

## Characterization of Phosphate Solubilizing Faba Bean (*Vicia faba L.*) Nodulating Rhizobia Isolated from Acidic Soils of Wollega, Ethiopia

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### Abstract

Some species of rhizobial bacteria nodulating faba bean are characterized by phosphate solubilization. In order to study their *in vitro* and symbiotic characteristics, twelve rhizobial isolates nodulating faba bean were collected from acidic soil of Wollega, Ethiopia. Solubilization index of the isolates ranges from 1.25 to 2.10. Mean Generation Time of the isolates were less than 2.34; and growth of isolates on Yeast Extract Mannitol agar with bromothymoleblue media were accompanied by change of color from blue to yellow. AUAVR-51 and AUAVR-52 were the highest in TCP-Solubilization Index. The two isolates were also recognized as stress tolerant when tested *in vitro* for extreme temperature, osmotic, acidic pH, intrinsic antibiotics, and acidic-aluminum as compared to the other isolates. However, there was no unique metabolic diversity and specialization of AUAVR-51 and AUAVR-52 isolates with respect to carbon and nitrogen source utilization. On the other hand, isolates AUAVR-51 and AUAVR-52 were characterized by effective and highly effective symbiosis on sterile potted sand growth, respectively. In general, phosphate solubilizing rhizobia nodulating faba bean from acidic soil are fast grower; and their solubilization potential varies. However, the contribution of these isolates as double fertilizer should be tested in the real acidic soil characterized by immobilized phosphorous.

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## INTRODUCTION

Faba bean (*Vicia faba L.*) is a leguminous crop grown in Ethiopia mainly in "Weyna Dega" climatic condition that ranges in altitude from 1800 to 2400 m.a.s.l. Grain seeds of the crop is known by high protein content. This is due to the effective symbiotic nitrogen fixing bacterial species generally called *Rhizobium leguminosarum* bv. *Viciae* that form association with roots of the crop (Zahran, 1999). Faba bean in association with the strain can fix up to 325 kg of N<sub>2</sub> h<sup>-1</sup> y<sup>-1</sup> (Somasegaran and Hoben, 1994). As a result, the crop is found to be very efficient N<sub>2</sub> fixer and can meet its entire nitrogen requirement through BNF (Abere Mnalku *et al.*, 2009; Zerihun Belay and Fassil Assefa, 2011 and Anteneh Argaw, 2012). This also improves soil nitrogen content that is in turn increases the yield of subsequent crops in cropping system.

Effective symbiotic association between rhizobial strain and host depends on different soil condition such as salinity, drought, acidity, and soil temperature (Zaharan, 1999). Although some parts of Wollega are major faba bean producing area, the symbiosis could be harnessed by a very low pH, a very high exchangeable acidity, low calcium, and potassium properties of the soil (Abdena Deressa *et al.*, 2013). The low pH of the soil results in immobilization of phosphorous by aluminum, and iron

ions. As a result, phosphorous availability as plant nutrient is limited, which in turn strap up energy acquisition for nitrogen fixation. However, there are legume nodulating bacteria such as *Rhizobium*, *Bradyrhizobium*, and *Mesorhizobium* strains possessing properties of solubilizing immobilized organic and inorganic phosphate sources (Halder *et al.*, 1990 and Peix *et al.*, 2001). Thus, besides the fixation of atmospheric nitrogen to its utilizable form, these bacteria also contribute to the growth of plants through solubilizing inorganic phosphates of low solubility. Specifically, the better potential of *Rhizobium leguminosarum* bv. *viciae* in solubilizing inorganic phosphate sources were reported by Alikhani *et al.* (2006).

On the other hand, there is variation in stress tolerance among different strains of *Rhizobium leguminosarum*. These variability is an important tools to measure the survival advantage of one strain over the other in the sever soil environment (Rice *et al.*, 1977). Strains resistant to different soil stresses have potential to improve the production of legumes grown on the area; and extend the ranges of soils upon which legumes adapted to grow (Munns, 1978). Metabolic versatility and specialization among rhizobial strains enables some strain

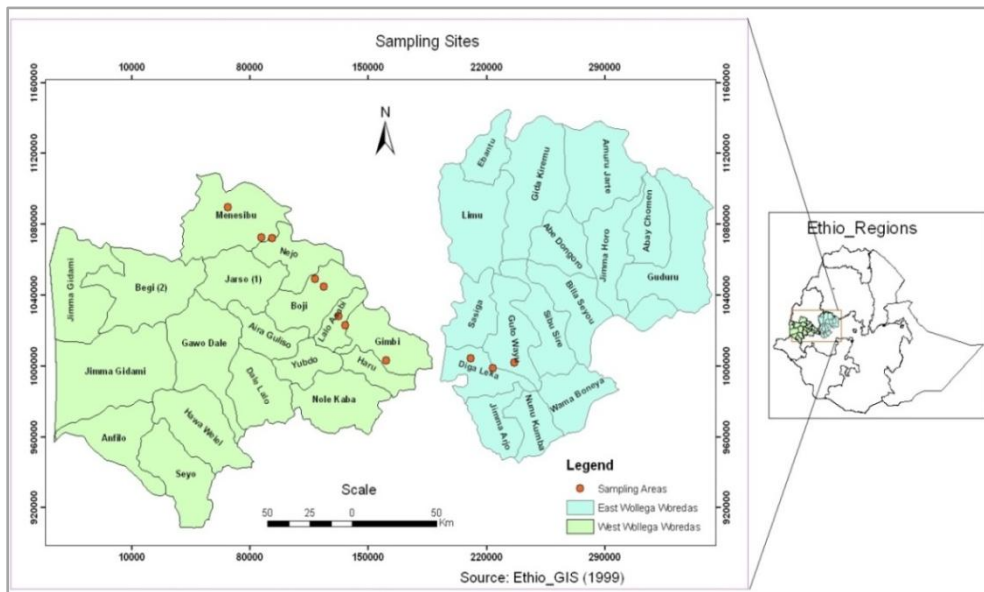
to persist in adverse environments and compete successfully with other bacteria (Mazur *et al.*, 2013). Therefore, the ability of legume-nodulating rhizobia to solubilise different inorganic phosphates sources, the potential to tolerate wide range of stress, and versatility in metabolism should be considered when selecting strains as biofertilizer. These properties are important for competitiveness and survival of inoculants with indigenous microflora. Thus, the main objective of this study was to isolate and characterize phosphate

solubilizing faba bean rhizobial isolates from acidic soil of Wollega.

**MATERIALS AND METHODS**

**Rhizobial Bacteria Isolation**

Faba bean root nodules were collected from farmers' field during its flowering stage in vials containing silica gel plugged with cotton. Totally 44 root nodule samples were taken from faba bean producing areas of Wollega (figure 1).



**Figure 1:** Sampling area.

Isolation and preservation of root nodule bacteria were carried out as described in Vincent (1970). Purity of the isolates was checked by Congo Red absorption on Yeast Extract Mannitol Agar (YEMA) (in g/l of distilled water; mannitol, 10; yeast extract, 1; K<sub>2</sub>HPO<sub>4</sub>, 0.5; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.2; NaCl, 0.1; agar, 15 and congoled, 0.025), and growth on peptone-glucose agar (in g/l distilled water, glucose, 5; peptone, 10; agar, 15 and pH, 6.7).

**Soil pH Measurements**

The collected soil samples from the sampling sites were dried and sieved to less than 2 mm; then 10 g of the soils were dissolved in 50 ml of deionized water (1:5 ratios). The container was shaken for about 3 min and the residue was allowed to settle for 2 min. Finally, pH readings were taken by pH meter.

**Phosphate Solubilization Test**

Tri-calcium phosphate solubilization (TCP) potential of the isolates was determined using Basal Sperber agar media (Sperber, 1958). Component of Basal Sperber agar media was (in g/l of distilled water: glucose, 10.0; yeast extract, 0.5; CaCl<sub>2</sub>, 0.1; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.25; and agar, 15.0; Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub>, 2.5; and pH adjusted at 7.2 before autoclaving). Solubilization Index (SI) was estimated according to Edi-premono *et al.* (1996).

**Authentication**

Authentication of the rhizobial isolates was conducted in Growth Pouch using faba bean called "Moti" obtained from Holeta Agricultural Research center. The procedure for seed surface sterilization was the same as the one described for nodule surface sterilized. The seedlings were inoculated with the rhizobial isolates grown on YEM broth (1ml/seedling) after appearance of secondary leaf. Growth Pouches containing uninoculated seedling were included as controls. All Growth Pouches were fertilized with quarter strength of Broughton and Dilworth N-free medium twice per week (Somasegaran and Hoben, 1994). Distilled sterile water was added whenever required. After 30 days of planting, the crops were checked for nodulation both in the inoculated and uninoculated Growth Pouches. The authenticated isolates

**Table 1:** Sampling area of the isolates.

Isolates	Sampling Sites			Soil pH
	Zone	Woredas	Kebeles	
AUAVR-12	EW	Gudeya Bila	Jawaja	4.8
AUAVR-13	"	Bila Sayo	Anno	4.8
AUAVR-14	"	"	Anno	4.6
AUAVR-29	"	"	Tilili	4.9
AUAVR-30	"	Sibu Sire	Chafe	4.9
AUAVR-34	"	"	Chafe	4.8
AUAVR-39	"	"	Moto	5.0
AUAVR-47	"	Gute	Warababo	5.2
AUAVR-49	WW	Gimbi	Enango	6.3
AUAVR-51	"	"	Enango	5.6
AUAVR-52	"	"	Lalisa Eyasus	5.3
AUAVR-53	"	Lalo Asabi	Jiru	5.3

EW (East Wollega); WW (West Wollega)

**Growth media, Incubation and Experimentation**

In the entire of this experiment the growth media was YEMA, and incubation temperature was 28±2°C for 5 days. All growth media were sterilized at 121°C for 15 minutes, and 72 hrs old liquid growth cultures with approximate viable cell number of 10<sup>9</sup> ml<sup>-1</sup> was used for inoculation unless stated otherwise.

were designated as AUAVR (Addis Ababa University Acidic soil *Vicia faba Rhizobium*) with different numbers to differentiate one isolate from the other.

### Morphological Growth & Biochemical Characteristics of the Isolates

The isolates were characterized by colony morphology and acid/alkaline production on YEMA plus 25 µgml<sup>-1</sup> Bromothymolblue (BTB) media (Ahmed *et al.*, 1984). Growth rate of the rhizobial isolates was estimated by measuring optical density (OD) using spectrometer from YEM-broth. OD readings were taken at 540nm beginning from time of inoculation (0hr) up to the 96<sup>th</sup> hour at every 6hrs interval. Finally, mean generation time (g) was calculated from the logarithmic phase as indicated on White (1995).

### Stress Tolerance Test

Tolerance of the isolates to extreme temperature, pH, and salt was checked according to Maatallah *et al.* (2002). Intrinsic antibiotics resistance (IAR) of isolates to Kanamycin sulfate, Streptomycin sulfate, Chloramphenicol, Ampicillin, Neomycin sulfate and Erythromycin with concentration of (µg/ml) 2.5, 5, and 10 was tested. Metals toxicity tolerance level of the isolates was checked at concentration of (µM) 50, 100, and 150 on Keyser solid media (Lupwayi and Haque, 1994). The metals were Aluminum (Al) in the form AlK(SO<sub>4</sub>)<sub>3</sub>.12H<sub>2</sub>O and Manganese(Mn) in the form of MnCl<sub>4</sub>.4H<sub>2</sub>O. Stock solutions of the antibiotics and metals was prepared; and sterilized by membrane filtration(pore size of 0.22µm). The required concentration was added to autoclaved media that was cooled to 50<sup>o</sup>c on water bath.

### Substrate Utilization Test

Growth of isolates on different Carbon and nitrogen sources was checked. The carbon sources were added at a final concentration of 1 g/l to a basal medium containing (per liter of distilled water: 1 g of K<sub>2</sub>HPO<sub>4</sub>, 1 g of KH<sub>2</sub>PO<sub>4</sub>, 0.01 g of FeCl<sub>3</sub>. 6H<sub>2</sub>O, 0.2 g of MgSO<sub>4</sub>.7H<sub>2</sub>O, 0.1 g of CaCl<sub>2</sub>, 1 g of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, and 15 g of agar. The amino acids were added at a concentration of 0.5 g/l to a similar media from which ammonium sulfate was omitted and mannitol was added at a concentration of 1 g/liter (Amarger *et al.* 1997). The carbohydrates sources were D-arabinose, D-galactose, D-mannose, Maltose, Dextrin, D-glucose, D-fructose, Sucrose and Mannitol. The nitrogen sources were L-asparagine, L-glutamate, L-tryptophan, L-tyrosine, L-alanine, L-arginine, Methionine, and Lysine. All of the substrates were filter sterilized using 0.22µm membrane and added to autoclaved basal media that was cooled to 50<sup>o</sup>C on water bath.

### Symbiotic Efficiency Test on Sterile Sand

Plastic pots (4kg capacity) were filled with acid washed and autoclave sterilized sand. Seed surface sterilization and seedling inoculation were done as described before for authentication. Both positive and negative controls were included as treatments. The positive control pots received 120 ml of 0.05% (w/v) of KNO<sub>3</sub> during inoculation and after 21 days as nitrogen source, whereas the negative control pots were devoid of both nitrogen sources and rhizobial isolates. After 45 days of planting, plants were harvested and the symbiotic effectiveness parameters such as nodule number(NN), nodule dry weight(NDW) (g/plant), and shoot dry weight(SDW) (g/plant) were recorded, and relative symbiotic

effectiveness(%SE) was calculated according to (Beck *et al.*, 1993). Using the formula set by Beck *et al.* (1993) percentage symbiotic effectiveness of the isolates was calculated. Shoot dry weight and nodule dry weight were measured after oven drying at 70<sup>o</sup>C for 48hrs.

### Data Analysis

Comparison among treatments was analyzed by one-way ANOVA (Turkey's and Tamhane's T2 tests) using the statistical program SPSS-15 software.

## RESULTS AND DISCUSSION

Faba bean root nodule inhabiting bacteria were isolated and tested for TCP-solubilization on agar media. Totally, twelve isolates were found to be TCP-solubilizer. Soil pH of sampling sites ranges from 4.8 to 6.3 (table 1). The isolates were gram negative and rod-shaped bacteria; and re-nodulated the host legume with pink/red color nodule. This proves that the bacterial isolates were rhizobia that form symbiotic association with faba bean. It is already identified that *Vicia faba* nodulating rhizobia are *Rhizobium leguminosarum* bv. *Viciae* (Somasegaran and Hoben, 1994). The rhizobial isolates changed the blue color of YEMA-BTB to yellow which is the characteristic of fast growing rhizobia due to acid production (Jordan, 1984). The change in color of YEMA-BTB from blue to Yellow by faba bean rhizobial isolates was also reported by Zerihun Belay and Fassil Assefa (2013).

Colony diameters of the isolates were in the range of 3 to 5 mm (table 2). The largest colony diameter was displayed by AUAVR-34. All isolates displayed large and mucoid colonies texture. Mean generation time of the rhizobial isolates was in the ranges of 1.43 to 2.34 hrs (table 2). Isolate AUAVR-49 was the lowest in mean generation time. Production of organic acid, large colony size, and short mean generation time is the characteristics of fast growing rhizobial isolates (Sadowsky *et al.*, 1983). Characteristic of the isolates such as colony diameter, mean generation time, colony texture, and growth properties on YEMA+BTB proved that all the isolates were fast-grower. The same was reported by Zerihun Belay and Fassil Assefa (2011) for *Vicia faba* rhizobia isolated from North Gonder, Ethiopia. Nevertheless, there was no correlation between soil pH of the sampling area, colony diameter, and mean generation time in the current research (table 2).

The Phosphate Solubilization Index (PSI) of the rhizobial isolates was in the range of 1.25 to 2.10 (table 2). AUAVR-51 and AUAVR-52 were the highest solubilizers. According to Alikhani *et al.* (2006), among the different rhizobial species collected from different kinds of host, a group of *Rhizobium leguminosarum* bv. *viciae* mobilized phosphate from TCP significantly than other species. The release of soluble phosphate significantly correlated with a drop in the pH of the culture filtrates, indicating the importance of acid production in the mobilization process (Song *et al.*, 2008). Unlike the fast grower- *rhizobium* species, the abundance of TCP-mobilizers among *bradyrhizobium* species are less common (Alikhani *et al.*, 2006). This indicates that TCP-mobilization is linked with acid productions, which is a typical characteristic of fast-grower rhizobia.

**Table 2:** cultural and symbiotic properties of TCP-solubilizer *Vicia faba* rhizobia

Strains	SI	CD	MGT(hr)	NN plant <sup>-1</sup>	NDW plant <sup>-1</sup>	SDW plant <sup>-1</sup>	SE%	
AUAVR-12	1.88	4.5	2.34	101 ±11.27 <sup>a-f</sup>	0.106±0.003 <sup>ab</sup>	1.523±0.473 <sup>b-k</sup>	68.94	Effective
AUAVR-13	1.25	4.5	2.22	98 ±13.12 <sup>a-g</sup>	0.100±0.004 <sup>ab</sup>	1.651±0.479 <sup>a-i</sup>	75.40	Effective
AUAVR-14	1.90	2.5	2.03	104 ±14.73 <sup>a-e</sup>	0.091±0.003 <sup>b</sup>	1.327±0.025 <sup>g-k</sup>	60.27	Effective
AUAVR-29	1.26	3.5	1.46	110 ±8.72 <sup>a-c</sup>	0.081±0.002 <sup>b</sup>	1.790±0.105 <sup>a-e</sup>	81.74	Highly Effective
AUAVR-30	1.27	4.5	1.99	90 ±13.65 <sup>b-h</sup>	0.055±0.004 <sup>b</sup>	1.760±0.131 <sup>a-f</sup>	78.54	Effective
AUAVR-34	1.50	5.0	1.53	107 ±10.58 <sup>a-d</sup>	0.074±0.006 <sup>b</sup>	1.426±0.042 <sup>d-k</sup>	64.84	Effective
AUAVR-39	1.50	4.0	1.5	98 ±12.58 <sup>a-f</sup>	0.063±0.005 <sup>b</sup>	1.407±0.084 <sup>e-k</sup>	64.38	Effective
AUAVR-47	1.31	4.0	1.81	97 ±7.81 <sup>a-g</sup>	0.108±0.012 <sup>ab</sup>	1.740±0.082 <sup>a-g</sup>	79.45	Effective
AUAVR-49	1.31	3.0	1.43	93 ±7.81 <sup>a-h</sup>	0.055±0.011 <sup>b</sup>	1.340±0.020 <sup>f-k</sup>	60.73	Effective
AUAVR-51	2.10	3.0	1.52	87 ±6.25 <sup>b-h</sup>	0.049±0.005 <sup>b</sup>	1.490±0.062 <sup>b-k</sup>	67.58	Effective
AUAVR-52	2.10	3.5	1.65	111 ±6.56 <sup>ab</sup>	0.065±0.007 <sup>b</sup>	1.860±0.062 <sup>a-c</sup>	84.93	Highly Effective
AUAVR-53	1.46	3.5	1.81	87 ±8.72 <sup>b-h</sup>	0.086±0.004 <sup>b</sup>	1.837±0.045 <sup>a-d</sup>	83.56	Highly Effective
+Ve control				-	-	2.190±0.118 <sup>a</sup>	100.0	Highly Effective
-Ve control				-	-	0.401±0.106 <sup>l</sup>	18.26	Less Effective

SI(Solubilization Index), CD(Colony Diameter), MGT(Mean Generation Time), NN(Nodule Number),NDW(Nodule Dry Weight), SDW(Shoot Dry Weight), %SE(Percentage symbiotic Effectiveness)

**Key:** Levels not followed by the same letter/letters were significantly different at  $P<0.05$  (Tukey's b test)

Although the optimum temperature for rhizobia on culture is between 27-39°C (Munevar and Wollum, 1981), some of the current isolates such as AUAVR-14, AUAVR 51, AUAVR 52, and AUAVR 53 were tolerant to low temperature to the level of 5°C. On the other hand, AUAVR 13, AUAVR 47, and AUAVR 53 were tolerant up to 45°C (table 3). Isolate AUAVR-52 showed growth in wide range of temperature (5-40°C). Low temperature tolerance of some isolates in laboratory could grant for inoculant production for the highland pulse crop particularly faba bean. Besides, the wide range of temperature tolerance of faba bean rhizobia could assist the isolates to cop up with current global climatic change.

However, there are contradictory reports on the reproducibility of *in vitro* and *in vivo* results on stress tolerance. According to Dadarwal *et al.* (1981) the tolerance of rhizobial isolates to different environmental stresses in *in vitro* condition has no positive correlation to *in vivo* nitrogen fixation potential. In contrast, Fitouri *et al.* (2012) stressed the selection of rhizobial bioinoculant for field application based on stress tolerance level the isolates during *in vitro* experiment. These contrasting research reports may depend on the type of cultivar, climatic condition of experimental area, and soil type. The two authors also concluded by emphasizing the need of similar trials in other sites using different types of hosts.

**Table 3:** Stress tolerance and substrate utilization properties of TCP solubilizer Rhizobia

Isolates	Temperature (°C)	NaCl (%)	pH		MaTL(µM) at pH 5		Maximum IAR Concentration						Carbohydrates		
	MiTL	MaTL	MiTL	MaTL	Aluminium	Manganese	Ampicillin	Kanamycin	Chloramphenicol	Erythromycin	Neomycin	Streptomycin	Arabinose	Starch	
AUAVR-12	20	40	2	5	10	50	200	5	2.5	10	5	<2.5	<2.5	-	+
AUAVR-13	20	45	0.5	5	9	75	200	10	5	10	<2.5	<2.5	<2.5	+	+
AUAVR-14	5	35	5	5.5	10	Nd	Nd	2.5	2.5	5	<2.5	<2.5	<2.5	+	+
AUAVR-29	15	35	4	5.5	10	Nd	Nd	2.5	2.5	10	10	<2.5	<2.5	-	+
AUAVR-30	10	35	5	6	10	Nd	Nd	5	2.5	5	10	<2.5	<2.5	-	-
AUAVR-34	15	40	3	6	10	Nd	Nd	10	2.5	5	10	<2.5	<2.5	-	-
AUAVR-39	20	30	4	6	10	Nd	Nd	10	2.5	5	10	<2.5	<2.5	-	-
AUAVR-47	15	45	1	5	10	25	200	10	2.5	5	<2.5	<2.5	5	+	-
AUAVR-49	15	30	6	5	10	0	25	10	5	10	<2.5	<2.5	5	+	-
AUAVR-51	5	30	6	4.5	10	75	200	10	5	10	<2.5	<2.5	5	+	-
AUAVR-52	5	40	6	4.5	10	75	200	10	5	10	<2.5	<2.5	<2.5	+	-
AUAVR-53	15	45	1	5.5	10	Nd	Nd	10	5	5	<2.5	<2.5	5	+	-

MiTL(Minimum tolerance level), MaTL(Maximum tolerance level), Nd(Not grow at pH 5); +(growth), -(no growth)

With regard to salt tolerance experiment, three faba bean rhizobial isolates such as AUAVR-49, AUAVR-51 and AUAVR-52, tolerated up to 6% NaCl on YEMA (table 3). This is in agreement with the research reports that stated the tolerance of fast growing rhizobial isolates to

NaCl is up to 5% NaCl (Zerihun Belay and Fassile Assefa, 2011; Mulisa Jida and Fassil Assefa, 2012). However, there is variation in osmotolerance among rhizobial isolates collected from different hosts (Fitouri *et al.*, 2012). Isolates also showed variation in tolerance to pH during

culturing on Keyser solid growth medium. All isolates grew on media adjusted at pH of 6-8 which could be considered as the optimum pH for the growth of the isolates. Although rhizobial isolates showed better tolerance to alkaline pH, only two isolates (AUAVR-51 and AUAVR 52) grew at pH 4.5 (table 3). Similar results were reported by Zerihun Belay and Fassil Assefa (2011) for *vicia faba* rhizobia and by Mulissa Jida and Fassil Assefa (2012) for rhizobia isolated from *Cicer arietinum* L.. According to the current result *Rhizobium leguminosarum* var *viciae* were tolerant on the alkaline growth media as compared to acidic growth media. This could be related to the additional extracellular organic acid release by the fast grower isolates which can aggravated their intolerance at low pH growth media (Sadowsky *et al.*, 1983)

The Intrinsic Antibiotic Resistance (IAR) of the TCP-solubilizer faba bean rhizobial isolates was studied to determine diversity among them. As indicated in table-3 isolates were sensitive to neomycin at all levels of concentration tested. Some isolates were resistant to streptomycine at 2.5 and 5 µg/ml. On the other hand, ampicillin and chloromphenicol were indiscriminatory tools to determine diversity among the isolates. AUAVR-49 and AUAVR-51 were the most resistant isolates to antibiotics as compared to the others. Differences in tolerance to different kinds of antibiotics was reported among strains belongs to same species of rhizobia (Amergar *et al.*, 1997). The difference in IAR among *Rhizobium leguminosarium* isolated from *Vicia faba* was also emphasized on the research report of Zerihun Belay and Fassil Assefa(2011). Young and Chao (1989) also reported variability in IAR of *rhizobium* isolated from same host plant at the same time. Thus, IAR test is change tool to study diversity among *rhizobium* isolated from the same host; in spite of the differences in the action antibiotics.

Isolates were also characterized by growth on basal media containing 12 different carbon sources and eight different nitrogen sources. All isolates showed visible colony on all tested carbon sources, except for arabinose and starch that supported only 58% and 42% of the isolates respectively (table-3). Two isolates (AUAVR-13 and AUAVR-14) utilized all the tested carbon sources. All isolates grew on all amino acids tested. The twelve different kinds of carbon sources and the eight nitrogen sources tested in this experiment failed to show diversity among the isolates. According to Amergar *et al.* (1997) utilization of some specific carbohydrates is the useful traits for differentiating species rhizobia that nodulates beans. However, some of the carbon sources recommended by the author were not included in the current experiment. In the same manner Cepeda and Lucia (2005) also reported that rhizobia that nodulates clover was able to metabolize L-alanine, L- proline and aminobutyric acid, but none of them utilized L-leucine. This indicates that there are some specific substrates that show diversity among phosphate-solubilizer rhizobia. Thus, in order test versatility of the rhizobial isolates with respect to metabolism, diverse kinds of carbohydrates and amino acid should be included.

Tolerance of TCP-solubilizer rhizobia to different concentration of Al and Mn was another parameter to study diversity. Five isolates were sensitive to all concentration levels of the toxic metals at pH 5(table-3). The maximum tolerance level of acidic-Al concentration was 75µM recorded for three isolates (AUAVR-13, 51, and 52). The impact of Mn on the growth of isolates was

insignificant up to the level of 200 µM (table 3). This result indicated that as concentration of Al increases at a specific acidic pH, sensitivity of the isolates increases. According to Keyser *et al.* (1979) maximum tolerance level of slow growing rhizobia for Al was 50 µM, and all strains tolerant to Al were tolerant to Mn also. The report stressed the toxicity of Mn to host plant as compared to the little or no influence on the rhizobial strains. The determinant effect of aluminum on rhizobia both in *in vitro* and *in vivo* condition was reported by Paudyal *et al.* (2007). According to Ayanaba *et al.* (1983) there is correlation between acid-Al sensitivity of isolates with their colony texture, as large-mucoid rhizobial colony were more resistant than dry-pinpoint colonies. Munns and Keyser (1981) observed the better tolerance of fast grower rhizobial isolates to Al toxicity as compared to the slow growers. The current research result also confirmed the former research reports.

The combination of different concentration Al and Mn had no unique effect on the growth of the isolates. Specifically, at a fixed concentration of Al, increase in concentration of Mn had no effect on the growth of isolates up to 200 µM (Data not shown). In contrary, the increase in Al concentration on a fixed concentration of Mn had a significant effect on the survival of the isolates on the culture media. In acid soils, Al toxicity and acidity itself are probably more important limiters of rhizobial growth than Mn toxicity and Ca deficiency (Keyser and Munns, 1979). The current research result is in agreement with the former reports. Thus, the response of rhizobial isolates to different levels of acidic-Al was the other parameter to study diversity that could exist among strains of the species.

Symbiotic effectiveness of the rhizobial isolates was measured in terms of NN, NDW, SDW and %SE. Symbiotic effectiveness of all isolates was categorized under either effective or highly effective. The correlation among TCP- SI, MGT, NN, NDW, SDW, and %SE was very weak at  $P \leq 0.05$  (table 2). Three isolates AUAVR-29, AUAVR-52 and AUAVR-53 were symbiotically highly effective similar to the positive control. Especially, the TCP-SI, NN, SDW, and %SE of isolate AUAVR-52 was the highest. The rhizobial isolate was also tolerant to different *in vitro* stresses. The lack of significant difference in symbiotic effectiveness (NN, NDW and SDW) among the TCP-solubilizer faba bean rhizobia isolates might be related to the sterile sand culture and the ideal greenhouse environment. According to this particular research there was positive correlation between *in vitro* stress tolerance and %SE of TCP solubilizer rhizobia isolate at  $P \leq 0.5$ . This result was similar to Sharma *et al.* (2013) who concluded that *invitro* stress tolerance is an indication for the survival and persistence of rhizobial isolates under severe and harsh desert conditions of real *in vivo* condition. Keyser *et al.* (1979) also reported the positive correlation between Al toxicity tolerances and symbiotic effectiveness of some strains. The symbiotic performance of stress tolerant-rhizobial isolates under sterile sand culture in green house may not ensure same conclusion for the real farm land environment. Thus, competitiveness and symbiotic effectiveness of the isolates should be checked under their respective stressed field environment so as to recommend as biofertilizer. In this study, percentage symbiotic effectiveness is good estimator of the symbiotic

compatibility between host and TCP-solubilizing rhizobial isolate.

## CONCLUSIONS

Acidic soil of Wollega contains Phosphate solubilizing faba bean rhizobia. The TCP solubilizing potential of the isolates varies. The difference in TCP-solubilization depends on some features of the isolates such as growth rate, amount of extracellular organic acid production, metabolic diversity and specialization, and their potential to tolerate different stress environments. TCP-solubilizing rhizobial isolates are fast-grower and organic acid producer. Isolates that have highest TCP-SI were tolerant to stresses such as extreme temperature, concentrated salts, acidic pH, acid-Al, and IAR. Moreover, these isolates also formed effective symbiotic association with the host plant. In this study, tests related to carbon and nitrogen substrate utilization didn't create meaning full result in order to correlate with stress tolerance and symbiotic effectiveness. Thus, the author recommends the succeeding researcher to use wide ranges of carbon and nitrogen sources to test diversity among TCP-solubilizing rhizobial isolates. Moreover, in order to recommend the isolates as biofertilizer repeated field trial should be conducted.

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