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Plant growth promoting properties of phosphate solubilizing *Bacillus* species isolated from the Aegean Region of Turkey

Pınar SÖZER BAHADIR[1,*], Fakhra LIAQAT[2], Rengin ELTEM[3]

[1]Ege University Central Research Test and Analysis Laboratories Research and Application Center (EGE MATAL), İzmir, Turkey
[2]Graduate School of Natural and Applied Sciences, Department of Biotechnology, Ege University, İzmir, Turkey
[3]Department of Bioengineering, Faculty of Engineering, Ege University, İzmir, Turkey

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Abstract: *Bacillus* species, due to their soil amendment properties, are important members of plant-growth–promoting rhizobacteria (PGPR). In this study, with the aim to discover potential biofertilizers, 440 *Bacillus* isolates from different sources were screened qualitatively for phosphate solubilizing and positive isolates were processed for quantitative estimation of solubilized phosphate and organic acid production. Organic acid production was initially detected by pH change of the media, whereas further confirmation and quantitative estimation were done by gas chromatography (GC). The results indicate that phosphate solubilization ranges from 6.9 ± 1.00 to 95.5 ± 1.83 μg mL^{-1} for *Bacillus* isolates and most of the isolates were able to produce more than one organic acid in substantial quantities range between 70.70 ± 1.90 and 619.20 ± 1.40 (ng μL^{-1}). Correlations among total organic acid concentration, final pH of the media, and soluble phosphate were statistically calculated. The six best phosphate-solubilizing isolates were further tested for indole-3-acetic acid (IAA) production, molecular identification, in vitro seed germination, and pot trials. All six strains produced IAA, significantly enhanced radicle and hypocotyl development, and considerably increased plant growth by improving growth of roots and stems. On the basis of results, these *Bacillus* strains can be considered as potential biofertilizers.

Key words: Phosphate solubilization, organic acids, indole-3-acetic acid (IAA), pot trials, biofertilizer

1. Introduction

Use of bacteria as a biofertilizer or biocontrol agent in agriculture became widespread in the late 1990s. Recently, the plant-growth–promoting and productivity enhancement properties of rhizobacteria have been studied worldwide. Agricultural policies that promote the use of environment friendly products, such as biofertilizers, have gained increasing importance nowadays (Chauhan et al., 2015). Commercial fertilizers containing strains of the genus *Bacillus* have been given considerable importance because they are more tolerant of extreme abiotic conditions, such as temperature, pH, and pesticides. *Bacillus*-based biofertilizers became a candidate for commercial production as they are not harmful to the environment and humans, play a role in biocontrol against pathogenic fungus, grow rapidly in the soil, and increase the nutrient uptake of plants to improve the productivity of plants (Kumar et al., 2011).

Phosphorus is a main vital macronutrient for plants known to perform many functions in their growth and metabolism. Numerous important cellular, metabolic, and reproductive mechanisms depend on adequate phosphorus supply (Chen et al., 2006; Kaymak, 2010). Although soils contain sufficient amounts of phosphate, only a very small quantity is accessible to plants. The ability of the microorganisms to solubilize phosphate is a key character related to plant phosphate nutrition (Hayat et al., 2010; Bhattacharrya and Jha, 2012; Sharma et al., 2013). The process of phosphate solubilization by phosphate-solubilizing bacteria (PSB) strains is linked with the production of low molecular weight organic acids, via which hydroxyl and carboxyl groups chelate cations bind to phosphate; thus soluble phosphate is obtained (Chen et al., 2006). Various types of microorganisms have been used as phosphate-solubilizing biofertilizer (Malboobi et al., 2009).

Production is a major property of plant growth–promoting bacteria (Mohite, 2013). IAA is one of the most physiologically active auxins. Bacteria synthesize auxins in order to perturb host physiological processes for their own benefit. The microorganisms isolated from the rhizosphere region of various crops can produce IAA. IAA

* Correspondence: pinarsozer@gmail.com

helps in the production of longer roots with an increased number of root hairs and root laterals, which are involved in nutrient uptake (Datta and Basu, 2000). With the aim to develop biofertilizers using indigenous *Bacillus* strains, this study was designed to be preliminary screening to get the best novel *Bacillus* isolates from the soil of this country. Furthermore, this study is an important step towards the development of biopreparates consisting of original indigenous strains. The application of indigenous strains as biofertilizers is more advantageous for the local soil and plants.

2. Materials and methods

2.1. Qualitative screening of phosphate solubilizing *Bacillus* spp.

In this study, a total of 440 *Bacillus* isolates present in the *Bacillus* Culture Collection of the Bioengineering Department, Ege University, previously recovered from different sources (Table 1) were screened for their ability to solubilize inorganic phosphate. National Botanical Research Institute's Phosphate (NBRIP) agar [$MgCl_2.6H_2O$ (5 g L^{-1}), $MgSO_4.H_2O$ (0.25 g L^{-1}), KCl (0.2 g L^{-1}), $(NH_4)_2SO_4$ (0.1g L^{-1}), $Ca_3(PO_4)_2$ (5 g L^{-1}), glucose (10 g L^{-1}), 1.5% agar, and pH 7.0] containing tricalcium phosphate/$Ca_3(PO_4)_2$ (TCP) as the sole phosphorus source was used for initial qualitative selection of all *Bacillus* isolates (Nautiyal, 1999). TCP was autoclaved separately and mixed aseptically with other sterile ingredients (Park et al., 2011). All of the isolates were growth activated on nutrient agar and transferred to NBRIP agar followed by incubation of 14 days at 30 °C. After incubation, the appearance of transparent zones around the microbial colonies was considered as a positive result for phosphate solubilization (Chen et al., 2006; Behera et al., 2014).

2.2. Quantitative determination of phosphate solubilization

According to the results of qualitative screening, positive isolates were selected and inoculated in NBRIP broth (predescribed composition without agar) at a concentration of 10^8 cfu mL^{-1} and incubated at 30 °C at 180 rpm for 10 days. The initial pH of the media was adjusted at 7.0. After incubation, the broth was centrifuged at 9000 rpm for 15 min to remove the biomass as well as insoluble suspended particles of TCP. The supernatant was further filtered through a 0.45-µm filter (MF-Millipore Membrane Filter, HAWP04700; Burlington, MA, USA). The amount of soluble phosphate was measured colorimetrically using the phosphomolybdate method (ascorbic acid method) (Murphy and Riley, 1962). At the end of the cultivation, the pH change of the media was also monitored.

2.3. Production of organic acids by PSB

After incubating isolates in NBRIP broth for 10 days, culture broth were centrifuged at 9000 rpm for 15 min and filtrated through a 0.22-µm filter (Millipore, Millex-GP Syringe Filter, SLGP033NB). For detection of organic acids, 1 µL of cell-free filtrate was injected to GC (6890N Agilent Technologies Network GC System; Santa Clara, CA, USA). Separation of organic acid was carried out with HP-FFAP 30 m × 0.25 mm capillary column J&W Scientific using a flame ionization detector. Helium was used as the carrier gas at a stable pressure (103 kPa). Initial temperature (70 °C) was automated to remain constant for 5 min, then to increase at a rate of 8 °C per min to reach 140 °C, and again reverted to 70 °C after 5 min. Retention time of 10 mm min^{-1} was programmed to get well separated peaks. Uninoculated broth served as a control (Vazquez et al., 2000). The organic acids were identified using known standards (acetic, propionic, butyric, caproic,

Table 1. Sources of 440 *Bacillus* isolates from different regions of Turkey.

Sources of isolates	Regions	Number of isolates
Vineyard soil	Saruhanlı, Bağlıca, Sarıgöl, Salihli, Alaşehir-Manisa; Menemen-Kemalpaşa-İzmir	239
Fig orchard soil	Germencik, Sultanhisar, Erbeyli, Umurlu, Efeler-Aydın	78
Pine nut forest soil	Bergama-İzmir	32
Cedar forest soil	Fethiye-Muğla	27
Sewage sludge	Çiğli-İzmir	22
Coastal area soil	Gulf of İzmir	18
Compost of mushroom (*Agaricus bisporus*, *Pleurotus* sp.)	İzmir	11
Garden soil	Bornova-İzmir	8
Paddy fields	Ergene Basin-Edirne	5

isobutyric, isovaleric, valeric, isocaproic, and heptanoic acids; Accustandard, New Haven, CT, USA).

2.4. Quantitative determination of IAA

Bacillus isolates exhibiting the best phosphate-solubilizing activities were selected and further tested for quantitative determination of IAA. Aliquots of 50 mL of nutrient broth (Merck, N7519) were sterilized by autoclaving and 2 mL of 0.5% filter sterilized L-tryptophan (25 μg mL^{-1}) was added. Test isolates were inoculated at a concentration of 10^8 cfu mL^{-1} (prepared according to McFarland Standard) and flasks were incubated for 48 h at 150 rpm and 30 °C. After the incubation, supernatant was collected by centrifugation at 9000 rpm for 15 min and 2 mL of the supernatant was mixed with 2 drops of orthophosphoric acid and 4 mL of Salkowski's reagent. IAA was measured by spectrophotometric method at 530 nm (Asghar et al., 2002).

2.5. Molecular identification of PSB

Bacillus isolates solubilizing high quantities of phosphate were selected for molecular identification. Molecular identification of selected bacterial strains was performed with 16S rDNA sequencing and 16S rDNA gene amplification was done with 27F and 1492R universal primers (Lane, 1991). Genes were amplified and sequenced by a commercial company with an ABI 3100 Genetic Analyzer (RefGen, METU Technopark, Ankara, Turkey). Sequences were adjusted and aligned by using the Geneious Bioinformatics Software (version 9.1) (BioMatters, Auckland, New Zealand) and a consensus sequence was obtained. An aligned contiguous consensus sequence of 16S rDNA was used for homology search with the basic local alignment search tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi - accessed on 15 March, 2016) algorithm from the National Center for Biotechnology Information (NCBI). The phylogenetic study of the 16S rDNA sequences of the isolates was conducted by Geneious Bioinformatics Software (version 9.1) using the neighbor-joining method. Data obtained after sequencing were submitted to the NCBI GenBank database to attain accession numbers.

2.6. Germination assay

Seeds of tomato (*Lycopersicon lycopersicum* cv. Target F1), pepper (*Capsicum annuum* L. var. cv. Kekova F1), and eggplant (*Solanum melongena* L. cv. Faselis F1) were kept in 0.1% $HgCl_2$ for 5 min, washed 5 times with sterile distilled water, and pregerminated in sterile wet cotton. The seeds were germinated for 2–3 days and, under aseptic conditions, were placed in petri dishes containing 1% agar. On each seed, the inoculum of *Bacillus* strains at 10^8 cfu mL^{-1} concentration was separately instilled. Sterile distilled water was instilled into the control seeds. Radicle (embryonic root) and hypocotyl lengths (mm) were measured in germinated seeds after 7 days of incubation in dark at 28 °C. Experiments were performed in three replicates for each plant type and each isolate (n = 9).

2.7. Pot trials

In order to determine the effects of phosphate-solubilizing strains on plant growth, tomato (*Lycopersicon lycopersicum* L. cv. Allegro F_1), pepper (*Capsicum annuum* L. cv. 37-04 RZ Sivri), and eggplant (*Solanum melongena* L. cv. Brigitte 10- 44 RZ F_1) seedlings collected from Grow Fide Production and Trade Co. (Antalya, Turkey) were used, and a 13-week trial was performed. For each plant, one seedling per strain was used in three replicates (n = 3). Bacterial suspension was prepared by inoculating the bacteria in nutrient yeast salt medium followed by 48 h of incubation at 30 °C and 180 rpm. After incubation, the bacterial biomass was recovered from the culture broth by centrifugation and washed twice with sterile distilled water. Biomass was suspended into sterile distilled water and the bacterial count was determined (Prabakaran et al., 2007). This bacterial suspension was diluted to get 4.0–8.0 × 10^8 cfu mL^{-1} and was transferred to a container under aseptic conditions. The rooted seedlings were placed in a container with bacterial suspension for 30 min to ensure bacterial contact with the roots. After the incubation, the seedlings were planted in a pot disinfected with a 1% hypochlorite solution. The pots were placed in a sunny open field and irrigated regularly. All the plant types were planted in three replicates per strain. For each plant, three control pots were planted, containing seedlings immersed in sterile water instead of the bacterial suspension (Orhan et al., 2006). Application of bacterial suspension was repeated after every 30 days for 13 weeks. In this preliminary experiment, the effects of the phosphate-solubilizing *Bacillus* strains on various plant growth parameters, such as number of fruits, product weight (g), height of plant, and number of leaves per plant, were studied. With the termination of the experiments and removal of the plants from the pots, root lengths were measured and dry weights of plants were calculated by drying them at 65 °C until a fixed weight was reached.

2.8. Statistical analysis

Statistical analysis was conducted to determine the effect of organic acid secretion and pH decrease for inorganic phosphate solubilization. For seed germination experiments, statistical analysis was performed to compare the radicle and hypocotyl length of seed treated with different isolates. For pot trials, statistical analysis was done to compare the weight and length of the stem plus roots of test plants. All the data were expressed as an average of three replicates. The significance of result for each experiment was established by one-way analysis of variance (ANOVA) and the means were separated by Tukey's test ($P \leq 0.05$). The signification level of the

acceptance limit was set to 95%. To compare the means of variables, $P < 0.05$ was considered statistically significant. Statistical analysis was performed by using the statistical software Minitab, version 17.0 (State College, PA, USA).

3. Results

3.1. Screening of phosphate solubilization

Out of 440 *Bacillus* isolates screened for phosphate solubilization, only 11.6% (n = 51) isolates had phosphate-solubilizing ability, based on a clear zone on NBRIP agar. Figure 1 shows the positive isolates, showing a clear zone of phosphate solubilization around the bacterial colony along with the negative isolates showing no zones. Quantitative estimation of phosphate solubilization of 51 positive isolates showed that the amount of solubilized phosphate range between 6.9 ± 1.00 μg mL^{-1} (by *Bacillus* sp. EGE-B-34.3) and 95.5 ± 1.83 μg mL^{-1} (by *Bacillus subtilis* EGE-B-24.4i).

According to GC analysis, nine different volatile organic acids (acetic acid, butyric acid, propionic acid, isobutyric acid, isocaproic acid, caproic acid, isovaleric acid, heptanoic acid, and valeric acids) were tested. GC analysis confirmed the production of organic acids in the broth by all the tested strains (Figure 2). Six different volatile organic acids were produced by 51 tested isolates (acetic acid, propionic acid, isobutyric acid, isocaproic acid, caproic acid, and heptanoic acid). The details are given in Table 2.

All the isolates synthesized acetic acid, and most of the isolates produced more than two organic acids. Heptanoic acid was not produced by any isolate except *Bacillus* sp. EGE-B-13.5i. Butyric, isovaleric, and valeric acids were not produced by any of the isolates. Total amounts of produced organic acids ranged from 70.70 ± 1.90 to 619.20 ± 1.40 ng μL^{-1}. The correlations between means of experimental data (total organic acid concentration and final pH; soluble phosphate and final pH; soluble phosphate and total organic acid) of selected strains were calculated (Table 3). One-way ANOVA and Tukey's multiple comparison test were employed at the significance level of 5%. The result was considered statistically significant ($P \leq 0.05$). A statistically negative correlation ($r = -0.459$, $P > 0.01$) was observed between soluble phosphate concentration and final pH. In addition, soluble phosphate concentration and total organic acid concentration were also highly positively correlated ($r = 0.555$, $P < 0.01$). There was no significant correlation between total organic acid concentration and the final pH of the media ($r = -0.230$, $P > 0.10$) (Table 3).

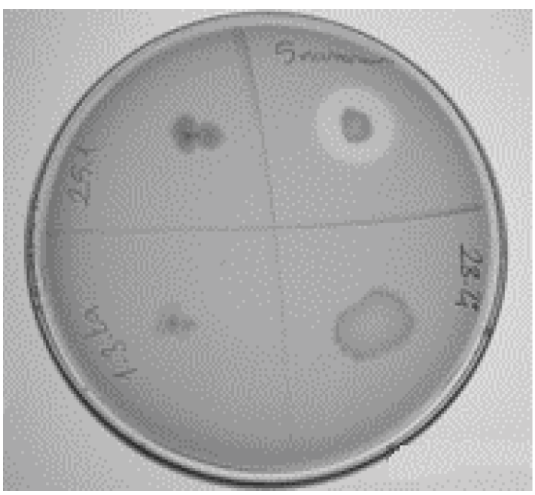

Figure 1. Clear zone of phosphate solubilization of *Bacillus subtilis* EGE-B-3.P.5 on NBRIP agar.

3.2. IAA production of selected isolates

All six isolates could produce IAA, and the quantities of IAA produced ranged from 0.80 ± 0.10 to 27.22 ± 1.76 μg mL^{-1} (Table 4).

3.3. Molecular identification

Isolates were molecularly identified using 16S rDNA sequencing. Two of the selected isolates were identified as *Bacillus megaterium*, three as *Bacillus subtilis*, and one as *Bacillus simplex* (Table 4). The phylogenetic studies of 16S rDNA sequence data of our isolates, plus the sequence data searched and retrieved from NCBI, were performed with Geneious Bioinformatics Software (version 9.1) (BioMatters), applying global alignment type with free end gaps, 65% similarity cost matrix, Tamura–Nei genetic distance model, and neighbor-joining tree build method (Figure 3).

The six best phosphate-solubilizing *Bacillus* strains were selected from different regions and soil sources. *B. megaterium* EGE-B-1.4.a was isolated from İzmir (compost of mushroom), *B. megaterium* EGE-B-10.3.F was isolated from Bergama-İzmir (pine nut forest soil), *B. simplex* EGE-B-1.2.k was isolated from Çiğli-İzmir (sewage sludge), *B. subtilis* EGE-B-24.4i was isolated from Erbeyli-Aydın (fig orchard soil), *B. subtilis* EGE-B-26.1 was isolated from Sarıgöl-Manisa (vineyard soil), and *B. subtilis* EGE-B-3.P.5 was isolated from garden soil in Bornova-İzmir.

3.4. In vitro germination assay

Seed germination experiments were performed by treating the pregerminated seeds of eggplant (*Solanum melongena* L. cv. Faselis F1), pepper (*Capsicum annuum* L. var. cv. Kekova F1), and tomato (*Lycopersicon lycopersicum* cv. Target F1) with suspensions of bacterial strains. Lengths of germinated radicle (cm) and hypocotyl (cm) were measured and compared to untreated control seeds. All the bacterial isolates exhibited positive effects on seed germination (Table 5).

3.5. Pot trials

Pot trials were performed by using the seedlings of eggplant (*Solanum melongena* L. cv. Brigitte 10-44 RZ F1), pepper (*Capsicum annuum* L. cv. 37-04 RZ Sivri), and

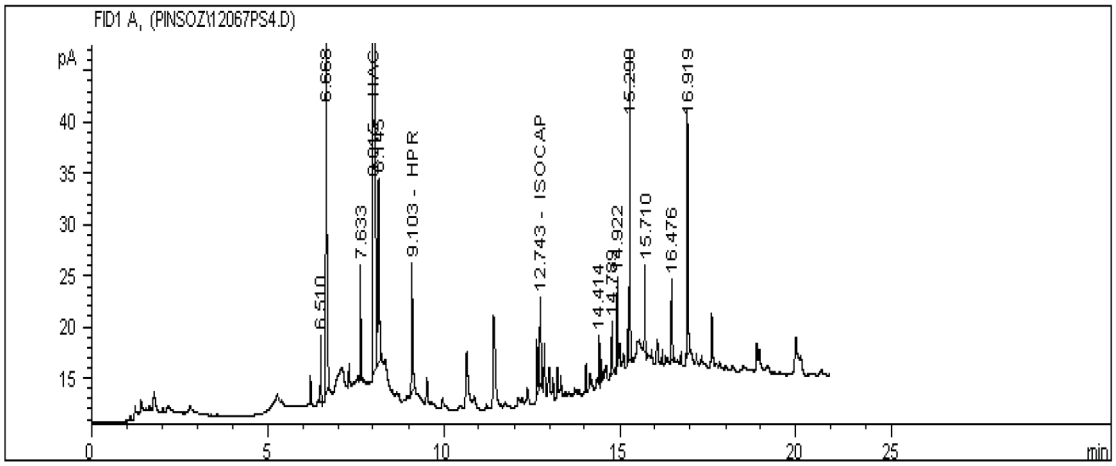

Figure 2. GC analysis of organic acid production.

tomato (*Lycopersicon lycopersicum* L. cv. Allegro F1). In the pot trials, three plants types were used for each bacterium in three replicates and one plant seedling was inoculated per pot. A total of 63 pots were inoculated (3 plants × 6 isolates with three replicates + 3 controls with three replicates). Dry root weight (g), dry stem weight (g), fresh root weight (g), fresh stem weight (g), and root length (cm) were measured at the end of the experiments. A significant increase in all the measures was observed for all the three test plants compared to the control plants (Table 6; Figures 4 and 5). Statistical analysis of data was done by Tukey's test ($P \leq 0.05$) and analysis showed that all measured vegetative growth parameters were promoted by *Bacillus* species. Although, for three different plants, each isolate showed a different plant-growth–promoting effect, as some isolates enhanced root growth while others were more effective for stem growth (Table 6).

4. Discussion

In recent years, sustainable agricultural production systems have used biofertilizers and organic products instead of agrochemicals (Bhattacharrya and Jha, 2012; Chauhan et al., 2015). Soil and plant rhizospheres contain a substantial diversity of PSB (Khan et al., 2014). In recent times, many studies performed on the microbial solubilization of phosphates as a substitute of chemical fertilizer (Vassilev et al., 2014; Oufdou et al., 2016). This study is different from other studies because, to the best of our knowledge, such large numbers of *Bacillus* isolates have not been screened before. Furthermore, the applications of indigenous strains to the soil as biofertilizers are more advantageous for the local soil and plants.

The plate-screening method is commonly used and it is an effective method for processing large numbers of isolates before quantitative measurement of phosphate in liquid media. Most studies use plate screening as their initial strategy to select potential PSB based on the formation of clear zones around the colonies (Sarkar et al., 2012; Teymouri et al., 2016). Because of complicated soil conditions, the appearance of a clear zone on a solid medium should not be the only method to test for phosphate solubilization. Further testing to assess phosphate solubilization should be undertaken simultaneously.

Qualitatively screening 440 *Bacillus* isolates for phosphate solubilization abilities showed that 389 of these isolates had no clear zone around bacterial growth in the agar plate. Quantitative phosphate measurements carried out in liquid media had contradictory results between clear zone diameters and phosphate solubilization activity: some isolates had smaller zones in plate screening but higher phosphate solubilization concentrations in quantitative detection and vice versa. Similar outcomes were reported by Sarkar et al. (2012).

There are many studies with *Bacillus* spp. using NBRIP broth with the presence of 0.5% TCP as the sole phosphate source (Nautiyal, 1999; Maitra et al., 2015; Teymouri et al., 2016). When analyzed, total phosphate solubilization in cultures of various *Bacillus* strains cultivated on insoluble phosphate substrates results showed that *B. amyloliquefaciens* produced 395 μg mL^{-1}, *B. polymyxa* 116 μg mL^{-1}, *B. megaterium* 82 μg mL^{-1}, *B. pulvifaciens* 54 μg mL^{-1}, and *B. circulars* 11 μg mL^{-1} (Rodríguez and Fraga, 1999; Chen et al., 2006). Erturk et al. (2012) examined the effect of inoculation of PGPR on many characteristics of strawberry. Four *Bacillus* strains were initially isolated from the rhizosphere in northeastern Anatolia and tested for phosphate solubilization capacity. *Bacillus* RC23 showed 23.8 ± 0.6 μg mL^{-1} phosphate solubilization. These results are comparable with our findings. Numerous different microorganisms can solubilize phosphate in the range of 30 to 900 μg mL^{-1}, depending on the insoluble phosphate composition, media components, and initial pH (Teymouri et al., 2016).

Table 2. Soluble phosphate concentrations, final pH of media, and organic acid concentrations for 51 *Bacillus* isolates.

	Organic acids (ng µL^{-1})						*Total organic acid (ng µL^{-1})	Final pH of media	Soluble-P (µg mL^{-1})
Bacillus isolates codes	HAC	HPR	ISOBUT	ISOCAP	HCAP	HEPTA			
EGE-B-13.4i	108.5 ± 4.31	15.3 ± 1.10	-	7.4 ± 0.20	-	-	131.20 ± 11.10	5.80 ± 0.29	7.5 ± 1.20
EGE-B-35.2	163.7 ± 1.30	21.9 ± 0.40	-	7.4 ± 0.040	-	-	193 ± 5.07	4.95 ± 0.07	32.1 ± 0.90
EGE-B-9.2.F	157.4 ± 5.10	20.2 ± 0.90	-	6.7 ± 0.20	-	-	184.40 ± 4.10	6.08 ± 0.22	9.5 ± 1.20
EGE-B-26.1**	166.5 ± 2.30	21.0 ± 0.50	-	13.7 ± 0.52	-	-	201.22 ± 2.60	4.92 ± 0.08	33.4 ± 1.42
EGE-B-2.1.F	163.2 ± 1.90	20.4 ± 0.50	-	10.9 ± 0.10	-	-	194.50 ± 1.90	5.38 ± 0.07	15.4 ± 2.85
EGE-B-1.1.F	157.6 ± 0.40	19.3 ± 0.30	-	11.5 ± 0.10	-	-	188.50 ± 0.60	5.12 ± 0.23	25.7 ± 1.90
EGE-B-34.3	145.9 ± 2.70	17.9 ± 0.30	-	9.9 ± 0.10	-	-	173.90 ± 0.70	5.99 ± 0.29	6.9 ± 1.00
EGE-B-32.1	131.9 ± 0.20	15.7 ± 0.10	-	10.1 ± 0.10	-	-	157.70 ± 0.90	5.27 ± 0.09	15.3 ± 3.50
EGE-B-1.7ka	191.3 ± 1.60	22.2 ± 0.60	-	11.3 ± 0.30	-	-	224.80 ± 1.50	5.16 ± 0.13	18.5 ± 0.70
EGE-B-1.11k	117.7 ± 2.10	14.1 ± 0.80	-	4.2 ± 0.40	-	-	136.00 ± 0.20	5.60 ± 0.48	13.2 ± 0.30
EGE-B-44.3	56.6 ± 1.40	-	-	14.1 ± 0.50	-	-	70.70 ± 1.90	4.20 ± 0.15	20.0 ± 0.20
EGE-B-44.4	77.8 ± 1.40	9.1 ± 0.10	-	15.9 ± 0.20	-	-	102.80 ± 0.10	4.77 ± 0.25	24.9 ± 1.10
EGE-B-1.2.F	95.2 ± 1.00	11.1 ± 0.50	-	16.6 ± 0.40	-	-	122.90 ± 1.90	5.50 ± 0.21	10.3 ± 0.20
EGE-B-37.3	112.9 ± 0.44	-	330.79 ± 1.81	-	8.27 ± 0.17	-	451.99 ± 0.79	5.80 ± 0.54	14.4 ± 0.20
EGE-B-30.5	133.37 ± 2.83	-	375.05 ± 0.55	-	10.73 ± 0.13	-	519.15 ± 1.95	5.45 ± 0.17	20.6 ± 0.90
EGE-B-40.5	130.21 ± 0.63	-	319.99 ± 0.61	-	8.06 ± 0.14	-	458.27 ± 1.03	5.80 ± 0.42	19.23 ±0.30
EGE-B-27.5	157.03 ± 4.73	-	442.80 ± 0.20	-	11.49 ± 0.16	-	611.32 ± 0.48	4.94 ± 01.8	8.25 ± 0.35
EGE-B-2.5d	48.79 ± 1.53	-	135.35 ± 1.15	-	-	-	184.14 ± 2.16	4.60 ± 0.20	23.8 ± 0.40
EGE-B-13.5i	39.68 ± 2.18	-	173.13 ± 1.47	-	3.65 ± 0.14	2.68 ± 0.58	219.13 ± 0.53	5.58 ± 0.33	11.2 ± 0.60
EGE-B-1.22k	169.89 ± 2.29	-	431.42 ± 0.58	4.83 ± 0.08	13.05 ± 0.05	-	619.20 ± 1.40	5.44 ± 0.26	11.5 ± 0.50
EGE-B-27.6	104.57 ± 0.67	-	299.88 ± 0.22	-	7.5 ± 0.10	-	411.98 ± 1.18	5.33 ± 0.12	15.7 ± 0.20
EGE-B-7.3.F	165.29 ± 1.06	-	435.97 ± 0.03	-	9.47 ± 0.05	-	610.74 ± 2.24	5.19 ± 0.19	15.6 ± 0.20
EGE-B-10.3.F**	155.6 ± 0.51	-	352.1 ± 1.00	-	6.57 ± 0.32	-	514.27 ± 1.38	4.96 ± 0.06	54.5 ± 1.65
EGE-B-23.5	140.18 ± 1.92	-	368.4 ± 0.90	-	10.02 ± 0.88	-	518.56 ± 3.66	5.17 ± 0.19	16.1 ± 0.30
EGE-B-1.d.4	124.86 ± 1.86	-	321.1 ± 0.60	-	10.5 ± 0.30	-	455.99 ± 1.19	5.21 ± 0.02	16.8 ± 0.90
EGE-B-1.2.k**	72.02 ± 0.58	-	213.14 ± 2.06	-	-	-	285.16 ± 17.26	4.99 ± 0.06	44.0 ± 1.31
EGE-B-11.7i	114.8 ± 0.30	-	295.76 ± 1.26	-	11.92 ± 0.32	-	422.49 ± 1.59	5.38 ± 0.11	14.3 ± 0.60
EGE-B-24.4i**	93.97 ± 2.13	-	242.91 ± 1.19	-	9.1 ± 0.50	-	345.98 ± 3.75	5.06 ±0.04	95.5 ± 1.83
EGE-B-1.3ka	165.88 ± 2.38	-	364.66 ± 1.54	-	8.42 ± 0.17	-	538.96 ± 8.64	5.28 ± 0.18	19.4 ± 0.40

Table 2. (Continue).

EGE-B-42.3	48.42 ± 0.22	-	138.06 ± 0.86	-	-	-	186.48 ± 2.95	5.42 ± 0.07	48.5 ± 1.25
EGE-B-3.4.F	83.20 ± 0.60	-	246.89 ± 1.69	-	-	-	330.10 ± 2.80	5.17 ± 0.06	8.7 ± 0.10
EGE-B-1.23k	58.19 ± 0.09	-	145.57 ± 0.87	-	-	-	203.76 ± 1.96	5.25 ±0.20	12.8 ± 0.70
EGE-B-45.2	37.98 ± 1.08	-	97.59 ± 0.41	-	-	-	135.58 ± 0.17	5.64 ±0.19	6.9 ± 0.80
EGE-B-13.4	116.02 ± 0.12	-	298.8 ± 0.70	-	12.2 ± 0.40	-	427.02 ± 1.22	5.45 ± 0.32	9.5 ± 0.60
EGE-B-2.14k	29.96 ± 1.46	-	101.8 ± 0.30	-	-	-	131.76 ± 2.16	4.33 ± 0.16	24.0 ± 0.50
EGE-B-21.4i	30.31 ± 0.21	-	70.66 ± 0.16	-	-	-	100.97 ± 1.83	5.33 ± 0.13	9.9 ± 0.70
EGE-B-1.4ka	31.06 ± 1.34	-	87.55 ± 0.35	-	-	-	118.62 ± 1.67	4.57 ± 0.42	14.9 ± 0.80
EGE-B-1.8.d	41.25 ± 0.75	-	97.01 ± 0.91	-	-	-	138.26 ± 1.81	4.15 ± 0.47	20.8 ± 0.80
EGE-B-5.4.F	65.95 ± 0.55	-	203.89 ± 2.29	-	-	-	269.84 ± 2.14	4.47 ± 0.24	17.4 ± 0.60
EGE-B-1.4.a**	152.76 ± 0.86	-	360.9 ± 1.20	-	7.15 ± 0.13	-	520.81 ± 6.49	5.51 ± 0.24	26.6 ± 1.31
EGE-B-3.P.5**	158.06 ± 1.14	-	438.68 ± 1.18	-	10.93 ± 0.27	-	607.67 ± 2.72	5.65 ± 0.06	86.1 ± 1.83
EGE-B-3.81	96.06 ± 0.46	-	249.64 ± 0.04	-	9.47 ± 0.09	-	355.17 ± 2.63	5.64 ± 0.24	12.1 ± 0.30
EGE-B-3.78	148.30 ± 1.10	-	379.1 ± 0.20	-	7.75 ± 0.19	-	542.69 ± 4.21	5.12 ± 0.11	23.2 ± 1.30
EGE-B-10.1.F	107.72 ± 0.48	-	294.54 ± 0.56	-	10.52 ± 0.68	-	412.78 ± 1.42	5.23 ±0.09	16.3 ± 0.20
EGE-B-2.2.d	118.28 ± 1.32	-	268.1 ± 0.50	-	6.49 ± 0.09	-	392.85 ± 0.55	5.24 ± 0.08	16.5 ± 0.30
EGE-B-8.1.F	118.68 ± 1.08	-	331.19 ± 0.91	-	6.88 ± 0.10	-	456.76 ± 0.84	5.82 ± 0.20	24.5 ± 0.40
EGE-B-2.8.a	87.42 ± 2.08	-	-	-	12.97 ± 0.28	-	100.39 ± 1.11	4.61 ± 0.38	9.8 ± 0.70
EGE-B-27.3	117.28 ± 0.18	-	138.45 ± 0.85	-	-	-	255.73 ± 1.07	4.18 ± 0.13	12.5 ± 0.50
EGE-B-1.2.d	69.67 ± 0.43	-	287.26 ± 0.74	-	-	-	356.93 ± 2.33	5.09 ± 0.03	7.4 ± 0.50
EGE-B-3.5.F	44.62 ± 0.68	-	97.86 ± 0.76	-	-	-	142.48 ± 3.32	4.04 ± 0.06	10.7 ± 1.83
EGE-B-1.1.d	35.57 ± 0.37	-	128.47 ± 0.57	-	-	-	164.04 ± 1.24	5.26 ± 0.90	8.9 ± 0.40

HAC: Acetic acid HPR: Propionic acid ISOBUT: Isobutyric acid
ISOCAP: Isocaproic acid HCAP: Caproic acid HEPTA: Heptanoic acid
Note: Values are given as means ± standard deviation (SD) for three replicate samples.
*Total organic acid was the sum of acetic acid, propionic acid, isobutyric acid, isocaproic acid, caproic acid, and heptanoic acid. - : Not detected.
** Six best phosphate-solubilizing *Bacillus* strains selected for further studies

Table 3. Correlation coefficients between soluble Phosphate, pH, and total organic acid.

	pH	Total organic acid (ng μL^{-1})
Soluble phosphate ($\mu g\ mL^{-1}$)	r = –0.459 (P > 0.01)	r = 0.555 (P > 0.01)
pH		r = –0.230 (P > 0.10)

Table 4. Characterization of six selected phosphate-solubilizing *Bacillus* strains.

Phosphate-solubilizing *Bacillus* strains	GenBank accession numbers	Final pH of media	Soluble P ($\mu g\ mL^{-1}$)	Total organic acids (ng μL^{-1})	Organic acids*	IAA ($\mu g\ mL^{-1}$)
Bacillus megaterium EGE-B-1.4.a	KU992628	5.51 ± 0.24a	26.6 ± 1.31f	520.81 ± 6.49b	HAC+ISOBUT+HCAP	3.30 ± 0.10cd
Bacillus megaterium EGE-B-10.3.F	KU992629	4.96 ± 0.06b	54.5 ± 1.65c	514.27 ± 1.38b	HAC+ISOBUT+HCAP	6.05 ± 0.20b
Bacillus simplex EGE-B-1.2.k	KU992627	4.99 ± 0.06b	44.0 ± 1.31d	285.16 ± 17.26d	HAC+ISOBUT	1.50 ± 0.30de
Bacillus subtilis EGE-B-24.4i	KU992626	5.06 ± 0.04b	95.5 ± 1.83a	345.98 ± 3.75c	HAC+ISOBUT+HCAP	27.22 ± 1.76a
Bacillus subtilis EGE-B-26.1	KU992630	4.92 ± 0.08b	33.4 ± 1.42e	201.22 ± 2.60e	HAC+HPR+ ISOCAP	3.78 ± 0.59c
Bacillus subtilis EGE-B-3.P.5	KU992625	5.65 ± 0.06a	86.1 ± 1.83b	607.67 ± 2.72a	HAC+ISOBUT+HCAP	0.80 ± 0.10e

Note: Values are given as means ± SD for three replicate samples. Mean values followed by the same letter(s) in each column indicates that there is no significant difference (Tukey, P ≤ 0.05) between the results of these isolates.
* Detected organic acids given at the end of Table 2.

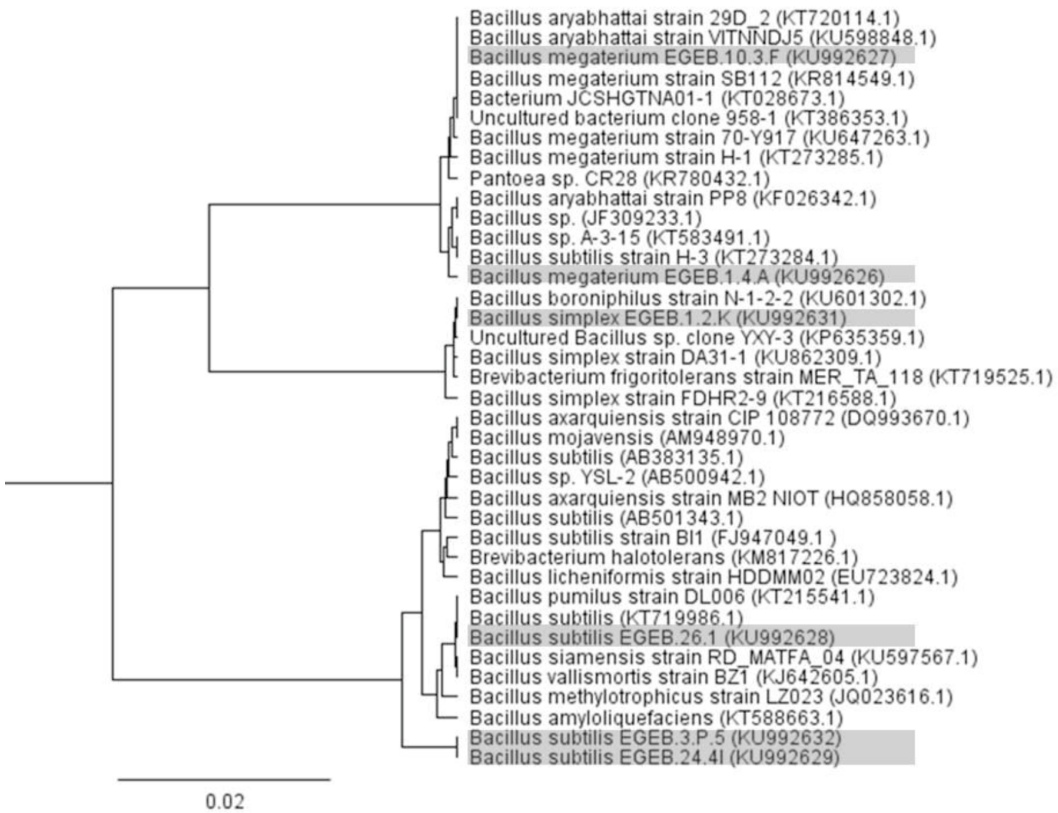

Figure 3. Phylogenetic tree showing the relationships among the *Bacillus* strains. The analysis was conducted using the neighbor-joining method.

Table 5. Seed germination experiment of *Bacillus* strains.

Test seeds	*Bacillus* strains	Radicle (cm)	Hypocotyl (cm)
Eggplant	*Bacillus megaterium* EGE-B-1.4.a	3.13 ± 0.31a	1.63 ± 0.49a
	Bacillus megaterium EGE-B-10.3.F	1.87 ± 0.29b	1.37 ± 0.15ab
	Bacillus simplex EGE-B-1.2.k	1.33 ± 0.32bc	0.87 ± 0.15ab
	Bacillus subtilis EGE-B-24.4i	3.33 ± 0.40a	1.43 ± 0.25ab
	Bacillus subtilis EGE-B-26.1	1.13 ± 0.59bc	1.00 ± 0.40ab
	Bacillus subtilis EGE-B-3.P.5	0.73 ± 0.15c	0.70 ± 0.10b
	Control	0.67 ± 0.21c	0.80 ± 0.10b
Pepper	*Bacillus megaterium* EGE-B-1.4.a	1.33 ± 0.61ab	0.67 ± 0.31ab
	Bacillus megaterium EGE-B-10.3.F	0.93 ± 0.06b	0.93 ± 0.21ab
	Bacillus simplex EGE-B-1.2.k	1.33 ± 0.15ab	0.47 ± 0.31b
	Bacillus subtilis EGE-B-24.4i	2.00 ± 0.36a	1.33 ± 0.32a
	Bacillus subtilis EGE-B-26.1	1.23 ± 0.12ab	1.17 ± 0.35ab
	Bacillus subtilis EGE-B-3.P.5	1.03 ± 0.45b	0.90 ± 0.10ab
	Control	0.77 ± 0.15b	0.43 ± 0.15b
Tomato	*Bacillus megaterium* EGE-B-1.4.a	4.80 ± 0.44ab	5.20 ± 0.20a
	Bacillus megaterium EGE-B-10.3.F	3.53 ± 0.31bcd	3.60 ± 0.44bc
	Bacillus simplex EGE-B-1.2.k	4.57 ± 0.21bc	4.53 ± 0.25ab
	Bacillus subtilis EGE-B-24.4i	6.77 ± 1.02a	3.97 ± 0.47b
	Bacillus subtilis EGE-B-26.1	3.07 ± 1.31bcd	2.73 ± 0.47cd
	Bacillus subtilis EGE-B-3.P.5	2.60 ± 0.56cd	2.40 ± 0.20d
	Control	2.40 ± 0.46d	2.43 ± 0.15d

Note: Values are given as means ± SD for three replicate samples. Mean values followed by the same letter(s) in each column indicates that there is no significant difference (Tukey, $P \leq 0.05$) between the results of these isolates.

As a well-known approach to hunt phosphate from insoluble sources, bacteria release organic acids or chelating agents into the media. In our study, a drop in pH in TCP-containing liquid media was observed for all PSB isolates, which shows an inverse relationship between pH and soluble phosphate concentration. The drop in pH of PSB cultures has also been reported by others (Rashid et al., 2004; Mehta et al., 2015). The drop in pH of the liquid media indicates the secretion of organic acids by different P-solubilizing microorganisms (Whitelaw, 1999; Rashid et al., 2004). The inverse correlation between the pH and soluble phosphate concentration illustrates that organic acid secretion by PSB strains has an important role in the acidification of the media (Zaidi et al., 2009). A similar inverse relationship between pH and soluble phosphate was reported earlier (Delvasto et al., 2008). Fifty-one *Bacillus* spp. isolates tested for volatile organic acid production in our study had a mixed pattern for production of six different organic acids. Studies have shown that the organic acids most commonly produced by different PSB are lactic, succinic, isovaleric, isobutyric, and acetic acids (Chen et al., 2006; Zaidi et al., 2016). Vazquez et al. (2000) reported that different *Bacillus* species (*B. licheniformis*, *B. amyloliquefaciens*, *B. atrophaeus*) have produced volatile and nonvolatile organic acids. *B. amyloliquefaciens* and *B. licheniformis* produced combinations of lactic, acetic, isovaleric, and isobutyric acids. Organic acids, including lactic, citric, glycolic, malonic, oxalic, tartaric, and succinic acid, have also been documented in the PSB (Rodríguez and Fraga, 1999; Vazquez et al., 2000; Chen et al., 2006).

Research has shown that the solubility of phosphate not only depends on pH, but also depends on the type and structure of the organic acid molecule produced by microorganisms (Zaidi et al., 2009). Furthermore, the simultaneous production of more than one organic acid is thought to increase the solubility of phosphate. The literature shows that the total amount of organic acids in the media varies in a range of 1.3 to 2803.77 g mL^{-1} (Ng et al., 2012). Alternatively, the absence of organic acids in the culture filtrates proves that acidification is possibly not the

Table 6. Effect of *Bacillus* inoculations on the root length and biomass of plants.

Seedlings	*Bacillus* strains	Dry root weight (g)	Dry stem weight (g)	Fresh root weight (g)	Fresh stem weight (g)	Root length (cm)
Eggplant	*Bacillus megaterium* EGE-B-1.4.a	41.32 ± 9.58b	9.31 ± 1.39ab	69.27 ± 16.81ab	53.84 ± 20.02ab	30.20 ± 1.10b
	Bacillus megaterium EGE-B-10.3.F	33.35 ± 11.17bc	7.96 ± 0.67abc	69.10 ± 20.30ab	54.20 ± 20.88ab	16.50 ± 1.50d
	Bacillus subtilis EGE-B-24.4i	18.75 ± 0.70bc	7.46 ± 0.44bc	32.03 ± 1.14bc	31.08 ± 1.58b	40.95 ± 2.25a
	Bacillus subtilis EGE-B-26.1	31.78 ± 12.88bc	8.82 ± 0.52ab	68.56 ± 15.29ab	51.98 ± 20.62ab	22.50 ± 0.50cd
	Bacillus subtilis EGE-B-3.P.5	82.90 ± 17.90a	10.12 ± 1.09a	107.00 ± 19.10a	74.6 ± 37.70a	39.40 ± 2.40a
	Bacillus simplex EGE-B-1.2.k	7.97 ± 4.10 c	6.17 ± 0.64 c	18.12 ± 7.38c	22.68 ± 7.10b	24.00 ± 5.00bc
	Control	5.81 ± 0.20c	6.58 ± 0.08c	14.06 ± 5.09c	22.59 ± 10.16b	9.35 ± 2.35e
Pepper	*Bacillus megaterium* EGE-B-1.4.a	5.17 ± 0.15b	6.29 ± 0.21abc	14.40 ± 1.86c	34.93 ± 0.65cd	23.80 ± 0.20a
	Bacillus megaterium EGE-B-10.3.F	2.49 ± 0.36b	6.94 ± 0.76ab	13.87 ± 3.81b	38.28 ± 2.80bc	12.40 ± 1.40bc
	Bacillus subtilis EGE-B-24.4i	14.40 ± 5.42a	8.12 ± 0.57a	16.02 ± 3.22b	45.64 ± 0.30a	24.50 ± 3.50a
	Bacillus subtilis EGE-B-26.1	4.50 ± 2.50b	6.01 ± 0.25abc	60.21 ± 4.93a	40.33 ± 0.37b	12.05 ± 0.25c
	Bacillus subtilis EGE-B-3.P.5	2.67 ± 0.77b	4.99 ± 1.16bc	9.30 ± 0.04bc	35.61 ± 2.46bcd	10.75 ± 1.25c
	Bacillus simplex EGE-B-1.2.k	5.71 ± 2.37b	5.07 ± 1.23bc	16.56 ± 2.36b	32.06 ± 2.10d	16.85 ± 0.85b
	Control	1.12 ± 0.70c	4.11 ± 0.71c	9.19 ± 1.79bc	24.72 ± 2.39e	6.08 ± 1.38d
Tomato	*Bacillus megaterium* EGE-B-1.4.a	14.55 ± 1.10a	7.77 ± 0.12de	23.76 ± 0.24b	50.66 ± 0.21bc	15.00 ± 2.00c
	Bacillus megaterium EGE-B-10.3.F	9.43 ± 0.09b	9.81 ± 0.04ab	28.56 ± 0.57a	51.70 ± 0.10b	19.50 ± 0.50a
	Bacillus subtilis EGE-B-24.4i	8.30 ± 0.20b	7.46 ± 0.19e	20.71 ± 0.27c	48.00 ± 1.00c	19.00 ± 1.00ab
	Bacillus subtilis EGE-B-26.1	13.70 ± 0.40a	10.33 ± 0.87a	29.58 ± 1.90a	59.37 ± 2.84a	16.00 ± 1.00bc
	Bacillus subtilis EGE-B-3.P.5	3.88 ± 0.64c	9.11 ± 0.12bc	13.90 ± 0.20d	32.45 ± 0.10e	18.00 ± 0.50abc
	Bacillus simplex EGE-B-1.2.k	8.68 ± 0.42b	8.77 ± 0.33cd	15.85 ± 0.10d	42.91 ± 0.02d	11.50 ± 0.10d
	Control	0.87 ± 0.02d	4.09 ± 0.01f	3.03 ± 0.07e	20.65 ± 0.20f	7.00 ± 1.50e

Note: Values are given as means ± SD for three replicates. Mean values followed by the same letter(s) in each column indicates that there is no significant difference (Tukey test, $P \leq 0.05$) between the results of these isolates.

key solubilization mechanism; phosphate solubilization was also accredited to chelation and reduction (Sharma et al., 2013).

Our study shows no significant correlation between the final pH of the media and the total organic acid concentration by the *Bacillus* strains. This suggests that our strains may produce other nonvolatile organic acids that were not within the scope of our study. Many studies with phosphate-solubilizing bacteria have revealed that various bacteria can produce lactic, citric, gluconic, malonic, oxalic, tartaric, and succinic acid to dissolve phosphate (Banik and Dey, 1982; Illmer and Schinner, 1992; Rodríguez and Fraga, 1999; Vazquez et al., 2000; Chen et al., 2006). In the present study, soluble phosphate concentration and total organic acid concentration were highly positively correlated ($r = 0.555$, $P < 0.01$); therefore, the results indicate that acidification might be the key strategy used by the PSB isolates for solubilizing phosphate.

IAA, a member of the group of phytohormones, is normally considered the most important native auxin. The most frequently reported mechanisms in plant growth promotion have been the production of the IAA. IAA promotes root size and distribution, ensuring an improved nutrient uptake from the soil. Plant roots contain tryptophan, which can be used by bacteria as a precursor for IAA production. Therefore, IAA measurement is considered a common trait among the characterization of plant-associated bacteria (Liaqat and Eltem, 2016). All the tested isolates in this study have the ability to produce IAA. Various similar studies have shown that IAA production is very common among PGPR (Ng et al., 2012; Islam et al., 2016; Zahid et al., 2015).

The six best phosphate-solubilizing *Bacillus* isolates were molecularly identified by 16s rDNA sequencing to confirm the species. Isolates belonging to same species have shown different phosphate-solubilizing abilities and organic acid production patterns, which indicate that each isolate is independent in their phosphate-solubilizing activities (Table 4). In earlier studies, *B. simplex* was isolated from rhizosphere and soil sources, while none of

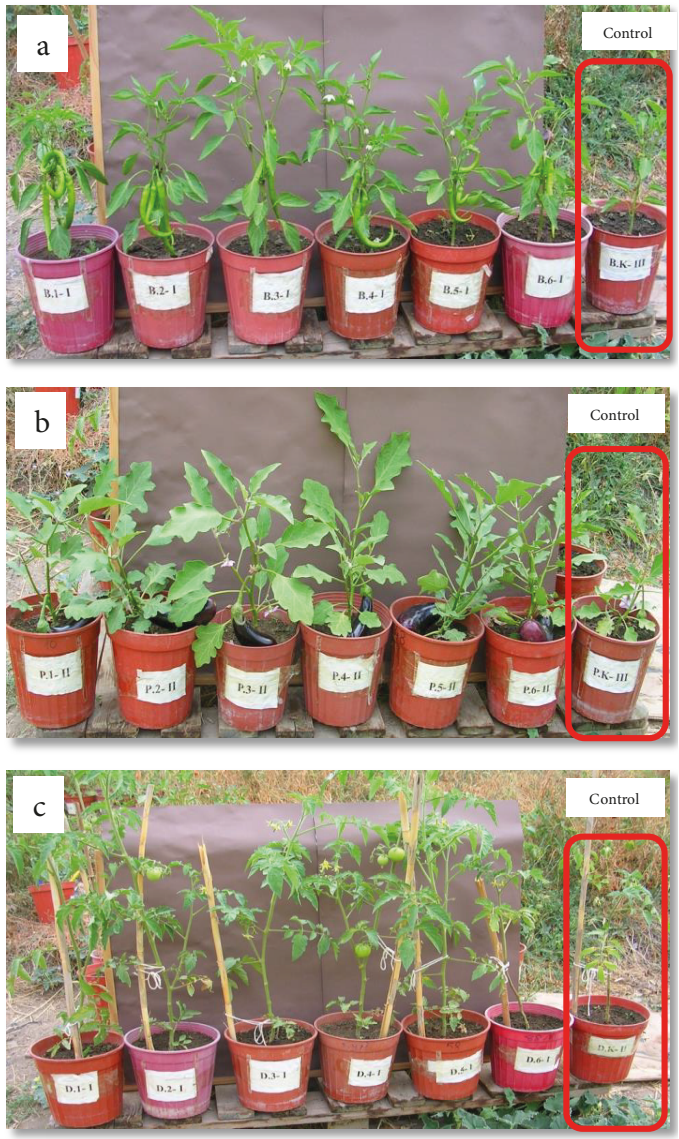

Figure 4. Pot trials with pepper, eggplant, and tomato seedlings (a, b, c respectively).

these isolates had phosphate-solubilizing ability (Erturk et al., 2012; Islam et al., 2016). In contrast, in the present study, the *Bacillus* strain identified as *B. simplex* has a good phosphate-solubilizing ability (44.0 ± 1.31 μg mL^{-1}).

In seed germination and pot trials, treatment with the *Bacillus* strains significantly promoted seed germination and growth of test plants. Root length and IAA production were highly positively correlated in tomato and pepper pot trials ($r = 0.488$, $P < 0.01$; $r = 0.629$, $P < 0.01$ respectively). Generally, an improvement in all the measured plant growth parameters was observed in pot trials. This may be attributed to inorganic phosphate solubilization, IAA production, and other plant-growth–promoting traits. Similar results have been reported in different studies (Bhattacharrya and Jha, 2012).

In the present experiments, different isolates had different effects on the seedlings of three different plants studied. Some isolates increased root and stem development, while others had positive effects on plant development and fruit production. These differences can be attributed to the different growth requirements of the plants, since in the mechanisms of vegetative plant growth each plant needs different basic substances (inorganic phosphate, nitrogen) and different phytohormones. In this study aiming to determine the effects of indigenous *Bacillus* strains on plant growth and development, different strains of *B. subtilis* (*B. subtilis* EGE-B-24.4i, *B. subtilis* EGE-B-26.1, *B. subtilis* EGE-B-3.P.5) had the best results on pepper, tomato, and eggplant in terms of all measured parameters. There are

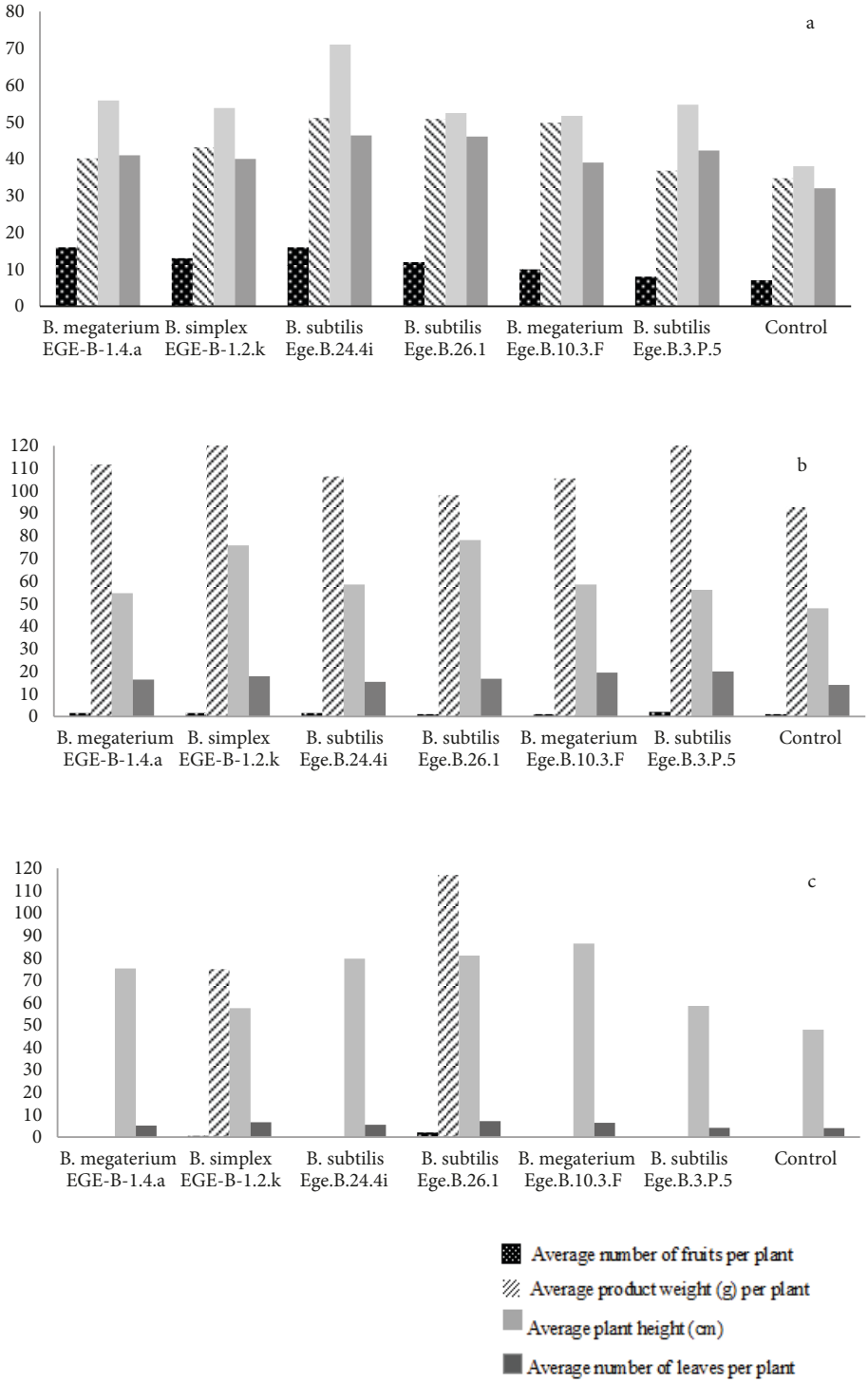

Figure 5. Growth-promoting effects of *Bacillus* strains on a) pepper, b) eggplant, and c) tomato seedlings.

several studies demonstrating the ability of *B. subtilis* strains to promote plant growth in various ways under different conditions using different plant types (Podile and Dube, 1988; Mena-Violante and Olalde-Portugal, 2007; Lamsal et al., 2012). This could be related to IAA production and phosphate solubilization in the strains; however, other mechanisms could also influence the beneficial effects obtained in vivo (Cabra Cendales et al., 2017).

Based on this study, a group of PSB could be considered proficient candidates for improving phosphate availability for crops. With more comprehensive studies related to plant growth promotion and pot trials, they will be endorsed as biofertilizers. The use of novel, natural potential-bearing soil bacteria instead of genetically modified strains is a more convenient approach for easier adaptation in agriculture. Consequently, further field experiments are necessary to increase the productivity and usage of plant-growth–promoting bacteria as biofertilizers.

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