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MESORHIZOBIUM CICERI AND *MESORHIZOBIUM PRULIFARIUM* ARE THE DOMINANT SYMBIOTICALLY EFFECTIVE STRAINS ON NATOLI AND ARERTI CHICKPEA HOST VARIETIES

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ABSTRACT: It is established that effectiveness in biological nitrogen fixation is a combination of the compatibility of legume host varieties and their endosymbionts working together under their respective agro-ecologies (environments), In order to fully realize the potential of nitrogen fixation and improve production and soil fertility, it is important to pre-screen rhizobial strains that are symbiotically effective and ecologically competitive on their hosts under *in vitro* environmental conditions. To this end, a total of 24 genetically diverse indigenous *Mesorhizobium* spp. were screened for their potential to ecological adaptations using standard methods and their symbiotic effectiveness on two chickpea varieties under greenhouse conditions. The data clearly separated ecological tolerant groups; *M. plurifarium* and *M. ciceri* and sensitive groups; *M. abyssinicae*, *M. gobiense*, *M. hawasiense*, *M. shonense* and *M. amorphae*. They also showed significant difference ($p<0.05$) in their nodulation features and growth characters on Natoli and Arerti, chickpea varieties. In general, 70% of the *Mesorhizobium* spp. were highly effective/effective on Natoli and Arerti plants. However, all *Mesorhizobium ciceri* and *Mesorhizobium plurifarium* were effective on Natoli variety compared with 71% and 83% on Arerti variety. The strains from both *M. hawasiense* and *M. gobiense* were ineffective on both varieties. Four strains: *M. ciceri* CPR67, *M. plurifarium* CPR112, *M. ciceri* CPR40 and *M. plurifarium* CPR3 were highly effective on both chickpea varieties (SE values 80–100%), and resistant to different *in vitro* ecological conditions. These strains may potentially improve chickpea production as inoculants, provided they are validated under different field conditions.

Key words/phrases: Arerti variety, Eco-physiological characteristics, Natoli variety, Stress tolerance.

INTRODUCTION

Chickpea (*Cicer arietinum* L.) is an important pulse crop cultivated on residual moisture in arid and semi-arid areas of the world (ICRISAT, 2013). Ethiopia is one of the secondary centres of the crop where over one million farmers produce chickpea over 240,000 hectares of land with a total

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production of 450,000 Mt (CSA, 2017). The crop is an important source of protein and cash, and it is integrated in low-input agriculture for it fixes atmospheric nitrogen to the tune of 90–180 kg N ha^{-1} (Werner, 2005), partly due to Biological Nitrogen fixation (BNF) in endosymbiotic association with different *Mesorhizobium* spp., mainly *M. ciceri* and *M. medeterraneum* (Nour *et al.*, 1994; Jarvis *et al.*, 1997).

However, symbiotic effectiveness in chickpea depends upon symbiotic and ecological competence of the *Mesorhizobium* strains, compatibility of the host varieties, their symbiosis under different environmental and soil conditions (Abi-Ghanem *et al*., 2012; Mohamed and Hassan, 2015; Zhang *et al*., 2017). These studies showed that ecological fitness of the endosymbionts can be screened through their *in vitro* ability to utilize different carbon and nitrogen substrates, their inherent resistance to different antibiotics, and tolerance to environmental factors such as acidity (pH), salinity (salt) and temperature under laboratory conditions.

Inoculation of chickpea with ecologically fit mesorhizobial strains improved nodulation, growth and yield components of chickpea varieties up to 70% yield increase over un-inoculated control plants, with a yield advantage equivalent to the application of 50 kg N/ha as fertilizer (El Hadi and Elsheikh, 1999; Romdhane *et al*., 2008; Mohamed and Hassan, 2015).

In Ethiopia, several studies were undertaken on phenotypic and symbiotic properties of chickpea rhizobia (Mulissa Jida and Fassil Assefa, 2012; Daniel Muleta and Fassil Assefa, 2015; Wondwosen Tena *et al.*, 2017; Wubayehu Gebremedhin *et al.*, 2018). These studies showed chickpea rhizobia have a predilection to moderately acidic to slightly alkaline pH (pH 5.5–8.0), temperature between 20°C–30°C, and <1% NaCl, characteristics of most root nodule bacteria, but few isolates are acid tolerant (growing at pH 4–5), thermo-tolerant (growing up to 45°C) and salt tolerant (3% NaCl). The authors also identified that several isolates combined tolerance to different *in vitro* stress conditions, nutritional versatility and symbiotic effectiveness comparable to nitrogen-fertilized control plants. Thus, the Ethiopian soils harbour a few, but highly competent and symbiotically effective strains among the indigenous chickpea rhizobia population that have a potential for commercial inoculant production.

However, most of the hitherto studies did not show the relationship of taxonomic groups with their symbiotic effectiveness properties. Although Mulissa Jida and Fassil Assefa (2012) and Wubayehu Gebremedhin *et al*. (2018) showed three and five phenotypic clusters (groups), they failed to

show the specific taxa with their pattern of symbiotic effectiveness. This shows that there is still a dearth of information to show the pattern of ecological competitiveness and symbiotic effectiveness of the different *Mesorhizobium* spp. with their host cultivars.

Therefore, this study was initiated to screen heterotrophically competent, stress tolerant and symbiotically effective strains from twenty four strains identified into seven genetically identified indigenous *Mesorhizobium* species under *in vitro* laboratory and greenhouse conditions.

MATERIALS AND METHODS

Source of rhizobia and growth conditions

The study included 24 chickpea nodulating rhizobial strains belonging to seven different *Mesorhizobium* spp. which were isolated from nodules collected from some parts of central and northern parts of Ethiopia (Table 1). The strains are deposited in culture collections at Addis Ababa University and University of Helsinki. The cultural and growth characteristics of the strains were undertaken by growing them to exponential phase on YEMA medium (5–7 days incubation at 28°C) and standardized to inoculum size of 10^6 cells/ml.

Table 1. Passport data of Mesorhizobial groups isolated from different parts of central and northern parts of Ethiopia.

No.	Strains	Species	Sampling site	Latitude	Longitude	Altitude (m)	Soil pH	ECe (dS/m)
1.	CPR3	*M. plurifarium*	Dembia	12° 27′ 07″N	37° 20′ 18″E	1927	6.51	0.46
2.	CPR31	*M. plurifarium*	Adet	11° 16′23″N	37° 29′45″E	1974	6.32	0.37
3.	CPR48	*M. plurifarium*	Enemay	10° 22′ 54″N	38° 11′ 17″E	2341	5.60	0.34
4.	CPR65	*M. plurifarium*	Becho	8° 85′ 38″N	38° 26′42″E	2082	6.05	0.25
5.	CPR99	*M. plurifarium*	Jari	11° 24′ 03″N	39° 39′ 12″E	1923	7.23	0.62
6.	CPR112	*M. plurifarium*	Sirinka	11° 59′ 25″N	39° 42′ 46″E	1720	7.76	0.92
7.	CPR131	*M. plurifarium*	Samre	13°11′ 03″N	39° 30′ 26″E	2040	6.09	0.68
8.	CPR20	*M. abyssinicae*	Fogera	12° 16′20″N	37° 29′18″E	1985	6.44	0.39
9.	CPR49	*M. abyssinicae*	Enemay	10° 27′ 32″N	38° 11′ 14″E	2341	5.60	0.34
10.	CPR75	*M. hawasiense*	Ada'a	8° 44′ 40″N	38° 59′ 27″E	1930	6.38	0.41
11.	CPR38	*M. shonense*	Adet	11° 26′26″N	37° 29′12″E	1920	6.32	0.37
12.	CPR82	*M. shonense*	Becho	8° 85′ 09″N	38° 26′ 11″E	1930	6.38	0.41
13.	CPR61	*M.ciceri*	Ada'a	8° 44′ 40″N	38° 59′27″E	2074	6.05	0.25
14.	CPR23	*M. ciceri*	Fogera	12° 16′20″N	37° 29′18″E	1985	6.44	0.39
15.	CPR40	*M. ciceri*	Adet	11° 16′26″N	37° 29′12”E	1974	6.32	0.37
16.	CPR67	*M. ciceri*	Becho	8° 85′ 09″N	38° 26′11”E	2082	6.05	0.25
17.	CPR92	*M. ciceri*	Jari	11° 23′ 03″N	390 39′ 12″E	1923	7.23	0.62
18.	CPR118	*M. ciceri*	Wukro	13° 83′ 67″N	39° 35′ 57″E	1955	7.68	0.89
19.	CPR11	*M. gobiense*	Dembia	12° 27′ 07″N	37°20′ 18″E	1927	6.51	0.46
20.	CPR8	*M. amorphae*	Dembia	12° 27′ 07″N	37° 20′ 18″E	1927	6.51	0.46
21.	CPR53	*M. amorphae*	Enemay	10° 27′ 32″N	38° 11′ 14″E	2341	5.60	0.34

No.	Strains	Species	Sampling site	Latitude	Longitude	Altitude (m)	Soil pH	ECe (dS/m)
22.	CPR63	*M. amorphae*	Becho	8° 85′ 09″N	38° 26′11″E	2082	6.05	0.25
23.	CPR103	*M. amorphae*	Sirinka	11° 5′ 25″N	39° 42′ 46″E	1923	7.76	0.92
24.	CPR117	*M. amorphae*	Wukiro	13° 83′ 67″N	39° 35′ 57″E	1955	7.68	0.89

Eco-physiological and nutritional characteristics of the strains

Tolerance to acidity, alkalinity, salinity, temperature, soil acidity and extracellular polysaccharide production

All experiments on tolerance to acidity, alkalinity, salinity and temperature were performed according to Laranjo and Oliveira (2011). The capacity of each rhizobial isolate to grow on acidic and alkaline media was determined by inoculating a loopful of each isolate on YEMA adjusted at a pH of 4.0, 5.0, and 9.0, using 1N HCl and NaOH before autoclaving. For salt tolerance, the isolates were transferred to YEMA plates supplemented with NaCl at concentrations of 1, 2, and 3 w/v. The ability of bacterial isolates to grow at high and low temperatures was monitored at incubation temperatures of 35, 37, 40 and 42°C. Tolerance to soil acidity related Al^{3+} and Mn^{2+} was tested at two different Al concentrations (25 µM and 50 µM) and two Mn concentrations (100 µM and 200 µM) using Keyser and Munns (KM) agar medium under acidic conditions (pH 5), according to Ayanaba *et al*. (1983). The ability of strains to produce extracellular polysaccharide (EPS) under acid stress was determined by ethanol precipitation method using the KM basal medium, according to Cunningham and Munns (1984).

Intrinsic antibiotic resistance

This intrinsic resistance of isolates was determined by inoculating (10^9 cells ml^{-1}) on solid YEMA medium containing five filter-sterilized (0, 22 mm Millipore filters) antibiotics at concentrations of 2.5, 5, 10 and 20 µg/ml of water according to Maatallah *et al*. (2002).

Nitrogen and carbon source utilization

The ability of isolates to utilize different nitrogen sources (10) and carbon sources (20) was determined following the method of Hungria *et al.* (2000). The nitrogen substrates 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 of mannitol. The plates were incubated at 28±2°C for 3–5 days. Similarly, the carbohydrates were prepared as 10% (w/v) solution in water. The carbohydrate-free YEMA medium was

modified by reducing the yeast extract to 0.05 g/l.

Inorganic phosphate solubilizing activity

The phosphate solubilizing (PS) activity was detected on Pikovskayas agar medium supplemented with tri-calcium phosphate. The formation of a clear halo around the bacterial colonies and solubilization index (SI) was recorded according to Alikhani *et al*. (2006).

Symbiotic performance on sand culture under greenhouse conditions

This experiment was carried out at the Department of Microbial, Cellular and Molecular Biology, Addis Ababa University. The two chickpea varieties; high yielding (Natoli, Desi type) and drought tolerant (Arerti, Kabuli type) were obtained from Debre Zeit Agricultural Research Centre (DARC). The experiment was undertaken following the procedures of Howieson and Dilworth (2016). Chickpea seeds were treated with 70% ethanol (5 sec), surface sterilized in 3% sodium hypo-chlorate (3 min) and then rinsed five times with sterile distilled water. Five sterilized seeds were germinated and sown in free-draining plastic pots (3 kg capacity) that had been surface disinfected by soaking in 70% ethanol and drying, and filled with acid treated sterile moistened sand. The germinating seeds were later thinned to three seedlings per pot after a week of planting.

One millilitre of liquid inoculum (10^9 rhizobia cells ml^{-1}) of the Mesorhizobial strains, and a reference inoculant strain, CpNSTC, obtained from National Soils Testing Centre were used separately to inoculate seeds at the sowing stage. The experiment was designed with three replications using randomized block design in the greenhouse with average day and night temperature of 26 and 15°C, respectively. All pots were fertilized with CRS (Centre for Rhizobium Studies, Australia) N-free nutrient solution with two days interval, and the N-fertilized pot received 70 mg KNO_3 L^{-1} $week^{-1}$ (Howieson and Dilworth, 2016) and watered every three days for eight weeks. Nodulation status; nodule number and nodule dry weight, and shoot dry weight were measured. Shoot nitrogen content was determined by modified “wet” Kjeldahl procedure according to Sahlemedhin Sertsu and Taye Bekele (2000).

Relative effectiveness of isolates was calculated according to the equation proposed by Date (1993) as follows:

Inoculated plant dry matter x 100 / N-fertilized plant dry matter with Nitrogen fixing effectiveness classified as ineffective <35%; lowly effective, 35 to 50%; effective, 50 to 80%; and highly effective, >80%.

Data analysis

All values are means of 3 replicates per treatment. The symbiotic data were analyzed using analysis of variance (ANOVA) in SPSS version 24, and mean comparison of treatments was analyzed using the Duncan's multiple range tests at $p \leq 0.05$ significance level. Pearson's correlations analysis was performed to examine the relationship between different test variables.

RESULTS AND DISCUSSION

Eco-physiological characteristics of the strains

All the *Mesorhizobium* strains were able to grow well up to 1% NaCl on YEMA medium, but only 25% of them were tolerant to 3% concentrations (Table 2). This result is comparable to previous works that fewer isolates were tolerant to 3% salt than the majority of the population (Mulissa Jida and Fassil Assefa, 2012; Daniel Muleta and Fassil Assefa, 2015). The most tolerant groups were, *M. plurifarium* CPR3, CPR99, CPR112 and CPR131 and *M. ciceri* strains CPR67 and CPR118 that grew well on YEMA medium adjusted to 3% NaCl.

Other studies also showed *M. ciceri* strains (Brigido and Oliveira, 2013) and *M. plurifarium* strains (Laranjo and Oliveira, 2011) are more tolerant to 3% NaCl concentrations than other chickpea strains such as *M. haukuii*/*M. amorphae* strains. Zahran (1999) reported root nodule bacteria that are tolerant to osmotic stress, in general, have the capacity to accumulate low molecular weight organic solutes in their cells.

Almost all strains grew at pH 5 (92%) than pH 9 (63%) (Table 2), indicating that they were tolerant to moderate acidity and sensitive to alkalinity. This is concurrent with different findings showing that chickpea rhizobial isolates from Ethiopia (Daniel Muleta and Fassil Assefa, 2015), Morocco (Maatallah *et al*., 2002), and Portugal (Brigido and Oliveira, 2013) are generally sensitive to low pH and grow well on moderate acidic conditions of pH 5 and pH 6.

The strains belonging to the dominant *M. plurifarium* and *M. amorphae*, and and *M. ciceri*, with the exception of strains CPR40 and CPR67, were sensitive to very low pH (did not grow at pH 4), contrary to the strains isolated from Turkey that were able to grow at pH 4 (Kucuk and Kivanc, 2008). The data indicate that strains which were isolated from relatively higher pH soils grew less vigorously at lower pH than strains originally isolated from acidic soil habitats (pH 5.2–6.5) (Table 1). This shows the interaction between site of nodule collection and pH of isolation to be highly

significant ($p<0.01$) as indicated in *Rhizobium meliloti* (*Sinorhizobium meliloti*) (Howieson *et al*., 1988) and *Mesorhizobium* spp. (Rodrigues *et al*., 2006; Brigido and Oliveira, 2013).

A number (>50%) of the local *Mesorhizobium* strains were tolerant to 40°C (Table 2), quite different from temperature-sensitive chickpea rhizobia (40–45°C) isolated from Ethiopia (Daniel Muleta and Fassil Assefa, 2015; Wubayehu Gebremedhin *et al*., 2018). However, a few studies from Spain (Nour *et al*., 1995), Turkey (Kucuk and Kivanc, 2008), and India (Rai *et al*., 2012) showed that some chickpea rhizobia were tolerant to high temperature that could be related to local adaptation.

Table 2. Eco-physiological characteristics of chickpea nodulating rhizobial strains grown on YEMA medium and incubated for 5–7 days.

		Eco-physiological characteristics								
		Salt tolerance			**pH tolerance**		**Temperature Tolerance**			
Rhizobia strains	**Species group**	**1.5%**	**2%**	**3%**	**pH 4**	**pH 5**	**pH 9**	**37°C**	**40°C**	**42°C**
CPR3	*M. plurifarium*	+	+	+	-	+	+	+	+	+
CPR31	>>	+	+	-	-	+	+	+	+	-
CPR48	>>	+	+	-	-	+	+	+	+	-
CPR65	>>	+	+	-	-	+	+	+	+	-
CPR99	>>	+	+	+	-	+	+	+	+	+
CPR112	>>	+	+	+	-	+	+	+	+	+
CPR131	>>	+	+	+	-	+	+	+	+	+
CPR20	*M. abyssinicae*	-	-	-	+	+	+	-	-	-
CPR49	>>	-	-	-	+	+	+	-	-	-
CPR75	*M. hawasiense*	-	-	-	+	+	+	-	-	-
CPR38	*M. shonense*	-	-	-	+	+	+	-	-	-
CPR82	>>	-	-	-	+	+	+	-	-	-
CPR23	*M. ciceri*	+	+	-	-	+	+	+	+	-
CPR40	>>	+	+	-	+	+	+	+	+	-
CPR61	>>	+	+	-	-	+	+	+	+	-
CPR67	>>	+	+	+	+	+	+	+	+	+
CPR92	>>	+	+	-	-	+	+	+	+	-
CPR118	>>	+	+	+	-	+	+	+	+	+
CPR11	*M. gobiense*	-	-	-	-	+	-	-	-	-
CPR8	*M. amorphae*	+	-	-	-	+	-	-	-	-
CPR53	>>	-	-	-	-	+	-	-	-	-
CPR63	>>	-	-	-	-	+	-	-	-	-
CPR103	>>	+	-	-	-	+	-	-	-	-
CPR117	>>	+	-	-	-	+	-	-	-	-
Total (%)		67	54	25	29	100	58	54	54	25

+ = tolerant (normal growth), - = no growth

The data also indicated *M. plurifarium* and *M. cicero* strains were more tolerant to (40–42°C); than the other species (Table 2) indicating there perseverance to heat stress compared to other strains (Alexandre *et al*., 2009; Laranjo and Oliveira, 2011). Adaptation to high temperature by rhizobia strains, in general (Zahran, 1999), and chickpea rhizobia, in particular (Rodrigues *et al*., 2006; Brigido and Oliveira, 2013), is due to overproduction of a set of proteins, termed heat shock proteins (HSPs), which are important for survival under stress conditions.

In general, the native *Mesorhizobium* strains showed significant difference in their tolerance to salinity, pH and heat stress *in vitro* that could be due to differences in their inherent genetic make-up and their adaptation to local conditions. The data clearly separated them into tolerant groups including *M. plurifarium* and *M. ciceri* groups and sensitive groups; including *M. abyssinicae*, *M. gobiense*, *M. hawasiense*, *M. shonense* and *M. amorphae* (Table 2). *M. ciceri* CPR67 showed marked inter-strain difference in all the tested eco-physiological parameters within the group compared to the other species or strains. Similarly, Wubayehu Gebremedhin *et al*. (2018) showed such a difference among the five clusters, where clusters 3 and 4 were very sensitive to most ecological factors compared to the relatively resistant Clusters 1, 2, and 5 that may represent different taxonomic groups.

Acidity-Al^{3+}/Mn^{2+} tolerance

The strains showed variable responses to Al^{3+} toxicity which is often associated with soil acidity (Table 3). Although strains were generally tolerant to Mn toxicity, fewer strains (29%) were resistant to 50 µM Al toxicity at pH 5. Unlike the aforementioned results, *M. abyssinicae* CPR20 and CPR49, *M. shonense* CPR38 and CPR82 that were sensitive to other ecological tests, were tolerant to the high concentration of Al (50 µM) at pH 5. This is similar to previous reports from Ethiopia (Mulissa Jida and Fassil Assefa, 2012) and Morocoo (Maatallah *et al*., 2002) that isolates were more resistant to Mn^{2+}, and sensitive to Al^{3+}.

Exopolysaccharide (EPS) production

The strains showed variations in EPS production under acidic conditions ranging from 36.5 to 121.4 µg ml^{-1} (Table 3). Seven strains (29%) from *M. ciceri*, *M. plurifarium*, *M. abyssinicae*, and *M. shonense*, CPR 38 produced more EPS (≥102.6 µg ml^{-1}) than the other strains (≤83.0 µg ml^{-1}) at pH 5 corresponding to their pattern of tolerance to acidity and Al^{3+} toxicity (Table 3) which is one of the adaptive responses to acid/Al stresses (Cunningham

and Munns, 1984; Graham *et al*., 1994).

Table 3.Tolerance to Al and Mn toxicity and exopolysaccharide (EPS) production of chickpea rhizobia strains grown on KM medium at pH 5 for 10–15 days. Phosphate solubilization (PS) of strains grown on Pikovskayas agar medium for 5–7 days.

		Al/Mn tolerance at pH 5				**EPS production (µg/ml)**	**PS**
Strains	**Species Group**	**Al (25 µM)**	**Al (50 µM)**	**Mn (100 µM)**	**Mn (200 µM)**	**At pH 5**	**SI**
CPR3	*M. plurifarium*	+	-	+	+	58.5	-
CPR31	>>	+	-	+	+	67.8	-
CPR48	>>	+	+	+	+	75.4	1.2
CPR65	>>	-	-	+	+	83.2	-
CPR99	>>	-	-	+	+	52.1	1.7
CPR112	>>	+	+	+	+	**104.7**	1.6
CPR131	>>	-	-	+	+	56.4	-
CPR20	*M. abyssinicae*	+	+	+	+	**108.9**	-
CPR49	>>	+	+	+	+	**116.3**	-
CPR75	*M. hawassense*	+	-	+	+	81.7	-
CPR38	*M. shonense*	+	+	+	+	102.6	1.7
CPR82	>>	+	+	+	+	**121.4**	1.3
CPR61	*M. ciceri*	+	-	+	+	82.6	1.4
CPR23	>>	+	-	+	+	**106.3**	-
CPR40	>>	+	+	+	+	83.0	1.5
CPR67	>>	+	+	+	+	**112.6**	2.1
CPR92	>>	+	-	+	+	69.4	-
CPR118	>>	+	-	+	+	78.6	2.4
CPR11	*M. gobiense*	-	-	+	-	36.5	-
CPR8	*M. amorphae*	+	-	+	+	63.4	-
CPR53	>>	+	-	+	+	46.7	-
CPR63	>>	-	-	+	+	53.6	-
CPR103	>>	+	-	+	+	65.8	-
CPR117	>>	+	-	+	+	62.3	-
Total (%)		67	21	100	92	-	29

+ = indicates well growth (tolerant); -= no growth/ sensitive to Al/Mn. PS = phosphate solubilization, SI = solubilization indices, - = also indicates no phosphate solubilization.

Phosphate solubilization of strains

It has been reported that certain isolates of *Rhizobium* can solubilize inorganic phosphates (Alikhani *et al*., 2006). Thus, nine strains (38%) were capable of solubilizing inorganic tri-calcium phosphate with solubilization indices (SI) of 1.2 to 2.4 (Table 3). The isolates were *M. plurifarium* CPR 48, 99, 112; *M. cicero* 40, 61, 67, 118; and *M. shonense* 38. Other studies in Ethiopia (Mulissa Jida and Fassil Assefa, 2012; Daniel Muleta and Fassil Assefa, 2015) also showed that 30–42% of the chickpea local isolates exhibited phosphate solubilizing characteristics, but with low solubilizing

efficiency (SI of 0.5 to 1.3). Rai *et al*. (2012) reported a number of phosphate solubilizers among chickpea nodulating rhizobia with dual benefits of P-mobilization and effective N_2-fixation.

Intrinsic antibiotic resistance (IAR)

All strains were resistant to nalidixic acid and novobiocin, and more than half of them were resistant to chloramphenicol, ampicillin, kanamycin and streptomycin (Table 4). On the contrary, chickpea rhizobia from Morocoo (Maatallah *et al*., 2002) and Turkey (Kucuk and Kivanc, 2008) were sensitive to these antibiotics, indicating large variability in antibiotic resistance of chickpea endosymbionts depending upon different agro-ecologies. *M. ciceri* and *M. plurifarium* strains were more tolerant to more antibiotics (90%), than *M. amorphae* (60%) and the other groups (30%). None of them were resistant to neomycin and spectinomycin which is similar to chickpea rhizobia isolated from Portugal (Alexandre *et al*., 2009).

Table 4. Intrinsic antibiotic resistance (IAR), tolerance to heavy metal Al and Mn, and pattern of carbon and nitrogen utilization of Mesorhizobial groups grown on YEMA medium incubated at 30°C for 5–7 days.

Parameter	Mp (n=7)	Ma (n=2)	Mh (n=2)	Ms (n=2)	Mc (n=6)	Mg (n=2)	Mam (3)
IAR							
Chl	7 (100%)	0	0	0	6 (100%)	0	0
Kan	7 (100%)	0	0	0	6 (100%)	0	0
Amp	4 (57%)	0	0	0	4 (67%)	0	3 (100%)
Str	6 (85%)	0	0	0	6 (100)	0	0
Ery	5 (70%)	0	0	0	5 (83%)	0	3 (100%)
Nov	7 (100%)	2 (100%)	2	2	6 (100%)	2	3 (100%
Nal	7 (100%)	2 (100)	2	2	6 (100)	2	3 (100%)
Average	87%	30%	30%	30%	90%	30%	60%
Al (50 µM)	2	2	0	2	2	0	0
Mn (200 µM)	7	2	2	2	6	2	3
Carbon	92%	75%	75%	75%	88%	70%	79%
Nitrogen	80%	50%	50%	50%	87%	40%	54%

N=number of strains; strains; Mp=*Mesorhizobium plurifarium*; Ma=*M. abyssinicae*; Mh=*M. hawasiense*; Ms=*M. shonense*; Mc=*M. ciceri*; Mg=*M. gobiense*; Mam=*M. amorphae*; Antibiotics: Amp=ampicillin; Chl= chloramphenicol; Ery= erythromycin; Kan= kanamycin; Nal=nalidixin; Nov=novobiocin; and Str=streptomycin

Utilization of different carbon and nitrogen substrates

The indigenous *Mesorhizobium* spp. were versatile in utilizing most of the monosaccharides, disaccharides, and amino acids (Table 4). However, fewer strains utilized dextrin and N-citrate, but all failed to utilize glycine (data not shown). The data showed that the strains were more versatile in utilizing carbohydrate sources (70–92%) than nitrogen sources (40–87%) which was contrary to the report of Mulissa Jida and Fassil Assefa (2012) that showed

isolates catabolized more nitrogen sources (average 85%) than carbohydrates (average 70%). Most of *M. plurifarium*, *M. ciceri*, and *M. amorphae* strains were more versatile in utilizing carbohydrates and amino acids (80–90%) than the other groups (70–75%).

All taken together, the *Mesorhizobium* species utilized a large number of carbohydrates and amino acids; and some strains representing *M. ciceri* and *M. plurifarium* strains catabolized citrate, dulcitol and lysine where others strains failed to do the same, which is similar to strains from Turkey (Kucuk and Kivanc, 2008). Some studies showed that high heterogeneity in substrate utilization is related to differences among strains and local adaptations (Nour *et al*., 1995; Maatallah *et al*., 2002). Soussi *et al*. (1999) also argued that the ability of rhizobia to metabolize a wide range of substrates is closely related to tolerance to stress environments and effectiveness in nodulation and N_2-fixing activities under the circumstances.

Symbiotic effectiveness of strains

The greenhouse trial on sand culture revealed all strains induced nodulation on both chickpea varieties (Table 5). The inoculated plants were significantly different in nodule number, nodule dry weight, shoot dry weight and N contents. The nodule number recorded was in the range of 14 per plant and 75 per plant (mean 55) on Natoli variety; and from 23 per plant to 66 per plant (mean 42) on Arerti variety; and the nodule dry weight was between 32.1 mg/p to 201.4 mg/p (mean, 90.0 mg/p) and 37.5 to 157.3 mg/P (mean=75.8 mg/P) on Arerti variety, respectively. Maximum and minimum shoot dry mass was scored;1.46 g/pl and 0.29 g/pl on Natoli variety and 1.34 g/pl and 0.34 g/pl on Arerti variety, respectively In all cases, the Natoli variety showed slightly, but not significantly, higher values than the Arerti variety.

On the basis of relative shoot dry matter accumulation by the inoculated plants in reference to the nitrogen-fertilized control (relative effectiveness), almost 70% of the *Mesorhizobium* strains showed effective and highly effective symbiosis on Natoli and Arerti plants of which 21%, were highly effective (accumulating more than 80% shoot dry weight compared to the N-fertilized control plants (Date, 1993) (Table 5). Most of the effective and highly effective strains were grouped under *M. ciceri* and *M. plurifarium*. This is similar to the report of Alexandre *et al*. (2009) that *M. ciceri* strains from Portugal were highly effective with symbiotic efficiency >75%.

Table 5. Symbiotic characteristics of chickpea rhizobia on "Natoli" and "Arerti" varieties grown on sand culture for sixty days under greenhouse conditions.

Strains	**Natoli**					**Arerti**				
	NN	**NDW**	**SDW**	**SN**	**SE**	**NN**	**NDW**	**SDW**	**SN**	**SE**
M. plurifarium										
CPR3	75a	127.3bc	1.28ab	35.6ab	90 (HE)	52bc	119.6bc	1.21ab	32.4b	82 (HE)
CPR31	52bc	75.4d	0.79c	18.2cd	56 (E)	34cd	38.8e	0.58cd	11.8d	39 (LE)
CPR48	56b	98.1cd	0.98bc	27.5bc	69 (E)	32cd	47.6de	0.63cd	13.5cd	42 (LE)
CPR65	72a	96.7cd	1.04b	29.7bc	73 (E)	53b	86.3cd	0.96bc	20.7c	65 (E)
CPR99	55b	92.3cd	0.93bc	25.4c	65 (E)	41c	84.7cd	0.83c	16.4c	57 (E)
CPR112	65ab	165.6ab	1.36°	39.7ab	96 (HE)	57b	137.2b	1.29°	35.9ab	88 (HE)
CPR131	52bc	89.3cd	0.91bc	23.3c	64 (E)	56b	100.6c	1.05b	24.6c	71 (E)
M. amorphae										
CPR8	63ab	76.6d	1.01b	28.6bc	71 (E)	45c	84.8cd	0.99bc	22.6c	67 (E)
CPR53	68a	73.8d	0.93bc	27.1bc	67 (E)	42c	63.9d	0.81c	16.3cd	55 (E)
CPR63	47bc	50.3de	0.62cd	13.7d	43 (LE)	33cd	37.5e	0.59cd	11.7d	40 (LE)
CPR103	60ab	77.5d	0.96bc	28.8bc	68 (E)	45c	84cd	0.95bc	21.9c	65 (E)
CPR117	56b	55.8de	0.58cd	15.4d	41 (LE)	29d	38.7e	0.57cd	13.4d	39 (LE)
M. ciceri										
CPR23	51bc	66.4d	0.92bc	22.3c	65 (E)	35cd	42.2de	0.62cd	13.8d	43(LE)
CPR40	59b	138.2b	1.23ab	39.5ab	87(HE)	46bc	124.6bc	1.22ab	35.3ab	83 (HE)
CPR61	46bc	68.3d	0.99b	27.8b	70 (E)	38cd	62.1d	0.80c	16.2cd	56 (E)
CPR67	63ab	201.4a	1.46°	46.3a	102 HE	55b	157.3ab	1.34°	37.6ab	91 (HE)
CPR92	56b	127.1bc	1.17ab	33.7b	82(HE)	39cd	75.8d	0.74c	15.7d	50 (E)
CPR118	52bc	92.7cd	1.09b	28.9bc	76 (E)	38cd	78.2d	0.92bc	21.4c	63 (E)
M. abyssinicae										
CPR20	56b	74.6d	0.87bc	21.6c	61 (E)	35cd	57.7de	0.74c	17.6cd	50 (E)
CPR49	62ab	57.2de	0.51d	11.4d	36 (LE)	31cd	40.9de	0.56cd	12.7d	38 (LE)
M. shonense										
CPR38	62ab	62.8d	0.57cd	13.6d	40 (LE)	61ab	76.5d	0.87bc	16.8cd	59 (E)
CPR82	58b	67.4d	0.61cd	14.2d	43 (LE)	66ab	113.8C	1.11b	28.6bc	76 (E)
M. hawasiense CPR75	57b	43.6de	0.35e	8.3de	25 (IE)	33cd	39.4e	0.38e	7.9d	26 (IE)
M. gobiense	14d	32.1e	0.29e	8.6de	21 (IE)	23d	37.7e	0.34e	9.3d	23 (IE)

Strains	**Natoli**					**Arerti**				
	NN	**NDW**	**SDW**	**SN**	**SE**	**NN**	**NDW**	**SDW**	**SN**	**SE**
CPR11										
Average	55b	90.0cd	0.85c	24.6c	17	42c	75.8d	0.84c	19.7cd	16
CpNSTC	63ab	81.4cd	1.02b	21.7c	72 (E)	54b	79.3cd	0.97bc	18.5cd	66 (E)
N-fertilized	-	-	1.42°	39.8ab	100	-	-	1.47°	43.7a	100
Un-inoculated	-	-	0.26e	4.8e	-	-	-	0.29e	5.2e	-

* Data on nodule number (NN), nodule dry weight (NDW), shoot dry weight (SDW) and shoot N are mean of 6 plants of 2 replicates; SE=relative symbiotic effectiveness.HE=highly effective; E=effective; LE=lowly effective; IE=ineffective (Date, 1993). Values in the same parameters followed by different letters shows significant differences at Duncan's multiple range test ($p<0.05$).

The percentage of highly effective strains recorded (21%) was significantly higher than 12–15% highly effective chickpea rhizobia isolates screened under greenhouse trials in Ethiopia (Mulissa Jida and Fassil Assefa, 2012; Daniel Muleta and Fassil Assefa, 2015; Wubayehu Gebremedhin *et al*., 2018), indicating possibilities to select more effective strains to improve N_2-fixation and yield of the crop.

The pattern showed that all strains from *M. ciceri* and *M. plurifarium* were highly effective and effective on Natoli variety compared to 83% and 71% of the strains that performed the same on Arerti variety. Although almost half of the strains from *M. abyssinicae* and *M. amorphae* nodulated both varieties with effective nodulation, none of the strains from *M. hawasiense* and *M. gobiense* were effective on their hosts. The data also showed that individual strains were not necessarily effective on both varieties, and a strain effective on one variety was ineffective on the other variety (Table 5). For example, strains like *M. ciceri* CPR92 and CPR23 and *M. plurifarium* CPR31 and CPR48 were more effective with Natoli than Arerti host variety; whereas *M. shonense* CPR38 and CPR82 were effective on Arerti but less effective on Natoli variety. That may be due to variations in their symbiotic compatibility between rhizobia and the host genotypes (El Hadi and Elsheikh, 1999; Romdhane *et al*., 2008; Gemuchu Keneni *et al*., 2012; Mohamed and Hassan, 2015).

The symbiotic effectiveness of the *Mesorhizobium* strains on the two host varieties was not significantly different compared to the recent work in Ethiopia. According to Wubayehu Gebremedhin *et al*. (2018), 87% of the rhizobia nodulated with effective fixation on the Desi seed type variety Natoli, compared to 13% of the same isolates that nodulated Kabuli seed type, variety Habru.

The symbiotic data in general showed that *M. plurifarium* and *M. ciceri*, and to some extent *M. amorphae* strains, performed better than the other groups. The combined evaluation of ecological competitiveness (*in vitro*) and the symbiotic effectiveness data shortlisted a total of 12 (50%); five strains from *M. ciceri* (CPR40, CPR61, CPR67, CPR92, CPR118) and four strains from *M. plurifarium* (CPR3, CPR48, CPR99, CPR112), two strains from *M. amorphae* (CPR8, CPR103), and one strain, *M. abyssinicae* 20, that performed better than the other strains. The best ones *M. ciceri* CPR67 and CPR40 and *M. plurifarium* CPR112 and CPR3 even out-performed the commercially available local strain CpNSTC on both plant varieties.

CONCLUSION

The study showed the existence of symbiotically effective naturally occurring chickpea rhizobia in Northern and central parts of Ethiopia, dominated by *Mesorhizobium ciceri* and *Mesorhizobium plurifarium*. They also displayed wide range of eco-physiological tolerance and nutritional versatility that could enable them to persist in the soil and compete with the possible influence of ineffective indigenous rhizobia for nodulation and nitrogen fixation. With a few exception, the dominant *Mesorhizobium ciceri* and *Mesorhizobium plurifarium* performed best irrespective of variety. Four strains: *M. ciceri* CPR67 and CPR40 and *M. plurifarium* CPR112 and CPR3 were superior in their ecological and symbiotic performance and compatibility with both chickpea varieties, and can be recommended as potential commercial inoculants, provided they can be tested in field trials and soil stress conditions to validate their symbiotic effectiveness. Ideally, such rhizobia inoculants could be used on different chickpea seed types, varieties, or cultivars and would be more interesting not only to reduce the financial burden to the inoculant industry, but also to increase the market share for crosscutting inoculants instead of formulating seed type, variety, or cultivar specific rhizobia inoculants.

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