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RESEARCH ARTICLE

PHENOTYPIC PROPERTIES OF SOME ACID TOLERANT FABA BEAN (*VICIA FABA* L.) RHIZOBIA FROM CENTRAL HIGHLANDS OF ETHIOPIA

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ABSTRACT: The study aimed to assess phenotypic properties of some selected acid-tolerant faba bean rhizobia from central highlands of Ethiopia. Thirty soil cores were taken from 0–20 cm and composited for soil analysis following standard procedures. The plant trap method was used to isolate rhizobia. The purified colonies were used for eco-physiological, plant growth promotion (PGP) traits, and seed germination test. The soils were strongly (4.8) to moderately acidic (6.25) with no risk of salinity. Organic matter was 2.6–4.69%, whilst total nitrogen was 0.11–0.37% and the phosphorous availability was 12.88–63.97 cmolc/kg. The CEC was in the order of Ca>Mg>K>Na where micronutrients were Fe>Mn>Zn>Cu and wider. Forty acid-tolerant isolates were obtained from five faba bean fields (collected from Fiche, Ginci, Gudar, Holota, and Midakegn). Of these, 15 isolates which showed 100% tolerance to pH 4 to 5 were selected for other multiple PGP traits. Three rhizobial isolates (MFB5, FFB1, and MFB4) showed positive response for 8 PGP traits while FFB37 and GuFB8 which responded for 7 PGP traits were considered as the most potential in plant growth promoting properties. Rhizobial isolate FFB25 produced the highest indole-3-acetic acid (IAA) (174.63 ± 0.19 µg/ml) followed by MFB5 with 173.13 ± 0.14 µg/ml. Moreover, isolate FFB27 was known phosphate solubilizer (2 ± 0.025a) followed by MFB4 (1.7 ± 0.08ab) with the highest halo zone formation measured in mm. Isolate obtained from Fiche (FFB37) was best in seed germination assay with 118.67% followed by FFB1 (86%) and FFB25 (75%). There was a variation in % seed germination (72% and 56%) and vigor index (219.60 and 91.28) among inoculated and non-inoculated faba bean seeds separately. This study suggests the potential of these rhizobial isolates for inoculum development following greenhouse and field trials as soil acidity alleviation tools to boost supply of food and feed crops in the region.

Key words/phrases: Acid tolerance, Faba bean, Phenotypic, Rhizobia.

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INTRODUCTION

Faba bean (*Vicia faba* L.) is one of the earliest domesticated food legumes and occupies nearly 3.2 million hectares worldwide (Torres *et al*., 2006). It is the most important pulse crop in terms of both area coverage and volume of annual production in Ethiopia. Currently, it occupies about 437,106 hectares of land with an annual national production of 1,016,068.2 tons, with a productivity of 2.11 ton ha^{-1}(Bachewe *et al*., 2018). The crop is mainly cultivated in mid and high-altitude areas, with an elevation ranging from 1,800–3,000 meters above sea level (Mussa Jarsso and Gemechu Keneni, 2006). It is an important grain legume in Ethiopia and is constrained by low production, and soil acidity, besides scanty information on optimum plant density and phosphorus nutrition (Kubure *et al*., 2016). The crop is integrated as a break crop or mixed crop in the low input agricultural systems to improve soil fertility since it fixes inorganic nitrogen through symbiosis with root nodule bacteria *Rhizobium leguminosarum* biovar *viceae* (Tian *et al*., 2010). The most common factors affecting biological nitrogen fixation and symbiosis activity are soil acidity, extreme temperature, water stress, salinity, pesticide application, quality of inoculants, and low soil fertility (Alemayehu Dabessa *et al*., 2018).

Soil acidity is one of the chemical soil degradation problems which affect soil productivity. It is estimated that 41% of Ethiopian highlands are affected by soil acidity (Schlede, 1989). Soil acidity hinders legume production more than any other crop as it affects the complex association of the legume host, the endosymbiont, and the symbiosis (Graham, 1992). Soil acidity affects the growth of crops because acidic soil contains toxic levels of aluminum and manganese and is characterized by the deficiency of essential plant nutrients such as P, Ca, K, Mg, and Mo (Wang *et al*., 2006). Due to this acidic nature, most of the soils of the highlands of Ethiopia are deficient in inherent available P and N content (Achalu Chimdi *et al*., 2012). Soil pH may also control biotic factors such as the activity and biomass composition of phyto-beneficial microorganisms and other micro-flora and fauna in the soils of both natural and agricultural lands (Bååth and Anderson, 2003). A study examining the effects of soil pH on cowpeas showed that poor growth of plants can sometimes be attributed to poor microorganism activity (Peet *et al*., 2003). It influences the complex interaction of the host plant, the survival, and the persistence of rhizobia in the soil.

Rhizobial isolates with PGP activity increased shoot dry weight by 40–55% and significantly elevated N_2 fixation potential and shoot P content in faba bean compared to isolate without PGP activity (Othman and Samih, 2016). Auxin (IAA) production by rhizobia is often considered to improve growth and N_2 fixation in many legumes including, beans, chickpea, and pea (Huang and Erickson, 2007; Anjum *et al*., 2011). It has been shown that enhanced P nutrition provided by P solubilizing rhizobia promotes overall plant growth, root development, and nitrogenase activity, all of which are positively connected with nodulation and N_2-fixation (Girmaye Kenasa *et al*., 2014). In addition, the PGP microbes contain useful variation for tolerating abiotic stresses like extremes of temperature, pH, salinity and drought, heavy metal, and pesticide pollution (Ojuederie *et al*., 2019). Mia *et al*. (2012) indicated that inoculation of rhizobia significantly increased the seedling emergence, seedling vigor, root growth namely root length, root surface area and volume of rice.

Inoculation of faba bean with acid-tolerant Rhizobium strains improved early nodulation and increased grain yield at all sites of acidic soil (Carter *et al*., 1994). Green gram (*Vigna radiata*) forms an effective symbiosis with some Rhizobium strains under acidic-stressed conditions. They result in biological nitrogen fixation and thereby reduction of the requirements for added nitrogenous fertilizer during the growing season (Vijila and Jebaraj, 2008). Rhizobium symbiosis with legumes produces 50% of 175 million tons of total biological N_2 fixation annually worldwide (Yadav and Verma, 2014). The failure of nodulation under acid soil conditions is common, especially in soils of pH less than 5 (Ibekwe *et al*., 1997). Highly effective rhizobial inoculation is a common practice in agricultural legume production (Da and Deng, 2003). Similarly, Mulissa Jida and Fassil Assefa (2014), reported that soil acidity and related stresses are among the major yield-limiting constraints for faba bean. Therefore, with the realization of the high limitation of N and P in most highlands of acidic soils in Ethiopia, inoculation of legumes with efficient rhizobia is one of the most important economic and eco-friendly practices used for the improvement of N fixation and soil acidity mitigation options. Hence, the study was intended to isolate and evaluate acid-tolerant Rhizobium and their impact on the adaptation of faba bean to stress in central highlands of Ethiopia.

MATERIALS AND METHODS

Description of the study area

Thirty soil samples from each sampling sites having 4 kg were sampled from acid-affected faba bean fields of the central highlands of Ethiopia (Fiche, Ginci, Gudar, Holota, and Midakegn) from September 2016 to August 2017. The coordinates and sampling points were recorded by using a GPS; altitude and other information is indicated in Table 1 and Fig. 1.

Table 1. Characteristics of soil sampling sites and identities of isolates obtained per site.

Site	Coordinates	Altitude (m)	Legumes history	Isolate identities (code)
Fiche	9° 45' 57" N 38° 42' 06" E	3,000	Common beans	FFB
Holeta	9° 03' 04" N 38° 26' 15" E	2,410	Common beans	HFB
Ginci	9° 00' 35" N 38° 60' 45" E	2,159	Common beans	GiFB
Gudar	8° 56' 21" N 37° 44' 49" E	2,072	Common beans	GuFB
Mida kegn	9° 08' 55" N 37° 28' 12" E	2,908	Common beans	MFB

Soil sampling and chemical analysis

Soils were sampled from topsoil 0–20 cm which had no history of inoculation. The soil samples were mixed thoroughly following a standard procedure to form a composite soil sample used for soil analysis and rhizobia trapping (Margesin and Schinner, 2005). Soil pH and electrical conductivity (EC) were measured in soil: a water ratio of 1:2.5 according to Joshi *et al*. (2009). Cation exchange capacity (CEC) was determined by Flame Emission Spectrophotometer (FES) (Sahlemedhin Sertsu and Taye Bekele, 2000). Exchangeable Ca and Mg were measured by EDTA titrimetric method and exchangeable K and Na by FES (Sahlemedhin Sertsu and Taye Bekele, 2000). Plant available potassium was determined by ammonium acetate (CH_3COONH_4) method (Bashour and Sayegh, 2007); while available phosphorus was extracted as described by (Modified ES ISO 11263:2015). Soil organic carbon (OC) and total nitrogen (TN) content were determined by dry combustion methods based on modified ES ISO 14235:2015 and ISO 11261 modified Kjeldahl method protocols, respectively. Soil organic matter (OM) was calculated by multiplying soil organic carbon by 1.724 assuming an average C concentration of OM of 58 % (%OM = %OC x 1.724). Available micronutrients (Cu, Fe, Mn, and Zn) of the soil were extracted with ammonium bicarbonate di-ethylene tri-amine Penta-acetic acid (DTPA) as described by Tan (1996).

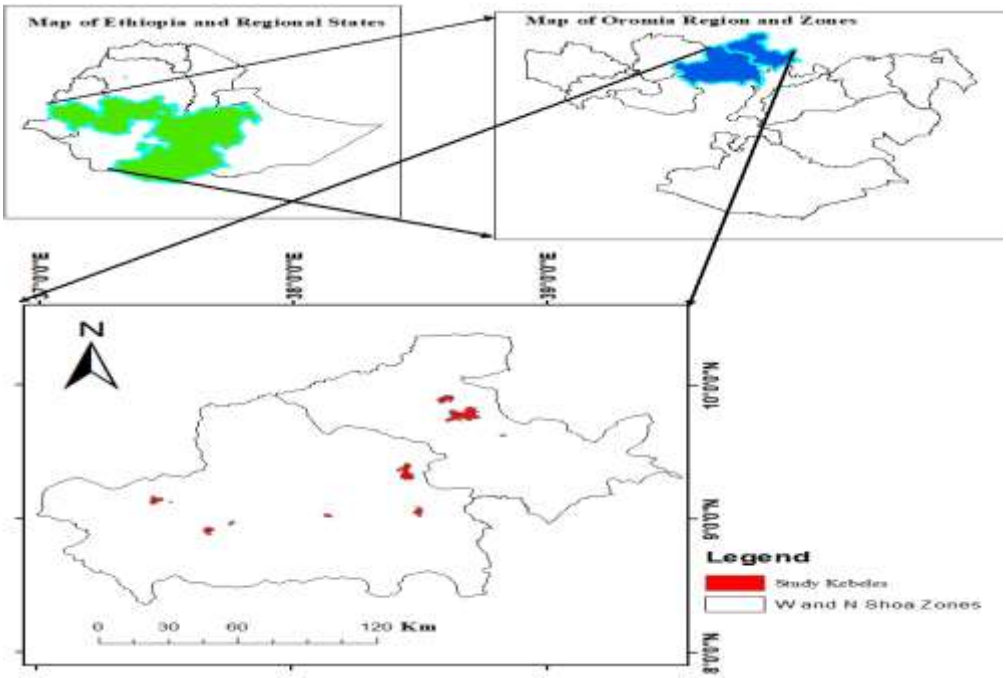

Fig. 1. Map of the study area.

Nodule trapping and *Rhizobium* isolation

Nodulation was induced by the plant infection method in the greenhouse at Addis Ababa University. A local landrace/cultivar of faba bean seeds preferred by farmers was chosen for this experiment. The seeds were surface sterilized in 70% (v/v) ethanol and then immersed in 3% (v/v) sodium hypochlorite and washed with sterile distilled water several times (Mothapo *et al*., 2013; Vincent, 1970). Plastic pots (20 cm diameter and 25 cm height) were filled with soil samples and watered with sterile water until adequately wet. Five seeds were planted in each pot allowing adequate space for germination but were later thinned to three. After 45 days of growth, plants were carefully uprooted and soil washed off the roots without detaching the nodules. Three to five well-formed and undamaged nodules were preserved in the refrigerator (4°C) for isolation of the rhizobial isolation. Isolation and purification of rhizobia were done following procedures of Somasegaran and Hoben (1994a) on Yeast Extract Mannitol Agar (YEMA) with Congo red (CR) per the following compositions (yeast extract, 1g/l; mannitol, 10g/l; $K_2HPO_4.7H_2O$, 0.5 g/l; $MgSO_4$. $6H_2O$, 0.2 g/l; NaCl, 0.1 g/l; agar-agar, 15 g/l and Congo red, 0.025 g/l) (Vincent, 1970).

A presumptive confirmation was done on YEMA with 0.025% bromothymol blue (BTB), glucose peptone agar (PGA), and ketolactose test, after incubation of 3 to 7 days at 28°C (Vincent, 1970).

A total of 140 rhizobial isolates were isolated from the five (Fiche, Ginci, Gudar, Holota, and Midakegn) and purified, characterized, and reduced to 15 isolates primarily based on pH tolerance and other PGP potentials, then stored temporarily using YEMA slant at 4°C until further analysis.

Growth and eco-physiological characterization

For each biochemical and physiological test, inoculation of a loop full of 48 hrs old broth culture was streaked onto the YEMA and incubated at 28 ± 2°C for 3–5 days (Somasegaran and Hoben, 1994a). Ultimately, the growth of each rhizobial isolate was determined (Solomon Legesse and Fassil Assefa, 2014). The ability of rhizobial isolates to grow in different concentrations of salt was tested by streaking them on a YEMA medium containing 0.5%, 1%, 3%, 5%, and 8% (w/v) NaCl (Romdhane *et al*., 2009), and differences in pH tolerance were tested in YE+MA by adjusting the pH to 4, 4.25, 4.5, 4.75, and 5 with either 1N NaOH or HCl (Küçük *et al*., 2006) and the range of growth temperature was examined by incubation of bacterial cultures in YEMA at 4°C, 20°C, 25°C, 30°C, 35°C, 42°C, and 55°C. All the plates were incubated at 28°C for 72 hours and YEMA medium plates were used as controls. The isolates were tested for single different antibiotics to determine their IAR pattern. The antibiotics used were (μg/ml): chloramphenicol, streptomycin, neomycin, tetracycline, gentamycin (10, 50, and 100), and ampicillin (100, 200, and 300). Isolates were grown in YEM broth in duplicate for 48 h and 10 μl of each isolate were inoculated on a YEMA medium and incubated at 28°C for 7 days (Assefa Keneni *et al*., 2010). The effects of a single different heavy metals (HMs) concentration on rhizobia were examined on a YEMA medium (Vincent, 1970). Lead (Pb) was applied as a compound of lead acetate ($Pb(CH_3COO)_2$ at concentrations of 10, 50, 100, and 200; Hg as $HgCl_2$ .4H2O, Cu as $CuSO_4.5H_2O$ and Ni as $NiSO_4.7H_2O$ at a concentration of 2, 10, 20, and 25 μg ml^{-1} were used.

Determination of plant growth promoting (PGP) traits

Ammonia and hydrogen cyanide (HCN) production

Ammonia production was done by growing the isolates in peptone water (5 ml) and incubated at 30°C for 4 days. Then 1 ml of Nessler's reagent was added to each tube. The development of faint yellow and deep yellow to

brown colour indicates ammonia production (Joseph *et al*., 2012). HCN production was done on nutrient agar amended with 4.4 g of glycine/l and bacteria were streaked on the nutrient agar plates. A Whatman filter paper soaked in the solution of 2% Na_2CO_3 and 0.5% picric acid solution was placed on the lid (on the top of the cover) of streaked plates and sealed with parafilm. Plates were incubated at 30 ± 2°C for 4 days. The change in the colour of filter paper from orange to red indicates HCN production (Joseph *et al*., 2012).

Starch hydrolysis and gelatinase test

Starch agar plates were prepared and streaked with the bacterial culture and incubated at 30 ± 2°C for 48 hours. After incubation plates were flooded with iodine solution and the clear zone around the streaked culture indicates the degradation of starch (Shahzad *et al*., 2012). Gelatinase test was performed on gelatin agar medium in g/l: (gelatin 12, beef extract 3, and peptone 5). A loop full of cultures was inoculated into gelatin tubes and incubated for 4 to 7 days at 37°C. Then the tubes were refrigerated at 4°C for half an hour if gelatinase is present, the liquid medium failed to solidify upon refrigeration (Shahzad *et al*., 2012).

Phosphate solubilization

Pikovskay medium was prepared for the phosphate solubilization test with the following composition in g/l; (glucose 10, tricalcium phosphate (TCP) 5, (NH4)2SO4 0.5, KCl 0.2, MgSO4 0.1, MnSO4 trace, FeSO4 trace, yeast extract 0.5 and agar 15). Plates were spot inoculated and incubated at 30 ± 2°C for 4–5 days. A clear zone of solubilization was formed around the colony that indicates phosphate solubilization (Sridevi and Mallaiah, 2009).

Protease, cellulase, and lipase activity

The qualitative assay for protease production was performed on sterile skim milk agar plates (skim milk 15, yeast extract 0.5, agar 9.13) in g/l. Isolates were spot inoculated and followed by incubation at 30°C and a zone of clearance around the colony indicating the enzymatic degradation of protease (Geetha *et al*., 2014). Each bacterial isolates were inoculated on carboxymethyl cellulose (CMC) agar plates with the following composition in g/l; (CMC 2, $NaNO_3$ 1, K_2HPO_4 1, KCl 1, $MgSO_4$ 0.5, $FeSO_4$ 0.01, yeast extract 5 and agar 15) and incubated at 30°C for 2 days. The plates were flooded with iodine. The clear zone formed by the isolates indicated their cellulase activity (Khianngam *et al*., 2014). The bacterial isolates were spot inoculated on lipase medium composed of (g/l): peptone, 10; NaCl, 5;

$CaCl_2$, 0.1; agar-agar, 20 and tween 20, 10 ml (v/v). The plates were then incubated at 28°C for 48 hr. Deposition around the bacterial colony indicates the activity of lipase (Kumari *et al*., 2010).

Indole-3-acetic acid (IAA) production

To test IAA production, bacterial cultures were grown for 48 h in culture broth at 28 ± 2ºC. A bacterial suspension (100 µl each) of fully grown bacterial culture was inoculated in a 5 ml broth culture medium in the presence of 500 µg/ml of tryptophan and incubated for 48 h at 28 ± 2ºC. Centrifugation of bacterial culture was done at 3000 rpm for 15 minutes and the supernatant (2 ml) was mixed with two drops of orthophosphoric acid and 4 ml of the Salkowski reagent (50 ml, 35% of perchloric acid, 1 ml 0.5M $FeCl_3$ solution). The development of a pink colour indicated IAA production, and the optical density (O.D) 530 nm was read using a spectrophotometer (Shaik *et al*., 2016).

Effect of *Rhizobium* on seed germination

Isolated *Rhizobium* were inoculated in yeast extract mannitol broth (YEMB) and allowed to grow over night at 28 ± 2°C. Faba bean seeds were surface sterilized by 70% ethanol and then treated with 1% sodium hypochlorite for 2 min followed by repeated washing with sterile water. Then, the seeds were soaked in the rhizobial culture broth for 5 h, which contain the bacterial suspension (10^8 CFU/ml) while seeds that were soaked in normal YEMB were kept as a control. Five seeds of each treatment were kept equidistance in sterilized Petri plates containing moist filter paper and cotton and the Petri plates. Both treated and untreated plates containing faba bean seeds were arranged in Complete Randomized Design (CBD) in triplicate and then incubated at 28 ± 2°C for 3–7 days. Seed germination and percent seedling emergence were calculated using the following formula (Mia *et al*., 2012):

$$\text{Germination rate \%} = \frac{\text{Number of seeds germinated}}{\text{Total number of seeds}} \times 100$$

Then, the radicle and plumule length of germinated seeds were taken for up to 7 days (Shiferaw Demissie *et al*., 2013; Gholami *et al*., 2009). The seed vigor index (VI) is calculated by determining the germination percentage and the total seedling length (mm) (radicle plus plumule length) of the same seed lot.

Vigor index (IV) = % germination x total plant length

Data analysis

All tests were set in triplicates and the data is average. Shoot dry weight, total nitrogen, nodule number, cell density mean separation, and soil physicochemical analysis were analyzed through one-way ANOVA. The plant growth-promoting traits were determined in percentage. Pearson correlation coefficient was calculated to check the relation between minimum pH tolerated in minimal salt medium and the isolates origin soil pH using (SPSS.V. 20).

RESULTS AND DISCUSSION

Soil chemical analysis

The mean pH of the study areas ranged from 4.8 to 6.25 and showed significant differences among the farming sites (ANOVA ($P \leq 0.05$) (Table 1). As per the ratings for Ethiopian soils by Tekalign Mamo *et al*. (1991), the soil pH was found to range from strongly acidic to slightly acidic. This variation might be due to the difference in parent material, topographic position, land use type, degree of removal of basic cations by crop harvest, and prevailing micro-climate conditions like rainfall intensity (Kedir Abate *et al*., 2016). The higher acidity of the soils for Midakegn was mainly due to the leaching of some basic cations. Similar finding was reported by Iwara *et al*. (2013) in Nigerian soil. The reason for this probably due to poorly managed cultivation, inappropriate use of ammonium-based fertilizers, and accelerated erosions that implied the deterioration of soil quality (Nega Emiru and Heluf Gebrekidan, 2013). Abayneh Esayas (2001) and Mohammed Assen *et al.* (2005) reported that soils at high altitudes and higher slopes had low pH values, probably suggesting the washing out of basic cations from these parts. In the study, soil pH shows minor changes with altitudes where low pH was recorded in higher altitudes as compared to lower altitudes (Fig. 2). Severe soil acidity problem has also been reported in the highland of Ethiopia (Abreha Kidanemariam *et al*., 2012). This is attributed to the aspect and slope that can control the movement of water and material on a hillslope and contribute to the spatial differences in soil properties (Tsui *et al*., 2004).

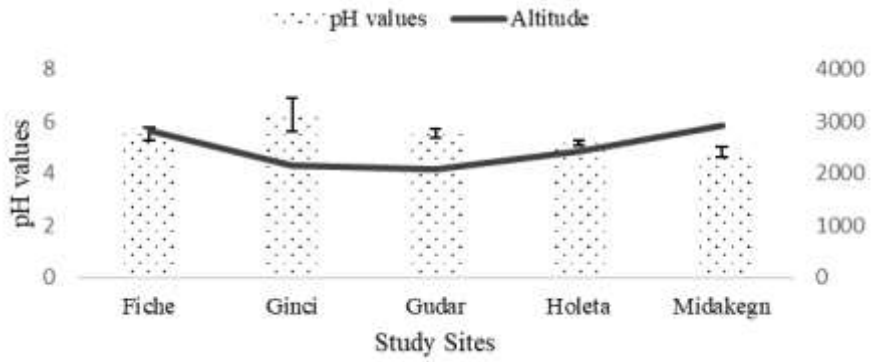

Fig. 2. The relationships between soil acidity along altitudes.

The study areas have low OM contents and showed a significant difference among sites ($P \leq 0.05$) (Table 2). The change in altitudinal gradients can influences SOM by controlling soil water balance, soil erosion, and geologic deposition processes (Tan *et al*., 2004). Similarly, TN contents of the soils of the sites ranged from low (0.11 ± 0.04) to high (0.37 ± 0.17) which was similar to those (Birhanu Debele, 1982; Tekalign Mamo *et al*., 1991). The TN content of the soils showed a significant difference among sites ($P \leq 0.05$) (Table 1). TN closely followed the trend of OM. Tekalign Mamo *et al*. (1998) and Mohammed (2003) reported that N is a deficient nutrient element in the soils of Ethiopia and thus called for the application of inorganic fertilizers and management of soil OM (Table 2).

Table 2. Soil chemical properties of the study areas (means ± standard error, n = 3).

Location	pH (1:2.5)	EC (1:2.5)	OC (%)	TN (%)	OM (%)	CEC	Av. P	C:N	Av. K	Ex. Na	Ex. K	Ex. Ca	Ex. Mg
Fiche	5.50 ± 0.26	0.13 ± 0.02	1.91 ± 0.12	0.11 ± 0.04	3.29 ± 0.41	53.5 5± 0.83	12.88 ± 0.34	17.73 ± 1.97	0.39 ± 0.04	0.12 ± 0.03	0.28 ± 0.04	31 ± 1.66	15.5 ± 0.67
Ginci	6.25 ± 0.62	0.17 ± 0.03	2.04 ± 0.09	0.18 ± 0.03	3.52 ± 0.07	60.49 ± 0.92	22.63 ± 0.88	11.30 ± 0.43	1.67 ± 0.11	0.15 ± 0.02	2.38 ± 0.04	48.84 ± 1.35	9.09 ± 0.82
Gudar	5.53 ± 0.15	0.11 ± 0.07	1.51 ± 0.26	0.14 ± 0.02	2.60 ± 0.18	27.82 ± 0.85	20.02 ± 1.02	10.80 ± 0.44	1.26 ± 0.1	0.12 ± 0.04	1.58 ± 0.12	9.6 ± 1.35	4.27 ± 0.92
Holeta	5.05 ± 0.08	0.14 ± 0.04	1.67 ± 0.52	0.18 ± 0.03	2.88 ± 0.42	26.57 ± 0.97	54.80 ± 0.86	9.30 ± 1.14	1.19 ± 0.03	0.09 ± 0.05	1.41 ± 0.04	7.44 ± 0.79	4.25 ± 0.63
Mida Kegn	4.80 ± 0.20	0.16 ± 0.05	2.72 ± 0.13	0.37 ± 0.17	4.69 ± 0.37	43.03 ± 0.24	63.97 ± 0.76	7.35 ± 1.37	0.58 ± 0.14	0.1 ± 0.04	0.8 ± 0.11	5.65 ± 0.45	5.65 ± 0.43
Sig.	0.003	0.548	0.003	0.028	0.000	0.00	0.00	0.000	0.00	0. 413	0.00	0.00	0.00

Units for CEC and exchangeable bases are in cmol (+)/kg, Av. P and cmol (+)/ kg in cmol (+)/ kg.

The CEC value ranged from high to very high and significantly varied from site to site (Table 2). Such variable ranges might be due to differences in soil type, land use type, and soil fertility management. These values indicate that the soils have adequate basic cations to support plant growth. In general terms, soils with large quantities of negative charge are more fertile because they retain more cations (McKenzie *et al*., 2004). The relative abundance of basic cations in the exchange complex was in the order of Ca>Mg>K>Na for soils collected from the study fields. The exchangeable K, Ca and Mg content of the soils are mostly within and sometimes a little above the critical values (Table 3). The phosphorus status of the present study is low for Fiche, medium for Ginci and Gudar, high for Holeta, and very high for Midakegn. The highest available phosphorus for Midakegn is likely due to the high OM content of the soil. The levels of available micronutrients in the soils of the study areas were found to be in Fe>Mn>Zn>Cu order (Table 3). The micronutrient contents of soils are influenced by several factors among which soil organic matter content, soil reaction, and clay content are the major ones (Fisseha Itanna, 1992). There was an inverse exponential relationship between soil pH and KCl extracted exchangeable Al in all soils (Fig. 3). This means that as the concentration of Al increases the pH of the soil decreases. The concentration of exchangeable Al decreased with increased soil pH (Chartres *et al*., 1990; Kariuki *et al*., 2007).

Table 3. Soil micronutrient status of the study site (means ± standard error, n = 3).

Location	Fe (mg/kg)	Mn (mg/kg)	Cu (mg/kg)	Zn (mg/kg)	Al (mg/kg)	PBS (%)
Fiche	112.77 ± 1.59	25.13 ± 0.90	13.18 ± 0.85	1.27 ± 0.28	0.12 ± 0.036	87.58
Ginci	23.48 ± 0.95	53.58 ± 1.42	2.26 ± 0.87	1.80 ± 0.17	0.06 ± 0.03	99.93
Gudar	51.32 ± 1.20	95.49 ± 1.39	1.52 ± 0.11	1.67 ± 0.23	0.1 ± 0.043	55.96
Holota	91.01 ± 1.66	130.39 ± 1.75	3.73 ± 0.27	2.41 ± 0.36	0.15 ± 0.026	49.64
Midakegn	107.10 ± 2.55	60.42 ± 0.69	2.14 ± 0.17	4.01 ± 0.69	0.69 ± 0.24	28.35
Total mean	77.13 ± 35.60	73.0 ± 37.69	4.56 ± 4.54	2.23 ± 1.04	0.22 ± 0.26	64.29
Sig.	0.00	0.002	0.01	0.00	0.00	

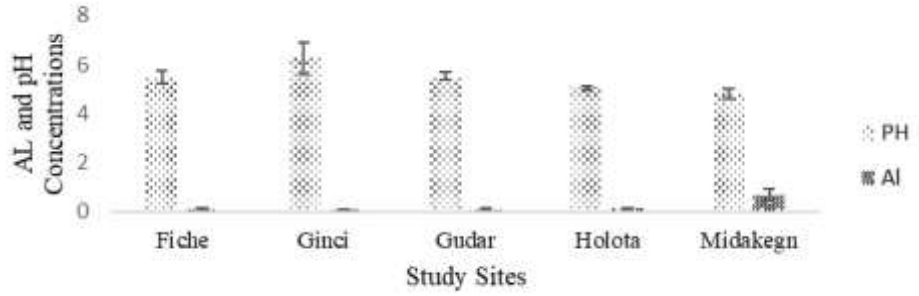

Fig. 3. pH and Al relationships of the study sites.

Isolation and authentication of rhizobia

One hundred forty isolates were obtained from the nodules of faba bean plant by streaking crushed nodule suspension on the YEMA medium plates. Of these, 40 low pH tolerant isolates were taken for further analysis and finally reduced to 15 rhizobial isolates. All the isolates were found to be Gram-negative rods, did not absorb Congo red, did not grow on PGA medium and there was the absence of 3-keto lactose in rhizobia. All the tested isolates changed the BTB color to yellowish indicating common characteristics of fast-growing *Rhizobium* sp. (Somasegaran and Hoben, 1994a). These results obtained from gram staining, growth on YEMA-CR, YEMA-BTB, and PGA medium preliminary confirm the standard cultural and morphological characteristics of *Rhizobium* species as described by Somasegaran and Hoben (1994a) and Vincent (1970). This result also agrees with previous observations on faba bean nodulating rhizobia (Zerihun Belay and Fassil Assefa, 2011; Alemayehu Workalemahu, 2009).

Eco-physiological characters

Salt, pH, and temperature tolerance

The results indicated that the isolates were tolerant to extremely low pH since they could survive and grew in the low pH, even at 4.0. The rhizobial isolates were tolerant to pH 4, 4.25, 4.5, 4.75 and 5 (80, 95, 100, 100 and 100%), respectively (Fig. 4). Results from root nodules of *Trifolium alexandrinum* confirmed 50% pH 4 tolerance in *Rhizobium*. Moreover, Jordan (1984) showed that most of the isolates are acid adapted, capable of surviving at pH values lower than the pH range between 4.5 and 9.5. Different strains of the same species may vary widely in their pH tolerance (Zahran, 1999). The fast-growing *Rhizobium* strains have generally been considered less tolerant to acid pH than slow-growing *Bradyrhizobium*

strains (Graham *et al*., 1994). However, Mpepereki *et al*. (1997) reported both fast and slow-growing *Bradyrhizobium* strains of *Vigna unguiculata* are tolerant to pH 4. *R. leguminosarum* biovar *Vicieae* of *V. faba* and *Pisum sativum* from Northwestern Ethiopia grew at pH 4.75 (Amha Gebremariam and Fassil Assefa, 2018). Slattery and Coventry (1995) isolated several *R. rrijblii* strains able to continue growing at pH 4.3 and the growth of some strains was unaffected by lower pH. Some rhizobial isolates were more sensitive to low pH than their host and this affects the establishment of the symbiosis (Zahran, 1999). The biological membrane is key to regulating acid stress by excluding protons from low external pH environments. Acid tolerant strains of many rhizobia species have been isolated with pH tolerance often being facilitated by proton exclusion, increased cytoplasmic buffering and acid-shock response mechanisms (Ferguson *et al*., 2013). Therefore, rhizobial isolates that survived in pH 4 are very important candidates as inoculants for acidic soils of most faba bean fields to improve their productivity.

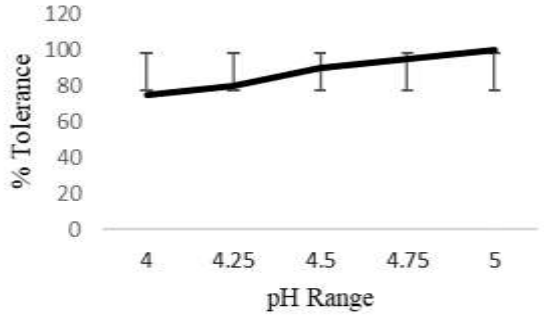

Fig. 4. pH tolerance of rhizobial isolates.

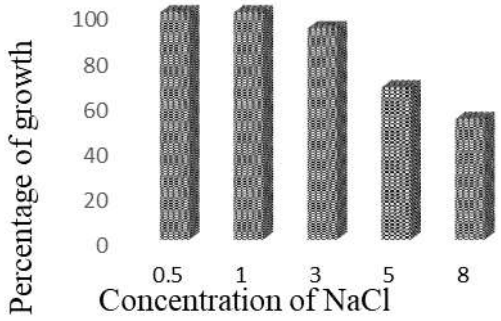

Fig. 5. Tolerance of faba bean rhizobial isolates to NaCl.

The luxuriant growth temperature where 100% of the isolates grew ranged from 25–35°C while the percentage of isolates that grew at 4, 20, 42, and 55°C was 13, 80, 19, and 0%, respectively. The variability in temperature tolerance may be due to the adaptation difference of the strains to the various temperature range and is highly strain-dependent for the genus Rhizobium (Jordan, 1984). Although the optimum temperature for rhizobia on culture is between 27–30°C (Munévar and Wollum, 1981), some were tolerant to a low temperature to the level of 20°C. Alternatively, rhizobial isolates of this study were tolerant up to 42°C (19%). This result is correlated with (Getaneh Tesfaye, 2008). Certain strains of *R. leguminosarum* bv *trifolii* can grow in artificial cultures, with growth responses up to 41°C (Giddens *et al*., 1982). Previous workers also confirmed that the optimum temperature for the growth of root-nodulating bacteria ranged from 25°C to 30°C (Bhargava *et al*., 2016) which is in line with the present finding. Further increase in temperature led to a noticeable decline in growth and at 45°C, most of the isolates demonstrated moderate growth.

Heavy metals (HMs) and IAR tolerance

All the isolates showed variability in their heavy metal tolerance (Fig. 6). There is a slight decrease in microbial growth as the concentrations of HMs increase. In this study, rhizobial isolates were resistant to Pb (82%) followed by Hg (70%) and Ni (45%) and all the isolates showed the least resistance to Cu (35%). The isolates were considered to be resistant when the growth occurred in the presence of heavy metals or sensitive if otherwise. The appearance of resistance levels in isolates against Pb may be due to the isolates containing the Pb-resistant gene (Sheng *et al*., 2008). Previous studies showed that increased concentrations of heavy metals can affect the growth, morphology, and activities of microorganisms in nitrogen fixation (Khan and Scullion, 2002). Furthermore, the tested isolates exhibited more sensitive against Cu than the other heavy metals. It has been shown that with increasing concentrations of heavy metals such as Cu, Zn, Ni, and Pb, the bacterial counts of *Rhizobium* sp. are reduced, and also the expression of nod genes was varied (Vasilica *et al*., 2011). In contrast, it was suggested that rhizobia can tolerate high heavy metal concentrations in different ways and may play a significant role in the restoration of contaminated soil (Teng *et al*., 2015). Isolation of rhizobia strains resistant to stresses like heavy metals is very important for efficient nitrogen fixation and improving plant productivity, especially in stressed environments.

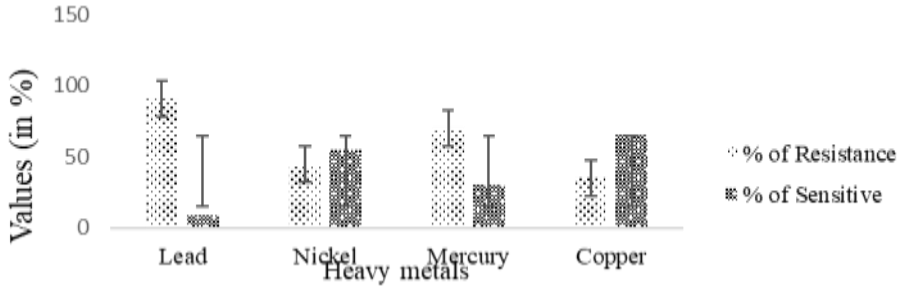

Fig. 6. Heavy metal tolerance of rhizobial isolates.

All the isolates were assessed for their IAR pattern against six different antibiotics. They showed resistance to low levels of at least one antibiotic. The evaluation of IAR showed that most of the tested isolates exhibited the highest resistance to streptomycin (93%), followed by gentamycin (82%), tetracycline (76%), neomycin (33%), and chloramphenicol (27%). On the other hand, ampicillin had the least antibacterial effect, the number of isolates showing resistance was four (Fig. 7). The overall pattern was shown by these antibiotics: streptomycin > gentamycin > tetracycline > neomycin > chloramphenicol > ampicillin. Hungria *et al*. (2001) stated that this character is due to three known determinants of bacterial permeability to antibiotics: hydrophobicity, electrical charge, and amount of the antibiotic. Streptomycin resistance is due to an alteration of a specific protein on the 30S ribosomal subunit to which streptomycin binds in the sensitive cell. The sensitivity of isolates to antibiotics may be due to these bacteria not being exposed to these antibiotics in natural environments. Depending on the difference in antibiotic resistance patterns, this technique could be successfully employed in the field of ecological studies, particularly in the recovery and enumeration of rhizobia introduced into the soil (Bedi and Naglot, 2011). Therefore, streptomycin-resistant in this study is effective and important in ecological field studies on strain competition.

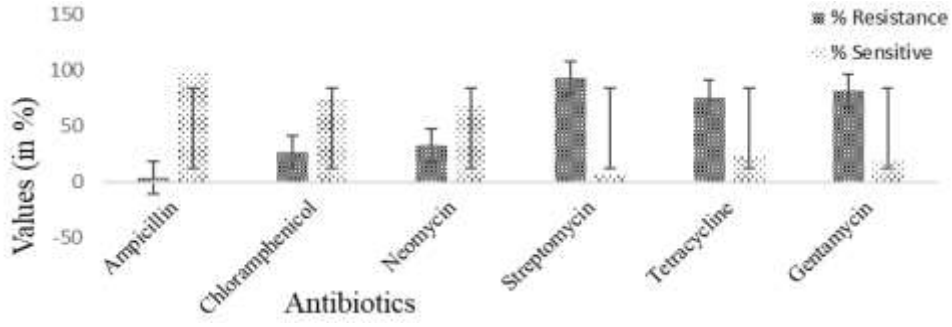

Fig. 7. Intrinsic antibiotic resistance (IAR) of faba bean rhizobial isolates.

Plant growth promoting (PGP) potential of rhizobial isolates

In this experiment, of the 15 faba bean rhizobial isolates, 93%, 47%, and 33% showed ammonia, HCN, and IAA production, respectively. Equally, 73%, 67%, 60%, 60%, and 40% of them showed CMC, gelatin, lipase, protease, and starch hydrolysis activity respectively while 53% exhibited phosphorus solubilization (Table 4). Isolate FFB27 takes the leading place in phosphate solubilization with 2 mm halo zone formation and followed by MFB4 rhizobial isolate with 1.7 mm solubilization index. Nearly, a similar result was reported by Manasa *et al*. (2017a) from different rhizosphere soils of India. Furthermore, Joseph *et al*. (2012) reported ammonia production in 74.2% *Rhizobium*. This finding is contrary to Deb *et al*. (2014) who reported that 20% of rhizobial isolates isolated from *Cajanus cajan* were found to be positive towards ammonia excretion. Accumulation of ammonia in soil may increase pH up to 9. It suppresses the growth of certain fungi and bacteria. Ammonia produced by bacteria is taken up by plants as a source of nitrogen for their growth (Ahmad *et al*., 2008). Phosphorus (P), the second important plant growth-limiting nutrient after nitrogen and is abundantly available in soils (Zaidi *et al*., 2009). Its availability to plants is generally low since it is found in insoluble forms. In this experiment, more than 50% of isolates showed a clear halo zone on pikovskaya's media containing tri-calcium phosphate. Solomon Legesse and Fassil Assefa (2014) retrieved twelve phosphate solubilizing isolates of faba bean from acidic soils of Wollega. The use of Rhizobium with PGP activities is becoming a popular approach for improving growth and N_2 fixation in legumes. Improved phosphorous nutrition by P solubilizing rhizobia was reported to influence overall plant growth, root development, and nitrogenase activity which is positively correlated with increased nodulation and N_2-fixation (Singh *et al*., 2014).

Hydrogen cyanide is a broad-spectrum antimicrobial compound involved in the biological control of root diseases by plant-associated nodulating bacteria (Ramette *et al*., 2003). In this study, 47% produced HCN and 53% failed to produce it. Earlier studies reported the role of HCN in disease inhibition *in vitro* conditions Shivakumar and Vijayendra (2006), and most of them produced HCN and helped in plant growth. IAA is the foremost phytohormone that accelerates plant growth and development by improving root/shoot growth and seedling vigor. The present finding found that the rhizobial isolates possesses relatively lower IAA producers 33% (Fig. 8). Nearly isolate FFB25 and FMB5 possesses the highest (174 µg/ml) IAA production while isolate FFB1 produces 158 µg/ml. However, it has been estimated that 80% of bacteria isolated from the rhizosphere can produce

IAA (Spaepen and Vanderleyden, 2011). On inoculation of *R. leguminosarum* bv. *viciae*, a 60-fold increase in IAA was observed in the nodules of vetch roots (Bhattacharyya and Jha, 2012). Additionally, about 73% of IAA production was estimated from different rhizosphere soils (Manasa *et al*., 2017b). Rhizobia employ the production of cell wall degrading enzymes as one of its strategies for antagonistic effect against plant pathogens. The results presented in (Fig. 8) indicated the ability of *Rhizobium* sp. the production cell wall degrading enzymes. It was reported by Oliveira *et al*. (2007) *Rhizobium* strains can utilize starch obtained from different sources. The production of lytic enzymes such as protease and lipase lyse the pathogenic fungal and bacterial cell walls (Ji *et al*., 2014). In agriculture, these protease-producing microorganisms have a role as biological control agents for the eradication of some fungal plant pathogens (Paul *et al*., 2001). Some of the proteases are involved in inactivating extracellular enzymes of phytopathogenic fungi (Elad and Kapat, 1999). Of the 15 rhizobial isolates, 11 of them produce cellulase enzyme which is indicated by degrading CMC as a sole source of carbon followed by gelatinase enzyme production. It has been reported that the production of bacterial cellulase in synergy with antibiotic production by other bacteria can suppress diseases caused by pathogenic fungi (Yu *et al*., 2007). Nevertheless, cellulase can also work synergistically with other antifungal compounds. *Paenibacillus ehimensis* KWN38 produces antifungal compounds in combination with lytic enzymes, especially cellulase and ß-glucanases, which protect crops against pathogenic oomycetes, such as strains belonging to the genus *Phytophthora* (Rastogi *et al*., 2009). Moreover, cellulase produced by this *Paenibacillus* species might help in rhizosphere soil decomposition, increasing the availability of nutrients for the plant (Trivedi *et al*., 2011). These and the other enzymes can play an important role in degrading fungal cell wall and considered as major antagonistic activity.

Table 4. Potential of faba bean rhizobia in plant growth promoting traits production.

Isolate	Site	Ammonia	CMC	Gelatin	HCN	Lipase	P. solubilization (mm) Mean ± SD	Protease	Starch	IAA (µg/ml) Mean ± SD
FFB1	Fiche	+	+	+	-	+	1.4 ± 0.05^{bc}	+	+	158.21 ± 0.074
FFB25	Fiche	+	+	+	-	-	1.5 ± 0.06^{bc}	+	-	174.63 ± 0.19
FFB27	Fiche	+	+	-	-	+	2 ± 0.025^{a}	-	+	124.54 ± 0.16
FFB37	Fiche	+	-	+	+	+	1.4 ± 0.03^{bc}	+	+	-
FFB39	Fiche	-	-	-	-	-	-	-	-	-

Isolate	Site	Ammonia	CMC	Gelatin	HCN	Lipase	P. solubilization (mm) Mean ± SD	Protease	Starch	IAA (µg/ml) Mean ± SD
MFB4	Mida kegn	+	+	+	+	+	1.7 ± 0.08^{ab}	+	+	-
MFB5	Mida kegn	+	+	+	+	+	1.2 ± 0.1^{cd}	-	+	173.13 ± 0.14
MFB7	Mida kegn	+	-	-	+	+	-	-	-	-
GuFB5	Gudar	+	+	+	-	-	-	-	+	-
GuFB8	Gudar	+	+	+	+	+	0.9 ± 0.36^{de}	-	-	132.83 ± 0.091
GiFB1	Ginchi	+	+	-	-	-	0.8 ± 0.4^{e}	+	+	-
GiFB2	Ginchi	+	+	+	+	+	-	-	+	-
GiFB3	Ginchi	+	+	+	+	-	-	-	-	-
HFB5	Holeta	+	-	-	-	-	-	-	-	-
HFB6	Holeta	+	+	+	-	+	-	+	+	-
Total %		93.33	73.33	66.67	46.67	60	53.33	40	60	33

Means with the same letter are not significantly different at p>0.05 using Duncan's multiple mean comparison tests.

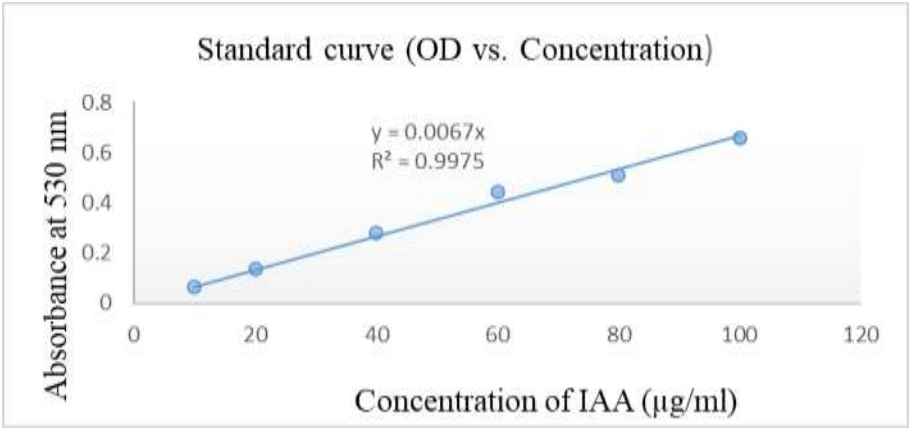

Fig. 8. Standard curve for IAA quantification.

Effect of Rhizobium on seed germination

Seedlings that emerged protruding around 2 mm were considered as emergence and were continued for a period of 120 h. Initially, the rhizobial treatments did not show any differences in emergence, however, at 24 to 72 h after inoculation, they showed higher percent emergence compared to the control. All the seeds showed maximum germination after 72 h. In a comparison of the control, the highest seed germination was obtained in seeds coated with *Rhizobium* culture. The number and length of the sprouts

were significant in the test as compared to the control (Fig. 9; Table 5). The highest germination 72% was recorded when seeds were treated with rhizobial isolates while the lowest germination 56% was recorded in control. Higher vigor index of seedling 219.60 was obtained from rhizobial treatment and the lowest was 91.28 in the untreated control. Initially, inoculated plants showed higher emergence which might be due to the production of phytohormone as phytohormone influences seed germination. Therefore, inoculation significantly influenced the seedling vigor and the highest value was found in the seeds when it was inoculated with *Rhizobium*. A similar result was reported by Mahanty *et al*. (2017). Also, the mean time and the mean daily germination was affected by *Rhizobium*. It was proved that germination ability was raised by *Rhizobium.* Biswas *et al*. (2000) and Noel *et al*. (1996) reported significant enhancement of early seedling root growth in non-legumes by seed inoculation with *R. leguminosarum* and attributed this effect to bacterial phytohormone production. The vigor index determines the state of the health of the seedling and ultimately the state of the productivity of the plant. The higher the vigor index, the better will be the yield of the plant. The present study revealed that significant improvement was made in seedling emergence and seedling vigor due to rhizobial inoculations. Higher vigor seeds are a prerequisite for the better establishment of the seedling. Seed vigor and viability are important components influencing seedling establishment and productivity (Miller and Copeland, 1997). The bacterial isolate FFB37 had the highest germination assay followed by isolate FFB1 while the lowest germination was recorded in isolate GiFB3 and similar trends were observed in the vigor index except for HFB6 which had the lowest vigor index.

Fig. 9. Effect of *in vitro* rhizobial isolate inoculation on seed germination.

Table 5. Effect of rhizobia on seed germination and vigor index.

Treatment	Day 1	Day 2	Day 3	% seed germination	Radicle length	Plumule length	Vigor index
FFB1	3.6	4.4	4.9	86	0.18	0.07	21.5 ± 0.052
FFB25	1.8	3.8	5.5	75	0.14	0.06	15 ± 0.056
FFB27	2.2	4	4.7	73.67	0.15	0.09	17.68 ± 0.042
FFB37	5.2	5.8	6.8	118.67	0.21	0.1	36.79 ± 0.078
FFB39	1.4	3.2	4.8	63.67	0.13	0.05	11.46 ± 0.056
MFB4	1.2	2.8	4.7	59	0.12	0.06	10.62 ± 0.042
MFB5	1.2	3	4.8	61	0.15	0.04	11.59 ± 0.078
MFB7	1.4	3.4	4.6	63.67	0.13	0.05	11.46 ± 0.056
GuFB5	1.6	3.6	4.4	64	0.14	0.06	12.8 ± 0.056
GuFB8	1.8	3.8	4.6	68	0.15	0.07	14.96 ± 0.056
GiFB1	1.6	2.8	4.9	62	0.17	0.05	13.64 ± 0.084
GiFB2	1.4	4.2	4.3	66	0.13	0.04	11.22 ± 0.064
GiFB3	1.2	3	3.9	55	0.14	0.03	9.35 ± 0.078
HFB5	1.6	3.6	4.9	68.33	0.16	0.05	14.35 ± 0.078
HFB6	1.8	3.4	4.5	65.67	0.07	0.06	8.54 ± 0.007
Sum	29	54.8	72.3	72	2.17	0.88	220.96
Mean ±SE	1.9 ± 1.17	3.65 ± 0.6	4.8 ± 0.4	71.98	0.14 ± 0.001	0.06 ± 0.0003	14.73 ± 48
Control	19	44	56	56	1.15	0.48	91.28
Mean± SE	0.42 ± 0.08	1.4 ± 0.91	1.6 ± 0.38		1.15 ± 1.23	0.48 ± 1.35	91.28

CONCLUSION AND RECCOMENDATIONS

The study demonstrated that different acid-tolerant rhizobia, isolated from soils obtained in faba bean fields, can withstand low pH levels. The acidic soils of the central highlands of Ethiopia contain acid-tolerant faba bean rhizobia (80%) at pH 4. This study showed that there was considerable variability in the level of stress tolerance of rhizobial isolates obtained from the different fields (NaCl, temperature, HMs, and IAR). Of the nine multiple PGP tests, three rhizobial isolates (MFB5, FFB1, and MFB4) showed positive response for 8 PGP traits while FFB37 and GuFB8 responded for 7 PGP traits and were considered effective as the most potential in plant growth promoting properties. Isolate obtained from Fiche (FFB37) was the best in seed germination assay with 118.67% followed by FFB1 (86%) and FFB25 (75%). There was a variation in % seed germination (72% and 56%) and vigor index (219.60 and 91.28) between rhizobial inoculated and non-inoculated faba bean seeds, respectively. Results suggested that rhizobia with PGP activity and seed germination and vigor potentials are more superior to those without such activities in promoting faba bean growth and are recommended for inoculum development following greenhouse and field trials. Hence, further evaluation of these acid-tolerant rhizobial isolates is needed to uncover their efficiency as plant growth promoting bacteria in the

greenhouse and soil-plant systems.

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