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Effects of Phosphate Solubilizing Rhizobia on the Growth of Teff (*Eragrostis teff*) under Greenhouse Conditions in Ethiopia

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Abstract

Phosphorus is the second most important nutrient for plant growth, but its availability is very low in the soil. This study was designed to identify phosphate-solubilizing Rhizobia from rhizosphere soil in Gorogutu and Deder districts of Ethiopia. The study evaluates the effects of phosphate-solubilizing Rhizobia on Teff (*Eragrostis teff*) growth under greenhouse conditions. The greenhouse experiment was assembled in a completely randomized design with 5 treatments including the control with three replications. A total of 30 Rhizobia isolates were obtained from rhizosphere soils in two districts (Gorogutu, and Deder) and screened for phosphate solubilization using Pikovskaya's Agar. Based on their phosphate solubilization indices, 4 potential isolates (PS-6, PS-22, PS-27, and PS-30) were selected for further study in the greenhouse. Phosphate solubilizing isolates in pot experiment under greenhouse were applied at a rate of 0.5 g per pot and Teff disinfected seeds were suspended in 30 ml thick cell suspension of phosphate solubilizing Rhizobia (10^{12} cells/ml) in the presence of 0.5% peptone (antidiuretic hormone for 30 minutes), but not the control. The result showed that almost all inoculants, after 30- and 90-days inoculation, have significantly improved the growth parameters of Teff (*Eragrostis teff*) under greenhouse conditions. Moreover, all the isolates showed a positive impact on all the growth parameters. The results from this experiment are useful in Ethiopia for biofertilizer development and for teff growth under organic farming systems. Therefore, isolates PS-6 and PS-22 are highly recommended to be prospective commercial biofertilizers at field conditions in different teff agroecologies.

Keywords

Teff; Phosphate rock; Phosphate Solubilizing Rhizobia; Biofertilizer

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Introduction

Phosphorus is the second mineral nutrient, next to nitrogen, that can limit the growth of plants. It is an essential element for plant development and growth (Alori**,** Glick and Babalola, 2017). Plants acquire phosphate from soil in a solution form as phosphate anions (Schubert, Steffens and Ashraf, 2020). However, phosphate anions are extremely reactive and immobilized by forming precipitation with cations such as calcium, magnesium, iron and aluminum depending on the properties of the soil. For this reason, soluble phosphate availability in the soil is usually in a small proportion (Wan *et al.*, 2020). However, different bacterial species acting as biofertilizers can solubilize insoluble phosphate compounds, such as tri-calcium phosphate, di-calcium phosphate, hydroxyl apatite and rock phosphate within the soil (Li *et al*, 2020). Biofertilizers are gaining importance as they are eco-friendly, non-hazardous and non-toxic products (Gichimu, Muthee and Nthakanio, 2020). They can also reduce and solve environmental problems related to abiotic factors such as temperature, pH and salt (Kshetri, Pandey and Sharma, 2018).

Many types of microorganisms are found in the rhizosphere and play an important role in plant growth and development. Phosphate solubilizing bacteria (PSB) have been used as biofertilizers since the 1950s (Sharon *et al*., 2016). These microorganisms secrete different types of organic acids, like carboxylic acid that lower the pH in the rhizosphere and consequently dissociate the bound forms of phosphate e.g., $Ca_3(PO_4)_2$ in calcareous soil (Sridhar *et al*, 2005). The role of these microorganisms in solubilizing inorganic phosphates in soil and making them available to plants is well documented (Corpas, Alche and Barroso, 2013). Rhizobia bacteria involved in symbiotic biological nitrogen fixation in the soil require phosphorus as energy for growth and survival (Sarker, Talukder and Islam, 2014).

Phosphate solubilizing microbes, for instance, play fundamental roles in biogeochemical phosphorus cycling in natural and agricultural ecosystems. Phosphate-solubilizing microbes can transform the insoluble phosphorus to soluble forms of HPO_4^{2-} and $H_2PO_4^-$ by acidification, chelating, exchange reactions, and polymeric substances formation (Lara, Sanes and Oviedo, 2013). Microbial phosphate cycling is greater in soils using organic fertilizers than soils receiving chemical fertilizers (Spohn and Widdig, 2017). Therefore, the use of phosphate solubilizing microbes can mobilize insoluble phosphorus in soils. Moreover, it offsets the high cost of manufacturing phosphate fertilizers. For instance, application of the phosphate-solubilizing microbes such as *Agrobacterium*, *Bacillus*, *Enterobacter*, *Pseudomonas*, *Aspergillus*, *Trichoderma* and *Glomus* around the roots of plants, in soils, and in fertilizers, has been shown to release soluble phosphorus, promote plant growth, and protect plants from pathogen infection (Shamseldin and Werner, 2005).

In general, phosphorus is a limited resource, and its efficient use should be the main focus in sustainable agriculture, because it is very liable to be lost from agricultural soils. Although farmers in Ethiopia have Indigenous knowledge in teff crop rotation with cereals and different legumes that indirectly enrich plant growth promoting Rhizobia (Gizaw *et al*, 2018), the application of biofertilizers (microbial inoculants) is not common. There is currently a growing interest in Ethiopia and other parts of the world to sustainably increase soil fertility and health through the application of microbial inoculants in order to minimize the cost and long-term environmental side effects of chemical fertilizers. Therefore, the aim of this study was to evaluate the effects of phosphate solubilizing Rhizobia isolated from cultivated rhizosphere soils on the growth of *Eragrostis teff* under greenhouse conditions.

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Materials and Methods

Study Site

This study was conducted from December 2019 to May 2021 at Haramaya University's main campus, Rare Research Field. The experimental site is geographically located in eastern part of Ethiopia located at the distance of about 25 km in the north-eastern direction from the town of Harar, which is situated at an altitude of 2006 meters above sea level, latitude of 9°24'53″ N latitude, and 42°2″9' E longitude (Figure 1). The site has a bimodal rainfall distribution pattern and is representative of a sub-humid, mid-altitude agroclimatic zone (Tamiru and Gedamu, 2019).

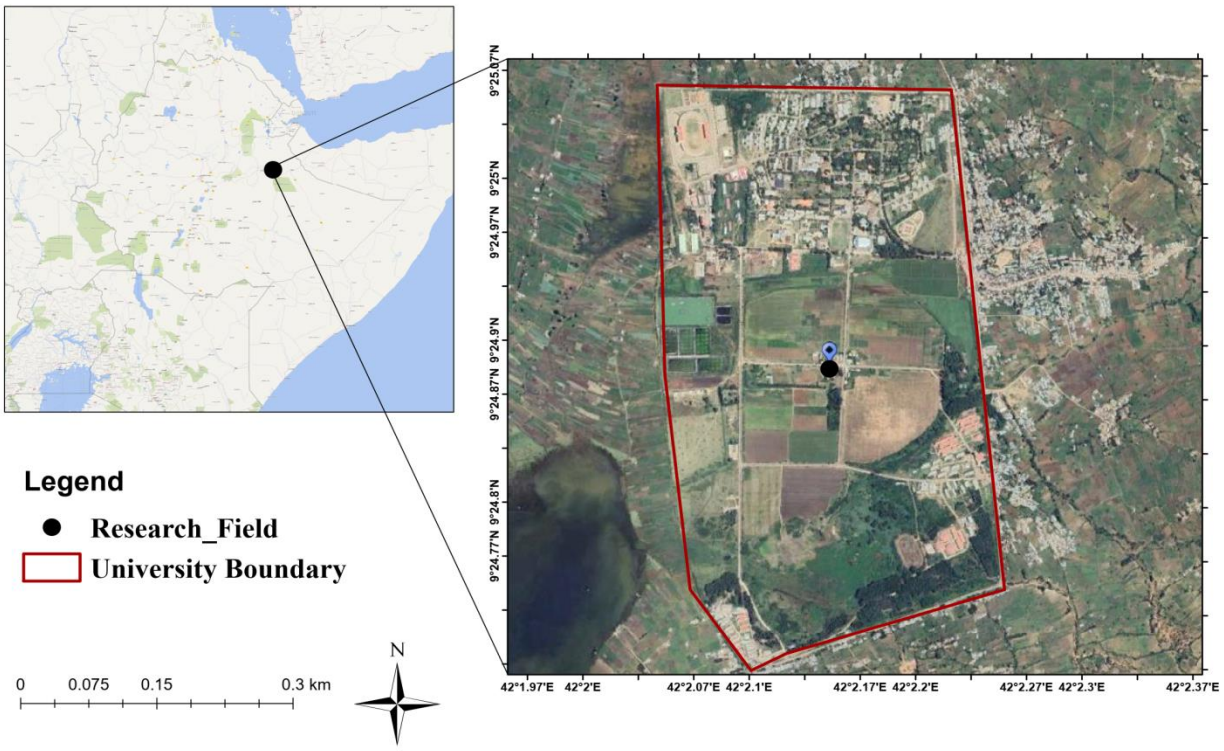

Figure: 1. Location of study area

Experimental Design

The study adopted a completely randomized design of three replicates within five treatment levels with isolates designated as PS (Phosphate solubilizing followed by isolate number), which are PS-6, PS-22, PS-27, PS-30, and the control by comparing the means between and within treatments.

Collection of Soil Samples

The phosphate solubilizing soil samples were collected using purposive sampling in two districts (Gorogotu, and Deder) from legumes-cultivated soils in eastern Hararghae highlands. Soil samples were collected at a depth of 0-15 cm. Each sample weighing 1.5 kg was thoroughly mixed to keep the uniformity of the distribution of microorganisms and transported to the laboratory within 24 hours.

Preparation and Analysis of Soil for the Pot Experiment

Rock phosphate rich soil samples were collected from Haramaya Woreda and used for the pot experiment under greenhouse conditions. The analysis of rock phosphate soil samples was done within 48 hours in Haramaya University's Central Laboratory for selected chemical and physical properties as shown in table1. The rock phosphate soil samples were crushed very well, air dried and passed through a 2 mm mesh size sieve before physicochemical analyses. The pH was determined from the filtered suspension of 1:2 soil to water ratio using a glass electrode attached to a digital pH meter. (Okalebo, Gathua and Woomer, 2002).

Table 1: Analysis of rock phosphate soils for pot experiment

Sample type	*% OC*	*% OM*	*% P*	*pH*	*Description*
Rock Phosphate Soil	0.818	5.674	0.948	4.5	- If % of OC is < 0.5, the amount of P is low - If % of OC is between 0.5 and 0.75, the amount of P is medium - If % OC > 0.75, the amount of P is high

OC = Organic Carbon, OM = Organic Matter and P = Phosphorus

Preparation of Culture Media

In this study, two kinds of culture media were used and prepared aseptically using standard methods. YEMA[1] as enrichment of isolates, and Pikovskaya's Agar Medium (PVK)[2] were used as a selective medium of phosphate solubilizing Rhizobia. In addition, YEMA with Congo red[3] was used to isolate, characterize and screen Rhizobia (Nakade, 2013).

Isolation of Phosphate Solubilizing Rhizobia

Phosphate solubilizing Rhizobia were isolated by serial dilution technique[4] (10^{-1} to 10^{-5}) from 1 g of soil sample. It was dispersed in 9 ml of autoclaved distilled water and poured in to screw cup test tube. Selection for phosphate solubilizing Rhizobia was done by culturing repeatedly on YEMA with Congo red. A single pure colony of each isolate was picked and placed at the center of Pikovskaya's Agar Medium (PVK) and incubated at 27±2ºC for 7-14 days. PVK was used for isolation of phosphate solubilizing Rhizobia due to havening insoluble tri-calcium phosphate (Tsegaye *et al*., 2018). After incubation, isolates forming colonies with clear halo zone were recorded as basic selective criteria of phosphate solubilizing Rhizobia. Then, the diameters of the halo zones (clear zones) were measured by a transparent ruler to calculate solubilization index using the formula indicated by Nagar for prepared inoculants (biofertilizers) under greenhouse experiment (Nagar, 2012).

Screening of Phosphate Solubilizing Rhizobia

The primary base for screening of phosphate solubilizing Rhizobia isolates was the phosphate solubilizing potential on PVK (containing insoluble tri-calcium phosphate) media. Then, from each isolates a loop full of pure culture was placed at the center of PVK agar media and incubated at 27±2ºC for 7-14 days to evaluate the extent of their ability to solubilize the insoluble tri-calcium phosphate (Abad, Sadaghiani and

[1] Yeast extract mannitol agar (YEMA) is the most commonly used medium for the culture of Rhizobia, and is made from either yeast water preparations, or commercial yeast extract powders and pastes.
[2] https://himedialabs.com/TD/M520.pdf
[3] https://pubchem.ncbi.nlm.nih.gov/compound/Congo-red#section=InChI-Key
[4] https://microbenotes.com/serial-dilution/

Barin, 2016). Phosphate solubilization was determined by measuring both colony and halo zone diameter with a transparent ruler and calculated by the formula recommended (Keneni, Assefa and Prabu, 2010). So, based on the formula, the four best phosphate solubilizing Rhizobia were recorded; above 3.5 mm Phosphate Solubilizing Index (PSI) were selected out of 30 total isolates for the next greenhouse experiment (Abad, Sadaghiani and Barin, 2016; Keneni, Assefa and Prabu, 2010).

Preparation of Inoculums for Pot Experiment

Single colonies of each isolate were transferred to 500 ml flaks containing nutrient broth and were then grown aerobically in flasks on a rotating shaker (150 rpm) for 48 hours at 30^0C. The bacteria suspension was diluted in sterile distilled water to a final concentration of 10^{12} CFU/ml. The resulting culture suspension was centrifuged in 50 ml capacity sterile plastic tubes at 5000 rpm for 10 minutes. The pellets were re-suspended in 50ml flask, and the suspension was adjusted to give a final concentration of 10^{12} CFU/ml (OD550 = 1) by relating the viable plate counts and optical density measurements using a standard curve.

The experiments were designed as a complete randomized design (CRD) with three replicates. Soil weight was 5 kg per pot. Teff grass seeds were planted in pots at seed rate of 0.51 gram per pot. During the experiment, a total of 30 ml of 10^{12} (Safari *et al*, 2020) thick cell suspension of phosphate solubilizing Rhizobia was prepared. Then, seeds were suspended in 30 ml thick cell suspension of phosphate solubilizing Rhizobia (10^{12} cells/ml) for 30 minutes with 0.5% peptone as adhesive and specimen having seeds without being suspended was used as the control. The seeds were air dried for 30 minutes in sterile petri-plates and were sown in the prepared pot experiment in the greenhouse. The seeds were watered twice per day and, after 30 days, plant growth parameters were measured and recorded (Sepehri and Shahbazi, 2017).

Preparation of Test Crop for Pot Experiment

The seeds of Quncho variety of Teff (DZ-Cr-387-RIL 355) were collected from Debrezeit Agricultural Research Center. Quncho Teff was developed and released by Debrezeit Agricultural Research Centre (Belay *et al*, 2006). The Quncho white seed cultivar Teff was selected because of its high yielding potential and adaptability to a wide range of factors (Tesfalem, 2014). After collection, the seeds were disinfected in 0.02% sodium hypochlorite for 2 minutes and were washed five times with distilled water before placing in the pot.

Statistical Data Analysis

The collected data were subjected to analysis of variance using the general linear model procedures of SAS version 9.1.3 (Shim, Billard and Pesti, 2014). Means of significant treatment effects were separated using the Least Significant Difference test[5] at $P<0.05$ level of significance.

Results and Discussion

Phosphate solubilization index was calculated using the formula indicated by Ponmurugan and Gopi (2006). Based on this, all 30 isolates were calculated. PS-27, PS-30, PS-6 and PS-22 were recorded the highest phosphate solubilization index. The standard deviation of phosphate solubilization index for PS-27, PS-30, PS-6 and PS-22 were 0.15, 0.29, 0.46, and 0.72, respectively. Therefore, PS-6, PS-27, PS-30 and PS-22 had the highest phosphate solubilizing index among the 30 Rhizobia isolates selected for the greenhouse experiments.

[5] https://www.statisticshowto.com/how-to-calculate-the-least-significant-difference-lsd/

Additionally, the mean values of halo zone for PS-6, PS-22, PS-27 and PS-30 were 17, 17, 12 and 13.4, respectively, and were the highest observed. However, PS-4 and PS-5 had the lowest mean values of phosphate solubilization index and halo zone. From colony diameter point of view, PS-9, PS-15, PS-16 and PS-21 had the highest colony diameters, while PS-22 and PS-24 showed the lowest colony diameters.

Table 2: Selection of Rhizobia Isolates Based on Phosphate Solubilizing Index

Isolates	*Phosphate Solubilization Index*	*Halo zone*	*Colony Diameter*
	Mean± SD	*Mean ± SD*	*Mean ± SD*
PS-1	2.10±0.12	0.50 ±0.35	4.00 ± 0.06
PS-2	3.30±0.38	8.00 ±0.58	3.50 ± 0.29
PS-3	2.13±0.15	5.00 ±0.69	4.20 ± 0.06
PS-4	1.40±0.06	2.00 ±0.06	4.70 ± 0.46
PS-5	1.17±0.03	1.00 ±0.00	4.50 ± 0.35
PSR-6	6.30±0.46	17.00 ±0.15	3.20 ± 0.06
PSR-7	2.30±0.17	5.00 ±0.17	4.00 ± 0.40
PS-8	2.27±0.09	6.00 ±0.06	4.60 ± 0.29
PS-9	1.50±0.06	3.00 ±0.17	5.30 ± 0.23
PS-10	1.80±0.21	3.50 ±0.29	3.50 ± 0.23
PS-11	2.83±0.66	3.70 ±0.17	3.70 ± 0.40
PS-12	1.90±0.06	3.00 ±0.06	3.30 ± 0.29
PS-13	2.00±0.12	5.00 ±0.52	4.80 ± 0.06
PS-14	2.00±0.06	4.00 ±0.23	3.90 ± 0.06
PS-15	2.77±0.03	9.00 ±0.23	5.00 ± 0.06
PS-16	2.37±0.03	7.00 ±0.17	4.90 ± 0.06
PS-17	1.90± 0.06	4.00 ± 0.00	4.20 ± 0.23
PS-18	2.30±0.10	5.00 ± 0.17	4.00 ± 0.06
PS-19	3.37±0.07	10.33 ± 0.60	3.70 ± 0.12
PS-20	3.00±0.17	6.50 ± 0.29	3.50 ± 0.35
PS-21	2.83±0.03	10.00 ± 0.58	5.37 ± 0.37
PS-22	6.70±0.72	17.00 ± 1.15	3.00 ± 0.17
PS-23	2.93±0.07	6.00 ± 0.23	3.10 ± 0.06
PS-24	2.30±0.23	4.00 ± 0.58	3.00 ± 0.06
PS-25	3.17±0.09	7.00 ± 0.17	3.20 ± 0.23
PS-26	1.90±0.00	4.00 ± 0.06	4.30 ± 0.12
PS-27	3.97± 0.15	12.00 ± 0.87	4.00 ± 0.12
PS-28	2.63±0.09	7.00 ± 0.17	4.20 ± 0.12
PS-29	2.50± 0.23	6.00 ± 0.58	4.00 ± 0.23
PS-30	5.47± 0.29	13.40 ± 0.47	3.00 ± 0.29

Values are given as means ±SD for triplicate samples, PS: phosphate solubilizing Rhizobia

Effect of PS Inoculation on Teff Growth Using Pot Experiment

The growth promoting ability of the inoculated PS isolates were determined based on data collected from the plant growth parameters (shoot length, root length, dry shoot, dry root and grain weight) after 30 and 90 days of seedling growth (Aamir *et al.*, 2013)**.**

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Data Collection after 30 Days of Seedling

After 30 days of Rhizobia inoculation, the teff seedling growth significantly increased on all teff growth parameters when compared with those of the control. Therefore, the highest root and shoot length was recorded by PS-22 (12 and 38.667 cm) and the highest root and shoot dry weight was (0.204 and 0.277 g), respectively. And the lowest root and shoot length were recorded by PS-27 (8.37 cm and 32.67 cm, respectively) while, the smallest root dry weight was observed within PS-2 (0.049 cm) and shoot dry weight within PS-27 (0.049 g) as seen in table 3. Similar results were reported by Gravel, Antoun and Tweddell (2007) who highlighted that inoculation of plants with plant growth promoting *Rhizobacteria* could significantly reflect changes in plant growth parameters, such as plant length and weight as well as yield (Gravel, Antoun and Tweddell, 2007). Many scholars have reported about plant growth promoting bacteria that can solubilize inorganic phosphate after been introduced into the soil or used as inoculants of plant seeds (Gull and Hafeez, 2012). According to Nie *et al*. (2002), plant growth promoting *Rhizobacteria* can enhance plant height and productivity by synthesizing phytohormones and increasing the local availability of nutrients.

The multiple comparison procedure for the selected isolates (PS-6, PS 22, PS-27 and PS-30) was used to determine which isolates are significantly different after obtaining a statistically significant result from an Analysis of Variance (ANOVA). The list square difference multiple comparison method showed significant mean difference with the root length, shoot length, dry root weight and shoot weight of treatments at level of alpha equal to 5%.

Table 3: Effects of inoculants on the growth of teff under greenhouse conditions after 30 days of seedling growth

Treatments	*Root length (cm) plant^{-1}*	*Shoot length (cm) plant^{-1}*	*Dry root weight (g) plant^{-1}*	*Dry shoot weight (g) plant^{-1}*
Control	4.000 ^{d} ± 1.000	11.000^{d} ± 1.000	0.008^{db} ± 0.006	0.048^{d} ± 0.062
PS-22	12.000^{a} ± 2.000	38.667^{a} ± 2.516	0.204^{a} ± 0.049	0.277^{a} ± 0.086
PS-6	11.200^{b} ± 2.206	37.000^{b} ± 6.557	0.180^{b} ± 0.065	0.206^{b} ± 0.036
PS-30	8.467^{ba} ± 1.550	36.000^{ba}± 1.732	0.107^{bc}± 0.078	0.146^{ba} ± 0.054
PS-27	8.367^{bc} ± 1.184	32.667^{bc} ± 2.886	0.049^{d} ± 0.053	0.049^{cb} ± 0.053
D.F.	4	4	4	4
P-value	0.0010	0.0001	0.0018	0.0032

Values are given as means ± SD for triplicate samples. Means followed by the different letter (s) in each column and row are significantly different at P<0.05, D.F.: Degree of Freedom, and PS: phosphate solubilizing Rhizobia.

Data Collection after 90 Days of Seedling

Results showed that, after 90 days, all the parameters were significantly affected by inoculation treatments over control (Table-4). The plant height increased progressively at successive observations with advancement in crop age. It was observed that the highest root length and shoot length, dry root and shoot weight as well as grain weight were recorded by PS-22 and the lowest one was at PS-27. Therefore, phosphate solubilizing *Rhizobacteria* reported to have increased root dry weight as well as yields (Singh, Pandey and Singh, 2011).

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Table 4: Evaluation of the effects of inoculants on the growth of Teff under greenhouse conditions after 90 days of seedling growth

Treatments	*Root length (cm) plant^{-1}*	*Shoot length (cm) plant^{-1}*	*Dry root weight (g) plant^{-1}*	*Dry shoot weight (g) plant^{-1}*	*Grain weight (g) plant^{-1}*
Control	2.900 ^{d} ± 1.014	13.000^{d} ± 2.645	0.100^{d} ± 0.000	0.011^{d} ± 0.009	0.009^{d} ± 0.006
PSR-22	9.667^{a} ± 3.332	41.667^{a} ± 5.663	0.467^{a} ± 0.057	0.333^{a} ± 0.032	0.378^{a} ± 0.258
PSR-6	8.675^{b} ± 0.599	41.333^{ab} ± 5.033	0.433^{b} ± 0.208	0.195^{b} ±0.067	0.367^{ab} ± 0.086
PSR-30	8.300^{ba} ± 1.212	38.750^{b} ± 2.629	0.433^{b} ± 0.152	0.144^{ba} ± 0.006	0.236^{ba} ± 0.255
PSR-27	8.000^{bc} ± 2.000	37.333^{ba} ±7.094	0.333^{c} ± 0.152	0.068cb ± 0.030	0.172^{bc} ± 0.034
DF	4	4	4	4	4
P-value	0.0143	0.0001	0.0352	0.3433	0.1991

Values are given as means ± SD for triplicate samples. Means followed by the same letter (s) in each column are not significantly different at P<0.05. DR: Degree of Freedom, and PSR: phosphate solubilizing Rhizobia

Conclusion

This study has provided significant evidence for the usefulness of a screening technique for the selection of phosphate solubilizing Rhizobia and testing its biological activity under greenhouse-controlled conditions. Therefore, application of the best isolates inoculums will be highly recommended to improve the growth or yield of crops in the greenhouse or field. This study discovered an isolation of potential phosphate solubilizer Rhizobia that can be beneficial for farmers as biofertilizers collected from legumes plant rhizosphere to increase the productivity of cereal crops in the field. Finally, this study will help researchers uncover the critical areas of using legume (nodule forming plants) as a source of phosphate-solubilizing bacteria to apply for the non-legume (cereal crops) that many researchers have not been able to explore.

Before recommending the use of these *Rhizobium* strains for improving yield of crops (field and greenhouse) further tests must be designed to examine their behavior under field conditions (competitiveness, persistence, etc.).

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Authors' Declarations and Essential Ethical Compliances

Authors' Contributions (in accordance with ICMJE criteria for authorship)

Contribution	Author 1	Author 2	Author 3	Author 4
Conceived and designed the research or analysis	Yes	Yes	Yes	No
Data collection	Yes	Yes	Yes	Yes
Contributed to data analysis and interpretation	Yes	Yes	Yes	Yes
Written the article/paper	Yes	Yes	No	No
Critical revision of the article/paper	Yes	Yes	Yes	No
Editing of the article/paper	Yes	Yes	Yes	Yes
Supervision	Yes	Yes	Yes	Yes
Project Administration	Yes	No	No	No
Funding Acquisition	No	No	No	No
Overall Contribution Proportion (%)	40	20	20	20

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