

Research Article

Sodium Hydrosulfide Enhances Drought Tolerance by Alleviating Oxidative Stress and Promoting Proline Accumulation in *Brassica napus* L.

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ABSTRACT

Drought is one of the environmental factors inhibiting plant productivity and growth, leading to oxidative damage. This study aims to identify the role of sodium hydrosulfide (NaHS) as a hydrogen sulfide (H₂S) donor in drought stress tolerance in *Brassica napus*. Drought-induced stress symptoms appeared eight days after treatment, showing wilted leaves and a significant reduction of leaf water potential. Drought-induced increase of lipid peroxidation was significantly reduced by NaHS application. NaHS-treated plants mitigated stress symptoms under drought conditions by reducing hydrogen peroxide (H₂O₂) content, confirmed with H₂O₂ localization in situ. Furthermore, NaHS promotes photosynthetic activity by maintaining chlorophyll and carotenoid content, thereby supporting plant growth under drought conditions. Pyrroline-5-carboxylate and proline contents were significantly increased by drought but further enhanced by NaHS treatment, indicating the important roles of proline accumulation in drought stress tolerance. In conclusion, this study provides valuable insight into the roles of NaHS in alleviating drought stress by reducing oxidative stress and promoting proline accumulation. Therefore, NaHS may serve as an effective strategy to enhance crop production under drought-stress conditions.

(Key words: *Brassica napus*, Drought stress, Proline, Sodium hydrosulfide)

I. INTRODUCTION

Abiotic stress is a worldwide environmental factor that limits plant productivity, particularly drought stress (Kour et al., 2020). Climate change is raising global temperature thus increasing savior drought conditions. Oilseed rape (*Brassica napus*) is a major oil source for industrial uses in the world (Kirkegaard et al., 2021). Drought stress is one abiotic stress factor that limits the global production of *B. napus*. Besides, drought stress caused the highest yield reduction in several growth stages (Bilibio et al., 2011). Furthermore, enhancing drought tolerance in agricultural crops, specifically *B. napus*, is not only essential for oilseed production but also has implications for forage plants and grassland ecosystems, thus supporting livestock and maintaining ecological balance (Hopkins and Del Prado, 2007).

Plants exposed to drought stress provoke water loss, commonly generating reactive oxygen species (ROS) such as hydrogen peroxide (H₂O₂) (La et al., 2019). Accumulation of H₂O₂ leads to cellular damage in plant cells, thus reducing

plant growth and productivity. In addition, malondialdehyde (MDA) is also known to induce damage in plant cells or activate plant protection depending on cellular production, scavenging, and signaling modulation (Morales et al., 2019).

Plants produce ROS due to defense mechanisms under stressful environmental conditions, thus producing osmoprotectants and antioxidants to maintain cellular homeostasis. Proline, widely known as an osmoprotectant functions osmotic pressure balance and maintains cellular membrane stability (Rejeb et al., 2014; Hasan et al., 2020; Lee et al., 2022). Several reports indicate that proline accumulation is associated with drought stress tolerance to reduce oxidative damage. Several environmental cues regulate proline biosynthesis through the glutamate pathway, where Δ^1 -pyrroline-5-carboxylate (P5C) acts as a critical intermediate (Kavi and Sreenivasulu, 2014).

Recently, gaseous signaling molecules have been reported to contribute to stress tolerance, particularly drought stress. Sodium hydrosulfide (NaHS), widely known as an H₂S donor improves plant growth and induces stress tolerance (Banerjee and Roychoudhury, 2021). Hydrogen sulfide (H₂S) was reportedly

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involved in stress tolerance by modulating antioxidant activity (Lai et al., 2014; Zhang et al., 2021). H₂S could activate plant defense mechanisms by modulating osmoprotectants, particularly proline (Azhar et al., 2018; Guo et al., 2023). Proline production could be a stress marker and or stress tolerance parameter in plants, thus mitigating oxidative damage and protecting membranes from peroxidation (Rhaman et al., 2024).

This study aims to investigate the role of NaHS in alleviating drought stress in *B. napus*. NaHS as an H₂S donor induces proline accumulation thereby inducing drought stress tolerance and promoting plant photosynthesis under drought conditions further improving livestock productivity.

II. MATERIALS AND METHODS

1. Plant materials and treatments

Oilseed rape (*Brassica napus* L. cv Mosa) was grown under greenhouse conditions for four weeks in 2-L pots containing a mixture of soil (70%) and perlite (30%). Four-leave stages plants were chosen and divided based on morphological similarity into three groups: (1) Control plants (200 mL water per day), (2) Drought-stress plants (50 mL water per day), and (3) NaHS + Drought plants (50 mL of 500 μ M NaHS per day). Three biological replications for each treatment were used in this experiment. The samples were collected on day eight following treatment.

2. Determination of leaf water potential and lipid peroxidation

Leaf water potential (LWP) was determined by Lee et al. (2023) using a pressure chamber (PMS Instrument, Corvallis, OR USA). Malondialdehyde (MDA), a represented lipid peroxidation, was determined by extracting a sample of fresh leaves (200 mg) with 0.1% trichloroacetic acid. After 10 min, samples were centrifuged at 12,000 \times g rpm for 10 min. Supernatants were mixed with 0.5% thiobarbituric acid in 20% trichloroacetic acid. Samples were gently mixed with vortex and incubated at 95°C. After 30 min, the reaction stopped in the ice and centrifuged at 12,000 \times g rpm for 15 min. Absorbance was measured at 532 nm, and the content of the MDA was calculated using the extinct coefficient 155 $\text{mM}^{-1}\text{cm}^{-1}$ (Hodges et al., 1999).

3. Determination of H₂O₂ content and H₂O₂ visualization

Fresh leaf samples (200 mg) were extracted using 1mL of 100 mM phosphate buffer and centrifuged at 10,000 \times g for 10 min. The supernatant was mixed with 0.1% titanium chloride (TiCl₄) in 20% sulfuric acid and centrifuged at 6,000 \times g for 3 min. Absorbance was read at 410 nm and calculated using the extinct coefficient of 0.28 $\mu\text{M}^{-1}\text{cm}^{-1}$ (Lin and Kao, 2001). H₂O₂ visualization was described by Lee et al. (2013) using the DAB (3,3-diaminobenzidine) staining method. Leaves were immersed in DAB solutions in 10 mM sodium phosphate buffer (pH 7) overnight. After that, the leaves were immersed in ethanol and boiled (100°C) to bleach the chlorophyll.

4. Determination of chlorophyll and carotenoid content

Chlorophyll and carotenoid content were determined using the method described by Richardson et al. (2002). Fresh leaves (approximately 100 mg) were immersed in 10 mL of DMSO and incubated for 48 h. After 48 h, supernatants were read in absorbance 645 nm and 663 nm for chlorophyll content, and 480 nm and 510 nm for carotenoid content using a microplate reader (Synergy H1 Hybrid reader; Biotech, Korea).

5. Determination of proline and P5C content

Leaf samples (200 mg) were extracted with 3% sulfosalicylic acid and centrifuged for 10 min at 12,000 \times g to determine the contents of proline and pyrroline-5-carboxylate (P5C). Afterward, supernatants were mixed with ninhydrin and 6M H₃PO₄ and incubated at 100°C. After 1 h, samples were cooled in the ice and added toluene. Samples were strongly vortexed and incubated overnight. Absorbance was determined at 520 nm and calculated using a proline standard curve. P5C content was determined by adding 10 mM 2-amino benzaldehyde into the supernatant and incubated for 2 h. Absorbance was recorded at 440 nm and P5C content was calculated using the extinction coefficient 2.58 $\text{mM}^{-1}\text{cm}^{-1}$ (Lee et al., 2009).

III. RESULTS

1. Physiological symptoms of plants

Plants subjected to drought stress for eight days, as outlined

in the experimental design (Fig. 1A). After eight days of treatment, drought-stressed plants showed wilting leaves due to water loss and reduced turgor pressure, there was a noticeable reduction in leaf sizes and stunted growth. NaHS-treated plants showed fewer wilting leaves and promoted better growth and plant vigor compared to drought-stressed plants (Fig. 1B)

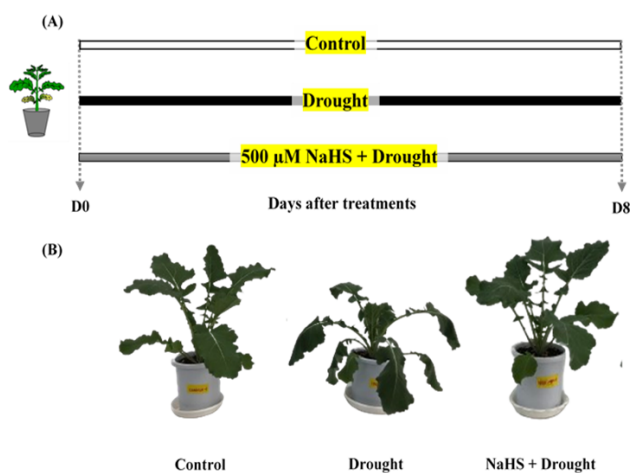


Fig. 1. Treatments during the experimental period (A) and plant morphology (B) after treatments.

2. Leaf water potential and MDA content

Drought stress significantly reduced leaf water potential (-1.38 MPa) compared to control plants. In contrast, the addition of NaHS under drought treatment induced leaf water potential by 63% compared to drought treatment (Fig. 2A). The concentration of malondialdehyde (MDA), a represented lipid peroxidation, in drought-stressed plants is $6.604 \mu\text{mol g}^{-1}$ FW was significantly higher than the control. NaHS-treated plants reduced MDA content compared to drought stress ($4.53 \mu\text{mol g}^{-1}$ FW) (Fig. 2B).

3. H_2O_2 visualization and H_2O_2 content

Drought-stressed plants show deep brown spot accumulation in DAB-stained leaves, while NaHS treatment plants reduce deep brown spots (Fig. 3A). Drought-stressed plants showed significant increases in the concentration of H_2O_2 by $56.67 \text{ nmol g}^{-1}$ FW. In contrast, NaHS-treated plants significantly reduced H_2O_2 content (48.9 nmol g^{-1} FW) compared to drought-stressed plants (Fig. 3B).

4. Chlorophyll and carotenoid content

Total chlorophyll and total carotenoid significantly decreased under drought-stressed treatment. In contrast, NaHS-treated plants induced chlorophyll and carotenoid content (compared to drought stress) under drought conditions (Fig. 4A and B)

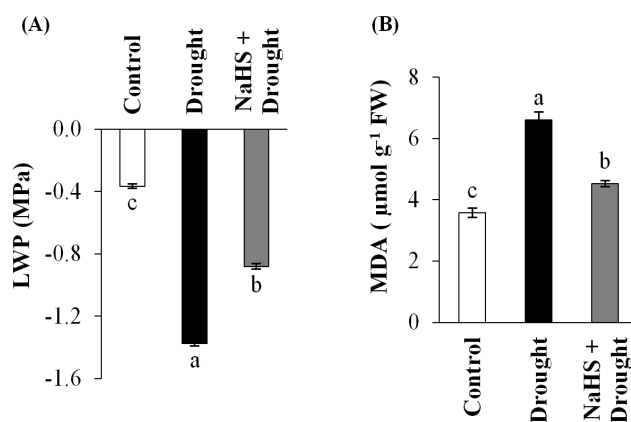


Fig. 2. Changes in leaf water potential (LWP, A) and MDA (B) in the leaves of *Brassica napus* under control, drought, or drought with NaHS treatments. The data is represented as mean \pm SE for $n=3$. Means denoted by the same different letter are significantly different at the $p < 0.05$ level according to Duncan's multiple range test.

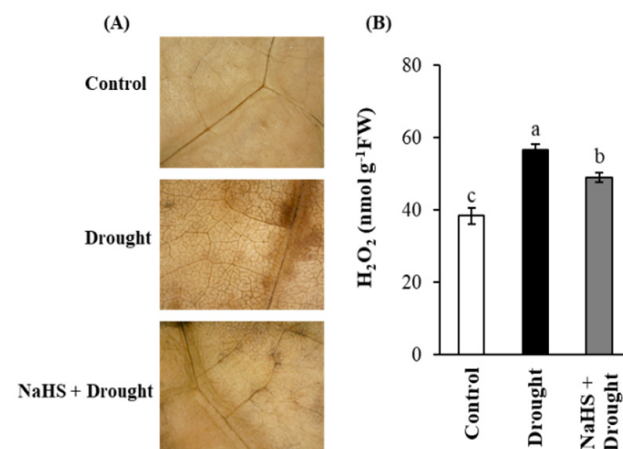


Fig. 3. Changes in H_2O_2 localization (A) and H_2O_2 content (B) in the leaves of *Brassica napus* under control, drought, or drought with NaHS treatments. The data is represented as mean \pm SE for $n=3$. Means denoted by the same different letter are significantly different at the $p < 0.05$ level according to Duncan's multiple range test.

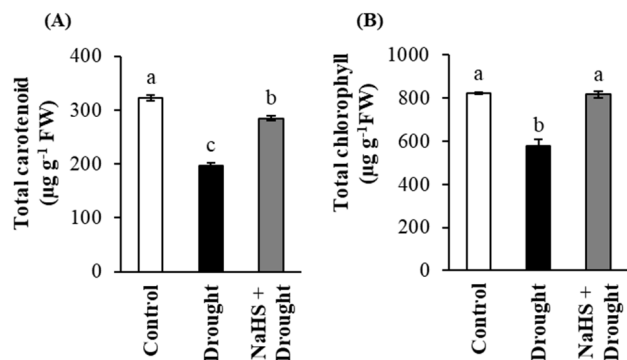


Fig. 4. Changes in the content of total carotenoid (A) and total chlorophyll (B) in the leaves of *Brassica napus* under control, drought, or drought with NaHS treatments. The data is represented as mean \pm SE for $n=3$. Means denoted by the same different letter are significantly different at the $p < 0.05$ level according to Duncan's multiple range test.

5. Proline and P5C content

Drought stress upregulated P5C content ($0.467 \mu\text{mol g}^{-1}$ FW) significantly higher compared to control. A similar response was found in NaHS-treated plants with P5C content ($0.479 \mu\text{mol g}^{-1}$ FW) significantly higher compared to control, but no significant difference compared to drought-stressed plants (Fig. 5A). Proline content is upregulated under drought stress ($125.39 \mu\text{g g}^{-1}$ FW) and much higher in NaHS treated plants ($166.31 \mu\text{g g}^{-1}$ FW) (Fig. 5B).

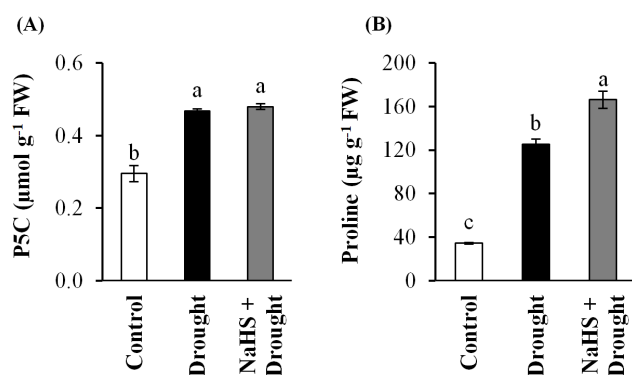


Fig. 5. Changes in the content of pyrroline-5-carboxylate (P5C, A) and proline (B) in the leaves of *Brassica napus* under control, drought, or drought with NaHS treatments. The data is represented as mean \pm SE for $n=3$. Means denoted by the same different letter are significantly different at the $p < 0.05$ level according to Duncan's multiple range test.

IV. DISCUSSION

Drought stress-induced oxidative damage in plant cells triggers protective responses to maintain cellular homeostasis by producing antioxidants and osmoprotectants (Hasanuzzaman and Fujita, 2022). Proline is widely recognized as an osmoprotectant involved in plant stress responses, with its accumulation playing a role in drought tolerance (La et al., 2020). However, proline accumulation can shift from being a hypersensitive response to serving as a stress tolerance parameter. In the present study, drought-induced stress symptoms were alleviated by NaHS treatment through additional proline accumulation. Moreover, NaHS-treated plants promote plant growth under drought conditions.

In this study, drought stress significantly decreased the leaf water potential compared to the control. In contrast, NaHS increased leaf water potential by 63% compared to drought (Fig. 2A). The reduction in leaf water potential caused by water deficit has proven to be a water stress indicator such as drought (Azhar et al., 2018; Park et al., 2021). Drought-induced MDA and H_2O_2 accumulation were alleviated by the addition of NaHS (Figs. 2B and 3A-B). NaHS may play an important role in alleviating drought stress by reducing stress symptoms and ROS accumulation. Moreover, drought inhibits plant growth by reducing chlorophyll and carotenoid content, while NaHS-treated plants induce chlorophyll and carotenoid production under drought (Fig. 4A and B). Drought stress can inhibit plant growth by reducing nutrient uptake, leading to elemental deficiencies and decreased photosynthesis activity (Salehi and Bakhshayeshan, 2016; Yang et al., 2021a).

Proline is well-documented as a cell membrane protector from oxidative stress damage caused by environmental stress, particularly drought (Yang et al., 2021b). Proline accumulation induces antioxidant activity and minimizes cell damage (Szabados and Savore, 2010). Several studies have also reported that proline helps plants maintain intercellular water content under water deficit and stabilizes plant cell membranes to maintain cellular integrity, functions, and structure. Proline can act as both a stress marker indicator and a tolerance mechanism (Venekamp et al., 2006; Szabados and Savore, 2010; Rehman et al., 2021; Yang et al., 2021b). In the present study, drought stress induced proline accumulation compared to control, while much higher proline content was observed in NaHS-treated plants (Fig. 5B). NaHS

may be involved in proline production, which in turn reduces H₂O₂ and MDA levels, further alleviating drought stress symptoms. Additionally, the elevated proline levels in NaHS-treated plants may be associated with an increase in chlorophyll and carotenoid levels, thereby improving growth under stress conditions. According to Banerjee and Roychoudhury (2021), proline production is likely a plant defense mechanism and induces plant stress tolerance. Proline accumulation is mainly regulated by key biosynthesis enzymes, pyrroline-5-carboxylate synthase (P5CS). Recent studies reported that the overexpression of P5CS in tobacco plants increases proline content, leading to osmotic stress tolerance in plants (Julia et al., 2014). In the present study, NaHS-induced P5C content further contributed to proline production, promoting drought tolerance.

In conclusion, NaHS, an H₂S donor, alleviated drought-induced stress symptoms through the induction of additional proline accumulation. This study provides evidence of the importance of proline accumulation in drought stress tolerance, particularly in *Brassica napus*. NaHS-induced proline accumulation reduces oxidative stress, thereby enhancing plant growth under drought-stress conditions. Moreover, this study provides insight into drought stress tolerance in forage plants, which in turn supports sustainable agricultural practices and sustaining livestock production. In addition to improving drought tolerance for forage crops, NaHS applications could be an effective strategy to boost agricultural productivity in water-deficit areas.

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VI. REFERENCES

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