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Review

Hydrogen sulfide: a new endogenous player in an old mechanism of plant tolerance to high salinity

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ABSTRACT

High salinity affects plants due to stimulation of osmotic stress. Cell signaling triggered by nitric oxide (NO) and hydrogen sulfide (H_2S) activates a cascade of biochemical events that culminate in plant tolerance to abiotic and biotic stresses. For instance, the NO/H_2S-stimulated biochemical events that occur in plants during response to high salinity include the control of reactive oxygen species, activation of antioxidant system, accumulation of osmoprotectants in cytosol, induction of K^+ uptake and Na^+ cell extrusion or its vacuolar compartmentation among others. This review is a compilation of what we have learned in the last 10 years about NO participation during cell signaling in response to high salinity as well as the role of H_2S, a new player in the mechanism of plant tolerance to salt stress. The main sources of NO and H_2S in plant cells is also discussed together with the evidence of interplay between both signaling molecules during response to stress.

Keywords: abiotic stress, cell signaling, hydrogen sulfide, nitric oxide, NO and H_2S biosynthesis, salt stress

Introduction

It is estimated that over 800 million hectares of land throughout the world are overloaded with salt, which represents more than 6 % of the world's total land area (Munns & Tester 2008). High salinity leads to osmotic stress, cell toxicity by ions excess and ultimately nutrition disorders and oxidative stress in plants (Munns & Tester 2008). A signaling cascade involving expression of specific genes and accumulation of certain metabolites is pivotal for plants successfully acclimating and tolerating high salinity (Gupta & Huang 2014). Nitric oxide (NO), and more recently hydrogen sulfide (H_2S), were recognized as important players in cell signaling triggered during plant response to biotic and abiotic stresses (Delledonne *et al.* 1998; Durner *et al.* 1998; Zhang *et al.* 2008). The role of these signaling molecules in salt stress has been explored over the past few years.

The NO is a gaseous free radical widely produced in living organisms. Its production was first reported in plants by Dr. Lowell Klepper by the end of the 1970s (Klepper 1979). Nevertheless, the advent of researches focusing on NO in plants took place 19 years later (Delledonne *et al.* 1998; Durner *et al.* 1998) when Drs. Robert F. Furchgott, Louis J.

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Ignarro and Ferid Murad were jointly laureate with the Nobel Prize for the disclosure of NO as an endothelium-derived relaxing factor in mammals. Since then, NO has been shown to influence plant response to salt stress by improving seed vigor and germination (Hayat *et al.* 2012) and controlling the cellular levels of reactive oxygen species (ROS) (Keyster *et al.* 2012; Ahmad *et al.* 2016), nutrient (Kong *et al.* 2016; Liu *et al.* 2016) and osmoprotectants (Wu *et al.* 2011; Tian *et al.* 2015). The NO biosynthesis in plant cells can occur by non-enzymatic and enzymatic means. Nitrite (NO_2^-) or nitrate (NO_3^-), in the presence of ascorbic acid (AsA), may be non-enzymatically converted to NO (Klepper 1990). The acidic condition of aleurone layers was also demonstrated to favor NO production in apoplast (Bethke *et al.* 2004). The light-dependent production of NO from nitrogen dioxide, assisted by carotenoids, has been reported (Cooney *et al.* 1994). On the other hand, the enzymatic mechanisms that drive NO production in plant cells are still under debate since a plethora of examples are reported in the literature.

The H_2S is a small and lipophilic molecule that was pointed out as a possible cellular signaling component in mammalians (Abe & Kimura 1996). Indeed, H_2S is considered the third gas transmitter in addition to NO and carbon monoxide (Wang 2002). Its ability to induce seed germination and relief copper stress was demonstrated in the late 2000s (Zhang *et al.* 2008). However, earlier H_2S was believed to be exclusively phytotoxic. Since then, H_2S was implicated in the ROS control through the activation of antioxidant system (Yu *et al.* 2013; Shan *et al.* 2014; da-Silva *et al.* 2017), maintenance of high K^+/Na^+ ratio (Lai *et al.* 2014; Deng *et al.* 2016) and accumulation of osmolytes (Shi *et al.* 2013) during plant response to high salt concentrations. Recent research demonstrates that H_2S is primarily produced in plant tissues from L/D-cysteine or sulfide (Li 2015).

This review describes the main enzymatic sources of NO and H_2S in plants and compiles what it is known from the past 10 years on the role of these signaling molecules during plants response to high salinity.

Biosynthesis of NO and H_2S in plant cells

Enzymes involved in NO biosynthesis

A body of evidence indicates that the production of NO in plant cells may come from both reductive and oxidative pathways (Fig. 1). Reductive mechanisms for NO synthesis include NO_3^- or NO_2^- as the primary substrates for nitrate reductase (NR), a plasma membrane-bound nitrite:NO reductase (NI-NOR), a mitochondrial protein system or xanthine oxidoreductase (XOR). The oxidative pathway comprises the polyamines and hydroxylamines metabolisms by still unknown mechanisms (Fig. 1).

The NR, a cytosolic enzyme, is able to catalyze directly or indirectly the NO production from NO_3^- (Yamasaki & Sakihama 2000; Modolo *et al.* 2005). The genes *Nia1* and *Nia2* encode for NR in *Arabidopsis thaliana* (Wilkinson

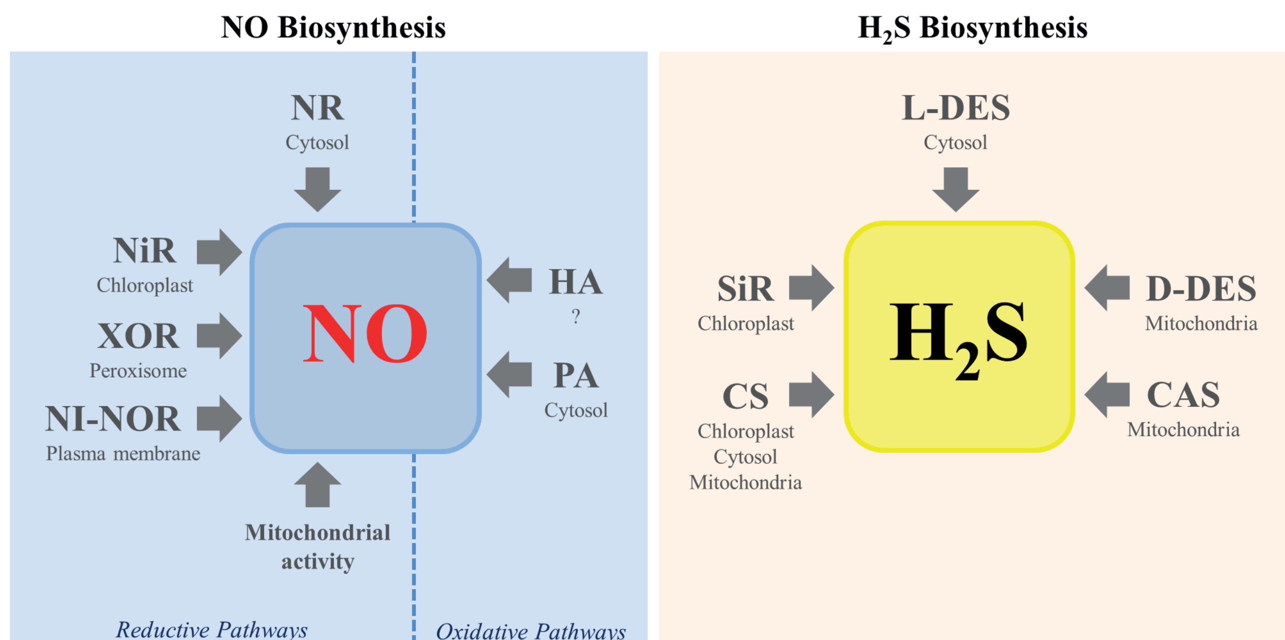

Figure 1. Schematic representation of the main pathways of NO and H_2S production reported in plant cells. **CAS**, β-cyanoalanine synthase; **CS**, cysteine synthase; **D/L-DES**, D/L-cysteine desulfhydrase; **HA**, hydroxylamines; **H_2S**, hydrogen sulfide; **NI-NOR**, plasma membrane-bound nitrite-nitric oxide reductase; **NiR**, nitrite reductase; **NO**, nitric oxide; **NR**, nitrate reductase; **PA**, polyamines; **SiR**, sulfite reductase; **XOR**, xanthine oxidoreductase.

& Crawford 1993). This enzyme promotes the NAD(P)H-dependent reduction of NO_3^- to NO_2^- and then to NO (Magalhaes *et al.* 2000; Yamasaki & Sakihama 2000). The direct production of NO from NR, however, represents only 1–2 % of the nitrate-reducing capacity of this enzyme (Rockel *et al.* 2002; Planchet *et al.* 2005). This is because relatively high amounts of NO_2^- (K_M = 100 µM) are required for NR reducing NO_2^- to NO, while the formation of NO is prevented in the presence of NO_3^- in amounts as low as 50 µM (Rockel *et al.* 2002). The NR can also indirectly contribute to NO production by catalyzing the formation of the substrate (NO_2^-) for other enzymatic systems. For instance, *in vitro* experiments showed that a plasma membrane-bound protein (NI-NOR) of tobacco (*Nicotiana tabacum*) roots was able to reduce NO_2^- to NO (Stöhr *et al.* 2001) that, in turn, yielded higher rates of tobacco root colonization by the arbuscular mycorrhizal *Glomus mosseae* (Moche *et al.* 2010). However, the gene that encodes for NI-NOR, the protein amino acid sequence and the electron donor that assists the NO_2^- reduction remain unknown. Mitochondrial activities were also determined to contribute to NO production from NO_2^- in Arabidopsis plants, tobacco cell suspensions and *Oryza sativa* (rice) (Modolo *et al.* 2005; Planchet *et al.* 2005; Stoimenova *et al.* 2007). The reduction of NO_2^- to NO has also been reported to be catalyzed by the peroxisomal enzyme XOR. This enzyme mainly catalyzes the formation of uric acid and O_2^- from xanthine oxidation. However, formation of NO from NO_2^- was observed to be assisted by XOR in the presence of NADH or xanthine as reducing agents (Li *et al.* 2004). Production of O_2^- or NO was recorded in pea (*Pisum sativum*) and attributed to XOR activity, depending on the cell redox state (Del Río *et al.* 2004) while white lupin (*Lupinus albus*) roots upon phosphate deficiency produced NO *via* XOR (Wang *et al.* 2010). The nitrite reductase (NiR) was also shown to be a possible source of NO in spinach (*Spinacia oleracea*) chloroplasts *via* reduction of NO_2^- assisted by ferredoxin. Then, NO would be a byproduct of the pathway that leads to NH_4^+ formation (Kuznetsova *et al.* 2004). The affinity of NO_2^- for mitochondrial NiR (mNiR) was determined to be very low, indicating that NO production *via* mNiR activity is only relevant under conditions in which NO_2^- accumulates in the organelle, such as hypoxia (Gupta *et al.* 2011).

Metabolization of polyamines constitutes an example of oxidative process that might drive NO production in plant cells. Arabidopsis supplemented with exogenous polyamines exhibited increased NO production in cells (Tun *et al.* 2006). Similar results were found in cadmium-stressed wheat (*Triticum aestivum*) (Groppa *et al.* 2008) and drought-stressed cucumber (*Cucumis sativus*) (Arasimowicz-Jelonek *et al.* 2009). The increment of arginase activity in tobacco leaves upon high salinity was recently determined to be accompanied of NO accumulation in cells (da-Silva *et al.* 2017). Arginase catalyzes the conversion of L-arginine to urea and L-ornithine, in which the latter may originate polyamines (*e.g.* putrecine, spermidine and/or spermine). The mechanism by which polyamines are oxidized to NO still remains to be elucidated. Another potential oxidative pathway that leads to NO production includes hydroxylamine as substrate. Treatment of NR-deficient tobacco cell cultures with exogenous hydroxylamine resulted in cellular accumulation of large amounts of NO (Rümer *et al.* 2009). The NO biosynthesis from the oxidation of hydroxylamine is believed to be involved in the regulation of ROS levels in plant cells, especially during the reoxygenation of anoxic tissues (Rümer *et al.* 2009). Nonetheless, both, the enzymatic system involved in the hydroxylamine-dependent NO formation and the site where such pathway takes place are still unknown. Many authors suggest that plant cells are also able to produce NO from L-arginine oxidation, with concomitant formation of L-citrulline, in a reaction catalyzed by a nitric oxide synthase (NOS)-like enzyme as it occurs in mammalian cells. Despite that, no gene or protein that encodes for an NOS-like enzyme has been so far isolated from plant cells. Likewise, the Nitric Oxide-Associated protein 1 (ATNOA1) of Arabidopsis (Guo *et al.* 2003) was initially believed to catalyze NO biosynthesis from L-arginine oxidation. Instead, evidence suggests that ATNOA1 somehow modulates NO accumulation in plant cells according to environmental conditions as *ATNOA1*-defective mutant plants may present normal NO levels (Moreau *et al.* 2008).

Enzymes involved in H_2S biosynthesis

Five enzymatic systems have been reported to contribute to H_2S biosynthesis in plant cells (Li 2015; Fig. 1). The majority of publications that deal with H_2S production in plants usually focus on the activity of L-cysteine desulfhydrase (L-DES) (Romero *et al.* 2013), a cytoplasmic enzyme that converts L-cysteine to pyruvate with release of H_2S and NH_4^+ (Harrington & Smith 1980; Álvarez *et al.* 2010; Li 2015), using pyridoxal phosphate as a cofactor (Calderwood & Kopriva 2014). The L-DES was also shown to regulate the L-cysteine homeostasis in Arabidopsis (Álvarez *et al.* 2010). Under physiological conditions, *DES1* expression, which encodes for L-DES, was induced by abscisic acid in Arabidopsis guard cells (Scuffi *et al.* 2014). Furthermore, the treatment of alfalfa (*Medicago sativa*) or tobacco with high NaCl concentrations enhanced L-DES activity (Lai *et al.* 2014; da-Silva *et al.* 2017). The L-DES was also stimulated in heat-stressed maize (*Zea mays*) plants incubated with salicylic acid or H_2O_2 (Li *et al.* 2015). Arabidopsis mutant plants exhibiting low expression of *LCD*, an L-DES encoding gene, presented low H_2S levels under drought conditions (Jin *et al.* 2013). In addition to L-DES, D-DES catalyzes the production of H_2S in plant cells by metabolizing D-cysteine, instead (Riemenschneider *et al.* 2005). Despite similar functions with discrimination of cysteine enantiomers, L-DES and D-DES are not related to

each other and their physiological implications remain to be clarified (Calderwood & Kopriva 2014). The expression of *DCD1* (a D-DES gene) increased in cadmium-stressed chinese cabbage (*Brassica rapa*), which resulted in H_2S accumulation in cells (Zhang *et al*. 2015). Increment of D-DES activity in a time-dependent manner was reported in salt-stressed alfalfa (Cui *et al*. 2014).

The mitochondrial enzyme β-cyanoalanine synthase (CAS) catalyzes the condensation of L-cysteine to cyanide (CN^-) to yield H_2S (Akopyan *et al*. 1975; Hatzfeld *et al*. 2000; Li 2015). Its activity helps plant to control the cell levels of CN^- during ethylene production as this anion is a potent inhibitor of mitochondrial respiratory chain. Cysteine synthase (CS), present in cytosol, mitochondria and chloroplasts, catalyzes the reversible reaction between L-cysteine and acetate to form *O*-acetyl-L-serine and H_2S (Wirtz & Hell 2006; Li 2015). It is also documented that high concentrations of NaCl stimulated CAS and CS activities in tobacco and resulted in H_2S accumulation in leaves (da-Silva *et al*. 2017). Besides these sources, plant cells are able to reduce SO_3^{2-} to H_2S in the presence of ferredoxin and sulfite reductase (SiR), a chloroplast enzyme (Nakayama *et al*. 2000; Li 2015). The SO_3^{2-} may originate from either SO_4^{2-} (through sulphur nutrition) or SO_2 (uptaken from atmosphere). In this sense, SO_4^{2-} is activated by ATP sulfurylase to form adenosine 5′-phosphosulfate (APS). The formed APS is further reduced to SO_3^{2-} *via* APS reductase activity (Nakayama *et al*. 2000; Li 2015). Notably, salt-stressed tobacco plants presented decreased SiR activity explained by the occurrence of stomatal closure, a condition that prevented SO_2 from entering into plant leaves (da-Silva *et al*. 2017). Therefore, the enzymes L-DES, CAS and CS, but not SiR, contribute to H_2S biosynthesis during tobacco response to high salinity.

Cell signaling in salt-stressed plants mediated by NO and H_2S

Integrated plant cell signaling must be orchestrated to provide with metabolic and structural changes for individuals survival and tolerance to salt stress. The next three sections will focus on current knowledge about NO and H_2S roles during plant responses to high salinity.

Nitric oxide

Important roles have been ascribed to NO in plants tolerance to abiotic stress, in which the increment of this signaling molecule in cells was associated to a variety of strategies used by plants to cope high salinity (Tab. 1). The *Atnoa1* Arabidopsis mutant plants, that exhibit impaired NO biosynthesis, were demonstrated to be highly sensitive to high salinity, being more vulnerable to oxidative stress and presenting lower germination and survival rates under such condition (Zhao *et al*. 2007).

Activation of the antioxidant system is one of the NO roles in plants under salt stress. Several findings indicate an improvement in the performance of enzymatic and non-enzymatic antioxidant systems in salt-stressed plants treated with NO donors. Cucumber seedlings hydroponically grown in medium containing 50 mM NaCl and 100 μM sodium nitroprusside (SNP; NO^+ donor) showed higher activity of superoxide dismutase (SOD), catalase (CAT), peroxidase and ascorbate peroxidase (APX) when compared to cucumber solely treated with 50 mM NaCl (Fan *et al*. 2007). As a result, cells from these seedlings presented lower membrane permeability and decreased levels of O_2^-, H_2O_2 and lipid peroxides (Fan *et al*. 2007). The NO was found to have dual role on SOD by positively modulating the *FeSOD* expression and negatively affecting *Cu/ZnSOD* one. With this, NO furnished a differential antioxidant protection to salt-stressed sunflower (*Helianthus annuus*) seedlings (Arora & Bhatla 2015). Likewise, the challenge of tobacco roots with NaCl led to accumulation of endogenous NO in leaves that was accompanied by an increment of SOD and CAT activities (da-Silva *et al*. 2017). The activity of monodehydroascorbate reductase, dehydroascorbate reductase (DHAR), glutathione reductase (GR), glutathione *S*-transferase, glutathione peroxidase (GPX), glyoxalase I and glyoxalase II (both related to methylglyoxylate detoxification) was also stimulated by SNP in wheat plants treated with 300 mM NaCl (Hasanuzzaman *et al*. 2011). Induction of non-enzymatic antioxidant system [AsA and reduced glutathione (GSH)] was observed in wheat seedlings treated with SNP prior to NaCl exposure (Hasanuzzaman *et al*. 2011). The treatment with SNP decreased ferritin levels in barley (*Hordeum vulgare*) seedlings, which contributed to the attenuation of oxidative stress triggered by high salinity (Li *et al*. 2008). Likewise, exogenous NO alleviated high-salinity-triggered oxidative stress in soybean (*Glycine max*; Simaei *et al*. 2012), mangrove (*Aegiceras corniculatum*; Chen *et al*. 2014), tomato (*Solanum lycopersicum*; Manai *et al*. 2014), cotton (*Gossypium hirsutum*; Dong *et al*. 2014), spinach (Du *et al*. 2015), sunflower (Kaur & Bhatla 2016) and bermudagrass (*Cynodon dactylon*; Liu *et al*. 2016).

The protective role of NO on plant photosynthetic apparatus is also documented. The SNP at 100 μM restored chloroplast pigments and maximum photochemical efficiency of photosystem II to normal levels in strawberries (*Fragaria* × *ananassa* cv. 'Camarosa') plants challenged with high salinity (Christou *et al*. 2014). Similar results were observed in salt-stressed cotton seedlings wherein application of 100 μM SNP to leaves improved plants photosynthetic performance (Liu *et al*. 2014). The treatment of chickpea (*Cicer arietinum*) plants with 100 mM NaCl and 50 μM of *S*-nitroso-*N*-acetylpenicillamine (SNAP; an NO donor) provided higher amounts of chlorophylls *a* and *b* and carotenoids in plant leaves in comparison with those solely treated with NaCl (Ahmad *et al*. 2016). Aspersion of salt-stressed cotton plants with SNP delayed leaf senescence

Table 1: Roles of nitric oxide (NO) during plant response to salt stress.

NO effect	Plant species
Activation of antioxidant system	*Aegiceras corniculatum* (Chen *et al.* 2014) *Cucumis sativus* (Fan *et al.* 2007) *Cynodon dactylon* (Liu *et al.* 2016) *Glycine max* (Simaei *et al.* 2012) *Gossypium hirsutum* (Dong *et al.* 2014) *Helianthus annuus* (Arora & Bhatla 2015; Kaur & Bhatla 2016) *Hordeum vulgare* (Li *et al.* 2008) *Nicotiana tabacum* (da-Silva *et al.* 2017) *Solanum lycopersicum* (Manai *et al.* 2014) *Spinacia oleracea* (Du *et al.* 2015) *Triticum aestivum* (Zheng *et al.* 2008; Hasanuzzaman *et al.* 2011)
Increase of K^+/Na^+ ratio	*Avicennia marina* (Chen *et al.* 2010) *Cucumis sativus* (Shi *et al.* 2007) *Cynodon dactylon* (Liu *et al.* 2016) *Gossypium hirsutum* (Liu *et al.* 2013; Kong *et al.* 2016) *Kandelia obovata* (Lang *et al.* 2014) *Aegiceras corniculatum* (Lang *et al.* 2014) *Limonium bicolor* (Ding *et al.* 2013) *Populus euphratica* (Zhang *et al.* 2007) *Triticum aestivum* (Tian *et al.* 2015)
Induction of osmoregulators accumulation	*Brassica juncea* (Zeng *et al.* 2011; Khan *et al.* 2012) *Cicer arietinum* (Ahmad *et al.* 2016) *Cucumis sativus* (Fan *et al.* 2013) *Gossypium hirsutum* (Liu *et al.* 2013) *Lycopersicom esculentum* (Wu *et al.* 2011) *Solanum lycopersicum* (Hayat *et al.* 2012) *Triticum aestivum* (Tian *et al.* 2015)
Induction of polyamines accumulation	*Cucumis sativus* (Fan *et al.* 2013)
Protection of photosynthetic apparatus	*Brassica juncea* (Fatma *et al.* 2016) *Cicer arietinum* (Ahmad *et al.* 2016) *Fragaria × ananassa* (Christou *et al.* 2014) *Gossypium hirsutum* (Kong *et al.* 2016) *Medicago truncatula* (Jian *et al.* 2016)
Stimulation of seed germination	*Arabidopsis thaliana* (Zhao *et al.* 2007) *Triticum aestivum* (Zheng *et al.* 2008) *Zea mays* (Bai *et al.* 2011)
Stimulation of plant growth	*Arabidopsis thaliana* (Liu *et al.* 2015) *Glycine max* (Egbichi *et al.* 2014; Vaishnav *et al.* 2016) *Gossypium hirsutum* (Liu *et al.* 2014)

and increased chlorophylls content and photosynthetic rate (Kong *et al.* 2016). The NO and sulfur nutrition were found to prevent chloroplasts damage in salt-exposed mustard (*B. juncea*) plants (Fatma *et al.* 2016). The SNP stimulated the expression of *AOX*, a component of plant mitochondrial electron transport, in barrelclover (*M. truncatula*) under high salinity thus, alleviating oxidative stress and photosynthetic damages (Jian *et al.* 2016).

Inhibition of plasma membrane H^+-ATPase and tonoplast H^+-PPase caused by NaCl was prevented by 50 µM SNP in cucumber plants (Shi *et al.* 2007). Additionally, the gene expression of a plasma membrane H^+-ATPase was stimulated by SNP in salt-stressed calluses of desert poplar (*Populus euphratica*), which in turn resulted in higher K^+/Na^+ ratio (Zhang *et al.* 2007). Plasma membrane H^+-ATPase and tonoplast Na^+/H^+ antiporter proteins were also induced by SNP in salt-stressed *Avicennia marina* and caused an increment of K^+/Na^+ ratio due to Na^+ efflux from cells towards salt glands (Chen *et al.* 2010). Similar results were observed in cotton (Kong *et al.* 2016), *Kandelia obovate* and *A. corniculatum* (Lang *et al.* 2014). Besides intense Na^+ secretion from sea-lavender (*Limonium bicolor*) leaves under stress, SNP caused an increment in the number of Na^+-loaded salt glands in salt-stressed plants (Ding 2013). In addition to increasing K^+/Na^+ ratio, SNP enhanced Ca^{2+} and Mg^{2+} uptake in salt-stressed plants (Liu *et al.* 2013; Tian *et al.* 2015; Liu *et al.* 2016).

Osmotic stress is a phenomenon also observed in plants under high salinity (Parihar *et al.* 2015). Soybean plants

incubated with SNP prior to salt stress exhibited higher relative water content (RWC) than salt-stressed plants devoid of NO treatment (Dinler *et al.* 2014). Exogenous NO also stimulated proline accumulation in several plant species (Wu *et al.* 2011; Zeng *et al.* 2011; Hayat *et al.* 2012; Khan *et al.* 2012; Fan *et al.* 2013; Liu *et al.* 2013). The activity of pyrroline-5-carboxylate synthetase and proline dehydrogenase, enzymes involved in L-proline biosynthesis, and L-proline accumulation were boosted by SNP in cucumber seedlings under high salinity (Fan *et al.* 2013). Then, cellular turgor was maintained at normal levels and seedlings overcame NaCl stress. Mustard plants subjected to salt stress exhibited higher amounts of glycine betaine when treated with SNP (Khan *et al.* 2012), while wheat plants accumulated soluble carbohydrates in cells (Tian *et al.* 2015). The NO released from SNAP also induced accumulation of L-proline, L-glycine betaine, soluble proteins and carbohydrates in leaves of salt-stressed chickpea (Ahmad *et al.* 2016).

The combined treatment of cucumber seedlings with NaCl and SNP caused an increment of spermine levels and (spermidine + spermine)/putrescine ratio, which in turn helped plant cells to cope with the abiotic stress imposed (Fan *et al.* 2013). Polyamines, such as spermine and spermidine allows for protein, nucleic acid and cell membrane stabilization, besides being great osmolytes and inducers of plant growth and development (Fan *et al.* 2013).

The SNP-induced germination of salt-stressed wheat seeds was attributed to the maintenance of K^+/Na^+ balance, increase of SOD and CAT activities and decrease of the lipid peroxides, H_2O_2 and O_2^- levels (Zheng *et al.* 2008). The SNAP, together with G-proteins, induced the protein accumulation, the antioxidant enzymes activity, the proteins related to cell defense, the energy metabolism and the cell division in salt-treated maize seedlings (Bai *et al.* 2011).

The application of an NO donor on NaCl-treated soybean improved plants growth and biomass accumulation in shoot, root and nodules (Egbichi *et al.* 2014; Vaishnav *et al.* 2016). Indeed, the NaCl-triggered disruption of *Pseudomonas simiae* (rhizobacteria) colonization in soybean was reverted by 100 µM SNP and allowed plant to tolerate salt stress (Vaishnav *et al.* 2016). Conversely, increased levels of NO triggered the decrease of root meristems growth through auxin depletion in NaCl-treated Arabidopsis (Liu *et al.* 2015). In fact, removal of endogenous NO from roots rescued, in part, *PIN* expression and destabilized IAA17 protein, involved in the repression of auxin signaling.

Hydrogen sulfide

Many physiological processes were also found to be regulated by H_2S in plants capable to tolerate different types of stress, including high salinity (Tab. 2).

Oxidative burst, an uncontrolled overproduction of ROS, is one of the first events elicited in plants cell upon salt stress, leading to intensification of electrolytes leakage, lipid peroxidation and protein oxidation. In fact, mitigation of oxidative stress in salt-stressed plants is one of the most studied roles of H_2S. The activities of SOD, CAT, APX, GR, GPX and DHAR in stressed cucumber seedlings were increased by treatment with NaHS (an H_2S-donor) while H_2O_2 and lipid peroxide levels decreased under the same experimental conditions (Yu *et al.* 2013). Undeniably,

Table 2: Roles of hydrogen sulfide (H_2S) during plant response to salt stress.

H_2S effect	Plant species
Activation of antioxidant system	*Cucumis sativus* (Yu *et al.* 2013; Sun & Luo 2014) *Cynodon dactylon* (Shi *et al.* 2013) *Fragaria × ananassa* (Christou *et al.* 2013) *Medicago sativa* (Wang *et al.* 2012; Lai *et al.* 2014) *Nicotiana tabacum* (da-Silva *et al.* 2017) *Oryza sativa* (Mostofa *et al.* 2015) **Triticum aestivum* (Khan *et al.* 2017) Zea mays (Shan *et al.* 2014)
Increase of K^+/Na^+ ratio	*Fragaria × ananassa* (Christou *et al.* 2013) *Hordeum vulgare* (Chen *et al.* 2015) *Medicago sativa* (Lai *et al.* 2014) *Triticum aestivum* (Deng *et al.* 2016)
Protection of photosynthetic apparatus	*Fragaria × ananassa* (Christou *et al.* 2013) *Oryza sativa* (Mostofa *et al.* 2015)
Stimulation of seed germination and plant growth	*Arabidopsis thaliana* (Li *et al.* 2014) *Cucumis sativus* (Sun & Luo 2014) *Cynodon dactylon* (Shi *et al.* 2013) *Medicago sativa* (Wang *et al.* 2012)
Induction of osmoregulators accumulation	*Cucumis sativus* (Sun & Luo 2014) *Cynodon dactylon* (Shi *et al.* 2013) *Oryza sativa* (Mostofa *et al.* 2015)

*Osmotic stress using PEG8000

the suppression of endogenous H_2S by infiltration of tobacco leaves with hypotaurine negatively affected the activity of SOD, CAT and APX in NaCl stress plants (da-Silva *et al.* 2017). The activity of enzymes involved in GSH (γ-glutamylcysteine synthetase) and AsA (L-galactono-1,4-lactone dehydrogenase) biosyntheses and further increment of GSH/oxidized glutathione and AsA/DHR ratios were stimulated by NaHS in leaves of salt-treated maize (Shan *et al.* 2014). Similarly to the observed for NO donors, NaHS controlled methylglyoxylate levels in rice by increasing the activity of glyoxalase I and glyoxalase II (Mostofa *et al.* 2015). In addition, NaHS decreased in plants the activity of lipoxygenase, an enzyme implicated in the formation of lipid peroxides. Alleviation of NaCl-induced oxidative stress by H_2S exogenous was also observed in alfalfa, bermudagrass, strawberry and cucumber (Wang *et al.* 2012; Christou *et al.* 2013; Shi *et al.* 2013; Lai *et al.* 2014; Sun & Luo 2014).

The maintenance of high K^+/Na^+ ratio in plant cells under salt-stress was also reported to be induced by H_2S. Wheat seedlings treated with 50 μM NaHS, followed by 100 mM NaCl exposure exhibited increased K^+/Na^+ ratio with augment of selective transport of K^+ over Na^+ through nonselective cation channels and salt overly sensitive 1 (SOS1), a plasma membrane Na^+/H^+ antiporter (Deng *et al.* 2016). Induction of plasma membrane Na^+/H^+ antiporter genes (*e.g. SOS2*-like, *SOS3*-like and *SOS4*) by NaHS was also described in strawberry plants under high salinity, indicating a role for H_2S in K^+ uptake (Christou *et al.* 2013). The K^+/Na^+ homeostasis in salt-treated alfalfa was shown to be maintained by NaHS through the prevention of K^+ efflux likely triggered by lower expression of shaker-like K^+ outward-rectifying channel genes (Lai *et al.* 2014). Similar results were shown in roots of salt-treated barley seedlings in the presence of NaHS (Chen *et al.* 2015). Remarkably, H_2S maintained low Na^+ levels in cells by increasing the transcription of genes that encode for plasma membrane H^+-ATPase, H^+-ATPase subunit β and vacuolar Na^+/H^+ antiporter and augmenting Na^+ compartmentation in vacuoles (Chen *et al.* 2015).

Germination of alfalfa seeds under 100 mM NaCl was stimulated by 100 μM NaHS (Wang *et al.* 2012). The improvement of seed germination rate caused by H_2S may be a result of the induction of starch break down in the endosperm as the activity of α-amylase and β-amylase increased in salt-stressed cucumber seeds upon treatment with NaHS and ultimately led to hypocotyl and radicle growth (Sun & Luo 2014). The inhibition of root growth in Arabidopsis under salt stress was abolished by NaHS (Li *et al.* 2014), while this H_2S donor improved the survival rate of salt-treated bermudagrass (Shi *et al.* 2013). The treatment of strawberry roots with NaHS prior to NaCl exposure resulted in increased photosynthetic rate, stomatal conductance and RWC in leaves in comparison with plants solely exposed to NaCl (Christou *et al.* 2013). Similarly, an H_2S donor increased chlorophyll, carotenoid and total protein contents in rice under salinity (Mostofa *et al.* 2015).

Exogenous H_2S also led to the accumulation of L-proline, sucrose and other soluble carbohydrates in NaCl-stressed bermudagrass cells (Shi *et al.* 2013). Soluble carbohydrates also accumulated in hypocotyl and radicle cells of cucumber plants stressed with sodium bicarbonate and treated with NaHS (Sun & Luo 2014).

Interplay between NO and H_2S during plant response to salt stress

It is unquestionable that NO and H_2S share roles in the signaling pathway that leads to plant tolerance to environmental stresses. Then, the extent of the cooperative function between these signaling molecules has received considerable attention recently.

Studies carried out with alfalfa seeds treated with NaCl for 24 h and barley seedlings challenged with NaCl for 48 h suggested that H_2S might induce NO production during the response to the stress (Tab. 2; Wang *et al.* 2012; Chen *et al.* 2015). The simultaneous treatment of alfalfa with 100 μM NaHS (H_2S donor) and 100 mM NaCl increased NO levels in cells by 30 %. This increment was accompanied of an increase in K^+/Na^+ ratio and transcription levels of SOD, CAT, APX and guaiacol peroxidase genes and a decrease in lipid peroxides (Wang *et al.* 2012). The use of a specific NO scavenger reversed the NaHS effects on alfalfa, clearly indicating the influence of exogenous H_2S on NO endogenous levels. The NaHS (100 μM) also boosted NO production in barley seedlings by 30 % in comparison to control, detected *in situ* using a fluorophore specific to NO (Chen *et al.* 2015). Meanwhile, NaHS maintained ionic homeostasis through the decrease of K^+ cell efflux and increase of Na^+ in vacuoles. The gene expression of an inward-rectifying potassium channel (HvAKT1) and a high-affinity K^+ uptake (HvHAK4) protein system also increased in barley upon concomitant treatment with NaCl and NaHS. Up-regulation of transcriptional levels of vacuolar Na^+/H^+ antiporter (HvVNHX2), H^+-ATPase subunit β (HvVHA-β) and protein expression of vacuolar Na^+/H^+ antiporter (NHE1) was also observed in barley under the experimental conditions tested (Chen *et al.* 2015). In contrast, the treatment of NaCl-exposed strawberries plants with NaHS (100 μM) decreased NO levels in plant cells by 1.7-fold (Christou *et al.* 2013; Tab. 2). The decrease in the NO levels in strawberry was attributed by the authors to a possible control of nitrosative stress. Likewise, the lipid peroxide levels decreased while increment of expression of genes encoding for antioxidant enzymes and biosynthesis of AsA, GSH and SOS was recorded. Another line of evidence shows that endogenous NO and H_2S stimulate the production of one another in tobacco leaves after NaCl stress for 10 days, as hypotaurine (an H_2S scavenger) compromised NO accumulation in ca. 1.3-fold and cPTIO (an NO scavenger) undermined H_2S production in ca. 1.6-fold (da-Silva *et al.* 2017; Tab. 2). Accumulation of NO and H_2S stimulated the activity of CAT and SOD, decreased stomatal conductance to

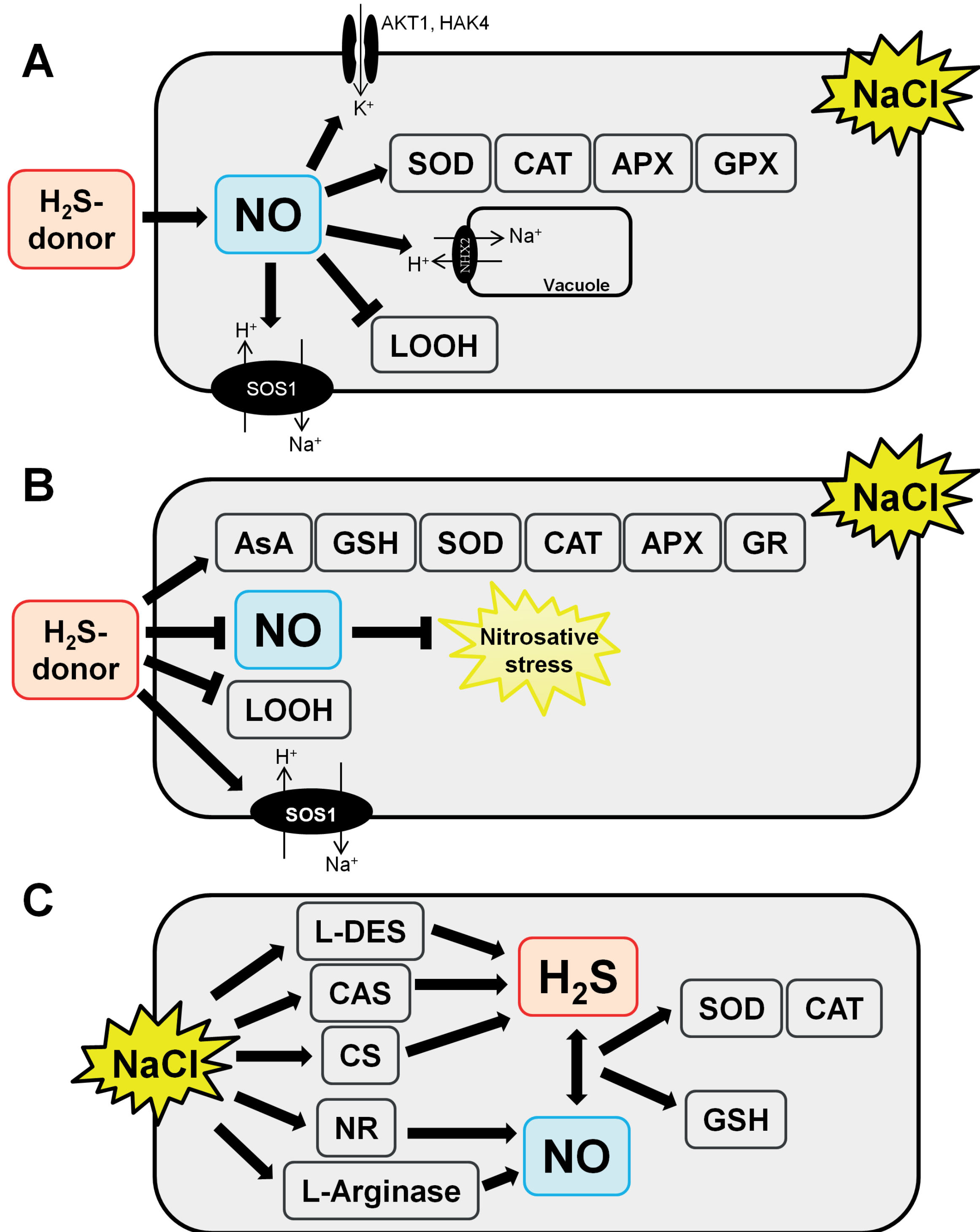

Figure 2. Events triggered by nitric oxide (NO) and hydrogen sulfide (H_2S) during plant response to high salinity. Panel **A:** main findings reported for alfalfa (Wang *et al*. 2012) and barley (Chen *et al*. 2015) in response to an H_2S donor; Panel **B**: main findings reported for strawberry (Christou *et al*. 2013) in response to an H_2S donor; Panel **C**: main findings reported for tobacco (da-Silva *et al*. 2017) highlighting the endogenous increment of both NO and H_2S in plants challenged with high salinity. Standard arrows indicate stimulation of a certain event while flat-headed arrows stand for repression of a certain event. **AKT1**, inward-rectifying potassium channel; **APX**, ascorbate peroxidase; **AsA**, ascorbic acid; **CAS**, β-cyanoalanine synthase; **CAT**, catalase; **CS**, cysteine synthase; **GPX**, glutathione peroxidase; **GR**, glutathione reductase; **GSH**, reduced glutathione; **HAK4**, high-affinity K^+ uptake system; **L-DES**, L-cysteine desulfhydrase; **LOOH**, lipid peroxides**; NHX2**, Na^+/H^+ antiporter**; NR**, nitrate reductase; **SOD**, superoxide dismutase; **SOS,** salt overly sensitive 1 protein.

prevent water loss and drove to the control of oxidative stress assisted by GSH. Additionally, 200 µM *S*-nitrosoglutathione (an NO donor) enhanced the levels of L-cysteine by 10 % and the activity of L/D-DES and CS that, in turn, led to 20 % higher amounts of H_2S in wheat seedlings under osmotic stress (Khan *et al*. 2017). The increase of H_2S, provoked by exogenous NO, controlled oxidative stress by improving SOD, CAT, APX, GR, NR and peroxidase activities in plant cells and relieving H_2O_2 and O_2^- effects. Accumulation of L-proline and glycine betaine was also observed in osmotic-stressed wheat seedlings supplemented with exogenous NO (Khan *et al*. 2017).

Figure 2 summarizes the known interactions between NO and H_2S, regardless of their origin (endogenous or not), determined during plant response to high salinity.

Concluding remarks

Both NO and H_2S may originate in plants from several pathways in which NR seems to be indirectly the main source of NO while the majority of H_2S produced comes from L-DES activity. The role of NO in the mitigation of oxidative burst in plants upon (a)biotic stress is known for roughly two decades and most recently, H_2S has emerged as a new player in such signaling pathway, orchestrating biochemical events that lead plants tolerance to high salinity. Recent studies show that NO and H_2S act together and influence the production of one another during plant response to relatively long periods of salt stress to improve plant antioxidant system, K^+ uptake over Na^+ and production of osmoprotective molecules. The extent of the interaction between these signaling molecules deserves more investigation, since there is still controversy with respect to which molecule triggers the cascade. Understanding this interplay will expand our knowledge on the complex biochemical cascade activated in plant cells with competence to cope with high salinity.

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