

## NITRIC OXIDE IN PLANTS: PROPERTIES, BIOSYNTHESIS AND PHYSIOLOGICAL FUNCTIONS\*

L. POPOVA\*\* AND T. TUAN

Institute of Plant Physiology and Genetics, Bulgarian Academy of Sciences,  
Acad. G. Bonchev str., Sofia, Bulgaria  
Email: lpopova@bio21.bas.bg

**Abstract** – Nitric oxide (NO) is an important signalling molecule with diverse physiological functions in plants. In the last years, many advances have been obtained regarding NO synthesis and its physiological effects in plants. It was found to play a crucial role in plant growth and development, starting from germination to flowering, ripening of fruit and senescence of organs, respiratory metabolism, as well as plant response to abiotic and biotic stressors. However, the molecular mechanisms underlying its effects remain poorly understood. This review discusses some aspects related to NO in plants, such as chemical properties, synthesis pathways, antioxidant action, physiological and biochemical changes that occur in plants under normal conditions and the possible functions of NO under changing environmental conditions. The interaction of NO with plant hormones, and cross-talk with salicylic acid are also discussed. The elucidation of such aspects represents a challenge to future studies.

**Keywords** – Nitric oxide, structure, synthesis, physiological functions, salicylic acid

### 1. BRIEF HISTORICAL NOTES AND DISCOVERY OF NITRIC OXIDE

Nitric oxide (NO) was first discovered as a colorless and toxic gas in 1772 by Joseph Priestly, who called it "nitrous air". This classification of NO as a toxic gas and air pollutant continued until 1987, when it was shown that NO is a natural component in the body. By 1987, nitric oxide's role in regulating blood pressure and relieving heart conditions was well-established [1]. During the 1990's, many people and scientists observed that purchase and distribution of noni (*Morinda citrifolia*) grow exponentially around the world started. This plant species grows naturally in Polynesia, Micronesia and the Hawaiian islands. The Polynesian people have been using noni for thousands of years as a cure-all plant. Later, from 1999 to 2000, Dr. Thomas Burke and other researchers found that noni fruit juice created nitric oxide in the body (cited from SCUIDOO). In 1992, nitric oxide was voted "Molecule of the Year" by Science magazine [2]. The importance of nitric oxide became front page news in 1998 when Louis J. Ignarro, Robert F. Furchgott and Ferid Murad were awarded the Nobel Prize for Medicine and Physiology. These scientists identified nitric oxide as a signaling molecule, opening up a new way of treatment for millions of patients.

### 2. CHEMICAL PROPERTIES OF NITRIC OXIDE

Nitric oxide (common name) or nitrogen monoxide (systematic name) is a chemical compound with the chemical formula NO. It is one of the smallest diatomic molecules with a high diffusivity ( $4.8 \times 10^5 \text{ cm}^2 \text{ s}^{-1}$  in  $\text{H}_2\text{O}$ ), exhibiting hydrophobic properties. NO may not only easily migrate in the hydrophilic regions of the cell, such as the cytoplasm, but also freely diffuse through the lipid phase of membranes [3]. The half-

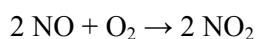
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\*\*Corresponding author

life of NO in biological tissues is estimated to be <6 s [4]. This short half-life reflects the highly reactive nature of NO, which reacts directly with metal complexes and other radicals and indirectly as a reactive nitrogen oxide species with DNA, proteins, and lipids [5]. It has been identified by mammalian biologists as a modulator of neurotransmission and as an effectors in immune responses [1]. By the late 1990's, accumulating evidence showed that NO is an important signal molecule involved in plant response to different environmental stresses [6-7]. These findings started an era of new and challenging studies.

Nitric oxide should not be confused with nitrous oxide (N<sub>2</sub>O), an anesthetic and greenhouse gas, or with nitrogen dioxide (NO<sub>2</sub>), a brown toxic gas and a major air pollutant. However, nitric oxide NO, being a very reactive species, in the presence of atmospheric oxygen forms other oxides, including NO<sub>2</sub>•, N<sub>2</sub>O<sub>3</sub> and N<sub>2</sub>O<sub>4</sub>, which may further react with cellular amines and thiols, or hydrolyse to NO<sub>2</sub><sup>-</sup> and NO<sub>3</sub><sup>-</sup> [8].

When exposed to oxygen, NO is converted into nitrogen dioxide.



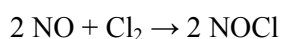
NO reacts with oxygen and water to form HNO<sub>2</sub> or nitrous acid.



NO readily reacts with the superoxide anion-radical (O<sub>2</sub>•<sup>-</sup>), as a result of which a peroxynitrite ion (ONOO<sup>-</sup>) is formed. In the physiological pH range ONOO<sup>-</sup> is unstable. Peroxynitrite anions may protonate, as a result of which peroxo-dioxonitric acid is formed, a source of nitrogen dioxide (NO<sub>2</sub>•) and a hydroxyl radical (HO•) [8].

In the physiological pH range complexes of NO, Fe<sup>2+</sup> and low-molecular thiols, referred to as dinitroso-iron complexes (DNICs), may also be formed [9]. DNICs are relatively stable molecules, in contrast to the high reactivity of NO and free iron, while the complex between NO and glutathione (GS-Fe-NO) is more stable than the complex with cysteine (Cys-Fe-NO). These compounds may function as cellular reservoirs of iron, thiols and NO, being degraded depending on the requirement of the cell for a given component [8-9].

NO forms complexes with all transition metals to give complexes called metal nitrosyls, such as nitrosyl chloride, nitrosyl iodite, or could be reduced to nitroxil (HNO) [10]. Nitrosyl iodide can form, but is an extremely short-lived species and tends to reform I<sub>2</sub>.



Moreover, the cation form as the electrophilic reagent may attack sulphur, iron, nitrogen and carbon centres of numerous organic compounds. In the biological tissue reversible nitrosylation of sulfhydryl groups of centres in proteins is crucial. Such modifications as the S-nitrosylation/denitrosylation of proteins affect the biological activity of these compounds, thus constituting an important element of signal transduction. NO induces different MAP kinase cascades, as well as regulating signalling proteins, e.g. protein kinase C, phosphatases, potassium channels and N-methyl-d-aspartate receptors [8].

Nitric oxide reacts with acetone and an alkoxide to a diazeniumdiolate or nitrosohydroxylamine and methyl acetate.

Nitric oxide in the air may convert to nitric acid, which has been implicated in acid rain. Furthermore, both NO and NO<sub>2</sub> participate in ozone layer depletion.

### 3. BIOSYNTHESIS OF NITRIC OXIDE

There are many possible sources of NO. The physiological role of each source depends on the species, type of tissue or cells, external conditions and potential activation of the signal pathway in the plant [11].

In biological systems, NO can be formed both enzymatically and nonenzymatically.

The enzyme responsible for NO generation in animal organisms is nitric oxide synthase (NOS) analyzing five-electron oxidation of one of the atoms in L-arginine ( $N^{3-}$  to  $N^{2+}$ ) with the participation of  $O_2$  and NADPH.

In plants, nitric oxide can be produced by any of four routes: (i) nitric oxide synthase, (ii) by plasma membrane-bound nitrate reductase, (iii) by mitochondrial electron transport chain, or (iv) by non-enzymatic reactions

Recently, in pea seedlings, using the chemiluminescence assay, Corpas et al. (2006) showed arginine-dependent NOS activity, which was constitutive, sensitive to an irreversible inhibitor of animal NOS, and dependent on the plant organ and its developmental stage [12].

Although NOS-like activity has been detected widely in plants, animal-type NOS is still elusive. There is no clear homologue of animal NOS in the genome of *A. thaliana*. Also, several studies provide evidence arguing for the existence of a NOS-like enzyme in plants. Other authors demonstrated the presence of a NOS-like proteins in plant tissues using immunoassays and immunocytochemical analyses with antibodies against animal NOS isoforms [13-15]. However, the results of immunoassays remain dubious, as antibodies against mammalian NOS may recognize many plant proteins not connected with NOS. Proteomic identification of candidate proteins showed that they were NOS-unrelated proteins [16]. The major origin of NO production in plants is probably through the action of NAD(P)H-dependent nitrate or nitrite (NiR) reductases [17]. Nitrate reductase (NR) provided the first known mechanism to make NO in plants. This enzyme normally reduces nitrate to nitrite, but it can also further reduce nitrite to NO [18]. NR is the only enzyme whose NO-producing activity has been rigorously confirmed both *in vivo* and *in vitro* [19-20]. Transformation of  $NO_2^-$  to NO occurs most probably on a molybdenum cofactor, similarly as in another NO-generating enzyme with a MoCo centre, xanthine oxidoreductase [21].

Production of NO, dependent on NR activity, was recorded in many plant species, e.g. in cucumber [22], sunflower, spinach, maize [23], *Arabidopsis* [24], wheat, orchid, aloe [25], tobacco [26], as well as *Chlamydomonas reinhardtii* [27]. NO generation via NR was demonstrated *in vitro* [27] and *in vivo* [23]. This synthesis was strictly dependent on nitrite and nitrate content in the tissue [17, 28].

Chandok et al. [29] identified, in tobacco, another candidate for NO enzymatic production in plants, the inducible NO synthase (iNOS), which is induced by pathogens and was identified as a variant of the P protein of the mitochondrial glycine decarboxylase complex. In addition, Godber et al. [30] suggested that xanthine oxidase, a ubiquitous molybdo-enzyme, could catalyse the reduction of nitrite to NO under hypoxia and in the presence of NADH. Stöhr et al. [31] reported a tobacco root-specific plasma membrane-bound nitrite: NO reductase (NI-NOR), which catalyzes the reduction of apoplastic nitrite into NO. Later, Stöhr and Stremlau [32] reported that NI-NOR may be involved in several physiological root processes, including development, response to anoxia, and symbiosis. Also, Besson-Bard et al. [33] stated that NI-NOR activity might be coordinated with those of a plasma membrane-bound NR (PM-NR) reducing apoplastic nitrate to nitrite. However, the identity of NI-NOR is currently unknown.

NOS functional activities have been detected in plant tissue extracts and purified organelles, including peroxisomes and mitochondria [33]. However, there is no direct experimental evidence that the radioactive products detected when assessing plant NOS activity *in vitro* is indeed L-citrulline [18]. Also, no protein or gene was identified that had any sequence similarity to the complete animal NOS proteins [18], and for review [34].

Xanthine oxidoreductase (XOR) is another MoCo-containing enzyme that has been found to produce NO in plants as well as in animals. Xanthine oxidoreductase occurs in two interconvertible forms: the superoxide producing xanthine oxidase and xanthine dehydrogenase [22]. XOR has been found in pea leaf peroxisomes

Another enzyme that can generate NO from nitrite is a plasma membrane-bound enzyme of tobacco roots (Ni-NOR) [35]. This enzyme has a higher molecular weight than nitrate reductase, but has to be characterized. Other good candidates for enzymatic generation of NO include horseradish peroxidases [17], cytochrome P450 [28], catalase, and hemoglobin.

Heme proteins that have been proposed as good candidates for the enzymatic generation of NO are cytochromes P450. These proteins are present in plants as well as in animal systems

NO may also be formed non-enzymatically in a reaction between nitrogen oxides and plant metabolites, or as a result of a chemical reduction of  $\text{NO}_2^-$  at acid pH. As has been documented, a sufficiently acid medium, required for  $\text{NO}_2^-$  reduction, is found in the apoplast of barley aleurone cells [4].

In bacteria, the primary role of NOS may not be producing NO, but rather synthesizing specific molecules. *Streptomyces turgidiscabies* NOS is needed to synthesize the phytotoxin-thaxtomine A, a nitrated dipeptide being required for plant pathogenicity [36].

It is well known that under physiological conditions plants are exposed to NO, produced with the participation of soil micro-organisms. Release of NO to the atmosphere occurs in the reaction of nitrification and denitrification, which may constitute an alternative source of NO for plants. Nitrification of  $\text{NH}_4^+$  is the primary source of  $\text{N}_2$  emitted to the atmosphere, where it oxidizes to NO and  $\text{NO}_2$  [10]. Nitrification/denitrification cycles provide NO as a by-product of  $\text{N}_2\text{O}$  oxidation into the atmosphere [37]. Plants not only react to the atmospheric or soil NO, but are also able to emit substantial amounts of NO. Thus, NO could be generated by non-enzymatic mechanisms, e.g. via chemical reduction of  $\text{NO}_2^-$  at acidic pH or by carotenoids in the presence of light [38] and at acidic pH in the presence of a reductant such as ascorbic acid [18].

The involvement of polyamines (Pas) in the NO synthesis is another important aspect to be considered [33, 39]. Tun et al. [40] added the polyamines spermidine and spermine to *Arabidopsis* seedlings and observed rapid production of NO in the elongation zone of the root tip and in primary leaves, especially in the veins and trichomes. Yamasaki and Cohen [41] stated that the PA-dependent NO production might be carried out by unknown enzymes or by PA oxidases.

#### 4. BIOLOGICAL FUNCTIONS

NO is one of the few gaseous signaling molecules known. It is a key vertebrate biological messenger, playing a role in a variety of biological processes. Nitric oxide is known as the 'endothelium-derived relaxing factor', or 'EDRF'. The endothelium (inner lining) of blood vessels uses nitric oxide to signal the surrounding smooth muscle to relax, thus resulting in vasodilation and increasing blood flow. The NO interaction with haemoglobin, also of plant origin, seems to be especially important. It was shown that under aerobic conditions plant haemoglobins may react with NO forming  $\text{NO}_3$ , which in this way modifies the NO level in the plant [2].

The high reactivity and free diffuses across membranes make NO ideal for a transient signal molecule between adjacent cells and within cells [42]. The production of nitric oxide is elevated in populations living at high-altitudes, which helps these people avoid hypoxia. Effects include blood vessel dilatation, neurotransmission, modulation of the hair cycle, and penile erections. Nitroglycerin and amyl nitrite serve as vasodilators because they are converted to nitric oxide in the body. Sildenafil, popularly known by the trade name Viagra, stimulates erections primarily by enhancing signaling through the nitric oxide pathway in the penis. Nitric oxide (NO) contributes to vessel homeostasis by inhibiting vascular smooth muscle contraction and growth, platelet aggregation, and leukocyte adhesion to the endothelium. Nitric oxide can contribute to reperfusion injury when an excessive amount produced during reperfusion (following a period of ischemia) reacts with superoxide to produce the damaging free radical peroxynitrite. In contrast,

inhaled nitric oxide has been shown to help survival and recovery from paraquat poisoning, which produces lung tissue damaging superoxide and hinders NOS metabolism [43-44].

Nitric oxide is also generated by macrophages and neutrophils as part of the human immune response. Nitric oxide is toxic to bacteria and other human pathogens. In response, however, many bacterial pathogens have evolved mechanisms for nitric oxide resistance.

## 5. NITRIC OXIDE FUNCTION IN PLANTS

In recent years, there has been much research into the diverse biological activities of NO in plants. Various studies have reported its presence in the plant kingdom and involvement in different cell processes such as growth and development, respiratory metabolism, senescence and maturation, as well as plant response to abiotic and biotic stressors. The early studies showing NO as a signaling molecule in plants appeared as late as 1998 [45-46] and as a consequence resulted in the intensification of research on the role of NO in plants. NO was classified as a phytohormone that might function as a gaseous endogenous plant growth regulator [47] as well as a nontraditional plant growth regulator [48].

## 6. EFFECT OF NO ON PLANT GROWTH AND DEVELOPMENT

Plant metabolism is highly influenced by NO, although some complications arise by the mode of its application. NO is a gas, but in most experiments it is applied in the form of donor compounds that release NO into solution, such as SNP, S-Nitrosoglutathione, and S-nitroso-N-acetylpenicillamin. These varieties of NO donors are often a source of discrepancies in the results. For this reason, the concentration of NO inside the plant tissue depends on some chemical factors (temperature, kinetics of release), the level of concentration, variety of plant systems, and altogether all these factors led to great variety of plant responses. It has been found that the treatment of spinach plants with a low concentration of NO gas (ambient atmosphere with  $200\text{nLL}^{-1}$  NO gas) significantly increased the shoot biomass of the soil-cultivated plants as compared with the control treatment (ambient atmosphere) [49]. In a rapidly growing pea foliage application, NO had a dual behavior. Low micromolar concentrations produced an increase in the rate of leaf expansion, whereas no promotive effect occurred at higher concentrations [50]. A similar dual behavior of NO donor SNP was also noted in wheat [51].

At extremely low concentrations (0.1 nM- 0.1  $\mu\text{M}$ ) a NO-releasing compound (SNP) induced maize root growth [52]. In cucumber, NO mediates their response, leading to adventitious root formation [53]. Treatment with 100  $\mu\text{M}$  NO (SNP) inhibits hypocotyl growth in potato, lettuce and *Arabidopsis* [48]. Correa- Aragunde et al. [54] reported that treatment with SNP led to the inhibition of primary root length and to a higher number of lateral roots of tomato seedlings. Exogenously applied NO (SNP) alleviates browning of tuber explants by reducing  $\text{H}_2\text{O}_2$  accumulation, thereby promoting a higher in vitro proliferation frequency of *Discorea opposita* [55].

## 7. EFFECT OF NITRIC OXIDE ON PHOTOSYNTHESIS AND CHLOROPHYLL CONTENT

Jasid et al. [56] suggested that at least two pathways for NO production are operative in chloroplasts of soybean, one dependent on NOS-like enzyme activity and another on nitrite. Under high NO concentration (i.e. high nitrite content in chloroplasts), the generation of reactive nitrogen species (i.e.  $\text{ONOO}^-$ ) may lead to impairment of the photosynthetic machinery. Takahashi and Yamasaki [57] have shown that NO is capable of inhibiting chloroplast electron transport in a reversible manner. Previously, studies of air pollution had suggested that NO may reduce  $\text{CO}_2$  assimilation [58]. Both photosynthesis and photorespiration have been found to be affected by NO in different plants [35]. Treatment with SNP

improved the rate of photosynthesis, chlorophyll content, transpiration rate and stomatal conductance in cucumber seedlings [59]. NO donor SNP has been found to decrease the level of enzymes that regulate photosynthesis in wheat. Lum et al. [60] reported that SNP decreased the amount of Rubisco activase and the  $\beta$ -subunits of the Rubisco subunit-binding protein in mung bean (*Phaseolus aureus*). NO is also able to influence the photosynthetic electron transport chain directly. Takahashi and Yamasaki [57] showed that NO (SNP) does not modify the maximal quantum efficiency, but inhibits the linear transport electron rate,  $\Delta pH$  formation across the thylakoid membrane, and decreases the rate of ATP synthesis. A moderate decrease in Fv/Fm was observed by SNP treatment in pea leaves [61-62].

One possible reason for the observed changes in the rates of photosynthesis and transpiration could be due to the effect of NO on stomatal behavior. The exogenous application of NO to both monocotyledonous and dicotyledonous through epidermal strips induced stomatal closure [63]. The observed effects of NO on stomatal movement is very often connected with ABA- induced stomatal closure [11] or with the involvement of nitrate reductase leading to NO production in guard cells [63].

NO have been found to enhance chlorophyll content in potato, lettuce, and *Arabidopsis* [48]. Treatment with SNP delayed yellowing and retarded the chlorophyll degradation in broccoli [64].

## 8. NITRIC OXIDE AND ENVIRONMENTAL STRESSES

In recent years, there has been much research into the diverse biological activities of nitric oxide (NO) in alleviating the harmful effects of environmental stresses in plants.

Some authors have considered NO as a stress-inducing agent [50], others have reported its protective role [65], depending on its concentration, the plant tissue or age, and the type of stress. An increase in NO production has been detected during both water and heat stress [50].

Literature data supply evidence showing that plant response to such stressors as drought [11], and cadmium [65], is regulated by NO.

It has also been found that exogenous NO protects potato plants against herbicide paraquat [66], and that treatment with NO increases salt adaptation of reed [67]. Nitric oxide is able to reduce the oxidative injury produced by drought on wheat seedlings and UV radiation on potato leaves.

All these effects seem to come from an NO-mediated reduction of the free amount of ROS. This suggestion was confirmed by Sun et al. (2007) [68] showing that NO can protect maize (*Zea mays* L.) plants from iron deficiency-induced oxidative stress by reacting with ROS directly or by changing activities of ROS-scavenging enzymes.

NO acts as a stress-coping factor for biotic stress in plants, like those infected with pathogen *Phytophthora infestans* potato leaves [45]. In a tobacco-*Pseudomonas* photosystem, NO is able to invoke a hypersensitive response [69]. As a result, the growth and development of the fungus was restricted and spreading to other parts of the plants was prevented.

It is now accepted that NO acts as a second messenger in plants. One of the most intriguing behaviors in NO biology is its dual function as a potent oxidant and effective antioxidant. This dual role of NO might depend on its concentration as well as on the status of the environment. 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 [70].

## 9. NITRIC OXIDE AND CROSS-TALK WITH SALICYLIC ACID

One of the fastest reactions of NO within biological systems is the combination with ROS. This function is very often described as either toxic or protective. Under severe stress conditions the production of ROS is predominate and under such conditions NO may act as a chain breaker and thus limit damage. NO itself is

a very reactive nitrogen species and therefore can start chain reactions that cause cell injury. This dual role of NO can be accomplished by a signal transduction pathway through a signaling cascade. Involvement of other signaling molecules, such as salicylic acid, jasmonic acid, abscisic acid, ethylene,  $\text{Ca}^{2+}$  activate a very complex network [71].

Several lines of evidence point to an inter-relationship between NO and salicylic acid (SA) in plant defence. It is well documented that both SA and NO play an important role in the activation of plant defense responses after pathogen attacks. However, the interrelationship between their respective signaling pathways is still unclear.

Treatment of tobacco and *A. thaliana* leaves with NO induces a substantial increase in endogenous SA [72]. Song and Goodman (2001) [73] suggest that NO is involved in both SA biosynthesis and action. It has also been shown that SA induced the production of ROS, such as  $\text{H}_2\text{O}_2$  and NO [74]. In addition, SA may mediate and/or potentiate NO effects by altering the activity of various NO-regulated enzymes.

The global picture of ROS-NO-SA interactions is far from being complete, but it has already been revealed as a fascinating cross talk of mechanisms able to fine tune resistance responses and other plant reactions to environmental stimuli, as well as important developmental aspects in the life of the plant.

### NOMENCLATURE

DNICs	dinitroso-iron complexes
NiR	nitrite reductase
NOS	nitric oxide synthase
NR	nitrate reductase
SNP	sodium nitropruside
XOR	xantine oxidoreductase

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