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Characterization of phosphate solubilizing rhizobacteria isolated from lentil growing areas of Ethiopia

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Lentil (*Lens culnaris* Medik.) is an invaluable source of dietary protein for the majority of Ethiopian population. The low availability of phosphorus is one of the major soil related stresses that limit the production of this crop. This study was aimed at isolation and characterization of phosphate solubilizing bacteria from lentil growing areas of Ethiopia and evaluating their effects on growth of this crop under greenhouse conditions. In total, 41 phosphate solubilizing bacterial strains were isolated from lentil rhizosphere and characterized using biochemical tests and 16S rDNA sequence analysis. The results showed the presence of genera *Acinetobacter, Agrobacterium, Bacillus, Burkholderia, Chryseomonas, Enterobacter, Pseudomonas, Ralstonia* and *Sphingomonas.* Phosphate solubilzing efficiencies of these isolates were evaluated in liquid medium using different insoluble P-sources and the result indicated that they released considerable amount of phosphates from tricalcium phosphate, rock phosphate and bone meal. Besides, some of these isolates exhibited multiple plant growth promoting characteristics such as indole acetic acid production and growth inhibition of *Fusarium oxysporum* under *in vitro* conditions. The effects of inoculation of *Enterobacter kobei* (PSBL5), *Bacillus subtilis* (PSBL21), *Enterobacter* species (PSBL26), *Bacillus* species (PSBL31) and *Pseudomonas fluorescence* (PSBL37) on lentil growth, nodulation, N and P content of shoot were evaluated under a greenhouse conditions and the results showed that these features were significantly increased over uninoculated control plants. Based on the results of this study, we conclude that lentil rhizosphere soil from producing areas of Ethiopia harbor diverse phosphate solubilizing bacterial strains with excellent phosphate solubilization and plant growth promotion ability. Thus, most of the promising strains should be tested in the field conditions under different agroecology of the country.

Key words: Rhizosphere soil, PGPR, rock phosphate, bone meal.

INTRODUCTION

Lentil (*Lens culnaris* Medik.) is an important pulse crop grown widely in Ethiopia over an area of 105,956 ha with an annual production of 123,777 tones (CSA, 2010). It is an invaluable source of dietary protein for the vast majority of the Ethiopian population. In addition, lentil restores and maintains soil fertility through its nitrogen-fixing symbiosis. Thereby, it ensures the sustainability of the crop producing system. Thus, it has been grown in

rotation with other major cereals in traditional low input system. However, the yields of lentil has been 1.17 tones ha^{-1} (CSA, 2010) that could be low compared to its potential (Hailemariam and Tsige, 2006; Jida and Assefa, 2011) due to soil infertility stresses which prevail under Ethiopian soil and climatic conditions (Ahmed and Ayalew, 2006; Fikre and Bejiga, 2006).

Phosphorus (P) is one of the essential macronutrients that limit plant growth in tropical soils (Vessey, 2003; Khan et al., 2007). Most soils in tropical and subtropical areas are predominantly acidic and have strong P sorption (fixation) ability (Khan et al., 2007). This leads to wide spread available P deficiency which is particularly the case for the large parts of Ethiopian soils (Beyene, 1982; Mamo et al., 1988). These low levels of root available P are mainly due to high reactivity of soluble P with Fe and Al oxides in acidic soils (Gyaneshwar et al., 2002). Hence, this has been one of the stresses that constrain the production of lentil and other pulse crops in the country.

On the other hand, the application of chemical fertilizer is highly limited by its cost intensity. Hence, its use for pulse crops production such as lentil is very low in Ethiopia. Moreover, up to 75% of the P-fertilizers added to the soil could be converted into insoluble forms by reacting with free Ca^{2+} or Fe^{3+} and Al^{3+} ions in high and low pH soils, respectively (Goldstein, 1986). These cause the urgent need for economically feasible and ecologically acceptable P-fertilizer sources. Thus, using microbial biofertilizers for improving the availability of P in the vicinity of plant roots represents an alternative to the use of chemical fertilizers (Naik et al., 2008). It has been well documented that phosphate solubilizing bacteria (PSB) are able to convert insoluble phosphate to soluble primary and secondary orthophosphate ions (Pal, 1998; Peix et al., 2001a; Chen et al., 2006; Vyas and Gulati, 2009).

Rhizopshere bacteria that solubilize P and enhance plant growth are referred to as plant growth promoting rhizobacteria (Glick, 1995). Compared to the bulk soil, the proportion of PSB is high in the rhizosphere and such bacteria are metabolically more active (Vazquez et al., 2000). Earlier studies indicated that different species of PSB were able to solubilize naturally occurring insoluble inorganic P sources such as, rock phosphate and old bone (Ivanova et al., 2006; Keneni et al., 2010). Besides increased P uptake, the production of phytohormones such as indoleacetic acid (IAA) by PSB could also play an important role in plant growth promotion (Naik et al., 2008; Shahab et al. 2009). In addition, PSB could also enhance plant growth by suppressing different soil borne fungal pathogens of crops (Peix et al., 2001b).

Even though the majority of Ethiopian soils are characterized by low available P (Beyene, 1982; Mamo et al., 1988), most of the hitherto studies have focused on the rate of P-fertilizer application. Consequently, there is little information on native PSB isolates recovered from the rhizosphere of lentil grown in Ethiopian soils. However, an array of bacteria with phosphate solubilizing potential have been found associated and within the rhizosphere of agriculturally important crops (Chen et al., 2006; Vyas and Gulati, 2009; Muleta et al., 2013). Moreover, several studies indicated the potential biotechnological application of such native bacteria in promotion of plant growth (Peix et al., 2001ab; Shahab et al., 2009). Therefore, the objectives of this study were isolation and characterization of PSB from lentil growing areas of Ethiopia and evaluation of their effects on the growth of lentil under greenhouse conditions.

MATERIALS AND METHODS

Study sites and soil samples collection.

Soil samples were collected from lentil grown fields in Amhara, Oromia and Tirgay regional states of Ethiopia in October, 2009. These regions represent the major lentil producing areas of the country. The areas were distributed in Central and Northern parts of the country with an altitude range of about 1799-3101 m above sea level (masl) and soil pH values ranging from moderately acidic (5.58) to slightly alkaline (7.18) (Table 1). Thirty soil samples were excavated from the 15-20 cm depth and collected in sterile plastic bags. The samples were stored at 4°C in a refrigerator until the time of analysis. Further analysis of the soil samples was performed in the Laboratory of the Stream of Applied Microbiology, Addis Ababa University.

Isolation of phosphate solubilizing rhizobacteria

Each soil sample was thoroughly mixed, air-dried in a greenhouse and then filled into surface sterilized (96% ethanol) plastic pots (4 kg capacity). Lentil seeds (*Teshale* cultivar) were selected and surface sterilized with 96% ethanol and 3% sodium hypochlorite solutions and rinsed five times with sterilized distilled water. Surface sterilized seeds were allowed to germinate on sterile water agar (1%) surface for three days at 25°C and five pre-germinated seeds were planted on each pot, which were thinned down to three seedlings 5 days after emergence (DAE). All pots were situated in a greenhouse over the table in randomized block design and watered to a field capacity every three days for 45 days after planting (DAP). Fourty-five DAP lentil seedlings with good growth and healthy appearance were selected from each pot and uprooted carefully with their rhizosphere soil. Plant roots with adhered rhizosphere soil were carefully transferred into sterile Petri dishes and 10 g of it was transferred to flasks (250 ml) containing sterilized 90 ml saline solution (0.85% w/v NaCl). The flasks were incubated on a gyratory shaker at 120 revolutions per minute (rpm) at room temperature for 30 min and then serially diluted. Aliquots of the appropriate dilution were spread plated on Pikovskaya medium (Pikovskaya, 1948). The plates were incubated for 5 days at 30°C.

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Table 1. Summary of the isolation sites and abundance of PSB in lentil rhizosphere soils.

Name of isolation site	pH of isolation soil	Number of PSB isolates obtained	Designation of PSB isolates
Adanaba (T)	7.03	1	PSBL29
Ambo (O)	6.75	1	PSBL49
Asgori (O)	6.20	2	PSBL05,12
Asketema (A)	7.04	1	PSBL100
Chatawa (A)	5.12	1	PSBL116
Chole I (O)	5.58	1	PSBL54
Chole II (O)	6.05	1	PSBL52
Debra Selam (A)	6.00	1	PSBL57
Dagam (O)	6.36	1	PSBL94
Fala'a (T)	7.00	1	PSBL28
Fiche (O)	6.83	1	PSBL33
Fogera meda (A)	6.00	2	PSBL95,96
Geshana (A)	5.80	1	PSBL21
Ginchi (O)	6.60	1	PSBL120
Goha Tsion (O)	6.3	1	PSBL39
Gurura (O)	7.01	3	PSBL51,53,113
Keyit (A)	6.38	2	PSBL90,92
Korem (T)	6.93	1	PSBL28
Makesagnit (A)	6.39	1	PSBL97
Obbi (O)	6.61	1	PSBL31
Sandafa (O)	6.00	1	PSBL58
Sheno I (O)	5.58	2	PSBL26, 45
Sheno II (O)	5.58	1	PSBL20
Tefki (O)	6.55	6	PSBL02, 04,14,19,37,50
Teji (O)	6.70	1	PSBL101
Tikana (A)	7.18	1	PSBL93
Wajel (A)	6.12	1	PSBL55
Woliso (O)	5.58	1	PSBL13
Wuchale (A)	6.58	1	PSBL99
Yetinora (O)	6.21	1	PSBL112

Numbers in the same column followed by the same superscript letter do not differ significantly ($p>0.05$) by DMRT; ND: not determined; A: Amahara regional state; O: Oromia regional state; T: Tigray regional state

Colonies surrounded with a large halo zone and different morphologies were selected and transferred to Pikovskaya (PK) broth separately. The test tubes were incubated at 30°C for 48 h. The isolates were purified by re-streaking on PK agar plates and maintained on PK agar slants for short term storage at 4°C and in 50% (v/v) glycerol for long term storage at -20°C. Isolates were designated as PSBL (Phosphate Solubilizing Bacteria from Lentil) 01- PSBL120 (Table 1).

Characterization and identification of the PSB Isolates

Biochemical characterization and identification by the API kits system

All isolates were characterized using the following tests: Gram reaction using Gregorson's KOH (Gregorson, 1978), oxidase (Kovacs, 1956), Oxidative/Fermentative (O/F) (Huge and Leifson, 1953). Based on the result of these tests isolates were assigned to different groups and identified to genus and/or species level using the API identification system assisted by API Plus computer software (bioMèrieux-SA, France) following manufacturer instructtions. Gram positive rods were further identified to species level using API 50CH test kits. Gram negative rods and oxidase negative bacteria with fermentative reaction in the O/F test were identified using the API 20E test kits. Gram negative rods with oxidative reaction in O/F test were identified by using API 20NE test kits. Moreover, isolates were selected for 16S rDNA sequence analysis based on their phosphate solubilization activity, abundance and the result of API identification system.

Identification by 16S rDNA sequencing

Genomic DNA extraction, PCR amplification of 16S rDNA and sequencing: All isolates were streaked on LB agar media and incubated for 24 h at 30°C. A single colony of each isolate was suspended in 30 µl sterile H_2O. The DNA from Gram negative

bacteria was extracted using thermal denaturation method (Mohran et al., 1998). All tubes that contain colony suspensions were incubated at 95°C for 10 min. The DNA from Gram positive isolates was extracted by heat thawing at 65°C for 3 minutes followed by freezing at -70°C for 3 min; the cycles were repeated three times. All the tubes were centrifuged at 13,000 *g* for 2 min and stored at 4°C.

Two microliters of extracted DNA was used as a template for PCR amplification using a thermal cycler. In addition to the DNA, the PCR reaction consisted of 0.4 µl 10 mM dNTP, 2.5 µl 10x PCR(Taq) buffer, 2.5 µl 25 mM $MgCl_2$, 0.2 µl (5 units/µl) of Taq polymerase, 1 µl (10 mM) of forward primer 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1 µl (10 mM) of reverse primer1492r (5'-GGTTACCTTGTTACGACTT-3'). The PCR program included a denaturation step of 5 min at 95°C, followed by 30 cycles of 95°C for 30 s, 50°C for 45 s, 72°C for 1.30 min, and an extension step of 10 min at 72°C. Amplified PCR products (1500 bp) were resolved on 1.0% agarose gel and stained with ethidium bromide (1 µg/ml). All amplified PCR products were sequenced with forward primer 27f by using Sanger sequencer (ABI-3730XL) at the Institute of Clinical and Molecular Biology, University of Kiel. The DNA sequences 850 bp were edited using Bioedit (www.mbio.ncsu.edu/BioEdit/) and analyzed by BLAST search (www.ncbi.nlm.nih.gov).

Phylogenetic analyses: A phylogenetic tree was constructed by using the neighbor joining method from distance matrices (Saitou and Nei, 1987). Bootstrap values (percentage of 500 replicates) are shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007).

Growth media and bacterial inoculum preparation

Bacterial isolates were grown in PK broth on a gyratory shaker at room temperature and 120 rpm for 72 h. The suspension was centrifuged in sterile plastic tubes (10 ml) at 5000 g for 10 min. The pellets were re-suspended in normal saline solution to give a final concentration of 10^8 cells ml^{-1} using the viable plate count method and optical density measurement at 600 nm. All bacterial inoculums were prepared like this unless otherwise stated. The pH of all growth media was adjusted to 7.0 if not stated.

Determination of phosphate solubilization efficiency of the PSB isolates

Phosphate solubilization efficiency of the PSB isolates was evaluated both qualitatively and quantitatively. PK agar plates were spot inoculated with 20 µl of culture suspension of each isolate. After incubation at 30°C for 5 days formation of a clear zone around the spot was checked and their solubilization index (SI) was calculated as described in Muleta et al. (2013).

Based on their phosphate solubilizing activities and abundance of a particular group, isolates were selected and used for quantitative estimations of solubilization efficiency in PK broth. Hundred ml of PK liquid medium without phosphorus sources was dispensed in a flask (250 ml) and supplemented with the following insoluble phosphate sources which contained equivalent amounts of P: $Ca_3(PO_4)_2$ (TCP) (500 mg); Egyptian rock phosphate (RP) (600 mg) and bone meal (BM) (400 mg) were added separately as a phosphorus source. All flasks were autoclaved and inoculated with 100 µl culture suspension of each isolate separately. The flasks were incubated at room temperature on a gyratory shaker at 120 rpm for 12 days. Five ml of samples were withdrawn from each treatment on day 4, 8, and 12 and analyzed for phosphorus solubilized and pH changes. The sample was centrifuged at 15,000 g for 15 min and the clear supernatant was analyzed for P content following the phospho-molybdate method (Murphy and Riley, 1962).

Screening for *in vitro* antagonistic activity against *Fusarium oxysporum*

The *in vitro Fusarium oxysporumf.*sp. *lentis* (FOL) growth inhibition of the isolates was tested using the dual culture technique (Landa et al., 1997). Twenty microliters of culture suspension of each isolate was equidistantly spotted on the margins of nutrient agar (NA) plates amended with glucose (1.0%) and incubated at 30°C for 24 h. A 4-5 mm diameter agar disc from potato dextrose agar (PDA) cultures of the fungal pathogens was placed at the centre of the NA plate inoculated with the bacterial isolate and incubated at 30°C for 5-7 days. The radii of the fungal colonies towards and away from the bacterial colonies were measured and the percentage of mycelia growth inhibition was calculated according to Idris et al. (2008).

Assay for Indole-3-acetic acid (IAA) production

Indole-3-acetic acid production was detected by modified method as described by Brick et al. (1991). Quantitative analysis of IAA was performed using the method of Loper and Scroth (1986). Cultures of the isolates were grown at 30°C in LB broth medium supplemented with 5 mM L-tryptophan for 48 h. The cultures were centrifuged (10,000 *g* for 15 min) and 2 ml supernatant was transferred to test tubes to which 100 µl of O-phosphoric acid and 4 ml of Salkowaski reagent (1 ml of 0.5M $FeCl_3$ in 50 ml 35% $HClO_4$) were added. The mixtures were incubated at room temperature for 25 min. Quantification of the IAA was carried out using spectrometric analysis at 530 nm.

Effects of inoculation of PSB isolates on the growth of lentil under greenhouse conditions

Bacterial isolates that showed high phosphate solubilization efficiency in PK broth were selected for their *in vivo* growth promotion evaluation under greenhouse conditions. Lentil seeds were surface sterilized, germinated and transplanted as described before. Prior to seedling transplantation, germinated seedlings were flooded with PSB culture grown in PK broth adjusted to 10^8 cells $seed^{-1}$ for one hour on separate sterilized Petri plates. Five inoculated seedlings were transferred to each pot containing 3 kg vertisol collected from Dima Guranda, around Sebeta. This soil has the following physicochemical characteristics: pH; 6.0, total N; 0.025%, organic carbon; 0.975, total P; 1667 ppm, available P; 2.99 ppm, available K; 25.33 ppm, Cation Exchange Capacity; 50 Cmol/Kg and loamy texture.

The seedlings were thinned down to three 5 DAE. The pots were irrigated with distilled sterilized water every three days. Uninoculated P-fertilized (20 mg Kg^{-1} as KH_2PO_4) pots were included as positive controls (PC) and uninoculated non P-fertilized pots as negative controls (NC). At sixty DAP all seedlings were carefully uprooted and washed under gently flowing tap water. The numbers of nodules found on the roots were recorded, and shoot dry weight was measured after drying at 70°C for 48 h. Total N of the shoot was determined using Kjeldahl methodas described in Sertsu and Bekele (2000). The P concentration of the shoot was determined by phospho-molybdate method (Murphy and Riley, 1962) after ashing 0.5 g of oven dried and ground shoot dry matter.

Data analysis

Statistical analysis of the data was carried out using analysis of variance (ANOVA) and the treatment means were compared relative to control following Duncan's multiple range test (DMRT) (Duncan, 1955). The relation between different parameters was evaluated by using the Pearson correlation coefficient using SPSS v.15 (SPSS Inc.2000).

RESULTS AND DISCUSSION

Isolation and Identification of PSB Isolates

Thirty soil samples were collected from different lentil producing areas found in central and northern parts of the country which represent major producing areas (Table 1). A total of 41 PSB isolates with higher phosphate solubilization activities (SI ≥1.34) were selected from the soil samples for further analysis (Table 1). The biochemical analysis revealed the presence of a wide array of Gram negative and Gram positive PSB in the rhizosphere of lentil. Gram negative rhizobacteria dominated the system accounting for 90% of isolates. Previous observation showed that the rhizosphere of many agriculturally important plants favor more Gram negative rhizobacteria than the Gram positives (Muleta et al., 2009). The results revealed the presence of 7 major genera: *Pseudomonas* (47.5%), *Burkholderia* (12.5%), *Bacillus* (10%), *Enterobacter* (10%), *Chrysomonas* (5%), *Sphingomonas* (2.5%) and *Agrobacterium* (2.5%). Members of the genus *Pseudomonas* were dominated by *Pseudomonas fluorescens* while all isolates of the genus *Burkholderia* were identified as *B. cepacia*. However, 12.5% them were left unidentified or showed low identification (<90%). This might be due to the limitations of using biochemical characteristics to identify the environmental isolates.

The partial 16S rDNA sequence analysis was also employed to identify the isolates to the genus and species level. The result showed the presence of genera such as, *Pseudomonas, Bacillus, Enterobacter, Acinetobacter, Ralstonia, Burkholderia and Agrobacterium.* Gram negative strains exhibited more diverse species compared to the Gram positive isolates. There were some disagreements between API kit identification and 16S rDNA gene sequence analysis which could be due to several reasons such as inoculum size and culture conditions. Moreover, genera *Ralstonia* and *Acientobacter* were identified only by sequences analysis indicating that this method is more powerful in identifying the environmental isolates.

Of the Gram negative strains three of them showed the highest similarity to different species of genus *Enterobacter*. The sequence analysis revealed that PSBL04 and PSBL92 were highly similar (100% sequence similarity) to *Enterobacter ludwigii* (FG859683), phosphate solubilizing bacteria isolated from tea rhizosphere and *Enterobacter cloacae* (GQ421477), antagonists of *Aspergillus flavus* from fermented beans, respectively while PSBL05 showed 99% identity similarity with *Enterobacter kobei* (NR 028993) (Table 2).

Two strains showed the closest match with species of *Pseudomonas*. PSBL57 showed 100% sequence similarity to *Pseudomonas costantinii* (EF111123) bacteria isolated from river while PSBL58 showed 99% sequence similarity with *Pseudomonas corrugate* (HQ242748) phosphate solubilizing bacteria from P-rich soil. According to API test kits identification of members of the *Pseudomonas* were dominated by *P. fluorescens* which was also confirmed by 16S rDNA sequences analysis since PSBL57 and PSBL58 showed good similarity with fluorescent pseudomonad species (*P. fluorescens (100%)* and *P. putida* (99%)).

Strain PSBL45 showed 100% sequence similarity with *Burkholderia cepacia* (JN208904) N-fixing symbionts of common bean and *P. fluorescens*, (HE610859) a drought resistance-promoting bacterium. Strain PSBL19 showed the highest (99%) sequence similarity to *Agrobacterium tumefaciens* (AB 681363) isolated from soil. PSBL39 showed the highest (99%) similarity to *Acinetobacter calcoaceticus* (JQ579640) phosphate solubilizing bacteria in paddy fields and endophytic bacteria in peanut, respectively.

Three members of Gram positive strains showed closest similarity to different species of genus *Bacillus*. PSBL12 showed 99% similarity with *Bacillus tequilensis* (JF 411313), bacteria isolated from disused river while PSBL21 and PSBL49 showed 99 and 100% sequence similarity, respectively with *Bacillus subtilis* (JN230358) bacterial endophytes from *Nicotiana attenuta*. The partial sequences of 16S rDNA genes of the isolates were multiple aligned with the nearest neighbors and other relevant bacterial sequences, and their identity and evolutionary relation was assessed by constructing phylogenetic tree (Figure 1). The phylogenetic tree showed the clustering of PSBL isolates with their respective genus with good bootstrap support values.

Phosphate solubilizing efficiency of the isolates on agar plates

The SI of each PSB isolate is presented in Table 2. Phosphate solubilizing activities of these isolates were assessed qualitatively on PK agar medium using SI as measure of their efficiency. The result showed a statistically significant variation among the isolates. The SI of the isolates ranged from 1.34 to 2.25 which were noted for isolate *Pseudomonas* sp. (PSBL101) and *A. tumefaciens* (PSBL19), respectively. This result indicates that *A. tumefaciens* (PSBL19) was the most efficient in phosphate solubilization on PK plates. Though it is not statistically significant, the result showed that there was positive correlation (r=0.5) between SI and the amount of soluble P released by the isolates in PK broth. Consequently, we should not rely only on SI to select efficient PSB isolates.

Table 2. Solubilization index (SI), identification and PGP characteristics of PSB isolated from lentil rhizosphere.

Isolate	Gram reaction	Solubilization Index (SI)±SD	O/F test	Oxidase	API identification	16S rDNA sequences identification
PSBL02	-	1.77±0.06^{e-h}	F	+	*Aeromonas hydrophila*	ND
PSBL04	-	1.82±0.08^{e-h}	F	-	*Pseudomonas fluorescens*	*Enterobacter ludwigii* (JX979114)
PSBL05	-	1.95±0.22^{c-e}	F	-	UI	*Enterobacter kobei* (JX979115)
PSBL12		1.95±0.21^{c-e}	O	+	*Bacillus sp.*	*Bacillus tequilensis* (JX979116)
PSBL13	+	1.86±0.10^{d-h}	O	+	*Bacillus sp.*	ND
PSBL14	-	1.79±0.07^{e-h}	F	+	*Aeromonas hydrophila*	ND
PSBL19	-	2.25±0.13^{a}	O	+	UI	*Agrobacterium tumefaciens* (JX979117)
PSBL20	-	1.87±0.13d^{e-h}	O	+	*Chryseomonas sp*	ND
PSBL21	+	1.88±0.05d^{e-g}	O	+	*Bacillus sp.*	*Bacillus subtilis* (JX979118)
PSBL26	-	1. 90±0.09^{de}	F	+	*Aeromonas hydrophila*	ND
PSBL28	-	1.85±0.09de^{f-h}	F	+	*Aeromonas hydrophila*	ND
PSBL29	-	1.81±0.06^{e-h}	O	+	*Burkholderia cepacia*	ND
PSBL30	-	1.80±0.07^{e-h}	F		*Chryseomonas luteola*	ND
PSBL31	+	1.88±0.08^{d-g}	O	+	*Bacillus sp.*	ND
PSBL33	-	1.84±0.03d^{e-h}	F	+	*Pseudomonas fluorescens*	ND
PSBL37	-	1.90±0.08^{de}	O	+	*Pseudomonas fluorescens*	ND
PSBL39	-	1.87±0.10^{d-h}	O	+	*UI*	*Acinetobacter calcoaceticus* (JX979119)
PSBL45	-	1.82±0.08^{e-h}	O	+	*Pseudomonas fluorescens*	*Pseudomonas koreensis* (JX979120)
PSBL49	+	2.21±0.10^{a}	O	+	*Bacillus sp.*	*Bacillus subtilis* (JX979121)
PSBL50	+	1.64±0.13^{g-i}	O	+	*Pseudomonas putida*	ND
PSBL51	-	1.87±0.17^{d-h}	O	+	*Sphingomonas paucimobils*	ND
PSBL52	-	1.86±0.20^{d-h}	O	+	*Pseudomonas sp.*	ND
PSBL53	-	1.60±0.15^{i}	O	+	*Pseudomonas fluorescens*	ND
PSBL54	-	1.65±0.14^{f-i}	O	+	*Pseudomonas sp.*	ND
PSBL55	-	1.45±0.18^{ij}	O	+	*Pseudomonas fluorescens*	ND
PSBL57	-	2.16±0.9^{a-c}	O		*Pseudomonas fluorescens*	*Pseudomonas costantinii* (JX979126)
PSBL58	-	2.13±0.12^{a-c}	O	+	*Pseudomonas fluorescens*	*Pseudomonas corrugate* (JX979127)
PSBL90	-	2.17±0.15^{a-c}	O	+	*Burkholderia cepacia*	ND
PSBL92	-	2.17±0.14^{a-c}	F	-	UI	*Enterobacter cloacae* (JX979128)
PSBL93	-	2.07±0.08^{a-d}	O	+	UI	*Ralstonia picketti* (JX979122)
PSBL94	-	2.16±0.06^{a-c}	O	+	*Pseudomonas sp.*	ND
PSBL95	-	2.14±0.15^{a-c}	O	+	*Pseudomonas sp.*	ND
PSBL96	-	2.14±0.15^{a-c}	O	+	*Burkholderia cepacia*	ND
PSBL97	-	1.86±0.11^{d-h}	O	+	*Pseudomonas sp.*	ND
PSBL99	-	1.88±0.10^{d-g}	O	+	*Burkholderia cepacia*	ND
PSBL100	-	2.18±0.12^{ab}	O	+	*Pseudomonas fluorescens*	ND
PSBL101	-	1.34±0.12^{j}	O	+	*Pseudomonas sp.*	ND
PSBL112	-	1.84±0.08^{d-h}	O	+	*Burkholderia cepacia*	ND
PSBL113	-	1.98±0.13^{b-e}	O	+	*Pseudomonas sp.*	ND
PSBL116	-	2.13±0.17^{a-c}	O	+	*Agrobacterium radiobacter*	ND
PSBL120	-	1.84±0.08^{d-h}	O	+	*Pseudomonas sp.*	ND

Numbers in the same column followed by the same superscript letter do not differ significantly (p> 0.05) by DMRT; **+**: the character present/gram positive; -: the character absent/gram negative; SD: standard deviation; FOL: *Fusarium oxysporum f.sp. lentis*; UI: unidentified; ND: not determined, O:Oxidative; F:Fermentative; parenthesis: accession number of the sequence in NCBI gene bank

Different insoluble P sources solubilization efficiency of the PSB isolates

The solubilization efficiencies of selected isolates were estimated in PK broth using TCP, RP and BM as insoluble P sources. Nine isolates were selected for this experiment based on their SI and abundance. Most tested isolates showed excellent TCP solubilization ability as they released considerable amounts of soluble phosphates in the medium until the last sampling day (Table 3). The amount of soluble P released was found to vary significantly among isolates and sampling days. After 12 days of incubation the highest amount of Preleased from TCP was 674 µg ml^{-1}. This indicates that *Enterobacter kobei* (PSBL5) is the most efficient in TCP solubilization in culture medium. On the other hand

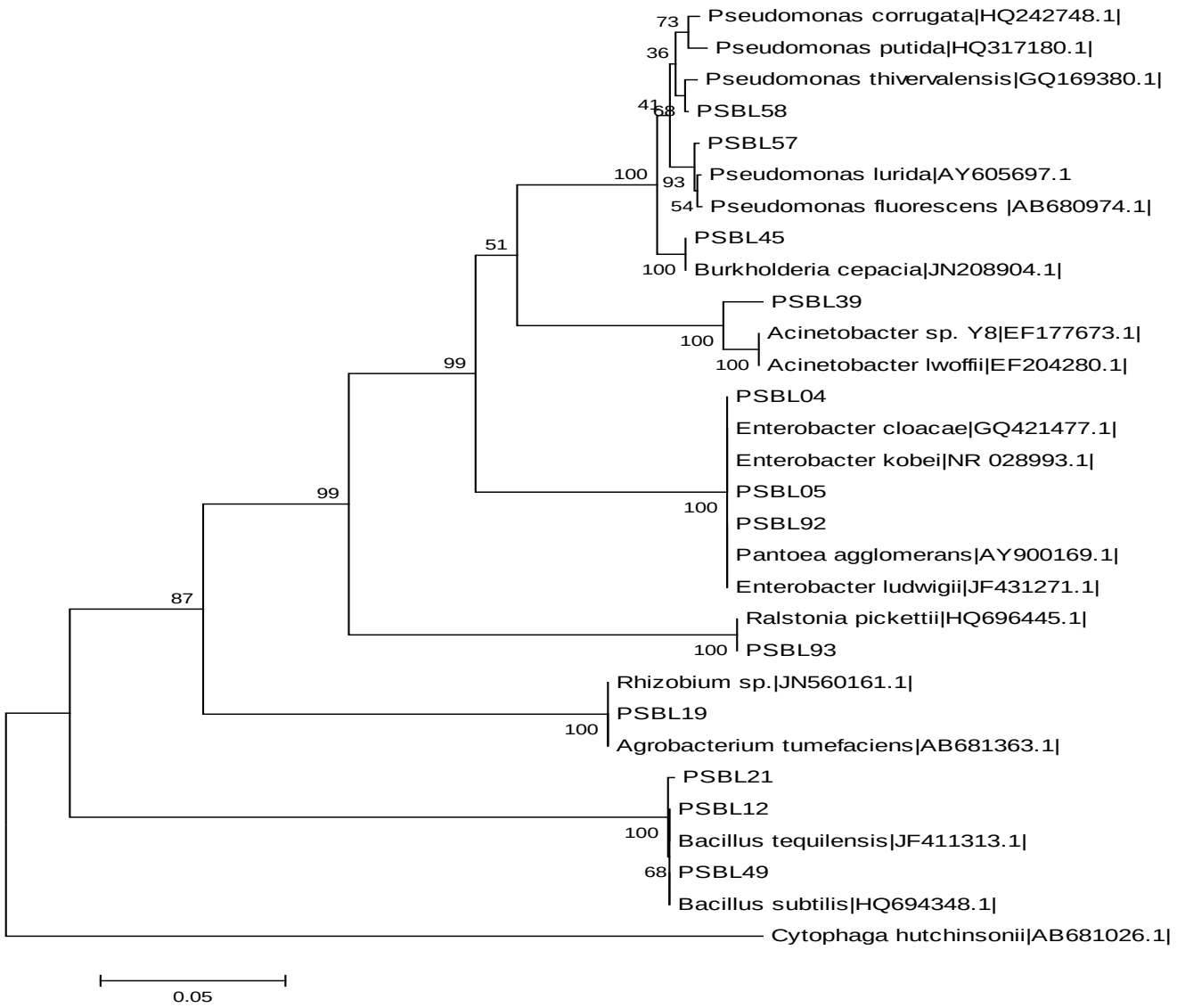

Figure 1. Neighbour-Joining phylogenetic dendrogram of 16S rRNA gene sequence showing the positions of different PSBL strains. Bootstrap values are shown at branching point. Cytophaga sequence is used as out group.

Pseudomonas fluorescens (PSBL33) was found to be poor in solubilization of TCP as it released only 52 µg ml^{-1} of P after 12 days of incubation. Generally, the amount of released P was found to increase progressively until the last sampling day for most isolates. However, it was also found to show a slight decline for *Enterobacter* species (PSBL28) and *P. fluorescens* (PSBL33) after the 8^{th} day of incubation while it was stabilized for *Pseudomonas koreensis* (PSBL45).

During the process of TCP solubilization all isolates showed a sharp drop in pH of the culture medium from 7.0 to 5.4-5.8 (Table 3). The lowest pH was recorded for isolate *P. koreensis* (PSBL45) that is, 5.4 while the highest was recorded for isolate *Enterobacter* species (PSBL26) which was 5.8 on 4^{th} day of incubation. After a sharp decline on day 4, a further decrease in pH was noted only for isolate *Bacillus* sp. (PSBL31) while all the others showed a slight increment. The correlation analysis showed that there is a statistically significant ($p \leq 0.05$) inverse relation between the amount of P released and pH of the broth ($r = -0.82$) indicating that acid production might be the main mechanism of TCP solubilization.

Similarly, the rock phosphate solubilization capacity of the isolates was investigated in PK broth and the isolates showed good solubilization ability of rock phosphate. The amount of soluble phosphorus released and the corresponding change in pH of the medium were varied among isolates and sampling dates (Table 3). The highest amount of soluble P was 78 µg ml^{-1} which was

Table 3. Different insoluble phosphate sources solubilization efficiencies PSB isolates obtained from lentil rhizosphere.

Isolates	Tricalcium phosphate						Rock phosphate						Bone phosphate					
	4th day		8th day		12th day		4th day		8th day		12thday		4th day		8th day		12thday	
	pH	P (µg ml^{-1})	pH	P (µg ml^{-1})	pH	P (µg ml^{-1})	pH	P (µg ml^{-1})	pH	P (µg ml^{-1})	pH	P (µg ml^{-1})	pH	P (µg ml^{-1})	pH	P (µg ml^{-1})	pH	P (µg ml^{-1})
PSBL04	5.6	378^{c}	5.6	483^{cd}	5.7	575^{c}	3.4	9^{f}	3.7	10^{g}	3.7	57^{b}	4.6	16^{g}	4.3	20^{f}	4.4	20^{e}
PSBL05	5.6	378^{c}	5.6	609^{a}	5.5	674^{a}	4.4	46^{d}	5.4	75^{a}	5.7	78^{a}	6.0	239^{b}	6.1	245^{b}	6.1	286^{a}
PSBL21	5.5	375^{c}	5.5	415^{e}	5.5	505^{d}	3.6	49^{c}	3.9	52^{d}	4.7	47^{e}	6.2	187^{d}	6.4	184^{c}	6.4	194^{b}
PSBL26	5.8	62^{d}	5.8	64^{f}	6.0	616^{b}	3.8	7^{f}	4.0	44^{e}	4.0	47^{e}	4.5	30^{f}	4.3	64^{e}	4.3	63^{d}
PSBL28	5.4	410^{ab}	5.4	529^{b}	5.4	492^{ef}	3.5	63^{a}	3.7	63^{b}	3.7	63^{b}	6.4	273^{a}	6.0	276^{a}	5.9	280^{a}
PSBL31	5.4	408^{ab}	5.3	474^{d}	5.2	670^{a}	5.2	44^{d}	5.6	61^{c}	5.0	65^{b}	5.3	201^{c}	5.2	248^{b}	5.3	297^{a}
PSBL33	5.4	52^{d}	5.5	49^{f}	5.8	52^{g}	3.7	60^{b}	3.8	60^{c}	3.9	62^{c}	6.4	204^{c}	6.3	180^{c}	6.4	187^{b}
PSBL37	5.5	380^{c}	5.5	401^{e}	5.5	478^{f}	5.5	41^{e}	5.6	39^{f}	5.6	38^{f}	6.2	191^{d}	6.1	180^{c}	6.1	187^{b}
PSBL45	5.4	398^{b}	5.5	491^{c}	5.6	495d^{e}	4.3	62^{a}	4.9	61^{c}	4.7	63^{bc}	5.9	126^{e}	5.9	143^{d}	5.9	150^{c}

Numbers in the same column followed by the same superscript letter do not differ significantly (p>0.05) by DMRT test.

released by *E. kobei* (PSBL5) while the least was 38 µg ml^{-1} which was recorded for *Pseudomonas fluorescens* (PSBL37) on the 12th days of incubation. Generally, the amount of soluble P in culture medium increased gradually up to the 12th days of incubation for most isolates except *Bacillus subtilis* (PSBL21) and *P. fluorescens* (PSBL37) which showed a slight decrease. Compared to other insoluble P sources the lowest amount of soluble P was obtained from rock phosphate dissolution by all tested isolates.

During the course of rock phosphate solubilization a sharp drop in pH of the growth medium was observed. The pH was varied from 3.4-5.2 which was noted for *Enterobacter ludwigii* (PSBL4) and *Bacillus* sp. (PSBL31), respectively. Compared to TCP and BM the lowest pH was recorded during the solubilization of rock phosphate. However, this low pH was not accompanied by a higher amount of soluble P as the lowest amount of it was obtained from rock phosphate compared to other insoluble P sources. Although statistically not significant the correlation analysis showed that there is an inverse relationship (r= -0.34) between the amount P released and the pH of the growth medium suggesting acid production as the mechanism of rock phosphate solubilization.

The amount of soluble phosphorus released from old bone meal and corresponding drop in pH of the culture medium by the PSB isolates is shown in (Table 3). All tested isolates showed BM solubilization ability with different level of efficiencies. There was a significant variation among isolates in terms of P solubilization and associated change in pH of the culture medium. Isolate *Bacillus* sp. (PSBL31) was comparatively more efficient in solubilizing BM than all other PSBL isolates, followed by *E. kobei* (PSBL5) and *Enterobacter* species (PSBL28) with 297, 286 and 280 µg ml^{-1} soluble P in culture medium, respectively. Isolate *E. ludwigii* (PSBL4) and *Enterobacter* species (PSBL26) were found to be the least efficient in solubilizing BM with 20 and 63 µg ml^{-1}soluble P, respectively. The amount of soluble P was found to increase progressively for all isolates except, PSBL37 and *P. fluorescens* (PSBL33) which showed a slight decrease after the fourth day.

Similar to other insoluble P sources a sharp drop in pH was observed during the course of BM solubilization by all isolates. On the fourth day of incubation drops in pH of the culture medium range from 4.5 to 6.4 which were recorded for isolates *Enterobacter* species (PSBL26) and *Enterobacter* species (PSBL28), respectively. The pH of the medium was found to decrease steadily until 12th day except for some isolates such as *B. subtilis* (PSBL21), *Enterobacter kobei* (PSBL5), *P. fluorescens* (PSBL33) and *P. koreensis* (PSBL45), which showed a slight increment or stability. Though it is not statistically significant there was positive correlation (r= 0.27) between soluble P and pH of the culture medium. This suggests that mechanisms other than acid production were involved in the dissolution of BM and hence further study on the solubilization mechanisms of BM is recommended. All PSB isolates were able to release significantly higher

amounts of soluble P from different insoluble phosphate sources into the culture medium. In general, the highest amount of soluble P was released from TCP followed by BM after 12 days of incubation. The lowest dissolution and sharpest drop in pH was obtained during solubilization of RP. Likewise, previous studies (Rodriguez and Fraga, 1999; Keneni et al., 2010) showed that phosphate solubilizing microorganisms release more soluble P from insoluble inorganic salts than naturally occurring phosphate sources such as rocks and bone meal that consists apatite.

All isolates displayed a significant drop in pH of the culture medium during the solubilization of all tested insoluble phosphate sources. In all cases phosphate solubilization was found to increase with the decreasing pH of the growth medium. Several studies have indicated that the drop in pH is due to the production of organic acids during bacterial growth (Chen et al., 2006; Ivanova et al., 2006; Muleta et al., 2013; Vyas and Gulati, 2009). The decrease in pH of the culture medium associated with the production of organic acids resulted in a considerable amount of P solubilization (Chen et al., 2006). An inverse relationship was established between the drop in pH of the culture medium and the amount of soluble P during solubilization of rock phosphate and TCP. These results are consistent with the report of earlier studies (Rodriguez Fraga, 1999; Naik et al., 2008; Vyas and Gulati, 2009) which showed that solubilization of insoluble phosphate sources were mediated particularly by the decreasing pH of the medium. Several studies showed that mineral phosphate solubilization by PSB isolates is through the release of low molecular weight organic acids (Goldstein, 1995; Kim et al., 1997; Vyas and Gulati, 2009). These organic acids chelate the cations bound to the insoluble phosphate through their hydroxyl or carboxyl groups, thereby converting it into soluble forms (Kim et al., 1997).

The fluctuations and differences in insoluble P sources solubilization efficiencies observed among isolates could be attributed to differences among test isolates in the amount of immobilized P (Muleta et al., 2013). When cells in the culture immobilize phosphate for microbial biomass production, the corresponding values decrease, creating such fluctuations (Muleta et al., 2013). Our observation, a decrease in soluble P and concomitant increase in the pH values of the growth medium indicates re-utilization of available P (Rashid et al., 2004; Tripura et al., 2007). In such cases the organic acids excreted to the growth medium could be reused by the isolates for their own metabolism (Tripura et al., 2007). This could be due to an exhaustion of the original carbon source (Rashid et al., 2004).

Our results indicate that PSB isolates native to Ethiopian soils have good rock phosphate solubilization ability. The dissolution of rock phosphate to a significant extent by these indigenous PSB isolates would give the opportunity to utilize rock phosphate deposits found in different parts of the country (Assefa 1991). In Ethiopia, studies (Bekele and Hofner, 1993) have indicated successful use of rock phosphate under greenhouse conditions. A study conducted elsewhere indicated that 50% of the costly superphosphate could be replaced by rock phosphate; a cheap source of P, when applied in conjunction PSB inoculants (Sundara et al., 2002). Thus, inoculation of native PSB isolates with superior rock phosphate solubilization ability could improve the productivity of lentil and other crops. Our results demonstrated that some PSB isolates released a considerable amount of P from insoluble animal bone; a rich source of P. Previous study (Postma et al., 2010) indicated that PSB strains showed excellent solubilization abilities of animal bone, a source of P that can be recycled. Furthermore, the superior BM solubilization of these isolates would also help to use the problematic waste accumulated around the abattoirs found in different urban areas of the country as P-fertilizer along with PSB inoculants. These could reduce our dependence on the very expensive chemical fertilizers.

Multiple PGP characteristics of PSB isolates from lentil rhizosphere

All PSB isolates obtained from lentil rhizosphere were screened for multiple PGP characteristics such as IAA production and inhibition of *F. oxysporum* growth under *in vitro* conditions. In addition to their superior phosphate solubilization activity, PSB isolates exhibited different PGP properties (Table 4). Dual culture assay indicated that PSBL31 and PSBL53 inhibited the growth of *F. oxysporum* with 41 and 32% of mycelia growth inhibition (Table 4). These isolates were found to be member of the genera *Bacillus* and *Pseudomonas*. Similarly, studies showed that PSB isolates obtained from different agriculturally important crops rhizosphere were found to be antagonistic to different fungal pathogens such as *Fusarium* species, *Phytium* species, *Rhizoctonia* species and others under *in vitro* conditions (Peix et al., 2001b; Naik et al., 2008). In Ethiopia lentil production has been constrained by wilt and root rot caused by different fungal plant pathogens (Ahmed and Ayalew, 2006). Of these wilt disease caused by *Fusarium oxysporum f*. sp. *lentis* has been responsible for major yield loss (Ahmed and Ayalew, 2006) and thus PSB isolates which inhibited the growth of this pathogen would give dual advantage as biofertilizer and biocontrol agents. Consequently, such isolates are highly recommended for greenhouse and field experiments.

In addition, 27.5% of the PSB isolates were found to be capable of producing IAA (Table 4). Quantitative analysis indicated that the highest amount of IAA produced was 48 µg ml^{-1} while the lowest was 14 µg ml^{-1} which was recorded for isolates PSBL19 and PSBL96, respectively (Table 4). The greatest number of IAA producer were

Table 4. Multiple PGP characteristics of PSB isolated from lentil rhizosphere.

Isolate	IAA (µg ml^{-1} ± SD)	*In vitro* inhibition of FoL growth (%)± SD
PSBL04	18.2±2.0^{cd}	-
PSBL19	47.5±1.6^{a}	-
PSBL21	16.7±2.5^{de}	-
PSBL28	14.7±1.5^{e}	-
PSBL31	-	41±2.5^{a}
PSBL33	-	-
PSBL37	17.9±1.9^{cd}	-
PSBL52	19.6±1.5^{c}	-
PSBL53	15.5±1.2^{e}	32±1.7^{b}
PSBL57	-	
PSBL92	20.7±1.2^{bc}	-
PSBL93	21.6 ±2.1^{bc}	-
PSBL96	14.3±0.7^{e}	-
PSBL97	14.7±0.3^{e}	-

Numbers in the same column followed by the same superscript letter do not differ significantly (p> 0.05) by DMRT; **+**: the character present/ -: the character absent, SD: standard deviation; FOL: *Fusarium oxysporum f.sp. lentis*

from the Gram negative isolates (90%) belonging to genera *Enterobacter, Pseudomonas, Agrobacterium, Ralstonia* and *Burkholderia*. The most capable strains belonged to the genera *Agrobacterium* and *Ralstonia.* Similarly, several previous studies demonstrated that PSB isolates obtained from the rhizosphere of different crops were able to exhibit different levels of IAA production (Naik et al., 2008; Shahab et al., 2009). These studies indicated that most of the studied isolates produced considerable amounts of IAA (Shahab et al., 2009). Such isolates were found to be excellent in promoting plant growth under greenhouse conditions (Shahab et al., 2009). The findings of the present investigation showed that IAA producing PSB isolates could be easily isolated from local soil and might be exploited as multipurpose inoculants after greenhouse and field tests.

Response of Lentil to inoculation PSB with different P sources

The effects of inoculation of selected PSB isolates on lentil growth, nodulation, and N and P content of shoot were evaluated under greenhouse conditions. During this experiment *Enterobacter kobei* (PSBL5), *Bacillus subtilis* (PSBL21), *Enterobacter* species (PSBL26), *Bacillus sp.* (PSBL31) and *Pseudomonas fluorescens* (PSBL37) were used as inoculants. The results showed that shoot dry matter yield of lentil was improved by inoculation of PSB isolates over the uninoculated plants (NC) control (Table 5). Their effect was comparable to P-fertilizer application. In addition to their phosphate solubilization two isolates (PSBL21 and PSBL37) were found to be IAA producers. However, further studies using IAA mutant strains of these isolates are needed to determine the exact contribution of IAA production in the promotion of plant growth when isolates of multiple PGP attributes are used as inoculants.

All PSB isolates except *Enterobacter* species (PSBL26) increased the number of nodules at a statistically significant level (*p≤0.05*) over the NC. This improvement was found to be comparable to the P-fertilized plant. The numbers of nodules were also found to vary considerably among the isolates. The highest number of nodules was induced by *E. kobei* (PSBL5) while the least was by *Enterobacter* species (PSBL26). These isolates increased nodule number by 43.9 and 38.2% over the NC, respectively. It is interesting to note that *E. kobei* (PSBL5) was superior to P-fertilization in terms nodulation induction. The result of our study indicated that the increase in nodule number is positively correlated with the increase in shoots dry weight (r=0.72).

All PSB isolates used in this experiment increased the concentration of N in the shoot of lentil significantly (*p≤0.05*) over the NC (Table 5). The highest N concentration was observed in *Enterobacter kobei* (PSBL5) and *Bacillus* sp. (PSBL31) inoculated plants and a 36.4% increase was obtained over the NC. The variations in N concentration obtained among isolates were not statistically significant. Their effect on N concentration was found to be comparable to P-fertilization. The results of our study indicated that the N content of shoot is positively correlated with the nodule number (r=0.67) and

Table 5. Effects of inoculation of PSB different on growth of lentil under glasshouse conditions.

Treatments	Nodule number/plant ± SD	Shoot dry weight(g)/Plant ± SD	Total Nitrogen(%) of shoot ± SD	Phosphorous concentration in shoot(mg/g SDW) ± SD
PC	34.3±2.5^{ab}	1.03±0.06^{a}	3.27±0.16^{a}	12.1±0.1^{ab}
NC	28.0±3^{c}	0.87±0.05^{b}	2.50±0.11^{b}	8.2±0.1^{c}
PSBL5	40.3±3.5^{a}	0.98±0.02^{ab}	3.41±0.15^{a}	12.8±0.1^{a}
PSBL21	36.0±4.0^{ab}	0.99±0.03^{ab}	3.31±0.16^{a}	11.3±0.15^{ab}
PSBL26	33±4^{bc}	0.96±0.14^{ab}	3.32±0.13^{a}	10.7±0.25^{abc}
PSBL31	38.7±3^{ab}	0.95±0.04^{ab}	3.41±0.17^{a}	12.0±0.1^{ab}
PSBL37	34.7±2.1^{ab}	0.97±0.08^{ab}	3.14±0.14^{a}	10.0±0.1^{bc}

Numbers in the same column followed by the same letter do not differ significantly (p> 0.05) by DMRT; PC: positive control; NC: negative control; SD: standard deviation

shoot dry weight (r=0.53) of the plants. Peix et al. (2001b) have also observed similar results in plants inoculated with PSB strain of *Burkholdera cepacia.* It has been documented that phosphorus deficiency in soils has a deleterious effect on the symbiotic interaction between rhizobia and legumes such as nodulation, N_2-fixation, and hence growth and productivity (Gyaneshwar et al., 2002). Several studies have demonstrated that inoculation of seedlings with PSB isolates increased the level of available phosphorus in the rhizosphere soil (Sundara et al., 2002; Vyas and Gulati, 2009). In this way, inoculation of lentil with efficient PSB could improve growth, nodulation and thus the nitrogen-fixation of this crop.

Similarly, all PSB isolates improved the concentration of P in the shoot of lentil at statistically significant level over the NC. The highest concentration of P was obtained by *Enterobacter kobei* (PSBL5) isolate inoculation followed by *Bacillus sp.* (PSBL31). These isolates increased P concentration by 56.1 and 46.3%, over the NC control, respectively. The P content of the plant was positively correlated with the N content (r=0.61) and shoot dry weight (r=0.72) of the plant. Previous studies have demonstrated that inoculation of PSB isolates increased the available P in soil (Sundara et al., 2002; Vyas and Gulati, 2009) and concomitantly improved P uptake of the plant (Peix et al., 2001a; Peix et al., 2001b; Vyas and Gulati, 2009).

Conclusion

In general, from the results of the present study it is possible to conclude that rhizosphere soils of lentil from different producing areas of Ethiopia harbor a considerably high number of PSB isolates. They belong to different bacterial genera: *Pseudomonas, Bacillus, Agrobacterium, Enterobacter, Burkholderia, Ralstonia, Chryseomonas* and *Acinetobacter.* The PSB isolates were able to release a considerable amount of P from TCP, RP and BM. The present investigation revealed the diversity of PSB with superior phosphate solubilizing capacity, multiple plant growth promoting traits and biocontrol properties. The greenhouse experiment showed that inoculation of lentil with PSB has improved the nodule number, dry matter yield, N and P content of the plant significantly. Some of the isolates such as *Enterobacter kobei* (PSBL5) and *Bacillus* (PSBL31) are of particular interest for further research and need to be tested in the field in different agroecological zones, as they could be used as efficient biofertilizer in the lentil production system. The knowledge generated on diversity and PGP characteristics of phosphate solubilizing bacteria from rhizosphere soil would be useful to design strategies to use phosphate solubilizing bacterial inoculants technology in the crop production systems of the country.

Conflict of interest

Author did not declare any conflict of interest.

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