



# Elemental sulphur as an induced antifungal substance in plant defence

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## Abstract

Man's oldest fungicide has probably long functioned in this role in plants, as a natural component of induced antifungal defence. Elemental sulphur ( $S^0$ ) is the only inorganic phytoalexin and the only phytoalexin produced by so many different taxa.  $S^0$  (detected by GC-MS as  $^{32}S_8$ ) is produced in representative species of Sterculiaceae (cocoa), Solanaceae (tomato, tobacco), Malvaceae (cotton), and Leguminosae (French bean) in response to xylem-invading fungal and bacterial pathogens. Production was more rapid and intensive in disease-resistant genotypes. Gene expression for  $S^0$  production may be xylem-specific as  $S^0$  was not present in leaves of six species undergoing hypersensitivity to *Pseudomonas syringae*. Anomalously, high constitutive  $S^0$  levels occurred in leaves of *Arabidopsis* and *Brassica oleracea*.  $S^0$  was highly toxic ( $ED_{50}$  1–3  $\mu\text{g ml}^{-1}$ ) to many fungal pathogens representing ascomycetes, basidiomycetes, and deuteromycetes, but not to an oomycete, *Phytophthora*, or to bacteria. Levels in cocoa and tomato xylem and *Arabidopsis* leaves were potentially inhibitory, but in other interactions were below theoretically toxic concentrations. However,  $S^0$  accumulation is highly localized, suggesting that the element is produced in sufficient amounts, at the right time and place to be effective. SEM-EDX revealed S in tomato and cocoa xylem walls, xylem parenchyma, and vascular gels and tyloses, all sites appropriate to counter vascular pathogenic *Verticillium dahliae*. Transient increases in sulphate, glutathione and cysteine occurred in tomato xylem. The sulphate may reflect the over-expression of sulphate transporters, but the thiols might be possible precursors. Analysis of differential gene expression should reveal what may be a novel biosynthetic pathway of  $S^0$  formation in eukaryotes.

Key words: *Arabidopsis*, defence, fungicide, hypersensitivity, pathogens, phytoalexin, sulphur, thiols, tomato, xylem.

## Introduction

There are many S-containing compounds, which have been linked, directly or indirectly, with the defence of plants against microbial pathogens; these include thionins, defensins, glucosinolates, crucifer phytoalexins, alliin, and glutathione (Hell, 1997). Before the recent discovery, to be outlined in this chapter, elemental sulphur ( $S^0$ ) was not considered as a component of disease resistance. This is partly because the element had never been detected in the numerous investigations of antimicrobial compounds in diverse plants.  $S^0$  may have been overlooked in thin layer chromatography (TLC) plate bioassays, the most common basis of discovery of new compounds. Because  $S^0$  is highly hydrophobic and often runs with or close to the solvent front, antifungal bands there are often dismissed as artefacts resulting from contamination from solvents or silica gel. Another likely reason for it remaining undetected for so long, is that  $S^0$  formation, although well known in certain specialized prokaryotes (Visser *et al.*, 1997; Reinartz *et al.*, 1998), has rarely been detected in eukaryotes (Kraus *et al.*, 1984; Pezet and Pont, 1977) and only three times in plants (Cooper *et al.*, 1996; Joyard *et al.*, 1988; Kylin *et al.*, 1994). The element is best known to Man as his oldest pesticide.

$S^0$  was first recommended for disease control by Forsyth (1802). By the early 20th century it was the most important fungicide, especially for fruit diseases, until organic S-fungicides such as maneb and captan were developed in the 1950s (Tweedy, 1981). Nevertheless, in the early 1970s, the use of  $S^0$  in the USA exceeded by 2.5 times that of any other fungicide (Hassall, 1990). Now, systemic fungicides

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with diverse but site-specific modes of action predominate, yet  $S^0$  is still used, often in combination with them; this is to increase their spectrum but also to increase their life-span, because of the development of pathogen resistance to compounds with single site action (Jolivet, 1993). Field resistance to  $S^0$  has not been reported, no doubt because it has multiple sites of action, although the precise mode(s) of action is still not known (Beffa *et al.*, 1987).

### Plant defences: a key role for phytoalexins

Plants possess a myriad of defences. Constitutive resistance includes physical barriers such as the cuticle and cell walls, and antimicrobial compounds (phytoanticipins) such as saponins and phenolics (Cooper, 1981; Kuhn and Hargreaves, 1987). Active defences in response to pathogen invasion include the formation of reaction oxygen species, localized cell wall reinforcement (by lignin, suberin, structural proteins, silicon, calcium), antimicrobial or lytic proteins, and antimicrobial compounds (phytoalexins). Many of these changes are linked to a rapid, apoptotic response, resulting in the death of one or a few invaded plant cells, known as the hypersensitive response (HR) (Hammond-Kosack and Jones, 1996).

Most plant families produce organic phytoalexins of diverse chemistry; these groups are often associated with a family, for example, sesquiterpenoids of Solanaceae, isoflavanoids of Leguminosae. Typically, there is a multiple response involving several related derivatives, such as up to nine wyerone (furanoacetylenic derivatives) forms in *Vicia faba*, and several forms of phaseollin in *Phaseolus vulgaris* and *Glycine max* (Keen and Kennedy, 1974; Mansfield, 2000). The compounds are formed *de novo* in living cells and accumulate to high levels, especially in HR cells. HR cells have lost the metabolic capacity to degrade them, unlike the producing cells in which there is a high turnover, perhaps because some phytoalexins are also toxic to plant cells. Pathogens are inhibited in or adjacent to HR cells and the accumulation of phytoalexins is one key component of creating an antimicrobial environment (Mansfield, 2000).

### Discovery of elemental sulphur as a phytoalexin in five plant families

An investigation (initially using a TLC bioassay) of chemical defences in resistant lines of *Theobroma cacao* (Sterculiaceae) against the xylem-invading fungal pathogen *Verticillium dahliae*, revealed a typical multiple phytoalexin response comprising four antifungal compounds (Cooper *et al.*, 1996; Resende *et al.*, 1996). Extracted xylem contained a terpenoid (arjunolic acid) and two phenolics (acetophenones). The most hydrophobic and fungitoxic compound unexpectedly proved to be elemental sulphur. This was confirmed by TLC co-chromatography,

GC-MS ( $S_8$  breaking down successively to  $S_2$ ), and X-ray crystallography. It was initially suspected to be artefactual, as a breakdown product of a S-rich organic compound. However, there was a lack of  $^1H$  or  $^{13}C$  NMR signals that would result from an organic S compound that undergoes thermolysis on GC-MS to give free S (Cooper *et al.*, 1996).

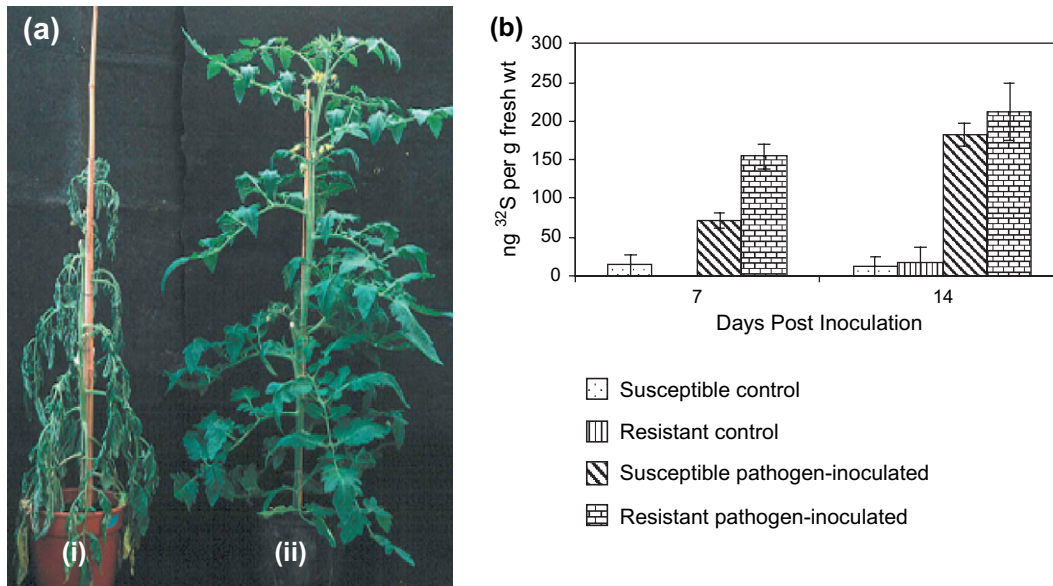
Subsequently  $S^0$  production has been reported to be linked with active defence in another three important plant families, and potentially with preformed defence in another family: Leguminosae, Malvaceae, Solanaceae, and Cruciferae (Williams *et al.*, 2002; Williams and Cooper, 2003). Accurate quantitative analysis was facilitated by the development of novel methods involving GC-MS using dilution analysis with an added  $^{34}S_8$  standard (Williams *et al.*, 2002).

$S^0$  was detected from xylem excised from plants inoculated with fungal vascular pathogens: cotton and tomato to *V. dahliae*, French bean and tobacco to *Fusarium oxysporum* formae speciales, and tomato to the bacterial vascular pathogen *Ralstonia solanacearum* (Fig. 1) (Williams and Cooper, 2003). The pattern and kinetics of  $S^0$  formation mirror that of many organic phytoalexins, with a faster and greater production in disease-resistant genotypes than in susceptible lines, and with no or negligible amounts in control tissue (Mansfield, 2000; Kuhn and Hargreaves, 1987). In view of this trend it was unexpected to find  $S^0$  in high constitutive amounts in leaves of *Arabidopsis* and in cotyledons of *Brassica oleracea*; in the latter hybrid, inoculation with an HR-inducing, incompatible race of the obligate downy mildew *Peronospora parasitica* did not increase  $S^0$  levels.

With the exception of members of the Cruciferae, the response may be specific to xylem.  $S^0$  was absent from leaves of six species showing hypersensitivity to incompatible isolates of *Pseudomonas syringae* pathovars. Other than lettuce, barley, and cabbage, these included species which had produced  $S^0$  in xylem challenged by vascular pathogens, i.e. tomato, tobacco, and French bean. Genes expressed specifically in xylem are well documented and those of possible relevance include group 1 and group 2 sulphate transporters, the former in response to *Verticillium* (Howarth *et al.*, 2003; Smith *et al.*, 1995; Takahashi *et al.*, 2000) and the I-2 gene in tomato for resistance to *F. oxysporum* (Mes *et al.*, 2000).

$S^0$  was not detected in xylem of strawberry challenged with *V. dahliae* or in maize leaves inoculated with *Erwinia stewartii*. A wider survey of  $S^0$  production is needed to confirm tissue specificity and occurrence in different taxa. However, the current methodology for accurate and unequivocal detection of  $S^0$  restricts the rate at which samples can be processed.

Because of its elemental nature, the likelihood of detection at the cellular level was possible. Few antimicrobial compounds in plants have been visualized at this level (Cooper *et al.*, 1996; Mansfield, 2000). Scanning electron



**Fig. 1.** (a) Symptoms expressed by a susceptible line (i) cv. Super Marmande and a resistant line (ii) cv. Hawaii 7996 of tomato 14 d after root inoculation with the bacterial pathogen *Ralstonia solanacearum*. (b) GC-MS analysis for  $\text{S}_8$  in xylem tissue excised from the two cultivars in (a). Plants were harvested after 7 d (initial symptoms in susceptible plants, none in resistant plants) and 14 d (severe water stress following systemic xylem colonization in the susceptible line, mild symptoms only on the lower leaves of the resistant line, as shown in (a)). Values are the mean with SE of three replicates comprising pooled samples from three plants. (From Williams and Cooper, 2003; copyrighted by *Physiological and Molecular Plant Pathology* and reprinted with permission).

microscopy combined with X-ray microanalysis (SEM-EDX) revealed that in *T. cacao* and in tomato, localized accumulations of S were present in locations relevant to inhibit a xylem-invading pathogen. i.e. in scattered xylem parenchyma cells, xylem vessel walls, and vascular occluding gels and tyloses (Fig. 2). The latter structures are probably produced as a defence response to occlude vessels and prevent systemic movement of the pathogen (Cooper, 2000). SEM-EDX also confirmed the absence of significant S levels in adjacent stem pith tissue in these two species (Cooper *et al.*, 1996; Williams and Cooper, 2003). The accumulation of S in xylem parenchyma cells could reflect accumulation in hypersensitive cells; the death of a proportion of these cells is typical for vascular diseases (Cooper, 1981). Other phytoalexins such as wyerone and phaseollin accumulate to high concentrations in necrotic areas (Mansfield, 2000).

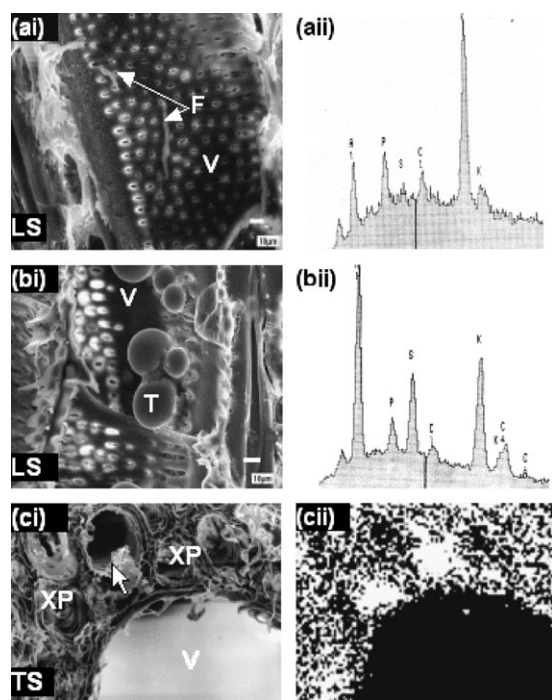
SEM-EDX showed S in *A. thaliana* leaves was present in all cell types, but the technique does not distinguish elemental from bound S and may merely reflect the many S compounds which characterize crucifers, such as glucosinolates and S-containing phytoalexins (Pedras *et al.*, 1997; Fahey *et al.*, 2001).

### Toxicity and pathogen spectrum of elemental sulphur

From previously published work, of which much was from the early 20th century, it is difficult to ascribe level and

range of toxicity of  $\text{S}^0$  to pathogens. Different formulations were used by investigators, such as sulphur flowers (sublimed  $\text{S}^0$ ), sulphur flour (ground  $\text{S}^0$ ), milk of sulphur (precipitated  $\text{S}^0$ ), colloidal  $\text{S}^0$  (wettable form of the previous types), lime sulphur (sulphide of lime containing  $\text{S}^0$ ), and liver of sulphur (potash containing  $\text{S}^0$ ) (Thatcher and Streeter, 1925; Sharvelle, 1961). Also different bioassays were used, but much data were derived from observations of disease control under field conditions (Williams and Cooper, 2004). Although minimum inhibitory concentrations and modes of action were not clear from these many studies, it is apparent that  $\text{S}^0$ -sensitive pathogens include powdery mildews (against which  $\text{S}^0$  is best known (Jolivet, 1993)), certain smut and rust fungi, anamorphic fungi with no described sexual stage such as *Verticillium* and *Fusarium*, and some fungi with known ascomycete stages such as *Stagonospora nodorum* and *Venturia inaequalis*. Therefore, the toxicity of  $\text{S}^0$  was reinvestigated using the pathogens from the host-pathogen survey and representatives of the major fungal groups, with a variety of bioassays designed to test effects in the short and long term, and to all relevant fungal stages. This was partly to relate the  $\text{S}^0$  levels detected *in planta* with a possible role in defence. Susceptibility of Gram-negative and -positive bacteria was also tested because of lack of information and because *R. solanacearum* triggered  $\text{S}^0$  formation in tomato.

In summary, all fungal species were highly sensitive to  $\text{S}^0$ . Spore germination (on glass) of most species was inhibited at *c.*  $3 \mu\text{g ml}^{-1}$  and the TLC bioassay (on silica



**Fig. 2.** (a, b) SEM-EDX detection and localization of S levels in xylem of susceptible and resistant *V. dahliae*-inoculated stems of tomato. Scanning electron images of longitudinal sections showing (ai) fungal hyphae (F) in a vessel (V) of a susceptible line (cv. GCR 26), and occluding tyloses (T) (bi) in a resistant line (GCR 218). X-ray analyses of these areas show very low levels of S in uninoculated control plants and in pith cells (not shown), low S in infected susceptible xylem (aii) and high S in resistant xylem (bii). Note the S peak in the resistant interaction is almost equivalent to (and sometimes exceeded) that for K, the predominant cytoplasmic ion; the Al peak derives from the coating evaporated onto the sample. (c). In some samples showing high S it was possible to localize the S to specific locations. A scanning electron image shows a transverse section of a xylem vessel and associated xylem parenchyma (XP) cells (ci). An X-ray dot map of the same area (cii) showing intense spots of S in XP cells. S localization was also detected in tyloses and vessel walls (not shown). (From Williams *et al.*, 2002. Copyrighted from American Society of Plant Biologists and reprinted with permission).

gel), which reflects growth of spores and hyphae, showed inhibition at *c.* 6–12  $\mu\text{g ml}^{-1}$ . Some fungi appeared highly sensitive, such as *Blumeria graminis* (cereal powdery mildew) and *Ustilago maydis* (maize smut), which were inhibited at  $<1 \mu\text{g ml}^{-1}$ . *Verticillium* and *Fusarium* species with a vascular mode of parasitism or with alternative pathogenic strategies (e.g. insect or nematode pathogens, or rot-inducing plant necrotrophs), were compared for  $\text{S}^0$  sensitivity. This was to test if xylem-invading species had acquired tolerance to  $\text{S}^0$  as a component of pathogenicity; tolerance to or detoxification of phytoalexins is often a prerequisite to pathogenicity (Morrissey and Osbourn, 1999; Van Etten *et al.*, 2001). However, there was no adaptation by vascular pathogenic forms, and this would agree with the absence of reports on pathogens developing resistance in the field to  $\text{S}^0$  following its use as a commercial fungicide.

There was no inhibition of the Oomycete *Phytophthora palmivora* even at 8  $\text{mg ml}^{-1}$ . Oomycetes resemble fungi in the form of their thalli, ecology, and the plant parasitic ability of some species and are traditionally studied by mycologists (Berbee and Taylor, 1999). However, they are not close relatives of fungi (according to flagella structure and small subunit rRNA). Nevertheless *Phytophthora* species are sensitive to certain antifungal phytoalexins, such as from soybean, sweet pepper, and potato (Williams and Cooper, 2004). The insensitivity of *P. parasitica* to  $\text{S}^0$  may explain the lack of data in the literature on the effects of  $\text{S}^0$  on this group.

Bacteria were also not inhibited by  $\text{S}^0$  concentrations up to 8  $\text{mg ml}^{-1}$ . This may reflect that  $\text{S}^0$  is produced by many specialized bacterial species: purple and green sulphur bacteria accumulate  $\text{S}^0$  globules when reduced S compounds are used as electron donors for anoxygenic photosynthesis, whereas, sulphur-oxidizing bacteria produce  $\text{S}^0$  aerobically (Hazeu *et al.*, 1988; Izac *et al.*, 1982; Prange *et al.*, 1999). The elicitation of  $\text{S}^0$  in tomato by *R. solanacearum* may therefore seem paradoxical, however, it shows that plants produce multiple defences, which are not entirely specific to the elicitation event, but part of a broad response.

#### Levels of elemental sulphur, its location and form in planta

$\text{S}^0$  appeared in *T. cacao* and tomato in a relevant tissue and cellular location, at the right time and in sufficient amounts to be implicated in a defence role, mediated by inhibition of the pathogen *V. dahliae*. Maximum amounts in *T. cacao* were  $>110 \mu\text{g g}^{-1}$  and in tomato  $>10 \mu\text{g g}^{-1}$ . In other interactions, in which sulphur was induced in resistant varieties to significant levels by fungal xylem-invading pathogens, the concentrations (max 100–250  $\text{ng g}^{-1}$ ) were below the theoretical levels for fungitoxicity (*c.* 1–12  $\mu\text{g ml}^{-1}$ ). Nevertheless, from many studies on organic phytoalexins, it is accepted that extracts from whole tissues can give substantial underestimates of phytoalexin levels present in localized areas of infection, such as HR cells (Mansfield, 2000). SEM-EDX analysis suggests that  $\text{S}^0$  can also be concentrated in this way. Levels of  $\text{S}^0$  (1.5–6  $\mu\text{g g}^{-1}$ ) in *Arabidopsis* leaves were also potentially inhibitory to fungi, but there is no evidence at this stage of cellular localization.

The inevitable anomaly exists as to how pathogens invade plants containing potentially toxic levels of constitutive antifungal compounds. The biotrophic pathogens of *Arabidopsis*, *Erysiphe orontii* (powdery mildew), and *Peronospora parasitica* (downy mildew) may not contact or release  $\text{S}^0$  because of their subtle mode of invasion of living cells, but necrotrophs are potentially vulnerable to preformed compounds because these aggressive pathogens release and encounter them following cellular

decompartmentalization and degradation (Morrissey and Osbourn, 1999). It may be coincidental, but to the authors' knowledge there are relatively few necrotrophs of *Arabidopsis*. It would be valuable to investigate those that do, such as *Botrytis cinerea* and *Alternaria brassicicola* (Thomma *et al.*, 1999), in terms of their sensitivity to  $S^0$  and cellular location with respect to sites of  $S^0$  accumulation.

As with other non-polar phytoalexins, the question must be considered, in what form does this hydrophobic element exist in the largely hydrophilic environment of the cell? This aspect has been studied in sulphur-producing bacteria in which sulphur globules are liquid and amorphous (Steudel *et al.*, 1990), whereas  $S^0$  is solid and usually crystalline at 20 °C. Proteins may act as surfactants, enveloping the sulphur droplets with their hydrophilic components interacting with the aqueous cytosol and the hydrophobic moieties directed to the globule surface, preventing crystallization (Brune, 1995; Steudel, 1989). However, sulphur globules have not been visualized in plant cells and  $S^0$  accumulations were also detected extracellularly (within xylem walls, gels, and tyloses). Other phytoalexins such as wyerone can also become extracellular, probably by secretion rather than passive diffusion (Kuhn and Hargreaves, 1987). Clearly much work remains as to the actual form *in planta* of  $S^0$  that is encountered by plant pathogens.

### Biogenesis of elemental sulphur

Sulphate, the major source of sulphur for plants, is reduced in a multistep pathway, predominantly in the chloroplasts, to sulphide. It then combines to form cysteine, of which some is converted to methionine or glutathione; the latter is the major stored and transportable form of non-protein reduced sulphur (Schmidt and Jäger, 1992; Hell, 1997; Leustek and Saito, 1999; Hawkesford and Wray, 2000). The production of elemental sulphur in eukaryotes is by an uncharacterized pathway. The origin of the  $S^0$  production in plants may be from glutathione or cysteine degradation or from oxidation of sulphide as a by-product. Therefore, these components were analysed in a model plant-pathogen system, in which  $S^0$  is produced in the resistant interaction, in order to provide possible clues for the biosynthetic route of  $S^0$ . Transient but significant increases of 2–3-fold were detected in concentrations of sulphate, glutathione, and cysteine in inoculated vascular tissues from stems of resistant but not susceptible lines of tomato after infection by *V. dahliae* (Williams *et al.*, 2002). Sulphate levels were also higher in root xylem and in leaves, and glutathione also increased in leaves. These changes in thiols and sulphate only occurred in plants supplied with a high sulphate regime (1.0 mM  $SO_4^{2-}$ ). Later comments on the frequency of sulphur deficiency in crops should be noted in the context of the capacity of stressed plants to produce defence-related compounds.

The increased sulphate levels may reflect the over-expression of sulphate transporters in response to the burden on metabolism to produce  $S^0$ . The transient peaks of cysteine and glutathione preceded or coincided with that of  $S^0$  and might implicate these S-containing compounds in the biogenesis of  $S^0$ . Leaves are the major site of S reduction, and glutathione would be involved in its transport in the xylem. The pool size of glutathione can also be elevated in response to the oxidative burst that typically accompanies defence to pathogens, because of the protective effect of reduced glutathione (Kömives *et al.*, 1998; May *et al.*, 1996). Localized glutathione accumulation and its subsequent degradation, together with chemical oxidation (Steudel *et al.*, 1986), could result in the observed depositions of  $S^0$ . An alternative route may involve sulphide oxidase postulated to result in  $S^0$  production in spinach chloroplasts (Joyard *et al.*, 1988), and oxidation by cytochromes has been suggested in the green alga *Chlorella fusca* (Kraus *et al.*, 1984). Both of these enzymes have been implicated in the production of bacterial  $S^0$  (Moriarty and Nicholas, 1970; Gray and Knaff, 1982; Pattaragulwanit *et al.*, 1998). It is possible that sulphide is a by-product of the degradation of these thiols and it is this sulphide that is oxidized to form  $S^0$  in a non-enzymic reaction (Steudel *et al.*, 1986). Cysteine degradation pathways are another possibility, for example, by a putative cysteine desulphydrase (Schmidt, 1987). Recently, a cysteine desulphurylase, targeted to plastids has been cloned from *Arabidopsis*, with the capacity to form L-alanine and elemental sulphur from cysteine as the substrate (Léon *et al.*, 2002). The cellular locations and regulation of different forms of this enzyme in plants which form  $S^0$  in response to pathogen attack would be of interest to evaluate the possible role of this gene family in  $S^0$  formation, as would the effect of gene disruption.

Novel components of a pathway leading to  $S^0$  accumulation, up-regulated following pathogen infection, may be identified by employing gene screening procedures relying on differential expression, such as differential display. These approaches are currently being applied to the tomato-*Verticillium* interaction. Biogenesis of constitutive  $S^0$  in crucifers may be by a different route, such as from the degradation of glucosinolates (Bones and Rossiter, 1996; Foo *et al.*, 2000). It is intended to deploy a mutant screen approach (to identify by chemical analysis  $S^0$ -deficient lines) as the first step to investigate  $S^0$  formation in *Arabidopsis*.

### Implications and applications

There is an irony that the discovery of this highly fungitoxic element in diverse plant species, coincides with sulphur deficiency as a major current nutrient disorder in Europe and other parts of the world (Schnug and Haneklaus, 1998). The application of sulphur in various forms to crops is now

advocated in order to compensate for this deficiency (Riley *et al.*, 2000). Treatment with elemental sulphur, such as in the micronized form (Jolivet, 1993) may provide the added bonuses of fungicidal action and enhancement of innate host defences based on S-containing compounds and, in some species, S<sup>0</sup> itself. S nutrition has been claimed to influence the resistance of *Brassica napus* and grapevine to fungal pathogens (Burandt *et al.*, 2001).

Genes for S<sup>0</sup> biosynthesis are not only likely to be novel, but may be transferable to S<sup>0</sup> non-producing species or, within a species able to accumulate S<sup>0</sup>, transferred to tissues other than xylem. S<sup>0</sup> formation should be a valuable addition to the defence armoury of plants, because it is toxic to most fungal pathogens and a trait that pathogens are unlikely to overcome.

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