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The era of nitric oxide in plant biology: Twenty years tying up loose ends

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ABSTRACT

Nitric oxide (NO) is an essential signal molecule to maintain cellular homeostasis in uni and pluricellular organisms. Conceptually, NO intervenes as much in sustaining basal metabolic processes, as in firing cellular responses to changes in internal and external conditions, and also in guiding the return to basal conditions. Behind these unusual capabilities of NO is the chemistry of this molecule, an unstable, reactive, free radical and short half-life gas. It is a lipophilic molecule that crosses all the barriers that biological membranes can impose.

The extraordinary impact that the elucidation of physiological processes regulated by NO has had on plants, is comparable to the consequences of the discovery in 1986 that NO is present in animal tissues, and the following deep studies that demonstrated its biological activity regulating blood pressure.

In this review, we have summarized and discuss the main discoveries that have emerged at Mar del Plata University over the past 20 years, and that have contributed to understand part of the biology of NO in plants. Besides, these findings are put in context with the progress made by other research groups, and in perspective, emphasizing that the history of NO in plants has just begun.

1. NO sources and homeostasis in higher plants

In plants, NO is produced from a variety of enzymatic and non-enzymatic sources, which are in continuous examination [1]. Nitric oxide can be produced non-enzymatically from NO_2^- in the presence of a reductant, such as ascorbate. As this reaction requires the undissociated acid form of NO_2 (HNO_2, pK = 3.2), chemical NO production is insignificant at physiological pH, but it might occur under acidic conditions in specific microenvironments like the apoplast, vacuoles and cell compartments under unbalanced redox conditions [2]. Cytosolic nitrate reductase (NR) has been described as one of the main enzymatic sources of NO biosynthesis in plants under aerobic conditions [3]. This NR-dependent pathway has been implicated in NO production during bacterial-induced defense responses [4], responses to disease in other plant–pathogen interactions [5], drought stress responses, cold acclimation and regulation of stomatal aperture [6–8], alleviation of symptoms derived from iron deficiency [9–11], and root growth associated processes [12,13].

In mammals, NO is produced mainly by the enzyme nitric oxide synthase (NOS; EC 1.14.13.39), which catalyzes the conversion of L-arginine (L-arg) to L-citrulline and NO in the presence of O_2. There are three different known animal NOS isoforms, two constitutive (neuronal [nNOS] and endothelial [eNOS], and one inducible [iNOS]). In animals, NO is an important regulatory molecule in the vasculature, and eNOS is responsible for most of the vascular NO produced. A functional eNOS requires dimerization of the enzyme, the presence of the substrate L-arg, and the essential cofactor (6*R*)-5,6,7,8-tetrahydro-L-biopterin (BH_4), one of the most potent naturally occurring reducing agents [14]. Although NOS-like activities in plants are sensitive to mammalian NOS inhibitors, no typical NOS sequence was found in more than one thousand sequenced transcriptomes of land plants [15]. Given the fast rate of genomes sequencing attained in the last years, many new NOS sequences have been found and annotated in photosynthetic acquatic microorganisms, prokaryotes and eukaryotes. This offers a new dimension for analyzing the evolution of those genes during the transition of organisms from water to land. At the end of this review, it will be described the last findings concerning the NOS genes found in photosynthetic microorganisms.

Beyond the main and secondary sources of NO formation in plants, there is a dynamic state of intra and extracellular NO that is continuously modulated by rates of diffusion of different forms of NO, levels of transport, scavenging and/or storage (Fig. 1).

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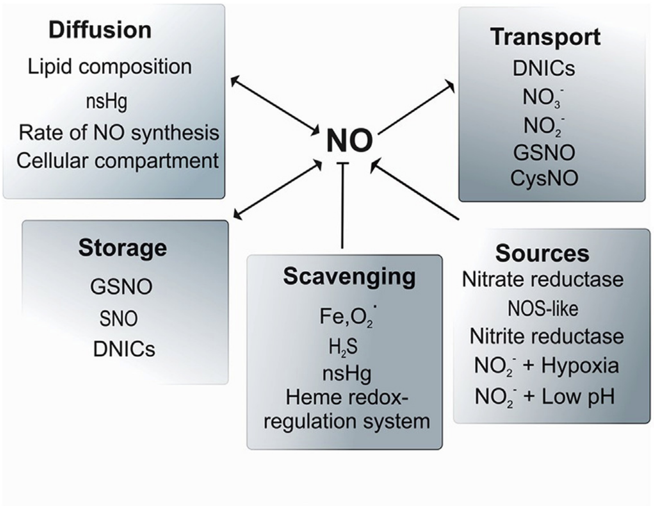

Fig. 1. Simplified schematic diagram summarizing NO interactions and homeostasis in plant cells. The source of NO by the reduction of nitrite can occur enzymatically, either through nitrate reductase (NR) or nitrite reductase (NiR) activities, or via non-enzymatic reactions (hypoxia and low pH conditions). Alternatively, NO formation can result by oxidative reactions from L-arg through NO synthase-like (NOS-like) activity detected in plants. The pool of NO is influenced by storage, scavenging, transport and diffusion. NO can react with reduced glutathione or thiol groups leading to the reversible formation of S-nitrosothiols (e.g., GSNO, S-nitroso glutathione) or DNICs (Dinitrosyl iron complexes). The NO can be scavenged by Fe, O_2, H_2S, by non-symbiotic hemoglobin (nsHb) dioxygenase activity, and Heme redox-regulation systems. The NO diffusion depends of cell membrane lipid composition, nsHem, rate of synthesis and subcellular compartmentalization of NO. In addition, NO can be transported through DNICs, NO_2^-, NO_3^- or S-NO.

2. Actions of NO in plants

2.1. NO counteracts oxidative stress conditions in plant cells

In 1998, three American pharmacologist scientists were awarded with the Nobel Prize in Medicine and Physiology, for their independent discoveries that allowed the identification of NO as the key molecule regulating blood pressure [16]. This fact meant the “Big Bang” in NO studies, because more and more works were done, not only in animals but also in plants. At that time, pioneering works showed the link between NO and reactive oxygen species (ROS), leading to the findings of NO-mediated plant protection against different stresses.

In 1997, it was demonstrated that NO induces the transcription of genes involved in plant defence mechanisms and preserves the level of chlorophyll in potato leaves infected with the pathogen *Phytophthora infestans* [17]. It was hypothesized that NO could mediate chlorophyll protection through their capability to scavenge ROS [18]. At low levels, ROS act as signals in different biological processes, however, higher amounts of ROS produced by uncontrolled generation, cause injuries like chlorophyll and protein breakdown, DNA fragmentation, ion leakage, lipid peroxidation, and, finally, cell death. The presence of an unpaired electron of the NO molecule makes it a reactive nitrogen species (RNS). When NO is combined with low amounts of superoxide anion ($O_2^{\cdot -}$), the formation of peroxynitrite ($ONOO^-$) damages lipids, proteins and DNA. On the other hand, if hydrogen peroxide (H_2O_2) accumulation is enough to induce toxicity, the presence of NO and $ONOO^-$ formation have a less toxic effect than $O_2^{\cdot -}$ itself and, under these conditions, NO might limit the damage and have a protective function. That is, essentially, the duality of NO action in biological systems; it can be toxic or protective for cell survival [19]. Also, additional effects between NO and ROS mediating plant defence activation against pathogens were reported [20,21]. Results showed that NO interacts synergistically with ROS to potentiate the induction of hypersensitive cell death in soybean (*Glycine max*) cells and the disease-resistance response of Arabidopsis leaves to the pathogen *Pseudomonas syringae* [20] In another pioneering work, it was demonstrated that NO together cyclic guanosine monophosphate (cGMP), and cyclic ADP-ribose act as essential signal molecules for the induction of defense genes in tobacco leaves infected by tobacco mosaic virus [21].

NO also participates in plant responses to wounding. Orozco-Cardenas et al. [22] showed that the treatment of young excised tomato plants with NO donors inhibited the expression of wound-inducible genes. Tomato plants accumulate high levels of H_2O_2 in response to wounding and elicitors, required for activation of wound signalling. It was proposed that NO might be acting as an antioxidant agent, protecting the plant cells and tissues from ROS damage. In another work, Paris et al. [23] demonstrated that NO was generated in potato leaflets after wounding and it was necessary to trigger the subsequent healing process.

In another set of experiments, it was demonstrated that extracellular adenosine triphosphate (eATP) stimulated NO and phosphatidic acid (PA) production in tomato suspension cells [24,25]. Then, it was suggested that balanced pools of reductants/oxidants are essential for the eATP-dependant hypocotyl elongation in *A. thaliana* seedlings [26]. Particularly, it was shown that eATP modulates a precise $O_2^{\cdot -}$ distribution which plays a prominent role during the hypocotyl elongation of etiolated seedlings [26]. In addition, the removal of NO reduced the hypocotyl elongation induced by eATP. These results indicate that eATP and NO, which regulates the pattern of O_2^- distribution, are required for the hypocotyl growth.

The ability of NO to capture ROS has been convincingly demonstrated in different plant experimental models. Treatments with diquat and paraquat, two methylviologen herbicides that generate an overproduction of $O_2^{\cdot -}$ in chloroplast, lead to a severe oxidative stress. NO treatments partially arrested several injuries induced by diquat and paraquat such as chlorophyll breakdown, ion leakage, protein and RNA degradation, necrosis and defoliation in potato leaves [19,27,28].

In another experimental model, NO prolongs the life of barley aleurone cells incubated in gibberellic acid (GA) during germination. The effects of NO can be mimicked by the antioxidant butylated hydroxy toluene (BHT), indicating that NO may act as an antioxidant in aleurone cells [29]. The results provided strong evidence supporting antioxidant NO activity in plants and they acted as starting point for a wide diversity of investigations around the world.

The ability of NO to promote adaptive responses to cope with water deficit conditions was also studied. It was demonstrated that NO treatments confer tolerance to water deficit in wheat leaves and seedlings subjected to drought stress conditions [30]. Particularly, the results indicated that ion leakage, a consequence of the oxidative stress, was significantly reduced in leaves treated with NO and exposed to a drought period with subsequent rehydration. We suggested that this protective role of NO may result, in part, from its interaction with lipid hydroperoxyl radicals or $O_2^{\cdot -}$, both of which promote lipid peroxidation [30].

Abscisic acid (ABA) is known as the stress hormone in plants, playing an important role in the induction of cellular mechanisms related to the antioxidant system. Zhou et al., [31] have demonstrated that ABA treatment increases the activities of superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX) in *Stylosanthes guianensis*, and that these responses were reversed in the presence of a NO-scavenger. These findings support the hypothesis that NO mediates the ABA-induced activation of antioxidant enzymes in plants.

Given that the salt stress induces the generation of an oxidative burst, Zhao et al. [32] examined if NO has the capacity to protect reed (*Phragmites communis*) calluses from high salt concentration. They showed that NO is produced under salt stress and it acts as a second messenger for the induction of PM H^+-ATPase expression that increases K^+ to Na^+ ratio, thus preventing oxidative damage and conferring increased salt tolerance. Other works have also reported that NO modulates pathways related to oxidative protection. Innocenti et al., [33] demonstrated that NO induces one of the main antioxidant paths in plants, the glutathione (GSH) synthesis in *Medicago truncatula* plants. All these evidences refer to different antioxidant activities mediated by NO in plants, either interacting directly with ROS or modulating pathways that avoids ROS production or detoxifies them.

2.2. The close interrelationship between NO, iron and proteins associated to iron metabolism

As stated before, it has been demonstrated that NO also counteracts the impact of iron deficiency on chlorophyll biosynthesis and steady state, and chloroplast development in both dicotyledonous and monocotyledonous species [9,10]. It was proposed that dinitrosyl iron complexes (DNICs) might be responsible of an increased iron transport and availability in NO-treated plants [9]. In parallel, Sun et al. [34] showed that NO reduces the accumulation of H_2O_2 and $O_2^{\cdot-}$ induced by iron deficiency in maize plants, protecting membrane lipids and proteins against oxidative damage. The authors suggested that NO can protect maize plants from iron deficiency-induced oxidative stress by reacting directly with ROS or by regulating activities of ROS-scavenging enzymes.

In the free ionic form, iron is toxic because it can catalyse the formation of ROS. Iron homeostasis is strongly dependent on ferritins, which are iron-storage proteins. Murgia et al., [35] showed that the NO-donor sodium nitroprusside (SNP) induces accumulation of ferritin both at mRNA and protein levels. By inducing ferritin accumulation and the subsequent iron free regulation, NO is therefore a key signalling molecule for modulating iron homeostasis and preventing oxidative damage. Frataxin is also essential for cellular iron homeostasis in mitochondria and cell survival during oxidative stress conditions. Frataxin deficiency causes increased ROS production, and interestingly, an Arabidopsis frataxin-deficient mutant displays increased total iron levels and NO accumulation [36]. Since NO is required for ferritin gene expression to diminish free iron, NO might be part of the homeostatic mechanisms working to counteract iron-mediated oxidative stress in the frataxin mutant. In addition, it has been demonstrated that NO has a relevant role to confront other stresses mediated by metals. Cadmium is a heavy metal that is toxic for humans, animals and plants, and it was found that NO reduces cadmium toxicity in rice leaves through its ability to scavenge ROS [37]. In agreement, Laspina et al., [38] demonstrated that the toxic effect induced by cadmium on sunflower (*Helianthus annuus*) leaves was counteracted by NO, suggesting that this effect might be due to the NO capability to scavenge ROS.

2.3. NO in the plant water management and drought tolerance

As stated, drought conditions, among other environmental constraints, generate oxidative stress in plants. In this context, NO alleviates symptoms of several deleterious consequences derived by oxidative burst such as chlorosis, DNA fragmentation, and apoptotic cell death when plants are challenged by (a)biotic stresses [18,20,26,27].

Encouraged by these results, in 2001, it was reported that SNP confers drought tolerance to both wheat detached leaves and seedlings. SNP was able to induce stomatal closure in both monocotyledonous and dicotyledonous [30]. This was the starting point of fundamental findings on the functions of NO in stomatal physiology.

Two articles published simultaneously [6,39] provided pharmacological evidence supporting that NO synthesis is required for ABA-induced stomatal closure. Later, it was demonstrated that NO selectively regulates Ca^{2+}-sensitive ion channels of *Vicia faba* guard cells by promoting Ca^{2+} release from intracellular stores to raise cytosolic-free Ca^{2+}. It is mediated via a cyclic guanosine monophosphate (cGMP)-dependent cascade [40]. Further research demonstrated that the NO-dependent signals can be modulated through protein phosphorylation upstream of intracellular Ca^{2+} release [41] and NO could regulate the Ca^{2+} insensitive outward rectifying K^+ channel, possible via S-nitrosylation [42]. Besides, evidence supported that NO and Ca^{2+} are active components in ABA inhibition of light-induced stomatal opening [43].

An earlier work [44] reported that ABA activates phospholipase D (PLD) and produces PA in guard cells, inhibiting the inward K^+ channel and reducing stomatal aperture. Based on that, Distéfano et al. [45], showed that the NO donor S-nitroso-N-acetylpenicyllamine (SNAP) increased the PA in *V. faba* epidermal peels to a maximum level within 30 min. It was also shown that PA can be generated by both PLD and phospholipase C (PLC) in concerted action with diacylglycerol kinase (DGK). Moreover, NO-induced stomatal closure was blocked when either PLC or PLD activities were inhibited. Thereby, it was demonstrated that PLC- and PLD-derived PA is a downstream component of NO signaling cascade during stomatal closure [45]. It was later shown that PLDδ, one of the 12 Arabidopsis PLDs isoforms, is required for ABA and NO-induced stomatal closure [46].

2.4. Hydrogen sulfide is a novel gasotransmitter involved in stomatal closure. Crosstalk with NO signaling

As proposed by Wang [47], the term 'gasotransmitters' refers to an emerging type of biological active molecules that can be grouped according to the following general criteria: (i) they are small molecules of gas; (ii) they can freely cross cell membranes; (iii) their effect do not rely on receptors; (iv) they are enzymatically generated and their production is regulated; (v) their functions can be mimicked by exogenous application of the gas, and (vi) their cellular effects may or may not be mediated by second messengers but should have specific cellular and molecular targets.

In addition to NO and carbon monoxide (CO), a third gasotransmitter hydrogen sulfide (H_2S), was shown to be active in animal systems [47,48]. García-Mata and Lamattina [49] present the first evidence that H_2S is a novel component of guard cell signaling in ABA-induced stomatal closure in *V. faba* and Arabidopsis. That work showed that the H_2S donor sodium hydrosulphide (NaHS) induced the stomatal closure in *V. faba* epidermal strips and the effect was fully blocked by the H_2S scavenger hypotaurine (HT).

In plants, H_2S is enzymatically produced in the cytosol through the activity of L-cysteine desulfhydrase (DES1) [50]. ABA is not able to close the stomata in guard cells of DES1 knockout Arabidopsis mutant plants (*des1*) [51] and the effect of ABA was restored by the exogenous application of H_2S. Results also showed that ABA induces *DES1* expression in guard cell-enriched RNA extracts from wild-type Arabidopsis plants. Furthermore, using epidermal strips from Arabidopsis mutated in the ABA receptor pyrabactin-resistant1 (*pyr1*)/pyrabactin-like1 (*pyl1*)/*pyl2*/*pyl4*, it was demonstrated that stomata are closed in response to exogenous H_2S, suggesting that this gasotransmitter is acting downstream ABA. In addition, the stomata from the Arabidopsis mutant ABA-insensitive1 (*abi1-1*) are insensitive to H_2S, suggesting that H_2S requires a functional phosphatase PP2A to induce stomatal closure. Regarding the crosstalk between H_2S and NO, results indicate that: (i) H_2S promotes NO production, (ii) DES1 is required for ABA-dependent NO production, and (iii) NO is downstream of H_2S in ABA-induced stomatal closure. Altogether, these data support that DES1 is a central component of ABA signaling in guard cells, and highlight the relevance of gasotransmitters and their interactions, as guard cell signaling molecules modulating leaf gas exchange [52].

2.5. NO mediates responses to UV-B radiation

Plants, as sessile organisms that absorb sunlight to grow and develop, are inevitably exposed to ultraviolet (UV)-B radiation (280–320 nm). High UV-B dose generates plant stress, since it produces DNA damage, protein crosslinking, membrane alterations, lipid peroxidation, increase of ROS, and impairment of pathogen resistance

[53,54].

Earlier reports described that exogenously added NO by means of different donors such as SNP, SNAP and nitrosoglutathione (GSNO), alleviates the damages generated by ROS increase and the stress symptoms induced by high UV-B doses [55–57]. It was also reported that both NO and UV-B absorbing compounds like phenylpropanoid (PP), increased as a consequence of the UV-B irradiation in plants [55,58–60]. Moreover, Mackerness et al. [61] showed that the induction of chalcone synthase (*CHS,* a PP biosynthetic gene) may be coordinately regulated by UV-B and NO.

Zhang et al. [62], reported that NO generation was triggered by ABA-induced H_2O_2 production in maize leaves. On the other hand, as described above, we demonstrated that NO is acting downstream of ABA in signaling cascade leading to stomatal closure [6,30]. Preliminary concept tests at Mar del Plata University, showed that potato leaves exposed to UV-B highly increased the production of NO and that exogenous addition of NO attenuated the necrosis generated by the exposition to high doses of UV-B. Thus, it was interesting to know if ABA could be involved in the signaling that control the NO-mediated plant responses to UV-B irradiation. Results showed that the ABA concentration increased two times in UV-B irradiated maize seedlings and it was later confirmed in Arabidopsis [63] and *Vitis vinifera* [64].

In parallel, He et al. [65–68] provided convincing genetic and pharmacological evidences indicating that UV-B increases the H_2O_2 production through the activation of NADPH oxidase (NOX), which provokes a NR-dependent NO increase, inducing the stomatal closure**.** To confirm the relevance of endogenous NO in plant responses to UV-B, the Arabidopsis *NOD* transgenic line expressing the inducible bacterial NO dioxygenase (NOD) was used. This Arabidopsis line allows to deplete endogenous NO and analyze its physiological role [69]. Thus, it was demonstrated that when endogenous NO was diminished, UV-B-irradiated Arabidopsis *NOD* plants exhibited more symptoms of damage as loss of weight, turgor and growth inhibition, compared to the wild type [69]. The ion leakage level was increased from 28 to 40%, and the chlorophyll concentration dropped from $1.2\,\mathrm{mg\,g^{-1}}$ FW to $0.8\,\mathrm{mg\,g^{-1}}$ FW in *NOD* plants compared to the wild type. Regarding UV-B absorbing compounds, anthocyanin content was increased in UV-B irradiated plants and it was dependent on NO since its depletion partially prevented the effect of UV-B on anthocyanin accumulation. Moreover, the PP biosynthetic genes *CHS* and chalcone isomerase (*CHI)* were highly expressed in UV-B irradiated plants, but severely repressed when NO was depleted [69]. All together, these results indicate that, in response to UV-B, endogenous NO modulates the expression of some genes involved in the flavonoid and anthocyanin biosynthesis in Arabidopsis helping to mitigate the damage provoked by the high UV-B irradiation.

3. NO acts like a plant growth regulator

3.1. Auxin and NO operate as a successful partnership for root growth and development

Auxin was discovered in 1934 in human urine and soon was detected in fungi and higher plants [70]. In plants, auxin acts not only on morphogenesis as a coordinating signal across the whole plant, but also as a local patterning signal. At the cellular level, the mechanisms underlying the auxin response are complex involving many components [71]. Auxins function as a regulator of cell division, elongation and differentiation. It has been established a long time ago that auxin transported from the shoot to root system is necessary for root growth [70].

3.2. Auxin, NO and adventitious root formation

Concerning NO and root growth, Gouvêa et al. [72] reported that NO could induce the elongation of maize root segments in a dose-dependent manner. Later, the linkage between auxin and NO has been reported for the first time in 2002, when NO was shown to be required for the adventitious root formation in cucumber [73]. After removal of the primary root system, adventitious root development was detected in the cucumber hypocotyls (explants) treated with the auxin indole acetic acid (IAA). In parallel experiments, two different NO donors (SNP, SNAP), applied to the explants were able to mimic the effect of the IAA. To go deep into the potential connection between IAA and NO, the specific NO scavenger 2-4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) was added to the IAA treatment and, surprisingly, the IAA-promoted adventitious root growth was completely blocked [73], suggesting that NO is a central partner of auxin for the promotion of adventitious rooting.

Then, in a series of experiments, it was demonstrated the occurrence of second messengers acting downstream NO and mediating the auxin signaling. Thus, it was found that NO operates downstream IAA promoting adventitious root development through pathways involving: (i) the guanylate cyclase (GC)-catalyzed synthesis of cGMP [74] and (ii) the mitogen activated protein kinase (MAPK) cascade [75]. Maximal MAPK activity was detected simultaneously with cell proliferation and adventitious root primordia formation in both NO- and IAA-treated explants. According to Mockaitis and Howell [76], this pathway could be mediating the expression of auxin-responsive genes.

Later, new findings allowed identifying the participation of Ca^{2+} and Ca^{2+}-dependent protein kinase (CDPK) in IAA- and NO-induced adventitious root formation in cucumber explants [77]. Different pharmacological approaches showed that the explants response to IAA and NO leading to adventitious roots formation depended on the availability of both intracellular and extracellular Ca^{2+} sources. On the other hand, the activity of CDPK was detected *in vitro* and *in gel* in soluble protein extracts of cucumber explants. After one day of either NO or IAA treatments, CDPK activity was induced and it extended up to the third day of treatment. Moreover, the presence of the NO-scavenger cPTIO prevented the activation of CDPK in both NO- and IAA-treated explants, suggesting that NO is required for the maximal and sustained activity of CDPK [77].

Following with the same experimental system, the involvement of phospholipid signaling during the auxin- and NO-induced adventitious root formation was studied. Explants were labeled with ^{32}P-inorganic and treated with IAA or the NO donor SNAP. Chromatographic analyses were used to separate and quantify phospholipid signal molecules. As a result, PA, phosphatidylinositol phosphate (PIP), and phosphatidylinositol bisphosphate (PIP_2) were identified to be accumulated after auxin or NO treatment leading to adventitious root formation. Auxin-induced PA, PIP, and PIP_2 accumulation relied on NO. Furthermore, it was demonstrated that auxin and NO trigger PA formation via PLD activity and that NO acts downstream of auxin to trigger PIP, PIP_2, and PA accumulation. Fig. 2 represents a schematic model that includes the main second messengers involved in the signaling pathways activated by auxin and NO, leading to adventitious rooting.

3.3. Auxin, NO and lateral root development

In parallel, Correa-Aragunde et al. [12] showed the first evidence on the inducing effect of NO on lateral root development in tomato. High NO concentrations were detected with the fluorescent probe DAF- 2DA in pericycle cells that will give place to a lateral root, indicating that NO is present during the early stages of lateral root development. Moreover, treatment with the NO-donor SNP increased lateral root number and, at the same time, decreased primary root length in a dose-dependent manner. When tomato seedlings were treated with the permeable auxin 1-naphthylacetic acid (NAA) resulted in the same root phenotype and architecture obtained with SNP. Concurrent treatment of NAA with the NO-scavenger cPTIO showed the blockage of responses due to NAA on lateral root formation, supporting a strict requirement of NO in the signal transduction cascade of auxin leading to lateral root

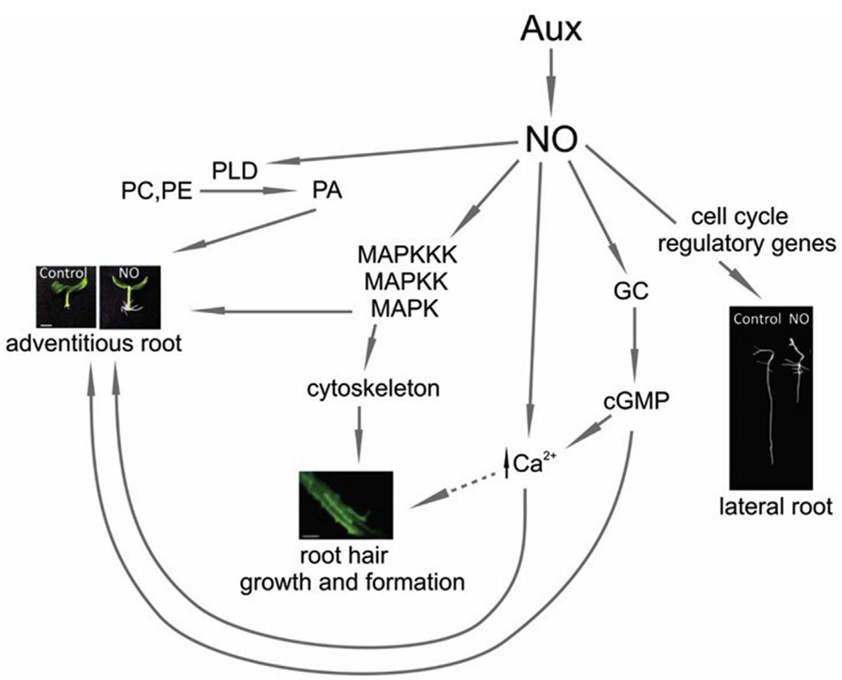

Fig. 2. Schematic illustration of the signaling networks involving auxin, NO and cellular messengers regulating lateral root development (LRD), adventitious root formation (ARF) and root hair growth and formation. Auxin induces NO signaling which has different targets. NO is responsible of: (i) control of cell cycle regulatory genes that induces LRD; (ii) activation of guanylate cyclase (GC) which increases cyclic GMP (cGMP) level regulating ARF; (iii) increase of Ca^{2+} concentration involved in ARF and root hair growth; (iv) increase of phospholipase D (PLD) activity that trigger phosphatidic acid (PA) involved in ARF; (v) activation of a mitogen activated protein kinase (MAPK) signaling cascade that targets root hair cytoskeleton and ARF. Grey arrows indicate links established with supporting experimental evidence and dashed arrow indicates suggested pathways not experimentally proved.

formation.

Following this line of research, Correa-Aragunde et al. [78] demonstrated that auxin-dependent cell cycle gene regulation was also relying on NO during lateral root formation in tomato. Auxin promotes lateral root initiation through the expression of cell cycle regulatory genes like cyclins and cyclin dependent kinases (CDK). Pretreatments with cPTIO and the auxin transport inhibitor naphthylphthalamic acid (NPA) were used to synchronize the formation of lateral root primordia. It was demonstrated that both NO and auxin induced the transcription of *CYCD3;1* [78]. On the contrary, the presence of auxin and NO strongly downregulated the cell cycle inhibitor *KRP2*. Altogether, the decrease of *KRP2* expression and the accumulation of *CYCD3;1* induce the progression from G1-to-S phase of the cell division cycle supporting the first periclinal and anticlinal cell divisions, and the establishment of lateral root primordia from founder cells in root pericycle.

3.4. Effects of auxin and NO on root hair physiology

Considering the effects of NO on growth and development of primary, lateral and adventitious roots, it was inevitable to predict a potential influence of NO mediating auxin signaling in processes modulating root hair physiology. Root hairs are specialized cell types that function in root anchoring and increasing the soil area exploitable by the plant. For its particular growing dynamic, root hair is taken as a model to study rapid cell growing processes. In 2006, Lombardo et al. [79] demonstrated that both auxin and NO regulated the root hair differentiation and elongation in lettuce. The results showed that, the addition of NAA or the NO-donor SNP, results in an increase of root hair density in lettuce seedlings grown in hydroponic conditions. In that growth conditions, auxin-induced root hair formation was NO-dependent [79].

As described in lettuce, an increase of NO-specific fluorescence detected with the probe DAF-FM DA was observed in Arabidopsis root hairs after NAA treatment, mainly located in the root hair files [79]. New approaches were useful to unravel the physiological mechanisms underpinning the NO-mediated effects on root hair growth. Thus, NO and auxin were involved in the rapid root hair growth through the formation of membrane vesicles during endocytosis process [80] and, more recently, it was demonstrated that ABA and NO modulate root hair growth and ectopic hair formation in Arabidopsis, through its coordinated action on the cytoskeleton organization [81].

The interaction between root and soil generates the physical environment named rhizosphere where is localized the more abundant concentration of microorganisms in soils that are deeply implicated in the plant life and survival. Creus et al. [82] and Molina-Favero et al. [83] demonstrated that *Azospirilum brasiliense*, a plant-growth-promoting rhizobacterium, produced directly or indirectly NO when colonizing tomato roots and it was involved in the lateral and adventitious roots formation. There, it was also reported that the NO scavenger cPTIO and the auxin competitive inhibitor *p*-chlorophenoxyisobutyric acid (PCIB) were capable of reducing the stimulatory effect produced by Azospirilum on lateral root formation in tomato suggesting that both auxin and NO are involved in the Azospirilum-induced effects. It is conceivable that auxin provokes an increase of NO concentration as previously reported by Pagnussat et al. [84].

4. Molecular mechanisms underpinning NO functions: S-nitrosylation

4.1. NO-mediated S-nitrosylation of MYB transcription factors

S-nitrosylation is an oxidative post-translational modification (PTM) of proteins resulting from covalent and reversible binding of NO to a cysteine residue in the target protein, forming a nitrosothiol (SNO). It is proposed that S-nitrosation is a more accurate term and that nitrosylation should be used for NO binding to a metal. However, in this review we will refer to S-nitrosylation as the convention for "ylation" in the signal transduction field refers to different PTMs (phosphorylation, sumoylation, glycosylation, etc). In animals, S-nitrosylation emerged as an important NO dependent PTM which regulates multiple physiological and pathophysiological processes [85,86]. In plants, the effort has been focused on the identification of S-nitrosylated proteins. Proteomic analysis in Arabidopsis allowed the identification of numerous candidate proteins from cell culture [87,88] and leaves [89]. These proteins belong to stress-related, redox-related, signaling/regulating, cytoskeleton and metabolic proteins. During hypersensitive response (HR), S-nitrosylated proteins were identified mostly intervening in intermediary metabolism, signaling and antioxidant defense, including ascorbate peroxidase (APX) and peroxiredoxin II E [90–92]. In another study, NO was shown to limit cell death development during HR by S-nitrosylation of the NADPH oxidase at Cys 890 inhibiting the production of ROS [93]. Several transcription factors (TF) have been shown to be modified by NO in animals [94], however there were no reports in plants [95] since 2007, when Serpa et al. [96] described for the first time the S-nitrosylation of the plant R2R3-MYB2. In this work, the effect of NO on the DNA binding ability of R2R3-MYB2 from Arabidopsis was evaluated. Results showed that the addition of either NO donors, SNP or GSNO, inhibited the DNA-binding capacity of the minimal DNA binding domain of the TF R2R3-MYB2 due to Cys53 S-nitrosylation [96]. Later, it was also described that S-nitrosylation of AtMYB30 in Cys49 and Cys53 promotes a conformational change in protein secondary structure inhibiting DNA binding [97]. These results established a relationship between the redox state and DNA-binding capacity of plant MYB TFs, and opened a new aspect of NO signaling in plants. Since then, many reports describing S-nitrosylation of plant TFs emerged [98,99]. For example, the TGA1 TF involved in the activation of pathogenesis related (PR) gene expression is S-nitrosylated after treatment with NO donor. NO increases TGA1 binding activity to the as-1 element of the PR1 promoter in the presence of the non-expresser of the pathogenesis-related gene 1 (NPR1) indicating an important role of S-nitrosylation in plant defense response [100]. Also, the TF ABI5, an ABA regulated growth repressor, is S-nitrosylated in Cys153 facilitating its degradation and promoting seed germination [101].

4.2. S-nitrosylation of TIR1, ASK1 and APX1 influences auxin signaling

As described above, many proteins involved in ABA and salicylic acid (SA) signaling are targets of S-nitrosylation. In 2012, the importance of S-nitrosylation in auxin signaling was described, specifically in the induction of auxin responsive genes [102]. The transport inhibitor response 1 (TIR1) is an auxin receptor that mediates degradation of Aux/IAA repressors. Arabidopsis s-phase kinase-associated protein (SKP)-like1 (ASK1) acts as a bridge between cullin-1 (CUL1) a subunit of the E3 ligase complex involved in protein ubiquitination and the TIR1/auxin-related F-box (AFBs) proteins. Degradation of Aux/IAA repressors by the 26S proteasome induces the expression of auxin responsive genes. Experiments with NO donors showed that Arabidopsis TIR1 is S-nitrosylated in Cys140 enhancing TIR1-Aux/IAA interaction and promoting Aux/IAA degradation. Subsequently, activation of auxin responsive gene expression is induced [102]. Very recently, the S-nitrosylation of Cys37 and Cys118 of ASK1 was described. This PTM enhances ASK1-TIR1-AFB2 and CUL1 interaction, modulating complex assembly and auxin response in a very sensitive way and, more interestingly, suggesting a putative trans-nitrosylating process operating in the regulation of the complex [103]. Taken together, these reports underline the importance of NO-mediated PTMs in auxin signaling.

NO-derived S-nitrosylation is not only involved in the initial steps of hormone signaling but also related to downstream events. Many enzymes involved in ROS production and metabolism have been described to be S-nitrosylated. As an example, S-nitrosylation of peroxiredoxin II E inhibits hydroperoxide-reducing peroxidase activity [91]. In peroxisomes, catalase and glycolate oxidase are S-nitrosylated and its activities are inhibited by NO donors [104]. In 2011, a proteomic investigation using the biotin switch method and labeling with isotope-coded affinity tags without the addition of a NO donor led to the identification of ascorbate peroxidase 1 (APX1) protein as a candidate for this PTM in Arabidopsis cells [88]. Two years later, the balance of S-nitrosylated/denitrosylated cytosolic APX1 was described during auxin mediated root growth. Through biotin switch assays, Correa-Aragunde et al. [105] demonstrated that auxin treatment induces APX1 denitrosylation and partial inhibition of its activity in Arabidopsis roots. As a consequence, H_2O_2 levels increased inducing lateral root formation. Results demonstrated that auxin- and NO-mediated pathways contribute to a tuned control of root development and architecture. The nicotinamide adenine dinucleotide phosphate (NADPH)-dependent thioredoxin reductase (NTR)-Thioredoxin (Trx) system is one mechanism that controls the balance between nitrosylation and denitrosylation process. In order to elucidate the role of auxin and NO in the activation of this system, Correa-Aragunde et al. [106] investigated the participation of the NTR-Trx system in auxin-mediated root development and its role in denitrosylating activity. Results showed that auxin induces NTR activity and that NO scavengers inhibit this activity. In addition, Arabidopsis roots treated with NTR inhibitors increases total nitrosothiol content and protein S- nitrosylation. These results indicate that auxin and NO induce NTR activity which correlates with protein denitrosylation [106] and may participate in APX1 denitrosylation (and partial inactivation) in Arabidopsis roots. In this sense, and in accordance with our results, Orman-Ligeza et al. [107]. reported that auxin is able to induce the expression of many respiratory burst oxidative homolog (RBOH) genes and that changes in ROS-related gene expression are associated with early steps of auxin-induced lateral root formation. Also, exogenous ROS treatment promotes developmental progression of existing lateral root pre-branch sites rather than inducing *de novo* initiation sites, conducing to an increase of lateral root growth [107]. On the other hand, it was reported that S-nitrosylation of APX during heat shock and H_2O_2-induced programmed cell death was responsible of a rapid decrease in its activity in tobacco cells [92]. Taken together all the results, APX1 nitrosylation/denytrosilation balance may have different effects depending on the species of analysis and the stress or combined stresses that are occurring. When analyzed, it can be found that many PTMs occur on APX1 [92], some of them simultaneously, that might regulate in a yet unknown way its activity under different physiological situations, bringing an exquisite and fine-tuned hub to control the H_2O_2 concentration in response to auxin signaling.

As suggested some years ago [86], new discoveries on S-nitrosylated plant proteins will extend our knowledge of the molecular bases underpinning NO functions in plant signaling.

5. NO synthases (NOS) in the photosynthetic organisms

5.1. The surprise came from the green algae Ostreococcus tauri: a canonical NOS enzyme with ultrafast NO producing activity

The first evidence that green algae belonging to the plant kingdom possess a NOS-like enzyme was reported in 2010 [108]. A putative gene showing a sequence similarity of near 40% to animal NOSs, was identified in the published genome of green alga *Ostreococcus tauri* [109]. This gene was named OtNOS and conserves the oxygenase (NOSoxy) and reductase (NOSred) domains of mammalian NOS including the binding motifs for heme, H_4B, NADPH, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) [108]. The NOSoxy and NOSred domains of OtNOS are joined by a calmodulin (CaM) binding domain. Despite the high similarity, some differences could be noted in the structure of the OtNOS with respect to animal NOS. CaM plays a critical role in activating NOS, since it triggers the electron transfer from flavin to heme. In eNOS and nNOS the electron transfer is triggered by CaM binding when Ca^{2+} levels are increased in cells while in iNOS, CaM is irreversibly bound independently of Ca^{2+} concentration. Indeed, OtNOS activity behaves like an intermediate between eNOS/nNOS and iNOS isoforms since in the absence of Ca^{2+}-CaM, OtNOS retains almost 70% of activity. Furthermore, OtNOS lacks the autoregulatory control element [108], indicating that it is close to the mammalian iNOS isoform. The autoregulatory control element (ACE) impedes CaM binding and enzymatic activation in constitutive NOSs. The increase in Ca^{2+} concentration triggers the binding of Ca^{2+}-CaM in constitutive NOS by displacing the ACE. The zinc (Zn) binding motif Cys-X_3-Cys in OtNOS is partially conserved compared to Cys-X_4-Cys in mammalian NOS. Even though the binding of Zn to OtNOS has not been experimentally proved, there are other examples of Zn binding motif consisting of Cys-X_3-Cys [108]. BH_4 cofactor is essential for NOS derived NO production in animals, since its absence uncouples the reaction leading to NADPH oxidation and O_2^- formation. *Ostreococcus* genome has been completely sequenced [109] and it lacks the genes encoding for the enzymes that synthesize BH_4, suggesting that OtNOS may bind another cofactor for catalytic activity. Similar to the bacterial NOS-like proteins, OtNOS does not conserve the extended N-terminal portion of mammalian NOSs consisting of the N-terminal hook and a BH_4-binding site. Once purified, recombinant OtNOS was replete with FAD and FMN and was shown to catalyse *in vitro* the synthesis of both NO and L-citrulline from L-arg. The Km of OtNOS for L-arg is about 12 μM and the activity is suppressed by the inactive L-arg analog N(ω)-nitro-L-arginine methyl ester (L-NAME) [108]. As demonstrated for bacterial NOS-like proteins [110], OtNOS can efficiently use BH_4 but also the pterin tetrahydrofolate (THF), both *in vivo* and *in vitro* [111]. A further characterization of the structural and electronic fingerprints of some OtNOSoxy reaction intermediates using a combination of spectroscopy techniques indicates that OtNOS is a genuine and ultrafast NO-producing enzyme [112].

Studies with *Ostreococcus tauri* cell cultures indicated that the activity of OtNOS depends on the phase of culture growth, with an optimum at the exponential growth phase. A significant increase of activity was also observed in response to photo inhibitory light intensities [108]. This latter finding suggested that NO may counteract the oxidative damages caused by high light irradiances as observed in higher plants [27]. In addition, the expression of OtNOS in transgenic Arabidopsis plants was shown to enhance several physiological traits such as germination rate, root and shoot development under high salinity conditions and the tolerance to drought and oxidative stresses [111].

The search of other NOSs in the one KP database and in publicly available plant genomes led to the identification of 15 NOS-like sequences in a total of 265 algal species [15]. Kumar et al. [113] identified two NOS-like sequences from the marine green algae *Bathycoccus prasinos* and *O. lucimarinus*. Phylogenetic analysis showed that the evolutionary history of NOS proteins is globally congruent with the organismal phylogenetic tree. Green algae and *Rhodochaete parvula* (red algae) are grouped together [112]. As stated, it was suggested [108] that NO produced from OtNOS counteracts the oxidative damages triggered by photo-inhibitory light intensities and, therefore, might confer a selective advantage under such stressful conditions. Another striking point concerns to the structural peculiarities observed in several algal NOS, and the evolutionary history linked to physiological functions derived of such divergences.

5.2. The characterization of a singular NOS from cyanobacteria

In bacteria, NO production has been primarily attributed to denitrification process, where inorganic nitrite is converted to NO and then to dinitrogen by action of several reductases [114]. Later, homologous sequences to animal NOSoxy were identified in several Gram-positive bacteria [115]. Most bacterial NOS enzymes only contain the NOSoxy domain and use non-specific cellular reductases as electron donors [116]. A peculiar bacterial NOS with both oxygenase and reductase domains was characterized in the Gram-negative bacterium *Sorangium cellulosum* [117].

Recently, the first NOS in cyanobacteria was characterized from *Synechococcus* PCC 7335 (SyNOS) [118], which has high similarity to animal NOS and OtNOS [108]. SyNOS contains the NOSoxy and NOSred domains present in eukaryotic and canonical NOSs. Furthermore, SyNOS presents a novel and unusual domain in its N-terminal end, which encodes a globin. Through phylogenetic analysis of NOSoxy domains, we could determine the presence of NOS sequence in four orders of cyanobacteria. Cyanobacterial NOS has two types of architectures. One possesses the typical bacterial NOS architecture with only the NOSoxy domain and the other has a globin connected to the NOSoxy and NOSred domains. SyNOS seems to have a typical NOS activity *in vivo*, since NO production is increased by addition of L-arg and inhibited by the inactive arg analog L-NAME. Furthermore, SyNOS inhibition affects normal *Synechococcus* growth, suggesting that the impairment of L-arg metabolism is critical for this cyanobacterium [118].

The heterologous expression of SyNOS in *E. coli* cells allows bacteria to grow in minimum media containing L-arg as the sole N source. Moreover, the expression of SyNOS confers *E. coli* higher growth rate than the wild type under N limited conditions [118]. It is hypothesized that the globin domain of SyNOS represents an evolutionary advantage, conferring NOS new functional capabilities for N metabolism. Bacterial globins convert NO into nitrate aerobically or into nitrous oxide anaerobically [119]. If globin domain of SyNOS may generate nitrate from the NO produced by SyNOS, it could provide more available nitrate to be incorporated into the N metabolism. Thus, SyNOS would allow a better growth by conferring an alternative route that improves the assimilation and metabolism of N [118].

6. Concluding remarks and future challenges in plant NO research

Science is a self-correcting activity where new discoveries lead to continually reviewing ancient findings and paradigms. The emergency of NO as a small gas molecule that is not just a toxic compound present in gases of biofuel combustion, but also produced and metabolized with functional properties in living organisms, was a shocking new at the end of the las century. This new attracted our attention to go fast into the exploration of NO functions in plant physiology. Fig. 3 summarizes the major contributions originated at the Mar del Plata University, on the functions, signaling, molecular mechanisms and sources of NO production in plants. Those findings contributed, in part, to understand the location and interactions of some pieces in the plant physiology and biochemistry puzzle, and also, the differences and similarities of the evolution of signaling between the different kingdoms of life.

Fig. 4 shows how the study of the NO biology in plants has grew during the last twenty years. It started with a very few 4 papers published in 1997 to more than 800, twenty years later, in 2017. When compared the production of papers in the plant NO field with papers published on the auxin hormone, which is extensively studied since Darwin inferred its presence in plants [120], and later when was isolated in 1926 [121], it can be seen the breakthrough that represented the emergency of the 'NO era' in plant research. During this period, new connections appeared linking environmental stimuli and plant responses through the actions mediated by NO. Many other plant physiological behaviors were more fully understood once new molecular interactions were discovered between NO, ROS and hormones that explained the control and changes of cell redox status, the regulation of gene expression, Ca^{2+} signaling, protein-protein interactions, modulation of enzymatic activities, among others, in a myriad of pathways orchestrated by NO in plants.

Even if a number of discoveries allowed describing many actions and targets of NO in plants, further research must be carried out to learn more about the connection between plant cell homeostasis and NO physiology. For instance, irrespective of the NO source, it is still not known the different threshold of NO concentrations for the different biological actions in different plant organs. To solve that, sensitive methods for intracellular NO detection and quantification should be developed, since there is no reliable and universal applicable method currently accepted.

Another intriguing aspect poorly studied yet is about how does NO work molecularly at many levels of the genome organization and expression, and how is NO specifically directed at specific DNA regions. Plant evolution is strongly influenced by epigenetic mechanisms. Epigenetic is heritable phenotypic changes that do not involve DNA sequence modification. Gene expression may be epigenetically regulated by histone variants, histone tail modifications, nucleosome positioning, DNA methylation and demethylation, and microRNAs (or noncoding RNAs). In animal cells, NO can impact acetylation and methylation of histones H3 and H4, as well as DNA methylation and microRNAs expression [122]. The study of the role of NO as epigenetic regulator in plants is still in its infancy. Two histone deacetylases (HDAC 2C and 2B) were identified as target of S-nitrosylation in

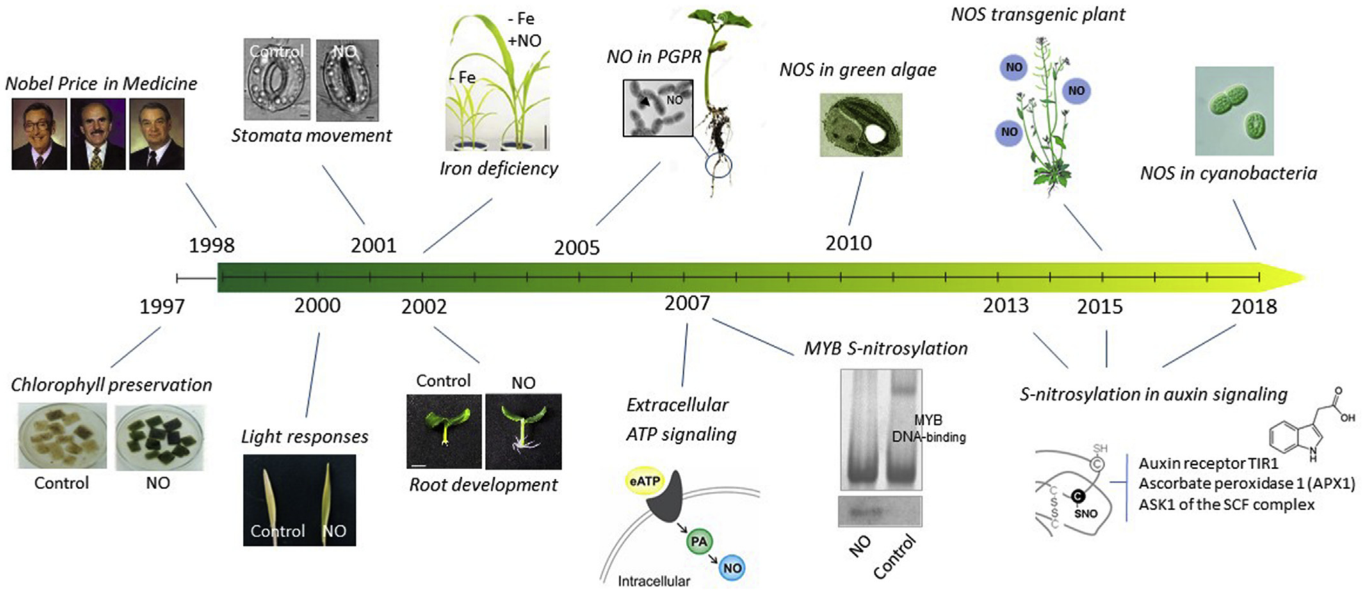

Fig. 3. Timeline of discoveries from NO research in plants at the Mar del Plata University, Argentina. The first evidences of NO effects in plants were published in 1997, one year before the Nobel Prize in Medicine was awarded to NO discoveries in humans. The first observation was that NO preserves the level of chlorophyll in leaves challenged by oxidative stress processes (pathogen attack, nutritional starvation) and participates in light-inducible responses. Later, it was found that NO acts as a signal molecule downstream of the phytohormones abscisic acid (ABA) and auxin that lead, respectively, to stomatal closure and root development. It was also found that NO produced by the plant growth promoting rhizobacteria (PGRP) *Azospirillum brasilense* influenced root development in plants. Extracellular ATP (eATP) was shown to induce phosphatidic acid (PA) and NO in plant cells. In 2007, it was reported the NO-mediated S-nitrosylation of plant R2R3-MYB transcription factor (TF). In 2010, the first NO synthase (NOS) in the plant kingdom was identified in the green algae *Ostreococcus tauri*. After that, transgenic plants expressing NOS from *O. tauri* were generated and analyzed. In the last years, S-nitrosylation of some components of the auxin signaling pathways, such as TIR1, ASK1 of the SCF complex, and APX1 were identified and characterized in auxin-mediated processes. The last finding was the characterization of first NOS in cyanobacteria from *Synechococcus* PCC 7335 (SyNOS). All references to works describing these findings are cited in the text.

Arabidopsis [98,123]. Rai et al. [124] have recently reported that DNA methylation is increased by treatment with SNP in heat-treated (40-42 °C) *Lablab purpureus* L. plants. Clearly, epigenetic regulation by NO opens new exciting perspectives into the study of NO signaling in plants.

It appears that NO response may be both local and systemic. But how the NO signal is transmitted? It's postulated that NO can be transported as GSNO by phloem cells, in a mechanism that involves ROS and Ca^{2+} [125]. However, additional biochemical and genetic evidences are required to confirm this idea and contrast it with locally-induced generation of NO.

Besides the interaction with auxins and ABA previously described in this review, NO interacts with other phytohormones like gibberellins, ethylene, salicylic acid, jasmonic acid, cytokinins and brassinosteroids. However, these data do not consider the non-linearity of the responses. It is known that NO regulates the hormone production and signaling and, at the same time, NO production and signaling is also modulated by hormones. Therefore, many efforts are still necessary to know the

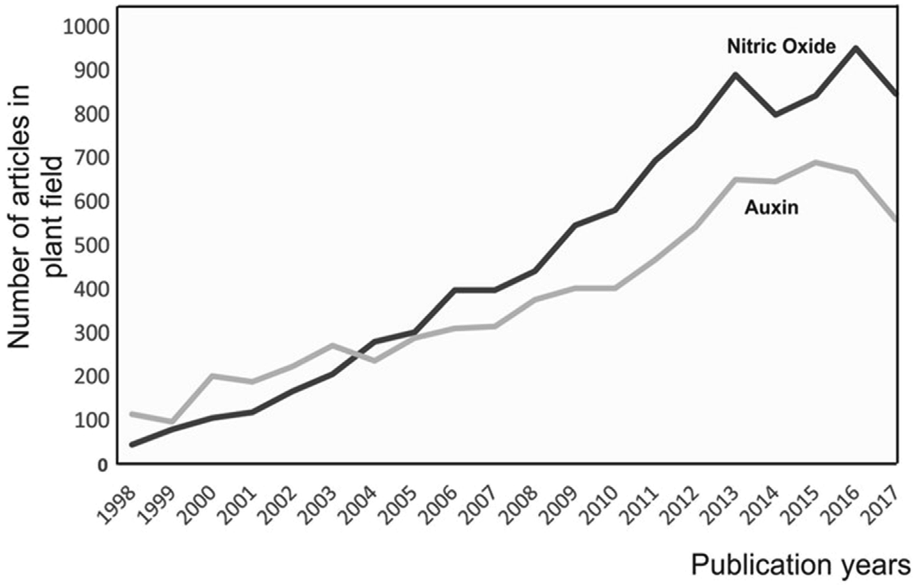

Fig. 4. Number of publications involving NO and auxin research in plants. The graphic shows the increase of the number of articles (indexed by PubMed) from 1998 to 2017.

simultaneity and hierarchy of these processes in time and space, for better understanding the crosstalk.

As previously described, S-nitrosylation is involved in the regulation of hormone effects, enzymatic activity and gene expression. Only few Cys in a protein are S-nitrosylated and targets of this PTM. But, how is this specificity achieved? It was postulated that a prerequisite is the proximity of the target Cys to the NO source. That was suggested for mammalian systems [126], but given the many ways in which NO can be generated in plants, this hypothesis requires a more careful consideration in plant systems. Even if some reports describe a consensus S-nitrosylaton sequence, the analysis of large-scale data sets raises doubts about the existence of such nitrosylation motif [127]. GAPDH, hemoglobin, caspase 3 and thioredoxin were reported to have nitrosylation activity in animals (and termed nitrosylases). That means they possess a transnitrosylating ability since they can transfer a NO moiety to a SH of a reduced Cys in a target protein. On the other hand, thioredoxin reductase and GSNO reductase (GSNOR) work favoring denitrosylation [128]. Both nitrosylases and denitrosylases are found in animals and plants. Therefore, it becomes more and more important to identify and characterize *in vivo* nitrosylated proteins in plants, their subcellular localization, as well as the potential activity of nitrosylases they may share.

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