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Biopriming with *Bacillus subtilis* Enhanced the Sulphur Use Efficiency of Indian Mustard under Graded Levels of Sulphur Fertilization

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Abstract: This study investigated the effect of bioinoculants (*Bacillus subtilis* and *Pseudomonas fluorescens*) as biopriming agents under varied sulphur (S) fertilizer levels (0, 20, 30, and 40 kg S ha^{-1}) to enhance sulphur use efficiency (SUE) in Indian mustard. The experiment was conducted during the 2018–19 and 2019–20 winter seasons at the research farm of the Institute of Agricultural Sciences, Banaras Hindu University, Varanasi (25°26′ N, 82°99′ E). A randomized block design was employed to assess the combined effect of biopriming and S fertilization on the partitioning of S in different parts of mustard plants, S uptake, SUE, and soil urease, dehydrogenase, alkaline phosphatase, and arylsulphatase activity. Results showed that the application of S fertilizers along with biopriming significantly increased the S content, uptake, and SUE by plants and enzymes involved in the S mineralization process. Application of 40 kg S ha^{-1} + *B. subtilis* resulted in the highest S content in the root (0.12%), stover (0.30%), and seed (0.67%), and the highest total S uptake (2.97 g m^{-2} in the first year and 3.37 g m^{-2} in the second year), agronomic use efficiency (8.80 g g^{-1}), apparent S recovery (22.37%), urease activity (156.68 µg NH_4^+ g^{-1} hr^{-1}), dehydrogenase activity (42.80 µg TPF g^{-1} 24 hr^{-1}), and arylsulphatase activity (39.94 µg pNP g^{-1} hr^{-1}). However, the highest alkaline phosphatase activity (129.17 µg pNP g^{-1} hr^{-1}) was found in the treatment that received 40 kg S ha^{-1} + *P. fluorescens*. Further, the different indices of SUE revealed that the effect of biopriming was more prominent in apparent recovery efficiency than agronomic SUE and physiological SUE. Conclusively, the present study demonstrated that seed biopriming with *B. subtilis* along with S fertilization is more rewarding and can promote sustainable production of Indian mustard.

Keywords: biopriming; *Bacillus subtilis*; *Pseudomonas fluorescens*; sulphur partitioning; arylsulphatase; sulphur use efficiency

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1. Introduction

Global crop production is highly dependent on the fertilizer sector. Continuous application of fertilizer is required to maintain and improve crop productivity for population demand. There is a parallel increase in fertilizer (NPK) consumption with food production (from 0.78 Mt in 1965–66 to 28.97 Mt in 2019–20) [1] which raised concern about the sustainability of the soil–plant–animal continuum. Indiscriminate nutrient use and overexploitation of resources affect the soil system by accelerating nutrient depletion, soil erosion, and soil acidity and salinity [2–5]. Moreover, these processes lower the nutrient use efficiency and in turn, increase the input requirement of fertilizers [6,7]. The contribution of

nitrogen (N), phosphorus (P), and potassium (K) fertilizers in total fertilizer consumption is greater than secondary and micronutrients, which is widening the negative balance of these nutrients in the soil and ultimately compromising food quality and human health [8]. Sulphur (S) deficiency is one common deficiency in the soil after NPK with respect to the extent of its deficiency [9].

The S element is the building block of various proteins and is essential for synthesizing S-containing amino acids such as cysteine, cystine, and methionine [10], which are vital to humans and animals [11]. Plants uptake S in the form of the sulphate ion (SO_4^{2-}) with the help of roots, and then it is transported to the leaves for sulphate reduction and assimilation [12]. Sulphur plays a crucial role in the synthesis of oil by enhancing glucosinolate content and percentage of oil content [13]. A deficiency of S leads to imbalanced nutrient uptake that ultimately results in loss of chlorophyll, stunted growth, and lower crop yields [14]. The key reasons for S deficiency include high-yielding crop varieties, non-judicious irrigation management, use of S-free fertilizers, etc. A recent study by The Sulphur Institute (TSI) mentioned that about 57–64 million hectares of net sown area in India is suffering from S deficiency [15]. After the 1980s, S deficiency in Indian soils became aggravated because of stringent pollution control measures to check the emission of sulphur dioxide from industrial chimneys. As a result, the production of S-carrying fertilizers increased in India from 607.8 (000 tonnes) in 1990–1991 to 950.1 (000 tonnes) in 2010–2011 [16]. Presently, Indian soils have a wide gap of N:P_2O_5:K_2O:S which is around 14.7:5.1:1.6:1, and thus, an advanced technique is critical for holistic S management and to improve the sulphur use efficiency (SUE) [17].

Biopriming is a novel seed treatment process used to improve seed germination and enhance crop nutrient and water uptake, growth, and yield [18,19]. Seed priming with living inoculums helps in enabling the adherence of bacteria to seeds, which improves the colonization of the rhizosphere and plant tolerance to adverse environmental conditions [20]. Beneficial microbes are commercially used for bioinoculation as they can influence plant growth positively by producing growth-promoting compounds and by solubilizing fixed forms of essential nutrients [21]. Various studies showed that biopriming promotes uniform seed germination, seedling vigor index, crop adaptability to adverse conditions (biotic and abiotic stresses), vegetative and reproductive growth, nutrient acquisition, yield, and quality of produce [22–25]. The biopriming agents commonly used are primarily live strains of bacteria and fungi such as *Mycorrhiza* spp., *Bacillus* spp., *Rhizobium* spp., *Agrobacterium* spp., *Azotobacter* spp., *Trichoderma* spp., *Azospirillum* spp., etc. [26,27]. Supplementation and enrichment of S in the soil solution by microbial mediation show promising effects under a fragile framework in the ecosystem with climatic variabilities. Harnessing the potential of inorganic fertilization in association with biopriming is key for integrated nutrient management (INM).

Indian mustard (*Brassica juncea*) is the most commonly grown oilseed crop in India [28]. In the *Brassica* family, S is of great importance for proper vegetative growth and the biosynthesis of protein and oil [29]. Studies have documented an increase in yield attributes and overall yield of Indian mustard with the use of S [30,31]. Generally, to obtain 90% of the potential yield in rapeseed-mustard, it needs 0.33 to 0.40% S in the leaf [32]. In addition, oilseeds vary in their sensitivity to S deficiency and S requirement [33]. However, improved and precise S management will significantly enhance the oil productivity of rapeseed-mustard while addressing the deficiencies [34]. Therefore, the optimum quantity of the S nutrient is of paramount importance to improve SUE in the mustard crop.

We hypothesized that the inclusion of seed biopriming in the INM technique can solve the problem of low SUE of Indian mustard. Therefore, the present investigation aimed to evaluate the effect of biopriming and graded S fertilization on SUE and enzymes involved in the mineralization of soil nutrients.

2. Material and Methods

2.1. Site Description

The field experiment was conducted at the research farm of the Institute of Agricultural Sciences, Banaras Hindu University, Varanasi, Uttar Pradesh, India, during two consecutive winter seasons of 2018–19 and 2019–20. The site of the experiment is situated at the Middle Gangetic Plains of Uttar Pradesh with a latitude of 25°26′ N and longitude of 82°99′ E and at an elevation of 80.7 m above mean sea level. Details on the physiochemical and biological properties of the experimental soil are presented in Table 1.

Table 1. Characteristics of the initial soil during the winter seasons of 2018 and 2019.

Particulars	Years		Method Followed
	2018	2019	
a. Physical properties			
Sand (g kg^{-1})	506.9	513.2	Bouyoucos [35]
Silt (g kg^{-1})	262.1	259.6	
Clay (g kg^{-1})	226.7	221.5	
Textural class	Sandy loam		
Bulk density (Mg m^{-3})	1.38	1.41	Black [36]
Particle density (Mg m^{-3})	2.63	2.61	
b. Chemical properties			
Organic carbon (g kg^{-1})	4.5	4.6	Walkley and Black [37]
pH (1:2.5 soil:water)	7.8	7.6	Jackson [38]
Electrical conductivity (dS m^{-1})	0.44	0.46	
Available N (kg ha^{-1})	202.7	208.4	Subbiah and Asija [39]
Available P (kg ha^{-1})	15.43	17.28	Olsen et al. [40]
Available K (kg ha^{-1})	237.4	239.8	Jackson [38]
Available S (mg kg^{-1})	8.7	9.9	Chesnin and Yien [41]
c. Biological properties			
Urease (µg NH_4^+ g^{-1} hr^{-1})	125.23	127.15	Douglas and Bremner [42]
Alkaline phosphatase (µg pNP g^{-1} hr^{-1})	96.42	88.64	Tabatabai and Bremner [43]
Dehydrogenase (µg TPF g^{-1} day^{-1})	25.57	25.16	Klein et al. [44]
Arylsulphatase (µg pNP g^{-1} hr^{-1})	19.82	20.83	Tabatabai and Bremner [45]

2.2. Treatment Details

There were twelve (12) treatment combinations of S fertilizer and seed priming with two bioinoculants, which were replicated thrice. The experimental design includes four (4) varied levels of S (0, 20, 30, and 40 kg ha^{-1}) and three (3) seed treatments (non-primed and primed with *Bacillus subtilis* and *Pseudomonas fluorescens*). A detailed description of the treatments is presented in Table 2.

Table 2. Treatment details used in the study.

Treatment Details	Notations Used
Bentonite sulphur @ 0 kg S ha^{-1} + No priming	T_1
Bentonite sulphur @ 0 kg S ha^{-1} + *Bacillus subtilis*	T_2
Bentonite sulphur @ 0 kg S ha^{-1} + *Pseudomonas fluorescens*	T_3
Bentonite sulphur @ 20 kg S ha^{-1} + No priming	T_4
Bentonite sulphur @ 20 kg S ha^{-1} + *Bacillus subtilis*	T_5
Bentonite sulphur @ 20 kg S ha^{-1} + *Pseudomonas fluorescens*	T_6
Bentonite sulphur @ 30 kg S ha^{-1} + No priming	T_7
Bentonite sulphur @ 30 kg S ha^{-1} + *Bacillus subtilis*	T_8
Bentonite sulphur @ 30 kg S ha^{-1} + *Pseudomonas fluorescens*	T_9
Bentonite sulphur @ 40 kg S ha^{-1} + No priming	T_{10}
Bentonite sulphur @ 40 kg S ha^{-1} + *Bacillus subtilis*	T_{11}
Bentonite sulphur @ 40 kg S ha^{-1} + *Pseudomonas fluorescens*	T_{12}

Note: Bentonite sulphur contains 90% elemental sulphur and 10% bentonite clay.

2.3. Preparation of Inoculum and Biopriming of Seeds

Pure culture of *P. fluorescens* and *B. subtilis* was collected from the Department of Mycology and Plant Pathology, Institute of Agricultural Sciences, Banaras Hindu University. The cultures were then inoculated in a nutrient broth and kept at 28 °C in a shaking incubator for 2 days. The bacterial pellets were harvested, and the final cell density was maintained at 4×10^8 CFU mL^{-1}. Seeds of mustard were firstly surface-sterilized for 1 min using 1% sodium hypochlorite. Then, the sterilized seeds were soaked in liquid culture comprising 2% carboxymethyl cellulose (adhesive agent) for 2 hr. After soaking the seeds in the culture for 2 hr at 28 ± 2 °C, they were subjected to air-drying at room temperature for 2 hr.

2.4. Crop Management

One deep ploughing was carried out by tractor followed by two ploughings by power tiller to obtain a good tilth. The weeds and stubble were removed, and clean leveling was performed. Irrigation/drainage channels were made. Bunds surrounding the seedbed were leveled at the proper height. The mustard seeds (cv. Giriraj) primed with *B. subtilis* (BHHU100) and *P. fluorescens* (OKC) were sown in furrows at a spacing of 30 cm. Extra seedlings were uprooted to maintain the desired spacing and population of the plot. The N, P, and K were applied in the ratio of 120:60:40 [46] through urea, diammonium phosphate, and muriate of potash as a basal dose. Bentonite S (as an S source) was applied 10 days before sowing. The water requirement was fulfilled at the critical stage of vegetative and siliqua formation. Hand weeding was conducted without disturbing the crop roots to reduce weed competition in the crop area. Finally, the crop was harvested at ground level when 80% of the siliquae matured.

2.5. Soil and Plant Sampling

For analysis, soils were collected from the upper layer (0–15 cm) after the harvest of the crop during both seasons. Soil samples at harvest were collected from 5 places, and a volume (500 g) was prepared using the quartering method. For analysis, fresh soil samples were stored in labelled zipper plastic bags at 2–4 °C. When each plot was harvested, five tagged plant samples (with roots) were collected at the same distance from the border of individual plots. Samples were washed with 0.1% detergent and 0.1 *N* HCl, and then with distilled water (DW) to remove the wax layer. Washed plant samples were oven-dried at 60 °C until samples became crispy. Dried plant samples were ground and stored in plastic bags at room temperature condition for evaluation of nutrient status.

2.6. Plant Analysis

Total S content in the root, stover, and seed was estimated from the digest obtained after diacid digestion. Ground plant samples were digested in a diacid mixture (HNO_3: $HClO_4$ at 9:4) on a hot plate, as described by Blanchar et al. [47], and digested samples were filtered through Whatman No. 1 filter paper and consolidated into a known volume for estimation of elemental contents. The digest was tested for total S by following the turbidimetric method in a spectrophotometer at 420 nm [41].

2.7. Computation of Sulphur Use Efficiency

The nutrient uptake (NU), agronomic use efficiency (AE_S), apparent recovery efficiency. (AR_S), and physiological use efficiency (PE_S) were calculated using the following formula:

$$\mathrm{NU}(\mathrm{kg\ ha^{-1}}) = \frac{\mathrm{NC} \times \mathrm{Y}}{100}$$

where NC = nutrient content in %; and Y = yield in kg ha^{-1}.

$$\mathrm{AE}\left(\mathrm{kg\ kg^{-1}}\right) = \frac{\mathrm{Y} - \mathrm{Yo}}{\mathrm{FA}}$$

where Y = yield of fertilized plot in kg ha^{-1}; Y_o = yield of unfertilized plot in kg ha^{-1}; and FA = rate of fertilizer applied in kg ha^{-1}.

$$\mathrm{AR}(\%) = \frac{\mathrm{NU} - \mathrm{NUo}}{\mathrm{FA}} \times 100$$

where NU = nutrient uptake in fertilized plot in kg ha^{-1}; NU_o = nutrient uptake in unfertilized plot in kg ha^{-1}; and FA = rate of fertilizer applied in kg ha^{-1}.

$$\mathrm{PE}(\mathrm{kg\ kg^{-1}}) = \frac{\mathrm{Y} - \mathrm{Yo}}{\mathrm{NU} - \mathrm{NUo}}$$

where Y = yield of fertilized plot in kg ha^{-1}; Y_o = yield of unfertilized plot in kg ha^{-1}; NU = nutrient uptake in fertilized plot in kg ha^{-1}; and NU_o = nutrient uptake in unfertilized plot in kg ha^{-1}.

2.8. Soil Analysis

Collected fresh soil samples were stored in plastic zipper bags at 2–4 °C for microbiological study.

2.8.1. Urease Activity

Soil samples were incubated with urea solution at 37 °C. Then, the remaining urea left after incubation was estimated. The urea hydrolyzed during incubation was calculated by subtracting the remaining amount from the added amount of urea. This value indicates the activity of the urease enzyme. About 10 g of fresh soil was placed in a conical flask, 5 mL of urea was added, and it was kept in an incubator for 5 hr at 37 °C. After incubation, 2 *M* potassium chloride-phenylmercuric acetate (KCl-PMA) extracting solution (50 mL) was added, followed by 1 hr of shaking [42]. The suspension was then filtered, and 1 mL of aliquot was placed in a 50 mL volumetric flask, to which the 2 *M* KCl-PMA solution and a coloring reagent (diacetyl monoxime and thiosemicarbazide) were added. The magenta color was developed after boiling the sample in a hot water bath. The color intensity was measured in a visible-spectrophotometer at 527 nm wavelength, and the urease activity was expressed in terms of μg NH_4^+ g^{-1} hr^{-1}.

2.8.2. Alkaline Phosphatase Activity

An assay of alkaline phosphatase activity in fresh soil samples involves the estimation of *p*-nitrophenol (pNP) released when a fresh soil sample is incubated with a buffered solution [43]. About 1 g of soil sample was placed in a test tube, and then about 0.2 mL of toluene, 4 mL of working modified universal buffer (MUB), and 1 mL of *p*-nitrophenol phosphatase solution were mixed with the soil. The soil mixture was then incubated at 37 °C for 1 hr, and about 1 mL of 0.5 *M* $CaCl_2$ and 4 mL of 0.5 *M* NaOH were added. The intensity of the yellow color of the filtrate was measured at 430 nm wavelength in a visible-spectrophotometer, and the alkaline phosphatase activity was expressed in terms of μg pNP g^{-1} hr^{-1}.

2.8.3. Dehydrogenase Activity

The assay of dehydrogenase activity was analyzed based on the transformation of triphenyl tetrazolium chloride (TTC) into triphenyl formazan (TPF) as mediated by the dehydrogenase enzyme [44]. About 6 g of fresh soil was placed in the test tube, and to this, 0.1 g of $CaCO_3$, 1 mL of 3% TTC solution, and 2.5 mL of DW were added. The samples were then incubated at 30 °C for 24 hr in the dark. After completion of the incubation period, 10 mL of ethanol was added, and the tube was tapped by hand. When the pink color developed, the suspension was filtered, and its intensity was measured at 485 nm wavelength using a visible-spectrophotometer. The results were expressed as μg TPF g^{-1} 24 hr^{-1}.

2.8.4. Arylsulphatase Activity

Arylsulphatase activity was estimated based on the release of SO_4^{2-} from sulphate ester. This enzyme act as an indicator of the S mineralization process in soil. About 1 g of soil passed through a 2 mm sieve was placed in a 100 mL conical flask, and to this, 0.2 mL of toluene, 4 mL of acetate buffer, and 1 mL of *p*-nitrophenol sulphate solution were added [45]. Then, the flask was swirled for a few minutes to mix the content, and samples were then incubated at 37 °C for 1 hr. After incubation, about 1 mL of 0.5 *M* $CaCl_2$ solution and 4 mL of 0.5 *M* NaOH solution were added. The yellow color of the filtrate was measured for its intensity with the help of a visible-spectrophotometer at 410 nm wavelength, and the results were expressed as μg pNP g^{-1} hr^{-1}.

2.9. Statistical Analysis

Experimental data were compiled and tested for one-way analysis of variance (ANOVA). The data were also subjected to Duncan's multiple range test (DMRT) at $p \leq 5\%$ significance level to significantly differentiate the variations among the treatments. Statistical Package for Social Science (SPSS) software (17.0 version) was used for the homogeneity test.

3. Results

3.1. S Content

Sulphur fertilization and biopriming had a significant ($p \leq 0.05$) effect on the S content in different parts of the mustard plant (Table 3). Pooled data from both years showed that the maximum content of S in the plant root was found in T_{11} (40 kg S ha^{-1} + *B. subtilis*) (0.12%) which was on par with T_8 (30 kg S ha^{-1} + *B. subtilis*) (0.11%) and T_{12} (40 kg S ha^{-1} + *P. fluorescens*) (0.11%). S content in the root was found to be increased with increasing doses of S (from 0 to 40 kg S ha^{-1}), and a significant effect of seed biopriming was observed over non-primed treatments. However, the lowest values of root S content were observed in the control, i.e., T_1 (0.05–0.06%). In the case of stover, S content ranged from 0.18 to 0.31%. Compared with T_1 (control) and T_{10} (40 kg S ha^{-1} + unprimed), the application of 40 kg S ha^{-1} + *B. subtilis* (T_{11}) increased the S content by 57.9% and 15.4%, respectively (Table 3). The highest content of S in the stover was recorded in T_{11} (0.30%), followed by T_{12} (0.29%) and T_8 (0.28%) as per pooled data. Application of 30 kg S ha^{-1} + *B. subtilis* and 30 kg S ha^{-1} + *P. fluorescens* (T_8 and T_9, respectively) showed greater accumulation of S

in the seed than the treatment that received 40 kg S ha^{-1} alone. Results further revealed that the application of S along with seed biopriming promotes greater assimilation of S in seed when compared with the S content in root and stover. The S content in the seed among different treatments followed the order of T_{11} (0.67%) > T_{12} (0.66%) > T_8 (0.66%) > T_9 (0.64%) > T_5 (0.63%) > T_{10} (0.61%) = T_6 (0.61%) > T_7 (0.59%) > T_2 (0.58%) > T_4 (0.57%) > T_3 (0.57%) > T_1 (0.54%). Conclusively, treatments T_{10}, T_{11}, and T_{12} showed enhanced seed S content by 12.9%, 19.4%, and 22.2% when compared with the control (T_1).

Table 3. Sulphur content of root, stover, and seed as influenced by the seed biopriming and varied levels of S fertilization in mustard.

Treatments	Sulphur Content (%)								
	Root			Stover			Seed		
	2018–19	2019–20	Pooled	2018–19	2019–20	Pooled	2018–19	2019–20	Pooled
T_1	0.05 ^{f}	0.06 ^{d}	0.06 ^{g}	0.18 ^{g}	0.20 ^{e}	0.19 ^{g}	0.52 ^{f}	0.56 ^{e}	0.54 ^{g}
T_2	0.07 ^{def}	0.08 ^{cd}	0.08 ^{efg}	0.23 ^{def}	0.25 ^{cd}	0.24 ^{de}	0.57 ^{de}	0.59 ^{cde}	0.58 ^{efg}
T_3	0.06 ^{ef}	0.07 ^{cd}	0.07 ^{fg}	0.21 ^{efg}	0.24 ^{cd}	0.23 ^{ef}	0.56 ^{de}	0.57 ^{de}	0.57 ^{fg}
T_4	0.06 ^{ef}	0.06 ^{d}	0.06 ^{g}	0.20 ^{fg}	0.22 ^{de}	0.21 ^{fg}	0.55 ^{ef}	0.58 ^{de}	0.57 ^{fg}
T_5	0.09 ^{abcde}	0.09 ^{abcd}	0.09 ^{bcde}	0.25 ^{bcd}	0.26 ^{bc}	0.26 ^{cd}	0.59 ^{cd}	0.66 ^{abc}	0.63 ^{bcd}
T_6	0.08 ^{bcdef}	0.09 ^{abcd}	0.09 ^{cdef}	0.24 ^{cde}	0.25 ^{bcd}	0.25 ^{cde}	0.58 ^{de}	0.63 ^{abcd}	0.61 ^{cde}
T_7	0.07 ^{cdef}	0.08 ^{bcd}	0.08 ^{efg}	0.23 ^{def}	0.24 ^{cd}	0.24 ^{de}	0.57 ^{de}	0.61 ^{bcde}	0.59 ^{def}
T_8	0.11 ^{ab}	0.11 $_{ab}$	0.11 ^{ab}	0.27 ^{ab}	0.29 ^{ab}	0.28 ^{ab}	0.62 ^{bc}	0.70 ^{a}	0.66 ^{ab}
T_9	0.10 ^{abc}	0.10 ^{abc}	0.10 ^{abcd}	0.26 ^{abc}	0.28 ^{abc}	0.27 ^{bc}	0.61 ^{bc}	0.67 ^{ab}	0.64 ^{abc}
T_{10}	0.09 ^{abcd}	0.10 ^{abc}	0.10 ^{abcd}	0.25 ^{bcd}	0.27 ^{bc}	0.26 ^{cd}	0.59 ^{cd}	0.64 ^{abcd}	0.61 ^{cde}
T_{11}	0.12 ^{a}	0.12 ^{a}	0.12 ^{a}	0.29 ^{a}	0.31 ^{a}	0.30 ^{a}	0.65 ^{a}	0.69 ^{a}	0.67 ^{a}
T_{12}	0.10 ^{abc}	0.11 ^{ab}	0.11 ^{abc}	0.28 ^{ab}	0.29 ^{ab}	0.29 ^{ab}	0.64 ^{ab}	0.68 ^{ab}	0.66 ^{ab}

T_1—0 kg S ha^{-1} + no priming; T_2—0 kg S ha^{-1} + *Bacillus subtilis*; T_3—0 kg S ha^{-1} + *Pseudomonas fluorescens*; T_4—20 kg S ha^{-1} + no priming; T_5—20 kg S ha^{-1} + *Bacillus subtilis*; T_6—20 kg S ha^{-1} + *Pseudomonas fluorescens*; T_7—30 kg S ha^{-1} + no priming; T_8—30 kg S ha^{-1} + *Bacillus subtilis*; T_9—30 kg S ha^{-1} + *Pseudomonas fluorescens*; T_{10}—40 kg S ha^{-1} + no priming; T_{11}—40 kg S ha^{-1} + *Bacillus subtilis*; T_{12}—40 kg S ha^{-1} + *Pseudomonas fluorescens*. Mean data followed by the same letters differ non-significantly ($p \leq 0.05$) within the column as per Duncan's test. ANOVA tables are provided in the Supplementary Materials (Tables S1–S3).

3.2. Sulphur Uptake

The highest S uptake by root was registered in T_{11} (0.15 g m^{-2}), which was on par with the results observed in T_8 (0.13 g m^{-2}) during 2018–19 (Figure 1a). The root S uptake in T_{12} (0.18 g m^{-2}) was found on par with T_{11} (0.19 g m^{-2}) during 2019–20. Seed biopriming in treatment T_{11} showed a significant increase in root S uptake by two to three times when compared with the control. Similarly, a significant increase in stover, seed, and total S uptake was also noticed in treatment T_{11}. The S uptake in stover ranged from 0.70 to 1.25 g m^{-2} in the first year and 0.79 to 1.43 g m^{-2} in the second year (Figure 1b). During both years, the S uptake by stover in T_{11} (40 kg S ha^{-1} + *B. subtilis*) was highest, but on-par results were recorded in T_{12} (40 kg S ha^{-1} + *P. fluorescens*). In the case of seed (Figure 1c), the maximum uptake was observed in T_{11} (1.57 and 1.75 g m^{-2}) and the lowest in T_1 (0.98 and 1.16 g m^{-2}) during the first and second year, respectively. Total S uptake (Figure 1d) varied from 1.73 to 2.97 g m^{-2} in the first year and from 2.02 to 3.17 g m^{-2} in the second year. Application of *B. subtilis* showed better results compared to *P. fluorescens*. Total S uptake was found to increase with increasing S levels, and this increase was greater with biopriming intervention. The S uptake in all parts of the plant was found to be increased among all the treatments during the second year compared to the first year.

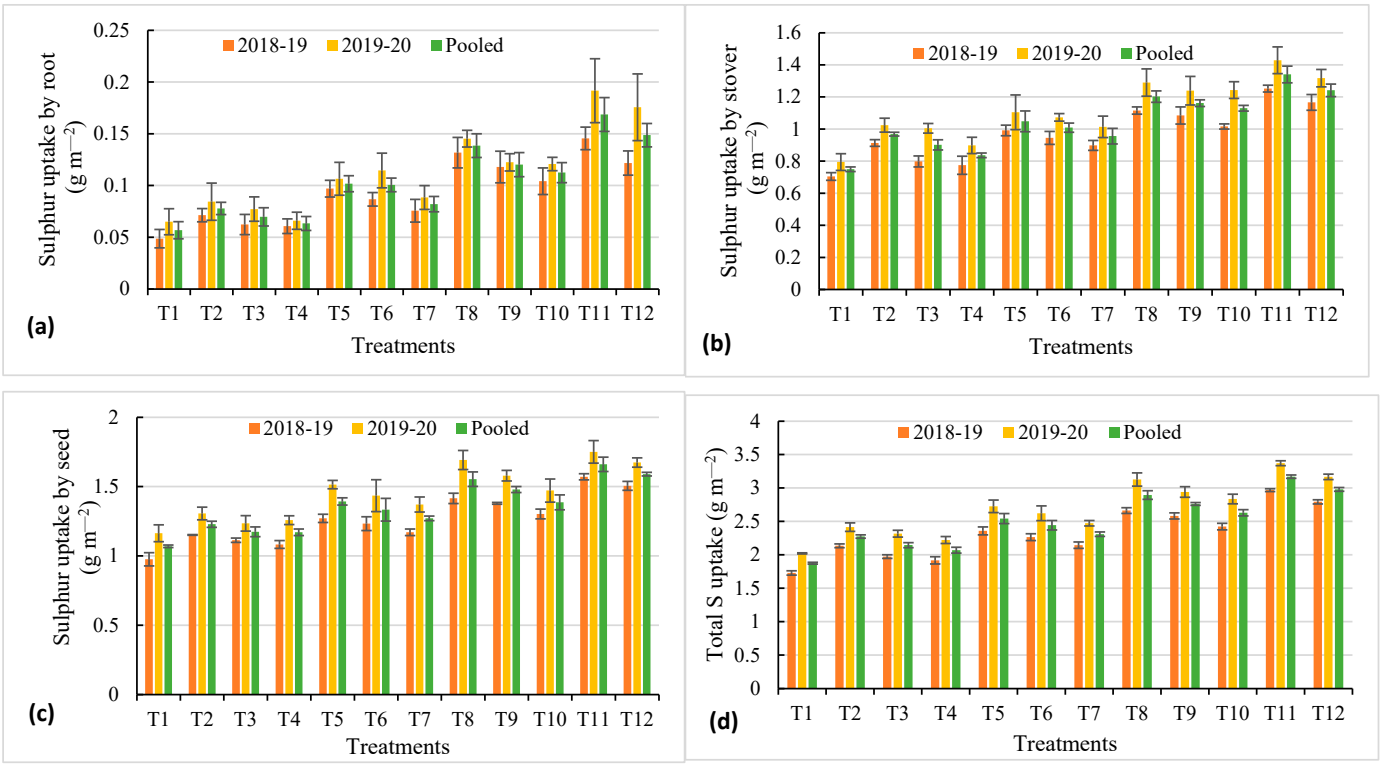

Figure 1. Sulphur uptake by root, stover, and seed as influenced by the seed biopriming and varied levels of S fertilization in mustard. (**a**) S uptake by root; (**b**) S uptake by stover; (**c**) S uptake by seed; (**d**) Total S uptake. Error bars indicate mean ± SE (n = 3). Treatments: T_1—0 kg S ha^{-1} + no priming; T_2—0 kg S ha^{-1} + *Bacillus subtilis*; T_3—0 kg S ha^{-1} + *Pseudomonas fluorescens*; T_4—20 kg S ha^{-1} + no priming; T_5—20 kg S ha^{-1} + *Bacillus subtilis*; T_6—20 kg S ha^{-1} + *Pseudomonas fluorescens*; T_7—30 kg S ha^{-1} + no priming; T_8—30 kg S ha^{-1} + *Bacillus subtilis*; T_9—30 kg S ha^{-1} + *Pseudomonas fluorescens*; T_{10}—40 kg S ha^{-1} + no priming; T_{11}—40 kg S ha^{-1} + *Bacillus subtilis*; T_{12}—40 kg S ha^{-1} + *Pseudomonas fluorescens*.

3.3. Urease Activity

Urease activity is an indicator of nitrogen mineralization as the urease enzyme hydrolyzes the urea. Urease activity, as influenced by the treatment combinations, varied from 138.73 to 154.53 µg NH_4^+ g^{-1} hr^{-1} in 2018–19 and 141.67 to 158.83 µg NH_4^+ g^{-1} hr^{-1} in 2019–20 (Figure 2a). Results revealed that the application of 40 kg S ha^{-1} + *B. subtilis* significantly ($p \leq 0.05$) improved the urease activity in soil by 11.8% and 4.7% over T_1 (control) and T_{10} (40 kg S ha^{-1}). However, T_{12} (30 kg S ha^{-1} + *P. fluorescens*) was statistically on par with T_{11} (40 kg S ha^{-1} + *B. subtilis*). Seed biopriming with *B. subtilis* and *P. fluorescens* showed higher urease activity than the application of S fertilization without biopriming. Application of S fertilizers along with *B. subtilis* enhanced the urease activity to a greater extent than S fertilizers along with *P. fluorescens*.

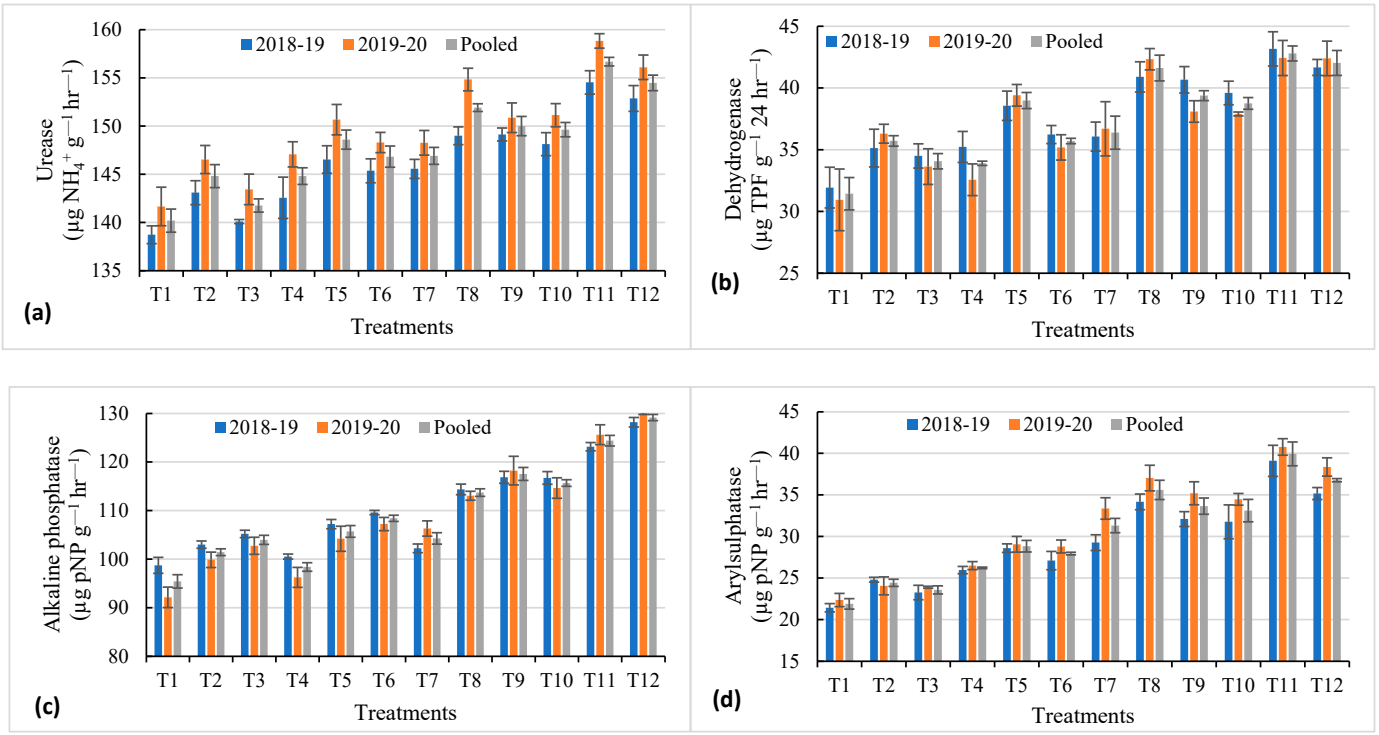

Figure 2. Soil enzymatic activities as influenced by the seed biopriming and varied levels of S fertilization in mustard. (**a**) Urease activity (**b**) Dehydrogenase activity (**c**) Alkaline phosphatase activity (**d**) Arylsulphatase activity. Error bars indicate mean ± SE ($n = 3$). Treatments: T_1—0 kg S ha^{-1} + no priming; T_2—0 kg S ha^{-1} + *Bacillus subtilis*; T_3—0 kg S ha^{-1} + *Pseudomonas fluorescens*; T_4—20 kg S ha^{-1} + no priming; T_5—20 kg S ha^{-1} + *Bacillus subtilis*; T_6—20 kg S ha^{-1} + *Pseudomonas fluorescens*; T_7—30 kg S ha^{-1} + no priming; T_8—30 kg S ha^{-1} + *Bacillus subtilis*; T_9—30 kg S ha^{-1} + *Pseudomonas fluorescens*; T_{10}—40 kg S ha^{-1} + no priming; T_{11}—40 kg S ha^{-1} + *Bacillus subtilis*; T_{12}—40 kg S ha^{-1} + *Pseudomonas fluorescens*.

3.4. Dehydrogenase Activity

Application of S fertilizers along with seed biopriming (*B. subtilis* and *P. fluorescens*) significantly ($p \leq 0.05$) improved the dehydrogenase activity compared to plants without seed biopriming (Figure 2b). The highest dehydrogenase activity was recorded in T_{11} (43.17 and 42.43 μg TPF g^{-1} 24 hr^{-1}) which is on par with T_{12} (41.67 and 42.40 μg TPF g^{-1} 24 hr^{-1}) during both the season of the experiment. According to pooled data, dehydrogenase activity was increased by 23.3%, 36.1%, and 33.7% in response to T_{10}, T_{11}, and T_{12}, respectively, when compared with T_1 (Figure 2b). Dehydrogenase activity is observed to increase with increasing levels of S from 0 to 40 kg S ha^{-1}.

3.5. Alkaline Phosphatase Activity

Mineralization of organic P in the soil can be correlated with the status of phosphatase activity in the soil. Application of T_{12} (40 kg S ha^{-1} + *P. fluorescens*) significantly ($p \leq 0.05$) enhanced the alkaline phosphatase activity compared with the control (T_1) and other treatments (Figure 2c). Though all priming agents with different fertilizer combinations increased the alkaline phosphatase activity, priming with *P. fluorescens* was the most efficient one in this respect. The efficiency of T_{11} (40 kg S ha^{-1} + *B. subtilis*) was on par with T_{12} (40 kg S ha^{-1} + *P. fluorescens*) in enhancing phosphatase activity. According to pooled data, the maximum alkaline phosphatase activity is noted in T_{12} (129.17 μg pNP g^{-1} hr^{-1}) followed by T_{11} (124.41 μg pNP g^{-1} hr^{-1}) (Figure 2c).

3.6. Arylsulphatase Activity

Arylsulphatase enzyme plays an important role in the S cycle and acts as an indicator of S availability to plants. Plots receiving 40 kg S ha^{-1} + *B. subtilis* (39.10 and 40.77 µg g^{-1} hr^{-1}) (T_{11}) recorded the highest arylsulphatase activity and the lowest was recorded in the plot without S fertilization and biopriming (35.17 and 38.37 µg pNP g^{-1} hr^{-1}) (T_1) during 2018–19 and 2019–20, respectively (Figure 2d). According to pooled data, the arylsulphatase activity was found to increase compared to the control (T_1) in the order of T_{11} (82.37%) > T_{12} (67.89%) ≥ T_8 (62.56%) ≥ T_9 (53.65%) ≥ T_{10} (51.23%) ≥ T_7 (43.01%) > T_5 (31.69%) ≥ T_6 (27.62%) ≥ T_4 (19.81%) ≥ T_2 (11.60%) ≥ T_3 (7.72%).

3.7. Sulphur Use Efficiency

Agronomic use efficiency (AE_s) of S varied from 5.23 to 8.80 g of above-ground part g^{-1} of S applied (Table 4). Application of S fertilizer without biopriming registered lower AE_s compared to primed treatments. All primed plots recorded an increase in AE_s compared to T_4 by 13.4, 16.2, 52.9, 45.5, 68.2, and 63.6%, respectively, in T_5, T_6, T_8, T_9, T_{11}, and T_{12} (Table 4). However, the overall effect of treatments was non-significant on the agronomic efficiency of S.

Table 4. Sulphur use efficiency as influenced by the seed biopriming and varied levels of S fertilization in mustard.

Treatments	Agronomic Use Efficiency (g g^{-1})			Apparent Recovery Efficiency (%)			Physiological Use Efficiency (g g^{-1})		
	2018–19	2019–20	Pooled	2018–19	2019–20	Pooled	2018–19	2019–20	Pooled
T_1	-	-	-	-	-	-	-	-	-
T_2	-	-	-	-	-	-	-	-	-
T_3	-	-	-	-	-	-	-	-	-
T_4	5.28	5.18	5.23	05.77 ^{c}	9.97 ^{b}	7.87 ^{d}	65.12	50.67	57.90
T_5	6.60	5.26	5.93	11.17 ^{bc}	15.56 ^{ab}	13.37 ^{cd}	74.28	66.45	70.37
T_6	6.78	5.37	6.08	14.52 ^{ab}	15.26 ^{ab}	14.89 ^{bcd}	48.91	45.85	47.38
T_7	6.78	5.18	5.98	11.39 ^{bc}	15.01 ^{ab}	13.20 ^{cd}	48.24	34.56	41.40
T_8	8.69	7.30	8.00	17.56 ^{ab}	23.76 ^{a}	20.66 ^{ab}	48.24	29.75	39.00
T_9	8.76	6.45	7.61	20.28 ^{a}	20.79 ^{ab}	20.54 ^{ab}	43.74	31.40	37.57
T_{10}	8.92	5.75	7.34	15.51 ^{ab}	20.31 ^{ab}	17.91 ^{abc}	50.12	27.51	38.82
T_{11}	9.57	8.03	8.80	20.82 ^{a}	23.92 ^{a}	22.37 ^{a}	46.12	35.05	40.59
T_{12}	9.40	7.74	8.57	20.48 ^{a}	21.25 ^{a}	20.87 ^{ab}	45.95	36.15	41.05

Treatments: T_1—0 kg S ha^{-1} + no priming; T_2—0 kg S ha^{-1} + *Bacillus subtilis*; T_3—0 kg S ha^{-1} + *Pseudomonas fluorescens*; T_4—20 kg S ha^{-1} + no priming; T_5—20 kg S ha^{-1} + *Bacillus subtilis*; T_6—20 kg S ha^{-1} + *Pseudomonas fluorescens*; T_7—30 kg S ha^{-1} + no priming; T_8—30 kg S ha^{-1} + *Bacillus subtilis*; T_9—30 kg S ha^{-1} + *Pseudomonas fluorescens*; T_{10}—40 kg S ha^{-1} + no priming; T_{11}—40 kg S ha^{-1} + *Bacillus subtilis*; T_{12}—40 kg S ha^{-1} + *Pseudomonas fluorescens*. Mean data followed by the same letters differ non-significantly ($p \leq 0.05$) within the column as per Duncan's test. ANOVA tables are provided in the Supplementary Material (Tables S4–S6). The results of Duncan's test for AE_s and PE_s have not been shown as they were statistically non-significant.

Apparent recovery efficiency (AR_S) can be well correlated with the transport of S from source to sink. The application of S fertilizer with seed biopriming significantly enhanced the AR_S (Table 4). During the first year, AR_S varied from 5.77 to 20.82%, and in the second year, it varied from 9.97 to 23.92%. Application of 40 kg S ha^{-1} + *B. subtilis* (T_{11}) increased the AR_S by 34.2 and 17.7% over the recommended dose of S without biopriming (40 kg S ha^{-1}) during the first and second year, respectively (Table 4). The increase in AR_S in response to seed inoculation was greater in the second year compared to the first year. Application of *P. fluorescens* also recorded low AR_S in comparison to *B. subtilis* at 40 kg S ha^{-1}. According to pooled data, AR_S was found in the order of T_{11} (22.37%) ≥ T_{12} (20.87%) ≥ T_8 (20.66%) ≥ T_9 (20.54%) ≥ T_{10} (17.91%) ≥ T_6 (14.98%) ≥ T_5 (13.37%) T_7 (13.20%) T_4 (7.87%).

Physiological use efficiency (PE_S) varied from 37.57 to 70.37 g of above-ground plant g^{-1} of S applied (pooled data) (Table 4). The highest PE_S of 74.28 and 66.45 g of above-ground plant g^{-1} of S applied were recorded with 20 kg S ha^{-1} + *B. subtilis* in

the first and second years, respectively. Results further revealed that the application of 30 kg S ha^{-1} + *P. fluorescence* (T_9) and 40 kg S ha^{-1} + no priming (T_{10}) registered the lowest PE_S of 43.74 and 27.51 g of above-ground plant g^{-1} of S applied in the first and second year, respectively (Table 4).

4. Discussion

In the present study, seed biopriming significantly ($p \leq 0.05$) improved the S content of root, stover, and seed. These observations were in accordance with earlier reports which showed increased S content in bioprimed plants compared to non-primed maize [48], wheat, and mustard plants [49]. The bioinoculants such as *B. subtilis* and *P. fluorescence* are reported to produce indole-3-acetic acid (IAA), siderophore, and hydrogen cyanide (HCN) and have P solubilization capacity that helps in improving plant growth [50–52]. We noticed higher S content in the root, stover, and seed with the higher dose of S fertilizer due to higher adsorption of the available form of S from the soil solution phase. Increased S content in plants indicates positive interaction of microbial agents with plants and improved S nutrition to plants through nutrient mineralization and enhanced root structures [53]. In the present study, the S content was recorded to be highest in the seed, followed by stover and root. This can be explained by the fact that the mobilization of S from root and stover to canola seed is highly necessary for oil synthesis [54]. As shown by Abdallah et al. [55], partitioning and remobilization of total S taken up in leaves, petioles, stems, and roots of oilseed rape varies with the S concentration in the soil. They observed that when plants were supplied with additional S, leaves were the sole export tissue, while the main sink tissues were stem (79%) and root (13%); in the case of S-deficient plants, 65% of S taken up is found in the roots and about 23% is found in leaves, with most of the latter distributed to young leaves. This indicates that oilseed crops prefer S, and during its growth, the uptake and mobilization of S to the tissues is more than the roots; as a result, we noticed more S accumulation in seeds. We observed higher S uptake in the bioprimed plants supplied with higher S doses. This is due to the development of pronounced root systems, higher microbial activities in the rhizosphere, and increased availability of S for assimilation by plant roots [56–58]. Plant growth-promoting rhizobacteria (PGPR) produce IAA which affects cell division, cell differentiation, and root development and suppresses pathogens. In our study, the INM technique resulted in a positive effect on nutrient uptake. Integrated nutrient management helps in mineralizing unavailable nutrient forms to plant-available nutrient forms and maintaining nutrient content in soil solution which consequently increases nutrient uptake [59].

Measurement of soil enzymatic activities, viz., urease, dehydrogenase, phosphatase, and arylsulphatase activity, is a valid indicator of the extent of microbial activity in the rhizosphere. In the present study, biopriming of seeds significantly ($p \leq 0.05$) enhanced the soil enzymatic activity. Seed priming with living inoculums helps in enabling the adherence of bacteria to seeds which improves the colonization of the rhizosphere [20]. Improved urease activity in response to bacterial inoculation of seed was reported by Kumar et al. [60] and Hridya et al. [61]. Higher urease activity in biopriming treatments in combination with S fertilizer compared to treatments without biopriming can be attributed to the crucial role of urease in N mineralization [62]. About a 27 to 29% increase in dehydrogenase activity in soil is due to the effect of integrated nutrient management [63]. Dehydrogenase enzyme activity can be well correlated with the ability of the soil microbial community to oxidize organic matter. Enhanced dehydrogenase activity in maize fields has been reported in response to nanophosphorus and phosphate-solubilizing bacteria [64]. The higher availability of substrate in integrated nutrient management for microbial nutrition might be the reason for higher dehydrogenase activity [65]. Previous studies reveal that the biopriming of mustard seed [66] with bacteria can improve soil phosphatase activity. Similarly, in our study, *B. subtilis* improved the phosphatase activity in the plant rhizosphere, and this could be attributed to the ability of PGPRs to improve microbial count in the rhizosphere and improve the physical and chemical properties of the soil [67]. Neetu et al. [68] reported

that *Glomus mosseae* + *P. fluorescens* inoculated linseed plants showed a maximum increase in phosphatase activity compared to non-inoculated plants. This enzyme (phosphatase) helps in the mineralization of bound P into a soluble form and consequently improves the P assimilation by plants. Bentonite S contains elemental S (S^o) which is oxidized into SO_4^{2-} form in the soil, and the process is majorly mediated by soil microbes [69]. Some reports suggest that the oxidation of S^o into SO_4^{2-} increases the S availability to microbes and thus enhances soil microbial activity and biomass [70]. Enhanced arylsulphatase activity was reported in soybean–wheat fields due to seed inoculation with *Pseudomonas* sp. strains [71].

Sulphur use efficiency can be well explained in its components such as AE, AR, and PE. Agronomic efficiency indicates the utilization of added fertilizer to produce potential crop yield [72]. The probable reason for higher AE_S with increasing S levels and biopriming is due to the greater availability of nutrients in the rhizosphere as mediated by soil microbes, improved root architecture, and increased crop yield. A similar increase in AE_P in sunflowers was reported by Sarwar et al. [73] in response to biopriming. The similar behavior of phosphate (PO_4^{3-}) and SO_4^{2-} in the soil can help in understanding the mechanism of increased SUE in light of PUE, as very few studies regarding the effect of biopriming on SUE are available in the literature. Apparent nutrient recovery defines the nutrient uptake by plants (seed) per unit of fertilizer added. In our study, the treatment showed a significant increase in AR_S while an insignificant increase in AE_S was observed. A similar effect on AE_P and AR_P in response to seed inoculation with microbial agents was recorded by Haokip et al. [74]. Inoculation of maize seed with S-oxidizing bacteria and varied S sources resulted in a profound increase in S uptake [75]. The ability of $PGPR_S$ (*B. subtilis* and *P. fluorescens*) to produce organic acids, growth hormones, and siderophores [59] might be the reason for increased SUE. Compared to the control, the application of *Azotobacter* and PSB improved the S uptake in mustard seed and stover [76]. A non-significant effect of integrated nutrient management on physiological use efficiency was reported by Sarkar et al. [72] in red cabbage. Yaseen and Malhi [77] reported that the application of P in a wheat field decreases the PE_P, similar to the results found in the present study. However, increased SUE and oil content in mustard due to bentonite S application at higher doses were reported in the *Terai* region [78,79].

5. Conclusions

The present investigation demonstrated that the biopriming of mustard seeds with *B. subtilis* and *P. fluorescens* is pivotal for increasing the use efficiency of S fertilizer (bentonite S) and improving soil enzymatic activity. Seed biopriming along with the application of S fertilizers significantly augmented the S content in the mustard crop (cv. Giriraj) compared to the solo application of S fertilizer. Application of 40 kg S ha^{-1} + *B. subtilis* resulted in the highest S content, S uptake, AE_S, AR_S, and soil enzymatic activity, which was on par with 40 kg S ha^{-1} + *P. fluorescens*. Our study also showed that the application of bioinoculants can reduce the generally recommended dose of S (40 kg S ha^{-1}) by 25%; that is, 30 kg S ha^{-1} + *B. subtilis* would be sufficient for growing mustard in the studied Inceptisol. Conclusively, biopriming proved to be a potential component of integrated nutrient management for improving SUE while maintaining and enhancing the microbial activity of the rhizosphere under mustard cultivation. This study also depicted the role of priming agents in the S dynamics of agroecosystems.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13040974/s1, Table S1: ANOVA Table for S content in root; Table S2: ANOVA Table for S content in grain; Table S3: ANOVA Table for S content in stover; Table S4: ANOVA table for Agronomic Use Efficiency; Table S5: Apparent Recovery Efficiency; Table S6: ANOVA Table for Physiological Use Efficiency.

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