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**Title:** Assaying the use of sodium thiosulphate as a biostimulant and its effect on cadmium accumulation and tolerance in *Brassica oleracea* plants

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**ABSTRACT**

An optimal uptake of mineral elements is crucial to ensure both crop yield and quality. The use of biostimulants is taking relevance to improve the nutrition of crops. Sulphur (S) is one of the elements with great potential within biostimulants. Furthermore, soil contamination by heavy metals such as cadmium (Cd) has become a serious environmental problem. Different studies have suggested the use of thiosulphate (TS) as a biostimulant and to increase the phytoremediation capacity of plants. Therefore, in the present study, we use a crop plant with high S requirements such as *Brassica oleracea*, to test whether TSserves as a biostimulant and whether affects Cd accumulation and tolerance. *B. oleracea* plantswere grown with two different TS doses (2 mM and 4 mM), under Cd toxicity, and with the combination of Cd toxicity and both TS doses. Parameters of biomass, mineral elements accumulation, and stress tolerance were analyzed. The results showed that TS reduced biomass of *B. oleracea* plants. The application of 2 mM TS increased Cd accumulation whereas the 4 mM dose reduced it. On the other hand, TS incremented micronutrient accumulation on plants subjected to Cd toxicity and increased Zn contents. Besides, the application of 2 mM to Cd-stressed plants enhanced photosynthesis performance and reduced oxidative stress. Finally, TS increased the antioxidant capacity of *B. oleracea* plants. Briefly, although TS can not be used as a biostimulant it could be used for Cd phytoremediation purposes and to enhance Zn accumulation in *B. oleracea* plants.

**Keywords**: Antioxidant, Cabbage, Cadmium, Mineral nutrients, Phytoremediation, Zinc

**Abbreviations**: Chl, chlorophyll; GSH, glutathione; GSSG, oxidized GSH; MDA, malondialdehyde; ROS, reactive oxygen species; TS, thiosulphate

**1. INTRODUCTION**

An optimal uptake of mineral elements is crucial to ensure a good crop yield and an adequate nutrient quality. One of the most important mineral elements is sulfur (S). S is an essential element for the nutrition and development of the plants due to the multitude of compounds of great interest that contain S, such as certain amino acids (cysteine and methionine), antioxidants such as glutathione (GSH), coenzymes, prosthetic groups, vitamins and secondary metabolites (Lou et al., 2017; Zhou et al., 2018). S is also related to the uptake, assimilation, and metabolism of other essential nutrients for plants mainly nitrogen (N). In addition, cysteine together with glycine and glutamate forms GSH a low molecular weight compound that acts as an antioxidant (Tao et al., 2018). However, the excessive use of fertilizers to supply S and other nutrients generates an ionic imbalance altering the characteristics of the soil, causing a nutritional deficit, as well as contamination of aquifers by leaching (FAO, 2017). Therefore, we must find alternatives for the fertilization of crops to obtain crops of high nutritional value with a lower contribution of fertilizers.

The use of biostimulants is taking relevance to improve the nutrition of crops. An agricultural biostimulant is any compound or set of compounds, whose function is to stimulate natural processes to benefit nutrient uptake, nutrient efficiency, tolerance to abiotic stress and/or crop quality, regardless of their nutrient content (Ricci et al., 2019). By improving the uptake of other nutrients and the metabolic efficiency of these, biostimulants increase the yield and nutritional quality of the crop, without the need to apply large amounts of fertilizers. This is of great importance since an increase in production is not always linked to an increase in the quality of the food obtained (Calvo et al., 2014). An example of biostimulants is thiosulphate (TS), an anion derived from thiosulphuric acid. However, there is great controversy about the effects of TS. When it is applied to the crop, it is rapidly oxidized to sulfate, an essential source of S for the plant. In this sense, TS can replace possible deficiencies of S (Alfaro et al., 2006). TS could be especially effective in species with higher S requirements such as *Brassica oleracea* (Khurana et al., 2015).

On the other hand, some studies suggest that TS could improve heavy metal tolerance and be useful to enhance phytoremediation of polluted soils (Grifoni et al., 2015; Liu et al., 2018). Soil contamination by heavy metals has become a serious environmental problem, as with increasing industrialization and agricultural activities, their accumulation is much higher and more frequent. One of the problems of heavy metals is that they cannot be degraded, so their persistence in nature is very high. Heavy metal contamination has caused health problems for some 10 million people worldwide, becoming a critical health hazard (Ashraf et al., 2019). Among the non-essential heavy metals, cadmium (Cd) is the most bioavailable and bioaccumulative due to its high solubility in soil and its easy absorption by plants. Cd is absorbed and accumulated in the roots, shoots and edible parts of the plants and enters the food chain (Zhou et al., 2018).

Cd reduces plant growth and productivity, affects nutrient uptake and photosynthetic machinery, induces oxidative stress and alters antioxidant defense systems (Lou et al., 2017; Zhou et al., 2018). One of the consequences of this toxicity is an increase in the production of reactive oxygen species (ROS), even though Cd is not a redox-active metal. ROS are by-products of aerobic metabolism and can act as cellular signals. However, in high concentrations, ROS cause oxidative damage to membranes (lipid peroxidation) increasing malondialdehyde (MDA) levels (Garg and Kaur, 2013). To counteract the effect of oxidative stress, plants increase the accumulation of antioxidant compounds and enhance the activity of antioxidant enzymes. Among the detoxification pathways that act under stress caused by heavy metals, the increase of S-rich compounds, such as reduced GSH, is considered to be of vital importance for the tolerance and survival of plants under stress (Pereira de Araújo et al., 2017).

Phytoremediation technique has been developed to clean soils contaminated with heavy metals using plants. Within phytoremediation, we can make use of phytoextraction thanks to the use of hyperaccumulator plants (Ashraf et al., 2019). The objective of phytoextraction is to remove metals from the soil through uptake by plants and subsequent harvesting (Wang et al., 2014). Plants from the genus *Brassica* can accumulate high heavy metals concentrations. Indeed, *B. oleracea* plants have been proved to be effective for the phytoremediation of heavy metals such as Cd (Szczygłowska et al., 2011). Normally in phytoextraction, the use of hyperaccumulator plants is accompanied by the addition of external agents to improve the plants' ability to extract metals (Grifoni et al., 2015). Thus, (Wang et al., 2014) and (Wang et al., 2018), proved that the addition of TS enhanced the efficiency of phytoextraction in Hg contaminated soils.

Furthermore, treatments with Na2SO3 and Na2SO4 improved the uptake of nutrients and the antioxidant enzymatic activity of Cd-stressed plants and reduced the MDA content in the leaves (Zhou et al., 2018). However, no studies have ever been carried out about the effects of TS, in terms of its capacity to increase or reduce Cd uptake by the plant and whether it can improve Cd tolerance. Therefore, in the present study, we use a widely widespread *Brassica* crop plant such as *B. oleracea*, to test whether TScan serve as a biostimulant and whether affects Cd accumulation and tolerance.

**2. MATERIAL AND METHODS**

*2.1. Plant material, growth conditions, and treatments*

*B. oleracea* cv. Bronco seeds were germinated and grown for 30 days in cell flats (cell size = 3 cm x 3 cm x 10 cm) filled with perlite mixture, and flats were placed on benches in a greenhouse. The 30-day-old seedlings were transferred to a growth chamber under controlled environmental conditions with a relative humidity of 60-80%, temperature of 22/18ºC (day/night) and 12/12-h photoperiod at a photosynthetic photon flux density (PPFD) of 350 µmol m-2 s-1 (measured at the top of plants with a 190 SB quantum sensor, LI-COR Inc., Lincoln, NE, USA). Plants were grown in hydroponic culture in lightweight polypropylene trays (60 cm diameter top, bottom diameter 60 cm, and 7 cm in height) with a volume of 3l. We used hydroponic culture to assure the control of the mineral elements supplied to the plants. Thus, throughout the experiment the plants received a growth solution composed of 4 mM KNO3, 3 mM Ca(NO3)2 •4 H2O, 2 mM MgSO4 •7 H2O, 6 mM KH2PO4, 1 mM NaH2PO4 •2 H2O, 2 μM MnCl2 •4 H2O, 10 µM ZnSO4, 0.25 μM CuSO4 •5 H2O, 0,1 μM Na2MoO4 •2 H2O, 5 μM Fe-chelate (Sequestrene;138FeG100) and 10 µM H3BO3. This solution, with a pH of 5.5–6.0, was changed every three days. Treatments were initiated 30 days after germination and were maintained for 21 days. Plants received six different treatments: Control (without Na2S2O3 nor CdCl2), 2 mM Na2S2O3 (2 mM TS), 4 mM Na2S2O3 (4 mM TS), 100 µM CdCl2 (Cd), 100 µM CdCl2 + 2 mM Na2S2O3 (Cd + 2 mM TS), 100 µM CdCl2 + 4 mM Na2S2O3 (Cd + 4 mM TS).

*2.2. Plant sampling*

Plant leaves were washed with distilled water, dried on filter paper, and weighed to obtain the fresh weight (FW). Half of the leaves from each treatment were frozen at −30ºC for later biochemical assays and the other half of the plant material was lyophilized to measure the dry weight (DW) and the concentration of the nutrients.

*2.3. Analysis of mineral elements*

Mineral elements were determined after a sample of 150 mg of dry material was subjected to a process of mineralization by wet digestion (Wolf, 1982). Dry leaves were ground and mineralized with a mixture of nitric acid (HNO3)/perchloric acid (HClO4) (v/v) and H2O2 at 30%. From the resulting mineralization, and after the addition of 20 ml of mili-Q H2O, elements concentrations were determined by ICP-MS (X-Series II; Termo Fisher Scientific Inc., Waltham, MA, USA). N concentration was determined by colorimetry based on the Berthelot reaction, as described by (Krom, 1980). The Cd and Zn contents were obtained by multiplying the concentrations of these elements by the leaf dry weight.

*2.4. SPAD Chl value and Chl a fluorescence*

The relative chlorophyll (Chl) content of leaves was measured by using SPAD chlorophyll meter SPAD-502 (Konica Minolta Sensing Inc., Japan). Three measurements were made in each leaf and the average was calculated.

To measure Chl *a* fluorescence, plants were adapted to dark for 30 min before measurements using special leaf clip holders that were allocated in each leaf. Chl *a* fluorescence kinetics was determined using the Handy PEA Chlorophyll Fluorimeter (Hansatech Ltd., King’s Lynn, Norfolk, UK); the fluorescence transients were induced by red light (650 nm) with 3000 µmol photons m-2s-1 light intensity and recorded by the instrument. Measurements were conducted with six plants of fully expanded leaves at midstem position. Handy PEA software provides the values of all parameters analyzed (Strasser et al., 2000).

*2.5.* *ROS and MDA concentrations*

The O2.− determination was based on the ability to reduce NBT. Optical density was measured at 580 nm (Kubiś, 2008). H2O2 concentration was colorimetrically measured as described by (Junglee et al., 2014). MDA concentration was determined according to Fu and Huang (2001) measuring absorbance at 532 nm. The non-specific absorbance value at 600 nm was obtained to correct the turbidity. MDA concentration was calculated using 155 mM-1 cm-1 as the extinction coefficient.

*2.6.* *GSH forms*

Oxidized GSH (GSSG), and total GSH (reduced GSH + GSSG) were analyzed according to Noctor and Foyer (1998). Reduced GSH levels were estimated as the difference between total GSH and GSSG. The extraction and quantification of total AsA, reduced AsA, followed the method of Law et al. (1983).

2.*7. Antioxidant capacity tests*

For Ferric Reducing Antioxidant Power (FRAP) and Trolox Equivalent Antioxidant Capacity (TEAC) tests, the asparagus spear was homogenized in methanol and centrifuged at 12,800 g for 2 min. FRAP assay was performed using 1 mM, 2,4,6-tripyridyl-2-triazine and 20 mM FeCl3 in 0.25 M CH3COONa, pH 3.6. The absorbance was then measured at 593 nm (Benzie and Strain, 1996).

TEAC assay was conducted according to (Re et al. (1999) and Cai et al. (2004) methods with modifications. Radical cation ABTS+ [2,2-azinobis-(3-acid-ethylbenzthiazoline-6-sulfonic)] was produced using 7 mM ABTS and 2.45 mM potassium persulfate incubate in the dark and at room temperature for 16 h. Then, the ABTS+ solution was diluted with methanol to an absorbance of 0.70 ± 0.02 at 734 nm. The supernatant and the diluted ABTS+ solution were mixed, kept at room temperature for 6 minutes and the absorbance was measured at 734 nm.

*2.8. Statistical analysis*

Data were subjected to a simple ANOVA at 95% confidence, using the Statgraphics Centurion XVI program. Means were compared by Fisher’s least significant differences (LSD). The significance levels were expressed as \* P<0.05, \*\* P<0.01, \*\*\* P<0.001, or NS (not significant).

**3. RESULTS**

*3.1. Plant biomass*

The application of TS and Cd stress caused a reduction of leaf DW compared to control conditions. The application of TS in plants subjected to Cd stress also reduced the DW to a greater extent in plants that received the 4 mM dose (Fig. 1A). Regarding root DW, TS also reduced its value in comparison to control plants. However, all plants treated with Cd showed a similar reduction in root DW regardless of the presence of TS (Fig. 1B).

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**Figure 1**. Effect of TS and Cd on leaf DW (A) and root DW (B). Values are expressed as means ± standard error (n=9). Columns marked with the same letters were not significantly different based on the LSD test (P < 0.05).

*3.2. Cd accumulation*

Plants subjected to Cd stress and treated with 2 mM TS showed a higher Cd concentration and content whereas plants treated with 4 mM TS presented lower values for these parameters in comparison to plants that were not treated with TS (Fig. 2).



**Figure 2**. Effect of TS on lead Cd concentration (A) and content (B) in plants treated with Cd. Values are expressed as means ± standard error (n=9). Columns marked with the same letters were not significantly different based on the LSD test (P < 0.05).

*3.3. Nutrients concentration*

Plants treated with TS showed lower N, K, Ca, and Mg concentrations in comparison to control plants. Specifically, plants treated with 2 mM TS registered lower P, S, and K levels whereas plants treated with 4 mM TS presented lower Ca concentration. Cd stress reduced N and Ca concentrations but increased K and Mg levels. The application of TS to Cd stressed plants reduced N, K, and Mg concentrations. 2 mM TS dose increased Ca and 4 mM enhanced P concentration in Cd-stressed plants (Table 1).

Regarding micronutrients, the application of TS significantly increased Zn concentration and Zn content regardless of the presence of Cd (Table 2; Fig. S1). In addition, plants that received 2 mM TS showed higher Mn concentration but 4 mM TS decreased Cu levels. Cd application increased B but reduced the concentration of the other micronutrients in comparison to control conditions. The 2 mM dose increased the accumulation of all micronutrients except B whereas the 4 mM TS increased Cu concentration in comparison to Cd-stressed plants without TS (Table 1).

**Table 1**. Mineral nutrients concentration in *B. oleracea* plants supplied with TS treated with Cd and the combination of both treatments.

|  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | N | P | S | K | Ca | Mg | B | Fe | Mn | Zn | Cu |
| Control | 83.83±7.90a | 10.12±0.51b | 19.26±0.96ab | 51.77±2.59bc | 36.42±1.82a | 6.99±0.35d | 187.35±9.37b | 64.59±3.23b | 114.47±5.72b | 27.22±1.36d | 31.23±1.56c |
| 2 mM TS | 76.97±7.81b | 8.82±0.44d | 17.12±0.86c | 39.01±1.95e | 28.02±1.40bc | 6.10±0.30e | 184.90±9.24b | 60.99±3.05bc | 124.00±6.20a | 43.40±2.17a | 27.08±1.35cd |
| 4 mM TS | 75.74±6.80b | 9.37±0.47bcd | 19.44±0.97ab | 44.86±2.24d | 23.78±1.19d | 5.49±0.27e | 172.20±8.61b | 64.05±3.20b | 107.92±5.40bc | 45.21±2.26a | 17.90±0.90e |
| Cd | 71.26±6.72bc | 9.72±0.49bc | 19.28±0.96ab | 60.11±3.01a | 25.62±1.28cd | 10.52±0.53a | 284.73±14.24a | 57.21±2.86c | 80.10±4.00d | 17.69±0.88e | 24.59±1.23d |
| Cd + 2 mM TS | 65.42±6.64c | 8.95±0.45cd | 17.84±0.89bc | 52.67±2.63b | 30.06±1.50b | 9.46±0.47b | 293.96±14.70a | 98.92±4.95a | 100.74±5.04c | 30.67±1.53c | 109.14±5.46a |
| Cd + 4 mM TS | 65.28±5.11c | 11.98±0.60a | 19.55±0.98a | 47.70±2.37cd | 25.99±1.30cd | 8.11±0.41c | 293.76±14.69a | 50.06±2.50d | 86.84±4.34d | 35.29±1.76b | 97.02±4.85b |
| *p*-value | \*\*\* | \*\*\* | \* | \*\*\* | \*\*\* | \*\*\* | \*\*\* | \*\*\* | \*\*\* | \*\*\* | \*\*\* |
| LSD0.05 | 6.53 | 0.88 | 1.67 | 4.42 | 2.55 | 0.71 | 21.57 | 6.03 | 9.20 | 3.07 | 5.62 |

N, P, S, Ca, and Mg are expressed as mg g-1DW, and B, Fe, Mn, Zn, and Cu are expressed as µg g-1DW. Values are means ± standard error (n=9) and differences between means were compared by Fisher’s least-significance test (LSD; P=0.05). Values with different letters indicate significant differences. The levels of significance were represented by p>0.05: NS (not significant), p<0.05 (\*), p<0.01 (\*\*) and p<0.001 (\*\*\*).

*3.3. SPAD and fluorescence parameters*

Plants of the different treatments showed no different SPAD values. The application of 2 mM TS reduced Fv/Fm values in comparison to control plants whereas the plants of the rest of the treatments presented similar Fv/Fm values. Plants supplied with 2 mM TS dose showed lower PIABS value whereas the application of this dose enhanced the PIABS index in plants subjected to Cd stress. Regarding RC/ABS, plants that received 2 mM TS showed lower values. Likewise, plants subjected to Cd stress also showed lower RC/ABS values although the application of TS increased RC/ABS value in Cd stressed plants, especially 2 mM dose (Table 2).

**Table 2**. SPAD and Chl *a* fluorescence parameters in *B. oleracea* plants supplied with TS, treated with Cd, and the combination of both treatments.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | SPAD | PIABS | Fv/Fm | RC/ABS |
| Control | 47.36±2.76a | 6.16±1.14b | 0.823±0.008b | 0.84±0.04ab |
| 2 mM TS | 45.96±2.72a | 4.11±0.59c | 0.807±0.004c | 0.72±0.03c |
| 4 mM TS | 46.07±2.16a | 6.07±0.70b | 0.828±0.003ab | 0.81±0.03b |
| Cd | 44.08±1.70a | 5.78±0.95b | 0.826±0.006ab | 0.69±0.05c |
| Cd + 2 mM TS | 45.50±2.89a | 8.19±0.99a | 0.829±0.001a | 0.87±0.08a |
| Cd + 4 mM TS | 44.71±2.79a | 5.78±0.93b | 0.830±0.004a | 0.78±0.05b |
| *p*-value | NS | \*\*\* | \*\*\* | \*\*\* |
| LSD0.05 | 3.29 | 1.04 | 0.006 | 0.06 |

Values are means ± standard error (n=9) and differences between means were compared by Fisher’s least-significance test (LSD; P=0.05). Values with different letters indicate significant differences. The levels of significance were represented by p>0.05: NS (not significant), p<0.05 (\*), p<0.01 (\*\*) and p<0.001 (\*\*\*).

*3.4. ROS and GSH levels, and antioxidant tests*

The application of TS increased MDA concentration in comparison to control conditions. Plants subjected to Cd stress showed the highest MDA levels and TS decreased its levels especially 2 mM dose. Plants treated with TS and Cd toxicity showed higher O⁠2˙‾ levels in comparison to control conditions. In addition, 4 mM TS dose increased O⁠2˙‾ in comparison to Cd-toxicity plants. Regarding H2O2, TS did not affect to H2O2 concentration in comparison to control conditions. However, plants subjected to Cd stress showed higher H2O2 levels. The application of 2 mM TS to these plants reduced H2O2 levels but 4 mM TS increased it (Table 3).

|  |  |  |  |
| --- | --- | --- | --- |
|  | MDA (μM g-1 FW) | O⁠2˙‾ (μg g-1 FW) | H2O2  (μg g-1 FW) |
| Control | 3.60±0.32d | 1.81±0.05d | 3.02±0.07d |
| 2 mM TS | 2.62±0.33e | 3.01±0.24c | 2.82±0.06d |
| 4 mM TS | 2.95±0.29e | 3.06±0.24c | 3.98±0.13d |
| Cd | 6.91±0.52a | 3.46±0.08bc | 33.84±3.23b |
| Cd + 2 mM TS | 4.78±0.54c | 3.89±0.08b | 24.19±3.00c |
| Cd + 4 mM TS | 5.78±0.32b | 4.71±0.17a | 41.09±2.47a |
| *p*-value | \*\*\* | \*\*\* | \*\*\* |
| LSD0.05 | 0.38 | 0.46 | 1.95 |

**Table 3**. MDA and ROS concentrations in *B. oleracea* plants supplied with TS, treated with Cd and the combination of both treatments.

Values are means ± standard error (n=9) and differences between means were compared by Fisher’s least-significance test (LSD; P=0.05). Values with different letters indicate significant differences. The levels of significance were represented by p>0.05: NS (not significant), p<0.05 (\*), p<0.01 (\*\*) and p<0.001 (\*\*\*).

TS application decreased total GSH and reduced GSH levels whereas Cd stress increased them in comparison to control plants. However, TS, especially 4 mM TS dose, decreased GSH concentration compared to Cd-stressed plants. Concerning GSSG, 4 mM TS reduced its levels whereas Cd toxicity increased GSSG levels. Besides, the application of TS to Cd-stressed plants reduced GSSG concentration. These results lead to a lower GSH/GSSG ratio in plants that received TS. Plants that received the Cd + 2 mM TS treatment showed the highest GSH/GSSG ratio whereas the Cd + 4 mM TS registered the lowest GSH/GSSG levels (Table 4).

**Table 4**. GSH forms and GSH/GSSG ration in *B. oleracea* plants supplied with TS, treated with Cd and the combination of both treatments.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | Total GSH  (μg g-1 FW) | GSH  (μg g-1 FW) | GSSG  (μg g-1 FW) | GSH/GSSG |
| Control | 37.67±6.46c | 35.36±4.13c | 4.65±0.77c | 7.72±1.12b |
| 2 mM TS | 27.59±3.07d | 25.23±2.20d | 4.02±0.95c | 6.50±1.12c |
| 4 mM TS | 14.98±3.39e | 13.97±2.84e | 2.50±0.52d | 5.60±0.30d |
| Cd | 68.17±5.50a | 60.17±4.36a | 8.00±1.28a | 7.62±0.74b |
| Cd + 2 mM TS | 60.02±5.75b | 54.12±5.26b | 5.90±0.65b | 9.21±0.68a |
| Cd + 4 mM TS | 29.80±5.36d | 24.26±4.63d | 5.54±0.79b | 4.37±0.35e |
| *p*-value | \*\*\* | \*\*\* | \*\*\* | \*\*\* |
| LSD0.05 | 4.81 | 3.83 | 0.82 | 0.75 |

Values are means ± standard error (n=9) and differences between means were compared by Fisher’s least-significance test (LSD; P=0.05). Values with different letters indicate significant differences. The levels of significance were represented by p>0.05: NS (not significant), p<0.05 (\*), p<0.01 (\*\*) and p<0.001 (\*\*\*).

Regarding the antioxidant test, plants supplied with 2 mM TS showed higher FRAP levels. On the other hand, Cd toxicity did not affect FRAP levels in *B. oleracea* plants although TS in combination with Cd stress increased FRAP levels especially 4 mM dose (Fig. 3A). With respect TEAC test, all treatments showed higher levels in comparison to control plants. Indeed, the application of TS increased TEAC levels when plants were subjected to Cd stress (Fig. 3B).

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**Figure 3**. Effect of TS and Cd on FRAP (A) and TEAC (B) antioxidant tests. Values are expressed as means ± standard error (n=9). Columns marked with the same letters were not significantly different based on the LSD test (P < 0.05).

**4. DISCUSSION**

*4.1. Plant biomass*

A proper S nutrition is crucial to ensure biomass production in *Brassica* crops (Santos et al., 2012).Several studies proved that the application of different S forms to *Brassica* plants grown in substrates contaminated with As and Cd enhanced the plant growth (Lou et al., 2017; Zhou et al., 2018). On the other hand, researchers observed different results when TS is applied to plants. Thus, TS enhanced growth in *Oxalis corniculate* (Liu et al., 2018), but it had no effect in *B. juncea* and *B. napus* and reduced biomass in combination with a high Hg dose (Wang et al., 2018). In the present experiment, the Na2S2O3 application reduced shoot biomass of *B. oleracea* plants under both control and Cd-toxicity conditions. This apparent toxicity may be due to an increment of salt concentration as proposed by Wang et al. (2012). Regarding root biomass,Steinitz and Bilavendran (2011) observed a positive effect of Na2S2O3 had against Ag and Cu toxicities in tomato roots. In the present experiment, we observed a negative effect of Na2S2O3 under control conditions but it did not cause any effect in Cd-stressed plants. Therefore, the biomass reduction produced by TS makes it incompatible with its use as a biostimulant at the doses tested in this study.

*4.2. Cd accumulation*

Several studies showed that S affects the accumulation of heavy metals in plants. This effect can be either positive or negative affecting the availability in the soil, the uptake by plants, and its transport to the shoot (Lou et al., 2017; Zhou et al., 2018). Regarding heavy metal accumulation, some studies proved the TS is effective in enhancing Hg and As accumulation in both roots and shoots (Grifoni et al., 2015; Wang et al., 2018; Liu et al., 2018). Thus, TS can complex with metal ions and increase its availability, the uptake, and its translocation through vascular tissue (Steinitz and Bilavendran, 2011).In the present study, the effect of Na2S2O3 depends on the dose applied. Thus, 2 mM TS increased Cd concentration by 82% and Cd content by 55% whereas 4 mM TS reduced Cd concentration by 41% and Cd content by 58%. The lower TS dose might promote the binding of Cd to TS and its transport to the shoot. On the other hand, the higher dose might saturate the transport systems that transport Cd to the shoot. These results, highlight the importance of the dose for the use of TS. Hence, the 2 mM Na2S2O3 dose could enhance the phytoremediation efficiency of Cd-polluted culture media, whereas 4 mM could prevent Cd accumulation in *B. oleracea* plants for food consumption.

*4.3. Nutrients concentration*

In general, other studies showed that TS and other S applications treatments improved the accumulation of S and other nutrients (Brodowska and Kaczor, 2009; Zhou et al., 2018). However, in our study TS application was not effective to increase S accumulation in the shoot. The extra S supply probably is not efficiently absorbed by roots or it is not transported to the shoot. In addition, TS reduced the accumulation of other macronutrients. This effect was especially observed in Ca and K accumulations. A reduction in K concentration was also observed by Wang et al. (2018) that suggested that TS causes damages to membranes of root cells leading to K leakage and thereby less K is available to the transport to the leaves. The lower K values in Cd + 4 mM TS plants suggest greater membrane damage which might hinder Cd uptake and limit Cd accumulation in these plants. On the other hand, under Cd stress, the negative effect of Na2S2O3 on macronutrient accumulation was lower and even enhanced Ca and P accumulations. Brodowska and Kaczor (2009) observed similar results as TS increased Ca accumulation in wheat and cocksfoot. Conversely, in our experiment, Na2S2O3 did not cause negative effects on micronutrients concentration and even considerably enhanced Zn accumulation. Thus, 2 mM and 4 mM increased Zn content by 14% and 34% respectively and this effect was also observed under Cd toxicity with roughly 45% of increment. In fact, Cd reduced micronutrient accumulation but TS, especially 2 mM dose, restored their accumulations. Zn, like Cd, is a harmful heavy metal when it is present in the environment at high concentrations (Szczygłowska et al., 2011); thereby TS could be useful for Zn phytoremediation. On the other hand, when Zn is not available for plants it leads to Zn deficiency being a widespread deficiency in food crops (Kabir et al., 2014). Therefore, the addition of TS could be beneficial in Zn biofortification programs.

*4.3. SPAD and fluorescence parameters*

A study on *Brassica campestris*, in an As-contaminated soil, showed that the addition of different S states (Na2SO3, Na2SO4, and S0) to the plants caused an increase in photosynthetic pigments (Zhou et al., 2018). In the present study, neither Na2S2O3 nor Cd showed a negative effect on chlorophyll accumulation because SPAD values in *B. oleracea* plants did not present significant differences between treatments. However, 2 mM Na2S2O3 dose affected negatively to photosynthesis performance when no Cd was applied whereas under Cd toxicity TS had a positive effect as increased RC/ABS and PIABS values. Hence, Na2S2O3 contributed to maintain active reaction centers and to maintain a normal photosynthetic performance under Cd stress conditions.

*3.4. ROS and GSH levels and antioxidant tests*

In the Lou et al. (2017) and Zhou et al. (2018) studies, the application of exogenous S in different forms to *Brassica* plants grown in a Cd-contaminated medium, reduced the MDA and H2O2 levels and increased GSH accumulation, which indicates a protective effect of S enhancing antioxidant defenses. In the present study, we observed an increase in oxidative stress indicators as a result of Cd toxicity. However, the 2 mM TS application reduced the values of all the analyzed stress indicators whereas the 4 mM was detrimental because increased ROS levels.

It is expected that the contribution of S to the plant favors the presence of GSH as observed in *B. campestris* (Zhou et al., 2018). In contrast, our results showed a considerable decrease in GSH concentration as a result of TS application and 4 mM TS showed the largest reduction. However, the better GSH/GSSG ratio observed in plants supplied with 2 mM Na2S2O3 might contribute to the lower stress indicators observed in these plants because the reduced form is the main form involved in antioxidant response (Gill and Tujeta, 2010). On the other hand, GSH and phytochelatins usually are accumulated under heavy metal stress to help in the sequestration of these metals in the vacuoles but at the same time could enhance the accumulation in the shoot (Sun et al., 2007). In the present experiment, GSH did not contribute to the higher Cd accumulation in plants that received 2 mM TS but the remarkable decrease in GSH in plant supplied with 4 mM might contribute to the lower Cd accumulation in these plants.

There are nutritional indicators that measure the antioxidant capacity of the plant such as the FRAP and TEAC tests. These tests reflect the amount of antioxidants compounds and the capacity of the antioxidant machinery (Song et al., 2010). The results showed that Na2S2O3 treatments significantly increased FRAP and TEAC levels. According to our data, authors such as Li et al. (2008) and Zhou et al. (2018) also showed an increase in FRAP and TEAC when different S forms were supplied. This could be due to the application of Na2S2O3 is causing oxidative stress in the plant, as noted above, and this could be the cause of the reduction in leaf biomass. Nevertheless, these tests also suggest that the application of Na2S2O3 could improve the nutritional quality in terms of antioxidant proprieties.

**5. CONCLUSION**

According to the results, TS was not effective as a biostimulant at the doses assayed because it reduced the biomass and had not positive effects on the nutrient accumulation of *B. oleracea* plants. Although TS enhanced the antioxidant capacity and considerably enhanced Zn accumulation. Thereby, TS could be used in Zn phytoremediation and Zn biofortification programs. In addition, the 2 mM TS dose increased Cd accumulation and could be effective for Cd phytoremediation purposes. Besides, on Cd stressed plants, 2 mM TS increased micronutrient accumulation and photosynthesis performance, and reduced oxidative stress. Briefly, although TS can not be used as a biostimulant it could be used for Cd phytoremediation purposes and to enhance Zn accumulation in *B. oleracea* plants. However, further research is needed to understand the relationship between TS and Cd accumulation in plants.

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**REFERENCIAS**

Alfaro, M., Bernier, R., Iraira, S., 2006. Effect of sulphur source effect on yield and quality of wheat and pasture ion two andisols. Agric. Técnica 66, 283–294.

Ashraf, Sana, Ali, Q., Zahir, Z.A., Ashraf, Sobia, Asghar, H.N., 2019. Phytoremediation: Environmentally sustainable way for reclamation of heavy metal polluted soils. Ecotoxicol. Environ. Saf. 174, 714–727. https://doi.org/10.1016/J.ECOENV.2019.02.068

Benzie, I.F.F., Strain, J.J., 1996. The Ferric Reducing Ability of Plasma (FRAP) as a measure of “Antioxidant power”: The FRAP assay. Anal. Biochem. 239, 70–76. https://doi.org/10.1006/ABIO.1996.0292

Brodowska, M.S., Kaczor, A., 2009. The effect of various forms of sulphur and nitrogen on calcium and magnesium content and uptake in spring wheat (*Triticum aestivum* L.) and cocksfoot (*Dactyhs glomerata* L.). J. Elem. 14, 641–647.

Cai, Y., Luo, Q., Sun, M., Corke, H., 2004. Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer. Life Sci. 74, 2157–2184. https://doi.org/10.1016/J.LFS.2003.09.047

Calvo, P., Nelson, L., Kloepper, J.W., 2014. Agricultural uses of plant biostimulants. Plant Soil 383, 3–41. https://doi.org/10.1007/s11104-014-2131-8

FAO, 2017. The future of food and agriculture. Rome.

Fu, J., Huang, B., 2001. Involvement of antioxidants and lipid peroxidation in the adaptation of two cool-season grasses to localized drought stress. Environ. Exp. Bot. 45, 105–114. https://doi.org/10.1016/S0098-8472(00)00084-8

Garg, N., Kaur, H., 2013. Response of antioxidant enzymes, phytochelatins and glutathione production towards Cd and Zn stresses in *Cajanus cajan* (L.) Millsp. genotypes colonized by arbuscular mycorrhizal Fungi. J. Agron. Crop Sci. 199, 118–133. https://doi.org/10.1111/j.1439-037X.2012.00533.x

Gill, S., Tujeta, N., 2010. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiol. Biochem. 48, 909–930. https://doi.org/10.1016/J.PLAPHY.2010.08.016

Grifoni, M., Schiavon, M., Pezzarossa, B., Petruzzelli, G., Malagoli, M., 2015. Effects of phosphate and thiosulphate on arsenic accumulation in the species *Brassica juncea*. Environ. Sci. Pollut. Res. 22, 2423–2433. https://doi.org/10.1007/s11356-014-2811-1

Junglee, S., Urban, L., Sallanon, H., Lopez-Lauri, F., 2014. Optimized assay for hydrogen peroxide determination in plant tissue using potassium iodide. Am. J. Anal. Chem. 05, 730–736. https://doi.org/10.4236/ajac.2014.511081

Kabir, A.H., Swaraz, A.M., Stangoulis, J., 2014. Zinc-deficiency resistance and biofortification in plants. J. Plant Nutr. Soil Sci. 177, 311–319. https://doi.org/10.1002/jpln.201300326

Khurana, M.P.S., Sadana, U.S., Bijay-Singh, 2015. Sulfur nutrition of crops in the Indo-Gangetic plains of South Asia. John Wiley & Sons, Ltd, pp. 11–24. https://doi.org/10.2134/agronmonogr50.c2

Krom, M.D., 1980. Spectrophotometric determination of ammonia: a study of a modified Berthelot reaction using salicylate and dichloroisocyanurate. Analyst 105, 305–316. https://doi.org/10.1039/AN9800500305

Kubiś, J., 2008. Exogenous spermidine differentially alters activities of some scavenging system enzymes, H2O2 and superoxide radical levels in water-stressed cucumber leaves. J. Plant Physiol. 165, 397–406. https://doi.org/10.1016/j.jplph.2007.02.005

Law, M.Y., Charles, S.A., Halliwell, B., 1983. Glutathione and ascorbic acid in spinach (*Spinacia oleracea*) chloroplasts. The effect of hydrogen peroxide and of Paraquat. Biochem. J. 210, 899–903. https://doi.org/10.1042/BJ2100899

Li, J., Zhu, Z., Gerendás, J., 2008. Effects of nitrogen and sulfur on total phenolics and antioxidant activity in two genotypes of leaf mustard. J. Plant Nutr. 31, 1642–1655. https://doi.org/10.1080/01904160802244860

Liu, Z., Wang, L., Ding, S., Xiao, H., 2018. Enhancer assisted-phytoremediation of mercury-contaminated soils by *Oxalis corniculata* L., and rhizosphere microorganism distribution of *Oxalis corniculata* L. Ecotoxicol. Environ. Saf. 160, 171–177. https://doi.org/10.1016/J.ECOENV.2018.05.041

Lou, L., Kang, J., Pang, H., Li, Q., Du, X., Wu, W., Chen, J., Lv, J., 2017. Sulfur protects Pakchoi (*Brassica chinensis* L.) seedlings against cadmium stress by regulating ascorbate-glutathione metabolism. Int. J. Mol. Sci. 18, 1628. https://doi.org/10.3390/ijms18081628

Noctor, G., Foyer, C.H., 1998. Simultaneous measurement of foliar glutathione, γ-glutamylcysteine, and amino acids by high-performance liquid chromatography: Comparison with two other assay methods for glutathione. Anal. Biochem. 264, 98–110. https://doi.org/10.1006/ABIO.1998.2794

Pereira de Araújo, R., Furtado de Almeida, A.-A., Silva Pereira, L., Mangabeira, P.A.O., Olimpio Souza, J., Pirovani, C.P., Ahnert, D., Baligar, V.C., 2017. Photosynthetic, antioxidative, molecular and ultrastructural responses of young cacao plants to Cd toxicity in the soil. Ecotoxicol. Environ. Saf. 144, 148–157. https://doi.org/10.1016/J.ECOENV.2017.06.006

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., Rice-Evans, C., 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic. Biol. Med. 26, 1231–1237. https://doi.org/10.1016/S0891-5849(98)00315-3

Ricci, M., Tilbury, L., Daridon, B., Sukalac, K., 2019. General principles to justify plant biostimulant claims. Front. Plant Sci. 10, 494. https://doi.org/10.3389/fpls.2019.00494

Santos, R.F., Borsoi, A., Secco, D., Souza, S.N.M. de, Frigo, E.P., 2012. Nitrogen and sulfur sources in the culture of *Brassica napus* L. van oleifera. J. Food, Agric. Environ. 10, 516–518.

Song, F.-L., Gan, R.-Y., Zhang, Y., Xiao, Q., Kuang, L., Li, H.-B., 2010. Total phenolic contents and antioxidant capacities of selected chinese medicinal plants. Int. J. Mol. Sci. 11, 2362–2372. https://doi.org/10.3390/ijms11062362

Steinitz, B., Bilavendran, A.D., 2011. Thiosulfate stimulates growth and alleviates silver and copper toxicity in tomato root cultures. Plant Cell, Tissue Organ Cult. 107, 355–363. https://doi.org/10.1007/s11240-011-9987-6

Strasser, R., Srivastava, A., Tsimilli-Michael, M., 2000. The fluorescence transient as a tool to characterize and screen photosynthetic samples, in: Yunus, M., Pathre, U., Mohanty, P. (Eds.), Probing Photosynthesis: Mechanism, Regulation and Adaptation. Taylor & Francis, London, pp. 443–480.

Sun, Q., Ye, Z.H., Wang, X.R., Wong, M.H., 2007. Cadmium hyperaccumulation leads to an increase of glutathione rather than phytochelatins in the cadmium hyperaccumulator *Sedum alfredii*. J. Plant Physiol. 164, 1489–1498. https://doi.org/10.1016/J.JPLPH.2006.10.001

Szczygłowska, M., Piekarska, A., Konieczka, P., Namieśnik, J., 2011. Use of *Brassica* plants in the phytoremediation and biofumigation processes. Int. J. Mol. Sci. 12, 7760–7771. https://doi.org/10.3390/ijms12117760

Tao, Z., Chang, X., Wang, D., Wang, Y., Ma, S., Yang, Y., Zhao, G., 2018. Effects of sulfur fertilization and short-term high temperature on wheat grain production and wheat flour proteins. Crop J. 6, 413–425. https://doi.org/10.1016/J.CJ.2018.01.007

Wang, J., Anderson, C.W.N., Xing, Y., Fan, Y., Xia, J., Shaheen, S.M., Rinklebe, J., Feng, X., 2018. Thiosulphate-induced phytoextraction of mercury in *Brassica juncea*: Spectroscopic investigations to define a mechanism for Hg uptake. Environ. Pollut. 242, 986–993. https://doi.org/10.1016/J.ENVPOL.2018.07.065

Wang, J., Feng, X., Anderson, C.W.N., Wang, H., Wang, L., 2014. Thiosulphate-induced mercury accumulation by plants: metal uptake and transformation of mercury fractionation in soil - results from a field study. Plant Soil 375, 21–33. https://doi.org/10.1007/s11104-013-1940-5

Wang, J., Feng, X., Anderson, C.W.N., Wang, H., Zheng, L., Hu, T., 2012. Implications of mercury speciation in thiosulfate treated plants. Environ. Sci. Technol. 46, 5361–5368. https://doi.org/10.1021/es204331a

Wolf, B., 1982. A comprehensive system of leaf analyses and its use for diagnosing crop nutrient status. Commun. Soil Sci. Plant Anal. 13, 1035–1059. https://doi.org/10.1080/00103628209367332

Zhou, J., Hao, M., Liu, Y., Huang, G., Fu, Q., Zhu, J., Hu, H., 2018. Effects of exogenous sulfur on growth and Cd uptake in Chinese cabbage (*Brassica campestris* spp. pekinensis) in Cd-contaminated soil. Environ. Sci. Pollut. Res. 25, 15823–15829. https://doi.org/10.1007/s11356-018-1712-0