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Physiological and Biochemical Mechanisms of Nitric Oxide Induced Abiotic Stress Tolerance in Plants

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Abstract: Abiotic stress is the major limiting factor of plant growth and crop yield. Better understanding of plant stress responses and tolerance is very important in the light of increasing intensities of stressors like salinity, drought, flooding, heavy metal, temperature extremes, high-light intensities, UB-radiation, herbicides, ozone and others, due to global climatic and other environmental changes. The role of Nitric oxide (NO) in stress responses in plants came in the focus of plant science in the last decade. NO is an important signaling molecule with diverse physiological and biochemical functions involving the induction of different intracellular plants processes, including the expression of defense-related and redox regulated genes against abiotic and biotic stress induced reactive oxygen species (ROS) detoxification. In spite of the significant progress that has been made in understanding NO biosynthesis and signaling in plant, several crucial questions remain unanswered. In this study, we reviewed the recent progress in NO research to reveal its diverse role in the physiological and biochemical processes in plants and the protective mechanisms towards abiotic stress tolerance.

Key words: Nitric oxide, reactive oxygen species, abiotic stress, signaling, antioxidant metabolism

INTRODUCTION

Abiotic stresses viz. salinity, drought, flooding, heavy metal, temperature extremes, high-light intensities, UB-radiation, herbicides, ozone are the major causes of yield loss in cultivated crops worldwide. The survival of plants under such a stressful condition depends on the plant's ability to perceive the stimulus, generate and transmit the signals and to initiate various physiological and biochemical changes (Bohnert and Jensen, 1996; Hossain and Fujita, 2009b; Hasanuzzaman *et al*., 2009). Nitric oxide (NO) is a highly reactive, membrane-permeable free radical which was earlier considered as a highly toxic compound. However, the discovery of NO signaling role in regulation of cardiovascular system has

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changed the paradigm concerning the cytotoxicity. Later, the discovery of its biological functions has been elucidated. Research on NO in plants has gained considerable attention in recent years mainly due to its function in plant growth and development and as a key signaling molecule in different intracellular processes in plants. The physiological function of NO in plants mainly involves the induction of different processes, including the expression of defense-related genes against abiotic and biotic stress and apoptosis/programmed cell death (PCD), maturation and senescence, stomatal closure, seed germination, root development and so on. NO can be produced in plants by non-enzymatic and enzymatic systems (Del Rio *et al*., 2004; Crawford and Guo, 2005; Delledonne, 2005; Arasimowicz and Floryszak-Wieczorek, 2007). However, the effects of NO on different types of cells have been proved to be either protective or toxic, depending on the concentration and situation.

NO triggers many kinds of redox-regulated defense-related gene expression directly or indirectly to establish plant stress tolerance (Polverari *et al*., 2003; Sung and Hong, 2010). Different reports have been published in recent years on the physiological function of NO (Bolwell, 1999; Wojtaszek, 2000; Beligni and Lamattina, 2001; Wendehenne *et al*., 2001; Neill *et al*., 2003; Lamattina *et al*., 2003) and particularly on NO signaling in the induction of cell death, defence genes and interaction with Reactive Oxygen Species (ROS) during plant defense (Van Camp *et al*., 1998; Durner and Klessig, 1999; Klessig *et al*., 2000; Delledonne *et al*., 2001; Wendehenne *et al*., 2001; Neill *et al*., 2003; Romero-Puertas and Delledonne, 2003). Exogenous application of NO confers tolerance to various abiotic stresses in plants by enhancing both enzymatic and non-enzymatic antioxidant defense system (Neill *et al*., 2002; Tian and Lei, 2006; Sheokand *et al*., 2008; Zheng *et al*., 2009; Singh *et al*., 2009; Xu *et al*., 2010). Several lines of study have shown that the protective effect of NO against abiotic stress is closely related to the NO-mediated reduction of ROS in plants (Beligni and Lamattina, 1999a; Wang and Yang, 2005).

In this review, we discuss recent progress in understanding the function of NO in plant responses and tolerance to abiotic stresses and in plant development. We explore the physiological and biochemical mechanisms of NO induced abiotic stress tolerance and the mechanisms by which it transduce signals into cellular responses towards stress tolerance.

HISTORICAL PERSPECTIVE OF NO

Nitric oxide was first described by Joseph Priestley in 1772, when it was considered as a highly toxic compound; indeed, it is a component of exhaust gas and industrial wastes. However, the discovery in the late 1980s of NO signaling role in regulation of cardiovascular system by R.F. Furchgott, L.J. Ignarro and F. Murad (Nobel Prize winners in Physiology and Medicine, 1998) has changed the paradigm concerning the cytotoxicity of free radical substances. The discovery and elucidation of its biological functions in the 1980s came as a surprise. NO was named Molecule of the Year in 1992 by the journal Science, a NO Society was founded and a scientific journal devoted entirely to NO was created (Delledonne, 2005). NO is a diffusible gaseous free radical. Its emission from plants has been reported several years ago in soybean plants (Klepper, 1979). Later, *in vivo* and *in vitro* Nitrate Reductase (NR) dependent NO production has been found in other plants such as sunflower and maize (Rockel *et al*., 2002). Although, NO synthase, the main enzyme that catalyses the *in vivo* synthesis of NO in animals has not been isolated in plants yet, NO has proved to be a functional metabolite in plants (Neill *et al*., 2002).

PRODUCTION OR GENERATION OF NO IN PLANTS

There are several sources of NO in nature and environment. As a pollutant, NO is produced by both automobile engines and power stations. NO is also emitted from plants under stress situations, such as herbicide treatment or pathogen attack, as well as under normal growth conditions (Wendehenne *et al*., 2004). In pea plants, wilting intensified the NO emission (Leshem and Haramaty, 1996) and in tobacco cells under heat, osmotic and salinity stresses, a rapid increase in NO production was observed (Gould *et al*., 2003). In leaves of *Arabidopsis*, wounding induced a fast accumulation of NO, as checked by Confocal Laser Scanning Microscopy (CLSM) and spin trapping Electron Paramagnetic Resonance (EPR) (Huang *et al*., 2004). These data led to postulate that NO could be a useful marker of plant stress (Magalhaes *et al*., 1999) and that NO generation, like that of the ROS, can occur naturally as a generalized response to different types of stress (Magalhaes *et al*., 1999; Gould *et al*., 2003). In cells, NO can exist in the form of three interconverting compounds: a free-radical nitric oxide ($NO^{\bullet}$), a nitrosonium cation (NO^{+}) and a nitroxyl anion (NO^{-}) (Hong *et al*., 2008).

In biological systems, NO can be generated enzymatically or non-enzymatically. The most extensively described NO-producing enzymes have been Nitric Oxide Synthase (NOS) and Nitrate Reductase (NR). Much early effort by plant scientists focused on searching for a plant NOS. The enzymic oxidation of L-arginine to yield NO and L-citrulline has been reported in extracts from pea (Leshem and Harmaty, 1996), lupin (Cueto *et al*., 1996), soybean (Delledonne *et al*., 1998), tobacco (Durner *et al*., 1998) and maize (Ribeiro *et al*., 1999). Competitive inhibitors based on L-arginine have been used to suppress NO production in soybean, *Arabidopsis* and tobacco (Delledonne *et al*., 1998; Durner *et al*., 1998) implicating NOS activity. NOS (Moncada *et al*., 1991) catalyses the two-step oxidation of L-arginine to NO and citrulline (L-arginine+NADPH+H+O_2 → N^{ω} hydroxyarginine +$NADP^{+}$+H_2O and thereafter N^{ω} hydroxyarginine + ½ NADPH + ½ H^{+} → Citrulline+NO^{+} ½ $NADP^{+}$ H_2O), a reaction that might also be catalysed by a cytochrome P450 (Boucher *et al*., 1992; Wojtaszek, 2000). NR generates NO from nitrite with NADPH as electron donor (Kaiser *et al*., 2002; Yamasaki *et al*., 1999). Zemojtel *et al*. (2004) postulated the discovery of a novel conserved family of NOS. The authors showed significant homology in NOS sequence in as divergent organisms as plants, snails and mammals. The discovery of a new class of NOS in *Arabidopsis thaliana* is a real breakthrough in the studies on NO occurrence and function in plants. Furthermore, it is now obvious that plants have evolved multiple routes of NO synthesis, different from those found in animals (Kopyra and Gwozdz, 2004).

Another enzyme involved in NO production is Nitrate Reductase (NR). Despite the tentative identification of a plant NOS gene, clear evidence shows that plants can produce NO from nitrite via NADPH-dependent NR (NO_3 → NO_2 → $^{\bullet}NO$+O_2). The application of high nitrite levels under conditions of anoxia increased NO production (Rockel *et al*., 2002). The formation of NO due to NR activity was reported in many plant species, such as sunflower, spinach, maize (Rockel *et al*., 2002), cucumber (De la Haba *et al*., 2001), *Arabidopsis thaliana* (Desikan *et al*., 2002), green alga *Chlamydomonas reinhardtii* (Sakihama *et al*., 2002) wheat, orchid and aloe (Xu and Zhao, 2003). Desikan *et al*. (2002) provided good evidence that NR-mediated NO generation in guard cells is required for abscisic acid-induced stomatal closure in *A. thaliana*. Xu and Zhao (2003) postulated that NR is a main source of endogenous NO in higher non-leguminous plants. NO content in leaves of wheat, orchid and aloe was reduced by 90% following the heat or microwave treatment, which indicates that NO is mostly enzymatically produced. Moreover, the reduction of NR activity and concomitant

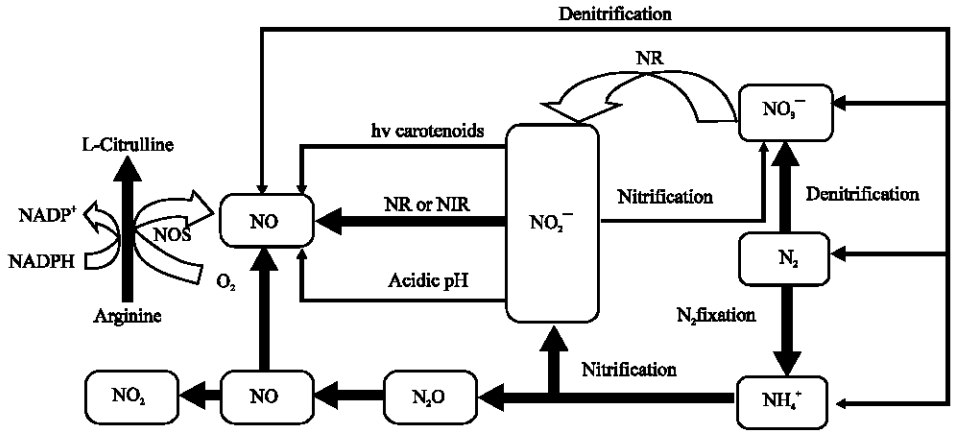

Fig. 1: Possible sources of NO in environment. NO is generated by the action of nitric oxide synthase (NOS). Major origins of NO are the reactions utilizing NO_2^-: non-enzymatic reductions either at acidic pH or light-driven in the presence of carotenoids and enzymatic catalysed by NAD(P)H-dependent Nitrate Reductase (NR) or nitrite reductases (NiR). It could also be a by-product of denitrification, nitrate assimilation and/or respiration. Nitrification of NH_4^+ is the major source of N_2O emitted to the atmosphere where it might be further oxidized to NO and NO_2 (Wojtaszek, 2000)

decrease in NO content were observed in wheat seedlings growing in a medium lacking molybdenum, which is the NR cofactor and after treatment with sodium tungstate, the NR inhibitor (Xu and Zhao, 2003).

Other enzymes that can generate NO are nitrite: NO-reductase (Ni-NOR), probably situated in the plasma membrane (Stöhr and Ullrich, 2002) and xanthine oxidoreductase (XOR) operating at low oxygen tensions and requiring molybdenum as a co factor (Neill *et al*., 2003).

In plants, NO can also be generated by non-enzymatic reduction of nitrite ($2NO_2^- + 2H^+ \rightarrow 2HNO_2 \rightarrow NO + NO_2 + H_2O$), but this is favored only under acid conditions such as found in the barley aleurone cell (Beligni *et al*., 2002; Bethke *et al*., 2004). Nitrification /denitrification cycles provide NO as a by-product of NO_2 oxidation into the atmosphere (Wojtaszek, 2000). Nitrite can also be chemically reduced by ascorbic acid at pH 3–6 to yield NO and dehydroascorbic acid (Henry *et al*., 1997). This reaction could occur at microlocalized pH conditions in the chloroplast and apoplastic space where ascorbic acid is known to be present (Horemans *et al*., 2000). Another non-enzymatic mechanism proposed of NO formation is the light-mediated reduction of NO_2 by carotenoids (Cooney *et al*., 1994). The possible sources of NO in the environment are illustrated in Fig. 1.

PROTECTION MECHANISM OF NO IN PLANT

Two mechanisms by which NO might abate stress have been postulated by Radi *et al*. (1991). First, NO might function as an antioxidant, by directly scavenging the ROS, such as superoxide radicals (O_2^-), to form peroxynitrite (Radi *et al*., 1991), which is considerably less toxic than peroxides and thus limit cellular damage. Second, NO could function as a signaling molecule in the cascade of events leading to changes of gene expression (Lamattina *et al*., 2003). Whereas some authors considered NO as a stress inducing agent (Leshem, 1996), others have reported its protective role (Beligni and Lamattina, 1999a, b; Hsu and Kao, 2004),

depending on its concentration, the plant tissue or age and the type of stress. When present at low amounts, NO acts as signals for the activation of defense responses, however, higher concentrations produced by uncontrolled ROS generation cause severe injury. The presence of an unpaired electron within the NO molecule makes it a reactive species and is also the origin of its duality. NO is generally toxic and in these conditions, when combined with low amounts of O_2^-, the formation of peroxynitrite ($ONOO^-$) was reported to be deleterious to lipids, proteins and DNA (Wink *et al*., 1993). However, whenever toxicity is incurred as a result of ROS damage, NO might act as a chain breaker and thus limit damage. In these situations, peroxides have proven to be much more toxic than NO and $ONOO^-$ and NO is considered to have a protective function (Wink *et al*., 1993). In addition, the reaction of NO with lipid alcoxyl (LO•) and peroxyl (LOO•) radicals is rapid, giving rise to the expectation that NO could also stop the propagation of radical-mediated lipid oxidation.

The NO-producing enzymes identified in plants are NR and several NOS-like activities, including one localized in peroxisomes which has been biochemically characterized. Recently, two genes of plant proteins with NOS activity have been isolated and characterized for the first time and both proteins do not have sequence similarities to any mammalian NOS isoform. However, different available evidence indicated that there are other potential enzymatic sources of NO in plants, including xanthine oxidoreductase, peroxidase, cytochrome P450 and some hemeproteins. In plants, the enzymatic production of the signal molecule NO, either constitutive or induced by different biotic/abiotic stresses, may be a much more common event than was initially thought (Del Rio *et al*., 2004). Most of the work on NO action in plant cells has focused on its ability to act in the same direction as ROS. This concept explains NO participation in the hypersensitive response (Van Camp *et al*., 1998), in the regulation of the expression of defense genes and in the increase in chlorophyll fluorescence (Leshem, 1996). Consequently, the participation of NO in the antioxidant cellular system of plants, as in animals, is a strong possibility. The main sources for NO-mediated cytoprotection within plant cells are shown in Fig. 2.

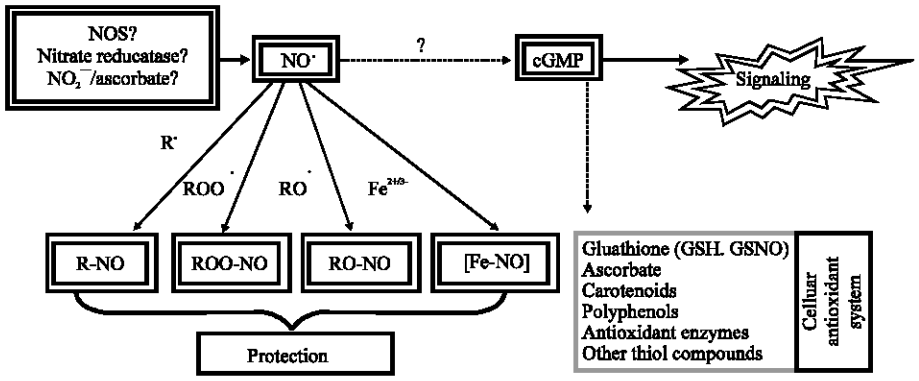

Fig. 2: Probable chemical reactions of NO in related to cytoprotection. NO reactivity with reactive oxygen species accounts for direct sources of both toxicity and protection. Indirect protection would come from the interaction between NO and the cellular antioxidant system. Signaling pathways, together with direct modification of target molecules could be mechanisms for other physiological functions of NO in plants. [Abbreviations: R•, non-oxygen free radicals; RO•, alcoxyl radicals; ROO•, peroxyl radicals] (Beligni and Lamattina, 1999b)

NO AS SIGNALING MOLECULE

NO acts as a signaling molecule within species from every biological kingdom, a feature reflecting its physical properties which give it an exceptionally rich chemistry. NO is highly reactive due to the presence of an unpaired electron and, as with oxygen, it can exist in a variety of reduced states, NO^- (nitroxyl ion), NO and NO^+ (nitrosonium ion), with each Reactive Nitrogen Intermediate (RNI) able to undergo specific interactions (Gow and Ischiropoulos, 2001). Every stressor triggers in the cell a signaling cascade leading to the triggering of specific defense responses. Recognition of the stress stimulus by the cell membrane receptor results in the formation of signaling molecules, which in turn leads to a change in the concentration or modulation of the so-called second messengers and as a consequence to the triggering of defense response.

In biological systems, NO affects signaling through a range of actions. Many NO effects are mediated by oxidative damage associated with the formation of the potent oxidant peroxynitrite via interaction with superoxide ($NO+O_2^- \rightarrow ONOO^-$). A more subtle action is the electrophilic attack by NO^- on thiol groups, particularly cysteine residues, resulting in *S*-nitrosylation of molecules such as glutathione or proteins. Protein *S*-nitrosylation can modulate protein activity (Lander *et al*., 1996) while, the kinase is inactivated (Park *et al*., 2000). Alternatively, NO can modify proteins by nitration, particularly of tyrosine residues (Radi, 2004). The effects induced by NO may be independent of cellular second messengers, although the biochemical mechanism of this effect has not been comprehensively clarified. The chemical nature of NO results in transition metals (e.g., Fe, Cu, Zn) and proteins containing thiol groups being important targets for this molecule (Wendehenne *et al*., 2001). Analogously as in the NO-guanylate cyclase interaction, NO may interact with iron present in other proteins. In this way, NO modifies activity of aconitase and Fe-S enzyme catalysing isomerization of citrate to isocitrate, in tobacco (Navarre *et al*., 2000). Inactivation of this enzyme decreases the cellular energy metabolism, which may results in reduced electron flow through the mitochondrial chain and a subsequent decrease in the ROS generation. Moreover, tobacco cytosolic aconitases have reasonably high homology to human iron regulatory protein (IRP-1), which suggests that it may possesses IRP activity and affect iron homeostasis in plants (Navarre *et al*., 2000). NO periodically inhibits also catalase and peroxidase, containing the haem system, which may potentially regulate ROS level in the cell, e.g., during programmed cell death (PCD) in xylem formation (Ferrer and Barcelo, 1999; Clark *et al*., 2000).

PHYSIOLOGICAL ASPECTS OF NO INDUCED ABIOTIC STRESS TOLERANCE

Nitric oxide (NO) is a relatively stable free radical gas which may act as a key signaling molecule in plants and mediates various physiological, biochemical and developmental processes including seed germination, stomatal closure, root development and hypersensitive responses (Delledonne *et al*., 2001; Neill *et al*., 2003). Garcia-Mata and Lamattina (2001) showed that exogenous NO (applied as sodium nitroprusside, SNP) reduced transpiration and induced stomatal closure in several species such as *Vicia faba*, *Salpichroa* and *Tradescantia* sp. and NO was indicated to be a component of ABA signaling pathways in ABA-induced stomatal closure. On the other hand, NO can also mediate plant growth regulators and ROS metabolism and increasing evidence has shown it is involved in signal transduction and responses to abiotic stress such as drought, low and high temperatures, UV-radiation. Some physiologican role of NO under abiotic stress condition are presented in Table 1.

Table 1: Outline of some important NO-mediating effect during abiotic stresses

Strain	NO-induced effect	Induced plant/organs	Reference
Salinity	Positive effect on antioxidant enzymes	Chickpea	Sheokand *et al.* (2008)
	Reduce mitochondrial oxidative damage	Wheat	Zheng *et al.* (2009)
	Increase salt tolerance by shift of the K^+/Na^+ ratio in favor of Na+ content	*Phragmites communis*	Cheng and Yuan (2009)
	Induce expression of Na^+/H^+ antiporter gene	Maize	Zhang *et al.* (2006)
Drought	Increase activities of antioxidant enzyme and proline accumulation	Rice	Farooq *et al.* (2009)
	Enhance capacity of antioxidants, improved stability of cellular membranes	Wheat	Tan *et al* (2008), Zhang *et al.* (2003a)
	Involving in ABA signaling, stomatal closure induction of ABA synthesis, LEA expression	*Pisum sativum*	Gould *et al.* (2003), Leshem and Haramaty (1996)
High temperature	Promote higher leaf photochemical activity and cell membrane integrity	Mung bean leaf discs	Yang *et al.* (2006)
	Increased tolerance of seedlings	*Medicago sativa*	Leshem *et al.* (1998)
Low temperature	Decline the ROS level	*Scenedesmus obliquus*	Mallick *et al.* (2000)
Heavy metal	Enhances cd tolerance by increasing pectin and hemicellulose contents	Rice	Xiong *et al.* (2009)
	Decrease Cd toxicity by uplifting the antioxidant enzymes activities	Sunflower	Laspina *et al.* (2005)
	Scavenge active oxygen species including	Rice leaves	Hsu and Kao (2004)
	H_2O_2 Promote antioxidant enzyme activities	Wheat leaves	Zhang *et al.* (2008b)
	Increased the root elongation; reduced the NOS activity	*Hibiscus moscheutos*	Tian *et al.* (2007)
Herbicides	Protection against cellular damage	Potato	Beligni and Lamattina (1999c)
	Promoted the activity of antioxidant enzymes	*Scenedesmus obliquus*	Mackerness *et al.* (2001)
	Prevent the ROS-mediated damages	Potato	Beligni and Lamattina (1999c)
UV-radiation	Induced the expression of CHS gene	*Arabidopsis thaliana*	Mackerness *et al.* (2001)
	Promote plant growth	*Maize*	An *et al.* (2005)
High light	Reduce light-induced electrolyte leakage, reduced ROS production through	*Chlamydomonas reinhardtii*	Sakihama *et al.* (2002)
	up-regulation of antioxidant enzymes	Tall fescue	Xu *et al.* (2010)

As a mediator of physiological processes, NO has an incredible number of beneficial effects; for example, it functions as a messenger in immune responses. But it can become very toxic under certain complex conditions determined, for example, by its rate of production and diffusion and the redox state of the cell (Murphy, 1999). In plant cells, NO and NO-derived molecules are involved in response to many abiotic stresses. Consequently, when a specific abiotic stress alters physiological NO metabolism causing damage to biological molecules, a nitrosative stress is generated (Corpas *et al*., 2007; Valderrama *et al*., 2007). In fact, NO interacts with ROS in various ways and might serve an antioxidant function during various stresses (Beligni and Lamattina, 1999b). Modulation by NO of superoxide formation (Caro and Puntarulo, 1998) and inhibition of lipid peroxidation (Boveris *et al*., 2000) also illustrate its potential antioxidant role, mainly due to its ability to maintain the cellular redox homeostasis and regulate ROS toxicity. Another key role of NO in abiotic stress response relies on its properties as a signaling molecule as described in previous heading.

SALINITY

Salinity is one of the most important stress factors which limit the growth and development of plant by altering their morphological, physiological and biochemical attributes. Under saline conditions, tolerant plant cells achieve ion homeostasis by extruding Na to the external medium and/or compartmentalizing into vacuoles, maintaining K uptake and high K and low Na in the cytosol. It has been proven that the activity of the plasma

membrane H^+-ATPase is a key index of plant adaptation to salt stress (Hasanuzzaman *et al*., 2009; Nahar and Hasanuzzaman, 2009). The protective role of NO in salt tolerance of plants is well documented. Zhang *et al*. (2004) reported that NO enhanced salt tolerance in maize seedlings, through increasing K^+ accumulation in roots, leaves and sheathes, while decreasing Na^+ accumulation (Zhang *et al*., 2004). Similarly, NO induced salt resistance of calluses from *Populus euphratica* also found by increasing the K^+/Na^+ ratio and this process was mediated by H_2O_2 and was dependent on the increased plasma membrane H^+-ATPase activity (Zhang *et al*., 2007). In maize, addition of exogenous NO increases tolerance to salt stress by elevating the activities of the proton-pump and Na^+/H^+ antiport of the tonoplast (Zhang *et al*., 2004). Additionally, pretreatment with NO donor (SNP) protected young rice seedlings, resulting in better plant growth and viability (Uchida *et al*., 2002), promoted seed germination and root growth of yellow lupine seedlings (Kopyra and Gwozdz, 2003) and increased growth and dry weight of maize seedlings (Zhang *et al*., 2006) under salt stress conditions. NO treated wheat (*Triticum aestivum* L.) leaves also showed less destruction of chlorophyll and plasma membrane permeability induced by NaCl treatment (Ruan *et al*., 2002). There is a wealth of evidence that NO induced salt tolerance is due to profound increase in both non enzymatic and enzymatic components.

DROUGHT

Drought is one of the most important abiotic stresses that causes significant reductions in crop yield and thus hinders the food security. Upon exposure to drought stress, plants exhibit a wide range of responses at the whole plant, cellular and molecular levels (Chaves *et al*., 2003; Shinozaki and Yamaguchi-Shinozaki, 2007; Hossain and Fujita, 2009b). The NO-synthesizing activity in wheat plants was found to increase under drought conditions. The newly synthesized NO together with H_2O_2 participated in the regulation of ABA-induced closing of stomata in various plant species (Neill *et al*., 2008). In addition, the protective role of NO in drought-stressed plants has been reported by several researchers. In a recent work, the activity of NOS in the cytosolic and microsomal fractions of maize leaves was determined (Sang *et al*., 2008). The results showed that water stress induced increases in NOS activity in the cytosolic and microsomal fractions and the NOS activity in the microsomal fraction was higher and more susceptible to water stress treatment than that in the cytosolic fraction of maize leaves. It was observed that exogenously applied NO, reduced water loss from detached wheat leaves and seedlings subjected to drought conditions, decreased ion leakage and transpiration rate and induced stomatal closure, thereby enhancing plant tolerance to drought stress (Garcia-Mata and Lamattina, 2001). Interestingly, a specific NO scavenger, cPTIO, reverted the above actions of NO (Garcia-Mata and Lamattina, 2001). Results of this experiment suggest that exogenous application of NO donors might confer on plants an increased tolerance to severe drought stress conditions. It was shown that treatment of plants with exogenous NO enhanced drought tolerance of cut leaves and seedlings of wheat (Tian and Lei, 2006). In addition, NO treatment enhanced wheat seedling growth and maintained relatively high water content and alleviated oxidative damage (Hao and Zhang, 2010). However, higher dose (2 mM SNP) aggravated the stress as a result of uncontrolled generation of ROS and ineffectiveness of antioxidant systems. Exogenous NO increased the activities of water stress induced subcellular antioxidant enzymes, which decreased accumulation of H_2O_2. These results suggest that NOS and NR are involved in water stress-induced NO production and NOS is the major source of NO. The potential ability of NO to scavenge H_2O_2 is at least in part due

to the induction of a subcellular antioxidant defense mechanism. NO alleviates the ROS-mediated cytotoxic process in potato leaves (Beligni and Lamattina, 1999a). The ROS-mediated damages caused by drought, including cell death, ion leakage and DNA fragmentation, were inhibited by exogenous NO and all of the protective effects were abolished by the treatment with PTIO (Beligni and Lamattina, 1999a). The protective effect of NO in osmotic stress was recently confirmed in two ecotypes of reed suspension cultures. Zhao *et al.* (2008) suggested that polyethylene glycol (PEG-6000) induced NO release in stress-tolerant but not sensitive ecotype reed, effectively protecting against oxidative damage and conferring an increased tolerance to osmotic stress (Zhao *et al.*, 2008). In wheat seedlings, the osmotic stress produced by treatment with 0.4 M manitol reduced leaf water loss while increasing the leaf ABA content. These effects were partially reversed by NO scavengers and NOS activity inhibitors (Xing *et al.*, 2004). In tomato detached leaves, the application of NO donors inhibited the synthesis of proteinase inhibitor I and the generation of H_2O_2 in response to mechanical wounding (Orozco-Cárdenas and Ryan, 2002).

EXTREME TEMPERATURE

Every plant has a critical temperature for its growth and development. Temperature, either very high or low, is harmful for plants. Research results indicated that NO also participates in plant response to high and low temperature stress. For example, high temperature treatment of lucerne cells resulted in an increase of NO synthesis, whereas, the application of exogenous NO increased cold tolerance in tomato, wheat and maize (Neill *et al.*, 2003). It was shown that both in tobacco leaf peels and suspension cells, high temperature generated a rapid and significant surge in NO levels (Gould *et al.*, 2003). Leshem (2001) reported that short term heat stress increased the NO production in alfalfa, which negatively correlated with ethylene production. NO pretreatment reduced heat-induced damage in rice seedlings and prevented the impairment of photosystem II (PSII). Additionally, NO pretreatment induces not only active oxygen scavenging enzyme activities but also expression of transcripts for stress related genes encoding sucrose-phosphate synthase and small heat shock protein (Uchida *et al.*, 2002). Lamattina *et al.* (2001) observed that treatment with NO increased the survival rate of leaves of wheat and maize seedlings (Lamattina *et al.*, 2001). The role of NO during extreme temperature stress might be to decrease the ROS level caused by heat or lower temperature (Neill *et al.*, 2002).

HEAVY METALS AND ALUMINUM

Heavy metal contamination of soils is an increasing problem worldwide and great environmental threats to biota as these metals are being accumulating in soils and plants in undesirable amounts. Heavy metal cause oxidative damages to plants when its concentration exceeds the limit (Hossain *et al.*, 2010). Interestingly, under heavy metal stress plant produces NO which further may protect the plants against damages due to stress (Hsu and Kao, 2004). In order to demonstrate the possible role of NO in response to heavy metals in the metal accumulator *Brassica juncea* and the crop plant *Pisum sativum*, researchers grew these plants in presence of 100 μM cadmium (Cd), copper (Cu), or zinc (Zn) (Bartha *et al.*, 2005). They obtained different NO levels with different heavy metal loads; the most effective metals were copper and cadmium, where the NO production doubled after 1 week of treatment. In case of copper treatment, two-phase kinetics was found, that is, a rapid NO burst in the first 6 h was followed by a slower, gradual increase. The fast

appearance of NO in the presence of cupric ions suggests that this can be a novel reaction hitherto not studied in plants under heavy metal stress. In relation to other abiotic stresses it was documented that exogenous NO reduces the destructive action of heavy metals, ethylene and herbicides on plants (Hung *et al*., 2002; Kopyra and Gwozdz, 2003).

In soybean plants exposed to an acute level of $CdCl_2$ (200 μM), the exogenous application of NO protected against oxidative damage caused by this metal stress, elevated levels of heme oxygenase-1 expression, as it occurs with other genes involved in the antioxidant defense system (Rao and Davis, 2001). In contrast, pretreatment of seedlings with 100 mM SNP protected sunflower leaves against Cd-induced oxidative stress (Orozco-Cardenas and Ryan, 2002). A similar effect has been described in *Lupinus* roots grown with 50 μM Cd^{2+} and it was proposed that the protective effect of NO could consist of stimulation of superoxide dismutase activity to counteract overproduction of superoxide radicals, thus avoiding formation of peroxynitrite from NO and O_2^- (Uchida *et al*., 2002). *Hibiscus moscheutos* exposed to 100 μM $AlCl_3$ experienced inhibition of root growth. This effect was accompanied by inhibition of NOS activity and reduced NO concentrations (Neill *et al*., 2008). Using fluorescent and laser scanning confocal microscopy, Kopyra and Gwozdz (2003) found that NO pretreatment significantly reduced O_2^--induced specific fluorescence in *Lupinus luteus* roots under heavy metals treatment. These results suggest that NO antioxidant function may be carried out by a scavenging of O_2^-. The detoxifying effect and antioxidative role of NO were also found in soybean cell cultures exposed to cadmium and copper treated *Chlorella* (Singh *et al*., 2004). Furthermore, a recent finding showed that NO alleviated the Al^{3+} toxicity in root elongation of *Hibiscus moschetuos* (Tian *et al*., 2007). In addition, mechanical damage was reported to elicit NO production from NOS activity in *Arabidopsis* leaves (Garces *et al*., 2001). In another study, *Cassia tora* plants pretreated for 12 h with 0.4 mM SNP and subsequently exposed for 24 h to 10 μM Al exhibited a significantly greater root elongation and a decrease in Al accumulation in root apexes as compared with plants without SNP treatment (Wang and Yang, 2005). All these data indicate the importance of exogenous NO in the uptake of micronutrients and in the protection against deleterious effects of toxic heavy metals such as Cd or Al (Corpas *et al*., 2006; Zhang *et al*., 2008a). Recently, Cu-induced NO generation and its relationship with proline synthesis in *Chlamydomonas reinhardtii* were investigated (Zhang *et al*., 2008b). However, the physiological implication of the plant endogenous NO in the response to heavy metal stress is still not well-known (Corpas *et al*., 2006).

HERBICIDES

More than 30 years ago, it was shown that the application of herbicides to soybean plants increased the NO production (Klepper, 1979). More recently, several studies have confirmed that the treatment with NO donors (SNP) protect plants from the deleterious herbicidal effects (Beligni and Lamattina, 1999c, 2002; Huang *et al*., 2002). NO treatment also protects chloroplast membrane, the diquat induced chlorophyll loss (Beligni and Lamattina, 1999c). Additionally, the paraquat induced protein content reduction was prevented by NO Cell death, ion leakage and DNA fragmentation, which are ROS-mediated damages resulting from *Phytophthora infestans* infection, were inhibited by NO donor and all those protective effects were abolished after treatment with cPTIO (Beligni and Lamattina, 1999c).

UV-RADIATION AND OZONE

The stratospheric ozone (O_3) layer is vital to life on earth because it is the principal agent absorbing the ultraviolet radiation in the earth's atmosphere. Since, 1990, the depletion

of the stratosphere O_3 layer due to anthropogenic and natural destruction is leading to increasing levels of solar ultraviolet-B (UV-B: 280-320 nm) radiation reaching the earth's surface (Kerr and McElroy, 1993; Russell *et al*., 1996). Ambient UV-B irradiance at low latitudes is also high due to the high solar angle and a relatively low stratospheric O_3 amount (Baker *et al*., 1980). Like other stress, exposure to UV-B leads to the generation of ROS. In addition,O_3 effects on plants are primarily induced by an increased production of ROS, both outside and inside the plant cell, which is a common feature of in plants.

Mackerness *et al*. (2001) showed the participation of NO in plant response to UV-B radiation, demonstrating an increase in NOS-type enzymatic activity and an elevation of NO level. Shi *et al*. (2005) suggested that NO may effectively protect plants against UV-B radiation, most probably through the increased activity of antioxidative enzymes. Qu *et al*. (2006) showed that UV-B radiation significantly induced NOS activity and promoted NO release. Zhang *et al*. (2003b) found that NO was a second messenger associated with developmental growth under UV-B radiation. It was found that UV-B radiation significantly induced NOS activities and accelerated the release of apparent NO of mesocotyl and that rhizospheric treatments to exogenous NO donors may mimic the response of the mesocotyl to UV-B radiation. 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). Moreover, UV-B induced stomatal closure, which was mediated by NO and H_2O_2 and the generation of NO was caused by a NOS-like activity (Ruan *et al*., 2004). However, other authors reported that NO generated in guard cells were produced by NR activity (Shi *et al*., 2007). NO treatment has been shown to increase levels of O_3-induced ethylene production and increase leaf injury (Rao and Davis, 2001). In tobacco plants, fumigated with O_3, the accumulation of hydrogen peroxide in mitochondria and early accumulation of NO and ethylene in leaf tissues have been described. He *et al*. (2005) reported that UV-B radiation induced stomatal closure by promoting NO and H_2O_2 production.

The treatment of *Vicia faba* leaves with SNP alleviated the injurious effect of UV-B, leading to the increased chlorophyll content and to the increase in potential and effective quantum yields of electron flow in photosystem II; the oxidative damage to thylakoid membranes was reduced (Shi *et al*., 2005). The alleviating effects of NO were also observed in experiments with an algal culture of *Spirulina platensis*, which was evident from protective action on total biomass and physiological parameters, such as the content of chlorophyll *a* and proline (Xue *et al*., 2006). Although SNP mitigated the inhibitory effect of UV-B irradiation, the endogenous NO was found to be the main factor responsible for inhibition of mesocotyle growth upon UV-B irradiation (Ederli *et al*., 2009).

BIOCHEMICAL MECHANISM OF NO INDUCED ABIOTIC STRESS TOLERANCE

A great variety of abiotic stresses including drought, salinity, ultraviolet light, heat, chilling, air pollutants and heavy metals cause molecular damage to plants, either directly or indirectly through ROS formation (Laspina *et al*., 2005; Ferreira and Cataneo, 2010), such as superoxide (O_2^-) and hydroxyl (·OH) radicals, hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) (Therond *et al*., 2000). Literature data supply evidence showing that plant response to such stressors as drought (Garcia-Mata and Lamattina, 2001; Zhao *et al*., 2001; Neill *et al*., 2002), salinity (Zhao *et al*., 2004, 2007), heavy metal (Kopyra and Gwozdz, 2003; Hsu and Kao, 2004; Wang *et al*., 2004), high light (Xu *et al*., 2010), UV-radiation (Mackerness *et al*., 2001), high temperature (Leshem *et al*., 1998; Yang *et al*., 2006), herbicide (Mallick *et al*., 2000;

Huang *et al*., 2002) is regulated by NO. In order to avoid ROS toxicity, aerobic cells are provided with a flexible set of enzymes and metabolites involved in ROS catabolism, which often acts at the site of ROS production (Shigeoka *et al*., 2002; Foyer, 2004; Mittler *et al*., 2004; De Pinto *et al*., 2006). Survival under these conditions depends on the capability of plants to increase specific pathways involved in ROS removal (Noctor and Foyer, 1998; Asada, 1999).

One of the most intriguing issues in NO biology is its dual function as a potent oxidant and an effective antioxidant (Beligni and Lamattina, 1999b). This dual role of NO might depend on differences in dose, bioprocesses, development stages, or species (Ferrer and Bacelo, 1999; Clark *et al*., 2000; Zeier *et al*., 2004). The cytoprotective role of NO is mainly based on its ability to maintain the cellular redox homeostasis and to regulate the level and toxicity of ROS. NO exerts a protective function against oxidative stress mediated by (1) reaction with lipid radicals, which stops the propagation of lipid oxidation; (2) scavenging the superoxide anion (O_2^-) and formation of peroxynitrite ($ONOO^-$) that is toxic for plants but can be neutralized by ascorbate and glutathione; (3) activation of antioxidant enzymes (SOD, CAT and POX etc.). One of the fastest reactions of NO within a biological system is its combination with superoxide anion (O_2^-) that leads to the formation of strong oxidant peroxynitrite ($ONOO^-$) (Neill *et al*., 2003; Wendehenne *et al*., 2001) that is one of the major toxic reactive nitrogen species that exerts deleterious effects on DNA, lipids and proteins (Stamler *et al*., 1992; Pryor and Squadrito, 1995; Yamasaki *et al*., 1999). The antioxidative protection offered by NO can be described under following headings:

REGULATION OF NON-ENZYMATIC ANTIOXIDANT CONTENT IN PLANTS BY NO AND STRESS TOLERANCE

Plants possess a variety of non-enzymatic molecules which play a substantial role in counteracting oxidative stress caused by stress. The non-enzymatic antioxidants include ascorbate, glutathione, tocopherols, carotenoids and flavanoids etc. (Noctor and Foyer, 1998; Tausz and Grill, 2000). They act coordinately with antioxidant enzymes to maintain the cellular redox state of the cell under stressful conditions.

Ascorbate(AsA)

In plant cell, AsA is the most abundant antioxidant and serves as a major contributor to the cellular redox state and protects plant against oxidative damage resulting from a range of biotic and abiotic stresses (Smirnoff, 2000; Hossain and Fujita, 2010). Due to the ability of AsA to donate electrons in a number of enzymatic and non-enzymatic reactions, it is considering to be the most popular and powerful ROS detoxifying compound. It is the substrate of cAPX and the corresponding organellar isoforms, which are critical components of the AsA-GSH cycle for H_2O_2 detoxification (Nakano and Asada, 1981; Dalton *et al*., 1986). AsA can directly quench $^1O_2$, O_2^- and ·OH and regenerate α-tocopherol from α-chromanoxyl radical thereby providing protection to membranes. Elevated levels of endogenous AsA in plants are necessary to offset oxidative stress in addition to regulating other plant metabolic processes (Smirnoff, 2000). Hsu and Kao (2004) reported that NO increase the levels AsA as a result of an increase in the capacity of NO to scavenge ROS in rice leaves treated with NO and $CdCl_2$ and might account in part for the lower contents of H_2O_2 observed in rice leaves treated with NO and $CdCl_2$. Laspina *et al*. (2005) reported that NO pretreatment before Cd exposure returned AsA contents to values close to the controls and NO-treated plants showed AsA content similar to controls.

Glutathione (GSH)

In higher plants, the redox active tripeptide glutathione (GSH) fulfils a plethora of functions. The chemical reactivity and high water solubility of the thiol group of GSH makes it particularly suitable to serve a broad range of biochemical functions to protect plants against oxidative stress (Hossain and Fujita, 2010; Hossain *et al*., 2010). These include its pivotal role for maintaining the cellular redox poise and its involvement in detoxification of heavy metals and xenobiotics. Intimately linked to these functions, GSH also acts as a cellular signal, mediating control of enzyme and/or regulatory protein activities, either directly or via glutaredoxins. GSH can participate not only in scavenging H_2O_2 through the AsA-GSH cycle but also in a direct reaction with other active oxygen species (May *et al*., 1998).

NO protects plant cells against oxidative processes by stimulating GSH synthesis. Increasing evidence indicates that the GSH biosynthetic pathway is stimulated in response to NO in plant and animal cells and increases oxidative stress tolerance (Moellering *et al*., 1998; Kim *et al*., 2004; Innocenti *et al*., 2007). The regulation of GSH synthesis by NO raises the question of the physiological roles that may be sustained by such a modulation. Several studies have evidenced the capacity of NO to counteract oxidative damages (Beligni and Lamattina, 1999c; Beligni *et al*., 2002; Wang and Wu, 2005). GSH may also play an important role in regulating NO bioactivity. Indeed, it can readily react with NO to form GSNO, which serves as a NO reservoir and a long-distance NO vector in mammals (Zhang and Hogg, 2004). In regard of recent reports indicating the importance of nitrosothiols in controlling plant responses to pathogens (Feechan *et al*., 2005), the stimulation of GSH synthesis by NO may provide an important regulatory loop for NO bioactivity. Laspina *et al*. (2005) observed a decrease in GSH level by Cd in sunflower leaves, but NO was able to counteract efficiently GSH depletion. In fact, Cd forms stable complexes with thiol groups such as GSH and phytochelatins (Cobbett, 2000) and this might be explaining, at least in part, GSH decrease. NO could be acting simply as an antioxidant (Beligni and Lamattina, 1999a; Beligni *et al*., 2002; Neill *et al*., 2002) or could be playing a role in the elevation of GSH levels, either by increasing the biosynthesis rate of this metabolite or through an increased supply of cysteine, the limiting substrate (Li *et al*., 1999). It was observed that NO pre-treated salt-stressed seedlings showed significant increase in GSH level as compared to the seedlings subjected to salt stress alone because GSH synthesis is enhanced by NO treatment (Moellering *et al*., 1998; Innocenti *et al*., 2007). The increased GSH pool was also found in wheat roots in response to NO (Groppa *et al*., 2008) which could be attributed to the increased NO levels. Several studies have evidenced that GSH biosynthetic pathway is stimulated in response to NO in animal cells and yeast (Moellering *et al*., 1998; Kim *et al*., 2004) and very recently, Innocenti *et al*. (2007) reported the effect of NO on the GSH/hGSH synthesis pathway was examined in roots of *Medicago truncatula*. Generation of NO was achieved by treatment of roots with NO and GSNO, two different NO donors with unrelated structures, which have been widely used to analyse gene expression in plants (Durner *et al*., 1998; Polverari *et al*., 2003; Murgia *et al*., 2004). This result provided the evidence that GSH synthesis is stimulated by NO in plants. This result is in resemblance with those of De Pinto *et al*. (2002), who reported a decrease of GSH content in NO treated BY-2 tobacco cells, suggesting a different response between cell culture and roots. Nevertheless, a similar response was previously reported for fission yeast and animal cells (Kuo and Abe, 1996; Moellering *et al*., 1998; Kim *et al*., 2004) and the present report extends the effect of NO on GSH synthesis pathway to plants. As for yeast and animals, NO triggered an increase of the endogenous GSH amount above control in *Medicago truncatula* roots through the

stimulation of GSH synthesis gene transcript accumulation. Whereas only γ-*ecs* gene stimulation was tested for GSH accumulation upon NO treatment in yeast and animals (Kuo and Abe, 1996; Kim *et al*., 2004).

REGULATION OF ANTIOXIDANT ENZYMATIC ACTIVITIES BY NO AND OXIDATIVE STRESS TOLERANCE

Apart from non-enzymatic antioxidant plant possess an array of antioxidant enzymes that maintains ROS homeostasis in all cellular compartments and regulates the adjustment of ROS levels according to the cellular needs at a particular time (Apel and Hirt, 2004; Gechev *et al*., 2006). These antioxidants include the enzymes, superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), glutathione peroxidase (GPX, EC 1.11.1.9), glutathione S-transferases (GST; EC 2.5.1.18), ascorbate peroxidase (APX; EC 1.11.1.11), dehydroascorbate reductase (DHAR; EC 1.8.5.1), glutathione reductase (GR; EC 1.6.4.2) and monodehydroascorbate reductase (MDHAR; EC 1.6.5.4). Extensive research findings supported the idea that coordinated induction and regulation of both enzymatic and non-enzymatic antioxidant defense pathway is necessary to obtain substantial tolerance against oxidative stress in plants.

Superoxide Dismutase (SOD)

Superoxide dismutase (SOD) is an important antioxidant enzyme and is the first line defense against oxidative stress in plants. SOD catalyses the dismutation of O_2^- to molecular oxygen (O_2) and H_2O_2 (Yu *et al*., 2005). It plays an important part in determining the concentration of O_2^- and H_2O_2 in plants hence performs a key role in the defense mechanism against free radical toxicity (Bowler *et al*., 1992). The induction of SOD in plant cells in response to different stressful conditions reflects its important role in the defense mechanism of plants. Stress tolerant plants have higher SOD activity as compared to sensitive plants (Shalata *et al*., 2001; Sekmen *et al*., 2007). As a signal molecule NO induces/stabilizes the expression of many antioxidative enzymes including SOD (Frank *et al*., 2000). Huaifu *et al*. (2007) found that exogenous NO increased the SOD activity of leaves under NaCl stress. Similarly, Shi *et al*. (2007) showed that application of NO significantly decreased the inhibition of SOD activity by salt stress, which suggested that application of NO could promote the conversion from O_2^- into H_2O_2 and O_2, which is an important step in protecting the cell. Additionally, Cheng *et al*. (2002) concluded that the inhibition of osmotic stress- and dehydration-enhanced senescences of rice leaves by NO is most likely mediated through an increase in SOD activity and a decrease in lipid peroxidation. In contrast, Laspina *et al*. (2005) observed 110% increase in SOD activity in Cd-treated plants, while plants treated with NO and subjected to Cd stress the SOD activity was also increased, but only to 59% over the control. NO prevented the paraquat-induced reduction in protein content, increase in level of MDA and decline in the activities of antioxidant enzymes including SOD. Therefore, increased SOD activity enhances stress tolerance of plants when other important antioxidant enzymes (APX, DHAR, MDHAR, GR, GSH and AsA) are also present in high levels. Because the over produced H_2O_2 must be scavenged efficiently, otherwise it can interact with O_2^- to form highly reactive hydroxyl radicals (·OH) that are thought to be primarily responsible for oxygen toxicity in the cell. There is a plenty of evidence that NO not only increases the SOD activity but also significantly increase the H_2O_2 degrading enzymes to maintain its level to perform intracellular signaling roles.

Ascorbate Peroxidase (APX)

Scavenging of H_2O_2 by APX is the first step of the AsA-GSH cycle (Asada, 1994). In the AsA-GSH cycle, APX catalyzes the reduction of H_2O_2 into H_2O with AsA serving as an electronic donor (Zhang *et al*., 2008a, b). On the other hand, it is known that APX is more efficient than CAT to detoxify H_2O_2, since it is widely distributed inside the cell and has high substrate affinities in the presence of AsA as reductant. In addition to H_2O_2 detoxification, cAPX isozymes have a dynamic function in redox signal modulation and gene expression under oxidative stress condition by modulating the concentration of H_2O_2 to adjust its activity for expression to a level sufficient for second messenger activity. NO could participate in a series of resistant physiological reaction by adjusting activities of APX and other relative enzymes containing hemachrome iron or by restraining activity of aconitase without hemachrome iron (Wang *et al*., 2004). The increase of APX activities reduced much production of ROS which makes it possible to increase osmotic adjustment ability and salt tolerance (Zhu, 2002). Exogenous NO reduced the membrane permeability and membrane lipid peroxidation and prevented the electrolyte leakage, which suggested exogenous NO possessed the functions of repairing and protecting the cell membrane to alleviate the injury in the cell membrane system. Farooq *et al*. (2009) reported that NO application improved the APX under drought stress conditions.

Chen *et al*. (2010) reported that NO treatment significantly elevated the depressed APX activity in barley seedlings after 10 and 15 days of $CdCl_2$ treatment. Thus, it might be deduced that NO indirectly scavenges ROS accumulation by elevating Cd-decreased APX activity which may account in part for its alleviating effect on Cd-induced oxidative damage in barley seedlings. In sunflower leaves treated with 0.5 mM Cd, APX activity increased 76% over the controls, but NO+Cd and NO treatments increased APX activity even more, 163 and 106% over the controls, respectively (Laspina *et al*., 2005). Additionally, pretreatment with SNP or SNAP resulted in remarkable increase in the activities of APX in the callus of Reed (Song *et al*., 2006). With cPTIO (NO scavenger) or in combination with SNP or SNAP treatments, the activities of APX kept at the level of heat treatment alone in callus whereas they declined markedly in callus compared with those under heat stress alone. Moreover, some antioxidant genes including APX were also found to be induced by NO in *Arabidopsis* suspension cells (Huang *et al*., 2002). Yang *et al*. (2006) reported that after heat shock, activity of APX decreased in water presoaked leaf discs and partially or fully recuperated due to SNP presoaking. Because the physiological role of APX is to break down H_2O_2 in the cell, decreases in activities of these two enzymes would result in H_2O_2 accumulation. A remarkable increase in the activity of APX was also observed with the treatment with NO in UV-B stressed bean plant (Shi *et al*., 2005). Mackerness *et al*. (2001) reported that indeed NO, upon UV-B treatment, can up- or downregulate different genes involved in the defense response or photosynthetic genes. Thus, it is highly possible that the protective effect of NO may be mediated by increased level of expression of genes encoding active oxygen scavenging enzymes under UV-B radiation. However, the effect of NO on peroxidase is somewhat controvertible; the lower concentration of NO increases peroxidase activity in *Brassica* whereas higher concentration proved inhibitory (Zanardo *et al*., 2005). Similarly, APX activity was inhibited by higher NO concentration in tobacco and canola (Ferrer and Barcelo, 1999). Generation of NO in *Arabidopsis* plants induces a decrease in the thylakoidal APX transcript accumulation; consistently, *Arabidopsis* plants over- or underexpressing on thylakoidal APX gene show increased or decreased sensitivity, respectively, to both NO-induced cell death and paraquat-induced oxidative stress (Murgia *et al*., 2004; Tarantino *et al*., 2005).

Monodehydroascorbate Reductase (MDHAR)

AsA is present in most cellular compartment and several pathways exist to ensure AsA recycling. With its ability to directly regenerate AsA from MDHA, MDHAR plays an important role in maintaining reduced pool of AsA and ascorbate redox state (Hossain *et al*., 2010). It has been suggested that regeneration of AsA is regulated in this cycle mainly by NADPH-dependent MDHAR activity (Mittova *et al*., 2000; Shalata *et al*., 2001). Recent studies showed that both MDHAR and DHAR are equally important in regulating AsA level and its redox state under oxidative stress condition (Eltayeb *et al*., 2006, 2007; Wang *et al*., 2010). There are very few reports about NO action on MDHAR activity in plants subjected to abiotic stresses. In H_2O_2-treated floral petals of *Phalaenopsis*, Tewari *et al*. (2009) reported that exogenous application of NO donors significantly enhanced the activity MDHAR.

Dehydroascorbate Reductase (DHAR)

Elevated AsA levels through increased DHAR activity as well as overexpression of DHAR in different sub-cellular compartments contribute significantly in enhancing plants tolerance to oxidative stress. In the absence of sufficient DHAR activity, DHA undergoes irreversible hydrolysis to 2, 3-dikitoglunic acid. DHAR allows the plant to recycle DHA, thereby capturing AsA before it is lost. Thus DHAR is a physiologically important reducing enzyme in the AsA-GSH cycle in higher plants (Hossain and Fujita, 2010). Ding *et al*. (2008) found a strong synergistic effect of NO under Fe deficiency in Chinese cabbage and concluded that addition of NO dramatically induced DHAR activity. Similarly, significant increase in DHAR activity was also observed in cucumber roots subjected to salt stress (Shi *et al*., 2007). However, Sheokand *et al*. (2008) reported that DHAR activity remained unchanged when treated with NO under salt stress conditions.

Glutathione Reductase (GR)

Biochemical and molecular studies have shown that GR plays an essential role in cell defense against reactive oxygen metabolites by sustaining the reduced state of GSH and AsA pools which in tern maintain cellular redox state under stress. It has been observed that stress-tolerant plants tend to have high activities of GR (Mittova *et al*., 2003; Sekmen *et al*., 2007). Additionally, overexpression of GR increases antioxidant activity and improves tolerance to oxidative stress (Potters *et al*., 2004). In contrast, decreased GR activity results in increased stress sensitivity (Noctor and Foyer, 1998). Increases in GR activity in NO treated seedlings were also reported in plants under various abiotic stress conditions (Sang *et al*., 2008; Xu *et al*., 2010). Application of NO significantly increased GR activity in salt-treated cucumber roots (Shi *et al*., 2007). Xu *et al*. (2010) reported that addition of exogenous NO significantly enhanced the GR activity under high-light stress and whereas a reversed pattern was found when the NO scavenger, PTIO was applied in tall fescue leaves. They also postulated the role of NO as a signaling molecule involved in inducing increases in the activities of antioxidant enzymes. However, Singh *et al*. (2008) showed lesser induction of GR activity by NO under short-term Cd-stress. In contrast, Sheokand *et al*. (2008) and Laspina *et al*. (2005) reported that GR activity was unchanged by exogenous NO pretreatment under salt and Cd-stress conditions.

Glutathione S-Transferase (GST)

GSTs constitute a family of multifunctional enzymes present in both plants and animals. These dimeric enzymes catalyze the conjugation of GSH to a variety of electrophilic, hydrophobic and often toxic substrates, thereby reducing their toxicity. GST have been

shown to confer tolerance of various plant species against abiotic oxidative stress (Hossain and Fujita, 2002; Fujita and Hossain, 2003a, b; Hossain *et al*., 2006a, b, 2009, 2010; Hossain and Fujita, 2009a-c, 2010). GPX is considered an important ROS scavenger because of its broader substrate specifications and stronger affinity for H_2O_2 than those of CAT (Brigelius-Flohe and Flohe, 2003). GSTs are considered detoxification enzymes since they metabolize a wide variety of exogenous toxic compounds. Thus, GST conjugates such compounds to the tripeptide glutathione (GSH, γ-glutamyl-cysteinyl-glycine), producing water-soluble conjugates of reduced toxicity (Marrs, 1996). However, our recent study showed that plants have different physiological GST inhibitor that decrease its activities (Hossain *et al*., 2006c, 2007, 2008; Rohman *et al*., 2009a, b). In a study Ferreira *et al*. (2010) soybean plants treated only with lactofen (an herbicide belonging to the diphenylether group) had higher GST activity, relative to NO-treated ones, except in the first evaluation, at 24 h after lactofen application (Ferreira *et al*., 2010). Plants pretreated with 50 μM SNP had a linear enzymatic activity increase over time, whereas those pretreated with the highest level (200 μM) presented a decrease in such activity after 24 h after lactofen application. Thus, the lower GST activity detected in SNP-pretreated plants, mainly those treated with the highest level (200 μM), was probably due to the NO antioxidant action in the scavenging of H_2O_2 originated as a consequence of lactofen action.

Glutathione Peroxidase (GPX)

In addition to CAT and APX, GPX is also reported to be the major H_2O_2-utilizing enzymes in plants (Asada, 1994). Laspina *et al*. (2005) observed that GPX activity, either in Cd or NO+Cd-treated plants, increased 31% over the controls. Similar increase of GPX activity by NO was observed in our laboratory in wheat seedlings subjected to salt stress (Hossain and Fujita, 2010). Shi *et al*. (2007) reported that application of NO did not increase GPX activity under salt stress on the 4th d of treatment but significantly enhanced GPX activity on the 8th d of treatment. Under normal conditions, application of NO also significantly increased GPX activity on both the 4th and 8th d of treatment (Shi *et al*., 2007). In contrast, Singh *et al*. (2008) found a decrease in GPX activity when Cd-stressed wheat roots were treated with NO.

Catalase (CAT)

Catalase is a key antioxidant enzyme present exclusively in perxoisomes which decomposes H_2O_2. The regulatory role of NO on CAT activity under abiotic stress condition has studied by several researchers. NO could significantly enhance antioxidative capacity by increasing the activities of CAT during wheat seed germination under osmotic stress was reported by Zhang *et al*. (2003a) and Farooq *et al*. (2009). Tu *et al*. (2003) reported that wheat leaves treated with NO reduced H_2O_2 by activating CAT in ageing wheat leaves. Ding *et al*. (2008) observed that addition of NO dramatically induced CAT activity under Fe deficiency whose activities were even beyond control. Huaifu *et al*. (2007) observed that NO treatment significantly increased the CAT activity when it was subjected to salt stress as compared to the seedlings exposed to salt alone. Exogenous NO treatment also significantly increased CAT activity in the mitochondria during germination under salt stress which might have contributed to the alleviated oxidative stress in the mitochondria of germinating wheat seeds and thereby improved germinating rate under salt stress (Zheng *et al*., 2009). However, Laspina *et al*. (2005) reported that CAT activity was strongly inhibited in sunflower plants treated with 0.5 mM Cd, showing a decay of 44% as compared to the controls. However, pretreatment with NO restored completely CAT activity, increasing its value 21% over the controls. NO-treated plants also increased the enzyme activity 22% over the controls. According to Yang *et al*. (2006), after heat shock, activity of CAT decreased in water

presoaked leaf discs and partially or fully recuperated due to presoaking with NO. Because the physiological role of CAT is to break down H_2O_2 in the cell, decreases in activities of these two enzymes would result in H_2O_2 accumulation. In rice seedlings, the supplementation of NO to Cd-treatment solution resulted in a significant decrease in induction levels of these scavenging enzymes compared to Cd treatment alone (Singh *et al*., 2008).

A large number of researches indicating that NO significantly increased the enzymatic and non-enzymatic components of antioxidant defense. However, Singh *et al*. (2009) reported that apart from upregulation of antioxidant enzymes, upon SNP addition the induction level of these scavenging enzymes were significantly lesser than in the treatments without SNP indicating its direct involvement as an antioxidant and quenching the ROS. However, the observed trend of changes in antioxidant enzymes upon NO supplement paralleled the changes in ROS species (O_2^-, H_2O_2 and MDA content).

INTERACTION OF NO WITH OTHER SIGNALING MOLECULES

Although, it is well established that H_2O_2 induces NO synthesis, there has been some inconsistencies in the reports regarding NO regulation of H_2O_2 synthesis (Lum *et al*., 2002; She *et al*., 2004; He *et al*., 2005; Bright *et al*., 2006). NO affected H_2O_2 concentration due to the inhibition of CAT and APX (Clark *et al.,* 2000), whereas exogenous H_2O_2 activated NO synthesis in tobacco (De Pinto *et al.,* 2006), suggesting a bidirectional interaction between the two compounds. An understanding of the signaling events that lie upstream and downstream of H_2O_2 or NO may be a clue in determining the relationships between these two molecules in the regulation of stomatal movements. Removal of H_2O_2 using antioxidants or inhibition of its synthesis by inhibiting NAD(P)H oxidase activity prevented both NO production and stomatal closure. Similarly, removal of NO by PTIO compromised the induction of stomatal closure by H_2O_2 or ABA. It is generally accepted that ABA-induced stomatal closure typically requires elevations in cytosolic calcium (Allen *et al*., 2000). Synthesis and action of calcium-mobilizing molecules such as cADPR regulate these elevations in calcium (Leckie *et al*., 1998). There are some evidences that both H_2O_2 and NO actions in the regulation of stomatal closure require calcium. Both NO and H_2O_2 were reported to activate calcium channels and inactivate K^+ channels in *Arabidopsis* or *Vicia faba* guard cells (Garcia-Mata *et al*., 2003; Pei *et al*., 2000; Zhang *et al*., 2001; Kohler *et al*., 2003). NO also influenced GSH synthesis, as demonstrated in *Medicago truncatula* roots in which the levels of GSH and γ-EC and Glutathione Synthetase (GS) gene expressions were increased by NO (Innocenti *et al.,* 2007; Xu *et al.,* 2010). During the interaction of GSH with NO, *S*-nitrosoglutathione (GSNO) is formed in a reaction that may interconnect the ROS- and NOS-based signaling pathways (Neill *et al.,* 2002).

CONCLUSIONS

The roles of NO in plant responses to abiotic stresses are studied through investigating the effects on plant physiological and biochemical changes under stress. NO has been found to play a crucial role in plant growth and development, starting from cell cycle regulation, differentiation and morphogenesis, including flowering and root formation. However, the most important and best documented function of NO is the up-regulation of antioxidant defense or directly functions as an antioxidant. Although several NO synthesis pathways in plants have been suggested, biochemical and molecular details of each pathway remain obscure and it is unclear how these identified pathways cooperate with each other in plants

and which pathway operates in each particular tissue or organ or at a specific time. Regarding NO biosynthesis, future studies should focus on how NO is produced in a particular tissue or organ (and in which pathway), at what time scale NO production is elicited by a developmental or environmental stimulus and how the above described pathways work in concert when/if they all work in the same tissue or organ at the same time scale.

In the last few years NO and H_2O_2 have emerged to be central players in the world of plant cell signaling, particularly under various stressful situations. The full range of biological functions for these two signaling molecules remain to be catalogued and determining the ways in which they interact, both together and with the ever-increasing array of signals known to be recognized by plants, will need to be elucidated (Neill *et al*., 2002). Other research priorities must include full characterization of the enzymes through which the intracellular concentrations of H_2O_2 and NO are regulated and where these enzymes are located in different cells and tissues. The intracellular signaling cascades that transduce H_2O_2 and NO perceptions into cellular responses have so far been characterized only superficially. Finally, there arises the question how H_2O_2 and NO are detected by cells. Such perception could conceivably involve direct interaction of H_2O_2 and NO with various cellular proteins, such as transcription factors, ion channels or enzymes. H_2O_2- and NO-sensitive enzymes could include signaling enzymes such as protein kinases and phosphatases (Neill *et al*., 2002). NOS deficient mutant and/or gene knock-out mutant are now available. Genomics tools are accelerating the discovery of NO producing genes on a global scale and are expanding our understanding of the oxidative stress response and the pleiotropic roles of NO in signaling, gene expression and plant stress tolerance.

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