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Research Article

Characterization of Rhizobia Isolated from Tigray Soil and Assessment of Their Effect on Germination and Seedling Vigor of Wheat and Field Pea

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Nowadays, the inoculation of plant growth-promoting rhizobia in leguminous and nonleguminous crops is given great emphasis as it improves germination and seedling vigor, resulting in increased yields. In this study, 32 rhizobia isolates were obtained from five different sampling sites in Tigray, Ethiopia. Based on morphological, biochemical, and confirmatory tests, including sugar fermentation, the isolates were identified as belonging to the rhizobia genera. *In vitro* assessment of plant growth-promoting properties revealed that all isolates produced indole-acetic-acid, ammonia, and solubilized phosphate, except TA8, which did not solubilize phosphate. Only 3 isolates (TA1, TA2, and TA8) produced hydrogen cyanide, so they can be used as biocontrol agents. Nineteen isolates showed a growth reduction activity against *Fusarium oxysporum*, with a percent inhibition range of 34.2%–65.8%. All isolates tolerated a pH range of 4.0–9.0. The isolates showed growth variations in various temperatures and salt concentrations. A few isolates were tolerant up to 45°C temperature and 6% (w/v) $CaCl_2$ and NaCl concentrations. Inoculation of the isolates to wheat seeds increased seed germination, seedling shoot/root length, and seedling vigor index compared to the positive and negative controls. Isolates KO3, KO4, ME3, and TA5 increased seed germination by 4%. KO1 (11.60 cm) and TA7 (11.70 cm) showed a significantly enhanced shoot length, and ME3 showed a maximum root length (13.90 cm). SH1, KO2, and the positive control showed a significant ($P \leq 0.05$) increase in pea seed germination (by 20%) compared to the negative control. The positive control had the longest field pea shoot (5.70 cm), and isolate TA9 had the longest field pea root (5.32 cm) compared to the negative control. Generally, the wheat and field pea seedlings responded differently to the inoculation of different isolates. This study shows that Tigray soils harbor a variety of rhizobia species, which can be used as plant growth-promoting and biocontrol agents.

1. Introduction

Rhizobia, a subdivision of the grand plant growth-promoting rhizobacteria (PGPR), are bacterial genera comprising *Rhizobium*, *Mesorhizobium*, *Bradyrhizobium*, and *Sinorhizobium* that are involved in plant growth and development as well as plant disease control [1, 2]. Rhizobia are primarily known for symbiotic interaction with leguminous plants, and as a result, they biologically fix the atmospheric nitrogen into readily available form to be used by the plants [3, 4]. In addition to biological nitrogen fixation, rhizobia are also widely investigated for other plant growth-promoting properties including the production of phytohormones, exopolysaccharides, hydrogen cyanide,

siderophores, antibiotics, enzymes, volatile compounds, and solubilization of minerals [2, 5–7], which are among the extensively studied plant growth-enhancing mechanisms used by rhizobia. Rhizobia are demonstrated to promote plant growth by direct mechanisms (that affect the growth of the plant) or indirect mechanisms (related to the plant defense systems) [1]. For instance, the inoculation of *Rhizobium leguminosarum bv. viciae* strain 33504-Alex1 has enhanced the faba bean resistance against yellow mosaic virus [7]. Various other findings also reported the antagonistic activities of rhizobia species against plant disease-causing pathogens including *Phytophthora capsici* [8], *Macrophomina* phaseolina and Fusarium solani [9], Fusarium oxysporum [10], and *Rhizoctonia solani* and *Sclerotium rolfsi* [11].

Studies revealed that rhizobia inoculation (alone or in combination with other bioinoculants) to legumes such as faba bean (*Vicia faba* L.), field pea (*Pisum sativum* L.), cowpea (*Vigna unguiculata* L.), lentil (*lens culinaris* L.), chickpea (*Cicer arietinum*), soya bean (*glycine max*), and groundnut (*Archis hypogaea* L.) positively influenced the plant growth parameters and the productivity [5, 12–15]. Increased root/shoot length, root/shoot weight, and seedling vigor index were reported in lentils [5]. *Rhizobium* inoculation and cross-inoculation brought a significant change in the shoot/root biomass, pod numbers, pod weight, straw yield, and grain yield of faba bean [12, 13, 16]. Pigeon peas inoculated with phosphate-solubilizing *Rhizobium* showed improved phosphorus uptake [17]. Furthermore, Ibrahim and El-Sawah [14] stated that the co-inoculation of *Rhizobium* with *Azotobacter* significantly enhanced pea (*Pisum sativum* L.) growth and yield.

Necessarily, rhizobia species are also extensively studied for plant growth promotion of nonleguminous crops. *Rhizobium pisi* was found to increase the growth and yield of wheat after being inoculated together with *Azospirillum brasilense* [18]. Maslennikova et al. [19] found that *Rhizobium leguminosarum* increased seed germination, seedling length, and biomass as well as the leaf chlorophyll content of wheat (*Triticum aestivum* L.). Root and shoot physiology including the chlorophyll content of wheat seedlings are improved by phosphate-solubilizing rhizobacteria [20]. The highest rice grains (4667 kg ha^{-1}) were obtained due to the inoculation of *Rhizobium* species of mungbean [21]. The potential of rhizobia to promote the growth of maize (*Zea mays* L.) was investigated and found to enhance the growth of the crop [22].

In Tigray, investigations regarding unraveling the plant growth prompting rhizobia and, in general, plant growth prompting rhizobacteria need to be explored at large [23, 24]. Farmers in Tigray tend to depend on the external inputs of chemical fertilizers, which are not sustainable and environmentally unfriendly [25]. Indeed, the application of rhizobial bioinoculants in agricultural systems is highly encouraged as they are eco-friendly, increase soil biodiversity, are cheaper in cost, and sustainably substitute for chemical fertilizers [16, 26]. The aim of this study was (1) to isolate and characterize rhizobia, (2) to assess the plant growth-promoting properties of the isolates, and (3) to evaluate the germination and seedling parameters of *dekoko* (*Pisum sativum* var. *abyssinicum*) and bread wheat (*Triticum aestivum* L.) inoculated with the rhizobia isolates and compare with uninoculated control groups. Ethiopian field pea locally known as *Dekoko* (minute seeded) is a highly nutritive and good nitrogen-fixing legume cultivar grown in northern highland regions of Ethiopia in which the symbionts of this plant are not well studied [27, 28].

2. Materials and Methods

2.1. Description of the Study Area. The laboratory work was carried out at the Microbiology and Molecular Biology Laboratory of Mekelle University College of Veterinary Science, which is located in Mekelle city. Mekelle is situated approximately 770 km away from the capital city, Addis Ababa. It is located between 39°26′60″ and 39°35′5″ E and 13°26′40″ to 13°33′0″ N and has an average elevation of 2151 meters above sea level [29]. The predicted average maximum temperatures in Mekelle range from 15°C to 28°C [30]. The soil type in Mekelle is cambisols, and the surface soil's textural class (the top 0.2 m) is silt-loam. The farming practice in the study area is both crop and livestock production, and the most commonly grown crops are wheat and barley [31]. Occasionally, pulses are also grown in Mekelle.

2.2. Collection of Soil and Seeds. Soil samples were collected from various locations in the Tigray region of Ethiopia, including Korem, Tsibet, Mekelle, Tanqua Abergele, and Shiraro. These locations were selected to represent the lowland, midland, and highland areas of the region, resulting in a diverse range of isolates. The samples were collected randomly from farmers' fields by excavating to a depth of approximately 20 cm and placed in sterile polyethylene sampling bags. The samples were then transported in an ice box to Mekelle University for experimentation, where they were stored in a +4°C-refrigerator. Additionally, plant seeds were collected, including bread wheat (*Triticum aestivum*) from Tigray Agricultural Research Institute (TARI) in Mekelle and Ethiopian field pea (*Pisum sativum* var. *abyssinicum*, also locally known as *dekoko*) from Alamata Agricultural Research Center (AARC) in Alamata, Tigray.

2.3. Sowing of Seeds. The procedure involved weighing the soil samples and placing them in plastic pots with a capacity of five liters. The field pea seeds were sterilized with using 70% ethanol for three minutes and then washed with distilled water. Next, the surface sterilized seeds (3-4 seeds per pot) were sown into the plastic pots containing soil samples. The pots were watered regularly every three days, and the seeds were allowed to grow for 45 days.

2.4. Reference Strain and Plant Pathogen Sources. Reference strain, *Rhizobium leguminosarum bv. Viciae*, used in this experiment was obtained from TARI. Likewise, the plant pathogen, *Fusarium oxysporum f. sp. ciceri*, was brought from the Ethiopian Biodiversity Institute (EBI), in Addis Ababa.

2.5. Isolation and Preservation of Rhizobia. Isolation of rhizobia from nodules was carried out according to the procedure of Somasegaran and Hoben [32] with minor modifications. Forty-five-day-old field pea plants were carefully uprooted from the plastic pots and repeatedly washed with tap water to remove the adsorbed soil. Then, the plants were contained in sterile plastic bags and transported to the microbiology laboratory on the same day. In the laboratory, about 10 healthy nodules were detached from the roots of each plant and washed with water. Then, the nodules were surface sterilized by soaking in 95% alcohol for 1 min and 3% H_2O_2 for 4 min. The nodules were immediately rinsed five times with sterile water to remove the traces of chemicals. Sterilized nodules were transferred into a sterile glass petri-plate containing 0.5 ml water and crushed using a sterile glass rod to obtain a milky bacterial suspension. Meanwhile, the soil sample from each area was weighed and serially diluted [33] in normal saline solution (0.85% w/v NaCI) to obtain 1×10^{-1} to 1×10^{-6} [34]. Then, the bacterial suspension from nodules and 0.1 ml from 10^{-3} and 10^{-4} diluted soil were separately cultured using the streak plate method on Yeast Mannitol Agar W/1.5% agar (yeast extract 1 g/L; mannitol 10 g/L; dipotassium phosphate 0.5 g/L; magnesium sulfate 0.2 g/L; sodium chloride 0.1 g/L; calcium carbonate 1 g/L agar 15 g/L; final pH (at 25°C) 6.8 ± 0.2), and incubated for 48 hours at 28 ± 2°C. The pH level of the medium was adjusted to be 6.5 to 6.9, which is the optimum range for the growth of rhizobia. The bacteria were subcultured multiple times until pure colonies were visible. The purified cultures were then stored at a temperature of +4°C for the next subsequent experiment. In addition, the isolates were stored at −20°C using 20% liquid glycerol [8] to protect the cells in case of emergencies.

As for Korem, Tsibet, Mekelle, Tanqua Abergele, and Shiraro, the isolates were assigned the designations KO, TS, ME, TA, and SH.

2.6. Rhizobial Confirmatory Tests. The three presumptive tests described by Somasegaran and Hoben [32] and two other distinguishing tests (Table 1) were carried out to confirm the isolates as rhizobial species. The confirmatory tests were also supported by Gram's staining using standard procedures.

2.7. Biochemical Characterization of Rhizobia. The following biochemical tests were carried out as per standard procedures. The different biochemical characterization tests are summarized in Table 2.

2.8. In Vitro Characterization of Rhizobia as Plant Growth Promoters

2.8.1. Production of Indole-3-Acetic Acid. Indole-acetic-acid (IAA) production was tested by growing cultures in a liquid Luria-Bertani (LB) medium. Exponentially growing isolates were inoculated into test tubes containing 10 ml LB broth. The tubes were incubated at 30°C for 6 days in a 140 rpm shaker incubator (THZ-300C, China). Bacterial cells were sedimented by centrifugation at 13000 rpm for 1 min. Subsequently, one ml of the supernatant was mixed with 2 ml of Salkowski's reagent prepared according to Muleta et al. [43] but in different ratios (1 ml of 0.5 M $FeCL_3$ $6H_2O$, 50 ml of distilled water, and 30 ml concentrated H_2SO_4). To take place a reaction between the reagent and the IAA in the broth, the mixture was incubated at room temperature in dark for 30 min. The development of pink or red color is an indicator of the production of IAA by the isolates [44].

2.8.2. Production of Ammonia. The qualitative ammonia production of the isolates was analyzed using the method described by Cappuccino and Sherman [40] and Nagalingam et al. [44]. The freshly growing isolates were inoculated into the test tubes containing 5 ml peptone water. The tubes were incubated for 4 days at 30°C in an incubator. After 4 days of incubation, 0.5 ml of Nessler's reagent was added to the bacterial suspension in each tube. Immediate development of a yellow to brown color will show a positive test for ammonia production [44].

2.8.3. Solubilization of Phosphate. The potential of the rhizobia isolates to solubilize the insoluble tri-calcium phosphate was assessed according to procedures noted by Menelih et al. [45]; Muleta et al. [43]; and Mbah et al. [46] even though the medium used was Basal Sperber agar formulated in the laboratory. The formulation of the medium was as follows: yeast extract 0.5 g/L, glucose 10 g/L, magnesium sulfate heptahydrate 0.25 g/L, Calcium chloride 0.1 g/L, tri-calcium phosphate 2.5 g/L, agar 15 g/L. The medium was steam sterilized and poured into 90-ml diameter petri-dishes. After cooling down, fresh cultures of the isolates were spot inoculated on the surface of the basal medium and incubated at 28 ± 2°C for 5 days. The formation of a clear halo zone around the colony indicated that the isolates were phosphate solubilizers. The phosphate solubilization index (PSI) was estimated by the following formula [43]:

$$\text{PSI} = \frac{\text{ZSD}}{\text{CD}}, \tag{1}$$

where PSI is the phosphate solubilization index, ZSD is the zone of solubilization diameter (sum of colony diameter and halo zone diameter), and CD is the colony diameter.

Table 1: Confirmatory tests used to identify Rhizobia isolates.

Confirmatory tests	Summary of the procedures	References
Bromothymol blue test	Fresh rhizobia isolates were streaked on yeast mannitol agar medium containing 5 ml of 0.5% bromothymol blue. After incubation, the yellow color indicated fast-growing rhizobia that produce acid, while the blue color indicated slow-growing rhizobia that produce alkaline	[15]
Growth on yeast mannitol agar with Congo red	Yeast mannitol agar was prepared by adding 0.0025% (w/v) Congo red powder. A 24-hour-old rhizobial culture was then spot inoculated on the medium and incubated at 28 ± 2°C for 48 hours. After the incubation period, the coloration of the bacterial colony was observed and recorded	[35]
Growth on glucose peptone agar medium	A glucose peptone agar medium was prepared with the following ingredients: glucose at 10 g/L, peptone at 20 g/L, NaCI at 5 g/L, and agar at 15 g/L. Bacterial cultures were then introduced onto plates containing this agar medium and incubated at a temperature of 28 ± 2°C. After 72 hours of incubation, the growth response of the bacteria was examined	[36]
Growth on Hofer's alkaline medium	Rhizobia isolates were streaked on plates containing the yeast mannitol agar medium with a high pH of 11.0. The pH of the medium was adjusted using 1N NaOH. A yeast mannitol agar plate with a normal pH of 6.8 was used as a control. The plates were then incubated for 48 hours at a temperature of 28 ± 2°C, and the growth of the isolates was observed	[37]
Ketolactase enzyme production test	The freshly prepared bacterial culture was spot-inoculated onto a lactose agar medium and incubated for 4 days at a temperature of 28 ± 2°C. After the incubation period, Benedict's reagent (5 ml) was added carefully to the petri-plates containing the bacterial culture. The mixture was then incubated at room temperature for an hour. The appearance of a yellow zone around the colony indicates contamination	Kaur et al. [38]

Table 2: Biochemical tests used to identify the Rhizobia isolates.

Biochemical tests	Summary of the procedures	References
Catalase test	The bacterial isolates were grown on the YEMA medium and mixed with 3% hydrogen peroxide. Appearance of bubbles indicated catalase-positive isolates	[15, 39–42]
Oxidase test	The test involved streaking actively growing bacterial cells on a filter paper containing drops of 1% Kovac's oxidase reagent. Oxidase enzyme-producing isolates changed the blue dye from dark purple to black within 5–10 seconds	
Methylene blue test	Methylene blue 0.1% (v/v) was added to the YEMA medium. The isolates were then streaked on the medium and incubated at 30°C for 48 hours before analyzing the growth of the isolates	
Citrate utilization test	Simmon's citrate agar medium was prepared, and the test isolates were streaked onto it. Then, it was incubated at 30°C for 24 hours to observe the growth. A blue coloration indicated a positive test, while citrate-negative isolates did not change the color of the medium and remained green	
Urease test	The urea agar medium (Christensen) incorporated with phenol red as pH indicator was inoculated with test organism and incubated at 30°C for 24–48 hrs. Urease enzyme production was observed by the color change of the medium from yellow to pink	
Gelatinase activity	The fresh cultures were inoculated in test tubes containing 10 ml yeast mannitol broth comprising 2% gelatin powder. Then, test tubes were incubated for 48 hrs at 28 ± °C Then, the test tubes containing culture broth were subjected to low temperature in the refrigerator (4°C) for 30 mins. After incubation at a low temperature, the culture broth was removed and observed for the presence of the gelatinase enzyme. The isolates that produced gelatinase enzyme (positive isolates) remained liquefied, whereas the negative isolates remained solid due to the presence of not degraded gelatin	
Starch hydrolysis test	The isolates were cultured on yeast mannitol Agar medium supplemented with 0.2% (w/v) starch powder. This medium was inoculated with bacterial isolates and incubated at 30°C for 24 hours. After incubation, 5 ml of iodine solution (0.340 grams iodine and 0.660 grams potassium iodide in 100 mm distilled water) was poured on the plates. The formation of a blue-black color due to the starch iodine complex and the observation of a clear zone around the colonies indicated that the isolates were starch degrading due to the production of amylase enzyme by the cells	
Triple sugar iron agar test	The fresh isolates were transferred and streaked onto triple sugar iron agar (TSI) slants. After 48 hours of incubation, the colors of the butt and slant were analyzed for each isolate	
Sugar fermentation test	The sugar fermentation test was performed in peptone water supplemented with phenol red indicator solution (0.01%). Eight different sugars (lactose, glycerol, galactose, maltose, fructose, sorbitol, sucrose, and glucose) with a final concentration of 1% were used in the experiment	

2.8.4. Antagonistic Test against Fusarium oxysporum. The isolates were tested *in vitro* against the phytopathogen *Fusarium oxysporum f. sp. ciceri* by dual inoculation assay. The pathogen was grown on Potato Dextrose Agar for 48 hours at 25°C. A fungal plug (6 mm diameter) was inoculated on the center of YEMA plates, and 24-hour-old rhizobia isolates were spot inoculated 3 cm away from the pathogen [8]. The pathogen alone incubated in a separate petri-plate was used as a control. All the plates were in triplicates and incubated at 28°C for 5 days to check the zone of inhibition. The growth inhibition zone was measured using a digital caliper in centimeters. The percent radial growth inhibition of the pathogen was calculated using the following equation [9]:

$$\%\ \text{inhibition} = \frac{\text{PC} - \text{PT}}{\text{PC}} \times 100, \quad (2)$$

where PC is the radial growth of the pathogen in control plates and PT is the radial growth of the pathogen in treatment plates.

2.8.5. Resistance to Environmental Factors. The isolates were, in vitro, evaluated for different abiotic stress resistance values including temperature, pH, and salinity. The test isolates were streaked on YEMA plates and incubated for five days at different temperatures, viz., 10°C, 20°C, 28°C, 37°C, and 45°C. The control was 28°C. The growth characteristics of the isolates were observed, recorded, and evaluated [45]. To test the isolate's growth under acidic and basic conditions, a YEMA medium was prepared with different pH values ranging from 4 to 9 using 1N HCl and 1N NaOH [36, 47]. The isolates were inoculated on the medium and then incubated at optimum temperature (28°C) for five days.

Salt tolerance was assessed at different concentrations of both sodium chloride (NaCl) and calcium chloride (CaCl2). Test isolates were spot inoculated on the YEMA medium containing 0.5%, 1%, 2%, 3%, 4%, 5%, and 6% sodium chloride and calcium chloride and incubated at 28°C for five days [23, 48].

2.9. In Vitro Bioassay for Seed Germination and Seedling Vigor. A seed germination experiment was done in the laboratory to evaluate the effect of rhizobial isolates on the germination of seeds and the vigorousness of the seedlings. Yeast mannitol broth was prepared, sterilized, and poured into glass test tubes. The rhizobial isolates were grown by inoculating into the test tubes of YEM broth and incubation in a 160 rpm shaking incubator (THZ-300C, China) at 28°C for 2 days. Healthy and similar size seeds (100) of bread wheat, called Dashen (HAR-408), and Ethiopian field pea (*Dekoko*) seeds (25) were surface sterilized by soaking in 0.1% mercuric chloride for 5 minutes [49]. Then, the seeds were immediately washed with distilled water 4 times to remove the traces of chemicals and allowed to air dry inside the safety hood. Sterilized seeds were soaked in the bacterial culture medium (1×10^9 CFU/ml) for 30 minutes [19, 50] followed by gentle mixing to allow colonization of bacterial cells on the seeds. Then, the seeds were aseptically transferred to 90-mm-diameter petri-dishes containing sterilized germination paper and evenly distributed on the surface of the petri-dishes. The petri-dishes were remained at room temperature in the dark for 7 days to allow the germination of seeds. Plates were triplicated, and uninoculated seeds soaked in sterile growth medium and distilled water served as a negative control. Furthermore, seeds bacterized with *Rhizobium leguminosarum bv. viciae* was used as a positive control. Germination was recorded up to 4 days since the radicle of each seed became half of the seed length. After 7 days, 10 random seedlings per replication were carefully uprooted from the germination paper, and the seedling root length and seedling shoot length of each plant were measured [19]. Finally, the percent germination and seedling vigor index were calculated as follows [8, 51]:

$$\text{percent germination} = \left(\frac{\text{number of germinated seeds}}{\text{total number of seeds}}\right) \times 100, \tag{3}$$

$$\text{seedling vigor index} = \%\ \text{germination} \times (\text{shoot length} + \text{root length}). \tag{4}$$

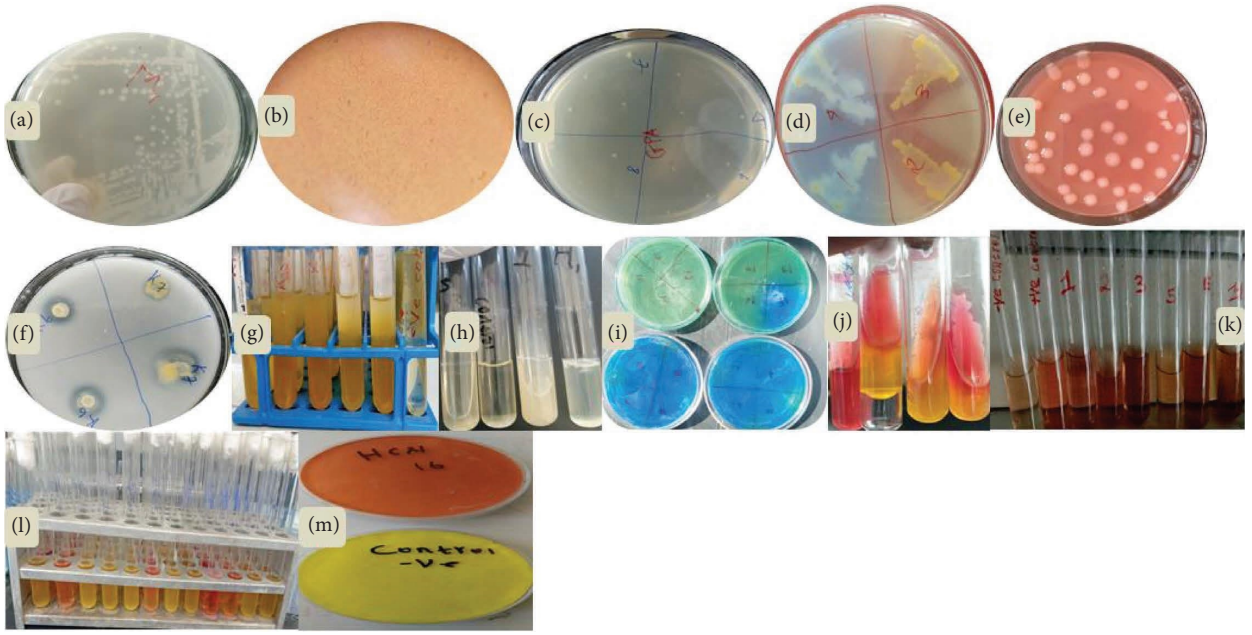
2.10. Data Analysis. The data were analyzed using R software version 4.2.3. A one-way analysis of variance (ANOVA) with the least significant difference (LSD) test was used to detect significant differences among isolates.

3. Result and Discussion

3.1. Isolation, Biochemical Characterization, and Confirmation of Rhizobia. The bacteria under study were isolated directly from soil and a legume crop (field pea). A total of 91 isolates were obtained from different geographical locations of the Tigray region on the YEMA medium. Out of these isolates, 32 isolates were selected based on different biochemical tests and preserved in 20-ml test tubes containing slants of the YEMA medium. Furthermore, the isolates were confirmed as rhizobia species using rhizobial confirmatory tests. The different morphological, cultural, and biochemical features exhibited by the rhizobia isolates are presented in Figure 1.

The biochemical tests revealed that all the isolates (100%) were positive for the catalase test, oxidase test, methylene blue test, and starch hydrolysis test (Table 3). However, 71.88% of the isolates utilized citrate, and 81.25% of the total isolates under study were effectively hydrolyzed urea. In addition, only 25% of the isolates produced gelatinase enzyme and showed degradation of gelatin. The majority of the tested isolates (93.75%) were positive for triple sugar iron agar. As it was observed from the agar slants, most of the tested isolates produced red slant and yellow butt showing only glucose was fermented in the triple sugar iron agar medium, which is in contradiction to the work of Singh et al. [42]. All the isolates were gram-negative and rod-shaped under microscopic observation (Figure 2). The isolates also exhibited transparent, creamy white colony color, different colony sizes, different colony elevations, and different degrees of mucus production (data not presented). Sticky mucus production is the best characteristic of rhizobia species as the rhizobia cells produce surface polysaccharides [52].

The data from the confirmatory tests showed that all the isolates had the features of rhizobia species (Table 4). Bromothymol blue test indicated that 15 isolates were acid producers (fast growers) as they changed the color of the YEMA medium, containing the bromothymol blue indicator, into yellow. The rest (17) isolates changed the color of the medium to blue, and they were alkali producers (slow growers). Gebremedhin [53] and Mohammed et al. [6] reported acid-producing rhizobia isolated from root nodules of field peas (*Pisum sativum* var. *abyssinicum*) and grass peas, respectively. However, in this experiment, diverse isolates of acid-producing and alkali-producing rhizobia were found because the isolation was performed from both the soil and root nodules. Jain et al. [54] reported both fast growers and slow growers of rhizobia. All the isolates did not grow on Hoffer's alkaline medium and lactose agar medium when incubated at 28 ± 2°C for 2 days and 4 days, respectively. Both Hoffer's alkaline medium and lactose agar medium were used to differentiate the rhizobial isolates from



Key: (a) Rhizobia colonies on YEMA medium (b) Rhizobia cells on microscope (c) growth on glucose peptone agar (d) bromothymol blue test (e) congo red test (f) Phosphate solubilization (g) ammonia production test (h) Gelatin hydrolysis test (i) citrate utilization test (j) triple sugar iron test (k) indole acetic acid test (l) carbohydrate fermentation test (m) hydrogen cyanide production test

Figure 1: Morphocultural and biochemical activities of the rhizobia isolates.

Table 3: Biochemical characteristics of Rhizobia isolated from different geographical locations.

Sampling site	Isolates	Biochemical tests								
		GS	CA	OX	MB	CU	UH	GE	SH	TSI
Shiraro	SH1	−ve, rod	+ve	+ve	+ve	+ve	+ve	−ve	+ve	+ve
	SH2	−ve, rod	+ve	+ve	+ve	−ve	+ve	−ve	+ve	+ve
	SH3	−ve, rod	+ve	+ve	+ve	+ve	+ve	−ve	+ve	+ve
	SH4	−ve, rod	+ve	+ve	+ve	+ve	+ve	−ve	+ve	+ve
	SH5	−ve, rod	+ve	+ve	+ve	+ve	+ve	−ve	+ve	+ve
Korem	KO1	−ve, rod	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
	KO2	−ve, rod	+ve	+ve	+ve	+ve	−ve	−ve	+ve	+ve
	KO3	−ve, rod	+ve	+ve	+ve	+ve	−ve	−ve	+ve	+ve
	KO4	−ve, rod	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
	KO5	−ve, rod	+ve	+ve	+ve	+ve	+ve	−ve	+ve	+ve
	KO6	−ve, rod	+ve	+ve	+ve	+ve	+ve	−ve	+ve	+ve
	KO7	−ve, rod	+ve	+ve	+ve	+ve	+ve	−ve	+ve	+ve
	KO8	−ve, rod	+ve	+ve	+ve	+ve	+ve	−ve	+ve	+ve
Tsibet	TS1	−ve, rod	+ve	+ve	+ve	+ve	+ve	−ve	+ve	+ve
	TS2	−ve, rod	+ve	+ve	+ve	+ve	+ve	−ve	+ve	−ve
	TS3	−ve, rod	+ve	+ve	+ve	+ve	+ve	−ve	+ve	+ve
	TS4	−ve, rod	+ve	+ve	+ve	+ve	+ve	−ve	+ve	+ve
Mekelle	ME1	−ve, rod	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
	ME2	−ve, rod	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
	ME3	−ve, rod	+ve	+ve	+ve	+ve	+ve	−ve	+ve	−ve
	ME4	−ve, rod	+ve	+ve	+ve	+ve	+ve	−ve	+ve	+ve
	ME5	−ve, rod	+ve	+ve	+ve	−ve	+ve	−ve	+ve	+ve
Tanqua Abergele	TA1	−ve, rod	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
	TA2	−ve, rod	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
	TA3	−ve, rod	+ve	+ve	+ve	−ve	+ve	−ve	+ve	+ve
	TA4	−ve, rod	+ve	+ve	+ve	−ve	+ve	−ve	+ve	+ve
	TA5	−ve, rod	+ve	+ve	+ve	−ve	+ve	+ve	+ve	+ve
	TA6	−ve, rod	+ve	+ve	+ve	−ve	−ve	−ve	+ve	+ve
	TA7	−ve, rod	+ve	+ve	+ve	−ve	−ve	−ve	+ve	+ve
	TA8	−ve, rod	+ve	+ve	+ve	−ve	−ve	−ve	+ve	+ve
	TA9	−ve, rod	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
	TA10	−ve, rod	+ve	+ve	+ve	−ve	−ve	−ve	+ve	+ve

GS: gram's staining; CA: catalase test; OX: oxidase test; MB: methylene blue test; CU: citrate utilization test; UH: urea hydrolysis test; GE: gelatinase test; SH: starch hydrolysis test; TSI: triple sugar iron test; +ve: positive reaction; −ve: negative reaction.

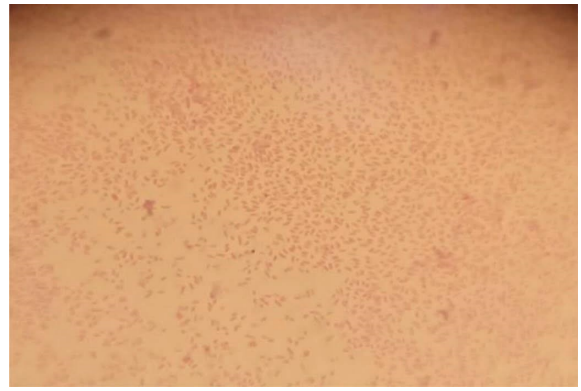


Figure 2: A 1000x total magnification of rhizobia cells using a compound microscope.

Table 4: Confirmatory tests and plant growth-promoting traits of Rhizobia isolates.

Sampling site	Isolates	BTB	HAM	KLT	YEMACR	GPA	IAA	AMM	HCN	Antagonistic
Shiraro	SH1	Alkali producer	−	−	−	+	+	+	−	+
	SH2	Alkali producer	−	−	−	+	+	+	−	+
	SH3	Alkali producer	−	−	−	+	+	+	−	+
	SH4	Alkali producer	−	−	−	+	+	+	−	+
	SH5	Alkali producer	−	−	−	+	+	+	−	+
Korem	KO1	Alkali producer	−	−	−	+	+	+	−	+
	KO2	Acid producer	−	−	−	+	+	+	−	+
	KO3	Acid producer	−	−	−	+	+	+	−	+
	KO4	Alkali producer	−	−	−	+	+	+	−	−
	KO5	Acid producer	−	−	−	+	+	+	−	+
	KO6	Alkali producer	−	−	−	+	+	+	−	+
	KO7	Alkali producer	−	−	−	+	+	+	−	−
	KO8	Acid producer	−	−	−	+	+	+	−	−
Tsibet	TS1	Alkali producer	−	−	−	+	+	+	−	+
	TS2	Alkali producer	−	−	−	+	+	+	−	−
	TS3	Alkali producer	−	−	−	+	+	+	−	+
	TS4	Alkali producer	−	−	−	+	+	+	−	−
Mekelle	ME1	Acid producer	−	−	−	−	+	+	−	−
	ME2	Acid producer	−	−	−	+	+	+	−	+
	ME3	Acid producer	−	−	−	+	+	+	−	−
	ME4	Acid producer	−	−	−	+	+	+	−	−
	ME5	Alkali producer	−	−	−	+	+	+	−	+
Tanqua Abergele	TA1	Alkali producer	−	−	−	+	+	+	+	+
	TA2	Alkali producer	−	−	−	+	+	+	+	+
	TA3	Acid producer	−	−	−	+	+	+	−	−
	TA4	Acid producer	−	−	−	+	+	+	−	−
	TA5	Acid producer	−	−	−	+	+	+	−	+
	TA6	Acid producer	−	−	−	+	+	+	−	−
	TA7	Acid producer	−	−	−	+	+	+	−	−
	TA8	Acid producer	−	−	−	+	+	+	+	+
	TA9	Alkali producer	−	−	−	+	+	+	−	−
	TA10	Acid producer	−	−	−	−	+	+	−	+

BTB: bromothymol blue; HAM: Hofer's alkaline medium; KTL: ketolactose medium; YEMACR: yeast extract mannitol agar + Congo red; GPA: glucose peptone agar; IAA: indole-acetic-acid; AMM: ammonia; HCN: hydrogen cyanide.

other contaminants mainly from *Agrobacterium* species. *Agrobacterium* species grow normally on an alkaline medium with pH 11.0, while rhizobia species do not. The ketolactose test is used to detect contamination, especially *Agrobacterium* contamination, during rhizobia isolation. Unlike *Agrobacterium*, when growing on the lactose medium, rhizobia does not have the 3-ketolactase enzyme, which is required for converting lactose into 3-ketolactose. This enzyme is also known as "hexapyranoside cytochrome c oxidoreductase." In addition, 100% of the isolates did not take up the Congo red dye from yeast extract mannitol agar medium. Congo red reaction in bacteria occurs because of

the dye adsorbed to the ions of the bacterial cell surface or the acid or alkaline produced on the surface of the cell during the growth of the bacteria. The Congo red test is widely used as an indicator in preliminary screening, identification, and biochemical characterization of rhizobia isolates in many papers [4, 25, 47, 55] in which if a certain bacteria (Rhizobia) did not absorb the dye or slightly absorb the dye, it confirms that it was the bacteria itself. The pigmentation of the dye in rhizobia is almost none.

Very poor growth was observed from 30 isolates after spot inoculation on the glucose peptone agar medium followed by incubation at $28 \pm 2^{\circ}$C for 72 hours. However, two isolates, ME2 and TA10, did not show growth on the medium. Many rhizobial strains exhibit poor growth or are unable to grow on this medium. This is due to the reason that rhizobia species cannot utilize the nitrogen source (peptone) of the medium. However, other microbes grow rapidly on glucose peptone agar medium. Similar studies have been conducted on rhizobia's growth reaction on glucose peptone agar [44, 56, 57].

A sugar fermentation test was carried out for the isolates using 8 different sugars, viz., lactose, glycerol, galactose, maltose, fructose, sorbitol, sucrose, and glucose. The isolates exhibited variations in consuming different carbohydrate sources (Figure 3). Among the 32 isolates, 14 isolates utilized lactose, 11 isolates utilized glycerol, and 19 isolates utilized sorbitol as a carbon source. Similarly, the majority of the isolates (31 and 29 out of 32) fermented galactose, maltose, fructose, sucrose, and glucose. On the other hand, the remaining isolates could not utilize the carbohydrates. Chen et al. [58] reported different carbohydrate fermenting rhizobia species, which agreed with our result. It is observed that the ability of the isolates to easily ferment the carbohydrates was slightly decreased from the monosaccharides (glucose, galactose, and fructose) to the disaccharides used in this study. Rhizobia species isolates from different parties of Ethiopia were found to utilize a broad range of carbohydrate sources [47].

3.2. In Vitro Characterization of Rhizobia as Plant Growth Promoters

3.2.1. Indole-Acetic-Acid Production, Ammonia Production, and Phosphate Solubilization. Rhizobacteria including rhizobia are microbial populations in soil that play a vital role in benefiting plant growth by directly supplying essential nutrients and indirectly as defense mechanisms [1]. In our experiment, almost all the isolates were found positive for the in vitro evaluation of plant growth-promoting properties except for hydrogen cyanide production (Table 4).

Apropos of indole-acetic-acid production, all 32 (100%) isolates were positive for the in vitro determination of the plant hormone production when the supernatant of the cell product was mixed and reacted with Salkowski reagent. The color of the mixture varied from pale pink to reddish, indicating that the rhizobial isolates had different intrinsic capabilities to produce indole-acetic-acid (Figure 1(k)). Jida and Assefa [47] reported indole-acetic-acid-producing rhizobia that were isolated from central and northern Ethiopia. Our result is greater than the reports from Jida and Assefa [47] who found 28.7% indole-acetic-acid producers out of 36 rhizobial isolates. Similarly, Muleta et al. [43] reported 40% indole-acetic-acid producers out of 62 mesorhizobial strains, which was still lower than the results we found.

Similar to plant hormone production, the isolates were able to produce ammonia and solubilize phosphate. Ammonia is the first stable product of biological nitrogen fixation by nitrogen-fixing bacteria including rhizobia. In this study, the observation of color development from the mixture of culture broth and Nessler's reagent showed that all the isolates were ammonia producers. Some of the isolates produce a yellow color and others produce a deep brown color (Figure 1(g)). The color variation inside the test tubes from yellow to deep brown indicated that the isolates had different potential to produce ammonia. The deep brown color is an indicator of the presence of much more ammonia concentration in the liquid medium. A similar result was reported by Chaudhary et al. [41] who tested *Rhizobium pusense* MB-17a for ammonia production in peptone water. Justin et al. [39] announced ammonia-producing isolates from root nodules of different plants with different amounts of ammonia production.

Regarding the phosphate solubilization, 96.9% (31) of the isolates solubilized the insoluble phosphate supplemented in the Basal Sperber Agar medium (Table 5). Figure 1(f) shows some of the isolates that solubilized phosphate and formed a clear halo zone around their colony. However, an isolate, TA8, from the Tanqua Abergele site did not show any halo zone around the colony, indicating that it was not a phosphate solubilizer. This is in line with the findings of Muleta et al. [43] who reported 70–100% phosphate-solubilizing rhizobia, specifically *Mesorhizobium* spp. isolated from 10 different sample collection sites in Ethiopia. However, it is much higher than the findings of Jida et al. [47, 59] and Justin et al. [39] who reported 16.7%, 44.4%, and 28.57% phosphate-solubilizing rhizobia, respectively. The numeric data from the qualitative determination of phosphate solubilization indicated that the isolates showed an average phosphate solubilization index (PSI) ranging from 1.08 (ME4) to 2.67 (KO3). Jida and Assefa [59] reported phosphate-solubilizing *Rhizobium leguminosarum bv. viciae* strains with solubilization index ranged from 1.14 to 1.3. Other similar works were also reported by Khairnar et al. [50]; Wdowiak-Wróbel et al. [35]; Jain et al. [56]; and Legesse and Assefa [23].

3.2.2. Hydrogen Cyanide Production and Antagonistic Activity. The activity of the isolates for hydrogen cyanide production (Table 4) and antagonism against the fungus *F. oxysporum* (Table 5) is very important in plant growth since they could be used as pathogen control strategies. From the tested isolates, only 3 isolates (TA1, TA2, and TA8) were able to produce hydrogen cyanide *in vitro*. These isolates exhibited a greater amount of HCN production so that they can be used against plant pathogens. These isolates originated from the same site, *Tanqua Abergele*. In the present study, very few isolates

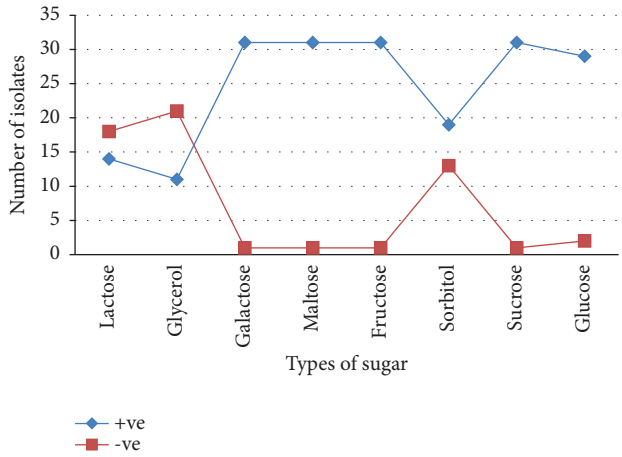


Figure 3: Carbohydrate fermentation. Key: +ve = positive reaction and −ve = negative reaction.

Table 5: Illustration of the phosphate solubilization features and *in vitro* antagonistic activities of the Rhizobial isolates.

Sampling site		Phosphate solubilization test				Antagonistic test		
	Isolates	CD (mm)	HZD (mm)	PSI	%SE	CZD (mm)	RFG (mm)	%IRG
Shiraro	SH1	12.33	4.67	1.38	37.9	7	18	52.6
	SH2	2.00	0.50	1.25	25	5	22	42.1
	SH3	8.33	2.83	1.34	34.0	3	20	47.4
	SH4	6.67	4.33	1.65	64.9	1	25	34.2
	SH5	6.17	3.00	1.49	48.6	2	25	34.2
Korem	KO1	4.00	4.00	2.00	100.0	0	0	0
	KO2	4.00	9.00	1.40	44.0	0	0	0
	KO3	3.00	5.00	2.67	60.0	1	14	63.2
	KO4	4.00	6.00	1.70	66.0	3	25	34.2
	KO5	7.67	6.67	1.87	87.0	3	24	36.8
	KO6	11.00	1.33	1.12	12.1	0	0	0
	KO7	10.00	1.67	1.17	16.7	0	0	0
	KO8	10.67	0.83	1.08	7.8	4	19	50
Tsibet	TS1	9.00	1.17	1.13	13.0	0	0	0
	TS2	10.00	2.17	1.22	21.7	1	20	47.4
	TS3	11.00	1.67	1.15	15.2	0	0	0
	TS4	10.00	5.67	1.57	56.7	1	20	47.4
Mekelle	ME1	5.00	5.00	2.00	100.0	1	15	60.5
	ME2	4.00	10.00	1.40	40.0	1	14	63.2
	ME3	9.67	3.67	1.38	38.0	0	0	0
	ME4	12.00	1.00	1.08	8.3	0	0	0
	ME5	4.67	5.33	2.14	114.1	9	19	50
Tanqua Abergele	TA1	3.00	5.00	1.40	60.0	1	21	44.7
	TA2	1.00	8.00	1.20	13.0	2	20	47.4
	TA3	5.00	7.00	1.70	71.0	0	0	0
	TA4	5.00	6.00	1.80	83.0	0	0	0
	TA5	2.00	3.00	1.60	67.0	3	20	47.4
	TA6	2.50	4.00	2.60	63.0	0	0	0
	TA7	1.00	5.00	1.20	20.0	0	0	0
	TA8	0.00	0.00	0.00	0.0	1	13	65.8
	TA9	2.00	3.00	1.60	67.0	0	0	0
	TA10	1.00	5.00	1.20	20.0	6	21	44.7

Key. CD: colony diameter; HZD: halozone diameter; PSI: phosphate solubilization index; %SE: present solubilization efficiency; CZD: clear zone diameter; RFG: radial fungal growth; %IRG: present inhibition in radial growth.

were positive for hydrogen cyanide production compared to the finding of Jain et al. [54] who reported 55 hydrogen cyanide-producing rhizobial isolates. Abdel-Lateif and Abd El-Ghany [9] also reported 50 rhizobia isolates that produced different amounts of hydrogen cyanide and suggested that the isolates can be used as biocontrol agents [44].

In an antagonistic test, more than half (19) of the isolates showed growth reduction activity against *F. oxysporum*. The isolates were able to show inhibition area around their colony after five days of incubation together with the *F. oxysporum* by the method of dual culture (Figure 4). However, none of the isolates showed complete inhibition against this pathogen. The diameter of the inhibition zone of the positive isolates ranged from 1 mm to 9 mm, and the percent inhibition was between 34.2% (SH4, SH5, and KO4) and 65.8% (TA8). The growth of the pathogen on control plates showed an average radial growth of 38 mm. Isolate TA8 was effective in both producing HCN and being highly prohibitive for the pathogen Jida and Assefa [47] observed that rhizobia isolates showed *in vitro* inhibition for *F. oxysporum*. Likewise, Muleta et al. [43] described that *Mesorhizobium* species are antagonistic to *Fusarium oxysporum f. sp. ciceris* with a growth inhibition of 9.2% to 57%. Besides, Abdel-Lateif and Abd El-Ghany [9] found rhizobia isolates inhibitory for other fungal pathogens namely *Fusarium solani* and *Macrophomina phaseolina*. The diversity of growth inhibition percentages, in our study, indicated that the different isolates had different capacities to inhibit the growth of the phytopathogen. The inhibited growth of the fungus in the medium by the isolates is because of the secretion of metabolic substances either antibiotics or enzymes that are hostile to the pathogen [44]. The difference in antagonistic behavior between the isolates against *F. oxysporum* emanates from the genetic makeup of the isolates, types of antibiotics, enzymes, and volatiles produced by the isolates as well as the cell wall structure of the test pathogen [9]. The isolates that did not exhibit antagonism, in the present experiment, may have different ways of defense mechanisms including bacteriocin production, interfering signal of the pathogen, and production of degrading enzymes (cellulase, chitinases, and proteases) to prohibit the plant pathogens [43].

3.3. Resistance to Environmental Factors. The sensitivity or tolerance of the isolates to different salt concentrations, temperature, and pH values is depicted in Table 6. All isolates in this study were pH tolerant, ranging from acidic pH 4 to basic pH 9. Consistent with our findings, Baye et al. [36] found that rhizobia isolates obtained from soil samples collected from eastern Ethiopia were resistant to pH ranging from 4.0 to 9.0, whereas all their isolates failed to resist pH 4, which contradicts our isolates. Jida and Assefa [47] also reported that rhizobia isolates are resistant to a pH range of 4.5 to 10.

Apropos of the temperature tolerance, our isolates showed growth variations. Hence, 11 (34.38%) isolates were tolerant to temperatures of 10°C, 20°C, 28°C, 37°C, and 45°C. On the other hand, 15 (46.89%) and 4 (12.5%) isolates were sensitive to 45°C and 10°C, respectively. Generally, the majority of the isolates grew well between 10°C and 37°C. Similarly, Legesse and Assefa [23] observed that all of their isolates from northwestern Tigray were temperature tolerant to the range of 15°C to 35°C, with 9 (26%) isolates adaptable to 45°C. Other studies also described that the majority of the rhizobia isolates collected from various parts of Ethiopia showed a mesophilic nature [60–63]. Interestingly, the majority of the thermophilic isolates (45°C tolerant isolates) in our study originated from the soil samples collected from the hot locations of *Shiraro* and *Tanqua Abergele*. Therefore, this nature of tolerance might be due to the high soil temperature of their habitat. These isolates could be important for application as an inoculant in temperature-stressed areas of Tigray since they are tolerant to high temperatures.

In addition, Table 6 shows that all isolates were tolerant to various NaCl concentrations of 0.5, 1, 2, 3, 4, 5, and 6% (w/v) except TS3 and ME5 that were sensitive to 5 and 6% NaCl concentrations, KO4 that was sensitive to 4 and 5% NaCl concentrations and KO7, TS2, and TS4 that were sensitive to 6% (w/v) NaCl concentration. Concerning $CaCI_2$, all isolates were well tolerated at concentrations of 0.5, 1, 2, and 3% (w/v). However, a great growth variation was observed for 4, 5, and 6% (w/v) $CaCI_2$ concentrations and the number of resistant isolates declined. Similar studies by Tsegaye et al. [60] and Amsalu et al. [61] indicated that *Rhizobium* isolates are tolerant of up to 6% (w/v) NaCl concentration. Contrary to the present study, Maâtallah et al. [48] reported that rhizobia isolates did not grow above 0.8% of $CaCI_2$ concentrations. Other works also isolated rhizobia species tolerant to slat concentrations of 7% NaCl [23] and 10% NaCl [63]. On the other hand, Baye et al. [36] found that all their rhizobia isolates (8 isolates) were sensitive to sodium chloride concentrations above 0.1%, which is highly contradictory to our study. Jida and Assefa [59] also found that rhizobia isolates did not grow at NaCl concentrations above 2%. This variation might be because of the soil type where the isolates were sampled from. However, the correlation between the isolates and their origin was not presented in our study. Isolates from saline soils are effectively grown at different salt concentrations whereas isolates from nonsaline soils are inhibited from growing in a medium containing various salt concentrations [48].

3.4. In Vitro Bioassay for Seed Germination and Seedling Vigor. In this experiment, the field pea (*P. sativum var. absynicum*) seeds were evaluated for germination, seedling shoot, and root growth parameters as a result of rhizobia inoculation. The data in Table 7 indicate that the isolates enhanced different degrees of seed germination and the root or shoot length of field pea seedlings after 7 days of *in vitro* germination. Seed germination was significantly ($P \leq 0.05$) enhanced by up to 20% as recorded from SH1 and KO2 as compared to the negative control but in line with the positive control. TS2 was the least isolate, which showed only a 1% seed germination increment. Regarding the shoot length, a significant increment was observed in all isolates relative to the uninoculated control. The highest shoot length was exhibited by the positive control (5.70 cm) followed by KO3 (5.70 cm) and TA9 (4.54 cm). In terms of the root length, TA9 had the highest value at 5.32 cm, followed by TA10 at 4.78 cm and TA8 at 4.30 cm. Inoculation of the two isolates,

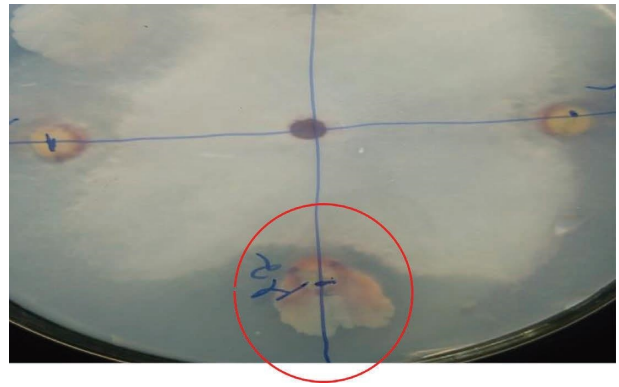


Figure 4: Inhibition potential of isolate SH2 against the growth of *F. oxysporum*.

Table 6: Growth features of rhizobia isolates at different pH, temperature, and salt concentrations.

Sampling site	Isolates	PH tolerant	Temperature (°C) tolerant	Temperature (°C) sensitive	NaCl tolerant (%)	NaCl sensitive (%)	$CaCl_2$ tolerant (%)	$CaCl_2$ sensitive (%)
Shiraro	SH1	4–9	10–45		0.5–6		0.5–5	6
	SH2	4–9	10–45		0.5–6		0.5–5	6
	SH3	4–9	10–45		0.5–6		0.5–5	6
	SH4	4–9	10–37	45	0.5–6		0.5–4	5-6
	SH5	4–9	10–37	45	0.5–6		0.5–4	5-6
Korem	KO1	4–9	10–37	45	0.5–6		0.5–4	5-6
	KO2	4–9	10–37	45	0.5–6		0.5–4	5-6
	KO3	4–9	10–37	45	0.5–6		0.5–4	5-6
	KO4	4–9	10–37	45	0.5–3	4–6	0.5–4	5-6
	KO5	4–9	10–45		0.5–6		0.5–6	
	KO6	4–9	10–45		0.5–6		0.5–5	6
	KO7	4–9	10–37	45	0.5–5	6	0.5–3	4–6
	KO8	4–9	10–45		0.5–6		0.5–4	5-6
Tsibet	TS1	4–9	10–37	45	0.5–6		0.5–3	4–6
	TS2	4–9	10–37	45	0.5–5	6	0.5–3	4–6
	TS3	4–9	10–37	45	0.5–4	5-6	0.5–3	4–6
	TS4	4–9	10–37	45	0.5–5	6	0.5–3	4–6
Mekelle	ME1	4–9	10–45		0.5–6		0.5–4	5-6
	ME2	4–9	20–37	10.45	0.5–6		0.5–4	5-6
	ME3	4–9	10–37	45	0.5–6		0.5–4	5-6
	ME4	4–9	10–37	45	0.5–6		0.5–3	4–6
	ME5	4–9	10–37	45	0.5–4	5-6	0.5–4	5-6
Tanqua Abergele	TA1	4–9	10–45		0.5–6		0.5–4	5-6
	TA2	4–9	10–45		0.5–6		0.5–4	5-6
	TA3	4–9	20–45	10	0.5–6		0.5–6	
	TA4	4–9	20–45	10	0.5–6		0.5–6	
	TA5	4–9	20–45	10	0.5–6		0.5–6	
	TA6	4–9	10–45		0.5–6		0.5–6	
	TA7	4–9	10–45		0.5–6		0.5–6	

KO1 and TS3, negatively affected the root length of the field pea. This can be due to lower indole-acetic-acid production by the isolates, which is important for root initiation [8]. The seeding vigor index of the pea ranged from 958 to 261. Generally, the pea seedlings respond differently to the inoculation of different isolates. To the best of our knowledge, we did not get papers related to *in vitro* bioassay for seed germination and evaluation parameters of 7-day-old Ethiopian field pea (*Pisum sativum* var. *abyssinicum*) seedlings.

The data regarding the wheat experiment revealed that the rhizobia isolates significantly ($P \leq 0.05$) increased wheat germination percentage compared to the control (NC and PC) groups. Hence, KO3, KO4, ME3, and TA5 were the elite isolates that increased seed germination by 4%, while SH4 was the least effective, which increased seed germination by only 0.33% (Table 8). Figure 5(a) shows wheat seed germination on a germination paper. Similar to our result, Maslennikova et al. [19] found a 4% increase in wheat seed germination by one of the *R. leguminosarum* strains. Fahsi

Table 7: Effect of rhizobia isolates on field pea seed germination and 7-day-old seedling growth parameters.

Isolates	GP	SSL (cm)	SRL (cm)	SVI
SH1	97.3 ± 2.30^{a}	3.48 ± 0.21^{efgh}	2.62 ± 0.37^{hij}	610 ± 44.0^{efg}
SH2	93.3 ± 2.30^{abcd}	3.22 ± 0.28^{fghi}	2.78 ± 0.31^{gh}	572 ± 34.8^{hi}
SH3	80.0 ± 4.00^{hi}	2.44 ± 0.26^{no}	2.66 ± 0.31^{hi}	408 ± 27.7^{qr}
SH4	90.7 ± 2.30^{bcde}	3.04 ± 0.08^{ijkl}	2.16 ± 0.08^{kl}	472 ± 15.1^{no}
SH5	82.7 ± 2.30^{gh}	2.72 ± 0.14^{mn}	2.24 ± 0.42^{jkl}	424 ± 32.6^{pq}
KO1	84.0 ± 4.00^{fgh}	2.88 ± 0.13^{jklm}	1.64 ± 0.24^{m}	395 ± 16.4^{qr}
KO2	97.3 ± 2.30^{a}	4.34 ± 0.11^{c}	3.72 ± 0.29^{d}	792 ± 42.7^{c}
KO3	94.7 ± 2.30^{abc}	5.70 ± 0.29^{b}	3.42 ± 0.42^{de}	805 ± 60.2^{c}
KO4	69.3 ± 2.30^{l}	1.68 ± 0.19^{p}	1.84 ± 0.20^{lm}	261 ± 8.2^{s}
KO5	84.0 ± 4.00^{fgh}	2.74 ± 0.16^{lmn}	2.16 ± 0.15^{kl}	406 ± 32.1^{qr}
KO6	96.0 ± 4.00^{ab}	3.96 ± 0.15^{d}	3.16 ± 0.27^{efg}	707 ± 30.1^{d}
KO7	78.7 ± 2.30^{hij}	3.90 ± 0.15^{d}	2.98 ± 0.20^{fgh}	535 ± 37.8^{jkl}
KO8	88.0 ± 0.00^{defg}	4.46 ± 0.37^{c}	2.88 ± 0.35^{gh}	640 ± 35.6^{e}
TS1	93.3 ± 2.30^{abcd}	3.20 ± 0.28^{ghi}	3.46 ± 0.38^{de}	613 ± 29.3^{efg}
TS2	73.3 ± 2.30^{jkl}	2.80 ± 0.29^{klm}	2.68 ± 0.31^{hi}	411 ± 52.9^{pq}
TS3	94.7 ± 2.30^{abc}	2.22 ± 0.22^{o}	1.66 ± 0.16^{m}	378 ± 11.8^{r}
TS4	86.7 ± 6.11^{efg}	3.52 ± 0.31^{ef}	2.28 ± 0.52^{ijk}	508 ± 66.2^{lm}
ME1	89.3 ± 2.30^{cdef}	3.12 ± 0.32^{ij}	3.62 ± 0.40^{d}	595 ± 33.9^{gh}
ME2	80.0 ± 0.00^{hi}	3.18 ± 0.23^{hij}	3.72 ± 0.33^{d}	565 ± 36.1^{hij}
ME3	92.0 ± 4.00^{abcde}	4.00 ± 0.21^{d}	2.30 ± 0.38^{ijk}	570 ± 31.0^{hi}
ME4	76.0 ± 4.00^{ijk}	2.78 ± 0.25^{lm}	2.74 ± 0.44^{gh}	440 ± 8.2^{op}
ME5	89.3 ± 2.30^{cdef}	3.74 ± 0.29^{de}	2.18 ± 0.23^{kl}	528 ± 70.2^{kl}
TA1	94.7 ± 2.30^{abc}	2.62 ± 0.23^{mn}	3.34 ± 0.35^{def}	546 ± 45.3^{ijk}
TA2	90.7 ± 2.30^{bcde}	3.50 ± 0.36^{efg}	3.52 ± 0.40^{de}	607 ± 32.7^{fg}
TA5	88.0 ± 4.00^{defg}	3.74 ± 0.26^{de}	3.48 ± 0.27^{de}	628 ± 53.5^{ef}
TA7	93.3 ± 4.61^{abcd}	3.10 ± 0.26^{ijk}	2.56 ± 0.43^{hijk}	493 ± 20.6^{mn}
TA8	92.0 ± 6.92^{abcde}	3.10 ± 0.24^{ijk}	4.30 ± 0.35^{c}	690 ± 9.7^{d}
TA9	93.3 ± 2.30^{abcd}	4.54 ± 0.32^{b}	5.32 ± 0.39^{a}	958 ± 42.6^{a}
TA10	93.3 ± 4.61^{abcd}	3.48 ± 0.32^{efgh}	4.78 ± 0.23^{b}	788 ± 73.1^{c}
NC	72.0 ± 4.00^{kl}	1.46 ± 0.15^{p}	2.20 ± 0.15^{jkl}	264 ± 28.0^{s}
PC	97.3 ± 2.30^{a}	5.70 ± 0.33^{a}	3.50 ± 0.47^{de}	918 ± 55.3^{b}
LSD (0.05)	5.48	0.31	0.42	31.71
SEm (±)	0.60	0.04	0.06	3.48
F-probability	<0.001	<0.001	<0.001	<0.001
CV (%)	3.81	7.47	11.43	3.43

Germination and shoot/root data are means of 3 replications and each replicate of the shoot/root length was an average of 10 field pea seedlings per petri-plates harvested after 7 days. Means in the same column that are followed by different letters are significantly different at $P \leq 0.05$ using Fisher's LSD test; LSD: least significant difference; CV: coefficient of variation (%); SEm: standard error of mean; NC: negative control; PC: positive control; GP: germination percentage; SSL: seedling shoot length; SRL: seedling root length; SVI: seedling vigor index.

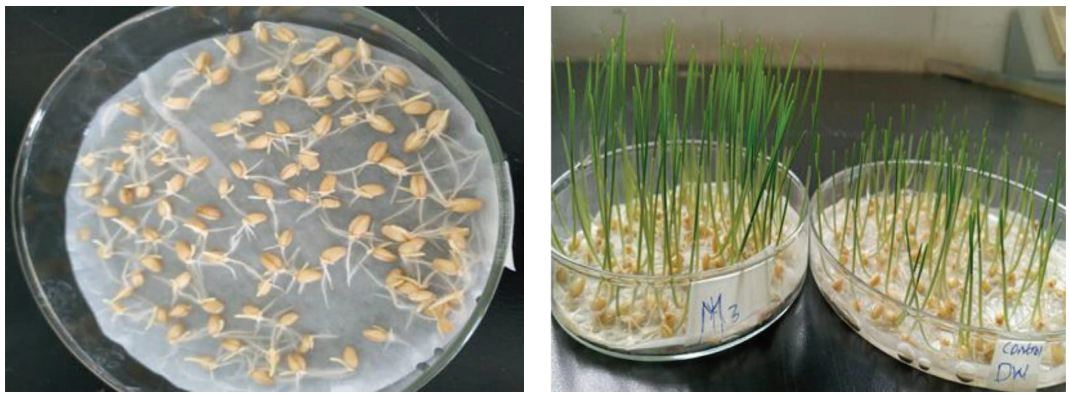
Table 8: Effect of rhizobia on wheat seed germination and 7-day-old seedling growth parameters.

Isolates	GP	SSL (cm)	SRL (cm)	SVI
SH1	97.0 ± 3.00^{def}	4.96 ± 0.55^{lmo}	6.52 ± 0.49^{hij}	1113 ± 3.5^{o}
SH2	99.0 ± 1.00^{abc}	6.43 ± 0.61^{ghij}	6.52 ± 0.57^{hij}	1283 ± 2.0^{i}
SH3	98.0 ± 0.00^{cd}	4.79 ± 0.31^{mno}	4.90 ± 0.44^{lmo}	951 ± 1.5^{r}
SH4	96.3 ± 1.15^{ef}	6.26 ± 1.28^{ghijk}	7.25 ± 0.62^{fgh}	1301 ± 0.9^{i}
SH5	95.8 ± 0.57^{f}	5.86 ± 0.41^{hijkl}	6.14 ± 0.52^{ijk}	1148 ± 4.5^{mn}
KO1	99.7 ± 0.57^{ab}	11.60 ± 0.51^{a}	8.57 ± 0.48^{de}	2012 ± 1.6^{b}
KO2	99.3 ± 0.57^{abc}	7.85 ± 0.53^{de}	9.28 ± 0.65^{cd}	1702 ± 7.2^{f}
KO3	100.0 ± 0.00^{a}	6.06 ± 0.54^{ghijk}	7.83 ± 0.55^{efg}	1389 ± 9.5^{h}
KO4	100.0 ± 0.00^{a}	9.86 ± 0.64^{b}	6.96 ± 0.71^{ghi}	1683 ± 11.4^{f}
KO5	99.3 ± 0.57^{abc}	7.56 ± 0.52^{def}	7.24 ± 0.64^{fgh}	1471 ± 11.0^{g}
KO6	99.0 ± 1.00^{abc}	4.33 ± 0.39^{o}	4.42 ± 0.52^{no}	866 ± 22.2^{t}
KO7	98.3 ± 1.52^{bcd}	5.81 ± 0.58^{ijkl}	5.73 ± 0.56^{jkl}	1136 ± 10.3^{no}
KO8	98.0 ± 1.00^{cd}	5.90 ± 0.49^{hijk}	5.14 ± 0.42^{lmo}	1082 ± 17.1^{p}
TS1	99.0 ± 1.00^{abc}	5.32 ± 0.56^{klmn}	5.46 ± 0.44^{klm}	1069 ± 23.7^{p}
TS2	98.0 ± 0.00^{cd}	6.29 ± 0.53^{ghij}	5.77 ± 0.41^{jkl}	1183 ± 12.1^{kl}
TS3	99.3 ± 0.57^{abc}	5.84 ± 0.49^{hijkl}	6.77 ± 0.55^{hi}	1253 ± 43.9^{j}

Table 8: Continued.

Isolates	GP	SSL (cm)	SRL (cm)	SVI
ME1	98.3 ± 0.57^{bcd}	4.82 ± 0.54^{mno}	5.25 ± 0.49^{klmn}	991 ± 15.6^{q}
ME2	99.7 ± 0.57^{ab}	6.97 ± 0.58^{efg}	10.13 ± 0.37^{c}	1704 ± 6.7^{f}
ME3	100.0 ± 0.00^{a}	8.39 ± 0.23^{cd}	13.90 ± 0.56^{a}	2230 ± 12.1^{a}
ME4	98.3 ± 0.57^{bcd}	5.82 ± 0.34^{ijkl}	6.42 ± 0.41^{hij}	1204 ± 28.9^{k}
ME5	97.3 ± 1.52^{de}	5.55 ± 0.67^{jklm}	6.14 ± 0.74^{ijk}	1138 ± 45.3^{no}
TA1	99.7 ± 0.57^{ab}	8.04 ± 0.35^{cd}	11.37 ± 0.61^{b}	1935 ± 6.8^{d}
TA2	99.7 ± 0.57^{ab}	8.81 ± 0.18^{c}	11.29 ± 0.65^{b}	2004 ± 7.8^{bc}
TA5	100.0 ± 0.00^{a}	7.48 ± 0.74^{def}	9.98 ± 0.52^{c}	1747 ± 12.5^{e}
TA7	99.0 ± 1.00^{abc}	11.70 ± 0.71^{a}	8.32 ± 0.33^{e}	1983 ± 3.4^{c}
TA8	99.7 ± 0.57^{ab}	5.32 ± 0.65^{klmn}	6.40 ± 0.68^{hij}	1170 ± 10.3^{lm}
TA9	99.0 ± 1.00^{abc}	6.69 ± 0.38^{fghi}	8.06 ± 0.67^{ef}	1461 ± 10.5^{g}
TA10	99.7 ± 0.57^{ab}	6.76 ± 0.53^{fgh}	8.11 ± 0.58^{ef}	1483 ± 8.2^{g}
NC	96.0 ± 0.00^{ef}	4.71 ± 0.59^{mno}	4.27 ± 0.63^{o}	862 ± 2.6^{t}
PC	99.3 ± 0.57^{abc}	4.61 ± 0.56^{no}	4.59 ± 0.62^{mno}	915 ± 3.0^{s}
LSD	1.65	0.94	0.92	26.25
SEm (±)	0.18	0.1	0.1	2.93
F-probability	<0.001	<0.001	<0.001	<0.001
CV (%)	1.02	8.6	7.75	1.16

Germination and shoot/root data are means of 3 replications and each replicate of the shoot/root length was an average of 10 wheat seedlings per petri-plates harvested after 7 days. Means in the same column that are followed by different letters are significantly different at $P \leq 0.05$ using Fisher's LSD test; LSD: least significant difference; CV: coefficient of variation (%); SEm: standard error of mean NC: negative control; PC: positive control; GP: germination percentage; SSL: seedling shoot length; SRL: seedling root length; SVI: seedling vigor index.



(a) (b)

Figure 5: Appearance of 4-day-old germinated wheat seeds (a) and 7-day-old wheat seedlings (b) inoculated with isolate ME3 alongside the uninoculated control.

et al. [64] also reported a significant increase in the rate of wheat seed germination by other plant growth-promoting rhizobacteria such as *Pseudomonas moraviensis*, *Pseudomonas frederiksbergensis*, *Bacillus halotolerans*, and *Enterobacter hormaechei*.

Regarding the wheat seedlings parameters, the isolates notably increased the shoot length as well as the root length of the plants (Figure 5(b)). The isolates KO1 (11.60 cm) and TA7 (11.70 cm) showed significantly ($P \leq 0.05$) higher values regarding the shoot length, and ME3 (13.90 cm) showed a maximum root length as compared to the uninoculated control and the positive control. Other isolates including the positive control also increased the shoot and root length at various levels but were not statistically significant (Table 8). The isolate KO6, with an SSL value of 4.33 cm and SRL value of 4.42 cm, was the least effective plant growth enhancer, which is similar to the positive and negative control groups. This indicated that wheat seedlings respond differently to the treatment of different rhizobial isolates [19]. The calculated seedling vigor index ranged from the highest value of 2230.00 given by ME3 to the lowest value of 865.68 given by KO6. This result was similar to the finding of Fahsi et al. [64].

4. Conclusion

In conclusion, the present study has shown the soil of Tigray harbors different rhizobia species used for plant growth-promoting activities by producing plant growth-promoting substances including the production of phytohormone, particularly indole-acetic-acid, ammonia, and phosphate-solubilization. Some of the isolates have also shown antagonism against the tested plant pathogen (*Fusarium*

oxysporum) and produced hydrogen cyanide gas, which is used as a biocontrol agent. The isolates showed tolerance to different pH, temperature, and salt concentrations. Majority of the isolates tolerated a pH of 4.0 to pH 9.0, temperature of 10°C to 37°C, and salinity concentrations of 0.5 to 3% (w/v). However, there are also beneficial isolates that were tolerated at higher temperature (45°C) and higher salt concentrations (6% w/v). The in vitro bioassay for wheat and field pea seeds showed significant ($P \leq 0.05$) increments in percent germination, seedling shoot length, and seedling root length due to the inoculation of the seeds with the isolates. In comparison with the negative control, but consistent with the positive control, isolates SH1 and KO2 enhanced field pea seed germination by 20%. The highest field pea shoot length (5.70 cm) was exhibited by the positive control, while isolate TA9 exhibited the highest field pea root length (5.32 cm). In the wheat experiment, the elite isolates were KO3, KO4, ME3, and TA5, which increased seed germination by 4% compared to the positive and negative controls. In addition, isolates TA7 (11.70 cm) and ME3 (13.90 cm) had significantly ($P \leq 0.05$) longer shoot and root lengths than the uninoculated (negative) and inoculated (positive) controls. Rhizobia isolates such as SH1, KO1, KO2, KO3, KO4, ME3, TA7, and TA9 can be used as inoculants for field pea and wheat crop production in the study area. Further studies are in progress to identify the isolates at the species level.

Data Availability

All data are included in the manuscript.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Authors' Contributions

All authors contributed to the study's conception and design. Material preparation, data collection, and analysis were performed by EM, AT, MY, MM, GG, BK, YT, TS, and RH. The first draft of the manuscript was written by EM, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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