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Potential of integrating sulphur-oxidizing bacteria and sulphur-coated urea on growth and yield of sunflower (*Helianthus annuus L.*)

Hafiz Faizan Ahmad[1], Azhar Hussain*[1-5], Hafiz Tanvir Ahmad[1], Farheen Nazli[2], Imran Khalid[3], Marium Fiaz[4] and Nazmi Mat Nawi[5]

[1]Department of Soil Science, Faculty of Agriculture & Environment, The Islamia University of Bahawalpur, 63100-Pakistan
[2]Institute of Agro-industry and Environment, Faculty of Agriculture & Environment, The Islamia University of Bahawalpur, 63100-Pakistan
[3]Department of Agriculture Extension Education, Faculty of Agriculture & Environment, The Islamia University of Bahawalpur, 63100-Pakistan
[4]School of Interdisciplinary Engineering & Sciences (SINES), National University of Sciences and Technology (NUST) Islamabad, Pakistan
[5]Department of Biological and Agricultural Engineering, Faculty of Engineering, Universiti Putra Malaysia, 43400, Serdang, Selangor, Malaysia

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Abstract

Sunflower (Helianthus annuus L.) is a significant oilseed crop due to its high oil content. The deficiency of sulphur and other nutrients causes a reduction in use efficiency and sunflower yields. Plant growth-promoting bacteria (PGPR) are most studied for enhancing nutrient use efficiencies in crops. Therefore, the present study evaluated the potential of sulphur-oxidizing bacteria (SOB) combined with sulphur-coated urea (SCU) for enhancing sulphur availability, nutrient use efficiency and yield of sunflower. Pre-isolated and pre-identified SOB strains were tested for sulphur oxidation potential and plant growth promoting attributes. The consortium of three most efficient strains, Enterococcus durans (MBT-TB1), Bacillus thuringiensis (MBT-TB2) and Bacillus cereus (MBT-TB3) was used for urea coating. A pot incubation study assessing sulphur and nitrogen release showed significantly increased urease activity and sulphur release over time, in T7 (SOB + SCU) treatment. Growth parameters like shoot length (11.0%), root length (18.75%), shoot dry weight (6.11%) and root dry weight (11.92%) were significantly improved as compared to the control in pot study. Yield components like head diameter, number of achenes and 100-seed weight increased by 14.46%, 10.96% and 15.08%. Grain yield parameters like nitrogen, phosphorus and potassium in grains increased by 7.63%, 12.93%, 7.89%, respectively. The macro and micronutrients such as sulphur, zinc and iron also increased by 13.27%, 15.45% and 8.03%, respectively. The combined use of SOB and SCU showed potential to improve sulphur availability, nutrient use efficiency, growth and yield of sunflower. However, these findings require validation through large scale field trials for wider adoption.

Keywords: Bio activated urea, controlled release fertilizer, nutrient uptake, oilseed productivity

Introduction

Agriculture is one of the key sectors of the Pakistani economy, contributing almost 24% to the GDP and employment to about 37.4% of the labor forces (Hamid and Akram, 2025). Oilseeds are one of the most economically significant agricultural products across the globe, serving as the main sources of edible vegetable oils, high-protein livestock feed, biodiesel and oleo chemical raw materials (Khan *et al.,* 2024). Among them, sunflower (*Helianthus annuus L.*) is considered as one of the important oilseed crops in the world because of its high content of oil (36-52 percent), favorable content of fatty acids and wide adaptability to agro-climate conditions in various parts of the world (Abiodun, 2017; Murphy, 2025).

The rising demand in developing economies is also a significant contribution to the consumption of vegetable oils. The national demand for edible oils is fulfilled by domestic

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production of oilseeds that is approximately 17% of it, making Pakistan one of the largest importers of edible oils worldwide (Hussain *et al.,* 2023). The country still depends on imported edible oil due to low productivity, climate stress, poor seed quality, pests and diseases and weak policy support (Khalid *et al.,* 2026). Low fertilizer application, inadequate irrigation practices, weed issues, pest and disease infestations and climate stresses like drought and heat are also causes of yield gaps in oil seed crops (Khan *et al.,* 2024).

Macro and micronutrients play a critical role in improving the yield of sunflowers. Plants require nitrogen, potassium and phosphorous in specific forms and at appropriate stages of growth for proper development (Irawan *et al.,* 2025). Sulphur is fourth essential macronutrient following nitrogen (N), phosphorus (P), and potassium (K) (Castro *et al.,* 2023). It is an essential part of the protein-forming sulphur-containing amino acids like cysteine and methionine and is required to form chlorophyll and regulate enzymes (Narayan *et al.,* 2023). The deficiency of sulphur (S) in soils is also a significant constraint to the productivity of oilseeds in Pakistan. The calcareous and alkaline characteristics of most of the agricultural soils limit the availability of sulphur (Khalid *et al.,* 2011).

Sulphur is important for oil seed crops because it is the precursor of glucosinolate biosynthesis that can modify seed characteristics (Borpatragohain *et al.,* 2019). Sulphur deficiency adversely affects oil biosynthesis in seeds and may reduce seed oil concentration up to 30%. It may also negatively affect fatty acid composition by lowering important fatty acids such as oleic and linoleic acids (Dobrokhotov *et al.,* 2023). Yield losses of up to 30-50% have been recorded in sulphur-deficient conditions in canola production systems, highlighting the critical nature of sulphur in promoting optimal oilseed productivity (Poisson *et al.,* 2019).

One of the significant limitations in agricultural production systems is also the inefficient use of nitrogen (N) fertilizers. Urea is exposed to massive losses (15-70 %) due to ammonia volatilization, nitrate leaching and denitrification at high temperatures and alkaline soils (Waqar *et al.,* 2022). Such losses significantly lower the efficiency of nitrogen use up to 20-35% in most of the developing nations (Govindasamy *et al.,* 2023). Therefore, it is necessary to ensure the improvement of nutrient use efficiency and yield potential in sunflower by optimizing the nitrogen and sulphur availability.

Sulphur oxidation rate is highly controlled by the presence of microbes and biochemical conditions of the soils. In calcareous soils having low organic matter, the population of sulphur oxidizing bacteria are often not adequate to support rapid sulphur mineralization in Pakistan. This is potentially leading to delayed sulfate availability and inefficient nutrient uptake (Nadeem *et al.,* 2023). Recent innovations in biofertilizer technology have suggested that the exogenous inoculation of SCU with SOB can enhance sulphur oxidation and improve the synchronized release of nitrogen and sulphur (Samantaray *et al.,* 2024). Microbial assisted nutrient management using PGPR, such as *Azotobacter* and *Bacillus ssp.,* has been reported to improve nitrogen and phosphorous use efficiency in cereal crops. However, the combined application of SOB and SCU remained less explored in oilseed production systems (Asghar *et al.,* 2022).

Therefore, the present study was conducted to assess the combined potential of SOB and SCU on growth, physiological performance and yield attributes of sunflower under pot conditions. We hypothesized that the combined use of SOB and SCU would enhance the synchronized release of nitrogen and sulphur. This would improve nutrient uptake, biomass accumulation and yield attributes of sunflower.

Materials and Methods

Characterization of sulphur oxidizing bacteria

Pre-isolated and pre-identified sulphur-oxidizing bacterial strains with accession numbers MBT-TB1 (OQ152632), MBT-TB2 (OQ711932) and MBT-TB3 (OQ804421) identified as *Enterococcus durans*, *Bacillus thuringiensis* and *Bacillus cereus,* respectively, were obtained from Microbial Biotechnologies Private Ltd. Lahore Pakistan. These strains were characterized for siderophore production, urease activity, gelatin liquefaction, catalase and oxidase activity in the Soil and Environmental Microbiology Laboratory, Department of Soil Science, The Islamia University of Bahawalpur. These SOB strains, based on their potential of sulphur oxidation and plant growth promoting characteristics such as sulfate production, pH reduction, urease activity and siderophore production, were used for the preparation of bio activated sulphur coated urea for the pot experiment.

The amount of sulfate (SO_4^{2-}) was measured in the cultures incubated in thiosulfate broth by the turbidimetric method of (SO_4^{2-}) with $BaCl_2$ by measuring the absorbance at 450 nm and calculating the concentration of the sulfate against a K_2SO_4 standard curve (Cha *et al.,* 1999; Kolmert *et al.,* 2000). To assess the pH reduction, 1 mL of fresh bacterial culture was inoculated into a thiosulfate medium (initial pH 8.0) supplemented with bromocresol purple indicator. The inoculated medium was incubated at 30°C for 16 days and the pH was measured with a calibrated pH meter. Indole production and citrate utilization were determined using standard diagnostic tests. Indole production was assessed on SIM agar slants (28 ± 2ºC, 72 h); adding 10 drops of Kovac's reagent yielding a red color. Citrate utilization was evaluated on Simmon's citrate agar (28 ± 2ºC, 24 h), where a green to blue color shift indicated a positive reaction (Reddy *et al.,* 2018). The production of siderophore was determined on Chrome Azurol S (CAS) agar in terms of the development of halo after incubation (Gopalakrishnan *et al.,* 2011). The standard protocols were used to test catalase, oxidase, gelatin liquefaction (Munde *et al.,* 2024). Gelatin liquefaction was evaluated on gelatin agar (30ºC, 72 h), where clear zones around growth after flooding with 12% $HgCl_2$ indicated a positive result. The catalase test was performed by adding 3% H_2O_2 to nutrient slants (30ºC, 24 h), with gas bubble formation. For the oxidase test, cultures grown on trypticase soy agar (28 ± 2ºC, 24 h) were treated with TPD, and a blue color shift confirmed a positive reaction. Urease activity was determined in Christensen's urea broth (Cappuccino and Sherman, 2005). Inoculated tubes were incubated at 30ºC for 24-48 hours, where a color shift from pale orange to bright pink confirmed a positive reaction.

Compatibility test and consortium development

The compatibility of the SOB strains (MBT-TB1, MBT-TB2, and MBT-TB3) with each other was tested by cross-streaking on LB agar plates and incubation at 30 ± 2°C, 48 h. Absence of an inhibition zone at the intersection point allowed the identification of compatible isolates (Fukui *et al.,* 1994). Since no antagonism was evident, the individual strains were grown to an optical density (OD_{600}) of 1.0 (1 x 10^8 CFU mL^{-1}). The SOB consortium was then made by combining the three strains in a 1:1:1 ratio to further formulation and crop testing.

Preparation of bio-activated sulphur coated urea

The SOB consortium was combined with finely ground commercial elemental sulphur, organic material and sulphur was coated on prilled urea at 5% (w/w) to prepare bio-activated sulphur-coated urea. Actively growing SOB cultures adjusted to an optical density (OD_{600} = 1.0 containing ~1 x 10^8 CFU mL^{-1}) in LB broth were mixed thoroughly with elemental sulphur, organic material and incubated at 28 ± 2ºC over a period of 72 h to enable the activation of the microbes. A uniform coating of the bio-activated sulphur on prilled urea was then done by a rotary drum at 50 rpm for 5 min. Coated granules were air-dried at room temperature and stored in airtight bags until use.

Microbial count on bio-activated sulphur coated urea

The standard serial dilution and pour plate method was used to determine the population of bacteria in the samples of the coated fertilizer. About 1 g of fertilizer sample was suspended in sterile distilled water and serially diluted up to 10^6 dilutions. These dilutions were plated on nutrient agar and allowed to incubate in 30 ± 2°C for 24 to 48 hours. The viable microbial count in terms of colony forming units was calculated next day after counting microbial colonies as CFU g^{-1} of fertilizer according to the procedure of Alexander (1982).

Release pattern studies of nitrogen and sulphur

To assess the release pattern of sulphur (S) and nitrogen (N) by the developed bio-activated sulphur-coated urea formulation, a pot incubation study was carried out in the Department of Soil Science. Before the experiment, soil samples were collected from the research farm for soil analysis. Pots were filled with air dried, ground, sieved and homogenized soil, then treated with the bio activated formulation containing SOB and SCU. Control treatment comprised of untreated pots that were neither coated nor inoculated. Treatments were arranged in a complete randomized design comprising three replications. The release pattern of nitrogen and sulphur was determined at 0, 15, 30, 45, 60 and 75 days after application with the help of soil samples.

Pre-sowing soil analysis

The physico-chemical characteristics of the soil were determined before sowing through standard laboratory procedures. The soil texture was estimated by using the Bouyoucos hydrometer method as described by the procedure of Moodie *et al.* (1951) and the USDA texture triangle was used to identify the soil textural class. The soil was classified as sandy loam, it contains 66.3% sand, 15.5% clay and 18.2% silt. The saturated soil paste extracts were used to determine the electrical conductivity (ECe) and pH using the methodology outlined in the U.S. Salinity Laboratory Staff (1954), showing ECe of 1.3 dS m^{-1} and pH of 8.2. The saturation percentage of the soil was 27%. The soil organic matter (0.56%) was obtained by the wet oxidation technique as described by Walkley and Black (1934). The total nitrogen (0.031%) was estimated by digesting the sample with sulphuric acid and distillation on Kjeldahl apparatus followed by titration as outlined by Jackson (1962). The available sulphur (7.3 mg kg^{-1}) was calculated by extracting calcium phosphate and measuring the turbidity using a turbidimetric analysis as recommended by Chesnin and Yien (1951). Available potassium (85 mg kg^{-1}) was obtained following ammonium acetate extraction with the use of flame photometer as outlined in U.S. Salinity Laboratory Staff (1954). The Watanabe and Olsen (1965) method was adopted to detect the extractable phosphorus (5.1 mg kg^{-1}) on spectrophotometer. Moreover, the soil had 0.60 mg kg^{-1} zinc and 0.65 mg kg^{-1} iron. Microbial population of rhizospheric soil samples at harvest was determined by the serial dilution and pour plate technique and counts of microorganisms were expressed as colony-forming units (CFU g^{-1}) by following the protocol of Alexander (1982).

Table 1: Treatment plan of the experiment with recommended doses of (NPK) added in each treatment

Treatment	Description
T0	Control
T1	Sulphur
T2	Sulphur Oxidizing Bacteria (SOB)
T3	SOB + Sulphur
T4	SOB Coated Urea
T5	SOB coated Urea + Sulphur
T6	Sulphur Coated Urea
T7	SOB + Sulphur coated Urea
T8	SOB + Sulphur coated Urea with 25% less Urea

Pot trial

Pot study was conducted under open environmental conditions at the Agricultural Research Farm Area, The Islamia University of Bahawalpur coordinates as (29°22'25.6"N 71°45'58.7"E) to determine the efficiency of microbially inoculated sulphur coated urea developed by using the pre-isolated and pre identified strains of sulphur oxidizing bacteria. For this purpose, sunflower (*Helianthus annuus* L.) Hysun-33 was grown in pots (12 x 18 in) during their respective growing seasons January to March in 2025, and the developed formulation was compared with conventional sulphur-coated urea and other sulphur treatments. The experiment consisted of nine treatments with three replications, making a total of 27 experimental pots. Each pot was filled with approximately 6-7 kg of homogenized pre-analyzed soil. Three healthy plants per pot were maintained until harvest. Recommended doses (120, 90, 60 kg ha^{-1}) of nitrogen, phosphorus and potassium were applied prior to sowing. Irrigation was applied according to plant requirement to maintain adequate soil moisture throughout the experiment. Every agronomic practice was kept to the same until harvest. Growth parameters were recorded at physiological maturity, and yield attributes were recorded at harvest. The design of the experiment was a completely randomized design with three replicates as shown in Table 1.

Plant analysis

In order to measure the physiological characters (chlorophyll SPAD value and relative water content), the analysis of plants was done at different growth stages. Growth parameters were taken at the maturity of the plant and yield parameters at harvest. Growth parameters including root length, shoot length, head diameter, both dry weight of shoots and roots were recorded from three randomly selected plants per treatment at harvest. Harvesting and threshing were followed by determining yield attributes such as the number of achenes $head^{-1}$ and weight of 100 seeds.

The chlorophyll content was determined with the help of SPAD-502 chlorophyll meter (Konica Minolta, Japan). Relative water content (RWC) was calculated as per Mayak *et al.* (2004) using turgid weight (TW), fresh weight (FW), and dry weight (DW) and calculated as:

$$RWC\ (\%) = \frac{(\mathrm{FW} - \mathrm{DW})}{(\mathrm{TW} - \mathrm{DW})} \times 100$$

In the case of mineral nutrient analysis, 0.2 g of the plant samples after oven drying and grinding were digested in a 100 mL conical flask using 6 mL concentrated sulphuric acid and left at room temperature overnight. Next day 1 mL H_2O_2 was added and the mixture was heated on a digestion plate at 250°C until a clear digest was formed. The digest was diluted to volume with distilled water and was utilized to determine the nutrients. Macro and micronutrients such as nitrogen (N), phosphorus (P), potassium (K), sulphur (S), zinc (Zn), and iron (Fe) were analyzed using standard methods (Ryan *et al.,* 2001). Nitrogen was determined by the Kjeldahl method (Jackson, 1962) using a Kjeldahl distillation unit. Watanabe and Olsen method was used to determine phosphorus by spectrophotometry. Potassium was determined by the flame photometry method (U.S. Salinity Laboratory Staff, 1954) using a flame photometer. Sulphur was determined by the turbidimetric analysis method using a spectrophotometer (Chesnin and Yien, 1951). Zinc and iron were determined using atomic absorption spectrophotometry.

The crude protein content was determined from the nitrogen content using the AOAC (2005) method as described by Thiex (2009):

$$\text{Protein}\ (\%) = \text{Nitrogen}\ (\%) \times 6.25$$

Table 2: Effect of sulphur oxidizing bacterial strains on siderophore production, oxidase activity, catalase activity, urease activity, gelatin liquefaction, indole production, and citrate utilization

Characteristics	*Enterococcus durans*	*Bacillus thuringiensis*	*Bacillus cereus*
Siderophore production	+	+	+
Oxidase activity	+	–	+
Catalase activity	+	+	+
Indole production	+	+	+
Urease activity	+	+	+
Citrate utilization	+	+	+
Gelatin liquefaction	+	–	+

Table 3: pH reduction ability of (SOB) sulphur oxidizing bacterial strains in Starkey broth and Thiosulphate broth after 15 days

Strains	Starkey broth	Thiosulphate broth
Control	5.71 ± 0.02a	5.84 ± 0.05a
Bacillus cereus	5.13 ± 0.03b	5.34 ± 0.05b
Enterococcus durans	4.47 ± 0.06d	4.73 ± 0.05c
Bacillus thuringiensis	4.74 ± 0.05c	4.83 ± 0.06c

Data shows the means of three replicates; same letter(s) in the means within the column indicate that they do not differ significantly according to the Tukey HSD test at $p \leq 0.05$.

Statistical analysis

For the determination of the significance of the findings, the data obtained from this research was analyzed by one-way ANOVA using Statistix 8.1 software for axenic and pot studies. The means of the treatment were computed for differences through Tukey HSD test at 5% probability and the AOV results for all variables are presented in supplementary data Table S1-S18 (Steel *et al.,* 1997).

Results

Characterization of pre-identified and pre-isolated sulphur oxidizing bacterial strains

Sulphur-oxidizing bacterial (SOB) strains (MBT-TB1) *Enterococcus durans* (MBT-TB2) *Bacillus thuringiensis* and (MBT-TB3) *Bacillus cereus* were characterized based on plant growth-promoting features such as siderophore production, urease activity, gelatin liquefaction, catalase and oxidase activity. Table 2 showed that all the tested strains were positive in several biochemical characteristics which are indicative of plant growth-promoting rhizobacteria. These characteristics indicate that they can improve nutrient availability and promote plant growth in soil conditions. In addition, cross-streaking by compatibility testing showed that there were no antagonistic interactions between the selected strains, which gives them a good chance to be used in the development of consortium. Following these results, the three strains of the SOB were found to be compatible and were then applied together in equal measures (1:1:1) in pot experiment.

pH reduction test

The capability of sulphur oxidizing bacterial strains to lower pH in growth media was tested to determine the activity of sulphur oxidation. The findings in Table 3 indicated that all the tested strains caused significant reduction of the pH of both Starkey and thiosulphate broth with the original pH of 8.0. MBT-TB1 was the highest pH reducing strain, which lowered the pH to 4.46 in Starkey broth and 4.72 in thiosulphate broth. This was succeeded by MBT-TB2 that lowered the pH to 4.72 and 4.85 in Starkey and thiosulphate broth, respectively. The minimal decrease was noticed in MBT-TB3 where the final pH was 5.05 and 5.25 in the respective media.

Sulphate ion production ability

The production of sulphate ions by sulphur-oxidizing bacterial strains was measured using thiosulphate broth and the data are shown in Figure 1. The ability to produce sulphur oxidase was confirmed by all the tested strains which produce more sulphate than the control (2.3 mM). MBT-TB2 was the most productive strain with a sulphate ion production of 5.92 mM, MBT-TB1 was the second most productive with 4.96 mM and MBT-TB3 was relatively less productive with a 4.16 mM production of sulphate ions.

Sulphur oxidation on the thiosulphate agar medium

The sulphur-oxidizing activity of the selected strains was confirmed on thiosulphate agar medium, and the results are presented in Figure 2. The tested strains (MBT-TB1)

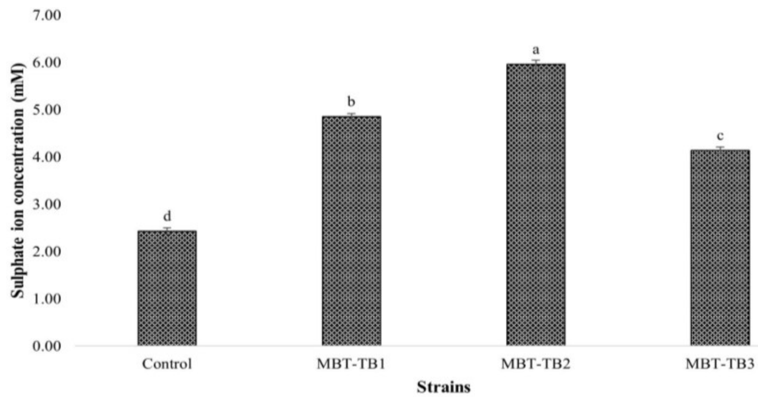

Figure 1: Effect of MBT-TB1, MBT-TB2 and MBT-TB3 on sulphate ion production

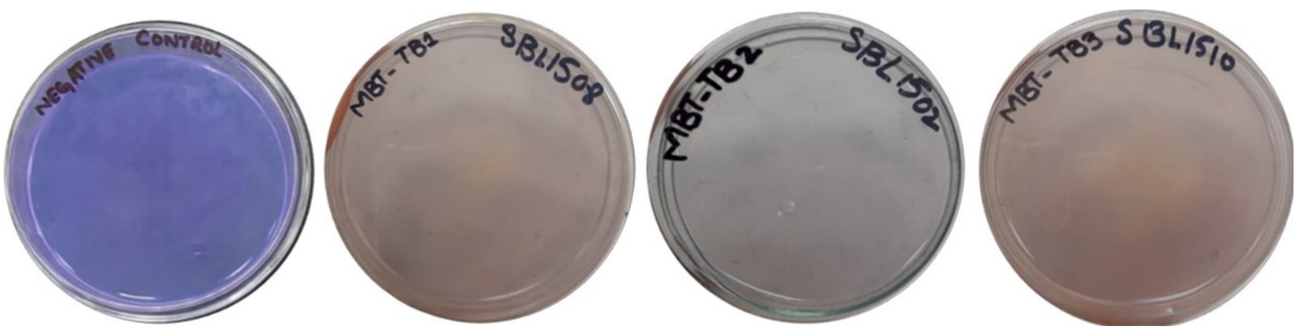

Figure 2: Sulphur oxidizing ability of (MBT-TB1) *Enterococcus durans,* (MBT-TB2) *Bacillus thuringiensis* and (MBT-TB3) *Bacillus cereus* bacterial strains on thiosulphate agar medium showing color change

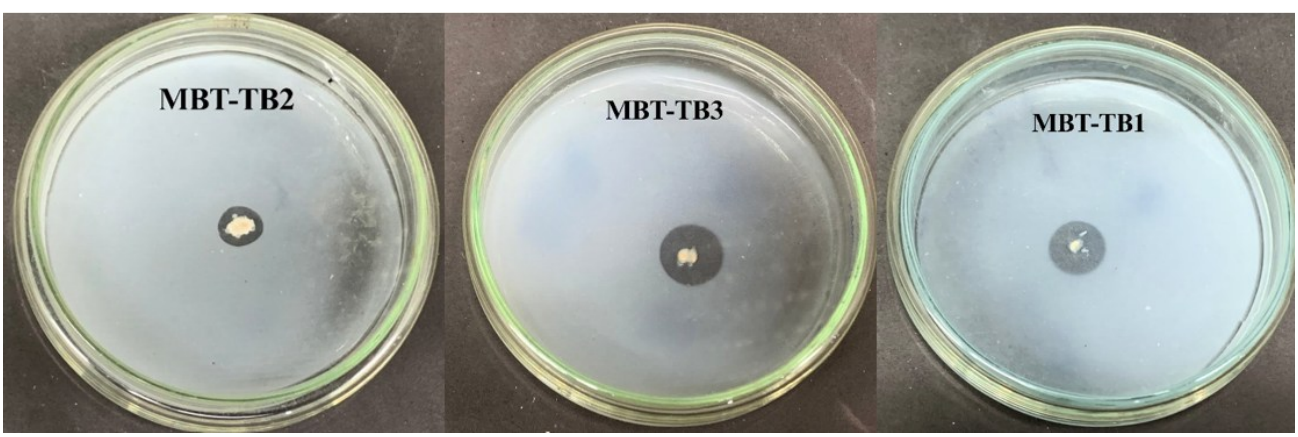

Figure 1: Zinc solubilizing ability of sulphur oxidizing bacterial strains (SOB) by MBT-TB1, MBT-TB2 and MBT-TB3 showing holozones in plates

Enterococcus durans, (MBT-TB2) *Bacillus thuringiensis,* and (MBT-TB3) *Bacillus cereus* showed a distinct potential of sulphur oxidation due to the appearance of yellow spots around bacteria colonies. The change from purple to yellow color showed the presence of acid produced as a result of the oxidation of thiosulphate to sulphuric acid. The active sulphur-oxidizing ability of all isolates was affirmed by the development of these zones.

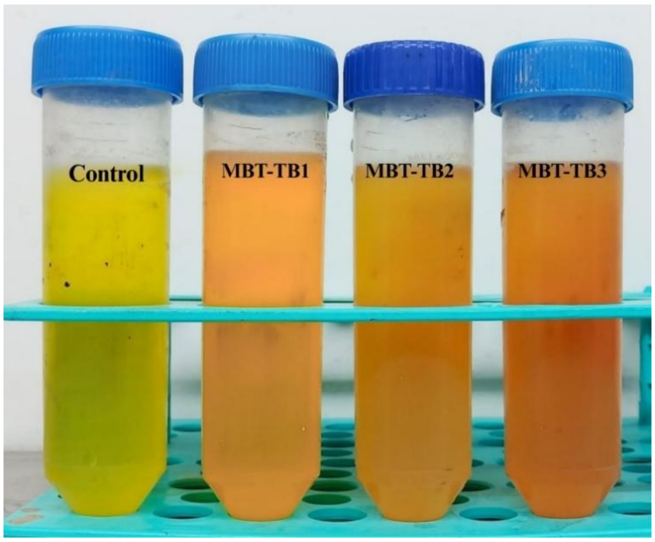

Figure 2: **Urease ability of (MBT-TB1) *Enterococcus durans,* (MBT-TB2) *Bacillus thuringiensis,* and (MBT-TB3) *Bacillus cereus* sulphur oxidizing bacterial strains showing changes in color for positive urease activity**

Compatibility test

The selected bacterial strains (MBT-TB1) *Enterococcus durans,* (MBT-TB2) *Bacillus thuringiensis,* and (MBT-TB3) *Bacillus cereus* were cross streaked on LB agar plates to determine compatibility. The results proved that the three strains were all compatible with one another because no inhibition zones were detected at the cross-section of streaks. Based on their sulphur oxidizing potential, plant growth promoting traits and mutual compatibility, the three selected SOB strains were combined as a consortium rather than used individually. This consortium was carried forward for the preparation of bio activated sulphur coated urea and subsequent evaluation in the sunflower pot trial.

Bacterial population determination in sulphur coated urea

Data presented in Figure 5 illustrates the bacterial population in sulphur coated urea under different treatments. The results showed significant variation in microbial population depending on sulphur coating and sulphur oxidizing bacteria inoculation. The maximum bacterial population was recorded in T7 (SOB + sulphur coated urea with NPK), followed by T5, T3 and T4. These treatments had shown that SOB inoculation greatly stimulated bacterial growth more than other treatments when used in combination with sulphur coating. A moderate bacterial count was also detected in T6 but relatively lower counts were detected in T8 and T2. The lowest bacterial populations were observed in T1 and control T0. The results indicate that SOB inoculation when combined with SCU, supported better bacterial survival and the successful bio activation of the coated fertilizer.

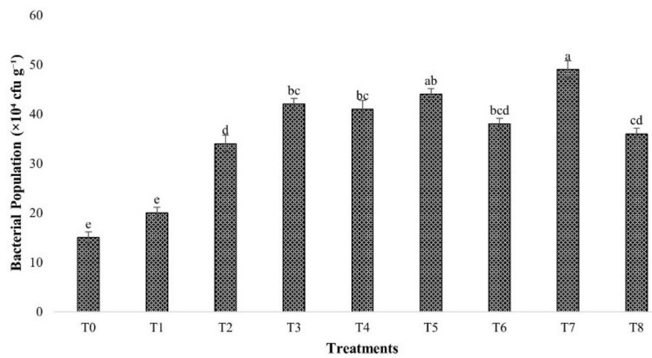

Figure 5: Bacterial population on sulfur & SOB coated urea under different treatments. Data represent mean ± SE, n = 3. Bars share similar letter(s) are not significantly different according to Tukey HSD test at $p \leq 0.05$

Treatment: T0 = Control; T1 = Sulphur; T2 = Sulphur Oxidizing Bacteria (SOB); T3 = SOB + Sulphur; T4 = SOB Coated Urea; T5 = SOB coated Urea + Sulphur; T6 = Sulphur Coated Urea; T7 = SOB + Sulphur coated Urea; T8 = SOB + Sulphur coated Urea with 25% less Urea

Sulphur and urease release of soil at various time interval

Sulphur release dynamics and urease activity in soil under different treatments over a 75-day incubation period is illustrated in Figure 6a and 6b. Findings showed that all treatments had an upward trend in the release of sulphur and urease activity relative to the control (T0) which had the lowest and almost stable level of sulphur and the lowest activity of the enzyme at any stage of the experiment. The highest and the longest sustained sulphur release was noted in T7, (SOB + S coated urea) where the increasing sulphur concentration was ranging between 7.2 mg kg^{-1} to 16.2 mg kg^{-1} at day 1 to 75 respectively, which translated to an increase of 95.97 percent over control as shown in Figure 6a.

Another similar upward trend was noted in T6, (S coated urea) and then T5, (SOB coated urea + S). This T8 treatment (SOB + S coated urea with 25% less urea) also had a similar release pattern meaning that there was efficient nutrient release despite reduced urea application.

The amount of sulphur released was found to be moderate with T4 (SOB coated urea) and T3 (SOB + sulphur) and comparatively lower with T2 (SOB) and T1 (sulphur), which means that either single treatment was not as effective as combined treatments. In general, sulphur coating and SOB inoculation treatment had a slow and prolonged release trend in comparison to the traditional applications. This was also seen with urease activity, with all treatments exhibiting an increasing tendency to mid-incubation and then an increasing or slight decrease at the end. In the initial stages (day 1), urease activity was consistent among treatments but over time, there were significant differences developed. The highest urease activity was recorded in T7, (SOB + S coated urea), reaching 31.7 mg $NH_4 - N\ kg^{-1}\ h^{-1}$, followed by T8 and T6, as illustrated in Figure 6b. On the contrary, control (T0) had the lowest urease activity during the incubation period. Combined use of sulphur coating and SOB treatments had an enhanced and sustained enzyme activity than individual ones, showing an enhanced ability to transform nitrogen and delayed urea hydrolysis.

Pot trial sunflower

Growth parameters

In pot trial of sunflower, the results of growth parameters revealed that the usage of sulphur, sulphur-coated urea, sulphur-oxidizing bacteria (SOB), and their combinations enhanced the parameters as compared to the control in terms of root length, shoot length, shoot dry weight, root dry weight, head diameter and SPAD value (Table 4). Notably, the maximum root length, shoot length, shoot dry weight, root dry weight, head diameter and SPAD value were

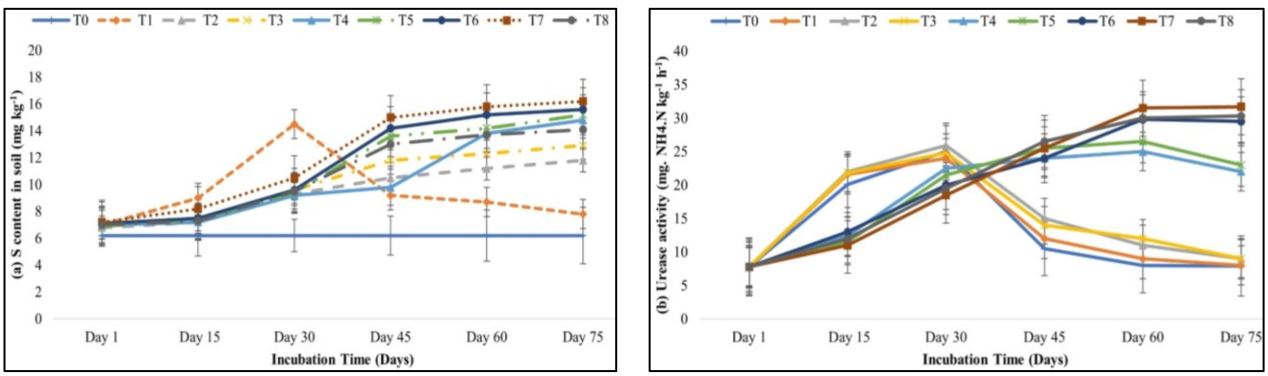

Figure 6: Effect of different sulfur, SOB and their urea coatings on the release patterns of sulphur (a) and urease activity (b) in soil at different time intervals

Treatment: T0 = Control; T1 = Sulphur; T2 = Sulphur Oxidizing Bacteria (SOB); T3 = SOB + Sulphur; T4 = SOB Coated Urea; T5 = SOB coated Urea + Sulphur; T6 = Sulphur Coated Urea; T7 = SOB + Sulphur coated Urea; T8 = SOB + Sulphur coated Urea with 25% less Urea

Table 4: Effect of different sulphur treatments and SOB on root length (cm), shoot length (cm), shoot dry weight (g plant^{-1}), root dry weight (g plant^{-1}), head diameter (cm) and SPAD value of sunflower. Data represent mean ± SE, n = 3. Bars sharing similar letter(s) are not significantly different according to Tukey HSD test at $p \leq 0.05$

Treatment	Root Length (cm)	Shoot Length (cm)	Shoot Dry Weight (g plant^{-1})	Root Dry Weight (g plant^{-1})	Head Diameter (cm)	SPAD Value
Control	8 ± 0.21d	88.6 ± 0.65d	52.33 ± 0.18e	17.33 ± 0.26d	7.79 ± 0.01d	34.6 ± 0.40d
Sulphur	8.33 ± 0.15cd	90.82 ± 0.61cd	52.93 ± 0.18de	17.60 ± 0.72cd	7.88 ± 0.02cd	35.16 ± 0.50cd
SOB	8.36 ± 0.19cd	91.08 ± 0.68cd	53.50 ± 0.17cd	18.00 ± 0.72b-d	7.96 ± 0.01cd	35.46 ± 0.38b-d
SOB + S	8.46 ± 0.12b-d	91.7 ± 0.55cd	54.20 ± 0.17bc	18.40 ± 0.73a-d	8.07 ± 0.05cd	35.7 ± 0.47b-d
SOB Coated Urea	8.6 ± 0.15b-d	92.59 ± 0.76bc	54.37 ± 0.20bc	18.60 ± 0.72a-c	8.15 ± 0.01c	36.1 ± 0.61b-d
SOB coated Urea + S	8.76 ± 0.12a-c	93.92 ± 0.68bc	54.73 ± 0.18ab	18.80 ± 0.72ab	8.48 ± 0.03b	36.7 ± 0.55a-d
Sulphur Coated Urea	8.96 ± 0.12a-c	95.25 ± 0.67ab	53.73 ± 0.18cd	18.20 ± 0.72b-d	8.48 ± 0.16b	37.6 ± 0.40a-c
SOB + SCU	9.5 ± 0.15a	98.35 ± 0.96a	55.53 ± 0.18a	19.40 ± 0.72a	8.91 ± 0.04a	38.93 ± 0.71a
SOB + SCU with 25% less Urea	9.2 ± 0.12ab	95.69 ± 0.76ab	54.10 ± 0.17bc	18.70 ± 0.73a-c	8.73 ± 0.09ab	37.86 ± 0.50ab
Tukey HSD ($p \leq 0.05$)	**0.7427**	**3.5216**	**1.2327**	**1.1613**	**0.3214**	**2.5432**

recorded in T7 (SOB + Sulphur coated Urea) over the control. T8 (SOB + Sulphur coated Urea) with 25% lower urea application also exhibited significant improvement in most of the growth parameters and was near T7. On the same note, T6 (Sulphur Coated Urea) application was better compared to T1 (sulphur) and T2 (SOB).

SPAD chlorophyll value

The effectiveness of different sulphur and SOB-based treatments on chlorophyll SPAD value of sunflower plants is presented in Table 4. The chlorophyll content improved significantly in all the treatments applied as compared to the control in which no other source of sulphur was applied other than recommended NPK. In T7 (SOB + Sulphur coated Urea (NPK)) the highest SPAD value of 38.93 was achieved that was 12.52% greater than that of the control. This was then succeeded by T8 and T6 which had an improvement of 9.44 and 8.67% over control, respectively. The T5, T4, T3, T2 and T1 also showed moderate increases but the improvement was less than the integrated treatments.

Yield attributes

The application of sulphur, SOB, sulphur-coated urea and combination of the integrated applications showed significant improvement in sunflower yield attributes. The highest number of achenes head^{-1} as shown in Figure 7a and 100-weight of the seed as shown in Figure 7b, was found in T7 (SOB + Sulphur coated Urea (NPK)). Similarly, the maximum head diameter as shown in Table 4, was also recorded in T7 as compared to the control. There was also significant improvement in yield attributes in the treatment T8, then T6 and T5. Conversely, when sulphur (T1) and SOB (T2) were applied individually, relatively smaller increases were realized, compared to the control. These findings revealed that SOB use in conjunction with sulphur-coated urea was more effective in enhancing the sunflower reproductive performance compared to individual treatments.

Nutrient contents in sunflower

The results showed that the application of sulphur, SOB, sulphur-coated urea, and their combinations significantly improved nitrogen, phosphorus and potassium contents in sunflower grains compared with the control (Figure 8a, 8b and 8c). The maximum levels of N, P and K were obtained in T7, (SOB + S coated Urea) as compared to the control. T8 treatment also showed similar values, then T6 and T5 which were again better than the individual applications of sulphur (T1) and SOB (T2). The increasing trend in macronutrient accumulation indicated that the combined use of SOB and sulphur-coated urea enhanced nutrient availability and uptake efficiency in sunflower plants.

Sulphur, zinc and iron content of sunflower

A similar trend was observed for sulphur and micronutrient contents, where all treatments showed improvement over the control. T7, (SOB + S coated Urea) had the highest levels of sulphur, zinc and iron as shown in Figure 9a, 9b and 9c. Treatments T8, T6 and T5 also showed significant improvement in grain nutrient enrichment, but relatively low values were in individual treatments. These findings distinctly show that combined use of SOB and sulphur-coated urea greatly enhanced the nutritional value and concentration of micronutrients in grains of sunflower.

Discussion

Nitrogen and sulphur are essential nutrients for sunflower growth, seed development and oil biosynthesis. Nitrogen supports vegetative growth and photosynthate production, whereas sulphur contributes to amino acid synthesis, protein formation and oil quality (Coêlho *et al.,* 2022). These realistic effects of sulphur in the soil and the plant improve yield and yield-contributing characteristics of sunflowers as explained by Asif *et al.* (2023). Sulphur is associated with the synthesis of proteins and amino acids, carbohydrates and oil production. The positive impact on growth, yield, and oil contents of sulphur was due to the synthesis of more carbohydrates, protein, and essential amino acids, which improved cell division, thus, improving plant height, diameter, leaf area, and other yield contributing parameters (Chowdhury *et al.,* 2020). In this research, isolated and pre identified sulphur oxidizing bacterial isolates were characterized for various plant growth promoting properties, such as pH reduction, sulphate ion production and sulphur oxidation activity. The improved growth and yield under T7 (SOB + SCU) may be attributed to better sulphur oxidation, improved nitrogen transformation and synchronized nutrient release. These findings were supported by Vishnu *et al.* (2024) which established that the chosen bacterial strains had high potential for sulphur oxidizing and promoting plant growth. The decrease in pH of broth media constituted active sulphur oxidation by these strains that can be linked to the formation of sulphuric acid as previously reported by Vidyalakshmi and Sridar (2013) and Ullah *et al.* (2016). This acidification process is important in increasing the nutrient availability in alkaline soils. The same outcome has been reported in a case by Behera *et al.* (2014) and Joshi *et al.* (2021) whereby sulphur oxidizing bacteria were found to reduce the soil pH by oxidizing elemental sulphur, which enhances the nutrient solubility and uptake.

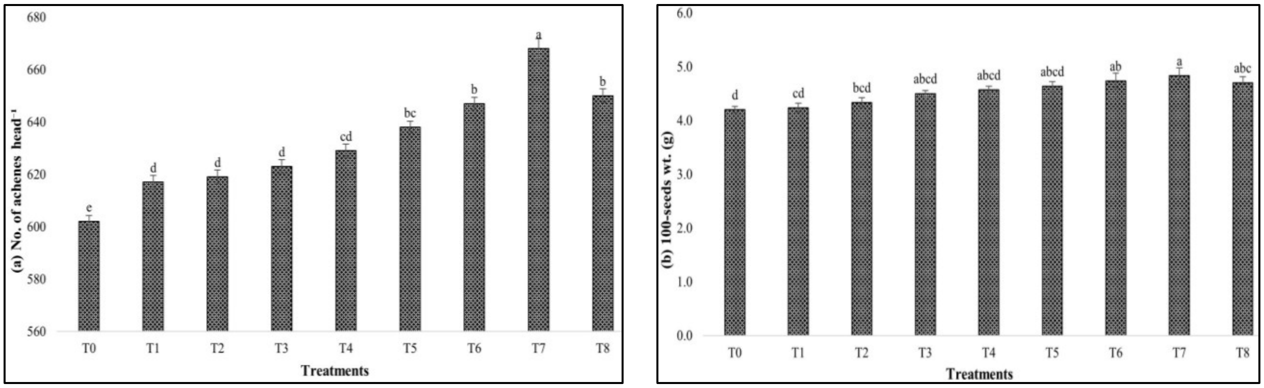

Figure 7: Effect of different sulphur treatments and SOB on sunflower yield attributes (a) No. of achenes head⁻¹ and (b) 100-seeds wt. (g). Data represent mean ± SE, n = 3. Bars share similar letter(s) are not significantly different according to Tukey HSD test at *p*≤0.05.

Treatment: T0 = Control; T1 = Sulphur; T2 = Sulphur Oxidizing Bacteria (SOB); T3 = SOB + Sulphur; T4 = SOB Coated Urea; T5 = SOB coated Urea + Sulphur; T6 = Sulphur Coated Urea; T7 = SOB + Sulphur coated Urea; T8 = SOB + Sulphur coated Urea with 25% less Urea

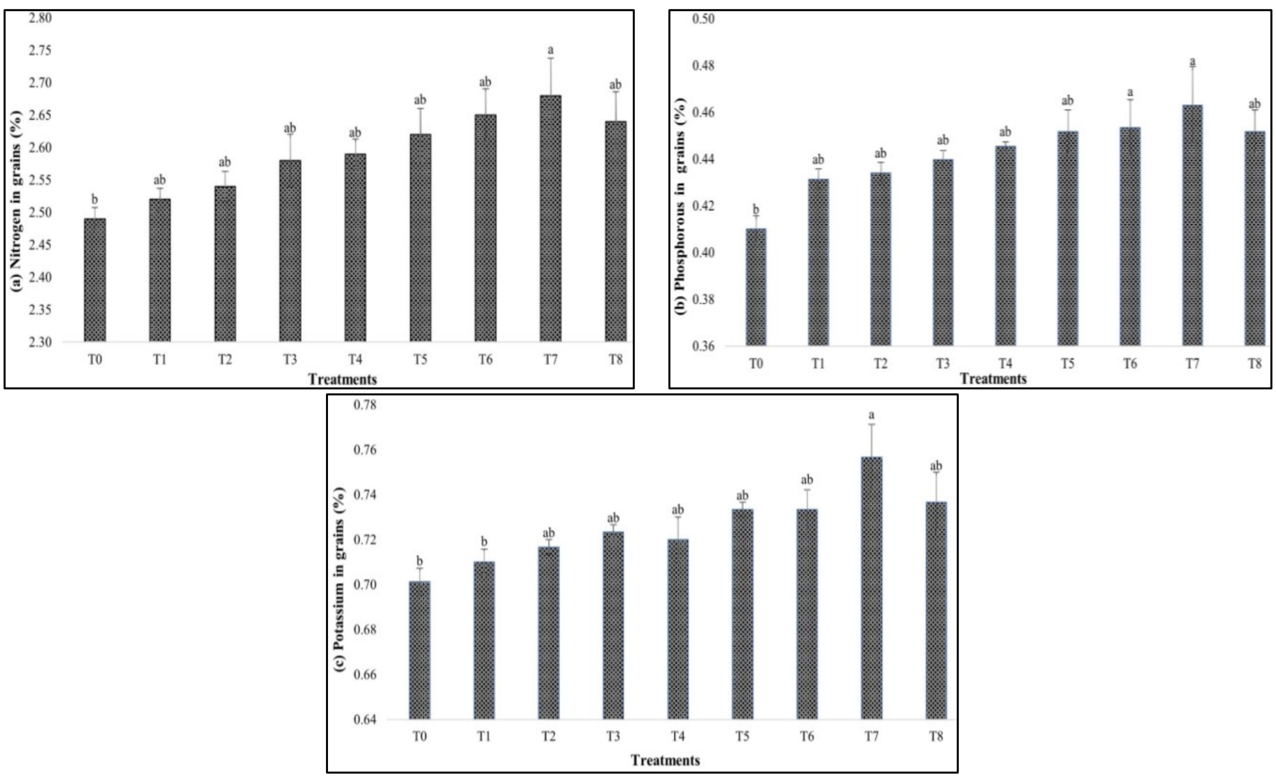

Figure 8: Effect of different sulphur treatments and SOB on (a) Nitrogen in grains (%), (b) Phosphorous in grains (%) and (c) Potassium in grains (%). Data represent mean ± SE, n = 3. Bars share similar letter(s) are not significantly different according to Tukey HSD test at *p*≤0.05

Treatment: T0 = Control; T1 = Sulphur; T2 = Sulphur Oxidizing Bacteria (SOB); T3 = SOB + Sulphur; T4 = SOB Coated Urea; T5 = SOB coated Urea + Sulphur; T6 = Sulphur Coated Urea; T7 = SOB + Sulphur coated Urea; T8 = SOB + Sulphur coated Urea with 25% less Urea

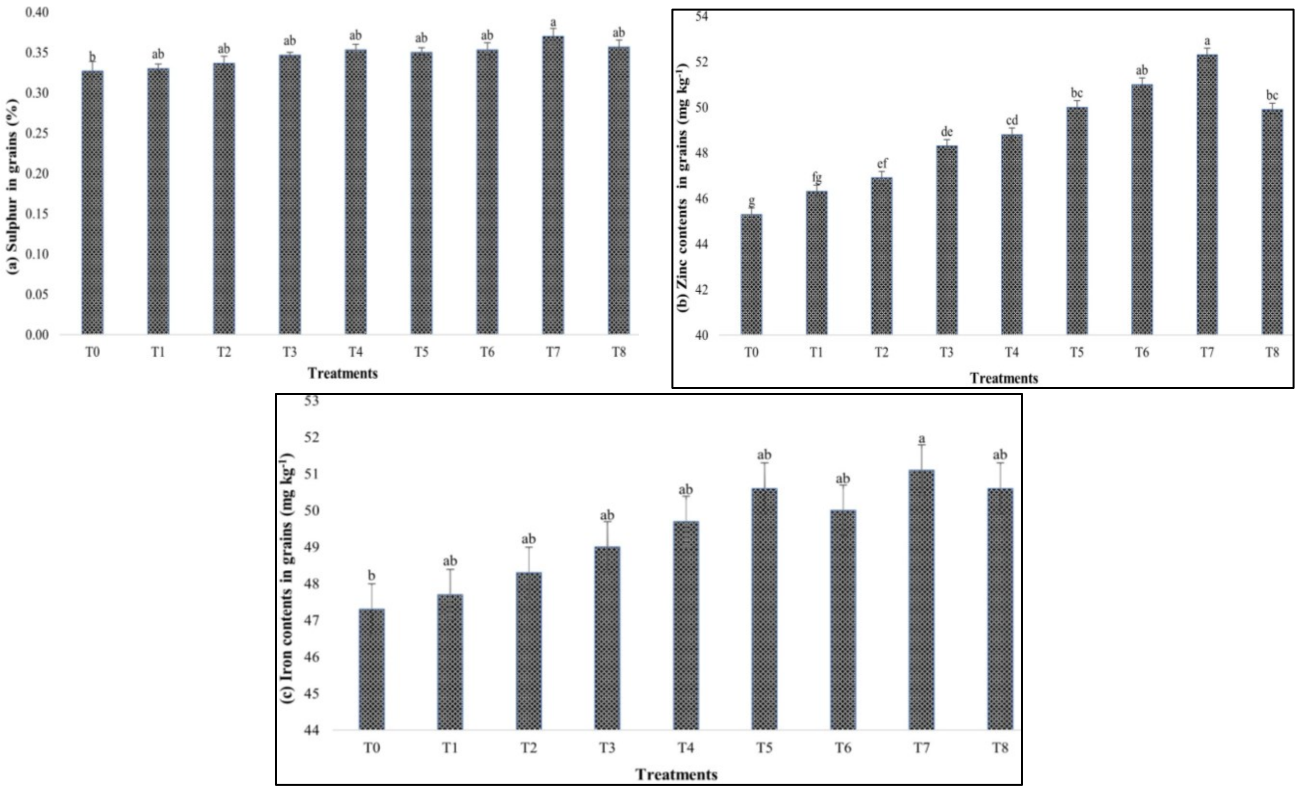

Figure 9: Effect of different sulphur treatments and SOB on (a) Sulphur in grains (%), (b) Zinc contents in grains (mg kg^{-1}) and (c) Iron contents in grains (mg kg^{-1}). Data represent mean ± SE, n = 3. Bars sharing similar letter(s) are not significantly different according to Tukey HSD test at *p*≤0.05

Treatment: T0 = Control; T1 = Sulphur; T2 = Sulphur Oxidizing Bacteria (SOB); T3 = SOB + Sulphur; T4 = SOB Coated Urea; T5 = SOB coated Urea + Sulphur; T6 = Sulphur Coated Urea; T7 = SOB + Sulphur coated Urea; T8 = SOB + Sulphur coated Urea with 25% less Urea

The selected SOB strains showed several plant growth promoting traits, including nutrient solubilization, urease activity, siderophore production and phytohormones related effects. These traits may have contributed to improved nutrient availability and plant growth in sunflower. Previous studies also reported that rhizobacteria enhance plant growth by increasing phosphorous and micronutrient solubility, producing IAA and supporting nutrient acquisition (Ansari *et al.,* 2023; Tariq *et al.,* 2023). Similarly, zinc solubilizing bacteria can improve enzyme activation and plant metabolism as reported by Saravanan *et al.* (2011), while siderophore producing bacteria enhance iron availability and nutrient uptake (Kumar *et al.,* 2019). This suggests that the chosen SOB strains in the current research could also add to the increased growth of plants due to the increased availability of micronutrients.

Moreover, SOB strains were positive in the urease activity, herein, the findings were consistent with those of Dodla *et al.* (2025) who found urease activities in sulphur oxidizing bacterial strains. The urease activity transforms naturally present or applied urea to ammonia and carbonate, thus boosting the availability of nitrogen in soil. This increased nitrogen conversion could have helped to improve plant growth which was witnessed in the current study. Like our findings, Nosheen and Bano (2014) also indicated that urease producing bacteria had an effect of increasing the efficiency of nitrogen utilization and uptake of plant nutrients.

The ability of SOB strains to produce sulphate ions further confirmed their efficiency in transforming unavailable sulphur into plant-available forms (Zhao *et al.,* 2017). Previous research has also indicated that sulphur oxidizing microorganisms oxidize reduced sulphur to sulphate by biological oxidation, which plays a significant role in the soil fertility and plant nutrition (Behera *et al.,* 2014). Furthermore, compatibility test conducted by Vishnu *et al.* (2024) also showed that all the chosen strains were not antagonistic in our work and could be easily mixed into a microbial consortium. These compatible consortia were believed to be better at field conditions because they interact synergistically and have better functional stability.

The incubation experiment also validated the performance of SOB and sulphur coated urea, in which a controlled and slow release of sulphur could be seen over a period of time (Dodla *et al.,* 2025). The maximum sulphur level was observed on treatments where SOB was applied with sulphur coated urea, where coated urea minimizes the loss of nitrogen as noted by Shivay *et al.* (2016), Essa *et al.* (2021) and Hindersah *et al.* (2021). This gradual release mechanism may support longer nutrient availability and reduce possible nutrient losses. They described that slower release of N in controlled release urea fertilizer may be most likely reason of low NH_3 volatilization compared to uncoated urea. Previous research has reported similar trends that coated fertilizers improved nutrient use efficiency by controlling nutrient release (Sattar *et al.,* 2021; Asghar *et al.,* 2022;). Moreover, the urease activity was more effective with combined treatments, which indicates the increase of nitrogen conversion in soil. Enhanced urease activity can be explained by the stimulation of microorganisms and enhanced biochemical activity in the soil, which promotes the effective hydrolysis of urea and the availability of nitrogen (Anwar *et al.,* 2025).

Growth parameters of sunflower as improved by the current study can be linked to the improved availability of nutrients and the improvement of soil microbial activity. Moreover, SOB inoculation must have enhanced root structure and efficiency in nutrient uptake, and thus, overall plant performance. These results were similar to those of the earlier research, which have shown that the addition of sulphur has a great impact on increasing growth parameters in sunflower. In the studies, Pavani *et al.* (2013) documented a significant growth in the height of plants when the amount of sulphur applied was 25 and 30 kg ha^{-1}. Equally, Hussain *et al.* (2023) and Senthamizhkumaran *et al.* (2018) found out that sulphur fertilization significantly enhanced vegetative growth in sunflower.

The weight gains in 100-seeds weight observed in the current study is consistent with the results of Usman *et al.* (2020) who indicated that the highest weight gain was recorded in 1000 achene weight when sulphur and boron were used. The growth in the size of the head in the current study is also corroborated by previous evidence where Saleem *et al.* (2019) reported that the size of sunflower heads increased with more sulphur applied whereas Devi *et al.* (2012) found that the size of the head significantly increased with the application of sulphur. The improved yield can be attributed to the presence of sulphur in the transformation of carbohydrates into oils and in the production of fatty acids as stated by Haq *et al.* (2021). Besides, the growth in achene yield observed in the current study is corroborated by the previous study in which Bonari *et al.* (2013) indicated a substantial growth in the grain yield when they applied sulphur. Correspondingly, Parveen *et al.* (2021) noted that the use of sulphur and nitrogen gave optimal achene yield as compared to the control. According to Asif *et al.* (2023), the application of sulphur at the right concentration had a great impact on the increasing sunflower yield.

The findings on the yield of the grain showed that the crude protein and macronutrient (N, P, K) contents increased markedly under the integrated treatments. The increased amount of nitrogen in grains was attributed to increased availability of nitrogen through increased urease activity and efficient use of nitrogen. Likewise, enhanced phosphorus and potassium absorption could be attributed to enhanced root development and augmented nutrient solubility when soil pH was low. Hammad *et al.* (2021) had previously made similar observations in sunflower. The NPK and S uptake of in sunflower was observed to be higher in the NPK and S treatments as compared to control (Shafiq *et al.,* 2021). Although temporal soil sampling was conducted to assess sulphur release and urease activity, but the plant N and S contents were measured at harvest only. Therefore, future studies should include multi-stage plant sampling to better understand the dynamics of N and S uptake during crop growth.

A similar increasing trend was observed for micronutrient Zn and Fe contents in sunflower grains. The maximum accumulation was observed in the treatments of SOB with sulphur coated urea, which showed enhanced nutrient bioavailability and uptake in accordance with the results of Joshi *et al.* (2021). SOB oxidation of sulphur results in acidic rhizosphere, increasing the solubility of micronutrients (zinc, iron, etc.). Such results are in line with earlier studies that have shown that nutrient management methods and microbial inoculation can significantly increase the enrichment of micronutrients in crops (Naveed *et al.,* 2021). Because the present study was conducted as a single site, single season pot experiment, the findings should be interpreted as preliminary evidence under controlled conditions. Multi location and multi field trials are required before making general agronomic recommendations for wider filed adoption.

Conclusion

The results suggested that integration of sulphur oxidizing bacteria (SOB) with sulphur coated urea showed potential as an effective approach for improving nutrient availability, enhancing crop growth, and increasing yield of oilseed crops. The findings of the current research revealed that SOB have the ability to oxidize sulphur, lower pH and transform it into plant available sulphate form, which enhances nutrient solubility and uptake in the soil conditions. Moreover, SOB and sulphur coated urea application as a combination produced greater growth parameters, yield attributes and nutrient contents in the sunflower than the control and individual treatments. Sulphur coated urea was used to ensure slower and prolonged release of sulphur and SOB inoculation increased its bioavailability by increasing the biological oxidation. This symbiotic relationship not only enhanced the efficiency of nutrient utilization, but minimized any nutrient loss possibilities. The integrated treatment was found to be more effective in the case of biomass production, achene, seed weight, and enhancement in the grain yield. The evidence of the current research suggests that the integrated use of SOB and sulphur coated urea is more effective than the traditional methods of fertilizers and may improve sunflower performance under pot conditions. Thus, it is concluded that this combined approach may serve as a promising nutrient management strategy under controlled conditions; however, field validation is required before wider recommendation.

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