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Phenotypic characterization and symbiotic effectiveness test of chickpea (*Cicer arietinum* L.) rhizobia isolated from Dejen and Aneded Districts, East Gojjam Zone, Amahara Region, Ethiopia

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Chickpea (*Cicer arietinum* L.) is an important leguminous crop grown in different parts of Ethiopia. It is a nutritionally valued and N_2-fixing legume which forms a symbiotic association with *Mesorhizobium*. This study was conducted to characterize and evaluate symbiotic efficacy of chickpea rhizobia isolated from soil samples collected from the study area. Seventeen chickpea rhizobia were isolated by soil host plant trap method and characterized for edaphic stress tolerance. Five of the isolates were grown at high temperature of 45°C and salt concentration of 6%, whereas seven were grown at acidic pH of 4, and four were grown at basic pH of 9. All isolates were not resistant to kanamycin, while fair resistance to erythromycin and streptomycin and modest resistance to ampicillin and azithromycin were observed. Furthermore, most of the isolates showed a variation in nodulation with higher (22 NN/p) and lowest (9 NN/p) scores. Shoot dry weight (SDW) of the plant ranged from 1.18 to 1.84 g/p and isolates showed effective (67%) to highly effective (100%) N_2-fixing performance. From these, four isolates showed multiple edaphic stress resistance and are recognized as promising candidate for chickpea production in stressed soil; however, further study in the filed is required.

Key words: Chickpea, *Rhizobium*, edaphic stress, symbiotic effectiveness.

INTRODUCTION

Chickpea (*Cicer arietinum* L.) is a cool season leguminous crop commonly grown in tropical, subtropical, temperate and semi-arid regions of the world (Miller et al., 2002; Singh et al., 2014). Ethiopia is considered as the center of secondary diversity for chickpea (Van der Maesen, 1987). Chickpea production ranks third among pulse crops grown in the country next to Faba bean (*Vicia faba*) and Field pea (*Pisum sativum*). Spatially, Amhara regional state takes the first share and is considered as a potential chickpea producer with 62% of annual production (IFPRI, 2010). Among the existing chickpea varieties, *Desi* type that was preferably grown in semi-

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arid tropics is the most dominant in Ethiopia (Naser et al., 2008). Chickpea cultivation in the country covers more than 208,388.6 ha of the land (CSA, 2011).

Chickpea is being valued for its high dietary nutrition and serves as an invaluable source of protein and other nutrients for consumers (Shiferaw and Teklewolde, 2007; Mohammed et al., 2011). Chickpea contains 29% protein, 59% carbohydrate, 3% fiber, 5% oil and 4% ash, and it is a good source of absorbable ions like Ca, P, Mg, Fe and K (Christodoulou et al., 2005). Therefore, it is commonly incorporated as part of the different Ethiopian dishes and used for balanced diet.

On the other hand, chickpea serves as cash generating crop in the country with 312,000 tons of annual production and has appreciated export markets (IFPRI, 2010). For instance, in Ethiopia from the 48% of the pulse exported volume, chickpea accounts for about 27% of the total quantity production, while the remaining is used for domestic market and household consumption (Shiferaw and Teklewolde, 2007).

Besides its nutritional quality and source of income, chickpea plays tremendous role in soil fertility by improvement of symbiotic N_2-fixation in association with Mesorhizobia bacteria (Werner, 2005; Funga et al., 2016). Improved soil fertility boosts crop production and maximizes chickpea yield (Jida and Assefa, 2012). The remaining plant biomass in the soil also increases nitrogen pool and serve as a nitrogen source for succeeding crops production by crop rotation cultivation process (Keneni et al., 2011; Beyene et al., 2013). Therefore, symbiotic N_2-fixation of chickpea is economically cost effective and environmentally friendly alternative to benefit farmers and help in sustainable crop production by shift cultivation of crops with limited use of synthetic fertilizer (Tena et al., 2017).

The N_2-fixation efficiency of chickpea infected by *Mesorhizobium* strains was determined by soil edaphic factors (Imran et al., 2015). Thus, the chickpea *Mesorhizobium* which were isolated from the local agro-ecology were expected to infect respective host plants and fix atmospheric nitrogen in a better way (Simon et al., 2014). This is because, indigenous Mesorhizobia are expected to have better adaptation mechanism of the localized soil ecological factors of a given farmland (Beyene et al., 2013). Hence, identifying efficient and superior N_2-fixing *Mesohizobium* strain from the local agro-ecology has paramount importance to enhance chickpea production and improve soil fertility.

Therefore, this study aimed to obtain efficient N_2-fixing Mesorhizobial isolates from chickpea rhizosphere soils and identify potential isolates which could be substitutes of synthetic fertilizer for chickpea cultivation.

MATERIALS AND METHODS

Study site and sample collection

This study was conducted in two purposely selected districts located in East Gojjam Zone, Amahara Regional state, Ethiopia because of their dominant production of chickpea (Dejen and Aneded). From those districts, the most potential chickpea grower kebeles (smaller administrative next to district) were identified during the field survey. In each selected kebele, one chickpea farm was taken as soil sample source. From these chickpea farms, triplicated soil samples were pooled by digging at 20 to 30 cm depth. Composite soil samples were collected using ethanol sterilized (70%) plastic bags in November 2015. Collected composite samples were then taken to Microbiology Laboratory, Department of Biology, Debre Markos University (DMU) for further work.

Table 1. Composition of YEMA medium autoclaved at 121°C for 15 min.

Component	Amounts used
Mannitol	10 g/l
K_2HPO_4	0.5 g/l
$MgSO_4$.$7H_2O$	0.2 g/l
NaCl	0.1 g/l
Yeast extract	0.5 g/l
Agar	15 g/l
Distilled water	1000 ml
pH	7±0.1

Nodule collection

Nodules were collected from chickpea by using soil-plant trap method in pots under greenhouse condition (Vincent, 1970). The chickpea plants grown in the collected soil samples for 45 days were uprooted and large sized, yellow colored nodules were picked and surface-sterilized (Somesagaran and Hoben, 1994).

Rhizobium isolation

Entrapped Mesorhizobia were isolated from the collected nodules after brief surface sterilization using 70% ethanol for 10 s and 5% local bleach for 3 min (Vincent, 1970). Then, treated nodules were rinsed five times by using sterilized water. Sterilized nodules were then crushed and loop full of sap was transferred onto yeast extract mannitol agar (YEMA) containing plates (Table 1). Inoculated plates were incubated in a bacteriological incubator adjusted at a temperature of 28°C for 3-5 days (Vincent, 1970). After growth, a single colony was picked up and purified periodically by re-streaking method on the fresh YEMA medium. Then, pure isolates were preserved on YEMA slants containing 0.3% (w/v) $CaCO_3$ and stored in refrigerator adjusted at a temperature of 4°C (Vincent, 1970).

Authentication of the isolates

Isolates infectivity of *Desi* types of chickpea was confirmed by inoculating them onto plant seedlings. Activated isolates were inoculated onto chickpea seedlings planted on sand filed plastic pots and allowed to grow for 45 days in the greenhouse. After 45 days of the growth, plant were uprooted and the existence of nodule were checked.

Characterization of the Isolates

All isolates were checked on YEMA medium containing 25 µg ml^{-1}

Congo red to evaluate their ability to absorb the dye. In addition, isolates were inoculated on medium containing 25 µg ml^{-1} bromothymol blue (BTB) to determine their ability to produce acid or base and color change of medium were observed (Lupwayi and Haque, 1994). Furthermore, appearance, color and size of the grown colonies were examined on YEMA plates.

Physiological characterization of isolates

All tests were carried out three times on YEMA plates and compared with control. The isolates growth was qualitativlly determined and recorded as (+) for growth, (±) for limited growth and (-) for no growth.

Salt tolerance

Isolates were tested for their salinity tolerance using YEMA medium supplemented with NaCl at concentrations of 0.1, 0.3, 0.5, 0.8, 1, 2, 3, 4, 5 and 6% (w/v) (Belay and Assfa, 2011).

Temperature tolerance

The ability of isolates growth at high and low temperatures were monitored using YEMA medium incubated at 5, 10, 15, 35, 40 and 45°C (Jida and Assefa, 2012).

pH tolerance

Isolates acid and alkaline tolerance were evaluated by growing them on the medium where pH was adjusted to 4, 4.5, 5, 5.5, 8 and 9 using sterile HCl and NaOH (Belay and Assefa, 2011).

Carbohydrates utilization by the isolates

Carbohydrate utilization by isolates was determined using the methods described by Somasegaran and Hoben (1994) on six carbohydrates. These carbohydrates were prepared as 10% (w/v) solution in water. Carbohydrate free medium, which is essentially similar to YEMA medium were modified by reducing yeast extract to 0.05 g/L. Heat-labile carbohydrate solutions were sterilized by membrane filtration method using Millipore with a pore size of 0.22 µm and added to the autoclaved basal medium. The heat-stable carbohydrates were autoclaved together with the medium. YEMA medium without carbon source and with mannitol was used as negative and positive controls, respectively.

Intrinsic antibiotic resistance (IAR)

The intrinsic antibiotic resistance of isolates was determined using some selected antibiotics. The tested antibiotics were ampicillin, streptomycin, kanamycin, erythromycin, azithromycin and chloramphenicol. These antibiotics were incorporated into YEMA medium after membrane filter sterilization using 0.22 µm size at the concentration of 2.5, 5 and 10 µg/ml (Beynon and Josey, 1980). Then, the isolates growth and failure were recorded.

Evaluation of isolates N_2-fixation effectiveness

The effectiveness of isolates was tested in a pot experiment conducted in greenhouse condition. 3 kg of carefully washed, sieved and HCl acid sterilized river sand were filled with alcohol-sterilized (70%) plastic pots. Chickpea seeds of uniform size and color were surface sterilized as described before and transferred to 0.75% (w/v) of water agar plates and allowed to germinate at 25°C for 3 days. Four chickpea seedlings were transferred into each pot, which were later thinned down to three. Each isolates grown in YEMA broth medium to logarithmic phase were adjusted to 10^9 cells ml^{-1}. Activated 1 ml of isolates were inoculated onto each seedling (1 ml/seedling) of the sand culture. The experiment set up was a complete randomized design (CRD) with three replicates. A plus –N with no inoculation and a non-inoculated with no N were used as the controls. The plus control contains 70 mg/L of N applied as a 0.05% KNO_3 (w/v) solution every week (Somasegaran and Hoben, 1994). Plants were supplied with tap water every two days and fertilized once a week with the quarter strength of N-free nutrient solution (Belay and Assefa, 2011). Plants growth were carried out in a greenhouse with a 12/12 h light/dark cycle. Finally, after 45 days of growth, all plants were harvested and the roots were scored for nodulation. The top plants and nodules were oven dried at 70°C for 48 h to determine the dry weight.

The percentage of isolates symbiotic effectiveness were calculated using equation proposed by Date et al. (1993) and indicated in Belay and Assefa (2011) with N_2-fixing effectiveness classified as ineffective <35%; lowly-effective, 35 to 50%; effective, 50 to 80%; and highly effective, >80%.

$$\text{SE}\ (\%) = \frac{\text{SDW of inoculated plants}}{\text{SDW of N}-\text{fertilized plants}} \times 100\%$$

SE = Symbiotic effectiveness; SDW = shoot dry weight.

Data analysis

Data analysis was done using one way analysis of variance (ANOVA) using version 20 SPSS statistical program. Mean separation was calculated using Tukey's HSD test when the value was significant at p = 0.05.

RESULTS AND DISCUSSION

A total of 17 chickpea bacteria were recovered from the rhizospheric soil collected from chickpea farms of two purposely selected Districts (Dejen and Aneded) by using soil-host plant trap method. All the isolates were authenticated as chickpea rhizobia by re-inoculation test using sterilized sand-filled pot experiment.

Colony characteristics and dyes absorption ability of the isolates are summarized in Table 2. After 72 h of growth on YEMA medium, colonies were found to be large in size (3.0 to 5.0 mm), diameter, and showed large mucoid, watery, flattened and raised appearance similar to the findings obtained by Singh and Bamania (2012). Most of them were colorless and transparent, while some became yellowish after 3 days of the growth. The staining experiment also confirmed that all bacterial cell wall were stained as pink as the color of safranin and grouped under Grahams' negative category (Agrawal et al., 2012). Furthermore, isolates showed considerable diversity on bromothymol blue (BTB) color conversion after 48 h of the growth. The isolates changed YEMA-BTB medium to yellow and deep yellow were categorized as fast growing

Table 2. Sample site, colony morphology and dye absorbance of isolates.

Sample collected kebele	Designated isolate	Colonies morphological characteristics				YEMA-BTB test	YEMA-CR test	Graham reaction
		Colony size (mm)	Colony appearance	Colony structure	Colony color	25 µg/ml	25 µg/ml	
Tike	DMU-1	3.0	D	F	CL	MY	NA	-ve
Yetnora	DMU-2	3.0	LM	R	Y	MY	NA	-ve
Terch	DMU-3	1.5	LM	R	CL	Y	NA	-ve
Konkoy	DMU-4	2.5	LW	F	CL	DY	A	-ve
Koncher	DMU-5	1.5	LM	R	Y	DY	A	-ve
Koncher	DMU-6	5.0	LW	R	CL	VLY	A	-ve
Enajima	DMU-7	3.2	LW	F	CL	N	A	-ve
Gudalima	DMU-8	5.0	LW	R	CL	DY	A	-ve
Sebshengo	DMU-9	4.0	LW	F	CL	MY	A	-ve
Denbukebay	DMU-10	3.0	D	F	CL	MY	A	-ve
Zemetin	DMU-11	2.0	LM	R	CL	DY	A	-ve
Denbukebay	DMU-12	4.5	LW	F	CL	Y	A	-ve
Gudalima	DMU-13	5.2	LW	F	CL	Y	A	-ve
Yetnora	DMU-14	5.3	LW	F	CL	MY	A	-ve
Terch	DMU-15	3.5	LW	F	Y	DY	A	-ve
Zemetin	DMU-16	3.0	LM	R	Y	DY	A	-ve
Sebshengo	DMU-17	4.5	LM	R	CL	MY	LA	-ve

D, Dry; LM, large mucoid; LW, large watery; R, raised; F, flatten; Y, yellow; CL, color less; MY, moderately yellow; DY, deep yellow; VLY, very less yellow; N, not changed; NA, not absorbed; A, absorbed; -ve, negative.

and others which changed to moderate yellow and did not show any color change were considered as a slow growing rhizobia. Chickpea rhizobia was reported to have both fast and slow growing strains (Nour et al., 1994). Moreover, isolates obtained from chickpea nodules failed to grow on BTB-medium (Wei et al., 2003). Most of the isolates were Congo red dyes absorbent, except the three DMU-1, DMU-2 and DMU-3.

Edaphic condition of the soil is the most determinant factor for successful symbiotic association of *Rhizobium* with their host plants. Temperature, pH, salinity, antibiotic tolerance and carbohydrates utilization are important parameters to characterize rhizobia by consideration as a phenotypic identification marker (Maatallah et al., 2002). Temperature and pH tolerance of isolates is presented in Table 3. Chickpea rhizobia in this study showed a variation in these parameters. Almost all the isolates were grown at a temperature range of 5 to 40°C. Temperature tolerance of chickpea rhizobia ranging from 10 to 42°C has been already reported in India (Rai et al., 2012). Only five (DMU-1, DMU-2, DMU-10, DMU-14 and DMU-15) isolates were tolerant to temperature at 45°C. These isolates were expected to have high temperature resistancy and considered as an important candidate to develop inoculants as a bio-fertilizer. Furthermore, all the isolates grew well at pH range of 4.5 to 8.5 and this report is in line with findings of Kucuk et al. (2006), Baoling et al. (2007) and Singh and Bamania (2012).

Most importantly, eight isolates (DMU-3, DMU-6, DMU-8, DMU-9, DMU-11, DMU-13 and DMU-17) showed their acidity tolerance by growing at pH 4. These isolates were considered as fast-growing strains as recognized from BTB-medium dyes conversion test and important candidate for acidic soil. This report is in agreement with findings of Gao et al. (1994) that showed that the rhizobia grown at pH as low as 4 were grouped under fast-growing strains whereas, four isolates such as DMU-6, DMU-7, DMU- 10 and DMU-14 were grown at pH 9. Some chickpea rhizobial isolates grew very well at pH 10 and tolerance to alkalinity increased at pH 11 (Singh et al., 2015). These alkaline condition preferring rhizobia were reported as slow-growing strains (Anand and Dogra, 1991). However, a number of reports indicated complete growth failures of chickpea rhizobia at pH of 9 (Kucuk et al., 2006; Baoling et al., 2007; Singh and Bamania, 2012). In this study, isolates showed edaphic factor tolerance diversity and similar with findings of Rai et al. (2012). Salinity test result also showed bacterial diversity towards different concentrations of the salt (Table 4). All the isolates were grown on the medium containing NaCl salt concentration ranging from 0.1 to 2%. Some isolates tolerated salt concentration upto 4%, while only a few isolates were grown at 5 and 6% of salt concentration. Most isolates are reported not to grow from 5% NaCl concentration and salt tolerance ability reduced with increase in salt concentration (Saraf and

Table 3. Evaluation of temperature and pH tolerance of the isolates.

Isolate	Temperature test (°C)						pH test					
	5	10	15	35	40	45	4	4.5	5	5.5	8.5	9
DMU-1	+	+	+	+	+	+	-	+	+	+	±	-
DMU-2	+	+	+	+	+	+	-	+	+	+	+	-
DMU-3	+	+	+	+	+	-	+	+	+	+	+	-
DMU-4	+	+	+	+	+	-	-	+	+	+	+	-
DMU-5	+	+	+	+	+	-	-	+	+	+	+	-
DMU-6	+	+	+	+	+	-	+	+	+	+	+	+
DMU-7	+	+	+	+	+	-	-	+	+	+	+	+
DMU-8	+	+	+	+	+	-	+	+	+	+	+	-
DMU-9	+	+	+	+	+	-	+	+	+	+	+	-
DMU-10	+	+	+	+	+	+	-	+	+	+	+	+
DMU-11	+	+	+	+	+	-	+	+	+	+	+	-
DMU-12	+	+	+	+	+	-	-	+	+	+	+	-
DMU-13	-	-	+	+	+	-	+	+	+	+	+	-
DMU-14	+	+	+	+	+	+	-	+	+	+	+	+
DMU-15	+	+	+	+	+	+	-	+	+	+	+	-
DMU-16	+	+	+	+	+	-	-	+	+	+	+	-
DMU-17	+	+	+	+	+	-	+	+	+	+	+	-

+, Growth of isolates; -, nongrowth of isolates; ±, growth of very few colonies.

Table 4. Salt tolerance and carbohydrates utilization test of isolates

Isolate	Salt concentration (%)										Carbohydrate utilization test						
	0.1	0.3	0.5	0.8	1	2	3	4	5	6	Fructose	Dextrose	Dextrin	Lactose	Maltose	Sucrose	Control
DMU-1	+	+	+	+	+	+	-	-	-	-	-	+	+	+	±	-	+
DMU-2	+	+	+	+	±	±	-	-	-	-	-	+	+	+	+	-	+
DMU-3	+	+	+	+	+	+	+	+	±	-	-	+	+	+	+	-	+
DMU-4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
DMU-5	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+
DMU-6	+	+	+	+	+	+	±	±	-	-	-	+	+	+	+	+	+
DMU-7	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+
DMU-8	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	±	+
DMU-9	+	+	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+
DMU-10	+	+	+	+	+	+	-	-	-	-	-	+	+	+	+	-	+
DMU-11	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	+
DMU-12	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+	+	+
DMU-13	+	+	+	+	+	±	-	-	-	-	+	+	+	+	+	+	+
DMU-14	+	+	+	+	+	+	±	±	±	-	+	+	+	+	+	+	+
DMU-15	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
DMU-16	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
DMU-17	+	+	+	+	+	±	-	-	-	-	+	+	+	+	+	+	+

+, Growth of isolates; -, nongrowth of isolates; ±, growth of very few colonies.

Dhandhukia, 2005; Singh and Bamania, 2012).

The isolates grown at 5 and 6% NaCl concentration were considered as salt tolerate and expected to have better adaptability to salty soil conditions. Therefore, isolates designated as DMU-4, DMU-5, DMU-11, DMU-15 and DMU-16 were grouped as salt tolerant rhizobial groups in this study.

Similarly, the salt tolerant isolates were better utilized

Table 5. Intrinsic antibiotic resistance test of the isolates.

Isolates	Antibiotics concentration range (µg)																	
	Ampicillin			Chromaphynicol			Erythromycin			Streptomycin			Azithromycin			Kanamycin		
	2.5	5	10	2.5	5	10	2.5	5	10	2.5	5	10	2.5	5	10	2.5	5	10
DMU-1	-	-	-	-	-	-	-	-	-	+	+	-	+	+	+	-	-	-
DMU-2	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
DMU-3	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
DMU-4	+	+	+	+	-	-	+	+	+	-	-	-	+	+	+	-	-	-
DMU-5	+	+	+	-	-	-	+	+	+	+	-	-	+	+	+	-	-	-
DMU-6	+	+	+	-	-	-	+	+	-	-	-	-	+	+	+	-	-	-
DMU-7	+	+	+	+	-	-	+	+	-	+	-	-	+	+	+	-	-	-
DMU-8	+	+	+	-	-	-	+	+	-	-	-	-	+	+	-	-	-	-
DMU-9	+	+	+	-	-	-	+	+	-	+	+	+	_	-	-	-	-	-
DMU-10	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-
DMU-11	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-
DMU-12	+	+	+	-	-	-	+	+	-	+	+	-	+	+	+	-	-	-
DMU-13	+	+	+	-	-	-	+	+	-	+	+	-	+	+	-	-	-	-
DMU-14	+	+	+	-	-	-	+	+	-	+	+	-	+	+	+	-	-	-
DMU-15	+	+	+	+	-	-	+	+	+	+	-	-	+	+	+	-	-	-
DMU-16	+	+	+	+	-	-	+	+	-	+	+	-	+	+	+	-	-	-
DMU-17	+	+	+	-	-	-	+	+	-	-	-	-	+	+	+	-	-	-

+, Growth of isolates; -, nongrowth of isolates.

among all tested carbohydrates. Only isolates DMU-5 and DMU-11 failed to grow on the disaccharide sugar, fructose, although tolerant to high salt concentration (Table 4). All the isolates were grown on dextrose, dextrin, lactose and maltose; however, isolates DMU-1, DMU-2, DMU-3, DMU-5, DMU-6, DMU-10 and DMU-11 failed to grow on the fructose and sucrose. With regards to this, there is well-established fact on *Rhizobium* utilization of various carbon sources for their growth and this is used as important tool to characterize the isolates (Maatallah et al., 2002). Rhizobial strains isolated from chickpea nodules were reported to utilize mannitol, lactose, sucrose, sorbitol, arabinose, galactose, mannose, maltose and raffinose as carbon sources (Singh and Bamania, 2012). Especially, fast-growing rhizobia were broadly recognized to grow on several types of carbon substrates, whereas slow growing rhizobia were grown only on very limited types of carbon sources. However, in this study, almost all isolates were grown on the tested carbohydrates and a broad range of carbohydrates was used as sources of carbon for growth. Hence in this regard, the result of this study is in line with the results of other studies (L'taief et al., 2007; Jida and Assefa, 2012). It is very interesting to note that chickpea *Mesorhizobium* can utilize a broad spectrum of carbohydrates for their cell growth and development. Such characteristics are usually used as diagnostic features for root nodule bacteria to test different carbon sources for their survival (Kucuk and Kıvanc, 2008).

On the other hand, as summarized in the Table 5, majority of the isolates failed to tolerant several types of antibiotics in different concentrations sspecially isolates that were completely susceptible to kanamycin and only a few isolates were tolerant to chloramphenicol at 2.5 μgl^{-1} concentration. However, most of the isolates were grown on different concentrations of Ampicillin, Erythromycin and Azithromycin antibiotics. Isolates grown on the Streptomycin showed very less tolerance with respect to concentration. Several studies reported the existence of broad variation among chickpea rhizobia with respect to the fate of their intrinsic antibiotics resistance (Maâtallah et al., 2002; Kucuk and Kıvanc, 2008). The isolates' sensitivity to antibiotics may be due to inability to resist exposed toxicity with less adaptation in natural environments (Singh and Bamania, 2012).

The legume food crop production was expected to be boosted by use of indigenous rhizobia as biofertilizer to supplement nitrogen requirement for cultivation of plants. Symbiotic effectiveness test was carried out to select the best N_2-fixing strains among the obtained isolates (Table 6). In this greenhouse experiment, all the 17 isolates showed variation in host plant nodulation, with scores lesser (9 N/p) up to higher (22 N/p) nodules per plant. Although, plants showed a variation on nodulation, N_2-fixation efficiency were found within effective up to highly effective ranges. Especially, shoot dry weight value was proved and considered as a direct indicator of isolates' N_2-fixation efficiency. Furthermore, this study showed the maximum (1.84 g/p) and minimum (1.18 g/p) shoot dry mass. For instance, isolate DMU-5, that scored high

Table 6. Symbiotic effectiveness evaluation of isolates in the greenhouse condition.

Isolates	NN p^{-1}	NDW g p^{-1}	SFW g p^{-1}	SDW g p^{-1}	NFE (%)	Score
DMU-1	15^{c}	0.102^{a}	5.85^{c}	1.30^{d}	74	E
DMU-2	22^{a}	0.112^{a}	7.17^{ab}	1.54^{b}	88	HE
DMU-3	17^{b}	0.072^{c}	7.39^{ab}	1.54^{b}	88	HE
DMU-4	14^{c}	0.091^{ab}	7.12^{ab}	1.69^{ab}	97	HE
DMU-5	21^{a}	0.093^{ab}	8.31^{a}	1.84^{a}	100	HE
DMU-6	17^{b}	0.056^{f}	5.95^{bc}	1.48^{bc}	85	HE
DMU-7	15^{c}	0.062^{e}	8.33^{a}	1.74^{a}	99	HE
DMU-8	12^{d}	0.075^{c}	7.07^{abc}	1.49^{bc}	85	HE
DMU-9	16^{c}	0.103^{a}	6.92^{b}	1.46^{c}	83	HE
DMU-10	12^{d}	0.054^{f}	4.79^{d}	1.32^{d}	75	E
DMU-11	11^{de}	0.062^{e}	5.28^{cd}	1.22^{de}	70	E
DMU-12	14^{c}	0.095^{ab}	6.63^{b}	1.47^{bc}	84	HE
DMU-13	10^{e}	0.082^{b}	6.23^{b}	1.39^{cd}	79	E
DMU-14	9^{e}	0.076^{c}	4.61^{d}	1.37^{cd}	78	E
DMU-15	12^{d}	0.097^{ab}	4.02^{de}	1.18^{e}	67	E
DMU-16	14^{c}	0.068^{d}	5.31^{c}	1.38^{d}	79	E
DMU-17	13^{d}	0.082^{b}	5.21^{cd}	1.40^{cd}	80	HE
Control N+			8.33^{d}	1.75^{a}		
Control N-			3.21^{e}	1.15^{f}		

NN p^{-1}, Nodule number per plants; NDW p^{-1}, nodule dry weight per plant; SFW p^{-1}, shoot fresh weight per plant; SDW p^{-1}, shoot dry weight per plant; NFE, N_2, fixation effectiveness; E, effective; HE, highly effective.

SDW of 1.84 g/p was highly effective (100%) and isolate, DMU-7 that scored SDW of 1.74 g/p was very effective (70%) on N_2-fixation performance. Nitrogen fixation performance was positively associated with plant SDW (Qureshi et al., 2013). Nine isolates, namely DMU-2, DMU-3, DMU-4, DMU-5, DMU-6, DMU-7, DMU-8, DMU-9, and DMU-12 were potential N_2-fixing isolates with highly effective (85 - 100%) fixation performance. The other isolates showed moderate percentage of N_2-fixation performance variation. Such variation in each evaluated parameters were expected to depend on the chickpea bacterial diversity in the soil (Sahgal and Johri, 2003).

Some of the isolates that showed two or more environmental stress tolerance has attracted the interest of investigators in this study. Especially, four isolates such as DMU-6, DMU-10, DMU-14, and DMU-15 showed multiple abiotic stress tolerance, namely high temperature, pH and salinity, with scoped effective (67 to 78%) to highly effective (85%) performance of N_2-fixation (Table 6).

Similarly, there are chickpea rhizobia which were reported from the alkaline condition of Indian soil (Singh et al., 2015). Therefore, these isolates were expected to be used as bio-fertilizer inocula in future, particularly in stressed farmlands to boost chickpea production and improve soil fertility. Inoculation with bacterial bio-fertilizer to farmland to improve crop production is not sole target rather it can have greate role to reduce the application of synthetic nitrogenous fertilizer on the farmland there by reducing pollution (Kennedy et al., 2004; Mia and Shamsuddin, 2010). However, biological N_2-fixation (BNF) use has an incredible role in substituting commercially synthetic N-fertilizer in cereal production thereby, reducing the environmental problem (Agrawal et al., 2012). The dependency and high amounts of synthetic fertilizers application are both costly for farmers and set the hazardous problem on nature and biodiversity. Therefore, BNF provides a better alternative to chemical fertilizers as the process, besides supplying nitrogen to crop, enriches soil nitrogen content and maintains soil health and productivity (Reddy and Reddy, 2004).

Conclusion

As shown in the study, chickpea rhizobia isolated from rhizosphere soil showed variation in agro-ecological stresses tolerance. These isolates which were tolerant to edaphic stresses could be the potential asset for an alternative source of environmentally friendly bio-fertilizer and potential resources for varied agro-ecology. Isolates from this study showed sounding tolerance to temperature, pH and salinity and could have potential to tolerate environmental toxicity and hence increase N_2-fixation effectiveness to enhance soil fertility in chickpea farming, thus increasing chickpea production. On the other hand, sensitive strains are least in tolerance to environmental toxicity and hence may not improve

chickpea production. From this study, it could be deduced that nodulation performance of the rhizobia strain is positively correlated to N_2-fixing effectiveness as well as higher shoots dry weight which confirms high assimilation of nitrogen of the chickpea seedlings. The isolates achievement on the N_2-fixing process is very high and had better nodulation, and effective to highly effective fixation performances. Thus, this confirms the presence of potentially efficient chickpea rhizobia candidates in the rhizospheric soil of the study area although further work on filed condintion is needed.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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