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Phosphate Solubilization Potential of Rhizosphere Soil Bacteria and their Possible Use as Biofertilizers

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PHOSPHORUS is second only to Nitrogen as the most limiting element for plant growth. The concentration of soluble P in soil is usually very low, however, more than 80% of P becomes immobile and unavailable for plant uptake. The development of a biological alternative to chemical fertilizers is of great importance for the improvement of agriculture as well as the protection of the environment. The present study investigated thirteen efficient inorganic-phosphate solubilizing bacteria (PSB) isolated from various rhizospheric soil samples collected from El-Bekaa, Lebanon. Two isolates (*Pseudomonas* sp. HD and *Escherichia* sp. HD), exhibited the highest phosphate solubilization percentages of 76.60 and 88.21%, respectively. Application of the Plackett-Burman design resulted in phosphate solubilization efficiency of 79.08 and 93.77% for Pseudomonas sp. HD and Escherichia sp. HD, respectively. *In vitro* cultivation of different plants was applied to reflect the relative importance of *Pseudomonas* sp. HD and *Escherichia* sp. HD as biofertilizers on growth parameter of melon (*Cucumis melo* var. *inodorus*), horse-bean (*Vicia faba*), cucumber (*Cucumis sativus*), Armenian cucumber (*Cucumis melo* var. *flexuosus*), bean (*Phaseolus vulgaris*) and squash (*Cucurbita pepo*). The best growth parameters of the studied plants where observed where seeds were soaked in water and $Ca_3(PO_4)_2$ with *Pseudomonas* sp. HD and *Escherichia* sp. HD.

Keywords: Biofertilizers, *Escherichia* sp., HD, HD El-Bekaa, Lebanon, Phosphate solubilization, *Pseudomonas* sp.

Introduction

Phosphorus (P) is an essential element in the nutrition of plant. Phosphorus is extremely important as a structural part of many compounds included in photosynthesis, energy transfer, signal transduction, and respiration (Khan et al., 2010). P occurs in soil mainly as insoluble mineral complexes which are not available form for root uptake. Some of these complexes appearing after frequent application of chemical fertilizers (Rengel & Marschner, 2005).

The extended use of chemical P fertilizers results in accumulation of harmful chemical residues, which may lead to serious environmental and health impacts (Tilman et al., 2001), therefore the search for environment friendly feasible alternative strategies for improving crop production in low or P-deficient soils is very important (Zaidi et al., 2009). Biofertilizers, are used to increase the fertility of soil, they help in promoting the growth of plants and trees by increasing the supply of essential nutrients to the plants. They are also responsible for continuous availability of nutrients from natural sources (Suhag, 2016).

Application of microbial biofertilizer is an important step in the biofertilizer technology.

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Phosphate solubilizing organisms are widely spread in the environment; they can belong to bacteria, yeasts and molds in addition to algae. These beneficial organisms are able to convert organic and inorganic insoluble phosphorus compounds to soluble form that can easily be assimilated by plants. Several species of phosphate-solubilizing bacteria (PSB) have been identified including *Pseudomonas* sp. (Rodriguez & Fraga, 1999; Sahandi et al., 2019), *Bacillus* (Ankit et al., 2011), *Rhizobium* (Halder & Chakrabartty, 1993), *Achromobacter, Aerobacter, Flavobacterium, Erwinia* (Goldstein, 1986; Rodriguez & Fraga 1999), *Citrobacter*, *Enterobacter, Proteus, Serratia* (Thaller et al., 1995; Farhat et al., 2009), *Klebsiella* (Ohtake et al., 1996), *Rhizoctonia solani* (Verma et al., 2014) and many other genera. It is well recognized that PSB produce organic acids (Halder et al., 1990). The second known organic acid identified in PSB is 2-ketogluconic acid, which is secreted by *Rhizobium leguminosarum* (Halder et al., 1990), *Rhizobium meliloti* (Halder & Chakrabartty, 1993). Lactic, isovaleric, isobutyric and acetic acids were identified in *Bacillus firmus* (Banik & Dey, 1982). PSB may act through mineralization of organic phosphorus and this is carried out by means of the action of several phosphatases which have the ability to mineralize organic phosphate (Rossolini et al., 1998). Some authors suggested the potential role of siderophores released from phosphate solubilizing microorganisms (Caballero-Mellado et al., 2007; Hamdali et al., 2008). According to Yi et al. (2008), exopolysaccharides (EPSs) excreted by some bacteria may have a role in P-solubilization.

According to Lebanon's first biennial update report to the UN Framework Convention on Climate Change (UNFCCC), consumption of chemical fertilizer in Lebanon increased progressively in recent years (MoE/UNDP/GEF, 2015). The present study was therefore undertaken to isolate P-solubilizing bacteria from the rhizosphere of some plants cultivated in various areas in El-Bekaa, Lebanon, evaluate their P-solubilization efficiency and identify the most potent P-solubilizing isolates. As well as, studying the effect of selected isolates, having high P-solubilizing potential, on the growth parameters and plant nutrition of some cultivated plants.

Materials and Methods

Samples

Thirty soil samples used for the isolation of the phosphate solubilizing bacteria (PSB) were collected from different rhizospheric soil samples at 30cm depth from various areas in Chtaura, El-Bekaa, Lebanon (33° 49' 0" North, 35° 51' 0" East).

Isolation, cultivation and selection of phosphate solubilizing bacteria

Phosphate-solubilizing bacteria were isolated from collected rhizospheric soil samples. One gm of each soil sample was suspended in 9mL sterile distilled water. One hundred µl of each serially diluted (up to 10^{-5}) suspension were plated on Pikovskaya medium (PVK) and National Botanical Research Institute's phosphate growth medium (NBRIP) supplemented with $Ca_3(PO_4)_2$ (TCP) as the sole P source. NBRIP composition was (gm/L): D-glucose, 10; $Ca_3(PO_4)_2$, 5; $MgCl_2 \cdot ٦H_2O$, 5; $MgSO_4 \cdot ٧ H_2O$, 0.25; KCl, 0.2; $(NH_4)_2SO_4$, 0.1; agar, 15; final pH: 7±0.2. PVK composition was (gm/L): D-glucose, 10; $Ca_3(PO_4)_2$, 5; $(NH_4)_2SO_4$, 0.5; NaCl, 0.2; $MgSO_4 \cdot ٧H_2O$, 0.1; KCl, 0.2; yeast extract, 0.5; $MnSO_4 \cdot H_2O$, 0.002; $FeSO_4 \cdot ٧H_2O$, 0.002; agar, 15; final pH: 7±0.2. Plates were incubated for 7 days at 30°C. Colonies showed clear zone around them were purified and preserved on slants containing the same medium of isolation (Mujahid et al., 2015; Li et al., 2019). Isolates were Gram stained.

Thirteen selected isolates were tested to compare their phosphate-solubilizing ability and detect the most potent strain. To prepare a seed culture, transfers from a bacterial slant (24hrs old) were inoculated into 250mL Erlenmeyer flasks containing 50 ml PVK or NBRIP. The culture was grown aerobically by shaking (170rpm) at 30°C for 18hrs until reached an absorbance equivalent to 1 ($A_{600nm} = 1$).

Qualitative estimation of phosphate solubilization

Phosphate solubilizing activity of each isolate was assayed qualitatively by spotting 10µL of each seed culture on the top of NBRIP and PVK media plates. The plates were incubated at 30°C for one week, then the clear halo around the colony was measured. Phosphate solubilization efficiency (PSE) and phosphate solubilization

index (PSI) were determined (Nguyen et al., 1992; Edi-Premono et al., 1996).

Solubilization efficiency (SE)= (Solubilization diameter - Colony diameter)/(Colony diameter) × 100

Solubilization index (SI)= (Solubilization diameter + Colony diameter)/(Colony diameter)

Quantitative determination of phosphate solubilization

Inocula (2%) from seed cultures of selected isolates were introduced to 50mL of liquid PVK or NBRIP dispensed in 250mL Erlenmeyer flasks then incubated at 30°C under shaken condition (170rpm) for 8 days. After incubation, cells were removed by centrifugation and the supernatant was used to determine the amount of soluble phosphorus. This was done by the molybdenum-blue method (Murphy & Riley, 1962). One ml of the supernatant was mixed with 200µL of the reagent (Ammonium molybdate (4%), ascorbic acid (0.1M), sulfuric acid (5N), and 50µL distilled water, heated in a water bath at 60°C for 30min, after cooling the formed blue colour was measured at 827nm against a blank prepared in the same manner using distilled water. A calibration curve was prepared using standard solution of potassium dihydrogen phosphate. The percentage of phosphate solubilization was calculated according to the following formula (Nandish, 2005):

Phosphate solubilisation (%)= (Initial phosphate concentration – Residual phosphate concentration)/(Initial phosphate concentration) × 100

Identification of the most potent isolate

The identification of the most potent isolates was carried using API 20 E (Biomerieux) and gene encodes 16S rRNA sequence analysis. From a single colony, the total genomic DNA was extracted and purified, then 16S rDNA was amplified using 8F as forward primer (AGA GTT TGA TCC TGG CTC AG) and *U1492R* as reverse primer (GGT TAC CTT GTT ACG ACT T) (Shaheen et al., 2016). The PCR product was purified and sequenced. The PCR product was purified using Gene JET™ PCR Purification Kit (Thermo K0701). The purified product was sequenced in GATC Biotech (Germany) using ABI 3730xl DNA sequencer. The sequences obtained were aligned with known 16S rDNA sequences in the Genbank database using NCBI BLAST (Basic local alignment search tool) to generate the percent homology with recorded identified strains. Nucleotide sequences were submitted to GenBank under the accession numbers obtained for each isolate.

Factors affecting phosphate solubilisation by the most potent isolates (PSB8 and PSB13)

Effect of incubation period and inoculum size

The most potent isolates (PSB8 and PSB13) were grown on liquid NBRIP and cultivated under shaken conditions (170rpm) at 30°C for 8 days. Quantitative determination of phosphate solubilisation and bacterial biomass assay were monitored at different incubation period (1-14 days) and when using various inoculum size (1-6%, v/v). The dry weight was determined by harvesting cells from cultures by centrifugation, cells were washed with 0.1N HCl to solubilize the remaining TCP, then washed with distilled water and re-centrifuged. The cell pellets were dried at 60°C until a constant weight was achieved using an analytical balance.

Plackett-Burman design

The Plackett-Burman experimental design, a fractional factorial design, was applied to reflect the relative importance of various factors on phosphate solubilization in liquid cultures. Total number of trials to be carried out according to Plackett-Burman was K + 1, where k was a number of independent variables (medium components and culture conditions). Each variable was represented at two levels, high and low, which were denoted by (+) and (-), respectively (Table 1). The number of positive signs and negative signs per trial were (k + 1)/2 and (k - 1)/2, respectively. Each column contained equal number of positive and negative signs. The main effect of each variable was determined using the following equation:

$$Exi = (\sum Mi+ - \sum Mi-) / N$$

where Exi is the variable main effect, Mi+ and Mi- are the percentages of phosphate solubilization in trials where the independent variable (xi) was present in high or low level, respectively, and N is the number of trials divided by two (Plackett & Burman, 1946). Using Microsoft Excel, statistical t-values for equal unpaired samples were calculated for determination of variable significance.

TABLE 1. Variables and their levels employed in Plackett-Burman design for screening of some factors affecting phosphate solubilization by *Pseudomonas* sp

Symbol	Variable	Low (-)	Basal (0)	High (+)
A	Glucose	5	10	15
B	$Ca_3(PO_4)_2$	3	5	7
C	$MgCl_2.6H_2O$	3	5	7
D	$MgSO_4.7H_2O$	0.15	0.25	0.35
E	KCl	0.1	0.2	0.3
F	$(NH_4)_2SO_4$	0.05	0.1	0.15
G	Volume (ml)	30	50	70

HD and *Escherichia* sp. HD grown on NBRIP

Application of PSB8 and PSB13 as biofertilizers on plants

In vitro cultivation of different plants was applied to reflect the relative importance of most potent isolates (PSB8 and PSB13) as biofertilizers on growth parameter of melon (*Cucumis melo var. inodorus*), horse-bean (*Vicia faba*), cucumber (*Cucumis sativus*), Armenian cucumber (*Cucumis melo var. flexuosus*), bean (*Phaseolus vulgaris*) and squash (*Cucurbita pepo*). Seeds were surface-sterilized with ethanol 95% for 1min, followed by 1% sodium hypochlorite for 30min, washed extensively with sterile water (Collavino et al., 2010). Seeds were grown in 500mL plastic pots with sterile or fresh soil. The seeds were soaked with bacterial cells at a density of about 10^8 colonies forming units (CFU) per milliliter. The experiment was performed under controlled environmental conditions (25±1/20±2°C day/ night temperature) and watered regularly. Treatments were designed in sterile and fresh soil as described in Table 2

TABLE 2. Different treatments applied as biofertilizers on studied plants

Symbol	Treatment
1	Water
2	water and TCP
3	water and TCP with PSB8
4	water and TCP with PSB13
5	water and TCP with PSB13 and PSB8
6	water with nitrogen, phosphorous and potassium (NPK) fertilizer
7	water and NPK with PSB8
8	water and NPK with PSB13
9	water ,water and NPK with PSB13 and PSB8

After 40 days, the plants were harvested. The roots and the shoots were removed and their lengths and dry weights were determined.

Results

Isolation and selection of the most potent phosphate solubilizing bacteria

Thirteen selected phosphate solubilizing isolates were rod-shaped Gram-negative bacteria; their colonies had smooth surface and creamy color. Data represented in Table 3 showed that the maximum phosphate solubilization index (PSI) (3.62) and the maximum phosphate solubilization efficiency (PSE) (162.85) on PVK, as well as the maximum PSI (7.00) and the maximum PSE (500.00) determined on NBRIP, were exhibited by PSB13. The efficiency of PSB13 was followed by that of PSB8 which showed high phosphate solubilization efficiency represented by PSI (3.00) and PSE (100.00) on PVK as well as PSI (5.66) and PSE (366.66) on NBRIP. Therefore, PSB13 and PSB8 were selected for further experiments.

Identification of PSB8 and PSB13 isolated strains

PSB8 was identified using API 20 NE as *Pseudomonas fluorescence* with 99.1 % similarity. PSB13 was identified using API 20 E as *Escherichia vulneris* with 84.2 % similarity. Whereas, the 16S rRNA sequences of PSB8 and PSB13 showed 91 and 96 % similarity with *Pseudomonas fluorescens* F113 (NC 016830.1) and *Escherichia vulneris* NBRC 102420 (GCF_000759795.1) recorded in the databases, respectively. Based on these biochemical and phylogenetic characterization results, the experimental bacterial isolates PSB8 and PSB13 were identified, res9-+pectively, as members of the genera *Pseudomonas* and *Escherichia*; thus, they were named *Pseudomonas* sp. HD (MW390992) and *Escherichia* sp. HD (MW392535), respectively.

TABLE 3. Phosphate solubilization index (PSI) and phosphate solubilization efficiency (PSE) of the bacterial isolates grown on PVK and NBRI

Medium	PSB	Solubilization zone diameter (mm) [Z]	Bacterial colony diameter (mm) [C]	Phosphate solubilization index (PSI)= (Z + C) / C	Phosphate solubilization efficiency (PSE)= (Z - C) / C × 100
PVK	1	10.50	8.20	2.28	28.04
	2	11.00	8.50	2.29	29.41
	3	11.20	9.00	2.24	24.44
	4	10.60	8.10	2.30	30.86
	5	10.50	7.50	2.40	40.00
	6	10.20	7.10	2.43	43.66
	7	12.00	8.10	2.48	48.14
	8	**8.20**	**4.10**	**3.00**	**100.00**
	9	11.00	9.00	2.22	22.22
	10	9.20	7.50	2.22	22.66
	11	10.10	6.10	2.65	65.57
	12	10.20	9.20	2.10	10.86
	13	**9.20**	**3.50**	**3.62**	**162.85**
NBRIP	1	9.20	6.00	2.53	53.33
	2	8.50	5.20	2.63	63.46
	3	10.00	6.00	2.66	66.66
	4	6.20	2.00	4.10	210.00
	5	7.50	2.00	4.75	275.00
	6	11.00	6.90	2.59	59.42
	7	6.00	1.50	5.00	300.00
	8	**7.00**	**1.50**	**5.66**	**366.66**
	9	9.00	5.50	2.63	63.63
	10	9.00	5.00	2.80	80.00
	11	8.10	2.00	5.05	305.00
	12	9.10	6.00	2.51	51.66
	13	**6.00**	**1.00**	**7.00**	**500.00**

Incubation time and inoculum size affecting phosphate solubilization by Pseudomonas sp. HD and Escherichia sp. HD

The findings displayed in Fig. 1 revealed that the highest percentage of phosphate solubilization (73.60 and 87.81%) and the highest biomass (24.05 and 30.05mg/mL) recorded for *Pseudomonas* sp. HD and *Escherichia* sp. HD, respectively, were obtained after incubation for 8 days. Shorter and longer time intervals exhibited least results. The best inoculum size that led to maximum phosphate solubilization by *Pseudomonas* sp. HD and *Escherichia* sp. HD was 4% v/v (76.60 and 88.21%, respectively) (Fig. 2). These conditions were set for further experiment.

Investigation of factors influencing phosphate solubilisation by Pseudomonas sp. HD and Escherichia sp. HD using multi-factorial statistical design: Plackett-Burman design

Phosphate solubilization percentage showed a wide variation throughout trials of the experiment. Trial 2 (Table 4) was found to have the highest influence on phosphate solubilisation. The modified culture medium of Trial 2 contained (gm/L): glucose, 15; $Ca_3(PO_4)_2$, 7; $MgCl_2.6H_2O$, 3; $MgSO_4.7H_2O$, 0.35; KCl, 0.1; $(NH_4)_2SO_4$, 0.05, the volume of culture medium was 70mL/

250mL flask inoculated with 4% v/v inoculum and incubated for 8 days at 30°C under shaken condition at 170rpm. Conditions of Trial 2 showed phosphate solubilization percentages of 79.08 and 93.77%, respectively for *Pseudomonas* sp. HD and *Escherichia* sp. HD which were greater than that recorded using the basal medium (76.60 and 88.21%, respectively for *Pseudomonas* sp. HD and *Escherichia* sp. HD).

The main effect (that was examined as a difference between both averages of measurements made at high level (+1) and the low level (-1) of the factor) of each examined factor affecting phosphate solubilization, was calculated for *Pseudomonas* sp. HD (Table 5) and for *Escherichia* sp. HD (Table 6). The analysis of the regression coefficients of the eight factors clarified that $MgCl_2.6H_2O$, KCl, $(NH_4)_2SO_4$ and culture volume showed a negative effect, whereas the other factors (glucose, $Ca_3(PO_4)_2$ and $MgSO_4.7H_2O$) resulted in a positive effect for *Pseudomonas* sp. HD and *Escherichia* sp. HD. Statistical analysis of the data is demonstrated for *Pseudomonas* sp. HD in Table 4 and for *Escherichia* sp. HD in Table 5 as t-value for the seven experimental variables. The significance level was determined using the student's t-test. The t-test for any individual effect allows an evaluation of the propability of finding the observed effect purely by chance. The factor which showed a high confidence percentage was glucose, where it was the most positive significant variable affecting phosphate solubilization by *Pseudomonas* sp. HD and *Escherichia* sp. HD.

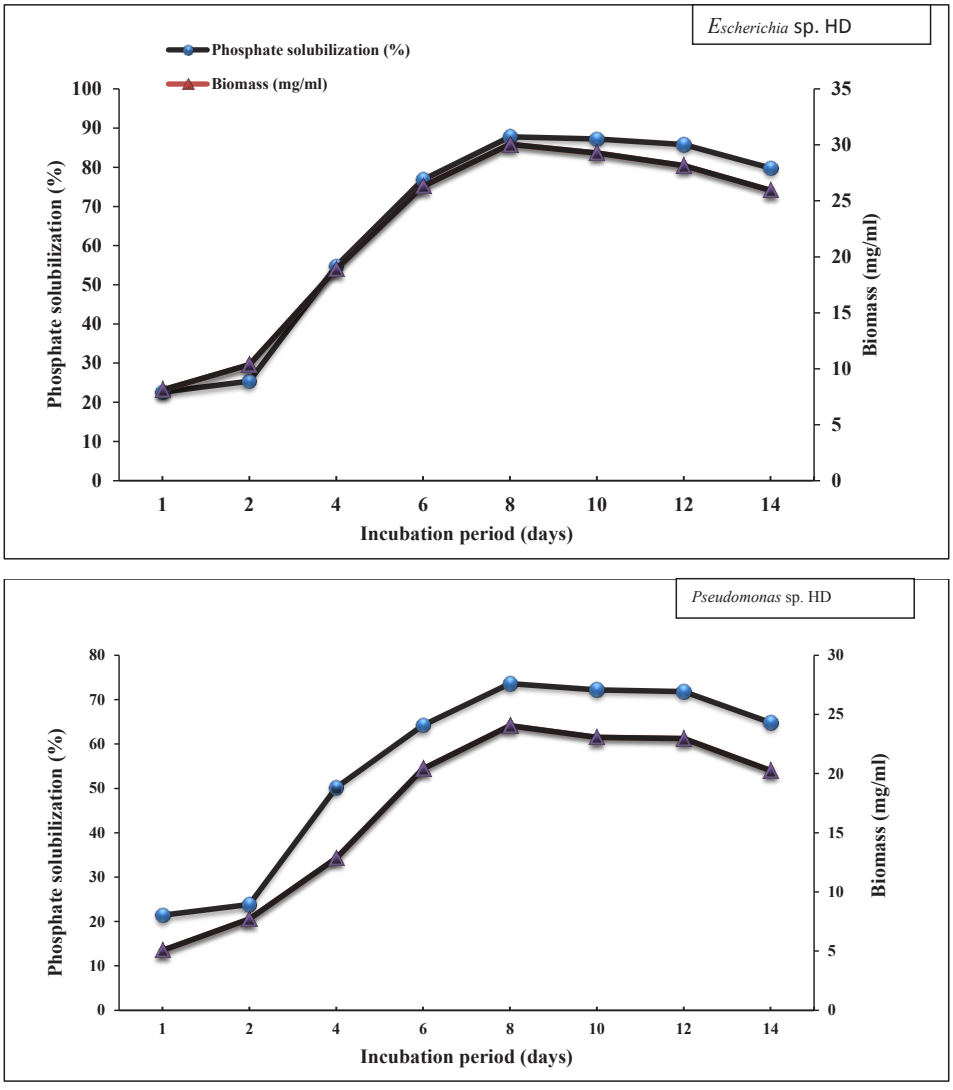

Fig. 1. Effect of incubation period on biomass and phosphate solubilization by *Escherichia* sp. HD and *Pseudomonas* sp. HD grown on NBRIP

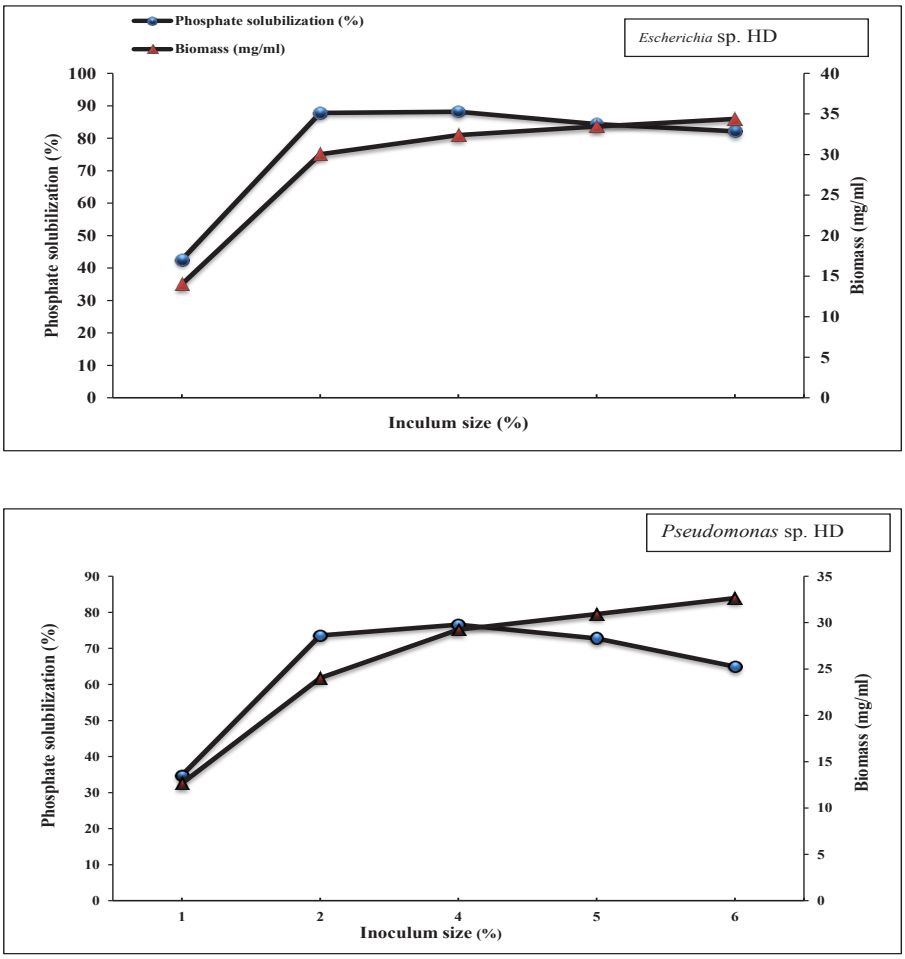

Fig. 2. Effect of inoculum size on biomass and phosphate solubilization by *Escherichia* sp. HD and *Pseudomonas* sp. HD and grown on NBRIP

Application of Pseudomonas sp. HD and Escherichia sp. HD as biofertilizers on plants

The obtained results of the application of *Pseudomonas* sp. HD and *Escherichia* sp. HD as biofertilizers on growth parameters of the studied plants are presented in Table 7. Generally, shoot length, root length, shoot dry weight and root dry weight for melon gave approximately the same results in fresh and sterile soil. In addition, the use of TCP with bacterial inoculants in all treatments gave more promising results than the use of a direct chemical fertilizer NPK. Moreover, the plant growth reached its maximum in treatment 5 (water and TCP with *Pseudomonas* sp. HD and *Escherichia* sp in seed) and its minimum in treatment 1 (water). Same results were observed for other plants.

Discussion

Most of the microorganisms used as biological fertilizers are isolated from the plant rhizosphere (Soumare et al., 2020). In the current study, phosphate solubilizing bacteria were isolated from the rhizosphere soil collected from various areas in El-Bekaa-Lebanon. Screening experiments were performed with thirteen phosphate solubilizing bacterial isolates to explore their potential to solubilize the insoluble tricalcium phosphate supplemented to NBRIP and PVK as the sole P source. The maximum phosphate solubilization efficiency, assessed by PSI and PSE, was exhibited by *Escherichia* sp. HD followed by *Pseudomonas* sp. HD. Kalayu (2019) reported the diverse organic acids released by Gram negative bacteria, which render Gram negative bacteria more effective in dissolving mineral phosphates than Gram-positive bacteria. He mentioned that *Pseudomonas* is among the most efficient P solubilizers.

TABLE 4. Randomized Plackett-Burman experimental design for evaluating factors influencing phosphate solubilization by *Pseudomonas* sp. HD and *Escherichia* sp. HD grown on NBRIP

Variable / Trial	A	B	C	D	E	F	G	P solubilization % by *Pseudomonas* sp. HD	P Solubilization % by *Escherichia* sp. HD	Biomass (mg/mL) by *Pseudomonas* sp. HD	Biomass (mg/mL) by *Escherichia* sp. HD
1	+	+	+	-	+	-	-	70.34	80.21	24.65	30.05
2	+	+	-	+	-	-	+	**79.08**	**93.77**	**26.45**	**33.10**
3	+	-	+	-	-	+	+	69.34	75.01	24.10	29.60
4	-	+	-	-	+	+	+	64.10	72.31	21.20	25.25
5	+	-	-	+	+	+	-	75.32	87.25	25.05	32.15
6	-	-	+	+	+	-	+	60.20	70.50	20.15	24.70
7	-	+	+	+	-	+	-	62.40	70.20	20.05	24.10
8	-	-	-	-	-	-	-	70.25	80.40	22.60	30.25
9	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**76.60**	**88.21**	**25.26**	**32.40**

TABLE 5. Degree of positive and negative effects of independent variables on phosphate solubilization (%) by *Pseudomonas* sp. HD grown on NBRIP according to levels in the Plackett-Burman experiment

Variable		Phosphate solubilization (%)				Mean	Main effect	T-value	Degree of significance (%)
A	+	70.34	79.08	69.34	75.32	73.52	9.2825	2.965372	95
	-	64.10	60.20	62.40	70.25	64.23			
B	+	70.34	79.08	64.10	62.40	68.98	0.2025	0.041204	95
	-	69.34	75.32	60.20	70.25	68.77			
C	+	70.34	69.34	60.20	62.40	65.57	-6.6175	-1.61158	95
	-	79.08	64.10	75.32	70.25	72.18			
D	+	79.08	75.32	60.20	62.40	69.25	0.7425	0.151349	95
	-	70.34	69.34	64.10	70.25	68.50			
E	+	70.34	64.10	75.32	60.20	67.49	-2.7775	-0.58074	95
	-	79.08	69.34	62.40	70.25	70.26			
F	+	69.34	64.10	75.32	62.40	67.79	-2.1775	-0.45044	95
	-	70.34	79.08	60.20	70.25	69.96			
G	+	79.08	69.34	64.10	60.20	68.18	-1.3975	-0.28625	95
	-	70.34	75.32	62.40	70.25	69.57			

TABLE 6. Degree of positive and negative effects of independent variables on phosphate solubilization (%) by *Escherichia* sp. HD grown on NBRIP according to levels in the Plackett-Burman experiment

Variable		Phosphate solubilization (%)				Mean	Main effect	T-value	Degree of significance (%)
A	+	80.21	93.77	75.01	87.25	84.06	10.7075	2.25728	95
	-	72.31	70.50	70.20	80.40	93.45			
B	+	80.21	93.77	72.31	70.20	79.12	0.8325	0.129238	95
	-	75.01	87.25	70.50	80.40	78.29			
C	+	80.21	75.01	70.50	70.20	73.98	-9.4525	-1.82871	95
	-	93.77	72.31	87.25	80.40	83.43			
D	+	93.77	87.25	70.50	70.20	80.43	3.4475	0.547645	95
	-	80.21	75.01	72.31	80.40	76.98			
E	+	80.21	72.31	87.25	70.50	77.56	-2.2775	-0.3568	95
	-	93.77	75.01	70.20	80.40	79.84			
F	+	75.01	72.31	87.25	70.20	76.19	-5.0275	-0.82212	95
	-	80.21	93.77	70.50	80.40	81.22			
G	+	93.77	75.01	72.31	70.50	77.89	-1.6175	-0.25208	95
	-	80.21	87.25	70.20	80.40	79.51			

Phosphate solubilizing microorganisms are able to use phosphate in their metabolic processes, they differ in their requirements for maximal activity. In the present study, *Pseudomonas* sp. HD and *Escherichia* sp. HD cultivated NBRIP medium showed the highest percentage of phosphate solubilization under shaken condition (170rpm) at 30°C. The percentage of phosphate solubilization was influenced by the incubation period; during the first days of incubation, phosphate solubilization percentage increased, to reach the maxiumum level at day 8 (73.60 and 87.81% for *Pseudomonas* sp. HD and *Escherichia* sp. HD, respectively), then decreased at longer incubation period. Such variations in phosphate solubilization were reported by Goenadi et al. (2000) and Laxmi et al. (2015). Reduction in release of soluble phosphorus during later phase of the incubation might be due to the depletion of nutrients in the culture medium, in particular, carbon source needed for the production of organic acids (Chaiharn & Lumyong, 2011). The increase of the inoculum size from 2 to 4 % enhanced the phosphate solubilization by *Pseudomonas* sp. HD and *Escherichia* sp. HD (76.60 and 88.21%, respectively). This may be attributed to the increase of bacterial biomass. Kolekar et al. (2017) found a positive correlation between the microbial biomass and phosphate solubilization.

Plackett-Burman design, applied in the current investigation, showed a great efficiency of phosphate solubilization, since the results demonstrated that the percentage of solubilization was enhanced in Trial 2 comparing with basal, from 76.60 to 79.08% for *Pseudomonas* sp. HD and from 88.21 to 93.77% for *Escherichia* sp. HD. The main effect of each variable was calculated according to the data recorded for the percentage of phosphate solubilization. The analysis of the regression coefficients of the eight factors clarified that $MgCl_2.6H_2O$, KCl, $(NH_4)_2SO_4$ and culture volume gave a negative effect, whereas the other factors (glucose, $Ca_3(PO_4)_2$ and $MgSO_4.7H_2O$) gave a positive effect, for *Pseudomonas* sp. HD and *Escherichia* sp. HD. Also, Verma & Ekka (2017) and Mujahid et al. (2015) have reported that glucose as carbon source and $(NH_4)_2SO_4$ as nitrogen source have the positive effect on phosphate solubilization by PSB.

TABLE 7. Effect of phosphate solubilizing bacteria on growth parameters of the studied plants

Treat-mentsnt	Parameters							
	Shoot length (cm)		Root length (cm)		Shoot dry weight (gm)		Root dry weight (g)	
	Fresh soil	Sterile soil	Fresh soil	Sterile soil	Fresh soil	Sterile soil	Fresh soil	Sterile soil
Melon (*Cucumis melo* var. *inodorus*)								
1	26.30	26.10	1.90	1.80	1.50	1.60	0.30	0.40
2	26.10	26.00	1.80	1.70	1.40	1.50	0.30	0.20
3	27.90	28.10	2.40	2.50	2.10	2.60	0.90	1.10
4	28.50	28.60	2.60	2.80.4	2.30	2.80	1.10	1.20
5	**28.60**	**28.90**	**2.80**	**3.206**	**2.40**	**2.90**	**1.20**	**1.50**
6	27.20	27.50	2.00	1.90	1.70	1.60	0.50	0.60
7	27.30	27.20	2.10	2.10	1.90	1.90	0.60	0.70
8	27.20	27.30	2.30	2.20	2.00	2.10	0.80	0.80
9	27.80	27.60	2.60	2.50	2.10	2.20	0.90	1.10
horse-bean (*Vicia faba*)								
1	27.50	27.00	3.10	3.00	1.50	1.60	0.50	0.60
2	27.30	26.90	2.80	2.70	1.40	1.30	0.30	0.40
3	28.90	28.90	3.70	3.90	2.20	2.20	1.00	1.10
4	29.70	29.50	3.80	4.10	2.30	2.40	1.30	1.40
5	**29.90**	**29.90**	**4.00**	**4.50**	**2.50**	**2.70**	**1.60**	**1.70**
6	27.80	27.90	3.30	3.50	1.70	1.70	0.70	0.70
7	28.30	28.40	3.60	3.60	1.90	1.90	0.80	0.80
8	28.50	28.70	3.80	3.80	2.10	2.10	1.00	1.00
9	28.80	28.90	3.90	3.90	2.20	2.20	1.20	1.20
Cucumber (*Cucumis sativus*)								
1	23.10	23.00	1.30	1.10	1.40	0.90	0.30	0.20
2	22.80	22.70	1.20	1.00	1.20	0.80	0.20	0.10
3	25.10	25.30	2.20	2.40	2.30	2.50	0.90	1.10
4	25.30	25.70	2.40	2.60	2.50	2.60	1.20	1.40
5	**25.60**	**25.90**	**2.70**	**2.90**	**2.80**	**3.00**	**1.50**	**1.70**
6	23.70	23.80	1.60	1.70	1.60	1.20	0.50	0.60
7	23.80	23.90	1.80	2.00	1.90	2.00	0.60	0.60
8	23.90	24.10	2.00	2.10	2.10	2.20	0.70	0.80
9	24.10	24.70	2.10	2.40	2.30	2.40	0.90	0.90
Armenian cucumber (*Cucumis melo var. flexuosus*)								
1	26.20	25.80	1.80	1.50	1.50	1.40	0.30	0.20
2	26.00	25.70	1.60	1.50	1.30	1.30	0.20	0.10
3	28.80	28.90	2.50	2.70	2.10	2.30	0.80	0.80
4	29.50	29.90	2.70	2.90	2.30	2.50	1.00	1.10
5	**29.90**	**30.60**	**2.90**	**3.10**	**2.70**	**2.70**	**1.10**	**1.20**
6	27.00	26.80	2.00	1.90	1.60	1.60	0.50	0.50
7	28.40	28.20	2.00	2.40	2.00	2.20	0.60	0.70
8	28.90	28.60	2.40	2.60	2.20	2.40	0.80	0.80
9	29.20	29.70	2.70	2.90	2.50	2.60	1.00	1.10

TABLE 7. Cont.

Treat-mentsnt	Parameters							
	Shoot length (cm)		Root length (cm)		Shoot dry weight (gm)		Root dry weight (g)	
	Fresh soil	Sterile soil	Fresh soil	Sterile soil	Fresh soil	Sterile soil	Fresh soil	Sterile soil
Bean (*Phaseolus vulgaris*)								
1	67.40	67.70	5.50	5.20	2.30	2.20	0.60	0.50
2	67.90	67.80	5.40	5.20	2.20	2.10	0.50/	0.50
3	69.50	69.70	5.80	5.90	2.50	2.60	0.70	0.80
4	69.70	70.50	5.90	6.10	2.70	2.80	0.90	0.90
5	**69.90**	**70.90**	**6.10**	**6.20**	**2.80**	**2.90**	**1.10**	**1.30**
6	68.50	68.90	5.60	5.70	2.40	2.50	0.60	0.70
7	68.50	69.10	5.60	5.70	2.30	2.40	0.60	0.70
8	68.80	69.50	5.80	5.90	2.50	2.70	0.80	0.80
9	69.10	69.90	5.90	6.10	2.60	2.80	1.00	1.10
Squash (*Cucurbita pepo*)								
1	21.50	20.90	2.50	2.30	1.70	1.50	0.50	0.40
2	20.10	20.30	2.10	2.00	1.80	1.30	0.40	0.30
3	23.60	23.80	2.60	2.80	1.90	1.90	0.70	0.80
4	23.60	23.80	2.20	2.50	1.70	1.60	0.60	0.70
5	23.80	24.30	2.40	2.70	1.90	1.80	0.60	0.80
6	23.40	24.10	2.10	2.40	1.70	1.70	0.30	0.80
7	24.20	24.30	2.30	2.50	1.70	1.80	0.60	0.70
8	24.60	25.10	2.50	2.70	1.80	1.90	0.70	0.70
9	25.20	25.90	2.60	2.90	2.10	1.90	0.90	1.00

(1) Water; (2) Water and TCP; (3) Water and TCP with *Pseudomonas* sp in seeds; (4) Water and TCP with *Escherichia* sp in seed; (5) Water and TCP with *Pseudomonas* sp. HD and *Escherichia* sp in seed; (6) Water with nitrogen, phosphorous and potassium (NPK) fertilizer; (7) Water and NPK with *Pseudomonas* sp in seed; (8) Water and NPK with *Escherichia* sp in seed and (9) Water and NPK with PSB8 and PSB13 in seed.

Development of biofertilizer technology was conducted to reduce the negative side effects of chemical fertilizer application (Aggani, 2013). Biofertilizers made of soil microorganisms gain importance for use in crop production because they are cheap, effective and environmental friendly. They have impact in restoring the soil's natural fertility and protecting it against drought, soil-borne diseases and therefore stimulate plant growth (Suhag, 2016). To minimize the dependence on phosphate fertilizers, inoculation of PSMs in soil or seed is known to enhance solubilization of applied and fixed phosphates in soil, resulting in better crop yield (Selvi et al., 2017). PSB (*Pseudomonas* sp. HD and *Escherichia* sp. HD) were *in vivo* testing as biofertilizers, they were used as microbial inoculants for six plants. Increased shoot length, root length, and shoot and root dry weight of the studied plants were recorded from the seedlings in treatment (5) where water and TCP with *Pseudomonas* sp. HD and *Escherichia* sp. HD were inoculated in seeds. In accordance with this finding, Sarker et al. (2014) found that *Pseudomonas* sp. PSB8 significantly enhanced growth and nutrient uptake by wheat seedlings. Moreover, Omer (2016) reported that maize seeds inoculated with PSB resulted in an increase in biological yield reaching 55.2 and 33% over control for *Pseudomonas geniculata* and *P. aeruginosa*, respectively.

Conclusion

In the present study, the isolated bacterial strains *Pseudomonas* sp. HD and *Escherichia* sp. HD were able to solubilize phosphate (after optimization of the nutritional and environmental factors affecting phosphate solubilization process) with percentages 79.08 and 93.77, respectively, within 8 days at pH 7, 30°C and under shaken condition (170rpm). They are excellent candidates for phosphate solubilization in soil to become available to plants. Regarding environmental

pollution due to excessive use of chemical fertilizers and high costs of P fertilizer production, these bacterial inoculants (*Pseudomonas* sp. HD and *Escherichia* sp. HD) tested may well be used as biofertilizers to enhance sustainable agricultural production.

Conflict of interests: The authors declare no conflict of interest.

Authors contribution: Dr. Salwa: Writing the article and following up its publication, in addition to participating in selecting the research point, planning the experiments and supervising their conduct. She carries out the experiment concerning the application of biofertilizers on plants. Dr. Hoda: Helping in the revision of the article and in revising the results of experiments. Hassan: Conducting the practical experiments of microbiology.

Ethical approval: Not applicable.

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كفاءة بكتريا التربة بنطاق الجذور في إذابة الفوسفات وإمكانية استخدا مها كأسمدة حيوية

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الفوسفات هو ثاني أهم العناصر الكيميائيّة بعد النّيتروجين، وهو ضروريٌّ من أجل نموالنّباتات وتطورها. وبما أنّ نسبة الفوسفات الذائب في التربة محددود جدًّا فإنّنا نحتاج إلى استعمال الأسمدة الكيميائيّة ذات التكلفة الباهظة الثّمن من جهة والّتي تسبّب تلوّث الطّبيعة من جهة أخرى. من هنا فإنّ اللّجوء إلى استعمال الطّرق البيولوجيّة لإذابة الفوسفات أثبتت فعاليّة وأفضليّة مقارنة بالأسمدة الكيميائيّة. ولقد كان الهدف الرّئيسيّ من البحث الحالي هو عزل سلالات من البكتريا قادرة على إذابة الفوسفات بفعاليّة. تمّ عزل ثلاثة عشر نوعاً من البكتريا القادرة على إذابة الفوسفات الغيرعضوي. وقد اظهرت *Pseudomonas* sp. HD و*Escherichia* sp. HD اعلى نسبة مئويّة لإذابة الفوسفات. تمّ تطبيق نظام Plackett-Burman الإحصائيّ بهدف توضيح أهميّة وتأثير بعض العوامل الغذائيّة والبيئيّة المؤثّرة على إذابة الفوسفات وزيادة كفاءة البكتريا في إذابته. فأوضحت النتائج أنّ زيادة الكلوكوز، $Ca_3(PO_4)_2$، $MgSO_4.7H_2O$ وحجم المنبت الغذائي في القارورة، هي العوامل الأكثر تأثيرًا في إذابة الفوسفات. وباستخدام المنبت الغذائي الشّبه مثاليّ تمكّنت *Pseudomonas* sp. HD من إذابة نسبة 79.08% و *Escherichia* sp. HD من إذابة 93.77% من الفوسفات. امتدّت الدّراسة لتقييم عمليّة إذابة الفوسفات بواسطة *Pseudomonas* sp. HD و *Escherichia* sp. HD وتأثيرهما على ستة أنواع من النبات وهي الشّمام، الفول، الخيار، القثّاء، الفاصولياء والكوسى. وقد استعملت خمس عشرة معالجة في حالات متباينة. وقد تبيّن بأنّ المعالجة الّتي تحتوي على الماء وهذان النّوعان من البكتيريا الملقّحة في البذور هي الأفضل من حيثُ النّتيجة الّتي ظهرت على طول الجذوع والجذور للنباتات وأيضًا على الوزن الصّافي لهم، وبناء على ذلك يمكن تطبيق استخدام هذه السلالة البكتريّة لمعالجة نسبة الفوسفات الذّائب في التّربة كأسمدة حيويّة.