Journal of the Korean Society of Grassland and Forage Science 43(1): 56~61, 2023
https://doi.org/10.5333/KGFS.2023.43.1.56

ISSN 2287-5824 (Print)
ISSN 2287-5832 (Online)

Research Article

Hydrogen Sulfide Alleviates Seed Germination Inhibition in Oilseed Rape (*Brassica napus* L.) Under Salt Stress

Muchlas Muchamad[1], Bok-Rye Lee[1,2], Sang-Hyun Park[1], Tae-Hwan Kim[1,*]

[1]Department of Animal Science, Institute of Agricultural Science and Technology, College of Agriculture & Life Science, Chonnam National University, Gwangju 61186, Republic of Korea

[2]Institute of Environmentally-Friendly Agriculture (IEFA), Chonnam National University, Gwangju 61186, Republic of Korea

ABSTRACT

The germination process is critical for plant growth and development and it is largely affected by environmental stress, especially salinity. Recently, hydrogen sulfide (H_2S) is well known to act as a signaling molecule in a defense mechanism against stress conditions but poorly understood regulating seed germination. In this study, the effects of NaHS (the H_2S donor) pretreatment on various biochemical (hydrogen peroxide (H_2O_2) content and amylase and protease activity) and physiological properties (germination rate) during seed germination of oilseed rape (*Brassica napus* L. cv. Mosa) were examined under salt stress. The seed germination and seedling growth of oilseed rape were inhibited by NaCl treatment but it was alleviated by NaHS pretreatment. The NaCl treatment increased H_2O_2 content leading to oxidative stress, but NaHS pre-treatments maintained much lower levels of H_2O_2 in germinating seeds under salt stress. Amylase activity, a starch degradation enzyme, significantly increased over 2-fold in control, NaHS pretreatment, and NaHS pretreatment under NaCl during seed germination compared to NaCl treatment. Protease activity was highly induced in NaHS-pretreated seeds compared to NaCl treatment, accompanied by a decrease in protein content. These results indicate that NaHS pretreatment could improve seed germination under salt stress conditions by decreasing H_2O_2 accumulation and activating the degradation of protein and starch to support seedling growth.

(Key words: Germination, Hydrogen sulfide, Oilseed rape, Salt stress)

Ⅰ. INTRODUCTION

Oilseed rape (*Brassica napus* L.) is an important oil crop that is widely cultivated around the world. However, its growth and productivity are severely affected by various environmental stresses including salt stress, which reduces seed germination (Kumar et al., 2020). Salinity conditions during seed imbibition increase the osmotic potential and inhibit water absorption, which is a critical process for germination, seedling establishment, and overall plant growth (Bentsink, 2018). Salinity can be harmful to seed germination by causing toxicity to the embryo due to elevated levels of sodium and chloride ions. It while also disrupts the normal cellular processes of plants and causing damage to cellular components through the accumulation of reactive oxygen species (ROS) induced by oxidative stress (Daszkowska-Golec, 2011; Goud and Kachole, 2011).

Hydrogen sulfide (H_2S) is a gas that occurs naturally in plants. H_2S functions as a signaling molecule modulating various plant processes such as root organogenesis, seed germination, stomata movement, transpiration, and senescence (Liu et al., 2019; Zhang et al., 2020; Zhou et al., 2020). In recent years, there has been increasing interest in investigating the application of NaHS, a donor of H_2S, to mature plants can improve their ability to endure different abiotic stresses, including drought (Zhou et al., 2020), extreme temperature (Pan et al, 2020), and high salt (Kaya et al., 2020). H_2S has been shown to mitigate the negative impact of salt stress on plant growth and development by reducing ROS accumulation and increasing antioxidant activity (Shen et al., 2013; Guo, 2018), positively affect on seed germination under salt stress. In addition, salt-stressed rice plants in H_2S application display higher antioxidants such as ascorbate, glutathione, and redox potential (Mostofa et al., 2015). Although H_2S has been reported to involve plant resistance mechanisms, the mechanisms of NaHS application to improve germination in oilseed rape

*Corresponding author: Tae-Hwan Kim, Department of Animal Science, Institute of Agricultural Science and Technology, College of Agriculture & Life Science, Chonnam National University, Gwangju 61186, Republic of Korea
Tel: +82-62-530-2126, E-mail: grassl@chonnam.ac.kr

under salt stress have not been fully elucidated.

Therefore, in the present study, the protein content, amylase and protease activity, and H_2O_2 accumulation were carried out to unveil the effects of H_2S pretreatment on oxidative stress induced by salt stress during seed germination of oilseed rape. The study will provide insights into the role of H_2S in regulating oxidative stress and improving seed germination under salt stress conditions, which could contribute to developing more salt-tolerant crops and sustainable agriculture practices.

Ⅱ. MATERIALS AND METHODS

1. Seed material and treatments

The seeds of oilseed rape (*Brassica napus* L. *cv.* Mosa) were sterilized with 70% EtOH for 3 min and washed with distilled water. The seeds were primarily divided into two groups: one group was immersed in water, and the other group was pre-treated with 500μM NaHS for 12 hours. The seeds were then allocated into Petri dish (9 cm in diameter and 1.2 cm in depth) and placed in an incubator at 28℃. To investigate the inhibitory effect of salt stress on seed germination, the water-treated group was divided into two subgroups: water (control) and 100 mM NaCl solution (NaCl group). The NaHS-pretreated group were also divided into two subgroups: one was watered with normal water (NaHS group), and the other was watered with 100 mM NaCl (NaHS+NaCl group). Samples were collected at 0, 12, 48, and 96 hours for analysis.

2. Determination of seed germination percentage and root length

The germination percentage of the seeds of oilseed rape was calculated as described previously (Samani et al., 2013). Germination percentage = S/T × 100 (S is the number of germinated seeds and T is the total number of seeds). The seeds were considered as germinated when the sprout's length reached or exceeded the half length of the seeds. Root length was measured using a ruler and expressed in millimeters.

3. Determination of H_2O_2 content

For H_2O_2 determination, a 100 mg seed was extracted with potassium phosphate (50 mM, pH 6.8) solution. The extracted solution was combined (1:1) with 0.1% titanium chloride in 20% (v/v) H_2SO_4 and centrifuged at 10,000 × g for 5 min. The absorbance of the supernatant was recorded at 410 nm. The H_2O_2 level was calculated by using an extinction coefficient of 0.28 μmol^{-1} cm^{-1}.

4. Determination of protein content, and amylase and protease activity

For measurement of protein content, seeds were extracted with 50 mM K-phosphate buffer (pH 7.8) and incubated for 30 min. Then the sample was centrifuged 12,000 × g for 10 min at 4℃. The supernatant was added (1:1) with Bradford solution, and the absorbance was measured at 595 nm.

For amylase activity checking, the seeds were crushed using homogenization in a 100 mM sodium acetate buffer (pH 5.5) and centrifuged at 12,000 × g for 10 min under 4℃. Then the supernatant was added with 1% soluble starch and incubated at 37℃ for 5 min. After incubation, DNS color reagent was added and left at boiling temperature for 5 min. The solution was kept at room temperature until cool down. Then absorbance was taken at 540 nm.

The protease was assayed according to the method described by Beyene (2006) with modifications. The 100 mg seeds were homogenized with of extraction medium (50 mM Tris-HCl, pH 9). The homogenate was centrifuged at 12,000 × g for 10 min at 4℃. The supernatant was added with 2% (m/v) azocasein which was dissolved in 50 mM Tris-HCl buffer (pH 9.0) and incubated at 37℃ for 30 min. An equal volume of ice-cold 10% (v/v) trichloroacetic acid (TCA) was added to the mixture solution.Uncleavedin was precipitated at 4℃, then was centrifuged at 12,000 × g for 10 min. The supernatant was added with 1 M NaOH, and the absorbance was taken at 440 nm.

5. Statistical analysis

The current experiment was conducted with a completely randomized design of three replications for each sampling day. Duncan's multiple range test was used to compare the means of separate replicates for each treatment day. The differences in letters were considered statistically significant at $p<0.05$. For statistical analysis, SAS 9.1.3 was used (SAS Institute Inc. in Cary, NC, United States).

Ⅲ. RESULTS

1. H_2S pretreatment effects on the seed germination of oilseed rape under salt stress

Seed germination was prominently inhibited by NaCl (100 mM) treatment (Table 1 and Fig. 1). Noticeably, salinity (NaCl) causes a delayed emergence of both shoot and root, compared with control plants (Table 1). NaHS pretreatment improved seed germination and root development compared to the other treatments. NaHS pretreatment alleviated the reduction of seed germination and root growth along with NaCl.

2. H_2S pretreatment effects on H_2O_2 contents during seed germination under salt stress

H_2O_2 content in control was gradually decreased during seed germination. H_2O_2 content was prominently increased with NaCl treatment along with restricted seed germination (Figs. 1 and 2) at 48 h and 96 h. However, NaCl along with NaHS pretreatment showed a lower H_2O_2 content at 48 h compared to NaCl treatment and a similar to control at 96 h (Fig. 2).

Table 1. Effect of NaHS pretreatment on seed germination of *Brassica napus* under salt stress.

Hours	Treatments	Germination percentage (%)	Root length (cm)
0	Control	0.00 + 0.00	0.00 + 0.00
12	Control	0.00 + 0.00	0.00 + 0.00
	NaHS	0.00 + 0.00	0.00 + 0.00
48	Control	$41.3 + 0.72^{b}$	$1.43 + 0.01^{a}$
	NaCl	$15.0 + 1.41^{d}$	$0.12 + 0.00^{c}$
	NaHS	$49.0 + 1.24^{a}$	$1.46 + 0.01^{a}$
	NaHS+NaCl	$35.7 + 1.52^{c}$	$0.36 + 0.02^{b}$
96	Control	$95.3 + 0.72^{a}$	$2.63 + 0.03^{b}$
	NaCl	$36.0 + 4.90^{c}$	$0.87 + 0.05^{d}$
	NaHS	$96.7 + 0.72^{a}$	$2.94 + 0.02^{a}$
	NaHS+NaCl	$73.0 + 0.47^{b}$	$1.73 + 0.00^{c}$

The data were presented as mean ± SE (n = 3). The different letters in the column at each sampling date are significantly different (p<0.05) according to Duncan's multiple range test.

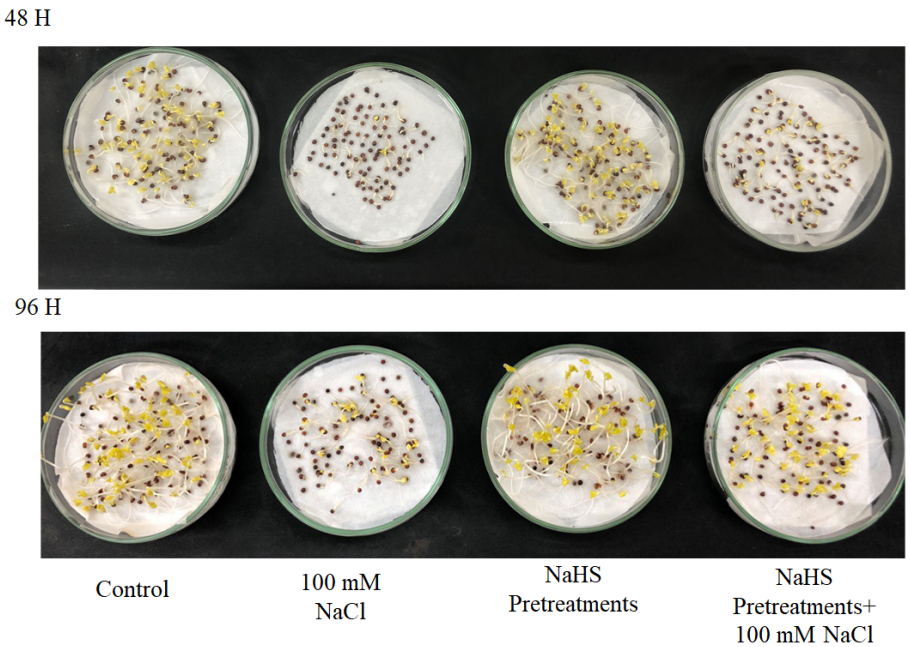

Fig. 1. Seed germination of *Brassica napus* during 96 h after NaHS pretreatment under salt stress.

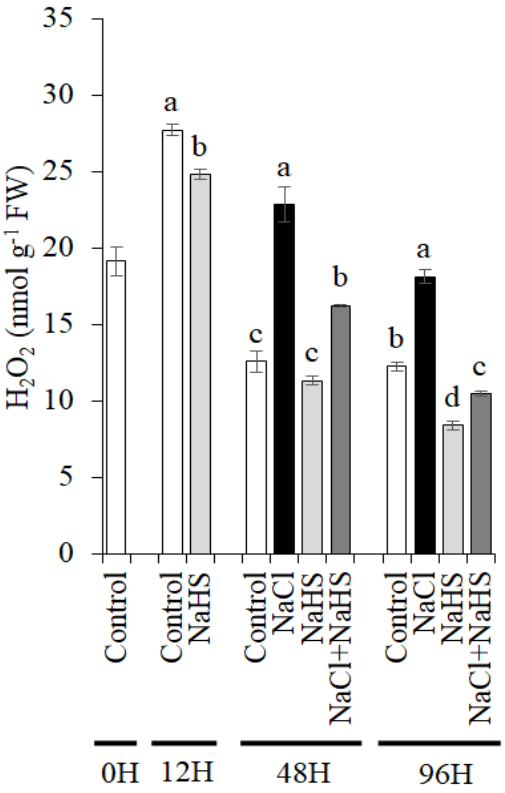

Fig. 2. Effects of NaHS pretreatment on H_2O_2 concentration during seed germination under salt stress. The data was presented as mean ± SE (n = 3). Bars labeled with the different letters are significantly different (p<0.05) according to Duncan's multiple range test.

3. Effects of NaHS pretreatment on the activities of amylase and protease, and protein content during seed germination under salt stress

Amylase and protease activities were remarkably increased in control and NaHS-pretreated plants under normal conditions. It was slightly or not increased in NaCl-treated plants but largely enhanced by NaHS pretreatment (Fig. 3A and B). Protease activity was inversely consistent with protein content during seed germination. Protein content in control and NaHS-pretreated plants gradually decreased under normal conditions as seeds were germinated (Fig. 3C). NaCl treatment maintained a higher level of protein content but less in NaHS-pretreated plants.

Ⅳ. Discussion

Numerous studies have reported the positive effects of H_2S on seed germination rate, seedling growth, and root length. Nevertheless, the underlying mechanism of H_2S to promote seed germination is still poorly elucidated. In the present study, NaHS was used as an H_2S donor. We confirmed that NaHS pretreatment alleviated the reduction of seed germination induced by salt stress.

In this study, salt stress caused by NaCl treatment

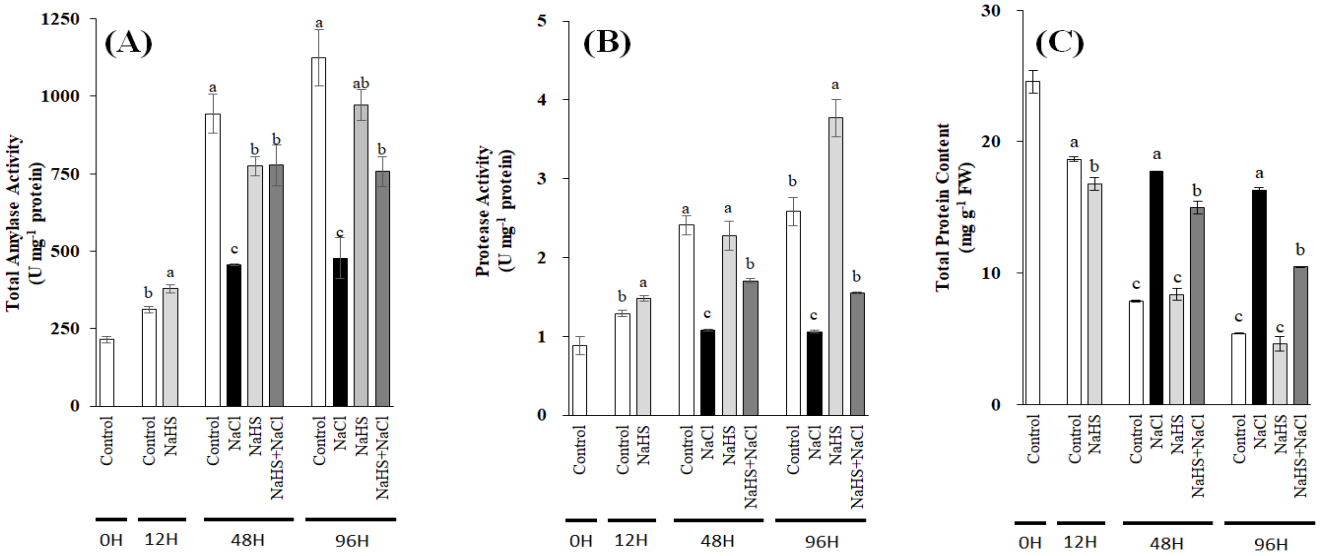

Fig. 3. Effects of NaHS pretreatment on total amylase activity (A), protease activity (B), and total protein content (C) during seed germination under salt stress. The data were presented as mean ± SE (n = 3). Bars labeled with the different letters are significantly different (p<0.05) at each sampling date according to Duncan's multiple range test.

significantly reduced seed germination and H_2O_2 accumulation (Fig. 1A and Table 1). These findings are consistent with other observations that high concentration of salt not only has toxic effects on specific ions but also decreases the water potential in the medium, making it difficult for germinating seeds to absorb water, ultimately leading to a reduction in germination (Tanyolac et al., 2007). However, NaHS-pretreated seeds were able to offset the inhibitory impact of salt stress on the germination process (Fig. 1A and Table 1) as well as H_2O_2 production (Fig. 2), indicating the positive effects of NaHS pretreatment on salt stress. The alterations in H_2O_2 levels that H_2S induced at lower concentrations may be linked to the activation of antioxidant systems that are relyon H_2S. Similar to our findings, salt stress response of H_2S were previously reported in alfalfa (Lai et al., 2014) and rice (Mostofa et al., 2015), with the increase of superoxide dismutase, glutathione reductase, and peroxidase activities. It has been suggested that H_2S triggers antioxidants by increasing glutathione and ascorbate levels, thereby increasing redox potential (Mostofa et al., 2015). These results indicate that H_2S plays a crucial role in the mechanisms that enable plants to withstand salt stress-induced oxidative stress during seed germination.

It has been reported that storage metabolites such as starch and proteins in the aleurone layers of seeds are hydrolyzed by amylase and protease enzymes to provide nutrients for seedling growth and development (Rahman et al., 2007). Previous physiological and biochemical studies have revealed that hydrolyzing starch increases metabolizable sugars by amylase promoted by gibberellin (Beck and Ziegler, 1989; Gulber et al., 1995). In this study, amylase and protease activities were gradually increased in germinating seeds (Fig. 3A and B), accompanied by a decrease in protein content (Fig. 3C) which provide amino acids to the seedlings before the onset of autotrophic growth (Zafar et al., 2005). Salt stress maintained protein content at a high level, concomitant with lower protease activity, leading to a delay in germination and seedling growth. However, NaHS pretreatment enhanced amylase and protease activities along with a decrease in protein content under salt stress. A similar pattern of changes was observed in barley aleurone layers of seeds in the presence of H_2S (Zhang et al., 2015). The results suggest that NaHS-induced increase of amylase and protease activity alleviates the seed germination delay induced by salt stress.

In conclusion, NaHS pretreatment could improve the germination of oilseed rape seeds exposed to salt stress. NaHS pretreatment significantly promoted oilseed rape seed germination and root growth, increased the activities of amylase and protease, restored the plasma membrane integrity in root tips damaged by osmotic stress, and reduced the accumulation of H_2O_2 in the presence of salt stress. The findings suggest that H_2S may serve as a novel antioxidant signal during oxidative stress, a function not previously observed in oilseed rape seeds.

Ⅴ. ACKNOWLEDGMENTS

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2022R1I1A3072357).

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(Received : March 23, 2023 | Revised : March 28, 2023 | Accepted : March 28, 2023)