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Alleviation of aluminum toxicity by hydrogen sulfide is related to elevated ATPase, and suppressed aluminum uptake and oxidative stress in barley

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ABSTRACT

Greenhouse hydroponic experiments were performed to evaluate potential role of H_2S on Al toxicity in barley seedlings. Seedlings pretreated with 200 μM NaHS as a donor of H_2S for 24 h and subsequently exposed to 100 μM $AlCl_3$ for 24 h had significantly longer roots than those without NaHS. The promoted root elongation was correlated with a substantial decrease in Al-induced overproduction of lipid peroxidation, electrolyte leakage and Al accumulation in roots, and a marked increase in Al-induced depress activities of Na^+K^+-ATPase and H^+-ATPase. The alleviating role of H_2S on Al-induced toxicity was also found in a time- and dose-dependent experiment. Addition of 200 and 400 μM NaHS to 100 μM $AlCl_3$ effectively alleviated Al-toxicity, markedly diminished Al-induced MDA accumulation, and increased chlorophyll content, net photosynthetic rate (Pn) and maximal photochemical efficiency (Fv/Fm) compared with Al alone. Exogenous H_2S significantly elevated depressed CAT activities, and further improved root POD activity. Moreover, NaHS decreased Al accumulation, but elevated concentrations of S, P, Ca, Mg and Fe in plants. These data suggest that H_2S-induced alleviation in Al toxicity is attributed to reduced Al uptake and MDA accumulation, improved uptake of P, Ca, Mg and Fe, and elevated ATPase and photosynthetic performance.

1. Introduction

Aluminum (Al) is a major yield-limiting factor in acidic soils, in which it becomes soluble into its ionic form causing phytotoxic effects even at micromolar level [1]. Moreover, excessive Al in the diet may impair kidney function, cause seizures and reduce mental alertness, as well as other chronic disorders [2]. Correspondingly, it is urgently necessary to elucidate the mechanism of Al accumulation/tolerance to develop approaches for preventing its accumulation in plants, so as to alleviate Al-toxicity in plants and minimize the associated health risks from exposure to high Al-containing foods.

Root growth inhibition is the primary symptom of Al toxicity [3]. Meanwhile, one of the earlier responses of Al toxicity is over accumulation in reactive oxygen species (ROS) and subsequent production of lipid peroxidation [4,5]. The high ROS scavenging ability by the activation of antioxidative system can be resulted into enhanced Al tolerance because adequate antioxidant enzymes and other antioxidant metabolites may help in removing excess ROS thus inhibiting lipid peroxidation [5]. It has been shown that elevated ROS level caused by Al stress activated expression of genes related to antioxidant enzymes such as superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), peroxidase (POD, EC 1.11.1.7) and glutathione-S-transferase (GST, EC 2.5.1.18) [6–8], resultantly conferring Al tolerance. In a recent study, Abu-Romman and Shatnawi [9] demonstrated that barley SOD gene (HvSOD) was involved in antioxidative pathway under various environmental stresses. Recently, the role of adenosine triphosphatases (ATPase), especially H^+-ATPases, in Al resistance in plants has been demonstrated [10,11]. Also Al-induced alteration of root surface potential causes reduction in Mg^{2+}-ATPase, which is the substrate for Mg-dependent ATPase of plant plasma membrane [12].

Hydrogen sulfide (H_2S), a colorless gas with foul odor of rotten eggs, is known as the third gaseous transmitter in mammalian cells after nitric oxide and carbon monoxide [13]. Recently, its role as to promote wheat seed germination [14], induce stomatal closure and to participate in abscisic acid-dependent signaling [15] was reported. Also it has antioxidative response against abiotic stresses such as copper, boron, drought and osmotic stresses

Abbreviations: APX, ascorbate peroxidase; ATPase, adenosine triphosphatase; CAT, catalase; Ci, intercellular CO_2 concentration; Fv/Fm, maximal photochemical efficiency of PSII; GR, glutathione reductase; Gs, stomatal conductance; GST, glutathione-S-transferase; H_2S, hydrogen sulfide; MDA, malondialdehyde; NaHS, sodium hydrogen sulfide; Pn, net photosynthetic rate; POD, guaiacol peroxidase; REL, relative electrolyte leakage; ROS, reactive oxygen species; RRE, relative root elongation; SOD, superoxide dismutase; SPAD, soil plant analysis development; TBA, thiobarbituric acid; Tr, transpiration rate.

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[14–17]. Hence, the questions arise whether H_2S participates in Al tolerance and does ATPase have any crucial role in response to Al stress? Thus further study is needed to test the hypotheses that external H_2S could act as a regulator or antioxidant intervention strategy in preventing Al toxicity, its alleviating role is related to ATPase, and to get a better understanding of how plants adjust to an adverse environment.

The present study demonstrates the potential role of H_2S in modulating Al-induced oxidative stress, Al uptake and translocation, ATPase and photosynthetic performance, and plant growth in barley plants coping with Al stress. Our results support the hypotheses that H_2S reduces Al concentration, suppresses ROS production and alleviates Al-induced oxidative damage in plants and helps maintain ATPase and photosynthetic performance and plant growth under Al stress.

2. Materials and methods

2.1. Plant material and growth condition

2.1.1. Short-term experiment

Hydroponic experiment was carried out on Huajiachi Campus, Zhejiang University, Hangzhou, China. Uniform healthy seeds of barley (*Hordeum vulgare* L. var. ZAU 3) were surface sterilized by soaking in 2% H_2O_2 for 30 min, rinsed with distilled water seven times and germinated on moist filter paper in an incubator at 20 ± 1 °C for 2 days. Germinating caryopses were transferred to net trays floated on containers filled with 5 L of basic nutrient solution [18] at pH 5.8 in greenhouse. Seven-day-old uniform seedlings with average tap root about 9.5 cm were transferred to 0.5 mM $CaCl_2$ solution at pH 4.3 containing with or without H_2S donor (NaHS, Sigma) [19] for 24 h prior to a further 24 h exposure to 0 or 100 μM $AlCl_3$. Al as $AlCl_3$ and H_2S as NaHS were added to $CaCl_2$ solution in corresponding containers to form five treatments: control, 48 h in 0.5 mM $CaCl_2$ solution without Al and NaHS; pre-S, 24 h pretreated with 200 μM NaHS, then transferred to 0.5 mM $CaCl_2$ solution without Al for 24 h; Al, 24 h growth in 0.5 mM $CaCl_2$ solution, then exposure to 100 μM $AlCl_3$ for 24 h; pre-S + Al, 24 h 200 μM NaHS pretreatment followed by 24 h 100 μM $AlCl_3$ exposure; and pre-Na_2SO_4 + Al, 24 h 200 μM Na_2SO_4 pretreatment followed by 24 h 100 μM $AlCl_3$. To verify the possible role of H_2S released by NaHS, 200 μM Na_2SO_4 was used as the control of NaHS. The experiment was laid in a completely randomized design with 4 replicates containing 15 plants each replicate per treatment. Root lengths were measured with a ruler keeping millimeter precision before and after treatment and then root elongation was calculated. Root samples were rinsed with 0.5 mM $CaCl_2$ solution, used immediately or frozen in liquid nitrogen and stored at −80 °C for analysis.

2.1.2. Long-term experiment

As to time and dose dependent experiment, seedlings were grown on net trays floating on containers as mentioned above, but each having approximately 9 L basal nutrient solution. After 10 days (two-leaf stage), Al as $AlCl_3$ and H_2S as NaHS were added to basal nutrient solution [18,20] in corresponding containers to form five treatments: control, basal nutrient solution without Al and NaHS; Al, 100 μM $AlCl_3$; Al + 100S, 100 μM $AlCl_3$ + 100 μM NaHS; Al + 200S, 100 μM $AlCl_3$ + 200 μM NaHS; Al + 400S, 100 μM $AlCl_3$ + 400 μM NaHS. The experiment was laid in a completely randomized design with three replicates containing 25 plants each replicate per treatment. Solution pH was adjusted to 4.3 ± 0.1 with NaOH or HCl on daily basis, as required. The solution was continuously aerated with pumps and renewed every 5 days. Plant samples for antioxidant enzyme activities were collected after 1, 5, 15 and 25 days Al exposure. Fresh samples were immediately frozen in liquid nitrogen and stored frozen at −80 °C for further analyses or directly used for biochemical assays.

2.2. Measurements of plant growth traits and S, Al and other elemental concentrations

After 25 days treatment, plants were uprooted and separated into roots and tops (shoots and leaves), plant height and root length were simultaneously measured, and then dried at 75 °C and weighed. Contents of Al and elements including S, P, K, Ca, Mg and Fe were determined by inductively coupled plasma atomic emission spectroscope (ICP-AES, IRIS/AP optical emission spectrometer, Thermo Jarrel Ash, San Jose, CA) after digesting samples with HNO_3–$HClO_4$ (2:1, v/v).

2.3. Examination of root Al distribution

After 24 h Al treatments, roots were immersed in 20 mM Na_2-EDTA solution for 15 min, washed in deionized water for 5 min, stained with 10 mM MES buffer (pH 5.5), containing 100 mM Morin (Sigma–Aldrich) for 30 min in dark. After a further wash in MES buffer, the images of longitudinal and transverse sections of root tips were obtained using a Zeiss confocal microscope (Axioplan 2 connected with LSM 510, Carl Zeiss, Oberkochen, Germany) at 488 nm/515 nm (Argon laser) excitation/emission wavelength [21].

For Al content, 10 root tips (0–1 cm) were excised and placed in a plastic tube containing 1.5 ml of 2 N HCl for at least 24 h shaking. The Al concentration in the solution was measured with ICP-AES [5].

2.4. Measurement of chlorophyll content and photosynthesis parameters

After 25 days of treatment, chlorophyll content (expressed as SPAD value measured with a chlorophyll meter Minolta SPAD-502, Japan) [13] and photosynthesis parameters were measured on the second uppermost fully expanded leaves with five replicates. A LI-6400 portable photosynthesis system (LI-COR, Lincoln, NE) was used to measure net photosynthetic rate (Pn), stomatal conductance (Gs), transpiration rate (Tr), and intercellular CO_2 concentration (Ci) [22]. The maximal photochemical efficiency of PSII (Fv/Fm, the ratio of variable fluorescence to maximal fluorescence) was synchronously measured using a portable pulse-modulated fluorometer (FMS-2 Hansatech Instruments Ltd., England).

2.5. Determination of relative electrolyte leakage (REL) and lipid peroxidation

Plasma membrane integrity in roots was measured in terms of electrolyte leakage. Root tissues (100 mg) were cut into small pieces and vibrated for 30 min in deionized water followed by measurement of conductivity of bathing medium (EC_1). The samples were boiled for 15 min and again measured the conductivity (EC_2) [5]. Percent relative electrolyte leakage (REL) was determined using the following formula:

$$\mathrm{REL}(\%) = \frac{EC_1}{EC_2} \times 100$$

The level of lipid peroxidation was measured as the amount of malondialdehyde (MDA) determined by the thiobarbituric acid (TBA) reaction [20].

2.6. Assays of antioxidant enzyme activities

Approximately 0.5 g of plant tissue was extracted for enzyme assays. SOD, CAT, POD, APX and GR activities were determined

according to Chen et al. [23]. Glutathione S-transferase (GST) activity was determined using a GST colorimetric activity assay kit (Jiancheng Bio Co., Nanjing, China). The reactions contained 50 mM potassium phosphate (pH 6.5) at 25 °C, aliquots of enzyme extract, 5 mM GSH, 0.4 mM CDNB, and 1% (v/v) ethanol in a final volume of 1 ml. Reactions were initiated with addition of the CDNB substrate in ethanol. Enzymatic formation of 2,4-dinitrophenyl-S-glutathione at 340 nm ($E = 9.6\ mM^{-1}\ cm^{-1}$) was monitored for 5 min and corrected for non-enzymatic controls. All spectrophotometric analyses were conducted on a Shimadzu UV-2410PC spectrophotometer. ATPase activity was determined by measuring the release of Pi using the activity assay kit (Jiancheng Bio Co., Nanjing, China; http://www.njjcbio.com/html/search.php).

2.7. Statictical analysis

Statistical analyses were performed with Data Processing System (DPS) statistical software package [24] using one-way ANOVA followed by the Duncan's Multiple Range Test (SSR) to evaluate significant treatment effects at significance level of $P \leq 0.05$.

3. Results

3.1. H_2S donor alleviated Al-induced inhibition of root elongation and biomass

The protective role of H_2S on A1-induced inhibition in barley was examined by pre-treating seedlings with 200 μM NaHS for 24 h, and subsequently subjecting to 100 μM $AlCl_3$ for 24 h (short-term experiment; Fig. 1). Al stress alone caused 63.6% reduction in root length compared with control. However, when pretreated with H_2S donor NaHS before Al exposure (i.e. pre-S + Al), the detrimental effects on root length were markedly declined. In contrast to NaHS pretreatment, seedlings pretreated with 200 μM of Na_2SO_4 had no obvious effects (Fig. 1A). Meanwhile, little visible difference of symptom was observed between Pre-S (pretreated with NaHS and then grown under normal condition) and control, conveying that NaHS itself conferred no toxicity to seedlings and showed no negative effect on plant growth. Also, H_2S by itself did not cause root inhibition.

In the long-term experiment, 25 days 100 μM Al exposure posed severe biomass reduction. Addition of NaHS in 100 μM Al solution significantly alleviated, with level-depending effect, Al-induced growth inhibition and symptoms of chlorosis and necrosis on leaves (Supplemental Table 2, Supplemental Fig. S1). Addition of 200 and 400 μM NaHS in 100 μM Al solution (Al + 200S, Al + 400S) increased plant height by 14.6%, 15.1%, root length by 18.7%, 106%, and biomass by 35%, 55.3%, respectively, compared with Al-alone treatment (Al). However, use of 100 μM NaHS was not effective in ameliorating Al toxicity on biomass.

3.2. H_2S suppressed Al uptake in barley plants under Al stress

Root Al concentrations were very low in plants grown in non-Al medium (both in control and pre-S alone), but increased to 4.3 nmol root tip^{-1} when treated with 100 μM Al. Presence of NaHS in Al-stressed medium (i.e. pre-S + Al) suppressed Al uptake, and root tip Al content reduced by 23% compared with Al alone treatment (Fig. 1B). Similar trend was observed in fluorescence images illustrating effect of pretreatment without or with 200 μM NaHS on Al localization in longitudinal and cross sections of root tips (Fig. 2). i.e. root tips from Al-alone solutions exhibited more fluorescence than NaHS pretreatment followed by Al exposure. The longitudinal section of root tips of pre-S + Al yielded 17% significantly less fluorescence intensity compared with Al-alone treatment (Fig. 2A and B). This decrease in fluorescence was parallel with significant reduction of Al content in root tips by pre-S + Al treatment (Fig. 1B).

In the long-term experiment, NaHS addition reduced Al concentration in shoots and roots compared with Al-alone treatment, with the largest reduction in Al + 400S treatment (cf. 66% and 37% reduction), followed by Al + 200S (57% and 22%) and Al + 100S (10% and 5%), respectively. On the basis of shoot Al accumulation, Al + 200S was the best treatment by reducing 43% Al accumulation in shoots as compared to Al alone. The result of 100 μM NaHS treatment was not statistically different from that of Al-alone treatment; and increasing NaHS to 400 μM was not effective on further reduction in shoot Al content as compared to 200 μM NaHS (Supplemental Table 2).

3.3. Effect of Al and H_2S on nutrient content

As shown in Table 1, exposure to 100 μM $AlCl_3$ alone caused significant reduction in P, Mg and Fe in both shoots and roots, while reduction in Ca concentration was only observed in roots. Application of NaHS along with 100 μM $AlCl_3$ promoted the concentration of P, Ca, Mg and Fe in plants. As compared with Al alone treatment, element concentration of Al + 200S/Al + 400S treatments in shoots enhanced P, Ca, Fe by 71%/107%, 61%/93%, 40%/44%; while in roots Ca, Mg, Fe respective increased 7%/9%, 37%/84%, 33%/34%. Al alone treatment decreased shoot S concentration. Addition of NaHS in 100 μM Al solution (Al + 100S, Al + 200S, Al + 400S) significantly increased S concentration by 109%, 223%, 277% in shoots and 80%, 86%, 86% in roots, respectively, compared with Al alone treatment.

3.4. Chlorophyll content and photosynthetic parameters

After 25 days of treatments, great variation in photosynthetic parameters was observed by Al toxicity and H_2S application. Leaf chlorophyll content (SPAD value) decreased by 30% in Al treatment compared with control. Application of NaHS (cf. Al + 100S, Al + 200S and Al + 400S treatments) reduced Al-induced chlorophyll inhibition by enhancing 8%, 29% and 34% SPAD value, respectively over Al alone treatment. Similar results were found in net photosynthetic rate (Pn), stomatal conductance (Gs), transpiration rate (Tr) and chlorophyll fluorescence (Fv/Fm). There were 37%, 143%, 43% and 26% reduction in Pn, Gs, Tr and Fv/Fm, respectively by Al treatment in comparison with control. Al + 200S and Al + 400S treatments improved Pn, Gs, Tr and Fv/Fm by 37%, 83%, 71%, 26%, and 45%, 94%, 81%, 18%, respectively, compared with Al alone treatment. However, no significant difference was found between Al + 100S and Al alone treatment. Contrary to these results, significant overproduction (12%) of the intercellular CO_2 concentration (Ci) by Al toxicity was noted compared to control; while Al + 200S and Al + 400S resulted in 6% and 8% reduction, respectively as against Al (Table 2).

3.5. H_2S reduced Al-induced electrolyte leakage and over accumulation of lipid peroxidation

A significant increase of 43% in relative electrolyte leakage (REL) in roots was observed in 24 h Al alone treatment compared with control. Pre-S + Al showed 20% decrease in REL over Al alone (Al) exposed roots. Pretreatment with Na_2SO_4 did not cause significant reduction in Al-induced REL (Fig. 3A).

Exposure to 100 μM $AlCl_3$ caused significant MDA overproduction, while Pre-S + Al significantly reduced MDA content. In short-term experiment, compared to control, 24 h Al exposure (Al) resulted in 65% increased root MDA contents (Fig. 3B), while Pre-S + Al lowered this overproduction to only 24%. Na_2SO_4 pretreatment was not effective in lowering MDA caused by Al toxicity (Fig. 3B). In time- and dose-dependent experiment, both root and

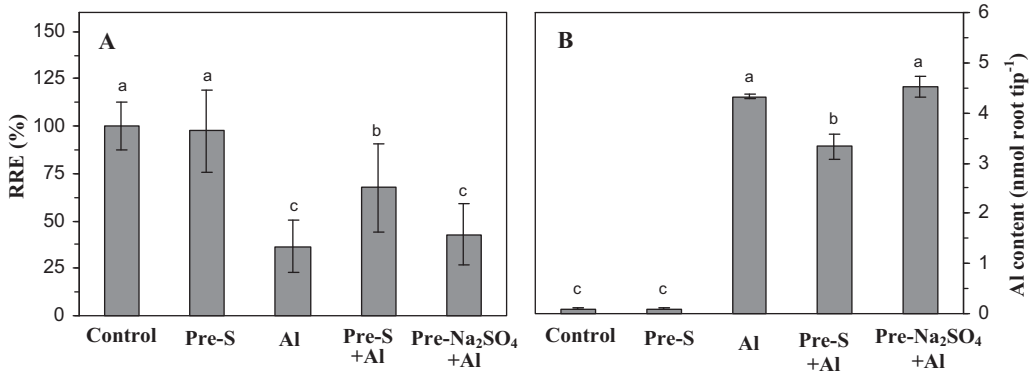

Fig. 1. Effect of H_2S-donor NaHS pretreatment on relative root elongation (RRE, A) and Al content (B) of barley roots under 24 h Al stress.
Seedlings were pre-treated with or without 200 μM NaHS for 24 h and then exposed to 100 μM $AlCl_3$ in 0.5 mM $CaCl_2$ solution for 24 h. Means with different letters are significantly different ($P < 0.05$). The five treatments represent as follows: control, 48 h in 0.5 mM $CaCl_2$ solution without Al and NaHS; Pre-S, pretreated with 200 μM NaHS in 0.5 mM $CaCl_2$ solution for 24 h then transferred to 0.5 mM $CaCl_2$ solution without Al and NaHS for further 24 h; Al, 24 h in 0.5 mM $CaCl_2$ solely solution prior to exposure to 100 μM $AlCl_3$ in 0.5 mM $CaCl_2$ for further 24 h; Pre-S + Al, 24 h 200 μM NaHS pretreatment followed by 24 h 100 μM $AlCl_3$ in 0.5 mM $CaCl_2$ solution; Pre-Na_2SO_4 + Al, 24 h 200 μM Na_2SO_4 pretreatment followed by 24 h 100 μM $AlCl_3$ in 0.5 mM $CaCl_2$ solution, respectively. RRE was calculated as percent of control.

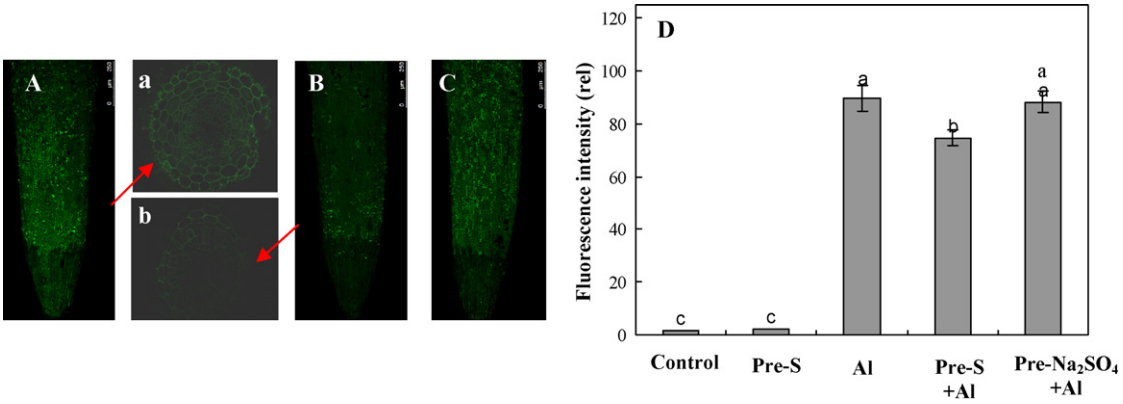

Fig. 2. Effect of the H_2S-donor NaHS pretreatment on fluorescence images illustrating effect of pretreatment without or with 200 μM NaHS on the localization of Al in longitudinal (A, Al; B, Pre-S + Al; C, Pre-Na_2SO_4 + Al) and cross (a and b) sections of the barley root tips exposed to 100 μM $AlCl_3$ for 24 h. Fresh root tips were taken from 0 to 10 mm behind the apex, and root cross-sections were taken from the root zone between 1 and 3 mm behind the apex. Bar = 250 μm. Relative fluorescence intensities (D) on longitudinal section were calculated using Image J software. Data are means ± SD ($n = 5$).

Table 1
Effect of the H_2S-donor NaHS on mineral nutrient content in barley seedlings after 25 days of Al exposure.

Treatment	S (mg g^{-1} DW)		P (mg g^{-1} DW)		K (mg g^{-1} DW)		Ca (mg g^{-1} DW)		Mg (mg g^{-1} DW)		Fe (μg g^{-1} DW)	
	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root
Control	1.0 c	2.0 b	4.6 a	2.3 a	6.3 a	3.7 b	0.9 c	1.1 a	0.8 a	1.1 a	98.2 a	417.9 a
Al	0.6 d	1.7 b	2.2 c	1.5 b	6.6 a	3.4 b	0.9 c	0.7 bc	0.4 b	0.4 d	49.7 c	260.1 c
Al + 100S	1.2 c	3.0 a	2.7 c	1.5 b	6.5 a	3.3 b	0.9 c	0.6 c	0.5 b	0.5 cd	44.9 c	277.6 c
Al + 200S	1.8 b	3.1 a	3.7 b	1.5 b	6.5 a	4.1 ab	1.4 b	0.8 b	0.5 b	0.6 c	69.5 b	344.8 b
Al + 400S	2.1 a	3.1 a	4.5 a	1.6 b	7.3 a	4.9 a	1.7 a	0.8 b	0.6 b	0.8 b	71.8 b	348.5 b

Control, and Al, Al + 100S, Al + 200S and Al + 400S correspond to basic nutrition solution without Al, and basic nutrient solution having 100 μM $AlCl_3$ and supplemented with 100, 200 and 400 μM NaHS, respectively. Means with different letters in the same columns are significantly different ($P < 0.05$).

Table 2
Effect of the H_2S-donor NaHS on SPAD value, the maximal photochemical efficiency (Fv/Fm), and photosynthesis parameters in barley leaves after 25 days of Al exposure.

Treatments	SPAD value	Fv/Fm	Pn (μM CO_2 m^{-2} s^{-1})	Tr (mM H_2O m^{-2} s^{-1})	Gs (mM m^{-2} s^{-1})	Ci (μM CO_2 M^{-1})
Control	44.4 a	0.791 a	18.6 a	2.24 a	259 a	654 d
Al	31.0 d	0.589 d	11.7 c	1.28 c	116 c	732 a
Al + 100S	33.8 c	0.630 c	12.8 c	1.57 b	127 c	715 ab
Al + 200S	40.1 b	0.741 b	16.0 b	2.18 a	213 b	691 bc
Al + 400S	41.6 b	0.733 b	16.9 ab	2.33 a	226 b	677 cd

Seedlings were grown in basic nutrient solution having 100 μM $AlCl_3$ and supplemented with 100, 200 and 400 μM NaHS for 25 days. Means with different letters in the same columns are significantly different ($P < 0.05$).
SPAD = soil plant analysis development, Pn = net photosynthetic rate, Gs = stomatal conductance, Tr = transpiration rate, Ci = intercellular CO_2 concentration.

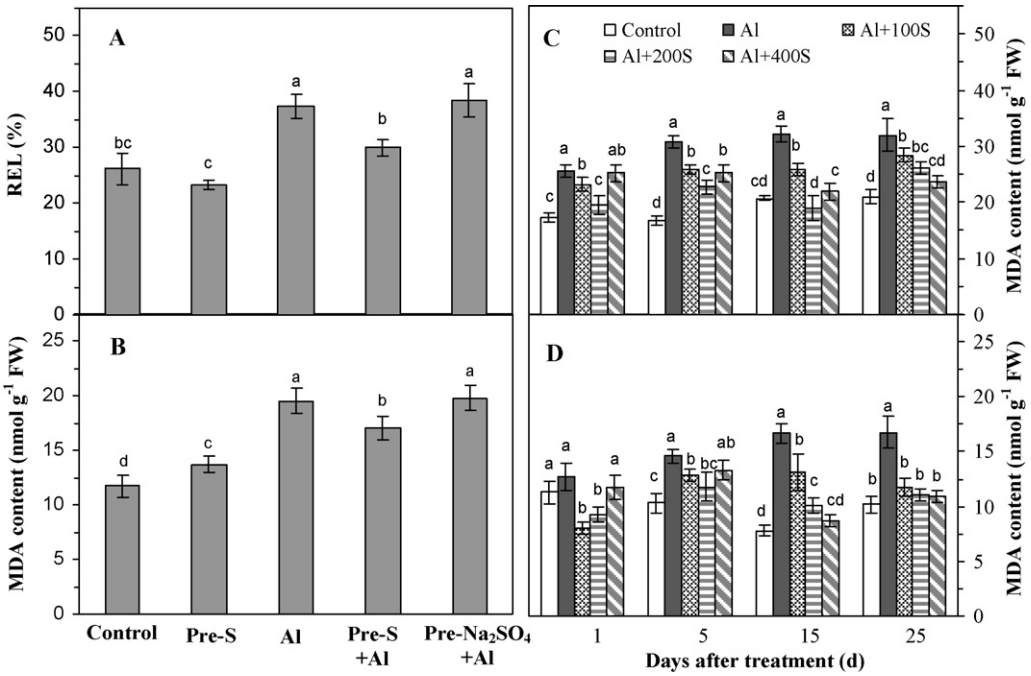

Fig. 3. Effect of H_2S-donor NaHS pretreatment on relative electrolyte leakage (REL, A) and MDA content (B) in barley roots after 24 h 100 μM Al stress; Effect of different levels of NaHS addition on MDA content in barley roots (C) and shoots (D) after 25 days 100 μM Al exposure. Control, and Al, Al + 100S, Al + 200S and Al + 400S (C and D) correspond to basic nutrition solution without Al, and basic nutrient solution having 100 μM $AlCl_3$ and supplemented with 100, 200 and 400 μM NaHS, respectively. Data are means with error bars indicating SD ($n = 3$). Different letters indicate significant differences ($P < 0.05$).

shoot showed significant enhancement in MDA contents by Al alone treatment; application of 100, 200 and 400 μM NaHS reduced MDA accumulation, except for day 1 of Al + 400S. On day 15, MDA contents in roots of Al + 200S and Al + 400S and in shoots of Al + 400S reduced to a level which was similar to the control. On day 25, NaHS addition in 100 μM Al solution diminished Al-induced MDA accumulation in shoots/roots and almost recovered to control level in shoots (Fig. 3C and D).

3.6. Response of antioxidant enzymes to Al and H_2S addition

There was a significant increase in activities of antioxidant enzymes (POD, SOD, CAT, APX and GR) in barley roots exposed to Al treatment (Supplemental Table 1). Pre-S + Al treatment increased GST activity by 25% compared to 24 h Al alone exposed roots. However, slight increase in POD, APX and GR activities was also observed by pre-S + Al. By contrast, CAT activity was significantly lowered in Pre-S + Al compared with Al alone treatment.

The time- and H_2S dose-dependent response pattern for CAT activity to Al and H_2S addition is shown in Fig. 4. Unlike short term experiment, shoot/root CAT activities were significantly reduced by Al exposure throughout the long term experiment. The SOD activity in both roots and shoots was increased by Al stress except for shoot SOD on day 1 and 25 exposure. Al + 100S lowered SOD activity in roots up to day 5 as compared with Al alone treatment (Al). On day 25, Al + 200S and Al + 400S resulted in 17% and 20% increase in root SOD activity than Al alone treatment (Al), respectively. In shoots, Al-induced SOD activity increase was decreased in Al + 200S and Al + 400S on day 5 and Al + 100S on day 15, but no difference was noticed on day 1 and 25 compared with Al alone treatment. Al stress resulted in significant enhancement of POD activity in both roots (except for day 25) and shoots in comparison with the control. H_2S supplementation to Al solution further boosted up POD activity in roots with H_2S-level. Whereas, NaHS addition resulted a decrease in shoot POD activities in comparison with Al alone. Dramatically, CAT activity in roots and shoots was lowered upon Al exposure, except for day 1 in shoots; and H_2S greatly enhanced CAT activity with increasing NaHS levels. On average of day 5, 15 and 25, CAT activity of Al + 100S, Al + 200S, Al + 400S treatments increased by 28%, 57%, 79% in roots and by 138%, 218%, 248% in shoots against Al alone treatment. Al exposure significantly increased APX activity in roots and shoots, except for day 1 in shoots. Addition of NaHS to Al solution lowered APX activity in roots up to day 5; but enhanced on day 25 with H_2S level, where Al + 400S caused 19% increase over Al alone treatment. In shoots, H_2S lowered APX activity on day 1; but caused significant increase after 5 days Al exposure.

Al stress elevated GR activity in roots of day 1 and in shoots of day 5, but no significant difference on the other days related to the control. Upon NaHS addition, GR activity both in roots and shoots significantly increased except for day 1 in shoots. NaHS induced GR increase was in linear fashion in NaHS dose dependent manner. After 25 days of treatment, GR activity of Al + 100S, Al + 200S, Al + 400S treatments enhanced by 29%, 55%, 76% in roots and 17%, 40%, 57% in shoots, respectively over Al alone treatment.

3.7. Effect of H_2S and aluminum on ATPase activity in barley roots

ATPase activity of roots was measured in short term experiment (Fig. 5). Aluminum stress caused significant inhibition of H^+-ATPase, Na^+K^+-ATPase and $Ca^{2+}Mg^{2+}$-ATPase activities, being 28%, 44% and 13% lower than that of control. Pretreatment of H_2S donor NaHS (Pre-S + Al) markedly up-regulated Al-induced decrease in H^+-ATPase and Na^+K^+-ATPase activities, but no significant effect on Al-dependent decrease in $Ca^{2+}Mg^{2+}$-ATPase activity. Pretreatment with Na_2SO_4 had no noteworthy effect on ATPase activity in barley roots under Al stress.

4. Discussion

Aluminum becomes highly phytotoxic in acidic environment thus resulting in alteration of various physiological and biochemical processes of plants [1]. Hydrogen sulfide is an emerging

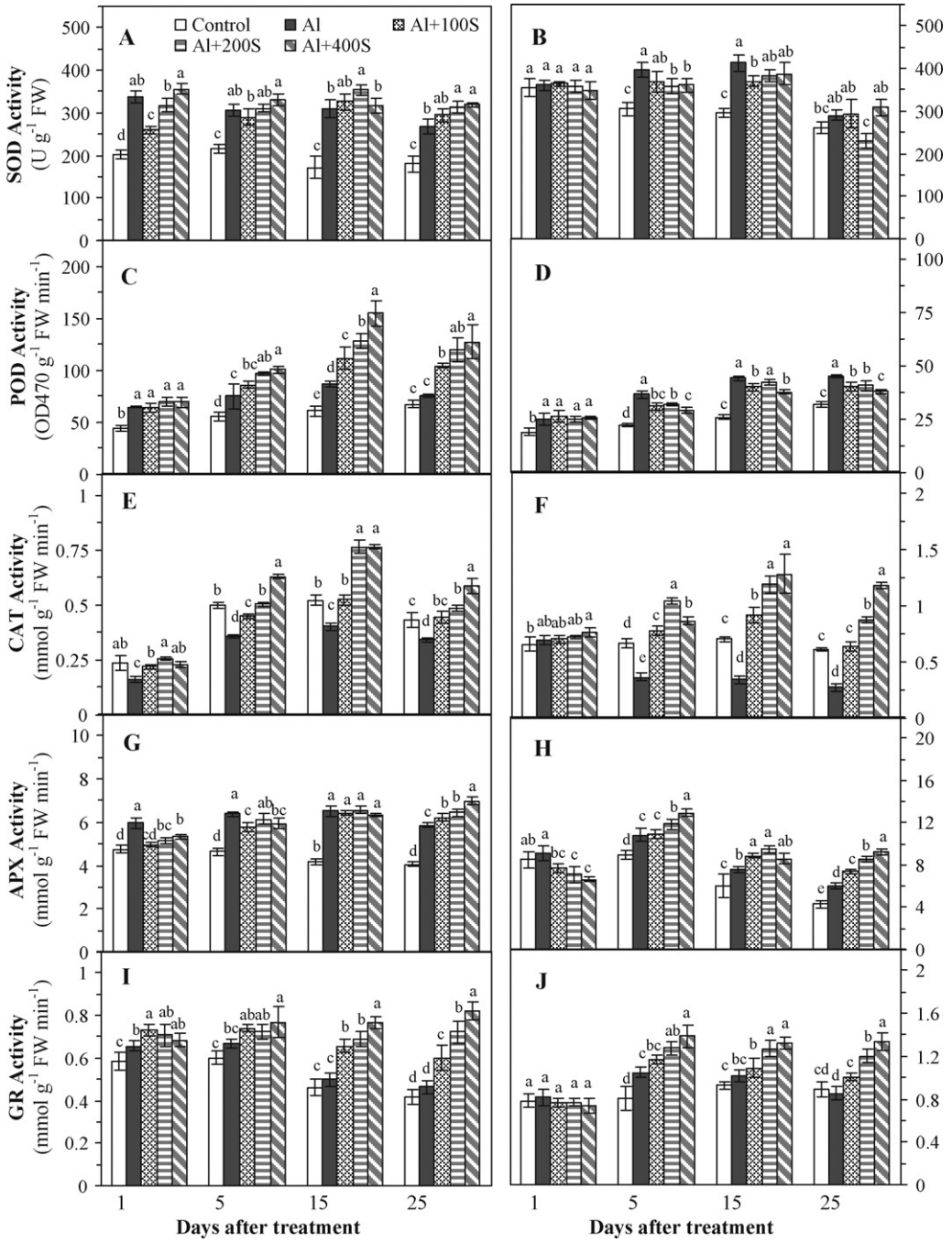

Fig. 4. Effect of NaHS on SOD, POD, CAT, APX and GR activities in roots (A, C, E, G, I) and shoots (B, D, F, H, J) of barley seedlings exposed to Al stress. Control, and Al, Al + 100S, Al + 200S and Al + 400S correspond to basic nutrition solution without Al, and basic nutrient solution having 100 μM $AlCl_3$ and supplemented with 100, 200 and 400 μM NaHS, respectively. Data are means with error bars indicating SD ($n = 5$). Different letters indicate significant differences ($P < 0.05$) among the 5 treatments and refer to each subset of data within each sampling date.

signaling molecule to regulate a variety of physiological processes in plants [15]. In this study, we analyzed the potential ameliorative role of H_2S against Al stress in barley seedlings by evaluating its performance on antioxidant defense system, ATPase activity and photosynthetic performance. Our results clearly demonstrated that pretreatment of barley seedlings with 200 μM NaHS for 24 h (pre-S + Al) decreased Al-induced root length inhibition and Al accumulation in root apices (Figs. 1 and 2). Our results suggest a potential role of NaHS application as an intervention strategy in mitigating Al stress and reducing Al uptake and translocation in barley plants. To test whether the ameliorative effect of NaHS on Al-dependent root elongation is via the production of HS^- or H_2S, the effect of 200 μM of Na_2SO_4 pretreatment on root elongation upon 100 μM Al exposure was also investigated. In contrast to NaHS, pretreated with 200 μM Na_2SO_4 had no significant effect on root elongation, Al accumulation in root apex, ion leakage and oxidative damage caused by Al exposure (Figs. 1–3). As NaHS is a donor of H_2S, therefore, our results further proved that NaHS-released H_2S, rather than other substances from decomposition of NaHS accounts for mitigating effect of Al induced inhibition of root elongation [19].

Aluminum stress resulted in oxidative stress measured in terms of MDA contents in barley roots/shoots (Fig. 3B–D), and caused significant changes in REL in roots (Fig. 3A), suggesting a negative

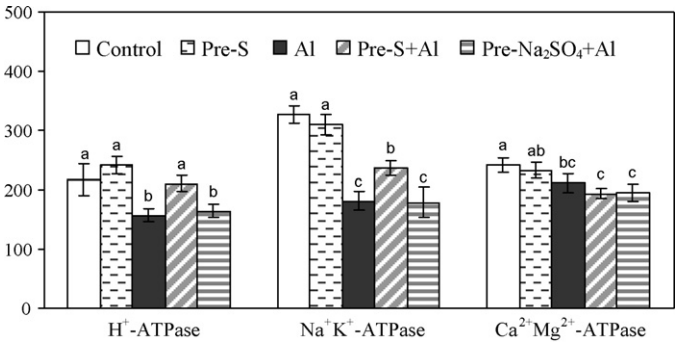

Fig. 5. Effect of pretreatment of 200 μM NaHS on ATPase activity (μmol Pi g^{-1} h^{-1}) on barley roots exposed to 100 μM $AlCl_3$ for 24 h. Data are means with error bars indicating SD ($n = 5$). Different letters indicate significant differences ($P < 0.05$) among the 5 treatments and refer to each subset of data.

impact on membrane integrity and thus membrane deterioration. Such alteration could affect normal ion exchange capacity of plasma membrane and all physiological activities linked to membrane functioning. Like other stresses, under Al stress, plant cells have evolved antioxidant enzymes system including POD, CAT, SOD, GST, GR and APX that are involved in cellular detoxification of ROS [25,26]. It has also been well documented that the genes encoding these antioxidant enzymes are also activated by Al stress [8]. Maron et al. [7], by employing microarray analysis showed a great variation in root gene expression under Al stress in maize and proposed that beside Al-activated citrate release, other possible mechanisms are likely to be operating in Al tolerance. There was varied expression of POD under Al stress in maize. Moreover, genes encoding GST and SOD were also upregulated, indicating their role in defense against Al stress. Abu-Romman and Shatnawi [9] showed that HvSOD gene is chloroplastic and is involved in antioxidative responses under environmental stresses. They proposed that upregulation of SOD gene in barley by drought and cold stress shows its importance in defense against environmental stresses. In our current study, Al stress suppressed CAT activity both in roots and shoots in the long term experiment (Fig. 4E and F); which was in agreement with reports from other plants such as rice [27]. This decline in CAT activity is a signal of oxidative stress creation, which might be due to inhibition of enzyme synthesis or due to a change in the assembly of enzyme subunits under Al condition [27]. However, Al stress caused increase in SOD, APX and POD activities (Fig. 4A–D, G and H). Enhanced SOD activity may function in signaling of Al induced oxidative stress, which can lead to the induction of antioxidant enzymes associated with H_2O_2 scavenging system [4,27]. The APX enzyme, which catalyses the reduction of H_2O_2 to water by using ascorbic acid as specific electron donor, was shown to be induced by Al both in roots and shoots as observed in rice [27]. Al-induced POD activity was in agreement with the observations by Wang et al. [28]. Therefore, it can be concluded that activated antioxidant system under Al stress may be beneficial for plants to remove excess ROS and inhibit lipid peroxidation.

Addition of NaHS (Al + 100S, Al + 200S, Al + 400S) markedly elevated CAT and further improved GR activity except for day 1 in shoots (Fig. 4E and F). The pattern of alterations in POD, SOD, APX and GR activities induced by Al stress was also affected by the presence of H_2S donor NaHS. Thus, it might be deduced that H_2S indirectly scavenges ROS accumulation via elevating Al-decreased CAT activities and further stimulating root/shoot GR and root POD activities, which may partly account for its alleviating effect on Al-induced oxidative damage in barley seedlings.

As an emerging signal molecule, H_2S has been documented to play a regulatory role including mitigation of oxidative stress and induction of antioxidant defense system in plants under various stressful conditions. It has been reported that H_2S enhances the activities of APX and GR in wheat seedlings under water stress [29]. Based on these evidences, it was concluded that H_2S may activate an antioxidant signaling pathway and play a protective role in plants against variety of abiotic stresses. This ability of H_2S to exert a protective function against Al-caused oxidative stress might be due to the following pathway: reaction with lipid radicals and then stop the propagation of lipid oxidation, and activation of antioxidant enzymes such as CAT and POD. Increasing evidences show that hydrogen sulfide (H_2S) can act as a signaling molecule similar to NO and CO in animals, and participating in various biological processes [19,30]. H_2S serves as a signal molecule to control thiol levels [31,32], and reduces NO accumulation in guard cells by causing stomatal opening in light and dark exposure as well [33]. However, the characterization and role of H_2S as a signal molecule and its molecular mechanisms of antioxidant adaptation are still limited. Therefore, further study is needed to verify the involved signaling pathways.

Al also alters membrane potential and ion channel activity [34], inhibition of proton adenosine triphosphatase (H^+-ATPase) [35] and lipid peroxidation. In present study, Al caused depression of root ATPase activity, especially H^+-ATPase and Na^+K^+-ATPase; however, pretreatment with NaHS resulted in up-regulation of both H^+-ATPase and Na^+K^+-ATPase activities. The plasma membrane Na^+K^+-ATPase are ubiquitous P-type membrane transport proteins, which couple the energy derived from ATP hydrolysis to drive transport of solutes against their electrochemical gradients and are involved in transport of protons [36]. Zhang and Han [37] reported that enhanced UV-B radiation reduced Na^+K^+-ATPase activity in mitochondria, chloroplasts and cellular solutes of wheat seedlings, UV-B radiation induced damage to wheat seedlings in terms of activity of Na^+K^+-ATPase in various organelles can be repaired in part by He-Ne laser irradiation.

Exposure to 100 μM $AlCl_3$ caused significant reduction in P, Mg and Fe in shoots/roots and Ca in roots. Therefore, excessive Al accumulation could affect the uptake and distribution of certain nutrients in the plants, and hence would be responsible for mineral deficiencies/imbalance and depression of plant growth. Addition of NaHS in Al treatments showed an Al-dose-dependent effect on mineral uptake. For example, addition of NaHS elevated concentrations of P, S, Ca, Mg, and Fe in plants, when compared with Al-alone treatment (Table 1). Thus, elevated uptake of P, Ca, Mg and Fe may be one of the mitigatory mechanisms of external NaHS.

Earlier investigations have demonstrated a notable reduction in the rate of photosynthesis (Pn) by Al in different plant species [38]. In our experiment, the results were consistent with the observations of Farquhar and Sharkey [39], who suggested that inhibition of photosynthesis, was caused by stomatal or non-stomatal factors, divided by intercellular CO_2 concentration (Ci). Although H_2S plays its role in absicic acid (ABA)-dependent induction of stomatal closure [15], in present study stomatal conductance (Gs) was enhanced by H_2S. As H_2S counteracts oxidative burst by reducing H_2O_2 concentration, it can be assumed that H_2S might be preventing H_2O_2 signaling in guar d cells. Hence, exogenous H_2S addition may have impaired the ABA-induced H_2O_2 mediated stomatal closure. Further, Chen et al. [40] also reported that 100 μM NaHS addition to *Spinacia oleracea* plants caused increase in Gs and enhanced the photosynthesis. However, further studies are needed to clarify the mechanism of H_2S induced enhancing Gs.

In conclusion, NaHS addition had significant beneficial effects on Al-exposed barley plants. It effectively decreased Al accumulation and alleviated Al-induced growth inhibition and toxicity. This alleviation was related to a significant reduction in Al uptake and MDA accumulation, and improvement in P, Ca, Mg and Fe uptake, ATPase and photosynthetic performance. In addition, CAT and POD activities, when concerning ROS scavenging systems, play

an important role in H_2S-induced alleviation of oxidative stress. H_2S-based lower lipid peroxidation might result in better functioning of plasma membrane, and reduce Al stress to barley plants as was evident by enhancement of root elongation, increased biomass and improvement of photosynthetic performance.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhazmat.2011.12.076.

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