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Research Article

Isolation, Characterization and Evaluation of Symbiotic Effectiveness of Rhizobia Nodulating Faba Bean (*Vicia faba.* L.)

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Abstract

Natural process of fixing molecular nitrogen to ammonium is called biological nitrogen fixation. By supplying nitrogen to legume plants, it helps to support sustainable agriculture globally. The most significant leguminous crop, faba beans, can coexist symbiotically with soil bacteria called rhizobia. The purpose of this study was to isolate, describe, and assess Rhizobium nodulating faba bean's symbiotic efficacy. YEMA-CR was used to isolate a total of 24 Rhizobium isolates. Every isolate was described using YEMA based on how they responded to phenotypic traits. Every isolate tested positive for BTB, catalase, and indole and was found to be motile, gram-negative, rod-shaped, and incapable of absorbing Congo red. The pattern of intrinsic antibiotic resistance revealed that whereas the majority of isolates were sensitive to tetracycline, the majority were tolerant to ampicillin and pencillin. Every isolate exhibited nodulation in sand culture. All examined metrics showed significant ($p<0.05$) improvements in plants, according to analyses of variance. Sixty-six percent of isolates were deemed highly successful based on symbiotic effectiveness. According to the results, the study region included Rhizobia bacteria that might be utilized as bio-fertilizer. Therefore, in order to better classify Rhizobium isolates and develop them for inoculants, more research on their field conditions and molecular characterization is required.

Keywords

Biological Nitrogen Fixation, Faba Bean, *Rhizobium*, Symbiotic Effectiveness

1. Introduction

One of the most restricting factors for plant growth and development worldwide is nitrogen [3]. Therefore, mineral N or biological N fixation (BNF) systems must be used to periodically replenish agricultural soils' nitrogen (N) stocks in order to maintain an adequate level for crop production [6]. It is impossible to overstate the importance of BNF as the primary mechanism for recycling nitrogen from unavailable inorganic forms to available forms in the bio-sphere. For small-holder farmers in underdeveloped nations, particularly in Ethiopia, where chemical N input is neither readily available nor reasonably priced; it represents a comparatively inexpensive supply of nitrogen [2].

In order to support sustainable agriculture globally, the nitrogenase enzyme naturally fixes molecular nitrogen to am-

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monia through a process known as biological N fixation (BNF). In addition to lowering energy costs, the procedure is becoming more significant in an effort to create sustainable agricultural production by conserving nitrogen fertilizers, lowering crop production costs, supplying nitrogen to subsequent crops, and increasing soil fertility [13].

Because it is high in protein, minerals, and vitamins, the faba bean (*Vicia faba* L.), an annual grain legume that is widely grown around the world, is used as food for both human and animal nutrition in many nations [16]. Similar to other legume crops, faba beans can develop a symbiotic relationship with a group of bacteria called *Rhizobium leguminosarum* bv. Vicia that nodulate roots [9]. The most significant legume crop in Ethiopia is the faba bean, which, like other legumes, can develop a symbiotic relationship with rhizobia, or soil bacteria [2].

It is simple and affordable to modify the Rhizobium-legume connection through inoculation under N-limiting conditions in order to increase crop productivity. According to [16] they are the answer to declining soil fertility, affordable, environmentally benign, and simple to use, and typically have no negative side effects. Notwithstanding these beneficial bacteria, the current investigation was started with the goals of isolating, characterizing, and assessing the symbiotic efficacy of Rhizobia nodulating faba beans (*Vicia faba* L.).

2. Materials and Methods

2.1. Description of Study Area

The study was carried out in Bench Sheko Zone's Sheybench District. It is about 565 kilometers away from Addis Ababa. The area is located at an elevation of 1420 meters above sea level (m. a. s. l.) between 6081'96" N latitude and 35082'04" E longitude. According to the SNNPR annual data abstract for 2012/2013, the woreda experiences temperatures between 20 and 32 ℃ and rainfall between 1200 and 2000 mm annually.

2.2. Experimental Design& Sampling Site

Three replications of each treatment were used in the fully randomized experimental setup. The study's chosen sample locations were located in the main faba bean-growing areas of the Sheybench district in the South West Regional State of Ethiopia's Bench Sheko zone.

2.3. Collection, Identification of Soil Sample and Induction of Nodulation

From the research region, three sample faba bean growing locations were chosen randomly. Three soil samples weighing three kilograms were taken from the fields of thirty farmers. Soil was collected at a depth of 5 to 15 cm at each sampling location. Sterile bags were used to collect the soil samples, which were then delivered to the Mizan Microbiology and Plant Pathology Regional Laboratory. Five Dosha bean seeds that had been surface sterilized were acquired from the Bonga Agricultural Research Center. Additionally, following germination, the number of plants thinned to three in each container. Healthy plants were removed after forty-five days, and root nodules were gathered [15].

2.4. Isolation of Rhizobia

The faba bean (*Vicia faba*), the diseased plant host, was carefully uprooted within each pot in the greenhouse, and the fresh, clean root nodules were gathered. After that, nodules were gathered, cleaned with tape water to get rid of any remaining soil particles, and then surface sterilized with 70% (v/v) alcohol for five seconds before being rinsed with sterile water once more. Following a three-minute surface sterilization with 3% sodium hypochlorite, the cleaned nodules were broken up with forceps in a drop of sterile water on a petridish and rinsed five times with sterile distilled water. YEMA-containing yeast extract manitol agar medium is streaked with the picking suspension using an inoculating loop. Following that, plates were incubated for three to five days at 28 ℃ in accordance with [18].

2.5. Purification and Preservation of Isolates

One well-isolated colony was selected for sub-culturing on different plates using sterile yeast extract manitol agar media in order to purify the cultures. Sub-culturing was done repeatedly until homogeneity and purity were preserved [18].

2.6. Presumptive Test

Pure isolates that displayed white and mucoid properties on YEMA media were subsequently examined on YEMA-CR, which served as an indicator to determine whether the isolates included any contaminants [15] The following presumptive tests were performed: Gram staining and the Bromothymol blue test [18].

2.7. Physiological and Biochemical Characteristics of Isolates

To confirm the characteristics of Rhizobia on Yeast Extract Manitol Agar media, the following selective physiological and biochemical tests were used to characterize the various isolates: salt tolerance, pH, temperature, intrinsic antibiotic, carbohydrate utilization, amino acid utilization, catalase, and indole acetic acid production [15].

2.8. Evaluation of Symbiotic Effectiveness of Isolates on River Sand Pot Experiment

As outlined by [14] authentication was carried out in pot experiments using sterilized river sand in the Mizan Plant Pathology and Microbiology Regional Laboratory in a greenhouse to ascertain the isolates' performance (symbiotic effectiveness) in nitrogen fixation. Twelve of the top isolates were chosen from a total of 24 isolates based on their morphological, physiological, and biochemical traits. Five pre-germinated seedlings were aseptically moved into each pot using sterile forceps. The seedlings were divided into three pots after a week. Using sterile micropipettes from pure broth culture, 1 ml of each isolate was fed to each seedling during the logarithmic (active) growth phase [15] Two treatments were employed as positive and negative controls.

2.9. Relative Effectiveness of Isolates

According to [10, 11] the efficiency of isolates in accumulating plant shoot dry matter was computed as follows:

$$\%SE = \frac{Plant\ Inoculated\ Plant\ D.W\ x\ 100}{N - Fertilized\ D.W}$$

Where SE stands for symbiotic effectiveness and D. W. for Dry weight.

The efficacy rate of nitrogen fixing is calculated as follows: According to the SE (%) values, >80% indicates high effectiveness, 50-80% indicates effectiveness, 35-50% indicates low effectiveness, and less than 35% indicates ineffectiveness.

2.10. Data Analysis

The SAS software version 9.2's General Linear Models Procedure was used to do a one-way analysis of variance (ANOVA) on the collected data. The least significant differences (LSD) test probability ($p<0.05$) level was used to determine the significance of the differences between the means of all treatments.

3. Results and Discussion

Three soil sampling locations yielded rhizobia isolates. All of the soil samples that were gathered and used to induce nodulation were successful in doing so. 24 nodule samples were successfully isolated utilizing YEM-CR throughout the course of three to five days of incubation, according to the isolation results. From the root nodules of the faba bean species (*Vicia faba*), 24 isolates have been found. Morphological tests were used to characterize each isolate. The isolates were rod-shaped, small to big mucoid in appearance, with a regular border. All of the isolates grew well on YEMA media, and their colonies ranged in diameter from 2.1 to 4.2 mm. large colonies on YEMA media within three to five days are therefore suggested to be a feature of rapidly developing Rhizobia [8, 12].

All of the isolates were discovered to be motile, rod-shaped, and gram negative upon microscopic examination, as demonstrated by the pinkish color of Gram's staining technique [5] observed similar findings. According to earlier research, [13, 6] none of the isolates absorbed Congo-red in dark conditions When streaked on yeast extract manitol Agar media containing bromothymol blue, isolates grew in three to five days, indicating that the rhizobia were developing quickly. Isolates that were inoculated showed growth and caused YEM media to turn yellow, indicating that the bacteria were generating acid [8] the use of the medium's sugar component for their growth is what causes this yellow color creation [11]. Every isolate exhibited tolerance to several physiological conditions, including temperature, salt, and pH. This outcome is consistent with [2].

Rhizobium isolates displayed varying growth states at varying NaCl concentrations. However, when the concentration of salt increased, the fraction of tolerant isolates declined. This result was consistent with [5 - 7] the isolates of Rhizobia were cultivated on YEMA medium at varying temperatures to test their capacity for temperature tolerance. These findings were consistent with earlier research showing that certain Rhizobium strains could grow at higher temperatures while others could grow at lower temperatures [16, 13] Although some strains may be able to withstand high temperatures, this does not necessarily mean that they are adapted at fixing nitrogen. We may draw the conclusion that the isolates that had withstood the excessive temperatures might aid in the development of inoculants that can be used in fields that experience extreme temperatures.

The isolates' only carbon sources for growth were 100% glucose, sucrose, fructose, galactose, maltose, and sorbitol. The rhizobial strains showed decreased growth variety in relation to the lactose. Lactose, which makes up 83.33% of isolates, was catabolized by all strains. Compared to strains with a degree of specificity in their requirements, those that can use a variety of carbon sources have an ecological advantage when colonizing the soil or rhizosphere [19] Similar results on *R. leguminosarum* bv. viciae's metabolism of carbohydrates have been documented by [17, 5], and [19]. After being exposed to hydrogen peroxide, all of the isolates produced oxygen bubbles surrounding the Rhizobial colony, which validated the Catalase test and supported the findings of [14, 17].

The culture covered with Whatman filter paper had a pink tint upon the addition of Salkawski reagent, indicating a successful indole production test [15]. As a result, the indole production test revealed that all of the chosen Rhizobial isolates were positive; this finding is consistent with [7, 10].

In a greenhouse pot experiment, the nitrogen fixation efficacy of twelve isolates derived from faba bean root nodules was evaluated on sterile and acid-treated sand using the

Dosha variety (local name) of faba beans. Every isolate developed nodules on the faba bean root under test, confirming their identity as Rhizobium. Rhizobium inoculation significantly *(P<0.05)* increased at all examined parameters, according to the findings of analyses of variance (Table 1). This result was consistent with the symbiotic traits of *R. leguminosarum* reported by [6]. The symbiotic efficacy of *Rhizobium leguminosarum* var. Viciea nodulating faba bean isolated from central Ethiopia was examined by [4].

All of the isolates in this investigation outperform the negative (un-inoculated) control in every parameter that was examined. Additionally, the experiment's negative control had yellow plant leaves, indicating a nitrogen deficit, whereas the other plants received isolates and KNO_3.

The Rhizobia isolate in this investigation displayed variation in nodule count. Since highly successful isolates had few nodules; nodule quantity is a less accurate measure of symbiotic efficiency. This suggests that a small number of efficient nodules on plant roots would be sufficient to fix nitrogen for the host plant's maximal advantage [14]. At the ($P < 0.05$) level of significance, there was a significant difference between the isolates and the controls in this investigation, as evidenced by the isolates' differences in nodule dry weight with Dosha faba bean (Table 1). WSH-13 and GOL-4 showed the highest and lowest mean nodule dry weights, respectively, of 0.109 and 0.05 (Table 1). In this investigation, the average nodule dry weight was 0.078g/p^{-1}. Comparably similar to the findings of other studies by [6, 7, 19].

Isolates HEB-28 recorded the highest mean shoot length of 47.46 cm/p, which was 8.6% longer than the positive control (N treated plants) and 42.75% longer than the negative control (Table 1). There was a notable difference in the mean shoot dry weight of the inoculated plants between the treatments. HEB-26 isolates had the highest mean shoot dry weight (2.91 g p^{-1}), while isolates HEB-21 had the lowest mean shoot dry weight (1.48 g p^{-1}).

The average shoot dry weight of 2.25 g p^{-1} was much higher than the mean shoot dry weight of 0.5 g p^{-1} previously reported by [5-7, 19] reported that shoot dry weight is a good measure of relative isolation effectiveness, but [1] recorded a mean shoot dry weight of 1.21g p^{-1}, which was much lower than the results. Compared to other investigator reports, more highly effective isolates were obtained in this investigation.

[6] Reported that 56% of the isolates were very successful in the Degaga and Dosha kinds that were gathered from Ethiopia's acidic soils. 16% of the faba bean isolates obtained from the acidic soils of Wollega, Western Ethiopia, were found to be highly effective by [7] whereas 23% of the isolates obtained from the main Northern Gonder, Ethiopia, were found to be highly effective by [19] There is a great deal of variation in the symbiotic efficacy of faba bean Rhizobium in Ethiopia [1] and the United States [10] Overall, the findings of this study and others indicate that the presence of viable naturally occurring faba bean rhizobial isolates in Ethiopia's various agro-ecological zones is crucial for improving the process of di-nitrogen fixation in faba beans.

Table 1. *Nodulation and Symbiotic Effectiveness of Isolates on River Sand.*

Isolates	NN (p^{-1})	NFW (g p^{-1})	NDW (g p^{-1})	SL (cm p^{-1})	SDW (g p^{-1})	SE%	Rate
GOL-1	85.66^{d}	0.184^{d}	0.084^{d}	46.37^{ab}	2.402^{f}	87.84	HE
GOL-2	69.66^{f}	0.153^{e}	0.096^{abcd}	43.33^{cd}	2.62^{cd}	97.49	HE
GOL-3	92.66^{bc}	0.155^{e}	0.098^{abcd}	44.66^{bc}	1.94^{g}	68.91	E
GOL-4	98.00^{b}	0.129^{g}	0.05^{e}	43.307^{d}	2.57^{cde}	93.62	HE
GOL-7	75.66^{e}	0.176^{cd}	0.105^{ab}	47.28^{a}	2.04^{g}	79.3	E
GOL-10	71.66^{ef}	0.146^{ef}	0.092^{bcd}	47.43^{a}	2.49^{def}	87.06	HE
WSH-13	97.33^{b}	0.261^{a}	0.109^{a}	43.00^{cd}	2.37^{f}	88.57	HE
WSH-18	94.33^{bc}	0.127^{g}	0.091^{bcd}	43.667^{cd}	2.42^{ef}	92.91	HE
HEB-21	63.33^{g}	0.136^{fg}	0.087^{cd}	45.243^{abc}	1.48^{h}	54.59	E
HEB-25	107.66^{a}	0.206^{a}	0.101^{abc}	46.58^{ab}	2.01^{g}	73.29	E
HEB-26	63.00^{g}	0.18^{cd}	0.091^{bcd}	41.58^{d}	2.91^{a}	107.99	HE
HEB28	91.66^{c}	0.174^{d}	0.101^{abc}	47.46^{a}	2.79^{ab}	103	HE
N+	0.00^{h}	0.00^{h}	0.00^{f}	43.38^{cd}	2.72^{bc}	100	-
N-	0.00^{h}	0.00^{h}	0.00^{f}	27.16^{e}	0.74^{j}	29.54	-
Mean	72.2	0.145	0.078	43.53	2.25	83.04	-
CV (%)	4.59	4.15	13.1	3.22	4.39	-	-

Isolates	NN (p^{-1})	NFW (g p^{-1})	NDW (g p^{-1})	SL (cm p^{-1})	SDW (g p^{-1})	SE%	Rate
LSD (0.05)	5.55	0.01	0.0173	2.34	0.165	-	-

Where; NN= nodule number, NFW= nodule fresh weight, NDW= nodule dry weight, SDW= shoot dry weight, SL=shoot length, SE=symbiotic effectiveness, p^{-1}=per plant. N^- = without chemical and inoculation, N^+ =with optimum amount of N-fertilizer, CV= Coefficient of variation, LSD= least significant difference. 0 = not found. Means within a column followed by the same letters are not significant at $p< 0.05$.

4. Conclusions

The investigation's presumptive and authentication test results demonstrated that every isolate was a genuine Rhizobium species found in faba beans. Rhizobium isolates' ability to grow at varying temperatures, salinities, pH levels, and antibiotics is one of its noteworthy traits. This ability is crucial for screening and creating inoculants that possess ecological competitiveness. This investigation demonstrated that nitrogen-fixing bacteria were present on the leguminous plant's root nodules. Compared to traditional chemical fertilizers, it would have no environmental effect. The isolates successfully infected the crop by symbiotically interacting with their host plant. The establishment of a sustainable agricultural system depends heavily on nitrogen fixation, which is particularly crucial for agriculture in less developed nations like Ethiopia.

Abbreviations

BTB	Bromothymol Blue
NDW	Nodule Dry Weight
NFW	Nodule Fresh Weight
SDW	Shoot Dry Weight
YEMA-CR	Yeast Extract Manitol Agar – Congo Red
YEMA	Yeast Extract Manitol Agar

Conflicts of Interest

The authors declare no conflicts of interest.

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