

## 11

### Nitric Oxide Signaling by Plant-Associated Bacteria

Michael F. Cohen, Lorenzo Lamattina, and Hideo Yamasaki

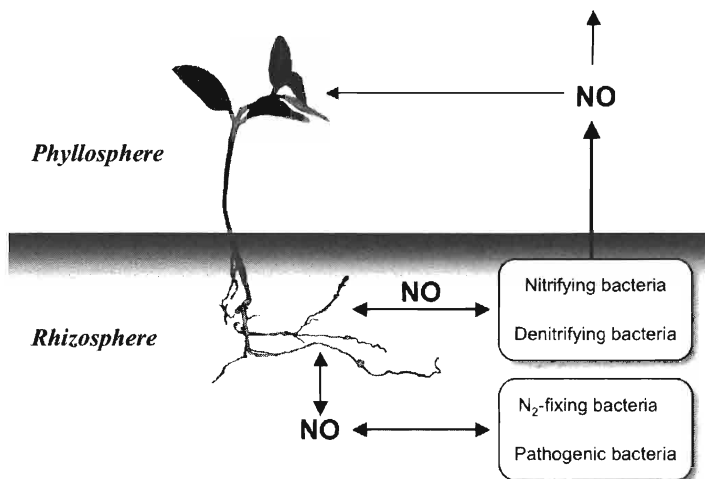
#### Summary

The primary source of NO in the biosphere is energy metabolism within nitrifying and denitrifying bacteria, which are common inhabitants of the rhizosphere. NO produced by these bacteria regulates their mobility and gene expression and can elicit responses in neighboring organisms, including plants. Many other bacteria employ bacterial nitric oxide synthase to generate NO. One clear function for bNOS is in oxidative defense signaling. An endophytic association of actinomycetes and other bNOS-containing bacteria, some of which may be vertically transmitted within seeds, has been demonstrated among diverse plant species. Actinomycetes possessing bNOS activity display increased capacity for rhizosphere colonization, and two species have been shown to upregulate bNOS activity in response to plant-derived disaccharides. As yet, evidence of an effect of bNOS-derived NO on the plant host has been indirect. In contrast, there is strong evidence that denitrification-derived NO from *Azospirillum brasilense* can stimulate root branching in tomato, and this path of NO production is also responsive to plant host compounds. Further investigations will likely reveal other influences of bacterial NO in known plant NO-responsive pathways.

#### 11.1

##### Introduction

The study of nitric oxide has entered the sphere of physiological ecology. Our understanding of NO roles in intercellular signaling within organisms has logically led to investigations of its capacity to mediate interactions between organisms. Surfaces and interiors of plants and other Eukarya are commonly colonized by bacteria, many of which can produce NO (Figure 11.1). In this chapter, we will focus primarily on findings that have come to light since a previous review of the field [1].

*Stratosphere*

**Figure 11.1** Major plant- and bacteria-related NO production sites and destinations. NO that escapes to the stratosphere is eventually oxidized and returns to earth as nitric acid (HNO<sub>3</sub>).

## 11.2

**Production of Nitric Oxide by Bacteria**

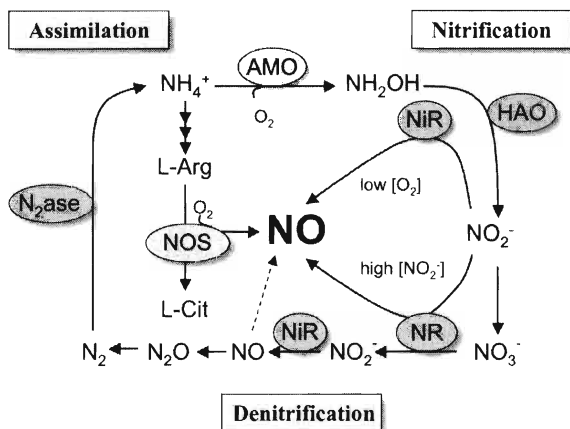
Most biogenic NO is released as a product of bacterial energy metabolism. Some bacteria also produce NO specifically for cell signaling and, in one group, for nitration of an important secondary metabolite. At a practical level, the near ubiquity of NO-producing bacteria on and within plants, including inside seeds, must be considered in studies of presumptive plant NO synthesis; care should be taken beyond simple surface disinfection of plant surfaces to establish bacteria-free conditions [1].

The section headings below are organized by mode of metabolism and not as bacterial classifications; many bacteria possess metabolic flexibility that resists facile categorization. For instance, *Nitrobacter vulgaris* can nitrify under aerobic conditions but can switch to denitrifying metabolism under anaerobic conditions [2, 3].

## 11.2.1

**Nitrification**

Nitrification is the extraction of energy by the sequential oxidation of nitrogen that occurs as ammonia (Figure 11.2). This process is by far the dominant source of NO emissions from soils [1, 4–8]. Complete oxidation to nitrate is carried out by the combined action of two metabolically distinct bacterial groups. The first group, termed the nitrosifiers or ammonia-oxidizing bacteria, oxidizes ammonia to nitrite, while the second group, the nitrifying bacteria, oxidizes the nitrite to nitrate.



**Figure 11.2** Pathways of bacterial NO production in relation to the nitrogen cycle. Selected enzymes shown in ovals are  $\text{N}_2\text{ase}$ , nitrogenase; AMO, ammonia monooxygenase; HAO, hydroxylamine oxidoreductase; NiR, nitrite reductase; NR, nitrate reductase; and NOS, nitric oxide synthase.

In the nitrosifiers, ammonia monooxygenase produces hydroxylamine, which is then oxidized to nitrite by hydroxylamine oxidoreductase. Maximal membrane potential is generated when the extracted electrons can flow to oxygen via a terminal cytochrome oxidase. However, under conditions where oxygen is limited, upregulation of nitrite oxidase activity consumes autogenic nitrite as a terminal electron acceptor, thereby releasing NO [9, 10].

### 11.2.2

#### Denitrification

In denitrification, nitrate is reduced in multiple steps to nitrogenous gases (Figure 11.2). This is the major mechanism whereby wetlands remove nitrogen from nitrate-rich treated municipal wastewaters [11, 12]. The complete denitrification process may take place in one species of microorganism or by successive action of multiple species. Many bacteria possess the first enzyme of the pathway, nitrate reductase (NR). In *Salmonella typhimurium*, when nitrate is not present, membrane-bound NR can reduce nitrite to NO [13], thus behaving similar to the NR of plants [14, 15]. Conversely, the NR of *Escherichia coli* does not directly catalyze NO formation; nitrate-cultivated *E. coli* cells produce NO from nitrite via a periplasmic nitrite reductase (NrfA) [16], which can also serve to detoxify NO to  $\text{NH}_4^+$  [17, 18]. Such studies of NO flux in enteropathogens are likely to have relevance to plant-NO research since it is increasingly apparent that these bacteria are able to grow and persist in association with plants [19, 20].

In bacteria that possess a more complete denitrification pathway, which may release either  $\text{N}_2\text{O}$  or  $\text{N}_2$  as the predominant product, NO is produced as a free intermediate by a periplasmic nitrite reductase [21, 22]. Concentrations of NO

sufficient to cause toxicity and stimulate NO-defensive gene expression in neighboring bacteria can be released by denitrifying bacteria *en masse* [23].

Taking an evolutionary perspective, all mitochondrion-bearing eukaryotes share a direct tie with the lineage of denitrifying bacteria. From sequence homology studies, it is apparent that the electron transport system of mitochondria descended from that of a denitrifying bacterium [24, 25]. The capacity to reduce nitrite to NO in the mitochondria of animals, fungi [26], algae [27, 28], and plants [29] underscores this evolutionary connection with bacterial denitrifiers.

### 11.2.3

#### Nitric Oxide Synthase

Certain bacteria employ bacterial nitric oxide synthase (bNOS) to produce NO as a regulatory signal or, in at least one lineage, as a biosynthetic unit of a secondary metabolite. The NOS enzymes found in bacteria, Archea, and Eukarya domains all produce NO from L-arginine, releasing L-citrulline as a by-product (Figure 11.2). Genetic confirmation of bNOS exists only for Gram-positive bacteria, including many common plant-associated species in the orders actinomycetales (*Mycobacterium*, *Nocardia*, *Rhodococcus*, and *Streptomyces*) and bacillales (*Bacillus*, *Geobacillus*, and *Paenibacillus*). The catalytic properties of bNOS are similar to the oxygenase domain of mammalian NOS but the bNOS lacks a reductase domain. In *Bacillus subtilis*, the reductase role may be taken by any of a variety of proteins; individual reductases capable of supporting sustained NO synthesis from bNOS *in vitro* [30] can be deleted without altering *in vivo* bNOS activity [31]. Caution should be taken in interpreting results of exposing live cells to traditional NOS inhibitors since some bacteria are capable of metabolizing these compounds, sometimes forming an NOS inducer in the process [32].

## 11.3

### Regulatory Roles for Nitric Oxide in Bacteria

NO can transiently bind numerous functional sites of proteins, including heme, iron–sulfur clusters, and thiols, thus enabling NO to impact cell activities from the transcriptional to posttranslational levels [33]. Its short half-life and ability to diffuse across membranes make NO ideal for use as a near real-time signal between cells. Moreover, one-electron reduction of NO can potentially be mediated by various components of respiratory electron transport systems under oxygen-deficient conditions [34, 35], giving rise to HNO, which has bioactivity distinct from that of NO, and is the most reactive of the nitrogen oxides [36].

### 11.3.1

#### Metabolic Regulation

In denitrifying bacteria, NO promotes its own elimination by inducing transcriptional activators of enzymes that reduce NO to N<sub>2</sub>O and inhibiting repressors (e.g.,

NsrR) of these genes [33]. Conversely, NO stimulates its own synthesis by inducing transcriptional activators of nitrite reductases. Thus, NO behaves as a signal to ensure coordinated respiratory flow of electrons through the denitrification pathway [33].

The influence of NO on gene expression in nitrifying bacteria is less well characterized [37]. Exposure of *Nitrosomonas europaea* to exogenous NO was found to induce NO production in this ammonia-oxidizing bacterium [38]. Consistent with this observation, acidification of a nitrite-containing medium, which results in nonenzymatic formation of NO [39], causes NsrR-dependent derepression of nitrite reductase (*nirK*) expression [40].

### 11.3.2

#### Regulation of Biofilm Formation

Nitric oxide has been shown to regulate biofilm formation in nitrifying and denitrifying bacteria. Biofilms confer several advantages to their resident bacteria, including increased resistance to antibiotics and a capacity for cooperative community metabolism [41]. In all of several species of ammonia-oxidizing species studied, NO was found to induce biofilm formation but at different concentrations [38]. Conversely, for the denitrifier *Pseudomonas aeruginosa*, NO caused biofilm dispersal and stimulated swarming motility [42].

Plant roots are common sites for biofilm formation [19]. Future research must seek to determine whether NO levels impact the dynamics of biofilm formation in the plant rhizosphere, which is commonly colonized by nitrifying and denitrifying bacteria [43, 44].

### 11.3.3

#### Stimulation of Oxidative and Nitrosative Defenses

Bacteria that produce NO or live near NO-emitting bacteria need to protect themselves against excessive NO levels and its decomposition products that can exert prooxidant effects. Under anaerobic conditions, NO can induce the expression of Nrf or a flavorubredoxin (FIRd), which reduces NO to ammonia or N<sub>2</sub>O, respectively. When oxygen is present, expression of the genes that encode these detoxification enzymes is repressed, and instead cells will oxidize NO via a flavohemoglobin (Hmp) [33]. Expression of Hmp in the phytopathogen *Erwinia chrysanthemi* is necessary for virulence [45], as it protects the bacterium against nitrosative stress and limits the ability of the plant host to mount a hypersensitive response [46].

Microbes that inhabit the phytosphere are faced with a multitude of oxidative stresses. In the phyllosphere, they are exposed to reactive oxygen species (ROS) generated by exposure to UV light [47]. While in the rhizosphere, they must deal with high levels of ROS generated through the activity of plant oxidases [48] and protozoan feeding [49, 50]. One clear function for bNOS is in oxidative defense signaling. In the leaf endophyte *Rhodococcus* sp. strain APG1, bNOS activity is associated with elevated catalase activity and tolerance of the bacterium to H<sub>2</sub>O<sub>2</sub> [51]. In *B. subtilis* and *Bacillus anthracis*, bNOS-derived NO exerts a cytoprotective effect by activating catalase and by

inhibiting enzymatic reduction of free cysteine, thereby suppressing the production of hydroxyl radicals via the Fenton reaction [52, 53]. The fact that bNOS activity is necessary for engulfed *B. anthracis* cells to survive the oxidative burst in macrophages [52] may be of relevance to the observation that *B. anthracis* reproduces and exchanges DNA in the oxidative rhizosphere of plants [54]. *Streptomyces* spp. displaying NOS activity were found to be significantly more prevalent in the rhizosphere of apple root compared to surrounding soil [55]. For now, the idea that bNOS activity may confer protection from plant-derived oxidative stresses remains an enticing speculation that awaits direct experimental demonstration.

## 11.4

### Bacterial Nitric Oxide in Plant–Bacteria Interactions

As described above, by acting as a signal to activate oxidative and nitrosative defense mechanisms, NO may improve the chances of bacterial survival in the phytosphere. In this section, we will describe examples of induction of bacterial NO synthesis in response to plants and known effects of bacterial NO on plant hosts.

#### 11.4.1

##### Production of NO in Response to Plant Products

Plant-derived disaccharides have been shown to upregulate NOS activity in two actinomycetes, *Streptomyces turgidiscabies* and *Rhodococcus* APG1.

*S. turgidiscabies*, a pathogen of tubers, induces bNOS activity in response to the cell wall-building block cellobiose, which is released in infected tissues through the action of the phytotoxin thaxtomin. NO from bNOS nitrates the indole group of thaxtomin, a modification necessary for its toxicity [56, 57]. In the root infection process, NO is produced in excess of what is needed for nitration of thaxtomin [58], an observation that should lead to further investigation of potential effects of the diffusible NO on the physiology and development of the host plant. Many beneficial *Streptomyces* secrete cell wall-degrading enzymes in the process of colonizing a plant host, thereby releasing cellobiose into their immediate environment [59, 60]. It remains to be seen whether NO is produced in the course of these interactions as well.

*Rhodococcus* strain APG1, isolated as an endophyte of *Azolla pinnata*, induces bNOS activity in response to sucrose [51], which is of potential physiological significance since sucrose is exuded into the symbiotic leaf cavities of *Azolla* spp. [61].

#### 11.4.2

##### Plant Responses to Bacterial NO: The *Azospirillum*–Tomato Interaction

The free-living diazotrophic *Azospirillum* is one of the best characterized plant growth promoting rhizobacteria (PGPR) [62]. *Azospirillum* can be considered a promiscuous plant colonizer since it is found in a wide range of habitats associated with roots of

both monocots and dicots. *Azospirillum* fixes atmospheric  $N_2$  through the nitrogenase complex under combined conditions of low nitrogen concentration and low  $O_2$  tension [63, 64]. However, there is a lack of evidence supporting the role of nitrogen fixation by *Azospirillum* in facilitating plant growth processes; no significant increase in the nitrogen content of *Azospirillum*-inoculated compared to noninoculated crops has been observed [65].

*Azospirillum* is able to exert beneficial effects on plant growth parameters under certain environmental and soil conditions, for example, water status, nitrogen concentration and availability, temperature, and so on [66, 67]. An ability to modify root architecture has remarkably been a consistently observed aspect of *Azospirillum*-induced plant growth promotion. The most characteristic contributions of *Azospirillum* in promoting plant root growth are (i) an increased number of lateral and adventitious roots and (ii) positive effects on root hair formation and development. These changes in architecture and total surface area of roots enhance a plant's capacity to explore the soil. Significant differences in coleoptile growth pattern have also been observed between *Azospirillum*-inoculated and noninoculated plant species [68, 69].

The beneficial effects exerted by the association of *Azospirillum* with plant roots rely on molecular mechanisms that are still poorly understood. Many working hypotheses have been based on the ability of *Azospirillum* to synthesize phytohormones. The synthesis of auxins, cytokinins, and gibberellins has been the most commonly invoked reason to explain the growth promoting activity of inoculation with PGPR in general, not just *Azospirillum*. Indeed, auxins are thought to play a central role in the activity of beneficial rhizobacteria to induce significant changes in root architecture.

In parallel, during the past decade, many seminal studies on the biology of NO in plant physiology have appeared (see Ref. [70]). In plants, NO is a versatile signal molecule that controls and synchronizes a complex network of auxin-, cytokinin-, ethylene-, and abscisic acid-stimulated processes. As a result, NO profoundly affects plant growth, development, and stress physiology. Among the diversity of NO-mediated effects in plants, NO acts downstream of auxins to modulate adventitious and lateral root formation, as well as root hair development [71–73]. These NO-ascribed effects show a surprising and remarkable overlap with *Azospirillum*-induced effects on root architecture. Thus, we sought to determine whether there is any involvement of NO in the *Azospirillum*–plant root association. The first challenge was to determine the ability of *Azospirillum* itself to produce NO, then to investigate its origin and relevance in inducing plant responses to the bacterial inoculation.

*Azospirillum* produces NO under both aerobic and anaerobic conditions, using ammonium or nitrate as nitrogen source. More importantly, *Azospirillum* produces substantial amount of NO that is easily detectable through electron paramagnetic resonance (EPR) spectra of the NO–MGD–Fe adduct. *Azospirillum* produces a  $6.4 \text{ nmol NO g}^{-1} \text{ FW}$  of bacteria growing in aerated OAB medium for 16 h [74]. This NO concentration is almost 10-fold higher than the NO concentration found in plants. When the NO was sequestered with the specific scavenger cPTIO, results clearly showed that the ability of *Azospirillum* inoculation to induce lateral root development in tomato was lost [74].

More recently, data from genetic approaches have given new insights that contribute to deciphering the function of *Azospirillum*-mediated NO production in its interaction with the plant root. First of all, it was demonstrated that *Azospirillum* generates more NO at the end of the log growth phase (stationary) than at the middle of log phase. Moreover, when nitrogen was supplied as nitrate, *Azospirillum* was able to generate NO by aerobic denitrification and produced 25-fold more NO than when nitrogen was supplied as ammonium. This NO concentration is quite high (more than 100  $\mu\text{M}$ ), and an endogenous NO-detoxifying system should be operational in *Azospirillum* when growing with nitrate. The periplasmic nitrate reductase isogenic mutant (Nap mutant *Faj164* [75]) of *Azospirillum brasilense* Sp245 was unable to generate high amounts of NO when growing with nitrate, confirming that a nitrate reductase in the periplasm is necessary for NO production in the presence of nitrate. Moreover, in contrast to the wild type, the *Faj164* mutant generated more NO when growing in ammonium than in nitrate [76]. In addition, the mutant *Faj009* is negative for indole-3-pyruvate decarboxylase activity and displays a 90% reduction in IAA synthesis [77] but is able to generate the same amount of NO as the wt strain Sp245 when growing in nitrate, confirming that the defect in auxin biosynthesis does not affect the activity of the periplasmic nitrate reductase [76]. It was also confirmed that addition of the nitrification intermediate hydroxylamine enhances NO production in the presence of  $\text{NH}_4^+$  as the nitrogen source, which is evidence of a heterotrophic nitrification pathway operating in *Azospirillum* [76]. These three isogenic strains, *A. brasilense* Sp245 wt, *Faj164* mutant, and *Faj009* mutant, are indeed powerful genetic tools for examining the ability of *Azospirillum* to modify root architecture in tomato and for gaining insight into the origin of NO in the association.

The wild-type strain of *A. brasilense* was compared with the two mutants *Faj009* and *Faj164* in their ability to induce adventitious and lateral roots in tomato. When the experiment was performed in water, the wild type and *Faj164* induced more lateral roots than the mutant impaired in IAA production, suggesting that auxins are required to attain some level of root branching [76]. The same results were reported in wheat [78, 79]. An interesting result was observed when the experiment was performed in the presence of nitrate. Under these conditions, both wild type and *Faj009* generated huge amounts of NO (25-fold more NO in nitrate than in ammonium). In contrast, mutant *Faj164*, impaired in the periplasmic NR, generated only 5% of NO compared to the wild type and *Faj009* in the presence of nitrate. *Faj164* was not able to induce adventitious and lateral root production even though this mutant synthesizes the same amount of IAA as the wild type. This result strongly supports the idea that NO produced by the bacteria might be critical for triggering the molecular mechanisms responsible for the metabolic and physiological changes that result in root branching [80].

The question of the number and relative importance of NO sources in the *Azospirillum*-plant root cell interaction is still open. Nitrite produced by Nap should serve as the substrate for NO production by the dissimilatory nitrite reductases found in *A. brasilense* [81]. Individual *A. brasilense* cells show induction of nitrite reductase expression on wheat root surfaces [81] and enhanced NO production upon exposure to seed extracts [82]. It is not yet known if *Azospirillum* inoculation triggers a



mechanism that induces NO synthesis by root cells. Furthermore, it cannot be ruled out that NO production occurs in the apoplast of roots through a nonenzymatic reaction as was previously described in the aleurone cell layer of germinating barley seeds [83].

In conclusion, the finding that the level of aerobic NO synthesis in *Azospirillum* correlates with the level of lateral and adventitious root formation in tomato plants constitutes the first evidence establishing a plant response to bacterial-derived NO. Taken together, the results summarized above imply the existence of two-way signaling between *Azospirillum* and the plant host. Further studies should address the regulatory *cis*- and *trans*-acting elements that control the aerobic and anaerobic denitrification pathway and the modulator role that plant root components may play in an intimate association of *Azospirillum* with its partner.

### 11.4.3

#### Perspectives

Plants have evolved as open systems in habitats perfused with bacteria and the products of bacterial metabolism, including nitric oxide [84]. Thus, given our understanding of the roles of NO in various plant physiological and developmental pathways, it is almost certain that many influences of bacterial NO on plants remain to be discovered. Likewise, plants may release NO as a means to exert influence over their bacterial contingent.

As we endeavor to elucidate the pathways of NO production and responses in plants, an appreciation of the spatial and evolutionary relations between plants and bacteria can help to guide our investigations.

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