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The role of soil microbes in plant sulphur nutrition

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Abstract

Chemical and spectroscopic studies have shown that in agricultural soils most of the soil sulphur (>95%) is present as sulphate esters or as carbon-bonded sulphur (sulphonates or amino acid sulphur), rather than inorganic sulphate. Plant sulphur nutrition depends primarily on the uptake of inorganic sulphate. However, recent research has demonstrated that the sulphate ester and sulphonate-pools of soil sulphur are also plant-bioavailable, probably due to interconversion of carbon-bonded sulphur and sulphate ester-sulphur to inorganic sulphate by soil microbes. In addition to this mineralization of bound forms of sulphur, soil microbes are also responsible for the rapid immobilization of sulphate, first to sulphate esters and subsequently to carbon-bound sulphur. The rate of sulphur cycling depends on the microbial community present, and on its metabolic activity, though it is not yet known if specific microbial species or genera control this process. The genes involved in the mobilization of sulphonate- and sulphate ester-sulphur by one common rhizosphere bacterium, *Pseudomonas putida,* have been investigated. Mutants of this species that are unable to transform sulphate esters show reduced survival in the soil, indicating that sulphate esters are important for bacterial S-nutrition in this environment. *P. putida* S-313 mutants that cannot metabolize sulphonate-sulphur do not promote the growth of tomato plants as the wild-type strain does, suggesting that the ability to mobilize bound sulphur for plant nutrition is an important role of this species.

Key words: Plant sulphur nutrition, *Pseudomonas*, rhizosphere, soil, sulphate ester, sulphonate, sulphur, sulphur cycling, XANES.

Introduction

Because of the increasing use of low sulphur fuels, and of enhanced emission controls, there has been a dramatic reduction in the atmospheric deposition of sulphur in recent years (Irwin *et al.*, 2002). This change has had an important impact in agriculture, since crop plants have become increasingly dependent on the soil to supply the sulphur that they need for the synthesis of proteins and a number of essential vitamins and cofactors. From the plant's perspective, the most important form of sulphur is inorganic sulphate, since this is the starting point for cysteine biosynthesis. However, inorganic sulphate forms only a very small part of the sulphur that is present in soils and, as a result, symptoms of sulphur deficiency are now frequently encountered in crop plants (Schnug and Haneklaus, 1998).

However, although inorganic sulphate generally makes up less than 5% of the sulphur present in agricultural soils, this does not mean that these soils contain limiting amounts of total sulphur. Most of the sulphur in soil environments (>95% of total sulphur) is bound to organic molecules, and is therefore not directly plant-available. This organic sulphur is present as a heterogeneous mixture of forms, partly included in microbial biomass and partly in the soil organic matter, and very little is known about the chemical identity of the specific sulphur-containing molecules. Traditionally, the types of sulphur species have been differentiated by their reactivity to reducing agents (Fig. 1), allowing the organosulphur pool to be divided up into three groups: (i) HI-reducible sulphur (thought to be primarily sulphate esters); (ii) Raney-nickel-reducible sulphur (mainly amino acids; Freney *et al.*, 1975); and (iii) residual carbon-bonded sulphur (thought to be largely sulphonates and heterocyclic sulphur). The identity of these groups has recently been confirmed by an independent

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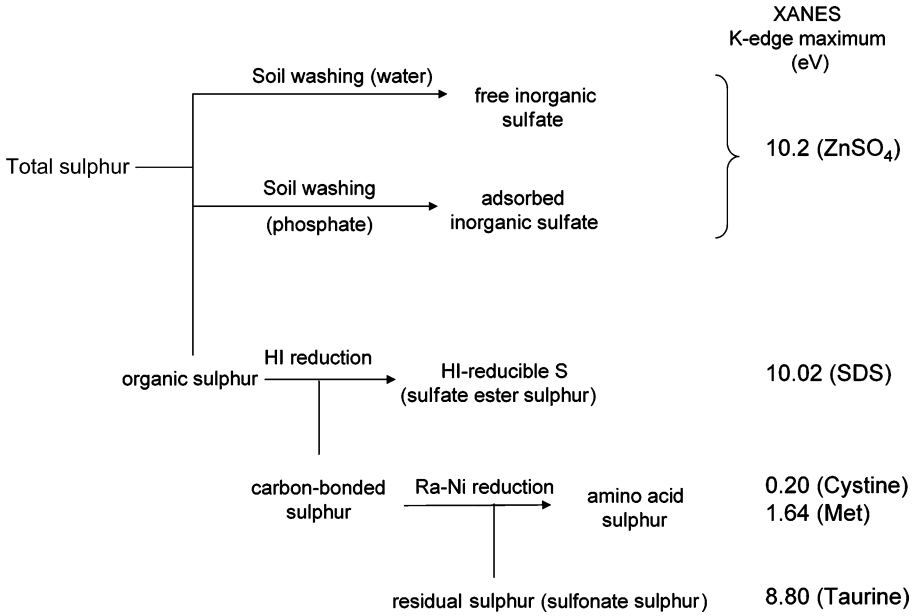

Fig. 1. Procedures for analysis of total soil sulphur (after Autry and Fitzgerald, 1990, and Freney *et al.*, 1975). 'Carbon-bonded sulphur' is calculated as the difference between total sulphur and HI-reducible sulphur. Similarly, residual sulphur is the calculated net organic sulphur after subtracting amino acid S and HI-reducible S. XANES K-edge values are given in eV relative to the value for elemental sulphur (MA Kertesz, P Mirleau, unpublished results, cf. Solomon *et al.*, 2003).

method, using X-ray near-edge spectroscopy (XANES) of soils and sediments (Jokic *et al.*, 2003; Prietzel *et al.*, 2003; Solomon *et al.*, 2003). Using XANES, the oxidation state and co-ordination environment of bound sulphur in soils can be compared with standard molecules, and spectral modelling is used to estimate the proportion of each sulphur form in the tested soil. Although the results obtained with XANES and 'wet' techniques are broadly similar, there are significant differences, especially in the sulphate ester fraction (Solomon *et al.*, 2003). The method has great potential as a non-invasive technique, allowing detailed analysis of sulphur dynamics, but the need for a synchrotron is possibly delaying its establishment as a routine technique.

Importantly, although some of the organosulphur present in soils is plant- and animal-derived (Kertesz, 1999), much is also synthesized *in situ*. The sulphur pools in soil are not static, but extremely dynamic. Inorganic sulphur forms are immobilized to organic sulphur, different organosulphur forms are interconverted, and immobilized sulphur is simultaneously mineralized to yield plant-available inorganic sulphur. These processes occur concurrently, and many of them are linked to the microbial biomass present in the soils. Especially in the rhizosphere, it is clear that microbes play a critical role as a link in allowing plants to access soil organosulphur. This review will consider the evidence for microbially mediated processes that catalyse sulphur cycling in the soil, and will summarize what is known about the organisms that catalyse these processes.

Sulphur immobilization processes

The two critical processes in sulphur cycling in soils, immobilization of inorganic sulphur and mobilization of organically bound sulphur, are both thought to be microbially mediated (Ghani *et al.*, 1992), but it is not yet known whether specific members of the microbial community play a dominant role in catalysing these processes. Immobilization has been studied in most detail using radiolabelled ^{35}S-sulphate, and measuring its incorporation into the different pools of bound sulphur. In an early study (Freney *et al.*, 1975), soils incorporated 35–44% of labelled sulphate into organosulphur within a period of 8 weeks, but this and other early reports applied the radiolabel together with carrier sulphate, modelling the changes in sulphur dynamics expected on the addition of sulphate fertilizers. However, the presence of carrier sulphate was found to retard the incorporation of ^{35}S into the organosulphur pool (Ghani *et al.*, 1993*a*), and later work (Eriksen, 1997*b*; Vong *et al.*, 2002) has used carrier-free ^{35}S-sulphate. Labelled sulphate is most rapidly incorporated into the sulphate ester pool (HI-reducible sulphur), and more slowly into the carbon-bonded S fraction (Ghani *et al.*, 1993*a*). Importantly, the rate at which added sulphate is immobilized depends critically on soil conditions, both on how the soil is preincubated before the addition of the ^{35}S-sulphate and on the carbon and nitrogen supplied to the soil at the time of label incorporation, implicating the soil microbial community as the major player in this process. Preincubation under moist conditions to encourage microbial growth prior to the

addition of labelled sulphate leads to slower inorporation of ^{35}S-sulphate into the sulphate ester pool than when the ^{35}S-sulphate is added directly to air-dried soils (Ghani *et al.*, 1993*a*), presumably because a burst of microbial growth on moistening encourages rapid sulphate immobilization. The addition of glucose as a readily utilized carbon source also encourages rapid bacterial growth, and leads to high levels of incorporation into the C-bonded S fraction (>70% of added ^{35}S in 20 d; Ghani *et al.*, 1993*a*), and a similar effect is seen with organic acids (succinate/malate) (Vong *et al.*, 2003), although in this case only total incorporation into organosulphur was measured. Similarly, the incorporation of sulphur into the organic S pool was increased dramatically by the incorporation of cellulose as an additional carbon source, with 40–50% incorporation observed in the presence of cellulose, and only 10–20% in its absence (Eriksen, 1997*b*). Microbial dry matter contains about 40% carbon and 1% sulphur, so the provision of an excess of bioavailable carbon stimulates microbial growth, but simultaneously leads to an enhanced requirement for sulphur. The addition of extra nitrogen (as ammonium nitrate) together with glucose slightly stimulated S immobilization above that of glucose alone (Vong *et al.*, 2003). The stimulation of bacterial growth in these supplemented systems may be regarded as similar to the stimulation of bacterial growth that occurs in the natural rhizosphere relative to the bulk soil (the so-called 'rhizosphere effect'), due to the release of organic acids and sugars in plant root exudates (Bertin *et al.*, 2003). Interestingly, it is evident that soil microbes actively compete with plants for the available sulphate under these carbon-enriched conditions. In soils supplemented with cellulose, for example, plant yield and plant sulphur content were both reduced (Eriksen, 1997*a*), suggesting that, over the time period studied, soil microbes were able to bind all the available sulphate into microbial biomass and thus deprive the plants of sulphur.

Mineralization of the soil organosulphur pool

Although many studies of sulphate immobilization have aimed at understanding the mobility and fate of sulphate in soils, a secondary purpose has often been to generate a labelled organic S pool, in order to evaluate the rate of mineralization, or remobilization, of the bound sulphur. It is clear from several studies that the most rapidly mineralized pool of organic S is the sulphur that has been most recently immobilized, and that immobilization and mineralization are taking place concurrently (Fig. 2). The reason for this is somewhat debated. Castellano and Dick have suggested that immobilized S makes its way initially into the sulphate ester pool, and is then slowly converted by microbial action into C-bonded S (Castellano and Dick, 1991). There are also reports of both bacteria and fungi catalysing immobilization of sulphate to choline sulphate, although this does not appear to have been studied in detail (Fitzgerald, 1976). Since sulphate ester-S is intrinsically more susceptible to hydrolysis (chemical or enzymatic) than is C-bonded S, it might therefore be expected to be more readily mineralized to sulphate. Eriksen, by contrast, has classified soil sulphur into forms that differ in the ease with which sulphur may be extracted from the soil particles (Eriksen *et al.*, 1995), and has shown that recently-formed organic S is less physically protected than aged organic S, and the latter is therefore released more slowly (Eriksen, 1997*b*). Both these reports represent field studies, whereas in a laboratory study it was reported that for recently immobilized S both sulphate ester and C-bonded S were rapidly mineralized (Ghani *et al.*, 1993*b*).

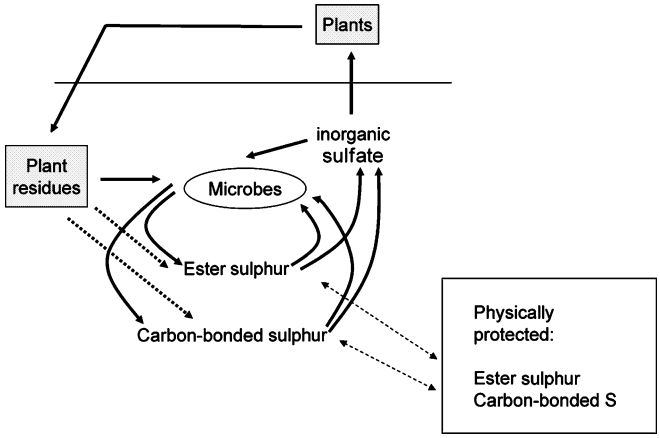

Fig. 2. Sulphur cycling within plant–soil–microbe systems, derived from the model of McGill and Cole (1981) as elaborated by Eriksen *et al.* (1998). The model has been described as oversimplified (Ghani *et al.*, 1992), mainly because the microbial biomass acts both as a catalyst of the transformations indicated and as part of the organic sulphur pool.

Probably the clearest finding regarding organosulphur transformations in soils is that the proportions of sulphate ester sulphur and C-bonded sulphur in a given soil, and the rates in which they are interconverted and mineralized, depend critically on the cropping of the soil concerned. The role of the plant in controlling sulphur transformations in the soil is thought to derive primarily from the increased microbial biomass present in the rhizosphere compared with the bulk soil (Castellano and Dick, 1991). Clear differences in sulphur transformations are observed for different plants, but until recently there has been little attempt to measure these within the rhizosphere separately from the bulk soil. Most studies have selected plant systems for investigation based on their perceived sulphur requirement, for example, a recent paper examined oilseed rape, radish, and wheat because of differences in their total S requirement and total S uptake (Hu *et al.*, 2002). However, the microbial community composition of the rhizosphere varies dramatically between different plant species (Gomes *et al.*, 2003; Kent and Triplett, 2002; Marschner *et al.*, 2001; Smalla *et al.*, 2001; van den Koornhuyse *et al.*,

2003). This effect is thought to be largely due to differences in the amount and composition of root exudates (Bertin *et al.*, 2003; Grayston *et al.*, 1998). Differences in sulphur transformations observed in soils grown with different plants may, therefore, be due to differences in the microbial community in the rhizosphere rather than purely a response to plant sulphur demand. It seems very likely that particular microbial species or genera in the rhizosphere play a greater role in sulphur cycling than others, but this functional specialization has not yet been investigated in any detail. A major stumbling block is that because most soil microbes cannot be grown in the laboratory with current techniques, cultivation-based techniques afford a very biased view of the microbial community (Rappe and Giovannoni, 2003). For a fuller understanding of the microbial role in sulphur cycling, a targeted functional diversity analysis will be required, using cultivation-independent techniques (e.g. by an adaptation of stable isotope probing techniques, Radajewski *et al.*, 2000).

Sulphate esters and microbial soil competence

Is soil organosulphur really necessary for soil microbes, or can they survive on the residual sulphate present in the soil? Initial studies have been carried out with a strain of *Pseudomonas putida*, which is a typical representative of the plant-associated microflora. The fluorescent pseudomonads have long been regarded as rhizosphere inhabitants *par excellence*, and they play an important role in many agricultural systems, since many *Pseudomonas* strains have plant growth-promoting effects, stimulating plant growth both directly (e.g. by auxin synthesis), and indirectly (e.g. by pathogen suppression) (Lugtenberg *et al.*, 2002; Persello-Cartieaux *et al.*, 2003). We chose to study rhizosphere sulphur metabolism in a *Pseudomonas putida* isolate, strain S-313R (Kahnert *et al.*, 2002). This strain is characterized by its ability to utilize an exceptionally broad range of organosulphur compounds as the sole sulphur source during *in vitro* growth, including many aromatic and aliphatic sulphonates and sulphate esters. A transposon mutant of this strain was isolated that was unable to desulphurize either arylsulphate esters or alkylsulphate esters *in vitro*, but grew normally with sulphonates and with sulphate (strain PH18). Its inability to grow with sulphate esters as a sulphur source was found to derive from a mutation in the SftR transcriptional activator protein, a member of the LysR-family that controls the expression of several sulphatases and sulphate ester transport systems, both in this strain and in other pseudomonads (Kahnert *et al.*, 2002). To assess the importance of sulphate esters for bacterial growth in soil and rhizosphere environments, survival of the wild-type strain was compared with that of the *sftR* mutant in microcosm experiments using three soils of different land-use histories from the same area (agricultural soil, forest soil, and a natural grassland soil). After incubation in washed, uncultivated soils, survival of the *sftR* mutant strain was significantly reduced relative to the wild-type strain (Kahnert *et al.*, 2002). In the *Arabidopsis* rhizosphere the effect was even more pronounced, since the wild-type strain was able to establish itself fairly stably, while the mutant strain died off significantly over 30 d. The ability to use sulphate esters is therefore critical for bacterial survival in the soil and rhizosphere (Kahnert *et al.*, 2002). This was also reflected in an experiment (MA Kertesz, P Mirleau, unpublished results), in which 100 bacterial strains were isolated at random from garden soil on non-selective medium, subjected to sulphate starvation and tested for arylsulphatase activity using X-sulphate as substrate. Sulphatase activity was observed for all colonies tested. This contrasts with similar experiments carried out with oral bacteria, where a much smaller proportion of the isolates were sulphatase-positive (Wyss, 1989), confirming the importance of sulphatases in the soil environment.

Arylsulphatase has been extensively studied as an important soil enzyme catalysing the hydrolysis of sulphate esters in the soil. The original model of McGill and Cole, which divided sulphur metabolism pathways in the soils into 'biological' pathways catalysed by micro-organisms and 'biochemical' pathways depending on free soil enzymes (McGill and Cole, 1981) relied heavily on the idea of arylsulphatase as an enzyme secreted by bacteria into the external environment as a response to sulphur limitation. Extracellular and intracellular sulphatase activities are distinguished by measuring enzyme activity before and after treatment with a plasmolytic agent (usually either toluene or chloroform fumigation; Klose and Tabatabai, 1999). It is interesting to note, however, that although many arylsulphatases from enteric bacteria are indeed extracellular, sulphatases identified in *Pseudomonas* species are almost exclusively intracellular (Kertesz, 2004), often coupled with active sulphate ester uptake systems. Nonetheless, total arylsulphatase activity in soils is correlated with microbial biomass (Klose *et al.*, 1999; Klose and Tabatabai, 1999; Vong *et al.*, 2003), and also with the rate of S immobilization (Vong *et al.*, 2003). Interestingly, the addition of exogenous arylsulphatase to soils did not appear to stimulate sulphate release from soil sulphate esters (Ganeshamurthy and Nielsen, 1990).

Can plants assimilate carbon-bound soil sulphur?

The data presented above suggest a model in which sulphate is immobilized to sulphate ester-sulphate, and subsequently converted by microbial action to carbon-bonded sulphur. The microbial survival data (Kahnert *et al.*, 2002) corroborate this by suggesting that the most important organosulphur pool for soil bacteria is the sulphate ester pool. Microbial C-bound sulphur may enter the soil

pool of C-bound sulphur after the death of the microorganism, or through protozoal predation releasing bacterial cellular contents into the soil environment. Although plants cannot access this C-bonded S directly, the bound sulphur in this pool is available to plants indirectly (Freney *et al.*, 1975; Ghani *et al.*, 1993*b*; Hu *et al.*, 2003; Shan *et al.*, 1997), via a process that is thought to be mediated by microbial action (Fig. 2). To test the importance of microbes in this process, a series of mutants of *P. putida* S-313 were generated that are unable to utilize the sulphur of aryl- and alkylsulphonates *in vitro* (Kahnert *et al.*, 2000; Vermeij *et al.*, 1999). These mutants were tested for their ability to stimulate the growth of tomato plants, after inoculation into the soil (Fig. 3). In the presence of *P. putida* S-313, tomato plant growth over 26 d (measured as shoot dry weight) was increased by 1.9-fold over that of the uninoculated control plants, indicating a significant plant-growth-promoting effect by this strain. Such plant-growth-promoting effects are not uncommon: the bacteria may stimulate plant growth by increased nutrient mobilization, protection against pathogens, or production of phytohormones (Bloemberg and Lugtenberg, 2001). The sulphatase-deficient mutant described above (strain PH18) showed the same plant-growth-promoting effect, demonstrating that although sulphate ester utilization is important for bacterial soil competence, it is not the limiting factor in the microbial stimulation of plant growth that is observed here. (Population analysis showed that the sulphatase-negative strain was still present in the rhizosphere at the end of the test period, despite showing reduced soil competence compared with the wild type.) Mutant strains that were unable to desulphurize aromatic or aliphatic sulphonates *in vitro* showed a different effect on plant growth: strain SN36, deficient in the *asfA* gene (required for arylsulphonate desulphurization; Vermeij *et al.*, 1999); strain SN34, deficient in the *ssuE* gene (required for aryl- or alkylsulphonate desulphurization; Kahnert *et al.*, 2000). Growth of tomato plants with either of these two strains in the rhizosphere did not lead to the growth stimulation that was observed with the wild-type strain. For the *asfA* mutant, plant growth was reduced to the level of the uninoculated control (Fig. 3), whereas the *ssuE* mutant led to an intermediate level of plant growth stimulation. The only known role of the *ssu* and *asf* loci is in sulphonate desulphurization and their expression is regulated by sulphur supply, so it seems unlikely that they are critical in, for example, phytohormone synthesis. This suggests that the plant-growth promoting effect of this strain is directly related to its ability to mobilize sulphonate-sulphur, but it remains to be clarified whether *P. putida* S-313 can deliver sulphonates directly to the plant, or whether the sulphonate-sulphur first enters the sulphate pool.

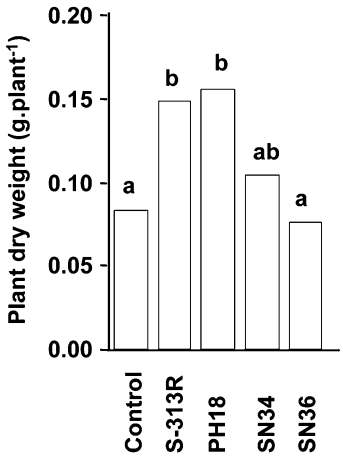

Fig. 3. Plant growth promotion by mutant derivatives of *Pseudomonas putida* S-313R (Kahnert *et al.*, 2002). Tomato plants were grown for 26 d in unsterilized compost either (a) without bacterial inoculation, (b) after inoculation with the wild-type strain S-313R, (c) after inoculation with an *sftR* mutant unable to use sulphate esters as S source *in vitro* (strain PH18), (d) after inoculation with an *ssuE* mutant unable to use aryl- or alkylsulphonates (strain SN34), or (e) after inoculation with an *asfA* mutant unable to use arylsulphonates (strain SN36). Plant dry weight was determined for five replicates. Letters show values that are statistically different from each other, determined using ANOVA (P=0.05) with Statview software.

Conclusions

Although it has been tempting to speculate that organosulphur inputs into soil environments are largely from external sources (e.g. plant sulpholipid in leaf litter), the evidence is now conclusive that there is active interconversion of organic and inorganic sulphur forms in the soil, and that this cycling is catalysed by microbial action. With the development of modern molecular techniques that do not rely on cultivation, more and more is being learnt about the composition of soil microbial communities, and how they change over time, but as yet little is known about the specific microbial species or genera that play important roles in the soil organosulphur cycle. The experiments described above are the first evidence of how one soil species may play a part in this, and the results re-emphasize the important role played by soil microbes in plant nutrition.

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