Indole Acetic Acid Producing And Phosphate Solubilizing Bacteria Native To Kenyan Soils Promote Growth of Common Bean (*Phaseolus Vulgaris* L.)

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Research Article

Keywords: Phaseolus vulgaris, co-inoculation, rhizobia, Indole acetic acid, phosphate solubilization, Bacillus, Phosphorous.

Posted Date: December 1st, 2021

DOI: https://doi.org/10.21203/rs.3.rs-1116404/v1

Abstract

Use of phosphate solubilizing bacteria (PSB) and rhizobia can have a positive effect on the growth of common bean. This study aimed at determining the mechanisms of action of native bacterial strains; and to determine their effect in enhancing growth of common bean. The strains were screened for their ability to solubilize insoluble inorganic phosphates and production of indole acetic acid *in vitro*. A greenhouse experiment was set up to evaluate the response of common bean to inoculation with selected bacterial strains. Six of the bacterial isolates tested showed a positive result for IAA production. *Rhizobium pusense* showed the greatest solubilization efficiency of 648 followed by *Bacillus megaterium* (322.3) and *Rhizobium phaseoli* (308.7). Inoculation of common bean with Rhizobia and PSB had a significant effect on the number of nodules per plant. The highest shoot biomass was observed when *Rhizobium phaseoli* was co-inoculated with *P. polymyxa* (4.3g plant^{-1}) compared to the single *Rhizobium phaseoli* inoculation (1.14 g plant^{-1}). The shoot tissue nitrogen and phosphorous concentration was increased as a results of co-inoculation up to 32.5% and 75.4% respectively. Therefore, tested bacterial strains have great potential in being formulated and used as biofertilizers that can be evaluated under varying field conditions.

Introduction

Over 200 million people in Sub-Saharan Africa (SSA) depend on common bean as a primary staple food[1]. The production of common bean (*Phaseolus vulgaris* L.) is, however, constrained by low soil fertility in many soils leading to a threat to food security. Nitrogen (N) and phosphorus (P) are the most limiting nutrients for plant growth. Phosphorus is generally deficient in some soils due to its ready fixation by iron and aluminum oxides[2,3]. The low level of production in SSA has been attributed partly to low levels of soil plant-available P and drought stress, caused by climate change variability[4,5]. Phosphorous is one of the most deficient nutrients for cultivation of common bean[4] because of the high P fixing soils[6]. Because common bean requires P to enhance energy for its metabolic activities, the crop possesses high requirements for P and is, hence sensitive to low plant-available P in soils[7].

Thus, one area of increasing interest is the use of microorganisms which act through a number of mechanisms such as nitrogen fixation, solubilization of phosphorous, production of indole acetic acid, cytokinins among others that facilitate nutrient acquisition[8,9]. Various soil microorganisms produce naturally occurring auxins, indole-3-acetic acid (IAA) as a direct mechanism to promote plant growth[10]. In particular, plant growth promotion and root nodulation are both affected by IAA. Bacterial IAA increases root surface area and length, and thereby provides the plant with greater access to soil nutrients. In addition, bacterial IAA loosens plant cell walls facilitating increased amount of root exudation that provides additional nutrients to support the growth of beneficial rhizosphere bacteria[11]. Most *Rhizobium* strains that have been examined have been found to produce IAA and several studies have suggested that increases in auxin levels in the host plant are necessary for nodule formation[11]. Nodule bacteria including *Rhizobium leguminosarum, R. undicolam, R. etlii*, *Sinorhizobium meliloti*, *R. phaseoli*, R.

pusense among others synthesizes IAA, thereby playing an important role in legume-rhizobia interaction[12–17]. It was reported that IAA acts as a signal molecule which is involved in plant signal processing, motility, or attachment of bacteria in root which help in legume-*Rhizobium* symbiosis[18].

Soil holds large amounts of phosphate, yet it is found in insoluble form. Phosphate solubilizing bacteria (PSB) are reported to solubilize the phosphate in the soil through acidification, chelation, or enzymatically[19]. These microorganisms mineralize organic phosphorus in soil by solubilizing complex-structured phosphates such as tricalcium phosphate to inorganic forms available to plants. Many of the PSB lower the pH of the medium by secretion of organic acids such as acetic, lactic, malic, succinic, oxalic and citric acids[2]. The ability of some microorganisms to convert insoluble phosphates to plant-available forms is an important characteristic for increasing plant yields[21]. Bhattacharyya and Jha[3] reported that bacterial genera like *Azotobacter*, *Bacillus*, *Beijerinckia*, *Burkholderia*, *Enterobacter*, *Erwinia*, *Flavobacterium*, *Microbacterium*, *Pseudomonas*, *Rhizobium* and *Serratia* as the most significant phosphate solubilizing bacteria. *Bacillus megaterium*, *B. circulans*, *B. coagulans*, *B. subtilis*, *Paenibacilus polymyxa*, *B. sircalmous*, and *Pseudomonas striata* are the most important strains[22–26].

Although the role of phosphorus in nodulation, nitrogen fixation and growth of common bean has been reported, the role of phosphate-solubilizing bacteria in phosphorus availability, growth promotion and also their interaction with N_2-fixing bacteria under tropical conditions requires thorough investigation. The mechanisms of action of the native Rhizobia and PSB need to be understood in order to select highly effective strains that can be exploited for inoculant production. This study aimed to assess IAA production and phosphate solubilization efficiency of locally isolated endophytic bacteria and to test their efficacy in growth promotion of common beans under greenhouse conditions.

Results

Production of Indole-3 acetic acid (IAA) by Rhizobium **species.** There was significance difference on the concentration of IAA produced by the different Rhizobia isolates. Four out of the ten bacterial isolates tested were considered high IAA producers (>0.85) while the rest were low producers. From the four high IAA producers, *R. pusense* (Busia) and *R. phaseoli* (Bungoma) produced higher levels of IAA with absorbance values of 1.33 and 1.14 respectively compared to the other isolates (Figure 1). Some of the isolates produced negligible amount of IAA (Figure 1). The colour development showed that the Rhizobia isolates had different ability to produce IAA (Photo 1).

In vitro **phosphate solubilization by the bacterial strain.** Significant ($p<0.05$) differences in the inorganic phosphate solubilization was observed among the different bacterial isolates (Table 1). *Rhizobium pusense* (Busia) showed the greatest solubilization efficiency of 648 and consequently the highest solubilization index of 7.3 (Table 1). It was followed by *B. megaterium* and *R. phaseoli* with an SE of 322.3 and 308.7 respectively. Based on the classification scale of Silva-Filho and Vider (2000), *R. pusense*, *B. megaterium* and *R. phaseoli* (S5) were classified as high solubilizers (SI>3.0). *Paenibacillus polymyxa*, *Pseodomonas sp*, *R. phaseoli* (B3), *B. aryabattai* and *B. megaterium* were considered medium

solubilizers (SI= 2.0-3.0); while the rest of the isolates were low solubilizers (Table 1). Photo 2 shows the solubilization diameter of some of the isolates.

Table 1
Phosphate solubilization efficiency (SE) and index (SI) of the different bacterial isolates

Strain	Solubilization efficiency (SE)	Solubilization index (SI)
R. pusense (B)	648.0 a	7.3a
B. megaterium	322.3 b	3.9bc
R. phaseoli (S)	308.7 b	4.2b
P. polymyxa	171.7 c	2.7bcd
Pseudomonas sp (B)	145.0 cd	2.5bcd
R. phaseoli (B)	108.3 cde	2.1cd
B. aryabhattai	104.0 cde	2.1cd
B. megaterium	100.0 cde	2d
R. leguminasorumi (S)	86.7 de	1.9d
B.subtilis	78.0 def	1.8d
R. leguminasorum (B)	74.7 def	1.7d
R. pusense (B)	64.7 ef	1.6d
R. pusense (B)	63.3 ef	1.6d
Paenibacillus sp (S)	50.0 ef	1.5d
Bacillus sp	49.3 ef	1.5d
B. megaterium	37.7 ef	1.3d
Pseudomonas sp (S)	0 f	1d
Tukey MSD ($\alpha \leq 0.05$)	79.2	1.8

Letters in brackets represent the location of isolation; Letters in brackets represent the location of isolation; S- Busia and B- Bungoma; and the numbers are codes given to the isolates. Means followed by different letters are significantly different at $\alpha \leq 0.05$.

Greenhouse Experiment

Effect of co-inoculation with Rhizobia and PSB on the nodulation of common bean. Inoculation of common bean with Rhizobia and PSB had a significant ($p \leq 0.05$) effect on the number of nodules per plant. The control treatment did not contain any nodules (Figure 2). In terms of the effect of co-inoculation, the results were varied depending on the specific Rhizobia-PSB interaction. Some of the interactions led to a synergistic effect on the nodulation while in other treatments, single inoculation with Rhizobia elicited a higher nodulation (Figure 2). For instance, the co-inoculation of *R. pusense* with *B. aryabhattai* (TRT-9) and *B. megaterium* (TRT-10) led to significantly higher number of nodules compared to the single *R. pusense* (TRT-1); whereas when co-inoculated with *P. polymyxa* (TRT-8), the nodule numbers are significantly lower (Figure 2). The co-inoculation of *R. phaseoli* with *P. polymyxa* (TRT-11) and *B. aryabhattai* (TRT-12) led to significantly high number of nodules than the single *R. phaseoli* (TRT-2) inoculation. However, there was depressed nodulation when it was co-inoculated with *B. megaterium* (Figure 2). Single inoculation with *R. pusense* (TRT-3) and *R. pusense* (TRT-4) had significantly higher number of nodules compared to the co-inoculation with the PSB. In terms of the nodule dry weight, a similar trend was observed in the inoculation effect as in the number of nodules. Correlation analysis showed a strong positive relationship ($R^2 = 0.995$; $p<0.001$) between the number of nodules and the nodule dry weight (Table 2).

Effect of co-inoculation with Rhizobia and PSB on the shoot and root biomass of common bean. Inoculation of common bean with Rhizobia and PSB generally increased the shoot biomass compared to the control (Figure 3). Specific Rhizobia-PSB co-inoculation had varied influence on the shoot biomass of the common bean. Some of the interactions were synergistic while others led to a lower biomass compared to when singly applied (Figure 3). A positive interaction was observed in most of the interactions except for the *R. pusense* co-inoculated with either *B. aryabhattai* or *B. megaterium*; *R. phaseoli* (B3) with *B. megaterium*; and co-inoculation of *R. pusense* with either *P. polymyxa* or *B. megaterium* (Figure 3). A noticeable positive co-inoculation effect was observed when *R. phaseoli* (B3) was co-inoculated with *P. polymyxa* (4.3g plant^{-1}) and with *B. aryabhattai* (3.4g plant^{-1}) compared to the single *R. phaseoli* inoculation with a biomass of 1.14 g plant^{-1} (Figure 3). Photo 3 shows a comparison on the above ground biomass of the bean crop between an inoculated and a control pot.

Inoculation of the common bean with the Rhizobia and PSB resulted in a significant ($p \leq 0.05$) increase in the root dry weight. The non-inoculated (control) treatment had significantly lowest root mass (0.9 g plant^{-1}) compared to the inoculated treatments except for the single *R. pusense* (B2) inoculation (0.8 g plant^{-1}) (Figure 4). In terms of the specific Rhizobia-PSB interactions, co-inoculation led to an increase in root biomass except for the *R. pusense* (S5) strain (Figure 4). Co-inoculation of *R. pusense* (B4) with *B. megaterium* resulted in significantly highest root biomass of 5.5g plant^{-1} (Figure 4). Pearson correlation analysis showed a positive significant ($p< 0.001$) relationship ($R^2 = 0.630$) between shoot and root biomass (Table 2). Comparison of the specific rhizobia-PSB co-inoculation effect on shoot and root biomass showed a similar trend except for the *R. pusense* (B2) and *R. phaseoli* + *P. polymixa* (compare Figure 3 and 4).

Table 2
Correlation analysis among the nodulation and growth parameters

	NN	SDW	RDW	NDW
NN	1	0.096^{ns}	0.209^{ns}	0.995^{***}
SB		1	0.630^{***}	0.110^{ns}
RB			1	0.226^{ns}
NDW				1

NN- Number of nodules; SDW-Shoot dry weight; RDW-Root dry weight; NDW- Nodule dry weight. Asterisks denotes the significance levels; *** significant at p< 0 0.001; ^{ns} – not significant.

Effect of co-inoculation on the tissue nitrogen (N) and phosphorous (P) concentration. Tissue N concentration was significantly affected by the inoculation of common bean with Rhizobia and PSB. Stimulatory effect was observed in specific Rhizobia-PSB interactions. For example, *R. phaseoli* + *B. aryabhattai* co-inoculation had significantly higher N concentration (2.38%) compared to the single *R. phaseoli* inoculation (2%) (Table 3). For the *R. pusense* co-inoculation, the highest N concentration was achieved when co-inoculated with *P. polymyxa* (2.38%). Similarly, co-inoculation of *R. pusense* (S5) with *P. polymyxa* led to significantly higher N concentration (2.73%) compared to the single *R. pusense* (S5) with 2.06% (Table 2). The highest tissue N concentration was observed in *the R. pusense* (B4) + *B. aryabhattai* co-inoculation (2.74%) that was significantly higher than the application of DAP with 2.48% N (Table 3).

For tissue P concentration, application of DAP resulted in the highest P accumulation of 0.98%. This was followed by co-inoculation of *R. pusense* (S5) + *P. polymyxa* (0.93%) despite the single *R. pusense* (S5) having the least %P concentration of 0.47% (Table 3). Co-inoculation of *R. phaseoli* (B3) with the PSB, *B. megaterium* led to significantly higher %P compared to the other two PSB and the single inoculation (Table 3). Similarly, *R. pusense* (B2) + *B. megaterium* had the highest %P concentration compared to the single inoculation and with the co-inoculation with *B. aryabhattai* and *P. polymyxa* (Table 3). Single inoculation with the PSB *P. polymyxa* led to the highest %P concentration (0.73%) compared to the other single inoculations (Table 3).

Table 3
Effect of co-inoculation on the tissue total nitrogen and total P concentrations in common bean

Treatment	Total N (%)	Total P (%)
	Singe inoculation	
Di-Ammonium phosphate (DAP)	2.48a	0.98a
R. pusense (B4)	2.36b	0.47b
B. megaterium	2.12c	0.59b
R. pusense (B2)	2.09cd	0.54b
R. pusense (S5)	2.06de	0.60b
B. aryabattai	2.03ef	0.48b
Control	2.02f	0.50b
P. polymyxa	2.00f	0.53b
R. phaseoli (B3)	2.00f	0.63b
	Co-inoculation	
R. pusense (B4) + *B. aryabattai*	2.74a	0.46g
R. pusense (S5) + *P. polymyxa*	2.73a	0.93b
R. pusense (B4) + *P. polymyxa*	2.49b	0.51f
Di-Ammonium phosphate (DAP)	2.48b	0.98a
R. phaseoli (B3) + *B. aryabattai*	2.38c	0.55e
R. pusense (B2) + *P. polymyxa*	2.38c	0.50fg
R. pusense (S5) + *B. aryabattai*	2.31d	0.65d
R. phaseoli (B3) + *B. megaterium*	2.10e	0.67d
R. pusense (B2) + *B. aryabattai*	2.09e	0.56e
R. pusense (S5) + *B. megaterium*	2.08e	0.72c
Control	2.02f	0.50fg
R. phaseoli (B3) + *P. polymyxa*	2.02f	0.49fg
R. pusense (B4) + *B. megaterium*	1.91g	0.51f
R. pusense (B2) + *B. megaterium*	1.55h	0.64d

Letters in brackets represent the location of isolation; S- Busia and B- Bungoma; and the numbers are codes given to the isolates. Means followed by different letters are significantly different from each other

at $\alpha \leq 0.05$.

Selected orthogonal contrast. In terms of the number of nodules, there was significant contrast among all the selected orthogonal contrasts. There was a highly significant ($p<0.001$) contrast between the control versus the inoculations (single and co-inoculation) and between the co-inoculation and application of DAP (Table 4). In terms of the shoot dry weight, there were no significant contrasts between the single inoculation versus the co-inoculation; and between the co-inoculation versus DAP application. Compared to the control, there were significant contrasts with the inoculation (both the single and co-inoculation) (Table 4). For the root dry weight, all the selected contrasts were significant except for the single versus the co-inoculation (Table 4). The orthogonal contrast between all the selected contrasts were not significant except for the co-inoculation versus control on the concentration of tissue N (Table 4). There was a significant contrast between the co-inoculation versus the control, inoculation versus control, and the control versus the rest on the tissue P concentration. There was no significant contrast between the single inoculation versus the control; and the co-inoculation versus DAP application (Table 4).

Table 4
Mean square values for selected orthogonal contrasts

Contrast	NN	SDW	RDW	%N	%P
Single vs co-inoculation	1.49^{*}	0.41^{ns}	1.67^{ns}	0.17^{ns}	0.02^{ns}
Co-inoculation vs +NP	9.29^{***}	3.01^{ns}	12.32^{*}	0.01^{ns}	0.004^{ns}
Co-inoculation vs control	9.29^{***}	10.83^{*}	17.04^{**}	0.26^{*}	0.32^{***}
Single vs control	5.88^{***}	8.52^{*}	8.08^{*}	0.08^{ns}	0.02^{ns}
Inoculation vs control	10.45^{***}	14.74^{**}	26.49^{***}	0.11^{ns}	0.11^{**}
Control vs the rest	3.25^{**}	13.56^{**}	20.97^{**}	0.11^{ns}	0.08^{*}

NN- Number of nodules; SDW-Shoot dry weight; RDW-Root dry weight; +NP- inorganic nitrogen and phosphorous as Di-ammonium phosphate (DAP). Asterisks denotes the significance levels; *, **, *** significant at $p< 0.05$, 0.01 and 0.001 respectively.

Discussions

Among the plant growth promoting hormones produced by microorganisms, indole acetic acid is the most common and physiologically active[27]. The results from the present study showed that the tested bacterial strains were able to synthesize IAA in vitro. Six of the bacterial isolates under study exhibited their positive reaction by developing pink colour when reacted with Salkowski's reagent which indicates positive result for IAA production. Further, the results indicated that the microbes differed in their ability to

produce IAA. Earlier studies have shown that IAA production by microbes differed between different species or even within strains of the same species[28,29,30]. From the ten bacterial species and strains tested in this study, the *Rhizobium* produced higher levels of IAA compared to the *Pseudomonas* and *Paenibaccillus* species. This concurs with Tsavkelova *et al*.[31], who reported that the strains of *Rhizobium* are among the most active IAA producers. Similarly Mandal *et al*.[32] reported that Rhizobia were the first group of bacteria, which are attributed to the ability of PGPR to release IAA.

The present study tested the *in vitro* phosphate solubilizing capacity of 17 bacterial isolates comprising of genus *Rhizobium*, *Bacillus*, *Paenibacillus* and *Pseudomonas*. Sixteen of the 17 isolates were able to solubilize the insoluble tricalcium phosphate by the formation of the halo zones. The size of the solubilization varied among the bacteria isolates tested. Similarly, Andrade *et al*.[33] reported a wide variations in solubilization indexes and zones among the tested isolates. The solubilization zone occurs due to the presence of some substances, such as organic acids, that are released by microorganisms into the medium that can form metal complexes with calcium, and thereby solubilize the P[34]. All the Bacillus strains tested in this study were able to solubilize the phosphate confirming what has been reported by previous studies indicating that they are efficient P solubilizers. For instance Andrade *et al*.[33], indicated that isolates of the genus *Bacillus* sp. were the most frequent P solubilizers and classified them as high efficiency solubilizers. *Bacillus megaterium* M510 was found to solubilize both aluminium phosphate and iron phosphate in addition to moderate the solubilization of tri-calcium phosphate[35]. Das *et al*.[36] indicated that Bacillus species isolated from rice rhizosphere solubilized phosphates, which was consistent with the results of our research for the isolate *B. megaterium*.

In addition to their beneficial nitrogen fixing activity with legumes, rhizobia can improve plant P nutrition by mobilizing inorganic and organic P. The present *in vitro* study showed that all the Rhizobium species were able to form solubilization zones with the tricalcium phosphate. Notably, the present results showed that *Rhizobium pusense* recorded significantly highest solubilization efficiency and solubilization index compared to the *Bacillus* strains. Earlier studies have also shown Rhizobium as efficient P solubilizers[37,38,39].

Results from the present study showed that inoculation with *Rhizobium* strains significantly affected the nodulation of common bean. This is in agreement with what has been previously reported by other authors. For instance Bastos[40] showed that inoculation with efficient rhizobial isolates promoted nodulation in common bean. The rhizobia strains used in this study were shown to have high IAA producing efficiency, thus resulting in enhanced nodule number and weights compared to the uninoculated control. Gosh *et al*.[12] reported that the number of effective nodules were increased when inoculated with IAA-producing rhizobia in *Cajanus cajan*. Similarly, Pii *et al*.[41] showed that IAA producing *Rhizobium* strains led to increased nodulation.

Inoculation of the four Rhizobium strains and their co-inoculation with the three PSB generally increased nodules compared with the uninoculated control. Co-inoculation of common bean with rhizobia and PSB

led to an increase in nodule number and nodule dry weight compared to the single rhizobia inoculation. This could be attributed to the multi-strain's ability to effectively nodulate and enhance solubilization of other essential soil minerals such as phosphorus[42]. Similar stimulatory effects on nodulation by co-inoculation of rhizobia and PSB has been reported by other authors[43,44,45].

On the other hand, the present study suggested that the coinoculation of Rhizobium and the PSB might not always increase nodules compared with the individual inoculation with one of the four Rhizobium strains. This results have been reported previously in white clover by[46], who showed that the co-inoculation of CHB1120 and G31 significantly increased nodules of white clover compared with the individual inoculation of CHB1120, but the co-inoculation of CHB1121 and two PGPR significantly decreased nodules in comparison with the individual inoculation of CHB1121. This suggests that the compatibility between these two kinds of microorganisms should be evaluated before application.

Results from this study showed that common bean in pots inoculated with either single *P. polymyxa* or *B. aryabhattai* developed nodules. Other than rhizobia, it was expected that the other bacterial strains will not elicit nodule formation. However, over the years, a vast number of bacteria other than rhizobia have been found in nodules[47,48,49]. A review by[50] highlighted that some of these non-rhizobial nodule endophytes have nif and nod genes and elicits nitrogen fixing nodules on nodules just like the rhizobia. A study by[51] reported that the diazotrophic bacteria used in their study were found to have the nitrogen fixing genes and nodulated and enhanced nodulation in chickpea plants under greenhouse conditions. There is therefore a need to test more of the non-rhizobial nodule endophytes for their nitrogen fixing ability and presence of nod genes to further understand their mechanisms of plant growth promotion.

Results from the present study showed that inoculation of common bean with rhizobia generally increased the shoot biomass and root dry weight compared to the control. The improved growth of plants subjected to *Rhizobium* inoculation is effectively attributed to its positive effect due to the symbiotic relationship between the rhizobia and the common bean[52]. Inoculation of seeds by phosphate solubilizing microorganisms is known to improve solubilization of insoluble phosphorus, which can therefore increase plant growth by enhancing the symbiotic efficiency of the common bean[53,54].

In the present study, combined inoculation of the common bean with the rhizobia and PSB resulted in higher shoot and root dry weights compared to the single rhizobia inoculation. This can be attributed to better establishment of *Rhizobium*-legume symbiosis due to more secretion of plant growth promoting hormones, and improved nutrient availability especially P[53],[55]. Similarly, Kumar *et al*.[56] reported a growth enhancement of common bean by application of *Bacillus* and their combination with *Rhizobium*. Khalifa and Almalki[57] showed that co-inoculation of phosphate-solubilizing *B. megaterium* and *Sinorhizobium meliloti* had a positive effect on the growth of common bean. Co-inoculation of *Rhizobium* MAP7 along with *Brevibacillus* MAP4 significantly increase the shoot dry weight compared to the treatment with *Rhizobium* MAP7 alone[58].

Similar to the results on nodulation, some of the co-inoculation did not lead to improved growth as compared to the single inoculation. For instance, the shoot biomass of common bean co-inoculated with *R. pusense* and *B. megaterium* was significantly lower compared to their individual inoculations. This suggest that the two strains were not compatible. Other studies have shown the lack of positive effects of co-inoculation in respect to single rhizobium inoculation[59,60]. Therefore, compatibility studies should be done before coming up with the right microbial consortia for formulations of biofertilizers to ensure enhanced crop growth and maximum benefits from the plant growth promotion of the introduced microorganisms.

Results from this study revealed that inoculation of the common bean with Rhizobia strains increased the shoot N content compared to the uninoculated control. This could be attributed to the formation of nodules by the Rhizobium that stimulated biological nitrogen fixation by the crop. Similarly, de Souza *et al*.[61] reported increased shoot N concentrations by Rhizobium when common beans were inoculated with *R. leguminosarum* strains. Similarly, shoot P concentration was increased as a results of inoculation with the PSB and Rhizobia. Earlier study by Chen *et al*.[62] showed that the use of phosphate solubilising bacteria as inoculants increases the P uptake by plants. These findings are similar to the study by Neila *et al*.[63] who observed that native rhizobia increase shoot phosphorus in bean. The present study showed a stimulatory effect in specific Rhizobia-PSB interactions in the total N and P concentration in the plant tissues. For example, *R. phaseoli* + *B. aryabhattai* co-inoculation had significantly higher N concentration compared to the single *R. phaseoli* inoculation. The enhancement in total N and P content of shoot in present study might be due to increase of nitrogen and phosphorous acquisition due to altering root structure and nodule formation in the crop[64]. Co-inoculation of common bean with *Rhizobium* and *Bacillus* strains was shown to improve nitrogen and phosphorus content compared to single *Rhizobium* inoculation[65]. A study by Nimnoi *et al*.[66] showed that the total N and P content of shoot was enhanced by co-inoculation of *Nocardia alba* strain S4301 with *Bradyrhizobium japonicum* USDA110 as compared to single inoculation of *Nocardia alba* strain S4301 and un-inoculated control treatments in soybean. The increased N content from the co-inoculation of Bacillus and Rhizobia strains could also be attributed to the nitrogen fixing ability of the Bacillus. A study on nitrogen fixing potential of diverse species of Bacillus has reported the presence of nifH gene and hence the capability to fix atmospheric nitrogen[67,68].

Native soil bacteria possesses ability to produce indole acetic acid growth hormones and to solubilize insoluble phosphate. Combined inoculation of rhizobia and PSB promoted the growth of common bean. Therefore, the phosphate solubilizing strains and the nitrogen fixing bacterial strains have great potential in being formulated and used as biofertilizers that can be tested under varying field conditions. This study highlights the importance of the use of phosphate solubilizing and IAA producer microorganisms as biofertilizers to enhance common bean growth.

Materials And Methods

Bacterial isolates description. Seven native plant growth promoting rhizobacteria (PGPR) strains were obtained from Soil Microbial Ecology Laboratory, Egerton University from the isolation work done earlier by Korir *et al*.[49]. The PGPR were isolated from root nodules of common beans and belongs to the genus Bacillus and Paenibacillus. The native rhizobia strains isolated from bean growing areas of Busia and Bungoma Counties. The isolated strains were characterized and molecularly identified at the University of Jena, Germany. The strains were coded as S1-S5 for the different isolates from Busia and B1-B5 for the isolates from Bungoma Counties respectively.

In vitro **screening of bacterial isolates for their plant growth promoting properties.**

Indole-3 acetic acid (IAA) Production. Indole-3 acetic acid (IAA) production was analysed using modified colorimetric method as described by[69]. Ten bacterial isolates were grown in yeast extract mannitol broth supplemented with tryptophan (0.1%) and incubated at 28°C for 2 to 3 days in a shaking incubator. Then, 3 ml of the log phase broth culture (10^9 cfu ml^{-1}) was centrifuged at 7826 x g for 15 minutes and 2 ml of the cell-free supernatant was transferred to a dry clean tube (15 ml capacity) to which 1 ml of 10 mM orthophosphoric acid and 4 ml of Salkowsky's reagent (1 ml of 0.5 M $FeCl_3$ in 49 ml of 35% Perchloric acid) was added and incubated in the dark at ambient temperature (25°C) for 25 minutes. The pink colour development was compared to the blank (sterile LB broth with 0.1% of tryptophan and reagents) at wavelength of 530 ηm and the absorbance was used as index of IAA production. Absorbance values of greater than 0.85 was considered high while those lesser than 0.85 considered low[69].

In vitro **phosphate solubilization.** The solubilization capacity of previously isolated Rhizobia and Bacillus strains was checked on Pikovskaya's medium ([70]Nautiyal, 1999). The growth and solubilization diameter was determined after incubation at 28 ± 2°C for seven days. The size of the halo of solubilization was obtained by subtracting the value of the colony diameter from the total halo solubilization diameter. On the basis of diameter of clearing halo zones, solubilization efficiency (SE) and solubilization index (SI) were calculated using the following formulae[71]:

$$\mathrm{SE} = \text{Solubilization diameter} \div \text{Growth diameter} \times 100$$

$$\mathrm{SI} = (\text{Colony diameter} + \text{Halozone diameter}) \div \text{Colony diameter}$$

The solubilization capacity was assessed based on the scale formulated by Silva-Filho and Vidor[72], where SI values under 1.0 were classified as very low solubilizers, values from 1.0 to 2.0 were classified as low solubilizers, values from 2.0 to 3.0 were classified as medium solubilizers, and values above 3.0 were classified as high solubilizers.

Greenhouse experiment. The plant growth promotion of the legume and stimulation of nodulation was tested for strains that showed capacity for phosphate solubilization and IAA production. Four Rhizobia and three Bacillus strains that exhibited high IAA production absorbance (>0.85) and phosphate solubilization efficiency (> 2.0) respectively were used in the greenhouse study.

Inoculum preparation. Rhizobium inoculum was prepared in yeast extract mannitol (YEM) medium and PSB in Pikovskaya's medium[70]. The bacterial cultures were inoculated in 500 mL conical flasks containing 150 ml of either the YEM or Pikovskaya's medium and incubated at 28 ±2^{o}C under shaking for three days to give an optical density of 0.5.

Treatment structure in the greenhouse. The experiment was conducted following guidelines by Figueiredo *et al*.[73]. Microbe-free vermiculite was obtained by autoclaving for 30 min at 121°C and 101 KPa, once a day for three consecutive days. Pots of 5.3 L capacity (15.0 cm inner diameter and 30 cm length), were filled with 3 kg of vermiculite. Two common bean seeds were planted per pot and thinned to one seedling per pot one week later, after which it was inoculated as per treatment i.e. un-inoculated, un-inoculated + inorganic NP source, PSB, *Rhizobium* and combination of PSB and *Rhizobium* (1:1). For single inoculation, 1ml of broth culture containing a *Rhizobium* or PSB (10^{9}cfu ml^{-1}) was inoculated per plant. For the co-inoculation, 0.5 ml of YEM broth containing a *Rhizobium* (10^{9}cfu ml^{-1}) plus 0.5 ml of nutrient broth containing the phosophobacteria (10^{8}cfu ml^{-1}) was applied per plant. Ten milliliters of nitrogen-free nutrient solution (Broughton and Dilworth, 1970) was applied to the pots once a week until flowering started. The treatment structure is shown in Table 4. The experiment was laid out in completely randomized design (CRD) with three replicates. The pots were watered regularly to maintain the substrate at field capacity.

Table 5
Treatment structure for the greenhouse co-inoculation study

Treatment ID	Description
TRT-1	*R. pusense* (B2)
TRT-2	*R. phaseoli* (B3)
TRT-3	*R. pusense* (B4)
TRT-4	*R. pusense* (S5)
TRT-5	*P. polymyxa*
TRT-6	*B. aryabhattai*
TRT-7	*B. megaterium*
TRT-8	*R. pusense* (B2) + *P. polymyxa*
TRT-9	*R. pusense* (B2) + *B. aryabhattai*
TRT-10	*R. pusense* (B2) + *B. megaterium*
TRT-11	*R. phaseoli* (B3) + *P. polymyxa*
TRT-12	*R. phaseoli* (B3) + *B. aryabhattai*
TRT-13	*R. phaseoli* (B3) + *B. megaterium*
TRT-14	*R. pusense* (B4) + *P. polymyxa*
TRT-15	*R. pusense* (B4) + *B. aryabhattai*
TRT-16	*R. pusense* (B4) + *B. megaterium*
TRT-17	*R. pusense* (S5) + *P. polymyxa*
TRT-18	*R. pusense* (S5) + *B. aryabhattai*
TRT-19	*R. pusense* (S5) + *B. megaterium*
TRT-20	Uninoculated + inorganic NP source
TRT-21	Uninoculated control

Data collection. At 50% flowering, the plants were uprooted to record number of nodules (NN), nodule dry weight (NDW), shoot dry weight (SDW) and root dry weight (RDW) after drying at 65°C for 72 hours to a constant weight. The shoots were analysed for nitrogen and phosphorous accumulation.

Data analysis. Data on *in vitro* IAA production and phosphate solubilization (SE and SI) was subjected to analysis of variance (ANOVA) and the means separated using Tukey least significant difference (α =

0.05). For the greenhouse experiment, data was first tested for normal distribution and the count data on nodule number was log transformed (Log_{10} x+1) before analysis so as not to violate the assumptions of ANOVA (Payton *et al*., 2006). To determine the effects due to inoculation, analysis of variance at $p < 0.05$ was done and means separated using the least significance difference (Tukey's test at $\alpha = 0.05$). Data was analysed using SAS Statistical Package Version 9.3[74].

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Photos

Photos 1, 2 and 3 are available in the Supplemental Files section

Figures

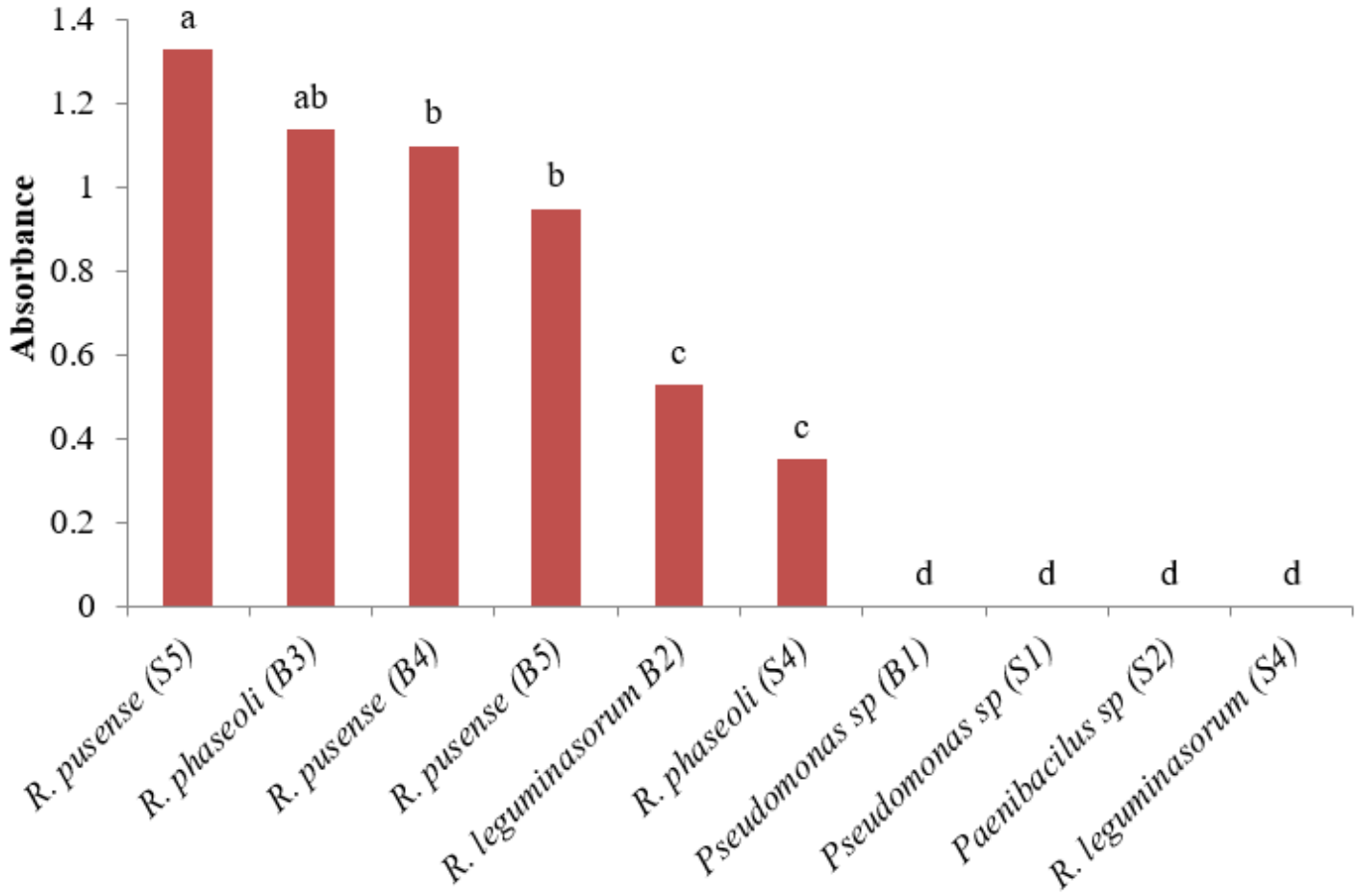

Figure 1

Absorbance (at 530 nm) values for different Rhizobium species using colorimetric method. Letters in brackets represent the location of isolation; S- Busia and B- Bungoma; and the numbers are codes given to the isolates. Means followed by different letters are significantly different from each other at $\alpha \leq 0.05$.

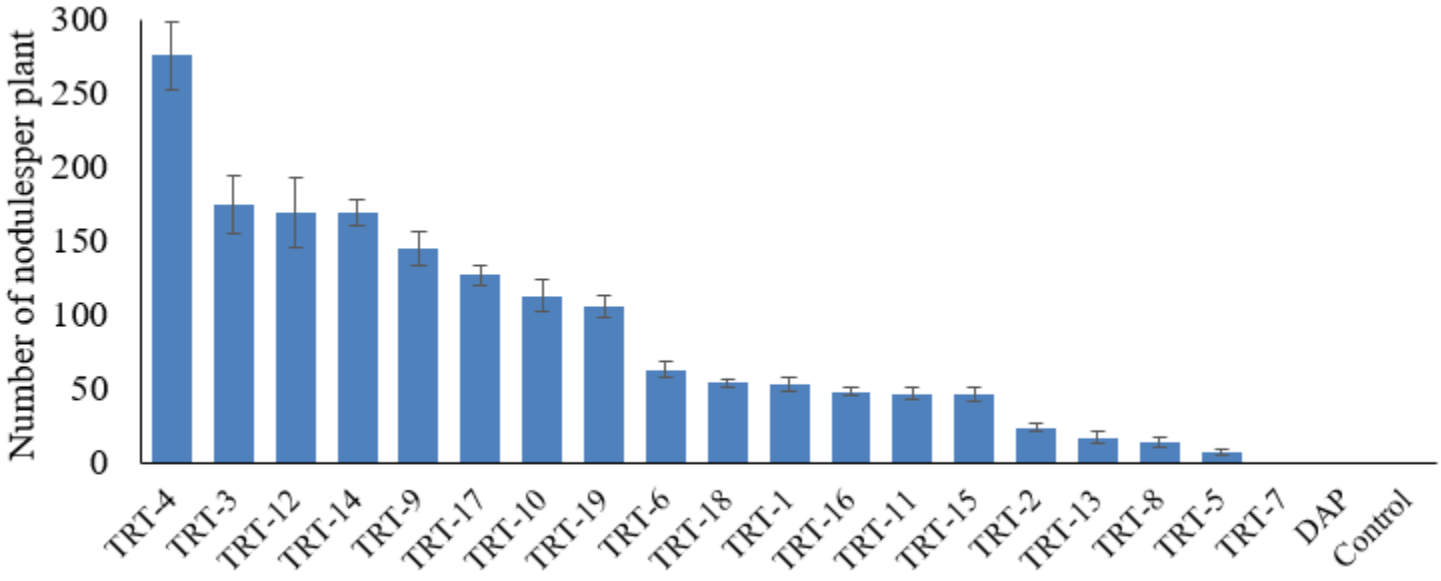

Figure 2

Effect of co-inoculation of common bean on number of nodules. Error bars represent the standard error of the means.

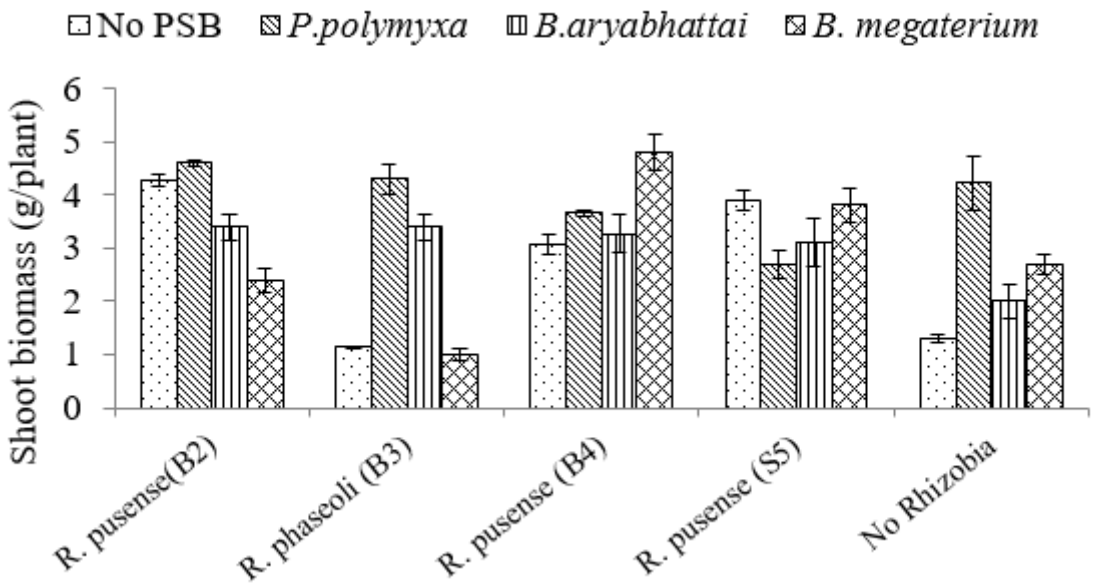

Figure 3

Effect of co-inoculation of common bean on the shoot biomass. Letters in brackets represent the location of isolation; S- Busia and B- Bungoma; and the numbers are codes given to the isolates. Error bars represent the standard error of the means

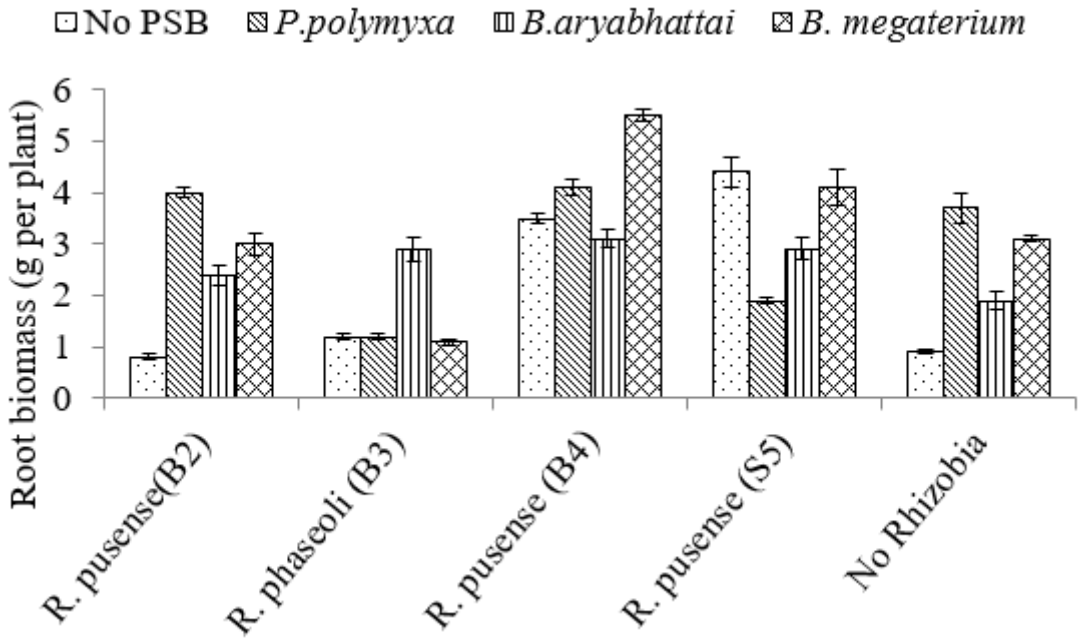

Figure 4

Effect of co-inoculation of common bean on the root biomass. Letters in brackets represent the location of isolation; S- Busia and B- Bungoma; and the numbers are codes given to the isolates. Error bars represent the standard error of the means

Supplementary Files

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- Photo1.png
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