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SCREENING FOR SYMBIOTICALLY EFFECTIVE AND ECOLOGICALLY COMPETITIVE CHICKPEA RHIZOBIAL INOCULANTS FROM ETHIOPIAN SOILS

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ABSTRACT: Chickpea is one the most important pulse crops grown for food and crop rotation in order to improve soil fertility because it fixes atmospheric nitrogen with root nodule bacteria. However, effectiveness in nitrogen fixation depends upon the host variety, the efficient endosymbiont and environmental conditions. This study was initiated to isolate and characterize chickpea rhizobia for their symbiotic effectiveness adapted to local environmental conditions. A total of seventy root nodule bacteria were isolated from different sampling sites in central and northern Ethiopia of which only 52% were rhizobia and the remaining were endophytes and non-nodulating rhizobia. All but two of the isolates were fast growers with generation time (<3 h) with large mucoid and large watery colony texture, and colony size (1-5 mm) and were able to change BTB-YEMA medium to yellow indicating that they were fast growing *Metarhizobium ciceri*. The isolates utilized different carbohydrates, except dulcitol, aesculine and citrate, and many amino acids, but none of the isolates assimilated aspargine and arginine, and a few isolates were able to utilize glycine and thymine. The isolates showed a pattern of tolerance to high pH (>8.0), high salt (4-6%) and medium temperature (20-30°C), and were sensitive to low pH (pH 4-4.5) and high temperature (40-45°C). Most isolates were resistant to several antibiotics and heavy metals, but relatively sensitive to chloramphenicol, tetracycline (51%). Of the tested antibiotics and heavy metals, streptomycin sulfate and mercury were found to be the most potent against the isolates. The symbiotic effectiveness test showed that only 20% of the isolates were able to increase the shoot dry mass as much as 50-100%. Of the nitrogen-fertilized control plants, two isolates (isolates NSCPR13 and NSCPR14) were highly effective (80%-100%). Based on their symbiotic effectiveness and wide physiological diversity and tolerance, the two best isolates; NSCPR13 and NSCPR24 could be tested in the field as future candidates for commercial inoculation.

Key words/phrases: Antibiotic resistance, Eco-physiological diversity, Heavy metal resistance.

INTRODUCTION

Chickpea is one of the most important pulse crops grown on marginal and low fertility soil with residual moisture after rainy seasons (EEPA, 2004). It originated from south-eastern Turkey where the wild subspecies *Cicer*

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arietinum reticulatum is widely distributed (Zohary and Hopf, 1993). It is now extensively grown in the Indian subcontinent, Mediterranean regions and Ethiopia. The seeds of chickpea contain high level of protein (20%), carbohydrate (55%) and fats (5%) making them highly nutritious (Nour *et al*., 1994). It is grown in crop rotation as part of low-input agriculture to improve soil fertility due to its ability to fix nitrogen in association with root nodule bacteria, known as rhizobia.

Chickpea has a marked specificity for the strain it forms an effective nodulation showing a loose cross inoculation relationship with some *Sesbania* species (Giller, 2001). *Rhizobia* isolated from chickpea nodules have a wide range of growth rates and are described as fast and slow-growing *Rhizobia*, and classified as *Mesorhizobium ciceri* and *M. mediterraneum* (Nour *et al*., 1994). More recently, *Sinorhizobium medicae* has also been found to form nodules on chickpea, but this symbiosis is ineffective (Martha *et al*., 2004).

According to Beck (1992), chickpea accumulates about 99 kg N ha^{-1}, of which about 81% is derived from the atmosphere (%Ndfa) through biological nitrogen fixation. Werner (2005) estimated that chickpea is capable of fixing 90-180 kg N ha^{-1}. However, efficiency in nitrogen fixation is generally influenced by the type of strain, host variety, and different environmental variations. Several works have shown that inoculation of chickpea cultivars with a selected *Rhizobium* strain resulted in a significant increment in nodule number per plant, nodule dry weight and crop yield (Elsheikh, 1992; Mahdi, 1992; Romdhane *et al*., 2009). El Hadi and Elsheikh (1999) also reported a 70% increase in chickpea yield with inoculation over the uninoculated control with a yield advantage of 72 and 70% which is equivalent to yield obtained through the application of 50 kg N ha^{-1}.

In Ethiopia, chickpea is mainly grown in the central, north, and northwest highlands of the country, mid-altitude 2100-2300 m a.s.l., with annual rainfall of 700-1300 mm (Asfaw Tilaye *et al*., 1994), and according to Central Statistics Authority, it is the fourth important pulse crop in terms of area coverage and production (CSA, 2008). It grows on predominantly brown and chestnut Vertisols, which are characterized by poor soil fertility (Angaw Tsigie and Asnakew Woldeab, 1994).

Chickpea productivity is also limited significantly due to the sensitivity of the legume-*Rhizobia* symbiosis and nodule formation to temperature, salinity and other environmental stresses (Graham, 1992; Hungaria *et al*.,

2000). Consequently, the ability of the isolates to utilize a wide range of carbon and nitrogen sources, acquire antibiotic and heavy metal resistance, and tolerance to different environmental conditions have significance not only for differentiating rhizobia groups but also for selecting isolates with competitive advantage over the aggressive, but often ineffective indigenous endosymbionts in order to persist and nodulate the host. Hence, isolates that are tolerant to these environmental constraints may be potential candidates for developing inoculants that can partly contribute to improving the fertility status of the soil and increasing the yield of the crop. However, in order to develop such inoculants, characterization and selection of symbiotically effective chickpea isolates from different growing regions is necessary.

In Ethiopia, previous studies on chickpea research focused on breeding and plant protection aspects of the host, and fertilizer trials to enhance production (Geletu Bejiga *et al*., 1996). Recently, several studies were undertaken on the genetic diversity symbio-agronomic characters of the different chickpea accessions to realize yield improvement (Gemechu Keneni *et al*., 2012), and plant growth promoting and symbiotic characteristics of chickpea rhizobia from some regions of Ethiopia (Mulissa Jida and Fassil Assefa, 2012). This particular study was, therefore, carried out to further evaluate phenotypic and symbiotic diversity of chickpea rhizobia from many sampling sites of some major chickpea growing areas in Ethiopia.

MATERIALS AND METHODS

Sampling site

Root nodules of chickpea and their corresponding soil samples were collected from a total of 70 sampling sites from farmers' fields of the major growing areas of Shewa, Gojam, Gondar, Tigray, and Wollo. The induction of nodulation was undertaken by growing chickpea cultivar called 'Ararti' (obtained from EIAR, Debre Zeit) on the representative soils using plant infection method (Somasegaran and Hoben, 1994).

Isolation and presumptive and definitive tests for root nodule bacteria

Nodules were surface sterilized with 95% ethanol and 3% sodium hypochlorite solutions (Somasegaran and Hoben, 1994) and crushed using alcohol flamed glass rod. Loopful of the extract was streaked on Yeast extract Mannitol Agar (YEMA) containing 0.0025% (w/v) Congo red (Vincent, 1970). The components of YEMA g/l: 0.5 K_2HPO_4, 0.2 $MgSO_4$, 0.1 NaCl, 10 Mannitol, 0.5 Yeast extract, 15 Agar (Vincent, 1970). All the

plates were incubated at 28°C for 4 to 6 days. From each plate, single typical rhizobia colony were picked and transferred to test tubes which contained 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 subjected to presumptive tests such as gram reaction using KOH test (Gregorson, 1978) and growth on Peptone Glucose Agar (PGA) (Somasegaran and Hoben, 1994), and grown on sand culture under greenhouse conditions to authenticate them as rhizobia according to Somasegaran and Hoben (1994). Pure isolates were then preserved on YEMA slants containing 0.3% $CaCO_3$ stored at 4°C for short-term storage and in glycerol (50% v/v) at −20°C for long-term storage. All isolates were designated as NSCPR (National Soil Chickpea Rhizobium) with different numbers representing each isolate.

Cultural and growth characteristics

The cultural and growth characterization of the isolates was performed by growing active cells (10^6/ml) into YEMA medium and incubating them at 28°C for 3-5 days. Each isolate was characterized by colony appearance, diameter, color and extracellular polysaccharide production according to Ahmed *et al*. (1984). The colonies were characterized as Small dry (SD), Large mucoid (LM) (opaque texture) and Large watery (LW) (transparent texture with production of copious amount of exo-polysaccharide on the medium). They were also grown on YEMA-BTB medium (0.125% bromothymol blue (BTB) indicator in YEMA to detect acid/alkaline production (Jordan, 1984). Growth rate was also determined by growing them on YEM broth for 3 days at 28°C on Shaker Incubator (INFROS AG, CH-4103) (120 rev/min). Samples were taken every 6 hours to measure their optical density (OD) at 540 nm using Spectrophotometer (Cecil, CE2040). Finally the generation time (g) was calculated from the logarithmic phase according to White (1995).

Nutritional and eco-physiological characteristics

All biochemical and physiological tests were carried out in triplicates, where loopful of overnight YEM broth culture of each isolate (adjusted to 10^6/ml) was transferred to YEMA plates and incubated at 28°C ± 2 for 3-5 days unless stated otherwise. Colony growth was checked visually and the results were scored qualitatively either as + for growth or − for no growth

(Somasegaran and Hoben, 1994).

Utilization of different C and N-sources

Isolates were tested for their utilization of different carbohydrates and amino acids as carbon and nitrogen sources, respectively according to Amarger *et al*. (1997). The carbon source substrates were separately added as a final concentration of 1 g/l to a basal medium containing (per litre) 1 g of K_2HPO_4, 1 g of KH_2PO_4, 0.01 g of $FeCl_3.6H_2O$, 0.2 g of $MgSO_4.7H_2O$, 0.1 g of $CaCl_2$, 1 g of $(NH_4)_2SO_4$ and 15 g agar. The carbohydrate sources were: 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. Similarly, the nitrogen sources were added at a concentration of 0.5 g/l to a similar basal medium except omitting ammonium sulfate and supplementing mannitol at a concentration of 1 g/litre; the amino acids were L-arginine, L-glycine, L-glutamate, L-leucine, L-lysine, L-phenylalanine, L-tyrosine, and L-thymine.

Salt, pH and temperature tolerance

The isolates were grown on YEMA at different incubation temperatures: 5, 15, 20, 35, 40 and 45°C and tested on the same medium containing sodium chloride (NaCl); 0.5%, 1.0%, 2.0%, 3.0%, 4.0%, 5.0%, 6.0%, 7.0%, 8.0%, 9.0% and 10.0% (w⁄v), and adjusted to pH of 4.0, 4.5, 5.0, 5.5, 7.5, 8.0, 8.5, 9.0, 9.5, and 10.0 (Maatallah *et al*., 2002).

Intrinsic antibiotic and heavy metal resistance

Intrinsic antibiotic and heavy metal resistance was determined on solid YEMA medium containing the following filter sterilized antibiotics or heavy metals (μg ml^{-1}): chloramphenicol; (μg ml^{-1}) (10), streptomycin (25 and 100, tetracycline (20), penicillin (5, 10), nystatine (5, 10), cephalexin (25,100), novobiocine (0.5, 1.5), trimetoprim (25, 100), AlK_2SO_4 (250), $CdCl_2$, $2H_2O$ (20), $CoCl_2$ (20), $CuCl_2$, $2H_2O$ (50), $HgCl_2$ (10), $MnSO_4$ (500), Pb $(CH_3COO)_2$ (250), and $ZnCl_2$ (50) (Maatallah *et al*., 2002).

Phosphate solubilization test

The isolates were tested for their phosphate solubilization ability by inoculating them to medium following the method of Alikhani *et al*. (2006). The ingredients of the Basal Sperber's agar medium (g/l) were: glucose, 10; yeast extract, 0.5; $CaCl_2$, 0.1; $MgSO_4.7H_2O$, 0.25; $Ca_3(PO_4)_2$, 2.5 and agar, 15. Inoculated plates were incubated at 28 ± 2°C for 3-5 days and the

solubilization indices (SI) was calculated.

Symbiotic properties of isolates

Greenhouse experiment

Symbiotic effectiveness of each isolate was undertaken on sand pot culture under greenhouse conditions according to Somasegaran and Hoben (1994). Five seeds of *Cicer arientinum* (var. Shasho) were surface sterilized and sown, each on 5 kg capacity pot filled with acid sand washed (river sand soaked into 95% H_2SO_4 for 24 hours on plastic container and rinsed repeatedly to remove the acid). The germinated seedlings were later thinned down to three seedlings per pot after germination. Each seedling was inoculated with 1 ml culture of each isolate, approximately 10^8 number of bacteria (0.93, OD 540) grown in YEM broth for 72 h.

The experiment was designed with three replications using a Complete Random Design in the glasshouse with average day and night temperature of 26 and 10°C, respectively. Each block contained a positive and a negative control. The positive control pots were fertilized with 70 mg/l nitrogen applied as 0.05 KNO_3 (w/v) solution per week without inoculation. The negative control was supplied only with the nitrogen free solution without the inoculum and the nitrogen fertilizer. All the pots were fertilized with quarter strength of Broughton and Dilworth N free medium (Broughton and Dilworth, 1971), and watered every three days and allowed to grow for eight weeks as described in Somasegaran and Hoben (1994). The plants were then harvested to count nodule number, and shoot dry weight, total nitrogen content, and symbiotic effectiveness (SE) were measured. Symbiotic Effectiveness (%) of each isolate was calculated according to Date (1993).

$$SE = 100 \times \frac{\text{shoot dry weight of plants inoculated with test isolate}}{\text{shoot dry weight of plants supplied with nitrogen}}$$

Symbiotic effectiveness was classified as ineffective, <35%; lowly-effective, 35-50%; effective, 50-80%; and highly effective, >80%.

The total nitrogen content of the plants was determined by modified "Wet" Kjeldahl method according to Sahlemedhin Sertsu and Taye Bekele (2000).

RESULTS AND DISCUSSION

A total of seventy root nodule bacteria were isolated from the sampling sites of Shewa (16), Gojam (12), Gondar (16), Tigray (11), and Wollo (15). Based on presumptive growth tests (CR-YEMA; PGA medium) Congo and authentication, only 37 isolates (52%) were found to be root nodule bacteria

forming nodules on the host upon reinoculation (Table 1). The pattern of nodulation across the sampling sites was also similar in that only 50-58% of the isolates were authenticated as root nodule bacteria (rhizobia).

Table 1. Sampling sites for soil and nodule collection.

No.	Isolate	Site	Latitude	Longitude	Altitude	Soil pH
1	NSCPR1	Gondar	N12° 47'51.4"	E037° 44'24.1"	2710	6.85
2	NSCPR2	Wollo	N11° 06'00.2"	E039° 48'37.7"	1720	6.45
3	NSCPR3	Shewa	N08° 18'42.9"	E037° 38'20.4"	1648	8.04
4	NSCPR4	Shewa	N09° 58'31.5"	E038° 18'41.8"	2540	7.34
5	NSCPR5	Gojam	N11° 27'57.6"	E037° 23'45.1"	1840	6.81
6	NSCPR6	Wollo	N11° 05'25.1"	E039° 46'53.1"	1850	6.55
7	NSCPR7	Wollo	N12°8'25.6"	E039°34'49.9"	1510	6.75
8	NSCPR8	Gojam	N10°42'36.6"	E038°09'50.1"	2560	6.8
9	NSCPR9	Gondar	N12°54'55.8"	E037°44'44.1"	2650	7.65
10	NSCPR10	Gondar	N12°46'46.2"	E037° 39'02.3"	2800	7.35
11	NSCPR11	Wollo	N11° 06'00.6"	E039° 40'31.6"	2030	6.55
12	NSCPR12	Gondar	N12° 10'31.6"	E037° 41'39.7"	1910	6.81
13	NSCPR13	Gojam	N10° 17'31.6"	E038°68'10.1"	2410	7.35
14	NSCPR14	Tigray	N13°59'46.6"	E038°12'08.9"	1810	6.81
15	NSCPR15	Tigray	N14°03'25.4"	E038°13'43.7"	1850	6.75
16	NSCPR16	Gondar	N12° 54'11.4"	E037° 44'24.1"	2700	7.35
17	NSCPR17	Wollo	N11°52'09"	E039°48'48.7"	1480	6.76
18	NSCPR18	Tigray	N13°07'50.2"	E039°29'49.7"	1990	6.71
19	NSCPR19	Gojam	N10° 23'55.1"	E038°09' 57.6"	2440	6.75
20	NSCPR20	Gojam	N11°31'39.2"	E037° 24'56.3"	1780	6.56
21	NSCPR21	Wollo	N11° 15'11.9"	E039° 40'55.6"	2140	6.75
22	NSCPR22	Wollo	N11° 04'13.2"	E039° 45'15.3"	1763	6.8
23	NSCPR23	Shewa	N08° 38'49.6"	E038° 09'34.8"	2285	7.98
24	NSCPR24	Gondar	N12° 20'14.9"	E037° 34.5'1.5"	1940	7
25	NSCPR25	Gojam	N11° 07'11.1"	E037° 46'08.8"	1740	7.18
26	NSCPR26	Shewa	N08° 16'59.6"	E037° 42'9.91"	1792	8.13
27	NSCPR27	Tigray	N13°52'04.2"	E039° 36'13.3"	2240	6.77
28	NSCPR28	Gondar	N12° 31'45.7"	E037°14' 45.8"	1950	7.25
29	NSCPR29	Tigray	N13° 52'45.0"	E038° 10'46.6"	1680	6.96
30	NSCPR30	Gojam	N11° 08'15.2"	E037°41' 0.71"	1880	6.42
31	NSCPR31	Shewa	N08° 42'35.1"	E039 °11'6.05"	2091	8.12
32	NSCPR32	Shewa	N09° 41'18.5"	E038° 49'35.3"	2650	8.33
33	NSCPR33	Shewa	N08° 47'11.9"	E039° 17'94.5"	7185	7.83
34	NSCPR34	Gondar	N12° 31'48.2"	E037° 17'27.1"	2020	7.32
35	NSCPR35	Wollo	N11°44'48"	E039°37'36.9"	1880	6.96
36	NSCPR36	Shewa	N08° 42'87.2"	E039° 00'87.4"	6148*	8.05
37	NSCPR37	Tigray	N12°43'05.3"	E039°32'13.7"	2490	6.95

With regard to cultural characteristics, all isolates (81%) showed similar colony texture of Large mucoid (LM) and Large watery types (LW) of colony with colony diameters of 2-5 mm with translucent texture within 2-3 days, fast doubling time of less than 3 h and produced acid on YEMA-BTB media. However, isolates NSCPR4 and NSCPR35 exhibited Small dry (SD)

colonies with colony size of 1 mm, generation time of 9-10 h, and changed the YEMA medium into blue colour (Table 2). This shows that the majority of the isolates fell into the category of fast-growing root nodule bacteria (Jordan, 1984). This is contrary to the report of Maatallah *et al*. (2002) where 78% of the chickpea isolates from Moroccan soils were slow and extra slow-growing bacteria.

Table 2. Some growth characteristics of chickpea rhizobia grown on YEMA and Serber's media incubated at 28°C for 3-5 days.

Isolates	**Site**	**Colony type**	**Colony diameter (mm)**	**Generation time (hrs.)**	**YEMA-BTB reaction**	**SI**
NSCPR1	Gondar	LM	2	2.3	Yellow	0
NSCPR2	Wollo	LM	5	1.7	Yellow	0
NSCPR3	Shewa	LM	3	2.0	Yellow	0
NSCPR4	Shewa	SD	1	10.3	Blue	0
NSCPR5	Gojam	LM	3	2.4	Yellow	1
NSCPR6	Wollo	LM	1	1.6	Yellow	0
NSCPR7	Wollo	LM	2	1.9	Yellow	0
NSCPR8	Gojam	LM	3	1.6	Yellow	0
NSCPR9	Gondar	LM	5	2.1	Yellow	0
NSCPR10	Gondar	LM	1	1.8	Yellow	0
NSCPR11	Wollo	LM	5	1.8	Yellow	0
NSCPR12	Gondar	LM	3	1.6	Yellow	0.5
NSCPR13	Gojam	LM	2	2.1	Yellow	1.22
NSCPR14	Tigray	LM	1	2.2	Yellow	1
NSCPR15	Tigray	SD	4	1.8	Yellow	1
NSCPR16	Gondar	LM	4	2.1	Yellow	0
NSCPR17	Wollo	LW	3	1.9	Yellow	0
NSCPR18	Tigray	LM	3	1.6	Yellow	1
NSCPR19	Gojam	LM	4	1.7	Yellow	0
NSCPR20	Gojam	LM	2	1.3	Yellow	1
NSCPR21	Wollo	LM	4	2.0	Yellow	0
NSCPR22	Wollo	LM	2	1.4	Yellow	0
NSCPR23	Shewa	LM	3	1.7	Yellow	0
NSCPR24	Gondar	SD	1	5.0	Yellow	1
NSCPR25	Gojam	LM	3	1.9	Yellow	0
NSCPR26	Shewa	LM	3	2.2	Yellow	0
NSCPR27	Tigray	LW	3	2.1	Yellow	0
NSCPR28	Gondar	LM	3	2.7	Yellow	0
NSCPR29	Tigray	LM	2	1.7	Yellow	0
NSCPR30	Gojam	LM	3	1.3	Yellow	1.3
NSCPR31	Shewa	LM	3	2.2	Yellow	0
NSCPR32	Shewa	LM	3	3.1	Yellow	0
NSCPR33	Shewa	LW	3	0.99	Yellow	1
NSCPR34	Gondar	LM	2	1.4	Yellow	0
NSCPR35	Wollo	LM	1	9.1	Blue	0
NSCPR36	Shewa	LM	3	1.8	Yellow	1
NSCPR37	Tigray	LM	2	1.7	Yellow	1

LM= Large mucoid, LW= Large watery, SD= Small dry, SI=solubilization index for phosphorus

In this study, 11 (30%) rhizobial isolates were found to solubilize tricalcium phosphate on Basal Sperber agar medium with solubilizing indices of 0.05-1.3 (Table 2). Mulissa Jida and Fassil Assefa (2012) also reported that 42% of the isolates solubilized with solubilization indices (SI) of 1.1-1.3. This, together with the findings of Halder *et al*. (1990) and Peix *et al*. (2001), showed that root nodule bacteria nodulating chickpea are powerful P solubilizers with dual benefits of P-mobilization and N-fixation.

Growth response of isolates to different carbon and nitrogen sources showed that most of the chickpea isolates were able to catabolize a large variety of carbon substrates, preferably glucose, lactose, maltose, galactose, xylose, cellobiose, sucrose and were limited in utilizing dulcitol, aesculine and citrate (Table 3). This is contrary to the report of Küçük and Kivanç (2008) who showed that none of chickpea nodulating *Rhizobia* from Turkey grew on citrate and all isolates showed weak growth on dulcitol, and according to Maatallah *et al*. (2002), a small proportion of chickpea isolates were selective and utilized acetate, aesculine, and arabinose. With regard to their preference to nitrogen sources, more than 90% of the isolates were able to catabolize alanine, isoleucine, leucine, phenylalanine tyrosine and tryptophan, and none of the isolates assimilated aspargine and arginine, and a few isolates were able to utilize glycine and thymine. On the contrary, Küçük and Kivanç (2008) reported that glycine was taken by all chickpea strains isolated from Turkey.

Isolates were grown between 10 and 30°C, pH 5.5-7.0, NaCl (0.5-2%), but differed in their tolerance to low temperature (5-10°C), low pH (4.0-4.5) and high temperature (40-45°C), high pH (9-10), and high NaCl concentration (3-6%). In general, chickpea isolates showed a pattern of more tolerance to high pH (60%), high salt concentration (70%), low temperature (5-10°C) (100%) than low pH (20%) and high temperature (14%). The data on salt tolerance was much higher than the previous report of Mulissa Jida and Fassil Assefa (2012) where more than 80% of chickpea rhizobia were tolerant to 0.5-2% NaCl.

Table 3. Nutritional and eco-physiological diversity of chickpea rhizobia grown on YEMA media at 30℃ for 5-7 days.

Isolate	Sampling site	Carbon*	Nitrogen*	NaCl	pH	T(℃)	IAR*	Heavy metal*
NSCPR1	Gondar	dul, cit	Lysine, thymine	5	5.5-9.5	10-35	All	Hg, Cu
NSCPR2	***Wollo***	All grow	Lysine, thymine	0.5	5.5-7	10-30	All	Hg
NSCPR3	Shewa	gly, dex, aesc, cit	Lysine, thymine	4	5.5-7.5	10-30	Str	“
NSCPR4	Shewa	aesc, cit	Lysine, thymine	6	5.5-10	10-30	Tet, Str	“
NSCPR5	Gojam	lac, aesc	All grow	5	5.5-10	5-30	Tet, Str	“
NSCPR6	Wollo	gly, dex, aesc, cit	Lysine,	5	5.5-10	10-35	All	“
NSCPR7	Wollo	gly, dex, aesc, cit	All grow	6	6.0-10	5-30	Str	“
NSCPR8	Gojam	gly, dex, aesc, cit	Glycine	4	5.5-10	10-45	Str	“
NSCPR9	Gondar	dul, aesc,dex	Thymine	4	5.5.-8.5	5-30	Str	Hg, Cu, Zn
NSCPR10	Gondar	gly, dex, aes, cit	All grow	2	5.5-9.5	10-30	All	Hg
NSCPR11	Wollo	dul, aesc	Lysine, thymine	4	6.0-10	10-30	Chl, Str, Tet	“
NSCPR12	Gondar	dul, aesc	Thymine	2	5.5-9.5	5-30	All	“
NSCPR13	Gojam	aes, dul	Thymine	2	4.5-8.5	5-40	Str	“
NSCPR14	Tigray	gly, dex, aesc, cit, cel	Lysine	4	5.5-8.5	10-30	Str	“
NSCPR15	Tigray	dul, aesc	Lysine, thymine	6	4.5-8.5	10-30	Chl ,Str, Tet	“
NSCPR16	Gondar	dul, aesc	Lysine, thymine	6	5.5-10	5-30	Chl ,Str, Tet	Hg, Cu,Zn
NSCPR17	***Wollo***	dul, aesc	Lysine	6	5.5-7.0	10-30	Str	Hg
NSCPR18	Tigray	dul, aesc	Thymine	5	5-8.5	5-35	All	“
NSCPR19	***Gojam***	dul, aesc	All grow	0.5	6.0-7.5	5-30	Chl, Str	Hg, Cu,Zn
NSCPR20	***Gojam***	aesc, dul	Thymine,	5	6.0-10	5-30	Tet,Str	Hg
NSCPR21	***Wollo***	dul, aesc, cel	Lysine, thymine	5	5.5-8.5	10-30	Chl,Tet,Str	“
NSCPR22	***Wollo***	gly, dex, aesc, cit	All grow	1	5.5-7	5-30	Str	“
NSCPR23	Shewa	dul	Lysine	6	6.0-10	10-30	Tet,Str	“
NSCPR24	Gondar	aesc, cit, dex	Thymine	4	4.5-10	10-30	Tet,Str	“
NSCPR25	***Gojam***	dex, lac, aesc	Lysine, thymine	4	4.0-10	5-45	Chl ,Str, Tet	“
NSCPR26	Shewa	aesc, dul	Lysine, thymine	3	5.5-8.5	10-30	Chl ,Str, Tet	“
NSCPR27	Tigray	dul, aesc	Lysine, thymine	6	5.5-10	10-35	Tet,Str	“
NSCPR28	Gondar	lac, aesc	All grow	5	5.5-10	5-30	Chl ,Str, Tet	“
NSCPR29	Tigray	dul, cit	Lysine, thymine	1	5.5-10	10-30	Tet, Str	“
NSCPR30	***Gojam***	aesc, dul	Lysine, thymine	1	4.0-10	10-40	Tet, Str	“

Isolate	Sampling site	Carbon*	Nitrogen*	NaCl	pH	T(°C)	IAR*	Heavy metal*
NSCPR31	Shewa	aesc, dul	Thymine		4.0-8.0	10-30	Str	"
NSCPR32	Shewa	aesc, dul	Lysine, thymine	5	5.5-8.0	10-45	Chl ,Str, Tet	"
NSCPR33	Shewa	cit, aesc	Lysine, thymine	5	5.5-10	10-30	Chl ,Str, Tet	"
NSCPR34	Gondar	All	Lysine	6	5.5-10	5-35	All	"
NSCPR35	***Wollo***	dul, aesc, cit	Lysine, thymine	6	4-10	5-30	Chl ,Str, Tet	"
NSCPR36	Shewa	gly, dex, aesc, cit	Thymine	5	6.0-10	5-35	All	Hg
NSCPR37	***Tigray***	dul, cit	Lysine	1	5.5-10	10-30	Tet, Str	Hg

- All but failed to grow; Carbon sources: Aesc= aesculine; cel=cellobiose; cit=citrate; dex=dextrin; dul=dulcitol; gly=glycerol; Chl=chloramphenicol; Lac=lactose; Str=Streptomycin; Tet=tetracycline

Similarly, earlier studies (Maatallah *et al*., 2002; L'taief *et al*., 2007) showed that chickpea rhizobia also exhibited a wide variation in their salt tolerance, even among isolates from the same site. The fact that more than half (54%) of the isolates were able to resist pH of 10 shows the baso-tolerant tendency of chickpea nodulating *Rhizobia* (Nour *et al*., 1994; Maatallah *et al*., 2002; Küçük and Kivanç, 2008; Mulissa Jida and Fassil Assefa, 2012) have also indicated that chickpea rhizobia exhibit moderately acidic and alkaline pH tolerance characteristics. Although the chickpea rhizobia in this study showed higher NaCl tolerance, they showed similar pattern of temperature profile (15-40) and pH profile (4.5-10) with the previous report (Mulissa Jida and Fassil Assefa, 2012).

Most of the chickpea isolates were resistant to penicillin, nystatine, cephalexin and novobiocin, trimetoprim. However, they were less resistant to chloramphenicol (70%) tetracycline (51%), streptomycin (25 µg ml^{-1}), respectively, at different concentrations. Almost all isolates were resistant to heavy metals; Co, Pb, Mn, Al, and Zn (data not shown), but very sensitive to Hg (100%) and Cu (16%) at a concentration of 10 µg and 50 µg ml^{-1}, respectively. Other studies also showed that chickpea rhizobia from Morocco (Maatallah *et al*., 2002), Turkey (Küçük and Kivanç, 2008) and Ethiopia (Mulissa Jida and Fassil Assefa, 2012) were relatively sensitive to tetracycline, chloramphenicol, and streptomycin. The study in Ethiopia also showed that chickpea isolates were resistant to zinc, but sensitive to lead (Pb). However, chickpea isolates from Morocco were resistant to low concentration of Hg and fewer isolates (20-60%) were resistant to Co, Pb, Al, Zn, and Cu compared to the present finding (Maatallah *et al*., 2002). This generally indicates that there is inherent antibiotic and heavy metal resistance characteristics of isolates based upon the origin of isolation and the concentrations of the chemicals to which they are exposed.

The symbiotic effectiveness test on sand pot culture showed the inoculated plants induced nodules with 12-42 nodules/plant, accumulated shoot dry matter (1.67-3.88 g/plant) and Total Nitrogen (TN) (1.47-2.12%)/plant. Although a few isolates induced higher nodule number and showed higher total nitrogen content, the inoculated plants, in general did not show significant difference in these parameters. The number of nodules induced by chickpea isolates in this study was significantly lower in the number of nodules (41-79), but significantly higher in the shoot dry weight (0.6-1.36 g/plant) than those reported by Mulissa Jida and Fassil Assefa (2012) indicating differences among host varieties and endosymbionts.

However, symbiotic effectiveness evaluation in relation to shoot dry matter accumulation by N-fertilized control plants showed that only 14 isolates (51%) were effective and highly effective (Table 4) according to Date (1993). The remaining were categorized as lowly effective (LE) and ineffective, indicating the Ethiopian soils did not only contain many effective chickpea rhizobia, but also their nodules harbour endophytes other than root nodule bacteria.

Table 4. Symbiotic performance of isolates inoculated on chickpea variety grown on sand culture for 45 days under greenhouse conditions.

No	Isolates	Sampling site	NN	SDW (g/plant)	% SE	Rate	TN%
1	NSCPR13	Gojam	45	3.88a*	100	Highly effective	2.12
2	NSCPR14	Tigray	38	3.06abc	84	Highly effective	1.94
3	NSCPR12	Gondar	40	2.64bcd	72	Effective	1.84
4	NSPR9	Gondar	25	2.43cde	67	Effective	1.59
5	NSCPR5	Gojam	38	2.33cdef	64	Effective	1.60
6	NSCPR30	Gojam	42	2.01defg	55	Effective	1.65
7	NSCPR3	Shewa	32	1.80defg	52	Effective	1.74
8	NSCPR4	Shewa	25	1.79defg	53	Effective	1.31
9	NSCPR21	Wollo	20	1.78defg	53	Effective	2.00
10	NSPRC27	Tigray	15	1.75defg	51	Effective	1.63
11	NSCPR37	Tigray	18	1.75defg	53	Effective	1.43
12	NSCPR15	Wollo	21	1.68defg	52	Effective	1.35
13	NSCPR35	Wollo	12	1.67defg	51	Effective	1.56
14	NSCPR24	Gondar	18	1.66defg	50	Effective	1.98
15	NSCPR28	Gondar	21	1.46g	41	Lowly effective	1.54
16	NSPRC16	Gondar	16	1.42g	40	Lowly effective	1.45
17	NSCPR7	Wollo	19	1.42g	40	Lowly effective	1.51
18	NSCPR31	Shewa	22	1.35gh	38	Lowly effective	1.60
19	NSCPR11	Wollo	15	1.31gh	37	Lowly effective	1.46
20	NSCPR29	Tigray	18	1.24h	35	Lowly effective	1.31
21	NSCPR36	Shewa	20	1.24h	35	Ineffective	1.42
22	NSCPR18	Tigray	21	1.10hi	30	Ineffective	1.45
23	NSCPR19	Gojam	16	1.10hi	30	Ineffective	1.40
24	NSCPR25	Gojam	12	1.1hi	30	Ineffective	1.47
25	Control(+)		0	3.55ab	-		2.12

SDW= Shoot dry weight/ plant (g/plant); %SE=% symbiotic effectiveness; TN =Total nitrogen (%N/gm); NN/plant= nodule number/plant; *= Numbers in the same column not connected by the same letters are significantly different at 0.05 levels (Tukey HSD).

Isolates NSCPR13 and NSCPR14 from Shebshengo (Gojam) and Lemlem (Tigray) showed the highest score (>80%) in effectiveness of symbiotic nitrogen fixation whereas the others were within the effectiveness scores of 50-80%. This is contrary to chickpea rhizobia from Morocco where 88% of

chickpea rhizobia were symbiotically effective (Maatallah *et al*., 2002). Previous works also showed that cultivar difference (Danso *et al*., 1995; El Hadi and Elsheikh, 1999) and endosymbiont-host cultivar interaction (O'Hara *et al*., 2002) affect recognition, infectivity (difference in nodule number) and effectiveness in legumes. Even though the nodule numbers didn't show statistical difference at 0.05 significance level, the highly effective and effective isolates displayed higher infectiveness. The same isolates also showed higher total nitrogen percentage accumulation per gram of plant sample (Table 4).

It is established that the performance of isolates under controlled greenhouse conditions may not necessarily corroborate with field conditions because of the influence of environmental conditions and the inherent genetic characteristics of the endosymbionts (Peoples *et al*., 1995). It is, therefore, necessary to extrapolate the nutritional versatility and eco-physiological tolerance of isolates under laboratory conditions with their symbiotic effectiveness in order to predict their performance under field conditions (Table 5). Accordingly, the symbiotically effective isolate NSCPR24 showed the highest score in the different parameters followed by the very effective NSCPR13, and effective isolates NSCPR30, and NSCPR15 indicating that the maximum effectiveness under greenhouse conditions may not guarantee the same performance under field conditions. This is simply because these extreme eco-physiological conditions, particularly, the existence of different secondary metabolites (antibiotics) produced by different soil bacteria and heavy metals in the soil adversely affect the survival and persistence of rhizobia under field conditions (Sharma *et al*., 2010). Under the circumstances, isolates NSCPR24 and NSCPR13 that combined characteristics of symbiotic effectiveness, nutritional versatility, and eco-physiological tolerance can be tested under field conditions.

Table 5. Eco-physiological and symbiotic properties of highly effective and effective chickpea isolates from different sampling sites.

Isolate	Region of isolation	Carbon source	Amino acid	T (°C)	Salt (%)	pH	IAR	Heavy metals	SI	RE	Score
NSCPR13	Gojam	2	1	1	2	2	1	2	1	VE	28
NSCPR14	Tigray	4	1	2	1	3	1	2	1	VE	25
NSCPR12	Gondar	2	1	2	2	3	1	2	1	E	26
NSPR9	Gondar	2	1	2	1	3	1	3	2	E	25
NSCPR5	Gojam	2	1	2	1	2	4	2	1	E	25
NSCPR30	Gojam	2	2	1	3	1	1	2	1	E	27
NSCPR3	Shewa	3	1	3	1	3	1	2	1	E	25
NSCPR4	Shewa	2	2	3	1	3	2	2	1	E	24
NSCPR21	Wollo	2	2	3	1	3	2	2	1	E	24
NSPRC27	Tigray	2	2	3	1	3	1	2	2	E	24
NSCPR37	Tigray	2	1	3	3	3	1	1	1	E	25
NSCPR15	Wollo	2	2	3	1	2	3	1	1	E	27
NSCPR35	Wollo	2	2	3	1	1	4	1	2	E	25
NSCPR24	Gondar	2	1	3	1	1	1	1	1	E	29

RE - Relative Effectiveness; Score 90-100%=1; 70-80%=2; 50-70%=3; 40-50%=4; IAR - Intrinsic Antibiotic Resistance; SI - Phosphate Solubilization Index

CONCLUSION

This study showed the existence of diversity of chickpea rhizobia in terms of their morphological, physiological and symbiotic characteristics from different parts of Ethiopia. It is interesting to note that 37 isolates (51%) were rhizobia of which 14% of the isolates were categorized as effective and highly effective, indicating that chickpea is not as receptive as other cool season legumes such as faba bean and field pea. This shows that there is a need for inoculation of chickpea crop by effective, infective and competitive isolates in the soil. Among the isolates, NSCPR24 and NSCPR13 showed a wide range of tolerance to different nutritional and eco-physiological characters that could presumably enable them to persist in the soil and out-compete the ineffective indigenous rhizobia for nodulation and maximum nitrogen fixation. If they perform well under field conditions, they can be used as commercial inoculants so as to fully realize their potential in the productivity of chickpea in the low-input agriculture in Ethiopia.

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