee et al., 2004). Indoleacetic acid production by microbial isolates varies greatly among different species and strains and

dictated by the availability of a substrate (Lee *et al*., 2004). Many rhizospheric bacteria produce IAA in the presence of a suitable precursor such as L-tryptophan (Karnwal 2009).

1.2.5.2.3 Lytic enzymes and HCN

The synthesis of a range of different lytic enzymes is the PGPR trait that is most often associated with the ability of the bacterium to prevent the proliferation of plant pathogens. Some biocontrol bacteria produce enzymes including chitinases, cellulases, β -1, 3 glucanases, proteases, and lipases that can lyse a portion of the cell walls of many pathogenic fungi (Glick, 2012). PGPR that synthesize one or more of these enzymes have been found to have biocontrol activity against a range of pathogenic fungi including Botrytis cinerea, Sclerotium rolfsii, Fusarium oxysporum, Phytophthora spp., Rhizoctonia solani, and Pythium ultimum (Peix *et al*., 2001b; Whipps, 2001; Abdel-Salam *et al*., 2007; and Naik *et al.*, 2008,).

Although cyanide acts as a general metabolic inhibitor, it is synthesized, excreted and metabolized by soil bacteria, as a mean to avoid competition. The host plants are generally not negatively affected by inoculation with cyanide producing bacterial strains (Zeller, 2007). Glycine is an HCN precursor where both carbons of glycine are used as sources of cyanide carbon (Castric, 1977). The level of HCN produced in root-free soil by *P. putida* and *A. delafieldii* generally increased with higher amounts of supplemental glycine, with *P. putida* typically generating more HCN (8–38 µM) at a given glycine level (Owen and Zdor, 2001). Wani *et al*. [143] tested the rhizosphere isolates for HCN producing ability in vitro to find that most of the isolates produced HCN and helped in the plant growth. *Pseudomonas fragi* CS11RH1 (MTCC 8984), produces hydrogen cyanide (HCN) and the seed bacterization with the

isolate significantly increases the percent germination, rate of germination, plant biomass and nutrient uptake of wheat seedlings (Selvakumar, 2009).

1.2.6 Metabolomics in the rhisosphere

In the rhizosphere, plant-microbe interactions play important roles in a number of vital ecosystem processes. These interactions have been shown to have many beneficial impacts on plants, including disease suppression (Mendes *et al.,* 2011), increased nutrient availability and uptake (Morrissey *et al.,* 2004), and increased immunity to abiotic (Zolla *et al*., 2013) and biotic stresses (Badri *et al.,* 2013b), each of which leads to increases in plant productivity (Berg, 2009). In turn, the plant provides the soil microbes with root exudates that are used as substrates and signaling molecules (Bais *et al.*, 2006).

Plant root-secreted phytochemicals mediate a wide variety of plant–microbe interactions in the soil. For example, the increased secretion of chlorogenic acid and caffeic acid and the decreased secretion of cinnamic acid by grafted-root watermelon improved its resistance to *Fusarium oxysporum f.sp. niveum* (Ling *et al*., 2013). Canavanine, secreted from the seed coat or roots of leguminous plants, acts as an antimicrobial for many rhizosphere bacteria but not rhizobia, suggesting that the host plant secretes this compound for selection of the beneficial microbes (Cai *et al*., 2009). In addition to these symbiotic interactions, root exudates are involved in the initiation of plant-PGPR interactions. Plant roots are likely to attract PGPR through the release of cues (root exudates) in which carbohydrates and amino acids predominantly act as chemoattractants. Recent studies have shown that arabinogalactan proteins (AGPs), which belong to the hydroxyproline-rich glycoprotein superfamily of plant cell wall proteins, play key

roles in various interactions between plant roots and rhizospheric microbes in the rhizosphere (Nguema-Ona *et al*., 2013). Plant root tips release living root border cells, border-like cells, and mucilage into the rhizosphere, which contains large amounts of AGPs (Cannesan *et al*., 2012). a plant arabinogalactan-like glycoprotein was found to be essential for the growth of bacteria on the roots of both legumes and non-legumes and was shown to promote the polar surface attachment by *Rhizobium leguminosarum* (Xie *et al*., 2012).

In addition to carbohydrates and amino acids, plants produce and release numerous secondary metabolites and hormones into the rhizosphere, many of which play a role in plant–microbe interactions. Plants use these compounds to attract beneficial soil microorganisms and defend themselves against pathogens (Neal *et al*., 2012). For instance, benzoxazinoids, found in the root exudates of maize, attract plant-beneficial rhizobacteria (Neal *et al*., 2012). Similarly, flavonoids act as chemoattractants to draw rhizobia to the root surface by regulating expression of the nod gene, which is responsible for the synthesis of Nod factors (lipochito-oligosaccharides) that play important roles in nodulation establishment (Abdel-Lateif *et al*., 2012). Rudrappa *et al*. (2008) demonstrated that malic acid released in the root exudates recruits the PGPR *Bacillus subtilis* to the rhizosphere upon infection with *A. thaliana* foliar pathogens. Further studies showed that the presence of *B. subtilis* invokes abscisic acid and salicylic acid signaling pathways in *A. thaliana*, resulting in the closure of stomata and the restriction of pathogen entry (Kumar *et al*., 2012).

Studies also suggested that plants select and attract specific microbes and, therefore, alter the composition and diversity of microbial communities in the rhizosphere in a plant-specific

manner (Houlden *et al*., 2008). For example, an *Arabidopsis* ABC transporter mutant that secreted more phenolics than sugars compared with the wild type caused significant changes to the natural microbial community (Badri *et al*., 2009a). These changes in root exudate composition were associated with beneficial bacterial communities enriched with PGPR, N_2-fixing bacteria, and metal remediation bacteria. Micallef *et al.* (2009b) showed not only that different *Arabidopsis* ecotypes exuded unique suites of compounds but also that these differences in root exudation supported distinct rhizosphere bacterial communities. Similarly, Badri *et al.,* (2013b) observed that the addition of distinct blends of natural chemicals derived from *Arabidopsis* root exudates added to the soil produced distinct rhizosphere microbial communities that appeared to have the ability to degrade atrazine or contained more symbiotic bacteria.

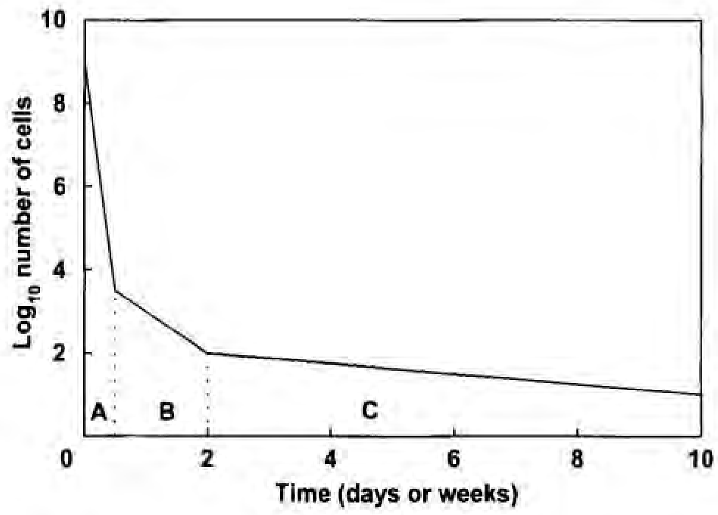
Phenolic compounds in plant root exudates influence microbial communities in the rhizosphere. A recent study showed that in the absence of the plant (*A. thaliana*), natural blends of phyto-chemicals present in the root exudates can modulate the soil microbiome and that these different groups of compounds impact the soil microbiome composition at various levels (Badri *et al.,* 2013a). A positive correlation was found with phenolic compounds and a higher number of unique operational taxonomic units compared with other groups of compounds, such as sugars, sugar alcohols, and amino acids, which implies that phenolic compounds act as specific substrates or signaling molecules for a large group of microbial species in the soil.

1.2.7 Inoculant formulations

Three general categories of materials used to formulate an inoculants: (1) soils, to include peat, coal and inorganic soils with and without various additives; (2) plant materials, to include plant

compost, bagasse, filter mud from sugar cane, farmyard manures and composts of these and other organic materials; and (3) inert materials, such as vermiculite, perlite, ground rock phosphate, calcium sulfate, polyamide and biopolymer gels, and alginate beads (Smith, 1992). Other forms include dried (lyophilized or freeze-dried, oil) and frozen (concentrated) cultures. Inoculants prepared from soils and plant materials are all of the traditional type in which the carrier is impregnated with a high count suspension of the rhizobia using simple mixing (non-sterilized) or aseptic injection (sterilized) of the carriers. In the inert group, inoculants prepared from polymers, oils or those prepared concentrated and frozen or freeze-dried require additional and more complex equipment. Even so, these forms are attractive for their potential to provide increased number of viable cells at the time of inoculation, for their larger shelf life and their adaptability to direct soil implant methods (Date, 2001).

It is important that inoculants contain high numbers of cells and that these cells remain viable when applied to the seed or soil, as this is the most vulnerable phase of the transfer (rooughley *et al* 1993). High quality product is available in several formulations but few of these provide adequate protection for good survival on the seed. In general, survival curves, for both the inoculants and the inoculum on the seed have a bi- or tri-phasic forms (Fig 1.1) (Date, 2001).



10
8
6
4
2
0
Log10 number of cells
A
B
C
2
4
6
8
10
Time (days or weeks)

osmotic and water of hydration factors, but the exact nature of this mechanism is not well understood. However, it is obvious that the lower the a_w the better the survival. Murnier and Jung (1985) state that 'drying kills 90% of the bacteria' but survival of polymer-entrapped bacteria is a function of the a_w of the storage medium. The addition of high molecular weight compounds (e.g. 6 carbon atoms, mannitol) significantly improved survival over that obtained with a lower molecular weight compound (e.g. 3-5 carbon atoms, glycerol and ribose). For compounds with the same number of carbon atoms viability is related to the functional chemical group on the molecule (Mungnier and Jung 1985). a_w is a prime factor in survival, particularly under high temperature conditions. The challenge then is to develop a formulation that maintains this condition at an industry and user level (Date, 2001).

Possibly, the most innovative technology for rhizobial inoculants in recent times involves the encapsulation of the bacteria in polymer microcapsules, beads or pellets (Date, 2001). Polyacrylamide and alginate are the 2 most commonly tested gels. The inoculant is formulated by mixing a culture of the bacteria with a hardening or gelling compound (adjuvant) in a polymer solution. Bulking or nutritive additives such as bran or clay can be added and the mixture added drop-wise into a solution containing calcium to solidify and form the gel pellets. Particles size and shape can be controlled by varying the drop-forming system. The particles are then dried for ease of packaging and handling. Hardness, moisture uptake or release and rate of degradation of the beads can be adjusted by varying the amount and grade of adjuvant and polymer used to prepare the pellets (Walter and Paau, 1993).

1.2.8 Coinoculation of PGPR and Rhizobia

Coinoculation studies with PGPR and Rhizobia showed increased nodulation and N fixation in legumes such as *Vigna radiata L.* (Sindhu *et al*., 2002), *Vigna unguiculata* L. (Silva *et al*., 2006), *Cajanus cajan* (Tilak *et al*., 2006), *Phaseolus valgaris* L. (Camacho *et al*., 2001; Figueiredo *et al.,* 2008,), and galega (Egamberdieva *et al*., 2010). A variety of rhizosphere microorganisms, including *Bacillus* and *Pseudomonas* species, are commonly found in the rhizosphere of leguminous and nonleguminous crops (Li and Alexander, 1988). The viability of co-inoculating soybean seeds with crude or formulated metabolites or with cells of *Bacillus subtilis*, to increase the contribution of the biological nitrogen fixation process was reported (Sharma *et al.,* 2013). Co-inoculation of the common bean with a *Rhizobium* and *Pseudomonas species* (Yadegari and Rahmani, 2010; Sánchez *et al.,* 2014) resulted in enhanced nodulation followed by higher shoot dry matter and seed yield production. Gross average of seed yield increased from 3000 kg/ha for *Rhizobium* alone to 4693 kg/ha after co-inoculation with *P. fluorescens* (Yadegari and Rahmani, 2010).

1.3 Objectives of the Study

1.3.1 General objective

The general objective of the study was to evaluate the diversity and screen effectiveness of rhizobia and phosphate solubilizing rhizobacteria from common bean inorder to select prime candidates for inoculant production so as to enhance growth and grain yield of common bean in Ethiopia.

1.3.2 Specific objectives

The specific objectives of the current study were to:

a) Isolate and characterize rhizobia from root nodules of common bean grown in different parts of Ethiopia and determine their phenotypic clusters based on numerical taxonomy
b) Screen the symbiotic effectiveness of common bean nodulating rhizobia under greenhouse condition
c) Isolate and characterize phosphate solubilizing bacteria from the rhizosphere of common bean and screening them for inorganic phosphate solubilizing abilities and other plant growth promoting characteristics
d) Investigate the diversity of phosphobacterial isolates from the rhizosphere of common bean and identify the two field tested *Rhizobium* isolates using partial 16S rRNA gene sequence analysis
e) Evaluate the effect of co-inoculation of selected rhizobial and phosphobacterial isolates on growth and yield of common bean varieties (Awash-1, Ibadoo and Nassir) under field trails

Chapter 2 Phenotypic diversity, PGP property and Symbiotic Effectiveness of Rhizobia from root nodules of common bean (*Phaseolus vulgaris* L.) in Ethiopia

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Abstract

Seventy six common bean nodulating rhizobia were isolated from the central, eastern, southern and western parts of Ethiopia and compared with nine reference strains by the numerical analysis of 75 phenotypic features. The isolates were authenticated for nodulation and effective nodule formation using pouch experiment. They were tested for utilizing various carbon and nitrogen sources and for tolerances to acidity, salinity, antibiotics and temperature on yeast extract manitol agar (YEMA). Selected isolates were evaluated for their symbiotic performance in plants grown on sterile sand in greenhouse. The isolates were also examined for additional plant growth promoting (PGP) characteristics. The common bean nodulating rhizobia showed diversity in utilizing various carbon and nitrogen sources and tolerance to acidity, salinity, antibiotics, and temperature. All the isolates except CBR039 and CBR151 reinfected the host plant and only 23% of them were symbiotically effective. CBR052 showed the highest symbiotic effectiveness (74.67%) by accumulating the largest shoot dry matter which exceeded the uninoculated plant by 109%. The numerical analysis indicated that the local isolates and the reference strains were linked at a similarity level of 83% forming four major diversity groups at a level of 89% relative similarity. The largest cluster (cluster II) consists of 38 isolates with two reference strains, *Rhizobium sp*. and *Rhizobium phaseoli.* Twenty seven isolates were grouped into cluster IV that were similar to *Rhizobium etli*, and 18 isolates formed cluster III that were *Rhizobium giardini*-like. Cluster I included 2 isolates related to *R. tropici*. Pertaining to the PGP properties, 61% were able to produce indole-3-acetic acid (IAA) (8.05-37.8µ/ml), 37% showed antagonistic activity against *Fusarium oxysporum f.sp. phaseoli* (14.4-37.5%), and 16% solubilized tricalcium phosphate (SI=0.57-2.22).This study therefore indicates that Ethiopian soils contain diverse group of common bean nodulating rhizobia, most areas harboring symbiotically less effective population and many edowed with different PGP characteristics.

Keywords:, common bean, numerical taxonomy, phenotypic diversity, *Rhizobium*

2.1 Introduction

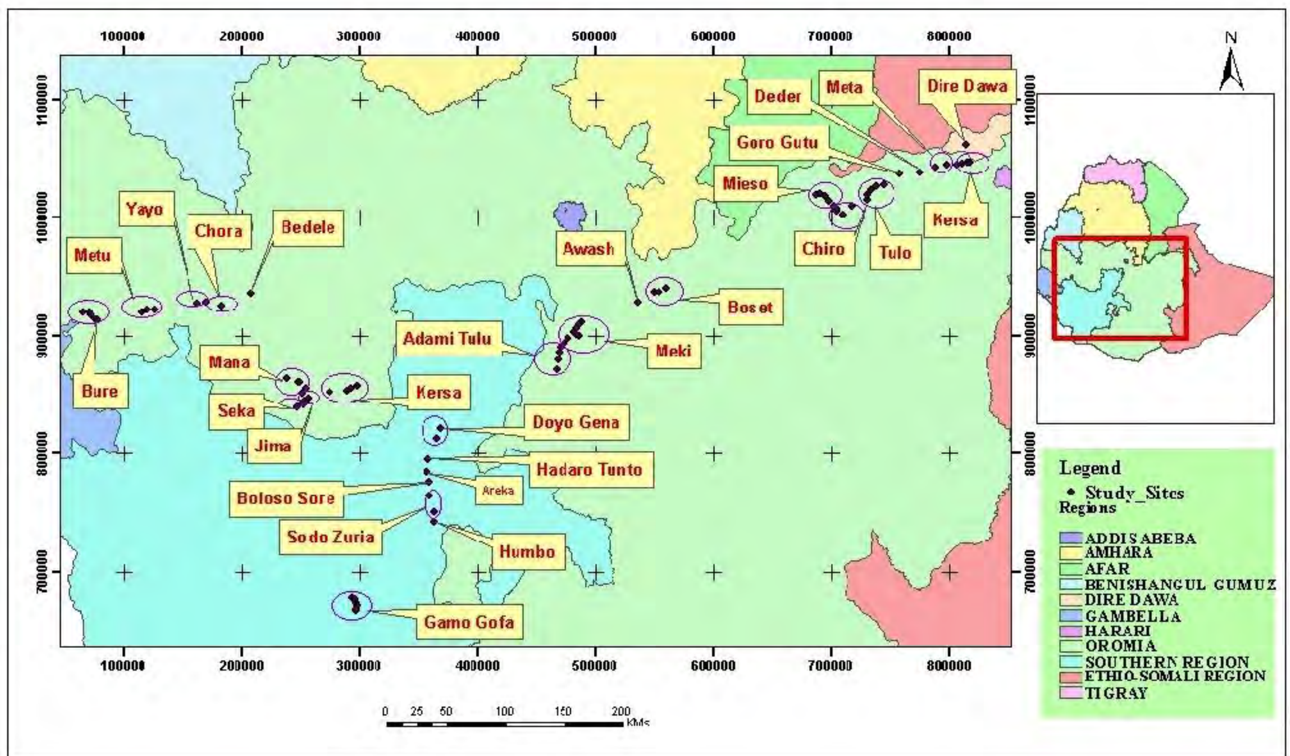
Common bean (*Phaseolus vulgaris* L.) is one of the most important leguminous crops which is used as a source for protein and calorie worldwide (Broughton *et al.,* 2003). It is widely cultivated in many parts of the tropics, sub-tropics and temperate regions (Singh, 1999). According to Wortmann (1998), it is a staple food for more than 100 million people in Africa with area coverage of over four million hectares of arable lands in more than 20 eastern and southern African countries. In Ethiopia, common bean is widely grown in areas with altitudes between 1200 and 2000 meter above sea level (Ohlander, 1977). Genetic and archaeological studies indicate that *P. vulgaris* is native to the Americas and domesticated in two distinct centres of Middle America and Andes with two highly differentiated gene pools (Koenig and Gepts, 1989; Gepts, 1998). More recent studies of domestication and the genetic diversity of *P. vulgaris* within the two gene pools identified the Oaxaca valley in the Middle Americas and southern Bolivia and northern Argentina in South America as the centres of domestication of common bean (Bitocchi *et al*., 2013).

Common bean is also grown as a break crop (crop rotation) since it replenishes soil fertility by fixing nitrogen with root nodule bacteria generally known as rhizobia. It is estimated that common bean production in Ethiopia covers 359, 235 ha with a total production of 0.42 million tons and an average yield of 1.2t/ha (CSA report, 2015). The country is exporting beans to Europe and Kenya and the export market is expanding to other countries (Ferris and Kaganzi, 2008). However, bean production could not be commensurate with the demand because of low production rate in the smallholder farming system that suffers from low soil fertility, mainly due to nitrogen deficiency (World Bank, 2006).

Common bean forms ymbiosis with a wide range of nitrogen fixing root nodule bacteria that include *R. leguminosarum sv. phaseolus* (Jordan, 1984), *R. tropici* (Martinez-Romero *et al.,* 1991), *R. etli* (Segovia *et al*., 1993), *R. giardini* and *R. gallicum* (Amarger *et al*., 1997) and the rhizobial species seem to have coevolved with their hosts at their centers of diversification (Martı´nez-Romero *et al*., 1996).The crop is usually considered as a poor nitrogen fixer due to its nodulation promiscuity (Michiels *et al*., 1998) and its association with highly competitive but poorly effective indigenous population of rhizobia in soil (Graham, 1981; Giller and Cadisch, 1995; Hungria and Vargas 2000). However, increases in nodulation, nitrogen fixation and grain yield have been observed when inoculated with selected effective strains (Hungria *et al.* 2000, 2003; Asadi Rahmani *et al*., 2005; Mnasri *et al.,* 2007).

It has been reported that rhizobia possess additional desirable plant growth promoting (PGP) traits such as pathogen inhibition, phosphate solubilization, and phytohormone production that could enhance host plant productivity (Rodriguez and Fraga 1999; Carson *et al*., 2000; Arora *et al*., 2001; Deshwal *et al.,* 2003a; Mia *et al.,* 2012; Mulissa Jida 2013). A number of studies confirmed that common bean nodulating rhizobia also exhibit these PGP characteristics (Chabot *et al*., 1996; Antoun *et al.* 1998; Ozkoc and Deleveli 2001; Mishra *et al*. 2006).

Several phenotypic and molecular taxonomic studies showed that soils in different parts of Ethiopia harbor diverse groups of resident bean-nodulating rhizobia: *R. phaseoli, R. etli, R. tropici, R. gardini*, and *Rhizobium sp.* as the major bean nodulating rhizobia in Ethiopia (Desta Beyene *et al.,* 2004; Alemayehu Workalemayehu *et al,* 2007; Anteneh Argaw, 2007; Aserse *et. al.*, 2012). However, the hitherto studies were limited to a few geographical areas against several


Metu
Yayo
Chora
Bedele
Bure
Mana
Seka
Jima
Kersa
Adami Tulu
Awash
Boset
Meki
Doyo Gena
Hadaro Tunto
Areka
Boloso Sore
Sodo Zuria
Humbo
Gamo Gofa
Deder
Meta
Dire Dawa
Goro Gutu
Mieso
Chiro
Tulo
Kersa
Legend
Study_Sites
Regions
ADDIS ABEBA
AMHARA
AFAR
BENISHANGUL GUMUZ
DIRE DAWA
GAMBELLA
HARARI
OROMIA
SOUTHERN REGION
ETHIO-SOMALI REGION
TIGRAY
N
KMs

3.2.2 Isolation and purification of bacteria from nodules

Rhizobia were isolated from root nodules following standard procedure (Somasegaran and Hoben, 1994). The desiccated nodules from each vial were allowed to imbibe water overnight for rehydration. Intact and undamaged nodules were surface sterilized in 95% ethanol for 10 seconds, transferred to a 3% (v/ v) solution of sodium hypochlorite for 3 minutes and rinsed in 5 changes of sterile water. Each nodule was separately crushed using glass rod in a drop of sterile water in a Petri dish from which a loopful of the suspension was streaked on Yeast Extract Manitol Agar (YEMA) and incubated at 28^0C for 3-5 days.

Distinct and well-isolated colonies were selected and purified by repeated sub-culturing on congo red-yeast extract manitol agar (CR-YEMA), after having diluted and shaken with Tween 80 buffer (Li *et al*., 2012). The pure isolates were maintained and preserved at -80 ^{0}C in YEM broth with 20% glycerol. Isolates were designated as CBR (Common bean rhizobia) with numerical orders.

3.2.3 Gram reaction

Gram staining was done to test the purity of isolates accoding to Gram's procedure (Somasegaran and Hoben, 1994). The heat-fixed smear of cells was flooded with crystal violet for 1 minute. After rinsing with tap water, the slide was flooded with Gram's iodine. Waiting for 1 minute, the slide was washed with a gentle and indirect stream of tap water for 2 seconds and flooded with alcohol. After waiting for 15 seconds, slides were flooded with safranin and recorded for the developing color after one minute.

3.2.4 Colony characterization and growth on YEMA, LB and PY-Ca media

The isolates were grown on YEMA medium to characterize their colony size and texture after 5 days of incubation (Somasegaran and Hoben 1994) and tested for acid or alkali reaction based on growth on YEMA supplemented with bromothymol (Jordan, 1984). Isolates were also tested for growth in Luria broth (LB) and Peptone yeast extract minus calcium (PY-Ca) media described in Martinez-Romero *et al*., (1991).

3.2.5 Generation time

Growth rate (mean generation time) of the isolates was assessed by inoculating 15 µl of the 48 hrs YEM broth culture (10^9cfu ml^{-1}) into Erlenmeyer flasks (200 ml) containing 50 ml YEM broth and incubated on a gyratory shaker at 120 rpm. Samples were taken every 6 hrs to measure optical density at 540 nm using spectrophotometer (Wagtech International, UK) and for viable count on YEMA medium after incubation at 28^0 for 3 days. Mean generation time was calculated from the exponential phase of the growth curve (Somasegaran and Hoben, 1994).

2.2.6 Authentication of rhizobia and preliminary screening for nodulation on pouch

Isolates were screened for nodulation using plastic pouches in greenhouse (Vincent, 1970). Seed variety of common bean, Nasir, which is popular for being used across different parts of Ethiopia, was provided by Awash Melkassa Agricultural Research Center for this experiment. The seeds were surface sterilized with 95% ethanol and 3% sodium hypochlorite solutions for 10 s and 3 min, respectively. The seeds were rinsed five times with sterilized distilled water and allowed to germinate on sterilized Petri plates containing moistened filter paper at room temperature. The seedlings with healthy radicle and plumule were transferred into growth pouches and inoculated with 1ml of rhizobial cultures (10^9 cfu ml^{-1}) grown in YEM broth to exponential phase. Non-inoculated pouches and 0.05% $KN0_3$ fertilised pouches were included as

negative and positive control, respectively. The experiment was conducted in three replications. Seedlings were alternatively fertilized with Broughton and Dilworth N-free medium (Appendix II) and irrigated with sterilized water following the protocol of Somasegaren and Hoben (1994). Six weeks after planting, plants were uprooted to check the vegetative conditions of the seedlings, the color of the leaves, appearance of the plants, and nodulation status of the roots. Green leaves and pink nodule indicated effective nitrogen fixation.

3.2.7 Cross inoculation test

Cross-nodulation was undertaken on a heterologous host, *Leucaena leucocephala* in plastic pouches under greenhouse condition according to Martinez-Romero *et al.,* (1991). The seeds of *L. leucocephala* were treated with concentrated sulfuric acid (98%) for 20 min and rinsed successively with water (Amarger *et al.,* 1994). The seeds were germinated on wet filter paper, transferred into pouches and inoculated with 1ml of active rhizobial cultures grown to exponential phase (10^9cfu ml^{-1}). The experiment was laid out in three replications. All pouches were fertilized with quarter strength Broughton and Dilworth N-free medium (Somasegaren and Hoben, 1994).

3.2.8 Phenotypic characteristerization of common bean rhizobia

3.2.8.1 Bacterial sources

A total of 85 bacterial isolates consisting of the 76 authenticated rhizobia and 9 reference common bean rhizobia strains from the culture collections of University of Helsinki (HU) were included. The referece strains from HU were HBR2 (*Rhizobium etli*), HBR4 (*Rhizobium etli*), HBR5 (*Rhizobium etli*), HBR9 (*Rhizobium phaseoli*), HBR11 (*Rhizobium phaseoli*), HBR17

(*Rhizobium phaseoli*), HBR21 (*Rhizobium giardini*), HBR22 (*Rhizobium. sp.*), and HBR24 (*Rhizobium etli*).

The isolates were tested for 75 different phenotypic characteristics for numerical analysis. Each test was made in triplicates by inoculating early log phase grown cultures on YEMA broth with 15 µl inoculum size (10^9cfu ml^{-1}) on YEMA plates adjusted to about pH 6.8 and incubated at 28^0C for 3 to 5 days unless stated otherwise (Amarger *et al*, 1997). The results were then converted to a binary dataset to estimate similarity matching. A dendrogram was constructed using a cluster analysis with unweighted pair groups with arithmetic means (UPGMA) based on the similarity matrix (Sneath and Sokal, 1973).

3.2.8.2 Tolerance of isolates to pH, salt and temperature

Isolates were inoculated to YEMA medium adjusted to pH 4, 4.5, 5, 5.5, 8.5, 9, and 9.5 with medium containing 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, and 5 % (w/v) NaCl. Isolates were also inoculated to the same medium and incubated at temperatures of 4, 10, 15, 20, 30, 35, 37, and 40^0C with the rim of the plates sealed with parafilm to avoid drying at higher temperatures (Amarger *et al*. 1997).

3.2.8.3 Carbon and nitrogen source utilization

Isolates were inoculated on different carbon and nitrogen sources on a basal medium containing (per litre) K_2HPO_4 (1g), KH_2PO_4 (1 g), $FeCl_3.6H_2O$ (0.01 g), $MgSO_4.7H_2O$ (0.2 g), $CaCl_2$ (0.1 g), $(NH_4)_2SO_4$ (1 g) agar (15 g), and distilled water (1L) according to Amarger *et al*. (1997). The carbon sources were: D-glucose, D-Fructose, sucrose, D-galactose, D-lactose, D-rhamnose, L-arabinose, D-xylose, D-trehalose, D-raffinose, D-mannose, D-dulcitol, dextrin, D-maltose,

malate, citrate, sorbitol, tartrate, glycerol, lactate, gluconate, starch, oxalate, and erythritol. Each carbon source was membrane filtered (0.2 µm) and added at a final concentration of 1g/l to the basal medium.

The isolates were also inoculated into the basal medium containing membrane filtered (0.2 µm) amino acids. The amino acids were Glycine, L-Proline, L-Phenylalanine, L-Leucine, L-Arginine, L-Isoleucine, L-Threonine, L-Histidine, L-Aspargine, L-Pyroglutamic acid, D-Serine, L-Glutamic acid, L-Alanine, L-Aspartic acid, cystine, methionine, valine, glutamine, tryptophan and tyrosine. Each added at a concentration of 0.5 g/liter to a similar basal medium from which ammonium sulfate was omitted and mannitol (1g/liter) was added as a carbon source.

3.2.8.4 Intrinsic antibiotic resistance (IAR)

The intrinsic antibiotic resistance of isolates was tested according to Amarger *et al*. (1997) by growing the isolates on YEMA medium containing filter sterilized (0.2 µm) solutions of 7 different antibiotics at different concentrations. The antibiotics were ampicillin, chloramphenicol, kanamycin, rifampin, spectinomycin, streptomycin, and nalidixic acid and prepared in distilled sterile water.

3.2.9 Screening for Plant growth promoting properties

3.2.9.1 Phosphate solubilization

Isolates were spot inoculated on Pikovskaya's (PKV) media consisting of ingredients in g/l: yeast extract (0.50), glucose (10.0), $Ca_3(PO_4)_2$ (5.0), $(NH_4)_2SO_4$ (0.5), NaCl (0.2), KCl (0.2), $MgSO_4.7H_2O$ (0.1), $MnSO_4.2H_2O$ (0.002), $FeSO_4.7H_2O$ (0.002), and agar (15.0) (Pikovskaya, 1948) and incubated at 28^0C for 7 days. The formation of clear zone around the colony indicated

P-solubilization and solubilization index (SI) was calculated as the ratio of total diameter (colony diameter + clear zone diameter) to colony diameter (Edi-Premono *et al*., 1996).

3.2.9.2 Indole-3 acetic acid (IAA) Production

Indole-3 acetic acid (IAA) production was tested using modified colorimetric method of Gordon and Weber (1951) as described by Hung and Annapurna (2004). Each isolate was grown in LB (Luria Bertani) broth supplemented with tryptophan (0.1%) and incubated at 28^{0}C for 2 to 3 days in a shaker (set at 130 rpm). Then 3 ml of the log phase broth culture (10^{9} cfu ml^{-1}) was centrifuged at 10000 rpm (Jouan A 14 centrifuge, Biostad, Qubec, Canada) for 15 minutes and two ml of the cell-free supernatant was transferred to a dry clean tube (15 ml capacity) to which 1 ml of 10 mM orthophosphoric acid and 4 ml of Salkowsky's reagent (1ml of 0.5 M $FeCl_3$ in 49ml of 35% $HClO_4$) were added and incubated in dark at ambient temperature for 25 minutes. The pink colour development was compared to the blank (sterile LB broth with 0.1% of tryptophan and reagents) using a spectrophotometer (Jenaway, Visible rang 6300, Angstrom advanced Inc.) at wavelength of 530 m and the absorbance was used as index of IAA production.

3.2.9.3 *In vitro* antagonistic effect against *Fusarium oxysporum*

The inhibitory effect of isolates against the pathogen, *Fusarium oxysporum f.sp. phaseoli,* was evaluated *in vitro* using the dual culture technique described by Skidmore and Dickinson (1976). The pathogen was provided by Haromaya University for this experiment. Culture suspension of 15 µl (48 hrs old) of each bacterial isolate was spotted at equidistant points along the perimeters

of YEMA plates supplemented with sucrose (0.5%) and incubated at 28º C for 2 days. Then a 4 mm diameter block of a 2-day grown pathogen culture on potato dextrose agar (PDA) was placed at the centre of the YEMA plate. All plates were incubated at 28ºC for 5 days. Plates with fungal block were included as a control to estimate the inhibition zone between the bacterial colony and and the radius of the fungal colony. Percentage inhibition of the fungal growth was calculated using the formula: 100 x (C - T) /C, where C is the radial growth of fungus in control, and T is the radial growth of the fungus in the dual culture (Gupta *et al*., 2006).

3.2.10 Evaluation of selected isolates for symbiotic effectiveness on sand culture

Rhizobial isolates from growth pouch experiment were selected and inoculated on a sand culture in order to evaluate their symbiotic effectiveness according to Somasegaran and Hoben (1994). Five pre-germinated seeds were planted on 3kg capacity plastic pots filled with nitrogen-free and acid washed sand (washed with strong sulfuric acid). Common bean seeds of Nasir variety were selected, surface sterilized and washed as before, and allowed to germinate on sterile water agar (1%) for three days at 25°C. All seedlings were inoculated with 1 ml of log phase bacterial culture (10^9 cfu ml^{-1}) and thinned down to three after five days of emergence (DAE). The experiment was statistically laid out with three replications using a complete randomized block design with non-inoculated and non nitrogen fertilized pots as negative and N fertilized (0.05% KNO_3 w/v solution) pots as positive control. All the seedlings were supplied with quarter-strength Broughton and Dilworth N-free medium (Somasegaran and Hoben 1994) once a week and watered every three days. Seedlings were grown under greenhouse conditions at a 12 hrs photoperiod with the mean minimum and mean maximum temperatures of 19 ± 2^0C and 30 ± 1^0C, respectively.

Forty five days after emergence, the plants were uprooted to record number of nodules (NN), nodule dry weight (NDW) and shoot dry weight (SDW). NDW and SDW after drying at 65^0C for 72 hours to a constant weight. Shoot N content was analyzed using the Kjeldahl digestion according to Sahlemedhin Sertsu and Taye Bekele, (2000) (appendix II).The relative symbiotic effectiveness (SE) was calculated by comparing the shoot dry weight of the inoculated plants with N fertilized control plants. The equation proposed by Date *et al*., (1993) cited in Pucino *et al*., (2000) was used for the computation.

$$\%\ \text{Symbiotic effectiveness} = 100 \times \frac{\text{Shoot dry weight of an inoculated plant}}{\text{Shoot dry weight of } KNO_3 \text{ fertilized plant}}$$

Isolates were rated as very effective (VE) when the percentage SDW was higher than 80%; effective (E) when the percentage of SDW was between 50%-80%; lowly effective (LE) between 35%-50%; and ineffective (I) when it was less than 35%.

2.2.11 Analysis of 16S rRNA gene sequences

2.2.11.1 DNA Extraction

Isolates, CBR052 and CBR141 were selected based on best symbiotic performance and streaked on YEM agar medium and incubated at 28^0C for three days. A single colony of each isolate was picked and inoculated in 4 ml sterile YEM broth in a test tube and grown until late log phase on a shaker-incubator adjusted at 28^0C. Total genomic DNA was extracted with the PureLink® Genomic DNA Kits (Thermo Fisher Scientific, Invitrogen, USA) following the manufacturer's instructions. The DNA integrity and absence of RNA contamination was checked on gel electrophoresis (1% agarose) that was visualized by UV illumination with a gel scanner (BioRad)

after staining with SYBR® Safe stain. All the purified DNA products were stored at minus 20^{0}C and used for amplification of the 16S rDNA.

2.2.11.2 PCR amplification of 16S rRNA genes

One µl of DNA was used as a template for PCR amplification using a thermo cycler (Biometra, Tprofessional basic thermocycler, Germany). In addition to the DNA, the PCR reaction mix consisted of 12.5µl Thermo Scientific DreamTaq PCR Master Mix (2X) which was a ready-to-use solution containing DreamTaq DNA Polymerase, optimized DreamTaq buffer, $MgCl_2$, and dNTPs, 12 µl sterile Milli-Q water, 0.25 µl of forward primer 9f (5' -GAG TTT GAT CIT IGCT CAG- 3') and 0.25µl of reverse primer 1512r (5 -ACGGYTACCTTGTTACACTT- 3). The PCR program included a denaturation step of 5 minute at 95 ^{0}C, followed by 36 cycles of 95^{0}C for 1 minute, 55 ^{0}C for 1 minute, 72 ^{0}C for 1 min, and finally an extension step for 5 minute at 72 ^{0}C.

2.2.11.3 Sequence Analysis

The quality of the sequence was checked and edited using Gap4 as implemented in Staden-package 1.7.0 (Staden, 1998). The 16S rRNA gene sequences of the two isolates were compared to the Genbank database by using the nucleotide blast program and sequences of each gene were aligned using ClustalW as implemented in Mega5 (Tamura, 2011) and manually corrected when necessary. Neighbor joining phylogenetic trees were constructed using MEGA version 5 using the Kimura's 2-parameter distance correction model (Kimira, 1980). Statistical support of the trees was calculated by boot strap analysis using 1000 replications and the percentage similarity of the genes was estimated using the Kimura-2 distance matrix correction model as implemented in MEGA version 5.

2.2.12 Data analysis

Data from phenotypic characterization were used for numerical taxonomic study, the presence (+) and absence (-) of growth was converted into binary data as 1 for bacterial growth and 0 for the absence of growth. A computer cluster analysis on 75 phenotypic variables for 85 test and reference bacteria was done by using NTSYS-pc program (Numerical Taxonomic System, Applied Biostatistics Inc version 21; New York, USA) and a dendrogram was constructed based on Unweighed Pair Group Method with Arithmetic mean (UPGMA). One way analysis of variance (ANOVA) for comparisons between the treatments was made using the statistical program SPSS version 20. Mean separation was calculated using the Turkey's HSD test value when the F-test was significant at $p = 0.05$ Percentage.

2.3 Results and Discussion

2.3.1 Growth and colony characteristics

A total of 76 bacterial isolates (Appendix IV) were collected from root nodule samples of common bean (*Phaseolus vulgaris*) grown at different agroecosystems (1268-2264 masl). Accordingly, 28 isolates from western, 26 isolates from eastern, 11 isolates from central and 11 isolates from southern parts of Ethiopia were collected. The isolates formed colony diameters between 2.0 and 5.0 mm within 2 to 5 days with growth rate of 0.75-3.35 h (Table 2.1). All the isolates were gram negative and rod shaped bacteria grown with little or no absorption of Congo red on CR-YEM medium. The colonies showed large watery translucent (LWT) or medium watery transluscent (MWT) characteristics on YEMA, and appeared glossy and circular colonies with entire margin and convex elevation on PY medium. The isolates turned the green YEMA-BTB mediim into moderately yellow and deep yellowish indicating that all the isolates are acid

producers. These growth features are characteristics of fast growing rhizobia (Jordan, 1984; Martinez-Romero *et al*., 1991).

Table 2.1 Distribution of common bean rhizobia from different sampling sites of Central, Eastern, Western, and Southern parts of Ethiopia

Sampling region	***Altitude***	***Number of isolates***	***Cultural characters***		
			Range of colony diameter	***Range of generation time***	***Texture***
Central Ethiopia	1639-1697	11	2.0-5.0 mm	0.8-2.3	MWT/LWT
Eastern Ethiopia	1268-2264	26	2.0-5.0 mm	0.9-3.2	MWT/LWT
Western Ethiopia	1496-2097	28	2.0-5.0 mm	0.78-3.25	MWT/LWT
Southern Ethiopia	1506-1966	11	2.5-5.0 mm	0.8-2.9	MWT/LWT

LWT: Large, Watery and Translucent; MWT: Medium, Watery and Translucent (L:>3 mm) (M:2-3mm) (S:<2 mm)

2.3.2 Authentication of isolates and screening for nodulation efficiency

Out of the 76 test isolates, all but two isolates CBR039 (Bure) and CBR151 (Elu Ababora) formed root nodules on the *P. vulgaris*. Only 34 isolates were found to be effective and moderately effective during screening for effectiveness on sand culture under greenhouse condition, the inoculated plants showed deep green and healthy leaves with pink and large nodules (2 -3 mm in diameter). More than half (55%) of the isolates were found to induce whitish and tiny nodules (2mm in diameter) where the inoculated plants were stunted with yellowish leaves (Appendix V). Albeit their appearance on plates was very similar to that of other test isolates that were able to nodulate the host plant, isolates CBR039 and CBR151 failed

to nodulate *P. vulgaris* up on reinoculation. Failure to nodulate the host plant was also reported by Anteneh Argaw, (2007) where four out of 62 isolates from common bean nodules couldn't reinfect the host plant. Spontaneous losses of the symbiotic plasmids (sym-plasmids) in rhizobia could contribute to the inability of the rhizobia to renodulate the host (Zhang *et al*., 2001).

2.3.3 Effect of temperature on growth of rhizobia

All isolates were able to grow between 15 and 35^0C (Table 2.2). There was a decline in growth of isolates at temperatures of < 15^0C and >35^0C, but seven isolates were able to grow at 40^0C. These results showed similar patterns with Anteneh Argaw (2007) where all the common bean isolates from western Ethiopia were grown between 15^0C and 35^0C and Martinez-Romero *et al.,* (1991), where rhizobia nodulating common bean were able to grow at temperature as high as 40^0C. The tendency of rhizobial isolates to grow at elevated temperatures on pure culture has strong correlation with their symbiotic performance under temperature stressed soil conditions (Hungria *et al.*, 2000), and it could help as an important characteristic to select rhizobia inoculants for tropical soils.

2.3.4 pH tolerance of isolates

Isolates were able to grow on the medium with pH range between 5.5 and 8.5 (table 2.2). Although the majority of the isolates were tolerant to moderately acidic and alkaline pH (5.5 - 8.5), only a few isolates (23.5%) were able to grow at pH 4.5 and pH 9.5. Though this result showed a difference from the report by Alemayehu Workalemahu and Fassil Assefa (2007) where all the isolates from southern Ethiopia were grown at pH 4.5, it is comparable to the results of Anteneh Argaw (2007) where about 24% of the 62 isolates from eastern Ethiopia were able to grow at pH 4.5.

Table 2.2 Physiological characteristics of rhizobial isolates from common bean

Isolate	Region	C- source utilization (%)	N- Source utilization (%)	Temperature tolerance	pH tolerance	Salt tolerance	IAR pattern
CBR002	WE	67	86	15–35	4.5–9.5	2.0	Nal, Str, Spe, Chl
CBR003	WE	92	86	15–35	5–9.0	0.5	Nal, Str, Kan
CBR005	WE	71	90	10–35	4.5–9.5	1.5	Nal, Rif, Spe, Chl
CBR006	WE	79	67	15–37	5.0–8.5	2.0	Nal, Rif, Spe, Chl
CBR007	WE	79	67	15–37	5.0–8.5	2.0	Nal, Rif, Spe, Chl
CBR008	WE	83	76	15–37	5.5–8.5	3.0	Nal, Chl, Rif, Str, Spe
CBR011	WE	67	86	15–40	4.5–9.5	2.5	Nal, Str, Spe, Chl
CBR013	WE	96	81	15–35	5–9.5	1.5	Nal, Kan, Amp
CBR015	WE	71	90	10–35	4.5–9.5	1.5	Nal, Rif, Spe, Chl
CBR018	WE	92	86	15–35	5–9.0	0.5	Nal, Str, Kan
CBR019	WE	71	90	10–35	4.5–9.5	1.5	Nal, Rif, Spe, Chl
CBR020	WE	83	76	15–37	5.5–8.5	3.0	Nal, Chl, Rif, Str, Spe
CBR023	WE	83	76	15–37	5.5–8.5	3.0	Nal, Chl, Rif, Str, Spe
CBR026	WE	75	71	15–37	5.5–8.5	2.5	Nal, spe, Chl
CBR028	WE	79	67	15–37	5.0–8.5	2.0	Nal, Rif, Spe, Chl
CBR031	WE	88	90	15–35	5.5–9.0	2.0	Nal, Str
CBR034	WE	92	86	15–35	5–9.0	0.5	Nal, Str, Kan
CBR035	WE	79	67	15–37	5.0–8.5	2.0	Nal, Rif, Spe, Chl
CBR036	WE	71	90	10–35	4.5–9.5	1.5	Nal, Rif, Spe, Chl
CBR039	WE	75	71	15–37	5.5–8.5	2.5	Nal, spe, Chl
CBR043	WE	83	76	15–37	5.5–8.5	3.0	Nal, Chl, Rif, Str
CBR045	WE	83	76	15–37	5.5–8.5	3.0	Nal, Chl, Rif, Str, Spe
CBR150	WE	75	71	15–37	5.5–8.5	2.5	Nal, spe, Chl
CBR151	WE	67	86	15–35	5.0–9.5	2.0	Nal, Str, Rif, Spe, Chl
CBR154	WE	63	71	10–40	4.5–8.5	4.5	Amp, Spe, Chl
CBR157	WE	83	76	15–37	5.5–8.5	3.0	Nal, Chl, Rif, Str
CBR158	WE	88	90	15–35	5.5–9.0	2.0	Nal, Str, Amp
CBR160	WE	92	86	15–35	4.5–9.5	0.5	Nal, Str, Kan
CBR046	CE	79	67	15–37	5.0–8.5	2.0	Nal, Rif, Spe, Chl
CBR049	CE	83	76	15–37	5.5–8.5	3.0	Nal, Chl, Rif, Str
CBR052	CE	88	90	15–35	5.0–9.0	3.0	Nal, Str, Amp
CBR053	CE	79	67	15–37	5.0–8.5	2.0	Nal, Rif, Spe, Chl
CBR055	CE	79	67	15–37	5.0–8.5	2.0	Nal, Rif, Spe, Chl
CBR056	CE	71	90	10–35	4.5–9.5	1.5	Nal, Rif, Spe, Chl
CBR059	CE	92	86	15–35	5–9.0	0.5	Nal, Str, Kan
CBR062	CE	96	81	15–35	5–9.5	1.5	Nal, Kan
CBR063	CE	83	76	15–37	5.5–8.5	3.0	Nal, Chl, Rif, Str
CBR066	CE	83	76	15–37	5.5–8.5	3.0	Nal, Chl, Rif, Str
CBR068	CE	75	71	15–37	5.5–8.5	2.5	Nal, spe, Chl
CBR075	EE	88	90	15–35	5.5–9.0	2.0	Nal, Str
CBR079	EE	71	90	10–35	4.5–9.5	1.5	Nal, Rif, Spe, Chl
CBR088	EE	88	90	15–35	5.5–9.0	2.0	Nal, Str
CBR094	EE	92	86	15–35	5–9.0	0.5	Nal, Str
CBR096	EE	92	86	15–35	5–9.0	0.5	Nal, Str, Kan
CBR097	EE	75	71	15–37	5.5–8.5	2.5	Nal, spe, Chl
CBR109	EE	92	86	15–35	5–9.0	0.5	Nal, Str, Kan
CBR110	EE	67	86	15–37	4.5–9.5	2.0	Nal, Str, Spe, Chl
CBR112	EE	88	90	15–35	5.5–9.0	2.0	Nal, Str, Amp

CBR114	EE	83	76	15–37	5.5–8.5	3.0	Nal, Chl, Rif, Str, Spe
CBR116	EE	75	71	15–37	5.5–8.5	2.5	Nal, spe, Chl
CBR117	EE	71	90	10–35	5.0 –9.5	1.5	Nal, Rif, Spe, Chl
CBR121	EE	88	90	15–35	5.5–9.0	2.0	Nal, Str
CBR122	EE	63	71	10–40	4.5–8.5	4.5	Amp, Spe, Chl
CBR124	EE	75	71	15–37	5.5–8.5	3.0	Nal, spe, Chl
CBR125	EE	67	86	15–35	4.5–9.5	2.0	Nal, Str, Rif, Spe, Chl
CBR127	EE	88	90	15–35	5.5–9.0	2.0	Nal, Str
CBR129	EE	67	86	15–40	4.5–9.5	2.0	Nal, Str, Rif, Spe, Chl
CBR130	EE	88	90	15–35	5.5–9.0	2.0	Nal, Str
CBR144	EE	83	76	15–37	5.5–8.5	3.0	Nal, Chl, Rif, Str, Spe
CBR147	EE	88	90	15–35	5.5–9.0	2.0	Nal, Str
CBR148	EE	75	71	15–37	5.5–8.5	2.5	Nal, spe, Chl
CBR161	EE	75	71	15–37	5.5–8.5	2.5	Nal, spe, Chl
CBR164	EE	88	90	10–35	5.5–9.0	2.0	Nal, Str
CBR169	EE	79	67	15–37	5.0–8.5	2.0	Nal, Rif, Spe, Chl
CBR172	EE	92	86	15–35	5–9.0	0.5	Nal, Str, Kan
CBR083	SE	67	86	15–40	4.5–9.5	2.0	Nal, Str, Rif, Spe, Chl
CBR089	SE	83	76	15–37	5.5–8.5	3.0	Nal, Chl, Rif, Str
CBR098	SE	83	76	15–37	5.5–8.5	3.0	Nal, Chl, Rif, Str, Spe
CBR104	SE	88	90	15–35	5.5–9.0	2.0	Nal, Str
CBR132	SE	83	76	15–37	5.5–8.5	3.0	Nal, Chl, Rif, Str
CBR133	SE	96	81	15–35	5–9.0	1.5	Nal, Kan
CBR137	SE	75	71	15–37	5.5–8.5	2.5	Nal, spe, Chl
CBR138	SE	67	86	15–40	4.5–9.5	2.5	Nal, Str, Spe, Chl
CBR140	SE	71	71	10–35	4.5–9.5	3.0	Nal, Rif, Spe, Chl
CBR141	SE	75	90	15–37	5.0–8.5	2.5	Nal, spe, Chl
CBR142	SE	67	86	15–40	4.5–9.5	2.5	Nal, Str, Spe, Chl
HBR11	–	75	71	15–37	5.5–8.5	2.5	Nal, spe, Chl
HBR17	–	83	76	15–37	5.5–8.5	3.0	Nal, Chl, Rif, Str
HBR02	–	92	86	15–35	5–9.0	0.5	Nal, Str, Kan
HBR21		71	90	10–35	4.5–9.5	1.5	Nal, Rif, Spe, Chl
HBR22	–	83	76	15–37	5.5–8.5	3.0	Nal, Chl, Rif, Str
HBR04	–	88	90	15–35	5.5–9.0	2.0	Nal, Str
HBR42	–	83	76	15–37	5.5–8.5	3.0	Nal, Chl, Rif, Str, Spe
HBR05	–	88	90	15–35	5.5–9.0	2.0	Nal, Str
HBR09	–	79	67	15-37	5.0–8.5	2.0	Nal, Chl, Rif, Str, Spe

Am: ampicillin; Chl: chloramphenicol, Kan: kanamycin, Nal: naldixic acid, Rif: rifampicin, Spe: spectinomycin, Str: streptomycin, IAR: intrinsic antibiotics resistance, WE: western Ethiopia, CE: central Ethiopia, EE: eastern Ethiopia, SE: southern Ethiopia.

2.3.5 Tolerance to different concentration of salt (NaCl)

With regard to salt tolerance, the majority of the isolates were grown between 1.5% and 3% but two isolates (CBR122 and CBR154) were able to grow at 4.5% NaCl concentration. Salinity of 0.5% allowed the growth of all the tested isolates. In this study, more isolates (75%) showed tolerance to higher levels of NaCl (2-4.5%) compared to the two isolates (11%) reported from

southern Ethiopia (Alemayehu Workalemahu and Fassil Assefa, 2007). Salinity is one of the factors that affect rhizobium-legume symbiosis and hence strategies to improve legume production in saline soils need to consider inoculation with salt-tolerant strains of rhizobia as an alternative approach to improve legume productivity.

2.3.6 Intrinsic antibiotic resistance of isolates

The isolates were resistant to nalidixic acid (97%) and chloramphenicol (68%) and were highly sensitive to kanamycin (87%) and ampcilin (92%). The isolates also showed moderate susceptibility to rifampicin (56.5%), streptomycin (43%), and spectinomycin (42%) (Table 2.2). Several reports showed that common bean rhizobia were resistant to nalidixic acid and chloramphenicol (Martinez-Romero *et al.,* 1991; Amarger *et al.,* 1997, Alemayehu Workalemahu and Fassil Assefa, 2007) but sensitive to spectinomycin, and rifampicin (Segovia *et al.,* 1993; Alemayehu Workalemahu and Fassil Assefa, 2007). Resistance/sensitivity to antibiotics is one of the most important taxonomic features among strains of rhizobia (Amarger *et al.,* 1997) and is one of the the methods to evaluating the ecological competitiveness of the strains in the soil (Kremer and Peterson, 1982).

It is also interesting to note that the majority of the isolates 84% were endowed with multiple resistances to 3-5 of the tested antibiotics. Five isolates from western Ethiopia (CBR008, CBR020, CBR023, CBR045), four isolates from eastern Ethiopia (CBR114, CBR125, CBR129, CBR144) and two isolates from southern Ethiopia (CBR082 and CBR098) showed multiple resistance to 5 antibiotics. The pattern of IAR was dominated by a combination of nalidixic acid, chloramphenicol, and spectinomycin followed by streptomycin and rifampicin. It is well

established that the multiple antibiotic resistances by the test isolates depends upon the soil habitat from where they were isolated.

2.3.7 Utilization of carbon and nitrogen sources

The isolates displayed high versatility in utilizating carbon and nitrogen sources (Table 2.2). Almost all isolates (95%) were capable of utilizing sucrose, maltose, D-mannitol, D-glucose, galactose, lactose, and trehalose as a sole carbon source. The majority of the isolates (85%) were grown on raffinose, D-glucoinic acid, raffinose, mannose, cellobiose, L-rhaminose, and glycerol. Likewise, other isolates from southern and estern Ethiopia (Alemayehu Workalemahu and Fassil Assefa 2007; Anteneh Argaw 2007) and from Southern America (Amarger *et al*., 1997; Hungria *et al*., 2000) showed similar pattern of carbohydrate utilization. This study also showed that only a few isolates were able to utilize sorbitol (18%) and dextrin (11%) as a sole source of carbon. This is also in line with the report that species of common bean rhizobia rarely utilize sorbitol and dextrin (Segovia *et al.,* 1993, Anteneh Argaw, 2007).

Likewise, the isolates showed variability in utilizing amino acids as a sole source of nitrogen (Table 2.2). All isolates utilized glutamine, tyrosine, leucine, methionine and isoleucine and the majority of the isolates (82%) utilized the rest of the amino acids with some level of variability, but fewer isolates utilized glycine (22%) and tryptophan (35%). Similarly, common bean rhizobia displayed high metabolic versatility on different carbon and nitrogen sources (Martinez-Romero *et al*., 1991; Amerger, 1997). This tendency of rhizobia to metabolize a wide variety of substrates could be viewed as a vital strategy for their saprophytic competition in the soil and hence an important approach for selection of competitive inoculants as biofertilizer. Moreover,

the tendency of the isolates to utilize various nitrogen and carbon sources is used as a diagnostic feature to evaluate strain diversity (Amarger *et al.,* 1997; Hungria *et al.*, 2001).

2.3.8 Plant growth promoting Properties

With regard to plant growth promoting properties of the isolates, 61% of the rhizobia were able to produce IAA (Appendix VI). Antoun *et al.,* (1998) also reported that 58% of the 266 rhizobial isolates from different pulse crops including common bean are capable of IAA biosynthesis. The current result also showed that IAA production by the isolates was positively correlated ($P<0.05$, $r=0.64$) with root size of inoculated plants and this is in line with the fact that IAA affects root system development in plants, which can increase or decrease radicle cell size, depending on the concentration as well as its interaction with other phytohormones (Dazzo and Yanni 2006).

Table 2.3 PGPR properties of rhizobial isolates

Sampling region	IAA producing isolates	P solubilizing isolates	*P. oxysporum* inhibiting isolates
WE	CBR003, CBR006, CBR011, CBR013, CBR018, CBR023, CBR026, CBR028, CBR035, CBR036, CBR043, CBR045, CBR151, CBR154, CBR157, CBR158	CBR005, CBR018, CBR019, CBR034, CBR150	CBR002, CBR007, CBR008, CBR013, CBR019, CBR023, CBR031, CBR035, CBR039, CBR045, CBR157, CBR160
CE	CBR046, CBR052, CBR055, CBR059, CBR062, CBR063, CBR066	CBR046, CBR056	CBR052, CBR053, CBR062, CBR068
EE	CBR075, CBR096, CBR097, CBR109, CBR114, CBR121, CBR122, CBR127, CBR130, CBR147, CBR148, CBR161, CBR164, CBR169	CBR096, CBR110, CBR125, CBR172	CBR088, CBR094, CBR112, CBR114, CBR117, CBR124, CBR129, CBR147, CBR164, CBR172
SE	CBR083, CBR089, CBR098, CBR104, CBR133, CBR137, CBR140, CBR141, CBR142	CBR140	CBR132, CBR142

WE:- Western Ethiopia, EE:- Eastern Ethiopia, CC:- Central Ethiopia, SE:- Southern Ethiopia

About 16% of the isolates were capable of solubilizing insoluble inorganic phosphate with solubilisation indices of 1.4 and 2.22. Although Alikhani *et al.,* (2006) catagorized common bean rhizobia as poor P-solubilizer compared to *R. leguminosarum sv. viciae* (SI=2.48); *Mesorhizobium. ciceri (SI=1.42); Mesorhizobium. mediterraneum (SI=1.42); and Sinorhizobium meliloti (SI=1.40),* this study showed that common bean rhizobia were as effective as other rhizobia in P solubilisation which was higher than *Rhizobium leguminosarum sv. phaseoli* (SI=0.96) reported by the same author.

The rhizobial isolates were also capable of supressing the growth of the pathogen *F. oxysporum* in a dual culture (Appendix VI). Accordingly, about 37% of the isolates inhibited the mycelia growth of the pathogen (Table 2.3**).** Similarly, Ozkoc and Deleveli (2001) reported that 29% of the tested strains of *Rhizobium phaseoli* inhibited mycelial growth of *Fusarium* sp. under *in vitro*, conditions indicating that besides their role in biological nitrogen fixation, species of common bean rhizobia do possess the potential to be used as a biocontrol agent against soil born root pathogens.

Although 23% of the isolates were symbiotically effective (>SE 50%), it is interesting to note that some of the symbiotically ineffective rhizobial isolates were endowed with other single or multiple PGP properties of phosphate solubilization (16%), growth hormone production (61%) and antagonism against fusarium wilt (37%). It is revealed that the isolates from central Ethiopia showed (27%) multiple properties followed by the isolates from western Ethiopia (25%), eastern Ethiopia (19%) and southern Ethiopia (18%).

2.3.9 Numerical analysis of the phenotypic characteristics

The dendrogram constructed for 85 bacterial strains using 75 phenotypic characteristics is shown in (Figure 2.2). The isolates in the cluster analysis, included 76 test isolates and 9 reference strains all linked at a similarity level of 83.6%. Four distinctive clusters were formed at a level of 89% similarity.

The largest of all groups was cluster II which contained 38 isolates and further diverged into two sub-clusters, IIa and IIb. Accordingly, 21 isolates, comprising 19 test isolates (CBR007, CBR028, CBR055, CBR169, CBR006, CBR035, CBR046, CBR053, CBR148, CBR026, CBR039, CBR097, CBR116, CBR141, CBR161, CBR150, CBR137, CBR068, and CBR124) and 2 reference *R. phaseoli* strains (HBR9 and HBR11) were grouped into subcluster IIa at 93% similarity. Similarly, 17 isolates including 14 test isolates (CBR023, CBR008, CBR045, CBR066, CBR157, CBR043, CBR132, CBR098, CBR144, CBR020, CBR114, CBR063, CBR089, CBR049) and 3 reference strains of which two were *Rhizobium sp.* (HBR22 and HBR42) and one *R. phaseoli* (HBR17) were clustered in subcluster IIb at a 93% similarity. None of the isolates in cluster II was able to use glycine as sole nitrogen source and all were sensitive to kanamycin and streptomycin but resistant to nalidixic acid. Their colonies appeared wet and translucent on YEMA and gummy on PY. None of them were able to grow on Luria Bertani (LB) medium. Most of the characteristics demonstrated by this group were congruent with the previous description by Martinez-Romero *et al*. (1991) on *Rhizobium leguminosarum sv. phaseoli*. Aserse *et al*. (2012) also reported that *R. phaseoli* and *Rhizobium* sp.from Ethiopian soils.

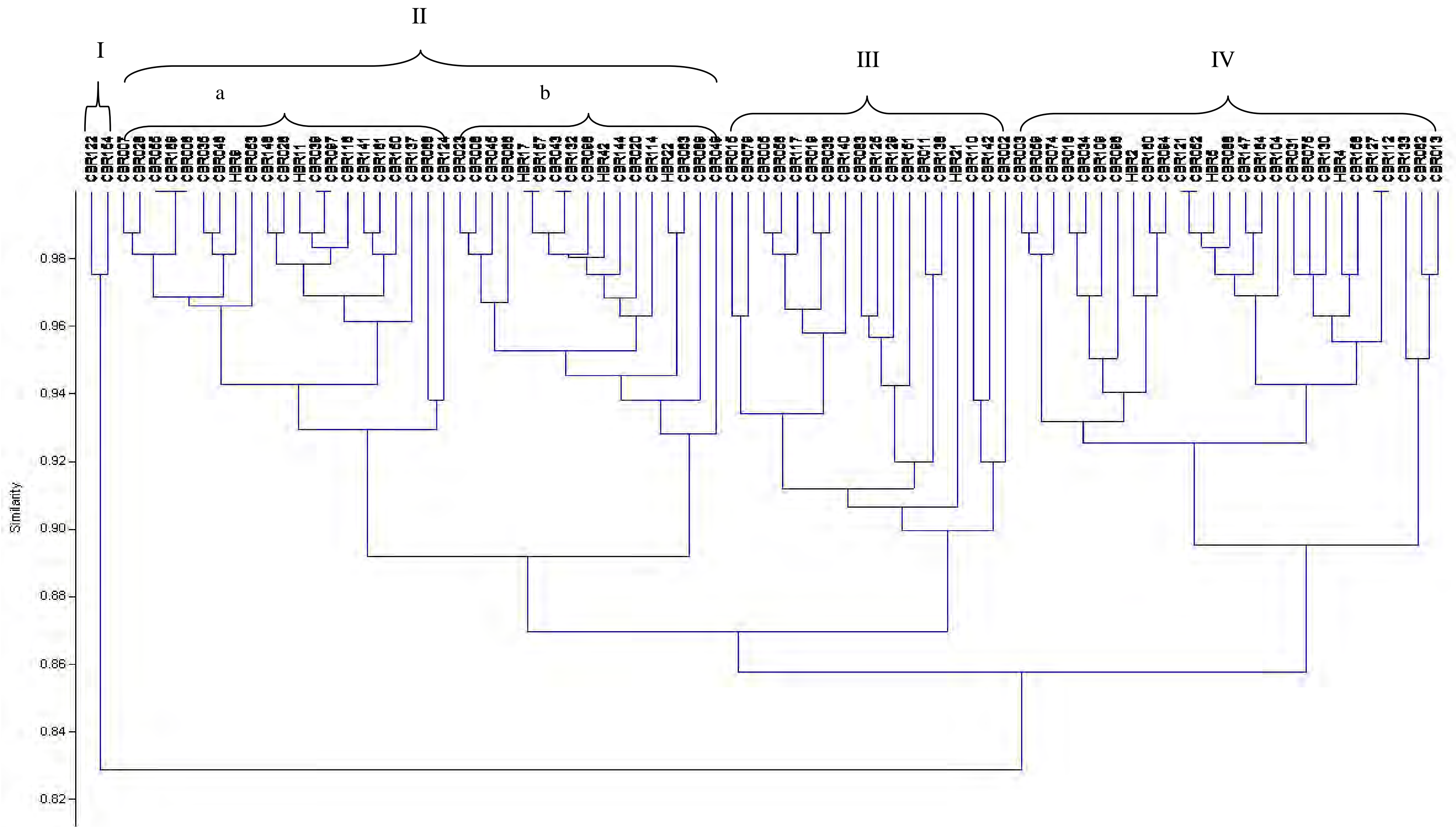


Figure 2.2 UPGMA dendrogram showing phenotypic relationships among rhizobia isolated from Phaseolus vulgaris in Ethiopia and reference strains. The cophenetic correlation (r) value was 0.82

The second larger group was represented by cluster IV and it consists of 27 isolates where 24 test bacteria (CBR003, CBR059, CBR172, CBR018, CBR034, CBR109, CBR096, CBR160, CBR094, CBR121, CBR052, CBR088, CBR147, CBR164, CBR104, CBR031, CBR075, CBR130, CBR158, CBR127, CBR112, CBR133, CBR062, and CBR013) were grouped with the three reference strains (HBR2, HBR4 and HBR5) classified as *R. etli.* All were linked together at a 90% similarity and further split into four sub-clusters at a boundary level of 93%. All the members in the cluster formed wet and translucent colonies on YEMA, gummy on PY and showed no growth on LB medium. All the isolates in this group were resistant to nalidixic acid, but failed to grow on spectinomycin, rifampin and chloramphenicol. Except the three isolates that formed one sub-cluster at 93% similarity, all members metabolized malate, and failed to grow on sorbitol as a sole carbon source. These characteristics were in good conformity with the isolates of *R. etli* describrd by Segovia *et al,* (1993) and Silva *et al*. (2003). *R. etli* was also reported as the dominant common bean rhizobia species in Ethiopia (Desta Beyene., 2004; Aserse *et al.,* 2012).

The third larger group, cluster III, consisted of 17 test isolates (CBR015, CBR079, CBR005, CBR056, CBR117, CBR019, CBR036, CBR140, CBR083, CBR125, CBR129, CBR151, CBR011, CBR138, CBR110, CBR142, CBR002) clustered with *R. giardini* reference strain (HBR21). All the members were characteristerized by wet and translucent colonies on YEMA and gummy on PY media. None of them were able to grow on LB medium. Unlike members of cluster II, isolates in this group were able to metabolize glycine. All the isolates were sensitive to kanamycin and half of them also showed susceptibility to streptomycin. All members of this group were able to grow well on lactate but failed to metabolize dulcitol as a sole carbon source.

These metabolic features were typical of *R. giardinii sv. phaseoli* strains as demonstrated by Amarger *et al.* (1997). Aserse *et al*. (2012) also identified species of *R.giardini* from Ethiopian soil using partial 16S rRNA gene sequence analysis.

The least number of isolates was included in cluster I which constitutes isolates CBR122 and CBR154 having no alignment with the reference strains. They showed the widest range of physiological tolerance to temperature (10^0C and 40^0C) and pH ranges of 4.5 to 8.5, and were tolerant to 4.5% NaCl where no other member of the clusters were able to grow. They were resistant to spectinomycin, chloramphenicol and ampcilin and exhibited growth on LB. Many of these features were in conformity with the typical attributes of *R. tropici* according to Martinez-Romero *et al*. (1991). However, none of them were able to nodulate *Leucaena leucocephala* in cross inoculation test which is one of the diagnostic characteristics of *R. tropici* (Anyango *et al*., 1995).

Based upon the numerical data from phenotypic characters, common bean rhizobia were tentatively classified into the fast growing common bean rhizobia under the Genus *Rhizobium* in four species. Many of the isolates (43%) were classified as *Rhizobium phaseoli/Rhizobium.* sp*.,* whereas the other isolates were grouped with *R etli* (32%), *R. gardiani* (22%), and *R. tropici*-like (3%). The isolates were distributed all across the different regions where *R. phaseoli* and *R. etli* showed moderate dominance in western and eastern Ethiopia, respectively.

The data from the tested characteristics and the dendrogram demonstrated the presence of diversity among the common bean nodulating rhizobia in Ethiopian soil. However, the study

used the morphological and physiological characterizations for the great majority of test isolates. The limited diagnostic yield of these techniques did not reveal the true picture of common bean rhizobia diversity at the species level. Therefore, there is a need to further studies using molecular techniques such as multi locus sequence analysis (MLSA) of some housekeeping genes, 16S rRNA gene sequence analysis and Amplified fragment length polymorphism (AFLP).

2.3.10 Symbiotic effectiveness of the selected test isolates

Isolate CBR141 induced the highest number of nodules (71 nodule/plant); whereas isolate CBR127 formed the least number of nodules (35 $plant^{-1}$) (Table 2.4). The inoculated plants showed nodule dry weight within the range of 0.046 gm (CBR043) and 0.136 gm (CBR052) per plant.

Table 2.4 Symbiotic traits of selected rhizobial isolates under greenhouse condition

Isolates	**Origin of isolates**		**Nodule number**	**Nodule dry wt. plant^{-1}**	**Shoot dry wt. (g plant^{-1})**	**Shoot nitrogen**	**SE%**	**Rate**
	Region	***Specific area***						
CBR002	WE	Jima	66.0±6.56a-c	0.065±0.006i-q	1.01±0.66n-r	1.24±0.066c-d	40.079	LE
CBR005	WE	Seka	59.33±4.04a-g	0.076±0.008g-l	1.26±0.05f-l	1.33±0.04b-c	50.000	E
CBR006	WE	Seka	63.67±5.51a-d	0.086±0.0053e-h	1.38±0.07d-g	1.36±0.06b-c	54.762	E
CBR013	WE	Mana	41.67±4.51h-j	0.053±0.0046o-q	1.11±0.03j-q	1.28±0.01b-c	44.048	LE
CBR018	WE	kersa	49.33±6.43c-j	0.068±0.0076h-p	1.06±0.02l-r	1.21±0.056c-d	42.063	LE
CBR023	WE	kersa	58.±5.29a-h	0.112±0.0046b-c	1.47±0.036d-e	1.42±0.08b-c	58.333	E
CBR026	WE	Metu	62.33±2.52a-f	0.085±0.0031e-i	1.11±0.037j-q	1.31±0.046b-c	44.048	LE
CBR031	WE	Metu	46.00.±3.61e-j	0.088±0.0067d-h	1.02±0.096m-r	1.17±0.026c-d	40.476	LE
CBR035	WE	Bure	53.33±4.04b-i	0.054±0.0044n-q	1.32±0.056e-i	0.83±0.07d	52.381	E
CBR043	WE	Bure	48.33±3.51d-j	0.046±0.0027q	1.0±0.046o-r	1.18±0.0343c-d	39.683	LE
CBR154	WE	EluAbabora	57.67±6.43a-h	0.068±0.006h-p	0.92±0.06q-r	1.18±0.076c-d	36.508	LE
CBR046	CE	Adami Tulu	51.00±4.58b-j	0.051±0.0067o-q	1.28±0.06e-k	1.26±0.03b-d	50.790	E
CBR049	CE	Ziway	63.00±4a-e	0.078±0.0037f-k	0.93±0.07q-r	1.36±0.11b-c	36.905	LE
CBR052	CE	Ziway	67.00±9.85a-b	0.136±0.0096a	1.88±0.08b	1.76±0.05b	74.603	E
CBR059	CE	Meki	45.33±2.08f-j	0.075±0.0052g-m	1.05±0.066l-r	1.23±0.01c-d	41.667	LE
CBR063	CE	Meki	41.00±2.00h-j	0.088±0.004d-g	0.98±0.07o-r	1.16±0.026c-d	38.889	LE
CBR079	EE	Kersa	61.33±5.51a-f	0.097±0.0046c-f	1.27±0.06e-k	1.18±0.087c-d	50.400	E
CBR088	EE	Kersa	52.00±3.61b-j	0.057±0.004i-q	1.55±0.06c-d	1.37±0.052b-c	61.508	E
CBR094	EE	Meta	38.33±5.61i-j	0.059±0.004j-q	0.99±0.07o-r	1.13±0.06c-d	39.286	LE
CBR096	EE	Deder	39.67±9.02i-j	0.064±0.0046j-q	1.12±0.06i-q	1.3±0.07b-c	44.444	LE
CBR109	EE	Tulo	53.00±4.58b-i	0.099±0.011c-e	1.57±0.06c-d	1.41±0.06b-c	62.302	E
CBR110	EE	Tulo	38.00±4.36i-j	0.047±0.0076q	1.26±0.04f-l	1.15±0.066c-d	50.000	E
CBR114	EE	Chiro	61.67±6.03a-f	0.074±0.0015g-n	1.27±0.07e-k	1.35±0.07a-c	50.400	E
CBR122	EE	Meiso	54.00±3.46b-i	0.108±0.009b-d	1.33±0.06e-h	1.41±0.03b-c	52.778	E
CBR127	EE	Boset	35.33±4.51j	0.089±0.0076d-g	0.95±0.036p-r	1.15±0.02c-d	37.698	LE
CBR130	EE	Boffa	46.00±3.61e-j	0.077±0.0056f-l	1.07±0.02k-r	1.32±0.056b-c	42.460	LE
CBR164	EE	Hirna	43.00±3g-j	0.055±0.0036m-q	1.42±0.16d-f	1.14±0.04c-d	56.349	E
CBR169	EE	Haromaya	48.00±7d-j	0.086±0.008e-h	0.94±0.056r	1.17±0.062c-d	35.714	LE
CBR172	EE	Haromaya	61.33±6.66a-f	0.071±0.00g-o	1.02±0.06m-r	1.22±0.036c-d	40.476	LE
CBR083	SE	Gamo Gofa	64.33±7.64a-d	0.068±0.003h-p	1.27±0.05e-k	1.21±0.04c-d	50.397	E
CBR098	SE	Gamo Gofa	43.00±4.00g-j	0.078±0.0042f-j	1.26±0.04f-l	1.19±0.04c-d	50.000	E
CBR133	SE	Hadaro tunto	50.33±6.51b-j	0.057±0.01k-q	1.31±0.05e-j	1.2±0.01c-d	51.984	E
CBR137	SE	Welayta	38.00±7i-j	0.048±0.008p-q	1.04±0.026m-r	1.25±0.03b-d	41.270	LE
CBR141	SE	Welayta	71.33±5.13a	0.122±0.011a-b	1.74±0.07b-c	1.64±0.046b-c	69.048	E
N-			0.00	0.0000	0.90±0.04q-r	1.13±0.03c-d	36.508	
N+			0.00	0.0000	2.52±0.05a	2.36±0.044a	100	

Levels not connected by same letter are significantly different at p 0.05. SE:- symbiotic effectiveness, WE:- Western Ethiopia, CE:- Central Ethiopia, EE:- Eastern Ethiopia, SE:- Southern Ethiopia, E:- effective, and LE:- lowly effective

The inoculated plants showed shoot dry matter per plant within the range of 0.92 g (CBR154) and 1.88 g (CBR052). Shoot dry weight is commonly used as indicator of symbiotic effectiveness (Amarger *et al.,* 1994; Somasegaran and Hoben 1994; Solaiman and Rabbiani,

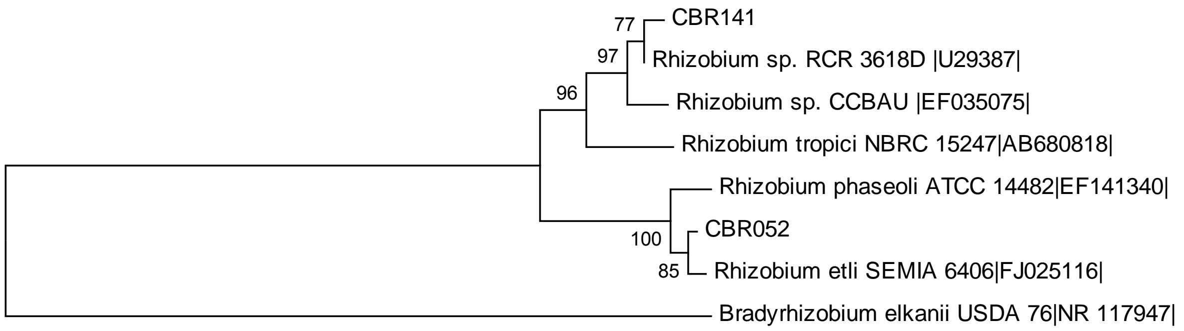
2004). Date (1993) also calculated the relative symbiotic effectiveness as % dry matter accumulation of inoculated plants in relation to the positive control. Accordingly, the relative symbiotic effectiveness of the isolates was between 35.7% and 74.6% of which 50% of the isolates induced effective symbiosis (50-74.6%) on the host plant. Among the effective isolates, CBR052 from Central Ethiopia showed the highest symbiotic effectiveness (74.6%) followed by CBR141 (69.0%) from Southern Ethiopia. None of the isolates were rated 'very effective' (>80%) (Table 2.4). The remaining 50% were rated lowly effective with < 50% relative symbiotic efficiency showing no significant ($p<0.05$) difference from the non-inoculated negative control. Based on the overall authentication tests in pouch and effectiveness screening on sand culture, fewer isolates (23%) were effective without having a highly effective symbiotic performance.

Previous studies in greenhouse (Alemayehu Workalemahu and Fassil Assefa, 2007; Aserse *et al.*, 2012) also showed that soils in various parts of Ethiopia harbor rhizobia that are able to nodulate *P. vulgaris* and some of them could effectively fix nitrogen. According to Alemayehu Workalemahu and Fassil Assefa (2007), 46% of isolates from southern Ethiopia were symbiotically effective where isolate AUPR2 from Konso area showed the highest relative symbiotic efficiency (72.6%) followed by AUPR1(67.7%) and AUPR7 (63%) from Arba Minch area. The isolate with the highest symbiotic effectiveness (74.6%) in this study was from Ziway (CBR052) and this is contrary to the report by Alemayehu Workalemahu and Fassil Assefa (2007) where isolates from the same location, AUPR17 and AUPR18 were rated lowly effective and ineffective, respectively.

The two isolates that ranked top in their relative symbiotic nitrogen fixation (CBR052 and CBR141) were also able utilize wide ranges of carbon and nitrogen sources and tested positive for the plant growth promoting hormone, IAA. Moreover, isolate CBR052 (*Rhizobium etli*) was able to suppress the radial growth of *Fusarium* phytopathogen by 24% (Appendix VI). Therefore the two isolates could be potential candidates for field trials so that their symbiotic performance and ecological competitiveness will be further examined across various soil and climatic conditions.

2.3.11 Identification of selected isolates by analysis of 16S rRNA gene sequences

The 16S rRNA gene sequences of the two field tested isolates showed the highest homology to the known species of bacteria in the database. Accordingly, CBR052 showed 99% match with *Rhizobium etli* RP212 |DQ406695| and CBR141 displayed 100% similarity to *Rhizobium sp*. R-26467 | AM231056|. The phylogenetic position of the isolates in relation to the reference strains is shown in Figure (Figure 2.3). Similarly, the taxonomic study on common bean rhizobia by Aserse *et al*., (2012) also reported the presence *R. etli* and *Rhizobium sp*. in addition to *R. giardinii and R. phaseoli* in the soils from various parts of Ethiopia.


77
CBR141
97
Rhizobium sp. RCR 3618D |U29387|
96
Rhizobium sp. CCBAU |EF035075|
Rhizobium tropici NBRC 15247|AB680818|
Rhizobium phaseoli ATCC 14482|EF141340|
100
CBR052
85
Rhizobium etli SEMIA 6406|FJ025116|
Bradyrhizobium elkanii USDA 76|NR 117947|
0.05

Chapter 3 Screening for phosphate solubilizing rhizobacteria associated with common bean (*Phaseolus valgaris* L.) and their effect on plant growth

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Abstract

A total of 54 rhizobacterial isolates were collected from common bean growing fields and screened for their potential to solubilize insoluble mineral phosphate and additional PGPR properties. The principal target was to screen for potential inoculants by evaluating the isolates for phosphate solubilizing efficiency using various media and greenhouse trial. Initial screening was conducted on Pikovskaya's (PKV) agar. Colorimetric quantification of free phosphate was performed in liquid media containging two phosphate sources. The impact of selected isolates on plant growth was assessed in greenhouse on common bean plants potted on soil amended with Egyptia rock phosphate (ERP). Additional plant growth promoting properties of isolates were also evaluated. The isolates displayed solubilization of tricalcium phosphate (TCP) with solubilization indices (SI) between 0.82 and 3.44. PGPR27 which is closely related to *Pseudomonas putida* strain NB2011 showed the highest efficiency by releasing 312.67 µg/ml of free phosphate after 96 hrs incubation in TCP. Plants inoculated with isolates PGPRC27, PGPRC44, PGPRC71 and PGPRC189 increased the nodule count, nodule dry weight, shoot dry weight, shoot nitrogen and shoot phosphorus as high as 103%, 42%, 98%, 65% and 81% , respectively compared to the uninoculated plants. Twelve of the isolates (22%) inhibited mycelial growth of the fungal phytopathogen, *Fusarium oxysporium f.sp. phaseoli*, by 19.9% to 51.3% *in vitro*. Thirty three of the isolates (61.1%) produced indole -3-acetic acid (IAA) between 9.5 and 53.23 $\mu g\ ml^{-1}$. The isolates were identified into 10 different genera that included *Bacillus, Arthrobacter, Brevibacterium, Lycinibacillus, Enterobacter, Acetinobacter, Pseudomonas, Klebsella, Devosia,* and *Chryseobacterium* based on 16S rRNA gene sequence analysis. This current study could therefore be important in proving the promising potential of indigenous common bean rhizosphere bacteria in enhancing plant growth and hence to be used as bioinoculants to ensure a sustainable cropping practice.

Keywords: Inoculants, phosphate solubilising rhizobacteria, tri calcium phosphate

3.1. Introduction

Phosphorus (P) is one of the major macronutrients that limit plant growth next to nitrogen (Goldstein, 1986). Infact the amount of phosphorus in the soil is generally quite high (often between 400 and 1, 200 mg kg^{-1} of soil), however, available phosphorus is scarce to support plant growth, due to either its existence as an insoluble inorganic mineral such as apatite or its pH mediated sorption or fixation (Khan *et al.*, 2007).

Microorganisms are involved in mineralizing organic phosphates and solubilising inorganic phosphates to improve the availability of soil P (Mardad *et al.*, 2013). These microorganisms, apart from miniralizing and solubilising insoluble phosphates, are also able to exert other beneficial effects upon plant growth and hence are known as 'plant growth promoting rhizobacteria' (PGPR) (Glicks 1995). PGPR possess one or more mechanisms that can simultaneously or independently become active at different stages of plant development and enhance growth and health by improving seedling emergence, plant vigour, and yield (Khan, 2006; Ahmed Idris *et al.,* 2008).

PGPR directly enhance plant growth by modulating phosphates in the soil and synthesizing plant growth hormones such as indoleacetic acid (IAA) (Pattern and Glick 1996; Gupta *et al.,* 2000). They also indirectly influence plant vigour by suppressing harmful microorganisms through the production of antibiotics, lytic enzymes and hydrogen cyanide (Gupta *et al.*, 2000).

Several studies showed that different species of bacteria are able to solubilize insoluble inorganic phosphate compounds such as tri-calcium phosphate, hydroxyapatite, rock phosphate and bone meal. The bacteria of the genera *Pseudomonas, Bacillus, Rhizobium, Burkholderia,*

Achromobacter, Agrobacterium, Microccocus, Aerobacter, Flavobacterium and *Erwinia* are well known for these properties (Ahmed Idris *et al*., 2008; Rodriguez and Fraga, 1999; Diriba Muleta *et al*., 2013). In addition to p solubilizing, some phosphobacteria are known to show other PGPR properties such as production of indole -3- acetic acid (IAA) and inhibition of different soil borne root pathogens (Peix *et al*., 2001b; Ponmurugan and Gopi, 2006; Naik *et al.,* 2008; Shahab *et al.,* 2009).

Application of phosphobacterial isolates is also reported to improve nodulation and nitrogen fixation in legume crops (Rodriguez and Fraga, 1999; Samavat *et al*., 2012) supplying the critically needed Phosphate for the rhizobial endosymbiosis and therefore recommended to be used as inoculant for mobilizing the fixed phosphorus in agricultural soils to improve legume productivity (Figueiredo *et al.,* 2008). Greenhouse and field trials also indicated that legume plants inoculated with phosphate solubilising rhizobacteria showed enhanced growth and yield (Peix *et al.,* 2001a; Shahab *et al*., 2009; Yadav *et al.,* 2010, Samavat, 2012).

The fact that these microorganisms contribute to plant growth and crop productivity has been widely accepted and being considered as a valuable input within the integrated soil fertility management approach (Harris *et al.,* 2006). Consequently, their potential as biofertilizers or biocontrol agents has been a centre of research interest worldwide for a number of years (Diriba Muleta *et al.,* 2007; Figueiredo *et al.,* 2008; Ahmed Idris *et al*., 2009; Mulissa Jida *et al.,* 2015).

In Ethiopia, some research activities have been undertaken on PGP properties of the rhizosphere bacteria from coffee (Diriba Muleta *et al.,* 2013), lentil and chickpea (Mulisa Jida, 2013), and sorghum (Ahmed Idris *et al.,* 2009). However, very little is known about the phytobeneficial aspects of the rhizosphere bacteria from common bean. This study, therefore, was aimed at isolating and characterizing common bean-associated phosphate solubilising rhizobacteria with multiple PGPR properties from soils of different part of Ethiopia as potential isolates for bioinoculant production.

3.2 Materials and Methods

3.2.1 Soil sample collection

Soil samples were collected from the rhizosphere of common bean grown in the central, eastern, southern and western Ethiopia between August and October, 2011. Plants were uprooted to collect the soil adhering to the roots into plastic bags, and stored at low temperature (4^0C).

3.2.2 Screening for phosphate solubilizing rhizobacteria

Each soil sample was transferred (10 gm) into 250 ml Erlenmeyer flasks containing sterilized 90 ml physiological saline (0.85% NaCl) to prepare a series of ten fold serial dilutions. Aliquots of serially diluted soil samples (0.1 ml) were spread plated on Pikovskaya (PKV) medium (Pikovskaya, 1948) in triplicates and incubated at 30^0C for 7 days. Colonies with clear haloes around were picked and purified by on the fresh PKV agar to be preserved on on nutrient agar slants at 4^0C. The isolates were suspended in 50% (v/v) glycerol and stored at -80^0C for long term preservation. Isolates were designated as PGPRC (Plant Growth Promoting Rhizobacteria from Common bean) with numerical orders (Table 3.1).

3.2.3 Identification of isolates using 16S rRNA gene sequence analysis

3.2.3.1 DNA extraction

Single colonies were picked from a 48 hrs culture grown on Luria-Bertani (LB) agar medium and inoculated in 4 ml sterile LB broth in a test tube and incubated to late log phase on a shaker-incubator (30^0C). Total genomic DNA of each isolate was extracted with the PureLink® Genomic DNA Kits (Thermo Fisher Scientific, Invitrogen, USA) following the manufacturer's instructions. The DNA integrity and absence of RNA contamination was checked on gel electrophoresis (1% agarose) and visualized by UV illumination with a gel scanner (BioRad) after staining with ethidium bromide. All DNA products were stored at -20^0C and used for amplification of the 16S rDNA.

3.2.3.2 PCR amplification of 16S rRNA genes

One µl of DNA was used as a template for PCR amplification using a thermo cycler (Biometra, Tprofessional basic thermocycler, Germany). The PCR reaction mix contained 12.5µl Thermo Scientific DreamTaq PCR Master Mix which was a ready-to-use solution containing DreamTaq DNA Polymerase, optimized DreamTaq buffer, $MgCl_2$, and dNTPs; 12 µl sterile Milli-Q water, 0.25 µl of forward primer 9f (5' -GAG TTT GAT CIT IGCT CAG- 3'), and 0.25 µl of reverse primer 1512r (5 -ACGGYTACCTTGTTACACTT- 3). The PCR program included a denaturation step of 5 minute at 95 ^{0}C, followed by 36 cycles of 95^0C for 1 minute, 55 ^{0}C for 1 minute, 72 ^{0}C for 1 min, and finally an extension step for 5 minute at 72 ^{0}C.

3.2.3.3 Sequence Analysis

The quality of the sequence was checked and edited using Gap4 as implemented in Staden-package 1.7.0 (Staden, 1998). The 16S rRNA gene sequences of the test isolates were compared

to the Genbank database by using the nucleotide blast program and sequences of each gene were aligned using ClustalW as implemented in Mega5 (Tamura, 2011) and manually corrected when necessary. Neighbor joining phylogenetic trees were constructed using MEGA version 5 using the Kimura's 2-parameter distance correction model (Kimura, 1980). Statistical support of the trees was calculated by boot strap analysis using 1000 replications and the percentage similarity of the genes was estimated using the Kimura-2 distance matrix correction model as implemented in MEGA 5.

3.2.4 Preparation of bacterial inoculum

For each test, fresh inocula were prepared by growing the isolates in nutrient broth for 48 hrs. The culture suspension was centrifuged (Wagtech International, UK) and the pellet was re-suspended in NaCl solution (0.85% w/v) to give a concentration of 10^8 cfu ml^{-1} (OD_{570} = 0.5-1.0).

3.2.5 Determination of SI and mobilized phosphate

Culture suspension (15 µl) of each test isolates was spot inoculated on PKV agar medium and incubated at 30ºC for 7 days according to Edi-Premono *et al*., (1996). Formation of a halo zone around the colony was checked and the solubilization indices (SI) were measured as a ratio of total diameter (colony diameter + clear zone diameter) to colony diameter.

Isolates with larger solubilization indices were selected and tested on liquid PKV basal mediim containing either of tricalcium phosphate (TCP) or Egyptian rock phosphate (ERP) as inorganic phosphate sources (Alikhani *et al*., 2006). One ml culture suspension (10^8cfu ml^{-1}) was transferred into 100 ml PKV broth containing 50 mg of TCP or ERP in a 250 ml Erlenmeyer flask in three replications. Uninoculated flasks with PKV basal medium containing inorganic

phosphate sources were included as controls. They were incubated at room temperature on a gyratory shaker (120 rpm) for 5-7 days.

Five ml sample suspension was withdrawn every 24 hours from culture flasks to measure pH and soluble P content. In each case the sample was centrifuged at 15,000 rpm (Wagtech International, UK) for 15 minutes to recover the supernatant. The amount of phosphorus was measured using the phospho-molybdate method (Murphy and Riley, 1962) where the absorbance of the developing blue color was read at 600 nm. The amount of soluble phosphorus was quantified from the standard calibration curve constructed using known concentrations of KH_2PO. The values of solubilized P detected in the uninoculated control were subtracted from their respective treatments.

3.2.6 Screening for additional Plant growth promoting properties of isolates

3.2.6.1 Indole-3-acetic acid (IAA) Production

Indole acetic acid (IAA) was determined by using the modified colorimetric method of Gordon and Weber (1951). One mL of 48 hrs bacterial culture (10^8 cfu ml^{-1}) was inoculated into 10 ml LB (Luria Bertani) broth containing filter sterilized L-tryptophan (1µg mL^{-1}) in 100 ml Erlenmeyer flasks and incubated at room temperature for 3 days on a gyratory shaker set at 120 rpm in triplicates. Non-inoculated control was included for comparison. The cultures were centrifuged at 10000 rpm (Wagtech International, UK) for 15 minutes from which 2.0 ml cell-free supernatant was mixed with 1 ml of 10 mM orthophosphoric acid and 4 ml of Salkowsky's reagent (1ml of 0.5 M $FeCl_3$ in 49 ml of 35% of $HClO_4$) in test tubes (12x120 mm), and incubated in dark at ambient temperature for 25 minutes. The intensity of pink colour was compared against a standard curve constructed with known concentration of IAA.

3.2.6.2 Screening for in vitro antagonistic activity against fungal growth

The antagonistic activity of isolates was screened against fungal pathogen - *Fusarium oxysporum f.sp. phaseoli* using dual petri-dish culture test as described by Skidmore and Dickinson (1976). Culture suspensions (15 µl) of bacterial isolates were spotted at equidistant points along the perimeters of nutrient agar plates supplemented with sucrose (0.5%) and incubated at 30º C for 2 days. The plates were then inoculated at the center with a 4 mm diameter PDA block with *Fusarium oxysporum f.sp. phaseoli* culture and incubated at 30ºC for 5 days in triplicates. Plates without bacteria were used as control. Percentage inhibition was calculated by measuring the radius of fungal growth towards the bacteria and radius of the control using the formula 100 x (C - T) /C, where C is the radial growth of fungus in control, and T the radial growth of the fungus in the dual culture (Gupta *et al*., 2006).

3.2.6.2.1 Screening for mechanisms involved in antagonism

3.2.6.2.1.1 Hydrolytic enzymes production assay

3.2.6.1.1.1 Screening for cellulolytic activity

Nutrient broth grown culture (10^8 cfu ml^{-1}) of each test isolate was spot inoculated on Carboxymethyl Cellulose (CMC) agar plates (0.2% $NaNO_3$, 0.1% K_2HPO_4 , 0.05% $MgSO_4$, 0.05% KCl, 0.2% carboxymethylcellulose sodium salt, 0.02% peptone, and 1.7% agar). The plates were incubated at 30^0C for 72 hours and flooded with Gram's iodine for 2 minutes to detect clear zone formation around colonies (Kasana *et al.,* 2008).

3.2.6.1.1.2 Screening for Proteolytic activity

Fourty eight hrs nutrient broth culture (10^8 cfu ml^{-1}) were spot plated on skim milk agar medium and incubated at 30^0C for 72 hours to observe halo zone formation around the colonies (Djanta *et al*. 2009). Ratio of holo zone to colony size was recorded as index of protease activity.

3.2.6.1.1.3 Screening for chitinolytic activity

Broth culture (10^8 cfu ml^{-1}) was spot inoculated on a nutrient agar medium containing 0.2% colloidal chitin. The plates were incubated at 30^0C for 72 to 96 hour to determine the formation of clear zone around the colonies and ratio of halo zone to colony size was recorded as index of chitinase activity (Hoster et al., 2005).

3.2.6.1.2 Hydrogen cyanide production

HCN production was tested using the method of Lorck (1948). Isolates from 48 hrs nutrient broth (10^8 cfu ml^{-1}) were streaked on King's B medium amended with 4.4 g glycine liter $^{-1}$. A Whatman filter paper no.1 impregnated in 0.5% picric acid and 2.0% sodium carbonate solution was placed at the upper lid of the Petri plate. Plates were sealed with parafilm and incubated at 30^0C for 96 hours. The colour formation in the presence of sodium picrate represented HCN production. A change of colour of the filter paper from yellow to light brown, brown or reddish-brown was recorded as weak (+), moderate (++) or strong (+++) reaction, respectively.

3.2.7 The effect of phosphobacterial isolates on growth of common bean under greenhouse condition

The effect of selected isolates on the growth of common bean was tested according to Figueiredo *et al.* (2008). The four PSR isolates: PGPRC27 (*Pseudomonas putida)*, PGPRC44 (*Bacillus thuringiensis)*, PGPRC71 (*Bacillus thuringiensis)* and PGPRC189 (*Pseudomonas poae*) were

evaluated based on their efficiency in solubilizing TCP and ERP in broth culture. Seeds of Nasir variety obtained from the Melkassa Agricultural Research Center /EIAR were surface sterilized with 95% ethanol and 3% sodium hypochlorite solutions for 10 seconds and 3 minutes, respectively (Somasegaren and Hoben 1994). The seeds were rinsed and germinated on water agar (1%). Five pregerminated seeds were transferred to 3kg capacity pots filled with air dried fine (5.0 mm sieved) composite top (0-20 cm) soil samples brought from Awash Melkassa, that were later thinned down to 3 after 5 days of emergence. The physical and chemical properties of the soil were pH (7.62), %TN 0.064), available P (mg/kg 5.63), TP (mg/kg 1137), available K (151.12 mg/kg), CEC (48 cmol/kg), %Clay (20), %Silt (47.51), and %Sand (32.48), population size of resident rhizobia ($2.6x10^1$). All the pots were supplied with the agronomically recommended amount of RP (200 kg ha^{-1}). Each plant including the controls was inoculated with 1ml of YEM broth culture (10^9 cfu ml^{-1}) of CBR052 (*Rhizobium etli)* per plant. The phosphobacterial treatments: PGPRC27, PGPRC44, PGPRC71, and PGPRC189 grown in nutrient broth (10^8 cfu ml^{-1}) were inoculated into plants (1 ml $plant^{-1}$). The treatments include the following in three replications.

T1= Seedling + RP + CBR052 (*Rhizobium etli)* + PGPRC27

T2= Seedling + RP + CBR052 (*Rhizobium etli)* + PGPRC44

T3= Seedling + RP + CBR052 (*Rhizobium etli)* + PGPRC71

T4= Seedling + RP + CBR052 (*Rhizobium etli)* + PGPRC189

T5= Seedling + RP + CBR052 (*Rhizobium etli)* + P (20 mg kg^{-1}as KH_2PO_4)

T6= Seedling + RP + CBR052 (*Rhizobium etli)*

The pots were arranged in triplicates in a completely randomized block design (CRBD) including pots phosphobacteria-uninoculated but fertilized with P (20 mg kg^{-1}as KH_2PO_4) as a positive control, whereas neither phosphobacteria-inoculated nor P-fertilized pots as a negative control. The seedlings were irrigated with water every three days.

After fourty five days of emergence, data were collected for nodule number, nodule dry weight, and shoot dry weight. Total shoot N and P contents were estimated using micro-kjeldahl (Sahlemedhin Sertsu and Taye Bekele, 2000) and phospho-molybdate methods (Murphy and Riley, 1962), respectively.

3.2.8 Statistical analysis

All the experiments were performed in triplicates to record average values with ±SD. Analysis of variance (ANOVA) was employed to analyze the data and differences among various treatment means against their respective controls were tested using SPSS version 20 and Tukey's HSD post hoc test at mean separation ($p < 0.05$). The correlation between different parameters was evaluated by using Pearson correlation coefficient test.

3.3 Results and Discussion

3.3.1 Isolation of phosphobacterial isolates and estimation of their abundance

In this study, the population density of phosphate solubilising rhizobacteria (PSR) in the soil samples of common bean growing areas was between $2.6x10^1$ and $9.2x10^3$ cfu g^{-1} of which the majority (68%) of the sampling sites harboured more than 10^2 PSB per gram soil (Table 3.1). This indicates that the population density of phosphobacteria in the rhizosphere of common bean is comparable to the phosphobacteria density under faba bean ($1.5x10^1$ - $1.8x10^3$ cfu g^{-1}) (Assefa Keneni *et al.,* 2010) but significantly lower than the phosphobacterial population under the rhizosphere soil of chickpea ($3.1x10^4$ - $5.6x10^5$ cfu g^{-1}) (Mulissa Jida, 2013).

Table 3.1: Distribution of Phosphobacteria isolated from common bean growing areas of Ethiopia

Origin of isolates	Sampling Region	Elevation of isolation site	Isolates selected from each site	PSB abundance (cfu/g) in soil of origin
Jimma	WE	1781	PGPRC6	1.2x10^3g-h
Seka	WE	1867	PGPRC11, PGPRC17	3.4x10^2j-k
Mana	WE	1876	PGPRC18, PGPRC19, PGPRC22	6.6x10^2h-k
kersa	WE	1696	PGPRC27,	2.7x10^3d-e
Metu	WE	1714	PGPRC32, PGPRC33, PGPRC38	5.3x10^1k
Bure	WE	1662	PGPRC43, PGPRC44	7.9x10^1k
Achibo	WE	1607	PGPRC179, PGPRC182	6.1x10^2h-k
Ziway	CE	1657	PGPRC106	4.7x10^2h-k
Meki	CE	1690	PGPRC117	3.2x10^3d
Awash	CE	1582	PGPRC171, PGPRC178	2.6x10k
Adami Tulu	CE	1649	PGPRC54	2.6x10^2j-k
Ziway	CE	1639	PGPRC57, PGPRC58, PGPRC66	8.5x10k
Meki	CE	1647	PGPBRC68, PGPRC71	3.2x10^3d
Dengego	EE	2143	PGPRC87	6.9x10^3b
Deder	EE	2171	PGPRC90, PGPRC94	8.4x10k
Chelenko	EE	2154	PGPRC95, PGPRC98	5.2x10^3k
Goro Gutu	EE	2264	PGPRC105	1.8x10^3f-g
Efa Bas	EE	2143	PGPRC108	1.1x10^3g-i
Chiro	EE	2123	PGPRC122	7.8x10k
Chiro	EE	2145	PGPRC123, PGPRC124	2.5x10^3h-j
Meiso	EE	1592	PGPRC131,PGPRC138,PGPRC139	5.6x10k
Boset	EE	1532	PGPRC144, PGPRC146	1.3x10^2k
Boffa	EE	1681	PGPRC153,	4.2x10^2i-k
Hirna	EE	1810	PGPRC186, PGPRC189	3.4x10k
Hadaro Tunto	SE	1740	PGPRC157	8.8x10k
Areka	SE	1712	PGPRC162, PGPRC165	1.9x10^2k
Humbo	SE	1827	PGPRC166,PGPRC167, PGPRC170	7.6x10^2k
Hanika	SE	1520	PGPRC183	9.2x10^3a
Karje	SE	1506	PGPRC185	2.3x10^3e-f
Kamba	SE	1864	PGPRC191,PGPRC192, PGPRC196	1.6x10^2k
Waraza Lasho	SE	1966	PGPRC56	5.2x10^3c

Levels in the same column not connected by same letter are significantly different at p 0.05. EE:- Estern Ethiopia, CE:- Southern Ethiopia, SE:- Southern Ethiopia and WE:- Western Ethiopia

3.3.2 Screening for Phosphate solubilisation on solid medium

A total of 58 phosphate solubilising rhizobacteria were collected from the rhizosphere of common bean of which 4 isolates lost their capacity for solubilisation after repeated sub-culturing. Reports from earlier studies (Rashid *et al*., 2004; Diriba Muleta *et al*., 2013) also showed loss of phosphate solubilizing activity of a few rhizobacteria following repeated sub-culturing on PKV agar plate. Out of the 54 P solubilising rhizobacterial isolates, 18 were collected from eastern Ethiopia, whereas 14, 12, and 10 isolates were recovered from western, southern and central Ethiopia, respectively, with SI ranging between 0.82 and 3.44 (Tables 3.2). This indicates that the phosphobacteria are almost evenly distributed in Ethiopian soils irrespective of the sampling site. The bacterial isolate PGPRC27 (*Pseudomonas putida)* showed the largest solubilization (SI = 3.44), followed by PGPRC189 (*Pseudomonas poae*) (SI = 3.36) and PGPRC71 (*Bacillus thuringiensis*) (SI = 3.28). The isolates with higher SI on solid medium (PKV) also released more P from insoluble phosphates in liquid cultures ($P< 0.05$, $r=0.66$). This is contrary to the report of Rashid *et al.,* (2004), where no correlation was observed between the size of clear zones (SI) and the solubilised P values in the liquid growth media. However, in Diriba Muleta *et al*., (2013) the correlation between the two variables in phosphobacterial isolates from coffee rhizosphere was strong ($r = 0.71$).

3.3.3 Identification of the isolates

The 16S rRNA gene sequences of the test strains showed 99% to 100% homology to the known species of bacteria when blasted on the NCBI database (Table 3.2). The data showed that both Gram-positive and Gram negative phosphobacteria occurred in the rhizosphere of *Phaseolus vulgris*. The gram positive bacteria constitute the largest proportion (65%), from which the genus

Bacillus was the most dominant (86%). The phylogenetic relationship of the isolates in relation to the reference strains is shown in (Figures 3.1 and 3.2).

A total of 10 phosphobacterial genera were recorded from the rhizosphere of common bean out of which 6 genera were gram negative, whereas 4 genera were isolates from gram positive bacteria indicating that the gram negative were more diverse than the other group. However, the gram positive bacteria were more abundant (65%) than the gram negative bacteria in which *Bacillus subtilis* constituted the highest (26%) percentage of the gram positive and *Acetinobacter* sp. constitutes the largest proportion (37%) from the gram negative bacteria. The 10 phosphobacteria genera were: *Bacillus, Arthrobacter, Brevibacterium, Lycinibacillus, Enterobacter, Acetinobacter, Pseudomonas, Klebsella, Devosia,* and *Chryseobacterium* (Table 3.2).

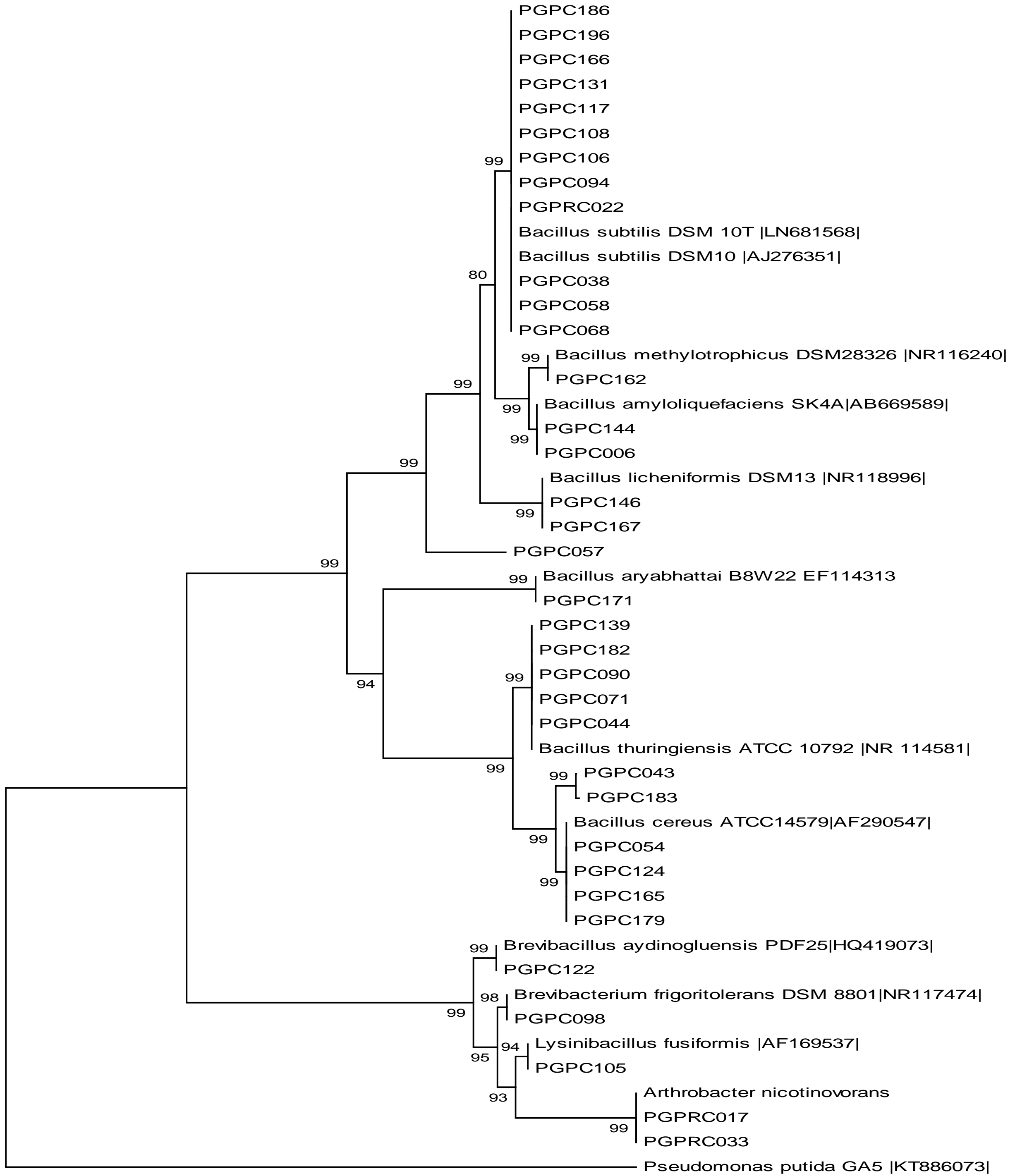
Table 3.2 Identification of Phosphobacterial isolates from the rhizosphere of common bean

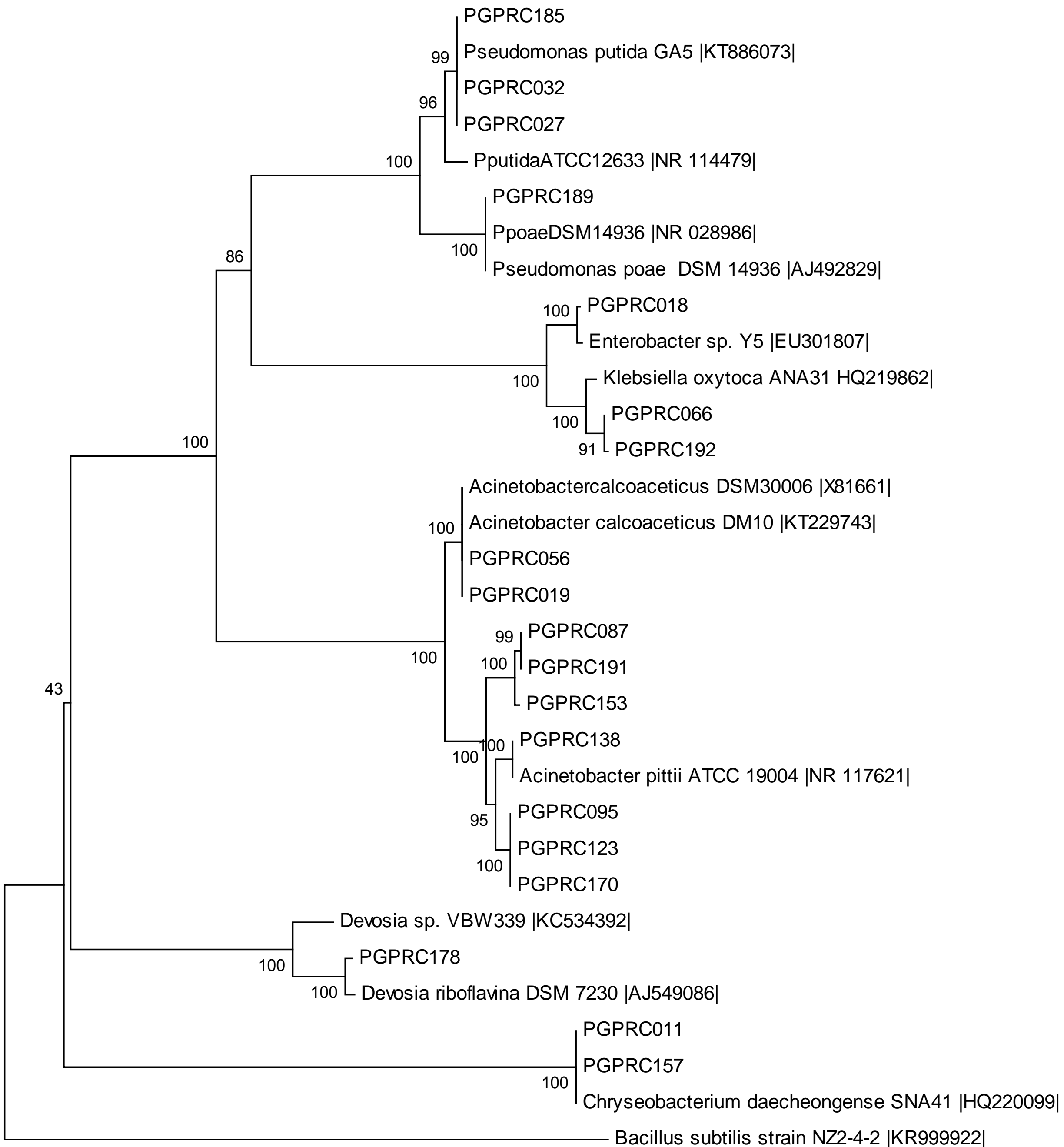
Isolate	*Sampling Region*	*Solubilization Index (SI)*	*Closest species based on 16S rRNA gene partial sequenceanalysis |accession number|*	*Gram reaction*	*Identity*
PGPRC6	*WE*	1.24±0.07±p-u	*Bacillus amyloliquefaciens* KSU-109|HM016080|	+	100%
PGPRC11	*WE*	2.51±0.11±e-h	*Chryseobacterium daecheongense* WR47 |JF700420|	–	99%
PGPRC17	*WE*	2.14±0.22±h-k	*Arthrobacter nicotinovorans* T258 |KC764990|	+	99%
PGPRC18	*WE*	1±0.12±s-v	*Enterobacter sp.* WS05 |JN210900|	–	99%
PGPRC19	*WE*	2.39±0.053±f-i	*Acinetobacter calcoaceticus* H3 |KJ149815|	–	100%
PGPRC22	*WE*	2.71±0.027±d-f	*Bacillus subtilis* NG4-6 KR999950|	+	100%
PGPRC27	*WE*	3.44±0.07±a	*Pseudomonas putida* NB2011	–	99%
PGPRC32	*WE*	2.3±0.097±g-j	*Pseudomonas putida* LB22 JF261631|	–	99%
PGPRC33	*WE*	2.15±0.19±h-k	*Arthrobacter nicotinovorans* TSWCSN20 |GQ284331|	+	99%
PGPRC38	*WE*	2.05±0.15±i-l	*Bacillus subtilis* CR8 |KR780412|	+	100%
PGPRC43	*WE*	1.75±0.04±k-n	*Bacillus cereus* BGSC 6A17 |EF210297|	+	99%
PGPRC44	*WE*	3.14±0.35±a	*Bacillus thuringiensis* YWC2-8 |CP013055|	+	100%
PGPRC179	*WE*	2.32±0.07±f-i	*Bacillus cereus* ML254 |KC692199|	+	99%
PGPRC182	*WE*	1.1±0.23±r-v	*Bacillus thuringiensis* RA2 |KU588387|	+	99%
PGPRC54	*CE*	1.75±0.04±k-n	*Bacillus cereus* OUC_Estg |KP161858|	+	99%
PGPRC57	*CE*	2.04±0.12i-l	*Bacillus safensis* KM8 |JF411308|	+	100%
PGPRC58	*CE*	2.83±0.16±c-e	*Bacillus sp*. ES1-5 |KJ878585|	+	99%
PGPRC66	*CE*	1.05±0.45±r-v	*Klebsiella oxytoca* SHD-1 |GU361112|	–	100%
PGPRC68	*CE*	1.41±0.06±n-r	*Bacillus sp*. CCBAU 10727 |EF377303|	+	100%
PGPRC71	*CE*	3.28±0.23±a-b	*Bacillus thuringiensis* |EB69 KP209387|	+	99%
PGPRC171	*CE*	1.11±0.91±r-v	*Bacillus aryabhattai* fwz21 |KF208483|	+	100%
PGPRC178	*CE*	1.48±0.58±n-s	*Devosia riboflavina* HPG62 |JQ291598|	–	99%
PGPRC106	*CE*	1.72±0.22±l-n	*Bacillus tequilensis* 111-4 |JX065214|	+	100%
PGPRC117	*CE*	1.18±0.08±q-v	*Bacillus subtilis subsp. inaquosorum* IS02 |JN856456|	+	100%
PGPRC87	*EE*	2.66±0.24±e-g	*Acinetobacter sp.* TRS9 |KJ631601|	–	99%
PGPRC90	*EE*	1.90±0.09±j-m	*Bacillus thuringiensis* A3-10 |JF496339|	+	100%
PGPRC94	*EE*	1.18±0.12±q-v	*Bacillus subtilis* BAB-2544 |KC443104|	+	100%
PGPRC95	*EE*	2.4±0.16±f-i	*Acinetobacter sp.* HN-7 |KT003253|	–	100%
PGPRC98	*EE*	2.23±0.09±h-j	*Brevibacterium frigoritolerans* MER_TA_56 |KT719460|	+	100%
PGPRC105	*EE*	2.2±0.6±h-j	*Lysinibacillus sp*. SSKSD12 |KF751677|	+	99%
PGPRC108	*EE*	0.96±0.62±t-v	*Bacillus sp.* B-AS-16 |JF901703|	+	100%
PGPRC122	*EE*	2.32±0.82±f-i	*Brevibacillus sp*. JS3 |AY372923|	+	100%
PGPRC123	*EE*	3.07±0.15±a-d	*Acinetobacter sp*. LMB-5 |KM981462|	–	100%
PGPRC124	*EE*	0.82±0.11v	*Bacillus cereus* SH06 KP027636|	+	100%
PGPRC131	*EE*	1.4±0.06±n-s	*Bacillus subtilis* strain NG3-7 |KR999941|	+	100%
PGPRC138	*EE*	2.29±0.72±g-j	*Acinetobacter sp*. LYC-1 |EU998912|	–	99%
PGPRC139	*EE*	3.16±0.43±a-c	*Bacillus thuringiensis* SN-17 |KR010173|	+	99%
PGPRC144	*EE*	1.05±0.55±r-v	*Bacillus amyloliquefaciens* ML581 |KC692179|	+	100%
PGPRC146	*EE*	1.28±0.06±o-t	*Bacillus subtilis* NG3-7 |KR999941|	+	99%
PGPRC153	*EE*	1.53±0.65±m-q	*Acinetobacter sp*. MSG8 |FJ848381|	–	99%
PGPRC186	*EE*	2.53±0.53±e-h	*Bacillus subtilis* NZ2-4-2 |KR999922|	+	100%
PGPRC189	*EE*	3.36±0.031±a	*Pseudomonas poae* BCHCNZ253 |GU188947|	–	99%
PGPRC56	*SE*	2.25±0.14±h-j	*Acinetobacter calcoaceticus* DM9 |KT229742|	–	99%
PGPRC157	*SE*	2.27±0.44±h-j	*Chryseobacterium sp.* WR21 |JF700394|	–	99%
PGPRC162	*SE*	2.91±0.31±b-e	*Bacillus methylotrophicus* MSL_3065 |KT719890|	+	100%
PGPRC165	*SE*	0.86±0.61±u-v	*Bacillus cereus* HYM89 |KT982246|	+	100%
PGPRC166	*SE*	2.67±0.07±d-g	*Bacillus subtilis* BJ-5 |GQ280015|	+	100%
PGPRC167	*SE*	2.61±0.25±e-g	*Bacillus tequilensis* RA1402 |KR819163|	+	100%
PGPRC170	*SE*	1.6±0.42±m-p	*Acinetobacter sp.* EN-41 |KP702928|	–	100%
PGPRC183	*SE*	2.23±0.741±h-j	*Bacillus cereus* RJ23 |KC990812|	+	100%
PGPRC185	*SE*	2.0±0.47±i-l	*Pseudomonas sp.* 242 |KT034415|	–	99%
PGPRC191	*SE*	1.15±0.34±q-v	*Acinetobacter sp.* MSG8 |FJ848381|	–	100%
PGPRC192	*SE*	1.72±0.86±l-n	*Klebsiella oxytoca* YNB101 |JQ039993|	–	99%
PGPRC196	*SE*	1.41±0.27±n-s	*Bacillus subtilis* PWK36 |KJ620422|	+	100%

Levels in the same column not connected by same letter are significantly different at p 0.05. +:- Gram positive, - :- Gram negative, CE:- central Ethiopia, EE:- eastern Ethiopia, SE:- southern Ethiopia, WE:- western Ethiopia

Generally, the diversity data indicate that a wide variety of phosphate solubilizing rhizobacteria inhabit the rhizosphere of common bean in Ethiopia. Similarly, earlier studies showed the rhizosphere soils of *Coffea arabica* (Diriba Muleta *et al*., 2007) and chick pea (Mulissa Jida, 2013) harbouring 11 and 9 genera of PSR, respectively.

The top nine isolates with higher P solubilization indices and four of which selected for greenhouse trial belonged to *Pseudomonas* and *Bacillus* species. These include PGPRC27 (*Pseudomonas putida),* PGPRC189 *(Pseudomonas poae)*, PGPRC71 (*Bacillus thuringiensis)*, and PGPRC44 (*Bacillus thuringiensis*) with SI of 3.44, 3.36, 3.28, and 3.14 respectively. Likewise, Tilak *et al.* (2005) stressed that the phosphate-solubilising microorganisms of superior performance belong to the bacterial genera *Bacillus* and *Pseudomonas*.


PGPC186
PGPC196
PGPC166
PGPC131
PGPC117
PGPC108
PGPC106
PGPC094
PGPRC022
Bacillus subtilis DSM 10T |LN681568|
Bacillus subtilis DSM10 |AJ276351|
PGPC038
PGPC058
PGPC068
Bacillus methylotrophicus DSM28326 |NR116240|
PGPC162
Bacillus amyloliquefaciens SK4A|AB669589|
PGPC144
PGPC006
Bacillus licheniformis DSM13 |NR118996|
PGPC146
PGPC167
PGPC057
Bacillus aryabhattai B8W22 EF114313
PGPC171
PGPC139
PGPC182
PGPC090
PGPC071
PGPC044
Bacillus thuringiensis ATCC 10792 |NR 114581|
PGPC043
PGPC183
Bacillus cereus ATCC14579|AF290547|
PGPC054
PGPC124
PGPC165
PGPC179
Brevibacillus aydinogluensis PDF25|HQ419073|
PGPC122
Brevibacterium frigoritolerans DSM 8801|NR117474|
PGPC098
Lysinibacillus fusiformis |AF169537|
PGPC105
Arthrobacter nicotinovorans
PGPRC017
PGPRC033
Pseudomonas putida GA5 |KT886073|
0.02


PGPRC185
Pseudomonas putida GA5 |KT886073|
PGPRC032
PGPRC027
PputidaATCC12633 |NR 114479|
PGPRC189
PpoaeDSM14936 |NR 028986|
Pseudomonas poae DSM 14936 |AJ492829|
PGPRC018
Enterobacter sp. Y5 |EU301807|
Klebsiella oxytoca ANA31 HQ219862|
PGPRC066
PGPRC192
Acinetobactercalcoaceticus DSM30006 |X81661|
Acinetobacter calcoaceticus DM10 |KT229743|
PGPRC056
PGPRC019
PGPRC087
PGPRC191
PGPRC153
PGPRC138
Acinetobacter pittii ATCC 19004 |NR 117621|
PGPRC095
PGPRC123
PGPRC170
Devosia sp. VBW339 |KC534392|
PGPRC178
Devosia riboflavina DSM 7230 |AJ549086|
PGPRC011
PGPRC157
Chryseobacterium daecheongense SNA41 |HQ220099|
Bacillus subtilis strain NZ2-4-2 |KR999922|
0.02

3.3.4 Efficiency of selected isolates for solubilizing TCP and ERP in liquid media

The solubilization efficiencies of nine selected isolates with SI of 2.71 to 3.44 were estimated in PKV broth using TCP and ERP as a sole P sources. Six of the nine isolates released their maximum level of P from TCP on the third day of sampling whereas the rest 3 reached their peak dissolution on day 4 sampling (Table 3.3). Except the three isolates (PGPRC44, PGPRC71 and PGPRC162) that were characterized by some decline from their maximal P level in the latter days of incubation, all the rest maintained their respective peak until the final day of sampling.

The 59.67 µg/ml dissolved P recorded on the 1st day of sampling by isolate PGPRC27 was progressively increased to a level of 312.67 µg/ml on the 4th day of sampling showing the most efficient and consistent performance in solubilizing TCP. This isolate released interestingly higher amount of P when compared to a highly effcient isolate (Moh305) from faba bean rhizosphere reported by Keneni Assefa *et al*. (2010) that released 260 µg/ml of P at its 5th day of incubation in a TCP broth. The lowest level of TCP solubilized in this study was recorded by PGPRC58 where the value of soluble P could not exceed 76.67 µg/ml all across its incubation period. By and large, the available P values in the TCP fortified liquid media of 4th day incubation stood in positive correlation ($P<0.05$, $r = 0.66$) with the SI.

Table 3.3 Efficiency of selected isolates for solubilizing TCP in liquid medium and the change in pH values

mIsolate	**Day 1**		**Day 2**		**Day 3**		**Day 4**		**Day 5**	
	P(µg/ml)	pH	P(µg/ml)	pH	P(µg/ml)	pH	P(µg/ml)	pH	P(µg/ml)	pH
PGPRC22	19±2c	6.51	33±2de	5.21	83.67±5ef	5.05	83.33±3de	5.09	83.33±4de	5.05
PGPRC27	59.67±3a	6.26	112±4a	4.40	273.33±9a	4.40	312.67±8a	4.40	312.33±8a	4.41
PGPRC44	36.33±3b	6.31	91.33±4c	4.23	166.67±2c	4.23	166.67±6b	4.42	151.67±8b	5.28
PGPRC58	22.33±2c	6.28	21.33±4e	5.41	76.67±6f	5.41	76.33±3e	5.48	76.67±6e	5.87
PGPRC71	21.33±3c	6.39	43.33±3d	4.58	126.67±5d	4.56	118.33±4c	4.57	111.67±4c	4.55
PGPRC123	14.67±3d	6.41	86.67±8c	5.06	78.67±4f	5.09	87.33±12de	5.07	87.3±7d	5.07
PGPRC139	12±1d	6.37	37.67±3d	4.48	96.67±9e	4.46	96.33±8d	4.48	96.33±8cd	5.41
PGPRC162	31±4b	6.38	102±5b	4.48	183.33±7b	4.44	156.67±7b	4.91	142±6bc	5.15
PGPRC189	14.67±4d	6.36	85±3c	4.35	139±5d	4.32	157.33±9b	4.30	157±10b	4.32
Control	1.61±0.04	7.10	1.93±0.05	7.10	2.42±0.03	7.10	3.05±0.06	7.10	3.53±0.04	7.10

Values in the same column not connected by same letter are significantly different at p 0.05. Values are means ± standard deviation (SD) for triplicates.

The pH value of the liquid growth media which was 7 at the beginning dropped to the range between 4.23 and 5.41 within the first two days of incubation by isolates PGPRC44 and PGPRC58, respectively. Five of the test bacteria namely, PGPRC22, PGPRC27, PGPRC71, PGPRC123, and PGPRC189 were able to maintain the once dropped pH values until the final day of incubation, whereas the rest four isolates brought about subsequent increase of pH values in their respective culture solutions. The data also showed an inverse correlation ($P<0.05$, $r= -0.59$) between the amount of P released and the concomitant pH values implying that the dissolution of the inorganic phosphate was prompted by the bacterially induced acidity in the culture broth.

With regard to solubilizing Egyptian rock phosphate in the liquid medium, the isolates did not release as much soluble P as they did from solubilization of TCP. However, statistically significant differences ($p < 0.05$) were observed among isolates in their P-solubilisation

efficiency on the various days of sampling. Except PGPRC189, all the test isolates progressively raised the level of solubilized P until the 6th day of sampling (Table 3.4). However, the measurement from the final day (day 7) sampling showed reduced level of solubilized phosphate in almost all the test isolates. On each of the sampling days, the maximum amount of dissolved P was recorded by PGPRC27, where the highest P concentration released from the rock phosphate being 117.22 µg/ml after a growth period of 6 days, followed by PGPRC189 (67 µg/ml) and PGPRC44 (64 µg/ml) after the same days of incubation. The P release by isolate PGPRC27 was remarkably higher than the rest of isolates in this experiment and it was 56% more efficient than PSBL05 that was isolated from lentil rhizosphere as reported by Mulissa Jida *et al*. (2015) for its highest ERP solublizing potential after five days of incubation.

The dissociation of the rock phosphate was also accompanied by a drop in pH value in the culture solution to acidic conditions from an initial value of 7.0 (Table 3.4). The most drastic fall in pH values was recorded at the 2nd day of measurement for isolates PGPRC27, PGPRC44, PGPRC71, and PGPRC123 and at the 3rd day of sampling for isolates PGPRC22, PGPRC58, PGPRC139, PGPRC162 and PGPRC189. All of the culture solutions maintained the dropped pH values until day 5, and most began to show an increase at day 6 and continued until 7th day of incubation. However, in broths with isolates PGPRC71 and PGPRC162, the declined pH values were sustained until the final day of sampling. After a growth period of 7 days, the highest (5.55) and lowest (4.57) pH values were recorded in the culture medium containing isolates PGPRC58 and PGPRC71, respectively.

Table 3.4 P-solubilization efficiency of selected isolates in liquid ERP medium and the change in pH values

Isolate	Day 1		Day 2		Day 3		Day 4		Day 5		Day 6		Day 7	
	P(µg/ml)	pH	P(µg/ml)	pH	P(µg/ml)	pH	P(µg/ml)	pH	P(µg/ml)	pH	P(µg/ml)	pH	P(µg/ml)	pH
PGPRC22	12.33±1cde	6.51	19.67±3d	5.88	21±2de	4.75	30.67±4d	4.72	26±3f	4.73	32.33±2e	5.23	29±5e	5.41
PGPRC27	27.67±2a	6.48	51.33±4a	4.35	72.67±2a	4.35	84.67±5a	4.35	98.14±6a	4.35	117.22±4a	4.85	91.14±8a	5.24
PGPRC44	22.67±1b	6.41	41.33±3b	4.38	45±2c	4.39	56.33±4b	4.37	61b±6c	4.38	64±2ab	4.77	56.33±3b	4.63
PGPRC58	12±1cde	6.53	20.67±1d	5.66	26.33±2d	5.23	30.33±5d	5.24	37.33±3e	5.23	42.33±5de	5.43	39.67±2de	5.55
PGPRC71	14±1cd	6.44	28±1c	4.55	54.67±3b	4.55	54.67±7b	4.55	52.33±4d	4.57	56±2bc	4.57	52±6bc	4.57
PGPRC123	11±2cde	6.39	20±3d	4.44	31.33±2d	4.42	28.33±d	4.44	32±3f	4.43	33±2e	5.05	31.67±2e	5.33
PGPRC139	9±1de	6.50	11.67±2e	6.02	16.33±1e	4.87	35±1cd	4.87	31.33±4f	4.89	38.33±3de	4.88	32.67±5e	4.88
PGPRC162	16.67±3c	6.16	31.33±6c	5.70	30±4d	4.48	45.33±2c	4.44	40.67±3e	4.46	48±5cd	4.74	44±2cd	4.92
PGPRC189	7±1e	6.41	15.33±1de	5.94	24.67±3de	5.14	40.33±5c	5.14	77±9b	5.11	67±7b	5.24	57±4b	5.41
Control	0.72±0.003	7.00	0.91±0.006	7.00	1.22±0.01	7.00	1.25±0.03	7.00	1.29±0.01	7.00	1.35±0.03	7.00	1.38±0.02	7.00

Values in the same column not connected by same letter are significantly different at p 0.05. Values are means ± standard deviation (SD) for triplicates

Some of the tested isolates showed erratic patterns in the level of dissociated P in the culture broths along the incubation periods (Table 3.3 and 3.4). This could be due to the difference in the rate of P released and uptaken by the bacterial isolates as incorporating the dissolved p into their own cellular biomass (immobilization) results in a decline of available P concentration in the medium (Rodríguez and Fraga, 1999). Phosphate regulation was also accounted for the fall of P level where it is induced by decreased production of organic acids once free phosphate was released into the medium (Krishnaraj *et al*., 2001).

The quantity of soluble phosphate was reduced towards the end of the incubation in most of the culture media and this could be a consequence of the available phosphate-precipitation by organic metabolites (Delvasto *et al*., 2006). The auto-consumption of soluble phosphate by the growing bacterial population (Rashid *et al*., 2004) could also be attributable to this P concentration drop. On the other hand, the bacterial isolates in the culture solution re-utilize their own organic acid secretions for metabolism (Tripura *et al.,* 2007) as the initial carbon sources get depleted (Rashid *et al*., 2004) in the latter growth periods and this could be responsible for the pH rise observed in most of the culture broths at the last hours of incubation in this study.

It was demonstrated that some of the phosphobacterial isolates obtained from the common bean rhizosphere are certainly capable of releasing dissolved phosphates when growing on poorly soluble sources such as TCP and RP. The property of these indigenous isolates to solubilize insoluble TCP and RP could be a good indicator that they are of paramount agronomic importance in releasing available P from the fixed forms of phosphate in the soil for plant use. It would also offer the opportunity to make use of the appetite deposits available in the country as

phosphorus fertilizer. Selecting effective isolates with superior phosphate solubilizing potential and applying them to the soil together with rock phosphate could ameliorate the soil fertility and hence augment crop yield. These could serve as viable alternative to mollify the growing pressure caused by the inflating price of synthetic fertilizers.

3.3.5 Additional PGPR properties

3.3.5.1 IAA production

The data showed that of the 54 phosphate solubilizing isolates, 74% acquired one or more additional PGP properties (Table 3.5) where 33 (61.1%) were able to produce the hormone IAA in liquid culture at concentrations ranging between 9.47 µg/ml (PGPRC179) and 53.23 µg/ml (PGPRC22) in the presence of 0.1% tryptophan indicating that the most potent isolate was five times more effective in IAA production than the least producing isolate. This is comparable to the report where 73.3% of rhizobacteria isolates from sorghum (Ahmed Idris *et al.,* 2009) and 48% of chickpea rhizobacteria (Mulissa Jida, 2013) were able to produce IAA.

3.3.5.2 Antifungal activity on dual culture assay

The antagonism study using the dual culture assay showed that 22% of the rhizobacterial isolates inhibited the mycelial growth of the fungal pathogen, *Fusarium oxysporum f.sp. phaseoli.* Accordingly, isolate PGPRC131 inhibited the pathogen by 51.3% followed by PGPRC58 with radial inhibition of 47.43% (Table 3.5). As corroborated by relevant tests in this study, the tendency of most of the isolates for antagonism was probably attributable to their ability to produce one or more of the lytic enzymes such as chitinase, cellulase, and protease (Table 3.5). In fact the most antagonistic isolate (PGPRC58) against the pathogen did not show any other PGPR property including cellwall degrading enzyme activities that may indicate that its

inhibitory activity may be attributable to other antagonistic features. Diriba Muleta *et al*. (2007) and Abdel-Salam *et al*. (2007) reported similar observations for *Pseudomonas* and *Bacillus* that suppressed *Fusarium* growth without. Another sudy by Peix *et al*. (2001) also indicated that a rhizobacterial strain of *Burkholderia cepacia* from common bean rhizosphere was able to antagonize *Fusarium oxysporum f.sp. phaseoli* pathogen by 10% inhibition of radial growth under *in vitro* conditions.

Table 3.5 Phosphobacterial isolates with additional PGP properties

	Sampling Region	*IAA production µgml⁻¹*	*F. oxysporum Inhibition*	*protease production*	*Cellulase production*	*Chitinase production*	*Cyanide production*
PGPRC6	*WE*	37.47±0.40e-g	–	–	–	–	–
PGPRC11	*WE*	–	19.6±0.88i-h	–	–	–	–
PGPRC18	*WE*	14.47±2.65s-t	–	–	–	–	–
PGPRC19	*WE*	–	41.53±0.49c-d	+	–	+	–
PGPRC22	*WE*	53.23±0.85a	–	–	–	–	–
PGPRC27	*WE*	34.4±0.85g-i	–	–	–	–	–
PGPRC32	*WE*	42.17±0.47d-e	–	–	–	–	–
PGPRC33	*WE*	41.43±1.96d-e	–	–	–	–	–
PGPRC38	*WE*	–	36.5±1.71e-f	+	+	–	+
PGPRC43	*WE*	37.37±0.55e-g	–	–	–	–	–
PGPRC44	*WE*	27.27±0.86k-m	–	–	–	–	–
PGPRC179	*WE*	9.47±1.65u	–	–	–	–	–
PGPRC182	*WE*	46.73±0.60a-c	35.2±0.36e-f	–	+	–	+
PGPRC57	*CE*	36.2±0.46f-h	24.33±3.30h	+	+	–	–
PGPRC58	*CE*	–	47.43±1.65a-b	–	–	–	–
PGPRC66	*CE*	30.2±0.40i-l	–	–	–	–	–
PGPRC171	*CE*	–	23.2±1.1h	–	+	+	–
PGPRC178	*CE*	22.47±0.35m-p	–	–	–	–	–
PGPRC106	*CE*	18.7±0.62p-s	–	–	–	–	–
PGPRC117	*CE*	35.5±0.44f-h	–	–	–	–	–
PGPRC94	*EE*	19.43±0.35o-r	–	–	–	–	–
PGPRC98	*EE*	43.33±4.8c-d	–	–	–	–	–
PGPRC105	*EE*	16.467±0.40q-t	32.5±0.40f-g	+	+	–	–
PGPRC108	*EE*	–	43.43±0.90c	+	+	–	–
PGPRC122	*EE*	21.4±0.95n-p	–	–	–	–	–
PGPRC123	*EE*	46.13±0.76a-c	–	–	–	–	–
PGPRC124	*EE*	33.5±0.92g-j	–	–	–	–	–
PGPRC131	*EE*	23.3±1.85m-p	51.3±0.26a	–	+	+	–
PGPRC139	*EE*	13.3±2.95t-u	–	–	–	–	–
PGPRC146	*EE*	34.67±1.00	–	–	–	–	–
PGPRC153	*EE*	31.53±0.40h-k	–	–	–	–	–
PGPRC186	*EE*	–	29.81±2.14g	+	–	+	–
PGPRC189	*EE*	29.13±0.85j-l	–	–	–	–	–
PGPRC56	*SE*	49.4±0.82a	–	–	–	–	–
PGPRC162	*SE*	25.63±0.91l-n	38.63±0.55d-e	–	+	–	+
PGPRC167	*SE*	48.7±0.82a-b	–	–	–	–	–
PGPRC183	*SE*	36.3±0.56f-h	–	–	–	–	–
PGPRC185	*SE*	15.43±0.25r-t	–	–	–	–	–
PGPRC191	*SE*	24.27±0.55m-o	–	–	–	–	–
PGPRC196	*SE*	20.87±0.31n-q	–	–	–	–	–

Values in the same column not connected by same letter are significantly different at p 0.05. Values are means ± standard deviation (SD) for triplicates. + :- growth present, - :- growth absent

3.3.5.2.1 Testing some traits used as mechanisms for antagonism

3.3.5.2.1.1 Production of lytic enzymes

Out of the 12 rhizobacterial isolates that were able to suppress the *in vitro* growth of *Fusarium* pathogen, all but PGPRC11 and PGPRC58 were able to show one or more of the possible inhibitory traits for antagonism (Appendix VII). Fifty percent of those with antagonistic properties: PGPRC19 (*Acinetobacter calcoaceticus)*, PGPRC38 (*Bacillus subtilis)*, PGPRC57 (*Bacillus safensis*), PGPRC105 (*Lysinibacillus* sp.), PGPRC108 (*Bacillus* sp.*)*, and PGPRC186 (*Bacillus subtilis*) were tested positive for the enzyme protease turning the opaquely whitish skim milk agar plate to clear and translucent halo around the colony up on bacterial protease activity. Diriba Muleta *et al*. (2007) also reported that the antagonistic effect of 10 rhizobacterial species of *Bacillus* recovered from the rhizosphere of *Coffea Arabica* was attributable to protease enzyme activity. Similarly, out of the six species that suppressed *Fusarium* in this study, the four with protease activity were *Bacillus*.

Among the bacterial isolates tested for hydrolysing chitin, four of them PGPRC19 (*Acinetobacter calcoaceticus)*, PGPRC131 (*Bacillus subtilis)*, PGPRC171 *(Bacillus aryabhattai)*, and PGPRC186 (*Bacillus subtilis*) (33.33%) had chitinolytic activity. Similarly, Muleta *et al.* (2007), reported that a rhizobacterial species of *Bacillus* from coffee rhizosphere produced chitinase enzyme that suppressed the *Fusarium* pathogen *in vitro*. An earlier study by Chet (1987) also substantiated that the role of certain bacterial isolates (*Bacillus* sp.) as biocontrol agents was due to production of chitinolytic enzyme which breaks down the fungal cell wall.

With regard to cellulase activity, 8 of antagonistically active isolates: PGPRC38 *(Bacillus subtilis)*, PGPRC57 *(Bacillus safensis)*, PGPRC105 *(Lysinibacillus* sp.), PGPRC108 *(Bacillus* sp.) PGPRC131 *(Bacillus subtilis*), PGPRC162 *(Bacillus methylotrophicus)*, PGPRC171 (*Bacillus aryabhattai)*, and PGPRC182 (*Bacillus thuringiensis*) were capable of hydrolyzing cellulose. Similarly, Diriba Muleta *et al.* (2007) reported that 7 rhizobacterial isolates (all *Bacillus* sp.) from coffee rhizosphere showed -1-3-glucanase activity.

3.3.5.2.1.2 Production of hydrogen cyanide (HCN)

Isolates PGPRC38 *(Bacillus subtilis)*, PGPRC162 *(Bacillus methylotrophicus)* and PGPRC182 (*Bacillus thuringiensis)* were able to synthesize HCN that turned the yellow coloured filter stripes to brownish colour (Appendix VII). Though the proportion is higher, Muleta *et al*. (2007) also reported that 50.7% of the antagonistically active rhizobacterial isolates against a fungal pathogen from coffee were cyanogenic. Killani *et al*. (2011) also demonstrated that the mycelial growth of soil borne fungal pathogens was supressed by *Bacillus* spp. under *in vitro* condition, and the suppresive activity of the *Baccillus* spp. was associated with biosynthesis of the poisonous HCN as a metabolite (Shobha and Kumudini, 2012).

Generally, out of the 54 phosphate solublizing bacteria, 61% were capable of IAA production, where as 22% were antagonistic to *Fusarium oxysporum (*Appendix VII*)*. However, only few isolates were capable of: chitinase activity (7.4%), protease activity (11%), cellulase activity (15%) and cyanide activity (6%). Ten P-solubilizing isolates showed multiple characteristics, where, the the isolates PGPRC38, PGPRC57, PGPRC105, PGPRC131, PGPRC182, and PGPRC162 displayed 2/3rd of the six PGP traits tested, whereas isolates PGPRC19, PGPRC108, PGPRC171, and PGPRC186 acquired 3 of the PGP features (Table 3.5).

3.3.6 Effect on growth of common bean under greenhouse condition

Effect of co-inoculation with selected phosphate solubilising rhizobacterial isolates PGPRC27 (*Pseudomonas putida)*, PGPRC44 (*Bacillus thuringiensis)*, PGPRC71 (*Bacillus thuringiensis)* and PGPRC189 (*Pseudomonas poae*) on growth and nitrogen fixation is shown in Table 3.6. Accordingly, the inoculated plants developed 31- 49 nodules plant^{-1} and nodule dry weight of 64-85 mg plant^{-1}. Compared to the control, the nodule count per plant showed a significant increase by 103%, 78%, 45% and 26% following application of the phosphobacterial isolates PGPRC27, PGPRC189, PGPRC44, and PGPRC71, respectively. Similar to this study, Samavat *et al.* (2012) also reported that inoculation with P solubilising *Pseudomonas* species increased the number of nodules in common bean twice as much as that of the negative control.

The PSR inoculated plants in this study significantly ($P<0.05$) increased shoot dry weights which is 40-98% over the control. Isolate PGPRC27 induced the highest shoot dry matter yield (1.62 g plant^{-1}) as compared to other PSR inoculated plants including the P-fertilized (1.48 g plant^{-1})(Table 3.6). A similar study to evaluate the effect of phosphate solubilizing *Pseudomonas* isolates on symbiotic nitrogen fixation in common bean by Samavat *et al.* (2012) showed 33-140% shoot dry matter increase over the negative control.

Table 3.6 Effect of inoculation with selected isolates on growth of common bean

Treatment	***Nodule No. per plant***	***Nodule dry weight per plant (mg)***	***Shoot dry weight per plant(g)***	***Total nitrogen (%)***	***Shoot P content (mg/g/sdw)***
PGPRC27	49.33±2.13a	85±5a	1.62±0.08a	1.68±0.06a	2.19±0.09a
PGPRC44	35.33±4.16abc	66±6bc	1.17±0.03b	1.34±0.05cd	1.44±0.04bc
PGPRC71	30.66±5.23cb	64±4bc	1.21±0.04b	1.27±0.04cd	1.66±0.06b
PGPRC189	43.33±3.12ab	72±3abc	1.50±0.02a	1.48±0.03bc	2.01±0.12a
P+	42.33±4.02ab	82±11ab	1.48±0.06a	1.72±0.07ab	2.11±0.11a
P-	24.33±2.08c	60±4c	0.82±0.05c	1.02±0.01d	1.21±0.07c

Values in the same column not connected by same letter are significantly different at p 0.05. Values are means ± standard deviation (SD) for triplicates.

The inoculated plants also increased in the total shoot N ranging between 25% (PGPRC71) and 65% (PGPRC27) compared to the uninoculated control (Table 3.6). The increases in shoot nitrogen showed strong positive correlations with the increase in both nodule number ($P<0.05$, $r=0.93$) and shoot dry weight ($P<0.05$, $r=0.94$). Similarly, correlations of the comparable magnitude were noticed among the growth indices of shoot nitrogen, nodule number and shoot dry weight of common bean that had been inoculated with phosphobacterial strains of *Bulkholderia cepacia* (Peix *et al*., 2001b). The applied phosphobacterial isolates probably increased the level of available phosphorus in the rhizosphere (Sundara *et al.,* 2002; Vyas and Gulati, 2009) and improved the symbiotic interaction between the *Rhizobium* and the host plant and hence the nitrogen fixation as P deficiency in soils hampers this interaction (Gyaneshwar *et al*., 2002). Therefore, nodulation and nitrogen-fixation in common bean could be augmented by inoculating the plant with bacterial isolates having better phosphate solubilizing capabilities.

The shoot P increase due to phosphobacterial treatments was as high as 81% (PGPRC27) and as low as 19% (PGPRC44) (Table 3.6). The P content of isolate PGPRC27 (2.19 mg/g) and PGPRC189 (2.01 mg/g) were statistically equivalent to the p-fertilized control (2.11 mg/p). Previous reports also demonstrated that plants inoculated with phosphobacterial isolates improved P uptake (Peix *et al*., 2001b; Vikram and Hamzehzarghani, 2008; Castango *et al*., 2011) and this could be attributable to the increased available P content of the soil from solubilization of insoluble P following inoculation (Vyas and Gulati, 2009). Generally, this greenhouse trial showed that the use of selected p solubililizing bacterial isolates significantly improved shoot dry matter, nodule number and dry weight, shoot N and P content which is in line with the fact that common bean has a higher internal P requirement for activities like nodule formation, optimum N accumulation and maintenance of good symbiotic interaction with rhizobia (Zaman-Allah *et al.,* 2007).

Chapter 4 Effect of co-inoculation with *Rhizobium* and phosphobacteria on growth and production of common bean (*phaseolus vulgaris* L.) under field condition

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Abstract

The co-inoculation effects of selected *Rhizobium* and phosphate solubilizing rhizobacteria (PSR) were determined both under greenhouse and field trials. Two *Rhizobium* isolates CBR052 (*Rhizobium etli*) and CBR141 (*Rhizobium* sp.) and two PSR isolates PGPRC27 *(Pseudomonas putida*) and PGPRC189 (*Pseudomonas poae*) were used on common bean varieties of Awash-1, Ibadoo and Nasir.The resident rhizobial population on the field was determined using most probable method. Data were recorded at 2 growth stages, 45 days after emergence and during harvesting (92 days after planting). Population density of the resident rhizobia at the experimental sites was 2.6×10^1 per gram of soil at Melkassa and 4.2×10^1 per gram of soil at Alem Tena before sowing. The coinoculation CBR052 + PGPRC27 in greenhouse increased nodule number, nodule dry weight, shoot dry weight and shoot nitrogen as much as 50%, 97%, 48% and 42%, respectively over the single inoculation of CBR052. Co-inoculation of *Rhizobium* species and PGPRC27 significantly ($P<0.05$) increased nodulation, soot dry weight, shoot nitrogen and grain yield of all the three varieties compared to the single *Rhizobium* inoculations and the uninoculated plants. The CBR052 + PGPR27 co-inoculation increased yield by 87.6% (2694.32 kg ha^{-1}) and 95.6% (2638.69 kg ha^{-1}) over the uninoculated control on Ibaddo variety at Melkassa and Alem Tena sites, respectively. The yield of variety Nasir was improved by 72.7% (2431.41kg ha^{-1}) at Melkasa and 76.9% (2370.69 kg ha^{-1}) at Alem Tena whereas Awash-1 variety increased by 40.5% (1988.87 kg ha^{-1}) at Melkassa and 35.6% (1882.56 kg ha^{-1}) at Alem Tena over the un-inoculated controls. The increase in grain yield was as much as 95%, 77% and 41% in Ibadoo, Nasir and Awash, respectively following co-inoculation of CBR052+PGPRC27. This indicates that co-inoculation of the proper plant varieties with rhizobia and PGPR could be important with respect to enhancing plant growth and grain yield of common bean in the low input sustainable farming system.

Keywords: Co-inoculation, PGPR, *Phaseolus valgaris L.*, *Rhizobium*

4.1 Introduction

Common bean (*P. vulgaris* L.), domestication of which occurred several thousand years ago in Mesoamerica and in the Andean region (Rodino *et al*., 2010), represents the most important grain legume in human diets, providing proteins and carbohydrates for more than 300 million people, especially in Latin America, the Caribbean and Africa (CGIAR, 2012).

Common bean is able to grow by assimilating mineral nitrogen or carry out biological nitrogen fixation through symbiosis with root nodule bacteria collectively called rhizobia. However, inferior nodulation and lack of responses to inoculation in field experiments have been frequently reported world-wide, raising uncertainties regarding the efficiency of bean inoculation (Buttery *et al.* 1987; and Hardarson 1993). The failure in some trials was mainly attributable to the intrinsic characteristics of the host plant to undergo promiscuous nodulation (Michiels *et al.*, 1998) and its association with highly competitive but poorly effective indigenous population of rhizobia in soil (Giller and Cadisch, 1995; Hungria and Vargas, 2000). Nevertheless, increase in nodulation, nitrogen fixation and grain yield was observed in common bean following single inoculation with selected efficient strains of rhizobia (Hungria *et al*., 2000, 2003; Asadi Rahmani *et al*., 2005; Mnasri *et al*., 2007; Asadi Rahmani *et al*., 2011) as well as co-inoculation with *Rhizobium* species and PGPR (Yadagari *et al.,* 2010; Stajkovi *et al*., 2011; Samavat *et al*., 2012, Sanchez *et al.,* 2014).

Plant growth-promoting rhizobacteria have been shown to increase the performance of rhizobia in nodulation and nitrogen fixation of common bean leading to increased plant growth. Greenhouse and field studies with PGPR and rhizobia strains have substantiated enhanced nodulation and nitrogen fixation in common bean (Figueiredo *et al.,* 2008). These synergistic

effects of *Rhizobium* - PGPR co-inoculations were also shown to increase the yield and yield components of common bean under field conditions in different parts of the world (Yadegari and Rahmani 2010; Sánchez *et al*., 2014).

Although the effects of native PGPR isolates on nodulation and nitrogen fixation of chickpea and lentl crops were assessed under greenhouse condition in Ethiopia (Mulisa jida, 2013), only few attempts were made to investigate whether such phytobeneficial soil bacteria are able to promote nitrogen fixation, plant growth and yield of common bean under natural field conditions. The objectives of this study, therefore, were to (a) assess the symbiotic effects of rhizobia on three popular bean varietis and (b) evaluate the synergistic effect of *Rhizobium* and phosphate solubilizing rhizobacteria on common bean under green house and field trials.

4.2 Materials and Methods

4.2.1 Plant material and bacterial sources

Three popular local varieties of common bean namely, Awash-1, Nasir, and Ibadoo were used throughout this experiment. The first two were kindly provided by Melkassa Agricultural Research Center, whereas the latter by Hawassa Agricultural Research Center, both centers are part of the Ethiopian Institute of Agricultural Research (EIAR).

Two *Rhizobium* isolates CBR052 (*Rhizobium etli)* and CBR141 (*Rhizobium* sp.) with high symbiotic nitrogen fixing potential and two phosphobacterial isolates PGPRC27 (*Pseudomonas putida*) and PGPRC189 (*Pseudomonas poae*) with good phosphate solubilizing abilities were obtained from the previous two studies.

4.2.3 Greenhouse experiment

The experiment was conducted in greenhouse following Figueiredo *et al.,* (2008). Pots were filled with composite top (0-20 cm) soil samples from Awash Melkassa. The physicochemical properties of the soil were pH (7.62), %TN (0.064), available P (mg/kg 5.63), TP (mg/kg 1137), Available K (151.12 mg/kg), CEC (48 cmol/kg), %Clay (20), %Silt (47.51), and %Sand (32.48). Microbe-free soil was obtained by autoclaving it for 30 min at 121^0C and 101 KPa, once a day for three consecutive days.

Seeds of the three common bean varieties (Awash-1, Nasir, and Ibadoo) were surface sterilized as before and treated with single inoculations and co-inoculations as follows.

T1 = Seed + CBR052 (*Rhizobium etli*)
T2 = Seed + CBR141 (*Rhizobium sp.*)
T3 = Seed + CBR052 (*Rhizobium etli*) + PGPRC27 (*Pseudomonas putida*)
T4 = Seed + CBR141 (*Rhizobium sp.*) + PGPRC27 (*Pseudomonas putida*)
T5 = Seed + CBR052 (*Rhizobium etli*) + PGPRC189 (*Pseudomonas poae*)
T6 = Seed + CBR141 (*Rhizobium sp.*) + PGPRC189 (*Pseudomonas poae*)
T7 = Seed + non inoculated + N and P fertilized (positive control)
T8 = Seed + non inoculated + non fertilized (negative control).

For single inoculation, 1ml of broth culture containing a *Rhizobium* (10^9cfu ml^{-1}) plus 1 ml of sterile nutrient broth were inoculated per plant. For the coinoculation, 1 ml of YEM broth containing a *Rhizobium* (10^9cfu ml^{-1}) plus 1 ml of nutrient broth containing the phosophobacteria (10^8cfu ml^{-1}) were applied per plant. The experiment was arranged in a completely randomized block design (CRBD) with three replications in the greenhouse. The pots were irrigated twice a week with tap water under temperature of $19±2^0$ mean minimum and $29±1^0$ mean maximum.

Plants were harvested 45 days after emergence (DAE) to record nodule number per plant (NN), nodule dry weight (NDW) and shoot dry weight (SDW) (after drying at 65^0C for 72 hours), and total shoot nitrogen (SN) following Kjeldahl digestion method (Appendix II).

4.2.4 Field study

4.2.4.1 Land preparation and inoculant formulation

The experiment was conducted on two field stations at Awash Melkassa (8°24'53"N 39°19'40"E) under Melkassa Agricultural Research Center and Alem Tena (8°18 N 38°57 E) under Debre Ziet Agricultural Research Center. The physical and chemical properties of the soils were, Melkassa: pH (7.62), %TN (0.064), available P (mg/kg 5.63), TP (mg/kg 1137), available K (151.12 mg/kg), CEC (48 cmol/kg), %Clay (20), %Silt (47.51), and %Sand (32.48); Alem Tena: pH (6.84), %TN (0.057), available P (mg/kg 7.03), TP (mg/kg 1082), available K (146.04mg/kg), CEC (55.52 cmol/kg), %Clay (33.71), %Silt (22.54), and %Sand (43.75). The experiment was carried out during the long rains of the year (July to October 2014).

The fields were ploughed, harrowed and ridged at a spacing of 0.3 m apart. Bean seeds of the three varieties were surface-sterilized and prepared as before, moistened with a 20% (w/v) solution of sucrose and mixed with the appropriate inoculants in a shade for immediate sowing (Yadagari *et al*., 2010). Accordingly, the *Rhizobium* isolates were grown to 10^9cfu ml^{-1} in yeast extract mannitol (YEM) broth on a rotary shaker for 72 hours at 28^0C. Likewise, the phosphobacterial isolates were grown in nutrient broth at 30^0C for 48 hours to get a population density of about 10^8 cfu ml^{-1}. Then 150ml of each bacterial culture was added to a polypropylene plastic bag containing 50 g of sterile powdered lignite and mixed thoroughly. The bean seeds

(1kg) were mixed with 10 g of each of lignite-based inoculants of *Rhizobium* isolates and the co-inoculated seeds received a 10 g additional lignite-based inoculant of phosphobacterium.

4.2.4.2 Estimation of indigenous rhizobial population

The presence and number of indigenous rhizobia nodulating common bean at the experimental sites was determined by plant infection test using the most probable number (MPN) technique (Vincent, 1970) with bean variety Nassir and statistical Tables of Somasegaran and Hoben (1994). Uniform and clean seeds of good viability were surface sterilized with alcohol and hydrogen peroxide as described by Somasegaran and Hoben (1994). The seeds were pre-germinated as before and transferred to plastic growth pouches arranged in four replications in a wooden rack and kept at the greenhouse awaiting inoculation. Fivefold dilution of each soil sample was made and one milliliter of the diluents was used to inoculate the seedling. The plants were fortified with sufficient Broughton and Dilworth N-free plant nutrient solution when required. Nodulation was assessed after twenty eight days. Positive and negative nodulation of growth units were recorded for all dilutions and the number of rhizobia was estimated in reference to MPN Table.

4.2.4.3 Experimental design and treatments

The field experiment was laid out in a randomized complete block design (RCBD) with three replications. Each plot was measured 3 m x 2.4 m with a 1 m alley between the plots and blocks to minimize contamination by run-off containing bacteria or fertilizer, caused by heavy rains. Each block was structured to incorporate 18 plots on which all the 6 treatments combined with the three common bean varieties were distributed randomly. Two seeds per spot were hand sown

at an inter and intra row spacing of 30 cm and 15 cm, respectively and later thinned down to one seedling per hill just two weeks after emergence.

Each plot included two controls, non-inoculated negative control (NI) and non-inoculated controls that received N and P fertilizer (NI+NP) for each of the three bean varieties tested on both sites. The fertilized control plants received urea (46% N) 30 kg of N ha^{-1} at sowing and 30 kg of N ha^{-1} at 25 days after emergence. Five days before sowing, the fertilized plots also received 84 kg ha^{-1}Phosphate fertilizer. The six treatments included in this study were:

T1 = Seed + CBR052 (*Rhizobium etli*)

T2 = Seed + CBR141 (*Rhizobium* sp.)

T3 = Seed + CBR052 (*Rhizobium etli*) + PGPRC27 (*Pseudomonas putida*)

T4 = Seed + CBR141 (*Rhizobium sp.*) + PGPRC27 (*Pseudomonas putida*)

T5 = Seed + Non inoculated + N and P fertilized (positive control)

T6 = Seed + Non inoculated + non fertilized (negative control)

4.2.4.4 Plant sampling and data gathering

Plant samples were taken at two growth stages. Forty five days after emergence, six plants were randomly collected per plot to evaluate nodulation (nodule number and dry weight per plant), shoot dry weight and total shoot nitrogen content as stated before.

Following physiological maturity, harvesting was conducted when plants and seeds were dry, 92 days after planting. All the plants were collected and their number per plot was counted. Six plant samples per plot were randomly taken and used for the determination of number of pods per plant, number of seeds per pod and weight of 100 seeds. Grains from the harvested area of

each plot were cleaned and weighed at 11% moisture content to estimate the grain yield of a plot per hectare. Accordingly, seed weight of a plot divided by the number of plants harvested from that plot was multiplied by the number of plants calculated per hectare.

4.2.5 Data analysis

Data collected from each experiment was subjected to tests of normality and homogeneity of variances for all the variables and to analysis of variance (ANOVA), taking P = 0.05 as a level of significance. Tukey HSD post hoc significance test was employed to compare means of the treatments using SPSS 20.0 for Windows (Lead Technology, Inc).

4.3 Results and Discussion

4.3.1 Inoculation and co-inoculation of common bean in greenhouse

The treatment of the three common bean varieties with the *Rhizobium* species alone and co-inoculation with the phosphobacteria isolates is shown in Table 4.1. Accordingly, the inoculated plants induced nodules (35.3-92.6 plant^{-1}) and nodule dry weight (0.027- 0.172 g) showing differences as much as twofold and six fold among nodule counts and nodule dry weights respectively. The data also showed the different inoculation treatments increased the shoot dry matter ranging from 1.29 - 2.83 g plant^{-1}compared to the uninoculated control (1.23-1.58 g). This indicates that the most effective treatment was able to bring a two fold increase in shoot dry matter when the least effective ones were as good as the uninoculated and non fertilized control plants. The data also showed that inoculation with *Rhizobium* isolates alone did not show significant ($P < 0.05$) difference in shoot dry weight on Awash-1 variety compared to the co-inoculated plants (Table 4.1).

Table 4.1 Nodule number (NN), Nodule dry weight (NDW), shoot dry weight (SDW), and Shoot nitrogen (SN) after inoculation and co-inoculation on common bean varieties in greenhouse

Treatments	*Nodule number*	*Nodule dry weight (g)*	*Shoot dry weight(g)*	*Shoot Nitrogen (%)*
Awash 1				
CBR052	44.33±2.52c-d	0.032±0.004b-c	1.39±0.038c-d	1.35±0.02c-d
CBR141	35.33±4.024d	0.027±0.019c	1.29±0.044d-e	1.32±0.04c-d
CBR052+PGPRC27	66.33±8.5a	0.063±0.0075a	1.62±0.042b	1.68±0.03b
CBR052+PGPRC189	57.00±4.16a-b	0.045±0.013b	1.43±0.05c	1.41±0.046c
CBR141+PGPRC27	46.33±5.13b-c	0.031±0.0095b	1.38±0.009c-d	1.42±0.036c
CBR141+PGPRC189	42.00±5.51c-d	0.033±0.005b-c	1.36±0.018c-d	1.38±0.095c
Fertilized	0.00	0.00	2.52±0.07a	2.44±0.035a
Non fertilized	0.00	0.00	1.23±0.012e	1.21±0.031d
Ibadoo				
CBR052	76.00±2.08b-c	0.092±0.025b-c	1.91±0.022c-d	1.92±0.11c
CBR141	67.00±6.03c	0.081±0.023c	1.73±0.033d-e	1.77±0.18c
CBR052+PGPRC27	92.67±6.81a	0.172±0.03a	2.83±0.014a	2.72±0.074a
CBR052+PGPRC189	83.33±7.21a-b	0.134±0.022a-b	2.33±0.015b	2.23±0.08b
CBR141+PGPRC27	68.67±3.03c	0.122±0.017b-c	2.11±0.11b-c	2.28±0.10b
CBR141+PGPRC189	71.33±4.04b-c	0.121±0.019b-c	1.88±0.2c-d	2.3±0.10b
Fertilized	0.00	0.00	2.93±0.023a	2.83±0.046a
Non fertilized	0.00	0.00	1.48±0.027e	1.34±0.62d
Nasir				
CBR052	61.67±5.03b-c	0.083±0.020b-c	1.595±0.020c-e	1.64±0.052d
CBR141	52.67±9.02c	0.063±0.015c	1.487±0.031d-e	1.65±0.021d
CBR052+PGPRC27	78.33±2.52a	0.131±0.014a	2.065±0.218b	1.97±0.046b
CBR052+PGPRC189	69.0±4.36a-b	0.104±0.003a-b	1.818±0.020c	1.78±0.032c
CBR141+PGPRC27	53.67±4.16c	0.111±0.020a-b	1.626±0.005c-d	1.69±0.040c-d
CBR141+PGPRC189	55.67±2.52b-c	0.103±0.019a-c	1.584±0.031d-e	1.67±0.010c-d
Fertilized	0.00	0.00	2.722±0.045a	2.56±0.041a
Non fertilized	0.00	0.00	1.378±0.023e	1.46±0.076e

Values in the same column not connected by same letter are significantly different at p 0.05. Values are means ± standard deviation (SD) for triplicates.

Though statistically not significant (P<0.05), plants inoculated with CBR052 (*Rhizobium etli*) showed higher nodule number, nodule dry weight, shoot dry weight and shoot nitrogen per plant than plants inoculated with CBR141 (*Rhizobium sp.*). Accordingly, co-inoculation treatments with CBR052 + PGPRC27 showed relatively the highest values in all the measured variables compared to other bacterial treatments on all varieties. In terms of shoot dry weight, the response of Ibadoo variety to this treatment (2.83 g plant^{-1}) was as much as the fertilized control plants (2.93 g plant^{-1}), while exceeding the unfertilized control by 91%. It is also interesting to note that

the same *Rhizobium* species when co-inoculated with PGPRC189 induced a shoot dry matter accumulation which exceeded the unfertilized control by 57% on the same variety showing a shortfall of 34% from CBR052 + PGPRC27. On the other hand, the Nasir and Awash-1 varieties accumulated 50% and 32% more shoot dry weight respectively over the unfertilized control in response to CBR052 + PGPRC27 co-inoculation (Table 4.1).

The data showed that variety Ibadoo performed the best in almost all evaluated parameters followed by Nasir and Awash-1 in all treatments, indicating that common bean varieties respond differently to bacterial inoculations. Rodiño *et al.* (2011) inoculated 158 common bean varieties with *R. tropici* (CIAT 899) in greenhouse and reported significant variation in nodule number (15 - 600 plant^{-1}) and nodule dry weight (0.05-5.97 mg plant^{-1}) indicating that genetic variability affects nodulation of common bean.

In general, dual inoculation of the common bean varieties with selected rhizobia and phosphobacteria significantly improved nodule numbers, nodule dry weight, shoot dry weight and shoot nitrogen compared to single inoculations with *Rhizobium*. Like wise, effect of co-inoculation with *Rhizobium* and PGPR in enhancing the symbiotic properties of legumes was reported in previous studies (Figueiredo *et al*., 2008). The same author reported that co-inoculation of CIAT899+DMS36 (*Paenibacillus polymyxa*) in common bean increased the nodule number by 87% over the single inoculation of CIAT899 (*R. tropici*).

4.3.2 Symbiotic effectiveness of common bean on field

4.3.2.1 Effect of *Rhizobium*-Phosphobacteria co-inoculation

The response of three common bean varieties to inoculation and co-inoculation of the effective inoculants was evaluated under two different field conditions (Tables 4.2, 4.3, 4.4 and 4.5). The rhizobial population in the soil was estimated to be $2.6x10^1$ and $4.2x10^1$ per gram of soil in the fields of Melkassa and Alem Tena, respectively indicating low population of resident rhizobia. The data showed that treatments did not show variation in measured parameters on Awash-1. However, the responses were variable on Ibadoo and Nasir varieties, with CBR052 (*R. etli*) + PGPRC27 (*P. putida*) combination by showing significantly increased values in the number and dry weight of nodule, shoot dry weight, and shoot nitrogen in all the three varieties (Table 4.2, 4.3). Accordingly, in plants co-inoculated with CBR052 + PGPRC27, the nodule number and nodule dry weight were increased by 49% and 89%, respectively over plants inoculated with CBR052 alone on Ibadoo variety at Melkassa. Similarly, Sánchez *et al*., (2014) reported that co-inoculation of common bean variety DOR-364 with *R. pisi (R40982)* + *P. monteilii* (R43453) increased nodule number and nodule dry weight by 55% and 133%, respectively over single inoculation with *R. pisi (R40982)*.

Table 4.2 Nodule number, Nodule dry weight, shoot dry weight, and Shoot nitrogen by common bean varieties in the field experiment at the Melkassa site

Treatments	Nodule number	Nodule dry weight (g)	shoot dry weight (g)	Total shoot N (%)
Awash 1				
CBR052	42.83±6.03a	0.026±0.014a	1.93±0.16b-c	1.53±0.032c-d
CBR141	42.12±2.53a	0.0193±0.003a-b	1.82±0.12c	1.45±0.041c-d
CBR052+PGPRC27	46.07±4.93a	0.027±0.014a	2.24±0.17b	1.71±0.066b
CBR141+PGPRC27	44.53±3.52a	0.028±0.011a	1.88±0.11c	1.54±0.036c
N+P+	11.40±3.0b	0.005±0.001b	2.72±0.069a	2.47±0.081a
N-P-	14.12±4.23b	0.008±0.002b	1.79±0.058c	1.38±0.119c-d
Ibadoo				
CBR052	56.54±5.13b-c	0.091±0.008c	2.53±0.066c	2.03±0.066b
CBR141	50.63±7.09c	0.080±0.013c	2.25±0.079d	1.79±0.090c
CBR052+PGPRC27	84.22±6.51a	0.172±0.007a	3.81±0.08a	2.67±0.060a
CBR141+PGPRC27	71.71±6.03b	0.113±0.009b	2.72±0.103b	2.19±0.091b
N+P+	13.00±1.73d	0.008±0.0006e	3.92±0.028a	2.61±0.072a
N-P-	15.43±3.06d	0.013±0.0015d	1.83±0.178e	1.37±0.095d
Nasir				
CBR052	49.53±6.51b-c	0.076±0.0062b	2.11±0.062c	1.81±0.071c
CBR141	43.61±4.51c	0.069±0.0095b	1.91±0.156d	1.69±0.042d
CBR052+PGPRC27	66.40±6.00a	0.120±0.0059a	3.28±0.057b	2.23±0.11b
CBR141+PGPRC27	58.21±8.19a-b	0.101±0.0078a	2.70±0.034c	1.74±0.075c
N+P+	9.00±1.00d	0.007±0.0016d	4.15±0.079a	2.53±0.092a
N-P-	14.62±1.53d	0.011±0.00058c	1.83±0.049d	1.35±0.121d

Values in the same column not connected by same letter are significantly different at p 0.05. Values are means ± standard deviation (SD) for triplicates.

Table 4.3 Nodule number, Nodule dry weight, shoot dry weight (SDW), and Shoot nitrogen (SN) by common bean varieties in the field experiment at the Alem Tena site

Test Isolates	Nodule number	Nodule dry weight (g)	shoot dry weight (g)	Shoot Nitrogen (%)
Awash 1				
CBR052	43.12±4.59a	0.025±0.0059a	2.04±0.143b-c	1.51±0.076b-c
CBR141	40.63±5.033a	0.022±0.004a	1.84±0.082c	1.47±0.057b-c
CBR052+PGPRC27	49.14±3.00a	0.026±0.0045a	2.34±0.115b	1.68±0.040b
CBR141+PGPRC27	46.63±2.52a	0.027±0.0031a	1.95±0.107c	1.53±0.036b-c
N+P+	8.47±1.53b	0.008±0.0025b	2.75±0.072a	2.45±0.035a
N-P-	12.00±2.00b	0.009±0.0015b	1.82±0.083c	1.39±0.093c
Ibadoo				
CBR052	54.23±6.03b-c	0.091±0.007c	2.61±0.066b	1.99±0.105b-c
CBR141	52.44±5.12c	0.098±0.013c	2.27±0.079d	1.74±0.031c-d
CBR052+PGPRC27	75.05±7.55a	0.167±0.004a	3.79±0.042a	2. 58±0.133a
CBR141+PGPRC27	67.82±4.00a-b	0.138±0.006b	2.69±0.118b	2.19±0.070b
N+P+	12.24±2.08d	0.008±0.002e	3.67±0.133a	2. 64±0.062a
N-P-	15.11±3.00d	0.014±0.001d	1.91±0.201e	1.41±0.154d
Nasir				
CBR052	47.21±3.12b	0.072±0.0084b	2.21±0.068d	1.733±0.087c
CBR141	45.00±4.0b	0.048±0.010c	2.10±0.061d	1.640±0.041c
CBR052+PGPRC27	64.23±6.51a	0.111±0.013a	3.35±0.08b	2.16±0.093b
CBR141+PGPRC27	55.04±7.55a-b	0.093±0.0047a	2.83±0.067c	1.68±0.042c
N+P+	8.60±1.21c	0.005±0.0012e	4.22±0.102a	2.563±0.110a
N-P-	11.33±1.53c	0.012±0.0006d	1.87±0.076e	1.350±0.098d

Values in the same column not connected by same letter are significantly different at p 0.05. Values are means ± standard deviation (SD) for triplicates.

The bacterial treatments elicited an increase in shoot dry weight (SDW) and shoot nitrogen (SN) (Tables 4.2, 4.3), forming strong correlation with the nodulation characteristics. Compared to the non-inoculated control, single inoculation of CBR052 on Ibadoo variety showed a 48% more shoot nitrogen accumulation at Melkassa site. However, the dual inoculation of CBR052 + PGPRC27 further increased the shoot nitrogen to 95%. Similarly, the single inoculation of CBR052 and coinoculation of CBR052 + PGPRC27 increased the nitrogen content by 41% and 83%, respectively on the field of Alem Tena. Single inoculation of the Ibadoo variety with CBR052 increased the SDW by 38% at Melkassa and by 36% at Alem Tena over the uninoculated controls and it was further increased to 108% and 98%, respectively due to coinoculation by CBR052 + PGPRC27. Likewise, Rahmani *et al.* (2011) showed the single

inoculation with *Rhizobium* Rb-130 on common bean where it increased SDW and N content by 44% and 51% over the uninoculated control on field. A coinoculation field trial by the same author on common bean also showed that SDW (26.2 g $plant^{-1}$) and N content (550 mg $plant^{-1}$) due to single inoculation of *Rhizobium* Rb133 were increased to SDW (32 g $plant^{-1}$) and N content (805 mg plant $^{-1}$) by the dual inoculation with *Rhizobium* Rb133+*P. fluorescens*.

The results also showed that the lowest nodulation of all the treatments was recorded in the chemical fertilized positive control plants. The downsides of increased soil nitrogen on nodulation of legumes have been described in the literature. The current results also showed conformity with the report of (Graham, 1981) that demonstrated nitrogen fertilization with the usually recommended rates of 40-60 kg N ha^{-1} supress nodulation.

4.3.2.2 Effect on yield and related parameters

Coinoculation of Ibadoo and Nasir varieties significantly ($P < 0.05$) improved weight of 100 seed and pod per plant compared to the non-inoculated control plants (Tables 4.4, 4.5). Co-inoculated treatments also brought about significant ($P<0.05$) changes on grain yield compared to both the single inoculations and non inoculated controls (Tables 4.4, 4.5). In the Melkassa soil, it was recorded that grain yield of Ibadoo variety under CBR052 inoculation was 2118.42 kg ha^{-1} which is 47.5% higher than the yield obtained from the non-inoculated control plant (1436.35 kg ha^{-1}). The combined inoculation of CBR052 and PGPRC27 increased the yield to 2694.32kg ha^{-1} which exceeded the noninoculated control by 87.6%, where the dual inoculation of CBR052 + PGPRC27 boosted the yield by 40.1% over the CBR052 single inoculation. A comparable

response was observed in the Alem Tena soil, with a 37.1% increase of grain yield by co-inoculation of CBR052 + PGPRC27 over the yield by CBR052 single inoculation.

In general, co-inoculated plants showed better agronomic characters and yield in both soil conditions and on all the tested varieties. In a similar field trial on common bean, Yadagari *et al*. (2010) reported seed yield production from co-inoculation of *Rhizobium* + *P. fluorescens* P-93 with a significant increase of 57%, 77%, and 73% over *Rhizobium* alone for cultivars 'Sayyad', 'Akhtar', and 'Goli', respectively. Hungaria *et al*. (2013) have also demonstrated that seed inoculation of common bean with *Rhizobium tropici* alone increased yield by 98 kg ha^{-1}, while co-inoculation with *A. brasilense* boosted the yeild to 285 kg ha^{-1}. The results also showed that besides its strong positive correlation ($P< 0.05$, $R=0.94$) with shoot dry matter, grain yield was also substantially correlated with NN ($P<0.05$, $R=0.96$) and NDW ($P<0.05$, $R=0.90$), indicating that the improved nodulations in common bean due to co-inoculation with *Rhizobium* + PSR were translated into plant biomass and then to seed production.

Table 4.4 Number of pod per plant, weight of 100 seed, and Seed yield per hectare in common bean varieties in the field experiment at the Melkassa site

Treatment	*Number of seed per pod*	*Weight of 100 seed (g)*	*Seed yield (kg ha^{-1})*
Awash1			
CBR052	4.8±0.2a	16.93±0.78b-c	1661.54±109c-d
CBR141	4.8±0.2a	16.57±0.46c	1554.79±52c-d
CBR052+PGPRC27	4.93±0.23a	18.83±0.55b	1988.87±113b
CBR141+PGPRC27	4.87±0.12a	17.47±0.67b-c	1788.96±105b-c
N+P+	5.07±0.23a	21.03±1.29a	3108.40±166a
N-P-	4.53±0.31a	16.40±0.27c	1415.39±41d
Ibadoo			
CBR052	4.27±0.12a	26.47±1.25b-c	2118.42±197c
CBR141	4.02±0.20a	25.23±0.72c	1914.57±85c
CBR052+PGPRC27	4.33±0.31a	28.57±0.60b	2694.32±136b
CBR141+PGPRC27	4.53±0.42a	27.27±0.64b-c	2481.34±130b
N+P+	4.53±0.12a	32.90±1.12a	3519.62±68a
N-P-	4.40±0.20a	22.50±0.26d	1436.35±31d
Nasir			
CBR052	4.53±0.31a	21.83±0.35b	1891.24±101c
CBR141	4.27±0.12a	21.77±0.31b	1732.74±42c
CBR052+PGPRC27	4.73±0.14a	22.73±0.85b	2431.41±31b
CBR141+PGPRC27	4.53±0.06a	22.70±1.01b	2211.35±162b
N+P+	4.80±0.00a	26.53±0.70a	3253.20±209a
N-P-	4.73±0.42a	18.70±1.73c	1407.67±148d

Values in the same column not connected by same letter are significantly different at p 0.05. Values are means ± standard deviation (SD) for triplicates.

Table 4.5 Number of pod per plant, weight of 100 seed, and Seed yield per hectare in common bean varieties in the field experiment at the Alem Tena

Treatment	*Number of pod per plant*	*Weight of 100 seed (gm)*	*Seed yield (kg ha^{-1})*
Awash1			
CBR052	8.83±0.25b	17.37±1.12b	1612.54±96b-c
CBR141	8.60±0.72b	16.83±1.76b	1502.47±193c
CBR052+PGPRC27	9.47±0.29b	18.40±1.01a-b	1882.56±96b
CBR141+PGPRC27	9.13±0.31b	17.77±0.65a-b	1706.63±50b-c
N+P+	13.37±1.15a	20.83±1.42a	3052.34±216a
N-P-	8.47±0.61b	16.03±0.46b	1388.09±88c
Ibadoo			
CBR052	8.20±0.95b-c	26.03±1.39b-c	1924.77±53.72d
CBR141	7.87±0.60b-c	25.67±0.61c	1822.71±22d
CBR052+PGPRC27	9.43±0.71a-b	28.23±0.95b	2638.69±68b
CBR141+PGPRC27	8.63±0.84a-b	27.67±0.64b-c	2369.91±131c
N+P+	10.17±0.56a	33.30±1.11a	3458.53±154a
N-P-	6.40±0.10c	22.27±0.61d	1348.95±39e
Nasir			
CBR052	8.77±0.40b-c	21.73±0.76c	1862.64±107c-d
CBR141	7.97±0.67c-d	21.53±1.42b	1677.16±146d-e
CBR052+PGPRC27	9.96±0.49b	23.43±0.81b	2370.67±109b
CBR141+PGPRC27	9.53±1.20b-c	22.83±1.36b	2161.90±186b-c
N+P+	11.37±0.42a	26.77±0.60a	3200.44±105a
N-P-	6.83±0.42d	18.17±1.00c	1340.30±124e

Values in the same column not connected by same letter are significantly different at p 0.05. Values are means ± standard deviation (SD) for triplicates.

The bean varieties showed significant ($P<0.05$) differences in yield in response to single inoculation/co-inoculation (Table 4.4, 4.5). Accordingly, Ibaddoo variety was the best responsive variety of all the common beans to both single inoculation and coinoculation treatments at both sites followed by Nassir and Awash-1. Furthermore, there was no significant bean variety - environment interaction and bean variety - bacteria interaction for grain yield and other associated parameters, indicating that there was no significant ($P<0.05$) difference between the two experimental sites of Melkassa and Alem Tena. Generally, the result showed that the varieties of common bean influenced the level of nodulation, shoot dry matter, shoot nitrogen and grain yield that are consequences of the symbiosis. Previous researches findings also

manifested the existence of variations in plant development responses among the common bean genotypes in relation to the rhizobial strains inoculated. Accordingly, Rodino *et al*. (2011) have included 64 common bean genotypes on a field experiment have verified significant differences in nodule number (1.5 - 600 $plant^{-1}$), shoot dry weight (2.7 - 12.7g $plant^{-1}$), dry weight per nodule (0.05 - 5.97mg). Similarly, Sanchez *et al*. (2014) reported that combined inoculation of common bean on a field with *Rhizobium pici* (R40982) and *P. monteilii* increased the NN by 76% in DOR-364, whereas only by 34% in BAT-477 variety over the *Rhizobium* alone. Therefore, inorder to get improved yield from inoculation and/or coinoculation with rhizobia and other PGP rhizobacteria, selection of responsive bean varieties would be very helpful.

In conclusion, although the level of response varied among the varieties used, all *Rhizobium*-PGPR co-inoculated plants manifested increased nodulation and grain yields than the single inoculated and non-inoculated control plants. This could demonstrate the potential of using combined inoculation for improved productivity of common bean. Good grain yield was recorded as result of dual application of CBR052 and PGRC27 on all common bean varieties, the effects being more pronounced on Ibadoo than the other two varieties at both experimental sites. More field trials are, therefore, required on more common bean varieties to identify more number of responsive host varieties so that the potential productivity of common bean could be fully realized in different agroecosystems of the country.

Chapter 5 Conclusion and Recommendation

5.1 Conclusion

The *Rhizobium* isolates collected from the root nodules of common bean grown in different regions of the country showed wide range of variability in their phenotypic characteristics such as tolerance to acidity, alkalinity, salinity and antibiotics as well as utilization of diverse carbon and nitrogen sources. Based on these phenotypic data, the numerical phylogeny of the isolates was determined whereby the 76 isolates formed four distinctive clusters that represent *Rhizobium etli* (24), *Rhizobium phaseoli*/*Rhizobium sp*. (33), *Rhizobium giardini* (17) and *Rhizobium tropici* (2).The procedures employed to screen the *Rhizobium* isolates for their relative symbiotic performance revealed that only 23% of them were symbiotically effective with rate of 50-74.6% Some of the selected isolates showed significant increase in nodulation, shoot dry weight, shoot nitrogen and grain yield over the uninoculated control. However, more than 77% of soil samples from common bean growing regions of central, eastern, southern and western Ethiopia harbour symbiotically less effective bean rhizobia and need to be augmented with inoculation with selected isolates.

The results also showed that an estimate of $2.6x10^1$ to $9.2x10^3$cfu $gram^{-1}$ soil of phosphate solubilizing rhizobacteria do exist in the rhizosphere of common bean in Ethiopia. Analysis of the partial 16S rRNA gene sequences enabled the identification of 10 genera of phosphobacteria namely, *Bacillus, Arthrobacter, Brevibacterium, Lycinibacillus, Enterobacter, Acetinobacter, Pseudomonas, Klebsiella, Devosia,* and *Chryseobacterium.* Some of the isolates were able to exhibit high potential to mobilize plant available phosphorus from the insoluble inorganic phosphate sources, which was as 312µg/ml from tri-calcium phosphate within 4 days and

117µg/ml from Egyptian rock phosphate within 6 days of incubation. Accordingly, inoculation of common bean with selected isolates enhanced the nodulation and symbiotic nitrogen fixation both under greenhouse and on field conditions. Therefore, application of selected phosphobacterial isolates to the P deficient soils of common bean growing areas would be a helpful strategy to improve the symbiotic interaction between the host plant and its rhizobial endosymbiont. In addition, out of the 54 phosphobacterial isolates, 61% were able to produce IAA whereas 22% showed antagonistic properties against *Fusarium oxysporum f.sp. phaseoli* where their potential role as a part of the integrated pest management would be of paramount importance.

The field trials on common bean varieties of Awash-1, Nasir, and Ibadoo revealed that the effect of co-inoculation was more pronounced than the single inoculations. Combined application of CBR052 and PGPRC27 produced the highest plant growth and grain yield in all the tested varieties on both experimental sites, Ibadoo being the best-responding variety with the highest yield followed by Nassir. Awash-1 was the least responsive to both inoculation and co-inoculation treatments. This indicates that growth, grain yield, and yield related responses of common bean to inoculation and co-inoculation are dependent upon type of the host plant varieties.

5.2 Recommendations

- The rhizobial isolate CBR052 and the phosphobacterial isolate PGPRC27 can be good candidates for the formulation of effective biofertilizer in the production system of common bean in Ethiopia. To this effect, devising ways to carry out subsequent field trials under various agroecologies of the country has to be planned out.

- Extending the co-inoculation tests on many more varieties of common bean is imperative.
- Cocktail of isolates that showed different PGP traits should be combined in different permutations for co-inoculation experiment with selected *Rhizobium* species.
- Testing the effective isolates with different doses of nitrogen and phosphorus on the field is necessary as part of the integrated soil fertility management.
- Genetic complementation and transformation of the symbiotically effective *Rhizobium* isolates should also be attempted by dual inoculation with the known isolates having strong P solubilizing abilities.
- Since the study was limited to few traits, the isolates should also be assessed further for additional indices of *in vitro* PGP characteristics such as production of ACC deaminase, siderophores, cytokines, lumichromes etc.
- Conventional procedures, which rely on phenotypic characteristics for assessing rhizobial diversity, may not warrant fully reliable conclusion about the actual diversity; and hence a polyphasic approach including MLSA of some housekeeping genes, 16S rRNA gene sequence analysis and AFLP should help to reveal a more accurate picture on the diversity of common bean rhizobia.

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Appendices

Appendix I Yeast Extract Mannitol Agar (YEMA) (Vincent, 1970)

Mannitol (10g), K_2HPO_4 (0.5g), $MgSO_4.7H_2O$ (0.2g), NaCl (0.1g), Yeast extracts (1g), Congo red (0.025ml), Agar (15g), Distilled water (1000ml).

Appendix II N-free Nutrient Solution (Broughton and Dilworth, 1970)

Stock Solution	Chemical	g/liter
1	$CaCl_2.2H_2O$	294.1
2	KH_2PO_4	136.1
3	$FeC_6H_5O7.3H_2O$	6.7
	$MgSO_4.7H_2O$	123.3
	K_2SO_4	87.0
	$MnSO_4.H_2O$	0.338
4	H_3BO_3	0.247
	$ZnSO_4.7H_2O$	0.288
	$CuSO_4.5H_2O$	0.100
	$CoSO_4.7H_2O$	0.056
	$Na_2MoO_2.2H_2O$	0.048

Taken from Somasegaren and Hoben (1994)

Appendix III Kjeldahl method for Plant nitrogen analysis

Nitrogen of plant matter is quantitatively determined by the modified Kjeldahl method using the manual produced by Sahlemedhin Sertsu and Taye Bekele (2000). At the beginning, 0.2 gm of ground samples was measured for analysis. Into the ground sample, 0.5gm of mixture from the preparation containing 10 gm of K_2SO_4, 2 gm of $CuSO_4$. H_2O and 0.2 gm of selenium was added

as a catalyst. This was immediately followed by addition of 7 ml of salicylic-sulfuric acid mixture and allowing it to react for 30 minutes. Then 0.5 gm of $Na_2S_2O_3 .5H_2O$ were added; agitated and allowed to react for five minutes. 0.5 gm salt and 7 ml of salicylic-sulfuric acid mixture was prepared for the blank. The whole mixture was then set for digestion at a temperature of 380^0 C for 3 hours until the color disappeared. Following digestion, the sample was distille by adding75ml of 40% NaOH and its nitrogen was collected with a flask containing 20 ml boric acid until the volume reached 110 ml. Eventually, the distillates became titrated using 0.1 N H_2SO_4 , and the reading of the burette recorded; nitrogen percentage was computed employing the formula below:

$$\% \text{ Nitrogen} = \frac{(V1 - V2) \times N \times 0.014 \times 100}{S}$$

Where:

V1 = ml of titrant (H_2SO_4) used for the sample V2 = ml of titrant (H_2SO_4) used for the blank

N= Normality of the H_2SO_4 acid

S= Weight of the plant material in g

0.014= meq weight of nitrogen in gram

Appendix IV Isolates of common bean rhizobia, site of isolation, growth and colony characteristics

Isolate	*Region*	*Origin of isolates*		*Elevation*	*Colony diameter*	*Mean generation time*	*Colony characteristic on YEMA*
		Specific location	*Major reference site*				
CBR002	WE	Kechema	Jima	1781	3.0	1.50	MWT
CBR003	WE	Kechema	Jima	1790	3.5	1.75	LWT
CBR005	WE	Buyo Kore	Seka	1759	5.0	0.85	LWT
CBR006	WE	Gibe Bosa	Seka	1867	4.0	1.15	LWT
CBR007	WE	Gibe Bosa	Seka	1983	2.5	1.25	MWT
CBR008	WE	Gudeta Bula	Mana	1876	4.5	1.02	LWT
CBR011	WE	Gube Muleta	Mana	2046	3.0	2.50	LWT
CBR013	WE	Gube Muleta	Mana	2097	5.0	0.75	LWT
CBR015	WE	Doya	Mana	1770	2.5	3.25	MWT
CBR018	WE	Bala Wajo	kersa	1702	4.5	1.26	LWT
CBR019	WE	Gelo	Kersa	1754	3.0	1.52	MWT
CBR020	WE	Kitimbila	kersa	1696	2.5	2.00	MWT
CBR023	WE	Babo Sarte	kersa	1801	4.5	1.25	LWT
CBR026	WE	Burusa	Metu	1524	2.0	1.45	MWT
CBR028	WE	Burusa	Metu	1661	3.5	2.05	LWT
CBR031	WE	Tulube	Metu	1714	4.0	0.95	LWT
CBR034	WE	Degoye	Bure	1680	3.0	1.00	MWT
CBR035	WE	Toli Cheka	Bure	1496	4.5	1.25	LWT
CBR036	WE	Obo Miriga	Bure	1654	4.0	0.85	LWT
CBR039	WE	Nabo Miriga	Bure	1662	2.0	3.35	MWT
CBR043	WE	Chore	Bure	1702	3.0	1.52	MWT
CBR045	WE	Bedelle	Bure	2018	5.0	0.76	LWT
CBR150	WE	Bedele	Bedele	1806	3.5	1.74	LWT
CBR151	WE	Geyi	EluAbabora	1690	4.0	1.85	LWT
CBR154	WE	Achibo	EluAbabora	1607	4.0	1.44	LWT
CBR157	WE	Wutete	EluAbabora	1632	3.5	1.90	LWT
CBR158	WE	Mana	EluAbabora	1910	4.5	0.84	LWT
CBR160	WE	Chora kombabi	EluAbabora	2023	3.0	1.33	MWT
CBR046	CE	Garbi Widana	Adami Tulu	1649	3.0	1.25	MWT
CBR049	CE	Abile Germama	Ziway	1639	4.5	0.95	LWT
CBR052	CE	Elka	Ziway	1657	4.5	1.55	LWT
CBR053	CE	Tuchi	Ziway	1667	3.0	1.25	MWT
CBR055	CE	Dodota Dembel	Ziway	1660	5.0	0.82	LWT
CBR056	CE	Korkadi	Meki	1647	3.5	1.75	LWT
CBR059	CE	Gemo	Meki	1641	3.0	2.22	MWT
CBR062	CE	Gemo	Meki	1644	3.5	1.05	LWT
CBR063	CE	Berta	Meki	1697	4.5	1.50	LWT
CBR066	CE	Berta	Meki	1687	2.0	2.28	MWT
CBR068	CE	Jewe Bote	Meki	1690	4.0	1.05	LWT
CBR075	EE	Woji Kebenga	Dengego	2143	3.5	1.95	LWT
CBR079	EE	Walawachu	Kersa	2029	4.0	0.88	LWT
CBR088	EE	Gale Mirga	Kersa	2022	3.5	2.25	LWT
CBR094	EE	Chelenko Lola	Meta	2154	3.0	1.77	MWT
CBR096	EE	Ademerer	Deder	2171	5.0	0.85	LWT

CBR097	EE	Chefe Anani	Goro Gutu	2264	2.5	1.45	MWT
CBR109	EE	Efa Bas	Tulo	2143	4.0	1.02	LWT
CBR110	EE	Lubu Dakab	Tulo	1779	3.5	2.05	LWT
CBR112	EE	Wachu Tateli	Asebe Teferi	2223	3.5	1.47	LWT
CBR114	EE	Kiliso	Chiro	2245	2.0	3.16	MWT
CBR116	EE	Medicho	Chiro	1622	2.5	2.35	MWT
CBR117	EE	Huse Mandera	Meiso	1492	4.0	1.08	LWT
CBR121	EE	Huse Demi	Meiso	1383	3.0	2.05	MWT
CBR122	EE	Fayo	Meiso	1342	2.0	3.15	MWT
CBR124	EE	Nanawa	Meiso	1364	2.5	2.75	MWT
CBR125	EE	Wedeyiti	Meiso	1701	3.5	2.01	LWT
CBR127	EE	Sarada Arada	Boset	1432	2.5	1.60	LWT
CBR129	EE	Kachachule Guja	Boset	1432	3.0	1.33	LWT
CBR130	EE	Foki	Boffa	1281	3.0	1.57	MWT
CBR144	EE	Awash	Awash	1542	4.0	1.14	LWT
CBR147	EE	Kombe Gugssa	Boset	1268	4.5	0.75	LWT
CBR148	EE	Awash	Awash	1582	3.0	1.50	MWT
CBR161	EE	Asebe Teferi	Asebe Teferi	1653	4.0	1.07	LWT
CBR164	EE	Hirna	Hirna	1810	4.5	0.99	LWT
CBR169	EE	Haromaya	Haromaya	1769	5.0	1.02	LWT
CBR172	EE	Haromaya	Haromaya	1768	4.0	1.51	LWT
CBR083	SE	Kamba	Gamo Gofa	1898	3.5	1.74	LWT
CBR089	SE	Kacho	Gamo Gofa	1866	5.0	0.75	LWT
CBR098	SE	Karje	Gamo Gofa	1506	3.0	1.78	MWT
CBR104	SE	Hanika	Gamo Gofa	1520	2.5	1.90	MWT
CBR132	SE	Songa	Gamo Fofa	1778	4.0	0.96	LWT
CBR133	SE	Lesho Mazoria	Hadaro tunto	1740	4.5	0.78	LWT
CBR137	SE	Areka	Welayta	1712	2.5	2.85	MWT
CBR138	SE	Dolla	Boloso Sore	1832	3.5	2.33	LWT
CBR140	SE	Waraza Lasho	Welayta	1966	3.0	2.00	MWT
CBR141	SE	Larena	Welayta	1815	4.5	1.08	LWT
CBR142	SE	Tebella	Humbo	1827	4.0	1.00	LWT

LWT: Large, Watery, and Translucent; MWT: Medium, Watery, and Translucent (L:>3mm) (M:2-3mm) (S:<2mm)

Appendix V Preliminary screening data of rhizobial isolates in Pouch culture

Isolate	*Taxonomic group*	*Region*	*Leaf colour*	*Nodule colur*	*Nodule size (mm)*	*Plant appearance*
CBR002	*R. giardini*	WE	Deep green	pink	2.5	+ + +
CBR003	*R. etli*	WE	Yellowy green	whitish	< 2.0	+ +
CBR005	*R. giardini*	WE	green	pink	2.2	+ + +
CBR006	*R. Phaseoli*	WE	Deep green	pink	3.0	+ + +
CBR007	*R. Phaseoli*	WE	Yellowy green	whitish	< 2.0	+ +
CBR008	*R. phseoli/sp.*	WE	Yellowy green	whitish	< 2.0	+ +

CBR011	*R. giardini*	WE	Yellowy green	whitish	< 2.0	+ +
CBR013	*R. etli*	WE	Deep green	pink	2.8	+ + +
CBR015	*R. giardini*	WE	Yellowy green	whitish	< 2.0	+ +
CBR018	*R. etli*	WE	Deep green	pink	2.4	+ + +
CBR019	*R. giardini*	WE	Yellowy green	whitish	< 2.0	+ +
CBR020	*R. phseoli/sp.*	WE	Yellowy green	whitish	< 2.0	+ +
CBR023	*R. phseoli/sp.*	WE	Deep green	pink	3.0	+ + +
CBR026	*R. Phaseoli*	WE	Deep green	pink	2.5	+ + +
CBR028	*R. Phaseoli*	WE	Yellowy green	whitish	< 2.0	+ +
CBR031	*R. etli*	WE	Deep green	pink	3.0	+ + +
CBR034	*R. etli*	WE	Yellowy green	whitish	< 2.0	+ +
CBR035	*R. Phaseoli*	WE	Deep green	pink	2.6	+ + +
CBR036	*R. giardini*	WE	Yellowy green	whitish	< 2.0	+ +
CBR039	*R. Phaseoli*	WE	Yellowy green	whitish	< 2.0	+ +
CBR043	*R. phseoli/sp.*	WE	Deep green	pink	2.8	+ + +
CBR045	*R. phseoli/sp.*	WE	Yellowy green	whitish	< 2.0	+ +
CBR150	*R. Phaseoli*	WE	Yellowy green	whitish	< 2.0	+ +
CBR151	*R. giardini*	WE	Yellowy green	whitish	< 2.0	+ +
CBR154	*R. tropici*	WE	Deep green	pink	2.7	+ + +
CBR157	*R. phseoli/sp.*	WE	Yellowy green	whitish	< 2.0	+ +
CBR158	*R. etli*	WE	Yellowy green	whitish	< 2.0	+ +
CBR160	*R. etli*	WE	Yellowy green	whitish	< 2.0	+ +
CBR046	*R. Phaseoli*	CE	Deep green	pink	2.5	+ + +
CBR049	*R. phseoli/sp.*	CE	Deep green	pink	2.7	+ + +
CBR052	*R. etli*	CE	Deep green	pink	3.0	+ + +
CBR053	*R. Phaseoli*	CE	Yellowy green	whitish	< 2.0	+ +
CBR055	*R. Phaseoli*	CE	Yellowy green	whitish	< 2.0	+ +
CBR056	*R. giardini*	CE	Yellowy green	whitish	< 2.0	+ +
CBR059	*R. etli*	CE	Deep green	pink	2.8	+ + +
CBR062	*R. etli*	CE	Yellowy green	whitish	< 2.0	+ +
CBR063	*R. phseoli/sp.*	CE	Deep green	pink	2.0	+ + +
CBR066	*R. phseoli/sp.*	CE	Yellowy green	whitish	< 2.0	+ +
CBR068	*R. Phaseoli*	CE	Yellowy green	whitish	< 2.0	+ +
CBR075	*R. etli*	EE	Yellowy green	whitish	< 2.0	+ +
CBR079	*R. giardini*	EE	Deep green	pink	2.7	+ + +
CBR088	*R. etli*	EE	Deep green	pink	2.6	+ + +
CBR094	*R. etli*	EE	Deep green	pink	2.2	+ + +
CBR096	*R. etli*	EE	Deep green	pink	2.4	+ + +
CBR097	*R. Phaseoli*	EE	Yellowy green	whitish	< 2.0	+ +
CBR109	*R. etli*	EE	Deep green	pink	2.8	+ + +
CBR110	*R. giardini*	EE	Deep green	pink	3.0	+ + +
CBR112	*R. etli*	EE	Yellowy green	whitish	< 2.0	+ +
CBR114	*R. phseoli/sp.*	EE	Deep green	pink	2.5	+ + +

CBR116	*R. Phaseoli*	EE	Yellowy green	whitish	< 2.0	+ +
CBR117	*R. giardini*	EE	Yellowy green	whitish	< 2.0	+ +
CBR121	*R. etli*	EE	Yellowy green	whitish	< 2.0	+ +
CBR122	*R. tropici*	EE	Deep green	pink	2.7	+ + +
CBR124	*R. Phaseoli*	EE	Yellowy green	whitish	< 2.0	+ +
CBR125	*R. giardini*	EE	Yellowy green	whitish	< 2.0	+ +
CBR127	*R. etli*	EE	Deep green	pink	3.0	+ + +
CBR129	*R. giardini*	EE	Yellowy green	whitish	< 2.0	+ +
CBR130	*R. etli*	EE	Deep green	pink	2.5	+ + +
CBR144	*R. phseoli/sp.*	EE	Yellowy green	whitish	< 2.0	+ +
CBR147	*R. etli*	EE	Yellowy green	whitish	< 2.0	+ +
CBR148	*R. Phaseoli*	EE	Yellowy green	whitish	< 2.0	+ +
CBR161	*R. Phaseoli*	EE	Yellowy green	whitish	< 2.0	+ +
CBR164	*R. etli*	EE	Deep green	pink	3.0	+ + +
CBR169	*R. Phaseoli*	EE	Deep green	pink	2.8	+ + +
CBR172	*R. etli*	EE	Deep green	pink	2.5	+ + +
CBR083	*R. giardini*	SE	Deep green	pink	3.0	+ + +
CBR089	*R. phseoli/sp.*	SE	Yellowy green	whitish	< 2.0	+ +
CBR098	*R. phseoli/sp.*	SE	Deep green	pink	2.7	+ + +
CBR104	*R. etli*	SE	Yellowy green	whitish	< 2.0	+ +
CBR132	*R. phseoli/sp.*	SE	Yellowy green	whitish	< 2.0	+ +
CBR133	*R. etli*	SE	Deep green	pink	2.0	+ + +
CBR137	*R. Phaseoli*	SE	Deep green	pink	2.4	+ + +
CBR138	*R. giardini*	SE	Yellowy green	whitish	< 2.0	+ +
CBR140	*R. giardini*	SE	Yellowy green	whitish	< 2.0	+ +
CBR141	*R. Phaseoli*	SE	Deep green	pink	3.0	+ + +
CBR142	*R. giardini*	SE	Yellowy green	whitish	< 2.0	+ +

+ + +:- very good , + +:- good, CE:- central Ethiopia, EE:- eastern Ethiopia, SE:- southern Ethiopia, WE:- western Ethiopia

Appendix VI PGPR properties of rhizobial isolates

Isolates	*Sampling Region*	*IAA production* µgml^{-1}	*P Solubilization (SI)*	*F. oxysporum Inhibition*
CBR002	WE	–	–	28.32±0.59d-e
CBR003	WE	21.06±1.65o-q		–
CBR005	WE	–	1.07±0.05e	–
CBR006	WE	19.24±0.74q-r	–	–
CBR007	WE	–	–	17.45±0.30i-m
CBR008	WE	–	–	23.53±0.37h-i
CBR011	WE	14.24±0.34v-w	–	–
CBR013	WE	31.16±0.59f	–	27.18±0.44d-f
CBR015	WE	–	–	–
CBR018	WE	8.05±0.25y-z	2.07±0.04b	–
CBR019	WE	–	1.42±0.06d	35.17±1.1b
CBR020	WE	–	–	–
CBR023	WE	23.23±0.35i-n	–	22.62±0.96i-j
CBR026	WE	17.66±0.57st	–	–
CBR028	WE	25.14±0.18i-k	–	–
CBR031	WE	–	–	24.32±0.42g-i
CBR034	WE	–	0.75±0.03f	–
CBR035	WE	26.73±0.25h-j	–	21.12±0.62j-k
CBR036	WE	13.12±0.32w-x	–	–
CBR039	WE	–	–	32.28±0.43c
CBR043	WE	16.02±0.11t-u	–	–
CBR045	WE	7.61±0.06y-z	–	15.36±0.56n-o
CBR150	WE	–	2.22±0.08a	–
CBR151	WE	23.72±0.66k-m	–	–
CBR154	WE	37.38±0.22a	–	–
CBR157	WE	6.21±0.49z	–	19.44±0.89k-i
CBR158	WE	21.46±0.71o	–	–
CBR160	WE	–	–	35.65±1.02ab
CBR046	CE	31.02±0.40d-e	1.63±0.05c	–
CBR049	CE	–	–	–
CBR052	CE	24.83±0.35j-i	–	24.16±0.33h-i
CBR053	CE	–	–	36.37±0.73a-b
CBR055	CE	12.44±0.53w-x	–	–
CBR056	CE	–	0.98±0.01e	–
CBR059	CE	26.18±0.41h-j	–	–
CBR062	CE	11.56±0.32x	–	28.41±0.72d-e
CBR063	CE	29.24±0.22g	–	–
CBR066	CE	23.22±0.41i-n	–	–
CBR068	CE	–	–	25.26±0.43f-h
CBR075	EE	18.48±0.19r-s	–	–
CBR079	EE	–	–	–
CBR088	EE	–	–	26.39±0.69e-j
CBR094	EE	–	–	18.17±0.49i-m
CBR096	EE	31.73±0.22e-f	1.74±0.04c	–
CBR097	EE	16.08±0.09t-u	–	–
CBR109	EE	15.35±0.61u-v	–	–
CBR110	EE	–	1.33±0.07d	–
CBR112	EE	–	–	37.53±0.65a
CBR114	EE	37.77±0.11b	–	16.31±0.62m-o
CBR116	EE	–	–	–
CBR117	EE	–	–	28.43±0.56d-e
CBR121	EE	21.92±0.25o-p	–	–

CBR122	EE	23.15±0.14i-n	–	–
CBR124	EE	–	–	22.37±0.14i-j
CBR125	EE	–	1.62±0.05c	–
CBR127	EE	13.61±0.53w	–	–
CBR129	EE	–	–	32.55±0.32c
CBR130	EE	16.42±0.17t-u	–	–
CBR144	EE	–	–	–
CBR147	EE	19.75±0.73p-r	–	34.28±1.01b
CBR148	EE	13.62±0.32w	–	–
CBR161	EE	26.13±0.55h-j	–	–
CBR164	EE	22.38±0.28m-o	–	28.82±0.91d
CBR169	EE	17.35±0.47s-t	–	–
CBR172	EE	–	1.45±0.03d	14.44±0.91o
CBR083	SE	21.63±0.08n-o	–	–
CBR089	SE	32.41±0.27d-f	–	–
CBR098	SE	34.820.12c	–	–
CBR104	SE	22.24±0.35m-o	–	–
CBR132	SE	–	–	25.41±0.26f-h
CBR133	SE	27.33±0.32h	–	–
CBR137	SE	9.11±0.18y	–	–
CBR138	SE	–	–	–
CBR140	SE	25.57±0.21i-k	0.57±0.01g	–
CBR141	SE	15.24±0.43u-v	–	–
CBR142	SE	34.63±0.22c-d	–	15.81±0.53n-o

+:- positive for the test, +:- negative for the test, WE:- Western Ethiopia, EE:- Eastern Ethiopia, CC:- Central Ethiopia, SE:- Southern Ethiopia

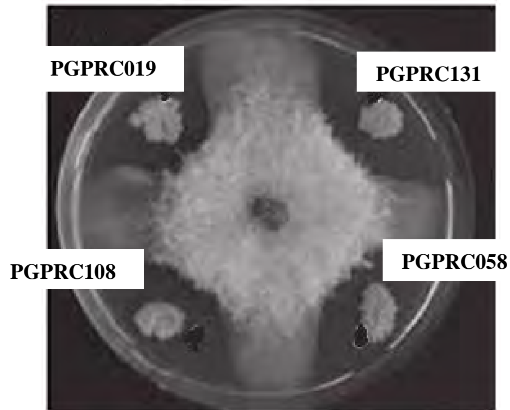
AppendixVII Phosphobacterial isolates tested for additional PGP properties

Isolate	*Solubilization of P (SI)*	*IAA production µgml^{-1}*	*F. oxysporum Inhibition*	*protease production*	*Cellulase production*	*Chitinase production*	*Cyanide production*
PGPRC6	1.24±0.07±p-u	37.47±0.40e-g	–	–	–	–	–
PGPRC11	2.51±0.11±e-h	–	19.6±0.88i-h	–	–	–	–
PGPRC17	2.14±0.22±h-k	–	–	–	–	–	–
PGPRC18	1±0.12±s-v	14.47±2.65s-t	–	–	–	–	–
PGPRC19	2.39±0.053±f-i	–	41.53±0.49c-d	+	–	+	–
PGPRC22	2.71±0.027±d-f	53.23±0.85a	–	–	–	–	–
PGPRC27	3.44±0.07±a	34.4±0.85g-i	–	–	–	–	–
PGPRC32	2.3±0.097±g-j	42.17±0.47d-e	–	–	–	–	–
PGPRC33	2.15±0.19±h-k	41.43±1.96d-e	–	–	–	–	–
PGPRC38	2.05±0.15±i-l	–	36.5±1.71e-f	+	+	–	+
PGPRC43	1.75±0.04±k-n	37.37±0.55e-g	–	–	–	–	–
PGPRC44	3.14±0.35±a	27.27±0.86k-m	–	–	–	–	–
PGPRC54	1.75±0.04±k-n	–	–	–	–	–	–
PGPRC56	2.25±0.14±h-j	49.4±0.82a	–	–	–	–	–
PGPRC57	2.04±0.12i-l	36.2±0.46f-h	24.33±3.30h	+	+	–	–
PGPRC58	2.83±0.16±c-e	–	47.43±1.65a-b	–	–	–	–
PGPRC66	1.05±0.45±r-v	30.2±0.40i-l	–	–	–	–	–
PGPRC68	1.41±0.06±n-r	–	–	–	–	–	–
PGPRC71	3.28±0.23±a-b	–	–	–	–	–	–
PGPRC87	2.66±0.24±e-g	–	–	–	–	–	–
PGPRC90	1.90±0.09±j-m	–	–	–	–	–	–
PGPRC94	1.18±0.12±q-v	19.43±0.35o-r	–	–	–	–	–
PGPRC95	2.4±0.16±f-i	–	–	–	–	–	–
PGPRC98	2.23±0.09±h-j	43.33±4.8c-d	–	–	–	–	–
PGPRC105	2.2±0.6±h-j	16.467±0.40q-t	32.5±0.40f-g	+	+	–	–
PGPRC106	1.72±0.22±l-n	18.7±0.62p-s	–	–	–	–	–
PGPRC108	0.96±0.62±t-v	–	43.43±0.90c	+	+	–	–
PGPRC117	1.18±0.08±q-v	35.5±0.44f-h	–	–	–	–	–
PGPRC122	2.32±0.82±f-i	21.4±0.95n-p	–	–	–	–	–
PGPRC123	3.07±0.15±a-d	46.13±0.76a-c	–	–	–	–	–
PGPRC124	0.82±0.11v	33.5±0.92g-j	–	–	–	–	–
PGPRC131	1.4±0.06±n-s	23.3±1.85m-p	51.3±0.26a	–	+	+	–
PGPRC138	2.29±0.72±g-j	–	–	–	–	–	–
PGPRC139	3.16±0.43±a-c	13.3±2.95t-u	–	–	–	–	–
PGPRC144	1.05±0.55±r-v	–	–	–	–	–	–
PGPRC146	1.28±0.06±o-t	34.67±1.00	–	–	–	–	–
PGPRC153	1.53±0.65±m-q	31.53±0.40h-k	–	–	–	–	–
PGPRC157	2.27±0.44±h-j	–	–	–	–	–	–
PGPRC162	2.91±0.31±b-e	25.63±0.91l-n	38.63±0.55d-e	–	+	–	+
PGPRC165	0.86±0.61±u-v	–	–	–	–	–	–
PGPRC166	2.67±0.07±d-g	–	–	–	–	–	–
PGPRC167	2.61±0.25±e-g	48.7±0.82a-b	–	–	–	–	–
PGPRC170	1.6±0.42±m-p	–	–	–	–	–	–
PGPRC171	1.11±0.91±r-v	–	23.2±1.1h	–	+	+	–
PGPRC178	1.48±0.58±n-s	22.47±0.35m-p	–	–	–	–	–

PGPRC179	2.32±0.07±f-i	9.47±1.65u	–	–	–	–	–
PGPRC182	1.1±0.23±r-v	46.73±0.60a-c	35.2±0.36e-f	–	+	–	+
PGPRC183	2.23±0.741±h-j	36.3±0.56f-h	–	–	–	–	–
PGPRC185	2.0±0.47±i-l	15.43±0.25r-t	–	–	–	–	–
PGPRC186	2.53±0.53±e-h	–	29.81±2.14g	+	–	+	–
PGPRC189	3.36±0.031±a	29.13±0.85j-l	–	–	–	–	–
PGPRC191	1.15±0.34±q-v	24.27±0.55m-o	–	–	–	–	–
PGPRC192	1.72±0.86±l-n	–	–	–	–	–	–
PGPRC196	1.41±0.27±n-s	20.87±0.31n-q	–	–	–	–	–

PGPRC27

PGPRC189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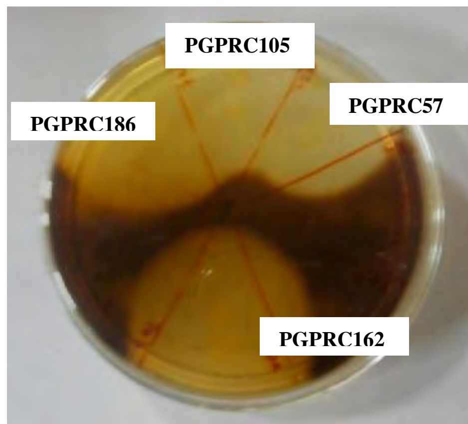
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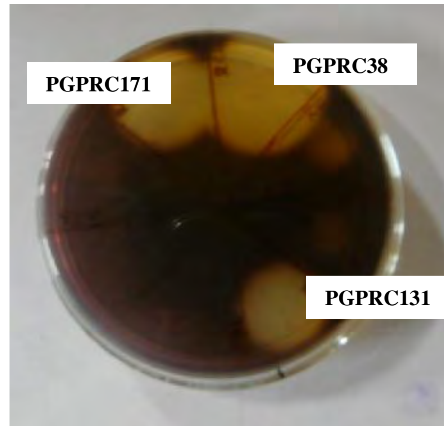
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