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Phosphate solubilization and the enhancement of chickpea growth by new rhizospheric microorganisms *Bacillus tequilensis* and *Trichoderma orientale*

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Abstract: Two *Trichoderma* strains and three *Bacillus* strains isolated from the rhizosphere of healthy chickpeas in Algeria were assessed for their phosphate solubilizing capacity *in vitro* as well as their growth effects on seedlings of the chickpea in pot experiments. The microorganisms tested had higher phosphate-solubilizing activities, with the solubilization index ranging from 2.41 to 7.40. The concentration of solubilized phosphate varied from 30.17 to 157.44 μg/mL. The maximum phosphate-solubilizing activity was observed in the two culture filtrates of *Bacillus tequilensis* Bt1 (157.44 μg/mL) and *Trichoderma orientale* T1 (143.33 μg/mL), accompanied by a decrease in pH of the growth medium from 4.51 to 5.75. The application of the strains (*B. tequilensis* Bt1 and *T. orientale* T1) separately and in combination had a beneficial effect on germination by promoting the development of the seeds and effectively enhancing plant growth. Chickpea seedlings showed better vegetative growth when treated with a mixture of *B. tequilensis* Bt1 and *T. orientale* T1 together than an individual treatment. To our knowledge, this is the first report of the phosphate-solubilizing potential of the combined microorganisms *B. tequilensis* and *T. orientale* and their capacity to promote plant growth in chickpeas.

Keywords: phosphate solubilizing microorganisms (PSMs), co-inoculation, *Bacillus tequilensis*, *Trichoderma orientale*, biofertilization

Abbreviations: phosphate-solubilizing microorganisms (PSMs); *Fusarium oxysporum* f. sp. *ciceris* (FOC); potato dextrose agar (PDA); tryptic soy broth (TSB); Pikovskaya agar medium (PVK); tricalcium phosphate (TCP); solubilization index (SI); analysis of variance (ANOVA); internal transcribed spacer regions (ITS); translation elongation factor-1 alpha (TEF-1α); stem length (SL); root length (RL); the fresh weight of the shoots (FWS); the fresh weight of the roots (FWR); the dry weight of the shoots (DWS); the dry weight of the roots (DWR); IAA (indole acetic acid)

INTRODUCTION

Chickpea (*Cicer arietinum* L.) is a prime pulse crop grown widely and consumed all over the world due to the very high nutritional value of its seeds. In several countries, chickpea production is limited by several stress factors, including the non-availability of nutrition in soils [1]. Plants need three primary nutrients, nitrogen (N), phosphorus (P), and potassium (K) in more significant amounts, and therefore, soil reserves of these elements must be regularly replenished to maintain good productivity. These nutrients are mostly supplied to plants in fertilizers [2,3].

Phosphorus is an essential nutrient for plant growth and is often present in soil in insoluble forms and, therefore, unavailable to plants [4]. Microorganisms like *Bacillus tequilensis* and *Trichoderma orientale* can solubilize these insoluble forms of phosphate into a soluble form that plants can readily uptake. This is typically achieved through the secretion of organic acids and enzymes by these microorganisms, which break down the phosphate compounds [5]. By solubilizing phosphate, these microorganisms make this essential nutrient more available to chickpea plants. This can lead to improved root development, increased nutrient uptake, and enhanced plant growth.

Trichoderma orientale is known for its biocontrol properties [6]. It can act as a biological control agent against soilborne pathogens, which can ben-

efit chickpea plants. Suppressing harmful pathogens' growth indirectly enhances plant growth and reduces the need for chemical pesticides. Some rhizospheric microorganisms, including *Bacillus* species, can also improve plant stress tolerance. This includes tolerance to abiotic stresses like drought and salinity. By promoting healthier root systems and nutrient uptake, these microorganisms can help chickpea plants better withstand environmental stresses [7].

The use of phosphate-solubilizing microorganisms like *Bacillus tequilensis* and *Trichoderma orientale* aligns with sustainable agriculture practices. It reduces the need for synthetic fertilizers, which can have negative environmental impacts, and promotes a more environmentally friendly and economically viable agricultural system. It is important to note that the effectiveness of these microorganisms can vary depending on factors such as soil type, environmental conditions, and the specific strain of the microorganism used. Field trials and research studies should typically be conducted to assess their performance in a specific agricultural setting.

Our research was carried out as an examination of an alternative strategy to chemical fertilizers. We aimed to develop an effective bioinoculant for solubilizing phosphate. For this reason, the present study was undertaken to evaluate qualitatively and quantitatively the efficiency of different isolates belonging to the *Bacillus* and *Trichoderma* genera in solubilizing phosphate. Furthermore, we explored the mechanisms of phosphate solubilization of two selected PSMs, *Bacillus tequilensis* Bt1 and *Trichoderma orientale* T1, in greenhouse conditions by single and combined seed applications to examine and evaluate their effect on chickpea plant growth and development.

MATERIALS AND METHODS

Fungal and bacterial isolates

Five strains listed in Supplementary Table S1 were used in this study: two *Trichoderma* isolates (T1 and T5) and three *Bacillus* isolates (Bs1, Bt1, and Rb29). All strains were initially isolated from the rhizosphere of healthy chickpeas in Algeria. *B. subtilis* MF352017 (Bs1) and *T. harzianum* KX523899 (T5) were previously found to exhibit, separately and in combination, biological control properties against fusarium wilt, a vascular disease of chickpea caused by *Fusarium oxysporum* f. sp. *ciceris* (FOC) [8]. *B. tequilensis* MF352019 (Bt1), *B. amyloliquefaciens* MF352007 (Rb29), and *B. subtilis* MF352017 (Bs1) were reported to induce systemic resistance against FOC [9]. However, the strain *Trichoderma orientale* MF410328 (T1) was isolated and identified in 2017 by A. A. Bekkar. The fungal cultures were kept on potato dextrose agar (PDA) at 4°C with periodic transfers. The bacterial strains were maintained at -80°C in tryptic soy broth (TSB) with 20% glycerol.

Determination of the solubilization index on the solid medium

The P solubilization ability of *Trichoderma* and *Bacillus* strains was evaluated qualitatively and quantitatively. All strains were tested under *in vitro* conditions for their phosphate-solubilizing ability on Pikovskaya agar medium (PVK) supplemented with insoluble tricalcium phosphate TCP $Ca_3(PO_4)_2$ at a concentration of 0.5% [10].

For qualitative estimation, 6 mm agar discs from *Trichoderma* cultures were placed in the center of the plate containing PVK agar medium supplemented with bromophenol blue. To assess the potential of each bacterial strain, the bacterial strains were cultured overnight on nutrient agar and then spotted onto PVK agar medium. Inoculated plates with *Trichoderma* or *Bacillus* were incubated at 28°C for 5 days. The plates were examined to see if clear zones (a halo) formed around colonies capable of solubilizing TCP and to calculate the solubilization index (SI). All observations were recorded in triplicate. Phosphate solubilization expressed in terms of mm diameter of the solubilization zone produced around the colony and the solubilization index (SI) were calculated by measuring the colony diameter and the halo zone and colony diameter, using the following formula of Edi-Premono et al. [11]:

$$SI = (\text{Colony diameter} + \text{Halo zone diameter}) / \text{Colony diameter}.$$

Phosphate solubilization efficiency in the liquid medium

Quantitative estimations and comparisons of the phosphate solubilization ability of the fungal and bacterial isolates were conducted using cultures grown in PVK.

For quantification of soluble phosphate produced by each strain, 100 µL of bacterial or fungal culture was added to 10 mL of PVK medium containing tricalcium phosphate as the sole phosphate source. The pH of the medium was adjusted to 7. Uninoculated broth served as a control. After incubation at 28°C in a rotary shaker (1.60×g/min) for 7 days, the supernatant was obtained by centrifugation at 704.34×g for 20 min, and the amount of soluble phosphate was measured following the phosphomolybdic blue color method of Olsen and Sommers [12]. The intensity of the blue color was measured on a spectrophotometer at 620 nm, and the quantity of solubilized phosphate was expressed as µg/mL. The pH of the filtrate was recorded at the end of the experiment. The experiments were done in triplicates.

Molecular identification and phylogenetic analysis of strains Bt1 and T1

Based on the *in vitro* phosphate solubilization potency, the efficient isolates Bt1 and T1 were identified through a molecular phylogenetic method. Strain Bt1 was identified by 16S rRNA gene sequencing using universal bacterial primers 27F and 1492R [13] (Supplementary Table S2). Strain T1 was phylogenetically analyzed based on the internal transcribed spacer region of the ribosomal DNA (ITS rDNA) and translation elongation factor-1 alpha (TEF-1α) sequences. Primers used for amplification (Supplementary Table S2) were ITS1 and ITS4 [14] and EF1-728F/EF-2 [15], respectively. A BLAST search was performed with the final obtained sequences using the NCBI BLAST Program (https://blast.ncbi.nlm.nih.gov/Blast.cgi Blast) to identify the two strains Bt1 and T1 based on their % homology with reference strains and according to the best-matched species and sequence identity. NCBI accession numbers were applied after submission to GenBank of the obtained sequences from two strains, Bt1 and T1. MEGA X software generated a neighbor-joining phylogenetic tree based on 1,000 bootstrap replications for each strain [16].

Pot experiment

The two most efficient isolates exhibiting greater phosphate-solubilizing activity in solid and broth medium were selected to test their performance under pot conditions in improving the development of chickpea plants. The chickpea seeds (*Cicer arietinum* var. ILC482) were supplied by the Technical Institute of Field Crops in Saïda, Algeria. They were surface-sterilized by sodium hypochlorite (2%) for 3 min, washed 3 times in sterile distilled water (SDW), and then dried. Three seeds were sown in each sterilized surface plastic pot filled with a previously disinfected substrate composed of a mixture of soil and peat (2v:1v) supplied with tricalcium phosphate (0.4 g/kg of substrate).

Fungal and bacterial inoculums as well as seed treatment procedures were according to Zaim et al. [8]. Seeds were treated by soaking for 30 min. Four treatments were set up as follows: treatment with a *Trichoderma* suspension (10^8 spores/mL); treatment with a *Bacillus* suspension with a final concentration of 10^8 bacteria/mL; treatment with a mixture of *Trichoderma* and *Bacillus* (V/V); treatment with sterile distilled water (used on untreated control seeds). The experiment was conducted in a completely randomized block design with 3 replicates/treatments. The plants were watered when required. After 4 weeks, the agronomic parameters related to vegetative development were measured for each treatment: stem length (SL) (cm), root length (RL) (cm), the fresh weight of shoots (FWS), and roots (FWR) (g/plant), the dry weight of shoots (DWS) and roots (DWR) (g/plant).

Statistical analysis

A completely randomized design was followed for the experiments, and the data were analyzed by ANOVA (analysis of variance). The Newman-Keuls test was used for the separation of means. The analyses were performed with a level of significance $P \leq 0.05$, and all analyses were carried out with the statistical software Statistica 8.0.

RESULTS

Qualitative estimation of phosphate solubilization

The phosphate solubilization test on Pikovskaya agar medium (PVK) supplemented with insoluble tricalcium phosphate (TCP) $Ca_3(PO_4)_2$ revealed that all tested strains were found to be potent phosphate solubilizers, showing a clear halo zone around their colonies after 5

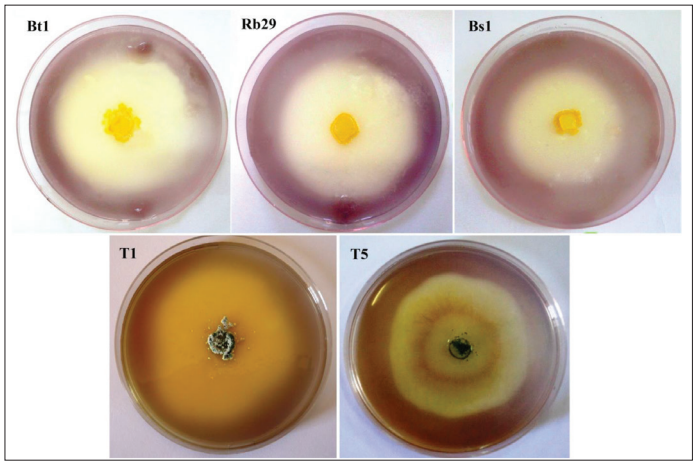

Fig. 1. Zone of insoluble phosphate (TCP) solubilization by *Bacillus* and *Trichoderma* isolates on Pikovskaya agar plates. All isolates tested produced a clear zone around the colony after 5 days of incubation at 28°C. Bt1 – *B. tequilensis*; Rb29 – *B. amyloliquefaciens*; Bs1 – *B. subtilis*; T1 – *T. orientale*; T5 –*T. harzianum*. The highest solubilization effect was noted in *B. tequilensis* Bt1. *T. orientale* T1 was the fungus with the highest levels of solubilization.

Table 1. Solubilization index of phosphate (IS) in solid medium; soluble phosphorus (µg mL^{-1}) in liquid medium, and pH of the medium produced by phosphate-solubilizing microorganisms.

Fungal and bacterial isolates			Phosphate solubilization		
			IS= (ø halo+ø colony) / ø colony	P_2O_5 (µg/mL)	pH
***Trichoderma* isolates**	**T1**	*T. orientale*	**2.81** ^{d}	**143.33** ^{b}	**4.51**
	T5	*T. harzianum*	**2.41** ^{d}	**48.85** ^{c}	**5.75**
***Bacillus* isolates**	**Bs1**	*B. subtilis*	**5.40** ^{c}	**42.02** ^{cd}	**5.33**
	Bt1	*B. tequilensis*	**7.40** ^{a}	**157.44** ^{a}	**4.78**
	Rb29	*B. amyloliquefaciens*	**6.36** ^{b}	**30.17** ^{d}	**5.01**

Means followed by different letters within a column are significantly different at P>0.05, according to the Newman-Keuls test.

days incubation at 28°C (Fig. 1). The estimation of the solubilization index (SI) made it possible to evaluate the solubilizing power of *Bacillus* and *Trichoderma* and to establish a comparison between the isolates tested. The SI varied among isolates and incubation times (Table 1). Specifically, the SI ranged from 2.41 to 7.40 on PVK plates supplemented with TCP. The efficiency of *Bacillus* isolates for phosphorus solubilization (SI ranging from 5.40 to 7.40) is higher than that of *Trichoderma* isolates (SI ranging from 2.41 to 2.41) (Table 1). The highest solubilization effect was noted in *B. tequilensis* Bt1 with a 7.40 rate. *T. orientale* T1 was the fungus with the highest levels of solubilization.

Quantitative estimation of phosphate solubilization

The isolates were positive for phosphate solubilization activity using PVK agar medium (Table 1). The concentration of solubilized phosphate determined after 7 days of incubation at 28°C was 30.17-157.44 µg/mL. All the strains produced a very good quantity of soluble phosphates by solubilizing the insoluble phosphates. The three bacterial strains exhibited high phosphate-solubilizing capacity. The strain with the highest capacity was *B. tequilensis* Bt1 with 157.44 µg/mL of solubilized phosphate. *T. orientale* T1 demonstrated higher levels of solubilization (143.33 g/mL) than *T. harzianum*. Among the isolates, *B. tequilensis* Bt1 and *T. orientale* T1 exhibited significantly ($P<0.05$) higher phosphate solubility and were selected for pot culture experiments.

Because solubilization of inorganic P is often associated with a reduction in pH by microorganisms, we measured the pH of PVK liquid medium inoculated with bacterial and fungal strains exhibiting phosphate-solubilization activities. Compared to the control, the pH of the inoculated medium decreased – indicating acid production. There were, however, significant differences in the pH recorded for the different tested isolates, where pH values ranged from 4.51 to 5.75 (Table 1), meaning that the amount of solubilized phosphate correlated positively with the decrease in pH of medium.

Molecular identification and phylogenetic analysis of strains Bt1 and T1

A molecular phylogenetic analysis was conducted to identify the selected efficient phosphate-solubilizing strains Bt1 and T1. Based on 16S rRNA gene sequencing, strain Bt1 was identified as *Bacillus tequilensis* with accession number MF352019 in the NCBI GenBank database. It showed 97.97% similarity to NR_104919 of *B. tequilensis*. The neighbor-joining tree was generated from a 16S rRNA gene sequence of the strain Bt1 and other related species (Fig. 2A).

Based on DNA sequences of the internal transcribed spacer (ITS) region and translation elongation factor 1-alpha (TEF1-α) gene, strain T1 was identified as *Trichoderma orientale* and deposited in GenBank under accession numbers MF410328 (ITS) and OR449362 (TEF1-α). The BLASTn search with the ITS and TEF sequences of strain T1 showed 99.60 and

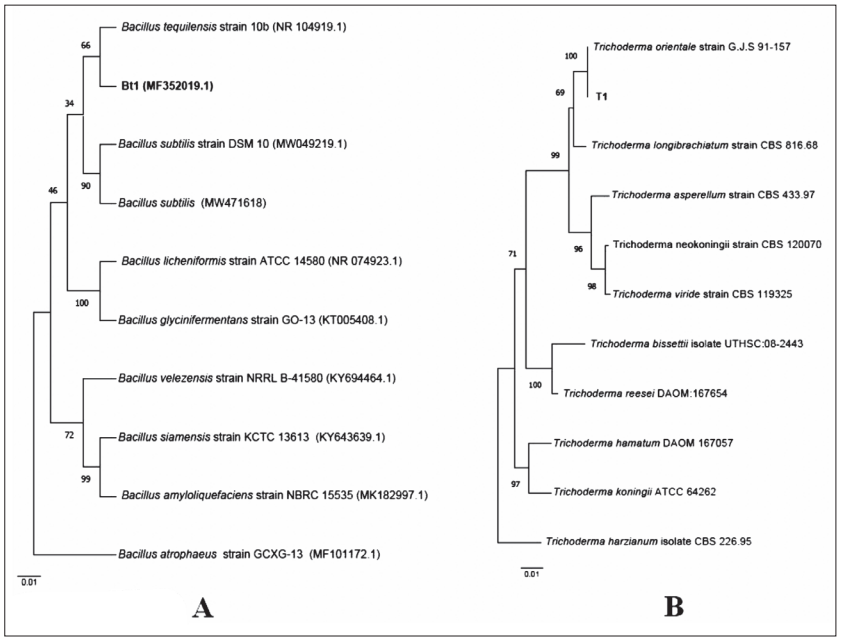

Fig. 2. Phylogenetic trees of the strains Bt1 (**A**) and T1 (**B**) inferred by the neighbor-joining method using MEGA X software. Relevant bootstrap values (expressed as a percentage of 1,000 replicates) are shown at branch point. The position of the strains Bt1 and T1 is indicated in bold text. **A –** Phylogenetic tree generated from the 16S rRNA gene sequence of the strain Bt1showing the phylogenetic relationships among *Bacillus* spp. **B –** Phylogenetic tree was constructed based on concatenated sequences of internal transcribed spacer (ITS) and translation elongation factor 1-alpha (TEF-1α) gene regions.

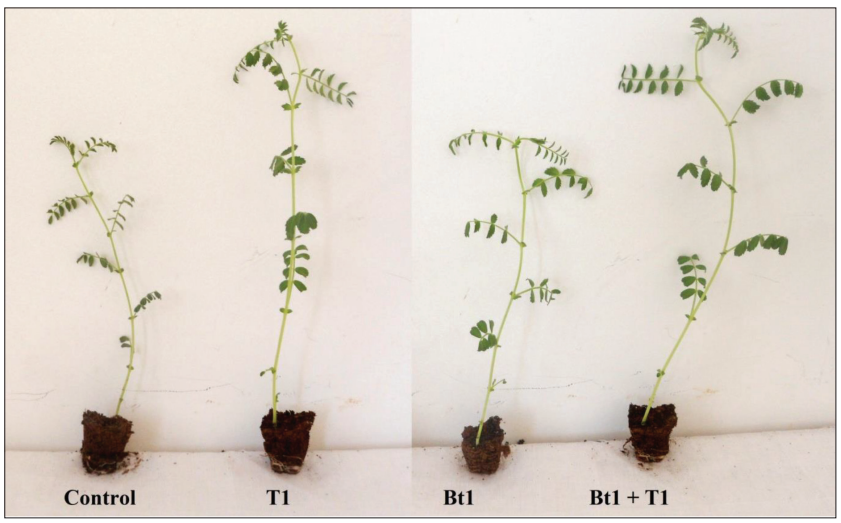

Fig. 3. Effect of selected strains *B. tequilensis* Bt1 and *T. orientale* T1, used alone or in combination, on seedling growth of chickpea compared to the control. The application of both strains (*B. tequilensis* Bt1 and *T. orientale* T1) separately and in combination has a beneficial effect on germination by promoting the development of the seeds as compared to untreated plants (control). Chickpea seedlings showed better vegetative growth when treated by a mixture of *B. tequilensis* Bt1 and *T. orientale* T1 together than an individual treatment.

99.64% similarities with the sequences of *T. orientale* NR_111317 (ITS) and EU401593 (TEF), respectively. The neighbor-joining method was used to construct a phylogenetic tree based on the combined ITS and TEF sequences of strain T1 with other *Trichoderma* species (Fig. 2B). A phylogenetic tree showed that strain T1 was clustered with *T. orientale* strain G.J.S91-157 with a high bootstrap value.

Effect of selected strains on chickpea growth

To test whether PSMs promote plant growth, we selected the two isolates (*B. tequilensis* Bt1 and *T. orientale* T1) with the highest P production and determined their effects on chickpea growth and development in culture pots. Compared to the non-inoculated control, the application of both PSMs, individually or in combination, significantly increased ($P<0.05$) seedling growth parameters.

The treatment of seeds with the two strains Bt1 and T1, separately and in combination, had a beneficial effect on germination by promoting seed development in comparison with untreated control seeds (Fig. 3). Comparative analysis of vegetative plant growth patterns, i.e., stem length, root length, the fresh weight of shoots and roots, the dry weight of shoots and roots, were recorded in triplicate from germination of seeds. Plant development data are presented in Table 2. The seedling height of chickpea inoculated with any of the two isolates was significantly greater than that of the uninoculated controls (Fig. 4A).

Seed treatment with T1 showed 113.71% and 122.60% increases in stem and root length, respectively. It also showed 136.53% and 163.15% increases in average shoot and root

Table 2. Variation of vegetative development parameters of chickpea plants treated with the strains Bt1 and T1, separately and in combination, compared to the control.

Treatments	Vegetative growth parameters					
	Length (cm)		Fresh weight (g/ plant)		Dry weight (g/ plant)	
	Stem (SL)	Root (RL)	Aerial part (FWA)	Roots (FWR)	Aerial part (DWA)	Roots (DWR)
Control	40.83 ^{c}	15.13^{d}	3.33^{d}	1.76^{d}	0.52^{d}	0.19^{b}
Bt1	45.86 ^{b}	18.13 ^{c}	4.21 ^{c}	3.01 ^{c}	0.67 ^{c}	0.29 ^{ab}
T1	46.43 ^{b}	18.55 ^{b}	4.33 ^{b}	3.21 ^{b}	0.71 ^{b}	0.31 ^{ab}
Bt1 + T1	48.09 ^{a}	18.89 ^{a}	4.66 ^{a}	3.41 ^{a}	0.75 ^{a}	0.34 ^{a}

SL – Stem length; RL – Root length; FWA – Fresh weight of the aerial part; FWR – Fresh weight of roots; DWA – Dry weight of the aerial part; DWR – Dry weight of roots. Means followed by different letters within a column are significantly different at P>0.05, according to the Newman-Keuls test.

weights, respectively, compared to the control. On the other hand, Bt1 inoculation increased stem and root lengths by 112.32% and 119.82%, respectively. It also increased average shoot and root weights by 128.84% and 152.63%, respectively, compared to the control (Fig. 3).

Inoculation with the mixture of Bt1 and T1 revealed a maximum stimulatory effect in terms of stem and root length and dry weight of chickpea plants (Table 2). The plants resulting from the seeds treated with the two species Bt1 and T1, separately or in combination, exhibited greater vegetative development compared to those of the untreated control. Similarly, comparison of the root systems of treated and untreated plants shows a clear difference between the two (Fig. 4B). Chickpea growth was greater when *B. tequilensis* Bt1 and *T. orientale* T1 were inoculated together than when plants were inoculated with either of them alone.

The ANOVA results showed a highly significant effect on stem length (SL), root length (RL), fresh weight of the shoots (FWS) and roots (FWR), and dry weight of shoots (DWS) and roots (DWR). A significant plant growth enhancement was observed in the presence of both isolates, alone and in combination. Compared to the control, all the treatments displayed higher and significantly different measurement values. The results of fresh weight (FWS and FWR) and dry weight (DWS and DWR, of the shoots and roots are highly significant in the case of chickpea seeds treated with the two isolates Bt1 and T1, alone or in combination, compared to the control.

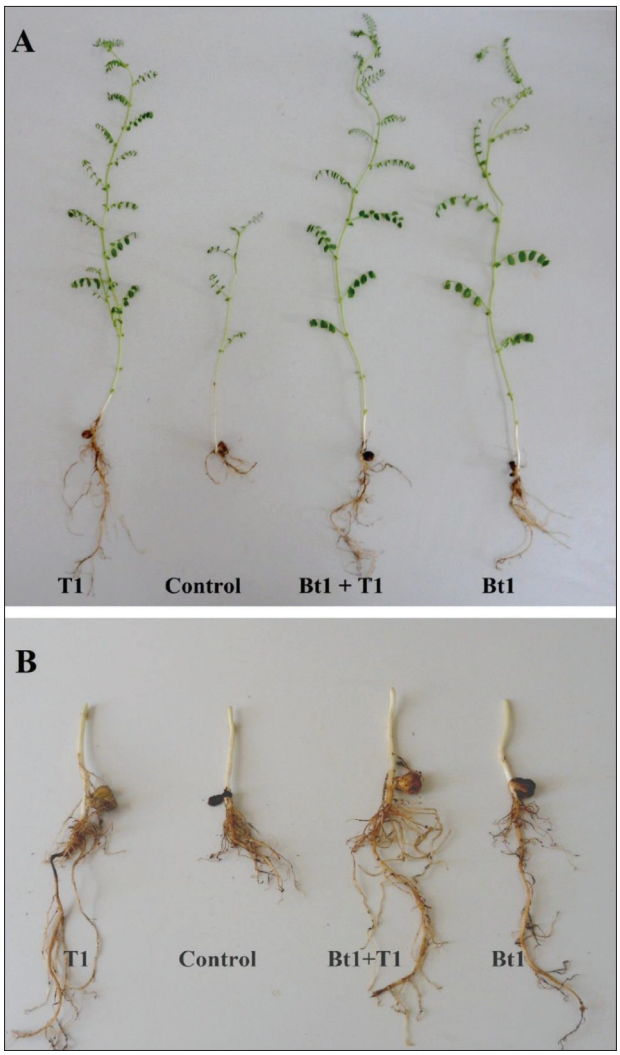

Fig. 4: Effect of selected strains *B. tequilensis* Bt1 and *T. orientale* T1 on aerial parts (**A**) and roots **(B)** of chickpea: **A –** Significant effect of plant growth enhancement in the presence of both isolates, alone or in combination, compared to the control; **B –** Comparison of the root systems of the treated plants with those of the untreated plants. During pot experiments, *T. orientale* T1 and *B. tequilensis* Bt1 inoculation increased chickpea growth parameters including shoot and root length in comparison with non-inoculated control plants.

DISCUSSION

In vitro screening in culture medium containing insoluble mineral phosphates as the sole source of phosphorus, such as tricalcium phosphate $Ca_3(P0_4)_2$, allows for the visual detection and quantitative estimation of phosphate solubilization capacity by microorganisms. An improved technique was developed by Gupta et al. [17] with a bromophenol blue medium. This study highlights the phosphate solubilizing potential

of *Trichoderma* and *Bacillus* isolated from chickpea rhizospheres. It was found that all isolates were capable of differentially utilizing TCP in PVK. This was indicated by the soluble phosphate concentrations (μg mL^{-1}) and the increase in acidity of the growth medium. However, the P-solubilizing efficiency was found to be higher in the isolates *T. orientale* T1 and *B. tequilensis* Bt1. The disappearance of TCP indicates the high potential of *Trichoderma* and *Bacillus* for solubilization of inorganic bound phosphate (TCP), which might have been subsequently taken up by the fungus for cellular processes.

In this medium, halos form around the colonies in response to the decrease in pH due to organic acid production, ion chelation, and exchange reaction [18-20]. Further, a gradual decrease in pH values is in agreement with the findings of Elias et al. [21], who also reported a decrease in pH by the nine isolates; the largest reduction of pH in the culture medium was from an initial value of 7.0 to 4.00, 4.05, 4.13, and 4.23 for the isolates JUHbF60 (*Aspergillus* spp.), JUCaF37 (*Aspergillus* spp.), JUHbF95 (*Aspergillus* spp.), and JUFbF59 (*Penicillium* spp.), respectively, after 10 days of incubation.

However, phosphate solubilization is a complex phenomenon that depends on several factors: nutritional, physiological, and crop growth conditions. In addition, several experimental protocols have demonstrated the role of organic acids in the solubilization of mineral phosphate. In most of the reported cases, the process of inorganic phosphate solubilization by microorganisms likely involves the secretion of organic acids, phosphatases, and phytases [19,20].

The quantity and type of acid produced, and the decrease in pH, vary with the genus and species of microbes considered. *Trichoderma* species can produce different organic acids including oxalic, citric, lactic, succinic, acetic, fumaric, formic, gluconic, glutaric, maleic, malic, tartaric, and propionic acids [18]. Among organic acid-producing fungi, strains of the genera *Aspergillus* and *Penicillium* were reported to produce oxalic, citric, gluconic, succinic, and propionic acids [22]. When compared to other fungi, *Trichoderma* was the most efficient in solubilizing phosphate [23]. In general, fungi show greater phosphate solubilization activity than bacteria. Phosphate-solubilizing fungi can reach a greater distance and show good attachment to insoluble phosphate particles due to their hyphal structure compared to bacteria and actinomycetes, which do not form hyphae [24].

Bacillus species, such as *B. tequilensis, B. subtilis*, *B. megaterium*, *B. amyloliquefaciens*, *B. licheniformis*, and *B. cereus* also exhibited efficient phosphate solubilizing through the secretion of organic acids, including oxalic, citric, succinic, malic, and propionic acids [17,20,22]. Vazquez et al. [25] and Yan et al. [26] reported that different organic acids, volatile and non-volatile, were released by *Bacillus atrophaeus*, *B. amyloliquefaciens*, *B. licheniformis*, and *B. subtilis* (volatile: acetate, isobutyrate, isovalerate, and valerate; non-volatile: lactate, fumarate, and succinate).

Under greenhouse conditions, the effect of PSM inoculation on growth and nutrient uptake in chickpea plants was also studied. Germination and seedling development are critical steps for successful crop establishment [27]. Seed treatments with various plant growth-promoting rhizobacteria (PGPR) and *Trichoderma* isolates, either individually or combined, improved seed germination (germination percentage and speed) and seedling vigor [28]. Similarly, Windham et al. [29] showed that the addition of *T. harzianum* and *T. koningii* to previously sterilized soil increased the germination percentage of tomato and tobacco seeds compared to the control. Several results are supported by the observations that *Trichoderma* species increased the rate of seed germination, accelerated seed germination, and increased seedling vigor [30].

PSMs have been shown to benefit plant growth in a variety of plant species [5,19]. It was demonstrated that plant inoculations with PSMs stimulate growth and increase yield and phosphorus uptake in pots and in the field in a variety of crops [19]. PSMs use various mechanisms to release phosphate, particularly the production of organic acids [19,20,22]. Marra et al. [31] reported that the amount of phosphate solubilization depends on the strain, carbon source, organic acids, and type of phosphate.

In this study, using *B. tequilensis* Bt1 and *T. orientale* T1 in combination resulted in a higher impact on phosphate solubilizing in plants. Similar studies have described the benefits of combined application of *Bacillus*, *Trichoderma* and other beneficial microorgan-

ism mixtures [32,33]. We suggest that the efficiency of mixtures is probably related to the complementary modes of action of combined organisms. This argument is consistent with the findings of Escalas et al. [34] who suggest that co-inoculation or mixed inoculation as a single inoculant could maximize a strain's performance.

Although there are studies indicating enhancement due to co-inoculation in plant biomass, grain yield, nodulation, and nitrogen fixation in chickpea [32,35], there is no report of using *B. tequilensis* and *T. orientale* in combination. To our knowledge, this is the first study of the P-solubilizing potential of the combined microorganisms *B. tequilensis* and *T. orientale* and their capacity to promote plant growth in chickpea. Our results show that the two tested isolates, *B. tequilensis* Bt1 and *T. orientale* T1, stimulated the growth parameters of chickpea plants to varying degrees. This stimulation essentially resulted in better axial growth and greater biomass. The stimulation of biomass was observed not only in aboveground plant parts but also in root parts. This corroborates the results of several authors [32,35,36]. Indeed, Elkoca et al. [36] and Gupta et al. [37] showed that treatment of chickpea seeds with *Bacillus* sp. or *Trichoderma* sp. improved biomass, nodulation, dry weight, and yield compared to the untreated control. The improvement in vegetative growth of plants under the action of *Trichoderma* spp. was observed by Windham et al. [29], who showed that the application of two species of *Trichoderma* to sterilized growing medium can improve the dry weight of the roots and aboveground plant parts of tomato and tobacco.

Many studies have explored the phosphate-solubilizing potential of various rhizospheric microorganisms and their ability to enhance the growth of chickpea plants. Peix et al. [38] found that inoculating chickpea plants with the rhizobacterium *Mesorhizobium mediterraneum* significantly increased plant phosphorus content and growth. Similarly, Rudresh et al. [32] reported that co-inoculating chickpea with *Rhizobium*, phosphate-solubilizing *Bacillus megaterium*, and *Trichoderma* spp. led to improved germination, nutrient uptake, nodulation, and yield.

Trichoderma has also been shown to solubilize phosphate and promote chickpea growth. Gull et al. [39] discovered that inoculating chickpea with phosphate-solubilizing bacteria and rhizobia increased phosphorus uptake, shoot length, shoot dry weight, and nodulation. Rudresh el al. [32] found that various *Trichoderma* species were able to solubilize tricalcium phosphate and increase phosphorus uptake, growth, and yield in chickpea. Singh et al. [40] showed that phosphate-solubilizing rhizobia enhanced nodulation, nitrogenase activity, and chickpea growth, especially in phosphate-amended soil.

Certain rhizospheric fungi also possess strong phosphate solubilizing potential. Kapri and Tewari [41] found that 14 strains of *Trichoderma* spp. isolated from forest tree rhizospheres solubilized tricalcium phosphate *in vitro*. The strain DRT-1 showed the highest phosphate-solubilizing ability. Under glasshouse conditions, DRT-1 increased chickpea growth parameters in phosphate-deficient soil.

Midekssa et al. [42] isolated 50 phosphate-solubilizing bacterial strains from chickpea rhizospheres, including genera such as *Acinetobacter*, *Bacillus*, *Burkholderia*, and *Pseudomonas*. Several strains increased the number of chickpea root nodules, shoot dry matter, and shoot nitrogen and phosphorus contents under glasshouse conditions.

In summary, inoculating chickpea with phosphate-solubilizing rhizospheric microorganisms such as *Mesorhizobium*, *Rhizobium*, *Bacillus*, *Trichoderma*, and other bacteria can increase phosphorus uptake, nodulation, growth, and yield. These microorganisms show potential for improving chickpea growth on phosphate-deficient soils.

Wani et al. [35] and Mouria et al. [43] suggested that the ability of *Bacillus* or *Trichoderma* species to stimulate plant growth independent of pathogens is due to the influence of *Bacillus* and *Trichoderma* strains on plant metabolism and enzyme activity, not just on the defense systems. *Bacillus* and *Trichoderma* also can produce phytohormones such as IAA that can act directly to improve plant growth. These phytohormones would act by strengthening or accelerating the development of both the root system and aboveground plant parts. Consequently, the root surface is increased, which intensifies the uptake of nutrients, water, and minerals by plants [44,45]. Several rhizospheric strains belonging to the genera *Trichoderma* and *Bacillus* have demonstrated efficiency in solubilizing phosphate and

promoting plant growth. Their rhizospheric origin could also be one of the contributing factors in the positive activity observed in chickpea plants [33,46].

CONCLUSIONS

The rhizosphere is the region that surrounds plant roots where root exudates are released, and the metabolic activities of the roots change the characteristics of the soil. Phosphate solubilizing microorganisms (PSMs) were isolated from chickpea rhizosphere. Our study study presents the first description of the combined use of *Bacillus tequilensis* and *Trichoderma orientale* to solubilize a considerable amount of P. It is concluded that the application of these isolates has the potential to significantly improve chickpea growth as more phosphorus is dissolved. The PSMs are effective as biofertilizers in enhancing crop yields in phosphate-deficient soils. These PSMs are likely to be one of the most important alternatives to using chemical fertilizers that can negatively impact human health and the environment. We suggest that future studies should explore the possibility of promoting these isolates as biofertilizers to improve P nutrition of several crops.

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Data availability: Data underlying the reported findings have been provided as a raw dataset which is available here: https://www.serbiosoc.org.rs/NewUploads/Uploads/Bekkar%20and%20Zaim_Dataset.pdf

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SUPPLEMENTARY MATERIAL

Supplementary Table S1. Strains of *Trichoderma* and *Bacillus* used in this study

Species	Isolates	GenBank Accession Number	Geographic Origin	Isolation year
Trichoderma orientale	T1	MF410328	Mascara	2017
Trichoderma harzianum	T5	KX523899	Mascara	2011
Bacillus subtilis	Bs1	MF352017	Mascara	2017
Bacillus tequilensis	Bt1	MF352019	Mascara	2017
Bacillus amyloliquefaciens	Rb29	MF352007	Constantine	2009

Supplementary Table S2. Primers used in this study.

Locus	Primer names	Orientation	Sequence (5' to 3')
16S	27F	Forward	AGAGTTTGATCCTGGCTCAG
	1492R	Reverse	GGTTACCTTGTTACGACTT
ITS	ITS1	Forward	TCCGTAGGTGAACCTGCGG
	ITS4	Reverse	TCCTCCGCTTATTGATATGC
TEF1	EF-728F	Forward	CATCGAGAAGTTCGAGAAGG
	EF-2	Reverse	GGARGTACCAGTSATCATGTT