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Nutritionally Versatile, Abiotic Stress Resistant and Symbiotically Effective Chickpea (*Cicer arietinum L.*) Root Nodulating Rhizobial isolates from Eastern, Southeastern and Southern Ethiopia.

Wubayehu Gebremedhin[1,2]*, Fassil Assefa[2], Moses Thuita[3], Cargele Masso[3]

1Ethiopian Institute of Agricultural Research Pawe Research Center, Pawe, Ethiopia
2Microbial, Cellular and Molecular Biology Program, Faculty of Life Science, Addis Ababa University, Ethiopia
3International Institute of Tropical Agriculture (IITA), Nairobi, Kenya

*Corresponding author. Tel: +251925513449; E-mail: wubsee6@gmail.com

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Research Article

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*. In this study a total of 39 root nodule bacteria were collected from nodules of 60 sampling sites from chickpea growing areas of the eastern, southeastern and southern parts of the country, of which 23 isolates (59%) were identified as chickpea root nodule bacteria based upon presumptive and definitive (authentication) tests. The isolates were also categorized into clusters based on their nutritional versatility, abiotic stress resistance and symbiotic effectiveness for preliminary taxonomic screening and as markers for selection of ecologically competent isolates *in vitro*. The isolates were fast growing and acid producing *rhizobia* with growth rate of 1-2.8 h, and changed the YEMA-BTB (Yeast Extract Mannitol Agar Bromothymol Blue) medium into yellow characteristics of many of the fast growing *rhizobia Mesothizobium spp*. isolated from chickpea. The chickpea root nodule bacteria induced 14-62 nodules plant^{-1} with nodule dry weight of 20-53.3 mg plant^{-1}; and shoot dry matter of 250-417 mg plant^{-1}, respectively. 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 cluster analysis including nutritional versatility and symbiotic effectiveness showed that *rhizobia* strains EIARCP7, EIARCP13, and EAIRCP19 were the most promising for effectiveness under variable sources of carbohydrates and amino-acids, which could represent an advantage to adapt under variable agro-ecological zones; these strains also showed high resistance to abiotic stresses.

Keywords: *Rhizobia* isolates; Nutritional versatility; Abiotic stress resistance; Symbiotic effectiveness; Chickpea

1. Introduction

Chickpea is the world's third most widely grown food legume next to bean and soybean and is grown in more than 50 countries, mainly in Asia (89.7% of the global production) [1]. The other chickpea production areas are found in Africa (4.3%), Americas (2.9%), Oceania, (2.6%) and Europe (0.4%). The global area under chickpea cultivation was about 11.0 million ha with a production of 8.8 metric tons and an average yield of nearly 800 kg ha^{-1} during the triennium 2004-2007 [1].

It is a strictly self-pollinated crop with two types of cultivars known as Desi and Kabuli in Ethiopia [2] and rich in protein (21.1%), carbohydrates (61.5 %), fats (4.5%) and other minerals such as calcium and iron [3]. In addition to nutritional quality and source of cash, chickpea restores and maintains soil fertility through its symbiotic nitrogen-fixation in association with root nodule *rhizobia* under conducive environment [4,5]. Traditionally, chickpea is considered as a restrictive host that is nodulated by two species

namely *Mesorhizobium ciceri* and *Mesorhizobium mediterraneum* [4]. However, other species such as *Mesorhizobium amorphae*, *Mesorhizobium huakuii* and *Mesorhizobium plurifarium* may effectively nodulate chickpea [6].

Hence, it obtains between 50% to 80% nitrogen derived from the atmosphere through nitrogen fixation (% Ndfa) [7,8], with a fixation rate of around 90–180 kg ha^{-1} under conducive environment [9]. *Rhizobia* inoculation of chickpea could improve its biological nitrogen fixation potential and yield as well [10-12]. It is reported that seed inoculation with rhizobium increased chickpea seed yields by 35% [10,11]. In Ethiopia, Chickpea is an important food and cash crop and cultivated in the northern highlands of Showa, Gojam, Tigray, Gondar, and Wello as well as in Arsi, Bale, Gamogofa and Hararge in the South and Southeastern part of the country [13]. It is mainly cultivated between 1400-2300 meters above the sea level (m.a.s.l) where annual rainfall ranges from 700-1200 mm. It accounts for about 17.3% of the total pulse production in the country [14]. However, its yield has remained very low with the national average yield of 1.1 metric tons ha^{-1} as compared to the average production rate of 2.61 metric tons ha^{-1} in other producing countries in Asia [15].

Recently, several research works were undertaken in Ethiopia to evaluate diversity and symbiotic effectiveness of chickpea cultivars from some parts of the country mainly from the northern, central highlands and some parts of the South [16-18]. Those studies showed chickpea *rhizobia* in the country are diverse in their symbiotic effectiveness across different agro ecological zones. It has also been established that successful inoculation of legumes depends on many factors, including matching *rhizobia*l strains and host cultivars and soil conditions. Sutton [19] reported that bacteria and bacteroids were susceptible to soil abiotic stress factors when released to new environment. In Ethiopia, studies assessing chickpea nodulating *rhizobia* resistance to abiotic stress such as soil acidity, soil salinity or alkalinity, temperature variability, various antibiotics, and heavy metals among others are however limited. The spatial variability of chickpea nodulating *rhizobia* and their effectiveness could be explained by the utilization of different carbohydrates or amino acids, which may vary according to local agro-climatic conditions. To these end investigation for effective nitrogen fixing chickpea *rhizobia* from eastern, south eastern and southern Ethiopia has not been exhaustively undertaken. Therefore, the objective of this study was to authenticate chickpea root nodulating *rhizobia* isolates and classify them based on nutritional versatility, resistance to abiotic stresses and symbiotic effectiveness as an indicator of their potential to perform under various agro-ecological zones and/or abiotic stresses.

2. Materials and Methods

2.1 Study sites and soil analysis

The study sites were selected based on the capability of the areas to produce chickpea as well as non-addressed areas with the previous researches of chickpea nodulating *rhizobia* effectiveness tests for better chickpea production.

Soil and nodule samples were collected in 10x10 m^2 bisect from more than 60 localities in chickpea growing areas of eastern (West Hararge), southeastern (Arsi, Bale) and southern (Gamogofa) zones of the Oromia and Southern Nations Nationalities and Peoples (SNNP) regional state of Ethiopia in 2014. Most of the nodule samples were from chickpea farms covered with Desi seed types, variety Natoli. The geographical locations of the sampling sites are shown in Figure 1. The sites are located between 1370-2553 m.a.s.l.

Soil samples were collected from topsoil (0-20 cm) at the same sites and put in alcohol sterilized polyethylene plastic bags. Each composite soil sample from each site was analyzed for physical and chemical properties (i.e. soil pH, Organic Carbon (OC), total N, available P, and texture) following standard procedures outlined in Sertsu and Bekele [20]. The study sites were selected to cover a wide range of soil pH, OC and nutrient levels based on secondary data.

2.2 Presumptive screening and authentication of pure cultures

The nodule and soil samples were collected between late October and early December, 2014. The root nodules were collected from field grown chickpea stands and from collected soil samples by trapping through plant induction in greenhouse. The root nodule bacteria were isolated using standard methods [21] by using Yeast Extract Mannitol Agar (YEMA) or selective media for root nodulating rhizo bacteria. The pure isolates were preserved at 4°C after it grew well on YEMA slant containing 0.3% (w/v) CaCO3 [21]. Duplicate samples were also stored at -200°C covered with 40% glycerol.

Cultures were examined for cell morphology and gram reaction after 3 days of growth in YEM broth medium. The colony morphology and purity of isolates were examined on YEMA containing Congo red plates

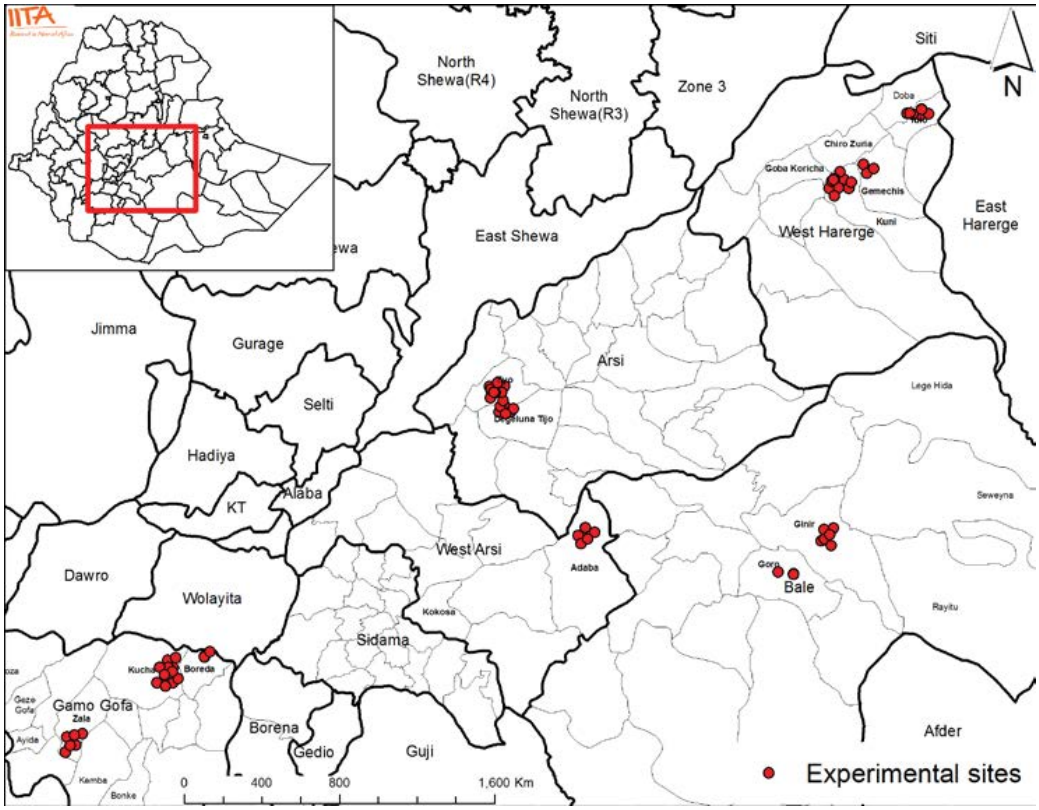

Figure 1. Geographic location of sampling sites.

after an incubation of 5 days at 28°C. They were also inoculated into the peptone-glucose medium [22] and Hofer's Alkaline Broth (HAB) medium [21] and incubated at 28°C for 3-5 days as presumptive tests for root nodule bacteria.

Individual colonies were characterized based on their appearance, shape, colony diameter, the capacity to produce exo-polysaccharide gum and their absorbance of the red color on YEMA-CR [21]. The production of acid or alkali was determined in YEMA medium containing 0.125 % Bromothymol Blue (BTB) [23]. Isolates were authenticated as root nodule bacteria by re-inoculating them on both host varieties namely desi seed type variety Natoli, and Kabuli seed type variety Habru using growth pouches.

2.3 Nitrogen and carbon utilization test

For nitrogen source utilization tests, 7 nitrogen sources were used (L-cysteine, thymine hydrochloride, L- asparagine, L-lysine, L- leucine, D-phenyl alanine and glycine). They were filter sterilized and added at a final concentration of 0.5 g/L to a basal media containing 1 g of KH_2PO_4; 1 g K_2HPO_4; 0.01 g $FeCl_3.6H_2O$; 0.2 g $MgSO_4.7H_2O$; 0.1 g $CaCl_2$; 15 g agar and supplemented with 1 g L-1 of mannitol [24]. The plates were incubated at 28 ± 2°C for 3-5 days.

Carbon utilization of isolates was determined following the method of Somasegaran and Hoben [25] on 10 carbohydrates namely lactose, D-mannitol, sucrose, galactose, D-maltose, sorbitol, arabinose, xylose, D-cellobiose, and inositol prepared as 10% (w/v) solution in water. The YEM media was modified by eliminating mannitol and reducing the amount of yeast to 0.05 g L^{-1} and replacing them with the tested C sources [26].

2.4 Assessment of resistance to abiotic stress

The negative impacts of non-living factors (abiotic stresses) for each *rhizobia*l isolates were tasted by their capability to grow or not were recorded and the ratio of their resistance or sensitivity were presented in percentage.

The intrinsic antibiotic and heavy metal resistance of isolates was determined by inoculating (10^8 cells mL^{-1}) on solid YEMA medium containing eight filter-sterilized (0, 22 mm Millipore filters) antibiotics at the concentrations shown in brackets in µg mL^{-1} of water : kanamycin (50), streptomycin (100), chloramphenicol (20), spectinomycin (100), tetracycline (20), bacitracin (30), penicillin G (30), gentamycin (10) and six sterilized heavy metals at concentrations in µg mL^{-1} of water (in brackets): AlK2SO4 (250), $CoCl_2$ (20), CuCl2 (50), $HgCl_2$ (10), $MnSO_4$ (500), and $ZnCl_2$ (50) on YEMA media at pH 6.8 according to Maatallah et al. [27].

The capacity of each *rhizobia*l isolate to grow on acidic or alkaline media was determined by inoculating a loopful of each isolate on YEMA adjusted at a pH of 4.5, 5.0, 8.0, 8.5, 9.0, 9.5, and 10.0 using 1 mole L^{-1} HCl or NaOH before autoclaving [28]. The range of pH 6.0 – 7.0, temperature of 25-30°C and salt concentration below 0.8% was not assessed as it is expected that the *rhizobia* isolates will grow optimally within this ranges [25] for salt tolerance, the isolates were transferred to YEMA plates supplemented with NaCl at the following concentrations in percentage (%): 0.8, 1.0, 1.5, 2.0, 2.5, 3.0, [27]. The ability of bacterial isolates to grow at high and low temperatures was monitored at incubation temperatures of 10, 20, 35, 40 and 45°C [29]. All experiments on abiotic stress resistance were performed in triplicate on YEMA agar plates.

2.5 Assessment of symbiotic effectiveness

The experiment on symbiotic effectiveness was carried out in a greenhouse at Holetta research center (EIAR). Plants were grown in free-draining plastic pots (3 kg capacity) that had been surface sterilized by soaking in 70% ethanol and drying. Sterile paper towels were inserted aseptically in the base of the pots to prevent loss of nutrients and filled with sterilized moisten sand. The chickpea (*C.arientinum*) Desi seeds variety Natoli was treated with 70% ethanol during 10 seconds, surface sterilized in 3% sodium hypo-chlorate (for 3 min) and then rinsed five times with sterile distilled water. Five disinfected seeds were sown in each pot and they were thinned to three seedlings per pot a week after planting. One mL of liquid inoculums (108 *rhizobia* cells mL^{-1}) of collected isolates that had induced nodulation during the authentication test was used separately to inoculate seeds at sowing. One commercialized chickpea strain in the country called CP-018 obtained from Ethiopian National Soil Testing Center was included as a standard check for effectiveness.

Treatments were arranged in a completely randomized design with three replicates each. Each pot received 100 mL of ¼ strength of Broughton and Dilworth [30] micro-nutrient N-free medium solution per day for the first 3 weeks, thereafter the pots received 200 mL nutrient solution application per day. Each positive control pots received 100 mL of 70 mg KNO_3 L^{-1} $week^{-1}$ [31]. Likewise, pots were watered every two days with sterile distilled water. Plants were harvested 60 days after planting for assessment of nodule number, nodule dry weight, and shoot dry weight.

2.6 Data analysis

Unweighted Pair Group Methods with Average (UPGMA) for phenotypic traits and Analysis of variance (ANOVA) for comparison between the treatments for shoot dry weight, nodule number, and nodule dry weight was performed using the statistical software SAS version 9.3. Mean separation was done using the Duncan Multiple Range Test (DMRT) value when the F-test was significant at $p \leq 5\%$. Relative effectiveness of isolates was calculated according to the equation (Eq. 1) by Gibson [32] as:

SE = ((Inoculated plant D.M. - control (-ve) D.M.)/ (N-Fertilized plant D.M. - control (-ve) D.M)) × 100)…….. Eq.1

With Nitrogen fixing effectiveness classified as ineffective: <35%; lowly-effective: 35 to 50%; effective: 50 to 80%; and highly effective: >80%.

The cluster membership between the *rhizobia* isolates was determined using hierarchical cluster analysis based on nutritional versatility, resistance to abiotic stresses, and symbiotic effectiveness using IBM SPSS Statistics Version 20. Three options of classification were assessed i.e. (i) nutritional versatility coupled with symbiotic effectiveness, (ii) resistance to abiotic stresses coupled with symbiotic effectiveness, and (iii) nutritional versatility coupled with resistance to abiotic stress and symbiotic effectiveness to determine the nutritional and resistance to abiotic stress characteristics of *rhizobia*l isolates found effective or highly effective. All parameters were expressed in percentage, and therefore no data transformation was performed. The classification method used was between group linkages, which were validated using the nearest neighbor based on intervals defined by the squared Euclidian distances to generate dendrograms. A single solution consisting of three high-level clusters including all *rhizobia* isolates was applied to define cluster membership.

3. Results

3.1 Aauthentication of *rhizobia* from the various sites

The soil pH levels in the study sites varied from slightly acidic (5.9) to moderately alkaline (7.75). Available P and total Nitrogen ranged from 4.1 to 61.6 mg kg^{-1} and 0.8 to 3.5 g kg^{-1} respectively. The organic carbon content of the study sites ranges from moderate to high levels. Majority of the sites were categorized under clay soil and a few of them were clay loam and sandy clay.A total of 39 root nodule bacteria were isolated from different sites of West Hararge, Gamogofa, Arsi, and Bale Zones; selected bacteria were found at various locations. All isolates were gram-negative rods, did not

grow on Peptone Glucose Agar with Bromocresol Purple (PGA-BCP) and on Hofer`s alkaline broth and failed to absorb Congo red under dark condition. During the authentication of each isolate through reinoculation on Desi seed types varieties Natoli and Kabuli seed types varieties Habru in growth pouches under greenhouse conditions, 23 isolates (59%) induced nodules on Desi seed types, varieties Natoli, and only three isolates namely EIARCP13 (from Arsi) and EIARCP10 as well as EIARCP12 (from Bale zone) nodulated both Desi seed types, varieties Natoli and Kabuli seeds types, varieties Habru in growth pouches. No other isolates did nodulate Kabuli seeds types, varieties Habru. We hypothesized that the 16 out the 39 bacteria isolated (41%) that did induce nodulation after re-inoculation were not rhizobia and could be other endophytes. Hence, the subsequent characterization did focus on the 23 bacteria (59%) that were considered as effective root nodulating rhizobial isolates. All the 23 rhizobia isolates formed large mucoid (LM) and large watery (LW) colonies with colony diameters between 2 and 5 mm and generation time between 1 - 2.8hr Table 1.

3.2 Nutrient sources utilization and abiotic stress resistance

The dendrogram obtained from Unweighted Pair Group Methods with Average (UPGMA) computer numerical analysis of 7 phenotypic characters (i.e. carbohydrates, amino acids, antibiotics, heavy metals, pH, temperature, and salinity at variable levels) on 49 different traits (Table 2) placed the isolates in five distinctive clusters Figure 2. Cluster I (C I; Figure 2) is the largest with 11 isolates. More than 72% of these isolates utilize more than 60% of tested carbohydrates, 57% of amino acids and were resistant to 37.5% of antibiotics. All isolates in this cluster were able to grow on more than 50% of the tested heavy metals and could withstand salinity up to 3% NaCl. The isolates in this cluster were poor in utilizing cellobiose, thymine, and glycine. They were sensitive to antibiotics like Chloramphenicol, Penicillin G, Kanamycin and Streptomycin, heavy metals like Cu and Co and high temperature >40°C.

Cluster II (Figure 2) comprised 5 isolates. In this cluster, more than 80% of the isolates catabolized 60% of the tested carbohydrates and 57% of amino acids. They were all resistant to bacitracin, gentamycin, Al, and Mn. They all tolerated temperature (10-35°C) and salt concentration of 0.8%. These isolates failed to catabolize cellobiose, arabinose, and thymine. They were all sensitive to chloramphenicol, spectinomycetes, glycine, mercury, temperature >40°C and a salt concentration greater than 0.8%.

Table 1. Colony Morphology, Colony Diameter, and Growth rate of the 23 *rhizobia* isolates obtained from different parts of Eastern, Southeastern and Southern Ethiopia.

No.	Isolates	Site (Zone)	Diameter (mm)	Appearance	Shape	MGT (hrs)
1	EIARCP5	Odabulto	4	LM	Domed	2.2
2	EIARCP6	Gemechis	3	LM	Domed	1.6
3	EIARCP7	Tulo	4	LM	Domed	1.5
4	EIARCP8	Ginir	4	LM	Domed	2.5
5	EIARCP9	Adaba	3	LM	Domed	1
6	EIARCP10	Ginir	4	LW	Flat	2.3
7	EIARCP11	Odabulto	3	LM	Domed	2.1
8	EIARCP12	Goro	3	LW	Flat	2.5
9	EIARCP13	Digelo & Tijo	5	LM	Domed	1.2
10	EIARCP14	Tulo	3	LM	Flat	2
11	EIARCP15	Tulo	4	LM	Domed	1.1
12	EIARCP16	Tulo	4	LM	Domed	2.5
13	EIARCP17	Tiyo	5	LW	Flat	1.7
14	EIARCP18	Goro	2	LM	Conical	2.3
15	EIARCP19	Gemechis	3	LM	Domed	1
16	EIARCP20	Zala	2	LM	Conical	1.9
17	EIARCP21	Boreda	3	LM	Domed	2.1
18	EIARCP22	Odabulto	4	LM	Domed	1.8
19	EIARCP23	Zala	5	LM	Domed	2.5
20	EIARCP24	Tulo	2	LM	Conical	2.8
21	EIARCP25	Ginir	5	LM	Flat	1.5
22	EIARCP26	Tiyo	4	LW	Flat	1.2
23	EIARCP27	Odabulto	4	LM	Domed	1.4

* MGT= Mean Generation Time

Table 2. Differentiation of phenotypic traits of the 23 chickpea isolates based on nutritional versatility and resistance to abiotic stresses.

Phenotypic Characters	CI (n=11)	CII (n=5)	CIII (n=3)	CIV (n=2)	CV (n=2)
1. Carbohydrates					
Cellobiose	4	2	2	2	2
Galactose	7	4	0	2	1
Xylose	7	5	2	0	2
Arabinose	10	2	2	2	2
Sucrose	10	3	2	0	2
Inositol	8	5	0	0	2
Lactose	7	3	0	0	1
Maltose	11	5	0	1	2
Sorbitol	10	5	0	2	2
Mannitol	11	5	3	2	2
2. N-sources					
Cystin	11	5	2	2	2
Thymine	1	2	1	0	0
Asparagine	7	5	2	1	2
Lycine	9	4	3	2	2
Leucine	11	4	2	2	2
Phenyl alanine	9	3	2	2	2
Glycin	4	1	1	0	1
3. Antibiotic resistance					
Chloramphenicol	0	0	0	0	1
Spectinomycin	7	1	0	0	0
Bacitracine	11	5	2	2	2
Penicillin G	2	3	1	0	2
Tetracycline	8	4	1	2	1
Gentamycine	9	5	2	1	1
Kanamycine	4	3	0	0	0
Streptomycin	4	3	0	0	1
4. Heavy Metal Resistance					
Al	11	4	3	1	2
Zn	8	3	1	0	0
Hg	0	1	1	0	0
Mn	10	5	3	0	2
Co	3	3	1	0	2
Cu	3	3	0	0	0
5. pH levels					
4.5	0	0	0	0	0
5	1	2	0	0	2
8	6	4	1	0	2
8.5	1	1	0	0	2
9	1	0	0	0	2
9.5	0	0	0	0	1
10	0	0	0	0	1
6. Temperature levels					
10	9	5	3	1	2
20	11	5	3	1	2
35	2	4	2	1	1
40	0	0	0	1	1
45	0	0	0	1	0
7. NaCl levels					
0.8	11	2	3	2	2
1	11	2	3	2	2
1.5	11	2	3	2	2
2	11	0	3	0	2
2.5	11	0	3	0	2
3	9	0	3	1	2

In general from all isolates in all clusters all utilizes mannitol, cystine, and leucine. More than 70% of them catabolized a wide spectrum of carbohydrates. Almost all isolates were resistant to bacitracin and many of them resistant to tetracycline and gentamicin. Almost all are sensitive to chloramphenicol, Hg, pH 4.5, and highest temperature levels (>40 oC).

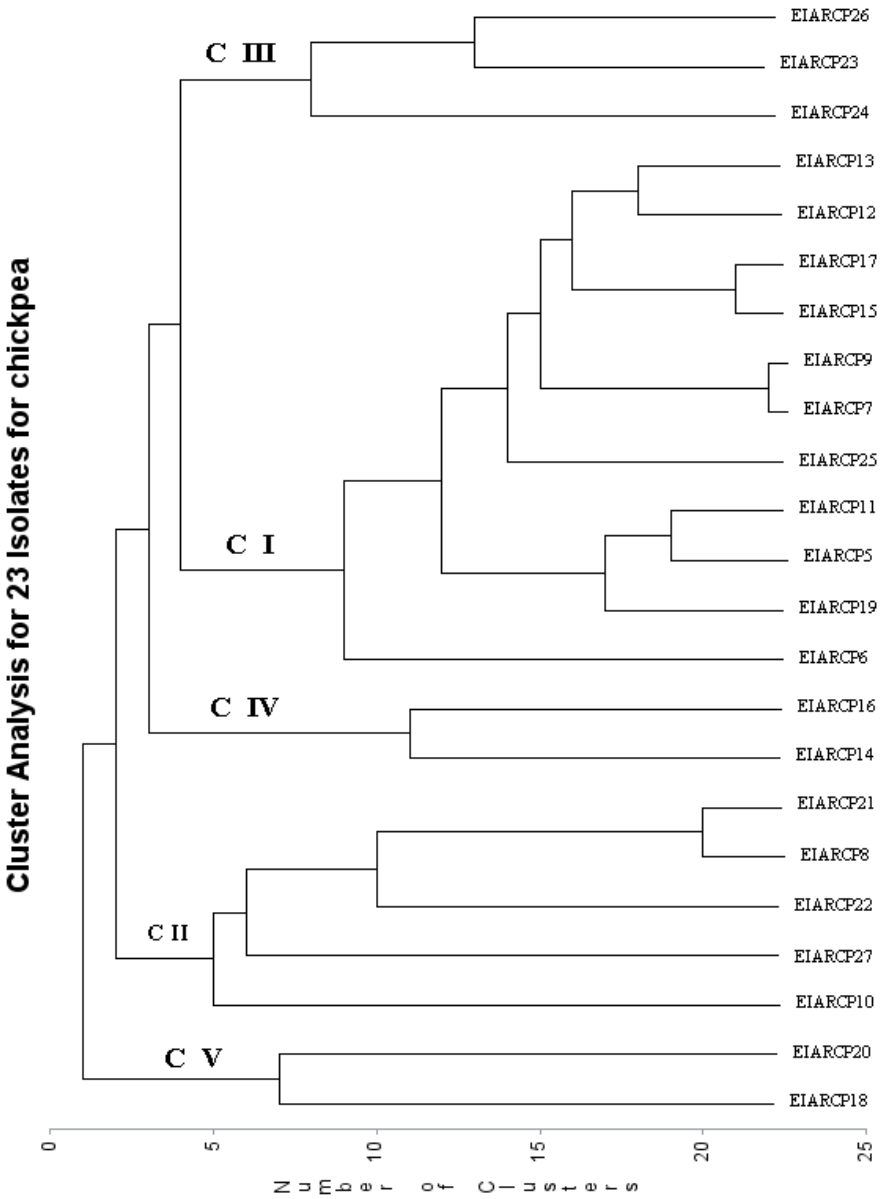

Figure 2. Dendrogram highlighting the phenotypic similarity among the test isolates.

Cluster III (Figure 2) contains 3 isolates. Two of them utilized cellobiose, xylose arabinose, and sucrose from carbohydrates and Phenylalanine, leucine, asparagines, cystine from amino acids. Two of them were also resistant to bacitracin and gentamycin. All isolates in this cluster showed resistance to Al and Mn, salt concentrations up to 3.0% and were only sensitive to a temperature above >40°C, except one that was already sensitive to 35°C. Isolates in this cluster were highly sensitive to antibiotics like chloramphenicol, spectinomycin, kanamycin and streptomycin, heavy metals like Cu, to all pH levels except pH 8 for one isolate.

Isolates in Cluster IV (C IV; Figure 2) utilized cellobiose, galactose, arabinose and sorbitol from carbohydrates as well as cysteine, lysine, leucine and phenylalanine from nitrogen sources. All isolates showed resistance to bacitracin and tetracycline from antibiotics, and one of these isolates were able to grow on all tested temperature levels (10-45°C). Most of these isolates were able to grow in all salt concentration levels. The isolates in this cluster failed to utilize sucrose, inositol, and lactose as well as thymine and glycine. The isolates were also sensitive to chloramphenicol, spectinomycin, penicillin G, kanamycin, and streptomycin. The isolates were highly sensitive to all tested heavy metals except Al for one of them, and to all pH levels tested.

All isolates in Cluster V (C V; Figure 2) were able to utilize all carbohydrate sources except galactose and lactose. Also, all utilized all amino acid sources except thymine and glycine. The isolates in this cluster

were able to grow on media containing bacitracin, penicillin G, Al, Mn or Co. They also grew within the pH range of 5-9 and temperature levels of (10-20 °C). All isolates in this cluster were tolerant to salt concentrations up to 3%. They showed sensitivity to spectinomycin, kanamycin, Zn, Hg Cu, low pH (4.5), and high temperature (45°C).

3.3 Symbiotic effectiveness tests

The isolates were also diverse in their symbiotic effectiveness (SE) on Desi seed type variety Natoli. The isolates induced nodulation with nodule numbers per plant ranging from 14 to 62. This represented more than the four-fold difference between the highly nodulating isolate (EIARCP7) and poorly nodulating isolate (EIARCP11) Table 3. The nodule dry weight varied between 20-53 mg plant^{-1} across isolates. The highest shoot dry weight of 417 mg plant^{-1} was recorded from the plant nodulated with EIARCP7, whereas the least shoot dry weight of 250 mg plant^{-1} was found for plants nodulated by EIARCP17. EIARCP7 not only showed the highest SE, but it was also utilizing most of C and N sources (90 and 71% respectively). It was also adapted to the range of alkaline tested and the normal temperature during the growing season in Ethiopian conditions (10-35%) and was resistant to 67% of the heavy metals assessed. However, it showed high sensitivity to extreme pH levels, and could be recommendable to soil with pH close to neutral, which is also consistent with the optimal pH ranges for chickpea growth (i.e. 6.0-8.0). The high sensitivity to antibiotics would not represent a significant hindrance given that their presence at a significant concentration in farmer fields could be negligible.

Table 3. The symbiotic performance of the *rhizobia* isolates on sand culture under greenhouse conditions at 60 days after planting.

No.	Isolates	Nodule Number (NN)Plant-1	Nodule Dry Weight (NDW) (mgPlant-1)	Shoot Dry Weight (SDW) (mgPlant-1)	SE (%)	Rate
1	EIARCP 7	62.0a	51a	417a	125	HE
2	EIARCP 13	50.0ab	43ab	383ab	108	HE
3	EIARCP 19	34.0bc	53a	350abcd	92	HE
4	EIARCP 6	56.0a	43ab	333bcde	83	HE
5	EIARCP 26	33.0bc	40abc	317bcdef	75	E
6	EIARCP 21	18cde	27bcdef	300cdefg	67	E
7	EIARCP 8	35bc	39abcd	283defgh	58	E
8	EIARCP 16	33bc	43ab	283defgh	58	E
9	EIARCP 10	20cde	32bcde	283defgh	58	E
10	EIARCP 15	23cd	25bcdef	283defgh	58	E
11	EIARCP 22	44ab	44ab	283defgh	58	E
12	EIARCP 11	14cde	27bcdef	267efgh	50	E
13	EIARCP 23	18cdef	25bdef	267efgh	50	E
14	EIARCP 18	18cdef	32bcde	267efgh	50	E
15	EIARCP 17	17cde	20def	250fgh	42	LE
16	EIARCP 20	9de	17efg	233ghi	33	IE
17	EIARCP 9	7de	8fg	217hij	25	IE
18	EIARCP 25	16cde	23cdef	217hij	25	IE
19	EIARCP 24	1e	0.g	217hij	25	IE
20	EIARCP 14	33bc	32bcde	217hij	25	IE
21	EIARCP 5	8de	18ef	167ij	0	IE
22	EIARCP 12	4de	27bcdef	150j	8	IE
23	EIARCP 27	23cd	22.5cdef	150j	8	IE
24	Control (+Ve)	23cd	20def	367abc	-	-
25	CP-018	19de	23cdef	300cdefg	67	E
26	Control (-Ve)	0e	0.00g	167ij	-	-

NB: Numbers in the same column followed by the same letter do not differ significantly at $p < 0.05$; HE = Highly Effective (SE > 80%); E = Effective (SE: 50-80%); LE = Less Effective (SE: 35-50%); and IE= Ineffective (SE < 35%).

The rating of the symbiotic effectiveness (SE) showed that four out of the 23 rhizobia isolates i.e. EIARCP07, EIARCP13, EIARCP19, and EIARCP6 were high effective, 10 were effective, and one was lowly effective, while the remaining eight were ineffective Table 3. Based on this rating, the highly effective isolates out-competed the commercially available strain CP-018. These four isolates, particularly EIARCP7 also showed high tissue dry weight, nodule numbers, and nodule dry weight. These four therefore represented good candidates for further screening under field conditions. Once these findings are validated at scale they could be recommended for rhizobia inoculant formulations for chickpea.

3.4 Classification of the rhizobia isolates based on combined phenotypic traits

For each rhizobia isolate, the percentage of the levels (i.e. phenotypic traits) of carbohydrates or amino acids that it could effectively utilize compared to the total levels for each of the two phenotypic characters was calculated Table 4. Similarly, the percentage of levels of each of the phenotypic characters related to abiotic stress (i.e. antibiotics, heavy metal, pH, salinity, and temperature) that each rhizobia isolate could tolerate compared to the total levels of each of them was also determined Table 4. For instance, 10 levels of C sources were used Table 2 and the rhizobia isolate EIARCP7 could utilize nine out of the ten carbohydrates i.e. 90% as shown in Table 4. Similarly, 6 levels of heavy metals were used Table 2 and the rhizobia isolate EIARCP7 was able to grow normally (i.e. tolerate) on four out of the six heavy metals i.e. 67% as shown in Table 4. The SE was calculated based on Eq.1. The information was then used in the hierarchical cluster analysis combining the various phenotypic characters to determine the cluster membership based on the proximity between various isolates (Figure 3).

When the three groups of phenotypic characters i.e. nutritional versatility, resistance to abiotic stress, and symbiotic effectiveness Table 4, were used for the classification of the isolates no clear trend was found. Isolates with variable phenotypic traits were found in the same clusters, mainly as a result of the variable level of nutritional versatility or resistance to abiotic stresses regardless of the symbiotic effectiveness (Figure 3).

When nutritional versatility was coupled with symbiotic effectiveness, a clear trend was observed in Figure 3. Cluster-1 included three isolates that were highly effective and could use over 80% and

Table 4. Nutritional versatility and abiotic stress resistance of Chickpea root nodulating *rhizobia* isolates from some parts of Eastern, Southeastern, and Southern Ethiopia.

Isolates	Site	Phenotypic characters							
		C Source (%)	N Source (%)	IAR (%)	IHMR (%)	pH %	NaCl %	T °C %	SE (%)
EIARCP7	West Hararge	90	71	37.5	67	22	100	67	125
EIARCP13	Arsi	80	71	62.5	33	22	86	50	108
EIARCP19	West Hararge	80	86	75	17	33	100	33	92
EIARCP6	West Hararge	60	43	37.5	50	33	100	33	83
EIARCP26	Arsi	20	57	37.5	33	22	100	67	75
EIARCP21	Gamogofa	60	71	87.5	67	33	14	67	67
EIARCP8	Bale	90	57	75	83	33	14	50	58
EIARCP16	West Hararge	60	57	37.5	17	22	57	83	58
EIARCP10	Bale	100	57	62.5	83	33	57	67	58
EIARCP15	West Hararge	100	43	37.5	17	33	86	50	58
EIARCP22	West Hararge	60	71	50	33	67	43	67	58
EIARCP11	West Hararge	80	57	87.5	67	22	100	50	50
EIARCP23	Gamogofa	40	43	25	50	33	100	50	50
EIARCP18	Bale	100	71	62.5	50	78	100	83	50
EIARCP5	West Hararge	70	71	62.5	50	33	100	50	0
EIARCP9	Bale	80	86	37.5	83	33	100	50	25
EIARCP12	Bale	80	86	25	50	44	100	50	8
EIARCP14	West Hararge	50	71	25	0	0	33	50	25
EIARCP17	Arsi	90	57	62.5	50	22	100	50	42
EIARCP20	Gamogofa	70	86	37.5	50	89	100	67	33
EIARCP25	Bale	70	71	37.5	67	0	100	40	25
EIARCP24	West Hararge	50	86	12.5	67	22	100	67	25
EIARCP27	West Hararge	80	86	25	50	33	0	67	8

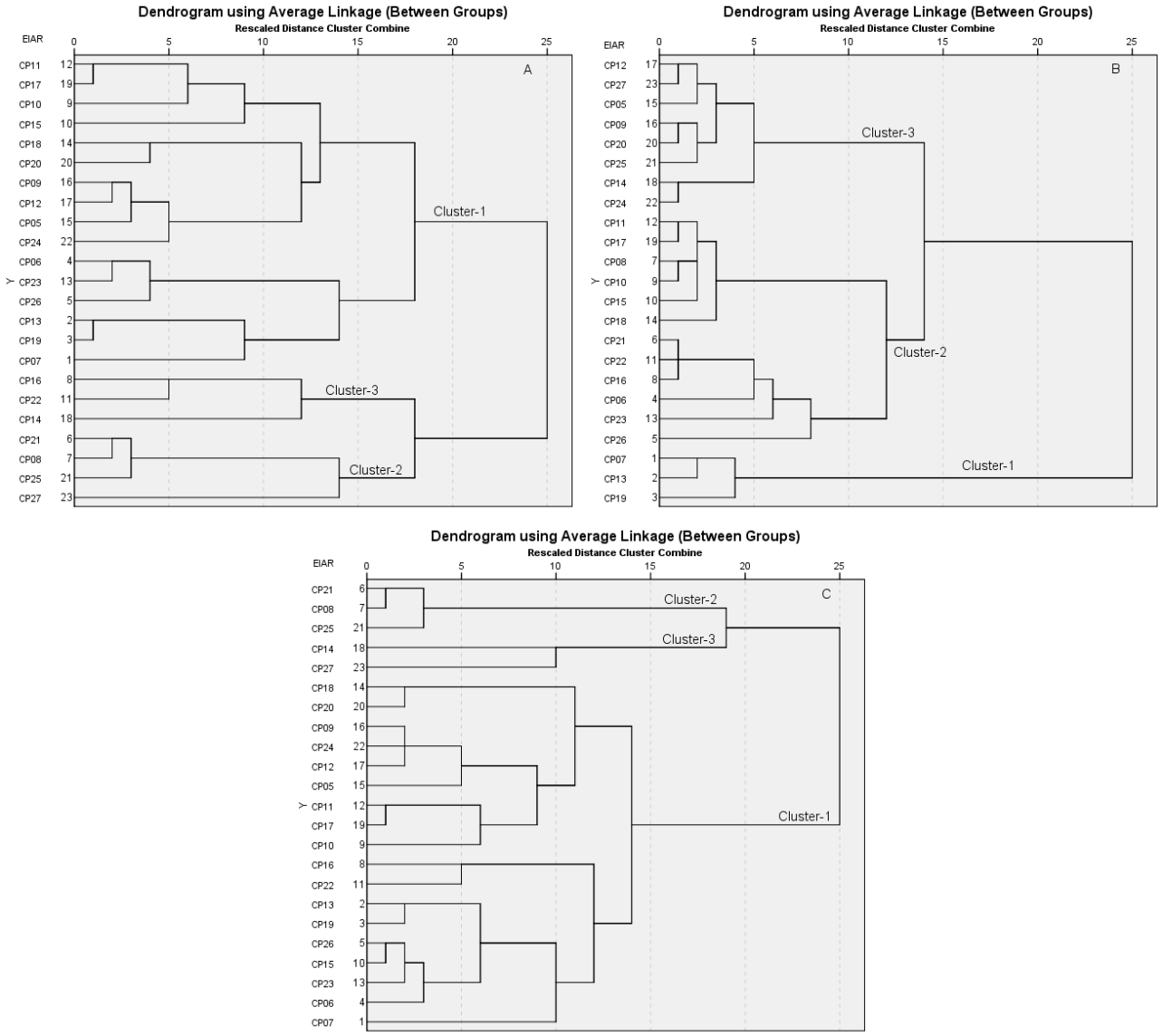

Figure 3. Classification of the *rhizobia* isolates based on nutritional versatility, resistance to abiotic stress and symbiotic effectiveness using hierarchical cluster analysis.

70% of the C and N sources respectively. The more an isolate can use various sources of C and N, the better for survival in various agro-ecological zones. Cluster-2 included 12 rhizobia isolates found effective based on the symbiotic effectiveness, and which could use 20-100% and 40-70% of the C and N sources respectively. Cluster-3 included isolates found ineffective (i.e. symbiotic effectiveness < 35%) and could use a variable range of C and N sources i.e. 50-90% and over 55% respectively. Hence, it would be crucial to couple rhizobia isolates symbiotic effectiveness with nutritional versatility when selecting promising isolates for use in variable agro-ecological zones.

When resistance to abiotic stress i.e. antibiotics, heavy metals, pH, salinity, and the temperature was coupled to symbiotic effectiveness in the hierarchical cluster analysis no clear trend was found (Figure 3), similar to the combination of nutritional versatility, resistance to abiotic stresses and symbiotic effectiveness. Cluster-1 includes 18 isolates of variable symbiotic effectiveness including ineffective, effective, and highly effective ones. Cluster-2 also included three isolates consisting of ineffective and effective ones. Cluster-3 included only two isolates, which were ineffective. Coupling the resistance to the abiotic stresses with symbiotic effectiveness was not useful to select promising isolates in this study.

4. Discussion

Presumptive and authentication tests are crucial to determine the actual rhizobia from bacteria isolated from chickpea nodules. In this study, only 23 out of 39 bacteria were confirmed as rhizobia isolates. This finding was consistent with that of [17] who reported only 37 out of 70 bacteria authenticated as

chickpea root nodulating rhizobia isolates (53%). The authentication result showed that most of the rhizobia isolates were seed-type specific, as 20 out of the 23 isolates (i.e. 87%) nodulated only Desi seed type variety Natoli, which was the original host-plant in field conditions and in the nodule trapping experiment in greenhouse conditions. Only three out of the 23 rhizobia isolates also nodulated Kabuli seed-type, variety Habru. Similarly, [33] reported that chickpea rhizobia are restrictive in nodulation depending upon cultivar/accessions. This represents a significant challenge to the development of rhizobia inoculants for chickpea given the seed-type, variety, and even cultivar specificity. Ideally, rhizobia inoculants that could be used on different chickpea seed-types, varieties, or cultivars would be more interesting not only to reduce the financial burden to the industry, but also to increase the market share for cross-cutting inoculants instead of formulating seed-type, variety, or cultuvar specific rhizobia inoculants. In the context of this study, the rhizobia isolate EIARCP13 that could nodulate both seed-types i.e. Desi and Kabuli, and was highly effective, and could utilize a wide range of carbohydrates and amino acids (N sources), and tolerate most of the abiotic stress, was very promising for consideration in rhizobia inoculant formulations for chickpea. Its performance has to be confirmed in field conditions at large scale.

The phenotypic characters related to nutritional versatility, resistance to abiotic stress and symbiotic effectiveness of the 23 chickpea rhizobia isolates revealed a wide diversity. Most of the rhizobia isolates could use a large range of carbohydrates and amino acids and they were fast growing bacteria. The ability of rhizobia isolates to catabolize a large number of monosaccharides and disaccharides, and most amino acids has been identified as one of the character of fast growing rhizobia [23] including Mesorhizobium Ciceri [16]. Similar versatility of C and N sources for selected chickpea nodulating bacteria isolates was reported in Morocco [27]. However, suitability of carbohydrates or amino acids would vary with rhizobia isolates and/or their origin. For instance, most of the chickpea rhizobia isolates obtained in this study could not grow on glycine and thymine contrary to isolates found in Turkey [34]. This confirms the importance of determining chickpea rhizobia strains suitable to a given region e.g. agro-climatic conditions. Similar trend has also been reported for the resistance to abiotic stress including spatial variability within and across countries based on data reported on India [29], Morocco [27], and Turkey [34], as well as other regions of Ethiopia [16-18]. This calls for further investigations to determine the historical exposure of given chickpea nodulating rhizobia isolates to antibiotics produced by diverse groups of bacteria, actinomycets or fungi and heavy metals based on the region of origin for the isolates and relate the information to the level of tolerance/ resistance to abiotic stress.

Over 91% of the chickpea nodulating rhizobia were found in soils with pH levels of 6.0-7.75. This pH range was consistent for optimum chickpea growth i.e. 6.0-8.0 [35]. Rhitu et al., showed that various chickpea rhizobial strains could grow well in wide range of pH from acid to alkaline i.e. 5-9.5 and this was consistent with chickpea production on calcareous soils in India [29]. Similarly, majority of the chickpea rhizobia isolates were salt tolerant (3%). The same pattern was previously reported by [17] where 94% of the chickpea rhizobia isolates could grow well at 3% NaCl. These Ethiopian isolates were more salt tolerant than the chickpea rhizobia isolates from Turkey [34] and from India [29] that could not tolerate pH >1%. This could be related to local adaptation since small farming is being done at the salinity soils of Ethiopia where chickpea is commonly grown along with inter cropping of wheat which is highly beneficial for the farmers [36].

From the present and previous studies, it can be concluded that the Ethiopian isolates were more sensitive to temperature (>35^{o}C). However, selected Turkish and Indian isolates have shown high temperature tolerance (>40^{o}C) [34, 29], which could again be related to local adaptation. The sampling areas in the Ethiopian highlands were cooler than those in the Turkish and Indian studies cited here. Chickpea rhizobia isolates that could withstand temperature up to 35°C are appropriate in Ethiopia as the temperatures rarely go beyond this temperature in the chickpea growing areas. However, in the context of climate change, it would be crucial to screen for chickpea rhizobia isolates that could tolerate high temperature (>35^{o}C) for potential adaptation to climate shocks.

In addition to nutritional versatility and resistance to abiotic stress, symbiotic effectiveness is an important phenotypic character of chickpea nodulating rhizobia. Three isolates EIARCP7, EIARCP13, and EIARCP19 were not only found highly effective, but also tolerant to most of the abiotic stress, and could utilize most of the C and N sources. These characters are crucial for potential use under various agro-ecological zones with different sources of nutrients and energy, and survival under various environmental stress including high temperature, acidity, alkalinity, salinity, antibiotics and heavy metals. Isolate EIARCP13 was particularly interesting as it could nodulate the two

seed-types used in this study. It would be important to further evaluate it using various chickpea seed-types, varieties, or cultivars in field conditions in different agro-ecological zones to determine its potential for formulation of chickpea rhizobia inoculants.

The hierarchical cluster analysis demonstrated that chickpea nodulating rhizobia isolates of various symbiotic effectiveness (i.e. ineffective, effective, or highly effective) could be found in the same cluster membership when classified based on nutritional versatility, resistance to abiotic stress, and symbiotic effectiveness. This implies that rhizobia of poor ability to effectively nodulate chickpea could still compete with high effective rhizobia for nutrients, energy sources, even under abiotic stress. Therefore, inoculation of chickpea with highly effective rhizobia strains would be crucial to increase their population and increase the potential of high nodule occupancy to improve chickpea productivity, as reported by [37]. According to Brockwell et al., competitive ability, persistence and survival in the soil are all important features that are required for the selection of elite strains for better nitrogen fixation and host productivity [37].

5. Conclusion

The authentication test demonstrated that only 23 out of the 39 bacteria found in the chickpea nodules were rhizobia. From the 23 rhizobia isolates, only 61 % of them were symbiotically effective. Four effective isolates (EIARCP7, EIARCP13, EIARCP19 and EIARCP6) outcompeted one of the commercially-available chickpea rhizobia strains i.e. CP-018. The highly effective chickpea rhizobia isolates also showed high nutritional versatility and resistance to abiotic stress, which represented a high potential to grow in areas with variable C and N sources and under various environmental stress including temperature, acidity, alkalinity, salinity, antibiotics and heavy metals. Since even less effective isolates showed significant nutritional versatility and resistance to antibiotic stress, inoculation of chickpea with highly effective rhizobia strains would be crucial to increase the population and consequently the ability to compete. Therefore, the highly effective rhizobia isolates identified in this study represent good candidates for further investigation in field conditions under variable agro-ecological zones to determine their potential for formulation of rhizobia inoculants for chickpea. This would be critical for the reduction of the current chickpea yield gap in the study areas.

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