International Journal of Agricultural and Natural Sciences
E-ISSN: 2651-3617 16(3): 223-230, 2023
doi: 10.5281/zenodo.10203691
Research Article

EFFECT OF RHİZOBACTERİA ON PLANT GROWTH HORMONE PRODUCTİON, PHOSPHATE SOLUBİLİZATİON AND GROWTH PERFORMANCE OF *Phaseolus vulgaris* L. UNDER *IN VİTRO* CONDİTİON

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(Received 19th September 2023; accepted 30th October 2023)

ABSTRACT. Bacterial inoculants are used as alternative bio-fertilizers because they can solubilize phosphate and increase plant growth and development. Therefore, the research was conducted to isolate phosphate solubilizing rhizobacteria (PSB) from the rhizosphere soil of faba bean and ground nut plants and examine their ability to solubilize phosphate and their effect on *Phaseolus vulgaris* seed germination and seedling growth. A total of 30 phosphate solubilization bacterial (PSB) isolates were collected from Babile, Meta, and Haramaya University farmlands, in Ethiopia. The laboratory experiment was based on bioassay to assess the seed germination percentage and seedling growth of *Phaseolus vulgaris*. To measure the zone of clearance for effective phosphate solubilization (PSB) as well as to examine their ability to produce Indole Acetic Acid (IAA), and the experimental design was (CRD) with factorial arrangements and three replications. The results of this study revealed the isolate PSB20 (6.250) showed the maximum solubilization index (SI), and PSB25 has the lowest SI (4.667) and PSB20 (2.967ppm) showed the highest IAA production as compared to PSB25 (2.197ppm). Among the treatments, PSB20 has a minimum date of germination (4 days) and a maximum length of plumule and radical (6.61cm) and (4.5cm), respectively. A significant reduction in day to germination was observed at PSB20 (4 days), while the maximum day-to-germination was observed at PSB25 (6.33 days). In the present investigation, PSB20 and PSB12 showed a better ability to solubilize phosphate than other isolates and enhance the seed germination percentage and seedling growth of *Phaseolus vulgaris*.

Keywords: *Plant growth, promoting rhizobacteria, solubilization ındex, auxin production*

INTRODUCTION

The sustainability of agricultural production systems is directly or indirectly dependent on microbial diversity and is considered essential for maintaining soil fertility. Using naturally occurring, free-living bacterial species that can protect and promote plant growth by colonizing and multiplying along the root surfaces of the inoculated plants is said to be one of the safest and most suitable alternatives [1]. The use of these microorganisms as environment-friendly bio-fertilizers helps to reduce the use of the much more expensive phosphate fertilizers. The rhizospheric soil contains diverse types of plant growth-promoting rhizobacteria (PGPR) communities, which exhibit beneficial effects on crop productivity, including numerous strains of the genera *Arthrobacter, Azospirillium, Azotobacter, Azoarcus, Bacillus, Burkholderia, Enterobacter, Rhizobium, etc.*[2]. Rhizobacteria lodging around or in the plant roots are more versatile in transforming, mobilizing, and solubilizing the nutrients compared to those from bulk soils. Therefore, the rhizobacteria are the dominant deriving forces in recycling soil nutrients, and consequently, they are crucial for soil fertility [3]. One of the significant essential macronutrients is phosphorous for plant growth and development.

Approximately 95-99% of soil phosphorous is present as insoluble phosphates and hence cannot be utilized by plants. Plants acquire phosphorous from soil solution as phosphate anions. However, phosphate anions are highly reactive and may be immobilized through precipitation with cations such as Ca_2+, Mg_2+, Fe_3+, and Al_3+, depending on the particular properties of the soil. In these forms, phosphorous is highly insoluble and unavailable to plants. As a result, the amount available to plants is usually a small proportion of this total [4]. Improving soil fertility by releasing bound phosphorous by microbial inoculants is an important aspect of increasing crop yield. Phosphate-solubilizing microorganisms play an essential role in plant nutrition by increasing the available phosphorous for uptake by plants. These plant growth-promoting microbes are also essential contributors to the bio-fertilization of crops. Apart from fertilization, microbial phosphorous mobilization would be the only possible way to increase available phosphate for plants.

The use of phosphorus-solubilizing microorganisms can increase crop yields by up to 70 percent. The phosphate-solubilizing bacteria as inoculants simultaneously increase P uptake by the plant and crop yield. The phosphate-solubilizing bacteria exhibiting multiple plant growth-promoting traits on soil-plant systems are needed to uncover their efficacy as effective bio-inoculants. The inoculation of PSB and plant growth-promoting rhizobacteria (PGPR) together could reduce 50% of P fertilizer application without any significant decrease in crop yield. The PSB is able to synthesize phytohormones like Indole acetic acid (IAA), Gibberellic acid (GA3), and siderophore PSB also enhances plant growth by increasing the efficiency of biological nitrogen fixation or enhancing the availability of other trace elements such as iron, zinc, *etc*. [5].

Although research on phosphorus-solubilizing microorganisms can be one of the best approaches to improve phosphorous uptake in haricot beans, the potential of phosphorus-solubilizing bacterial inoculation in the simultaneous increase of phosphorus uptake and growth of haricot beans has not been sufficiently evaluated. To this effect, the objective of this study was to isolate phosphate-solubilizing rhizobacteria isolates from rhizosphere soil and examine their ability to solubilize phosphate and production of indole acetic acid. The *in vitro* evaluation of their effects on seed germination traits of haricot bean.

MATERIALS AND METHODS

Source of Rhizobacteria and Sample Collection

The soil samples were collected from the rhizosphere of agricultural lands of Haramaya University, Deder, and Babille. A total of 30 soil samples were collected from these sites in polyethylene bags. These soil samples were collected from up to a depth of 15 cm of the topsoil found near the roots of two selected leguminous plant species i.e., faba bean and groundnut. The rhizosphere soil samples from each of these selected plant species were collected using a shovel, soils were then transferred to polyethylene bags and transported to the Soil Microbiology Laboratory of the School of Plant Sciences at Haramaya University and stored at 4^oC until further analysis.

Preparation of Inoculum from Soil Samples and Inoculation

Soil samples were dissolved in sterile distilled water and then serially diluted up to 10^{-5}. Then, samples from the serial dilutions were spread-plated on Pikovskaya's Agar Medium (PVK) and incubated at 30^{oC} for 24 hrs. The bacterial colonies with a clear phosphate solubilization zone were observed after incubation and were selected and streaked on new PVK agar plates to get isolated pure colonies. Further purification was carried out by re-streaking on the same media.

Isolation of Phosphate Solubilizing Rhizobacteria and Determination of Solubilization Index Under In Vitro Conditions

Detection and estimation of the phosphate solubilization ability of microorganisms were done using the Petri plate screening method. Phosphate solubilizing bacteria (PSB**)** were isolated from each sample using a serial dilution technique followed by spread plating on Pikovskaya's agar medium (PVK). One gram of soil sample was suspended in 9 ml of distilled water, thoroughly shaken, and kept on a rotary shaker adjusted at 125rpm for 30 min. 1mL of the resulting soil suspension was transferred to 9 ml of sterile distilled water to form 10^{-2} dilution. Finally, 10^{-3}, 10^{-4}, and 10^{-5} dilutions were made for each soil sample. From the appropriate serial dilutions, 0.1ml of the sample was spread on Pikovskaya's agar medium (PVK) and incubated at 30^{0C} for 7 days. The appearance of clear halo zones around the microbial colonies in media was an indication of the presence of phosphate solubilizing bacteria (PSB) and hence these colonies were isolated and aseptically transferred to Pikovskaya's agar medium (PVK) to produce pure cultures. The phosphate solubilization index of PSB was calculated using the formula provided [6]. A loop-full of 24 h old cultures was spread on Picovskaya's agar plate and incubated at 30^0C for 7 days. The diameters of both the colony and the halo zone were measured by a transparent ruler and used for the calculation of the solubilization index (SI) as indicated below.

$$\mathrm{SI} = \frac{\text{colony diameter} + \text{halozone diameter}}{\text{colony diameter}}$$

In-Vitro Screening of Rhizobacteria for Indole Acetic Acid Production

The screening of rhizobacteria for plant growth-promoting activities was done by testing their ability to produce auxin (IAA equivalent). Bacterial indole acetic acid production was examined by growing isolates in nutrient broth supplemented with tryptophan[7]. For this purpose, 25 ml of glucose peptone medium was added in 100ml of Erlenmeyer flask; autoclaved, and cooled; L- Tryptophan solution was sterilized and added at a desired concentration, i.e. 1g/L to the liquid medium. The flask contents were then inoculated by adding 1ml of 5 days old bacterial suspension in broth culture. Then the flasks were incubated at 30ºC for 48 hrs. An untreated control was also prepared similarly but without tryptophan and used for comparison. After incubation, the contents were filtered through Watman No.1 filter paper. The amount of auxin (IAA equivalents) was determined by a spectrophotometer using a Salkowiski coloring reagent [8]. To measure the auxin concentration (IAA- equivalents), 3.0 mL of culture filtrate and 2 mL of Salkowiski reagent were added to test tubes and allowed to stand for half an hour for color development.

Seed Germination Bioassay

Phaseolus vulgaris seeds were surface sterilized with 0.1% HgCl2 for 3 min and washed with distilled water 4-5 times. Seeds were soaked for 20-30 min in 48 hrs old bacterial broth cultures containing at least 108 cells/ml. The seeds were kept on sterilized filter paper (in Petri plates) and incubated at 30^{0C} for 6 to 8 days. After incubation, the seedlings' percent seed germination, root, and shoot lengths were recorded. 120 haricot bean seeds were dipped into a nutrient broth culture of PSB isolates for five hours, containing the bacterial suspension of (108 CFU mL-1) while fifteen (15) seeds were dipped in distilled water and served as control. Five seeds per plate of inoculated haricot beans were placed in Petri dishes with one Whatman No.1 filter paper layer. Both treated and untreated plates containing haricot bean seeds were arranged in a complete randomized design (CRD) triplicate and incubated at 300C for 3-7 days. The percent of germinated seeds for 1-3 days, radical and plumule length of germinated seeds were taken up to 7 days [9]. The vigor index of germinating seeds and germination percentage were determined [10].

RESULTS AND DISCUSSION

Isolation and Identification of Rhizobacteria

A total of 30 bacterial isolates that exhibited clear zones on the PVK agar medium containing insoluble tri-calcium phosphate from soil samples were selected as phosphate-solubilizing rhizobacteria. Out of 30 microbial isolates, 8 isolates (PSB2, PSB3, PSB4, PSB12, PSB15, PSB18, PSB20, and PSB25) were selected as efficient phosphate-solubilizing rhizobacteria. It showed a high Phosphate Solubilization Index (PSI) ranging from 4.667 to 6.250 after 7 days of incubation. Phosphate-solubilizing microbes were detected by the formation of clear halos around their colonies. The halo zone was produced due to the solubilization of insoluble phosphates, which in turn was mediated via the production of organic acids in the surrounding medium. The results indicated that isolate PSB20 (6.250) showed the maximum solubilization index, which differed significantly from the next three highest solubilization indices demonstrated by isolates PSB12, PSB2, and PSB3 (Table 1). The isolates were ranked for phosphate solubilization as PSB20>PSB12>PSB2>PSB3>PSB4=PSB18>PSB15>PSB25. The change in the pH of the culture medium reflected the difference in the phosphate solubilization. The most efficient isolates, PSB20 and PSB2, had lower pH of the medium (5.6 and 5.9, respectively). An inverse relationship was observed between the pH value of the culture medium and the concentration of phosphate solubilized, indicating the organic acid secretion.

Table 1. *Phosphate solubilizing activities of selected phosphate solubilizing rhizobacteria*

Isolates	Colony diameter(mm)	Halozone diameter(mm)	Solubilization index(SI)	pH of culture media
Control	4.000^{d}	0.00^{f}	1.000^{e}	6.7
PSB2	4.167^{cd}	20.00^{c}	5.790^{ab}	5.9
PSB3	4.333b^{cd}	19.00^{cd}	5.433^{bc}	6
PSB4	5.000^{abc}	21.33^{b}	5.267^{bc}	6
PSB12	3.500^{d}	16.33^{e}	5.800^{ab}	5.8
PSB15	5.067^{ab}	19.67^{c}	4.883^{cd}	6.5
PSB18	5.333^{a}	22.67^{a}	5.267^{bc}	5.7
PSB20	3.667^{d}	19.00^{cd}	6.250^{a}	5.6
PSB25	5.000^{abc}	18.33^{d}	4.667^{d}	6

Means followed by the same letter(s) with the column are not significantly different at P=0.05

This finding in accordance with Khiari, [11] indicates the production of organic acids by PSB in the medium lowers the pH of the medium. In this experiment the highest solubilization index was observed in the media inoculated with PSB20, and the corresponding pH during the 7 days of incubation was 5.6. The result revealed a perfect inverse correlation between the amounts of tricalcium solubilized and the pH of the medium inoculated with PSB cultures. In line with this, [12] indicated the production of organic acids by PSB in the medium lowers the pH of the medium.

Auxin Production by Phosphate Solubilizing Rhizobacterial Isolates

All of the selected eight Phosphate Solubilizing Rhizobacteria (PSB) isolates used in this study were found to produce auxin. Table 3 shows the auxin production by the isolates expressed as IAA equivalents. All selected (8) PSB isolates used in this study exhibited the capacity to produce indoleacetic acid (IAA); therefore, this might have contributed to enhanced shoot and root length through cell elongation and multiplication. The test results of the isolates indicated that isolates of PSB varied greatly in the amount of auxins they produced in the broth medium in the presence of L-TRP (Table 2). Among the 8 PSB isolates, PSB2, PSB3, and PSB20 produced higher levels of auxin (ranging from 2.917 to 2.987 ppm IAA equivalents). Isolate PSB25, on the other hand, showed the lowest auxin production (2.197 ppm). This finding is similar to the work of [13], which observed an increase in plant height, number of branches, number of pods, grain weight, and eventually higher seed and straw yields in mung bean plants after inoculation of *B. megaterium* in salt-affected soils due to the productions of auxin.

Table 2. *The amount of auxin produced (expressed as IAA equivalents) by phosphate solubilizing rhizobacterial isolates*

Isolates	IAA (ppm)
PSB2	2.917^{a}
PSB3	2.987^{a}
PSB4	2.80^{b}
PSB12	2.613^{c}
PSB15	2.403^{d}
PSB18	2.717^{bc}
PSB20	2.967^{a}
PSB25	2.197^{e}

Means with the same letter in the columns are not significantly different, LSD: Least significant difference at P=0.05, IAA= Indole Acetic Acid

Effect of Phosphate Solubilizing Rhizobacteria Inoculation on Seed Germination and Seedling Growth of Haricot Bean

The results of haricot bean inoculation with Phosphate Solubilizing Rhizobacteria (PSB) isolates on growth parameters were presented in table-3. All of the inoculated treatments significantly ($p<0.05$) decreased the germination date and increased the plumule and radical length of the haricot bean compared to the control. The number of days for seed germination was shorter with PSB inoculants. Among the treatments, PSB20 has a minimum date of germination (4 days) and a maximum length of plumule and radical (7.00cm) and (5.17), respectively, followed by PSB12(4.33 days) and a maximum length of plumule and radical (5.833cm and 4.17 cm) respectively while PSB25 with a date to germination of 6.33 was not significantly different from the control. But, still, they had better radical and plumule lengths (4.5cm) and (6.67cm), respectively. Most inoculated treatments gave the highest length of plumule and radical compared to the control. Significant ($P<0.05$) differences in both plumule and radical length of haricot beans were observed due to inoculation with PSB. As the result in Table 3 indicates, plumule and radical length varied from PSB15 (5.333 cm) to PSB20 (7.00 cm) and PSB4 (3.83 cm) to PSB20 (5.17 cm), respectively. Among the treatments, the highest mean value of plumule length was recorded in PSB20 (7.00 cm) followed by PSB25 (6.667 cm), whereas the least was observed in PSB15 (5.333 cm) while the highest mean value of radical length was recorded in PSB20 (5.17 cm) followed by PSB25 (4.50 cm) while the most negligible value was observed in PSB4 (3.83 cm).

Table 2. *Effect of phosphate solubilizing rhizobacteria on germination of haricot bean seeds*

Treatments	ED	RadL(cm)	Plu L(cm)	GP	VI
Control	6.667^{a}	3.67^{d}	5.000^{c}	86.7	751.67
PSB2	5.000^{cd}	4.50^{b}	5.833^{bc}	93.3	964.07
PSB3	5.333^{c}	4.00^{c}	5.667^{bc}	86.7	838.13
PSB4	5.333^{c}	3.83^{c}	6.000^{abc}	90.0	884.70
PSB12	4.333^{de}	4.17^{c}	5.833^{bc}	93.3	933.28
PSB15	5.667^{bc}	4.17^{c}	5.333^{c}	80.0	760.24
PSB18	5.000^{cd}	4.50^{b}	5.667^{bc}	86.7	881.48
PSB20	4.000^{e}	5.17^{a}	7.000^{a}	90.0	1095.3
PSB25	6.333^{ab}	4.50^{b}	6.667^{ab}	93.3	1041.88

Means with the same letter in the columns are not significantly different, LSD: Least significant difference at P=0.05, ED= Emergence Date, RadL=Radicle length, GP=Germination Percentage, VI=Vigor Index

PSB3, PSB12, and PSB25 showed the highest germination percentage (GP) (93.3% each) and they are significantly the same, while PSB 15 has the least GP (80.0%), which was even less than the control. This is, therefore, because of the stimulatory effects of PSB on seed germination of plants. In the present study, the inoculants PSB20 and PSB12 increased the percent of seed germination, which is compared with the result of Ghanem [14] reported the increment of maize seed germination by (18.5%) over control due to plant growth-promoting rhizobacteria inoculants. The increment of seed germination with inoculants could be due to the isolate's ability to synthesize seed germination hormone. [15] also reported that the inoculation injection of PSB enhanced wheat's radical and root length. PSB20 showed the highest vigor index, followed by PSB25 compared to other treatments. PSB15 has a minimum vigor index (760.24) and is not significantly different from the control. This present investigation confirms the earlier works, which revealed that under in vitro conditions, seed treatment with PGPR strains improved seed germination, seedling vigor, and seedling emergence over the control. Similar

improvement of seed germination parameters by rhizobacteria has been reported in other cereals, such as sorghum [16]. The inoculated plants resulted in better germination, early development, and flowering and an increase in dry weight of both the root and upper plant parts. Furthermore, the inoculants increased vigor index, radical, and plumule length compared to control. To this effect, this result reported by Ghanem[14] the inoculation of PSB enhanced wheat's radicle and root length compared to individuals. This could be because of more growth-promoting substances released by inoculants. These results suggest that treatment with PSB is beneficial as a general increase in seed germination as well as radicle and plumule length as compared to control observed in all cases. This study revealed that under *in vitro* conditions, seed treatment with PSB strains improved seed germination, seedling vigor, seedling emergence, and overcontrol.

CONCLUSION

In this study, out of 30 microbial isolates, 8 isolates (PSB2, PSB3, PSB4, PSB12, PSB15, PSB18, PSB20, and PSB25) were selected as efficient phosphate-solubilizing rhizobacteria. It showed a high Phosphate Solubilization Index (PSI) ranging from 4.667 to 6.250 after 7 days of incubation. The isolate PSB20 (6.250) showed the maximum solubilization index, which differed significantly from the next three highest solubilization indices demonstrated by isolates PSB12, PSB2, and PSB3. Finally, the isolates were ranked based on phosphate solubilization as PSB20>PSB12>PSB2>PSB3>PSB4=PSB18>PSB15>PSB25. Among the 8 PSB isolates, PSB2, PSB3, and PSB20 produced higher levels of auxin (ranging from 2.917 to 2.987 ppm IAA equivalents). Among the treatments, PSB20 has a minimum date of germination (4 days) and the maximum length of plumule and radical (7.00cm) and (5.17), respectively followed by PSB12(4.33 days) and the maximum length of plumule and radical 5.833cm and 4.17 cm. These results suggest that treatment with PSB is beneficial as a general increase in seed germination as well as radicle and plumule length as compared to control. The present study, suggests that using PSB isolates as bio-inoculants might be beneficial for haricot bean cultivation. Microbial inoculants play a significant role in regulating the dynamics of organic matter decomposition and the availability of plant nutrients.

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