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Inoculation with Elite Strains of Phosphate-Solubilizing Bacteria Enhances the Effectiveness of Fertilization with Rock Phosphates

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ABSTRACT

Eight bacterial strains expressing high-levels of rock phosphate solubilization were isolated from Tunisian agricultural soils. 16S rDNA sequencing assigned these strains to four genera, *Pseudomonas, Bacillus, Serratia, and Burkholderia*. Both qualitative and quantitative assays were performed to study their plant-growth promoting abilities. Five strains released inorganic phosphorus up to 600 µg ml^{-1} with rock phosphate as sole phosphorus source. They displayed additional capabilities of producing phytases (16.1–24.8 Uml^{-1}), IAA (till 39.6 µg ml^{-1}) and siderophores (9–81.1%). When tested on *Medicago truncatula* on sterile stand, two strains were discarded since they did not show significant enhancement. The remaining bacterial strains, *Pseudomonas corrugata* SP77, *Pseudomonas koreensis* LT62, and *Pseudomonas frederiksbergensis* G62, were further tested on *Medicago* in two phosphorus-deficient soils amended with Tunisian rock phosphate. The three strains induced significant enhancement in shoot dry weight and nodule fresh weight; however, the best results were observed with SP77 and the consortium. The increase in shoot dry yield ranged from 40 to 134% and from 13 to 87% for soil 1 and soil 2, respectively. The biomineralization of rock phosphates by these elite strains will constitute an eco-friendly alternative to chemical fertilizer, mainly in neutral and alkaline soils.

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Introduction

Phosphate fertilization is a major agricultural research topic. Apart from the fact that phosphorus is the second major nutrient element required by plants, it is one of the most interesting current research issues because of its scarcity, it is considered to be the most limiting nutrient factor for growth and development of legumes in subtropical regions (Kuhad et al. 2011; Raghothama and Karthikeyan 1999). With the concomitant increase in global food demand, securing enough phosphorus will be critical for food security in the future decades (Cordell et al. 2011). Under alkaline conditions, only 10–20% of applied phosphorus fertilizers are reported to be exploited by plants in the year of application (Kaleem Abbasi and Manzoor 2018). The rest reacts with ions, immobilize on the soil matrix or precipitate. Therefore, it cannot be used for crops and might cause environmental pollution (Liu et al. 2018). In order to ensure food security in developing countries, the application of phosphate rocks seems a cheap alternative that can replace the use of expensive chemical inputs. However, in many cases, the application of inorganic P fertilizers alone seems unsustainable (Kaleem Abbasi and Manzoor 2018) due to their low reactivity. Therefore, proper utilization of rock phosphate mixed with a bio-preparation containing viable and sufficient number of efficient phosphate-solubilizing microorganisms (PSM) could play an important role in optimizing the availability of P for the plant. Indeed, PSM adopt various strategies for the mobilization and transformation of inorganic P (Khan et al. 2014). Under in vitro conditions, the solubilization of phosphate compounds by PSM is very common. However, when these isolates are further tested for the direct contribution of phosphorus to the plants, their performance has been contradictory, only very few are true PSM (Bashan et al. 2013; Khan et al. 2007). Hence, the viable alternative of mixed P fertilizers composed with PSM and rock phosphate is the area requiring further work. Therefore, our overall objective of this study is to find a valuable natural and sustainable alternative phosphorus fertilizer mix that could be both inexpensive for farmers and sustainable to minimize the environmental threats.

Because Tunisia is endowed with rock phosphate resources and almost soils are alkaline, it appears interesting to associate PSM with the source of phosphorus in mineral soils of origin. To do so, our specific objectives were to (i) isolate and identify phosphate solubilizing bacteria from

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different Tunisian soils, (ii) characterize their plant growth-promoting abilities *in vitro* and (iii) validate the *in vitro* results *in vivo* on *Medicago truncatula* in different soils.

Materials and methods

Screening by enrichment of phosphate solubilizing bacteria

Phosphate solubilizing bacteria (PSB) were isolated from soil samples obtained from different geographic Tunisian sites (Table 1). One gram of soil sample was incubated on National Botanical Research Institute's phosphate (NBRIP) broth medium (Nautiyal 1999) with shaking for 7 days at 28 °C and then serially diluted. Appropriate soil dilutions (10^{-6}) were plated in triplicates on the same medium supplied with agar and containing 5 g of tricalcium phosphate $Ca_3(PO_4)_2$ (TCP) as a sole phosphorus source. Colonies forming clear halos were selected as phosphate solubilizing bacteria. A solubilization index was calculated according to Premono et al. (1996) as follows: SI = (diameter of the colony + diameter of the halo)/diameter of the colony. All the isolates were conserved in glycerol at −80 °C for further use.

Quantitative estimation of soluble phosphate

Tunisian rock phosphate (TRP) extracted from the geological deposit of Gafsa belongs to the carbonate-fluorapatite group. It can hold up to 30% P_2O_5 and it is characterized by a high reactivity and CaO content of 45–50%. In order to quantify the bio-mineralization of TRP by selected bacterial strains, Erlenmeyer flasks containing 25 ml of NBRIP broth supplemented with TRP (5 g/l) were inoculated with the phosphate solubilizing bacterial isolates (10^9 CFU ml^{-1}) in triplicate. Non-inoculated medium served as control. Mineralization of tricalcium phosphate (TCP) was used as a reference. The flasks were incubated in a shaker incubator at 28 °C and 150 rpm. At the 15th day, 5 ml of bacterial culture samples were collected and centrifuged at 10000 rpm for 10 min. The supernatant was used to estimate released phosphate spectrophotometrically at 880 nm according to the standard molybdenum blue method of Murphy and Riley (1962). Phosphate content was estimated by comparison with a standard curve prepared using serial dilutions of KH_2PO_4.

Molecular identification of phosphate solubilizing bacteria

Phosphate solubilizing bacteria were identified on the basis of their 16S rDNA gene sequences. Primers fD1 and rD1 (Weisburg et al. 1991) were used to amplify 16S rRNA genes as previously reported (Mhamdi et al. 2002). PCR-amplified products were purified from agarose gels using the EZ-10 spin column DNA gel extraction kit (Bio Basic Inc.) following the manufacturers' instructions. Purified fragments were sequenced on an ABI3130 genetic analyzer (Applied Biosystems). Sequences were assembled by the CAP program (http://doua.prabi.fr/software/cap3) and checked manually. The accession numbers are given in Table 1. The BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to search for sequence similarities in DNA databases.

In vitro characterization of plant growth-promoting abilities

Determination of phytase activity

Isolated strains were subjected to quantitative screening for the phytase production using Bergersen minimal medium (Bergersen 1961) in a rotator shaker for 7 days at 37 °C. The phytase activity assay was carried out using the supernatant according to Singh et al. (Singh et al. 2013). The reaction consisted of incubating 0.2 ml of the supernatant enzyme solution with 0.8 ml of 2 mM acetate buffer pH 5.5 containing 1 mM sodium phytate. After incubation at 37 °C for 30 min, the reaction was stopped by adding 10% trichloroacetic acid. The absorbance of the liberated inorganic phosphate was quantified by spectrophotometry at 660 nm. One enzyme unit (U) is the amount of enzyme liberating 1 µg of inorganic phosphate in 1 min.

Indolic acids and siderophore production

Bacterial strains were incubated for 5 days at 28 °C with shaking at 150 rpm in 10 ml of sucrose minimal salts medium (SMS) (sucrose, 10 $g{\cdot}L^{-1}$; $(NH_4)_2SO_4$, 1 $g{\cdot}L^{-1}$; K_2HPO_4, 2 $g{\cdot}L^{-1}$; $MgSO_4{,}7H_2O$, 0.5 $g{\cdot}L^{-1}$; yeast extract, 0.5 $g{\cdot}L^{-1}$; $CaCO_3$, 0.5 $g{\cdot}L^{-1}$; NaCl, 0.1 $g{\cdot}L^{-1}$ and pH 7.2). Another set was supplemented with 0.5 mg ml^{-1} of tryptophan (Gordon and Weber 1951). Cells and supernatants were separated by centrifugation at 13000 g for 10 min and 1 ml of supernatant was mixed with 100 ml of orthophosphoric acid (10 mM) and 2 ml of Salkowski reagent (2% 0.5 M $FeCl_3$ in 35% perchloric acid) and incubated at 25 °C in darkness for 30 min (Gordon and Weber 1951).

Table 1. Identification of phosphate solubilizing isolates based on 16S rDNA gene.

Isolate	Origin	pH	Accession number	Closest type strain	Similarity%
LR88	Khnis (35° 35.71′N, 10° 10.81E)	7.91	KX374898	*Serratia liquefaciens*ATCC 27592^T	99.86
LT58	Khnis	–	KX374895	*Pseudomonas spp.*	100
LT62	Khnis	–	KX374894	*Pseudomonas koreensis* ***	99.2
SP77	Khnis	–	KX374896	*Pseudomonas corrugata*CFBP 5454^T	99.85
65B	Tabarka (8° 45.29′E, 36°57.16′N)	6.32	KX374897	*Bacillus safensis****	100
1A	Tabarka	–	KX374893	*Burkholderia spp.*	100
G62	Gafsa (34° 34.31′N, 8° 8.43E)	7.9	KX374899	*Pseudomonas frederiksbergensis* DSM 13022^T	99.7
W62	Siliena (36° 0.383′N, 9° 26.04′E)	7.64	KX374900	*Bacillus circulans* NBRC 13626^T	99.61

Development of pink-red coloration indicates IAA related compounds production, the optical density (OD) was recorded at 530 nm. The concentration in the supernatant was determined using a standard IAA solution. Siderophore production was quantified using Chrome Azurol Sulfonate (CAS) medium (Schwyn and Neilands 1987). Bacteria were grown in Bergersen minimal medium at 27 °C to reach about 10^9 cells ml^{-1}. Fresh bacterial supernatant was mixed with CAS solution by vortexing (1:1; *v:v*) and incubated at room temperature in the darkness for 20 min. The absorbance was measured at 630 nm, Bergersen medium was used as blank. Siderophore production was estimated following the formula described by Castellano-Hinojosa et al. (2016) as follow:

% siderophores units
= [(absorbance of the reference solution
− absorbance of the sample)/absorbance of the reference solution]/100

Hydrogen cyanide (HCN) production

Bacterial hydrogen cyanide production was measured qualitatively according to Feigl and Anger (Feigl and Anger 1966) with some adaptations. Feigl-Anger discs were prepared by dipping sterile Whatman filter paper in chloroform supplemented with copper ethylacetoacetate and tetra base (4,4′ tertrame-thyldiaminodiphenylmethane). Bacteria were streaked on Luria-Bertani (LB) agar plates. Discs were placed on the lid of the Petri dishes, sealed with parafilm and incubated at 28 °C for 5 days. A change from white to blue color of the Feigl-Anger discs indicated positive HCN production.

Tolerance against abiotic stresses

A pre-culture of each strain was prepared in yeast extract mannitol broth medium (YEM) by shaking at 150 rpm at 28 °C for two days. Then, an equivalent of 0.04 U of OD was inoculated to 100 ml YEM supplemented with varying concentrations of NaCl or PEG6000 (Mrabet et al. 2011). NaCl concentrations tested were 0.6, 1, 1.5, and 1.7 M. PEG6000 concentrations tested were 15, 20, 25, and 30%. Growth on different pHs from 3 to 11 was also tested. Non-inoculated YEM medium was used as control. Bacterial growth was checked by measuring the optical density at 620 nm in a spectrophotometer after 72 h. Four replicates were considered for each treatment.

Antibiosis test

In vitro inhibition of the strains against each other was evaluated by the overlay plate technique (Oresnik et al. 1999). For each strain, a culture of 48 h (OD_{630nm}=1) in liquid YEM medium was diluted to 10^3 in 5 ml of soft YEM medium and transferred on solid YEM layer in Petri dishes (indicative strain). Up to solidification, 3 ml of a saturated culture of each of the other strains was spotted on the agar surface (producing strains). Results were observed after 5 days of incubation at 28 °C. Antibiosis inhibition was detected with the presence of a halo surrounding the spotted strains. Each antibiosis test consisted of three replicates in two independent experiments.

Greenhouse experiments

Effect of PSB on Medicago truncatula grown on sterilized sand

To complement the *in vitro* study, a controlled experiment was conducted in plastic pots containing 2 kg of sterilized sand to evaluate the effect of the selected PSB as bio-inoculants for promoting growth and nodulation of *Medicago truncatula.* Five independent bacterial strains and the consortium were tested in two sets with four replicates for each treatment (control, LR88, LT62, SP77, G62, W62, and consortium of the five strains). In the first set, *Medicago truncatula* plants were grown on sterilized sand without any fertilization. In the second set, plants were fertilized with TRP at the rate of 150 Kg ha^{-1}. This fertilization rate was retained on the basis of previous experiments (Trabelsi et al. 2017). Seeds of *M. truncatula* cv. Jemalong were surface disinfected by submerging in sulfuric acid H_2SO_4 (95%) for 3 min and washed abundantly with distilled water. After 6 h, soaked seeds were transferred in Petri dishes with filter paper and kept in darkness at 4 °C overnight and at room temperature during 2 days for germination (Jalali et al. 2018). PSB selected strains were grown in 100 ml of yeast extract mannitol broth medium (YEM) and incubated in a rotator shaker (150 rpm) for 48 h at 28 °C. The number of cells was counted with a Flow cytometer (C6 cube, Sysmex). Bacterial inoculants containing approximately 10^9 cells ml^{-1} were used to inoculate 10 days old plants. *Ensifer meliloti* strain TII7 was used as a basic rhizobial inoculant for all treatments. Plants were kept in a growth chamber under controlled conditions (25 °C/20 °C (day/night), a relative humidity of 60% and a photoperiod of 16/8 h) and watered with modified Hogland nutrient solution with Pi (0.13 mM) and free of nitrogen (Arnon and Hoagland 1940). Plants were harvested at flowering stage after three months and subjected to nodule and shoot dry matter weighting. The estimation of the plant response to inoculation with PSB in terms of biomass enhancement was calculated using the following formula:

Growth enhancement : GE (%)
= (shoot dry weight of treated plants
– shoot dry weight of control plants)/
shoot dry weight of control plants × 100

Effect of combined TRP-PSB on Medicago truncatula in phosphorus deficient soils

In order to assess the capacity of PSB in releasing soluble phosphate from TRP under soil conditions, a complementary second experiment was conducted with the best bacterial strains selected from the first experiment on sand. TRP was applied for all the treatments at the rate of 150 Kg ha^{-1}

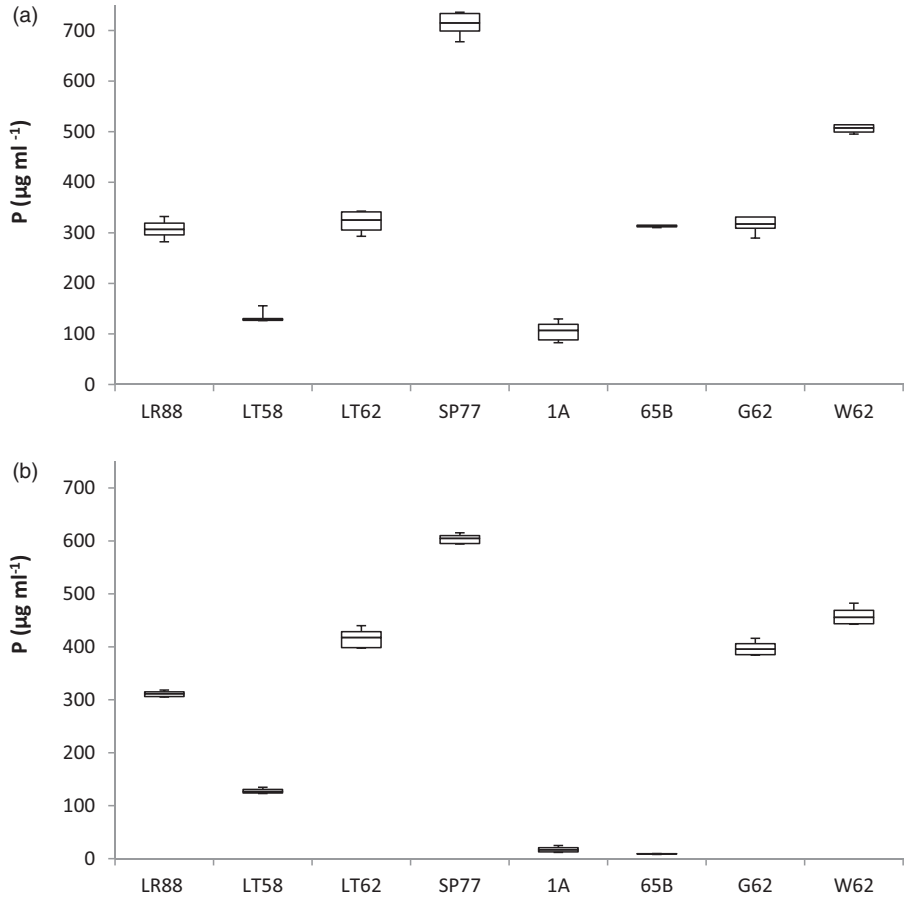

Figure 1. Boxplots representing quantitative estimation of soluble phosphate by bacteria after 15 days of incubation in NBRIB medium with different phosphorus forms (a) tri-calcium phosphate $Ca_3(PO_4)_2$, (b) Tunisian rock phosphate. Error bars represent standard error; medians are displayed together with 25th and 75th quartiles.

four weeks before seeding to assist dissolution. Five treatments were conducted (Non-inoculated control, LT62, SP77, G62) with four replicates for each treatment. The experiment was conducted under controlled conditions in the glasshouse (65% relative humidity, 28 °C and 16/8 h photoperiod). *Medicago truncatula* plants were grown in 2 kg plastic pots containing phosphorus-deficient soils collected from the region of Siliana (clay loam, pH 7.64, total N (%) 1.36, P (mg Kg^{-1}) 14, C (%) 1.08 and EC (µs cm^{-1}) 214) and the region of Takelsa (sandy clay loam, pH 6.89, total N (%) 0.45, P (ppm) 13, C (%) 0.62 and EC (µs $cm^{-1)}$ 169). After 10 days, plants were inoculated with 10^9 CFU ml^{-1} of the efficient PSB selected from the previous experiment and watered twice a week by sterile distilled water. After three months, (flowering stage, period of maximum P demand), nodule fresh weight and shoot dry weight were determined. Phosphorus content was assessed from dry shoots with Olsen method following the protocol reported by Zasoski and Burau (1977).

Statistical analysis

Four replicates were considered for plant growth promotion parameters (Phosphorus solubilization, phytase, siderophore, HCN, and indolic acids). Three replicates were considered for shoot dry weight, nodule fresh weight, and shoot phosphorus content. Results were subjected to one-way analysis of variance (ANOVA) and mean comparison by the Tukey's HSD ($p < 0.05$) test using the statistical software SPSS 20.

Results

The in vitro selection of elite PSB

A total of 170 bacterial isolates exhibiting phosphorus solubilizing activity were obtained from seven different soils in NBRIP plates. According to their solubilizing index, eight bacterial strains having SI >2.2 were selected. Sequencing of 16S rRNA genes allowed the classification of the eight strains of PSB in four genera: *Pseudomonas*, *Bacillus*, *Serratia,* and *Burkholderia* (Table 1). PSB was then screened in NBRIP broth medium supplied with two phosphorus forms (TCP and TRP) to evaluate their phosphate solubilizing efficiency. The quantitative estimation of the phosphate solubilizing ability of some strains significantly varied according to the P source (Figure 1). The phosphate solubilizing ability of the strains ranged from 107 µg ml^{-1} to 715 µg ml^{-1} with TCP as phosphorus source and from 9.2 µg ml^{-1} to 605 µg ml^{-1} with TRP. *Pseudomonas corrugate* SP77 expressed the highest values of solubilized phosphorus with the two phosphorus forms (TCP and TRP). However, no P solubilization was observed with

Table 2. Plant growth promoting abilities of selected phosphate solubilizing isolates.

Strains	TCP Solubilization ($\mu g\ ml^{-1}$)	Phytase activity ($U\ L^{-1}$)	Total indoles ($\mu g\ ml^{-1}$)	Siderophore production (%)	HCN	Growth on 1M NaCl	Growth on PEG 30%	Growth on pH 4
Pseudomonas corrugata SP77	714.96	23.02	39.6	81.1	+	+	+	+
Bacillus circulans W62	507.4	16.11	25	50.16	−	+	−	+
Pseudomonas koreensis LT62	325.21	22.85	31.63	52.65	+	+	+	−
Pseudomonas frederiksbergensis G62	317.33	18.12	31.11	63	+	+	−	−
Serratia liquefaciens LR88	306.74	24.84	11.52	9	−	+	+	+

(−) test achieved but result is negative.

*Burkholderia sp.*1A and *Bacillus safensis* 65B with TRP as a sole phosphorus source. Five isolates showing solubilization levels of TRP higher than 300 $\mu g\ ml^{-1}$ (strains SP77, LR88, LT62, G62, and W62) were selected for the *in vitro* assessment of plant growth-promoting abilities (Table 2). All strains were found to be positive for the production of phytases (16.1–24.8 $U\ ml^{-1}$) and indolic acids (11.5–39.6 $\mu g\ ml^{-1}$). Three strains demonstrated the capacity to synthesize HCN and four were able to produce and secrete high concentrations of iron-chelating compounds (CAS). Interestingly, *Pseudomonas corrugate* SP77 exhibited the highest plant growth-promoting abilities in terms of phosphorus solubilization, indolic acids, phytases, and siderophores.

In vitro antibiosis assays showed that the five retained PSB are neither sensitive to each other nor to the *Ensifer meliloti* TII7 used as rhizobial inoculant (data not shown). Thus their use independently or conjointly is supposed to not cause any undesirable interference.

The in planta selection of PSB

The five PSB strains selected on the basis of their *in vitro* PGPR abilities were assessed further on *M. truncatula* grown on sterilized sand with or without additional fertilization with TRP. The five strains were used separately or conjointly as a consortium (Figure 2). Without TRP fertilization, four strains out of the five induced significant increase in shoot dry yield. *Pseudomonas corrugata* SP77 and the consortium showed the most significant effects on plant growth. However, *Serratia liquefaciens* LR88 did not induce any significant enhancement.

Under TRP fertilization, only three strains out of the five and the consortium induced a significant increase in shoot dry yield comparing to the non-inoculated control. Likewise, the most active P-solubilizing strain SP77 and the consortium gave the best results in shoot dry yield and nodule fresh weight. *Serratia liquefaciens* LR88 and *Bacillus circulans* W62 did not induce any significant increase in plant growth, albeit showing potentialities *in vitro* including the liberation of important quantities of soluble phosphorus from TRP.

Effect of combining TRP and PSB on M. truncatula grown on phosphorus-deficient soils

The most effective strains from the previous experiment on sterile sand (LT62, SP77 and G62) were selected to further assess their effect on *M. truncatula* grown on phosphorus deficient soils amended with TRP. Inoculation with the three PSB significantly increased shoot dry yield of *M. truncatula.* The most effective strains were SP77 and G62 together with the consortium of the three strains. Both strains and consortium increased also nodule fresh weight and phosphorus content (Figure 3).

Discussion

Phosphorus solubilization is one of the most important mechanisms through which PGPR promote plant growth. PGPRs also increase root area to facilitate the uptake of plant nutrients from the soil (Castellani-Hinojosa and Bedmar 2017). In the present study, we have obtained a collection of eight phosphate solubilizing bacteria isolated from different Tunisian alkaline soils. No antagonist effects were found between them. The TCP solubilization by these isolates ranged from 107 $\mu g\ ml^{-1}$ to 715 $\mu g\ ml^{-1}$. Among the isolates, *Pseudomonas corrugata* SP77 was the best P-solubilizing bacterium. To our knowledge, SP77 exhibited the highest capacity reported for solubilizing TCP in liquid NBRIP medium. In their study, Kumar et al. (2013) obtained a collection of PSB from Himalayan soils with phosphorus solubilization values ranging from 193 to 642 $\mu g\ ml^{-1}$. Similarly, Oves et al. (2013) stated solubilization of 417 $\mu g\ ml^{-1}$ for *Pseudomonas aeruginosa* strain isolated from the heavy metal contaminated water. From maize rhizosphere, Zhao et al. (2013) reported a maximum of 452 $\mu g\ ml^{-1}$ with *Burkholderia cepacia*. However, for *Serratia sp.* isolated from mangrove soils, Behera et al. (2017) have reported a maximum of phosphate solubilizing activity of 45 $\mu g\ ml^{-1}$.

For better quantification of the phosphorus solubilization efficiency of the selected PSB, we qualified a second phosphorus form, TRP. Indeed, TCP is an amorphous mineral easily battered by microorganisms commonly used in literature, while Tunisian igneous rocks contain apatite crystalline structures which are much more resistant to corrosion and recalcitrant to chemical weathering (Bashan et al. 2013).

The higher P release was produced by *Pseudomonas corrugata* SP77. Nevertheless, *Burkholderia sp.* strain 1 A and *Bacillus safensis* strain 65B did not solubilize TRP, albeit they solubilized TCP. Indeed, several bacterial strains produce organic acid from the metabolism of sugars by digesting the glucose to strong gluconic or 2-ketogluconic acids (Bashan et al. 2013). Consequently, they might be able to dissolve TCP but fail to dissolve more complex forms of

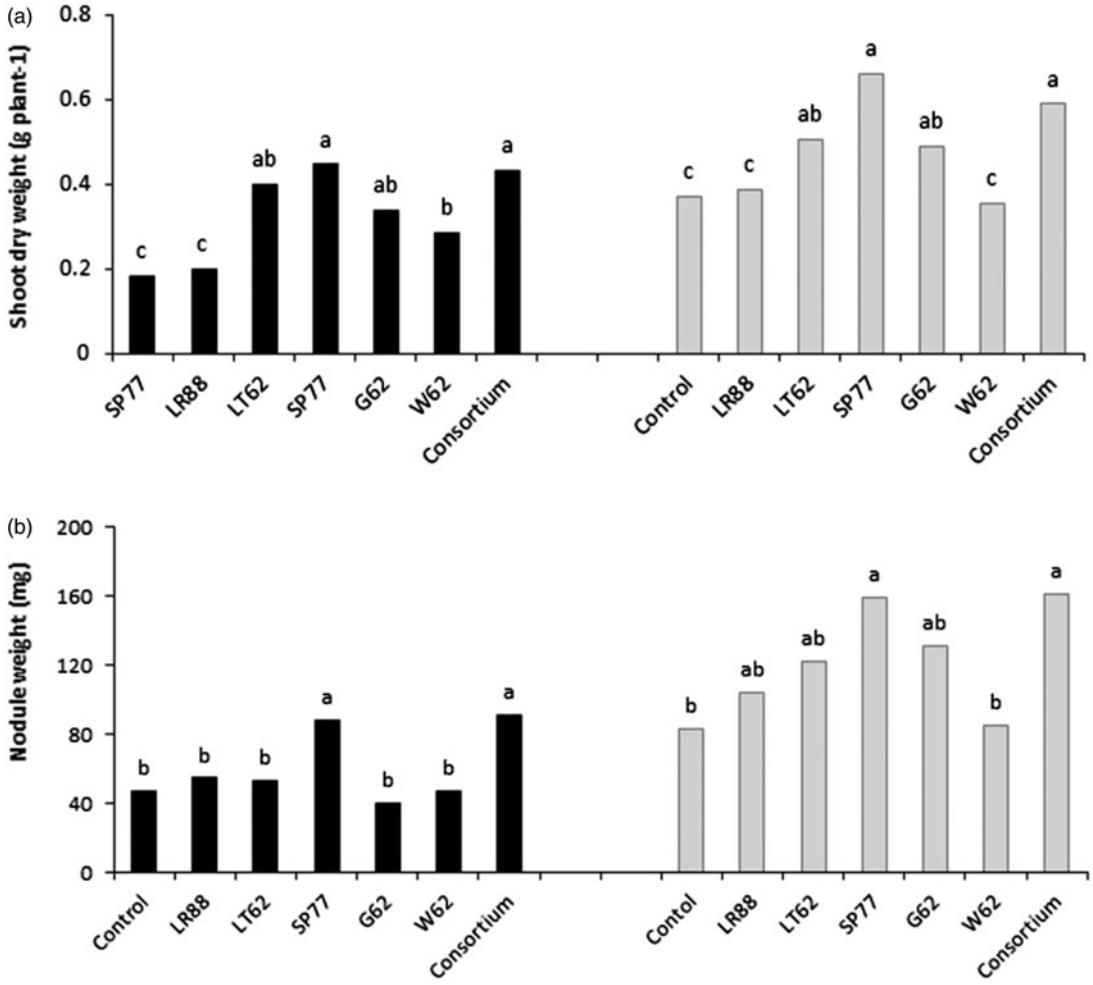

Figure 2. Effect of the inoculation of *Medicago truncatula* with selected PSB strains on: (a) shoot dry weights and (b) nodule weight. Non fertilized treatments are represented in black and treatments fertilized with Tunisian rock phosphate (TRP) are represented in grey. Means with different letters are significantly different (HSD test, $p < 0.05$, $n = 3$).

phosphorus and therefore they might be not able to promote plant growth. Several authors have reported that is no correlation between potential PSB isolated on TCP and their ability to promote plant growth (Bashan et al. 2013; Collavino et al. 2010; Taurian et al. 2010).

Besides P-solubilizing capacity of PSB, it is recognized that the phosphatases and phytases are one of the key enzymes involved in P mineralization which can contribute substantially to increase available P to plants. In the last few years, more emphasis has been given to the phytase production by microorganisms since it is the biggest potential source of phytase production (Simoes Nunes and Kumar 2018). Our results showed a higher phytase activity for *Serratia liquefaciens* LR88 (24.8 U L^{-1}) and *Pseudomonas corrugata* SP77 (23 U L^{-1}). Recently, Jorquera et al. (Jorquera et al. 2017) reported extracellular phytase activities ranging from 0.2 to 55.6 U L^{-1} for bacteria from hydrothermal environments.

Our strains showed also other potential features including phytohormone production. The production of auxins was one of the principal PGPR traits used to discriminate between strains. Indeed, auxins stimulate plant root development and root hair elongation which increase the absorptive surface area of plants (Walpola and Yoon 2013). In our study, the estimation of IAA level in bacterial broth cultures revealed that all selected bacteria produced indolic acids in higher quantities in the presence of the precursor L-tryptophan. *Pseudomonas corrugata* SP77 was the best producer of indolic acids with a rate of 39.6 µg ml^{-1}. This level of production of indolic acids is considered very high according to levels commonly reported in the literature. Arfaoui et al (2018) described the highest auxin production of 28 µg ml^{-1} for *Bacillus* and *Streptomyces* after seven days of growth. Also, from phytase producing bacterial isolates, Singh et al. (2014) reported a maximum of auxin production of 35 µgml^{-1} by *Advenella*. On the other hand, the production of siderophores was detected in all isolates and ranged from 6 to 81%. *Pseudomonas corrugata* SP77 had the highest production. The production rate of siderophores by this strain is considered high even when compared to levels commonly reported in bibliography. Moreover, the results of the qualitative test of HCN production showed that just 3 isolates were able to produce HCN. This volatile compound acts as a biocontrol agent and contributes to plant resistance (Takos et al. 2010). In oligotrophic alpine environments, HCN was involved in mineral mobilization and especially increased the availability of phosphorus (Rijavec and Lapanje 2016). Recently, HCN production ability was revealed in different bacterial genera (Shameer and Prasad 2018). Moreover, the

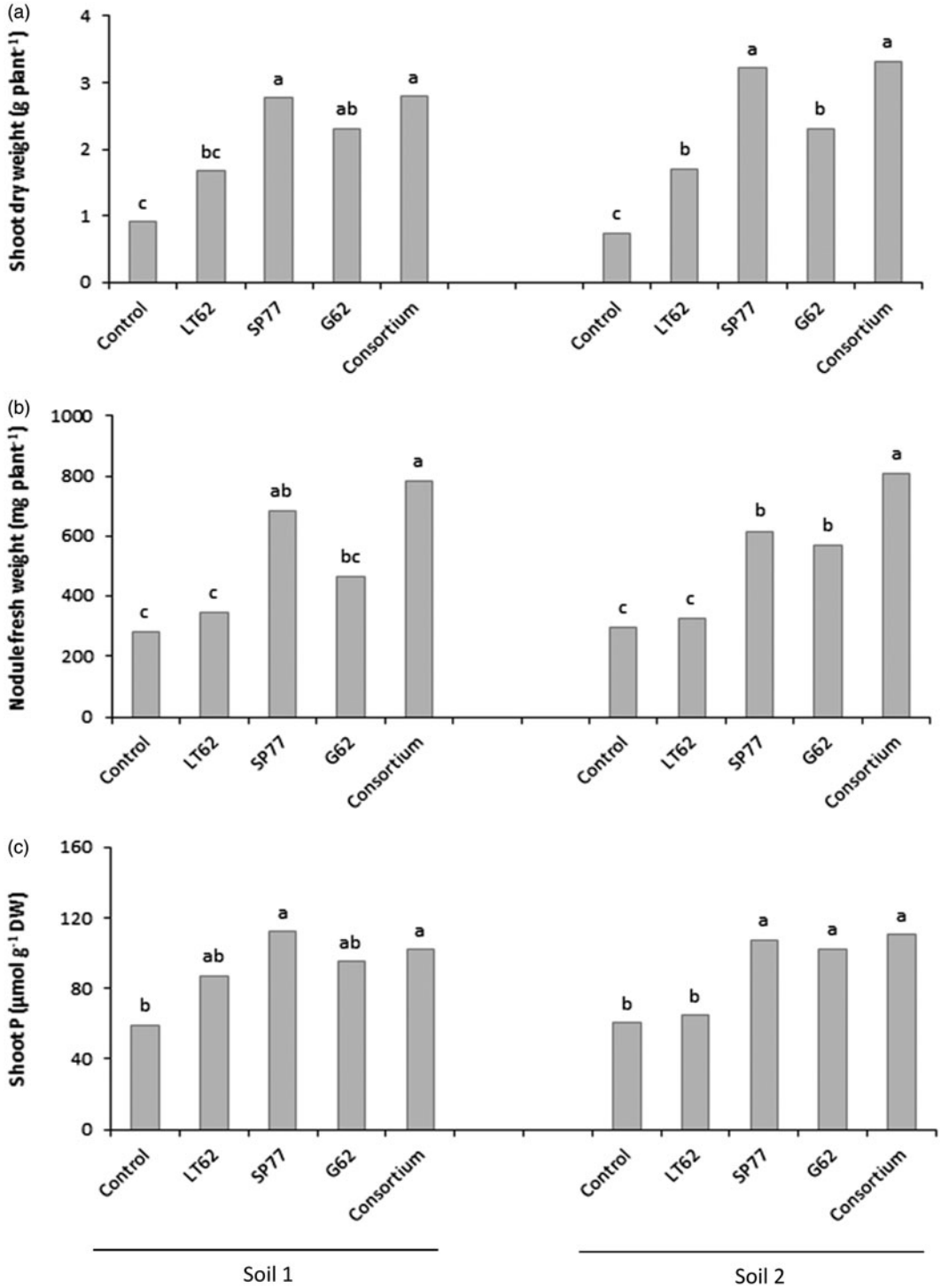

Figure 3. Effect of the inoculation of *M. truncatula* with selected PSB strains on shoot dry weights (a) nodule weight (b) and phosphorus content (c) in two phosphorus deficient soils: Soil 1 (Siliena) and Soil 2 (Takelsa). All treatments were fertilized with the inorganic phosphorus (TRP). Means with different letters are significantly different (HSD test, $p < 0.05$, $n = 3$).

selected PSB showed to be tolerant to NaCl and PEG-induced osmotic stress. They may be suitable for use in soils showing these constraints. However, their effect under these conditions should be investigated further.

The goal of this study was to develop eco-friendly viable biofertilizers as an alternative to chemical P fertilizers. Therefore, the efficiency of these PSB candidates on plants was then carried-out with or without TRP in controlled conditions. The outcome of the consortium inoculation to *M. truncatula* contributed to an increase in shoot dry yield up to +136% on sterile sand without TRP fertilization proving its high potentialities in promoting plant growth. When used under TRP fertilization, the consortium of three PSB performed better and allowed and enhancement in plant growth on sterile sand of 222% compared with the non-inoculated and non-fertilized control. Growth enhancement of 59% was observed compared with the inoculated and non-fertilized plants. This enhancement points out the plant growth ability component of the inoculant. Consequently, the use of bacterial consortia containing several bacterial strains to assist rock

phosphate solubilization will be more suitable for enhancing plant productivity. The interaction plant-PSB might result in more appropriate ecological balance.

Furthermore, PSB inoculation enhanced nodulation in *Medicago* plants. Indeed, P is a key element in the energetic plant metabolism, it is an essential nutrient for nitrogenase (Meena et al. 2018). Therefore, the nitrogen fixation in legumes is promoted by an optimum phosphorus level in host tissues (Dhakal et al. 2016). Tagore et al. (2013) reported that PSB inoculation increased leghemoglobin content in nodules of *Cicer arietinum* under field conditions.

Results from soil inoculation tests with the most promising bacteria (strains LT62, SP77, and G62) selected from the first experiment revealed that the single inoculation with SP77 as well as with the consortium showed the most significant effects on shoot dry yield up to +137% an +87% for soil 1 and soil 2 respectively.

The results on two different soils were positively correlated with the first experimentation on sterilized sand. However, the single inoculation with G62 *Pseudomonas frederiksbergensis* in soil 2 was not efficient as in the first soil. It might be due to the outcome of other mechanisms such as antibiosis effect with other indigenous rhizospheric microflora which compete for space and nutrients. It has been reported that inoculation with PGPR is significantly influenced by microbial rhizospheric communities, soil characteristics and host plant (Antoun 2012; Buragohain et al. 2018). Therefore, using inoculants composed of multiple strains to cumulate mechanisms at once and to reinforce the installation of the consortium in the rhizosphere would guarantee the success of inoculation on different soils and conditions better than using single inoculants.

For the use of natural reserves of phosphate rocks, the utilization of P-solubilizing bacteria as biofertilizer has enormous potentials; it is a low-cost input with high benefits in agriculture. Nevertheless, to distinguish elite PSB, the selective medium should be well considered. In this study, the choice of the selected bacteria was based on their solubilization in NBRIB medium with RP as a sole phosphorus source. Some supposed PSB selected bacteria had the capacity to solubilize just the TCP form and this might be related to other mechanisms cited above. Inoculation with elite PSB endowed with PGPR abilities increases the efficacy of the inoculants. For large-scale application, the single inoculation may either have antagonist effect with rock phosphate or could be not efficient in some soils and could cause a broad spectrum of issues. The use of multispecific and polyvalent consortia of PSB conjointly with TRP could be a promising management strategy to improve plant growth and conserve soil dynamic equilibrium. This study should be complemented with multi-crop field trials to provide undeniable evidence for the usefulness of this consortium and eventually its commercial application.

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