ADDIS ABABA UNIVERSITY
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Phenotypic and Symbiotic effectiveness of Chickpea (*Cicer arietinum* L.) Root nodulating Rhizobia from Some Selected Parts of Southeastern Ethiopia.

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A thesis submitted to the school of graduate studies of Addis Ababa University in partial fulfillment of the requirement for the degree of masters of Science in Applied Microbiology

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Declaration

I the under signed declare that this thesis is my original work. It has never been submitted in any institution and all sources of materials used have been fully acknowledged.

Name Wubayehu Gebremedhin

Signature_____

Date_____

Affectionately Dedicated

To

My Beloved Mother

Late W/ro Etabez Getaneh

Who scarified her entire life for her son`s success!!

`` May God rest her soul in peace``

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Table of Contents Page

List of tables Page

List of figures Page

List of Appendices

List abbreviations

ANOVA	Analysis of Variance
BCP	Bromo cresol purple
BNF	Biological Nitrogen Fixation
BTB	Bromothymole blue
CEC	Cat ion exchange capacity
CFU	Colony forming unit
C I – C V	Cluster one to Cluster five
CR	Congo red
D.M	Dry matter
EIAR	Ethiopian Institute of Agricultural Research
HAB	Hoffer`s Alkaline Broth
HMR	Heavy Metal Resistance
IAR	Intrinsic Antibiotics Resistance
ICARDA	International Center for Agricultural Research in the Dry Areas
m.a.s.l	Meter above sea level
MB	Mega Bases
MGT	Mean generation time
Ndfa	Nitrogen derived from atmosphere
Nif gene	Nitrogen fixing gen
NSTC	National soil testing center
OC	Organic carbon
OD	Optical Density
SE	Symbiotic effectiveness
SNNP	Southern Nation, Nationality and peoples

TN	Total Nitrogen
UPGMA	Un weighed Pair Group Methods with Average
Var	Variety
YEMA	Yeas Extract Manitol Agar

ABSTRACT

Chickpea is the world's third most important food legume next to bean and soybean. Ethiopia is the largest producer of chickpea in Africa. However, chickpea production is very low due to poor soil fertility, poor nodulation and lack of inoculation with effective rhizobia. Although breeding for improved cultivar for better production and disease resistance has been undertaken for quite a long time in the country, there was limited research on selection of effective and competitive chickpea rhizobia for inoculant production to fully realize the benefits of biological nitrogen fixation. In this study, soil and nodule samples were collected from 60 sampling sites from chickpea growing areas of the eastern, southeastern and southern parts of the country to collect 39 root nodule bacteria. Based upon presumptive and definitive (authentication tests), only 23 isolates (59%) were root nodule bacteria. Out of the authenticated rhizobia, 14 isolates (61%) were categorized as either highly effective (17%) and effective (44%) with shoot dry matter accumulation of 80-100% and 50-80% in relation to the nitrogen-fertilized control plants, respectively. The inoculated plants showed diversity in nodule number, nodule dry weight and shoot dry weight. Accordingly, the isolates induced 14-62 nodules/plant with nodule dry weight of 20-53.3 mg/pt; and shoot dry matter of 250-417mg/Pt, respectively. The isolates were fast growing and acid producing rhizobia with growth rate of 1-2.8 hr, and changed the YEMA-BTB medium into yellow that can be tentatively grouped into the genus *Mesorhizobium*. They failed to solubilize inorganic phosphate on Pikovskaya's medium. The isolates were also screened *in vitro* for their nutritional versatility and eco-physiological tolerance for preliminary taxonomic screening and as markers for selection of ecologically competent isolates for inoculation study in the field. Accordingly, the isolates utilized most of the tested carbohydrates and amino acids except lactose and glycine. The chickpea rhizobia, with few exceptions were tolerant to moderately acidic and near neutral pH (pH 6.5-8.0), relatively salt tolerant (1-3% NaCl) and mildly temperature resistant (10-30^{o}C). Most of the isolates were sensitive to the antibiotics chloramphenicol and Penicillin G, to heavy metals Hg and Cu. However they showed inherent resistance to Bacitracine, gentamycine, Al and Mn. Based on the combined pattern of nutritional and ecological tolerance with symbiotic effectiveness, isolates EIARCP7, EIARCP13, EIARCP8, EIARCP10, EIARCP11, and EIARCP18 combined the best match of high symbiotic effectiveness with a wide range of resistance to eco-physiological stresses under laboratory conditions and may qualify to be potential candidates for a field trial in the future.

Key words: - Endosymbionts, Isolates, *Mesorhizobia*, Nodulation efficiency

1. INTRODUCTION

Chickpea is the world's third most important food legume next to bean and soybean (Namvar and Sharifi, 2011) in terms of production. It is grown in more than 50 countries of which 89.7% is mainly cultivated in Asia followed by 4.3% in Africa, 2.6% in Oceania, 2.9% in Americas and 0.4% in Europe. In Asia more than 64% of chickpea is grown in India. The global area under chickpea cultivation was about 11.0 million ha with a production of 8.8 m tons and average yield of nearly 800 kg ha^{-1} (Gaur *et al.*, 2010).Chickpea is a strictly self-pollinated crop with two types of cultivars, *Desi* and *Kabuli*. *Desi* cultivars are characterized by various combinations of brown, yellow, green and black colors, having small and angular shape with a rough surface and thick seed coat. The *Desi* cultivars account for more than 85% of world`s production. It is grown in India, Ethiopia, Mexico and Iran (Muehlbauer and Tullu, 1997). On the contrary, the *Kabuli* Chickpea is characterized by white color, ram`s head shape, thin seed coat and smooth seed surface and grown in Afghanistan, North Africa, southern Europe and Americas (Gaur *et al.*, 2010).

Chickpea fresh green leaves are eaten as vegetable and its flour is used in the preparation of various types of sweets. It contains 21.1% protein, 61.5 % carbohydrates, 4.5% fat and is rich in calcium, iron and niacin (Kiran Yadav, 2009). In addition to its nutritional quality and source of cash, chickpea restores and maintains soil fertility through its symbiotic nitrogen-fixation in association with root nodule bacteria from the genus *Mesorhizobium* species (Jarvis *et al*., 1997). Traditionally, chickpea is considered as a restrictive host nodulated by two species namely *Mesorhizobium ciceri* and *Mesorhizobium mediterranean* (Nour *et al.*, 1995). They are characterized by a wide range of growth rates as fast, slow and extra slow growing Rhizobia (Maatallah *et al*., 2002). Laranjo *et al*. (2004) have showed that other *Mesorhizobium* species may effectively nodulate chickpea.

It was estimated that chickpea obtains between 60 % to 100% nitrogen derived from atmosphere (Ndfa) with an average of 78% Ndfa (Aslam *et al*., 2003). Werner (2005) has estimated that chickpea fix N to the rate of 90 – 180 kg ha^{-1}. These values were consistent with published estimates of chickpea N_2 fixation, % Ndfa range of 55–80% for winter-sown chickpea in Syria and France (Beck *et al.*, 1991). According to Bhuiyan *et al*. (1998) seed inoculation with *Rhizobium* increased chickpea nodulation and seed yields up to 35%. This indicates the need for

selection of compatible and effective inoculants to fully realize the potential of N fixation to boost chickpea production.

Ethiopia is the largest producer of chickpea in Africa accounting for about 46% of the continent's production during 1994-2006 (FAOSTAT, 2012). It is also the seventh largest producer worldwide and contributes about 2% to the total world chickpea production. It is cultivated in the northern highlands of Showa, Gojam, Tigray, Gondar, and Wello as well as in Arsi and Bale, Gamogofa and Hararge of south eastern Ethiopia (Geletu Bejiga *et al.*, 1996). It is mainly cultivated between 1400-2300 m.a.s.l with annual rainfall ranges from 700and 1200 mm.
In Ethiopia, chickpea is an important food and cash crop with high acceptability and wider use. It accounts about 16% of the total pulse production of the country (Kassie Menale *et al*., 2009). However, its yield has remained very low (Geletu Bejiga *et al*., 2006) with the national average yield of 1.1-t ha^{-1} (CSA, 2012) compared to the average production rate of 2.61-t ha^{-1} in other chickpea producing countries in Asia (Maredia and Akibode, 2011).
Hence, for several decades (1974-2005) many research activities were undertaken in Ethiopia to improve chickpea cultivars with respect to their yield, tolerance to different biotic and abiotic stresses consequently, 11 improved chickpea varieties (six *kabuli* and five *desi*) were released by Ethiopian Institute of Agricultural Research (EIAR) (Shiferaw Bekele and Teklewolde Hailemariam, 2007). However, research on plant nutrition improvement and that of harnessing the potential of soil microbes to improve nitrogen fixation and phosphorous solubilization for increasing chickpea production were very limited in southeastern Ethiopia.
Recently, several research works have been initiated in Ethiopia to prepare inoculants for chickpea and other pulse crops at Ethiopin National Soil Testing Center (NSTC) (unpublished report). Several studies were also have undertaken on phenotypic diversity and symbiotic effectiveness of chickpea rhizobia from some growing areas of northern and central highlands of the country (Ahmed, 2010; Mulisa Jida and Fasil Assefa, 2012; Daneil Muleta, 2009). In addition Ibsa Aliyi, (2013) also tested agronomic and symbiotic characteristics of chickpea and Gemechu Keneni *et al*. (2012) have evaluated isolates from NSTC on selected chickpea landraces and improved variety and showed improvement in their symbioagronomic characteristics. But, surveying for effective nitrogen fixing chickpea rhizobia from southeastern Ethiopia has not been exhaustively undertaken. The characterization of the phenotypic and symbiotic properties of chickpea rhizobia from the above locations is helpful to develop

nutritionally versatile, ecologically competent and symbiotically effective inoculants for chickpea production and soil amelioration.

1.1. General objective:-

- To study the phenotypic and symbiotic properties of chickpea rhizobia from some parts of southeastern Ethiopia so as to select nutritionally versatile, ecologically competent, and symbiotically effective inoculants for chickpea production and soil amelioration.

1.2. Specific objectives:-

- To isolate chickpea root nodulating rhizobial isolates from nodules of field grown chickpea and nodules from chickpea plants grown on the field collected soils from some parts of Southern Ethiopia.
- To characterize the nutritional and eco physiological tolerance of the isolates.
- To authenticate and evaluate the symbiotic effectiveness of isolates under greenhouse conditions.
- To select symbiotically effective and ecologically competent isolates *in vitro*.

2. LITERATURE REVIEW

2.1. Nitrogen fixation

Although nitrogen gas (N_2) accounts for about eighty percent of the Earth's atmosphere it is the most limited nutrient to plant growth. This is because the N_2 molecule is very stable chemically and it has to be "fixed" into ammonium (NH_4^+) and nitrate (NO_3^-) so as to be assimilated by plants (Fisher and Newton, 2002).

Nitrogen can be chemically fixed by lightning, combustion and volcanism. The enormous energy of lightning ionizes the molecules in the atmosphere and enables them to combine and form nitrogen oxides and contributes to 10% of the total annual yield of fixed nitrogen (Fisher and Newton, 2002). Nitrogen can be synthetically fixed by Haber and Bosch process by combining

atmospheric N_2 gas (freed from contaminating O_2) and hydrogen (H_2) gas (usually derived either from natural gas or petroleum) under a high pressure and high temperature to form ammonia (NH_3) in large scale production of nitrogen fertilizer (urea) (Fisher and Newton, 2002). The process produces an equilibrium mixture that contains approximately 20% ammonia (Bockman, 1997).The production of fertilizer ammonia together with high yielding crop varieties contributed to the green revolution of the 1960`s (William, 2000).

The second most important source of fixed nitrogen derives from the activity of certain soil bacteria (biological nitrogen fixation) that absorb atmospheric N_2 gas and convert it into ammonium. The biological process contributes nearly 79 % of the total annual yield of fixed nitrogen globally (Burns and Hardy, 1975).

2.2. Biological nitrogen fixation

Biological nitrogen fixation (BNF) is a process by which N_2^{14} in the atmosphere is reduced into a biologically useful, combined form of N-ammonia by nitrogen fixing bacteria (Giller, 2001). The most important N fixing agents in agricultural systems are the symbiotic associations between legumes and the microsymbiont rhizobia via the formation of nodules (Giller, 2001). Nodules are formed on roots or, in some cases, stem (Tamimi and Timko, 2003).

Each year, about 175 million ton of N is contributed by BNF globally (Burns and Hardy, 1975), of which nearly 79% is accounted for by terrestrial fixation. Therefore, symbiotic nitrogen fixation is of great importance not only in the production of leguminous crops but also in the global nitrogen cycle (Ben Romdhane *et al*., 2008). Both nodulation and phenological traits of chickpea were improved by inoculation, consequently resulting in improved yield and yield related traits indicating the positive impact of biological nitrogen fixation on the crop productivity (Ibsa Aliyi, 2013). The rhizobial population plays a major role in meeting the nitrogen requirements of the plants.

2.3. Rhizobia–legume symbiosis

The interaction between rhizobia and their corresponding specific legume host plants leading to nodule formation is a complex process that requires a continuous and adequate signal exchange

between the plant and the bacteria (Perret *et al*., 2000). During this interaction, rhizobia are attracted by root exudates and colonize plant root surfaces. Flavonoids, the plant signal compounds present in the exudates, trigger the transcription of bacterial nodulation (*Nod*) genes leading thereby to the synthesis of *lipochito-oligosaccharide* signals called *Nod* factors.

These signal compounds (*lipochito-oligosaccharide)* in turn cause the legume root hairs to curl. *Nod* factors together with additional microbial signals, such as polysaccharides and secreted proteins, allow bacteria attached to root hairs to penetrate the root through a tubular structure called the infection thread, through which the rhizobia enter, move into root hair, and subsequently reach to the dividing cortical cells. When the thread reaches the primordium, the bacteria are released into the plant cytoplasm, where they differentiate into endosymbiotic form, the N_2 -fixing bacteroids. Inside the central nodule cells, rhizobia are housed as symbiosome that are horizontally acquired organelles and are involved in the enzymatic reduction of atmospheric nitrogen to ammonia and make this N accessible to their hosts. In return, the bacteria are supplied with carbohydrates in a protected environment. The host plant, however, regulates the number of nodules formed, the maturation of nodules, and the N_2 fixation of the nodules dependent upon available nitrogen (Fabio *et al.,* 2000).

Up to 25% of a legume's net photosynthate may be required for nitrogen fixation by rhizobia (Minchin *et al*., 1981). Faster fixation rates (mol nitrogen per s) can be beneficial for hosts, but carbon costs can also be important. Rhizobia that fix more nitrogen per carbon respired could free more carbon for other functions, including the option of supporting more nodules with the same amount of photosynthate.

2.4. Nodulation in the leguminosae

Most legumes genera are able to form rhizobial symbioses. This is perhaps not surprising considering the vastness and diversity of the family, currently estimated to contain 16,000–19,000 species in about 750 different genera (Allen and Allen, 1981). The family *Leguminosae* is divided into three subfamilies: the *Papilionoideae*, the *Mimosoideae* and the *Caesalpinioideae* From these the *Caesalpinioideae* is accepted as the most primitive group and the *Papilionoideae* and *Mimosoideae* are likely to have evolved from a common, nodulated caesalpinioid ancestor

(Sprent, 2001). *Papilionoideae* is the biggest subfamily that contains most of the legume tribes such as *Vicieae, Cicereae, and Phaseoleae*.

The *Mimosoideae* and the *Caesalpinioideae* are almost completely restricted to the tropics. The *Papilionoideae* contains the majority of the most important grain legumes. Nodulation capacity has been surveyed in several thousand species, representing about 20% of leguminous species and including members of about 60% of the legume genera (Sprent, 2001). Of these, 97% of the examined *papilionoid* species form nodules, as do more than 90% of *mimosoid* species. In contrast, only 23% of the examined *Caesalpinioid* species nodulate. These species all fall within eight genera – seven in the tribe *Caesalpinieae* and one sole genus, *Chamaecrista*, within the tribe *Cassieae* (De Faria *et al*., 1989).

Rhizobia, in general, produce both indeterminate and determinate types of nodules. Indeterminate nodules are characterized by different zones: (1) the distal meristem, where bacteria are internalized, (2) an inter zone with amyloplast accumulation and differentiation of bacteroids, and (3) a fixation zone that includes plant cells and a senescent zone (Pawlowski and Bisseling, 1996). In comparison, determinate nodules are typically round shaped and are derived from the cessation of meristem activity after nodule initiation and growth of the nodule mainly by cell expansion (Jeroen *et al*., 2006) (Fig 1.)

Figure 1. Determinate and indeterminate types of nodules

a) Determinate nodules (Soybean)

b). Indeterminate nodules (Chickpea)

Source: - a) microbewiki.kenyon.edu

b) Captured from west Hararge site.

The survey by Corby (1988) recognized three types of indeterminate nodule: *Caesalpinioid*, *Crotalarioid* and *Lupin* type; and two types of determinate nodule: *aeschynomenoid* and *desmodoid*.

2.5. The nod genes and legume promiscuity in rhizobia

In the 1970s, it was discovered that symbiosis and pathogenicity genes are harbored in plasmids,. These plasmids are conjugative and found in both genera *Agro bacterium* (Ledeboer *et al*., 1976) and *Rhizobium* (Zurkowski and Lorkiewicz, 1979). The symbiotic genes included those involved in legume nodulation (*nod*) and in nitrogen fixation (*nif)*. The nod genes are responsible for the synthesis of nod factors (*lipochitin-oligosaccharides*) that are receptors for the plant flavonoid signal (Broughton *et al*., 2000). The *nod*D is a regulatory gene of the operon *nod*ABC whose genes are determinants of the host range (Perret *et al*., 2000). The *nif* genes are involved in nitrogen fixation and are carried by rhizobia but also by free-living nitrogen fixing bacteria (Zehr *et al*., 2003). Symbiotic genes are harbored in plasmids in fast and in some intermediate-growing species of rhizobia, where as these genes are integrated in the chromosome in the intermediate and slow-growing rhizobia, harbored in symbiotic islands (Young *et al*., 2006). Symbiotic genes also named "auxiliary" or "accessory" genes, are commonly included in species description of rhizobia. From these genes most commonly studied are *nod*D, *nod*A, *nod*C, and *nif*H (Laguerre *et al*., 2001). Nevertheless, these genes are not useful in taxonomy because of their ability to be transferred in nature from plasmids to chromosomal islands (Nakatsukasa *et al*., 2008), from bacteria to plants (Broothaerts *et al*., 2005), and among bacteria (Rogel *et al*., 2001). Therefore, the analysis of symbiotic genes is overall useful to identify new-rhizobial species forming nodules and to carry out bio geographical studies of legume endosymbionts. Particularly, the nodulation genes are useful to define biovars within rhizobial species (Leon Barrios *et al*., 2009).

Within rhizobia, the concept of biovar is directly linked to the concept of legume promiscuity. It is known for many years that legume has different promiscuity degree and whereas some of them can be nodulated by several species of *rhizobia* such as *Macroptilium* and others are restrictive hosts for nodulation such as *Cicer* (Perret *et al*., 2000). In the same way, rhizobial strains can have broad or narrow host range. It is clearly identified that the *R. leguminosarum* bv. *Trifolii* can only nodulate plants of genus *Trifolium* whereas *Rhizobium* sp. NGR234 nodulates over 100

legumes as well as the non legume *Parasponia* (Pueppke and Broughton, 1999). Within these genes, *nod*C has been widely analyzed in rhizobial strains and found related with the host range of rhizobia and the promiscuity degree of the hosts (Iglesias *et al*., 2008). Moreover it was described the biovar *Ciceri* based on the *nod*C gene analysis (Rivas *et al*., 2007) concluded that *Cicer arietinum* is a very restrictive host, because although it can be nodulated by several species of *Mesorhizobium*, all of them carry nearly identical *nodC* genes. By contrast *P. vulgaris* is a very promiscuous legume since it is nodulated by the highest number of taxonomic species, which carry very divergent symbiotic genes (Zurdo Pineiro *et al*., 2009).

2.6. Taxonomy of rhizobia

Rhizobia are bacteria capable of forming nodules on leguminous plants. The description of the first rhizobial species was mainly based on the legume, which acted as host. Bacterial genome is consisting of two parts. The basic genome is composed of housekeeping genes that are needed under almost all growth conditions, they are carried on the chromosome, their organization is reasonably stable, and they are predominantly inherited vertically from mother to daughter cells. By contrast, the accessory genome consists of genes that are entirely selfish or offer adaptations to special circumstances, these are carried on plasmids, islands, transposons and phages, they undergo frequent rearrangement and are often transferred horizontally between cells (Fabio *et al.*, 2000).

In rhizobia three sizes of replicon are generally distinguished: plasmids (<1MB), mega plasmids (1-2MB) and chromosome (>4MB, so far as known). The organization differs: *Rhizobium leguminosarum* has a chromosome and 2-8 plasmids, *Sinorhizobium meliloti* has a chromosome, two mega plasmids, and sometimes 1-2 plasmids, *Mesorhizobium* has a chromosome, but may lack other independent replicons. In each case the nodulation genes are carried in the accessory genome: on a plasmid, a mega plasmid, and a symbiotic island inserted in the chromosome, respectively (Fabio *et al.*, 2000). Early *Rhizobium* taxonomy has been mainly based on the nodulating host range, although overlapping host ranges have already been reported more than fifty years ago. The development of molecular techniques accelerated the taxonomic evaluation and led to the identification of many new rhizobial genera. Based on the sequence of the 16S

rRNA gene; rhizobia could be grouped in the alpha subdivision of the Proteobacteria and several genera have been defined including *Rhizobium, Bradyrhizobium, Azorhizobium, Allorhizobium, Sinorhizobium* and *Mesorhizobium* (Young and Haukka, 1996). .

The identification of *M. amorpha* (Wang *et al*., 1999) showed the clear existence of symbiotic plasmids containing *nif* and *nod* genes in *Mesorhizobium*. *M. loti* and *M. plurifarium* have these genes in the chromosome and in *M. loti*; symbiotic islands have been described (Sullivan and Ronson, 1998). This finding adds support to *Mesorhizobium* being intermediate between *Rhizobium* (having mainly symbiotic plasmids) and *Bradyrhizobium* (with symbiotic determinants in chromosome).

The first described *Rhizobium* species, namely *R. leguminosarum* with current taxonomy of rhizobia can be grouped in to three biovars: *R. leguminosarum* bv. *trifolii* that nodulated clover, *R. leguminosarum* bv. *Viciae* that nodulate pea and faba bean, and *R. leguminosarum* bv. *Phaseoli* nodulating common bean (Jordan, 1984).

2.7. Quality aspect of legume inoculants and carriers

Inoculants are commercially prepared Rhizobium bacteria designed for application. There are many different species of Rhizobium bacteria and of those species, there are many different strains. Much of these are found naturally in the soil in very small amounts. To assure an adequate amount of Rhizobium bacteria is present in the soil to benefit the crop; the producer is recommended to apply an inoculant to the legume seed before planting (Burton, 1984). Normally inoculants are designed for application to seeds because this is an easy and convenient way of putting the rhizobia in the root zone of the developing seedling where infection of the root hairs can occur and nodules develop. Inoculants are available in dry, granular, or liquid formulations and may be applied to the seed by either one of the following methods (Duvauchelle, 2014):

1. Slurry Method – The inoculant is mixed with a liquid sticker the resulting slurry is mixed with the seed. Immediately after coating, spread the seed out and allow it to dry in a cool, shady area before planting. This method is generally considered to be the most effective.
2. Dry Sprinkle Method –The dry inoculant is sprinkled over the seed and thoroughly mixed in a container or seed hopper. This may be a quick way to inoculate seed, but considered not as effective as the slurry method because the inoculant does not adhere well to the seed.

Effective liquid stickers include 10-20% solutions of gums or sugars mixed with water, depending on the type of seed. There are also commercial sticker products available.

Due to the different formulations and carriers used in developing the inoculant, different manufacturer will have a different requirement for the amount needed. Peat is the most commonly used solid carrier in making legume inoculants. It is also the most dependable because rhizobia in a peat carrier remain viable longer both in the package and on the seed. Chemical and physical analyses of carrier materials are helpful but do not confirm the quality of a carrier. The quality can be determined only by placing viable rhizobia in the material and monitoring the growth and survival of rhizobia over a period of 6 months or longer (Burton, 1984). The important qualities of a carrier material in legume inoculant production are: Good absorption capacity, Easy to dry and grind, Nontoxic to rhizobia, Free of abrasive minerals, Low in content of soluble salts, Easy to sterilize, Available in adequate amounts at a reasonable cost (Paczkowski and Berryhill, 1979). Many other materials such as bagasse, sugar cane filter mud, coir dust, coal, lignite, charcoal, straws, various compost mixtures, clays, and minerals such as apatite and vermiculite, have all been tested and are acceptable (Date and Roughley, 1977).

2.8. Environmental factors affecting BNF

The interaction between the microsymbiont and the legume host plant are known to be complicated by unfavorable edaphic, climatic and management factors (Broughton *et al*., 2003). It is believed that any environmental or physical stress that reduces host plant growth and development may also negatively affect the associated bacterial strain and, thereby, the amount of nitrogen fixation. It is a 'rule of thumb' that symbiotically fixed nitrogen alone may not increase production if some other nutrients are limiting unless the latter are ameliorated (Bohlool *et al*., 1992).

The soil environment is under a constant state of change and can be relatively stressful for both macro- and microorganisms. Fluctuations in pH, nutrient availability, temperature, and water status, among other factors, greatly influence the growth, survival, and metabolic activity of soil microorganisms and plants, and their ability to enter into symbiotic interactions. Many diverse biological associations contribute to N_2 fixation (BNF) in both soil and aquatic systems (Sprent, 1984). However, in most agricultural systems, the primary source of biologically-fixed N via the

symbiotic interactions of legumes and soil bacteria of the genera *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, *Allorhizobium*, *Mesorhizobium*, and *Azorhizobium* (Sadowsky and Graham, 1988). The other 20% is contributed mainly by the actinorrhizal (by Frankia) and Anabena-Azolla types of symbiotic interactions.

Rhizobia can exist in two fundamentally different modes. Either as free-living saprophytic heterotrophs or as legume-host-specific nitrogen- fixing symbionts. The later mode of existence gives rhizobia advantages with respect to survival and persistence over most other soil bacteria. The growth of rhizobia in the rhizosphere may be stimulated by plant root exudates rhizobia in soils are associated with aggregates gives them some degree of protection from perturbations by environmental and biotic factors. Nodules can contain more than 10^{10} rhizobia g^{-1} (Dermott *et al*., 1987). Nodule senescence at the end of the growing season leads to the release of a large number of rhizobia into soils.

Bottomley (1992) have shown that a legume host is not needed for persistence (saprophytic competence) of rhizobia in soils although; nodule bacteria and bacteroids, after release into the environment are often susceptible to osmotic and other soil stress factors. Any environmental factor that negatively influences either the growth of rhizobia or the host plant itself has a dramatic impact on symbiotic N_2 fixation. It can negatively influence the nodulation process itself indirectly affect nitrogen fixation, or directly influence plant growth and vigor during post-nodulation events and so affect the efficient functioning of the nitrogenase enzyme complex. Some factors such as soil temperature, soil pH stress, nutrient stress, desiccation tolerance as well as soil water content and stress are mentioned as the major environmental stress factors affecting BNF (Sadowsky, 2005). Fixing atmospheric nitrogen depends on the environmental conditions, the availability of nutrients in the soil, cultural practices, and mainly the number and effectivity of native rhizobia (Rachna and Dudeja, 2009).

2.9. Chickpea *(Cicer arietinum)*

Chickpea was first produced in the Middle East about 7400 BC. Larger seeds with some evidence for domestication have been found from 6500 BC (Zohary and Hopf, 1993). Hence, it is said to be one of the oldest pulses known and cultivated from ancient times both in Asia and in

Europe. Chickpea constitutes 20% of the world's pulse production. It is currently grown on about 11 million hectares worldwide with average annual production of 8.8 million tons (Gaur et al., 2010). About 95% of chickpea cultivation and consumption is in the developing countries (Kiran Yadav, 2009).

Common names for chickpeas are Bengal gram in India, garbanzo in Latin America, Hamaz in the Arab world, Nohud in Turkey, and Shimbra in Ethiopia (Muehlbauer and Tullu, 1997). The center of their origin is probably western Asia; the Caucasus and Persia, India and Afghanistan. The germplasm of chickpea is maintained at the two international centers, ICRISAT in India and ICARDA in Syria, and also in the USDA-ARS Regional Plant Introduction Station at Pullman, U.S.A. Rhizobia isolated from chickpea nodules were found to have a wide range of growth rates and were described as fast- and slow-growing rhizobia (Giller, 2001**)**. Taxonomic studies later led to the description of two chickpea-nodulating rhizobial species: *Mesorhizobium ciceri* and *M. mediterraneum* (Nour *et al*., 1995). Chickpea nodules have indeterminate growth and can be very large.*Mesorhizobium ciceri* strain is a motile, Gram negative, non-spore-forming rod shaped bacteria. It is fast to moderately fast growing and forming 2-4 mm diameter colonies within 3-4 days, Colonies are slightly domed, moderately muccoid with smooth margins. The organism tolerates a pH range between 5.5 and 9.0 (Jarvis *et al*., 1997).

3. MATERIALS AND METHODS

3.1. Meteorological data of the sites

Bale and west Hararge have a range of temperatures at 20-25^{o}C and of rain fall at 400-700mm (Atkins and Ash, 2009). The mean annual rainfall of Arsi Zone sites varies between 800 mm and 1400 with average Temperatures 15-20^{o}C (Abate Feyissa, 2009). Average temperature of the study sites in Gamogofa zone is 29°C and the average annual rainfall is 900 mm. (SNNPRs Investment Expansion Process).

3.2. Soil and nodule collections

From sampling sites in chickpea growing areas of eastern, southeastern and southern parts of Ethiopia the root nodules and soil samples were collected from late October to early December, 2014. The geographical locations of the sampling sites were recorded using GPS (Table 3). From each site 10x10m transect was plotted from which five healthy and good stand plants were randomly selected and uprooted to collect pink nodules and kept in sealed vials containing a desiccant (Silica gel) covered with 1cm of cotton wool (Somasegaren and Hoben, 1994).

Table 1.The study sites GPS coordinates and origins of chickpea rhizobial isolates collected from different sampling sites of West Hararge, Arsi, Bale and Gamogofa

No.	Strain Designation	Zone	District	Latitude (N)	Longitude (E)	altitude
1	EIARCP5	W/Hararge	Odabulto	08°53`41.6``	040 °44`18.9``	1720
2	EIARCP6	W/Hararge	Gemechis	08° 56`33.7``	040°49`59.8``	1880
3	EIARCP7	W/Hararge	Tulo	09° 10`00.7``	041°09`43.3``	1922
4	EIARCP8	Bale	Ginir	07° 10`20.8``	040°42`45.6``	1913
5	EIARCP9	W/ Arsi	Adaba	7.16412529	39.53504875	2488
6	EIARCP10	Bale	Ginir	07° 10`20.8``	040°42`45.6``	1913
7	EIARCP11	W/Hararge	Odabulto	08°53`41.6``	040 °44`18.9``	1720
8	EIARCP12	Bale	Goro	6.98164615	40.53950359	1701
9	EIARCP13	Arsi	Digelo & Tijo	7.76819372	39.16293886	2553
10	EIARCP14	W/Hararge	Tulo	09°12`45.8``	041°06`20.5``	1789
11	EIARCP15	W/Hararge	Tulo	09 °13`09.2``	041°05`20.4``	1819
12	EIARCP16	W/Hararge	Tulo	09°12`45.3``	04 °05`31.6``	1801
13	EIARCP17	Arsi	Tiyo	07 °51`39.5``	039 °04`40.5``	2312

14	EIARCP18	Bale	Goro	6.99234741	40.46282309	1810
15	EIARCP19	W/Hararge	Gemechis	08°56`33.7``	040 °49`59.8``	1880
16	EIARCP20	Gamogofa	Zala	06°08`56.7``	037 °00`51.2``	1370
17	EIARCP21	Gamogofa	Boreda	06°36`17.1``	037 °41`44``	1562
18	EIARCP22	W/Hararge	Odabulto	08 °53`28.3``	040 °44`01.0``	1711
19	EIARCP23	Gamogofa	Zala	06 °12`00.9``	037° 02`05.6``	1326
20	EIARCP24	W/Hararge	Tulo	09°14`00.5``	041°09`42.5``	1923
21	EIARCP25	Bale	Ginir	7.15181286	40.68928418	1948
22	EIARCP26	Arsi	Tiyo	07°49`14.9``	039°07`26.2``	2556
23	EIARCP27	W/Hararge	Odabulto	040°43`20.8``	08° 43`26.3``	1702

Soil samples at rhizosphere of the uprooted plants from the same site were composited and collected into 1Kg plastic bags. The soil samples were analyzed for their physical and chemical properties (Soil pH, OC, total N, available P, K, CEC and texture of the soil) (Sahlemedhin Sertsu and Taye Bekele, 2000).

3.3. Induction of nodulation by rhizobia under greenhouse conditions

Nodulation was induced by using 'plant induction' method (Vincent, 1970) at Holeta Research Center (EIAR) green house. Each representative soil samples were thoroughly mixed and sieved using 2 mm sieve and filled into 3 kg capacity, 95% alcohol and 3 % (v/v) sodium hypochlorite sterilized pots. Five *Natoli* seeds were surface sterilized as before and plated on each pots and later thinned down to three per pot.

The pots were arranged in a complete random design to allow plant growth in a greenhouse with 12/12h light/dark cycle. The pots were watered twice a week at full field capacity. After 60 days of planting, plants were uprooted and nodules were randomly picked, surface sterilized as before, crushed as before and were inoculated on Yeast extract Mannitol Agar (YEMA) and incubated at 28 ± 2 °C for 3-5 days (Vincent, 1970).

3.4. Isolation of rhizobia from nodules

The desiccated nodules were soaked in distilled water using 150 ml beakers overnight for rehydration, surface sterilized with 95% ethanol for 10 seconds and with 3 % (v/v) solution of sodium hypochlorite for 3 minutes and rinsed six times according to Somasegaran and Hoben (1994). They were finally crushed with normal saline solution (0.85% NaCl) and loopful of the suspension was streaked on Yeast Extract Mannitol Agar (YEMA) and incubated at 28 ± 2 ^{o} C for 3 to 5 days (Vincent, 1970).

3.5. Purification and preservation of isolates

Single colonies from each plate were picked up and periodically purified by re-streaking on YEMA plates and incubating at 28 ± 2 ^{o} C for 3-5 days until pure and uniform colony types were obtained. The pure isolates were preserved at 4^{o}C on YEMA slant tubes containing 0.3 % (w/v) $CaCO_3$ (Vincent, 1970). Duplicate samples were also stored at -20^{0} C covered with 40% glycerol for long term preservation.

3.6. Designation of isolates

All isolates were designated as **EIARCP** the letters stand for **E**thiopian **I**nstitute of **A**gricultural **R**esearch **CP** (chickpea) isolates with different numbers.

3.7. Presumptive screening of root nodule bacteria

3.7.1. Gram staining test

All isolates were gram stained as a rapid means of identification of contaminants (Lupwayi and Haque, 1994) as follows:-

1. Using a sterile loop, a loopful of the samples was transferred on two different slides.
2. The specimens were allowed to dry on the slide at room temperature.
3. After the specimen were dried, it was heat-fixed
4. The Stain bacterial smears were stained by Gram's method as follows:

Crystal Violet----------1 minute

It was washed gently in tap water for 2-3 seconds.

Gram's Iodine (I2-KI) ----------1 minute

It was washed gently in tap water, shake off excess water.

95% alcohol-acetone----------10 seconds

It was washed gently in tap water, shake off excess water.

Safranin (counter stain) ----------20 seconds

Finally it was washed in tap water and blot dried.

Then it was examined with oil immersion optics using electron microscope Gram-negative organisms showed pink to red where as gram positive bacteria's showed purple/blue color.

3.7.2. Congo red absorption

Isolates were inoculated on YEMA medium containing Congo red (0.0025%) and dark-incubated at 28 ± 2 ^{o}C for 3-5 days (Vincent, 1970).

3.7.3. Peptone - glucose – test

Isolates were also inoculated into peptone-glucose medium by dissolving 5 g of glucose, 10 g of peptone, 15 g of agar and 10 ml of Bromocresol purple (BCP) in a liter of distilled water and the pH was adjusted to 6.8 with 1N NaOH and HCl according to Lupwayi and Haque (1994). The inoculated plates were incubated at 28 ± 2 ^{o}C for 3-5 days.

3.7.4. Hofer's alkaline broth test

Isolates were also inoculated into Hofer's alkaline broth (HAB) in 250 ml Erlenmer flask to determine their growth (Vincent, 1970). The medium contained 0.5 g K_2HPO_4, 0.2 g $MgSO_4$, 0.1 g NaCl, 0.05 g $CaCO_3$ 1 g yeast extract and 10 g mannitol in a liter of distilled water and adjusted to pH 11±0.2. The flasks were incubated on orbital shaker at 120 rev min^{-1} at room temperature for 3 days.

3.7.5. Cultural characteristics

The isolates were evaluated for their cultural characteristics such as colony type, texture, and diameter according to Ahmed *et al*. (1984). A loop-full of 72 hours old YEM broth cultures (10^8 cells/ml) were inoculated into YEMA plates and incubated at 28 ± 2 ^{o}C for 3-5 days. They were characterized as large mucoid (LM) and large watery (LW) colonies with exo-polysaccharide production.

3.7.6. Acid/ base production

The ability of isolates to release acid or base to change the medium to yellow or blue was determined by inoculating 72 hr old culture (10^8 cells/ml) on YEMA medium containing 0.125% bromothymol blue (BTB) indicator at pH 6.8 and incubating them at 28^0C for 3-5 days according to Jordan (1984).

3.8. Growth rate determination

Each isolate was grown on 100 ml sterilized YEM broth in 250 ml Erlenmeyer flask and kept on orbital shaker at 120 rev min^{-1} at room temperature for 3 days. Samples were transferred into 5 ml cuvette every 6 hours to measure turbidity using spectrophotometer (UV-7804C, Ultraviolet Visible spectrophotometer) at optical density (OD_{540}nm). Simultaneously, samples were serially diluted from which 0.1 ml of the diluents was inoculated into YEMA plates to count the colony forming units (CFU) (Somasegaren and Hoben, 1994). Finally the generation time (g) was calculated from the logarithmic phase according to White (1995) using the formula:

$$g = \frac{\log 2\ (t)}{\text{Log X- log Xo,}}$$

where: **g** is generation time

t is time elapsed

Xo is first OD reading in logarithmic phase

X is second OD reading in logarithmic phase

3.9. Physiological and biochemical characterization

All the tests except for phosphate solubilization were performed in triplicates on YEM agar medium containing plates divided into 10 equal squares at pH 6.8 unless stated otherwise. Each square was spot-inoculated using 10 µl of the 72 hour old YEM broth (10^8 cells/ml) and incubated at a temperature of 28 ± 2 ^{o}C for 3-5 days unless stated otherwise. Colony growth was determined qualitatively as (+) for positive growth but for no growth (-) (Somasegaran and Hoben, 1994).

3.9.1. Carbohydrate utilization

The ability of isolates to utilize different carbon sources was tested on modified YEMA containing different carbon sources. The carbohydrates were prepared at 1% (w/v) in place of manitol and the yeast extract was replaced by NH_4Cl (0.1%, w/v) according to Mohamed *et al.* (2000). The heat stable carbohydrates (lactose, D- manitol, and sucrose) were sterilized together with the modified YEMA medium, whereas the rest heat labile (galactose, D-maltose, sorbitol, arabinose, xylose, D- cellobiose, and inositol) were filter sterilized using 0.2 µm membrane filter and added to sterilized YEMA medium.

3.9.2. Nitrogen utilization

Isolates were also tested for their ability to grow on different nitrogen sources on modified YEMA medium containing filter sterilized amino acids like, L-cystine, thymine hydrochloride, L- aspargine, L-lysine, L- leucine, D-phenyl alanine and glycine as nitrogen sources. The N sources were prepared as 10 % w/v and 80 µl was added to 5 ml YEMA, where yeast extract was reduced to 50 mg/l (Kumar *et al*., 1999).

3.9.3. Intrinsic Antibiotic Resistance (IAR)

Intrinsic Antibiotics Resistance (IAR) of isolates was tested on solid YEMA medium containing the following filter sterilized antibiotics in µg/ml; kanamycin (50), streptomycin (100), , chloramphenicol (20), spectinomycin (100), tetracycline (20), bacitracine (30), penicillin G (30), gentamycine (10) as described by Maatallah *et al*. (2002). The stock solution of each antibiotic was prepared by dissolving 2 g of each antibiotic in 100 ml of water.

3.9.4. Intrinsic heavy metal resistance

All isolates were also tested for resistance to the following heavy metals (at the concentrations µg ml^{-1} in the parenthesis): AlK_2SO_4 (250), $CoCl_2$ (20), $CuCl_2$ (50), $HgCl_2$ (10), $MnSO_4$ (500), and $ZnCl_2$ (50) in YEMA media at pH 6.8 (Maatallah *et al*., 2002).

3.9.5. pH tolerance

The ability of the isolates to grow at different pH was tested on YEM agar medium adjusted to pH 4.5, 5.0, 8.0, 8.5, 9.0, 9.5, and 10.0 (Bernal and Graham, 2001).

3.9.6. Temperature tolerance

Tolerance of isolates to temperature was tested by inoculating them on YEM agar plates and incubating them at temperatures of 10, 20, 35, 40 and 45 °C (Rhitu *et al*., 2012).

3.9.7. Salt tolerance

Tolerance of isolates was also assessed by observing their growth on YEMA medium supplemented with 0.8, 1.0%, 1.5%, 2.0%, 2.5%, 3.0%, 4.0%, 5.0%, 6.0%, (w ⁄ v) NaCl (Maatallah *et al.*, 2002).

3.9.8. Phosphate solubilization test

The phosphate solubilizing ability of the isolates was tested on Pikovskaya's agar medium containing (per litter) 15.0 g agar, 10.0 g glucose, 5.0 g $Ca_3(PO_4)$, 0.5 g yeast extract, 0.5 g $(NH_4)_2SO_4$, 0.2 g KCl, 0.1 g $MgSO_4.7H_2O$, 0.1 mg $MnSo_4.2H2O$, 0.1 mg $FeSo_4$ according to Atlas (2010). After mixing and autoclaving these components 72 h old fresh culture broth ($10^8 ml^{-1}$) were spot inoculated on the medium and incubated at 28±2^oC for 7 days.

3.10. Authentication and symbiotic properties of isolates

3.10.1. Authentication of isolates on growth pouch

Isolates were authenticated as root nodule bacteria by re inoculating them on both host varieties namely *desi* seed type *var. natoli* and *Kabuli* seed type *var. Habru* obtained from Debrezeit Research Center (EIAR).

The above seeds were surface sterilized with 95% alcohol and 3 % (v/v) sodium hypochlorite and germinated on Petri dish then transferred to grow in pouches/ three seeds for each. After three days of planting, each seedling was inoculated with 1ml culture with approximately 10^8 of bacteria per ml (0.93, OD_{540}). The plants were fertilized with N free nutrient medium (Broughton and Dilworth, 1970) every week and watered every three days as described by Somasegaran and Hoben (1994).

Table 2. N-free nutrient solutions (Broughton and Dilworth, 1970)

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

The experiment was laid out with three replications in a completely randomized design. Each replication contained two control treatments for comparison, which were used as negative and positive controls. Positive control pouches was fertilized with nitrogen, at a rate 0.05 KNO_3 (w/v) solution per week without inoculation. The negative control pouches were supplied only with the nitrogen free solution without inoculation and the nitrogen fertilizer. The plants were grown in a greenhouse with a 12 h photoperiod under 23 °C/15 °C day/night temperature. All pouches were allowed to grow for 60 days to observe the presence or absence of nodulation.

3.10.2. Evaluation of isolates for symbiotic effectiveness on sand

Isolates that showed good performance during authentication were selected for evaluating their symbiotic effectiveness on sand culture according to Somasegaren and Hoben (1994). Three Kg capacity pots were filled with acid washed and autoclave sterilized sand. Seeds of chickpea variety (*Desi* seed var *natoli*) were surface sterilized as before and germinated on Petri dishes for 3 days from which five pre germinated seeds were transferred to each pot that were later thinned down to three. After three days of planting, each seedling was inoculated with 1ml of 72 hrs culture, (approximately 10^8 of bacteria per ml (0.93, OD_{540})). Commercialized chickpea rhizobia *CP-018* obtained from Ethiopian National Soil Testing Center was used as reference inoculant.

This experiment was laid out in completely randomized design with three replications. Each replication contained two control treatments (negative and positive controls) and one treatment with reference strain (CP-018). The positive control pots were fertilized with 0.05 KNO_3 (w/v) nitrogen solution per week without inoculation. The negative control pots were supplied only with the nitrogen free solution without the inoculum and the nitrogen fertilizer. The plants were harvested 60 days after planting to record number of nodules, shoot dry weight and nodule dry weights.

3.10.3. Relative effectiveness of the isolates

Symbiotic effectiveness of isolates was determined according to Gibson (1987). It was an estimate as the ratio between the positive control and each treatment, using shoot dry weight values. The value of the negative control was subtracted from both N fertilized dry weight and from inoculated dry matter as follows:

$$SE = \frac{(\text{Inoculated plant D.M. - control (-ve) D.M.}) \times 100}{\text{N-Fertilized plant D.M. - control (-ve) D.M.}}$$

Where, D.M. = dry matter, S.E. = symbiotic effectiveness

The rate of nitrogen fixing effectiveness is evaluated as: Highly effective > 80%, Effective 50-80%, Lower effective 35-50% and Ineffective <35%.

3.11. Data analysis

Un weighed Pair Group Methods with Average (UPGMA) for phenotypic traits and Analysis of variance (ANOVA) for comparisons between the treatments with shoot dry weight, nodule number and nodule dry weight per plant was done using the statistical program SAS version 9.3. Mean separation was calculated using the LSD test value when the F-test was significant at p = 0.05%.

4. RESULTS

4.1. Soil chemical properties

It is clearly indicated that in Table 3 west Hararge sites ranges from slightly acidic to neutral pH. Arsi site showed moderately acidic pH, soils from Bale were grouped as slightly alkaline to neutral and Gamogofa soils were in range between slightly acidic to slightly alkaline pH. Additionally available P in west Hararge soils was very high and it was low in other study sites.

The organic carbon and total N rates of west Hararge, Bale and Gamogofa soils were grouped as low N. whereas Arsi soils ranges moderate to high N levels. Also organic Carbon at west Hararge and Bale soils were moderate and it was high level at Arsi and low at Gamogofa soils.

The West Hararge soils contained ultimate C: N ratio where as in the other sites it was slightly lower hence the micro organisms must acquire enough carbon and nitrogen from the environment in which they live to maintain that ratio of carbon and nitrogen in their bodies. CEC (Cation Exchange Capacity) rating of Gamogofa, Arsi and Bale soils were high in where as west Hararge soils ranges from high to very high.

Table 3. Soil analysis for sampling sites of the rhizobial isolates obtained from different parts of south eastern Ethiopia.

Site	pH	P	% OC	K	TN	C:N	CEC	Texture
W/ Hararge	6.22-6.85	5.87-61.62	1.67-2.61	0.52-1.09	0.06-0.12	22:1 - 28:1	26.3-53.98	Clay & Sandy Clay loam
Arsi	5.44-6.35	6.5-11.82	2.46-4.36	0.73-1.35	0.19-0.35	12:1-13:1	33.36-36.32	Clay & clay loam
Bale	6.95-7.47	6.73-14.28	1.29-2.03	0.12-1.18	0.08-0.17	12:1-16:1	54.24-60.4	Clay
Gamogofa	6.46-7.41	5.47-10.58	0.78-1.55	0.8-1.35	0.06-0.1	13:1-16:1	42.1-51.54	Clay

4.2. Isolation, presumption test and authentication of rhizobia

A total of 39 root nodule bacteria were isolated from 60 chickpea nodule samples from different sites of West Hararge, Gamogofa, Arsi, and Bale Zones. All isolates were gram negative rods, did not absorb Congo red under dark condition, did not grow on peptone glucose agar with Bromocresol Purple (PGA-BCP) and on Hofer`s alkaline broth. From these 23 isolates (59%) induced nodules on the host upon reinoculation under greenhouse condition. All the 23 isolates were able to nodulate *Desi* seed types, var *Natoli*, and three isolates namely EIARCP13 (Arsi) and EIARCP10 as well as EIARCP12 (Bale zone) nodulated both *Desi* seed types, var *Natoli* and *Kabuli* seeds types, var *Habru* in growth pouches.

4.3. Characterization of the isolates

4.3.1. Cultural and growth rate characterizations

The colony diameter ranges from the lowest 2 mm to largest 5 mm. The majority of the isolates (83%) were large muccoid (LM) and the remaining isolates were large watery (LW). Most of the isolates were characterized by domed shape (61%) followed by colonies with flat shape (27%) and the remaining few (12%) were showed a conical shape. All the isolates changed the color of YEMA- BTB medium to yellow and none of them absorbed Congo red (Appendix 8).

The isolates (74%) were within the range of 1.5-2.8 hrs of doubling time (Table 4). The rest of the isolates were with the doubling time of 1-1.5 hr. The isolates EIARCP9 and EIARCP19 from Bale and west Hararge respectively were the fast growing isolates with a doubling time of 1 hour whereas isolate EIARCP24 from west Hararge showed the slowest doubling time of 2.8 hrs. All the isolates were categorized as fast growers since all display generation time below 3 hours (Table 4).

Table 4. Colony morphology, colony diameter, and growth rate of the isolates obtained from different parts of southeastern Ethiopia.

No.	Isolates	Colony appearance	Shape	Diameter (mm)	MGT (in hours)
1	EIARCP5	LM	Domed	4	2.2
2	EIARCP6	LM	Domed	3	1.6
3	EIARCP7	LM	Domed	4	1.5
4	EIARCP8	LM	Domed	4	2.5
5	EIARCP9	LM	Domed	3	1.0
6	EIARCP10	LW	Flat	4	2.3
7	EIARCP11	LM	Domed	3	2.1
8	EIARCP12	LW	Flat	3	2.5
9	EIARCP13	LM	Domed	5	1.2
10	EIARCP14	LM	Flat	3	2.0
11	EIARCP15	LM	Domed	4	1.1
12	EIARCP16	LM	Domed	4	2.5
13	EIARCP17	LW	Flat	5	1.7
14	EIARCP18	LM	Conical	2	2.3
15	EIARCP19	LM	Domed	3	1.0
16	EIARCP20	LM	Conical	2	1.9
17	EIARCP21	LM	Domed	3	2.1
18	EIARCP22	LM	Domed	4	1.8
19	EIARCP23	LM	Domed	5	2.5
20	EIARCP24	LM	Conical	2	2.8
21	EIARCP25	LM	Flat	5	1.5
22	EIARCP26	LW	Flat	4	1.2
23	EIARCP27	LM	Domed	4	1.4

Legend: - LM= Large Muccoid, LW= Large Watery

4.3.2. Biochemical and physiological characterization

4.3.2.1. Physiological characterization

None of isolates solubilized tri- calcium in Pikoviskaya`s medium.

4.3.2.2. Carbohydrate utilization

All the isolates catabolized mannitol (Appendix 2). Majority of the isolates utilized sorbitol (83%), maltose (83%), arabinose (74%), sucrose (78%), galactose (70%), xylose (70%), inositol (65%), cellobiose (61%) and more than half of them lactose (52%) (Table5). Generally, rhizobial isolates (80-100 %.) were catabolized mannitol, sorbitol and maltose.

4.3.2.3. Nitrogen source utilization

The isolates were capable of metabolizing a variety of amino acids as their nitrogen source (Table 5). Almost all of the isolates used cystine, leucine and lysine as their sole nitrogen source. Many isolates catabolized aspargine and phenylalanine. Additionally, a few isolates utilized thymine and glycine. The isolates such as EIARCP9, EIARCP12, EIARCP19, EIARCP20, EIARCP24 and EIARCP27 were versatile in utilizing 86% of the tested N sources.

Table 5. Carbon and Nitrogen Utilization of the rhizobial isolates obtained from different parts of south eastern Ethiopia.

No.	Isolate	% C utilized	Pattern of C utilization	% N utilized	Pattern of N utilization
1	EIARCP5	70	All but Galactose, Inositol & Lactose	71	All except Thymine & Glycin
2	EIARCP6	70	All but Galactose, Inositol & Sorbitol	43	Utilizes only Cystin, Leucine & Phenyl alanine
3	EIARCP7	90	All but Cellobiose	71	All except Thymine & Glycin
4	EIARCP8	90	All but Cellobiose	57	All except Thymine, Lycine & Phenyl alanine
5	EIARCP9	80	All but Cellobiose & xylose	86	All except Thymine
6	EIARCP10	100	All	57	All except Leucin, Phenyl alanine& Glycin
7	EIARCP11	80	All but Arabinose & lactose	57	All except Thymine, Lycin & Glycin
8	EIARCP12	80	All but Cellobiose & xylose	86	All except Thymine
9	EIARCP13	80	All but Cellobiose & xylose	71	All except Thymine & Glycin
10	EIARCP14	50	All but Xylose, Sucrose, Inositol, lactose & Maltose	71	All except Thymine & Glycin
11	EIARCP15	100	All	43	Utilizes only Cystin, Lycine & Leucine
12	EIARCP16	60	All but Xylose, Sucrose, Inositol & Lactose	57	All except Thymine, Aspargine &Glycin

13	EIARCP17	90	All but Cellobiose	57	All except Thymine, Aspargine &Glycin
14	EIARCP18	100	All	71	All except Thymine and Glycin
15	EIARCP19	80	All but Inositol & Lactose	86	All except Thymine
16	EIARCP20	70	All but Galactose, Arabinose & Lactose	86	All except Thymine
17	EIARCP21	70	All but Cellobiose, Arabinose & Lactose	71	All except Thymine and Glycin
18	EIARCP22	70	All but Cellobiose, Arabinose & Lactose	71	All except Thymine and Glycin
19	EIARCP23	40	Utilizes only Cellobiose, Arabinose, Sucrose & Mannitol	43	Utilizes only Cystin, Aspargine & Lycine
20	EIARCP24	50	All but Galactose, Inositol, Lactose, Maltose & sorbitol	86	All except Aspargine
21	EIARCP25	70	All but Cellobiose, Xylose &sucrose	71	All except Aspargine and Phenyl alanine
22	EIARCP26	20	Utilizes only Xylose and Mannitol	57	All except Cystin, Thymine and Glycin
23	EIARCP27	80	All but Arabinose and Sucrose	86	All except Glycin

4.3.2.5. Intrinsic Antibiotics Resistance (IAR)

Almost all (96%) of the isolates showed resistance to antibiotic bacitracin (Appendix 4). The majority of the isolates, 78% and 69%, were resistant to gentamycin and tetracycline, respectively (Table 6). However, a few isolates (30-35%) were resistant to spectinomycin, streptomycin, Penicillin G and Kanamycin. But all the isolates except EIARCP18 were sensitive to chloramphenicol (Appendix 4).

4.3.2.4. Heavy metal resistance

All isolates except EIARCP14 and EIARCP22 grew on YEMA media containing Al and the majority of isolates were resistant to Mn (87%) and Zn (52%). The data (Table 6) showed variation in heavy metal resistance pattern ranges from isolates resistant to several heavy metals (83%) to very sensitive isolates to all tested heavy metals. EIARCP14 did not grow on all of tested heavy metals. In addition to this 74% and 61% of isolates were sensitive to Co and Cu, respectively and all the isolates except EIARCP10 failed to grow on Hg (Appendix 5).

Table 6. Intrinsic Antibiotic resistance (IAR) and heavy metal resistance (HMR) of chickpea rhizobial isolates obtained from different parts of South eastern Ethiopia.

No.	Isolate	% IAR	Pattern of antibiotic resistance (IAR)	% Heavy metal resistance	Pattern of HMR
1	EIARCP5	63	All except Chlor a., Spec. and Gentam.	50	All except Zn, Hg and Co
2	EIARCP6	38	Resist only Spec, Bacitracine and Gentam.	50	All except Hg,Co and Cu
3	EIARCP7	38	Resist only Spec, Bacitracine and Gentam.	67	All except Hg and Cu
4	EIARCP8	75	All except Chlor a. and Spec.	83	All except Hg
5	EIARCP9	38	Resist only Spec, Bacitracine and Gentam.	83	All except Hg
6	EIARCP10	63	All except Chlor a., Spec., and Penic G.	83	All except Cu
7	EIARCP11	88	All except Chlor a.	67	All except Hg and Cu
8	EIARCP12	25	Resist only Bacitracine and Tetracycline.	50	All except Hg, Co and Cu
9	EIARCP13	63	All except Chlor a., Penic G., and Strept.	33	Resist only Al and Mn
10	EIARCP14	25	Resist only Bacitracine and Tetracycline.	0	Sensitive to All tested heavy metals
11	EIARCP15	38	Resist only Bacitracine, Tetracycline and Gentam.	17	Resist only Al
12	EIARCP16	38	Resist only Bacitracine, Tetracycline and Gentam.	17	Resist only Al
13	EIARCP17	63	All except Chlor a., Penic G. and Kan m.	50	All except Hg, Co and Cu

14	EIARCP18	63	All except Spec, Kan m. and Strep.	50	All except Zn, Hg and Cu
15	EIARCP19	75	All except Chlor a. and Penic G.	17	Resist only Al
16	EIARCP20	38	Resist only Bacitracine, Penic G. and Strept.	50	All except Zn, Hg and Cu
17	EIARCP21	88	All except Chlor a.	67	All except Hg and Co
18	EIARCP22	50	Resist Bacitracine, Penic G., Tetracycline and Genta m.	33	Resist only Mn and Cu
19	EIARCP23	25	Resist only Bacitracine and Genta m.	50	All except Zn, Hg and Cu
20	EIARCP24	13	Resist only Penic G.	67	All except Co and Cu
21	EIARCP25	38	Resist only Bacitracine, Tetra C. and Geta m.	67	All except Hg and Co
22	EIARCP26	38	Resist only Bacitracine, Tetra C. and Geta m.	33	Resist only Al and Mn
23	EIARCP27	25	Resist only Bacitracine and Genta m.	50	All except Zn, Hg and Cu

NB:- Chlor a.= Chloramphenicol, Spec.= Spectinomycin, Genta m.= Gentamycin, Strept. = Streptomycin, Penic G. = Penicillin G

4.3.2.6. Salt tolerance

Tolerance of rhizobial isolates to NaCl concentration showed variation among strains (Table 7). All isolates grew in control culture (in YEMA containing 0.01% NaCl). But showed a steady decrease in growth when they were inoculated into the medium containing 4 to 6% salt concentration (Appendix 6). Consequently, 61% isolates were grown on YEMA supplied with 3% salt concentration. On the other hand, 82% and 78% of isolates grew on 1.5% and 2% salt concentration, respectively. Most isolates (87 %) of them showed growth on YEMA medium containing 0.8% and 1% NaCl concentration. Most isolates salt tolerance showed broad range of resistance except growth of isolates EIARCP8, EIARCP21 and EIARCP27 that were restricted to grow only in a control culture (0.01% NaCl) (Table 7 and Appendix 6).

4.3.2.7. pH tolerance

The pH tolerance physiological trait indicated a wide diversity amongst these rhizobia from the summarized results (Table 7). Over 56 % and 22% of the isolates were found to be tolerant to pH 8 and pH 8.5, respectively. Additionally, 17 % and 13% of the isolates grew at pH 9 and pH 9.5, respectively. The only isolate grown on YEMA medium containing pH 10 was EIARCP20, while none of the isolates grew on pH 4.5. Majority of the isolates grew at pH levels 6.8-8. Also isolates EIARCP20 and EIARCP18 tolerated wider pH levels (5-9.5) (Table 7).

4.3.2.8. Temperature tolerance

The result obtained from this work revealed that the tolerance of chickpea rhizobia isolates to temperature stress was very diverse (Table 7). At 20º C, the isolates growth was 95.65%, while growth at 10º C was 86.96 %. Most isolates tolerated cold stress better than the heat stress. From the 23 tested isolates, EIARCP16 was the most tolerant isolate to both (40º C and 45º C) continuous heat as well as EIARCP18 was grew on 40º C. On the other hand, isolates EIARCP6 and EIARCP19 are highly sensitive to both too hot and too cold temperatures. A total of 87% of the isolates grew on temperature ranges 10-30^{o}C (Table7).

Table 7. Patterns of salt, temperature and pH tolerance of chickpea rhizobial isolated from some parts of southeastern Ethiopia.

No.	Isolates	NaCl (%)	pH levels	Temperature (^{o}C)
1	EIARCP5	3	6.8-8	10-30
2	EIARCP6	3	6.8-8	20-30
3	EIARCP7	3	6.8-7	10-35
4	EIARCP8	0.01	6.8-8	10-35
5	EIARCP9	3	6.8-8	10-35
6	EIARCP10	1.5	6.8-8.5	10-35
7	EIARCP11	3	6.8-7	10-30
8	EIARCP12	3	6.8-9	10-30
9	EIARCP13	2.5	6.8-7	10-30
10	EIARCP14	1	6.8-7	10-30
11	EIARCP15	2.5	6.8-8	10-30
12	EIARCP16	1.5	6.8-7	20-45
13	EIARCP17	3	6.8-8	10-30
14	EIARCP18	3	5-9.5	10-40
15	EIARCP19	3	6.8-8	20-30
16	EIARCP20	3	5-10	10-35
17	EIARCP21	0.01	6.8-8	10-35
18	EIARCP22	1	6.8-9.5	10-35
19	EIARCP23	3	6.8-8	10-30
20	EIARCP24	3	6.8-7	10-35
21	EIARCP25	3	6.8-7	10-30
22	EIARCP26	3	6.8-7	10-35
23	EIARCP27	0.01	6.8-8	10-35

4.3.3. Numerical analysis

The phenotypic traits of the 23 chickpea isolates were summarized as all strains grew in the control cultures (28^{o}C, pH 7.0 and 0.01% NaCl). The physiological traits studied indicated wide diversity among these rhizobia. The dendogram obtained from the numerical analysis of 23 phenotypic traits placed these strains in five distinctive clusters (Figure 2).

Cluster I was the largest with 11 strains originating from various locations. The Isolates exhibited all levels of effectiveness even though the highest effective isolates were dominantly included in this cluster. All the isolates in Cluster I commonly share characteristics such as their potential to resist the presence of Al and bacitracin in their growth media. They also have ability to catabolize maltose and utilize cystine as their carbohydrate and amino acid sources, respectively. All these isolates were tolerant for salt concentration that ranged from 0.8% to 2.5%. On the contrary all of them were not able to grow on high temperatures (40^{o} C and 45^{o} C).

Strains belonging to cluster II had an average infectivity and effectiveness except EIARCP27 which was the poorest with its effectiveness. These cluster commonly shared catablolization of 50% tested carbohydrates, had Mn resistance from heavy metals and bacitracin and gentamycin from antibiotics. Additionally, they utilized cystine and asparagine. They showed better growth at 10^{o}C and 20^{o}C temperatures. This cluster was sensitive to chloramphenicol, salt concentration more than 2% and high temperatures (40 ^{o} C and 45^{o} C).

Cluster III contains three fast-growing isolates originating from different locations. They displayed good symbiotic efficiency except EIARCP24 which showed ineffective performance. This cluster showed salt tolerance upto 3% concentration and exhibited better growth at 35^{o}C similar to isolates in cluster II.

Isolates in cluster IV utilized half of the tested carbohydrate and N sources. Additionally, EIARCP16 from this cluster showed average effectiveness, antibiotics resistance such as bacitracin, tetracycline and gentamycin. It showed better temperature tolerance at 35^{o}C to 45^{o}C. This cluster neither grew on alkaline nor on acidic pH but performed well on neutral pH.

Cluster V were the collection of least symbiotic effective isolates but exhibited wider heavy metal resistance, carbohydrate utilization and N source utilization efficiency.

This numeric analysis showed the clustering of isolates did not correlate with their geographical origin since isolates from same origin were included in different clusters and isolates from diverse origins were found in the same group (Fig. 2).

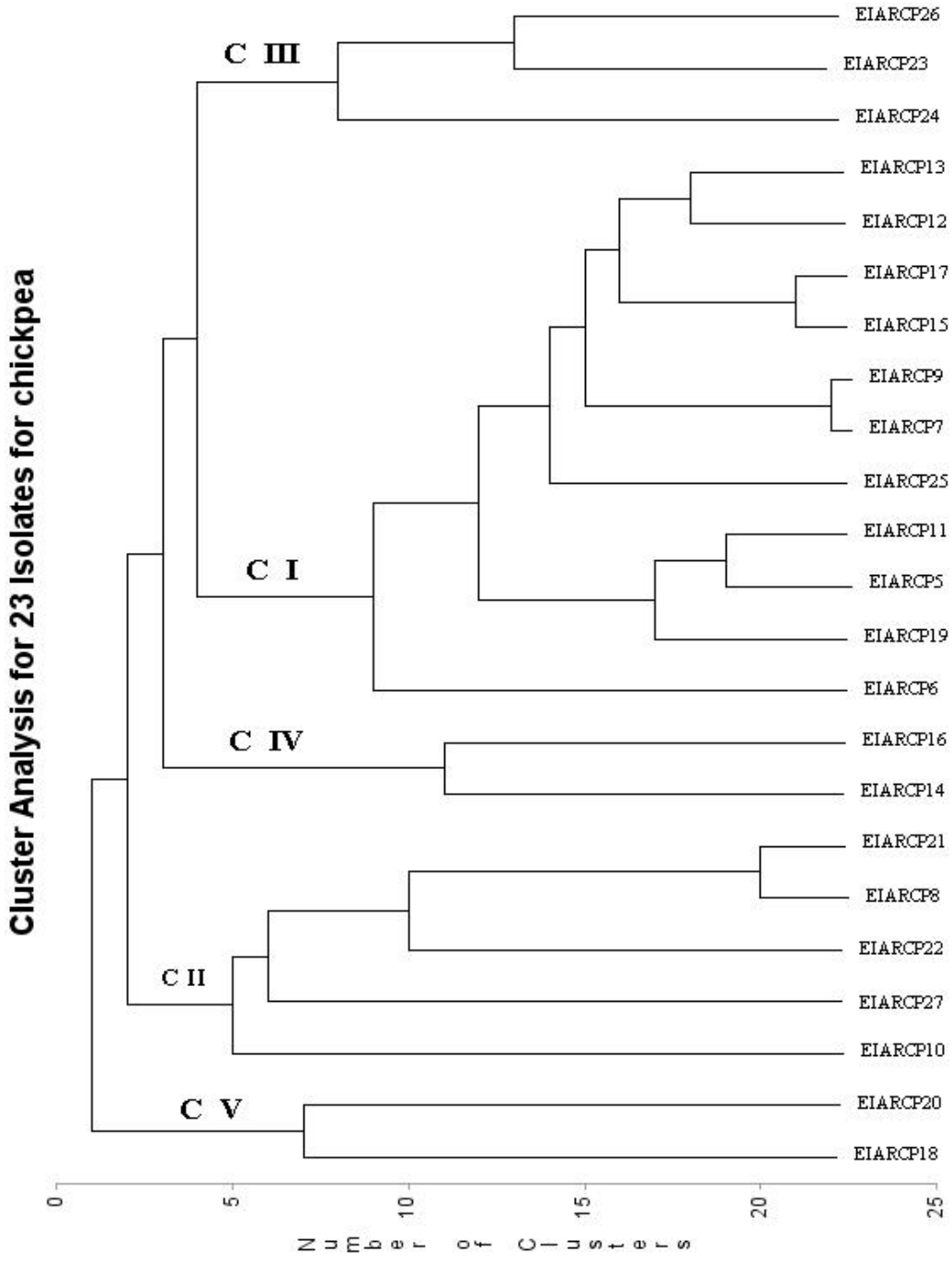

Figure 2. Dendrogram highlighting the phenotypic similarity among the test isolates.

4.3.4. Symbiotic effectiveness test on sand culture.

All isolates were tested for symbiotic effectiveness using *Desi* seed type variety *Natoli* (Appendix 10). They showed variations in symbiotic effectiveness, nodule number, nodule dry weight and shoot dry biomass (Table8). The isolates induced nodulation with nodule numbers ranging from 14 nodules per plant to 62 nodules per plant, a fourfold difference between the highly nodulating isolate EIARCP7 and poorly nodulating isolate EIARCP11. They also showed variation in nodule dry weight between 20-53 mg/plant. The highest shoot dry weight of 417 mg/plant was recorded from the plant nodulated with EIARCP7, whereas the least shoot dry weight of 250 mg/plant was obtained from plants nodulated by isolate EIARCP17. Although the data showed a fourfold difference in nodule numbers between the inoculated plants with high and low nodule number, their differences in nodule dry weight and shoot dry weight were more than twofold. A strong correlation was observed between nodule number and nodule dry weight with correlation coefficient (r) value of 0.86 and 0.75 respectively. On the other hand moderate correlation (r= 0.64) were obtained between shoot dry weight and nodule dry weights.

Table 8. Symbiotic performance of the isolates obtained from different parts of southeastern Ethiopia.

No.	Isolates	NN Plant^{-1}	NDW(mg/Pt)	SDW(mg/Pt)	SE (%)	Rate
1	EIARCP 7	62.0a	51a	417a	125.00	HE
2	EIARCP 13	50.0ab	42abc	383ab	108	HE
3	EIARCP 19	34.0bcd	53a	350abcd	92	HE
4	EIARCP 6	56.0a	43ab	333bcde	83	HE
5	EIARCP 26	33.0bcd	40abc	317bcdef	75	E
6	EIARCP 21	18cdefg	27cdef	300cdef	67	E
7	EIARCP 8	35bc	39abcde	283defg	58	E
8	EIARCP 16	33bcd	43ab	283defgh	58	E
9	EIARCP 10	20cdef	32bcdef	283defgh	58	E
10	EIARCP 15	23cde	25def	283defgh	58	E
11	EIARCP 22	44ab	44ab	283defgh	58	E
12	EIARCP 11	14efg	27cdef	267efgh	50	E
13	EIARCP 23	18cdefg	25def	267efgh	50	E
14	EIARCP 18	18cdefg	32bcdef	267efgh	50	E
15	EIARCP 17	17defg	20fg	250fgh	42	LE
16	EIARCP 20	9efg	17fg	233ghi	33	IE
17	EIARCP 9	7efg	8gh	217hij	25	IE
18	EIARCP 25	16defg	23efg	217hij	25	IE
19	EIARCP 24	1g	0.h	217hij	25	IE
20	EIARCP 14	33bcd	32bcdef	217hij	25	IE

21	EIARCP 5	8efg	18fg	167ij	0	IE
22	EIARCP 12	4fg	27cdef	150j	8	IE
23	EIARCP 27	23cde	22.5fg	150j	8	IE
24	Control (+Ve)	23cde	20fg	367abc	100	HE
25	CP-018	19efg	23efg	300cdefg	67	E
26	Control (-Ve)	0g	0.00h	167ij	0	IE
	LSD	18	16	68		

Legend - HE= Highly Effective E= Effective LE= Less Effective IE= Ineffective

* Means with the same letter in the same column are not significantly different.

In addition to the statistically analyzed results visible morphological differences like, deep green leaf, pink nodule color and vigorous growth of chickpea was observed with plants inoculated with symbiotically effective isolates (Fig. 3).

Similarly, the symbiotic effectiveness (SE) of inoculated plants in relation to the positive and negative control plants showed difference (p=0.05) from the lowest effective isolates (EIARCP17) having SE of 42% to that of isolates such as EIARCP7, EIARCP13, EIARCP19 and EIARCP6 that were highly effective with symbiotic effectiveness rate of 80-100 (Table8). In addition, the other 43.5% were effectively display SE rate between 50-80%, whereas isolate EIARCP17 was lower effective with symbiotic efficiency of 35-50%. The rest, 35% were ineffective with symbiotic effectiveness below 35%.

The data showed the highest effective isolates were accompanied with higher tissue dry weight, nodule numbers and nodule dry weight. The commercial strain (CP-018) was significantly (p=0.05) less effective in all nodulation parameters and shoot dry weights compared to the highly effective isolates.

Figure 3. Symbiotic effectiveness of isolates as compared to standard check on sand

Inoculated (EIARCP6) **Control (-Ve)** **Control (+)** **(at early stage)**

Table 9. Summary of Eco-physiological and symbiotic properties of highly effective and effective chickpea nodulating isolates

No.	Isolates	Site	Colony character	Utilized C-source (%)	Utilized N source (%)	Tolerated antibiotics (%)	Tolerated Heavy metal (%)	Tolerated pH levels	Tolerated salt conc. (%)	Tolerated temp. (^{o}C)	Rate
1	EIARCP 7	West Hararge	LM	90	71	37.5	67	6.8-7	3	10-35	HE
2	EIARCP 13	Arsi	LM	80	71	62.5	33	6.8-7	2.5	10-30	HE
4	EIARCP 19	West Hararge	LM	80	86	75	17	6.8-8	3	20-30	HE
5	EIARCP 6	West Hararge	LM	60	43	37.5	50	6.8-8	3	20-30	HE
6	EIARCP 26	Arsi	LW	20	57	37.5	33	6.8-7	3	10-35	E
8	EIARCP 21	Gamogofa	LM	60	71	87.5	67	6.8-8	0.01	10-35	E
9	EIARCP 8	Bale	LM	90	57	75	83	6.8-8	0.01	10-35	E
10	EIARCP 16	West Hararge	LM	60	57	37.5	17	6.8-7	1.5	20-45	E
11	EIARCP 10	Bale	LW	100	57	62.5	83	6.8-8.5	1.5	10-40	E
12	EIARCP 15	West Hararge	LM	100	43	37.5	17	6.8-8	2.5	10-30	E
13	EIARCP 22	West Hararge	LM	60	71	50	33	6.8-9.5	1	10-35	E
14	EIARCP 11	West Hararge	LM	80	57	87.5	67	6.8-7	3	10-30	E
15	EIARCP 23	Gamogofa	LM	40	43	25	50	6.8-8	3	10-30	E
16	EIARCP 18	Bale	LM	100	71	62.5	50	5-9.5	3	10-30	E

It is clearly indicated that, 50% of effective isolates utilized 80-100% of the tested carbohydrates (Table 9). Also 69% of effective isolates utilized 57-71% of the tested N sources and were resistant to 63-88% of the tested antibiotics. More over 50% of effective isolates showed resistance to 50-83% of the tested heavy metals. Additionally, 56% of effective isolates showed resistance for wider pH ranges between 6.8-9.5 and also EIARCP18 and EIARCP22 grown between 5-9.5 and 6.8-9.5, respectively. With regard to salt tolerance, 69% of effective isolates grew on salt concentration of 3% NaCl. Furthermore, 88% of effective isolates grown at temperatures 10-35^{o}C, whereas EIARCP16 and EIARCP10 were grown at temperatures 20-45^{o}C and 10-40^{o}C, respectively. From the listed effective isolates 25% of isolates were highly effective and the rest 75% were effective in their symbiotic potential under greenhouse condition.

5. DISCUSSION

In the present study soil texture triangle 90% of the study sites were clay soil and the remaining 10 % sites were clay loam and sandy clay loam. Similarly Tate (2000) the mentioned principal soil physicochemical properties were adequate to obtain better growth of pulses, and for the survival and diversity of the indigenous rhizobia.

All isolated 39 rhizobial isolates were identified as gram negative-rods with no absorption of Congo-red on YEMA-CR medium under dark incubation, mediums turned the BTB supplemented YEMA medium into yellowish. These results obtained from gram staining, growth on YEMA-CR, YEMA-BTB and PGA medium preliminary confirm the standard cultural and morphological characteristics of *Rhizobium* species as described by Somasegaran and Hoben, (1994) and Vincent (1970). None of the isolates showed growth on peptone-glucose-agar medium indicating they are free of other contaminants other than rhizobia (Subba Rao, 1988). Based on the definitive test, however, only 23 isolates (59%) were authenticated as root nodule bacteria by re nodulating on the two host varieties. Accordingly, 87 % of the isolates were able to nodulate *Desi* seed types, var *Natoli*, and 13% of them nodulate both *Desi* seed types, var *Natoli* and *Kabuli* seeds types, var *Habru* on growth pouches. This indicates that chickpea rhizobia are restrictive in nodulation depending upon cultivar/accessions (Gemechu Keneni *et al.* 2012)

Daniel Muleta (2009) reported that the majority (81%) of chickpea isolates were LM in appearance and the majority (92%) were fast growers (92-96%) from Ethiopian soils with doubling time of below 3 hours, and all of them acidified the YEMA-BTB medium. Based on their growth rate and acid reaction on their growth medium according to Jordan (1984), these isolates were fast growers and acid producers. None of the isolates solubilized inorganic calcium phosphate (Ca-P) in Pikoviskaya`s medium. Similarly, Ahmed Said (2010) has studied on chickpea rhizobia of South and North Wello zones. However, Mulisa Jida and Fasil Assefa (2012), Daniel Muleta (2009) and Wendwosen Tena (2016) showed 42%, 30% and 33% of chickpea isolates respectively solubilized inorganic phosphate. Similarly, Rhitu *et al.* (2012) showed that 10% of strains isolated from Indian soils were found to be phosphate solubilizers.

More than 78% of the isolates were catabolyzed a wide spectrum of carbohydrates sources. This indicates that the isolates were more versatile in utilizing the carbohydrates compared to the findings of Maatallah *et al*. (2002); Daniel Muleta (2009) and Mulisa Jida and Fasil Assefa (2012) where only 55%, 43% and 29% of their isolates respectively utilized 70% of the tested carbon sources.

With regard to pattern of amino acid utilization, most of the isolates utilized cystine, lysine, leucine, asparagine and phenylalanine as their sole nitrogen source, but most failed to grow on glycine and thymine. However, Küçük and Kıvanç (2008) have observed that rhizobial isolates failed to utilize lysine and grew better on glycine. Daniel Muleta (2009) showed the same pattern of utilization of leucine and phenylalanines, except a few of their isolates (16%) were able to utilize lysine. This score showed that chickpea isolates from Ethiopian soils showed poor growth on glycine where as better growth on leucine, cystine and phenyl alanine with an exception of the work by Mulisa Jida and Fasil Assefa (2012) that showed isolates poor growth on phenyl alanine.

All isolates exhibited variations in their intrinsic antibiotic resistance (IAR) to different types and concentrations (10-100µg/ml). However, many of antibiotics concentrations in this work were greater than almost all of antibiotics concentrations on the previous works except chloramphenicol concentration of Küçük and Kıvanç (2008) and Rhitu *et al*. (2012), better resistance of these isolates to tetracycline was obtained compared to less than 16% of chickpea isolates resistance by Mulisa Jida and Fasil Assefa (2012); Ahmed Said (2010); Rhitu *et al*., (2012). The isolates were highly sensitive to chloramphenicol (below 16% growth) similar to the isolates of Maatallah *et al.* (2002) and Rhitu *et al*. (2012). However, Küçük and Kıvanç (2008); Daniel Muleta (2009); Ahmed Said (2010); Mulisa Jida and Fasil Assefa (2012) and Wendwosen Tena (2016) showed better resistance of (52-89% growth) on chloramphenicol. In general, the pattern of resistance and sensitivity of chickpea rhizobial to different types and concentrations of antibiotics was diverse between countries (Maatallah *et al.* 2002; Küçük and Kıvanç, 2008; Rhitu *et al*., 2012; Mulisa Jida and Fasil Assefa, 2012) and within locations of the same country (Daniel Muleta and Fasil Assefa, 2014; Wondwosen Tena, 2016). From this it is clearly shown that the rhizobial isolates have a diverse response to different antibiotics in their natural environments.

All isolates were able to grow well up to 1.0% NaCl on YEMA plates and majority of the isolates were able to grow at 3% NaCl concentration which is comparable to the findings that more than 94% of chickpea rhizobial isolates from northern and central parts of Ethiopia (Daniel Muleta, 2009) could tolerate up to 3% of NaCl. Küçük and Kıvanç (2008) have reported 46-61% of the isolates grew on YEMA medium containing salt concentration of 0.1-0.5% NaCl and agreed with the work of Maatallah *et al.* (2002). Mulisa Jida and Fasil Assefa (2012) reported 75% of the tested rhizobia grew well in 1% NaCl. However, only 11.1% of the isolates tolerated 5% NaCl. Wendwosen Tena (2016) reported that all of the isolates were able to grow on media containing 2% NaCl and almost all isolates were sensitive to NaCl ≥ 3%. According to Daniel Muleta (2009), all the isolates grew with 2% of sodium chloride with 96.4% of their isolates managed to grow at 3% NaCl. Therefore, the results of this report and that of previous works (Daniel Muleta, 2009; Wendwosen Tena, 2016) illustrated that chickpea isolates showed better salt tolerance with a maximum limit of 3% NaCl concentration, however, a few data were reported on isolates growth at NaCl >3% (Mulisa Jida and Fasil Assefa, 2012).

Almost all the Isolates were sensitive to acidity pH 4-5.0, and grow at near neutral and moderately alkaline environment (pH 6.8-9.5), except EIARC P18 and EIARCP20 that were tolerant to moderately acidic and alkalinity (pH 5.5 to 10). Ahmed Said (2010) also showed that 59% of the isolates from south and north Wollo tolerated wide range of pH 4.5-9.5. Similarly Wendwosen Tena (2016) reported that almost all of the isolates grew on pH 5.5-9 as well as 46% and 56% were able to grow on pH 9.5 and 10, respectively. However, the rhizobial isolates grew well in moderately acidic pH (5.5) to neutral pH and slightly alkaline pH (8.0) (Mulisa Jida and Fasil Assefa, 2012). Similarly, Rhitu *et al*. (2012) have showed that almost all of chickpea rhizobial strains grew within the pH range of 5-9.5 and according to his narration this tolerance to alkaline pH correlates with calcareous soil where chickpea is generally grown.

All rhizobial isolates were tolerant to temperature of (10 - 30 ^{o}C). The isolates in this work showed temperature tolerance ranges (10-30^{o}C). This result was in agreed with the work by Daniel Muleta (2009). In the present study showed that the wider temperature tolerance range as compared to chickpea rhizobial isolates (20-30^{o}C tolerance) conducted by Mulisa Jida and Fasil Assefa (2012), Ahmed Said (2010) and Wendwosen Tena (2016). But only few isolates (13%) were able to grow at high temperature (40^{o} C). Mulisa Jida and Fasil Assefa (2012) and Daniel

Muleta (2009) showed that only 17% and 8%, respectively of chickpea isolates were tolerant to the same temperature. Although these studies in Ethiopia showed that chickpea rhizobia are sensitive to higher temperatures (>35^{o}C), a considerable number of isolates (up to 39% from *M. ciceri* and *M. Mediterraneum* type strains isolated from different countries were tolerant to 40^{o}C (Nour *et al.*, 1995; Küçük and Kıvanç, 2008).

Symbiotic effectiveness is commonly used to assess the ecological and evolutionary relationship between rhizobia and their host (Brockwell *et al.*, 1998). Accordingly, the inoculated plants showed diversity in their physical appearances; nodule number, nodule dry weight and shoot dry weight. The nodule number recorded from the authenticated plants ranged from lowest 14 per plant for isolate EIARCP11 to 62 per plant for isolate EIARCP7 and the range of nodule dry mass was between 20 mg/p for less effective isolate EIARCP17 and 53.3 mg/pt for isolates EIARCP19, respectively.

Maximum and minimum mean shoot dry mass of 417mg/pt and 250 mg/pt was scored by EIARCP7 and EIARCP17, respectively. Isolates from northern Ethiopia (Mulisa Jida and Fasil Assefa (2012), Central Ethiopia (Daniel Muleta, 2009) showed maximum and minimum mean shoot dry mass of 0.6g/pt and 1.36 g/pt, 1.08 g/pt and 0.3.88 g/pt, respectively indicating that the difference may emanate from effectiveness of individual endo-symbionts, host variety and planting time in the greenhouse. The isolates in this study showed moderate to strong correlation in their symbiotic and nodulation parameters obtained by Deborah (2016). Such correlations are usually used routinely as an indicator of relative effectiveness (Somasegaran and Hoben, 1994). In this experiment, a significant (p=0.05) difference was recorded in shoot dry weight, nodule number and nodule dry weight among the infective isolates.

On the basis of relative shoot dry matter accumulation by the inoculated plants in reference to the nitrogen-fertilized control as a measure of effectiveness (Gibson, 1987), 61% of the isolates were symbiotically effective. From these effective isolates 17% of them i.e. EIARCP6, EIARCP 7, EIARCP 19 (all from West Hararge), and EIARCP 13 (from Arsi) were rated as highly effective with symbiotic effectiveness between 83% and 125%. Likewise, almost 50% of the isolates were rated effective by scoring relative effectiveness scale between 50-80%. In general the 61% of chickpea isolates were rated as effective and the rest of the isolates (39%) were rated as lowly effective and ineffective with relative effectiveness scale below 50%. The result also showed that

the highly effective isolates especially EIARCP7 from west Hararge and EIARCP13 from Arsi zone significantly increased nodule number, nodule dry weight, and shoot dry mass by 33%, 23% and 14% ($p<0.05$), respectively compared to the commercialized isolate (CP-018).

In this study the number of highly effective and effective isolates was higher than the symbiotic effective isolates when compared to the result obtained (17%) symbiotically effective isolates by Daniel Muleta (2009) and isolates (38%) from Portugal (Laranjo *et al*., 2008). However, the symbiotic rate was lower than the symbiotic effectiveness rate of chickpea isolates from Morocco (77%) (Maatallah *et al.* 2002), from India (83%) (Rhitu *et al*., 2012) and from the Ethiopia (89%) (Mulisa Jida and Fasil Assefa, 2012).

Based on their eco-physiological and nutritional versatility isolates; EIARCP7, EIARCP8, EIARCP10, EIARCP11, and EIARCP18 showed better rank of versatility than the other isolates especially compared with highly effective isolates EIARCP6 and EIARCP26. Although the later isolates were highly effective *in vitro* growth performance under different stress conditions was very poor compared to other isolates. However, Brockwell *et al.* (1982) have observed that more than 10 features that are required in the selection of elite strains (inoculants) for better nitrogen fixation and host productivity, competitive ability, persistence and survival in the soil are all the more important. Consequently, the rhizobial isolates EIARCP7, EIARCP8, EIARCP10, EIARCP11, and EIARCP18 combined the best match of high symbiotic effectiveness with a wide range of resistance to eco-physiological stresses under laboratory conditions and may qualify to be potential candidates for a field trial to fully realize biological nitrogen fixation for chickpea production under low-input agriculture in Ethiopia.

6. CONCLUSION AND RECOMMENDATION

6.1. Conclusion

The southeastern highlands of Ethiopia is one of the important sites of chickpea production, from 65% of collected nodule samples the isolates which presumably found to be rhizobial isolates contributing in need of inoculants were isolated. From isolated 39 chickpea rhizobial isolates 59% of them were authenticated as true root nodulating chickpea rhizobials. From these isolates 61 % of them were symbiotically effective since they showed best symbiotic effectiveness on sand culture.

From these the highly effective isolates were collected from west Hrarge and Arsi zones. The effectiveness test of isolates EIARCP7, EIARCP13, EIARCP19 and EIARCP6 on sand culture was better than commercialized isolate (CP-018). Consequently, isolates EIARCP7, EIARCP8, EIARCP10, EIARCP11, and EIARCP18 combined the best match of high symbiotic effectiveness with a wide range of resistance to eco-physiological stresses under laboratory conditions.

Though the presence of diversity among the isolates revealed that the possibility of getting potentially effective adaptable rhizobial isolate that enhance chickpea productivity, the weak symbiotic properties observed in some sampling sites during isolation and nodulation status survey might partly be responsible for nodulation and shoot dry weight variation in the cropping systems.

6.2. Recommendations

- Isolation and characterization of symbiotically effective isolates on sand under greenhouse condition may not give the same result in field condition so this must be supported by trial in field condition to realize inoculants development for large scale production.

- On different variety of *Desi* and *Kabuli* seed types the response of rhizobial isolates may show different pattern in nodulation efficiency hence better to do further works by integrating the association of different host cultivars with that of phenotypic, symbiotic and genetic characteristics of a given rhizobial isolates.

- To get symbiotically effective and ecologically adaptive legume rhizobial isolate studies should be undertaken in different parts of the country.

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APPENDICES

Appendix 1. Soil chemical analyses results

Isolates	pH	P(ppm)	% OC	K (meq/100 g)	% TN	CEC (meq /100 g)	Texture			Textural class
							%Clay	% Silt	% Sand	
EIARCP5	6.25	12.77	1.67	0.72	0.09	50.9	58.75	31.25	10	Clay
EIARCP6	6.66	11.2	1.67	0.52	0.08	53.72	38.75	36.25	25	Clay loam
EIARCP7	6.35	61.62	1.75	0.76	0.12	26.3	35	17.5	47.5	Sandy Clay loam
EIARCP8	7.49	8.69	1.29	1.18	0.08	59.98	62.5	12.5	25	Clay
EIARCP9	6.95	8.27	1.95	1.44	0.15	60.4	72.5	18.75	8.75	Clay
EIARCP10	7.49	8.69	1.29	1.18	0.08	59.98	62.5	12.5	25	Clay
EIARCP11	6.25	12.77	1.67	0.72	0.09	50.9	58.75	31.25	10	Clay
EIARCP12	7.75	4.1	1.99	1.16	0.13	58.86	62.5	22.5	15	Clay
EIARCP13	5.95	11.82	2.46	1.22	0.19	36.32	55	27.5	17.5	Clay
EIARCP14	6.75	31.2	2.61	0.94	0.06	40.74	53.75	27.5	18.75	Clay
EIARCP15	6.22	14.28	2.03	0.78	0.09	54.24	65	22.5	12.5	Clay

EIARCP16	6.85	21.78	1.74	0.88	0.1	53.88	61.25	26.25	12.5	Clay
EIARCP17	5.44	6.5	3.43	1.35	0.24	33.38	57.5	26.25	16.25	Clay
EIARCP18	7.47	6.73	1.71	0.12	0.17	56.82	60	30	10	Clay
EIARCP19	6.66	11.2	1.67	0.52	0.08	53.72	38.75	36.25	25	Clay loam
EIARCP20	7.41	9.13	1.48	1.37	0.1	50.44	60	23.75	16.25	Clay
EIARCP21	6.46	5.47	0.78	0.83	0.06	42.1	61.25	15	23.75	Clay
EIARCP22	6.52	5.87	2.14	0.71	0.12	53.84	58.75	26.25	15	Clay
EIARCP23	6.78	10.58	1.55	1.35	0.08	51.54	72.5	15	12.5	Clay
EIARCP24	6.74	17.91	1.52	1.09	0.1	33.82	55	22.5	22.5	Clay
EIARCP25	7.3	8.62	2.1	1.07	0.13	60.84	65	22.5	12.5	Clay
EIARCP26	6.35	8.11	4.36	0.73	0.35	33.36	40	38.75	21.25	Clay loam
EIARCP27	6.31	14.65	1.87	0.61	0.09	53.98	63.75	21.25	15	Clay

Appendix 2. Carbohydrate utilization of the isolates

No.	Isolates	Cellobiose	Galactose	Xylose	Arabinose	Sucrose	Inositol	Lactose	Maltose	Sorbitol	Manitol	CHO utilized (%)
1	**EIARCP5**	+	-	+	+	+	-	-	+	+	+	70
2	**EIARCP6**	+	-	+	+	+	-	+	+	-	+	70
3	**EIARCP7**	-	+	+	+	+	+	+	+	+	+	90
4	**EIARCP8**	-	+	+	+	+	+	+	+	+	+	90
5	**EIARCP9**	+	-	-	+	+	+	+	+	+	+	80
6	**EIARCP10**	+	+	+	+	+	+	+	+	+	+	100
7	**EIARCP11**	+	+	+	-	+	+	-	+	+	+	80
8	**EIARCP12**	-	+	-	+	+	+	+	+	+	+	80
9	**EIARCP13**	-	+	-	+	+	+	+	+	+	+	80
10	**EIARCP14**	+	+	-	+	-	-	-	-	+	+	50
11	**EIARCP15**	+	+	+	+	+	+	+	+	+	+	100
12	**EIARCP16**	+	+	-	+	-	-	-	+	+	+	60

13	**EIARCP17**	-	+	+	+	+	+	+	+	+	+	90
14	**EIARCP18**	+	+	+	+	+	+	+	+	+	+	100
15	**EIARCP19**	+	+	+	+	+	-	-	+	+	+	80
16	**EIARCP20**	+	-	+	-	+	+	-	+	+	+	70
17	**EIARCP21**	-	+	+	-	+	+	-	+	+	+	70
18	**EIARCP22**	-	+	+	-	+	+	-	+	+	+	70
19	**EIARCP23**	+	-	-	+	+	-	-	-	-	+	40
20	**EIARCP24**	+	-	+	+	+	-	-	-	-	+	50
21	**EIARCP25**	-	+	-	+	-	+	+	+	+	+	70
22	**EIARCP26**	-	-	+	-	-	-	-	-	-	+	20
23	**EIARCP27**	+	+	+	-	-	+	+	+	+	+	80
	(+) Isolates %	60.87	69.57	69.57	73.91	78.26	65.22	52.17	82.61	82.61	100	

NB: - + = grow, - = no growth.

Appendix 3. N sources utilization of the isolates

No.	Isolates	Cystine	Thymine	Aspargine	Lycine	Leucine	Pheny A	Glycin	N source utilized (%)
1	**EIARCP5**	+	-	+	+	+	+	-	71
2	**EIARCP6**	+	-	-	-	+	+	-	43
3	**EIARCP7**	+	-	+	+	+	+	-	71
4	**EIARCP8**	+	-	+	-	+	-	+	57
5	**EIARCP9**	+	-	+	+	+	+	+	86
6	**EIARCP10**	+	+	+	+	-	-	-	57
7	**EIARCP11**	+	-	+	-	+	+	-	57
8	**EIARCP12**	+	-	+	+	+	+	+	86
9	**EIARCP13**	+	-	+	+	+	+	-	71
10	**EIARCP14**	+	-	+	+	+	+	-	71
11	**EIARCP15**	+	-	-	+	+	-	-	43
12	**EIARCP16**	+	-	-	+	+	+	-	57
13	**EIARCP17**	+	-	-	+	+	+	-	57
14	**EIARCP18**	+	-	+	+	+	+	-	71
15	**EIARCP19**	+	-	+	+	+	+	+	86
16	**EIARCP20**	+	-	+	+	+	+	+	86
17	**EIARCP21**	+	-	+	+	+	+	-	71
18	**EIARCP22**	+	-	+	+	+	+	-	71
19	**EIARCP23**	+	-	+	+	-	-	-	43
20	**EIARCP24**	+	+	-	+	+	+	+	86

21	**EIARCP25**	+	+	-	+	+	-	+	71
22	**EIARCP26**	-	-	+	+	+	+	-	57
23	**EIARCP27**	+	+	+	+	+	+	-	86
(+) Isolates %		95.65	17.39	73.91	86.96	91.3	78.26	30.43	

NB: - + = grow, - = no growth.

Appendix 4. Antibiotics resistance of the isolates

No.	Isolates	Chlor a.	Spec	Bacitre	Penicillin	Tetracycl	Gentamyc	Kanamyci	Strept	Resistance (%)
1	**EIARCP5**	-	-	+	+	+	-	+	+	62.5
2	**EIARCP6**	-	+	+	-	-	+	-	-	37.5
3	**EIARCP7**	-	+	+	-	-	+	-	-	37.5
4	**EIARCP8**	-	-	+	+	+	+	+	+	75
5	**EIARCP9**	-	+	+	-	-	+	-	-	37.5
6	**EIARCP10**	-	-	+	-	+	+	+	+	62.5
7	**EIARCP11**	-	+	+	+	+	+	+	+	87.5
8	**EIARCP12**	-	-	+	-	+	-	-	-	25
9	**EIARCP13**	-	+	+	-	+	+	+	-	62.5
10	**EIARCP14**	-	-	+	-	+	-	-	-	25
11	**EIARCP15**	-	-	+	-	+	+	-	-	37.5
12	**EIARCP16**	-	-	+	-	+	+	-	-	37.5
13	**EIARCP17**	-	+	+	-	+	+	-	+	62.5
14	**EIARCP18**	+	-	+	+	+	+	-	-	62.5

15	**EIARCP19**	-	+	+	-	+	+	+	+	75
16	**EIARCP20**	-	-	+	+	-	-	-	+	37.5
17	**EIARCP21**	-	+	+	+	+	+	+	+	87.5
18	**EIARCP22**	-	-	+	+	+	+	-	-	50
19	**EIARCP23**	-	-	+	-	-	+	-	-	25
20	**EIARCP24**	-	-	-	+	-	-	-	-	12.5
21	**EIARCP25**	-	-	+	-	+	+	-	-	37.5
22	**EIARCP26**	-	-	+	-	+	+	-	-	37.5
23	**EIARCP27**	-	-	+	-	-	+	-	-	25
	+ Isolates(%)	4.35	34.78	95.65	34.78	69.57	78.26	30.43	34.78	

NB: - + = grow, - = no growth

Appendix 5. Heavy metal resistance of the isolates

No.		Al	Zn	Hg	Mn	Co	Cu	Resistance (%)
1	**EIARCP5**	+	-	-	+	-	+	50
2	**EIARCP6**	+	+	-	+	-	-	50
3	**EIARCP7**	+	+	-	+	+	-	67
4	**EIARCP8**	+	+	-	+	+	+	83
5	**EIARCP9**	+	+	-	+	+	+	83
6	**EIARCP10**	+	+	+	+	+	-	83
7	**EIARCP11**	+	+	-	+	+	-	67
8	**EIARCP12**	+	+	-	+	-	-	50
9	**EIARCP13**	+	-	-	+	-	-	33
10	**EIARCP14**	-	-	-	-	-	-	0
11	**EIARCP15**	+	-	-	-	-	-	17
12	**EIARCP16**	+	-	-	-	-	-	17
13	**EIARCP17**	+	+	-	+	-	-	50
14	**EIARCP18**	+	-	-	+	+	-	50
15	**EIARCP19**	+	-	-	-	-	-	17
16	**EIARCP20**	+	-	-	+	+	-	50
17	**EIARCP21**	+	+	-	+	-	+	67
18	**EIARCP22**	-	-	-	+	-	+	33
19	**EIARCP23**	+	-	-	+	+	-	50
20	**EIARCP24**	+	+	+	+	-	-	67
21	**EIARCP25**	+	+	-	+	-	+	67
22	**EIARCP26**	+	-	-	+	-	-	33
23	**EIARCP27**	+	-	-	+	+	-	50
	+Isolates(%)	91.3	52.17	8.7	86.95	39.13	26.09	

NB: - + = grow, - = no growth.

Appendix 6. Salt and pH tolerance of the isolates

		Salt tolerance of the isolates										Isolates pH tolerance								
No.	Isolates	0.80%	1%	1.50%	2%	2.50%	3%	4%	5%	6%	Tolerated NaCl (%)	pH 4.5	pH 5	pH 8	pH 8.5	pH 9	pH 9.5	pH 10	Tolerated pH levels
1	**EIARCP5**	+	+	+	+	+	+	-	-	-	0.01-3	-	-	+	-	-	-	-	6.8-8
2	**EIARCP6**	+	+	+	+	+	+	-	-	-	0.01-3	-	-	+	-	-	-	-	6.8-8
3	**EIARCP7**	+	+	+	+	+	+	-	-	-	0.01-3	-	-	-	-	-	-	-	6.8-7
4	**EIARCP8**	-	-	-	-	-	-	-	-	-	0.01	-	-	+	-	-	-	-	6.8-8
5	**EIARCP9**	+	+	+	+	+	+	-	-	-	0.01-3	-	-	+	-	-	-	-	6.8-8
6	**EIARCP10**	+	+	+	-	-	-	-	-	-	0.01-1.5	-	-	-	+	-	-	-	`6.8-8.5
7	**EIARCP11**	+	+	+	+	+	+	-	-	-	0.01-3	-	-	-	-	-	-	-	6.8-7
8	**EIARCP12**	+	+	+	+	+	+	-	-	-	0.01-3	-	-	-	+	+	-	-	6.8-9
9	**EIARCP13**	+	+	+	+	+	-	-	-	-	0.01-2.5	-	-	-	-	-	-	-	6.8-7
10	**EIARCP14**	+	+	-	-	-	-	-	-	-	0.01-1	-	-	-	-	-	-	-	6.8-7
11	**EIARCP15**	+	+	+	+	+	-	-	-	-	0.01-2.5	-	-	+	-	-	-	-	6.8-8
12	**EIARCP16**	+	+	+	-	-	-	-	-	-	0.01-1.5	-	-	-	-	-	-	-	6.8-7
13	**EIARCP17**	+	+	+	+	+	+	-	-	-	0.01-3	-	-	+	-	-	-	-	6.8-8
14	**EIARCP18**	+	+	+	+	+	+	-	-	-	0.01-3	-	+	+	+	+	+	-	5-9.5
15	**EIARCP19**	+	+	+	+	+	+	-	-	-	0.01-3	-	-	+	-	-	-	-	6.8-8

16	**EIARCP20**	+	+	+	+	+	+	-	-	-	0.01-3	-	+	+	+	+	+	+	5-10
17	**EIARCP21**	-	-	-	-	-	-	-	-	-	0.01	-	-	+	-	-	-	-	6.8-8
18	**EIARCP22**	+	+	-	-	-	-	-	-	-	0.01-1	-	-	+	+	+	+	-	6.8-9.5
19	**EIARCP23**	+	+	+	+	+	+	-	-	-	0.01-3	-	-	+	-	-	-	-	6.8-8
20	**EIARCP24**	+	+	+	+	+	+	-	-	-	0.01-3	-	-	-	-	-	-	-	6.8-7
21	**EIARCP25**	+	+	+	+	+	+	-	-	-	0.01-3	-	-	-	-	-	-	-	6.8-7
22	**EIARCP26**	+	+	+	+	+	+	-	-	-	0.01-3	-	-	-	-	-	-	-	6.8-7
23	**EIARCP27**	-	-	-	-	-	-	-	-	-	0.01	-	-	+	-	-	-	-	6.8-8
	+Isolates(%)	86.95	86.95	82.6	78.26	73.91	60.87	0	0	0		0	8.7	56.52	21.74	17.39	13.04	4.35	

NB: - + = grow, - = no growth

Appendix 7. Temperature tolerance of the isolates

No	Isolates	10 ^{o}C	20 ^{o}C	35 ^{o}C	40 ^{o}C	45 ^{o}C	Tolerated T (^{o}C)
1	**EIARCP5**	+	+	-	-	-	10-30
2	**EIARCP6**	-	+	-	-	-	20-30
3	**EIARCP7**	+	+	+	-	-	10-35
4	**EIARCP8**	+	+	-	-	-	10-35
5	**EIARCP9**	+	+	+	-	-	10-35
6	**EIARCP10**	+	+	+	-	-	10-35
7	**EIARCP11**	+	+	-	-	-	10-30
8	**EIARCP12**	+	+	-	-	-	10-30
9	**EIARCP13**	+	+	-	-	-	10-30
10	**EIARCP14**	+	+	-	-	-	10-30
11	**EIARCP15**	+	+	-	-	-	10-30
12	**EIARCP16**	-	+	+	+	+	20-45
13	**EIARCP17**	+	+	-	-	-	10-30
14	**EIARCP18**	+	+	+	+	-	10-40
15	**EIARCP19**	-	+	-	-	-	20-30
16	**EIARCP20**	+	+	+	-	-	10-35
17	**EIARCP21**	+	+	+	-	-	10-35
18	**EIARCP22**	+	+	+	-	-	10-35
19	**EIARCP23**	+	+	-	-	-	10-30
20	**EIARCP24**	+	+	+	-	-	10-35
21	**EIARCP25**	+	+	-	-	-	10-30
22	**EIARCP26**	+	+	+	-	-	10-35
23	**EIARCP27**	+	+	+	-	-	10-35
	+ Isolates (%)	86.96	95.65	43.48	8.7	4.35	

NB: + =grow, - =no growth

Appendix 8. Purified colony of Chickpea Rhizobia

Sample of pure isolate (EIARCP19) on YEMA media

Appendix 9. Authentication Highlights on growth pouches

Effective nodulation on growth pouch

Ineffective nodulation on growth pouch

Appendix 10. Inoculated Chickpea performance at harvest on sand culture

Strains EIARCP16, EIARCP 26, EIARCP19, EIARCP13 on sand culture as T no. labeled on the pots (at Harvest stage)

Appendix 11. USDA soil textural triangle

https://www.google.comnesoil.com/properties/texture/sld005.htm

Soil Texture Triangle

-Lab data needed

-Percent by weight of fine earth fraction