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Sulfur fertilization and waterlogging stress resistance: Elucidation of the underlying mechanisms in wheat and rapeseed

Dissertation

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Chapter 1

General introduction

The adverse effects of the environment on plant growth, development, and productivity are known as abiotic stress (Zhang et al., 2022). There are several common abiotic stresses that affect plants, viz. waterlogging, extreme temperatures, drought, salinity, heavy metals, etc. Waterlogging is one of the issues that has attracted researchers' attention in recent decades because of the intensity of extreme weather events (Li et al., 2021; Trnka et al., 2014). There is no doubt that water plays an important role throughout the life cycle of plants, but excessive soil saturation can result in waterlogging (Meng et al., 2018). In addition, climate change has also caused more unpredictable rainfall during winter and spring throughout the world, particularly in Europe, the United States, India, China, Pakistan, Argentina, and some other irrigated regions (Feyen et al., 2012; He, 2014; Kreuzwieser et al., 2009; Pang et al., 2007b; Ploschuk et al., 2018; Shabala et al., 2014). A global study estimates that 17 million square kilometers of land are affected by waterlogging each year, which represents 10-12 percent of agricultural land worldwide (Kaur et al., 2019; Shabala et al., 2014). Therefore, waterlogging affect plant growth and development, which ultimately results in yield losses or harvest failures, that could severely compromise food security by 2050, when 10 billion people are expected on this planet (Armstrong and Drew, 2002; Moustafa-Farag et al., 2020; Pan et al., 2021). Furthermore, a meta-analysis by Tian et al. (2021) presented that waterlogging decreased global crop yield by 32.9% on average. There are several factors that determine the response of plants to waterlogging, including the stage of growth, the duration of exposure, and the extent of aridity (Ding et al., 2020a; Setter et al., 2003; Wu et al., 2019). In contrast, Kaur et al. (2019) mentioned that yield losses due to waterlogging stress range from 1 to 100% depending on the crop, the waterlogging duration, and the crop growth stage. Thus, almost all widely cultivated crops, such as wheat, corn, rapeseed, soybean, etc., are affected by waterlogging (Ding et al., 2020b; Men et al., 2020).

Throughout the world, including Europe, North America and Asia, rapeseed (*Brassica napus* L.) is seriously affected by waterlogging (Pachauri et al., 2014). Besides being a major source of edible vegetable oil, rapeseed oilcake, which is a byproduct of oil production, is also an excellent source of protein for animal feeding (Hussain et al., 2016). Having a low saturated fat content and a high amount of healthy unsaturated fats, rapeseed oil has many health benefits. In addition, it contains omega-3 fatty acids, which are good for heart health (Zeb et al., 2021). As a rotation crop, rapeseed is still a favorable choice in Asia (Shen et al., 2023). Lack of aerenchyma formation makes oilseed rape (*Brassica napus* L.) highly susceptible to waterlogging (Voesenek et al., 1999). Over the past decades, waterlogging has hindered rapeseed growth, resulting in yield reductions of more than 50% (Herzog et al., 2016; Wollmer et al., 2017). Further, waterlogging altered the lipid biosynthesis metabolic pathway as well as increased erucic acid and glucosinolate content in oil (Xu et al., 2014).

Aside from rapeseed, wheat is also affected by waterlogging worldwide despite the capability of forming aerenchyma (Ploschuk et al., 2018). Approximately 10–15 million hectares land of wheat are affected each year due to waterlogging, resulting in yield losses of between 20 and 50% (Pais et al., 2023). Globally, wheat is considered as the third most produced cereal crop and supplied around 20% of energy and protein requirements (Hawkesford et al., 2013). A well-drained soil is essential for wheat production; therefore, waterlogging can cause it to fail (De San Celedonio et al., 2022).

Causes of waterlogging

Over the whole life cycle, water is a vital component for plants, but excessive soil saturation in the root zone leads to waterlogging (Meng et al., 2018). The main causes include extreme climate, increasing precipitation events, rainfall taking place after irrigation, poor external drainage (runoff) as well as internal drainage (shallow water table), soil compaction, and land topography (Hanna et al., 1982; Kirkpatrick et al., 2006). When soil is poorly structured due to human activity or natural factors, for example heavy traffic or puddling, water cannot drain properly in the soil profile (Batey, 2009). Generally, waterlogging is caused by soil compaction and texture, like in heavy clay soils or duplex soils (coarse textured topsoil covering compacted clay subsoil) and can be severe if they occur at low elevations (Blessitt, 2007; Cox et al., 1990). Additionally, the slope of the land is also involved in waterlogging events because high slopes increase runoff, especially when the soil is saturated to the surface and water cannot penetrate. As a result of high slopes, the soil is prone to lateral seepage (Batey, 2009).

1.2 Fate of waterlogged soil

Waterlogging can occur when soil water goes beyond field capacity (Pampana, 2016). As a result of waterlogging stress, the air is removed from soil pores, resulting in an imbalance between solid: pore material ratio from 50:50 to 75:25, resulting in a blocked exchange of gases between atmosphere and soil (Morales-Olmedo et al., 2015; Pais et al., 2023). However, due to the limited oxygen transmission in water ($1/10^4$ the rate as air) waterlogging stress is often associated with hypoxic and anoxic conditions (Pan et al., 2021). In addition, in waterlogged soils, root respiration and microbial activity consume soil oxygen, stimulating a hypoxia/anoxic situation in the rhizosphere, which results in both decreased O_2 levels and toxic CO_2 concentrations in soil (Sasidharan et al., 2017). As a consequence, waterlogging creates oxygen shortages, which cause soils to undergo different changes, namely an increase in ethylene levels and anaerobic metabolism products (e.g., Mn^{2+} , H_2S , Fe^{2+} , carboxylic acids etc.) (Jackson, 2005; Ponnampereuma, 1984; Smith and Russell, 1969). Apart from these factors, soil that become waterlogged tend to sharply drop redox potential (Eh) due to the shortage of oxygen (Mobilian et al., 2022; Sajwan and Lind, 1986). Consequently, the threshold redox potential of less than +300 mV may drop between -100 and -200 mV based on waterlogging duration and intensity (Mobilian et al., 2022). The decline of Eh below +300 mV indicates anaerobic conditions, while a further decrease below 150 mV causes the reduction of Fe^{3+} to Fe^{2+} , and SO_4^{2-} to H_2S below -100 mV (Husson et al., 2013; Pepper & Gentry, 2015). Apart from these processes, soil waterlogging modifies soil pH in a way that it tends toward neutrality, though decreasing in alkaline and increases in acidic soils which have a major influence on nutrient solubility, mobility, and uptake (Husson et al., 2013; Pias et al., 2023).

1.3 Physiological, metabolic and mineral nutrients change in waterlogged plants

1.3.1 Reactive oxygen species (ROS)

Waterlogging and anaerobic respiration that last for a prolonged period can accumulate toxic compounds such as lactic acid, ethanol, and aldehydes (Xu et al., 2014; Zhang et al., 2019).

Upon exposure to ethylene, waterlogging stress enhances the expression of respiratory burst oxidase homolog (RBOH), generating reactive oxygen species (ROS) including superoxide, singlet oxygen, hydrogen peroxide, and hydroxyl radical (Qi et al., 2019). In response, plants

activate their antioxidant defense mechanisms to scavenge excessive ROS (Teoh et al., 2022). Since the cellular antioxidant defense system cannot completely combat ROS generation due to waterlogging stress, excessive ROS formation occurs, resulting in oxidative stress (Hasanuzzaman et al., 2017; Keya et al., 2022). Additionally, plants close their stomata during waterlogging to prevent water loss, thus downregulating their photosynthetic processes due to the light reaction both of which lead to the higher formation of reactive oxygen species (ROS) (Park et al., 2019; Yetisir et al., 2006). Nevertheless, overproduction of ROS causes photooxidative damage to various cellular entities namely lipoperoxidation, protein degradation, enzyme inactivation and disruption of DNA strands (Nadarajah et al., 2020; Pan et al., 2021). Besides their damaging effects, ROS are also regarded as a signaling molecule that affect plant growth, stress-responsive genes, and ultimately leads to programmed cell death (PCD) (Gill and Tuteja, 2010). In order to cope with oxidative stress, plants produce antioxidant compounds, such as ascorbate, glutathione as well as antioxidant enzymes, such as superoxide dismutase (EC 1.15.1.1), ascorbate peroxidase (EC 1.11.1.11), catalase (EC 1.11.1.6), and glutathione reductase (EC: 1.6.4.2) (Anee et al., 2019). The results of some studies reported that the activity of antioxidant enzymes can be increased under waterlogging (Yin et al., 2009), while others have demonstrated reduced antioxidant enzyme activities in waterlogged plants (Tan et al., 2008).

1.3.2 Impacts on physiological performance

Waterlogging stress potentially impairs net photosynthesis, e.g., stomatal closure, reduced transpiration, reduction of photosynthetic (Herzog et al., 2016; Jackson et al., 1988). A waterlogged soil is unable to exchange gas with the atmosphere due to air being removed from pores; at the same time, waterlogging may increase CO₂ concentrations in the root zone and decrease hydraulic conductance, leading to rapid stomatal closure and lower net photosynthesis and transpiration rates (Anee et al., 2019; Zhu et al., 2016). In addition to damaging the photosynthetic apparatus, waterlogging lowered the ratio of variable to maximal fluorescence (Fv/Fm), a sign of photo-inhibitory impairment under stress (Kaur et al., 2019).

1.3.3 Nutrient uptake impairment due to waterlogging

In waterlogged soil, oxygen is rapidly exhausted, which causes the roots to switch from aerobic to anaerobic respiration, increasing the concentrations of CO₂ and ethylene. This result in a

severe reduction of ATP synthesis since plants switch to fermentation mode to produce ATP as a rescue mechanism for root cells, which affects the efficiency of ATP production: i.e., 3 ATP molecules against 39 ATP molecules from hexose respiration (Drew, 1997; Kaur et al., 2020; Pampana et al., 2016; Sairam et al., 2008). During such an anaerobic condition, reduced root hair formation, lowering the ability to absorb water and mineral nutrients are observed (Ashraf et al., 2011; Shabala et al., 2014; Teoh et al., 2022; Wollmer et al., 2017). In addition, a sharp reduction in ATP production impairs cellular metabolism at the root level by impairing aquaporin phosphorylation that controls water flux within the cell, reducing root hydraulic conductivity and further impairing water and nutrient absorption (Geigenberger, 2003; Malik et al., 2002; Pang et al., 2007a). Furthermore, due to low ATP concentrations, H^+ -ATPase activity is reduced in roots, causing partial membrane depolarization, which impaired inorganic cation uptake (Schubert and Yan, 1999; Steffens et al., 2005; Zhang et al., 2019). Further, by reducing H^+ -ATPase activity in O_2 -deficient roots it is likely that the proton motive force, mediated by plasma membrane H^+ -ATPases, is also reduced (Elzenga & van Veen, 2010). Accordingly, nutrient channels become blocked, reducing, e.g., xylem loading by making it difficult for water to get into the xylem and surrounding cells, thereby lowering ion mobility (Colmer & Greenway, 2011; Krieger, 2005). Nonetheless, in waterlogged soils, organic matter decomposes, producing organic acids and other potentially toxic metabolites that inhibit root respiration, thereby reducing nutrient uptake (Pais et al., 2023). In barley, for example, Colmer et al. (2011) reported adverse effects of organic acids on K^+ fluxes. Nutrient uptake by mass flow was also found to be reduced, due to the stomatal closure which dropped transpiration and affected the mass flow of nutrients (McGrath et al., 2013). Above all, waterlogging creates a paucity of soil mineral nutrients especially nitrate and sulfate because of denitrification and desulfurization processes which promote nitrogen (N) and sulfur (S) losses by both leaching and volatilization (Malik et al., 2002).

1.4 Plant adaptation to waterlogging stress

A variety of morphological, anatomical, and physiological modifications exist in plants to cope with waterlogging stress, referred to as low-oxygen escape and low-oxygen quiescence strategies (Voesenek and Bailey-Serres, 2015). In the low-oxygen escape strategy, a variety of plant species prevent oxygen deficiency by developing features that favor gas transport, including the development of adventitious roots, creation of airspace (aerenchyma) in the root cortex and leaves, radial root oxygen barrier by producing lignin/suberin barrier to limit the

radial loss of oxygen in root, stem enlargement (hypertrophy), formation of gas films on hydrophobic leaf surfaces that facilitate gas exchange between the submerged shoot and surrounding water (Ambro et al., 2018; Sasidharan and Voesenek, 2015). In contrast, some plant species adapt to waterlogging by following the low-oxygen quiescence strategy, requiring significant metabolic changes by suppressing growth and development until waterlogging recede. Towards this goal, energy consumption is minimized by turning off energy-intensive processes such as ribosome biogenesis and the synthesis of cellular walls, and induction of fermentation processes mostly involving lactic acid and ethanol fermentation (Shabala et al., 2014; van Dongen & Licausi, 2015; Wittig et al., 2020; Yamauchi et al., 2018). Adventitious roots, however, contain more aerenchyma than primary lateral roots, which assists the plant in supplying oxygen to the root tip and rhizosphere as well as removing gases (CO₂, ethylene, and methane) from the root and soil. The ability of different species to generate adventitious roots with aerenchyma can vary; for example, root porosity can range from 19–30% in wheat (Huang et al., 1994) and 7–23% in barley (Pang et al., 2007a), while the roots of rapeseed (Voesenek et al., 1999) as well as field pea (Healy and Armstrong, 1972) do not produce aerenchyma under oxygen deficit conditions.

1.5 Role of exogenous nutrient application for waterlogging adaptation

Nutrition-induced changes in waterlogging tolerance have been thoroughly investigated. It has been shown that plant nutrients can effectively reduce waterlogging damage by improving the photosynthetic components, antioxidant defenses, increasing nutrient uptake, ensuring cell turgidity, regulating metabolite patterns, changing metabolite concentrations, and acting as precursors for various enzymes and hormones (Cakmak et al., 2005; Marschner, 2001; Wang et al., 2013). Therefore, exogenous nutrients supplies either to soil and/or foliar can modify plant responses to waterlogging stress. For example, the application of phosphorus in the soil increases the growth of *Pinus taeda* under waterlogging conditions (Hook et al., 1983). In contrast, Pang et al. (2007b) demonstrated that leaf nutrient application ameliorates the detrimental effect of waterlogging on barley. Nonetheless, Ashraf et al. (2011) concluded that exogenous K applied as a foliar spray and in the soil alleviated the adverse effects of waterlogging in cotton plants. Furthermore, Yang et al. (2016) revealed that exogenous Ca modulated osmotic substance levels, antioxidant system, root respiration and metabolism, thereby reducing waterlogging-induced damage to pepper plants. Likewise, supplemental nitrogen treatment leads to higher growth, nitrogen accumulation, enhanced antioxidant system

as well as photosynthetic performance of winter rapeseed, corn and cotton under waterlogging stress (Chen et al., 2010; Guo et al., 2009; Men et al., 2020). In addition to these, potassium also displayed a scavenging ability towards waterlogging stress by maintaining membrane stability, high photosynthetic rate, and higher nutrients uptake and growth, in different crops (Ashraf et al., 2011; Mamun et al., 2022; Wang et al., 2013). Besides macronutrients, micronutrients also postered waterlogging tolerance (Kumari et al., 2021). For example, the foliar application of boron was demonstrated to be more effective to mitigate the adverse effects of waterlogging by increasing the stability of leaf membranes, chlorophyll contents, soluble sugars, soluble proteins and amino acid contents (Sayed et al., 1998). Further, plants tend to be more tolerant to waterlogged conditions when Zinc was applied to Zn deficiency plants to restore the activities of the ADH (Porwal et al., 2021).

1.6 Role of sulfur in mitigating abiotic stress

Sulfur and its derivatives are found to have protective functions in response to abiotic stresses (Cao et al., 2014; Noctor, 2006). Sulfur (S) is necessary for plants to synthesize glutathione (GSH). After being absorbed as sulfate, sulfur is incorporated into cysteine (Cys) which serves as a precursor for several key compounds, including methionine, S-adenosylmethionine, glutathione and others (Li, 2020). In diverse abiotic stress conditions, an exogenous supply of S and its derivatives have been shown to benefit plants' survival by stabilizing metabolic processes, cellular acclimatization, strengthening antioxidant defense system and adaptability to adverse conditions (Anjum et al., 2015; Fatma et al., 2014; Hasanuzzaman et al., 2018; Waraich et al., 2022). Likewise, Bano et al. (2022) demonstrated that sulfur alleviated arsenic phytotoxicity in *Brassica napus* by upregulating antioxidant systems, thiol biosynthesis, S-assimilation, and photosynthesis. In addition, Keya et al. (2022) and Siddiqui et al. (2022) reported that S derivatives (GSH) can alleviate waterlogging stress in sesame and rice. Further, Li et al. (2021) and Cheng et al. (2013) demonstrated that H₂S can alleviate waterlogging stress in different plants.

Objectives

In the first part of this study, the impact of early (Feekes Stage 6) and late (Feekes Stage 10.1) waterlogging on S-uptake, distribution, and yield quality of commercially available inbred and hybrid wheat cultivars was investigated. Up to now, the differences between commercial high-yielding inbred and hybrid wheat genotypes regarding waterlogging tolerance, S uptake and

yield quality are still scarce. Considering this, the objective of the first study was to evaluate the comparative performance of hybrid and inbred winter wheat varieties in terms of yield, yield components, S-uptake and partitioning, and grain storage proteins (glutenins and gliadins) under early and late waterlogging conditions.

Based on our initial findings, it was noticed that waterlogging reduced S uptake, which in turn reduced yield and quality. Therefore, it can be assumed that exogenous S supply can be a game changer for waterlogging stress mitigation and that responses will vary with supplementation levels. To assess this statement, high sulfur demanding rapeseed (*Brassica napus* L.) was selected as a crop of choice. Although several studies have confirmed that S posterred tolerance of different abiotic stresses in a variety of crops, to the best of my knowledge, there is rare evidence of whether soil supplementation with S can enhance waterlogging stress tolerance in rapeseed. Therefore, the second study was designed to investigate whether waterlogged plants supplemented with different S levels (no-S, low-S, medium-S and high-S) can improve S-assisted antioxidant defense and offset waterlogging-induced adverse effects on the growth and yield of rapeseed.

From the second study, it was concluded with convincing results that soil supplementation with high-S can accelerate waterlogging tolerance to rapeseed. However, some convincing reports of foliar feeding that can provide rapid and efficient treatment for deficiency symptoms, particularly when problems relating to soil fixation occurred which restrict nutrient uptake from soil drive into third study of this thesis (Noreen et al., 2018; Oosterhuis et al., 2010). Therefore, the third study was conducted to investigate the ability of foliar-S to attenuate waterlogging stress. It was hypothesized that foliar-S upsurses glutathione contents which will then be utilized for boosting the antioxidant system of both S-fertilized (+S) and unfertilized (-S) waterlogged plants. In this regard, the objective of the third study was to understand the effect of S supplementation as foliar S and/or soil S-fertilized waterlogged plants on physiological responses, growth and nutrients uptake.

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Chapter 2

Sulfur uptake and distribution, grain yield, and quality of hybrid and inbred winter wheat (*Triticum aestivum* L.) varieties under early and late waterlogging

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RESEARCH ARTICLE

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Abstract

Background: Waterlogging at early and later growth stages could affect sulfur (S) uptake, distribution, yield, and quality of winter wheat. We hypothesized that the hybrid winter wheat variety (Hyvento), with a high degree of heterosis and genetic diversity, is likely to uptake and utilize S more efficiently and withstands waterlogging better than the inbred wheat variety (Kredo).

Aims: The objective of the present study was to investigate the effect of 2-week waterlogging at the end of tillering stage (Feekes Stage 6) and/or ear emergence stage (Feekes Stage 10.1) on grain yield, yield components, S-uptake and its partitioning, and composition of grain storage proteins (glutenins and gliadins) of hybrid (Hyvento) and inbred (Kredo) winter wheat varieties.

Methods: Plants were grown in large containers (120 L) in a semi-controlled greenhouse and harvested three times immediately after each waterlogging treatment and at agronomic maturity. Grain yield, S concentration in different plant organs, total protein content, and patterns of gluten proteins (gliadin and glutenin) and their subunits (by sodium dodecyl sulfate–polyacrylamide gel electrophoresis) in grains were evaluated.

Results: Grain yield of the inbred variety was more negatively affected under early and late waterlogging than the hybrid variety and was associated with reduced grain size. The decline in grain yield was significantly higher under late waterlogging (30%–31%) than early waterlogging (17%–18%). Compared to the hybrid variety Hyvento, the waterlogging-induced decline in S concentration in grains, husk, and straw was markedly lower in the inbred variety Kredo. While early waterlogging did not affect total grain protein content in either of the varieties, late waterlogging significantly reduced the total grain protein content (18%) in the inbred variety Kredo. Similarly, the grain gliadin content of the hybrid variety was not affected by both waterlogged conditions, whereas 32% and 23% reductions were recorded for the inbred variety under early and late waterlogging, respectively. Nevertheless, waterlogging significantly increased the proportion of S-poor high-molecular-weight glutenin subunits and ω -gliadin in the inbred variety.

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Conclusion: It could be inferred from the present study that hybrid winter wheat variety is relatively resistant to waterlogging than the inbred wheat variety, and thereby produces higher grain yield. Efficient uptake and metabolism of S could be one of the mechanisms conferring waterlogging resistance in winter wheat.

KEYWORDS

genetic diversity, hybrid and inbred wheat, sulfur uptake protein accumulation pattern, waterlogging resistance

1 | INTRODUCTION

Waterlogging (WL) is one of the major abiotic stresses that impede the production and quality of crops (Arata et al., 2019). Because of global climate change, the magnitude and frequency of WL risk are predicted to increase, especially in the high- and mid-latitude regions (Easterling et al., 2000; García et al., 2007; Jiang et al., 2008; Schumacher & Johnson, 2006). Moreover, the occurrence of flash floods is also expected to increase in Europe due to an intensified hydrological cycle by global warming (Feyen et al., 2012; IPCC, 2014). Annually, about 10% of the world's land area and 10–15 million ha of the world's wheat-growing regions, including 15–20% of wheat cultivated area, are affected by WL (Hossain & Uddin, 2011).

In Germany, heavy rainfall events have become more intense, and the average annual precipitation has been significantly higher over the last 20 years (Deumlich & Gericke, 2020). Accordingly, winter (December to February) and summer (June to August) precipitations in northern Europe have increased up to 70 mm (Maraun, 2013) and 18 mm per decade, respectively (Van den Besselaar et al., 2013). Therefore, wheat is likely to experience WL stress during the end of tillering (Feekes Stage 6) and ear emergence (Feekes Stage 10.1) stages, which are critical for crop establishment, grain yield, and quality.

Under WL conditions, ethylene production rapidly increases inside the plants as a primary signal for acclimation to this adverse condition (Pan et al., 2021). This stress can result in anaerobiosis, which may cause leaf senescence, reduced root growth, and tillering, ultimately affecting biomass and grain yield. In addition, WL restricts photosynthesis and carbohydrate synthesis both in the shoot and root (Xie et al., 2003; Yamauchi et al., 2013). These effects depend on the duration of WL, crop development stage, and the WL-resistance capacity of the crop species/variety (Arata et al., 2019; Arduini et al., 2016; De San Celedonio et al., 2014). Coincident with the susceptibility of wheat to WL, a grain yield decline ranging from 20% to 50% has been reported (Arduini et al., 2016; Belford, 1981; Cao et al., 1996; Ding & Musgrave, 1995; Zhang et al., 2006) depending upon the duration and timing of WL (Musgrave & Ding, 1998; Xie et al., 2003).

WL decreases the uptake and transport of ions through roots causing nutrient deficiencies (Setter et al., 2009; Wollmer et al., 2019; Zhang et al., 2011). Sulfur (S) deficiency is among the most probable nutrient deficiencies that might occur under WL conditions due to the reduction of sulfate into hydrogen sulfide and its subsequent volatilization or precipitation as sulfides of micronutrient cations (Fe,

Cu, Mn, and Zn) (McGrath et al., 1996; Ponnampereuma, 1972; Steffens et al., 2005). Apart from the precise effects on crop growth and grain yield, soil S deficiency can also exert a considerable influence on wheat quality due to the alteration of S-rich prolamins (α - and γ -gliadins, low-molecular-weight glutenin subunits [LMW-GS]) and S-low/S-poor prolamins (high-molecular-weight glutenin subunits [HMW-GS], ω -gliadins) (Wollmer et al., 2018). Thus, the proteins responsible for the breadmaking process, gliadins and glutenins, will be affected (Jiang et al., 2008). Glutenins are multimeric aggregates of HMW-GS and LMW-GS subunits. On the other hand, gliadins are mainly monomeric proteins and divided into four structural types, that is, α -, β -, γ -, and ω -gliadins. The quality of wheat grains is affected by the proportion of each protein fraction constituting that grain protein (Payne et al., 1987; Wieser, 2007).

Tolerance of the winter cereal to WL is related mainly to (1) the duration of the WL event, (2) the crop development stage at which WL occurs, and (3) the sensitivity of the species or variety (Brisson et al., 2002; de San Celedonio et al., 2014). For instance, WL for 20 days can reduce the grain yield of winter wheat by 32%–94% (de San Celedonio et al., 2014). Regarding the development stages of wheat, it was revealed that the order of susceptibility to WL at different stages was as follows: booting stage > jointing stage > tillering stage > grain filling stage (Bao, 1997). Furthermore, there are pieces of evidence of genotypic differences among wheat genotypes' intolerance to WL (Mano & Oyanagi, 2009). Therefore, hybrid wheat production is a potential strategy to alleviate yield depressions and fluctuations caused by weather extremes and associated plant stresses (Mühleisen et al., 2014). Hybrid genotypes showed more tolerance against different abiotic stresses, including WL, drought, frost, salinity, and heat stresses (Adee, 2016; Cao et al., 1992; Carretero et al., 2014; Collaku & Harrison, 2005; Longin et al., 2013; Ünay & Şimşek, 2020; Zhou et al., 2007). For example, hybrid wheat (Ibrahim et al., 2014; Jatoi et al., 2014) and maize (Adee et al., 2016; Singh et al., 2013) varieties were found more tolerant to drought stress and hybrid wheat to salinity and heat stresses (Yildirim et al., 2010).

The differences between the commercial high-yielding inbred and hybrid wheat genotypes regarding tolerance, yield, and yield quality under WL are still scarce, especially under west-European conditions. In addition, the WL-induced effects on inbred and hybrid wheat varieties, S uptake, and distribution and grain quality concerning protein composition have not so far been examined to draw a factual and sound inference (Ashraf, 2014). This justifies the integrated analysis



TABLE 1 pH, organic matter, and nutrient contents of the topsoil and subsoil used in the experiment.

Parameter	Topsoil	Subsoil
Texture	Loamy sand	Sandy loam
pH _{1:2.5} (CaCl ₂)	6.0	5.8
Organic matter (g kg ⁻¹)	15.0	4.0
Total nitrogen (N, %)	0.09	0.03
Potassium (K, mg kg ⁻¹)	99.6	33.2
Phosphorus (P, mg kg ⁻¹)	48.0	21.8
Sulfur (SO ₄ ²⁻ , mg kg ⁻¹)	160.0	70.0

of these variables under WL stress. Therefore, we consider it a reasonable hypothesis that the hybrid wheat variety, with a high degree of heterosis and genetic diversity, uptakes and utilizes S more efficiently and withstands WL better than the inbred wheat variety at Feekes Stage 6 and/or Feekes Stage 10.1. Accordingly, the objective of the present study was to assess the comparative performance of commercial hybrid and inbred winter wheat varieties regarding grain yield, yield components, S-uptake and its partitioning, and grain storage proteins (glutenins and gliadins) composition under early and late WL conditions.

2 | MATERIALS AND METHODS

2.1 | Container experiment

Seeds of hybrid (Hyvento) and inbred (Kredo) winter wheat (*Triticum aestivum* L.) varieties were obtained from Nordsaat Saatucht GmbH, Langenstein, Germany. The experiment was carried out in 120-L plastic containers (height: 0.9 m, area: 0.16 m²) with draining valves at the bottom (Hohmann et al., 2016), filled with the soil derived from the experimental farm of Kiel University, Germany. The soil is classified as a Cambisol following the criteria described by WRB (IUSS Working Group WRB, 2015). After drying and homogenization, the soil was filled in three steps in the containers: (1) gravel layer as drainage, (2) mixture of sandy loam subsoil and sand (1:1 [w/w]), and (3) mixture of loamy sand topsoil with sand (9:1 [w/w]). The pH, organic matter content, and nutrient contents of loamy sand topsoil and sandy loam subsoil are depicted in Table 1. Before seed sowing, each container was applied with the following fertilizers as basal dose (per kg of soil): 44 mg Ca(H₂PO₄)₂, 156 mg K₂SO₄, 23 mg MgSO₄·7H₂O, 150 mg urea (CH₄N₂O), and 25 mg (NH₄)₂SO₄. The experimental containers were placed in a nontemperature greenhouse for the whole growing period. The experiment consisted of the following three treatments: (1) control (moisture level equivalent to 60% soil water holding capacity [WHC]), (2) early WL (EWL; at first node visible stage [Feekes Stage 6; Large, 1954]), and (3) late WL (LWL; at beginning of ear emergence [Feekes Stage 10.1]). For both WL treatments, approximately 3-cm layer of water was kept above the soil surface for 14 days. The WL treatments were terminated by draining the water through the faucets at

the bottom of each container. After termination of WL treatments, the moisture level in the containers was maintained equivalent to the control, that is, 60% WHC. The water level in the containers was adjusted to 60% soil WHC by weighing the containers twice a week. The experimental treatments, each having four replicates, were arranged in a completely randomized design.

2.2 | Soil sampling and analysis

Soil samples (each of approximately 250–300 g) were collected from all the containers by digging three soil cores near the center to a depth of 50 cm before seed sowing and after the WL treatments have been implemented. The holes so formed in the pots were filled with the soil taken from the surrounding areas. After that, the soil samples were kept at –20°C, and later analyzed for SO₄²⁻-S contents by inductively coupled plasma optical emission spectrometry (ICP-OES) following their extraction with 0.0125 M CaCl₂ (soil to solution ratio of 1:4) as described by Bassler and Hoffmann (1997).

2.3 | Plant sampling and analysis

Plant samples were collected immediately after the termination of WL and at maturity. Five plants were randomly selected for the first sampling. From the top, three leaves were separated as younger leaves, while the rest of the leaves were collected as older leaves, and shoot samples were also collected separately. For sampling at maturity, plants in each container were harvested as one biological replicate and separated into straw, husk, and grains. The samples were oven-dried (65°C) to a constant weight, and their dry weights were recorded. For further analysis, samples were milled to a fine powder with a ball mill (MM 200, Retsch GmbH, Haan, Germany).

2.3.1 | Determination of total S and N

For determining S and N, the ground fine powdered samples were weighed (5–10 mg each) and mixed with 10 mg wolfram-(IV)-oxide. Subsequently, the samples were put into aluminum capsules, and total N and S contents were determined by a CNS elemental analyzer (Flash EA 1112 NCS, Thermo Fisher Scientific, Waltham, MA, USA) following the Dumas combustion method. Raw protein concentration of the wheat flour was obtained by multiplying N concentration by 5.81 (Fujihara et al., 2008).

2.3.2 | Extraction and quantification of storage protein fractions

Wheat grain storage proteins were extracted stepwise following a published protocol with modifications (Wollmer et al., 2018). In brief, 100 mg of whole flour was extracted with 1 mL extraction buffer

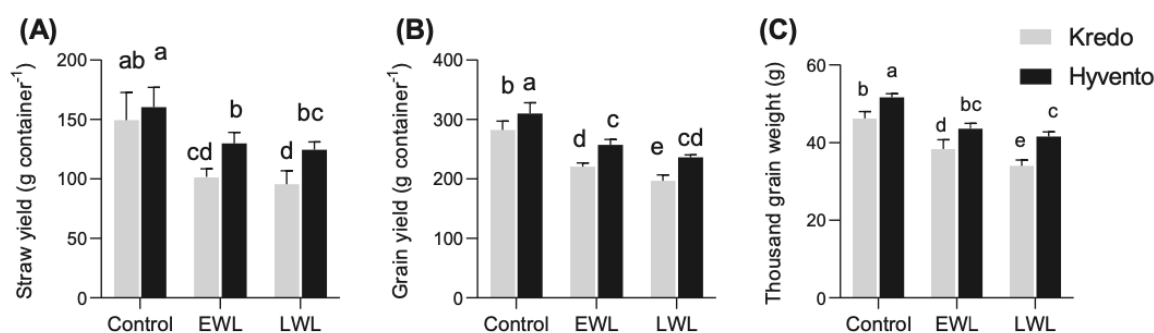


FIGURE 1 Effect of early waterlogging (EWL) and late waterlogging (LWL) on straw yield (A), grain yield (C), and thousand grain weight of the inbred (Kredo) and hybrid (Hyvento) varieties of winter wheat. Data are means of four replicates. Error bars represent standard deviations. Data with different letters are significantly different as measured by Duncan's multiple range (DMR) test ($p \leq 0.05$).

(pH 7.6) containing 0.4 M NaCl, 67 mM Na₂HPO₄, and 7.7 mM KH₂PO₄ for obtaining the albumin-globulin fraction. Thereafter, gliadins were extracted from the residue with 0.5 mL 60% (v/v) ethanol and then glutenins with 1 mL extraction buffer containing 50% 1-propanol (v/v), 2 M urea, 1% dithiothreitol (DTT) (w/v), and 0.1 M Tris-HCl (pH 7.5). A chloroform/methanol solution was used to precipitate the proteins of each fraction. Finally, protein concentration was determined following the Bradford method (Bradford, 1976).

2.3.3 | Fractionation and determination of protein subunits

One-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (1D SDS-PAGE) was performed to quantify gliadin and glutenin subunits following the protocol described by Laemmli (1970). Based on molecular weights or S contents, the gliadin was separated into S-rich γ -gliadins and α/β -gliadins, and S-poor ω -gliadins. Similarly, glutenin was separated into LMW-GS, namely, D-type, B-type, and C-type, and HMW-GS (D'ovidio & Masci, 2004). Gels were prepared using 15% polyacrylamide gels (15% separating gels and 6.3% stacking gels) with a 7 cm \times 8.6 cm \times 0.1 cm in a Bio-Rad Mini-PROTEAN Vertical Electrophoresis Cell (Bio-Rad Laboratories, Hercules, USA). Each gel was loaded with 3 μ g gliadin and glutenin per slot, with 70 volts for 120 min. A molecular weight standard (Rotimark 10–150, Roti-Mark, Roth, Crailsheim, Germany) was used for comparison. Proteins were fixed to the SDS-PAGE gels overnight using 40% ethanol and 10% acetic acid. After that, gel staining was done using "PhastGelTM Blue R" and then removed and stained with 10% acetic acid until the gel background was transparent. After destaining, all gels were scanned with an image scanner (Epson PerfectionV 700) at 300 dpi and 16 bits per pixel as TIF format. Gels were analyzed according to Wollmer et al. (2018) with modifications. In this study, all bands were calculated using Gel Analyzer 19a software. However, Gel lanes and bands were detected automatically and selected manually. The raw volumes of all bands in each lane were calculated with a different software than the above-referenced workers and adjusted manually where needed. Relative percentage of each band was used to equalize the differences

between each staining. In this process, the sum of all band's raw volumes was set to 100% and used to calculate the percentage of each band of a lane.

2.4 | Statistical analysis

The data were statistically analyzed by two-way ANOVA, and the significance of differences among means was determined by Duncan's multiple range (DMR) test at $p \leq 5\%$. The analytical software Statistix 10 (Tallahassee, Florida, USA) was used to perform the statistical analysis.

3 | RESULTS

3.1 | Plant growth and yield

The straw dry matter, thousand-grain weight, and grain yield of both varieties were significantly affected by WL treatments (Figure 1). WL-induced reduction in straw yield of Kredo was almost double (32%/37%) that of Hyvento (18%/22%) under both WL treatments (Figure 1A). Following the same trend, a significantly higher reduction in grain yield of Kredo (22%/44%) was recorded as compared to Hyvento (16%/31%) with both EWL and LWL treatments (Figure 1B). The reduction in grain yield was associated with thousand grain weight, which also decreased in the same proportion (Figure 1C). However, the negative effect of LWL on straw yield, grain yield, and thousand grain weight of both the varieties was markedly higher than the EWL treatment (Figure 1A–C).

3.2 | Changes in soil sulfate and plant S concentrations

The concentration of plant-available SO₄²⁻ in soil was significantly reduced by WL treatments, causing a 51%–60% reduction in SO₄²⁻ concentration in waterlogged soils compared to the control (Figure 2).

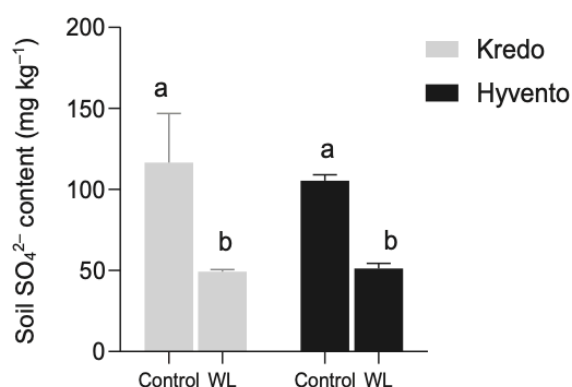


FIGURE 2 Effect of early waterlogging (EWL) and late waterlogging (LWL) on soil sulphate content. Data are means of four replicates. Error bars represent standard deviations. Data with different letters are significantly different as measured by Duncan's multiple range (DMR) test ($p \leq 0.05$).

Accordingly, WL had a drastic effect on S contents in different parts of waterlogged plants, collected immediately after the termination of WL treatments and at maturity. The highest significant differences were recorded in the younger leaves, showing reductions in S concentration by 47% and 50% in Kredo and 36% and 31% in Hyvento with EWL and LWL treatments, respectively, compared to the control (Figure 3A). Similarly, S concentration in the shoots decreased by 46% and 44% in Kredo and 27% and 37% in Hyvento under EWL and LWL, respectively (Figure 3B). WL at both stages caused significant changes in the S concentrations of grain and husk of both varieties. Sulfur concentration in the grains of Kredo and Hyvento reduced by 29% and 25% under EWL and 42% and 30% under LWL, respectively (Figure 4A). The highest reduction in S concentration was recorded in the husk for Kredo (60%–65%) and Hyvento (50%–57%) under both WL treatments (Figure 4C). Surprisingly, neither of the WL treatments affected S concentration in the straw of Hyvento (Figure 4B). In contrast, both WL treatments reduced S concentration in the

TABLE 2 Nitrogen to sulfur (N/S) ratio in grains of hybrid (Kredo) and inbred (Hyvento) varieties of winter bread wheat grown under control (no waterlogging), early waterlogging (EWL), and late waterlogging (LWL) treatments.

Treatment	Kredo	Hyvento
Control	14.28b	14.34b
EWL	19.40ab	17.94ab
LWL	21.21a	20.21ab

Note: Data presented are the means \pm SD of four replicates. Different letters indicate a significant difference from each other in all combinations (Duncan's multiple range test, $p \leq 0.05$).

straw of Kredo by 44% under EWL and 60% under LWL (Figure 4B). Though the N/S ratio of grains between varieties was unaffected at EWL, the N/S ratio of Kredo (inbred) significantly increased at LWL (Table 2).

3.3 | Grain total protein and protein compositions

WL at both growth stages tended to decrease the total grain protein concentration of Kredo (9%–10%) and Hyvento (15%–19%) (Figure 5A); however, the effect was significant only for Kredo with the LWL treatment (Figure 5A). Among the storage proteins, the amount of gliadin in Kredo was significantly reduced by both EWL (32%) and LWL (23%), but it remained unaffected in Hyvento (Figure 5C). The glutenin content in grains remained unchanged with EWL, but LWL significantly increased the amount of glutenin in grains of both varieties (Figure 5B). In Kredo, the ratio of gliadin to glutenin was reduced significantly by 35% and 38% compared with the control, respectively, whereas this response was absent in Hyvento (Figure 5F).

Nine protein bands were detected for the gliadin fractions, and their relative quantities were analyzed in both varieties. In Kredo, both WL treatments significantly increased S-poor ω -gliadins and decreased S-rich γ -gliadins compared to the control (Figure 5D).

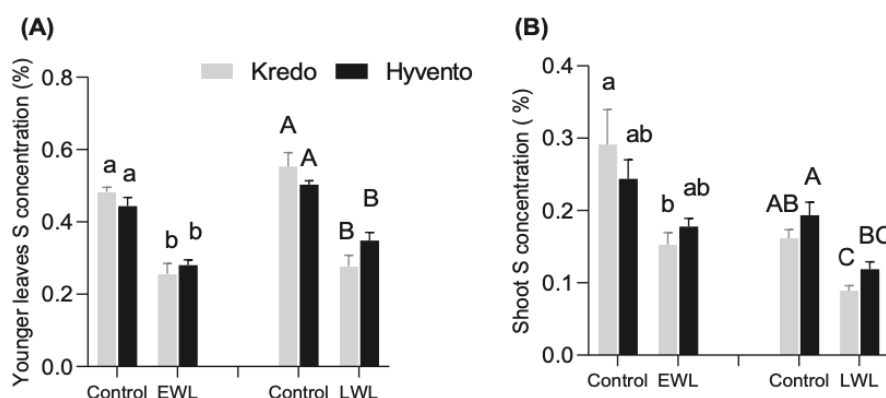


FIGURE 3 Effect of early waterlogging (EWL) and late waterlogging (LWL) on sulfur (S) concentration in younger leaves (A) and shoot (B) of the inbred (Kredo) and hybrid (Hyvento) varieties of winter wheat. Data are means of four replicates. Error bars represent standard deviations. Data with different letters are significantly different as measured by Duncan's multiple range (DMR) test ($p \leq 0.05$).

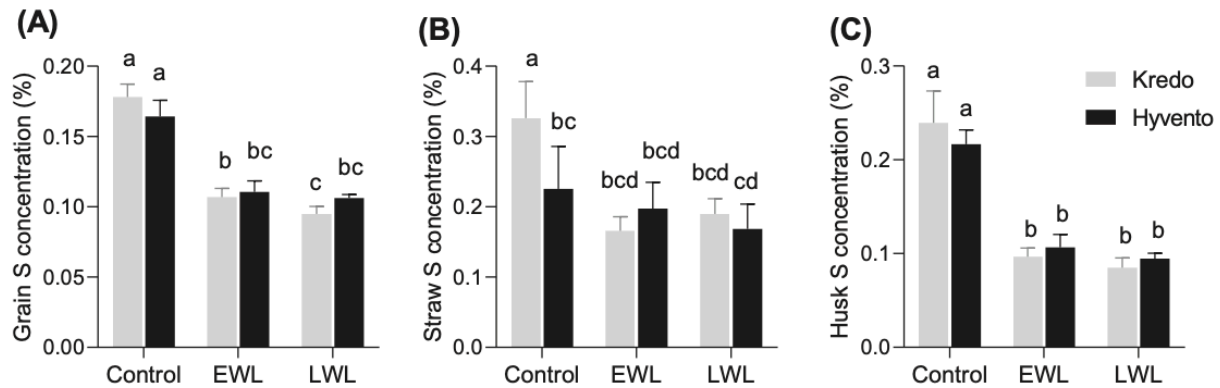


FIGURE 4 Effect of early waterlogging (EWL) and late waterlogging (LWL) on sulfur (S) concentration in postharvest grain (A), straw (B), and husk (C) of the inbred (Kredo) and hybrid (Hyvento) varieties of winter wheat. Data are means of four replicates. Error bars represent standard deviations. Data with different letters are significantly different as measured by Duncan's multiple range (DMR) test ($p \leq 0.05$).

TABLE 3 Relative abundance (%) of high molecular weight (HMW-GS) and subfractions (D-type, B-type, C-type) of LMW-GS glutenin in grains of inbred (Kredo) and hybrid (Hyvento) varieties of winter wheat grown under control (no waterlogging) early waterlogging (EWL) and late waterlogging (LWL) treatments.

Subunit/type	Kredo			Hyvento		
	Control	EWL	LWL	Control	EWL	LWL
HMW-GS	17.11de	21.25bc	23.15a	16.06e	17.66d	20.64c
D-type	13.67c	15.90b	16.61b	20.60a	21.67a	21.16a
B-type	26.67a	22.54b	20.89c	18.49d	15.54e	14.92e
C-type	45.90ab	42.01cd	39.43d	46.48a	43.74abc	43.58abc

Note: Data presented are the means \pm SD of four replicates. Different letters within a row indicate a significant difference from each other in all combinations (Duncan's multiple range test, $p \leq 0.05$).

However, these responses were not identified in the hybrid variety Hyvento (Figure 5E).

For the glutenin subfractions, 13 bands were detected in Kredo, and 12 bands were detected in Hyvento. In Kredo, S-low HMW-GS increased by 25% and 36% under EWL and LWL, respectively. However, in Hyvento, HMW-GS increased only by 10% and 28% under EWL and LWL, respectively (Table 3). Under EWL and LWL, there was a statistically significant and similar drop in the relative abundance of S-rich B-type LMW-GS subfraction both in Kredo (15% and 22%, respectively) and in Hyvento (16% and 19%, respectively) (Table 3). Nevertheless, the relative abundance of LMW-GS C-type subfraction in Kredo was decreased by 8% and 14% in EWL and LWL, respectively (Table 3). In contrast, Hyvento did not show a similar response under either of the WL treatment (Table 3).

4 | DISCUSSIONS

Although many researchers have evaluated the effects of WL on the growth, physiological behavior, and ionic homeostasis in different

crops, studies on the possible differences in WL tolerance between hybrid and inbred wheat varieties and the underlying mechanisms are lacking. To contribute to this platform, we evaluated the WL tolerance in two winter wheat varieties, a high-yielding inbred variety (Kredo) and a commercial hybrid variety (Hyvento), on the basis of growth, yield, yield quality as well as S uptake pattern. The present study revealed high genotypic differences for WL tolerance, whereby Hyvento (hybrid) wheat variety proved relatively tolerant to WL than Kredo (inbred) variety. These observations agree with previous work on other crops showing that hybrid varieties' tolerance to different abiotic stress is higher than inbred varieties (Adee, 2016; Carretero et al., 2014; Collaku & Harrison, 2005; Ünay & Şimşek, 2020; Yildirim et al., 2010). Other studies have shown that high-yielding wheat genotypes are more affected by WL than lower yielding one because they could not maintain high tillering and kernels per spike (Collaku & Harrison, 2002; Setter et al., 2003). However, in our study, the inbred variety Kredo showed a higher reduction in grain yield than the hybrid Hyvento, and the decline in yield was associated with a decrease in kernel size. Likewise, Sharma et al. (2018) noted substantial genotypic variation for grain yield and biomass under WL treatments. Compared to EWL for both varieties, grain yield decreased drastically at LWL. However, other published results also noted a substantially higher reduction in grain yield (92%) of wheat exposed to WL at the Feekes Stage 10.1 (de San Celedonio et al., 2014).

Our results showed that WL caused a significantly higher S decrease in all plant tissues (younger leaves, shoot, straw, husk, and grains) of the inbred variety Kredo than Hyvento (Figure 3). Moreover, the reduction in S concentration in plant tissues was higher in the case of EWL than in LWL treatment. Therefore, we assumed that WL induced a higher reduction of S concentrations in Kredo that would have more adversely affected the yield and grain quality than Hyvento. Similarly, Singh et al. (2017) noticed that average leaf S concentration was reduced by 61% relative to drained conditions, diminishing yield by 27%. Some other researchers also observed that S deficiency caused yield losses of up to 55% (De Ruiter & Martin, 2001; Flaete et al., 2005; Järvan et al., 2008; Sahota, 2006). The decline in tissue S concentration

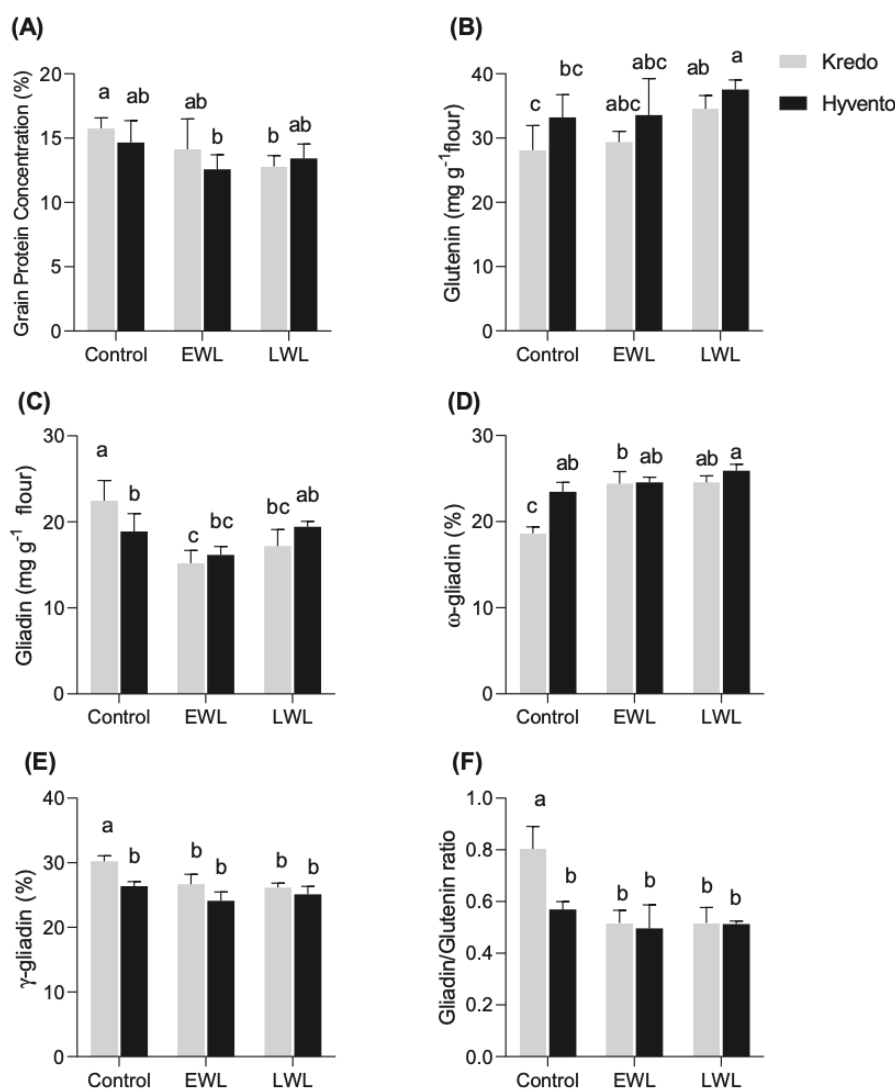


FIGURE 5 Effect of early waterlogging (EWL) and late waterlogging (LWL) on grain protein concentration (%) (A), glutenin (mg g^{-1} flour) (B), gliadin (mg g^{-1} flour) (C), gliadin/glutenin ratio (D), ω -gliadin (%) (E), and γ -gliadin (%) (F) of the inbred (Kredo) and hybrid (Hyvento) varieties of winter wheat. Data are means of four replicates. Error bars represent standard deviations. Data with different letters are significantly different as measured by Duncan's multiple range (DMR) test ($p \leq 0.05$).

was associated with a 54%–65% decline in SO_4^{2-} concentration in the WL soils (Figure 2).

WL-induced S deficiency in plants also affects the contents and composition of crude grain protein (Jiang et al., 2009; Zörb et al., 2009). Though recent studies demonstrate that WL affects the protein compositions, especially gliadin subunits of winter wheat varieties (Wollmer et al., 2018), the comparative effect of WL on inbred and hybrid varieties has not been studied so far. In this study, we observed that the grain protein concentrations and the gliadin and glutenin subfractions and their subunits pattern in the inbred variety Kredo were more severely affected than the hybrid variety Hyvento in response to EWL and LWL. Based on the criteria given by Randall et al. (1981), the grains of both varieties produced under EWL and LWL regimes could be

classified as S deficient ($S < 0.12\%$, N/S ratio $> 17:1$; Table 2). Accordingly, we can infer that this S deprivation, in turn, was underpinned by low grain gluten proteins as well as gliadin and glutenin subunits, thus, increasing the relative pools of S-low/poor HMW-GS and ω -gliadin. Indeed, it has been indicated that S deficiency was correlated with a significant increase in S-poor ω -gliadins and S-low HMW-GS as well as a substantial decrease in S-rich LMW-GS and γ -gliadin (Anderson et al., 1997; MacRitchie & Gupta, 1993; Moss et al., 1981; Shewry et al., 2001; Wieser et al., 2004; Zhao et al., 1999). Thus, this statement supports that WL negatively affected the S uptake, translocation, and remobilization from shoots and roots, which affects yield and storage proteins, because S is essential for the biosynthesis of wheat storage proteins (Yu et al., 2021). Under such circumstances, a remarkable shift

in grain storage protein composition is likely to occur because gliadins and glutenins comprise S-containing amino acids in different proportions (Shewry et al., 2000). A similar trend was reported under various abiotic stresses (DuPont et al., 2006; Zörb et al., 2017). Accordingly, gluten proteins and their subfractions were less affected in Hyvento under WL due to the lesser reductions in S concentration in grains, shoots, and leaves. We concluded that efficient uptake of S and its utilization into different grain proteins have partially conferred WL tolerance in hybrid winter wheat Hyvento.

5 | CONCLUSIONS

The results of this study proved that late water logging has more drastic negative effects on S uptake, grain yield, and protein patterns of both the varieties than early water logging. The hybrid variety Hyvento proved to be more efficient in S uptake and its utilization in different proteins than inbred variety Kredo. Moreover, unaffected total protein content and gliadin fractions in Hyvento under WL treatments, leading to a higher proportion of S-rich LMW-GS and γ -gliadin than Kredo, imply that this variety is also efficient in S utilization in different proteins. Thus, an efficient S uptake, its translocation to shoots, and subsequent metabolization could be among the key mechanisms conferring waterlogging tolerance in hybrid winter wheat.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Chapter 3

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

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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 (Voesenek 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_{CaCl2} 6.5) (Table 1). Based on the standard field fertilizer application, the following four S-levels (as MgSO₄ • 7H₂O) were applied to the soil 5 days prior to sowing: 0 mg S kg⁻¹ soil, no-S (S₀); 35 mg S kg⁻¹ soil, low-S (S₁); 70 mg S kg⁻¹ soil, medium-S (S₂); and 140 mg S kg⁻¹ soil, high-S (S₃). Basal fertilization was carried out as follows (in g kg⁻¹ soil): 0.47 NH₄NO₃, 0.74 KH₂PO₄, 0.46 MgCl₂ • 6H₂O, 0.3 CaCl₂ • 2H₂O, 0.015 Fe-EDTA, 0.006 CuSO₄ • 5H₂O, 0.011 ZnSO₄ • 7H₂O, 0.015 MnSO₄ • H₂O, 0.009 H₃BO₃, 0.0006 (NH₄)₆Mo₇O₂₄ • 4H₂O. 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 silique, 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 Hasanuzzaman 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 + WL), low-S (S_1 + WL), and medium-S (S_2 + 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 + 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 + WL), medium-S (S_2 + WL), and high-S (S_3 + WL) had 6%, 60% and 71% lesser senesced leaves compared with no-S (S_0 + WL) plants (Fig. 2C).

Although compared with controls the number of siliqua in no-S (S_0 + WL) and low-S (S_1 + WL) waterlogged plants did not differ statistically, medium-S (S_2 + WL) and high-S (S_3 + 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 + 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 + 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 + WL) increased by 3.7-, 5.2- and 1.3-fold compared to no-S (S_0 + WL), low-S (S_1 + WL), and medium-S (S_2 + WL) supplemented plants, respectively (Table 2). The application of high-S (S_3) proved ineffective in increasing S concentration compared to medium-S

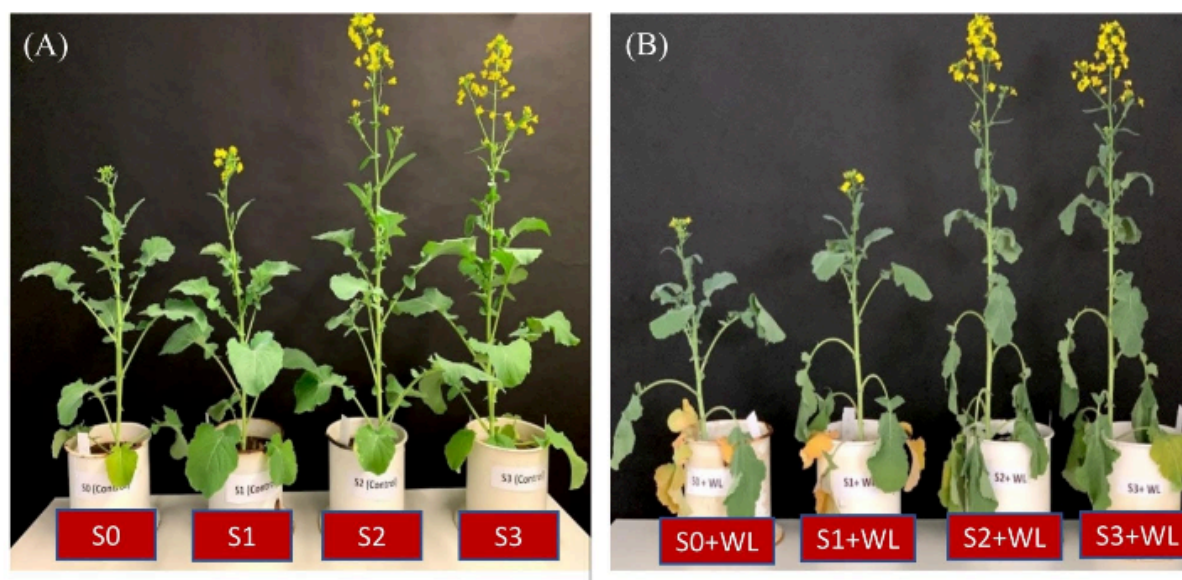
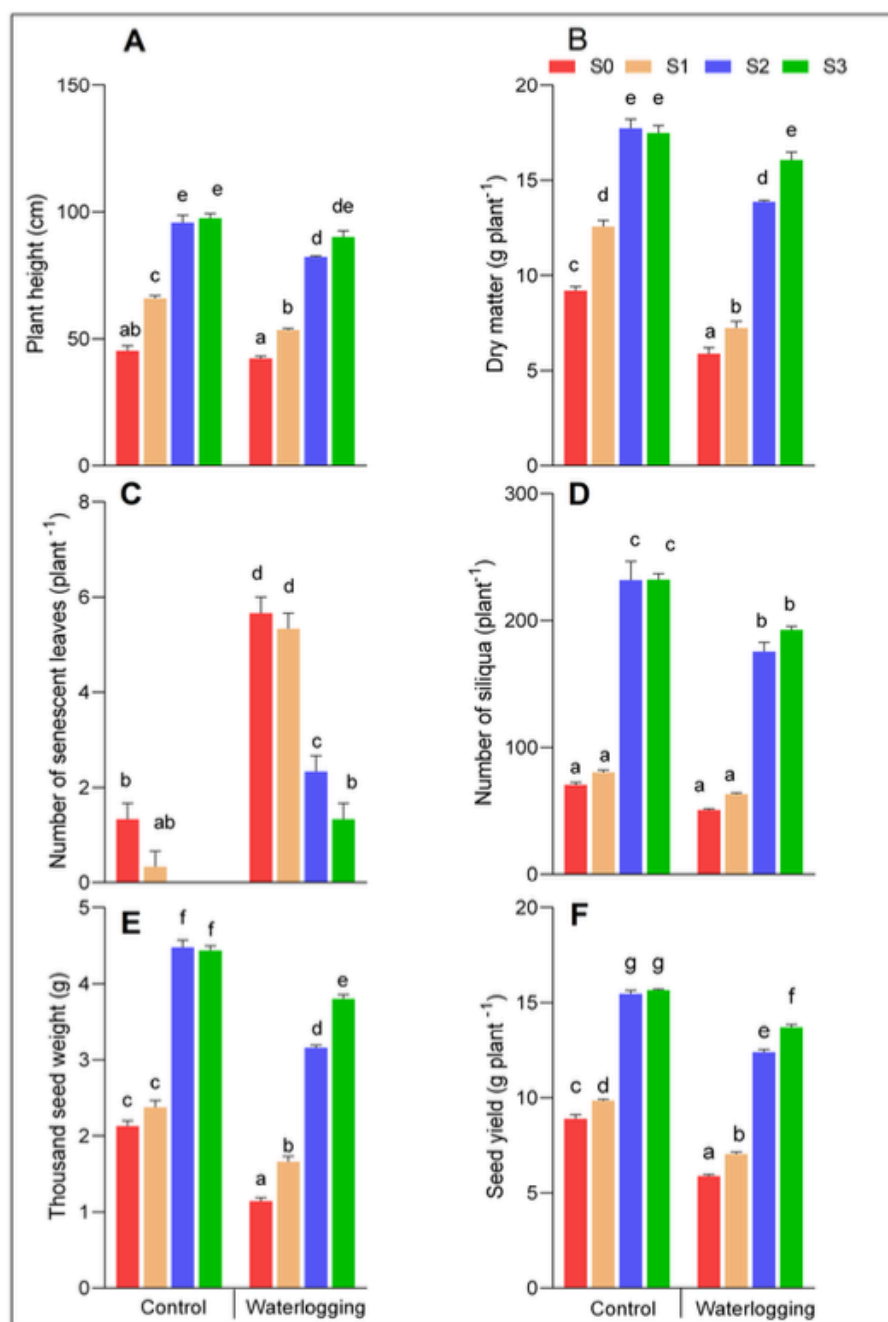


Fig. 1 Effect of different levels of soil-applied sulfur (0, 35, 70 and 140 mg S kg^{-1} soil) on the growth of normal (A) and waterlogged (B) plants of rapeseed (*Brassica napus* L.)

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).

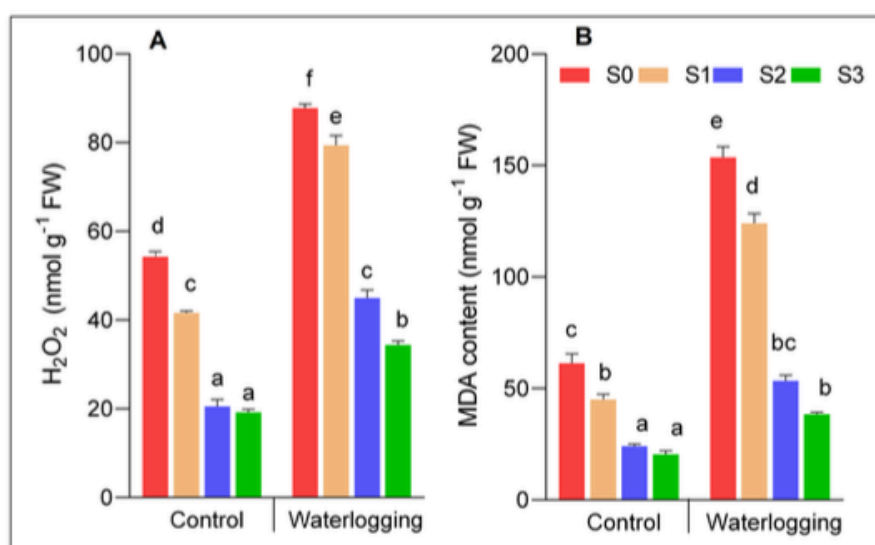
High-S (S_3 +WL) supplemented plants increased nitrate (NO_3^-) concentration by 17-, 10-, and 1.1-fold compared with no-S (S_0 +WL), low-S (S_1 +WL) and medium-S (S_2 +WL) treatments under waterlogging stress,

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 ^c	19.71 ± 0.49 ^c	3.42 ± 0.63 ^a	5.55 ± 0.45 ^{ab}	16.69 ± 1.29 ^d	19.27 ± 0.87 ^c
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; $p \leq 0.05$)



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

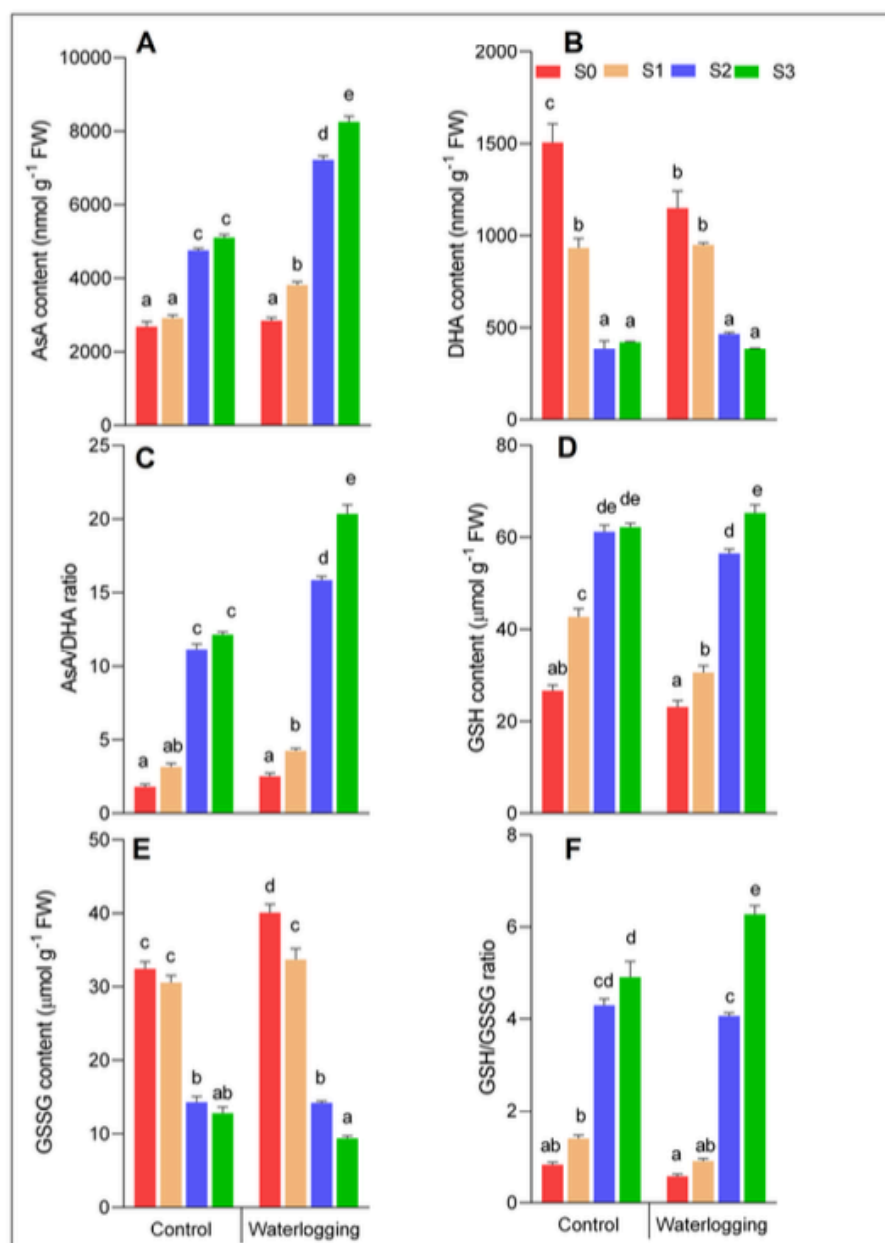
The waterlogging stress resulted in significantly higher H₂O₂ contents than the respective controls. Compared to no-S (S₀ + WL) treated plants under waterlogging condition, low-S (S₁ + WL), medium-S (S₂ + WL), and high-S (S₃ + WL) supplementation notably decreased H₂O₂ content by 10%, 49% and 61%, respectively (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).

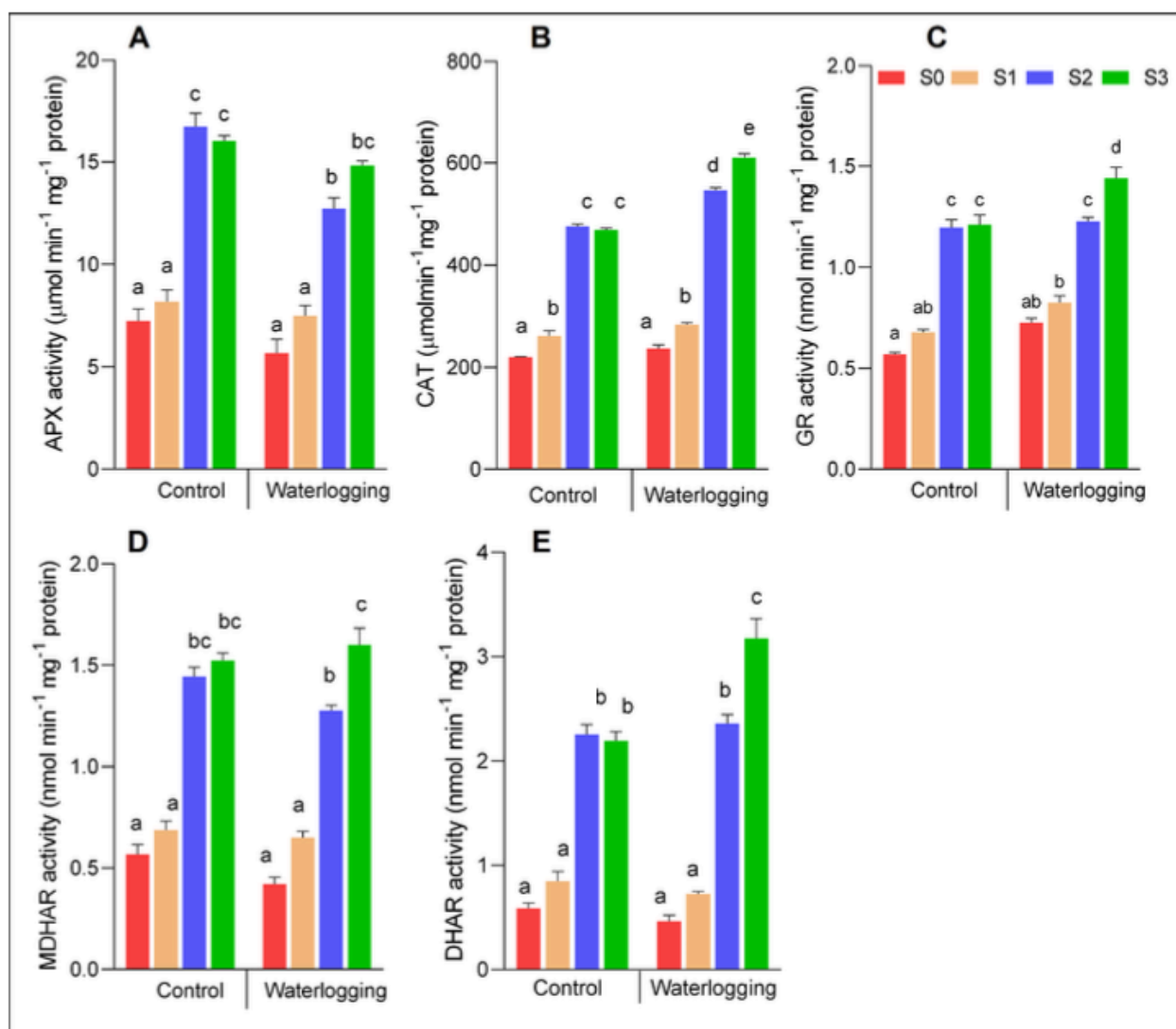


Fig. 5 Effect of different levels of soil-applied S (S₀, No-S application; S₁, 35 mg S kg⁻¹ soil; S₂, 70 mg S kg⁻¹ soil; S₃, 140 mg S kg⁻¹ soil) on the activity of APX (A), CAT (B), GR (C), MDHAR (D) and DHAR (E) in leaves of rapeseed (*Brassica napus* L.) grown under con-

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

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; $p \leq 0.05$)

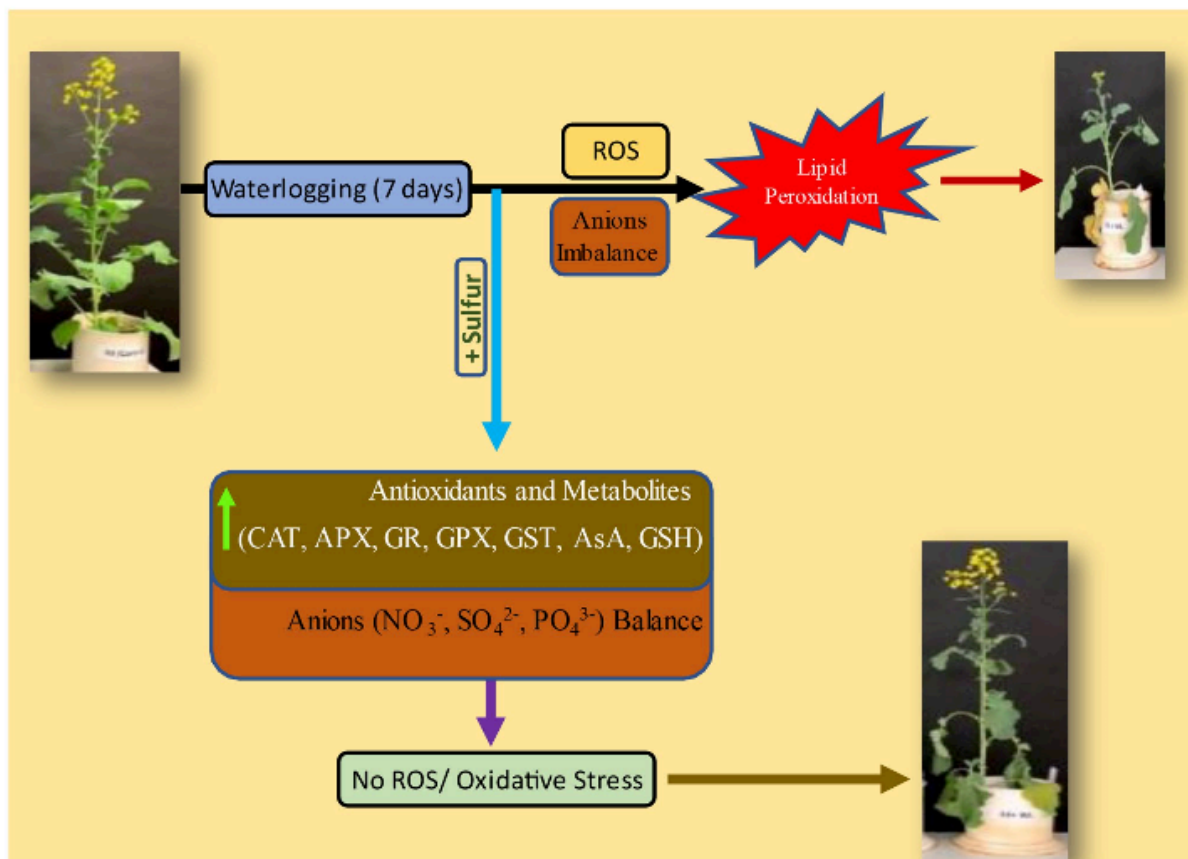
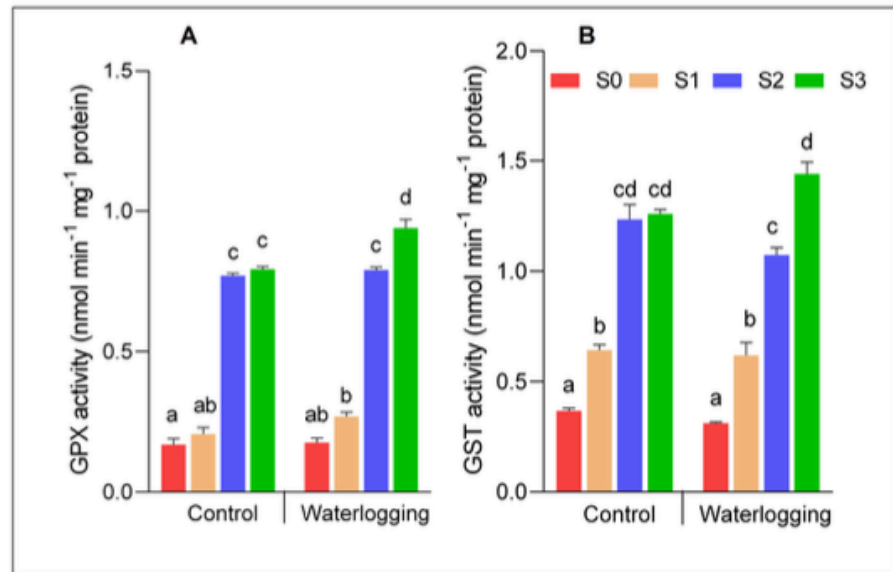


Fig. 7 Schematic illustration for possible mechanism of S-assist tolerance of rapeseed (*Brassica napus* L.) under control and waterlogged soils. Here, ASA, ascorbate; GSH, glutathione; ROS, Reactive oxygen

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; Nocitor 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 H_2O_2 . The underlying mechanism is oxygen radical scavenging; GPX reduces H_2O_2 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 H_2O_2 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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Chapter 4

Foliar-applied sulfur is supplemental to its soil application in alleviating waterlogging stress through the mitigation of oxidative stress and ion homeostasis in rapeseed (*Brassica napus* L.)

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Foliar-applied sulfur is supplemental to its soil application in alleviating waterlogging stress through the mitigation of oxidative stress and ion homeostasis in rapeseed (*Brassica napus* L.)

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Abstract

Under changing climate, waterlogging (WL) has appeared as a serious issue for agricultural production around the world in recent years. Sulfur (S), a macronutrient, postered attenuation on the negative effects of diverse abiotic stresses. Thus, a pot experiment was executed to observe the effectiveness of supplementation of S in soil or/and foliar as MgSO₄ on the growth, photosynthesis, antioxidants, and nutrients uptake to ameliorate the deleterious impacts of WL-stress in *Brassica napus*. Waterlogging stress was applied at vegetative stage (BBCH-31) for 7 days and S supplementations were managed by applying sufficient S-fertilized (+S) to soil, whereas foliar S (300 ppm) was sprayed two days prior to the onset of waterlogging treatment. The results demonstrated that waterlogged plants showed elevated generation of hydrogen peroxide (H₂O₂) and malondialdehyde (MDA) contents, reduced nutrients uptake which led to the reduction of net photosynthesis and plant growth. However, the repressing effects of WL-stress were alleviated by both soil and foliar S-fertilization. Moreover, mitigation of waterlogging effects was more pronounced in soil- plus foliar S-fertilized plants, and the least was observed in unfertilized (without S) and untreated (without foliar-S) plants. The greater waterlogging tolerance of combined soil S-fertilized and foliar-S treated plants was attributed to corresponding enhanced antioxidant enzymes (SOD, CAT, GR, APX), metabolites (AsA, GSH), nutrients uptake (S, P, Mg, Zn), net photosynthesis rate and decreased H₂O₂ and MDA contents. Collectively, the observations of present study exhibit the beneficial role of foliar-S application to soil S-fertilized waterlogged *Brassica napus* plants and the underlying physiological and biochemical tolerance mechanisms, as well as ion homeostasis.

Keywords: rapeseed, reactive oxygen species (ROS), glutathione (GSH), foliar sulfur (S), waterlogging, antioxidant enzymes

Introduction

Heavy rainfall events, poor soil drainage, uneven topography, and soil compaction are the factors responsible for making waterlogging a significant abiotic stress for crop plants; around 16% of fertile areas worldwide experience regular waterlogging (Kaur et al., 2020). In response to global warming, heavy rainfall events are estimated to enhance by approximately 7% (IPCC, 2022). During waterlogging, oxygen in soils is depleted either partially (hypoxic) or completely (anoxic), which negatively affect nutrient absorption, plant physiology, enzyme activity and plant development, eventually lead to diminished yields (Dossa et al., 2019; Keya et al., 2022; Pan et al., 2021). To confront WL-stress, plants have generated many adaptational mechanisms. For example, the establishment of aerenchyma tissues, stems and roots transport oxygen from leaves to the other organs of the plant and prevent its oxygen deficiency (Ambro et al., 2022; Manik et al., 2022). Additionally, some plant species have formed ROL (radial oxygen loss) blockades to curtail the radial loss of oxygen transported from leaves to the roots under waterlogging stress (Pedersen et al., 2021).

Oilseed rape (*Brassica napus* L.) is remarkably sensitive to WL-stress due to the dearth of aerenchyma (Voesecek et al., 1999). Therefore, despite expanding rapeseed cultivation in recent decades in many areas notably Canada, China, India, Australia and Europe, yield is not increasing because waterlogging is significantly affecting its production (FAO, 2021; Pachauri et al., 2014). In recent decades, waterlogging has hampered rapeseed growth by negatively affecting physiological functions, e.g., by altering lipid biosynthesis pathways (Ghobadi et al., 2017), resulting in yield reductions as well as yield quality (Wollmer et al., 2017). Moreover, waterlogging leads to a rapid reduction in soil redox potential, causing substantial alterations to its plant available nutrient profile and increasing the concentrations of toxic substances. As a result, plants' nutrient uptake is hampered, and leads to an ionic imbalance in cells.

Under oxygen-limited state, plant metabolism shifts to anaerobic fermentation from aerobic respiration, shrinking energy by nearly 37.5% (Keya et al., 2022). Furthermore, waterlogging can increase CO₂ levels within the root area and lower hydraulic conductance, triggering rapid stomatal closure and lowering net photosynthetic along with transpiration rates (Anee et al., 2019; Zhu et al., 2016). The impaired net photosynthesis in WL-stressed plants produces ROS

(reactive oxygen species) namely hydroxyl radical, hydrogen peroxide, superoxide etc. that harm cellular components, causing lipid peroxidation and other physiological changes (Zhang et al., 2019). Under non-stressed conditions, enzymatic and non-enzymatic antioxidants tightly control ROS formation and consumption. In waterlogged conditions, ROS production surpasses the proficiency of antioxidative machinery to eradicate them, which results in oxidative stress. Several mechanisms have evolved in plants that reduce ROS accumulation to alleviate oxidative stress (Noctor and Foyer, 1998). In both chloroplasts and cytosol, superoxide dismutase, catalase together with the ascorbate-glutathione cycle enzymes, function as antioxidant enzymes (Hasanuzzaman et al., 2017). The non-enzymatic antioxidant includes glutathione (GSH), ascorbate (AsA) and many other phenolic compounds (Kumari et al., 2022).

Plants require sulfur (S) to synthesize cysteine (Cys) after being incorporated as sulfate and is assimilated into glutathione (GSH). Cysteine then serves as a donor or precursor of crucial substances like methionine, glutathione, S-adenosylmethionine and other S-containing compounds (Li Q., 2020). These sulfur derivatives play pivotal roles in different abiotic stress counteraction, cellular acclimatization as well as resilience to unfavorable environments (Cao et al., 2014; Noctor, 2006). In other words, sulfur contributes to the generation of both disulfide (S-S) and sulfhydryl (S-H) bonds which stabilize plant's protein structures (Saito, 2000). Moreover, S-containing compounds like Fe-S cluster proteins play integral part in numerous biological mechanisms, for instance photosynthesis, metabolism, and energy production (Nakai et al., 2020; Yadav et al., 2021).

Sulfur (S) paucity is one of the most certain nutrient deficiencies which arise under waterlogged state as sulphate, which is plant available form of S in soil, is highly mobile in the soil-water phase and prone to leaching and reduction (Bloomfield, 1969). It is therefore not surprising that waterlogging can result in the contraction of sulfate towards hydrogen sulfide up to 60% and its afterward precipitation or volatilization (Hussain et al., 2022). Moreover, in humid climate regions e.g., northern European countries, S is considered a limiting nutrient (Lewandowska et al., 2008). In this context, S fertilization has been consistently notified of reducing negative responses to heavy metal toxicity, salinity stress and drought stress (Bashir et al., 2015; Fatma et al., 2014; Lou et al., 2017). Soil-applied S and its derivatives have been proven beneficial in alleviating waterlogging stress; however, its positive effects are limited by the above-described soil conditions (Hussain et al., 2023; Keya et al., 2022; Xiao et al., 2020).

In this situation, foliar application of S, which is considered to be quick and efficient method to treat S deficiency, could help induce waterlogging tolerance in plants. Some convincing evidence suggest that foliar-applied nutrients can reduce abiotic stresses by improving antioxidant capacity and nutrient absorption (Gai et al., 2022; Noreen et al., 2018; Naeem et al., 2017). In addition to abiotic stress tolerance, S enhances plant nutrients uptake by forming a synergistic relationship with most nutrients (Mehmood et al., 2021). Nevertheless, the impacts of foliar-applied S on the growth, nutrients uptake and physiological functioning of waterlogged *Brassica napus* plants, and the probable underlying physiological mechanisms have not been studied yet. Moreover, it is not clear yet if foliar-applied S has additive effect to soil-applied S in mitigating waterlogging stress. In this context, it was assumed that foliar-applied S could be absorbed and transported which will balancing ions of waterlogged plants. Thus, we hypothesized that combination of soil and leaf applied S can be a worthwhile for nutrients uptake and physiological augmentation, that will ultimately mitigate waterlogging stress. Therefore, in the current study, we focused on combined and separate effects of soil and foliar S supplementation on growth, biochemical and physiological attributes, and ion homeostasis under waterlogging stress.

2. Materials and methods

2.1 Plant culture, growth environment, and treatments

Seeds of spring oilseed rape (*Brassica napus* L. cv. Campino) were obtained from Norddeutsche Pflanzenzucht Lembke (NPZ), Hohenlieth, Germany. After soaking overnight in distilled water, the seeds were sown in 6 kg pots filled with sandy loam soil. The pots were placed in the greenhouse set to the following conditions: day/night temperatures of 22 °C/18 °C, 12 h photoperiod with a light intensity of 350 $\mu\text{M photon m}^{-2} \text{s}^{-1}$ (recorded by a light meter, Li-198, Lincoln, NE, USA) and 62-70% relative humidity. Physico-chemical properties of the experimental soil presented in Table 1. The following fertilizers were applied as a basal dose (g kg^{-1} soil): $\text{NH}_4\text{NO}_3 = 0.47$, $\text{KH}_2\text{PO}_4 = 0.74$, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O} = 0.46$, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O} = 0.3$, Fe-EDTA = 0.015, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O} = 0.011$, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O} = 0.006$, $\text{MnSO}_4 \cdot \text{H}_2\text{O} = 0.015$, $\text{H}_3\text{BO}_3 = 0.009$, $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O} = 0.0006$. The experiment had following three factors, each having two levels: 1) Waterlogging; no waterlogging (control) and 07 days of waterlogging (WL), 2) Soil S application; unfertilized (-S) and S-fertilized (70 mg S kg^{-1} soil; +S) as $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$,

3) Foliar S application; untreated and foliar-S treated. These factors were studied in all possible combinations, yielding following eight treatments:

- (1) Control plants with unfertilized and untreated (no foliar-S); no soil S (-S)
- (2) Control plants with S-fertilized and untreated; sufficient soil S (+S)
- (3) Control plants with unfertilized and foliar-S treated (-S+F)
- (4) Control plants with S-fertilized and foliar-S treated (+S+F)
- (5) Waterlogged plants with unfertilized and untreated (-S+WL)
- (6) Waterlogged plants with S-fertilized and untreated (+S+WL)
- (7) Waterlogged plants with unfertilized and foliar-S treated (-S+WL+F)
- (8) Waterlogged plants with S-fertilized and foliar-S treated (+S+WL+F)

The non-waterlogged plants (control) were grown at 80% of soil water holding capacity, while the waterlogged plants were put through waterlogging at the vegetative stage (BBCH-31) for seven days by managing water level approximately 3 cm beyond the soil surface in the pots. To maintain a constant water level, pots were positioned in buckets (15-L) loaded with tap water. The foliar fertilization of approximately 15 mL of 300 ppm MgSO_4 solution was carried out on each plant. The concentration of MgSO_4 used for foliar fertilization is the highest concentration that does not burn the leaves of rapeseed, and it was determined from a pretrial using a range of concentrations varying from 50 to 350 ppm. A similar amount of deionized water (dH_2O) was sprayed on plants receiving no foliar S fertilization (untreated). Foliar treatments were conducted in the evening, when the greenhouse's temperature was relatively low, to prevent the prompt evaporation of the sprayed solution. As a surfactant, Silwet® Gold (0.1%) was used to assured optimum adherence of treated solution to the surface of the leaves. While spraying, the pots were concealed with aluminum foil to evade S addition of the soil. The experimental treatments had two sets, each consisting of four replications. One set was used in fresh form for biochemical analyses while the second set was used for determining growth parameters and destructive analysis of nutrients. The pots were completely randomized during the experimental period.

Table 1. The physio-chemical properties of the experimental soil

Soil properties	Value
pH _{1:2.5} -(CaCl ₂)	6.5
Organic matter (%)	2.1
Available sulfur (S, mg kg ⁻¹)	6.5
Available nitrogen (N, %)	0.09
Available potassium (K, mg kg ⁻¹)	69
Available phosphorus (P, mg kg ⁻¹)	94
Available magnesium (Mg, mg kg ⁻¹)	3.2
Mechanical composition	
Sand (%)	85
Silt (%)	10.9
Clay (%)	4.1
Textural class	Loamy sand
DTPA extractable micronutrients	
Zinc (Zn, mg kg ⁻¹)	1.6
Manganese (Mn, mg kg ⁻¹)	33.6
Iron (Fe, mg kg ⁻¹)	348.1
Copper (Cu, mg kg ⁻¹)	1.1

2.2 Gas exchange measurements and harvesting of plants

Parameters associated with gas exchange were carried out on 7th day of the waterlogging period. A gas-exchange analyzer (Li-Cor 6400, USA) was operated for the assessment of stomatal conductance (gs) and net photosynthesis (Pn). The instrument automatically adjusts the CO₂ concentration at 400 $\mu\text{mol mol}^{-1}$, which adapted by CO₂ inoculation in the reference chamber with the inclusion of airflow (300 mmol s^{-1}) and block temperature (25°C). The uppermost fully extended leaves of all treated plants were considered, and measurements were made during 9:00–11:00 h on a 6 cm² middle portion of leaf. The net photosynthesis (Pn) and stomatal conductance (gs) were conveyed as $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ and $\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$, respectively.

Plants were obtained 7 days after the commencement of waterlogging treatments. From the 1st set, leaves were separated, flushed with deionized water three times to erase the foliar S potentially sticking to the surfaces, and immediately chilled in liquid nitrogen and preserved at -20°C for biochemical analysis. On the other hand, plants from the second set were drenched

3 times with deionized water and finally dried at 65°C in an oven for three consecutive days. Afterward, dry matter yield was documented, and plant samples were crushed to a powdered substance with Foss Tecator (Cyclotec 1093, Höganäs, Sweden) and ball mill (MM 200, Retsch GmbH, Germany).

2.3 Determination of macro and micronutrients

The total sulfur content in leaves was measured following the Dumas combustion method (ICC 167). Shortly, 5-10 mg of powdered samples together with same amount of wolfram-(IV)-oxide was combined in tin capsules. Following that, total S concentration was quantified with elemental analyzer (Flash EA 1112 NCS, Thermo Fisher Scientific, Waltham, MA, USA). For quality assurance, accredited reference material from wheat flour (IVA 33802156; IVA Analysis Technology GmbH & Co., Germany) was included with each batch.

To determine mineral content, 200 mg dried leaf powder was assimilated with 10 mL of 69% HNO₃ for 45 min in a microwave (MARS 6 Xpress; CEM, MC, USA). Thereafter, the assimilated samples were poured with deionized water up to 100 mL and preserved at 4°C. Afterward, macronutrients (P and Mg) and micronutrients (Zn, Fe, Mn) concentrations were quantified by utilizing inductively coupled plasma-mass spectrometry (ICP-MS; Agilent - 7700, Böblingen, Germany). Analyses were conducted utilizing NCS DC 73350, an accredited material (poplar) as explained by Jezek et al. (2015).

2.4 Determination of H₂O₂ and malondialdehyde content

Hydrogen peroxide content was quantified as narrated by Velikova et al. (2000). Frozen leaf (0.2 g) was blended in an ice-cold mortar with 1.6 mL 0.1 % trichloroacetic acid (TCA). Following that, the blended solution was centrifuged at 12,000 g for 20 min at 4°C. The reaction assay comprised of 500 µL each of the aliquot and 0.1 M K-P buffer (potassium phosphate buffer; pH 7.8), and 1 mL of 1 M KI (potassium iodide). Later, the mixture was quickly vortexed and kept for 1 h in the dark room at room temperature. Finally, the absorbance of the mixtures was recorded at 390 nm using a spectrophotometer (Thermo Helios Gamma, 9423 UVG 1000E, UK). The content of H₂O₂ was determined using the standard curve and presented as µmol g⁻¹ fresh weight.

As a measure of lipid peroxidation in leaves, malondialdehyde (MDA), an outcome of lipid peroxidation, was quantified following to Heath and Packer (1968). Briefly, frozen leaf powder (0.25 g) was homogenized in 1 mL of 5 % (w/v) trichloroacetic acid (TCA) solution. After that, the blended mixture was centrifuged at 12,500 g for 15 min, and 1 mL of the aliquot was combined to 4 mL of 0.5 % (w/v) TBA prepared in 20 % TCA. Later, the reaction mixture was incubated in hot water bath at 95°C for 30 min. Afterward, the reaction was terminated by keeping the tubes immediately in an ice box. Then, the absorbance of the reaction assay was monitored at 532 nm and 600 nm. Finally, the value for non-specific absorption was removed by subtracting 600 nm values from 532 nm values. The MDA contents were assessed by applying an extinction coefficient of $155 \text{ mM}^{-1} \text{ cm}^{-1}$ and stated as nmol g^{-1} fresh weight.

2.5 Extraction and measurement of ascorbate and glutathione content

Total ascorbate and reduced ascorbate (AsA) were evaluated adopted the technique of Kampfenkel et al. (1995). Dehydroascorbic acid (DHA) was obtained by subtracting total and reduced ascorbate (AsA). Whereas reduced glutathione (GSH) was derived by deducting total glutathione with oxidized glutathione (GSSG). Afterward, 0.5 g frozen leaf sample was homogenized in 4 mL of 5 % (w/v) TCA (trichloroacetic acid). Then, the blended mixture was centrifuged at 12,000 g for 15 min at 4°C. Later, the decanted liquid was utilized for ascorbate (AsA), and glutathione (GSH) quantification. To assess total ascorbate, the first step involved the preparation of neutralized solution by using 0.2 mL of supernatant with 0.3 mL of 0.5 M K-P buffer (pH 7.0), after that 0.1 mL of 0.1 M DTT (dithiothreitol) was poured to minimize the oxidized fraction. On the other hand, for preparing to neutralize the solution of reduced ascorbate (AsA), the same volume of deionized water was used instead of 0.1 M DTT. After that, the reaction combination was kept for 40 min at room temperature. Later, the reaction components were designed employing ascorbate oxidase, K-P buffer (pH 6.5), and neutralized mixture. Finally, 0.5 units of AO (ascorbate oxidase) was poured, and the absorbance was documented at 265 nm. Conversely, the total ascorbate and ascorbate (AsA) amount were measured applying a standard curve of different concentrations of commercial ascorbate. Dehydroascorbic acid (DHA) was obtained by subtracting total ascorbate and ascorbate (AsA). Thus, ascorbate (AsA) content was displayed as nmol g^{-1} fresh weight.

Total glutathione and oxidized glutathione content were measured as narrated by Hasanuzzaman et al. (2014) with smaller adjustments (increased incubation duration).

According to enzymatic recovery, glutathione is oxidized by DTNB (5,5'-dithiobis (2-nitrobenzoic acid)) as well as reduced by NADPH in the existence of glutathione reductase. The amount of total glutathione was determined by the change of absorbance at 412 nm of NTB (2-nitro-5-thiobenzoic acid), which is derived by DTNB reduction (Anderson, 1995). Briefly, GR (baker yeast, type III, Sigma–Aldrich, USA), NADPH and DTNB were prepared with reaction buffer (0.1 M K-P buffer, pH 7.0; 5 mM EDTA). After that, the supernatant (20 μ L) was stirred with 0.38 mL reaction buffer, 0.1 mL of GR (20 IU mL⁻¹), together with 0.1 mL of DTNB (2.4 mg mL⁻¹). Afterward, oxidized glutathione (GSSG) was estimated upon eradication of GSH by supplementing 2-vinyl pyridine at room temperature for 1.5 hr. Then, the reaction was commenced by combining with 0.1 mL of NADPH (1.9 mg mL⁻¹). Lastly, the rate of absorption variations was inspected at 412 nm. Standard curves with recognized concentrations of available commercial glutathione (GSH) and glutathione disulfide (GSSG) along with 5% meta phosphoric acid were applied to calculate total GSH and GSSG contents. Lastly, the glutathione content was estimated by subtracting the values of oxidized glutathione (GSSG) from the total glutathione and represented as μ mol g⁻¹ fresh weight.

2.6 Antioxidant enzyme assays

2.6.1 Superoxide dismutase (SOD) activity

The activity of the superoxide dismutase enzyme was assessed by utilizing the nitro blue tetrazolium (NBT) test (Senthilkumar et al., 2021). Briefly, 0.5 g of frozen leaves were homogenized in 5 mM phosphate buffer to extract plant samples. The blended mixture was centrifuged at 11,500 g at 4°C for 10 min and supernatant was utilized for the measurement of SOD activity. A reaction combination confined 1.3 mL of 50 mM sodium carbonate buffer (pH 10.2), 0.01 mL TritonX-100 (0.06%), 0.5 mL NBT (96 μ M) together with 20 μ L EDTA (0.1 mM). The reaction was ignited by inserting 0.1 mL hydroxylamine hydrochloride (20 mM; pH 6). Finally, the plant extract was added, and optical density was estimated at 560 nm. However, the reagent blanks and control blanks were handled in the uniform way but without plant extract. The quantity of enzyme inhibiting by 50% the photo-reduction of NBT under the given assay conditions was defined as one unit of the enzyme.

2.6.2 Extraction of crude plant extract

For collecting crude plant extract, all the procedures were executed at 4°C. Frozen leaf powder (0.4 g) was blended in 1 mL K-P buffer (50 mM; pH 7.0), consisting of 100 mM KCl, 1 mM AsA, 5 mM β -mercaptoethanol, and 10% (w/v) glycerol. After that, plant extracts were centrifuged at 12,000 g for 15 min at 4°C, and the supernatants were utilized for enzyme activities. All the spectrophotometric assessments were executed using a spectrophotometer (UV-vis; Thermo Helios Gamma, 9423 UVG 1000E, UK).

2.6.3 Total proteins

The method of Bradford (1976) was implemented to calculate total soluble proteins. As the protein standard, BSA (Bovine serum albumin) solution was utilized, and the absorbance was assessed at 595 nm.

2.6.4 Assay of antioxidant enzymes

Catalase (CAT; EC: 1.11.1.6) activity was quantified following to Aebi (1984), with minor changes as narrated by Hasanuzzaman et al. (2011). The CAT activity was quantified by observing the reduction of hydrogen peroxide (H_2O_2) in the presence of the plant extract. Afterward, the reaction was commenced by combining 50 mM K-P buffer (pH 7.0) and 15 mM H_2O_2 with plant extract (final volume 0.7 mL). The drop-in absorbance was observed at room temperature for 1 min at 240 nm. Finally, catalase activity was computed utilizing an extinction coefficient of $0.039 \text{ mM}^{-1} \text{ cm}^{-1}$ and one unit of enzyme was demonstrated as $1 \mu\text{M}$ of the substrate that interacted per mg protein per minute.

Ascorbate peroxidase (APX; EC: 1.11.1.11) activity was assayed following the technique of Nakano and Asada (1981). Nonetheless, the estimation of APX activity is based on ascorbic acid (AsA)-dependent reduction of hydrogen peroxide (H_2O_2). The assay mixture contained plant extract, 50 mM K-P buffer (pH 7.0), 0.1 mM H_2O_2 , 0.1 mM EDTA and 0.5 mM AsA in an ultimate volume of 0.7 mL. Later, the reaction mixture was launched by adding H_2O_2 , and the absorbance was measured at 290 nm for one minute. Lastly, APX activity was quantified by applying an extinction coefficient of $2.8 \text{ mM}^{-1} \text{ cm}^{-1}$ and conveyed as μmol of H_2O_2 reacted $\text{min}^{-1} \text{ mg}^{-1}$ protein.

Glutathione reductase (GR; EC: 1.6.4.2) activity was determined according to the procedure narrated by Mohsin et al. (2019). Here, the oxidation of glutathione-dependent NADPH (nicotinamide adenine dinucleotide phosphate) was observed at 340 nm. Then, the reaction

mixture was formulated with 1 mM GSSG, 1 mM EDTA, 0.2 mM NADPH, 0.1 M K-P buffer (pH 7.8) together with plant extract in a total volume of 1 ml. Lastly, the reaction was started by adding GSSG and the reduction in absorbance was monitored for one min. Finally, GR activity was computed by applying an extinction coefficient of $6.2 \text{ mM}^{-1} \text{ cm}^{-1}$ and indicated as $\text{nmol min}^{-1} \text{ mg}^{-1}$ protein.

Glutathione peroxidase activity (GPX; EC: 1.11.1.9) was estimated as explained by Hasanuzzaman et al. (2011). For monitoring GPX activity, the change in absorbance as the result of oxidation of NADPH to NADP^+ was quantified at 340 nm for one min. The reaction combination included 100 mM of K-P buffer (pH 7.0), 1 mM sodium azide (NaN_3), 1 mM EDTA, 0.12 mM NADPH, 2 mM GSH, 0.6 mM H_2O_2 , 1-unit GR, and the plant extract. Later, GPX activity was evaluated by considering the extinction coefficient of $6.62 \text{ mM}^{-1} \text{ cm}^{-1}$ and presented as unit. One unit expressed as 1 nM of substrate interacted per minute per mg of protein.

Glutathione S-transferase (GST; EC: 2.5.1.18) activity was measured following the method outlined by Hasanuzzaman et al. (2017). The binding of the thiol group of glutathione to the 1-Chloro-2,4-dinitrobenzene (CDNB) substrate was measured as the change in absorbance at 340 nm for one min. The reaction assay contained 1.5 mM GSH, 1 mM CDNB and 100 mM Tris–HCl buffer (pH 6.5). However, GST activity was estimated by applying the extinction coefficient of $9.6 \text{ mM}^{-1} \text{ cm}^{-1}$ and indicated as unit. One unit expressed as 1 nM of substrate interacted per minute per mg of protein.

2.7 Statistical analysis

The data were analyzed using R (version 4.0.2). The studied parameters were correlated by utilizing Pearson correlation analysis. Moreover, all the graphs were outlined with GraphPad Prism 8 (version 8.2.1). The variances among the studied parameters were evaluated at 5% probability level with the Tukey's HSD test.

3. Result

3.1 Plant growth and gas exchange attributes

Both unfertilized (-S) and S-fertilized (+S) plants showed notable reduction in height and total dry matter production under waterlogging compared to the non-waterlogged control condition (Fig. 1). Height of both unfertilized and S-fertilized waterlogged plants receiving no foliar S treatment reduced by about 19% compared with their non-waterlogged counterparts (Fig. 1B). On the other hand, height of foliar S treated unfertilized waterlogged plants decreased by only 14%, while that of S-fertilized plants remained unaffected compared with their non-waterlogged counterparts. The dry matter of untreated unfertilized and S-fertilized plants reduced by 21% and 23%, respectively by waterlogging compared to their respective non-waterlogged controls (Fig. 1C). The foliar S treatment alleviated the inhibitory effects of waterlogging stress on both unfertilized and S-fertilized plants, leading to dry matter reduction of only 12% and 17%, respectively. Other way around, it is inferred that foliar S treatment to unfertilized and S-fertilized plants under waterlogging increased plant height by 5% and 44%, and dry matter by 13% and 34%, respectively.

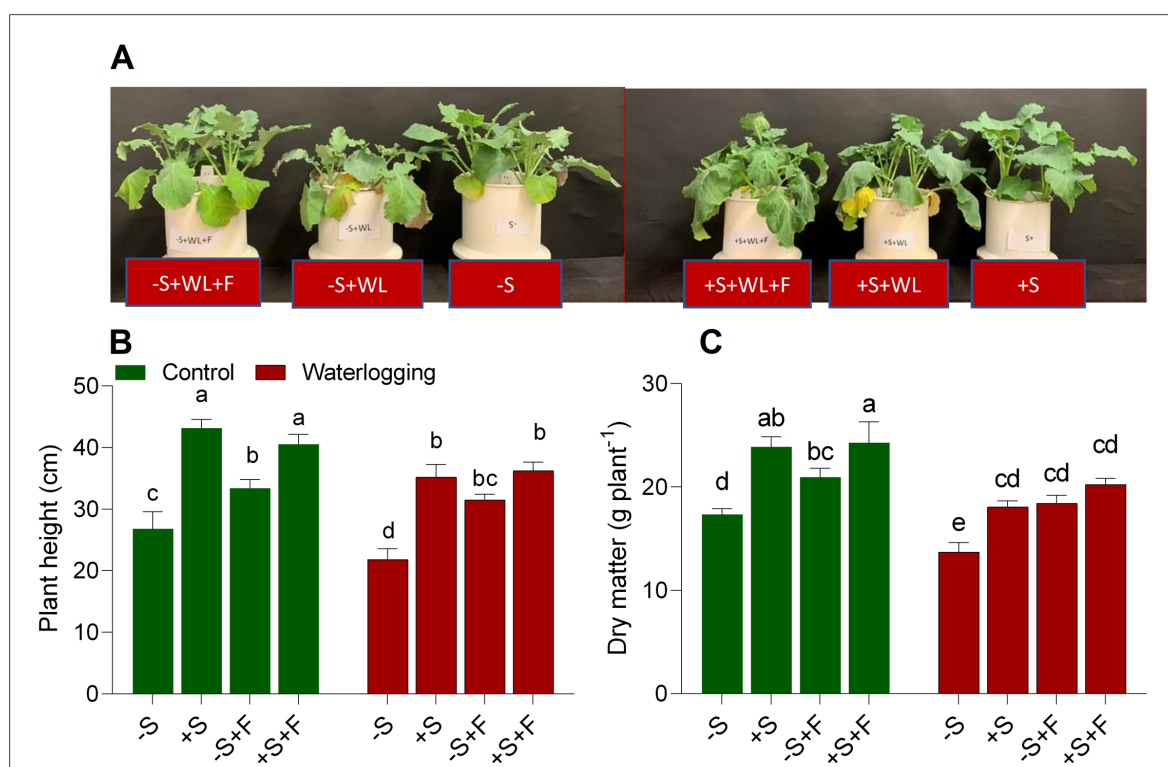


Fig 1. Effect of soil- and/ or foliar-applied S on phenotype (A), plant height (B) and dry matter (C) of *Brassica napus* plants grown under control (80% soil water holding capacity) and waterlogged conditions. The data are means \pm SE of four independent pot replicates. Bars

followed by different letters indicate significant differences between the treatment groups (Tukey's test; $p \leq 0.05$).

Photosynthesis rate of S-fertilized, both untreated and foliar S treated waterlogged plants reduced notably, while unfertilized waterlogged plants of both nontreated and foliar S treated remain unaffected compared with their control groups (Fig. 2A). Under waterlogging, both soil S-fertilization and foliar S treatment, alone or in combination equally improved photosynthesis rate. However, under waterlogging condition improvement in photosynthetic rate of unfertilized and S-fertilized plants induced by foliar S treatment was substantially higher by 53% and 19%, respectively than untreated plants (Fig. 2A). The effect of foliar S treatment on stomatal conductance was similar to that on photosynthesis rate (Fig. 2B).

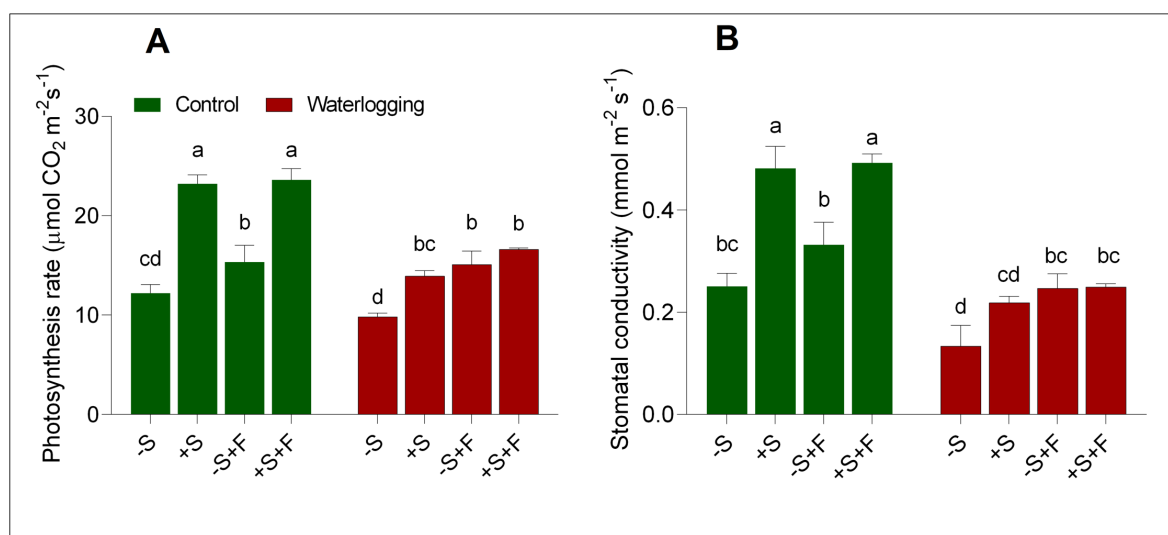


Fig 2. Effect of soil- and/ or foliar-applied S on photosynthesis (A) and stomatal conductance (B) of *Brassica napus* plants grown under control (80% soil water holding capacity) and waterlogged conditions. The data are means \pm SE of four independent pot replicates. The bars followed by different letters indicate significant differences between the treatment groups (Tukey's test; $p \leq 0.05$).

3.2 Oxidative stress markers (H₂O₂ and Lipid Peroxidation)

Waterlogging increased MDA content of unfertilized and S-fertilized plants receiving no foliar S treatment by 101% and 84%, respectively compared with the respective controls (Fig. 3A). Foliar S treatment considerably decreased MDA level in waterlogged plants, by 35% in unfertilized plants and 20% in S-fertilized plants, compared with their untreated counterparts

(Fig. 3A). Eventually, under waterlogged condition, the lowest MDA level was recorded in plants receiving both soil S and foliar S fertilization (Fig. 3A).

Similarly, waterlogging stress significantly increased H_2O_2 accumulation compared with the controls in all the treatments (Fig. 3B). However, foliar S treated waterlogged plants had 15% (+S) and 35% (-S) lower H_2O_2 level than respective untreated plants, depicting an additive negative effect of soil and foliar S fertilization (Fig. 3B).

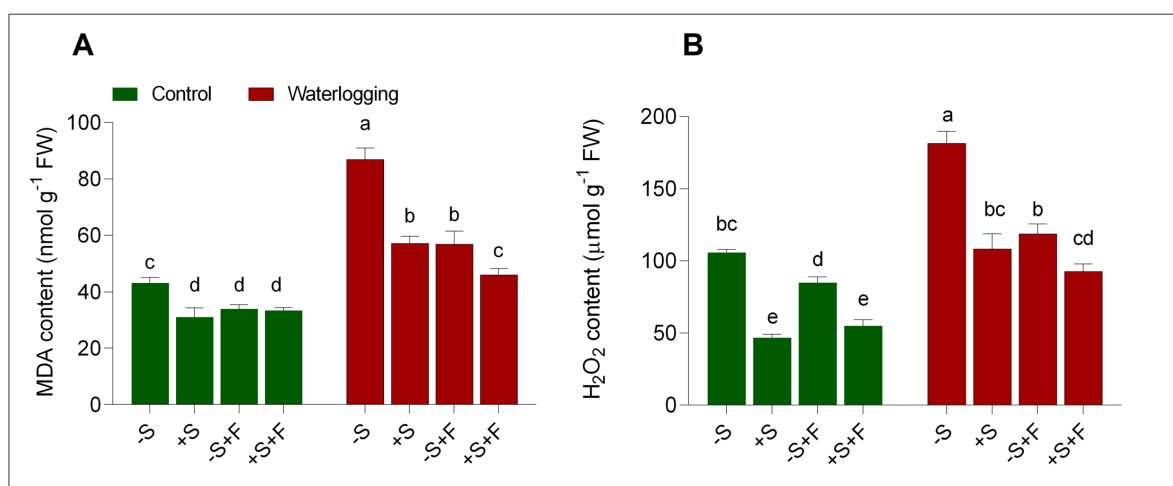


Fig 3. Effect of soil- and/ or foliar-applied S on MDA contents (A) and H_2O_2 contents (B) in leaves of *Brassica napus* plants grown under control (80% soil water holding capacity) and waterlogged conditions. The data are means \pm SE of four independent pot replicates. The bars followed by different letters indicate significant differences between the treatment groups (Tukey's test; $p \leq 0.05$).

3.3 AsA and GSH contents

Compared with the respective controls, the waterlogging increased AsA content by 25% and 64% in untreated unfertilized and S-fertilized plants, respectively (Fig. 4A). The waterlogging induced increase in AsA content of foliar S treated plants was higher than untreated plants, i.e., 62% and 73% for unfertilized and S-fertilized plants, respectively. Overall, foliar S application mitigated the waterlogging stress by increasing AsA by 43% and 13% in unfertilized (-S) and S-fertilized (+S) plants respectively (Fig. 4A).

When exposed to waterlogging stress, the DHA content in all treatments increased significantly, except for unfertilized foliar-S treated plants which had statistically similar DHA contents compared with their respective controls (Fig. 4B). However, compared with untreated

plants, foliar S treated unfertilized and S-fertilized plants had 47% and 5% less DHA content, respectively under waterlogging condition (Fig. 4B).

Waterlogging decreased GSH contents of untreated unfertilized and S-fertilized plants by 14% and 50% as compared to control (Fig. 4C). Interestingly, under waterlogging condition foliar S treatment increased the GSH contents significantly (by 75%) in unfertilized and non-significantly (by 28%) in S-fertilized plants compared with untreated plants (Fig. 4C). Therefore, both soil S-fertilization and foliar S treatment, alone or in combination improved GSH contents under WL-stress. However, foliar S treated, unfertilized, and S-fertilized waterlogged plants had 30% and 9% lower GSSG contents than untreated waterlogged plants (Fig. 4 D).

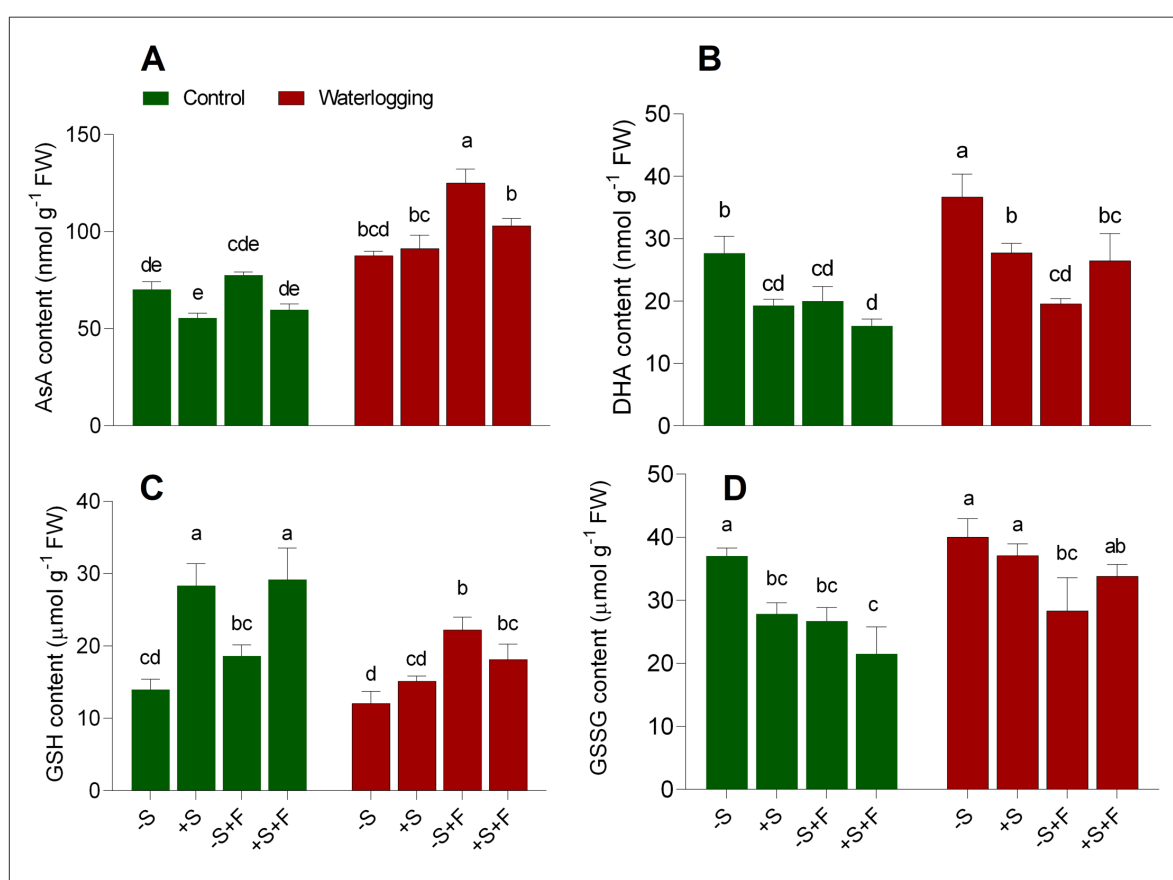


Fig 4. Effect of soil- and/ or foliar-applied S on the contents of AsA (A), DHA (B), GSH (C) and GSSG (D) in leaves of *Brassica napus* plants grown under control (80% soil water holding capacity) and waterlogged conditions. The data are means \pm SE of four independent pot replicates. The bars followed by different letters indicate significant differences between the treatment groups (Tukey's test; $p \leq 0.05$). AsA; ascorbate, DHA; dehydroascorbate, GSH; reduced glutathione, GSSG; oxidized glutathione.

3.4 Antioxidant enzymes activities

As shown in Fig. 5A, waterlogging did not affect APX activity of all treatments, except for foliar S treated plants, which had higher APX activity than control plants (Fig. 5A). Conversely, when waterlogged plants were treated with foliar S, APX activity of unfertilized and S-fertilized plants increased by 77% and 18% respectively, compared with untreated plants (Fig. 5A). Waterlogging increased the CAT activity in all treatment groups compared to their respective controls (Fig. 5B). The CAT activity of S-fertilized waterlogged plants was notably higher compared with unfertilized waterlogged plants. Similarly, CAT activity of foliar S treated unfertilized and S-fertilized plants was 33% and 15%, respectively higher than untreated waterlogged plants (Fig. 5B).

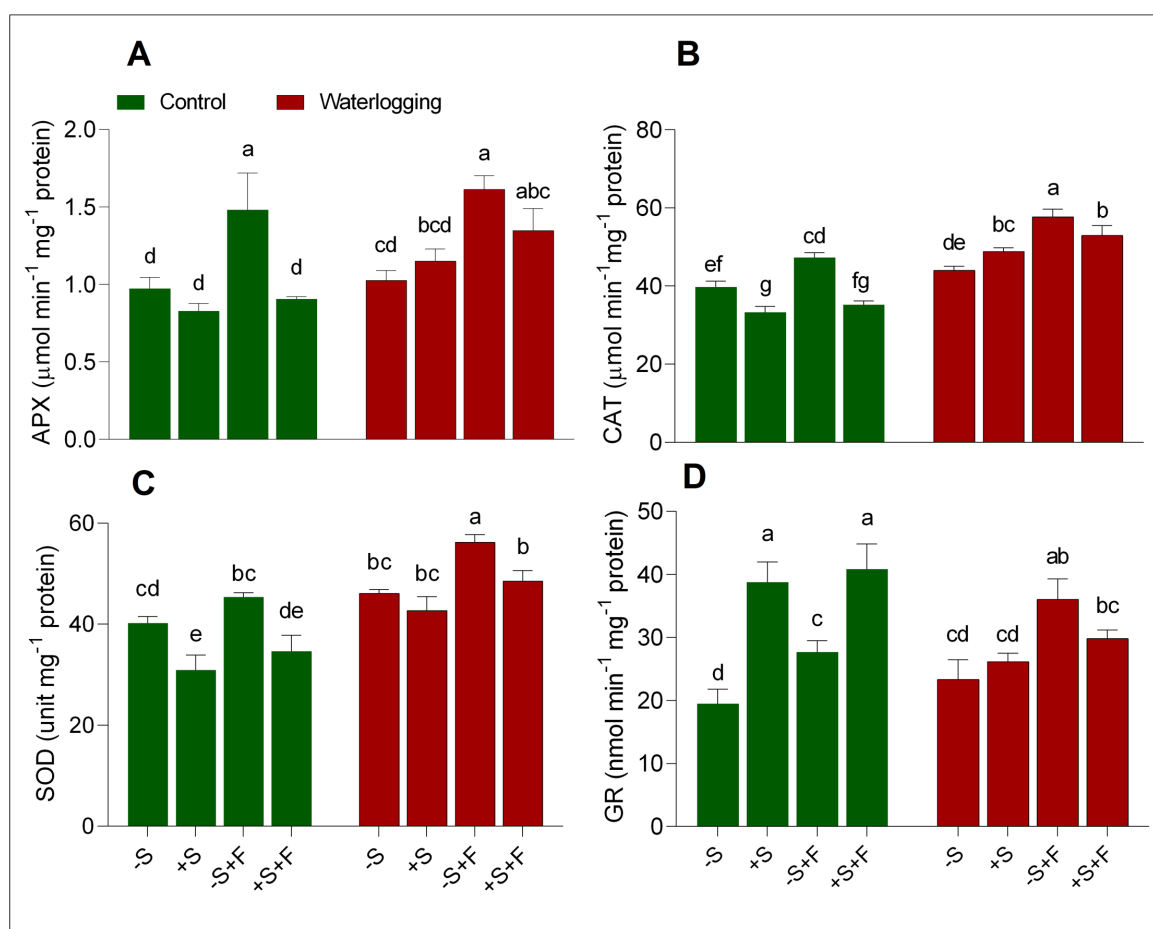


Fig 5. Effect of soil- and/or foliar-applied S on the activities of APX (A), CAT (B), SOD (C) and GR (D) in leaves of *Brassica napus* plants grown under control (80% soil water holding capacity) and waterlogged conditions. The data are means \pm SE of four independent pot replicates. The bars followed by different letters indicate significant differences between the

treatment groups (Tukey's test; $p \leq 0.05$). APX; Ascorbate peroxidase, CAT; Catalase, SOD; Superoxide dismutase, GR; Glutathione reductase.

The SOD activity of all treatments increased significantly under waterlogging, except for unfertilized untreated plants, which showed slightly higher (by 15%) SOD activity compared with their control counterpart (Fig. 5C). Conversely, under waterlogging condition, foliar S treatment of both unfertilized and S-fertilized plants increased SOD activity by 20 % and 15%, respectively compared with the untreated plants (Fig. 5C). Although the activity of GR in untreated unfertilized waterlogged plants remained unchanged but decreased significantly in S-fertilized waterlogged plants compared with control groups (Fig. 5D). The GR activity of both unfertilized and S-fertilized waterlogged plants receiving foliar S increased significantly by 54% and 14% respectively, compared with their untreated waterlogged plants (Fig. 5D). Significantly higher activities of GPX and GST in all treatments were observed under waterlogging stress compared with their control counterparts (Fig. 6). The GPX activity of foliar-S treated waterlogged plants was higher in unfertilized (by 37%) and S-fertilized plants (by 12%) compared with untreated waterlogged plants (Fig. 6A). Similarly, in contrast with untreated plants, foliar S treated unfertilized and S-fertilized waterlogged plants had 49% and 10%, respectively higher GST activity (Fig. 6B).

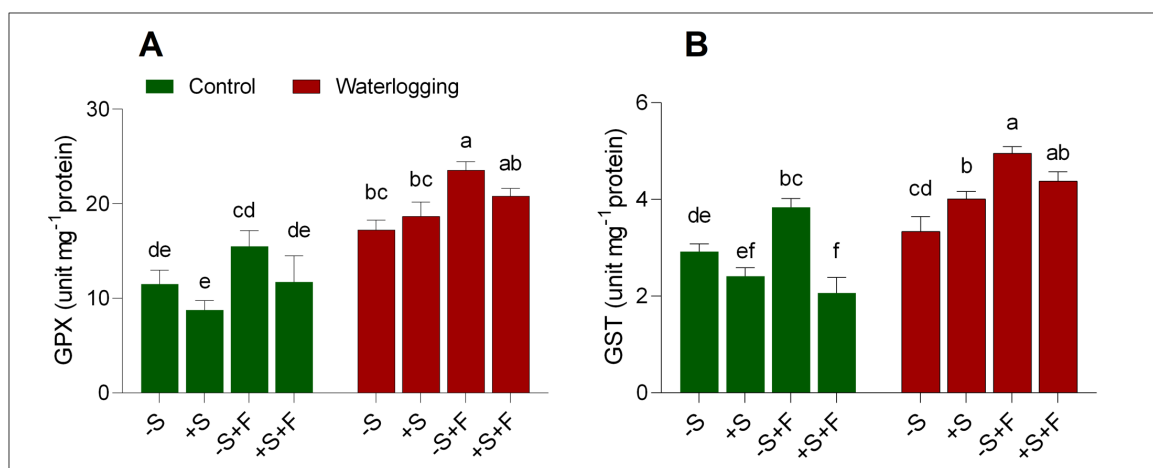


Fig 6. Effect of soil- and/ or foliar-applied S on the activities of GPX (A) and GST (B) in leaves of *Brassica napus* plants grown under control (80% soil water holding capacity) and waterlogged conditions. The data are means \pm SE of four independent pot replicates. The bars followed by different letters indicate significant differences between the treatment groups (Tukey's test; $p \leq 0.05$). GPX; glutathione peroxidase, GST; glutathione S-transferase.

3.5 Macro and micronutrients contents

The concentration of the macronutrients S, P and Mg in untreated S-fertilized plants was remarkably suppressed by 43%, 40% and 27% compared with control groups (Table 2). Conversely, in S-fertilized, foliar S treated plants displayed waterlogged reduction of S, P and Mg decrease by 40%, 37% and 20%, respectively compared with respective control groups (Table 2). Interestingly, in unfertilized plants under waterlogging condition the concentrations of S and Mg in both untreated and foliar S applied plants remained unaffected but the concentration of P reduced significantly compared with their control counterparts (Table 2). However, in contrast with untreated plants, foliar-S treated both unfertilized and S-fertilized plants showed higher S (by 43% and 8%), Mg (by 30% and 9%) and P (by 69% and 18%) concentrations under waterlogging condition (Table 2).

On the other hand, Fe and Mn concentrations in waterlogged plants of all treatments increased markedly compared with respective control groups (Table 2). Conversely, Fe and Mn concentrations increased in foliar S treated unfertilized plants (by 32% and 22%, respectively) but decreased (by 21 % and 8%) in S-fertilized plants compared with untreated waterlogged plants (Table 2). However, although under WL-stress Zn concentrations of both untreated and foliar S treated with S-fertilized plants reduced significantly but displayed statistically similar in unfertilized plants compared with their respective controls (Table 2). Furthermore, in contrast with untreated waterlogged plants, foliar S treated both unfertilized and S-fertilized waterlogged plants had 44% and 13%, respectively higher Zn concentration (Table 2).

Chapter 4

Table 2. Effect of soil- and foliar-applied S on the concentration of total S, P, Mg, Zn, Fe and Mn in leaves of *Brassica napus* grown under control (80% soil water holding capacity) and waterlogged conditions

Nutrient	Control				Waterlogged			
	+S		-S		+S		-S	
	No-Foliar S	Foliar S	No-Foliar S	Foliar S	No-Foliar S	Foliar S	No-Foliar S	Foliar S
Total S (%)	0.99 ± 0.05 a	1.05 ± 0.04 a	0.41 ± 0.01c	0.58 ± 0.02b	0.57 ± 0.02b	0.62 ± 0.01b	0.39 ± 0.02c	0.56 ± 0.01b
P (mg g ⁻¹ DM)	6.39 ± 0.23 a	6.64 ± 0.25 a	4.05 ± 0.039 bc	4.79 ± 0.09 b	3.83 ± 0.09c	4.15 ± 0.10 bc	3.04 ± 0.02d	3.96 ± 0.21c
Mg (mg g ⁻¹ DM)	5.89 ± 0.34 ab	6.39 ± 0.16 a	2.65 ± 0.33 e	4.14 ± 0.32 cd	4.32 ± 0.051cd	5.09 ± 0.11bc	2.25 ± 0.17e	3.81 ± 0.34 d
Zn (µg g ⁻¹ DM)	77.6 ± 1.70 a	78.8 ± 1.48 a	46.0 ± 1.41 de	53.8 ± 1.23 cd	56.9 ± 1.04 bc	64.30 ± 0.72 b	39.37 ± 1.22e	56.6 ± 2.92 bc
Fe (µg g ⁻¹ DM)	84.7 ± 3.13 c	87.8 ± 1.68 c	51.3 ± 2.31 d	64.4 ± 0.44 d	135.2 ± 2.91a	107.4 ± 6.05 b	83.3 ± 3.26 c	109.8 ± 5.92b
Mn (µg g ⁻¹ DM)	138.5 ± 4.85 b	143.9 ± 4.83 b	62.5 ± 2.39d	95.5 ± 4.46 cd	237.6 ± 5.12 a	218.0 ± 9.78 a	108.8 ± 4.7 bc	132.4 ± 3.72 bc

+S represents sufficient-S fertilized while -S, represent unfertilized respectively. Data are means ± SE of four independent pot replicates. Values followed by different letters in a row indicate significant differences between the treatments (Tukey's test; $p < 0.05$).

4 Discussion

Waterlogging stress leads to decrease in the concentration of nutrient elements in plants, disrupting their metabolism and expected growth rate. Finally, it initiates cascades of physiological, biochemical, and morphological changes that limit the productivity of plants. Nonetheless, sulfur showed protective roles in conferring plant tolerance to several abiotic stresses (Liu et al., 2021). In the current study, the effect of foliar-applied S in inducing waterlogging tolerance in unfertilized and S-fertilized *Brassica* plants was investigated.

4.1 Growth impairment in response to waterlogging stress

Our results showed that exposure to 7 days of waterlogging brought phenotypic and morphological changes, such as leaf chlorosis, reduction of biomass and plant height of *Brassica napus* compared with control conditions (Fig. 1). It has been reported that waterlogging reduces the growth of rapeseed and several crops like wheat, barley, sesame, and rice (Anee et al., 2019; Keya et al., 2021). In the present study, total dry biomass and plant height were significantly lower under waterlogging than controls (Fig. 1B- 1C). The possible causes of retarded growth of plants under waterlogging stress could lead to increased ROS production (Teoh et al., 2022), inadequate nutrient uptake (Wollmer et al., 2018), and declines in photosynthetic rate (Kumari et al., 2022), which were also found in the current study (Fig. 2, 3; Table 2). However, all adverse effects on growth were partially or entirely reversed by both soil S- fertilized (+S) and foliar-S treated waterlogged *Brassica* plants. As known, rapeseed (*Brassica napus*) plants need adequate S (Li et al., 2020), thus after waterlogging, leaf S concentration was reduced (Table 2) due to the leaching of sulphate concentration in the soil (Hussain et al., 2022). Therefore, both soil and foliar S supplementation to the WL-stressed plants maximize the uptake of nutrients (Table 2) as S has a synergistic relationship with most of the nutrients (De Souza Silva et al., 2014; Shah et al., 2022). Besides correcting photosynthetic impairment (Fig. 2A-B), S supplementation reduced ROS (Fig. 3A-B), all together promoting plant growth.

4.2 Physiological response of waterlogged rapeseed

In this study, the WL-stress reduced net photosynthetic rate and stomatal conductance in all treatments, suggesting that the photosynthetic apparatus was most responsive to WL stress (Fig. 2). The probable reason might be the WL-stress caused anaerobic respiration, which

decreases root hydraulic conductivity, thereby inhibiting water uptake (Kaur et al., 2019). As a result, to prevent such water loss in the plant, the stomata close, thereby lowering the net photosynthetic rate (Holbrook and Zwieniecki, 2003). Moreover, the reduction of mineral nutrients in leaves especially S (Table 2) also affects photosynthetic apparatus and net photosynthesis (Li et al., 2020; Lunde et al. 2008). Nonetheless, the combined treatments of S-fertilized (+S) with foliar-S treated plants showed notable improvement in net photosynthesis and stomatal conductance under waterlogging conditions, indicating that S plays a crucial role in maintaining photosynthetic activity (Fig. 2). The chloroplast contains proteins rich in sulfur, so a sulfur deficiency causes leaf chlorosis by altering chloroplast morphology, affecting photosynthesis profoundly and can only be corrected by adding external sources of sulfur (Terry N., 1976). The plausible explanation for the improvement in photosynthetic rate is that supplementation of S increased the S content in leaf tissues (Table 2), which enhanced the uptake of key nutrients such as Mg (Table 2), crucial for chlorophyll stability (Skudra et al., 2017; Kumawat, 2006). Moreover, S contributes to Fe availability in leaves (Table 2), which affects Fe-S cluster, a major component of photosynthetic and electron transport chain (Ibrahim et al., 2020; Lu 2018; Vigani et al., 2018; Zuchi et al., 2009). Our findings are supported by several studies observed in rapeseed (Fatma et al., 2014), wheat (Shi et al., 2022), sesame (Keya et al., 2022), and rice (Siddiqui et al., 2021) under different abiotic stresses, those revealed the scavenging functions of S against photosynthetic pigment degradation and photosynthetic activity.

4.3 Differential ROS association with foliar S and untreated WL-stressed plants

In waterlogged conditions, plants experience reduced oxygen availability, resulting in an increase in reactive oxygen species (ROS) (Teoh et al., 2022). As a consequence, excessive formation of ROS in stressed cells results when the cellular antioxidant defense system can't combat the ROS generation which leads to oxidative stress (Keya et al., 2022). As a response to this oxidative stress, plants deploy various antioxidant defense mechanisms (Siddiqui et al., 2020). A first line of defense against waterlogging stress, SOD, CAT, APX, and GR are key antioxidant enzymes that can help to mitigate plants from waterlogging stress by trapping and detoxifying ROS (Anee et al., 2019). The present study demonstrated that to counteract oxidative stress, plants activities of antioxidant enzymes viz. APX, GR, SOD and CAT were augmented (Fig. 5), but concurrently, H₂O₂ content also elevated that made the membrane more vulnerable to leakage by peroxidation of membrane lipids which was confirmed by an increase

in MDA content (Fig. 3). Conversely, foliar S along with soil S supplementation of WL-stressed plants further increased the activities of these antioxidant enzymes (Fig. 4-5) as well as minimized MDA and H₂O₂ contents substantially (Fig. 3). The fact is that detoxifications of ROS take place through, SOD dismutates O₂^{·-} radicals to H₂O₂, whereas APX, GR, CAT convert H₂O₂ into H₂O and O₂ (Arbona et al., 2008; Hasanuzzaman et al., 2019). However, under WL-stress without soil S supplementation or foliar S, up-regulation of antioxidant enzymes was insufficient to detoxify ROS (Fig. 3) that affected the stressed plants with excessive accumulation of H₂O₂ content and enhanced lipid peroxidation in plants with concomitant higher MDA accumulation (Arbona et al., 2008). Nonetheless, several studies reported that exogenous S can reduce H₂O₂ and MDA levels in plants when they were subjected to oxidative stress (Anjum et al., 2015; Talukdar et al., 2015). This is due to fulfilment of S, which was insufficient in WL-stressed plants (Schnug and Haneklaus, 2005), which spikes the demand for S for the synthesis of GSH and other S derivatives to increase tolerance (Anjum et al., 2015; Freitas et al., 2019; Liu, 2021). However, the role of GSH in protecting plants from waterlogging stress has been postulated widely (Keya et al., 2022; Zheng et al., 2021). According to these findings, the highest enhancement of GSH was exhibited with the foliar-S treated combined with S-fertilized plants (Fig. 4C), which directly contributes to the maintenance of ASA-GSH cycle that scavenged H₂O₂. Likewise, the highest AsA contents of both S-fertilized and foliar-S treated plants due to the highest GSH contents, as GSH is utilized by DHAR to convert DHA to AsA through the GSH-AsA cycle (Foyer and Noctor, 2011). The present study also showed reduced GSSG contents and increased GR activity with the supplementation of S (soil or/and foliar), in that during the ROS detoxification process, GSH acts as an efficient electron donor, therefore its oxidized to GSSG (Asada, 1994). Further, GSH can be regenerated by GR (Fig. 5) using NADPH as the substrate (Noctor and Foyer, 1998). Nonetheless, GSH not only detoxifies ROS, but also modulates antioxidant enzymes and the antioxidant defense system, which was also observed in this experiment. Aside from AsA and GSH, GPX also assists in the detoxification of hydrogen peroxide, while GST detoxifies a wide range of xenobiotics and endogenous substances (Hasanuzzaman et al., 2019). Moreover, higher GPX and GST activities of combined S supplementation of WL-stress plants conferred more tolerability by downregulating the peroxidation of cell membranes (Fig. 6). Therefore, in the present study, supplementation of S combined with soil and foliar not only improved growth, physiology, and nutrient uptake, but also increased the antioxidant enzymes (Fig. 4-5), while decreasing H₂O₂ and MDA content (Fig. 3) of leaf tissue of *Brassica napus* under WL-stress.

4.4 Retrieval of nutrient concentrations

In the current experiment, WL stress decreased the concentrations of S, P, K, Mg and Zn in the leaves, indicating the reduction of nutrient uptake (Table 2). The underlying mechanism could be oxidative stress, which blocks the nutrients channels, so that the stressed plants have difficulty in getting water from the soil to the xylem and surrounding cells, leading to a decreased ions' mobility (Hasanuzzaman et al., 2013; Krieger et al., 2005). Moreover, waterlogging stress reduce H⁺-ATPase activity (Schubert and Yan, 1999; Zhang et al., 2017) resulted in partial membrane depolarization, which impaired inorganic cation uptake (Steffens et al., 2005). Additionally, the present study found that the WL-treated *Brassica* leaf contained high concentrations of Fe and Mn (Table 2), but not that much to consider as toxicity (Millaleo et al., 2010; Zahra et al., 2021). In waterlogging condition due to the higher soil temperature could result in a faster release of soil-bound nutrients such as Mn²⁺ or Fe³⁺ (Ponnamperuma, 1984). Interestingly, supplementation of S as foliar to the unfertilized and S-fertilized plants not only increased leaf S concentration, but also increased P, Mg and Zn nutrients concentration (Table 2). Noteworthy, these results are validated by several studies that previously confirmed the synergistic effects of S with Mg, P and Zn on nutrient uptake, in different crops (Abdin et al., 2003; Dash et al., 2015; Ejraei et al., 2010; Farhad et al., 2010; Freitas et al., 2019; Ismail et al., 2005; Yadav et al., 2011; Zenda et al., 2021).

Conclusion

The findings of this study led to conclude that WL-stress exhibited differential responses to untreated and foliar-S treated S-fertilized and unfertilized *Brassica napus* plants in terms of growth, gas exchange attributes, enzymatic and non-enzymatic antioxidant system, and nutrients uptake. More precisely, better growth, higher net photosynthesis, and lower ROS in the foliar-S treated *Brassica* plants exhibited the mitigation of the negative effect of waterlogging. Thus, the present investigation also revealed that a better antioxidative defense system of combined soil S-fertilized and foliar-S treatments conferred a lesser reduction of WL-induced damage. However, these results will be helpful for a deeper understanding of the precise mechanism of foliar-S-assist tolerance in rapeseed at genetic and molecular levels.

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Chapter 5
General Discussion

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Adaptation mechanisms play a key role in plant endurance and productivity when exposed to abiotic stress. Increasing evidence suggests that plants' mineral nutritional status modifies their ability to adjust to unfavorable stress conditions. Accordingly, in this thesis, studies were conducted to investigate whether persistent waterlogging affects sulfur uptake, partitioning, and how such alteration of sulfur can affect seed storage protein compositions. Additionally, in contrast to previous studies under waterlogging stress, sulfur supplementation has been considered as a mitigation strategy either as soil and/or foliar supplement in high S-demanding crops like rapeseed (*Brassica napus* L.).

5.1 Waterlogging affects the growth and development of wheat and rapeseed

The presence of waterlogging over a period of 14 days (Chapter 2) and 7 days (Chapter 3 and 4) resulted in several phenotypic as well as morphological aberrations, namely stunted growth, reduced total biomass and leaf chlorosis (Chapter 2 and Chapter 3; Fig. 1 & 2) of both wheat and rapeseed compared with control counterparts. The biomass reduction of rapeseed (35-65%) (Chapter 3 and Chapter 4) was higher compared with wheat (18-33%) (Chapter 2) under waterlogging stress. Since rapeseed is regarded as a more waterlogging-sensitive plant compared with wheat due to the lack of generating adventitious roots with aerenchyma when in an oxygen-deficient medium (Voesenek et al., 1999), observing higher reduction in this study was imperative. The causes of retarded growth and development of plants under waterlogging include increased ROS production (Chapter 3; Fig. 3), improper nutrient uptake (Chapter 4; Table 2) and declines in photosynthetic rate (Chapter 4; Fig. 2). Interestingly, high genotypic differences for waterlogging tolerance were observed between the wheat varieties where the hybrid variety Hyvento proved relatively tolerant to waterlogging compared with the inbred variety Kredo (Chapter 2, Fig. 1 and 3). Waterlogging inhibits a wide range of developmental processes, including energy metabolism (Pan et al., 2021), therefore with a high degree of heterosis as well as genetic diversity, hybrids more efficiently withstand abiotic stresses than the inbred varieties (Adee et al., 2016; Sing et al., 2013). Surprisingly, higher S supplementation in soil from no-S to high-S posterred reduced growth retardation (Chapter 3; Fig. 1 and 2). With the same trend, foliar applied S also reduced the growth retardation of both

unfertilized (-S) and sufficient S-fertilized (+S) treated plants (Chapter 4; Fig. 1). It can be reasoned that under waterlogging stress, the reduction of S (Chapter 3, Table 2) in the plant certainly augments the demand for its supplementation for improving photosynthetic apparatus and cellular metabolism, which finally leads to increase nutrient uptake and growth (Fatma et al., 2014; Narayan et al., 2022). Additionally, S can promote cell enlargement, cell division, and tissue formation, thereby enhancing growth attributes (Chapter 3,4) and ultimately better nutrient uptakes (Shah et al., 2022; Li et al., 2020).

In the present studies, it was also noticed that S supplementation increased mineral nutrients uptake, which plays a role in the reduction of plant growth retardation (Chapter 3, Table 2; Chapter 4, Table 2).

5.2 Waterlogging affects the S-uptake, yield and yield quality

The results revealed a substantial decrease in the yield of both wheat and rapeseed (Chapter 2, 3), especially the protein compositions of wheat cultivars (Kredo and Hyvento). Due to waterlogging, storage protein compositions were more adversely affected in Kredo than in Hyvento (Chapter 2; Fig. 5). The quality of wheat (*Triticum aestivum* L.) is strongly influenced by grain storage protein compositions, especially gluten proteins (Yu et al., 2018). In response to waterlogging of early or late stage, S-rich gliadin and glutenin subunits in Kredo were more severely affected than those in Hyvento (Chapter 2; Fig. 5, Table 3). The composition of grain storage proteins is likely to change because gliadins and glutenins contain large amount of S-containing amino acids (Shewry et al., 2000). It was also observed that waterlogging caused higher S concentration reduction in all plant tissues of Kredo compared to Hyvento (Chapter 2, Fig. 3 and 4). At the same time, it also affirmed notable sulphate reduction by 54% (Kredo) and 65% (Hyvento) due to waterlogging condition (Chapter 2; Fig. 2). This finding means that waterlogging creates a paucity of soil mineral nutrient availability, especially sulphate (Chapter 2), due to the losses by leaching. This is why the waterlogging affected yield and yield quality more adversely in Kredo than in Hyvento (Chapter 2; Fig. 1). Therefore, it is reasonable that higher S reduction due to the waterlogging stress in Kredo is linked with low grain gluten proteins, thereby enhancing the relative pool of S-low/poor HMW-GS, ω -gliadin and reducing S-rich LMW-GS and γ -gliadin (%) compared with Hyvento (Chapter 2; Fig. 5; Table 3). Furthermore, higher N/S ratio (Chapter 2, Table 2) in the grain of both varieties due to the waterlogging events was observed, which reflects the imbalance of N and S within the plant tissue; and a high N:S ratio ($> 17:1$) can indicate a potential sulfur deficiency (Randall et al.,

1981). Additionally, S observed a crucial factor involving sulfur-nitrogen interactions in protein biosynthesis (Luo et al., 2000). Due to the decline of sulfur-containing amino acids like methionine and cysteine, protein biosynthesis is inhibited, causing an accumulation of arginine and asparagine, both of which are nitrogenous compounds (Hoefgen et al., 2008; Luo et al., 2000). Moreover, the results of this study revealed that among the two waterlogging treatments, late waterlogging has more drastic negative effects on S uptake, protein patterns and grain yield of both the wheat varieties than early waterlogging (Chapter 2, Fig. 3,4). As previously explained, late waterlogging often coincides with the reproductive stage of wheat, which is a critical period for yield formation. Therefore, in this stage, wheat plants require adequate soil moisture for grain filling, but excessive waterlogging can lead to oxygen deprivation in the roots, resulting in reduced photosynthesis, and nutrient uptake, which ultimately affect yield and yield quality (Ding et al., 2020a; Shao et al., 2013; Wollmer et al., 2017). However, the findings of this study partially contradict those observed by Ploschuk et al. (2018), who reported drastic effects of late waterlogging on growth, yield and physiological apparatus in barley and rapeseed, but not in wheat. By contrast, de San Celedonio (2014) demonstrated higher yield loss in wheat of up to 92% when waterlogging stress was imposed during the anthesis period (LWL). Nevertheless, besides duration of waterlogging and growth stage, the tolerance to waterlogging depends on the combination of genotypes and environmental conditions (Herzog et al., 2016). Accordingly, it was decided that effective uptake of S and its utilization into various grain proteins have partially invested waterlogging tolerance in hybrid winter wheat Hyvento compared with Kredo. Likewise, in rapeseed, a considerable reduction of yield and yield components (number of siliqua, 1000-seed weight) was observed due to waterlogging (Chapter 3, Fig. 2). Reduction of S-uptake (total S and SO_4^{2-}) due to the waterlogging effects on the yield and yield-contributing components of rapeseed. Such imbalance in nutrient uptake followed in reduced seed yield, and yield-contributing traits of all S-levels supplemented waterlogged plants (Chapter 3; Fig. 2). Therefore, the present results suggest that enough sulfur is a key factor for crucial metabolites for better waterlogging resistance (Chapter 3,4).

5.3 Supplementation of Sulfur increase mineral nutrient uptake of waterlogged plants

As very few studies have dealt with the supplementation of S either in soil or foliar as a mitigation approach in crop such as rapeseed, under waterlogging stress. Therefore, in these studies (chapter 3,4), rapeseed plants were subjected to the supplementation of sulfur as

MgSO₄·7H₂O in soil from no to high doses (Chapter 3) and as foliar feeding to unfertilized and sufficient S-fertilized plants for a 7-day waterlogging stress period. Seven days of waterlogging postured drastic alteration of mineral nutrients in rapeseed (Chapter 3, 4). Especially, the reduction of NO₃⁻, SO₄²⁻ and PO₄³⁻ accumulation except for Cl⁻, which notably increased in waterlogged rapeseed leaves (Chapter 3; Table 2). Such imbalance in nutrient uptake affected growth, seed yield, and yield-contributing(related) traits of all S-levels supplemented waterlogged plants (Chapter 3; Fig. 2). Conversely, S supplementation in soil (from low to high S) increased NO₃⁻, SO₄²⁻, and PO₄³⁻, but decreased Cl⁻ uptake (Chapter 3; Table 2). The decreased Cl⁻ concentrations of high S supplementation can be explained as the antagonistic interaction of Cl⁻ with SO₄²⁻ and NO₃⁻ (Geilfus, 2018; Sagervanshi et al., 2020). At the same time, elevated Cl⁻ concentrations and subsequent S reduction under waterlogging stress negatively affected NO₃⁻, SO₄²⁻ and PO₄³⁻ uptake. Besides these, in the present experiment, it was also noticed that WL-stress notably reduced P, Mg and Zn uptake (Chapter 4; Table 2). Due to oxidative stress (Chapter 3,4), nutrients channels become blocked, so stressed plants have difficulty getting water from the soil to the xylem and surrounding cells, leading to a decrease in ions' mobility (Hasanuzzaman et al., 2013; Krieger, 2005). Another reason for reduced nutrient uptake is downgraded H⁺-ATPase activity due to low ATP concentrations in roots (Schubert and Yan 1999; Zhang et al., 2019), resulting in partial membrane depolarization, which impaired nutrients uptake (Steffens et al., 2005). Additionally, the investigation also observed that the WL-treated *Brassica* leaf contained high concentrations of Fe²⁺ and Mn³⁺ (Chapter 4; Fig. 5). However, all these adverse effects were partially or entirely reversed by supplementation of S as foliar to both unfertilized (-S) and S-fertilized treated waterlogged *Brassica* plants (Chapter 4). As known, rapeseed (*Brassica napus*) plants need quite a bit of S, thus after waterlogging those were deprived of S as soil sulfate was leached (Chapter 2, Fig. 2) and availability of mineral sulphate concentrations in the soil were reduced (Hussain et al., 2022). Moreover, several reports confirmed the interactive effects of S with Mg, P, K and Zn on nutrient uptake, stimulating growth and yield in different crops (Dash, 2015; Elzenga et al., 2010; Farhad et al., 2012; Freitas, 2019; Yadav, 2011; Zenda et al., 2021). Therefore, the present results suggest that the S supplementation of either soil (Chapter 3) or/and foliar (Chapter 4) can modulate nutrient uptake in the rapeseed under waterlogging stress.

5.4 Sulfur supplementation improved the antioxidant defenses system of waterlogged plants

The present studies displayed that waterlogging causes increased H₂O₂ and malondialdehyde (MDA) contents than their control counterparts (Chapter 3 and 4), indicating ROS-induced oxidative stress (Amnan et al., 2022). In waterlogged conditions, plants experience reduced oxygen availability, thus the limited availability of O₂ triggers leakage of electrons from mitochondria, which together with impaired photosynthesis (Chapter 4; Fig. 2), induces a burst of excessive ROS production, causing oxidative damage to the WL-stressed plants (Xu et al., 2013). Surprisingly, S supplementation from no (zero) to high (Chapter 3) as well as either in soil or/and foliar (Chapter 4) to waterlogged plants significantly dropped H₂O₂ and MDA contents and boosted antioxidant enzymes CAT, APX, and GR activities; thus, it can be reasoned that S played an important role in reducing oxidative stress and providing protection to cell membranes. After being taken up by the roots as sulfate (SO₄²⁻), S is transformed into cysteine (Cys). Later, Cys serves as a precursor or donor for key S compounds like methionine (Met), glutathione (GSH), phytochelatins (PCs) and other S-derivatives, which play a role in plant's stress adaptation processes (Anjum et al., 2008; Cheng et al., 2013; Khan et al., 2014; Kumari et al., 2022). Therefore, in the S-assimilatory pathway, GSH synthesis is controlled and linked to ascorbate-glutathione (AsA-GSH) cycle enzymes. As a result, the S assimilation-regulated antioxidant system (Chapter 3, 4) helps to eliminate ROS by repairing cellular damage (Han et al., 2013). Similar to these results, higher levels of H₂O₂ and MDA induced by different abiotic stresses were found to be significantly decreased in exogenous S-treated plants. For example, Min et al. (2016) presented that S helps to mitigate heat stress by reducing H₂O₂ and MDA contents and increasing activities of SOD, CAT and APX in *Arabidopsis*. Interestingly, in these experiments, S-supplementation in soil (Chapter 3; Fig. 4) and/or foliar (Chapter 4, Fig. 4) to waterlogged plants increased AsA, which is most likely due to the increase in GSH content (Chapter 3, 4). The increasing trend of GSH and AsA to the supplemented S-treated waterlogged plants indicated that the utilization of S to synthesize GSH by stressed plants (Chapter 4; Fig. 4). These findings are similar to those of Lunde et al. (2008), who found that rice plants deficient in sulfur had 70% lower levels of GSH. This mechanism can be explained by the Mehler-peroxidase reaction, where GSH plays a vital role in the regeneration of AsA from dehydroascorbate (DHA) resulting in the oxidation of GSH to glutathione disulfide (GSSG) (Li et al., 2010; Noctor and Foyer, 1998). Therefore, exogenous S increased the contents of GSH and AsA in higher soil S-supplemented (Chapter 3) and foliar-S supplemented unfertilized and/ or S-fertilized waterlogged plants (Chapter 4).

5.5 Supplementation of sulfur improved leaf physiology in waterlogged plants

Waterlogging-induced impairment on the gas exchange parameters also observed a sharp reduction in net photosynthesis and stomatal conductance (Chapter 4; Fig. 2). As a result of prolonged waterlogging condition, enzymes that are responsible for photosynthesis are inhibited (Wu et al., 2019); chlorophyll synthesis in leaves decreases, leading to leaf senescence (Chapter 3, Fig. 2); as a result, the net photosynthetic rate decreases (Pan et al., 2021). Moreover, the rapid reduction of soil oxygen concentrations during waterlogging creates an energy-deficient environment (Adegoye et al., 2023). To maintain leaf turgor during energy-deficient waterlogging conditions, stomata compensate for the decline in water absorption caused by an increase in root resistance, ultimately affecting net photosynthesis (Jackson et al., 1987). Moreover, nutrients reduction due to waterlogging (Chapter 2, 3,4) can also adversely affect photosynthesis. It can restrict CO₂ uptake, limiting carbon assimilation and thus restricting the metabolic need for S (Malcolm et al., 2008) and ultimately reducing photosynthesis. For example, Adegoye et al. (2023) reported that 15 days of waterlogging decreased the photosynthetic rate by 42%, and reduced mineral nutrients resulted in lower biomass and seed yield of soyabean. Meanwhile, Lunde et al. (2008) observed that S deficiency severely affects photosynthetic apparatus, and chlorophyll content was reduced by 49%. In addition, electron transport in the Calvin cycle decreased during stress conditions, while electron leakage during photosynthesis increased. The combined effect of these factors results in higher respiration rates, reduced photosynthesis (Chapter 4, Fig. 2), and elevated levels of ROS (Chapter 3,4) in stressed plants (Noctor et al., 2006). The concomitant alterations of photosynthetic apparatus and Calvin cycle led to the significant reduction in photosynthesis. The chloroplast is involved vital reactions, making it essential that these organelles are robust against ROS, which is accomplished by antioxidant enzymes and metabolites (AsA and GSH) that quench excess ROS production (Nadarajah, 2020). Conversely, rapeseed plants treated with supplemented S (foliar) and/or unfertilized and S-fertilized waterlogged plants showed notable improvement in net photosynthesis and stomatal conductance (Chapter 4, Fig. 2), indicating that S plays an essential role in maintaining photosynthetic activity. The explanation for the improvements in photosynthetic pigment is that supplementation of S increases the S content in leaf tissues (Chapter 3, 4; Table 2), which stimulates the production of porphyrin (an intermediate in chlorophyll synthesis), increasing nutrients uptake especially Mg (Chapter 4, Table 2). All of these are crucial for photosynthetic apparatus (Kumawat, 2006; Shah et al., 2022; Skudra et al., 2017). Moreover, the chloroplast contains proteins rich in sulfur, so a sulfur

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deficiency causes leaf chlorosis by altering chloroplast morphology, affecting photosynthesis profoundly and can only be corrected by adding external sources of sulfur (Terry, 1976).

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**Chapter 6
Summary**

Chapter 6

Summary

Global climate change has significantly increased rainfall amounts during winter and spring resulting in waterlogging. Throughout the world, waterlogging is one of the most serious abiotic stress factors, causing numerous yield losses each year.

The displayed thesis consists of three independent experiments. The first study figured out the drastic effect of waterlogging on S-uptake, growth, yield, and yield quality of inbred (Kredo) and hybrid (Hyvento) wheat cultivars in two crucial stages of growth, namely tillering stage/early waterlogging (Feekes Stage 6) and/or ear emergence stage/late waterlogging (Feekes Stage 10.1). Intriguingly, waterlogging significantly reduced the concentration of plant-available SO_4^{2-} in waterlogged soil (51%–60%) compared to non-waterlogged soils. Although results demonstrate that waterlogging affects both studied varieties, the hybrid (Hyvento) variety showed a lesser decline in S concentration and uptake compared with inbred (Kredo) in different parts of plant as well as growth, yield, and yield quality. Surprisingly, due to waterlogging, total protein content and gliadin fractions were not affected in the hybrid (Hyvento), whereas a significant reduction in grain gliadin content was noticed in the inbred variety. Due to waterlogging imposition, the proportion of S-low to poor HMW-GS and ω -gliadin in inbred variety increased significantly compared to the control. Waterlogging at the late waterlogging (Feekes Stage 10.1) could drastically affect S uptake, yields, and protein patterns in both varieties, compared with tillering stage/early waterlogging (Feekes Stage 6).

In the second experiment, different levels of supplemented S in soil, ranging from no-S to high-S was applied to investigate the S-induced stress resistance of oilseed rape (*Brassica napus* L.) plants under waterlogging condition. Thus, the effect of four S treatments, viz. no-S, low-S, medium-S and high-S on growth, recovery, physiological responses, anion relations, oxidative damage and changes in non-protein thiol (glutathione) was investigated in control and waterlogged *Brassica napus* plants. The results demonstrate that waterlogging had significant adverse effects on the growth of *Brassica napus* as indicated by their reduced biomass, yield, higher malondialdehyde and H_2O_2 content as well as an inefficient antioxidative defense system. Although sulfur supplementation from medium to high levels had negligible effects on the antioxidant system, morphological and yield traits of well-watered control rapeseed plants. Interestingly, in waterlogging condition, S supplementation, in soil from medium-S to high-S

levels significantly improved the antioxidative enzymes activities and metabolites, growth, yield, and decreased the chloride, malondialdehyde and H_2O_2 level, so that their growth and yield were improved linearly with the increasing supplemented S levels. These results reveal that waterlogging-induced oxidative stress was ameliorated by high-S supplementation mainly through biochemical adjustments as observed in increasing antioxidant systems. Nonetheless, high-S supplementation ameliorating WL-stress 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. Therefore, high sulfur supplementation alleviates waterlogging stress to rapeseed primarily through an upsurging antioxidant defense system and anions adjustments.

In the third experiment, the effect of S supplementation as foliar-S and/or soil S-fertilized plants on growth, photosynthetic response, antioxidant defense system, and impact on ion homeostasis in *Brassica napus* plants grown under waterlogging was investigated. As observed, foliar S alone or combined with soil S-fertilized plants prevented the waterlogging-induced decline. However, foliar S protected rapeseed plants from waterlogging-induced adversities through restricting ROS-induced cellular damage by raising the activities of antioxidant enzymes (CAT, APX, GR, SOD, GPX and GST), the levels of non-enzymatic antioxidants (e.g., GSH and AsA) and accumulating lesser Fe and Mn, but more P, S, Zn in *Brassica napus* plants. The findings of this study led to the conclusion that despite waterlogging stress on rapeseed plants, exogenous S as soil or combined with foliar can effectively modulate multiple physiological and biochemical mechanisms to enhance the WL tolerance of *Brassica napus*. Therefore, the application of S as a foliar spray at a concentration of 300 ppm was shown to have a clear contribution to ameliorating waterlogging stress.

However, these studies shed light on the responses of plants to waterlogging stress and the mechanism of ameliorating waterlogging stress by using sulfur fertilization.

Zusammenfassung

Der globale Klimawandel ist ursächlich für viele neuartige Wetterphänomene und hat so beispielsweise die Niederschlagsmengen im Winter und Frühjahr derart erhöht, dass Staunässe die Folge ist. Weltweit zählt Staunässe heute zu einer der schwerwiegendsten abiotischen Stressfaktoren, die jedes Jahr zu hohen Ertragseinbußen führt.

Ziel dieser Arbeit war es daher, in drei unabhängigen Experimenten, herauszufinden, wie den negativen Folgen von Staunässe begegnet werden kann. Im ersten Teil dieser Arbeit wurden die Auswirkungen von Staunässe auf die S-Aufnahme, das Wachstum, den Ertrag und die Ertragsqualität von Inzucht- (Kredo) und Hybridweizensorten (Hyvento) in zwei entscheidenden Wachstumsstadien, nämlich im Bestockungsstadium/frühe Staunässe (Feekes Stadium 6) und/oder im Ährenaufgang/späte Staunässe (Feekes Stadium 10.1) untersucht. Interessanterweise verringerte Staunässe die Konzentration von pflanzenverfügbarem SO_4^{2-} in staunassen Böden erheblich (51–60 %) im Vergleich zu nicht staunassen Böden. Obwohl die Ergebnisse zeigen, dass Staunässe beide untersuchten Sorten beeinträchtigt, zeigte die Hybridsorte (Hyvento) im Vergleich zur Inzuchtsorte (Kredo) einen geringeren Rückgang der S-Konzentration und der S-Aufnahme in verschiedenen Pflanzenteilen, sowie des Wachstums, des Ertrags und der Ertragsqualität. Dies führte bei der Inzuchtsorte zu einem signifikanten Rückgang des Gliadinegehalts im Korn, und der Anteil an S-armem bis schwachem HMW-GS und ω -Gliadin stieg bei der Inzuchtsorte im Vergleich zur Kontrolle deutlich an. Überraschenderweise wurde bei der Hybridsorte (Hyvento) der Gesamtproteingehalt und die Gliadinfraktionen durch Staunässe nicht beeinträchtigt. Letztlich lässt sich aus diesen Ergebnissen schließen, dass späte Staunässe im Stadium des Ährenschiebens (Feekes-Stadium 10.1) die S-Aufnahme, den Ertrag und das Proteinmuster in beiden Sorten stärker beeinflusst, verglichen mit früher Staunässe im Stadium der Bestockung (Feekes-Stadium 6).

Im zweiten Versuch wurde die S-induzierte Stressresistenz von Rapspflanzen (*Brassica napus* L.) unter Staunässebedingungen bei verschiedenen S-Gehalten im Boden untersucht. Insgesamt wurden die Auswirkung von vier S-Stufen (kein S, niedrig, mittel, hoch) auf Wachstum, Gesundheit, physiologische Reaktionen, Anionenbeziehungen, oxidative Schäden und Veränderungen bei Nicht-Protein-Thiolen (Glutathion) unter Kontroll- und Staunässe-Bedingungen bei *Brassica napus* untersucht. Die Ergebnisse zeigen, dass die negativen Auswirkungen auf das Wachstum von Raps unter Staunässe erheblich sind, was sich in einer geringeren Biomasse, einem geringeren Ertrag, einem höheren Malondialdehyd- und H_2O_2 -

Gehalt sowie einem ineffizienten antioxidativen Abwehrsystem widerspiegelt. Hierbei war eine Schwefelergänzung in mittlerer bis hoher Konzentration von lediglich vernachlässigbarer Wirkung auf das antioxidative System, die morphologischen Eigenschaften und den Ertrag von gut bewässerten Kontrollpflanzen. Interessanterweise verbesserte jedoch die S-Supplementierung unter Staunässebedingungen die Aktivität antioxidativer Enzyme und Metabolite, das Wachstum und den Ertrag signifikant und verringerte den Chlorid-, Malondialdehyd- und H_2O_2 -Gehalt im Boden, so dass Wachstum und Ertrag linear mit steigendem S-Gehalt verbessert wurden. Diese Ergebnisse zeigen, dass der durch Staunässe ausgelöste oxidative Stress durch eine Supplementierung mit hohem S-Gehalt hauptsächlich durch biochemische Anpassungen gemildert wurde, wie sie bei der Erhöhung der antioxidativen Systeme beobachtet wurden. Nichtsdestotrotz verbessert eine hohe S-Supplementierung die negativen Folgen von Staunässe in Rapspflanzen und wird mit der Regulierung der Anionenaufnahme in Verbindung gebracht, beispielsweise mit einer Verringerung der Cl^- -Aufnahme und einer Erhöhung der Aufnahme von Sulfat, Nitrat und Phosphat. Es kann daher geschlussfolgert werden, dass eine hohe Schwefelzufuhr den Staunässe-Stress bei Raps in erster Linie durch ein ansteigendes antioxidatives Abwehrsystem und die Anpassung der Anionen abmildert.

Im dritten Versuch wurde die Wirkung einer S-Supplementierung nach Blattapplikation und/oder Bodengabe auf das Wachstum, die photosynthetische Reaktion, das antioxidative Abwehrsystem und die Auswirkungen auf die Ionenhomöostase bei *Brassica napus* unter Staunässe. Dabei wurde beobachtet, dass eine alleinige S-Blattapplikation oder eine Gabe in Kombination mit S-Bodenapplikation den durch Staunässe verursachten Wachstumsrückgang verringerte. Dieses ist vor allem darauf zurückzuführen, dass die Aktivitäten der antioxidativen Enzyme (CAT, APX, GR, SOD, GPX und GST) und der Gehalt an nicht-enzymatischen Antioxidantien (z.B. GSH und AsA) erhöht wurden und sich weniger Fe und Mn, dafür aber mehr P, S und Zn in den Rapspflanzen anreicherte. Die Ergebnisse dieser Studie lassen den Schluss zu, dass trotz Staunässe-Stress bei Raps exogene S-Zufuhr über den Boden oder in Kombination über das Blatt mehrere physiologische und biochemische Mechanismen wirksam modulieren kann, um die Staunässe-Toleranz von Raps zu verbessern. Es hat sich gezeigt, dass die Anwendung von S als Blattgabe in einer Konzentration von 300 ppm einen deutlichen Beitrag zur Verbesserung von Staunässe-Stress leistet.

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Zusammenfassend kann aus allen drei Experimenten geschlossen werden, dass eine Schwefeldüngung eine wirksame Maßnahme ist, die Stresssymptomatik bei verschiedenen Kulturpflanzen abzumildern.

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