Sulfite Oxidase Activity Level Determines the Sulfite Toxicity Effect in Leaves and Fruits of Tomato Plants

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Abstract: Increasing plant tolerance to sulfites/\(\text{SO}_2\) can lead to the development of tolerant crops to biotic and abiotic stresses. Plant sulfite oxidase (SO) is a molybdo-enzyme that oxidizes excess \(\text{SO}_2\)/sulfite into non-toxic sulfate. The effect of toxic sulfite on leaves and fruits was studied in tomato plants with different SO expression: wild-type, SO overexpression (OE) and SO RNA interference (Ri). Sulfite-dipped ripe-fruits and sulfite treated leaf discs of Ri plants impaired in SO activity were more susceptible, whereas OE plants were more resistant, as revealed by remaining chlorophyll and tissue damage levels. Application of molybdenum further enhanced the tolerance of leaf discs to sulfite by enhancing SO activity in OE lines, but not in wild-type or Ri plants. Notably, incubation with tungsten, the molybdenum antagonist, overturned the effect of molybdenum spray in OE plants, revealed by remaining chlorophyll content and SO activity. The results indicate that SO in tomato leaves and ripe fruits determines the resistance to sulfite and the application of molybdenum enhances sulfite resistance in OE plants by increasing SO activity. Overall, the results suggest that SO overexpression can be employed, with or without molybdenum application, for developing fruit and vegetable crops tolerant to sulfite/\(\text{SO}_2\) containing pre- and postharvest treatments.

Keywords: sulfite oxidase; molybdenum; tungsten; sulfite toxicity; tomato; SO expression

1. Introduction

Sulfur is the least abundant macronutrient in plants, comprising approximately 0.1% of the dry matter. As a structural component, like nitrogen and carbon [1], sulfur is found in amino acids (cysteine and methionine), oligopeptides (such as glutathione and phytochelatins), vitamins and cofactors (such as biotin, thiamine, CoA and S-adenosyl methionine), and in a variety of secondary products such as glucosinolates [2].

Sulfate (\(\text{SO}_4^{2-}\)) is the primary source of sulfur in plants [3,4]. Sulfate is transported from soil into roots where it can remain or be distributed [5]. The reduction of sulfate by the sulfate reduction pathway into cysteine is initiated by its adenylation catalyzed by ATP sulfurylase (ATPS) in both chloroplast and cytosol. The resulting adenosine 5-phosphosulfate (APS) forms a branching point in the pathway as acted by different enzymes [4,6]. In the primary sulfate assimilation, APS is first reduced by APS reductase (APR) to sulfite, which is further reduced to sulfide by the chloroplast-localized, ferredoxin-dependent sulfite reductase (SiR) [7–9]. The \(\text{O-Acetyl-L-serine (OAS)}\) synthesized from serine and acetyl-Coenzyme A catalyzed by serine acetyltransferase (SAT) together with sulfide is catalyzed into cysteine by OAS (thiol) lyase (OASTL) [9].

Sulfite generated by APR or obtained from atmospheric \(\text{SO}_2\) is a highly toxic intermediate. The bulk of sulfite is normally channeled in the assimilatory reduction pathway to generate sulfide catalyzed by SiR, yet
excess sulfite is oxidized effectively back to sulfate by the peroxisome localized sulfite oxidase (SO), acting as the safety valve in the sulfate reduction pathway [10]. Unlike SiR, which depends on energy devoted to ferredoxin reduction to act as the donor of six electrons for sulfite reduction [8,9], the peroxisome localized SO is much less energy dependent. In the reaction catalyzed by SO, two electrons are transferred from sulfite to the molybdenum cofactor (Moco) redox center, which are subsequently transferred to molecular oxygen with simultaneous production of hydrogen peroxide (H$_2$O$_2$) and sulfate [11]. The generated H$_2$O$_2$ can further oxidize another sulfite molecule to sulfate nonenzymatically or by the peroxisomal catalase upon low cellular sulfite concentration to H$_2$O and O$_2$ [12]. The sulfate generated by sulfite oxidation either is accumulated in vacuoles to be employed later upon sulfur shortage, or immediately enters the sulfate reduction pathway as described above.

SO$_2$ is a gaseous pollutant. It is a major atmospheric contaminant resulting from the combustion of sulfur-rich fossil fuels and from natural sources such as microbial activities, forest fire and volcanic eruptions. SO$_2$ enters plants via their stomata and readily hydrates to form the sulfite ions, HSO$_3^-$ and SO$_3^{2-}$ [13]. At sub-toxic levels, plants are able to utilize SO$_2$. Indeed, sulfur assimilation and biomass production are reported to be positively correlated with SO$_2$ in the air [14]. Yet, above a certain threshold, sulfite toxicity leads to visible symptoms of chlorosis and necrosis causing reduction in plant growth, which leads to severe loss in yield [15]. Sulfite anions (HSO$_3^-$ and SO$_3^{2-}$) are nucleophilic agents that are able to attack DNA, proteins and lipids and thus affect plant growth and vitality [16]. Sulfites can interfere with the thiol/disulphide functional groups and disrupt the regulation of key metabolic processes, including photosynthesis and respiration [17,18], by inactivation of proteins like thioredoxins [18]. Sulfite can degrade lipids by oxidation, which leads to lipid peroxidation, resulting in cell membrane dysfunction and damage [16]. The susceptibility to SO$_2$ differs between the plant species in combination with the duration and concentration of SO$_2$ in the atmosphere [12,19].

Studies related to exogenous applied sulfite oxidation by plant SO are mainly based on Arabidopsis plants [10–12,20,21] and rarely, tomato plants [10]. Moreover, no studies have investigated the effect of SO expression level on ripe tomato fruits dipped in toxic sulfite. Additionally, the effect of molybdenum (Mo) spray on leaves of tomato plants with various SO expression has rarely been examined as a means to increase leaf tolerance to toxic sulfites. A study to identify ways to increase the tolerance of tomato plants to toxic sulfite/SO$_2$ levels may lead to the development of crops more tolerant to higher concentrations of sulfite/SO$_2$, thus facilitating pre- and postharvest treatments such as dipping ripe tomato fruit in sulfite solution to avoid insects/pathogen damage.

By the use of tomato wild type, two SO overexpression (OE) and two SO RNA interference (Ri) independent lines it was shown in this study that SO level determines the resistance to toxic sulfites in tomato leaves and ripe fruits and the application of Mo further enhances the sulfite resistance in OE tomato plants by increasing SO activity.

2. Results

2.1. Chlorophyll Content and SO Activity

The effect of SO expression levels on the capacity to detoxify toxic levels of sulfite was investigated in wild type and SO modified tomato lines. Leaf discs were treated with 7 mM sodium sulfite for 24 h as was done previously with Arabidopsis and tomato plants [10]. Chlorophyll content, a sensitive indicator of leaf health, was monitored on the leaf discs after treatment. SO enzyme activity was determined in the crude protein extracted from leaves of wild type and SO modified plants.

Leaf discs from the first fully developed leaf of wild type (RR), SO OE (OE 13-6/6 and OE 12-5/7) and SO Ri (Ri 421 and Ri 131) were taken for the experiment. No damage to the leaf discs was observed in the control treatment (Figure 1A), while symptoms of chlorosis and damage from the periphery were observed in the leaf discs incubated with 7 mM Na$_2$SO$_3$ (Figure 1B). The least damage occurred in the leaf discs of both the OE lines, followed by the wild type and Ri lines. In the wild type, the observed damage was intermediate, while Ri lines were strongly affected by the sulfite treatment.
The results show that the tomato lines with higher SO expression levels were less affected by the sulfite treatment as compared to the lines with lower SO expression levels (Figure 1B).

Additionally, remaining chlorophyll content was measured in the leaf discs 24 h after sulfite treatment. A higher amount of remaining chlorophyll was found in OE lines, whereas it was lower in Ri lines as compared to the wild type (Figure 2). Remaining chlorophyll content in the RR line was 61% of the control. In SO OE lines, remaining chlorophyll content in OE 13-6/6 and OE 12-5/7 was 22% and 33%, respectively, which was higher than the wild type. While in SO Ri lines, the remaining chlorophyll content was reduced to 52% and 56% for line Ri 421 and Ri 131, respectively, compared to the wild type (Figure 2). The results show that the remaining chlorophyll content in the leaf discs after sulfite treatment was higher for the tomato lines with higher SO expression levels and lower for those with lower or no SO expression.

**Figure 1.** Effect of sulfite application on wild type and SO modified tomato lines. Leaf discs of wild type (RR), SO OE lines (OE 13-6/6, OE 12-5/7) and SO Ri lines (Ri 421, Ri 131) were subjected to (A) 0 mM and (B) 7 mM Na$_2$SO$_3$ and photographed 24 h after starting the treatment.

**Figure 2.** Remaining chlorophyll in wild type and SO modified tomato plants after sulfite treatment. Leaf discs from wild type (RR) and SO OE (OE 13-6/6 and OE 12-5/7) and SO Ri (Ri 421 and Ri 131) tomato lines were subjected to 0 mM (control) and 7 mM Na$_2$SO$_3$ for 24 h. The remaining chlorophyll content was expressed in percentage of control. Values are means ± SE (n = 5). Means denoted with different letters were significantly different (p < 0.05).
The effect of SO expression levels on SO enzyme activity was examined in wild type (RR) and SO modified tomato lines employed by us before [10]. A significant effect of SO expression levels on SO enzyme activity ($p < 0.001$) was found. The enzyme activity of SO OE lines was significantly higher as compared to wild type and SO Ri lines (Figure 3). As compared to wild type, enzyme activity in OE 13-6/6 and OE 12-5/7 lines was about 2-fold higher. Compared to the activity in wild type leaves, the SO activity level was only 26% in Ri 421 and Ri 131 lines (Figure 3). The results are in agreement with the activity shown before in these Ri and OE mutants to be less than 30% and about 2-fold higher, respectively, as compared with wild type plants [10]. The results demonstrate the higher SO activity in the tomato lines with higher SO expression levels, and the lower SO activity in the tomato Ri lines, as the result of RNA interference [10].

**Figure 3.** SO enzyme activity in wild type and SO modified tomato lines. Wild type (RR), SO OE (OE 13-6/6 and OE 12-5/7) and SO Ri (Ri 421 and Ri 131) lines were analyzed for SO enzyme activity using 10 µg of crude protein per assay. Values are means ± SE ($n = 8–12$). Means denoted with different letters were significantly different ($p < 0.001$). The presented data show one of the two experiments that yielded essentially identical results.

2.2. Effect of Mo Spray on SO Modified Plants

The effect of Mo was investigated in wild type (RR), SO OE (OE 13-6/6) and SO Ri (Ri 421) tomato lines. Since Mo is present in the molybdenum cofactor redox centre of SO, its effect on SO enzyme activity, especially in the overexpression line was of interest. Plants were sprayed with 3 µM Na$_2$MoO$_4$ and the response of leaf discs on sulfite treatment, remaining chlorophyll after sulfite and SO enzyme activity were studied.

Twenty-four hours after the treatment with 7 mM Na$_2$SO$_3$, no damage of the leaf discs was observed in the control, but clear damage symptoms were visible in the sulfite treated leaf discs (Figure 4B). Irrespective of the Mo spray, the Ri 421 line was most damaged by sulfite, showing the symptoms of chlorosis, while OE 13-6/6 was least affected by the sulfite treatment, especially when sprayed with Mo (Figure 4B). After the sulfite treatment, the RR line showed intermediate damage, fitting between OE and Ri lines.

Additionally, remaining chlorophyll content in the leaf discs after sulfite treatment was found to be highest in the OE 13-6/6 line, which was 66% for the Mo sprayed leaf discs and 56% for non-sprayed leaf discs (Figure 5). Remaining chlorophyll content in the RR line (40%) was found to be higher than in the Ri 421 line and lower than in the OE 13-6/6 line. In the wild type, remaining chlorophyll content was slightly higher, but not significantly different in Mo sprayed plants as compared to doubly distilled water (DDW) sprayed plants. However, with Mo spray, remaining chlorophyll content in the leaf discs
of the OE 13-6/6 line was significantly higher than that without Mo spray. Remaining chlorophyll content in the Ri 421 line was lowest (27%) as compared to the other lines with no observed effect of the Mo spray (Figure 5).

**Figure 4.** Response of Mo spray on the effect of sulfite treated SO modified tomato lines. Leaf discs from wild type (RR) and SO OE (OE 13-6/6) and SO Ri (Ri 421) tomato lines with (Mo) and without Mo treatment were subjected to (A) 0 mM and (B) 7 mM Na$_2$SO$_3$ and photographed 24 h after starting the treatment.

**Figure 5.** Remaining chlorophyll in Mo sprayed plants after sulfite treatment. Leaf discs from wild type (RR) and SO OE (OE 13-6/6) and SO Ri (Ri 421) tomato lines with and without Mo treatment were subjected to 0 mM and 7 mM Na$_2$SO$_3$ for 24 h and the remaining chlorophyll content was expressed in percentage of control. Values are means ± SE ($n = 5$). Means denoted with asterisk were significantly different ($p < 0.05$) within the line.
The effect of Mo spray on SO enzyme activity was investigated in SO modified plants. In general, SO enzyme activity was found to be highest in the OE 13-6/6 line followed by the RR and Ri 421 lines (Figure 6). SO enzyme activity in OE 13-6/6 was higher in the Mo sprayed leaves as compared to the non-sprayed plants. The RR line showed a non-significant tendency for higher SO activity level in the Mo sprayed leaves. No Mo effect was observed in both the RR line and the Ri 421 line (Figure 6).

Figure 6. Effect of sulfite application on Mo sprayed and non-sprayed tomato lines. The shoot tips of control and Mo sprayed plants were kept in a 3 mM Na₂SO₃ solution for 8 h. SO enzyme activity was analyzed by using 2 µg of crude protein per assay. Values are means ± SE (n = 4–6). Different letters show significant differences, p < 0.05. Upper case letters represent the effect between tomato lines and the lower case letters represent the effect between pretreatments within the line.

In addition, after sulfite treatment of the Mo sprayed plants, no further changes in SO activity were observed for either of the tomato lines (Figure 6).

2.3. Effect of Molybdenum and Tungsten Application on Wild Type and SO Modified Plants

The effect of Mo and tungsten (W) application was studied in wild type (RR), SO OE (OE 13-6/6) and SO Ri (Ri 421) tomato lines. Since application of Mo significantly increased the SO enzyme activity in SO OE lines, it was of interest to verify that the increased SO enzyme activity in Mo sprayed plants was due to Mo application. W can substitute for Mo in Moco and therefore inactivate the Mo enzymes. Thus, the effect on SO enzyme activity was investigated by employing the first fully developed leaf, which was dipped in DDW, 500 µM Na₂MoO₄ or 300 µM H₂WO₄ for 24 h at room temperature. The response of leaf discs, remaining chlorophyll and SO enzyme activity after sulfite treatment was studied.

The strongest damage after sulfite treatment was found in H₂WO₄ (W) incubated leaf discs followed by DDW incubated discs and less symptoms of damage and chlorosis were observed in Na₂MoO₄ (Mo) incubated discs (Figure 7). In all DDW, Mo or W spray treatments, the Ri 421 line was more affected by the sulfite treatment than other genotypes, which led to symptoms of chlorosis and damage on the discs, while the OE 13-6/6 line was least damaged as compared to the RR and Ri line. The symptoms of damage observed in the RR line were intermediate between Ri 421 and OE 13-6/6 lines. However, in the OE 13-6/6 line, the severity of damage followed the order Mo, DDW and W, with stronger symptoms in the W incubated leaves. In the RR line, the damage observed in DDW and Mo incubated leaves was similar, but more severe in the W incubated leaf. Leaf discs of the Ri 421 line equally suffered from the sulfite treatment, irrespective of the pretreatments (Figure 7).
between the pretreatments (Figure 8).

ff chlorophyll content between DDW and Mo pretreatment, while it was significantly lower after W incubation. In the Ri 421 line, no effect of Mo and W pretreatments was observed on SO enzyme activity (Figure 9).

Additionally, the effect of DDW, Mo and W pretreatment on SO enzyme activity was studied in wild type and SO modified tomato lines. Since application of Mo significantly increased the SO enzyme activity in SO OE lines, it was of interest to verify that the increased SO enzyme activity in Mo sprayed plants was due to Mo application. W can substitute for Mo in Moco and therefore inactivate the Mo enzymes. Thus, the effect on SO enzyme activity was investigated by employing the first fully wild type and SO modified plants. SO enzyme activity was found to be highest in the OE 13-6/6 line, while no effect was observed in leaves pretreated with Mo as compared to control leaves (DDW incubation), while it was significantly lower after W incubation. No significant difference was observed in the remaining chlorophyll content in Ri 421 between either of the pretreatments (Figure 8).

Furthermore, in the OE 13-6/6 line, remaining chlorophyll content was significantly higher in leaves pretreated with Mo as compared to control leaves (DDW incubation), while it was significantly lower in leaves incubated with W. In the RR line, there was no significant difference in the remaining chlorophyll content between DDW and Mo pretreatment, while it was significantly lower after W incubation. No significant difference was observed in the remaining chlorophyll content in Ri 421 between either of the pretreatments (Figure 8).

**Figure 7.** The response of molybdenum, tungsten and DDW pretreatments to toxic sulfite levels in wild type and SO modified tomato lines. Leaves from wild type (RR) and SO OE (OE 13-6/6) and SO Ri (Ri 421) tomato lines were incubated in DDW, 500 µM Mo or 300 µM W for 24 h, then leaf discs were subjected to (A) 0 mM and (B) 7 mM Na2SO3 for 24 h and photographed.

**Figure 8.** Effect of molybdenum, tungsten and DDW pretreatment on the remaining chlorophyll content in leaf discs after sulfite treatment. Leaves from wild type (RR), SO OE (OE 13-6/6) and SO Ri (Ri 421) tomato lines were incubated in DDW, 500 µM Mo or 300 µM W for 24 h. Leaf discs were subjected to 0 mM and 7 mM Na2SO3 for 24 h. The remaining chlorophyll was expressed in percent of control. Values are the mean ± SE (n = 7). Different letters show significant differences, p < 0.05. Upper case letters represent the effect between tomato lines and the lower case letters represent the effect between pretreatments within the line.
Additionally, the effect of DDW, Mo and W pretreatment on SO enzyme activity was studied in wild type and SO modified plants. SO enzyme activity was found to be highest in the OE 13-6/6 line followed by the RR line and Ri 421 line, respectively (Figure 9). Mo pretreatment significantly increased SO enzyme activity in the OE 13-6/6 line, while no effect was observed in leaves pretreated with W. In the RR line, an increasing, but not significant, tendency in SO enzyme activity was observed in Mo treated leaves. In the Ri 421 line, no effect of Mo and W pretreatments was observed on SO enzyme activity (Figure 9).

**Figure 8.** Effect of molybdenum, tungsten and DDW pretreatment on the remaining chlorophyll content in leaf discs after sulfite treatment. Leaves from wild type (RR), SO OE (OE 13-6/6) and SO Ri (Ri 421) tomato lines were incubated in DDW, 500 µM Mo or 300 µM W for 24 h. Leaf discs were subjected to 0 mM and 7 mM Na_2SO_3 for 24 h. The remaining chlorophyll was expressed in percent of control. Values are the mean ± SE (n = 7). Different letters show significant differences, p < 0.05. Upper case letters represent the effect between tomato lines and the lower case letters represent the effect between pretreatments within the line.

**Figure 9.** Effect of DDW, molybdenum and tungsten pretreatment on SO enzyme activity in wild type and SO modified tomato lines. Leaves from wild type (RR), SO OE (OE 13-6/6) and SO Ri (Ri 421) tomato lines were incubated in DDW, 500 µM Mo or 300 µM W for 24 h. SO enzyme activity was analyzed by using 2 µg of crude protein per assay. Values are means ± SE (n = 2). Different letters show the significant differences at p = 0.001.

**2.4. Effect of Sulfite Application on Ripe Fruits**

Ripe tomato fruits with intact calyx were dipped in solutions containing 0 or 200 mM sulfite for two hours, gently dried with blotting paper and the effect of sulfite on the fruits was observed in wild type and SO-modified tomato lines 24 h later. No damage was observed in the control fruits dipped in water, but the sulfite treatment resulted in the degradation of the tissues in the vicinity of the calyx in the form of an indentation and wrinkling of the tissue (Figure 10). Fruits of SO OE lines (OE 13-6/6 and OE 12-5/7) showed only minor damage as compared to the water dipped SO OE fruits. In wild type fruits (RR), these damages were slightly enhanced as compared to the water dipped wild type fruits, while fruits of the SO Ri lines (Ri 421 and Ri 131) were considerably damaged by the sulfite dipping treatment, showing a further spread of tissue damage near the calyx, with line Ri 421 showing a higher severity of the symptoms than line Ri 131.
The sulfite content in the pericarp was analyzed 24 h following the dipping treatment. Wild type and both OE lines did not differ significantly after sulfite treatment as compared to their water dipped counterparts (Figure 11), whereas SO Ri lines displayed a significant augmentation in pericarp sulfite levels after sulfite dipping. The sulfite induced stronger tissue damage observed in the calyx vicinity of Ri fruits compared to wild type fruits, and the absence of damage in OE fruits, with no differences in pericarp sulfite level, indicate that the intact tomato fruit peel efficiently protects the fruits from sulfite penetration into the pericarp. Additionally, the results indicate that 200 mM sulfite damaged the tomato fruit tissue at the vicinity of the fruit calyx only in wild type and Ri, but not in SO OE fruits.

**Figure 10.** Effect of sulfite dipping on the fruits of wild type and SO modified plant’s appearance. Fruits of wild type (RR), SO OE (OE 13-6/6 and OE 12-5/7), and SO Ri (Ri 421 and Ri 131) lines were dipped for two hours in 200 mM Na$_2$SO$_3$, subsequently kept at room temperature for 24 h and photographed. The damaged regions are indicated by arrows in the sulfite treated RR fruit. Fruits dipped in water served as a control.

**Figure 11.** Pericarp sulfite content after sulfite dipping treatment. Fruits of wild type (RR), SO OE (OE 13-6/6 and OE 12-5/7), and SO Ri (Ri 421 and Ri 131) lines were dipped in 200 mM Na$_2$SO$_3$ for two hours, subsequently kept at room temperature for 24 h and sampled. Fruits dipped in water served as a control. The values are means ± SE (n = 4–6). Asterisks indicate significant differences at p < 0.05 between the treatments within the tomato lines.
3. Discussion and Conclusions

The SO gene was thought to possess a housekeeping function that was revealed by its basic expression levels in *Arabidopsis* plant organs [10 and references therein]. In addition, plant SO is thought to play a role in protecting the thioredoxin system from damage [18]. It is further speculated that SO plays a role in stress signaling demonstrated in *Arabidopsis* plants, since it has the ability to produce H$_2$O$_2$, as a reaction product [12]. However, when *Arabidopsis* and tomatoes plants are exposed to elevated SO$_2$ conditions, SO was demonstrated to be a major enzyme to protect the plants against SO$_2$ toxicity by oxidizing sulfite to sulfate [10,20]. Moreover, it has been shown that SO in co-regulation with APR plays a role in driving an internal sulfate–sulfite cycle for fine-tuning of sulfur flux in *Arabidopsis* plants [21]. Overexpressing APR, a key enzyme in the sulfur assimilation pathway, that generates sulfite, resulted in adverse effects in *Arabidopsis thaliana* transgenic plants, including chlorosis and inhibition of growth [22]. In the current study, the effect of Mo application on the resistance to applied toxic sulfite was studied in tomato plants with high, low and normal expression levels of SO.

Firstly, we showed that leaf discs sampled from leaves of plants impaired in SO activity (Ri lines), treated with 7 mM sulfite solution for 24 h, were susceptible to the externally applied sulfite, showing the symptoms of chlorophyll degradation and damage from the periphery (Figure 1). Leaf discs of SO OE lines showed the least damage, whereas wild type, leaf discs were more damaged than the OE and less than Ri lines (Figure 1). Remaining chlorophyll content, a sensitive indicator of leaf health, supports this result, as it was 27% higher in OE lines and 54% lower in Ri lines as compared to wild type (Figure 2). This resistance to the sulfite in OE lines is likely provided by a higher SO activity level and the susceptibility of Ri lines to sulfite is due to SO activity impairment (Figure 3). The Ri lines, nevertheless, did demonstrate some SO activity, which is likely to be attributed to non-specific activity. Such activity has been reported in the non-leaky molybdenum co-factor mutants of *Nicotiana plumbaginifolia*, which should have no SO activity [11]. The results shown in the current study are in agreement with previous results [10,20,21] supporting the notion that SO is a major enzyme in protecting plants against sulfite toxicity.

Mo is an essential micronutrient in plants and animals. The requirement of Mo for plant growth was first reported by Arnon and Stout [23] using hydroponically grown tomato. It itself is not biologically active, but is rather predominantly found to be an integral part of the organic pterin complex called molybdenum cofactor, functioning as an active center in certain enzymes [24]. These molybdoenzymes, amongst the SO, catalyze important transformations in the global sulfur, nitrogen and carbon cycles [25]. Leaf discs sampled from the Mo sprayed OE plants, were more tolerant to the sulfite treatment, showing less damage and higher remaining chlorophyll content compared to leaf discs sampled from water sprayed plants (Figures 4 and 5). Foliar application of Mo has been shown to improve plant performance. Mo foliar application on Mo-deficient plants effectively rescued the activity of the molybdoenzyme nitrate reductase (NR) in grapes [26]. Mo applied as molybdate in the nutrition medium or as a foliar spray increased the biomass accumulation of the seawater grown halophyte *Salicornia europaea*, by enhancing the activity of the molybdoenzymes NR and xanthine dehydrogenase [27]. Mo sprayed OE plants exhibited a significant increase in SO activity compared to non-sprayed plants, whereas no significant enhanced activity was noticed in Mo sprayed wild type and Ri leaves (Figure 6). We further investigated the effect of sulfite treatment on SO activity in Mo sprayed plants. Incubation of leaves in 3 mM Na$_2$SO$_3$ for 8 h revealed increased SO activity in Mo sprayed OE leaves compared to the activity in control plants treated in the absence of both sulfite and Mo. Such a difference in SO activity was not noticed in wild type and Ri leaves. Interestingly, no difference was noticed in SO activity in Mo treated plants compared to plants treated with Mo together with sulfite, in all the genotypes examined (Figure 6). The results clearly indicate that unlike wild type and Ri, the OE plants grew in the current study below Mo optimum conditions, since the Mo level supplied to the plants in the nutrient solution was not sufficient to generate an higher SO activity. The supplement of additional Mo by the foliar application increased the SO activity resulting in better resistance of OE plants to sulfite toxicity (Figures 4–6). These results indicate a discrepancy
between the excess SO apoprotein level, resulting from the SO OE system in the modified plants, and the inadequate content of the Mo cofactor, limited by the Mo level in the OE plants, but not in wild type and Ri plants. To support this notion, we investigated the SO activity in wild type and SO modified plants after incubation pretreatment with DDW, Mo and W of the leaf cuttings. Due to the comparative electrochemistry of Mo and W elements, Mo can be substituted by W [28]; however, the enzyme with W in its cofactor cannot perform biological functions [29]. A study performed in rats that were fed with tungstate or molybdate followed by subsequent measurement revealed that both Mo and W could be incorporated into the pterin cofactor of sulfite oxidase and xanthine dehydrogenase, but the W containing enzymes were completely inactive [30].

Incubation of leaf cuttings for 24 h in Mo resulted in an increase in tolerance to sulfite with a significant increase in SO activity of OE (Figures 7–9). This suggests that in SO OE plants, the ‘empty’ Mo cofactor of the apoprotein was occupied by the applied Mo at the metal binding site, resulting in increased capacity of SO activity. Similarly, an increase in the activity of the molybdoenzymes AO and XDH has been reported in barley roots in response to Mo supplement to the nutrient medium [31]. In contrast, this effect was not observed after W pretreatment, suggesting a Mo specific increase in SO activity in the OE line in the absence of W pretreatment (Figure 9). Moreover, the remaining chlorophyll content was lower in the W treatment than in the control, in wild type and OE, but not in the Ri line (Figures 7 and 8). The decrease in remaining chlorophyll in wild type and OE by W treatment, and the absence of improvement or deterioration in remaining chlorophyll level compared to the sulfite treated control in Ri leaf discs in response to Mo or W, further supports the notion of a Mo specific increase in SO activity in the OE line. It has been reported that W application in barley resulted in decreased AO and XDH activities, with reduction in growth [31]. Thus, pretreatment of Mo significantly increased the SO activity in the SO OE line, which however was not achieved by W. In the latter case, W might be bound to the binding site in the Mo cofactor resulting in a non-functional cofactor, which cannot participate in sulfite reduction. Since the W incubation time in our experiment was limited to 24 h, Mo already bound to the cofactor could not be substituted with W, resulting in similar SO activity as in the control treatment (DDW) (Figure 9).

Rationally, when studying plant genotypes responses to sulfite toxicity, one should desire minimum sulfite levels used (one level is the best) to distinguish between genotypes. In tobacco, the leaf discs method was employed and leaf discs exposed to 20 mM sulfite exhibited higher chlorophyll damage in wild type compared to mutants overexpressing cysteine synthase (the complex of OASTL and SAT) [15]. Exposing tomato and Arabidopsis leaf discs to 7 mM sulfite, as performed in the current experiment, resulted in differences in remaining chlorophyll between genotypes, with the highest in OE, whereas Ri exhibited lower remaining chlorophyll content than the wild type (see Figure S1 in [10]). Significantly, 5 and 7.5 mM sulfite infiltration into tomato wild type leaves resulted in tissue damage of 5% and 10%, respectively, while 15% and 30% damage were noticed in Ri leaves (see supplemental Figure 5A in [32]). These results support the selection of 7 mM sulfite used in the current study. Additionally, the use of 200 mM sulfite solution for the two-hour tomato fruit dipping is supported by the absence of damage in OE and the higher damage in Ri compared to wild type fruits (Figure 10).

SO₂/SO₃ has been used extensively in post-harvest treatment of the fruits and vegetables in order to keep the quality, such as to prevent browning or to disinfect the products [33]. SO₂ fumigation was shown to be very effective for prevention of fungal decay such as Botrytis cinerea in grape berries and blueberries [34,35]. However, the effectiveness of SO₂ strongly depended on concentration and timing of exposure [36]. Thus, the toxicity of SO₂/SO₃ for both plants and humans is considered as a limiting factor of the usage of SO₂. Here, we suggest that the sulfite treatment of fruits can be effectively used for the treatment of the fruits of OE lines or species with naturally high expression of SO (Figure 10). Moreover, effective sulfite utilization in the fruits of OE lines would allow balancing of the sulfite level in treated fruits (Figure 11).

Several studies showed that similar to the peroxisomal SO, the chloroplast localized SiR can significantly protect plants against sulfite toxicity [37–39], indicating that both are major players in
sulfite detoxification. Co-suppression of SiR activity by ca 50% resulted in enhanced sulfite in tobacco leaves [38], whereas ZmSiR-overexpressing Arabidopsis plants (Arabidopsis carrying maize SiR under the control of 35-S promoter) were more tolerant to severe SO2 stress than wild type, showing a significantly higher chlorophyll content and lower damage than in wild type [39]. SiR OE Arabidopsis and tomato plants were impressively more tolerant to toxic sulfite than the wild type plants [37], suggesting that one should examine the feasibility of employing a SiR overexpression system to generate crop resistance to high sulfite in a future study. Yet, the high metabolic cost of a six-electron transfer for sulfide generation and the acetyl-coenzyme A used for O-Acetyl-L-serine synthesis, both catalyzed into cysteine [8,9], should be considered. Significantly, the enhancement of SiR expression and activity, in SO Ri tomato plants [10,32], led to the enhancement in total (free and protein bound) cysteine and methionine levels in plant leaves compared to wild type plants, grown under control conditions. Yet, the high level of the S-amino acids was shown to be degraded, generating enhanced levels of toxic sulfites in SO Ri leaves exposed to dark stress conditions [32]. Accordingly, a scenario should be considered where the overexpression of the SiR pathway may lead to a futile pathway in which the toxic sulfite is inserted into the sulfur assimilation pathway and might again be released as a result of sulfur-containing metabolite turnover.

On the other hand, a drawback for plants that have high SO activities seems unlikely. Biomass accumulation of the canopy, as well as the total tomato fruits accumulated in OE were similar to those in wild type, while both genotypes exhibited significantly higher accumulated biomass and fruits than Ri plants (Supplementary Figures S1 and S2). Additionally, total soluble solids (TSS), which indicate the sweetness of the tomato fruits, was similar in in all genotypes (Supplementary Table S1). Moreover, while free cysteine and glutathione were similar in wild-type compared to OE plants in Arabidopsis grown under normal unstressed conditions, [21], ZmSO-overexpressing tobacco plants exhibited a higher level of reduced glutathione (GSH) under normal growth conditions. Yet, under drought stress, ZmSO-overexpressing tobacco plants exhibited higher tolerance as a result of enhanced GSH, and lower reactive oxygen species and malondialdehyde levels in the mutant compared to wild type plants [40]. The similar or higher glutathione content shown in Arabidopsis OE and ZmSO-overexpressing tobacco plants compared to wild type plants indicates that overexpression of SO is not likely to interfere with biosynthesis of S-amino acids.

Taken together, we conclude that SO is important for normal physiological processes in plants. From the observation of fewer symptoms of sulfite toxicity on the leaf discs of SO OE plants, we show here that SO is an important enzyme for protecting plants against the exogenously applied sulfite. Application of Mo increased SO activity in the OE line and therefore enhances the resistance to sulfite toxicity. No increased SO activity in SO OE lines after W incubation suggests that the enhanced tolerance of SO OE lines to sulfite treatment after Mo application was due to increased SO activity in SO OE lines. These results suggest that SO overexpression can be employed, with or without Mo application, for developing fruits and vegetable crops tolerant to sulfite/SO2 containing pre- and postharvest treatments.

4. Materials and Methods

4.1. Plant Material and Growth Conditions

Tomato plants (Solanum lycopersicon Mill.) of the wild type (Rheinlands Ruhm, RR) and SO modified plants were used for the experiment. SO modified plants were altered as described by Brychkova et al. [10]. Plants were modified to increase the expression levels of SO (overexpression, OE), while in RNA interference lines (Ri) the SO gene was abrogated. Two overexpression independent lines (OE 13-6/6 and OE 12-5/7) and two Ri independent lines (Ri 421 and Ri 131), used by us before (see Figure S1 in [10]), were used for the experiments to ensure reliable data from the mutants as compared to wild type plants.
Germination of seeds was carried out in Petri dishes lined with wet filter paper at room temperature in the dark. The seedlings were transferred to soil in pots (12 cm × 8 cm × 6 cm) at the hook stage (10 seedlings per pot) in which they were allowed to grow for about 2 weeks. Kanamycin resistance of both types of SO modified lines (SO OE and SO Ri) was verified before each experiment by spraying the plants at least 3 times with 0.05% kanamycin sulfate in 0.01% Triton.

Ten plants per line were grown randomly in a transgenic greenhouse at Ben-Gurion University in Beersheva, Israel. Temperature was maintained between 25 °C in the daytime and 18 °C at night. Tomato plants were grown in plastic pots (10-L) filled with commercial potting mixture HR-1 (Shacham Givat Ada, Ltd., Israel) and irrigated with a drip irrigation system according to the plant requirements. Nutrients were supplied using the fertilizer Ply-feed 23:7:23 (NPK) containing Fe, Zn, Mn, Cu, and Mo (1000, 150, 500, 110, 70 ppm, respectively, https://www.haifa-group.com/) with the irrigation water. Temperature in the greenhouse, irrigation schedule and fertilizer amount were controlled by a computer system. Plants were trained to single shoots by timely removal of the lateral buds.

4.2. Treatment of Plants and Leaves with Molybdenum, Tungsten or Sulfite

Six to eight similar sized plants from each line at the 4-leaf stage were chosen for the Mo spray treatment. Plants were sprayed with 3 µM Na₂MoO₄ in 0.004% Triton twice a week for the duration of two weeks. Control plants were sprayed with doubly distilled water (DDW) in 0.004% Triton. Then, the first fully developed leaf was sampled either for the leaf discs sulfite treatment or frozen in liquid nitrogen and stored at −80 °C for SO activity. Additionally, shoot tips were cut 2 cm below the first fully developed leaf and immediately placed in a 3 mM Na₂SO₃ solution. The shoot tips were kept in the treatment solution for eight hours, and subsequently the first fully developed leaf was sampled for SO enzyme activity as described below.

In additional experiments, the first fully developed leaf from wild type (RR), SO OE (13-6/6) and SO Ri (421) tomato lines were incubated either in DDW, 500 µM Na₂MoO₄ or 300 µM H₂WO₄ for 24 h at room temperature under constant light with photon flux of 100 µmols⁻¹m⁻². For the subsequent treatment, the leaves were used for the leaf discs sulfite treatment or were immediately frozen in liquid nitrogen for further enzyme analysis.

4.3. Leaf Discs Experiments

Leaf discs were sampled from the youngest first fully developed leaf randomly from 6–8 tomato plants and placed in DDW on a wet filter paper under photon flux of 100 µmols⁻¹m⁻². Six to seven leaf discs of 7 mm diameter per treatment and plant line were punched from the leaf lamina excluding the main veins. The discs were then transferred into the treatment solution containing 0 and 7 mM Na₂SO₃. Treated and control leaf discs were kept sealed in a Petri dish at room temperature in a 16-h light regime with photon flux of 100 µmols⁻¹m⁻². The solutions were changed 16 h after the initial start of the treatment. The symptoms of chlorosis and damage on the leaf discs were photographed 24 h after the initial treatment and quantified by measuring the chlorophyll content. Each leaf disc experiment was replicated at least twice.

4.4. Chlorophyll Content Determination

Chlorophyll content was determined by extracting the leaf disc samples in 80% ethanol for 48 h at 4° C in the dark. The extract was centrifuged at 12,000 g for 10 min. The resulting supernatant was diluted 10 times in 80% ethanol and the color was measured as the absorbance at 652 nm using a UV/VIS spectrophotometer (JASCO, model V-530). Total chlorophyll content was calculated as described by Arnon [41] and remaining chlorophyll content was expressed as % of control.

4.5. Protein Extraction from the Leaf Samples

Crude proteins were extracted as done before [32] by grinding leaf samples in an extraction buffer at the ratio of 1:6 (w/v) with approximately 10 mg polyvinylpyrrolidone (PVPP). The extraction
buffer was composed of 0.1 M Tris-acetate (pH 7.25) with 0.05 µM phenylmethylsulphonyl fluoride (PMSF). The resulting extract was centrifuged at 12,000 g for 15 min at 4 °C. Six molar ammonium sulfate was added to the resultant supernatant at a ratio of 1:10 and centrifuged for 10 min at 12,000 g. Subsequently, the supernatant was transferred to a round bottomed tube, containing ammonium sulfate at a ratio of 2:1 (v/w) and centrifuged again at 12,000 g for 10 min at 4 °C. The supernatant was discarded and the pellet was re-dissolved in 0.1 M Tris-acetate (pH 7.25). The resulting solution was then passed through a 2.8 cm Sephadex G-25 column and subsequently eluted with 0.1 M Tris-acetate (pH 7.25) using a double volume of the protein solution.

4.6. Determination of Protein Concentration

Total soluble protein content was estimated by a modified Bradford procedure, using crystalline bovine serum albumin as a reference [42]. Briefly, protein extracts from different lines of tomato plants were diluted at a ratio of 1:25 with DDW and mixed with Protein Assay at a ratio of 1:10. The resulting color was measured as the absorbance at 595 nm in UV/VIS spectrophotometer (JASCO, model V-530) and the protein concentration was calculated.

4.7. Assessment of SO Enzyme Activity

SO enzyme activity was determined in the crude protein extracts of the leaf. The activity was determined using 2 or 10 µg of soluble protein. The Fuchsine color reagent was composed of a fresh mixture of reagent A, B and DDW at a ratio of 1:1:7. Reagent A was a 0.04% pararosaniline solution discolored in 2.3 M H₂SO₄ and reagent B was composed of 3.2% formaldehyde.

The reaction was started by adding soluble protein to 0.1 mM freshly prepared Na₂SO₃ and then incubated for 5 min at 30 °C. The reaction was terminated by adding the color reagent into the reaction mixture. The reaction in another set of samples was immediately stopped after the addition of the substrate (Na₂SO₃) with the color reagent. The absorbance of the resulting color was measured at 540 nm in a spectrophotometer (Sunrise, Tecan, Pharmatec instrumentation ltd, Israel). The readings were compared with the known standard of freshly prepared Na₂SO₃. The final SO activity was expressed in µmol SO₃ per mg protein per minute.

4.8. Sulfite Treatment of Fruits

Ripe fruits from wild type and SO modified plants were harvested randomly from ten plants per line with intact calyx. Similar fruits were selected in respect to the color, size and calyx freshness. The fruits were dipped completely in the treatment solution containing 0 or 200 mM Na₂SO₃ for two hours. After removal from the treatment solution, the fruits were wiped gently with blotting paper to remove the excess solution. Subsequently, the fruits were kept at room temperature for 24 h. Appearing symptoms of the sulfite treatment were observed on the fruits and photographed (Nikon Coolpix-4500) after removing the calyx for precise symptom observation. Additionally, 200-mg fruit samples were taken from the pericarp, snap frozen in liquid nitrogen and stored at −80 °C for further examination. The sulfite level in the tomato fruits was determined as described above. Briefly, tomato fruit samples were extracted with DDW in the ratio of 1:4 (w/v). The resulting extract was centrifuged for 15 min at 12,000 g. The supernatant was collected and kept on ice. The sulfite content was determined colorimetrically by using the Fuchsin color reagent as described above. The color reagent was added to the plant extract in the ratio of 1:3 and the resulting color was measured after 10 min at 540 nM in a spectrophotometer (Sunrise, Tecan. Pharmatec Instrumentation ltd, Israel). Sulfite content was determined against a known standard solution of Na₂SO₃ and expressed in nmol per g fresh weight.

4.9. Determination of Biomass Accumulation, Fruit Yield and TSS

Total biomass of the canopy was measured after harvesting the fruits at the end of the experiment, 93 days after transplanting. Fruits, harvested from each plant, were recorded and quantified to get
the total yield per plant of each tomato line. For total soluble solids (TSS) determination, fruits were extracted in a ratio of 1:5 (w/v), using DDW and then centrifuged for 10 min at 12,000 g at 4°C. TSS was determined in the fruit extract using a refractometer (Atago Digital Refractometer, model: PR-1, Atago Co., Ltd., Japan; https://www.atago.net/en/).

4.10. Statistical Analysis

Significant differences between treatments were analyzed by appropriate single or multi-factorial analysis of variance (ANOVA) using the JumpIn 5.0.1a software package. When ANOVA indicated significance, multiple comparison of treatment means was performed according to Tukey–Kramer HSD or Student’s t-test.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/10/5/694/s1,
Figure S1: Total biomass of tomato wild type (RR), SO OE (OE 13-6/6 and OE 12-5/7) and SO Ri (Ri 421 and Ri 131) lines was measured after harvesting the fruits at the end of the experiment, 93 days after transplanting. The values are averages ± SE (n = 9). Different letters show significant difference at p < 0.05; Figure S2: Total fruit yield per plant was recorded for wild type (RR), SO OE (OE 13-6/6 and OE 12-5/7) and SO Ri (Ri 421 and Ri 131) lines. The values are averages ± SE (n = 10). Different letters show the significant difference at p < 0.05; Table S1: Total soluble solids (TSS) were detected in wild type (RR), SO OE (OE 13-6/6 and OE 12-5/7), SO Ri (Ri 421 and Ri 131) lines. The values are means ± SE (n = 10). Different letters show significant differences at p < 0.05.

Author Contributions: M.S. conceived the idea and coordinated the work. U.S., A.B. and Y.V. designed the experiments. U.S. performed all experimental work. Y.V. and M.S. supervised the work. U.S. and Y.V. analyzed the data. All authors have read and agreed to the published version of the manuscript.

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