



High Sulfur (S) Supplementation Imparts Waterlogging Tolerance to Oilseed rape (*Brassica napus* L.) Through Upregulating S Metabolism and Antioxidant Pathways

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Abstract

Waterlogging significantly impacts plant growth and development by altering nutrient uptake and antioxidant enzyme functions, resulting in reduced yield. Plants need sulfur (S) to produce reduced glutathione (GSH), a thiol compound that combats abiotic stresses. It is hypothesized that supplying S to high S-demanding oilseed rape plants beyond its growth requirements can reduce the adverse effect of waterlogging stress. Therefore, this study evaluated the comparative effect of different S doses (mg kg⁻¹ soil), i.e., no-S (S₀), low-S (S₁, 35), medium-S (S₂, 70), and high-S (S₃, 140) on growth, yield and antioxidant defense systems of normal growing and waterlogged oilseed rape plants. Waterlogging was imposed at the inflorescence emergence stage for 7 days by retaining a 3-cm layer of water above the soil surface. Waterlogged plants supplemented with high-S showed improved growth and higher yield than those supplemented with lower S levels, and this response was associated with improved activity/contents of antioxidants, including ascorbate (AsA), GSH, ascorbate peroxidase, catalase, glutathione reductase, glutathione peroxidase, glutathione S-transferase, monodehydroascorbate reductase, and dehydroascorbate reductase, with concomitant lowering of hydrogen peroxide, dehydroascorbate and malondialdehyde content. Furthermore, leaf S concentration was enhanced in waterlogged plants treated with high-S, while anions were regulated whereby Cl⁻ uptake was decreased. However, under control conditions, high-S did not provide additional benefits of improved plant growth, yield, and antioxidant activities compared to the medium-S application. Thus, it is concluded that additional S supplementation mitigates the adverse effects of waterlogging stress on oilseed rape, and needs to be employed as a potential strategy to alleviate the negative effects of this abiotic stress.

Keywords Waterlogging tolerance · Sulfur · Antioxidants · S metabolites · Rapeseed · Reduced Glutathione

Introduction

Anthropogenic activities in the past and present are responsible for the ever-increasing global warming, resulting in a substantial increase in dry periods worldwide. However, not only drought but the frequency and intensity of extreme climate events, such as heavy rainfalls, have also increased (Alifu et al. 2022; Tian et al. 2021). Together with excessive

irrigation and river overflow, high precipitation can increase the probability of long periods of waterlogged soil conditions (Tabari et al. 2020). The recent trend analyses of excessive rain across Europe revealed an increasing tendency in the magnitudes of extreme rainfall, mainly concentrated in winter (4–10%) (Madsen et al. 2014). Although trends vary regionally and seasonally, heavy rains in Germany have become more erosive and significantly higher in the past 20 years (Deumlich et al. 2020; Maraun 2013).

The primary effect of waterlogging is the impediment of gas diffusion through saturated soil pores (10,000 slower diffusion of gases in water than in air), which causes hypoxia and then dysfunction in the roots (Jackson and Ricard 2003). Waterlogging can potentially affect physiological processes, such as suppressing the activities of antioxidant enzymes and reducing the photosynthetic capability

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of crop plants (Simova-Stoilova et al. 2012). Moreover, it can inhibit plant growth and development by inducing nutrient deficiencies/toxicities and promoting the production of reactive oxygen species (ROS), mainly hydrogen peroxide (H_2O_2) (Teoh et al. 2022; Zhang et al. 2015). High levels of ROS cause membrane lipid peroxidation, protein oxidation, and enzyme inhibition, resulting in cell death (Tanou et al. 2009). Thus, the antioxidant defense mechanism is of prime importance for plants' survival under waterlogging through scavenging ROS and protecting cells from oxidative stress (Doupis et al. 2017).

An adaptation to waterlogging stress involves morphological changes in the root system, the development of aerenchyma, and adventitious roots that improve the exchange of gases inside the roots (Sauter 2013). Due to the lack of aerenchyma, oilseed rape (*Brassica napus* L.) is highly prone to waterlogging (Voeselek et al. 1999). Thus, globally, including in Europe and North America, one of the most extreme abiotic stresses affecting rapeseed production is waterlogging (Pachauri et al. 2014). This is even more alarming, as oilseed rape, after soybeans, is the world's second-largest oilseed crop (FAO 2021). In addition to edible oil, rapeseed has many other uses, e.g., cattle feed, a source of biodiesel, or bioethanol (Kdidi et al. 2019). Over recent decades, rapeseed production expanded across most major production regions, including Canada, Europe, China, India, and Australia (FAO 2021). Still, rapeseed is commonly grown in Asia as a rotation crop on rice fields (Zou et al. 2015). Consequently, rapeseed naturally experiences waterlogging stress during growth and development. In the past decades, rapeseed growth was severely hampered due to waterlogging, resulting in more than 50% yield reductions (Wollmer et al. 2018; Herzog et al. 2016). Moreover, waterlogging affected the oil quality by increasing erucic acid and glucosinolate content as well as altering lipid biosynthesis metabolic pathways (Xu et al. 2015).

It is well known that sulfur (S) alone or its derivatives can positively regulate antioxidant defense under abiotic stress

conditions and thus mitigates oxidative stress (Bashir et al. 2015). In this context, for example, GSH acts as a cell redox regulator which scavenges reactive oxygen species. Keeping a favorable redox state for the detoxification of H_2O_2 depends on the balance between glutathione (GSH) and oxidized glutathione (GSSG) (Arbona et al. 2008). Although many researches have focused on the effects of waterlogging on growth, yield, ion homeostasis, antioxidant system, and some other physiological as well as molecular traits of the rapeseed (Ding et al. 2022; Ambro et al. 2022; Lee et al. 2014), the attenuation of the adverse effects of abiotic stresses by S supplementation has been inadequately studied. Increased elemental S in soil decreased salt toxicity in *B. juncea* (Fatma et al. 2014; Tong et al. 2014), the adverse effects of freezing (4 °C) (Lou et al. 2017), heavy metal (Cd) exposure (Min et al. 2016) and heat stress in plants (Shi et al. 2015). To the best of our knowledge, there is rare evidence of whether soil supplementation with S can enhance waterlogging stress tolerance in rapeseed. Therefore, this study aimed to investigate whether waterlogged plants supplemented with excess S can improve S-assisted antioxidant defense and offset waterlogging induced adverse effects of oxidative stress on the growth and yield of rapeseed. We hypothesized that supplementing plants with high-S augments GSH levels which alleviates waterlogging stress by upregulating the antioxidant system.

Materials and Methods

Plant Material and Plant Cultivation

Four overnight-soaked seeds of oilseed rape (*Brassica napus* L. cv. campino; Norddeutsche Pflanzenzucht Lembke, Hohenlieth, Germany) were sown in Mitscherlich pots, filled with 6 kg of a loamy sand soil (85% sand, 10.9% silt, and 4.1% clay, 6.5 mg S kg^{-1} soil, pH_{CaCl_2} 6.5) (Table 1). Based on the standard field fertilizer application, the following four S-levels (as $MgSO_4 \cdot 7H_2O$) were applied to the soil 5 days prior to sowing: 0 mg S kg^{-1} soil, no-S (S_0); 35 mg S kg^{-1} soil, low-S (S_1); 70 mg S kg^{-1} soil, medium-S (S_2); and 140 mg S kg^{-1} soil, high-S (S_3). Basal fertilization was carried out as follows (in g kg^{-1} soil): 0.47 NH_4NO_3 , 0.74 KH_2PO_4 , 0.46 $MgCl_2 \cdot 6H_2O$, 0.3 $CaCl_2 \cdot 2H_2O$, 0.015 Fe-EDTA, 0.006 $CuSO_4 \cdot 5H_2O$, 0.011 $ZnSO_4 \cdot 7H_2O$, 0.015 $MnSO_4 \cdot H_2O$, 0.009 H_3BO_3 , 0.0006 $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$. After germination,

only two plants were retained in each pot. The soil moisture level in all the pots was equally maintained at 60% water holding capacity (WHC) for six weeks after sowing. Thereafter, waterlogging treatment was imposed at the inflorescence emergence stage by keeping approximately a

Table 1 The physio-chemical properties of soil used in the experiment

Parameter	Value
Texture	Loamy sand
$pH_{1:2.5}(CaCl_2)$	6.5
Sand (%)	85
Silt (%)	10.9
Clay (%)	4.1
Organic matter (%)	2.1
Sulfur (S, mg kg^{-1})	6.5
Total nitrogen (N, %)	0.09
Potassium (K, mg kg^{-1})	69
Phosphorus (P, mg kg^{-1})	94
Zinc (Zn, mg kg^{-1})	1.6
Manganese (Mn, mg kg^{-1})	33.6

3 cm layer of water above the soil surface in the pots for seven consecutive days. The pots were placed in 15 L plastic buckets which were pre-filled with water to achieve a water depth of 3 cm above the soil in pots. Throughout the experiment, a control was maintained at 60% WHC for each S level. Plants were grown under controlled conditions in the greenhouse with the following conditions: Day/night temperature 22/18°C, photoperiod 14/10 h (day/night), relative humidity 62–70%, light intensity 350 $\mu\text{M photon m}^{-2} \text{s}^{-1}$ (recorded by a light meter, Li-198, Lincoln, NE, USA). The experiment consisted of two sets of treatments, each having four replications, which were arranged according to completely randomized design. One set was used for growth and yield data recording as well as analysis of nutrients, while the other set was used for biochemical analyses.

Morphological Assessment and Plant Harvest

Plants from the first set of the experiments (four replications) were harvested 7 days after the onset of waterlogging treatment. Before the harvest, the number of senesced leaves was recorded in each treatment based on visual observation. The fresh shoot samples were frozen in liquid N_2 , and stored at -80°C until used for biochemical analysis. The waterlogging treatment of the second set of plants was finished after seven days, and further were grown under normal soil water (60% WHC) for the rest of the growth period; plants were harvested at agronomic maturity. Plant height was recorded, and the plants were cut at the base. The number of siliqua, thousand seed weight, and seed yields were recorded. The plant samples were dried at 65°C for 72 h, and their dry matter was recorded. The dried samples were ground to a fine powder with a ball mill (MM 200, Retsch GmbH, Haan, Germany).

Determination of Inorganic Anions

The extraction of inorganic anions was done according to Cataldi et al. (2000) with a few modifications. Briefly, approximately 20 mg of dried and powdered leaf samples were dissolved and boiled in 1.5 mL deionized water for 5 min. The samples were then thoroughly mixed and subsequently placed in an ice-water bath for 30 min. The supernatants were collected after centrifugation at $17,500 \times g$ at 4°C for 10 min. For the precipitation of protein from the supernatant, 200 μL chloroform was added and centrifuged at $17,500 \times g$ at 4°C for 5 min. To purify the supernatant, it was passed through a strata C-18 column (Phenomenex, Torrance). Afterward, the anions were determined by isocratic ion chromatography (IC-5000 Capillary Reagent-Free IC System, Thermo Scientific).

Determination of Total Sulfur

The total sulfur of the leaf was determined following the method of Hussain et al. (2022). Briefly, 5–10 mg of finely ground samples were weighed into tin capsules along with 10 mg wolfram-(IV)-oxide. Subsequently, total S concentration was determined by the Dumas combustion method with CNS elemental analyzer (Flash EA 1112 NCS, Thermo Fisher Scientific, Waltham, MA, USA).

Quantification of Malondialdehyde Content

Malondialdehyde (MDA) content was measured following the method of Heath and Packer (1968). In brief, frozen leaf tissues were homogenized in 5% trichloroacetic acid (TCA) using mortar and pestle. After centrifuging at $11,500 \times g$ and 4°C for 15 min, 1 mL of the aliquot and 4 mL of 20% TCA containing 0.5% TBA were mixed. After heating at 95°C for 30 min, the mixture was cooled promptly on an ice bath and centrifuged at $10,000 \times g$ for 10 min. To correct for nonspecific turbidity, the absorbance of the supernatant was estimated at 532 nm and subtracted from the absorbance of the same at 600 nm. The contents of MDA were calculated based on the extinction coefficient ($155 \text{ mM}^{-1} \text{ cm}^{-1}$), and the concentration was expressed as nmol g^{-1} fresh weight.

Determination of H_2O_2 Production Level

Leaves H_2O_2 concentration was determined following the method described by Velikova et al. (2000). Leaf samples (0.2 g) were homogenized in 1.6 mL of 0.1% trichloroacetic acid (TCA) in an ice bath for 30 min and centrifuged at $12,000 \times g$ at 4°C for 20 min. Next, 0.5 mL of 0.1 M potassium phosphate buffer (K-P buffer; pH 7.8) and 1 mL of 1 M potassium iodide (KI) were added to 0.5 mL of supernatant and kept in the dark for 1 h. The absorbance of the mixture was measured at 390 nm. Quantification of H_2O_2 was done using a standard curve, and the concentration was presented as $\mu\text{mol g}^{-1}$ fresh weight.

Extraction and Measurement of Ascorbate and Glutathione

For the analysis of ascorbate and glutathione, frozen leaves (0.5 g) were homogenized in 5% meta-phosphoric acid containing 1 mM ethylene diamine tetra acetic acid (EDTA) and centrifuged at $11,500 \times g$ and 4°C for 15 min, and the supernatant was collected. Ascorbate content was determined following Anee et al. (2019). In brief, 0.3 mL of 0.5 M K-P buffer (pH 7.0) and 0.1 mL of 0.1 M dithiothreitol were added to reduce the oxidized fraction. Finally, the reaction mixture was formulated using K-P buffer (pH 6.5)

and ascorbate oxidase with a neutralized solution. In the presence of 0.5 units of ascorbate oxidase (AO), a change in absorbance was measured at 265 nm in 100 mM K-P buffer (pH 7.0). The standard curve used to determine total ascorbate and AsA was subtracted from total ascorbate to calculate dehydroascorbate (DHA). The ascorbate (AsA) content was expressed as nmol g^{-1} fresh weight.

Reduced glutathione (GSH) and oxidized glutathione (GSSG) were assayed following Yu et al. (2003). Shortly, the extract solution was neutralized by adding 0.5 M K-P buffer (pH 7) and dH_2O for total glutathione. In the meantime, the GSH was oxidized by DTNB [5,5-dithiol-bis (2-nitrobenzoic acid)] and reduced by nicotinamide adenine dinucleotide phosphate (NADPH) with GR. Changes in absorbance were photometrically measured at 412 nm. Standard curves were made with the known concentrations ranging from 5 to 40 μM of reduced glutathione (GSH) and oxidized glutathione (GSSG). The content of GSH was calculated by subtracting GSSG from the total GSH and expressed as $\mu\text{mol g}^{-1}$ fresh weight.

Antioxidant Enzymes Assay

The steps involved in preparing crude plant extract were carried out at 4 °C. Frozen leaf tissue (0.3 g) was homogenized with a 2 mL extraction mixture containing 0.5 M K-P buffer (pH 7.0), 100 mM KCl, 1 mM AsA, 5 mM β -mercaptoethanol, and 10% (w/v) glycerol. The mixture was then centrifuged at $12,000 \times g$ for 20 min. The crude extract was stored at -20 °C until further analysis. Spectrophotometric measurements were performed to determine antioxidant enzymes with a UV visible spectrophotometer (Thermo Helios Gamma, 9423 UVG 1000E, United Kingdom).

Protein Quantification

The protein concentration of the samples was measured as described by Bradford (1976). In this case, bovine serum albumin was used as a standard, and absorbance was measured at 595 nm.

Catalase (CAT; EC: 1.11.1.6) activity was measured according to Aebi (1984) with slight modifications. Initiation of the reaction was accomplished by adding 15 mM H_2O_2 with mixture of 50 mM K-P buffer (pH 7.0) and plant extract. The drop-in absorbance was monitored for up to 1 min at 240 nm. The calculation was performed using an extinction coefficient of $39.4 \text{ M}^{-1} \text{ cm}^{-1}$. One unit of catalase was expressed as 1 μM of substrate reacted per minute per mg protein.

Ascorbate peroxidase (APX; EC: 1.11.1.11) was observed by the decrease in absorbance at 290 nm following

of H_2O_2 dependent oxidation of ascorbate (Nakano and Asada 1981). The assay mixture contained 50 mM K-P buffer (pH 7.0), 0.1 mM EDTA and 0.1 mM H_2O_2 . The activity of APX was calculated using the extinction coefficient of $2.8 \text{ mM}^{-1} \text{ cm}^{-1}$, and one unit of APX was defined as $\mu\text{mol H}_2\text{O}_2 \text{ min}^{-1} \text{ mg}^{-1}$ protein.

Glutathione reductase (GR; EC: 1.6.4.2) activity was measured following the method described by Mohsin et al. (2019). The oxidation of glutathione-dependent NADPH was monitored at 340 nm. There were five components in the assay mixture: phosphate buffer (0.5 M, pH 7), 1 mM EDTA, 1 mM GSSG, 0.2 mM NADPH, and crude plant extract. For calculating GR activity, $6.2 \text{ mM}^{-1} \text{ cm}^{-1}$ was used as the extinction coefficient. The activity of GR was defined as 1 nM of substrate reacted per minute per mg of protein ($\text{nmol min}^{-1} \text{ mg}^{-1}$ protein).

Monodehydroascorbate reductase (MDHAR, EC 1.6.5.4) activity was determined using the method described by Hosain et al. (1985). The decrease in absorbance due to NADH oxidation was monitored at 340 nm. The reaction assay contained 50 mM Tris-HCl buffer (pH 7.5), 0.2 mM NADPH, 2.5 mM AsA, 0.5 units of ascorbate oxidase (AO) and crude plant extract. The activity of MDHAR was calculated using the extinction co-efficient of $6.2 \text{ mM}^{-1} \text{ cm}^{-1}$ and expressed as $\text{nmol min}^{-1} \text{ mg}^{-1}$ protein.

The activity of dehydroascorbate reductase (DHAR, EC 1.8.5.1) was measured following the method of Anee et al. (2019). The enhanced absorbance due to GSH-dependent AsA generation was measured spectrophotometrically. To prepare reaction mixtures, 50 mM K-P buffer (pH 7.0), 2.5 mM GSH, 0.1 mM EDTA, 0.1 mM dehydroascorbate (DHA), and enzyme extract were used. The change in absorbance was measured at 265 nm for 1 min, and DHAR activity was expressed as $\text{nmol min}^{-1} \text{ mg}^{-1}$ protein.

The activity of glutathione peroxidase (GPX, EC: 1.11.1.9) was measured following Elia et al. (2003). The oxidation of NADPH to NADP+ was marked by a reduction in absorbance at 340 nm that presented a spectrophotometric means for monitoring GPX activity. The assay mixture contained 100 mM of K-P buffer (pH 7.0), 1 mM EDTA, 1 mM sodium azide (NaN_3), 0.12 mM NADPH, 2 mM GSH, 1 unit GR, 0.6 mM H_2O_2 (as a substrate), and plant crude extract. The absorbance of the mixture was monitored for 1 min, and the extinction coefficient used for the calculation was $6.62 \text{ mM}^{-1} \text{ cm}^{-1}$. The activity of GPX was defined as 1 nM of substrate reacted per minute per mg of protein.

Glutathione S-transferase (GST, EC: 2.5.1.18) activity was determined using the method described by Hasanuz-zaman et al. (2019). The conjugation of the thiol group of glutathione (GSH) to the CDNB substrate was detected with the increased absorbance at 340 nm. The reaction mixture contained 2 mM GSH, 0.25 mM K-P buffer (pH 6.5), 30

mM CDNB, and crude plant extract. The extinction coefficient $9.6 \text{ mM}^{-1} \text{ cm}^{-1}$ was utilized to estimate GST activity, where the unit of GST activity was expressed as $\text{nmol min}^{-1} \text{ mg}^{-1}$ protein.

Statistical Analysis

The data were statistically analyzed following two-way ANOVA using R (version 4.0.4) software. The differences among the treatments were compared with Tukey's HSD test at 5% probability level.

Results

Sulfur Enhances Growth Performance Under Waterlogging Stress

Waterlogging stress significantly inhibited the growth performance and biomass production of *Brassica napus* plants (Fig. 1). The waterlogged plants applied with no-S ($S_0 + \text{WL}$), low-S ($S_1 + \text{WL}$), and medium-S ($S_2 + \text{WL}$) showed reduced height and dry matter production as compared to respective controls (Fig. 2A and B). On the other hand, waterlogging-induced reduction in dry matter was not observed in high-S supplemented plants ($S_3 + \text{WL}$) compared to its corresponding control (Fig. 2B). Conversely, S application significantly mitigated the waterlogging-induced decline in dry matter production: S_1 , S_2 , and S_3 treated waterlogged plants exhibited dry matter enhancement by 1.5, 4.8 and 5.6-fold,

respectively, compared to S_0 treatment (Fig. 2B). With the same trend, waterlogged plants supplemented with low-S ($S_1 + \text{WL}$), medium-S ($S_2 + \text{WL}$), and high-S ($S_3 + \text{WL}$) had 6%, 60% and 71% lesser senesced leaves compared with no-S ($S_0 + \text{WL}$) plants (Fig. 2C).

Although compared with controls the number of siliqua in no-S ($S_0 + \text{WL}$) and low-S ($S_1 + \text{WL}$) waterlogged plants did not differ statistically, medium-S ($S_2 + \text{WL}$) and high-S ($S_3 + \text{WL}$) supplemented waterlogged plants exhibited a significant decline (Fig. 2D). However, the thousand-seed weight and seed yield of S_0 , S_1 , S_2 and S_3 treated waterlogged plants markedly decreased compared to respective controls (Fig. 2E F). Although high-S ($S_3 + \text{WL}$) treated waterlogged plants showed a slight increase in the number of siliqua, but displayed a significant increase of 20% and 11% in thousand seed weight and seed yield respectively, compared to medium-S ($S_2 + \text{WL}$) supplied waterlogged plants (Fig. 2E). Surprisingly, number of siliqua, 1000-seed weight and seed yield of high-S (S_3) and medium-S (S_2) supplied control plants were similar (Fig. 2D F).

Sulfur Balances Anions Contents Under Waterlogging Stress

Total S concentrations in leaves of waterlogged plants supplied with high-S ($S_3 + \text{WL}$) increased by 3.7-, 5.2- and 1.3-fold compared to no-S ($S_0 + \text{WL}$), low-S ($S_1 + \text{WL}$), and medium-S ($S_2 + \text{WL}$) supplemented plants, respectively (Table 2). The application of high-S (S_3) proved ineffective in increasing S concentration compared to medium-S

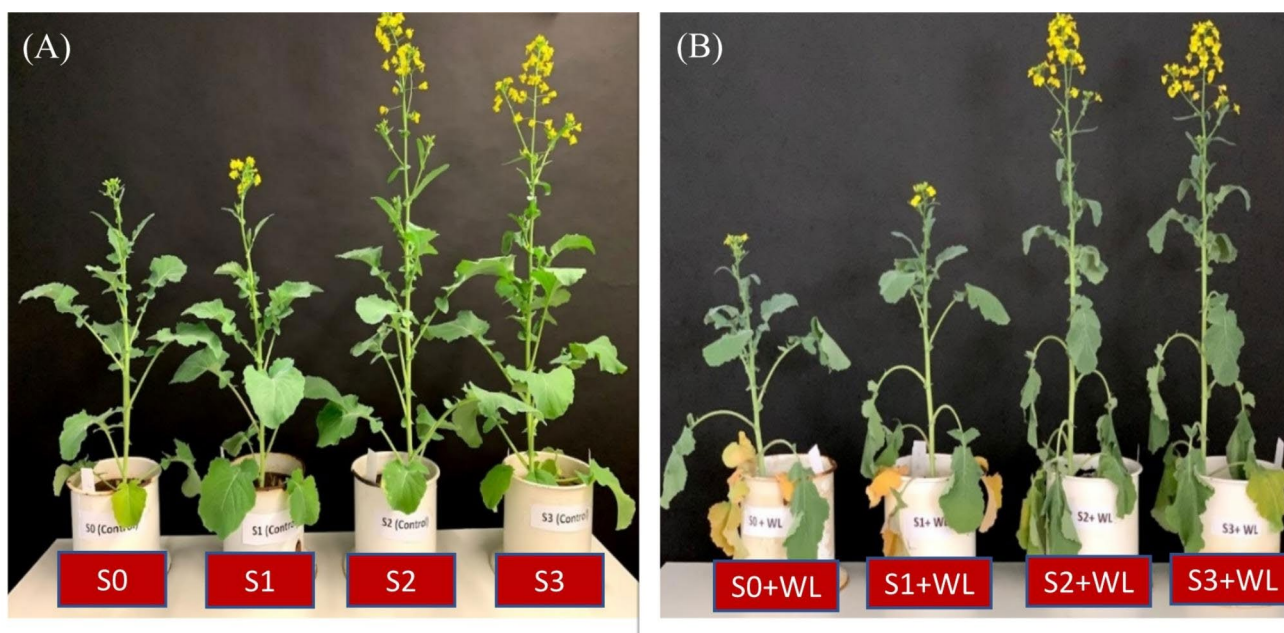
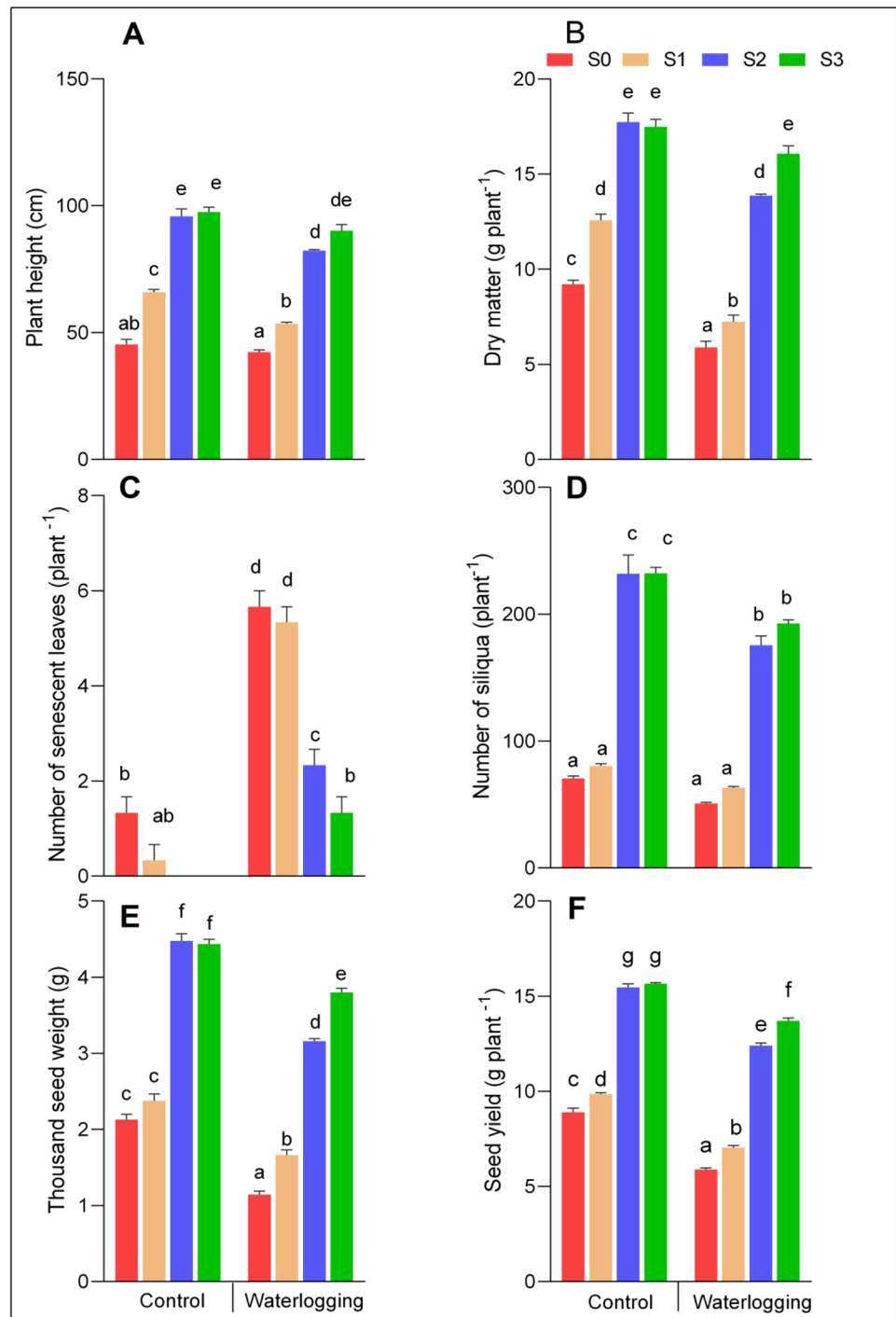


Fig. 1 Effect of different levels of soil-applied sulfur (0, 35, 70 and 140 mg S kg^{-1} soil) on the growth of normal (A) and waterlogged (B) plants of rapeseed (*Brassica napus* L.)

Fig. 2 Effect of different levels of soil-applied S (S_0 , No-S application; S_1 , 35 mg S kg^{-1} soil; S_2 , 70 mg S kg^{-1} soil; S_3 , 140 mg S kg^{-1} soil) on plant height (A), dry matter (B), number of senescent leaves (C), number of siliqua (D), thousand seed weight (E) and seed yield (F) of rape-seed (*Brassica napus* L.) grown under control (normal irrigated) and waterlogged condition. The data are means \pm SE of four independent pot replicates. Different letters indicate significant differences between the treatment groups (Tukey's test; $p \leq 0.05$)



(S_2) under control conditions. Similar to total S contents, waterlogging decreased water-soluble sulfate (SO_4^{2-}) concentration in *Brassica napus* leaves by 70%, 60%, and 21% in no-S (S_0 +WL), low-S (S_1 +WL) and medium-S (S_2 +WL) treatment, compared to their corresponding controls, respectively (Table 2). Conversely, waterlogging did not affect SO_4^{2-} concentration in the leaves of high-S (S_3 +WL) supplied plants compared with its control (S_3)

(Table 2). Moreover, in contrast to medium S supplied waterlogged plants (S_2 +WL), high S supplied waterlogged plants (S_3 +WL) had 15% higher SO_4^{2-} concentration in leaves (Table 2).

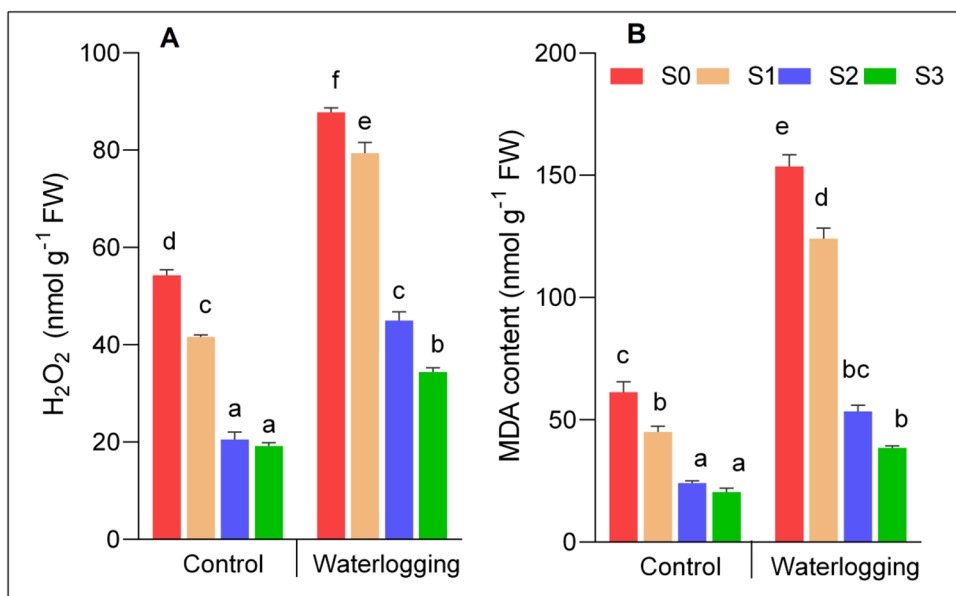
High-S (S_3 +WL) supplemented plants increased nitrate (NO_3^-) concentration by 17-, 10-, and 1.1-fold compared with no-S (S_0 +WL), low-S (S_1 +WL) and medium-S (S_2 +WL) treatments under waterlogging stress,

Table 2 Concentration of total S, and water-extractable sulfate (SO_4^{2-}), nitrate (NO_3^-), phosphate (PO_4^{3-}) and chloride (Cl^-) in leaves (*Brassica napus* L.) of rapeseed grown under control and waterlogged soils treated with different S levels

Leaf S/Anion concentration	Control				Waterlogged			
	S ₀	S ₁	S ₂	S ₃	S ₀	S ₁	S ₂	S ₃
Total S (%)	0.68 ± 0.01 ^b	0.92 ± 0.02 ^c	1.97 ± 0.11 ^f	2.02 ± 0.12 ^{ef}	0.53 ± 0.03 ^a	0.67 ± 0.05 ^{bc}	1.71 ± 0.02 ^d	2.00 ± 0.11 ^e
Sulphate (SO_4^{2-}) (mg g ⁻¹ DM)	5.81 ± 0.09 ^b	8.80 ± 0.58 ^c	20.28 ± 1.44 ^e	19.71 ± 0.49 ^e	3.42 ± 0.63 ^a	5.55 ± 0.45 ^{ab}	16.69 ± 1.29 ^d	19.27 ± 0.87 ^e
Nitrate (NO_3^-) (mg g ⁻¹ DM)	0.67 ± 0.07 ^b	0.86 ± 0.86 ^b	3.39 ± 3.39 ^c	3.42 ± 3.42 ^c	0.18 ± 0.18 ^a	0.31 ± 0.31 ^a	2.75 ± 0.03 ^c	3.07 ± 0.09 ^d
Phosphate (PO_4^{3-}) (mg g ⁻¹ DM)	1.36 ± 0.19 ^b	1.42 ± 0.33 ^b	3.37 ± 0.12 ^d	3.27 ± 0.15 ^d	0.74 ± 0.03 ^a	0.92 ± 0.08 ^{ab}	2.19 ± 0.23 ^c	2.56 ± 0.18 ^c
Chloride (Cl^-) (mg g ⁻¹ DM)	23.1 ± 2.09 ^c	18.60 ± 0.58 ^b	13.27 ± 0.41 ^a	13.57 ± 0.34 ^a	33.37 ± 1.65 ^d	26.61 ± 2.56 ^c	14.31 ± 1.47 ^a	12.44 ± 0.09 ^a

S₀, represents no-S application while S₁, S₂ and S₃ represent S application @ 35, 70 and 140 mg kg⁻¹ soil, respectively. Data are means ± SE of four independent pot replicates. Different letters in a row indicate significant differences between the treatments in a row (Tukey's test; p < 0.05)

Fig. 3 Effect of different levels of soil-applied S (S₀, No-S application; S₁, 35 mg S kg⁻¹ soil; S₂, 70 mg S kg⁻¹ soil; S₃, 140 mg S kg⁻¹ soil) on H₂O₂ content (A) and malondialdehyde content (B) in leaves of rapeseed (*Brassica napus* L.) grown under control and waterlogged soils. Data are means ± SE of four independent pot replicates. Bars followed by different letters indicate significant differences between the treatment groups (Tukey's test; p ≤ 0.05)



respectively (Table 2). In contrast, NO_3^- concentration of control plants treated with medium-S (S₂) and high-S (S₃) was statistically similar (Table 2). Application of high-S (S₃+WL) improved phosphate (PO_4^{3-}) concentration by 16% compared to the medium-S (S₂+WL) supplied waterlogged plants (Table 2). Additionally, waterlogging declined PO_4^{3-} concentration by 22% in high-S (S₃+WL) and 35% in medium-S (S₂+WL) supplied plants compared to the respective controls (Table 2).

Leaf chloride (Cl^-) concentration of high-S (S₃+WL) treated waterlogged plants was significantly declined by 63% and 53% compared with no-S (S₀+WL) and low-S (S₁+WL) treated waterlogged plants respectively, but slightly (by 13%) reduced compared with medium-S (S₂+WL) treated waterlogged plants (Table 2). Furthermore, compared with control counterparts Cl^- concentrations in low-S (S₁+WL) and no-S (S₀+WL) plants were notably elevated by 43% and 44% respectively, whereas

medium-S (S₂+WL) and high-S (S₃+WL) treated plants did not differ statistically under waterlogging condition (Table 2).

Sulfur Mitigates Waterlogging stress-mediated hike in H₂O₂ and MDA Content

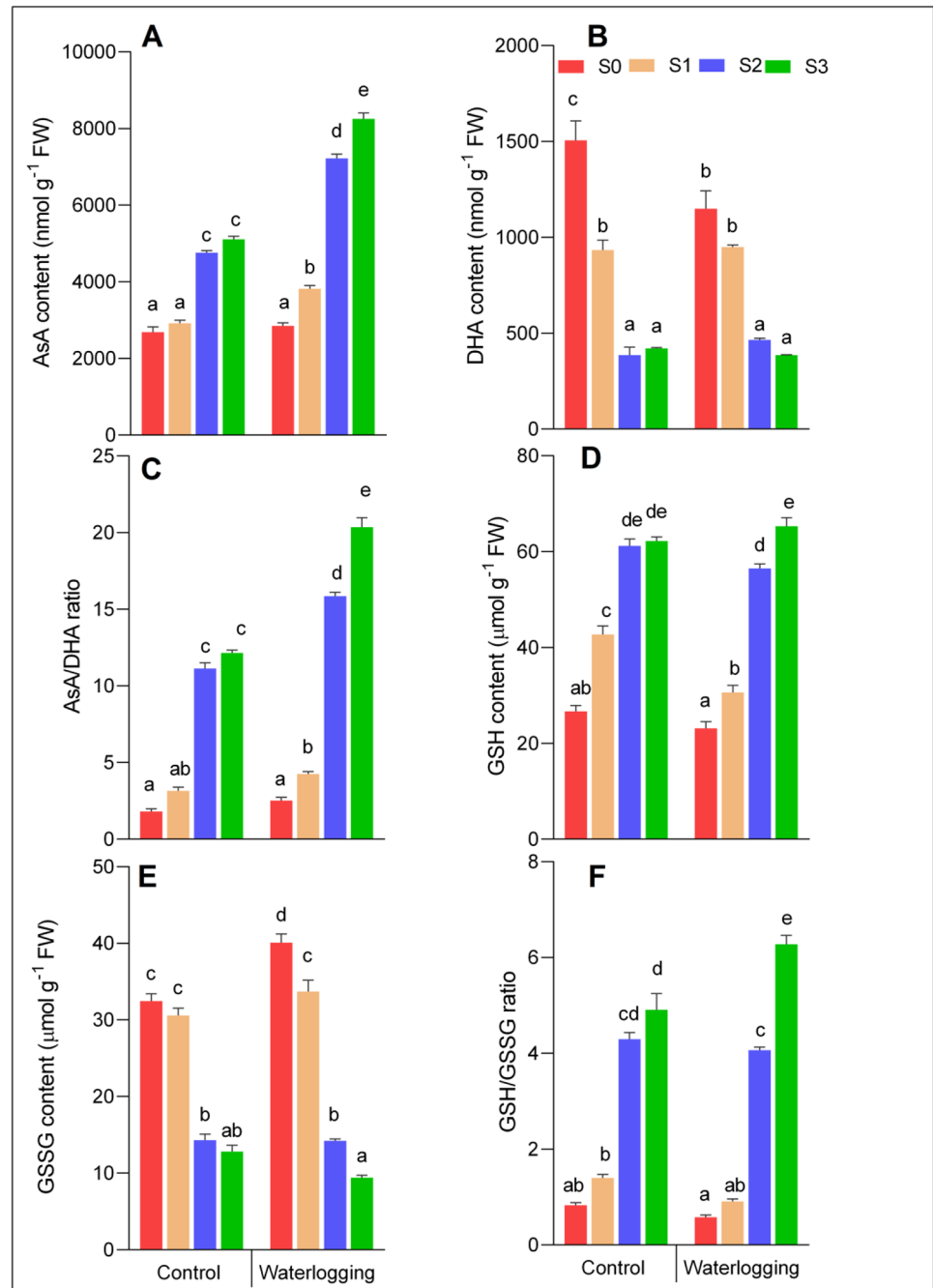
The waterlogging stress resulted in significantly higher H₂O₂ contents than the respective controls. Compared to no-S (S₀+WL) treated plants under waterlogging condition, low-S (S₁+WL), medium-S (S₂+WL), and high-S (S₃+WL) supplementation notably decreased H₂O₂ content by 10%, 49% and 61%, respectively (Fig. 3A). Although, H₂O₂ content did not differ between high-S (S₃) and medium-S (S₂) supplemented plants under control condition, whereas high-S (S₃+WL) supplemented plants H₂O₂ contents declined significantly by 23% than medium-S (S₂+WL) supplemented plants under waterlogged

conditions (Fig. 3A). Under waterlogged conditions, MDA content of all treatments exhibited a similar trend as that of H_2O_2 content (Fig. 3B). Across all S treatments, the waterlogged plants had significantly higher MDA contents than the respective control plants (Fig. 3B). Under waterlogging, low-S ($S_1 + WL$), medium S ($S_2 + WL$), and high-S ($S_3 + WL$) supplementation decreased MDA content markedly by 19%, 65% and 73% respectively, compared to no-S ($S_0 + WL$) treated plants (Fig. 3B).

Sulfur Supplementation Enhances the Activities of Enzymatic and non-enzymatic Antioxidants Under Waterlogging

In comparison with no-S treated waterlogged plants ($S_0 + WL$), low-S ($S_1 + WL$), medium-S ($S_2 + WL$), and high-S ($S_3 + WL$) treated plants had 1.3-, 2.5- and 3-fold higher AsA content, respectively (Fig. 4A). Moreover, AsA contents in no-S ($S_0 + WL$) treated waterlogged plants was similar to control, while that of low-S ($S_1 + WL$), medium-S ($S_2 + WL$) and high-S ($S_3 + WL$) supplemented waterlogged

Fig. 4 Effect of different levels of soil-applied S (S_0 , No-S application; S_1 , 35 mg S kg^{-1} soil; S_2 , 70 mg S kg^{-1} soil; S_3 , 140 mg S kg^{-1} soil) on ascorbate (AsA) content (A), dehydroascorbate (DHA) content (B), AsA/DHA ratio (C), glutathione (GSH) content (D), oxidized glutathione (GSSG) content (E), and GSH/GSSG ratio (F) in leaves of rapeseed (*Brassica napus* L.) grown under control and waterlogged soils. Data are means \pm SE of four independent pot replicates. Bars followed by different letters indicate significant differences between the treatment groups (Tukey's test; $p \leq 0.05$)



plants were 0.3, 1.5- and 1.7-fold higher than the respective controls (Fig. 4A). Although DHA content notably decreased in no-S ($S_0 + WL$) waterlogged plants, it remained unchanged in low-S ($S_1 + WL$), medium-S ($S_2 + WL$) and high-S ($S_3 + WL$) supplemented waterlogged plants compared to their control group (Fig. 4B). In addition, the DHA content of high-S ($S_3 + WL$) supplemented plants dropped by 66% and 60% compared with no-S ($S_0 + WL$) and low-S ($S_1 + WL$) treated plants, while exhibiting statistically similar values with medium-S ($S_2 + WL$) treated plants under waterlogging condition, respectively (Fig. 4B). Accordingly, in high-S ($S_3 + WL$) treated waterlogged plants, AsA/DHA ratio was 67% higher than respective control (S_3) and 27% higher than medium-S ($S_2 + WL$) treated waterlogged plants (Fig. 4C).

The glutathione (GSH) content of both control and waterlogged plants linearly increased with S supplementation, with the exception of a non-significant difference between high-S (S_3) and medium-S (S_2) treated control plants (Fig. 4D). On the other hand, a non-significant increase (only by 5%) of glutathione content (GSH) was observed in high-S ($S_3 + WL$) supplemented waterlogged plants compared to control (Fig. 4D). Although the oxidized glutathione (GSSG) contents of no-S ($S_0 + WL$) waterlogged plants increased by 23% over control, there was no difference in GSSG contents between control and waterlogged plants of low-S (S_1), medium S (S_2) and high-S (S_3) supplemented plants (Fig. 4E). The GSH/GSSG ratio of high-S supplemented ($S_3 + WL$) waterlogged plants was 18% higher than the respective control (S_3), and 35% higher than medium-S treated ($S_2 + WL$) waterlogged plants (Fig. 4F).

The activity of APX in high-S ($S_3 + WL$) plants was considerably higher by 124% and 161% than low-S ($S_1 + WL$) and no-S ($S_0 + WL$) treated plants, while compared with medium-S ($S_2 + WL$) treated plants the APX activity increased non-significantly by 30% under waterlogging condition (Fig. 5A). Conversely, under waterlogging conditions the activity of APX in medium-S ($S_2 + WL$) supplemented plants declined notably by 26%, while it declined slightly (by 8%) in high-S ($S_3 + WL$) supplemented plants compared to the control group (Fig. 5A). Catalase (CAT) activity in medium-S ($S_2 + WL$) and high-S ($S_3 + WL$) supplemented waterlogged plants was 15% and 30% higher compared to respective controls (Fig. 5B). Furthermore, CAT activity of high-S ($S_3 + WL$) waterlogged plants increased significantly by 157%, 115%, and 12% compared with no-S ($S_0 + WL$), low-S ($S_1 + WL$) and medium-S ($S_2 + WL$) supplemented waterlogged plants, respectively (Fig. 5B).

Glutathione reductase (GR) activity of waterlogged plants supplemented with high-S ($S_3 + WL$) increased by 17%, while of no-S ($S_0 + WL$), low-S ($S_1 + WL$) and medium-S ($S_2 + WL$) waterlogged plants did not significantly increased

in comparison with the control counterparts (Fig. 5C). However, glutathione reductase (GR) activity of high-S ($S_3 + WL$) treated waterlogged plants increased was 17% higher than that of medium-S ($S_2 + WL$) waterlogged plants (Fig. 5C). By contrast, GR activity of high-S (S_3) supplemented control plants did not differ statistically compared with medium-S (S_2) supplemented control plants (Fig. 5C). Furthermore, MDHAR activity of no-S ($S_0 + WL$), low-S ($S_1 + WL$) and medium-S ($S_2 + WL$) treated waterlogged plants decreased non-significantly by 25%, 6% and 12% respectively, whereas in high-S ($S_3 + WL$) treated plants it increased slightly by 8% compared with respective controls (Fig. 5D). Compared with medium-S ($S_2 + WL$) waterlogged plants, high-S ($S_3 + WL$) treated waterlogged plants had 20% higher MDHAR activity (Fig. 5D). On the other hand, the MDHAR activity of high-S (S_3) supplemented control plants had no significant difference from that of medium (S_2) supplemented control plants (Fig. 5D).

The DHAR activity of no-S ($S_0 + WL$) and low-S ($S_1 + WL$) waterlogged plants slightly reduced by 21% and 14% compared with respective controls (Fig. 5E). Conversely, the DHAR activity of high S ($S_3 + WL$) treated waterlogged plants increased considerably by 43%, whereas medium S ($S_2 + WL$) supplemented waterlogged plants increased non-significantly (by 6%) compared to respective controls (Fig. 5E). Moreover, high-S waterlogged plants ($S_3 + WL$) exhibited 34% increase in DHAR activity compared to medium S ($S_2 + WL$) supplemented waterlogged plants (Fig. 5E). However, the DHAR activity of control plants that received medium-S (S_2) and high-S (S_3) showed was statistically similar (Fig. 5E).

Under waterlogging conditions, GPX activity of high-S ($S_3 + WL$) treated waterlogged plants increased by 18%, while that of no-S ($S_0 + WL$), low-S ($S_1 + WL$) and medium-S ($S_2 + WL$) treated waterlogged plants was statistically similar to respective controls (Fig. 6A). Strikingly, high-S supplemented waterlogged plants ($S_3 + WL$) showed 25% higher GPX activity compared to the medium-S supplemented waterlogged plants ($S_2 + WL$) (Fig. 6A). On the other hand, control plants receiving medium-S (S_2) and high S (S_3) had statistically similar GPX activities (Fig. 6A).

GST activity of no-S ($S_0 + WL$), low-S ($S_1 + WL$) and medium-S ($S_2 + WL$) and high-S ($S_3 + WL$) was statistically similar to respective non-waterlogged controls (Fig. 6B). However, GST activity of high-S treated waterlogged plants ($S_3 + WL$) increased considerably by 34% compared to medium-S ($S_2 + WL$) supplemented waterlogged plants, while medium S (S_2) and high-S (S_3) treated control plants had statistically similar GST activity (Fig. 6B).

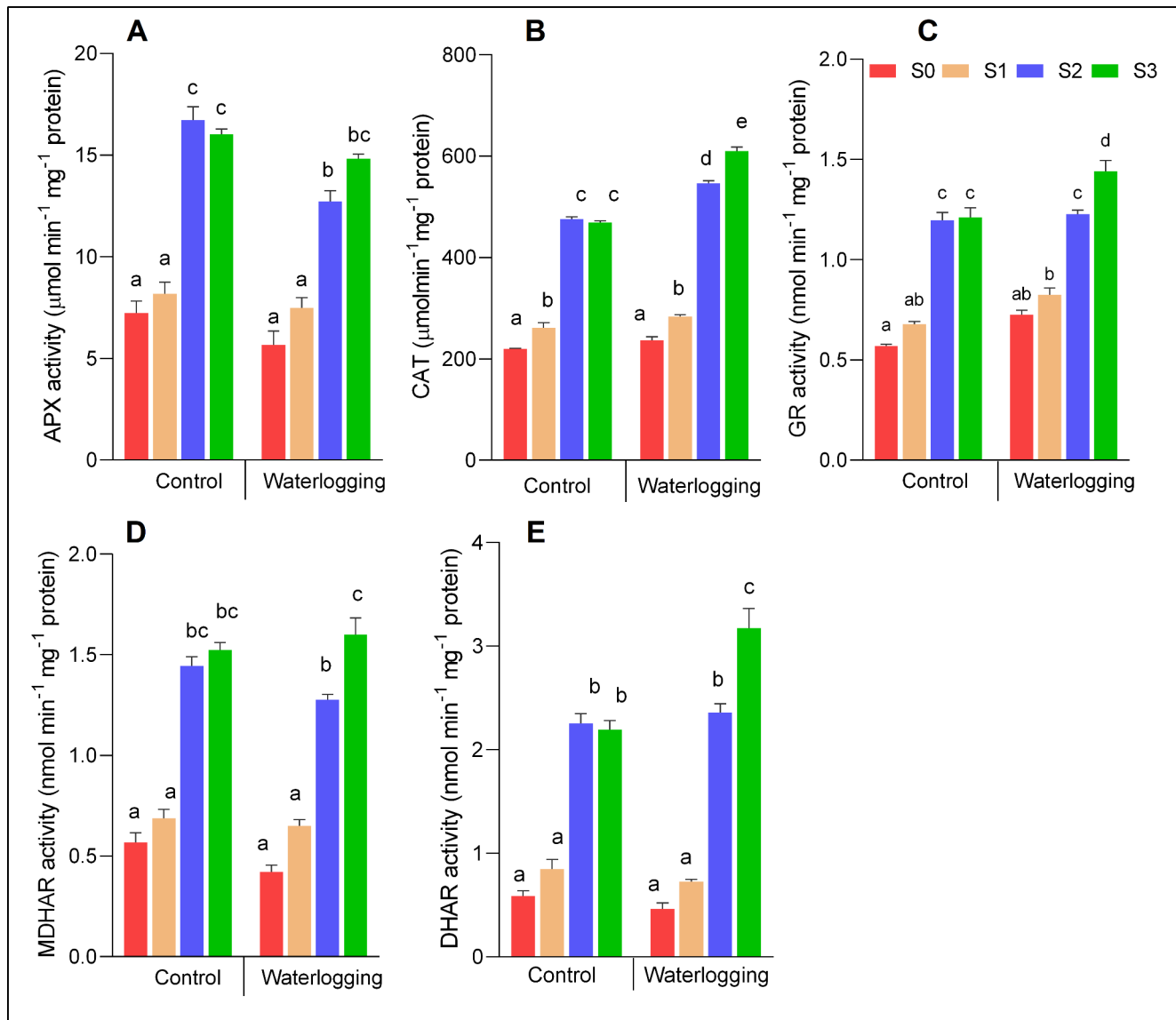


Fig. 5 Effect of different levels of soil-applied S (S₀, No-S application; S₁, 35 mg S kg⁻¹ soil; S₂, 70 mg S kg⁻¹ soil; S₃, 140 mg S kg⁻¹ soil) on the activity of APX (A), CAT (B), GR (C), MDHAR (D) and DHAR (E) in leaves of rapeseed (*Brassica napus* L.) grown under con-

trol and waterlogged soils. Data are means ± SE of four independent pot replicates. Bars followed by different letters indicate significant differences between the treatment groups (Tukey's test; $p \leq 0.05$)

Discussion

In plants, metabolism is a prerequisite for better productivity and higher yields, but it generally gets disturbed under abiotic stress conditions, mainly due to the excess production of reactive oxygen species (ROS). Therefore, redox balance is one of the key features by which oxidized products are reduced, and harmful effects such as lipid peroxidation are mitigated (Anee et al. 2019). As such, waterlogging exerts a detrimental effect due to the formation of hypoxic or even anoxic conditions, subsequently being followed by an impairment of the photosynthetic machinery that can promote the overproduction of ROS. To alleviate such stress

responses, S supplementation is reported to be an effective strategy for crop plants (Lou et al. 2017; Boldrin et al. 2016). Thus, in the current study, the influence of different levels of S supplementation (no-S, low-S, medium-S, and high-S) on the growth, recovery, and antioxidant defense mechanisms of rapeseed plants grown under waterlogged conditions was evaluated.

Supplementation of S to Waterlogged Plants Stimulates Growth, Yield, and Nutrient Status

The results showed that rapeseed is highly susceptible to waterlogging, depicting hampered plant performance

Fig. 6 Effect of different levels of soil-applied S (S_0 , No-S application; S_1 , 35 mg S kg^{-1} soil; S_2 , 70 mg S kg^{-1} soil; S_3 , 140 mg S kg^{-1} soil) on the activity of GPX (A) and GST (B) in leaves of rapeseed (*Brassica napus* L.) grown under control and waterlogged soils. Data are means \pm SE of four independent pot replicates. Bars followed by different letters indicate significant differences between the treatment groups (Tukey’s test; $p \leq 0.05$)

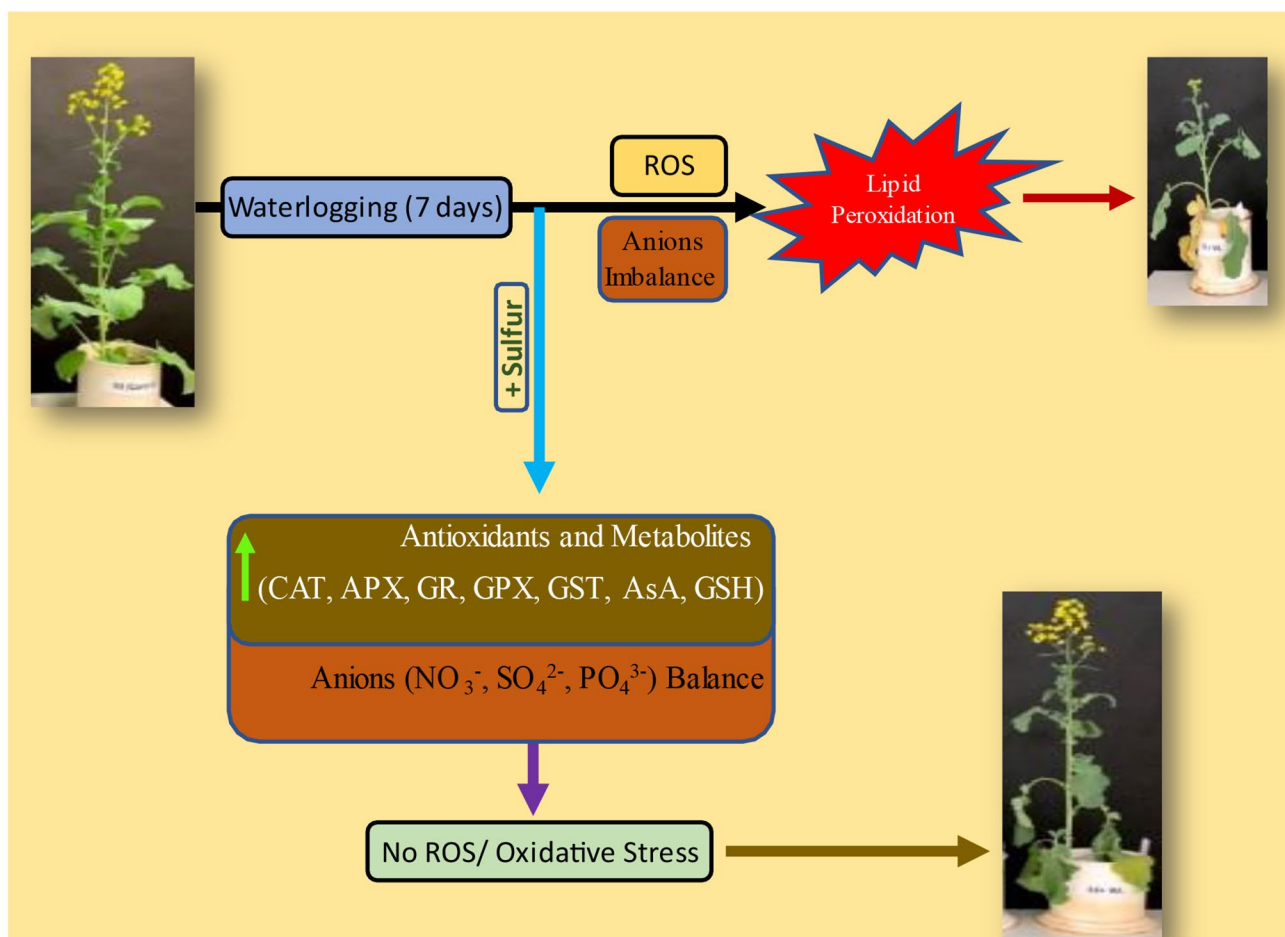
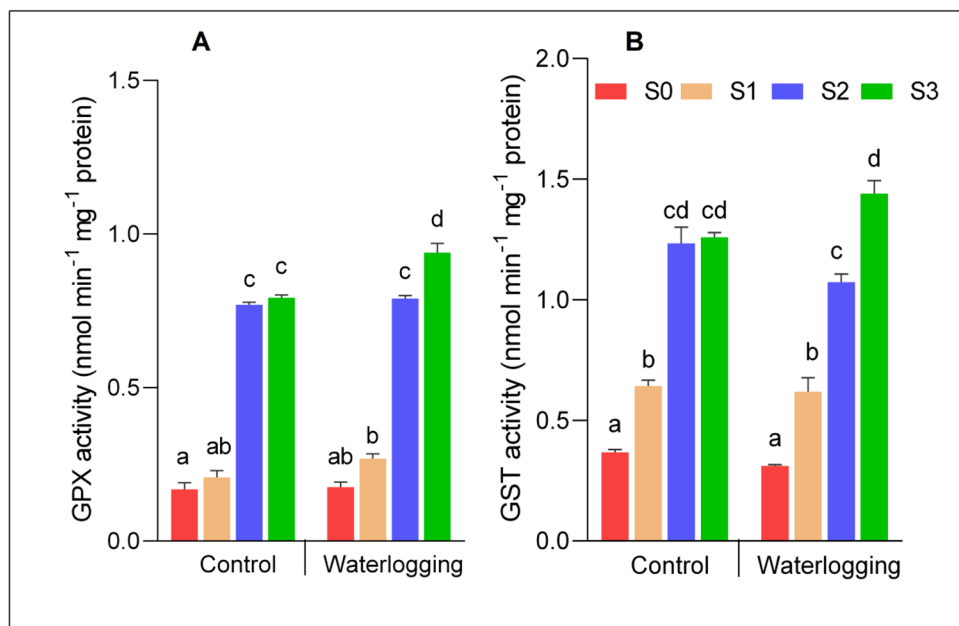


Fig. 7 Schematic illustration for possible mechanism of S-assist tolerance of rapeseed (*Brassica napus* L.) under control and waterlogged soils. Here, ASA, ascorbate; GSH, glutathione; ROS, Reactive oxygen

species; MDA, malondialdehyde; APX, ascorbate peroxidase; CAT, catalase; GPX, glutathione peroxidase; GST, glutathione S-transferase; GR, glutathione reductase

under only seven days of waterlogging stress (Figs. 1 and 2). Besides yellowing and chlorosis of older leaves, younger leaves also showed discoloration (Fig. 1). Waterlogged leaves showed symptoms of a serious S deficiency and low NO_3^- , SO_4^{2-} and PO_4^{3-} accumulation (Table 2). Such reduction in nutrient uptake resulted in reduced growth (Fig. 2A), seed yield, and yield-contributing traits (Fig. 2B C) of waterlogged plants (Hussain et al. 2022; Wollmer et al. 2018). On the other hand, S supplementation increased S content together with increased NO_3^- , SO_4^{2-} and PO_4^{3-} uptake (Table 2), possibly contributing to retrieval of waterlogging-induced loss in growth, yield, and yield contributing traits (Fig. 2A F). It can be reasoned that under abiotic stresses, the reduction of S in the plant certainly increases the demand for its supplementation for foiling photosynthetic apparatus and cellular metabolism, which finally leads to increased growth and development (Narayan et al. 2022; Fatma et al. 2014). Additional S, as supplemented here under waterlogging, was used as an alternative electron donor, as oxygen is depleted and soil microbes switch from using O_2 as an electron acceptor to SO_4^{2-} (Madigan et al. 2003). In this study, it was found that under control (normal water) conditions, high application of S beyond medium-S level did not benefit S accumulation, growth, yield and antioxidant metabolites (Figs. 2, 4 and 5). This finding is consistent with the early reports that high-S doses did not benefit non-stressed control plants (Rehman et al. 2013; Ahmad et al. 2011). It is assumed that plants do not accumulate S beyond an adequate S application level, and further increases in S application would not benefit the growth and yield. On the other hand, waterlogged plants need excess S application due to reduced S availability in soil and the increased demand for S to synthesize compounds containing reduced S (Fatma et al. 2014).

In the present investigation, it was also observed that chloride (Cl^-) concentration under waterlogging stress was much higher in the *Brassica napus* leaf (Table 2), possibly due to its increased transport rates in plants (Barrett-Lennard 2003). As a result of higher Cl^- concentrations and subsequent S deprivation under waterlogging stress, NO_3^- , SO_4^{2-} and PO_4^{3-} uptake was strongly negatively affected (Table 2). Supplemental S, especially high-S level, decreased Cl^- concentrations in waterlogged plants (Table 2), which might be attributed to the efficient Cl^- exclusion because Cl^- efflux has a positive correlation with stress tolerance (Sagervanshi et al. 2020; Li et al. 2016). Antagonistic interaction between Cl^- and SO_4^{2-} could also explain the reduction in Cl^- by increased S supplementation (Geilfus 2018).

Waterlogged Plants Reduced H_2O_2 and MDA with Supplemented S

Waterlogging treatment increased H_2O_2 content, which resulted in an elevation of MDA content in rapeseed leaves, indicating a high degree of lipid peroxidation (Fig. 3A and B). It is logical to infer that the antioxidant defense system could not mitigate the ROS surplus generated as a result of waterlogging stress (Wang et al. 2021). The antioxidant systems in plants possibly suffer from a lack of adequate S supply required for synthesizing antioxidant enzymes and other molecules, such as GSH, to suppress ROS production (Bashir et al. 2015). In contrast, S supplementation to waterlogged plants significantly decreased H_2O_2 and malondialdehyde (MDA) contents, possibly by enhanced antioxidant activities (Fig. 3A and B). In the S-assimilatory pathway, GSH synthesis is controlled and linked to ascorbate-glutathione (AsA-GSH) enzymes. As a result, the S assimilation-regulated antioxidant system helps eliminate H_2O_2 and repair cellular damage (Han et al. 2013).

Supplementation of S Enhances Antioxidant System in Waterlogged Plants

In this study, a considerable linear increase in the content of AsA was observed in plants supplemented with increasing S doses, which is most likely due to the increase in GSH content of S-supplemented waterlogged plants (Fig. 4D). This mechanism can be explained by the Mehler-peroxidase reaction where GSH plays a vital role in the regeneration of AsA from dehydroascorbate (DHA) via the enzyme dehydroascorbate reductase (DHAR), resulting in oxidation of GSH to glutathione disulfide (GSSG) (Li et al. 2010; Nector and Foyer 1998). It is clear from the findings that sulfur supplementation was associated with higher waterlogging tolerance, as AsA can directly scavenge ROS (Gill and Tuteja 2010). Again, in this study, the increasing trend of DHAR and MDHAR, together with APX and GR activity of the S-supplemented waterlogged plants (Fig. 5), highly supports this reasoning. As found in this study, compared with low- and medium-S, high-S applied waterlogged plants had higher CAT and APX activities (Fig. 5A and B), being involved in the reduction of H_2O_2 into H_2O (Teoh et al. 2022). The increase in APX and CAT activities could be attributed to maintaining the balance of the GSH–AsA cycle with the augmentation of GSH (Gomes-Junior et al. 2006).

An essential indicator of the amount of oxidative stress experienced by the cell is the ratio of AsA and DHA; an increase in DHA and a decrease in AsA indicate the inhibition of ascorbate synthesis. In this study, a reduction in DHA content was recorded with increasing S application to the waterlogged plants (Fig. 4B). Therefore, relatively

higher waterlogging tolerance of high-S supplemented plants could also be related to higher AsA/DHA and GSH/GSSG ratios, which maintain the redox state in plant cells under ominous stress conditions (Mohsin et al. 2019). It is assumed that the addition of S beyond plant needs tends to restore GSH levels and maintain the redox state of the cells under waterlogging conditions. Higher GSH/GSSG ratio, as well as GR activity (Fig. 5C) in S-supplemented plants, led to the inference that the former attribute is directly related to the latter one. This interpretation is supported by Mohsin et al. (2019), who observed a similar effect of increasing GR activity on balancing the redox state while elevating salt stress in wheat. Furthermore, these findings are consistent with the observation of Bano et al. (2022), indicating that higher S supplementation reduced arsenic (As) toxicity by boosting the antioxidant system and thus mitigated oxidative stress in *Brassica napus* plants. Aside from that, the higher tolerance of high-S supplemented waterlogged plants over medium-S waterlogged plants is attributed to higher GPX and GST activity (Fig. 6A–B), which are responsible for the detoxification and reduction of H₂O₂. The underlying mechanism is oxygen radical scavenging; GPX reduces H₂O₂ via glutathione (GSH) to create water and glutathione disulfide (Li et al. 2020). Thus, the increased production of GSH in S-supplemented waterlogged plants accelerates GPX and GST activities to inhibit H₂O₂ accumulation (Dixon 2010). The results of this study showed that an efficient antioxidant system is correlated with increased resistance to waterlogging stress and the removal of ROS (Fig. 7). Waterlogged plants supplemented with lower amounts of S were found to have a reduced synthesis of GSH and lower antioxidant capacity, which led to a lower ability to counteract waterlogging stress. On the other hand, a higher S accumulation in high-S-supplemented plants resulted in an upsurge in GSH production, allowing the plants to balance ion uptake and cope with waterlogging (Fig. 7).

Conclusion

In *Brassica napus*, waterlogging stress negatively affected plant growth and yield, possibly sparking oxidative stress due to the overproduction of reactive oxygen species. In contrast, supplementing waterlogged plants with high levels of S proved beneficial for the growth and development of plants, due to a remarkable increase in ROS scavenging capabilities, especially at the highest rate of application. Waterlogging-induced oxidative stress was ameliorated by high-S supplementation through increasing antioxidant metabolites (AsA and GSH) and enzyme activities (APX, CAT, GR, MDHR, DHAR). Moreover, it was found that high-S induced waterlogging resistance in rapeseed plants

was also linked with the regulation of anion uptake, i.e., reduction in Cl⁻ uptake and increase in the uptake of sulfate, nitrate, and phosphate. Overall, this study provided valuable insight about the S-assisted waterlogging tolerance in *Brassica napus* L.

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Author Contributions MAH conducted the experiments, measurements, calculations, analyses, and wrote the paper. AN assisted with the designing and setting-up of the experiment, supervised the measurements, and reviewed the manuscript. BP evaluated the measurements, reviewed the manuscript. KHM reviewed the manuscript, designed and supervised the study.

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Declarations

Conflict of interest The authors have declared that they do not have any conflicts of interest.

Consent for publication This manuscript does not provide data from any individual person, so it is “not applicable.”

Research Involving Human and Animal Participants We all certify that manuscripts reporting studies do not include any human participants, human data, or human tissue. So, it is not applicable.

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