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

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Abstract

Plant sulfite oxidase (SO) is a molybdo-enzyme responsible for the oxidation of excess SO_2/sulfite into non-toxic sulfate. The effect of toxic sulfite level on leaves and fruits was studied in tomato plants with different SO expression levels: wild-type (WT), SO overexpression (OE) and SO RNA interference (Ri) plants. Leaf discs and ripe fruit of plants lacking SO were more susceptible, whereas SO OE plants were more resistant as revealed by remaining chlorophyll content and tissue damage levels. Application of molybdenum further enhanced the tolerance of leaf discs to sulfite by enhancing SO activity in SO OE lines, but not in WT or Ri plants. Notably, incubation with tungsten, the molybdenum antagonist, overturned the effect of molybdenum spray in SO OE plants, revealed by remaining chlorophyll content and SO activity. The results indicate that SO determines, in tomato leaves and ripe fruits, the resistance to toxic sulfite and the application of molybdenum enhances sulfite resistance in OE plants by increasing SO activity. The results suggest that overexpressing SO

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mechanism can be employed in agriculture with or without molybdenum application, for the development of more tolerate crops and vegetables to higher concentrations of sulfite/SO_2 containing postharvest treatments.

Keywords

sulfite oxidase**,** molybdenum, tungsten, sulfite toxicity, tomato, SO expression

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1. Introduction

Sulfur is the least abundant macronutrient in plants, comprising approximately 0.1% of the dry matter. As a component of a molecule it is mostly involved in catalytic or electrochemical functions rather than as a structural component such as are 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 (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 initiates 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) [7,8]. The *O*-Acetyl-L-serine (OAS) synthesized from serine and acetyl-Coenzyme A catalyzed by serine acetyltransferase together with sulfide is catalyzed into cysteine by OAS (thiol) lyase (OASTL) [9].

Sulfite generated by APR or obtained from atmospheric SO_2 is a highly toxic intermediate. A bulk of sulfite is normally channeled in the assimilatory reduction pathway to generate sulfide catalyzed by sulfite reductase (SiR), yet excess sulfite is be oxidized effectively back to sulfate by the peroxisome localized sulfite oxidase (SO), acting as the safety valve in the sulfate reduction pathway [10]. SO is catalyzing the reaction in which two electrons are

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transferred from sulfite to the molybdenum cofactor (Moco) redox center, which are subsequently transferred to molecular oxygen with simultaneous production of hydrogen peroxide (H_2O_2) and sulfate [11]. The generated H_2O_2 can further oxidize another sulfite molecule to sulfate nonenzymatically or acted by the peroxiosomal catalase upon low cellular sulfite concentration to H_2O and O_2 [12]. 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 enter 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 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].

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 sulfite in tomato leaves and ripe fruits and the

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application of molybdenum (Mo) further enhances the sulfite resistance in OE tomato plants by increasing SO activity. The results suggest that the overexpressing SO mechanism can be used in agriculture for the development of SO transformed lines in crops and vegetables, which can tolerate higher concentrations of pesticides or sulfur containing postharvest treatments of fruits and vegetables; and application of Mo on them for better results.

2. Results

2.1. Chlorophyll content and SO activity

The effect of SO expression levels of the plants 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 hours. 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 of the leaf discs was observed in the control treatment (Fig. 1A), while symptoms of chlorosis and damage from the periphery were observed in the leaf discs incubated with 7 mM Na_2SO_3 (Fig. 1B). The least damage occurred in the leaf discs of both the OE lines, followed by the wild type and Ri lines. In 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 (Fig. 1B).

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Additionally, remaining chlorophyll content was measured in the leaf discs 24 hours 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 (Fig. 2). Remaining chlorophyll content in RR line was 60.7% of the control. In SO OE lines, remaining chlorophyll content in OE 13-6/6 and OE 12-5/7 was 22.4% and 32.5%, respectively, higher as compared to the wild type. While in SO Ri lines, the remaining chlorophyll content was reduced to 51.5% and 55.9% for line Ri 421 and Ri 131, respectively, compared to the wild type (Fig. 2). The results show the remaining chlorophyll content in the leaf discs after sulfite treatment was higher for the tomato lines having higher SO expression levels and vice versa.

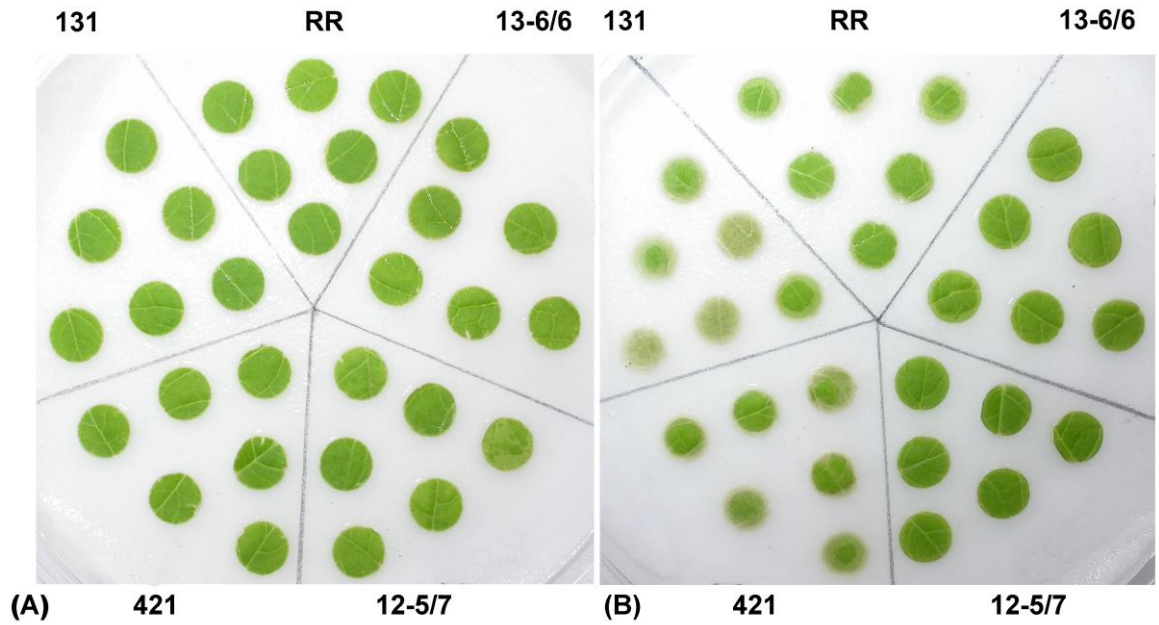

Figure 1. Effect of sulfite application on wild type and SO modified tomato lines. Leaf discs of wild type (RR), SO OE lines (OE 13-6/6, OE 12-5/7) and SO Ri lines (Ri 421, Ri 131) were subjected to (A) 0 mM and (B) 7 mM Na_2SO_3 and photographed 24 hours after starting the treatment.

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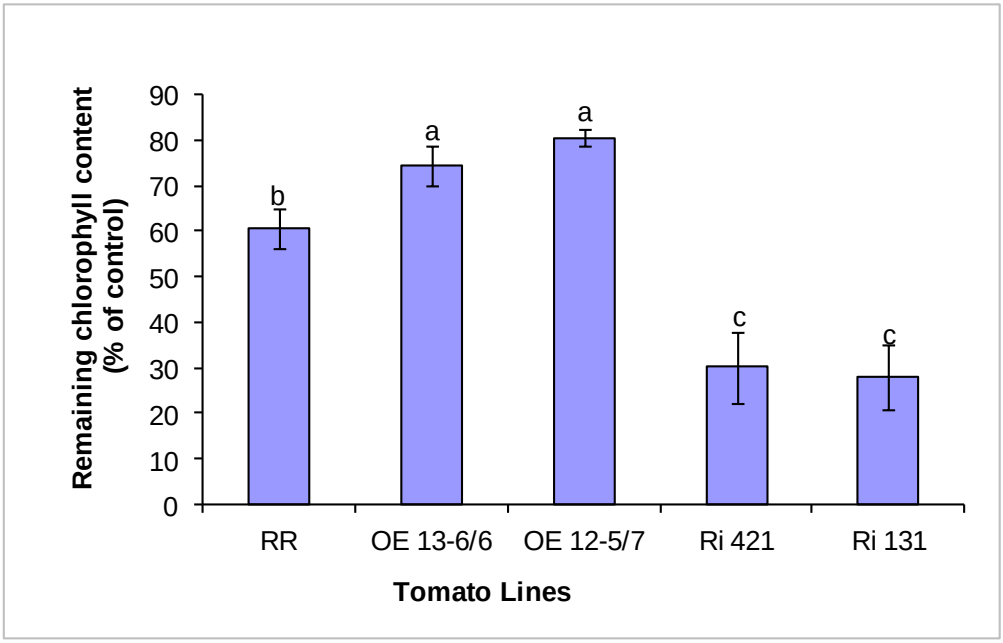

Figure 2. Remaining chlorophyll in wild type and SO modified tomato plants after sulfite treatment. Leaf discs from wild type (RR) and SO OE (OE 13-6/6 and OE 12-5/7) and SO Ri (Ri 421 and Ri 131) tomato lines were subjected to 0 mM (control) and 7 mM Na_2SO_3 for 24 hours. The remaining chlorophyll 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. 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 (Fig. 3). As compared to the wild type, enzyme activity in OE 13-6/6 and OE 12-5/7 line was 2.0 and 2.1 fold, respectively. In SO Ri lines, SO activity level was much lower as compared to wild type; only 25.5% in Ri 421 line and 26.0% in Ri 131 line, respectively (Fig. 3). The results show the higher SO enzyme activity

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for the tomato lines with higher SO expression levels, whereas the SO enzyme activity level was lower for the tomato lines having lower SO expression levels.

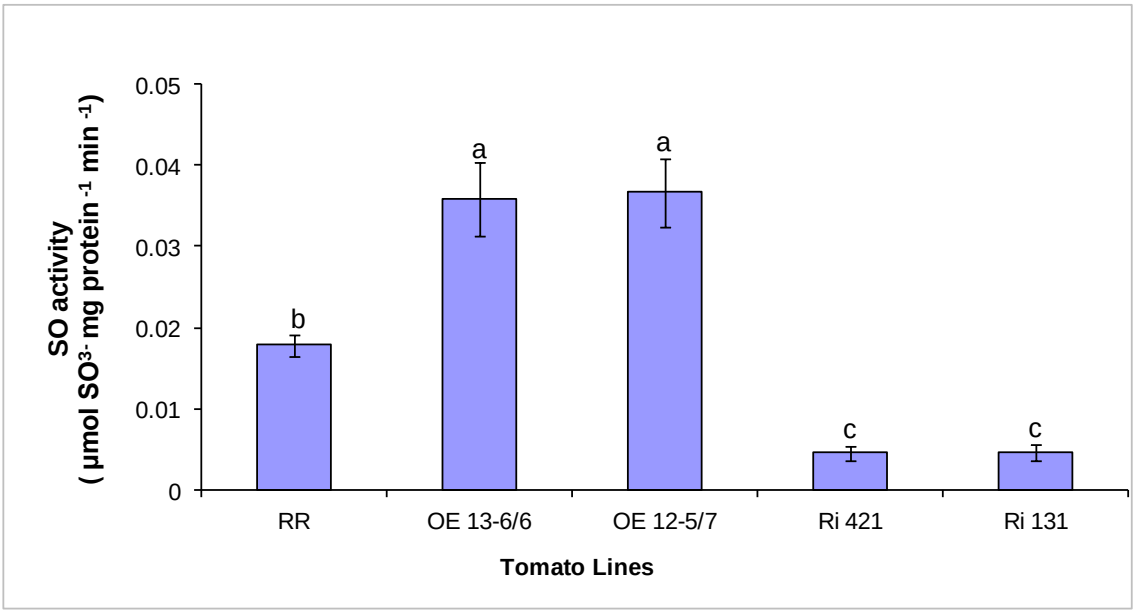

Figure 3. SO enzyme activity in wild type and SO modified tomato lines. Wild type (RR), SO OE (OE 13-6/6 and OE 12-5/7) and SO Ri (Ri 421 and Ri 131) lines were analyzed for SO enzyme activity using 10 µg of crude protein per assay. Values are means ± SE (n = 8-12). Means denoted with different letters 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_2MoO_4 and the response of leaf discs on sulfite treatment, remaining chlorophyll after sulfite treatment and SO enzyme activity was studied.

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Twenty four hours after the treatment with 7 mM Na_2SO_3 no damage of the leaf discs was observed in the control, but clear damage symptoms were visible in the treatment (Fig. 4B). Irrespective of the Mo spray, Ri 421 line was most damaged, showing the symptoms of chlorosis, while OE 13-6/6 was least affected by the sulfite treatment, especially when sprayed with Mo (Fig. 4B). After the sulfite treatment, 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 OE 13-6/6 line, which was 66.1% for the Mo sprayed leaf discs and 55.9% for non sprayed leaf discs (Fig. 5). Remaining chlorophyll content in RR line (39.7%) was found to be between that of OE 13-6/6 and Ri 421 lines, being higher than Ri 421 line and lower than OE 13-6/6 line. In the wild type, remaining chlorophyll content was slightly higher, but not significant in Mo sprayed plants as compared to non sprayed plants. However, with Mo spray, remaining chlorophyll content in the leaf discs of OE 13-6/6 line was significantly higher than that without Mo spray. Remaining chlorophyll content in Ri 421 line was lowest (26.8%) as compared to the other lines with no observed effect of the Mo spray (Fig. 5).

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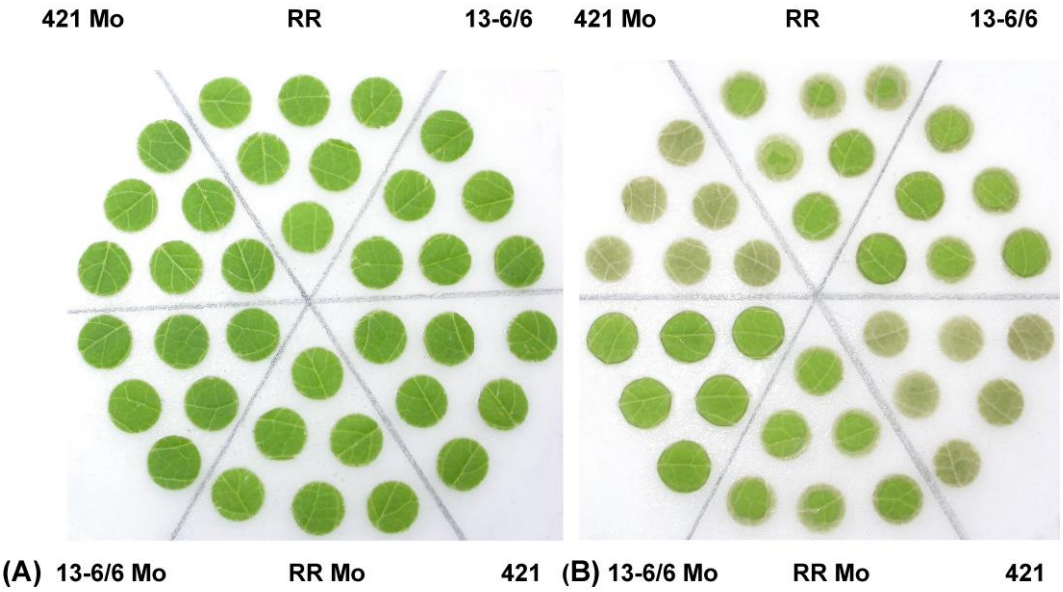

Figure 4. Response of Mo spray on the effect of sulfite treated SO modified tomato lines. Leaf discs from wild type (RR) and SO OE (13-6/6) and SO Ri (421) tomato lines with (Mo) and without Mo treatment were subjected to (A) 0 mM and (B) 7 mM Na_2SO_3 and photographed 24 hours after starting the treatment.

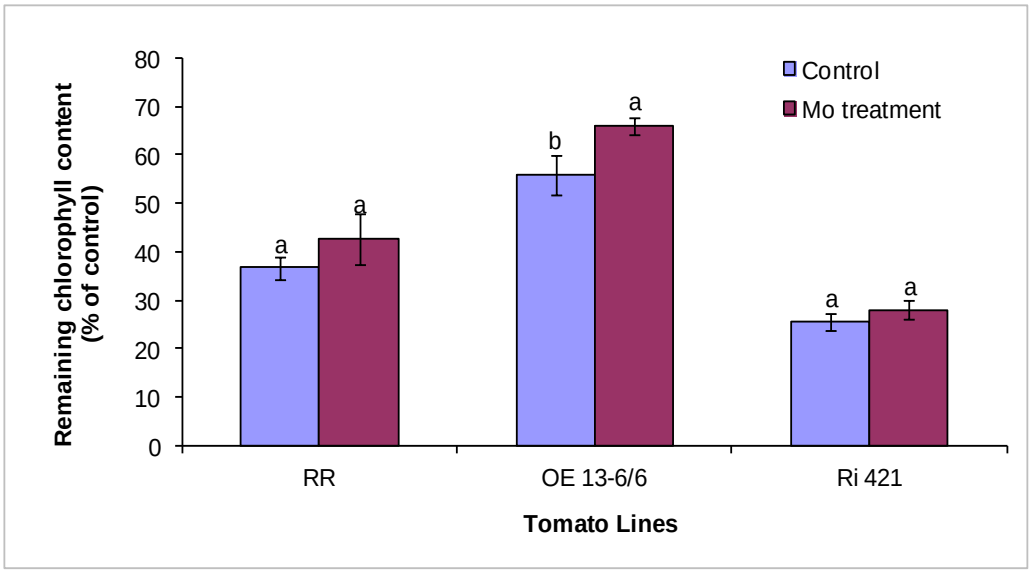

Figure 5. Remaining chlorophyll in Mo sprayed plants after sulfite treatment. Leaf discs from wild type (RR) and SO OE (OE 13-6/6) and SO Ri (Ri 421) tomato lines with and without Mo treatment were subjected to 0 mM and 7 mM Na_2SO_3 for 24 hours and

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the remaining chlorophyll content was expressed in percentage of control. Values are means ± SE (n = 5). Means denoted with different letters were significantly different ($p < 0.05$) 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 OE 13-6/6 line followed by RR line and Ri 421 line (Fig. 6). SO enzyme activity in OE 13-6/6 was higher in the Mo sprayed leaves as compared to the non sprayed plants. RR line showed a non significant tendency for higher SO activity level in the Mo sprayed leaves. No Mo effect was observed in Ri 421 line (Fig. 6).

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

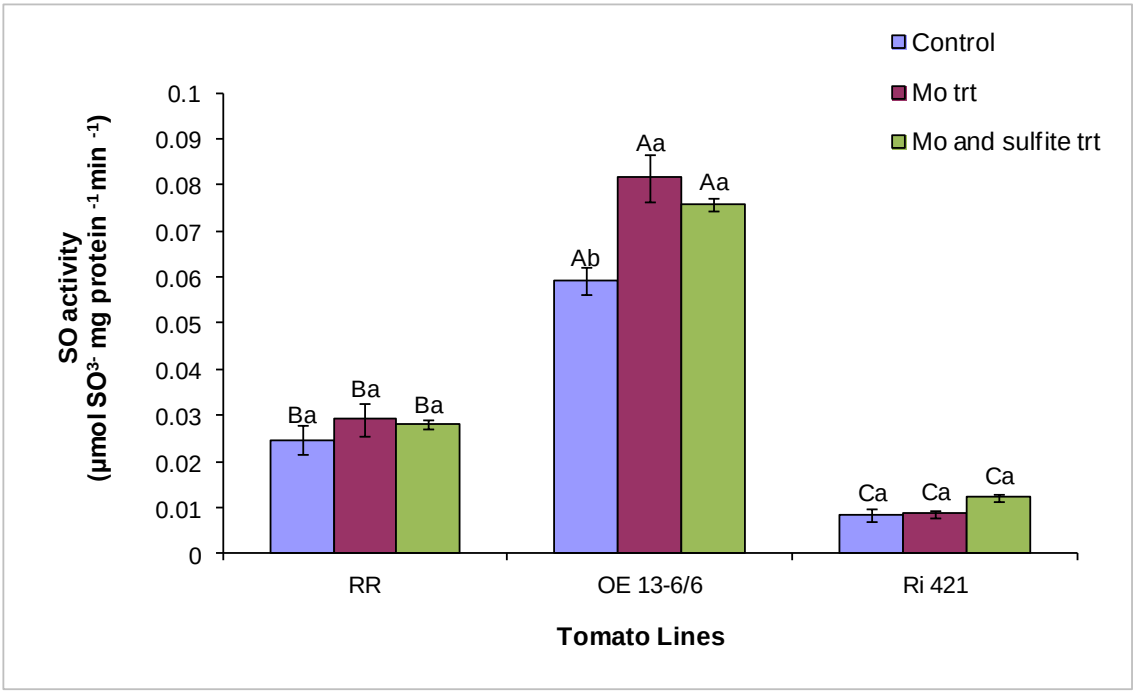

Figure 6. Effect of sulfite application on Mo sprayed and non-sprayed tomato lines. The shoot tips of control and Mo sprayed plants were kept in a 3 mM Na_2SO_3 solution for 8 hours. SO enzyme activity was analyzed by using 2 µg of crude protein

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per assay. Values are means ± SE (n = 4-6). Means denoted with different letters were significantly different ($p < 0.05$).

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 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 Na_2MoO_4 or 300 µM H_2WO_4 for 24 hours at room temperature. The response of leaf discs, remaining chlorophyll and SO enzyme activity after sulfite treatment were studied.

The strongest damage after sulfite treatment was found in H_2WO_4 (W) incubated leaf discs followed by DDW incubated discs and less symptoms of damage and chlorosis were observed in Na_2MoO_4 (Mo) incubated discs (Fig. 7). In all DDW, Mo or W incubation treatments, Ri 421 line was more affected by the sulfite treatment, which led to symptoms of chlorosis and damage on the discs, while OE 13-6/6 line was least damaged as compared to RR and Ri line. The symptoms of damage observed in RR line were intermediate between Ri 421 and OE 13-6/6 line. 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

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was similar, but more severe in the W incubated leaf. Leaf discs of Ri 421 line equally suffered from the sulfite treatment, irrespective of the pretreatments (Fig. 7).

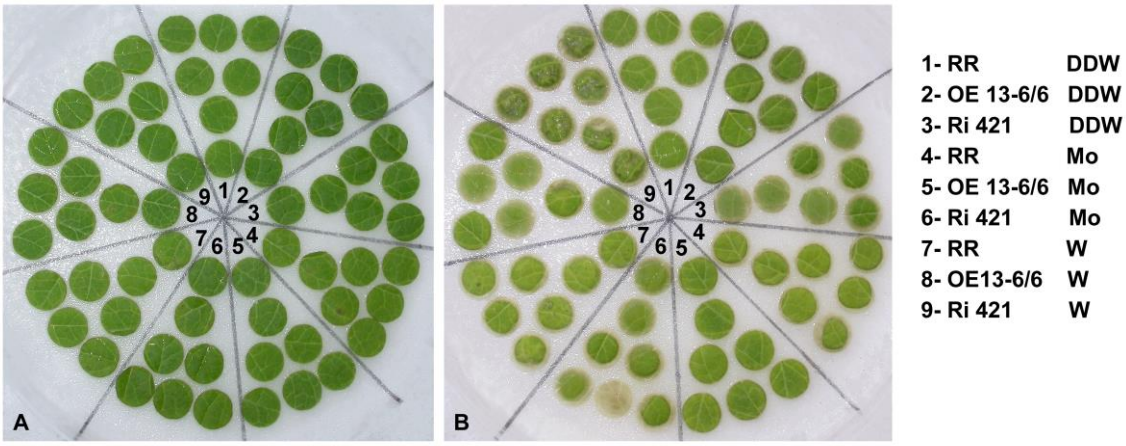

Figure 7. The response of molybdenum, tungsten and DDW pretreatments to toxic sulfite levels in wild type and SO modified tomato lines. Leaves from wild type (RR) and SO OE (13-6/6) and SO Ri (421) tomato lines were incubated in DDW, 500 µM Mo or 300 µM W for 24 hours , then leaf discs were subjected to (A) 0 mM and (B) 7 mM Na_2SO_3 for 24 hours and photographed.

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 (Fig. 8).

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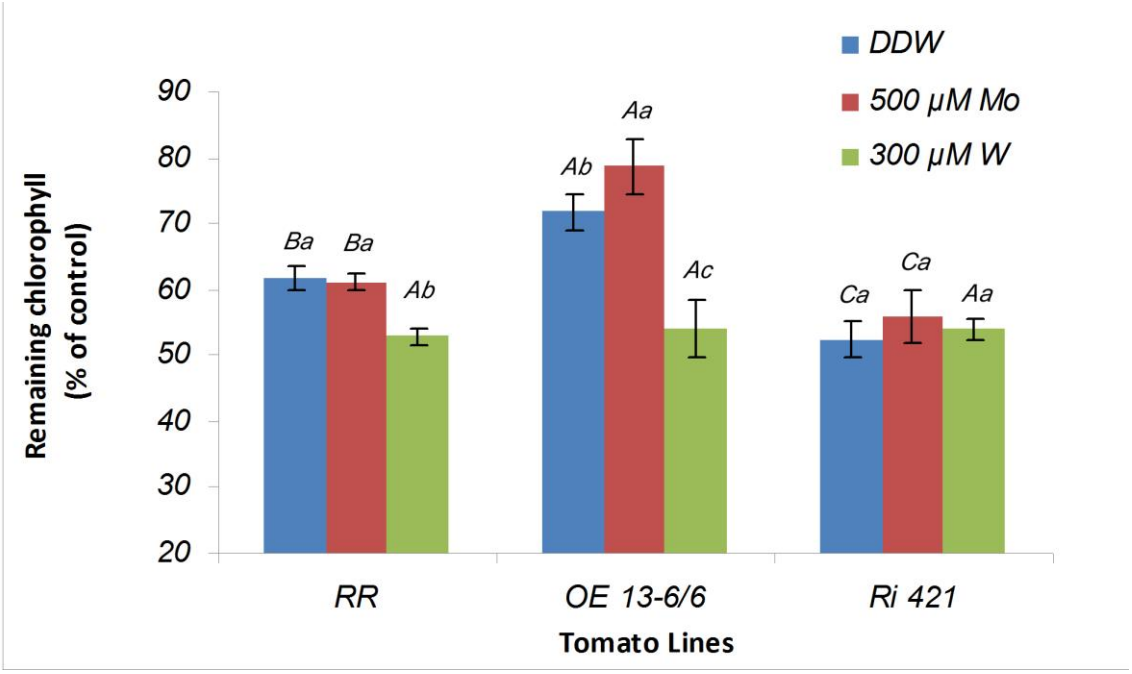

Figure 8. Effect of molybdenum, tungsten and DDW pretreatment on the remaining chlorophyll content in leaf discs after sulfite treatment. Leaves from wild type (RR), SO OE (13-6/6) and SO Ri (421) tomato lines were incubated in DDW, 500 µM Mo or 300 µM W for 24 hours. Leaf discs were subjected to 0 mM and 7 mM Na_2SO_3 for 24 hours. 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 RR line and Ri 421 line respectively (Fig. 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 (Fig. 9).

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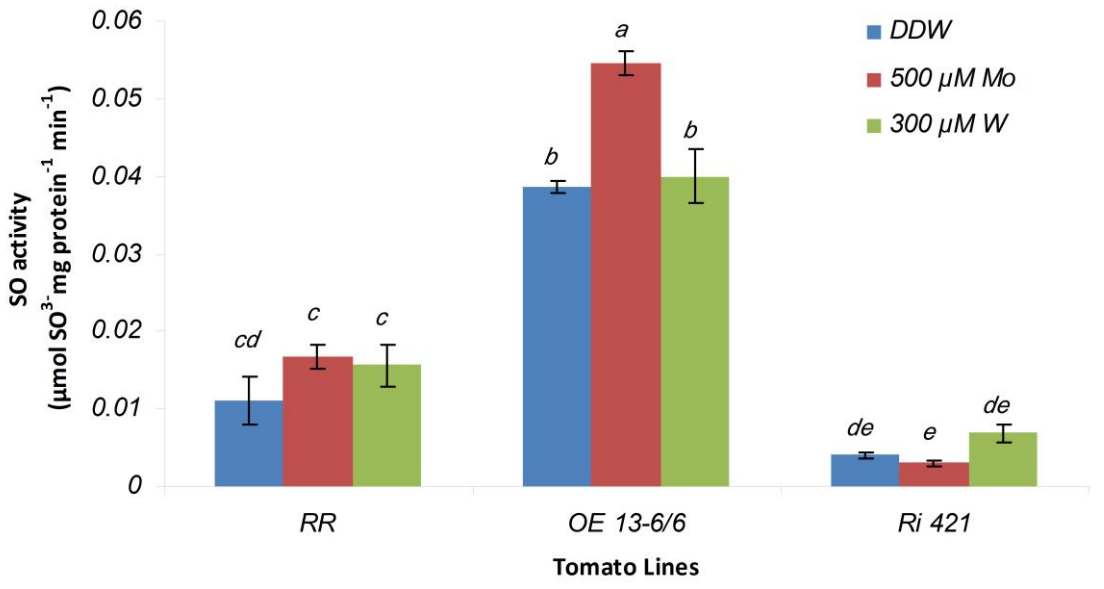

Figure 9. Effect of DDW, molybdenum and tungsten pretreatment on SO enzyme activity in wild type and SO modified tomato lines. Leaves from wild type (RR), SO OE (13-6/6) and SO Ri (421) tomato lines were incubated in DDW, 500 µM Mo or 300 µM W for 24 hours. SO enzyme activity was analyzed by using 2 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

The effect of sulfite on ripe fruits was observed in wild type and SO-modified tomato lines. No damage was observed in the control fruits dipped in water, but the sulfite treatment resulted in the degradation of the tissues at the region below the calyx in form of an indentation and wrinkling of the wounded tissue (Fig. 10). Fruits of SO OE lines (OE 13-6/6 and OE 12-5/7) showed only minor damages as compared to the water dipped plants. In wild type fruits (RR) these damages were slightly enhanced, while fruits of the SO Ri lines (Ri 421 and Ri

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131) were considerably damaged by the sulfite dipping treatment, with line Ri 421 showing a higher severity of the symptoms than line Ri 131.

Figure 10. Effect of sulfite dipping on the fruits of wild type and SO modified plants appearance. Fruits of wild type (RR), SO OE (OE 13-6/6 and OE 12-5/7), SO Ri (Ri 421 and Ri 131) lines were dipped for two hours in 200 mM Na_2SO_3, subsequently kept at room temperature for 24 hours and photographed. The damaged regions are indicated by arrows in the sulfite treated RR fruit. Fruits dipped in water served as a control.

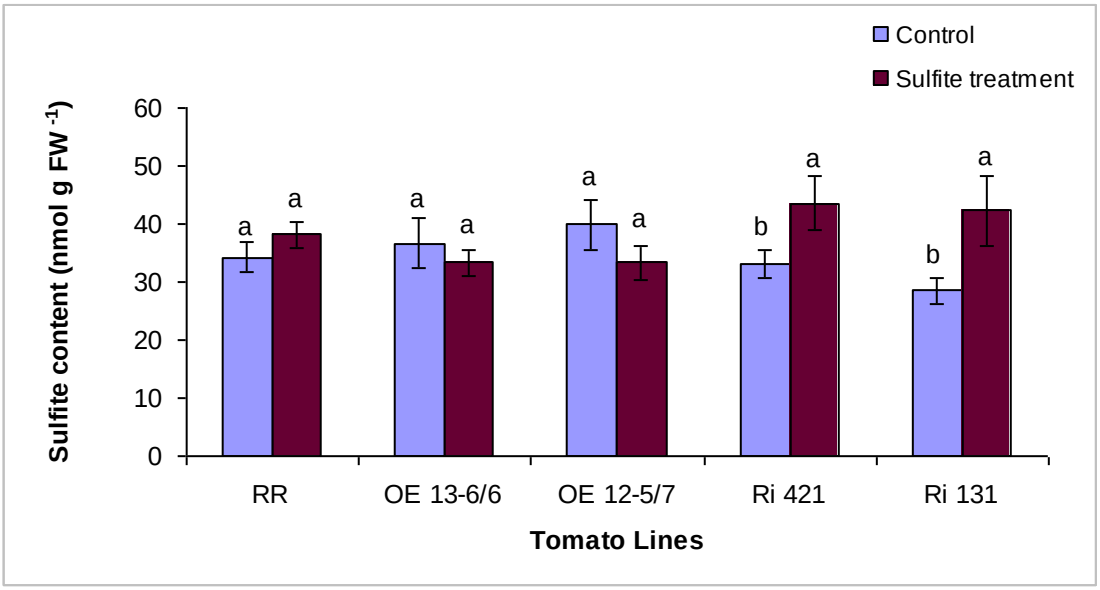

Figure 11. Pericarp sulfite content after sulfite dipping treatment. Fruits of wild type (RR), SO OE (OE 13-6/6 and OE 12-5/7), and SO Ri (Ri 421 and Ri 131) lines

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were dipped in 200 mM Na_2SO_3 for two hours, subsequently kept at room temperature for 24 hours and sampled. Fruits dipped in water served as a control. The values are means ± SE (n= 4-6). Different letters show significant differences at $p < 0.05$ between the treatments within the tomato lines.

The sulfite content in the pericarp was analyzed following the dipping treatment. Wild type and both OE lines did not differ significantly after sulfite treatment as compared to their water dipped counterparts (Fig. 11), whereas, SO Ri lines displayed a significant augmentation in pericarp sulfite levels after sulfite dipping.

3. Discussion and Conclusions

SO in plants is a molybdenum cofactor containing enzyme. The SO gene was thought to possess a housekeeping function that was revealed by its basic expression levels in *Arabidopsis* plant organs [10]. In addition, plant SO is thought to play a role in the cellular sulfur turnover [12,17] or in protecting the thioredoxin system from damage [18]. It is further speculated that SO plays a role in stress signaling, since it has the ability to produce H_2O_2, as a reaction product [12]. However, when plants are exposed to elevated SO_2 conditions, SO is the major enzyme to protect the plants against SO_2 toxicity by oxidizing sulfite to sulfate [10,20]. Moreover, it has been shown recently that SO in co-regulation with APR plays a role in driving an internal sulfate-sulfite cycle for fine tuning of sulfur flux in the plants [21]. Nevertheless, overexpressing APR, an enzyme in the sulfur assimilation pathway, resulted in adverse effects in the transgenic plants including chlorosis and inhibition of growth [22]. In this study

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the effect of Mo on SO enzyme activity was investigated on wild type, SO OE and SO Ri tomato lines.

Sulfite is a toxic metabolite that is formed during the sulfur reduction pathway [7]. Sulfite thus formed is normally reduced in the sulfur assimilation pathway catalyzed by sulfite reductase in the chloroplast to form sulfide, which is then incorporated into sulfur containing amino acids [7]. Contrasting to this pathway, sulfite can be oxidized to sulfate by the molybdenum cofactor containing enzyme SO [12]. Here, we showed that the leaf discs from plants lacking SO (Ri lines) were more susceptible to the externally applied sulfite, showing the symptoms of chlorophyll degradation and damage from the periphery (Fig. 1). Moreover, the leaf discs of SO OE lines showed the least damage. The wild type, where SO expression was not altered was more damaged than the OE lines and less than Ri lines (Fig. 1). Remaining chlorophyll content, a sensitive indicator of leaf health, supports this result, where it is 27% higher in OE lines and 54% lower in Ri lines as compared to wild type (Fig. 2). This resistance to the sulfite in OE lines may be provided by a higher SO activity level and the susceptibility of Ri lines to sulfite is due to lack of SO. These results are supported by a 2 fold increase in SO activity in OE lines compared to wild type (Fig. 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]. These results support the findings of Brychkova et al. [10] and Lang et al. [20] who showed a major role of plant SO for protecting plants against SO_2/sulfite toxicity. Thus, plant SO is the major

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enzyme to act on the externally applied sulfite and its level in the plants determines the level of toxicity in plants.

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 [24]. These molybdoenzymes catalyze important transformations in the global sulfur, nitrogen and carbon cycles [25]. Leaf discs of the Mo sprayed OE 13-6/6 plants in our experiment were more tolerant to sulfite treatment with less damage, retaining more chlorophyll content compared to non sprayed (Figs. 4 and 5). Indeed, foliar application of Mo on Mo-deficient plants effectively rescued the activity of the molybdoenzyme NR in grapes [26]. Moreover, application of molybdate in the nutrition medium or as a foliar spray increased the yield accumulation of the seawater grown halophyte *Salicornia europaea* by enhancing the activity of the molybdoenzymes NR and XDH [27]. Therefore, we determined the SO activity in Mo sprayed plants. As expected, a significant increase in SO activity was achieved in OE 13-6/6 line after Mo application as compared to non-sprayed, but no effect was observed in wild type and Ri 421 line (Fig.6). We further investigated the effect of sulfite pretreatment on SO activity. Incubation of leaves in 3 mM Na_2SO_3 for 8 hours revealed increased activity in Mo sprayed OE 13-6/6 leaves and not in wild type or Ri 421 line. However, after sulfite pretreatment, no further increase in SO activity was found in wild type and SO modified lines (Fig. 6). Similar results have been reported by Brychkova et al. [10], where SO protein and transcript levels were not highly sensitive to SO_2 exposure, rather, SiR transcripts were highly induced in a SO

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dependent manner. This suggests that under non-toxic dose of sulfite, SiR may first reduce the sulfite to reductive pathway. However, under higher sulfite conditions that cannot be used by SiR, SO is the only enzyme to detoxify the sulfite to protect the plants. Therefore, SO in plants probably serves as a physiological "safety valve" for sulfite toxicity.

The application of Mo increased the tolerance to sulfite toxicity in OE 13-6/6 line by increasing the SO activity (Fig. 6). This may be due to an excess of SO apoprotein in OE plants, which was functional after the application of Mo. To test this hypothesis, we investigated the SO activity in wild type and SO modified plants after incubation pretreatment with DDW, Mo and W of the leaf cuttings. W, a higher congener of Mo possesses similar structural and functional properties like Mo. So, due to the comparative electrochemistry of Mo and W compounds, Mo can be substituted by W [28], however, the enzyme with W in its cofactor cannot perform biological functions [29]. For example, an experiment performed in rats that were fed with tungstate or molybdate followed by subsequent measurement of the metal content in sulfite oxidase and xanthine oxidase, revealed that both metals could be incorporated into pterin cofactor of the enzymes, but the tungsten containing enzymes were completely inactive [30].

Incubation of leaf cuttings for 24 hours in Mo resulted in an increase in tolerance to sulfite with a significant increase in SO activity of OE 13-6/6 line (Figs. 7, 8 and 9). This might suggest that in SO OE plants the MO 'empty' Moco was occupied by the Mo supplied by Mo pretreatment at the metal binding site, and after incorporation into the apoenzyme resulted in the increased SO activity. An increase in activity of molybdoenzymes AO and XDH has been

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reported in barley roots with the Mo application in the nutrient medium [31]. However, this effect was not observed in W pretreatment suggesting a Mo specific increase in SO activity in the OE line (Fig. 9). Moreover, the leaf pre-treated with W resulted in no differences for the remaining chlorophyll content between wild type, OE and Ri lines of W pre-treated leaves (Figs. 7 and 8). This might reflect the W toxicity in plants. It has been reported that W treatment in barley for 9 days resulted in decreased activity of molybdoenzymes AO and XDH in the leaves with reduction in growth [31]. Thus, pretreatment of Mo significantly increased the SO activity in SO OE line, which however was not achieved by W. In the latter case, W might be bound to the metal binding site in molybdenum 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 hours, the cofactor which already bound Mo might not be shifted by W, resulting in similar SO activity as in the control treatment (DDW) (Fig. 9).

SO_2/SO_3 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 [32]. SO_2 fumigation was shown to be very effective for prevention of fungal decay such as *Botrytis cinerea* in grape berries and blueberries [33,34]. However, the effectiveness of SO_2 was strongly depend on concentration and timing of exposure [35]. Thus, the toxicity of the SO_2/SO_3 for both, plants and humans, is considered as a limiting factor of the usage of SO_2. 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

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of SO (Fig. 10). Moreover, effective sulfite utilization in the fruits of OE line would allow balancing the sulfite level in treated fruits (Fig. 11).

Taken together, sulfite oxidase is important for normal physiological processes in plant. 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 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 to of SO OE lines to sulfite treatment after Mo application was due to increased SO activity in SO OE lines. These results suggest that the mechanism of overexpressing SO can be used in agriculture for the development of SO transformed lines in crops and vegetables, which can tolerate higher concentrations of pesticides or sulfur containing postharvest treatments of fruits and vegetables; and application of Mo on them for better results.

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

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421 and Ri 131) were used for the experiments to ensure reliable data from the mutants as compared to WT 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.

Plants were grown in a transgenic greenhouse at Ben-Gurion University in Beer Sheva, Israel. Temperature was maintained between 25º C in daytime and 18º C at night. Tomato plants were grown in plastic pots (10-liter) 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

Similar sized plants from all lines at the 4-leaf stage were chosen for the Mo spray treatment. Plants were sprayed with 3 µM Na_2MoO_4 in 0.004% Triton twice a week for the duration of two weeks. Control plants were sprayed with

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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 -80º C for SO activity. Additionally, shoot tips were cut 2 cm below the first fully developed leaf and immediately placed in a 3 mM Na_2SO_3 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 Na_2MoO_4 or 300 µM H_2WO_4 for 24 hours at room temperature under constant light with photon flux of 100 $\mu mols^{-1}m^{-2}$. 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 experiment

Leaf discs were sampled from the youngest first fully developed leaf and placed in DDW on a wet filter paper under photon flux of 100 $\mu mols^{-1}m^{-2}$. Leaf discs of 7 mm diameter were punched from the leaf lamina excluding the main veins. The discs were then transferred into the treatment solution containing 0 and 7 mM Na_2SO_3. Treatment and control were kept sealed in a Petri dish at room temperature in a 16 hour light regime with photon flux of 100 $\mu mols^{-1}m^{-2}$. The solutions were changed 16 hours after the initial start of the treatment. The symptoms of chlorosis and damage on the leaf discs were photographed 24 hours after the initial treatment and quantified by measuring the chlorophyll content.

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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 minutes. 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 [36] and remaining chlorophyll content was expressed as % of control.

4.5. Protein extraction from the leaf samples

Crude proteins were extracted as done before [37] 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 minutes at 4º C. Six molar Ammonium sulfate was added to the resultant supernatant at a ratio of 1:10 and centrifuged for 10 minutes 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 minutes at 4º 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.

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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 [38]. 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 H_2SO_4 and reagent B was composed of 3.2% formaldehyde.

The reaction was started by adding soluble protein to 0.1 mM freshly prepared Na_2SO_3 and then incubated for 5 minutes at 30º C. The reaction was terminated by adding the color reagent into the reaction mixture. Another set of samples were immediately stopped after the addition of the substrate (Na_2SO_3) 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 Na_2SO_3. The final SO activity was expressed in µmol SO_3 per mg protein per minute.

4.8. Sulfite treatment of fruits

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Ripe fruits from wild type and SO modified plants were harvested 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 Na_2SO_3 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 hours. 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 -80º C for further examination. 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 minutes 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 minutes at 540 nM in a spectrophotometer (Sunrise, Tecan. Pharmatec Instrumentation ltd, Israel). Sulfite content was determined against a known a standard solution of Na_2SO_3 and expressed in nmol per g fresh weight.

4.9. 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

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treatment means was performed according to Tukey–Kramer HSD or Student's t-test.

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