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# Hydrogen Sulfide Alleviates Kiwifruit Ripening and Senescence by Antagonizing Effect of Ethylene

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***Abstract.*** **Kiwifruit (*Actinidia deliciosa*) is a typical climacteric fruit, and its ripening is closely associated with ethylene. In this study, we present evidence that $H_2S$ alleviated ethylene-induced ripening and senescence of kiwifruit. Kiwifruit were fumigated with ethylene released from 0.4 g·L$^{-1}$ ethephon solution or $H_2S$ with 1 mM sodium hydrosulfide (NaHS) as the donor or in combination. Fumigation with ethylene was found to accelerate kiwifruit ripening and $H_2S$ treatment effectively alleviated ethylene-induced fruit softening in parallel with attenuated activity of polygalacturonase (PG) and amylase. Ethylene + $H_2S$ treatment also maintained higher levels of ascorbic acid, titratable acid, starch, soluble protein, and reducing sugar compared with ethylene group, whereas suppressed the increase in chlorophyll and carotenoid. Kiwifruit ripening and senescence under ethylene treatment was accompanied by elevation in reactive oxygen species (ROS) levels, including $H_2O_2$ and superoxide anion and malondialdehyde (MDA), but combined treatment of ethylene plus $H_2S$ alleviated oxidative stress in fruit. Furthermore, the activities of antioxidative enzymes catalase (CAT) and ascorbate peroxidase (APX) were increased by ethylene + $H_2S$ treatment in comparison with ethylene alone, whereas the activities of lipoxygenase (LOX) and polyphenol oxidase (PPO) were attenuated by $H_2S$ treatment. Further investigations showed that $H_2S$ repressed the expression of ethylene synthesis-related genes *AdSAM*, *AdACS1*, *AdACS2*, *AdACO2*, and *AdACO3* and cysteine protease genes, such as *AdCP1* and *AdCP3*. Taken together, our findings suggest that $H_2S$ alleviates kiwifruit ripening and senescence by antagonizing the effect of ethylene through reduction of oxidative stress and inhibition of ethylene synthesis pathway.**

Fruits are consumed by people for food, either as edible products, for culinary ingredients, or for medicinal use for a long time. They are genetically a very diverse group and play a major role in modern society and economy. Fruits are natural sources of vitamins, phytochemicals, and minerals (Canan et al., 2016; Ercisli, 2009; Zorenc et al., 2016). Among them, kiwifruit (*A. deliciosa*) is a highly nutritious berry, which is rich in ascorbic acid, dietary fiber, phenolics, flavonoid, carotenoids, and minerals (Celik et al., 2007; Du et al., 2009). Unripe kiwifruit with hard texture and high acidity is harvested to avoid mechanical injury (Park et al., 2006). Because of rapid postharvest ripening and senescence, consumers are concerned about its appearance and textual quality. As a typical climacteric fruit, kiwifruit postharvest ripening and senescence are very sensitive to ethylene, and even extremely low concentrations (0.1 μL·L$^{-1}$) can stimulate fruit ripening and softening (Harman and McDonald, 1989; Yin et al., 2008). Postharvest fruit ripening and senescence result in profound changes in fruit sensory, texture, and nutritional quality because of cell wall degradation, membrane deterioration, cell structure modification, and cell death (Kumar et al., 2016). Thus, development of new storage protocols for kiwifruit to delay fruit softening as well as to ensure good texture and nutrients is of commercial importance for producers.

Ethylene or ethephon (2-chloroethyl phosphoric acid) is applied commercially to accelerate the ripening of climacteric fruit (Korsak and Park, 2010). Ethylene biosynthesis has been clarified in plant, which involves the conversion of methionine to S-adenosyl methionine (SAM) by SAM synthetase, SAM to 1-aminocyclopropane-1-carboxylic acid (ACC) catalyzed by ACC synthase (ACS), and ACC to ethylene catalyzed by ACC oxidase (ACO) (Adams and Yang, 1979; Mworia et al., 2010). Besides ethylene, ROS and oxidative stress are also responsible for fruit senescence (Tian et al., 2013). ROS is highly reactive and causes oxidative damage to plant cells (Apel and Hirt, 2004), including protein breakdown and lipid peroxidation. Therefore, developing strategies to attenuate ethylene synthesis and inhibit ROS production or promote ROS metabolism could be effective for reducing quality deterioration and extending the storage life of kiwifruit.

Hydrogen sulfide ($H_2S$) is emerging as a new gaseous signaling molecule in diverse organisms, such as bacteria, fungi, worms, human, and plants (Wang, 2012). $H_2S$ participates in multiple processes in plants, including seed germination, stomatal movement, root organogenesis, and photosynthesis (García-Mata and Lamattina, 2010; Jin et al., 2013; Zhang et al., 2009). Furthermore, evidence indicates that $H_2S$ can prolong postharvest shelf life of many fruits and vegetables, including strawberry, kiwifruit, and broccoli by regulating ROS metabolism, antioxidant system, and senescence-related genes expression (Gao et al., 2013; Hu et al., 2012; Li et al., 2014, 2017; Zhu et al., 2014). Moreover, our previous data showed that ethylene synthesis genes, such as *BoACS2* and *BoACS3*, were downregulated by $H_2S$ in dark-induced senescence of broccoli (*Brassica oleracea* L.) (Li et al., 2015). However, there is still a lack of information about the molecular interaction between $H_2S$ and ethylene signaling in fruit ripening.

In the present study, ethylene (released by ethephon), $H_2S$ or in combination was applied to kiwifruit, and the effects of $H_2S$ signal on fruit softening, ROS metabolism, and ethylene synthesis were investigated. The results confirmed the regulatory role of $H_2S$ in ethylene-promoted kiwifruit ripening and senescence.

## Materials and Methods

*Plant materials and treatments.* In this study, kiwifruit (*A. deliciosa* cv. Qinmei)

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were kindly supplied by the Anhui Academy of Agricultural Sciences, Hefei, Anhui province, China. The fruit were harvested in the same day with similar size and maturity (80%) and sorted without physical damages or infections. Kiwifruit were fumigated with different concentrations (0, 0.4, 0.6, and 0.8 g·L$^{-1}$) of ethephon solutions (in 50 mM phosphate saline buffer, pH 7.0) for 8 d, and the concentration of 0.4 g·L$^{-1}$ was selected for the following experiments. Kiwifruit were divided into four groups with 15 kiwifruit for each group. The first group, i.e., the control group which includes five kiwifruit in three replicates was stored in sealed containers (volume 3 L) at 25 °C with a relative humidity of 85% to 90%. The second group ($H_2S$ group) in the container was fumigated with $H_2S$ released from 150 mL of 1 mM NaHS. The third group (ethylene group abbreviated as ETH group) was fumigated with ethylene released from 100 mL of 0.4 g·L$^{-1}$ ethephon solution. The fourth group (ETH + $H_2S$ group) was stored in a container containing 150 mL of 1 mM NaHS and 100 mL of 0.4 g·L$^{-1}$ ethephon solution which were stored in two separate beakers. NaHS and ethephon solutions were renewed daily, and fruit flesh (without peel and seeds) was sampled at different time points for assays.

*Determination of fruit firmness.* Fruit firmness was measured at the equatorial part of each intact kiwifruit by a 2-mm diameter flat probe with a texture analyzer (Model *TA-XT plus*; Stable Micro System, England, UK). The penetration depth was 15 mm, and the cross-head speed was 1.5 mm·s$^{-1}$. When the probe was penetrating the fruit, the firmness of the peel and flesh was obtained sequentially. Fruit firmness values were an average of eight replicates ±standard deviation (SD).

*Determination of the activities of PG and amylase.* Polygalacturonase (PG) (EC 3.2.1.15) activity was determined according to Pathak and Sanwal (1998), and 1 unit (U) of activity was defined as 1 μmol galacturonic acid generated per 1 g fresh weight (FW) per hour. Amylase activity in kiwifruit samples (5.00 ± 0.05 g) was determined using the starch–iodine method according to Collins et al. (1972). One unit of activity was calculated by taking the quantity of the enzyme to reach 50% of the original color intensity.

*Analysis of chlorophyll, carotenoid, ascorbic acid, titratable acidity (TA), starch, soluble protein, and reducing sugar in kiwifruit.* The contents of chlorophyll and carotenoid in kiwifruit were determined in accordance with the methods of Lichtenthaler and Wellburn (1983) and Nath et al. (2011), respectively. 5.0 ± 0.5 g of kiwifruit flesh were sampled. Each analysis was repeated three times, and the results of chlorophyll and carotenoid were expressed as mg·g$^{-1}$ FW.

Ascorbic acid content was determined by the 2,6-dichloroindophenol titrimetric method in accordance with the Association of Official Analytical Chemists method (AOAC, 1984). The assay was repeated in triplicate, and the results were expressed as mg·g$^{-1}$ FW.

The TA of kiwifruit juice was determined by titration with 0.1 M NaOH to pH 8.1 (Jin et al., 2014), and the TA value was expressed as percent of citric acid in kiwifruit juice.

For the determination of starch content in kiwifruit, fruit tissue (5.00 ± 0.05 g) was ground in a cooled mortar with 4 mL of 80% $Ca(NO_3)_2$, and the homogenate was centrifuged at 12,000 *g* for 15 min. The supernatant was discarded and the pellet washed twice with 5 mL of sterile water. Starch content was determined by following the method described by Sanz et al. (1987).

For the determination of reducing sugar and soluble protein, fruit samples (5.00 ± 0.05 g) were ground with 2 mL of 200 mM phosphate buffer (pH 7.0) containing 0.15 M NaCl. Then, the homogenate was centrifuged at 12,000 *g* for 30 min at 4 °C. Reducing sugar was measured by the method of dinitrosalicylic acid according to Miller (1959). The supernatant (0.2 mL) was mixed with 1.5 mL of 3,5-dinitrosalicylic acid and 1.8 mL of $dH_2O$, then the mixture was heated at 100 °C for 5 min and cooled immediately. After adding 25 mL of $dH_2O$ to the mixture, the content of reducing sugar was determined at 540 nm by using a spectrophotometer. Coomassie brilliant blue was used to determine the content of soluble protein. Absorbance was recorded at 595 nm by the method of Bradford (1976). The calibration curves were prepared by using glucose and bovine serum albumin as the standard, respectively. The contents of reducing sugar and soluble protein were expressed as mg·g$^{-1}$ FW.

*Determination of hydrogen peroxide ($H_2O_2$), superoxide anion ($\cdot O_2^-$), and MDA.* For the determination of $H_2O_2$, fresh kiwifruit flesh samples (5.00 ± 0.05 g) were homogenized with 2 mL of chilled acetone and then centrifuged at 12,000 *g* for 30 min at 4 °C and 0.5 mL of the supernatant fraction was mixed with 1.5 mL of $CHCl_3$ and $CCl_4$ (1:3, v/v) mixture. After adding 2.5 mL of distilled water, the mixture was centrifuged at 12,000 *g* for 1 min and the aqueous phase collected for $H_2O_2$ determination (Zhang et al., 2011). $H_2O_2$ content was indicated as mmol·g$^{-1}$ FW.

The generation rate of $\cdot O_2^-$ was determined using hydroxylamine method (Elstner and Heupel, 1976). Fresh kiwifruit flesh samples (5.00 ± 0.05 g) were ground with 3 mL of 50 mM Tris-HCl buffer (pH 7.8), and the homogenate was centrifuged at 12,000 *g* at 4 °C for 30 min. The reaction mixture (0.5 mL) contained 50 mM Tris-HCl buffer (pH 7.5), 0.5 mM XTT [sodium, 3-1-(phenylamino-carbonyl)-3, 4-tetrazolium-bis (4-methoxy-6-nitro), and benzenesulfonic acid hydrate], and 50 μL of sample extracts. Corrections were made for the background absorbance in the presence of 50 U of superoxide dismutase. The value of $\cdot O_2^-$ generation rate was expressed as μg·g$^{-1}$·min$^{-1}$ FW.

MDA content was analyzed by following the method of Zhang et al. (2011). Kiwifruit flesh samples (5.00 ± 0.05 g) were ground in liquid nitrogen and extracted in 3 mL of 0.1% trichloroacetic acid. An extinction coefficient of 155·mM$^{-1}$·cm$^{-1}$ was used to calculate MDA content and the value was expressed as μmol·g$^{-1}$ FW.

*Activity assay of CAT, APX, and guaiacol peroxidase (POD).* Flesh tissue of kiwifruit (5.0 ± 0.05 g) was ground in liquid nitrogen and homogenized with 3 mL 0.2 M phosphate buffer (pH 7.5) supplemented with 1 mM ethylene diamine tetraacetic acid, 1 mM phenylmethylsulfonyl fluoride, 5% polyvinyl pyrrolidone and 1 mM dithiothreitol. The homogenate was centrifuged for 30 min at 12,000 *g* at 4 °C, and the supernatant was collected for enzyme activity analysis. The activity of CAT (EC 1.11.1.6) was measured based on the decrease in $H_2O_2$ concentration (Havir and McHale, 1987). APX (EC 1.11.1.11) activity was determined spectrometrically by the method of Nakano and Asada (1981). POD (EC 1.11.1.7) activity was determined based on the method of Zhang et al. (2008).

*Analysis of activities of LOX and PPO.* For LOX (EC 1.13.11.12), kiwifruit flesh samples (5.00 ± 0.05 g) were homogenized with 5 mL of 200 mM phosphate buffer (pH 6.0) (Hu et al., 2012). The homogenate was centrifuged at 12,000 *g* at 4 °C for 10 min, and 50 μL of the supernatant enzyme extract was assayed in a mixture of 3 mL containing 200 mM borate buffer (pH 6.0), 0.25% linoleic acid and 0.25% *Tween*-20. The reaction was carried out at 25 °C for 5 min, and the activity of LOX was determined by monitoring the changes in absorbance at 234 nm.

Activity of PPO (EC 1.10.3.1) was assayed based on the method by Benjamin and Montgomery (1973). Kiwifruit samples (5.00 ± 0.05 g) were homogenized with 3.0 mL of sodium phosphate buffer (50 mM, pH 6.8). After centrifugation, PPO activity in supernatant was assayed with catechol as substrate. One unit of PPO activity was defined as an increase in 0.01 optical density (OD) value in absorbance at 410 nm·min$^{-1}$. The results were expressed on a FW basis as U·g$^{-1}$.

*RNA extraction and semiquantitative polymerase chain reaction (PCR) analysis.* Total RNA was extracted from 0.15 g flesh tissue of kiwifruit samples using *TransZol Plant* kit (*TransGene*, Beijing, China). First-strand cDNA was synthesized using a reverse transcription kit (*Prime Script™ RT Master Mix*; *Takara*, Tokyo, Japan) from 2.5 μg total RNA. cDNA fragments were amplified by reversed transcript PCR (RT-PCR) with Prime Script RT Master Mix (*TaKaRa*, Tokyo, Japan). Primers used for RT-PCR are shown in Table 1. The expression of actin gene *AdACT* was used as a control. PCR conditions were initially denatured at 94 °C for 5 min, followed by appropriate cycles of 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 30 s. Then, the products of PCR underwent electrophoresis in 1% agarose.

*Statistical analysis.* The data in the article are based on three replicates in each experiment, and the experiments were repeated independently for three times. Statistical significance was tested by one-way analysis of variance using IBM SPSS Statistics (*SPSS* version 20.0; Armonk, NY), and the results

Table 1. Primers used in reverse transcription-polymerase chain reaction.

| Gene name | Forward primer (5′→3′) | Reverse primer (5′→3′) | Accession number |
|---|---|---|---|
| *AdACT* | CAGTGTTTCCCAGTATTGTTG | TTGTAGAATGTATGATGCCAGAT | EF063572 |
| *AdSAM* | GGCTATGCCACTGATGAA | TACTGAGCAGGGACGACA | U17240.1 |
| *AdACS1* | CTCCTGCTCACGTTCATCAC | TGGAGTGGCTACTGTCCTTTA | AB007449 |
| *AdACS2* | TCAGATTCACCAATGACAAAAA | GTACGAGTAAACTATACCGACCC | AB005722 |
| *AdACS3* | GGTTGAAGCGATTAGGTATAGA | TGACAGGAAGATCCAGGAGTTA | Achn317341 |
| *AdACO1* | GCATCATCCTCCTCTTCCA | TGACATTCTGTTGCCATCC | AB003514 |
| *AdACO2* | AAGAAGGCTTTCAGTGGC | GTGTTTCATTGGCGGGAC | HQ293205 |
| *AdACO3* | GATGTCCCGCCAATGAAA | GCCATCCGTCTGAGCAAT | HQ293207 |
| *AdCP1* | AAGGGAAATCACAAACTCACC | AGTCCAGCAAGATCCACAAT | EF530141 |
| *AdCP2* | GACGAGATGGCATTGAAGAA | CACCAGTGAATACACCCGATA | EF530142 |
| *AdCP3* | GGAATGTTTGCGGAGTTG | CTGGCTTGGGAGTATTATCTG | EF530143 |
| *AdCP4* | TTCCTCTGCTTCGCCTTC | TGGTGAGCCACTTCTCGTAA | EF530144 |
| *AdCP5* | GATTGGAGGAAGTATGGGATT | CGTTGGCTAAGGCATTTATT | EF530145 |
| *AdCP6* | ATTCCTCCAGATCCTTCACC | ACATCGCCATCACTTCGTC | EF530146 |

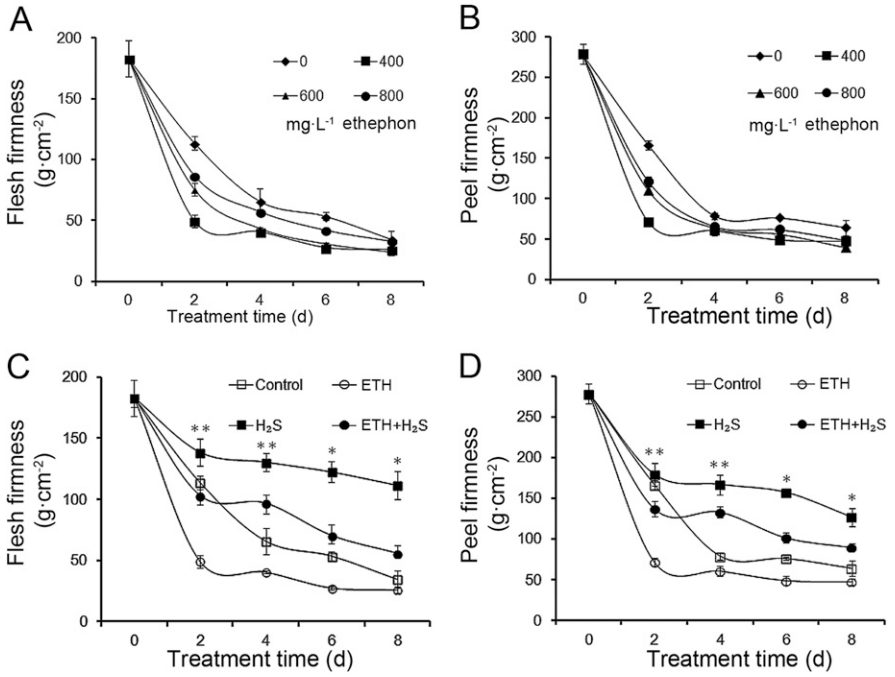


Fig. 1. Effects of ethylene and $H_2S$ on flesh and peel firmness of kiwifruit. Kiwifruit were fumigated with different concentrations (0, 400, 600, and 800 mg·L$^{-1}$) of ethephon aqueous solutions at 25 °C, and the firmness of flesh (**A**) and peel (**B**) was determined on Days 0, 2, 4, 6, and 8. Kiwifruits were then treated with ethylene, $H_2S$ or in combination and the firmness of flesh (**C**) and peel (**D**) was determined on Days 0, 2, 4, 6, and 8. Control: $H_2O$; $H_2S$: 1 mM NaHS; ETH: 0.4 g·L$^{-1}$ ethephon solution; ETH + $H_2S$: 0.4 g·L$^{-1}$ ethephon solution plus 1 mM NaHS. Data are presented as means ± standard deviation ($n = 8$). The symbols * and ** stand for significant difference between ETH and ETH + $H_2S$ at $P < 0.05$ and $P < 0.01$, respectively.

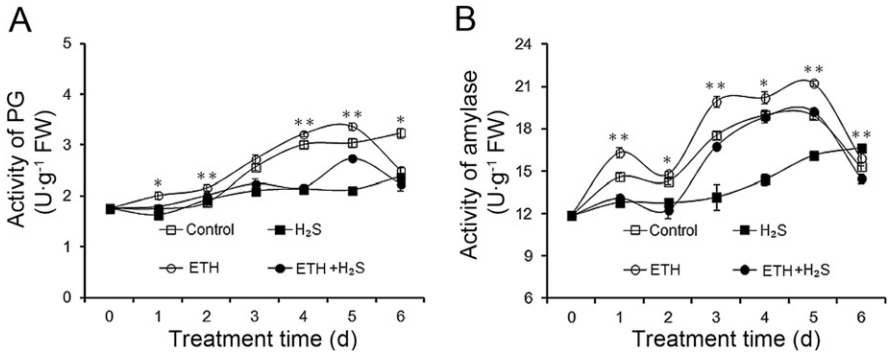


Fig. 2. Effects of ethylene, $H_2S$ and combined treatment on the activities of polygalacturonase (PG) (**A**) and amylase (**B**) in kiwifruit. The activities were determined daily on Days 0 to 6 in kiwifruit treated with water, ethylene, $H_2S$, or in combination at 25 °C. Control: $H_2O$; $H_2S$: 1 mM NaHS; ETH: 0.4 g·L$^{-1}$ ethephon solution; ETH + $H_2S$: 0.4 g·L$^{-1}$ ethephon solution plus 1 mM NaHS. Data are presented as means ± standard deviation ($n = 3$). The symbols * and ** stand for significant difference between ETH and ETH + $H_2S$ at $P < 0.05$ and $P < 0.01$, respectively.

were expressed as the means ± SD. The significant differences were calculated after a significance ($P < 0.01$ or $P < 0.05$) *t* test.

## Results

*Effect of ethylene and $H_2S$ on kiwifruit softening.* Kiwifruit were fumigated with different concentrations (0, 0.4, 0.6, and 0.8 g·L$^{-1}$) of ethephon solutions and the firmness of the flesh and peel was determined (Fig. 1A and B). The firmness values of the flesh and peel decreased gradually in all ethephon treatments and control fruit. A significant decrease in firmness was observed in 0.4 g·L$^{-1}$ ethephon treatment, suggesting that the ripening of kiwifruit was strongly stimulated by external application of 0.4 g·L$^{-1}$ ethephon. To study the possible role of $H_2S$ in ethylene-induced fruit ripening, $H_2S$ released from 1 mM NaHS aqueous solutions was applied to kiwifruit alone or in combination with ethylene. Flesh and peel softening of kiwifruit was markedly accelerated by exogenous ethylene treatment especially during the first 2 d of storage, whereas was inhibited significantly by combined treatment of $H_2S$ and ethylene (Fig. 1C and D). Besides, $H_2S$ could maintain a higher value of flesh and peel firmness compared with control, ethylene, and $H_2S$ +ethylene group. The results indicated that $H_2S$ has an obvious effect in maintaining the firmness of flesh and peel when applied alone or in combination with ethylene.

*Effects of ethylene and $H_2S$, alone and in combination, on PG and amylase activities.* Then, the enzyme activities required for nutrient degradation and fruit softening were determined. PG activity in control and ethylene group rose continuously except a drop for ethylene group on Day 6 (Fig. 2A). By contrast, treatment with $H_2S$ or ethylene + $H_2S$ significantly prevented the increase in PG activity compared with control or ethylene treatment. Amylase activity increased gradually in control, ethylene, and ethylene + $H_2S$, whereas the increase was attenuated in $H_2S$ treatment (Fig. 2B). Besides, ethylene + $H_2S$ maintained a significantly lower level of amylase activity compared with ethylene alone during the whole storage, suggesting that $H_2S$ could alleviate starch degradation in kiwifruit by attenuating amylase activity.

*Effects of ethylene and $H_2S$, alone and in combination, on contents of chlorophylls, carotenoid, ascorbic acid, TA, starch, soluble protein, and reducing sugar in kiwifruit.* To test the effects of $H_2S$ in delaying ethylene-induced ripening and senescence in kiwifruit, functional and nutritional components, including chlorophyll, carotenoid, ascorbic acid, starch, TA, soluble protein, and reducing sugar, were determined. The content of total chlorophyll increased gradually in all treatments during kiwifruit storage (Fig. 3A). However, chlorophyll content in ethylene-treated fruit was significantly higher than that of the control, $H_2S$ and ethylene + $H_2S$ groups after 2 d of storage. $H_2S$ treatment alone maintained a significantly lower level of chlorophyll than other three groups since 3 d of storage. The changes of chlorophyll *a* and *b* showed a similar change in pattern to that of total chlorophyll (Fig. 3B and C).

Carotenoid content in the control group increased continuously with the treatment time, whereas ethylene induced higher accumulation of carotenoid which peaked on Day 3 followed by a decline (Fig. 3D). $H_2S$ alone or in combination with ethylene prevented the accumulation and maintained a significantly lower level of carotenoid compared with control and ethylene group during the whole storage (Fig. 3D).

Ascorbic acid is a natural antioxidant in kiwifruit. The content of ascorbic acid decreased gradually in all treatments during storage (Fig. 3E). However, ethylene treatment induced the lowest ascorbic acid content in kiwifruit compared with control or ethylene + $H_2S$. In all, the results showed that $H_2S$ could alleviate the reduction in ascorbic acid during kiwifruit storage when applied alone or in combination with ethylene (Fig. 3E).

TA decreased progressively with storage time in all samples (Fig. 3F). Ethylene treatment induced a faster decrease in TA compared with other groups, whereas combined treatment of ethylene + $H_2S$ alleviated the decrease especially on Days 2 and 5. Starch content in kiwifruit decreased continuously in all treatments (Fig. 3G). Starch was degradated faster in ethylene-treated group, whereas ethylene + $H_2S$ treatment significantly counteracted the degradation.

Soluble protein content in control kiwifruit fluctuated during the first 5 d of storage followed by a decline on Day 6, whereas ethylene treatment triggered a dramatic decrease in soluble protein after 3 d of storage (Fig. 3H). Ethylene + $H_2S$ and $H_2S$ treatment tended to sustain stable level of soluble protein except a decrease on Day 5 for ethylene + $H_2S$ and the values were significantly higher than that of ethylene group on Days 4 to 6 (Fig. 3H). Reducing sugar content in the

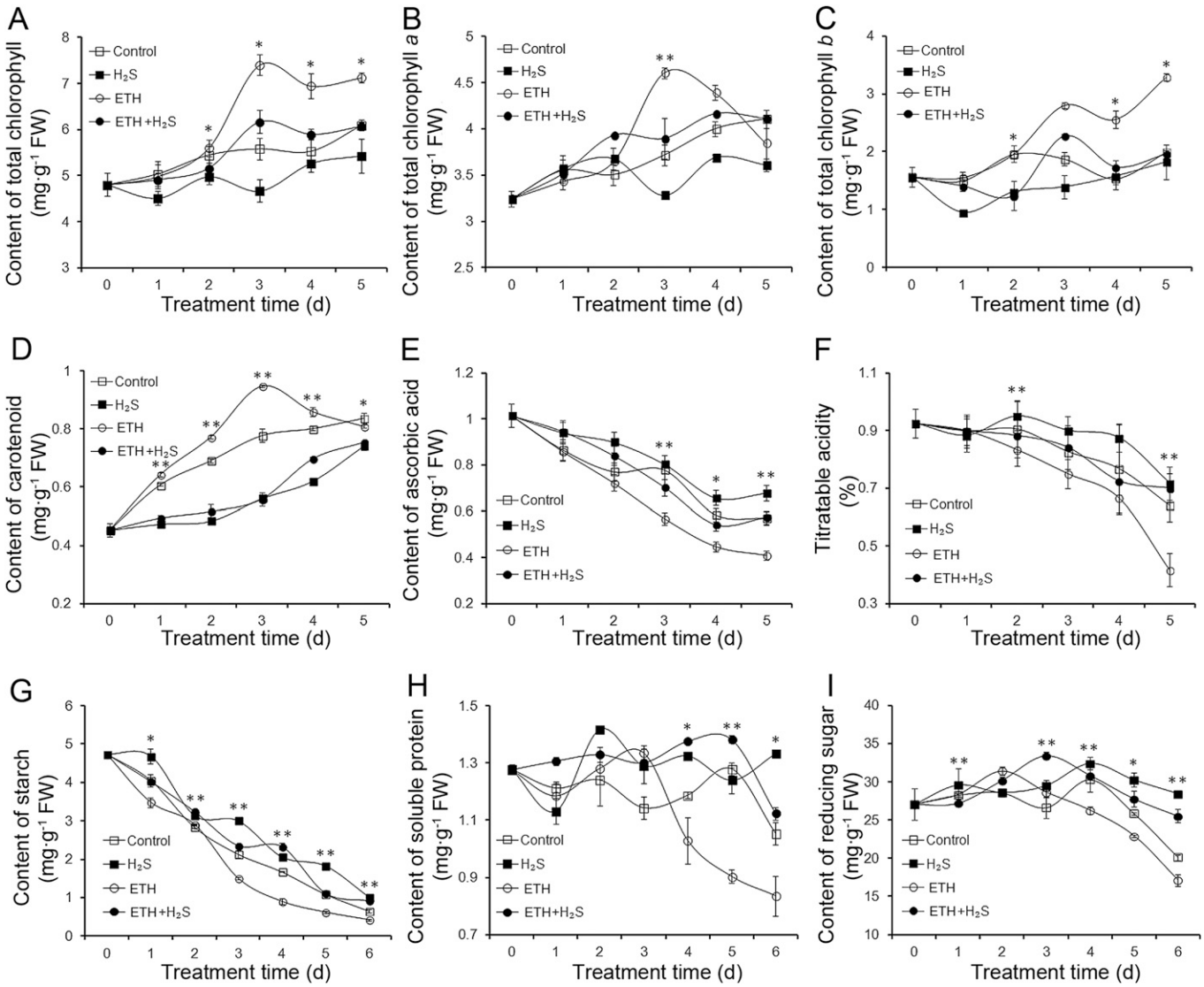


Fig. 3. Effects of ethylene, $H_2S$, and combined treatment on the contents of total chlorophyll (**A**), chlorophyll *a* (**B**), chlorophyll *b* (**C**), carotenoid (**D**), ascorbic acid (**E**), titratable acidity (**F**), starch (**G**), soluble protein (**H**) and reducing sugar (**I**) in kiwifruit. Kiwifruits were treated with water, ethylene, $H_2S$, or in combination at 25 °C, and the data were determined on Days 0 to 5 or 6. Control: $H_2O$; $H_2S$: 1 mM NaHS; ETH: 0.4 g·L$^{-1}$ ethephon solution; ETH + $H_2S$: 0.4 g·L$^{-1}$ ethephon solution plus 1 mM NaHS. Data are presented as means ± standard deviation ($n$ = 3). The symbols * and ** stand for significant difference between ETH and ETH + $H_2S$ at $P < 0.05$ and $P < 0.01$, respectively.

control kiwifruit fluctuated till Day 4 followed by a decrease, whereas ethylene induced a decrease after 2 d of storage (Fig. 3I). Ethylene + $H_2S$ alleviated the reduction in reducing sugar and induced significant higher level since 3 d of treatment compared with ethylene group (Fig. 3I).

*Effects of ethylene and $H_2S$, alone and in combination, on contents of $H_2O_2$ and MDA, and $\cdot O_2^-$ production in kiwifruit.* As ROS are involved in the senescence process of kiwifruit, levels of $H_2O_2$ and MDA, and production of $\cdot O_2^-$ were determined. As indicated in Fig. 4A, $H_2O_2$ content increased gradually during storage time in all treatments. Ethylene treatment induced rapid accumulation of $H_2O_2$, whereas ethylene + $H_2S$ suppressed the accumulation after 2 d of storage. Similarly, an increase in $\cdot O_2^-$ production was observed in all groups. The formation rate of $\cdot O_2^-$ increased rapidly in ethylene treatment, whereas ethylene + $H_2S$ significantly alleviated the increase after 1 d of storage. In addition, $H_2S$ treatment alone induced significantly less $\cdot O_2^-$ production compared with control after 3 d of treatment (Fig. 4B).

MDA, an indicator of lipid peroxidation, showed an consistent increase in all samples during the first 3 d of storage (Fig. 4C). In ethylene group, content of MDA increased dramatically and peaked on Day 3 followed by a decrease, whereas the increase was significantly attenuated by ethylene + $H_2S$ treatment. On Days 2 and 3, MDA content in ethylene sample was about 2- and 3-fold of that in ethylene + $H_2S$ treatment respectively. However, because of decline in MDA content for control and ethylene treatment at later stage of storage, the content in $H_2S$ and ethylene + $H_2S$ treatment was significantly higher than that of control or ethylene group.

*Effects of ethylene and $H_2S$, alone and in combination, on the activities of CAT, APX, POD, LOX, and PPO.* Then, the changes in

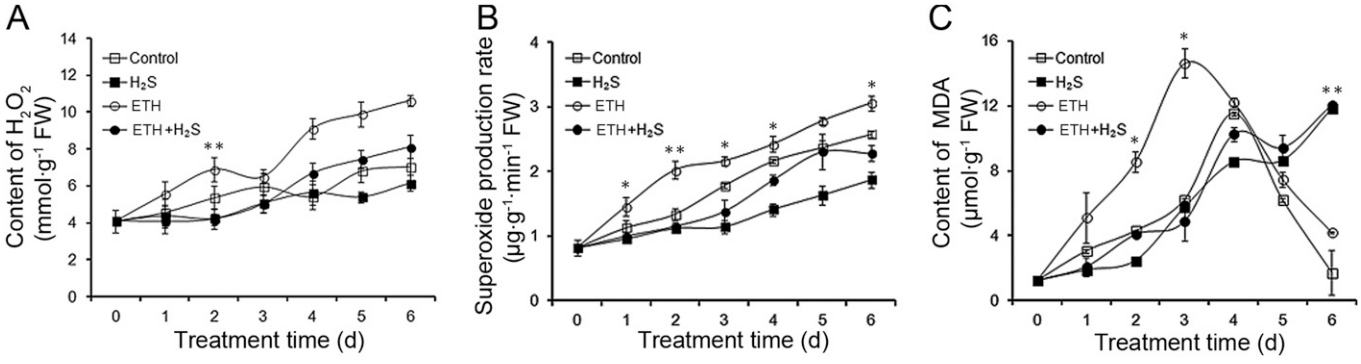


Fig. 4. Changes in oxidative stress of kiwifruit during storage. The content of hydrogen peroxide ($H_2O_2$) (**A**), production of superoxide anion ($\cdot O_2^-$) (**B**) and content of malondialdehyde (MDA) (**C**) were determined on Days 0 to 6 in kiwifruit treated with water, ethylene, $H_2S$, or in combination at 25 °C. Control: $H_2O$; $H_2S$: 1 mM NaHS; ETH: 0.4 g·L$^{-1}$ ethephon solution; ETH + $H_2S$: 0.4 g·L$^{-1}$ ethephon solution plus 1 mM NaHS. Data are presented as means ± standard deviation ($n$ = 3). The symbols * and ** stand for significant difference between ETH and ETH + $H_2S$ at $P < 0.05$ and $P < 0.01$, respectively.

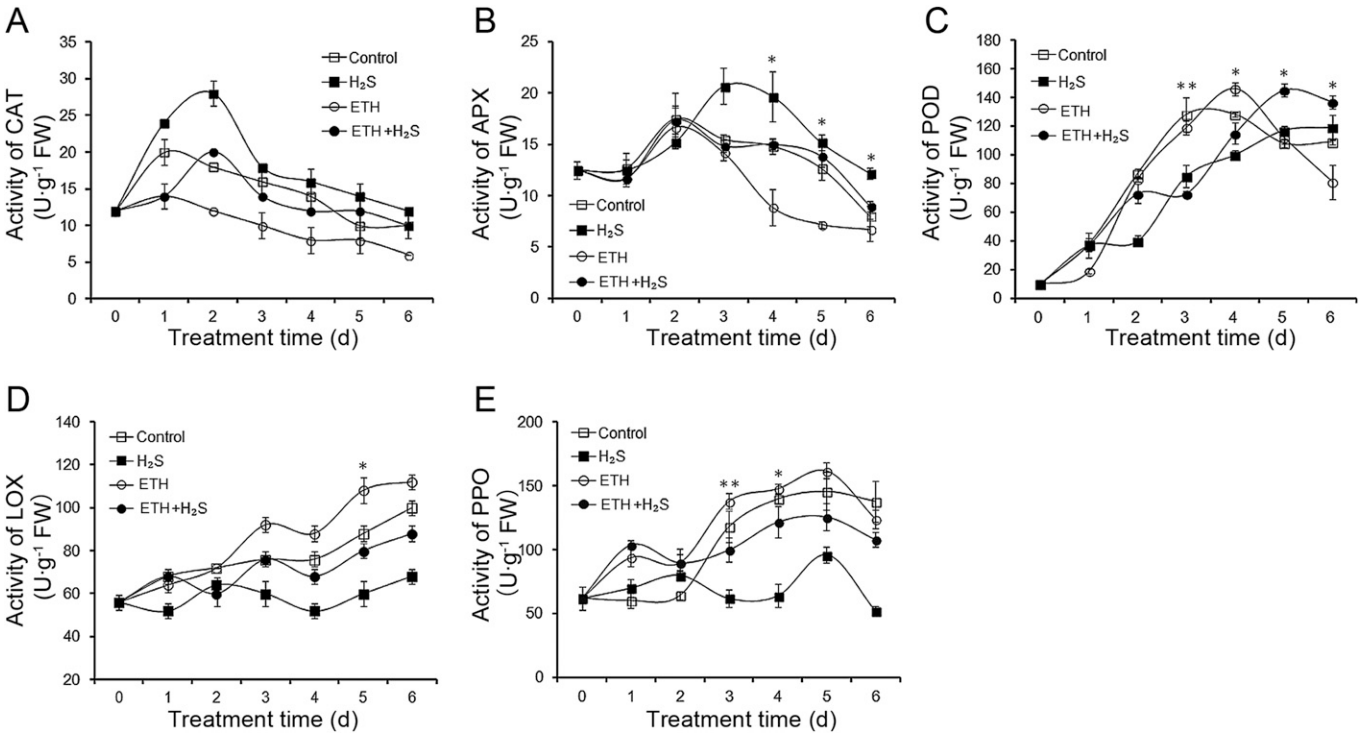


Fig. 5. Effects of ethylene, $H_2S$, and combined treatment on the activities of catalase (CAT) (**A**), ascorbate peroxidase (APX) (**B**), guaiacol peroxidase (POD) (**C**), lipoxygenase (LOX) (**D**), and polyphenol oxidase (PPO) (**E**) in kiwifruit. The activities were determined daily on Days 0 to 6 in kiwifruit treated with water, ethylene, $H_2S$, or in combination at 25 °C. Control: $H_2O$; $H_2S$: 1 mM NaHS; ETH: 0.4 g·L$^{-1}$ ethephon solution; ETH + $H_2S$: 0.4 g·L$^{-1}$ ethephon solution plus 1 mM NaHS. Data are presented as means ± standard deviation ($n$ = 3). The symbols * and ** stand for significant difference between ETH and ETH + $H_2S$ at $P < 0.05$ and $P < 0.01$, respectively.

activities of antioxidative enzymes were investigated. As shown in Fig. 5A, the activity of CAT in control sample increased on Day 1 followed by a gradual decrease, whereas $H_2S$ treatment alone induced higher CAT activity compared with other groups. The activity was attenuated by ethylene treatment, whereas the combined treatment with $H_2S$ induced higher CAT activity after 2 d of storage.

APX activity in control, ethylene, and ethylene + $H_2S$ groups increased and peaked on Day 2 followed by a gradual decrease, whereas the activity in ethylene treatment was significantly lower than other three groups on Days 4 and 5 (Fig. 5B). APX in $H_2S$ group increased till Day 3 followed by a decrease, whereas the activity was still significantly higher than control on Days 3 to 5. Activities of POD increased rapidly for all treatments during the first 4 d of storage followed by a decrease for control and ethylene treatment. POD activity in ethylene treatment was significantly higher than ethylene + $H_2S$ on Days 3 to 4 but significantly lower than the combined treatment on Days 5 and 6. Figure 5D showed that LOX activity in $H_2S$ treatment fluctuated during storage, whereas that of other three groups increased consistently. However, the rapid increase in LOX activity in ethylene treatment was attenuated in ethylene + $H_2S$ after 2 d of storage. As shown Fig. 5E, a consistent increase in PPO activity was observed in the control, ethylene, and ethylene + $H_2S$ except Day 6, whereas the activity in $H_2S$ group displayed a fluctuating pattern. Treatments with $H_2S$ and ethylene + $H_2S$ inhibited the increase in PPO compared with the control or ethylene treatment after 3 d of storage.

*Effects of $H_2S$ on expression of genes involved in ethylene synthesis and protein degradation in kiwifruit.* Kiwifruit ripening and senescence are accompanied by ethylene production and protein degradation. To study whether $H_2S$ has an effect on ethylene synthesis pathway and protein degradation, expressions of *AdSAM*, ACS genes *AdACS1–3* and ACO genes *AdACO1–3*, and cysteine protease genes *AdCP1–6* were measured on Day 1 and 3 of storage. Gene expression of *AdSAM* in the control sample increased on Days 1 and 3 compared with Day 0, whereas $H_2S$ downregulated the expression (Fig. 6A). Besides, $H_2S$ also attenuated the expression of *AdACS1 and AdACS2* on Days 1 and 3, respectively, whereas the expression of *AdACS3* was not detected in both control and $H_2S$ treatment. *AdACO2* and *AdACO3* were highly expressed in control samples, whereas $H_2S$ treatment significantly repressed their expressions on Days 1 and 3. $H_2S$ did not show obvious effect on the expression of *AdACO1*. The expression of cysteine protease genes *AdCP1* and *AdCP3* increased during kiwifruit storage, whereas $H_2S$ significantly repressed their expressions (Fig. 6B). The expressions of other cysteine protease genes were not significantly changed by $H_2S$.

## Discussion

Kiwifruit is highly perishable after postharvest ripening, leading to short storage and shelf life. Flesh firmness is an important characteristic for defining postharvest quality of kiwifruit. In the present work, we found that ethephon at 0.4 $g \cdot L^{-1}$ is effective in accelerating peel and flesh softening in kiwifruit, and the combination of $H_2S$ alleviated ethylene-induced fruit ripening (Fig. 1). Fruit softening is highly associated with physiological events, such as release of galactose from pectic polymers catalyzed by PG and starch hydrolysis catalyzed by amylase (Gao et al., 2013; Hu et al., 2012). Consistently, increased PG and amylase activities are observed in both control and ethylene treatment, whereas $H_2S$ significantly alleviated the increase (Fig. 2). Proteases, which were initially considered to be purely degradative enzymes involved in intracellular protein turnover, are shown to participate in the regulation of many critical physiological and cellular processes (Ehrmann and Clausen, 2004). A recent work found that a number of cysteine protease genes increase their expression during tomato (*Solanum lycopersicum*) fruit ripening, suggesting the role of protein degradation in fruit ripening (Wang et al., 2017). In our work, $H_2S$ significantly repressed the expression of cysteine protease genes *AdCP1* and *AdCP3*, suggesting the alleviated protein degradation in $H_2S$-treated fruit (Fig. 6B).

Fruit ripening and senescence are a complex process associated with physiological and biochemical changes. The contents of several functional or antioxidant components, including chlorophyll, carotenoid, ascorbic acid, soluble protein, and reducing sugar, are determined. During postharvest storage, we observed an obvious decrease in ascorbic acid, soluble protein, and reducing sugar in ethylene treatment, whereas combined treatment with $H_2S$ could alleviate such a decrease (Fig. 3). During kiwifruit storage, chlorophyll and carotenoid increased continuously in ethylene treatment, but $H_2S$ tended to inhibit the increase. Consistent to previous report that ethephon treatment decreased TA value in kiwifruit (Zhang et al., 2012), descending TA value was also observed in ethylene treatment during kiwifruit storage (Fig. 3F). However, $H_2S$ or ethylene + $H_2S$ sustained higher TA value compared with ethylene treatment.

Fruit ripening and senescence are highly related to the overproduction of superoxide anion ($\cdot O_2^-$) and $H_2O_2$ (Tian et al., 2013). With fruit ripening and senescence, ethylene induced higher levels of $H_2O_2$ and $\cdot O_2^-$ compared with control, whereas ROS accumulation was attenuated in ethylene + $H_2S$ treatment (Fig. 4A and B). Augmented level of ROS induces oxidative stress, but plants have an efficient enzymatic system and a number of low-molecular-mass antioxidants for detoxification of these oxygen radicals. Antioxidant enzymes CAT, APX, and POD are required for $H_2O_2$ decomposing

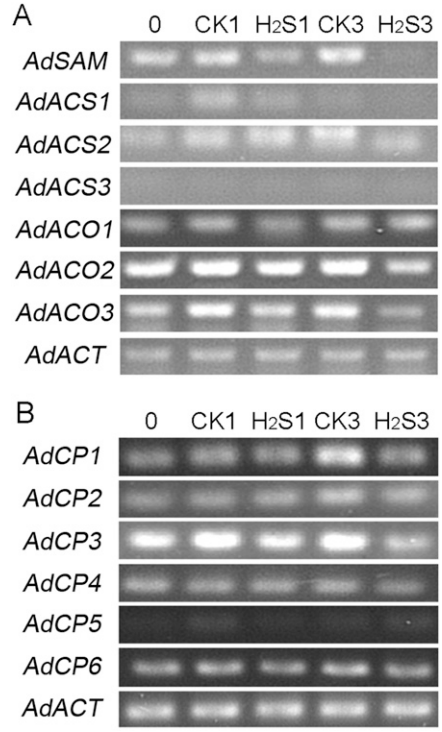


Fig. 6. Effects of $H_2S$ on relative expression of ethylene biosynthesis genes (**A**) and protein degradation related genes (**B**). The expression of *AdACT* gene was used as a control. Ethylene pathway genes, including S-adenosyl methionine synthetase (*AdSAM*), 1-aminocyclopropane-1-carboxylic acid (ACC) synthase genes (*AdACS1*, *AdACS2*, and *AdACS3*), ACC oxidase genes (*AdACO1*, *AdACO2*, and *AdACO3*), and cysteine protease genes *AdCP1–6* were assayed on Days 0, 1, and 3 in postharvest kiwifruit subjected to water or 1 mM NaHS treatment at 25 °C. CK: water control; $H_2S$: 1 mM NaHS.

to water and thus constitute the primary antioxidant defense (Gill and Tuteja, 2010). In the present study, ethylene + $H_2S$ enhanced the activities of CAT and APX compared with ethylene alone (Fig. 5A and B). Besides, an increased level of ascorbic acid also helped to scavenge $H_2O_2$ in ethylene + $H_2S$ treatment (Fig. 3E). Lipid peroxidation mediated mainly by LOXs is involved in fruit ripening and senescence, and LOXs are of a large family of plant enzymes that catalyze the hydroperoxidation of polyunsaturated fatty acids and lead to the production of MDA (Havir and McHale, 1987). In our study, ethylene induced MDA accumulation in the first 4 d of storage, whereas ethylene + $H_2S$ sustained lower levels of MDA and LOX activity, suggesting that lipid peroxidation stress in postharvest kiwifruit was alleviated by $H_2S$ (Figs. 4C and 5D). Besides, the activity of PPO, responsible for the enzymatic browning of fruit by catalyzing polyphenols to quinines (Tomás-Barberán and Espín, 2001), was inhibited by $H_2S$ treatment (Fig. 5E). These observations suggest that $H_2S$ delayed fruit ripening and senescence by inhibiting the accumulation of ROS and alleviating oxidative stress in postharvest kiwifruit.

Ethylene plays an important role in climacteric fruit ripening and senescence. In the present work we found that $H_2S$ repressed the expression of ethylene synthesis related genes *AdSAM*, *AdACS1*, *AdACS2*, *AdACO2*, and *AdACO3* compared with control. In consistent, $H_2S$ was found to downregulate the expressions of *BoACS2* and *BoACS3* in broccoli (Li et al., 2015). A recent work also provides evidence that $H_2S$ delays senescence of green leafy vegetable, pak choy (*Brassica rapa* subsp. *Chinensis*), by inhibiting both the production of ethylene and the action of ethylene (Al Ubeed et al., 2017).

In conclusion, our results indicated that $H_2S$ could alleviate postharvest ripening and senescence of kiwifruit and maintain high fruit quality by decreasing ROS accumulation, improving natural antioxidant contents, and reducing lipid peroxidation. Besides, we provided strong evidence that $H_2S$ may play an antagonizing role in the pathway of ethylene by inhibiting the expression of ethylene biosynthesis genes.

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