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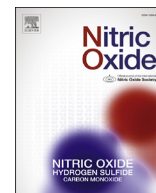

Nitric oxide-induced synthesis of hydrogen sulfide alleviates osmotic stress in wheat seedlings through sustaining antioxidant enzymes, osmolyte accumulation and cysteine homeostasis

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ABSTRACT

Nitric oxide (NO) and hydrogen sulfide (H_2S) have been shown to act as signaling molecules in various physiological processes, play significant roles in plant cellular processes, and also mediate responses to both biotic and abiotic stresses in plants. The present investigation was carried out to test the effect of exogenous NO on endogenous synthesis of H_2S in osmotic-stressed wheat (*Triticum aestivum* L.) seedlings. The results show that application of NO to wheat seedlings, suffered from PEG8000-induced osmotic stress, considerably enhanced the activities of H_2S-synthesizing enzymes L-cysteine desulfhydrase (LCD) and D-cysteine desulfhydrase (DCD) leading to enhanced level of endogenous H_2S content. At the same time exogenous NO also enhanced the activity of cysteine (Cys)-synthesizing enzyme *O*-acetylserine(thiol)lyase (OAS-TL) and maintained Cys homeostasis under osmotic stress. NO and H_2S together markedly improved the activities of antioxidant enzymes viz. ascorbate peroxidase (APX), glutathione reductase (GR), peroxidase (POX), superoxide dismutase (SOD) and catalase (CAT). Furthermore, NO and H_2S caused additional accumulation of osmolytes proline (Pro) and glycine betaine (GB), all these collectively resulted in the protection of plants against osmotic stress-induced oxidative stress. On the other hand, NO scavenger cPTIO [2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide] and H_2S scavenger HT (hypotaurine) invalidated the effect of NO on endogenous H_2S levels and Cys homeostasis which resulted in weak protection against osmotic stress. Application of *N*-ethylmaleimide (NEM) suppressed GR activity and caused an increase in oxidative stress. We concluded that NO in association with endogenous H_2S activates the defense system to the level required to counter osmotic stress and maintains normal functioning of cellular machinery.

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1. Introduction

The existence of hydrogen sulfide (H_2S), a colourless gas with a characteristic unpleasant smell of rotten egg, has been substantiated for its toxicity with fatal effects and root cause for life destructions and extinctions on the planet earth over millions of years [1,2]. However, in recent years H_2S has emerged as a third gaseous transmitter after nitric oxide (NO) and carbon monoxide [3,4]. Strong evidence of H_2S emission from plants was reported by Wilson et al. [5], when they quantified H_2S from leaves of cucumber, pumpkin, cantaloupe, corn, soybean and cotton. Rennenberg [6] further confirmed the release of H_2S by higher plants. In plants, H_2S is considered to be a by-product of cysteine (Cys) degradation. The enzymes L-cysteine desulfhydrase (LCD; EC 4.4.1.1) and D-cysteine desulfhydrase (DCD; EC 4.4.1.15) desulfurylate L-Cys and D-Cys, respectively into H_2S, ammonia, and pyruvate [7,8]. However, *O*-acetylserine(thiol)lyase (OAS-TL; EC 2.5.1.47), a cysteine synthase-like protein synthesizes Cys from *O*-acetylserine (OAS) and sulfide (Fig. 6). Thus, Cys is the key sulfur containing precursor of various biomolecules including antioxidants and other defense compounds [9,10].

Information on the role of H_2S as signaling molecule in plants is limited compared to those in animals. However, several studies showed that H_2S plays crucial role in a plethora of plant cellular processes including seed germination, root morphogenesis, photosynthesis, flower senescence and guard cell signaling [11–14].

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Exogenous application of sodium hydrosulfide (NaHS), an H_2S donor, has been shown to mitigate adverse effects of various abiotic stresses [14–19], functions as a potent antioxidant [20] and signaling molecule [21,22]. Since, Cys is the source molecule of H_2S and other defense molecules, therefore maintenance of Cys homeostasis is vital for normal functioning of cellular system under stressful conditions.

Synthesis of NO in plants is mainly carried out by nitrate reductase (NR)-catalyzed reduction of nitrite (NO_2^-) and L-arginine-dependent nitric oxide synthase-like activity [23–27]. NO has been shown to regulate several functions including seed germination, adventitious root formation, flowering, stomatal closure and senescence [28,29] and exhibit antioxidant and cytoprotective effects with DNA, lipids, proteins and chlorophyll [30,31]. In addition, NO mediates responses to both biotic and abiotic stresses in plants [32,33], acts as a second messenger in signaling cascades involving cytosolic Ca^{2+}, cyclic adenosine diphosphate ribose (cADPR), salicylic, jasmonic and abscisic acids, hydrogen peroxide (H_2O_2), cyclic guanosine 5′-monophosphate (cGMP) and mitogen activated protein kinase (MAPK) [34–38]. A significant number of studies show that exogenous application of NO elevates activities of antioxidant enzymes [31,39–41] and gives protection to the plants against various abiotic stresses [14,31,32,42].

Manifestation of osmotic stress is one of the primary effects of several abiotic stresses including drought. Osmotic stress affects various physiological processes and causes stomatal closure, turgor loss, reduced photosynthetic activity and suppressed carbon assimilation [43,44]. In plants, stressful conditions create an imbalance between production and scavenging of reactive oxygen species (ROS) that results in over production of ROS. Excessive accumulation of ROS creates oxidative stress and results in the oxidation of lipids, proteins and nucleic acids [44,45] leading to reduced carbon assimilation and crop yield. Being sessile in nature, plants have no choice to escape stress-induced detrimental effects, the character which makes plants a unique example with the inherent quality of not to quit under any stressful conditions. Plants resist osmotic stress by accumulating osmolytes such as proline (Pro) and glycinebetaine (GB). These osmolytes stabilize the quaternary structure of proteins, maintain membrane stability and protect plants against various abiotic stresses through osmotic adjustment [46–49]. To cope with the detrimental effects of oxidative stress plants are equipped with a defense system, orchestrated by various antioxidant enzymes such as ascorbate peroxidase (APX), glutathione reductase (GR), peroxidase (POX), superoxide dismutase (SOD) and catalase (CAT). Timely and precise activation of these defense systems prior to the onset of damage is of prime importance for the endurance of plants under stressful conditions. Therefore, precise response to stress stimulus is vital and it is transmitted by a network of signaling molecules.

Although, in recent years the role of NO and H_2S in abiotic stress tolerance of plants gained much attention, little or vague information is available on the effect of exogenous NO on endogenous synthesis of H_2S and Cys homeostasis under osmotic stress. Considering the important roles of NO and H_2S in plants, the present work was planned to test the effect of NO on the synthesis of H_2S and maintenance of Cys homeostasis and their role in the tolerance of wheat to osmotic stress.

2. Materials and methods

2.1. Plant material and treatments

To test the proposed hypothesis a sand culture pot experiment was performed using wheat cultivar ‘Irena’. Healthy and uniform seeds of wheat (*Triticum aestivum* L. cv. Irena) were surface sterilized with 0.1% $HgCl_2$ for 10 min. After 10 min the seeds were vigorously rinsed with double distilled water (DDW). Surface sterilized seeds were sown in plastic pots containing acid washed sand and kept for 14 days under natural illuminated conditions with average day/night temperature $25/10 \pm 2$ °C. All the pots were supplied with 50 mL of Raukura's nutrient solution [50] every day.

After 14 days the seedlings were uprooted and divided into five groups which were transferred to five respective Petri dishes and treated with: (i) DDW (Control), (ii) 15% (w/v) PEG8000 (PEG) (iii) 0.2 mM GSNO+15% (w/v) PEG8000 (GSNO + PEG), (iv) 0.2 mM GSNO+15% (w/v) PEG8000 + 0.2 mM cPTIO (GSNO + PEG + cPTIO) and (v) 0.2 mM GSNO+15% (w/v) PEG8000 + 1 mM HT (GSNO + PEG + HT). Petri dishes were sealed to avoid the NO gas letting out the air and were kept at 20 °C for 12 h. To test the effect of GR activity on the tolerance of plants to oxidative stress, *N*-ethylmaleimide (NEM) was used as: (i) 0.02 mM NEM (ii) 15% (w/v) PEG+0.02 mM NEM, and (iii) 15% (w/v) PEG+0.2 mM GSNO+0.02 mM NEM. Polyethylene glycol 8000 (PEG8000) was used as osmotic stress inducer, *S*-nitrosoglutathione (GSNO) was used as NO donor, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) and hypotaurine (HT) were used as NO and H_2S scavengers, respectively. NEM is an alkylating reagent that strongly reacts with thiol group (—SH) and inhibits GR activity [51,52]. Seedlings treated with DDW only were considered as control. Each treatment was replicated three times, and each replicate was consisted of three seedlings.

After 12 h the seedlings (without cotyledons) were used for the analyses of relative water content (RWC), ion leakage, H_2O_2 and $O_2\cdot$ content, thiobarbituric acid reactive substances (TBARS), and Pro and GB content. Activities of NO-synthesizing enzyme nitrate reductase (NR), H_2S-synthesizing enzymes LCD and DCD, and Cys-synthesizing enzyme OAS-TL were estimated. Endogenous levels of NO and H_2S were also quantified Activities of antioxidant enzymes were studied in terms of APX, GR, POX, SOD, and CAT.

2.2. Estimation of relative water content (RWC) and ion leakage

For each treatment, RWC was determined by the method of Yamasaki and Dillenburg [53] using the following formula:

$$\text{RWC}\ (\%) = \frac{\text{FW} - \text{DW}}{\text{TW} - \text{DW}} \times 100$$

Fresh weight (FW) was measured after 12 h of treatments. To determine turgid weight (TW) the seedlings were kept in double distilled water (DDW) inside a covered petri dish for 4 h. After gently wiping the water from the surface with tissue paper, the seedlings were weighed, and dry weight (DW) was determined by drying at 80 °C for 24 h.

Membrane permeability was assessed in term of ion leakage (%) by the method of Lutts et al. [54]. After 12 h of treatment, seedlings were placed in sealed vials containing DDW and incubated on a rotary shaker for 24 h, subsequently, the conductivity (EC1) of solution was determined. Samples were then autoclaved at 120 °C for 20 min and electrical conductivity (EC2) was measured again after cooling the solution at room temperature. The ion leakage (%) was calculated as [(EC1/EC2) × 100].

2.3. Estimation of hydrogen peroxide (H_2O_2) and superoxide ($O_2\cdot$) content and TBARS

Hydrogen peroxide (H_2O_2) content was determined according to Velikova et al. [55]. The homogenate was centrifuged at 12,000 rpm for 15 min, and 0.5 mL of the supernatant was added to 0.5 mL of 10 mM potassium phosphate buffer (pH 7.0) and 1 mL of 1 M

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