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Isolation and Biochemical Characterization of Rhizobium Strains from Fababean (*Vicia faba L*) Nodule from Arsi Zone of Ethiopia

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Abstract **Original Research Article**

Nitrogen (N) deficiency is one of the most common factors for reduction in yield of legume crops particularly in Ethiopia. The utilization of biological nitrogen fixation (BNF) by legume-*Rhizobium* symbiosis is most prominent symbiosis found in nature; so for increasing legume production and soil fertility we should use organic fertilizer such as bio fertilizer. The aim of this study was to isolate, identify and characterize the fababean nodulating *Rhizobium* isolates. Fababean (*Vicia faba L.*) rhizobia were isolated from nodules collected from four fababean growing area of Arsi zone of Ethiopia. A total of twenty rhizobia isolates were collected and characterized based upon their morphological and biochemical characteristics. Out of total, twelve isolates were selected as representative rhizobia samples. The result of this study showed that all isolates were fast-growing and failed to absorb Congo red. Cell size ranged from 2.1 to 5.2 mm. The smallest and largest colony diameter (2.1 and 5.2 mm) was recorded in isolates BKFB3 and MRFB10 respectively on YEMA medium. Isolates were capable of utilizing 50-100% of the tested carbohydrates. 75% of all isolates metabolized all the nitrogen sources and all isolates were utilized glutamine. All isolates were sensitive to neomycin and ampicillin at 20µg/ml concentration.

Keywords: *Rhizobia*, Fababean, Isolates, Nitrogen fixation Ethiopia.

INTRODUCTION

Leguminous plants (pulses) are important as human food and animal feed they provide not only high quality protein but also a variety of nutrients such as vitamins, minerals, and other nutrients. Most species of the Leguminosae form symbiotic associations with nitrogen-fixing root nodule forming bacteria known as *rhizobia*. They fulfill most of their N requirements through this process. Therefore, the legumes are an essential part of the terrestrial nitrogen cycle and used to sustain ecosystem functioning (Sprent, 2001). They occupy 12-15% of the earth's arable land and account for a third of human dietary protein needs and for up to 2/3 of subsistence livelihood (Graham and Vance, 2003). Legume plants (Fababean, field pea, chickpea, soybean, etc.) show a vast diversity in morphology, habitat and ecology. However, all legume species can be distinguished from non-*leguminous* plants as they produce pods as a common feature.

Fababean (*Vicia faba L*) is one of the earliest domesticated cool season food legumes in Ethiopia. Ethiopia is the second largest fababean producer in the world next to China (Teklay *et al*., 2014). Fababean plays a great role in every aspect of Ethiopian life not only as food but also the straw and the seed as feed for animals, haulms as firewood, green manuring and for compost preparation (Comlanvi, 2011). It plays an important role in the restoration of soil fertility through atmospheric nitrogen fixation, which provides agricultural sustainability (Ronner *et al*., 2013).

Rhizobia are a genetically diverse and physiologically heterogeneous group of bacteria (Somasegaran and Hoben, 1994) and they are able to bring out nodule formation on legumes are called *rhizobia*. *Rhizobia* are a ubiquitous part of the soil micro-flora in a free-living state in the rhizosphere of legumes until the point where nodulation becomes possible. *Rhizobia* are bacteria that selectively infect the roots of some legumes and have the following characteristics; gram negative, motile rod-shaped (approximately 0.5-0.9 µm in width and 1.2-3.0 µm in length) and heterotrophic (Somasegaran and Hoben, 1994).

Root nodule bacteria generally grow under the following conditions 25-30°C (optimum) in the pH range of 6-7. *Rhizobium* growth normally occurs under aerobic conditions. When fixing nitrogen, low levels of oxygen are required to protect the enzyme nitrogenase

and hence, *Rhizobium* is able to grow in microaerophilic conditions (Somasegaran and Hoben, 1994).

Rhizobia are of great importance for nitrogen acquisition through symbiotic nitrogen fixation in a wide variety of leguminous plants. These bacteria differ from most of other soil microorganisms by taking dual forms, i.e., a free-living form in soils and a symbiotic form inside of host legumes. Therefore, they should have a versatile strategy for survival, whether inhabiting soils or root nodules formed through *rhizobia*-legume interactions. The objective of this study was to isolate, identify and biochemical characterization of root nodulating *rhizobium* isolates from fababean (*Vicia faba L*) nodules.

MATERIAL AND METHODS

The experiments were carried out at the Department of Microbiology, College of Natural Science, and Addis Ababa University, Ethiopia.

Collection of samples

Plant samples were collected from different locations of Arsi zone fababean growing area such, Bekoji, Meraro, Kersa and Munesa. A total of 20 (five for each area) samples of fababean nodules were collected and big sized and pink colored nodule preferably on tap root were selected and transported to the laboratory for further investigation by following the method given by Vincent (1970).

Isolation of *Rhizobium* from root nodules

In the laboratory nodule samples were tagged properly and named as BKFB1-BKFB5 for Bekoji, MRFB6-MRFB10 for Meraro, KRFB11-KRFB15 for Kersa and MUFB16-MUFB20 for Munesa. The nodules (collected from inoculated host fababean) was surface sterilized with 95% ethanol for 10 seconds, and transferred to 3% (v/v) solution of sodium hypo chlorate for 2-minutes (Lupwayi and Haque, 1994).The surface sterilized nodules was then rinsed with sterile distilled water six times to completely remove the sterilizing chemicals. The nodules were crushed with sterile glass rods in1 drop of sterilized 0.85% NaCl. The crushed nodules were transferred to YEMA medium (DIFCO). They were then incubated at 28±2^{o}c and periodically checked for colony formation.

Table-1: YEMA Composition

Chemicals	Amount (gm/l)
$MgSO_4.7H_2O$	0.2g
KH_2PO_4	0.5g
NaCl	0.2g
Yeast extract	0.5g
D-manitol	10g
Agar	15g
Distil water	1000ml

Autoclaved at 121^{o}C for 15 minutes, pH adjusted to 7, Taken from –Lupwayi and Haque. (1994)

Purification and preservation

Colonies of isolates was picked with sterile inoculating loop and streaked on sterile YEMA plates and incubated at 28±2^{0}C. The purity and uniformity of the colony type was carefully examined through repeated re-streaking and a single well isolated colony picked to YEMA slant containing 0.3% (W/V) $CaCO_3$ in a culture tube and incubated at 28±2^{0}C. After sufficient growth the culture slant were preserved at 4^{0}c (Somesagaran and Hoben, 1994).

Presumptive test

1. Gram staining test

Gram stain reaction was carried out to confirm that all isolates were gram negative and does not contain any gram positive bacteria or contaminants. Gram's procedure was done as indicated in Lupwayi and Haque, (1994).

2. Congo red absorption

Colonies was tested on Congo-Red (CR-YEMA) (Somasegaran and Hoben, 1994). Stock solution of Congo Red (CR) prepared by dissolving 0.25g of CR in 100ml sterile distilled water.10ml from this stock solution added to one liter of YEMA. The broth culture suspension inoculated into YEMA-CR medium, and the plates were wrapped with aluminum foil to provide a dark condition and incubated to at 28±2 ^{0}C observe CR absorption or not.

3. Acid and alkaline production on BTB

They were tested for production of acid or alkaline by incorporating bromothymol blue (BTB) as reaction indicator on yeast extract mannitol agar (YEMA) according to Somassegaran and Hoben (1994). After 48 hours of growth, a loop full of *Rhizobium* culture (10^{5}cells/ml) streaked on YEMA-BTB plate, fast growers changed the BTB in to yellow color, but slow growers changed into blue color, and results were recorded after 3-5 days of incubation.

YEMA----------1 liter
BTB-(0.5 % w/v in 95% ethanol) ----------5ml
pH----------6.8

4. Growths on Peptone Glucose Agar (PGA) Medium

Isolates inoculated on PGA incorporated with bromocresol purple in order to check a change in pH of the medium associated with the presence of contaminants in the preserved culture further checked on PGA medium with 10 µ g/ml Bromocresol purple (BCP) dye.

Composition of PGA medium
Glucose…….5g
Peptone……10g
Agar…….15g
BCP stock solution..... 10ml
PH……6.7
Distilled H2O……..1liter
Autoclaved at 121^{0}C for 15 min. Source: Somasegaran and Hoben (1994)

The BCP (Bromo cresol purple) prepared as stock solution by dissolving 1g/100ml of ethanol (Somasegaran and Hoben, 1994). The pH adjusted at 6.8 by 1N NaOH and HCl (Lupwayi and Haque, 1994). The bacterial culture suspension was inoculated on the medium and incubated at 28±2^{o}c; finally presence/absence of bacterial colonies was checked.

Colony Morphology

The cultural characteristics of the isolates were performed according to (Lupwayi and Haque, 1994). Each bacterial isolates From YEMB were inoculated on the YEMA medium and incubated for 3-5 days. A single colony of each isolate was characterized by colony appearance, diameter, color, shape and extra cellular polysaccharide production.

Biochemical characteristics

Carbohydrate Utilization

Isolates were checked for their ability to utilize different carbohydrate sources. Different sources of carbohydrate can be checked up. This may include glucose, maltose, D-fructose, glycerol and Gluconate. The test was carried out according to Somasegaran and Hoben (1994). Ten percent distilled water solution of each carbohydrate (w/v but v/v for glycerol) was prepared and heat stable carbohydrates (glucose and D-fructose) were autoclaved together with the medium, but heat labile carbohydrates (gluconate, maltose and glycerol) were filter sterilized using disposable membrane filter of 0.22µm sizes and added to the basal medium (YEMA) after sterilization when the medium temperature was reduced to 50 ^{O}C. Finally, a loop full of 72 hours old YEM broth culture of each rhizobia isolate was streaked on the plates of incorporated carbohydrates under test and incubated at 28±2 ^{O}C for 3 to 5 days and growth was recorded as (+) for positive growth and (-) for no growth in relation to the positive control YEMA plates.

Amino Acid Utilization

The isolates were streaked on different amino acids including: Glutamine, Peptone, Glycine, alanine and L-lysine in order to determine the ability of the isolates to utilize the amino acids as a nitrogen source. They were added at concentration of 0.5g /l to a basal medium (Somasegran and Hoben, 1994). Finally 48hr old rhizobia suspensions were inoculated in to these basal media and incubated at 28±2 ^{0}C for 3-5 days.

Intrinsic antibiotic resistance (IAR)

The resistance of isolates to antibiotics was tested by streaking them on solid YEMA medium containing freshly prepared filter sterilized antibiotics using 0.22 µm sized membrane filters: Tetracycline, Erythromycin, Streptomycin, Penicillin and Neomycin and two concentrations (10 and 20 µg/ml). The stock of each antibiotic solution was first prepared by dissolving 2g of each antibiotic in 100ml of water as described in Lupiwayi and Haque, (1994). Erythromycin was dissolved in ethanol and the other was dissolved in sterilized distilled water. The filter sterilized solutions of each antibiotic was added to sterile YEMA cooled to 50 ^{0}c and mixed thoroughly. The isolates then tested by streaking the culture on each cooled plate and incubated at 28 + 2^{o}C for 3-5 days. The results were recorded qualitatively either as +/- for growth and no growth, respectively.

RESULT AND DISCUSSION

Isolation and Morphological characteristics of rhizobia

A total of 20 root nodule samples collected from different fababean growing areas from Arsi zone. The data in Table 1 shown that among 20 samples tested, 12 samples were found positive for the presence of *Rhizobium* on the basis of white mucoid growth on YEMA medium when incubated for 24 h at 28±2^{o}c. Pure colonies of rhizobia isolates were microscopically examined to determine cell shape and size, Gram stain reaction and motility in liquid culture. Colony morphology of rhizobia isolates was studied to determine the opacity and viscosity. The data in Table (2) show that all rhizobia isolates tested had fast growth on YEMA medium, non-capsulated, short rod, Gram negative and motile. Cell size ranged from 2.1 to 5.2 mm. The smallest and largest colony diameter (2.1 and 5.2 mm) was recorded in isolates BKFB3 and MRFB10 respectively on YEMA medium (Table 2). According to Somasegaren and Hoben, 1994 classification those isolates were classified as fast growing root nodule bacteria. Similar results were reported by Mona H. A. *et al*., 2016 on rhizobia isolated from fababean colonies of the isolates were appeared a sticky natural, indicating the production of mucous substances which is one of the characteristics of *Rhizobia* (Singh *et al*., 2013).

All the isolates were failed to absorb Congo red pigment when grown on YEMA-CR medium. The isolates change the color of Bromocresol purple, when grown on peptone glucose agar medium and changed the YEMA-BTB medium. This result is similar to the results of Abere Minalku *et al*., 2009 and Zerihun Belay and Fassil Assefa (2011) on isolation and characterization of rhizobia from fababean. Among all the isolates MRFB6, MRFB7, MRFB10, KRFB12, KRFB13, MUFB16, MUFB18 and MUFB19, showed large mucoid growth on YEMA medium, isolates

BKFB2, BKFB3, KRFB14, and MUFB20, showed small mucoid growth on YEMA media (Table 2).

Table-2: Morphological characteristics of rhizobia isolated from fababean nodule

Isolates	Colony size (mm)	colony type	colony shape	colony texture	Colony color	Gram rxn test	YEMA-BTB	YEMA-CR	PGA
BKFB2	2.5	SM	rod	buttery	White	-	Yellow	+	+
BKFB3	2.1	SM	rod	elastic	White	-	Yellow	+	+
MRFB6	3.5	LM	rod	elastic	White	-	Yellow	+	+
MRFB7	4.8	LM	rod	buttery	White	-	Yellow	+	+
MRFB10	5.2	LM	rod	elastic	Yellow	-	Yellow	+	+
KRFB12	4.2	LM	rod	buttery	White	-	Yellow	+	+
KRFB13	4.2	LM	rod	elastic	White	-	Yellow	+	+
KRFB14	3.2	SM	rod	elastic	Yellow	-	Yellow	+	+
MUFB16	3.5	LM	rod	elastic	White	-	Yellow	+	+
MUFB18	4.5	LM	rod	elastic	White	-	Yellow	+	-
MUFB19	4.8	LM	rod	elastic	White	-	Yellow	+	-
MUFB20	2.8	SM	rod	elastic	White	-	Yellow	+	+

LM= large mucoid, SM= small mucoid

Biochemical characteristics of rhizobia isolates

Carbohydrate utilization

The data on table 3 showed that isolates were capable of utilizing 50-100% of the tested carbohydrates. All isolates utilized glucose and most of the isolates (85.3%, 58.3% and 50%) were capable of utilized glycerol, D-fructose, gluconate and maltose. Girmaye *et al*. (2014) result more than 50% the fababean isolates were able to utilize and grow well on most carbon sources. The result, in general showed that the majority of isolates were able to use a broad range of carbon sources.

Amino acid utilization

The results of utilization of nitrogen sources showed that more than 75% of all isolates metabolized all the nitrogen sources and all isolates were utilized glutamine (table 3). The result which revealed that similar with Getahun Negash (2015) and Girmaye *et al*. (2014) who were identified that 37.1% and 48% of the isolates were able to metabolize all the nitrogen sources respectively.

Table-3: Carbohydrate and amino acid utilization of rhizobia isolates

Isolates	Carbohydrate utilization					Amino acid utilization				
	Glucose	Maltose	D-fructose	Glycerol	Gluconate	Glutamine	Peptone	l-lysine	Glycine	Alanine
BKFB2	+	+	+	+	+	+	+	+	+	+
BKFB3	+	+	-	+	+	+	+	+	+	+
MRFB6	+	+	+	+	-	+	-	-	-	-
MRFB7	+	-	-	+	+	+	+	+	+	+
MRFB10	+	-	-	+	-	+	+	-	+	+
KRFB12	+	+	+	+	-	+	-	-	+	-
KRFB13	+	-	-	-	-	+	+	+	-	-
KRFB14	+	+	+	+	+	+	-	+	+	+
MUFB16	+	+	+	+	+	+	-	-	-	-
MUFB18	+	-	-	-	-	+	+	+	+	+
MUFB19	+	-	+	+	-	+	+	-	+	+
MUFB20	+	-	+	+	+	+	+	+	+	+
Utilization%	100	50	58.3	83.3	50	100	66.6	58.3	83.3	66.6

Antibiotics resistance test

Results presented in (Table 4) indicated different antibiotic resistance patterns among the rhizobia isolates. All isolates (100%) were resistant to tetracycline and streptomycin at concentration of 10µg/ml and most of isolates were resistance to erythromycin (75%), penicillin (25%) and neomycin (41.6%) at the concentration of 10µg/ml. All isolates were sensitive to neomycin and ampicillin at 20µg/ml concentration. In a similar study, fababean rhizobia showed sensitivity to ampicillin and kanamycin than other types of antibiotics (Girmaye *et al*., 2014). Finally, antibiotic resistance test indicated that all the isolates showed variation in tolerance to 20µg/ml concentration of tested antibiotics (Table 4).

Table-4: Antibiotics resistance test

Isolates	Antibiotics resistance									
	Tetracycline		Erythromycin		Streptomycin		Ampicillin		Neomycin	
	10µg/ml	20µg/ml	10µg/ml	20µg/ml	10µg/ml	20µg/ml	10µg/ml	20µg/ml	10µg/ml	20µg/ml
BKFB2	+	-	+	+	+	-	+	-	-	-
BKFB3	-	-	+	-	+	-	-	-	+	-
MRFB6	+	-	+	-	+	-	-	-	-	-
MRFB7	+	-	+	-	+	+	-	-	-	-
MRFB10	+	-	+	-	+	+	-	-	-	-
KRFB12	+	+	+	-	+	-	+	-	+	-
KRFB13	-	-	+	-	+	-	-	-	-	-
KRFB14	+	+	+	+	+	-	-	-	-	-
MUFB16	-	-	+	+	+	-	+	-	+	-
MUFB18	+	-	+	-	+	+	-	-	-	-
MUFB19	+	-	+	-	+	+	+	-	+	-
MUFB20	+	-	+	+	+	+	-	-	+	-
Resistance%	75	16.6	100	25	100	41.6	25	0	41.6	0

CONCLUSION

The results from this study concluded that isolation and biochemical characterization of rhizobia isolates from fababean nodules were to select and screen superior isolates for preparation of effective strains. From the result of this study rhizobia isolates were fast growing type and did not absorb red color when cultured in YEMA containing congo red medium. The tested isolates were utilized different carbon and nitrogen sources and able to tolerate wide range of antibiotics. Therefore, the presence of diversity from the study areas showed that getting effective rhizobial strains for the production of fababean. Hence, studies needed molecular characterization for obtaining superior rhizobia strains from different area for production of bio fertilizer.

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Conflict of interest

The authors do not declare any conflict of interest.

REFERENCE

- Minalku, A., Gebrekidan, H., & Assefa, F. (2009). Symbiotic effectiveness and characterization of Rhizobium strains of Faba bean (Viciae faba L.) Collected from eastern and Western Harareghe Highlands of Ethiopia. *Ethiopian J. Nat. Resour*, *11*(2), 223-244.
- Akibode, C. S. (2011). *Trends in the production, trade, and consumption of food-legume crops in sub-Saharan Africa* (No. 1097-2016-88694).
- Getahun Negash, T. (2015). *Symbiotic and phenotypic characteristics of indigenous rhizobia nodulating faba bean (Vicia faba L.) growing in some parts of Wello, Northern Ethiopia* (Doctoral dissertation, MSc thesis Hawassa University).
- Kenasa, G., Jida, M., & Assefa, F. (2014). Characterization of phosphate solubilizing faba bean (Vicia faba L.) nodulating rhizobia isolated from acidic soils of Wollega, Ethiopia. *Science, Technology and Arts Research Journal*, *3*(3), 11-17.
- Lupwayi, N. Z., & Haque, I. (1994). Legume-Rhizobium technology manual. *ILCA Environmental Sciences Working Document (ILCA). no. 29.*
- Hussein, M. H., Zaghloul, R. A., Abou Aly, H. A., Abdel-Rahman, H. M., & Abotaleb, H. H. Isolation and identification of rhizobial strains from faba bean nodules.
- Ronner, E., Ken E Giller., & Esther, R. (2013). Agronomy, Farming Systems and Ongoing Projects on Grain Legumes in Ethiopia.
- Singh, N. K., Luthra, U., & Desai, N. (2013). Phenotypic and genotypic characterization of Rhizobium species isolated from the root nodules of Sesbania sesban found in Mumbai and its suburban areas. *Indian Journal of Applied Research*, *3*(7), 2249-555X.
- Somasegaran, P., & Hoben, H. J. (2012). *Handbook for rhizobia: methods in legume-Rhizobium technology*. Springer Science & Business Media.
- Sprent, J.I. (2001). Nodulation in Legumes. Royal Botanic Gardens, Kew, 87.
- Abebe, T., Birhane, T., Nega, Y., & Workineh, A. (2014). The prevalence and importance of faba bean diseases with special consideration to the newly emerging faba bean gall in Tigray, Ethiopia. *Discourse Journal of Agriculture and Food Sciences*, *2*(2), 33-38.
- Callow, J. A. (1971). A Manual for the Practical Study of Root-Nodule Bacteria.
- Belay, Z., & Assefa, F. (2011). Symbiotic and phenotypic diversity of Rhizobium leguminosarum bv. viciae from Northern Gondar, Ethiopia. *African Journal of Biotechnology*, *10*(21), 4372-4379.