

Chapter 2

Functional Role of Nitric Oxide Under Abiotic Stress Conditions

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Abstract Nitric oxide (NO), a free radical in living organisms, is considered a phytohormone and a key signalling molecule functioning in various physiological processes of plants. These physiological processes include germination, growth, senescence, and photosynthesis as well as response mechanisms to specific environmental stresses. Plants under abiotic stress conditions experience oxidative and nitrosative stress; the latter mainly elicited by regulation of NO production. Nitrosative stress describes the molecular or cellular damage promoted by imbalance in NO homeostasis and other reactive nitrogen species. Additionally, depending on its concentration and location in plant cells or tissues, NO might function as an antioxidant and scavenge some other reactive intermediates. Direct or indirect involvement of NO in response mechanisms under water stress, drought, salinity, heavy metal stress, high or low temperature extremities, and ultraviolet radiation has been reported. In this work, the recent findings and current knowledge on the function of NO in plants under abiotic stress conditions are reviewed briefly.

Keywords Nitrosative stress • Oxidative stress • Phytohormone • Signalling

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2.1 Introduction

Nitric oxide (NO) is a gaseous signalling molecule which has attracted much attention because of its diverse functional roles in physiological processes and response mechanisms to various environmental stresses. NO functions in cellular protection against toxicity of reactive oxygen species (ROS), defense response, and tolerance to abiotic stress (Lamattina et al. 2003; Corpas et al. 2007; Besson-Bard et al. 2008a; Neill et al. 2008). In plant cells, endogenous NO can be produced by either L-arginine-dependent nitric oxide synthase (NOS)-like activity or nitrate reductase (NR) activity (Moreau et al. 2008). There are also few other enzymatic and non-enzymatic processes which have been proposed to contribute to cellular NO content. NO and a family of related molecules are designated as reactive nitrogen species (RNS) which include *S*-nitrosothiols (SNOs), *S*-nitrosogluthathione (GSNO), peroxynitrite (ONOO^-), dinitrogen trioxide (N_2O_3) and nitrogen dioxide (NO_2) (Corpas et al. 2007).

To clarify the effects of NO in plants under environmental stresses, extensive studies have been conducted using indirect approaches such as exogenous application of NO donors [e.g. sodium nitroprusside (SNP)], NO scavengers [e.g. 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO)] and enzyme inhibitors. Under adverse environmental circumstances, imbalance of RNS leads to nitrosative stress (Corpas et al. 2011). Certain downstream signalling pathways, employing calcium ion (Ca^{2+}), cyclic guanosine monophosphate (cGMP), and cyclic adenosine diphosphoribose (cADPR), were proposed to be involved in NO-mediated molecular events in plant cells (Neill et al. 2003; Besson-Bard et al. 2008b). NO can exert its effects through regulating the generation of ROS or modulating components of antioxidative system (Laspina et al. 2005). NO can also modulate biological responses by direct modification of proteins, reacting with cysteine residues (*S*-nitrosylation), tyrosine residues (nitration), or iron and zinc in metalloproteins (metal nitrosylation) (Besson-Bard et al. 2008a). Schematic representation of functional roles of NO under abiotic stresses is displayed in Fig. 2.1.

Available data indicates that plant response to stressors such as drought, high or low temperature, salinity, heavy metals and oxidative stress, is regulated by NO (Uchida et al. 2002). The recent findings and current knowledge on the function of NO in plants under different abiotic stress conditions are reviewed in this chapter.

2.2 Nitric Oxide and Abiotic Stress

2.2.1 Heavy Metal Toxicity

Heavy metals include a group of metals and metalloids that are toxic to plants at extremely low concentrations. Although some members of this group, specifically the micronutrients, are required for normal growth and development, others do

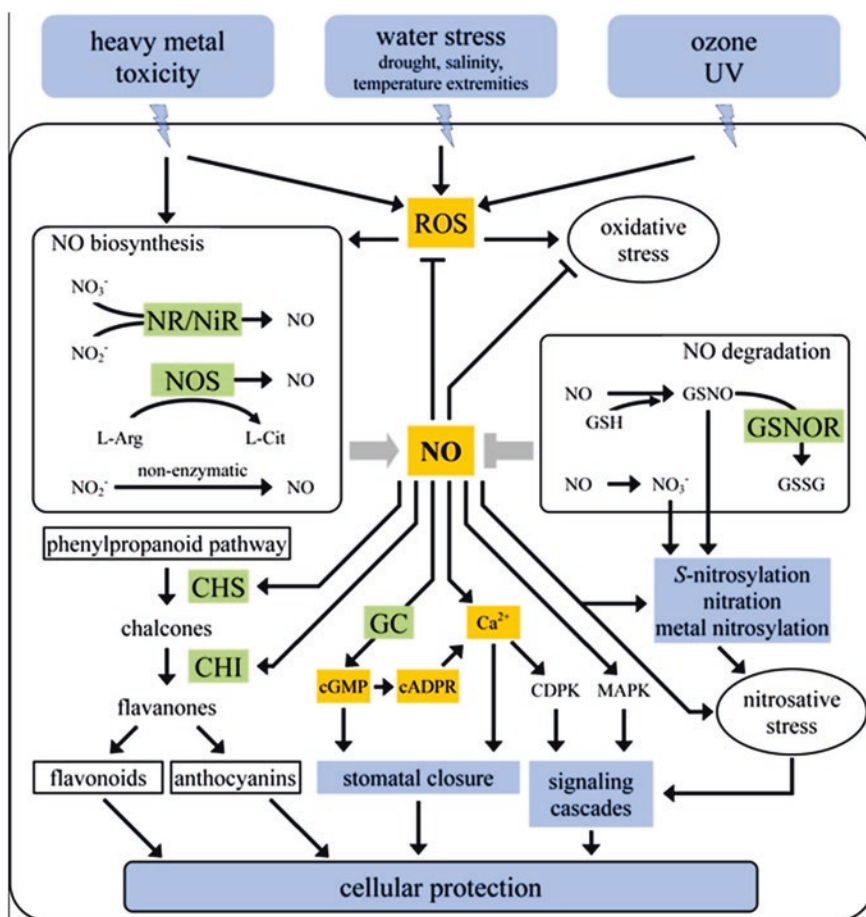


Fig. 2.1 Schematic representation showing functional role of nitric oxide under abiotic stresses. The signalling molecules include nitric oxide (NO), reactive oxygen species (ROS), calcium ion (Ca^{2+}), cyclic guanosine monophosphate (cGMP), and cyclic adenosine diphosphoribose (cADPR). The enzymes include nitric oxide synthase (NOS), nitrate reductase (NR), nitrite reductase (NiR), S-nitrosoglutathione reductase (GSNOR), guanylyl cyclase (GC), chalcone synthase (CHS), and chalcone isomerase (CHI). Arrows and T-bars indicate activation and inhibition, respectively. [Ultra violet (UV), nitrate (NO_3^-), nitrite (NO_2^-), L-arginine (L-Arg), L-citrulline (L-Cit), reduced glutathione (GSH), oxidized glutathione (GSSG), S-nitrosoglutathione (GSNO), calcium-dependent protein kinase (CDPK), mitogen-activated protein kinase (MAPK)]

not perform any known physiological function in plant cells. Nevertheless, at high concentrations all heavy metals can accumulate over time, inhibit growth, and cause toxicity in plant tissues (Benzarti et al. 2008; Rascio and Navari-Izzo 2011).

In order to cope with various heavy metal stresses, plants possess mainly two defense strategies: (1) They adjust cellular free metal content by metal exclusion,

cell wall binding, chelation in the cytosol, and compartmentation in the vacuole; and (2) regulate cellular and molecular responses by induction of antioxidative defense, up-regulation of defensive gene expression, and recovery of stress-damaged proteins (Hall 2002). Accumulating data clearly indicated involvement of NO in cellular response against heavy metal toxicity. Although the information on molecular mechanisms is limited, it has been well established that NO functions in tolerance as a key signalling molecule besides ROS, Ca^{2+} , GSH, and others (Fig. 2.1) (Thapa et al. 2012).

Cadmium (Cd) is one of the most common heavy metals found in soil and considered one of the most phytotoxic anthropogenic pollutant (Benavides et al. 2005; Arasimowicz-Jelonek et al. 2011). Cd toxicity limits growth, photosynthetic attributes, and ultimately yield components. However, exogenous application of NO improves all these parameters in Cd-treated plants (Jhanji et al. 2012). The molecular mechanisms of Cd cytotoxicity in plants are not fully understood, however, certain studies reported participation of NO under Cd stress and alleviation of Cd-induced oxidative damage by application of exogenous NO. Cd-induced increase in endogenous NO level has been observed in cell suspension cultures of soybean (Kopyra et al. 2006) and *Arabidopsis thaliana* (De Michele et al. 2009) and roots of yellow lupine (*Lupinus luteus* L.) seedlings (Arasimowicz-Jelonek et al. 2012).

However, several studies reported reduction of NO or GSNO, a natural reservoir for NO, under Cd toxicity (Barroso et al. 2006; Rodríguez-Serrano et al. 2009). Furthermore, Barroso et al. (2006) observed a decrease in the glutathione (GSH) content with concomitant reduction in the activity and expression level of the catabolic enzyme GSNO reductase (GSNOR) in pea leaves. In a similar study, Rodríguez-Serrano et al. (2009) observed a severe decline in the NOS-dependent NO production under Cd stress.

According to another approach interestingly, exogenous NO counteracts Cd toxicity by regulating cellular distribution of excess Cd and accumulation in cell wall. Xiong et al. (2009) attributed the NO-induced Cd tolerance to the distribution of Cd in the cell walls of rice (*Oryza sativa*) roots. NO also activates the Osmotic Stress-Activated Protein Kinase (OSAK) (Kulik et al. 2012) and Mitogen-Activated Protein Kinase (MAPK) during programmed cell death (Ye et al. 2013), through regulation of gene expression and signal transduction (Zago et al. 2006). Moreover, NO protects plants from Cd-induced oxidative stress (Hsu and Kao 2004; Lasplina et al. 2005) either by directly scavenging ROS or by activating antioxidative enzymes (Arasimowicz-Jelonek et al. 2011). Wang et al. (2013) observed that exogenous application of NO donor SNP significantly decreased the level of ROS and lipid peroxidation and increased the activities of superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), and ascorbate peroxidase (APX) in ryegrass (*Lolium perenne* L.) seedlings exposed to Cd.

Although a certain number of studies indicated a role for exogenous NO in protection against Cd toxicity, it was also claimed that endogenous NO production is necessary for implementation of toxicity (Groppa et al. 2008; Besson-Bard et al. 2009; De Michele et al. 2009). It has been noticed that a short-term

Cd treatment in cell suspension might promote NO burst whereas a long treatment might decrease NO generation. Additionally the concentration, subcellular or tissue localization, and site of action of NO could also account for the observed variations. Alleviation of Cd toxicity by NO also depends on Cd concentrations, duration of treatment, and developmental stage of the plant (Groppa et al. 2008). Arsenic (As) is a potentially pollutant metalloid component of a wide range of minerals. Under As toxicity, plants suffer alterations at various levels including uptake and transport of As, intracellular metabolism, and gene expression (Verbruggen et al. 2009). Strategies employed by plants against As toxicity include reduction of As to arsenite (AsIII), chelation of AsIII with glutathione and phytochelatins, and sub-sequestration of complexes in vacuoles (Gupta et al. 2011). Accumulating data suggest that metabolism of ROS and oxidative stress might be involved in As toxicity (Sharma 2012). Additionally, protein S-nitrosylation induced by NO was demonstrated to be key in regulating the activity of proteins involved in the metabolism of ROS, such as glycolate oxidase, CAT (Ortega-Galisteo et al. 2012), and NADPH oxidase (Yung et al. 2011). Recently it was suggested that As might cause oxidative stress by inducing glycolate oxidase. However, NADPH oxidase did not appear to participate in ROS overproduction but could be critical in regulating antioxidant defenses as well as the transport and translocation of As and other macro- and micro-nutrients (Gupta et al. 2013).

Studies analyzing function of NO under As toxicity in higher plants are limited. In roots of rice (*O. sativa*), exogenous application of SNP provided tolerance against As toxicity and ameliorated the As-induced decrease in length. Furthermore, SNP reduced As-induced accumulation of malondialdehyde (MDA), superoxide ion (O_2^-), and H_2O_2 (Singh et al. 2009; Jin et al. 2010), and an increase in the activities of SOD, CAT and APX was recorded in the presence of SNP under As stress (Jin et al. 2010). In mung bean (*Vigna radiata*) exogenous application of NO as SNP (75 μ M) significantly improved seed germination, growth and CAT activity and decreased As accumulation and activity of SOD (Ismail 2012).

In *Arabidopsis*, exposure to As resulted in a significant growth reduction and caused lipid peroxidation. The changes in growth parameters were accompanied by an alteration of antioxidative enzymes including CAT and glutathione reductase. Additionally, changes in NO metabolism such as a significant increase in NO content, GSNOR activity and protein tyrosine nitration as well as a reduction in GSH and GSNO content were observed. Thus, it was concluded that As stress in *Arabidopsis* provoked both oxidative and nitrosative stress (Leterrier et al. 2012).

It was proposed that As stress is transduced through MAPK signalling cascade in rice (Rao et al. 2011). Toxic As concentration produced ROS and NO in rice roots and severely retarded growth of seedlings. Activity assays were used for determination of As-mediated activation of a 42 kDa MAPK in leaves and 42 and 44 kDa MAPK in roots. Transcript analysis of MAPK family and immunokinase assay in As-treated rice seedlings revealed significant level of induction in OsMPK3 transcripts in leaves and OsMPK3 and OsMPK4 transcripts in roots. Among MAPK kinase (MKKs) gene family, OsMKK4 transcripts were induced

in As-treated rice leaves and roots. The results together with in silico analysis indicated that OsMPK3 and OsMPK4 were the kinases responsible for the signal transduction along with OsMKK4 as an upstream element of the cascade (Rao et al. 2011). However, genetic and mutant analyses need to be carried out to confirm these results.

Although aluminum (Al) is not a heavy metal, it limits crop growth and yield in acid soils by inhibiting cell division and uptake of water and nutrients (Ma et al. 2002). In *Hibiscus moscheutos*, exposure to Al rapidly inhibited the root elongation, but the inhibitory effect of Al on root elongation was alleviated by application of SNP. Further, it was proposed that Al toxicity might inhibit NOS-like activity and might disrupt NO homeostasis, leading to low levels of endogenous NO and inhibition of root elongation in plants (Tian et al. 2007; He et al. 2012a). Accumulating evidences point towards the alleviation of Al toxicity by exogenous NO treatments through activation of antioxidative capacity that may eliminate ROS (He et al. 2012a). Exogenous SNP prevented Al-induced accumulation of MDA in roots and leaves, and alleviated inhibition of growth and impairment of photosynthetic electron transport chain in sour pummelo (*Citrus grandis*) seedlings (Yang et al. 2012) and improved photosynthetic efficiency of Al-stressed *Artemisia annua* (Aftab et al. 2012). Wang et al. (2010) observed a significant role of NR-dependent NO production in alleviating Al-induced oxidative stress in the roots of red kidney bean (*Phaseolus vulgaris*). However, He et al. (2012b) proposed that NO might reduce Al accumulation and enhance tolerance to Al toxicity by regulating hormonal equilibrium in root apices of rye (*Secale cereale* L.) and wheat (*Triticum aestivum*).

The effects of different heavy metals on endogenous NO content in different plant species and tissues as well as the effects of exogenous NO supply on tolerance to different heavy metals are a matter of continuous research. An increase in NO accumulation has been observed in *Arabidopsis* cell suspension cultures under stress imposed by 300 μ M iron (Fe) (Arnaud et al. 2006). Zinc (Zn)-induced NO production promoted ROS accumulation in roots of *Solanum nigrum* primarily by modulating the expression and activity of antioxidative enzymes (Xu et al. 2010). In *Pogonatherum crinitum* root cells, lead (Pb) treatment enhanced nitrate reductase activity and induced rapid NO generation (Yu et al. 2012). On the other hand, NO donor, SNP, effectively reduced copper (Cu)-induced toxicity in the mountain ginseng (*Panax ginseng*) adventitious roots (Tewari et al. 2008) and manganese (Mn) toxicity in excised rice leaves (Srivastava and Dubey 2012).

In most of the studies exogenous NO was proposed to alleviate heavy metal toxicity and attenuate oxidative stresses by decreasing the H₂O₂ content and enhancing the activity of antioxidant enzymes like SOD, CAT, POX and APX. On the other hand, heavy metal-induced accumulation of NO was reported to be responsible for heavy metal toxicity. Further detailed studies may facilitate understanding of the networks involved in plant defenses against heavy metal stress and the roles of NO in regulating both ion homeostasis and cellular responses to heavy metals (Xiong et al. 2010).

2.2.2 Drought Stress

Drought is an important stress affecting crop productivity and yield. It was exhibited that exogenous NO improved drought tolerance and enhanced net photosynthetic rate in wheat (Garcia-Mata and Lamattina 2001; Boyarshinov and Asafova 2011) and rice (Farooq et al. 2009) by improving stability of membrane, enhancing activities of antioxidant enzymes and reducing H₂O₂ and MDA contents. It has been suggested that protective effect of NO on cell membranes might depend on activation of antioxidant enzymes (Boyarshinov and Asafova 2011) as NO has a high affinity for iron-containing enzymes. There are several studies that support NO-induced stimulation of major antioxidant enzymes such as APX, CAT and SOD under stress conditions (Sang et al. 2008; Uchida et al. 2002; Tian and Lei 2007). Hao et al. (2008) reported that treatment with NO prevented water loss and oxidative damage by enhancing SOD activity in maize leaves under water deficit conditions. Application of exogenous SNP prevented drought-induced decrease in growth performance, relative water content and membrane stability by enhancing proline accumulation and activation of antioxidant enzymes accompanied by a decrease in lipid peroxidation and H₂O₂ content under drought stress (Lei et al. 2007; Nasibi and Kalantari 2009). Transgenic *Arabidopsis* lines expressing rat neuronal NOS showed higher drought tolerance with reduced rate of water loss, reduced stomatal aperture, and altered proline and MDA contents (Shi et al. 2012).

The presence of NO donors and ROS increased the abscisic acid (ABA) synthesis in wheat roots under water deficit (Zhao et al. 2001). Additionally, NO accumulation was shown to be essential in ABA-induced closure of stomata in *Vicia faba* plants (Garcia-Mata and Lamattina 2002). It was also indicated that stress-induced ABA and NO accumulation and stomatal closure were additionally dependent on H₂O₂ synthesis in *Arabidopsis* plants (Bright et al. 2006; Neill 2007; Neill et al. 2008). Neill et al. (2008) suggested that cADP ribose (cADPR) might be involved in NO synthesis and signal transduction pathways. Since the changes in stomatal conductance seemed to be closely related to both ABA and NO increase after drought stress in grapevine plants, it was assumed that NO has a potential role in signalling pathway in grapevines under drought (Patakas et al. 2010). Treatment with NO scavenger, cPTIO, increased the NOS activity while the application of NO donor, SNP, reduced NOS activity in maize seedlings which further confirms involvement of NO in drought signalling pathway and function of NO as a cellular messenger to mediate the adaptive responses to drought stress (Hao et al. 2008).

2.2.3 Salinity

Salt stress takes place in the presence of excessive accumulation of soluble salts in the soil. It suppresses plant growth and productivity due to its negative effects

on ion homeostasis and osmotic balance (Munns and Tester 2008). In addition to ionic and osmotic components, salt stress, like other abiotic stresses, leads to oxidative stress through an increase in ROS. Plant adaptations to salinity are mainly of three distinct types; (1) osmotic stress tolerance, (2) ion exclusion, and (3) tissue tolerance to accumulated ions (Munns and Tester 2008). The involvement of NO in salt tolerance has recently been investigated extensively, and function of NO in tolerance has been demonstrated in many plant species (Uchida et al. 2002; Zhang et al. 2006; Tanou et al. 2012; Khan et al. 2012).

Exogenous application of NO increases tolerance to salinity by inducing the activities of antioxidative enzymes in *Citrus aurantium* L. (Tanou et al. 2012) and tomato (Hayat et al. 2012). Besides, enhancing the activities of antioxidative enzymes, NO also protects plants from salinity by boosting the contents of antioxidant metabolites, e.g. ascorbate and reduced glutathione, and the osmolytes, e.g. proline and soluble sugar (Wu et al. 2011). It has been shown that combined application of NO donor, SNP, and calcium chloride (CaCl_2) plays a role in enhancing the tolerance of plants to salt stress by improving antioxidative defense system, osmolyte accumulation and ionic homeostasis (Khan et al. 2012).

Maintenance of K^+ to Na^+ ratio is another strategy to counter the damaging effects of salt stress. In the calluses of reed (*Phragmites communis*) under 200 mM NaCl treatment, addition of NO donor, SNP, stimulated the expression of plasma membrane H^+ -ATPase and induced salt resistance by increasing the K^+ to Na^+ ratio (Zhao et al. 2004). In sunflower seedlings, it was shown that NO provoked biochemical adaptation during seedling growth under salinity conditions. The Na^+/K^+ ratio increased 4-fold in roots, and Na^+ was rapidly transported to the cotyledons (David et al. 2010). *Arabidopsis* mutant *Atmoa1* with reduced endogenous NO level was more sensitive and exhibited lower survival rates compared to wild type under NaCl stress (Guo et al. 2003; Zhao et al. 2007). *Atmoa1* mutants displayed a greater Na^+/K^+ ratio in shoots than the wild type after exposure to NaCl, but SNP treatment attenuated this elevation of the Na^+/K^+ ratio (Zhao et al. 2007).

The available data on the involvement of NO in response mechanism to salinity are contradictory, solely due to the plant species and the severity of the salinity treatment used. A proteomic analysis was used to determine 85 leaf proteins that underwent quantitative variations in citrus plants directly exposed to salt stress. The results exhibited a crosstalk between signalling pathways of H_2O_2 and NO in acclimation to salinity (Tanou et al. 2009). Existence of crosstalk between ROS and NO has been documented by others as well (Zago et al. 2006; Neill 2007; Rodríguez-Serrano et al. 2009). Recently it was reported that oxidative and nitrosative signalling and associated post-translational modifications including protein carbonylation, nitration and S-nitrosylation orchestrate the acclimation of citrus plants to salinity stress (Tanou et al. 2012). Family of protein kinases was proposed as a key component of signalling process mediated by NO during salinity response. In cell suspensions of tobacco exposed to salt stress, *Nicotiana tabacum* Osmotic Stress-Activated Protein Kinase (NtOSAK) was activated by NO. Analysis of NtOSAK revealed that the activation was not due to a process of

S-nitrosylation, but instead due to a process of phosphorylation of two residues located in the kinase activation loop. Additionally, it was demonstrated that NtOSAK interacted with glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which underwent a process of *S*-nitrosylation which affected neither GAPDH activity nor interaction with NtOSAK. Overall, the results indicated that both proteins were regulated directly or indirectly by NO (Wawer et al. 2010).

The interaction between NO and polyamines, and their role in defense responses against abiotic and biotic stress conditions have been investigated (Wimalasekera et al. 2011). It was reported that NO enhanced the tolerance of cucumber seedlings to NaCl stress by regulating the content and proportions of different types of free polyamines (Fan et al. 2013).

2.2.4 Heat Stress

High temperature is one of the most important abiotic stresses that negatively affect plant growth and development. Heat stress results in overproduction of ROS (Kotak et al. 2007) and NO. The rate of NO emission was increased under heat stress in adaxial epidermal cells and cell suspensions of tobacco (Gould et al. 2003). NO donor SNP activated antioxidant enzymes (SOD, CAT, and soluble POX) and elevated heat tolerance in wheat coleoptiles. SNP-induced heat tolerance was dependent on Ca^{2+} and ROS, whose production was probably boosted by activation of NADPH oxidase (Karpets et al. 2011). It was suggested that the activation of already available NADPH oxidases was initiated by NO (Karpets et al. 2012).

However, in sunflower seedlings, heat treatment (38 °C for 4 h) reduced NO production, inhibited GSNOR activity, increased protein nitration, and caused accumulation of SNOs and peroxynitrite (Chaki et al. 2011). Studies with *Arabidopsis* mutant *hot5* deficient in GSNOR activity showed the correlation of this enzyme with heat stress response. It was indicated that GSNOR modulates the intracellular level of SNOs, enabling thermo tolerance (Lee et al. 2008). When pea plants were exposed to heat stress (38 °C for 4 h), NO content was reduced in leaves but NOS-like activity was not affected significantly. It was also shown that the SNO content increased in stressed pea plants (Corpas et al. 2008). After heat treatment of tobacco suspension cells at 35 °C or 55 °C, it was determined that NO production in cells shocked at 35 °C was lower compared to ones shocked at 55 °C (Locato et al. 2008). Song et al. (2006) reported that calluses of two ecotypes of reed (*P. communis*) showed much higher activities of SOD, CAT and APX in the presence of SNP under heat stress than those under heat stress alone. On the other hand, the activity of lipoxygenase was down-regulated accordingly in calluses of reed (Song et al. 2006). Although exogenous application of H_2O_2 or SNP prior to treatments with salinity or heat alleviated the deleterious effects of salt or heat stress in young rice plants, high concentrations of H_2O_2 or SNP treatment decreased growth rate (Uchida et al. 2002).

The effects of NO on non-photochemical fluorescence quenching (NPQ) of chlorophyll *a* fluorescence were investigated under heat stress (Hossain et al. 2011). When intact leaves of wild type and Δ GLB3 (lacks truncated hemoglobin gene) *Arabidopsis thaliana* were compared under heat stress, it was found that NPQ of Δ GLB3 was severely declined. The effect was also found to be mimicked by chemical NO donors, and it was completely prevented by the NO scavenger, cPTIO. It was suggested that NO can be involved in the decline of NPQ under heat stress (Hossain et al. 2011). Overall, it can be concluded that the modulation of intracellular NO depends on the degree of heat treatment and plant tissues or species used.

2.2.5 Cold Stress

Changes in temperature have important effects on plant growth and development. In nature, some plants acquire enhanced freezing tolerance by cold acclimation, which increases the chance of survival at subsequent freezing temperatures. Although low temperature induces changes in expression of hundreds of genes (Seki et al. 2002; Shinozaki et al. 2003; Sharma et al. 2005; Zhu et al. 2007; Winfield et al. 2010), the information related with the effect of NO and nitrogenous molecules on tolerance to cold stress is very limited.

When NO was applied exogenously, tolerance to cold conditions was improved in various plant species such as wheat, maize and tomato (Neill et al. 2003). Both NR and NOS-like enzyme have been suggested as sources of NO in response to cold (Corpas et al. 2008; Zhao et al. 2009, 2011; Cantrel et al. 2011). The synthesis of NO under low temperature stress has been reported in *Lotus japonicus*, *Pisum sativum* and *Arabidopsis thaliana* (Shimoda et al. 2005; Corpas et al. 2008; Cantrel et al. 2011). However, Airaki et al. (2012) reported a significant decrease in NO content in pepper leaves under low temperature (8 °C for 24 h) stress. It was proposed that NO induction under cold stress played a role in freezing tolerance through proline synthesis (Zhao et al. 2009). In *Brassica juncea* low temperature-induced S-nitrosylation caused inactivation of carboxylase activity of Rubisco, and this may indirectly indicate a role for NO after chilling exposure (Abatand Deswal 2009). It was suggested that NOS-like activity and subsequent NO accumulation participates in decreased cold-responsive pollen germination, inhibited tube growth, and reduced proline accumulation, partly via cGMP signaling pathway in *Camellia sinensis* (Wang et al. 2012).

NO was proposed as a critical regulator of synthesis of phosphatidic acid, a phospholipid derived signal, in plants (Laxalt et al. 2007). While exogenous application of NO donors led to phosphatidic acid synthesis in tomato (*Solanum lycopersicum*), *Vicia faba* and cucumber (*Cucumis sativus*), phosphatidic acid synthesis in response to xylanase and auxins was impaired by treatments with the NO scavenger, cPTIO (Laxalt et al. 2007; Distefano et al. 2008; Lanteri et al. 2008). Cantrel et al. (2011) demonstrated that the rise in phosphatidic acid levels was not

mediated by cold-triggered NO production. It was shown that sphingolipids were transiently phosphorylated in response to cold exposure and NO served as a negative regulator of this phosphorylation. It was also indicated that phytosphingosine phosphate and ceramide phosphate were transiently synthesized after chilling, and synthesis was negatively regulated by NO in *A. thaliana*. It was proposed that NO might be considered as an intermediate in gene regulation and lipid-based signaling during cold transduction (Cantrel et al. 2011). Since NO plays a central role in modulating the synthesis of sphingolipid signals, it might be a key player in the plant response to cold stress.

2.2.6 Ozone

Ozone (O₃) is an extremely active form of oxygen. Air pollutants can liberate ozone during photochemical reactions. Ozone has negative effects on plants as well as protective effects on cellular damage by ultraviolet (UV) radiation. Air pollutant ozone enters to the leaf through open stomata. The effects of ozone on plants depend on concentration and exposure time. While a low level of ozone exposure reduces photosynthesis and growth, a high concentration of ozone provokes cell death with visible injuries in the leaves. After ozone treatment, NOS activity was induced, salicylic acid was accumulated, and cell death was observed (Rao and Davis 2001; Ahlfors et al. 2009). High concentrations of ozone treatment in *Arabidopsis* caused a burst of ROS and NO (Ahlfors et al. 2009) but decreased contents of ascorbate and glutathione (Mahalingam et al. 2006).

When tobacco plants were exposed to ozone, accumulation of H₂O₂ and NO was observed. Up-regulation of alternative oxidase gene (AOX) expression, decrease in cytochrome-c content and accumulation of ethylene were also detected under ozone exposure. Depending on the results, it was suggested that NO is coordinated together with ethylene for the regulation of AOX expression (Ederli et al. 2006). NO was indicated as indispensable for activation of *AOX1a* encoding a protein involved in scavenging of mitochondrial ROS (Ederli et al. 2006, 2008). Ozone and NO caused transcriptional activation of *AOX1a*, glutathione peroxidase (GPX) and induction of ethylene production through aminocyclopropanecarboxylic acid synthase (ACS2) which were cGMP independent. However, NO-induced accumulation of transcripts of phenylalanine ammonia lyase (*PALa*) and pathogenesis-related protein (*PR1a*) was cGMP dependent (Pasqualini et al. 2009). Additionally, cGMP has been reported to increase in response to NO (Durner et al. 1998). Treatment with ozone induced increases in NO levels in plants (Ahlfors et al. 2009). Ozone also stimulates a salicylic acid induced protein kinase (SIPK) in a NO-dependent manner and Ca²⁺ is essential, but not sufficient, to promote NO accumulation in ozone-treated tobacco (Pasqualini et al. 2012).

Ozone exposure induced flavonol accumulation and NO generation in *Ginkgo biloba* cells, however, pretreatment with NO specific scavenger cPTIO inhibited ozone-induced flavonol production. It was also indicated that NR activity

was enhanced with ozone exposure. NR-mediated NO signalling is found to be involved in ozone-induced flavonol accumulation in *G. biloba* cells (Xua et al. 2012). It can be concluded that ozone prompts a general response where NO, ROS and several hormones interact. NO can modify signalling, hormone biosynthesis and gene expression in plants exposed to ozone.

2.2.7 UV-B Radiation

Ultraviolet B (UV-B) radiation (280–320 nm) is absorbed by stratospheric ozone, but a small proportion is transmitted to the earth surface. Since atmospheric pollutants cause the destruction of ozone layer, UV-B radiation on the earth surface rises. High doses of UV-B radiation induce production of ROS, causing damage to proteins, lipids and DNA, and hence affect the cell integrity, morphology, and physiology of plants. Under UV-B stress, the NO produced in plants is able to protect the cells from the deleterious effects of ROS and oxidative stress (Frohnmeier and Staiger 2003).

Besides oxidative pathways, UV-B radiation strongly induced NOS-like activity and caused a decrease of both leaf biomass and exo- or endo- β -glucanase activities (An et al. 2005). In contrast, an NOS inhibitor and a NO donor largely decreased the activity of NOS in non-irradiated seedlings. Tossi et al. (2009a) observed that apocynin (an inhibitor of NADPH oxidase) reduced UV-B-induced oxidative damage by reducing chlorophyll breakdown caused by H_2O_2 , and this was correlated with NO production mediated by NOS activity. Pretreatment with diphenyleneiodonium (an inhibitor of NADPH oxidase) and NG-nitro-L-arginine methyl ester (L-NAME, an inhibitor of NOS) partially blocked the NO accumulation (Tossi et al. 2009b). In bean seedlings subjected to UV-B radiation, exogenous NO partially alleviated the UV-B effect characterized by a decrease in chlorophyll content and oxidative damage to the thylakoid membrane (Shi et al. 2005).

Various NOS-like enzymes were characterized in plants, but their molecular basis is still doubtful in higher plants (Moreau et al. 2008). Zhang et al. (2011) have proved that NO derived from NR plays a role in conferring UV-B stress tolerance. Direct association between NR activity, NO emission and nitrite (NO_2^-) content was determined in silver birch (*Betula pendula*) under UV-B radiation. It was confirmed that NR activity and NO emission are required for flavonoid accumulation in silver birch, and NO scavenger cPTIO abolished NO. Although a role for an NOS-like source for NO during UV-B stress was suggested in maize and *G. biloba* (Hao et al. 2009; Tossi et al. 2009a), the effect of mammalian NOS inhibitors on UV-B-induced NO generation cannot be shown by Zhang et al. (2011).

The involvement of NO in response to UV-B radiation (An et al. 2005; Qu et al. 2006) appears to act through ABA-mediated pathways (Tossi et al. 2009b). Tossi et al. (2012a) proposed that UV-B perception in plant cells leads to an increase in ABA levels which triggers elevation of cytosolic Ca^{2+} concentration leading to NO production through induction of NOS and/or NOS-like activities.

This enhancement of NO production contributes to tolerance to high doses of UV-B by protecting cell redox homeostasis from uncontrolled generation of ROS and associated deleterious effects provoked by UV-B radiation. In *A. thaliana* an NOS inhibitor and a NO scavenger partially blocked UV-B-mediated induction of chalcone synthase (*CHS*) gene involved in producing chalcones that participate in defense mechanisms and production of protective pigments such as flavonoids (Fig. 2.1) (Mackerness et al. 2001). It was found that irradiation of silver birch leaves with UV-B induces both flavonoid accumulation and NO generation (Zhang et al. 2011). Systemic accumulation of NO through UV-B radiation in maize seedlings was also reported by Tossi et al. (2012b). Up regulation of maize P (*ZmP*) gene expression and its target genes *CHS* and chalcone isomerase (*CHI*) by NO, leads to an increased flavonoid biosynthesis in non-irradiated leaf regions. This systemic response to UV-B perception involves enhanced levels of NO and flavonoids. It was also indicated that NO is involved in secondary metabolite production. Recently, it was suggested that UV-B-enhanced NO levels in plant cells can also protect microtubule organization as well as microtubule-related processes of root growth and development against disrupting effects of UV-B (Krasylenko et al. 2012).

2.2.8 Flooding

Flooding is a major obstacle affecting plant survival in various regions of the world. Non-enzymatic formation of NO depends on acidic pH and extracellular NO_2^- which are conditions that occur during anaerobiosis (Dat et al. 2004). When cells are under anaerobic conditions like flooding, NO_2^- can accumulate intracellularly. Subsequently, NO_2^- is used as a substrate by cytosolic NR for generation of NO (Botrel et al. 1996; Rockel et al. 2002).

Various reactions involved in production of NO in roots are stimulated under anaerobic conditions. It was shown that side-reaction of NR is the reduction of NO_2^- to NO (Yamasaki et al. 1999). Since NO formation by NR requires high NO_2^- concentration, it is suggested that NO can be formed by NR and accumulates only when the cells are in transition to the unfavorable anaerobic conditions (Sairam et al. 2008). Dordas et al. (2003) proposed that in hypoxic maize cell cultures and alfalfa root cultures, one of the functions of hypoxic stress-induced haemoglobin (Hb) is to modulate NO levels in the cell. High nitrate (NO_3^-) concentrations together with the induction of stress-induced Hb in *Arabidopsis* may also relate to a requirement to modulate NO levels (Wang et al. 2000). When plant roots were exposed to hypoxia, NR activity was increased, with NO_2^- reduction being suppressed at the nitrite reductase step (Botrelet et al. 1996). For the immediate survival of plant root cells, Hb may be pivotal to regulate the levels of NO. Hypoxia results with a decline in mitochondrial respiration, an increase in NADH and a drop in ATP levels. Then, Hb gene expression and activation of NR occurs, together with production of NO (Igamberdiev et al. 2005). This would prevent

the cell death and maintain ATP levels. Sanchez et al. (2010) suggested that NO formed by copper-containing nitrite reductase in soybean nodules after flooding has a negative effect on expression of nitrogenase. They proposed that plant oxygen carrier leghemoglobin (Lb) has a major role in detoxifying NO and NO₂⁻ in response to flooding conditions.

2.2.9 Wounding

Different types of abiotic and biotic stresses, including herbivores, wind or rain, can produce mechanical injury in plants. In the damaged zone, plants respond with a cascade of events and signals that induce various genes and molecular responses (Schilmiller and Howe 2005). There are studies providing evidence for the involvement of NO and other nitrogenous metabolites in response to wounding. In *A. thaliana*, mechanical wounding induced a rapid accumulation of NO which was proposed to be involved in jasmonic acid-associated defense response (Huang et al. 2004). In pea (*P. sativum*) leaves, mechanical wounding induced accumulation of NO after 4 h. This was accompanied by induction of NOS and GSNOR activities and an increase in the content of SNOs. However, pattern of tyrosine nitration of proteins was not affected by mechanical injury (Corpas et al. 2008). It was reported that wounding triggered accumulation of certain SNOs, specifically GSNO, but did not affect NO content in sunflower hypocotyls. GSNO accumulation was indicated as an outcome of down-regulation of GSNOR activity in sunflower (Chaki et al. 2011). Overall, involvement of NO or other reactive nitrogen species (RNS) might be critical for induction of responses to mechanical injury in plants.

2.3 Conclusion

It is well known that abiotic stresses (salinity, water deficit, extreme temperatures, toxic metals, air pollutants etc.) limit plant growth and productivity. Abiotic stress is estimated to be the primary cause of worldwide crop loss. Several studies have been performed to understand tolerance mechanisms of plants in order to overcome the negative effects of these stresses on yield. There are also studies in literature supporting the relevance of NO in plants under abiotic stress conditions. Application of exogenous NO provides certain level of resistance against several types of stresses by activating different biochemical pathways. NO may help plants to survive stressful conditions through its function as a signalling molecule in the activation of antioxidative enzymes or its direct reaction with active oxygen, nitrogen and lipid radicals. Further genetic and proteomic analyses and additional physiological approaches will be required to understand the details of NO metabolism and function in plants. The acquired data will shed light on the sources of NO

and factors affecting its synthesis under abiotic stress, and also will provide in depth information on different strategies which this multifaceted molecule adopts in facing the detrimental effects of abiotic stress.

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