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Development of Biological Sulfur Fertilizer for the Improvement of Desert Soil Fertility in Kuwait

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ABSTRACT

Purpose: The alkaline nature of the Kuwait desert soil results in the disturbed of plant germination, absorption of water and fertilizers during growth, leading to an environment, where plants can hardly be grown. The local soil is alkaline with pH normally above 9.00. This is too high for many plant species. With the utilization of newly developed elemental sulfur and sulfur-oxidizing bacteria biofertilizer, it is hypothesized that soil is acidified enough to the extent of improving soil property and thus increasing nutrient availability and crop productivity.

Methods: In house sulfur-oxidizing microbes were screened, enriched and isolated in Kuwait. The most promising strains were optimized through growth and nutrient modification. The production process for biofertilizer pellets utilizing byproduct sulfur and an inoculum of sulfur-oxidizing microbes were developed. The effectiveness of the pellets as soil enhancers was tested with Alfalfa plant in greenhouse and field conditions.

Result: In general, increasing application rates of sulfur, lead significantly to pH decrease and Alfalfa dry matter production increase under greenhouse conditions. The highest application rate of 20 g S kg^{-1} has caused two unit reductions in the pH of the growth medium within 45 days. On the other hand, the field evaluation for the biofertilizer produced has slightly reduced soil pH compared to the greenhouse pH reduction.

Conclusion: The use of the biological sulfur fertilizer has produced positive responses in terms of changing pH of the growth medium in greenhouse and field test as well as the growth responses by the test crop used in this investigation.

INTRODUCTION

Sustainable farming has been identified bio-fertilizer as an alternative to chemical-based fertilizers for increasing soil fertility and crop production. As bio-fertilizers beneficial microbes have become of utmost importance in the agricultural sector owing to their potential role in food safety and sustainable crop production [1-4]. Biofertilizers application leads to improved nutrients and water uptake, plant growth and plant tolerance to abiotic and biotic factors. These potential biological fertilizers can play a key role in the productivity and sustainability of soil and in protecting the environment as environmentally friendly and cost-effective inputs for the farmers.

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In soil, microorganisms play a major role in sulfur oxidation. Microbial sulfur oxidation is, for the most part, beneficial to soil fertility, causing the formation of sulfate, the major S-ion utilized by plants, where the acidity produced by oxidation of the element can be used to improve highly alkaline soils [5]. A wide spectrum of microorganisms is capable of oxidizing sulfur in the environment, including members of the genus *Thiobacillus*, a number of heterotrophs, photosynthetic sulfur bacteria, and the colorless, filamentous sulfur bacteria. Of these, only the thiobacilli and heterotrophs play an important part in S oxidation in most agricultural soils [5], exceptions are flooded soils, in which the microorganisms that are found are normally regarded as aquatic, e.g., *Beggiatoa*, but that can be isolated from both agricultural and nonagricultural soils [6,7].

Thiobacillus is the best-known genus of colorless bacteria [8,9]. They are usually pseudomonad-like organisms, although the genus also contains nonmotile rods and coccoid organisms. They have one common ability, namely, to use energy from the oxidation of reduced sulfur compounds. Within the genus, there are two major groups: (1) the obligate chemolithotrophs, which gain energy from the oxidation of S and use CO_2 as their main carbon source; and (2) the facultative autotrophs, or mixotrophs, that can grow autotrophically, mixotrophically, or as heterotrophs [10].

However, S-oxidizing microorganisms live in soils in consortia, possibly even in mutualistic associations. The process involves a succession of groups of microorganisms [11]. In neutral to alkaline soils, heterotrophs are the primary S-oxidizers between pH 7.5 and 6. *Thiobacillus thioparus* then takes over, and *T. thiooxidans* becomes dominant below pH 5. Consortia existing between sulfate reducers and sulfide-oxidizing bacteria was found that behave like the prokaryotic equivalent of the differential multicellular organism [12]. Similarly, heterotrophic bacteria have been found to be associated with *T. ferrooxidans* isolated from coal shales [13]. It was suggested that these so-called "satellite" organisms are beneficial to the thiobacilli, consuming products such as acetate and pyruvate, which may inhibit chemolithotrophs. Similarly, satellites have been reported as being associated with nitrifying bacteria [14]. It is also interesting that the desulfurization of coal and coke oven effluents is more efficient when mixed cultures, including heterotrophs, are used [5]. Synergism between S-oxidizers growing on solid media has been reported [5].

In calcareous soils, poor availability of nutrients rather than low nutrient content is one of the major factors for plant nutrient deficiency incident. Numerous soil factors are governed nutrient uptake of a plant. Among them, high soil pH and $CaCO_3$ contents are the main factors responsible for the low availability of plant nutrients [15]. Application of N, P and K fertilizer can't resolve the nutrient deficiency under unfavorable soil conditions with high pH and $CaCO_3$.

The alkaline nature of the Kuwait desert soil results in the disturbed plant germination, water absorption, and fertilizers during growth, leading to an environment where plants can hardly be grown. Such a problem can be solved by neutralization of the alkaline components in the soil. One approach for neutralization of alkaline soil is the application of sulfur materials and sulfur-oxidizing bacteria as a soil amendment. Sulfur materials are oxidized by sulfur-oxidizing microbes in the soil to produce sulfate ion, and then the alkaline components are neutralized, at the same time soluble sulfur which is an essential component for plants can be supplied. The aim of the present study was to develop and evaluate elemental sulfur (a by-product of petroleum hydrodesulphurization processes) and sulfur-oxidizing bacteria biofertilizer product for improving vegetation in Kuwait desert alkaline soil.

MATERIALS AND METHODS

Screening of Sulfur-Oxidizing Bacteria

Fifty-one soil samples were screened for sulfur-oxidizing bacteria, collected from a sulfur contaminated area in Kuwait. The pH for these samples was determined and was used for the inoculation of Starkey media. The media consisting of 3 gL^{-1} KH_2PO_4, 0.5 gL^{-1} $MgSO_4.7H_2O$, 2 gL^{-1} $(NH_4)SO_4$, 0.25 gL^{-1} $CaCl_2$, 0.01 gL^{-1} $FeSO_4$, 0.00075 gL^{-1} $Na_2MoO_4.2H_2O$, 10 gL^{-1} S, 0.01 gL^{-1} bromothymol blue, pH 7. The samples were screened for sulfur-oxidizing bacteria at 37°C and 50°C.

Growth Condition Optimization

The optimal condition for biomass production four two selected isolates was optimized using different concentrations of elemental sulfur (0% to 2.5%), yeast extract (0% to 0.25%), different pH (3-9) and temperature (30°C to 60°C), using Starkey media.

Soil Sulfur Biofertilizer Production

Soil biofertilizer was produced by mixing sulfur, sewage sludge, clay minerals (<0.05 mm) and high polymer chemicals (Bentonite, Hiroshima Wako Co. Ltd.). The shape of the sulfur biofertilizer was designed as a pellet with a diameter of 5 m to 10 m **(Figure 1)**, taking the usage into account. The target of the compression strength of the pellet was set to be more than 0.5 $kgfcm^{-2}$.

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Figure 1. Sulfur biofertilizer pellet with a diameter of 5 m to 10 m.

Development of Soil Biofertilizer and Evaluation

The soil biofertilizer production was carried out with two kinds of scale. In the small scale (about 300 g), soil amendment was produced by the Kneader and the Pushing Type Casting machine. In the large scale (about 10 kg), soil amendment was produced by the Mixer with a roller and the Briquetting Machine. First, by using Kneader or Mixer, dry sludge and binder were mixed in the dry form and then, they were mixed again after the addition of water. After that, they were put into the Pushing Type Casting machine or Briquetting machine to make the pellet. Compression strength and water stability were measured. The destruction halftime for the soil biofertilizer in water was measured as the index of water stability. In this measurement, 10 g of soil amendment and 100 ml of water were used.

Greenhouse Experiment

The experiment was carried out in a 4 H × 7 W × 15 L m fiberglass (3 mm) greenhouse. The greenhouse was equipped with moveable benches for space-saving and cooled through an evaporative fan-and-pad cooling system. Ambient temperatures of the greenhouse have been in the range 15°C to 33°C. The soil biofertilizer (in a form of pellets) consist of 30% elemental S, 50% dry sludge, 10% bentonite, 10% sand and 1% bacteria (KNPCN and KNPCF (1:1), 5×10^6-5×10^7 g^{-1} (against dry soil)) binding to attapulgite. The product was incorporated into finely sieved local soil, which was mixed with horticultural grade perlite at a ratio of 50:50 (v:v) to improve the physical property of the root medium. The local soil is sandy in texture (sand: silt: clay, 92%:4%: 4%), with a pH of around 8.0. The experiment was laid out in a randomized complete design with six S rates: 0, 1.3, 2.5, 5.0, 10 and 20 $gSkg^{-1}$ local soil and eight replications per soil treatment. The initial pH of soil at the time of planting the seeds was 9.18. A 1 g of Alfalfa seeds were planted in 20 cm diameter pots. Necessary nutrients have been included in the irrigation water. It is composed of the following chemicals with concentrations used in the nutrient solution: Macronutrients: calcium nitrate ($CaNO_3$)$2.4H_2O$, 2.5 mM), potassium nitrate (KNO_3. 3.0 mM), magnesium sulfate ($MgSO_4.7H_2O$, 1.5 mM), potassium phosphate (KH_2PO_4, 0.2 mM) and iron as EDTA complex (6.0 mg L^{-1}). Micronutrients: (Hoagland micronutrient solution): boric acid (H_3BO_3, 2,860.0 mgL^{-1}), manganese chloride ($MnC_1.4H_2O$, 1,810.0 mgL^{-1}), zinc sulfate ($ZnSO_4.7H_2O$, 220.0 mgL^{-1}), copper sulfate ($CuSO4.7H_2O$, 80.0 mgL^{-1}), and molybdate ($H_2MoO_4.H_2O$, 90.0 mgL^{-1}). The changes in pH were monitored on a weekly basis and plant tissue samples were taken for dry matter weight as well as analyses for important nutrient elements.

Field Trials

Plots size of 5 × 4 m (20 m^2) were irrigated by a sprinkler irrigation system with desalinated water. The plot fertilization schedule and dosages provided were applied according to the following: 1/2 kg per plot, and 3/4 kg per plot. The sulfur biofertilizer pellets consisted of S° (50%), dry sludge (10%), sand (20%) and bentonite (20%) and were incorporated into the top 10 cm to 15 cm of soil. Sulfur biofertilizer rates applied were 0, 16 or 26.5 kg per plot (0, 0.8 or 1.325 kgm^{-2}). This is equivalent to 0, 0.4 or 0.663 $S°kgm^{-2}$. The experiment was laid out in a randomized complete design with eight replications.

Chemical Analysis

The elemental compositions of organic matter in the soil (%Carbon, %Hydrogen, and %Nitrogen) were determined with an elemental analyzer (Thermo Flash 2000 CE EA1112). The total contents of inorganic matter and /or macro- and micro-elements in plant tissue and soil samples were determined by Inductively Coupled Plasma-Atomic Emission Spectrometry (ICP-AES, Varian, Australia; method 6010C). X-ray diffraction (XRD) patterns were measured with a diffractometer (MiniFlex, Rigaku, Tokyo, Japan).

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RESULTS

Screening of Sulfur-Oxidizing Bacteria and Growth Condition Optimization

Forty-five gram-negative *Coccobacilli* bacteria were isolated, 26 isolates at 37°C and 19 isolates at 50°C. Isolates with the lowest incubation time, between three to seven incubating days, were selected for further studies. The isolates growth and pH curves were studied. Maximum growth and pH drop were determined for each isolate **(Table 1)**. At 37°C maximum growth was found with isolate 6 (18×10^7 CFU), after four incubation days, with a maximum pH drop equal to 3.7. On the other hand, a maximum drop in pH at 37°C was found with isolate 10 (pH=2.1), with maximum growth equal to 14×10^7 CFU after eight of incubation days. Isolates 10 and 2 (max growth=6×10^7 CFU, max pH drops=4.6) were selected for growth condition optimization at 37°C and 50°C, respectively. Isolate 10 was labeled as KNPCF and isolate 2 was labeled as KNPCN.

Table 1. Maximum growth and maximum pH drop for the selected isolates.

Isolate ID	Soil pH	Max CFU (X107)	Max pH drop	Incubation Temperature	Incubation Time (Day)
1	7.05	5+0.3	4.7+0	50°C	4
2	8.24	6 +0.6	4.6+0	50°C	3
3	8.03	7 +0.4	4.2+0	37°C	14
4	3.45	6+0.4	4.2+0	37°C	15
5	7.94	7+0.4	4.2+0	37°C	15
6	7.46	18+6.0	3.7+0	37°C	4
7	7.86	4+0.6	3.1+0	37°C	9
8	6.89	11+1.0	2.7+0.1	37°C	9
9	9.4	7+0.2	2.4+0.3	37°C	11
10	6.36	14+0.5	2.1+0.1	37°C	8

Optimized Condition for Isolates KNPCF and KNPCN

The growth condition for biomass production for KNPCF **(Figure 2)** and KNPCN **(Figure 3)** was optimized at 37°C and 50°C, respectively. Both strains had the same optimal temperature (45°C), sulfur concentration (2%) and yeast extract concentration (0.15%). However, they have a different optimal growth pH; optimal pH for KNPCF is 3 whereas for KNPCN is 9. The strains were grown under the optimized condition, mixed in 1:1 ration and bind to attapulgite. They were identified as *Acidithiobacillus thiooxidans* KNPCF and Thiobacillus sp. KNPCN by MIDI Labs laboratories (U.S.A) using 16s rRNA gene sequence similarity, which was performed using a Perkin-Elmer Applied Biosystem's MicroSeqTM microbial analysis software and database. The sequence determined for the 16s rRNA gene resulted in 91% and 99% match with Acidithiobacillus thiooxidans and *Thiobacillus* sp. respectively (GeneBank accession no. MK085107and MK085108 respectively).

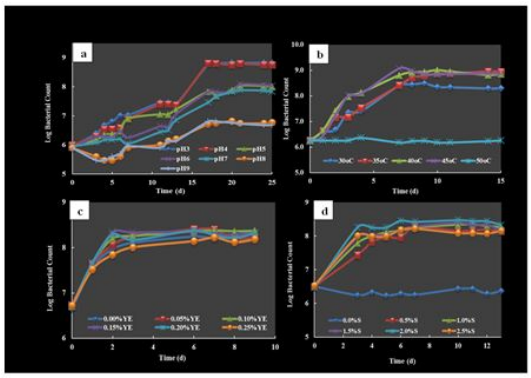

Figure 2. Growth condition optimization for biomass production for KNPCF. (a): pH; (b): Temperature; (c): Yeast extract; (d): Elemental Sulfur.

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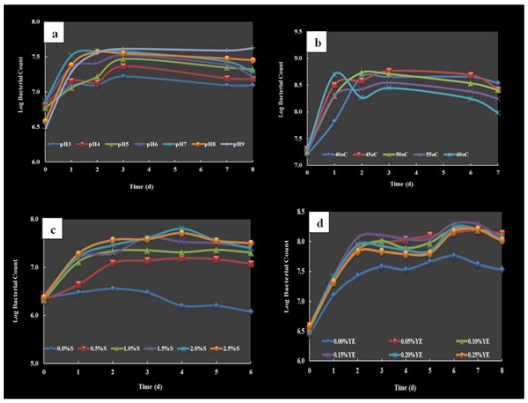

Figure 3. Growth condition optimization for biomass production for KNPCN. (a): pH; (b): Temperature; (c): Yeast extract; (d): Elemental Sulfur.

Sulfur Biofertilizer Production, Development and Evaluation

Table 2 shows the chemical analysis result for the materials used in the sulfur biofertilizer. Kuwait soil as a binder does not contain organic compounds, and the main component is Quartz (SiO_2), furthermore, it contains Calcite ($CaCO_3$) and Anorthite ($CaAl_2Si_2O_8$). Bentonite also does not contain organic compounds, and it is the mixture of the Rectorite ($K1.2Al_4Si_8O_20(OH)4.4H_2O$) and Quartz ($SiO_2$). The dry sludge contains 32% of organic carbon, 3.2% of organic nitrogen and 11% of phosphoric acid (P_2O_5). The main component is Quartz (SiO_2) and there are no crystalline materials except for Quartz.

Table 2. Chemical analysis result of the materials used in the sulfur biofertilizer.

Materials		Kuwait soil	Bentonite	Sulfur	Dry sludge
Organic matter (wt%)	C	0.2	0.3	0.2	32.1
	S	0.1	0.2	99.8	1
	N	<0.1	<0.1		3.2
Inorganic matter (wt%)	SiO_2	82.5	67.8		30
	Al_2O_3	6	23.1		7.7
	CaO	5.8	1.5		7.8
	Fe_2O_3	1.3	1.3		2
	K_2O	1.6	0.4		1.1
	MgO	1.7	3.1		2.9
	Na_2O	0.9	2.1		2
	P_2O_5	<0.1	<0.1		11
	TiO_2	<0.1	0.3		<0.1
X-ray diffraction Results		Quartz	Rectorite		Quartz
		(Main)	(Main)		(as mineral)
		Calcite	Quartz		
		Anorthite	(Main)		

Table 3 shows the results for the effect of using Kuwait soil and bentonite, as a binder, on the compression strength and water stability in the small-scale sulfur biofertilizer produced. As shown in this table, Kuwait soil could be used as a binder for sulfur biofertilizer production. In the presence of the same amount of Bentonite and Kuwait soil, compression strength was increased and the stability in the water was also increased. Compression strength was higher than the target (0.5 $kgfcm^{-2}$).

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Table 3. Effect of Kuwait soil and Bentonite on the compression strength and water stability for the small-scale sulfur biofertilizer produced in the presence of organic compounds (PVA).

Amendment No.	Components (wt%)					Compression Strength (kgf)	Water Stability (days)
	Sulfur	Sludge	Soil	Bentonite	PVA		
L1	30	50	20	0	4	2.7	21
L2	30	50	10	10	4	4.2	25
L3	30	50	0	20	4	3.6	21

Table 4 shows the results for the effect of using Kuwait soil and bentonite, as a binder, on the compression strength in the large-scale soil amendment produced. In the presence of the same amount of Bentonite and Kuwait soil and the absence of the organic compounds PVA, compression strength was increased. In this case, also, compression strength was higher than the target (0.5 $kgfcm^{-2}$).

Table 4. Effect of Kuwait soil and Bentonite on the compression strength for the large-scale sulfur biofertilizer produced in the absence of organic compounds.

Amendment No.	Components (wt%)				
	Sulfur	Dry sludge	Soil	Bentonite	Compression Strength (kgf)
L1	30	50	20	0	1.7
L2	30	50	20	0	1.5
L3	30	50	0	20	2.3
L4	30	50	10	10	3

Sulfur Biofertilizer Pellet Test in the Greenhouse

The growth of Alfalfa plant at different sulfur biofertilizer application rate at day 45 was greatest at the two highest levels (l0 $gSkg^{-1}$ (33.5g) and 20 $gSkg^{-1}$ (66.7 g)) **(Figure 4)**. The difference may be due to the change in pH of the growth medium as the amount of S^0 increased **(Figure 5)**. Application of 20 $gSkg^{-1}$ was effective in reducing pH by approximately 2.0 units in 45 days in the present pot experiment. **Figure 6** shows the results of fitting a second order polynomial model to describe the relationship between Dry Matter (DM) production of the Alfalfa plant and the S^0 treatment. There was a statistically significant relationship between DM and S^0 at the 99% significance level with an R^2 of 87.9%. Furthermore, polynomial regression analysis of the relationship between DM production of Alfalfa plant and pH of the growth medium reveals that the lower the pH of the growth medium, the higher DM yield **(Figure 7)**.

Figure 4. Effect of increasing sulfur biofertilizer application rate on Alfalfa plant growth.

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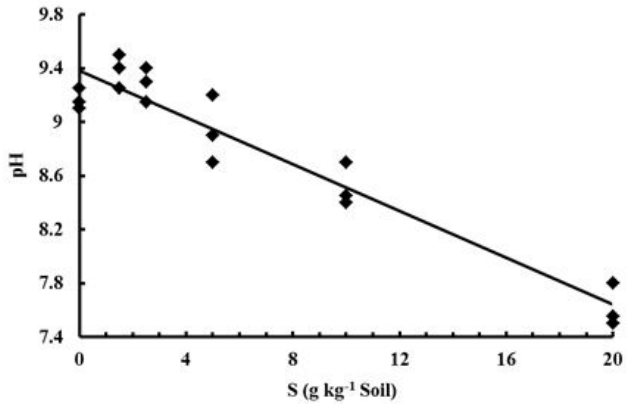

Figure 5. Effect of sulfur biofertilizer application rate on pH of growth medium.

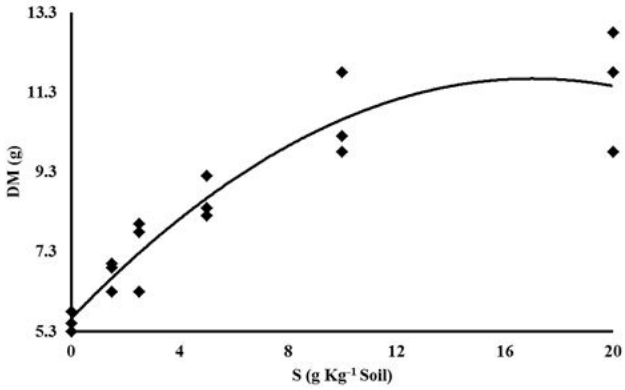

Figure 6. Effect of sulfur biofertilizer application rate on dry matter yield of Alfalfa.

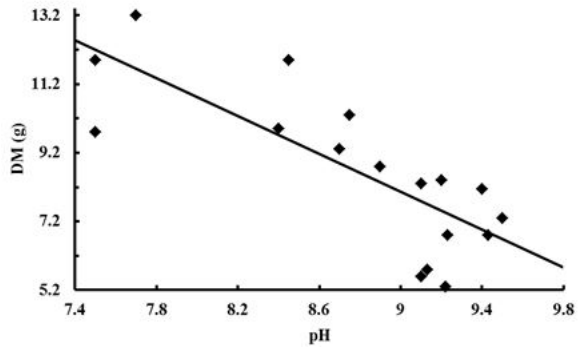

Figure 7. Effect of soil pH alteration on DM of Alfalfa (45 days after planting).

Alfalfa plant tissue samples were analyzed for the concentration of macro- and micro-elements **(Table 5)**. Of particular importance was the significant SO_4 concentration in plant tissues in the 5.0 S^0 treatment, in which the SO_4 concentration in Alfalfa plant tissues grown in the presence of sulfur-oxidizing bacteria was over six times higher than that grown in the absence of the bacteria. Comparisons of Alfalfa plant growth in pots grown in the presence or absence of sulfur-oxidizing bacteria in the 1.3 $gSkg^{-1}$ treatment vs. control (0 $gSkg^{-1}$ soil) after 20 and 30 days seeding in pots in the greenhouse environment illustrate that growth in the presence of bacteria was greater than that in their absence.

Table 5. Macro and micro-elements[a] in Alfalfa plant tissues grown at different concentrations of sulfur in the biofertilizer in the presence (P) or absence (A) of sulfur-oxidizing bacteria (B).

S^{ob}	B	Mg	P	Ca	K	SO_4	Cu	Fe	Mo	Zn
0	A	3131	2081	18603	32995	9160	11.7	554	<1	16

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	P	2635	3768	21253	41160	14428	9	305	<1	13
1.3	A	1863	1442	21613	38147	10743	7	467	<1	44
	P	2533	2891	19273	40166	11910	18	602	<1	17
2.5	A	2925	2021	22237	34529	16188	8	687	<1	18
	P	2802	3238	17374	27143	19062	24	1168	<1	17
5	A	2181	1152	26848	36823	14228	23	1634	<1	20
	P	2230	2568	24408	40428	90597	5.5	949	<1	14
[a] (mg kg); [b] g S kgsoil										

Sulfur Biofertilizer Pellet Test in the Field

In field evaluation, sulfur biofertilizer pellet showed slightly reduced soil pH. This probably due to the high buffering capacity of the soil in the experimental site caused by the relatively high content of $CaCO_3$ and temperature variation during the day. In a sunflower plots experiment, the soil pH decrease as the application rate of S increased from 0 to 0.8 $kgSm^{-2}$ with a simultaneous increase of the SO_4 concentration of the soil solution **(Figure 8)**. As well, the concentration of SO_4 in sunflower tissues increased similarly. Similar results were also obtained for com and Alfalfa. The root system is taken up divalent anion SO_4^{2-} at the physiological pH range, and long-distance transport of sulfate occurs mainly in the xylem. Within the plant, the reduction of sulfate is necessary to incorporate sulfur into amino acids, proteins, and coenzymes.

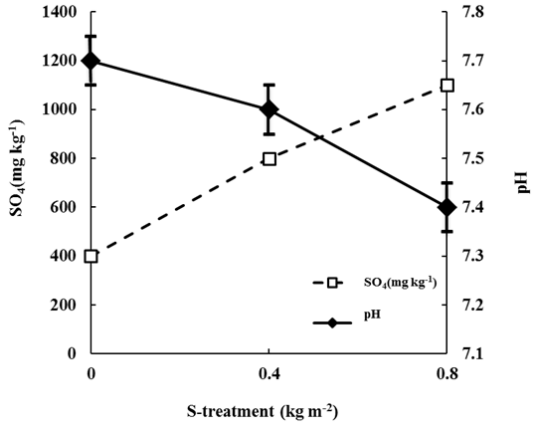

Figure 8. Effect of sulfur biofertilizer on pH and SO_4 concentration of soil at sunflower harvest.

DISCUSSION AND CONCLUSION

Alkali soils formed as a result of carbonate and bicarbonate deposition which occur in several parts of the world [16]. It has been characterized by high pH (>8.5), high exchangeable sodium percentage (ESPs>15) and high levels of carbonates and bicarbonates, all of which are detrimental to the physicochemical properties of the soil. Gypsum and elemental sulfur (S^0), have been used for years as soil amendments [17]. S^0 the application was found to increase the chemically available phosphorous from native soil apatite or added rock phosphate in some calcareous soils [18]. In Saudi Arabian, Falih [19] reported that the amendment of soils with S^0 led to a large decline in pH and $CaCO_3$, and an obvious increase in Mn and Fe, with a slight increase in total soluble salts in most tested soils. Furthermore, in Arkansas, USA, the application rates of 1,100 $kgha^{-1}$ of S^0 improved rice grain yield at one of three locations in Arkansas, nevertheless the application of 4,400 $kgha^{-1}$ S^0 resulted in stand loss and reduced yields caused by soil salinity [20]. Many researchers [21-25], though, have suggested that the response to the S^0 amendment is dependent on the buffering capacity and the amount of $CaCO_3$ present in the soil. Soils amended with S^0 was significantly decreased in soil pH and increased soil SO_4 levels upon the addition of organic matter compared with S^0 alone or organic matter alone. Sewage sludge has been reported to enhance sulfur oxidation by up to >50% of the applied S^0 within six weeks [26].

Biologically oxidized S^0 by *Thiobacillus thiooxidans* to H_2SO_4 under aerobic conditions is frequently applied to reduce soil pH and dissolve insoluble micronutrients and PO_4. In soil, first soil bases neutralizing and lowering pH directly and second dissolving native soil $CaCO_3$ to form gypsum ($CaSO_4.2H_2O$). In Iranian calcareous soils pot experiments, the application of *Thiobacillus*

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inoculants along with S^0 had significant effects on growth parameters of *Zea mays* and on the pH of soil under greenhouse conditions [27].

In this study, we found that the use of the sulfur biofertilizer product produced somewhat positive responses in terms of changing pH of the growth medium in the greenhouse and, to a lesser extent, in the field soil as well as the growth responses by the tested crops. S oxidation rate in the biofertilizer is governed primarily by the microorganisms. Therefore, S oxidation rate is regulated by the physical factors like soil temperature and moistness which play an important role in regulating microorganisms' growth [28]. For future research work in this area, further optimization tests for the sulfur biofertilizer product are needed and more crop and/or ornamental species should be tested. Also, the long-term environmental effects of the applied sulfur in soils also need to be investigated.

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CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

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RESEARCH INVOLVING HUMAN PARTICIPANTS AND/OR ANIMALS

This article does not contain any studies with human participants or animals performed by any of the authors.

INFORMED CONSENT

Informed consent was obtained from all individual participants included in the study.

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