Exogenous hydrogen sulfide enhanced Al stress tolerance in tea plant [Camellia sinensis (L.)]

Anqi Xing1#, Zaifa Shu2#, Peifang Huang1, Yang Zhang3, Xueyan Sui3,4, Shuai Wan1, Shujing Liu1, Xuan Chen1, Xinghui Li1 and Yuhua Wang1*

1 College of Horticulture, Nanjing Agricultural University, Nanjing 210095, China
2 Lishui Institute of Agriculture and Forestry Sciences, Lishui 323000, China
3 Jiangsu Land Consolidation and Rehabilitation Center, Nanjing 210017, China
4 Jiangsu Donghai and Yixing Land Consolidation and Ecological Protection Field Scientific Observation and Research Station, Ministry of Natural Resources, Yixing 214200, China

# These authors contributed equally: Anqi Xing, Zaifa Shu
* Corresponding author, E-mail: wangyuhua@njau.edu.cn

Abstract

Al is an essential element for the growth of tea plants roots, but excessive Al affects growth and development of Camellia sinensis. The underlying mechanism, particularly regulation of gas signaling molecule H2S, remains unclear. This study aims to uncover the function of H2S on C. sinensis under Al stress by treating hydroponic tea seedlings with different Al concentration, Na2S (H2S donor) and DL-Propargylglycine (PAG, synthesis inhibitor). High concentration of Al inhibits growth of tea roots, while H2S significantly improves the effects caused by Al stress. Whether it is 2 mM Al3+ or 4 mM Al3+, H2S reduces content of Al in the entire plant and roots, increases root activity, further promotes root growth, increases fresh and dry weight, regulates ion homeostasis, improves cell structure, increases chlorophyll content, and thus reduces the damage of Al toxicity in C. sinensis. Moreover, in response to the stress of 2 mM Al3+, H2S simultaneously alleviates Al stress by regulating substances related to antioxidant pathways, increasing content of GSH and GSSG, enhancing activity of GST, GR, LCD, and key components of tea, in order to alleviate Al stress. These approaches have effectively improved Al tolerance of C. sinensis, providing a new perspective for the study of H2S enhancing Al tolerance.

Introduction

Tea plant [Camellia sinensis (L.) O. Kuntze], is suitable for growing in acidic soil with pH 4.5-6.5. Aluminum (Al) toxicity is currently a crucial factor limiting plant growth in acidic environments, because when the pH of the soil is less than 5, Al can be transformed into phytotoxic trivalent cation (Al3+) that are readily absorbed by plants, thereby affecting plant growth[1]. As an Al hyperaccumulating plant, C. sinensis can contain up to 30,000 mg·kg−1 of Al in its mature leaves without showing symptoms of Al toxicity[2]. Appropriate Al concentration promotes the growth and development of tea plants. Once it exceeds 1 mmol·L−1, C. sinensis suffer from a negative effect on its normal growth[3].

Various strategies for plants to cope with Al toxicity include external exclusion mechanism such as increasing Al chelation and reducing Al uptake by plants, as well as increasing antioxidant enzyme activity and reducing toxic substances caused by reactive oxygen species and free radicals, among other internal detoxification mechanisms[4]. Meanwhile, excessive Al also has a certain impact on the tea quality components of tea polyphenols, catechins, amino acids, caffeine and other substances[5]. Not only that, tea consumption also increases dietary intake of Al, which is thought to be associated with Alzheimer’s disease[6]. Therefore, it is urgent to explore measures to reduce content of Al in C. sinensis is of great significance in alleviating Al stress and improving tea quality.

Hydrogen sulfide (H2S) has classically been regarded as a poisonous gas and atmospheric pollutant, but it was subsequently found to be the third gaseous signaling molecule after nitric oxide (NO) and carbon monoxide (CO)[7]. And synthesizes endogenous H2S mainly through L-cysteine desulphydrase (LCD), which is widely present in plants[8]. Recently, research on H2S has begun to reveal the role of these molecules in regulating plant abiotic and biotic stress resistance responses. Through the exogenous application of H2S donors, H2S has been proven to regulate plant growth and increase plant tolerance to drought, salt, temperature, and metal stress. It can be seen that H2S plays vital roles in facilitating plant with tolerance to environmental stresses[9]. However, the role of H2S in alleviating Al stress of C. sinensis is still unclear.

There are many studies on Al enrichment in tea plants, but currently there is a lack of research on H2S signaling molecules for Al tolerance in tea plants. Now, through different hydroponic treatments (0.4Al, H2S + 0.4Al, PAG + 0.4Al, 2Al, H2S + 2Al, PAG + 2Al, 4Al, H2S + 4Al, PAG + 4Al), we investigated the effects of H2S preapplication on the biomass, the content and transfer rate of Al and other elements in different tissues, the content of chlorophyll, photosynthetic indices, the ultrastructure, the antioxidant enzyme activity and tea quality compo-
ments under Al stress. Preliminary exploration of the role of exogenous H₂S in the physiological response of tea plants to Al stress provides new ideas for further research on alleviating Al stress and reducing Al accumulation in tea plants.

Materials and methods

Plant material and experimental treatments

For the experiment, annual cutting seedlings of C. sinensis cv. ‘Zhongcha 108’ were obtained from the Nanjing (Ya Run Tea Co., Ltd., Jiangsu Province, China). The tea seedlings were firstly pre-cultured in water for 5 days, then transferred to 1/8, 1/4, and 1/2 total nutrient solutions to culture for 5 days in each strength nutrient solution, and finally transferred to total nutrient solutions for 10 days (culture medium was replaced every 5 days) [19]. The seedlings with consistent growth were used to carry out the subsequent treatment assays with H₂S or PAG and Al³⁺ as shown in Table 1. For treatments, Al₂(SO₄)₃:18H₂O, Na₂S·9H₂O and DL-propargylglycine (PAG) were the Al³⁺ donor [17], H₂S donor [18] and L-cysteine desulfurase (L-CDS) inhibitor [19], respectively. And solution pH was adjusted to 4.5 ± 0.1 with 1.0 mol·L⁻¹ NaOH or 1.0 mol·L⁻¹ HCl. The experiments were performed in the Intelligent Greenhouse of Nanjing Agricultural University (China), controlled growth room at 25 °C/22 °C with 16 h light/8 h dark cycle, 30000 lx light intensity and a 16 h light/8 h dark cycle, 30000 lx light intensity and a 16 h light/8 h dark cycle.

Fresh and dry weight analysis

Plants were collected and separated into young leaf (the first and second leaf from the top of plants), mature leaf (remaining leaves), stem and root. Fresh weight (FW) of seedlings were measured by weighing instantly after harvesting and then placed into an oven at 105 °C for 30 min and then baked at 80 °C until biomass became stable. The dry weight (DW) immediately weighed after removal from the oven.

Root activity assessments

Root activity was measured using the 2,3,5-tripheyl tetrazolium chloride (TTC) method [16]. About 0.5 g of fresh root tips were placed in a mixture of 5 mL 1% TTC and 5 mL phosphate buffer for 1 h at 37 °C in the dark. The assays were terminated by adding 2 mL 1.0 mol·L⁻¹ H₂SO₄ to the reaction mixture. The reduced TTC was extracted with 3-4 mL ethyl acetate, then absorbance was determined at 520 nm.

Transmission electron microscopy

Leaf fragments without veins were collected from randomly selected plants, then fixed 24 h in 2.5% glutaraldehyde solution and stored at 4 °C. Samples were rinsed three times with the same phosphate-buffered saline (PBS, pH 7.2), and post-fixed in 1% osmium oxide for 1 h, washed three times with distilled water. The samples were dehydrated in a graded series of ethanol (50, 70, 80 and 100%) and at the end treated with absolute acetone for 24 h. Ultra-thin sections (≤ 100 nm) of specimens were prepared for viewing.

Measurement of chlorophyll content and photosynthesis parameters

Chlorophyll a and chlorophyll b of randomly selected mature leaves per treatment were measured as described previously [15]. Samples were completely immersed with 10.0 mL mixture of acetone-95% ethanol-water (9:2:1, v: v) and transferred into tubes placed in a dark place until the leaves turn completely white. The OD₆₆₅ and OD₉₇₃ values were used to calculate chlorophyll content. A LI-6400XT portable photosynthesis system (Li-Cor Biosciences, Lincoln, Nebraska, USA) was used to measure net photosynthetic rate (Pn), stomatal conductance (Gs), transpiration rate (Tr), and intercellular CO₂ concentration (Ci) with 1200 μmol·m⁻²·s⁻¹ illuminance and 500 μmol·m⁻²·s⁻¹ flow rate.

Assay of Al and other elemental concentrations

The plant samples with 0.2 g were placed into the digestion vessels, mixed with HNO₃, HClO₄, (4:1, v: v) and digested in microwave digestion system. The concentrations of Al, calcium (Ca), magnesium (Mg), manganese (Mn), iron (Fe) and zinc (Zn) in the filtrate were determined using inductively coupled plasma optical emission (ICP-OES, PerkinElmer Inc.) following a standard procedure.

Analysis of lipid peroxidation and proline content

Lipid peroxidation was measured in terms of malondialdehyde (MDA) content according to Alatawi [16]. Fresh leaves (0.1 g) were ground and extracted in 1 mL of 10% trichloroacetic acid (TCA), then the supernatant was collected by centrifuging at 5,000 rpm for 10 min. 0.5 mL supernatant (0.5 mL distilled water as control) were homogenized in 0.5 mL of 0.6 % 2-thiobarbituric acid (TBA) and heat in boiling water for 15 min, then cooled until room temperature. The absorbance of the supernatant was measured at 532 nm, 600nm, 450nm.

Proline content was determined by acid ninhydrin method [20]. First, 0.1 g of leaf samples was added to 1 mL of 3% sulfoalicylic acid solution and extracted in a boiling water for 10 min, then centrifuged at 5000 rpm for 10 min. Next, 0.2 mL of supernatant was homogenized and mixed with 0.2 mL of acetic acid and 0.2 mL of 2.5% acid ninhydrin and kept at boiling water for 30 min, after cooled until room temperature, 0.4 mL of toluene treated and then oscillated by the vortex for 30 seconds. After 10 min, supernatant centrifuged at 3000 rpm for 5 min. Finally, the absorbance was scored at 520 nm.

Determination of GSH, GSSG and enzyme activity

The glutathione (GSH) and oxidized glutathione (GSSG) were measured by GSH and GSSG kit (NO. BC1170, NO. BC1180; Beijing Solarbio Science & Technology Co., Ltd., China). LCD enzyme was detected by referring to the LCD kit (NO. MBE21193; Nanjing Malbo Biotechnology Co., Ltd., China). The activities of glutathiones-transferase (GST) and glutathione reductase (GR) was determined following the description by kit (NO. BC0350, NO. BC1160; Beijing Solarbio Science & Technology Co., Ltd., China). Superoxide dismutase (SOD), peroxidase

**Table 1.** Description of 9 experimental treatments.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Days 1-15</th>
<th>Days 15-30</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.4A1 (control)</td>
<td>0.4 mmol·L⁻¹ Al³⁺</td>
<td>0.4 mmol·L⁻¹ Al³⁺</td>
</tr>
<tr>
<td>H₂S + 0.4A1</td>
<td>100 μmol·L⁻¹ H₂S + 0.4 mmol·L⁻¹ Al³⁺</td>
<td>0.4 mmol·L⁻¹ Al³⁺</td>
</tr>
<tr>
<td>PAG + 0.4A1</td>
<td>1 mmol·L⁻¹ PAG + 0.4 mmol·L⁻¹ Al³⁺</td>
<td>0.4 mmol·L⁻¹ Al³⁺</td>
</tr>
<tr>
<td>2A1</td>
<td>0.4 mmol·L⁻¹ Al³⁺</td>
<td>0.4 mmol·L⁻¹ Al³⁺</td>
</tr>
<tr>
<td>H₂S + 2A1</td>
<td>100 μmol·L⁻¹ H₂S + 0.4 mmol·L⁻¹ Al³⁺</td>
<td>0.4 mmol·L⁻¹ Al³⁺</td>
</tr>
<tr>
<td>PAG + 2A1</td>
<td>1 mmol·L⁻¹ PAG + 0.4 mmol·L⁻¹ Al³⁺</td>
<td>0.4 mmol·L⁻¹ Al³⁺</td>
</tr>
<tr>
<td>4A1</td>
<td>0.4 mmol·L⁻¹ Al³⁺</td>
<td>0.4 mmol·L⁻¹ Al³⁺</td>
</tr>
<tr>
<td>H₂S + 4A1</td>
<td>100 μmol·L⁻¹ H₂S + 0.4 mmol·L⁻¹ Al³⁺</td>
<td>0.4 mmol·L⁻¹ Al³⁺</td>
</tr>
<tr>
<td>PAG + 4A1</td>
<td>1 mmol·L⁻¹ PAG + 0.4 mmol·L⁻¹ Al³⁺</td>
<td>0.4 mmol·L⁻¹ Al³⁺</td>
</tr>
</tbody>
</table>

Xing et al. Beverage Plant Research 2024, in press
(POD) and catalase (CAT) was performed according to instructions of the kits (NO. R22262, NO. R3031, NO. R22072; Shanghai Yuanye Biotechnology Co., Ltd, China), respectively.

**Determination of tea components content**

The contents assay viz. tea polyphenols, catechins, amino acids and caffeine was measured according to GB/T 8313-2018, GB/T 8314-2013 and GB/T 8312-2013[18-20], respectively.

**Data statistics and analysis**

All the data were from three independent experiments with three biological repeats. The experimental data were statistically processed using Excel 2016, GraphPad Prism 8.0 and variance analysis software SPSS 20.0 (SPSS Inc. version 22.0, Chicago, IL, USA, 2013). Different lowercase letters on the graphs indicate that the mean values among different H$_2$S conditions under the same Al concentration treatment were statistically different at $p < 0.05$ level, and different uppercase letters represent significant differences among different Al concentration treatments under the same H$_2$S condition at $p < 0.05$ level.

**Results**

**Effects of different treatments of H$_2$S and Al on C. sinensis**

As expected, new root of C. sinensis treated with 2Al and 4Al was less than that of normal 0.4Al culture, but early application of H$_2$S compared to alone Al treatment effectively promoted the root development, while PAG + Al significantly inhibited root growth (Fig. 1). Moreover, application of PAG not only inhibited normal development of root system, but also inhibited the growth of leaves (Fig. 1c, 1f and 1i). Chlorosis, even leaf abscission symptoms in leaves have also occurred (Fig. 1c, 1f and 1i).

To further clarify whether H$_2$S is beneficial for tea root growth under different Al conditions, we explored root activity. We observed higher concentrations (2Al and 4Al), resulted in a greatly decrease in root activity (Fig. 1). And an increase of 37.59%, 58.42%, and 19.55% in root activity under H$_2$S pretreatment compared to the separate 0.4Al, 2Al and 4Al treatments, respectively (Fig. 1). However, exogenous PAG treatment significantly inhibited root activity compared to various Al concentrations (Fig. 1).

**Effects different treatments on fresh and dry weight**

Overall, the total fresh weight (FW) and dry weight (DW) of tea plants were both increased by early application of H$_2$S, while the use of PAG reduced the FW and DW of C. sinensis (Fig. 2e and 2j). Moreover, the results showed that, except for H$_2$S + 4Al, which did not increase FW in the leaves compared to 4Al, the FW of other different tissues under H$_2$S + Al treatments showed an increase in FW compared to the single Al treatment (Fig. 2a–2d). In addition, the DW of other tissues increased under H$_2$S + Al treatments compared to single Al treatment for tea seedlings, except for H$_2$S + 4Al which showed decrease in DW of old leaves compared to 4Al (Fig. 2f–2j).

**Effect of H$_2$S on accumulation and translocation factor of Al in C. sinensis**

There was no significant decrease in content of Al between pre-applied H$_2$S treatment and single Al treatment in young leaves (Table 2). Nevertheless, compared with 0.4Al treatment, content of Al in roots markedly increased when H$_2$S was applied in advance, while accumulation of Al in roots was dramatically reduced when H$_2$S was applied in advance to the 2mM Al and 4mM Al treatments (Table 2). Meanwhile, compared to other treatments within the group, content of Al was the highest in roots when PAG-pretreated was applied in advance, with similar performance in total Al content (Table 2).

Unusually, pretreatment with H$_2$S increased content of Al in mature leaves compared to Al treatment alone, and there was a similar trend of Al accumulation in stems (Table 2). Under normal Al concentration, the translocation factor (TF) of Al of 0.4Al is the highest, which is 1.7 times that of H$_2$S + 0.4Al and 10.625 times that of PAG + 0.4Al (Table 2). Whereas, TF of Al demonstrated H$_2$S + Al > Al > PAG + Al after 2Al and 4Al treatment (Table 2).

**H$_2$S affects ion homeostasis of C. sinensis after different treatments**

Content of Ca increased in the solution with H$_2$S or PAG pre-
Hydrogen sulfide enhanced Al stress in tea plant

Fig. 2  Fresh and dry weight in young leaves (a, f), mature leaves (b, g), stems (c, h), roots (d, i), and total content of Al (e, j) of C. sinensis cultured with different treatments. Different lowercase letters represent significant differences among different H2S conditions under the same Al concentration treatment, and different uppercase letters represent significant differences among different Al concentration treatments under the same H2S condition (p<0.05), as determined by the Duncan test.

Table 2. Effects on content and translocation factor (TF) of Al in C. sinensis under different treatments.

<table>
<thead>
<tr>
<th>Elements</th>
<th>Treatments</th>
<th>YL (mg·kg−1)</th>
<th>ML (mg·kg−1)</th>
<th>S (mg·kg−1)</th>
<th>R (mg·kg−1)</th>
<th>Total content (mg·kg−1)</th>
<th>TF (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Al</td>
<td>0.4Al</td>
<td>551.30 ± 25.52Bb</td>
<td>1240.96 ± 324.00Aa</td>
<td>427.13 ± 12.20Bb</td>
<td>2566.89 ± 96.52Bc</td>
<td>4750.29 ± 207.35Bc</td>
<td>0.85 ± 0.14Aa</td>
</tr>
<tr>
<td></td>
<td>0.4Al+H2S</td>
<td>474.49 ± 57.74Ba</td>
<td>1605.65 ± 30.85Ba</td>
<td>558.06 ± 69.96Ca</td>
<td>5323.33 ± 506.90Cb</td>
<td>7961.52 ± 573.30Bb</td>
<td>0.50 ± 0.04Bb</td>
</tr>
<tr>
<td></td>
<td>0.4Al+PAG</td>
<td>291.67 ± 33.05Cb</td>
<td>1125.82 ± 345.62Aa</td>
<td>537.35 ± 118.21Aa</td>
<td>2352.28 ± 1412.99Bb</td>
<td>2547.12 ± 1623.32Ba</td>
<td>0.08 ± 0.01Bc</td>
</tr>
<tr>
<td></td>
<td>2Al</td>
<td>700.68 ± 14.51Aa</td>
<td>1232.58 ± 102.65Ab</td>
<td>735.12 ± 40.15Ab</td>
<td>15651.00 ± 387.50Ab</td>
<td>18319.37 ± 478.25Ab</td>
<td>0.17 ± 0.01Bb</td>
</tr>
<tr>
<td></td>
<td>2Al+H2S</td>
<td>758.33 ± 51.39Ab</td>
<td>2000.13 ± 209.09Aa</td>
<td>976.59 ± 29.70Aa</td>
<td>8574.85 ± 700.31Bb</td>
<td>12309.90 ± 638.39Ac</td>
<td>0.44 ± 0.05Ba</td>
</tr>
<tr>
<td></td>
<td>2Al+PAG</td>
<td>999.06 ± 45.47Aa</td>
<td>1101.78 ± 48.02Ab</td>
<td>696.60 ± 131.31Ab</td>
<td>28361.41 ± 199.73Aa</td>
<td>31131.86 ± 296.48Ac</td>
<td>0.10 ± 0.01AAb</td>
</tr>
<tr>
<td></td>
<td>4Al</td>
<td>771.52 ± 123.22Ab</td>
<td>1342.59 ± 60.73Aa</td>
<td>675.39 ± 120.29Ab</td>
<td>14741.91 ± 2122.85Ab</td>
<td>17531.41 ± 2218.98Ab</td>
<td>0.19 ± 0.03Bb</td>
</tr>
<tr>
<td></td>
<td>4Al+H2S</td>
<td>819.06 ± 20.35Aa</td>
<td>1475.13 ± 107.29Ab</td>
<td>732.93 ± 62.82Bb</td>
<td>10066.06 ± 835.49Ac</td>
<td>13093.18 ± 954.89Ab</td>
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<tr>
<td></td>
<td>4Al+PAG</td>
<td>1285.44 ± 106.37Ab</td>
<td>1086.46 ± 135.20Ab</td>
<td>756.13 ± 104.12Aa</td>
<td>26682.34 ± 3130.59Ab</td>
<td>29810.37 ± 3150.15Aa</td>
<td>0.12 ± 0.02AAb</td>
</tr>
</tbody>
</table>

Values are the mean ± SD (n = 3). Different lowercase letters represent significant differences among different H2S conditions under the same Al concentration treatment, and different uppercase letters represent significant differences among different Al concentration treatments under the same H2S condition (p<0.05), as determined by the Duncan test.

applied, and this increase was more elevated in 0.4Al than 2Al, 4Al in young leaves, while more increased in 2Al and 4Al than 0.4Al in mature leaves (Table 3). In stems, application of PAG remarkably enhanced the concentrations of Ca under 0.4Al, but decreased content of Ca in 2Al and 4Al (Table 3). Moreover, results in roots showed that content of Ca under H2S + 0.4Al was 2.71 times that of 0.4Al, and content of Ca in PAG + 0.4Al was 4.5 times that of 0.4Al, but H2S + 4Al and PAG + 4Al inhibited content of Ca compared to 4Al, and changes in content of Ca between 2Al, H2S + 2Al, and PAG + 2Al groups were relatively small (Table 3). After H2S combined 2Al significantly improved content of total Ca, while a little effect on 0.4Al and 4Al (Table 3). In addition, the TF of Ca exhibited 0.4Al > H2S + 0.4Al > PAG + 0.4Al, while H2S + 2Al and PAG + 2Al have no significant effect on the TF of Ca compared to 2Al, only H2S + 4Al significantly promoted TF of Ca compared to 4Al (Table 3).

Content of Mg in young leaves were found to significantly inhibited only in H2S + 4Al and PAG + 4Al compared to 4Al, but there was no significant change in content of Mg between treatments at only 4Al in mature leaves (Table 3). The application of exogenous PAG contributed to increase in content of Mg in stems, but H2S had a small effect on the level of Mg compared to Al alone in stems (Table 3). However, H2S significantly increased Mg levels in roots (Table 3). It was found that the change in total content of Mg was not significant under H2S + 4Al compared to 4Al, while content of Mg under other H2S + Al treatments significantly increased compared to Al alone (Table 3). However, the TF of Mg was inhibited by 73.66% under H2S + 0.4Al compared to 0.4Al, 23.82% under H2S + 2Al compared to 2Al, and 30.84% under H2S + 4Al compared to 4Al.
It was demonstrated that $\text{H}_2\text{S}$ promoted an increase in content of Zn in different tissues (Table 3). Meanwhile, the total content of Zn also showed that $\text{H}_2\text{S}$-pretreated significantly promoted the accumulation of Zn in $\text{C. sinensis}$. The effects of $\text{PAG}$ and $\text{H}_2\text{S}$ on TF of Zn under different Al concentrations were also inconsistent. Significantly inhibited TF of Zn was observed in exogenous $\text{H}_2\text{S}$ or $\text{PAG}$ followed by $0.4\text{Al}$, however, TF of Zn showed significant performance as $2\text{Al}>2\text{Al}+\text{H}_2\text{S}+2\text{Al}$, but there was no significant difference in the effect of exogenous $\text{H}_2\text{S}$ or $\text{PAG}$ on TF of Zn at $4\text{Al}$ (Table 3).

Content of Mn further increased after applying $\text{H}_2\text{S} + 2\text{Al}$ and $\text{H}_2\text{S} + 4\text{Al}$ to young leaves, while content of Mn was decreased but not significant in $\text{H}_2\text{S} + 0.4\text{Al}$ compared with simple Al treatment (Table 3). $\text{H}_2\text{S} + \text{Al}$ that dramatically increased Mn levels compared to Al in mature leaves, consistent with the performance in stems (Table 3). In roots, it was $\text{PAG} + \text{Al}$ that significantly increased total content of Mn in tea plants compared to Al alone, but its effects of $\text{H}_2\text{S}$ followed by $0.4\text{Al}$, $0.4\text{Al} + \text{PAG}$ followed by $0.4\text{Al}$, and $0.4\text{Al} + \text{H}_2\text{S}$ followed by $0.4\text{Al}$ were not significant in $\text{H}_2\text{S}$ under different Al concentration treatments and different uppercase letters represent significant differences among different Al concentration treatments under the same $\text{H}_2\text{S}$ concentration treatment, and different lowercase letters represent significant differences among different Al concentration treatments under the same $\text{H}_2\text{S}$ condition ($p < 0.05$), as determined by the Duncan test.

(Tables 3).
Chlorophyll content and photosynthetic parameters analysis

An increase was observed in chl a content under \( \text{H}_2\text{S} \) as compared to Al treatment alone, however, reduction of chl a showed in exogenous PAG, and chl b content has the same performance (Fig. 4a and 4b). Furthermore, total chlorophyll content also has the same trend, and with the increase of Al concentration, the total chlorophyll content of \( \text{H}_2\text{S} + \text{Al} \) increases by 21.15%, 11.59%, and 17.64% compared to Al, respectively (Fig. 4c). Nevertheless, the results of chl a/chl b showed the opposite, namely PAG + Al > Al > \( \text{H}_2\text{S} + \text{Al} \) (Fig. 4d).

Pn under 0.4Al was significantly promoted by application of \( \text{H}_2\text{S} \), but pretreatment with PAG significantly decreased Pn (Fig. 5a). Differently, the effect of applying \( \text{H}_2\text{S} \) and PAG on Pn showed an opposite trend at 2Al, and exogenous application of \( \text{H}_2\text{S} \) and PAG showed significant inhibition compared to 4Al alone (Fig. 5a). Gs showed a consistent trend at 0.4Al and 4Al, with \( \text{H}_2\text{S} + \text{Al} \) > Al > PAG + Al (Fig. 5b). Ci were different under different treatments with different Al concentrations, namely \( \text{H}_2\text{S} + 0.4\text{Al} > 0.4\text{Al} > \text{PAG} + 0.4\text{Al} \), 2Al > \( \text{H}_2\text{S} + 2\text{Al} \) > PAG + 2Al, and PAG + 4Al > 4Al > \( \text{H}_2\text{S} + 4\text{Al} \) (Fig. 5c). The results of Tr under normal Al concentration were consistent with those of Pn, Gs and Ci, but at high concentrations of Al, they showed 2Al > PAG + 2Al > \( \text{H}_2\text{S} + 2\text{Al} \), 4Al > \( \text{H}_2\text{S} + 4\text{Al} \) > PAG + 4Al, respectively (Fig. 5d). Tr agreed with Pn, Gs, Ci results at normal Al concentration, but exhibited 2Al > PAG + 2Al > \( \text{H}_2\text{S} + 2\text{Al} \) and 4Al > \( \text{H}_2\text{S} + 4\text{Al} \) > PAG + 4Al, respectively, at Al stress concentrations (Fig. 5).

Effects of \( \text{H}_2\text{S} \) on Proline, MDA content and

![Fig. 3](image-url) Different changes in ultrastructure of C. sinensis after different treatments (a: 0.4Al, b: \( \text{H}_2\text{S} + 0.4\text{Al} \), c: PAG + 0.4Al, d: 2Al, e: \( \text{H}_2\text{S} + 2\text{Al} \), f: PAG + 2Al, g: 4Al, h: \( \text{H}_2\text{S} + 4\text{Al} \), i: PAG + 4Al). PE: chloroplast membrane, Ch: chloroplast, SG: starch granules, Th: matrix lamellae, OG: osmiophilic granule. Scale bar = 1.0 μm.
Interestingly, MDA content in leaves of H$_2$S pretreatment was inhibited by 3.61% compared to 2Al, whereas preincubation of PAG significantly increased MDA content (Fig. 6a). Proline content significantly accumulated in Al stress compared to 0.4Al, and its content increases by 2.82% under H$_2$S + 2Al compared to 2Al, while pretreatment with H$_2$S before 4Al treatment did not inhibit lipid peroxidation through proline content (Fig. 6b).

**enzyme activity under Al conditions**

Similar tendency was observed in roots and leaves under normal Al, with H$_2$S + 0.4Al compared to 0.4Al not significantly increasing CAT activity by 15% and 16.67%, respectively (Fig. 7a and 7b). CAT showed the highest activity of H$_2$S + 2Al in leaves, but the lowest activity in roots under H$_2$S + 2Al (Fig. 7a and 7b). And CAT activity of leaves at 4Al was higher than that of H$_2$S + 4Al at 4Al and PAG + 4Al, while the CAT activity in roots treated with PAG + 4Al was higher than that of 4Al and H$_2$S + 4Al (Fig. 7a and 7b).
Similarly, POD activity showed the same trend in roots and leaves only under normal Al, with POD activity in PAG + 0.4Al greater than that in 0.4Al and H$_2$S + 0.4Al (Fig. 7c and 7d). Meanwhile, it is noteworthy that POD activity after H$_2$S + 2Al is 3.56 times compared to 2Al in leaves, while the lowest POD activity was observed in the roots at H$_2$S + 2Al, and the same was showed PAG + Al > Al > H$_2$S + Al at 4 Al (Fig. 7c and 7d).

However, there was no significant difference between the treatments at 4Al for the leaves (Fig. 7c).

Compared with 0.4Al treatment, H$_2$S + 0.4Al treatment increased SOD activity in leaves and roots (Fig. 7e and 7f). However, there was no significant difference in SOD activity after applying H$_2$S at 4 mM Al in the roots and leaves (Fig. 7e and 7f).

Fig. 6  The effect of different treatments on MDA (a) and proline content (b) in tea leaves. Different lowercase letters represent significant differences among different H$_2$S conditions under the same Al concentration treatment, and different uppercase letters represent significant differences among different Al concentration treatments under the same H$_2$S condition ($p < 0.05$), as determined by the Duncan test.

Fig. 7  C. sinensis on antioxidant enzyme activities in leaves (a, c, e) and roots (b, d, f) with different treatments. Different lowercase letters represent significant differences among different H$_2$S conditions under the same Al concentration treatment, and different uppercase letters represent significant differences among different Al concentration treatments under the same H$_2$S condition ($p < 0.05$), as determined by the Duncan test.
But the application of H₂S and PAG under 2Al conditions in leaves failed to stimulate the activity of SOD, and pretreatment with H₂S or PAG in roots dramatically decreased the activity of SOD (Fig. 7e and 7f). Furthermore, there was no significant difference in SOD activity among different treatments at 4Al in leaves and roots (Fig. 7e and 7f).

GSH content in leaves under normal Al and 2Al all exhibited H₂S + Al > Al > PAG + Al, but GSH exhibited Al > H₂S + Al > PAG + Al in 4Al (Fig. 8a). The content of GSSG was decreased in PAG-treated at 0.4Al and 2Al, but was increased in PAG + 4Al cultured (Fig. 8b). Noteworthy, no significant change of GSH/GSSG was discovered when H₂S or PAG was added together with Al treatment (Fig. 8c).

It was found that GST activity in leaves was higher in H₂S + 0.4Al than under 0.4Al and PAG + 0.4Al treatments (Fig. 8d). And GST activity exhibited the highest in H₂S + Al, followed by 2Al, and the lowest in PAG + 2Al. Unlike under 4Al where the activity of GST was inhibited by 4Al treatment with H₂S and PAG, although the level of decrease was not significant (Fig. 8d).

Tea leaves exposed to H₂S + 0.4Al treatment exhibited a significant increase of GR activity in comparison with 0.4Al alone and PAG + 0.4Al samples (Fig. 8e). PAG + 2Al and H₂S + 4Al treatments had the lowest GR activity compared with 2Al and 4Al, respectively (Fig. 8e).

LCD activity only showed H₂S + 0.4Al > 0.4Al > PAG + 0.4Al under normal Al concentration in leaves, and there was a significant difference among different treatments (Fig. 9a). However, the application of high concentration Al showed no significant difference under early application of H₂S or PAG (Fig. 9a). What is different in root is that except for the insignificant difference in LCD activity between H₂S + 2Al, 2Al and PAG + 2Al, all other groups showed significant differences, and LCD activity showed H₂S + Al > Al > PAG + Al (Fig. 9b).

**Response of tea components to different treatments**

The synthesis of tea polyphenols was drastically promoted by H₂S + 0.4Al, but slightly deduced by PAG + 0.4Al (Fig. 10a). Compared to 2Al, H₂S + 2Al increased tea polyphenol content, while PAG + 2Al decreased tea polyphenol content, both of which were not significant (Fig. 10a). Similarly, the effects of various treatments based on 4Al on tea polyphenols were not significant (Fig. 10a).

H₂S + 0.4Al treatment induced the highest amount of amino acids after treatment, significantly higher than both Al and PAG + 0.4Al (Fig. 10b). H₂S +2Al and H₂S +4Al did not significantly affect the amino acid content when compared to 2Al and 4Al, respectively (Fig. 10b). With PAG+0.4Al treatment, amino acid content increased compared to 0.4Al, but amino acid content inhibited in PAG + 4Al, and no significant difference between PAG + 2Al and 2Al (Fig. 10b).

Caffeine content at normal Al concentrations showed no significant difference in caffeine content among H₂S + 0.4Al, 0.4Al, PAG + 0.4Al. And H₂S + 2Al, 2Al, PAG + 2Al were the same (Fig. 10c). The caffeine content only after being subjected to PAG + 4Al was greater than that of H₂S + 4Al and 4Al (Fig. 10c).

Results showed that the most abundant one was epicatechin (EC), along with epigallocatechin (EGC), epicatechin gallate (EGCG), gallocatechin (GC), galloloycatechin gallate (GCG), epicatechin gallate (ECG) and catechin (C) detected in tea leaves (Table 4). Compared to 0.4Al, H₂S + 0.4Al increased the total catechin content by 9.48%, while H₂S + 4Al has a 14.45% increase in total catechin content compared to 4Al (Table 4). In each component, C, G and EGC under H₂S + 2Al were increased compared to 2Al. C and EC contents can be generally stimulated under H₂S + 0.4Al and PAG + 0.4Al, while C and EC contents were reduced by H₂S + 4Al (Table 4). Although the contents of EGCG, ECG and GCG of ester catechins were

Fig. 8 / Effect of different treatments on GSH content (a), GSSG content (b), GSH/GSSG (c), GST activity (d) and GR activity (e) in tea leaves. Different lowercase letters represent significant differences among different H₂S conditions under the same Al concentration treatment, and different uppercase letters represent significant differences among different Al concentration treatments under the same H₂S condition (p < 0.05), as determined by the Duncan test.
relatively low, early application of H₂S was still sufficient to stimulate an increase in EGCG, ECG, and GCG at 0.4Al and 2Al (Table 4). It was found that H₂S + 0.4Al increased EGCG by 19.35% compared to 0.4Al, and H₂S + 2Al increased EGCG by 8.70% compared to 2Al. Interestingly, even with early application of H₂S, EGCG, ECG, and GCG were still repressed by 4Al (Table 4).

Discussion

**H₂S induced a well-developed C. sinensis and improved root activity**

It is easy to accumulate too much availability Al³⁺ in the rhizosphere environment of *C. sinensis* suitable for planting in acid soil. Al actually has been regarded as an essential element with dose-dependent effect, which is first reflected in root growth and development [6]. Root growth is stimulated in low concentrations of Al, while high concentrations of Al growth of the root and the plant is delayed [21]. In present study, we also demonstrated that the effects on root development was strongly dependent on the Al concentration, the root system was damaged and new roots were failed to generate by Al stress concentration (Fig. 1a–i). At the same time, it showed that H₂S broke the restriction of Al stress on root development, but PAG promoted the root development hindered by Al stress (Fig. 1a–i). Moreover, pre-treatment with H₂S increased total FW, total DW and root activity of *C. sinensis* to cope with excessive Al inhibition (Fig. 1j and Fig. 2). Recent research has demonstrated that H₂S alleviates the inhibition of plant growth under metal stress in various crop plant species, including mungbean [22], soybean [23] and Miscanthus sacchariflorus [24]. These results indicated that H₂S can effectively alleviate the growth and development of *C. sinensis* under Al stress.

**H₂S promotes plant ion absorption of *C. sinensis* under Al stress**

Maintaining constant intracellular ion homeostasis is crucial for plant adapting to stress environments. Most of Al in *C. sinensis* was contained in root after Al stress (Table 2), affecting the root growth attributes more than the shoot growth attributes, which ultimately limited the growth and development of plants. Similar results were also observed in previous studies [25–26]. H₂S alleviated the enrichment of Al in roots and promoted the TF of Al under Al stress, while PAG increased the accumulation of Al and inhibited the TF of Al (Table 2). Moreover, H₂S application helped to maintain ion homeostasis by accumulating Ca in mature leaves, Mg, Zn and Mn in above-ground parts and increasing the TF of Fe under Al stress (Table 3). It has also been reported that H₂S improves nutrients uptake under Al stress [27]. The results showed that H₂S directly mitigated inhibitory effect of Al toxicity on root growth by decreasing content of Al in root system, thus pre-application of H₂S promoted the root growth and development of *C. sinensis*. Therefore, an increased uptake of Ca, Mg, Zn and Mn has been explained as a consequence of the stimulation of root growth under H₂S.

**H₂S enhances chlorophyll synthesis and ultrastructural stability under Al stress**

We confirmed that excessive accumulation of Al disrupted
HYDROGEN SULFIDE ENHANCED AL STRESS IN TEA PLANT

Xing et al. Beverage Plant Research 2024, in press

Table 4. Effect of different treatments on catechins in C. sinensis

<table>
<thead>
<tr>
<th>Treatment</th>
<th>GC (%)</th>
<th>EGC (%)</th>
<th>C (%)</th>
<th>EC (%)</th>
<th>EGCG (%)</th>
<th>ECG (%)</th>
<th>GCG (%)</th>
<th>Total catechins (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.4Al</td>
<td>0.81 ± 0.03Aa</td>
<td>2.92 ± 0.41Aa</td>
<td>0.24 ± 0.00Ab</td>
<td>3.68 ± 0.42Ab</td>
<td>0.93 ± 0.09Ab</td>
<td>0.38 ± 0.01Aa</td>
<td>0.62 ± 0.06Aa</td>
<td>9.81 ± 1.00Aa</td>
</tr>
<tr>
<td>0.4Al+H2S</td>
<td>0.80 ± 0.01Aa</td>
<td>2.91 ± 0.06Aa</td>
<td>0.24 ± 0.00Aa</td>
<td>4.30 ± 0.17Aa</td>
<td>1.11 ± 0.04Aa</td>
<td>0.39 ± 0.00Aa</td>
<td>0.73 ± 0.01Aa</td>
<td>10.74 ± 0.16Aa</td>
</tr>
<tr>
<td>0.4Al+PAG</td>
<td>0.82 ± 0.01Aa</td>
<td>2.44 ± 0.22Aa</td>
<td>0.25 ± 0.00Aa</td>
<td>3.95 ± 0.13Aab</td>
<td>0.94 ± 0.05Aa</td>
<td>0.37 ± 0.01Aa</td>
<td>0.59 ± 0.04Aa</td>
<td>9.61 ± 0.23Aa</td>
</tr>
<tr>
<td>2Al</td>
<td>0.76 ± 0.01Ba</td>
<td>2.30 ± 0.35Aa</td>
<td>0.24 ± 0.00Aa</td>
<td>3.44 ± 0.29Aa</td>
<td>0.92 ± 0.05Aa</td>
<td>0.37 ± 0.01Aa</td>
<td>0.60 ± 0.04Aa</td>
<td>9.41 ± 1.30Aa</td>
</tr>
<tr>
<td>2Al+H2S</td>
<td>0.79 ± 0.02Aa</td>
<td>2.80 ± 0.40Aa</td>
<td>0.24 ± 0.01Ba</td>
<td>3.35 ± 0.64Aa</td>
<td>1.00 ± 0.15Aa</td>
<td>0.37 ± 0.01Ba</td>
<td>0.62 ± 0.07Ba</td>
<td>8.91 ± 0.76Aa</td>
</tr>
<tr>
<td>2Al+PAG</td>
<td>0.76 ± 0.04Aa</td>
<td>2.58 ± 0.31Aa</td>
<td>0.24 ± 0.00Aa</td>
<td>3.76 ± 0.62Aa</td>
<td>1.06 ± 0.17Aa</td>
<td>0.38 ± 0.01Aa</td>
<td>0.67 ± 0.09Aa</td>
<td>9.91 ± 1.19Aa</td>
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<tr>
<td>4Al</td>
<td>0.75 ± 0.02Ba</td>
<td>2.31 ± 0.45Aab</td>
<td>0.24 ± 0.01Aa</td>
<td>3.59 ± 0.81Aab</td>
<td>0.96 ± 0.11Aa</td>
<td>0.37 ± 0.01Aa</td>
<td>0.57 ± 0.06Aa</td>
<td>7.82 ± 0.03Aab</td>
</tr>
<tr>
<td>4Al+H2S</td>
<td>0.77 ± 0.02Aa</td>
<td>2.11 ± 0.09Bb</td>
<td>0.23 ± 0.00Bb</td>
<td>2.75 ± 0.04Ba</td>
<td>0.82 ± 0.03Bb</td>
<td>0.36 ± 0.00Ca</td>
<td>0.53 ± 0.01Bb</td>
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<tr>
<td>4Al+PAG</td>
<td>0.76 ± 0.01Ba</td>
<td>2.80 ± 0.32Aa</td>
<td>0.25 ± 0.00Aa</td>
<td>4.11 ± 0.51Aa</td>
<td>1.16 ± 0.11Aa</td>
<td>0.33 ± 0.00Bb</td>
<td>0.70 ± 0.04Aa</td>
<td>10.26 ± 0.99Aa</td>
</tr>
</tbody>
</table>

Data are mean values ± SD (n = 3). Different lowercase letters represent significant differences among different H2S conditions under the same Al stress concentration, and different uppercase letters represent significant differences among different Al concentration treatments under the same H2S condition (p < 0.05), as determined by the Duncan test.

Ultrastructural and inhibited several processes, such as chlorophyll contents and photosynthesis. Meanwhile, application of exogenous H2S enhanced chlorophyll contents under Al stress conditions (Fig. 4), which was also reported by Ali[27], who determined that H2S increased chlorophyll a and chlorophyll b by reducing damage to thylakoids in the chloroplast of Brassica napus. It is well known that the chlorophyll content and photosynthetic rate are closely correlated in plants. However, this result indicates that H2S failed to promote photosynthesis in C. sinensis under Al stress (Fig. 5), suggesting that H2S mitigates Al toxicity mainly through the increase of chlorophyll content and ultrastructural stabilization rather than regulating photosynthetic parameters.

H2S regulates the antioxidant system of C. sinensis to resist Al stress

Plants suffering from Al toxicity often exhibit symptoms associated with membrane lipid peroxidation, which result in accumulation of MDA[28]. As previously studied[29], the present results indicated that H2S reduce accumulation of MDA in leaves at 2Al (Fig. 6a). Proline participates in removing membrane lipid peroxidation under stress conditions[30]. However, using exogenous H2S at 2Al concentrations only increases proline content in tea leaves by 2.82% on compared to 2Al alone (Fig. 6b). CAT, POD and SOD are the main antioxidant enzymes in plants, all of which are involved in the inhibition of oxidative stress and lipid peroxidation[31] in plants under excessive Al conditions, thus mitigating Al toxicity in plants[32]. CAT and POD played a role in the leaves under H2S + 2Al, because the activities of CAT and POD in H2S + 2Al were significantly higher than those in 2Al (Fig. 7a and 7c). There is also evidence indicating that H2S-induced alleviation in Al toxicity is attributed to elevated CAT and POD activities, but in barley roots[33]. At the same time, H2S + 2Al and H2S + 4Al reduced CAT, POD and SOD activities in roots, compared with 2Al and 4Al, respectively (Fig. 7b, 7d and 7f). When concerning reactive oxygen species scavenging systems, it is speculated that H2S may alleviate Al toxicity through elevated CAT and POD activities in leaves, while the root system mainly alleviates Al injury through other ways, thus the activities of CAT, POD and SOD decreased. Taken together these data supports the idea that H2S reduces MDA and increases proline levels by regulating antioxidant enzyme activity to alleviate stress in 2Al treatment in leaves.

GSH, the major non-enzymatic antioxidants in the ASA-GSH cycle contributing to plant antioxidant defense[34]. Consistent with previous research results[35], the GSH content in leaves significantly increased after exposure to Al stress. Although exogenous H2S reduced the GSH content in barley leaves[33], it did not decrease GSH content in tea leaves under H2S + 2Al, and only decreased the GSH content under H2S + 4Al (Fig. 8a), indicating that H2S responds to 4Al toxicity by altering GSH content in leaves, triggering the ASA-GSH cycle and improving antioxidant capacity. Consistently, levels of GSSG, which is reduced to GSH, enhanced in leaves during Al stress exposure, and H2S reduce the content of GSSG only in 4Al (Fig. 8b). The GSH/GSSG ratio is also an important indicator of intracellular redox homeostasis within cells. Exogenous H2S modulated the GSH/GSSG ratios by altering GSH and GSSG to varying levels, but resulting in a little change in GSH/GSSG compared to Al stress alone (Fig. 8c). These outcomes are consistent with the findings of previous studies on bermudagrass[36] and rice[37]. GST has been found to catalyze the chelation of GSH with metals and reduce the toxicity of metals to plants[38]. The GST activity under H2S + 2Al not H2S + 4Al stress was significantly enhanced (Fig. 8d), plants rely on the binding to minimize damage, which was consistent with the study of Miscanthus sacchariflorus[39].

GR regulates the redox state of glutathione by converting GSSG into GSH, and also responsible for combating a large amount of reactive oxygen species in plants[39]. The GR activity in this study shown an increase under Al stress which is similar to the observations made in this study[40]. Higher GR activity after H2S + 2Al and lower GR activity under H2S + 4Al were observed, respectively in comparison to 2Al and 4Al (Figure 8e). The above results confirmed that H2S alleviates 2Al stress by regulating substances derived from antioxidant system, whereas the mechanism was complex, resulting in a small pattern of changes in H2S + 4Al compared with 4Al stress alone.

LCD is primarily responsible for catalyses the decomposition of cysteine to H2S. Further enzyme analysis indicated that the externally applied H2S enhanced the activity of LCD relative to Al alone stress, which was especially significant in roots. In Spinacia oleracea also clearly showed an increase in LCD activity with application H2S[41], and an early H2S signal might promoted higher LCD activity than Al stress after 3 hours[42]. Taken together, LCD activity regulates the internal H2S pathway in C. sinensis and plays a more effective role in roots rather than leaves.

H2S altered tea components during Al stress

Various components of the tea plant, including tea polyphenols, amino acids, caffeine, catechins, are not only closely
related to the flavor of the tea plant, but also have an effect when *C. sinensis* is exposed to stress. The synthesis of amino acids, caffeine, and catechins is regulated by Al. In this study, compared with normal Al concentration, the changes in tea polyphenol content under Al stress were not significant, while the content of free amino acids was significantly reduced and the content of caffeine was significantly increased (Fig. 9). At normal Al concentration, early application of H$_2$S increases the content of these substances (Fig. 9), which may be related to the promotion of tea roots growth by H$_2$S. As a major component of the ester type catechins, EGCG has been reported to chelate Al, thus conferring Al tolerance to plants. It was found that the EGC content increased by 8.70% under H$_2$S + 2Al compared to 2Al, and excessive stress of 4Al may lead to a decrease in EGC content, and even with the addition of exogenous H$_2$S, the changes in content remains small under 4Al stress (Table 4). Combined with the above results, it providing further evidence that the part of H$_2$S that promotes the increase of components may have chelated with too much Al at H$_2$S + Al, resulting in a decrease in the final content, or may be caused by severe stress at 4Al.

Conclusions

Our results indicated that H$_2$S may be a pivotal actor in enhancing the resistance of *C. sinensis* to Al stress. Increasing biomass, promoting root activity, reducing accumulation of Al in roots and increasing TF of Al, regulating the content of Ca, Mg, Zn, Mn and Fe and their TF in different tissues, increasing chlorophyll content, maintaining ultrastructural homeostasis, regulating substances related to antioxidant pathways and tea plant components all play key roles in the ameliorating effect. Moreover, compared to 4Al, H$_2$S can better alleviate the stress caused by 2Al.

Author contributions

The authors confirm contribution to the paper as follows: Conceptualization: Shu Z, Sui X and Wang Y; investigation: Shu Z, Huang P and Wan S; data curation: Zhang Y and Xing A; project administration: Shu Z and Wang Y; supervision: Wang Y; resources: Li X; formal analysis: Xing A; visualization: Xing A; writing—original draft preparation: Xing A; writing—review and editing: Xing A, Liu S, Chen X, Li X and Wang Y; funding acquisition: Chen X and Wang Y. All authors read and approved the final manuscript.

Data availability

All data generated or analyzed during this study are available within the article.

Acknowledgments

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Conflict of interest

The authors declare that they have no conflict of interest.

Dates

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Hydrogen sulfide enhanced al stress in tea plant


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