Research article

Ecology Journal

ISSN (Print): 2957-4471, ISSN (Online): 2708-6364, Website: https://journal.esbangladesh.org
Ecol. J. (2022) 4 (1) : 79-89

EFFECTS OF SODIUM HYDROSULFIDE ON PHYSIOLOGICAL RESPONSES IN A SALT-TOLERANT SOYBEAN GENOTYPE 'AGS313' UNDER SALINE CONDITIONS

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Received: 17 May 2022, Revised: 28 May 2022, Accepted: 05 June 2022

ABSTRACT

Hydrogen sulfide (H_2S) has been recently found to be a signaling molecule to environmental stress tolerance. This study was conducted to investigate the effects of irrigation (Experiments 1 and 2) and foliar application (Experiment 3) of sodium hydrosulfide (NaHS), as a H_2S donor, under NaCl stress. A Bangladeshi salt-tolerant soybean genotype 'AGS313' was compared with a salt-sensitive Japanese variety 'Fukuyutaka'. While the shoot dry weight was improved by NaHS application in the experiment 1, the vegetative growth was not improved by NaHS application in the experiments 2 and 3. NaHS irrigation decreased leaf Cl content, while NaHS foliar application increased photosynthetic rate. These results showed that NaHS application had slightly positive effects on soybean under NaCl stress conditions.

Keywords: *Glycine max*, oxidative stress, photosynthetic rate, sodium chloride, stomatal conductance

Introduction

Salinity is an environmental stress that can greatly reduce crop productivity. Salinity can occur under all climatic conditions, and is relatively more common in arid, semi-arid, and coastal areas. The coastal area in Bangladesh is the main soybean (*Glycine max* L.) production area, where salinity threats economic crop production (Mannan *et al*. 2013). In Bangladesh, Mannan *et al*. (2012) evaluated the salt tolerance of 170 soybean genotypes and 'AGS313', the salt-tolerant genotype, has been introduced in salinity area (Karim *et al*. 2012). Cultivation methods to reduce salinity have also been studied (Fujii and Higuchi 2021), but are not yet fully established. Under salt stress, plants are subjected to osmotic stress due to high salinity in the rhizosphere and ionic stress due to the accumulation of excess Na and Cl (Munns and Tester 2008). As a result, reactive oxygen species (ROS) such as O^{2-} and H_2O_2 are generated. Excessive ROS accumulation damages to proteins, DNA, lipids, membrane tissues, and enzyme activity.

In recent years, hydrogen sulfide (H_2S) has gained attention as a gaseous signaling molecule in the response of plants to biotic and abiotic stresses, like nitric oxide and carbon monoxide (da-Silva and Modolo 2017). H_2S was reported to be involved in physiological processes such as blood pressure regulation, anti-inflammatory effects, and vasorelaxation in mammalian studies (Wagner 2009). As the effects of H_2S, activation of photosynthesis (Ali *et al*. 2014, Chen *et al*. 2011, Duan *et al*. 2015), stomatal opening and closure (Papanatsiou *et al*. 2015, Jin *et al*. 2017, Shen *et al*. 2020), seed germination (Zhang *et al*. 2008), root formation (Zhang *et al*. 2009, Jia *et al*. 2015, Mei *et al*. 2019), and fruit maturation (Ge *et al*. 2017, Munoz-Vargas *et al*. 2018) have already been reported. Even under salt stress, the activation of antioxidant systems (Yu *et al*. 2013, Christou *et al*. 2013, Shan *et al*. 2014), maintenance of K/Na (Chen *et al*. 2015, Lai *et al*. 2014, Deng *et al*. 2016), activation of photosynthesis (Christou *et al*. 2013, Mostofa *et al*. 2015), and accumulation of osmotic regulators (Sun and Luo, 2014, Shi *et al*. 2013) have also been reported.

To understand the effectiveness of H_2S application on soybean growth under saline stress conditions and the saline stress mitigating mechanism of H_2S application, information on responses of leaf mineral contents and physiological parameters, such as relative chlorophyll content (SPAD), photosynthetic rate, stomatal conductance and maximum quantum efficiency of PSII photochemistry (Fv/Fm), would presumably be useful. Thus, the effects

of sodium hydrosulfide (NaHS) irrigation, as a H_2S donor, under different NaCl stress, such as 100 mM (Experiment 1) and 50 mM (Experiment 2) stress, and those of foliar application by spray under 150 mM NaCl stress (Experiment 3) on vegetative growth, leaf mineral contents, and physiological responses, were investigated in a salt tolerant soybean genotype 'AGS313'. In the experiments 2 and 3, salt-sensitive variety 'Fukuyutaka' was also used as a comparison with 'AGS313'.

Materials and Methods

Experiment 1

Thirty-two salt-tolerant soybean plants 'AGS313' were grown in a greenhouse at Kyoto University (35.0 °N, 135.8 °E). 'AGS313' was reported to be a salt tolerant soybean genotype (Karim *et al.* 2012, Fujii and Higuchi, 2019). The seeds were sown in plastic pots on March 26, 2021. The diameter and height of the pots were 12 cm and 10 cm, respectively. It was filled with expanded vermiculite for germination. After germination, the plants were transplanted to 4 L plastic pots filled with 1: 1 mixture of sandy soil and bark compost by volume. During the experiment, 600 mL of nutrition solution containing NH_4-N: 0.7 mM, NO_3-N: 8 mM, K: 4 mM, P: 0.7 mM, Ca: 2 mM, Mg: 1 mM, S: 1 mM was applied three times a week. One time of these applications, micronutrients (Fe: 57 μM, Cu: 0.6 μM, Zn: 0.9 μM, Mn: 9.9 μM, B: 47.2 μM, Mo: 0.08 μM) were added.

When the 5th leaf emerged, on May 10, the 32 plants were divided into 4 groups with 8 replications. Each plant was applied with 600 mL of tap water (Control treatment) or the solution, containing 100 mM NaCl (NaCl treatment), 100 mM NaCl + 0.1 mM NaHS (0.1 mM NaHS treatment), or 100 mM NaCl + 0.2 mM NaHS (0.2 mM NaHS treatment), twice a day on sunny days and 0 to 1 time a day on cloudy or rainy days instead of irrigation water. To prevent severe NaCl damage due to a sudden NaCl increase, NaCl concentration in the solutions was increased by 20 mM every day from May 10 to 14. From May 14, 100 mM NaCl was applied for 18 days.

Plant height, number of leaves, relative chlorophyll content (SPAD), and maximum quantum efficiency of PSII photochemistry (Fv/Fm) were measured on May 10, 20, 26, and June 1. The SPAD was measured five times for each plant and the average values were recorded, using a chlorophyll meter (SPAD-502, Konica Minolta Sensing Inc., Japan). Fv/Fm was measured three times for each plant and the average values were recorded, using a chlorophyll fluorometer (OS-30P, Opti-Sciences, Inc., USA) under a dark condition at 2100-2200 h. Photosynthetic rate and stomatal conductance were measured on May 10, 26, and June 1. Photosynthetic rate was measured at 1000-1200 h, using a photosynthetic rate measurement system (MIC-100, Masa International Corp., Japan). The measurement conditions of photosynthetic rate were as follows: PAR 1200 μmol/m^2/s, stabilization time 3 sec, measurement start CO_2 concentration 400 ppm, and measurement CO_2 span 20 ppm. When the measurement was not finished within 60 second, it was regarded as below the lower limit of measurement. Stomatal conductance was measured using a SC-1 leaf porometer system (SC-1, Meter Group, Inc., USA). The 3rd- 5th youngest leaves were used for the measurements of photosynthetic rate and stomatal conductance. After the end of treatment, the plants were sampled on July 4 and oven-dried at 70 °C for 3 days. Then the shoot dry weight and root dry weight were measured. To determine leaf Na, K, Ca, Mg, and P contents, the leaves sampled on May 27 and June 4 were oven-dried at 70 °C for 3 days and grounded. After wet-ashing with nitric acid, Na, K, Ca, Mg, and P contents were determined using an inductively coupled plasma spectrophotometer (ICPE-9000, Shimadzu Corp., Japan). To determine the Cl and NO_3 contents in leaves, those were extracted from grounded leaves with 40 °C water. The Cl concentration was measured using a Cl ion electrode (CL-2021, DKK-TOA Corp., Japan) with ion/pH meter (IM-22P, DKK-TOA Corp., Japan), and the NO_3 concentration was determined using the method of Cataldo *et al*. (1975). Soil water content at a depth of 0 to 5 cm was measured at 1500-1530 h on June 1, using a soil moisture meter (EC5, Meter Group, Inc., USA).

Experiment 2

Two soybean varieties, 'AGS313' and 'Fukuyutaka', were sown on April 24, 2021, transplanted on June 10, and cultivated in the same way as in the experiment 1. When the 5th leaf emerged, on June 23, the 30 plants were divided into 3 groups with 10 replications for each variety. Each plant was applied with 600 mL of tap water

(Control treatment) or solutions containing 50 mM NaCl (NaCl treatment) or 50 mM NaCl + 0.1 mM NaHS (NaHS treatment) twice a day on sunny days and 0 to 1 time a day on cloudy or rainy days instead of irrigation water. NaCl concentration in the solutions was increased by 10 mM every day from June 23 to June 27. From June 27, 50 mM NaCl was applied for 26 days. Plant height, number of leaves, SPAD, Fv/Fm, and length and width of middle leaflet at the 3rd youngest leaf were measured on July 22. The area of middle leaflet at the 3rd youngest leaf was estimated using the following calibration equations, which was established in advance for each variety. A, L, and W denote area, length, and width of middle leaflet.

AGS313: $A = 0.62\ LW + 1.42$ $r^2 = 0.99$ *** (n = 100)

Fukuyutaka: $A = 0.64\ LW + 1.83$ $r^2 = 0.99$ *** (n = 100)

Photosynthetic rate and stomatal conductance were measured on July 21 at 1200-1330 h using the same devices and methods in the experiment 1. After the end of treatment, the plants were sampled on July 24 and oven-dried at 70 °C for 3 days. Then the shoot dry weight and root dry weight were measured.

Leaf mineral contents were measured in the same way as in the experiment 1. The leaves were sampled on July 24. On the same day, 50 mg of fresh leaves for each plant was sampled. Fresh leaf was soaked to 10 mL of distilled water and incubated in an incubator at 28 °C for 2 hours. Then electrical conductivity (EC1) was measured using a conductivity meter (LAQUAtwin, HORIBA Advanced Techno, Co., Ltd., Japan). After the measurement, the sample was incubated at 100 °C for 20 min. After cooling to room temperature, electrical conductivity (EC2) was measured again. Then electrolyte leakage (EL: EC1/EC2 × 100) was calculated.

Experiment 3

Two varieties, 'AGS313' and 'Fukuyutaka', were sown on July 20, 2021, transplanted on August 5, 2021, and cultivated in the same way as in the experiments 1 and 2. When the 2nd leaf emerged, on August 12, the 27 plants were divided into 2 groups with 9 and 18 plants for each variety, 10 mL NaHS solution was sprayed to leaves for 9 plants (NaHS treatment) and 10 mL of tap water was sprayed for the rest 18 plants every day. From August 15, 600 mL of 150 mM NaCl solutions was applied to the NaHS treatment and 9 of 18 plants sprayed with tap water (NaCl treatment) twice a day on sunny days and 0 to 1 time a day on cloudy or rainy days. To the rest 9 plants 600 mL of tap water was applied (Control treatment).

Plant height, number of leaves, SPAD, Fv/Fm, and length and width of middle leaflet at the 3rd and 4th youngest leaves were measured on August 24, and the area of those leaflets were calculated using the same calibration equations in the experiment 2. Photosynthetic rate and stomatal conductance were measured on August 24 at 1100-1300 h using the same devices and methods in the experiments 1 and 2. After the end of treatment, the plants were sampled on August 26 and dried at 70 °C for 3 days. Then the shoot dry weight and root dry weight were measured. Leaf mineral contents and EL were measured in the same way as in the experiment 2 using leaves sampled on August 26.

Statistical analysis

For the experiment 1, the data were analyzed by analysis of variance (ANOVA). The data with statistical differences among treatments as found by ANOVA were further analyzed by Tukey's multiple comparison tests ($p<0.05$). For the experiments 2 and 3, the data were analyzed by two-way ANOVA (variety × treatment). After that, statistical differences among treatments were subjected to further analysis using Tukey's multiple comparison tests ($p<0.05$).

Results

Experiment 1

Shoot dry weight was decreased by NaCl application and 0.1 mM NaHS application lessened the detrimental effect of the NaCl application (Table 1). Root dry weight was also decreased by NaCl application and not significantly enhanced by NaHS application. Soil water content was increased by NaCl application. Plant height and number of leaves were also decreased by NaCl application and not significantly enhanced by NaHS application (Figure 1). Photosynthetic rate and stomatal conductance were decreased by NaCl application, and not enhanced by NaHS application. SPAD was increased by NaCl application, and it was the highest in 0.1 mM NaHS on May 26. Fv/

Table 1. Effects of sodium chloride and sodium hydrosulfide application on shoot and root dry weights in 'AGS313' soybean and soil water content 22 days after treatment.

Treatment[x]	Shoot dry weight (g)	Root dry weight (g)	Soil water content (%)
Control	27.4 a	5.4 a	21.1 b
NaCl	11.6 c	2.0 b	35.5 a
0.1mM NaHS	17.8 b	2.5 b	34.2 a
0.2mM NaHS	14.6 bc	2.7 b	34.7 a

x: Each plant was applied with 600 mL of tap water (Control) or solution, containing 100 mM NaCl (NaCl), 100 mM NaCl+ 0.1 mM NaHS (0.1 mM NaHS), or 100 mM NaCl+ 0.2 mM NaHS (0.2 mM NaHS), twice a day on sunny days and 0 to 1 time a day on cloudy or rainy days instead of irrigation water. Different letters within the same column indicate statistically different at $p<0.05$ by Tukey's multiple comparison tests (n=8).

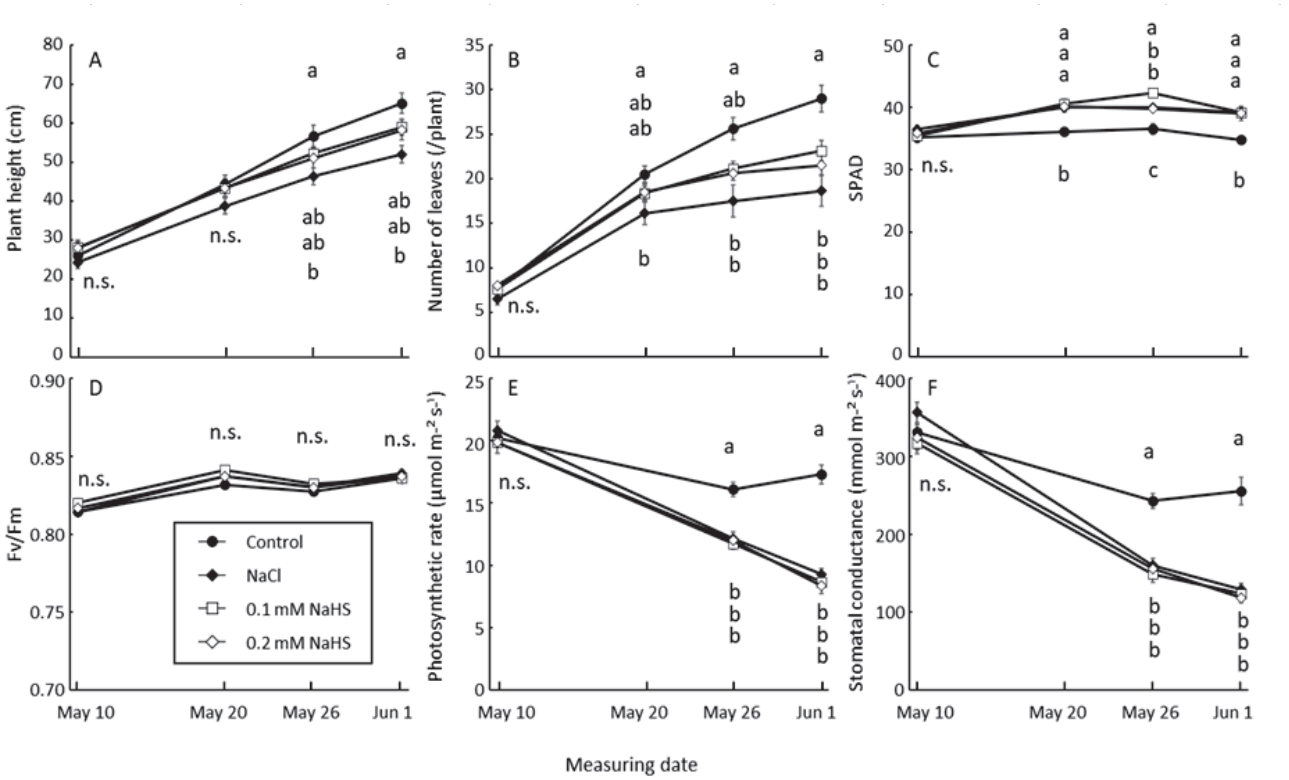

Figure 1. Effects of sodium hydrosulfide irrigation on plant height (A), number of leaves (B), SPAD (C), Fv/Fm (D), photosynthetic rate (E), and stomatal conductance (F) in 'AGS313' soybean under 100 mM NaCl. Bars indicate standard error (n=8), and different letters within the same date indicate statistically different at $p<0.05$ by Tukey's multiple comparison tests.

Fm was not affected by the application of NaCl or NaHS throughout the experiment.

Leaf Na content was not affected by the 17 day-application of NaCl or NaHS (Table 2). Leaf Cl content was increased by NaCl application and suppressed by the 17 day-application of 0.2 mM NaHS. Leaf K content was decreased by NaCl application. Leaf Ca, Mg, P, and NO_3 contents and K/Na were not affected by the 17 day-application of NaCl or NaHS. Leaf Na content was the highest in 0.2 mM NaHS application and the lowest in control 22 days after application, on June 4 (Table 3). Leaf Cl content was increased by the 22 day-application of 0.2 mM NaHS. Leaf K, Ca, Mg, and NO_3 contents were not affected by the 22 day-application of NaCl or NaHS. Leaf P content was increased by the 22 day-application of NaCl. Twenty-two days after the application, leaf K/Na was the highest in control and the lowest in 0.2 mM NaHS application.

Experiment 2

Vegetative growth, such as plant height, number of leaves, area of middle leaflet at the 3rd youngest leaf, shoot dry

Table 2. Effects of sodium chloride and sodium hydrosulfide application on leaf mineral contents in 'AGS313' soybean 17 days after treatment.

Treatment[x]	Na (mg g^{-1}D.W.)	Cl (mg g^{-1}D.W.)	K (mg g^{-1}D.W.)	Ca (mg g^{-1}D.W.)	Mg (mg g^{-1}D.W.)	P (mg g^{-1}D.W.)	NO_3 (mg g^{-1}D.W.)	K/Na
Control	0.79	7.2 c	23.8 a	13.8	4.77	4.81	24.4	33.4
NaCl	1.29	12.1 a	17.2 b	20.7	6.24	5.76	25.0	19.5
0.1mM NaHS	0.67	10.9 ab	20.3 ab	15.7	4.98	5.98	19.2	34.0
0.2mM NaHS	1.09	10.0 b	18.3 ab	23.6	7.95	5.82	23.9	21.2

x: Each plant was applied with 600 mL of tap water (Control) or solution, containing 100 mM NaCl (NaCl), 100 mM NaCl+ 0.1 mM NaHS (0.1 mM NaHS), or 100 mM NaCl+ 0.2 mM NaHS (0.2 mM NaHS), twice a day on sunny days and 0 to 1 time a day on cloudy or rainy days instead of irrigation water. Different letters within the same column indicate statistically different at $p<0.05$ by Tukey's multiple comparison tests (n=8).

Table 3. Effects of sodium chloride and sodium hydrosulfide application on leaf mineral contents in 'AGS313' soybean 22 days after treatment.

Treatment[x]	Na (mg g^{-1}D.W.)	Cl (mg g^{-1}D.W.)	K (mg g^{-1}D.W.)	Ca (mg g^{-1}D.W.)	Mg (mg g^{-1}D.W.)	P (mg g^{-1}D.W.)	NO_3 (mg g^{-1}D.W.)	K/Na
Control	2.68 b	6.8 c	17.6	10.9	3.72	3.75 b	12.6	7.9 a
NaCl	4.43 ab	13.9 b	19.9	13.7	4.77	5.32 a	10.6	4.9 ab
0.1mM NaHS	4.20 ab	13.2 b	19.5	10.3	3.84	4.58 ab	11.4	4.8 ab
0.2mM NaHS	5.34 a	16.0 a	18.9	13.7	4.65	4.71 a	12.3	3.9 b

x: Each plant was applied with 600 mL of tap water (Control) or solution, containing 100 mM NaCl (NaCl), 100 mM NaCl+ 0.1 mM NaHS (0.1 mM NaHS), or 100 mM NaCl+ 0.2 mM NaHS (0.2 mM NaHS), twice a day on sunny days and 0 to 1 time a day on cloudy or rainy days instead of irrigation water. Different letters within the same column indicate statistically different at $p<0.05$ by Tukey's multiple comparison tests (n=8).

weight, and root dry weight, were decreased by NaCl application, and NaHS application did not mitigate the detrimental effect of NaCl (Table 4).

Photosynthetic rate and stomatal conductance were decreased by NaCl application and were not improved by NaHS application (Table 5). There were interactions between variety and treatment of NaCl and NaHS applications in SPAD, Fv/Fm, and EL. SPAD and Fv/Fm of 'AGS313' were not affected by the application of NaCl or NaHS, while those of 'Fukuyutaka' were decreased by NaCl application and not increased by NaHS application. EL of 'AGS313' was not affected by NaCl application, while that of 'Fukuyutaka' was increased by NaCl application. NaHS application did not improve SPAD, Fv/Fm and EL.

Leaf Na content of 'AGS313' was not affected by the applications of NaCl or NaHS, while that of 'Fukuyutaka' was increased by NaCl application (Table 6). Leaf Cl content of 'AGS313' was not affected by the applications of NaCl or NaHS while that of 'Fukuyutaka' was increased by NaCl application and was suppressed by NaHS application. Leaf K content was decreased by NaCl application in 'AGS313' and increased in 'Fukuyutaka'. Leaf Ca content was not affected by variety and application. Leaf Mg content was increased by NaCl application. Leaf P content was increased by NaCl application in 'AGS313' and was not affected by the application of NaCl or NaHS in 'Fukuyutaka'. Leaf NO_3 content was not affected by the application. Leaf K/Na was decreased by NaCl application in both varieties.

Experiment 3

Leaf number, area of middle leaflet at the 3rd and 4th youngest leaf, shoot dry weight, and root dry weight were decreased by NaCl application and were not improved by NaHS foliar application by spray (Table 7). There was an interaction between variety and treatment of NaCl and NaHS applications in plant height, while it was decreased by NaCl application and not improved by NaHS foliar application in both varieties.

Photosynthetic rate was decreased by NaCl application, and improved by NaHS foliar application (Table 8). Stomatal conductance was also decreased by NaCl application and not improved by NaHS foliar application.

Table 4. Effects of sodium chloride and sodium hydrosulfide application on vegetative growth in 'AGS313' and 'Fukuyutaka' soybean 28 days after treatment.

Variety	Treatment[x]	Plant height (cm)	Leaf number (/plant)	Leaf area[y] (cm^2)	Shoot dry weight (g)	Root dry weight (g)
AGS313	Control	109.4	35.7	94.0	38.1	12.8
	NaCl	89.0	27.1	84.0	27.1	9.5
	NaCl+NaHS	93.8	28.0	81.1	26.7	7.9
Fukuyutaka	Control	64.0	36.1	94.2	39.3	12.2
	NaCl	59.8	31.0	82.8	23.9	6.6
	NaCl+NaHS	61.5	29.6	75.7	22.8	7.0
Average						
AGS313		97.4 a	30.3	84.2	30.6	10.1 a
Fukuyutaka		61.8 b	32.2	86.4	28.7	8.6 b
	Control	86.7 a	35.9 a	94.1 a	38.7 a	12.5 a
	NaCl	74.4 b	29.1 b	83.4 b	25.5 b	8.0 b
	NaCl+NaHS	77.7 ab	28.8 b	78.4 b	24.8 b	7.5 b
Variety［V］		**	n.s.	n.s.	n.s.	**
Treatment［T］		*	**	**	**	**
［V］×［T］		n.s.	n.s.	n.s.	n.s.	n.s.

x: Each plant was applied with 600 mL of tap water (Control) or solutions containing 50 mM NaCl (NaCl) or 50 mM NaCl + 0.1 mM NaHS (NaCl+NaHS) twice a day on sunny days and 0 to 1 time a day on cloudy or rainy days instead of irrigation water. y: Leaf area was measured at middle leaflet of the third youngest leaf. **, *, and n.s. indicate significant differences at $p<0.01$, $p<0.05$, and not significant, respectively by two-way ANOVA. Different letters within the same column indicate statistically different at $p<0.05$ by Tukey's multiple comparison tests (n=10).

Table 5. Effects of sodium chloride and sodium hydrosulfide application on physiological responses in 'AGS313' and 'Fukuyutaka' soybean 28 days after treatment.

Variety	Treatment[x]	Photosynthetic rate ($\mu mol\ m^{-2}\ s^{-1}$)	Stomatal conductance ($mmol\ m^{-2}\ s^{-1}$)	SPAD	Fv/Fm	Electrolyte leakage (%)
AGS313	Control	18.9	553	39.9 a	0.84 a	9.6 a
	NaCl	16.4	403	40.8 a	0.84 a	10.8 a
	NaCl+NaHS	16.6	480	40.9 a	0.84 a	9.9 a
Fukuyutaka	Control	17.0	595	39.7 a	0.84 a	14.8 b
	NaCl	8.6	283	30.3 b	0.80 b	28.8 a
	NaCl+NaHS	9.9	247	29.3 b	0.81 b	31.9 a
Average						
AGS313		17.3 a	479	40.5	0.84	10.1
Fukuyutaka		11.8 b	375	33.1	0.82	25.2
	Control	18.0 a	574 a	39.8	0.84	12.2
	NaCl	12.5 b	343 b	35.5	0.82	19.8
	NaCl+NaHS	13.2 b	363 b	35.1	0.82	20.9
Variety［V］		**	n.s.	**	**	**
Treatment［T］		**	**	**	**	**
［V］×［T］		n.s.	n.s.	**	**	**

x: Each plant was applied with 600 mL of tap water (Control) or solutions containing 50 mM NaCl (NaCl) or 50 mM NaCl + 0.1 mM NaHS (NaCl+NaHS) twice a day on sunny days and 0 to 1 time a day on cloudy or rainy days instead of irrigation water. **, *, and n.s. indicate significant differences at $p<0.01$, $p<0.05$, and not significant, respectively by two-way ANOVA. Different letters within the same column indicate statistically different at $p<0.05$ by Tukey's multiple comparison tests (n=10).

Table 6. Effects of sodium chloride and sodium hydrosulfide application on leaf mineral contents in 'AGS313' and 'Fukuyutaka' soybean 30 days after treatment.

Variety	Treatment[x]	Na (mg g^{-1}D.W.)	Cl (mg g^{-1}D.W.)	K (mg g^{-1}D.W.)	Ca (mg g^{-1}D.W.)	Mg (mg g^{-1}D.W.)	P (mg g^{-1}D.W.)	NO_3 (mg g^{-1}D.W.)	K/Na
AGS313	Control	1.29 a	5.1 a	13.0 a	18.6	5.21	2.56 c	11.3	10.1
	NaCl	1.41 a	6.5 a	9.3 b	21.3	6.08	3.10 b	10.1	6.8
	NaCl+NaHS	1.34 a	6.1 a	9.2 b	22.3	6.10	3.61 a	9.0	7.0
Fukuyutaka	Control	0.72 b	7.3 c	13.4 b	18.8	4.73	3.37 a	13.7	20.1
	NaCl	1.51 a	19.3 a	17.1 a	22.3	6.32	2.97 a	15.3	14.6
	NaCl+NaHS	1.21 ab	13.9 b	16.8 a	21.0	6.13	3.22 a	15.0	15.4
Average									
AGS313		1.35	5.9	10.5	20.7	5.80	3.09	10.1 b	8.0 b
Fukuyutaka		1.15	13.5	15.8	20.7	5.73	3.19	14.7 a	16.7 a
	Control	1.00	6.2	13.2	18.7	4.97 b	2.96	12.5	15.1 a
	NaCl	1.46	12.9	13.2	21.8	6.20 a	3.04	12.7	10.7 b
	NaCl+NaHS	1.27	10.0	13.0	21.6	6.12 a	3.42	12.0	11.2 b
Variety［V］		n.s.	**	**	n.s.	n.s.	n.s.	**	**
Treatment［T］		**	**	n.s.	n.s.	**	**	n.s.	*
［V］×［T］		*	**	**	n.s.	n.s.	**	n.s.	n.s.

x: Each plant was applied with 600 mL of tap water (Control) or solutions containing 50 mM NaCl (NaCl) or 50 mM NaCl + 0.1 mM NaHS (NaCl+NaHS) twice a day on sunny days and 0 to 1 time a day on cloudy or rainy days instead of irrigation water. **, *, and n.s. indicate significant differences at $p<0.01$, $p<0.05$, and not significant, respectively by two-way ANOVA. Different letters within the same column indicate statistically different at $p<0.05$ by Tukey's multiple comparison tests (n=10).

Table 7. Effects of foliar application of sodium hydrosulfide on vegetative growth in 'AGS313' and 'Fukuyutaka' soybean 13 days after treatment under NaCl application.

Variety	Treatment[x]	Plant height (cm)	Leaf number (/plant)	Leaf area[y] of the 3rd youngest leaf (cm^2)	Leaf area of the 4th youngest leaf (cm^2)	Shoot dry weight (g)	Root dry weight (g)
AGS313	Control	81.9 a	10.8	74.9	64.0	4.5	0.5
	NaCl	63.4 b	7.4	46.5	39.4	2.3	0.3
	NaCl+NaHS	63.4 b	7.4	51.3	42.7	2.6	0.4
Fukuyutaka	Control	56.9 a	14.8	63.3	61.6	5.3	0.7
	NaCl	51.1 b	9.8	46.0	40.4	2.9	0.5
	NaCl+NaHS	54.0 ab	11.4	46.0	54.8	3.4	0.5
Average							
AGS313		69.6	8.6 b	57.6 a	48.7	3.2 b	0.4 b
Fukuyutaka		54.0	12.0 a	51.8 b	52.3	3.9 a	0.6 a
	Control	69.4	12.8 a	69.1 a	62.8 a	4.9 a	0.6 a
	NaCl	57.3	8.6 b	46.3 b	39.9 b	2.6 b	0.4 b
	NaCl+NaHS	58.7	9.4 b	48.7 b	48.8 b	3.0 b	0.4 b
Variety［V］		**	**	*	n.s.	**	**
Treatment［T］		**	**	**	**	**	**
［V］×［T］		**	n.s.	n.s.	n.s.	n.s.	n.s.

x: Each plant was applied with 600 mL of tap water (control) or solutions containing 150 mM NaCl (NaCl) twice a day on sunny days and 0 to 1 time a day on cloudy or rainy days instead of irrigation water. Half plants applied with NaCl was foliar applied with 10 mL NaHS solution (NaCl+NaHS) every day. y: Leaf area was measured at middle leaflet. **, *, and n.s. indicate significant differences at $p<0.01$, $p<0.05$, and not significant, respectively by two-way ANOVA. Different letters within the same column indicate statistically different at $p<0.05$ by Tukey's multiple comparison tests (n=9).

SPAD and Fv/Fm were increased by NaCl application. EL was not affected by the applications of NaCl or NaHS.

Leaf Na content was the highest at NaCl treatment and the lowest at control in both varieties (Table 9). Leaf Cl content in 'AGS313' was not affected by the applications, while that in 'Fukuyutaka' was increased by NaCl application and it was not decreased by NaHS foliar application. Leaf K content was not affected by the applications. Leaf Ca content in 'AGS313' was not affected by the applications and that in 'Fukuyutaka' was the highest at NaCl treatment and the lowest at control. Leaf Mg content was not affected by the applications. Leaf P content was increased by NaCl application. Leaf NO_3 content was decreased by NaCl application. Leaf K/Na was decreased by NaCl application and not increased by NaHS foliar application.

Discussion

Vegetative growth was not greatly improved by NaHS application in the 3 experiments except for some indexes under saline conditions. In the experiment 1, shoot dry weight was improved by 0.1 mM NaHS application while plant height, number of leaves, and root dry weight were not significantly improved (Figure 1, Table 1). In the experiments 2 and 3, there were no positive effects of NaHS application on vegetative growth (Table 4, Table 7). Thus, NaHS had slightly positive effects on vegetative growth in soybean under NaCl stress.

In the experiment 1,17 day-application of 0.2 mM NaHS improved leaf Cl content in 'AGS313' (Table 2). In the experiment 2, 30 day-application of 0.1 mM NaHS improved leaf Cl content in 'Fukuyutaka' (Table 6). NaHS irrigation may have acted on root, inhibiting Cl absorption from soil to root and Cl transport from root to shoot. It is well known that regulation of Cl to shoot is important for salt tolerance of soybean (Valencia *et al*. 2008, Onodera *et al*. 2019, Fujii and Higuchi 2019). Soybean expressing the Cl-regulated genes, *GmCLC1* and *Ncl*, in the root increased salt tolerance by suppressing Cl transport to shoot (Wei *et al*. 2016, Do *et al*. 2016). NaHS was reported to be a signal substance to *SOS1*, a gene that regulates Na transport, leading to maintenance

Table 8. Effects of foliar application of sodium hydrosulfide on physiological responses in 'AGS313' and 'Fukuyutaka' soybean 13 days after treatment under NaCl application.

Variety	Treatment[x]	Photosynthetic rate ($\mu mol\ m^{-2}\ s^{-1}$)	Stomatal conductance ($mmol\ m^{-2}\ s^{-1}$)	SPAD	Fv/Fm	Electrolyte leakage (%)
AGS313	Control	14.8	635	36.3	0.83	4.2
	NaCl	5.7	129	39.9	0.84	8.2
	NaCl+NaHS	8.5	105	40.9	0.84	6.3
Fukuyutaka	Control	16.9	669	36.1	0.83	7.2
	NaCl	7.2	114	37.7	0.85	9.6
	NaCl+NaHS	8.2	83	39.3	0.84	8.8
Average						
AGS313		9.6	290	39.1 a	0.84 b	6.2 b
Fukuyutaka		10.8	289	37.7 b	0.84 a	8.5 a
	Control	15.9 a	652 a	36.2 b	0.83 b	5.7
	NaCl	6.5 c	121 b	38.8 a	0.84 a	8.9
	NaCl+NaHS	8.4 b	94 b	40.1 a	0.84 a	7.6
Variety［V］		n.s.	n.s.	*	*	*
Treatment［T］		**	**	**	**	n.s.
［V］×［T］		n.s.	n.s.	n.s.	n.s.	n.s.

x: Each plant was applied with 600 mL of tap water (control) or solutions containing 150 mM NaCl (NaCl) twice a day on sunny days and 0 to 1 time a day on cloudy or rainy days instead of irrigation water. Half plants applied with NaCl was foliar applied with 10 mL NaHS solution (NaCl+NaHS) every day. **, *, and n.s. indicate significant differences at $p<0.01$, $p<0.05$, and not significant, respectively by two-way ANOVA. Different letters within the same column indicate statistically different at $p<0.05$ by Tukey's multiple comparison tests (n=9).

Table 9. Effects of foliar application of sodium hydrosulfide on leaf mineral contents in 'AGS313' and 'Fukuyutaka' soybean 13 days after treatment under NaCl application.

Variety	Treatment[x]	Na (mg g^{-1}D.W.)	Cl (mg g^{-1}D.W.)	K (mg g^{-1}D.W.)	Ca (mg g^{-1}D.W.)	Mg (mg g^{-1}D.W.)	P (mg g^{-1}D.W.)	NO_3 (mg g^{-1}D.W.)	K/Na
AGS313	Control	1.06	6.1 a	11.7 a	4.04	4.47	12.2	13.3	11.6
	NaCl	2.58	7.8 a	10.4 a	3.52	6.58	12.8	11.5	7.4
	NaCl+NaHS	1.71	8.1 a	10.0 a	3.86	6.43	13.1	11.6	8.8
Fukuyutaka	Control	1.03	7.1 b	13.5 b	4.89	4.57	15.8	15.2	15.5
	NaCl	2.19	11.2 a	17.2 a	5.17	5.51	15.6	11.6	8.4
	NaCl+NaHS	1.75	10.4 a	16.2 ab	5.00	5.74	16.1	12.2	9.6
Average									
AGS313		1.78	7.3	10.7	3.81 b	5.83 a	12.7 b	12.1	9.2 b
Fukuyutaka		1.66	9.6	15.7	5.02 a	5.27 b	15.9 a	13.0	11.2 a
	Control	1.04 b	6.6	12.6	4.46	4.52 b	14.0	14.2 a	13.5 a
	NaCl	2.39 a	9.5	13.8	4.34	6.05 a	14.2	11.6 b	7.9 b
	NaCl+NaHS	1.73 ab	9.3	13.1	4.43	6.09 a	14.6	11.9 b	9.2 b
Variety［V］		n.s.	**	**	**	*	**	n.s.	*
Treatment［T］		*	**	n.s.	n.s.	**	n.s.	**	**
［V］×［T］		n.s.	**	**	n.s.	n.s.	n.s.	n.s.	n.s.

x: Each plant was applied with 600 mL of tap water (control) or solutions containing 150 mM NaCl (NaCl) twice a day on sunny days and 0 to 1 time a day on cloudy or rainy days instead of irrigation water. Half plants applied with NaCl was foliar applied with 10 mL NaHS solution (NaCl+NaHS) every day. **, *, and n.s. indicate significant differences at $p<0.01$, $p<0.05$, and not significant, respectively by two-way ANOVA. Different letters within the same column indicate statistically different at $p<0.05$ by Tukey's multiple comparison tests (n=9).

of K/Na (Deng *et al*. 2016). As well as Na regulation, for Cl regulation, in soybean NaHS might have acted as a signal substance to express genes such as *GmCLC1* and *Ncl*, which suppress Cl absorption and transport. On the other hand, the NaHS foliar application in the experiment 3 did not reduce leaf Cl content (Table 9). H_2S may not have been transferred to the roots by foliar application and had no effect on Cl absorption and transport. H_2S may be difficult to be transferred from leaf to root.

In the experiment 1, leaf Cl content was decreased by 17 day-application of 0.2 mM NaHS (Table 2), while it was increased by 22 day-application (Table 3). Appropriate concentration of NaHS application was reported to promote root formation and elongation (Mei *et al*., 2019). On the other hand, an excess H_2S in the soil negatively affects root activity in general. In the experiment 1, 22 day-application of NaHS concentration at 0.2 mM may have been slightly excess and negatively affected root activity, while 17 day-application of 0.2 mM may have been appropriate and inhibited Cl absorption and transport.

In the experiment 3, photosynthetic rate was enhanced by NaHS foliar application, while stomatal conductance and Fv/Fm were not (Table 8). On the other hand, NaHS application enhanced photosynthetic rate, stomatal conductance, and Fv/Fm (Zhang *et al*. 2010; Christou *et al*. 2013, Jiang *et al*. 2019). Therefore, photosynthetic rate was not enhanced by stomata and photosystem II as factors in the experiment 3. A possible factor of enhancing photosynthesis might have been the higher rate of CO_2 fixation due to the activation of dark reaction systems such as RuBisCO. NaHS application also increased photosynthetic rate in rice and spinach by increasing the activity of RuBisCO (Duan *et al*. 2015, Chen *et al*. 2011). Further studies are needed to investigate the activity of the dark reaction system, such as the initial slope of the A/Ci curve (Kondo *et al*. 2017) and the activity of RuBisCO. NaHS foliar application increased photosynthetic rate in the experiment 3, while NaHS irrigation application did not increase it in the experiments 1 and 2. In the experiments 1 and 2, where H_2S may not have been transferred to the

leaves. H_2S might be as difficult to be transferred from root to leaf as it is from leaf to root. Further studies are needed on the transferability of H_2S in plants.

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