

Review article

NO to drought-multifunctional role of nitric oxide in plant drought: Do we have all the answers?



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ABSTRACT

Nitric oxide (NO) is a versatile gaseous signaling molecule with increasing significance in plant research due to its association with various stress responses. Although, improved drought tolerance by NO is associated greatly with its ability to reduce stomatal opening and oxidative stress, it can immensely influence other physiological processes such as photosynthesis, proline accumulation and seed germination under water deficit. NO as a free radical can directly alter proteins, enzyme activities, gene transcription, and post-translational modifications that benefit functional recovery from drought. The present drought-mitigating strategies have focused on exogenous application of NO donors for exploring the associated physiological and molecular events, transgenic and mutant studies, but are inadequate. Considering the biphasic effects of NO, a cautious deployment is necessary along with a systematic approach for deciphering positively regulated responses to avoid any cytotoxic effects. Identification of NO target molecules and in-depth analysis of its effects under realistic field drought conditions should be an upmost priority. This detailed synthesis on the role of NO offers new insights on its functions, signaling, regulation, interactions and co-existence with different drought-related events providing future directions for exploiting this molecule towards improving drought tolerance in crop plants.

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1. Introduction

Crops growing in arid- and semi-arid regions are constantly confronted with water deficit conditions resulting in compromised yields. Since frequent and severe drought can lead to crop damage, exploring the mechanisms of drought tolerance in plants can substantially improve crop production. Nitric oxide (NO), being a small diffusible free radical that plants use as a gaseous signaling molecule adapts them to stressful conditions by modulating various physiological processes, thereby enhancing their survival [1–3]. NO emission from plants was demonstrated as early as 1979 by air purging of herbicide-treated soybean leaves, and thereafter NO has long been of major interest in both plant and animal research [4]. Initial findings implicated NO as a modulator of plant defense during plant pathogen interactions [2,3]. Thereafter, it has gained increasing attention by plant researchers because of its involvement in diverse physiological processes from promoting seed germination [5] to regulation of plant maturation and senescence [6]. NO is involved in mediation of stomatal movement [7,8], and light-mediated greening and suppression of floral transition [1,2], besides having a prominent role in regulation of a plethora of abiotic and biotic stresses such as drought, high light, salinity, cold, heat, and pathogen infection [3,9,10]. Increasing evidence supports its role in early nodulation in legumes and symbiotic interactions involving arbuscular mycorrhizal fungi, along with its well-established function in controlling root organogenesis, development of lateral roots and root hairs [1]. Considering the rapidity of publications in this area of research, NO seems now to be accepted as an important signaling molecule in regulating various cellular processes in different plant organs, from roots to fruits and responses to a range of endogenous signals and exogenous stimuli [1–3].

Despite the growing knowledge about NO-mediated plant functions, detailed information on its functional status with respect to individual stress conditions has so far been illusive. Although NO is recognized as multitasked molecule with innumerable functions in plant drought responses, many questions remain unanswered [1]. Queries on the origin and signaling of NO during water deficit, identifying the target molecules for its action, and the physiological and molecular processes involved in NO-mediated drought stress amelioration still need to be addressed. Another important aspect that remains elusive is to what are the commons and contradictions in the results of NO-drought studies? While, NO pretreatment has been shown to activate plant defenses so as to better prepare the crop to defend itself when actual stress occurs [7,9]. How far the application of pharmacological compounds reflects the true physiological effect of NO? is also debatable.

It is difficult to draw any meaningful conclusions from many of the individual studies, since most of them are rather descriptive without exploring the underlying signaling cascades. Hence, this review aims to bring together these scattered sets of data from various published studies to synthesize meaningful conclusions and cautions in NO-drought studies.

2. NO and water deficit responses in plants

Many studies have reported an increased production of NO in drought-stressed plants [[11–24]; Table 1]. Drought stress-induced NO in a wide variety of plant species including vegetables, horticultural plants, epiphytes etc. suggest universal requirement of NO during drought stress signaling. The accumulation of NO also depends on the duration and severity of the given drought stress as observed in the case of *Cucumis sativus* where the roots had a slight enhancement in NO synthesis when subjected to <10 h water

deficit, while increased to a greater extent with prolonged drought of up to 17 h [17,18].

2.1. Exogenous NO donors in drought stress amelioration

Owing to the gaps in our knowledge on the molecular identities and mechanism of NO generation in plants, the current research on NO in plants mostly relies on exogenous application of NO-donors and inhibitors/scavengers ([25–39]; Table 2). Most commonly used NO donors in plant drought stress are sodium nitroprusside (SNP; Na₂ [Fe(CN)₅NO]·2H₂O), S-nitroso-N-acetylpenicillamine (SNAP), S-nitrosoglutathione (GSNO), and diethylamine NONOate sodium (DEA-NONOate). These differ substantially in their bio-response due to their ability to release NO in different redox forms, such as NO* from SNAP and GSNO, NO⁺ from SNP (Table 2). Although accumulation of NO during drought stress appears to be a general response in diverse plant species and tissues, its specificity has only been established by using various inhibitors/scavengers such as c-PTIO [2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide] or L-NAME [(N(G)-nitro-L-arginine-methyl ester)] that reverse the process(s) [7,14,17,18,23,25,30,38,39]. Similarly, the kinetics of NO release from donors depends on many factors such as the reactivity of donor, surrounding environment, chemical nature of tissue, light, concentration and active duration of exposure etc. NO release by SNP is a result of photochemical reactions, while GSNO can release NO even in the dark, both processes being slow and stable in contrast to DEA-NONOate induced quick NO burst that decays rapidly. Nonetheless, while using NO donors in drought stress studies, a potential consideration is to elucidate the complete NO release mechanisms and bioactivities of their by-products. For example, SNP the most often used NO donor in drought stress studies leaves cyanide and iron ions as by products [40]. Cyanide being bioactive is involved in inhibition of nitrate reductase (NR) and cytochrome c oxidase that regulates the NO production. However, the amount of NO released from such donors and their time course is often not taken into consideration while interpreting the results. Similarly, the stability of these molecules in the given experimental conditions, and their interaction with other molecules in vitro and in vivo needs to be addressed to understand the cause and effects before making any conclusions. Generating release profiles of a range of NO donors through systematic assessment using adequate controls with byproducts will counter these concerns, to allow critical and better comparison of data across NO-drought studies.

2.2. NO generation and signaling under water deficit

In spite of growing evidence demonstrating the induction of NO in plants by water deficit, pathways responsible for the NO production are not yet completely identified. NR that converts nitrite to NO in a NAD(P)H-dependent manner is the well-known NO-generating enzyme in plants under water deficit [14,18,30,41]. NR is encoded by *NIA1* and *NIA2* in *Arabidopsis*, while the double mutant of *nia* genes resulted in little NO generation in the guard cells in response to ABA [20]. The synthesis of NO by NR activity has been corroborated in many plant species under dehydration [[1,8,17,18,30,42]; Table 2]. Although, the plasma membrane-bound nitrite:NO reductase and xanthine oxidoreductase are also known to reduce nitrite to NO, the contribution of these pathways in drought-induced NO generation is still not validated [41,42].

An arginine-dependent NO production involving NOS-like enzyme in drought-induced NO generation has been demonstrated by measuring NOS activities and by suppressing NO production by mammalian NOS inhibitors [[11,17,19,30,38,43,44]; Table 2]. While several reports support an arginine-dependent NO production in higher plants, the existence of a NOS gene is debatable

Table 1
Drought-induced nitric oxide (NO) generation in plant tissues and methods used for its detection.

Plant Species	Experimental system	Tissue	Method of detection	NO accumulation	Reference
<i>P. sativum</i>	Dehydration at 24 °C for 2 h in dry air	Seedlings	NO specific probe	160 nM g ⁻¹ FW ^a	[9]
<i>Oryza sativa</i>	15% PEG ^a for 24 h/200 mM mannitol for 24 h	Leaves	Fluorometric (DAF-2DA) ^a	3- fold over control	[11,12]
<i>Zea mays</i>	12% PEG for 10 d	Root tips/leaves/seedlings	Griess reagent	6 and 2 nM g ⁻¹ DW ^a	[13,14]
<i>Ailanthus altissima</i>	Withholding water for 14 d	Leaves	Griess reagent	0.3 μM g ⁻¹ FW	[15]
<i>Medicago truncatula</i>	Withholding water for 9 d	Seedlings/roots/leaves	Griess reagent	0.7 and 3 nM g ⁻¹ FW	[16]
<i>Cucumis sativus</i>	Withholding water for 5–17 h	Seedlings/roots/leaves	Fluorometric (DAF-2DA)	300 A.U. ^a	[17,18]
<i>Poncirus trifoliata</i>	Dehydration for 6 h	Leaves	NO assay kit	40 μM g ⁻¹ protein	[19]
<i>Arabidopsis thaliana</i>	Withholding water for 21 d	Leaves	Hemoglobin assay	2.5 μM g ⁻¹ FW	[20,21]
<i>Vitis vinifera</i>	Withholding water for 7 d	Leaves	Fluorometric	2-fold over control	[22]
<i>Citrus aurantium</i>	13% PEG for 12 d	Leaves	Griess reagent	0.03 μM g ⁻¹ FW	[23]
<i>Guzmania monostachia</i>	30% PEG for 7 d	Leaves	Spectrofluorimetry (DAR4M) ^a	40000 A.U.	[24]

^a DAF-2DA (diaminofluorescein diacetate); DAR4 M (diaminorhodamine-4 M); PEG (polyethylene glycol); DW (dry weight); FW (fresh weight); A.U (arbitrary units).

[45]. Interestingly, the T-DNA insertional mutant (*atnos1*) of *Arabidopsis thaliana*, that was initially identified as the first plant NOS based on its homology to a snail protein [41,42,45] failed to show any NO synthase activity in the recombinant AtNOS1 protein and enzymes in maize and rice bearing its gene orthologs. This indicated the *A. thaliana nitric oxide associated* (*AtNOA1*) as a regulator of NO rather than the molecule of synthesis [45,46]. Subsequently, two genes from the green algae, *Ostreococcus tauri* and *Ostreococcus lucimarinus* were considered to be the first NO synthase genes identified in the plant kingdom based on structural modeling, having 44–45% homology to animal NO synthase genes [47]. While NR and NO synthase enzymes contribute predominantly to NO production under water deficit conditions, other molecules such as polyamines are also involved [18,41]. Interestingly, NO can also be released non-enzymatically by the interaction of two nitrous acid (HNO₂) molecules derived from protonated nitrite under low pH, by reduction of NO₂ to NO by carotenoids under light, or by oxidation of hydroxylamine and salicylhydroxamate in plant cell cultures [1,42].

While chloroplasts have been hypothesized to be the sites of NO production in *A. thaliana*, *Nicotiana tabacum* and *Brassica juncea* for over a decade [22], recent evidence indicates peroxisomes being the source of NOS activity and NO production and the role of cytoplasm still needs further elucidation [2,17,48]. Future studies should target the where and how of NO origin and signaling to understand the functional relevance of local signaling, particularly under water deficit conditions.

There has been sufficient data suggesting NO as an endogenous signal that mediates plant responses to various stimuli. While the NO dependent protein modifications have been identified for specific regulatory proteins, no general mechanism that coordinates NO sensing across multiple plant processes have been identified. A unifying mechanism for NO sensing in plants based on targeted proteolysis of plant-specific group VII ERF transcription factors has been recently elucidated in *A. thaliana* showing that the N-end rule pathway proteolysis is essential for NO perception throughout the plant life cycle [49]. This was based on the observed NO insensitivity in seedlings of Arg/N-end rule pathway mutants (*prt6* and *ate1ate2*) and transgenic plants (*promRAP2.3::MA-RAP2.3*) to the donors. These findings identified VII ERFs as central hubs for the perception of both NO and oxygen and thus identifying the N-end rule pathway as a key integrator of multiple gaseous and other signals in plants. [49]. Investigations on, whether NO is perceived by similar mechanism or by any other means during drought are critical to identify the NO sensing mechanism in plants undergoing water stress.

Free radical nature and the ability to diffuse across membranes leads to a wide range of interactions with target proteins via direct chemical modifications in a redox and concentration-dependent fashion. NO can rapidly react with transition metal-containing proteins of a wide functional spectrum such as receptors, transcription

factors, and cellular messengers. Though the NO signaling pathway under drought stress is not fully defined several components depend on NO for stress response. These include secondary messengers such as Ca²⁺, cyclic guanosine monophosphate (cGMP), hydrogen peroxide (H₂O₂), phytohormones such as abscisic acid (ABA), protein kinases such as serine/threonine protein kinase (OST1), and mitogen-activated kinases (MAPK) which, in turn can modulate expression of target genes that are involved in stress recovery [8,50]. A possible role of NO as a signaling intermediate involved in guard cell movements have been laid out [8].

Water stress causes cellular dehydration resulting in loss of turgor that triggers ABA synthesis that activates H₂O₂ generation followed by NO generation via a signaling pathway involving ABA receptors, Ca²⁺/calmodulin, the OST1 protein kinase etc [51]. NO enhances antioxidant enzyme activity and induces stomatal closure through steps that require MAPKs, cGMP and Ca²⁺, though signaling pathways are not clearly defined. Besides MAPK, NO also activates other protein kinases such as osmotic stress-activated kinase, NtOSAK, in *N. tabacum* [2,50]. NO alters protein phosphorylation and altered calcium flux to have normal NO responses to occur in the guard cells [8]. Both salt and osmotic stresses induce a rapid increase in the cGMP content of *A. thaliana* seedlings [1]. In addition, cGMP-independent post-translational protein modifications such as S-nitrosylation or tyrosine nitration of various proteins and phytohormones is also emerging as a potential way by which NO may have its global effects, especially under stress conditions [50]. Several genomic and proteomic techniques have recently been deployed to identify the key NO interacting molecules in plants [52]. Nevertheless, whether NO has its own signaling pathway or acts by influencing other signaling molecules and pathways resulting in physiological response to the given conditions still need clarification.

Of course, a major challenge is to develop accurate and sensitive methods for the detection and measurement of NO concentration in planta. A major portion of drought-induced NO release is demonstrated by in vivo deployment of NO-sensitive cell-permeable fluorophore diamino-fluorescein forms (DAF-2DA or DAF-FM DA) [17,18]. Other methods such as 'Griess reagent' assay based on measuring nitrite, a stable NO metabolite, via the Griess reaction, and hemoglobin assay based on conversion of oxyhemoglobin to methaemoglobin also contribute to the available data (Table 1) [18,19,20,22]. Methods such as Griess reaction and hemoglobin assay are destructive and detect NO only after cell extraction. In vivo bio-imaging using fluorophores such as DAF depend on the oxidation of NO may not reflect the specific quantity of NO production in the cell. Technical issues associated with the specificity and accuracy of probes and methods can be partially resolved by using at least two different methods for measuring NO in plant drought stress [53]. Moreover, measuring overall changes in NO production under water deficit may not be sufficient to understand the spatiotemporal effects of NO as a drought-signaling molecule. Fur-

Table 2
Various nitric oxide (NO) donors, scavengers\inhibitors and methods for the detection of NO used for drought tolerance studies in plants under imposed drought stress in vitro using polyethylene glycol (PEG) or by withholding water in pots.

Plant species	Drought imposition	Source of NO	Scavenger\inhibitor	Method of detection	Response	References
<i>Oryza sativa</i>	Water withholding	150 μ M SNP ^a	200 μ M cPTIO	NM ^a	Maintenance of tissue water potential and enhanced capacity of antioxidants, improved stability of cellular membranes, and enhanced photosynthetic capacity	[12,25]
<i>Triticum aestivum</i>	Water withholding	150, 200, 300 μ M SNP	500 μ M cPTIO ^a	NM	Stomatal closure, enhanced capacity of antioxidants, improved stability of cellular membranes, enhanced proline content	[26,27,28]
<i>Zea mays</i>	PEG ^a	100 μ M SNP	400 μ M cPTIO; 25 μ M L-NAME ^a , 100 μ M NaN ₃ ^a	Griess reagent	Enhanced capacity of antioxidants, induced glycine-betaine accumulation	[14]
<i>Vicia faba</i>	Water withholding	200 μ M SNP	400 μ M cPTIO	;Griess reagent, DAF-2DA ^a	Stomatal closure, reduced ion leakage and cell injury index	[7,29]
<i>Medicago truncatula</i>	PEG	500 μ M DEA-NONOate ^a	250 μ M cPTIO, 500 μ M L-NAME	DAF-2DA	Stomatal closure, no accumulation of proline, reduced seed germination	[30]
<i>Cucumis sativus</i>	PEG	100 μ M SNP ^a , 100 μ M GSNO, 1.0 mM spermine 1.0 mM spermidine	200 μ M cPTIO, 200 μ M L-NAME, 100 μ M Tungstate	DAF-2DA	Enhanced capacity of antioxidants	[17,18]
<i>Poncirus trifoliata</i>	Water withholding	100 μ M SNP	100 μ M L-NAME	Griess reagent	Antioxidant defence, stomatal closure	[19]
<i>Arabidopsis thaliana</i>	Water withholding	50, 100, 300, 1000 μ M SNP, 300 μ M SNAP	100 μ M cPTIO, 500 μ M L-NAME	DAF2-DA	Stomatal closure, altered gene transcription, enhanced seed germination, transcriptomic analysis of nNOS transgenic plants	[20,21,31]
<i>Malus hupehensis</i>	Water withholding	300 μ M SNP	NU ^a	NM	Enhanced photosynthesis	[32]
<i>Populus przewalskii</i>	Water withholding	>500 μ M SNP	NU	NM	Enhanced photosynthesis, enhanced capacity of antioxidants, enhanced proline and amino acids	[33]
<i>Phaseolus vulgaris</i>	Water withholding	100 μ M SNP	NU	NM	Reduced ion leakage and cell injury index, enhanced stomatal conductance, enhanced capacity of antioxidants	[34]
<i>Vigna unguiculata</i>	Water withholding	100 μ M SNP	NU	NM	Enhanced stomatal conductance, reduced ion on leakage and and cell injury index, enhanced capacity of antioxidants	[34]
<i>Citrus aurantium</i>	PEG	NA ^a	300 μ M cPTIO	Griess reagent, DAF-2DA	Enhanced capacity of antioxidants, enhanced S-nitrosylation, enhanced gene expression	[23]
<i>Solanum lycopersicum</i>	Water withholding	100 μ M SNP	NU	NM	Enhanced capacity of antioxidants, increased carbonic anhydrase activity and photosynthesis	[35]
<i>Tradescantia</i>	Water withholding	150 μ M SNP	300 μ m cPTIO	Griess reagent	Stomatal closure, reduced ion leakage and cell injury index	[7]
<i>Ginkgo biloba</i>	Water withholding	250 μ M SNP	Hemoglobin	NM	Rise in proline, soluble sugar, flavonoid and ginkgolide content.	[36]
<i>Agrostis stolonifera, Lolium arundinaceum</i>	Water withholding	150 μ M SNP	NU	NM	Reduced ion leakage, high chlorophyll and proline content, enhanced antioxidant defence	[37]
<i>Tagetes erecta</i>	PEG	10 μ M SNP	200 μ M cPTIO, 25 μ M -L-NAME	NM	Increased leaf chlorophyll content, chlorophyll fluorescence parameters, adventitious rooting, increased soluble carbohydrate and protein content, decreasing starch content	[38]
<i>Dendrobium huoshanense</i>	PEG	50 μ M SNP	50 μ M cPTIO	NM	high level of relative water content, lower content of malondialdehyde (MDA), reduced DNA methylation	[39]

^a GSNO (S-nitrosoglutathione); SNP (Sodium nitroprusside); SNAP (S-nitroso-N-acetylpa (ennicillamine); DEA-NONOate (Diethylamine NONOate sodium); DAF-2DDiaminofluorescein diacetate); NaN₃ (Sodium azide); NM (Not measured), NU (Not used); DAF-2DA (diamino-fluorescein di acetate), L-NAME (N(G)-nitro-L-arginine-methyl ester), cPTIO (2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide).

ther investigations should focus on revealing precise biochemical description at the organ level, or cell fractions across the duration of stress to elucidate the kinetics of NO production and associated physiological response.

2.3. NO as a modulator of stomatal movement

Stomatal closure is widely used as a surrogate in water deficit studies since stomates act as gateways for transpiration, having immense effect on minimizing water loss under water deficit [7,8]. Stomatal movements act as a convergence point between developmental and stress tolerance in plants, and are under the control of an array of signaling components such as ROS, NO, Ca²⁺, protein kinases etc. [18]. While the involvement of plant growth regulator like abscisic acid is well-studied under drought stress, the exact mechanisms by which NO controls stomatal closure under drought stress is still not very clear [54]. NO accumulates in the guard cells of *Vicia faba* epidermal strips during dark-induced stomatal closure [55], which is reduced up to 50% by NO scavenger treatment, asserting the role of NO in stomatal closure. Subsequently, several studies suggested the generation of NO in guard cells in response to drought and ABA [30,34,54]. The accumulation of NO has often been visualized in guard cells in response to water deficit by using a NO-sensitive fluorescent dye DAF-2DA [10,15,16,23,40]. The increase in NO production correlated significantly to the decrease in stomatal conductance in potted *Vitis vinifera* under drought stress [22]. Exogenous treatment with SNP also resulted in reduction in stomatal opening in epidermal strips of *Salpichroa organifolia* and *Tradescantia* sp. [7] that gets reversed by cPTIO treatment [8].

NO has been implicated as an important player in ABA-mediated signaling pathways during stomatal closure [1,24,54]. Stomata close in response to NO or ABA during drought, while inhibitors/scavengers of NO synthesis reverse this process confirming the importance of NO as an intermediate in ABA-mediated stomatal closure in many plants [8,20,56]. ABA failed to induce stomatal closure in *A. thaliana* NO mutant nitric oxide associated1 (Atnoa1) and nitrate reductase defective double mutant nia1nia2 having reduced endogenous NO levels, providing genetic evidence for the indispensable requirement of NO during ABA-mediated closure of stomatal guard cells [20,54]. This also suggested the involvement of NR as a source of NO generation in guard cells of *A. thaliana* during ABA-induced stomatal closure [8]. Furthermore, treatment with SNP also induces increase in the content of ABA by up-regulating the expression of a key gene such as 9-cis-epoxycarotenoid dioxygenase involved in ABA biosynthesis [10]. NO is not only involved in drought-induced ABA synthesis, but can also influence its signaling that negatively regulates the ABA sensitivity during drought. NO-deficient triple mutant nia1nia2noa1-2 plants were hypersensitive to dehydration and ABA treatment in stomatal closure [20]. Recently, GSNO reductase deficiency in *Arabidopsis* gsnor1-3 mutant- that resulted in NO over-accumulation in guard cells promoting constitutive S-nitrosylation of open stomata 1 (OST1)/sucrose non-fermenting 1 (SNF1)-related protein kinase 2.6 (SnRK2.6) and impairment of ABA-induced stomatal closure emphasizes the role of NO in desensitizing the ABA signaling after a period of drought [51]. While these studies reinforce that NO is a key intermediate in ABA-induced stomatal closure, whether the increased levels of NO in stomatal guard cells during dehydration stress is a direct consequence of stress or indirect effect of ABA needs to be further studied in depth.

Stomatal movement involves a set of players including calcium, kinases, H₂O₂ and plant growth regulators that possibly interact with NO. In *A. thaliana*, H₂O₂ accumulation either precedes or parallels the accumulation of NO in response to ABA, light or drought. NO production in *Pisum sativum* guard cells appears to occur downstream of ROS production during stomatal closure [10,55,57].

Mutation in NADPH oxidase resulted in reduced NO production and stomatal closure in respiratory burst oxidase homolog mutant of *A. thaliana* [55]. In addition, H₂O₂ induces alkalization in the cytosol by inhibiting K⁺ channel activity and stimulates NO signaling in guard cells in the presence of ABA, that suggests a link between ROS generation and NO production during stomatal closure [21]. NO promotes Ca²⁺ transients during ABA-induced stomatal closure in response to stress and non-stress conditions [3,51,56]. NO induces Ca²⁺ release from intercellular Ca²⁺ stores through cGMP/cyclic ADP Ribose (cADPR) dependent signaling pathway [56,21]. This elevation in free cytosolic Ca²⁺ leads to stomatal closure either by disabling K⁺_{in} channels and/or by stimulating Cl⁻ ion channels [21,29]. In parallel, ABA-induced NO activates phospholipase C and/or phospholipase D pathways to generate polyamines that in turn can induce Ca²⁺ release from internal stores. Phospholipase D hydrolyses the structural phospholipid to phosphatidic acid (PtdOH) that acts as secondary messenger during stomatal closure. Phospholipase D mutants of *A. thaliana* lacking the ABA-induced phosphatidic acid production failed to close their stomata in response to NO, thereby implying the involvement of phospholipase D in NO signaling during stomata closure [8]. Adding to the complexity, phytohormones such as jasmonic acid, ethylene, auxin and cytokinin are also known to regulate stomatal closure [58]. While ABA being the dominant player acts in concert with NO and jasmonic acid during stomatal closure, the role of ethylene is still ambiguous as it can regulate stomatal movement in both ways under drought stress. Ethylene releasing compounds such as ethephon and ethylene precursor 1-aminocyclopropane-1-carboxylic acid not only inhibit NO accumulation but also the ABA-and dark-and light-induced stomatal closure [31,57]. On the other hand, it can induce stomatal closure by stimulating H₂O₂ production. While auxin and cytokinins act as positive regulators of stomatal opening at normal physiological concentrations, they tend to induce stomatal closure at high physiological concentrations [58]. Further studies need to connect these scattered sets of events and molecules associated with NO to generate the full length signaling pathway models that regulate stomata under drought stress.

With the currently available information, it is clear that the regulation of stomatal closure is one of the well studied NO responses in plants, and most studies suggest its role in the induction of stomatal closure [7]. In some studies however, two-way regulation of stomatal movements by NO has also been observed [29]. Although nitrite or SNAP treatment increased the stomatal pore size in *V. faba* leaf peels as a result of enhanced levels of nitrate reductase-dependent NO production in guard cells [29], stomatal movements under dehydration were not studied. It seems that in *A. thaliana*, stomatal closure by ABA is independent of NO since the requirement of NO was important during well-hydrated conditions but not during dehydration [31]. In this study, scavenging NO did not inhibit ABA-induced stomatal closure in the leaves under water deficit. The kinetics of stomatal closure in wild type and nitrate reductase double mutant (nia1nia2) plants was similar in response to ABA and nitrite under dehydration, suggesting NO-independent stomatal closure by ABA [20,31]. NO concentration also appears to be a deciding factor under stress as high concentrations facilitate stomata opening [29,31,57]. New insights into protein interactions, the co-expression of genes, and metabolic factors that are involved during NO-mediated stomatal movements could be very valuable. Most of our understanding of NO-mediated stomatal movements under drought is based on fluorophore in detached leaves using sensitive techniques such as confocal microscopy and scanning microscopy. Though they offer high resolutions, they are expensive and not readily available to many researchers. The accuracy of other stomatal aperture measurement techniques used in drought experiments is questionable. Moreover, how far the NO data gen-

erated through detached leaf assays and epidermal peels integrate with the drought-induced changes in the surrounding tissues in an intact plant also needs to be investigated.

2.4. NO and the regulation of photosynthesis

Water deficit is the most obvious reason for yield loss due to a strong link between transpiration and photosynthesis. Plants restrict transpiration under water deficit that prevents water loss, but leads to yield penalty due to reduced CO₂ availability. While it is well-known that NO can modulate transpiration through its effect on stomatal movements, more insights into the role of NO in photosynthetic reactions under drought is likely to be valuable.

While chloroplasts are one of the endogenous NO cellular sources during water deficit [48], it has been assumed that there are binding sites for NO within the photosystem II (PSII) between the primary and secondary quinone acceptors [48,59]. Water stress promotes the dissociation of PSII proteins, thereby impairing photosynthetic activity by affecting the steady state contents of its primary functional protein complexes [60]. One mechanism of NO-mediated regulation of photosynthesis has been partly attributed to the protection of critical functional proteins in PSII complex during drought stress. For example, in *Triticum aestivum* SNP was shown to restrict the drought-induced reduction in transcription of *psbA* gene encoding for D1 protein of PSII complex. This helped in quick turnover of D1 protein leading to an increased capacity to replace inactivated reaction centers of PSII during stress, thereby securing the photochemical reactions during grain filling [27,61]. Drought significantly decreases the maximal utilization of the quantum efficiency by PSII centers and photochemical quenching. Exogenous application of SNP during adventitious rooting in explants of *Tagetes erecta* reduced drought-induced reduction in photochemical quenching, thus facilitating the participation of more excited light-energy in photochemical reactions [38]. Treatment with GSNO enhanced the photosynthetic rate in *Rumex* leaves under osmotic stress, apparently due to enhanced CO₂ assimilation that reduced the generation of ROS by inhibiting Mehler reaction [62]. Additionally, exogenous NO ameliorates drought stress by altering chlorophyll fluorescence and photosynthesis [32,61]. The influence of exogenous NO on photosynthesis was studied in *Malus hupehensis* seedlings growing in nutrient solution under osmotic stress where drought induced adverse effects on photosynthesis and chlorophyll content that were alleviated by SNP treatment [32]. Intriguingly, NO can reversibly bind to PSII and inhibit electron transport, thereby reducing the net photosynthetic activity in isolated thylakoids of *Spinacia oleracea* [38,59,63]. Although, the photochemical efficiency of PSII (F_v/F_m) in the leaves of *Populus przewalskii* increased as an outcome of increasing NO levels by SNP treatment, these effects however decreased by prolonging the drought stress duration [33]. These examples indicate the ability of NO to inhibit photosynthesis in a reversible manner that depends on its concentration and the severity of stress [63].

One important question that needs attention is on how does NO stimulate photosynthesis while closing stomata under drought? Universally, crops when exposed to water deficit start to senesce that result in limiting photosynthesis and yield. However, SNP-treated leaves had more chlorophyll content when compared to their respective controls, which is likely due to enhanced iron uptake and its availability in the treated plants through the formation of dinitrosyl iron complexes [38,64]. The availability of iron from the dissociation of SNP is also another possibility for enhanced chlorophyll content and PSII electron transport rate. Besides SNP, other NO donors such as GSNO and DEA-NONOate can also positively regulate the expression of major iron transporter genes such as iron-regulated transporter 1, root plasma membrane ferric reductase 2, nicotianamine synthase 4, and Fer-like Fe deficiency-

induced transcription factor and also enhance its bioavailability by forming deferoxamine-Fe (III) complex [64]. In addition, NO combats drought-induced leaf senescence by antagonizing the effects of ethylene [65]. NO can enhance or inhibit the activities of various enzymes by directly reacting with the transition metals such as iron present in proteins and enzymes. NO can decrease mitochondrial respiration by selective inhibition of key tricarboxylic acid cycle enzymes and acts as a strong stimulator of alternative oxidase that switches on alternative pathway for respiration [31,66]. Micromolar levels of SNP in *Lycopersicon esculentum* resulted in increased activity of carbonic anhydrase that catalyzes the interconversion of CO₂ and HCO₃⁻, and thus indirectly maintain constant supply of CO₂ to ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCo). However, high NO concentrations of 1 M resulted in decreased carbonic anhydrase as well as RuBisCo activities [35,66]. Clearly, NO at nanomolar to micromolar concentrations, either through exogenous application or by endogenous production can enhance the net photosynthesis, and ameliorate the stress effects on chloroplasts [67]. In contrast, at micromolar to millimolar concentrations, NO accelerates deterioration of chloroplasts and the degradation of plastid pigments, thereby destroying photochemistry and photosynthesis [35,63]. NO S-nitrosylates RuBisCo in a dose-dependent manner and increasing concentrations correlated well with reduced RuBisCo activity [67]. Functionality of the photosynthetic machinery is conceivably better maintained in the SNP-treated plants under drought stress [27,32,38]. Moreover, lowered cellular ATP levels triggers NR that subsequently results in enhanced nitrites and NO leading to increased contents of nitrogen and protein, and enhanced crop biomass [61].

There is some evidence to show that NO effects are dependent on the nature of NO donor used. SNP seems to be the only donor that increases the overall PSII electron transport rate, and does not act as an uncoupler [32,40]. It is speculated that the spent donors such as cyanide and iron ions released during photolysis of SNP may contribute to the phenomenon working well in contrast to other donors of NO. Chlorophyll fluorescence experiments with *P. sativum* leaves demonstrate that both the photolytic products reduce photochemical activity of PSII in vivo [40], with NO scavenger treatment only partially restoring the effects mediated by SNP. However, SNP-induced stimulation of PS II electron transport rate and reduction in oxygen evolution in *P. sativum* thylakoids does not occur in KCN and NaNO₂-treated samples, demonstrating that the observations resulted from the activity of NO only. The stimulating effect of SNP on PSII photochemistry has been correlated with an increase in the proportion of the open PSII reaction centers. Enhanced oxygen consumption at increasing SNP concentrations could be another possible explanation of its reported protective effect [27,33,65,66]. Nevertheless, uncertainties not only on how much NO directly reaches the photosynthetic apparatus, but also the products of photolysis of NO donors under drought stress need to be studied thoroughly. This calls for good experimental designs with all controls and multiple NO donors for validating the role of NO in different plant species [40].

2.5. NO as an antioxidant

The ROS produced during water deficit stress can cause an imbalance between oxidative free radicals and antioxidant machinery, with excessive quantities of ROS resulting in aberrant cell signaling, membrane damage, and death [33,35,68]. Rapidly reducing oxidative free radicals result in less membrane damage which subsequently reduces the cell acidity and toxicity, thereby stabilizing cellular metabolism by maintaining the integrity of macromolecules. The potential of a plant to detoxify ROS can contribute to its enhanced drought tolerance.

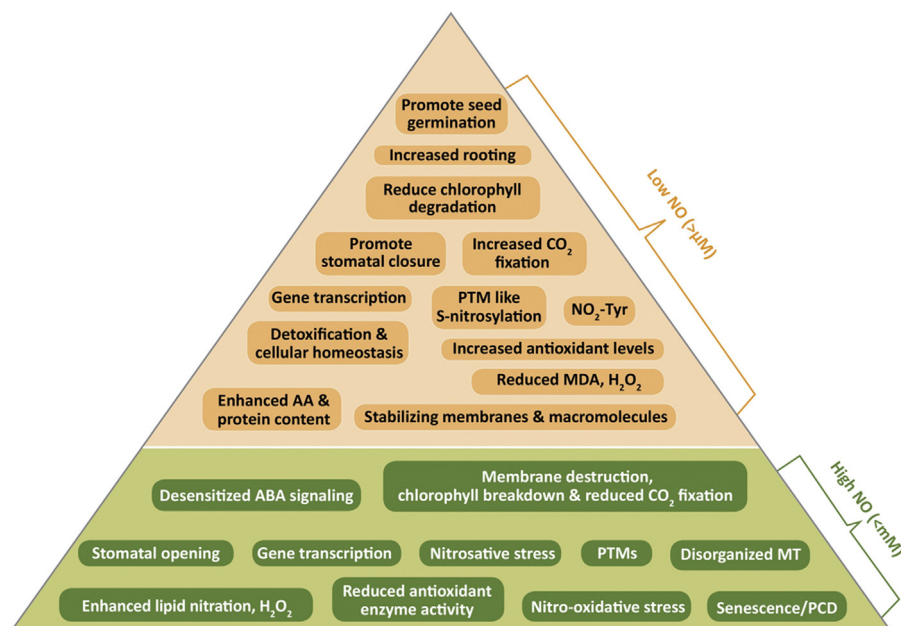


Fig. 1. Concentration-dependent biphasic effects of NO during water deficit in plants, the events not being sequential in order of their appearance [PTM: posttranslational modification; NO₂-Tyr; tyrosine nitration; PCD: programmed cell death; MDA: malonaldehyde; ABA: abscisic acid; AA: aminoacids; MT: microtubules; H₂O₂: hydrogen peroxide].

NO, as a free radical can form various reactive nitrogen species such as nitrosonium ions (NO⁺), nitroxyl anion (NO⁻), S-nitrosothiols (SNOs), peroxyxynitrite (ONOO⁻) and nitrogen oxides (NO_x), which are involved in various physiological processes in plants [68,69]. The generation of NO occurs in cellular compartments such as mitochondria, plastids and peroxisomes where ROS accumulate during stress [1,48,55]. The concomitant generation of these molecules indicates a possible link between these events that synergistically or antagonistically regulate the synthesis and action of each other. While many reports have demonstrated significant crosstalk between NO and ROS, yet their clear relationship in drought stress responses remains elusive [68]. Exogenous NO treatment under drought stress often results in reduced H₂O₂ content and lipid peroxidation in plants [14,17,25,26]. Pre-treatment with NO donor generally prepare plants to forthcoming stress conditions either by stimulating antioxidant machinery or by inducing endogenous NO that can in turn induce set of stress ameliorative events even after removal or degradation of the NO donor [18,25,35]. An antioxidant function was often attributed to NO due to its ability to protect plants from stress-induced oxidative damage [2,14,34]. NO alleviated the ROS-mediated cytotoxic processes in *Solanum tuberosum* leaves and inhibited cell death, ion leakage, and DNA fragmentation [35]. However, all these protective functions were abolished by treatment with a NO scavenger. The effect of SNP was recently investigated in two turf grass species-*Agrostis stolonifera* and *Lolium arundinaceum*, where it could maintain significantly higher relative water content and reduced ion leakage during drought stress [37]. NO treatment resulted in higher superoxide dismutase (SOD) and ascorbate peroxidase (APX) activity under drought, while no significant differences were found between treated and control plants for SOD activity during recovery. Interestingly, APX activity in NO donor-sprayed plants was higher than in the control plants during recovery phase, suggesting stage-specific effects of NO during drought stress [37]. NO can be considered as double-edged sword due to its biphasic effects [35,69] where this duality originates from the presence of unpaired electron within NO molecule. Even short alterations in NO concentration can lead to the biphasic effects (Fig. 1). At low concentrations (nanomolar to micromolar), NO can act as cyto-

protective by interrupting lipid peroxidation and inducing the expression of antioxidant enzymes, besides scavenging superoxide (O₂⁻) and free radicals (R[•]). NO can act as a chain breaker during lipid peroxidation by interacting with lipid alcoxyl and peroxy radicals. In contrast, NO in milli to molar concentrations seems to cause nitrosative stress leading to protein, nucleic acid and membrane damage in plant cells [38,62] owing to its reaction with (O₂⁻) forming peroxyxynitrite which can destroy the structure and function of biological macromolecules [70]. Water deficit can induce both oxidative and nitrosative stress in plants where NO and ROS acts as signaling molecules. Water deficit induced a differential distribution of oxidative and nitrosative stress in *Lotus japonicus* where the oxidative stress was more pronounced in leaves while roots had more nitrosative stress [71]. Additionally, excess NO can act synergistically with ROS and result in nitro-oxidative stress and elicit undesirable toxic effects in plant cells. Some of the molecules like GSNO [formed by non-enzymatic reaction of NO with reduced glutathione (GSH) in the presence of oxygen] involved in NO homeostasis in plants and animals act as a mobile reservoir of NO bioactivity [1,68,70,72]. Loss-of-function mutations in S-nitrosogluthathione reductase in *A. thaliana* led to increased cellular NO content and reduced basal defense [70,72]. Many other molecules such as gamma-tocopherol, carotenoids, flavonoids and plant hemoglobins are also known to maintain NO homeostasis enzymatically in plants under stress conditions [70,72].

Antioxidant enzyme activities can be stimulated or inhibited by NO-mediated oxidation, S-nitrosylation or nitration, depending on the physiological condition of the cell [52,67]. Tyrosine nitration of proteins is a hallmark of various stress conditions as manifested during mechanical wounding in *Helianthus annuus* hypocotyls and *P. sativum* leaves, cold stress in *Piper nigrum* leaves, and water stress in *L. japonicus* [71]. NO-treated *Dendrobium huoshanense* plants maintained high levels of antioxidant enzyme activities and less lipid peroxidation under drought stress [39]. In *Oryza sativa*, enhanced activities of SOD, peroxidase and glutathione reductase was observed following foliar application of SNP. This NO-mediated stimulation of antioxidant machinery also resulted in reduced lipid peroxidation under drought stress [14,25,26,29]. Similarly, exogenous NO treatment delayed the accumulation of ROS by stimulating

peroxidase and SOD activities in *P. przewalskii* and higher SOD activity in *Zea mays* compared to control plants. This effect got reversed by a NO scavenger (c-PTIO) under drought stress [17,33,39]. Thus, stimulating the activity of antioxidant enzymes has displayed the potential antioxidant ability of NO in many higher plants under different stress conditions [2,3,25].

While many studies have shed light on the antioxidant and stress ameliorative potential of exogenous NO donors in plants, it is critical to dissect the possible mechanisms underlying these responses. The ability of NO to combat oxidative stress can be explained by several ways: (1.) NO limits ROS accumulation by inhibiting the ROS-producing enzyme *NADPH oxidase* by S-nitrosylation [39,67,69], (2.) NO acts as an antioxidant at low doses and reacts with ROS such as superoxide resulting in chemical generation of peroxynitrite that may subsequently be scavenged by other cellular processes [1,37,68]. During this process, the toxic ROS are replaced by short-lived peroxynitrite (ONOO⁻) in the cellular environment. Still at higher physiological levels, peroxynitrite can destroy the structure and function of biological macromolecules [69], (3.) NO reportedly stimulates the expression of antioxidant genes resulting in higher enzyme activities, possibly by post-translational modifications that renders the plants drought tolerant [25,39], (4.) NO also acts by targeted inactivation of *aconitase*, a key enzyme of tricarboxylic acid cycle, which converts citrate to isocitrate [66]. Inactivation of *aconitase* down-regulates unwanted turn over of the tricarboxylic acid by reducing mitochondrial electron flow and thus reducing the ROS generation. This contributes to further reduction in stressed cells and offer protection against additional oxidative stress, and (5.) additionally, NO stimulates an alternative oxidase to switch the electron flow from mitochondrial cytochrome c pathway into alternative oxidative pathway that maintains leaf respiration rates even under stress conditions [66]. Thus, NO not only reduces the level of oxidative stress resulting from decreased photosynthesis, but also help in maintaining high vacuolar concentrations of osmotically active solutes and amino acids. Nonetheless, uncontrolled NO levels resulting from severe stress conditions can shift the cellular conditions from a mild oxidative stress to a severe nitroso-oxidative stress which can lead to cell death [68,70]. Low levels of NO have a potential to enhance the antioxidant capacity and help in cell survival under stress [25,37,39].

2.6. NO and proline

Proline is a well-known osmoprotectant that accumulates in many plants in response to the imposition of a wide range of biotic and abiotic stresses. While its involvement in stress physiology is still unresolved, it has been suggested to enhance drought stress tolerance by protecting the protein turnover machinery from stress damage [12,15,73]. Proline has been shown to protect *nitrogenase* activity from water deficit stress in *Glycine max* [74]. SNP treatment resulted in 2- to 3-fold increase in proline content in drought-stressed *Ginkgo biloba* [36] and *P. przewalskii* plants [33]. NO promoted drought-induced free proline accumulation in *O. sativa* and *T. aestivum* [25,27] with conflicting reports about NO-induced proline accumulation in *O. sativa* [12,25]. Proline levels increased in response to either foliar applications of SNP or by enhancing endogenous NO levels by transgenic in drought-stressed *O. sativa* leaves [11,25] in one study, but not in another under similar conditions [12]. The observed discrepancies can be due to differences in the concentration of applied NO donor and its duration, developmental stage, as well as the method of drought imposition. SNP had little effect on proline accumulation under well-watered conditions [36]. While short-term water deficit could induce NO and proline accumulation, the NO-induced proline accumulation seems to be stress- and dose-specific, but not a general

response to NO donor treatment. For example, at low concentration, both GSNO and SNP reduced proline accumulation under water deficit in *C. sativas* suggesting the importance of the given exogenous donor concentration in proline accumulation [17]. Interestingly, neither quenching endogenous NO by cPTIO nor inducing by NO donor (DEA-NONOate) had significant effect on the accumulation of proline in *Medicago* seedlings. Since there is no effect on the expression of proline-metabolism and catabolism genes (ornithine δ -aminotransferase and proline dehydrogenase), it further confirms the independent regulation of proline and NO during osmotic stress in *Medicago truncatula* [30]. While both NO and proline accumulation appears to be important during drought stress, their interdependency needs further exploration. Apart from proline, NO also mediates the accumulation of glycine betaine in enhancing osmotic ability under water deficit in the leaves of *Z. mays* where exogenous application of SNP enhanced the accumulation of glycine betaine by stimulating the activity of betaine aldehyde dehydrogenase [14]. Glycine betaine content negatively correlated with the application of either cPTIO or L-NAME or the combined application of NO inhibitors NaN₃ and L-NAME.

2.7. NO effects on seed germination under water deficit

NO is a key signaling component in the breaking of seed dormancy [5]. Exogenous application of NO-releasing compounds such as nitrites, SNP stimulates seed germination in various plant species [75]. Further substantiating this phenomenon, NR and NOS activities were detected in the embryo as well as in the aleurone layer in *A. thaliana* [5,54,75]. NO is implicated in stimulating β -amylase activity, oxidation of NADPH, and accelerating glucose catabolism by stimulating the pentose phosphate pathway while increasing the rate of germination [5]. The germination-promoting effect of NO is partly due to its effect on expression of ABA catabolic genes that release seeds from dormancy [54]. NR-deficient mutants of *A. thaliana* *nia1nia2noa1-2* are hypersensitive to ABA with enhanced seed dormancy and resistance to water deficit [20]. Besides, these mutant plants had greater sensitivity to ABA throughout their life cycle. Interestingly, these ABA-mediated inhibitory actions were complemented by exogenous application of NO, indicating the regulation of ABA sensitivity by endogenous NO levels. Aquaporins, the water channel proteins may be the targets of NO in inducing seed germination since in *O. sativa* the exogenous application of SNP promoted seed germination under drought by stimulating the transcription of several water channel proteins such as plasma membrane intrinsic proteins (*OsPIPI1*; *1-OsPIPI1*; *3* and *OsPIPI1*; *8*) [76]. NO donor (DEA-NONOate) inhibited seed germination in *M. truncatula* under water deficit stress [30]. Indeed, the cyanide released from SNP is the likely causative agent for its observed stimulating effect on seed germination. The germination rate of *M. truncatula* seeds was significantly reduced after PEG treatment, while quenching NO by cPTIO increased the rate of germination under water deficit [30], thereby drawing speculations on the role of scavenger that allow better respiratory activity, fully active metabolism, and hence leading to higher rate of germination under water deficit.

3. Deciphering NO-drought effects at molecular level

3.1. NO-responsive genes and functional validation studies

Deciphering the molecular mechanisms by which NO exerts its multiple biological functions has been of major interest. While drought stress decrease the DNA methylation levels in *D. huoshanense*, NO increased the demethylation ratio of methylated sites indicating that NO can trigger gene expression under drought [39]. Many studies used high throughput technologies to identify the NO-responsive genes following exogenous application of NO

donors or inhibitors [52,77,78]. The emerging picture indicates that NO can modulate plant stress responses at the genomic, proteomic and post-proteomic levels [52,78]. While a great deal of molecular information has been generated regarding plant responses to drought stress, molecular level insights into the NO-mediated drought stress tolerance has not yet been elucidated. So far, no genome-scale study has been conducted in exploring NO responsive genes under drought stress. Genome level studies to establish transcriptomic and proteomic data sets using the high throughput technologies such as micro-arrays, RNA-sequencing and quantitative proteomics focusing on both model and non-model plants will provide global insights on NO mediated drought stress amelioration. However, the expression studies of genes that are potentially involved in the production of NO in the leaves of *Citrus aurantium* under drought [23] indicated transcript abundance of the up regulated genes viz. nitrate reductase (NR), nitrite reductase (NiR), polyamine oxidase (PAO), and *NADH: Nitro BT oxidoreductase* (NADHox), in contrast to down regulation of alternative oxidase (AOX), diamine oxidase (DAO), and S-nitrosoglutathione reductase (GSNOR). Similarly, SNP-pretreated leaves of *T. aestivum* exhibited a 2-fold accumulation of the late embryogenesis abundant protein 3 transcripts compared with the control after 2 h of drought [7]. As mentioned in previous sections, the expression of *psbA* gene was upregulated by SNP treatment and 1-pyrroline-5-carboxylate Synthetase gene was up-regulated by cPTIO under dehydration [27,30].

Few studies have been undertaken to modulate the endogenous NO levels either by mutations or using transgenic approaches. Although, few *A. thaliana* mutants such as *Atnoa1* that are defective in functional cGTPase, and mutants of nitrate reductase *genia1 nia2* with altered NO levels were characterized physiologically to some extent during water deficit, although their genetic and molecular dissections are not yet complete [8,20,31]. Moreover, due to the multiple roles of NO in plant development, mutants with altered NO levels are expected to have pleiotropic effects on metabolism. For instance, mutants such as *atnoa1*, *atnia1atnia2*, and *atnox1* have negative influence on plant development even under control conditions [20]. On the other hand, few studies have tried the ectopic expression of these mutations to further understand their role in plants. A *NiR* antisense mRNA expressed under the control of a double 35S promoter in *N. tabacum*, and mutated *nitrate reductase* gene for the regulatory phosphorylation site, Ser 521 to Asp under the control of cauliflower mosaic virus 35S promoter in *N. plumbaginifolia* resulted in higher NO emission [79,80]. NO production is also modulated through the overexpression or antisense suppression of *GSNOR* in *A. thaliana* or by overexpression of non-symbiotic hemoglobins in *M. sativa* root cultures overproducing *M. sativa* hemoglobin in *Nicotiana* or by induced expression of the *Escherichia coli* flavohemoglobin Hmp, an enzyme functioning as a NOD in *A. thaliana* [81]. However, none of these transgenic plants were tested under drought stress. Research efforts using transgenic plants have been limited due to the dearth of molecular identities related to NO synthesis and signaling in plants. So far, most of the transgenic work in plant drought focuses on the constitutive expression of NOS genes in plants. Transgenic plants of *A. thaliana* and *O. sativa* overexpressing the rat *neural nitric oxide synthase* gene (*nNOS*) were more tolerant to abiotic and biotic stresses than their untransformed controls [11,43]. These transgenic plants had significantly improved drought tolerance, enhanced levels of antioxidants, and osmolytes during the dehydration phase, quick recovery from stress and improved survival during re-hydration period due to the enhanced in vivo NO concentration. A detailed microarray analysis under drought revealed significant up-regulation of several stress-related genes that are commonly responsive to ABA and NO, partly explaining the observed tolerance [43]. Expression of the mammalian neuronal NO synthase under the constitutive CaMV35S promoter in *N.*

tabacum enhanced NO production that resulted in enhanced resistance to biotic stress. The expression of jasmonic acid, salicylic acid, ethylene-related genes and genes encoding pathogenesis-related proteins were up-regulated while inhibiting catalase gene expression in these transgenic plants [44]. These plants also had elevated levels of salicylic acid. Similar to mammalian NOS, transgenic expression of nitric oxide synthase from the green alga *O. tauri* (OtNOS) in *A. thaliana* under the control of stress- and ABA-inducible promoter of *H. annuus*, Hahb-4 gene resulted in higher NO accumulation and enhanced tolerance to salt, drought and oxidative stress [82]. Moreover, OtNOS transgenic lines also exhibited better stomatal development compared with control plants. Knockout mutations of arginine amide hydrolase (AtARGAHs) also resulted in enhanced synthesis of polyamines and NO in *A. thaliana* due to the enhanced availability of arginine. The knockout lines of AtARGAHs (*argah*) accumulated significantly higher concentrations of NO, and hence exhibited improved water deficit, while the overexpression of AtARGAHs showed an opposite effect on stress tolerances [83]. Moreover, AtARGAHs lines did not show any apparent negative effects on plant development (plant height and dry weight) under control conditions, unlike *nNOS*-overexpressing transgenic lines. With such evidences, the modulation of NO production by trans-genomics may expand the possibilities to alleviate drought stress tolerance in higher plants. Nonetheless, complete identification of target genes or proteins will provide opportunities to establish an in vivo experimental system to study the consequences of modulating NO levels in plants under drought. However, for precise genetic manipulations, molecular techniques such as antisense, RNA interference gene silencing or genome editing tools such as transcription activator-like effector nucleases (TALENs), zinc-finger nucleases (ZFNs) and clustered regularly interspaced short palindromic repeats (CRISPRs) could also be deployed in achieving the target-specific editing of NO biosynthetic and signaling pathways under water deficits. Moreover, selecting the right promoters will be an important consideration to assure proper expression of the transgenes. For example, in *N. plumbaginifolia* transgenics using NR promoter with a reporter gene or structural NR gene in NR-deficient mutant often led to no or very low expression [79,80]. While differences in stress tolerance across species may be due to differential sensitivity to the endogenous NO levels, transgenic technology has a potential to provide genetic evidence for the importance of NO levels in modulation of gene expression and enhancing plant fitness under drought stress. A step forward now will be to explore more NO-responsive genes under drought at the whole genome level, since it will be difficult to modulate multiple pathways either by mutations or transgenics without their complete knowledge.

3.2. NO target proteins and post-translational modifications

Plant proteome is flexible and subject to changing levels of synthesis, degradation and post-translational modifications. Comparative proteome profiles of leaf samples of *Gossypium hirsutum* treated with SNP and NO scavenger followed by NO donor treatment revealed 166 differentially expressed proteins belonging to different cellular compartments and involved in diverse pathways. Of these, 47 were upregulated, 82 were down-regulated and 37 were condition-specific [84]. The emerging experimental evidence demonstrates that NO possibly operates through post-translational modification of proteins, mainly via S-nitrosylation, metal nitrosylation, carbonylation and tyrosine nitration [54,67,70,85]. In protein S-nitrosylation, the co-valent attachment of the NO group to the thiol site of protein cysteine is the most common post-translational modification affecting protein activity in a reversible manner depending on the physiological condition [54,85]. Detection and functional analysis of

S-nitrosylated proteins is demanding under drought because of its instability and reversibility. A number of S-nitrosylated proteins were identified in *A. thaliana*, *B. juncea*, *C. aurantium* and *Z. mays* after stress treatment [85,86]. GSNO detected notably under biotic and abiotic stress conditions is also an important example of S-nitrosylation. In addition to S-nitrosylation, an irreversible reaction of a nitrating agent with a tyrosine residue of any target protein leads to tyrosine nitration. It is now recognized as an important redox-mediated post-translational modification in plants. These redox modifications act as molecular switches that allow the target proteins/molecules suitable for changing cellular status during stress. Several NO-mediated post-transcriptional modifications (nitrosylation, nitration, and carbonylation) have been reported to influence ascorbate peroxidase activity during drought providing a way to mitigate the H₂O₂ concentration in plant cells [87]. The K⁺ channel at the guard cell plasma membrane or a closely associated regulatory protein is modified through S-nitrosylation facilitating stomatal closure during drought [56]. NO fumigation of *A. toxicaria* prevented the inactivation of the antioxidant enzymes by S-nitrosylation, and thus reduced H₂O₂ levels, thereby increasing desiccation tolerance of seeds [86,87]. It is evident that NO-mediated transcriptomic, proteomic, and post-translational modifications during drought stress in plants are still unexplored areas, possibly due to the complexities of drought and NO treatments and modification of high number of proteins, thereby masking the effect of abundant proteins on other proteins.

Defining proteins that are modified by NO under drought, and mechanisms of NO-mediated protein modifications and their significance in the context of drought is essential to understand how NO regulates biological functions. One temporary fix for these problems is enriching the low abundant candidate proteins by cellular fractionations coupled with new high-throughput and sensitive techniques which could facilitate the identification of target proteins and their post-translational modifications. The proteomic analysis of drought remains largely untapped, but more research in this direction will facilitate research aimed at the identification of protein candidates in water deficit condition.

4. What is pivotal in NO-drought research?

Although a large amount of convincing evidence has accumulated in support of the role for NO in various drought stress responses, most of the studies have relied on in vitro systems and glasshouse conditions, and none under field conditions (Table 2). Furthermore, majority of the studies used PEG to create moderate to severe drought simulations that cause osmotic stress rather than drought stress. Although these studies using seedlings, protoplasts or detached leaves or whole plants in growth chamber do provide some basic information, these must be up-scaled to the whole plant level to understand the actual role of NO under field conditions to achieve practical prospects. Several studies indicate that NO potentially enhances plant survival under water deficit conditions mostly by increasing cellular antioxidant defenses. However, laboratory conditions are substantially different from the actual field and hence, improved plant survival under PEG-induced drought may not always correlate with the gains in the field. Moreover, antioxidative defense have shown to occur at much later stage of stress tolerance in field crops such as *Cicer arietinum*, *Arachis hypogaea* and *Pennisetum glaucum*, bearing no correlation with the actual yield under drought [88]. Nevertheless, the influence of NO on stomatal closure is indeed beneficial for maintaining plant water status, and hence holds relevance. Considering this, NO studies toward plant drought stress tolerance must be focused on traits that directly influence water status such as water uptake and conservation capacities under water limiting conditions. Exploring the

influence of NO on traits that influence water conservation such as transpiration efficiency and photosynthesis under drought would hold greater value in terms of plant-water economics. Such field studies would greatly aid the understanding on how the NO signaling pathways function in open environments. Hence, a shift from in vitro to whole plant studies or even to field studies is required for better impact. Undoubtedly, the consequences of high NO content may be very different from the low doses of NO in plants (Fig. 1), with optimal dosage resulting in stress reduction and increased growth efficiency, whereas, at high physiological levels NO may lead to cytotoxicity and adverse effects. Keeping this in view, a systematic approach must be followed for deciphering cumulative responses at a given NO concentration under stress, and not merely focusing on one positively regulated plant process. Moreover, merely addressing the physiological role of NO in drought responses is not sufficient and requires unravelling of the critical molecular mechanisms to precisely understand its potential role in plant stress physiology. Furthermore, developing models for the traits that are either individually controlled or in combination by NO and drought will open up new possibilities to identify new NO targets and strategies leading to multi-stress resistance in crop plants. Besides, the origin and signaling information about NO need to be further resolved, and the rudimentary NO biosynthesis pathway(s) fully defined at the molecular level, either based on the functional homology to known animal counterparts, or newly elucidated pathways following validation in planta. Another pertinent question that needs clear answers is how the small NO molecule can influence modification of massive number of molecules that enhance plants tolerance to drought? Of course, many crosstalk events are evident between NO and other molecules from published studies, the integration of these signaling events with respect to individual stress response is very critical for identifying the missing links. Similarly, knowledge on whether NO acts as a modifier of other hormone responses or independently deploys other signaling molecules, would be useful besides information on how it gets recruited by drought stress at the right time and right place to integrate with various other signaling molecules and signaling events. Clearly, to answer some of these critical questions, high throughput transcriptomic and proteomic studies are indispensable to identify specific NO targets under various stress conditions. A complete characterization of NO-mediated post-translational modifications under drought stress conditions ought to bring clarity not only in complex NO signaling mechanisms, but also establishing direct relationship between protein modifications and functional changes that occur under drought. Further, validation and physiological interpretations of these identified NO target molecules under drought stress condition should be a high priority for comprehensive NO research. Although our knowledge of the role of NO in drought stress is not yet conclusive, we find enormous potential in the ongoing experiments in identifying molecular targets as a key resource for field-level studies as well as laboratory-based NO research. While manipulation of endogenous NO levels, mostly by exogenous donors have an immense ameliorative effect under water deficit, it has been hard to draw any clear conclusions from many of these studies, since most being rather superficial without exploring the underlying signaling pathways. A combined genetic and pharmacological approach would steer the future direction of plant NO research under drought conditions.

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