

# Unravelling ties in the nitrogen network: polyamines and nitric oxide emerging as essential players in the signaling roadway

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## Abstract

Nitrogen (N) is a central mineral nutrient essential for plant development and growth. It is usually scarcely found in soils, so the knowledge of the overall plant N metabolism deserves substantial attention. Polyamines (PAs) are N-containing low molecular weight compounds of polycationic nature, involved in essential processes all throughout the life of plants whereas nitric oxide (NO) is a gaseous free radical involved in signaling cascades related to many physiological events. Polyamines and NO share signaling functions and interact with each other in several biological functions, mainly in stress responses. Biosynthesis pathways of PAs and NO are overlapped; PAs induce NO formation, but it is still but it is still not completely defined whether PAs act as substrates, cofactors, or signals for promoting NO synthesis and

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also, which are the mechanisms involved in NO regulation of PAs metabolism. Polyamine levels are of vital importance in the regulation of the network of N-metabolizing pathways in plants, as they are components of the core of the overall N metabolism. In light of the importance of improving the efficiency of N uptake and distribution, it is time to elucidate the intricate relationship among N as a nutrient with PAs and NO as emerging signaling molecules. The close cooperation among these players in the whole N metabolism is an interesting target for the development of biotechnological tools for sustainable agriculture

**Keywords**

Nitric oxide, nitrogen, polyamines, signaling cascades, stress responses

## Introduction

After diverging from the animal and fungal lineages, plants became photosynthetic organisms by acquiring a single-cell photosynthetic bacterium into their eukaryotic cells. The competence to convert sunlight energy into chemical energy has allowed plants to occupy a niche highly consistent to our demands as aerobic organisms; while photosynthesizing, they produce all the oxygen that animals need to live on earth. But they do many other things, one of them being crucial for us right now: directly or indirectly, they provide all the food we need. They also provide pharmaceuticals and nutraceuticals, offer wood, fiber and renewable fuels, protect us from the sun, give us colors, scents and flavors, produce antibodies, vaccines or useful proteins for human health (O'Brien et al., 2016).

### 1 | Nitrogen in plant growth and development

All living organisms require nitrogen (N), and it is crucial for plant development and growth. Since it is a central building block of almost all vital biomolecules such as nucleic acids, amino acids, chlorophyll, cofactors, enzymes, vitamins, secondary metabolites and hormones, it is essential for plant metabolism. (Lea & Mifflin, 2011). Despite its abundance in the atmosphere, plants cannot use  $N_2$  directly and depend on soil microorganisms to obtain inorganic N as nitrate ( $NO_3^-$ ) or ammonium ( $NH_4^+$ ) from the organic matter; leguminous plants can additionally obtain N through biological fixation. Nitrogen is required by most of plants in greater quantities than any other mineral because of the vast amount of N invested in the photosynthetic machinery.

This crucial role of N throughout the plant life cycle makes it the main conditioning factor for plant growth and crop yields.

For that reason, improving plant nitrogen use efficiency (NUE) remains one of the major challenges of our times in the field of plant nitrogen metabolism. Plant breeding and agricultural innovations have provided several strategies to guarantee that crop yields continually increase to feed our ever-growing population. However, crop plants remove nutrients from the soil to metabolize them into essential proteins and other compounds. Each harvest depletes nutrients from the soil, which must be replenished regularly (Hawkesford & Howarth, 2011). So, the demand to meet the future growth in population will require even greater increases in agricultural yields. The use of fertilizers is the first election when nutrients have to be replaced to soils. But it is well known that they have an environmental impact when they are overused. Excess of nutrients that are not taken up into the plants drain to nearby land, rivers, and lakes, leading to damages or even destruction of these ecosystems and causing substantial ecological problems (Hirel et al., 2007; Perchlik & Tegeder, 2017; Vidal et al., 2020). Residual amounts of N can also escape into the atmosphere, contributing to an increase in greenhouse gases. The nitrogen gases include various oxides of nitrogen, like nitric oxide (NO), nitrogen dioxide (NO<sub>2</sub>), dinitrogen pentoxide (N<sub>2</sub>O<sub>5</sub>) and nitrous oxide (N<sub>2</sub>O), that react with other contaminants to compromise air quality (Socolow, 1999)

The study of the overall plant N metabolism deserves substantial attention in the present and future days. The first step in plant N metabolism is N acquisition by the root using an active transport across the plasma membrane of root epidermal and cortical cells. After being absorbed by the roots, N is moved to the rest of the plant via

a well-efficient transport system. In *Arabidopsis thaliana*, at least four protein families of nitrate transporters are encoded by four gene families, *NRT1 (NPF)*, *NRT2*, *CLC* and *SLAC1/SLAH*, and four ammonium transporters (AMTs) are involved in N uptake from the soil (Krapp, 2015; Wang et al., 2018). In the plant cells,  $\text{NO}_3^-$  can be stored in the vacuole where it is important for cation charge balance and as an osmoticum for cell expansion or growth (Orcel & Miller, 2011). Once in leaves,  $\text{NO}_3^-$  is reduced to nitrite ( $\text{NO}_2^-$ ) by the activity of NAD(P)H-dependent cytosolic nitrate reductase (NR, EC: 1.7.1.1). The major part of  $\text{NO}_3^-$  is reduced in leaves, though reduction also occurs in roots. Nitrite, in turn, is promptly removed from cells or transported to plastids where it is reduced by nitrite reductase (NiR, EC 1.7.7.1) into  $\text{NH}_4^+$  for further assimilation into organic compounds, like amino acids by the glutamine synthetase (EC 6.3.1.2)/glutamine-2-oxoglutarate aminotransferase (EC 1.4.1.13) (GS/GOGAT cycle) (Crawford, 1995; Kishorekumar et al., 2020). For further details in N uptake and assimilation and the regulation of N metabolism, see the relevant revisions (Krapp A, 2015; Lea & Mifflin, 2003, 2011; Masclaux-Daubresse et al, 2010; O'Brien et al., 2016; Vidal et al., 2020).

This vital pathway of N uptake and assimilation is tightly regulated by N itself, but also by carbon (C) signaling. Plants rely on both local and long distance signaling mechanisms that inform the roots about the actual external  $\text{NO}_3^-$  concentration, and communicate N nutrient status across different tissues and organs to guarantee the efficiency of root  $\text{NO}_3^-$  uptake (Lillo, 2008; Gojon et al., 2009). A transcriptome profiling in response to N treatments revealed that only a minor proportion of the differentially expressed genes is specifically regulated by N signals, but most N-

responsive genes are also under the control of hormone and C signaling pathways (Gutierrez et al., 2007; Krouk et al., 2009; Nero et al., 2009).

Many internal and environmental factors can modify  $\text{NO}_3^-$  acquisition by the plant. For example,  $\text{NO}_3^-$  concentration in the soil solution usually varies several orders of magnitude both seasonally and from place to place within the soil (Miller et al., 2007). Therefore, plants must constantly modulate the efficiency of root  $\text{NO}_3^-$  uptake, not only to compensate the fluctuations of external  $\text{NO}_3^-$  availability, but also to adjust the rate of  $\text{NO}_3^-$  intake to the 'N demand' for growth (Ruffel et al., 2014). Two major mechanisms coordinately modulate nitrate uptake. One is related to the nitrate primary response, which is characterized by a rapid induction of the expression of nitrate transporters shortly after nitrate treatment (Medici & Krouk, 2014). The second one is a negative feedback exerted by the N status that down-regulates the expression of nitrate transporters. This repression is relieved when the organisms experience N limitation, resulting in a strong increase in nitrate uptake capacity (Chaput et al, 2020; Miller et al., 2008). Using a split-root approach in *Medicago truncatula* plants, it was observed that N acquisition by one side of the root system is repressed by high N supply to the other side, regardless of the nature of the N source, suggesting that not only root  $\text{NO}_3^-$  uptake, but also root  $\text{NH}_4^+$  uptake and nodule  $\text{N}_2$  fixation are under the control of systemic signals related to the N status of the whole plant, and so raising the possibility of a common regulatory mechanism involved in the control of these pathways of N acquisition. Besides, *Arabidopsis* mutants altered in the NRT2.1 response to high N provision (*hni*, for high nitrogen insensitive) displayed reduced down-regulation of both NRT2.1 expression and high-affinity  $\text{NO}_3^-$  influx under repressive conditions, and induced changes in amino acid, organic acid, and sugars

pools, suggesting a possible role of these metabolites in the control of  $\text{NO}_3^-$  uptake by the plant (Girin et al., 2007).

This interplay between uptake and demand is finely represented in the case of NR, the cytosolic enzyme that catalyzes the first step of the  $\text{NO}_3^-$  assimilation pathway and is a crucial component in the long journey that starts with N acquisition from soils and continues with distribution and assimilation in all plant organs (Crawford & Glass, 1998; Loqué et al, 2003). In higher plants, NR is rapidly inactivated/activated by phosphorylation/dephosphorylation of a Ser residue, in response to environmental stimuli and various treatments. But it is after 14-3-3 proteins bind to phosphorylated NR when the enzyme is actually inhibited (Kaiser & Huber, 2001; Huber et al., 2002). Sugars, cytosolic acidification, and anaerobiosis are factors all known to activate NR in leaves and roots (Provan and Lillo, 1999). Enzymatic inhibition is observed in the presence of cations, being  $\text{Mg}^{2+}$  and polyamines the most important ones (Provan et al., 2000). The post-translational modification (PTM) of NR by phosphorylation is essential for controlling nitrite accumulation and subsequent deleterious effects in conditions where nitrite reduction is limited (Lillo et al., 2004).

Nitrate reductase is considered as a controlling step in  $\text{NO}_3^-$  assimilation. Its gene expression is induced by  $\text{NO}_3^-$  and repressed by amino acids (Provan & Lillo, 1999; Vincentz et al., 1993), and the  $\text{NO}_3^-$  induction is completely abolished during C starvation (Matt et al., 2002). NR regulatory mechanisms have been mainly studied at the level of root  $\text{NO}_3^-$  uptake and transport and they depend on both local and long-range signals (Chaput et al., 2020). In *Arabidopsis thaliana*, as in many other plant species including *Nicotiana*, *Triticum*, *Medicago* or *Lycopersicum*, the catalytic activity of NR is conferred by the genes *NIA1* and *NIA2* (Wilkinson & Crawford, 1993), which

are expressed as the two isoforms of the enzyme, NIA1 and NIA2. In Arabidopsis, the transcription pattern of the two isoforms NIA1 and NIA2 is similar after nitrate induction (Lin & Cheng, 1997). Double mutant *nia1nia2* plants show scarce growth on media with nitrate as the sole N source, in part due to the lack of N incorporation into amino acids (Zhao et al., 2016). Surprisingly, gene expression of *NRT1.1* and *NRT2.1* transporters is constitutively upregulated in roots of *nia1nia2* plants, suggesting that NR activity or a nitrogen-containing metabolite, derived from nitrate reduction, feedback regulate uptake systems (Lejay et al., 1999). However, the identity of this regulatory metabolite remains undetermined up to now (Frungillo et al., 2014; Salgado et al., 2013). Altogether, these regulations enable plants to activate genes involved in N-assimilation when C skeletons are abundant and internal levels of organic N are low, or to inhibit N assimilation when levels of photosynthate are low or internal levels of organic N are high, underlying the importance of C/N ratio signaling in the regulation of N metabolism (Chaput et al, 2020). These events clearly indicate that N availability, acquisition, distribution and use during the life cycle need to be tightly controlled at the level of regulation of all these processes.

## 2 | Nitric oxide, a side product of NR activity, is a key signaling molecule

An extensive work in the animal systems has led to the acceptance of nitric oxide (NO) as a critical component of second messenger cascades of hormone and physiological signals (Lamattina et al., 2003; Tuteja et al., 2004). Our understanding of plant physiology has been deeply impacted after the discovery of this bioactive small gaseous molecule in mammals (Furchgott & Zawadzki, 1980). However, it was in 1979



when Klepper first mentioned NO as a small molecule actually produced by plants (Klepper, 1979). This free radical has become more and more important after being involved in an astonishing diversity of plant functions, starting from seed germination, plant growth and development to flowering and senescence, stomatal closure, iron homeostasis, immunity as well as the pivotal role in plant responses to biotic or abiotic stresses (Buet et al., 2019; Corpas & Palma, 2018; Fancy et al., 2017; Galatro et al., 2020; Lamattina et al., 2003; Wendehenne & Hancock, 2011; Yu et al., 2014).

The role of NO in N uptake and assimilation in plants has been highlighted, suggesting that NO is an important signaling molecule of the nitrate-sensing pathway (Sanz-Luque et al., 2013; Sun et al., 2015). However, little is known about the mechanisms of NO-regulated N assimilation in response to different N sources. It is essential to remark that increased NO level has been reported to increase in different plant tissues following alterations in nutrient supply due to modifications in its synthesis or degradation rates (Buet et al., 2019;; Lamattina et al., 2003; Rockel et al., 2002; Sun et al., 2015). A very interesting role for NO production in maize root response to  $\text{NO}_3^-$  was postulated due to the observation of a coordinate spatiotemporal expression of NR and nonsymbiotic hemoglobins (nsHbs) involved in NO synthesis and scavenging, respectively (Perazzolli et al., 2004, 2006). Plant hemoglobins (HBs) are heme proteins that reversibly bind molecular oxygen ( $\text{O}_2$ ), first identified as symbiotic HBs (sHb) in legume root nodules, and later as nsHbs (Garrocho-Villegas et al., 2007). Moreover, nsHbs have been reported to be induced by nitrate in *Arabidopsis* and *Lotus japonicus* (Shimoda et al., 2005; Wang et al., 2000). These findings suggested that they could be involved in the early perception and signaling of  $\text{NO}_3^-$  in the rhizosphere (Manoli et al., 2014; Trevisan et al., 2011). The NO-related events that follow the NO increase

constitute an exciting field of research to understand how plants may adapt to environmental conditions of nutrient shortage.

Several enzymes have been involved in NO biosynthesis in photosynthetic organisms (Gupta et al. 2011; Mur et al. 2013). Many of them have in common to be molybdenum enzymes (Chamizo-Ampudia et al., 2013; Tejada-Jiménez et al., 2019). In line with this and in addition to its main role in nitrate reduction, NR performs the function of being responsible for the generation of NO (Chamizo-Ampudia et al., 2017; Yamasaki et al., 1999; Yamasaki & Sakihama 2000). The conversion of nitrite to NO by NR was documented in 1988 (Dean & Harper, 1988) in *Leguminosae* and has been successively demonstrated in detail in other plant species, implying a putative responsibility for NR as signal emitter. For this reason, in addition to its physiological function in N assimilation, NR may be required for the control of the production of reactive nitrogen species (RNS) (Trevisan et al., 2011; Yamasaki & Sakihama, 2000)

It is remarkable that the two NRs in *Arabidopsis thaliana* are not functionally identical in NO production. It has been reported that NIA1 is the most efficient NiR while NIA2 exhibits higher nitrate reductase activity, which supports the hypothesis that each isoform has special functions in the plant (Mohn et al., 2019). Moreover, *Arabidopsis nia1* mutants show reduced levels of NO even though they still harbor a functional NIA2 gene, so NIA1 seems to be more related to NO production than the functionally redundant NIA2 (Wilson et al., 2009)

Despite NR is the best understood pathway for NO synthesis in plants up to now, and although several studies using different experimental plant models or *in vitro* approaches have suggested that NR represents the major enzymatic source of NO in several plant species, like wheat (Rosales et al, 2011, 2012), *Arabidopsis* (Chen et al.,

2016; Hao et al., 2010; Zhao et al, 2016), tomato (Jin et al., 2009) or maize (Manoli et al., 2014), some data do not match with this idea (Rockel et al., 2002). The low efficiency of NO production by NR, that *in vitro* accounts for a small fraction (1%) of its nitrate reduction activity at saturating NADH and nitrite concentrations (Miller & Smith, 2008; Rockel et al., 2002), suggests that other NO sources should be active plant cells. Chamizo-Ampudia et al. (2016) have reported that NR is unable to produce NO *in vitro* from nitrite when nitrate is present in the medium. Moreover, it seems that NR could participate in NO production in variable quantities depending on NR activity, its activation state, and the relative amounts of nitrite and nitrate in the cytosol. In *Chlamydomonas reinhardtii*, it has been reported that NR transfers electrons from NAD(P)H to the molybdoenzyme amidoxime reducing component (ARC, renamed as NOFNiR) (Chamizo-Ampudia et al., 2016), which synthesizes NO from nitrite, both *in vitro* and *in vivo*, mediated by its diaphorase activity. In addition, NAD(P)H-NR can also transfer electrons to the truncated hemoglobin THB1, which catalyzes the conversion of NO into nitrate by its dioxygenase activity (Sanz-Luque et al., 2015). This dual system for NO synthesis formed by ARC and NR can catalyze NO production from nitrite in the presence of millimolar concentrations of nitrate. High nitrate concentrations in the cell represents a very unfavorable condition for NR alone to produce NO owing to the fact that nitrate strongly inhibits the NiR activity of NR (Chamizo-Ampudia et al., 2016; Rockel et al., 2002). So, this could be a major way to produce NO, as mentioned by the authors. However, further research on the conditions that regulate NO or NO<sub>2</sub><sup>-</sup> production (such as the factors which favor the activity of NOFNiR and hemoglobins over NR) is required.

Not only NR is a source of NO but, in turn, its activity is regulated by NO levels (Sun et al., 2017) The NR substrate, nitrate, is a particular molecule because of its dual role as a nutrient and a signal that regulates plant N acquisition and metabolism (Ho et al., 2009; Ruffel & Gojon, 2017)). However, it appears to have no direct effects on the modulation of NR activity but through the alteration of endogenous NO level (Du et al., 2008; Rosales et al., 2012; Sun et al., 2017). Rosales et al (2011) reported that NR activity in wheat leaf was negatively modulated by NO released from a NO donor (SNP, sodium nitroprusside or GSNO, nitrosoglutathione); simultaneously nitrate content significantly increased, indicating that the substrate for NR activity was present in sufficient quantities for NR activity. Frungillo et al (2014) showed that NO feedback regulates flux through nitrate assimilation pathways and controls its bioavailability and its own consumption by modulating the expression of NRTs and the activity of NR.

Nitrite, that is the product of NR activity, might also form NO through the membrane-bound nitrite reductase (Ni:NOR; Stöhr et al., 2001; Stöhr & Ullrich, 2002), by xanthine oxidoreductase (Corpas et al., 1997; Cantu-Medellin & Kelley, 2013), or, by the non-enzymatic way, through the action of the chloroplast and mitochondrial electron transport chain under anaerobic conditions (Gupta et al., 2011). As has been documented for *Chlamydomonas*, in vitro studies have recently shown that in both, humans and plants, ARC are able to catalyze the reduction of nitrite to NO (Sparacino-Watkins et al. 2014; Yang et al. 2015).

Besides the nitrite-dependent routes, the possibility that plants also produce NO through the activity of a nitric oxide synthase (NOS) has been widely considered. In animals, NO is produced by the enzyme NO synthase (NOS; EC 1.14.13.39), which is present in three isoforms that co-exist, namely neuronal, endothelial and inducible

NOSs (nNOS, eNOS and iNOS, respectively) (Stuehr & Vasquez-Vivar, 2017). However, attempts to identify a plant NO synthase that catalyzes oxidative synthesis of NO from arginine (Arg) (as in animals) have not been successful up to now (Mur et al., 2013; Sanz-Luque et al., 2015). This is surprising since plants show an Arg-dependent NO synthesis (Flores et al., 2008). Corpas & Barroso (2017) postulated that in plants, the L-Arg-dependent NO synthesis could be due to cooperation among discrete proteins, resulting in the formation of a protein complex with requirements for an enzyme activity that are similar to those of the animal NOS. This would explain the lack of success in finding canonical NOS proteins at the molecular level. In the plant lineage, an extensive *in silico* study demonstrated that NOS homologs could not be found in the transcriptome of >1000 different photosynthetic organisms, with the exception of ~12 algae species (Jeandroz et al., 2016; León & Costa-Broseta, 2019), including the recently characterized NOS from the photosynthetic unicellular *Ostreococcus tauri* (Foresi et al., 2010; Weisslocker-Schaetzel et al., 2017). *O. tauri* is a single-celled green alga, which shares a common ancestor with higher plants and shows 42% similarity to human NOSs and is considered part of an early diverging class within the green plant lineage. This discovery points to a loss of this enzyme at the beginning of plant evolution.

Even though it has been well demonstrated that NR is partially responsible for NO formation in plants, together with NOS-like enzyme activity, the *Arabidopsis thaliana* triple mutant *nia1nia2noa1* still generates NO (León & Costa-Broseta, 2019; Lozano-Juste & León, 2010). This fact leads to the idea that another enzymatic pathway for NO formation exists in plants. Decoding the complex mechanisms responsible for NO

production in higher plants is a challenging issue of great interest for a better understanding of the role of NO in plant physiology.

### 3 | Polyamines: stars in the N metabolism screenplay

Nowadays, there are no doubts remaining that N is a central milestone essential for plant growth and development. Among the great variety of nitrogenous compounds that exist in plants, polyamines (PAs) are undoubtedly fundamental players. However, the functions that PAs display in N metabolism have not been completely clarified yet. Polyamines are very appealing and still mysterious nitrogenous compounds, discovered about three centuries ago by Anton van Leeuwenhoek, who noted deposition of satellite crystals with aging of sperm when studying human semen (Cohen, 1998). By 1920's, these crystals were known to be ubiquitous bioactive compounds, occurring in almost all types of living organisms including microorganisms, plants, animals and even viruses (Cohen, 1998). However, it was not until the beginning of the twentieth century that PAs began to attract the attention of plant physiologists due to their wide spectrum of physiological functions, and nowadays their role in controlling plant growth and development is recognized beyond all doubt (Alcázar et al., 2010; Bagni & Tassoni, 2001; Benavides et al., 2005; Chen et al., 2018; Fuell et al., 2010; Kusano et al., 2008; Tiburcio et al., 2014).

Polyamines, also known as biogenic amines, are low molecular weight compounds of polycationic nature (Kusano et al., 2008). Major free PAs in plants include putrescine (Put), spermidine (Spd), spermine (Spm), and its structural isomer thermospermine (tSpm). Agmatine (Agm) and cadaverine (Cad) are also found. Putrescine, Spd and Spm

differ from each other in the number of positive charges exhibited at the physiological pH of the cell (Gill & Tuteja 2010). These three main PAs are required for cell survival and growth, due to the importance of their molecular interactions with nucleic acids (affecting transcription and translation processes) and participation in cellular membrane stability (Benavides et al., 2018; Minocha et al., 2014;) and also because essential signaling molecules, like H<sub>2</sub>O<sub>2</sub>, NO or  $\gamma$ -aminobutyric acid (GABA), are produced as a result of their catabolism and play primary roles in antioxidant defense promotion (Agurla et al., 2018; Mellidou et al., 2017; Recalde et al., 2018). Although many of their specific cellular functions in plants remain uncharacterized, they have been implicated in a variety of physiological responses and molecular interactions (Alcázar et al., 2020, and references therein), the majority of which are undoubtedly shared to those described for NO. They take part in seed germination, (Huang et al., 2017), photosynthesis (Ioannidis and Kotzabasis, 2007), cell division (Yamashita et al., 2017), pollen tube growth (Wu et al., 2010), membrane fluidity (Benavides et al., 2018), protein regulation (Baron & Stasolla, 2008; Takahashi & Kakehi, 2010), root and shoot growth, fruit development and ripening, floral development (Guo et al., 2015, 2018; Imamura et al., 2015), senescence (Sobieszczuk-Nowicka, 2017), ion homeostasis (Yuan et al., 2019) and responses to the diverse array of signals from abiotic or biotic stresses (Groppa et al., 2001; 2007; Groppa & Benavides, 2008; Handa et al., 2018; Seo et al., 2019; Tiburcio et al., 2014).

The cellular concentrations of PAs usually vary depending on growth or the stage of plant development, nutritional status, particularly the type of N form supplied to the plant, as well as in response to various environmental signals. As PAs are implicated in the regulation of C:N ratio (Wuddineh, et al., 2018), and because important signaling

molecules are formed as a result of their catabolism, plants have evolved robust control mechanisms for maintaining PAs homeostasis, which is mainly accomplished by their synthesis, conjugation, transport to other tissues/organs and their catabolism or turnover (Mattoo et al., 2006; Moschou et al., 2012; Wuddineh et al., 2018).

Almost all eukaryotes synthesize Put directly from ornithine (Orn) in a reaction catalyzed by ornithine decarboxylase (ODC, EC 4.1.1.17), which removes the carboxyl group of the C1 atom of Orn to form Put and CO<sub>2</sub> (Pegg, 2006). The ODC pathway occurs in almost all plant species, except for many species of the Brassicaceae, as *Arabidopsis thaliana*, where ODC is absent (Hanfrey et al., 2001), indicating that the ornithine pathway is not essential for normal growth (Tiburcio et al., 2014). In plants missing *ODC*, duplication of arginine decarboxylase (*ADC*) genes might be a compensatory mechanism (Hanfrey et al., 2001). Early in the evolution of the Brassicaceae, the duplication of an ancestral *ADC* generated two paralogs (*ADC1* and *ADC2*) that have been maintained even after the genome shrinkage in species like *Arabidopsis thaliana* (Galloway et al. 1998). In animals, the production of Put through ODC is considered the key step for limiting PA biosynthesis, whereas in plants *ADC* (EC 4.1.1.19) performs this role (Hanfrey et al., 2001). In this route, Put is synthesized from L-arginine (L-Arg) by the action of *ADC* followed by two successive steps catalyzed by agmatine iminohydrolase (AIH, EC 3.5.3.12) and N- carbamoyl-Put amidohydrolase (CPA, EC 3.5.1.77), to form Put, CO<sub>2</sub>, and NH<sub>3</sub> (Fuell et al., 2010) (Fig. 1). The triamine spermidine (Spd) and the tetraamines spermine (Spm) and thermospermine (tSpm) are synthesized by aminopropyl transferases (Spd synthase (SPDS, EC 2.5.1.16) for Spd, and Spm synthase (SPMS, EC 2.5.1.22) or tSpm synthase (tSPMS, EC 2.5.1.79) for Spm or tSpm, respectively, using Put and aminopropyl residues, which are gradually



provided by decarboxylated S-adenosylmethionine (dcSAM or dcAdoMet) (Shao et al., 2012; Vuosku et al., 2018; Wu et al., 2007). The enzyme SAM decarboxylase (SAMDC, EC :4.1.1.50 or AdoMetDC ) produces dcSAM from SAM; it is often believed that this reaction is a rate limiting step in the biosynthesis of the higher PAs (Hu et al., 2005; Shao et al., 2012).

It is well known that PAs are catabolized by two major categories of cell type-specific amine oxidases, the homodimeric copper-containing amine oxidases (CuAOs, EC 1.4.3.6) also named diamine oxidases (DAO EC 1.4.3.22), and the monomeric flavin-containing PA oxidases (PAOs, EC 1.5.3.11) (Cona et al., 2006). CuAOS catalyzes the breakdown of Put to GABA via the intermediates 4-aminobutanal and  $\Delta^1$ -pyrroline. There is a general opinion that indicates that plants have two types of PAOs: one catalyzes a terminal catabolic reaction oxidizing Spd or Spm/tSpm (as in barley and maize) producing N<sup>-</sup>(3-aminopropyl)-4-aminobutanal and 4-aminobutanal, respectively, as well as 1,3-diaminopropane and H<sub>2</sub>O<sub>2</sub> in both reactions, whereas the most recently characterized *Arabidopsis* and rice PAOs oxidize PAs in an alternative pathway, they back-convert them into respective lower amines (Put and Spd, respectively) (Angelini et al., 2010; Cona et al., 2006). Polyamine catabolism results in the formation of three essential signaling molecules: H<sub>2</sub>O<sub>2</sub>, major player in PAs-induced regulation of various biological processes (Gupta et al., 2016); GABA, derived from 4-aminobutanal and involved in many physiological functions (Bown & Shelp, 2016), and NO, that plays a vital role in plant growth and development as well as in responses to biotic and abiotic stresses. The most updated information on plant CuAOs and PAOs is provided by Moschou et al. (2012) and Kusano et al. (2015).

Polyamine levels are of vital importance in the regulation of the network of N-metabolizing pathways in plants, as they are components of the core of the overall N primary metabolism (Fig. 1). Taking into account that Orn is formed from glutamate (Glu) through pyrroline-5-carboxylate (P5C) in a reaction catalyzed by ornithine aminotransferase (OAT, EC 2.6.1.13), and it is also synthesized from Arg through arginase (AN, EC 3.5.3.1) releasing urea, and both amino acids are the source for Put, Glu can be considered as an initial precursor for PAs biosynthesis (Bagni & Tassoni, 2001; Recalde et al., 2018; Wuddineh et al., 2018). Glutamate is a central molecule in amino acid metabolism. In plants, Glu can be principally synthesized via GS/GOGAT cycle. As a product of this pathway, Glu is the major donor of amine groups for most of the other amino acids as well as other nitrogenous compounds in plants aside from PAs, like glutamine (Gln), proline (Pro) and GABA, which constitute a crucial cooperating pathway for C and N assimilation (Forde & Lea 2007; Majumdar et al., 2016; Wuddineh et al., 2016). Therefore, the form of N absorbed by plants ( $\text{NO}_3^-$  or  $\text{NH}_4^+$ ) may have major effects on the metabolism of PAs (Moschou et al., 2012; Wudinehh et al., 2016). Polyamines are often found in high concentration in plants (up to millimolar levels). Such high levels turn them into potential strong sinks for N excess within the cell, thus helping to reduce cytotoxicity due to  $\text{NH}_4^+$  (Moschou et al., 2012; Serapiglia et al., 2008). It has also been demonstrated that excess of  $\text{NH}_4^+$  nutrition results in increased Put biosynthesis and accumulation in *Vitis vinifera* (Primikiriou et al., 2000). It is known that the type of N fertilizer impacts the PA metabolic pathway through its effect on the rate of  $\text{NH}_4^+$  assimilation, as reported by Zhu et al. (2018), who observed that Put level was significantly higher in the roots of rice cultivars irrigated with  $\text{NH}_4^+$  compared to  $\text{NO}_3^-$ , and Houdusse et al. (2005), who showed that

ammonium nutrition was associated with significant increases in Put content and plant growth reduction, suggesting that Put biosynthesis might be related to Pro degradation by a specific pathway related to ammonium detoxification.

The biosynthesis of PAs relies in part on how much N can be assimilated by the cell, which makes the activity of GS a major factor in PA synthesis (Bhatnagar et al., 2001).

In the notion that Glu is an alternative precursor of PAs biosynthesis through Orn, it might be logical that, the N which flows through the GS/GOGAT pathway might impact on PAs concentration within the cell. In addition, methionine sulfoximine, an inhibitor of GS, reduced Put levels in poplar cells, supporting this idea (Bhatnagar et al., 2001). It is postulated that Orn is not only a central molecule in the Arg, Glu and Pro pathways but also a master regulator of the flux of Glu diverging C and N into all these metabolites (Majumdar et al., 2013; Page et al., 2016), reinforcing the idea that PAs are interconnected with the major N pools. Accordingly, poplar cells overexpressing ODC show increased Put levels and reduced contents of Glu and Gln (Mohapatra et al., 2010). These findings suggest that PAs exert a feedback regulation of the major N pools. The interplay between PAs metabolism and GS/GOGAT cycle needs to be addressed in order to clarify not only the involvement of the ratio  $\text{NO}_3^-/\text{NH}_4^+$  and N availability in the regulation of PAs metabolism, but also the influence of PAs in the regulation of N assimilation.

The key role of Orn in Put production in plants has been barely studied. As mentioned above, PAs can be formed from Glu through Orn (Wuddineh et al., 2018). Considering that in most plants not only the cellular concentration of Orn is much lower compared with Arg or Glu, but also Orn is an intermediate in the Glu to Arg pathway, and both Glu and Arg are often present in large quantities, Orn significance in the regulation of

Put production and in relation to N metabolism deserved to be discussed (de Oliveira et al., 2018; Mayer & Michael, 2003). In view of this issue, Majumdar et al. (2013) demonstrated that not only does the cellular Orn concentration play a key role in Put production but also its own biosynthesis responds actively to its demand in the PA biosynthetic pathway, thus affecting the general N metabolism. It is suggested the occurrence in plants of an Orn-sensing and signal transduction mechanism, which regulates its own biosynthesis and also plays a role in regulating Arg, Pro and GABA production (Majumdar et al., 2013). To add evidence in the same direction, Majumdar et al. (2016) showed that the overall conversion of Glu to Arg was enhanced by an increased utilization of Orn for PAs biosynthesis in transgenic *Arabidopsis thaliana* plants overexpressing the mODC gene (mammalian ODC cDNA), which produce several-fold higher concentrations of Put. Considering all different aspects about the relationship between PAs and N metabolism discussed before, it could be contemplated the fact that manipulation of Put biosynthesis through multiple approaches should contribute to an improved assimilation of N and C in plants, thus favouring NUE and diminishing the use of fertilizers.

#### **4 | Polyamines and NO: the still mysterious dialogue among essential N metabolites involved in multiple responses in plants**

Another relevant connection that links PAs and N metabolism is the relationship between PAs and NO. It was discussed earlier that both molecules can influence N major hubs (and vice versa). Moreover, they play significant roles in highly diverse metabolic, developmental, physiological and environmental responses, and it

has been revealed that they act together under several circumstances. It has been demonstrated that exogenously applied PAs induced NO production in *Arabidopsis* (Tun et al., 2006) or wheat (Groppa et al., 2008; Rosales et al., 2012), indicating NO as a potential mediator of PA actions through a mechanism still undeciphered, filling a gap between many known physiological effects of PAs and amelioration of stresses (Wimalasekera et al., 2011a; Yamasaki et al., 2006, Sequera-Mutiozabal, et al., 2017). Furthermore, PAs and NO form a variety of conjugates under stress conditions and have reactive oxygen and nitrogen species (ROS and RNS) scavenging function (Sakihama et al., 2003) and changes in PAs and NO generation depend on the type and duration of stressors (Arasimowicz-Jelonek et al., 2009). In this sense, a concerted action of PAs and NO has been described in several physiological events related to growth and developmental processes as well in plant responses to environmental signals. It is of high relevance to elucidate this relationship to move forward in the direction of better understanding this intriguing relationship between these two essential N compounds.

#### 4.1 | Polyamines and NO in growth and development

In *Rosa canina* L., the increase in Put oxidation through CuAO activity led to an increase in H<sub>2</sub>O<sub>2</sub> production, followed by an enhancement in NOS-like gene expression, ending in a signal that resulted in stomatal closure (Adamipour et al., 2020). However, the authors did not evaluate NO formation, to establish a relationship between the increase of a putative NOS-like gene expression with the level of NO, and also to confirm if this NO was upstream or downstream H<sub>2</sub>O<sub>2</sub> in the signaling road to regulate stomatal closure. In *Arabidopsis thaliana*, both NO and ROS were essential signaling

components during Put, Spd, and Spm-induced stomatal closure, and PAs-induced ROS production was mediated by both NADPH oxidase and amine oxidase, while the rise in ROS appears to be upstream of NO (Agurla et al., 2018). Nitric oxide production was suggested to come from NOS-like activity, as L-NAME (N omega-Nitro-L-arginine methyl ester hydrochloride, a NOS inhibitor) completely reversed the stomatal closure caused by PAs. Moreover, NO coming from NR was discarded because tungstate (a NR inhibitor) had no effect in stomatal closure, and there was no change on PAs-induced stomatal closure in *nia* mutants. It is quite reasonable to expect that H<sub>2</sub>O<sub>2</sub>, a product of PAs oxidation, can elevate NO. Further work is required to understand if PAs have a direct or indirect effect on the production of NO and ROS in stomatal guard cells (Agurla et al., 2018).

Polyamines-NO crosstalk during seed dormancy release and seed germination has been documented in *Malus domestica* (apple) (Krasuska et al., 2014). Nitric oxide, Put and Spd induced dormancy breakage and germination of apple embryos, in line with the stimulation of urea cycle and high free Arg pool in seedlings roots, whereas NO favoured embryos germination by increasing the level of free PAs (mostly Put) (Krasuska et al., 2014). High activity of arginase was observed in axes of embryos treated with Put or Spd, thus confirming a stimulatory effect of both PAs on N metabolism during apple embryos germination, whereas the opposite effect (low arginase activity) was shown for embryos imbibed in the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1- $\beta$ -oxyl-3-oxide (cPTIO) or Spm. These results indicated that dormancy release in embryonic axes strongly depends on NO and particularly, on PAs (Krasuska et al., 2016). In *Araucaria angustifolia*, it has been demonstrated that Spd and Spm supplementation into the culture medium of

embryogenic suspension cultures reduced growth and NO release; on the contrary, Put induced NO biosynthesis in embryonic cells (Silveira et al., 2006).

Low concentrations of exogenously added Put, Spd or Spm differently affected tobacco pollen germination and pollen tube growth through their influence on ROS and NO production. Putrescine had a certain positive effect on pollen tube emergence, but a negative one on its further elongation, Spd increased both processes, while spermine had a negative effect on pollen germination but did not influence pollen tube growth (Benkő et al., 2020). The authors concluded that PAs regulated pollen germination primarily via regulating the ROS level, while tube elongation mainly influencing the NO level.

The delayed dark-induced senescence in *Arabidopsis* PA back-conversion mutants, where conversion of Spm to Spd, and/or Spd to Put did not occurred, was associated with accumulation of Spm levels, a reduced production of ROS and, interestingly, an increase in the levels of NO (Sequera-Mutiozabal et al., 2016). It was suggested that Spm is a “signaling” metabolite involved in a strategy to cope with damage during senescence and against stress through metabolic conversions, involving redox state transitions, changes in sugar and nitrogen metabolism, cross-talk with hormones and mitochondrial electron transport chain modulation (Sequera-Mutiozabal et al., 2016).

In olive plants, the use of PAs biosynthesis inhibitors like cyclohexylamine (CHA) and methylglyoxalbis(guanylhydrazone) (MGBG), inhibitors of SPDS and SAMDC activity, respectively, significantly induced abscission of olive mature fruits by decreasing endogenously formed NO. In contrast, PA treatments induced NO production in the olive fruit abscission zone, being Spd the PAs that stimulated higher NO production in one of the cultivars compared with the Put treatment (Parra-Lobato et al., 2011).

These results demonstrated that PAs play a positive role in NO production during mature fruit abscission, indicating putatively PA-dependent NO biosynthesis as a potential link of NO and abscission, suggesting the presence of an unknown enzyme that converts PA directly to NO and other products, in agreement with previous data (Arasimowicz-Jelonek et al., 2009; Tun et al., 2006; Yamasaki & Cohen, 2006).

Filippou et al. (2013) performed a global analysis leading to a better understanding of the role of the NO donor SNP in the regulation of PAs metabolism in mature (40 day) and senescing (65 day) *Medicago truncatula* plants. The authors found that in long-term (24 h) treatments, 2.5 mM SNP increased Put, as a result of an increase in biosynthetic ADC. DAO activity increased significantly in correlation with Put levels, whereas PAO activity, involved in spermidine/spermine degradation, increased slightly. These results suggested that SNP-derived NO affected PAs metabolism in a time and developmental stage-dependent mechanisms that have to be further elucidated.

One novel aspect of PAs effects in close relationship with NO and H<sub>2</sub>O<sub>2</sub> is the Spm-induced autophagy in wheat roots, where Spm induced autophagy in a process accompanied by an increased generation of ROS and NO, which seem to play a signal role and are required to trigger autophagy in root cells (Dmitrieva et al., 2018).

#### 4.2 | Polyamines and NO: intimate friends in the signaling road to abiotic stress defense

In *Solanum lycopersicum* L., it has been shown that exogenous application of Spd, but not Put, induced NO accumulation and increased stress tolerance in leaves under chilling stress, by increasing the photosynthetic efficiency of photosystem II (Fv/Fm), reducing electrolyte leakage and inducing gene expression and activity of



antioxidant enzymes. After Spd application, the increase in NR and NOS-like activity observed was reversed by adding L-NAME or tungstate, concluding that Spd enhanced NO levels through the increase in NOS-like and NR activities. Remarkable data is that Spd, but not Put, also induced H<sub>2</sub>O<sub>2</sub> generation, and when plants were treated with catalase (CAT) prior to Spd, NO increase did not occur, suggesting that H<sub>2</sub>O<sub>2</sub> could be generated as a consequence of Spd catabolism and acts upstream of NO to enhance its accumulation (Diao et al., 2016).

When applied exogenously to mung bean plants exposed to cadmium (Cd), both Put and NO could enhance plant stress tolerance by diminishing Cd uptake and translocation to shoots, and reducing oxidative damage, either directly by scavenging ROS and also by improving the antioxidant system, which suggests that PAs and NO are interlinked through the biosynthetic and metabolic pathways (Nahar et al., 2016). The authors mentioned that, for the first time, it was reported that Put and/or SNP enhanced Cd tolerance by inducing methyl glyoxal detoxification through an improved glyoxalase system.

Application of exogenous melatonin improved tomato seedlings response to heat stress, through inducing PAs and NO accumulation and adjusting redox balance (Jahan et al., 2019). The effect of PAs and NO was evidenced by enhancing the activity of the antioxidant system, reducing malondialdehyde (MDA) content and membrane injury. Apparently, the increase in NO content was mediated by the up-regulation of NR and NOS-like activities and gene expression (Jahan et al., 2019). In the same way, Zhou et al. (2016) demonstrated that exogenous melatonin improved plant iron (Fe) deficiency tolerance by increasing PAs-mediated NO accumulation. In *adc2-1* mutants defective in Put biosynthesis, and in plants treated with D-Arg (ADC inhibitor), Fe content did not

increase after melatonin application, and iron deficiency symptoms could not be relieved, highlighting the important role of PAs in this response. Moreover, melatonin also failed in inducing Fe-deficiency tolerance in both *nia1nia2noa1* (*noa*: nitric oxide associated) mutants and cPTIO-treated plants, thus suggesting that NR is involved in NO production, and PAs somehow regulated this pathway (Zhou et al., 2016).

In tomato plants under alkaline stress, treatment with Spm or SNP could alleviate oxidative stress symptoms and improve growth parameters (Gong et al., 2014), whereas both compounds increased NO content, the addition of cPTIO reduced or eliminated NO or Spm-derived protection, thus suggesting that NO itself is the molecule that triggers tolerance mechanisms. Moreover, Spm induced NR gene expression, pointing out that this enzyme could be responsible for NO formation (Gong et al., 2014). Stress also induced the expression of *S/ADC S/ODC S/SAMDC S/SPDS S/SPMS* genes of PAs metabolism, being even higher with SNP or GSNO treatment, and this effect was abolished when cPTIO was co-applied with the NO donors. Gupta et al. (2013) combined the data available for PAs, DAO, PAO, and NO in salt stress, and speculated that PA-induced NO generation, possibly through DAO and PAO activity, might be an intermediate candidate involved in salt-stress tolerance, whereas Peng et al. (2016) reported that, in *Trifolium repens*, Spd played a role in drought stress-activated pathways associated with NO release, which mediated antioxidant defense, contributing to drought tolerance in this plant.

Exogenous application of Spd or Spm induced a significantly higher production of NO at 12 h and 24 h of chilling stress in tomato seedlings, accompanied by an induction of NR and NOS-like activities, and also DAO and PAO activities. In contrast, treatment with Put did not induce neither NO accumulation nor the induction of these enzyme

activities (Diao et al., 2017). It was suggested that NO generation upon Spd or Spm application was mediated by a H<sub>2</sub>O<sub>2</sub>-dependent NR or NOS-like increased activity and Put could improve chilling tolerance via activation of abscisic acid (ABA) synthesis.

In soybean callus, Spd, Spm and Put increased DAO activity and NO content, as well as H<sub>2</sub>O<sub>2</sub>, but addition of catalase or DMTU (dimethylthiourea, an H<sub>2</sub>O<sub>2</sub> scavenger) previous to the treatment with PAs, reduce H<sub>2</sub>O<sub>2</sub> and reverse PA-induced NO increase, suggesting that H<sub>2</sub>O<sub>2</sub> is necessary to PA-induced NO generation (Yang et al., 2014). Besides, adding aminoguanidine (AG, a DAO inhibitor) previous to PA treatment, reduced NO formation, which correlated with a decrease in DAO activity. However, considering that AG can inhibit both DAO and animal NOS, the authors cautiously concluded that DAO might be involved in PA-induced NO biosynthesis and that H<sub>2</sub>O<sub>2</sub>, which was required in PA-induced NO generation, might be derived from PA oxidative degradation (Yang et al., 2014).

As well as there is compelling evidence that demonstrates that PAs induce NO accumulation and this NO mediates PAs-induced stress tolerance, some authors have also demonstrated that NO can modulate PAs metabolism leading to an improved stress response. For instance, it was observed that exogenous application of NO (as SNP) enhanced chilling tolerance of banana fruits and bamboo shoots, by up-regulating ADC and ODC genes and enzyme activities and thus increasing PAs content, in a response also mediated by GABA and Pro (Wang et al., 2016, 2017). It was also observed higher Spm levels in sunflower seedling cotyledons under salt stress treated with NO as compared with saline treatment alone, due to upregulation of the PA biosynthetic enzymes ADC and SAMDC (Tailor et al., 2019). Although NO donor application significantly increased the production of Spm, it did not alter the level of

free Spm but enhanced the proportion of conjugated and bound Spm, irrespective of salt stress. In cucumber seedlings under NaCl stress, NO treatment reduces Put and Spd content, and increases Spm content, thus restoring (Spd + Spm)/Put to control values, consequently improving growth parameters (Fan et al., 2013).

## 5 | Polyamines as a novel source of NO

Given that PAs and NO share common functions in physiological and stress responses, and considering that it has been demonstrated that they act together, at least under certain circumstances, a question that arises is how one could induce the synthesis (or accumulation) of the other, and therefore act synergically or be the link by which they could execute their actions. Since Tun et al. (2006) documented for the first time the increased NO release in *Arabidopsis* seedlings after treatment with Spd and Spm, it has been proposed the presence of an unknown enzyme responsible for the direct conversion of PAs to NO. In this work, it was observed that Spm and Spd, but not Put, induced NO biosynthesis in *Arabidopsis* root tip and in primary leaves, but not in cotyledons, suggesting that this induction is tissue specific, and also mediated by an enzyme because there is no lag phase between the addition of Spm and the NO increase. Since Arg did not cause a significant increase in NO level compared to control, the contribution of NOS-like activity was discarded. Accordingly, Wimalasekera et al. (2011b) suggested that NO could be directly generated from PAs by CuAO, since *Arabidopsis CuAO1* mutants were impaired in NO accumulation in response to PAs and ABA treatment compared to wild type plants.

It has been widely discussed that the mechanism by which PAs induce NO accumulation involves  $H_2O_2$  as a mediator in the regulation of NOS, NR or both. Wang et al. (2010) reported that DAO and PAO might be responsible for generating NO from PAs, but they also produce  $H_2O_2$ , a known signal to act upstream of NO biosynthesis in response to certain stimuli. A different explanation was proposed by Groß et al. (2017) in *CuAO8* Arabidopsis mutants, where a lower NO accumulation compared to wild type plants was observed in response to salt stress. The authors observed that the impairment in NO production was not caused by a change in NR activity but it could have been due to a higher arginase activity in *CuAO8* mutants, which cause a lower Arg bioavailability affecting NO formation via a NOS-like pathway (Groß et al., 2017).

In spite of the proposed hypothesis explaining NO generation from PAS mentioned above, several differences have been observed concerning the behavior of each PA with respect to NO formation or the precise circumstances in which PAs induce NO formation, either affecting NR, NOS-like or both proteins. Moreover, the mechanisms involved in  $H_2O_2$  regulation of NR or NOS-like-dependent NO formation, as well as to know if  $H_2O_2$  is essential to regulate this process are points that remain unanswered to date. Another question to be resolved is to find out if DAO or PAO enzymes are really potential sources of NO, in which case, the role of  $H_2O_2$  in this pathway becomes questionable.

## 6 | PAs-NO and posttranslational modifications

Among the multiple functions attributed to NO in the road of plant signaling, it is now evident that NO performs this function through protein post-translational

modifications (PTMs), with nitration and S-nitrosylation evolving as the most important ones described until now (Corpas & Barroso, 2013; Gallego & Benavides, 2019; Lounifi et al., 2013; Sandalio et al., 2019). In view of the close relationship between PAs and NO at multiple levels (formation, biosynthesis regulation, responses against stress, involvement in plant growth and development, among others), it is of major interest to understand the crosstalk of PAs and NO in PTMs, a knowledge that is still limited concerning how this PTM is regulated by factors that regulate PAs and NO signaling in plants.

S-nitrosylation of proteins occurs when sulfhydryl group (–SH) of cysteine residues of proteins or peptides reacts with NO (Astier et al., 2012; Fu et al., 2018), whereas tyrosine (Tyr) nitration is the reaction of a nitrating agent with a Tyr residue of a target protein (Kolbert et al., 2017; Mata-Perez et al., 2016). Polyamine regulation of nitrosative signaling remains uncertain, particularly during stress conditions (Filippou et al., 2013; Parra-Lobato & Gómez-Jiménez 2011; Wimalasekera et al., 2011b). In citrus plants treated with NaCl, alone and in combination with PAs, Tanou et al. (2014) performed S-nitrosylated proteome analysis, finding that tyrosine nitration is depressed by Spd or Spm, while protein S-nitrosylation increases in response to all PAs. Spermidine plus NaCl diminished the accumulation of nitrated proteins compared with NaCl treatment, whereas Spm plus NaCl lowered nitration to levels close to unstressed control plants, indicating that PAs were able to modulate nitrosative status. Furthermore, S-nitrosylation was enhanced by PAs, particularly by Spd, showing a substantial modulation of nitrosylation by PAs (Tanou et al., 2014). On the other hand, *Arabidopsis* T-DNA insertional mutants deficient in *CuAO1* display lower NO production, whilst enhanced S-nitrosylation following PAs treatments were detected in

Col WT plants, decreasing S-nitrosylated proteins of 1 mM Spd-treated seedlings when they were pre-treated with 200  $\mu\text{M}$  cPTIO (Wimalasekera et al., 2011b). These observations suggest that the possible relationship between PAs and NO-originated nitrosative signaling could regulate stress responses in plants. The biological significance of these changes in protein modification should be explored in the future.

In apple embryos, a transient increase in total soluble nitrated proteins level was detected after 2 days of embryo imbibition in Put or Spd, in accordance with changes in superoxide anion ( $\text{O}_2^-$ ) and NO levels (Krasuska et al., 2014). As the culture was prolonged,  $\text{NO}_2^-$  concentration and also total soluble nitrated proteins level were decreased in axes of apple embryos germinating in Put or Spd. An altered pattern of nitrated proteins during dormancy release of apple embryos, and NO-induced decline in total amount of these proteins have been reported (Krasuska et al., 2016). The authors concluded that some of nitrated proteins belong to storage proteins and nitration of Tyr marked them to degradation, suggesting that protein nitration level could be the indicator of progression of dormancy breakage and probably of growth initiation of embryonic root. The question is, if Tyr-nitrated or S-nitrosylated proteins lose their ability to bind PAs, and become more prone to degradation (Krasuska et al., 2016).

## 7 | Concluding remarks and future perspectives

In this review we have updated the main published results about the crosslinked responses involving two essential participants in N metabolism, PAs and NO, in multiple processes related to plant growth, development and environmental

challenges (Fig. 1). The mechanisms associated to the close relationship between NO and PAs that explain their interdependence are still puzzling. It is unclear whether polyamines act as substrates, cofactors, or signals for promoting NO synthesis. Despite there being much evidence supporting the fact that NO and PAs can stimulate each other's production, it appears that PAs depend on NO as a downstream signal to trigger various stress responsive mechanisms to improve stress tolerance, not exerting their effects by themselves, at least under certain circumstances. Several reports have demonstrated that exogenous PA treatment result in a higher level of NO in plants (Tun et al., 2006; Groppa et al., 2008; Rosales et al, 2011, 2012; Diao et al., 2016, 2017; Recalde et al, 2018), whereas it was shown that NO influences PAs metabolism (Diao et al., 2016, 2017; Gong et al., 2014; Groppa et al., 2008; Wang et al., 2016, 2017; Tailor 2019).

There are many unanswered questions that still need to be responded: if NO is exerting its signaling function downstream of PAs when PAs are exogenously added, when they increased in response to environmental factors, or in both cases; if PAs regulate NO formation always by affecting NR or NOS-like enzymes activity and, in this case, H<sub>2</sub>O<sub>2</sub> the molecule that mediates this regulation; if, in addition, NO is formed from PAs in an enzymatic route catalyzed by an unidentified enzyme or an oxidation process mediated by CuAO or PAO; if NO, a product of NR activity or PAs catabolism, is the most important molecule that operates at the interface between nitrate perception and transduction, so it is a principal player in the overall physiological and developmental plant adaptation to N nutrition

Last, but not least, even though a dual signaling role for PAs, exerted through NO and H<sub>2</sub>O<sub>2</sub> under normal physiological conditions or under abiotic stress has been revealed,



many pieces of the puzzle are still missing. Modification of a single step in N or PAs metabolism could impact greatly in the metabolic grid of the cell. The elucidation of PAs and NO- dynamic roles in the signaling route connecting the components of the complex network of N metabolism constitutes an exciting field of research to understand how PAs and NO interact to manage N flux for growth and development in changing environmental conditions.

In light of the importance of N for plant growth and the environmental costs of acute fertilization as well as the increasing importance of improving the efficiency of N uptake, distribution and use, it is time to elucidate the intricate relationship among N as a nutrient with PAs and NO as emerging signaling molecules in the heart of the N hub. The close cooperation among these players in the whole N metabolism is essential to know the molecular mechanisms underlying plant adaptation to N fluctuations and is a primary objective for the development of biotechnological tools for sustainable agriculture in developing countries

#### **Declaration of Competing Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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