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Effects of Acidity on Growth and Symbiotic Performance of *Rhizobium leguminosarum* bv. *viciae* Strains Isolated from Faba Bean Producing Areas of Ethiopia

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Abstract

Faba bean is one of the legume crops commonly grown in Ethiopia. It is important source of dietary protein to the majority of population in the country. Soil acidity and related stresses are among the major yield limiting constraints for this crop. This study was conducted with the aim of evaluating acidity tolerance of *Rhizobium leguminosarum* bv. *viciae* strains isolated from faba bean growing regions of the country and their symbiotic performance under different acidic conditions. Four strains isolated from root nodules of faba bean were tested for tolerance to acidity in a defined liquid media. The results indicated that none of the tested strains was tolerant to pH 4.0 while two of them (AUFR46 and AUFR100) were found to be tolerant of pH 4.5. When tested at pH 5.0 only one isolate (AUFR58) was sensitive. The results of the present study also showed that all acid tolerant strains were recovered from highly acidic soil (4.8- 5.2) and the acid sensitive strain was isolated from neutral soil. A positive correlation (r= 0.92) was observed between minimum pH tolerated in culture media and pH of origin soil of the strains. Nodulation and nitrogen fixation abilities of these strains were evaluated on sand culture. The results indicated that at pH 4.5 and 5.0 nodulation was totally inhibited and only one isolate (AUFR58) could not be able to induce nodule formation on host plant roots at pH 5.5. The results also showed that acidity (pH 5.5) reduced shoot dry weight, nodules number, total nitrogen at a highly significant level (P<0.01) compared to plants grown at pH 6.5 and 7.0. In pot experiment with soil of different pH, inoculation of the rhizobial strains improved the growth, nodulation and nitrogen content of the plants significantly over the uninoculated controls. Besides, acid tolerant strains showed better performance over acid sensitive strains in acidic soils and thus, they are highly recommended for field test in acidic soil.

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INTRODUCTION

Faba bean is one of the major pulse crops commonly grown in Ethiopia, and it ranks first in area and production. It occupies areas of 459, 183.5 ha land with annual production of 697, 798.3 tons (CSA, 2011).This grain legume is important source of dietary protein and daily food supplements to the majority of Ethiopian population. The seeds are mostly boiled and used as snack in the daily food of the rural people. They are also used in the preparation of local dishes such as '*shiro wot* 'and '*kik wot*' to be consumed with cereal *injera.* Moreover, they provide large cash for farmers and foreign exchange for the country (Desta Beyene, 1988).

In low-input agriculture systems of Ethiopia, chemical fertilizers are rarely used in the production of faba bean and other pulse crops; instead, these crops are used as a restorer of soil fertility for the following cereal crops (Asfaw Telaye *et al*., 1994; Mulissa Jida and Fassil Assefa, 2012). Thus, rotation of faba bean and other pulses with cereal has been used to improve both soil fertility and structure. Therefore, the potential of this food legume as a supplier of biologically fixed nitrogen for non-leguminous crops might be more important than their potential as food or cash. In addition, this food legume and othersare predominantly cultivated in marginal agricultural areas where the other cereal crops fail to grow (Asfaw Telaye *et al*., 1994). Despite its multifaceted benefits the productivity of faba bean has remained very low compared to the potential. Soil related stresses such as acidity and associated low phosphate availability are among the major yield limiting constraints (Asfaw Telaye, 1985; Asfaw Telaye *et al*., 1994).

Soil acidity is among the common problems that limits the production of faba bean in Ethiopia (Asfaw Telaye, 1985; Asfaw Tilalye *et al*., 1994; Aynaebeba Adamu *et al*.,

2001; Asefa Keneni *et al*., 2010). Acid soil infertility is caused by toxicities of hydrogen ion, aluminum, and manganese, and deficiency of calcium, molybdenum and phosphorus (Graham, 1992; Chen *et al*., 1993). Soil acidity can adversely affects survival, growth and nitrogen-fixation efficiency of the rhizobia, formation of symbiotic association between rhizobia and their host legumes, (Chen *et al*., 1991, Chen *et al*., 1993, Graham, 1992; Zahran, 1999).

Generally, *Rhizobium* strains vary markedly in their acid tolerance and ability to nodulate their host legume on acid soil (Zahran, 1999). Several studies indicated that some rhizobia strains are acid tolerant when grown in acidic laboratory medium (Chen *et al*., 1993; Del Papa *et al*., 1999; Asefa Keneni *et al*., 2010; Mulissa Jida and Fassil Assefa, 2011). Though there is no basis to support that a higher acid and/or soil acid tolerance of bacteria corresponds to a better symbiotic performance under acidic conditions, Howieson *et al.* (1988) found that acid tolerant *Sinorhizobium meliloti* strains enhanced the establishment of medic pastures in mildly acidic soils. Del Papa*et al*. (1999) have obtained acid tolerant strains of rhizobia, which formed nodules with low rate of nitrogen-fixation. Although acid tolerance and symbiotic effectiveness are both desirable bacterial traits, they are not necessarily linked (Howieson *et al*., 1988). Hence, selection of acid tolerant together with effective rhizobial strains is important to improve the production of legumes on acid soils.

Several studies showed that Ethiopian soils harbored symbiotically effective rhizobia which are tolerant to different stresses such as acidity (Zerihun Belay and Fassil Assefa, 2011; Anteneh Argaw, 2012). In order to overcome low production of faba bean on acidic soils, different methods can be employed ranging from liming of soils (Marschner,1995; Achalu Chimdi *et al*., 2013) to that of selecting tolerant host, tolerant and effective rhizobia to achieve effective symbiosis (Carter *et al*.,1994; Mulissa Jida and Fassil Assefa, 2011). However, little research attentions have been given to acid tolerant rhizobia and evaluation of their symbiotic performance under acidic conditions. Hence, this study was conducted to evaluate acidity tolerance of selected *Rhizobium leguminosarum* bv *viciae* strains isolated from different faba bean growing regions of Ethiopia with different soil pH, and their symbiotic performance under different acidic conditions.

MATERIALS AND METHODS

Test Bacterial Strains

Rhizobium leguminosarum bv*. viciae* strains used in this study were AUFR7, AUFR46, AUFR58 and AUFR100 (Table 1). They were selected out of 100 *Rhizobium leguminosarum* bv. *viciae* culture collection, Applied Microbiology Laboratory, Addis Ababa University (AAU), which were previously isolated from different faba bean growing areas of Ethiopia. Selection was based on their effectiveness and pH of the origin soil of the strains. The strains were maintained on Yeast Extract Mannitol Agar) (YEMA) slant at 4^0C (Vincent, 1970). Their purity was checked regularly by streaking on YEMA plates.

Table 1: Test rhizobial strains and their isolation site geographical location.

Isolate	Isolation site	Regional state	Zone	pH of soil
AUFR7	Alelitu chole	Oromiya	Northern Shewa	6.2
AUFR46	Debera Zebit	Amhara	Southern Gonder	5.5
AUFR58	Ashengie	Tigray	Southern Tigray	7.0
AUFR100	Holeta	Oromiya	Western Shewa	4.8

Determination of Acid Tolerance of the Rhizobial Strains

Buffered defined growth medium described by Wood *et al*., (1988) was used to determine the acidity tolerance levels of the rhizobial isolates. The pH of the medium was adjusted to 4.0, 4.5, 5.0, 5.5, 6.0, and 6.5 with 1N HCl and NaOH using pH meter before autoclaving. Hundred ml of the medium was dispensed in 250 flasks and autoclaved. The flasks were inoculated with 1ml exponential phase rhizobial culture grown in YEM broth at 28°C for 3 days and adjusted to give an initial inoculum density of 10^3 cells ml^{-1}. The strains were grown for 120 hrs on a rotary shaker (120rpm). Bacterial growth was followed by measuring optical density (using spectrometer at 540 nm) and spreading 0.1 ml of the culture on YEMA plate severy 12 hrs, and counting colony forming unit (CFU). Late log phase cell density $ml^{-1}$was calculated from the growth curve and used for comparisons.

Symbiotic Effectiveness Test on Sand Culture under Different pH

Symbiotic effectiveness of the strains was evaluated on sand culture under different acidic pH. About 3 kg of carefully acid washed and sterilized sand was added into plastic pots (3.5Kg capacity).The faba bean cultivar called '*Mesay*' obtained from Ethiopian Institute Agricultural Research (EIAR) was used in this study. Faba bean seeds were surface sterilized with 95% ethanol and 3% sodium hypochlorite solutions for 10 s and 3 min., respectively (Vincent, 1970) and rinsed five times with sterilized distilled water to remove traces of sterilizing chemicals. Surface sterilized seeds were allowed to germinate on a Water Agar (0.75 %**)** for three days at 25^0C. The rhizobial strains were cultured in YEM broth for three days. Germinated seedlings on the agar surface were flooded with rhizobial culture adjusted to 10^9 cells per seed for one hour. Five inoculated seedlings were transferred on each pot which was later on thinned down to three after 10 days of planting

The pots were irrigated with nitrogen free plant nutrient solution as described by (Somasegren and Hoben, 1994). The pH of the solution was adjusted to 4.5, 5.0, 5.5, 6.0., 6.5 and 7.0 with 1N HCl and NaOH prior to autoclaving and checked after autoclaving and did not vary significantly. Each isolate was used as inoculants at each pH treatments. Uninoculated nitrogen-fertilized (0.05% KNO_3 solution) pots were included as control. The experiments were set in triplicate. All pots were arranged in a completely randomized block design in a glasshouse found at College of Natural Sciences, AAU. The pots were watered with sterilized distilled water adjusted to their respective pH once a week and the pH of the sand culture was monitored regularly.

Sixty days after planting seedlings were carefully uprooted, and nodule number was recorded. Shoot dry weight was determined after drying at 70^0C for 48 hrs to the constant weight. Relative symbiotic effectiveness of each isolate was calculated by using the formula (100 × inoculated plant shoot dry weight/ N-fertilized plant shoot dry weight) of Gibson (1987). Total nitrogen of shoot was determined by modified wet Kjeldhal method as described in Sahlemedhin Sertsu and Taye Bekele (2000).

Soil Sample Collection

Composite soil samples of 0-30cm depth were collected from Gonder (Debrezebit), Shewa (Alelitu Chole and Holeta), and Tigray (Ashenge). Each soil sample was thoroughly mixed and air-dried in a glasshouse. Air-dried soil samples were ground and passed through a 2mm sieve to remove stones and large pieces of organic matter. The physicochemical characteristics of the soil samples were analyzed at Ethiopian National Soil Testing Center, Addis Ababa (Table 2).

Table 2: Soil physical and chemical properties.

Parameters	Alelitu	Gonder	Holeta	Tigray
pH H_2O 1:2.5	6.3	5.2	4.8	6.9
EC (dsm)	0.187	0.125	0.088	0.980
Class	Clay	Clay loam	Clay	Loam
Na meq/100 gm	1.140	0.4	0.540	0.460
K meq/100 gm	2.52	1.62	1.79	1.32
Ca meq/100 gm	31.87	33.98	10.08	22.61
Mg	10.37	13.17	4.12	11.36
T.E .bases	45.90	49.17	16.53	35.75
CEC meq/100	46.67	50.40	22.00	36.40
T.N %	0.09	0.118	0.088	0.112
O.C %	0.998	1.317	0.738	1.197
C/N ratio	11	11	8	11
Av.P(ppm)	4.54	19.18	1.92	10.64
Fe(ppm)	26.64	23.92	9.44	18.92
Mn (ppm)	33.3	26.80	80.1	18.84
Zn (ppm)	2.30	2.10	1.50	1.50
Cu (ppm)	1.68	0.88	0.88	1.08

E.C: Electrical conductivity, CEC: Cation exchange capacity, T.N.: Total nitrogen, O.C: Organic carbon, C/N: Carbon to nitrogen ratio, Av.P: Available phosphorus, T.E: Total exchangeable bases.

Symbiotic Effectiveness Test in Different soils

The pot experiments were carried out in a glasshouse under 12 hrs (light and dark period). In order to determine the effectiveness of the strains at different soil conditions, pot experiments were carried out in a glasshouse. Three kg of air-dried and sieved soil was placed in plastic pots. Faba bean seeds sterilization, germination, inoculation and sowing were done as described before. Uninoculated pots were included as controls. The experiments were set in triplicates as described before. All pots were watered with distilled water twice a week. Sixty days after planting seedlings were carefully uprooted, and nodule number was recorded. Shoot dry weight and total nitrogen were analyzed as described before.

Data Analysis

All tests were set in triplicates and the data is average these. Shoot dry weight, total nitrogen, nodule number, and cell density mean separation were analyzed by Tukey's HSD test. Pearson correlation coefficient was calculated to check the relation between minimum pH tolerated in minimal salt medium and the strains origin soil pH using (SPSS.V.15.).

RESULTS

Four selected strains of *R. leguminosarum* bv. *viciae* strains were tested for growth at different acidic pH in a buffered defined liquid medium. The results showed that none of the tested strains grew at pH 4.0 where as all of them grew very well at pH≥5.5 (Table 3). Two strains (AUFR46 and AUFR100) were found to be to lerant to pH 4.5 while AUFR7 and AUFR58 were sensitive to this pH. Of the tested strains only AUFR58 was sensitive to pH 5.0. Although all tested strains grew at pH≥5.5, a considerable variation was observed with respect to cell number (Table 3).

Table 3: Growth pattern of rhizobial strains at different acidic pH.

pH	AUFR7 $\log_{10}$ CFU ml^{-1}	AUFR46 $\log_{10}$ CFU ml^{-1}	AUFR58 $\log_{10}$ CFU ml^{-1}	AUFR100 $\log_{10}$ CFU ml^{-1}
4.5	NG	7.62 ± 0.03c	NG	8.41 ± 0.10b
5.0	8.77 ± 0.02b	7.88 ± 0.1c	NG	8.42 ± 0.20b
5.5	8.91 ± 0.01a	8.41 ± 0.12b	8.98 ± 0.02a	8.45 ± 0.02b
6.0	8.95 ± 0.02a	8.87 ± 0.03a	9.00 ± 0.03a	8.95 ± 0.03a
6.5	8.97 ± 0.15a	8.89 ± 0.10a	9.00 ± 0.40a	8.99 ± 0.10a

Numbers in the same column and row followed by the same letter do not differ significantly at p= 0.05 by Tukey's HSD test, NG: no growth

Cell number of strain AUFR7was decreased with increased acidity level (Table 3). However, only some of the differences were statistically significant (P<0.05). The number of cells obtained at pH 5.0 was significantly lower than that of pH 6.0 and 6.5 (Table 3). Strain AUFR46 was found to grow slowly compared with other strains. The cell number of strain AUFR46 was affected at a highly significant level (P<0.01) by acidity at pH≤ 5.5. However, the cell number obtained at pH 6.0 and 4.5 did not vary significantly from that obtained at pH 6.5 and 5.0, respectively (Table 3). Cell number of strain AUFR58 reduced at pH 5.5 and 6.0 compared with pH 6.5 (Table 3). However, the differences were not statistically significant (P>0.05). Cell number displayed by strain AUFR100 at pH≤ 5.5 was found to be lower than that of pH 6.5 and 6.0 at a highly significant level (P<0.01).

The effect of acidity on symbiotic performance of the four rhizobial strains was investigated by growing the host plant at pH 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0 in sand culture. The mean shoot dry matter accumulation of inoculated faba beans was found tovary significantly with pH (Table 4). All isolates failed to nodulate their host plant at pH 4.5 and 5.0 while only AUFR58 failed to form nodules on host plant at pH5.5 (Table 4).

Table 4: Shoot dry weight and total nitrogen as influenced by inoculation of rhizobial strains.

		Shoot Dry Weight (g plant^{-1})		
Treatment	**pH 5.5**	**pH 6.0**	**pH 6.5**	**pH 7.0**
KNO3	1.04±0.04^{a}	1.24±0.04^{a}	1.35±0.04^{a}	1.45±0.05^{a}
AUFR7	0.84±0.5^{a}	0.96±0.04^{a}	1.4±0.06^{a}	1.45±0.09^{a}
AUF46	0.88±0.02^{a}	0.98±0.2^{a}	1.3±0.06^{a}	1.42±0.1^{ab}
AUFR 58	NG	0.72±0.02^{c}	1.3±0.04^{a}	1.46±0.05^{a}
AUFR100	0.73±+0.03^{b}	0.86±0.01^{b}	1.2±0.1^{b}	1.25±0.07^{b}
		Total Nitrogen (%)		
Treatment	**pH 5.5**	**pH 6.0**	**pH 6.5**	**pH 7.0**
KNO3	3.2±0.012^{a}	3.3±0.12^{a}	3.4±0.5^{a}	3.4±0.1^{a}
AUFR7	2.2±0.07^{b}	2.3±0.08^{b}	2.7±0.1^{b}	2.8±0.05^{b}
AUF46	2.3±0.1^{b}	2.5±0.05^{b}	2.5±0.05^{c}	2.6±0.1^{c}
AUFR 58	NG	1.3±0.06^{d}	2.4±0.^{ac}	2.6±0.08^{c}
AUFR100	1.27±0.05^{d}	1.4±0.1b	2.5±0.1^{c}	2.5±0.06^{c}
		Number of Nodules Plant^{-1}		
Treatment	**pH 5.5**	**pH 6.0**	**pH 6.5**	**pH 7.0**
KNO3	NG	NG	NG	NG
AUFR7	34±4b	64±6^{a}	67±7^{b}	34±4^{c}
AUF46	42 ±4^{a}	67±7^{a}	78±8^{a}	42 ±4^{b}
AUFR 58	NG	10±3^{b}	93±4^{a}	95±3^{a}
AUFR100	7.3±2^{c}	8.0±2^{b}	18±4^{c}	17±2^{d}

Numbers in the same column followed by the same letter do not differ significantly at p= 0.05by Tukey's HSD test, NG: no growth

The nodulating isolates showed variation in the mean shoot dry matter accumulation in relation with the different pH treatments (Table 4). The highest dry matter accumulation of 0.88 g plant^{-1} was recorded by the isolate AUFR46 followed by isolate AUFR7 with 0.84 g plant^{-1} at pH 5.5; consequently they displayed very effective symbiotic effectiveness of 84.6 and 80.8%, respectively, compared to the N-fertilized positive control (Table 5). These isolates were found to be significantly different from isolate AUFR100 with only 0.73 gplant^{-1} shoot dry matter yield and 70.2% effectiveness from the control plants. The isolates were found to accumulate shoot dry matter ranging from 0.72 to 0.98 g plant^{-1} at pH 6.0. In all cases a steady increase in shoot dry matter yield and concomitant increase in symbiotic effectiveness (86% - 100%)was observed as the pH increase.

Isolate AUFR46 induced the highest number of nodules (42 nodules plant^{-1}) followed by AUFR7 with 34 nodules plant^{-1} with significant difference from each other at pH 5.5. The least nodule number, i.e. 7.3 nodules plant^{-1} was recorded for AUFR100 inoculated host plants. In all cases there was a significant increase in nodule number as the pH of growth medium increase. The total nitrogen content of the host plants also showed variations at ≥5.5. In all cases N-fertilized plants displayed the highest N content of shoot, 3.2% to 3.4% (Table 4). However, the rhizobial inoculated plants showed discrepancies in % of N-content at different pH treatments. At pH 5.5 and 6.0, isolate AUFR7 and AUFR46 were found to accumulate N content of 2.2% and 2.3% which was about 30% less than N-fertilized plants (Table 4).

The results of pot experiment using different soils indicated that the performance of isolate AUFR58 and AUFR100 with regard to N-fixation and efficiency in shoot dry matter yield was similar, except that isolate AUFR100 was found to be as efficient as AUFR7 and AUFR46 on Tigray soil (Table 5). In all cases the performance of all isolates was lower on Holeta soil (Table 5). However, these isolates (AUFR7 and AUFR46) were found to be performing better than isolates AUFR58 and AUFR100 in all soil types, except Holeta soil.

Table 5: Relative symbiotic effectiveness of the rhizobial isolates under different pH

Relative symbiotic effectiveness (%)				
Treatments	**pH 5.5**	**pH 6.0**	**pH 6.5**	**pH 7.0**
KNO3	100	100	100	100
AUFR7	80.8	77.4	103.7	100
AUF46	84.6	79.0	96.3	97.9
AUFR 58	NG	58.1	96.3	100.7
AUFR100	70.2	69.4	88.9	86.2

Relative symbiotic effectiveness=100 × inoculated plant shoot dry weight/ N-fertilized plant shoot dry weight(Gibson (1987), NG; no growth

The data also showed that isolate AUFR7 induced the highest shoot dry matter accumulation on all the soil types with significant and non-significant differences among the different treatments (Table 5). This isolate was found to be 1.3 to 2 times more effective in shoot dry mass compared to the uninoculated control plants. Similarly, plants inoculated with isolate AUFR46 were found to perform as well as AUFR7, except on Alelitu and Holeta soil. Although shoot dry matter accumulation of the host plant with different isolate and soil types was variable, the difference in N uptake and accumulation was not significantly different. The data showed that the plants with different treatments accumulated N content ranging from 1.73-3.38%. In general, it appears that isolate AUFR7, AUFR46 and AUFR58 showed variation in N content with different soil types (Table 6).

Table 6: Shoot dry weight and total nitrogen as influenced by rhizobial inoculation

	Shoot dry Weight (g plant^{-1})			
Treatment	**Alelitu**	**Gonder**	**Tigray**	**Holeta**
AUFR7	1.5±0.04^{a}	1.2±0.05^{a}	1.2±0.05^{a}	1.0±0.06^{a}
AUF46	1.24±0.4^{b}	1.16±0.06^{a}	1.15±0.07^{a}	0.8±0.07^{b}
AUFR 58	0.94±0.03^{c}	0.86±0.06^{b}	0.95±0.05	0.76±0.03^{c}
AUFR100	0.97±0.03^{c}	0.91±0.04^{b}	1.15±0.06^{a}	0.84±0.05^{b}
Unioculated	0.74±0.04^{d}	0.78±0.07^{c}	0.76±0.04^{c}	0.72±0.02^{c}
	Total Nitrogen (%)			
Treatment	**Alelitu**	**Gonder**	**Tigray**	**Holeta**
AUFR7	2.87±0.12^{b}	3.19±0.12^{a}	2.59±0.03^{b}	2.17±0.07^{c}
AUF46	2.92±0.18^{a}	2.73±0.10^{b}	3.19±0.13^{a}	2.76±0.10^{a}
AUFR 58	2.81±0.22^{b}	2.40±0.10^{c}	3.38±0.5^{a}	2.48±0.05^{b}
AUFR100	2.52±0.10^{b}	2.62±.10^{b}	2.22±0.07^{d}	1.73±0.03^{d}
Unioculated	2.25±0.12^{c}	2.38±0.05^{c}	2.46±0.1.6^{c}	1.29±0.05^{e}
	Number of Nodules Plant^{-1}			
Treatment	**Alelitu**	**Gonder**	**Tigray**	**Holeta**
AUFR7	159±10^{a}	103±8^{a}	152±11^{a}	53±8^{b}
AUF46	121±23^{a}	65±7^{b}	96±6^{b}	24±5^{c}
AUFR 58	85±10^{b}	36±6^{c}	97±8^{b}	27±8^{c}
AUFR100	99±10^{b}	63±5^{b}	117±16^{a}	80±10^{a}
Unioculated	81±7^{b}	23±3^{d}	59±10^{c}	23±10^{c}

Numbers in the same column followed by the same letter do not differ significantly at p= 0.05 by Tukey's HSD test.

DISCUSSION

The acidity tolerance study in a defined liquid medium at different acidic pH showed that none of the faba bean rhizobia was found to grow at pH 4.0.However, isolate AUFR46 and AUFR100 were found to be tolerant of pH 4.5. At pH 5.0 only isolate AUFR58 was found to be sensitive. All isolates showed growth at pH≥ 5.5. According to the classification of Del Papa *et al*. (1999) AUFR7, AUFR46, and AUFR100 can be grouped as acid tolerant whereas one isolate AUFR58 can be considered as mild-acid tolerant.

Lindstrom and Myllyneimi (1987) have found that the lowest pH at which growth was observed for *Rhizobium leguminosarum* was varied between 4.7 and 4.9. In contrast, the present study found that the lowest pH at which growth was observed for *Rhizobium leguminosarum* bv. *viciae* varied considerably. It was found to be between 4.5 and 5.5. The result of this study indicated that the lowest pH at which growth was observed has a positive correlation with the pH of isolation site. All strains which showed growth at the lowest pH and considered as acid tolerant were recovered from highly acidic soil (pH 4.8 and 5.2) and strains which grew at pH 5.5 was isolated from neutral soil (pH 7.0). Similarly Del Papa *et al* (1999) have reported that all acid tolerant strains of alfalfa-nodulating rhizobia were recovered from acidic soils between 5.0 and 6.5, and most mildly acid tolerant and acid sensitive strains were obtained from soils above this pH. Furthermore; our result demonstrated

the existence of similar population of acid tolerant faba bean nodulating *Rhizobium* strains in geographically distant regions that have soil acidity as a common feature. The occurrence of acid tolerant strains in acid soil may relate to a better adaptation of these rhizobia to their habitat.

The ability of the isolates to induce nodulation and nitrogen-fixation was tested under different acidic conditions. The data showed that nodulation was totally inhibited at 4.5 and 5.0. All isolates induced nodulation at pH5.5, except AUFR58. The superior acidity tolerance of isolate AUFR46 and AUFR100 was not associated with their nodulation ability at the same pH. Evans *et al*. (1980) observed that nodulation of *Pisum sativum* was more susceptible to acidity than either rhizobia multiplication or plant growth. Furthermore it was shown that multiplication in liquid culture is not an indicator for nodulating ability under acidic conditions; instead it improves their competitive ability under acidic conditions (Cooper *et al*., 1985; Wood *et al*. 1988).

The results of this study also demonstrated that variations amongst the acid tolerant inoculants with regard to number of nodule, shoot dry matter and total nitrogen contents of the inoculated plants. Low pH (5.5) reduced nodules number, shoot biomass and total nitrogen of plants inoculated with isolate AUFR7, AUFR46, AUFR100 at a highly significant level (P<0.01) when compared with their corresponding plants grown at pH 7.0. Vassileva *et al*. (1997) obtained that number of nodules, nitrogenase activity, and fresh weight and dry weight of nodules were affected to a greater extent by acidity. Similarly, Paulino *et al*. (1987) have found that low pH (5.2) decreased nodule number and acetylene reduction in *Rhizobium-Pisum sativum* associations. Effect of low pH on plant dry weight and total nitrogen can be associated with restricted nodulation and nitrogenase activity.

In the present work symbiotic performance of the four rhizobial isolates on sand culture of different acidic pH was found to be different, that is the more acid tolerant strains in laboratory media performed more than the less tolerant isolate at low pH (5.5 and 6.0). Similarly, Lindstrom *et al*. (1985) have observed the performance of the *Rhizobium leguminosarum* bv. trifolii-*Trifolium pretense* symbiosis were best when the Rhizobial strains were acid tolerant strains.

The pot experiments showed that the faba bean cultivar itself suffered from pH stress imposed at pH 4.5, 5.0, and 5.5 (Table 3). This is evidenced form reduced shoot dry matter and total nitrogen contents of plants compared with the host plants grown at pH 7.0. Similar result was reported for *Trifolium partense* (Lindstrom and Myllyeimi, 1987). Under acidic conditions growth of the legumes appears to be one of the limiting factors for legume symbiosis. Yan *et al*. (1992) have demonstrated that low pH of rooting medium limited dry matter production during vegetative plant growth, and broad bean (*Vicia faba* L.) was found to be particularly sensitive low pH (Schubert *et al*., 1990).

According to Desta Beyene and Angaw Tsige (1989) based on their nitrogen status; Gonder and Tigray soils can be classified as medium whereas Alelitu and Holeta can be classified as low (Table 2). Based on their available phosphorus Gonder soil can be classified as high, Tigray soil as medium, and Alelitu and Holeta soil as very low (Ngeborg, 1986). Consequently, the low number of nodules produced by plants grown on Gonder soil can be attributed to high nitrogen content and that of Holeta soil to low available phosphate and highly acidic soil pH.

The data of our pot experiment showed that inoculation of faba bean with the Rhizobial strains improved plant growth, nodulation and total nitrogen over uninoculated controls. In addition, our result indicated that in acidic soil acid tolerant isolate showed comparative advantage over acid sensitive isolate in the ability to nodulate the faba beans and to fix nitrogen. The symbiotic performance of all strains except AUFR58 was found to be less when compared the plants grown on other soils. This was may be due to high concentration of Mn, low available phosphorus, and highly acidic pH and other related factors.

Lindstrom and Myllyneimi (1987) have observed that in pot experiment with acidic soil inoculation with the best strain improved the yields the plants fourteen fold compared with uninoculated controls**.** Hartel and Buton (1989) have observed that acid tolerant *Medicago sativa* L inoculated with acid tolerant rhizobia produced greater top growth, nodule number and weight, and acetylene reduction value in acid soil than the same plants inoculated with acid sensitive strains. The present findings are consistent with this result.

It is difficult to relate the tolerance of the strains to acid stress in liquid culture to its performance in acid soil, because of the difficulties in measuring the pH encountered by the bacteria in the rhizosphere (Wood *et al*., 1988). The concept of concentration of ions is complicated in soils that are characterized by exchange of reaction, and the pH value of soil is not well-defined measurement (White, 1969). In our pot experiment, the pH value of some of the soil was below the limit of multiplication for some strains. Nevertheless, all strains produced nodules on faba bean in all tested soils. This may be explained the acidity of the rhizosphere may be different from that in the soil away from the roots (Jarvis and Roson, 1983, Marschner, 1995). However, the result of this study suggests that pH of isolation site of the strains could be used to predict their performance in acidic soils.

CONCLUSIONS

In conclusion *Rhizobium leguminosarum* bv. *viciae* strains recovered from highly acidic soils were highly acid tolerant, isolate recovered from mildly acidic soils was found to be moderately acid tolerant, and strains which come from neutral soil was acid sensitive or less tolerant. Hence, it can be suggested that pH of isolation site can be used to predict acid tolerance of the rhizobial strains in liquid laboratory media.

In sand culture where the stress factor was only H^+ ions the symbiotic performance of the *Rhizobium leguminosarum* bv. *viciae* strains tolerance in laboratory were found to be related with their symbiotic performance in acidic sand culture. Most acid tolerant strains where performed well at acidic pH and acid sensitive strains did not nodulated faba bean at pH 5.5, but performed best at neutral pH. For all tested *Rhizobium* strains-faba bean symbiosis shoot length, shoot dry weight, number of

nodules and dry weight, and total nitrogen were highly reduced by decreasing pH from 7.0 to 5.5.

In pot experiment, inoculation of faba beans improved growth and nitrogen content of the plants when compared with uninoculated controls. Thus, there is a potential of using rhizobial isolate to improve the yields of faba bean in acidic soils. Based on the above conclusion the following can be recommended: more screening and then selection of acid tolerant strains with superior symbiotic effectiveness which would be used as inoculant should be done from all acidic soils and faba bean producing areas of the country. Furthermore, studies on molecular basis of the acidity tolerance of *Rhizobium leguminosarum* bv *viciae*, and their interaction with faba bean at acidic soils are very important. In order to use the best strain for inoculation of faba bean on acid soils appropriate field test is highly needed.

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