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RESEARCH ARTICLE

Assessment of the Potential of *Rhizobium leguminosarum bv.viciae* on Two Different Soils with the Ashebka faba Bean Variety (*Viciae faba* L.) as the Host Plant

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Abstract

Objectives: To assess the effectiveness of the potential of the *Rhizobium leguminosarum bv.viciae* strain in two different soils with the Ashebka faba bean variety as the host plant. **Method**: Soil physicochemical analysis and the most probable number were done according to their standard procedure. The pot was laid out in a complete randomized design with three replications. Three top strains were selected as inoculants for faba beans grown on the slightly acidic Shentia soil and the slightly neutral Dabat soil with their control. The symbiotic effectiveness of the strains was evaluated based on plant agronomy and the total nitrogen of the plant. The results of the strains were analyzed by SPSS version 26. **Findings**: The results of shoot dry weight show that all strains accumulated 81mg/p for isolate WK1E1, followed by 85mg/p for isolate GR1E1, and finally 87mg/p^{-1} for isolate co-inoculant on Dabat soil, and 78mg/p for isolate WK1E1, followed by 82mg/p for GR1E1 and finally 85mg/p^{-1} for co-inoculant Shentia soil. The nodule number record range from 138mg/p^{-1}, and 173mg/p^{-1} for isolate WK1E1 and co-inoculate, respectively for Dabat site. 139mg/p^{-1} for isolate WK1E1 and 165 mg/p^{-1} for isolate co-inoculate Shentia site. Maximum mean shoot dry mass (91mg/p) was scored by positive nitrogen and the minimum (29mg/p) by the negative control nitrogen treated control for Dabat. The maximum mean shoot dry mass (86mg/p) was scored by the positive nitrogen treated control and the minimum (29mg/p) by the negative control for Shentia. For Dabat soil, the relative effectiveness expressed as a percentage of shoot dry mass of inoculated over total nitrogen control, showed that 89, 93, and 95.6 and Shentia soil the relative effectiveness expressed as a percentage of shoot dry weight of inoculants over nitrogen treated control, showed that 90.6, 95.3, and 98.8 for isolates WK1E1, GR1E1, and co-inoculant, respectively for both soils. Positive correlations were also observed concerning nodule numbers with other agronomic parameters. **Novelty**: No such testing

was attempted in that study area before, and this new idea came because the *Rhizobium leguminosarum bv.viciae* isolates tested their survival, compatibility, and effectiveness in two different soils with Ashebka faba bean as the host.
Keywords: Ashebka faba bean, most probable estimation, *Rhizobium leguminosarum bv.Vicae*, inoculums, symbiosis

1 Introduction

Faba bean is a major staple crop in eastern, southern Asian, Middle Eastern, European, South American, and central Africa. It is among the most dietary proteins in humans [1]. Faba bean production in Ethiopia including Dembiya, Debark, and Dabat woredas is below its production potential but the crop is still widely grown in most regions of the country. The production of faba beans decreases from time to time due to poor soil fertility [2]. This is due to the fact that this crop is mostly grown by poor small-scale farmers who are not able to purchase expensive inorganic fertilizer and face negative effects from its use [3].

Currently, more emphasis is placed on the use of biological nitrogen fixation technology in agricultural systems to overcome the problems associated with soil fertility depletion and excessive use of inorganic nitrogen fertilizer [4]. Biological nitrogen fixation by *Rhizobium* is considered as an inexpensive and environmentally friendly alternative to improve crop yield compared to legumes with counterpart of legumes with *Rhizobium* inoculants. It is the most used biological nitrogen fixation technology in agriculture as evidenced in previous studies, but not well established in most African countries including Ethiopia [2]. This technology may be successful if the isolation and characterization of indigenous *Rhizobium leguminosarum bv.viciae* found in the soil are done properly, as this may lead to the discovery of stronger strains for inoculant production. Additionally, it is emphasized that prior to the potential assessment of *Rhizobium leguminosarum bv.viciae* isolate inoculants in the pot experiment, the identification potential strains for inoculants production should be done to enhance faba bean yield and determine the most suitable locations for inoculation.

2 Methodology

2.1 Description of study sites

The soil for the pot experiment was taken from Shentia, Central Gondar Zone, and the Dabat site, North Gondar Zone, Amhara Regional State, Ethiopia. The experimental sites are located at geographical coordinates of 12.5894° N latitude and 37.4442° E Longitude at an altitude of 2133 meters above sea level for the Shentia site, College of Agriculture, University of Gondar, and Latitude12° 59’ 1.54”N, and longitude 37° 45’ 55.66”E at an altitude range from about 1000 meters to over 2500 meters above sea level for the Dabat site, Gondar Agricultural Research Centre in 2015.

2.2 Study design, study period, and sampling method

2.2.1 Study design and study period

A randomized complete design (CRD) was used to conduct this study. The study was conducted from May to July 2023 at the College of Agriculture, University of Gondar, Gondar, Ethiopia.

2.3 Analysis of a soil sample

In this study, physicochemical analyses of the study site soil samples, such as pH, organic carbon, cation exchange capacity, and exchangeable bases (K, Ca, and Mg), and total nitrogen from the representative bulk soil sample before planting were analyzed. Soil analyses for the aforementioned physicochemical parameters were done at the Gondar Agricultural Research Center. Soil sample pH, cation exchange capacity, and electrical conductivity analysis were done accordingly[5]. The availability of phosphorus[6], potassium, total nitrogen, organic matter[7], sodium, magnesium, and calcium were done based on the standard soil analysis method.

2.4 Estimation of native *Rhizobium* in soil, inoculum preparation, and pot experiments

2.4.1 Method for Determining the Population of Indigenous Rhizobia in the Soil

The plant infection count, also known as most probable number (MPN) counts, was used to determine the number of viable and infective *Rhizobium*. Ten grams of soil were diluted in aseptic conditions in 90ml of sterilized distilled water up to 10^{-6} and used to inoculate a faba bean seedling adequately grown in acidic treated and sterilized sand using a plastic cup in four replications. Nodule observation were made 30 days after inoculation, and positive, and negative nodulation of growth units was recorded for all dilutions and converted to the number of rhizobia g^{-1} soil using the most probable table[8].

2.4.2 Preparation of inoculums

A single pure colony was transferred into yeast extract mannitol broth medium and kept at 30 °C for 3 days on a rotary shaker at 120 rpm[9]. Four hundred ml of liquid medium containing *Rhizobium* was added to 1kg of carrier (sterile activated charcoal), mixed through, and then packed in plastic bags. The packed plastic bag was stored in room temperature for 2-3 weeks, and the number of *Rhizobium* per gram of activated charcoal was checked.

2.5 Pot experiment

The symbiotic effectiveness of the new *Rhizobium leguminosarum bv.vicea* strains was evaluated. To perform this research, soil is needed. The soil found in Shentia are located at the border of the Tewodross campus, the University of Gondar, and Dabat, Dabat woreda, North Gondar Zone, Ethiopia. Plastic pots were bought from the local market. The seed of the Ashebka faba bean variety were collected from the Amhara Regional Research Institute, Gondar. Regarding isolates with their WK1E1, and GR1E1 and other combinations of them isolated from the Microbial and Molecular Biology Laboratory, Institute of Biotechnology, University of Gondar, Gondar, Ethiopia, and the standard *Rhizobium* inoculant "EAL110" as a positive control obtained from Mengsha Biofertilizer producing PLC Addis Ababa, Ethiopia, and urea obtained from Amhara Regional Research Institute, Gondar. Others were left non- inoculated as a negative control.

1. **Assessment of N_2-fixing potential**

After strains have been screened for symbiotic effectiveness under sterile conditions, their performance can be assessed in sterile soils. Soil chemical status such as pH, CEC, TN, and EC can provide useful information about the soil. The highly effective isolates were selected, and their symbiotic effectiveness was further determined through a pot soil experiment in a greenhouse. The sterile soil was properly mixed, sieved and air-dried and a 3 kg of soil was distributed in plastic pots. The Ashebeka Faba bean variety, which was obtained from the Gondar Agricultural Research Centre, was surface sterilized using 95 % ethanol for 10 min and 3 % sodium hypochlorite for 3 min and rinsed with sterile water. Five ungerminated seeds were sown in each pot and later thinned down to three after germination for one week. After a week, each seedling was inoculated with 1 ml of each isolate grown for 3 days in YEM broth on a rotary shaker at 120 rpm. The experiment was set up in triplicate under a greenhouse.

The pots were arranged in a complete random design (CRD), with each consisting of a negative control (without nitrogen and inoculate) and a positive control (standard *Rhizobium* and urea). The nitrogen fertilizer was given at a concentration of 0.05 g / l per week until the plants were harvested. All the pots were watered every two days.

After 45 days of growth, the whole plant was carefully uprooted to determine nodulation number, nodule colour, nodule dry weight, nodule fresh weight, shoot length, shoot fresh weight, and shoot dry weight and plant total nitrogen by the Kejhalde method. Soil nitrogen was analyzed before planting. The effectiveness of the isolates was selected based on symbiotic effectiveness and accumulating plant record agronomy data, as described[10].

1. **Data management and analyses:**

The generated data were subjected to statistical analysis to determine the mean variations between the treatments. The analysis of variance (ANOVA) at $p < 0.05$ was considered significant for all analysis by using SPSS software version 26. Symbiotic effectiveness plant agronomic parameter data were analyzed by a two-way analysis of variance using SPSS software.

3 Results and Discussion

3.1 Soil analysis

The physiochemical analysis of soil samples revealed that the two study sites have following results. Similar soil parameters were exhibited except for p/ppm and pH. Sheinta soil is twofold higher for p/ppm than Dabat soil. Slightly acidic soil was noted with Shentia soil and slightly neutral for Dabat soil. The pH analyses indicated that the pH values of soil samples were 6.5 and 7.42 recorded from the Shentia and Dabat sites, respectively. Slightly acidic soil was noted with Shentia soil, and slightly neutral soil for Dabat soil [11]. Similarly, electrical conductivity (EC) analysis showed that soil samples have significantly different electrical conductivity, with the highest value of 0.094 obtained from the Dabat and a lower value of 0.073 obtained from the Shentia soil sample. Regarding macro-elements and organic matter analysis, soil samples have different levels of organic matter and microelements. The organic matter content ranged between 2.273 and 1.741% recorded from the Dabat and Shentia site soil samples, respectively. Likewise, the concentration of macro-elements such as phosphorus, calcium, potassium, sodium, and magnesium are significantly different. Whereas the amount of total nitrogen recorded was 0.199 for Dabat and 0.164 for Shentia site soil samples.

3.2 Determining the Population of Indigenous Rhizobia in the Soil

The most likely number of rhizobia specific to faba bean was calculated by the standard method. The MPN result showed that, both the highest and lowest population sizes of Indogioues *Rhizobium* were observed at the Dabat and Shentia sites, respectively. The highest *Rhizobium* population size was 5.8 $x10^2$ cells g^{-1} soil, while the lowest was 1 $x10^2$ cells g^{-1} soil observed at Shentia and Dabat soil, respectively. Using this method, a rhizobial population of colony forming units per gram of soil was estimated, indicating a low number of native rhizobia able to nodulate the faba bean. But both of the soils needed to be inoculated because $<10^3$. In addition, the soil analysis showed that, the study area has high deficiency in nitrogen and phosphorus. *Rhizobium leguminosarum bv.vicea* able to solubilize phosphorus and fix atmospheric nitrogen [12].

3.3 Pot experiment

3.3.1 Evaluation of the Symbiotic Effectiveness of the Strains on Different Soils

The findings of this study showed that *Rhizobium leguminosarum bv.vicea* isolates had a considerable effect on agronomic properties such as plant height, number of nodules, and dry weight parameters as compared to the negative control and standard *Rhizobium* and urea, as indicated in S**upplementary table A** and Figure 1. Three top strains were used as inoculants for faba beans grown on the slightly acidic Shentia soil and the slightly neutral Dabat soil. The nodule number records range from 138 to 173 for isolate WK1E1 and co-inoculate, respectively. For the Dabat site 139 forisolates WK1E1, and 165for isolates andco-inoculate for Shentia site. This result is greater than the Mekonnen result [13] who sates the maximum nodule number is 156.85 from stain EHHFR1C and the minimum is 72.31 from strain ECHFR3D. The result also greater than Argaw report who reported that the minimum 66.7 from strain NSFBR-12 and the maximum score of number of nodule was 150 from strain NSFBR-30 on Holleta soil but greater than on kullumsa soil who state 186.7 nodule number from NSFBR-1. This difference may be due to strain potential difference.

The nodule dry weight records range from 17.2, and 15.1 for isolate co-inoculate, Shentia, and Dabat soil, respectively. This result is less than Allito [14], who states that maximum nodule dry weight 242.0g from stain NSFBR-15 but greater than Mekonne, who reports that 0.2938 nodule dry weight was obtained from EHHFR1 strain. And also this result less than belay report who report that maximum result 5.4g from strain AUFR114 but greater than Argaw report. This difference may came due to inheritance of soil property and compatibility of plant with *Rhizobium leguminosarum bv.vicea*.

The shoot dry weight results show that all strains accumulated 81mg/p for isolate WK1E1, followed by 85mg/p for isolate GR1E1, and finally 87mg/p^{-1} for isolate co-inoculant on Dabat soil, and 78mg/p for isolate WK1E1, followed by 82mg/p for GR1E1, and finally 85mg/p for co-inoculant Shentia soil. This result is less than that of Allito who reported 262.7G/P obtained from NSFBR-15, but greater than that of Mekonne, who reported 1.10g p^{-1} nodule dry weight obtained from the EHHFR2A strain. This result less than belay report. This implied that nodulation enhanced shoot dry weight accumulation.

The maximum mean shoot dry mass (91mg/p) was scored by positive nitrogen and the minimum (29mg/p) by the negative control nitrogen control for Dabat. The maximum mean shoot dry mass (86mg/p) was scored by positive nitrogen control

Fig 1. The shoot height of the different treatment inoculant with two positive (urea and standard *Rhizobium*) and negative control (without any treatment) on Shentia and Dabat soil culture, respectively

and the minimum (29mg/p) by the negative control for Shentia. This result is greater than the Mekonnen result report that EHHFR(1C&4C) were the isolates that scored the highest shoot dry weight (7.44 and 7.43 g plant^{-1}), followed by isolate EHHFR3D with a shoot dry weight of 7.2 g/p. Inoculation with rhizobial cultures increased shoot dry weight by 66.6% for Dabat and 65.8% for Shentia over negative controls. Some inoculated plants produced greater shoot length and shoot dry weight compared with nitrogen treated plants. This might be because some rhizobia isolates produce plant-growth-promoting hormone in addition to fixing nitrogen [15].

For Dabat soil, the relative effectiveness expressed as a percentage of shoot dry mass inoculated over nitrogen treated control, showed that 89, 93, and 95.6 and Shentia soil the relative effectiveness expressed as a percentage of shoot dry weight of inoculants over nitrogen treated control, showed that 90.6, 95.3 and 98.8 for isolates WK1E1, GR1E1, and co-inoculant, respectively, for both soils were found to be highly effective (80-100%), effective (50-80%), lowly effective (35-50%), and ineffective (<35%) (**Supplementary Table A**). The highest score of 80-100% effectiveness of symbiotic nitrogen fixation were displayed by all isolates by WK1E1, followed by GR1E1, and co-inoculant with shoot dry weight. Shoot dry weight >81mg/P for Dabat soil, and >78mg/P for Shentia (**Supplementary Table A**). This indicates the highest scores of symbiotic nitrogen fixation.

The first criterion for a *Rhizobium leguminosarum bv.vicea* used as a biofertilizer or nitrogen inoculum is that it must be superior in its symbiotic effectiveness and have a highly effective nitrogen-fixing ability, forming a symbiotic association with the host legume [16]. For Dabat soil, the relative effectiveness expressed as a percentage of shoot dry mass inoculated over control,

showed that 89, 93, and 95.6, and Shentia soil, the relative effectiveness expressed as percentage of shoot dry weight of inoculants over control, showed that 90.6, 95.3, and 98.8 for isolates WK1E1, GR1E1, and co-inoculant, respectively, for both soils were found to be highly effective (80-100%), effective(50-80%), lowly effective (35-50%), and ineffective (<35%) (**Supplementary Table A**). The highest score of 80-100% effectiveness of symbiotic nitrogen fixation was displayed by all isolates. WK1E1 followed by GR1E1, and co-inoculant with shoot dry weight. Shoot dry weight >81mg/P for Dabat soil and >78mg/P for Shentia (**Supplementary Table A**). The highest SE% was found with the isolate co-inoculant, which gave a SE% value of 98.8% for Shentia soil, and 95.6 for Dabat soil. These findings indicate their potential to fix atmospheric nitrogen, as indicated SE% value.

Co-inoculation had the highest shoot length of 51cm and 52 cm for Shentia, and Dabat soil, respectively. This result was greater than that of Benmoussa who stated that 47cm obtained from NSFBR-41. This promotional effect of inoculation treatments is in line with results obtained by Benmoussa who stated that vegetative growth parameters of faba bean were significantly increased by inoculation treatments compared with control. Rhizobial inoculation increased shoot length up to 42.3% for Shentia soil compared with the negative control for both soils. A similar result was obtained on common vetch (*Viciae sativa* L.), in which inoculation increased shoot length over uninoculated plants. Inoculation of seed with *Rhizobium* significantly increased plant height in faba beans. This result also less than Argaw report who state that the maximum shoot length 60.3 was recorded.

The maximum total nitrogen was 3.2 and 3.0 for co-inoculant and GR1E1 and WK1E1, respectively, for the Dabat site, and similarly for 3.1 and 3.0 for WK1E1 and GR1E1 and co-inoculant, respectively, for the Shentia site. This result is less than the maximum recording of the Mekonnen reported of 5.3 obtained from ECHFR3D the minimum 3.6 obtained from EHHFR1C. But greater than Alito report who report that 2.4 was obtained from EHHFR3C. The result less than Argaw report who state that the maximum plant nitrogen 4.5 from strain NSFBR-48and the minimum 3.42 from strain NSFBR-44[17]. But greater than Belay report who state that the maximum plant total nitrogen 2.8 and minimum 1.9 form strain ahfr132 and AUFR 128, respectively. This difference may be due to soil physicochemical properties, bacteria potential and their compatibility[18].

3.3.2 Correlation analysis

The study observed strong positive correlations between the number of nodules and various plant parameters in both Dabat and Shentia soils. In Dabat soil, the number of nodules was strongly positively correlated with nodule fresh weight, nodule dry weight, shoot length, shoot fresh weight, and shoot dry weight. Similarly, in Shentia soil, the number of nodules exhibited strong positive correlations with nodule fresh weight, shoot length, shoot fresh weight, shoot dry weight, and plant total nitrogen as shown in Tables 1 and 2. The research indicated that nodulation status positively correlates with agronomic parameters and plant tissue nitrogen, aligning with previous findings of Aragw report. The correlation results demonstrated that *Rhizobium* inoculation significantly increased all investigated parameters (plant height, number of nodules, nodule dry weight, and symbiotic effectiveness) at $p<0.05$ compared to positive and negative controls.

Table 1. Correlation coefficients among investigated parameter in Ashebka faba bean variety on Dabat soil

Correlations							
	NN	**NFW**	**NDW**	**SL**	**SFW**	**SDW**	**TN**
NN	1	.999**	.999**	.595**	.529*	.529*	0.258
NFW	.999**	1	.999**	.598**	.535*	.529*	0.259
NDW	.999**	.999**	1	.595**	.531*	.530*	0.252
SL	.595**	.598**	.595**	1	.965**	.977**	.818**
SFW	.529*	.535*	.531*	.965**	1	.980**	.795**
SDW	.529*	.529*	.530*	.977**	.980**	1	.818**
TN	0.258	0.259	0.252	.818**	.795**	.818**	1

* Correlation is significant at the 0.05 level.

Additionally, in Dabat soil, shoot length showed a strong positive correlation with shoot fresh weight, shoot dry weight, and total nitrogen. Indicated in his work that nodulation status correlated with plant agronomic parameter[19]. Shoot fresh weight was strongly positively correlated with shoot dry weight and total nitrogen. Similarly, in Shentia soil, shoot length had a strong positive correlation with shoot fresh weight and shoot dry weight, and a positive correlation with total nitrogen. Shoot fresh weight in Shentia soil showed a strong positive correlation with plant total nitrogen, while shoot dry weight exhibited a positive correlation with total nitrogen as shown in Tables 1 and 2.

Table 2. Correlation coefficients among investigated parameter in Ashebka faba bean variety on Shentia soil

Correlations							
	NN	**NFW**	**NDW**	**SL**	**SFW**	**SDW**	**TN**
NN	1	.999**	.998**	.549*	.503*	.577*	0.012
NFW	.999**	1	.999**	.548*	.493*	.577*	0.019
NDW	.998**	.999**	1	.546*	.490*	.577*	0.024
SL	.549*	.548*	.546*	1	.946**	.992**	.696**
SFW	.503*	.493*	.490*	.946**	1	.941**	.609**
SDW	.577*	.577*	.577*	.992**	.941**	1	.705**
TN	0.012	0.019	0.024	.696**	.609**	.705**	1

** Correlation is significant at the 0.01 level.
* Correlation is significant at the 0.05 level.

4 Conclusion

The result of these studies showed that two *Rhizobium leguminosarum bv.vicea* isolates and other combinations of them had considerable effect on agronomic properties such as plant height, nodulation number, nodule colour, nodule dry weight, nodule fresh weight, shoot length, shoot fresh weight, shoot dry weight, and symbiotic effectiveness. Isolates co-inoculant followed by GR1E1 followed by WK1E1 showed significantly ($p<0.05$) higher shoot dry weight, isolates that induced the highest shoot dry weight (87mg/p and 8mg/p) followed by isolate GR1E1 with shoot dry weight of 81mg/p and 82mg/p for Dabat and Shentia sites, respectively. The nodule number record range from 138mg/p, and 173mg/p for isolate WK1E1 and co-inoculate, respectively for Dabat site .139mg/pisolate WK1E1 and 165 mg/pfor isolate co-inoculate Shentia site. Positive correlations were observed concerning shoot dry weight and dry weight of nodules ($r = 0.999$, $p<0.05$). Moreover, symbiosis efficacy was evaluated using slightly acidic Shentia soil and slightly neutral Dabat soil. Moreover, this study makes known the abundance and existence of effective faba bean rhizobial isolates in these specific areas of Dabat and Shentia, Gondar, Ethiopia. This research indicates the potential of these isolates to be effective on two different soils, which could give us an indication for potential nitrogen fixing. Thus, based on their symbiotic efficiency at the greenhouse level, these faba bean nodulating rhizobia isolates were recommended to be used as candidates for the future development of faba bean rhizobial inoculants after being tested on field conditions.

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