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Enhancing chickpea yield through the application of sulfur and sulfur-oxidizing bacteria

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Plant growth-promoting microorganisms can enhance sulfur uptake and boost crop production. This study was conducted to evaluate the changes in physiology, metabolism, and yield of chickpeas following the application of sulfur and two microbial consortia: (1) *Thiobacillus* sp., *Bacillus subtilis*, *Paraburkholderia fungorum*, and *Paenibacillus* sp.; and (2) *Enterobacter* sp. and *Pseudomonas* sp. The soil amendment involving a combination of sulfur and sulfur-oxidizing bacteria (SOB) in any quantity had positive effects on the availability of phosphorus, nitrogen, and potassium in the soil. A combination of 90% sulfur with *Enterobacter* sp. and *Pseudomonas* sp. resulted in a decrease in soil pH after harvesting in both years. Both years showed a strong correlation between soil pH and soil macronutrient concentration. In both years, the maximum grain yield was achieved through a combination of increased sulfur levels and SOB. The results reveal that sulfur application and SOB can increase nutrient availability, nutrient uptake, and yield of chickpea growth in calcareous soils.

Keywords Bio-fertilizer, Calcareous soils, Nitrogen, Soil pH, Sulfur

Chickpea (*Cicer arietinum* L.) is a significant legume crop that is widely cultivated and consumed around the world[1]. In 2022, it was cultivated on approximately 18 million hectares, resulting in an annual production of 17.2 million tons. The average productivity, or grain yield, ranged from 1,200 to 1,300 kg per hectare[2]. Chickpeas are an excellent source of nutrients, carbohydrates, protein, fiber, minerals, and essential amino acids[3,4]. Furthermore, chickpeas have natural features such as leaf fall, biological nitrogen fixation, and improved rhizosphere functions, which are vital for enhancing soil health[5]. In Iran, chickpeas are primarily cultivated in calcareous soils, which are known for their high levels of calcium carbonate ($CaCO_3$). However, these soils present significant challenges to farmers due to their high pH and excessive $CaCO_3$ content, which can restrict the availability of essential nutrients such as phosphorus and micronutrients. Consequently, the growth and yield of plants may be negatively affected[6].

Sulfur is an essential element for all organisms and serves various functions. Plant sulfur nutrition is essential because plants are our primary source of essential amino acids such as methionine and cysteine, glutathione, vitamins (biotin and thiamine), phytochelatins, chlorophyll, coenzyme A, and S-adenosyl-methionine[7–9]. Sulfur deficiency affects the growth, development, disease resistance, and performance of plants and significantly impacts the nutritional quality of crops[7]. Using sulfur is an effective method for lowering soil pH and enhancing nutrient uptake. This approach is both cost-efficient and widely utilized for this purpose. It is particularly beneficial in arid and desert regions, such as Iran, where calcareous soils and high pH levels can limit nutrient availability to plants. In these areas, the application of sulfur-containing fertilizers can improve soil fertility and promote healthier crop growth[10–12] Each mole of sulfur dioxide, when oxidized, produces two moles of hydrogen ions (H^+) in the soil solution, leading to a decrease in soil pH near the plant roots. This decrease enhances the availability of soil nutrients[13]. Applied sulfur will be effective when adequately incorporated into the soil, leading to oxidation by microorganisms[14]. Important microorganisms involved in sulfur oxidation include a group of bacteria from the genera *Acidithiobacillus* and *Thiobacillus*, as well as heterotrophic bacteria such as *Cytobacillus firmus*, *Enterobacter cloacae*, *Enterobacter ludwigii*, *Klebsiella oxytoca*, *Phytobacter diazotrophicus*, and *Pseudomonas stutzeri*[15,16].

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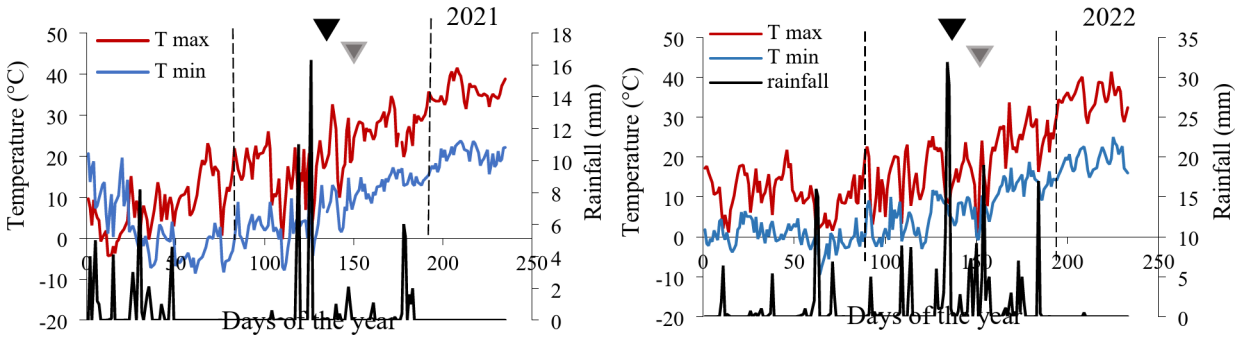

Fig. 1. Growing conditions (meteorological data) for chickpea crops resulting from the sowing dates to harvest time. The range between the two points shows the period of chickpea growth. Arrowheads mean flowering time (black) and pod set (gray).

Year*	Soil acidity	Electrical conductivity (dS m^{-1})	Texture	Mineral Carbon (%)	Organic carbon (%)	Nitrogen%))	Phosphorus (mg kg^{-1})	Potassium (mg kg^{-1})
2021	8.01	2.88	Loam	1.05	0.61	0.063	15	119
2022	8.12	1.98	Loam	1.25	0.65	0.068	16.3	126

Table 1. Some physical and chemical properties of soil at the experimental site. *The study was conducted in two different plots each year.

The supplementation of sulfur-containing mineral fertilizers in agriculture has been reported to address many soil and plant nutrition issues by enhancing soil biological and physical properties, reducing pH values, and increasing the availability of plant nutrients[17,18]. For example, the addition of sulfur to nodulated legumes has been shown to not only increase the synthesis of sulfur-containing amino acids but also enhance the amounts of N_2 fixed in soils and plant tissues[8,9]. It has been proven that *Thiobacillus* sp. enhances plant growth most efficiently in unfavorable growth conditions[19]. Application of sulfur and *Thiobacillus* sp. to sesame (*Sesamum indicum* L.) and mung bean (*Vigna radiata* L.) under field conditions showed improved yield and growth indices for both crops[20]. Janmohammadi et al.[21] demonstrated that the use of sulfur significantly extended vegetative growth and postponed maturity. In addition, optimizing sulfur nutrition before the flowering stage is necessary for achieving maximum vegetative growth, which will directly impact yield potential[22]. According to Jabbar et al.[23], the application of sulfur and sulfur-oxidizing bacteria (SOB) significantly increased the concentrations of phosphorus, nitrogen, potassium, and sulfur compared to the control. Also, Soaud et al.[24] and Amin et al.[25] demonstrated that soils inoculated with SOB significantly affect the concentrations of phosphorus, sulfate, and micronutrients. Therefore, they suggested that sulfur plays a crucial role in amending calcareous soils. The application of sulfur and sulfur-oxidizing bacteria enhances the availability of phosphorus, nitrogen, and potassium through various mechanisms[24,25]. Sulfur-oxidizing bacteria convert elemental sulfur into sulfuric acid, which lowers soil pH and improves phosphorus availability by reducing its fixation and increasing its solubility. Additionally, sulfur oxidation contributes to the mineralization of organic matter, releasing nitrogen and phosphorus into the soil in forms that are readily available to plants. Finally, sulfur-oxidizing bacteria can help solubilize potassium by producing organic acids, thereby increasing its availability for plant uptake[21,24,25].

In many regions of the world, including Iran, alkaline soils are commonly found in areas with arid and semi-arid climates, such as in parts of the central plateau and southern regions[26]. Alkaline soils frequently have high pH levels, usually above 8.5, which can impact plant growth by restricting nutrient availability and causing toxicity problems with certain plants. To address alkaline soils in Iran, agricultural practices such as soil amendment with organic matter, sulfur, or acidifying agents can be used to lower the pH and improve soil fertility. The aims of this study were (i) to observe the effect of different SOB and sulfur chemicals on the biochemical changes of the leaf, (ii) to observe the effect of different SOB and sulfur chemicals on the concentration of macronutrients (nitrogen, phosphorus, potassium) and pH in soil, (iii) to assess the effects of different SOB and sulfur chemicals on yield, and (iv) to evaluate the response of soil nutrition, soil pH, and leaf biochemical composition to yield. By conducting this experiment, we can uncover the correlation between soil pH, nutrient concentration, and crop yield, paving the way for effective sulfur application and increased crop production.

Materials and methods

Experimental locations and growing conditions

A split-plot experiment was conducted using a completely randomized design (Fig. 1 Supplementary). The research was carried out at the Faculty of Agriculture, Ferdowsi University of Mashhad, Iran, in experimental fields located at a latitude of 36°15′N and a longitude of 59°38′E, at an elevation of 985 m above sea level. The study was carried out from March 2021 to July 2022 and included three replications. Figure 1 presents the rainfall, minimum and maximum temperatures recorded in 2021 and 2022, as well as the long-term averages obtained from the Meteorological Organization. Table 1 provides details of the experimental soil sites.

Experimental setup

Biological treatments were categorized into three levels: control (without SOB) (B_1), a microbial consortium featuring *Thiobacillus* sp., *Bacillus subtilis*, *Paraburkholderia fungorum*, and *Paenibacillus* sp. under the Dayan® trademark (B_2), and another microbial consortium comprising *Enterobacter* sp. and *Pseudomonas* sp. under the Mehr Asia® trademark (B_3) in the main plots (Table 2). Sulfur was applied at four different levels, organized as sub-plots: control (no sulfur application) (F_1), 175 kg ha^{-1} of sulfur (70% bentonite sulfur; 70% bentonite sulfur is a type of fertilizer composed of a mixture that contains 70% elemental sulfur and 30% bentonite clay) (F_2), 225 kg ha^{-1} of sulfur (90% bentonite sulfur; 90 elemental sulfur and 10% bentonite clay) (F_3), and 250 kg ha^{-1} of sulfur (99% bentonite sulfur; 99 elemental sulfur and 1% bentonite clay) (F_4).

Chickpea seeds were sown in plots measuring 2 × 3 m^2, with an inter-row spacing of 50 cm and an intra-row spacing of 6.5 cm, at a depth of 5 cm. Microbial inoculation was conducted in three phases: at the time of planting when the seeds were sown, during the second irrigation (7 days after sowing, DAS), and at the flowering stage. Each liquid biofertilizer contained 10^9 colony-forming units (CFU) per milliliter. The source of sulfur was bentonite sulfur, which was applied before planting. In this experiment, the surface irrigation method was applied. The crops were irrigated immediately after sowing the seeds and subsequently at 14-day intervals until harvest. Weeding was performed by hand only twice before the crop canopy closed.

Data collection

Photosynthetic pigment content

The photosynthetic pigment content was quantified using the 96% ethanol extraction method. According to the method, optical density was measured at 664 nm and 648 nm for chlorophyll a and chlorophyll b, as described by Lichtenthaler[27]. The results were described in milligrams per gram of fresh weight (mg g^{-1} fw).

$$\text{Chlorophyll } a = (13.36\ (A\,664) - 5.19\ (8))$$

$$\text{Chlorophyll } b = (27.43\ (A\,648) - 8.12\ (64))$$

Leaf-soluble carbohydrates content (SC)

To measure the leaf SC, the method described by Dubois et al.[28] was followed. Initially, 100 mg of the fresh leaf was homogenized in 70% ethanol and stored at 4 °C overnight. The solids were then removed by centrifuging the sample at 3000 x g for 5 min. Next, the supernatant was mixed with phenol and sulfuric acid, and the sample was subjected to a hot water bath at 100 °C for 30 min. Finally, the absorbance was measured at 480 nm using D-glucose as a standard.

Leaf phenol content

To determine the total phenol content, 100 mg of fresh leaf weight was homogenized in 96% ethanol. After centrifuging at 3000 x g for 5 min, a proportional amount of the supernatant was mixed with 1 mL of double-distilled water and 20 µL of Folin–Ciocalteu reagent. After 5 min, 300 µL of 20% w/v sodium carbonate was added, and the mixture was kept at 40 °C for 30 min. The total phenol content was determined based on the absorbance at 765 nm using a gallic acid standard and reported as mg.g^{-1} fresh weight, following the method described by Singleton et al.[29].

1,1-diphenyl-2-picrylhydrazyl (DPPH)

To determine the DPPH radical scavenging activity, the method described by Sanna et al.[30] was followed. A 05mM solution of DPPH in 96% ethanol was prepared, and 4 ml of this solution was mixed with 1 ml of an ethanolic extract at varying concentrations (0.02–0.1 mg ml^{-1}). The mixture was thoroughly vortexed and then incubated in the dark for 30 min. The absorbance was measured at 517 nm using a spectrophotometer. Ascorbic acid was used to establish the standard curve.

Analysis of soil pH and mineral nutrients

The soil pH is measured at different development stages: vegetative stage (30 DAS), flowering stage (80 DAS), pod set stage (95 DAS), and after harvesting (115 DAS). Measurements are taken promptly, both immediately and after sampling, using a pH meter equipped with a highly sensitive probe. The samples collected from each plot were analyzed to estimate the soil's nitrogen, phosphorus, and potassium concentrations. The dried samples were ground. For nitrogen concentration determination, a finely ground sample was digested in concentrated sulfuric

Sulfur-oxidizing bacteria	set of strains	Application	Company
Solpho Bacter Dayan (liquid biofertilizer*)	*Thiobacillus* sp. and *Bacillus subtilis* NCBI Accession No.MK968145, *Paraburkholderia fungorum* NCBI Accession No. MK968146 and *Paenibacillus* sp.NCBI Accession No. MT102427	Bacterial treatments at the rate of 5 L ha^{-1} were applied separately at each bacterial plot	Dayan Company in Iran
Biosulfur (powder biofertilizer)	*Enterobacter* sp. and *Pseudomonas* sp.	Bacterial treatments at the rate of 6 Kg ha^{-1} were applied separately at each bacterial plot	Mehr Asia Biotechnology Company in Iran

Table 2. The supplementary bacterial content of biofertilizer used in this study. *The liquid biofertilizer contained 10^9 colony-forming units (CFU) per milliliter.

acid (H_2SO_4) with a digestion mixture comprising copper sulfate, potassium sulfate, and mercuric oxide[31]. The nitrogen concentration was determined using the Kjeldahl method[31]. To determine the concentration of phosphorus and potassium, the sample was digested with a di-acid mixture of nitric acid (HNO_3) and chloric acid ($HClO_3$). The digestion methods for phosphorus and potassium were chosen to determine the total content of these elements in the soil. Phosphorus concentration in the extract was measured at 436 nm using vanadate/molybdate as a colorimetric agent in a spectrophotometer (Visible-UV Jenway Model 5630). Potassium was quantified using the flame emission photometry method (Jenway PFP7, England)[32].

Harvest

Whole plants (12 treatments × 3 replicates = 36 plots) were harvested at maturity, defined as when 95% of the pods were brown. After that, considering the marginal effect, the remaining plants were harvested to evaluate grain yield (GY).

Statistical analysis

Initially, a combined analysis of variance was conducted using the General Linear Model (GLM) procedure of the Statistical Analysis System. The analysis included sulfur and SOB as fixed effects and 'year' as a random effect. However, the data did not show significance ($p > 0.05$). In the subsequent step, data from each year were analyzed separately using the General Linear Model ($p \leq 0.05$). Statistical analyses were performed using SAS v. 9.1 software (SAS Institute Inc., Cary, NC, USA), which included analysis of variance, comparison of means using LSD tests, and calculation of correlation coefficients between traits. Additional graphical analyses were conducted using JMP v.4.0, R (package ggplot2), and GraphPad Prism v.9.0 software.

Results

Leaf biochemical properties

Free radical scavenging activity (DPPH)

According to the results, the interaction effects of SOB and sulfur on the DPPH of chickpeas were significant ($p \leq 0.05$) (Table 3). During the vegetative stage, inoculation with bio-fertilizers significantly increased the DPPH content. The highest DPPH level was obtained in B_2F_3 (1.17 mg gfw^{-1}) in 2021 and in B_3F_1 (1.47 mg gfw^{-1}) in 2022 (Table 3). In the flowering stage, the highest DPPH levels in 2021 were found in B_2F_3 (5.71 mg gfw^{-1}), and in 2022 in B_3F_1 (6.70 mg gfw^{-1}), respectively. Comparison of the mean of this characteristic in the pod stage also showed that B_2F_1 (8.60 mg gfw^{-1}) in the first year of the study and B_3F_1 (10.93 mg gfw^{-1}) in the second year had the highest DPPH values (Table 3).

Total phenol content

In this study, the interaction effects of SOB and sulfur on the total phenol content of chickpeas were significant ($p \leq 0.05$) (Table 3). The total phenol content is influenced by the application of sulfur on SOB. In the vegetative stage, the total phenol content was significantly higher for B_3F_4 in 2021 and 2022 (88 and 100 mg gfw^{-1}, respectively) compared to the other treatments (Table 3). In the flowering stage, B_2F_2 (111 mg gfw^{-1}) in the first year and B_3F_2 (124 mg gfw^{-1}) in the second year, as well as in the pod stage, B_3F_2 (124 mg gfw^{-1}) in the first year and B_3F_4 (161 mg gfw^{-1}) in the second year, exhibited the highest phenol content (Table 3).

Water-soluble carbohydrates content (WSC)

The response of WSC content varied among different treatments. In this way, during the vegetative stage, the highest WSC content in the first year of the study was found in B_3F_3 (0.49 mg gfw^{-1}), and in the second year, it was in B_2F_2 (0.50 mg gfw^{-1}) (Table 3). In the flowering stage, B_3F_3 (4.24 mg gfw^{-1}) had the highest WSC content in the first year, while B_3F_4 (4.05 mg gfw^{-1}) had the highest content in the second year. In the pod stage, B_2F_3 (5.62 mg gfw^{-1}) had the highest WSC content in the first year, and B_3F_3 (5.88 mg gfw^{-1}) had the highest content in the second year (Table 3).

Chlorophyll contents

Photosynthetic pigments (chlorophyll a and chlorophyll b) were significantly affected by various fertilizer treatments (Fig. 2). An increase in the levels of both types of chlorophyll was observed with the application of bio-fertilizer and sulfur during growth compared to growth without bacteria and sulfur application. The maximum chlorophyll a content was recorded in the leaves of B_3F_4 and B_3F_3, surpassing the control levels in 2021 and 2022, respectively. Similarly, the content of Chlorophyll b in the leaves of B_2F_1 and B_2F_2 increased compared to the control in 2021 and 2022, respectively, at the vegetative stage. In response to the B_2F_3 treatment, the content of chlorophyll a increased during the flowering stage compared to the control in 2021 and 2022, while the content of chlorophyll b peaked at the B_2F_2 and B_2F_3 treatments. In the filling pod stage, the highest concentrations of chlorophyll a and chlorophyll b were observed in B_2F_3 and B_2F_1 in the treated sets, indicating an increase compared to the control.

Soil nutrients and soil pH

In this study, the application of sulfur and bio-fertilizer significantly enhanced the levels of nitrogen, phosphorus, and potassium ($p \leq 0.05$) (Figs. 3 and 4 and 5). The nitrogen concentration is influenced by the application of sulfur on SOB. The nitrogen concentration was significantly higher in all treatments compared to pre-experimental levels. In the vegetative stage, the nitrogen concentration peaked at 0.065% and 0.059% for B_3F_4 in 2021 and 2022, respectively (Fig. 3a). During the flowering stage, the highest nitrogen concentration was observed in B_3F_3, and in 2022 in the B_3F_4 treatment (Fig. 3b). The highest nitrogen uptake in the soil at the pod stage and after harvesting was associated with the B_3F_1 treatment in 2021, which increased more than the control

Treatment	Vegetative stage			Flowering stage			Pod set stage		
	DPPH (mg gfw^{-1})	Phenol (mg gfw^{-1})	Carbohydrates (mg gfw^{-1})	DPPH (mg gfw^{-1})	Phenol (mg gfw^{-1})	Carbohydrates (mg gfw^{-1})	DPPH (mg gfw^{-1})	Phenol (mg gfw^{-1})	Carbohydrates (mg gfw^{-1})
2021									
B1F1	0.92^{e}	72.22^{cd}	0.31^{f}	4.14^{ef}	83.04^{f}	1.71^{e}	4.45^{de}	86.90^{f}	3.99^{d}
B1F2	0.80^{e}	82.84^{a-c}	0.37^{e}	4.09^{ef}	103^{bc}	2.32^{d}	4.81^{ce}	108^{cd}	4.20^{d}
B1F3	0.85^{de}	85.53^{ab}	0.38^{de}	4.40^{de}	110^{a}	2.56^{d}	4.54^{de}	107^{d}	4.54^{b-d}
B1F4	0.87^{c-e}	73.72^{bc}	0.41^{cd}	4.37^{ef}	101^{cd}	3.56^{b}	4.82^{c-e}	108^{cd}	4.82^{a-d}
B2F1	0.95^{c-e}	59.98^{d}	0.38^{de}	4.44^{de}	103^{bc}	2.77^{cd}	8.60^{a}	94^{e}	5.35^{a-c}
B2F2	1.01^{b-d}	79.58^{a-c}	0.44^{b}	5.82^{cd}	111^{a}	3.75^{ab}	4.49^{de}	107^{cd}	5.30^{a-c}
B2F3	1.17^{a}	70.79^{cd}	0.38^{de}	5.70^{a}	99.53^{cd}	3.47^{b}	5.71^{b-d}	113 ^{bc}	5.62^{a}
B2F4	1.01^{b-d}	82.59^{a-c}	0.42^{bc}	5.32^{ab}	95.00^{de}	2.61^{d}	6.26^{b}	106 ^{d}	4.90^{a-d}
B3F1	0.86^{de}	73.30^{bc}	0.33^{f}	3.93^{f}	94.69^{de}	1.73^{e}	5.45^{b-e}	106^{d}	4.45^{cd}
B3F2	1.03^{a-c}	76.78^{a-c}	0.42^{bc}	4.39^{de}	97.49^{c-e}	2.81^{cd}	5.01^{b-e}	124^{a}	5.43^{ab}
B3F3	1.13^{ab}	80.30^{a-c}	0.49^{a}	5.16^{bc}	100^{cd}	4.24^{a}	6.02^{bc}	118^{b}	4.33^{d}
B3F4	0.89^{c-e}	87.84^{a}	0.47^{a}	4.20^{ef}	107^{ab}	3.25^{bc}	4.31^{e}	118^{ab}	5.51^{a}
B×F	*	*	**	**	*	**	*	*	*
CV%	9.75	9.35	4.82	5.64	4.10	12.27	3.91	3.92	11.06
2022									
B1F1	1.01^{e}	61.32^{e}	0.28^{e}	5.94^{bc}	86.90^{f}	2.57^{e}	9.08^{c}	126^{d-g}	4.79^{b}
B1F2	0.86^{f}	75.62^{b-d}	0.49^{a}	5.01^{ef}	106de	3.17^{cd}	7.69^{e-g}	119^{f-h}	5.12^{ab}
B1F3	0.88^{f}	73.81^{cd}	0.47^{ab}	4.68^{f}	109^{ce}	3.40^{a-d}	7.26^{fg}	128^{d-e}	5.82^{a}
B1F4	0.72^{g}	70.77^{c-e}	0.38^{b-d}	3.88^{g}	108^{ce}	3.55^{a-d}	6.88^{g}	130de	5.44^{ab}
B2F1	1.25^{bc}	66.57^{de}	0.31^{ed}	5.75^{b-d}	94.35^{f}	3.14^{cd}	8.62^{cd}	115^{h}	5.45^{ab}
B2F2	0.87^{f}	85.05^{b}	0.50^{a}	4.98^{ef}	107de	2.06^{de}	7.86^{d-f}	117^{gh}	4.96^{ab}
B2F3	0.99^{e}	75.13^{b-d}	0.45^{ab}	5.59^{cd}	113^{b-d}	3.13^{cd}	8.56^{c-e}	122^{e-h}	5.44^{ab}
B2F4	0.76^{g}	77.93^{bc}	0.42^{a-c}	4.75^{f}	106de	3.32^{b-d}	7.42^{f-g}	134^{cd}	5.38^{ab}
B3F1	1.47^{a}	71.76^{cd}	0.31^{de}	6.69^{a}	104^{e}	3.27^{cd}	10.93^{a}	121^{e-h}	5.16^{ab}
B3F2	1.19^{cd}	71.78^{cd}	0.40^{a-d}	6.15^{b}	124^{a}	3.8^{1ab}	10.05^{ab}	141^{bc}	5.79^{a}
B3F3	1.15^{d}	78.94^{bc}	0.35^{de}	6.07^{bc}	115^{bc}	3.65^{a-c}	8.75^{cd}	146^{b}	5.88^{a}
B3F4	1.30^{b}	100^{a}	0.43^{a-c}	5.35^{de}	120^{ab}	4.05^{a}	9.48^{bc}	161^{a}	5.74^{a}
B×F	**	**	*	*	*	*	*	**	*
CV%	4.92	7.99	13.77	5.21	3.83	9.47	6.92	4.52	9.44

Table 3. The analysis of variance of the interactive effects of using sulfur and sulfur-oxidizing bacteria of plant characteristics of chickpea in 2021 and 2022. Similar letters are not significant based on the LSD test at 5% of probability. *B1* no sulfur-oxidizing bacteria, *B2* Solpho Bacter Dayan, *B3* Biosulfur, *F1* without adding sulfur, *F2* 70% bentonite sulfur, *F3* 90% bentonite sulfur, *F4* 99% bentonite sulfur. B×F: significant interactive effects, $^{*}P \leq 0.05$, $^{**}P \leq 0.01$.

(Fig. 3c and d). A significant increase in nitrogen levels in the soil was observed with B_3F_4 during the pod stage and after harvesting (Fig. 3c and d).

A significant disparity in the phosphorus content available in the soil between treatments was observed at all stages of plant growth. In the vegetative stage, the highest phosphorus concentration in B_3F_4 is observed in 2021 and 2022, respectively (Fig. 4a). In the flowering stage (60 DAS), the highest phosphorus concentrations were 14.8 and 15.1 mg kg^{-1} for B_3F_2 and B_3F_4 treatments in 2021 and 2022, respectively (Fig. 4b). The highest phosphorus uptake in the pod stage was associated with the B_3F_2 and B_3F_4 treatments in 2021 and 2022, respectively, which were higher than the control (Fig. 4c). After the harvest, the absorbable phosphorus has decreased due to the plant's uptake and stabilization in the soil (Fig. 4d).

The application of sulfur and bio-fertilizer significantly increased the concentration of potassium compared to the control without sulfur application ($p \leq 0.05$) (Fig. 5). The highest potassium uptake in the soil during the vegetative stage was associated with the B_3F_3 treatment in 2021 and 2022, showing a significant increase compared to the control (Fig. 5a). The highest concentration of potassium increased from 82 to 89 mg kg^{-1} for the control to 124 and 122 mg kg^{-1} for the B_3F_3 treatments in 2021 and 2022, respectively, at 60 DAS (during the flowering stage, as shown in Fig. 5b). In contrast, the potassium content at 80 DAS during the pod stage showed the highest potassium uptake in the soil, with levels of 115 mg kg^{-1} related to B_3F_4 in 2021 and 2022, respectively, which were higher than the control (Fig. 5c). The results indicated that amending the soil with sulfur significantly increases the potassium content compared to the control after the harvesting stage (Fig. 5d).

The data analysis in Fig. 5 revealed significant differences when adding the inoculant and sulfur fertilizer to the soil's pH content ($p \leq 0.05$). The results also revealed that soil pH content decreased significantly compared to the values before the experiment (Fig. 6), indicating that the reduction is related to the post-harvest period.

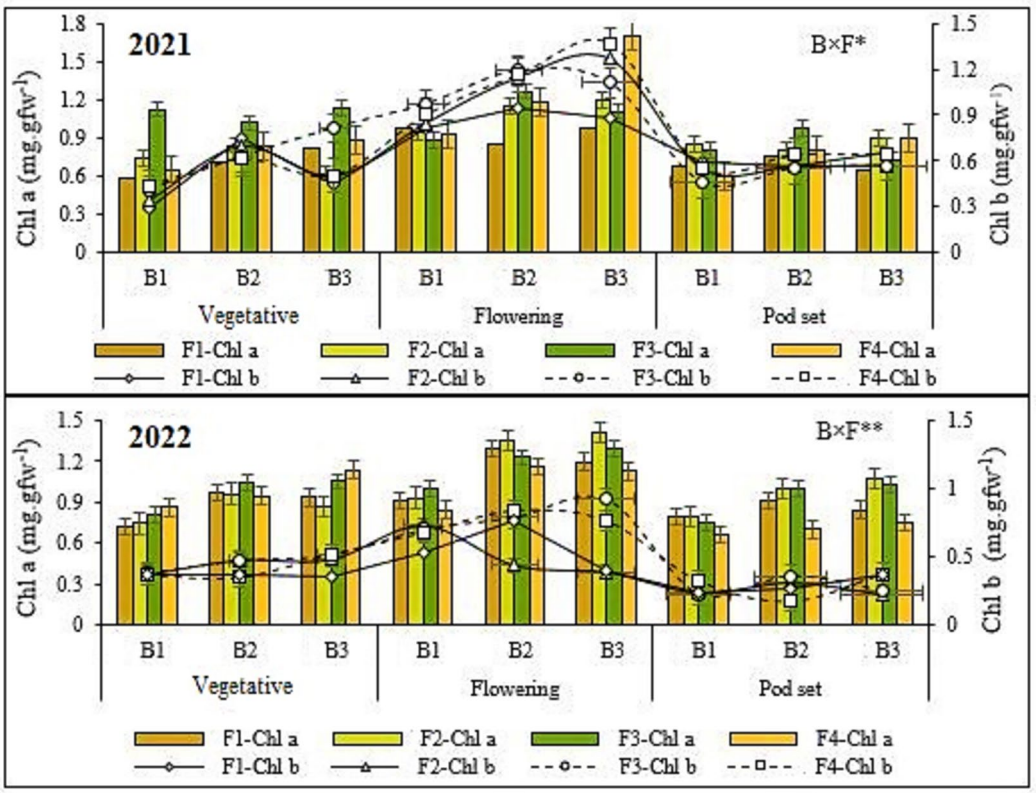

Fig. 2. The effect of sulfur and sulfur-oxidizing bacteria on chlorophyll contents in chickpea at different development stages. The details of the set of strains are provided in Table 2. Vertical bars indicate the standard error of means. B: Sulfur-oxidizing bacteria, F: Sulfur; B×F: Significant interactive effects, $^{*}P \leq 0.05$, $^{**}P \leq 0.01$. *Chl a* chlorophyll a; *Chl b* chlorophyll b; *B1* without sulfur-oxidizing bacteria; *B2* Solpho Bacter Dayan; *B3* biosulfur; *F1* without adding sulfur; *F2* 70% bentonite sulfur; *F3* 90% bentonite sulfur; *F4* 99% bentonite sulfur.

Grain yield

Box plots for GY are shown in Fig. 7a. The interaction effect between SOB and sulfur was significant in 2021 and 2022 ($P \leq 0.05$) (Fig. 7a). In the first year (2021), the maximum GY obtained with B3F4 (134 g m^{-2}) fertilizer increased by 83% compared to the control, followed by B_3F_2 and B_3F_3. In the second year (2022), there was a significant 45% increase in GY for B_2F_3 compared to the control (Fig. 7a). The histogram distribution of yield in 2022 was more spread out than in 2021. The value of grain yield in 2021 was lower compared to 2022 (Fig. 7b).

Pearson correlation

In 2021, there was an increase in soil nitrogen uptake and a decrease in pH (R^2 = -0.40) (Table 4). Significant responses of soil pH were observed in relation to the concentration of phosphorus (R^2 = -0.44) and potassium (R^2 = -0.57), respectively. In the year 2022, there was a significant correlation between nitrogen, phosphorus, and potassium concentrations (R^2 = -0.52, -0.56, and −0.44, respectively) (Table 4). In 2021, there was a significant and positive correlation between the GY and nitrogen, phosphorus, and potassium concentrations (R^2 = 0.72, 0.69, and 0.52, respectively) (Table 4). Also, there was a significant positive correlation between the GY and the concentrations of nitrogen, phosphorus, and potassium (R^2 = 0.57, 0.66, and 0.55, respectively) in 2022 (Table 4). The data analysis revealed a positive and significant correlation between GY and the levels of chlorophyll a and chlorophyll b in 2021 (R^2 = 0.67 and 0.58, respectively). In 2022, there was a correlation between GY and the concentration of chlorophyll a (R^2 = 0.54). Also, there was a significant correlation between phenol content and GY in 2021 and 2022 (R^2 = 0.50 and 0.42, respectively) (Table 4).

Discussion

The alkaline soil's calcareous nature and high bicarbonate content create precipitation and fixation issues, limiting nutrient use efficiency, especially for phosphorus and micronutrients. Effective soil nutrition management can enhance fertilizer use efficiency, leading to improved plant growth and yields. The study's findings demonstrate that the addition of sulfur to soil resulted in significant improvements in the availability of essential nutrients, such as phosphorus, nitrogen, and potassium. In the control, the average soil available phosphorus was approximately 10.3 mg kg^{-1} at 30 DAS. However, this value increased to 14.8 mg kg^{-1} at 60 DAS in the B3F4 treatment (Fig. 6). Fox[33] reported that the addition of sulfur to alkaline soils could enhance phosphorus availability through oxidation. This process can decrease soil pH and convert the unavailable

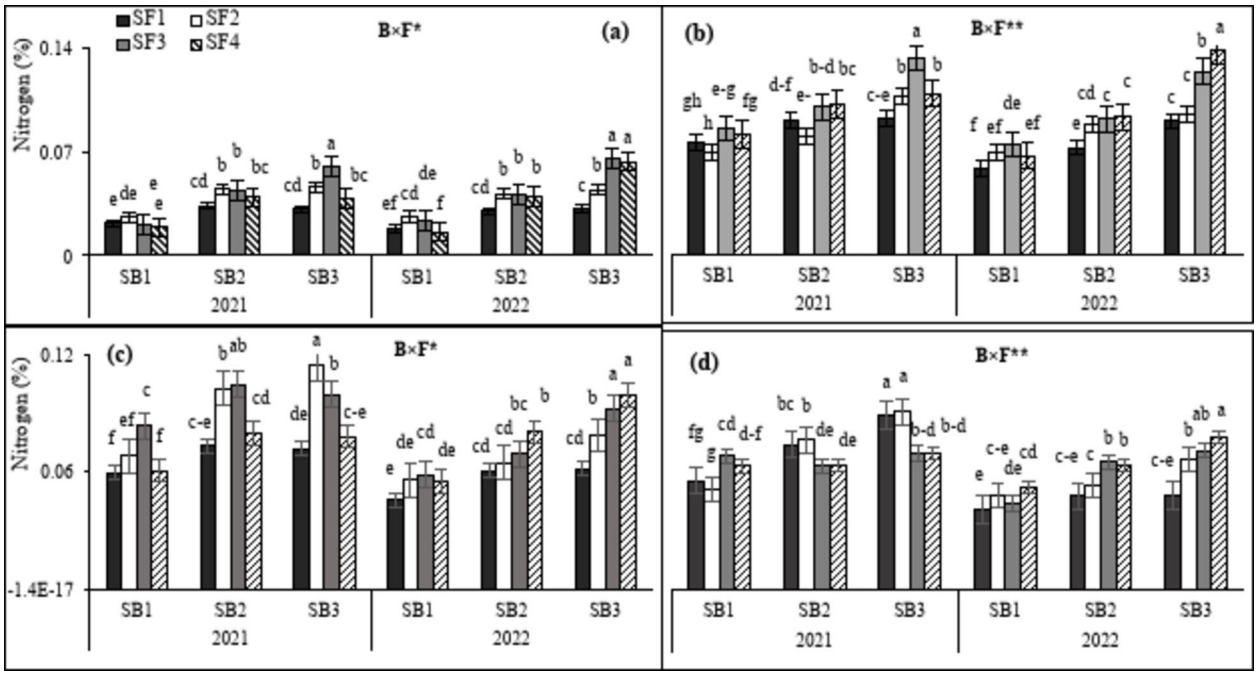

Fig. 3. The effect of sulfur and sulfur-oxidizing bacteria on nitrogen in chickpea at different development stages (**a**) Vegetative stage, (**b**) Flowering stage, (**c**) Pod set stage, (**d**) After harvesting. The details of the set of strains are provided in Table 2. Vertical bars indicate the standard error of means. *SB* sulfur-oxidizing bacteria, *F* sulfur; *B*×*F* significant interactive effects, $^{*}P \leq 0.05$, $^{**}P \leq 0.01$. *SB1* without sulfur-oxidizing bacteria; *SB2* Solpho Bacter Dayan; *SB3* biosulfur; *SF1* without adding sulfur; *SF2* 70% bentonite sulfur; *SF3* 90% bentonite sulfur; *SF4* 99% bentonite sulfur.

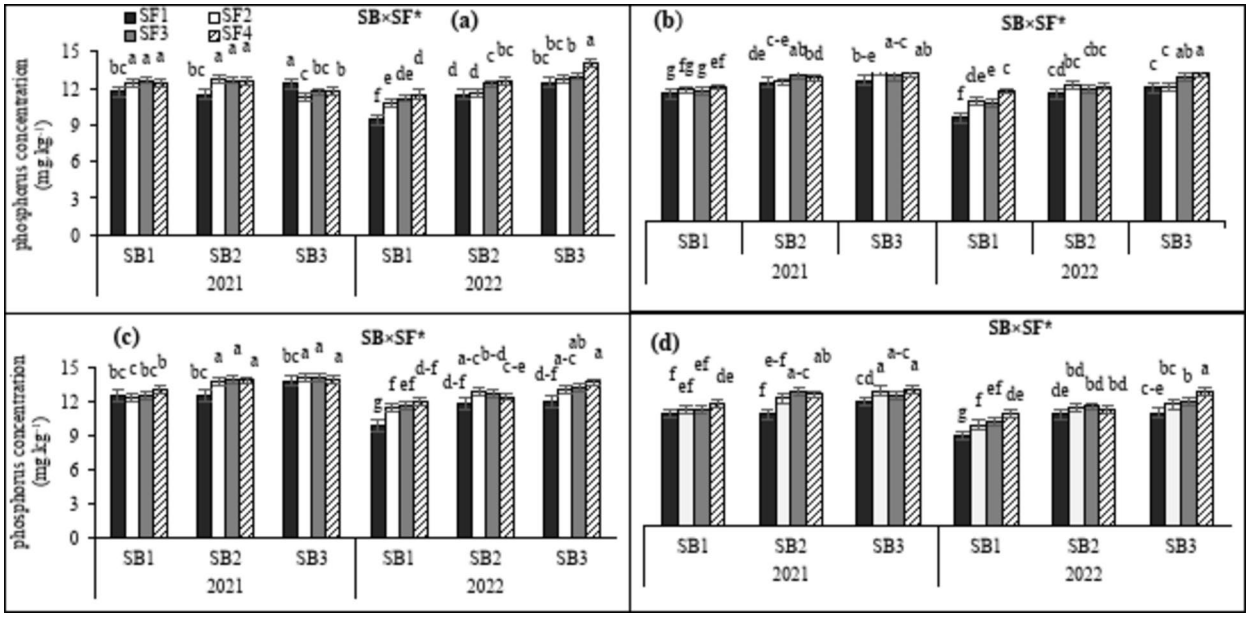

Fig. 4. The effect of sulfur and sulfur-oxidizing bacteria on phosphorus concentration in chickpea at different development stages (**a**) Vegetative stage, (**b**) Flowering stage, (**c**) Pod set stage, (**d**) After harvesting. The details of the set of strains are provided in Table 2. Vertical bars indicate the standard error of means. SB: Sulfur-oxidizing bacteria, F: Sulfur; B×F: Significant interactive effects, $^{*}P \leq 0.05$, $^{**}P \leq 0.01$. *SB1* without sulfur-oxidizing bacteria; *SB2* Solpho Bacter Dayan; *SB3* biosulfur; *SF1* without adding sulfur; *SF2* 70% bentonite sulfur; *SF3* 90% bentonite sulfur; *SF4* 99% bentonite sulfur.

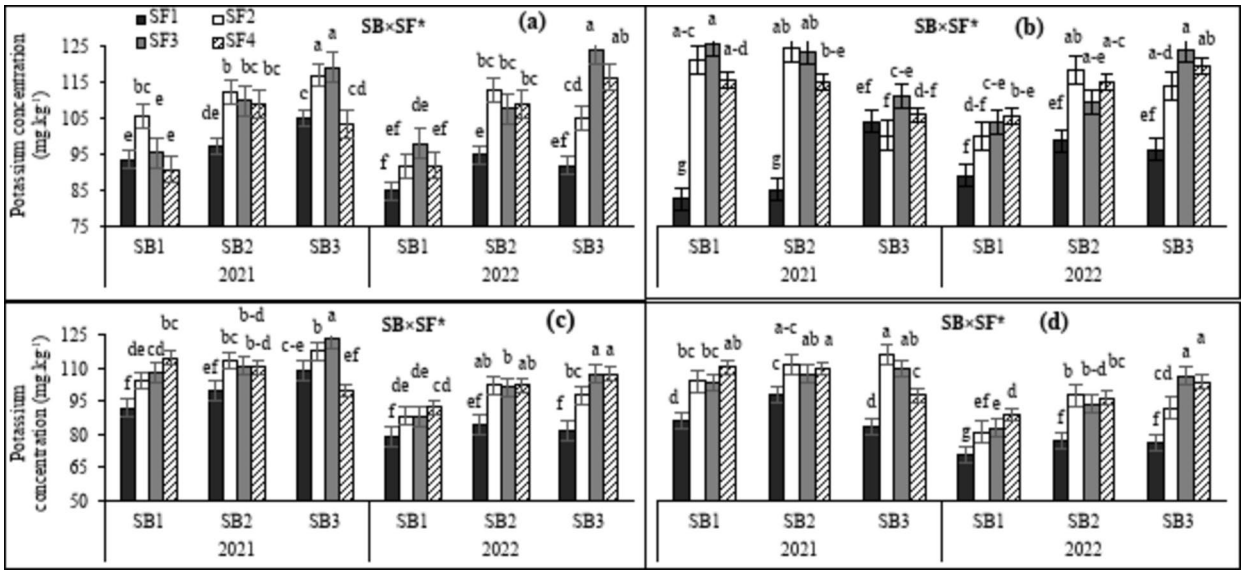

Fig. 5. The effect of sulfur and sulfur-oxidizing bacteria on potassium concentration in chickpea at different development stages (**a**) Vegetative stage, (**b**) Flowering stage, (**c**) Pod set stage, (**d**) After harvesting. The details of the set of strains are provided in Table 2. Vertical bars indicate the standard error of means. *SB*: Sulfur-oxidizing bacteria, *F* Sulfur; *B*×*F* Significant interactive effects, $^{*}P \leq 0.05$, $^{**}P \leq 0.01$. *SB1* Without sulfur-oxidizing bacteria; *SB2* Solpho Bacter Dayan; *SB3*: Biosulfur; *SF1* Without sulfur; *SF2* 70% bentonite sulfur; *SF3* 90% bentonite sulfur; *SF4* 99% bentonite sulfur.

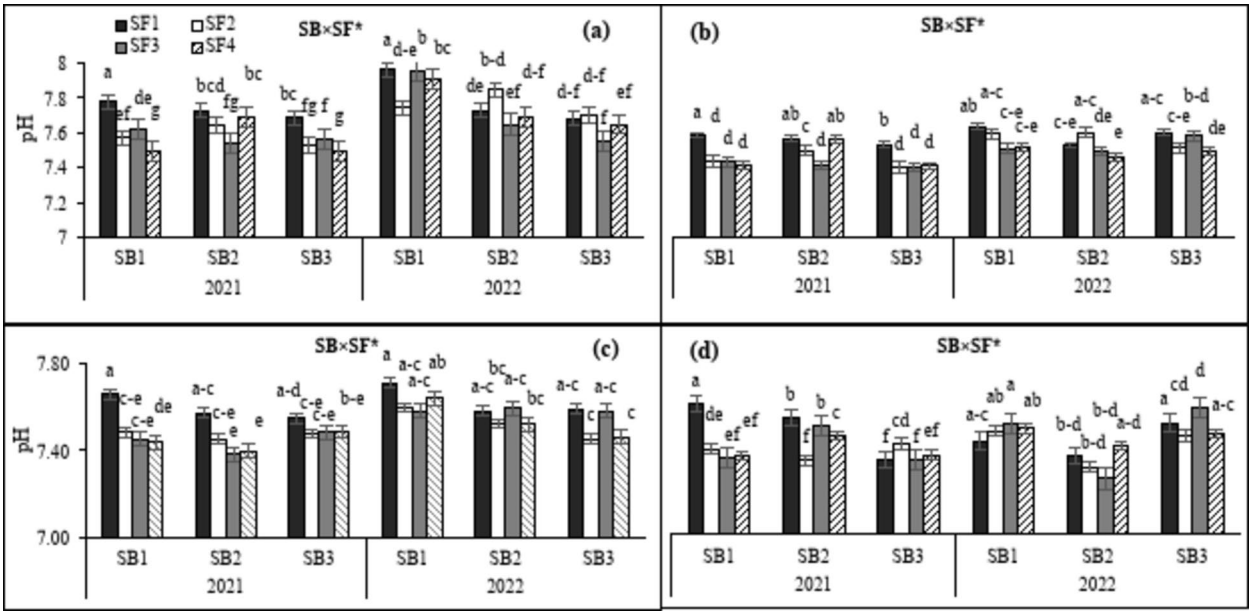

Fig. 6. The effect of sulfur and sulfur-oxidizing bacteria on soil pH at different development stages (**a**): Vegetative stage, (**b**): Flowering stage, (**c**): Pod set stage, (**d**): After harvesting. The details of the set of strains are provided in Table 2. Vertical bars indicate the standard error of means. *SB*: Sulfur-oxidizing bacteria, *F* Sulfur; *B*×*F* significant interactive effects, $^{*}P \leq 0.05$, $^{**}P \leq 0.01$. *SB1* Without sulfur-oxidizing bacteria; *SB2* Solpho Bacter Dayan; *SB3*: Biosulfur; *SF1* Without sulfur; *SF2* 70% bentonite sulfur; *SF3* 90% bentonite sulfur; *SF4* 99% bentonite sulfur.

form of nutrients into an available form for plant uptake[34]. Adding sulfur can affect phosphorus availability through mechanisms such as substituting phosphate (PO_4) with sulfate (SO_4) from exchange sites, releasing PO_4 by association with calcium, and decreasing soil pH[35,36]. Furthermore, the inoculation of calcareous soils with SOB has been shown in several studies to enhance the sulfur oxidation process and improve phosphorus availability[37,38]. In the same soil with available phosphorus, higher levels of elemental sulfur with SOB recorded

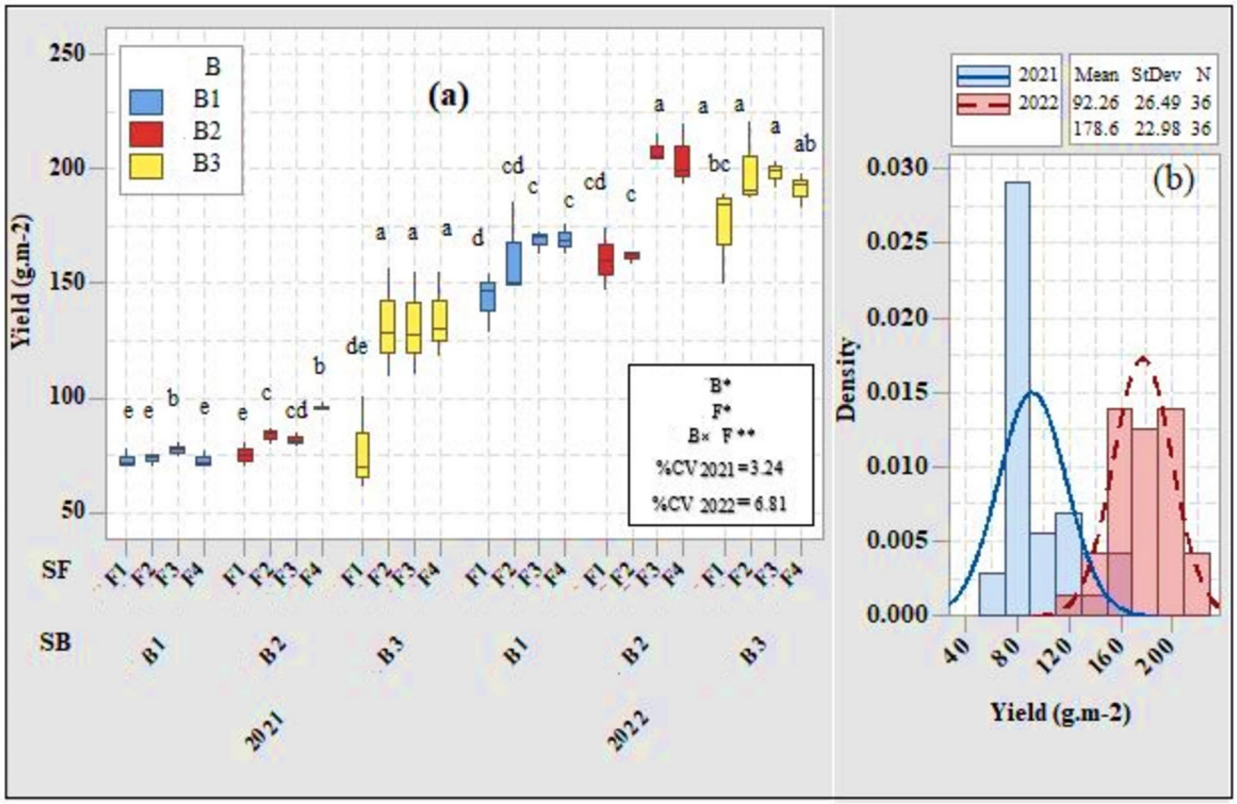

Fig. 7. Grain yield box plot for different level of sulfur-oxidizing bacteria and sulfur application (**a**). Box edges represent upper and lower quartiles, with the median value shown as a bold line in the middle of the box. Different letters indicate significant differences between sulfur-oxidizing bacteria and sulfur ($P \leq 0.05$). Histogram showing the distribution of grain yield across 2021 and 2022 years (**b**). *B* sulfur-oxidizing bacteria, *F* Sulfur; *B*×*F* significant interactive effects, $^{*}P \leq 0.05$, $^{**}P \leq 0.01$. *B1* without sulfur-oxidizing bacteria; *B2* Solpho Bacter Dayan; *B3* biosulfur; *SF1* without sulfur; *SF2* 70% bentonite sulfur; *SF3* 90% bentonite sulfur; *SF4* 99% bentonite sulfur.

NO.	Traits	1	2	3	4	5	6	7	8	9	10
1	pH	1	− 0.51**	0.54**	− 0.76**	− 0.26	− 0.37*	0.53**	− 0.40*	− 0.44*	− 0.57**
2	Yield	− 0.58**	1	− 0.23	0.50**	− 0.23	0.67**	0.58**	0.72**	0.69**	0.52**
3	DPPH	0.37*	− 0.67**	1	− 0.42*	− 0.02	− 0.37	− 0.28	− 0.36*	− 0.31	− 0.38*
4	Phenol	− 0.42*	0.42*	− 0.72**	1	0.18	0.50**	0.62**	0.51**	0.55**	0.61**
5	Carbohydrates	0.32	− 0.37*	0.36*	− 0.24	1	0.00	0.07	0.01	0.11	0.19
6	Chl a	− 0.47**	0.54**	− 0.73**	0.79**	− 0.46**	1	0.43*	0.65**	0.72**	0.48**
7	Chl b	− 0.35	0.23	− 0.28	0.25	− 0.13	0.14	1	0.55**	0.41*	0.60**
8	Nitrogen soil	− 0.52**	0.57**	− 0.85**	0.80**	− 0.34	0.73**	0.40*	1	0.77**	0.81**
9	Phosphorus soil	− 0.56**	0.66**	− 0.90**	0.77**	0.44*	0.68**	0.32	0.88**	1	0.62**
10	Potassium soil	− 0.44*	0.55**	− 0.87**	0.73**	− 0.41*	0.80**	0.24	0.84**	0.81**	1

Table 4. Pearson's correlation between soil pH, yield, leaf physiological composition, and soil nutrition of chickpeas in 2021 (top of triangle) and 2022 (bottom of triangle). *Chl a* chlorophyll a, *Chl b* chlorophyll b, Asterisks denote significant differences: $^{*}p \leq 0.05$; $^{**}p \leq 0.01$.

high nitrogen and potassium concentrations in soils. The average nitrogen and potassium in control treatment were 0.20% and 94 mg kg^{-1}, respectively. However, they increased to 0.62% and 122 mg kg^{-1}, respectively, in the B_3F_4, B_2F_3, and B_3F_3 treatments (Figs. 5 and 7).

Due to the increased absorption of nutrients from the soil by the plant during the vegetative and reproductive growth stages, the amount of nutrients in the soil decreases over time. The most significant decrease in nutrient availability in the soil occurred after harvest time (Figs. 5 and 6, and 7). Research studies, such as those conducted by Zhao et al.[39], Becana et al.[9], Zenda et al.[18], and Chaudhary et al.[8], have shown that the addition of

sulfur to legumes can improve the synthesis of sulfur-containing amino acids and increase the nitrogen value in plants and soil. In addition, a study conducted by Mohamed and Gomaa[40] revealed that the use of SOB in combination with sulfur to inoculate cowpea (*Vigna unguiculata*) led to significant improvements in soil fertility, soil characteristics, and nutrient availability. The results of the experiment by Amini et al.[41] also showed that the utilization of plant growth-promoting bacteria enhances the physiology and performance of moldavian balm (*Dracocephalum moldavica* L.).

Figure 6 illustrates the impact of repeated applications of sulfur in combination with SOB on soil pH. The initial soil pH values in 2021 and 2022 were 7.9 and 7.9, respectively. During the growth period, the application of sulfur led to a significant decrease in soil pH. The pH value decreased by 0.53 units with the addition of sulfur in B_3F_4 in 2021 and by 0.57 units in B_3F_4 in 2022 at 60 DAS (Fig. 6). The soil pH decreased by 0.76 and 0.81 units in B_3F_3 in 2021 and 2022, respectively, following sulfur and SOB applications after harvesting. Therefore, the most significant reduction is associated with the post-harvest period. A drop in soil pH resulting from the oxidation of sulfur can lead to increased solubilization of other nutrients, such as phosphorus and potassium, in the rhizosphere. In addition, a decrease in soil pH can also increase the electrical conductivity of the soil[42,43]. In this study, soil pH reduction by a combination of sulfur and SOB influenced the availability of nitrogen, phosphorus, and potassium in soils (Figs. 2 and 3, and 4). This phenomenon was also reported by Ye et al.[6].

The application of sulfur and SOB enhanced the GY in B_3F_4 (134 g m^{-2}) and B3F3 (198 g m^{-2}) compared to the control in 2021 and 2022. According to Fig. 7a, the average GY in 2022 increased by 47% compared to 2021. This improvement can be attributed to favorable weather conditions, including a significant increase in rainfall in 2022, and a decrease in temperature during the flowering and podding stages compared to 2021 (Fig. 1). Overall, increased rainfall and optimal temperature conditions during crucial growth stages led to a significant boost in GY for the 2022 season. The same results were also reported by Fattah et al.[44] and Besharati[10]. Salimpour et al.[37] documented a 22.2% increase in canola (*Brassica napus* L.) seed yield after treating plants with sulfur plus *Thiobacillus* sp. A study conducted by Balloei et al.[45] showed that the application of 150–300 kg ha^{-1} of sulfur improved the seed yield, biomass, and harvest index of soybean (*Glycine max* L.). The study conducted by Mohamed and Gomaa[40] found that inoculating cowpea with SOB led to a significant increase in dry weights, as well as in various minerals such as sulfur, potassium, nitrogen, phosphorus, iron, manganese, zinc, and copper. There was a strong and positive correlation between GY and nitrogen, phosphorus, and potassium (Table 4). Additionally, linear regression analysis revealed a negative and significant correlation between pH and the increase in GY. This suggests that the yield rises as the pH decreases.

The inoculation with SOB significantly increased the chlorophyll a content during the flowering stage in B_3F_4 and B_3F_2 compared to the control in 2021 and 2022, respectively. (Fig. 2). At 60 DAS, higher chlorophyll b contents were obtained with the combined treatment of F_2B_3 and F_3B_3 compared to the control in 2021 and 2022. The availability of a sufficient amount of sulfur in plants leads to an increase in photosynthetic activity, robust metabolism, and protein synthesis[46]. In both years, the utilization of sulfur and bacteria significantly influenced the levels of nitrogen, phosphorus, and potassium in carbohydrates, phenols, and DPPH compared to the control. This effect was notable because it occurred during the pod formation stage, coinciding with exposure to high temperatures at the beginning of summer (Fig. 1), resulting in increased phenol levels. The bacterial treatments resulted in a more significant increase in phenol compared to the control, suggesting the potential role of sulfur compounds in alleviating abiotic stresses.

Although the application of sulfur and SOB has shown promise in enhancing chickpea yields, several factors may limit its effectiveness. These factors include soil conditions such as pH, temperature, moisture, and organic matter content, all of which can impact the performance of sulfur-oxidizing bacteria. Furthermore, the oxidation process is influenced by environmental factors like temperature and moisture, which can potentially slow down or inhibit the process. Lastly, the availability and cost of sulfur-oxidizing bacteria, along with their proper handling and storage requirements, can pose challenges for farmers, particularly in developing countries.

Conclusion

The environmental advantages of compounds with non-toxic, natural origins, and biodegradable properties make them excellent alternatives to chemical fertilizers in sustainable agroecosystems. Therefore, due to the favorable effect of SOB in enhancing the efficiency of the photosynthetic apparatus and plant metabolism, ultimately leading to increased seed yield, its application in chickpea fields is recommended. Furthermore, future efforts to enhance chickpea yield through the application of sulfur and SOB should concentrate on overcoming existing limitations and optimizing methodologies. This can be accomplished by isolating and characterizing novel bacterial strains, investigating various sulfur sources and application techniques, exploring synergistic applications with other beneficial microorganisms, and conducting field trials in diverse agricultural environments.

Data availability

The necessary information is available from the corresponding author on reasonable request.

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Author contributions
J.N & A.Y: Conceptualization, Methodology, Supervision; A.Y, A.H, Z.N & N.K: Field work, Data collection, A.Y & A.M.K: Conceptualization, Methodology, Data analysis, Writing; A.H: Reviewing, Editing.All authors reviewed the manuscript.

Declarations

Competing interests
The authors declare no competing interests.

Ethical approval
The experiments conducted on the studied plant were in compliance with all relevant institutional, national, and international guidelines and legislation. Seeds were provided from the Mashhad Chickpea Collection Research Center for Plant Sciences at Ferdowsi University of Mashhad, Iran.

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