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Research Article

Morphological Variation and Basic Characteristics of Selected Indigenous *Rhizobia* Isolated from Major Chickpea (*Cicer arietinum L.*) Growing in Regions of Ethiopia

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ABSTRACT

Chickpea *(Cicer arietinum L.)* is a major legume crop in Ethiopia and provide multiple benefits, due to high nutritive value as well as the ability of the crop to enrich nitrogen poor soils due to biological nitrogen fixation with different *Rhizobia* isolates. However, the effectiveness of the isolates varies due to inherent feature, stress tolerance and substrate utilization characteristics of the isolates. This necessitates the screening of the basic properties of the isolates under *in vitro* laboratory conditions. To this effect, 15 indigenous isolates from chickpea growing regions were tested for *in vitro* basic features, stress tolerance and substrate utilization properties. There were variations in morphological features, stress tolerance and nutritional diversity among isolates. The isolates also formed colony with circular shape, entire margin, white large creamy mucoid to watery small creamy mucoid texture. Chickpea isolates showed broad range (0-100%) salt tolerance to different NaCl concentrations. The isolates grown in moderately acidic pH 4.5 to alkaline pH 7.5 ranging from (25-50%). Subsequently the isolates were grown at optimum temperature up to 37°C level range from (25-100%). The isolates were more tolerant to the tested antibiotics (0-75%) and resistance to the heavy metal (0-100%). In addition, chickpea isolates better utilized the carbohydrates (0-100%) and similarly, the amino acids (25-75%). All taken together, the data provided an important complement to select representative isolates competitive in the soil which is a one of the desirable characteristics for inoculant isolates selection for effective nitrogen fixation.

Keywords: Chickpea; Characteristics; Isolate; Morphology; *Rhizobia*

INTRODUCTION

Chickpea is one of the most important pulse crops used for food and feed in more than 60 countries worldwide mostly in the semi-arid tropics of sub-Saharan Africa and South Asia [1] and contributes to more than 20% of world pulse production [2]. It is integrated in soil cropping systems in the tropics for soil fertility management, for it fixes nitrogen with root nodule bacteria of the genus *Mesorhizobium* [3]. It is estimated that chickpea can produce up to 176 kg N ha-1 annually depending on cultivar, bacterial strain, and environmental factors [4]. Although chickpea is considered for long as very host specific to *M. ciceri* and *M. mediterraneum* [5].

The *Mesorhizobium* spp are saprophytic soil microbiota that have to persist and compete with other microorganisms to survive in the soil so as to effectively nodulate their host [6]. Their survival in the soil and their symbiotic performance are influenced by various abiotic factors temperature, soil pH, salinity, nutrients and environmental pollutants such as heavy metals and antibiotic produced by other microorganisms in the soil [7]. Stress tolerance tests and substrate utilization capability *in vitro* characteristics of *rhizobial* isolates is vital to obtain information about the adaptation to the intended soil environment of the *Rhizobia* in the organism's habitat that may beneficially influence plant growth and development of the host plant [8].

This necessitates the primary screening of the isolates to test their tolerance to abiotic stresses such as acidity (pH), salinity (salt) and temperature in the laboratory conditions. Apart from abiotic factors, carbon and nitrogen utilization also showed their *in vitro* ability to metabolize à wide range of respiratory substrates. That will give a more-clear picture on environmental fitness of isolates in the soil for establishing N_2-fixing symbiosis under adverse conditions.

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MATERIALS AND METHODS

Source of isolates and growth conditions

Fifteen test isolates were obtained as part of an earlier study by Greenlon A, et al. [9] and Damtew Z [10] and presented in Table 1. The isolates were kept in 20% (v/v) glycerol stocks at-80°C and retrieved on Yeast Extract Agar (YEMA) Medium at 28°C for 4-5 days for this characterization. Each isolate was characterized by colony appearance, diameter, color and extracellular polysaccharide production. The colonies were characterized as small creamy mucoid, large mucoid and watery less creamy mucoid. Growth rate were assessed by inoculating 1 ml of active rhizobial culture approximately (~109 cells/ml) in 10 ml of YM broth in flask and incubated on shaker for at least 4 to 5 days [11].

Then optical density of each respective isolate was measured at 540 nm using spectrophotometer starting at the time of inoculation and every 6 hours interval. After incubation viable colony forming units were determined through serial dilutions of the culture in sterile water and spread 0.1 ml of each dilution on YMA media in triplicate plate using sterilized glass. Plates contained 20-200 number of colonies were preferred to calculating the numbers of viable colony forming units (cfu) and generation time was calculated from the logarithmic exponential phase of the growth curve [12].

Reaction of *rhizobial* isolates were tested on media supplemented with Congo red at concentration (25 µg/ml). Acid or alkali production test was performed on YEMA-BTB medium (0.5%) bromothymol blue (BTB) indicator [13]. The ability of isolates to produce acid or alkaline in this media were evaluated by observed the color change around growing colonies; fast growing *rhizobial* strains produce acid in this medium, turning the medium yellow and slow growing *Rhizobia* produce alkali, which turns the medium blue. The isolates were also tested for growth on peptone glucose medium [14].

Tolerance of the isolates to salinity was tested on YEAM medium supplemented with, 1, 2 and 3 % (w/v) NaCl and that of acidity/alkalinity on the same medium adjusted to pH of 4.5, 5 and 7.5. They were also grown on the same medium incubated at 25, 30 and 35°C to evaluate their tolerance to heat [15]. The intrinsic antibiotic resistance (IAR) of the strains was performed according [16]. The antibiotics were prepared at different concentrations (µg/ml); Ampicillin (10), penicillin (10), kanamycin (15) and Rifampicine (10). Resistance to heavy metal was tested on YEMA medium containing; $K_2Cr_2O_7$ 50, $MnCl_2$ 50, $AlK(SO_4)_3$ 25 and $MnSO_4$ 75 (µg/ml) of water by Maatallah J, et al. [16].

The ability of strains to utilize different carbohydrates (1% (w/v) as the sole carbon source was tested on basal media containing (g/l); K_2HPO_4 (1), KH_2PO_4 (1), $FeCl_3.6H_2$ (0.01), $MgSO_4.7H_2O$ (0.2), $CaCl_2$ (0.1; $(NH_4)_2SO_4$ (1) and agar (15). Carbon sources such as galactose, maltose, lactose and raffinose were used. Likewise, methionine, tyrosine, thiamine and riboflavin as nitrogen substrates were tested on the same basal medium after replacing ammonium sulfate (1g/l) and reducing mannitol to a final concentration of 0.5 % (w/v) according to Amarger N, et al. [17].

RESULTS AND DISCUSSION

Morphology and related growth characteristics

The selected tested isolates exhibited colony diameter ranges from 1.5- 4.1 mm and the largest diameter development at isolate 29P4S2-a and 87P1S1were more pronounced (Table 2). The isolates illustrate gram negative nature which ensures that isolates free from gram positive bacteria. The isolates also formed colony with circular shape, entire margin, mobile motility, white large creamy mucoid to watery small creamy mucoid texture with different level of mucus production. Which conform the typical feature of Mesorhizobium isolates characteristics (Table 2) (Figure 1).

All isolates changed the color of YEMA supplemented with BTB to yellow indicating that they are acid producers [18]. The CR absorption test also indicated that none of the isolates absorbed CR in YEMA plates; this is distinctive character of rhizobia with only few exceptions [11]. On the other hand, none of the tested isolates manage to grow on PGA plates.

The isolates revealed doubling time ranging from 1.5 h to 3.4 h. The isolate 13P3S2 and ET1were typically grown faster with mean generation time (1.2, 1.6h) respectively, whereas 76P3S1 were found to be moderately fast grower with mean generation time (1.6 h). Isolate 20P2S1 and ET13 had intermidiate growth rate.

Table1: List of isolates and regions of culture collection.

No	Isolates	Region	Latitude	Longitude	Elevation	Soil pH
1	ET1	Amhara, South Gonder	11°27'58.3"N	38°12'46.6"E	2795	6.9
2	ET3	Oromia, East Shewa	8°49'31.7"N	38°59'25.4"E	1943	6.7
3	56P2S1	Amhara, South Gonder	11°55'9.3"N	37°52'36.9"E	1995	5.4
4	29P4S2-a	Amhara, East Gojam	10°42'47.3"	38°10'30.6"E	2541	6.4
5	20P2S1	Oromia, West Shewa	8°39'21.1"N	38°29'2.8"E	2207	7.3
6	ET13	Amhara, North Gondar	12°11'15.4"N	37°40'24.8"E	1889	7.0
7	58P2S1	Amhara, South Gonder	11°29'16.6"N	38°12'45.8"E	2870	6.2
8	13P3S2	Oromia, West Shewa	8°46'10.9"N	38°39'5.6"E	2231	7.5
9	76P3S1	Amhara, North Gondar	12°20'59.4"N	37°11'57.9"E	1808	7.8
10	68P1S1	Amhara, North Gondar	12°26'13.2"N	37°30'25.3"E	1930	6.8
11	87P1S1	Oromia, West Shewa	8°43'6.1"N	38°15'53.5"E	2127	6.3
12	ET7	Amhara, North Gondar	12°28'35.9"N	37°22'57.5"E	1988	7.4
13	ET19	Amhara, South Gonder	12°5'20.4"N	37°45'17.9"E	1865	6.4
14	42P3S1-a	Amhara, North Gondar	12°28'35.9"N	37°22'57.5"E	1988	5.8
15	ET14	Amhara, North Gondar	12°11'14.7"N	37°40'24.8"E	1888	6.4

Table2: General colony growth characteristic of 15 rhizobia isolates from major chickpea growing regions of Ethiopia.

No	Isolates	Colony size (mm)	Colony appearance	Shape	BTB reaction	MGT (hrs)	Peptone glucose
1	ET1	2.4	Large creamy mucoid	Domed	Yellow	1.5	NG
2	ET3	2.7	Large creamy mucoid	Domed	Yellow	2.0	NG
3	56P2S1	2.2	Small creamy mucoid	Domed	Blue	2.5	NG
4	29P4S2-a	4.1	large creamy mucoid	Domed	Yellow	3.4	NG
5	20P2S1	1.5	Small creamy mucoid	Flat	Yellow	1.7	NG
6	ET13	2.0	Waterless creamy mucoid	Domed	Yellow	1.7	NG
7	58P2S1	2.1	Small creamy mucoid	Conical	Yellow	3.2	NG
8	13P3S2	1.5	Small creamy mucoid	Domed	Blue	1.2	NG
9	76P3S1	1.5	Milky large mucoid	Domed	Blue	1.6	NG
10	68P1S1	1.6	Large creamy mucoid	Flat	Yellow	3.3	NG
11	87P1S1	3.5	Large creamy mucoid	Conical	Yellow	2.1	NG
12	ET7	2.0	Milky less mucoid	Domed	Yellow	3.4	NG
13	ET19	2.8	Large creamy mucoid	Flat	Blue	1.9	NG
14	42P3S1-a	1.8	Small creamy mucoid	Domed	Yellow	2.8	NG
15	ET14	1.6	Small creamy mucoid	Domed	Yellow	2.2	NG

Stands for: MGT=Mean Generation Time; NG=No Growth.

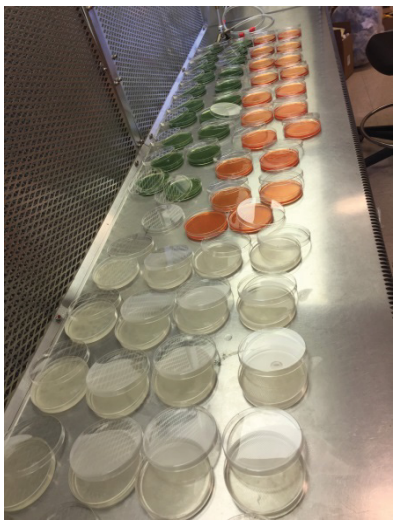
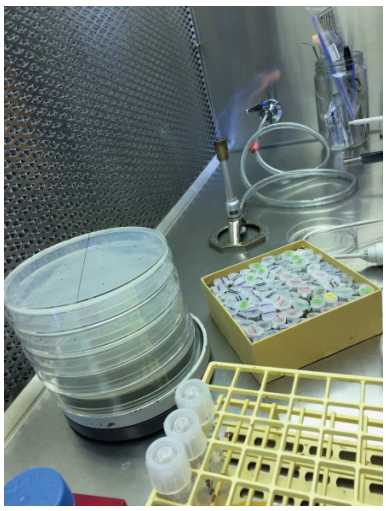
(A)   (B)

Figure 1: (a) Size, Shape, appearance and gram reaction and (b) biochemical characteristics.

All isolates revealed none absorbance of Congo red and did not grow on Peptone glucose agar with Bromocresol Purple, that was an indicative of *rhizobia* characteristics. Most of the isolates changed the colour of YMA medium supplemented with BTB to yellow and four isolates displayed blue that predicts the ability of the strains in acid or alkali production.

Most of the different isolates showed similar growth pattern to previous study in chickpea *rhizobia*. Thus, the report of Romdhane SB, et al. [19] described that all of chickpea nodulating *rhizobia* isolates from Tunisia showed the same colony morphology and growth rate on YMA medium. Whereas on other study *Mesorhizobium ciceri* [3] were found specific chickpea symbionts distinguished as slow growing *rhizobia*. According to Sharma A, et al. [20] the isolates which produced yellow colour colonies would have been classified as acid producer/fast growers, whereas isolates produced blue colour colonies indicated alkali producer/slow growers.

Most isolates (87%) were able to grow at 1% NaCl concentration, whereas 73% and 60% of the isolates were tolerant to 1%,2% and 4% NaCl, respectively (Table 3). A previous study in Portugal showed that Chickpea isolates showed a better growth with 1.5% NaCl, but inhibited at 3% NaCl concentrations [21]. More isolates grew at pH 7.5 (93%) than at pH 5 (66%), but fewer strains were tolerant to pH 4.5 (33%) (Table 3). Other studies showed that chickpea *rhizobia* were tolerant to pH 5 to 9.5 [22]. About 40% of the isolates grew at optimum temperature up to 35°C, while 80% were tolerated 25°C. Chickpea *Mesorhizobia* tolerant to different temperature levels between 15°C, 37°C were isolated elsewhere [23].

Most isolates were resistant to high kanamycin (80%), Rifampicine (60%) and Ampicillin (11%) (Table 3). Majority of the isolates exhibited (66%) resistance to the heavy metal $MnSO_4$, but fewer isolates were tolerant $K_2Cr_2O_7$ (53%), followed by $MnCl_2$ (46%). Muletta D and Assefa F [24] reported that most Ethiopian chickpea *rhizobia* isolate were resistant to common antibiotic and heavy metals. The pattern of utilization of carbon substrates showed that most isolates (60%) were able to grow using galactose as carbon sources; followed by a number of isolates (66%, 45%) utilizing

Table3: Differentiation of stress tolerance, carbon and nitrogen substrate utilization pattern of isolates.

Characteristics	ET1	ET3	56P2S1	29P4S2-a	20P2S1	ET13	58P2S1	13P3S2	76P3S1	68P1S1	87P1S1	ET7	ET19	42P3S1-a	ET14	Total (%)
Salt Tolerance																
1%	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	87
2%	+	+	-	-	+	-	-	+	+	+	+	+	+	+	+	73
3%	+	+	-	-	+	+	+	-	-	-	-	+	+	+	+	60
pH Tolerance																
pH 4.5	-	-	-	+	-	+	-	+	-	-	+	-	+	-	-	33
pH 5.0	+	+	+	-	+	-	-	-	+	+	+	+	-	+	+	66
pH 7.5	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	93
Temperature																
25°C	+	+	+	+	+	+	+	+	-	-	+	+	+	+	-	80
30°C	+	-	+	-	+	-	-	+	+	+	+	+	+	+	+	73
35°C	-	+	+	-	+	-	+		-	+	-	-	-	-	+	40
Antibiotics																
kanamycin	+	+	+	+	-	+	+	+	+	-	+	+	+	+	-	80
Rifampicine	-	-	+	-	+	-	+	+	+	-	+	+	+	+	-	60
Penicillin	+	+	-	+	+	+	-	-	-	+	-	-	-	-	-	40
Ampicillin	-	-	-	-	-	-	+	-	-	+	-	+	-	-	-	11
Heavy Metals																
$MnSO_4$	+	+	-	+	+	+	-	+	-	+	+	+	-	-	+	66
$K_2Cr_2O_7$	+	-	-	+	+	+	+	+	-	+	-	-	-	+	-	53
$MnCl_2$	+	-	-	-	-	-	+	-	-	-	+	+	+	+	+	46
$AlK(SO_4)_3$	+	+	-	-	-	+	-	-	-	-	+	-	-	-	-	26
Carbohydrate	100	75	50	0	75	100	75	75	0	75	75	75	50	50	25	
Amino acid	50	50	50	25	25	25	25	75	25	50	50	50	50	50	50	

maltose and lactose, respectively cellulose (data not shown). Similarly, the majority of isolates better utilized the amino acids methionine (60%) and tyrosine (53%). Other studies showed chickpea isolates displayed high utilization of carbon and nitrogen substrates [16,24].

CONCLUSION

The study showed the presence of a predominant chickpea *Rhizobia* accompanying tolerance to various stress conditions and nutritional versatility. Isolates such as; ET1 and ET3 displayed wide range of stress tolerance and versatile substrates utilization. In general, the tested isolates showed dominance stress tolerance, carbon and nitrogen assimilation features, inherent antibiotic and heavy metal resistance. This might provide a greater tolerance to adverse environments and compete against the possible influence of ineffective indigenous *Rhizobia* for nodulation and nitrogen fixation. Thus, the results of this study highlighted the potential of such isolates to further evaluate at green house and wide environment field trials for the selection of elite inoculant strains that could presumably enable to applied under different soil and environmental conditions.

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