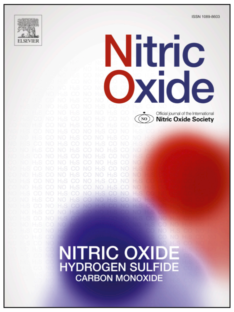

A forty year journey: The generation and roles of NO in plants

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A Forty Year Journey: The Generation and Roles of NO in Plants

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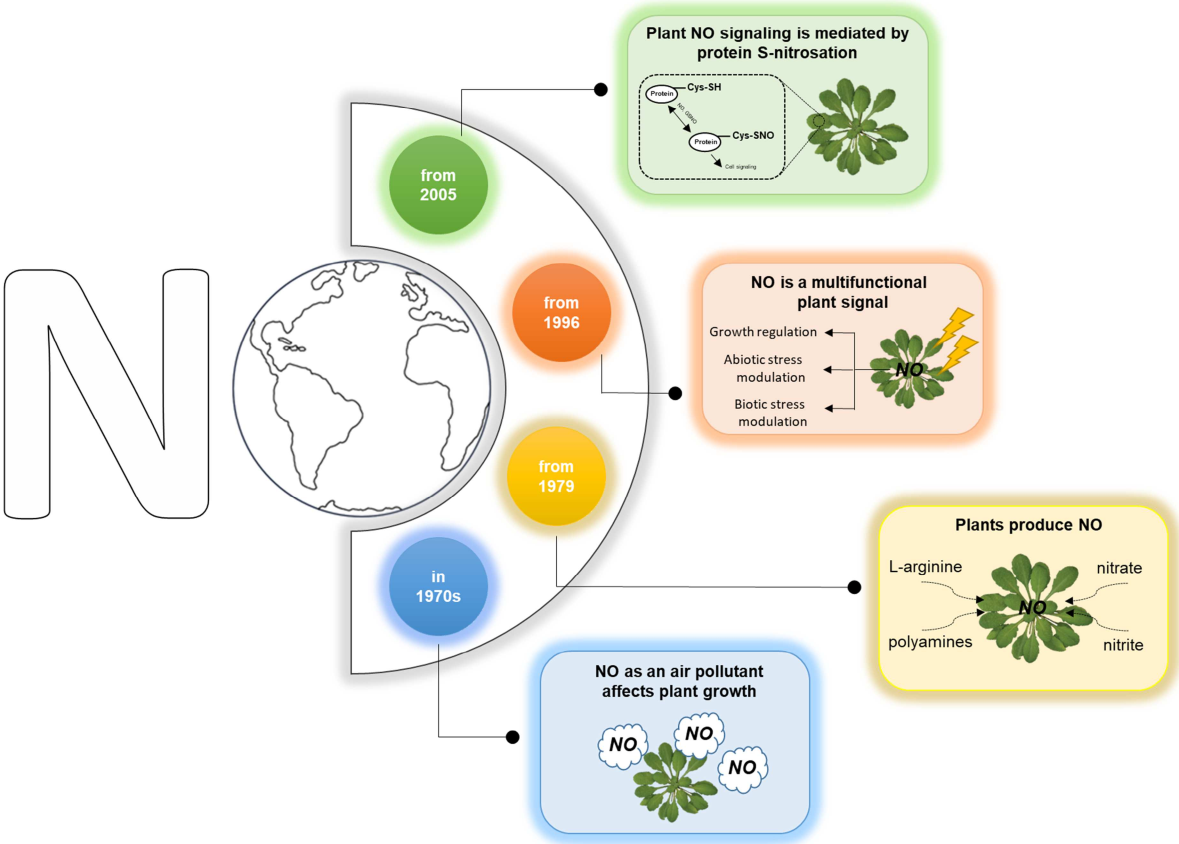
Abstract

In this year there is the 40th anniversary of the first publication of plant nitric oxide (NO) emission by Lowell Klepper. In the decades since then numerous milestone discoveries have revealed that NO is a multifunctional molecule in plant cells regulating both plant development and stress responses. Apropos of the anniversary, these authors aim to review and discuss the developments of past concepts in plant NO research related to NO metabolism, NO signaling, NO's action in plant growth and in stress responses and NO's interactions with other reactive compounds. Despite the long-lasting research efforts and the accumulating experimental evidences numerous questions are still needed to be answered, thus future challenges and research directions have also been drawn up.



Keywords: abiotic stress, nitric oxide, pathogen challenge, plant development, plant reproduction, symbiosis.

Abbreviations: AOX, alternative oxidase; Cys, cysteine; COX, cytochrome oxidase; CK, cytokinin; DAF-2 DA, 4,5-diaminofluorescein diacetate; DAF-FM DA, 4-Amino-5-methylamino- 2',7'-difluorofluorescein diacetate; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethyl urea; EDRF, endothelium-derived relaxation factor; ET, ethylene; GSH, glutathione; GSNO, *S*-nitrosoglutathione; GSNOR, *S*-nitrosoglutathione reductase; H_2, hydrogen gas; H_2S, hydrogen sulfide; HR, hypersensitive response;

Lbs, leghemoglobins; L-NAME, *N*(G)-Nitro-L-arginine methyl ester; L-NNA, N^G-nitro-L-arginine; LPS, lipopolysaccharide; MAP kinase, mitogen-activated protein kinase; N_2, nitrogen gas; NiR, nitrite reductase; NO, nitric oxide; NO_2, nitrogen dioxide, $NO^{+,}$ nitrosonium cation, NO^-, nitroxyl anion; NO_3^-, nitrate; nitrite, NO_2^-; N_2O, dinitrogen oxide; N_2O_3, dinitrogen trioxide; NOD, NO dioxygenase; NOFNiR, NO forming nitrite reductase; NO_2-FAs, nitro-fatty acids; eNOS, endothelial nitric oxide synthase; iNOS; inducible nitric oxide synthase, nNOS; neuronal nitric oxide synthase, nitrate reductase, NR; $ONOO^-$, peroxynitrite; Phytogbs1, Phytoglobins1; PTM, posttranslational modification; PTS, peroxisomal targeting signal; RLS, reactive lipid species; ROS, reactive oxygen species; RNS; reactive nitrogen species; SA, salicylic acid; SAR, systemic aqcuired resistance; Ser, serine; cGC, soluble guanylate cyclase; SHAM, salicylhydroxamic acid; SNAP, *S*-nitroso-*N*-acetylpenicillamine; SNO, *S*-nitrosothiol; SNP, sodium nitroprusside; SOD, superoxide dismutase; Thr, threonine; Trx, thioredoxin.

1. Introduction

The history of nitric oxide (NO) in biological systems is often dated back to the 1980s, when the acetylcholine-induced relaxation of the smooth muscle was shown to be dependent on the presence of endothelial cells (Furchgott, 1980). It was also found that endothelial cells release a chemical signal (endothelium-derived relaxation factor, EDRF) which appeared to be very labile. Further experiments revealed that EDRF is no other than the gaseous free radical, NO (Furchgott, 1987; Ignarro, 1987; Palmer *et al.,* 1987). From this remarkable finding, active research began to explore the synthesis, roles and signaling of NO especially in relation to cardiovascular and other human health issues. The scientific journal 'Science' assigned NO as the "Molecule of the Year" in 1992 and the discovery of NO as EDRF and revealing its signal interactions in the vasculature resulted in the award of the Nobel Prize in Physiology and Medicine in 1998 (Howlett, 1998). Meanwhile, indeed prior to this, research on NO in relation to plants was being carried out. The earliest studies examined NO as an air pollutant that comes into contact with aerial plant parts and influences physiological processes (Wellburn *et al.,* 1972; Benett and Hill, 1973; Taylor, 1974; Zeewart, 1976; Anderson and Mansfield, 1979; Caporn *et al.,* 1991; Morgan, 1992).

The intriguing fact that plants emit NO into their environment was first published 40 years ago by Lowell A. Klepper (Klepper, 1979; Fig 1). He based his studies on the observation that photosynthesis-inhibiting herbicides block light-dependent nitrite reduction, leading to the accumulation of nitrite in treated plant parts (Klepper, 1974). He applied two experimental systems: soybean leaf discs were floated on herbicide solutions and leaves were sprayed with solutions of herbicides such as 2,4-dichlorophenoxy acetic acid (2,4-D). Interestingly, from herbicide-treated leaves, NO emissions were 15 times higher than nitrogen-dioxide (NO_2) emissions, explained by the weaker water solubility of NO compared to NO_2. NO emissions could immediately be detected after treatment (with no lag period) and was directly proportional to applied herbicide concentrations. In addition, the ratios of NO emissions were closely related to the nitrite (NO_2^-) content of the leaf. In this milestone publication, Klepper (1979) revealed that herbicide-induced NO emission is dependent on the presence of light, as NO emissions were higher under dark conditions, but decreased rapidly in light, suggesting that light-dependent nitrite reduction eliminates nitrite as a substrate for NO emissions. The author mentioned that plants are able to bind and thereby eliminate nitric oxides (NO_x) from the atmosphere, while also being able to generate and emit these gases in case their metabolic balance is disturbed. In this early publication, Klepper already outlined a possible explanation for NO release from NO_2^-, but only further studies could explain that. Purging of nitrogen gas (N_2) during the *in vivo* nitrate reductase (NR) assay of soybean leaves also caused NO_x formation from accumulated NO_2^- implying the possibility that an enzymatic reaction was responsible for the NO evolution (Harper, 1981). In a further study, gas chromatography mass spectrometry (GC-MS) was applied to identify NO and dinitrogen oxide (N_2O) as dominant NO_x species; both originated from nitrate (NO_3^-) reduction in soybean leaves (Dean and Harper, 1986).

As seen from above, the NO concept in plant biology research has expanded over time. Initially NO gas was considered as an air pollutant and its effects on plants were primarily examined, but since 1979, NO was studied as an endogenous plant NO product and in some of these early publications plant NO emissions were linked to NR activity (Yamasaki 2000). Without exception, early studies were conducted on legume species (*Glycine ssp, Psophocarpus tetragonolobus, Neonotonia wightii, Pueraria ssp*), known to possess special nitrogen metabolism. However, a second phase of plant NO research was launched in 1996 (e.g. Millar and Day, 1996; Cueto *et al.,* 1996; Noritake *et al.,* 1996; Leshem and Haramaty, 1996), where experimental plant species were more diverse (e.g. soybean, lupine, potato, flowers, fruits, etc.), and methodological approaches were more novel (as detailed in thematic subchapters).

Apropos of the 40th anniversary of plant NO research, the aim here is to commemorate the milestone results of the past decades (Fig 1) and to discuss the developments and changes of concepts over time.

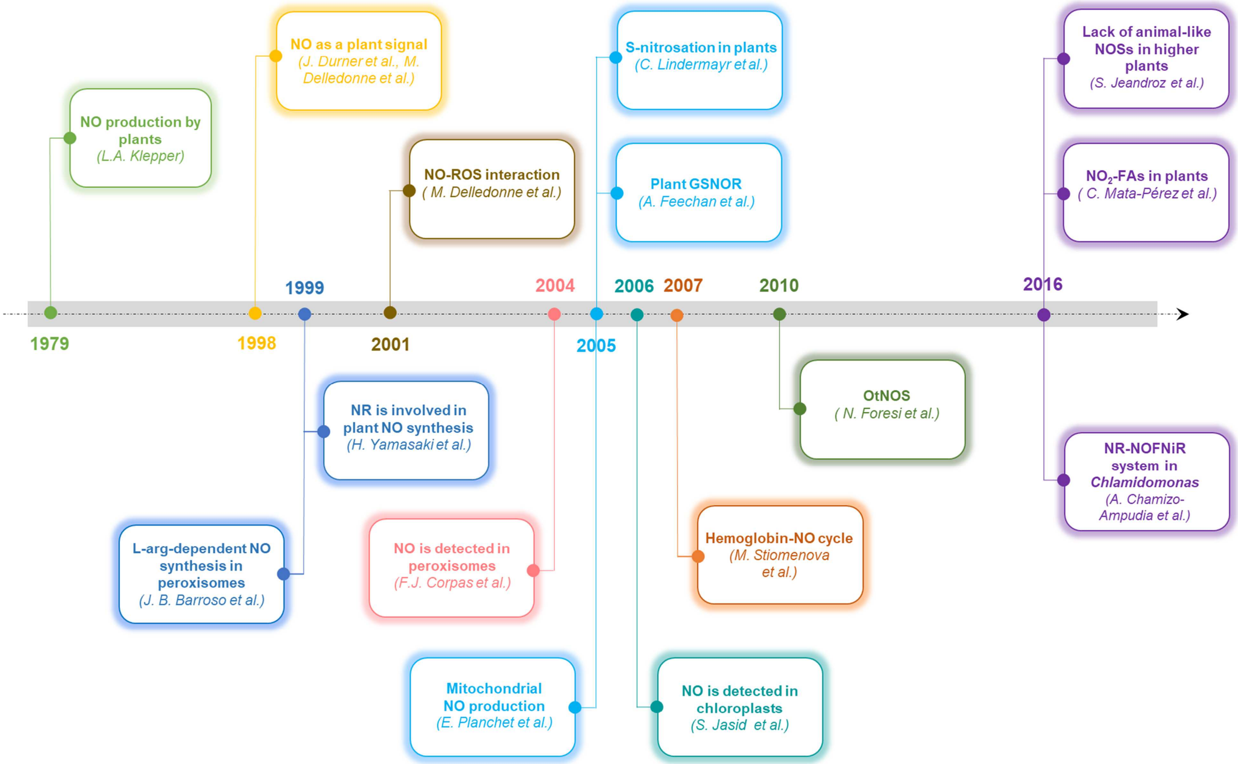


Fig 1 Milestone publications in the 40-year history of NO research. Nitric oxide production by plants was first described by Klepper (1979). Almost twenty years later, NO was identified as a signal in plant immunity by Durner *et al.* (1998) and Delledonne *et al.* (1998). One year later, peroxisomal L-arginine-dependent NO synthesis was published by Barroso *et al.* (1999). In the same year, the involvement of nitrate reductase (NR) in plant NO synthesis was also revealed (Yamasaki *et al.*, 1999). Soon, the interaction of NO with reactive oxygen species (ROS) was demonstated (Delledonne *et al.*, 2001). In 2004, the first evidence of peroxisomal NO production was provided by Corpas and coworkers. In the following year, the presence of *S*-nitrosated proteins was firstly published by Lindermayr *et al.* (2005) and also *S*-nitrosoglutathione reductase (GSNOR) enzyme was identified (Feechan *et al.*, 2005). In the same year, the involvement of mitchondrial NO production was also evidenced (Planchet *et al.,* 2005). Soon after, the hemoglobin-NO cycle regulating NO levels was discovered (Stoimenova *et al.,* 2007). Three years later, mammalian-like nitric oxide synthase (NOS) was characterized in *Ostreococcus tauri* (Foresi *et al.*, 2010). Recently, the NR-NOFNiR enzyme system was observed in *Chlamidomonas* (Chamizo-Ampudia *et al.*, 2016) and in the same year, the presence and the signaling role of nitro-fatty acids (NO-FAs) were evidenced in *Arabidopsis* (Mata-Pérez *et al.*, 2016). Furthermore, it was confirmed that land plants do not

possess typical animal NOSs in contrast to several algal species, suggesting that a loss of this gene during evolution (Jeandroz *et al.*, 2016).

2. Plant NO metabolism

One of the oldest and still hot topics in plant NO research is the synthesis and removal of this gaseous molecule. Historically, two enzymes are relevant in relation to plant NO synthesis: nitric oxide synthase (NOS) and nitrate reductase (NR).

2.1. Do plants possess NOS?

NOS represents one of a few heme-containing enzymes producing NO. It is active as homodimers, catalysing the synthesis of NO and citrulline from L-arginine (L-arg) *via* the intermediate N-hydroxy-L-arg (Bredt and Snyder 1990; Stuehr *et al.,* 2004). Mammals possess three NOS isoforms encoded by three distinct genes: neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). Both nNOS and eNOS are constitutive and are involved in signaling processes. iNOS is controlled at the transcriptional level and is classically related to immune responses/inflammation. NOS is present in many life forms (Santolini *et al.*, 2019) and its role in catalysing NO synthesis in plants was mooted in the 1990s. Several studies reported the measurement of NOS activities in plant tissue, cellular and organellular extracts. Two publications reported the identification of candidate enzymes catalyzing this activity in land plants. None of these proteins had similarity to animal NOS isoforms. The first candidate was identified as a variant of the P protein of the glycine decarboxylase complex (Chandok *et al*., 2003). However, it turned out that the recombinant *A. thaliana* variant P protein had no NO-synthesizing activity, thus questioning the reliability of the data. The manuscript was later retracted (Klessig *et al*., 2004). The second candidate, named AtNOS1, was identified in *A. thaliana* based on its similarity with a protein associated to NO synthesis in the snail *Helix pomatia* (Guo *et al*., 2003). The corresponding T-DNA mutants showed a reduced production of NO, both constitutively and in response to abscisic acid (ABA). However, doubts about the capacity of this protein to display a NOS activity have been further raised. In particular, Zemojtel *et al*. (2006) failed to reproduce the NOS activity of AtNOS1 reported by Guo *et al*. (2003) and demonstrated that animal homologues of AtNOS1 did not display such activity. Accordingly, it was shown later that in contrary to NOS, AtNOS1 neither binds nor oxidizes arginine to NO and, rather, displays a GTPase activity (Moreau *et al.,* 2008). The protein was renamed NO-associated protein 1 (AtNOA1) but the original publication of Guo *et al*. (2003) was not retracted. More generally, the specificity of the measurement of NOS activity and of NOS inhibitors in plants has also been questioned (Rasul *et al.*, 2012; Tischner *et al.,* 2007; Butt *et al.,* 2003). Furthermore, the plant genomes sequenced so far did not reveal any sequences encoding NOS. Overall, the title of Fröhlich and Durner's paper (2011) "The hunt for plant nitric oxide synthase (NOS): Is one really needed?" summarized the situation well.

A recent investigation clarified this never-ending debate. Using the transcriptome database generated by the 1000 plants (1KP) international multidisciplinary consortium, as well as publicly available plant genomes, Jeandroz *et al.* (2016) searched for the presence of sequences showing identity with human nNOS in over 1300 species. No NOS homologs were found in the genomes and/or transcriptomes of land plants. A similar conclusion was recently reached by Hancock and Neill (2019) also searching for plant proteins homologous to mammalian NOS using a bioinformatic approach. Nevertheless, 15 typical NOSs were found in the 265 algal species screened (Jeandroz *et al.*, 2016). This data confirmed the pioneer work of the group of L. Lamattina (Foresi *et al.*, 2010; Fig 1, Foresi *et al.,* 2015) who characterized a functional NOS in the green alga *Ostreococcus tauri*. Most of these NOSs were distributed in green algae but, surprisingly, did not correspond to phylogeny. In term of structure, these enzymes display classical NOS features with both the N-terminal Oxy and C-terminal Red domains. The presence of a functional CaM-binding site was more questionable.

Further *in silico* structural analyses on candidate algal NOSs (Santolini *et al.*, 2017) revealed that compared to their mammalian counterparts, the algal NOSs present singularities such as the absence of the N-terminal hook and the Zn/S cluster motif involved in the homo-dimer interface. Furthermore, the presence of residue inserts and the substitution of residues involved in key NOS properties (such as NO release at the end of the catalytic process and H_4B binding) were also noticed. These particularities suggest that these proteins might not be genuine NOSs but could display original biochemistry and functions. Accordingly, the recombinant *O. tauri* NOS is characterized by an ultrafast NO-producing capacity as compared to mammalian NOSs (Weisslocker-Schaetzel *et al.,* 2017).

The studies briefly summarized here indicate that land plants do not possess typical NOS and, inevitably, raise the question of the enzymatic processes underlying the L-arg-dependent NO synthesis measured in those organisms. It should be noted that model animals are able to survive without NOS; however, the total absence of all NOS isoforms is associated with a variety of disorders, as demonstrated in the triple NOSs null mice suffering from metabolic as well as malfunctions of cardiovascular, renal, lung and bone tissues (Tsutsui *et al.,* 2015). Beside the NOS-catalyzed oxidative pathway of NO production from L-arginine, reductive pathways of NO production from nitrate/nitrite have been recognized as universally present mechanisms contributing with a similar importance to the regulation of NO levels in eukaryotic cells, mediated namely by Mo-containing enzymes such as sulphite, xanthine and aldehyde oxidases in animals or nitrate reductase in plants (reviewed in Bender and Schwartz, 2018).

2.2. NR role in plant NO synthesis

In addition to NOS, the other widely-researched enzyme related to NO synthesis is NR which is a well characterised enzyme involved in plant nitrogen metabolism. This cytosolic enzyme converts nitrate to nitrite by transferring two electrons from NAD(P)H to nitrate (Planchet *et al.,* 2005, Fig 1). Nitrite is further reduced to ammonium in plastids by plastidial nitrite reductase (NiR). NR uses molybdopterin, heme and FAD as cofactors (Hageman 1990). In *Arabidopsis,* NR is encoded by two genes *NIA1* and *NIA2.* Early evidence suggested that NR is involved in NAD(P)H-dependent reduction of nitrite to NO (Yamasaki *et al.*, 1999; Rockel *et al.*, 2002). NR affinity towards nitrite is low (K_m ~ 100 µM), and considering the average concentration of nitrite in plant tissues (of the µM order), nitrite is a limiting factor for NO production (Planchet *et al.,* 2005). Under standard conditions the nitrite reduction is 1% of total NR activity (Rockel *et al.*, 2002) suggesting a minor portion of activity contributes to NO production.

Since the early 2000s, there has been a growing number of studies reporting involvement of NR-dependent NO in biotic and abiotic plant stress respones. Under hypoxic conditions cytoplasmic acidosis takes place due to increased fermentation. Under such conditions NiR is inhibited (Kaiser and Brendle-Behnisch, 1995), leading to increased nitrite and concomitant NO production. Accordingly, an antisense line of NiR in tobacco (*Nicotiana tabacum*) generated NO constitutively (Planchet *et al.,* 2005). Nitrite produced has beneficial roles in reducing cytoplasmic acidosis (Allègre *et al.*, 2004; Libourel *et al.,* 2006). Thus, transgenic tobacco plants with low root NR activity were more sensitive to root anoxia (Stoimenova *et al.*, 2003). Under hypoxia, feeding plants with ammonium as an N source caused inhibition of NR activity, NO production and reduced ATP, suggesting a role for NR-dependent NO in hypoxia and anoxia tolerance (Wany *et al.,* 2019). Lillo *et al.* (2004) demonstrated that post-translational regulation of nitrate reductase plays a role in NO production. NR-kinase phosphorylates a conserved serine residue and enabling NR to bind to 14-3-3 proteins. NR then becomes inactive and is subjected to proteolytic degradation (Kaiser and Huber, 2001). The mutation in NR phosphorylation site results in high nitrite accumulation and NO emission (Lea *et al.*, 2004). Some recent evidence indicated that the key process of NO synthesis indirectly involves the activity of NR. The NR enzyme transfers electrons from NAD(P)H to the NO forming nitrite reductase (NOFNiR) which catalyses the reduction of nitrite to NO *in vitro* and *in vivo* (Chamizo-Ampudia *et al.,* 2016; Fig 1). This observation was made in *Chlamydomonas* but authors suggest that the NR-NOFNiR system can be a relevant NO source also in higher plants (Chamizo-Ampudia *et al.,* 2017). In addition to its role in hypoxic responses, important discoveries revealed that NR-dependent NO plays a role in plant development and various stress responses. Indeed, Desikan *et al.* (2002) found that NR-mediated NO is essential for ABA-induced stomatal closure in *Arabidopsis*. Application of ABA to epidermal peels led to rapid NO synthesis and stomatal closure. The NR double mutant *nia1nia2* that fails to synthesize NO does not respond to exogenous ABA, whereas the stomata of this mutant responded to exogenous NO, suggesting an important role of this enzyme in stomatal function. NR-dependent NO also plays a role in auxin-induced NO production (Kolbert *et al.,* 2008), floral transition (Seligman *et al.*, 2008), root hair development (Lombardo and Lamattina, 2012) and stem cell homeostasis (Wany *et al.,* 2018). Either using pharmacological suppression of NR-dependent NO, or by using a *nia1nia2* mutant, it was demonstrated that NR-dependent NO plays a role in freezing, cold and osmotic and hypoxic tolerance (Zhao *et al.,* 2009; Kolbert *et al.,* 2010; Xie *et al.*, 2013; Blokhina and Fagerstedt, 2010). Recently, it was demonstrated that down-regulation of NR-dependent NO causes stabilization of *ERF-VII* group transcription factors in response to multiple abiotic stresses (Vicente *et al.,* 2017). NR-dependent NO also plays a role in induction of antioxidant metabolism to increase plant tolerance to stress (Sang *et al.,* 2008). Finally, NR was shown to be involved in *Pythium* and *Phytophthora* elicitor-induced NO production (Yamamoto-Katou *et al.,* 2006), *Trichoderm*a induced NO production (Gupta *et al.,* 2014) and in *Pseudomonas* induced hypersensitive response in tobacco (Gupta *et al.,* 2012; Modolo *et al.,* 2005) and *Verticillium dahlia* induced NO production (Shi *et al.,* 2008). It was shown that NR is required for transcriptional modulation and bactericidal activity of NO during defense against pathogenic *Pseudomonas syringae* (Vitor *et al.*, 2013).

As seen from above, the mechanism of NO synthesis by NR has been characterized which was followed by biochemical and genetic studies revealing the role of NR-associated NO synthesis in plant development and stress responses. According to the newest findings, the involvement of NR in NO synthesis seems to be indirect.

Meanwhile, NO synthesis has also started to be investigated at the subcellular level and further, the mitochondrion, peroxysome and chloroplast seem to have prominent roles in relation to NO production.

2.3. NO production from mitochondria

Mitochondria are one of the sources for NO production. Nohl *et al.* (1999) first discovered that mammalian mitochondria recycle nitrite to NO at Complex III (bc1 complex), an activity sensitive to the Complex III inhibitor myxothiazol (Kozlov *et al.,* 1999) which inhibits the reduction of Complex III from UQH_2, leading to the formation of ubisemiquinone anion which then reacts with nitrite to form NO (Gupta *et al.,* 2018). In plants, the involvement of mitochondria in NO production was first reported by Tischner *et al.* (2004). These authors demonstrated that *Chlorella sorokiniana* is able to generate NO under anoxic conditions when supplied with nitrite. Interestingly these authors found that ammonium grown *Chlorella sorokiniana* produce NO which is sensitive to the alternative oxidase (AOX) inhibitor salicylhydroxamic acid (SHAM), suggesting that AOX also plays a role in nitrite-dependent NO production under anoxia. Planchet *et al.* (2005) demonstrated that isolated mitochondria from tobacco cell suspensions are able to generate NO from nitrite. Gupta *et al.* (2005) reported that isolated root mitochondria from pea, barley and tobacco are able to reduce nitrite to NO *in vitro* and *in vivo*, and that myxothiazol and SHAM inhibit this production. Interestingly, it was found that potato and cauliflower mitochondria produce much less NO. Gupta *et al.* (2005) found that oxygen is inhibitory for nitrite-dependent NO production (with Ki_{O2}= 0.05% and $K_{m\ nitrite}$ = 175 µM). Later, Stoimenova *et al.* (2007) demonstrated that rice and barley root mitochondria, under anaerobic conditions, have the capacity to use nitrite as an electron acceptor to oxidize cytosolic NAD(P)H and generate NO. In *Medicago truncatula* root nodules under hypoxia, NO production was increased by nitrite addition and inhibited by myxothiazol and antimycin A, indicating that nodule mitochondria participate in NO production at the expense of nitrite (Horchani *et al.,* 2011).

Recently it was demonstrated that nitrite reduction to NO helps in the protection of mitochondrial structure and function (Gupta *et al*., 2017). Nitrite addition to anoxic mitochondria leads to increased NO and reduced ROS levels, lipid peroxidation, along with increased ATP. Nitrite-dependent NO also plays a role in formation of super complexes of mitochondria. In contrast, under hypoxia the mitochondria are scavengers of NO under normoxia. Alber *et al.* (2017) found that under normoxia, inhibition of Complex III led to increased NO production. Electron pressure in Complex III results in the generation of NO while AOX removes excess NO under normoxia (Cvetkovska *et al.,* 2014). Cytochrome oxidase (COX; Complex IV) is also involved in NO production. The addition of KCN to isolated mitochondria leads to inhibition of nitrite dependent NO (Stoimenova *et al.,* 2007; Planchet *et al.,* 2005). In animal systems, the mechanism of NO production by COX under anoxia was shown to be linked to oxidation of iron by nitrite after its binding at the fully reduced $Fe_{a3}Cu_B$ centre (Castello *et al.,* 2006). In plants, the mechanism remains to be demonstrated.

2.4. Enzymatic NO generation in peroxisomes and chloroplasts

Although the enzymatic NO source in higher plant cells is still controversial (Astier *et al.,* 2018), there are accumulating data which indicate that some organelles have endogenous NO generation dependent on either L-arginine (oxidative pathway) or nitrate/nitrite (reductive pathway).

Peroxisomes are single-membrane bound organelles that have a versatile metabolism sharing different metabolic pathways with chloroplasts, mitochondria or lipid bodies such as photorespiration, glyoxylate cycle or β-oxidation. In fact, these organelles establish physical contact to facilitate the metabolic interchange amongst themselves (Oikawa *et al.,* 2015; Cui *et al.,* 2016). Plant peroxisomes were found to have an active ROS metabolism and consequently a prominent oxidative metabolism. Besides this, these organelles have the enzymatic capacity to generate NADPH, an essential electron donor in NO generation by animal NOS isoenzymes.

In this context, using isolated leaf peroxisomes from pea plants and based on the reaction catalyzed by animal NOSs (L-arginine + 2 NADPH + 2 $O_2 \longrightarrow$ L-citrulline + NO + 2 NADP + H_2O) the assay of NOS activity monitoring the generation of L-[^{3}H]citrulline provided a NOS-like activity which required Ca^{2+} and which was strictly dependent of NADPH as an electron donor (Barroso *et al.,* 1999, Fig 1). Consequently, this was the first plant organelle where the putative presence of NOS-like activity with similar requirements and inhibitor sensitivity to animal NOS was reported (Table 1). Although not in plants, from this first report, two further papers demonstrated the presence of an iNOS in peroxisomes from rat hepatocytes whose protein expression increased under sepsis conditions (Stolz *et al.,* 2002; Loughran *et al.,* 2005), supporting the notion that such organelles contain this enzymatic activity.

However, doubts were raised about this finding due to the inexistence of a plant gene encoding a typical animal NOS in the Arabidopsis Genome Initiative published in 2000, and the unspecificity of the determination method of the NOS activity based on L-citrulline metabolism, since it was reported that L-citruline could be also generated by chloroplastic ornithine transcarbamylase through the L-Arg biosynthesis pathway (de Ruiter and Kollöffel, 1985). Therefore, further work was required and a year later, using ozone chemiluminiscence approach to determine direct NO generation instead of L-citrulline, a NOS-like activity strictly dependent on NADPH, calcium, calmodulin, and BH_4 was reported in isolated leaf peroxisomes (Corpas *et al.,* 2004). It was also found that this peroxisomal NOS-like activity was downregulated (72%) during natural senescence of pea leaves. Additionally, the presence of NO was corroborated by other techniques: EPR spectroscopy using the spin trap $Fe(MGD)_2$ and fluorometric analysis with DAF-2 DA (Corpas *et al.*, 2004, Fig. 1). These data provided further clear evidence of L-Arg dependent-NOS like activity in plant peroxisomes.

Moreover, it has been demonstrated that the import of proteins responsible for plant peroxisome NO generation has a peroxisomal targeting signal (PTS) type 2 and that this import is dependent on Ca^{2+} and calmodulin (Corpas and Barroso, 2014b, 2018). So far, there is no evidence for alternative NO sources in peroxisomes, either enzymatic (i.e. nitrate reductase or xanthine oxidoreductase) or non-enzymatic. All-in-all, the available data support that plant peroxisomes have an active nitro-oxidative metabolism which is modulated under physiological and stress conditions (Corpas and Barroso, 2017b).

The chloroplast is exclusive for plant green tissues and it has an active reactive oxygen species (ROS) metabolism as a consequence of the photosynthetic activity. One of the first lines of evidence regarding the production of NO in chloroplasts was reported by Cooney *et al.* (1994) based on the non-enzymatic light-dependent conversion of NO_2 to NO by the participation of carotenoids. However, stronger evidence of the NO production in chloroplasts came from the analyses of purified chloroplasts from soybean leaves (Jasid *et al.,* 2006, Fig. 1) using EPR spectroscopy with the spin trap $Fe(MGD)_2$. Unlike peroxisomes, data provided solid evidence of two potential sources in chloroplasts: from arginine and in a nitrite-dependent manner. As part of the characterization of the L-Arg-dependent NO generation, it was found that the NO production was inhibited by typical inhibitors of animal NOS (L-NAME or L-NNA), depended on NADPH as electron donor, but it was independent of calcium and calmodulin (Jasid *et al.,* 2006). The presence of NO in chloroplasts has been also observed by confocal laser microscopy *in vivo* soybean cotyledons using a NO specific fluorescence probe (DAF-FM DA). The NO signal in chloroplast was significantly affected by different herbicides such as 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU) and paraquat (methyl viologen) (Galatro *et al.,* 2013). Other reports have provided some controversial data supporting that chloroplast NO is exclusively generated from L-Arg (Tewari *et al.*, 2013). Moreover, the application of exogenous NO showed that chloroplast functions are also significantly affected by this gas (Misra *et al.,* 2014). For instance, NO released from the donor molecule SNAP affected the function of chloroplasts through the inhibition of photophosphorylation (Takahashi and Yamasaki, 2002).

Table 1 provides a summary of the main requirements of the enzymatic systems responsible of the endogenous NO in peroxisomes and chloroplasts.

Table 1. Summary of the biochemical requirements of the NO producing enzymatic sources in plant peroxisomes and chloroplasts.

Organelles	NO generation (nmol NO · min^{-1} · mg^{-1} prot)	Cofactors	Inhibitors	Reference
Peroxisomes				
L-Arg dependent	5.6 $^{(a)}$ 4.9$^{(b)}$	NADPH, Ca^{2+}, CaM, FMN, FAD, BH_4	Aminoguanidine L-NMMA, L-NAME, thiocitrulline	Barroso *et al.,* 1999; Corpas *et al.,* 2004
Chloroplasts				
L-Arg dependent	0.76$^{(c)}$	NADPH	L-NAME, L-NNA	Jasid *et al.,* 2006
Nitrite dependent	3.2 $^{(c)}$	-	DCMU	Jasid *et al.,* 2006

$^{(a)}$Arginine-citrulline assay. $^{(b)}$Ozone chemiluminiscence assay. $^{(c)}$ Spin trapping EPR assay. DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethyl urea. (L-NAME, Nω-nitro-L-Arg methyl ester hydrochloride.

L-NMMA, Nω-Methyl-L-Arg acetate salt. L-NNA, Nω -nitro-L-Arg.

2.5. NO scavenging through the Phytoglobin-NO cycle

Non-symbiotic hemoglobins are class 1 hemoglobins. These are known as Phytoglobins1 (Phytogbs1, Hill *et al.*, 2016) and have a very high affinity to oxygen. Phytogb1, first described by Taylor *et al.* (1994), was shown to be up-regulated under hypoxia as well as in response to low ATP and nitrate (Nie and Hill, 1997). Later it was found that NO is an inducer of Phytogb expression (Ohwaki *et al.,* 2005). Phytogbs1 are scavengers of NO using traces of oxygen (Igamberdiev *et al.,* 2004a; 2006) with a K_m value of 2 nM (Smagghe *et al.,* 2009). This is at least two orders of magnitude lower than required for the saturation of COX (Gupta and Igamberdiev, 2011), hence this biochemical property permits Phytogb1 to scavenge NO at low oxygen content. Under hypoxic conditions, nitrite reduction occurs at Complex III, Complex IV and AOX sites (Gupta *et al.,* 2018) with subsequent NO crossing the membranes and diffusing into the cytosol (Stoimenova *et al.,* 2007). Oxygenated

Phytogb1 converts NO to nitrate and becomes metPhytogb1, while this protein is subsequently reduced by methemoglobin reductase (Igamberdiev *et al.,* 2006). The nitrate generated becomes a substrate for NR leading to formation of nitrite which then enters in mitochondria to become a substrate for NO production. This nitrate-nitrite-NO recycling is called the Phytoglobin-NO '(Phytogb-NO) cycle. Operation of this cycle leads to the production of limited amount of ATP (Stoimenova *et al.,* 2007, Fig 1). Under hypoxic conditions energy becomes depleted so the Phytogb-NO cycle can contribute to anoxic ATP formation, together with fermentation (Gupta and Igamberdiev, 2011). This cycle becomes important for reoxidation of accumulated NAD(P)H under hypoxia, and helps maintenance of NADH/NAD$^+$, NADPH/NADP$^+$ and ATP/ADP ratios (Igamberdiev *et al.,* 2004b). Horchani *et al.* (2011) reported that this cycle plays a role in generation of ATP in N_2-fixing nodules. Interestingly, it was found that both plant and bacterial electron transport chains participate in the production of NO through the operation of the Phytogb-NO cycle in plant cells, and of the denitrification pathway in bacteroids (Horchani *et al.,* 2011). In the nucleus, Phytogbs are thought to be maintained in the functional (ferrous) form by reduced flavins that are abundant in this compartment (Sainz *et al.,* 2013), possibly facilitating their potential role in the control of NO-regulated gene expression. In the context of plant immunity, this could occur through either the well-established route of Non-expresser of Pathogenesis-Related 1 genes (NPR1) and TGACG sequence specific binding protein1 (TGA1) (Tada *et al.,* 2008; Lindermayr *et al.,* 2010) and/or the more recently identified system of *S*-nitrosothiol Regulated Gene1 (SRG1) (Cui *et al.,* 2018). Recently Martinez-Medina *et al.* (2019) demonstrated that tight regulation of NO and PHYTOGB1 (class 1 hemoglobin) plays a role in plant mycorrhizal interaction. They showed that overexpression of PHYTOGB1 leads to increased AM colonization and that PHYTOGB1 can be regulated via NO concentration.

3. Plant NO signaling

Despite an increasing tranche of data implicating a role for NO in numerous plant cellular processes during the early 2000s, the associated molecular mechanism(s) linked with NO bioactivity remained obscure. In mammals, NO produced by NOS had been shown to promote the activity of soluble guanylate cyclase (sGC), through NO binding to the prosthetic heme (Wedel *et al.,* 1994; Stone and Marletta, 1994).

Subsequently, NO-activated mammalian sGC produced the intracellular messenger, cGMP, whose effects are mediated by cGMP-dependent protein kinases and cGMP-regulated ion channels (Russwurm and Koesling, 2004) integral to physiological processes like smooth muscle relaxation (Waldman and Murad, 1987; Moncada *et al.*, 1991). Further, this signal could be diminished by cGMP degrading phosphodiesterases (Mullershausen *et al.,* 2001). However, while a plant protein with potential GC activity was reported, there was no associated heme domain (Ludidi and Gehring, 2003). Thus, in the early 2000s no plant homologues of mammalian NO-modulated sGCs and cGMP phosphodiesterases had been identified and this continues to be the case (Jeandroz *et al.,* 2016; Astier *et al*., 2019). Thus, despite low levels of cGMP being detected in plants and exogenous cGMP application and constitutive accumulation of cGMP in GC overexpressing *Arabidopsis* being implicated in a number of plant processes (Durner *et al.,* 1998; Pagnussat *et al.,* 2003; Szmidt-Jaworska *et al.,* 2004, Hussain *et al*., 2016), a sGC-cGMP-dependent route for the transfer of NO bioactivity appears unlikely. Accordingly, a bio-informatic search for components of the prototypic NO/cGMP cascade found in animals (eg. sGC, cGMP-dependent protein kinases, cyclic nucleotide-gated channels and cGMP phosphodiesterases) in over 1000 plant species strongly supports the possibility that plants do not mediate NO signalling through this signalling module (Astier *et al*., 2019).

Therefore, how might NO-based signals be conveyed in plants? Further clues to this conundrum were again available from mammalian studies: in a ground-breaking paper, Stamler and Loscalzo (Stamler *et al.,* 1992) identified a process termed *S*-nitrosylation. This redox-based, post-translational modification (PTM) encompassed the covalent attachment of NO to the sulphur of a rare, highly reactive protein cysteine (Cys) thiol (S-H) forming an *S*-nitrosothiol (*S*NO) (Stamler *et al.,* 1992), with the biochemical properties of sulphur perfectly facilitating this process (Umbreen *et al.*, 2019). This modification was subsequently shown to regulate protein structure in an allosteric fashion modulating protein function (Kim *et al.,* 2002). This mechanism to convey NO bioreactivity was therefore independent of sGC activity and subsequent downstream signaling. It should be mentioned that although *S*-nitrosylation has been extensively used in plant research, the term *S*-nitrosation is chemically more precise (see Heinrich *et al.*, 2013).

During the mid-2000s a number of key papers demonstrated, for the first time, that plant proteins could also be *S*-nitrosylated *in vitro* (Lindermayr *et al.,* 2005; Fig 1, Lindermayr *et al.,* 2006) and *in vivo* (Perazzolli *et al.*, 2004; Tada *et al.,* 2008; Wang *et al.,* 2009). For example, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) enzyme activity was regulated by addition of NO donors to plant extracts (Lindermayr *et al.,* 2005) and salicylic (SA) binding and carbonic anhydrase activity was controlled by *S*-nitrosation of SABP3 *in vivo* (Wang *et al.*, 2009). Collectively, these findings established that NO bioactivity could also be conveyed by the *S*-nitrosation of proteins in plants and further, this redox-based, post-translational modification could directly modulate protein function.

A key feature of cellular signaling networks is an associated mechanism to terminate the transduction process when appropriate, to ameliorate the chances of excessive activation of target processes. Thus, the next fundamentally important question was how might NO signaling through *S*-nitrosation be curtailed? Glutathione (GSH), a major cellular antioxidant (Schafer and Buettner, 2001), had been found to react with NO in mammalian cells to form a compound termed, *S*-nitrosoglutathione (GSNO) through *S*-nitrosation (Gaston *et al.,* 1993). GSNO could therefore act as a reservoir of NO bioactivity, by functioning as a natural NO donor driving protein *S*-nitrosation (Corpas *et al.,* 2013*a*). The content of GSNO in plants is thought to be in the low nmol range, based on the determination of low molecular weight SNOs (Feechan *et al.,* 2005, Fig 1). A landmark paper in 2002 characterized an enzyme in plants, termed *S*-nitrosoglutathione reductase (GSNOR), that could control the GSNO content and subsequently the global *S*-nitrosation levels *in planta* (Sakamoto *et al.,* 2002). GSNOR enzyme is now appreciated as a highly conserved master regulator of NO signaling (Spadaro *et al.,* 2010; Leterrier *et al.,* 2011, Jahnová *et al.*, 2019). Loss-of-function mutations in GSNOR increased global *S*-nitrosation and compromised multiple modes of plant disease resistance. Conversely, mutations that resulted in overexpression of GSNOR led to decreased global *S*-nitrosation and enhanced, broad-spectrum disease resistance. Importantly, these findings provided the first genetic evidence for NO function in plants, uncovered a key *in vivo* role for *S*-nitrosation in the transfer of NO bioactivity and provided a mechanism that could diminish NO signaling indirectly, by turning over GSNO. Subsequently, two forward genetic screens identified a central role for GSNOR in plant adaptation to high temperatures and in herbicide resistance (Lee *et al.*, 2008; Chen *et al.*, 2009), further expanding our appreciation of *S*-nitrosation signaling functions.

The race was then on to identify the protein targets of *S*-nitrosation that underpin NO signaling in a diverse range of plant cellular processes. This was supported by advances in protein mass spectrometry and critically by the biotin-switch technique (Jaffrey and Snyder 2001), which enabled protein SNOs to be replaced with a biotin tag, facilitating purification of the labelled proteins by streptavidin columns/beads and their subsequent identification by MS. This approach led and continues to lead to an increasing catalogue of *S*-nitrosated proteins implicated in a diverse set of environmental and developmental responses (Abat and Deswal, 2009; Wang *et al.*, 2009; Fares *et al.,* 2011; Kwon *et al.,* 2012).

The next key step was to ascribe *S*-nitrosation at a given Cys to a specific biological function. In this context, studies uncovered a role for SNO formation at Cys260 and Cys266 of the transcription factor, TGA1, in the regulation of SA signaling required to establish systemic acquired resistance (SAR) (Lindermayr *et al.,* 2010). Also, *S*-nitrosation of the transcriptional co-activator, NPR1, at Cys156 promoted NPR1 oligomer formation, reducing the translocation of NPR1 monomer to the nucleus and the associated activation of SAR (Tada *et al.,* 2008). Furthermore, *S*-nitrosation of the NADPH oxidase responsible for the pathogen-induced oxidative burst, respiratory burst oxidase homologue D (RBOHD), was found to be at Cys890. This specific PTM was found to reduce RBOHD generated ROS curbing the extent of hypersensitive response (HR) cell death development at the late stages of the plant immune response. Interestingly, this mechanism was found to be conserved across phylogenetic kingdoms (Yun *et al.,* 2011). More recently, in an elegant study, *S*-nitrosation of GSNOR has been shown to induce the selective autophagy of this enzyme during hypoxia. *S*-nitrosation of GSNOR at Cys10 induces a conformational change, exposing an AUTOPHAGY-RELATED8 (ATG8)-interacting motif accessible to the autophagy machinery. Upon binding by ATG8, GSNOR is recruited into the autophagosome and degraded in an AIM-dependent manner (Zhan *et al.,* 2018). Collectively, these findings show that SNO formation at specific Cys residues of target proteins regulates distinctive biological processes, providing selective routes for NO signaling.

An important feature of cell signaling systems is the direct reversal of the modulating PTM. For example, in phosphorylation signal cascades, phosphatase enzymes remove a phosphate moiety from a serine (Ser) or threonine (Thr) residue previously modified by a Ser/Thr kinase (Johnson and Barford, 1993). Building on previous studies from mammals (Benhar *et al.,* 2008), Thioredoxin (Trx) h5 was identified as a specific de-nitrosylase for a subset of plant proteins, including NPR1 (Kneeshaw *et al.*, 2014). Thus, at least two distinct strategies have evolved in plants to terminate NO-mediated signaling via *S*-nitrosation: (1) indirect turnover of the NO reservoir, GSNO, by GSNOR (Feechan *et al.,* 2005; Lee *et al.,* 2008; Chen *et al.,* 2009; Malik *et al.,* 2011) and (2) direct, selective protein de-nitrosylation by Trxh5 (Kneeshaw *et al.,* 2014).

Future challenges in this increasingly important area, include understanding how *S*-nitrosation might interface with other PTM mechanisms. In this context, exciting research has revealed that SNO formation might function as an important regulator of the plant epigenetic machinery. Two plant histone deacetylases (HDT2 and HDT3), which function as “erasers” of epigenetic marks, have been identified as targets of *S*-nitrosation (Chaki *et al.,* 2015) and nuclear histone deacetylase activity was found to be inhibited by exogenous GSNO (Mengel *et al.,* 2017). In addition, SUMO conjugating enzyme 1 (SCE1) is thought to be *S*-nitrosylated *in vivo* to regulate plant immune function. This also provides a novel strategy to control this PTM: the modulation of global SUMOylation levels, this is distinct from previous well-established mechanisms that operate at a local level, to regulate the addition of SUMO to a single target protein (Skelly *et al.,* unpublished data). The mechanisms underpinning the signaling specificity of SNO formation also warrant further attention. Surprisingly, the emerging evidence suggests that GSNO and NO have genetically additive functions. Thus, these two related redox signaling molecules may have both distinct and shared protein targets (Yun *et al.,* 2016). Therefore, over the history of NO plant biology, *S*-nitrosation has emerged as the prototypic, NO-based, PTM, serving to stabilise and diversify NO-dependent signals, supporting ubiquitous signaling networks targeting a plethora of plant proteins. However, there are many key outstanding questions, beyond the scope of this review, that urgently need to be addressed. Consequently, exciting times lie ahead for this important redox-based PTM, which is becoming increasingly appreciated as a central regulator of key plant cellular processes.

4. NO bioactivity in plants

With 40 years of research behind us, we can confidently state that NO is a multifunctional regulator in plant cells. It influences plant growth and development, and also regulates various plant environment responses.

4.1. Nitric oxide in vegetative growth, development and hormonal interactions

The first evidence for the growth regulating effect of NO was published more than 30 years ago. Then Leshem and Haramaty (1996) revealed the simultaneous release of NO and ethylene (ET) during pea leaf senescence. In addition, depending on its applied concentration, NO mitigated stress or inhibited leaf growth. The beneficial action of low NO concentrations was explained by its reducing effect on ET levels, which was the first evidence of an NO-phytohormone interaction (Leshem and Haramaty, 1996). These early results raised the possibility of using NO in postharvest management (Leshem *et al.,* 1998; Leshem and Wills 1998) and substantiated further research of practical significance (e.g. Lai *et al.*, 2011; Kang *et al.,* 2016; Hao *et al.,* 2016). The concentration-dependent effect of NO on growth was confirmed by its induction of corn root elongation (Gouvea *et al.,* 1997). It was also found that NO promotes de-etiolation but inhibits hypocotyl elongation in lettuce (Beligni and Lamattina, 2000). Remarkably, NO was also found to be involved in salicylic acid (SA)-associated processes, since NO induced SA-dependent gene expression in tobacco (Durner *et al.*, 1998, Fig 1). It was also discovered that NO mimics the effect of cytokinin (CK) on betalain accumulation in the *Amaranthus* system and NOS inhibitors prevent CK action (Scherer and Holk, 2000); however, these findings were later questioned by Romanov *et al.* (2008). The effect of CK treatment on NO formation was described a year later by Tun *et al.* (2001). Based on the early discovered overlaps between the actions of NO and plant hormones (ET, CK), the question has arisen whether NO could be considered as a phytohormone (Beligni and Lamattina, 2001a,b; Shapiro, 2005; Lamattina and Polacco, 2007). Since the signal function of NO is independent of specific receptors and the range of its effective concentration is higher than those of established phytohormones, presently we do not consider NO as a classic hormone. Rather, NO may function as a non-traditional growth regulator that acts in combination with traditional phytohormones during growth and development.

The research group of L. Lamattina contributed greatly to the exploration of NO’s role in root development. As recently reviewed by Del Castello *et al.* (2019), between

2002 and 2008, numerous associated studies revealed the role of NO in adventitious root, lateral root and root hair development. Around this time, the role of NO in gravitropic bending (Hu *et al.,* 2005) as well as in xylem differentiation (Gabaldón *et al.,* 2005) had been clarified. All of the above-mentioned studies have been conducted on crops such as soybean, pea, tomato, maize, lettuce or cucumber and used biochemical approaches, meaning that the effect of modified endogenous NO levels were observed. In 2003, the characterization of the first *Arabidopsis* mutant (*Atnos1:* later renamed *Atnoa1*) with modified NO levels revealed that insufficient NO content results in deficient root, shoot and inflorescence development (Guo *et al.,* 2003). However, as discussed above, some years later Moreau *et al*. (2008) showed that AtNOA1 protein is not an NOS but a GTPase with a pleiotrophic phenotype including diminished NO production. Subsequently, further *Arabidopsis* (He *et al.,* 2004; Lee *et al.,* 2008; Kwon *et al*., 2012; Lozano-Juste and León, 2010; Frungillo *et al.,* 2014) and rice (Lin *et al.,* 2012) mutants possessing modified NO/SNO levels were phenotyped which enabled the biochemical assessments to be complemented by genetic approaches. A good example of complementarity between biochemical and genetic methodology is the work of Fernández-Marcos *et al.* (2011), where the inhibitory effect of NO (both exogenous donor treatment and *Arabidopsis* mutants) on root meristem activity and PIN1-mediated auxin transport was demonstrated.

Current research is focusing on the molecular mechanisms of NO’s action during growth. The NO-dependent *S*-nitrosation of molecules involved in hormonal signaling such as e.g. NON-EXPRESSER OF PATHOGENESIS-RELATED GENE1 (NPR1, Tada *et al.*, 2008), salicylic acid binding protein (SABP3, Wang *et al.*, 2009), the auxin receptor TRANSPORT INHIBITOR RESPONSE 1 (TIR1, Terrile *et al.,* 2012), the cytokinin signal transducer HISTIDINE PHOSPHOTRANSFER PROTEIN 1 (AHP1) (Feng *et al.,* 2013), the ABA-insensitive 5 (ABI5) transcription factor (Albertos *et al.,* 2015) and the auxin-related S-phase kinase-associated protein 1 (SKP1, Iglesias *et al.,* 2018) have been revealed. Comprehensive overviews on the integration of NO in the plant hormonal system have been given by several authors (Freschi, 2013; Mur *et al.*, 2013; Parí *et al.*, 2013; Sanz *et al.*, 2015).

4.2. NO in plant reproduction

Beyond vegetative growth, NO has been found to be instrumental in many facets of plant reproduction, from the development of flowers (Zafra *et al.*, 2010; Zhou and Zhang, 2014) to the germination of seeds (Šírová *et al.,* 2011).

Some of the earliest work was on seed germination. Early reports (Beligni and Lamattina, 2000) on this phenomenon appeared only two years after papers on NO and host defense in plants (Lazalt *et al.,* 1997; Delledonne *et al.*, 1998; Durner *et al.,* 1998, Fig 1). Two NO donors, SNP or SNAP, induced germination in lettuce (*Lactuca sativa* L. cv. Grand Rapids) while no effect was seen with nitrate or nitrite. Bethke *et al*. (2004) also investigated seed germination using *Arabidopsis thaliana* (L.) Heynh. and barley (*Hordeum vulgare* L.). Here, SNP was used and shown to break dormancy of seeds but higher concentrations (250 µM) inhibited germination. It was also suggested that ABA was downstream of NO in the breaking of dormancy. With the worry that SNP effects were actually mediated by cyanide (a SNP by-product) further studies were carried out and it was confirmed that NO was instrumental in breaking seed dormancy (Bethke *et al.,* 2006). This was later confirmed by the use of NO gas, delivered directly to the seed rather than through a donor molecule (Libourel *et al.,* 2006). This research team carried on being instrumental in this field, for example showing the importance of the aleurone layer in mediating NO effects (Bethke *et al.*, 2007). Using two NO donors, SNP or SNAP, embryonic dormany in apple was shown to be broken by NO and this was correlated to ethylene production (Gniazdowska *et al.,* 2007).

Early work on flowering saw the appearance of mutants which over-produced NO or generated less NO and the authors stated that increased NO delays flowering (He *et al.,* 2004). Gene expression was modulated and the authors suggested that NO regulates the photoperiod. Also, in that year NO was found to be a key regulator of pollen tube growth (Prado *et al.*, 2004). By exposing pollen tubes to NO and using pharmacological agents they showed that pollen tube orientation was mediated by NO and was also dependent on cGMP signaling. This work was also the focus of a review paper in that year (Feijó *et al.,* 2004).

Further research soon followed which confirmed such work on seeds and flowers. Simpson (2005) showed modulation of NO levels altered gene expression which mediates flowering, in particular a repressor of flowering, FLC. Others continued to use SNP treatment of seeds, for example of wheat (*Triticum aestivum* L), where this NO donor induced an increase in activity of β-amylase but had no effect on α-amylase, and as this effect was also seen in other species the authors suggested that this was a universal effect of NO (Zhang *et al.,* 2005). For example, seed germination experiments were carried out in a range of species including *Suaeda salsa* (Li *et al.,* 2005). It was suggested that compounds such as γ-tocopherol affect the rate of NO production in seeds (Desel and Krupinska, 2005) while other compounds like gibberellic acid nitrite have their effect by being NO donors (Jovanović *et al.,* 2005).

At approximately the same time Hiscock's group published a paper on peroxidase in stigmas (McInnis *et al.*, 2005) and the following year reported on ROS localization in that tissue from Senecio (McInnis *et al.*, 2006a). Interestingly, it appeared that the ROS generated at the stigma was reduced by the presence of pollen and it was suggested that there was a crosstalk between the ROS and NO signaling. ROS may serve as a protection to the stigma, while NO may lower this resistance and allows pollen to germinate (McInnis *et al.*, 2006b). The commonality of pollen growth and fern spore germination was explored in a review in 2007 (Bushart and Roux, 2007) where there was a particular focus on the interplay between NO and calcium ion signaling. Although not directly using NO, the effect of other gases on pollen germination and function was also investigated that year (Chichiriccò and Picozzi, 2007). Here, NO_2, CO, and O_3 were found to reduce pollen germination.

Work with the NR double mutant (*nia1nia2*) soon followed when it was shown that this enzyme is important in flower development, at least in *Arabidopsis* (Seligman *et al.*, 2008). Progressing the work on pollen, the orientation of pollen tubes was further investigated and it was shown that NO was certainly involved, controlling the growth to the ovule's micropyle, and by using imaging techniques the mediation of pollen tube growth by calcium ions could be investigated (Prado *et al.,* 2008). Interestingly ATP as an extracellular signal has been shown to inhibit both pollen germination and elongation (Reichler *et al.,* 2009). Extracellular ATP-ɣ-S (which is poorly hydrolysed) induced NO generation. The effects of ATP-ɣ-S were lower in plants lacking NR (*nia1nia2* mutants), antagonists of guanylyl cyclase had an effect and it was concluded that NO was partly mediating the effects of extracellular nucleotides. In the same year, work on the cell walls of pollen tubes showed that NO altered F-actin organization which was mediated by NO regulation of extracellular calcium ion influx (Wang *et al.*, 2009). Also in 2009, studies using fluorescent probes and confocal microscopy showed that pollen could generate NO and nitrite (Bright *et* *al.,* 2009) and later Zafra *et al.* (2010) looked at the localisation of both NO and ROS in reproductive tissues of olive. Stigma and anther tissues, along with the pollen showed the most NO and ROS accumulation but the style and ovary showed no NO or ROS.

Flower senescence also involves the action of NO. It was shown that the application of NO reduced xanthine oxidase activity, as well as superoxide dismutase (SOD) activity. With also alterations of antioxidant capacity, the result was a lowering of superoxide and hydrogen peroxide levels. Taken together it was concluded that NO was important for the control of flower senescence having an effect on several redox couples and the non-protein thiol status of cells (Tewari *et al.,* 2009).

Therefore, historically it can be seen that NO has a range of impacts on plant reproduction, mediating flower development, being made by pollen, mediating pollen tube growth, breaking dormancy and being involved in flower senescence. Much research in this area has continued unabated in the last decade. Examples include the role of NO in programmed cell death which facilitates self-incompatibility and prevention of self-fertilization (Serrano *et al.,* 2015), while others using pollen tubes also revealed the crosstalk of NO pathways with other signaling components, such as calcium ions, ROS, and Mitogen Activated Protein (MAP) kinases (Wang *et al.,* 2016) and antioxidants such as ascorbate (Senthil Kumar *et al.,* 2016). The field has also adopted up-to-date methods along the way. For example, recently the *S*-nitroso and nitro-proteomes of olive (*Olea europaea* L) pollen have been studied (Carmona *et al.,* 2017; Jimenez-Quesada *et al.,* 2017) showing some of the molecular effects of increased in NO.

There have been relatively recent reviews on many of the area of plant reproduction, including flower development (Zhou and Zhang, 2014) and seed germination (Šírová *et al.,* 2011; Signorelli and Considine, 2018).

4.3. NO in symbiotic interactions

First experiments in the 1980s on NO production in legume species were continued later and researchers began to characterize the roles of NO in symbiotic interactions of legumes.

Prior to the pioneer study of Cueto *et al.* (1996) in *Lupinus* nodules, the occurrence of NO complexed to leghemoglobins (Lbs) was reported by EPR techniques in crude preparations from soyabean and cowpea root nodules (Maskall *et al.,* 1977) and in nodules of nitrate-treated cowpea and pea (Kanayama and Yamamoto, 1991). The role of NO in establishing symbiosis was later suggested by observations in intact soybean nodules, where a major component of EPR spectra attributed to a NO-Lb complex, was absent in senescent nodules (Mathieu *et al.,* 1998). These finding were in apparent contrast to detected inhibitory effects of NO on nitrogenase from soybean bacteroids (Trinchant and Rigaud, 1982); however, soon specific roles were recognized of controlled NO production by both plant and bacteria as symbiotic partners in different stages of their interactions, with a crucial role for hemoglobins in NO removal (reviewed in Hichri *et al.,* 2015; Berger *et al.,* 2019).

Plant NOS-like activity (Leach *et al.,* 2010) and NR (Boscari *et al.,* 2013) were suggested as NO sources in the first steps of symbiotic interactions, whereas NOS-like (Cueto *et al.,* 1996) as well as both plant and bacterial NR and respiratory chains might be additional NO sources in N_2-fixing nodules (Horchani *et al.,* 2011). Interestingly, MtNOA1 affects the establishment and functioning of symbiotic interactions of *M. truncatula* with *Sinorhizobium meliloti*, but had no influence on NO production in the nodules (Pauly *et al.,* 2011).

A significant finding was that symbiotic rhizobia respond to NO by upregulation of more than 100 genes, including *hmp*, encoding a putative flavohemoglobins (Meilhoc *et al.,* 2010). A *S. meliloti* hmp mutant displayed a higher sensitivity toward NO in culture and reduced N_2-fixation efficiency (Cam *et al.,* 2012). Lipopolysaccharides (LPS) from the cell surface of *Mesorhizobium loti*, involved in plant-*Rhizobium* recognition, were identified as NO-inducing factors in *Lotus japonicus* roots (Murakami *et al.,* 2011). An important advance in understanding the role of NO in differential responses of plants to symbiotic and pathogenic microbes was brought by Nagata *et al.* (2008), who found NO production and Phytogb1 expression in the roots of *L. japonicus* were not affected by non-symbiotic and only transiently increased by symbiotic rhizobia, whereas inoculation with plant pathogens induced continuous NO production but not of Phytogb1. NO was observed to induce gene expression of Phytogb1 in *Lotus japonicus* (Shimoda *et al.,* 2005). In subsequent studies NO was detected in different sites during the infection process of *M. truncatula–S. meliloti* interactions, including nodule primordia, where NO depletion caused a significant delay in nodule appearance (Del Giudice *et al.,* 2011). Microarray analysis of NO-responsive genes in *M. truncatula* roots brought further evidence that NO might regulate symbiosis establishment and nodule development

(Ferrarini *et al.,* 2008). Further transcriptomic analysis confirmed NO involvement in the repression of plant immunity, and induction of cell cycle and protein synthesis genes, allowing the beneficial plant–microbe interactions (Boscari *et al.,* 2013).

NO was also detected in the N_2-fixing zone of functional nodules in *M. truncatula–S. meliloti* symbiosis, but not in meristematic, infection and senescence zones (Baudouin *et al.,* 2006). The question was raised of the toxic effects versus signaling/metabolic functions of NO in nodules. On the one hand, NO production is linked, via a Phytogb-NO respiration process, with improved capacity of the nodules to maintain their energy status under hypoxic conditions (Horchani *et al.*, 2011). On the other hand, beside nitrogenase, enzyme components of the N_2-fixing machinery can be modulated by NO–dependent posttranslational modifications, as shown for glutamine synthetase irreversibly inactivated by tyrosine nitration (Melo *et al.,* 2011). Due to NO inhibitory effects on nitrogenase and many enzymes of nitrogen and carbon metabolism, possibly through *S*-nitrosation modifications (Puppo *et al.,* 2013), N_2-fixation efficiency in mature nodules is decreased by high levels of NO which was postulated to be a signal in developmental as well as stress-induced senescence (Cam *et al.,* 2012; Fukudome *et al.*, 2018).

NO has been also recognized to play similar roles in other symbiotic interactions (reviewed in Hichri *et al.,* 2016; Martinez-Medina *et al.*, 2019), including actinorhizal symbiosis of *Alnus* sp. (Sasakura *et al.,* 2006), mycorrhizal symbiosis in olive seedlings (Espinosa *et al.,* 2014) and symbiotic interaction during lichen rehydration (Catalá *et al.,* 2010; Expósito *et al.*, 2019). Recent years thus witnessed great advances in our understanding of the role of NO in plant-microbe symbiosis, including NO sources, targets and molecular mechanisms of its action in plant cells as well as in their non-pathogenic microbial associates, in parallel to discoveries of the conserved roles of NO in microbiome interactions in the animal and human fields (Vanhatalo *et al.*, 2018; Seth *et al.*, 2019).

4.4. NO as a stress modulator

The role of ROS in plant stress has been known for some time (Osswald and Elstnerk, 1987; reviewed by Mittler, 2006) and it was a couple of years before this that Rowland *et al.,* (1985) were investigating the effects of nitrogen species, including NO, on plant growth. But the turning point for NO research in plants came with the publication of papers on the potential role of NO in pathogen interactions of plants (Lazalt *et al.,* 1997; Delledonne *et al.,*1998; Durner *et al.,* 1998, Fig 1) (see 4.4.1). Three years later the role of NO in mitigating other plant stresses was being reported: UV-light (A.-H.-Mackerness *et al.,* 2001) and drought (Garcia-Mata and Lamattina, 2001; Zhao *et al.,* 2001). A year later both heat and salt stress were being studied with a focus on the role of NO and hydrogen peroxide (Uchida *et al.,* 2002). Flooding and hypoxia were the focus of work on Alfalfa (Dordas *et al.,* 2003) and in the same year cadmium and lead ions and the alleviation of stress by NO was reported (Kopyra and Gwóźdź, 2003). The same paper also reported on the role of NO in salt stress. From then onwards there has been a range of stresses investigated which involve or are attenuated by NO. These include an assortment of metal ions and other abiotic stresses as listed in Table 2.

The role of NO in plant stress has been the subject of several recent reviews (Sahay *et al.,* 2017; Saddhe *et al.,* 2019; Sami *et al.,* 2018), hence we indicate only the groundbreaking first papers here to show historical context (Table 2)

Stress response mediated by NO	Plant species used	Citation
Atmospheric NOX		Rowland *et al.,* 1985
Pathogen/biotic	Potato Soybean *Arabidopsis* Tobacco	Lazalt *et al.,* 1997 Delledonne *et al.,* 1998 Durner *et al.,* 1998
UV light	*Arabidopsis thaliana*	A –H. –Mackerness *et al.,* 2001
Drought	*Triticum aestivum* *Tradescantia* sp. *Salpichroa organifolia* *Vicia faba* *Wheat*	García-Mata and Lamattina, 2001 Zhao *et al.,* 2001
Salt	*Oryza sativa* *Lupinus luteus* *Phragmites communis* Trin.	Uchida *et al.,* 2002 Kopyra and Gwóźdź, 2003 Zhao *et al.*, 2004
Heat	*Oryza sativa*	Uchida *et al.,* 2002

	Phragmites communis Trin	Song *et al.,* 2006
Flooding/hypoxia	Alfalfa	Dordas *et al.*, 2003
Cadmium ions	*Lupinus luteus* *Pisum sativum* L *Pisum sativum* L *Arabidopsis*	Kopyra and Gwóźdź, 2003 Barroso *et al*., 2006 Rodríguez-Serrano *et al.*, 2006 Besson-Bard *et al.,* 2009
Lead	*Lupinus luteus* *Triticum aestivum* L	Kopyra and Gwóźdź, 2003 Yang *et al.,* 2010
Copper	Chlorella	Singh *et al.,* 2004
Zinc	*Solanum nigrum*	Xu *et al.,* 2010
Osmotic	*Triticum aestivum* L. Yangmai 158	Wang *et al.,* 2004
Aluminum	*Cassia tora* L *Arabidopsis*	Wang and Yang, 2005 Illés *et al.,* 2006
Ozone	Phragmites	Velikova *et al.,* 2005
Cold	*Brassica juncea* *Eriobotrya japonica* Lindl	Abat and Deswal, 2009 Wu *et al.,* 2009
Arsenic	*Oryza sativa* Fescue	Singh *et al.,* 2009 Jin *et al.,* 2010

Table 2: Early evidences of plant stress responses found to be mediated by NO.

4.4.1. NO in plant-pathogen interactions

NO research in plant biotic interactions was boosted by two seminal papers published in 1998; the year of the NO Nobel Prize awards. A study on soya bean cell culture by Lamb's group found NO to potentiate ROS-induced cell death within the hypersensitive response of *A. thaliana* plants to virulent *P. syringae*, which could be compromised by decreasing NO levels (Delledonne *et al.,* 1998). Increased NO production was observed in Klessig's lab in a resistant genotype of tobacco infected with tobacco mosaic virus, where experiments using tobacco cell culture revealed cGMP-dependent and independent NO signaling in induction of plant defence genes (Durner *et al.,* 1998). Importantly, these results appeared in line with observations in the vertebrate immunity (Mayer and Hemmens, 1997; Patel *et al.,* 1999), and immediately raised a wide interest within the NO community (Dangl, 1998; Bolwell, 1999). It was noted that the antimicrobial action of NO mediated by nitrosative stress might be counterbalanced by flavohemoglobins evolved in microbial pathogens (Hausladen and Stamler, 1998). Already at this early stage, GSNO was suggested as the long-distance signaling molecule in the plant systemic acquired resistance (Durner and Klessig, 1999).

Similarly to other NO fields, great advances were enabled by introduction of diaminofluorescein-based probes developed for *in vivo* NO imaging. They were exploited to record the NO burst induced in tobacco cells by cryptogein, a proteinaceous elicitor from *Phytophthora cryptogea* (Foissner *et al.,* 2000). Further research showed that HR cell death in soybean culture was not activated by NO interactions with superoxide, like in animals, but with hydrogen peroxide (H_2O_2) produced by SOD (Delledonne *et al.,* 2001). In contrast, NO induced programmed cell death in *Arabidopsis* cell culture independent of ROS via a cGMP-dependent pathway involving MAP kinases (Clarke *et al.,* 2000). The interrelation of NO signaling with that of salicylic acid were gradually recognized, as SA-induced protein kinase was identified downstream of SA in the NO signaling in tobacco defence responses (Kumar and Klessig, 2000). A newly uncovered mechanism of NO-ROS crosstalk included the capacity of NO and peroxynitrite ($ONOO^-$) to inhibit two major H_2O_2-scavenging enzymes, catalase and ascorbate peroxidase (Clark *et al.,* 2000). De Gara's group reported NO- and ROS-dependent modulation of redox balance, governed by the ascorbate and glutathione redox couples, formed part of the transduction signaling pathways that trigger cell death and plant defence responses in tobacco BY-2 cells (de Pinto *et al.,* 2002). However, in some instances NO was not observed as an early signaling component in HR initiation, such as in *Arabidopsis* leaves where NO was reported to serve rather as an intercellular signal in HR spreading (Zhang *et al.,* 2003). Likewise, NO and ROS were not essential mediators of the HR initiation in oat responses to a avirulent crown rust fungus, but participated in apoptosis induction in cells adjacent to the HR dead cells (Tada *et al.,* 2004). A rapid burst of NO was implicated in mechanisms of innate resistance in *Arabidopsis* plants in response to bacterial LPS (Zeidler *et al.,* 2004). Importantly, this and other studies exploited newly available *atnos1* (later renamed as *atnoa1*) mutant plants showing decreased levels of NO, however, after NOA1 protein was uncovered to be only indirectly related to NO production and also multiple pleiotropic effects of its down-regulation demonstrated in *atnoa1* mutant plants, its further use in plant NO studies has been discouraged (Moreau *et al*., 2008). A different experimental approach used *Arabidopsis* plants expressing a bacterial NO dioxygenase (NOD), which showed impaired NO signaling in incompatible plant-pathogen interactions (Zeier *et al.,* 2004), similar to tobacco overexpressing alfa alfa hemoglobin (Seregelyes *et al.,* 2003).

Nevertheless, this and other studies readdressed the quest for NO sources in plant biotic interactions. Modolo *et al.,* (2005) found mitochondrial nitrite reduction to contribute in cooperation with NOS and NR activities to NO generation in *A. thaliana*–*P. syringae* interactions. In *N. benthamiana*, NR was reported as the source of NO induced by infestin, the major elicitin of *P. infestans* (Yamamoto-Katou *et al.,* 2006). Further studies using *N. benthamiana* widened the knowledge on MAP kinases and their role in the regulation of NO- and NADPH oxidase-dependent ROS burst (Asai *et al.,* 2008). Biosynthesis of flavin, the important prosthetic group of active flavoproteins, such as the NOS-like enzyme, NR and RBOH, was found to be required for both NO and ROS production and HR cell death in *N. benthamiana*, and to influence its susceptibility to oomycete and ascomycete pathogens (Asai *et al.,* 2010).

In 2005 *S*-nitrosation, emerging as a key cGMP-independent mechanisms of NO biological activity, made its appearance into the plant defence field. Loake's group reported *Arabidopsis* mutants in GSNOR, the key regulatory enzyme of *S*-nitrosation, were compromised in the basal and non-host disease resistance, whereas increased GSNOR activity activated resistance to the virulent pathogen (Feechan *et al.,* 2005, Fig 1). Contrasting results obtained by Martínez's group on *Arabidopsis* plants with decreased GSNOR showing enhanced basal resistance against the oomycete *Peronospora parasitica* (Rustérucci *et al.,* 2007) were probably caused by the differential effects of using an antisense strategy for GSNOR downregulation. However, depletion of GSNOR function by RNAi resulted in disease susceptibility in tomato (Hussain *et al*., 2019). Sunflower cultivars resistant to downy mildew were found to induce GSNOR activity to avoid nitrosative stress, which is characterized by pathogen-induced NO production, *S*-nitrosothiol accumulation and protein nitration (Chaki *et al.,* 2009).

Regulatory nitrosative modifications were revealed for ROS-producing enzymes and components of SA signaling, when several reports demonstrated crosstalk between NO and glutathione through *S*-nitrosation of NPR1, a master regulator of SA-mediated defence genes, which promoted its nuclear accumulation and activation of PR genes (Tada *et al.,* 2008; Sun *et al.,* 2012; Kovacs *et al.,* 2015). Immune responses elicited by oligogalacturonides in *Arabidopsis* induced a NR-dependent NO production, which modulated NADPH oxidase-mediated ROS production (Rasul *et al.,* 2012). Under high *S*-nitrosothiol levels, NO negatively regulates the HR by *S*-nitrosation of the NADPH oxidase at conserved Cys890, inhibiting its ROS-generating activity (Yun *et al.,* 2011). Recently, *S*-nitrosation was revealed also as a host strategy disarming pathogen effector, as shown for the *S*-nitrosation-dependent inhibition of the bacterial effector HopAI1 targeting host MAP kinase signaling (Ling *et al.,* 2017). Current knowledge suggests NO and GSNO show additive functions in plant immunity with distinct or overlapping molecular targets (Yun *et al.,* 2016).

In parallel, NO produced by microbial pathogens was identified as a crucial factor of pathogen development and interactions with host cells. An early reports identified a bacterial NOS mediating the nitration of a dipeptide phytotoxin required for pathogenicity of *Streptomyces turgidiscabies* (Kers *et al.,* 2004; Johnson *et al.,* 2008). In this phytopathogen, NO production increased in response to cellobiose, a plant cell wall component. NO was found important for race-specific HR in a barley genotype resistant to *Blumeria graminis* (Prats *et al.,* 2005), where NO was also generated by pathogen cells as a pathogenesis determinant (Prats *et al.,* 2008). Bacterial flavohemoglobins, such as HmpX in *Erwinia chrysanthemi*, can scavenge NO and thus protect the pathogen cells from nitrosative stress and attenuate host HR (Boccara *et al.,* 2005). Nowadays, functions of NO in development and growth of plant pathogen as well as in their virulence and survival are widely recognized (reviewed in Arasimowicz-Jelonek and Floryszak-Wieczorek, 2014). It is thus increasingly evident that NO is involved in multiple steps of plant-pathogen interactions ranging from early pathogen recognition to late host cell responses, in gene expression regulation and defence metabolites production. However, this is in a highly specific manner depending on life strategies of diverse phytopathogens and resistance mechanisms available in distinct plant species and genotypes. As the major part of the current knowledge has been obtained on model plant species like *Arabidopsis* and tobacco and their available mutant lines, further progress is needed to transfer this into practical applications of increased pathogen defence in agriculturally important crops.

4.5. Interactions of NO with other redox molecules and nitrosative stress

NO, along with other RNS, is likely to be generated in cells at the same time and in the same subcellular location as several other small reactive signaling molecules (reviewed recently by Hancock and Neill, 2019), including ROS, hydrogen sulfide (H_2S) and hydrogen gas (H_2). As discussed in 4.4, NO production increases under biotic or abiotic stress conditions (Beligni and Lamattina, 2000, 2001; Chaki *et al.*, 2009; Airaki *et al.*, 2012; Ziogas *et al.*, 2013; Krasuska *et al.,* 2015), and such production of ROS and NO can generate a range of NO-derived molecules such as peroxynitrite ($ONOO^-$), nitrogen dioxide (NO_2), dinitrogen trioxide (N_2O_3), and other related molecules such as SNOs and GSNO, (Begara-Morales *et al.*, 2018). Along with other forms of NO, ie the nitrosonium cation (NO^+) and the nitroxyl anion (NO^-), such nitrogen-based compounds are often referred to as reactive nitrogen species (RNS). As the over-accumulation of ROS leads to oxidative stress so the over-generation of RNS can lead to nitrosative stress (Corpas *et al.*, 2007; Valderrama *et al.,* 2007), a term which was first used in plant sciences in 2003 (Neill *et al.,* 2003). Since then, numerous other papers have used the term to discuss the damage that RNS and ROS might inflict on plant cells (papers such as Jasid *et al.,* 2006; Lee *et al.,* 2008; Corpas *et al.,* 2007). In this regard, processes such as lipid peroxidation, protein carbonylation or sulfhydryl oxidation have been widely considered as markers of oxidative stress (Dalle-Donne *et al.*, 2003; Waszczak *et al.*, 2014; Akter *et al.*, 2015). However, RNS transmit their bioactivity mainly through post-translational modifications such as tyrosine nitration, *S*-nitrosation and nitroalkylation, which can regulate protein function and can be therefore considered as key regulators of oxidative and nitrosative signaling mechanisms (Mur *et al.*, 2013; Lindermayr and Durner, 2015; Mata-Pérez *et al.*, 2016). Of course, oxidative stress and nitrosative stress are not mutually exclusive and the term nitro-oxidative stress was subsequently suggested (Corpas and Barroso, 2013b).

However, many of the potential interactions will not be plant specific and therefore a broad look at the history of the literature is required here. The chemical generation of $ONOO^-$ (recently reviewed by Radi, 2019) has also been reported (Blough and Zafiriou, 1985) and was discussed by Beckman *et al.* (1990) where there was a focus on its disintegration, as shown in Equation 1:

Equation 1:

$$O_2^{\cdot-} + NO^{\cdot} \rightarrow ONOO^- \text{ (peroxynitrite, pKa=6.8)} + H^+ \leftrightarrows ONOOH \rightarrow HO^{\cdot} + NO_2^{\cdot} \rightarrow NO_3^- + H^+$$

Therefore, if NO and superoxide anions are generated at the same time in plant cells there is the potential for $ONOO^-$ production and it is known that this molecule has signalling properties (Speckmann *et al.,* 2016). $ONOO^-$ is able to nitrate tyrosine residues of target proteins and thus regulating their function. In this regard, it can, for instance, regulate superoxide dismutases and consequently influencing the accumulation of other reactive species such as ROS (Holzmeister *et al*., 2015). Similar to $ONOO^-$, the role of GSNO in cells has also been known at least since the 1980's (Park, 1988). This is produced by the reaction of NO with glutathione as shown in Equation 2, and for over twenty years it has been suggested as a way to transport NO around organisms (Hogg *et al.*, 1996) as well as being mooted as important at mediating NO effects (Yun *et al.,* 2016).

Equation 2:

$$NO^{\cdot} + GSH \rightarrow GSNO \text{ (}S\text{-nitrosoglutathione, pKa=2.212)} + H^+ + e^-$$

The redox nature of NO has also been reported for a long time. Koppenol (1997) reported on the mid-point potentials of many redox couples relevant to biological systems, including several involving the radical form $NO^{\cdot}$. Two years later, Hughes published a paper on the relationships between nitric oxide, nitroxyl ion and nitrosonium cation, and also $ONOO^-$ (Hughes, 1999). The reduction potential of NO was the subject of a paper (Bartberger *et al.,* 2002) which was published shortly after the paper on the influence of glutathione on intracellular redox poise (Schafer and Buettner, 2001), the latter highlighting the influence redox has on cellular function and how the multiple factors influencing it, such as increases in the rate of NO production, need to be considered. Therefore, the interaction of NO with the cellular redox status is important (recently revisited by Hancock and Whiteman, 2018).

The influence of NO on proteins came to the fore as methods for its assay were reported (Jaffrey and Synder, 2001). The *S*-nitrosation of proteins, as discussed in section 3, could be determined but of particular relevance here is that some of these proteins such as RBOHD can themselves produce reactive signaling molecules (Yu *et al*., 2012). A second RNS-based PTM is tyrosine nitration, as also mentioned above. Here, the binding of a NO_2 moiety with the tyrosine aromatic ring leads to the formation of 3-nitrotyrosine (Gow *et al.*, 2004; Radi, 2004, 2013). Such activity has been reported to be important in abiotic stress tolerance in plants (Arora *et al.,* 2016) and as mentioned, it has been suggested that it could be a good marker of nitro-oxidative stress conditions.

Other interactions of NO with reactive signals were also reported, particularly with H_2S (Whiteman *et al.,* 2006) (Equation 3). The nitrosothiol produced can itself be used as a signaling molecule and so influence the activity of plant cells. Buntkowsky *et al.* (2006) reviewed the physical characteristics of molecular hydrogen with a view to its influence on other reactive compounds such as NO. Physical properties, rather than classical chemistry, was also the focus of a paper by Akdaq *et al.* (2007) who was looking at how magnetism can influence NO generation, albeit in rats and not plants, although the potential effects can be extrapolated across cell types, as mooted much later (Hancock and Hancock, 2018).

Equation 3:

$$NO^{\cdot} + H_2S \rightarrow HSNO\ (S\text{-nitrosothiol}) + H^{+} + e^{-}$$

More recently the role of H_2 in signaling has been highlighted (Hancock and Hancock, 2019) and the influence of NO on this signaling has been reported, especially in adventitious root formation (Zhu *et al.,* 2016a; 2016b). This is a subject which will undoubtedly get more attention in the future as H_2 has been suggested to be useful for improved agriculture (Zeng *et al.,* 2014).

Although cGMP itself is an important signal, the nitrated cGMP derivative 8-nitro-cGMP was suggested to functions in guard cell signaling (Joudoi *et al*., 2013), showing another aspect of the influence of NO on the regulation of cellular function (Sawa *et al*., 2013). The interaction of RNS is not restricted to small molecules and proteins. Interestingly, the interaction of RNS and fatty acids is getting more attention in last years in animal systems (reviewed by Schopfer *et al.*, 2018) and its relevance to plants has been investigated more recently (Mata-Pérez *et al.*, 2016, Fig 1.). Thus, the reactive lipids species (RLS) resulting from the interaction of non-saturated fatty acids with NO and derived species, such as $^{\cdot}NO_2$ and $ONOO^-$, are called nitro-fatty acids (NO_2-FAs), nitrolipids or nitroakenes (Freeman *et al.*, 2008). More recently, the implication as signaling molecules in the development and responses to abiotic stress processes in plants has been described (Mata-Pérez *et al.*, 2016, 2017; Padilla *et al.*, 2017). These molecules can also release NO and modulate the expression of genes associated with antioxidant responses (Schopfer *et al.*, 2005; Gorczynski *et al.*, 2007; Mata-Pérez *et al.*, 2016; Padilla *et al.*, 2017). Furthermore, NO_2-FAs are powerful biological electrophiles which can react with biological nucleophiles such as glutathione (Jobbagy *et al.*, 2019) and certain protein amino acid residues. Thus, the adduction of NO_2-FAs to protein targets generates a reversible post-translational modification called nitroalkylation (Geisler and Rudolph, 2012) and can be considered a novel NO-PTM similar to *S*-nitrosation (Jobbagy *et al.*, 2019; Aranda-Caño *et al.,* 2019).

Therefore, it can be seen that NO does not work in isolation, and over the last thirty years the interactions of NO with ROS, H_2S, H_2, proteins, fatty acids and redox potential have all been investigated. There have been several recent reviews on how NO interacts with signaling, especially by other reactive molecules (Hancock and Whiteman, 2014; Hancock, 2019; Hancock and Neill, 2019).

5. Conclusions and future challenges of plant NO research

NO research in plant sciences now spans back over forty years. It has seen several ups and downs but there is no doubt that evidence has been accumulated which shows that NO is a major player in plant cell metabolism and signaling. NO can be measured in and from plant cells, and there are myriad of responses which are mediated, perhaps in part, by NO.

There are a range of plant sources of NO, including NR, an enzyme usually associated with nitrogen assimilation. It appears that higher plants lack a true NOS enzyme, although homologues can be seen in algae. Researchers continue to find that NOS substrates and inhibitors have actions in plants so the future may see the identification of novel NO generating enzymes.

Downstream most of the effects of NO seem to be mediated by the modification of thiol groups in a process commonly known as *S*-nitrosation. A range of proteins have been found to undergo this PTM, including ones involved in metabolism and gene expression. However, NO can also cause nitration of proteins, giving it a second arm of influence.

A wide range of physiological activities involve NO in plants, ranging from seed germination, through growth modulation and stomatal aperture control, to senescence and programmed cell death. Furthermore, during the life of a plant NO also aids in the war against pathogens and amelioration of a plethora stresses.

Of course, NO does not work alone and much of the work has been carried out in relation to other signaling molecules, such as ROS, H_2S and H_2. Interactions with such molecules will yield further components useful in cell control, such as peroxynitrite and nitrosothiols. NO can also be involved in fatty acid signaling through the formation of NO_2-FAs. Therefore, NO should be seen in the context of a complex network of molecules, together orchestrating the function of plant cells.

Over production of RNS, and indeed ROS, will lead to nitrosative and oxidative damage to cells so understanding the generation and cellular use of NO is important. Future work will no doubt focus of the methods cells use to generate NO under defined conditions, how that NO leads to downstream effects and how this can be modulated by endogenous treatments. The spatial and temporal accumulation of NO will be crucial to understand in individual cells and organelles. So too will be the inter-cell effects of NO, perhaps mediated by compounds such as GSNO.

There has been much work carried out on NO in plants over the last forty years, but numerous questions remain. The past and present concepts as well as future challenges of plant NO research are summarized in Fig 2. NO continues to be an exciting molecule for plant scientists to investigate. Understanding how NO fits into the immensely complex metabolism of plant cells will lead to treatments which will eventually contribute to improved plant growth, better crop protection and enhanced post-harvest protection of plant products, yielding a potential socio-economic impact.

Collectively, the last 40 years of research has established the birth and glory of this existential plant molecule. We now look forward to the next, potentially even more exciting, 4 decades of NO research.

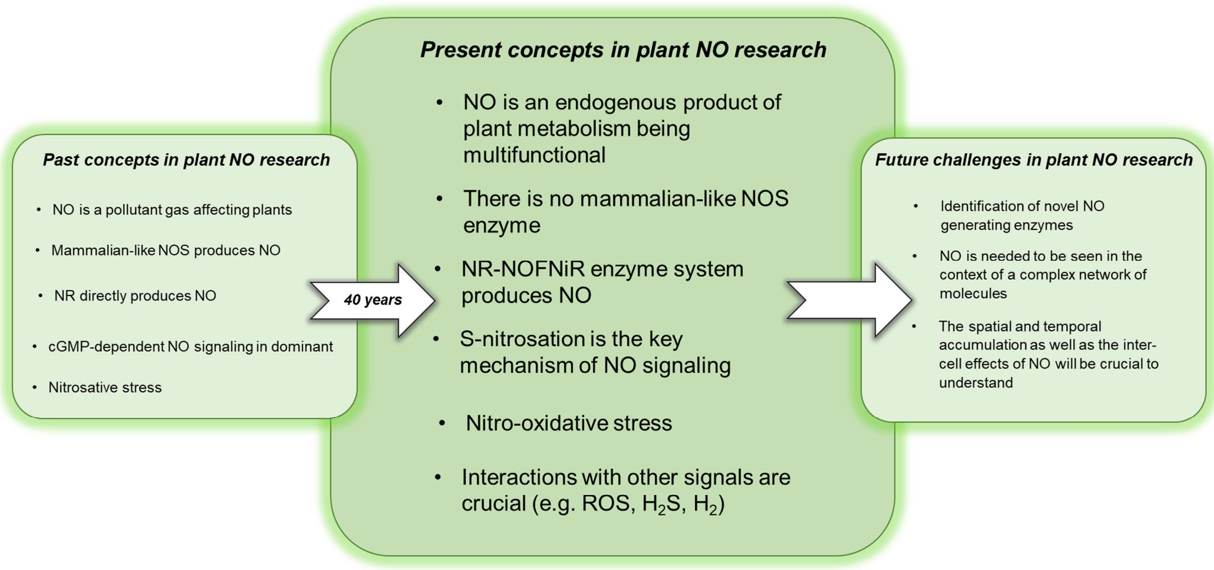


Fig 2. Past and present concepts and future challenges of plant NO research.

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Highlights_R2

- A review of the milestones from the last forty years of NO research in plants
- An overview of the generation and metabolism of NO in plants
- An overview of how NO controls plant reproduction, development and stress responses
- A review of the roles of NO in plant cell signalling
- A discussion of the future challenges and research directions of plant NO research