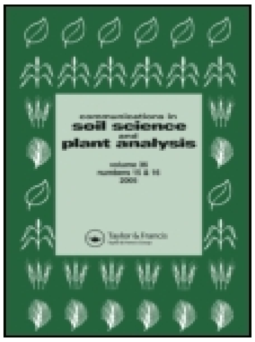

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# Bioresource Efficacy of Phosphate Rock, Sulfur, and Thiobacillus Inoculum in Improving Soil Phosphorus Availability

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**ABSTRACT**
In order to improve the effectiveness of phosphate rock as phosphorus fertilizer, elemental sulfur and Thiobacillus have been evaluated as amendments. First, Thiobacillus was isolated from different soil samples. Then, a greenhouse pot experiment was conducted using a completely randomized factorial design with three factors included: elemental sulfur at four levels of 0, 1000, 2000, and 5000 mg kg$^{-1}$; phosphate rock at three levels of 0, 1000 and 2000 mg kg$^{-1}$; four Thiobacillus inoculums ($T_1$, $T_2$, $T_3$, $T_4$) and without inoculation ($T_0$) in three replications. Results showed that all the four Thiobacillus inoculums increased significantly extractable soil-P. Combined application of phosphate rock and sulfur in equal proportion (1:1) along with inoculum Thiobacillus had a significant effect in improving phosphorus availability in soil. Combined application of sulfur (at rates of 1000 and 2000 mg kg$^{-1}$) and Thiobacillus significantly increased phosphorus uptake by plants as compared to the control.

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**KEYWORDS**
Biofertilizer; phosphate rock; sulfur oxidation; Thiobacillus

## Introduction

Phosphorus (P) is the second most limiting soil nutrient in crop production in Iran. Most of the agricultural lands of Iran are calcareous soils. When soluble P fertilizers such as single superphosphate or triple superphosphate are added to these soils, phosphorus is strongly fixed with Ca in the soil solution and with soil solid, so it is changed to unavailable form phosphate ($PO_4^{3-}$) for plant uptake. While total concentration of phosphorus in soils is about 0.05%, only 0.1% of that amount is plant available (Illmer and Schinner 1995). Critical levels of P in soil and plant depend on plant species and soil properties. Due to high cost of soluble phosphorus fertilizers, the use of phosphate rock had been recognized as a valuable low-cost alternative for the conventional water-soluble P fertilizers. However, due to their low solubility in calcareous soils, direct application of rock phosphates is not recommended. Several strategies have been used to enhance phosphorus availability from rock phosphates such as partial acidulation of phosphate rock (Rajan and Marwaha 1993), mixing phosphate rock with composting organic wastes, using phosphate rock along with some microorganism such as P-solubilizing bacteria, endomycorrhizae or ectomycorrhizae, and application of acidifying materials such as elemental sulfur along with sulfur-oxidizing bacteria (Babana, Samaké, and Maïga 2011). Among the biological means, using phosphate-solubilizing bacteria and arbuscular mycorrhiza usually is not suitable for orchard trees. Seeds of crops should be inoculated with P-solubilizing bacteria before planting; also use of mycorrhizal inoculum was done only at the sowing stage of crops by inoculum of crop seeds. Based on this, tree seeds should be inoculated prior to planting in nursery beds and when the seedlings are colonized by mycorrhizae, they

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are transplanted into the restoration site. Moreover, currently, small-scale inoculum production is expensive due to overhead and labor costs. Elemental sulfur would be efficient due to some favorable properties such as ability to act as a fungicide and acaricide and ability to oxidize and produce acid that helps in reducing the alkalinity of soils. However, elemental sulfur is an insoluble hydrophobic solid that microbes play a key role in the sulfur oxidation (Malhi, Schoenau, and Grand 2005). Most soils contain a wide range of soil microorganisms that are able to oxidize sulfur (S). Among them, the group of bacteria belonging to the genus Thiobacillus are the most important and common organisms in S-oxidization (Tabatabai 1986; Wainwright 1984). Most agricultural land, especially calcareous soils, contain very few Thiobacillus (Babana, Samaké, and Maïga 2011; Rupela and Tauro 1973; Swaby and Fedel 1973) and the population of Thiobacillus increases by application of sulfur in the soil (Germida and Janzen 1993). The soils with low numbers of Thiobacillus generally show rates of slowed sulfur oxidation; although when the soil inoculated with Thiobacillus bacteria, population of these organisms increase and are further accelerated by sulfur oxidation (Germida and Janzen 1993). Many studies for bioconversion of phosphate rock revolve around inoculating Thiobacillus in liquid salts media containing elemental sulfur or some of inorganic reduced sulfur and phosphate rock while soils make up a complicated and heterogeneous system that in these complex environments impact of chemical and biological interfacial interactions are affected by many factors such as bio-solubilization of phosphate rock. Since, it will increasingly become important, faced with complex environmental problems and attempt to overcome them, the current study was planned to: (i) study the presence of Thiobasillus isolate and characterize these bacteria in different samples, and (ii) determine the effects of bio-solubilization of different levels of phosphate rock and elemental sulfur on P availability in a calcareous soil by Thiobasillus inoculum application.

## Materials and methods

### *Isolation and Identification of Thiobacillus Bacteria Genus*

Twenty different samples of agriculture soils, industrial wastewater solid and liquid and soil and waters sulfur hot springs were collected from different cities of Iran. The samples were stored in a refrigerator at 4°C, until to time of isolation. Bacteria were grown in a sterile Postgate medium (Postgate 1966). For neutrophilic Thiobacillus growth, the pH of medium was adjusted to 7 with 2 potassium hydroxide (N KOH) but for acidophilic Thiobacillus, the initial pH of medium did not change. To isolate Thiobacillus, 1 g or 1 ml of each sample was inoculated into 100 ml of sterile Postgate and were incubated at room temperature with shaking. Change in the color of the medium may be due to bacterial growth. Then, each sample were spread onto plate dilution Postgate agar incubated at 27°C. Later, morphologically different single colonies were purified using repeating these stages.

Gram staining of the isolates were performed and observed under microscope. The sulfur oxidation experiment were tested by inoculating 1 ml of suspension of bacteria to the medium with the same composition of Postgate only instead of sodium thiosulfate pentahydrate ($Na_2S_2O_3.5H_2O$), 1% (w/v) tyndalized elemental sulfur powder was used. The heterotrophic culture with same composition of Postgate only instead of $Na_2S_2O_3.5H_2O$ was used. We also used 10 g yeast extract and also nutrient agar medium was applied to test the ability of the isolates to utilize a carbon source for heterotrophic growth.

### *The Greenhouse Experiments*

The phosphate rock that contains 44.46% calcium oxide (CaO) and 15.39% phosphorus pentoxide ($P_2O_5$) was crushed and sieved to a diameter of <60 mesh. The elemental sulfur powder was purchased from Merck Company. A calcareous soil sample with low available P was collected from surface horizon (0–30 cm) in Davaran, Kerman province, Iran. The soil sample was air-dried and ground to pass through a 2 mm sieve and mixed with acid-washed sand (2:1). Soil pH and electrical conductivity (EC) were determined using saturation extract of soil. Equivalent calcium carbonate was determined by titration with acid (Richards 1954). Available P was measured by Olsen extraction method (Olsen et al. 1954). These soil properties were as follows: EC, 1.7dS $m^{-1}$; pH, 7.85; lime, 21.8% and available P, 5 mg $kg^{-1}$. The soil was slightly alkaline and low in EC and available phosphorous. Two greenhouse investigations were performed consecutively on the same pots to evaluate the effects of treatments on soil phosphorus availability and phosphorus uptake by alfalfa. First experiment was arranged in a completely randomized factorial design with three replications under greenhouse condition. The treatments comprised phosphate rock at three rates (($PR_0$) 0, ($PR_1$) 1000, and ($PR_2$) 2000 mg per kg of soil)); elemental sulfur at four rates (($S_0$) 0, ($S_1$) 1000, ($S_2$) 2000, and ($S_3$) 5000 mg sulfur per kg of soil)); and five Thiobacillus inoculum ($T_0$, $T_1$, $T_2$, $T_3$, and $T_4$). Each pot was filled with 400 g of processed soil and treated with respective levels of phosphate rock and elemental sulfur and prepared uniformly. Thiobacillus inoculums ($T_1$, $T_2$, $T_3$, and $T_4$) were grown on liquid Postgate medium. All 36 pots were individually inoculated with $T_1$, $T_2$, $T_3$, and $T_4$ suspension. For 36 pots that did not receive Thiobacillus inoculums ($T_0$), the same volumes of sterile liquid postage medium were employed. The experiment was conducted for 50 days. During the control experiment time, distilled water was added to the pots to keep them at 70% of water-holding capacity. At the end of the experiments, soil samples were air-dried and sieved (2 mm) and the available soil phosphorus was extracted using sodium bicarbonate ($NaHCO_3$) (Olsen et al. 1954). Data were analyzed using MINITAB and MSTATC software. Significant differences of the means ($P < 0.05$) were determined by Duncan's Multiple Range Test. Then, the second greenhouse experiment was conducted based on the results of soil P availability in order to investigate the effects of the treatments on phosphorus uptake by alfalfa. The seeds were sown in the previous pots applied in the greenhouse experiment (I) with the same of levels of sulfur (($S_0$) 0, ($S_1$) 1000, ($S_2$) 2000, and ($S_3$) 5000 mg sulfur per kg of soil)) and phosphate rock (($PR_0$) 0, ($PR_1$) 1000, and ($PR_2$) 2000 mg per kg of soil)) and using $T_1$ inoculum and $T_0$ (without inoculation) in three replications. The greenhouse experiment (II) was conducted in a completely randomized design with factorial layout. Soil moisture was maintained near 70% of field capacity by adding distilled water. After 45 days, plants were harvested and then shoots and roots were separated and dried (70 $^{\circ}$C) for determination of phosphorus uptake. Data were analyzed using SAS software. Significant differences of the means ($P < 0.05$) were determined by Duncan's Multiple Range Test.

## Results and discussion

### *Isolation and Identification of Thiobacillus Bacteria Genus*

In different samples, 29 isolates were separated. All of them were chemolithotroph, Gram negative, and neutrophilic, while no acidophilic Thiobacillus was detected. Among neutrophilic Thiobacillus, eight isolates could oxidize sulfur as the sole source of energy that separated from only three samples. Out of eight isolates, only $T_1$ was able to grow as heterotrophy and others were obligately chemolithotrophic. Swaby and Fedel (1973) isolated T. thioparus and T. thiooxidans only in 5 and 1 out of 56 Australian soils, respectively. Likewise, similar results were observed by Lee, Boswell, and Watkinson (1988) and McCaskill and Blair (1987), who were unable to detect T. thiooxidans from New Zealand and Australian soils. Lawrence and Germida (1991) and Chapman (1990) also reported similar results that acidophilic Thiobacillus sp. was absent, whereas neutrophilic bacteria such as T. thioparus was detected. Probably it is due to soil pH that it isn't suitable for acidophilic Thiobacillus to develop in neutral conditions. Based on the information, in this study, we could isolate only neutrophilic Thiobacillus, while no Thiobacillus acidophilic was detected. However, most of agricultural soil in Iran was calcareous and appears that neutrophilic Thiobacillus can better adapt with these conditions. Due to the inability of Thiobacillus bacteria to produce spores along with some soil unfavorable factors including high pH, high base status, and apparent lack of available energy substrates especially in soil that had not been previously fertilized with sulfur lead low bacteria populations in most of agricultural soil.

The results of the present study were in conformity with those recently recorded by other researchers that the population of Thiobacillus bacteria in most agricultural soils is low (Babana, Samaké, and Maïga 2011).

## Phosphorus Availability

The analysis of variance of the greenhouse experiment (I) is shown in Table 1. The results showed that the main effect of Thiobacillus, sulfur, phosphate rock, and the interactive effects between Thiobacillus–sulfur, Thiobacillus–phosphate rock, and Thiobacillus–phosphate rock–sulfur had a significant effect on Olsen P extractable. Inoculation of Thiobacillus bacteria showed a significant effect on availability of soil phosphorous. Olsen P extractable increased significantly in all inoculated treatments (Figure 1). This may be due to sulfuric acid production during bacterial oxidation of elemental sulfur. The highest concentration of P was obtained for $T_1$ treatment. As seen from Figure 1, $T_2$, $T_3$, $T_4$ have increased Olsen P extractable, but there is no significant difference among them. It appears that they had similar ability to produce sulfuric acid and dissolve phosphorus compound. In this sense, Babana, Samaké, and Maïga (2011) have reported also that the capacity of acid production by bacteria depends on the species of Thiobacillus.

**Table 1.** Analysis of variance of treatments on soil P avilability

| | | Mean square |
|---|---|---|
| Source | DF | Available P |
| Sulfur | 3 | 130.55** |
| *Thiobacillus* | 4 | 488.16** |
| Phosphate Rock | 2 | 306.55** |
| *Thiobacillus**sulfur | 12 | 65.77** |
| Sulfur*Phosphate Rock | 6 | 20.41$^{ns}$ |
| Phosphate Rock* *Thiobacillus* | 8 | 78.88** |
| Sulfur*Phosphate Rock* *Thiobacillus* | 24 | 138.16** |
| Error | 120 | 20.94 |
| C.V% | - | 25.69 |

** Significant differences at $p < 0.001$ and ns: no statistically significant differences

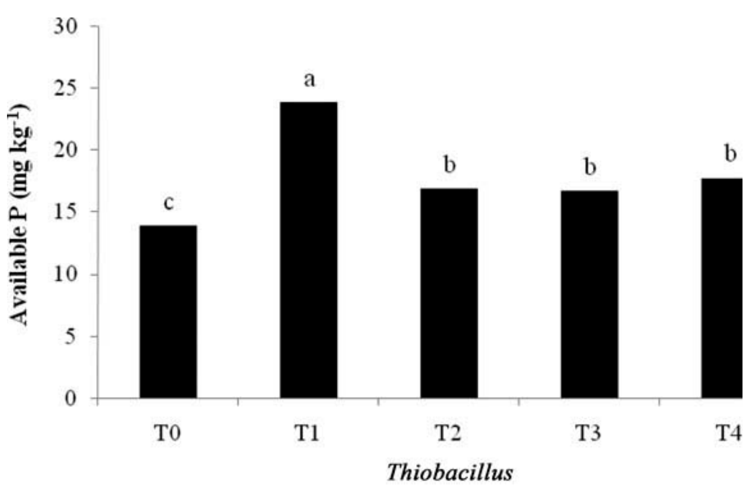


**Figure 1.** Effect of *Thiobacillus* inoculum on available phosphorus in soil.

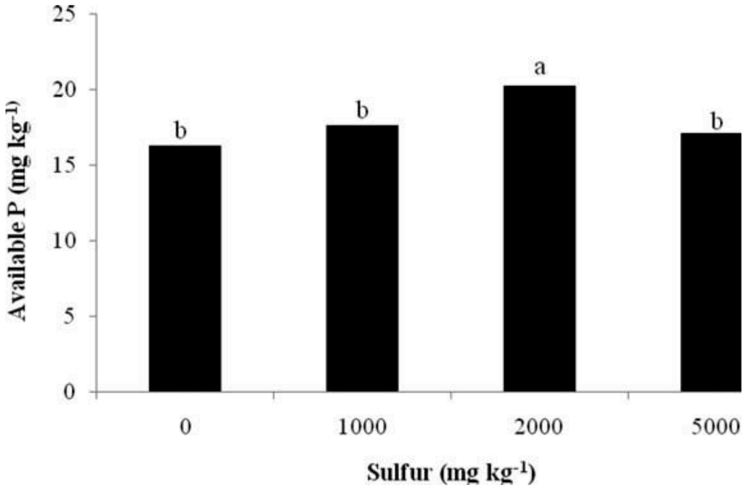


**Figure 2.** Effect of sulfur on available phosphorus in soil.

The main effects of elemental sulfur are shown in Figure 2. It appears that application of elemental sulfur at rates of 1000 and 5000 mg kg$^{-1}$ were not suitable amounts to enhancing availability of phosphorus significantly. Soil, crop, and agro-climatic conditions are the effective parameters on the magnitude of P and S responses (Bharose et al. 2011). It appears that $S_2$ could be optimum level of sulfur to enhance P availability. Mohammady Aria et al. (2010) recorded that sulfur had a significant effect on water-soluble phosphorus and in their study the highest solubility rate of phosphate rock was obtained in 20% of S ($S_3$) treatment and it was 2.4 times more than $S_1$ (0%) treatment.

The effects of phosphate rock application on Olsen's P extractable are shown in Figure 3. The effect of phosphate rock on Olsen P was significant, although at a rate of 1000 mg kg$^{-1}$, Olsen P was similar to untreated soil. Increasing the level of phosphate rock from 1000 to 2000 mg kg$^{-1}$ has resulted in a significant increase in the Olsen's P extractable from 16.2 mg kg$^{-1}$ to 25.7 mg kg$^{-1}$. A similar finding was reported by Stamford et al. (2007). It has been reported that the greater P sorption capacity of soils, which caused depletion of soil solution phosphorus and more dissolution

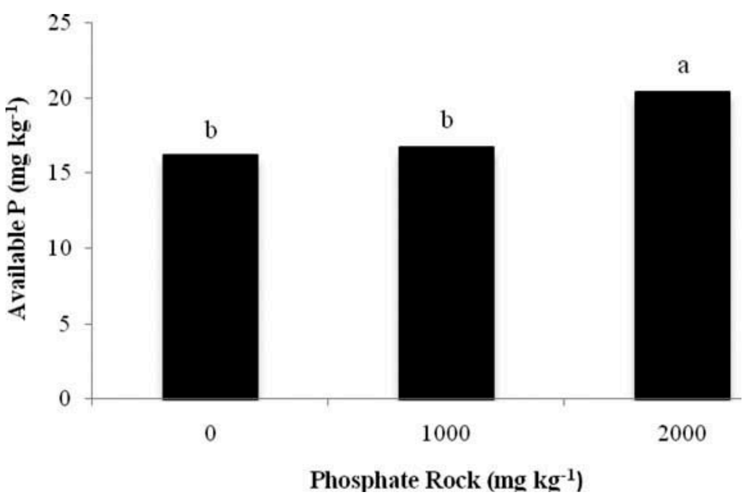


**Figure 3.** Effect of phosphate rock on available phosphorus in soil.

of phosphate rock. Although the greater P sorption capacity of soils can increase the solubility of phosphate rock, the availability of soil phosphorus will depend on the initial state of soil P and the amount of phosphate rock added to the soil. When small amounts of phosphate rock are added to the phosphorus deficient-soils, all of the dissolved phosphorus may be fixed by soil solid phases.

However, if the greater levels of phosphate rock apply, as the solution phosphorus concentrations rises above the threshold concentrations for net P uptake by plants, crop yield increases. Soil used in this experiment had slightly soluble P, so it appears that almost all of 1000 mg phosphate rock adsorbed by the soil, but double application of phosphate rock increased dissolved P in the soil solution.

Olsen P was found to be significantly affected by the interaction between Tiobacillus inoculation and S levels (Figure 4). $T_1$ treatment had the greatest Olsen P in all levels of S treatment and elemental S is a fundamental substrate for sulfur-oxidizing bacteria, since the microbial oxidation of S by Thiobacillus was an acid-producing process the production of sulfuric acid ($H_2SO_4$) is responsible for the increased solubility of apatite mineral. These results are in agreement with the findings of Ghani, Rajan, and Lee (1994), who observed that a combination of elemental S and Thiobacillus could increase extractable P to nine times relative to the untreated phosphate rock. Ullah et al. (2013) also recorded the use of Thiobacillus along with elemental sulfur-enhanced P in the soil by solubilizing present insoluble calcium bounded P fractions such as octacalcium phosphate (Ca8-P) and apatite (Ca10-P). The interaction between Thiobacillus and phosphate rock on this characteristic was significant (Figure 5). Combination of application of $T_1$ and $PR_2$ markedly increased the Olsen's P extractable compared to application of T and P alone. Bojinova et al. (1997) reported that the decomposition of the phosphorite crystal structure occurred by bioconversion of phosphorite using Aspergillusniger. Data in Table 2 indicated higher P availability for $T_3PR_2S_2$, followed by $T_1PR_0S_2$, $T_1PR_1S_3$, $T_1PR_1S_2$, and $T_1PR_2S_1$. These treatments have maintained the highest-level Olsen's P extractable status among all treatments. The enhancing P availability may be due to the interaction of phosphate rock with the produced sulfuric acid by Thiobacillus spp. (Rajan 1983). This result coincides with the findings of Bhatti and Yawar (2010). who observed that A. Ferrooxidans, A. Thiooxidans as well as a mixed culture of these bacteria solubilized significant amounts of P from phosphate rock in bioleaching process as compared with pure culture alone. Babana, Samaké, and Maïga (2011) also showed that all the isolates of Thiobacillus can produce sulfuric acid by oxidizing elemental S and, therefore, solubilize phosphate rock. Similar results were recorded by Costa, Medronho, and Pecanha (1992) and Chi, Xiao, and Gao (2006) for P

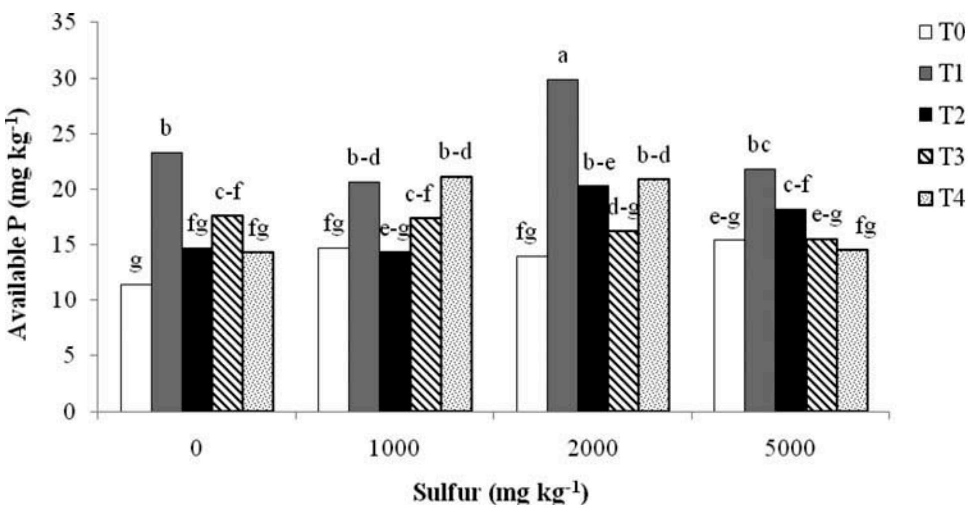


Figure 4. Combined effects of *Thiobacillus* inoculum and elemental sulfur on available phosphorus in soil.

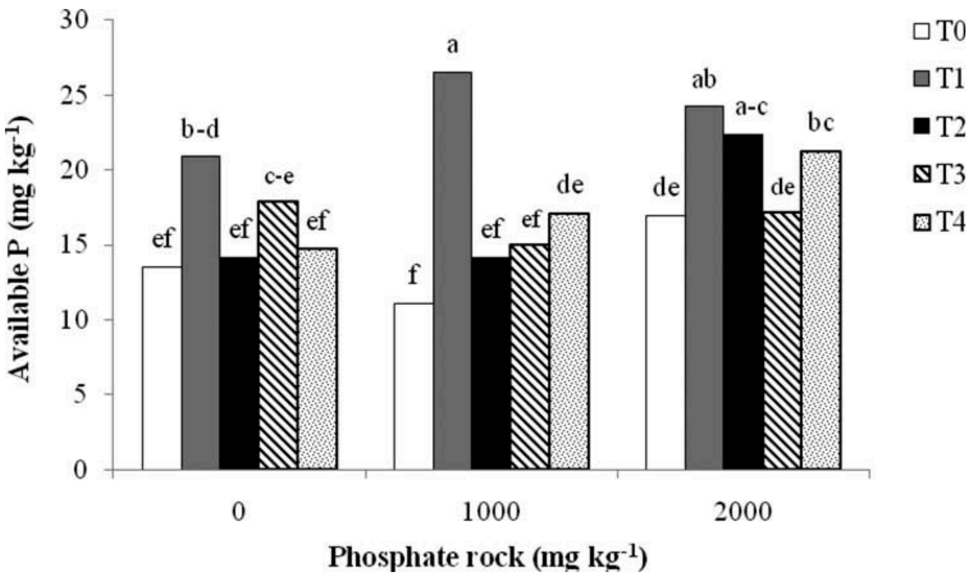


**Figure 5.** Combined effects of *Thiobacillus* inoculum and phosphate rock on available phosphorus in soil.

**Table 2.** Interactive effects of *Thiobacillus* inoculum, phosphate rock, and sulfur on available phosphorus in soil

| | Phosphate Rock (mg kg$^{-1}$) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | | | | 1000 | | | | 2000 | | | |
| *Thiobacillus* | Sulfur (mg kg$^{-1}$) | | | | Sulfur (mg kg$^{-1}$) | | | | Sulfur (mg kg$^{-1}$) | | | |
| | 0 | 1000 | 2000 | 5000 | 0 | 1000 | 2000 | 5000 | 0 | 1000 | 2000 | 5000 |
| $T_0$ | 10.4 $^{lo}$ | 12.3 $^{ms}$ | 17$^{do}$ | 14 $^{fo}$ | 12 $^{ms}$ | 8.9 $^{rs}$ | 9.5$^{qs}$ | 14 $^{ks}$ | 12 $^{ns}$ | 22.8 $^{dl}$ | 15.3 $^{js}$ | 17.93 $^{fr}$ |
| $T_1$ | 25.87 $^{bg}$ | 8.73 $^{rs}$ | 34.3 $^{ab}$ | 15 $^{ks}$ | 19 $^{eq}$ | 27.6 $^{ae}$ | 27.8 $^{ad}$ | 32 $^{ac}$ | 25 $^{ci}$ | 25.5 $^{ch}$ | 27.3$^{ae}$ | 18.73 $^{dq}$ |
| $T_2$ | 10.27 $^{qs}$ | 18.5 $^{eq}$ | 21.3 $^{dm}$ | 8.9 $^{rs}$ | 17 $^{do}$ | 19 $^{eq}$ | 21 $^{dm}$ | 12 $^{ns}$ | 16 $^{is}$ | 26.3 $^{bf}$ | 20 $^{dn}$ | 22.8 $^{dl}$ |
| $T_3$ | 11 $^{qs}$ | 14.4 $^{ks}$ | 13.6 $^{ls}$ | 18 $^{fr}$ | 16 $^{hr}$ | 12 $^{ms}$ | 12 $^{ns}$ | 16 $^{hs}$ | 17 $^{gr}$ | 16.5 $^{hr}$ | 35.5 $^{a}$ | 20.67 $^{dn}$ |
| $T_4$ | 22.47 $^{dl}$ | 23 $^{dk}$ | 10.7 $^{ps}$ | 15 $^{js}$ | 10 $^{qs}$ | 20 $^{do}$ | 22 $^{dl}$ | 7.1 $^{s}$ | 20 $^{dp}$ | 9 $^{rs}$ | 15.5 $^{js}$ | 24.27 $^{cj}$ |

Values with a common letter are not significantly different (p = 0.05)

solubilization from phosphate rock containing pyrites with acidophilic sulfur-iron-oxidizing bacteria. Stamford et al. (2007) also reported similar results in increasing of P available of soil when they applied 150 and 200 g sulfur kg$^{-1}$ phosphate rock inoculated with Acidithiobacillus.

## *Phosphorus Uptake by Alfalfa*

The analysis of variance of the experiment (II) is shown in Table 3. The results showed that the main effects of phosphate rock and sulfur on the phosphorus uptake were significant. The interactive effects between Thiobacillus and sulfur were significant on the phosphorus uptake by plant. Phosphorus uptake increased by 32% with 2000 mg kg$^{-1}$ phosphate rock (Figure 6). Application of elemental sulfur at rates of 1000 and 2000 mg kg$^{-1}$ significantly increased phosphorus uptake by plants (Figure 7). Combined application of sulfur (at rates of 1000 and 2000 mg kg$^{-1}$) and Thiobacillus significantly increased phosphorus uptake by plants as compared to the control (Figure 8). Mohammady Aria et al. (2010) reported that the highest concentrations of water-soluble phosphorus were obtained in treatments with 20% sulfur inoculated with T. thiooxidans. Increased P uptake by plants in the presence of sulfur and Thiobacillus was in compliance with other reports (El-Fayoumy and El-Gamal 1998; El-Tarabily et al. 2006).

**Table 3.** Analysis of variance of treatments on P uptake by alfalfa

| Source | DF | Mean square P uptake |
|---|---|---|
| Sulfur | 3 | 0.00058430** |
| *Thiobacillus* | 1 | 0.00000141 ns |
| Phosphate Rock | 2 | 0.00057604** |
| *Thiobacillus**sulfur | 3 | 0.00009408* |
| Sulfur*Phosphate Rock | 6 | 0.00004851ns |
| Phosphate Rock* *Thiobacillus* | 2 | 0.00027623** |
| Sulfur*Phosphate Rock* *Thiobacillus* | 6 | 0.00005847 ns |
| Error | 45 | 0.000032 |
| C.V% | - | 17.81 |

* and ** Significant differences at $p < 0.05$ and $p < 0.01$ respectively and ns: no statistically significant differences.

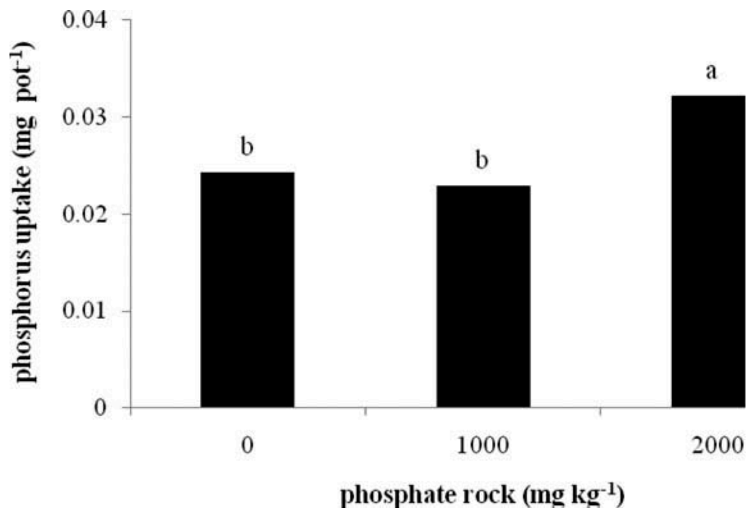


**Figure 6.** Effect of phosphate rock on phosphorus uptake by alfalfa.

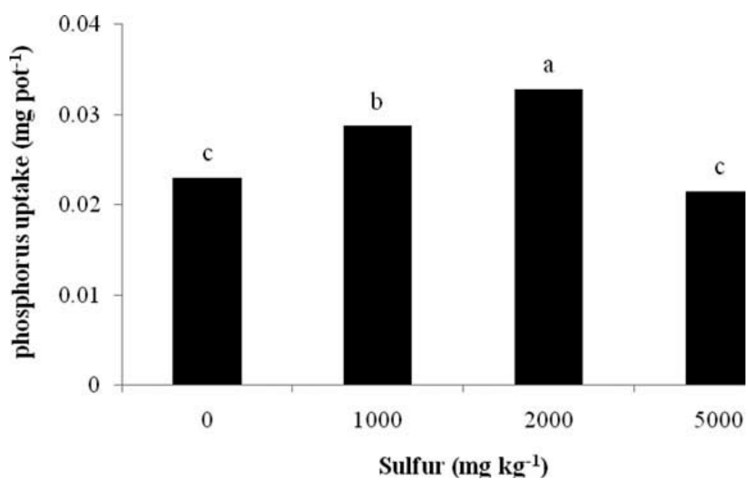


**Figure 7.** Effect of elemental sulfur levels on phosphorus uptake by alfalfa.

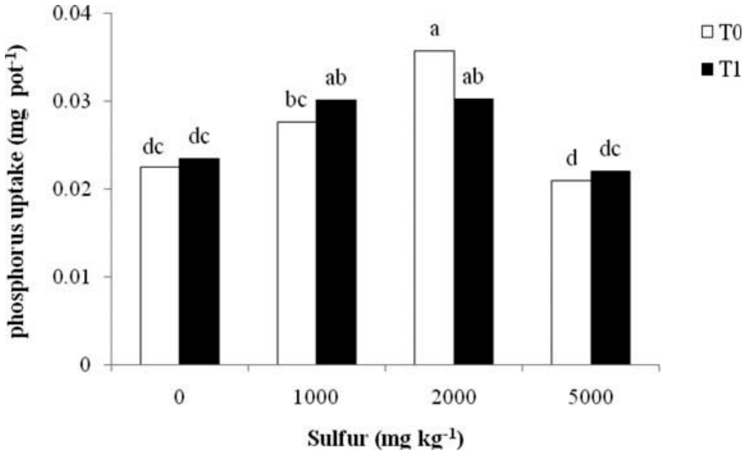


**Figure 8.** Combined effects of *Thiobacillus* inoculum and elemental sulfur on phosphorus uptake by alfalfa.

## Conclusions

Since most of calcareous soil contain a few Thiobacillus population, it is important to use Thiobacillus inoculum along with sulfur to improve bioavailable phosphorus from phosphate rock to achieve sustainable agriculture. Inoculation of Thiobacillus bacteria showed a significant effect on availability of soil phosphorous. Olsen P extractable increased significantly in all inoculated treatments. Sulfur had a significant and positive effect on the concentration of Olsen P extractable. The results also showed that the highest uptake of P by alfalfa was obtained in treatments with sulfur at rates of 1000 and 2000 mg kg$^{-1}$ inoculated with Thiobacillus.

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