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Hydrogen Sulfide Affects the Root Development of Strawberry During Plug Transplant Production

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Received: 7 December 2019; Accepted: 3 January 2020; Published: 5 January 2020

Abstract: Hydrogen sulfide (H_2S) is endogenously produced in plant cells and plays an essential role in root development. Given its potential for future agricultural applications, the optimal concentration of sodium hydrosulfide (NaHS, an H_2S donor) and the potential mechanisms for root development in the strawberry 'Seolhyang' were investigated in this study. The results showed that NaHS with a concentration of 1.250 mM had a positive effect on root development in strawberry. Further experiments showed that exogenous NaHS elevated the H_2S content in the root. The dry root weight was increased by the 1.250 mM NaHS treatment, but was reduced by the hypotaurine (an H_2S scavenger) treatment. Similar changes were found between H_2S and soluble sugar contents, indicating that H_2S enhanced the accumulation of soluble sugar. Therefore, it is suggested that the accumulation of soluble sugar induced by H_2S is either directly or indirectly involved in root development in strawberry during plug production. Moreover, superoxide dismutase was shown to have contributed to the elevated H_2O_2 contents. These results contribute to our understanding of the role that H_2S plays and some of the relevant mechanisms in which H_2S regulates root development.

Keywords: antioxidant enzyme; hydrogen peroxide; hydrogen sulfide; root development; soluble sugar

1. Introduction

Runner tips have been widely used to produce strawberry plug transplants because of their advantages, which include their reduced pesticide requirements and soil-borne diseases, ease of transplanting, and low water requirements [1]. A well-rooted plant is essential for shipping, transplanting, and special conditioning. Studies have been conducted to determine the optimal cell-tray sizes [2], rooting media [3], and temperatures [4,5] for root development in strawberry. Moreover, the rooting time was found to affect the plant morphology. Early-rooted strawberry plants had more nodes, runners, and higher total fresh and dry weights [6]. In Korea, farmers propagate strawberry plants in both the summer and winter. The root development is adversely affected by the low temperature in winter. Therefore, it is important to explore a variety of strategies to obtain high-quality transplants.

Hydrogen sulfide (H_2S) has been studied as a gaseous transmitter for the roles it plays in plant growth, development, and tolerance to biotic and abiotic stresses. H_2S can be synthesized via enzymatic reactions in plant cells or be absorbed from the rhizosphere. It has been reported that H_2S inhibits the primary root elongation, but promotes the lateral root initiation, in *Arabidopsis* [7]. The positive effects

of H_2S on root formation in tomato [8–10] and cucumber [11] have also been reported. In strawberry, H_2S has been shown to prolong the postharvest shelf life [12,13] and induce systemic tolerances to heat, salinity, and non-ionic osmotic stresses [14,15]. However, the roles of H_2S in the root development of strawberry have not yet been identified. Furthermore, the concentrations of H_2S used for horticultural crops have ranged from 0.2 μM to 10 mM in previous studies [16]. The optimal concentration of H_2S for root development in strawberry has not been established.

Rooting is a physiological process of high energy demands, which requires high carbohydrate, starch, and protein contents [17–20]. It has been shown that the number of roots increased in a sugarcane bagasse medium in in-vitro propagated strawberry [21]. Moreover, it has been observed that H_2S increases the soluble sugar content by up-regulating the genes involved in the synthesis of sugar [22]. Therefore, the soluble sugar, starch, and soluble protein contents were investigated as they are appropriate indicators of the rooting capacity.

Numerous studies have addressed the roles of antioxidant enzymes in rooting [23–26]. In an enzymatic anti-oxidant system, superoxide dismutase (SOD) converts superoxide ions to hydrogen peroxide (H_2O_2). The H_2O_2 is scavenged by peroxidases such as glutathione peroxidase (GPX), catalase (CAT), and ascorbate peroxidase (APX). Although higher concentrations of H_2O_2 cause oxidative damage in plant cells, H_2O_2 in low concentrations can serve important biological roles in cell division, differentiation, and migration [27]. The involvement of H_2O_2 in root development has been reported in tomato [10], mung bean [28,29], and cucumber seedlings [30].

Multiple H_2S donors are currently being studied for their potential uses in biological and clinical applications [31]. Given the potential for future commercial applications, we sought to investigate the optimal concentration and effects of sodium hydrosulfide (NaHS) on the root development in strawberry. The results of this study may also help to elucidate the role that H_2S plays in regulating root development.

2. Materials and Methods

2.1. Plant Materials and Chemical Treatments

Unrooted runner plants of the strawberry (*Fragaria* × *ananassa* Duch.) 'Seolhyang' were used in this study. For experiment 1, the runner plants with 20 cm long and three fully expanded leaves were obtained from a strawberry farm (Sugok-myeon, Jinju, Gyeongsangnam-do, Korea) and stuck in a BVB Medium (Bas Van Buuren Substrates, EN-12580, De Lier, The Netherlands) containing NaHS (0.010, 0.125, 0.250, 1.250, 2.500, 12.500, 25.000, and 37.500 mM; an H_2S donor) or distilled water (the control) in 21-cell zigzag trays (21-Zigpot/21 cell tray, Daeseung, Jeonju, Korea). Experiment 1 was conducted in December of 2017, and the mean air temperature was 10 °C. For experiment 2, the runner plants with 15 cm long and three fully expanded leaves were stuck and grown in a BVB Medium containing 0.125, 1.250, and 12.500 mM of NaHS; 50 μM of hypotaurine (HT, an H_2S scavenger); or distilled water (the control). Experiment 2 was conducted in December of 2018, and the mean air temperature was 15 °C. Plants were kept on a mist propagation bench with a relative humidity of 80% for 10 days, followed by cultivation in a glasshouse at Gyeongsang National University, Jinju, Korea, for another 10 days. The experiment was conducted based on a completely randomized design with three replicates and each replicate contained seven runner plants. The plants were treated again with the chemicals mentioned above one day before measuring and sampling.

2.2. Measurement of Root Development and Sampling

The substrate was washed to expose the roots. The number of roots, length of the longest root, and fresh root weight were then measured. The root dry weight was measured after drying the roots in an oven at 65 °C for 3 days. The roots were immersed in liquid nitrogen and ground into a fine powder for the subsequent analyses.

2.3. H_2S and H_2O_2 Quantification

The H_2S level was measured according to the method introduced by Nashef et al. [32]. In brief, 0.2 g of root samples was homogenized in 1 mL of a 100 mM potassium phosphate buffer (pH 7.0) containing 10 mM of ethylenediaminetetraacetic acid and then centrifuged at 13,000 *g* for 15 min at 4 °C. The reaction mixture was made in a total volume of 2 mL containing with 100 µL of the supernatant, 20 µL of 20 mM 5,5′-dithiobis (2-nitrobenzoic acid), and 1880 µL of extraction buffer. The absorbance was read at 412 nm after incubating the mixture at room temperature for 2 min. The H_2S content was quantified based on a standard curve of known NaHS concentrations. H_2O_2 quantification was performed as described by Loreto and Velikova [33]. In brief, 0.2 g of root samples was homogenized in 1 mL of 0.1% (*w*/*v*) trichloroacetic acid and then centrifuged at 13,000 *g* for 15 min at 4 °C. The reaction mixture was made in a total volume of 2 mL containing 0.5 mL of the supernatant, 0.5 mL of 10 mM potassium phosphate buffer (pH 7.0), and 1 mL of 1 M potassium iodide. The absorbance of the mixture was read at 390 nm. The H_2O_2 content was calculated based on a standard curve of known H_2O_2 concentrations.

2.4. Measurements of Soluble Sugar, Starch, and Soluble Protein Contents

Soluble sugar and starch contents were measured via the anthrone-sulfuric acid method introduced by Xue et al. [34]. In brief, 0.3 g of root samples was homogenized with 25 mL of distilled water to extract the soluble sugar, and the residue was homogenized with 20 mL of distilled water and 2 mL of 9.2 M perchloric acid for measurement of the starch content. The contents of soluble sugar and starch were calculated based on a standard curve of known concentrations of sucrose and starch, respectively.

Soluble protein was measured using a Bradford Reagent Kit (Sigma-Aldrich, St. Louis, MO, USA), according to the manual. The soluble protein content was calculated based on a standard curve of a known concentration of bovine serum albumin.

2.5. Determination of the Antioxidant Enzyme Activities

The activities of antioxidant enzymes SOD, GPX, CAT, and APX were determined by the method introduced by Manivannan et al. [35]. In brief, samples were ground in a 100 mM sodium phosphate buffer (pH 7.0) for the GPX activity assay and in a 50 mM sodium phosphate buffer (pH 7.0) containing 1 mM of ethylenediaminetetraacetic acid, 0.05% Triton X 100, and 2% polyvinylpyrrolidone for SOD, CAT, and APX activity assays. The SOD assay was made in a total volume of 3.0 mL containing 2.5 mL of sodium phosphate buffer (50 mM), 0.4 mL of riboflavin (2 mM), and 0.1 mL of enzyme extract. The GPX assay was made in a total volume of 3.00 mL containing 2.80 mL of sodium phosphate buffer (50 mM), 0.05 mL of guaiacol (20 mM), 0.05 mL of H_2O_2 (30 mM), and 0.10 mL of enzyme extract. The CAT assay was made in a total volume of 3.0 mL containing 2.8 mL of sodium phosphate buffer (50 mM), 0.1 mL of H_2O_2 (30 mM), and 0.10 mL of enzyme extract. The APX assay was made in a total volume of 3.0 mL containing 2.8 mL of sodium phosphate buffer (50 mM containing 0.5 mM of ascorbic acid), 0.1 mL of H_2O_2 (30 mM), and 0.10 mL of enzyme extract. The specific enzyme activity was represented as the unit·μg^{-1} protein.

2.6. Statistical Analysis

After the normality and homoscedasticity of data were verified, the SAS statistical software, Release 8.2 (SAS Inst., Cary. N.C., USA), was used for the statistical analysis, followed by an analysis of variance (ANOVA) and Duncan's multiple range test ($p \leq 0.05$).

3. Results

3.1. The Optimal Concentration of NaHS for Strawberry (Experiment 1)

NaHS at appropriate concentrations promoted the root development in strawberry runner plants (Figure 1A). The length of the longest root was increased significantly by the 0.250, 1.250, and 2.500 mM NaHS treatments compared to that of the control ($p \leq 0.05$; Figure 1C). The dry weights of the roots were significantly increased by the 1.250 mM NaHS treatment ($p \leq 0.05$; Figure 1D,E). Even though the mean values of the number of roots and fresh weight of roots were highest in the 2.500 mM NaHS treatment and 1.250 mM NaHS treatment, respectively, there were no statistically significant differences (Figure 1B,C). Overall, the exogenous NaHS at the concentration of 1.250 mM had an overall positive effect on the root development, and was selected as the optimal concentration for the following experiments.

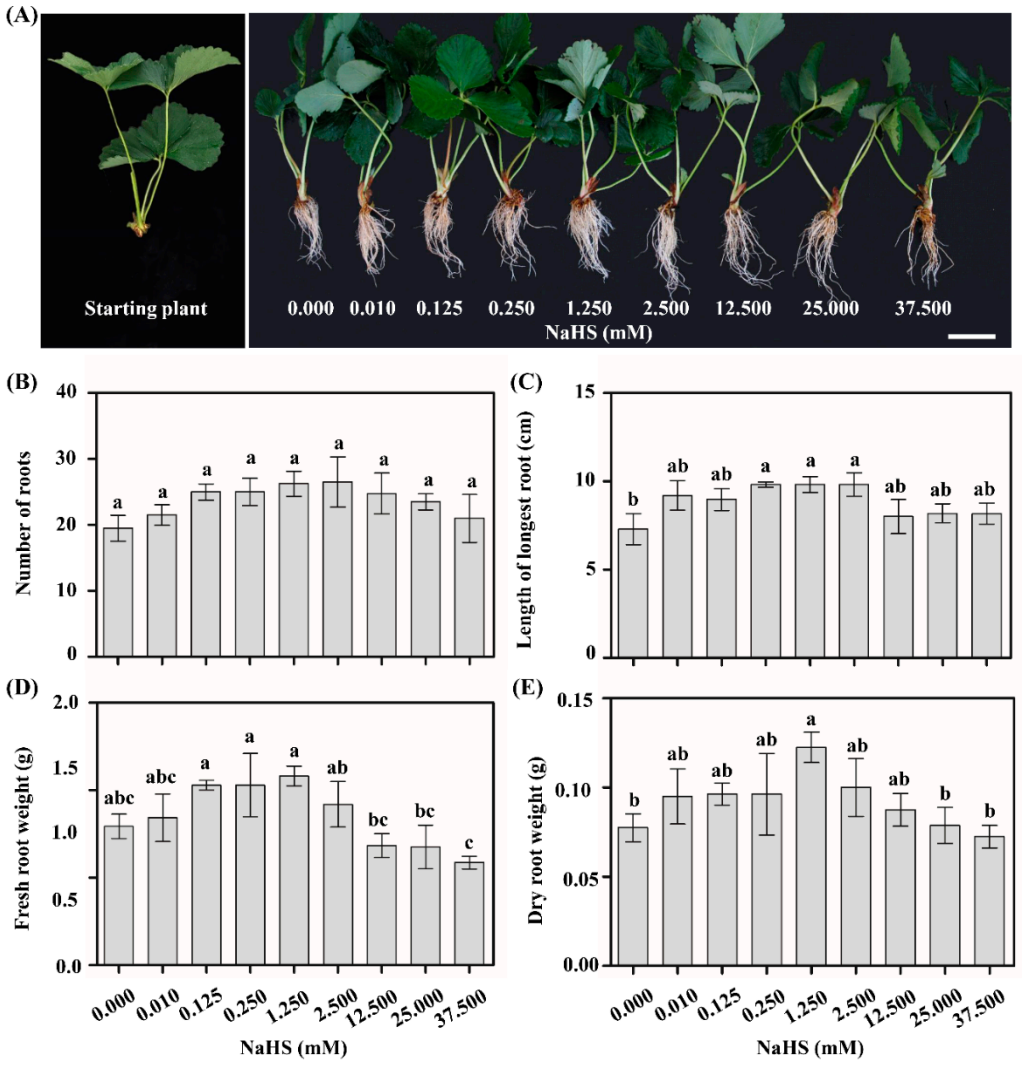

Figure 1. The effects of different sodium hydrosulfide (NaHS) concentrations (mM) on the morphology (**A**), number of roots (**B**), length of the longest root (**C**), fresh root weight (**D**), and dry root weight (**E**) of strawberry runner plants. The data were collected 20 days after the first treatment and are represented as the mean ± S.E of six biological replicates. Means accompanied by different letters are significantly different according to Duncan's multiple range test at a 5% significance level. Scale bar = 5 cm.

3.2. Root Development Induced by H_2S (Experiment 2)

The runner plants were treated with HT, H_2O, and NaHS to investigate the effects of H_2S on the root development (Figure 2A). The H_2S content in strawberry was significantly elevated with the 1.250 mM NaHS treatment, but significantly reduced with the 12.500 mM NaHS treatment ($p \leq 0.05$), compared to that for the HT treatment and the control (Figure 2B).

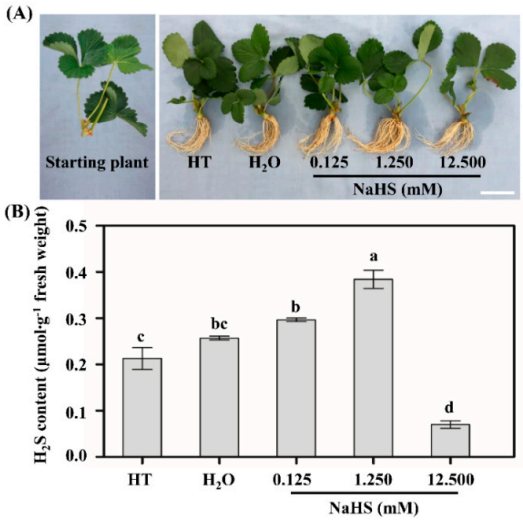

Figure 2. The effects of hypotaurine (HT), H_2O, and NaHS on the morphology (**A**) and H_2S content (**B**) of strawberry runner plants. The data were collected 20 days after the first treatment and are represented as the mean ± S.E of three biological replicates and three technical replicates. Means accompanied by different letters are significant differences according to Duncan's multiple range test at a 5% significance level. Scale bar = 5 cm.

Correspondingly, as the H_2S content increased, the fresh and dry weights of roots also increased (Figure 3C,D). However, the number of roots and length of the longest root in the HT and the NaHS treatments showed no significant differences compared with the control ($p \leq 0.05$; Figure 3A,B).

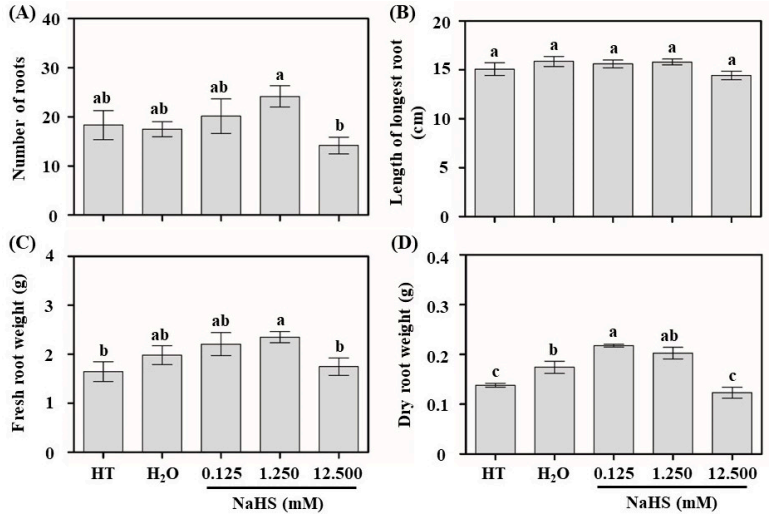

Figure 3. The effects of HT, H_2O, and NaHS on the number of roots (**A**), length of the longest root (**B**), fresh root weight (**C**), and dry root weight (**D**) in strawberry runner plants. The data were collected 20 days after the first treatment and are represented as the mean ± S.E of six biological replicates. Means accompanied by different letters are significant differences according to Duncan's multiple range test at a 5% significance level.

3.3. Soluble Sugar, Starch, and Soluble Protein Content

The soluble sugar content was significantly increased by the 0.125 and 1.250 mM NaHS treatments ($p \leq 0.05$, Figure 4A). The starch and soluble protein contents in the roots of strawberry runner plants showed no significant variations among the treatments (Figure 4B,C).

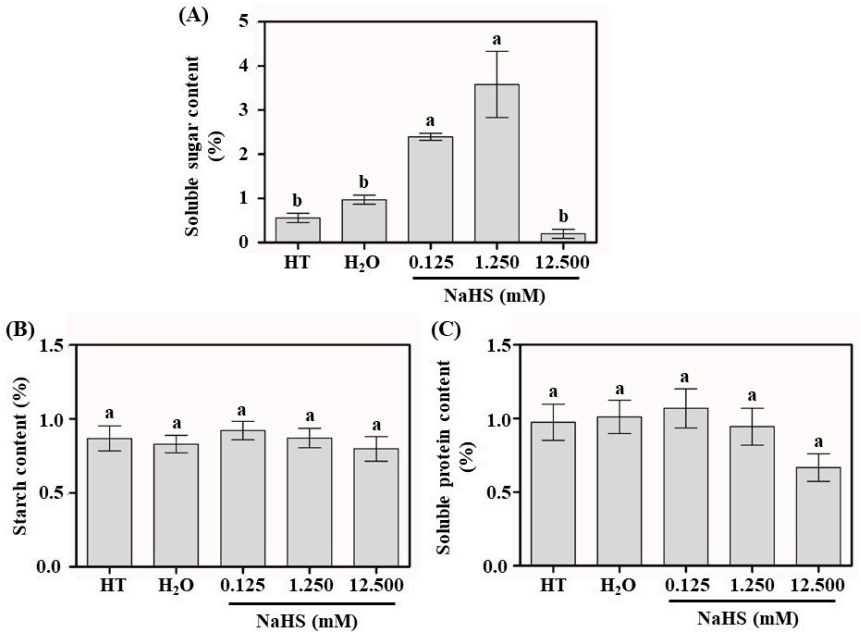

Figure 4. The effects of HT, H_2O, and NaHS on the soluble sugar (**A**), starch (**B**) and soluble protein (**C**) contents in the roots of strawberry runner plants. Samples were collected 20 days after the first treatment, and the data are represented as the mean ± S.E of three biological replicates and three technical replicates. Means accompanied by different letters are significant differences according to Duncan's multiple range test at a 5% significance level.

3.4. H_2O_2 Content

The H_2O_2 contents in the roots of strawberry runner plants were significantly increased by the 0.125 and 1.250 mM NaHS treatments, compared to that in the HT treatment ($p \leq 0.05$). However, no significant differences were found in the roots of strawberry runner plants grown in the 0.125 and 1.250 mM NaHS treatments and in the control (Figure 5).

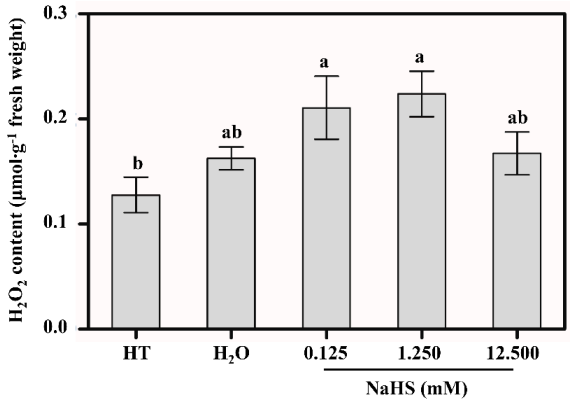

Figure 5. The effects of HT, H_2O, and NaHS on the H_2O_2 content in the roots of strawberry runner plants. Samples were collected 20 days after the first treatment, and the data are represented as the mean ± S.E of three biological replicates and three technical replicates. Means accompanied by different letters are significant differences according to Duncan's multiple range test at a 5% significance level.

3.5. Antioxidant Enzyme Activities

The SOD activity in the roots of strawberry runner plants was significantly increased by the 0.125 treatment compared to that in the HT treatment ($p \leq 0.05$, Figure 6A). The GPX activity was the highest in the HT treatment and the lowest in the 12.500 mM NaHS treatment (Figure 6B). The CAT activity was significantly inhibited by the HT and NaHS treatments compared to the control ($p \leq 0.05$, Figure 6C). The APX activity was not significantly changed by different treatments (Figure 6D).

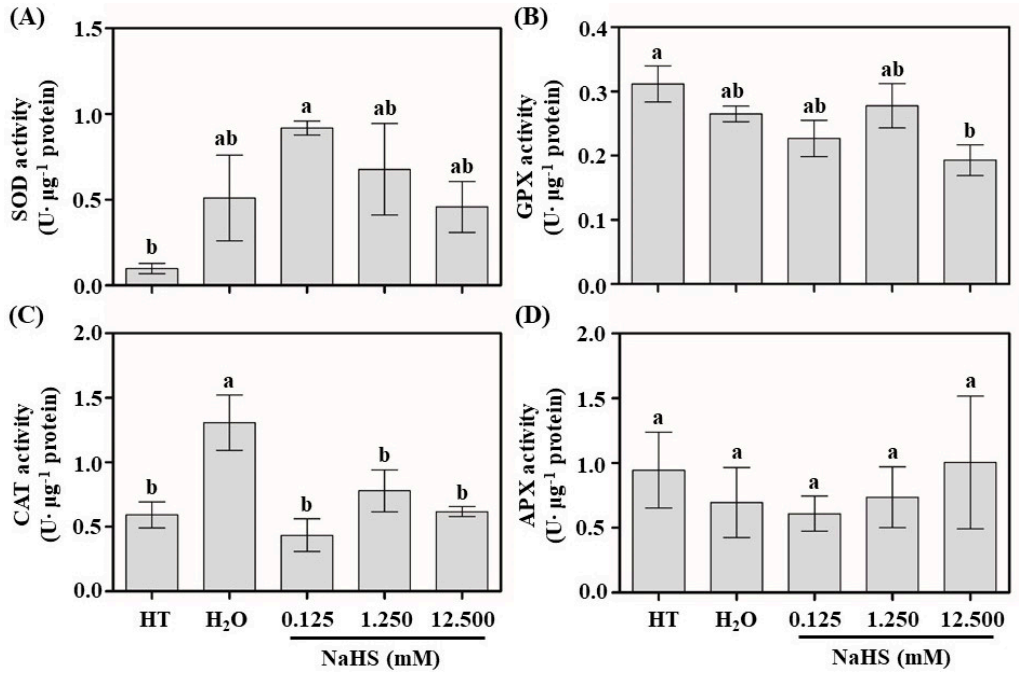

Figure 6. The effects of HT, H_2O, and NaHS on the activity of superoxide dismutase (SOD) (**A**), glutathione peroxidase (GPX) (**B**), catalase (CAT) (**C**), and ascorbate peroxidase (APX) (**D**) in the roots of strawberry runner plants. Samples were collected 20 days after the first treatment, and the data are represented by the mean ± S.E of three biological replicates and three technical replicates. Means accompanied by different letters are significant differences according to Duncan's multiple range test at a 5% significance level.

4. Discussion and Conclusions

H_2S can be endogenously generated in plant cells or obtained from water-soluble donors such as NaHS. Endogenous H_2S has beneficial effects on plant growth and defense responses [16,36]. Several investigators have used concentrations between 0.010 and 10.000 mM of NaHS to evaluate the effects of H_2S on the root development in cucumber and tomato [8,37]. In our study, the root development in strawberry was promoted by the 1.250 mM NaHS treatment. Moreover, the H_2S content in the roots of strawberry ranged from 0.26 (the control) to 0.38 (1.250 mM NaHS treatment) μmol·g^{-1} fresh weight. In the roots of *Arabidopsis*, the H_2S content was about 0.02 and 0.23 μmol·g^{-1} fresh weight in the control and 1.000 mM NaHS treatment, respectively [7]. In the roots of tomato, the highest H_2S content was observed nine hours after the treatments, when it was about 0.70 (the control) and 1.80 (100 nM naphthalene acetic acid treatment) μmol·g^{-1} fresh weight. Twelve hours after the treatments, the H_2S content decreased to approximately 0.50 (the control) and 0.60 (100 nM naphthalene acetic acid treatment) μmol·g^{-1} fresh weight [8]. In summary, the endogenous concentrations of H_2S in the roots of plants are found to be approximate based on the published data.

Since the H_2S content can be increased initially and then decreased after the NaHS treatments, the plants were treated again with chemicals one day before sampling. It was found that the H_2S content was significantly increased by the 1.250 mM NaHS treatment, while there was no significant difference

between the HT treatment and the control. As the dry root weight was significantly decreased by the HT treatment, it is suggested that temporal changes of H_2S content in the HT treatment need to be further investigated. In addition, the results of two experiments were inconsistent. For example, the length of the longest root was significantly increased in the 1.250 mM NaHS treatment in experiment 1, while there were no significant differences between the 1.250 mM NaHS treatment and the control in experiment 2. This might have been caused by the different air temperatures at the time when the experiments were conducted.

The soluble sugar content in strawberry root was increased by the 0.125 and 1.250 mM NaHS treatments, which is consistent with the H_2S content in different treatments. However, the starch and soluble protein contents were not affected by the treatments. These results indicate that soluble sugar accumulation might be induced by H_2S in the roots of strawberry. It has been revealed that H_2S up-regulates sugar biosynthesis-related genes, such as sucrose phosphate synthase (*SPS1*), fructose-1,6-bisphosphatase (*FBPase*), and trehalose-6-phosphate synthase (*T6PS*), under drought stress in *Spinacia oleracea* seedlings [22]. In addition, soluble sugar changes the genes and metabolites involved in the indole-3-acetic acid (IAA) biosynthetic pathways [38]. Therefore, soluble sugar might be induced by H_2S through a change of sugar biosynthesis-related genes, and further promotes the root development by inducing IAA biosynthesis.

The H_2O_2 content and SOD activity were promoted by the 0.125 mM NaHS treatment compared with the HT treatment. Therefore, the SOD activity might have contributed to the accumulation of H_2O_2 [39]. Studies have reported that H_2S contributes to higher activities of reactive oxygen species (ROS)-scavenging enzymes under abiotic stress [40–42]. In this study, the CAT activity in the roots of strawberry runner plants was inhibited in the NaHS treatments, and it was also inhibited in the HT treatment compared to the control. Therefore, the change of CAT activity in the roots of strawberry runner plants might not be caused by H_2S. Moreover, the GPX and APX activities in the roots of strawberry runner plants showed no significant differences among the treatments. Therefore, it could be deduced that H_2O_2 was in physiological concentrations, and thus did not activate the peroxidases.

In this study, the contents of H_2S, soluble sugar, and H_2O_2 reached optimal concentrations only after the application of 1.250 mM NaHS. The 12.500 mM NaHS treatment significantly decreased the dry weight of roots compared with the 1.250 mM NaHS treatment. Therefore, high concentrations of NaHS might have interfered with the metabolism of strawberry plants. Multiple studies have also reported the toxic effect of NaHS at high concentrations [43–45]. Moreover, the concentrations of NaHS applied in this study should decrease over time due to fogging, and the plants were treated again with chemicals one day before sampling. It is a shortcoming that we failed to determine the final concentrations of NaHS.

Overall, soluble sugar and H_2O_2 accumulation was found to be induced by H_2S. It could be deduced that both types of accumulation are either directly or indirectly involved in the root development in strawberry during plug transplant production. Further works are needed to investigate the temporal changes of metabolism and biochemistry, as well as the genes that are involved in the interplay of H_2S and H_2O_2 signaling when plants are treated with NaHS.

Author Contributions: Conceptualization, B.R.J. and Y.L. (Ya Liu); methodology, B.R.J. and J.H.; formal analysis, J.H. and Y.L. (Yali Li); resources, B.R.J.; data curation, J.H., Y.L. (Yali Li), D.I.K., and H.W.; writing—original draft preparation, J.H.; writing—review and editing, B.R.J.; project administration, B.R.J.; funding acquisition, B.R.J., D.I.K., H.W., J.H., and Y.L. (Yali Li). All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Agrobio-Industry Technology Development Program, Ministry of Food, Agriculture, Forestry and Fisheries, Republic of Korea (Project No. 315004-5).

Acknowledgments: This study was supported by the Agrobio-Industry Technology Development Program, Ministry of Food, Agriculture, Forestry and Fisheries, Republic of Korea (Project No. 315004-5). Jiangtao Hu, Yali Li, Ya Liu, Dong Il Kang, and Hao Wei were supported by the BK21 Plus Program, Ministry of Education, Korea.

Conflicts of Interest: The authors declare no conflicts of interest.

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