REVISÃO DE LITERATURA

SULFUR IN AGRICULTURE[1]

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SUMMARY

Sulfur (S) deficiency in soils is becoming increasingly common in many areas of the world as a result of agronomic practices, high biomass exportation and reduced S emissions to the atmosphere. In this review, the incidence and commercial exploitation of S pools in nature are discussed, as well as the importance of S for plants and the organic and inorganic S forms in soil and their transformations, especially the process of microbiological oxidation of elemental sulfur (S^0) as an alternative to the replenishment of S levels in the soil. The diversity of S^0-oxidizing microorganisms in soils, in particular the genus *Thiobacillus*, and the biochemical mechanisms of S^0 oxidation in bacteria were also addressed. Finally, the main methods to measure the S^0 oxidation rate in soils and the variables that influence this process were revised.

Index terms: fertilization, agriculture, microbial diversity, soil fertility.

RESUMO: *ENXOFRE NA AGRICULTURA*

A deficiência de enxofre (S) nos solos vem se tornando cada vez mais comum em várias áreas do mundo em razão de práticas agronômicas, alta exportação de biomassa e redução das emissões atmosféricas. Nesta revisão são abordados a incidência, a exploração comercial e estoques de S na natureza, a importância do S para as plantas, as formas orgânicas e inorgânicas no solo e suas transformações, assim como, principalmente, o processo de oxidação microbiológica do enxofre elementar (S^0) como alternativa para a reposição dos níveis de S do solo. Também é abordada a diversidade de microrganismos oxidantes de S^0 nos solos, com destaque para o gênero Thiobacillus*, bem como os mecanismos bioquímicos de oxidação do S^0 em bactérias. Por fim, foram revisados os principais métodos para determinação da taxa de oxidação do S^0 nos solos e as variáveis que influenciam esse processo.*

Termos de indexação: adubação, agricultura, diversidade microbiana, fertilidade do solo.

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INTRODUCTION

The importance of sulfur (S) in agriculture has been recognized for more than a century (Bogdanov, 1899; Hart & Peterson, 1911). However, the continual use of concentrated nitrogen (N) and phosphorus (P) fertilizer formulations that do not contain S, reduced use of S-containing pesticides, greater export of S from soil in high crop yields, reduced S input through rainwater, and the reduction of emissions of S dioxide (SO_2) from fossil fuel burning to the atmosphere has led to an increase of S deficiency in soils (Wainwright, 1984). In some specific areas, such as the Brazilian cerrado, S deficiency in soils can occur naturally (McClung & Freitas, 1959; Ribeiro Jr. et al., 2001).

A sustainable agricultural management to ensure an adequate S supply for plants requires the understanding and quantification of S transformations in the soil, including the predominant microbial processes of S immobilization, mineralization, oxidation, and reduction. The use of elemental sulfur (S^0) as fertilizer is a cheap alternative to replenish S lost from the soil and allows the utilization of concentrated commercial forms of N and P. However, S^0 replenishment also has to be well-adjusted to meet the plant demand for sulfate (SO_4^{2-}) in a short period while avoiding soil and water pollution, since S^0 oxidation depends on the microbial diversity and on soil and environmental characteristics (Wainwright, 1984).

In this article the occurrence of S stocks in nature, the general S transformations in soils and the quantification methods are reviewed. In addition, the microbiological oxidation of S^0 and implications for S^0 as fertilizer are discussed.

SULFUR OCCURRENCE AND TRANSFORMATIONS

Natural occurrence and commercial exploitation of S

Sulfur, a non-metallic chemical element with the molecular formula S8, is naturally present in three forms (alpha, beta and gamma). It is yellow (alpha) or pale yellow (beta and gamma), insoluble in water, and with varying solubility in organic solvents (Albuquerque et al., 2008). The earth's crust contains between 0.06 and 0.10 % of S (Havlin et al., 2005), found in native form in volcanic deposits, bedded evaporites and salt domes. Sulfur can also be associated with minerals in the form of sulfides such as chalcopyrite, pyrrhotite, sphalerite, galena, arsenopyrite, and pyrite, and sulfates, such as anhydrite, barite and gypsum. Natural gas, oil, coal, bitumen sands and pyrobitumen shale also contain S. Sulfur can also be found as hydrogen sulfide (H_2S) in anoxic soils and as a by-product of industrial activities (Albuquerque et al., 2008; Fonseca & Bacic, 2009).

The global supplies of S are estimated to be in the range of 5 billion tons contained in natural gas, oil, metal sulfides, salt domes, and volcanic deposits, approximately 600 billion tons in coal and pyrobitumen shale, and virtually unlimited amounts in the form of sulfates such as gypsum or anhydrite (Albuquerque et al., 2008). The S reserves in Brazil are estimated at 48.5 million tons, or 1.2 % of the world reserves (Fonseca & Bacic, 2009). The S main reserves in Brazil are associated with the refining of petroleum and natural gas. Additionally, S is found in the form of pyrobitumen shale from the Irati formation in the Paraná basin, in coal in southern Brazil, especially in Santa Catarina (75 % pyrite and 25 % coal), as a by-product from the mining of metal sulfides of zinc, nickel and gold in Minas Gerais and of copper in Bahia, Goiás and Pará, and in its native form in stratiform sediments (7.1 % S) in Sririri, in the State of Sergipe (Fonseca & Bacic, 2009).

According to the Brazilian National Department of Mineral Production (Fonseca & Bacic, 2009), the world production of S in 2008 reached approximately 69 million tons. The countries with the highest production were Canada (13.5 %), the United States (13 %), China (12 %), Russia (10 %), Japan (4.5 %), Saudi Arabia (4.5 %) and Kazakhstan (4 %). The Brazilian production of S in the same year was 513 thousand tons, accounting for 0.7 % of the world production. Of the S produced in Brazil, 33 % was derived from petroleum refining and bituminous shales and 67 % from by-products of mining and metallurgy. However, the Brazilian production of S is believed to increase because of the recovery of S forms associated with petroleum refining and the natural gas basins of the newly discovered pre-salt reservoirs and because of public policies requiring the reduction of S content in fuels. Of all S consumed in the world, 55 % was used for the production of fertilizers. In Brazil, this percentage exceeds 65 %. Most S is used in the form of sulfuric acid for the solubilization of rock phosphate and production of ammonium sulfate. In addition, S is also used in pigments, chemicals, paper and steel manufacturing, pulp fibers, photography, carbon disulfide production, insecticides, fungicides, explosives, rubber vulcanization, and other applications (Albuquerque et al., 2008). In Brazil, in 2008, 2.1 million tons of S^0 were imported at a cost of 1.03 billion US dollars, aside from 508,000 tons of sulfuric acid, creating a trade deficit of US$ 1.1 billion (Fonseca & Bacic, 2009). The trend of increasing domestic production and sinking of international prices may reduce this trade deficit in the future, however.

The importance of S for plants

Sulfur is a vital element for all organisms due to its important role in methionine and cysteine biosynthesis. Cysteine is not only an important

constituent of proteins, but is also essential to determine the structural conformation of proteins and metal binding, and contributes to the catalysis of enzymatic reactions (Kertesz et al., 2007). Sulfur is also essential for the synthesis of coenzyme A, which is important for fatty acid biosynthesis and oxidation, amino acid uptake, oxidation of intermediates of the citric acid cycle, and for ferredoxin oxidation, which is vital in photosynthesis and biological N fixation. Furthermore, S is important in vitamin synthesis (Havlin et al., 2005).

Although S uptake by plant roots occurs preferentially in the form of sulfate (SO_4^{2-}), S can also be absorbed as thiosulfate ($S_2O_3^{2-}$). Leaves can additionally absorb small amounts of SO_2 (Havlin et al., 2005). Vitti et al. (2007) have also reported foliar assimilation of S^0 in soybean which, regardless of the dose and nature of the source, resulted in increased N and S levels in the leaves as compared to S^0 supplied to the soil.

The S concentration in plant tissues varies between 0.1 and 0.5 %, with decreasing concentrations in plants of the orders *Cruciferae*, *Leguminosae* and *Gramineae*, respectively. Symptoms of S deficiency in plants are characterized by reduced plant growth and occurrence of uniform chlorosis on younger leaves (Havlin et al., 2005).

Organic sulfur in soil

Inorganic S is a readily available fraction for root uptake, but represents on average less than 5 % of the total S in the soil. The majority of S (> 95 %) in soil is bound to organic molecules and is only indirectly available to plants (Kertesz & Mirleau, 2004). Traditional chemical methods allow for the fractionation of soil S in three large fractions of organic S: (a) organic S not directly bound to carbon (C), which is reduced to H_2S by hydriodic acid (HI); (b) organic S directly bound to C (C-S), which is reduced to H_2S by Raney nickel, and (c) residual C bonded S. Organic S not directly bound to C is composed primarily of sulfate esters (C-O-S), such as phenol sulfate, sulfated lipids and sulfated polysaccharides, among others. The fraction of organic S directly bound to C consists of the S-containing amino acids, thiols, disulfides, sulfones, and sulfonic acids. The third fraction of organic S is probably composed of sulfonates, sulfoxides, and heterocyclic S (Freney, 1967; Tabatabai, 1984; Kertesz et al., 2007; Eriksen, 2008).

More recent studies using sulfur K-edge X-ray absorption near edge spectroscopy (XANES) directly on soil samples or humic fractions demonstrated the separation of organic S into the following five fractions based on its oxidation state: two reduced S fractions (poly, di, and monosulfides, thiols and thiophenes), two S fractions in intermediate oxidation states (sulfoxides and sulfonates) and one fraction containing highly oxidized S (sulfate esters) (Solomon et al., 2003; Zhao et al., 2006; Kertesz et al., 2007).

Mineralization and immobilization of S in soil

According to Kertesz & Mirleau (2004), the S pool in the soil is controlled by the balance between immobilization of soluble S and mineralization of organic S. The factors controlling immobilization/mineralization include S concentration in organic matter, moisture, pH, presence of plants, cultivation time, type of management and, particularly, the microbial diversity and soil enzymatic activity (Eriksen et al., 1998; Havlin et al., 2005; Schoenau & Malhi, 2008). In general, in oxic soils, organic matter with a C:S ratio of >400 induces a net temporary immobilization of SO_4^{2-} readily available to plants, whereas organic residues with a C:S ratio of <200 promote net mineralization. Organic materials with a C:S ratio between 200 and 400 result in no net changes in SO_4^{2-} concentration in the soil solution (Dick et al., 2008). Sources of mineralizable organic S include animal and vegetable wastes; soil microbial biomass and metabolites, and humus (Schoenau & Malhi, 2008).

The S mineralization rate is extremely low at temperatures below 10 °C and above 40 °C, with an optimum temperature around 30 °C. The optimal moisture content for mineralization is approximately 60 % of field capacity, with a pH of 6-7. Rhizospheric microbial activity positively affects mineralization when compared to non rhizospheric soil. Although the S content in soils can drop dramatically after the first cultivation, equilibrium can be reached over time depending on weather conditions, management practices and local soil characteristics (Havlin et al., 2005). The C:N:S ratio of undisturbed soils is higher than that of cultivated soils. The reduction of this relationship after successive cultivation cycles suggests that S is less mineralizable than C and N (Tabatabai, 1984). However, the use of conservative management practices, e.g., no-tillage, can avoid nutrient loss, resulting in increased soil organic matter content and S mineralization rates (Schoenau & Malhi, 2008). The S in the sulfate ester fraction can reach up to 60 % of the total soil organic S, and can be mineralized by the activity of sulfatases, such as arylsulfatase, which is produced by a wide variety of heterotrophic microorganisms, especially *Pseudomonas*. Other families of sulfatases have been identified in soil bacteria, such as alkylsulfatase, serine-dependent alkylsulfatases and arylsulfotransferase (Kertesz et al., 2007). However, recent studies have shown that although S compounds directly bound to C, e.g., S-containing amino acids, are more reduced, they are mineralized more rapidly than sulfate ester compounds (Solomon et al., 2003; Kertesz et al., 2007).

Inorganic sulfur in soil

In nature, sulfur can be found in different oxidation states ranging from -2 to +6, where sulfide (HS^-) is

the most reduced and sulfate (SO_4^{2-}) is the most oxidized form (Figure 1). Elemental sulfur (S^0) is the immediate product of hydrogen sulfide (H_2S) oxidation and the most stable form of S (Suzuki, 1999).

As previously mentioned, the inorganic fraction of S represents less than 5 % of the total S in soil and is derived from the mineralization of organic S, atmospheric inputs (acid rain), pesticides, and mineral fertilizers. According to Schoenau & Malhi (2008), between 1 and 5 % of the total soil organic S can be converted to SO_4^{2-} by mineralization. In addition, anthropogenic SO_2 emissions into the atmosphere have been reduced over the past 30 years in many European countries and in the USA (Lehmann, 2008). After the adoption of air pollution control laws in 1990, the amount of sulfuric acid in acid rain in the eastern areas of the USA was reduced by 50 % (Malakoff, 2010).

The main S inputs into the soil are indirect, mainly through NPK fertilizers. The most common forms of sulfate in fertilizers are ammonium sulfate (24 % S), single superphosphate (12 % S), gypsum (14-18% S), potassium sulfate (18 % S) and potassium magnesium sulfate (22 % S). The most concentrated S source is S^0 (100 % S), either linked to bentonite (90 % S) or suspended in clay (40-60 % S). Some fertilizers can be S-enriched by S^0 coating, as in the case of S^0 coated urea (10-20 % S).

Losses of inorganic S in soils occur through the adsorption of sulfate on Fe, Al oxides and clays, leaching, erosion, crop exportation and, to a lesser extent, volatilization. Volatilization losses may be more relevant in flooded soils due to the microbial reduction of oxidized S forms to volatile H_2S (Eriksen et al., 1998).

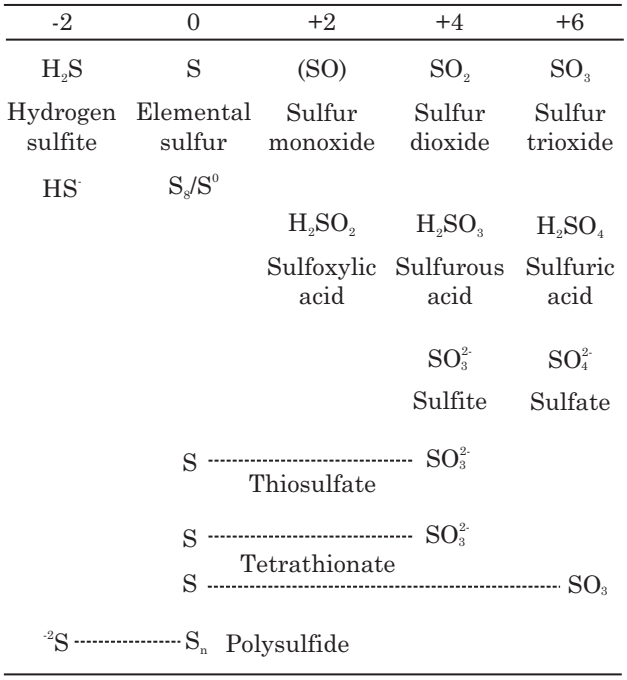

Figure 1. Oxidation state of diverse inorganic sulfur compounds. Extracted from Suzuki (1999).

MICROBIAL OXIDATION OF S^0 IN SOIL

The genus *Thiobacillus*

The first reported use of S^0 in agriculture occurred in 1877 in South Carolina, in the USA. Charles F. Panknin suggested incorporating a mixture containing 95 parts of bones or finely ground mineral phosphate with 5 parts of finely powdered elemental S into the soil to solubilize phosphorus with the sulfuric acid produced by S^0 oxidation. In the same year, he patented his discovery, recorded as part of "Letters Patent No. 193,890" (Lipman et al., 1916). According to Lipman et al. (1916), Panknin had the knowledge that S^0 in soil was oxidized to SO_4^{2-} producing sulfuric acid. However, he did not realize the process was microbiological.

As the field of microbiology expanded, several important new discoveries related to microbial S metabolism were made. For example, Winogradsky (1887) reported that *Beggiatoa* bacteria were able to use H_2S as an energy source and to fix atmospheric CO_2. Later, Beijerinck (1904) isolated the S-oxidizing bacteria *Thiobacillus denitrificans* and *Thiobacillus thioparus*. Armed with this new information, Lipman et al. (1916) tested the oxidation capacity of sterilized and unsterilized soil samples treated with S^0. Although the responsible organism was not isolated at the time, the authors concluded that the S^0 oxidation process was microbiological.

Waksman & Joffe (1922), Lipman's colleagues at the New Jersey Agricultural Experiment Station, isolated the bacteria *Thiobacillus thiooxidans* from a mixture of soil, rock phosphate and S in inorganic culture media, attributing an ability to oxidize S^0 into sulfuric acid to this isolate. Starkey (1925) published a detailed study on the physiology and main factors affecting the oxidative process of the newly discovered bacterium. In 1935, Starkey isolated *Thiobacillus novellus*, a new facultative heterotrophic bacterium able to use thiosulfate as energy source and in 1951, another important species of the genus *Thiobacillus*, *T. ferrooxidans*, was isolated from an acidic iron-rich drainage lake of a coal mine in the USA (Temple & Colmer, 1951). The new bacterium described was very similar to *T. thiooxidans* and had an ability to oxidize Fe and thiosulfate, but was unable to grow on S^0.

Although the mixture of rock phosphate and S^0 described by Panknin and Lipman was not normally used in agriculture at that time, it stimulated interest in S transformations and the biochemistry of S-oxidizing bacteria in soils (Starkey, 1966).

Whereas *T. novellus, T. thioparus* and *T. denitrificans* were normally detected in soils with pH close to 7.0, *T. thiooxidans* and *T. ferrooxidans* were rarely detected under the same conditions. Additionally, several studies failed to isolate these species from agricultural soils, even after the use of S^0 enrichment procedures (Starkey, 1966). Further studies regarding S^0 oxidation in many countries showed variable results. For example, whereas *T. thiooxidans* was detected in two thirds of the soil samples analyzed in Australia (Vitolins & Swaby, 1969) and New Zealand (Lee et al., 1987), it was not detected in soil samples from Canada (Lawrence & Germida, 1991) or Scotland (Chapman, 1990). When Vitolins & Swaby (1969) tested the *in vitro* ability to oxidize S^0 and thiosulfate of 206 strains of bacteria isolated from Australian soils, they predominately identified facultative chemolithotrophic or heterotrophic organisms rather than chemolithotrophic *Thiobacillus*.

With the increasing occurrence of soil S deficiency in large areas of Australia and New Zealand and the need for fertilization mainly of pasture lands, Swaby (1975) used the same approach as Panknin, but inoculated the mixture of rock phosphate and S^0 with *T. thiooxidans* and pelleted it, naming the product Biosuper. Several studies have shown the efficiency of this preparation, with equal or superior results in some cases to treatments with soluble phosphate fertilizers (Partridge, 1980; Rajan, 1981; Robbins et al., 1984; Besharati et al., 2007). In Brazil, biofertilizers containing rock phosphate, S^0 and *T. thiooxidans* have been applied to various crops, including cowpea, sugarcane, grapes, and melon (Stamford et al., 2003; Stamford et al., 2007; Moura et al., 2007; Stamford et al., 2008) showing similar results as with soluble S sources.

In 2000, a reclassification of the 17 species of the genus *Thiobacillus* was proposed, based on the sequence of the 16S rRNA gene and DNA-DNA hybridization (Kelly & Wood, 2000). The new classification proposed three new genera (*Acidithiobacillus*, *Halothiobacillus* and *Thermithiobacillus*) and the reclassification of other species in the existing genera. *T. thiooxidans* and *T. ferrooxidans* (*Gammaproteobacteria*) were renamed *Acidithiobacillus thiooxidans* and *Acidithiobacillus ferrooxidans*, whereas *T. novellus* (*Alphaproteobacteria*) was renamed *Starkey novella* (Robertson & Kuenen, 2006).

Diversity of S-oxidizing soil microorganisms

Reduced S forms can be oxidized in the soil by chemolithotrophic microorganisms that derive energy from the oxidation of inorganic compounds and use CO_2 as carbon source, such as *A. thiooxidans*, anoxygenic photoautotrophic (purple and green sulfur bacteria) and heterotrophic bacteria and fungi that derive carbon and energy from organic substances. Chemolithotrophic and heterotrophic organisms are prevalent in well-drained soil (Germida & Janzen, 1993).

According to Czaban & Kobus (2000), bacteria are more efficient in oxidizing S than fungi in experiments with soils treated with S^0 and antibiotics. Wainwright & Killham (1980) demonstrated the *in vitro* ability of *Fusarium solani* to oxidize S^0, similar to heterotrophic bacteria in sterile and non-sterile soil samples. However, *F. solani* was less efficient in oxidizing S^0 than *A. thiooxidans*, an obligate chemolithotrophic bacterium. Other fungi species described as being able to oxidize S^0 include *Aspergillus niger*, *Mucor flavus*, *Trichoderma harzianum* (Grayston et al., 1986), *Saccharomyces,* and *Debaryomyces* (Vitolins & Swaby, 1969). By sequencing the internal transcribed spacer (ITS) region, Li et al. (2010) identified 18 fungal isolates of the genera *Penicillium*, *Aspergillus*, *Paecilomyces*, *Fusarium*, *Bipolaris,* and *Pleosporales* with the ability to oxidize S^0 *in vitro*.

In the domain Bacteria, the obligate chemolithotrophic genera most commonly observed in soil belong to *Betaproteobacteria* (*Thiobacillus*) and *Gammaproteobacteria* (*Acidithiobacillus*). *Alphaproteobacteria*, such as *Paracoccus,* are often mixotrophic (Ghosh & Dam, 2009). Representatives of the phyla *Actinobacteria* (Anandham et al., 2008) and *Firmicutes* (Jiang et al., 2008) also have the ability to oxidize reduced forms of S in soils.

Paracoccus, which is a Gram-negative, spherical, facultative chemolithotrophic, aerobic bacterium, but capable of reducing nitrate under heterotrophic anaerobic growth and of oxidizing reduced S compounds, is one of the most well-studied genera of S oxidizers (Friedrich et al., 2001; Friedrich et al., 2005; Ghosh et al., 2006). Although mostly isolated from anaerobic digesters and activated sludge, representatives of this genus have been isolated from rhizospheric soils (Ghosh at al., 2006). One soil isolate from India able to oxidize thiosulfate in both mineral and organic culture media showed high similarity to *Paracoccus versattus* and *P. alcaliphilus*, based on the sequence of the 16S rRNA gene (Deb et al., 2004). Bacterial isolates from the rhizospheric soil of leguminous plants similar to *P. bengalensis*, *P. pantotrophu* and *P. thiocyanatus* were able to oxidize different forms of S, including thiosulfate, tetrathionate, thiocyanate, sulfide and elemental S (Ghosh et al., 2006; Ghosh & Roy, 2007b).

Recent studies have reported the isolation of novel mixotrophic genera of bacteria capable of oxidizing reduced forms of S in the soil, including genera before associated with S oxidation. El-Tarabily et al. (2006) published the first report on the isolation of *Rhizobium* spp. strains able to oxidize S^0 in calcareous soils from the United Arab Emirates. Another nitrogen-fixing bacterium isolated from the rhizosphere of a herbaceous legume, *Mesorhizobium thiogangeticum*, has also been described as capable of oxidizing S^0 and

thiosulfate (Ghosh & Roy, 2006). New *Azospirillum* and *Pseudoxanthomonas* strains were also isolated from legume rhizosphere (Ghosh & Roy, 2007a). Stubner et al. (1998) isolated strains of facultative chemolithotrophic *Ancylobacter aquaticus*, *Xanthobacter* sp, *Bosea thiooxidans* and the obligate chemolithotrophic *Thiobacillus thioparus* from the rhizosphere of rice. Other species isolated and associated with S oxidation in rice fields were phylogenetically related to *Mesorhizobium loti*, *Hydrogenophaga* sp., *Delftia* sp, *Pandoraea* sp., *Achromobacter* sp (Graff & Stubner, 2003) and *Methylobacterium oryzae* (Anandham et al., 2007). Heterotrophic, chemolithotrophic and mixotrophic growth of *Burkholderia* and *Alcaligenes* (*Alphaproteobacteria*), *Pandoraea* (*Betaproteobacteria*), *Dyella* and *Halothiobacillus* (*Gammaproteobacteria*) and *Microbacterium* and *Leifsonia* (*Actinobacteria*), on thiosulfate, was also described (Anandham et al., 2008).

Mechanisms of S oxidation in bacteria

Thiosulfate is an important reduced form of S present in different environments; it is used as a source of electrons for energy generation in photosynthetic and respiratory systems of a wide variety of bacteria. Another reduced form of S widely used by chemolithotrophic bacteria is tetrathionate, which can also be produced as an intermediate sulphur compound in the oxidation of thiosulfate (Ghosh & Dam, 2009). Over time, two oxidation processes for sulfide, thiosulfate and elemental S have been proposed, one involving the formation of tetrathionate as intermediate sulphur compound and the other involving the direct oxidation of thiosulfate to sulfate (Kelly et al., 1997).

Several obligate chemolithotrophic *Beta-* and *Gammaproteobacteria*, such as *Acidithiobacillus*, produce tetrathionate as intermediate (S_4I), whereas photo and chemolithotrophic *Alphaproteobacteria*, such as *Paracoccus*, use the mechanism known as "Paracoccus sulfur oxidation" or "PSO pathway" also known as Kelly-Friederick pathway, which is controlled by the *sox* operon (Kelly et al., 1997; Friederich et al., 2001; Friederich et al., 2005). A third mechanism of thiosulfate oxidation in anaerobic photo or chemolithotrophic bacteria that deposits sulfur intracellularly is known as the "branched thiosulfate oxidation pathway" (Ghosh & Dam, 2009).

The Sox pathway is the most studied and well understood S oxidation pathway, even though several steps are still unclear, mostly in relation to the oxidation of S^0. In this process, a multienzyme complex, encoded by the *soxTRS-VW-XYZABCDEFGH* operon drives the oxidation of sulfide, thiosulfate, sulfite and S^0 (Mukhopadhyaya et al., 2000; Bagchi & Roy, 2005; Lahiri et al., 2006; Ghosh et al., 2009). The *soxXA* genes encode a *c*-type cytochrome, *soxYZ* genes encode proteins that covalently bind sulfur and sulfur compound-chelating proteins, respectively, *soxB* encodes a monomeric, dimanganese-containing protein, and $sox(CD)_2$ genes encode a sulfur dehydrogenase (Ghosh & Dam, 2009). The Sox complex covalently binds to a molecule of thiosulfate by means of a cysteinyl residue located at the C terminus of the SoxY subunit of the SoxYZ protein and oxidizes the S atoms by transferring electrons to a *c*-type cytochrome without the formation of intermediate compounds. Other forms of S are introduced into the system in the relative position of its oxidation state through enzymatic or non-enzymatic conjugation with SoxY (Ghosh & Dam, 2009). The *sox* operon is widely distributed within the domain Bacteria; the occurrence and evolution of this enzyme complex in nature has been evaluated predominantly through amplification and phylogenetic analysis of the *soxB* gene (Ghosh et al., 2001; Meyer et al., 2007; Anandham et al., 2008).

The S_4I route, with the formation of tetrathionate as an intermediate in the oxidation of thiosulfate is not yet as well understood as the Sox pathway due to the controversial identification of several proteins of the system (Ghosh & Dam, 2009). The most recently proposed mechanism was described for the bacterium *Tetrathiobacter kashmirensis* (Dam et al., 2007). In this case, a periplasmic oxidation system performs the oxidation of thiosulfate to tetrathionate, whereas the complete oxidation of tetrathionate occurs on the membrane. Sulfite is oxidized in the cytoplasm by a sulfite dehydrogenase, which involves a ubiquinone-cytochrome *b*-mediated transfer of electrons to oxygen (Dam et al., 2007; Ghosh & Dam, 2009).

METHODS FOR DETERMINATION OF S^0 OXIDATION IN SOIL

According to Wainwright (1984), the majority of the studies focusing on S^0 oxidation involve the incubation of soil samples with S^0 under laboratory conditions for several weeks, followed by the analysis of ionic S species and/or changes in the population of S-oxidizing bacteria. The S^0 oxidation rate in the soil can be evaluated and determined directly by quantifying the S^0 remaining after the incubation period (Watkinson et al., 1987; Watkinson & Lee, 1994) or indirectly based on the quantification of sulfate formed as a final product of S^0 oxidation (Massoumi & Cornfield, 1963; Janzen & Bettany, 1987). Other indirect measurement methods include pH determination, which tends to decrease with the formation of sulfuric acid (Massoumi & Cornfield, 1963), respirometry (Baldensperger, 1976), and $^{14}CO_2$ uptake (Belly & Brock, 1974).

The remaining S^0 can be determined by high pressure liquid chromatography (HPLC) analysis of chloroform soil extracts, using a reverse phase column and an isocratic gradient of methanol and chloroform (Lauren & Watkinson, 1985; Watkinson et al., 1987).

Several variations of the HPLC method for determination of S^0 in hydrocarbons, soil, metal sulfides and water have been published (Clarck & Lesage, 1989; Rethmeier et al., 1997; McGuire & Hamers, 2000; Hurse & Abeydeera, 2002). A gas chromatography method has also been described as an alternative for S^0 quantification in soil samples (Richard et al., 1977). The amount of S^0 dissolved in acetone can also be determined using turbidimetry after replacing the solvent with water, dispersing the colloidal S^0 and determining absorbance at 420 nm (Hart, 1961). The S^0 can also be extracted from soil with chloroform, treated with HNO_3-KNO_3, and determined by inductively coupled plasma atomic emission spectroscopy (ICP-AES) (Zhao et al., 1996) or sulfur K-edge X-ray absorption spectroscopy (Burton, 2009).

Soluble SO_4^{2-} in soil is determined mainly by the turbidimetric method, where sulfate is extracted from the soil with monocalcium phosphate or ammonium acetate, and precipitated with barium chloride. The turbidity of the suspension is proportional to the amount of sulfate in the sample, and the absorbance is measured by spectrophotometry at 420 nm (Massoumi & Cornfield, 1963; Vitti, 1988; Cantarella & Prochnow, 2001).

Prochnow et al. (1997) described the use of ion exchange resin for the extraction of SO_4^{2-} from soil samples, with results similar to extraction with ammonium acetate. The amount of sulfate in soil can also be determined using ion exchange chromatography (Ohira & Toda, 2006; Yang et al., 2010).

According to Watkinson & Blair (1993), the determination of the remaining S^0 is more accurate than the determination of $S0_4^{2-}$ for the evaluation of S^0 oxidation in soils. The authors argue that during long incubation periods, the soil organic S may be mineralized and/or $S0_4^{2-}$ can be immobilized, decreasing the efficacy of the methodology. To minimize this effect, Janzen & Bettany (1987) suggested a shortened incubation period of six days prior to sulfate determination. Another drawback of determining the remaining S^0 is the use of toxic solvents such as toluene, chloroform and acetone for S^0 extraction from soil samples (Barrow, 1968). In addition, the extraction of S^0 with chloroform may be incomplete, depending on the levels of soil moisture or sample drying temperature (Barrow, 1970). Soil particle aggregates cannot be properly dispersed in chloroform, difficulting the solubilization of occluded S^0 and interfering with the accuracy of the analysis (Watkinson et al., 1987).

Variables affecting the oxidation of S^0 in soil

The main environmental factors influencing S^0 oxidation in soils are temperature, moisture, aeration, pH, and microbial diversity. Other factors, such as particle size, dispersion in soil and the fertilizer formulation also affect the S^0 oxidation rate.

In general, the rate of S^0 oxidation is minimal in temperatures below 10 °C and above 40 °C (Freney, 1967). Nor & Tabatabai (1977) observed an increase in the oxidation rate with an increase in incubation temperature of soils in Iowa. The average percent of S^0 oxidation after 57 days of incubation was 8 % at 5 °C, 22 % at 15 °C and 47 % at 30 °C.

The relationship between S^0 oxidation and soil moisture is parabolic, with minimal oxidation occurring when water availability is low, rising when moisture availability increases up to a peak and then decreasing again when the moisture content exceeds optimal levels (Janzen & Bettany, 1987). The optimum moisture for maximum S^0 oxidation is near the soil field capacity since it allows for good soil aeration (Wainwright, 1984). Solberg et al. (2005) recovered between 32 and 53 % and between 72 and 106 % of the soil SO_4^{2-} when the moisture content was 40 and 90 % of the field capacity, respectively. Limiting levels of water potential for S oxidation vary according to the soil type, depending on texture and degree of aeration (Janzen & Bettany, 1987).

The oxidation of S^0 results in H^+ generation during the process. However, the degree of acidification varies depending on the amount of applied S^0 and the soil buffering capacity (Yang et al., 2008). Several studies have shown an increase in S^0 oxidation in alkaline soils or in response to the addition of $CaCO_3$ (Freney, 1967; Adamczyk-Winiarska et al., 1975; Nor & Tabatabai, 1977; Czaban & Kobus, 2000; Yang et al., 2008). According to Adamczyk-Winiarska et al. (1975), calcium carbonate could improve the conditions for the development of S-oxidizing microorganisms in the soil. Czaban & Kobus (2000) suggested that under acidic conditions, S^0 oxidation is driven predominantly by soil fungi.

Vogler & Umbreit (1941) proved the need for a direct contact between *T. thiooxidans*, (actually *A. thiooxidans*) and S^0 particles for efficient oxidation. The oxidation rate is related to the total area of S^0 particles, which in turn increases with decreasing S^0 particle size (Freney, 1967). To obtain a greater efficiency when S^0 is used as fertilizer, the size of the applied particles must be between 80-1,000 mesh or smaller (Wainwright, 1984). The particle shape also influences the total surface area; the more spherical the shape, the smaller the mass/area ratio (Germinda & Janzen, 1993). Several mathematical models have been described for the determination of the oxidation rate depending on the size and shape of S^0 particles over time, and there is an ongoing debate over which is the best model (Blair, 1987; Janzen & Bettany, 1987; McCaskill & Blair, 1987; Blair et al., 1993; Watkinson, 1993; Watkinson & Blair, 1993).

As mentioned above, the oxidation rate is inversely proportional to the S^0 particle size; however, the application of very small particles in the field is very difficult, partly because of the fire risk due to sulfur flammability. Additionally, an inadequate dispersion of S^0 particles in the soil dramatically decreases the oxidation rate (Germida & Janzen, 1993). The same

authors comment that oxidation was limited when S^0 dispersion in the soil was low (< 50 g soil g^{-1} S^0) and maximal when S^0 was diluted to 1,000 g soil g^{-1} S^0. However, optimal conditions may vary according to the soil type. The use of S^0 in bentonite, covered urea or mixed with rock phosphate facilitates field application. In contrast, an increase in surface area decreases the oxidation rate in the soil (Boswell & Friesen, 1993; Boswell et al., 1996).

Finally, a previous S^0 application to a particular area has a positive effect on the S^0 oxidation rate observed after the second application. Solberg et al. (2005) observed that the SO_4^{2-} recovery was between 1.88 to 3.13 times greater after the second S^0 application compared to the first. However, according to Li et al. (2005), in soils that already had a high oxidation rate after the first application, a second S^0 application did not necessarily improve this rate. In contrast, in soils with low initial oxidation rates, an increase in oxidation can be expected after the second S^0 application. One possible explanation for this phenomenon is the proliferation of oxidizing microorganisms in the soil after successive S^0 applications.

FINAL CONSIDERATIONS

Worldwide, S deficiency in soil is on the rise, due to agricultural practices and declining atmospheric sulfur inputs. Since S is essential for the development and nutritional quality of crops it must be replenished to avoid yield losses. The majority of S in soil is present in organic forms not readily available for plant uptake. Although the utilization of S^0 is an economical alternative for the rapid and cheap replenishment of sulfur levels, it must first be oxidized to sulfate prior to plant uptake. Soil and environmental factors influence the efficiency of S^0 oxidation as well as the size of the sulfur particles applied and the diversity of the soil microbiota. The biotechnological application of S^0 oxidizing microorganisms is an opportunity for developing new biofertilizers and increasing of S^0 oxidation rates in soils.

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