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Early plant growth promotion of maize by various sulfur oxidizing bacteria that uses different thiosulfate oxidation pathway

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The objective of the work was to evaluate the plant growth promotion of maize by thiosulfate-oxidizing bacteria possessing the tetrathionate intermediate (S4I) and/or paracoccus sulfur oxidation (PSO) pathway for thiosulfate oxidation. *Halothiobacillus* sp. possessing the S4I and PSO pathways for thiosulfate oxidation recorded the highest phosphate solubilization ($480 \mu\text{g ml}^{-1}$), followed by other S4I pathway bearing bacteria (*Microbacterium phyllosphaerae* and *Pandoraea sputorum*). All the tested bacterial strains such as *Halothiobacillus* sp., *Dyella thiooxydans*, *M. phyllosphaerae*, *Pandoraea sputorum*, and *Pandoraea* sp., consumed the glucose and thiosulfate simultaneously, indicating that these organisms had a mixotrophic metabolism. Results of growth chamber study revealed that inoculation of *D. thiooxydans* and *M. phyllosphaerae* (both holding the S4I pathway) significantly enhanced the maize root length (73 and 67%, respectively), shoot length (27 and 31%), and shoot biomass (58 and 45%), along with the nutrient uptake of P, K, S, Mn, Ca, Cu and Na. Therefore, it was concluded that *D. thiooxydans* and *M. phyllosphaerae* possessing the S4I pathway are effective early plant growth promoting rhizobacteria for maize when compared with other bacteria possessing the S4I and PSO pathway for thiosulfate oxidation.

Key words: Maize, mixotrophic growth, paracoccus sulfur oxidation pathway, phosphate solubilization, tetrathionate intermediate pathway, sulfur nutrition.

INTRODUCTION

As sulfur (S) is an essential nutrient for plant growth, sulfur-deficient conditions causes severe losses in crop

yield. Yet, sulfur nutrition has received little attention for decades, since atmospheric inputs have provided

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Abbreviations: S, Sulfur; S4I, S4 intermediate pathway; PSO, paracoccus sulfur oxidation; MST, mineral salt thiosulfate; TCP, tri calcium phosphate.

Table 1. Details of bacterial strains with their thiosulfate oxidation pathway and nutritional type (Anandham et al., 2008b).

Classification of the strains based on carbon source utilization	Bacterial strains	Source ^c	NCBI accession No.	Presence of <i>soxB</i>	Thiosulfate oxidation pathway
Obligate chemolithoautotroph	<i>Halothiobacillus</i> sp. (ATSB2) ^a	<i>Oryza sativa</i> ssp. <i>Japonica</i>	EF397569	+	S4I and PSO
Facultative chemolithoautotroph	<i>Dyella thiooxydans</i> (ATSB10) ^b	<i>Helianthus annuus</i>	EF397574	-	S4I
Facultative chemolithoautotroph	<i>Microbacterium phyllosphaerae</i> (ATSB31) ^b	<i>Glycine max</i>	EF397588	-	S4I
Facultative chemolithoautotroph	<i>Pandoraea</i> sp. (ATSB30) ^b	<i>Glycine max</i>	EF397587	+	S4I and PSO
Facultative chemolithoautotroph	<i>Pandoraea sputorum</i> (ATSB28) ^b	<i>Capsicum annum</i>	EF397586	-	S4I

^aStrain could grow only chemolithoautotrophically with reduced sulfur compounds (obligate chemolithoautotroph). ^bBacterial strains could grow chemolithoautotrophically, chemoorganotrophically and heterotrophically (facultative chemolithoautotroph). ^cBacteria were isolated from rhizosphere soils of plants cultivated at fields, located in Kimphae, Jung ha- dong (E 128° 49' - 128° 56', N 35°10' - 35° 17'), Kyung Nam Province of Republic of Korea. All the tested organisms could use thiosulfate, tetrathionate, sulfur, sulfite, sulfide and trithionate. +, Positive; -, negative; S4I, tetrathionate intermediate pathway; PSO, paracoccus sulfur oxidation pathway.

adequate amounts. However, recent reductions in sulfur inputs from atmospheric depositions have resulted in a negative sulfur balance in arable soils, making crop plants increasingly dependent on the soil for the sulfur requirement (Kertesz and Mirleau, 2004). Thus, to alleviate this deficiency, sulfur fertilizers are invariably added to soils, usually in a reduced form, such as elemental sulfur. Yet, reduced form of S in the fertilizers must be oxidized by bacteria to sulfate (available form of S) (Wainright, 1984; Grayston and Germida, 1991; Scherer, 2001). In a previous study, the combined application of elemental sulfur and rock phosphate (RP) significantly increased the available P when inoculated with *Acidithiobacillus* sp. It oxidized elemental sulfur into sulfuric acid, which has in turn promoted RP solubilization and also improved the plant growth (Stamford et al., 2007). The inoculation of Thiobacilli with sulfur and whey has also been found to enhance the solubilization of RP (Ghani et al., 1994). In another study, the rate of phosphate solubilization was increased based on the glucose concentration and type of phosphate solubilizing bacteria used (Son et al., 2006; Anandham et al., 2007b).

Two major biochemical pathways for sulfur oxidation have been identified in sulfur oxidizers (Kelly et al., 1997). The first is the 'S4 intermediate' pathway (S4I), which includes the oxidation of tetrathionate or trithionate or polythionate and sulfur from thiosulfate, while the second is the 'paracoccus sulfur oxidation' (PSO) pathway that directly oxidizes thiosulfate into sulfate (Kelly et al., 1997). In the PSO pathway, the thiosulfate oxidation is carried out by a thiosulfate-oxidizing-multi-enzyme system (TOMES), where sulfate thiol esterase or sulfate thiol hydrolase is coded by *soxB*, which contains a prosthetic manganese cluster in the reaction center, and is essential for thiosulfate oxidation, by *Paracoccus pantotrophus*

(Friedrich et al. 2001). The existence of more than one thiosulfate oxidation system within a single bacterium has already been documented (Hensen et al., 2006; Anandham et al., 2008b).

While several studies have reported on the enhancement of sulfur availability, rock phosphate solubilization, and the plant growth promotion of sulfur-oxidizing bacteria (Grayston and Germida, 1991; Stamford et al., 2002; El-Tarabily et al., 2006; Anandham et al., 2007a, 2008a), none of these studies have correlated the thiosulfate oxidation pathway with sulfur oxidation and plant growth promotion. In a previous study, several thiosulfate-oxidizing bacteria were isolated from the rhizosphere of crop plants and documented their thiosulfate oxidation pathway (Anandham et al., 2008b). Accordingly, this study examined the solubilization of tri-calcium phosphate resulting from the oxidation of thiosulfate and early plant growth promotion of maize when inoculating thiosulfate-oxidizing bacteria possessing the S4I and/or PSO pathway for thiosulfate oxidation.

MATERIALS AND METHODS

Bacterial strains and culture conditions

The bacterial strains used in this study and their mode of thiosulfate oxidation are listed in Table 1. Unless otherwise stated, all the bacterial strains were cultivated in a mineral salts thiosulfate (MST) medium containing (g l⁻¹): NH₄Cl, 1.0; K₂HPO₄, 4.0; KH₂PO₄, 1.5; MgSO₄·7H₂O, 0.5; Na₂S₂O₃·5H₂O, 5.0; yeast extract, 0.05; and trace element solution 5 ml; pH 6.5 at 30°C for 4 days (Mukhopadhyaya et al., 2000).

Tri-calcium phosphate (TCP) solubilization

The TCP solubilization ability of the bacteria with different thio-

sulfate oxidation pathways was examined in a slightly modified Waksman and Joffe medium that contained (g l^{-1}): $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$, 5.0; $(\text{NH}_4)_2\text{SO}_4$, 2.0; MgSO_4 , 0.5; FeSO_4 , 0.01; KH_2PO_4 , 5.0; TCP, 5.0; and glucose (0, 5.0 and 10.0) (Waksman and Joffe, 1922). A quantitative assessment of the solubilization was carried out in Erlenmeyer flasks (250 ml) containing 100 ml of the liquid medium inoculated with 1 ml of the thiosulfate-oxidizing bacteria (1×10^7 cfu ml^{-1}) and incubated at 30°C under constant shaking (120 rpm). An autoclaved uninoculated medium served as the control. The soluble P content in the culture supernatant was assayed using the method of Murphy and Riley (1962). The thiosulfate consumption was assayed spectrophotometrically using a cyanolytic method (Kelly and Wood, 1994), and the glucose concentration in the growth medium was assayed according to the method reported by Nelson (1944).

Determination of growth and nutritional parameters

This experiment was conducted to determine the potential of thiosulfate-oxidizing bacteria with various biochemical pathways for thiosulfate oxidation to stimulate maize growth by increasing the plant-available sulfate and phosphate (through rock phosphate solubilization) in the rhizosphere through sulfur oxidation. The bacterial strains were grown in the MST medium, harvested by centrifugation, washed twice, and suspended in saline (0.85% NaCl). Meanwhile, the maize seeds (*Zea mays*) (Hungnong Seed Co. Ltd., Korea and Seminis Korea Inc., Korea) were surface disinfected in 70% ethanol for 1 min, immersed in 0.5% NaOCl for 2 min, and washed 4 times with sterilized distilled water. The surface-sterilized seeds were then shaken in the bacterial suspension (1×10^7 cfu ml^{-1}) for 2 h, asexically dried seeds were placed in a Petri dish with moist sterile filter paper, and incubated for 7 days at $25 \pm 1^\circ\text{C}$ under darkness. On day 7, the germinated maize seeds were transplanted into a pot containing acid-washed autoclaved sand (350 g) amended with elemental sulfur ($50 \mu\text{g g}^{-1}$ of sand) as the sulfur source. Two milliliters (1×10^7 cfu ml^{-1}) of the different bacterial strains was also placed in a 2-cm hole made in the pots before transplanting. Control treatment consists of pre-germinated maize seeds that had been previously soaked in saline to aid germination. It was transplanted into sand either unamended with sulfur or amended with Na_2SO_4 ($50 \mu\text{g SO}_4\text{-S g}^{-1}$ of sand). The control without sulfur was included to determine the growth of the maize under sulfur-deficient conditions, whereas the control with Na_2SO_4 as the sulfur source were included to determine the growth of the maize in the presence of readily available sulfate and the treatment details are given in Table 5. For each treatment, 30 pots were maintained, each with a single plant, were arranged in a completely randomized block design with three replications. The pots were placed in a growth chamber at $25 \pm 1^\circ\text{C}$ with a photoperiod of 12 h dark followed by 12 h light ($18 \mu\text{mol m}^{-2} \text{S}^{-1}$). The plants were watered regularly with sterile distilled water. Each pot received the following basal nutrients: 68 mg N [$26 \text{ mg NH}_4\text{NO}_3$, 28 mg $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, and 14 mg Mg $(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$]; 10 mg P as K_2HPO_4 or 83.3 mg RP that supplies 10 mg P; 160 mg Ca as Ca $(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$; 48 mg Mg as Mg $(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$; 1 mg Mn as $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$; 0.2 mg Zn as $\text{Zn}(\text{C}_2\text{H}_3\text{O}_2)_2$; 0.01 mg Cu as $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$; 2 mg Fe as Fe-EDTA; and 0.005 mg Mo as $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ (Histuda et al., 2005). After 30 days, the plants were harvested to determine the root and shoot length and dry weight. The nutrient contents in the plant material were also analyzed. The total nitrogen (Kjeldahl) was determined as per the procedures outlined by Page et al. (1982). After nitric and perchloric acid digestion of the plant samples, the P, K, Mg, Mn, Cu, Ca, Na, Zn, and Fe were determined using an ICP-OES (Optima 5300DV,

Perkin Elmer, USA). Finally, the total sulfur content in the plant samples was determined using a turbidometric analysis after wet oxidation with magnesium nitrate and perchloric acid (Nes, 1979).

Statistical analysis

The data were analyzed by an analysis of variance (ANOVA) using the general linear model version 9.1; SAS institute Inc, Cary, NC, USA. The means were compared using the least significant difference (LSD). The significance levels were within confidence limits of 0.05 or less.

RESULTS

TCP solubilization

The inoculation of the thiosulfate-oxidizing bacteria significantly enhanced the TCP solubilization, and the soluble P level in the Waksman and Joffe medium increased with the incubation time (Table 2). Statistical analysis revealed maximum thiosulfate consumption and TCP solubilization in the medium unamended with glucose, followed by the media containing 0.5 and 1.0% glucose (Tables 2 and 3). The supplied (20 mM) thiosulfate was almost completely consumed in the medium devoid of glucose and hence the experiment was terminated on day 18 (Table 3). The medium amended with thiosulfate and glucose and inoculated with the thiosulfate-oxidizing bacteria utilized both the thiosulfate and glucose (Tables 3 and 4). Among the tested bacterial strains, *Halothiobacillus* sp. with the S4I and PSO pathways consumed the maximum thiosulfate (19.89 mM) and showed the highest soluble P in the growth medium devoid of glucose ($480.49 \mu\text{g P ml}^{-1}$) (Tables 2 and 3). Similarly on day 18, the facultative chemolithoautotrophic *Pandoraea sputorum* (possessing the S4I pathway for thiosulfate oxidation) inoculated into the medium containing 20 mM of thiosulfate plus 0.5 or 1% glucose recorded the highest soluble P, followed by *Halothiobacillus* sp. and *Pandoraea* sp. (including the S4I + PSO pathways) (Table 2).

Early plant growth promotion in maize

Five thiosulfate-oxidizing bacteria with different thiosulfate oxidation pathways were tested for their potential to promote maize growth. *Halothiobacillus* sp. with the S4I and PSO pathways had no effect on the early maize growth. However, the growth was stimulated when sulfur was added to the sand in either reduced form (elemental sulfur) with the inoculated bacteria or oxidized form (Na_2SO_4). In this experiment, sulfur-deficiency symptoms (yellowing of young leaves) were noticed in the plants in the control treatment. Meanwhile, *D. thiooxydans* and *M. phyllosphaerae* with the S4I pathway significantly enhanced the root length (73 and 67%, respectively), shoot length (27 and 31%), and shoot biomass (58 and 45%) when compared with the uninoculated control (Table 5). Also, *Pandoraea* sp. and *Pandoraea sputorum* significantly enhanced the plant biomass (Table 5). However, the plant growth promotion effect was not significant for the treatment amended with RP as compared to the treatment supplied with readily available

Table 2. Tri-calcium phosphate solubilization by thiosulfate oxidizing bacteria.

Bacterial strains/ growth condition	Soluble P ($\mu\text{g ml}^{-1}$)		
	Incubation day		
	6	12	18
No Glucose			
<i>Halothiobacillus</i> sp.	86.88 $\pm$ 4 ^a	207.15 $\pm$ 2 ^a	480.49 $\pm$ 5 ^a
<i>Dyella thiooxydans</i>	75.41 $\pm$ 2 ^b	195.77 $\pm$ 2 ^{cd}	405.28 $\pm$ 6 ^f
<i>M. phyllosphaerae</i>	69.47 $\pm$ 6 ^c	205.53 $\pm$ 4 ^{ab}	422.56 $\pm$ 4 ^c
<i>Pandoraea</i> sp.	65.65 $\pm$ 3 ^d	203.09 $\pm$ 5 ^{ab}	429.67 $\pm$ 9 ^b
<i>Pandoraea sputorum</i>	65.22 $\pm$ 5 ^d	186.83 $\pm$ 6 ^{efg}	417.48 $\pm$ 7 ^{de}
0.5% Glucose			
<i>Halothiobacillus</i> sp.	74.14 $\pm$ 5 ^b	199.84 $\pm$ 9 ^{bc}	420.53 $\pm$ 10 ^{cd}
<i>Dyella thiooxydans</i>	66.92 $\pm$ 3 ^{cd}	185.20 $\pm$ 5 ^{gh}	405.28 $\pm$ 5 ^f
<i>M. phyllosphaerae</i>	66.50 $\pm$ 5 ^d	205.53 $\pm$ 4 ^{ab}	404.27 $\pm$ 4 ^f
<i>Pandoraea</i> sp.	65.65 $\pm$ 2 ^d	195.77 $\pm$ 5 ^{cd}	418.50 $\pm$ 6 ^{fde}
<i>Pandoraea sputorum</i>	66.07 $\pm$ 8 ^d	181.95 $\pm$ 3 ^{ghf}	430.69 $\pm$ 5 ^b
1.0% Glucose			
<i>Halothiobacillus</i> sp.	66.89 $\pm$ 3 ^{cd}	191.71 $\pm$ 6 ^{def}	406.30 $\pm$ 6 ^f
<i>Dyella thiooxydans</i>	64.37 $\pm$ 4 ^d	173.82 $\pm$ 3 ^j	379.88 $\pm$ 9 ^g
<i>M. phyllosphaerae</i>	65.65 $\pm$ 5 ^d	192.52 $\pm$ 2 ^{de}	403.25 $\pm$ 3 ^f
<i>Pandoraea</i> sp.	60.98 $\pm$ 2 ^e	174.63 $\pm$ 4 ^{ij}	416.46 $\pm$ 4 ^e
<i>Pandoraea sputorum</i>	59.70 $\pm$ 2 ^e	180.33 $\pm$ 9 ^{ih}	420.53 $\pm$ 5 ^{cd}
LSD ($P \leq 0.05$)	2.93	2.71	3.86

Tri-calcium phosphate solubilization of thiosulfate oxidizing bacteria due to production of sulfuric acid while oxidation of thiosulfate in Waksman and Joffe medium amended with or without glucose were determined. Values are the mean of three replications $\pm$ SD. Values in each column followed by same letter (s) are not significantly different.

Table 3. Thiosulfate consumption by thiosulfate oxidizing bacteria.

Bacterial strains/ growth condition	Thiosulfate consumed (mM)		
	Incubation day		
	6	12	18
No Glucose			
<i>Halothiobacillus</i> sp.	11.07 $\pm$ 1 ^{abc}	18.40 $\pm$ 0.4 ^a	19.89 $\pm$ 0.9 ^a
<i>Dyella thiooxydans</i>	9.25 $\pm$ 0.5 ^{de}	12.09 $\pm$ 1 ^{cde}	16.32 $\pm$ 0.52 ^d
<i>M. phyllosphaerae</i>	10.33 $\pm$ 0.83 ^{cde}	13.32 $\pm$ 0.82 ^c	15.67 $\pm$ 0.87 ^{de}
<i>Pandoraea</i> sp.	10.33 $\pm$ 1 ^{cde}	16.24 $\pm$ 0.49 ^b	19.02 $\pm$ 0.55 ^{abc}
<i>Pandoraea sputorum</i>	12.46 $\pm$ 0.96 ^a	17.80 $\pm$ 1.48 ^a	19.99 $\pm$ 0.09 ^a
0.5% Glucose			
<i>Halothiobacillus</i> sp.	10.72 $\pm$ 1 ^{bcd}	17.55 $\pm$ 1 ^{ab}	19.11 $\pm$ 0.27 ^{abc}
<i>Dyella thiooxydans</i>	9.05 $\pm$ 0.46 ^e	11.20 $\pm$ 0.7 ^{ef}	15.85 $\pm$ 1 ^{de}
<i>M. phyllosphaerae</i>	10.12 $\pm$ 0.62 ^{cde}	11.74 $\pm$ 0.84 ^{def}	14.93 $\pm$ 1.43 ^{ef}
<i>Pandoraea</i> sp.	10.84 $\pm$ 1 ^{abcd}	11.10 $\pm$ 0.6 ^{ef}	18.15 $\pm$ 0.25 ^c
<i>Pandoraea sputorum</i>	12.21 $\pm$ 0.46 ^{abc}	16.90 $\pm$ 1 ^{ab}	19.61 $\pm$ 0.29 ^{ab}
1.0% Glucose			
<i>Halothiobacillus</i> sp.	10.10 $\pm$ 1 ^{cde}	17.00 $\pm$ 1 ^{ab}	18.55 $\pm$ 1b ^c

Table 3. Contd.

<i>Dyella thiooxydans</i>	8.81 ± 1.01 ^e	10.01 ± 0.51 ^f	15.69 ± 0.94 ^{de}
<i>M. phyllosphaerae</i>	9.88 ± 0.5 ^{cde}	10.72 ± 1 ^{ef}	13.94 ± 1 ^f
<i>Pandoraea</i> sp.	11.32 ± 0.8 ^{abc}	10.51 ± 1.04 ^f	14.42 ± 0.92 ^f
<i>Pandoraea sputorum</i>	12.05 ± 1 ^{ab}	12.86 ± 1 ^{cd}	16.64 ± 0.74 ^f
LSD ($P \leq 0.05$)	1.64	1.50	1.24

Thiosulfate consumption of thiosulfate oxidizing bacteria in Waksman and Joffe medium amended with and without glucose. Values are the mean of three replications ± SD. Values in each column followed by same letter (s) are not significantly different.

Table 4. Glucose consumption by thiosulfate oxidizing bacteria.

Bacterial strains/ growth condition	Glucose consumed (mg 100 ml ⁻¹)	
	Incubation day	
	6	18
0.5% Glucose		
<i>Halothiobacillus</i> sp.	0.397 ± 0.07 ^e	0.444 ± 0.04 ^e
<i>Dyella thiooxydans</i>	0.426 ± 0.03 ^{de}	0.464 ± 0.02 ^d
<i>M. phyllosphaerae</i>	0.415 ± 0.01 ^e	0.485 ± 0.02 ^c
<i>Pandoraea</i> sp.	0.481 ± 0.05 ^c	0.484 ± 0.03 ^c
<i>Pandoraea sputorum</i>	0.453 ± 0.03 ^{cd}	0.495 ± 0.01 ^c
1.0% Glucose		
<i>Halothiobacillus</i> sp.	0.874 ± 0.03 ^b	0.938 ± 0.02 ^a
<i>Dyella thiooxydans</i>	0.897 ± 0.04 ^{ab}	0.904 ± 0.02 ^b
<i>M. phyllosphaerae</i>	0.888 ± 0.02 ^{ab}	0.909 ± 0.01 ^b
<i>Pandoraea</i> sp.	0.915 ± 0.02 ^a	0.931 ± 0.02 ^a
<i>Pandoraea sputorum</i>	0.901 ± 0.02 ^{ab}	0.903 ± 0.02 ^b
LSD ($P \leq 0.05$)	0.03	0.02

Glucose consumption of thiosulfate oxidizing bacteria in Waksman and Joffe medium amended with glucose 0.5 and 1% glucose. Values are the mean of three replications ± SD. Values in each column followed by same letter (s) are not significantly different.

Table 5. Effect of thiosulfate oxidizing bacterial inoculation on biomass production of maize plant on 30 days after planting.

Treatment	Root Length (cm)	Shoot Length (cm)	Root Biomass (mg plant ⁻¹)	Shoot Biomass (mg plant ⁻¹)
<i>Halothiobacillus</i> sp.+S ⁰	8.3 ± 2.2 ^{cd}	28.8 ± 2.0 ^{gf}	50 ± 2.0 ^f	192 ± 1.0 ^l
<i>D. thiooxydans</i> +S ⁰	14.6 ± 2.1 ^a	36.1 ± 1.0 ^a	183 ± 5.0 ^a	346 ± 3.0 ^a
<i>M. phyllosphaerae</i> +S ⁰	14.1 ± 2.7 ^{ab}	37.3 ± 3.0 ^a	180 ± 2.0 ^a	318 ± 2.0 ^c
<i>Pandoraea</i> sp.+S ⁰	7.5 ± 2.3 ^d	34.1 ± 1.0 ^b	120 ± 2.0 ^{bc}	279 ± 3.0 ^d
<i>P. sputorum</i> +S ⁰	11.5 ± 2.4 ^{abc}	27.8 ± 2.0 ^g	72 ± 1.0 ^{ef}	230 ± 2.0 ^h
<i>Halothiobacillus</i> sp.+S ⁰ +RP	8.6 ± 1.3 ^{cd}	28.3 ± 2.0 ^g	82 ± 2.0 ^{de}	224 ± 2.0 ^l
<i>D. thiooxydans</i> +S ⁰ +RP	11.0 ± 1.0 ^{bcd}	33.3 ± 3.0 ^{bc}	101 ± 4.0 ^{cd}	246 ± 2.0 ^f
<i>M. phyllosphaerae</i> +S ⁰ +RP	6.5 ± 1.8 ^{cd}	27.4 ± 3.0 ^g	60 ± 2.0 ^{ef}	260 ± 2.0 ^e
<i>Pandoraea</i> sp.+S ⁰ +RP	9.0 ± 1.6 ^{cd}	30.6 ± 2.0 ^{de}	58 ± 3.0 ^{ef}	237 ± 3.0 ^g
<i>P. sputorum</i> +S ⁰ +RP	6.5 ± 0.4 ^{cd}	30.1 ± 2.0 ^{ef}	76 ± 1.0 ^{def}	229 ± 2.0 ^h
S ⁰ +RP	14.6 ± 1.3 ^a	32.0 ± 1.0 ^{cd}	78 ± 2.0 ^{de}	222 ± 1.0 ^j
Na ₂ SO ₄	11.8 ± 1.7 ^{abc}	36.9 ± 4.0 ^a	142 ± 6.0 ^b	329 ± 2.0 ^b
Control	8.5 ± 0.3 ^{cd}	28.5 ± 3.0 ^g	50 ± 4.0 ^f	219 ± 1.0 ^k
LSD ($P \leq 0.05$)	3.6	1.6	25.2	2.8

RP, rock phosphate; S⁰, elemental sulfur. Values in each column are the mean of three replications of ± SD. Values in each column followed by the same letter (s) are not significantly different.

Table 6. Effect of thiosulfate oxidizing bacteria inoculation on macro and secondary nutrient uptake of maize plant on 30 days after planting.

Treatment	N (%)	P K S		
		(µg g ⁻¹ plant tissue)		
<i>Halothiobacillus</i> sp.+S ⁰	4.22 ± 0.02 ^{jk}	35.02 ± 0.15 ^c	21.28 ± 0.25 ^j	3.84 ± 0.06 ^k
<i>D. thiooxydans</i> +S ⁰	4.11 ± 0.11 ^k	42.65 ± 0.30 ^a	21.67 ± 0.55 ⁱ	12.17 ± 0.15 ^a
<i>M. phyllosphaerae</i> +S ⁰	5.27 ± 0.07 ^a	35.27 ± 0.27 ^c	26.79 ± 0.45 ^d	7.64 ± 0.05 ^e
<i>Pandora</i> sp.+S ⁰	4.71 ± 0.03 ^{de}	35.96 ± 0.90 ^b	21.27 ± 0.27 ^j	6.64 ± 0.04 ^{hg}
<i>P. sputorum</i> +S ⁰	4.33 ± .03 ^{hijk}	31.00 ± 0.50 ^e	22.21 ± 0.21 ^{hg}	9.13 ± 0 ^c
<i>Halothiobacillus</i> sp.+S ⁰ +RP	4.72 ± 0.04 ^{de}	21.13 ± 0.13 ^l	21.93 ± 0.60 ^{ih}	4.90 ± 0.26 ^j
<i>D. thiooxydans</i> +S ⁰ +RP	4.43 ± 0.04 ^{ghij}	27.79 ± 0.24 ^h	30.71 ± 0.33 ^b	9.55 ± 0.04 ^b
<i>M. phyllosphaerae</i> +S ⁰ +RP	4.68 ± 0.05 ^{def}	28.45 ± 0.45 ^g	22.47 ± 0.11 ^g	6.20 ± 0.21 ⁱ
<i>Pandora</i> sp.+S ⁰ +RP	5.12 ± 0.12 ^{ab}	22.43 ± 0.30 ^k	24.62 ± 0.09 ^f	8.68 ± 0.07 ^d
<i>P. sputorum</i> +S ⁰ +RP	4.29 ± 0.17 ^{ijk}	24.77 ± 0.26 ^j	22.01 ± 0.10 ^{ih}	7.21 ± 0.16 ^f
S ⁰ +RP	4.57 ± 0.05 ^{defg}	26.87 ± 0.50 ⁱ	31.05 ± 0.17 ^b	6.63 ± 0.11 ^g
Na ₂ SO ₄	4.46 ± 0.03 ^{fghi}	35.07 ± 0.42 ^c	32.90 ± 0.95 ^a	6.43 ± 0.07 ^{hi}
Control	4.79 ± 0.29 ^{cd}	30.15 ± 0.15 ^f	29.02 ± 0.24 ^c	0.10 ± 0.08 ^l
LSD (P≤ 0.05)	0.23	0.34	0.39	0.06 ± 0.08

RP, rock phosphate; S⁰, elemental sulfur. Values in each column are the mean of three replications of ± SD. Values in each column followed by the same letter (s) are not significantly different.

P. The analysis of the nutrient contents in plant tissues revealed that the thiosulfate-oxidizing bacterial inoculation significantly improved the nutrient uptake. The *D. thiooxydans* inoculated plants showed a significant increase in the nutrient uptake including P, K, S, Mn, Ca, Cu, and Na, followed by *M. phyllosphaerae* (Tables 6 and 7). *Pandora* sp. and *Pandora* *sputorum* inoculated plants significantly improved the plant uptake of P, S, Mn, Zn and Na as compared to control (Tables 6 and 7).

DISCUSSION

Thiosulfate-oxidizing bacteria are known to have at least two thiosulfate oxidation pathways for oxidizing thiosulfate into sulfate. Besides, the existence of a common mechanism for thiosulfate oxidation in all thiosulfate-oxidizing bacteria has also been proposed (Kelly et al., 1997; Friedrich et al., 2001). However, this study demonstrated the effect of sulfur oxidizing bacteria possessing different thiosulfate oxidation pathway on TCP solubilization and early plant growth promotion of maize. Thiosulfate was used to test the lithotrophic process since; thiosulfate is used as electron donor by most of the sulfur oxidizing bacteria including chemolithotrophs and chemoheterotrophs (Mukhopadhyaya et al., 2000). In the present study, chemolithoautotrophic *Halothiobacillus* sp. consumed highest thiosulfate as compared to other strains. Earlier studies have also reported that chemolithoautotrophic thiosulfate oxidizers consumed the maximum thiosulfate as compared to other facultative chemolithoautotrophs and chemoheterotrophs (Kelly et al., 1997; Sievert et al., 2000).

High phosphate sorption in soils is a serious limiting factor for plant productivity and phosphate fertilization efficiency. In the current study, TCP solubilization due to the oxidation of thiosulfate into sulfuric acid by thiosulfate-oxidizing bacteria was examined in a medium amended with and without glucose. The TCP solubilization only increased in the medium devoid of glucose, which can be explained by the fact that the prolonged metabolic state of the cells with the increased substrate (glucose) repressed the formation of the enzyme or enzymes responsible for thiosulfate oxidation (Pepper and Miller, 1978; Wood et al., 2004). In the present study, the process of preparing the partially acidulated rock phosphate was also found to take a relatively long time when the RP and sulfur mixture was amended with organic substrates and inoculated with chemolithotrophic thiosulfate-oxidizing bacteria. However, in previous studies, amendments with whey and glucose were found to increase the RP and TCP solubilization (Ghani et al., 1994; Son et al., 2006; Anandham et al., 2007b), yet these studies used heterotrophic thiobacilli and heterotrophic phosphate-solubilizing bacteria, which could explain the anomaly. *Halothiobacillus* sp. possessing the S4I and PSO pathways for thiosulfate oxidation recorded the highest soluble P, whereas the other S4I pathway bacteria (*M. phyllosphaerae* and *Pandora* *sputorum*) and S4I + PSO pathway bacterium (*Pandora* sp.) were statistically at par with each other. In this study, all the tested strains consumed the glucose and thiosulfate, indicating that these bacteria have a mixotrophic metabolism (Anandham et al., 2007c). Mixotrophic growth (the utilization of organic and inorganic substrates) may in fact be metabolically advantageous for these bacteria. Since low

Table 7. Effect of thiosulfate oxidizing bacteria inoculation on micro nutrient uptake of maize plant on 30 days after planting.

Treatment	$(\mu\text{g g}^{-1} \text{ plant tissue})$			$(\text{mg kg}^{-1} \text{ plant tissue})$			
	Fe	Mg	Mn	Zn	Ca	Cu	Na
<i>Halothiobacillus</i> sp.+S ⁰	1.17 ± 0.10 ⁱ	3.54 ± 0.28 ^f	0.40 ± 0.02 ^c	0.872 ± 0.002 ^b	6.94 ± 0.25 ^f	21.74 ± 0.5 ^m	0.291 ± 0.003 ^k
<i>D. thiooxydans</i> +S ⁰	2.41 ± 0.30 ^{cd}	5.45 ± 0.20 ^a	0.71 ± 0.03 ^a	0.102 ± 0.001 ^f	10.56 ± 0.56 ^a	124.38 ± 0.60 ^a	0.951 ± 0.028 ^a
<i>M. phyllosphaerae</i> +S ⁰	1.36 ± 0.33 ^{ih}	4.70 ± 0.30 ^b	0.46 ± 0.06 ^b	0.167 ± 0.003 ^d	8.38 ± 0.38 ^d	121.72 ± 0.72 ^b	0.829 ± 0.002 ^d
<i>Pandoraea</i> sp.+S ⁰	1.54 ± 0.25 ^{gh}	4.11 ± 0.11 ^{de}	0.44 ± 0.04 ^b	0.046 ± 0.001 ^g	5.79 ± 0.45 ^g	41.9 ± 0.45 ^h	0.756 ± 0.005 ^f
<i>P. sputorum</i> +S ⁰	1.44 ± 0.22 ^h	4.11 ± 0.15 ^{de}	0.29 ± 0.04 ^d	0.019 ± 0.001 ⁱ	5.78 ± 0.23 ^g	27.36 ± 0.26 ⁱ	0.471 ± 0.001 ⁱ
<i>Halothiobacillus</i> sp.+S ⁰ +RP	3.17 ± 0.14 ^b	4.21 ± 0.15 ^d	0.40 ± 0.05 ^c	0.104 ± 0.003 ^f	6.78 ± 0.50 ^f	44.07 ± 0.23 ^f	0.794 ± 0.004 ^e
<i>D. thiooxydans</i> +S ⁰ +RP	3.60 ± 0.30 ^a	4.13 ± 0.17 ^d	0.22 ± 0.02 ^e	0.953 ± 0.003 ^a	8.62 ± 0.55 ^{cd}	47.97 ± 0.28 ^d	0.894 ± 0.002 ^b
<i>M. phyllosphaerae</i> +S ⁰ +RP	2.55 ± 0.55 ^c	3.91 ± 0.24 ^e	0.12 ± 0.01 ^f	0.103 ± 0.002 ^f	9.26 ± 0.13 ^b	45.92 ± 0.46 ^e	0.170 ± 0.002 ^m
<i>Pandoraea</i> sp.+S ⁰ +RP	2.01 ± 0.12 ^e	4.15 ± 0.15 ^d	0.47 ± 0.02 ^b	0.041 ± 0.003 ^h	10.53 ± 0.27 ^a	32.11 ± 0.11 ^j	0.656 ± 0.006 ^g
<i>P. sputorum</i> +S ⁰ +RP	1.72 ± 0.26 ^{gf}	3.20 ± 0.20 ^g	0.14 ± 0.01 ^f	0.205 ± 0.005 ^c	7.69 ± 0.25 ^e	28.61 ± 0.42 ^k	0.396 ± 0.003 ^j
S ⁰ +RP	2.05 ± 0.11 ^e	4.15 ± 0.15 ^d	0.19 ± 0.02 ^e	0.011 ± 0.001 ^k	8.31 ± 0.26 ^d	34.50 ± 0.50 ⁱ	0.222 ± 0.001 ⁱ
Na ₂ SO ₄	2.43 ± 0.43 ^{cd}	4.45 ± 0.45 ^c	0.22 ± 0.02 ^e	0.126 ± 0.006 ^e	7.14 ± 0.14 ^f	43.22 ± 0.22 ^g	0.856 ± 0.003 ^c
Control	2.29 ± 0.10 ^d	5.74 ± 0.13 ^a	0.03 ± 0 ^g	0.001 ± 0 ^l	10.46 ± 0.38 ^a	47.89 ± 0.25 ^d	0.174 ± 0.002 ^m
LSD ($P \leq 0.05$)	0.22	0.20	0.03	0.003	0.36	0.66	0.34

RP, rock phosphate; S⁰, elemental sulfur. Values in each column are the mean of three replications of $\pm$ SD. Values in each column followed by the same letter (s) are not significantly different.

concentrations of sulfur compounds can limit growth, the usage of organic carbon for biomass synthesis or even the co-oxidation of sulfur compounds together with organic substrates may ensure the growth and better survival of sulfur-oxidizing bacteria in the rhizosphere (Anandham et al., 2007c). To the best of our knowledge, this is the first report on the mixotrophic growth of *D. thiooxydans*, *M. phyllosphaerae*, and *Pandoraea* spp.

The effect of thiosulfate-oxidizing bacteria on sulfur nutrition during the early growth of maize was tested since sulfur-deficiency symptoms usually occur during the early stages of crop growth and may disappear later. However, sulfur deficiency symptom could easily be noted in maize plant, hence it is considered as indicator plant for assessing the sulfur deficiency of soils (Grayston and

Germida, 1991). Inoculation experiments with microbes initially are performed under gnotobiotic condition in sand to assess their colonization potential (Egamberdieva, 2010). Hence, in the current study, plant growth promoting potential of thiosulfate oxidizing bacteria was assessed in sand under gnotobiotic condition. Previous studies reported the plant growth promotion of wheat and maize were assessed through inoculation of *Pseudomonas* sp. and nitrogen fixers, respectively in sand under gnotobiotic condition (Mehnaz and Lazarovits, 2006; Egamberdieva, 2010). In the growth chamber study, sulfur is normally used instead of thiosulfate, as it is considered an effective fertilizer or soil acidulant in a wide range of agricultural soils (Janzen and Bettany, 1987). The application of sulfur and/or inoculation of the thiosulfate-oxidizing bacteria enhanced the shoot

and root length and the plant biomass of the maize. Several earlier reports have also noted the beneficial effect of sulfur application for the crop growth of groundnut, canola and maize (Hago and Salama, 1987; Blake-Kalff et al., 1998; Histuda et al., 2005; El-Tarabily et al., 2006). Deficiency symptom was also observed with the control treatments, where all the nutrients, except for sulfur, were amended, thereby implying that the application of NPK alone is not sufficient for overall plant growth. While the sulfur oxidation potential of *Halothiobacillus* sp. has already been well documented under laboratory conditions, the inoculation of *Halothiobacillus* sp. in this study did not promote plant growth (Anandham et al., 2008a, 2008b). For the plants treated with the S4I pathway bacteria (*D. thiooxydans* and *M. phyllosphaerae*), the root length was longer and the root biomass was

greater than the treatment in which plants that received readily available sulfate. This may be due to the fact that these bacteria in addition to S oxidation also have various plant growth promoting traits that were previously demonstrated (Anandham et al., 2008a). Thus, these two bacteria may have enhanced the maize growth via two (or more) mechanisms simultaneously. The plant growth promotion effect was not significant for the treatment amended with RP as compared to the treatment supplied with readily available P. Since, sulfur is oxidized into thio-sulfate and other intermediate products (Grayston and Germida, 1991), and the conversion of these intermediate products into sulfuric acid takes quite a long time, this likely delayed the release of P_2O_5 from the rock phosphate (Janzen and Bettany, 1987; Anandham et al., 2008b). Previously, inoculation of plant growth promoting bacteria in maize significantly improved the maize growth and yield (Gholami et al., 2012).

In addition to increasing the sulfur and nitrogen uptake in maize, the inoculation of *D. thiooxydans* and *M. phyllosphaerae* also increased the uptake of Fe, Mn, Zn, Ca, Cu and Na, probably the inoculated bacteria acidified the microenvironment around the maize roots and solubilized the nutrients ultimately taken up by the plants (Grayston and Germida, 1991). Inoculation of sulfur-oxidizing bacteria increased the availability of phosphorus, iron and zinc in calcareous soils (Heydarnezhad et al., 2012). The thiosulfate-oxidizing bacteria with the S4I pathway for thiosulfate oxidation (*D. thiooxydans* and *M. phyllosphaerae*) increased the soil sulfate content and promoted the maize plant growth as compared to bacteria possessing the S4I and PSO pathways for thiosulfate oxidation. During the process of sulfur oxidation by the thiosulfate-oxidizing bacteria, thiosulfate, trithionate, tetrathionate and polythionates were probably accumulated in the sand. These reduced inorganic sulfur compounds may have then supported the chemolithoautotrophic growth of the bacteria including the S4I pathway for thiosulfate oxidation, thereby enhancing the survivability of the inoculated bacteria and ultimately the maize plant growth. Meanwhile, the lack of plant growth promotion by the inoculated *Halothiobacillus* sp., *Pandoraea* sp. and *P. sputorum* including the S4I and PSO pathways and S4I pathway, respectively, may have been due to a strain-dependant phenomenon or poor colonization potential, however, this needs to be investigated through further studies.

Conclusion

This study demonstrated that the thiosulfate-oxidizing bacteria *D. thiooxydans* and *M. phyllosphaerae* including the S4I pathway were both effective early plant growth promoting rhizobacteria for maize. The findings of the present study will be further investigated by generating transposon insertion mutants with impaired thiosulfate oxidation to study the correlation between the thiosulfate

oxidation pathway and sulfur oxidation-mediated plant growth promotion. In addition, the exact mechanism of the maize plant growth promotion by these thiosulfate-oxidizing bacteria will be studied without any sulfur amendment, and in comparison with other plant growth promoting bacteria that lack the sulfur oxidation trait.

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