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Research Article

Isolation and Biochemical Characterization of *Rhizobium* strains from Root nodules of Field Pea *Pisum sativum var. abyssinicum*

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Abstract

A study was conducted to isolate and characterize indigenous *Rhizobium* strains isolated from root nodules of field pea (*Pisum sativum var. abyssinicum)*. The selected Rhizobial isolates were originated from southern Tigray having long history of field pea growing with uninoculation. All the morphological, physiological and biochemical characteristics of the Rhizobium species were done in Mekelle soil research center, soil microbiology laboratory following the standard procedures. The morphological characteristics of the *Rhizhobium* sp. was rod shaped, gram negative and mucous producing. All the rhizobial isolates had recorded positive response to the biochemical characteristics of catalase, lysine, lipase, Hofer's alkaline Broth and BTB test. They were found to be temperature and pH resistance at 15-25^{o}C and 5.5-6.5 respectively. More than half of the isolates were sensitive to salinity levels beyond 2% and all of the tested isolates were resistance to metal salts of $ZnSO_4.7H_2O$ and $MnSO4.4H_2O$. Field pea (Pisum sativum var. abyssinicum) rhizobial isolates were assessed against various antibiotics resistance including Ampicillin, Erythromycin, Rifampicin and Streptomycin. Rifampicin and Ampicillin were more resistance comparing to the remaining antibiotics. The rhizobial population of the study sites recorded was $3.1x10^4$ and $1.0x10^5$ per gram of soil which indicates dominantly enough for nitrogen fixation.

Keywords: *Rhizobium* strains, *Pisum sativum var. abyssinicum,* biochemical characteristics, antibiotics resistance.

Introduction

Field pea (*Pisum sativum* L.) is one of the most important highland pulses grown in Ethiopia followed to faba bean (*Vicia faba* L.) and chickpea (*Cicer arietinum)* in terms of production and area coverage (CSA, 2015). Accordingly the agency reported that the area coverage for production and its total yield production of field pea in 2014/15 cropping season has been 230,660.38 ha and 342,636,780 kg respectively. Although field pea is an important cool season pulse grown in Ethiopia, while its productivity is very low compared to other growing areas in the world (Kelley *et al*., 2000). The major constraints of this low production of field peas are poor soil fertility, susceptibility to pests (*Bruchus pisorum* L.) and disease (Mulusew *et al*., 2010). Among them, soil fertility is one of the major constraints for low field pea production in Ethiopia (Tsigie and Woldeab, 1994). To alleviate the production up to 61% by N and P inorganic fertilizers application several researches were done (Tsigie and Woldeab, 1994). However inorganic N application alone had no significant effect on most agronomic measurements in Bale and Arsi research areas (SnARC, 2010). On the other hand, field peas have the inherent ability to obtain much of its nitrogen (N) requirement from the atmosphere by forming a symbiotic relationship with Rhizobium bacteria in the soil (Schatz and Endres, 2009). Accordingly, in the soil system it accounts so many microorganisms in 1 g of soil which contains a community of 10^9 microorganisms and *Rhizobia* representing around 0.1% of soil microbes or 10^6 g-1 soil (Zeenat *et al*., 2017). *Rhizobia* are one of the most efficient bacterial symbionts of legumes that fix atmospheric nitrogen by the process of biological nitrogen fixation (BNF). They able to metabolize atmospheric nitrogen and convert it into plant usable form In return, *Rhizobia* utilize the carbon substrates derived from the plant photosynthesis. In agricultural land 80% of the biologically fixed nitrogen were comes from the symbiosis concerning to the leguminous plants and bacteria of family *Rhizobiaceae* (Zeenat *et al*., 2017). Field peas obtain part of their nitrogen (N) requirement from the atmosphere by forming a symbiotic relationship with a root nodule forming *bacterium.* Under favourable conditions, field pea can derive 65-70% of accumulated N from the atmosphere through the action of biological N_2 fixation (Jensen, 1997). *Rhizobium*-legume symbiosis has been widely studied as a model of mutualistic associations and as a beneficial association for sustainable agriculture.

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With increasing use of *Rhizobium* and other beneficial microbes as bio fertilizers, reduction in the need for chemical fertilizers can be observed. According to Vessey (2006) and Erman *et al.* (2011) report, utilization of biofertilizer has great importance in decreasing environmental pollution and deterioration of nature. Inoculation of seeds with *Rhizobium* strains increased nodulation, N uptake, and growth and yield parameters of legume crops (Erman *et al.*, 2011). There is huge amount of literature available, reporting rhizobia from different pulses (Hou *et al*., 2009; Wadhwa *et al*. 2011; Riah *et al*. 2014) but limited studies are there about the biochemical characterization of rhizobia inhabiting field pea. Therefore, this study was aimed to isolate and test the biochemical characterization of *Rhizobium* strains from Root nodules of Field pea (*Pisum sativum var. abyssinicum*) for better agriculture growth.

Materials and methods

Collection and extraction of root nodules: Root nodules of field pea (*pisum sativum var. abyssinicum*) were collected from Southern Tigray Emba alaje and Endamohoni districts by considering the cropping history of *Rhizobium* inoculation of nodulating field pea. The root nodules were washed with tap water and pink color nodules were detached from the plant roots and kept in vial containing silica gel covered with a cotton plug until isolation. After a week of sampling, the detached nodules were washed in tap water to remove the adhering soil particles from the nodule surface and surface sterilized briefly with 70% ethanol and 4% (v/v) sodium hypochlorite for 10 sec and 3 min respectively, and then rinsed five times with sterilized distilled water (Somasegaren and Hoben, 1994). Sterile nodules were crushed immediately with the help of flamed glass rod in 0.1 N NaCl to obtained milky suspension of bacteriods and streaked to newly prepared yeast extract manitol (YEM) agar medium containing Congo red (CR) (25 mg L^{-1}). The plates were labeled as F1 to F3 to represent the sampling sites and incubated at BOD incubator as of 28±2°C which is optimum growth temperature for microorganisms for 3-5 d then re-streaked on newly prepared YEMA+CR to obtain pure culture. Pure and single colony isolates were picked from plates, labeled and stored on YEM agar slants containing 0.3% (W/V) $CaCO_3$ at 4°C refrigerator for further characterization. The isolates were coded as MSRCDRI (Mekelle Soil Research Center Dokoko Rhizobial Isolates) for identification and stored.

Serial dilutions of the extracted root nodules: After the extraction of bacteroid solution from the root nodules of field pea (*Pisum sativum var. abyssinicum*), serial dilution was made starting from 10^{-1} to 10^{-8}. About 2 mL of sterilized root nodule bacteroids solution was taken in to 90 mL sterilized distilled water and serially diluted up to 10^{-6} dilution. For identification of the colonies, 10^{-4} to 10^{-6} dilution of nodule extract were placed on YEMA Congo red agar media plates and then kept in BOD incubator at 28 ± 2°C for 3-5 d. After the plates were taken out of the incubator colony morphology (its size and texture) was identified using soil microbiology manual standard procedures carried out. All bacteriological isolation and the entire process of biochemical tests were carried in the laminar air flow to maintain the sterility.

Enumeration of Rhizobia: According to Somasegaren and Hoben (1994), enumeration of indigenous rhizobium was counted using different counting techniques. There are few new techniques with which to quantify rhizobia, in this study the most probable number (MPN) plant infection (indirect method) was used to enumerate rhizobia capable of nodulating field pea (*Pisum Sativum var. Abyssinicum*) from the collected districts following the standard procedure made by Somasegaren and Hoben (1994). A 10-fold serial dilution was used; one gram of 2 mm sieved soil samples was diluted in aseptic conditions in to 9 mL sterilized distilled water. About 1 mL from 1st dilution was transferred in to 9 mL sterilized water up to 10^{-8} cells mL^{-1} and was used to inoculate the seedlings aseptically from the 1st dilution to 10^{-8} cells mL^{-1} separately which was grown in acid treated and autoclaved river sand. Nodule observations were made after 30 d of inoculation. Positive and negative nodulation of growth were recorded for all dilution and converted in to number of rhizobia per gram using MPN table (Somasegaren and Hoben, 1994) following the formula indicated below.

$$X = \frac{m \times d}{V}$$

Where:
X= the number of Rhizobia from the soil
m=Likely number from the MPN table for the lower dilution of the series
d=Lowest dilution (first unit used in the tabulation)
V=Volume of aliquot applied to plant

Gram staining of the bacterial strain: The pure cultures of bacterial strains were put for gram staining for more specific identification of the colonies. The gram staining was done in laminar air flow hood. The slides were firstly washed with ethanol and colonies were marked on the slides with the help of inoculating needle and were heat fixed. Then smears were stained in following steps:
a) First applied crystal violet on each slide and kept for 1 min.
b) Distilled water wash.

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c) Iodine on the slides as mordant (1 min) then 95% alcohol wash (30 sec) and then washed with distilled water.
d) Safranin was applied on the slides and then washed with distilled water and air dried the slides.

Effect of salt: The salinity tolerance was assessed by culturing the bacteria on YEMA medium containing different salt concentration 1%, 2%, 3%, 4% and 5% (w/v) NaCl (Lupwayi and Haque, 1994).

pH variation assay: The ability of *Rhizobial* isolate to grow at different pH was tested in YEMA medium by adjusting the pH to 4.0, 5.5, 6.5, 8.5, 9.0 and 9.5 with NaOH and HCl (Lupwayi and Haque, 1994).

Temperature tolerance: Temperature tolerance was investigated by assaying the growth of bacterial cultures in YEMA medium at different temperature levels 5, 15°C, 25°C, 35°C and 40°C (Lupwayi and Haque, 1994).

Effect of metal salts: The isolate was tested for their sensitivity to different concentrations of metal salts by supplemented to YEM solid media plates containing $ZnSO_4.7H_2O$ at 0.125, 0.250, 0.500, 1.0, 2.0 mg/L and $MnSO_4.4H_2O$ at 0.75, 1.5, 3.0, 6.0, 12 mg/L levels. Each isolates were streaked in laminar flow to allow contaminations and incubating at 28±2°C for 3-7 d (Ausili *et al*., 2002). After 7 d of incubation the effect of metal salts was determined by assaying *Rhizobial* growth.

Intrinsic Antibiotic Resistance (IAR): The IAR of isolates to different antibiotics at different concentration were determined by streaking each isolate on solid YEM medium containing filter sterilized antibiotics using 0.22 µm size membrane filters (mg/L) Ampicillin (2.5 and 5), Erythromycin (0.1 and 2.5), Rifampicin (2.5 and 5) and Streptomycin (2.5 and 5) (Beynon and Josey, 1980). Erythromycin was dissolved in ethanol, whereas the others were dissolved in sterilized distilled water (Somasegaren and Hoben 1994). The stock solutions of antibiotics were added to autoclaved YEMA media cooled approximately to 45°C.

Biochemical studies

Catalase test: The catalase test of the isolates was performed to test the presence of enzymatic catalase in which the *Rhizobium* strains to hydrolyze hydrogen peroxide (H_2O_2) into water (H_2O) and oxygen (O_2). A loop full of the tested isolates was smear in clean and autoclaved glass slide separately, and then a few drops of H_2O_2 were added to the slide. Production of gas bubbles and effervescence showed a positive test.

Bromothymol blue test: It selectively identifies fast and slow growing isolate of *Rhizobium*. A loop full of 48 h old culture broth of the isolates were streaked on YEMA medium containing 0.025% BTB indicator and incubated for 3-7 d for acid or alkaline reaction at 28±2°C after 7 d of incubation colour changes were measured (Jordan, 1984).

Lipase test: The field pea rhizobial isolates was tested for their presence of lipase around bacterial colonies by supplementing YEM solid media with 1% (w/v) Tween 80. The tested isolates were tested in triplicates.

Lysine decarboxylase test: In this test, *Rhizobium* strains were streaked on Bromocresol Purple Falkow medium containing peptone 5 g, yeast extract 3 g, glucose 1 g, Bromocresol purple 0.02 g/L of distilled water. Then *Rhizobial* strains were streaked on the media and were kept for incubation at 34°C for 24 h.

Hofer's Alkaline Broth test: In this study, the *Rhizobium* strains were added to Hofer's alkaline broth to distinguish *Rhizobium* from *Agrobacterium radiobacter* (Vincent, 1970; Gupta, 2000). Hofer's alkaline broth medium contains (0.5 g KHPO4, 0.2 g MgSO4, 0.1 g NaCl, 0.05 g CaCO3, 1.0 g yeast extract and 10 g mannitol in a litter of distilled water). The pH of the stock solutions were adjusted to 11.0 using 1N NaOH and 1 ml of 1.6% thymol blue.

Results and discussion

Field pea is one of the most common legume crops with long history in Ethiopian agriculture, which is known to form symbiosis with nitrogen fixing bacteria. However, *Rhizobium* bacteria associated with this particular crop have not been investigated so far. A total of 10 field pea bacterial strains were isolated from root nodules of field pea (*pisum sativum var. abyssinicum*), which is obtained from southern Tigray, Ethiopia.

Enumerating indigenous Rhizobia nodulating field pea: The indigenous rhizobia population per gram of soil which is nodulating field pea (*pisum sativum var. abyssinicum*) and soil physico-chemical properties of the study sites were also evaluated. The most likely number of rhizobia specific to the host plant was calculated using the tenth fold most probable number table indicated by Somasegren and Hoben (1994). Indigenous soil rhizobial population is one of the most important factors in determining a response to inoculation with a rhizobial strains (Thies *et al*., 1991). The soils collected from the study areas were contained a compatible native rhizobial isolates against native field soil rhizobia. The MPN count of rhizobia in the soils of Emba-alaje and Enda-mohoni were $3.1x10^4$ and $1.0x10^5$ per gram of soil respectively. This indicates that areas having long history of growing legumes have enough background rhizobial population and particularly the study sites have enough rhizobial nodulating field peas (Table 4).

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Similarly Bayou (2015) reported that the density of indigenous rhizobia able to nodulate a particular legume species can vary from 10-10^7 g^{-1} soil and may be attributed to cropping history of the soil.

Morphological characteristics: All the tested isolates were found to be gram negative and did not absorb Congo red from CR-YEMA medium (Table 1). The tested isolates were changed BTB color in to yellowish after culturing on YEMA-BTB medium (Table 1; Fig. 1). This could be considered as an indicator of production of acidic and verifying the common characteristics of fast growing *Rhizobium* sp. (Somasegaren and Hoben, 1994). According to Jordan, 1984 classification those isolates were classified as fast growing root nodule bacteria. Similar results were found by Fano (2010), Aregu *et al*. (2012) and Kassa *et al*. (2015) from previous observations on filed pea nodulating rhizobia. 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). After 3 days of incubation colonies seemed rounded and raised in shape and looks white in color. Eighty percent (80%) of the isolates displayed large mucoid with colony diameter ranging from 1.35-5.33 mm, whereas others which constitute 20% of the test isolates formed large watery with colony diameter ranging from 4.33-5 mm (Table 1). Similar results were reported on rhizobia of field pea isolated from Ethiopian soils (Fano, 2010; Aregu *et al*., 2012; Kassa *et al*., 2015). All the rhizobial isolates originated from the study area had recorded positive response to the biochemical characteristics of catalase, lysine, lipase, Hofer's alkaline Broth and BTB test (Table 1; Fig. 1). The tested isolates were positive response on catalase test by bubble formation around bacterial colonies. Lysine decarboxylase test was performed using bromocresol purple falkow media and color change was observed (Fig. 3). According to Datta *et al.* (2015), lysine decarboxylase test was observed color change in the medium inoculated with *Rhizobium phaseoli*, *Rhizobium trifolii* and *Bradyrhizobium japonicum,* contrary to this no such color change was found in the medium inoculated with *Rhizobium leguminosarum*.

Microscopic Observations: Gram's staining of the isolates was detected by microscopic observations and the *Rhizobium* strains were found to be gram negative (Table 1). Similar results were reported by Aregu *et al*. (2012) and Kassa *et al*. (2015) on rhizobial field pea from Ethiopian soils.

Effect of salt, pH, temperature and metal salts on Rhizobium growth: The physiological tolerance of rhizobia in response to various environmental stresses that compromise survival could assist in selection of robust strains more compatible with seed coating application (Deaker *et al*., 2004).

Fig. 1. Effect of Biochemical tests (Lipase test, BTB, Lysine and H_2O_2) on growth of *Rhizobium* isolates.

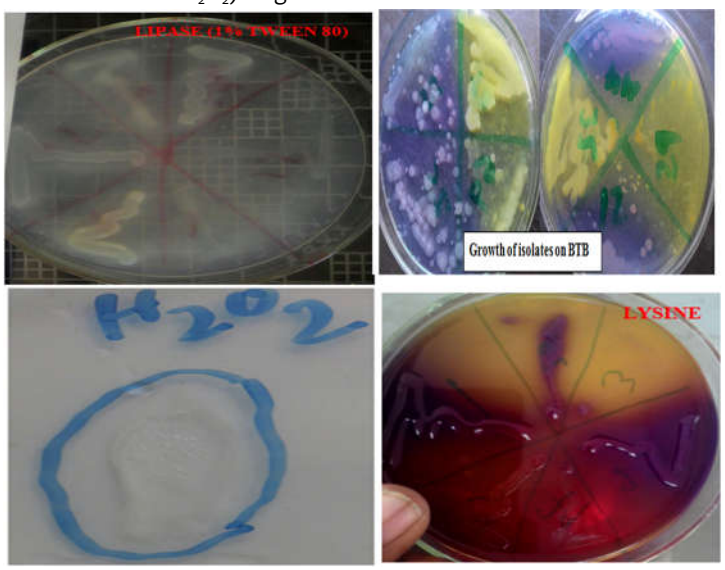

Fig. 2. Effects of NaCl, pH and different Temperature levels on rhizobial isolates.

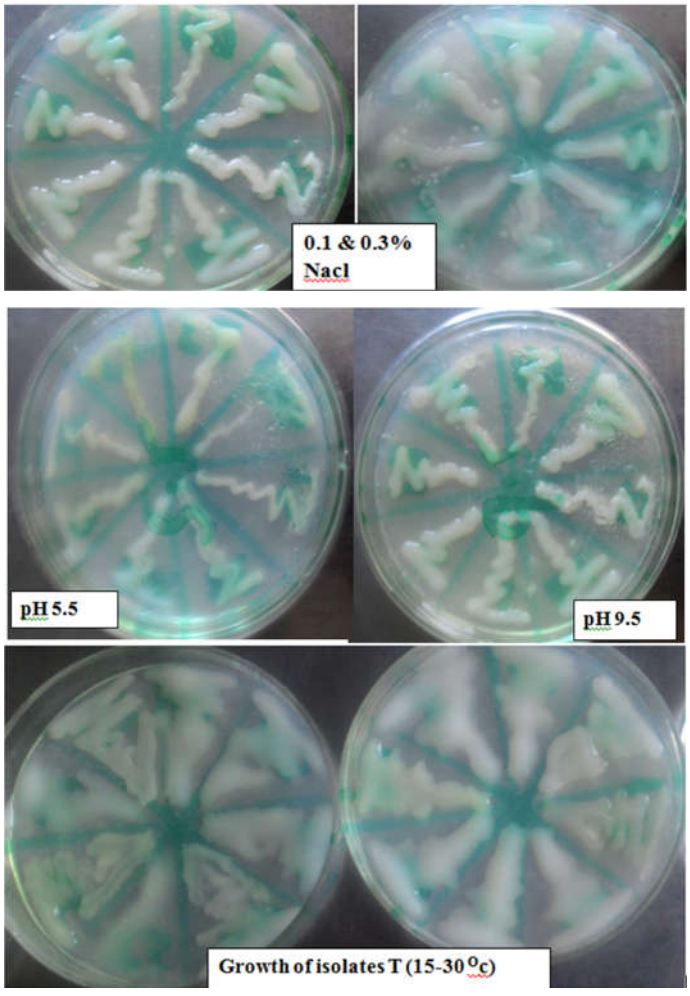

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Table 1. Morphological and biochemical characteristics rhizobial isolates.

Isolates	CM	CS (mm)	GBTB	YEMA-CR	Catalase test	Lipase test	Lysine tset	HAB	Gram stain
MSRCDRI-1	LM	1.42	Y	CL	+	+	+	-	-
MSRCDRI-3	LM	2.35	Y	CL	+	+	+	-	-
MSRCDRI-20	LM	5.33	Y	CL	+	+	+	-	-
MSRCDRI-21	LW	5.00	Y	CL	+	+	+	-	-
MSRCDRI-23	LM	4.23	Y	CL	+	+	+	-	-
MSRCDRI-25	LM	3.33	Y	CL	+	+	+	-	-
MSRCDRI-30	LW	4.33	Y	CL	+	+	+	-	-
MSRCDRI-52	LM	4.23	Y	CL	+	+	+	-	-
MSRCDRI-54	LM	5.00	Y	CL	+	+	+	-	-
MSRCDRI-79	LM	3.67	Y	CL	+	+	+	-	-

Where; MSRCDRI = Mekelle Soil Research Center Dokoko Rhizobial Isolate, LM= large mucoid, LW= large watery, CM= colony morphology, CS= colony size, GBTB = growth on bromotymole blue, Y= yellow, CL= color less, HAB= Hofer's Alkaline Broth.

Table 2. Physiological and metal salts effects on field pea *Rhizobium*.

Temperature (°C)	5	15	25	35	40
Total isolates	0	10	10	7	1
%	0	100	100	70	10
pH	4.0	5.5	6.5	8.5	9.5
Total isolates	0	10	10	10	10
%	0	100	100	100	100
NaCl (%)	1	2	3	4	5
Total isolates	6	5	5	4	3
%	60	50	50	40	30
Metal salts					
$ZnSO_4.7H_2O$ (mg/L)	0.125	0.250	0.50	1.0	2.0
Total isolates	10	10	6	5	3
% resistance	100	100	60	50	30
$MnSO4.4H_2O$ (mg/L)	0.75	1.5	3.0	6.0	12
Total isolates	9	7	5	4	4
% resistance	90	70	50	40	40

Table 3. The effect of antibiotics on field pea *Rhizobium* strains.

Antibiotics	% of resistance isolates	
	2.5 µg/mL	5.0 µg/mL
Ampicillin	70	70
Erythromycin	40	20
Rifampicin	90	80
Streptomycin	20	10

Table 4. The soil physico-chemical and most probable number.

District	pH	OM	Total N	Ava. P	CEC meq/100g soil	Texture	MPN
E/Alaje	6.42	1.72	0.09	18.78	40.2	SCL	$3.1x10^4$
E/Mohoni	6.38	1.92	0.14	17.7	43.40	SCL	$1.0x10^5$

Where; OM= organic matter, MPN= most probable number, SCL= sandy clay loam.

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All the isolates (100%) grew well in the range of 15-25°C and none of them were grown at 4°C (Table 2; Fig. 2). Different findings on field pea isolates from Ethiopian soils indicated that almost all isolates were tolerant to 5-38°C while up to 50% of the isolates were also grown at 5-10°C (Fano, 2010; Aregu *et al*., 2012; Kassa *et al*., 2015). In the soil environment the condition is highly different. All the native rhizobial strains were able to survive well in the various soils adjusted to pH (5.5-9.5) and none of the isolates were grew at pH 4 (Table 2; Fig. 2). This indicates that the isolates were applicable site specific, sites/soils having low soil pH (pH <4) doesn't recommend those rhizobial isolates. This study was in agreement with Fano (2010) almost all the isolates grew on pH range of 5-9 of the same cultivar. Contrary to this study, Aregu *et al*. (2012) and Kassa *et al*. (2015) reported that all isolates were grown at pH range of 4.5-9. It is known that high salt concentration can affect the growth of *Rhizobia* (Hanaa and Mustapha, 2009). In this study, *Rhizobial* isolates were tested on salt concentration ranging from 1-5 % (w/v) Nacl (Table 2; Fig. 2). The native *Rhizobial* strains isolated from the study areas were able to grow variably throughout the different sodium chloride range tested from 1-5% indicating the fact that native *Rhizobial* strains were more adaptive to soils highly concentrated with various forms of cations and anions (Table 2). Similarly Aregu *et al*. (2012) also reported that *Rhizobium* isolates were capable of growing at NaCl concentrations up to 6% the same cultivar. Contrary to this study, Kassa *et al*. (2015) isolated field pea rhizobia from eastern, Ethiopia that were very sensitive to NaCl concentrations more than 0.1%. The bacterial isolates of the current study also tested to different harmful metal salts having different concentrations of $ZnSO_4.7H_2O$ and $MnSO_4.4H_2O$ (Table 2). Hundred percent of the tested isolates were more resistance to $ZnSO_4.7H_2O$ with 0.125 and 0.250 mg/L concentrations, while as the metal salt concentration increases 0.5 mg/L the resistance of the rhizobial isolates were decreased (Table 2). The growth of the isolates on different $MnSO_4.4H_2O$ metal salt concentrations indicated that 70-90% of the isolates were resistance to 0.75 and 1.5% $MnSO4.4H_2O$ concentrations respectively. Similarly the resistance of rhizobial isolates decreased as the metal salt concentration of $MnSO4.4H_2O$ increase beyond 3.0% (Table 2).

Intrinsic Antibiotic Resistance (IAR) spectra: Filed pea (*pisum sativum var. abyssinicum*) rhizobial isolates were assessed against various antibiotics including *Ampicillin*, *Erythromycin*, *Rifampicin* and *Streptomycin* having two levels (2.5 and 5 µg/mL) (Table 3). All of the tested isolates were relatively resistance to the respective antibiotics accordingly; *Rifampicin* and *Ampicillin* were more resistance to the tested *Rhizobial* isolates, while most of the *Rhizobial* isolates were sensitive to *Streptomycin*.

The degree of rhizobial resistance was decrease as the concentration of antibiotics increased. Ninety percent (90%) of the isolates were grown on Rifampicin at 2.5 µg/mL and 20% of them were grown on Streptomycin with the same antibiotics concentration. Relatively 80% and 10% of the isolates were grown at 5 µg/mL Rifampicin and Streptomycin respectively (Table 3). This variation of growth in different antibiotics concentration indicates that the diversity among the rhizobial isolates. The highest competition ability may directly influence the N_2-fixing ability of the plant correlated with the increase in the yield (Thies *et al*., 2001).

Conclusion

From this study, it can be concluded that the screening of the effective isolates on biochemical tests are important for further investigation on field pea growing areas of Ethiopia. Most of the tested isolates were able to tolerate wide range of pH, NaCl, temperature, antibiotics as well as utilized wide range of biochemical testes. Therefore, the presence of diversity from the study areas revealed the possibility of getting potentially effective indigenous rhizobial isolates that elevate field pea production. Hence, studies need for inoculation and factors responsible for poor nodulation need to be undertaken to realize the importance of biological nitrogen fixation in Tigray region, particularly in the study area.

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