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Full Length Research Paper

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

Mulissa Jida[1,2]* and Fassil Assefa[1]

[1]Microbial, Cellular and Molecular Biology Program, Faculty of Life Science, Addis Ababa University, Ethiopia.
[2]Biological Science Program, Faculty of Natural Science, Wollega University, Ethiopia.

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Chickpea (*Cicer arietinum* L.) is one of the major sources of dietary protein for majority of Ethiopian population. It also maintains soil fertility through its symbiotic nitrogen-fixation in association with *Mesorhizobium* species. Therefore, this study was aimed at isolation, characterization and selection of symbiotically effective native chickpea nodulating rhizobia endowed with different plant growth-promoting (PGP) characteristics. Hence, phenotypic and plant growth promoting characteristics of thirty-six rhizobia isolates recovered from root nodules of chickpea grown in soils collected from different chickpea producing areas found in Central and Northern part of Ethiopia were investigated. The result of our study indicated that chickpea rhizobial isolates have shown wide diversity in their different C and N-sources utilization pattern and tolerance to salinity, high temperatures, acid and alkaline pH, heavy metals and antibiotics. Symbiotic and morphological characterization also showed a wide diversity among tested isolates. Moreover, screening for PGP characteristics indicated that 44.4% of the isolates were phosphate solubilizer while 27.8% of them were found to be indole-3-acetic acid (IAA) producer. Furthermore, 19.4% tested isolates showed antagonistic activity against *Fusarium oxysporum* in dual culture assay. Generally, the present study indicates that Ethiopian soils contain symbiotically effective chickpea nodulating rhizobia which are endowed with different PGP characteristics.

Key words: Chickpea, *Mesorhizobium*, plant growth promoting, Ethiopia, symbiotic nitrogen fixation.

INTRODUCTION

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

In addition to nutritional quality and source of cash, chickpea restores and maintains soil fertility through its symbiotic nitrogen-fixation in association with *Mesorhizobium* species (Nour et al., 1994, 1995; Jarvis et al., 1997). It is capable of fixing 90-180 kg N ha^{-1} (Werner, 2005) and therefore, grown in rotation with major cereals such as tef (*Erograstic tef*), wheat (*Triticum sp.*), barley (*Horduem vulgare*) in traditional low-input agricultural system. However, its yield has remained very low (Bejiga and Daba, 2006; Keneni et al., 2011a) and thus, many research activities have been undertaken to improve chickpea cultivars with respect to their yield, tolerance to different biotic and abiotic stresses (Anbessa and Bejiga, 2002; Ahmed and Ayalew, 2006; Keneni et al., 2011a; Keneni et al., 2011b). Consequently, many improved

*Corresponding author. E-mail: mulaeabageda@gmail.com

cultivars were released by Ethiopian Agriculture Research Organization (EARO) (Shiferaw and Teklewolde, 2007). Nevertheless, these alone could not improve the extremely low productivity of chickpea. One of the strategies which has been given less attention was exploiting the benefits of its symbiotic nitrogen fixation by selecting effective rhizobia.

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

Mesorhizobium strains naturally vary in their nitrogen fixing capacity and adaptation to prevailing environmental stresses (Zharan, 1999; Maâtallah et al., 2002; L'taief et al., 2007). Consequently, selection of symbiotically efficient rhizobial strains which are tolerant to locally prevailing stresses is highly desirable. In other parts of the world where chickpea is commonly grown several studies have been conducted on chickpea rhizobia characterization and selection of best strains for inoculant production (Maâtallah et al., 2002; L'taief et al., 2007; Küçük and Kıvanç, 2008). However, in Ethiopia most of the hither to studies were committed to breeding the host plant for adaptation to different stresses (Anbessa and Bejiga, 2002; Ahmed and Ayalew, 2006; Keneni et al., 2011a, b) and thus, there is no information about the characteristics of chickpea rhizobia. These necessitates for research activities devoted to investigate different characteristics of chickpea rhizobia isolated from producing areas of the country. Hence, this study was aimed at isolation, characterization and selection of symbiotically effective native chickpea rhizobia isolates endowed with different PGP characteristics.

MATERIALS AND METHODS

Study sites and soil samples collection

Soil samples were collected from chickpea grown farmer's field found in Showa, Gonder, Gojam, Wallo and Tirgay areas of Ethiopia (Figure 1). The areas are distributed in central and northern parts of the country with an altitude ranging from 1526 (Alamata) to 2840 (Sheno) meter above sea level (masl) and pH from moderately acidic (5.6) to alkaline (7.9) (Table 1). About 3 kg of soil samples were excavated from 15-20 cm depth from each site. A total of 36 soil samples were collected in sterile plastic bags and carefully transported to Applied Microbiology laboratory, Addis Ababa University, for further work in October, 2009.

Isolation of rhizobia

The rhizobia were isolated from soil samples by inducing nodulation on chickpea cultivar called 'Ararti' (obtained from EARO, Debra Zeit) using plant infection method (Vincent, 1970). Each soil sample was filled into surface sterilized (95% ethanol) plastic pots. Chickpea seeds were selected and surface sterilized with 95% ethanol and 3% sodium hypochlorite solutions for 10 s and 3 min, respectively. The seeds were rinsed five times with sterilized distilled water to remove traces of sterilizing chemicals and allowed to germinate on sterile water gar (1%) surface for three days at 25℃. Five pre-germinated seeds were planted on each pot. The seedlings were thinned down to three after 5 days of emergence (DAE). All pots were situated in glasshouse over the table and watered to a field capacity every three days for 60 days after planting (DAP).

Sixty DAP all the plants were carefully uprooted from the pots and washed under gently flowing tap water to remove soil particles. Large and reddish nodules were separately collected from each pot on separate sterile Petri dishes and surface sterilized as described before, and crushed using alcohol flamed glass rod. Loopful of the extract was streaked on Yeast extract Mannitol Agar (YEMA) containing 0.0025% (w/v) Congo red (Vincent, 1970). The components of YEMA g/L: 0.5 K_2HPO_4, 0.2 $MgSO_4$, 0.1 NaCl, 10 Mannitol, 0.5 Yeast extract, 15 Agar (Vincent, 1970). All the plates were incubated at 28℃ for 4 to 6 days. From each plate, single typical rhizobia colony were picked and transferred to test tubes which contain sterile Yeast extract Mannitol Broth (YEMB) (Vincent, 1970). The test tubes were incubated at room temperature on a gyratory shaker at 120 revolution (r) minute $(m)^{-1}$ for 3 days and purified by re-streaking on new YEMA plates for growth. The pure cultures were further confirmed by presumptive tests such as gram reaction using KOH test as described by Gregorson (1978) and growth on Peptone Glucose Agar (PGA) (Somasegaran and Hoben, 1994). Pure isolates were then preserved on YEMA slants containing 0.3% $CaCO_3$ stored at 4℃ for short-term storage (Vincent, 1970) and in glycerol (50% v/v) at -20℃ for long-term storage. All the rhizobial isolates were designated as CR1-50(C: Chickpea; R: Rhizobia) (Table 1).

Rhizobial inoculum preparation

Rhizobial isolates were grown in YEMB on a gyratory shaker at room temperature and 120 r m^{-1} for 72 h. The suspension was centrifuged in sterile plastic tubes (10 ml) at 5000 r m^{-1} for 10 min. The pellets were re-suspended in normal saline (0.90% w/v of NaCl) solution to give a final concentration of 10^8 CFU/ml using the viable plate count method and optical density measurement by spectrophotometer at 540 nm. All inoculums were prepared like this unless otherwise stated.

Authentication and preliminary symbiotic characterization of the isolates

All rhizobial isolates were authenticated and characterized symbiotically by re-inoculating on their host plant on sand culture. About 3 kg of washed and autoclave sterilized sand was placed in

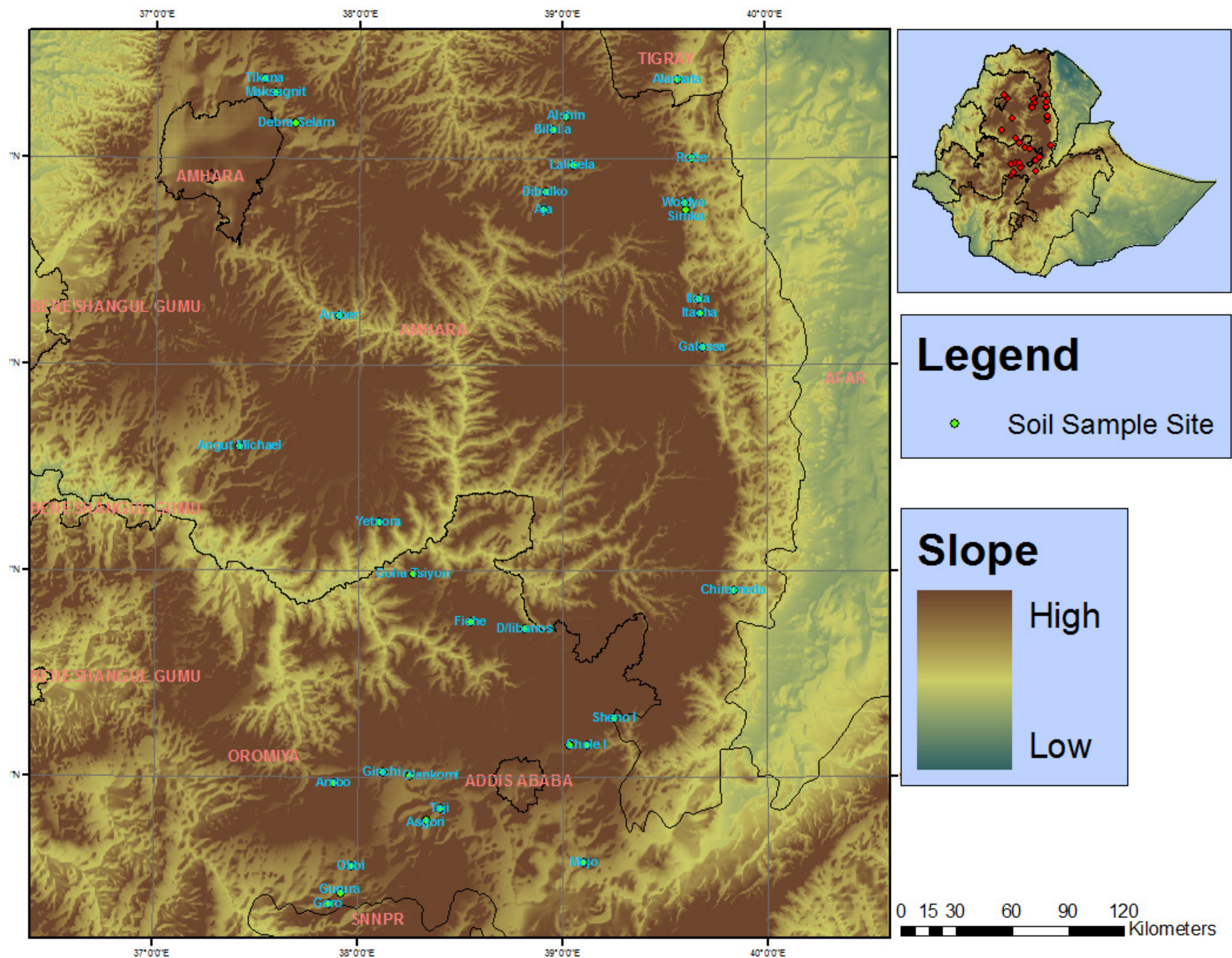

Figure 1. Soil sampling sites.

plastic pots (3 kg capacity). The chickpea cultivar called 'Ararti' seeds were surface sterilized and germinated as described before. The germinated seedlings were flooded with each rhizobial culture adjusted 10^9 cells per seed for 1 h on separate autoclave sterilized plates. Five inoculated seedlings were transferred to each pot which was later thinned down to three after 5 DAE. The pots were irrigated with nitrogen free plant nutrient solution as described by Somasegaran and Hoben (1994). Uninoculated but nitrogen-fertilized pots were included as positive (TN) control and uninoculated and non fertilized (T0) pots as negative control. The experiment was statistically laid out with three replications using randomized block design. As control, each block contained two pots (T0 and TN) with uninoculated seedlings. Plants were supplied with water every three days and once a week with a nitrogen-free nutrient solution (Somasegaran and Hoben, 1994). Furthermore, TN control received weekly 0.05% (w/v) KNO_3 as nitrogen source weekly. Sixty DAP all seedlings were carefully uprooted, nodule number counted, nodule dry weight and shoot dry weight were measured after drying at 70°C for 48 h to a constant weight. Relative symbiotic effectiveness of each isolate was calculated by using the formula (100 × inoculated plant shoot dry weight/ N-fertilized plant shoot dry weight) of Gibson (1987).

Colony morphology, growth and biochemical characteristics

Colony morphology was evaluated by streaking a loop of the initial inoculum on YEMA plates and allowing the isolates to grow at 28°C for 5 days (Vincent, 1970; Sinclair and Eaglesham, 1984). Growth rate of the isolates was assessed by inoculating 20 µl of the inoculum into YEMB test tubes incubated in a gyratory shaker at 120 r m^{-1}, by measuring the optical density at 540 nm using spectrophotometer every 6 h and spread plating 0.1 ml diluted culture on YEMA plates. The generation time (GT) was calculated from the logarithmic phase of growth curve as described by Somasegaran and Hoben (1994). Acid or alkali production test was carried out by growing isolates on YEMA medium containing Bromothymol blue (BTB)(Somasegaran and Hoben, 1994).

Physiological characteristics

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

Table 1. Chickpea rhizobila isolates, site of isolation, growth and colony characteristics.

S/N	Isolate	Isolation site	pH of isolation site	Altitude of isolation site (masl)	Colony characteristics	Colony diameter(mm)	GT (h)
1	CR01	Fiche	6.8	2748	LMM	3.2	4.8
2	CR02	Alem Tena	7.1	1637	LWM	2.4	4.8
3	CR03	Debra Selam	6.0	1896	LMM	3.4	5.0
4	CR04	Sandafa	6.0	2554	LCM	3.2	5.1
5	CR05	Galessa	7.6	2017	LWM	2.7	4.5
6	CR06	Chirameda	6.6	1747	LWM	4.5	2.8
7	CR08	Ginchi	6.6	2378	LMM	2.3	4.7
8	CR09	Goro	6.6	1832	LMM	3.2	5.2
9	CR11	Angut Michael	6.3	1850	LWM	2.6	4.9
10	CR12	Maksagnt	6.4	1978	LWM	2.3	4.6
11	CR14	Chole	6.3	2612	LWM	4.3	3.2
12	CR16	Olankomi	6.6	2378	LMM	3.3	4.4
13	CR18	Sirnka	6.9	1843	LWM	2.5	4.1
14	CR19	Dibulko	6.8	1992	LWM	3.7	4.7
15	CR20	Lalibela	7.4	2138	LMM	3.7	4.8
16	CR21	Yetinora	6.4	2437	LMM	3.7	4.1
17	CR23	Gurura	7.0	1906	LMM	3.1	4.7
18	CR24	Fogera	6.4	1931	LMM	2.8	5.1
19	CR25	Alshin	6.7	2082	LWLM	2.2	5.0
20	CR28	Bilbila	6.6	2069	SWLM	1.9	5.4
21	CR29	Amber	6.3	2454	LMM	4.0	2.8
22	CR31	Asgori	6.6	2078	LWhLM	2.2	4.7
23	CR32	Aja	6.8	2023	LMM	3.2	4.4
24	CR34	Woldya	6.6	2074	SWLM	1.9	5.2
25	CR36	Alamata	7.9	1526	LWM	3.3	4.5
26	CR37	Robe	7.8	1658	LWhLM	3.7	5.2
27	CR38	Itacha	6.8	2134	LWM	2.3	4.8
28	CR39	Tikana	7.2	1942	LWhLM	2.0	5.2
29	CR40	Ilala	7.6	1924	LWLM	2.0	5.3
30	CR42	Goha Tsion	6.2	2517	LMM	2.0	4.2
31	CR44	Ambo	6.7	2170	LWM	2.5	3.8
32	CR45	Obbi	6.6	2108	SWLM	1.5	4.8
33	CR46	Mojo	6.8	1774	LMM	2.1	5.4
34	CR47	Sheno	5.6	2840	LCM	2.2	4.6
35	CR48	Teji	6.7	2065	LWM	2.4	4.5
36	CR50	Debra Libanos	7.3	2594	LMM	2.3	4.8

LCM: Large, creamy, mucoid; LWM: Large, watery, mucoid; LMLM: Large, milky, less mucoid; LWLM: Large watery, less mucoid; LWhLM: Large, white less mucoid; SWLM: Small watery less mucoid; GT: Generation time.

carried out in triplicates.

Salt, pH and temperature tolerance

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

Intrinsic antibiotic and heavy metal resistance

This intrinsic antibiotic and heavy metal resistance was determined on solid YEMA medium containing the following filter sterilized antibiotics or heavy metals (µg.ml $^{-1}$): Ampicillin (5 and 10), chloramphenicol (5 and 10), erythromycin (5 and 10), nalidixic acid (5 and 10), streptomycin (10 and 50), neomycin (5 and 10) and

tetracycline (5 and10); $AlK(SO_4)_3.12H_2O$ (10, 25), $K_2Cr_2O_7$ (50), $CoCl_2$ (10), $CuCl_2.2H_2O$ (10), $HgCl_2$ (5), $MnCl_2$ (50, 75), $NiSO_4$ (10), $Pb(CH3COO)_2$ (10), and $ZnCl_2$ (50).

Utilization of different C and N-sources

Different carbohydrates were added as described by Amarger et al. (1997) at final concentration of 1 gL^{-1} to the basal medium containing (gL^{-1}): K_2HPO_4, 1; KH_2PO_4, 1; $FeCl_3$. $6H_2O$, 0.01; $MgSO_4.7H_2O$, 0.2; $CaCl_2$, 0.1; $NH_4(SO_4)_2$, 1; and 15 g of agar. The following filter sterilized sole carbon sources were added after autoclaving: Citrate, D-sorbitol, D-mannose, D-maltose, D-galactose, D-arabinose, gluconate, raffinose, xylose, dulcitol, cellobiose, anoditol, inulin, aesculin, trehalose and inositol. D-mannitol, D-glucose, α-lactose, D-fructose, glycerol, α-cellulose, sucrose, starch, tartarate, gelatin and dextrin were added before autoclaving. Filter sterilized L-tryptophan, methionine, L-tyrosine, leucine, L-asparagine, DL-β-phenylalanine, L-arginine, glutamic acid, L-lysine, analanine, serine, glycine, thiamine, niacin and riboflavin were used as sole nitrogen source for isolates by adding a final concentration of 0.5 gL^{-1} to the above basal medium from which NH_4 $(SO_4)_2$ was omitted and mannitol was added after autoclaving (Amarger et al., 1997). All inoculated palates were incubated at 28°C and results were observed after 5 days.

Determination of PGP Properties of the Isolates

Phosphate solubilization

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

Screening for in vitro antagonistic activity against Fusarium oxysporum

The *in vitro* mycelial growth inhibition of *F. oxysporum f.sp. ciceri* (obtained from EARO, Debra Zeit) by the rhizobial isolates was tested using the dual culture technique as described by Landa et al. (1997). Twenty microliter (20 µl) of each isolates inoculum was equidistantly spotted on the margins of YEMA plates amended with sucrose (0.5%) and incubated at 28°C for 24 h. A 4 to 5 mm diameter agar disc from Potato Dextrose Agar (PDA) cultures of the fungal pathogens was placed at the centre of the YEMA plate for each bacterial isolate and incubated at 28°C for 5 days. The radii of the fungal colony towards and away from the bacterial colony were measured and the presence of clear inhibition zone between the bacterial isolates and growing fungi was considered as positive.

IAA production

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

Data analysis

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

RESULTS AND DISCUSSION

A total of 36 chickpea rhizobial isolates were recovered from as many sampling sites from Central and Northern Ethiopia with altitude ranging from 1526 (Alamata) to 2840 masl (Sheno), and soil pH from moderately acidic (pH 5.6) to slightly alkaline (pH 7.9) (Table 1). The distribution pattern showed that most of the sample sites were from mid altitude with 1750 to 2500 masl and mildly acidic soil pH of 6.0 to 6.9.

Symbiotic characteristics

All tested isolates of chickpea rhizobia showed great variation in their capacity to induce the formation of nodule on the host plant root under glasshouse conditions. The mean nodule number per plant varied from 41 to 79 which were induced by isolate CR14 and CR08, respectively (Table 2). In general, 83% of the isolates induced the formation of more than 50 nodule number on the roots of their host indicating that chickpea rhizobial isolates from Ethiopian soils are the most infective strains. The highest nodule dry weight was recorded for isolate CR32, that is, 120 mg/plant whereas the lowest was 56 mg which was recorded for isolate CR02 and CR50 (Table 2).

Similarly, all tested chickpea rhizobia exhibited high diversity in their capacity to fix atmospheric nitrogen and thus shoot dry matter accumulation. In comparison with TN control which represents 100% level of shoot dry matter and T0 control which represents 19.1%, all isolates showed shoot dry matter yield ranging from 0.60 to 1.36 g per plant (Table 2). The relative effectiveness, which is expressed in percent of TN control showed that isolates CR06, CR38, CR45 and CR47 were the most efficient with more than 80% dry matter yield while CR01, CR08 and CR40 were the least efficient with 44.1% relative effectiveness (Table 2). These isolates were also particularly most infective and highly effective and hence must be taken into consideration for chickpea inoculation trail at different zones under Ethiopian soil and climatic conditions. It has been demonstrated that inoculation of selected rhizobial inoculants on chickpea has beneficial effect on yield (Romdhane et al., 2009).

Overall, the mean comparison showed several overlapping groups for both shoot and nodule dry matter yield and nodule numbers (Table 2). In this study, a correlation between the increase of dry matter and the number or the dry weight of nodules was not found to be statistically

Table 2. PGP and Symbiotic characteristics of chickpea nodulating rhizobial isolates.

Treatment (rhizobial isolates)	Nodule Number/plant ± SE	Nodule dry weight (mg)/plant ± SE	Shoot dry weight(g)/plant ± SE	Relative effectiveness (%)	P.S. activit y(S.I.)	IAA produc tion	*In vitro* inhibition of *F. oxysporum*
CR01	45.8±3.0^{i}	78±8^{b}	0.60±0.02^{k}	44.1	-	+	-
CR02	57.3±3.2^{b-i}	56±5^{b}	0.91±0.02^{c-i}	66.9	1.2	+	-
CR03	49.3±1.9^{h-j}	75±2^{b}	0.69±0.03^{i-k}	50.7	-	-	-
CR04	51.8±3.2^{d-j}	69±8^{b}	0.81±0.05^{d-k}	59.6	-	+	-
CR05	58.3±2.6^{b-i}	114±3^{a}	0.82±0.1^{d-k}	60.3	-	-	-
CR06	58. 7±2.5^{b-i}	92±20^{b}	1.13±0.04^{bc}	83.1	1.14	-	+
CR08	79.3±3.2^{a}	115±9^{a}	0.60±0.02^{k}	44.1	1.23	-	-
CR09	60.8±1.6^{b-h}	101±7^{a}	0.86±0.08^{c-k}	63.2	1.15	-	-
CR11	61.2±2.5^{b-h}	99±2^{a}	0.75±0.01^{fg-k}	55.1	1.25	-	-
CR12	43.3±1.2^{ij}	80±9^{b}	0.63±0.03^{jk}	46.3	-	-	-
CR14	41.2±1.5^{j}	76±4^{b}	0.92±0.03^{c-i}	67.6	-	-	-
CR16	43.8±1.4^{ij}	116±15^{a}	0.88±0.04^{c-k}	64.7	-	-	-
CR18	60.8±2.8^{b-g}	82±9b	0.99±0.05^{c-f}	72.8	-	+	-
CR19	51.2±1.6^{e-j}	92±4b	0.97±0.04^{c-i}	71.3	-	+	-
CR20	60.51.0^{b-h}	99±2^{a}	1.03±0.01^{c-e}	75.7	1.21	-	-
CR21	54.5±3.0^{c-j}	89±10^{b}	0.72±0.04^{g-k}	52.9	-	-	-
CR23	59.3±2.5^{b-h}	75±5^{b}	0.81±0.04^{d-k}	59.6	1.13	-	+
CR24	66.0±2.0^{a-g}	96±4^{a}	0.72±0.01^{g-k}	52.9	1.23	-	-
CR25	62.5±2.0^{b-g}	102±6^{a}	0.95±0.09^{cg-i}	69.8	-	-	+
CR28	64.8±2.5^{a-e}	97±2^{a}	0. 80±0.05^{d-k}	58.8	1.12	-	-
CR29	49.0±3.1^{h-j}	95±9^{b}	0.80±0.03^{d-j}	58.8	-	+	-
CR31	68.7±3.5^{a-c}	105±6^{a}	0.98±0.01^{c-g}	72.1	1.3	+	-
CR32	70.2±1.8^{ab}	120±3^{a}	1.04±0.02^{c-e}	76.5	1.13	-	-
CR34	51.0±1.5^{e-j}	77±6^{b}	0.81±0.01^{d}-k	59.6	-	-	-
CR36	46.2±2.9^{h-j}	98±7^{a}	0.89±0.05^{c-j}	65.4	-	-	-
CR37	62.0±4.5^{b-g}	100±20^{a}	0.70±0.07^{h-k}	51.5	1.17	+	-
CR38	64.5±1.6^{a-f}	104±30^{a}	1.36±0.12^{a}	100	1.2	-	-
CR39	62.5±1.0^{b-g}	99±30^{a}	0.81±0.12^{d-k}	59.6	-	-	-
CR40	67.0±4.8^{a-d}	74±8^{b}	0.60±0.01^{k}	44.1	-	+	-
CR42	63.5±3.8^{b-g}	90±6^{b}	0.81±0.02^{d-k}	59.6	-	-	-
CR44	61.3±5.9^{b-h}	96±1^{b}	0.76±0.04^{e-k}	55.9	1.21	-	-
CR45	63.0±1.2^{b-g}	94±3^{b}	1.22±0.06^{b}	89.7	-	-	+
CR46	62.0±2.8^{b-g}	75±6^{b}	0.70±0.01^{h-k}	51.5	1.15	-	+
CR47	71.0±1.2^{ab}	100±3^{a}	1.11±0.07^{b}-^{d}	81.6	-	-	+
CR48	50.8±2.0^{e-j}	95±7^{b}	0.85±0.05^{c-k}	62.5	-	+	-
CR50	57.3±3.2^{b-i}	56±5^{b}	0.75±0.06^{f-k}	55.1	-	-	+
T0	-	-	0.26±0.004^{l}	19.1	-	-	-
TN	-	-	1.36±0.03^{a}	100	-	-	-

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

significant (p>0.05). Similarly, Maâtallah et al. (2002) has also reported that there was no positive correlation between the increase of shoot dry matter or the number and the dry weight of nodules. Dudeja et al. (1981) demonstrated that the dry matter yield was rather correlated with the nodule leghaemogblobin concentration than with the number or the dry weight of nodules.

Growth characteristics and colony morphology

Based on their mean generation times, 88.9% of the isolates were moderately slow growers with generation time (GT) 4.1 to 5.4 h, whereas only 11.1% of the isolate were found to be fast grower with GT 2.8 to 3.8 h (Table 1). Previous studies indicated that *Mesorhizobium ciceri*

(Nour et al., 1994) and *Mesorhizobium mediterraneum* (Nour et al., 1995), which are the specific chickpea symbionts are moderately slow growing rhizobia.

All isolates formed colony with circular shape, entire margin, milky-to-watery translucent to creamy and white opaque features with different level of mucus production. Most isolates (89%) exhibited copious production of mucus while the remaining isolates showed less mucus production (Table 1). The colony diameter of the isolates was ranged from 1.5 to 4.5 mm. Out of all isolates only 8% of them had colony diameter of less than 2 mm indicating that all isolates could be able to form medium to large colony on YEMA surfaces at optimum pH of growth media (6.7) and incubation temperature 28°C after 5 days of incubation (Table 1). Such characteristics are wide spread among rhizobia (Jordan, 1984).

All isolates changed the color of YEMA supplemented with BTB to yellow indicating that they are acid producers (Vincent, 1970). The CR absorption test also indicated that none of the isolates absorbed CR in YEMA plates; this is distinctive character of rhizobia with only few exceptions (Somasegaran and Hoben, 1994). On the other hand, none of the tested isolates manage to grow on PGA plates. In addition, all tested isolates were KOH test positive indicating that they were Gram negative. These indicate that all isolates were not contaminant rather rhizobia (Vincent, 1970; Somasegaran and Hoben, 1994).

Physiological characteristics

Tolerance of acidic and alkaline pH

As shown in Table 3, the chickpea nodulating rhizobia isolated from different Ethiopian soils exhibited a wide diversity in their different pH tolerance. All tested isolates grew well in moderately acidic pH (5.5) to neutral pH and slightly alkaline pH (8.0) (Table 3). Some isolates exhibited an acid tolerant character since 31 and 56% of them grew at pH 4.5 and 5, respectively. Similarly, some isolates showed alkaline tolerant character as 25 and 22% of the isolates grew at pH 9.5 and 10, respectively. Several studies (Nour et al., 1994; Maâtallah et al., 2002; Küçük and Kıvanç, 2008) have also indicated that chickpea rhizobia exhibit moderately acidic and alkaline pH tolerance characteristics.

It is interesting to note that large variation was observed among chickpea rhizobial isolates with regard to growth in relation to pH of the medium. There might be a relation between pH of origin of isolates and their acid and alkaline pH tolerance. However, in this study such kind of correlation was not statistically significant.

Moreover, metal (Al and Mn) toxicity tolerance of isolates was tested at pH 5.0 and 19% of them were found to be tolerant to very low concentration of Al while about 45% of them tolerated both high and low concentrations of Mn at pH 5.0 (Table 3). Since most Ethiopian soils are acidic like any other tropical soils where associated metal toxicity problem is expected to avail such characteristics of these isolates are very desirable to use them as inoculants on acidic soils. One of the environmental stresses which pose a significant constraint to chickpea and other legume crops production in Ethiopia is soil acidity (Tilaye, 1985). It has been demonstrated that acidity limits nodulation and the nitrogen fixation (Graham, 1992); hence, rhizobia strains tolerant to acidity and associated metal toxicity might be used to improve this crop yield when inoculated on acidic soil.

Temperature tolerance

Temperature conditions have a great effect on rhizobial growth and symbiotic performance (Zahran, 1999). As shown in Table 3, maximum growth of all tested isolates was obtained between 20 and 30°C. Below and above these values, the percentage of isolates that grew decreased to reach 27.8% at 10°C and 42% at 35°C. None of the tested isolates could be able to tolerate and grow at 4°C while17% of them could grow at 40°C. It has been reported that some chickpea rhizobia were thermo-tolerant which could be able to grow at temperature of 40°C (Maâtallah et al., 2002; Küçük and Kıvanç, 2008). Increased temperature optima of these isolates may be beneficial for its application in temperature stressed conditions as symbiotic performance of different rhizobial strains under temperature stress has been correlated with their ability to grow in pure culture at elevated temperature (Hungria et al., 2000). Though correlation between climatic region of origin area of each isolates and tolerance to low or high temperature was not carried out in this study, their temperature tolerance might be related to their origin soil temperature.

Salt tolerance

The data in Table 3 showed that chickpea nodulating rhizobia exhibited high diversity in their salt tolerance. The salt inhibitory concentrations tolerance varied among the strains. In our study, high tolerance to sodium chloride (NaCl) was observed since 75% of the tested rhizobia could grow well with 1% NaCl. However, at higher concentrations the percentage of tolerant isolate decreased with increasing salt concentration as only 11.1% of the isolates tolerated 5% NaCl. Similarly, earlier studies (Maâtallah et al., 2002; L'taief et al., 2007) observed that chickpea rhizobia also exhibited a wide variation in their salt tolerance, even among isolates from the same site. Though it is often believed that saline soils naturally select strains more tolerant to salinity, such kind of correlation was not carried out in our study.

Table 3. Physiological characteristics of chickpea nodulating rhizobial isolates.

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

Am: Ampicillin; Na: Naldixic acid; Er: Erythromycin; Ch: Chloramphenicol; St: Streptomycin; Ne: Neomycin; -: Absent.

Intrinsic antibiotics and heavy metals resistance

The evaluation of intrinsic resistance to antibiotics of chickpea rhizobia showed that 47.2 and 75% of the tested isolates exhibited high resistance to nalidixic acid and erythromycin, respectively (Table 3). In the presence of ampicillin, Chloroamphincol, neomycin or streptomycin only 5.6 to 30.6% (according to antibiotic and their concentrations) of isolates were resistant. All tested isolates were found to be sensitive to low concentration of tetracycline. Several studies (Maâtallah et al., 2002; Küçük and Kıvanç, 2008) also observed great variation among chickpea rhizobia with respect to their intrinsic antibiotics resistance pattern (IAR). The IAR can be used for the identification of rhizobial strains that occupy nodules in studies designed to evaluate the ecological competitiveness (Kremer and Peterson, 1982). In addition, the pattern of antibiotics resistance has been used to identify diversity among strains of rhizobia (Somasegaran and Hoben, 1994). Consequently, it could be used as supplementary diagnostic character for different rhizobial strains (Amarger et al., 1997).

A comparable pattern was also observed with heavy metals resistance (Table 3). More than 16.7 and 69.4% of isolates showed good tolerance to Zinc and Chromium, respectively. Only 11% of the isolates were found to be resistant to Lead. None of the isolates exhibited an intrinsic resistance to the remaining heavy metals indicating that they are highly inhibitory to the isolates. Previous studies also reported that some *Mesorhizobium* species were found to be tolerant to few heavy metals (Maâtallah et al., 2002; Küçük and Kıvanç, 2008). Though metals concentrations in soils origin of the isolates were not determined, their metal toxicity tolerance might be related to their adaptation at their soil of isolation sites. In fact, this requires further investigation. The heavy metal resistance traits of the rhizobial isolates would be used as invaluable positive markers during genetic studies (Küçük and Kıvanç, 2008). The high level of Zinc and Chromium resistance suggest that these metals could be used as selective agents for some *Mesorhizobium strains* (Sinclair and Eaglesham, 1984; Küçük and Kıvanç, 2008).

C and N-sources utilization

Most of the chickpea rhizobia strains were able to catabolize a large variety of carbon substrates (Table 3). All tested strains grew on D-glucose, D-mannitol, galactose, maltose, lactose, raffinose, cellobiose and sucrose. All tested isolates were unable to utilize citrate as sole source of carbon. Graham and Parker (1964) found that utilization of citrate as sole sources of carbon was restricted to slow-growing bradyrhizobia. As reported by earlier studies (Graham and Parker, 1964; Sadowsky et al., 1983), fast-growing rhizobia were able to grow on a large variety of carbon substrates whereas slow-growing rhizobia were more limited in their ability to use diverse carbon sources. However, our result shows that the majority of tested slow-growing chickpea rhizobia were able to use a broad range of carbohydrates. This is in line with the result of other studies (Nour et al., 1994; Matalah et al., 2002; L'taief et al., 2007). It is very interesting to notice that the types of carbohydrates utilized also varied among chickpea rhizobia. Such characteristics are usually used as diagnostic features for root nodule bacteria (Küçük and Kıvanç, 2008; Hungria et al., 2001).

Chickpea rhizobia also exhibited diversity in utilizing different amino acids and vitamins as sole N-sources (Table 3). Methionine, tyrosine, thiamine and riboflavin were utilized by all isolates whereas niacin was least preferred N-source by most isolates. Except phenylalanine and glycine, most isolates metabolized the remaining N-sources tested. This is in line with the previous studies (Amarger et al., 1997; Küçük and Kıvanç, 2008). The ability of isolates to utilize wide range of N-sources would give more ecological competence in the soil and it is also one of the desirable characteristics for isolates selected for field studies. Furthermore, utilization of different compounds by strains, as nitrogen sources is one of the most useful traits for their differentiation (Hungria et al., 2001).

PGP properties of the isolates

Chickpea nodulating rhizoia showed PGP characteristics such as auxin production, inorganic phosphate solubilization, and *F. oxysporum* inhibition under *in vitro* conditions (Table 2). Out of all tested isolates 27.8% of them were found to be auxin producer while only 44.4% were insoluble inorganic phosphate solubilizer with solubilization index ranging from 1.3 to 1.12. Whether present in the rhizosphere as molecules from bacterial saprophytes or present in plant tissues as products released by endophytes, auxin can massively proliferate root hair production (Yanni et al., 2001) and thus enhance the root's absorptive capacity and nutrient uptake of the crops (Dakora, 2003). Besides, inoculation of plants with phosphate solubilizing bacteria often stimulates plant growth by increasing phosphorus uptake (Chabot et al., 1996). Thus, this ability makes them more important as multi-purpose inoculants for this crop production (Jida and Assefa, 2011). Furthermore, 19.4% tested chickpea nodulating rhizobia were found to inhibit the growth of *F. oxysporum* in dual culture assay. It has also been documented that chickpea rhizobial strains showed a strong inhibitory effect against other fungal pathogen of chickpea such as *Ascocyhta rabiei* (Küçük and Kıvanç, 2008) and *Rhizoctonia solani* (Hemissi et al., 2011) under *in vitro* conditions. Consequently, these strains could be used as biocontrol agents against root rot and wilt causing fungal pathogens of chickpea. Nevertheless, further investigations need to be carried out on the mechanism of inhibition and evaluation of their activity under glasshouse and field conditions.

Several studies showed that different strains of *Mesorhizobium* species are endowed with PGP characteristics (Antoun et al., 1998; Alikhani and Yakhchali, 2009; Etesami et al., 2009; Hemissi et al., 2011). When there is no legume host rhizobia enjoy saprophytic life. They become attracted to the roots of non-legume crops and nourish root exudates in the rhizosphere (Dowling and Broughton, 1986). Consequently, rhizobia which have PGP characteristics would increase the yield of non-legume crops which could be grown in rotation or mixed cropping with legumes (Antoun et al., 1998; Alikhani and Yakhchali, 2009; Etesami et al., 2009). Thus, root colonization pattern and PGP activity of such isolates when inoculated to wheat, tef and barley which are commonly grown in rotation with chickpea under Ethiopian conditions needs to be investigated.

Based on the result of our study the following isolates CR06, CR18, CR19, CR20, CR31, CR32, CR38, CR45 and CR47 are highly recommended for field test and

ecological competitiveness studies under different Ethiopian soil and climatic conditions (Table 3), since these isolates have exhibited interesting features such as wide range of C and N-sources utilization, tolerance of acidic pH, metal toxicity and antibiotics. In addition, they exhibited PGP features such as auxin production, *F. oxysporum* growth inhibition, inorganic phosphate solubilization, and highly effective nitrogen fixation. In general, from the present study, it can be concluded that Ethiopian soils harbors highly efficient nitrogen-fixing chickpea nodulating rhizobia which are diverse in their morphological, physiological, symbiotic and PGP characteristics. In addition to *Mesorhizobium ciceri* (Nour et al., 1994) and *M. mediterraneum* (Nour et al., 1995) some bacterial species have been described to nodulate chickpea with different symbiotic efficiency (Laranjo et al., 2004; Rivas et al., 2007). However, during this study methods used for characterizing and distinguishing rhizobial strains were morphological, physiological and symbiotic. These traditional methods of rhizobial characterization frequently fail to identify strains to a species level. Hence, this study should be corroborated by polymerase chain reaction (PCR) based molecular methods such as restriction fragment length polymorphism (RFLP), rapid amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and 16S rRNA sequence analysis to evaluate their genotypic diversity and identify the isolates to the species level.

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