Journal of King Saud University - Science 36 (2024) 103175

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Journal of King Saud University - Science

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Full Length Article

# Mitigation of lead toxicity in *Brassica juncea* L. by sulphur application – Via various biochemical and transcriptomic strategies

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ARTICLE INFO

*Keywords:*
Antioxidants
*Brassica juncea*
Gene expression
Lead stress
qRT-PCR
Sulphur
Transcriptomics

ABSTRACT

The present study was designed to elucidate whether exogenously applied sulphur (S) (S1; 100 ppm, S2; 200 ppm S) alleviate lead (Pb)-induced (Pb1; 100 ppm, Pb2; 200 ppm and Pb3; 300 ppm Pb) stress in the leaves of *Brassica juncea*. To study this effect, oxidative stress biomarkers (hydrogen peroxide and malondialdehyde), enzymatic antioxidants (ascorbate peroxidase (APX), glutathione reductase (GR)), non-enzymatic antioxidants (chlorophyll and carotenoids) and S-metabolic enzymes like adenosine triphosphate sulfurylase (ATPS), O-acetylserine(thiol) lyase (OASTL) were studied. Pb treatment dramatically inhibited plant development by increasing lipid peroxidation and hydrogen peroxide ($H_2O_2$) accumulation by 34.82 and 238.89 % respectively at 90 days plant. The overproduced oxidative biomarkers alter plant cell homeostasis. In contrast, APX (25.56 %) and GR (32.45 %) activities, chlorophyll (15.51 %) and carotenoids (30 %) contents increased under similar Pb treatments and regulated ROS-antioxidant balance. Exogenous S application, maintained the redox status of cell by further increasing the enzymatic activities. Pb treatment increased S assimilation in *Brassica* by elevating enzyme activities of ATPS and OASTL, which was further augmented by S supplementation to Pb-stressed plants. Besides gene expression of *BjSULT*, *BjOASTL* and *BjGR1* exhibited slight increase under Pb stress, S application to the stressed plants doubled the expression and assisted the stress resistance. Overall, our findings demonstrate that S protect Pb-stressed *Brassica* seedlings, implying that S could be an ideal choice for reducing Pb toxicity in crops.

## 1. Introduction

Environmental pollution by heavy metals and deposition in soil is of great solicitude in agricultural production owing to the destruction on crop growth heavy metals were deposited in agricultural areas as a result of fast industrialization, improper trace metal consumption, disposal, and dumping into the environment, as well as excessive fertilizer and pesticide use (Amel et al., 2016) that posed a threat to human and environmental health. Plants succumb to stress and remodel their metabolic systems and gene expression in response to these metals absorption (e.g., lead, arsenic, cadmium) (Dixit et al., 2016). Under Pb stress, enhanced reactive oxygen species (ROS) production cause damage to cellular organelles (Hasan et al., 2019). The disturbed cellular homeostasis is regulated by APX and GR antioxidants. Besides, overproduced $H_2O_2$ and malondialdehyde (MDA) is counterattacked by chlorophyll and carotenoids (Dixit et al., 2016, Kohli et al., 2019).

Sulphur is an important macronutrient that assists in heavy metal detoxification, particularly Pb. Sulphur is transferred into plants by sulphate transporter genes (*SULT*) and assimilates into compounds like cysteine (Cys) and methionine (Met) by a enzymatic processes mediated by ATP sulfurylase (ATPS) and O-acetylserine (thiol) lyase (OASTL) enzymes (Ma et al., 2018). The GSH is a significant redox buffer and precursor of phytochelatins (PC), which bind to heavy metals and detoxifies in the form of PC-metal complexes, and transported to vacuoles through *ABC* transporters for metal sequestration (Song et al., 2010). Furthermore, multiple studies have discovered that providing additional

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https://doi.org/10.1016/j.jksus.2024.103175
Received 30 August 2023; Received in revised form 17 March 2024; Accepted 19 March 2024
Available online 20 March 2024

S to plants, enhanced their antioxidant system, thiol metabolism, and metal toxicity (Liang et al., 2016). Further, to Cd toxic pakchoi, exogenous S promoted sulphur assimilation and boosted critical S genes involved in S metabolism and thiol pool (Lou et al., 2017).

Additionally, sulfate deficiency might make plants more stressed by heavy metals, whereas a sufficient sulfate supply can lessen the toxicity of heavy metals (Na and Salt, 2011; Astolfi et al., 2014). However, physiological processes that underpin the interactions between heavy metals and S is largely unknown. In plants, a number of genes transcriptional levels also regulate lead accumulation and sulfate absorption. In *Arabidopsis thaliana* L. ATP-binding cassette subfamily C transporter 1 (*ABCC1*) plays a key role in uptaking of heavy metals and translocating into vacuoles (Köhler and Neuhaus, 2000; Cao et al., 2009). Heavy metal exposure has been connected to alterations in the genetic level of herbaceous plants (Takahashi et al., 2011), but the transcriptional regulation of genes in mustard when exposed to Pb and/or varied S treatments remain mostly unknown.

Mustard (*Brassica juncea* (L.) Czern & Coss) is an important Indian oilseed. *Brassica* hyperaccumulates heavy metals, making it vulnerable to metals and damaging pigments, growth, and yield. Sulphur improved antioxidants and S-assimilating enzymes, reducing metal toxicity (Liang et al., 2016; Singh et al., 2020). Till date, S relationship to Pb remains unknown. Thus, current study examined how sulphur affects antioxidant enzyme activities, stress biomarkers, photosynthetic pigment concentration, and gene expression in lead-stressed Indian mustard (*Brassica juncea* (L.) Czern & Coss).

## 2. Materials and methods

### 2.1. Crop raising

Indian mustard [*Brassica juncea* (L.)] variety of RH-749 was grown in the screen house, Department of Biochemistry. Stress conditions were achieved by treating various concentrations (100, 200 and 300 ppm) of Pb [$Pb(NO_3)_2$] dissolved in water. To ameliorate Pb-stress, two different concentrations (100 and 200 ppm) of sulphur [$Zn(SO_4).7H_2O$] was sprayed. The experiment was established with 12 treatments (three replicates each treatment): control, Pb1 (100 ppm Pb), Pb2 (200 ppm Pb), Pb3 (300 ppm Pb), S1 (100 ppm S), S2 (200 ppm S) and following combinations were made for present study: Pb1 + S1, Pb1 + S2, Pb2 + S1, Pb2 + S2, Pb3 + S1 and Pb3 + S2. Here, Pb1, Pb2 and Pb3 treatments are with Pb alone, S1 and S2 treatments are with sulphur alone. Lead spray was done 5 days prior to sampling and sulphur spraying was done 3 days prior to sampling. Leaf samples were collected at vegetative stage −30 days after sowing (DAS), flowering stage (60 DAS) and pod filling stage (90 DAS) for further experimental analysis.

### 2.2. Oxidative stress biomarkers

#### 2.2.1. Hydrogen peroxide ($H_2O_2$)

The content of hydrogen peroxide was determined following the Velikova et al. (2000). Extract was centrifuged with trichloroacetic acid (TCA, 0.1 %), phosphate buffer (10 mM) and 1 M potassium Iodide was added and absorbance was recorded at 390 nm. Standard curve of 10–100 μmoles of $H_2O_2$ was used to find $H_2O_2$ content.

#### 2.2.2. Lipid peroxidation

Lipid peroxidation was determined by TBARS content following Heath and Packer (1968) method. Extract was centrifuged with TCA (20 %), to 1 ml supernatant TCA containing thiobarbituric acid (0.6 %) was added. Mixture was heated at 95 °C for 30 min, absorbance was taken at 532 nm and at 600 nm. The peroxidation of lipids was estimated by the extinction coefficient 155 $mM^{-1}cm^{-1}$.

### 2.3. Enzymatic antioxidants

#### 2.3.1. Preparation of enzyme extract

The leaves of *B. juncea* at three different stages *viz.*, 30, 60 and 90 DAS were used for enzymatic studies. The leaves of 1 g was homogenized 5 ml phosphate buffer containing EDTA, β-mercaptoethanol and PVP. Whereas for ascorbate peroxidase the extraction buffer also contained ascorbate. Homogenate was centrifuged and supernatant was carefully transferred into the tubes and used for further analysis.

#### 2.3.2. Glutathione reductase (GR) (EC: 1.6.4.2)

Glutathione reductase activity was estimated according to Schaedle and Bassham (1977). To the 200 μl extract, phosphate buffer, oxidized glutathione (GSSG), NADPH was added. The reaction was initiated by adding NADPH, absorbance measured at 340 nm and GR activity was estimated by using the extinction coefficient of NADPH 6.22 $nM^{-1}$ $cm^{-1}$.

#### 2.3.3. Ascorbate peroxidase (EC 1.11.1.11)

Ascorbate peroxidase was estimated according to Nakano and Asada (1981). To 0.1 ml extract, phosphate buffer (pH 7.0), AsA, EDTA, $H_2O_2$ was added. Absorbance recorded at 290 nm. The APX activity was estimated by the extinction coefficient of ascorbate 2.8 $mM^{-1}$ $cm^{-1}$.

### 2.4. Sulphur assimilating enzymes

#### 2.4.1. Atp-sulfurylase (ATPS/APS) (EC: 2.7.7.2)

ATPS (EC2.7.7.4) was estimated following Lappartient and Touraine (1996) procedure. Fresh tissue (0.1 g) was ground in Tris-HCl (pH 8.0) constituting EDTA, DTT and PVP and centrifuged at 20,000*g* for 10 min at 4 °C. The reaction contains $MgCl_2$, $Na_2MoO_4$, $Na_2ATP$ and sulfate-free inorganic pyrophosphate in Tris-HCl (pH 8.0). A second portion was prepared without $Na_2MoO_4$. Phosphate present in mixture was estimated spectrophotometrically by incubating mixture for 15 min at 37 °C.

#### 2.4.2. O-acetyl serine thiol lyase (OASTL) (EC: 4.2.99.8)

OASTL (EC 4.2.99.8) was estimated following Riemenschneider et al. (2005) method. Tissues were homogenized in ice-cold Tris-HCl before being centrifuged. To the 50 μl extract, OAS, $Na_2S$, DTT, Tris-HCl (pH 7.5) was added and incubated (30 min, 37 °C). Afterwards, acidic ninhydrin addition terminates reaction and Cysteine was determined. Finally, colored solution was neutralized by 900 μl, 70 % ethanol addition to sample and absorbance recorded at 560 nm.

### 2.5. Non-enzymatic antioxidants

#### 2.5.1. Carotenoid

The carotenoid content in the leaves was estimated according to Wellburn (1994). The carotenoids extracted in the DMSO were measured by reading the absorbance at 480 nm.

#### 2.5.2. Chlorophyll

By using SPAD 502 chlorophyll meter, the chlorophyll content of the plants was measured instantly to reduce the risk of yield limiting deficiencies.

### 2.6. Gene expression analysis

The fresh seedlings of 30, 60 and 90 days *B. juncea* plants was used for RNA extraction. Trizol method was followed to extract the RNA. Treatment of RNA with RNAse free DNase was done before gene expression analysis and RNA was quantified by Nanodrop spectrophotometer. Out of total RNA first 1 μg was used for the synthesis of cDNA by using the qscript cDNA synthesis kit. For gene expression, actin gene in *B. juncea* used to normalise gene expression of the selected genes and the primers used are presented in Table 1. At each time point, the

**Table 1**
List of primers used for RT-PCR.

| S. No. | Primer | Forward primer sequence (5′-3′) | Reverse primer sequence (5′-3′) |
|---|---|---|---|
| 1 | *BjSULT* | CGAAGGAAGGTTTGCTGAAG | TTGTACTCTCTGGCCCATCC |
| 2 | *BjPb* | GCAAGCATGGAGGAAACTG | AGGCCACCGTGTAACCAAGT |
| 3 | *BjABCC* | AGAATCGTGGAGCTGGAGAA | AAAAGAACCGGTGACTGTGG |
| 4 | *BjATPS* | GGATGCTGTCTTTGCTTTCC | ACCTTCTCGTGCTGCTTCAT |
| 5 | *BjOASTL* | CTTGAATCACCATGGCCTCT | CTTAGCAGCAACACGACCAA |
| 6 | *BjGR1* | GGACGTCCCTTCATTCCTGA | CCCATTAAAGATGCCCGCAA |
| 7 | *Actin* | CAGTACTGCTGACTGAGGCG | TGCGTCCACTAGCATATAGGG |

expression of a subset of genes was compared to that of the reference gene (β-actin gene), and the resulting expression ratio was calculated in terms of change in the fold expression. The PCR conditions used were initial denaturation at for 5 min, 40 cycles of denaturation at 95 °C for 15 sec, annealing at 59 °C for 30 sec and extension at 65 °C for 1 min. The qRT-PCR was done on Biorad system (CFX96 Touch system) by One-step real-time detector and using SYBR Green PCR Master Mix.

### 2.7. Statistical analysis

The research data was analyzed with SPSS 24.0 statistical software package, as mean of three replicates using two factor analysis of variance (ANOVA) by completely randomized design (CRD) to evaluate the significant difference of means, using 5 % level of significance.

## 3. Results

### 3.1. Oxidative biomarkers

#### 3.1.1. Hydrogen peroxide

Lead stress of 100, 200 and 300 ppm caused significant $H_2O_2$ increase by 69.43, 201.59 and 303.34 % (30 DAS), 97.90, 193.13 and 317.39 % (60 DAS), 73.48, 144.89 and 238.89 % (90 DAS), respectively, in comparison to control (Fig. 1A). The S1 sulphur spray has decreased

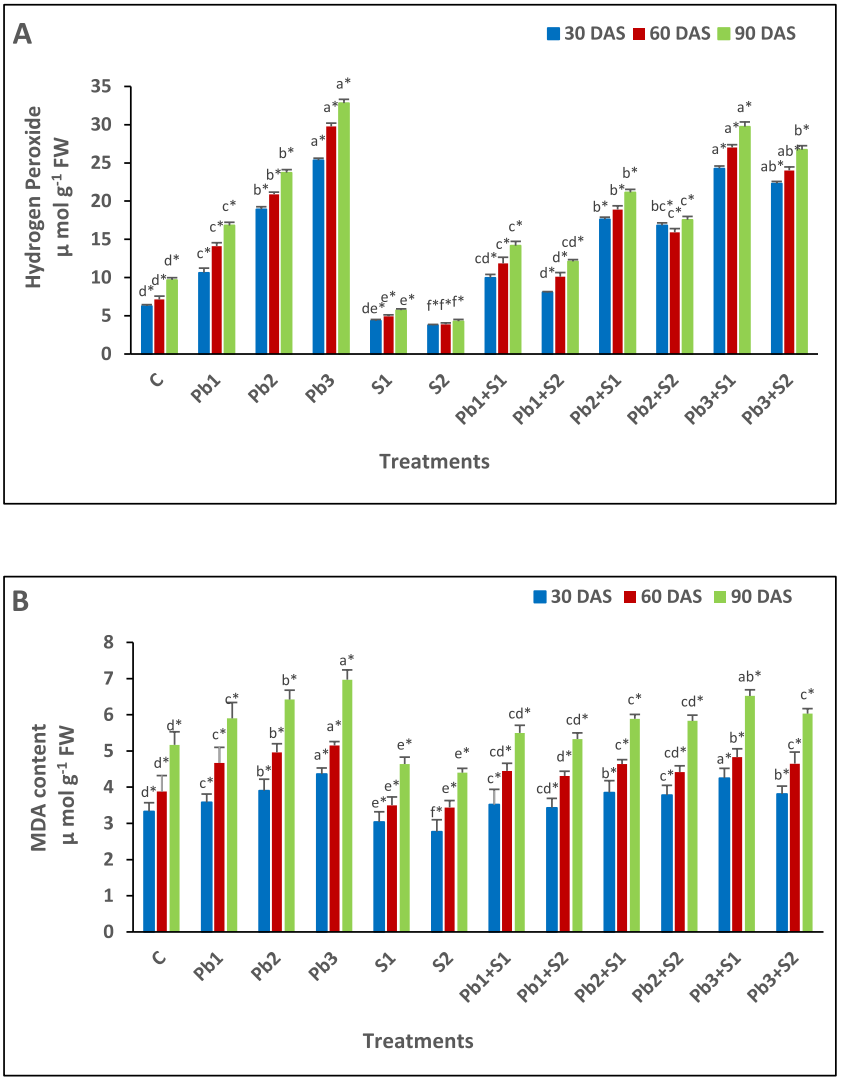


**Fig. 1.** Determination of the impact of exogenous sulphur under lead stress on oxidative biomarkers (**A**) Hydrogen peroxide; (**B**) Malondialdehyde (MDA) in Indian mustard. Various letters indicate statistically significant difference ($P < 0.05$) between treatments within a developmental stage.

$H_2O_2$ by 30.73, 31.00 and 40.87 % at S2 spray $H_2O_2$ decreased by 40.13, 45.72 and 55.73 %, respectively at 30, 60 and 90 DAS, in respect to control. Maximum reduction of $H_2O_2$ was observed under S2 spray at Pb1 + S2 as 24.15, 28.35 and 27.96 % in comparison to Pb1 stress, whereas at Pb2 + S2, 11.19, 23.83 and 26.00 % in comparison to Pb2 stress, and at Pb3 + S2, 11.92, 19.29 and 18.62 % in comparison to Pb3 stress, at 30, 60 and 90 DAS, respectively.

### 3.1.2. Malondialdehyde

The MDA content under 100, 200 and 300 ppm lead stress was increased by 7.51, 17.12 and 30.93 % at 30 DAS, 20.36, 27.84 and 32.73 % at 60 DAS and at 90 DAS, MDA content was enhanced by 14.12, 24.18 and 34.82 %, respectively, in respect to control. S1 spray reduced MDA content by 8.71, 9.79 and 10.25 % and 16.82, 11.34 and 14.89 % at S2 spray at 30, 60 and 90 DAS, respectively, in comparison to control. Sulphur treatment of 100 and 200 ppm to the Pb3 + S1, Pb3 + S2, MDA content showed a decrease of 2.52 and 12.61 % (30 DAS), 6.21 and 9.71 % (60 DAS), 6.46 and 13.49 % (90 DAS), respectively, in respect to Pb3 stress (Fig. 1B).

## 3.2. Enzymatic antioxidants

### 3.2.1. Ascorbate peroxidase (APX)

The Pb treatments of 100, 200 and 300 ppm has significantly increased APX activity by 22.75, 33.56 and 57.84 % at 30 DAS, and 14.35, 32.66 and 46.47 % increase at 60 DAS and at 90 DAS 8.34, 15.21 and 25.56 % increase, respectively, compared with control (Fig. 2A). APX activity increased by 14.84, 14.78 and 4.86 % at S1 spray, and 27.60, 23.55 and 7.03 % at S2 spray, respectively, at 30, 60 and 90 DAS, with respect to control. However, S2 enhanced APX activity by 10.40, 7.96 and 7.20 % at Pb1 + S2, and 13.19, 6.86 and 5.90 % at Pb2 + S2, and 8.26, 10.96 and 5.29 % in correspondence to Pb1, Pb2 and Pb3 stress, at 30, 60 and 90 DAS, respectively.

### 3.2.2. Glutathione reductase (GR)

Lead treatments of 100, 200 and 300 ppm significantly increased GR content by 18.82, 32.30 and 51.97 % (30 DAS), whereas 9.91, 23.42 and 32.99 % (60 DAS) and 9.01, 19.74 and 32.45 % (90 DAS) respectively, in comparsion to control (Fig. 2B). S1 spray elevated GR content by 17.13, 12.14 and 14.70 % and S2 spray by 23.88, 19.32 and 21.19 % in comparison to control, at 30, 60 and 90 DAS respectively. S1 spray to Pb-

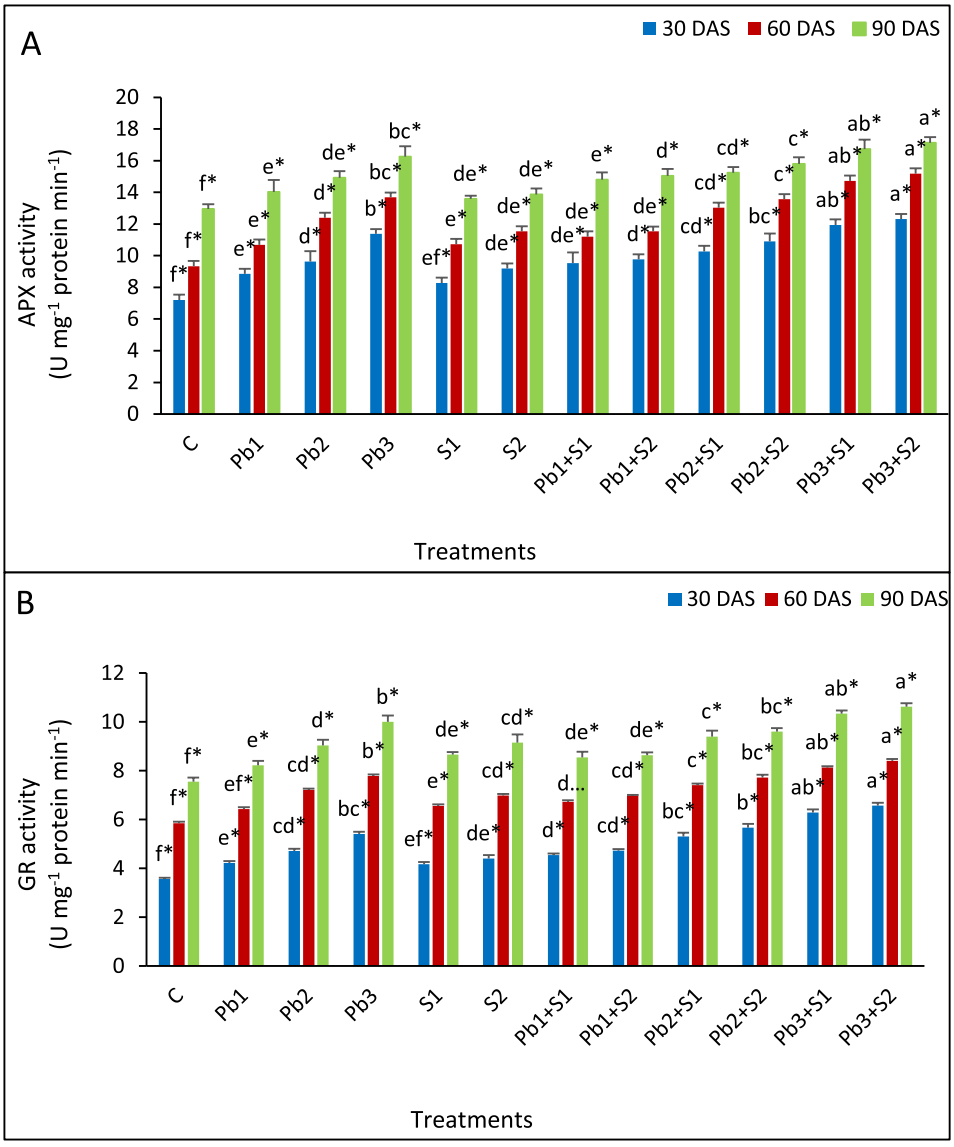


**Fig. 2.** Determination of the impact of exogenous sulphur under lead stress on antioxidative enzymes (**A**) Ascorbate peroxidase (APX); (**B**) Glutathione reductase (GR) in Indian mustard leaves. Various letters indicate statistically significant difference ($P < 0.05$) between treatments within a developmental stage.

stressed *Brassica* showed highest enhancement at Pb3 + S1 stress by 16.27, 4.63 and 3.40 % in comparison to Pb3 stress at 30, 60 and 90 DAS, respectively. S2 spray to Pb-stressed *Brassica* showed an enhancement of 21.44, 7.97 and 6.30 %at Pb3 + S2 in correspondence to Pb3 stress at 30, 60 and 90 DAS, respectively.

### 3.3. Sulphur-assimilating enzymes

#### 3.3.1. ATP sulfurylase (ATPS)

The ATPS activity was increased by 3.53, 6.96 and 6.11 % (Pb1), 14.12, 13.04 and 10.69 % (Pb2), 21.18, 22.30 and 14.50 % (Pb3) at 30, 60 and 90 DAS, respectively, with respect to control (Fig. 3A). The ATPS activity was augmented upon exogenous S application of 100 and 200 ppm, by 11.76 and 14.12 % (30 DAS), 8.70 and 14.78 % (60 DAS) and by 3.82 and 8.40 % (90DAS), respectively, with respect to control. Further ATPS activity increased under S2 by 7.95, 5.69 and 2.88 % at Pb1 + S2, similarly, 5.49, 4.62 and 3.45 % increase at Pb2 + S2, and 7.77, 4.35 and 7.33 % increase at Pb3 + S2, the increase was in respect to Pb1, Pb2 and Pb3 stress, respectively, at 30, 60 and 90 DAS.

#### 3.3.2. O-acetylserine (thiol) lyase (OASTL)

Under Pb1, Pb2 and Pb3 stress, OASTL activity increased by 7.58, 15.15 and 21.21 % (30 DAS), 5.13, 11.54 and 17.95 % (60 DAS), 11.36, 14.77 and 25 % (90 DAS), respectively, in comparison to control. At S1 spray, OASTL activity increased by 4.55, 2.58 and 3.41 %, S2 spray showed 9.09, 7.69 and 5.68 % at 30, 60 and 90 DAS, respectively, in respect to control. Highest increase in OASTL activity under S1 spray to lead stressed plants was reported at Pb3 + S1 by 3.75, 3.26 and 3.64 % in respect to Pb3 stress, respectively at 30, 60 and 90 DAS. S2 application to Pb1 + S2 plants, activity increased by 7.14, 6.10 and 1.08 %, in respect to Pb1 stress at 30, 60 and 90 DAS. Similar increase was observed under Pb2 + S2 and Pb3 + S2.

### 3.4. Non enzymatic antioxidants

#### 3.4.1. Chlorophyll

The graphical representation (Fig. 4A) shows that chlorophyll decreased under lead stress by 11.14, 24.92 and 34.60 % (30 DAS), 4.68, 12.89 and 22.76 % (60 DAS) and 1.59, 7.30 and 15.51 % (90DAS) respectively, at 100, 200 and 300 ppm Pb, in respect to control. Further, S1 and S2 application increased chlorophyll by 9.54 and 16.83 %, 7.98

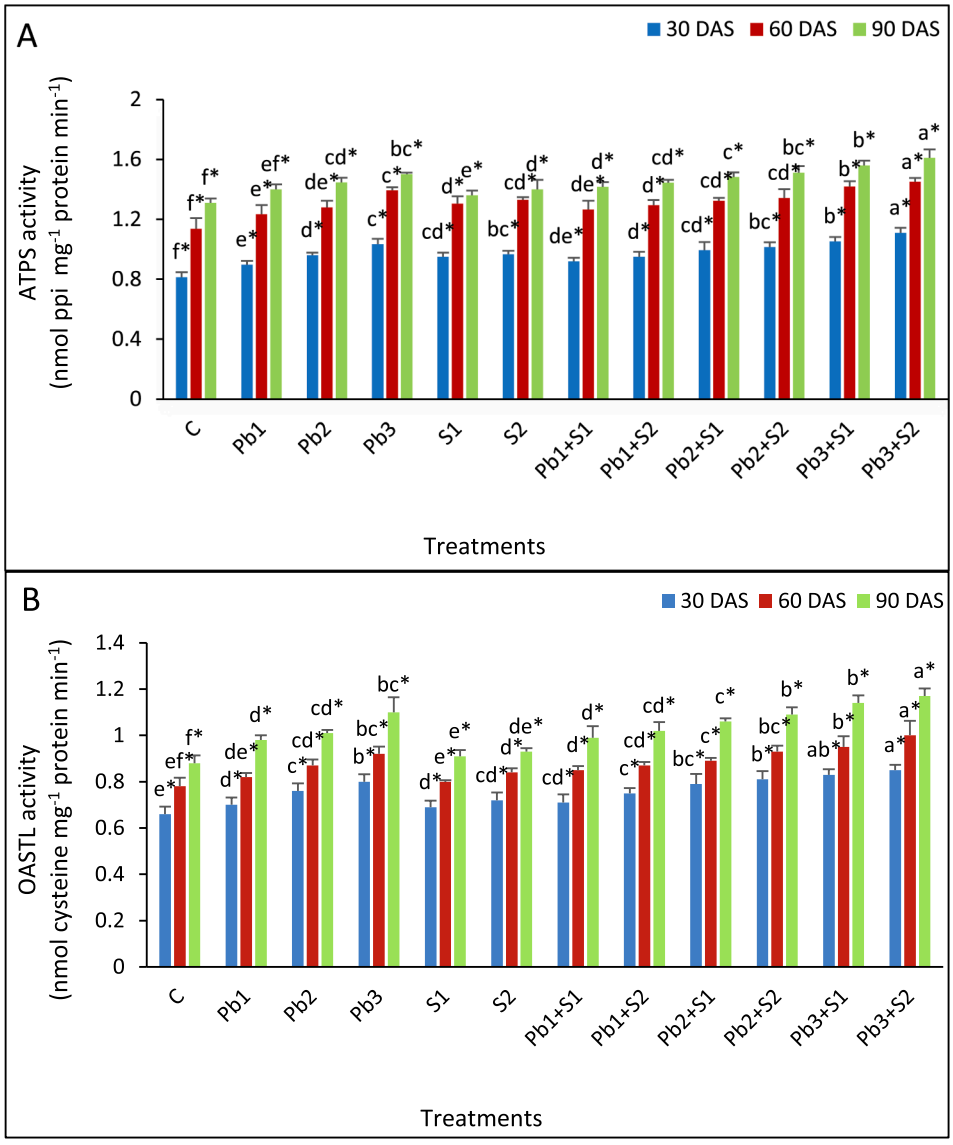


**Fig. 3.** Determination of the impact of exogenous sulphur on S assimilating enzymes (**A**) ATP-Sulfurylase (ATPS); (**B**) O-acetylserine (thiol) lyase (OASTL) in Indian mustard leaves under lead stress. Various letters indicate statistically significant difference ($P < 0.05$) between treatments within a developmental stage.

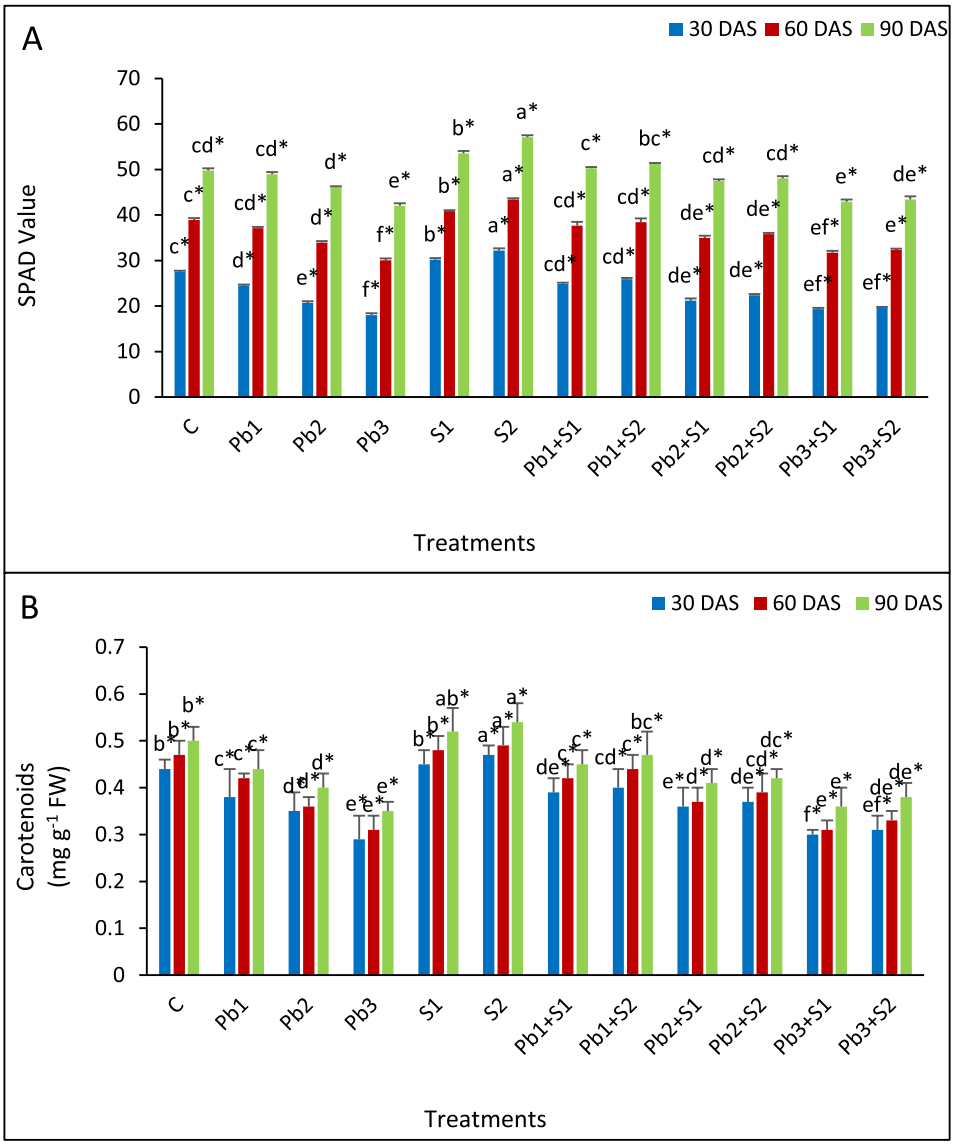


**Fig. 4.** Determination of the impact of exogenous sulphur on non enzymatic antioxidants (**A**) Chlorophyll; (**B**) carotenoids in the leaves of Indian mustard under lead stress. Various letters indicate statistically significant differences ($P < 0.05$) between treatments within a developmental stage.

and 11.53 %, 7.58 and 14.81 %, respectively, at 30, 60 and 90 DAS in comparison to control. Highest increase of chlorophyll content was seen at Pb3 + S1 and Pb3 + S2 treated plants as 7.04 and 8.99 %, 5.49 and 7.48 %, 2.16 and 3.28 % at 30, 60 and 90 DAS, respectively, in comparison to Pb3 stress.

### 3.4.2. *Carotenoids*

Lead treatment of 100, 200 and 300 ppm resulted in decrease (Fig. 4B) of carotenoid content and highest decrease was 12.00, 20.00 and 30.00 % at 90 DAS respectively, in respect to control. Further, sulphur application increased carotenoid content by 2.27, 2.13 and 4.00 % at S1 spray, and at S2 spray 6.82, 4.26 and 8.00 %, increased respectively, at 30, 60 and 90 DAS, in respect to control. S spray to Pb-stressed plants showed an increased carotenoid and highest enhancement of carotenoids was noticed at Pb3 + S2, by 6.90, 6.45 and 8.57 %, respectively, at 30, 60 and 90 DAS, in comparison to Pb3 stressed plants.

## 3.5. *Gene expression analysis*

The gene expression of *BjSULT* showed the highest relative values of 0.95 and 0.91 fold under Pb2 and Pb3 stress respectively, compared to control of 30 DAS. S1 and S2 spray showed 1.82 and 1.95-fold increase at 90 DAS. Sulphur spray of 100 and 200 ppm on Pb3 + S1 and Pb3 + S2 resulted highest gene expression levels of 1.26 and 1.32 fold, respectively, at 90 DAS. The highest *BjPb* expression was seen at 90 DAS, with 2.42 and 2.83-fold increase under Pb2 and Pb3 stress, compared to control. S1 and S2 spray resulted a decreased *BjPb* expression. The *BjPb* expression had greatest reduction at 60 DAS when S2 was applied from a 2.58-fold increase under Pb3 stress to a 2.15-fold drop under Pb3 + S2 stress, relative to the control. The *BjABCC* expression was highest at 90 DAS, with fold changes of 1.39 and 1.46, respectively, under Pb2 and Pb3 stress, comparison to control. S1 and S2 spray resulted an increased expression 1.39 and 1.41 fold (90 DAS), respectively. Specifically, at 90 DAS, highest expression of *BjABCC* found to be 1.49 fold higher under Pb3 + S2 stress compared to control. The relative gene expression of *BjATPS* and *BjOASTL* increased by 1.78 and 1.84-fold, and 1.63 and 1.69-fold, respectively, under Pb2 and Pb3 stress at 90 DAS compared to control. Further spray of S1 and S2 at 90 DAS, showed a fold increase of 1.73 and 1.85 for *BjATPS*, and 1.56 and 1.61 for *BjOASTL*, respectively. Sulphur treatment to Pb-stressed *Brassica* resulted in greatest expression at 90 DAS, with fold increases of 2.03 and 2.42 at Pb3 + S1 and Pb3 + S2 stress, respectively, compared to the control. The gene *BjGR1* exhibited

greatest level of gene expression at Pb3 stress as 1.75-fold increase compared to 90 DAS control. The *BjGR1* gene showed high upregulation of 1.84 folds change under S2 sulphur spray at 90 DAS. S2 application under Pb3 + S2 stress resulted in the greatest level of *BjGR1* expression, with a fold increase of 1.81 at 90 DAS. Fig. 5A-F displays the findings of the investigation of gene expression.

## 4. Discussion

The presence of lead has detrimental effects on plant development, impacting several aspects such as physiological, biochemical, and molecular processes (Hasanuzzaman et al., 2018). *Brassica* is a very effective bioremediant, a nourishing cow supplement, and a valuable reservoir of beneficial phytochemicals such as vitamins and minerals (Xin et al., 2013). Hence, it was imperative to investigate the effects of Pb in *Brassica* plants and crucial elements involved in maintaining cell structure and the process of assimilating sulphur. The decrease in *Brassica* development is clear sign of lead poisoning, which can be attributed to elevated levels of Pb that generate ROS. The ROS might have disrupted the delicate balance between the production of ROS and the antioxidant defence mechanism that eliminates ROS (Ahmad et al., 2019). The elevated $H_2O_2$ and MDA levels of results corresponds with findings of Bharwana et al. (2013) and Singh et al. (2020) where an increased $H_2O_2$ and MDA levels were reported under Pb stress in *Gossypium* and *Brassica juncea*. Exogenous sulphur spray to *Brassica* plants under lead stress resulted in reduction in $H_2O_2$ and MDA levels, hence minimising oxidative damage to the cells. The use of sulphur spray effectively reduced the MDA by decreasing the accumulation of $H_2O_2$ therefore promoting growth of *B. juncea* under Pb stress. Sulphur spray has decreased the $H_2O_2$ and MDA in rice (Dixit et al., 2016) and maize (Cui and Wang, 2006) plants under cadmium and arsenic stress. The use of zinc sulphate as a foliar spray resulted in decrease of $H_2O_2$ and MDA levels in *Brassica* leaves that were treated with lead as shown in a study conducted by Ahmad et al. (2017). This corroborates usage of zinc sulphate as sulphur supplement to the *Brassica* plants.

Present study shows that *Brassica* exposed to lead stress showed an increase in APX and GR activity. The activity of APX increased following the administration of sulphur, indicating that the detoxification of $H_2O_2$ was effective. Ahmad et al. (2015) and Liang et al. (2016) reported that addition of S boosted APX activity in *Brassica* plants that were exposed to Pb and Cd. Sulphur enhanced activities of APX and GR in order to safeguard the leaf tissues of *B. juncea* from harmful effects of metal stress, as reported by Singh et al. (2020). S2 spray enhanced GR and APX activities in *B. juncea*. *Vicia faba* and tartary buckwheat exhibited comparable enhancement of metal stress defence when treated with sulphur (Wu et al., 2018, Lu et al., 2019). This study demonstrates that

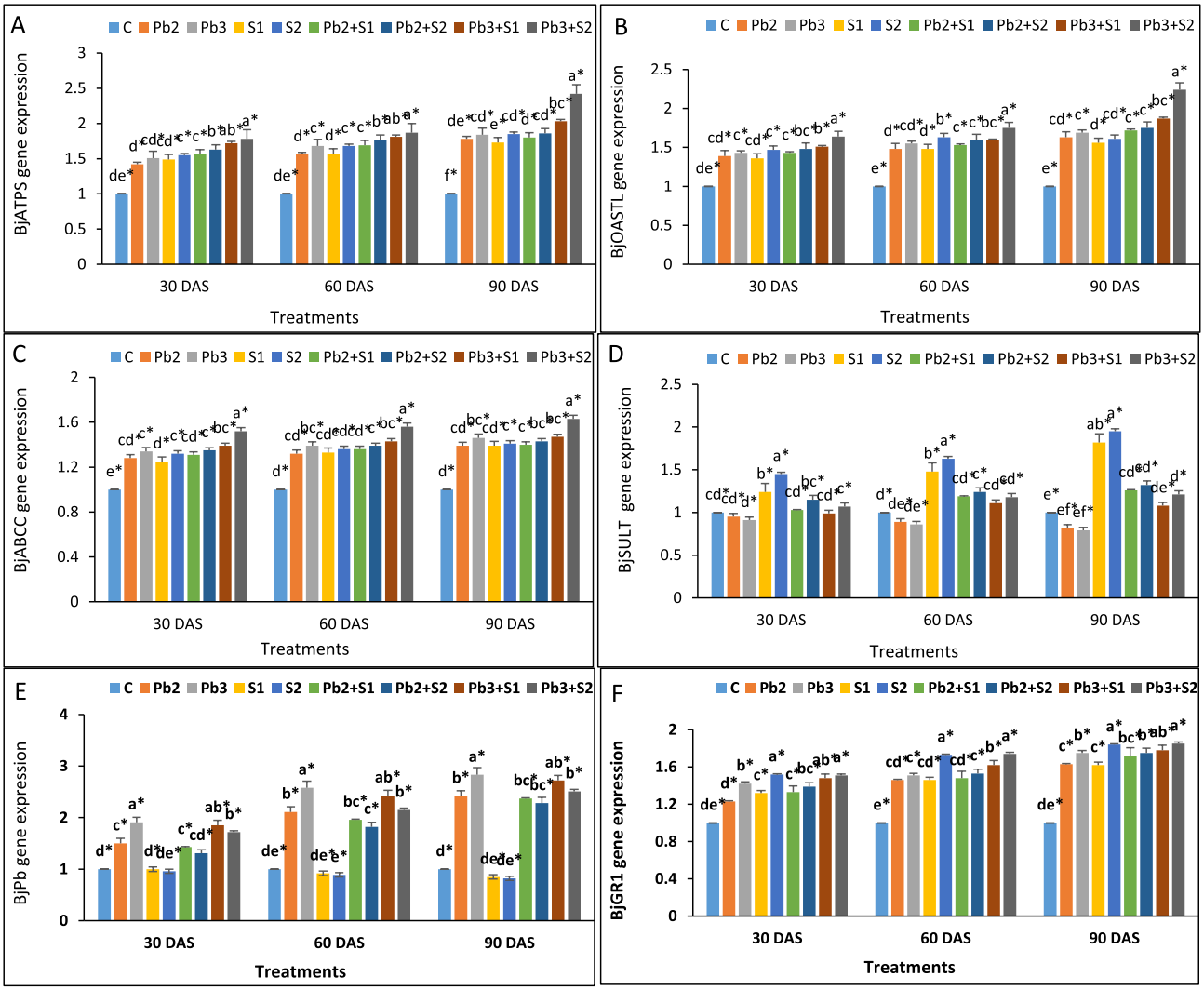


**Fig. 5.** Effects of exogenous sulphur on transcript levels of the six genes encoding sulphur assimilation enzymes in the leaves of Indian mustard under lead stress. Transcript levels of *BjSULT*, *BjABCC*, *BjATPS* and *BjOASTL* (A,C,D,E) genes play a role in sulphur assimilation pathway, *BjPb* (B) gene in lead regulation and *BjGR1* (F) gene in antioxidative system. Various letters indicate statistically significant differences ($P < 0.05$) between treatments within a developmental stage.

plants further treated with sulphur have enhanced ability to regulate oxidative stress. Finally this finding provides evidence that application of S enhances the activity of antioxidant enzymes, so offering a feasible approach to enhance plant tolerance against lead contamination.

The present study demonstrates a significant decrease in chlorophyll and carotenoid levels, in response to lead stress. Shahid et al. (2015) observed a reduction in chlorophyll levels in *Vicia faba* plants under Pb stress. Reduction in Chlorophyll is due to the change of chl central atom magnesium with Pb (Dixit et al., 2016). Exogenous sulphur spray mitigated harmful effects of Pb and enhanced the levels of photosynthetic pigments in *Brassica* plants, in comparison to plants that were not treated with sulphur spray. Similarly, the administration of S to plants under Pb stress led to an increase in the levels of chlorophyll and carotenoid content. Bharwana et al. (2014) found that treating *Gossypium* with Pb resulted in a reduction in its carotenoid concentration. The possible rationale for the augmentation of pigments in response to sulphur is the need to stabilize biomolecules using sulfolipids and integrate Fe-S clusters into apoproteins by repairing protein complexes that include Fe-S. Thus sulphur application has enhanced the activity of photosynthetic pigments n *Brassica*.

Plant life in severe settings relies heavily on the uptake and assimilation of sulphur (Fig. 6). The present investigation found that ATPS and OASTL activities increased slightly when *B. juncea* was exposed to Pb stress. This study corroborates the previous reports of *Brassica juncea* L. under Selenium stress (Gupta and Gupta, 2016) and *Brassica chinensis* L. cadmium stress (Liang et al., 2016). ATPS was the first enzyme to incorporate sulphur in plants (Lappartient and Touraine, 1996), whereas OASTL synthesizes cysteine, a precursor to glutathione (GSH) and other S-containing compounds (Takahashi et al., 2011). The enhancement of ATPS and OASTL enzyme activity under stress is evident with the elevation in transcript level of *BjATPS* and *BjOASTL* gene. However, elevated ATPS and OASTL activities in S2 spray, suggest that *BjATPS* and *BjOASTL* expression enhances S assimilation in high S-applied *Brassica* to minimize Pb toxicity. Various research found that sulphur-assimilating ATPS and OASTL enzymes enhanced heavy metal tolerance (Lou et al., 2017). *ATPS* gene overexpression in Indian mustard increased tolerance to Cd-stress trough phytoextraction (Wangeline et al., 2002). Even while *OASTL* did not affect S assimilation, overexpression of various *OASTL* isoforms in metal-stressed plants increases Cd tolerance (Nakamura et al., 2014). The results of the present study shows an increased *BjSULT* expression under lead stress and sulphur translocation in plants depends on the *SULT* gene (Takahashi et al., 2000) and found that sulfate-assimilating transcriptional genes such *SULTR1;1* and *SULTR2;2* help plants to translocate sulphur. Hence, we can conclude that increased *BjATPS* and *BjOASTL* expression under Pb stress was due to increased sulphur translocation through *BjSULT* transporters. Further, sulphur spray to stressed plants increased sulphur assimilation through *BjSULT* transporters and paved way for the enhancement of *BjATPS* and *BjOASTL* genes to ameliorate the lead toxicity.

The current study found that *BjPb* expression increased under Pb stress and showed a reduced expression under sulphur spray, indicating that sulphur spray reduced lead transport into the plant's cellular system and increased Pb tolerance through the augmentation of *BjATPS* and *BjOASTL* genes. In present study, expression of *BjABCC* vacuole transporter enhanced under Pb stress. Exogenous sulphur spray to the lead stressed plants, increased *BjABCC* transporter expression resulting more sequestration of lead into the vacuoles. Similar results were found upon S supplementation to *P. deltoides* where, *ABCC1.1* transporter sequestered PCs-Pb into the vacuole (Ma et al., 2018). The results of increased GR activity correlated with the increased *BjGR* transcript under lead stress. Similar, enhanced *GR* expression results was observed by Kaur et al. (2018) in *B. juncea* to various salt stress. In present study, sulphur spray to lead treated plants exhibited an increased expression of *BjGR* transcripts. Sulphur spray has mitigated the ROS content by enhancing gene transcript levels of BjGR in Cd stressed *B. chinensis* (Lou et al.,

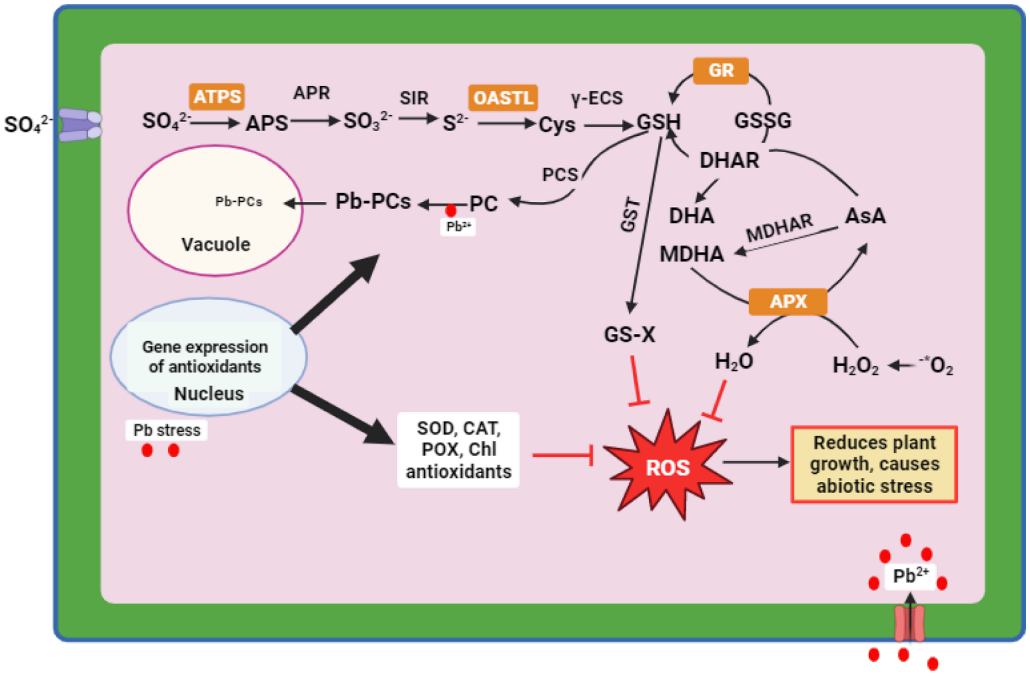


**Fig. 6.** Schematic presentation of antioxidant defense mechanism and sulphur role in regulating lead accumulation and tolerance. $SO_4^{2-}$available form of sulphur, ATPS-ATP sulfurylase, APS-adenosine 5′-phosphosulfate, APR-APS reductase, $SO_3^{2-}$-sulfite, SIR-sulfite reductase, $S^{2-}$-sulfide, OASTL-O-acetyl serine(thiol)lyase, Cys-cysteine, γ-ECS-γ-glutamylcsyteine synthetase, GSH-reduced glutathione, GSSG-oxidized glutathione, GR-glutathione reductase, PCS-phytochelatin synthetase, PC-phytochelatins, Pb-PCs-lead associated with phytochelatins, GST-glutathione-S-transferase, GS-X-glutathione containing compounds, DHAR-dehydroascorbate reductase, DHA-dehydroascorbate, AsA-ascorbate, MDHA-mono dehydroascorbate, MDHAR-mono dehydroascorbate reductase, APX-ascorbate peroxidase, $H_2O$-water, $H_2O_2$-hydrogen peroxide, $^{\bullet 2}O_2$-superoxide anion, ROS-reactive oxygen species, SOD-superoxide dismutase, CAT-catalase, POX-peroxidase, Chl-chlorophyll, Pb-lead, $Pb^{2+}$-ionic form of lead.

2017). Enhanced lead content entered plants through *BjPb* transporters and led to increased ROS. Sulphur application in present study might be the possible reason for the enhanced expression of *BjGR* and have lowered ROS and increased efficiency of photosynthetic pigments. Further sulphur treatment, S entered plants through *BjSULT* transporters and incorporated sequestration of Pb by enhancing *BjATPS* and *BjOASTL* level. This significant change in the alteration of S-assimilation pathway resulted in the excess sequestration of Pb through S-mediating enzymes and might be the reason for the reduced Pb content under sulphur spray.

## 5. Conclusions

In the current study, high sulphur application has resisted the Pb toxicity by decreasing the stress biomarkers and enhanced plant growth by strengthening antioxidant defense system. These data demonstrated that sulphur supplementation fine-tuned *Brassica* plants response to excess Pb including enhanced control of Pb distribution and ROS removal. Further S application has also alleviated the sulphur assimilating enzymes and sulfur related genes; therefore, S played a major role in maintaining the redox status of the cell. Furthermore, experiments are needed to explore the S combinational secondary metabolites at proteomic expression level in plants under different stress condition.

**Declaration of Funding:** No external funding.

**Ethics approval:** Not applicable.

## CRediT authorship contribution statement

**Hemanthkumar Manne:** Conceptualization, Formal analysis, Methodology, Writing – original draft. **Nisha Kumari:** Data curation, Project administration, Supervision. **Shikha Yashveer:** Methodology, Supervision, Visualization. **Sonia Nain:** Investigation, Resources, Writing – review & editing. **Jyoti Duhan:** Visualization, Writing – review & editing. **Ram Avtar:** Investigation, Software. **Sushil:** . **Minakshi Jattan:** Investigation, Methodology. **Babita Rani:** Software, Validation. **Abdulaziz Abdullah Alsahli:** Conceptualization, Writing – original draft, Writing – review & editing. **Sajid Ali:** Methodology, Validation.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Acknowledgement

The research was financially supported by the Oilseeds section, Department of Genetics and Plant breeding and Department of Biochemistry, CCSHAU, India. The authors would also like to extend their sincere appreciation to the Researchers Supporting Project Number (RSP2024R236), King Saud University, Riyadh, Saudi Arabia.

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