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Plant Growth Promoting Properties of Rhizobacteria Isolated from Chickpea and Lentil Producing Areas of Ethiopia: Implication for Productivity in Low-inputs Agricultural system

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

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Abstract

Chickpea (*Cicer arietinum* L.) and lentil (*Lens culnaris* Medik.) are among the major sources of dietary protein for the majority of population in Ethiopia. They also restore and maintain soil fertility through their symbiotic nitrogen-fixation in association with rhizobia. This study was intended to investigate plant growth promoting properties of chickpea and lentil nodulating rhizobia and phosphate solubilizing bacterial isolates from major producing areas of the country. Accordingly, a total of 157 bacterial isolates were obtained and characterized using different standard methods. Phenotypic and plant growth promoting characteristics of 66 rhizobial isolates recovered from root nodules of chickpea and lentil grown in soils collected from producing areas of the country were investigated. These rhizobial isolates showed wide diversity in their different C and N-sources utilization pattern and tolerance to salinity, high temperatures, acid and alkaline pH, heavy metals and antibiotics. Symbiotic and morphological characterization also showed a wide diversity among these rhizobial isolates. Moreover, some of these rhizobial isolates exhibited plant growth promoting characteristics such as phosphate solubilization, IAA production and antagonistic activity against *Fusarium oxysporum*. In addition, a total of 91 phosphate solubilizing bacterial isolates were obtained from chickpea and lentil rhizosphere soil samples collected from different producing areas of the country. These isolates were identified using the API biochemical test kits and partial 16S rDNA sequences analysis. The result showed the presence of genera such as *Acinetobacter, Aeromonas, Agrobacterium, Bacillus, Brevibacillus, Burkholderia, Chryseomonas, Empedobacter, Enterobacter, Pseudomonas, Ralstonia, Sphingomonas and Stenotrophomonas*. Phosphate solubilizing efficiency of the isolates was assessed qualitatively using solubilization index and quantitatively. Quantitative

estimation of the phosphate solubilization efficiency of different insoluble phosphorus sources showed that most isolates have good solubilization ability of tricalcium phosphate, rock phosphate and bone meal. Furthermore, some of these isolates showed multiple plant growth promoting characteristics; production of indole acetic acid and fluorescent siderophore, and inhibition of *Fusarium oxysporum* growth under *in vitro* conditions. The effects of inoculation of isolates with multiple plant growth promoting properties on chickpea and lentil growth in pot culture showed significant improvement in number of nodules, shoot dry matter, shoot nitrogen and phosphorus contents. Generally, the present study indicated that Ethiopian soils contain symbiotically effective chickpea and lentil rhizobia which are endowed with different plant growth promoting attributes. Moreover, chickpea and lentil rhizosphere harbor phosphate solubilizing bacteria which are diverse in their taxonomy and phosphate solubilizing efficiency. Some of these isolates are of particular interest for further research under different field conditions.

Key words: IAA, *Mesorhizobium* species, PGPR, PSB, *Rhizobium* species

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1. Mulissa Jida and Fassil Assefa (2011). Phenotypic and plant growth promoting characteristics of *Rhizobium leguminosarum bv. viciae* from lentil (*Lens culnaris* Medik.) growing areas of Ethiopia. *Afr. J. Microbiol. Res.* **5**: 4133-4142.
2. Mulissa Jida and Fassil Assefa (2012). Phenotypic diversity and plant growth promoting characteristics of *Mesorhizobium* species isolated from chickpea (*Cicer arietinum* L.) growing areas of Ethiopia. *Afr. J. Biotechnol.* **11:** 7483-7493.
3. Mulissa Jida, Lösche, C., Schmitz-Streit, R., Fassil Assefa (2012). Phosphate solubilization efficiency and plant growth promoting properties of rhizobacteria isolated from lentil (*Lens culnaris* Medik.) growing areas of Ethiopia. Submitted.
4. Mulissa Jida, Lösche, C., Schmitz-Streit, R., Fassil Assefa (2012). Diversity, phosphate solubilization efficiency and plant growth promotion of rhizobacteria from chickpea *(Cicer aeritinum L.)* producing areas of Ethiopia. Submiited.

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Chapter 1 Introduction

1.1. General Introduction

Chickpea (*Cicer aeritinum* L.) and lentil (*Lens culinaris* Medk.) are among the major legume crops widely grown in Ethiopia. They are very important both interms of area coverage and total production (CSA, 2011). These legume crops are an important source of dietary protein and are the daily food supplements to majority of the Ethiopian population. In low-input agricultural systems of Ethiopia, chemical fertilizers are rarely used in the production of food legumes; instead, these crops are used as a restorer of soil fertility for the following cereal crops (Asfaw Telaye *et al.*, 1994). Thus, rotation of legume crops such as chickpea, lentil, and others with cereal is commonly used to improve soil fertility and structure, break disease cycle, and thus ensure sustainability and profitability of the crop production systems. Hence, promoting production of legume crops such as chickpea and lentil is very important to improve productivity of cereal crops which are commonly grown in rotation with them.

Though the area of production of chickpea and lentil is very high their yields remained very low compared to their potential (Geletu Bejiga and Ketema Daba, 2006; Gemachu Keneni *et al.*, 2011a). The major constraints to the production of these crops are low available phosphate in soil (Asfaw Tilaye, 1985) and wilt and root rot diseases caused by different fungal pathogens (Seid Ahmed and Melkamu Ayalew, 2006). Among these wilt diseases caused by soil-borne fungi, *Fusarium oxysporum* is the most important biotic stresses of these crops (Seid Ahmed and Melkamu Ayalew, 2006) and it has the potential to cause severe yield losses. Currently, the use of plant growth promoting rhizobacteria (PGPR) inoculants appears to be the most practical, environmentally friendly and economically feasible way of improving phosphorous nutrition and

controlling wilt disease (Ponmurugan and Gopi, 2006; Peix *et al.* 2001b). Hence, screening and selection of rhizobacteria with the potential to be used as inoculants in the production systems of these crops is highly necessitated in the country.

Plant growth promoting rhizobacteria are beneficial bacteria that colonize plant roots and enhance plant growth through a wide variety of mechanisms (Kloepper and Schroth, 1978). The growth promoting activities of rhizobacterial on crops may be manifested directly or indirectly (Glick, 1995; Biswas *et al.*, 2000). The indirect mechanisms include production of siderophore and antimicrobial compounds which may hinder colonization of hosts by phytopathogens, thereby; suppressing the disease they cause (Dakora, 2003).

The direct mechanisms of plant growth promoting activities include nitrogen fixation, solubilization of precipitated mineral nutrients such as phosphate and magnesium, and production of plant growth regulators such as indole acetic acid that induce additional root hair and lateral root formation, thereby enhancing the plant's ability to take up nutrients from soil and increase yield (Biswas *et al.*, 2000). In the context of increasing concern for food and environmental quality, the use of PGPR for reducing chemicals input in agriculture is potentially an important issue. Thus, research in this area is escalating more than ever in the world.

In Ethiopia, the hitherto research activities on chickpea and lentil have given much attention to the genetics studies and variety selection with respect to resistance to different biotic and abiotic stresses (Geletu Bejiga and Yadeta Anbessa, 1999; Yadeta Anbessa and Geletu Bejiga, 2002;

Seid Ahmed and Melkamu Ayalew, 2006; Edossa Fikiru *et al.*, 2007; Gemachu Keneni *et al.*, 2011a; Gemachu Keneni *et al.*, 2011b). Nevertheless, these alone could not result in intended improved productivity of these crops.

Currently, several studies revealed that PGPR could play the vital roles in the production of different crops elsewhere in the world (Antoun *et al.*, 1998; Peix *et al.*, 2001ab; Yanni *et al.*, 2001; Alikhani and Yakhchali, 2009). Despite these only few attempts were made in isolating and characterizing PGPR from Ethiopian soil, and evaluating their potential as biofertilizer and biocontrol agents (Diriba Muleta *et al.*, 2007; Ahmed Idris *et al.*, 2008; Ahmed Idris *et al.*, 2009; Diriba Muleta *et al.*, 2009; Delelegn Woyessa and Fassil Assefa, 2011). These studies indicated that rhizobacteria isolated from rhizosphere of coffee (*Coffee arabica*), sorghum (*Sorghum bicolour*) and tef (*Erograstis tef)* significantly inhibited growth of different phytopathogenic fungi under *in vitro* conditions and reduced incidence and disease severity of wilt and stem rot. In addition, these isolates have also demonstrated that different plant growth promoting traits such as phosphate solubilization and phytohormone production, and significant growth promotion of sorghum (Ahmed Idris *et al.*, 2009).

Similarly, studies (Asefa Keneni *et al.*, 2010; Diriba Muleta *et al.*, 2013) showed that rhizobacteria isolated from faba bean (*Vicia faba*) rhizosphere and natural coffee forests agroforestry systems released a considerable amount of soluble phosphate from insoluble sources. However, little efforts have been made in screening, selecting and evaluating PGPR isolates from chickpea and lentil producing areas of Ethiopia. Thus, this study was initiated to

study plant growth promoting (PGP) properties of rhizobacteria from chickpea and lentil producing areas of Ethiopia.

1.2. Literature Review

1.2.1. Pulse crops production in Ethiopia

Pulses are an important component of agricultural and food systems all over the world. In Ethiopia, pulse crops occupy 11.5% of the total cultivated land and 9.6% of the total production of major crops (CSA, 2011). They are cheap source of protein in the diets of the Ethiopian population. In addition, these crops also play a significant role in export market and enhancing soil fertility. Chickpea and lentil are among the major pulses widely cultivated in the country.

1.2.2. Chickpea

Chickpea (*Cicer arietinum* L.) belongs to family *Leguminoseae* and tribe *Cicereae*. It most probably originated in an area that comprised present-day south-eastern Turkey and adjoining northern Syria (van der Maesen, 1987). This crop is the third most important pulse gown widely in the world (Joshi *et al.*, 2001). Currently, it is common in over 40 countries represented in all continents (Menale Kassie *et al.*, 2009). However, the most important chickpea producing countries are India, Turkey, Pakistan, Iran, Mexico, Australia, Ethiopia, Myanmar, and Canada. In Ethiopia, the earliest finding of chickpea is reported in 1520 BC (Joshi *et al.*, 2001). Hence, it is an ancient crop that has been grown in the country since antiquity.

Chickpea has been widely grown in Ethiopia over 208,388.6 ha of land with annual production of 322,838.8 t and average national productivity 1.55 t ha^{-1} (CSA, 2011). Recently, study indicated that producing area can be extended to the drier agroecological zones of the country

under supplemental and full irrigation (Geletu Bejiga and Ketema Daba, 2006). In addition, Ethiopia is considered as center of diversity for chickpea (Geletu Bejiga and Ketema Daba, 2006; Gemachu Keneni *et al.*, 2011b).

Chickpea seed has high protein content. The seeds are eaten as green vegetable (green immature seed), *Kollo* (soaked and roasted seeds) and *nifro* (boiled seeds). The sauces made of chickpea; shiro (powdered seeds) and *kik* (split seeds) are commonly eaten with *injera* (pancake) made of cereals. The ground seeds could be used to make bread. This crop can also be used in mixture with cereals and root crops in the preparation of childhood food (Geletu Bejiga *et al.* 1996). The residue of this crop is also valuable animal feed. Moreover, it is one of the major export commodities and good source of foreign currency for the country (Geletu Bejiga and Ketema Daba, 2006; Shiferaw Bekele and Teklewolde Hailemariam, 2007).

There are two types of chickpea in the world: the *Desi* type, with small angular brownish colored seeds, widely cultivated in Ethiopia; and the *Kabuli* type, with large, ram shaped, white or pale cream or yellow colored seeds, grown only in small plots in some areas of the country (Shiferaw Bekele and Teklewold Hailamariam, 2007). Morphologically, *desi* types have pink flowers while the *kabuli* types are characterized by white flowers (Menale Kassie *et al.*, 2009). The crop grows in several regions of the country with altitude range of 1400-2300 m.a.s.l. and annual rain fall of 700-2000 mm. It grows on stored soil moisture after the end of rainy season on clay soil. This permits farmers to grow a second crop and secure an additional source of income and protein through efficient use of the residual moisture in black soils at the end of the rains (Geletu Bejiga *et al.*, 1996).

The nitrogen fixed by the crop enriches soil nutrients for the subsequent cereal crops that follow in the rotation system (Shiferaw Bekele *et al.*, 2007). This makes the crop highly integrated into the farming system and highly ecologically friendly for growing in many areas that suffer from soil nutrient depletion. The yield potential of chickpea is as high as 6 t ha^{-1} (Menale Kassie *et al.*, 2009). However, the national average grain yield in Ethiopia is only 1.55 t ha^{-1} (CSA, 2011). This low yield is caused by different biological and physical constraints such as low soil fertility and diseases (Asfaw Tilaye, 1985; Seid Ahmed and Melkamu Ayalew, 2006).

1.2.3. Lentil

Lentil (*Lens culnaris Medik.*) belongs to the *Viceae* tribe in the *Legumnosae* (*Fabaceae*) family, commonly known as the legume family. The center of origin of lentil is the Near East and was first domesticated in the Fertile Crescent around 700BC (Zohary, 1972). It first spread to the Nile from the near east, to Central Europe and then to the Indian Subcontinent and the Mediterranean Basin by the end of the Bronze Age (Cubero, 1981). It is currently an important pulse crop grown widely throughout the Indian Subcontinent, Middle East, Northern Africa, Southern Europe, North and South America, Australia and West Asia (Ford and Taylor, 2003). Ethiopia is among the major lentil producing countries and has been the top producer in Africa. Lentil is widely grown in diverse agroecological zones of central highlands, southeastern, western, northern and northeastern parts of Ethiopia (Asnake Fikre and Geletu Bejiga, 2006).

Lentil is among the major cool season pulses widely cultivated in Ethiopia. The crop covers an area of 77,334.2 ha with annual production of 80,951.7 t and average national productivity of 1.0

t ha^{-1} (CSA, 2011). Its productivity is very low compared to the other producing countries such as Egypt, where the productivity is 1.7 t ha^{-1} (Anteneh Argaw, 2012). In Ethiopia, lentil grows between 1700-3000 m.a.s.l with annual rainfall ranging from 700-2000 mm (Asnake Fikre and Geletu Bejiga, 2006). The country is amongst the centers of diversity for lentil (Edossa Fikiru *et al.*, 2007; Edossa Fikiru *et al.*, 2010).

Lentil is important source of protein for the majority of Ethiopian population. It is consumed in different preparations as split or whole grain sauces with *injera* (pancake) made of cereals, and sometimes as roasted or boiled whole grain snack alone or often mixed with cereals. The relatively high level of lysine in lentil compensates for low concentration in cereal grains hence when consumed in combination gives nutritionally well balanced diet. The straw/haul is an important source of feed for animals.

Besides, lentil is good source of cash for farmers amongst the field crops. It is commonly grown in rotation with cereals to break cereal disease cycles and fix atmospheric nitrogen, thus reducing the demand of other cereal crops for chemical fertilizers (Edossa Fikiru *et al.*, 2007). Hence, its production has been expanding in different areas of the country (Asnake Fikre and Geletu Bejiga, 2006). However, its productivity has been constrained by low soil fertility (Getachew Agegnehu *et al.*, 2006) and susceptibility of the landraces to various diseases and their inherently low yield potential.

1.2.4. Ethiopian soils

Most Ethiopian soils are predominantly characterized by low fertility. Hence, low soil fertility is among the major factors for low productivity of legume crops in Ethiopia. Nitrogen (N) deficiency is one of the most important factors limiting productivity in most Ethiopian soils (Desta Beyene and Angaw Tsigie 1989). Low available P in soil is also among the most limiting soil fertility related factors for crop production in the country (Desta Beyene, 1982; Asfaw Tilaye, 1985; Tekalign Mamo *et al.*, 1988; Dawit Solomon *et al.*, 2002). This deficiency is mainly caused either by the inherent characteristics of the parent material or by the strong fixation, which turns large proportions of total soil P into unavailable forms. The problem is further exacerbated by nutrient mining due to the low-input agriculture practiced in the region (Dawit Solomon *et al.*, 2002).

Application of chemical fertilizers could play a significant role in increasing productivity. However, the increasing cost of chemical fertilizers is becoming unaffordable for farmers of developing countries such as Ethiopia. Therefore, enhancing soil fertility through biological methods such as nitrogen fixation and phosphate solubilization is becoming vital for increasing crop productivity and ensuring food security. Thus, understandings of the characteristics of the rhizobial and PSB populations reside in Ethiopian soils and selection of effective strains which will be used as inoculants is highly necessitated.

1.2.5. The rhizosphere

The term rhizosphere describes the narrow zone of soil surrounding the roots where microbial populations are stimulated by root activities (Verma *et al.*, 2010). The original concept has been

extended and now the rhizosphere can be defined as the volume of soil influenced by plant root and/or in association with roots, root hairs and plant produced material (Andrade *et al.*, 1997; Mahafee and Kloepper, 1997). This includes soil bound to plant roots, often extending a few milli meter (mm) from the root surface (Mahafee and Kloepper, 1997) and the plant root epidermal layers (Verma *et al.*, 2010). The rhizosphere can be demarcated into endorhizosphere, rhizoplane and ectorhizosphere (Bhavdish *et al.*, 2003). Endorhizosphere refers to the internal root area extending generally to the cortical region but which now appears to harbor rather large populations of bacteria with varied functions (Lynch, 1990). The rhizoplane and ectorhizosphere represent surface of the root and adjoining soil component, respectively which often appear as a continuum (Verma *et al.*, 2010).

Plant roots release exudates into the rhizosphere which contain sugars and amino acids, providing rich sources of energy and nutrients for rhizobacteria, resulting in bacterial population greater in this area than the bulk soil (Bhavdish *et al.*, 2003; Verma *et al.*, 2010). The other sources of nutrients include various kinds of secretions, lysates, sloughed-off cells, mucigel, and dead biotic components (Bhavdish *et al.*, 2003). Several biochemical interactions and exchanges of signal molecules between plants and soil microorganisms occur in this zone (Werner, 2001). These interactions can significantly influence plant growth and crop yields. In the rhizosphere, bacteria are the most abundant microorganisms (Antoun and Prevost, 2005) and hence most likely to influence the growth of plants.

1.2.6. Plant Growth Promoting Rhizobacteria and their mechanisms of action

Rhizosphere can be colonized by both beneficial and deleterious bacteria. Beneficial bacteria which are able to colonize rhizosphere and promote plant growth are referred to as plant growth promoting rhizobacteria (PGPR) (Kloepper and Schroth, 1978). They can enhance plant growth through direct or indirect mechanisms (Glick, 1995). PGPR stimulate plant growth directly either by synthesizing phytohormones or by promoting nutrition processes such as nitrogen fixation, phosphate solubilization, or even by accelerating the mineralization processes, and siderophore production, which facilitate iron uptake from soil (Lippmann *et al.*, 1995; Verma *et al.*, 2010). They can also stimulate growth indirectly by protecting the plant against soil-borne fungal pathogens or deleterious bacteria, by competing with them for iron through their siderophores, and by producing antibiotics and/or chitinases and glucanases, which in turn promote the lysis of microbial cells (O'Sullivan and O'Gara, 1992; Somers and Vanderleyden, 2004). Hence, in broad sense, PGPR include the nitrogen-fixing rhizobia, an endosymbiont of the legume (Verma *et al.*, 2010).

Plant growth promoting rhizobacteria associations may range in degree of bacterial proximity to the root and intimacy of association. These associations can be categorized as extracellular PGPR (ePGPR), existing in the ectorhizosphere, on the rhizoplane or in the space between cells of the root cortex and interacellular PGPR (iPGPR), which exists inside root cells including nodules (Verma *et al.*, 2010). The ePGPR include genera such as *Bacillus, Pseudomonas, Burkholderia, Enterobacter, Serratia, Erwinia, Arthrobacter, Flavobacterium, Agrobacterium, Azotobacter, Azospirillum* and others (Gray and Smith, 2005). The iPGPR include a wide range of soil bacteria such as rhizobia producing nodules and others which reside inside the roots

without forming any nodules. Soil bacteria in the genera *Rhizobium, Bradyrhizobium, Sinorhizobium, Azorhizobium, Mesorhizobium* and *Allorhizobium* which invade plant roots and form nodules are well characterized endophytes (Verma *et al.*, 2010).

1.2.6.1. Biological nitrogen fixation

One of the most widely studied beneficial plant-microbe interactions is the symbiotic relationship between legumes and rhizobia. The term rhizobia collectively represent the genera *Rhizobium, Bradyrhizobium, Sinorhizobium, Mesorhizobium, Allorhizobium,* and *Azorhizobium.* Interactions between rhizobia and legume roots result in formation of root nodules, in which rhizobia use energy from the host plant to fix atmospheric N_2 into plant-available forms of nitrogen. The amount of N_2 fixed by a legume crop varies widely because it depends on the legume genotype, rhizobia strain and the soil environment (Lupwayi *et al.*, 2011).

The ability of legumes to fix N_2 allows farmers to grow them with minimal inputs of N-fertilizer. Non-legume crops grown in association or in rotation with them usually have reduced fertilizer N-requirement, which has both economic and environmental benefits. Some of the N_2 fixed by legumes is recycled, mostly during decomposition of above-ground and below-ground crop residues (Lupwayi *et al.*, 2011). Nitrogen sparing is another way in which legume crops contribute N to crops grown intercropped or rotationally. Since part of their N-requirement is met by N_2 fixation, legumes utilize less of the available soil N than cereals, thereby conserving inorganic N for the intercrop or following crop (Herridge *et al.*, 1995).

As a legume, chickpea and lentil can obtain a significant portion of its N requirement through symbiotic N_2 fixation when grown in association with effective and compatible *Rhizobium* and *Mesorhizobium* strains. The proportion of N derived from N_2 fixation could be low particularly where native rhizobial populations are low or inefficient (Beck, 1992). These caused by multitude of biotic and abiotic factors that can influence the success of the legume-*Rhizobium* association. In particular, it is generally agreed that the symbiotic association operates at the highest efficiency in N-limiting systems, particularly if other potentially limiting factors are supplied in adequate quantities (Abaidoo *et al.* 1990).

1.2.6.2. Rhizobia as PGPR

Rhizobia are symbiotic bacterial partners forming nitrogen-fixing nodules on legumes. Appart from this, several studies reported that these bacteria share several plant growth promoting characteristics with other PGPR strains (Antoun *et al.*, 1998; Yanni *et al.*, 2001; Vergas *et al.*, 2009). In addition to their symbiotic N_2 fixation, they are capable of producing phytohormones, siderophores and HCN. They also exhibit antagonistic effects towards many plant pathogenic fungi (Antoun *et al.*, 1998). Rhizobia can directly affect plant growth through the production of phytohormones (Lippmann *et al.*, 1995) since they release growth-promoting molecules such as IAA, gibberellins and cytokinins (Dakora, 2003). Whether present in the rhizosphere as molecules from bacterial saprophytes or present in plant tissues as products released by endophytes, these compounds can massively proliferate root hair production and thus enhance the root's absorptive capacity and nutrient uptake in both legume and non-legume components of cropping systems(Yanni *et al.*, 2001).

These endophytic rhizobia and other bacteria have been found to increase yields of non-legume crops such as rice and maize (Biswas *et al.*, 2000; Lupwayi *et al.*, 2011). These bacteria increase yields by stimulating plant growth, increasing disease resistance, or improving the plant's ability to withstand environmental stresses like drought (Lupwayi *et al.*, 2011). The rhizobia act as plant growth-promoting rhizobacteria (PGPR) that have been shown to expand the root architecture of the crop, enabling those plants to accumulate more N, P and other nutrients than control plants (Yanni *et al.*, 2001). Therefore, rhizobia contribute to the rotational benefits of legumes in cropping systems in more ways than just fixing nitrogen (Lupwayi *et al.*, 2011).

1.2.6.3. Phosphate solubilization

Phosphorus (P), the second most important macronutrient required by plants. It plays an important role in transfer of high energy, cell division, photosynthesis, biological oxidation, metabolism, growth, reproduction and nutrient uptake in plants (Sashidhar and Podile, 2010). P is required in optimum amounts for the growth and development of the plants (Bagyaraj *et al.*, 2000). The plants obtain their P requirements from soil pool. It occurs in soil as inorganic phosphate produced by weathering of parent rocks or as organic phosphate derived from decaying plant, animal and microorganisms (Bagyaraj *et al.*, 2000).

Most soils contain substantial reserves of total P; large part of it relatively remains inert and only less than 10% of soil P enters the plant-animal cycle (Kucey *et al.*, 1989). Hence, most soils have inadequate supply of available P and likely to induce deficiency of this mineral. This problem has been circumvented through application of chemical phosphatic fertilizers. Most of the

applied phosphatic fertilizers are also reprecipitated into insoluble mineral complexes and are not efficiently taken up by the plants (Sashidhar and Podile, 2010).

1.2.6.3.1. Phosphate solubilizing bacteria (PSB)

There has been a continuous search for viable alternatives to the chemical phosphate fertilizers. There are soil microorganisms which could solubilize the insoluble soil P and make it available to the plants (Pal, 1998; Hilda and Fraga, 1999). These microorganisms bring about the solubilization of fixed soil phosphate, resulting in higher crop yields, and therefore are used as biofertilizers. Rhizobacteria from genera such as *Acinetobacter, Agrobacterium, Bradyrhizobium, Burkholderia, Achromobacter, Bacillus, Enterobacter, Erwinia, Flavobacterium, Pseudomonas, Ralstonia, Rhizobium, Mesorhizobium* and *Serratia* are highly efficient in solubilizing unavailable complexed phosphate into available inorganic phosphate ion (Rodriguez and Fraga 1999; Sashidhar and Podile, 2010). The ecological role of these bacteria in soil is also very important because they take part in biogeochemical cycles of P in the ecosystems (Saha and Biswas, 2009).

1.2.6.3.2. Mechanisms of inorganic phosphate solubilization

Phosphate solubilizing bacteria use different mechanisms to solubilize the insoluble forms of the phosphate. Organic acids which are released by the PSB are good chelators of divalent cations of Ca^{2+} accompanying release of phosphates from insoluble phosphatic compounds (Sashidhar and Podile, 2010). These organic acids could also form soluble complexes with metal ions associated with insoluble 'P', thus releasing the phosphate. Many of the PSB lower the pH of the medium either by H^+ extrusion (Illmer and Schinner, 1995) or by secretion of organic acids such as

acetic, lactic, malic, succinic, tartaric, gluconic, 2-ketogluconic, oxalic and citric acids (Kucey *et al.* 1989; Bolan *et al.*1994).

1.2.6.3.3. Rock phosphate, animal bone and PSB as biofertilizer

Use of rock phosphate as fertilizer for P-deficient soils has received significant interest in recent years since they are natural, inexpensive and available fertilizers. However its solubilization rarely occurs in non-acidic soils (Caravaca *et al.*, 2004). Physical and chemical treatment like thermal alteration and partial acidification, for increasing the P availability from rock phosphate (RP) are cost intensive and makes the environment health worse (Kang *et al.*, 2008). An alternative has been the use of microorganisms with the capability to solubilize RP and release soluble P. Biosolubilization of RP and its use in agriculture is receiving greater attention during the last decade, and several reports have indicated that some microorganisms are capable of solubilizing insoluble RP and releasing soluble P (Hilda and Fraga, 1999; Ivanova et al., 2006: Asefa Keneni *et al.*, 2010).

Animal bones are also good source of P that can be recycled. They have been little used in agriculture (Warren *et al.*, 2009). It mainly consists of P and Ca, and could be used as a P fertilizer. However, its low solubility limits direct application of bone to soil. Studies indicated that its P solubility could be improved by simultaneous application of phosphate solubilizing bacteria (Asefa Keneni *et al.*, 2010; Postma *et al.*, 2010). Technologies producing safe P fertilizers from animal bones will facilitate the recycling of P from food waste. The risk of transmitting diseases could be minimized by carbonization of animal bone meal, i.e. high heat treatment, results in a porous product which is called animal bone charcoal. Furthermore, the

porous structure and large specific surface area of animal bone charcoal make it an ideal carrier to harbor PSB (Postma *et al.*, 2010).

1.2.6.4. Indole acetic acid (IAA) production

Phytohormones production is among the direct mechanisms of plant growth promotion by PGPR (Glick, 1995). Indole acetic acid (IAA), a member of the group of phytohormones, is generally considered to be the most important auxin in plants (Ashrafuzzaman *et al.*, 2009). IAA is involved in controlling many important physiological processes including cell enlargement and division, tissue differentiation, and responses to light and gravity (Leveau and Lindow, 2005). Production of the IAA is widespread among plant-associated bacteria (Patten and Glick, 1996; Arshad and Frankenberger, 1993). Promotion of root growth is one of the major markers by which the beneficial effect of plant growth-promoting bacteria is measured (Glick, 1995). Rapid establishment of roots, whether by elongation of primary roots or by proliferation of lateral and adventitious roots, is advantageous for young seedlings as it increases their ability to anchor themselves to the soil and to obtain water and nutrients from their environment, thus enhancing their chances for survival (Patten and Glick, 2002; Leveau and Lindow, 2005).

Many rhizobacteria isolated from the rhizosphere have the capacity to synthesize IAA *in vitro* in the presence of physiological precursors, mainly tryptophan (Costacurta and Vanderleyden, 1995; Lee *et al.*, 2004; Diriba Muleta *et al.*, 2009; Mulissa Jida and Fassil Assefa, 2012). Microbial isolates from the rhizosphere of different crops appear to have a greater potential to synthesize and release IAA as secondary metabolites because of the relatively rich supply of substrates (Barazani and Friedman, 1999, Costacurta and Vanderleyden, 1995; Lee *et al.*, 2004).

Production of IAA by microbial isolates varies greatly among different species and strains and depends on the availability of substrate (Lee *et al.*, 2004).

1.2.6.5. Siderophore production

Though iron is the most abundant element in the earth's crust its insolubility highly constrains its availability for microbial assimilation. The availability of iron in highly heterogeneous environments such as rhizosphere is extremely limiting for microbial growth and survival (Lugtenberg and Kamilova, 2009). Thus, their ability to survive and proliferate in such environment depends on their ability to scavenge sufficient iron from a limiting pool. In aqueous solution iron exists in ferrous (Fe^{2+}) or ferric (Fe3+) form. The latter being less soluble and predominantly exists in highly oxidized and aerated soils. Consequently, microorganisms secrete iron-binding ligands called siderophores to survive in such environments (O'Sullivan and O'Gara, 1992; Lugtenberg and Kamilova, 2009). These compounds can bind the ferric iron and make it available to the host microorganisms.

Soil pseudomonads generally produce fluorescent, yellow-green, water-soluble siderophores that can be classified as pyoverdin or pseudobactin (O'Sullivan and O'Gara, 1992). These fluorescent siderophores differ in composition, number and configuration of amino acids in their peptide backbone (Neilands, 1986). The production of these compounds has been implicated in the disease suppression ability of fluorescent *Pseudomonas* species (Loper and Buyer, 1991). The mechanism of siderphore-mediated disease suppression is that these compounds are secreted by fluorescents *Pseudomonas* species under low-iron growth conditions (O'Sullivan and O'Gara, 1992). These ligands form complex with ferric iron which is available to the producing strains

through specific receptors on the outer surfaces of their cell membrane (Buyer and Leong, 1986). In this way, fluorescent pseudomonads restrict the growth of deleterious bacteria and fungi in the rhizosphere. It has been reported that plants are capable of obtaining iron from some microbial siderophores (O'Sullivan and O'Gara, 1992).

1.2.6.6. PGPR as Biological control of plant diseases

Plant diseases are responsible for significant losses of crops yield globally. Resistant plants and synthetic chemicals are often used to control plant disease. Resistance does not exist against all diseases and the breeding of resistant plants takes many years (Lugtenberg and Kamilova, 2009). The use of agrochemicals is negatively perceived by consumers. These initiate the search for alternative methods to control plant diseases. The use of microbes to control diseases, which is termed as biological control, is an environment-friendly approach. PGPR produces secondary metabolites on or near the plant surface, i.e., the site where it should act. Moreover, the molecules of biological origin are biodegradable compared with many agrochemicals that resist degradation by microbes (Lugtenbergand Kamilova, 2009). Mechanisms responsible for this biocontrol activity include competition for nutrients, niche exclusion, induced systemic resistance, and the production of anti-fungal metabolites (Bloemberg and Lugtenberg, 2001).

1.2.6.7. *Fusarium* wilt of chickpea of lentil

Fusarium wilt caused by *Fusarium oxysporum* is globally the major disease of chickpea and lentil (Bakhsh *et al.*, 2007). It has been the major yield-limiting factor in Ethiopia and other producing countries which could cause up to 40% yield loss (Nene *et al.*, 1984; Seid Ahmad *et al.*, 1990; Bakhsh *et al.*, 2007; Ahmad *et al.*, 2010). Chemical control of wilt is not much

effective and economical because the pathogen is soil as well as seed-borne in nature and is difficult to eradicate (Bakhsh *et al.*, 2007). Furthermore, fungal chlamydospores survive in soil up to 6 years even in the absence of the host plants (Haware *et al.*, 1996; Singh *et al.*, 2008). The use of resistant cultivars is an eco-friendly and economically viable strategy to minimize losses caused by wilt (Ahmad *et al.*, 2010; Nene and Haware, 1980; Bakhsh *et al.*, 2007; Singh *et al.*, 2008). However, this is limited due to the prevalence of location specific races of the wilt pathogen (Singh *et al.*, 2008). Furthermore, cultivars with high yield and wilt resistance are not yet widely available to farmers in many countries (Ahmad *et al.*, 2010). Thus, an integrated disease-management approach is essential to combat fusarium wilt. Such disease-management strategy should include PGPR biocontrol agents.

1.2.7. Status of PGPR research in Ethiopia

In Ethiopia, most research activities on biofertilizer have focused mainly on symbiotic nitrogen fixation of major legume crops. Several studies were carried out to identify and characterize symbiotically effective indigenous rhizobial strains isolated from faba bean, field pea, chickpea and lentil (Ayneabeba Adamu *et al.*, 2001; Asfaw Hailemariam and Angaw Tsige, 2006; Zerihun Belay and Fassil Assefa, 2011; Anteneh Argaw, 2012). Attempts were made in mass producing and distributing selected strains of rhizobia to farmers in various parts of the country (Asfaw Hailemariam and Angaw Tsige, 2006; Adane Abraham, 2009). However, there is no report on their PGP properties apart from nitrogen fixation.

Several studies attempted to isolate and characterize PGPR from Ethiopian soils, and evaluating their potential as biofertilizer and biocontrol agents (Diriba Muleta *et al.*, 2007; Ahmed Idris *et*

al., 2008; Ahmed Idris *et al.*, 2009; Diriba Muleta *et al.*, 2009; Delelegn Woyessa and Fassil Assefa, 2011). Similarly, studies (Asefa Keneni *et al.*, 2010; Diriba Muleta *et al.*, 2013) showed that rhizobacteria isolated from faba bean (*Vicia faba*) rhizosphere and natural coffee forests systems released a considerable amount of soluble phosphate from insoluble sources. However, the biofertilizers and biocontrols selected locally are not yet commercialized (Adane Abraham, 2009).

1.3. Objectives of the Study

The General Objective of this study was: to study plant growth promoting properties of rhizobacteria isolated from chickpea and lentil producing areas of Ethiopia and to determine their role in these crop production systems.

The Specific objectives of the current work were:

- To isolate and identify rhizobacteria with plant growth promoting activities from rhizosphere and root nodules of chickpea and lentil.
- To evaluate phenotypic diversity of rhizobia isolated from root nodules of chickpea and lentil
- To investigate phosphate solubilizing capacity of the rhizobacteria isolated from chickpea and lentil.
- To evaluate IAA producing ability of different rhizobacteria isolated from chickpea and lentil.
- To investigate the *in vitro* inhibitory activities of the rhizobacterial isolates against *Fusarium oxysporum.*
- To evaluate the effects of inoculation of selected rhizobacterial isolates on the growth of chickpea and lentil under glasshouse conditions.

Chapter 2 Phenotypic and plant growth promoting characteristics of *Rhizobium* species from lentil Growing areas of Ethiopia

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Abstract

Lentil (*Lens culnaris* Medik.) is an invaluable source of protein for the vast majority of Ethiopian population. It also maintains soil fertility through symbiotic nitrogen-fixation in association with *Rhizobium species*. The aim of this study was selecting symbiotically efficient native lentil nodulating rhizobia endowed with different plant growth-promoting (PGP) characteristics. A total of 30 lentil nodulating rhizobial isolates were retrieved from soils collected from different farmer fields found in central and northern parts of the country. All isolates were characterized based on morphological, physiological, symbiotic and PGP characteristics. The result of this study showed that these isolates exhibited interesting features such as wide range of carbon-sources and nitrogen sources utilization, tolerance to acidic and alkaline pH, metal toxicity and antibiotics. Symbiotic characterization indicated that all the tested isolates have showed great diversity in their capacity to nodulate their host plant and produce shoot dry matter yield under glasshouse conditions. In addition, they showed PGP characteristics such as IAA production and inorganic phosphate solubilization. Out of all the tested isolates 36.7% of them were IAA producer while only 16.7% were insoluble inorganic phosphate solubilizers. In general, from the present study, it can be concluded that Ethiopian soils harbor highly efficient nitrogen-fixing lentil nodulating rhizobia which are diverse in their morphological, physiological and symbiotic characteristics.

Key words: *IAA production, Nodulation, N_2-fixation, PGP, Phosphate solubilization*

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Introduction

Lentil (*Lens culnaris* Medik.) is an important pulse crop grown widely through out the world (Erskine, 1997; Ford and Taylor, 2003). It is also one of the principal cool season food legumes widely grown in Ethiopia over an area of 77, 334.2 ha with annual production of 80, 951.7 tones (t); the average national productivity being about 10.5 t/ha (CSA, 2011) which is very low compared to its potential (Asfaw Hailemariam and Angaw Tsige, 2006). Nutritionally lentil contains 23 - 24% of protein (Werner, 2005) for the vast majority of Ethiopian population.

Lentil is consumed in different preparations, as a split or whole grain with the cereal *injera* (pan cake) and sometimes as roasted or boiled whole grain snack alone or often mixed with cereals. The relatively high level of lysine in lentil compensates for low concentration in cereal grains, hence when consumed in combination gives nutritionally well balanced diet (Muelbauer *et al.*, 1985) and thus contribute for food security. Besides, lentil is leading in fetching the local market price and giving significant export market option out of field crops (Asnake Fikre and Geletu Bejiga, 2006). The straw/haul is also an important source of feed for animals fattening.

In addition to it's nutritionally quality and source of cash, lentil restores and maintains soil fertility through its symbiotic nitrogen-fixation in association with *Rhizobium leguminosarum* (Jordan, 1984). This legume symbiotic nitrogen fixation is often the most feasible and economical N input for resource-poor Ethiopia farmers. It is capable of supplying 80 kg N ha^{-1} to the soil (Werner, 2005). Consequently, it is grown in rotation with major cereals such as tef

(*Erograstis tef*), wheat (*Triticum* sp.), barley (*Horduem vulgare*) and others as restorer of soil fertility instead of using chemical fertilizer for its production. Hence, its yield has remained very low and thus many researches have been undertaken to improve lentil cultivars with respect to their yield, tolerance to different diseases and environmental stresses (Edossa Fikiru *et al.*, 2007; Asnake Fikre and Geletu Bejiga, 2006; Geletu Bejiga and Yadeta Anbessa, 1999; Geletu Bejiga and Yadeta Anbessa, 1995). Nevertheless, these alone could not improve the extremely low productivity of lentil. One of the research areas which have been given less attention was exploiting the benefits of its symbiotic nitrogen fixation by selecting effective *Rhizobium* strains. Therefore, research attention should also be given to characterization, selection and utilization of its rhizobia to improve the amount of nitrogen fixed so as to increase yield.

Several studies showed that some *Rhizobium* species strains possess plant growth promoting characterstics in addition to their symbiotic N_2 fixation and are considered as plant growth promoting rhizobacteria (PGPR) (Antoun *et al.*, 1998; Yanni *et al.*, 2001; Somers and Vanderleyden, 2004). Rhizosphere bacteria that are capable of stimulating plant growth by colonizing roots are known as PGPR (Kloepper and Schroth, 1978). PGPR stimulate plant growth directly either by synthesizing phytohormones such as indole-3-acetic acid (IAA) or by promoting nutrition processes such as phosphate solubilization and siderophore production, which facilitate phosphorus and iron uptake, respectively from soil (Lippmann *et al.*, 1995). They can also stimulate growth indirectly by protecting the plant against soil-borne fungal pathogens (O'Sullivan and O'Gara, 1992). Several studies showed that *Rhizobium* species share characteristics such as phosphate solubilzation, phytohormones and siderophore production, and biocontrol activity with PGPR strains (Antoun *et al.*, 1998; Yanni *et al.*, 2001; Vergas *et al.*,

2009). Such kind of rhizobial strains could be used as broad-range and multipurpose inoculants for both legume and non-legume crops grown rotationally or concurrently. Thus, local rhizobial isolates must be screened for their plant growth-promoting (PGP) activity in addition to their excellent symbiotic performance.

Rhizobium species strains naturally vary in their nitrogen fixing capacity and adaptation to prevailing environmental stresses (Zharan, 1999). Consequently, selection of symbiotically efficient rhizobial strains which are tolerant to locally prevailing stresses is very important. Several studies were conducted on lentil rhizobia characterization and selection of best strains for inoculant production elsewhere in the world where this crop is commonly grown (Athar, 1998; Sajjad *et al.*, 2008; Rashid *et al.*, 2009). Athar (1998) studied drought tolerance pattern of lentil rhizobia isolated from arid and semiarid areas and indicated that strains originated from saline areas showed significantly better survival under low water potential. In addition, Sajjad *et al.* (2008) studied the genetic diversity of lentil nodulating rhizobia elsewhere by using random amplified polymorphic DNA (RAPD) markers and obtained a considerable genetic diversity among them. Rashid *et al.*(2009) also characterized lentil rhizobia using both physiological and molecular methods and showed that there was a considerable variation among different strains.

However, very little attention has been given to lentil rhizobia characterization and utilization in Ethiopia. These necessitates the need for research activities devoted to isolation and characterization of lentil root nodule bacteria which eventually leads to the selection of strains which are adapted to local environmental conditions. Hence, this study was initiated with the aim

of investigating phenotypic and PGP characterstics of lentil nodulating rhizobia isolated from major producing areas of Ethiopia.

Materials and methods

Study sites and soil samples collection

Soil samples were collected from the lentil grown fields found in Amhara, Oromiya and Tigray Regional States of Ethiopia, which are the major lentil producing areas of the country (Table 2.1). The areas are distributed in central and northern parts of the country (Fig 2.1). Thirty-five samples were collected in October, 2009. Lentil agricultural fields with no previous history of inoculation with rhizobia were selected from each site and soil samples were excavated from the upper15-20 cm depth, pooled together and collected in sterile plastic bags. About three kg of composite soil samples collected from each sites were carefully transported to the Applied Microbiology Laboratory, Addis Ababa University, for further work.

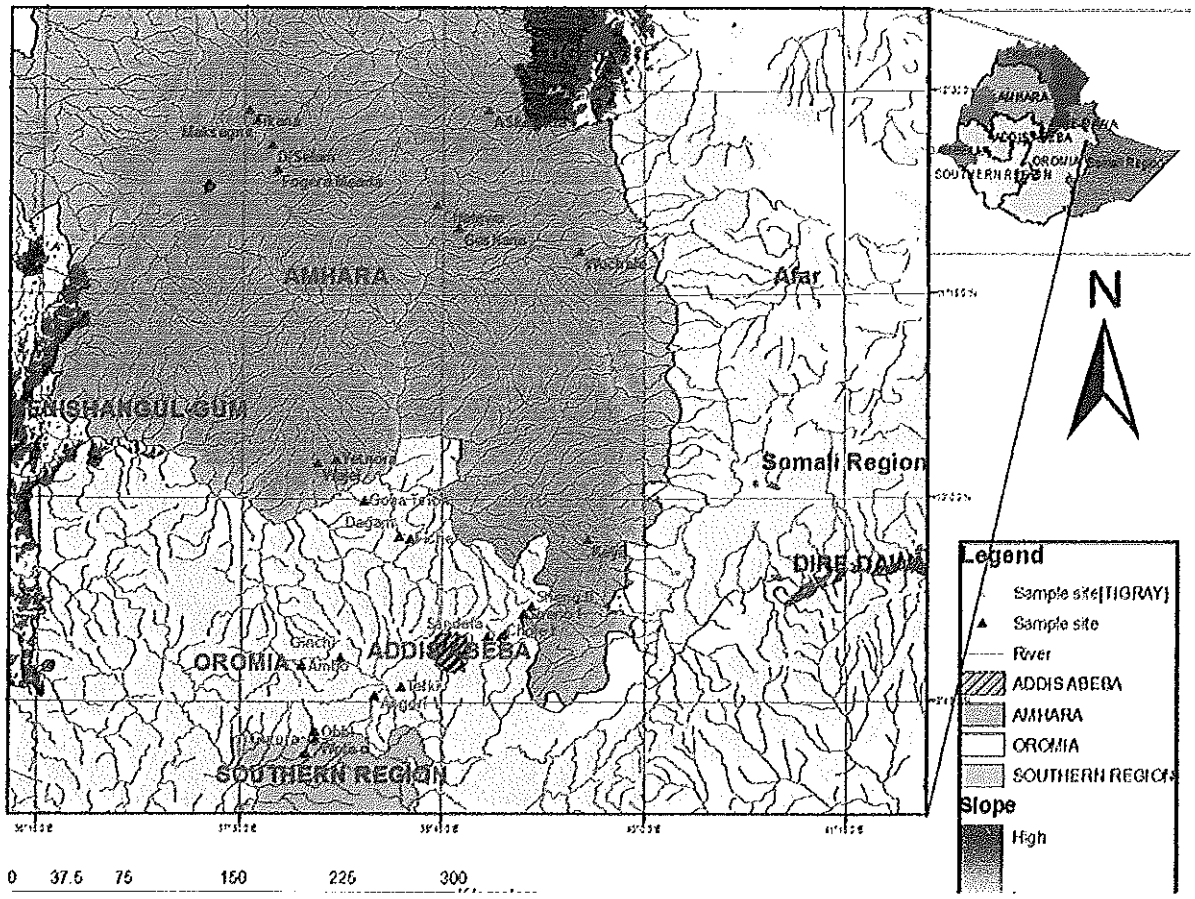

Figure 2.1 Lentil Soil sample collection sites

Isolation of rhizobia

Rhizobia were isolated from soil samples by inducing nodulation on lentil cultivar called 'Gudo' obtained from Debre Zeit, Ethiopian Instituite of Agriculture Research (EIAR) using plant infection method (Vincent, 1970). Each soil sample was thoroughly mixed and air-dried in a glasshouse. Air-dried soil samples were grinded and passed through a 2 mm sieve to remove stones and large pieces of organic matter, and then filled into 3 kg capacity surface sterilized plastic pots.

Lentil seeds were selected and surface sterilized with 95% ethanol and 0.1% acidified mercuric chloride solutions for three minutes with each (Vincent, 1970) and rinsed five times with sterilized distilled water to remove traces of sterilizing chemicals. Surface sterilized seeds were allowed to germinate on sterile water agar (1%) surface for three days at 25^0C and five pre-germinated seeds were planted on each pot, which were thinned down to three after 5 days of emergence (DAE). All pots were situated in glasshouse and watered to a field capacity every three days for 45 days after planting (DAP).

Forty-five DAP, all the plants were carefully uprooted from the pots and washed under gently flowing tap water to remove soil particles. Large and pink nodules were separately collected from each pot on separate sterile Petri dishes and surface sterilized as described before, and crushed using alcohol flamed glass rod. Loopful of the extract was streaked on Yeast Extract Mannitol Agar (YEMA) containing 0.0025% (w/v) Congo red (CR) (Vincent, 1970). The components of YEMA g/l: 0.5 K_2HPO_4, 0.2 $MgSO_4$, 0.1 NaCl, 10 Mannitol, 0.5 Yeast Extract, 15 Agar (Vincent, 1970). All the plates were incubated at 28±2 °C for 5 days. From each plate, single typical rhizobial colony was picked and transferred to a test tube which contained sterile Yeast extract Mannitol Broth (YEMB) (Vincent, 1970). The test tubes were incubated at 28 ±2^{0}C for 3 days and purified by re-streaking on YEMA plates for growth. The pure cultures were further confirmed by presumptive tests such as gram reaction and growth on Peptone Glucose Agar (PGA) (Vincent, 1970). Pure isolates were then preserved on YEMA slants containing 0.3% $CaCO_3$ stored at 4°C for short-term (Vincent, 1970) and 50% glycerol at -20°C for long-term preservations. All the rhizobial isolates were designated as LR1-30 (L: Lentil; R: Rhizobia) (Table 2.1).

Colony morphology, Growth and Biochemical Characterizations

Colony morphology (colour, mucoidty, transparency, and borders) was evaluated by streaking a loop of the initial inoculum on YEMA plates and allowing the bacteria to grow at 28°C for 5 days (Vincent, 1970; Somasegaran and Hoben, 1994). Bacterial growth was assessed by inoculating 20 µl of the initial inoculums into 100 ml YEMB in flask (250 ml). The flasks were incubated on a gyratory shaker at120 revolution min^{-1}(rpm). The growth rate was monitored by measuring the optical density at 540 nm every 6 hrs and spread plating 0.1 ml diluted culture on YEMA. The generation time (GT) was calculated from the logarithmic phase of growth. Acid or alkali production ability of the isolates was tested by growing isolates on YEMA medium containing bromothymol blue (BTB) (Somasegaran and Hoben, 1994).

Rhizobial inoculum preparation

Rhizobial isolates were grown to exponential phase in YEMB on a gyratory shaker at room temperature and with 120 rpm. The suspension was centrifuged in sterile plastic tubes (10 ml) at 5000 rpm^{1} for 10 min. The pellets were re-suspended in normal saline (0.85% w/v of NaCl) solution to give a final concentration of 10^8 CFU/ml using the viable plate count method and optical density measurement using spectrophotometer at 540 nm. All inoculums were prepared like this unless stated.

Physiological characteristics

All tests, except carbohydrate and N-source assimilation, were carried out on YEMA plates. Petri dishes containing YEMA medium were inoculated with 10 µl of the initial inoculum. After 5 days of incubation at 28 °C, bacterial growth was compared with the controls. Three replicates were included per treatment.

Salt, pH and Temperature tolerance

Salt tolerance of the isolates was determined on YEMA plates containing from 0 to 2% (w/v) NaCl concentrations. Tolerance to acid or alkaline pH was tested on medium set at different pH values using 1N HCl and 1N NaOH whereas temperature tolerance was evaluated on agar plates inoculated as described above and incubated at temperatures from 4 to 40° C.

Intrinsic antibiotic and heavy metal resistance

This intrinsic antibiotic and heavy metal resistance was determined on medium containing the following filter sterilized antibiotics or heavy metals (µg ml^{-1}): ampicillin (5 and 10), chloramphenicol (5 and 10), erythromycin (5 and 10), nalidixic acid (5 and 10), rifampcin (5 and 10), streptomycin (10 and 50), neomycin (5 and 10) and tetracycline (5 and10); $AlK(SO_4)_3.12H_2O$ (10, 25), $K_2Cr_2O_7$ (50), $CoCl_2$ (10), $CuCl_2.2H_2O$ (10), $HgCl_2$ (5), $MnCl_2$ (50, 75), $NiSO_4$ (10), $Pb(CH_3COO)_2$ (10), and $ZnCl_2$ (50). Aluminium and manganese toxicity tolerance test was carried in YEMA medium at pH 5.0 only for isolates which grew at this pH value and below.

Utilization of different C-sources and N-sources

The isolates were tested for their capacity to utilize different substartes on agar plates. The plates were inoculated with 10 µl of the initial inoculum. Different carbohydrates were added as described by Amarger et al. (1997) at final concentration of 1 mgl^{-1} to the basal medium containing (gl^{-1}): K_2HPO_4, 1; KH_2PO_4, 1; $FeCl_3$.6H2O, 0.01; $MgSO_4.7H_2O$, 0.2; $CaCl_2$, 0.1; NH_4SO_4, 1; and 15 gm of agar. The following filter sterilized (0.22µm milipore) sole carbon sources were added after autoclaving: Citrate, D-Sorbitol, D-mannose, D-glucose D-maltose, D-galactose, D-arabinose, gluconate, raffinose, xylose, dulcitol, cellobiose, adonitol, inulin, aesculin, trehalose and inositol. The following carbon souces were added before autoclaving: D-mannitol, α-lactose, D-fructose, glycerol, α-cellulose, sucrose, and dextrin. Filter sterilized L-Tryptophan, Methionine, L-Tyrosine, Leucine, L-Asparagine, DL-β-Phenylalanine, L-Arginine, Glutamic acid, L-Lysine, Analanine, Serine, Glycine, Thiamine, Niacin and Riboflavin were used as sole nitrogen source for the isolates by adding a final concentration of 0.5 g/l to the above basal medium from which $(NH_4)_2SO_4$ was omitted and mannitol was added (Amarger *et al.*, 1997). Inoculated palates were incubated at 28 °C and the results were observed after 3-5 days.

Numerical analysis

Physiological tests such as different substrates utilization and intrinsic antibiotic and heavy metals resistance were used to construct phenogram. Traits were coded 1 for positive and 0 for negative. The final matrix contained 30 isolates and 62 traits. A computer cluster analysis of 62 phenotypic variables was carried out using Ward's linkage and Euclidian distance as a measure of dissimilarity coefficient(Ward,1963) and a phenogram was constructed PAST ver.2.17c (Hammer *et al.*, 2001).

PGP characterstics of rhizobial isolates

Phosphate solubilization

Phosphate solubilizing (PS) ability of the isolates was determined using Pikovaskya (Pikovskya, 1948) agar plates which were spot inoculated with a 10 µl of the initial inoculum. After incubation at 28±1°C for 5 days, formation of a clear zone around the spot was recorded and their solubilization index (S.I.) was calculated as the ratio of colony and halo zone diameter to colony diameter (Edi–Premono *et al.*, 1996).

Screening for In Vitro Antagonistic Activity against Fungal Pathogens

The *in vitro* mycelial growth inhibition of the isolates against *Fusarium oxysporum f.sp. lentis* (obtained from Debre Zeit, EIAR) was tested using the dual culture technique as described by Landa *et al.*(1997). Ten µl of the inoculum of each rhizobial isolates was equidistantly placed on the margins of YEMA plates amended with sucrose (0.5%) and incubated at 28 °C for 24 h. A 4-5 mm agar disc from PDA cultures of the fungal pathogens was also be placed at the centre of the YEMA plate for each bacterial isolate and incubated at 28 ± 2 °C for 5-7 days. The presence of inhibition was observed.

IAA production

The inoculum (10 µl) of rhizobial isolates were inoculated into YEMB medium (10 ml) amended with 5 mM L-tryptophan allowed to grow for 72 h (Bric *.et al.*, 1991). The cultures were centrifuged at 10,000 rpm for 15 min and 2 ml supernatant of the culture was transferred to a new tube (10 ml) to which 100 µl of 10 mM O-phosphoric acid and 4 ml of Salkowaski reagent (1 ml of 0.5 mM $FeCl_3$ in 35% $HClO_4$) were added. The mixtures were incubated at room temperature for 25 min and observed for pink color development.

Authentication and symbiotic characterization of the isolates

All rhizobial isolates were authenticated by re-inoculating on their previous host, cultivar Gudo. Simultaneously, the symbiotic effectiveness of the strains was also evaluated in sand culture. About 3 kg of acid washed and autoclave sterilized river sand was placed in plastic pots (3 kg capacity). Lentil seeds were selected and surface sterilized, and germinated as described before. Germinated seedlings were transferred into sterile Petri plates and flooded with each rhizobial inoculum adjusted to 10^9 cells per seed for one hour. Five inoculated seedlings were transferred to each pot and thinned down to three after 5 DAE.

The pots were irrigated with nitrogen free plant nutrient solution as described by (Somasegaran and Hoben, 1994). Uninoculated nitrogen-fertilized pots were included as positive (TN) and uninoculated (T0) pots as negative controls. Furthermore, TN control received 0.05% (w/v) KNO_3 as nitrogen source weekly while all other pots received the same initially as starter nitrogen source only once. The experiment was statistically laid out with three replications using randomized block design. Plants were supplied with distilled water every three days. Sixty DAP all seedlings were carefully uprooted, and nodule number counted, and nodule dry weight and shoot dry weight were measured after drying at 70^0C for 48 hrs to the constant weight. The relative symbiotic effectiveness of the isolates was calculated by using the formula (100 × inoculated plant shoot dry weight/ N-fertilized plant shoot dry weight) according to Gibson (1987).

Data analysis

Symbiotic characterization data was analyzed by analysis of variance and the treatment means were compared following Duncan's test (DT) by using SPSS (V.17). Correlations among some parameters were checked by using bivariate regression analysis using SPSS.

Results and discussion

Growth characteristics and Colony morphology

On the basis of their generation times 80% of the isolates were fast growers with mean generation time (MGT) 1-3 h, whereas 20% of them were relatively slow growers with 3h<MGT<4h (Table 2.1). There was no correlation($r<0.5$; $p>0.05$) between growth rate of the isolates and their distribution in acid, alkaline and neutral soil pH of different sampling sites. Similarly, Maâtallah *et al.*(2002) also showed that fast, slow and very slow growing rhizobia from chickpea were found in the same soil.

All isolates formed colony with circular shape, entire margin, milky-to-watery translucent to creamy opaque features with different level of mucus production (Table 2.1). Most isolates exhibited copious production of exoplosaccharide while the remaining isolates showed less mucus production (Table 2.1). The colony diameter of the isolates was ranged from 2.5 mm to 3.9 mm indicating that all isolates could be able to form large colony on YEMA surfaces after 5 days of incubation. Smilarly, Anteneh Argaw (2012) observed that lentil nodulating rhizobial isolates obtained from Ethiopian soils formed colonies with dome-shaped, shiny appearance, buttery texture, gelatinous due to the production of exopolysaccharides, and colony diameter ranged from 3.5 to 4.5 mm after 5 days incubation. According to Jordan (1984) such

characteristics are common among fast growing rhizobia in general and *Rhizobium leguminosarum* bv. *viciae* cross inoculation group in particular.

Table 2.1 Distribution and charactersitcs of lentil rhizobial isolates from different sampling sites of Ethiopia

Isolate	Name of isolation site	Altitude of isolation site (masl)	pH of isolation soil	Colony characteristics	Colony diameter (mm)	MGT (hour)
LR1	Shenoll	2868	5.58	LMLM	2.6	3.7
LR2	Ambo	2170	6.75	LCM	3.1	2.5
LR3	Keyit	2931	6.38	LMM	3.9	2.1
LR4	Wajel	2432	6.12	LCLM	3.1	2.6
LR5	Sandafa	2554	6.00	LMLM	3.3	2.7
LR6	D/Selam	1896	6.00	LCM	3.7	1.9
LR7	Fogera meda	1799	6.00	LCM	3.6	2
LR8	Tefki	2047	6.55	LCM	3.1	2.8
LR9	Makesagnit	1978	6.39	LWM	2.5	3.4
LR10	Yetinora	2437	6.21	LWM	3.0	2.3
LR11	Teji	2065	6.70	LCM	3.2	2.2
LR12	Shenol	2032	5.58	LCM	3.3	2.1
LR13	Asgori	2078	6.20	LCM	2.8	2.9
LR14	Chatawa	2912	5.12	LWM	2.9	2.3
LR15	Cholell	2647	6.05	LCM	3.1	2.4
LR16	Tikana	1942	7.18	LWM	3.2	2.5
LR17	Wuchale	1980	6.58	LMM	3.1	2.6
LR18	Obbi	2108	6.61	LWLM	2.5	3.8
LR19	Gurura	1906	7.01	LCM	3.3	2.3
LR20	Cholel	2612	5.58	LWM	3.4	2.4
LR21	Asketema	2499	7.04	LCM	3.6	2.2
LR22	Dagam	3101	6.36	LWM	2.6	3.5
LR23	Goha Tsion	2517	6.3	LCM	3.5	2.1
LR24	Woliso	2008	5.58	LWM	3.4	2
LR25	Adanaba	2533	7.03	LMLM	2.6	3.6
LR26	Fiche	2748	6.83	LWM	3.1	2.3
LR27	Korem	2482	6.93	LWM	3.0	3.2
LR28	Geshana	2907	5.80	LWM	3.2	2.4
LR29	Ginchi	2250	6.60	LWM	3.0	2.4
LR30	Fala'a	2549	7.00	LWM	3.1	2.8

LCM: large, creamy, mucoid; LWM: large, watery, mucoid; LMLM: large, milky, less mucoid; LWLM: large watery,less mucoid; LCLM:large, creamy less mucoid,, MGT: mean generation time

All isolates changed the color of YEMA supplemented with BTB to yellow (data not shown) indicating that they are acid producers and hence possible to categorize them as fast-growers. The CR absorption test also indicated that none of the isolates absorbed CR on YEMA plates (data not shown). This is a distinctive character of rhizobia with only few exceptions (Somasegaran and Hoben, 1994). Similarly, previous studies (Anteneh Argaw, 2012; Zerihun Belay and Fassil Assefa, 2011) indicated that *R. leguminosarum* isolates recovered from Ethiopian soils did not absorb CR and changed the BTB color to yellow on YEMA medium. On the other hand none of the tested isolates grew on PGA plates. In addition, all tested isolates were gram negative (Table 2.1). These indicate that all isolates were not contaminant rather fast growing rhizobia (Somasegaran and Hoben, 1994).

Physiological characteristics

Tolerance to acidic and alkaline pH

As shown in Table 2.2, the lentil nodulating rhizobia showed varation in their different pH tolerance. All tested isolates grew in mildly acid pH (5.5) to neutral pH and slightly alkaline pH (8.0). However, isolate LR1, LR10, LR15, LR17, LR18, LR20, LR21 and LR27 were tolerant to pH 4.5 indicating that only 26.7 % of the isolates exhibited an acid tolerant character. On the other hand only 13.3 % of the isolates (LR16, LR19, LR21, LR25, LR27 and LR30) grew at pH 8.5. None of these isolates could grow at pH 9.0. Rashid *et al.* (2009) also found that lentil nodulating rhizobia grow well at acidic pH as low as pH 4 and alkaline pH as high as pH 10.

Table 2.2 Physiological characteristics of lentil rhizobial isolates

Isolate	pH tolerated	NaCl % tolerated	Temperature tolerated	C-sources % utilized	N-sources % utilized	IAR pattern	Heavy metals resistance
LR1	4.5-8.0	1	15-35	92.3%	86.7	St,Na, ch	Cr
LR2	5.5-8.0	0.75	20-30	88.5	80.0	Na, Ch	Cr
LR3	5.5-8.0	0.75	20-30	38.5	60	ch	-
LR4	5.5-8.0	0.75	15-35	92.3	86.7	Na, ch	Cr,Zn
LR5	5.5-8.0	0.75	20-30	30.8	33.3	-	-
LR6	5.5-8.0	0.75	15-35	92.3	78.6	St,Na, ch	Cr
LR7	5.5-8.0	0.75	20-35	92.3	86.7	Ch,Na	Cr
LR8	5.5-8.0	0.75	20-30	92.3	78.6	Na, ch	Cr
LR9	5.5-8.0	0.75	20-30	50	60	Am, ch, Na,Er	Cr
LR10	4.5-8.0	1	10-35	92.3	73.3	Ch, Na	Cr
LR11	5.0-8.0	1	20-30	76.9	73.3	Ch	Cr, Zn
LR12	5.0-8.0	1	20-30	46.2	53.3	Ch, Er	-
LR13	5.0-8.0	1	20-30	53.8	66.7	-	Cr
LR14	5.5-8.0	0.75	20-30	53.8	46.7	-	-
LR15	4.5-8.0	1	15-35	92.3	66.7	Am,Ch, Na, Er	Cr
LR16	5.5-8.5	0.75	20-30	50.0	13.3	Na, Er	-
LR17	4.5-8.0	1	15-35	88.5	86.7	Na, Er	Cr
LR18	4.5-8.0	1	15-35	88.5	86.7	Am,Ch, Na, Er	Cr, Zn
LR19	5.5-8.5	0.75	10-30	88.5	86.7	Na, Er	Cr, Zn
LR20	4.5-8.0	1	15-35	88.5	86.7	-	-
LR21	4.5-8.5	1	10-35	92.3	86.7	Er	Cr
LR22	5.5-8.0	0.75	20-30	80.8	73.3	Na, Er	Cr
LR23	5.5-8.0	0.75	20-30	61.5	53.3	Na	-
LR24	5.5-8.0	0.75	20-30	80.8	73.3	Na	-
LR25	5.5-8.5	0.75	20-30	92.3	86.7	Ch	Cr
LR26	5.5-8.0	0.75	20-30	88.5	86.7	Na,ch	Cr,Zn
LR27	4.5-8.5	1	15-35	76.9	86.7	Na	-
LR28	5.5-8.0	0.75	20-30	50	46.7	-	-
LR29	5.5-8.0	0.75	20-30	53.8	53.3	-	-
LR30	5.5-8.5	1	20-30	61.5	60	-	-

Am: ampicillin; Na: naldixic acid, Er: erytheromycin, Ch: chloramphenicol, St: streptomycin, -: not tolerant/resistant, IAR: intrinsic antibiotics resistance

Generally, large variation was observed among fast-growing rhizobial isolates with regard to growth in relation to pH of the medium (Graham and Parker, 1964). There might be a relation between pH of origin of isolates and their acid and alkaline pH tolerance. However, in this study such kind correlation was not statistically significant($r<0.5$; $p>0.05$). Moreover, metal (Al and Mn) toxicity tolerance of isolates was tested at pH5.0 and all tested isolates were found to be sensitive to very low concentration of Al (Table 2.3). Isolate LR1, LR10, LR15, LR17, LR18, LR20 and LR27 tolerated both high and low concentrations of Mn at pH 5.0 while LR11, LR12, LR13 and LR21 were found to be sensitive to low concentration of Mn. Since most Ethiopian soils are acidic where associated metal toxicity expected to prevail such isolates are very important to use as inoculants.

Table 2.3. Soil acidity related metals (Al & Mn) tolarance of rhizobial isolates

Isolate	Mn		Al
	50 μg ml^{-1}	75 μg ml^{-1}	10 μg ml^{-1}
LR1	+	+	-
LR10	+	+	-
LR11	-	-	-
LR11	-	-	-
LR12	-	-	-
LR13	-	-	-
LR15	+	+	-
LR17	+	+	-
LR18	+	+	-
LR20	+	+	-
LR21	-	-	-
LR27	+	-	-

+: growth present, -: growth absent

Temperature tolerance

As shown in Table 2.2, maximum growth of all tested strains was obtained between 20 and 30 °C. However, isolate LR1, LR4, LR6, LR15, LR17, LR18, LR20 and LR27 showed tolerance to a wider range of temperature values (15-35°C). Isolate LR10 and LR21 were found to be tolerant to widest range (10-35°C) of temeperature values. None of the tested isolates could be able to tolerate and grow at 4°C and 40°C. Likewise, Rashid *et al.* (2009) observed that all lentil nodulating rhizobia strains grew well at 33°C and one strain could grow at 38°C. Increased

temperature optima of these isolates may be beneficial for its application in temperature stressed conditions as symbiotic performance of different rhizobial strains under temperature stress has been correlated with their ability to grow in pure culture at elevated temperature (Hungaria, 2000).

Salt tolerance

The data in Table 2.2 showed that lentil rhizobia exhibited less diversity in their salt tolerance. Generally, less tolerance to sodium chloride (NaCl) was observed among the rhizobial isolates. Most (60%) of the isolates could not grow on the medium containing 1% NaCl. However, LR1, LR10, LR11, LR12, LR13, LR15, LR17, LR18, LR20, LR21, LR27 and LR30 tolerated 1% NaCl. Several studies also reported that rhizobial isolates exhibit a large range of sensitiveness to salinity (Singleton *et al.*, 1982). The growth of lentil rhizobia with various salt concentrations was positively correlated($R=0.8$; $P<0.05$) with their isolation site soil pH. This indicates that saline soils naturally select strains which are more tolerant to salinity. Fast-growing rhizobia strains which could tolerate 2% NaCl were capable of growing at pH 9.5 (Sadowsky *et al.*, 1983). Similarly, an apparent positive correlation was observed between salt tolerance of the isolates and their alkaline pH tolerance($R=0.8$; $p<0.05$).

Intrinsic antibiotics and heavy metals resistance

The evaluation of intrinsic resistance to antibiotics of lentil rhizobia showed that most (50-80%) of the tested isolates exhibited high resistance to chloramphenicol and nalidixic acid (Table 2.2). In the presence of ampicillin, streptomycin, or erythromycin, only few isolates were resistant. Out of all tested isolates and different antibiotics 23.3% of the isolates were found to be sensitive

to all tested antibiotics even at low concentration while 56.7% of them resist two and above types of antibiotics. All tested isolates were found to be sensitive to low concentration of tetracycline, neomycin and rifampcin. Previous study (Rashid *et al.*, 2009) also observed great variation among lentil rhizobia with respect to their in intrinsic antibiotics resistance pattern. The intrinsic resistance to antibiotics can be used for the identification of rhizobial strains that occupy nodules in studies designed to evaluate the ecological competitiveness (Kremer and Peterson, 1982). In addition, the pattern of antibiotics resistance has been used to identify diversity among strains of rhizobia. As a result, it could be used as supplementary diagnostic character for different rhizobial strains (Amarger *et al.*, 1997).

Heavy metals resistance pattern of the rhizobial isolates is presented in (Table 2.2). The results showed that most (60%) isolates were found to be tolerant chromium whereas only some (16.7%) could grow in the medium containing zinc. The remainig heavy metals were found to be inhibitory to all rhizobial isolates. Though the concentrations of chromium in the soil samples wase not determined during this study their chromium tolerance might be related to their adaptation to the metals at their isolation sites. The heavy metal resistance traits of the rhizobial isolates would be used as invaluable positive markers during genetic studies (Küçük and Kıvanç, 2008). The high level of Zn and Cr resistance suggest that these metals could be used as selective agents for some *Rhizobium leguminosarum bv. viciae* strains (Küçük and Kıvanç, 2008; Sinclair and Eaglesham, 1984)

C-Sources and N-Sources utilization pattern

Most of the lentil rhizobia strains were able to catabolize a large variety of carbon substrates (Table 2.2). All tested strains grew on D-glucose and D-mannitol. More than 70% of the isolates utilized all tested carbohydrates as C-source. The most metabolically versatrile isolates were LR1,'LR4, LR6, LR7, LR8, LR10, LR15, LR21 and LR25 that grew well on 92.3% of the carbohydrates tested. Isolate LR3, LR5 and LR12 were found to be fastidious that grow on <50% of the carbohydrates tested.

Generally, the rhizobial strains exhibited a large diversity in utilizing different carbohydrates. All tested isolates were unable to utilize citrate as sole source of carbon. Graham and Parker (1964) found that utilization of citrate as sole sources of carbon was restricted to slow-growing rhizobia. As reported by earlier studies (Graham and Parker, 1964; Sadowsky *et al.*, 1983), fast-growing rhizobia were able to grow on a large variety of carbon substrates. However, our results show the majority of tested lentil rhizobia were able to use a broad range of carbohydrates. It is very interesting to notice that the types of carbohydrates utilized also vary from monosaccharides to polysaccharides like starch and cellulose among lentil rhizobia. Similarly, other studies (Glenn and Dilworth, 1981; Sadowsky *et al.*, 1983) observed that disaccharides such as cellobiose, lactose, maltose, trehalose and sucrose, and trisaccharide such as raffinose were catabolized by fast-growing strains of rhizobia.

Lentil rhizobia also exhibited diversity in utilizing different amino acids and vitamins as sole N-sources (Table 2.2). Serine and riboflavin were utilized by all isolates where as phenylalanine and glycine were not utilized by any isolates tested. Except tryptophan and niacin more than 80% of the isolates metabolized the remaining N-sources tested. The most efficient isolates were LR1, LR4, LR7, LR17, LR18, LR19, LR20, LR21, LR25, LR26 and LR27 that utilized 86.7% of the N-sources tested. The least efficient isolates were LR5, LR14, LR16 and LR28 that were capable of utilizing only ≤46.7% of the tested N-sources. Previous studies (Amarger *et al.*, 1997; Küçük and Kıvanç, 2008) also reported that rhizobia displayed wide diversity in their N-sources utilization pattern. The ability of isolates to utilize wide range of N-sources would give more survival advantage in the soil and it is one of the desirable characteristics for isolates to be selected for field studies.

Numerical analysis

Numerical analysis multiple phenotypic traits categorized the 30 lentil rhizobial isolates into four clusters (C1, C2, C3 and C4) indicating the phenotypic diversity exists among lentil nodulating rhizobial group (Figure 2.2). This crop was previously believed to be nodulated by *R. leguminosarum* only. However, recent works indicated that lentil could also be nodulated by *R. etilli* (Rashid *et al.*, 2012). Cluster C1 was the largest of all with 10 members followed by C2 which comprised 9 members. Cluster C3 was the smallest with three members. Isolate LR7 and LR8 obtained from different geographic location showed 100% similarity (Figure 2.2).

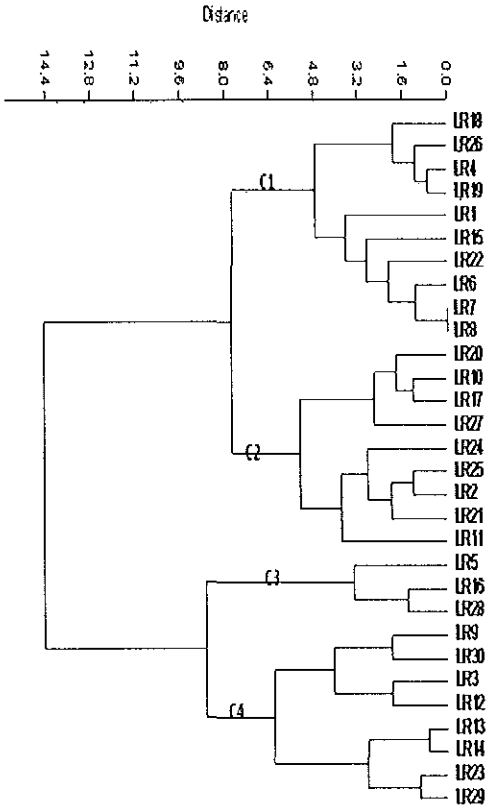

Figure 2.2 Phenogram showing phenotypic similarity among lentil rhizobia isolates reterived fom lentil producing areas of Ethiopia

PGP properties of the isolates

Lentil nodulating rhizoia showed very interesting characteristics such as auxin production and inorganic phosphate solubilization (Table 2.4). Out of all tested isolates 36.7% of them were auxin producer while only 16.7% were insoluble inorganic phosphate solubilizer with solubilization index ranging from 1.3 to 1.14 which was recorded for isolate LR25 and LR15, respectively. Several studies reported that different strains of *Rhizobium leguminosarum* are endowed with these characteristics (Antoun *et al.*, 1998; Alikhani *et al.*, 2006; Sajjad *et al.*, 2008; Alikhani and Yakhchali, 2009; Etesami *et al.*, 2009). Contrary to the previous study

(Antoun *et al.*, 1998) none of the tested isolates showed any antagonistic activity against test fungal pathogens in dual culture assay.

Rhizobia enjoy saprophytic life in soil when there is no legume host and they non-specifically attracted to the roots of non-legume crops and nourish root exudates in the rhizosphere (Dowling and Broughton, 1986). This would give the advantage of increasing the yield of non-legume crops by inoculating legume with symbiotically effective rhizobia which have PGP characteristics such as IAA production and PSB activity (Matiru and Dakora, 2004). Several studies demonstrated that such kind of rhizobia would increase the yield of non-legume crops grown in rotation or mixed cropping with legumes (Antoun *et al.*, 1998; Alikhani and Yakhchali, 2009; Etesami *et al.*, 2009). Thus, root colonization pattern and PGP activity of such isolates when inoculated to wheat, teff and barley which is grown in rotation with lentil under Ethiopian conditions needs to be investigated.

Symbiotic characteristics of the isolates

All tested isolates of lentil rhizobia showed great variation in their capacity to induce nodule formation on the host plant and produce nodule dry weight under glasshouse conditions. The mean nodule number per plant was ranged from 20 for isolate LR15 and LR29 to 62 for isolate LR26 (Table 2.4). Generally, 83% of the isolates formed more than 30 nodule number on the roots of their host. The highest nodule dry weight was recorded for isolate LR4 i.e. 112 mg whereas the smallest was 9 mg which was recorded for isolate LR28 (Table 2.4). Anteneh Argaw (2012) also reported that lentil rhizobia isolated from Ethiopian soils were showed nodule numbers varying between 68 and 17 plant^{-1}and nodules dry weights between 0.015 and 0.0016 g plant^{-1}under controlled conditions using pouch experiment.

Table 2.4 *In vitro* PGP activity of the rhizobial isolates and their Symbiotic characteristics under glasshouse conditions

Isolates	Nodule Number/plant ± SE	Nodule dry weight(mg) / plant ± SE	Shoot dry weight(g)/plant ± SE	Relative effectiveness %	Solubilization index	IAA production
LR1	49.5±4.3a-d	74±6a-g	0.71±0.02a-c	97.3	-	-
LR2	39.8±3.6b-g	33±3c-g	0.73±0.1a	100	-	+
LR3	34.7±2.6c-h	50±3a-g	0.41±0.07h-k	56.2	-	-
LR4	34.5±4.4c-h	112±99a	0.66±0.03a-d	90.4	-	-
LR5	57.7±4.6a-c	82±3a-d	0.40±0.02i-k	54.8	-	+
LR6	32.7±2.8d-h	15±3e-g	0.66±0.01a-d	90.4	-	-
LR7	34.7±2.2c-h	25±1d-g	0.45±0.01g-k	61.6	-	-
LR8	32.5±2.6d-h	96±43a-c	0.73±0.05a	100	-	-
LR9	33.0±1.8d-h	11±2e-g	0.35±0.03jk	47.9	-	-
LR10	34.2±0.9c-h	9±3fg	0.57±0.03a-g	78.1	-	-
LR11	38.3±2.2b-h	9±1fg	0.44±0.04g-k	60.3	1.13	+
LR12	27.7±4.2e-h	4±1g	0.32±0.01k	43.8	1.18	-
LR13	40.5±2.7b-f	7±1g	0.59±0.01a-g	80.8	-	-
LR14	34.8±1.9c-h	5±1g	0.34±0.02k	46.6	-	-
LR15	20.3±3.2h	10±1e-g	0.45±0.01g-k	61.6	-	-
LR16	27.2±3.4e-h	10±1e-g	0.44±0.02g-k	60.3	1.14	-
LR17	24.2±2.9f-h	25±8d-g	0.36±0.02jk	49.3	-	+
LR18	52.7±4.9a-c	84±3a-e	0.44±0.01g-j	60.3	-	+
LR19	49.0±7.2a-d	90±12a-c	0.51±0.01d-j	69.9	-	+
LR20	25.7±2.9f-h	17±3e-g	0.46±0.04g-k	63.0	-	-
LR21	47.5±1.3a-d	38±4b-g	0.45±0.04g-k	61.6	-	+
LR22	50.2±6.8a-d	18±1e-g	0.51±0.01d-j	69.9	-	+
LR23	39.3±2.5b-h	15±2e-g	0.56±0.01b-i	76.7	1.15	+
LR24	37.5±2.8c-h	21±3d-g	0.63±0.04a-f	86.3	1.17	+
LR25	45.3±2.9a-e	22±1d-g	0.73±0.01ab	100	-	-
LR26	62.3±4.5a	110±25a	0.67±0.01a-d	91.8	1.13	-
LR27	32.7±3.2d-h	22±3d-g	0.47±0.04f-k	64.4	-	+
LR28	34.3±2.2c-h	9±1fg	0.63±0.03a-f	86.3	-	-
LR29	20.5±0.8gh	15±3e-g	0.54±0.02c-i	74	-	-
LR30	33.3±2.5c-h	17±1e-g	0.58±0.01a-h	79.5	-	-
TN	-	-	0.73±0.08a	100		
T0	-	-	0.25±0.01l	34.2		

Numbers in the same column followed by the same letter do not differ significantly at p= 0.05 by DT; +: present; -: absent, SE: standard error

Similarly, all tested isolates of lentil rhizobia exhibited high diversity in their shoot dry matter accumulation. In comparison with TN control which represents 100% level of shoot dry matter and T0 control which represented 34%, all tested isolates showed shoot dry matter yield ranging from 0.32 g to 0.73 g per plant which is higher than T0 control (0.25 g/plant). The relative effectiveness, which is expressed in percent of TN control, showed that isolates LR2, LR8 and LR25 were the most efficient with 100% dry matter yield while LR9, LR12, LR14 and LR17 were the least efficient with less than 50% relative effectiveness (Table 2.4). Out of 30 isolates tested 12 (40%) isolates did not vary significantly ($p<0.05$) from TN control in shoot dry matter yield. Their relative effectiveness ranges from 78% to 100%. These isolates were also particularly most infective with mean nodule number ranging from 32 to 62 and highly effective.

Similarly, earler studies in Ethiopia (Ayneabeba Adamu *et al.*, 2001; Zerihun Belay and Fassil Assefa, 2011) showed that most *Rhizobium leguminosarum* isolates nodulating faba bean were characterized as symbiotically effective isolates when tested in a controlled environment using a sterilized sand culture. Anteneh Argaw (2012) also observed that rhizobial inoculations to the lentil significantly increased shoot dry weight over the uninoculated control and most of these isolates were found to be very effective (20.9%) and effective in (77.4%) .

Based on the result of the current study the following isolates LR1, LR2, LR6, LR8, LR23, LR24, LR25 and LR26 are highly recommended for field experiment and ecological competitiveness studies under different Ethiopian soil and climatic conditions. These isolates exhibited interesting features such as wide range of carbon-sources and nitrogen sources utilization, tolerance of acidic pH, metal toxicity, antibiotics, PGP features such as auxin

production and inorganic phosphate solubilization, and highly effective nitrogen fixation (Table 2.2, 2.3 and 2.4). In general, from the present study, it can be concluded that Ethiopian soils harbor highly efficient nitrogen-fixing lentil nodulating rhizobia which are diverse in their morphological, physiological and symbiotic characteristics.

This study also indicated that some of these isolates possessed special characteristics such as IAA production and phosphate solubilization activities which can make them candidate for multipurpose inoculants production for the lentil production system in Ethiopia. During this study, methods used for characterizing and distinguishing rhizobial strains were morphological, physiological and symbiotic. However, these traditional methods of rhizobial characterization frequently fail to identify strains within a species. Hence, such kind of study must be substantiated by PCR based molecular methods such as RFLP, RAPD, AFLP and 16S rRNA sequence analysis so as to obtain a better understanding of microbial diversity and strain identification.

Chapter 3 Phenotypic diversity and plant growth promoting characteristics of *Mesorhizobium* species isolated from chickpea (*Cicer arietinum L.*) growing areas of Ethiopia

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Abstract

Chickpea (*Cicer arietinum* L.) is one of the major sources of dietary protein for the majority of Ethiopian population. It also maintains soil fertility through its symbiotic nitrogen-fixation in association with *Mesorhizobium* species. Therefore, this study was aimed at isolation, characterization and selection of symbiotically effective *Mesorhizobium* species endowed with different plant growth-promoting (PGP) characteristics from chickpea producing areas of Ethiopia. Hence, phenotypic and plant growth promoting characteristics of thirty-six rhizobia isolates recovered from root nodules of chickpea grown in soils collected from different producing areas found in central and northern part of the country were investigated. The result of our study indicated that chickpea rhizobial isolates showed wide diversity in their different C and N-sources utilization pattern and tolerance to salinity, high temperatures, acid and alkaline pH, heavy metals and antibiotics. Symbiotic and morphological characterization also showed a wide diversity among the isolates. Moreover, screening for PGP characteristics indicated that 44.4% of the isolates were phosphate solubilizer while 27.8% of them were found to be IAA producer. Furthermore, 19.4% tested isolates showed antagonistic activity against *Fusarium oxysporum* in dual culture assay. Generally, the present study indicated that Ethiopian soils contain symbiotically effective chickpea nodulating rhizobia which are endowed with different PGP characteristics.

Key words; Antagonistic, IAA, nodulation, phosphate solubilization, symbiotic nitrogen fixation

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Introduction

Chickpea (*Cicer aeritinum* L.) is one of the major food legume crops grown widely in tropics, sub-tropics and temperate regions of the world. It is also one of the principal food legumes which has been widely grown in Ethiopia over an area of 208,388.6 ha (CSA, 2011). Nutritionally chickpea seed contains 19.8 % protein and substantial amount of other nutrients (Werner, 2005). In Ethiopia, it serves as an invaluable source of dietary protein which is commonly consumed in different forms as supplementary food. Besides, chickpea is one of the major export commodities amongst the field crops (Geletu Bejiga and Ketema Daba, 2006; Shiferaw Bekele and Teklewolde Hailemariam, 2007).

In addition to nutritional quality and source of cash, chickpea restores and maintains soil fertility through its symbiotic nitrogen-fixation in association with *Mesorhizobium* species (Nour *et al.*, 1994, Nour *et al.*, 1995; Jarvis *et al.*, 1997). It is capable of fixing 90-180 kg N ha^{-1}(Werner, 2005). It is, therefore, grown in rotation with major cereals in traditional low-input agricultural system. However, in Ethiopia the yield of chickpea has remained very low (Geletu Bejiga and Ketema Daba, 2006, Gemachu Keneni *et al.*, 2011a) and thus, many research activities have been undertaken to improve chickpea cultivars with respect to their yield, tolerance to different biotic and abiotic stresses (Yadeta Anbessa and Geletu Bejiga, 2002; Seid Ahmed and Melkamu Ayalew, 2006; Gemachu Keneni *et al.*, 2011a; Gemachu Keneni *et al.*, 2011b). Consequently, many improved cultivars were released by Ethiopian Instituite of Agriculture Research (EIAR) (Shiferaw Bekele and Teklewolde Hailamariam, 2007). One of the strategies which have been given less attention to this end was exploiting the benefits of its symbiotic nitrogen fixation by selecting effective rhizobia.

Recently, some strains of rhizobial species were found to exhibit plant growth promoting (PGP) characteristics since they promoted the growth of some crops through mechanisms that are independent of biological nitrogen-fixation (Antoun *et al.*, 1998; Peix *et al.*, 2001a; Yanni *et al.*, 2001; Alikhani and Yakhchali, 2009). These mechanisms include stimulating plant growth directly either by synthesizing phytohormones such as indole-3-acetic acid (IAA) or by promoting nutrition processes such as phosphate solubilization and siderophore production, which facilitate phosphorus and iron uptake, respectively from soil. They can also stimulate growth indirectly by protecting the plant against soil-borne fungal pathogens. Several studies showed that *Mesorhizobium* species also exhibit such characteristics (Peix *et al.*, 2001a; Alikhani and Yakhchali, 2009; Hemissi *et al.*, 2011). Such kind of rhizobial strains could be used as multipurpose inoculants for both legume and non-legume crops grown rotationally or simultaneously. Thus, native rhizobial isolates must be screened for their PGP activity in addition to their symbiotic effectiveness.

Mesorhizobium strains naturally vary in their nitrogen fixing capacity and adaptation to various environmental stresses (Zharan, 1999; Maâtallah *et al.*, 2002; L'taief *et al.*, 2007). So, selection of symbiotically efficient rhizobial strains which are tolerant to locally prevailing stresses is highly desirable. To this end, several studies have been conducted on chickpea rhizobia characterization and selection of best strains for inoculant production elsewhere in the world (Maâtallah *et al.*, 2002; L'taief *et al.*, 2007; Küçük and Kıvanç, 2008). However, in Ethiopia very little is known about phenotypic and symbiotic characteristics of chickpea nodulating

Mesorhizobium. Thus, there is no enough information about their PGP characteristics. These necessitates for research activities devoted to investigate different characteristics of chickpea rhizobia isolated from producing areas of the the country. Hence, this study was aimed at isolation, characterization and selection of symbiotically effective native chickpea rhizobia isolates endowed with different PGP characteristics.

Materials and methods

Study sites and soil samples collection

Soil samples were collected from chickpea grown farmers' field found in Amhara, Oromia and Tirgay regional states of Ethiopia (Fig 3.1). The areas are distributed in central and northern parts of the country with an altitude ranging from 1526 (Alamata) to 2840 (Sheno) meter above sea level (masl) and with soil pH from moderately acidic (5.6) to alkaline (7.9) (Table 3.1). A total of 36 soil samples were excavated from 15-20 cm depth and collected in sterile plastic bags. The soil samples were carefully transported to Applied Microbiology laboratory, Addis Ababa University, for further analysis in October, 2009.

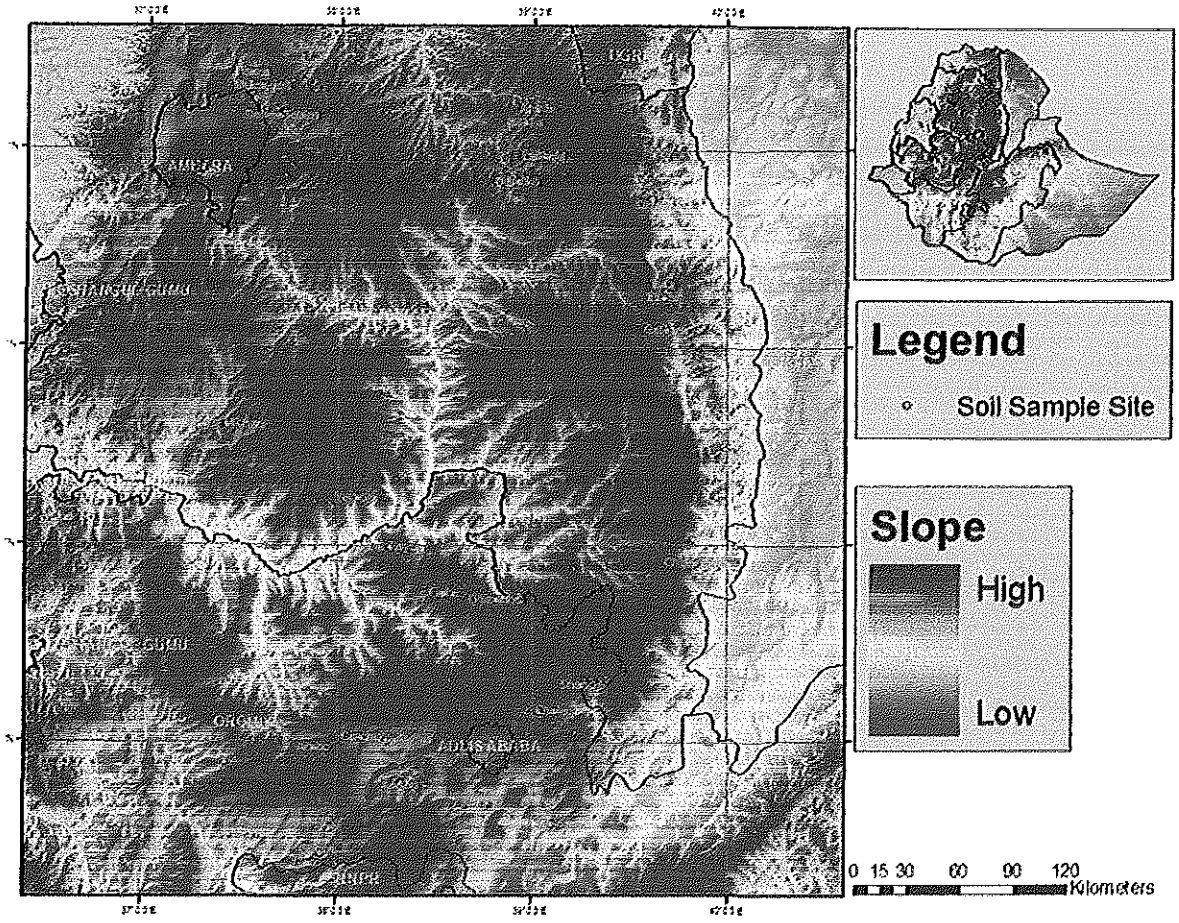

Figure 3.1 Chickpea soil sample collection sites

Isolation of rhizobia

The rhizobia were isolated from soil samples by inducing nodulation on chickpea cultivar called 'Ararti' (obtained from EIAR, Debre Zeit) using plant infection method (Vincent, 1970). Each soil sample was filled into surface sterilized (95% ethanol) plastic pots. Chickpea seeds were selected and surface sterilized with 95% ethanol and 3% sodium hypochlorite solutions for 10 seconds and 3 minutes, respectively. The seeds were rinsed five times with sterilized distilled water to remove traces of sterilizing chemicals and allowed to germinate on sterile water gar

(1%) surface for three days at 25°C. Five pre-germinated seeds were planted on each pot. The seedlings were thinned down to three after 5 days of emergence (DAE). All pots were situated in glasshouse and watered to a field capacity every three days for 60 days after planting (DAP).

Sixty DAP the plants were carefully uprooted from the pots and washed under gently flowing tap water to remove soil particles. Large and reddish nodules were separately collected from each pot on separate sterile Petri dishes and surface sterilized as described before, and crushed using alcohol flamed glass rod. Loopful of the extract was streaked on Yeast extract Mannitol Agar (YEMA) containing 0.0025% (w/v) Congo red (Vincent, 1970). The components of YEMA g/l: 0.5 K_2HPO_4, 0.2 $MgSO_4$, 0.1 NaCl, 10 Mannitol, 0.5 Yeast Extract, 15 Agar (Vincent, 1970). All the plates were incubated at 28°C for 6 days. Single typical rhizobia colony were picked from each plate and transferred to test tube which contained sterile Yeast Extract Mannitol Broth (YEMB) (Vincent, 1970). The test tubes were incubated at room temperature on a gyratory shaker at120 revolution (r) minute $(m)^{-1}$ for 3 days and purified by re-streaking on YEMA plates. The pure isolates were further confirmed by presumptive tests such as Gram reaction using KOH test as described by Gregorson (1978) and growth on Peptone Glucose Agar (PGA) (Somasegaran and Hoben, 1994). Pure isolates were then preserved on YEMA slants containing 0.3% $CaCO_3$ stored at 4°C for short-term storage (Vincent, 1970) and in glycerol (50% v/v) at -20°C for long-term storage. All the rhizobial isolates were designated as CR1-50 (C: Chickpea; R: Rhizobia) (Table 3.1).

Colony morphology, growth and biochemical characteristics

Colony morphology was evaluated by streaking a loop of the initial inoculum on YEMA plates and allowing the isolates to grow at 28°C for 5 days (Somasegaran and Hoben, 1994). Growth rate of the isolates was assessed by inoculating 20 µl of the inoculum into YEMB containing flasks (250 ml) incubated on a gyratory shaker at120 r m^{-1}. Then optical density was measured at 540 nm using spectrophotometer every 6 h and 0.1ml diluted culture was spread plated on YEMA plates. The generation time (GT) was calculated from the logarithmic phase of growth curve as described by Somasegaran and Hoben (1994). Acid or alkali production test was carried out by growing isolates on YEMA medium containing Bromothymol blue (BTB) (Somasegaran and Hoben, 1994).

Rhizobial inoculum preparation

Rhizobial isolates were grown in YEMB on a gyratory shaker at room temperature with 120 rpm for 72 hours (h). The suspension was centrifuged in sterile plastic tubes (10 ml) at 5000 rpm for 10 min. The pellets were re-suspended in normal saline (0.85% w/v of NaCl) solution to give a final concentration of 10^8 CFU/ml using the viable plate count method and optical density measurement by spectrophotometer at 540 nm.

Physiological characteristics

All tests, except C and N-source assimilation, were carried out on YEMA plates. Petri dishes containing defined medium were inoculated with 20 µl of the inoculum. After 5 days of incubation at 28°C, bacterial growth was compared with the controls. All tests were carried out in triplicates.

Salt, pH and temperature tolerance

Salt tolerance of the isolates was determined on YEMA plates containing 0 to 5% (w/v) NaCl concentrations. Tolerance to extreme pH was tested on YEMA medium set at different pH (4.5-10) values using 1N HCl and 1N NaOH. Temperature tolerance was evaluated on YEMA plates inoculated as described above and incubated at temperatures from 4 to 40° C.

Intrinsic antibiotic and heavy metal resistance

This intrinsic antibiotic and heavy metal resistance was determined on solid YEMA medium containing the following filter sterilized antibiotics or heavy metals ($\mu g.ml^{-1}$): ampicillin (5 and 10), chloramphenicol (5 and 10), erythromycin (5 and 10), nalidixic acid (5 and 10), streptomycin (10 and 50), neomycin(5 and 10) and tetracycline (5 and10); $AlK(SO_4)_3.12H_2O$ (10, 25), $K_2Cr_2O_7$ (50), $CoCl_2$ (10), $CuCl_2.2H_2O$ (10), $HgCl_2$ (5), $MnCl_2$ (50, 75), $NiSO_4$ (10), $Pb(CH3COO)_2$ (10), and $ZnCl_2$ (50). Aluminium and manganese toxicity tolerance test was carried in YEMA medium adjusted at pH 5.0 only for isolates which grew at pH values $\leq$5.0.

Utilization of different C and N-sources

Different carbohydrates were added as described by Amarger *et al.* (1997) at final concentration of $1gl^{-1}$ to the basal medium containing (gl^{-1}): K_2HPO_4, 1; KH_2PO_4, 1; $FeCl_3.6H_2O$, 0.01; $MgSO_4.7H_2O$, 0.2; $CaCl_2$, 0.1; $NH_4(SO_4)_2$, 1; and agar, 15. The following filter sterilized sole carbon sources were added after autoclaving: Citrate, D-sorbitol, D-glucose, D-mannose, D-

maltose, D-galactose, D-arabinose, gluconate, raffinose, xylose, dulcitol, cellobiose, adonitol, inulin, aesculin, trehalose and inositol. D-mannitol, α-lactose, D-fructose, glycerol, α-cellulose, sucrose, starch, tartarate, malonic acid and dextrin were added before autoclaving. Filter sterilized L-tryptophan, methionine, L-tyrosine, leucine, L-asparagine, DL-β-phenylalanine, L-arginine, glutamic acid, L-lysine, analanine, serine, glycine, thiamine, niacin and riboflavin were used as sole nitrogen source for isolates by adding a final concentration of $0.5gL^{-1}$ to the above basal medium from which $(NH_4)_2(SO_4)$ was omitted and mannitol was added after autoclaving (Amarger *et al.*, 1997). All inoculated palates were incubated at 28°C and the results were observed after 5 days.

Numerical analysis

Physiological tests such as different substrates utilization and intrinsic antibiotic and heavy metals resistance were used to construct phenogram. Traits were coded 1 for positive and 0 for negative. A computer cluster analysis of 62 phenotypic variables was carried out using ward linkage and Euclidian distance as a measure of dissimilarity coefficient and a phenogram was constructed PAST ver.2.17c (Hammer *et al.*, 2001).

Screening for PGP Properties of the Isolates

Phosphate solubilization

Phosphate solubilizing ability of the isolates was determined using Pikovaskaya (Pikovskaya, 1948) agar plates were spot inoculated with 20 µl of the inoculum. After incubation at 28°C for 5 days, formation of a clear zone around the spot was recorded and solubilization index (SI) was calculated as described by (Edi-Premono *et al.*, 1996) for positive isolates.

IAA production

Exponentially grown cultures of each isolates were inoculated onYEMB medium supplemented with 5 mM L-tryptophan for 72 h (Bric *et al.*, 1991) to determine their IAA production ability. Supernatant of the strains were collected by centrifugation at 10,000 rpm^1 for 15 min and 2 ml supernatant of each was transferred separately to a fresh tube to which 100 µl of 10 mM O-phosphoric acid and 4 ml of Salkowaski reagent (1 ml of 0.5 mM $FeCl_3$ in 35% $HClO_4$) were added. Mixtures were incubated at room temperature for 25 min and observed for the development of pink color.

Screening for *in vitro* antagonistic activity against *Fusarium oxysporum*

The *in vitro* mycelial growth inhibition of *Fusarium oxysporum f.sp. ciceri* (obtained from EAIR, Debre Zeit) by the rhizobial isolates was tested using the dual culture technique as described by Landa *et al.* (1997). Twenty µl the inoculum of each rhizobial isolates was equidistantly spotted on the margins of YEMA plates amended with sucrose (0.5%) and incubated at 28°C for 24 h. A 4-5 mm diameter agar disc from Potato Dextrose Agar (PDA) cultures of the fungal pathogens was placed at the centre of the YEMA plate for each bacterial isolate and further incubated at 28°C for 5 days. The radii of the fungal colonies towards and away from the bacterial colonies were measured and and the *in vitro* inhibition was calculated following Ahmed Idris *et al.* (2008).

Authentication and symbiotic characterization of the isolates

All rhizobial isolates were authenticated and characterized symbiotically by re-inoculating them on their host plant on sand culture. About 3 kg of washed and autoclaved sterilized sand was placed in plastic pots (3 kg capacity). Seeds of chickpea cultivar called 'Arati' surface sterilized and germinated as described before. The germinated seedlings were flooded with each rhizobial isolate adjusted to 10^9 cells per seed for 1 h on separate sterilized Petri plates.

Five inoculated seedlings were transferred to each pot which was later thinned down to three after 5 DAE. The pots were irrigated with nitrogen free plant nutrient solution as described by Somasegaran and Hoben (1994). Uninoculated but nitrogen-fertilized (0.05% w/v KNO_3) pots were included as positive (TN) and uninoculated and not nitrogen-fertilized (T0) pots as negative controls. The experiment was laid out with three replications using randomized block design. The seedlings were supplied with water every three days and once a week with a nitrogen-free nutrient solution (Somasegaran and Hoben, 1994). Sixty DAP all plants were carefully uprooted, nodule number counted, and nodule dry weight and shoot dry weight were measured after drying at 70^0C for 48 h to a constant weight. Relative symbiotic effectiveness of each isolate was calculated by using the formula (100 × inoculated plant shoot dry weight/ N-fertilized plant shoot dry weight) as described by Gibson (1987).

Data analysis

All experiments were set in triplicate and the data is average of three. Symbiotic data was analyzed by ANOVA and the treatment means were compared following Duncan's test (DT) by using SPSS (V.17). Correlations among some parameters were checked by using linear regression analysis.

Results and discussion

A total of 36 chickpea rhizobial isolates were recovered from as many sampling sites from central and northern Ethiopia with altitude ranging from 1526 (Alamata) to 2840 masl (Sheno), and soil pH from moderately acidic (pH 5.6) to slightly alkaline (pH 7.9)(Table 3.1). The distribution pattern showed that most of the sample sites were from mid altitude with 1750-2500 masl and mildly acidic soil pH of 6.0-6.9.

Table 3.1 Chickpea rhizobila isolates, site of isolation, growth and colony characteristics

isolate	isolation sites	pH of isolation site	Altitude of isolation site (masl)	Colony characteristics	Colony diameter(mm)	MGT (h)
CR01	Fiche	6.8	2748	LMM	3.2	4.8
CR02	Alem Tena	7.1	1637	LWM	2.4	4.8
CR03	D/Selam	6.0	1896	LMM	3.4	5.0
CR04	Sandafa	6.0	2554	LCM	3.2	5.1
CR05	Galessa	7.6	2017	LWM	2.7	4.5
CR06	Chirameda	6.6	1747	LWM	4.5	2.8
CR08	Ginchi	6.6	2378	LMM	2.3	4.7
CR09	Goro	6.6	1832	LMM	3.2	5.2
CR11	Angut Michael	6.3	1850	LWM	2.6	4.9
CR12	Maksagnt	6.4	1978	LWM	2.3	4.6
CR14	Chole	6.3	2612	LWM	4.3	3.2
CR16	Olankomi	6.6	2378	LMM	3.3	4.4
CR18	Sirnka	6.9	1843	LWM	2.5	4.1
CR19	Dibulko	6.8	1992	LWM	3.7	4.7
CR20	Lalibela	7.4	2138	LMM	3.7	4.8
CR21	Yetinora	6.4	2437	LMM	3.7	4.1
CR23	Gurura	7.0	1906	LMM	3.1	4.7
CR24	Fogera	6.4	1931	LMM	2.8	5.1
CR25	Alshin	6.7	2082	LWLM	2.2	5.0
CR28	Bilbila	6.6	2069	SWLM	1.9	5.4
CR29	Amber	6.3	2454	LMM	4.0	2.8
CR31	Asgori	6.6	2078	LWhLM	2.2	4.7
CR32	Aja	6.8	2023	LMM	3.2	4.4
CR34	Woldya	6.6	2074	SWLM	1.9	5.2
CR36	Alamata	7.9	1526	LWM	3.3	4.5
CR37	Robe	7.8	1658	LWhLM	3.7	5.2
CR38	Itacha	6.8	2134	LWM	2.3	4.8
CR39	Tikana	7.2	1942	LWhLM	2.0	5.2
CR40	Ilala	7.6	1924	LWLM	2.0	5.3
CR42	Goha Tsion	6.2	2517	LMM	2.0	4.2
CR44	Ambo	6.7	2170	LWM	2.5	3.8
CR45	Obbi	6.6	2108	SWLM	1.5	4.8
CR46	Mojo	6.8	1774	LMM	2.1	5.4
CR47	Sheno	5.6	2840	LCM	2.2	4.6
CR48	Teji	6.7	2065	LWM	2.4	4.5
CR50	D/Libanos	7.3	2594	LMM	2.3	4.8

LCM: large, creamy, mucoid; LWM: large, watery, mucoid; LMLM: large, milky, less mucoid; LWLM: large watery, less mucoid; LWhLM: large, white less mucoid; SWLM: small watery less mucoid; MGT: mean generation time

Growth characteristics and colony morphology

The isolates showed doubling time ranging from 2.8 h to 5.4 h. Based on their mean generation times 88.9% of the isolates were moderately slow growers with mean generation time (MGT) 4.1-5.4 h, whereas only 11.1% of the isolate were found to be fast grower with GT 2.8-3.8 h (Table 3.1). Previous studies indicated that *Mesorhizobium ciceri* (Nour *et al.*, 1994) and *Mesorhizobium mediterraneum* (Nour *et al.*, 1995), which are the specific chickpea symbionts, are moderately slow growing rhizobia.

All isolates formed colony with circular shape, entire margin, milky-to-watery translucent to creamy and white opaque features with different level of mucus production. Most isolates (89%) exhibited copious production of mucus while the remaining isolates showed less mucus production (Table 3.1). The colony diameter of the isolates was ranged from 1.5 mm to 4.5 mm. Out of all isolates only 8% of them had colony diameter of less than 2 mm indicating that all isolates could be able to form medium to large colony on YEMA surfaces at optimum growth conditions after 5 days of incubation (Table 3.1). Such characteristics are wide spread among rhizobia (Jordan, 1984).

All isolates changed the color of YEMA supplemented with BTB to yellow indicating that they are acid producers (Vincent, 1970). The CR absorption test also indicated that none of the isolates absorbed CR in YEMA plates; this is distinctive character of rhizobia with only few exceptions (Somasegaran and Hoben, 1994). On the other hand none of the tested isolates grew on PGA plates. In addition, all tested isolates were KOH test positive indicating that they were

Gram negative. These indicate that all isolates were not contaminant rather rhizobia (Vincent, 1970; Somasegaran and Hoben, 1994).

Physiological characteristics

Tolerance of acidic and alkaline pH

Chickpea nodulating rhizobia isolated from different Ethiopian soils exhibited a wide diversity in their different pH tolerance (Table 3.2). All tested isolates grew well in moderately acidic pH (5.5) to neutral pH and slightly alkaline pH (8.0)(Table 3.2). Some isolates exhibited an acid tolerant character since 31% and 56% of them grew at pH 4.5 and 5, respectively. Similarly, some isolates showed alkali tolerant character as 25% and 22% of the isolates grew at pH 9.5 and 10, respectively. Several studies (Nour *et al.*, 1994; Maâtallah *et al.*, 2002; Küçük and Kıvanç, 2008) have also indicated that chickpea rhizobia exhibit moderately acidic and alkaline pH tolerance characteristics.

Table 3.2 Physiological characteristics of chickpea nodulating rhizobial isolates

Isolate	pH tolerated	NaCl % tolerated	Temperature tolerated	C-sources% utilized	N-sources% utilized	IAR pattern	Heavy metals resistance
CR01	5.0-10	1	15-35	67	93	Na, Ch	Cr,Zn
CR02	4.5-8.0	5	15-35	56	87	Na, Ch,Er	Cr
CR03	5.5-10.0	3	15-40	78	80	Er	-
CR04	5.0-10	5	15-35	93	93	Na, Ch,Am,St	Cr,Zn
CR05	4.5-8.0	5	15-35	93	87	St,Ch,Ne,Na	Cr
CR06	5.0-10	1	10-30	82	93	St,Na, Ch,Am,Er	Cr,Zn
CR08	5.5-8.0	0.5	15-35	82	87	Ch,St,Er	Cr
CR09	4.5-8.0	1	15-30	56	87	Er,Na	Cr
CR11	5.5-8.0	1	15-30	56	87	Na,Er	Cr
CR12	5.0-9.5	2	15-35	56	87	Er, Na	Cr,Pb
CR14	4.5-9.0	1	15-30	85	87	Na,Er,St	Cr, Zn
CR16	5.0-9.0	4	15-35	59	87	-	Cr,Zn
CR18	4.5-8.0	1	10-30	48	73	Na,Er,Ne	Cr
CR19	4.5-8.0	2	10-30	52	80	Na,Er	Cr
CR20	5.5-8.5	0.5	15-30	67	87	Na, Er	Cr
CR21	4.5-8.0	1	15-35	56	87	Na, Er,Ch	Pb, Cr,Zn
CR23	4.5-8.0	0.5	20-30	59	80	Na, Er	-
CR24	4.5-8.0	1	15-40	52	87	Ch, Na, Er	Cr
CR25	5.5-10.0	5	15-40	85	87	Ch, Er	-
CR28	5.5-8.0	0.5	10-35	63	87	Na,Er	Cr
CR29	4.5-8.5	2	15-30	52	87	Er,Na,Ch	Cr
CR31	4.5-8.0	1	20-30	82	80	Na, Er	Cr
CR32	4.5-9.0	1	10-30	59	80	Na,Er	Cr
CR34	5.5-8.0	0.5	10-30	67	87	Na,Er	-
CR36	5.5-8.5	1	15-30	59	87	Er	-
CR37	5.5-8.0	1	15-30	59	87	Na,Er	Cr
CR38	5.5-8.5	0.5	20-30	48	80	Na,Er	Cr
CR39	4.5-8.0	2	10-30	56	87	Na,Er	Cr
CR40	5.0-10	3	15-35	78	87	-	-
CR42	5.5-8.5	1	15-30	63	87	Na,Er	Cr
CR44	5.0-10	1	15-40	78	87	Na,Er	Cr
CR45	4.5-8.0	0.5	15-35	56	80	-	Pb
CR46	5.5-8.0	1	20-30	59	80	Na,Er	-
CR47	5.5-8.0	1	10-30	82	80	Na,Er	-
CR48	5.0-10	2	15-40	70	100	Na	Cr,Zn
CR50	5.5-8.0	0.5	15-30	37	87	-	-

Am: ampicillin; Na; naldixic acid; Er; erythromycin; Ch: chloramphenicol; St: streptomycin; Ne: neomycin; -: no growth

Moreover, soil acidity related metals (Al and Mn) toxicity tolerance of acid tolerant isolates which grew at pH≤5.0 value was tested at pH5.0 and isolate CR04, CR06, CR29, CR39 and CR48 were found to be tolerant to very low concentrations of Al (25 μg ml^{-1}) while most of them tolerated both high and low concentrations of Mn (50 μg ml^{-1}) at pH 5.0(Table 3.3). One of the environmental stresses which pose a significant constraint to chickpea and other legume crops production in Ethiopia is soil acidity (Asfaw Tilaye, 1985). It has been demonstrated that acidity limits nodulation and the nitrogen fixation (Graham 1992); hence, rhizobia strains tolerant to acidity and associated metal toxicity might be used to improve this crop yield when inoculated to acidic soil.

Table 3.3 Soil acidity related metals (Al and Mn) toxicity tolerance of rhizobial isolates

Isolate	Al		Mn	
	10 µg/ml	25 µg/ml	50 µg/ml	75 µg/ml
CR01	-	-	+	+
CR02	-	-	+	+
CR04	+	+	+	+
CR05	-	-	+	+
CR06	+	+	+	+
CR09	-	-	+	+
CR12	-	-	+	+
CR14	-	-	+	+
CR16	-	-	+	+
CR18	-	-	+	+
CR19	-	-	+	+
CR21	-	-	+	+
CR23	-	-	-	-
CR24	-	-	+	-
CR29	+	+	+	+
CR31	-	-	-	-
CR32	-	-	+	-
CR39	+	+	+	+
CR40	-	-	-	-
CR44	-	-	+	+
CR45	-	-	-	-
CR48	+	+	+	+

+: growth present, -: growth absent

Temperature tolerance

Temperature conditions have a great effect on rhizobial growth and symbiotic performance (Zahran, 1999). As shown in Table 3.2, maximum growth of all tested isolates was obtained between 20 and 30 °C. Below and above these values, the percentage of isolates that grew decreased to reach 27.8 % at 10°C and 42% at 35°C. Isolate CR03, CR24, CR44 and CR48 grew over a wide range of temperature (15°C- 40°C). None of the tested isolates could be able to tolerate and grow at 4°C while isolate CR03, CR24, CR25, CR44 and CR45 could grow at 40°C and can be considered as thermo-tolerant. It has been reported that some chickpea rhizobia were thermo-tolerant which could be able to grow at temperature of 40°C (Maâtallah *et al.*, 2002; Küçük and Kıvanç, 2008). Increased temperature optima of these isolates may be beneficial for its application in high temperature stressed conditions. Hungria *et al.* (2000) showed that symbiotic performance of different rhizobial strains under temperature stress has been correlated with their ability to grow in pure culture at elevated temperature.

Salt tolerance

The data in Table 3.2 shows that chickpea nodulating rhizobia exhibited high diversity in their salt tolerance. The inhibitory salt concentrations tolerance was varied among strains. In our study high tolerance to sodium chloride (NaCl), was observed since 75% of the tested rhizobia could grow well with 1% NaCl. However, at higher concentrations the percentage of tolerant isolate decreased since only 11.1% of the isolates tolerated 5% NaCl. Isolate CR8, CR20, CR23, CR28, CR34, CR38, CR45 and CR50 showed low tolerance level to salt as they grew only in 0.5% NaCl containing medium. Isolate CR02, C404, CR05 and CR25 were the most tolerant which

grew well on medium containing 5% NaCl. Similarly, earlier studies (Maâtallah *et al.*, 2002; L'taief *et al.*, 2007) observed that chickpea rhizobia also exhibited a wide variation in their salt tolerance, even among isolates from the same site. Though it is often believed that saline soils naturally select strains more tolerant to salinity such kind of correlation was not carried out in our study.

Intrinsic antibiotics and heavy metals resistance

The evaluation of intrinsic resistance to antibiotics of chickpea rhizobia showed that most of the tested isolates exhibited high resistance to nalidixic acid and erythromycin (Table 3.2). In the presence of ampicillin, chloroamphincol, neomycin or streptomycin only 5.6 to 30.6% of isolates were resistant. All tested isolates were found to be sensitive to low concentration of tetracycline. Among the isolates CR16, CR40, CR45 and CR50 were the most sensitive isolates that did not grow on any antibiotics. Isolate CR06 was the most antibiotics resistant which grew on 5 antibiotics followed by CR04 and CR05 which showed resistance to 4 antibiotics.

Several studies (Maâtallah *et al.*, 2002; Küçük and Kıvanç, 2008) also observed great variation among chickpea rhizobia with respect to their intrinsic antibiotics resistance pattern (IAR). The IAR can be used for the identification of rhizobial strains that occupy nodules in studies designed to evaluate the ecological competitiveness (Kremer and Peterson, 1982). In addition, the pattern of antibiotics resistance has been used to identify diversity among strains of rhizobia (Somasegaran and Hoben, 1994). Consequently, it could be used as supplementary diagnostic character for different rhizobial strains (Amarger *et al.*, 1997).

Variations were also observed among the isolates in their heavy metals resistance pattern (Table 3.2). The result showed that 72% of the isolates were found to be resistant to Cr followed by Zn which was resisted by 19.4% of the isolates. Only 8.3% of the isolates were found to be resistant to lead. None of the isolates exhibited an intrinsic resistance to the remaining heavy metals indicating that they are highly inhibitory to the isolates. Isolate CR21 was the most resistant that grew on three heavy meatls whereas isolate CR03, CR23, CR25, CR34, CR36, CR40, CR46, CR47 and CR50 were the most sensitive that did not grow on any of the tested heavy metals.

Previous studies also reported that some *Mesorhizobium* species were found to be tolerant to few heavy metals (Maâtallah *et al.*, 2002; Küçük and Kıvanç, 2008). Though metals concentrations in soils samples were not determined their metal toxicity tolerance might be related to their adaptation at their isolation sites. The heavy metal resistance traits of the rhizobial isolates would be used as invaluable positive markers during genetic studies (Küçük and Kıvanç, 2008). The high level of Zn and Cr resistance suggest that these metals could be used as selective agents for some *Mesorhizobium strains* (Sinclair and Eaglesham, 1984; Küçük and Kıvanç, 2008).

C-sources and N-Sources utilization pattern

Most of the chickpea rhizobia strains were able to catabolize diverse carbon substrates (Table 3.2). All tested strains grew on D-glucose D-mannitol, galactose, maltose, lactose, raffinose, cellobiose and sucrose. All tested isolates were unable to utilize citrate as sole source of carbon. Isolate CR04 and CR05 were the most versatile isolates that could grow on 93% of the tested

carbon sources whereas CR18, CR19, CR24, CR29, CR38 and CR50 where the most fastidious isolates which coluld utilize only 37-52% of the tested crabon substartes. Graham and Parker (1964) found that utilization of citrate as sole sources of carbon was restricted to slow-growing bradyrhizobia.

Earlier studies (Graham and Parker, 1964; Sadowsky *et al.*, 1983) showed that fast-growing rhizobia were able to grow on a large variety of carbon substrates whereas slow-growing rhizobia were more limited in their ability to use diverse carbon sources. However, our result showed that the majority of tested slow-growing chickpea rhizobia were able to use a broad range of carbohydrates. This is in line with the result of other studies (Nour *et al.*, 1994; Maâtallah *et al.*, 2002; L'taief *et al.*, 2007). It is very interesting to notice that the types of carbohydrates utilized were also varied among chickpea rhizobia. Such characteristics are usually used as diagnostic features for root nodule bacteria (Küçük and Kıvanç, 2008; Hungria *et al.*, 2001).

Chickpea rhizobia also exhibited diversity in utilizing different amino acids and vitamins as sole N-sources (Table 3.2). Methionine, tyrosine, thiamine and riboflavin were utilized by all isolates whereas niacin was least preferred N-source by most isolates. Except phenylalanine and glycine, most isolates metabolized the remaining N-sources tested. Isolate CR48 was the most efficient isolate that could grow on all tested N-sources followed by CR01, CR04 and CR06 which could utilize 93% of the tested substrates. Similarly, previous study (Küçük and Kıvanç, 2008) showed that chickpea rhizobia could be able to utilize different substrates as N-sources. The ability of

isolates to utilize wide range of N-sources would give more ecological competence in the soil and it is also one of the desirable characteristics for isolates to be selected for field studies.

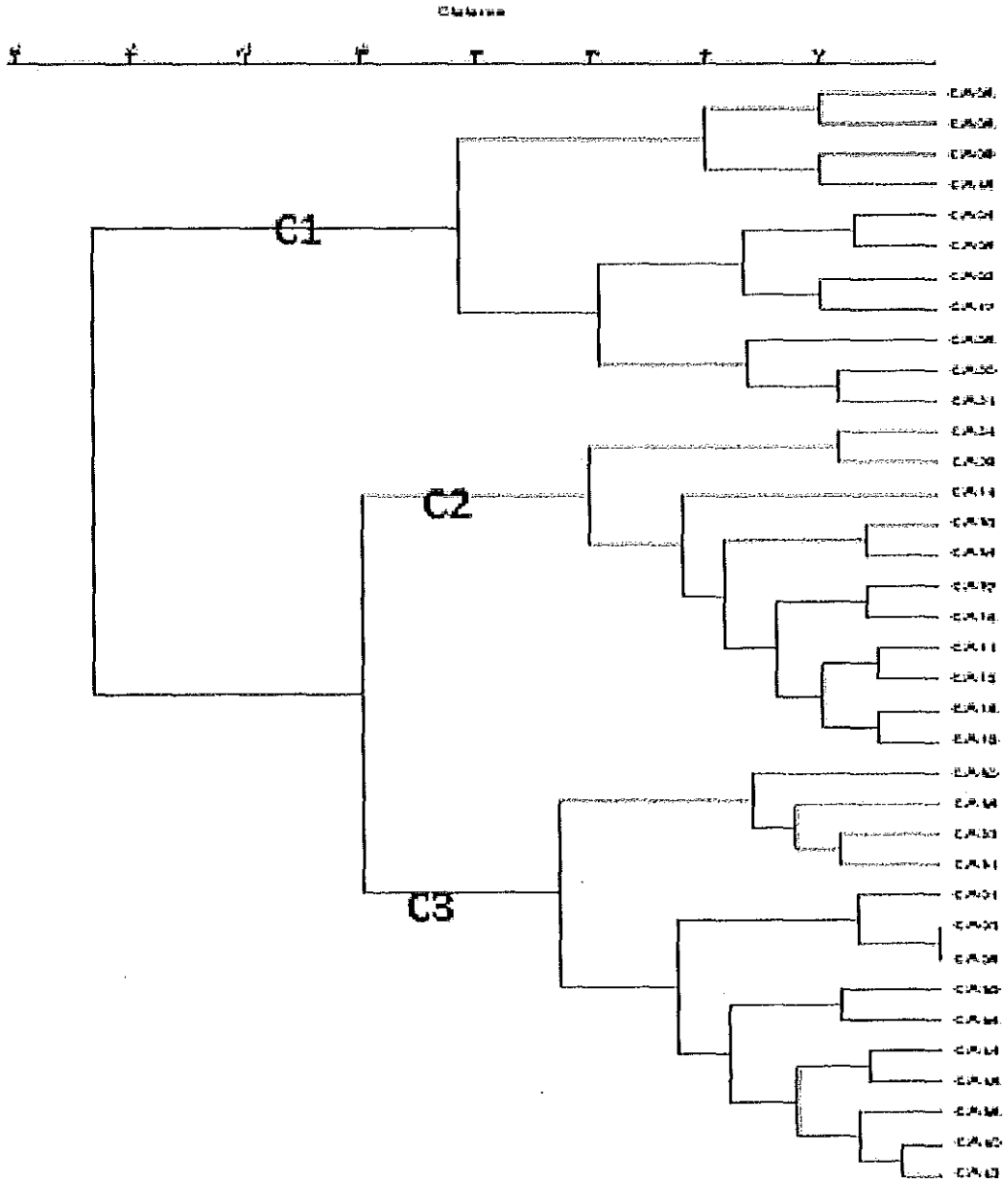

Figure 3.2 Phenogram showing phenotypic similarity of chickpea rhizobia isolated from Ethiopian soils

Numerical analysis

Cluster analysis grouped the 36 chickpea rhizobia into three clusters (C1, C2 and C3)(Figure 3.2) indicating the diversity of this rhizobial group. The isolates were clustered irrespective of the geographic locations of their isolation sites. The number of isolates varied from cluster to cluster. Cluster C3with a total of 14 members is the largest where as the remaining clusters (C1 and C2) contained equal member (11 isolates). Isolate CR03 (Debere Selam) and CR25 (Alshin) obtained from different geographical location showed 100% similarity.

PGP properties of the isolates

Chickpea nodulating rhizoia showed PGP characteristics such as auxin production, inorganic phosphate solubilization, and *F. oxysporum* inhibition under *in vitro* conditions (Table 3.4). Out of all tested isolates 27.8% of them were found to be auxin producer while only 44.4% were insoluble inorganic phosphate solubilizer with solubilization index ranging from 1.3 to 1.12. Whether present in the rhizosphere as molecules from bacterial saprophytes or present in plant tissues as products released by endophytes, auxin can massively proliferate root hair production (Yanni *et al.*, 2001) and thus enhance the root's absorptive capacity and nutrient uptake of the crops (Dakora,2003). Besides, inoculation of plants with phosphate solubilizing bacteria often stimulates plant growth by increasing phosphorus uptake (Chabot *et al.*, 1996). Thus, this ability makes them more important as multi-purpose inoculants for this crop production.

Table 3.4 PGP characteristics of chickpea nodulating rhizobial isolates

rhizobial isolates	S.I.	IAA production	*In vitro* inhibition of *F.oxysporum*
CR01	-	+	-
CR02	1.2	+	-
CR03	-	-	-
CR04	-	+	-
CR05	-	-	-
CR06	1.14	-	+
CR08	1.23	-	-
CR09	1.15	-	-
CR11	1.25	-	-
CR12	-	-	-
CR14	-	-	-
CR16	-	-	-
CR18	-	+	-
CR19	-	+	-
CR20	1.21	-	-
CR21	-	-	-
CR23	1.13	-	+
CR24	1.23	-	-
CR25	-	-	+
CR28	1.12	-	-
CR29	-	+	-
CR31	1.3	+	-
CR32	1.13	-	-
CR34	-	-	-
CR36	-	-	-
CR37	1.17	+	-
CR38	1.2	-	-
CR39	-	-	-
CR40	-	+	-
CR42	-	-	-
CR44	1.21	-	-
CR45	-	-	+
CR46	1.15	-	+
CR47	-	-	+
CR48	-	+	-
CR50	-	-	+

+: the character present; -: the character absent; SE: standard error; S.I. : solubilization index

Furthermore, 19.4% tested chickpea nodulating rhizobia were found to inhibit the growth of *F. oxysporum* in dual culture assay. It has also been documented that chickpea rhizobial strains showed a strong inhibitory effect against other fungal pathogen of chickpea such as *Ascocyhta rabiei* (Küçük and Kıvanç, 2008) and *Rhizoctonia solani* (Hemissi *et al.*, 2011) under *in vitro* conditions. Consequently, these strains could be used as biocontrol agents against root rot and wilt causing fungal pathogens of chickpea. Nevertheless, further investigations need to be carried out on the mechanism of inhibition and evaluation of their activity under glasshouse and field conditions.

Some of the isolates also showed dual PGP charactersitcs. Isolate CR02, CR31 and CR37 showed both phosphate solubilization and IAA production abilities whereas isolate CR06, CR23 and CR46 were both phosphate solubilization and antagonistic activities against the test pathogen. None of the isolates could display both IAA production and antagonistic activities against the test pathogen. Several studies showed that different strains of *Mesorhizobium* species endowed with PGP characteristics (Antoun *et al.*, 1998; Alikhani and Yakhchali, 2009; Etesami *et al.*, 2009; Hemissi *et al.*, 2011). When there is no legume host rhizobia could exist as saprophytes. As a result, they become attracted to the roots of non-legume crops and nourish root exudates in the rhizosphere (Dowling and Broughton, 1986). Consequently, rhizobia which have PGP characteristics would increase the yield of non-legume crops which could be grown in rotation or mixed cropping with legumes (Antoun *et al.*, 1998; Alikhani and Yakhchali, 2009; Etesami *et al.*, 2009). Thus, root colonization pattern and PGP activity of such isolates when inoculated to wheat, tef and barley which are commonly grown in rotation with chickpea under Ethiopian conditions needs to be investigated.

Symbiotic characteristics of the isolates

All tested isolates of chickpea rhizobia showed great variation in their capacity to induce the formation of nodule on the host plant root under glasshouse conditions. The mean nodule number per plant was varied from 41 to 79 which were induced by isolate CR14 and CR08, respectively (Table 3.5). In general, 83% of the isolates induced the formation of more than 50 nodule number on the roots of their host. In similar study, Maâtallah *et al.*(2002) reported that the mean nodule number per plant was ranged from 11 to 62. This indicates that chickpea rhizobial isolates from Ethiopian soils are the most infective strains which could form high number of nodules. The highest nodule dry weight was recorded for isolate CR32 i.e. 120 mg/plant whereas the lowest was 56 mg which was recorded for isolate CR02 and CR50 (Table 3.5).

Table 3.5 Symbiotic characteristics of chickpea nodulating rhizobial isolates

Treatments (rhizobial isolates)	Nodule Number/plant ± SE	Nodule dry weight(mg)/plant ± SE	Shoot dry weight(g)/plant ± SE	Relative effectiveness %
CR01	45.8±3.0i	78±8b	0.60±0.02k	44.1
CR02	57.3±3.2b-i	56±5b	0.91±0.02c-i	66.9
CR03	49.3±1.9h-j	75±2b	0.69±0.03i-k	50.7
CR04	51.8±3.2d-j	69±8b	0.81±0.05d-k	59.6
CR05	58.3±2.6b-i	114±3a	0.82±0.1d-k	60.3
CR06	58. 7±2.5b-i	92±20b	1.13±0.04bc	83.1
CR08	79.3±3.2a	115±9a	0.60±0.02k	44.1
CR09	60.8±1.6b-h	101±7a	0.86±0.08c-k	63.2
CR11	61.2±2.5b-h	99±2a	0.75±0.01f-k	55.1
CR12	43.3±1.2ij	80±9b	0.63±0.03jk	46.3
CR14	41.2±1.5j	76±4b	0.92±0.03c-i	67.6
CR16	43.8±1.4ij	116±15a	0.88±0.04c-k	64.7
CR18	60.8±2.8b-g	82±9b	0.99±0.05c-f	72,8
CR19	51.2±1.6e-j	92±4b	0.97±0.04c-i	71.3
CR20	60.51.0b-h	99±2a	1.03±0.01c-e	75.7
CR21	54.5±3.0c-j	89±10b	0.72±0.04g-k	52.9
CR23	59.3±2.5b-h	75±5b	0.81±0.04d-k	59.6
CR24	66.0±2.0a-g	96±4a	0.72±0.01g-k	52.9
CR25	62.5±2.0b-g	102±6a	0.95±0.09c-i	69.8
CR28	64.8±2.5a-e	97±2a	0. 80±0.05d-k	58.8
CR29	49.0±3.1h-j	95±9b	0.80±0.03d-j	58.8
CR31	68.7±3.5a-c	105±6a	0.98±0.01c-g	72.1
CR32	70.2±1.8ab	120±3a	1.04±0.02c-e	76.5
CR34	51.0±1.5e-j	77±6b	0.81±0.01d-k	59.6
CR36	46.2±2.9h-j	98±7a	0.89±0.05c-j	65.4
CR37	62.0±4.5b-g	100±20a	0.70±0.07h-k	51.5
CR38	64.5±1.6a-f	104±30a	1.36±0.12a	100
CR39	62.5±1.0b-g	99±30a	0.81±0.12d-k	59.6
CR40	67.0±4.8a-d	74±8b	0.60±0.01k	44.1
CR42	63.5±3.8b-g	90±6b	0.81±0.02d-k	59.6
CR44	61.3±5.9b-h	96±1b	0.76±0.04e-k	55.9
CR45	63.0±1.2b-g	94±3b	1.22±0.06b	89.7
CR46	62.0±2.8b-g	75±6b	0.70±0.01h-k	51.5
CR47	71.0±1.2ab	100±3a	1.11±0.07b-d	81.6
CR48	50.8±2.0e-j	95±7b	0.85±0.05c-k	62.5
CR50	57.3±3.2b-i	56±5b	0.75±0.06f-k	55.1
T0	-	-	0.26±0.004l	19.1
TN	-	-	1.36±0.03a	100

Numbers in the same column followed by the same letter do not differ significantly at p= 0.05 by DT; +: the character present; -: the character absent; SE: standard error

Similarly, all tested chickpea rhizobia exhibited high diversity in their capacity to accumulate shoot dry matter yield. In comparison with TN control which represented 100% level of shoot dry matter and T0 control which represented 19.1%, the isolates showed shoot dry matter yield ranging from 0.60g to 1.36g per plant (Table 3.5). This indicates that the most effective isolate could accumulate twice as much shoot dry mass as that of the least effective isolates. The relative effectiveness showed that isolate CR06, CR38, CR45 and CR47 were the most efficient with 80%- 100% dry matter yield while isolates CR01, CR08 and CR40 were the least efficient with 44.1% relative effectiveness (Table 3.5). These isolates were also particularly most infective and highly effective. Previous study (L'taief *et al.*, 2007) showed that the relative effectiveness of chickpea nodulating rhizobia was varied from 41% to 86%.

In our study a correlation ($r<0.5$; $p>0.05$) between the increase of dry matter and the number or the dry weight of nodules was not found to be statistically significant ($p>0.05$). Similarly, Maâtallah *et al.* (2002) also reported that there was no positive correlation between the increase of shoot dry matter or the number and the dry weight of nodules. Dudeja *et al.*, (1981) demonstrated that the dry matter yield was rather correlated with the nodule leghaemogblobin concentration than with the number or the dry weight of nodules.

Based on the result of our studies the following isolates CR06, CR18, CR19, CR20, CR31, CR32, CR38, CR45 and CR47 are highly recommended for field test and ecological competitiveness studies under different Ethiopian soil and climatic conditions. Since these

isolates have exhibited interesting features such as wide range of C and N-sources utilization, tolerance of acidic pH, metal toxicity and antibiotics(Table 3.2, 3.3, 3.4 and 3.5). In addition, they exhibited PGP features such as auxin production, *F. oxysporum* growth inhibition, inorganic phosphate solubilization, and highly effective nitrogen fixation. In general, from the present study, it can be concluded that Ethiopian soils harbor highly efficient nitrogen-fixing chickpea nodulating rhizobia which are diverse in their morphological, physiological, symbiotic and PGP characteristics.

In addition to *Mesorhizobium ciceri* (Nour *et al.*, 1994) and *Mesorhizobium, mediterraneum* (Nour *et al.*, 1995) some bacterial species have been described to nodulate chickpea with different symbiotic efficiency (Laranjo *et al.*, 2004; Rivas *et al.*, 2007). However, during this study methods used for characterizing and distinguishing rhizobial strains were morphological, physiological and symbiotic. These traditional methods of rhizobial characterization frequently fail to identify strains to a species level. Hence, this study should be corroborated by PCR based molecular methods such as RFLP, RAPD, AFLP and 16S rRNA sequence analysis to evaluate their genotypic diversity and identify the isolates to the species level.

Chapter 4 Phosphate solubilization efficiency and plant growth promoting properties of rhizobacteria isolated from lentil (*Lens culnaris* Medik.) growing areas of Ethiopia

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Abstract

Lentil (*Lens culnaris* Medik.) is an important source of protein for the majority of Ethiopian population. The low availability of phosphorus in soil is one of the major soil related stresses that limit the production of this crop. This, study was aimed at isolation and characterization of phosphate solubilizing bacteria from lentil growing areas of Ethiopia and assessing their effects on growth of this crop under glasshouse conditions. A total of 41 phosphate solubilizing bacterial isolates were obtained from lentil rhizosphere and identified using biochemical tests and 16S rDNA sequence analysis. The result showed the presence of genera *Acinetobacter, Aeromonas, Agrobacterium, Bacillus, Burkholderia, Chryseomonas, Enterobacter, Pseudomonas, Ralstonia* and *Sphingomonas.* Phosphate solubilzing efficiencies of these isolates were determined in liquid medium using different insoluble P sources and the result indicated that they released significant amount of phosphates from tricalcium phosphate, rock phosphate and bone meal. Besides phosphate solubilization, some of these isolates exhibited multiple plant growth promoting characteristics such as indole acetic acid production and fungal pathogen growth inhibition. Over 26% of the isolates were capable of producing indole acetic acid and 4.9% of them inhibited the growth of *Fusarium oxysporum* under *in vitro* conditions. The effects of inoculation of *Enterobacter kobei* (PSBL5), *Bacillus subtilis* (PSBL21), *Aeromonas hydrophila* (PSBL26), *Bacillus* species (PSBL31) and *Pseudomonas fluorescence* (PSBL37) on lentil growth, nodulation, nitrogen fixation and P uptake were investigated under a glasshouse conditions and the results showed that these features were significantly increased over uninoculated control plants. It is possible to conclude that lentil rhizosphere soils from producing areas of Ethiopia harbor diverse phosphate solubilizing bacterial isolates with excellent phosphate solubilization and plant growth promotion ability. Thus, some of these isolates need to be tested in the field conditions under different agroecology of the country.

Key words: Rock phosphate, Bone meal, PGPR, IAA, Rhizosphere soil

Introduction

Lentil (*Lens culnaris* Medik.) is an important pulse crop grown widely in Ethiopia over an area of 105,956 ha with annual production of 123,777 tones (CSA, 2010). It is an invaluable source of dietary protein for the vast majority of Ethiopian population. In addition, lentil restores and maintains soil fertility through its symbiotic nitrogen-fixation and thereby ensures sustainability of the crop production system. Consequently, it has been grown in rotation with major cereals in traditional low input system. However, its yield has remained very low compared to its potential (Asfaw Hailemariam and Angaw Tsige, 2006; Mulissa Jida and Fassil Assefa, 2011) as result of soil fertility related stresses which prevail under Ethiopian edaphoclimatic conditions (Seid Ahmed and Melkamu Ayalew, 2006; Asnake Fikre and Geletu Bejiga, 2006).

Phosphorus (P) is one of the essential macronutrients that limit plant growth in tropical soil (Vessey, 2003; Khan *et al.*, 2006). Most tropical and subtropical area soils are predominantly acidic and extremely deficient in available phosphorus dueto their strong P-fixation ability (Khan *et al.*, 2006). These low levels of available P are mainly duc to high reactivity of soluble P with Fe and Al oxides in acidic soils (Gyaneshwar *et al.*, 2002). Similarly, most Ethiopian soils are also acidic and predominantly characterized by low available P (Desta Beyene, 1982; Tekalign Mamo *et al.*, 1988). Hence, low available P in soil has been one of the stresses that constrain the production of lentil and other pulse crops in the country (Asfaw Tilaye, 1985; Getachew Agegnehu *et al.*, 2006). Application of chemical fertilizer is highly limited by its very expensive cost and for this reasons its use for pulse crops production such as lentil is very low in Ethiopia (Samia Zekaria, 2006). Furthermore, up to 75% of the P fertilizers added to the soil could be converted to insoluble forms by reacting with free Ca^{+2} or Fe^{+3} and Al^{+3} ions in alkaline and

acidic soils, respectively (Goldstein, 1986). These cause an urgent need for economically feasible and ecologically acceptable fertilizer sources. Thus, using microbial biofertilizer for improving the availability of P in the vicinity of plant roots represents a feasible alternative to the use of chemical fertilizers (Naik *et al.*, 2008). It has been well documented that phosphate solubilizing bacteria (PSB) are active in conversion of insoluble phosphate to soluble primary and secondary orthophosphate ions (Pal, 1998; Peix *et al.* 2001a; Chen *et al.*, 2006; Vyas and Gulati, 2009).

Bacteria that colonize the rhizosphere and plant root, and enhance plant growth are referred to as plant growth promoting rhizobacteria (PGPR) (Kloepper and Schroth, 1978). Phosphate solubilization by rhizobacteria that make the P available in soil solution for plant growth is considered as one of the important attributes of PGPR (Chen *et al.*, 2006). The proportion of PSB is very high in the rhizosphere and such bacteria are metabolically more active than those isolated from bulk soil (Vazquez et al., 2000). This pertains to plants grown in P-deficient soils (Khan *et al.*, 2006). Several studies showed the presence of PSB in the rhizosphere of different agriculturally important crops (Kim *et al.* 1998; Peix *et al.* 2001a; Alam *et al.*, 2002; Ponmurugan and Gopi, 2006; Rajapaksha and Senanayake, 2011) and their pivotal role in conversion of insoluble P to the orthophosphate ions which can be utilized by plants (Chabot *et al.*, 1993; Pal, 1998; Ponmurugan and Gopi, 2006). Earlier studies indicated that different species of PSB were able to solubilize naturally occurring insoluble inorganic P sources such as, rock phosphate and old bone (Ivanova *et al.*, 2006; Asefa Keneni *et al.*, 2010). Besides increased P uptake, the production of phytohormones such as indole acetic acid (IAA) by PSB could also play an important role in plant growth promotion (Ponmurugan and Gopi, 2006; Naik *et al.*,

2008; Shahab *et al.*, 2009). In addition, PSB could also enhance plant growth by suppressing different soil borne fungal pathogens of crops (Peix *et al.* 2001b).

Though the majority of Ethiopian soils are mainly characterized by low available P (Desta Beyene, 1982; Tekalign Mamo *et al.*, 1988), most of the hitherto studies have been concerned with the rate of P-fertilizer application (Getachew Agegnehu *et al.*, 2006). Consequently, there is little information about native PSB isolates recovered from the rhizosphere of lentil grown in Ethiopian soils. Therefore, the main purpose of this study was to isolate and characterize PSB from lentil growing areas of Ethiopia and evaluate their effects on the growth of lentil under glasshouse conditions.

Materials and methods

Study Sites and Soil Samples Collection.

Soil samples were collected from lentil growing farmers' fields in Amhara, Oromia and Tirgay Regional States of Ethiopia in October, 2009. These regions represent the major lentil producing areas of the country (Fig 2.1). The areas are distributed in central and northern parts of the country with an altitude range of about 1799 – 3101 meters above sea level (masl) and soil pH from moderately acidic (5.58) to slightly alkaline (7.18) (Table 4.1). The soil samples were excavated from the 15-20 cm depth and collected in sterile plastic bags. A total of 35 soil samples were carefully transported to the Applied Microbiology Laboratory, Addis Ababa University, for further work.

2.2. Isolation and Enumeration of Phosphate solubilizing Rhizobacteria

The soil sample was thoroughly mixed, air-dried in a glasshouse and then filled into 3 kg capacity surface sterilized plastic pots. Lentil seeds (*Teshale* cultivar), obtained from Ethiopian Institute of Agricultural Research (EIAR), Debre Zeit were selected and surface sterilized first with 95% ethanol and then with 3% sodium hypochlorite solutions for 10 seconds and 3 minutes, respectively and, rinsed five times with sterilized distilled water. Surface sterilized seeds were allowed to germinate on sterile water agar (1%) surface for three days at 25°C and five pre-germinated seeds were planted in each pot, which were thinned down to three 5 days after emergence (DAE). All pots were situated in glasshouse over the table and watered to a field capacity every three days for 45 days after planting (DAP).

Fourty-five DAP healthy lentil seedlings with good growth were selected from each pot and uprooted carefully with their rhizosphere soil. Plant roots with adhered rhizosphere soil were carefully transferred into sterile Petri dishes and 10 g of it was transferred to flasks (250 ml) containing sterilized 90 ml saline solution (0.85% w/v NaCl). The flasks were incubated on a gyratory shaker at 120 revolutions per minute (rpm) at room temperature for 30 minutes and then serially diluted. Suspensions of the appropriate dilution were spread plated on Pikovskaya medium (PK) (Pikovskaya, 1948) and the plates were incubated at 30°C for 5 days. All colonies surrounded with apparent clear zone were counted and colonies with a good halo zone and of different morphologies were selected and transferred to PK broth separately. The test tubes were incubated at 30°C for 48 hours (hrs). The isolates were purified by re-streaking on PK agar plates and maintained on PK slants for short term storage at 4°C and 50% (v/v) glycerol for long term

storage at -20°C. Isolates were designated as PSBL01 (Phosphate Solubilizing Bacteria from Lentil) - PSBL120 (Table 4.1).

Identification of the PSB isolates

Biochemical characterization and identification by the API kits system

All isolates were preliminarily characterized using the following tests: Gram reaction using Gregorson's KOH (Gregorson, 1978), 'cytochrome oxidase (Kovacs, 1956), Oxidative/Fermentative (O/F) test (Huge and Leifson, 1953) and endospore formation tests. Based on the result of these tests isolates were assigned to different groups and identified to species level using the API identification system assisted by API Plus computer software (bioMèrieux_SA, Marcy-l'Etiole, France) following manufacturer's instructions. Gram positive, endospore forming rods were identified to species level using API 50 CH test kits. Gram negative rods and cytochrome oxidase negative isolates with fermentative reaction in the O/F test were identified using the API 20E test kits. Gram negative rods with oxidative reaction in O/F test were identified by using API 20NE test kits. Moreover, isolates were selected for 16S rDNA sequence analysis based on their phosphate solubilization activity, abundance of particular group and the result of API identification system.

Identification of isolates with 16S rDNA sequencing

Genomic DNA extraction, PCR amplification of 16S rDNA and Sequencing

All isolates were streaked on LB agar medium and incubated at 30°C for 24 hrs. A single colony of each isolate was picked using a sterile tooth pick and suspended in 30 µl sterile H_2O in eppendrof tubes. The DNA from gram negative bacteria was extracted using thermal denaturation method (Mohran *et al.* 1998). All tubes that contain colony suspensions were incubated at 95°C for 10 minutes. The DNA from gram positive isolates was extracted by heat thawing at 65°C for 3 minutes followed by freezing at -70°C for 3 minutes; the cycles were repeated three times. All the tubes were centrifuged at 13,000 rpm for 2 minutes and stored at 4°C.

Two µl of extracted DNA was used as a template for PCR amplification using a thermal cycler. In addition to the DNA, the PCR reaction consisted of 0.4 µl 10mM dNTP, 2.5 µl 10x PCR(Taq) buffer, 2.5 µl 25 mM $MgCl_2$, 0.2 µl (5 units/µl) of Taq polymerase, 1 µl (10 mM) of forward primer 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1µl (10 mM) of reverse primer1492r (5'-GGTTACCTTGTTACGACTT-3'). All primers were synthesized by Fermentas (MWG, Germany). The PCR program included a denaturation step of 5 min at 95°C, followed by 30 cycles of 95°C for 30 sec, 50°C for 45 sec, 72°C for 1.30 min, and an extension step of 10 min at 72°C. Amplified PCR products were resolved on 1.0% agarose gel and stained with ethidium bromide (1 µg/ml). All amplified PCR products were stored at -20°C and sequenced with forward primer 27f by using Sanger sequencer (ABI-3730XL) at the institute of Clinical and Molecular Biology, University of Kiel. PCR products that generated unreadable sequences were

reproduced as described above. The DNA sequences were edited using Bioedit (http://www.mbio.ncsu.edu/BioEdit/). BLAST search of National Center for Biotechnology Information Gen Bank (NCBI) (*www.ncbi.nlm.nih.gov/*) data libraries was used to establish the identity of the isolates.

Phylogenetic analysis

The sequences and standard sequences of bacterial lineage from the NCBI were aligned using Clustal W (Bioedit). A phylogenetic tree was constructed by using neighbor joining method from distance matrices. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004) and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset. There were a total of 507 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura *et al.*, 2007).

Bacterial inoculum preparation

Bacterial isolates were grown in PK broth on a gyratory shaker at room temperature and 120 r pm for 72 hrs. The suspension was centrifuged in sterile plastic tubes (10 ml) at 5000 rpm for 10 min. The pellets were re-suspended in normal saline solution to give a final concentration of 10^8 cells ml^{-1} using the viable plate count method and optical density measurement at 600 nm. All bacterial inoculums were prepared like this unless otherwise stated.

Determination of phosphate solubilization efficiency of the PSB isolates

Phosphate solubilization efficiency of the PSB isolates was determined qualitatively using solubilization index (SI). PK agar plates were spot inoculated with a 20 µl of culture suspension of each isolates. After incubation at 30°C for 5 days formation of a clear zone around the spot was checked and their SI was calculated using the formula, *SI= (colony + halo zone diameter)/ colony diameter* (Edi-Premono *et al.*, 1996).

Based on their phosphate solubilizing activities and abundance isolates were selected and used for quantitative estimation of P solubilization efficiency in PK broth. Hundred ml of PK liquid medium without phosphorus sources was dispensed in a 250 ml flask and supplemented with the following insoluble phosphate sources which contains equivalent amount of P. The insoluble phosphate sources were $Ca_3(PO_4)_2$ (TCP); Egyptian rock phosphate (RP) and bone meal (BM) (obtained from EAIR, Holeta) were added separately as a sole phosphorus source. All flasks were autoclaved and inoculated with 100 µl culture suspension of each isolate separately. The flasks were incubated at room temperature on a gyratory shaker at 120 rpm for 12 days. Five ml of samples were withdrawn from each treatment on day 4, 8, and 12 and analyzed for phosphorus solubilized and pH changes. The sample was centrifuged at 15,000 rpm for 15 minutes and the clear supernatant was used for P analysis. The amount of solubilized P was quantitatively determined following the phosphor-molybdate method (Murphy and Riley, 1962) as described in Sahelmedhin Sertsu and Taye Bekele (2000). The concentrations of solubilized P was calculated

using the standards of known concentrations of KH_2PO_4.The amount of solubilized P obtained from the control was deducted from their respective treatments to get the final results.

Screening for *in vitro* antagonistic activity against *Fusarium oxysporum*

The *in vitro* growth inhibition of the isolates against *Fusarium oxysporum f.sp. lentis* (FOL), obtained from EAIR, Debre Zeit, was tested using the dual culture technique (Landa *et.al.*, 1997). Twenty µl of culture suspension of each isolate was equidistantly spotted on the margins of nutrient agar (NA) plates amended with glucose (1.0%) and incubated at 30 °C for 24 h. A 4-5 mm diameter agar disc from potato dextrose agar (PDA) cultures of the fungal pathogen was placed at the centre of the NA plate inoculated with the bacterial isolate and incubated at 30°C for 5-7 days. The radii of the fungal colonies towards (r) and away (R) from the bacterial colonies were measured and and the *in vitro* inhibition was calculated following Ahmed Idris *et al.* (2008).

Fluorescent siderophore and IAA production test

Cultures of the isolates were grown at 30°C in LB broth medium supplemented with 5 mM L-tryptophan for 48 hrs (Bric *et al.*, 1991) to determine their IAA producing ability. Supernatant of the strains were collected by centrifugation at 10,000 rpm for 15 min and 2 ml supernatant of each was transferred to tubes to which 100 µl of 10 mM O-phosphoric acid and 4 ml of Salkowaski reagent (1 ml of 0.5M $FeCl_3$ in 50 ml 35% $HClO_4$) were added. Mixtures were incubated at room temperature for 25 minutes and observed for the development of pink color. Quantitative estimation of the IAA was carried out by measuring the intensity of the color

at 530 nm using spectrometer (Gordon and Weber, 1951). The concentrations of IAA were calculated using standard curve drawn with known concentrations of indole-3-acetic acid (Sigma-Aldrich). Fluorescent siderophore production was tested by streaking the bacterial cultures on King's B medium (King, 1954) and incubated at 30^0C for 48 hrs. The yellow green fluorescence pigment of the bacterial colonies that diffuse in the surrounding agar was evaluated using an UV lamp at 365 nm. Formation of fluorescent pigment was considered as an indication of siderophore production.

Effects of inoculation of PSB isolates on the growth of lentil under glasshouse conditions

Bacterial isolates that showed high phosphate solubilization efficiency in PK broth were selected for their *in vivo* growth promotion evaluation under greenhouse conditions. Lentil seeds were surface sterilized, germinated and transplanted as described before. Prior to transplantation, germinated seedlings were flooded with PSB culture grown in PK broth adjusted to 10^8cells $seed^{-1}$ for one hour on separate sterilized Petri plates. Five inoculated seedlings were transferred to each pot containing 3 kg vertisol collected from farm land Dima Guranda, around Sebeta. This soil has the following physicochemical characteristics: pH; 6.0, total N;0.025%, organic carbon;0.975, total P;1667 ppm, available P;2.99 ppm, available K;25.33 ppm, Cation Exchange Capacity;50 Cmol/Kg and loamy texture. The seedlings were thinned down to three 5 DAE. The pots were irrigated with distilled sterilized water every three days. Uninoculated P-fertilized (20 mg kg^{-1}as KH_2PO_4) pots were included as positive controls (PC) and uninoculated non P-fertilized pots as negative control (NC). Isolates PSBL05 (*Enterobacter kobei*), PSBL21 (*Bacillus subtilis*), PSBL26 (*Aeromonas hydrophila*), PSBL31 (*Bacillus species*) and PSBL37

(*Pseudomonas fluorescens*) were used as inoculants. In addition, two isolates (PSBL05 and PSBL31) which released higher amount of soluble P from RP and BM were used as inoculants in conjunction with the agronomically recommended amount of RP (200 kg ha^{-1}) or BM (75 kg ha^{-1}). At sixty DAP all seedlings were carefully uprooted and washed under gently flowing tap water. Then nodules of the plants were counted, and shoot dry weight was measured after drying at 70^0C for 48 hrs. Total N of the shoot was determined using kjeldahl method as described in Sahalmedhin Sertsu and Taye Bekele (2000). The P concentration of the shoot was determined by phospho-molybdate method (Murphy and Riley, 1962) after ashing 0.5 g of oven dried and ground shoot dry matter. The concentration of P in shoot was calculated from the standards drawn with known concentrations of KH_2PO_4.

Data Analysis

Data was analyzed using analysis of variance (ANOVA) and the treatment means were compared relative to control following Duncan's test (DT). The relation between different parameters was evaluated by using Pearson correlation coefficient using SPSS v.15.

Result and discussion

Isolation, Enumeration and Identification of PSB Isolates

A total of 30 soil samples were collected from different lentil producing areas found in central and northern parts of the country which represents major producing areas of the country (Table 4.1). The sampling sites were distributed in different parts of the country with an altitude range of about 1799 – 3101 meters above sea level (masl) and pH from moderately acidic (5.58) to

slightly alkaline (7.18). Most (25) of the sampling sites soils were acdic with pH value ranging from 5.12 -6.93.

The number of PSB varied from 2.4×10^3 to 2.1×10^5 CFU g^{-1} of rhizosphere soil, which was recorded for soil samples from Teji and Asketema, respectively (Table 4.1). Asefa Keneni *et al.*(2010) reported that the number of PSB in the rhizosphere of faba bean was ranged from 15 to $1.8x10^3$ CFU g^{-1}.This indicates that the proportions of such rhizobacteria are very high in lentil rhizosphere soils from producing areas of Ethiopia. Similarly, previous studies indicated that the proportions of PSB were very high in the rhizosphere of some agriculturally important crops such as maize, sorghum, rice, barley and chickpea, and thus they could play an essential role in P nutrition of the plants (Alam *et al.*, 2002; Ponmurugan and Gopi, 2006; Rajapaksha and Senanayake, 2011; Peix *et al.* 2001a; Kim *et al.* 1998). Furthermore, it has been demonstrated that PSB are also common in bulk soil and play central role in the natural P cycle as well (Chen *et al.*, 2006; Ponmurugan and Gopi, 2006; Vikram *et al.*, 2007). However, PSB obtained from rizosphere soil are metabolically more active than those isolated from bulk soil (Vazquez *et al.*, 2000).

Table 4.1 Distribution and abundance PSBL in the farmers' fields of lentil producing areas of Ethiopia

Name of isolation site	Altitude of isolation site (masl)	pH of isolation soil	Abundance of PSB(CFU/g) in the rhizosphere soil	Number of PSB isolates obtained	PSB isolates obtained
Adanaba	2533	7.03	1.4×10^5a-d	1	PSBL29
Ambo	2170	6.75	4.0×10^3d	1	PSBL49
Asgori	2078	6.20	ND	2	PSBL05,12
Asketema	2499	7.04	2.1×10^5ab	1	PSBL100
Chatawa	2912	5.12	3.6×10^4cd	1	PSBL116
Chole I	2612	5.58	4.8×10^4cd	1	PSBL54
Chole II	2647	6.05	ND	1	PSBL52
D/Selam	1896	6.00	6.1×10^4b-d	1	PSBL57
Dagam	3101	6.36	1.9×10^5ab	1	PSBL94
Fala'a	2549	7.00	1.4×10^5a-d	1	PSBL28
Fiche	2748	6.83	ND	1	PSBL33
Fogera meda	1799	6.00	1.0×10^5b-d	2	PSBL95,96
Geshana	2907	5.80	1.5×10^5a-d	1	PSBL21
Ginchi	2250	6.60	5.8×10^4b-d	1	PSBL120
Goha Tsion	2517	6.3	7.3×10^3d	1	PSBL33
Gurura	1906	7.01	3.3×10^3d	3	PSBL51,53,113
Keyit	2931	6.38	1.2×10^5a-d	2	PSBL90,92
Korem	2482	6.93	1.0×10^5b-d	1	PSBL28
Makesagnit	1978	6.39	5.3×10^4cd	1	PSBL97
Obbi	2108	6.61	ND	1	PSBL31
Sandafa	2554	6.00	3.8×10^4cd	1	PSBL58
Sheno I	2032	5.58	ND	2	PSBL26, 45
Sheno II	2868	5.58	ND	1	PSBL20
Tefki	2047	6.55	2.9×10^3d	6	PSBL02, 04,14,19,37,50
Teji	2065	6.70	2.4×10^3d	1	PSBL101
Tikana	1942	7.18	2.1×10^4d	1	PSBL93
Wajel	2432	6.12	7.8×10^4b-d	1	PSBL55
Woliso	2008	5.58	2.8×10^3d	1	PSBL13
Wuchale	1980	6.58	1.8×10^5a-c	1	PSBL99
Yetinora	2437	6.21	3.3×10^4cd	1	PSBL112

ND: not determined, Numbers in the same column followed by the same letter do not differ significantly at p= 0.05 by DMRT

A total of 41 PSB isolates with higher phosphate solubilization activities (SI ≥1.34) were selected from the soil samples for further analysis (Table 4.2). These isolates were characterized biochemically using some preliminary biochemical tests and API test kits. The result revealed the presence of an array of gram negative and gram positive PSB isolates in the rhizosphere of

lentil. They were dominated by gram negative rhizobacteria which accounts for 87.8% of isolates (Table 4.2). Similarly, previous study reported that the rhizospheres of many agriculturally important plants were found to favor more Gram negative rhizobacteria than Gram positive (Diriba Muleta *et al.*, 2013).

All selected isolates were subjected to the API kits identification system and the result revealed the presence of 7 major genera including *Pseudomonas* (46.3%), *Burkholderia* (12.2%), *Bacillus* (9.8%), *Aeromonas* (9.8%), *Chrysomonas* (4.9%), *Sphingomonas* (2.4%) and *Agrobacterium* (2.4%) (Table 4.2). The result of API kits indicated that members of the genus *Pseudomonas* were dominated by *P. fluorescens* while all isolates of the genus *Burkholderia* were identified as *B. cepacia*. Despite this about 12.2% them were left unidentified or showed low identification (<90%). This might be due to the limitations of using biochemical characteristics to identify the environmental isolates.

The partial 16S rDNA sequence analysis was also employed to identify the isolates to the genus and species level. The result showed the presence of genera such as, *Pseudomonas, Bacillus, Enterobacter, Acinetobacter, Ralstonia, Burkholderia and Agrobacterim.* Gram negative starins exhibited more diverse species compared to the gram positive isolates. There were some disagreements between API kit identification and 16S rDNA gene sequence analysis which could be due to several reasons such as inoculum size and culture conditions. Moreover, genera *Enterobacter, Ralstonia* and *Acientobacter* were identified only by sequences analysis indicating that this method is more powerful in identifying the environmental isolates.

Table 4.2 Solubilization index (SI) and identification of PSB isolated from lentil rhizosphere

Isolate	Gram reaction	Solubilization Index (SI)±SE	API identification	16S rDNA sequences identification
PSBL02	-	1.77±0.06e-h	*Aeromonas hydrophila*	ND
PSBL04	-	1.82±0.08e-h	*Pseudomonas fluorescens*	*Enterobacter ludwigii*(JX979114)
PSBL05	-	1.95±0.22c-e	UI	*Enterobacter kobei*(JX979115)
PSBL12		1.95±0.21c-e	*Bacillus species*	*Bacillus tequilensis* (JX979116)
PSBL13	+	1.86±0.10d-h	*Bacillus species*	ND
PSBL14	-	1.79±0.07e-h	*Aeromonas hydrophila*	ND
PSBL19	-	2.25±0.13a	UI	*Agrobacterium tumefaciens* (JX979117)
PSBL20	-	1.87±0.13de-h	*Chryseomonas species*	ND
PSBL21	+	1.88±0.05de-g	*Bacillus species*	*Bacillus subtilis* (JX979118)
PSBL26	-	1. 90±0.09de	*Aeromonas hydrophila*	ND
PSBL28	-	1.85±0.09d-h	*Aeromonas hydrophila*	ND
PSBL29	-	1.81±0.06e-h	*Burkholderia cepacia*	ND
PSBL30	-	1.80±0.07e-h	*Chryseomonas luteola*	ND
PSBL31	+	1.88±0.08d-g	*Bacillus species*	ND
PSBL33	-	1.84±0.03de-h	*Pseudomonas fluorescens*	ND
PSBL37	-	1.90±0.08de	*Pseudomonas fluorescens*	ND
PSBL39	-	1.87±0.10d-h	*UI*	*Acinetobacter calcoaceticus* (JX979119)
PSBL45	-	1.82±0.08e-h	*Pseudomonas fluorescens*	*Pseudomonas koreensis* (JX979120)
PSBL49	+	2.21±0.10a	*Bacillus species*	*Bacillus subtilis* (JX979121)
PSBL50	+	1.64±0.13g-i	*Pseudomonas putida*	ND
PSBL51	-	1.87±0.17d-h	*Sphingomonas paucimobils*	ND
PSBL52	-	1.86±0.20d-h	*Pseudomonas species*	ND
PSBL53	-	1.60±0.15i	*Pseudomonas fluorescens*	ND
PSBL54	-	1.65±0.14f-i	*Pseudomonas species*	ND
PSBL55	-	1.45±0.18ij	*Pseudomonas fluorescens*	ND
PSBL57	-	2.16±0.9a-c	*Pseudomonas fluorescens*	*Pseudomonas costantinii* (JX979126)
PSBL58	-	2.13±0.12a-c	*Pseudomonas fluorescens*	*Pseudomonas corrugate* (JX979127)
PSBL90	-	2.17±0.15a-c	*Burkholderia cepacia*	ND
PSBL92	-	2.17±0.14a-c	UI	*Enterobacter cloacae* (JX979128)
PSBL93	-	2.07±0.08a-d	UI	*Ralstonia picketti* (JX979122)
PSBL94	-	2.16±0.06a-c	*Pseudomonas species*	ND
PSBL95	-	2.14±0.15a-c	*Pseudomonas species*	ND
PSBL96	-	2.14±0.15a-c	*Burkholderia cepacia*	ND
PSBL97	-	1.86±0.11d-h	*Pseudomonas species*	ND
PSBL99	-	1.88±0.10d-g	*Burkholderia cepacia*	ND
PSBL100	-	2.18±0.12ab	*Pseudomonas fluorescens*	ND
PSBL101	-	1.34±0.12j	*Pseudomonas species*	ND
PSBL112	-	1.84±0.08d-h	*Burkholderia cepacia*	ND
PSBL113	-	1.98±0.13b-e	*Pseudomonas species*	ND
PSBL116	-	2.13±0.17a-c	*Agrobacterium radiobacter*	ND
PSBL120	-	1.84±0.08d-h	*Pseudomonas species*	ND

Numbers in the same column followed by the same letter do not differ significantly at p= 0.05 by DT; +: the character present/gram positive; -: the character absent/gram negative; SE: standard error; FOL: *Fusarium oxysporum f.sp. lentis*; UI: unidentified; ND: not determined, parenthesis: accession number of the sequence in NCBI gene bank

Of the Gram negative strains three of them showed the highest similarity to different species of genus *Enterobacter*. The sequence analysis revealed that PSBL04 and PSBL92 were highly similar (100% sequence similarity) to *Enterobacter ludwigii* (FG859683), phosphate solubilizing bacteria isolated from tea rhizosphere and *Enterobacter cloacae* (GQ421477), antagonists of *Aspergillus flavus* from fermented beans, respectively while PSBL05 showed 99% identity similarity with *Enterobacter kobei* (NR 028993) (Table 4.2).

Two strains showed the highest closest match with species of *Pseudomonas*. PSBL57 showed 100% sequence similarity to *Pseudomonas costantinii* (EF111123) bacteria isolated from river while PSBL58 showed 99% sequence similarity with *Pseudomonas corrugate* (HQ242748) phosphate solubilizing bacteria from P-rich soil. According to API test kits identification of members of the *Pseudomonas* were dominated by *P. fluorescens* which was also confirmed by 16S rDNA sequences analysis since PSBL57 and PSBL58 showed good similarity with fluorescent pseudomonad species (*P. putida* and *P. fluorescens*). They also showed a high similarity to *P. fluorescens (100%)* and *P. putida (99%)*.

PSBL45 showed 100% sequence similarity with *Burkholderia cepacia* (JN208904) rhizobial symbionts effective N-fixing symbionts of common bean and *P. fluorescens*, (HE610859) a drought resistance-promoting bacterium. PSBL19 showed the highest (99%) sequence similarity to *Agrobacterium tumefaciens* (AB 681363) isolated from soil. PSBL39 showed the highest (99%) identity similarity to *Acinetobacter calcoaceticus* (JQ579640) phosphate solubilizing bacteria in paddy fields and endophytic bacteria in peanut, respectively.

Three members of Gram positive strains showed closest similarity to different species of genus *Bacillus*. PSBL12 showed 99% similarity with *Bacillus tequilensis* (JF 411313), bacteria isolated from disused River while PSBL21 and PSBL49 showed 99% and 100% sequence similarity, respectively with *Bacillus subtilis* (JN230358) bacterial endophytes from *Nicotiana attenuta*. The partial sequences of 16S rDNA genes of the isolates were multiple aligned with the nearest neighbors and other relevant bacterial sequences, and their identity and evolutionary relation was assessed by constructing phylogenetic tree (Figure 4.1). The phylogenetic tree showed the clustering PSBL isolates with their respective genus with good bootstrap support values.

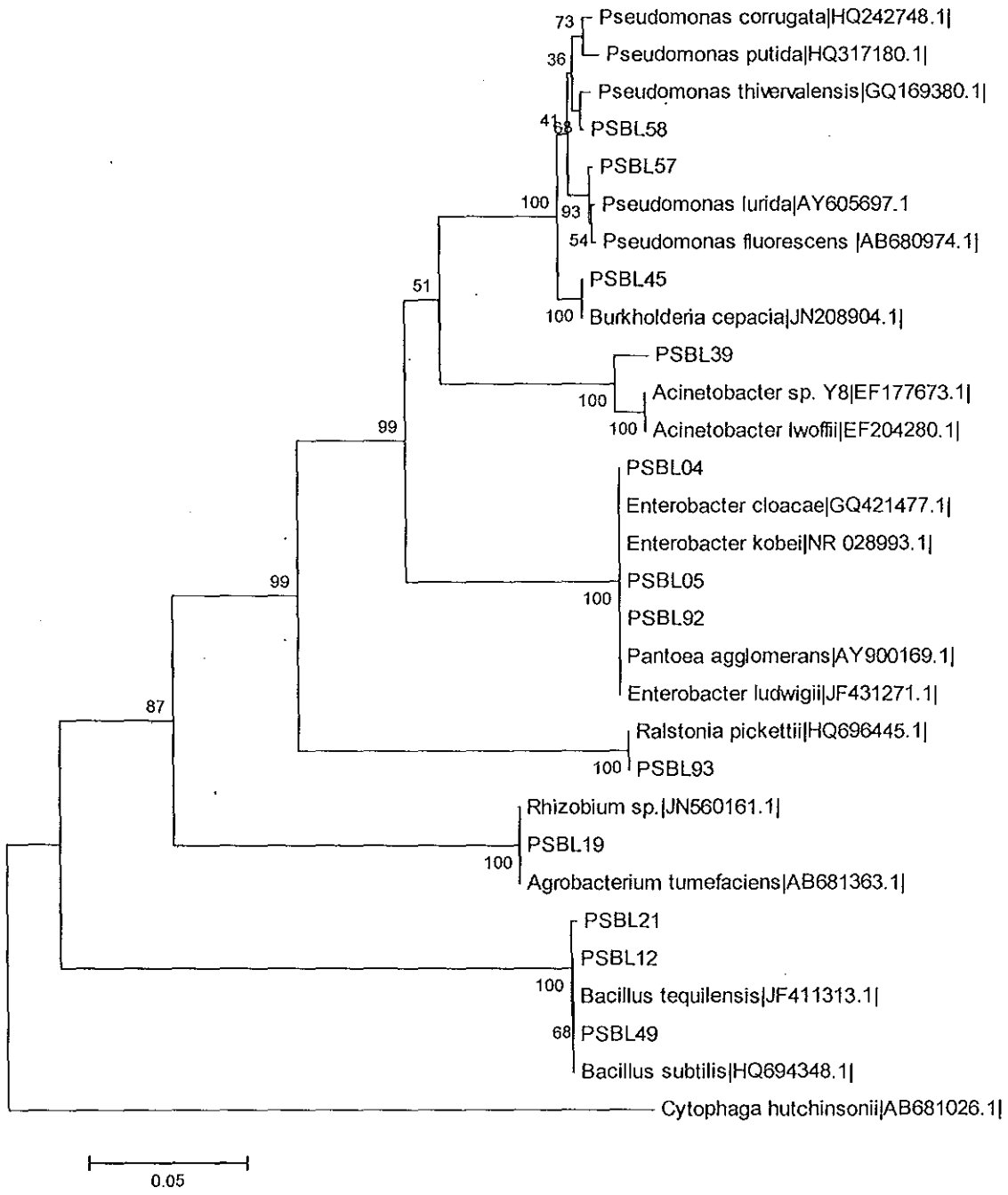

Figure 4.1 Neighbour-Joining phylogenetic dendrogram of 16S rRNA gene sequence showing the positions of different PSBL strains. Bootstrap values are shown at branching point

4.2. Phosphate solubilizing efficiency of the isolates on agar plates

Phosphate solubilzing activities of these isolates were assessed qualitatively on PK agar medium using SI as measure of their efficiency (Table 4.2). The result showed statistically significant ($p>0.05$) variation among the isolates. The SI of the isolates ranged from 1.34 to 2.25 which were recorded for isolate PSBL101 and PSBL19, respectively. This result indicates that PSBL19 was the most efficient phosphate solubilizer on PK plates. Generally, the highest SI was produced by gram negative isolates as compared to gram positive indicating that they are more efficient in tricalcium phosphate solubilization on PK agar medium. Previous investigators have also demonstrated that gram negative PSB strains of different species were efficient in solubilization of TCP on PK plates (Diriba Muleta *et al.*, 2013). Though it is not statistically significant ($p>0.05$) the result showed that there was positive correlation ($r=0.5$) between SI and the amount of soluble P released by the isolates in PK broth. Consequently, we should not rely only on SI to select efficient PSB isolates.

4.3. Different insoluble P sources solubilization efficiency of the PSB isolates

Different insoluble P sources solubilization efficiencies of selected isolates were estimated in PK broth using TCP, RP and BM as insoluble P sources. Most tested isolates exhibited good solubilization ability of TCP, BM and RP. Statistically significant ($p\leq0.05$) variation was observed among isolates in terms of dissolved P and pH drop of the growth medium after 12 days of incubation (Table 4.3).

Most of the isolates released considerable amounts of soluble phosphates from TCP into the medium until the last sampling day (Table 4.3). The amount of soluble P released was found to vary significantly among isolates and sampling days. After 12 days of incubation the highest amount of P released from TCP was 674 μg ml^{-1} which was released by PSBL5 indicating that this isolate is the most efficient in TCP solubilization. On the other hand PSBL33 was found to be the poorest in solubilization of TCP as it released only 52 μg ml^{-1} of P after 12 days of incubation. Generally, the amount of released P was found to increase progressively until the last sampling day for most isolates. However, it was also found to show a slight decline for PSBL28 and PSBL33 after the 8^{th} day of incubation while it was stabilized for PSBL45.

During the process of TCP solubilization all isolates showed a sharp drop in pH of the culture medium from 7.0 to 5.4-5.8 (Table 4.3). The lowest pH was recorded for isolate PSBL45 i.e. 5.4 while the highest was recorded for isolate PSBL26 which was 5.8 on 4^{th} day of incubation. After a sharp decline on day 4, a further decrease in pH was noted only for isolate PSBL31 while all the others showed slight increment. The correlation analysis showed that there is a statistically significant ($p \leq 0.05$) inverse relation between the amount of P released and pH of the broth ($r = -0.82$) indicating that acid production might be the main mechanism of TCP solubilization.

Table 4.3 Different insoluble phosphate sources solubilization efficiencies PSB isolates obtained from lentil rhizosphere

Isolates	Tricalcium phosphate						Rock phosphate						Bone phosphate					
	4th day		8th day		12th day		4th day		8th day		12thday		4th day		8th day		12thday	
	pH	P (µg ml^{-1})	pH	P (µg ml^{-1})	pH	P (µg ml^{-1})	pH	P (µg ml^{-1})	pH	P (µg ml^{-1})	pH	P (µg ml^{-1})	pH	P (µg ml^{-1})	pH	P (µg ml^{-1})	pH	P (µg ml^{-1})
PSBL04	5.6	378c	5.6	483cd	5.7	575c	3.4	9f	3.7	10g	3.7	57b	4.6	16g	4.3	20f	4.4	20e
PSBL05	5.6	378c	5.6	609a	5.5	674a	4.4	46d	5.4	75a	5.7	78a	6.0	239b	6.1	245b	6.1	286a
PSBL21	5.5	375c	5.5	415e	5.5	505d	3.6	49c	3.9	52d	4.7	47e	6.2	187d	6.4	184c	6.4	194b
PSBL26	5.8	62d	5.8	64f	6.0	616b	3.8	7f	4.0	44e	4.0	47e	4.5	30f	4.3	64e	4.3	63d
PSBL28	5.4	410ab	5.4	529b	5.4	492ef	3.5	63a	3.7	63b	3.7	63b	6.4	273a	6.0	276a	5.9	280a
PSBL31	5.4	408ab	5.3	474d	5.2	670a	5.2	44d	5.6	61c	5.0	65b	5.3	201c	5.2	248b	5.3	297a
PSBL33	5.4	52d	5.5	49f	5.8	52g	3.7	60b	3.8	60c	3.9	62c	6.4	204c	6.3	180c	6.4	187b
PSBL37	5.5	380c	5.5	401e	5.5	478f	5.5	41e	5.6	39f	5.6	38f	6.2	191d	6.1	180c	6.1	187b
PSBL45	5.4	398b	5.5	491c	5.6	495de	4.3	62a	4.9	61c	4.7	63bc	5.9	126e	5.9	143d	5.9	150c

Data is average of triplicate, Numbers in the same column followed by the same letter do not differ significantly at p= 0.05 by DT test

Similarly, the rock phosphate solubilization capacity of the isolates was investigated in PK broth supplemented with rock phosphate. The amount of soluble phosphorus released was varied among isolates and sampling dates (Table 4.3). The highest amount of soluble P was 78 μg ml^{-1} which was released by PSBL5 while the least was 38 μg ml^{-1} which was recorded for PSBL37 on the 12^{th} days of incubation. The amount of soluble P in culture medium increased gradually up to the 12^{th} days of incubation for most isolates except PSBL21 and PSBL37 which showed a slight decrease. Compared to other insoluble P sources the lowest amount of soluble P was obtained from rock phosphate dissolution by all tested isolates.

During the course of rock phosphate solubilization a sharp drop in pH of the growth medium was observed. The pH was varied from 3.4 - 5.2 which was measured for PSBL4 and PSBL31, respectively. Compared to TCP and BM the lowest pH was recorded during the solubilization of rock phosphate. However, this low pH was not accompanied by a higher amount of soluble P as the lowest amount of it was obtained from rock phosphate compared to other insoluble P sources. The correlation analysis showed that there is an inverse relationship ($r = -0.34$) between the amount P released and the pH of the growth medium suggesting acid production as the mechanism of rock phosphate solubilization.

The amount of soluble phosphorus released from old bone meal and corresponding drop in pH of the culture medium by the PSB isolates are shown in (Table 4.3). All tested isolates showed BM solubilization ability with different level of efficiencies. There was a significant variation among isolates in terms P solubilization and associated change in pH of the culture medium. Isolate PSBL31 was comparatively more efficient in solubilizing BM than all of the other PSBL

isolates, followed by PSBL5 and PSBL28 with 297, 286 and 280 μg ml^{-1} soluble P in culture medium, respectively. Isolate PSBL4 and PSBL26 were found to be the least efficient in solubilizing BM with 20 and 63 μg ml^{-1} solubilized P, respectively. The amount of solubilized P was found to increase progressively for all isolates except, PSBL37 and PSBL33 which showed a slight decrease after 4^{th} days.

Similar to other insoluble P sources sharp drop of pH was observed during the course of BM solubilization by all isolates. On 4^{th} days incubation drop in pH of the culture medium range from 4.5 to 6.4 which were noted for isolate PSBL26 and PSBL28, respectively. The pH of the medium was found to decrease steadily until 12^{th} day except some isolates such as PSBL21, PSB5, PSBL33 and PSBL45 which showed a slight increment or stability. There was positive correlation (r= 0.27) between soluble P and pH of the culture medium. This suggests that mechanisms other than acid production were involved in the dissolution of BM and hence further study on the solubilization mechanisms of BM is recommended.

All PSB isolates were able to release significantly higher amounts soluble P from different insoluble phosphate sources into culture medium. In general, the highest amount of soluble P was released from TCP followed by BM after 12 days of incubation. The lowest dissolution and sharpest drop in pH was obtained during solubilization of RP. Likewise, previous studies (Rodriguez and Fraga, 1999; Asefa Keneni *et al.*, 2010) showed that phosphate solubilizing microorganisms release more soluble P from insoluble inorganic salts than naturally occurring phosphate sources such as rocks and bone meal which are made of apatite.

All isolates displayed significant drop in pH of the culture medium during the solubilization of all tested insoluble phosphate sources. In all cases phosphate solubilization was found to increase with the decreasing pH of the growth medium. Several studies indicated that the drop in pH is due to the production of organic acids during bacterial growth (Chen *et al.*, 2006; Ivanova *et al.*, 2006; Vyas and Gulati, 2009; Diriba Muleta *et al.*, 2013). The decrease in pH of the culture medium associated with the production of organic acids resulted in considerable amount of P solubilization (Chen *et al.*, 2006). A clear inverse relationship was established between drop in pH of the culture medium and the amount of P solubilized. These results are consistent with the report of earlier studies (Rodriguez Fraga, 1999; Whitelaw, 2000; Naik *et al.*, 2008; Vyas and Gulati, 2009) which showed that solubilization of insoluble phosphate sources were mediated specially by the decreasing pH of the medium. It has been documented that mineral phosphate solubilization by PSB isolates is through the release of low molecular weight organic acids (Goldstein, 1995; Kim *et al.*, 1997; Vyas and Gulati, 2009). These organic acids chelate the cations bound to the insoluble phosphate through their hydroxyl or carboxyl groups, thereby converting it into soluble forms (Kim *et al.*, 1997).

The fluctuations and differences in insoluble P sources solubilization efficiencies observed among isolates could be attributed to differences among test isolates in the amount of immobilized P (Diriba Muleta *et al.*, 2013). When cells in the culture immobilize phosphate for microbial biomass production, the corresponding values decrease, creating such fluctuations (Diriba Muleta *et al.*, 2013; Rashid *et al.*, 2004). Our observation, a decrease in soluble P and concomitant increase in the pH values of the growth medium indicates re-utilization of available P (Rashid *et al.*, 2004; Tripura *et al.*, 2007). In such cases the organic acids excreted to the

growth medium could be reused by the isolates for their own metabolism (Tripura *et al.*, 2007). This could be due to an exhaustion of the original carbon source (Rashid *et al.*, 2004).

Our results indicated that PSB isolates native to Ethiopian soils have good rock phosphate solubilization ability. The dissolution of rock phosphate to a significant extent by these indigenous PSB isolates would give the opportunity to utilize rock phosphate deposits found in different parts of the country. Study conducted elsewhere indicated that 50% of the costly superphosphate could be replaced by rock phosphate; a cheap source of P, when applied in conjunction with PSB inoculants (Sundara *et al.*, 2002). Thus, inoculation of native PSB isolates with superior rock phosphate solubilization ability could improve the productivity of lentil and other crops.

Our results demonstrated that some PSB isolates released a considerable amount of P from insoluble animal bone; a rich source of P. Study indicated that PSB strains showed excellent solubilization abilities of animal bone, a source P of that can be recycled (Postma *et al.*, 2010). Furthermore, the superior BM solubilization of these isolates would also help us to use the problematic waste accumulated around the abattoirs found in different urban areas of the country as P fertilizer along with PSB inoculants. These could reduce our dependence on the very expensive chemical fertilizers.

4.4. Multiple PGP characteristics of PSB isolates from lentil rhizosphere

All PSB isolates obtained from lentil rhizosphere were screened for multiple PGP characteristics such as IAA production, inhibition of *F. oxysporum* growth and fluorescent siderophore production under *in vitro* conditions (Table 4.4). The result indicated that in addition to their phosphate solubilization activity, some PSB isolates exhibited different PGP properties. Out of them 10% were shown to be capable of producing fluorescent siderophore and all of these isolates were identified as *Pseudomonas fluorescens* by API test kits. Earlier study (Naik *et al.*, 2008) also showed that fluorescent pseudomonas strains possess siderophore production ability in addition to their phosphate solubilizing activity and thus, they could serve as biocontrol agents against fungal phytopathogens.

Dual culture assay indicated that two isolates (PSBL31 and PSBL53) inhibited the growth of *F. oxysporum* with inhibition of 41 and 32%, respectively (Table 4.4). These isolates were belonged to the genus *Bacillus* and *Pseudomonas*. Several studies have reported that PSB isolates obtained from different agriculturally important crops rhizosphere where found to be antagonistic to different fungal plant pathogens such as *Fusarium* species, *Phytium* species, *Rhizoctonia* species and others under *in vitro* conditions (Peix *et al.*, 2001b; Naik *et al.*, 2009). In Ethiopia, lentil production has been constrained by wilt and root rot caused by different fungal plant pathogens. Of these wilt disease caused by *Fusarium oxysporum f.sp. lentis* has been responsible for major yield loss (Seid Ahmed and Melkamu Ayalew, 2006) and thus PSB isolates which inhibited the growth of this pathogens would give dual advantage as biofertilizer and biocontrol agents. Consequently, such isolates are highly recommended for greenhouse and field trail.

In addition, 26.8% of the PSB isolates were found to be capable of producing IAA. Quantitative estimation indicated that the highest amount of IAA produced was 48 µg ml^{-1} and the lowest was 14 µg ml^{-1} which was recorded for isolate PSBL19 and PSBL96, respectively (Table 4.4). The greatest number of IAA producer were gram negative bacteria (90%) belonged to different genus *Enterobacter, Pseudomonas, Agrobacterium, Ralstonia, Aeromonas* and *Burkholderia.* The most capable strains belonged to the genus *Agrobacterium* and *Ralstonia.* Similarly, several previous studies demonstrated that PSB isolates obtained from different crops rhizosphere were able to exhibit different IAA production ability (Ponmurugan and Gopi, 2006; Naik *et al.*, 2008; Shahab *et al.*, 2009). These studies indicated that most of the isolates produced considerable amount of IAA (Ponmurugan and Gopi, 2006; Shahab *et al.*, 2009). Such isolates were found to be excellent in promoting plant growth under greenhouse experiments (Shahab *et al.*, 2009). PSBL31, PSBL33, PSBL37 and PSBL53 showed multiple PGP characterstics. These isolates are worth of testing under field and greenhouse conditions.

Table 4.4 Multiple PGP characteristics of PSB isolated from lentil rhizosphere

Isolate	IAA (µg ml^{-1} ± SE	Fluorescent Siderophore production	*In vitro* inhibition of FoL growth (%)± SE
PSBL02	-	-	-
PSBL04	18.2±2.0cd	-	-
PSBL05	-	-	-
PSBL12	-	-	
PSBL13	-	-	-
PSBL14	-	-	-
PSBL19	47.5±1.6a	-	-
PSBL20		-	-
PSBL21	16.7±2.5de	-	-
PSBL26		-	-
PSBL28	14.7±1.5e	-	-
PSBL29	-	-	-
PSBL30	-	-	-
PSBL31	-	-	41±2.5
PSBL33	-	+	-
PSBL37	17.9±1.9cd	+	-
PSBL39	-	-	-
PSBL45	-		-
PSBL49	-	-	-
PSBL50	-	-	-
PSBL51	-	-	-
PSBL52	19.6±1.5c	-	-
PSBL53	15.5±1.2e	+	32±1.7
PSBL54	-	-	-
PSBL55	-	-	-
PSBL57	-	+	
PSBL58	-		-
PSBL90	-	-	-
PSBL92	20.7±1.2bc	-	-
PSBL93	21.6 ±2.1bc	-	-
PSBL94	-	-	-
PSBL95	-	-	-
PSBL96	14.3±0.7e	-	-
PSBL97	14.7±0.3e	-	-
PSBL99	-	-	-
PSBL100	-	-	-
PSBL101	-	-	-
PSBL112	-	-	-
PSBL113	-	-	-
PSBL116	-	-	-
PSBL120	-	-	-

Numbers in the same column followed by the same letter do not differ significantly at p= 0.05 by DT; +: the character present/ -: the character absent, SE: standard error; FOL: *Fusarium oxysporum f.sp. lentis*

4.5. Effects of inoculation of PSB isolates on the growth of lentil under glasshouse conditions

The effects of inoculation of selected PSB isolates on lentil growth, nodulation, N and P content of shoot were investigated under glasshouse conditions. During this experiment *Enterobacter* (PSBL5), *Bacillus* (PSBL21), *Aeromonas* (PSBL26), *Bacillus* (PSBL31) and *Pseudomonas* (PSBL37) were used as inoculants. The results showed that shoot dry matter yield of lentil was improved by inoculation of PSB isolates over the uninoculated plants (NC) control (Table 4.5). However, only *Enterobacter* (PSBL5) (12.6%) and *Bacillus* (PSBL21) (13.8%) isolates increased shoot dry matter yield at statistically significant level ($p \leq 0.05$) over the NC control. The effects of PSB isolates inoculation on shoot dry matter yield of lentil did not vary significantly ($p<0.05$) among isolates. Their effect was almost comparable to P-fertilizer application. In addition to their phosphate solubilization two isolates (PSBL21 and PSBL37) were found to be IAA producer. However, further studies using IAA mutant strains of these isolates are needed to explore the exact contribution of IAA production in the promotion of plant growth.

Nodulation of lentil was one of the parameters measured and used to evaluate the effect of different PSB isolates inoculation. All PSB isolates except *Aeromonas* (PSBL26) increased the average number of nodules at statistically significant level ($p \leq 0.05$) over the NC. This improvement was found to be comparable to P-fertilized plant. The numbers of nodules were also found to vary considerably among the isolates. The highest number of nodules was induced by *Enterobacter* (PSBL5) while the least was induced by *Aeromonas* (PSBL26). These isolates

increased nodule number by 43.9% and 38.2% over the NC, respectively. It is very interesting to note that *Enterobacter* (PSBL5) was superior to P-fertilization in terms nodulation induction. The result of our study indicated that the increase in nodule number is positively correlated with the increase in shoots dry weight(r=0.72).

All PSB isolates used in this experiment increased the concentration of N in shoot of lentil significantly ($p \leq 0.05$) over the NC (Table 4.5). The highest N concentration was observed in *Enterobacter* (PSBL5) and *Bacillus* (PSBL31) inoculated plants and 36.4% increase was obtained over the NC. The variations in N concentration obtained among isolates were not statistically significant ($p<0.05$). Their effect on N concentration was found to be comparable to P-fertilization. The results of our study indicated that the N content of shoot is positively correlated with the nodule number (r=0.67) and shoot dry weight (r=0.53) of the plants. Peix *et al* (2001b) have also observed similar result in plants inoculated with PSB and antagonistic strain of *Burkholderia cepacia.* It has been documented that phosphorus deficiency in soils has a deleterious effect on symbiotic interaction between rhizobia and legumes such as nodulation, N_2-fixation, and hence growth and productivity (Gyaneshwar *et al.* 2002). Several studies have demonstrated that inoculation of seedlings with PSB isolates increased the level of available phosphorus in the rhizosphere soil (Sundara *et al.*, 2002; Vyas and Gulati, 2009). In this way, inoculation of Lentil with efficient PSB could improve growth, nodulation and thus nitrogen-fixation process of this legume.

Likewise, all PSB isolates improved the concentration of P in shoot of lentil at statistically significant level over the NC. The highest concentration of P in shoot was obtained by PSBL5

isolate inoculation while the lowest was by PSBL37. These isolates increased P concentration by 56.1% and 21.9%, over the NC control, respectively. The P content of the plant was positively correlated with the N(r=0.61) content and shoot dry weight(r=0.72) of the plant. These results are in line with the earlier report by other investigators (Peix *et al.*, 2001b). Inoculation of PSB isolates improved the availability of soluble P in the rhizosphere soil and hence the P uptake of the plant (Vikram and Hamzehzarghani, 2008). Previous studies have demonstrated that inoculation of PSB isolates increased the available P in soil (Sundara *et al.*, 2002; Vyas and Gulati, 2009) and concomitantly improved P uptake of the plant (Peix *et al.*, 2001a; Peix *et al.*, 2001b; Vyas and Gulati, 2009).

In addition, *Enterobacter* (PSBL5) and *Bacillus* (PSBL31) were used as inoculants together with agronomically recommended amount of BM (75kg ha^{-1}) and RP (200kg ha^{-1}). The result demonstrated that application of RP and BM in conjunction with inoculation of these isolates improved some growth parameters significantly over plants inoculated with their respective isolates alone. Our results showed that dry matter yield and P concentration were increased at statistically significant level over inoculation of their respective PSB isolates alone. In contrast, inoculation of *Enterobacter* (PSBL5) and *Bacillus* (PSBL31) along with BM did not improve the growth parameters such as shoot dry weight, nodule number, nodule dry weight and N and P concentration of lentil over their respective PSB isolates alone inoculated plants. The presence or absence of rock phosphate and bone meal had no significant effect on the dry matter yield, N concentration and nodule number of lentil. This might be due to the fact that the soil used for the glasshouse experiment contained high amount of total P i.e. 1667 ppm.

In general, from the result of the present study it is possible to conclude that rhizosphere soils of lentil from different producing areas of Ethiopia harbor considerably high number of PSB isolates. Our study also demonstrated that they belong to different bacterial genera: *Pseudomonas, Bacillus, Agrobacterium, Acientobacter, Enterobacter, Burkholderia, Ralstonia, Aeromonas, Chryseomonas and Acinetobacter*. The PSB isolates were able to release considerable amount P from TCP, RP and BM. The present investigation revealed the diversity of PSB with superior phosphate solubilizing capacity, multiple plant growth promoting traits and biocontrol properties. Result of the greenhouse experiment showed that inoculation of lentil with PSB has improved the nodule number, dry matter yield, N and P content of the plant significantly. When inoculated along with RP or BM they may improve on soil with low total P. Some of the isolates such as *Enterobacter* (PSBL5) and *Bacillus* (PSBL31) are of particular interest for further research and need to be tested in the field under different agroecological zones as they could be used as efficient biofertilizer in lentil production system. The knowledge generated on diversity and PGP characteristics of phosphate solubilizing bacteria from rhizosphere soil would be useful to design strategies to use phosphate solubilizing bacterial inoculants in the crop production systems of the country.

Table 4.5 Effects of inoculation of PSB and different insoluble P-source on growth of lentil under glasshouse conditions

Treatments	Nodule number/plant ± SE	Shoot dry weight(g)/Plant ± SE	Total Nitrogen(%) of shoot ± SE	Phosphorous concentration in shoot(mg/g SDW) ± SE
PC	34.3±2.5cd	1.03±0.06abc	3.27±0.16ab	12.1±0.1bc
NC	28.0±3e	0.87±0.05d	2.50±0.11c	8.2±0.1d
PSBL5	40.3±3.5ab	0.98±0.02bc	3.41±0.15a	12.8±0.1b
PSBL21	36.0±4.0bcd	0.99±0.03bc	3.31±0.16ab	11.3±0.15bc
PSBL26	33±4de	0.96±0.14cd	3.32±0.13ab	10.7±0.25bcd
PSBL31	38.7±3abc	0.95±0.04cd	3.41±0.17a	12.0±0.1bc
PSBL37	34.7±2.1cd	0.97±0.08cd	3.14±0.14ab	10.0±0.1cd
PSBL5+RP	43.0±3.6a	1.10±0.1a	3.27±0.14ab	16.3±0.15a
PSBL31+RP	41.7±3.5ab	1.12±0.01a	3.36±0.17a	15.3±0.15a
PSBL5+BM	39.7±4abc	1.01±0.03abc	3.04±0.15b	11.3±0.15bc
PSBL31+BM	40.3±3abc	0.96±0.03cd	3.36±0.18a	11.7±0.15bc

Data is average of triplicate, Numbers in the same column followed by the same letter do not differ significantly at p= 0.05 by DT; PC: positive control; NC: negative control; RP: rock phosphate, BM: bone meal, SE: standard error

Chapter 5 Diversity, phosphate solubilization efficiency and plant growth promotion of rhizobacteria from chickpea *(Cicer aeritinum L.)* producing areas of Ethiopia

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Abstract

Chickpea is one of the principal legume crops grown widely in Ethiopia. The low availablility of phosphorus in soil is one of the stresses which constrain the production of this crop in the country. Therefore, this study was aimed at isolation and characterization of phosphate solubilizing bacteria from chickpea rhizosphere. Accordingly, a total of 50 phosphate solubilizing bacterial isolates were obtained and identified using the API biochemical test kit systems and partial 16S rDNA sequences analysis. The result showed the presence of different genera such as *Acinetobacter, Aeromonas, Bacillus, Brevibacillus, Burkholderia, Empedobacter, Enterobacter, Pseudomonas, Ralstonia, Sphingomonas and Stenotrophomonas.* Phosphate solubilizing efficiency of the isolates was assessed qualitatively using solubilization index (SI) and their SI was ranged from 1.40 to 3.06. Quantitative estimation of the phosphate solubilization efficiency of different insoluble phosphorus sources showed that most isolates have good solubilization ability of $Ca_3(PO_4)_2$, rock phosphate and bone meal. The isolates were also screened for different plant growth promoting attributes and the result showed that 44.9 % and 12.2% of them were capable of producing indole acetic acid and fluorescent siderophore, respectively. Furthermore, 18.4% of them were capable of inhibiting the growth of *Fusarium oxysporum* under *in vitro* conditions. The effects of inoculation of *Bacillus flexus* (PSBC17), *Pseudomonas fluorescence* (PSBC33), *Aeromonas hydrophila* (PSBC35), *Enterobacter sakazaki* (PSBC79) and *Aeromonas hydrophila* (PSBC81) on chickpea growth in pot culture showed significant improvement of number of nodules, shoot dry matter yield, nitrogen and phosphorus concentration of shoot. Based on the result of the present study, it is possible to conclude that chickpea rhizosphere harbor phosphate solubilizing bacteria which are diverse in taxonomy and phosphate solubilizing efficiency. Some of these isolates are of particular interest for further research under different field conditions.

Key words: *PGPR, IAA, rhizosphere soil, rock phosphate, bone meal*

Introduction

Chickpea (*Cicer aeritinum* L.) is grown widely in tropics, sub-tropics and temperate regions of the world. It is also grown in Ethiopia over an area of 208,388.6 ha (CSA, 2011). This crop is an invaluable source of dietary protein for the majority of Ethiopian population. In addition, chickpea restores and maintains soil fertility and therefore, grown in rotation with major cereals such as tef (*Erograstis tef*), wheat (*Triticum sp.*), barley (*Horduem vulgare*) in traditional low-input agricultural system. This is due to its ability to fix nitrogen in association with root nodule bacteria known as *Mesorhizobium* (Nour *et al.*, 1994). However, its yield has remained very low compared to the potential due to several environmental stresses which prevail under Ethiopian edaphoclimatic conditions (Geletu Bejiga and Ketema Daba, 2006; Gemachu Keneni *et al.*, 2011a).

Phosphorus (P) is one of the macronutrients required for growth and development of chickpea. It has been documented that most Ethiopian soils are acidic and mainly characterized by low available P (Desta Beyene, 1982; Tekalign Mamo *et al.*, 1988). This is partly due to acid mediated P-fixation in the soil (Goldstein, 1986). Hence, low available P in soil is one of the stresses which constrain the production of chickpea and other pulse crops in the country (Asfaw Tilaye, 1985; Getachew Agegnehu *et al.*, 2006). The most common method of lessening low avilable P problem and hence increasing crop production is application of chemical fertilizer. However, this is highly limited by its ever increasing cost and for this reasons its application for pulse crops production in general and chickpea production in particular is very low in Ethiopia (Samia Zekaria, 2006). Consequently, there is an urgent need to llok for for economically feasible and ecologically acceptable P-fertilizer sources. Thus, using microbial biofertilizer for improving the availability of P in the rhizosphere of plant could represent practical option to the

use of chemical fertilizers (Naik *et al.*, 2008). Several studies revealed that phosphate solubilizing bacteria (PSB) are active in conversion of insoluble phosphate to soluble primary and secondary orthophosphate ions which can be taken up and utilized by plants (Pal, 1998; Rodriguez Fraga, 1999; Chen *et al.*, 2006; Naik *et al.*, 2008; Vyas and Gulati, 2009).

Bacteria that colonize the rhizosphere and plant root, and enhance plant growth are referred to as plant growth promoting rhizobacteria (PGPR) (Kloepper and Schroth, 1978). Phosphate solubilization by rhizobacteria that makes the P available in soil for plant growth is considered as one of the important attributes of PGPR (Chen *et al.*, 2006). Several studies have showed the presence of PSB in the rhizosphere of different agriculturally important crops and their pivotal role in conversion of insoluble P to the orthophosphate ions (Chabot *et al.*, 1993; Pal, 1998; Peix *et al.*, 2001a; Alam *et al.*, 2002; Ponmurugan and Gopi, 2006; Rajapaksha and Senanayake, 2011).

Studies have revealed that rhizobacteria from genera such as *Agrobacterium, Bacillus, Burkholderia, Enterobacter, Erwinia, Pseudomonas, Rhizobium, Mesorhizobium, Pantoea* and *Serratia* are highly efficient in solubilizing unavailable phosphate into available inorganic phosphate ion (Illmer *et al.*, 1995; Illmer & Schinner, 1995; Rodriguez Fraga, 1999; Naik *et al.*, 2008; Castagno *et al.*,2011). It has been documented that different species of PSB were able to solubilize insoluble inorganic P sources such as tri-calcium phosphate, hydroxyl apatite, rock phosphate and old bone (Asefa Keneni *et al.*, 2010; Mulissa Jida and Fassil Assefa, 2012; Diriba Muleta *et al.*, 2013). Recently, increasing evidences indicated that the production of phytohormones such as indole acetic acid(IAA) and suppression of different soil borne

phytopathogens ability of PSB could also play an imperative role in plant growth promotion (Peix *et al.* 2001b; Ponmurugan and Gopi, 2006; Naik *et al.*, 2008; Shahab *et al.*, 2009). Several workers reported that inoculation of PSB which exhibited multiple plant growth promotion activities significantly enhanced plant growth and yield under glasshouse and field conditions (Peix *et al.* 2001a; Shahab *et al.*, 2009; Yadav *et al.*, 2010).

In view of their multiple plant growth promoting attributes PSB could maintain soil quality and health. Therefore, study committed to isolation, characterization and selection of efficient PSB with different plant growth promoting properties from chickpea rhizosphere soils is highly desirable to design strategies to use native isolates for the development of inoculant technology for organic agriculture. Despite the benefits that phosphate solubilizing bacteria could present to legume cultivation, there is very limited information about indigenous bacterial isolates with these properties from the rhizosphere of chickpea in Ethiopian soils. Therefore, the aim of the present study was to isolate and characterize phosphate solubilizing bacteria from chickpea rhizosphere and evaluate their plant growth promoting activities under glasshouse conditions.

Materials and methods

Study sites and Soil Samples Collection

Soil samples were collected from chickpea grown fields found in central and northern parts of Ethiopia with altitude ranging from 1526 (Alamata) to 2840 masl (Sheno) with soil pH from moderately acidic (pH 5.6) to slightly alkaline (pH 7.9) in October, 2009 (Table 5.1). These are the major chickpea producing parts of the country (Figure 3.1). About 3 kg of soil samples were excavated from 15-20 cm depth from each site and collected into sterile polyethylene bags. A total of 36 soil samples were collected and carefully transported to Applied Microbiology laboratory, Addis Ababa University for further analysis.

Isolation and Enumeration of Phosphate solubilizing bacteria

Each soil sample was thoroughly mixed, air-dried and then filled into 3kg capacity surface sterilized (96% ethanol) plastic pots. Chickpea seeds (*Akaki* cultivar, obtained from the Ethiopian Institute of Agricultural Research (EIAR), Debre Zeit) were selected and 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 to remove traces of sterilizing chemicals and allowed to germinate on sterile water gar (1%) surface for three days at 25°C. Five pre-germinated seeds were planted in each pot. The seedlings were thinned down to three after 5 days of emergence (DAE). All pots were arranged in glasshouse and watered to a field capacity every three days for 45 days after planting (DAP).

Fourty-five DAP chickpea seedlings with good growth and healthy appearance were selected from each pot and uprooted carefully with their rhizosphere soil. The roots with tightly adhered soil were carefully transferred into sterile Petri dishes. Ten gram of rhizospheric soil with adhered roots was transferred to flasks which contain sterilized 90 ml solution of NaCl (0.85% w/v). The flasks were incubated on gyratory shaker at 120 revolutions per minute (rpm) at room temperature for 30 minutes and then serially diluted. Aliquots of the appropriate serial dilution were spread-plated on Pikovskaya (PK) medium (Pikovskaya, 1948). All plates were incubated at 30 °C for 5 days. Colonies surrounded with apparent clear zone were counted and those with large halo zone and different morphological appearances were selected, and transferred to PK broth separately. The test tubes were incubated at 30°C for 48 hours (hrs). The isolates were purified by re-streaking on PK plates and persistence of their phosphate-solubilizing capacity was confirmed by three successive subcultures in the same medium. Selected pure isolates were maintained on PK agar slants for short term storage at 4°C and 50% (v/v) glycerol in PK broth for long term storage at -20°C. Isolates were designated as PSBC (Phosphate Solubilizing Bacteria from Chickpea) 01- PSBC135 (Table 5.1).

Bacterial inoculum preparation and determination of solubilization index (SI) of the isolates

Bacterial isolates were grown in PK broth on a gyratory shaker at room temperature and 120 rpm for 48 hrs. The suspension was centrifuged in sterile plastic tubes (10 ml) at 5000 rpm for 10 min. The pellets were re-suspended in NaCl (0.85% w/v) solution to give a final concentration of 10^8 colony forming units (CFU)/ml using the viable plate count method and optical density measurement at 600 nm. All bacterial inoculums were prepared like this unless otherwise stated.

The agar plates (PK) were spot inoculated with 20 µl of culture suspension of each isolates. After incubation at 30°C for 5 days, formation of a clear zone around the spot was checked and their SI was measured using the formula, *SI= (colony + halo zone diameter)/ colony diameter* (Edi-Premono *et al.,* 1996).

Quantitative estimation of phosphate solubilization efficiency of selected PSB isolates

Based on their phosphate solubilizing activities and abundance of a particular group, isolates were selected and used for quantitative estimation of solubilization efficiency in PK broth. Hundred ml of PK liquid medium without phosphorus sources was dispensed in a 250 ml Erlenmeyer flask and supplemented with the following insoluble phosphate sources which contained equivalent amount of P. The insoluble phosphate sources were tricalcium phosphate (TCP); Egyptian rock phosphate (RP) and bone meal (BM) (obtained from EIAR, Holeta) were added separately as sole phosphorus source. All flasks were autoclaved and inoculated with 100 µl culture suspension of each isolate separately. Uninoculated PK broths supplemented with the above insoluble P sources were included as control. The flasks were incubated at room temperature on a gyratory shaker at 120 rpm for 12 days. Samples were withdrawn from each treatment on day 4, 8, and 12. Each sample was centrifuged (Wagtech International, UK) at 15,000 rpm for 15 minutes and the supernatant was used for determination of pH and soluble P released into the culture. The amount of solubilized P was quantitatively determined following the phospho-molybdate method (Murphy and Riley, 1962) as described in Sahlemedhin Sertsu and Taye Bekele (2000). The quantity of solubilized P was calculated from the standard curve generated using KH_2PO_4. The amount of solubilized P obtained from the control was deducted from their respective treatments.

Identification of phosphate solubilizing bacterial isolates

Morphological, biochemical and API characterization of the isolates

The isolates were characterized using the following preliminary tests: Gram reaction (KOH test) (Gregorson, 1978), oxidative/fermentative (O/F) test (Huge and Leifson, 1953), endospore formation test and API biochemical test kits system assisted by API Plus computer software (bioMèrieux, France) following manufacturer's instructions. Gram positive, endospore forming rods were identified to species level using API 50 CH test strips. Gram negative rods with fermentative reaction in the O/F test were identified using the API 20E test strips while Gram negative rods with oxidative reaction in O/F test were identified by using API 20NE test strips. Moreover, isolates were subject to 16S rDNA sequence analysis based on the result of API identification system.

Identification by 16S rDNA sequence analysis

Genomic DNA extraction

All isolates were streaked on Luria-Bertani (LB) agar medium and incubated for 48 hrs at 30°C. A single colony of each isolates was picked using sterile tooth pick and suspended in 30 µl sterile H_2O in eppendrof tube. The DNA from gram negative bacteria was extracted by using thermal denaturation method (Mohran *et al.*, 1998). All tubes which contain colony suspension were incubated at 95°C for 10 minutes. The DNA from gram positive was extracted by heat thawing at 65°C for 3 minutes followed by freezing at -70°C for 3 minutes, the cycles were repeated for three times. All the tubes were centrifuged at 13,000 rpm for 2 minutes and stored at 4°C.

PCR amplification of 16S rDNA and Sequencing

Two µl of the extracted DNA was used as a template for PCR amplification using a thermal cycler. In addition to the DNA, the PCR reaction consisted of 0.4 µl 10mM dNTP, 2.5 µl 10x PCR (Taq) buffer, 2.5 µl 25 mM $MgCl_2$, 0.2 µl (5 units/µl) of Taq polymerase, 1 µl (10 mM) of forward primer 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1µl (10 mM) of reverse primer 1492r (5'-GGTTACCTTGTTACGACTT-3'). All primers were synthesized by Fermentas (MWG, Germany). The PCR program included a denaturation step of 5 min at 95°C, followed by 30 cycles of 95°C for 30 sec, 50°C for 45 sec, 72°C for 1.30 min, and finally an extension step of 10 min at 72°C. Amplified PCR products were resolved in 1.0% agarose gel and stained with ethidium bromide (1 µg/ml). All amplified PCR products were stored at -20°C and used for sequencing.

PCR products were sequenced with forward primer 27f by using Sanger sequencing (ABI-3730XL), Sequencing facilities available at the institute of Clinical and Molecular Biology, University of Kiel. Those PCR products that generated unreadable sequences were reproduced as described above. DNA sequences were edited using Bioedit program (http://www.mbio.ncsu.edu/BioEdit/). BLAST search of National Center for Biotechnology Information GenBank (NCBI) (*www.ncbi.nlm.nih.gov/*) data libraries was used to establish the identity of the isolates.

Phylogenetic analyses

Sequence data were multiple aligned using Clustal W (Bioedit) and compared with available sequences of bacterial lineage in the NCBI obtained using BLAST. A phylogenetic tree was constructed by using neighbor joining method from distance matrices using Mega4 program (Tamura *et al.*, 2007). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) was shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004) and were in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset. There were a total of 575 positions in the final dataset.

In vitro* antagonistic activity test against *Fusarium oxysporum

The *in vitro* growth inhibition activity of the isolates against *Fusarium oxysporum f.sp. ciceri* (FOC), obtained from EIAR, Debre Zeit, was tested using the dual culture technique (Landa *et.al.* 1997). Twenty µl of culture suspension of each isolates was equidistantly spotted on the margins of nutrient agar (NA) (Oxoid) plates amended with glucose (1.0%) and incubated at 30 °C for 24 h. A 4-5 mm diameter agar disc from potato dextrose agar (PDA) (Oxoid) cultures of the fungal pathogens was placed at the centre of the NA plate inoculated with the bacterial isolate and incubated at 30°C for 5 days. The radii of the fungal colony towards and away from the bacterial colony were measured and the *in vitro* inhibition was calculated following Ahmed Idris *et al.* (2008).

IAA production and Fluorescent siderophores production test

Exponentially grown cultures of the isolates were grown separately on LB broth medium supplemented with 5mM L-tryptophan for 48 hrs (Bric *et al.*, 1991) to determine their IAA production ability. The culture of each isolates were centrifuged at 10,000 rpm for 15 min and 2 ml supernatant of each was transferred separately to a new tube to which 100 µl of 10 mM O-phosphoric acid and 4 ml of Salkowaski reagent (1 ml of 0.5M $FeCl_3$ in 50 ml 35% (v/v) $HClO_4$) were added. The mixtures were incubated for 25 min at room temperature and the development of pink color and absorbance was measured at 530 nm. Quantitative estimation of the IAA was carried out calorimetrically (Gordon and Weber, 1951). IAA concentration was determined against standard curve constructed from different concentrations of indole-3-acetic acid (Sigma-Aldrich).The ability of isolates to produce fluorescent siderophores was tested by streaking bacteria on King's medium B (King, 1954) and incubating for 2 days at 30°C. Plates were inspected for the production of yellow green fluorescence pigment around the bacterial colonies that diffuse in the surrounding agar under 366-nm UV light in dark room.

Effects of inoculation of PSB isolates on the growth of chickpea under glasshouse conditions

Sterilization, germination, inoculation and planting of Chickpea

Bacterial isolates which showed high phosphate solubilization efficiency in PK broth were selected for their *in vivo* growth promotion test under the greenhouse conditions. Chickpea seeds were surface sterilized, germinated and transplanted to plastic pots as described before. Prior to seedling transplantation, germinated seedlings were flooded with PSB culture grown in PK broth

adjusted to 10^8cells per seed for one hour on separate sterile Petri plates. Five inoculated seedlings were transferred on each pot containing 3 kg soil with the following physical and chemical (pH; 6.0, total N;0.025%, organic carbon;0.975, total P;1667 ppm, available P;2.99 ppm, available K;25.33 ppm, CEC;50 Cmol/kg and loamy texture) properties. The soil sample was analyzed at the laboratory of Addis Ababa Environmental Protection Agency. The seedlings were thinned down to three 5 DAE. The pots were arranged in completely randomized block design and irrigated with distilled sterilized water every three days. Uninoculated P-fertilized (20 mg kg^{-1}as KH_2PO_4) pots were included as positive control (PC) and uninoculated non P-fertilized pots as negative control (NC). In addition, three isolates (PSBC17, PSBC35 and PSBC81) were used as inoculants in conjunction with agronomical recommended amount of RP (200 kg ha^{-1}) or BM (75 kg ha^{-1}). Sixty DAP all seedlings were carefully uprooted and washed under gently flowing tap water. Then nodule number counted, and shoot dry weight was measured after drying at 70^0C for 48 hours. Total N and P concentration of the shoot was determined using micro-kjeldahl and phospho-molybdate methods (Murphy and Riley, 1962), respectively at the laboratory of Addis Ababa Environmental Protection Agency.

Data Analysis

Data were analyzed using analysis of variance (ANOVA) and the treatment means were compared relative to control following Duncan's test (DT). The correlation between different parameters was evaluated by using Pearson correlation coefficient test using SPSS V.15.

3. Result and discussion

3.1. Isolation, Enumeration and Identification of PSB Isolates

The number of phosphate solubilizing bacteria found in each soil samples was determined by using plate count method. The abundance of PSB was found to range from 2.3×10^3 to 5.6×10^5 CFU g^{-1} of the rhizosphere soil which was obtained from Alamata or Sandfa and Gurura, respectively (Table 5.1). Previous study (Asefa Keneni *et al.*, 2010) reported that the abundance of PSB was varied from $1.8x10^3$ CFU g-1 soil in the rhizosphere of faba bean. The number of PSB was $\geq3.1\times10^4$ in 80.5% of the samples indicating that the proportions of such bacteria are very high in chickpea rhizosphere soils collected from producing areas of Ethiopia. Similarly, several investigators indicated that the proportions of such bacteria were very high in the rhizosphere soils of some agriculturally important crops such as maize, sorghum, rice, barley, groundnut and chickpea, and thus they could play crucial role in P nutrition of the plants (Chabot *et al.* 1996; Kim *et al.* 1998; Alam *et al.*, 2002; Ponmurugan and Gopi, 2006; Rajapaksha and Senanayake, 2011; Peix *et al.* 2001a).

Table 5.1 Abundance and distribution of Phosphate solubilizing bacteria isolated from chickpea producing areas of Ethiopia

Isolation sites	pH of isolation site	Altitude of isolation site (masl)	Abundance of PSB (CFU/g) in soil isolation site soil	Number of isolates selected	Isolates obtained from each site
Galessa	7.6	2017	3.7×10^{4}de	1	PSBC01
Alamata	7.9	1526	1.5×10^{5}bcde	1	PSBC02
Robe	7.8	1658	6.9×10^{4}cde	1	PSBC05
Woldya	6.6	2074	1.3×10^{5}bcde	1	PSBC06
Aja	6.8	2023	5.6×10^{5} a	2	PSBC07,41
Debre Selam	6.0	1896	6.1×10^{4}cde	1	PSBC10
Alem Tena	7.1	1637	5.9×10^{4}cde	1	PSBC13
Chirameda	6.6	1747	3.5×10^{4}de	1	PSBC14
Sirnka	6.9	1843	3.6×10^{4}de	2	PSBC15, 16
Angut Michael	6.3	1850	1.1×10^{5}cde	2	PSBC17,18
Maksagnt	6.4	1978	6.1×10^{4}cde	2	PSBC28,29
Yetinora	6.4	2437	7.8×10^{4}cde	2	PSBC30,31
Lalibela	7.4	2138	4.8×10^{4}de	2	PSBC33,40
Olankomi	6.6	2378	2.2×10^{5}bc	1	PSBC35,39
Debre Libanos	7.3	2594	2.2×10^{5}bc	2	PSBC42,46
Dibulko	6.8	1992	4.8×10^{4}de	1	PSBC45
Ginchi	6.6	2378	2.9×10^{3}e	2	PSBC61,62
Ambo	6.7	2170	4.0×10^{3}e	1	PSBC63
Fiche	6.8	2748	1.9×10^{5}bc	1	PSBC67
Tikana	7.2	1942	5.9×10^{4}cde	2	PSBC68,126
Fogera	6.4	1931	4.8×10^{4}de	1	PSBC69
Alshin	6.7	2082	3.1×10^{4}de	1	PSBC70
Bilbila	6.6	2069	3.4×10^{4}de	1	PSBC71
Amber	6.3	2454	6.0×10^{4}cde	1	PSBC72
Gurura	7.0	1906	2.3×10^{3}e	2	PSBC74,79
Goro	6.6	1832	2.9×10^{3}e	1	PSBC81
Teji	6.7	2065	1.4×10^{5}bcde	2	PSBC86,89
Asgori	6.6	2078	4.0×10^{3}e	1	PSBC97
Itacha	6.8	2134	4.6×10^{4}de	1	PSBC99
Ilala	7.6	1924	6.2×10^{4}cde	1	PSBC108
Goha Tsion	6.2	2517	4.6×10^{4}de	1	PSBC109
Mojo	6.8	1774	3.7×10^{4}de	1	PSBC123
Obbi	6.6	2108	5.9×10^{3}ce	1	PSBC125
Sandafa	6.0	2554	1.5×10^{5}bcde	2	PSBC131,132
Sheno	5.6	2840	ND	2	PSBC133,134
Chole	6.3	2612	4.8×10^{4}de	2	PSBC135

ND; not determined, PSB abundance data are average of triplicates and data in the same column followed by the same letter do not differ significantly at p=0.05 using Duncan's test.

Based on their halo zone size and colony morphology, a total of 50 PSB isolates with SI ≥1.40 were obtained from 36 soil samples (Table 5.1). These isolates were categorized into different groups using some preliminary tests and then subjected to their respective API test kit system for identification. The result revealed the presence of a diverse group of phosphate solubilizing bacteria in the rhizosphere of chickpea. The isolates were dominated by Gram negative rhizobacteria which accounts for 85.7% of the isolates. Gram positive isolates were only 14.3% indicating that they are the minority among phosphate solubilizing bacteria in the rhizosphere. Likewise, Diriba Muleta *et al.* (2007) showed that the rhizospheres of coffee arabica were found to favor more Gram negative rhizobacteria than Gram positive. Furthermore, 11 major genera were identified by using biochemical API kits. The genera were *Burkholderia* (28.6%), *Pseudomonas* (22.5%), *Bacillus* (12.3%), *Aeromonas* (4.1%), *Brevibacillus* (2%), *Enterobacter* (2%), *Sphingomonas* (2%), *Stenotrophomonas* (4.1%) and *Ralstonia* (2%). As far as species diversity is concerned *P. fluorescens* (80%) was the dominant species among the genus *Pseudomonas* while all isolates of the genus *Burkholderia* were identified as *B. cepacia.* However, 22.5% the isolates were left unidentified or showed low identification level (<90%) which might be due to the limitation of biochemical characteristics and thus API kits to identify environmental isolates with diverse nature.

The partial 16S rDNA sequences analysis was also employed to identify the isolates to the genus and species level. Based on the abundance of a particular group, API kit result and PGP activity 20 isolates were selected for sequence analysis. The result showed the presence of genera such as *Pseudomonas, Bacillus, Acinetobacter, Ralstonia, Burkholderia and Empedobacter.* There were some unlikenesses between API kit and 16S rDNA sequence identification which could be due to

several reasons such as inoculum size and culture conditions. Genera *Empedobacter* and *Acientobacter* were identified only by sequences analysis (Table 5.2).

Table 5.2 Identification of PSB isolated from chickpea rhizosphere

Isolate	SI± SE	Gram reaction	API identification	16S rDNA sequence identification
PSBC01	1.7±0.10i-n	-	*Pseudomonas fluorescens*	ND
PSBC02	3.06±0.42a	-	UI	*Ralstonia picketlii(JX979097)*
PSBC05	2.25±0.25d-f	-	UI	*Burkholderia phytofirmans(JX979098)*
PSBC06	1.57±0.15k-n	-	UI	*Acinetobacter lwoffii(JX979099)*
PSBC07	2.66±0.34bc	-	*Burkholderia cepacia*	*Burkholderia terricola(JX979100)*
PSBC10	1.54±0.19l-n	-	*Burkholderia cepacia*	ND
PSBC13	2.28±0.25d-f	-	*Pseudomonas species*	*Pseudomonas lini(JX979123)*
PSBC14	1.44±0.06n		*Burkholderia cepacia*	ND
PSBC15	2.88±0.18ab	-	*Stenotrophomonas maltophila*	"
PSBC16	2.83±0.17a-c	+	*Bacillus species*	"
PSBC17	2.53±0.38b-e	+	*Bacillus species*	*Bacillus flexus(JX979101)*
PSBC18	1.94±0.07f-j	-	*Burkholderia cepacia*	ND
PSBC28	2.85±0.17a-c	+	*Bacillus species*	*Bacillus subtilis(JX979102)*
PSBC29	1.65±0.13j-m	+	*Bacillus stearothermophilus*	*Bacillus subtilis(JX979103)*
PSBC30	2.48±0.13c-e	-	*Burkholderia cepacia*	ND
PSBC31	1.91±0.10g-k	+	*Brevibacillus brevis*	*Bacillus subtilis(JX979104)*
PSBC33	2.20±0.20e-g	-	*Pseudomonas fluorescence*	ND
PSBC35	2.50±0.10c-e	-	*Aeromonas hydrophila*	"
PSBC40	1.90±0.10g-l	-	UI	*Empedobacter brevis(JX979105)*
PSBC41	1.45±0.04n	-	*Pseudomonas fluorescens*	*Pseudomonas corrugate(JX979106)*
PSBC42	1.52±0.14mn		*Burkholderia cepacia*	ND
PSBC45	2.00±0.07f-j	-	*Burkholderia cepacia*	"
PSBC46	2.12±0.13f-h	-	*Burkholderia cepacia*	"
PSBC61	2.12±0.12f-h	-	UI	"
PSBC62	2.06±0.17f-i	-	UI	"
PSBC63	2.50±0.25c-e	-	UI	*Empedobacter brevis(JX979107)*
PSBC67	2.07±0.12f-i	-	*Pseudomonas fluorescens*	*Pseudomonas lini(JX979108)*
PSBC68	1.69±0.27j-n	-	*Pseudomonas fluorescens*	*Pseudomonas lini(JX979124)*
PSBC69	1.83±0.11h-m	-	*Pseudomonas aeruginosa*	*Pseudomonas lini(JX979109)*
PSBC70	1.51±0.15mn	-	*Pseudomonas fluorescens*	-
PSBC71	2.08±0.29f-h	+	*Bacillus species*	*Bacillus subtilis (JX979110)*
PSBC72	2.07±0.12f-i	-	*Pseudomonas fluorescens*	ND
PSBC74	2.62±0.13b-d	-	*Burkholderia cepacia*	"

PSBC79	2.58±0.14b-d	-	*Enterobacter sakazaki*	"
PSBC81	1.43±0.07n	-	*Aeromonas hydrophila*	-
PSBC86	1.95±0.25f-j	-	*Sphingomonas paucimobils*	-
PSBC89	1.43±0.06n	-	UI	*Acinetobacter lwoffii(JX979111)*
PSBC97	1.87±0.08g-m	-	*Ralstonia pickettii*	*Ralstonia pickettii (JX979112)*
PSBC99	1.46±0.05n	-	*Burkholderia cepacia*	*Burkholderia terricola (JX979113)*
PSBC108	1.52±0.17mn	-	*Stenotrophomonas maltophila*	ND
PSBC109	1.86±0.13g-m	-	*Burkholderia cepacia*	-
PSBC123	1.46±0.12n	-	*UI*	*Acinetobacter johnsonii (JX979126)*
PSBC125	1.81±0.15h-m		*Pseudomonas fluorescens*	ND
PSBC126	1.40±0.16n	-	*Burkholderia cepacia*	-
PSBC131	1.80±0.17h-m	+	*Bacillus sp.*	-
PSRB132	1.85±0.11g-m	-	*Burkholderia cepacia*	-
PSRB133	1.66±0.13j-n	-	UI	-
PSRB134	1.44±0.06n	-	*Burkholderia cepacia*	-
PSRB135	1.43±0.05n	-	UI	-

ND; not determined, +; Gram positive or positive for the test, -; Gram negative or negative for the test, FOC; *Fusarium oxysporum f.sp. ciceri*, SE;standard error, parenthesis: accession number in NCBI Gene bank, Means in the same column followed by the same letter do not differ significantly at p=0.05 using DT test

The phylogenetic relationship of the isolates in relation to the reference strains is shown in figure 5.1. Gram negative isolates exhibited more diverse genera and species compared to the gram positive isolates. Isolates PSBC13, PSBC67, PSBC68 and PSBC69 showed 99% similarity with *Pseudomonas lini* (EU221401), PGPR isolated from wheat rhizosphere while PSBC41 found to be 99% similar with *Pseudomonas corrugate* (HQ242748) phosphate solubilizing bacteria. On the other hand, PSBC97 showed 100% identity similarity with denitrifying bacteria *Ralstonia pickettii* (HE575958) isolated from soil whereas PSBC02 showed 99% homology with *Ralstonia pickettii* strain QL-A6 (HQ267096) obtained from tomato rhizosphere. Isolate PSBC05 was found to be 99% similar with *Burkholderia phytofirmans* (HQ242761) phosphate solubilizing rhizobacteria isolated from P-rich soil while PSBC07 and PSBC99 showed 99% homology with *Burkholderia terricola* (FM209484) salt tolerant bacteria isolated from faba bean nodules.

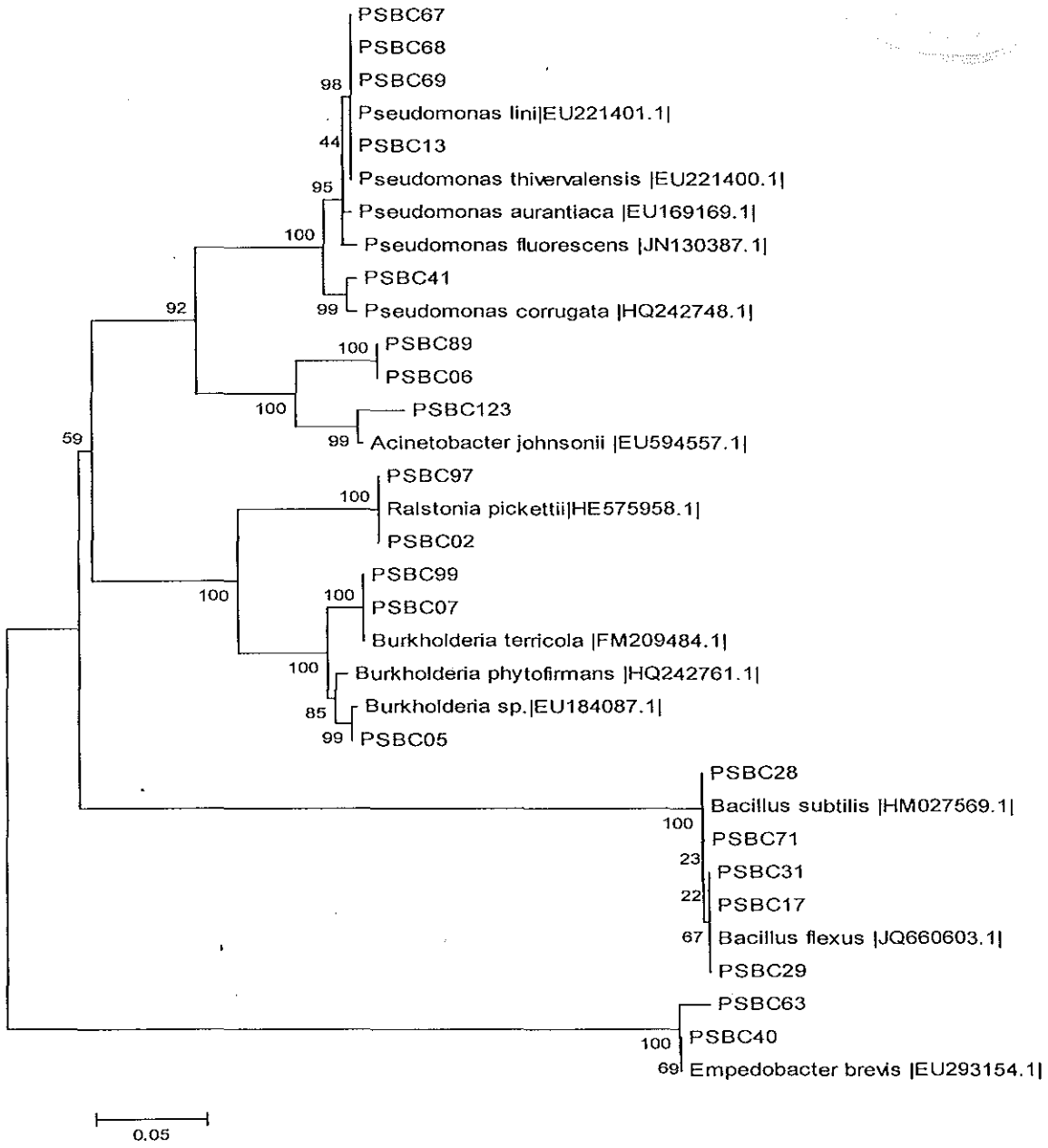

Figure 5.1 Neighbour-Joining phylogenetic tree of 16S rRNA gene sequence showing the positions of different PSBC strains. Bootstrap values are shown at branching point.

Isolates PSBC06 and PSBC89 were found to be 99% similar with *Acinetobacter lwoffii* (FN393792) and *Acinetobacter lwoffii* (EF204280) obtained from different environment, respectively while PSBC123 showed 99% homology with *Acinetobacter johnsonii* (EU594557), an endophytic bacteria obtained from sugar beet. Isolates PSBC40 and PSBC63 were found to be 99% and 100% similar with *Empedobacter brevis* (EU293154) isolated from soil, respectively. *Empedobacter brevis,* formerly designated *Flavobacterium breve* is a Gram-negative rod belonging to the family of *Flavobacteriaceae*. Several studies indicated that different species from genus *Pseudomonas, Ralstonia, Burkholderia, Flavobacterium* and *Acientobacter* are efficient phosphate solubilizing bacteria (Rodriguez Fraga, 1999).

Isolates PSBC31 and PSBC71 were found to be 99% similar with *Bacillus subtilis* (EF656456) endophytic bacteria from wheat and antagonistic to wheat sharp eyespot disease and *Bacillus subtilis* (HM027569) soil-born PGPR, respectively whereas PSBC28 showed 99% homology with soil *Bacillus subtilis* (HM027569). Isolate PSBC17 showed 99% similarity with *Bacillus flexus* (JQ660603) obtained from verimcompost. Moreover, the partial sequences of 16S rDNA of the isolates were multiple aligned with the nearest neighbors and other relevant bacterial sequences, and their identity and evolutionary history was inferred by constructing phylogenetic tree (Figure 5.1). The phylogenetic tree showed the clustering of PSB isolates with their respective genus with high bootstrap support values.

Qualitative estimation of insoluble phosphate solubilizing efficiency of the isolates

The SI of each PSB isolate is depicted in Table 5.2. Phosphate solubilizing efficiency of these isolates was determined on PK agar medium qualitatively by using SI as measure of their efficiency. The data showed that the isolates displayed SI ranging from 1.40 to 3.06 (Table 5.2). The most efficient isolate was PSBC02 (*Ralstonia pickettii*) followed by PSBC15 (*Strenotrophomonas maltophila*), with SI of 3.06 and 2.88, respectively whereas PSBC126 (*Burkholderia cepacia*) was found to be least efficient with SI of 1.40.

Quantitative estimation of insoluble phosphates solubilization efficiency of the PSB isolates

Quantitative estimation of different insoluble P-sources solubilization efficiencies of selected isolates were carried out in PK broth using TCP, RP and BM as sole P sources. Most isolates exhibited considerable solubilization ability of TCP, BM and RP. Significant ($p<0.05$) variation was also observed among isolates in terms of the amount of dissolved P and drop in pH of the growth medium after 12 days of incubation (Table 5.3 and 5.4).

Table 5.3 Calcium phosphate and rock phosphate solubilization efficiency PSB isolates from chickpea rhizosphere

Isolate	$Ca_3(PO4)_2$						Rock phosphate					
	4th day		8th day		12th day		4th day		8th day		12th	
	pH	P(µg/ml) ±SE	pH	P(µg/ml) ±SE	pH	P(µg/ml) ±SE	pH	P(µg/ml) ±SE	pH	P(µg/ml) ±SE	pH	P(µg/ml) ±SE
PSBC05	5.2	13±8c	5.8	174±9b-d	6.3	145±12c	4.2	28±1b	4.7	42±1c	4.9	44±2b
PSBC17	4.8	26±10c	4.9	200±12b-d	5.9	207±9b	5.1	14±1cd	5.3	53±2a	5.6	56±1a
PSBC28	4.8	30±4c	5.7	172±44b-d	5.9	161±53c	4.1	10±1d	4.2	7±1h	4.5	7±1f
PSBC29	4.9	15±6c	4.7	70±20e	5.9	137±44c	4.6	14±1cd	5.0	20±1	5.1	14±3e
PSBC31	5.0	21±7c	5.3	148±42c-e	5.9	148±58c	5.8	12±4cd	5.8	15±1f	5.8	15±1e
PSBC33	4.6	41±4c	5.1	295±39a	5.8	315±50a	4.1	36±3a	4.1	38±3d	4.9	38±1c
PSBC35	5.4	120±32b	5.8	263±42a	6.1	379±54a	4.9	31±3ab	5.8	46±4b	6.1	57±3a
PSBC79	5.6	171±17a	5.8	238±44ac	6.2	346±28a	4.3	32±2ab	4.6	34±3e	5.0	33±3d
PSBC81	4.9	48±10c	5.2	193±12b-d	6.1	299±48a	4.1	35±9a	4.7	38±1d	5.0	35±4cd
PSBC97	5.0	53±14c	5.6	110±42c-e	6.0	143±37c	6.9	17±1c	5.6	12±1g	6.1	15±1e

SE; standard error, the data is average of triplicates, Numbers in the same column followed by the same letter do not differ significantly at p= 0.05 by DT test, the amount P (mg) is in 100 ml

All tested isolates released greater amounts of soluble phosphates into the culture medium until the last sampling day (Table 5.3). The amount of solubilized P was found to vary significantly ($p<0.05$) among isolates and sampling days. After 12 days of incubation the highest amount of P released from TCP was 379 µg/ml which was solubilized by PSBC35 indicating that this isolate was the most efficient in TCP solubilization (Table 5.3). On the other hand, PSBC29 was found to be the poorest in solubilization of TCP as it released only 137 µg/ml of P after 12 days of incubation. Generally, the amount of solubilized P was found to increase progressively until the last sampling day for most isolates. However, it was found to show a slight decline for PSBC5 after the 8th day of incubation.

During solubilization of TCP the isolates showed a sharp decline of in pH of the growth medium on 4th day of incubation (Table 5.3). The acidic pH was ranged from 4.8 to 5.6 which were recorded for isolate PSBC33 and PSBC79, respectively. Then after, all isolates exhibited progressively increase in pH of the growth medium until the 12th days of incubation. The correlation analysis showed that there was statistically significant ($p<0.05$) inverse relation between the concentration of solubilized P and concomitant drop in pH of the growth medium ($r= -0.82$) suggesting that acid production is the mechanism of TCP solubilization.

Similarly, selected isolates were tested for their rock phosphate solubilization and most of them showed good solubilization ability. The amount of soluble phosphate released and corresponding change in pH of the medium were varied among isolates and sampling dates (Table 5.3). The highest amount of solubilized P was 57 µg/ml which was released by PSBC35 while the least was 7 µg/ml which was recorded for PSBC28 on 12th day of incubation. The amount of solubilized P increased gradually up to the 12th days of incubation for most isolates (PSBC17, PSBC5, PSBC31 and PSBC33) while all the rest showed a slight decline (Table 5.3). There was a sharp drop of pH from 7.0 to acidic during RP solubilization. The lowest pH of the growth medium was recorded on 4th day for all isolates except PSBC97. Then after, the pH of the growth medium was found to increase gradually until the 12th day of incubation. The correlation analysis showed that there was an inverse relation($r= -0.34$; $p>0.05$) between solubilized P and associated drop in pH of the culture medium indicating that acidification of the growth medium as mechanism of rock phosphate solubilization.

Table 5.4 Bone phosphate solubilization efficiencies PSB isolates obtained from chickpea rhizosphere

Isolate	4th day		8th day		12th day	
	pH	P(µg/ml) ±SE	pH	P(µg/ml) ±SE	pH	P(µg/ml) ±SE
PSBC17	6.0	155±1b	6.1	161±6c	6.2	162±3c
PSBC33	6.1	119±7d	6.1	115±1e	6.1	120±4e
PSBC35	6.1	124±2c	6.1	131±3d	6.3	139±1d
PSBC79	5.0	33±11f	5.5	182±8b	5.7	195±1b
PSBC81	6.0	211±5a	6.4	217±3a	6.5	232±1a
PSBC97	4.6	66±2e	5.0	87±9f	4.8	102±4f

The data is average of triplicates, Numbers in the same column followed by the same letter do not differ significantly at p= 0.05 by DT test, the amount P (mg) is in 100 ml

The amount of soluble phosphorus released from old BM and corresponding drop in pH of the medium by the PSBC isolates are shown in Table 5.4. All tested isolates solubilized BM with different level of efficiencies. There were significant ($p<0.05$) variations among isolates in terms P solubilization and concomitant change in pH of the culture medium. Isolate PSBC81 (232 µg/ml) was comparatively more efficient in solubilizing BM than all of the other isolates, followed by PSBC79 (195 µg/ml). The amount of solubilized P was found to increase progressively with days of incubation.

Similar to other P sources, drop in pH of the growth medium was observed during the course of BM solubilization by all isolates. On day 4 drop in pH of the culture medium was ranged from 4.6 to 6.1 which were measured for isolate PSBC97 and PSBC33, respectively. Then after, the pH of the medium was found to increase gradually until the last sampling day. There was positive correlation (r: 0.27; $p>0.05$) between soluble P and decrease in pH of the culture medium. This suggests that mechanisms other than acid production were involved in the

dissolution of BM. This is in line with the result of the previous study (Asefa Keneni *et al.*, 2010) and hence further study on the solubilization mechanisms of BM is highly recommended.

All PSB isolates recovered from chickpea rhizosphere soils showed considerably higher solubilization ability of different insoluble phosphates in broth culture. Generally, the highest amount of P was released from TCP followed by BM after 12 days of incubation. The lowest dissolution and sharpest drop in pH was obtained during solubilization of RP. Likewise, several studies (Rodriguez and Fraga, 1999; Asefa Keneni *et al.*, 2010) have shown that phosphate solubilizing microorganisms mobilize more P from insoluble inorganic salts than from naturally occurring phosphate sources such as rocks and bone meal which are made of apatite.

All isolates displayed significant drop in pH of the culture media during the solubilization of all tested insoluble phosphate sources. Several studies indicated that the drop in pH is due to the production of organic acids during bacterial growth (Illmer *et al.*, 1995; Kim *et al.*, 1997; Chen *et al.*, 2006; Vyas and Gulati, 2009; Diriba Muleta *et al.*, 2013). The decreasing pH and production of organic acids resulted in considerable amount of solublized P (Chen *et al.*, 2006; Naik *et al.*, 2008; Vyas and Gulati, 2009). This could be through the action of their hydroxyl or carboxyl groups that chelate the cations bound to the phosphate, thereby converting it into soluble forms (Kpomblekou and Tabatabi, 1994; Kim *et al.*, 1997).

The fluctuations and differences in insoluble P sources solublization efficiency observed among isolates could be attributed to differences among test isolates in the types and amount of organic

acids produced (Vyas and Gulati, 2009). Alternatively, it could also be explained by the differences in rate of P release and immobilization (Prasanna *et al.*, 2011). When the bacterial cells in the culture immobilize more phosphate for biomass production, the corresponding values decrease, creating such fluctuations (Rashid *et al.*, 2004; Diriba Muleta *et al.*, 2013). A decrease in soluble P and concomitant increase in the pH values of the growth medium indicates re-utilization of available P (Rashid *et al.*, 2004; Tripura *et al.*, 2007). In such cases excreted organic acids could be reused by the isolates for their own metabolism (Tripura *et al.*, 2007) which is highly associated with an exhaustion of the original carbon source (Rashid *et al.*, 2004) as phosphate solubilization is a complex process which depends on many factors such as nutritional, physiological and growth conditions of the culture (Reyes *et al.*, 1999).

Our results indicated that some PSB isolates native to Ethiopian soils released considerable amounts of soluble P from RP. The dissolution of rock phosphate to a significant extent by these indigenous PSB isolates would give the opportunity to utilize rock phosphate deposits found in different parts of the country as cheap P fertilizers. Thus, inoculation of native PSB isolates with superior rock phosphate solubilzation ability along with application of rock phosphate could improve the productivity of chickpea and other crops. Furthermore, the superior BM solobilization of these isolates would also help to use the waste accumulated around the abattoirs found in different urban areas of the country as P fertilizer along with PSB inoculants. These could reduce the dependence on the very expensive chemical fertilizers.

3.4. PGP characteristics of PSB isolates from chickpea rhizosphere

Some PSB isolates exhibited different multiple PGP attributes besides their phosphate solubilization activity. Out of all tested isolates some (12.2%) of them were shown to be capable of producing fluorescent siderophore and all of these isolates were identified as *Pseudomonas fluorescens* by API test kit (Table 5.2 and 5.5). Earlier studies (Naik *et al.*, 2008.) also showed that fluorescent pseudomonas strains possess siderophore production ability in addition to their phosphate solubilizing activity and could be used as biocontrol agents against soil borne phytopathogens. This feature has been related to improving plant iron nutrition and to biocontrol capacity (Khan *et al.* 2006); therefore they could provide several advantageous for plant.

Table 5.5 Multiple PGP characteristics of PSB isolated from chickpea rhizosphere

Isolate	FOC inhibition (%)± SE	Fluorescent siderophore	IAA (μg ml^{-1}) ± SE
PSBC01	41.2±1.3b	-	-
PSBC02	-	-	17.6±0.8d
PSBC05	38.5±1.1c	-	-
PSBC06	-	-	12.3±1.1f
PSBC07	-	-	-
PSBC10	-	-	-
PSBC13	-	-	19.2±1.0d
PSBC14	-	-	19.7±1.2d
PSBC15	-	-	-
PSBC16	-	-	21.4±0.2d
PSBC17	-	-	61.2±1.2a
PSBC18	-	-	-
PSBC28	-	-	20.5±0.7d
PSBC29	-	-	16.4±0.8e
PSBC30	-	-	-
PSBC31	-	-	16.3±0.6e
PSBC33	-	-	37.7±1.2b
PSBC35	-	-	
PSBC39			25.8±0.2c
PSBC40	-	+	22.1±1.4d
PSBC41	-	-	-
PSBC42	-	-	-
PSBC45	-	-	-
PSBC46	-	-	-
PSBC61	-	-	18.2±0.9d
PSBC62	25.6±1.5e	-	-
PSBC63	-	-	-
PSBC67	-	+	19.4±1.4d
PSBC68	40.3±1.8b	+	13.4±1.3ef
PSBC69	44.2±1.6a	-	16.8±1.5d
PSBC70	45.8±1.9a	+	18.7±1.6d
PSBC71	-	-	19.6±1.3d
PSBC72	-	-	14.6±0.4e
PSBC74	-	-	-
PSBC79	-	-	15.5±0.7e
PSBC81	-	-	58.0±2.0a
PSBC86	-	-	-
PSBC89	-	-	-
PSBC97	-	-	18.6±1.5d
PSBC99	-	-	-
PSBC108	-	-	-
PSBC109	-	-	-
PSBC123	26.3±2.1e	-	14.3±0.6e
PSBC125	-	+	-
PSBC126	-	+	15.2±1.0e
PSBC131	-	-	-
PSRB132	-	-	-
PSRB133	-	-	-
PSRB134	40.5±1.4b	-	-
PSRB135	30.3±2.1d	-	-

+; positive for the test, -; negative for the test, FOC; *Fusarium oxysporum f.sp. ciceri,* Means in the same column followed by the same letter do not differ significantly at p=0.05 using DT test

Dual culture assay indicated that some (18.4%) of the isolates inhibited the growth of *F. oxysporum* under *in vitro* conditions. Their *in vitro* inhibition % was ranged 25.6 to 45.8 (Table 5.5). These isolates were belonged to the genus *Pseudomonas* (44.4%), *Burkholderia* (22.2%) and *Acinetobacter* (11.1%) while the remaining isolates were among the unidentified group. All antagonistic isolates from *Pseudomonas* group were identified as *Pseudomonas fluorescens* by API test kits. Several studies have indicated that phosphate solubilizing bacterial isolates obtained from different agriculturally important crops rhizosphere where found to be antagonistic to different fungal phytopathogens such as *Fusarium* species, *Phytium* species, *Rhizoctonia* species and others under *in vitro* conditions (Peix *et al.*, 2001b; Naik *et al.*, 2008).

In Ethiopia chickpea production has been constrained by wilt and root rot caused by different fungal plant pathogens. Of these wilt disease caused by *Fusarium oxysporum* has been responsible for major yield loss (Seid Ahmed and Melkamu Ayalew, 2006) and thus isolates which inhibited the growth of this pathogens would give dual advantage as biofertilizer and biocontrol agents. Consequently, such isolates are highly recommended for greenhouse and field experiments.

Besides, 49.0 % of the PSB isolates were capable of producing IAA indicating that this ability is common among rhizosphere bacteria (Table 5.5). These result suggested that indigenous IAA producing phosphate solubilizing bacteria could be easily isolated from local soil and might be exploited for local use. The majority of these isolates were members of genus *Pseudomonas* (12.3%) and *Bacillus* (27.2%) while all the rest belonged to genera *Ralstonia, Empedobacter,*

Acientobacter, Burkholderia, Aeromonas and *Enterobacter*. Quantitative estimation indicated that the highest (61µg ml^{-1}) amount of IAA produced by isolate PSBC17 whereas the lowest (14µg ml^{-1}) was which was recorded for isolate PSBC68. The most capable strains belonged to the genus *Bacillus* and *Aeromonas*.

Similarly, several previous studies demonstrated that PSB isolates from different crops rhizosphere were found to exhibit different IAA production ability (Ponmurugan and Gopi, 2006; Vikram *et al.*, 2007; Naik *et al.*, 2008; Shahab *et al.*, 2009; Yadav *et al.*, 2010). The large variation in the amount of IAA produced by different strains has been attributed to the variability in the metabolism of different strains of PSB (Leinhos and Vacek, 1994). Such isolates were found to be excellent in promoting plant growth under greenhouse experiments (Shahab *et al.*, 2009; Yadav *et al.*, 2010). Thus, these isolates could provide additional plant growth promoting activities apart from solubilizing insoluble P sources and hence further studies are required to explore the exact contribution of IAA production in the promotion of plant growth.

3.5. Effects of inoculation of PSB isolates on the growth of chickpea under glasshouse conditions

The effects of inoculation of PSB isolates on chickpea growth parameters such as number of nodules, nodule dry weight, shoot dry matter yield, N and P concentration of shoot were investigated under a glasshouse conditions. Shoot dry matter yield of the plant was significantly increased by inoculation of all isolates except PSBC33 compared to the uninoculated control. PSBC81 and PSBC35 were found to be the most effective isolates in terms of increasing shoot dry weight of chickpea (Table 5.6). They increased shoot dry weight by 96% and 88%,

respectively over the uninoculated control. These enhancements were found to be significantly higher than shoot dry weight of P fertilized chickpea. Likewise, Yadav *et al.* (2010) reported that most of PGPR isolates resulted in a significant increase in dry matter production of shoot and root of chickpea seedlings under growth chamber conditions.

All tested isolates significantly improved nodulation of chickpea over the uninoculated control. The variation observed among some isolates was also found to be significant ($p<0.05$). Isolate PSBC81 was found to be the most efficient in inducing nodulation of chickpea followed by isolate PSBC35. They increased the number of nodules by 78.8% and 66.8%, respectively (Table 5.6). These superior increments were also found to be significantly ($p<0.05$) higher than the number of nodules obtained from P fertilized plants. Inoculation of all isolates improved nitrogen fixation and hence higher N concentration was recorded in shoot of inoculated plants than the uninoculated control. However, only PSBC35, PSBC79 and PSBC81 resulted in significant increase over the uninoculated control. The highest concentration of N was obtained in plants inoculated with PSBC35 followed by PSBC79 with the concentrations of total N were 2.48 and 2.43%, respectively.

The result of this study indicated that the increase in nodule number is positively correlated with the increase in shoots dry weight ($r=0.93$; $p<0.05$) and N content ($r=0.70$; $p<0.05$). Peix *et al* (2001b) have also established similar correlation among the growth parameters in common bean inoculated with phosphate solubilizing strain of *Burkholderia cepacia.* Phosphorus deficiency in soils could have a deleterious effect on symbiotic interaction between rhizobia and legume crops, thus affecting its growth and productivity (Gyaneshwar et al. 2002). Several studies have

demonstrated that inoculation of seedlings with PSB isolates increased the level of available phosphorus in the rhizosphere soil (Sundara *et al.*, 2002; Vyas and Gulati, 2009). In this way, inoculation of chickpea with selected phosphate-solubilizing bacteria improves nodulation and nitrogen-fixation processes of this legume crop.

Likewise, the effect of inoculation of PSB isolates on the concentration of P in shoot of chickpea was investigated and most of them showed statistically significant improvement over the uninoculated control plants. Inoculation of PSBC79, PSBC81 and PSBC35 significantly ($p<0.05$) increased the concentration of P in chickpea shoot over the uninoculated control (Table 4.6). In contrast, P concentration of PSBC33 and PSBC17 inoculated plants P concentration did not vary significantly from uninoculated control plants. The highest (2.01 mg/g) concentration of P was obtained in PSBC79 inoculated plant where as the lowest (1.16mg/g) was measured in PSBC33 inoculated plant. Several studies have revealed that inoculation of PSB isolates increased the available P in soil (Sundara *et al.*, 2002; Vikram and Hamzehzarghani, 2008; Vyas and Gulati, 2009) and concomitantly improved P uptake of the plant (Peix *et al.*, 2001a; Peix *et al.*, 2001b; Vikram and Hamzehzarghani, 2008; Vyas and Gulati, 2009; Castango *et al.*, 2011).

The result of the present study indicated that inoculation of seed with selected PSB isolates significantly increased number and dry weight of nodules; shoot dry weight and, N and P concentration of shoot (Table 5.6). Significant variations were also observed among isolates with respect to their effects on the growth, nitrogen fixation and P nutrition of chickpea. Though it is difficult to estimate its contribution we suggest that production of growth promoting substances

IAA by the all the isolates used as inoculants might also contributed to their stimulatory effects on plant growth.

In addition, PSBC17, PSBC35 and PSBC81 were used as inoculants together with agronomically recommended rate of BM and RP. The result demonstrated that application of RP and BM combined with inoculation of these isolates improved some parameters significantly over plants inoculated with their respective isolates alone (Table 5.6). The effect of inoculation of PSBC17, PSBC35 and PSBC81 along with RP was investigated and the result showed that dry matter yield, number of nodules, N and P concentration were increased at statistically ($p<0.05$) significant level over plants treated with rock phosphate only (Table 5.6). Application of all isolates along with rock phosphate except PSBC35 significantly ($p<0.05$) increased shoot dry weight, number of nodules and N concentration of shoot over the plants treated with their respective isolates alone. Inoculation of PSBC35 together with rock phosphate improved only N concentration of shoot while P concentration was increased by PSBC81 application along with fine ground rock phosphate. Several studies indicated that utilization of rock phosphates as P-fertilizer was successful when applied in combination with phosphate solubilizing bacteria isolates which are capable of releasing P from rock phosphate under in vitro conditions.

In addition, inoculation of PSBC17, PSBC35 and PSBC81 along with BM significantly improved nodulation of chickpea over their respective PSB isolates alone inoculated plants (Table 5.6). Inoculation of PSBC17 simultaneously with application of bone meal significantly increased all the growth parameters measured. This would give us the opportunity to utilize old bone, the most commonly available waste as P-fertilizer and clean our environment.

In general, the result of the present study showed that chickpea rhizosphere soils from different producing areas of Ethiopia harbor considerably high number of PSB isolates. Our study has also demonstrated that they belong to different bacterial genera: *Acinetobacter, Aeromonas, Bacillus, Brevibacillus, Burkholderia, Chryseomonas, Enterobacter, Empedobacter, Pseudomonas,* and *Ralstonia, Sphingomonas* and *Stenotrophomonas.* The PSB isolates were able to release significantly higher amount of Phosphate from different insoluble inorganic phosphates such as TCP, RP and BM. The present investigation revealed the diversity of PSB with the potential of solubilizing phosphate, multiple plant growth promoting traits and biocontrol properties. The greenhouse experiment showed that inoculation of chickpea with PSB has improved the nodule number, dry matter yield, N and P uptake of the plant significantly. The result of the current study indicated that some isolates are of particular interest for further research and need to be tested on the field under different agroecological zones. The knowledge generated on diversity and PGP characteristics of phosphate solubilizing bacteria from rhizosphere soil will be useful to design strategies to use these strains as inoculants in sustainable agriculture.

Table 5.6 Effects of inoculation of PSB with different P-source on growth of Chickpea

Treatments	Nodule number plant^{-1} ±SE	Shoot dry weight(g) Plant^{-1} ±SE	Total Nitrogen(%) of shoot± SE	Phosphorous concentration in shoot(mg/g SDW) ± SE
UIA	25.0±2.6d	0.75±0.02f	1.34±0.07d	1.38±0.059c
P+	32. 7±4.0c	1.09±0.22cd	2.02±0.48b	1.55±0.140c
PSBC17	32. 7±2.5c	0.92±0.02def	1.44±0.05d	1.33±0.051d
PSBC33	33.0±3.6c	0.89±0.06ef	1.63±0.12cd	1.36±0.031d
PSBC35	41. 7±2.6b	1.41±0.16ab	2.48±0.19a	1.75±0.261bc
PSBC81	44.7±2.5ab	1.47±0.15a	2.03±0.02bc	1.42±0.158.5c
PSBC79	32.3±3.2c	0.92±0.03def	2.43±0.15ab	2.01±0.49ab
RP	25.3±2.5h	0.95±0.07de	1.31±0.12d	1.35±0.158de
RP+PSBC17	46.7±3.2ab	1.54±0.18a	2.01±0.45bc	1.34±0.028.5d
RP+PSBC35	35.3±3.1c	1.19±0.02c	2.31±0.20a	1.74±0.044.5b
RP+PSBC81	49.7±3.5a	1.52±0.08a	2.27±0.11ab	2.24±0.191a
BM	31.0±2.7c	1.04±0.09de	1.49±0.54d	1.32±0.123d
BM+PSBC17	42.0±3.0b	1.19±0.02c	2.67±0.01a	1.73±0.421bc
BM+PSBC35	44.3±4.2ab	1.42±0.10ab	2.44±0.31ab	1.75.0±0.278bc
BM+PSBC81	48.0±3.0a	1.42±0.02ab	2.38±0.01ab	1.42.0±0.034.5c

SE; standard error, The data is average of triplicates, Numbers in the same column followed by the same letter do not differ significantly at p= 0.05 by DT, SDW; shoot drey weight

Chapter 6 Conclusion and recommendation

Ethiopian soils collected from chickpea and lentil producing areas contain these crop nodulating and symbiotically effective rhizobial population. Most of these rhizobial isolates have exhibited a wide range of substrate utilization and tolerance to salinity, acidic and alkaline pH, metal toxicity, and antibiotics. Furthermore, some of the isolates showed PGP characterstics such as auxin production, inorganic phosphate solubilization and antagonistc activites against *F. oxysporum*. In general, from the present study, it can be concluded that Ethiopian soils harbor chickpea and lentil nodulating rhizobia which are diverse in their morphological, physiological, PGP and symbiotic characteristics.

In addition, the results of the present study showed that chickpea and lentil rhizosphere soils from different producing areas of Ethiopia harbor considerably high number of PSB. Our study has also demonstrated that they belonged to different bacterial genera: *Pseudomonas, Bacillus, Acientobacter, Agrobacterium, Enterobacter, Empedobacter, Burkholderia, Ralstonia, Aeromonas,* and *Chryseomonas.* These isolates were capable of releasing significantly higher amount of P from different insoluble phosphates sources such as TCP, RP and BM. The present investigation revealed that diverse group of PSB with multiple plant growth promoting traits and biocontrol properties exist in Ethiopian soils. The greenhouse experiments showed that inoculation of chickpea and lentil with PSB improved the nodule number, dry matter yield, N and P content of the plant significantly. The knowledge generated on diversity and PGP characteristics of phosphate solubilizing bacteria from rhizosphere soil will be useful to design strategies to use these strains as multi purpose inoculants in low-input agriculture.

Recommendations

Based on the results of the present study we recommend the following:

- Lentil nodulating rhizobial isolates (LR1, LR2, LR6, LR8, LR23, LR24, LR25, LR26) and chickpea nodulating mesorhizobial isolates (CR06, CR18, CR19, CR20, CR31, CR32, CR38, CR45, CR47) showed tolerance to different stresses, wide sources of substrates utilization, excellent symbiotic performance and PGP chraracteristics and hence are highly recommended for field trail and ecological competitiveness studies under different Ethiopian soil and climatic conditions.

- During this study methods used for characterizing rhizobial strains were morphological, physiological and symbiotic. However, these traditional methods of rhizobial characterization frequently fail to identify isolates to a species level. Hence, such kinds of study must be substantiated by PCR based molecular methods such as RFLP, RAPD, AFLP and 16S rRNA sequence analysis so as to obtain a better understanding of microbial diversity and strain identification.

- Rhizobia which have PGP characteristics would increase the yield of non-legume crops which could be grown in rotation or mixed cropping with legumes. Thus, root colonization pattern and PGP activity of such isolates when inoculated to wheat, tef and barley which are commonly grown in rotation with chickpea and lentil under Ethiopian conditions needs to be investigated.

- The result of the current study indicated that PSB isolates such as PSBL5, PSBL31, PSBC81, and PSBC35 are of particular interest for further research and need to be taken to the field under different agroecological zones as they could be used as efficient biofertilizer in chickpea and lentil production system in Ethiopia.

- Co-inoculation of efficient PSB and rhizobia might synergetically enhance plant growth and hence, such kinds of studies are highly recommended using selected PSB and rhizobial isolates of both chickpea and lentil.

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