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Diversity, Symbiotic Effectiveness and Plant Growth Promoting Characteristics of Rhizobia and Rhizospheric Bacteria on Growth and Production of White lupin (*Lupinus albus* L.) under Greenhouse and Field Conditions in North Western Ethiopia

BY
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Abstract

White Lupin (*Lupinus albus* L.) is a traditional legume crop in Ethiopia that can play complementary or alternative roles as sources of organic fertilizers to replenish nitrogen depleted soils in farming systems as it forms symbiotic associations with soil bacteria that have atmospheric nitrogen fixation and other plant growth promoting abilities. This study was aimed to investigate the diversity and symbiotic effectiveness of rhizobia and rhizobacteria on growth and production of white lupin (*Lupinus albus* L.) from major producing areas of the country. A total of 100 bacterial isolates were obtained from the root nodules of White lupin and characterized based on their phenotypic, biochemical and plant growth promoting characteristics using various standard methods. Accordingly, 39 isolates were confirmed as rhizobia on the basis of presumptive and authentication tests. These rhizobial isolates showed wide diversity in their symbiotic and cultural characteristics as well as heterotrophy, such as C and N-substrates utilization, tolerance to metal toxicity, antibiotics, pH, salt and temperature. Moreover, some of the isolates showed interesting PGP traits (IAA, siderophore and HCN production, P solubilization and antagonistic activity against *Fusarium oxysporum*) which can make them prior candidates for the production of inoculants for enhancing White lupin production in the country. Furthermore, the total of 136 phosphate solubilizing bacterial isolates were obtained from rhizospheric soils of White lupin producing area of the country and 40 effective phosphate solubilizing bacterial isolates were selected on the basis of their solubilization index (SI). The phosphate solubilizing bacterial isolates showed variations in their cultural characteristics, doubling times, Gram reactions and C and N-sources utilization patterns. Quantitative estimation of phosphate solubilizing efficiency of the phosphate solubilizing rhizospheric bacterial isolates on different inorganic phosphate sources showed that some of the isolates have better solubilization potential of tricalcium, aluminum and iron phosphates in Pikosvikya broth upon 5 days of incubation. In addition, they showed good

tricalcium phosphate solubilization potential under different stress conditions, such as various salt concentrations, pH and temperature ranges and most of them showed multiple plant growth promoting characteristics. Furthermore, three rhizobial and two phosphate solubilizing rhizospheric bacterial isolates that showed better relative symbiotic effectiveness, plant growth promoting features, stress tolerance and heterotrophic competence were selected to study the effect of single and co-inoculation on the growth and nodulation of the White lupin under greenhouse and field conditions. Accordingly, co-inoculated white lupin plants resulted in higher nodule number, nodule and shoot dry matter accumulation, percent nitrogen and improved relative symbiotic effectiveness than single rhizobial inoculation, and N-fertilized plants on sand and soil cultures under greenhouse conditions. Similarly, inoculation of White lupin plants either with the selected rhizobial isolates alone or co-inoculation of rhizobial and phosphate solubilizing rhizospheric bacterial isolates showed improved performance on the nodulation, growth and yield of the crop compared to the negative control under field conditions. In general, the present study showed that Ethiopian soils contain symbiotically effective White lupin rhizobia and efficient phosphate solubilizing rhizospheric bacteria with multiple plant growth promoting traits to enhance growth and production of the crop under natural conditions. However, repeated tests should be carried out on different field conditions in order to use these rhizobial and phosphate solubilizing bacterial isolates for inoculant production.

Key words: Effectiveness, Lupin, PSRB, PGP, Rhizobia.

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Chapter 1: Introduction

1.1. General Introduction

Pulses are members of the leguminous plants and the second most important crops in the national diet of Ethiopia after cereals occupying 13% of the total cultivated cropland and 12% of the total production of major crops in the country (Geletu Bejiga and Ketema Daba, 2006). Some of the cool season pulse crops are faba bean, chickpea, field pea, lentil, and lupin that are grown between 1800-3000masl.They are important components of crop production in smallholders' agriculture in Ethiopia, providing alternative source of protein, cash income, and food security. They are also important constituents in the field cropping rotation and to enhance soil fertility through symbiotic association with N-fixing rhizobium. The importance of selection, commercialization and application of rhizobia (inoculants) with host pulse crops has been well known for decades (Giller, 2001). Microbial inoculants are the promising components for integral solutions to agro-environmental problems because they promote plant growth by enhancing nutrient availability and uptake, as well as maintaining the health of the plant (Korir *et al*., 2017). This requires for screening compatible host, endosymbionts and appropriate environmental conditions.

Phosphorus is also the second most limiting plant growth, production and biological nitrogen fixation (BNF) by leguminous crops. Different groups of microorganisms are involved in the mineralization and solubilization of organic and inorganic phosphorus sources in the rhizosphere of legumes and other crops. These groups of microorganisms are generally known as Plant Growth promoting Rhizobacteria (PGPR) and involved in improving growth and health of plants through various direct and indirect mechanisms. Plant growth promoting rhizobacteria (PGPB) and rhizobia have multitude applications in enhancing agricultural production through the generation of phytohormones, increasing mineral availability in the soil, improving disease resistance and

decreasing susceptibility to stress conditions. They have the ability to promote growth and inhibit plant disease, and as they are in intimate contact with the plant, they are attractive choices as plant growth promoters and biological control agents (Kloepper *et al*., 2004, Weller, 2007, Adesemoye *et al*., 2008). For the last few decades, there has been a lot of interest in the selection and application of these microorganisms to boost productivity of crops.

Low soil fertility remains a problem in many areas of Ethiopia; especially as traditional land management practices have given way to more intensive cropping. This is neither available nor affordable to the resource poor farmers in Ethiopia. Thus, it is very challenging for farmers to supplement N and P fertilizers in the soil to avoid the nutrient deficiencies. Given the increasing costs of chemical fertilizers, application of rhizobia and plant growth promoting rhizobacteria (PGPR) is a strategic option for sustainable agricultural practices. White lupin (*Lupinus albus* L.) is one the four species originated from the Mediterranean area or "Old World" that has been cultivated for its agronomic importance in the World (Uzun *et al*., 2007). It is estimated that *Lupinus* species fix and assimilate nitrogen in the range of 145 to 208 kg N/ha/year (Jansen, 2006). The high nitrogen yield of lupins is exploited as sources of organic fertilizer to regain nitrogen depleted soils in agricultural systems.

The white lupin in Ethiopia is locally known as 'gibto'. It is produced by smallholder subsistent farmers in two regional states of Ethiopia; Amhara and Benishangul-gumuz, the former being the largest producer. It is grown in elevations ranging between 1500-3000 m.a.s.l. in summer and Meher seasons (June-December). It was reported that in the year 2008, a total of about 17, 000 tons of lupin, with a mean productivity of 1.4 t/ha, was produced in these two major lupin producing regional states (ECSA, 2018).

However, White lupin is mostly produced as a sole crop with minimum agronomic practices with zero tillage and no weeding activities which are the main causes of low and inconsistent

productivity status of the crop. In this regard, Ethiopian Central statistics Agency (ECSA) 11 years' (2004-2014) assessment reports on lupin crop production and area coverage indicated inconsistent condition for total productivity of the crop (Hibistu Azeze *et al*., 2016). According to Francis (1999), the white lupin variety grown in North-western Ethiopia is a bitter variety due to its high alkaloid content. It is a neglected orphan crop mostly grown as a break crop to a continuously cultivated land, as forage, input for traditional alcohol production and hypertension treatment (Zerihun Nigussie, 2012). Mixed crop-livestock production and rotation and/or fallowing are the typical farming systems to regain soil fertility in the North-western part of Ethiopia (Yeheyis Likawunt *et al*; 2010).

For many years now, a lot of focus has been given to study of diversity and symbiotic properties of rhizobia to some extent rhizobacteria of several pulse crops such as faba bean, field pea, chickpea, lentil in Ethiopia (Zerihun Belay and Fassil Assefa, 2011; Mulisa Jida and Fassil Assefa, 2012). The authors showed that the Ethiopian soils harbor as effective inoculants as the commercialized ones that could improve production of different types of pulse crops in the country. However, there is no comprehensive research carried out on the search and integrated application of rhizobium and rhizobacteria from white lupin except a screening study on phosphate solubilizing ability from the rhizosphere of lupin from limited sampling sites (Dereje Haile *et al*., 2016). Hence, the objective of this study was to evaluate diversity and effectiveness of rhizobia and PGPR associated with White lupin for their biotechnological applications in biological nitrogen fixation, control of plant pathogens and plant growth promotion.

1.2. Literature Review

1.2.1. Pulse Crops in Ethiopia

Ethiopia has been known as the home land and domestication of several crop plants. Agriculture is the basis for the entire socioeconomic structure of the country and has a major influence on all other economic sectors and development processes of the country (Kindie Tesfaye, 2016). Cereals and pulses are important food and cash crops for farmers and rural households in Ethiopia, and accounts approximately to about 60% of rural employment and 80% of total cultivated land.

Pulses or grain legumes are the second most important crops in the national diet after cereals occupying 13% of the total cultivated cropland and 12% of the total production of major crops in the country. They are the major sources of protein in cereal based diets, and exported in green, dried and processed forms. They contribute to the sustainability of soil fertility, in the dry land cropping systems because of nitrogen fixation and reducing the requirements for inorganic commercial fertilizers (Karanja, 2016).

They are categorized into cool season and tropical grain legumes distributed in the highlands and lowlands depending upon the climatological conditions. Cool season crops are grown widely at altitudes from 1800-3,000 meters in the northern and central highlands of Ethiopia (Geletu Bejiga and Ketema Daba, 2006). The major cool season grain legumes are; Faba bean (horse beans) (*Vicia faba*), Field pea *(Pisum sativum*) Chickpeas (*Cicer aretinum* L.), Lentils (*Lens esculenta* L.), Peas (*Pisum sativum*) and Adzuki Beans (*Paseoulus angularis*) and white lupin (EEPA, 2004). White lupin (*Lupinus albus* L.) is also one of the pulse crops produced by smallholder subsistent farmers in two regional states of Ethiopia: Amhara and Benishangul-gumuz, the former being the largest producer (ECSA, 2012).

1.2.2. White Lupin (*Lupinus albus* L.)

Lupin belongs to the genus *Lupinus*, family Leguminosae and subfamily Papilionaceae. It is a cool season legume that includes more than 450 species worldwide of which only four species (White lupin (*L. albus* L.), blue lupin (*L. angustifolius* L.), Yellow lupin (*L. luteus* L.) and Andrean lupin (L. *mutabilis* L.)) have been cultivated because of their agronomic importance in the World (Uzun *et al*., 2007; Zerihun Nigusie, 2012). The first three species were originated from the Mediterranean area or "Old World" while the fourth one belongs to the South America or "New World".

Originally, the name of the genus Lupinus descends from the Latin word "Lupus" meaning 'wolf', because Romans' believed that Lupin robbed nutrients in the soil in the same way that a wolf would 'steal' domestic animals. However, the opposite is true as it is among the legumes that can replenish soil fertility (Small, 2012). It is known by different local names in different countries, such as "Gibito" in Ethiopia (Zerihun Nigusie, 2011), 'Termes' in ancient Greeks, 'Turmus' in most Arab countries and India, 'Termiye'or 'Acibakla' in Turkey (Yorgancilar *et al*; 2009).

White lupin (*Lupinus albus* L.) originates from South-Eastern Europe and Western Asia where wild types still occur. The species *albus* in the scientific name *Lupinus albus* is Latin in origin and implies for the white flower. White Lupin (*L. albus* l.) was cultivated in Mediterranean countries as a subsistence crop for thousands of years; especially by the ancient Egyptians (hence the occasional name Egyptian Lupin). It is an economically and agriculturally valuable plant as its seeds are used as a protein source for animal and human nutrition in various parts of the world and numerous cultivars have been selected and widely grown for local consumption and export in Australia (Small, 2012).

The traditional orphan pulse crop originally grown around the Mediterranean, the black and the Nile Valley extending to Sudan and Ethiopia, has been extended to grow in other countries such as, Kenya, Tanzania, Zimbabwe, South Africa, Mauritius, United States and South America (mainly Brazil and Chile) (Jansen, 2006).

It is highly adaptable to marginal soils and climates and performs well on disturbed areas, and sites with reduced competition (Kohajdova *et al*, 2011). It grows well in acidic soils but tolerates mildly alkaline and slightly calcareous soils. It prefers soils with pH6.5 or less i.e. soil acidity is less critical for lupin production than for other legumes such as alfalfa and soybean (Brebaum and Boland, 1995), and some white lupin cultivars are more tolerant to salinity and heavy soils than other crops (Jansen, 2006). Growth is hampered on heavy clay, waterlogged and strongly alkaline soils. White lupin seeds have been used in human nutrition and treatment of various diseases for several thousands of years. For the last 20 years, quiet new properties of white lupin have been discovered as functional food groups because of the unique profile of protein, fatty acids with a desirable ratio of omega-6 to omega-3 acids, oligosaccharides and antioxidants or non-starch carbohydrates that make white lupin an excellent component in many healthy diets (Prusinski, 2017).

These components have health benefits in the human body include treatment of diabetes, hypertension, obesity, cardiovascular diseases, glycaemia, appetite, insulin resistance, and colorectal cancer. Seeds of White lupin, together with other crops, are used for the production of gluten-free flour, bacterial and fungal fermented products and pasta products, as substitutes of meat, egg protein and sausages. Moreover, seeds of the crop are cooked, roasted and ground and mixed with cereal flour in the production of bread, crisps, pasta and dietary dishes (Prusinski, 2017).

1.2.3. Importance of White Lupin (*Lupinus albus* L.) in Ethiopia

White lupin is produced by smallholder subsistence farmers in two regional states of Ethiopia; Amhara and Benishangul-gumuz, the former being the largest producer. It is grown in elevations ranging between 1500-3000 masl in summer season (mid-June to mid-December). It is grown in mixed crop-livestock production and rotation and/or fallowing are the typical farming systems to regain soil fertility. The smallholder farmers grow the crop using zero-tillage or plowing their land only once without applying any type of fertilizer and weed management systems (Yihenew Gebreselassie, 2002; Yeheyis Likawent, 2012).

The variety of White lupin cultivated in Ethiopia is locally known as 'Gibto'. In Ethiopia, White lupin is a multipurpose underutilized legume crop with a diversified array of importance. For instance, the crop plays significant roles as green manure, cover crops, forage production, traditional hypertension treatment, pharmaceutical use, food additives, and as feed for animals, ecto-parasite control, phytoremediation use, maintaining soil fertility, Ornamentation, erosion control, pest control, live fencing, and crop rotation (Jansen, 2006; Zerihun Nigusie, 2012).

Its seed is used as a snack, for the preparation of local alcoholic drink, 'Areqi' and as 'Shiro' flour for the people living in the north western part of Ethiopia especially West Gojam, Awi and South Gondar zones. Before consumption, the seeds are roasted and soaked up to 3-7 days in running water to remove the bitter and toxic alkaloids (Yeheyis Likawent *et al*; 2010). It was reported that in the year 2011/12, a total of about 443,705 quintal of lupin, with a mean productivity of 1.2 t/ha, was produced (ECSA, 2012). According to Francis (1999), White lupin variety grown in North-western Ethiopia is bitter due to its high alkaloid content.

1.2.4. Integration of Leguminous Crops in low in put Agriculture in Ethiopia

Low availability of N and P in the soil is a problem that limits crop production in most parts of Ethiopia (Desta Beyene and Angaw Tsigie, 1989, Dawit Solomon *et al*., 2002). Legumes have long been recognized and valued as "soil building" pulse crops as legume-based systems improve several aspects of soil fertility, such as N and P availability (Jansen *et al*., 2012). Legumes are incorporated in low in put cropping systems in the form of crop rotation and mixed cropping systems in the country because they fix inorganic nitrogen in association with rhizobia. Apart from that, microbial inputs from other rhizosphere microorganisms have been found to improve plant growth, health and productivity of legumes and other crops through direct and indirect mechanisms including mineralization and solubilization of phosphorus.

Hence, improving soil fertility and crop production through the application of environmental friendly sources of nitrogen and phosphorus such as biological nitrogen fixation and phosphate solubilization by selecting effective strains of bacteria from Ethiopian soils would represent an economically beneficial and ecologically sound technology to enhance production in low in put agriculture (Mulisa Jida and Fassil Assefa, 2012).

1.2.5. Rhizosphere

The rhizosphere is the volume of soil under the influence of the exudates of plant roots (sugars, organic acids and amino acids), where very important and intensive microbe–plant interactions take place. These interactions can both significantly influence plant growth and crop yields and have biotechnological applications (Hernandez-Leon *et al*., 2015, Santoyo *et al*., 2017).

The microbes colonizing rhizosphere comprise bacteria, fungi, acticomycetes, protozoa, and algae. However, bacteria are the most abundant microbial groups that are not evenly distributed in the

environment. They present in the rhizosphere with a concentration approximately ranging from 10–1000 times higher than in bulk soil (Olanrewaju et al., 2017., Guada *et al*., 2018).

The rhizosphere microbiome is considered as a potential source for the entry of bacteria into plant roots that can provide different benefits to plants through various mechanisms, such as enhancing plant growth, provision of nutrient via nitrogen fixation and phosphate solubilization, removing soil contaminants and reducing or avoiding the deleterious effects of phytopathogens (Koch *et al*., 2010, Marquez-Santacruez *et al*., 2010). These microorganisms are generally known as Plant growth-promoting rhizobacteria (PGPR).

In recent years, the use of PGPR have gained great attention and acceptance worldwide for their agricultural benefits (increase crop yield) through the application of combinations of different modes of action as they help in alleviating dependence on the application of synthetic agro-chemicals (Figueiredo *et al*., 2016). Thus, the rhizosphere is the most preferential area for producing new agricultural inputs as bioinoculants or biopromoters for plant production that do not have damaging impacts on the environment or human or animal health (Owen *et al*., 2015).

1.2.6. Plant Growth Promoting Rhizobacteria

Plant growth promoting rhizobacteria can be categorized into two main groups as extracellular plant growth promoting rhizobacteria (e-PGPR) and intracellular plant growth promoting rhizobacteria (i-PGPR) (Viveros *et al*., 2010). The e-PGPR inhabits the rhizosphere or in the spaces between the cells of the root cortex, whereas i-PGPR predominantly dwells inside the specialized nodular structures of root cells and within the interior tissues of a plant.

They comprise various strains of rhizobia and other endophytic bacteria. The major genera considered as e-PGPR are *Azotobacter*, *Serratia*, *Azospirillum*, *Bacillus*, *Caulobacter*, C*hromobacterium*, *Agrobacterium*, *Erwinia*, *Flavobacterium*, *Arthrobacter*, *Micrococcous*, *Pseudomonas*, and *Burkholderia*. The bacterial genera, *Allorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, and *Rhizobium*, as well as *Frankia* species, which can establish truly mutualistic interactions with their hosts and fix atmospheric nitrogen, belong to i-PGPR (Bhattacharyya and Jha, 2012, García-Fraile *et al*., 2015).

Regarding symbiotic bacteria, they can integrate their physiology with the plant, resulting in the formation of specialized structures known as nodules (Vajan *et al*, 2016). The best known mutualistic symbiotic bacteria are the rhizobia, which establish symbiotic associations with leguminous crop plants and fix atmospheric nitrogen for the plant in certain root structures known as nodules. In addition, *Frankia* are other groups of mutualistic bacteria associated with higher or actinorrhizic plants, such as *Alnus* trees, where bacterial nitrogen fixation takes place (García-Fraile *et al*., 2015).

PGPR inhabit the rhizosphere for nutrients from plant root exudates (vitamins, sugars, proteins, carbohydrates, organic acids, amino acids and mucilage). They, in turn, provide various advantages for the plant by increasing plant growth through different direct and indirect mechanisms. In addition, the PGP microbes help plants tolerate abiotic stresses like extremes of temperature, pH, salinity and drought. Plant growth promoting bacteria (PGPR) can be classified as biofertilizers, rhizoremediators, phytostimulators and stress controllers. Bacterial fertilizer is referred to the bacteria that supply nutrition to the associated plant. They may benefit plants by providing nutrients such as N, P, Fe, and others which are required for the proper growth and different metabolic processes (Lugtenberg and Kamilova, 2009).

1.2.7. Plant Growth Promotion Mechanisms by PGPR

PGPR improve plant growth and crop yield through direct and indirect mode of actions that include nutrient mobilization, production of phytohormones, lessening the impacts of biotic and abiotic stresses, inducing the production of volatile organic compounds and enzymes to prevent various plant diseases (Zakry *et al.,* 2012, Gupta *et al*., 2015). Nevertheless, these direct and indirect mode of actions carried out by PGPR are influenced by a number of factors that include biotic factors (plant genotypes, plant developmental stages, plant defense mechanisms, other members of the microbial community) and abiotic factors (soil composition, soil management and climatic conditions) (Garcia-fraile *et al*., 2015).

1.2.7.1. Direct mechanisms of plant growth promotion

PGPR considered as direct plant growth and development facilitators due to their tendency to increase the accessibility and concentration of nutrients through increasing the flux of resources at the root surface from the environment The mechanism includes the increase in accessibility of nitrogen, phosphorous and iron through the mechanisms of N-fixation, phosphate solubilization and iron chelation via siderophores, or production of plant hormones including auxin, cytokinin and ethylene (Zakry *et al.,* 2012, Gupta *et al*., 2015).

1.2.7.1.1. Biological nitrogen fixation

Nitrogen is one of the most important macronutrients essential for the growth of all living organisms including plants and bacteria. Although, nitrogen is the abundant element in the atmosphere that accounts more than three quarters of the atmosphere in the form of nitrogen gas (N_2), it cannot be utilized by plants, animals and microorganisms. Hence, nitrogen first must be

reduced and converted into either ammonia or nitrate through the process known as biological nitrogen fixation (BNF) to be utilized by plants (Peoples *et al*., 2009).

The process of biological nitrogen fixation (BNF) is one of astonishing biological processes that provide approximately two-thirds of fixed nitrogen through symbiotic association between leguminous plants and the root nodule bacteria generally known as rhizobia. (Sulieman and Tran, 2014). Currently, bacteria nodulating legumes belong to the *Proteobacteria* class. The majority of them belong to the genera of *Rhizobium*, *Mesorhizobium*, *Bradyrhizobium*, *Azorhizobium, Allorhizobium* and *Ensifer* (formerly *Sinorhizobium*). It has been estimated that biological nitrogen fixation produces roughly 200 million tonnes of nitrogen per year. The process is controlled by various *nif* genes along with other structural genes that involved in activating iron protein, donating electrons, biosynthesizing the iron molybdenum cofactor, and many other regulatory genes which are mandatory for the synthesis and activity of the nitrogenase (Reed *et al*., 2011). Application of efficient biological N_2-fixing rhizobia either alone or in combination on crop fields improves nitrogen level in agricultural soils that subsequently enhances plant growth as well as crop production and the process of disease management (Damam *et al*., 2016). Thus, the interaction between the two partners offers an economically attractive and ecologically sound means of reducing external inputs and improving internal resources.

Nitrogen fixing bacteria are housed in special root organs called nodules where they fix atmospheric nitrogen gas to ammonia, in which the plant can assimilate via glutamine synthase to form glutamine. In return, the bacteria obtain plant carbohydrates, mainly as malate for food and energy source for nitrogen fixation. The process of nitrogen fixation begins only after formation of symbiosome because membrane envelopes are the site of primary reaction of nitrogen fixation and production of nitrogenase by bacteroids.

Rhizobia are Gram negative bacteria that colonize plant root cells and totally dependent upon their plant hosts for nutrients when they live within the nodule. Subsequently, Photosynthetic products such as glucose, sucrose and other organic compounds translocated from the leaves to nodules and serve as substrates to facilitate nitrogen fixation by bacteria. Moreover, the bacterial β-hydroxybutyrate dehydrogenase helps to generate the reducing power of ATP for the support of nitrogen fixation (Trainer and Charles, 2006).

In addition to symbiotic nitrogen fixation, free living bacteria also have nitrogen-fixing capacity in the soil. The free-living nitrogen-fixing bacteria are widely distributed among phylogenetically diverse bacterial genera such as *Acetobacter*, *Arthrobacter*, *Azoarcus*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Herbaspirillum*, *Klebsiella* and *Pseudomonas* associated with some agronomically important crops. However, in practice most free-living bacteria fix only a limited amount of nitrogen and are therefore not employed commercially (Olanrewaju *et al*., 2017).

1.2.7.1.1.1. Current Rhizobial Taxonomy

Bacteria that form nitrogen-fixing symbiosis with legume plants belonging to groups of α- and β-proteobacteria are collectively called rhizobia. Initially, rhizobia were classified cross-inoculation tests between their host plants. Moreover, they were classified as fast and slow growing strains on the basis of their growth rate on the culture medium. However, such classification mechanisms were inconsistent due to doubts on the validity of such classification (Rao *et al*., 2018).

The classification of rhizobia has been gone through a considerable change in recent years due to the addition of several new genera and species to this important bacterial group. Recent studies have shown the existence of a great diversity among nitrogen-fixing bacteria isolated from

different legumes that comprise more than 98 species of rhizobia with 14 genera of α- and β-*proteobacteria* groups (Berrada and Fikri-Benbrahim, 2014). On the basis of 16S rDNA sequence , legume symbionts belong to three main distinct phylogenetic sub-classes: i) Sub-Class *α-proteobacteria* that includes the genera, *Rhizobium*, *Mesorhizobium*, *Ensifer* (formerly *Sinorhizobium*), *Bradyrhizobium*, *Phyllobacterium*, *Microvirga*, *Azorhizobium*, *Ocrhobactrum*, *Methylobacterium*, *Devosia*, *Shinella* (Zakhia *et al*., 2004), Subclass *β-proteobacteria* with the genera *Burkholderia*, *Cupriavidus* (formerly *Ralstonia*) (Moulin *et al*., 2001) and iii) *γ-proteobacteria* represented by *Pseudomonas* sp.(Shiraish *et al*., 2010).

According to Berrada and Fikri-Benbrahim (2014), there is certainly much opportunity to discover new rhizobial species, since only 23% of known legumes were identified specifically for symbiotic relationship up to date as the discovery of new symbionts associated with legumes is necessary to gain deep insight into the taxonomy of the rhizobia. The same authors explained that the current taxonomy of root nodulating rhizobia is rapidly progressing because of the latest advances in molecular biology-based omics technology, such as genomics, proteomics, transcriptomics and metabolomics. This obviously implicated that the use of whole genome sequencing based taxonomy (genomotaxonomy) will again change the current concepts of this important group of bacteria. Apart from this, the increase in legume explorations as well as advanced studies of novel legume species in different geographical regions offers yet more opportunities to isolate and characterize more novel rhizobial species.

Moreover, Kairos evolution (non-nodulating rhizobia may became symbiotic) as nodulation genes could spread from inoculated rhizobia into native population of non nodulating bacteria. When the bacteria are not dormant, the accessory genomes for symbiotic interactions with plants and other

kinds of niche adaptation of indigenous soil rhizobia probably undergo frequent Kairos evolution, which further results in diversified symbiotic efficiency in soil rhizobia. Kairos evolution is fast, takes place under special conditions, and leads to niche adaptation in soil rhizobia (Rao *et al*., 2018).

1.2.7.1.2. Phosphate Solubilization

Phosphorus is the second most important macronutrient needed by plants, after nitrogen as it affects plant structure at cellular level and stimulates plant growth as well as productivity. Despite of the large reservoir of inorganic and organic phosphates in most agricultural soils, about 95 - 99% of phosphorus is in the insoluble, immobilized or precipitated forms and it is difficult to be accessed by plants (Chauhan *et al*., 2017). Only very low concentration of P is available to plants, and it is commonly deficient in most natural soils as it is fixed as insoluble calcium phosphates in alkaline soils or iron and aluminum phosphates in acidic soils. Plants absorb phosphate only as monobasic ($H_2PO_4^{-1}$) and the dibasic (HPO_4^{-2}) forms (Pérez-Montano *et al*., 2014).

In order to alleviate p scarcity in the soil, it is usually added to soils as chemical fertilizers synthesized through high-energy-intensive processes. However, plants can use only insignificant amount of this P as most of it is involved in the formation of metal–cation complexes, and rapidly becomes fixed in the soil. Thus, solubilization and mineralization of P by phosphate-solubilizing bacteria (PSB) and other soil microorganisms are the two major mechanisms in the bio-geochemical cycling of phosphate in the soil (Sharma *et al*., 2013).

Phosphate solubilizing bacteria convert inorganic soil phosphates, such as $Ca_3(PO_4)_2$), $FePO_4$ and $AlPO_4$ and organic phosphates (inositol phosphate, phosphomonoesters and phosphotriesters) to a form that can be readily accessible to plants through the synthesis of low molecular weight organic

acids, siderophores, hydroxyl ions and phosphatases. Production of organic acids, particularly gluconic and carboxylic acids is one of the well-studied mechanisms utilized by various phosphate solubilizing soil bacteria (Sharma *et al*., 2013). These organic acids bind phosphate with their hydroxyl and carboxyl groups thereby chelating cations and also inducing soil acidification, resulting in the release of soluble phosphate (Bhattacharyya and Jha 2012).

Phosphate solubilizing PGPB included in the genera *Arthrobacter*, *Bacillus*, *Beijerinckia, Burkholderia, Enterobacter, Erwinia, Flavobacterium, Microbacterium Pseudomonas*, *Rhizobium*, *Rhodococcus*, and *Serratia* have attracted the attention of agriculturists as soil inoculums to improve plant growth and yield (Oteino *et al*., 2015). The role of phosphate solubilization in plant growth promotion is often supplemented with other plant beneficial activities (nitrogen fixation, IAA production, and iron sequestration). Hence, it is highly beneficial to carryout inoculation with phosphate solubilizing bacteria that have multiple biosynthesis activity or in mixed inocula with other rhizospheric microbes with multiple plant growth promoting properties (Gupta *et al*., 2015). Hence, inoculation of soils with potential PSB is the best option in order to intensify the availability of soluble phosphorus in the rhizosphere and its absorption by the plant.

1.2.7.1.3. Siderophore production

Iron is an essential micronutrient for all life forms as it is involved in diverse and essential biological processes, such as photosynthesis, respiration, chlorophyll biosynthesis. Although it is a third abundant element on the earth's crust, it is hardly soluble and therefore not readily accessible by living organisms. In aerobic conditions, iron commonly exists in nature in the form of Fe^{3+}, which is highly insoluble and inaccessible to living organisms. Plants produce and excrete chelators (phytosiderophores) which bind Fe^{3+} and transport it to the root surface where it is either

reduced to Fe^{2+}, which is subsequently taken up by the plant, or it is absorbed as a Fe^{3+}-phytosiderophore complex by the plant (Lemanceau *et al.*, 2009).

Bacteria living under low Fe^{3+} concentrations can overcome Fe limitation by using chelator agents called siderophores. Thus, siderophore production is one of the PGP properties of rhizobacteria. Siderophores are low-molecular weight secondary metabolites produced by microorganisms under iron limiting conditions (Goswami *et al*. 2016). They have side chains and functional groups with high-affinity to bind ferric ions and transport them through the cell membrane (Niehus *et al*., 2017). According to their structural features, functional groups, and ligand types, siderophores produced by microorganisms are classified into four main groups as carboxylate, hydroxamates, catecholates, and mixed type (Kumar *et al*., 2017). Siderophores generated by PGPR also play key roles in the inhibition of phytopathogens because of iron starvation or competitive exclusion in iron deficient conditions (Dalvi and Rakh, 2017). These siderophores bind most of the Fe^{3+} present in the soil and make it unavailable to the fungal and bacterial pathogens in the host plant rhizosphere.

In general, iron deficiency in most soils is a major global issue as it forms insoluble complexes with other organic and inorganic molecules in the soil. Hence, Siderophores produced by PGPR are the most important alternatives to solubilize and chelate iron from the complexes in order to fulfill iron requirement of plants (Arora *et al*., 2013; Singh *et al*., 2017).

1.2.7.1.4. Phytohormone production

PGPR are also implicated with altering root architecture and promote plant development with the production of different phytohormones. The major phytohormones produced by PGPR include IAA, cytokinins, gibberellins and Ethylene, certain volatiles and the cofactor pyrroquinoline

quinine (PQQ)that can affect cell division in the root by increasing the number of lateral roots and root hairs with a subsequent escalation of nutrient and water uptake (Han *et al*. 2005, Kloepper *et al*., 2007).Different researchers demonstrated that about 80% of rhizobacteria can synthesize Indole acetic acid (IAA) from tryptophan by various pathways and is proposed to act in conjunction with endogenous IAA to stimulate cell proliferation and enhance the host's uptake of minerals and nutrients from the soil (Spaepen *et al*., 2007). Indole acetic acid affects multitude of plant development activities; plant cell division, increases the rate of vegetative and root growth, affects photosynthesis, biosynthesis of various metabolites, and resistance to stressful conditions.

IAA secreted by rhizobacteria interferes with various plant developmental processes because the endogenous pool of plant IAA may be altered by the acquisition of IAA that has been secreted by soil bacteria (Glick, 2012). Evidently, IAA also acts as a reciprocal signaling molecule affecting gene expression in several microorganisms. Consequently, IAA plays a very important role in rhizobacteria-plant interactions (Spaepen and Vanderleyden, 2011). Moreover, down-regulation of IAA as signaling is associated with the plant defense mechanisms against a number of phytopathogenic bacteria (Spaepen and Vanderleyden, 2011).

Plant growth promotion as a result of IAA has been documented in several plants in recent years (Spaepen *et al.*, 2007). However, beneficial effects of bacterial IAA depend upon the optimum concentration, which may vary for different plants. The role of Phytohormone produced by associative bacteria in the promotion of plant growth hormones during stress conditions such as salinity or drought has also been demonstrated (Egamberdieva, 2012). Furthermore, the role of IAA in response to stress is evident from its increased production of IAA in *Azospirillum* sp. during carbon limitation and acidic pH (Spaepen *et al*., 2007).

1.2.7.2. Indirect mechanisms

The contribution of plant growth promoting bacteria in enhancing plant growth and crop yield is associated with various indirect mechanisms. These include production of different hydrolytic enzymes (chitinases, cellulases, proteases...etc.), synthesis of various antibiotics against fungal and bacterial pathogens, induction of systemic resistance against various phytopathogens and pests, production of siderophores, volatile organic compounds such as HCN, and exopolysaccharides (Gupta *et al*. 2015, Gamalero and Glick, 2015). These mechanisms enable plants to grow actively under different environmental biotic and abiotic stresses.

Plant growth promoting rhizobacteria involved in sustaining plant growth through the production several enzymes, such as β-1, 3-glucanase, ACC-deaminase, and Chitinase in response to phytopathogens that are generally involved in lysing cell walls and neutralizing pathogens (Goswami *et al.,* 2016). Most of the fungal cell wall constituents are made of β-1, 4-N-acetyl-glucoseamine and chitin in which β-1, 3- glucanases and Chitinase producing bacteria control their growth by breaking down these cell wall components.

Many rhizobacteria have been reported to produce antifungal metabolites like, HCN, phenazines, pyrrolnitrin, 2, 4-diacetylphloroglucinol, pyoluteorin, viscosinamide and tensin (Bhattacharyya and Jha, 2012). Interaction of some rhizobacteria with the plant roots can result in plant resistance against some pathogenic bacteria, fungi, and viruses. This phenomenon is called induced systemic resistance (ISR).

Moreover, ISR involves jasmonate and ethylene signaling within the plant and these hormones stimulate the host plants' defense responses against a variety of plant pathogens (Glick, 2012). Many individual bacterial components induce ISR, such as lipopolysaccharides (LPS), flagella,

siderophores, cyclic lipopeptides, 2, 4-diacetylphloroglucinol, homoserine lactones, and volatiles like, acetoin and 2, 3-butanediol (Lugtenberg and Kamilova, 2009).

Among the PGPR, *Pseudomonas fluorescens* LPK2 and *Sinorhizobium fredii* KCC5 are known to produce beta-glucanases and Chitinase that have a potential of preventing *Fusarium oxysporum* and *Fusarium udum* that cause fusarium wilt (Ramadan *et al*., 2016). Some groups of PGPB also produce different antibiotics in response to proliferation of plant pathogens. The production of one or more antibiotics is the mechanism most commonly associated with the ability of plant growth promoting bacteria to act as antagonistic agents against phytopathogens.

The application of microorganisms to control diseases, which is a form of biological control, is an environment-friendly approach. In general, competition for nutrients, niche exclusion, induced systemic resistance and production of antifungal metabolites are the chief modes of biocontrol activity in PGPR (Glick, 2012).

1.2.8. Rhizobium-PGPR Co-inoculation

The use of synthetic agro-chemical inputs such as chemical fertilizers and pesticides in the low-input agriculture in developing agriculture has not been sustainable for many years now. Therefore, searching for an easily available and affordable technology towards using effective rhizobia and other plant growth-promoting rhizobacteria (PGPR) as biofertilizers and/or as biocontrol agents is all the more important to enhance plant growth, increase yield, and suppress diseases in a wide range of agricultural crops (Hassen *et al*., 2016). Kumar *et al*, (2011) reported that inoculation of Groundnut with a mixture of PGPR containing *Rhizobium* strain Tt9 with a phosphate solubilizing PGPR *Bacillus megaterium var phosphaticum* resulted in improved nodulation, nitrogen fixation and crop yield as the phosphate solubilizing co-inoculant fulfilled about 50 % the phosphorus

fertiler requirement of the crop. Hence, the application of the consortium of rhizobia and PGPR with multiple plant growth promoting traits is a promising alternative for the development of sustainable agriculture in future as it resulted in enhanced plant growth and crop yield with no negative environmental impact.

According to Saharan and Nehra, (2011), combined inoculation of rhizobia with phosphate solubilizing bacteria (PSB) showed synergetic effect on the symbiotic parameters, such as nodule number and plant biomass which resulted in improved grain yield of legumes. Co-inoculation of Rhizobium with PGPR is an interesting alternative that has recently become more popular and gained huge attention for improving nodulation, nitrogen fixation and yield of several legumes in many parts of the World. Recently, co-inoculation of *Bradyrhizobium* and PGPR significantly enhanced the growth and yield of Soybean as compared to single *Bradyrhizobium* inoculation (Htwe *et al*., 2018). Such enhancement of growth and yield of legumes due to co-inoculation is attributed to the growth enhancement by non-rhizobial PGPR.

1.2.9. Role of Microbial Inoculants against Environmental Stresses

The number of plant growth promoting microorganisms that are found in the soil is not high enough to compete with other indigenous microorganisms commonly found in the rhizosphere. Hence, inoculation of plants with selected target microorganisms at a much higher concentration than those normally found in the soil is indispensable to take advantage of their beneficial properties for the enhancement of plant growth and crop yield (Gouda *et al*., 2018). Knowing and understanding the causes for the failures of the inoculants in the field may lead to the development of better bio-formulations that are effective in natural conditions.

In the environment, microbial cells are subjected to a range of adverse abiotic and biotic conditions. Therefore, the success of the application of microbial inoculants highly depends on whether the environmental condition is favorable to the survival and functioning of the microbes or available productive niches. The dynamics of inoculant population is responsible for the effectiveness and inconsistent performance of inoculated microbes in the soil. Some of the major abiotic and biotic factors that influence the performance of microbial inoculants in the soil are discussed as follows:

1.2.9.1. PGPR and abiotic stresses

Abiotic stresses, such as extreme temperature, drought, salinity, acidic soils and metallic toxicity seriously affect the legume-rhizobium symbiosis and crop production. Depending on the type of crop, such abiotic stresses result in 50 % - 82% yield loss which causes food insecurity problems worldwide (Vejan *et al*., 2016). In order to overcome these problems, plants carry out a variety of responses through the synthesis of 1- aminocyclopropane 1-carboxylic acid (ACC) that served as a precursor for the synthesis of ethylene which, in turn, help to induce multiple physiological and metabolic changes in the plants at molecular level (Sharma *et al.,* 2013).

Avery small amount of ethylene is required by plants in the course of their growth to break seed dormancy and high level of the compound following germination is inhibitory to root elongation (Hassen *et al*., 2016). However, in order to lessen this problem, many PGPR strains in the soil are able to produce the enzyme ACC-deaminase involved in the cleavage of plant ethylene precursor ACC, into ammonia and α-ketobutyrate to lower the level of ethylene and it associated stress in plants. Furthermore, ACC-deaminase produced by PGPR strains Pseudomonas*, Azospirillum, Bacillus, Burkholderia, Enterobacter,* and *Kluyvera*) is highly important in

protecting plants against a wide range of abiotic stresses including drought, salinity, heat flooding and water logging (Blaha *et al*., 2006, Hassen *et al*., 2016).

Hence, to alleviate the inhibitory effect of these abiotic stresses on plant growth and yield, especially in arid areas, selection and development of inoculants with stress tolerant ACC-deaminase producing PGPR could be the best strategy (Ali *et al*., 2014, Gontia-Mishra *et al*., 2014, Glick, 2012). Apart from the production of ACC- deaminase, many PGPR are also naturally endowed with other stress tolerance traits such as production of exopolysaccharides (EPS), osmolytes, cytokinin, and antioxidant enzymes.

Inoculation of plants with exopolysaccharide producing PGPR results in significantly minimized salt stress by binding cations such as Na^+ and decreasing the level of Na^+ available for uptake (Grover *et al*., 2011). Moreover, research reports indicated that some free living PGPR strains induce osmolytes which assist plants to relieve salt stresses by scaling up their osmotic potential within the cells (Gururani *et al*., 2013).

Abiotic stresses resulting from water deficiency and drought have a serious negative effect on nodule initiation and N_2 fixation in legumes and limit plant growth and productivity. To solve such problem, Grover *et al*., (2011), reported that inoculating plants suffering from oxidative stress with a PGPR *Azotobacter chrococcum* strain, that produce cytokinin and antioxidants, resulted in the accumulation of abscisic acid (ABA) that resulted in the degradation of reactive oxygen species (ROS). Similarly, Karthikeyan *et al.* (2012) and Agami *et al*. (2016) reported the positive effect of treating stressed plants with the diazotrophic bacteria *Azospirillum* and *Azotobacter* with the antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POX), and catalase (CAT) in alleviating water deficiency and drought stresses.

Low or high temperature is another most serious abiotic stress that limits nodulation, nitrogen fixation, and plant growth and productivity by adversely affecting the effectiveness of rhizobia and plant growth and development. However, selecting temperature tolerant strains of rhizobia and PGPR could maintain effective symbiotic nitrogen fixation and crop yield by producing heat shock proteins (Hungria and Kaschuk, 2014).

Regarding soil pH stress, soil acidity is a significant problem in hindering agricultural production in many areas of the world and limits legume productivity. Most leguminous plants require a neutral or slightly acidic soil for growth, especially on symbiotic N_2 fixation dependent environments (Bordeleau and Prevost, 1994). Legumes and their rhizobia exhibit varied responses to acidity (Graham *et al*., 1994). The highest level of nodulation occurs at pH ranges between 5.5 and 7.2, while it becomes reduced below pH 5.5, except some acid-tolerant strains of rhizobia that demonstrate a comparative advantage over acid-sensitive strains in the ability to nodulate their host legume at pH 4.5.

Some strains of rhizobial species vary widely in their pH tolerance. Most fast-growing rhizobia have generally been considered less tolerant to acid pH than slow growing strains of *Bradyrhizobium.* Differences in LPS composition and structure of the outer membrane, proton exclusion as well as extrusion, accumulation of cellular polyamines, and synthesis of acid shock proteins have been associated with the growth of rhizobium at acidic conditions. Therefore, selection of acid-tolerant rhizobia to inoculate legume hosts under acidic conditions will ensure the establishment of the symbiosis and also successful performance of the legume under acidic soils (Correa and Barneix, 1997).

Heavy metals are also the most deleterious abiotic stresses that have toxic effects on plants and soil microorganisms (Tak *et al*., 2013). Some PGPR are used to reduce plant stress in heavy metal contaminated soils by sequestering heavy metals or bioremediating contaminated areas. Such plant associated bacteria can be potentially used to improve phytoextraction activities by altering the solubility, availability and transport of heavy metals by either reducing soil pH or releasing chelators (Ma *et al*., 2011). For instance, PGPR strains, such as *Pseudomonas putida* and *Pseudomonas fluorescens* were found to neutralize the toxic effect Cd pollution on barely plants due to their potential to scavenge Cd ions from the soil (Baherlouei *et al*., 2011).

1.2.9.2. PGPR against biotic stresses

Various pathogenic bacteria, viruses, fungi, nematodes, protists, insects, and viroids severely affect plant growth and agricultural yield. According to Strange and Scott (2005), food production suffers a loss of about 15% worldwide mainly due to phytopathogens. Biotic Stresses are major challenges to crop yield and thereby inspires breeding of resistant crops due to the vast economic loss. Ngumbi and Kloepper, (2016) argued that Plants inoculated by soaking their roots or seeds overnight in cultures of PGPR exhibit enormous resistance to different forms of biotic stresses.

1.3. Objectives of the Study

The general objective of the study was: to evaluate diversity, plant growth promoting characteristics and symbiotic effectiveness of rhizobia and rhizobacteria on growth and production of White lupin (*Lupinus albus* L.) under greenhouse and field conditions in Ethiopia.

The specific objectives of this study were to:

- Determine the Eco-physiological and biochemical characteristics of rhizobia isolated from the root nodules of white lupin.
- Evaluate plant growth promoting traits of Rhizobia isolated from the root nodules of the White lupin (*Lupinus albus* L.).
- Evaluate the symbiotic effectiveness of white lupin rhizobia on sand culture under greenhouse conditions.
- Investigate the phosphate solubilizing efficiency and PGP traits of rhizobacteria isolated from the rhizosphere of white lupin (*Lupinus albus* L.).
- Evaluate the growth response of White lupin to single and co-inoculation with the selected rhizobial and phosphate solubilizing rhizospheric bacterial (PSRB) isolates under greenhouse and field conditions.

Chapter 2 Phenotypic, Symbiotic and Plant Growth Promoting Characteristics of Rhizobia Isolated from Root Nodules of White Lupin (*Lupinus albus* L.) from North-western Ethiopia

Abstract

White lupin (*Lupinus albus* L.) is a legume with great agronomic potential as it can maintain soil fertility through symbiotic fixation of atmospheric nitrogen in association with soil bacteria of the genus *Rhizobium* and *Bradyrhizobium*.This study was undertaken with the aim of phenotypic, PGP and symbiotic characterization of rhizobia collected from root nodules of white lupin grown on soils of North-western Ethiopia. A total of 100 bacteria were isolated by using standard procedures from the root nodules of well grown White lupin plants from North-western parts of the country, of which 39 of them were confirmed as root nodule rhizobia based on presumptive and authentication tests. Rhizobial isolates showed wide variations in their colony morphology (62 % large white mucoid, 23 % small white mucoid and 15 % yellow large mucoid) with doubling times ranging from 2.0 – 6.5 h. On the basis of acid/base reaction, 29 (74 %) of the isolates were fast growing (acid producers), while 10 (26 %) of them were slow growing (base producers). Moreover, the rhizobial isolates showed relative variations in the utilization of carbon and nitrogen source substrates, tolerance to acidic and alkaline pH, high salt, high temperature, antibiotics and metal toxicity. With regard to relative symbiotic effectiveness (RSE), 9 (23 %) were found to be highly effective (RSE $\geq$ 80 %), 26 (67 %) effective (RSE = 50 $\leq$ x < 80 %) and 4 (10 %) lowly effective (RSE = 35 $\geq$ x < 50) under greenhouse conditions. Furthermore, the rhizobial isolates showed multiple plant growth promoting features, such as production of IAA (82 %), siderophores (51 %), HCN (10 %), tricalcium phosphate solubilization (69 %) and antagonistic activity against *Fusarium oxysporum* (3 %). In general, the present study indicated the presence of highly efficient nitrogen fixing White lupin nodulating rhizobia with diverse morphological, physiological, symbiotic and PGP characteristics in Ethiopian soils and hence, it is recommended to use rhizobial inoculants in the production of White lupin upon further testing under field conditions.

Key words: Nodulation, PGP, Rhizobia, RSE

2.1. Introduction

Rhizobia are groups of bacteria that form nodules with most leguminous plants to fix nitrogen in a process known as Biological Nitrogen Fixation (BNF). The amount of biologically fixed nitrogen is 1.72 x 10^8 tons per year of which more than 70% is contributed by the endosymbiotic association of these bacteria with leguminous plants (Coyne, 1999). Apart from biological nitrogen fixation, rhizobia are endowed with various plant growth promoting properties (PGP) (Alkani *et al*, 2006, Nithyakalyani *et al*., 2016). Pongsip (2012) showed that *Rhizobia* are the most important groups of plant growth promoting rhizobacteria that provide multiple growth promotion advantages through direct and indirect mechanisms, including production of phytohormones, siderophores, HCN, hydrolytic enzymes and solubilization of insoluble phosphates.

The most effective nitrogen fixation is achieved by selectively efficient rhizobial strains, which can be native to the soil or introduced in the form of commercial inoculants. Inoculation of legumes with effective rhizobia has been found to improve grain yields of leguminous crops (Zengenia *et al.,* 2006; Nkwiine and Rwakaira-Silver, 2007).

Selection of effective rhizobia with competitiveness against different environmental stresses such as pH, NaCl, inherent antibiotic resistance (IAR) in *in vitro* is essential for production of inoculants needed for sustainable agricultural systems. Similarly, Correa and Barneix (1997) suggested that if isolates of rhizobia show better symbiotic performance under laboratory conditions and are superior in competitiveness under natural ecological conditions, they could become useful inoculants under field conditions. Thus, screening of effective rhizobia with stress tolerance and additional plant growth promoting properties in the form of commercial inoculants has been found to improve grain yield of leguminous crops (Zengenia *et al.,* 2006; Nkwiine and Rwakaira-Silver, 2007).

White lupin (*Lupinus albus* L.) is one of the traditional pulse crops mostly produced and consumed by smallholder farmers and is widely used as a break crop to maintain soil fertility in North western parts of Ethiopia (Yehiyis Likawunt, 2010., Zerihun Nigusie, 2012), because of its prolific nitrogen fixation (Jansen, 2006).

Lupin can potentially fix and accumulate a total of 145 to 208 kg/ha nitrogen per year into usable forms for itself and the companion or succeeding crop (Jansen, 2006). It is nodulated by both fast and slow-growing rhizobia (Miller and Pepper, 1988; Kuykendall *et al*., 1991; Bottomley *et al*., 1994; Barrera *et al*., 1997). Nevertheless slow-growing rhizobia are more frequently isolated from this legume. Moreover, root nodulating endophytes were also isolated from the nodules of white lupin. According to the rRNA gene sequences, these endophytes were subsequently identified as members of the genus *Ochrobactrum* within the subclass of *Proteobacteria*. This genus belongs to the family Brucellaceae with a single species, *Ochrobactrum anthropi* (Holmes *et al*., 1988). The genus *Ochrobactrum* also contains the species *lupini* and *cytisi* (Trujillo *et al*., 2005, Zurdo-Pineiro *et al*., 2007).

For many years now, extensive studies were undertaken on diversity, symbiotic effectiveness and PGP properties of many cool season legumes in Ethiopia (Mulisa Jida and Fassil Assefa, 2012). However, despite the agronomic, nutritional and ecological importance of White lupin (*Lupinus albus* L.), little attention was given to this crop with respect to its significance in soil fertility and symbionts. Therefore, this study was undertaken with the aim of characterization of the phenotypic, PGP and symbiotic properties of rhizobia collected from root nodules of white lupin grown on soils of North-western Ethiopia.

2.2. Materials and Methods

2.2.1. Sample Collection Sites

The study was conducted in three major White lupin producing Administrative zones of Amhara (West Gojam, Awi, and South Gonder) and Benishangul-gumuz National Regional states in eight districts of Northwestern Ethiopia (Fig. 2.1) from September - October, 2013.

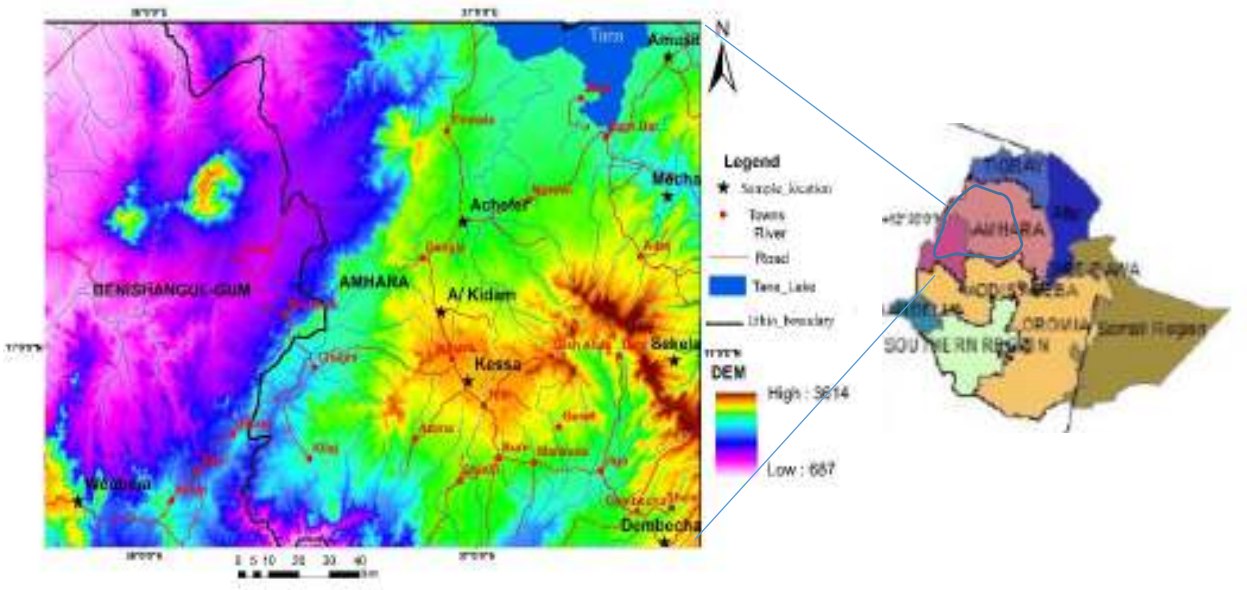


Figure1.1 White Lupin Nodule and Soil Sample Collection Sites

2.2.2. Collection of Nodules from the Roots of White Lupin (*Lupinus albus* L.)

Five to six healthy plants were randomly selected before flowering from each sampling site (field) and uprooted carefully from which 10 pink nodules were sub-sampled and collected in closed vials with silica gel (desiccant) (Somasegaran and Hoben, 1994). They were collected from four Zones with altitude ranging from 1926 – 2636 masl and soil pH from moderately acidic (pH 5.72) to slightly alkaline (pH 7.10) (Table 2.1).Then, the samples were brought immediately to the Applied Microbiology Laboratory of AAU and stored at 4 ^{0}C for the isolation of rhizobia.

Table2. 1 Test isolates, isolation sites, altitude and soil pH of the study area

Isolates	Isolation sites		Altitude (masl)	Location		Soil pH
	Zones	District		N	E	
LUR1	South Gonder	Amusit	1943	11° 47' 64"	37° 34'04"	5.95
LUR2		"	1958	11° 47' 63"	37° 34'04"	6.43
LUR5		"	1934	11° 47' 72"	37° 34'13"	5.72
LUR6		"	1926	11° 47' 79"	37° 34'13"	6.64
LUR8		"	1939	11° 47' 77"	37° 34'10"	6.82
LUR12	West Gojam	Mecha	1989	11° 24' 89"	37° 34'10"	6.82
LUR20		"	1988	11° 24' 91"	37° 34'10"	6.93
LuR21		"	1985	11° 24' 89"	37° 34'10"	7.10
LUR22		"	2002	11° 24' 92"	37° 34'10"	6.82
LUR24		Achefer	1983	11° 21' 04"	36° 56'69"	6.72
LUR25		"	1986	11° 21' 01"	36° 56'80"	6.89
LUR26		"	1986	11° 20' 99"	36° 56'77"	5.97
LUR27		"	1980	11° 20' 97"	36° 56'10"	6.68
LUR28		"	1983	11° 20' 90"	36° 56'90"	6.67
LUR29		"	1981	11° 21' 07"	36° 56'73"	7.03
LUR30		Sekela	2582	10° 58' 40"	37° 34'89"	6.87
LUR32		"	2550	10° 58' 38"	37° 34'87"	6.54
LUR36		"	2561	10° 58' 52"	37° 09'89"	6.62
LUR37		"	2560	10° 58' 49"	37° 09'90"	6.67
LUR38		"	2582	10° 58' 53"	37° 09'90"	6.48
LUR42		"	2587	10° 58' 51"	37° 09'87"	6.72
LUR43		"	2570	10° 58' 37"	37° 09'86"	6.97
LUR46		Dembecha	2394	10° 29' 01"	37° 33'53"	6.90
LUR47		"	2397	10° 29' 01"	37° 33'52"	7.01
LUR48		"	2391	10° 29' 01"	37° 33'54"	7.02
LUR54		"	2396	10° 29' 99"	37° 33'54"	7.06
LUR56		"	2395	10° 29' 99"	37° 33'55"	7.04
LUR57		"	2390	10° 29' 97"	37° 33'54"	6.91
LUR58	Awi	Addis Kidam	2419	11° 05' 84"	36° 52'72"	7.04
LUR62		"	2417	11° 05' 85"	36° 52'88"	7.10
LUR67		"	2416	11° 05' 87"	36° 52'87"	7.06
LUR80		"	2422	11° 05' 86"	36° 52'89"	6.93
LUR84		Kessa	2501	10° 54' 15"	36° 58'50"	7.04
LUR85		"	2508	10° 54' 26"	36° 58'16"	6.87
LUR87		"	2512	10° 54' 67"	36° 58'14"	7.09
LUR90	Benishangul gimuz	Wenbera	2060	11° 38' 82"	35° 47' 64"	6.84
LUR91		"	2540	10° 34' 60"	35° 47' 60"	7.01
LUR93		"	2500	11° 38' 82"	35° 47' 67"	6.96
LUR96		"	2636	10° 59' 07"	35° 47'62"	7.02

LUR = Lupin nodulating rhizobia, N = north, E = east, masl = meter above sea level

2.2.3. Isolation and Purification of Rhizobia from Nodules

The collected intact and undamaged white lupin root nodules were first washed with tap water and then surface-sterilized with 95% ethanol for 10 sec and transferred to a 3% solution of sodium hypochlorite (NaOCl) for 3 min. Then, the nodules were immediately washed 5-6 times with sterile distilled water to completely rinse off and remove the surface sterilizing chemicals. The nodules were crushed with alcohol flamed sterile glass rod in a drop of normal saline solution (0.85% NaCl) under aseptic conditions as indicated in Somasegaran and Hoben (1994). Loopful of crushed nodule suspensions were streaked on Yeast Extract Mannitol agar (YEMA) plate and incubated at 28±2 ^{0}C for 3-7 days (Vincent, 1970). Distinct and well isolated colonies were selected and purified by repeated streaking on YEM agar for purity. The pure isolates were preserved at -80 ^{0}C in YEM broth containing 50 % glycerol for future use. The isolates were designated as LUR (Lupin Rhizobia) with numbers.

2.2.4. Confirmatory Tests

Different presumptive tests such as growth on YEMA-Cr (yeast extract mannitol with Congo red), and PGA (Peptone glucose agar) were performed to differentiate rhizobial isolates from other non-rhizobial bacteria (Somasegaran and Hoben, 1994). A loopful of bacterial broth was streaked on the respective media and incubated at 28 + 2 ^{0}C for 3-7 days. Gram staining was carried out by streaking a loopful of pure culture on YEMA medium and stained as per the standard Gram's procedure (Somasegaran and Hoben, 1994). They were also inoculated on YEMA medium containing 1% (w/v) BTB (Bromothymol blue) and incubated as before according to Jordan (1984) for their acid/base production test. Isolates that changed the medium to yellow were grouped as acid producers (fast growing) whereas those that turned the medium to blue were considered as base producers (slow growing).

2.2.5. Cultural and growth characteristics of the isolates

Rhizobial isolates maintained on the YEMA slant were streaked on YEMA plates and incubated at 28 $\pm$ 2 ^{0}C for 3-7 days. Then, the cultural characteristics were evaluated on the basis of colony size, color, and texture according to Ahmed (1984).

Mean generation time of the isolates was studied by transferring a single colony into 10ml Yeast Extract Mannitol Broth (YEMB) for 24 hrs, from which 1ml was inoculated into 100ml of YEM broth into 250 ml Erlenmeyer flasks. They were incubated on orbital shaker at 150 rpm for 3-7 days at room temperature. One ml of cell suspensions was taken every 6 hrs to determine growth (optical density) at OD540) using spectrophotometer (Jenway, 6405 Uv/vis spectrophotometer). Samples were taken simultaneously from appropriate dilution (10^{-1} to 10^{-10}) from which 0.1ml of each diluent was dispensed on to sterilized YEMA plates to determine the colony forming units (Somasegaran and Hoben, 1994). Finally, the generation time (g) was calculated from the logarithmic phase according to White (1995).

2.2.6. Physiological Characteristics of the Isolates

The isolates were investigated for their ability to utilize different carbon and nitrogen sources, inherent antibiotic resistance (IAR) and heavy metals resistance, tolerance to salt, temperature and pH. Except, the carbon test that was carried out by using kits, all other tests were done by inoculating the isolates on YEMA plates containing the test chemicals and substrates and adjusting to different pH and temperatures. All tests were incubated at 30^{o}C and for 5-7 days, unless stated otherwise.

2.2.6.1. Salt, Temperature, and pH Tolerance Tests

The ability of isolates to grow on acidic or basic media was determined on YEM agar plates adjusted to pH 4, 5, 8 and 9.0 by using 1 N HCl or NaOH. They were also inoculated to YEMA solid medium supplemented with NaCl at concentrations of 1- 8% (w/v) and incubated at 5, 15, 30, 37, 40 and 45°C to evaluate their salt and temperature tolerance, respectively (Hungria *et al*., 2001). Presence of growth at each salt concentration, temperature and pH was designated as positive (+) and negative (-) for the presence and absence of growth, respectively.

2.2.6.2. Intrinsic Antibiotic Resistance

The isolates were tested for antibiotic sensitivity by Kirby Bauer disc diffusion method on YEMA medium (Bauer *et al*., 1966). Eight antibiotic disks; Penicillin (10 µg), Chloramphenicol (30 µg), Kanamycin (30 µg), Tetracycline (25 µg), Ampicillin (10 µg), Streptomycin (10 µg), Gentamycin (30 µg), and Amikacin (10 µg) were used for examining the inherent antibiotic resistance (IAR) of Rhizobium isolates. Then, 0.1 ml of the log phase culture of each isolate was inoculated over the entire surface of YEMA medium. Antibiotic disks were placed equidistantly at the center of the plates and were incubated at 30 ^{0}C for 3-7 days. Resistance to antibiotic was detected by inhibition zone formed around the disks.

2.2.6.3. Effect of Heavy Metals on the Growth of Rhizobial Isolates

All the isolates were tested for their sensitivity to heavy metals according to Cervates *et al*., (1986). The isolates were transferred into freshly prepared YEMA plates amended with heavy metal salts i.e., $K_2Cr_2O_7$ (50 µg), $CoCl_2$ (10 µg), $CuCl_2.2H_2O$ (10 µg), $HgCl_2$ (5 µg), $MnCl_2$ (50, and 75 µg), $NiCl_2$ (10 and 50 µg), and $ZnCl_2$ (50 µg). They were incubated at 30 ^{0}C for 3-7 days.

2.2.7. Utilization of Carbon and Nitrogen Sources

The ability of isolates to utilize different carbon sources was tested on HiCarboTM kits (Part A, B, and C) of HIMEDIA that contain 34 different carbohydrates with a control. Kit part A contained 12 carbohydrates (Lactose, Xylose, Maltose, Fructose, Dextrose, Galactose, Raffinose, Trehalose, Melibiose, Sucrose, L-Arabinose and Mannose), Kit part B also contained 12 carbohydrates (Inulin, Sodium Gluconate, Glycerol, Salicin, Dulcitol, Inositol, Sorbinol, Mannitol, Adonitol, Arabitol, Erythritol and α-Methyl-D-glucoside), whereas Kit Part C contained 10 carbohydrates (Rhamnose, Cellobiose, Melezitose, α-Methyl-D-Mannoside, Xylitol, ONPG, Esculin, D-Arabinose, Malonate and Sorbose).

The test was performed according to the Manufacturer's protocol. Pure cultures were grown in YEM broth medium at 30^{o}C until inoculum turbidity reached > 0.5 OD at 620nm. Then, each well of the kit was inoculated with 50 µl of log phase of the rhizobial suspension and incubated at 30 ^{0}C for 24 hours. Finally, the result was recorded as positive, and negative according to result interpretation chart provided by the Manufacturer.

The rhizobial isolates were inoculated into a basal medium containing K_2HPO_4, 1g; $FeCl_3.6H_2O$, 0.01g; $MgSO_4.7H_2O$, 0.2g; $CaCl_2$, 0.1g; Mannitol, 1g; and 15 g of agar containing different amino acids to determine their ability to assimilate different nitrogen sources (Amarger *et al.,*1997). The amino acids were; Glycine, Asparagine, L- Glutamine, Phenylalanine, L- Tryptophan, Methionine, L-Arginine, L-Lysine, Leucine, and L-Proline and incorporated to the basal medium at a concentration of 0.5g/liter. The plates were incubated at 30 ^{0}C for 3-7 days and presence as well as absence of growth was recorded as positive and negative in that order.

2.2.8. Plant Growth Promoting Properties of the Rhizobial Isolates

2.2.8.1. Phosphorus solubilization

The potential of rhizobial isolates for phosphate solubilization was screened by using the procedure described by Pikovskaya (1948). 10 µl of each rhizobial isolate was streaked on Pikovskaya medium (glucose, 10g., $Ca_3(PO_4)_2$, 5g., $(NH_3)_2SO_4$, 0.5g., NaCl, 0.2g., $MgSO_4.7H_2O$, 0.1g., KCl, 0.2g., $Fe_2(SO_4)_3.7H_2O$., $MnSO_4$, 0.002g., yeast extract, 0.5g., agar, 20g., Distilled water, 1000ml, pH 7.0) and incubated at 30 ^{0}C for 3-7 days. The isolates that showed clear zone around their colonies were considered as P solubilizers and their solubilization index (SI) was calculated as the ratio of colony and halo zone diameter to colony diameter (Edi–Premono *et al*., 1996).

2.2.8.2. IAA production

Production of Indole acetic acid (IAA) was studied on YEM broth medium supplemented with 3mg/mL of L-tryptophan and incubated at 28 $\pm$ 2 ^{0}C for 3- 7 days according to the method by Gordon and Weber (1951). Two ml of the bacterial cultures was then transferred into sterile Eppendorf tubes and centrifuged at 10,000 rpm for 10 min, from which1ml of supernatant was mixed with a drop of Ortophosphoric acid and 2ml of Salkowsky reagent (2% of 0.5 M $FeCl_3$ in 35% perchloric acid). The solution was allowed to settle for 25 min and development of pink color considered positive for IAA production. The intensity of the color formation was determined using a spectrophotometer at 535 nm and quantified based on the standard curve computed over a known concentration of IAA at concentrations of 0, 5, 10, 20, 50, and 100 µg ml^{-1}.

2.2.8.3. Siderophore production test

The rhizobial isolates were screened for the production of Siderophore by chrome azurol S (CAS) assay method as described by Schwyn and Neilands (1987). The blue agar CAS medium was prepared by adding 850 mL of autoclaved MM9 salt medium (added with 32.24 g piperazine- N,

N'-bis 2- ethanesulfonic acid (PIPES) at pH 6), 100 mL of blue dye, 30 mL of filter sterilized 10 % Casaminoacid solution and 10 mL of 20 % glucose solution. Then, the blue agar medium was aseptically poured on to sterile plates and allowed to solidify. All the bacterial isolates obtained were inoculated into the CAS medium and incubated at 28 ^{0}C for 24 h. Development of yellowish orange halo around the colonies was considered as the indication for the production of siderophores.

2.2.8.4. Antagonistic activity

Rhizobial isolates were screened for antagonistic activity against *Fusarium oxysporum* by using dual plate technique (Ganesan and Gnanamanickam, 1987). 10 µL of the inoculum of each rhizobial isolates was equidistantly placed near to the edge of YEMA plates amended with 0.5 % (w/v) of sucrose and incubated for 24hrs at 30 ^{0}C. A 3-4 mm agar disc from PDA cultures of the fungal pathogens was also be placed at the center of the YEMA plate for each bacterial isolate and incubated at 28 ± 2 °C for 5-7 days. The antagonistic activity of each isolate was scored as low (+), moderate (++) and high (+++) depending on the zone of inhibition.

2.2.8.5. Hydrogen Cyanide (HCN) Production

To determine the production of HCN, rhizobial isolates were streaked onto YEMA medium plates supplemented with glycine (4.4 g/L). The plates containing the cultures were inverted after a piece of Whatman number 1 filter paper soaked with 0.5% picric acid and 2% sodium carbonate was placed on the upper lid. Each Petri plate was wrapped with parafilm and incubated at 30 ^{0}C for 7 days. Alteration of filter paper color from yellow to red was indicator of HCN production (Dye, 1962).

2.2.9. Authentication and Screening Symbiotic Effectiveness of Isolates on Sand Culture

Thirty-nine rhizobial isolates that qualified the confirmatory test were further tested for authentication and evaluating their symbiotic effectiveness in plastic pots using sterilized sand in the greenhouse (Somasegaran and Hoben, 1994). Acid washed sand and repeatedly washed by tap water until the pH is 7.2 was filled in 3-kg plastic pots. White lupin seeds (bitter variety) of uniform size and color from West Gojam were surface sterilized using 3% H_2O_2 for 10 minutes in petri plates and then washed with several changes of distilled water (Vincent, 1970). The surface sterilized seeds were germinated in 1% water agar petridishes and five germinated seeds were transferred into the pots that were later thinned down into 3 per pot. After a week of planting, each plant was inoculated with 10^9 cells/ml of inoculum.

The pots were arranged in randomized complete block design (RCBD) with three replications in the greenhouse with 12 hours photoperiod. Treatments without nitrogen and inoculants were included as negative control, while uninoculated pots supplied with 0.05% KNO_3 (w/v) nitrogen source served as positive control (Somasegaran and Hoben, 1994). Furthermore, both the control and experimental plants were supplied with distilled water every two days, and quarter strength of N-free nutrient solution once a week.

After 90 days of planting, each plant was carefully uprooted to measure nodule number (NN), nodule dry weight (NDW) and shoot dry weight (SDW) of the plants. Furthermore, the relative symbiotic effectiveness (RE) of the isolates was computed according to Gibson (1987) by using the formula (inoculated plant shoots dry matter/ N-fertilized plant shoots dry matter x 100).

2.2.10. Data Analysis

All the symbiotic experiments were carried out in triplicate and analyzed by one-way analysis of variance (ANOVA) and the treatment means were compared relative to controls following Duncan's test (DT) by using SPSS (Ver.20). The relation between different parameters was also evaluated by using Pearson correlation coefficient at $p \leq 0.05$.

2.3. Results and Discussion

2.3.1. Isolation and confirmatory tests of the root nodule bacteria

Pinkish nodules were collected from the roots of 80 well grown white lupin plants from four major white lupin producing administrative zones of Amhara (West Gojam, Awi, and South Gonder), and Benishangul-gumuz National Regional States of Northwestern Ethiopia.

A total of 100 root nodule bacteria were screened from the root nodules of white lupin (*Lupinus albus* L.) by using Yeast extract agar (YEMA) medium, of which 39 isolates (39%) were confirmed as root nodule rhizobia based on presumptive and authentication tests, while the rest 61 (61 %) of the test isolates were rejected as they failed to fulfil the characteristics of rhizobia (data not shown). The isolates were characterized on the basis of their cultural and mean generation time (MGT) after they were incubated on yeast extract mannitol agar (YEMA) plate at $28 \pm 2\ ^{0}C$ for 3-7 days. Accordingly, most isolates, 25 (64%) were large, mucoid and white in appearance, and others which constituted 10 (26%) of the tested isolates formed small and white mucoid colonies, whereas the rest 4(10 %) isolates showed yellow colonies with large and mucoid appearances on YEMA plate (Table 2.2).

Table2. 2 Cultural characteristics and Mean Generation Time (MGT) of the Isolates

Isolates	Isolation sites	Colony characteristics	Colony Size (mm)	Growth on YEMA-BTB	GMT (Hrs.)	Taxonomic Groups
LUR1	Amu-SG	SMW	1.6	Blue	6.3	Slow growing
LUR2	Amu-SG	LMW	3.2	Deep Yellow	2.1	Fast growing
LUR5	Amu-SG	LMY	3.6	Deep yellow	3.0	Fast growing
LUR6	Amu-SG	SMW	1.2	Blue	5.4	Slow growing
LUR8	Amu-SG	SMW	0.9	Blue	4.6	Slow growing
LUR12	Mec-WG	LMY	3.8	Moderate Yellow	2.3	Fast growing
LUR20	Mec-WG	SMW	1.4	Blue	6.5	Slow growing
LuR21	Mec-WG	LMW	2.7	Deep Yellow	2.2	Fast growing
LUR22	Mec-WG	LMW	3.4	Deep Yellow	2.4	Fast growing
LUR24	Ach-WG	LMW	2.5	Deep Yellow	2.7	Fast growing
LUR25	Ach-WG	LMW	2.7	Moderate yellow	3.0	Fast growing
LUR26	Ach-WG	LMW	2.9	Moderate Yellow	2.4	Fast growing
LUR27	Ach-WG	LMW	2.8	Deep Yellow	2.4	Fast growing
LUR28	Ach-WG	LMW	2.7	Moderate yellow	2.8	Fast growing
LUR29	Ach-WG	LMW	3.4	Deep Yellow	2.0	Fast growing
LUR30	Sek-WG	LMW	2.7	Deep Yellow	3.0	Fast growing
LUR32	Sek-WG	LMW	2.6	Moderate Yellow	2.0	Fast growing
LUR36	Sek-WG	LMW	2.8	Moderate Yellow	2.1	Fast growing
LUR37	Sek-WG	LMW	3.1	Moderate yellow	2.9	Fast growing
LUR38	Sek-WG	SMW	1.3	Blue	4.9	Slow growing
LUR42	Sek-WG	LMW	2.6	Deep Yellow	2.3	Fast growing
LUR43	Sek-WG	SMW	1.5	Blue	3.6	Slow growing
LUR46	Dem-WG	LMW	3.5	Deep Yellow	2.4	Fast growing
LUR47	Dem-WG	LMW	2.6	Deep Yellow	2.4	Fast growing
LUR48	Dem-WG	LMW	3.3	Moderate Yellow	3.0	Fast growing
LUR54	Dem-WG	SMW	0.8	blue	4.3	Slow growing
LUR56	Dem-WG	LMY	2.7	Moderate Yellow	2.9	Fast growing
LUR57	Dem-WG	LMY	3.1	Moderate Yellow	3.0	Fast growing
LUR58	AdK-AW	LMW	2.8	Deep yellow	3.0	Fast growing
LUR62	AdK-AW	LMW	3.3	Moderate yellow	3.0	Fast growing
LUR67	AdK-AW	LMW	2.9	Deep Yellow	2.4	Fast growing
LUR80	AdK-AW	LMW	2.4	Deep yellow	3.8	Fast growing
LUR84	Kes-AW	LMW	3.3	Deep Yellow	2.6	Fast growing
LUR85	Kes-AW	LMW	2.8	Moderate Yellow	3.0	Fast growing
LUR87	Kes-AW	SMW	1.2	Blue	5.6	Slow growing
LUR90	Wen-BG	LMW	3.2	Deep Yellow	2.0	Fast growing
LUR91	Wen-BG	SMW	1.8	Blue	5.4	Slow growing
LUR93	Wen-BG	SMW	0.5	Blue	6.5	Slow growing
LUR96	Wen.BG	LMW	3.7	Deep Yellow	3.0	Fast growing

LUR =Lupin nodulating rhizobia, Amu-SG= Amusit-South Gonder, Mec-WG = Mecha - West Gojam, Ach-WG = Achefer-West Gojam, Sek-WG = Sekela – West Gojam, Dem-WG = Dembecha – West Gojam, Adk-AW = Addis Kidam-Awi, Kes-AW = Kessa- Awi, Wen-BG = Wenbera-Benishangulgumuz, SMW =small mucoid white, LMW =Large mucoid white, LMY = Large mucoid yellow.

The isolates displayed colony diameter between 0.5-3.8mm within 3-7 days of incubation in which the majority 29(74 %) of them formed large colonies on YEMA plates. However, 10 (26 %) of them showed < 2mm colony diameter and grouped as slow growers (Table 2.2). The findings of Martha *et al*. (2005) showed that the colonies of two fast growing White lupin rhizobial isolates (LUP21^{T} and LUP23) were white mucoid on YEMA medium. EI Hilali *et al.* (2016) indicated that all the tested rhizobial isolates of lupin (159 isolates) formed circular gummy and creamy colonies of 1 to 3mm in diameter within 2-3 days of incubation on YEMA medium. Generally, white mucoid and semitransparent colony texture on YEMA medium are common characteristics of rhizobial isolates of legumes (Young, 2001). But, our study showed 4(10%) of the isolates (LUR5, LUR12, LUR56 and LUR57) formed large mucoid yellow colonies.

Regarding mean generation time, isolates showed different growth rate with doubling times ranging from 2.0 - 6.5 h, from which 74% (29) of the isolates showed shorter doubling times ranging 2.0 - 3.0 h and 26% (10) of the isolates displayed a relatively longer doubling times of 3.6-6.5 h and were categorized as slow growers. However, Martha *et al.* (2005) indicated that the mean generation time of two fast growing lupin nodulating rhizobia ranged from 3-4 hours. Moreover, EI Hilali *et al.* (2016), showed that 10% of the rhizobial isolates were very fast growing with a growth rate < 1h and 73 % of the rhizobial strains showed doubling time between 1 and 2 h. The same authors explained that two rhizobial strains of lupin showed a doubling time greater than 3h and the strains were more related to fast growing.

Most of the isolates 29 (74%) changed the color of the YEMA_BTB medium into yellow indicating acid production by rhizobial isolates whereas the remaining turned the medium into blue showing that they were alkali producers (Table 2.2). The characteristics of isolates on acid/alkali production and fast/slow growth fit into the categorization of the isolates into fast and slow growing. Puleko and Zarinik (2010) also reported that lupins were nodulated by both fast and slow growing strains of rhizobia in which 77.6 % of them were acid producers while 22.4% were alkalizing isolates.

2.3.3. Physiological Characteristics of the Isolates

2.3.3.1. C-sources utilization of the isolates

White lupin rhizobial isolates were diverse in the ability to catabolize carbon substrates (Table 2.3). All isolates metabolized the majority of the tested carbohydrates, only fewer isolates 6(15%) utilized Alpha-Methyl-D- Mannoside and Sorbose as carbon sources; whereas less than 50% of the isolates metabolized Dulcitol, Sodium Gluconate and Malonate (Table 2.3). Five fast growing rhizobial isolates (LUR27, LUR57, LUR58, LUR84 and LUR90) utilized >90% of the tested carbohydrates, and only two isolates (LRI26 and LRI28) utilized comparatively less number of carbohydrates (65 %) of the tested C-sources. However, the slow growing rhizobial isolates (LUR1, LUR6, LUR8, LUR20, LUR38, LUR43, LUR54, LUR87, LUR91 and LUR93) utilized >75 % of the tested carbohydrates.

In general, White lupin rhizobial isolates were versatile in the utilization of different carbon substrates which may implicate their high competitiveness or saprophytic capacity in the natural environments. Miller and Pepper (1988) showed that fast growing rhizobial strains of White lupin isolated from the Sonoran Desert, were unable to utilize Fructose, and Arabinose whereas Galactose was only consumed by fast growing strains as a carbon source.

However, this study showed that these carbohydrates were utilized almost by fast and slow growing rhizobial isolates. Similarly, EI Hilali *et al.* (2016) indicated that lupin nodulating rhizobial strains revealed good growth on carbohydrates such as L-arabinose, Fructose, Ribose and Mannitol and regarding the assimilation of Arabinose, Chakrabati *et al*. (1981) explained that only lupin nodulating strains were able to assimilate among all the *Bradyrhizobium* they have been tested. Moreover, Martha *et al*. (2005) reported that white lupin nodulating rhizobial strains showed assimilation of multiple carbon substrates.

2.3.3.2. N-sources utilization tests

Rhizobial isolates were able to utilize different amino acid substrates as a source of nitrogen (Table 2.3). Accordingly, the amino acids: Asparagine, L-Lysine, and L-Glutamine were utilized by all isolates as N-source substrates. Most isolates (34 (87%)) showed growth on the medium amended with L-Proline, followed by Leucine and L-Arginine (32 (82%) isolates), Methionine (30 (77 %) isolates) and Phenylalanine (21 (54 %) isolates), but none of them catabolized Glycine. The result indicated that only 13 (33 %) of the isolates catabolized more than 80 % of the tested N-source substrates from which 8 (21 %) of them were slow growing, whereas 26 (67%) of the isolates utilized 44 – 78% of the tested amino acids (Table 2.2). However, Miller and Pepper (1988) reported that the fast growing rhizobial strains of lupin grew better than slow growing strains when asparagine was in the growth medium and glutamine was not generally a satisfactory N-source for all types of rhizobial strains. Mulisa Jida and Fassil Assefa (2011) showed that more than 80 % of the rhizobial isolates obtained from Lentil metabolized Methionine, L-Tyrosine, Tyrosine, Leucine, Asparagine, Arginine glutamine and L-Lysine as a N-sources and they reported that Phenylalanine and Glycine were not utilized as N-source by all isolates.

Table2. 3 In *vitro* Ecological and Heterotrophic competence of White Lupin (*Lupinus albus* L.) Rhizobial Isolates

Isolates	Isolation sites	C and N-sources Utilized (%)		HMR	IAR pattern	ST (%)	TT (^{0}C)	pH tolerance
		C	N					
LUR1	Amu-SG	79.4	89	Mn, Zn, Cu,	Ch, Am, P	5	10-45	4-8
LUR2	Amu-SG	76.5	78	Mn, Zn, Ni, Cu	Am, p	4	10-35	5-8
LUR5	Amu-SG	79.4	78	Mn, Zn	Ch, Am, P	5	5-35	6-8
LUR6	Amu-SG	85.5	89	Mn, Zn	P	5	10-35	6-8
LUR8	Amu-SG	79.4	78	Mn, Zn, Ni	Ch, Gn, P	4	10-40	6-8
LUR12	Mec-WG	82.4	89	Mn, Zn, Cu	Tet, Ka, Gn, P	5	10-45	6-9
LUR20	Mec-WG	79.4	89	Mn, Zn, Cr,	Ch, Am, P	5	5-35	5-8
LUR21	Mec-WG	79.4	78	Mn, Zn	P	3	10-35	5-8
LUR22	Mec-WG	82.4	89	-	P	3	10-35	6-9
LUR24	Ach-WG	76.5	55	Mn, Zn, Cu	P	5	10-35	5-8
LUR25	Ach-WG	73.5	78	Mn, Zn, Cr, Ni	Ch, P	5	10-35	5-8
LUR26	Ach-WG	64.7	55	Mn, Zn, Cr, Cu	P	1	10-35	6-8
LUR27	Ach-WG	94.1	78	Mn, Zn, Cr	Am, Ka, P	5	5-40	6-8
LUR28	Ach-WG	64.7	44	Mn, Zn, Ni, Cu	Ka, P	5	10-35	5-8
LUR29	Ach-WG	76.5	67	Mn, Zn, Cu	P	5	10-40	6-8
LUR30	Sek-WG	79.2	78	Mn, Zn, Ni	P	4	10-35	6-8
LUR32	Sek-WG	70.6	78	Mn, Zn, Ni, Cu	P	4	10-35	6-8
LUR36	Sek-WG	73.5	67	Mn, Zn, Cr,	-	3	10-35	6-8
LUR37	Sek-WG	82.4	67	Mn, Zn	P	4	10-35	6-8
LUR38	Sek-WG	85.3	89	-	P	4	10-35	6-8
LUR42	Sek-WG	85.3	67	Mn, Zn	Ch, Tet, Am, P	4	5-35	5-8
LUR43	Sek-WG	85.3	67	Mn, Zn	Tet, Am, P	4	10-40	5-8
LUR46	Dem-EG	73.5	78	Mn, Zn	Am, P	4	10-35	4-8
LUR47	Dem-EG	79.4	89	Mn, Zn, Cr, Cu	Ch, Tet, Am, P	5	10-35	6-8
LUR48	Dem-EG	85.3	89	Mn, Zn, Cr	P	3	10-35	4-8
LUR54	Dem-EG	79.4	89	Mn, Zn, Cr	P	4	10-35	5-8
LUR56	Dem-EG	79.4	67	Mn, Zn	Ka, Am, P	3	10-35	6-8
LUR57	Dem-EG	79.4	89	Mn, Zn, Cu	P	3	5-40	6-8
LUR58	AdK-AW	91.1	78	Mn, Zn, Cu	-	5	10-35	5-8
LUR62	AdK-AW	94.1	78	Mn, Zn, Cr	Ch, Tet, Ka, P	5	10-40	6-9
LUR67	AdK-AW	79.4	78	Mn, Zn, Cr	P	2	5-40	6-8
LUR80	AdK-AW	85.3	78	Mn, Zn, Cr	Ch, AK, P	6	10-35	6-9
LUR84	Kes-AW	94.1	78	Mn, Zn, Cu	Ch, Tet, Am, P	5	10-40	5-8
LUR85	Kes-AW	88.2	67	Mn, Zn, Ni, Cu	Ch, Ak, P	5	10-35	6-8
LUR87	Kes-AW	79.4	89	Mn, Zn, Ni, Cu	Ka, Am Ak, P	5	5-45	5-8
LUR90	Wen-BG	97.0	89	Mn, Zn, Cr, Cu	Ch, Tet, Am, P	6	10-45	6-9
LUR91	Wen-BG	82.4	67	Mn, Zn, Ni	P	3	10-35	6-8
LUR93	Wen-BG	76.5	89	-	Ch, P	4	10-35	6-8
LUR96	Wen.BG	70.6	44	Ni, Cu	Ch, P	1	5-40	5-8

HMR = Heavy metal resistance, IAR = intrinsic antibiotic resistance, ST `= Salt tolerance, TT = Temperature tolerance, Mn = Manganese, Zn = Zinc, Cu = copper, Ni = Nickel, Cr = Chromium, Ch = Chloramphenicol, Am = Ampicillin, P = Penicillin, Tet = Tetracycline, Ka = Kanamycin, Gn = Gentamycin, AK = Amikacin.

2.3.3.3. Salt, Temperature, and pH Tolerance Tests

Regarding salt tolerance, the rhizobial isolates showed a wide variation in their growth on the YEMA medium containing different concentrations of salt (NaCl) (Table 2.3). Accordingly, all rhizobial isolates were tolerant to 1 - 2% concentration of NaCl, but less than half of the isolates (41 %(16)) showed growth at the concentration of 5% of NaCl, and only two rhizobial isolates (LUR80 and LUR90) withstood salt concentration of 6%, while LUR26 and LUR96 were the least tolerant isolates as they grew on the medium containing salt concentration of 1 %.

In general, slow growing rhizobial isolates showed growth on the medium containing 3-5% salt, whereas fast growing isolates grew on 1-6 % salt containing growth medium. Raza *et al*. (2001) reported slow growing White lupin rhizobia (Bradyrhizobium strains) which were able to tolerate greater than 5% NaCl concentration. This study indicated that rhizobial isolates of White lupin have relatively a high tolerance to salinity tolerance compared to the rhizobial isolates of other legumes such as chickpea which were found to be tolerant to 1% NaCl concentration (Mulisa Jida and Fassil Assefa, 2011). Graham *et al.,* (1992) revealed as there is marked variation in salt tolerance among the Rhizobium and Bradyrhizobium strains obtained from different legumes.

All isolates showed better growth at the temperature ranges of 10 ^{0}C -35 ^{0}C., but fewer isolates were able to grow at the temperatures greater than 35 ^{0}C and the percentage of isolates reduced to 23 % at 40 ^{0}C and 10 % 45 ^{0}C. Hilali *et al*. (2016) indicated the presence of lupin rhizobial isolates that grew at temperatures between 4 ^{0}C and 42 ^{0}C.

Concerning pH tolerance, all the rhizobial isolates showed maximum growth at nearly neutral pH ranging from 6-8. The growth of the isolates decreased with increase in acidity or alkalinity of the growth medium. Accordingly, 33% (13) isolates (LUR2, LUR20, LUR21, LUR24, LUR25, LUR28, LUR42, LUR43, LUR54, LUR58, LUR84, LUR87 and LUR96) grew at pH range of 5.0 to 8.0, and 21%(8) of the isolates were able to grow at pH of 4.0, and pH of 9.0. With regard to this, Raza *et al.* (2001) indicated that the rhizobial strains isolated from Egyptian White lupin were tolerant to pH range from 4 to 10 and they explained such growth of the isolates at higher pH may be due to their isolation from nearly alkaline soils.

2.3.3.4. IAR Pattern of the Isolates

Regarding intrinsic resistance to antibiotics, almost all isolates showed resistance to 10 µg of Penicillin, except two isolates (LRI36 and LRI58), and some of the isolates (13 (33%)) were resistant to 10 µg of Ampicillin and 30 µg of Chloramphenicol (Table 2.3). They were also mildly resistant to 25 µg of Tetracycline and 30 µg of Kanamycin where 18% (7) and 15% (6) of the isolates grew on the media containing the two antibiotics, respectively. All the rhizobial isolates were sensitive to 10 µg of Streptomycin followed by 10 µg of Amikacin and 30 µg of Gentamycin, except isolates LUR80, LUR85 and LUR87 (Amikacin), and LUR8 and LUR25 (gentamycin). Generally, Streptomycin, Amikacin, Gentamicin, Kanamycin, Tetracycline, and Chloramphenicol were found to be the most growth inhibitor antibiotics for the tested rhizobial isolates of White lupin in that order (Table 2.3, Appendix table 3).

Likewise, El Hilali *et al*. (2016) showed that Tetracycline, Streptomycin and kanamycin, inhibitors of the plasmic membrane and protein synthesis, had the most drastic effect on the growth of rhizobial strains of lupin rhizobia. Similarly, Miller and Pepper (1988) revealed that these three antibiotics were inhibitors for certain fast growing rhizobial strains of lupin and according to Zeghari *et al*. (2000), Kanamycin was reported to be highly devastative for slow growing rhizobial species. Moreover, Raza *et al*. (2001) and Young and Chao (1998) reported that both fast and slow growing strains of rhizobia from different legumes showed diverse variation in resistance to different antibiotics.

2.3.3.5. Heavy Metals Resistance

Rhizobial isolates showed a wide difference in their resistance to the tested heavy metals. With the exception of four isolates (LUR22, LUR38, LUR87 and LUR93), all isolates grew on the growth medium containing the given concentrations of Mn and Zn. However, some isolates were tolerant to Cu (41%), Cr (31%), and Ni (26%), respectively (Table 2.3). Nine isolates namely, LUR12, LUR25, LUR26, LUR28, LUR32, LUR47, LUR85, LUR90, LUR96 were relatively resistant to more than half of the tested heavy metals. All isolates were sensitive to Co and Hg, similar to earlier report showing the negative effect of Cobalt on the growth of lupin symbionts (Riley and Dillworth, 1985). On the contrary, EI Hilali *et al*. (2016) reported lupin rhizobial isolates showed growth on the growth medium supplemented with Hg and Co.

2.3.4. Plant Growth Promoting Properties of the Rhizobial Isolates

White lupin nodulating rhizobial isolates showed interesting PGP characteristics, such as IAA production and inorganic phosphate solubilization. Accordingly, 32 (82%) of the isolates produced an average of 21.5 µg/ml IAA (Table 2.4). The highest amount of IAA (37.8 µg/ml) was produced by isolate LUR20, while the lowest amount (6.1 µg/ml) produced by LUR37. The average amount

of IAA produced by White lupin nodulating rhizobial isolates was lower than the mean IAA (33.7 µg/ml) produced by cowpea nodule bacterial isolates (Girmaye Kenena and Fassil Assefa, 2017) by 36 %, but the number of IAA producing White lupin nodulating rhizobial isolates was markedly higher than IAA producing Lentil rhizobia (Mulisa Jida and Fassil Assefa, 2011) and Cowpea (Girmaye Kenena and Fassil Assefa, 2017) nodulating rhizobial isolates by 45%. Among the IAA producers, 30 (77%) were fast growing while 2(5%) of them were from the slow growing groups indicating that fast growing rhizobial isolates of White lupin are good IAA producers than the slow growing ones. Alikhani and Yakhachai (2009) reported that large numbers of IAA producers were fast growing nodule bacterial species than the slow growing ones. However, Girmaye Kenena and Fassil Assefa, (2017) reported IAA production by cowpea nodule bacteria was irrespective of their growth properties as both slow and fast-growing isolates induced IAA. Previous research also reported IAA production as common features of several physiologically active legume nodulating rhizobia with variations on the type of host plants (Antoun *et al*., 1998, Gravel *et al*., 2007).

As indicated from table 2.4, 27 (69.2%) of the isolates were able to solubilize insoluble TCP on Pikovskaya medium with solubilization index ranging from 1.2 to 1.83 after 5 days of incubation. Larger solubilization index (SI) was recorded from the isolates LUR20 and LUR29 followed by the isolates LUR30, LUR62, LUR12 and LUR48 with solubilization index of 1.74, 1.73, 1.71 and 1.7, respectively. However, Dereje Haile *et al*, (2016) reported that rhizobial isolates of White lupin displayed solubilization index (SI) of 2-3 upon 4 days of incubation which was significantly higher than the present finding. In addition to N_2- fixing potential, several rhizobia improve legume phosphate nutrition by solubilizing insoluble phosphate due to their ability to release organic acids (Alikhani *et al*., 2006). Compared to the solubilization index (SI) of other rhizobial strains obtained from Lentil (1.13 – 1.18) (Mulisa Jida and Fassil Assefa, 2011), Chick pea (1.13 -1.3) (Mulisa Jida

and Fassil Assefa, 2012) and Soybean (1.3 – 1.5) (Diriba Temesgen and Fassil Assefa, 2017), white lupin rhizobial isolates showed relatively better solubilization index.

Regarding siderophore production, white lupin rhizobial isolates showed the capacity to sequester iron with iron chelating substance on CAS blue agar assay. Accordingly, 48% (14) of fast growing and 60% (6) of slow growing isolates were siderophore producers. Abd-alla (1999) reported that 33 % of Bradyrhizobium (Lupin) produced iron chelating siderophore on CAS agar medium. Waheed *et al*, (2014) showed that 57% of rhizobium strains isolated from Pea crop produced siderophores that can help plants in iron acquisition and protection against phytopathogenic microbes.

Concerning HCN production and antagonistic activity, only 4 (10 %) of rhizobial isolates (LUR8, LUR12, LUR48 and LUR85) showed HCN production and LUR85 was the only isolate that revealed antagonistic activity against the fungal pathogen in a dual culture assay. Likewise, Singh *et al*., (2014) reported only one rhizobial isolate (RASH6) from chick pea that showed HCN production. However, Manasa *et al*. (2017) reported 53 % of rhizobial isolates from crops, such as Ground nut, Maize, Black gram and Soy bean produced HCN and 20 % of them exhibited inhibition potential against fungal pathogens under *in vitro* conditions.

Table2. 4 Plant Growth Promoting Properties of White Lupin Rhizobial Isolates

Isolates	IAA (µg/ml)	PS	SI	Siderophore production	HCN production	Antagonistic activity
LUR1	-	+	1.6	+	-	-
LUR2	17.7±0.69^{ab}	-	-	+	-	-
LUR5	20.8±2.25^{ab}	-	1.4	+	-	-
LUR6	29.9±2.21^{ab}	+	1.5	-	-	-
LUR8	-	+	1.4	-	+	-
LUR12	32.6±2.63^{ab}	+	1.7	+	+	-
LUR20	37.8±2.15^{a}	+	1.8	+	-	-
LUR21	11.4±1.59^{ab}	+	1.2	-	-	-
LUR22	25.0±1.75^{ab}	-	-	+	-	-
LUR24	12.3±2.79^{ab}	-	1.3	+	-	-
LUR25	21.4±2.17^{ab}	+	1.3	-	-	-
LUR26	23.1±2.37^{ab}	-	-	-	-	-
LUR27	37.7±2.53^{a}	+	1.4	-	-	-
LUR28	26.3±2.68^{ab}	+	1.4	+	-	-
LUR29	26.3±4.04^{ab}	+	1.8	+	-	-
LUR30	36.2±26.27^{ab}	+	1.7	-	-	-
LUR32	24.8±3.31^{ab}	+	1.3	+	-	-
LUR36	7.7±2.28^{ab}	-	-	-	-	-
LUR37	6.1±2.11^{b}	-	-	-	-	-
LUR38	-	+	1.2	+	-	-
LUR42	20.4±3.83^{ab}	+	1.5	-	-	-
LUR43	-	+	1.4	-	-	-
LUR46	23.0±6.36^{ab}	-	-	-	-	-
LUR47	8.9±1.94^{ab}	+	1.6	-	-	-
LUR48	26.3±2.45^{ab}	+	1.7	-	+	-
LUR54	18.8±3.18^{ab}	-	1.3	-	-	-
LUR56	22.6±2.75^{ab}	-	-	+	-	-
LUR57	36.9±3.80^{a}	+	1.8	-	-	-
LUR58	14.2±2.37^{ab}	+	1.3	-	-	-
LUR62	9.7±1.49^{ab}	+	1.7	+	-	-
LUR67	8.6±1.40^{ab}	-		-	-	-
LUR80	19.7±1.49^{ab}	+	1.2	+	-	-
LUR84	20.2±5.17^{ab}	+	-	+	-	-
LUR85	8.9±0.76^{ab}	+	-	+	+	+
LUR87	-	+	1.5	+	-	-
LUR90	21.4±1.62^{ab}	+	1.3	+	-	-
LUR91	-	+	1.4	+	-	-
LUR93	-	-	-	+	-	-
LUR96	32.2±2.92^{ab}	+	1.6	-	-	-

IAA = Indole acetic acid, PS =phosphate solubilization, SI = Solubilization index, HCN = Hydrogen cyanide

2.3.5. Authentication and Screening Symbiotic Effectiveness of Isolates on Sand Culture

The data showed that all of the 39 isolates induced nodulation upon reinoculation on the host plant on sand culture under greenhouse conditions with significant variations in their ability to infect the host plant evidenced from the difference in the nodule number, nodule dry weight and shoot dry weight plant^{-1}at $p < 0.05$ level of significance (Table 2.5).The rhizobial isolates induced nodulation on the host plant ranging from 7- 47 nodules plant^{-1} at $p < 0.05$ level of significance showing the different potential of the rhizobial isolates to infect the host plant. The plants inoculated with isolate LUR62 formed higher nodule number (47 nodules plant^{-1}). In general, the average number of nodules formed by White lupin plants in this study was 20 nodules per plant which was much higher than 8 nodules per plant reported by Moulin *et al* (2001) and Martha *et al*. (2005) and less than 26 nodules per plant obtained by Abdalla (1999) indicating that nodulation in White lupin is different in various countries.

The isolates also showed significant difference in nodule dry weight within the range of 0.03g and 0.26g/plant. The average nodule dry weight obtained from this study was 0.11g/plant which was slightly less than the average NDW (0.15 g/plant) obtained from Faba bean (Anteneh Argaw, 2012), but greater than NDW of Lentil (0.03gm/plant) (Mulisa Jida and Fassil Assefa, 2011) and Chickpea (0.09gm/plant) (Mulisa Jida and Fassil Assefa, 2012).The data also showed the highest and lowest shoot dry weight of 1.36gm/plant (LUR87) and 0.47gm/plant (LUR26) indicating more than 2-fold difference between the most highly effective and lowly effective rhizobial isolates (Table 2.5). The average shoot dry weight of the inoculated white lupin plants was found to be 0.86gm/plant which was greater than the average values of 0.430gm, 0.45gm and 0.42gm/plant obtained by Miller and Pepper (1988), Moulin *et al.* (2001) and Martha *et al*. (2005), respectively indicating the presence of better rhizobial strains of White lupin in the soils of Ethiopia.

Table2. 5 Nodulation and symbiotic effectiveness of representative white lupin rhizobial isolates on the host plant grown under greenhouse conditions for 45 days

Isolates	NN Plant^{-1}	NDW (g Plant^{-1})	SDW (g/plant)	RE (%)	Rate
LUR1	37.00±4.93^{b-d}	0.21±0.02b	1.04±0.05^{e}	83.9	HE
LUR2	27.67±2.33^{e-h}	0.07±.00^{i-m}	0.84±0.03^{h-k}	67.7	E
LUR5	30.33±4.37^{c-g}	0.16±.00^{c}	0.94±0.03^{f}	75.8	E
LUR6	14.67±1.45^{j-o}	0.07±.00^{i-m}	0.55±0.05^{r}	44.4	LE
LUR8	35.67±2.33^{b-e}	0.08±.00^{i-k}	1.06±0.03^{de}	85.5	HE
LUR12	40.33±1.76^{ab}	0.26±0.04^{a}	1.23±0.01^{ab}	99.2	HE
LUR20	24.33±2.91^{f-g}	0.07±0.01^{i-l}	0.79±0.05^{j-n}	63.7	E
LUR21	22.67±1.20^{f-k}	0.29±.00^{a}	0.83±0.02^{h-e}	66.9	E
LUR22	14.67±1.20^{j-o}	0.08±.00^{i-k}	0.84±0.03^{h-k}	67.7	E
LUR24	11.67±2.19^{i-o}	0.06±.00^{j-m}	0.85±.00^{g-k}	68.5	E
LUR25	18.33±3.67^{h-n}	0.12±.00^{c-g}	0.56±0.04^{r}	45.2	LE
LUR26	13.67±1.76^{k-o}	0.06±.00^{i-m}	0.47±0.02^{s}	37.9	LE
LUR27	20.33±2.67^{g-m}	0.13±0.02^{c-e}	0.82±0.02^{i-m}	66.1	E
LUR28	22.33±4.70^{f-l}	0.14±0.02^{cd}	0.74±0.02^{m-o}	59.7	E
LUR29	6.67±1.20^{op}	0.03±.00^{i-n}	0.86±0.05^{f-k}	69.4	E
LUR30	8.33±2.03^{n-p}	0.03±.00^{mn}	0.71±0.02^{n-p}	57.3	E
LUR32	9.67±1.33^{m-p}	0.05±.00^{j-m}	0.94±0.01^{fg}	75.8	E
LUR36	9.67±2.96^{m-p}	0.03±.00^{l-n}	0.78±0^{k-o}	62.9	E
LUR37	17.67±5.21^{h-n}	0.09±0.02^{h-k}	0.65±0.02^{pq}	52.4	E
LUR38	8.33±1.76^{n-p}	0.07±.00i^{-k}	0.86±0.01^{f-j}	69.4	E
LUR38	10.33±1.45^{m-o}	0.05±.00^{k-m}	0.69±0^{o-q}	55.6	E
LUR42	11.00±2.00^{m-o}	0.09±0.01^{g-k}	0.86±0.01^{f-j}	69.4	E
LUR43	11.67±3.18^{i-o}	0.07±0.01^{i-k}	0.76±0.01^{l-o}	61.3	E
LUR46	12.00±2.65^{k-o}	0.08±0.02^{i-k}	0.65±0.01^{pq}	51.6	E
LUR47	12.67±1.45^{k-o}	0.10±0.01^{l-i}	0.87±.00^{f-i}	70.2	E
LUR48	19.00±2.08^{h-n}	0.09±.00^{f-j}	0.91±0.01^{f-h}	74.2	E
LUR54	26.67±2.91^{f-i}	0.13±0.01^{c-f}	0.85±0.01^{h-k}	68.5	E
LUR56	14.00±3.06^{j-o}	0.10±0.02^{d-i}	0.75±.00^{l-o}	60.5	E
LUR57	16.67±1.86^{i-o}	0.12±.00^{c-h}	0.92±.00^{f-h}	74.2	E
LUR58	14.67±5.36^{j-o}	0.09±0.01^{f-i}	0.62±.00^{qr}	50.0	E
LUR62	47.33±8.84^{a}	0.20±0.02^{b}	1.07±0.04^{c-e}	86.3	HE
LUR67	6.00±1.00^{op}	0.03±.00^{l-n}	0.56±0.01^{r}	45.2	LE
LUR80	28.00±3.46^{d-h}	0.10±.00^{e-i}	1.24±0.01^{a}	100	HE
LUR84	38.33±1.86^{a-c}	0.13±0.01^{c-e}	1.14±0.05^{bc}	91.9	HE
LUR85	29.67±4.26^{c-g}	0.20±.00^{b}	1.10±0.01^{c-e}	88.7	HE
LUR87	31.67±1.45^{b-f}	0.12±.00^{c-h}	1.36±0.03^{a}	109.7	HE
LUR90	27.67±2.03^{d-h}	0.10±.00^{d-i}	1.05±0.04^{de}	84.7	HE
LUR91	16.33±2.91^{i-o}	0.07±0.01^{i-k}	0.91±0.01^{f-h}	73.4	E
LUR93	12.67±4.81^{k-o}	0.08±0.02^{i-k}	0.71±0.01^{n-p}	57.2	E
TN	0±0	0±0	1.24±0.03^{a}	100	
T0	0±0	0±0	0.17±0.02^{t}	13.5	

NN = module number, NDW = nodule dry weight, SDW = Shoot dry weight, RE = Relative effectiveness, E = effective, LE = lowly effective, HE = highly effective, same letters are not significantly different at LSD $p<0.05$ level and all the data are the means of triplicates.

Correlation between nodule number, nodule dry weight and shoot dry weight was observed. However, nodule dry weight measurements were relatively better correlated (r = 0.719) to shoot dry weight than nodule number (r = 0.388). Such correlation of nodule dry weight with shoot dry weight indicates the N fixation efficiency of isolates and the importance of these parameters to determine symbiotic effectiveness of legume nodulating rhizobia (Peoples *et al.,* 2002).

The relative symbiotic effectiveness of the isolates was computed in reference to the TN control, and isolates LUR12, LUR80 and LUR87 were as equally efficient in dry matter accumulation as the N-fertilized control plants (Table 2.5). The overall performance of the isolates showed that 90% of the isolates performed as good as 50-109% shoot dry matter accumulation from which 9(23%) of the isolates were highly effective, whereas 26 (67%) and 4 (10%) were effective and lowly effective, respectively according to Gibson (1987). Consequently, isolates LUR1, LUR8, LUR12, LUR62, LUR80, LUR84, LUR85, LUR87 and LUR90) were the most effective rhizobial isolates with nodule numbers ranging from 27 to 47/plant, and with symbiotic effectiveness between 85% to 100% of shoot dry matter accumulation in relation to the positive control. There was no significant difference in symbiotic effectiveness (distribution of ineffective and highly/effective) between the fast and slow growing rhizobia.

Although there are no previous findings regarding the symbiotic effectiveness of white lupin nodulating rhizobia, the results of this research reflected the presence of symbiotically effective white lupin nodulating rhizobia in soils where white lupin is widely grown for years as a break crop. Earlier studies in Ethiopia (Mulisa Jida and Fassil Assefa, 2011 Zerihun Belay and Fassil Assefa, 2011) showed that 33 % and 24 % rhizobia isolated from lentil and Faba bean, respectively were characterized as symbiotically highly effective under greenhouse conditions. Moreover, Mulisa Jida and Fassil Assefa (2012) and Anteneh Argaw (2012) reported that about 11 % (from

Chickpea) and 21 % (from Lentil) of rhizobial isolates were highly effective on sterile sand culture in the same order.

In conclusion, 9 isolates (LUR1, LUR8, LUR12, LUR62, LUR80, LUR84, LUR85, LUR87, and LUR90) showed better qualities (features) in heterotrophy, such as C and N-substrates utilization, tolerance to metal toxicity, antibiotics, pH, salt and temperature (Tables 2.2 and 2.3) and hence, they are highly recommended for ecological competitiveness and efficiency studies under field conditions in Ethiopian soils. Moreover, these rhizobial isolates showed interesting PGP traits (Tables 2.4 and 2.5) which can make them prior candidates for the production of inoculants for enhancing White lupin production in the country.

Chapter 3 Isolation and Characterization of Phosphate Solubilizing Bacteria Associated with the Rhizosphere of White Lupin (*Lupinus albus* L) from Northwestern Part of Ethiopia

Abstract

Phosphorus is a vital macronutrient for plant growth and production. However, a great portion of it becomes insoluble and unavailable to plants because of its pH-mediated fixation and precipitation in the soil. Efficient phosphate solubilizing bacteria can improve the accessibility of phosphate in the plant rhizosphere. This research was aimed at the isolation and characterization of phosphate solubilizing bacteria associated with the rhizosphere of white lupin (*lupinus albus* L) from Northwestern part of Ethiopia. A total of 40 effective phosphate solubilizing bacterial isolates associated with the rhizosphere of White lupin were selected and showed variations in their morphological characteristics, doubling times (1.5 – 5.8 h), Gram reactions (55 % Gram negative and 45 % Gram positive), C and N-sources utilization patterns and phosphate solubilization indexes (SI). Accordingly, seven best phosphate solubilizing bacterial isolates were selected on the basis of their SI for the determination of amount of solubilized phosphate from different sources ($Ca_3(PO_4)_2$, $AlPO_4$ and $FePO_4$) in Pikosvikya broth medium and plant growth promoting characteristic of the isolates. The average amount of solubilized phosphate from $Ca_3(PO_4)_2$, $AlPO_4$ and $FePO_4$ was found to be 113.2µg, 56.1µg and 48.3µg/ml after 5 days of incubation. Some of the isolates also showed good tricalcium solubilization potential under different stress conditions, such as various salt concentrations (2-10 % NaCl), low/high pH (4-12) and low/high temperature (10 ^{0}C – 45 ^{0}C). Besides, most of them showed multiple plant growth promoting characteristics: Indole acetic acid (71 %), siderophore (71 %), hydrogen cyanide (57 %) and ammonia (100 %) production and antagonistic activity against *fusarium oxysporum* (57 %) under *in vitro* conditions. Hence, it is possible to conclude that white lupin rhizosphere soils comprise efficient phosphate solubilizing bacteria with multiple plant growth promoting abilities that can be considered as good candidates for field applications under phosphate limited agro-ecologies of the country.

Key words: Lupin, PGP, Rhizosphere, SI

3.1. Introduction

Phosphorus is the second most limiting element next to nitrogen for crop production. However, the availability of P to plants in the soil is a serious problem because of its pH-mediated fixation and precipitation in the soil. Several studies showed that most (95-99%) of the chemical P fertilizers used in modern agriculture becomes insoluble and unavailable to plants as it forms calcium phosphates in alkaline soils and iron and aluminum phosphates in acidic soils (Zahid *et al*., 2015, Pandey *et al*., 2017). Under P limited circumstances, the use of chemical phosphorous sources is not sustainable because the applied phosphorus can be easily converted into an insoluble and unavailable form leaving only 5% or less of the total amount of applied P in the soil for plant growth and production (Pandey *et al*., 2017).

Microorganisms play a very important role in the P cycle in the soil for they mineralize organic phosphorus using the enzyme phytase. The Phosphate solubilizing potential of PSB is attributed to their ability to reduce the pH by releasing various organic acids that play important roles in the solubilization of insoluble phosphate in the rhizosphere of plants through anion exchange or chelating Fe and Al ions associated with phosphates (Korrir *et al*., 2017).

This process helps to convert insoluble forms of phosphate into a soluble monobasic ($H_2PO_4^-$) and dibasic (HPO_4^{2-}) ions that can be utilized by plants (Hariprasad and Niranjana, 2008, Banerjee *et al*., 2010). Moreover, a number of phosphate solubilizing microorganisms have plant growth promoting properties through various direct and indirect mechanisms such as, production of phytohormones, siderophores, hydrogen cyanide (HCN), ammonia, hydrolytic enzymes and associative nitrogen fixation. Apart from P solubilization, these bacteria also enhance plant growth and health through different direct and indirect mechanisms, and thus are known as Plant Growth Promoting Rhizobacteria (PGPR). Some of the most important phosphate solubilizers include

Alcaligenes sp., *Aerobactor aerogenes*, *Achromobacter sp*., *Actinomadura oligospora*, *Burkholderia sp*., *Pseudomonas sp*., *Bacillus sp*., *Serratia*, *Azospirillum, Flavobacterium*, *Arthrobacter*, *Micrococcous*, (Pandey *et al*., 2017). Selection of PGP and phosphate solubilizing rhizobacteria (PSB) is preferable to chemical fertilizers because microbial inoculants that possess P solubilizing abilities in soils are considered as environmental-friendly inputs than the application of chemical P fertilizers to attain maximum crop production (Situmorang et al., 2015).

However, the Plant growth promoting potential of phosphate solubilizing microorganisms is affected by various environmental stresses, such as high salt, high or low pH and temperature that necessitate the search for phosphate solubilizing bacteria that can solubilize insoluble phosphate under various environmental stresses (Majeed *et al*.,2015). Phosphate solubilizing microorganisms are found in the rhizosphere of every crop to maintain plant growth and productivity. However, the importance of PSB to legumes is by far more important since the crop require more P for different physiological function and nitrogen fixation.

For many years now, different studies were undertaken on BNF and plant growth promoting properties of leguminous crops from different parts of Ethiopia. These include: chick pea and lentil (Mulisa Jida and Fassil Assefa, 2012), cowpea (Diriba Temesgen and Fassil Assefa, 2017), and soybean (Girmaye Kenena and Fassil Assefa, 2017). The studies showed these legumes harbored different effective PSB with multiple growth promotion properties that enhanced plant production with co-inoculation of the hosts twice more than the yield produced with single inoculation of the nitrogen fixing endosymbionts. Hence, this study was aimed to isolate and characterize phosphate solubilizing bacteria with multiple PGP properties from White lupin (*Lupinus albus* L.) rhizosphere in north western parts of Ethiopia.

3.2. Materials and Methods

3.2.1. Soil sampling and study sites

Soil samples for the isolation of PGPR were collected from 20 agricultural fields from white lupin (*Lupinus albus* L.) growing areas of Amhara and Benishangul-gumuz National Regional Sates found in the Northwest parts of Ethiopia from the altitude range 1926 – 2636 masl and soil pH from 5.7 (moderately acidic) to 7.10 (slightly basic) from September - October, 2013. The soils strongly adhered to the roots of the plant were collected after excavating from 5-20 cm depth and carefully transferred into sterile Polyethylene bags and stored in the ice box. Then, the soil samples were transported to the Applied Microbiology Laboratory of Addis Ababa University and stored at 4 ^{0}C for the isolation of PGPR and soil analysis.

3.2.2. Isolation of phosphate solubilizing rhizobacteria

The soil samples were air dried and grounded into smaller parts and sieved with 2mm mesh size from which 10gm of fine soil was added to 90ml of sterile distilled water in conical flasks and shaken for about 30min in rotary shaker. They were then prepared to appropriate dilution (10^{-2} – 10^{-6}) and 1ml of each of the suspension was streaked on Pikovskaya medium (Pikovskaya, 1948), and incubated at 30 ^{0}C for 4-8 days. Different colony types with apparent halo zone were purified by sub-culturing on NA medium and preserved on NA agar slant at 4^{0}C for short term storage and at -80 ^{0}C for long term storage in NA broth containing 50% (v/v) glycerol. The isolates were designated as PSRL (Phosphate Solubilizing Rhizobacteria from Lupin) followed by different numbers.

3.2.3. Biochemical characterization of the isolates

3.2.3.1. Gram reaction

The isolates were examined for their gram reaction following the method outlined by Aneja (2001). A loopful of culture suspension was smeared on a clean glass slide stained with crystal violet, and counter stained with safranin. Based on retention or loss of initial stain color the isolates were classified as Gram positive or Gram negative.

3.2.4. Physiological characteristics of phosphate solubilizing rhizobacteria (PSRB)

3.2.4.1. Carbon and nitrogen sources utilization

HiCarbo^{TM} kits (Part A, B, and C) of HIMEDIA that contain 34 different carbohydrates with a control were used to test the carbon source utilization pattern of the bacterial isolates. Kit part A contains 12 carbohydrates (Lactose, Xylose, Maltose, Fructose, Dextrose, Galactose, Raffinose, Trehalose, Melibiose, Sucrose, L-Arabinose and Mannose), Kit part B also contains 12 carbohydrates (Inulin, Sodium Gluconate, Glycerol, Salicin, Dulcitol, Inositol, Sorbinol, Mannitol, Adonitol, Arabitol, Erythritol and α-Methyl-D-glucoside), whereas Kit Part C contains 10 carbohydrates (Rhamnose, Cellobiose, Melezitose, α-Methyl-D-Mannoside, Xylitol, ONPG, Esculin, D- Arabinose, Malonate and Sorbose).The test was performed according to the Manufacturer's protocol. Pure cultures were grown in nutrient broth medium at 30 ^{0}C until inoculum turbidity reached $\geq$ 0.5 OD at 620nm. Then, each well of the kit was inoculated with 50 µl of log phase of the suspension by surface inoculation method and incubated at 30 ^{0}C for 24 hours. Finally, the result was recorded as positive, or negative according to result interpretation chart provided by the Manufacturer.

3.2.5. Evaluation of phosphate solubilizing index (SI) of PSRB

The qualitative phosphate solubilization efficiency of the isolates was determined on Pikovskaya agar medium containing (glucose, 10g., $Ca_3(PO_4)_2$., 5g, $(NH)_2SO_4$, 0.5g., NaCl, 0.2g., $MgSO_4.7H_2O$, 0.1g., KCl, 0.2g., $Fe_2(SO_4)_3.7H_2O$., $MnSO_4$, 0.002g., yeast extract, 0.5g., agar, 20g., Distilled water, 1000ml, pH 7.0). 20 µl of each isolate was spot inoculated on PK agar plates and incubated at 30 ^{0}C for 3-9 days and their solubilization index (SI) was computed by using the formula: $SI = \frac{Colony\ Diameter + Halo\ zone\ diameter}{Colony\ Diameter}$ (Edi–Premono *et al*., 1996).

3.2.6. Evaluation of solubilized phosphate on liquid PK medium

The amount of P released from solubilization by the isolates was determined in 100ml Pikovskaya basal broth medium (PK) in 250 ml Erlenmeyer flasks and supplemented with 0.005g/ml tricalcium phosphate as insoluble phosphate source. All the flasks were autoclaved and inoculated with 0.1ml of each bacterial culture from appropriate dilution ($10^{-2} – 10^{-6}$) separately and PK flasks containing tricalcium phosphate were included as control. Flasks were incubated on rotary shaker at 120 rpm for 5 days at room temperature. Moreover, the isolates were also inoculated into flasks containing the same basal medium containing 0.004g/ml AlPO4 and 0.006 g/mlFePO4 as inorganic phosphate sources and incubated for the same days as before. $AlPO_4$ and of $FePo_4$ were added into separate 250 ml Erlenmeyer flasks containing slightly modified 100ml of PK broth medium with 0.0005g/ml of $CaCO_3$ to avoid lowering of pH in the broth as described in Banerjee *et al*., (2010).

10ml of each culture was taken out from each flask after 3 and 5 days of incubation, and centrifuged at 15,000rpm for 15 minutes to determine soluble P released and pH of the culture supernatant according to the Molybdophosphoric acid blue method described by Jackson (1973).

Hundred microliters of the supernatant were mixed with 10ml of Chloromolybdic reagent (15g of Ammonium molybdate in 400 ml distilled water and 342 ml of conc. HCl, total volume was made 1L), vigorously shaken and further diluted to 40ml with water. Then, 5 drops of Chlorostannous acid (10g $SnCl_2$: 25ml conc. HCl) was added slowly and the final volume was made to 50ml with distilled water. Absorbance was measured at OD 660nm using spectrophotometer (Jenway, 6405 Uv/vis) against the blank, and the amount of solubilized P (µg ml^{-1}) was calculated from the standard curve constructed with known concentrations of KH_2PO_4.

3.2.7. Estimation of stress induced phosphate solubilizing capacity of PSRB

The ability of PS of isolates under stress conditions such as salt, pH, and temperature, was undertaken in 100 ml Pikovskaya broth containing 0.05 % (W/V) $Ca_3(PO_4)_2$ and adjusted to different concentrations of NaCl (1%, 2%, 3%, 5%, and 6%, w/v). Similarly, the effect of pH on P solubilization of the isolates was carried out by adjusting the pH of Pikovskaya broth to pH (4, 5, 6, 8, 9 and 10) using 1N HCl or 1M NaOH. The flasks for salt and pH stress tests were incubated at 30 ^{0}C for 5 days. Moreover, the effect of temperature on phosphate solubilization was undertaken on Pikovskaya broth and incubated at different temperatures of 35 ^{0}C, 40 ^{0}C and 45 ^{0}C, and incubated for 5 days. In all cases, the quantity of solubilized P was measured colorimetrically as described above.

3.2.8. Multiple plant growth promoting (PGP) properties of the PSRB isolates

3.2.8.1. IAA production

Production of Indole acetic acid (IAA) by the bacterial isolates was studied according to Gordon and Weber (1951). They were inoculated on nutrient broth medium supplemented with 3mg/mL of L-tryptophan and incubated at 30 ^{0}C for 2-5 days. Two ml of the bacterial culture was centrifuged at 10,000 rpm for 10 min, from which 1ml of supernatant was mixed with a drop of Ortophosphoric acid and 2ml of Salkowsky reagent (2% of 0.5 M FeCl3 in 35% perchloric acid). The solution was allowed to settle for 25 min for the development of pink color. Optical density (OD) was measured using a spectrophotometer (Jenway, 6405 Uv/vis) at 535 nm and the amount of IAA produced by the bacterial isolates was determined by using a standard curve graph prepared from known concentrations of pure IAA at 0, 5, 10, 20, 50, and 100 μg mL^{-1}.

3.2.8.2. Siderophore production

The bacterial isolates were screened for the production of Siderophore by chrome azurol S (CAS) assay method as described by Schwyn and Neilands (1987). The blue agar CAS medium was prepared by adding 850 mL of autoclaved MM9 salt medium (added with 32.24 g piperazine- N, N'-bis 2- ethanesulfonic acid (PIPES) at pH 6), 100 mL of blue dye, 30 mL of filter sterilized 10 % Casaminoacid solution and 10 mL of 20 % glucose solution. The bacterial isolates were inoculated into the CAS medium and incubated at 28 ^{0}C for 24 h. Development of yellowish orange halo around the colonies was indicator of siderophore production.

3.2.8.3. Antagonistic activity

Phosphates solubilizing bacterial isolates were screened for antagonistic activity against *Fusarium oxysporum* (obtained from EIAR, Ethiopian Institute of Agricultural Research) by using dual plate technique (Ganesan and Gnanamanickam, 1987). Accordingly, 10 µL of the inoculum of each PSB isolates was equidistantly placed near to the edge of nutrient agar (NA) plates amended with 0.5 % (w/v) of sucrose and incubated for 24hrs at 30 ^{0}C. A 3-4 mm agar disc from PDA cultures of the fungal pathogens was also be placed at the center of the NA plate for each bacterial isolate and incubated at 28 ± 2 °C for 72h. Then, percentage of inhibition of radial growth (PIRG) was calculated as PIRG = [(C-T)/C] ×100, where C is radial growth of fungus on control plates, and T is the radial growth of the fungus in the dual culture.

3.2.8.4. Hydrogen cyanide (HCN) production

The production of HCN by the bacterial isolates was carried out by streak plating onto nutrient agar medium supplemented with glycine (4.4 g/L) according to Dye, (1962). Then pieces of Whatman number 1 filter paper soaked with 0.5% picric acid and 2% sodium carbonate was placed on the under sides of the lid of each plate. The plates were wrapped with parafilm and incubated at 30 ^{0}C for 4 days. Change of orange to red color of the filter paper was considered to be a positive test for the production of HCN.

3.2.8.5. Production of ammonia

All isolates were tested for ammonia production by inoculating the cultures in 10 ml peptone water and incubated for 72 h at 36°C. After incubation, 0.5 ml of nessler's reagent was added to each tube. Development of brown to yellow color was taken as a positive test for production of ammonia (Cappuccino and Sherman, 1992).

3.2.9. Data Analysis

All the data for plant growth parameters were carried out in triplicate and analyzed by one-way analysis of variance (ANOVA) and the treatment means were compared relative to controls following Duncan's test (DT) by using SPSS (Ver.20). The relation between different parameters was also evaluated by using Pearson correlation coefficient at $p \leq 0.05$.

3.3. Results and Discussion

3.3.1. Isolation and enumeration of phosphate solubilizing rhizospheric bacteria (PSRB)

A total of 136 phosphate solubilizing rhizospheric bacterial isolates were obtained from 39 soil samples (soil pH ranged from slightly acidic (5.95) to moderately basic (7.10) using PK medium after 3-7 days of incubation at 30 ^{0}C. The population density of PSRB from the different sampling sites was within the range of 3.03 x 10^4 to 1.93 x 10^6 CFU g^{-1} soil (Table 3.1). The highest number of PSB isolates (morphotypes) 24 was recorded from Dembecha (West Gojam); whereas the least number of 12 PSB was recovered from Mecha (West Gojam) and Kessa (Awi zone), indicating a two-fold difference between the two sampling sites irrespective of the population density of the sampling sites.

Table3. 1 Sampling Sites, Locations, Distribution and Abundance of PSRB and soil pH of the study area

Isolation Sites	Abundance of RB (cfu/gm of soil)	Number of PSB isolates	Soil pH
Amusit (South Gonder)	1.50 x 10^5f	18	5.72-6.84
Mecha (West Gojam)	4.10 x 10^4bc	12	6.82-7.10
Achefer (West Gojam)	4.83 x 10^5b	20	5.97-7.03
Sekela (West Gojam)	1.93 x 10^6ef	21	6.48-6.97
Dembecha (West Gojam)	6.97 x 10^4a	24	6.90-7.42
Addis Kidam (Awi Zone)	3.03 x 10^4cd	15	6.93-7.06
Kessa (Awi Zone)	2.50 x 10^5d-f	12	6.87-7.09
Wenbera (Benishangul)	2.87 x 10^5de	14	6.84-7.02

RB = rhizospheric bacteria, PSB = phosphate solubilizing bacteria

Dereje Haile *et al*., (2016) also reported that the number of PSRB in the rhizospheric soil of the lupinus crop was between 1.47 x 10^5 and 2.19 x 10^5 from Dembecha and Merawi, respectively which was slightly higher than the present study. The data also showed that the number of PSRB isolated from some rhizosphere soil samples of white lupin was slightly higher than the PSRB isolated from other legume crops such as faba bean from 1.5 to 1.8 x 10^3 (Assefa Keneni *et al*., 2010) CFU g^{-1} of soil, but similar to the number of PSRB isolated from Lentil with the range from 2.4 x 10^4 to 2.1 x 10^5 (Mulisa Jida Fassil Assefa, 2012). In general, the abundance of phosphate solubilizing bacteria is higher in the rhizospheric soils of agriculturally important crops than the bulk soils and they could play significant role in improving the availability of P for the growth and productivity of crops (Rajapaksha and Senanayake, 2011).

3.3.2. Phosphate Solubilizing Efficiency of the Isolates

In this study, 40 relatively effective PSRB were selected on the basis of the SI and are shown on Table 3.2. Colony diameter of the selected phosphate solubilizing isolates was from 1.8 to 4.5mm, whereas the diameter of halo zone produced by these isolates ranged from 2.7 to 9.6mm upon 5 days of incubation. Thus, the isolates displayed solubilizing index (SI) ranged from 2.2 to 4.3mm. Two isolates (PSRL38, and PSRL40) were found to have phosphate solubilizing index (SI) of >4 followed by PSRL58 (SI = 3.9), PSRL60 (SI = 3.8) and PSRL112 (SI =3.7) whereas isolate PSRL123 was found to have the lowest SI (2.2).

Table 3. 2 Screening for effectiveness of PSB based on solubilization indices grown on Pkovskaya's medium incubated at 30 ^{0}C for 5 days.

Isolates	Sampling site	Site pH	CD (mm)	HZD (mm)	SI	Average SI/Site
PSRL2	Amusit (South Gonder)	6.82	2.5	5.3	3.1	2.8
PSRL12		5.95	2.8	4.2	2.5	
PSRL19	Mecha (West Gojam)	5.87	1.8	2.7	2.5	2.7
PSRL22		5.82	2.6	4.2	2.6	
PSRL26		6.65	2.8	5.8	3.1	
PSRL33	Achefer (West Gojam)	5.53	2.7	5.2	2.9	3.2
PSRL34		5.67	2.0	3.7	2.9	
PSRL38		5.84	2.5	8.0	4.3	
PSRL40		5.57	2.5	4.5	2.8	
PSRL41		6.08	2.5	8.2	4.3	
PSRL45		5.92	2.0	3.5	2.8	
PSRL49		5.76	2.4	3.8	2.6	
PSRL52	Sekela (West Gojam)	6.54	2.5	4.3	2.7	3.2
PSRL53		5.87	3.5	5.8	2.7	
PSRL58		6.26	2.6	7.6	3.9	
PSRL60		7.12	2.7	7.5	3.8	
PSRL62		6.75	3.0	5.0	2.7	
PSRL63		5.98	2.5	5.4	3.2	
PSRL67		7.03	2.8	6.8	3.4	
PSRL70		5.97	2.8	6.0	3.1	
PSRL73	Dembecha (West Gojam)	6.94	3.5	5.6	2.6	2.7
PSRL74		7.21	2.7	4.3	2.6	
PSRL76		6.78	2.6	4.5	2.7	
PSRL77		7.31	3.5	5.2	2.5	
PSRL79		5.89	3.4	5.4	2.6	
PSRL84		6.34	3.4	5.3	2.6	
PSRL88		7.14	4.5	9.6	3.1	
PSRL89		6.58	2.5	4.3	2.7	
PSRL90		5.97	3.0	5.2	2.7	
PSRL95		6.36	3.0	5.0	2.7	
PSRL97	Addis Kidam (Awi)	6.93	2.5	4.3	2.7	2.9
PSRL100		7.04	1.5	3.5	3.3	
PSRL108		6.67	2.0	3.5	2.8	
PSRL112	Kassa (Awi)	6.87	2.5	6.8	3.7	3.2
PSRL118		6.93	2.6	8.4	3.2	
PSRL121		7.13	2.0	3.4	2.7	
PSRL123	Wenbera (Benishangulgumuz)	6.96	3.0	3.6	2.2	2.6
PSRL128		7.11	4.5	6.8	2.5	
PSRL130		6.87	3.5	5.2	2.5	
PSRL135		6.98	2.4	4.7	3.0	
Average		6.48	2.9	5.3	2.9	

CD = Colony Diameter, HZD = Halo zone Diameter, SI = Solubilization index, SE = Solubilization Efficiency and PSRB = Phosphate solubilizing Rhizospheric Bacteria.

Moreover, the average colony diameter (CD), halo zone diameter (HZD) and solubilizing indexes (SI) of the isolates were found to be 2.9mm, 5.3mm, and 2.9 after 5 days of incubation, respectively. PSRB isolates from Achefer, Sekela and Kessa sites showed higher average solubilization index (SI = 3.2) followed by isolates from Addis Kidam and Amusit that showed average SI of 2.9 and 2.8 in the same order, whereas the least average solubilization index (SI= 2.6) was obtained from PSRB isolates of Wenbera followed by SI of 2.7 from isolates of Mecha and Dembecha Dereje Haile *et al*. (2016) also reported that most phosphate solubilizing rhizospheric bacteria associated with the roots of White lupin (*Lupinus albus* L.) showed increased halo zone diameter upon 2-8 days of incubation and two isolates (HUBSB-35T_1 and HUBSB-45K_2) displayed phosphate solubilizing index (SI) of 4.5 at 8 days of incubation. Likewise, Sen and Joshi (2017) reported phosphate solubilizing bacteria isolated from the rhizosphere of plants grown in Tailing Dam of Zawar Mines, India showed maximum SI 3.18 to 4.5 after 5 days of incubation. This indicates that efficiency of PS is dependent upon differences in incubation time and sampling sites.

Other studies also showed that variation in the solubilizing index and efficiency of the isolates may be due to the difference in the ability of the isolates to secrete different types of extracellular organic acids that play great role in the solubilization of insoluble phosphate (Mursyyida *et al*., 2015). Moreover, Dereje Haile *et al*., (2016) reported that variation in the phosphate solubilizing index (SI) of the different isolates could possibly be due to the difference in isolates type, natural environments and agricultural practices undertaken in the area. Accordingly, seven isolates (PSRL67, PSRL112, PSRL60, PSRL58, PSRL38, PSRL118 and PSRL41) were selected on the basis of their phosphate solubilizing index for further characterization.

3.3.3. Morphological Characteristics and MGT of Phosphate Solubilizing Bacteria

Each phosphate solubilizing bacterial isolates were characterized on the basis of their colony morphology and biochemical characteristics on nutrient agar plates after 3-7 days of incubation at 30 ^{0}C (Table 3.3). Regarding their morphological characteristics, 22 (55%) of them were found to be white in colony color, while 18 (45%) of the isolates were yellow in appearance. In addition, 20 (50%), 15 (37.5%) and 5 (12.5%) of the isolates revealed flat, raised and pulvate in colony elevation in the same order, whereas 31 (77.5%), 6 (15%) and 3 (7.5%) of them showed entire smooth, undulate and lobate in their colony margin, respectively and 22(55 %) of the PS rhizospheric bacterial isolates were gram negative whereas the remaining 18 (45 %) were gram positive. Although there are no sufficient data regarding the morphological characteristics of phosphate solubilizing rhizobacteria associated with the roots of White lupin (*Lupinus albus* L.), Dereje Haile *et al*., (2016) reported phosphate solubilizing rhizospheric bacteria that were gram negative motile rods with round and irregular round colony margin from the same plant. The same authors explained that the colony color of some isolates was white while some others were yellow with differences in transparency in which some were mucoid opaque and others were transparent.

Table3. 3 Morphological Characteristics and MGT of PSRB

Isolates	Colony morphology				MGT (Hrs)	GR
	Color	Shape	Elevation	Shape		
PSRL2	W	LC	Flat	Entire	1.5	+
PSRL12	W	LC	Flat	Entire	2.6	-
PSRL19	Y	LC	Raised	Entire	5.6	-
PSRL22	Y	LC	Flat	Entire	4.3	
PSRL26	Y	LI	Flat	Undulate	3.3	+
PSRL33	W	LC	Raised	Undulate	4.8	-
PSRL34	W	LC	Raised	Entire	5.0	+
PSRL38	Y	LC	Pulvinate	Entire	3.1	-
PSRL40	Y	LC	Raised	Entire	3.6	-
PSRL41	Y	LC	Pulvinate	Entire	3.2	+
PSRL45	W	LC	Flat	Undulate	5.1	-
PSRL49	W	LI	Flat	Entire	5.3	-
PSRL52	W	LI	Raised	Lobate	2.2	+
PSRL53	Y	LC	Raised	Entire	2.4	-
PSRL58	Y	LC	Pulvinate	Entire	4.3	+
PSRL60	W	LC	Flat	Entire	4.1	-
PSRL62	W	LC	Pulvinate	Entire	3.2	+
PSRL63	W	LC	Raised	Entire	3.8	
PSRL67	W	LC	Raised	Entire	3.4	+
PSRL70	Y	LI	Raised	undulate	5.2	-
PSRL73	Y	LC	Flat	Entire	2.3	+
PSRL74	W	LI	Flat	Lobate	2.6	+
PSRL76	Y	LI	Flat	Entire	2.2	+
PSRL77	Y	LC	Flat	Entire	1.8	-
PSRL79	W	LC	Raised	Entire	3.9	
PSRL84	W	LI	Flat	undulate	4.4	+
PSRL88	W	LC	Flat	Entire	3.5	+
PSRL89	W	LI	Flat	Entire	4.6	-
PSRL90	W	LI	Raised	Entire	5.8	+
PSRL95	Y	LI	Raised	Entire	3.4	-
PSRL97	Y	LC	Flat	Lobate	3.7	-
PSRL100	Y	LC	Flat	Undulate	2.1	-
PSRL108	Y	LC	Flat	Entire	1.8	-
PSRL112	Y	LC	Raised	Entire	4.8	
PSRL118	W	LC	Flat	Entire	2.8	+
PSRL121	W	LI	Pulvinate	Entire	3.5	-
PSRL123	W	LI	Flat	Entire	4.1	+
PSRL128	W	LC	Flat	Entire	3.2	-
PSRL130	Y	LC	Raised	Entire	2.6	+
PSRL135	W	LC	Raised	Entire	3.2	+

W = white, Y = yellow, LC = large circular, LI = large irregular, MGT = mean generation time, GR – gram reaction.

As it is shown in table 3.3, the mean generation time (MGT) of the isolates was found to be in the range of 1.5 – 5.8. Isolate PSRL2 showed the shortest doubling time (1.5 hrs) while the longest doubling time 5.8 hrs) was recorded from isolate PSRL90. Similarly, Dereje Haile *et al*., (2016) reported that the mean generation time of phosphate solubilizing rhizobacteria isolated White lupin ranged from 4.1 – 5.8 hrs.

3.3.4. Quantitative Estimation of Solubilized Phosphate and pH Changes by PSRB

The selected phosphate solubilizing rhizospheric bacterial isolates were analyzed for their quantitative $Ca_3(PO_4)_2$ (TCP), $AlPO_4$ and $FePO_4$ solubilizing potential in PK broth upon 5 days of incubation and seven best PSRB isolates were selected for further characterization (Table 3.4).

Table 3. 4 The solubilization efficiency of PSRB isolates on a basal Pikovskaya broth with different inorganic phosphate sources; $Ca_3(PO_4)_2$, $AlPO_4$ and $FePO_4$ and pH changes of the medium incubated at 30 ^{0}C for 5 days (µg ml^{-1})

	TCP			Al-P			Fe-P		
Isolates	P released	Rate g/day	pH	P released	Rate g/day	pH	P released	Rate g/day	pH
PSRL38	88.8±0.3^{d}	17.8±.1^{d}	5.4±.2^{ab}	36.3±3.7^{cd}	7.3±.7^{cd}	5.6±.1^{b}	22.8±2.6^{cd}	4.6±.5^{cd}	5.6±.2^{ab}
PSRL41	185.7±1.4^{a}	37.1±.3^{a}	4.8±.2^{b}	132.7±1.6^{a}	26.5±.3^{a}	4.6±.2^{cd}	114.6±2.8^{a}	22.9±.6^{a}	4.5±.2^{b}
PSRL58	102.2±1.2^{c}	20.4±.2^{c}	5.2±.6^{b}	46.6±4.7^{c}	9.3±.9^{c}	5.9±.3^{ab}	49.5±18.7^{bc}	9.9±3.8^{bc}	5.2±.1^{b}
PSRL60	97.2±0.8^{c}	19.4±.2^{c}	5.5±.3^{ab}	33.8±4.3^{c-e}	6.8±.8^{cd}	5.2±.2^{b-d}	28.5±5.3^{cd}	5.7±1^{cd}	5.7±.3^{ab}
PSRL67	67.2±1.3^{e}	13.4±.3^{e}	5.8±.5^{ab}	18.9±2.4^{ef}	4.6±1de	5.2±.2^{b-d}	28.7±4.0^{cd}	5.7±.8^{cd}	5.4±.5^{b}
PSRL112	86.4±1.0^{d}	17.3±.2^{d}	5.6±.1^{ab}	26.9±3.1^{de}	5.4±.6de	5.5±.2^{bc}	19.6±4.4^{cd}	3.9±.9^{cd}	5.8±.3^{ab}
PSRL118	158.7±0.6^{b}	31.7±.1^{b}	4.9±.2^{b}	97.7±4.7^{b}	19.5±1^{d}	4.5±.2^{d}	74.3±3.3^{b}	14.9±.7^{b}	5.2±.3^{b}
Mean	112.3	22.4	5.3	56.1	11.3	5.2	48.3	9.7	5.3
Control	21.1±3.6^{f}	4.2±.7^{f}	6.9±.0^{a}	8.9±1.5^{f}	1.8±.3^{a}	6.9±.0^{a}	7.7±1.4^{d}	1.6±.3^{d}	6.9±.0^{a}
CV (%)	5.16	5.16	8.94	17.52	18.88	6.25	27.88	28.13	8.13
LSD	0.012	0.050	1.88	0.041	0.059	0.147	0.161	0.173	0.270

Data are means of three replicates. Same letters are not significantly different at LSD $P<0.01$ level. Different letters within column indicate significant difference

The amount of soluble P from TCP, $AlPO_4$ and $FePO_4$ was ranged 67.2 – 185.7µg/ml, 18.9 - 132.7µg/ml and 19.6 – 114.6µg/ml with the average production of 112.3, 56.1 and 48.3µg/ml of P after 5 days of incubation in the same order (Table 3.4). The highest amount of P (185.7 µg/ml from TCP, 132µg/ml from $AlPO_4$ and114µg/ml from $FePO_4$) was released by isolate PSRL41 from Achefer district followed by isolate PSRL118 from Kessa district that produced 158.7 and 97.7 and 74.3 µg/ml of P from TCP, $AlPO_4$ and $FePO_4$, respectively. On average, PSRB isolates produced 22.4, 11.3 and 9.7µg of P per day indicating that these isolates were the best P solubilizing bacteria from the tested insoluble phosphate sources. Generally, the results of this study showed that the selected PSRB isolates from the roots of White lupin can solubilize $Ca_3(PO_4)_2$ better than $AlPO_4$ and $FePO_4$.

As indicated in table 3.4, all the isolates released significantly ($P < 0.05$) higher P from the tested sources compared to the control. Among the selected PSB isolates, PSRL112 and PSRL67 were found to be relatively the weakest in the solubilization of TCP as they released only 86.4 and 67.2µg/ml of P, followed by PSRL38 and PSRL60 that released 88.8 and 97.2 µg/ml of P at 5 days of incubation in the same order. In the case of $FePO_4$, isolates PSRL112, PSRL38, PSRL60 and PSRL67 were found to have the lowest potential of releasing soluble P as they produced only 19.6, 22.8, 28.5 and 28.7 20µg/ml of P in the same order, whereas PSRL76 released the lowest P (18.9µg) from $AlPO_4$ followed by PSRL112 that produced 26.9 µg of P upon 5 days of incubation (Table 3.4). However, the most efficient isolate PSRL41 released about 3 times higher phosphate than the weakest isolate PSRL67 and twice than PSRL38 on TCP.

Generally, isolates PSRL41 and PSRL118 solubilized significantly higher amount of phosphate from all insoluble phosphate sources. Likewise, several studies indicated that various phosphate solubilizing rhizospheric bacterial isolates showed variations in phosphate solubilization potential due to differences in organic acid secretion and released more soluble P from insoluble TCP in liquid PK medium after 10 days of incubation (Oliveira *et al*., 2009). Aarab *et al*. (2013) reported that PSB isolated from the rhizosphere of *Lupinus hirsutus* L in Morocco, solubilized insoluble TCP in the range of 81.94 – 298.66 mg/L of P after 7 days of incubation with a fall in pH to 4.83. Moreover, Gupta *et al*. (2012), Padder *et al*. (2017), Wang *et al*. (2017), Banerjee *et al.* (2010) also reported that most phosphate solubilizing rhizospheric bacterial isolates showed variations in their P solubilizing potential and released relatively higher amount of soluble P from TCP than other insoluble phosphates such as $AlPO_4$ and $FePO_4$. Several research results indicated that as the cells in the culture immobilize soluble phosphate for biomass production, a corresponding decrease in soluble P and increase in pH value of the growth medium was observed (Tripura *et al*., 2007b, Diriba Muleta *et al*., 2012).

Regarding pH change, a notable decrease in the pH of PK broth was observed with a progressive increase in the amount of soluble phosphate. All the isolates showed a sharp decline in pH of the growth medium on 5^{th} day of incubation. The pH at the 5th day was ranged from 4.8 -5.8 for TCP, 4.5 – 5.9 for $AlPO_4$ and 4.5 – 5.8 for $FePO_4$. An inverse relationship was observed between pH value of culture medium and concentration of solubilized phosphate, indicating that the difference in P solubilization potential among the tested bacterial isolates was as a result of variation in the secretion of organic acids and change in the pH of the culture medium. Rashid *et al* (2004) also explained the inverse relationship between pH value and quantity of soluble phosphate in liquid PK medium upon different days of incubation. Similarly, various studies (Mulisa Djida and Fassil Assefa., 2012, and Poul and Sinha, 2017), reported that the solubilization of tri-calcium phosphate by PSB in liquid PK medium was

accompanied by a significant decline in pH after 4 days of incubation period and maximum drop in pH was correlated with elevated levels of soluble phosphate (13 to 171 µg ml^{-1} and 219.64µg ml^{-1}, respectively after 4 days of incubation). The acidification of culture supernants clearly indicated the production of organic acids and considered to be the major mechanism for inorganic phosphate solubilization (Rajkumar and Frietas, 2009).

3.3.5. Physiological Characteristics of the Phosphate Solubilizing Rhizobacteria

3.3.5.1. C and N-sources Utilization of the Isolates

The selected Phosphate solubilizing rhizospheric bacterial (PSRB) isolates of White lupin were able to utilize diverse sources of carbon (Figure 3.1). All the isolates consumed more than 85 % of the tested carbon source substrates. Isolates PSRL58 and PSRL118 were found to catabolize 33 (97 %) of the tested carbon sources, except Sorbose followed by isolates PSRL41 and PSRL67 that were found to utilize 32 (94 %) of carbohydrates with the exception of α – Methyl-D-Mannoside and Sorbose. The rest three isolates (PSRL38, PSRL60 and PSRL112) were also found to utilize 30 (88 %) of the tested carbohydrates without showing growth on the medium supplemented with Sodium Gluconate, Dulcitol, α –Methyl-D-Mannoside and Sorbose. All the tested isolates were unable to metabolize Sorbose as a sole source of carbon. Similarly, Dereje Haile et *al*., (2017) reported the presence of phosphate solubilizing rhizospheric bacterial isolates from roots of White lupin that utilize all the tested carbon sources. Nautiyal (2000) also argued that the bacterial strains that have a potential of utilizing a wide range of carbon sources are considered to be the most efficient ones as they can produce different organic acids that can lower the pH of the growth medium in order to enhance insoluble phosphate solubilization.

All the PSRB isolates were assessed for their utilization of nitrogen sources by using ten amino acids and all the tested amino acids were found to be assimilated by 3(43%) of isolates (PSRL41, PSRL58, and PSRL118) whereas PSRL38, PSRL67 and PSRL112 utilized 9 (90 %)

of the tested nitrogen source substrates with the exception of Glycine which was not utilized by these three PSRB isolates of White lupin (Figure 3.1). The only isolate that found to utilize relatively less N sources was isolate PSRL that utilized 8 (80 %) of the tested amino acids. Generally, PSRB isolates were found to utilize multiple sources of C and N that is assumed to be enhancing the phosphate solubilization potential of the isolates. Sharma *et al*., (2013) also explained C and N sources greatly influence the P solubilization potentials of phosphate solubilizing microbes (PSMs).

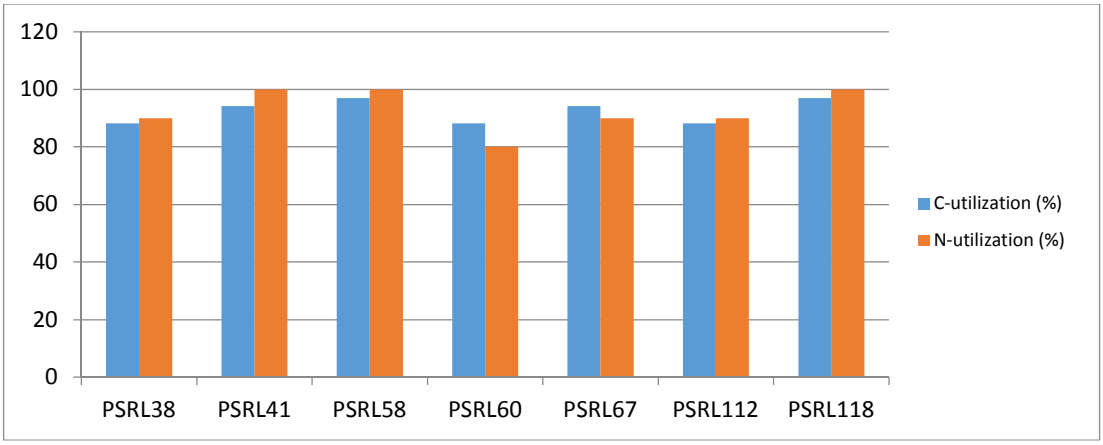


Figure 3. 1 C and N-sources utilization of PSRB

3.3.6. Stress induced phosphate solubilizing capacity of PSRB

The tested isolates showed different levels of TCP solubilization under different stresses at 5 days of incubation (Figure 3.2). All isolates released relatively high amount of P at 2% salt concentration with a sharp drop as concentration of salt increased indicating that moderate salt concentration may stimulate P solubilizing potential of PSB isolates. However, isolate PSRL41 showed efficient phosphate solubilization at relatively at wide range of salt concentration (2% to 8%) with the amount of solubilized phosphate exceeding 260µg/ml followed by isolate PSRL118 that released 263, 227 and 214 µg/ml of P at 2%, 3% and 5% NaCl concentration in the same order. Isolate PSRL67 exhibited relatively minimum P solubilization potential through all salt concentrations followed by isolate PSRL60 (Figure 3.2).

Regarding the effect of temperature on P solubilization, all the isolates showed increased levels of P solubilization at the temperature ranges from 15 ^{0}C to 35 ^{0}C and lower temperature (10 ^{0}C) clearly inhibited their P solubilizing potential, except isolates PSRL41 and PSRL118 that showed maximum P solubilization potential (127µg/ml and 138µg/ml, respectively). Higher temperature (45 ^{0}C) never showed negative impacts on P solubilization efficiency of isolate PSRL41 as it released maximum P (194µg/ml) at this temperature. Isolate PSRL118 was also the second-high temperature resistant P solubilizing rhizospheric bacteria that showed a steady increment in the amount of solubilized P (138µg/ml to 162µg/ml) at 10 ^{0}C to 35 ^{0}C with slight decrease at 45 ^{0}C (154µg/ml) and minimum P (22µg/ml and 12µg/ml was released by isolate PSRL67 at both lower and elevated temperatures (10 ^{0}C and 45 ^{0}C), respectively.

Concerning the impact of pH, all isolates showed maximum production of soluble phosphate at pH 8 with progressive decrease at pH values 10 and 12. Isolate PSRL41 released maximum amount of P (394µg/ml) at pH 8 followed by isolates PSRL118, PSRL58 and PSRL60 that produced 262, 212, and 179µg/ml of P at the same pH. Moreover, PSRL41 was the only isolate that released significantly higher soluble P (123µg/ml) at pH 4 showing that this isolate had better tolerance to acidic and alkali stresses than the others. In contrary, isolate PSRL67 was found to be the least acidic and alkali tolerant bacteria as it released only 20 and 17 µg/ml of phosphate at pH 4 and pH 12, respectively.

Phosphate solubilizing rhizobacteria are always challenged with numerous environmental stresses. The ability to tolerate adverse environmental factors such as high salinity, high/low pH and temperature is highly important not only for the survival of rhizobacteria in agricultural soils, but also to be used as biofertilizer (Banerjee *et al*., 2010). In this study, we found two promising isolates (PSRL41 and PSRL118) that had high P solubilizing potential at wide range of salt concentrations (2% to 8%), temperature (15 ^{0}C to 45 ^{0}C) and pH 8. They had positive application prospects as biofertilizers in saline–alkaline soils and high temperature

environments. However, Dereje Haile *et al*., (2016), reported one PSB isolate (HUPSB-57) isolated from the rhizospheric soil of White lupin (*Lupinus albus* L.) that showed salt tolerance up to 10% and another isolate (HUPSB-27) that showed growth at wide range of temperature (10 ^{0}C to 40 ^{0}C) at all tested pH values (4-10).

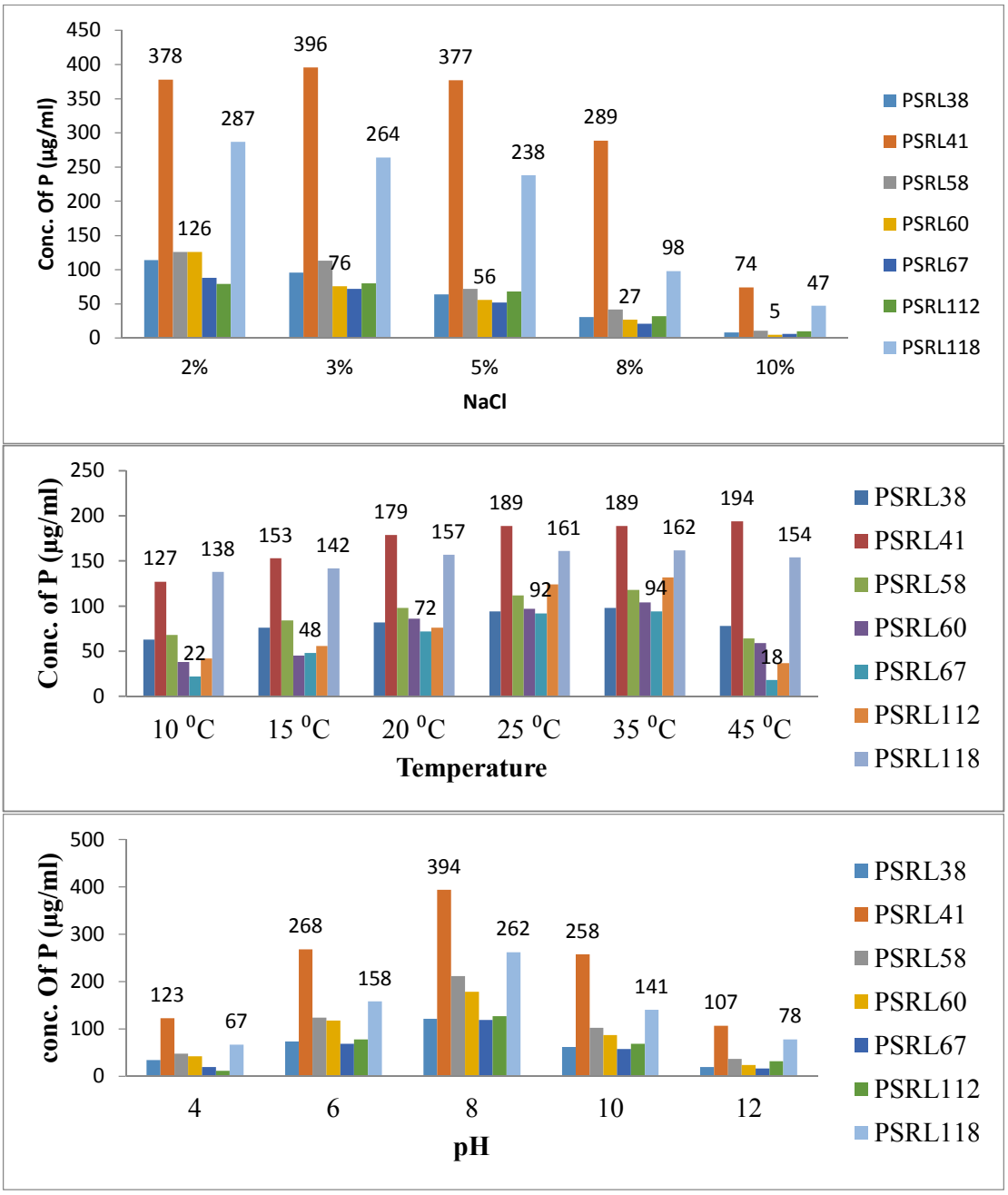


Figure 3. 2 Stress induced solubilization of TCP in liquid PK medium. Each value is the mean of three replicates.

3.3.7. Multiple PGP characteristics of the selected PSB isolates from White lupin rhizosphere

In addition to phosphate solubilization activity, all the selected PSB isolates obtained from the rhizosphere of White lupin were studied for their other PGP traits such as antifungal activity, IAA, siderophore, HCN and ammonia production (Table 3. 5).

Plant growth promoting hormone, IAA was determined by using L-tryptophan supplemented nutrient broth under laboratory conditions. Among the tested isolates 5 (71 %) of them produced IAA ranging from 12.3 to 55.8µg/ml. Maximum IAA production (55.8µg/ml) was obtained from isolate PSRL41 followed by PSRL118 and PSRL38 that produced 42.7 and 27.1µg/ml IAA, respectively whereas minimum IAA production was recorded to be 12.3µg/ml followed by 16.7µg/ml which were produced by PSRL112 and PSRL67 in the same order. However, isolates PSRL58 and PSRL 67 never produced IAA in the growth medium supplemented with the precursor. Joseph *et al*. (2007) reported that IAA production was shown in all (100 %) of the PGP bacteria isolated from the rhizosphere of Chickpea, whereas Mulisa Jida *et al.* (2012) showed that only 26.8 % of the PSB isolated from the rhizospheric soils of Lentil produced IAA in the range of 14 – 48 µg/ml and Routray and Khanna (2018) also reported that 25 % of rhizobacterial isolates from Mung bean produced 6.06 – 32.13 µg/ml of IAA indicating variations in the production of IAA by PSB isolates from different host plants.

Table 3. 5 Multiple PGP Characteristics of the Selected PSB Isolates from White Lupin Rhizosphere

Isolates	IAA (µg/ml)	Siderophore production	HCN Production	*In vitro* Inhibition of *F. oxysporum* (%)	Ammonia Production
PSRL38	27.1±2.48[c]	+	_	-	+
PSRL 41	55.8±3.94[a]	+++	++	62.5±2.56[a]	+
PSRL 58	_	-	-	-	+
PSRL 60	16,7±2.34[d]	-	_	-	+
PSRL 67	_	+	+	18.5 ±2.34[c]	+
PSRL 112	12.3±1.07[d]	++	+	14.6±1.38[c]	+
PSRL 118	42.7±2.34[b]	+++	++	39.7 ±4.14[b]	+

Production of Siderophore by the selected PSB isolates was tested qualitatively using Chrome Azurol S (CAS) agar medium and 5(71 %) of the isolates exhibited production of siderophores which are likely play an important role in the acquisition of iron to the plants and suppression of fungal pathogens in the rhizosphere by chelating iron. Various researches indicated that PSB isolates have different potential of siderophore production. Aarab *et al*. (2013) reported that none of the bacterial isolates from the rhizosphere of *Lupinus hirsutus* L produced siderophores. However, Joseph *et al*. (2007) showed that about 74.2 % of *Pseudomonas spp.* isolated from rhizospheric soils of Chickpea produced Siderophores. Dastager *et al*., (2010) also explained that production of siderophores is another most important trait of plant growth promoting microbes for healthy plant growth by suppressing fungal pathogens via iron chelating and augmenting the availability of P by the solubilization of iron-bound phosphate. Moreover, the production of siderophores by PGPR and their importance in maintaining heath plant growth by making iron unavailable to the phytopathogens was also reported by Ahmad *et al*., (2008).

Regarding the antifungal activity of the selected PSB isolates, PSRL41, PSRL67, PSRL112, and PSRL118 showed inhibition activity against *F. oxysporum* ranging from 14.6 to 62.5 % inhibition (Table 3.5). PSRL41 induced larger inhibition (62.5%) against the tested pathogenic fungus followed by PSRL118 that showed 39.7% of inhibition (Table 3.5). Isolates PSRL 67 and PSRL112 induced 18.5 and 14.6% inhibition, respectively whereas the rest 3 (42.9%) of the isolates failed to induce antifungal activity at all. The results of this study indicated that the antifungal activity of the selected test isolates revealed a close relationship between the production of siderophores and HCN because Siderophore and HCN production and antifungal activities were simultaneously exhibited by isolates PSRL41, LUR67, LUR112 and LUR118, except isolate PSRL38 that showed moderate siderophore production but not HCN production and antifungal activity.

Ahmad *et al*., (2008) showed that antifungal activity of the bacterial isolates might be due to the production of siderophores and HCN or synergistic interaction of these two or other metabolites. Moreover, Diby (2004) reported that production of HCN along with siderophore is the major cause of biocontrol activity for protection of Black pepper and ginger.

Ammonia released by PGPR is another most important trait which directly or indirectly benefits plant growth. All the tested PSB isolates were able to produce ammonia (Table 3.5). Ammonia production can help in satisfying the nitrogen demand of host plant and in excess reduces the colonization of plants by pathogens (Babalola, 2010, Mbai *et al*., 2013).

To sum up, the selected phosphate solubilizing bacterial isolates showed variations in their cultural characteristics, doubling times, gram reactions and C and N-sources utilization as well as in the amount of phosphate they solubilized from different inorganic phosphate sources. Some of the isolates showed better solubilization potential of tricalcium, aluminum and iron phosphates in Pikosvikya broth upon 5 days of incubation. In addition, they showed good TCP

solubilization potential under different stress conditions, such as various salt concentrations, pH and temperature ranges. Moreover, most of them showed multiple plant growth promoting characteristics: Indole acetic acid, siderophore, hydrogen cyanide and ammonia production and antagonistic activity against *fusarium oxysporum* under *in vitro* conditions. Two isolates (PSRL41 and PSRL118) showed significantly ($P< 0.05$) better multiple plant growth traits in addition to their good phosphate solubilization ability under stressed conditions. Hence, they are highly recommended to be used as inoculants under greenhouse and field conditions.

Chapter 4 Response of White Lupin (*lupinus albus* L.) to Single and Mixed Inoculation with Rhizobia and PSRB of North-west Ethiopia under Field Conditions

Abstract

White lupin (*Lupinus albus* L) is a legume with great agronomic potential for forage and human consumption. However, its yield is low and inconsistent as it is mostly produced with minimum agronomic practices. The present study was conducted to evaluate the growth response of White lupin to single and co-inoculation with the selected rhizobial and phosphate solubilizing rhizospheric bacterial (PSRB) isolates under greenhouse and field conditions. The field experiment was carried out at the Farmers Training Center (FTC) of Achefer district in Western Gojam. Three rhizobial (LUR12, LUR80 and LUR87) and two phosphate solubilizing rhizospheric bacterial (PSRL41 and PSRL118) isolates were selected for the experiment under greenhouse and field conditions. Accordingly, co-inoculated white lupin plants resulted in significantly ($P < 0.05$) higher nodule number, nodule dry weight, shoot dry matter accumulation, percent nitrogen and relative symbiotic effectiveness by 66.2 %, 95.8 %, 43.5%, 56.5 % and 43.5 % than single rhizobial inoculation in the same order under greenhouse conditions on sand culture. Moreover, Co-inoculated treatments generally showed an average increase in nodule number, nodule dry weight, shoot dry weight and percent nitrogen by 35%, 70%, 17% and 22% on Achefer soil and 40%, 107%, 20% and 11% on Holeta soil, respectively compared to single inoculated treatments under greenhouse. Similarly, both single and mixed inoculants showed markedly improved symbio-agronomic performances under field conditions in which plants co-inoculated with LUR87 and PSRL41 induced significantly ($P < 0.05$) higher nodule number (67 plant-1), nodule dry weight (0.40 g plant-1), shoot dry weight (5.83g plant-1) percent nitrogen (5.74 %) and grain yield (8691 kg ha-1) compared to single inoculated, negative and positive treatments under field conditions. In general, the study showed that agronomic performance of White lupin was significantly improved by either single or mixed inoculations and it is attributed to be an alternative method to enhance production of the crop in the country, where farmers cannot afford to buy chemical fertilizers.

Key words: Agronomic performance, Co-inoculation, Grain yield, PSRL

4.1. Introduction

Pulse crops are the second most important food crops produced in different amounts next to cereals both in area coverage and volume of production in Ethiopia (ECSA, 2015). Various legume species, including faba bean (*Vicia faba* L.), field pea (*Pisum sativum* L.), chickpea (*Cicer arietinum* L.), grass pea (*Lathyrus sativus* L.), and white lupin (*Lupinus albus* L.) are widely grown by farmers in the north western parts of the country (ECSA, 2015). Mixed crop-livestock production and rotation and/or fallowing are the typical farming systems used to regain soil fertility in the country.

Lupinus is a leguminous crop assumed to be originated in the Mediterranean region and distributed to the different parts of the world including Ethiopia along the Nile valley. The crop is grown in elevations between 1500 – 3000 m.a.s.l and it prefers well drained, light to medium textured and moderately acidic or neutral soils with a pH range of 4.5–7.5 (Hibistu Azeze, 2016). It is grown for green manuring, forage, human consumption and health. White lupin (*Lupinus albus* L.) is a traditional legume crop in Ethiopia that is produced for thousands of years in smallholder farming systems mainly for its grain and soil fertility maintenance values importances (Yeheyis Likawent *et al*., 2010, Zerihun Nigusie, 2012, Yilkal Tadele, 2015)

Intensive farming practices to achieve high yields require continuous application of chemical fertilizers that are costly and major causes of severe environmental problems. This regular application of chemical fertilizers in agro-ecosystems is currently under debate due to environmental concern and fear of consumer's health. Consequently, in order to augment the natural fertility of soils and develop better approaches to minimize the need for chemical fertilizers and pesticides, application of microbial inoculants or plant growth promoting rhizobacteria for the enhancement of sustainable agricultural production is becoming a more cost effective and environmentally-friend alternative in intensive agricultural practices in many

parts of the World (Majeed *et al*., 2015). Lupins obtain their nitrogen sources through symbiotic association with nitrogen fixing *Rhizobia* can fix and accumulate a total of 145 to 208 Kg N/ha/year (Jansen, 2006). This denotes that lupins can play complementary or alternative roles as sources of organic fertilizers to replenish nitrogen depleted soils in farming systems as they form symbiotic associations with various plant growth promoting rhizobacteria that have atmospheric nitrogen fixation and other plant growth promoting abilities.

Apart from nitrogen fixation, other plant growth promoting rhizobacteria are found in the rhizosphere to enhance plant growth and development (Dasgupta *et al*., 2015). *Rhizobia* and plant growth promoting rhizospheric bacteria can enhance the growth and development of plants through several mechanisms. Many reports indicated that bioinoculants have been shown to increase yield, health and nodulation of various leguminous plants when co-inoculated with plant growth promoting bacteria, compared to inoculation with rhizobium alone under field conditions. Stajkovic *et al*. (2011) indicated that co- inoculation with potential plant growth-promoting rhizobia, increases growth and yield of large number of plants including legumes as PGPB can increase nitrogen uptake, synthesis of phytohormones, solubilization of insoluble mineral salts and iron chelation. They also play a great role in the suppression of soil-borne pathogens by producing antimicrobial metabolites such as HCN and ammonia or competing for nutrients and/or niches (Stajkovic *et al*., 2011, Marinkovic *et al.,* 2016).

Therefore, application of effective bacterial strains as biofertilizers to improve legume production is an important approach in sustainable agriculture as they can provide nitrogen through nitrogen fixation and can make insoluble mineral salts readily available to the plants (Sánchez *et al*., 2014). Hence, production of cheaper and environmentally friend bioinoculants from native rhizobia and plant growth promoting rhizospheric bacteria of legumes such as

White lupin that have high capacity of nitrogen fixation and multiple plant growth promoting properties is one of the alternatives for increasing agricultural production. However, no research has been attempted so far on the production of bioinoculants from symbiotic bacteria of White lupin in Ethiopia. The main objective of this study, therefore, was to evaluate the effects of rhizobia and phosphate solubilizing rhizospheric bacteria of White lupin on its growth and yield under greenhouse and field conditions.

4.2. Materials and Methods

4.2.1. Sources of Rhizobial and Phosphate Solubilizing Rhizospheric Bacterial Isolates

Rhizobial and phosphate solubilizing rhizospheric bacterial isolates were previously collected from the nodules and rhizospheric soils of White lupin from North-western parts of Ethiopia and preserved at -80 ^{0}C with 50% glycerol in Addis Ababa University applied microbiology laboratory for further studies. Rhizobium isolates (LUR12, LUR80 and LUR87) and two phosphate solubilizing rhizospheric bacterial isolates (PSRL41 and PSRL118) that showed better PGP traits, stress tolerance and heterotrophic competence were selected for this study under greenhouse and field conditions (table 4.1).

Table4. 1 Physiological and PGP traits of the selected isolates for field Trial

Physiological and PGP traits		Isolates				
		LUR12	LUR80	LUR87	PSRL41	PSRL118
MGT (hrs)		2.3	3.8	5.6	3.2	2.8
Stress Tolerance	Salt tolerance (%)	1-5	1-6	1-5	2-8	2-5
	pH tolerance	6-9	6-9	5-8	4-10	4-10
	Temp. tolerance (^{0}C)	10-45	10-35	5-45	10-45	10-45
	IAR (%)	4(50)	3 (38)	4 (50)	5 (63)	4 (50)
	HMT (%)	3 (43)	3 (43)	4 (57)	5 (71)	6 (88)
Heterotrophic Competence	C-source (%)	82.4	85.3	79.4	94.1	97
	N-source (%)	63.6	81.8	63.6	100	100
Plant growth promoting traits	P solubilization (TCP)	+	-	+	383±0.61 a	313±1.78b
	IAA production (µg/ml)	32.6±2.63^{ab}	19.7±1.49^{ab}	-	39.7 ±4.14b	42.7±2.34 b
	Siderophore production	+	+	+	+++	+++
	HCN production	-	-	-	++	++
	Antagonistic Activity	-	-	-	62.5±2.56a	39.7 ±4.14b
RSE (Sand culture) (%)		99.2	100	100.8	-	-

MGT = Mean generation time, IAR = Intrinsic antibiotic resistance, HMT = Heavy metal tolerance, RSE = Relative symbiotic effectiveness.

4.2.2. Effect of single and co-inoculation on growth and nodulation of White lupin Under Greenhouse

The effect of single or co-inoculation of the isolates on the growth and nodulation of White lupin was examined using sterilized sand under greenhouse conditions according to Somasegaran and Hoben (1994). River sand obtained from Ethiopian National Soil Testing Center (ENSTC) was immersed in 1N H_2SO_4 and repeatedly washed by tap water to remove the residual acid. Then, the sand was autoclaved using autoclavable plastic bags at 121 ^{0}C for an hour and cooled to room temperature. Then, 3kg of sand was transferred into 5kg capacity plastic pots which were surface sterilized with 70% ethanol according to Subba Rao (1999).

White lupin seeds of uniform size and color from Achefer district were surface sterilized with70% ethanol and 3% hypochlorite. After repeated washing, seeds were allowed to germinate on 1% water agar and inoculated either with rhizobial isolates alone, or in combination with phosphate solubilizing bacterial (PSRB) isolates in the ratio of 1:1 that were grown on YEM and nutrient broths, respectively to their exponential phase and adjusted to10^9 cfu/ml. Then, six inoculated seedlings were planted per pot that were later thinned down to three after two weeks. The seedlings were inoculated with single and co-inoculations as follows.

T_1. Seed + LUR87
T_2. Seed + LUR12
T_3. Seed + LUR80
T_4. Seed + LUR87 + PSRL41
T_5. Seed + LUR87 +PSRL118
T_6. Seed + LUR12 +PSRL41
T_7. Seed + LUR12 + PSRL118
T_8. Seed + LUR80 +PSRL41
T_9. Seed + LUR80 + PSRL118
T_{10}. Non-inoculated seed + N fertilizer (+ve control)
T_{11}.Non-inoculated seed + No N source (-ve control)

The experiment was designed with inoculated treatments with three replications in a randomized complete block design (RCBD) system. The experiment included controls without inoculation, but with N source (+ve control) and with no N-source and inoculation (-ve control)) for comparison. All pots were treated with N-free plant nutrients except the positive control pots that were treated with nutrient containing 70 mg L^{-1} of KNO_3 as nitrogen source twice a week for three months (Broughton and Dilworth, 1971). Then, plants were harvested after ninety days to record the symbiotic properties, such as nodule number (NN), nodule dry weight (NDW), shoots dry weight (SDW), and relative symbiotic effectiveness (RE) according to Date (1993) as:

$$RE = \frac{\text{Inoculated plants SDM}}{\text{+ve control plants SDM}} \text{ x } 100$$

Then, Symbiotic Effectiveness (SE) values of each isolate was rated as ineffective (≤35%), lowly-effective (35-50%), effective (51-80%) and highly effective for SE > 80%.

4.2.3. Symbiotic effectiveness of isolates on different soil conditions

The effect of single and co-inoculation of rhizobial and PSRB isolates on the growth of White lupin was studied in pot experiments under greenhouse conditions, using non-sterile soils. The bulk soil for pot experiment was collected from the farm lands of Achefer and Holeta districts. 3 kg of finely sieved and homogenized soil samples were filled in 5kg capacity of surface sterilized plastic pots (Somasegaran and Hoben, 1994). Before planting, the soils were watered to approximately 75% field capacity. Healthy selected seeds of white lupin were surface sterilized, washed and germinated as before.

Cultures of rhizobial and PSRB isolates were prepared as before and 1ml of each culture suspension (10^{-9} cfu/ml) was flooded on each seedling for an hour. Then, six inoculated seedlings were planted on each pot, which were later thinned down to three. There were two control treatments: the negative control, which was without nitrogen and inoculants while the positive control was supplied with 70mg N/liter as a 0.05% KNO_3 (w/v) solution every week as described in Somasegaran and Hoben, (1994). The experiment was done in triplicates and arranged in Randomized Complete Block Design (RCBD). The pots were watered every two days to field capacity of 75%. After 90 days of growth, the whole plant was carefully uprooted to determine nodulation score, nodule dry weight, shoot dry mass and total nitrogen.

The selected chemical properties of the two soils were also determined after they were air dried, crushed and sieved by 2mm mesh sieve. Soil parameters determined were soil pH (1:2 soils to water ratio suspension), organic carbon (wet oxidation/dichromate digestion), total nitrogen (Modified micro-Kjeldhal procedure), C: N and available P (Olsen extraction method). All the parameters were determined following the manual produced by Sahlemedhin and Taye (2000).

4.2.4. Estimation of indigenous rhizobial population of the Soils

The population density of indigenous rhizobia from Achefer and Holeta soils which could nodulate *Lupinus albus* L. was determined by plant infection technique using growth pouches containing N-free sand as indicated by Somasegaran and Hoben (1994). The soils were selected on the basis of their cropping history in the past. In Achefer soil, the legume white lupin had been grown for the past many years whereas no white lupin had been grown in Holeta soil in history. Lupin seeds were surface sterilized, washed and germinated as before and stored at 28+2 ^{0}C. Fivefold soil serial dilutions (10^{-1} -10^{-5}) were prepared from both soils and 1ml of each dilution was applied to the roots of the test plants with four replications for each dilution. One uninoculated control pouch following each group of inoculated plants was used.

Observations for the presence of nodules were made after 45 days of planting and the most probable number (MPN) of indigenous rhizobia in each soil was calculated on the basis of positive nodulating units. Then the results were compared with standard most probable table (Vincent, 1970). The MPN was calculated by using the following formula:

$$X = \frac{m \times d}{V}$$

Where, X = MPN per gram of soil, m = Likely number from the MPN table for the lowest dilution of the series, d = lowest dilution (first unit used in the tabulation), v = volume of aliquot applied to plant.

4.2.5. Compatibility test and Preparation of inoculants

Compatibility of the selected rhizobial (LUR12, LUR80 and LUR87) and PSRB (PSRL41 and PSRL118) isolates was tested by spread plate method on YEMA medium according to Anandaraj and Delapierre (2010). The PSRB isolates were spot inoculated on the medium containing each rhizobium and incubated at 30 ^{0}C for 72 h. Then the absence of inhibition zone and growth of the spot inoculated PSRB indicated compatibility between the co-inoculants. The selected inoculants were prepared by injecting 40 ml of the actively grown broth cultures (10^9cfu/ml) of the rhizobial isolates into 130g of sterile peat in plastic bags. Simultaneously, the co-inoculants (rhizobial and phosphate solubilizing rhizospheric bacterial isolates) were prepared by injecting equal proportion of the rhizobial and the PSRB isolates into the bag containing 130g of the carrier material, sterilized peat. The bags were sealed, mixed thoroughly, and incubated at 28°C for two weeks.

4.2.6. Symbio-agronomic performance of the inoculants under field conditions

The Symbio-agronomic performance of the isolates under field conditions was performed at the farmers' training center of Achefer district (FTC) in Western Gojam zone from August to December, 2017. Some of the chemical properties of the soils of the study site were analyzed

at Ethiopian National Soil Testing Center and found to be: pH 5.8, organic carbon (%) 2.7, total nitrogen (%) 0.16, C: N 10.4 and available phosphate (AP) 3.7mg/kg.

The experimental field was ploughed and prepared with three blocks at 0.5m gap between them in which each block containing 11 plots and a randomized complete design block (RCBD) was used in the selected farm land in triplicate. The treatments included inoculation of the three rhizobial isolates (LUR12, LUR80 and LUR87) individually and dually with the selected two best phosphate solubilizing rhizospheric bacteria (PSRL41 and PSRL118). Uninoculated and nitrogen fertilizer supplied seeds were used as positive controls whereas both non-fertilized and non-inoculated seeds used as negative controls. A total of 11 treatments were used under greenhouse study. The positive controls were treated with 100 kg urea and 50kg phosphate fertilizer per hectare. The area of each experimental plot was 2 m by 3 m (6 m^2) with 1m space between them. The space between plants, rows, and blocks was 15 cm, 30 cm, and 0.5 m, respectively.

White lupin seeds were surface-sterilized and prepared as before and moistened with a 20% (w/v) solution of sucrose and mixed with the appropriate inoculants in a shade for immediate sowing (Yadagari *et al*., 2010). During planting, 1g of peat-based inoculants was mixed with 100g of White lupin seeds that were rinsed with 15% sucrose (Somasegaran and Hoben, 1994) and regular weeding was made as necessary.

4.2.7. Data collection from the field experiment

Field data were collected at two stages of plant growth. Growth characteristics such as nodule number (NN), nodule dry weight (NDW), shoot dry weight (SDW) and shoot total nitrogen content (SNC) were recorded after 90 days of emergence (at 50% of the crop's flowering stage) from 5 plants per plot and the average number for all samplings was calculated. The rest growth parameters such as number of pods per plant (PN), number of seeds per pods (SN), number of

seeds per plant, White lupin grain yields (GY) and 100 seed weight (HSW) were recorded at full maturity stage of White lupin. GY was measured as kg ha^{-1} based on 14% moisture content.

4.2.8. Plant Total Nitrogen Analysis

Soot total nitrogen content of the plants was quantitatively determined by the modified "Wet" Kjeldahl method which is based on the principle of treating plant material with concentrated H_2SO_4 to oxidize the nitrogen into ammonium sulphate. The mixtures of K_2SO_4 (10gm), $CuSO_4.5H_2O$ (2gm) and selenium (0.2gm) was used as catalyst and 2gm of the resulting mixture was added into the test tube containing 0.3gm of the crushed sample of each plant shoot. Seven ml of sulphuric acid and 3ml of hydrogen per oxide were added and allowed to react for 30 minutes. For blank, a mixture of 2gm of the above salt mixture, 7ml of sulphuric acid and 3ml of hydrogen per oxide was prepared and placed in a separate test tube. Then, digestion of the sample was carried out at 380 ^{0}C for 3hrs until a clear solution remained.

The digested sample was distilled by adding 40ml of 40% NaOH and ammonia liberated in the distillation process with NaOH was trapped by adding 20ml of boric acid (Sahlemedhin and Taye, 2000).

Eventually, the distillates were titrated by 0.1N H_2S0_4 and the percentage of total nitrogen was calculated using the following formula:

$$\text{Nitrogen (\%)} = \frac{(a - b)x\ N\ x\ 0.014}{S}\ x\ 100$$

Where, a = ml of $H_2\ S0_4$ required for titration of sample

b = ml of boric acid required for titration of blank

S = Sample weight in gm

N = Normality of H_2So_4 0.014= meq. Weight of nitrogen in mg

4.2.9. Data Analysis

All the collected field data were analyzed by one-way analysis of variance (ANOVA) and the treatment means were compared relative to controls following Duncan's test (DT) by using SPSS (Ver.20). The relation between different parameters was also evaluated by using Pearson correlation coefficient at $p \leq 0.05$.

4.3. Results and Discussions

4.3.1. Effect of Single and co-inoculation on the growth of White lupin on Sand culture under greenhouse conditions

Most rhizobial and PSRB isolates of White lupin never showed significant variations ($P<0.05$) in their symbiotic properties such as NN, NDW, SDW, and plant tissue nitrogen content (N %) in both single and co-inoculations on sand culture under greenhouse conditions (Table 4.2). The inoculated plants induced nodules in the range of 28.00 - 61.33 plant^{-1}showing more than twofold differences among them. Similarly, they produced NDW in the range of 0.10 – 0.38g/plant with a more than three-fold difference between the isolate accumulating the highest and the lowest NDW which were recorded from plants inoculated with LUR80 and LUR12 + PSRL41, respectively.

Table4. 2 Nodulation and relative symbiotic effectiveness of white lupin (*Lupinus albus* L.) rhizobial and PSB isolates after alone and co-inoculation on sand culture

Treatments	NN plant^{-1}	NDW (gm/plant)	SDW (gm/plant)	Shoot Nitrogen (%)	RE (%)
LUR12	40.33±1.86^{cd}	0.26±0.12^{b}	1.23±0.01^{f}	1.36±0.05^{f}	99.2
LUR80	28.00±0.58^{e}	0.10±0.06^{cd}	1.24±0.01^{e}	1.45±0.03^{f}	100.0
LUR87	31.67±0.88d^{e}	0.12±0.06^{c}	1.25±0.01^{e}	1.61±0.04^{e}	100.8
LUR12 + PSRL41	61.33±2.40^{a}	0.38±0.02^{a}	1.76±0.04^{bc}	2.16±0.01^{c}	142.0
LUR12 + PSRL118	56.67±1.45^{ab}	0.32±0.02^{ab}	1.67±0.03^{cd}	1.88±0.05^{d}	134.7
LUR80 +PSRL41	52.00±1.15^{ab}	0.33±0.02^{ab}	1.81±0.01^{b}	2.34±0.02^{b}	146.0
LUR80 + PSRL118	47.67±1.45^{bc}	0.30±0.02^{ab}	1.64±0.03^{d}	2.14±0.01^{c}	132.3
LUR87 + PSRL41	59.67±2.73^{a}	0.30±0.03^{ab}	1.96±0.01^{a}	2.87±0.03^{a}	158.1
LUR87 + PSRL118	55.00±4.04^{ab}	0.25±0.03^{b}	1.83±0.02^{b}	2.40±0.02^{b}	147.6
TN	0.00	0.00	1.24±0.01^{e}	1.36±0.02^{f}	100.0
T0	0.00	0.00	0.43±0.01^{f}	1.14±0.02^{g}	34.7

NN = Nodule number, NDW = Nodule dry weight, SDW = Shoot dry weight, SN = Shoot nitrogen content, RE = Relative effectiveness, TN = N-fertilized treatment, T0 = none fertilized control. Values in the same column with the same letters are not significantly different at $p\leq0.05$. Values are means ± standard deviation (SD) for triplicates.

Co-inoculation of LUR12 with PSRL41 produced the highest nodule number (61.33) compared to the plants inoculated with LUR12 alone (40.33), followed by the plants co-inoculated with LUR87 and PSRL41 that produced 59.67 nodules per plant compared to the number of nodules produced by the plants inoculated with LUR87 alone (31.67). In general, all the plants co-inoculated with the rhizobial and PSRB isolates indicated a significant ($P<0.05$) increase in nodule numbers compared to plants inoculated with rhizobial isolates alone.

Co-inoculation of LUR87 with PSRL41 revealed the highest increase (88.4%) in nodule number followed by the plants co-inoculated with LUR80 + PSRL41 (85.7%), LUR87 + PSRL118 (75.6%) and LUR80 + PSRL118 (70.25%) compared to the plants inoculated with the rhizobial isolates alone. However, co-inoculation of LUR12 with PSRL118 showed only 40.5% increment in nodule number followed by 52% increase by the plants co-inoculated with LUR12 and PSRL41. In general, single inoculated plants produce average NN of 33/plant while coinoculated plants produce average NN of 55 plant^{-1}.

This Increase in nodule number as a result of dual inoculation might be associated with proliferation in root length and branch due to additional plant growth substances provided by PSRB isolates in which more active sites for nodulation by the rhizobial isolates are created. Korir *et al*., (2017) reported a significant increase in nodule number as a result of co-inoculation of common bean with rhizobium and PGPR (*Bacillus megaterum*) and explained that this might be due to the improvement of plant growth as a result of extra merits obtained from the co-inoculant *Bacillus megaterum*. Similarly, Sharma *et al*., (2013) explained that some PGPR possess ability of phosphate solubilization and other plant growth promotion traits that could be useful in improvement of legume production by enhancing nodulation and N fixation.

In terms of nodule dry weight, the co-inoculated treatments showed significant variation ($p<0.05$) compared to plants inoculated with the rhizobial isolates alone and it was found

positively correlated with nodule number (r =0.927) scored by the inoculated plants. The highest nodule dry weight (0.38g/plant) which was 46.2% higher than the plants inoculated with LUR12 alone obtained from plants co-inoculated with LUR12 and PSRL41 followed by plants co-inoculated with LUR80 + PSRL41 (0.33g/plant), LUR12 + PSRL118 (0.32g/plant), LUR80 + PSRL118 (0.30g/plant), LUR87 + PSRL41 (0.30g/plant) and LUR87 + PSRL118 (0.25g/plant) that showed 230%, 23.1%, 200%, 150% and 108.3% increment of NDW compared to the non-coinoculated plants in that order. The average NDW of co-inoculated plants was o.31/plant which is almost twice as much as the NDW (0.16/plant) of single inoculated plants with rhizobia.

The positive effect of combined inoculation of rhizobium with PGPRB can be attributed to a general improvement in root development and an increase in the number of nodules which again results in the increase in the biomass accumulation of nodules (Sa *et al*., 2012).

Plants co-inoculated with rhizobial and the selected phosphate solubilizing rhizospheric bacterial isolates were found to accumulate a significantly ($P<0.05$) higher shoot mass as compared to positive controls and single inoculation. As indicated from table 4.2, the SDW of the inoculated plants was in the range of 1.23 - 1.96g/plant that indicated more than 50% difference between the highest and lowest SDW. The highest SDW (1.96g/plant) was recorded from plants co-inoculated with LUR87 and PSRL118 which was 57 % and 58.1% higher than the SDW obtained from LUR87 alone inoculation and positive control followed by PSRL118 that showed 46.4% and 47.6% increase in SDW compared to single inoculation, respectively and dual inoculation of LUR80 and PSRL41 showed 46% increase in SDW compared to both single inoculation and positive control. Relatively lower SDW increase was recorded from the plants co-inoculated with LUR12 + SRL118 (1.67g/plant) and LUR80 + PSRLPSRL118 (1.64g/plant) that showed 35.8% and 32.3% increase compared to single inoculation and 34.7%

and 32.3% more than the positive control. Generally, co-inoculated plants produced an average of 43 % more SDW compared to single inoculated plants.

In general, combined inoculation of rhizobium with PSRB boosted the shoot dry weights as compared to rhizobium inoculation alone and positive controls. All the co-inoculated treatments showed a significant increase (32.3% - 58.1%) in shoot dry mass accumulation compared to the positive controls and single inoculation. Mishra *et al.,* (2014) noted that enhancement legume nitrogen fixation by co-inoculation with PGPB is a way to improve plant nutrition and plant growth. Different research results also explained that high biomass accumulation is due to phosphate solubilization and release of other important plant growth promoting secondary metabolites by the co-inoculant rhizobacteria and nitrogen fixation by the rhizobia in response to the rich supplies of the substrates (Verma *et al*., 2012, Korir *et al.*, 2017).

All the co-inoculated plants showed significant ($p<0.05$) increase in their percent nitrogen accumulation that ranged from 1.88 %- 2.87 %. The co-inoculated plants showed an average increase of twice compared with the average nitrogen accumulation of single inoculated and positive control plants. Plants co-inoculated with LUR87 and PSRL41 accumulated significantly ($p<0.05$) the highest percent of nitrogen (2.87%) while co-inoculated plants with LUR12 + PSRL118 showed relatively lower percent N (1.88%) compared to the rest co-inoculated plants.

However, plants inoculated with rhizobium isolates alone never showed significant variation ($p<0.05$) in terms of the positive controls, except plants inoculated with LUR87 alone that showed statistically higher percent N that is 18.4% more than the positive control indicating co-inoculated PSRB isolates have positive effects on the enhancement of symbiotic performance of LUR87.

All the rhizobial isolates co-inoculated with the PSRB isolates showed 32 % - 57% increase in their relative symbiotic effectiveness compared to rhizobial inoculation alone and N-fertilized plants. The maximum RE was 158.1% scored by the co-inoculation of LUR87 with PSRL41 that showed 57 % higher relative symbiotic effectiveness compared to N-fertilized plants. However, the RE obtained from rhizobial inoculation alone was found to be almost the same as N-fertilized control and more than two-fold in terms of the negative control.

Various researches also indicated that co-inoculation of rhizobia with PGPR improves growth and yield compared to single inoculation as co-inoculated plants get better balanced nutrition, and improved absorption of nitrogen, phosphorus, and mineral nutrients (Araújo *et al*., 2009). Bashir *et al*., (2011) also explained that co-inoculation of rhizobia with PSB bacteria increases Phosphorous availability, the number and size of nodules and the amount of nitrogen assimilated per unit weight of nodules, increasing the percent nitrogen in the harvested portion of the host legume. In general, the results of this study indicated that co-inoculated white lupin plants resulted in higher NN, NDW, SDW, percent nitrogen and improved relative symbiotic effectiveness than single rhizobial inoculation, and N-fertilized plants which implies that co-inoculation is more advantageous over single inoculation on the improvement of growth parameters of plants.

4.3.3. Evaluation of Symbiotic effectiveness of Single and Co-inoculation on the Growth of White Lupin on Different Soils under Greenhouse Conditions

The symbiotic effectiveness of single and mixed inoculation with selected rhizobial isolates (LUR12, LUR80 and LUR87) and PSRB isolates (PSRL41 and PSRL118) on two different soils collected from Achefer (West Gojam) and Holeta (Oromia region) under greenhouse is shown in Table 4.3. The data showed that single and mixed inoculants markedly improved the nodulation, nodule dry weight, shoot dry weight and percent nitrogen of White lupin on both Achefer and Holeta soils compared to the uninoculated negative controls (Table 4.3).

Table 4. 3 Comparative effectiveness of single and co-inoculation of selected white lupin rhizobial PSRB isolates on Achefer and Holeta Soils

Soil sources	Treatments	NN plant^{-1}	NDW (g/plant)	SDW (g/plant)	Shoot total N (%)
Achefer	LUR12	50.67±4.33^{b-d}	0.27±0.01^{de}	2.66±0.03^{d}	2.54±0.04^{fg}
	LUR80	44.33±1.86^{d}	0.14±0.00^{f}	2.63±0.03^{d}	2.42±0.04^{g}
	LUR87	45.67±4.06^{cd}	0.18±0.01^{ef}	2.80±0.23^{b-d}	2.93±0.04^{d}
	LUR12 + PSRL41	68.67±4.06^{ab}	0.38±0.01^{a-c}	3.34±0.04^{ab}	3.36±0.32^{c}
	LUR12 + PSRL118	64.33±2.60^{abc}	0.33±0.03^{b-d}	3.27±0.01^{a-c}	2.98±0.13^{d}
	LUR80 +PSRL41	56.33±5.61^{a-d}	0.28±0.04^{cd}	2.73±0.06^{cd}	2.82±0.05^{de}
	LUR80 + PSRL118	53.33±1.86^{a-d}	0.28±0.04^{d}	2.68±0.03^{d}	2.67±0.21^{ef}
	LUR87 + PSRL41	70.67±2.40^{a}	0.42±0.01^{a}	3.77±0.07^{a}	3.92±0.04^{a}
	LUR87 + PSRL118	66.67±4.81^{ab}	0.39±0.00^{ab}	3.50±0.14^{a}	3.59±0.02^{ab}
	TN	19.33±2.91^{e}	0.09±0.02^{f}	3.24±0.05^{a-c}	3.66±0.03^{b}
	T0	42.00±4.16^{d}	0.14±0.00^{f}	2.27±0.11^{d}	2.63±0.08^{e-g}
Holeta	LUR12	44.00±1.15^{b-d}	0.18±0.01^{cd}	2.10±0.07^{cd}	2.18±0.06^{f}
	LUR80	32.33±1.45^{d}	0.11±0.00^{e}	1.90±0.12^{de}	2.23±0.34^{ef}
	LUR87	38.67±1.20^{cd}	0.14±0.00^{de}	2.18±0.04^{cd}	2.55±0.06^{d}
	LUR12 + PSRL41	60.33±3.38^{a}	0.33±0.02^{ab}	2.45±0.06^{bc}	2.51±0.04^{d}
	LUR12 + PSRL118	54.67±5.84^{ab}	0.27±0.01^{b}	2.32±0.15^{bc}	2.34±0.10^{e}
	LUR80 +PSRL41	53.00±1.53^{ab}	0.21±0.01^{c}	2.21±0.02^{cd}	3.23±0.16^{b}
	LUR80 + PSRL118	51.33±2.03^{ab}	0.19±0.00^{cd}	2.11±0.01^{cd}	3.18±0.02^{b}
	LUR87 + PSRL41	54.33±0.88^{ab}	0.38±0.03^{a}	3.07±0.07^{a}	3.68±0.04^{a}
	LUR87 + PSRL118	48.33±1.45^{bc}	0.35±0.00^{a}	2.71±0.04^{ab}	2.98±0.11^{c}
	TN	.00	.00	2.67±0.07^{ab}	3.63±0.30^{a}
	T0	.00	.00	1.53±0.12^{e}	1.85±0.06^{g}

NN = Nodule number, NDW = Nodule dry weight, SDW = Shoot dry weight, SN = Shoot nitrogen content, RE = Relative effectiveness, TN = N-fertilized treatment, T0 = none fertilized control. Values in the same column with the same letters are not significantly different at $p \leq 0.05$. Values are means ± standard deviation (SD) for triplicates.

Co-inoculation treatments generally showed an average increase in nodule numbers on both soils by 35 % - 51% compared to single inoculation treatments, and negative controls. The highest numbers of nodules (66.67 -70.67nodules plant$^{-1)}$ were formed on the root systems of White lupin inoculated with co-cultures of LUR87 + PSRL41, LUR12 + PSRL41 and LUR87 + PSRL118 in pot experiment on Achefer soils followed by plants co-inoculated with LUR12 + PSRL118 that formed 64.33 nodules plant^{-1}. Generally, inoculated plants indicated 20 % - 64 % increase in NN than rhizobium inoculation alone in in both soils.

Co-inoculation treatments on Holeta soils also induced relatively higher number of nodules over single inoculation treatments. Among the co-inoculated treatments, LUR80 + PSRL41 increased nodule numbers by 64% than LUR80 inoculation alone followed by co-inoculation of the same rhizobial isolate with PSRL118 that showed 58.8% increase over LUR80 inoculation alone. The rest mixed culture inoculation treatments (LUR12 + PSRL41, LUR12 + PSRL118, LUR87 + PSRL41 and LUR87 + PSRL118) showed 37.1%, 24.25%, 40.5% and 25% increment in nodule number compared to their respective single inoculation on pot cultures of Holeta soils.

Improvement of nodulation by the mixed cultures may be attributed to the provision of the rhizobial isolates with some synergistic substances such as IAA, solubilized phosphate, siderophores and other plant growth promoting substances that can enhance root development and proliferation for more infection site access for rhizobia. A number of evidences have been reported by several researchers on the enhancement of nodulation by PGPR as they create more infection sites on the root systems of legume plants (Wani *et al*., 2007, Verma *et al*., 2010, Badawi *et al* 2011, EI-Nahrawy and Omara, 2017). Moreover, Sa *et al.* (2012) also explained the positive effect of synergistic inoculation of endophytic bacteria with *Rhizobium spp*. in improving nodule numbers may be attributed to a general enhancement in root development as a result of extra merits obtained from effective endophytic bacteria.

Generally, Co-inoculation of rhizobial and PSRB isolates led to a significant increase in nodule dry weights compared to single inoculations, N-fertilized and negative controls.

The nodule dry mass of single and co-inoculated plants was in the range of 0.14 – 0.42gm/plant on pot cultures of Achefer and Holeta soils. Accordingly, co-inoculation of LUR87 with PSRL41 and LUR87 with PSRL118 induced the highest nodule dry weight (0.42gm and 0.39gm/plant on Achefer soil and 0.38 gm and 0.35gm/plant on Holeta soil, respectively)

followed by LUR12 + PSRL41 that induced 0.38gm and 0.33gm/plant nodule dry weights on Achefer and Holeta soils in the same order. On Achefer soils, co-inoculated treatments showed 41% to 133% and 100 % to 200% increment in NDW over rhizobium inoculation alone and negative controls, respectively, while 72.7% to 171% increment was obtained from Holeta soils compared to single inoculation treatments.

Regarding shoot dry weight, co-inoculation of rhizobium with PSRB isolates improved shoot dry matter production over rhizobium inoculation alone, negative and N-fertilized treatments on Achefer soils. The highest shoot dry matter production (3.77gm/plant) was obtained from the co-cultures of LUR87 and PSRL41 followed by 3.27gm/plant produced by the same rhizobium co-inoculated with PSRL118 on Achefer soils. Combined inoculations of LUR87 + PSRL41, LUR87 + PSRL118, LUR12 + PSRL41 and LUR12 + PSRL118 increased SDW by 44 – 66 % over uninoculated negative control and 1-16 % increase compared to N-fertilized control on Achefer soils. Moreover, all the co-inoculated treatments increased SDW in the range of 2% - 35% over rhizobium inoculation treatments alone.

Different research results also indicated that co-inoculation of some elite PGPB along with effective rhizobia stimulated nodulation, nitrogen fixation and growth of plants (Neumann and Laing, 2006, Verma *et al*., 2012). Rhizobia have been shown to greatly improve the productivity and quality of many legumes, when they are coinoculated with PGPRB. This synergistic effect may be revealed by their potential to enhance the N_2-fixation performance, as well as availability and uptake of nutrients from soil due to the production of substances like phytohormones, siderophores, phosphate solubilization and improvement of nutrients and water uptake (Badawi *et al*., 2011).

The combined inoculations of White lupin with rhizobial and PSRB isolates increased SDW of plants in respect to the rhizobium sole inoculation and uninoculated negative treatments on

pot cultures of Holeta soils. LUR87 + PSRL41 recorded a significantly ($p < 0.05$) higher SDW (3.07gm/) compared to LUR87 alone (2.18gm/plant) and uninoculated negative control (1.53gm/plant) followed by LUR87 + PSRL118 that produced 2.71gm of SDW per plant. Generally, treatments consisting LUR87 + PSRL41 induced 40.8%, 100.7% and 15% SDW over the sole rhizobium inoculation, negative and positive treatments for the same order while LUR87 + PSRL118 surpassed the rhizobium inoculation alone, negative and positive treatments by 24.3%, 77% and 1.5%, respectively. Moreover, combined treatments of LUR12 + PSRL41, LUR12 + PSRL118, LUR80 + PSRL41 and LUR80 + PSRL118 showed 10.5% - 16.7% and 38% - 60% increase in SDW over sole rhizobium inoculation and negative treatments in the same order, while rhizobium alone inoculation treatments revealed 24.2% - 42.5% SDW increment compared to the negative treatments on pot cultures of Holeta soils.

However, compared to shoot dry mass accumulations obtained from Achefer soils, all the single as well as co-inoculation treatments found to induce lesser SDW accumulation by 22% - 48% on pot cultures of Holeta soils indicating that the performance of inoculants could be associated with soil characteristics such as biotic and abiotic factors of soil environment. Several studies showed that various biotic and abiotic environmental factors can influence symbiotic establishment of rhizobia and PGPRB with the host plant and reducing the survival of bacteria in the soil (Nascimento *et al*., 2012, Sanchez *et al*., 2014).

White lupin plants inoculated with LUR87 + PSRL118 were found to produce significantly ($p <0.05$) highest shoot nitrogen content (3.92 % on Achefer soil and 3.68 % on Holeta soil) which showed a 7.1% - 49 % and 1.4% - 99% increase on Achefer and Holeta soils, respectively compared to rhizobium alone inoculation, negative and positive treatments followed by LUR87 + PSRL118 and LUR80 + PSRL41 that produced 3.59% and 3.23% shoot nitrogen on pot cultures of Achefer and Holeta soils in the same order. This variation in

shoot N (%) among the inoculants could be associated with differences in adapting the soil environment at which inoculation is carried out. Khare and Arora (2014) reported that different physical, chemical and biotic factors of the soil can influence the performance and population dynamics of inoculants. The same authors also explained that trophic competitions and antagonistic interactions with the native microbial and fauna populations determine the efficacy of inoculants in various soils. Earlier works by Jensen *et al*. (2005) and MA *et al*. (2004) also reported that the competition potential of the inoculants and adaptation to the stress during early symbiotic interactions in the new environment have been shown to contribute for the variations of the outcomes of N-fixation.

4.3.4. Symbiotic effectiveness of Single and Co-inoculants in Sand and soil pot cultures

Relative symbiotic effectiveness of the single and co-inoculation of the selected isolates was tested on pot sand and soil cultures under greenhouse condition. Accordingly, inoculations with LUR87, LUR12 + PSRL41, LUR80 + PSRL41, LUR87 +PSRL41 and LUR87 + PSRL118 revealed highly effective symbiosis (HE) on pot sand and soil cultures (Table 4. 4).

Table 4. 4 Relative symbiotic effectiveness of single and co-inoculants on sand and soil cultures under greenhouse conditions

Inoculants	Sand culture	Achefer Soil	Holeta Soil
LUR12	HE	HE	E
LUR80	HE	HE	E
LUR87	HE	HE	HE
LUR12 + PSRL41	HE	HE	HE
LUR12 + PSRL118	HE	HE	HE
LUR80 +PSRL41	HE	HE	HE
LUR80 + PSRL118	HE	HE	E
LUR87 + PSRL41	HE	HE	HE
LUR87 + PSRL118	HE	HE	HE

HE = highly effective (RSE ≥ 80), E = effective (RSE = 50 < x < 80)

All the single and co-inoculations formed highly effective (RSE ≥ 80) symbiosis on pot sand and soil cultures of Achefer soil. However, single inoculation of LUR12 and LUR80 and co-inoculation of LUR80 with PSRl118 were found to be effective (RSE = 50 < x < 80) on the pot cultures of Holeta soils. The decline in the symbiotic effectiveness of the inoculants (LUR12, LUR80 and LUR80 + PSRL118) on Holeta soils may be attributed to the decline in the size of populations of active cells due to physical, chemical, and physicochemical nature of the soil and influence of indigenous microorganisms on the inoculant population both quantitatively and qualitatively.

Regarding the effects of soil environment on the efficacy of microbial inoculants, Khare and Arora (2014) explained that soil abiotic as well as biotic factors exert their direct impact on inoculant population dynamics and success on their symbiotic performance by imposing stresses. Skei-Boahen *et al*., (2017) also mentioned that the competitive advantage of microbial inoculants may depend on the characteristics such as tolerance to drought, high temperature, low pH and other soil factors.

4.3.5. Effects of Single and co-inoculation on the Performances of White lupin under field conditions

4.3.5.1. Effect of Single and Co-inoculation on Nodulation and Biomass Production of White lupin (*Lupinus albus* L.) under Field conditions

Inoculation of White lupin plants either with the selected rhizobial isolates alone or co-inoculation of rhizobial and PSRB isolates showed improved performance on the nodulation and other growth parameters of the crop, such as NDW, SDW and SNC (N %) compared to the uninoculated and non-fertilized as well as fertilized controls (Table4. 5).

Table 4. 5 Mean symbiotic performance of inoculants on White lupin under field conditions at Achefer District

Treatments	NN/plant	NDW (gm/plant)	SDW (gm/plant)	SNC (N %)
LUR12	43.7±3.7^{cd}	0.24±0.07^{bc}	3.48±0.35^{d}	3.26±0.43^{cd}
LUR80	36.3±4.2^{d}	0.12±0.03^{c-e}	2.91±0.10^{de}	3.17±0.30^{cd}
LUR87	48.4±2.4^{cd}	0.21±0.02^{b-d}	4.42±0.31^{bc}	4.23±0.17^{bc}
LUR12 + PSRL41	54.3±2.8^{bc}	0.29±0.04^{ab}	3.83±0.12^{cd}	3.85±0.38^{bc}
LUR12 + PSRL118	48.5±4.6cd	0.27±0.04^{ab}	3.64±0.31^{cd}	3.46±0.56^{cd}
LUR80 +PSRL41	50.8±6.c3^{c}	0.27±0.07^{ab}	3.13±0.14^{de}	3.37±0.65^{cd}
LUR80 + PSRL118	46.5±2.5^{cd}	0.25±0.03^{bc}	2.97±0.21^{de}	3.24±0.32^{cd}
LUR87 + PSRL41	67.4±5.6^{a}	0.40±0.02^{a}	5.83±0.44^{a}	5.74±0.50^{a}
LUR87 + PSRL118	62.8±2.5^{ab}	0.34±0.07^{ab}	5.18±0.22^{ab}	4.83±0.26^{ab}
TN	12.3±1.9^{e}	0.06±0.02^{e}	5.13±0.19^{ab}	5.02±0.40^{ab}
T0	15.6±3.1^{e}	0.09±0.01de	2.46±0.46^{e}	2.44±0.34^{d}

NN = nodule number, NDW = nodule dry weight; SDW = shoot dry weight; SNC = shoot total nitrogen content. Values within a column sharing the same letter are statistically non-significant at $P \leq 0.05$.

In respect to nodulation, the NN of plants inoculated with single or mixed cultures was found to be in the range of 36.3 to 67.4 per plant (average 51). Plants inoculated with rhizobial alone or mixtures of rhizobial and PSRB isolates revealed statistically significant ($P \leq 0.05$) increase in NN (about 3 to 4.5 times higher) than the plants nodulated by indigenous rhizobia (positive and negative control plants). However, there was no significant variation ($P \leq 0.05$) in NN between most plants inoculated with rhizobium alone and mixed cultures. However, co-inoculation of White lupin plants with LUR87 and PSRL41 was found to induce significantly ($P \leq 0.05$) the

highest NN (67.4 nodules plant^{-1}) followed by plants co-inoculated with LUR87 and PSRL118 that produced 62.8 nodules plant^{-1} compared to the rest plants (Table 4.5).

The highest nodule number (67.4 nodules plant^{-1}) recorded from the co-inoculation of LUR87 and PSRL41 exceeded NN of the rest single and co-inoculated plants by 7.3 % - 85.7 %, whereas NN obtained from the co-inoculation of LUR87 with PSRL118 surpassed other single and co-inoculated plants by 15.7 % - 73% indicating that co-inoculation of LUR87 with two phosphate solubilizing rhizospheric bacterial isolates (PSRL41 and PSRL118) performed better compared to the rest inoculants. Moreover, high positive correlation (r = 0.853) was noted between NN and Nodule dry weight (NDW) of both single and co-inoculated plants.

Although inoculation of plants did not indicate significant variation ($p \leq 0.05$) in NN and NDW between single and co-inoculated plants, all the plants revealed increased NN and NDW due to co-inoculation of the rhizobium with PSRB isolates in respect to inoculation with rhizobium alone in White lupin. However, compared to the positive and negative controls, all the single and combined inoculated plants indicated statistically significant ($P \leq 0.05$) higher NN and NDW. The NDW of both single and dual inoculated plants was in the range of 0.12 – 0.40gm per plant that was about four-fold difference between the lowest and the highest NDW (Table 4.5). The highest NDW (0.40gm plant^{-1}) was induced by the co-inoculation of LUR 87 with PSRL41 followed by 0.34gm plant^{-1} obtained from the co-inoculation of the same rhizobium isolate with PSRL118 (Table 4.5).

In general, the study revealed increased number of nodules and nodule dry weight as a result of combined inoculation of rhizobial and PSRB isolates compared to single inoculation, positive and negative controls. The positive effect of combined inoculation of effective PSRB with rhizobial isolates can be attributed to enhancement of early nodulation or increase in the numbers of nodules due to improvements in root development resulted from easily access of

soil nutrients such as Fe and P as well as secretion of growth promoting hormones facilitated by the synergy of co-inoculants (Sa *et al*., 2012, Korir *et al*., 2017). Moreover, Stajkovic *et al*., (2011) reported that mixed inoculation of legumes such as common bean plants, with *Rhizobium Spp*. and effective *Bacillus* strains positively improved nodule number (5.6 % - 96.3%) as well as nodule dry weight (2.7 % - 70 %))than rhizobium alone inoculation.

Similarly, Htwe *et al*., (2018) mentioned that co-inoculation of nitrogen fixing bacteria (*Bradyrhizobium japonicum S*AY3 with *Streptomyces griseoflavus* P4 from Soybean) has become more common than single inoculation with rhizobia or PGPRB because of their synergic advantage in improving nitrogen fixation and crop yield. The same authors also explained that the improvement of nodulation in co-inoculated treatments may be associated with the competitive advantage of inoculated stains over the indigenous rhizobia for nodule formation.

With regard to shoot dry weight (SDW) and shoot nitrogen accumulation (SNC), plants inoculated either with rhizobium alone or dual inoculation achieved higher SDW and SNC than the negative control plants indicating their nitrogen fixing potential. Co-inoculation of White lupin plants with LUR87 and PSRl41 significantly increased ($p \leq 0.05$) SDW and SNC of plants by 32 % – 137 % and 36 % - 135 % compared to the other single, co-inoculated and negative control plants in the same order followed by plants co-inoculated with LUR87 + PSRL118 that increased SDW and SNC by 17.2 % – 110.5 %

and 14.2 % - 98 %, respectively**.** Moreover, plants co-inoculated with LUR87 + PSRL41 showed significant increase in SDW and SNC over the N-fertilized control plants by 13.6 % and 14.3 % in the same order.

As the selected PSRB (PSRL41 and PSRL118) possess the potential of plant growth promoting traits (P solubilization, Fe chelation through siderophore production, IAA production), they could be useful in White lupin production improvement via boosting the availability of nutrients (P and Fe) in the soil as well as production of plant growth hormones. Marinkovic *et al*. (2016) reported that NN, NDW and SDW of co-inoculated Soybean plants showed a marked mean increase by 76.1 %, 51.6 % and 32.4 % over the negative treatment plants. Moreover, Girmaye Keneni and Fassil Assefa (2017) reported that the average percent nitrogen of Cowpea (2.9%) as a result co-inoculation was higher than single inoculated and negative as well as positive treatment plants indicating that effective PGPR have enormous potential in the formulation of bioinoculants to be used in enhancing the performance of rhizobia by increasing nutrient availability in the soil that in turn improve crop yield.

4.3.5.2. Effect of Single and Co-inoculation on the Mean Performance of Inoculants on White lupin (*Lupinus albus* L.) under Field conditions

Table4. 6 Mean agronomic performance of the inoculants on White lupin under field conditions at Achefer district

Treatments	PN ($plant^{-1}$)	PN/m^2	SN (pod^{-1})	SN ($plant^{-1}$)	HSW (gm^{-1})	GY (Kg ha^{-1})
LUR12	20.3 ± 2.03^{bc}	248.3 ± 27.4^{ab}	$3.3\pm0.88^{a-d}$	65.7 ± 13.40^{cd}	33.6^{b-d}	2786.0 ± 314^{cde}
LUR80	18.7 ± 1.15^{bc}	217.4 ± 27.3^{b}	2.3 ± 0.33^{d}	46.7 ± 7.06^{d}	32.4^{b-d}	2590.8 ± 200^{cde}
LUR87	24.7 ± 2.31^{abc}	305.7 ± 14.6^{ab}	$5.0\pm0.58^{a-c}$	$85.3\pm23.70^{b-d}$	37.9^{a-c}	5832.5 ± 299^{b}
LUR12 + PSRL41	23.3 ± 0.88^{abc}	280.5 ± 19.0^{ab}	$4.0\pm0.58^{a-d}$	$89.7\pm13.93^{b-d}$	36.3^{a-c}	4072.4 ± 276^{c}
LUR12 + PSRL118	20.7 ± 0.67^{bc}	256.5 ± 42.6^{ab}	$3.7\pm0.33^{a-d}$	76.0 ± 8.33^{cd}	34.5^{a-d}	3271.6 ± 520^{cd}
LUR80 +PSRL41	21.3 ± 1.45^{abc}	258.4 ± 40.3^{ab}	2.7 ± 0.33^{cd}	51.3 ± 4.37^{d}	35.3^{a-d}	2741.4 ± 381^{cde}
LUR80 + PSRL118	19.0 ± 1.20^{bc}	227.1 ± 36.0^{ab}	$3.0\pm0.58^{b-d}$	47.7 ± 12.25^{d}	32.7^{b-d}	2629.9 ± 354^{cde}
LUR87 + PSRL41	29.7 ± 2.03^{a}	362.2 ± 23.7^{a}	5.7 ± 0.33^{a}	176.6 ± 19.70^{a}	40.8^{a}	8691.3 ± 351^{a}
LUR87 + PSRL118	25.3 ± 2.52^{abc}	312.5 ± 14.3^{ab}	5.3 ± 0.33^{ab}	$131.7\pm6.01^{a-c}$	39.1^{ab}	6515.0 ± 302^{b}
TN	26.7 ± 2.31^{ab}	328.3 ± 15.5^{ab}	5.7 ± 0.33^{a}	148.7 ± 20.54^{ab}	37.7^{a-c}	7025.9 ± 331^{b}
T0	17.3 ± 1.45^{c}	208.2 ± 7.7^{b}	2.3 ± 0.33^{d}	40.0 ± 3.46^{d}	28.9^{d}	1403.3 ± 52^{e}
Average	22.6	274.3	3.9	85.6	35.8	4347.8

PN = pod number; SN = seed number; HSW = hundred seed weight; GY = grain yield. Values within a column sharing the same letter are statistically non-significant at $P \leq 0.05$.

As it is indicated in Table 4.6, the effects of rhizobium alone and combined inoculation of rhizobial and PSRB isolates on pod numbers (PN plant^{-1} and PN m^{-2}), seed numbers (SN /pod and SN plant^{-1}), and 100-seed weight (HSW) were studied under field conditions, and most of them did not show significant variation ($P \leq 0.05$) among the treatments. However, single and combined inoculation of white lupin in the field, increased grain yield ranging from 2590 by LUR80 alone to 8691kg/ha with mixed culture of LUR87 and PSRL41, showing 33.4% - 235.6% increase over the other single and combined inoculated treatments, whereas it resulted in 23.7 % and 519.5% increase against the positive and negative treatments, respectively.

Compared to single inoculation alone, grain yield increase due to dual inoculation was up to 48.5%. Compared to the mean yield of white lupin (840kg ha^{-1}) which is reported by ECSA, (2009) in Amhara and Benishangulgumuz Regional states, the current grain yield of the crop as a result of inoculation was found to be tenfold higher. Moreover, the current grain yield was also found to be higher than the seed yield (500 -5000 kg ha^{-1}) reported by Jansen (2006). In comparison to the mean grain yield of other legumes such as Cowpea (2184.44 kg ha^{-1}) reported by Girmaye Keneni and Fassil Assefa (2017), the average grain yield of White lupin (4347.8 kg ha^{-1}) was almost higher by twofold indicating inoculation of the crop with effective rhizobial and PSB inoculants is the most important method in boosting the yield of the crop in Ethiopia.

In general, such an increase in grain yield due to inoculation may be attributed to the high competency of the introduced inoculants against the native rhizobial population in the soil of the study field and the positive synergic effect of co-inoculation of White lupin with effective rhizobia and PSRB. Similar to this work, different researchers explained the synergetic effects of PGPR with multiple plant growth promoting traits and elite *Rhizobium* spp. on nodulation and yield of legumes, for instance, inoculated Soybean plants induced average NN and grain yield of 28 plant^{-1} and 4208 kg ha^{-1} (Marinkovic *et al*., 2016). Qureshi *et al*., (2009) also explained that inoculation of effective rhizobial species with other PGPR is highly important

in increasing signal exchange between host legumes and introduced rhizobia resulting in more N_2 fixing sites and higher yield of legumes.

Chapter 5 Conclusion and recommendations

5.1. Conclusion

The isolation and characterization of superior *Rhizobium* from soils of legume growing areas is very important because the effective rhizobial strains are used as inoculants to ensure effective nodulation so as to contribute sufficient N to legumes and enhance grain yields. Root nodules of White lupin (*Lupinus albus* L) grown on soils of Ethiopia harbor both fast and slow growing rhizobia with diverse morphological, physiological, PGP and symbiotic characteristics. Accordingly, most of these rhizobial isolates showed a wide range of eco-physiological competences: C and N substrate utilization, tolerance to salinity, low/high temperature and pH, antibiotics as well as metal toxicity. In addition, some of the rhizobial isolates showed multiple plant growth promoting characteristics, such as IAA, siderophore, and HCN production, inorganic phosphate solubilization, and antagonist activities against *Fusarium oxysporum*. These rhizobial isolates of White lupin also showed significantly higher nodulation, dry mass accumulation and relative symbiotic effectiveness under controlled environments.

The results of the study also showed that White lupin rhizosphere soils comprise considerably higher number of phosphate solubilizing bacteria with diverse morphology, heterotrophy and plant growth promoting characteristics. The isolates were able to release markedly higher amount of soluble phosphate from different inorganic phosphate sources, such as tricalcium, aluminum and iron phosphates indicating that they can perform better both in acidic or basic soils of Ethiopia. Furthermore, the present study showed that the PSB isolates induced considerably higher amount of soluble phosphate from TCP under salinity, low/high pH and temperature stress conditions and the presence of efficient PSB endowed with multiple plant growth promoting characteristics (IAA, siderophore, HCN and ammonia production as well as antagonistic activity against fungal pathogens) apart from phosphate solubilization.

Regarding the effect of inoculants on the growth and production of White lupin, improved nodulation, nitrogen fixation and yield parameters were achieved by single rhizobial isolate inoculation, while mixed inoculation of rhizobial and PSRB isolates had better effect on nodulation, dry mass accumulation, and gain yield both under controlled and field conditions. All the single as well as co-inoculated treatments showed positive effect on White lupin yield with the highest gain yield obtained from the co-inoculation of rhizobial isolates with PSRB isolates attributed to their compatibility and their positive synergism towards enhancing growth and yield of white lupin in Ethiopia.

5.2. Recommendations

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

- White lupin nodulating rhizobia isolates (LUR12, LUR80 and LUR87) and phosphate solubilizing rhizospheric bacterial isolates (PSRL41 and PSRL1187) showed markedly diverse characteristics in the utilization of C and N source substrates, tolerance to different stresses, good symbiotic performance and multiple plant growth promoting features. However, such qualities of the isolates in laboratory may not be shown in adverse conditions of the external environment. Hence, repeated evaluation of their consistent performance through successive field trials under different agro ecologies of the country is highly recommended.
- Conventional methods used for characterizing the morphological, physiological, symbiotic effectiveness and PGP traits of the rhizobial as well as PSRB isolates may not be reliable to assess their actual diversity and identify them to a species level, and hence diversity studies by PCR based molecular techniques, such as RAPD, AFLP, 16s rRNA gene sequence analysis and RFLP should be performed so as to obtain a better strain identification and understanding about rhizobia and rhizospheric bacteria associated with White lupin.

- Nodule occupancy, Survival and performance of the selected effective indigenous rhizobial and PSRB isolates should be tested under field conditions with higher number of the isolates using different formulations.
- The result of the current study indicated that co-inoculation of efficient rhizobial and PSRB isolates (especially LUR87 + PSRL41) significantly improved the growth and production of White lupin under field conditions. Therefore, it is highly recommended to use such mixed inoculation of rhizobia with PSRB as White lupin inoculants upon further testing under different agro ecological fields in Ethiopia.
- The selected isolates should also be assessed further Assessing the impact of inoculated soil on the growth and production of accompanied crops after white lupin production is also imperative.

- It is also highly recommended to educate and encourage farmers to use efficient inoculants for the production of crops
- for their additional PGP characteristics, such as production of ACC-deaminase and hydrolytic enzymes.

6. References

Aarab, S., Ollero, F. J., Megías, M., laglaoui, A., Bakkali, M., Arakrak, A. (2013). Isolation and Identification of Potential Phosphate Solubilizing Bacteria from the Rhizosphere of *Lupinus hirsutus* L. in the north of Morocco. *Moroccan J. of Biol,* **10**: 7-13.

Abd-Alla, M.H. (1999). Nodulation and nitrogen fixation of Lupinus species with Bradyrhizobium (lupin) strains in iron-deficient soil. *Biol Fertil Soils*, **28**: 407–415.

Adesemoye, A.O., Torbert, H.A. and Kloepper, J.W. (2008). Enhanced plant nutrient use efficiency with PGPR and AMF in an integrated nutrient management system. *Can J. Microbiol.* **54**:876–886.

Ahmed, M. H., Uddin, M.R. and McLuaughlin, W. (1984). Characterization of indigenous rhizobia from wild legumes. *FEMS Microbiol. Lett.* **24**:197-203.

Agami, R.A., Medani, R.A., Abd El-Mola, I.A. and Taha, R.S. (2016). Exogenous application with plant growth promoting rhizobacteria (PGPR) or proline induces stress tolerance in basil plants (Ocimum basilicum L.) exposed to water stress. Int. J. Environ. Agric. Res. 2 (**5**), 78–92.

Ahmad, F., Ahmad, I. and Khan, M.S. (2008). Screening of free-living rhizospheric bacteria for their multiple plant growth promoting activities. *Microbiological Research* **163**:173-181.

Ali, S.Z., Sandhya, V. and Rao, L.V. (2014). Isolation and characterization of drought tolerant ACC deaminase and exopolysaccharide producing fluorescent *Pseudomonas spp*. *Ann Microbiol*, **64**:493–502.

Alikhani, H.A., Saleh-Rastin, N. and Antoun, H. (2006). Phosphate Solubilization Activity of Rhizobia Native to Iranian Soils. Plant and Soil, **287**: 35-41.

Alikhani, H.A. and Yakhchali, B. (2009). Potential use of Iranian rhizobial strains as plant growth promoting rhizobacteria and effects of selected strains on growth characteristics of Wheat, Corn and Alfalfa. Desert 14:27-35.

Amarger, N., V. Macheret, and G. Aguerre, 1997. *Rhizobium gallicum* sp. nov. and *Rhizobiumgiardinii* sp. nov., from *Phaseolus vulgaris* Nodules *Int. J. Syst. Bacteriol*. **47**:996–1006.

Aneja, K.R. (2001). Biochemical activities of Microorganisms: In experiments in Microbiology, Plant pathology, tissue culture and Mushroom production Technology Third Edition, New International Publishers, 245-281.

Anandaraj, B. and Delapierre, L. R. (2010). Studies on Influence of Bioinoculants (*Pseudomonas fluorescens, Rhizobium sp., Bacillus megaterium*) in Green gram *Anandaraj etal, j Biosci Tech,* **1(2)**: 95-99

Anteneh Argaw, (2012). Characterization of Symbiotic Effectiveness of Rhizobia Nodulating Faba bean (*Vicia faba* L.) Isolated from Central Ethiopia. *Research Journal of Microbiology,* **7**: 280-296.

Antoun, H., Beauchamp, C.J., Goussard, N., Chabot, R. and Lalande, R. (1998). Potential of Rhizobium and Bradyrhizobium species as plant growth promoting rhizobacteria on non-legumes: Effect on radishes (Raphanus sativus L.). Plant Soil 204(1):57-67.

Araújo, F., Sérgio, A., and Figueiredo, M. V. B. (2009). "Role of plant growth promoting bacteria in sustainable agriculture chapter 10," in Sustainable Agriculture: Technology, Planning, eds A. Salazar and I. Rios (Hauppauge: Nova Science Publishers).

Arora, N.K, Tewari, S. and Singh, R. (2013). Multifaceted plant-associated microbes and their mechanisms diminish the concept of direct and indirect PGPRs. In: Arora NK (ed) Plant microbe symbiosis—*fundamentals and advances*. Springer, India, 411–449.

Babalola, O. O. (2010). Beneficial bacteria of agricultural importance. *Biotechnology Letters*. **11**: 1559-1570.

Badawi, F S. F., Biomy, A.M.M. and Desoky, A.H. (2011). Peanut plant growth and yield as influenced by co-inoculation with Bradyrhizobium and some rhizo-microorganisms under sandy loam soil conditions. *Annals of Agricultural Science*, **56**: 17–25.

Baharlouei, J., Pazira, E., Khavazi, K. and Solhi, M. (2011). Evaluation of inoculation of plant growth-promoting rhizobacteria on cadmium uptake by canola and barley. *2nd Int. Conf. Env. Sci. Tech*. **2:** 28–32.

Banerjee, S., Palit, R., Sengupta, C. and Standing, D. (2010). Stress induced phosphate solubilization by Arthrobacter sp. and Bacillus sp. isolated from tomato rhizosphere. *AJCS*. **6**:378-382, SSN:1835-2707.

Barrera, L.L., Trujillo. M. E. Goodfellow. M., García.F.J., Hernández-Lucas.I., Dávila. G., van Berkum, P. and Martínez-Romero, E. (1997). Biodiversity of bradyrhizobia nodulating Lupinus spp. *International Journal of Systematic and Evolutionary Microbiology*, 47 (**4**): 1086-1091.

Bashir K, Ali, S. and Umair, A. (2011). Effect of different phosphorus levels on xylem sap components and their correlation with growth variables of mash bean. *Sarhad Journal of Agriculture*, 27, (**4**).

Bauer, A.W., Kirby, M.M., Sherris, J.C. and Truck, M. (1966). Antibiotic susceptibility testing by a standardized single disk method. *Am J Clin Pathol*, **45**: 493-496.

Berrada, H. and Fikri-Benbrahim, K. (2014). Taxonomy the Rhizobia; Current prospective. *British microbiology research Journal* 4(6) 616 -639.

Bhattacharyya, P.N. and Jha, D.K. (2012). Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture. *World J. Microbiol. Biotechnol*. **28**: 1327–1350.

Blaha, D., Prigent-Combaret, C., Mirza, M.S. and Moenne- Loccoz, Y. (2006). Phylogeny of the 1- aminocyclopropane- 1-carboxylic acid deaminase encoding gene *acdS* in phytobenefi cial and pathogenic Proteobacteria and relation with strain biogeography. *FEMS Microbiol Ecol,* **56**:455–470.

Bottomley, P., Cheng, H.H. and Strain, S.R. **(**1994). Genetic structure and symbiotic characteristics of a *Bradyrhizobium* population recoverd from a pasture soil. *Appl. Environ. Microbiol*. **60:**1754–1761.

Bordeleau, L. M., and Prévost, D. (1994). Nodulation and nitrogen fixation in extreme environments. *Plant Soil* **161**:115-124.

Broughton, W.J. and Dilworth, M.J. (1971). Control of leghemoglobin synthesis in snakebean. *Biochem. J.* **125**: 1075-1080.

Capuccino, J.G. and Sherman, N. (1996). Microbiology: A Laboratory Manual. The Benjamin/Cunning Publishing Company Inc. Menlopark, California.

Cervantes, C., J. Chavez, N.A. Cardova, P. De Na Mora and Velasco, J.A. (1986). Resistance to Metal by *Pseudomonas aerugenosa Clinical Isolates. Microbiol*. **48** :159- 163.

Chakrabati S.K., Lee, M.S. and Gibson, A.H. (1981). Diversity in the nutritional requirements of the strains of various Rhizobium strains. *Soil Biol. Biochem* **13**: 349-354.

Chauhan, A., Guleria, S., Balgir, P.P., Walia, A., Mahajan, R., Mehata, p. and Shirkot, K. (2017). Tricalcium phosphate solubilization and nitrogen fixation by newly isolated *Aneurinibacillus aneurinilyticus* CKMV1 from rhizosphere of *Valeriana jatamansi* and its growth promotional effect. Brazilian Journal of Microbiology, 48(**2**):294-304.

Correa, O. S. and Barneix, A. J. (1997). Cellular mechanisms of pH tolerance in *Rhizobium loti*.World J. *Microbiol. Biotechnol*. **13**:153–157.

Coyne, S. M**. (**1999). Soil Microbiology: an exploratory approach. Delmar publisher. NewYork, 279-289.

Damam, M., Kaloori, K., Gaddam, B. and Kausar, R. (2016). Plant growth promoting substances (phytohormones) produced by rhizobacterial strains isolated from the rhizosphere of medicinal plants. Int. J. Pharm. Sci. Rev. 37 (**1**): 130–136.

Dasgupta, D., Ghati, A., Sarkar, A., Sengupta, C. and Paul, G. (2015). Application of Plant Growth Promoting Rhizobacteria (PGPR) Isolated from the Rhizosphere of *Sesbania bispinosa* on the Growth of Chickpea (*Cicer arietinum L.*). *Int.J.Curr.Microbiol.App.* 4(**5**): 1033-1042.

Date, R. A., Williams, R.W. and Bush,H.V.(1993). Screening cropland pasture for effective nitrogen fixing association. *CSIRO, Division of Tropical crops and pastures*, **17**:3-5.

Dastager, S.G., Deepa, C. K. and Pandey, A. (2010). Isolation and characterization of novel plant growth promoting *Micrococcus* sp NII-0909 and its interaction with cowpea. Plant Physiol. Biochem., **48**: 987-992.

Dawit Solomon, Lehmann, J., Tekalign Mamo, Fritzsche, F. and Zech, W. (2002). Phosphorus forms and dynamics as influenced by land use changes in the sub-humid Ethiopian highlands. *Geoderma*, **105**: 21–48.

Diby P. (2004). Physiological, Biochemical and Molecular Studies on the Root Rot (Caused by *Phytophthora capsici*) Suppression in Black Pepper (*Piper nigrum* L.) by Rhizosphere Bacteria. Ph.D. thesis, University of Calicut, Calicut.

Diriba Muleta, Fassil Assefa, Börjesson, E. and Granhall, U. (2013). Phosphate-solubilizing rhizobacteria associated with *Coffee arabica* L. in natural coffee forests of southwestern Ethiopia. *Journal of the Saudi Society of Agricultural Sciences*. **12**:73-84.

Diriba Temesgen and Fassil Assefa (2017). Screening, Identification, and Characterization of Soybean rhizobia and Rhizobacteria for their Symbiotic, Multiple Plant Growth Enhancement Properties, and Ecological Adaptations for Enhancing Productivity of the Crop under Low-input Agriculture. PhD dissertation, School of Graduate Studies, Addis Ababa University.

Desta Beyene and Angaw Tsigie (1989). Conserving microorganisms in the soil*: soil science research in Ethiopia*. Proceedings of the First Soil Science Research Review Workshop; February 11–14 1986; Addis Ababa, Ethiopia.

Dereje Haile, Firew Mekbib, and Fassil Assefa (2016). Isolation of Phosphate Solubilizing Bacteria from White Lupin (Lupinus albus L.) Rhizosphere Soils Collected from Gojam, Ethiopia. *J Fertil Pestic* **7**: 172. doi:10.4172/2471-2728.1000172.

Dye, D.W. (1962). The inadequacy of the usual determinative tests for identification of *Xanthomonas sp*. *New Zealand Journal of Science.* **5**: 393-416.

ECSA. (2012). Report on area and production of major crops. Statistical Bulletin, Ethiopian Central Statistical Agency, Addis Ababa, Ethiopia.

ECSA, (2018)**.** Report on area and production of crops (Private peasant holdings, Meher season). ECSA (Ethiopian Central Statistical Agency), Addis Ababa, Ethiopia, pp.

ECSA. (2015). Report on area and production of crops (Private peasant holdings Meher season). Ethiopian Central Statistical Agency, Addis Ababa, Ethiopia.

Edi-Premono, M., Moawad, A.M. and Vlek, P.G. (1996). Effect of Phosophate Solubilizing *Pseudomonas putida* on the growth of maize and its survival in the rhizosphere. *Indonesian J.Crop Sci.***11**:13-23.

EEPA. (2004). Report on Ethiopian Pulses Profile. Ethiopia: Land of Crops Diversity, Ethiopian Export Promotion Agency, Addis Ababa.

El Hilali I., El Jamali, J., Alami, I. T. and Maltouf, A. F. (2016). Characterization and Biodiversity of a Fast-Growing Rhizobacterial Population Nodulating Lupine in Morocco. *International Journal of New Technology and Research (IJNTR***),** 2 **(12):** 27-37.

El-Nahrawy, S. and Omara, A.E. (2017). Effectiveness of Co-inoculation with *Pseudomonas koreensis* and Rhizobia on Growth, Nodulation and Yield of Common Bean (*Phaseolus vulgaris* L.). *Microbiology Research Journal International*, **21**: 1-15.

Egamberdieva, D. (2012). *Pseudomonas chlororaphis*: a salt-tolerant bacterial inoculant for plant growth stimulation under saline soil conditions. *Acta Physiol Plant*, 34(**2**):751–56. doi:10.1007/s11738-011-0875-9.

Figueiredo, M.V.B., Bonifacio, A., Rodrigues, A.C. and de Araujo, F.F. (2016). Plant Growth-Promoting Rhizobacteria: Key Mechanisms of Action, review, 3-37.

Francis, C. (1999). New crops and oilseeds from Ethiopia and elsewhere. Australian new Crops newsletter.11: www.newcrops.uq.edu.au/newslett/ncnl1117.htm. Accessed on November 12, 2017.

Gamalero, E. and Glick, B.R. (2015). Bacterial modulation of plant ethylene levels. *Plant Physiol* 169(**1**):13–22.

Ganesan, P. and Gnanamanicka, S. S. (1987). Biological control of *Sclerotium rolfsii Sacc*. In peanut by inoculation with *P. fluorescens. Soil Biology and Biochemistry*. **19**: 35-38.

García-Fraile, P., Menéndez, E. and Rivas, R. (2015). Role of bacterial biofertilizers in agriculture and forestry. Review, *Bioengineering*, **3**: 183-205.

Ganesan P. and Gnanamanicka, S. S. (1987). Biological control of Sclerotium rolfsii Sacc. In peanut by inoculation with *P. fluorescens. Soil Biology and Biochemistry*. **19**: 35-38.

Geletu Bejiga and Ketema Daba (2006). Breeding chickpea for wide adaptation. In: *Food and forage legumes of Ethiopia progress and prospects*, 59-66, ICARDA, Addis Ababa, Ethiopia.

Gibson A.H. (1987). Evaluation of nitrogen fixation by legumes in the greenhouse and growth chamber. **In**: *Symbiotic Nitrogen Fixation Technology*, pp. 321–363, (Gibson, A.H., ed.), New York, Marcel Dekker.

Giller, K.E., 2001. Nitrogen fixation in tropical cropping systems. Second Edition. CABI publishing, Wallingford, UK.

Girmaye Kenasa and Fassil Assefa (2017). Diversity, Plant Growth Promoting and Symbiotic Properties of Cowpea (*Vigna unguiculata* L. walp.) Root Nodule Bacteria from Ethiopian Soils. PhD Dissertation, School of Graduate Studies**,** Addis Ababa University**.**

Glick, B.R., (2012). Plant growth-promoting bacteria: mechanisms and applications. *Scientifica,* 1–15, doi:10.6064/2012/963401.

Gontia-Mishra, I., Sasidharan, S. and Tiwari, S. (2014). Recent developments in use of 1-aminocyclopropane-1- carboxylate (ACC) deaminase for conferring tolerance to biotic and abiotic stress. *Biotechnol Lett*, **36**:889–898.

Gordon, S.A., Weber, R.P. (1951). Colorimetric estimation of indole acetic acid. *Plant Physiol.* **26:** 192-195.

Goswami, D., Thakker, J.N. and Dhandhukia, P.C. (2016). Portraying mechanics of plant growth promoting rhizobacteria (PGPR): a review. *Cogent Food Agric*. **2**:1–19.

Gouda, S., Kerry, R.G., Das, G., Paramithiotis,S., Shin, H.S. and Kumar Patra, J.K. (2018). Revitalization of plant growth promoting rhizobacteria for sustainable development in agriculture. *Microbiological research*, (**206**): 131-140.

Grover, M., Ali, S.Z., Sandhya, V., Rasul, A. and Ventakeshwarlu, B. (2011). Role of microorganisms in adaptation of agricultural crops to abiotic stress. *World J Microbiol Biotechnol,* **27**:1231–1240.

Gupta, G, Parihar, S.S., Ahirwar, N.K., Snehi, S.K. and Singh, V. (2015). Plant Growth Promoting Rhizobacteria (PGPR): Current and Future Prospects for Development of Sustainable Agriculture. *J Microb Biochem Technol*. **7:** 096-102.

Gururani, M.A., Upadhyaya, C.P., Upadhyaya, C.P., Baskar. B., Venkatesh, J., Nookaraju, A. and Park, S.W/ (2013). Plant growth promoting rhizobacteria enhance abiotic stress tolerance in *Solanum tuberosum* through inducing changes in the expression of ROS scavenging enzymes and improved photosynthetic performance. *J Plant Growth Regul*, **32:**245–258.

Graham, P. H., Draeger, K., Ferrey, M. L., Conroy, M. J., Hammer, B. E., Martinez, E., Naarons, S. R., and Quinto, C. (1994). Acid pH tolerance in strains of *Rhizobium* and *Bradyrhizobium*, and initial studies on the basis for acid tolerance of *Rhizobium tropici* UMR1899. *Can. J. Microbiol*. **40**:198-207.

Graham, P.H. (1992). Stress tolerance in *Rhizobium* and *Bradyrhizobium*, and nodulation under adverse soil conditions. *Can. J. Microbiol*. **38**:475-484.

Hall, R.S., Johnson, S.K., Baxter, A.L. and Ball, M.J. (2005). Lupin kernel fibre-enriched foods beneficially modify serum lipids in men. *European Journal of Clinical Nutrition*. **59**:325-333.

Hariprasad, P. and Niranjana, S. R. (2008). Isolation and characterization of phosphate solubilizing rhizobacteria to improve plant health of tomato. *Plant Soil*. **316**:13–24, DOI 10.1007/s11104-008-9754-6.

Hassen, A.I., Bopape, F.L. and L. K. Sanger, (2016). Microbial Inoculants as Agents of Growth Promotion and Abiotic Stress Tolerance in Plants. Review, *Microbial Inoculants in Sustainable Agricultural Productivity*, 23-36. DOI 10.1007/978-81-322-2647-5_2.

Hernández-León R, Rojas-Solís D, Contreras-Pérez M. (2015). Characterization of the antifungal and plant growth-promoting effects of diffusible and volatile organic compounds produced by Pseudomonas fluorescens strains. *Biol Cont*, **81**: 83-92.

Hibstu, Azeze (2016). Genetic diversity and association of traits in white lupin (*lupinus albus* l.) accessions of Ethiopia, PhD dissertation, School of Graduate studies, Haramaya University.

Holmes, B., Popoff, M., Kiredjian,M. and Kersters,K. **(**1988). *Ochrobactrum anthropi* gen. nov., sp. nov. from human clinic specimens and previously known as group Vd. *Int. J. Syst. Bacteriol*. **38:**406–416.

Htwe, A.Z., Moh, S. M., Moe, K. and Yamakawa, T. (2018). Effects of co-inoculation of Bradyrhizobium japonicum SAY3-7 and Streptomyces griseoflavus P4 on plant growth, nodulation, nitrogen fixation, nutrient uptake, and yield of soybean in a field condition. *Soil Science and Plant Nutrition*, DOI: 10.1080/00380768.2017.1421436.

Hungria M, Chueire LMO, Coca RG, Megias M (2001). Preliminary characterization of fast growing rhizobial strains isolated from soybean nodules in Brazil. *Soil Biol. Biochem*. **33**: 1349-1361.

Hungria, M., and Kaschuk, G. (2014). Regulation of N2 fixation and NO3−/NH4+ assimilation in nodulated and N-fertilized *Phaseolus. vulgaris* L. exposed to high temperature stress. *Environ. Exp. Bot*. **98**:32-39.

Jackson, M.L. (1973). Soil chemical Analysis, Prentice Hall of India, New Delhi, India, 134 – 182.

Jansen, P. C. M. (2006). *Lupinus albus L*. [Internet] Record from Protabase. Brink M and Belay G (Editors). PROTA (Plant Resources of Tropical Africa Resourcesvegetablesdel'Afriquetropicale),Wageningen,Netherlands. http://database.prota.org/dbtwwpd/exec/dbtwpub.dll?AC=QBE_QUERY&BU=http://database.prota.org/search.htm&TN=PROTAB~1&QB0=AND&QF0=Species+Code&QI0=Lupinus+albus&RF=Webdisplay, Accessed on March 2017.

Jensen J.B O.Y., Ampomah, R. Darrah, N.K. Peters and T.V. Bhuvaneswari, (2005). Role of trehalose transport and utilization in *Sinorhizobium meliloti-alfalfa* interactions. *Mol. Plant-Microbe Interact.* **18:** 694-702.

Jensen, E.S., Peoples. M.B., Boddey, R.M., Gresshoff, P.M., Hauggaard-Nielsen, H., Alves, B.J. and Morrison, M.J. (2012). Legumes for mitigation of climate change and the provision of feedstock for biofuels and biorefineries. A review. *Agron Sustain Dev*. 32:329–364.

Jordan, D. C. (1984). Family III. *Rhizobiaceae*, *In* N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology. The Williams &Wilkins Co., Baltimore, Md. p. 235–255.

Joseph, B., Patra, R.R., and Lawrence, R. (2007). Characterization of plant growth promoting rhizobacteria associated with chickpea (*Cicer arietinum* L.) *International Journal of Plant Production,* 2: 141-152.

Karanja, D. (2016). Pulse crops grown in Ethiopia, Kenya and United Republic of Tanzania for local and Export Market. International Trade Centre, Eastern Africa Grain Council. Kenaf cultivars for fiber yield using GGE biplots in different environments of Ethiopia.

Karthikeyan, B., Joe, M.M., Islam, M. D. R. and Sa, T. (2012). ACC deaminase containing diazotrophic endophytic bacteria ameliorate salt stress in *Catharanthus roseus* through reduced ethylene levels and induction of antioxidative defense systems. *Symbiosis* **56**:77–86.

Kindie Tesfaye (2016). Description of Cropping Systems, Climate and Soils in Ethiopia. Global Yield Gap Atlas, http://www.yieldgap.org/ethiopia, accessed on 19 June, 2017.

Kloepper, J., Ryu, C. and Zhang, S. (2004). Induced systemic resistance and promotion of plant growth by *Bacillus* Spp. *Phytopathology*.**94**:1259–1266.

Kloepper, J.W., Gutierrez-Estrada, A. and Mclnroy, J.A. (2007). Photoperiod regulates elicitation of growth promotion but not induced resistance by plant growth-promoting rhizobacteria. *Can J Microbiol,* 53(**2**):159–167.

Koch, M., Delmotte, N., Rehrauer, H., Vorholt, J.A., Pessi, G. and Hennecke, H. (2010). Rhizobial adaptations to hosts, a new facet in the legume root-nodule symbiosis. *Mol Plant-Microbe Interact* **23:** 784-790.

Kohajdova, Z., Karovičova, J. and Schmidt, Š. (2011). Lupin composition and possible use in bakery– a review. *Czech J. Food Sci*. **29**: 203–211.

Korir, H., Mungai, N.W., Thuita, M., Hamba, Y. and Masso, C. (2017). Co-inoculation Effect of Rhizobia and Plant Growth Promoting Rhizobacteria on Common Bean Growth in a Low Phosphorus Soil. *Front. Plant Sci*. **8**:141. doi: 10.3389/fpls.2017.00141.

Kumar, V., Menon S., Agarwal, H., and Gonpalakrishnan, D. (2017). Characterization and optimization of bacterium isolated from soil samples for the production of siderophores. *Resource Efficient Technologies*, 3(**4)**:434-439, doi:10.1016/j. reffit.2017.04.004.

Kumar, A., Prakash, A. and Johri, B.N. (2011). Bacillus as PGPR in crop ecosystem. In: Maheshwari DK (ed) Bacteria in agrobiology: *crop ecosystem*. Springer, Berlin/ Heidelberg, pp 37–59.

Kuykendall, L. D., Saxena, B., Devine, T. E. and Udell, S. E. (1991). Genetic diversity in *Bradyrhizobium japonicum* Jordan 1982 and a proposal for *Bradyrhizobium elkanii* sp. nov. *Can.J. Microbiol*. **38:**501–505.

Kyei-Boahen, S., Savala, C. E. N., Chikoye, D. and Abaidoo, R. (2017). Growth and Yield Responses of Cowpea to Inoculation and Phosphorus Fertilization in Different Environments. *Frontiers in Plant Science*,***8***:6 46.

Lemanceau, P., Bauer, P., Kraemer, S. and Briat, J.F. (2009). Iron dynamics in the rhizosphere as a case study for analyzing interactions between soils, plants and microbes. *Plant and Soil*. **321**:513–535.

Lugtenberg, B. and Kamilova, F. (2009). Plant-growth-promoting Rhizobacteria. Annual Review. *Microbiol*. **63**:541–56.

Ma, Y., Prasad, M.N.V., Rajkuma, M. and Freitas, H. (2011). Plant growth promoting rhizobacteria and endophytes accelerate phytoremediation of metalliferous soils. *Biotechnol. Adv*. **29**: 248–258.

Majeed, A., Abbasi, M.K., Hameed, S., Imran, A. and Rahim, N. (2015). Isolation and characterization of plant growth-promoting rhizobacteria from wheat rhizosphere and their effect on plant growth promotion. *Front. Microbiol*. **6**:198.

Manasa, K., Subhash Reddy, R., riveni, S., Kranthi Kumar, B. and Gowri Priya, N. (2017). Characterization of *Rhizobium* Isolates and their Potential PGPR Characteristics of different Rhizosphere Soils of Telangana Region, India. *Int.J.Curr.Microbiol.App.Sci, 6*(**5**): 2808-2813.

Marinković, J., Bjelić, D., Tintor, B., Miladinović, J., Đukić, V. and Đorđević, V. (2016). Effects of soybean co-inoculation with plant growth promoting rhizobacteria in field trial. *Romanian Biotechnological Letters, X: 1-9.*

Marquez-Santacruz, H.A., Hernandez-Leon, R., Orozco- Mosqueda, M.C., Velazquez-Sepulveda, I. and Santoyo, G. (2010). Diversity of bacterial endophytes in roots of Mexican husk tomato plants (*Physalis ixocarpa*) and their detection in the rhizosphere. *Genetics and Molecular Research*, **9**: 2372-80.

Mbai, F. N. (2013). Isolation and characterization of bacterial root endophytes with potential to enhance plant growth from Kenyan Basmati rice. *American International Journal of Contemporary Research*. **4**:25-40.

Miller, and Schlinkert, M. (1985). Physiological and biochemical characteristics of a fast· growing strain of lupine rhizobia isolated from the sonoran desert. PhD, Dissertation-Reproduction (electronic), The University of Arizona.

Mishra, P.K., Bisht, S.C., Jeevanandan, K., Kumar,S., Bisht, J.K. and Bhatt, J.C. (2014). Synergistic effect of inoculating plant growth-promoting Pseudomonas spp. and

Rhizobium leguminosarum-FB1 on growth and nutrient uptake of rajmash (*Phaseolus vulgaris* L.), *Archives of Agronomy and Soil Science*, 60 (**6**): 799-815.

Moulin L.., A. Munive, B. Dreyfus and C. Bolvin- Masson, 2001. Nodulation of legumes by members of the β-subclass of *proteobacteria*. *Nature* **411**: 948–950.

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.

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.

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, Submitted.

Nascimento, F., Brígido, C., Alho, L., Glick, B. and Oliveira, S. (2012). Enhanced chickpea growth-promotion ability of *Mesorhizobium cicero* strain expressing an exogenous ACC deaminase gene. *Plant Soil*. **353**:22 -230.

Nautiyal, C.S., Bhadauria, S., Kumar, P., Lal, H. and Mondal, R. (2000). Stress induced phosphate solubilization in bacteria isolated from alkaline soils. *Fed Eur Micr Societies* **182**: 291-296.

Neumann, B. and Laing, M. (2006). Trichoderma: an ally in the quest for soil system sustainability: Biological Approaches to Sustainable Soil Systems. *CRC Press*, Taylor and Farncis Group, Boca Raton, London, New York, pp. 491–500.

Niehus, R., Picot, A., Oliveira, N. M., Mitri, S. and Foster, K. R. (2017). The evolution of siderophore production as a competitive trait. *Evolution* ,**112**: 1443–1455.

Nithyakalyani, V., Kannan, M. and Anandan, R. (2016). Insecticide and Salt Tolerance of Plant Growth Promoting Root Nodule Bacteria. *Int.J.Curr.Microbiol.App.Sci*, **4**: 942-956.

Nkwiine, C. and Rwaira-silver, M.C. (2007). Status of research on soil microsymbionts in Uganda. Afr.J Ecol. **45**:27-35.

Ngumbi, E. and Kloepper, J. (2016). Bacterial-mediated drought tolerance: current and future prospects. *Appl. Soil Ecol*. **105**: 109–125.

Olanrewaju, O.S., Glick, B.R. & Babalola, O.O. (2017). Mechanisms of action of plant growth promoting bacteria. A review, *World J Microbiol Biotechno*, 33: 197.

Oliveira, C.A.; Alves, V.M.C.; Marriel, I.E.; Gomes, E.A.; Scotti, M.R.; Carneiro, N.P.; Guimarães, C.T.; Schaffert, R.E.; Sá, N.M.H. (2009). Phosphate solubilizing microorganisms isolated from rhizosphere of maize cultivated in an oxisol of the Brazilian Cerrado Biome. *Soil. Biol. Biochem*. **41:** 1782–1787.

Oteino, N., Lally, R.D., Kiwanuka, S., Lloyd, A., Ryan, D., Germaine, K.J. and Dowling, D.N. (2015). Plant growth promotion induced by phosphate solubilizing endophytic Pseudomonas isolates. *Front. Microbiol.* 6: 745.

Owen, D., Williams, A.P., Griffith, G.W. and Withers, P.J.A. (2015). Use of commercial bio-inoculants to increase agricultural production through improved phosphorous acquisition *Appl. Soil Ecol.,* **86**:41-54.

Pandey, P.K., Singh,M.C., Siddhartha Singh, S., Singh,A.K., Kumar, M., Pathak,M., Shakywar, R.C. and Pandey, A.K. (2017). Inside the Plants: Endophytic Bacteria and

their Functional Attributes for Plant Growth P romotion. *Int.J.Curr.Microbiol.App.Sci*, 6(**2**): 11 – 21.

Peoples, M.B., Brockwell, J., Herridge,D.F., Rochester, I.J., Alves, B.I.R., Urquiaga, S., Boddey, R.M., Dakora, F.D., Bhattarai, S., Maskey, S.L., Sampet, C., Rerkasem, B., Khans, D.F., Hauggaard-Nielsen, H. and Jensen, B.S. (2009). The contributions of nitrogen-fixing crop legumes to the productivity of agricultural systems. A review, *symbiosis,* 48: 1-17.

Pérez-Montano, F., Alías-Villegas, C., Bellogín, R.A., del Cerro, P., Espuny, M.R., Jiménez-Guerrero, I., López-Baena, F.J., Ollero, F.J. and Cubo, T. (2014). Plant growth promotion in cereal and leguminous agricultural important plants: From microorganism capacities to crop production. *Microbiol. Res*. 169:325-336.

Pikovskaya, R. I. (1948). Mobilization of phosphates in soil in connection with the vital activities of some microbial species. *Mikrobiologia*., **17**: 362 - 370.

Pongsip, N. (2012). Symbiotic Variation and Plant-Growth-Promoting Traits of Rhizobia. Review, **20**:73-92.

Paul, D. and Sinha, S.N. (2017). Isolation and characterization of phosphate solubilizing bacterium *Pseudomonas aeruginosa* KUPSB12 with antibacterial potential from river Ganga, India. *Annals of Agrarian Science,* **15:** 130 -136.

Prusinski, J. (2017). White Lupin (*Lupinus albus* L.) Nutritional and Health Values in Human Nutrition – a Review. *Czech J. Food Sci.,* 35(**2**): 95–105.

Pudełko, K. and Żarnicka, J. (2010). Diversity in symbiotic specificity of bacterial strains nodulating lupins in Poland. *Polish Journal of Agronomy*, **2**: 50–56.

Qureshi, M.A., Ahmad, Naveed, M., Iqbal, A., Akhtar, N., Niazi, K.H. (2009). Co-inoculation with *Mesorhizobium cicero* and *Azotobacter chroococcum* for improving growth, nodulation and yield of chickpea (*Cicer arietinum* L.). *Soil Environ* **28**:124-129.

Rajapaksha, C.P. and Senanayake A.P. (2011). Potential use of rock-phosphate-solubilizing bacteria associated with wild rice as inoculants for improved rice (*Oryza sativa). Arch.Agron.Soil Sci.***57**:775-788.

Ramadan, E.M., AbdelHafez, A.A., Hassan, E.A. and Saber, F.M. (2016). Plant growth promoting rhizobacteria and their potential for biocontrol of phytopathogens. *Afr. J. Microbiol. Res,* **10**: 486–504.

Rashid, M. S., Khalili, N., Ayub, S., Alam and Latif, F. (2004). Organic acid production and phosphate solubilization by phosphate solubilizing microorganisms (PSM) under in vitro conditions. *Pakistan Journal of Biological Sciences*, **7**: 187-196.

Raza S., B. Jornsgard, H. Abou-Taleb and J.L. Christiansen, 2001. Tolerance of *Bradyrhizobium* sp (*Lupinus*) strains to salinity, pH, CaCO3 and antibiotics. *Letters in Applied Microbiology,* **32**: 379-383.

Rao, D.L.N., Mohanty, S.R., Acharya, C., and Atoliya, N. (2018). Rhizobial Taxonomy-*Current status. IUNFC Newsletter*, (3) 1.

Reed, S.C., Cleveland, C.C. and Townsend, A.R. (2011). Functional ecology of free-living nitrogen fixation: a contemporary perspective. *Annu. Rev. Ecol. Environ. Syst*, **42**: 489–512.

Riley, I.T, and Dillworth, M.J. (1985). Cobalt status and its effects on soil population of rhizobium lupini, rhizosphere colonization, and nodule initiation. *Soil bacteriology and Biochemistry*, **17**: 81-85.

Routray, S. and Khanna, V. (2018). Characterization of Rhizobacteria for Multiple Plant Growth Promoting Traits from Mung Bean Rhizosphere. *Int.J.Curr.Microbiol.App.Sci,* 7(**1**): 2264-2269.

Sa, A. L. B., Dias, A. C. F., Teixeiria, M. A. and Vieira, R. F. (2012). Contribution of N_2 fixation for the world agriculture, in Bacteria in Agrobiology: *Plant Probiotics*, ed. D. K. Maheshwari (Berlin: Springer-Verlag), pp: 33–42.

Saharan, B.S. and Nehra, V. (2011). Plant growth promoting rhizobacteria: a critical review. Life Sci Med Res *LSMR,* **21**:1–29.

Sahlemedhin Sertsu and Taye Bekele (2000). *Procedures for soil and plant analysis*. National Soil Research Center, Ethiopian Agricultural Research Organization, Addis Ababa, Ethiopia, pp.110.

Sánchez, A.C., Gutiérrez, R.T., Santana, R.C., Urrutia, A.R., Fauvart, M., Michiels, J. and Vanderleyden, J. (2014). Effects of co-inoculation of native Rhizobium and Pseudomonas strains on growth parameters and yield of two contrasting *Phaseolus vulgaris* L. genotypes under Cuban soil conditions. *European Journal of Soil Biology*, **62**: 105 – 112.

Santoyo, G., Hernández-Pacheco, C., Hernández-Salmerón, J., Hernández-León, R. (2017). The role of abiotic factors modulating the plant-microbe-soil interactions: toward sustainable agriculture. A review. *Spanish Journal of Agricultural Research*, **15**, e03R01.

Sen, M. and Joshi, H. (2017). Characterization of Phosphate Solubilizing Bacteria Isolated from Mine Tailings of Zawar Mines, Udaipur, India. *Int.J.Curr.Microbiol.App.Sci*, (2017) 6 (**8**): 588-596.

Schwyn, B. and Neilands, J.B. (1987). Universal chemical assay for the detection and determination of siderophores. *Anal Biochem.* 160(**1**):47–56.

Sharma, A., Johri, B.N., Sharma, A.K. and Glick, B.R. (2013). Plant growth-promoting bacterium *Pseudomonas sp*. strain GRP3 influences iron acquisition in mung bean (*Vignaradiata L. Wilzeck*). *Soil Biol. Biochem*. **35**: 887–894.

Sharma, S.B., Sayyed, R.Z., Trived, M.H. and Gobi, T.A. (2013). Phosphate solubilizing microbes: sustainable approach for managing phosphorus deficiency in agricultural soils. *SpringerPlus*, **2**:587.

Singh, S., Gupta, G., Khare, E., Behal, K.K. and Arora, N.K. (2014). Phosphate solubilizing Rhizobia Promote the Growth of Chickpea under buffering Conditions. *Int. J. Pure App. Biosci*. 2(5):97 -106.

Singh, R., Pandey, D.K., Kumar, A. and Singh, M. (2017). PGPR isolates from the rhizosphere of vegetable crop Momordica charantia: characterization and application as biofertilizer. *Int J Curr Microbiol App Sci* ,6(**3**):1789–1802.

Shiraishi, A., Matsushita N., Hougetsu, T. (2010). Nodulation in black locust by the *Gamma proteobacteria Pseudomonas* sp. and the *Beta-proteobacteria Burkholderia* sp. Syst. *Appl. Microbiol*, **33**:269-274.

Situmorang, E. C., Prameswara, A., Sinthya, H.C., Toruan-Mathius, N. and Liwang, T. (2015). Indigenous Phosphate Solubilizing Bacteria from Peat Soil for an Eco-friendly Biofertilizer in Oil Palm Plantation. KnE Energy, **1**: 65-72.

Small, E. (2012). Lupins – benefit and harm potentials, Biodiversity, (38) 13: 54-64.

Somasegaran, P. and H.J. Hoben, 1994. *Handbook for Rhizobia*. Springer-Verlag, P.380.

Spaepen, S. and Vanderleyden, J. and Remans, R. (2007). Indole-3-acetic acid in microbial and microorganism-plant signaling. *In: Unden F (ed) FEMS microbiol rev*. Blackwell Publishing Ltd., New York, 1–24.

Spaepen, S. and Vanderleyden, J., (2011). Auxin and plant-microbe interactions. *Cold Spring Harb. Perspect. Biol*.3(**4**): a001438.

Strange, R.N. and Scott, P.R. (2005) Plant disease: a threat to global food security. *Ann. Rev. Phytopatho* **43**: 1–660.

Stajkovic, O., Delic, D., Josic, D., Kuzmanovic, D., Rasulic, N., and Vukcevic, K. J. (2011). Improvement of common bean growth by co-inoculation with Rhizobium and plant growth-promoting bacteria. *Rom. Biotechnol. Lett*. **16**: 5919–5926.

Subba Rao, N.S., (1999). Soil Microbiology. Fourth edition of Soil microorganisms and Plant Growth. Oxford and IBH Publishing Co. PVVT.LTD, New Delhi, Kolkata, 166-228.

Sulieman S. and Tran, L.S.P. (2014). Symbiotic Nitrogen Fixation in Legume Nodules: Metabolism and Regulatory Mechanisms. *Int. J. Mol. Sci. **15***: 19389-19393.

Tak, H.I., Ahmad, F. and Babalola, O.O. (2013). Advances in the Application of Plant Growth Promoting Rhizobacteria in Phytoremediation of Heavy metals A review, *Environmental contamination and Toxicology* (**223**)*,* 33 -52.

Trainer, M.A. and Charles, T.C. (2006). The role of PHB metabolism in the symbiosis of rhizobia with legumes. *Appl. Microbiol. Biotechnol.* **71**: 377–386.

Tripura, C.B., Sudhakar Reddy, P., Reddy, M. K., Sashidhar, B.and Podile, A.R. (2007b). Glucose dehydrogenase of a rhizobacterial strain of *Enterobacter asburiae* involved in mineral phosphate solubilization shares properties and sequence homology with other members of enterobacteriaceae. *Indian J Microbiol* **47**: 126–131.

Trujillo, E.M., A. Willems, A. Abril, A.M. Planchuelo, R. Rivas, D. Ludena, P.F. Mateos, E. Martinez-Molina and E. Velazquez, (2005). Nodulation of Lupinus albus by strains of *Ochrobactrum lupine* spnov. *Appl. Environ. Microbiol*. **71**: 1318–1327.

Uzun, B., Arslan, C., Karhan, M. and Toker, C. (2007). Fat and fatty acids of white lupin (*Lupinus albus L*.) in comparison to sesame (*Sesamum indicum* L.). *Food Chemistry*. **102**: 45–49.

Vejan, P., Abbdillah, R., Khadira, T., Ismail, S. and Boyce, A.N. (2016). Role of Plant Growth Promoting Rhizobacteria in Agricultural Sustainability—A Review. *Molecules*. **21**: 573.

Verma, J. P., Yadav, J. and Tiwari, K. N. (2012). Enhancement of nodulation and yield of chickpea by co-inoculation of indigenous *mesorhizobium spp*. and Plant Growth–Promoting Rhizobacteria in Eastern Uttar Pradesh. *Commun. Soil Sci. Plant Anal*. **43**: 605–621.

Verma, J.P., Yadav, J., Tiwari, K.N. and Singh, V. (2010). Impact of plant growth promoting rhizobacteria on crop production. *Int. J. Agric. Res*. **5**: 954–983.

Vincent, J. M., (1970). A Manual for the Practical Study of Root Nodule Bacteria. Blackwell, Oxford and Edinburgh, P.164.

Viveros, O.M., Jorquera, M.A., Crowley, D.E., Gajardo, G. and Mora, M.L. (2010). Mechanisms and practical considerations involved in plant growth promotion by rhizobacteria. *J. Soil Sci. Plant Nutr.* **10**: 293–319.

Waheed, A., Afzal, A., Sultan, T., Rahman, I.U., Sohail, Ijaz, F., Manan, S., Asma, Zia, M.A., Khan, K. and Shah, A. (2014). Isolation and biochemical characterization of rhizobium from pea crop at Swabi. International Journal of Biosciences (IJB), 4, (**8**) :231-240.

Wani, P.A. and Irene, O.L. (2014). Screening of Microbes for Their Metal, Antibiotic Resistance and Plant Growth Promoting Activity. *Current Research in Bacteriology,* **7**: 22-31.

Ward, J.H. (1963). Hierarchical grouping to optimize an objective function. *Journal of American Statistical Association* **58**: 236-244.

White, D. (1995). The physiology and Biochemistry of Prokaryotes. Oxford University. Oxford, pp. 378.

Weller, D.M. (2007). Pseudomonas biocontrol agents of soil borne pathogens: looking back over 30 years. *Phytopathology*. **97**:250–256.

Yihenew Gebreselassie. (2002). Selected Chemical and Physical characteristics of Soils of Adet Research Centre and its Testing Sites in North Western Ethiopia. *Ethiopian Journal of Natural Resources*, 4 (**2**):199 - 215.

Yeheyis Likawent, Kijora, C., Solomon Melaku, Anteneh Girma and Peters, K.J. (2010)**.** White lupin (*Lupinus albus* L.), the neglected multipurpose crop: Its production and utilization

in the mixed crop-livestock farming system of Ethiopia. *Livestock Research for Rural Development.* Volume 22, (**7**4).

Yilkal Tadele (2015). White Lupin (*Lupinus albus* L.) grain, a potential source of protein for ruminants: A review, *Research Journal of Agriculture and Environmental Management*. 4(**4**): 180-188.

Yorgancilar, Babaoglu, M., M., Hakki, E.E. and Atalay, E. (2009). Determination of the relationship among old world lupin (*Lupinus* sp.) species using RAPD and ISSR markers. *Afr. J. Biotechnol*., **8**: 3524-3530.

Young, C.C. and Chao, C.C. (1989). Intrinsic antibiotic resistance and competition in fast- and slow-growing soybean rhizobia on a hybrid of Asian and US cultivars. *Biology Fertile Soils,* 8, 66 - 70.

Young, J.M., Kuykendall, L.D. E.Martinez-Romero, Kerr, A. and Sawada, H. (2001). A revision of *Rhizobium* Frank 18889, with an embedded description of the genus, and the inclusion of all species of *Agrobacterium conn* 1942 *Allorhizobium undicola* delajudi *et al.,* 1998 as new combinations: *Rhizobium radiobacter, rhizobium rhizogenes, R. rubi,r. undicola* and *R. vitis.Int J Syst evol microbial.* **51**:89-103.

Zahid, M., Abbasi, M.K., Hammed, S. and Rahim, N. (2015). Isolation and identification of indigenous plant growth promoting rhizobacteria from Himalayan region of Kashmir and their effect on improving growth and nutrient contents of maize (*Zea mays* L.). *Front. Microbiol.* **6**:207.

Zakhia, F., Jeder, H., Domergue, O., Willems, A., Cleyet-Marel, J.C., Gillis, M., Dreyfus, B., de Lajudie, P. (2004). Characterization of wild legume nodulating bacteria (LNB) in the infra-arid zone of Tunisia. Syst. *Appl. Microbiol*. **27**:380-395.

Zakry, F.A.A., Shamsuddin, Z.H., Khairuddin, A.R., Zakaria, Z.Z. and Anuar, A.R. (2012). Inoculation of Bacillus sphaericus UPMB-10 to young oil palm and measurement of its uptake of fixed nitrogen using the N isotope dilution technique. *Microbial. Environ*. **27**: 257–262.

Zengenia, R., Mpepereki, S. and Giller, K.E. (2006). Manure and soil properties affect survival and persistence of Soya bean nodulating rhizobia in small holder soils of Zimbabwe. *Applied Soil Ecol.* **32**: 232-242.

Zerihun Belay and Fassil Assefa (2011). Symbiotic and phenotypic diversity of *Rhizobium leguminosarum bv. viciae* from Northern Gondar, Ethiopia. *Afr. J. Biotechnol*. **10**: 4372- 4379.

Zerihun Nigusie (2011). Contribution of White Lupin (Lupinus albus L.) for Food Security in North-Western Ethiopia: A Review. Asian Journal of Plant sciences **11**: 200-205.

Zerihun Nigussie. (2012). Contribution of White Lupin (*Lupinus albus* L.) for Food Security in North-Western Ethiopia: A Review. *Asian Journal of Plant Sciences.* **11**: 200-205.

Zeghari, K., Aurag, J., Khbaya, Kharchaf D., Filali-Maltouf A. (2000). Phenotypic characteristics of rhizobia isoltes nodulating Acacia species in the arid and Saharan regions of Morocco. *Letters in Appl. Microbiol,* **30**: 351-357.

Zurdo-Pin˜eiro, J.L., Rivas, R., Trujillo, M.E.,Vizcaı´no, N., Carrasco,J.A., Chamber,M., Palomares, A., Mateos,P.F., Martı´nez-Molina, E. and Vela´zquez, E. (2007). *Ochrobactrum cytisi* sp. nov., isolated from nodules of Cytisus scoparius in Spain. *International Journal of Systematic and Evolutionary Microbiology*, (57**)** 784–788.

7. APPENDICES

7.1. Appendix Tables

Appendix Table-1. N-free Nutrient Solution (Broughton and Dilworth, 1970)

Solutions	Nutrient	Form	**$g.l^{-1}$**
1	Ca	$CaCl_2.2H_2O$	294.1
2	P	KH_2PO_4	136.1
3	Fe	Fe-Citrate	6.7
	Mg	$MgSO_4.7H_2O$	123.0
	K	K_2SO_4	87.0
	Mn	$MnSO_4.H_2O$	0338
	B	H_3BO_3	0.247
4	Zn	$ZnSO_4.7H_2O$	0.288
	Cu	$CuSO_4.5H_2O$	0.100
	Co	$CoSO_4.7H_2O$	0.056
	Mo	$Na\ MoO_3.H_2O$	0.048

Taken from Somasegaran and Hoben (1994)

Appendix Table-2a. Carbohydrate utilization of the isolates (Kit Part A)

Isolates	Carbohydrates											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
LUR1	+	+	+	+	+	+	+	+	+	+	+	+
LUR2	+	+	+	+	+	+	+	+	+	+	+	+
LUR5	+	+	+	+	+	+	+	+	+	+	+	+
LUR6	-	+	+	+	+	+	+	+	+	+	+	+
LUR8	+	+	+	+	+	+	+	+	+	+	+	+
LUR12	+	+	+	+	+	+	+	+	+	+	+	+
LUR20	-	+	+	+	+	+	+	+	+	+	+	+
LUR21	+	+	+	+	+	+	+	+	+	+	+	+
LUR22	+	+	+	+	+	+	+	+	+	+	+	+
LUR24	+	-	+	-	+	+	+	+	+	+	+	+
LUR25	+	+	+	+	+	+	+	+	+	+	+	+
LUR26	+	-	+	+	-	+	+	+	+	-	+	+
LUR27	+	+	+	+	+	+	+	+	+	+	+	+
LUR28	-	-	-	+	+	-	-	+	+	+	+	+
LUR29	-	+	+	+	+	+	+	+	+	+	+	+
LUR30	+	+	+	+	+	+	+	+	+	+	+	+
LUR32	+	+	+	+	+	+	+	+	+	+	+	+
LUR36	+	+	+	+	+	+	+	+	+	+	+	+
LUR37	+	+	+	+	+	+	+	+	+	+	+	+
LUR38	+	+	+	+	+	+	+	+	+	+	+	+
LUR42	+	+	+	+	+	+	+	+	+	+	+	+
LUR43	-	+	+	+	+	+	+	+	+	+	+	+
LUR46	-	-	+	+	+	+	+	+	+	+	+	+
LUR47	+	+	+	+	+	+	+	+	+	+	+	+
LUR48	+	+	+	+	+	+	+	+	+	+	+	+
LUR54	+	+	+	+	+	+	+	+	+	+	+	+
LUR56	+	+	+	+	+	+	+	+	+	+	+	+
LUR57	+	+	+	+	+	+	+	+	+	+	+	+
LUR58	+	+	+	+	+	+	+	+	+	+	+	+
LUR62	+	+	+	+	+	+	+	+	+	+	+	+
LUR67	+	+	+	+	+	+	+	+	+	+	+	+
LUR80	+	+	+	+	+	+	+	+	+	+	+	+
LUR84	+	+	+	+	+	+	+	+	+	+	+	+
LUR85	+	+	+	+	+	-	+	+	+	+	+	+
LUR87	+	+	+	+	+	+	+	+	+	+	+	+
LUR90	+	+	+	+	+	+	+	+	+	+	+	+
LUR91	-	+	+	+	+	+	+	+	+	+	+	+
LUR93	+	+	+	+	+	+	+	+	+	+	+	+
LUR96	+	+	+	+	+	+	+	+	+	+	+	+

1. Lactose 2. Xylose 3. Maltose 4. Fructose 5. Dextrose 6. Galactose 7. Raffinose 8. Trehalose 9. Melibiose 10. Sucrose 11. L-Arabinose 12. Mannose

Appendix Table-2b. Carbohydrate utilization of the isolates (Kit Part B)

Isolates	Carbohydrates											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
LUR1	+	-	+	+	-	+	+	+	+	-	+	+
LUR2	+	+	+	+	-	-	+	+	+	-	-	-
LUR5	+	+	+	+	-	+	+	+	+	-	-	-
LUR6	+	-	+	+	-	+	+	+	+	-	+	+
LUR8	+	+	+	+	-	+	+	+	+	+	+	-
LUR12	+	-	+	+	-	+	+	+	+	+	-	+
LUR20	+	-	+	+	-	+	+	+	+	-	+	+
LUR21	+	-	+	+	-	+	+	+	+	+	+	+
LUR22	+	-	+	+	-	+	+	+	+	+	+	+
LUR24	+	-	+	+	-	+	+	+	+	+	-	+
LUR25	+	-	+	+	-	+	+	+	-	-	-	-
LUR26	+	-	+	+	-	+	+	+	+	-	+	-
LUR27	+	+	+	+	-	+	+	+	+	+	+	+
LUR28	+	+	+	+	+	+	+	+	+	+	+	+
LUR29	+	+	-	+	-	+	+	+	+	-	-	+
LUR30	-	-	+	+	-	+	+	+	+	+	-	+
LUR32	-	-	+	+	-	+	+	+	+	+	-	-
LUR36	-	-	+	+	-	+	+	+	+	-	+	-
LUR37	+	-	+	+	-	+	+	+	+	+	-	-
LUR38	+	-	+	+	-	+	-	+	+	+	+	+
LUR42	+	-	+	+	-	+	+	+	+	+	+	+
LUR43	+	+	+	+	+	+	+	+	+	+	+	+
LUR46	+	+	+	+	-	+	+	+	+	+	+	-
LUR47	+	-	+	+	-	+	+	+	+	-	-	+
LUR48	+	+	+	+	-	+	+	+	+	-	-	+
LUR54	+	+	+	+	-	+	+	+	+	-	+	-
LUR56	+	_-	+	+	-	+	+	+	+	-	-	+
LUR57	-	-	+	+	+	+	+	+	+	+	+	+
LUR58	+	+	+	+	+	+	+	+	+	+	+	+
LUR62	+	+	+	+	+	+	+	+	+	+	+	+
LUR67	+	-	+	+	-	+	+	+	+	-	+	+
LUR80	+	+	+	-	+	+	+	+	+	+	+	+
LUR84	+	+	+	+	+	+	+	+	+	+	+	+
LUR85	+	+	+	+	+	+	+	+	+	+	+	+
LUR87	+	-	+	+	-	+	+	+	+	+	+	-
LUR90	+	+	+	+	+	+	+	+	+	+	+	+
LUR91	+	-	+	+	+	+	+	+	+	-	+	+
LUR93	+	-	+	+	-	+	+	+	-	-	-	+
LUR96	-	-	+	+	+	+	+	+	+	+	+	+

1.Inuline 2. Sodium Gluconate 3. Glycerol 4. Salicin 5. Dulcitol 6. Inositol 7. Sorbinol 8. Mannitol 9. Adonitol 10. Arabitol 11. Erythritol 12. Alpha-Methyl –D-glucoside

Appendix Table-2c. Carbohydrate utilization of the isolates (Kit Part C)

Isolates	Carbohydrates									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
LUR1	+	+	+	-	-	+	+	+	-	-
LUR2	+	+	+	-	+	+	+	+	-	-
LUR5	+	+	-	-	+	+	+	+	+	-
LUR6	+	+	+	+	+	+	+	+	+	-
LUR8	-	-	-	-	+	+	+	+	+	-
LUR12	+	-	+	-	+	+	+	+	+	-
LUR20	+	+	+	-	+	+	+	+	-	-
LUR21	-	-	+	-	+	+	+	+	-	-
LUR22	-	+	+	-	+	+	+	+	-	-
LUR24	+	+	+	-	-	+	+	+	-	+
LUR25	+	+	+	-	+	+	+	+	-	-
LUR26	+	+	-	-	+	-	+	+	-	-
LUR27	+	+	+	+	+	-	+	+	+	+
LUR28	-	-	-	-	-	-	+	+	-	-
LUR29	+	+	+	-	+	+	+	+	+	-
LUR30	+	+	+	+	-	+	+	+	-	-
LUR32	-	+	+	-	+	-	+	+	-	-
LUR36	+	+	-	-	+	+	+	+	-	-
LUR37	-	+	+	-	+	+	+	+	+	+
LUR38	+	+	+	-	+	+	+	+	-	-
LUR42	+	+	-	-	+	+	+	+	+	-
LUR43	+	+	-	-	+	+	+	+	-	-
LUR46	+	+	+	+	+	+	+	+	+	-
LUR47	+	-	-	-	+	+	+	+	-	-
LUR48	+	+	-	-	+	+	+	+	-	-
LUR54	+	+	+	-	+	+	+	+	+	-
LUR56	+	+	+	-	+	+	+	+	-	-
LUR57	-	+	-	-	+	+	+	+	-	+
LUR58	+	+	+	-	+	+	+	+	-	-
LUR62	+	+	+	-	+	+	+	+	+	-
LUR67	+	+	+	-	+	-	+	+	-	-
LUR80	+	-	+	-	+	+	+	+	-	-
LUR84	+	+	+	+	+	-	+	+	-	+
LUR85	+	+	+	-	+	+	+	+	-	-
LUR87	+	+	-	-	+	+	+	+	-	-
LUR90	+	+	+	+	+	-	+	+	+	+
LUR91	+	+	-	-	+	+	+	+	+	-
LUR93	+	+	-	-	+	+	+	+	+	-
LUR96	-	+	-	-	-	-	+	-	+	-

1.Rhamnose 2. Cellobiose 3. Melezitose 4. Alpha-Methyl-D-Mannoside 5. Xylitol 6. ONPG 7. Esculin 8. D Arabinose 9. Malonate 10. Sorbose

Appendix Table-3. Amino acid utilization of the isolates

Isolates	N-sources (Amino acids)									
	Gly	Asp	L-Glu	Phen	L-Lys	Met	L-Arg	Leu	L-Pro	% utilized
LUR1	-	+	+	+	+	+	+	+	+	89
LUR2	-	+	+	-	+	+	+	+	+	78
LUR5	-	+	+	-	+	+	+	+	+	78
LUR6	-	+	+	+	+	+	+	+	+	89
LUR8	-	+	+	-	+	+	+	+	+	78
LUR12	-	+	+	+	+	+	+	+	+	89
LUR20	-	+	+	+	+	+	+	+	+	89
LUR21	-	+	+	-	+	+	+	+	+	78
LUR22	-	+	+	+	+	+	+	+	+	89
LUR24	-	+	+	-	+	+	-	-	+	55
LUR25	-	+	+	-	+	+	+	+	+	78
LUR26	-	+	+	-	+	+	-	-	+	55
LUR27	-	+	+	-	+	+	+	+	+	78
LUR28	-	+	+	-	+	-	-	-	+	44
LUR29	-	+	+	+	+	-	-	+	+	67
LUR30	-	+	+	+	+	+	+	+	-	78
LUR32	-	+	+	-	+	-	+	+	+	78
LUR36	-	+	+	+	+	+	+	-	-	67
LUR37	-	+	+	-	+	-	+	+	+	67
LUR38	-	+	+	+	+	+	+	+	+	89
LUR42	-	+	+	+	+	-	+	-	+	67
LUR43	-	+	+	+	+	+	+	-	-	67
LUR46	-	+	+	-	+	+	+	+	+	78
LUR47	-	+	+	+	+	+	+	+	+	89
LUR48	-	+	+	+	+	+	+	+	+	89
LUR54	-	+	+	+	+	+	+	+	+	89
LUR56	-	+	+	+	+	-	-	+	+	67
LUR57	-	+	+	+	+	+	+	+	+	89
LUR58	-	+	+	+	+	+	+	+	-	78
LUR62	-	+	+	-	+	+	+	+	+	78
LUR67	-	+	+	+	+	+	+	+	-	78
LUR80	-	+	+	-	+	-	+	+	+	78
LUR84	-	+	+	-	+	+	+	+	+	78
LUR85	-	+	+	-	+	+	-	+	+	67
LUR87	-	+	+	+	+	+	+	+	+	89
LUR90	-	+	+	+	+	+	+	+	+	89
LUR91	-	+	+	-	+	-	+	+	+	67
LUR93	-	+	+	+	+	+	+	+	+	89
LUR96	-	+	+	-	+	-	-	-	+	44

Gly = Glycine, Asp = Asparagine, L-Glu = L-Glutamine, Phen = Phenylalanine, Met = Methionine, L-Arg = L-Arginine, L-Lys = L-Lysine, Leu = Leucine, L-Pro = L-Proline.

Appendix Table 4. Tolerance of white lupin rhizobia to different antibiotics

Isolates	P (10 µg)	Ch (30 µg)	Ka (30 µg)	Tet (25 µg)	Am (10 µg)	Str (10 µg)	Gn (30 µg)	Ak (µg)
LUR1	+	+	-	-	+	-	-	-
LUR2	+	-	-	-	+	-	-	-
LUR5	+	+	-	-	+	-	-	-
LUR6	+	-	-	-	-	-	-	-
LUR8	+	+	-	-	-	-	+	-
LUR12	+	-	+	+	-	-	+	-
LUR20	+	-	-	-	+	-	-	-
LUR21	+	_	-	-	-	-	-	-
LUR22	+	-	-	-	-	-	-	-
LUR24	+	-	-	-	-	-	-	-
LUR25	+	+	-	-	-	-	-	-
LUR26	+	-	-	-	-	-	-	-
LUR27	+	-	+	-	+	-	-	-
LUR28	+	-	+	-	-	-	-	-
LUR29	+	-	-	-	-	-	-	-
LUR30	+	-	-	-	-	-	-	-
LUR32	+	-	-	-	-	-	-	-
LUR36	-	-	-	-	-	-	-	-
LUR37	+	-	-	-	-	-	-	-
LUR38	+	-	-	-	-	-	-	-
LUR42	+	+	-	+	+	-	-	-
LUR43	+	-	-	+	+	-	-	-
LUR46	+	-	-	-	+	-	-	-
LUR47	+	+	-	+	+	-	-	-
LUR48	+	-	-	-	-	-	-	-
LUR54	+	-	-	-	-	-	-	-
LUR56	+	-	+	-	+	-	-	-
LUR57	+	-	-	-	-	-	-	-
LUR58	-	-	-	-	-	-	-	-
LUR62	+	+	-	+	-	-	-	+
LUR67	+	-	-	-	-	-	-	-
LUR80	+	+	-	-	-	-	-	+
LUR84	+	+	-	+	+	-	-	-
LUR85	+	+	-	-	-	-	-	-
LUR87	+	-	+	-	+	-	-	+
LUR90	+	+	-	+	+	-	-	-
LUR91	+	-	-	-	-	-	-	-
LUR93	+	+	-	-	-	-	-	-
LUR96	+	+	-	-	-	-	-	-

P = Penicillin, Ch = chloramphenicol, Ka = Kanamycin, Tet =Tetracycline, Am =Ampicillin, Str =Streptomycin, Gn = Gentamycin, Ak = Amikacin.

Appendix Table 5: ANOVA for the different parameters on sand culture under greenhouse conditions

Parameters		Sum of Squares	Df	Mean Square	F-value
Nodule Number	Between groups	14778.5	40	369.463	12.690
	Within groups	2387.333	82	29.114	
	Total	17165.870	122		
Nodule dry Mass	Between groups	.469	40	0.012	31.084
	Within groups	.031	82	0.000	
	Total	.500	122		
Shoot Dry Mass	Between groups	6.063	40	0.152	73.896
	Within groups	.168	82	0.002	
	Total	6.231	122		

df= degree of freedom

Appendix Table 6: Correlation coefficient (r) of selected parameters on sand culture under greenhouse conditions.

Parameters		NN	NDW	SDW
NN	Pearson Correlation	1	.540**	.719**
	Sig. (2-tailed)		.000	.000
	N	123	123	123
NDW	Pearson Correlation	.388**	1	.719**
	Sig. (2-tailed)	.000		.000
	N	123	123	123
SDW	Pearson Correlation	.540**	.388**	1
	Sig. (2-tailed)	.000	.000	
	N	123	123	123

**. Correlation is significant at the 0.01 level (2-tailed).

Appendix Table 7: Nodulation scenario of MPN determination on Achefer and Holeta soils.

Soils	Dilution Level	Nodulation Under Replication				
		I	II	III	IV	Nodulation Units*
Achefer	10^{-1}	+	+	+	+	4
	10^{-2}	+	+	+	+	4
	10^{-3}	+	+	+	+	4
	10^{-4}	+	+	+	+	4
	10^{-5}	+	+	+	+	4
	10^{-6}	+	+	+	+	4
	10^{-7}	+	+	_	+	3
	10^{-8}	+	_	_	_	1
	10^{-9}	_	_	_	_	0
	10^{-10}	-	-	-	-	0
	Total	8	7	6	7	28
Holeta	10^{-1}	+	+	+	_	3
	10^{-2}	+	+	_	+	3
	10^{-3}	_	+	_	+	2
	10^{-4}	_	_	_	+	1
	10^{-5}	_	_	_	_	0
	10^{-6}	_	_	_	_	0
	10^{-7}	_	_	_	_	0
	10^{-8}	_	_	_	_	0
	10^{-9}	_	_	_	_	0
	10^{-10}	-	-	-	-	0
	Total	2	3	1	3	9

(+) is for the presence of nodulation, (-) is for the absence of nodulation and * is for total nodulation at each dilution level.

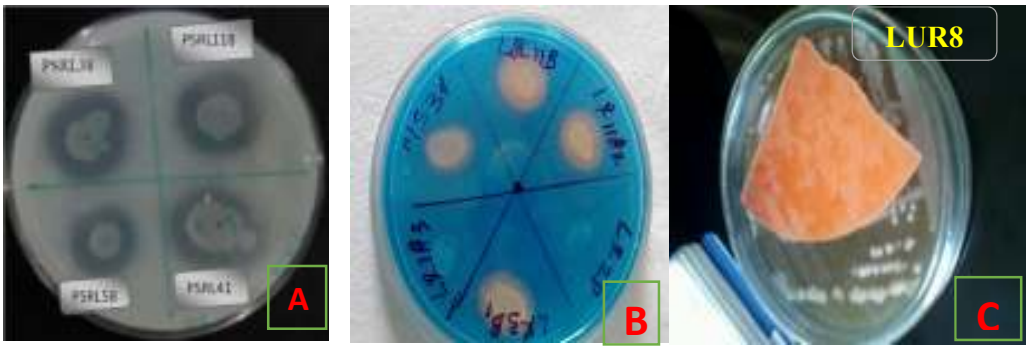
7.2. Appendix Figures

LUR12 LUR62

Appendix Figure 1. Nodules ofs isolates (HULR4, HULR17 and HULR62) on sand culture Under greenhouse conditions

Appendix Figure 2. Stand of white lupin plants inoculated with different isolates on sand cultureunder greenhouse conditions .

Appendix Figure 3. Stand of white lupin plants inoculated with selected highly effective isolates on Achefer (A) and Holeta (B) soils under greenhouse conditions.



Appendix Figure 4. Phosphate solubilization (A), Siderophore Production (B) and HCN production (C) characteristics of some PGPR from White Lupin.