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Symbiotic and phenotypic characterization of *Rhizobium* isolates nodulating fenugreek (*Trigonellafoenum-graecum* L.) from North and East Shewa, Ethiopia

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Abstract

Fenugreek (*Trigonella foenum-graecum L.*), a crop with a long history in Ethiopian agriculture, is known to form a symbiosis with nitrogen-fixing bacteria (*Rhizobia*). In the current study, *Rhizobium* bacteria nodulating fenugreek in the East and North Shewa of Ethiopia, where the plant is commonly grown, were collected and characterized. All isolated strains were fast growing on Yeast Extract Mannitol Agar (YEMA) plates. After 3 days of incubation at 30°C, the rounded colonies had mucous transparent to creamy appearance. Most isolates were able to produce acid after 72 h of incubation and only three isolates slightly absorbed Congo red. Heterotrophic competence among isolates was also observed from their utilization capabilities of various carbon and nitrogen sources. Interestingly, the isolates showed strong tolerance to different eco-physiological parameters (pH, temperature, salt concentration, and intrinsic antibiotic resistance). Plants inoculated with different isolates showed differences with respect to nodule number and dry weight and shoot dry weight. Two isolates (AAUFR-26 and AAUFR-30) from North Shewa, representing 12.5% of the isolates, were highly effective (> 80%) in forming symbiosis with fenugreek whereas, 67% were found to be effective (50-80%). More shoot dry weight were observed for whichisolates from North Shewa were inoculated, indicating that some soil-related factors such as pH might have affected the symbiotic effectiveness. Overall, the present study provides a baseline data for further investigations on nitrogen fixation capability of fenugreek associated *Rhizobial* strains so that efficient isolates may be used as biofertilizers in areas where indigenous *Rhizobium* failed to nodulate fenugreek.

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Introduction

Fenugreek (*Trigonella foenum-graecum L.*), the leguminous plant commonly used as condiments and medicinal purposes, has a long history of being used as a potent anti-diabetic, digestive, antipyretic, lactogogue, hypolipidemic, antioxidant and many other purposes (Bhatti and Khan, 1996; Puri *et al.*, 2002; Platel and K.Srinivasan, 2004; Hamden *et al.*, 2010 ; Helmy, 2011). Its cultivation and economic importance in the Ethiopian agriculture dated back to a long period of history (Alevtina and Zerihun, 2009). According to the 2012 CSA estimates of Ethiopia, fenugreek cultivation covers about 39,965 hectares of agricultural land with over 362,939 quintals of fenugreek seed per year, which makes fenugreek the sixth most produced highland pulses in the country (Fikreselassie *et al.*, 2012). Fenugreek is also a good soil renovator and widely used as a green manure (Saeed and Elsheikh, 1995; Abdelgani *et al.*, 1999), which is associated with its ability in forming symbiosis with *Rhizobia* and nitrogen fixation (Abdelgani *et al.*, 1999).

Nitrogen is the most important element for plant growth and its availability has a major impact on both crop yield and product quality in agriculture(Stougaard, 2000). Plants lack the genes encoding the nitrogenase enzymes which are used to reduce atmospheric dinitrogen into biologically utilizable form. Hence, some plants form symbiosis with nitrogen-fixing microorganisms. *Rhizobium* is among the bacteria that can form nodules on roots or stems of leguminous plants including fenugreek, in which biological nitrogen fixation takes place. Fenugreek can drive about 48% of its nitrogen demand by fixing atmospheric nitrogen (Desperrier *et al.*, 1985) whereas the remaining demand would be met from the soil nitrogenpool. However, fenugreek may not always fix nitrogen as it is dependent on the presence of the right bacteria (*Sinorhizobium meliloti*) in the soil(Willems, 2003). Low pH and other soil-related factors can also severely affect the survival, nodulation and nitrogen fixation of indigenous *Rhizobia*, although such problems can be resolved by inoculation of effective *Rhizobia*.

Despite the large scale production of fenugreek in Ethiopia as well as its role in atmospheric nitrogen fixation, to the best of our knowledge, no information about the indigenous *Rhizobial* strains present in its nodules has been reported so far. Hence, the current work aimed to isolate, phenotypically characterize and evaluate the symbiotic performance of *Rhizobia* from root nodules of fenugreek plants from some selected fenugreek growing areas of East and North Shewa of Ethiopia.

Materials and methods

Samples and sampling sites

Soil and nodule samples were collected from two different parts of Ethiopia where fenugreek is commonly grown. Soil samples were collected randomly from ten farmer's fields in the Eastern Shewa of Ethiopia where fenugreek has grown previously. Multiple soil samples taken from 10-15 cm depth were pooled and sealed in alcohol sterilized plastic bags. On the other hand, nodule samples of fenugreek plants from Northern Shewa of Ethiopia were collected, pooled and kept in closed alcohol sterilized plastic vials with silica gel at room temperature until the isolation of *Rhizobial* strains were conducted at the laboratory of microbiology, Addis Ababa University.

Induction of nodulation

Induction of nodulation was performed using plant trap method (Vincent and Humphrey, 1970). Briefly, each soil sample was weighed and added to 3 kg capacity surface sterilized (95% ethanol) plastic pots. Undamaged and uniform fenugreek seeds variety name "Chala" (obtained from Ethiopian Agricultural Research Institute, Debrezeit) were surface sterilized by 70% ethanol followed by 3% sodium hypochlorite, each for 3 minutes (Somasegaran and Hoben, 1994). The seeds were then repeatedly washed using sterile distilled water to avoid any possible effects due to the sterilizing chemicals. Five pre-germinated seeds (incubated in sterile water at 25 ^{o}C for 3 days) were sown and later thinned down to three and allowed them to grow for 45 days with regular watering every three days after planting.

Collection and isolation of Rhizobia

Isolation of *Rhizobia* from soil samples collected from East Shewa were performed 45 days after planting. Isolation was carried out after careful uprooting of the plants and aseptic collection of the respective nodules. On the other hand, nodule samples collected from North Shewa were imbibed in water for 24 hours before crushing. Then, the undamaged nodules from both sampling sites were sterilized by 70% ethanol for 10 s followed by 3% solution of hydrogen peroxide for 3 min. In order to remove the effect of sterilizing chemicals, nodules were rinsed repeatedly in sterilized water and the sterile nodules were crushed using sterile glass rod in normal saline solution (0.85% NaCl) as indicated previously(Somasegaran and Hoben, 1994). The suspension was streaked on Yeast Extract Mannitol Agar (YEMA) plates containing 0.0025% (w/v) Congo red(Vincent and Humphrey, 1970). The components of YEMA were as reported earlier(Somasegaran and Hoben, 1994); which contained g/l 10 Mannitol, 0.5 K2HPO4, 0.2 MgSO4.7H2O, 0.1 NaCl, 1 Yeast Extract, and 15 Agar. Pure cultures of the isolates were obtained through repeated sub-culturing of the colonies on YEMA plates and well isolated colonies were picked and transferred to YEMA slants containing 0.3% (w/v) $CaCO_3$ in culture tubes and incubated at 30 °C. When sufficient growth was observed, cultures were preserved at 4 °C for future use.

Presumptive test of the isolates

Gram staining and growth on peptone-glucose medium

Gram staining were conducted for all isolates as a means for rapid identification of gram-positive contaminants as indicated by(Lupwayi and Haque, 1994). Three days old yeast extract mannitol broth cultures were streaked on two different media; YEMA containing 25 ppm Congo red (YEMA-Cr) and glucose-peptone agar (PGA) containing 25 ppm bromocresol purple (BCP). The components of the peptone-glucose medium were g/l, 5 peptone, 10 agar, 15 Glucose(Lupwayi and Haque, 1994). Plates were then incubated at 30 °Cfor 3-5 days and colony growth characteristics were observed.

Authentication and preliminary screening of the isolates

All the purified isolates were screened for infectivity and efficiency in 3 kg capacity pots containing 3 kg of sterilized and nitrogen-free sand. Plastic pots were surface-sterilized with 95% ethanol whereas the river sand was sterilized with concentrated sulfuric acid (H_2SO_4) and autoclaved(Lupwayi and Haque, 1994). Five surface sterilized and pre-germinated seeds were transferred to the pots, which were then thinned down to three seedlings per pot after a week of germination. Each *Rhizobium* isolate was grown on YEM broth to approximatly10^9 cells/ml and inoculated into each seedling. The experiment was statistically laid out with three replications as Randomized Complete Block Design (RCBD). Each block contained two pots, negative control (T0) and positive control (TN) with uninoculated seedlings. The TN was supplied with N weekly at a rate of 0.05% KNO_3 (w/v) solution. Plants were also fertilized with the nitrogen-free nutrient solution once a week and received water every three days (Broughton and Dilworth 1970), cited in(Somasegaran and Hoben, 1994). After 45 days of planting, the plants carefully uprooted and nodule number were counted, nodule dry weight and shoot dry weight were also measured after drying at 70 °C for 48 h. The relative symbiotic effectiveness of isolates was carried out as previously described (Assefa, 2011; Argaw, 2012).

Effectiveness for atmospheric nitrogen fixation was evaluated by comparing it with the N-fertilized positive control as: Highly effective > 80%, Effective 50-80%, poorly effective 35-50% and ineffective <35%(Jida and Assefa, 2014).

Colony morphology of isolates

A loop full of test isolates from broth culture of 72 h were streaked on YEMA medium and incubated at 28+2 °C. After 5-7 days, colony diameter and morphology of the isolates were carefully recorded.

Determination of mean generation time (MGT)

Each isolate was streaked on YEMA plates and a single colony from the respective isolates was transferred into test tubes containing 10 ml YEM broth, and incubated, on a rotary shaker (125 rev /min) at room temperature for 48 h. Then, one ml of cell suspension from each culture broth was transferred into 250 ml Erlenmeyer flasks containing 100 ml of YEM broth and incubated on a rotary shaker (125 rev/min) at 28ºC. Turbidity was measured every 6 h at 540 nm using UV-7804C spectrophotometer (Sunny Optical Technology, Yuyao, Zhejiang, China). Mean generation time or doubling time was calculated from the logarithmic phase.

Acid-base production test

The ability of isolates to produce acid or base in the medium were evaluated by inoculating on YEM broth containing the Bromothymol blue (BTB) (0.125%) as a pH indicator. A color change of the colonies was observed after 3-7 days of incubation.

Utilization of different C and N sources

Utilization of various carbon sources by the isolates was determined following the method of (Somasegaran and Hoben, 1994) on sixteen carbohydrates. Carbohydrates were prepared as10% (w/v) solution in sterilized water. Heat labile carbohydrates (D-sorbitol, citrate, D-mannose, D-maltose, galactose, D-arabinose, raffinose, cellobiose, trehalose and rhamnose) were sterilized by membrane filtration using Millipore with pore size of 0.45µm (Hach, USA) and added to the autoclaved carbohydrate free basal medium, which is essentially similar to YEMA medium; but modified by reducing the yeast extract to 0.05 g/l. On the other hand, the heat-stable carbohydrates (D-mannitol, lactose, D-fructose, glycerol, sucrose, and starch) were autoclaved together with the basal medium. YEMA medium without carbon source and with mannitol were used as negative and positive controls, respectively. A variety of amino acids; alanine, L-arginine, L-asparagine, L-glutamate, L-leucine, L-lycine, L-methionine, L-phenylalanine, L-tryptophane, L-valine, and L-tyrosine were used in this experiment in order to determine the ability of the isolates to utilize the amino acids as a nitrogen source. These amino acids were added at a concentration of 0.5 g/l to a basal media source that lack NH_4 $(SO_4)_2$ and supplemented with 1 g/l of mannitol. The membrane filter sterilized amino acids were added to the autoclaved and cooled (approximately at 55 ºC) basal media. Finally, 48 h old *Rhizobial* suspensions were inoculated into these basal media and incubated at 30 ºC for 3-5 days. In both cases, the growth of the isolates on the respective media was observed.

Salt, temperature and pH tolerance

All isolates were tested for their tolerance to salinity in YEMA medium supplemented with 0.1-6% (w/v) NaCl concentrations(Lupwayi and Haque, 1994). The ability of isolates to grow at high and low temperatures was also determined using YEMA medium incubated at temperatures ranging from 15-40 ºC(Lupwayi and Haque, 1994).The ability of the isolates to grow in acid and alkaline media was tested by inoculating them into YEMA medium adjusted to pH 4.5-9.5.0 using 1N HCl and 1N NaOH(Bernal and Graham, 2001).

Antibiotic resistance (AR)

The AR of isolates to different antibiotics at different concentration were evaluated by streaking each isolate on YEMA containing filter sterilized antibiotics (µg/ml) ampicillin (3 and 10), chloramphenicol (5 and 10), nalidixic acid (5 and 10), erythromycin (5 and 10), gentamycin (3 and 5), kanamycin (3 and 5) and streptomycin (3 and 5). Erythromycin was dissolved in ethanol, whereas the others were dissolved in sterilized water. The stock solution of each antibiotic was filter sterilized using Millipore filter sized (0.45 µm) and aseptically added to autoclaved YEMA media cooled approximately to 45 ºC.

Data analysis

Observed and measured characteristics of the isolates were presented in table and figures. Tolerance of

isolates to different stresses and utilization of carbohydrate was shown in dendrogram which was constructed using NTSYS software version 2:1. Comparison between treatments was analyzed by one-way ANOVA (Turkey' using GMP-5) software.

Results and discussion

Fenugreek, one of the crops with a long history in Ethiopian agriculture, is known to form symbiosis with nitrogen-fixing bacteria. However, *Rhizobium* bacteria associated with fenugreek have not been investigated so far. Upon targeting the East and North Shewa of Ethiopia where fenugreek is abundantly grown, the present study isolated and characterized, for the first time, fenugreek associated *Rhizobial* strains. A total of sixteen fenugreek-associated *Rhizobial* isolates (9 from field-collected nodules and 7 from nodules induced under greenhouse conditions) were obtained and characterized. As the ability to form nodules along with the subsequent capacity of fixing nitrogen is widely used as a means to evaluate the natural links between the *Rhizobia* and their host plants(Brockwell *et al.*, 1995), *Rhizobial* isolates in the current study were assessed for phenotypic and symbiotic characteristics.

Table 1. Morphology and growth characteristics of isolates from fenugreek grown on YEMA and incubated at 30°C.

Rhizobial isolates	CS	CC	CD	MGT	Presumptive taste		
					Growth-on YEMA-CR	Growth on YEMA-BTB	Growth on PGA-BCP
AAUFR-03	NS	SM	3.5	2.8	light red	yellow	-
AAUFR-04	NS	SM	3	2.1	Colorless	yellow	-
AAUFR-13	NS	LM	3.5	1.8	Colorless	yellow	-
AAUFR-14	NS	SC	2.5	5.6	Colorless	blue	-
AAUFR-17	NS	LM	4	1.4	Colorless	yellow	+
AAUFR-20	NS	LM	4	1.5	Colorless	yellow	-
AAUFR-26	NS	SC	2.5	3.2	Colorless	yellow	-
AAUFR-27	NS	LM	3.5	2.3	Colorless	yellow	-
AAUFR-30	NS	SC	2	1.9	light red	yellow	-
AAUFR-45	ES	LM	3.5	6.3	Colorless	blue	-
AAUFR-46	ES	LM	5	1.9	Colorless	yellow	-
AAUFR-47	ES	LM	4.5	2.1	light red	yellow	+
AAUFR-48	ES	LM	3.5	3.1	Colorless	yellow	-
AAUFR-49	ES	SC	2.5	3.3	Colorless	yellow	-
AAUFR-50	ES	LM	4	2.2	Colorless	yellow	+
AAUFR-51	ES	LM	3.5	2.1	Colorless	yellow	-

Keys: CS = Collection site; CC= Colony characteristic; CD = Colony diameter; MGT = Mean generation time; SM = Small mucoid, LM=Large mucoid, SC=Small creamy, ES=East Shewa, NS=North Shewa.

Colonies of the isolates appeared to have a sticky nature (Table 1), indicating the production of mucous substances which is indeed one of the characteristics *Rhizobia*(Singh *et al.*, 2013). After 3-4 days of incubation, colonies seemed rounded in shape (diameter, 2.5-4.5 mm). Despite all colonies looked white, few isolates (AAURF-3, AAURF-27, and AAUFR-30) turned yellowish after 4 days of incubation at 30 °C (Table 1), which is consistent with the appearance of other isolates recovered elsewhere from fenugreek plants(Baljinder *et al.*, 2008). As expected, all of the isolates were Gram negative.

Phenotypic and symbiotic characteristics generally varied among the isolates. All isolates, except AAUFR-03, AAUFR-30 and AAUFR-47 (those slightly

absorbed), did not absorb Congo red, showing the variation in absorption capacities of Congo red among the isolates (Kneen and Larue, 1983; Kawaka *et al.*, 2014). On the other hand, isolates AAUFR (17, 47 and 50) showed poor growth on peptone glucose agar media (PGA) (Table 1). Indeed, it is well known that *Rhizobia* grow poorly if at all, with little change in pH on PGA were many other bacteria can grow and acidify the medium (Somasegaran and Hoben, 1994). All but two (AAUFR-14 and AAUFR-45) changed the green color of BTB-YEM broth to yellow, indicating the production of acid which is one of the characteristic of fast-growing *Rhizobial* strains (Talukder *et al.*, 2008). However, isolate AAUFR-45 and AAUFR-14 changed the color into blue which is typical of base production by slow growing *Rhizobia*(Talukder *et al.,* 2008). Consistently, the doubling time of most isolates was found to be relatively fast (1.8 - 3.3 h). Isolates AAUFR-17 and AAUFR-20 exhibited the fastest generation time to double its population (Table-1). According to the classification scheme of the family *Rhizobiaceae*(Jordan, 1984; Somasegaran and Hoben, 1994), isolates of*Rhizobia* in the current study might show typical properties of fast-growing *Rhizobium*. Based on the criterion of cross-nodulation host pattern, isolates might be categorized into *Sinorhizobium meliloti*. This is consistent with the characteristics of *Rhizobium* isolated from alfalfa plant in Ethiopia(Shimekite, 2006), likely indicating the similarities of *Rhizobial* isolates modulating the two plants. Moreover, (Bernal and Graham, 2001)reported that isolates of *R. meliloti*, like other fast-growing *Rhizobium* species, usually have a generation time of 2 to 4 h and are acid producers on YEM growth medium.

Table 2. Physiological characteristics of fenugreek nodulating *Rhizobial* isolates.

Rhizobial isolates	Site	No of Carbon sources utilized	No of N sources utilized	NaCl % tolerated	pH tolerated	T(C°)tolerated	Antibiotics(14*)resisted	% SE	Rate	Values of HC and ET
AAUFR-27	NS	15	8	0.1-6	4.5-9.5	15-40	9	73	E	13
AAUFR-46	ES	14	10	0.1-5	4.5-9.5	20-40	10	54	E	15
AAUFR-13	NS	15	11	0.1-3	4.5-9.5	15-37	7	69	E	15
AAUFR-49	ES	14	10	0.1-3	4.5-9.5	15-30	10	67	E	16
AAUFR-03	NS	15	10	0.1-3	4.5-9.5	15-37	5	71	E	18
AAUFR-14	NS	14	10	0.1-3	5.5-9.5	15-37	6	67	E	19
AAUFR-47	ES	15	10	0.1-3	4.5-9.5	25-37	7	59	E	19
AAUFR-30	NS	12	11	0.1-3	5.5-9.5	15-30	4	84	HE	20
AAUFR-26	NS	14	10	0.1-3	4.5-9.5	20-37	6	80	HE	20
AAUFR-04	NS	15	11	0.1-3	5.5-9.5	20-37	6	64	E	20
AAUFR-20	NS	15	7	0.1-3	4.5-9.5	15-30	6	64	E	21
AAUFR-50	ES	15	11	0.1-3	5.5-9.5	25-40	4	72	E	21
AAUFR-51	ES	15	11	0.1-3	5.5-9.5	25-37	4	66	E	22
AAUFR-17	NS	15	9	0.1-4	5.5-9.5	20-30	5	65	E	23

Key: SE= symbiotic effectiveness, HC=heterotrophic competence, ET=Eco-physiological tolerance.

It is known that high salt concentrations can affect the growth of *Rhizobial* (Hanaa and Mustapha, 2009). In the current study, *Rhizobial* isolates generally grow on media with salt concentrations ranging from 0.1 to 3% (w/v) NaCl (Table-2). Except AAUFR-27, AAUFR-46, AAUFR-17 and AAUFR-30 that tolerated 6%, 5%, 4% and 4% (w/v), respectively of NaCl, all other isolates were sensitive to salt concentrations above 3% w/v, implicating the effect of soil salinity on the efficiency of the isolates. Despite a report by Baljinder *et al.*(2008) indicated that *Rhizobial* isolates from fenugreek plants were unable to grow salinities above 1% NaCl concentrations, such variations from the current isolates might be associated with the type of soil where the isolates adapted to grow. Indeed, a previous work by (Shimekite, 2006) from alfalfa plants of Ethiopian demonstrated the tolerance of many of the *Rhizobial* isolates for 1-5% NaCl concentrations. Another study (Keneni *et al.*, 2010)also showed that *Rhizobial* strains isolated from faba bean of Wollo region of Ethiopia were able to grow variably under different

sodium chloride concentrations (0.5% to 5%). Moreover, some *Rhizobial* isolates from bean have been shown to grow under high salt conditions (4-5%)(Kucuk *et al.*, 2006). In the current work, the fast growing isolates generally seemed more tolerant to high NaCl concentrations than slow growing isolates.

Table 3. Intrinsic antibiotic resistance of isolates at different antibiotic concentrations grown on YEMA medium incubated at 30°C for 3-7 days.

Antibiotics	% Resistance of isolates		
	3 .0 µg/ml	5.0 µg/ml	10 µg/ml
Ampicillin	88	-	69
Chloramphenicol	-	81	38
Erythromycin	-	50	25
Kanamycin	0	0	-
Nalidixic acid	-	100	100
Streptomycin	38	19	-
Gentamycin	13	0	-

Table 4. The effect of *Rhizobia* on the nodule number (NN), nodule dry weight (NDW) and shoot dry weight (SDW) of fenugreek grown on sand culture under greenhouse conditions.

Isolates	**NN/Plant± SE**	**NDW(mg/plant±SE)**	**SDW(mg/plant±SE)**	**%SE**	**Effectiveness**
AAUFR-03	24±3.28^{abcd}	14.67±1.76^{abcd}	71.67±4.18^{bcd}	71	E
AAUFR-04	19±2.19^{bcd}	12.00±3.51^{bcde}	65.33±4.06^{bcde}	64	E
AAUFR-13	29±3.76^{abc}	20.33±1.20^{abcd}	70.33±0.88^{bcd}	69	E
AAUFR-14	22±2.91^{bcd}	18.33±1.86^{abcd}	68.00±4.62^{bcd}	67	E
AAUFR-17	17±1.45^{bcd}	13.33±2.96^{abcde}	66.00±5.29^{bcde}	65	E
AAUFR-20	22±3.05^{bcd}	16.33±2.91^{abcd}	65.33±3.52^{bcde}	64	E
AAUFR-26	41±6.64^{a}	27.33±3.28^{a}	80.67±1.76^{bc}	80	HE
AAUFR-27	33±3.84^{ab}	24.67±2.96^{ab}	74.33±6.12^{bcd}	73	E
AAUFR-30	30±2.40^{ab}	25.00±2.64^{ab}	85.66±2.19^{ab}	84	HE
AAUFR-45	11±3.61^{cd}	10.00±2.08^{cde}	46.33±3.38^{ef}	45	LE
AAUFR-46	16±3.06^{bcd}	10.67±4.18^{bcde}	55.67±3.48^{de}	54	E
AAUFR-47	20±4.73^{bcd}	16.33±2.19^{abcd}	60.67±1.20^{cde}	59	E
AAUFR-48	10±2.08^{d}	6.00±0.57^{de}	46.33±1.86^{ef}	45	LE
AAUFR-49	25±3.46^{abcd}	17.33±2.03^{abcd}	68.00±1.53^{bcd}	67	E
AAUFR-50	22±1.76^{bcd}	20.33±5.24^{abcd}	73.33±7.31^{bcd}	72	E
AAUFR-51	28±2.33^{abc}	22.67±3.84^{abc}	67.00±1.53^{bcd}	66	E
TN	0	0	101.67±4.48^{a}	100	
To	0	0	29.00±4.62^{f}	28	

Key: HE= effective; E = effective; %SE= % of symbiotic effectiveness, numbers in the same column followed by the same letter(s) are not significantly different at p≤0.05.

Except 44% of isolates that failed to grown at pH 4.5, the remaining were able to grow at pH 4.5-9.5 (Table 2) with abundant growth from pH 6.5 to 8.5, suggesting the preference of the isolates for higher

pH. Consistently, tolerance of *Rhizobial* isolates from alfalfa from pH 5-9 (Shimekite, 2006) and from chickpea in the pH range of 5.5-8 where 25% grew at pH 9.5(Jida and Assefa, 2012)have been demonstrated.

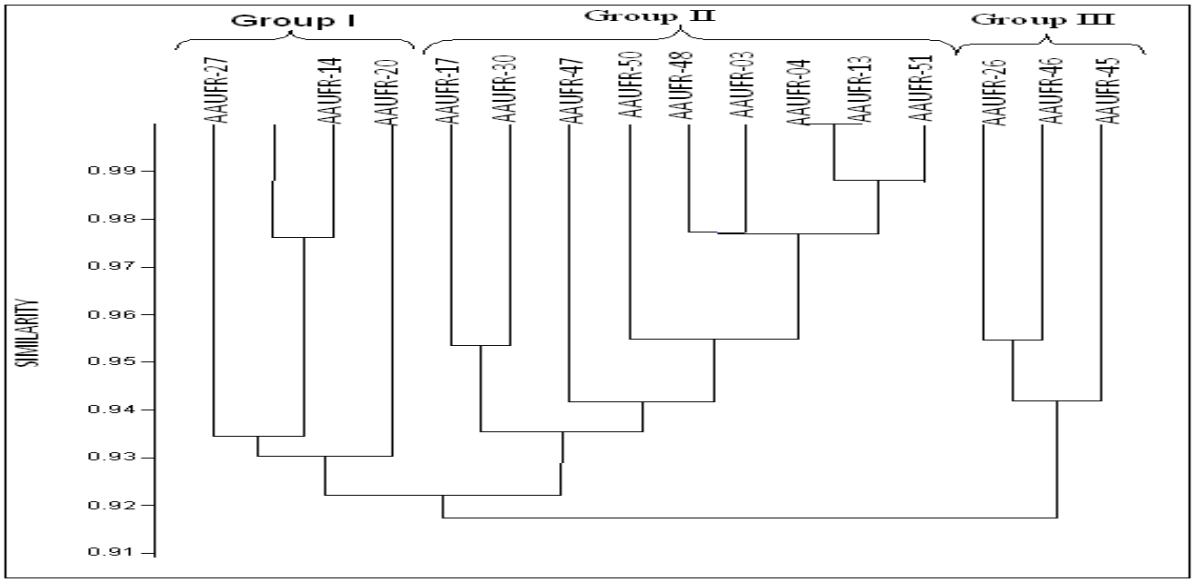

Fig. 1. Dendrogram showing the relationship of fenugreek associated *Rhizobial* isolates with respect to their tolerance to various stresses and carbohydrate utilization capabilities.

In the temperature tolerance analysis, maximum growth of all tested isolates were observed between 25 and 30 °C (Table 2). The growth of isolates below and above these values decreased to reach 75% at 20 °C and 37 °C. Only 50% and 19% of the tested isolates could be able to tolerate temperatures of below 20 °C and above 40 °C respectively, which might suggest the mesophilic nature of most of the isolates. The growth of some of the isolates at 40 °C is not surprising as previous studies already identified thermotolerant isolates from chickpea and alfalfa that can grow at 40 °C(Shimekite, 2006; Jida and Assefa, 2012). Therefore, the conclusion made by Kucuk and his colleagues (Kucuk *et al*., 2006) that 29.4 °C as the optimum for growth of the *Rhizobia* and by Baljinder and his colleagues (Baljinder *et al*., 2008)that no isolates of fenugreek *Rhizobium* are able to grow at 37 °C may need further confirmation, although other growth factors may have influences on the growth temperature.

All of the tested isolates were able to catabolize L-tryptophan, L-tyrosine, and L-arginine (Table 2). Methionine supported the growth of about 75 % of the isolates whereas other amino acids used in the growth media supported the growth of more than 80% of the isolates. According to previous reports, most of *Rhizobia* nodulating common bean are able to utilize most nitrogen sources(Sessitsch *et al*., 1997b; Argaw, 2012). Similar results have been reported by(Workalemahu, 2006) for isolates of *Rhizobia* from the common bean in South Ethiopia, indicating the wide options of nitrogen sources for *Rhizobia*(El-Akhal *et al*., 2009).

Except starch on which isolates showed large diversity in growth, most all of *Rhizobial* isolates grew on the tested carbohydrate. Starch supported the growth of 75 % of the isolates, which is consistent with other reports that demonstrated utilization of starch by many isolates of *Rhizobia*, for instance, about 80% of isolates from common bean (Argaw, 2012) and 69% of isolates from grass pea (Adal, 2009) for growth and energy. On the other hand, none of the isolates grew on citrate (Table 2), which is also in line with other reports from studies in which 61% of the isolates from fababean and 69% of the isolates from alfalfa did not metabolize citrate as sole

source of carbon(Belay, 2006; Shimekite, 2006). Despite utilization of citrate as a sole source of carbon was proposed to be restricted in slow-growing *Bradyrhizobia*(Graham and Parker, 1964), the fast-growing isolates in the current study and *Rhizobia*l isolates of Jordan (Jordan, 1984)clearly demonstrated the successful utilization of a wide range of carbohydrates as a carbon source.

In order to select the best isolates for further work, heterotrophic competence and eco-physiological tolerance in relation to the symbiotic effectiveness of the isolates were evaluated. Generally, wide variations in tolerance to a variety of physiological characteristics were observed (Table 2). Isolate AAUFR-27 showed the smallest value (13) for its heterotrophic competence and eco-physiological tolerance followed by isolates AAUFR-13 and AAUFR-46, for which a value of 15 was recorded. However, isolates AAUFR-26 and AAUFR-30, which were highly effective isolates, were found to have a narrow range of tolerance to different environmental conditions both with heterotrophic competence and eco-physiological tolerance (20). Utilization of wide range of carbon and nitrogen sources by isolates and adapting to different environmental conditions would give more ecological competence in the soil and used for their differentiation and it is also one of the required characteristics for selection of isolates for preparation of inoculants (Hungriaa *et al*., 2001; Küçük and Kıvanç, 2008).

In the AR experiments, isolates exhibited random susceptibility to different types and concentrations of antibiotics (Table 3). Most of the *Rhizobial* cultures significantly resisted many of the tested antibiotics. The AR in most isolates decreased with increasing the concentrations given in the culture medium. At higher concentrations (10 µg/ml) of the respective antibiotics, bacterial growth was suppressed whereas at lower concentrations (5-3 µg/ml) isolates generally tolerated ampicillin, chloramphenicol, nalidixic acid, erythromycin, gentamicin and streptomycin by 88, 81, 100, 50, 13 and 38%, respectively. However, at higher antibiotic concentrations, isolates poorly tolerated ampicillin (69%), chloramphenicol (38%), and erythromycin (25%). Interestingly, nalidixic acid was 100% tolerated by all isolates at both concentrations (Table 3). However, kanamycin and gentamicin have a distinct suppressive effect on most isolates. This is not surprising as sensitivities of various *Rhizobial* isolates to different antibiotics have already been determined by a number of researchers (Shimekite, 2006; Keneni *et al*., 2010; Argaw, 2012).
The preliminary screening for the ability to form nodules and the ability to form enough nitrogen by the isolates were evaluated on the basis of nodule number, nodule dry weight and shoot dry weight. All tested isolates of fenugreek associated *Rhizobia* showed variation in their capacity to induce the formation of nodule on roots of the host plant under glasshouse conditions. The mean nodule number per plant varied from 10 to 41, which were induced by isolate AAUFR-48 and AAUFR-26, respectively (Table 4). On the other hand, the highest nodule dry weight (27.33 mg/plant) was measured for the isolate AAUFR-26 whereas the lowest (6 mg/plant) was recorded for the isolate AAUFR-48 (Table 4). Likewise, all tested fenugreek *Rhizobia* exhibited high diversity in their capacity to fix atmospheric nitrogen and thus shoot dry matter accumulation.

Fenugreek associated *Rhizobium* isolates had shoot dry matter yield ranging from 46 to 85 mg per plant (Table 4). The relative effectiveness, which is expressed in percent of TN control, showed that isolates AAUFR-30 and AAUFR-26, both from North Shewa, were the most efficient with more than 80% dry matter yield whereas isolates AAUFR-45 and AAUFR-48 that were both from East Shewa were the least efficient (both 45%) relative effectiveness, indicating variations due to regional differences. Consistently, about 56% of the isolates from North Shewa and 31% of the isolates from East Shewa induced effective nodulation and nitrogen fixation and shoot dry weight accumulation (Table 4), further confirming the variation of isolates due to regional differences which would be associated with the respective environmental factors.

In the current study, although there was inconsistency in nodule number and weight among the treated plants, a significant difference existed with respect to their shoot dry matter at P=0.05. Consistently, Jida and Assefa (2012) reported that no positive relationship between the increase in shoot dry matter or number with the dry weight of nodules. However, Dudeja *et al.*(1981) demonstrated that the dry matter yield was relatively correlated with the nodule leghaemogblobin concentration rather than with the number or the dry weight of the plant.

Numerical analysis based on different phenotypic characteristics puts the isolates into different groups (Fig.1). AAUFR-26, AAUFR-45, and AAUFR-46 clustered separately while other isolates clustered into two main groups at a boundary level of 0.92 average distance. Cluster I comprises (25%) AAUFR-14, AAUFR-20, AAUFR-27 and AAUFR-49 whereas cluster II, the biggest cluster (56%), comprised of 9 isolates (AAUFR-03, AAUFR-04, AAUFR-13, AAUFR-17, AAUFR-30, AAUFR-47, AAUFR-48, AAUFR-50, and AAUFR-51) that displayed two sub–clusters with 94% relative similarity. Isolate AAUFR-14, AAUFR-50 and AAUFR-27 were clustered separately at 93, 94 and 94% level of similarity, respectively. Such variations are indeed consistent with the above results that showed variations towards salinity, temperature, pH and antibiotics.

Conclusion

The phenotypic and symbiotic characteristics of fenugreek associated *Rhizobial* isolates studied in the current work might categorize them under the cross-inoculation group of *Sinorhizobium meliloti*. The resistance of some isolates to extremes of pH, temperature and salt concentrations, the ability of most isolates to utilize a wide variety of carbon and nitrogen sources, and the considerable resistance to many of the tested antibiotics, may favor the establishment of the *Rhizobia* in the various types of soils, which would be an advantage to be used as inoculants in fenugreek cultivation.

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