Nitric oxide, other reactive signalling compounds, redox and reductive stress

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Highlight Statement

Nitric oxide is a key signalling molecule in plant cells, but its role will be influenced by the redox status of the cell, which may undergo oxidative or reductive stress.

Abstract

Nitric oxide (NO) and other reactive nitrogen species (RNS) are key signalling molecules in plants, but they do not work in isolation. NO is produced in cells, often increased in response to stress conditions, but many other reactive compounds used in signalling are generated and accumulate spatially and temporally together. This includes the reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), and hydrogen sulfide (H_2S). Here, the interactions with such other reactive molecules is briefly reviewed. Furthermore, along with ROS and H_2S, NO will potentially contribute to the overall intracellular redox of the cell. However, RNS will exist in

redox couples and therefore the influence of the cellular redox on such couples will be explored. In discussions of the aberrations in intracellular redox it is usually oxidation, so called oxidative stress, which is discussed. Here, the notion of reductive stress is looked at and how this may influence the signalling which may be mediated by NO. By getting a more holistic view of NO biology, the influence on cell activity of NO and other RNS can be more fully understood, and may lead to the elucidation of methods for NO-based manipulation of plant physiology, leading to better stress responses and improved crops in the future.

INTRODUCTION

There is no doubt that nitric oxide (NO) is now considered to be an immensely important signalling molecule in both animals and plants. Along with other nitrogen-based reactive compounds in biological systems, NO is considered to be part of a group of molecules referred to as reactive nitrogen species (RNS). NO research in plants has spanned forty years (Kolbert *et al.,* 2019).

The signalling in which NO is involved in animals is well established. In humans, NO is generated by three nitric oxide synthase enzymes (NOS: Tejero *et al*., 2019) as well as other less studied sources, such as xanthine oxidase (Tropea *et al*., 2018). Downstream NO is known to activate the enzyme guanylyl cyclase, leading to increased cGMP and further downstream effects. Alternatively, it can lead to post-translational modifications of proteins including the alteration of cysteine thiols and tyrosine residues. However, in plants, the system is not so well established. There is still controversy over the presence of a higher plant NOS, with papers claiming that it does not exist, or if it does, that is very hard to find (Jeandroz *et al*., 2016; Hancock and Neill, 2019), although a NOS has been reported in algae (Foresi *et al*., 2010; Astier *et al*., 2018). Downstream signalling through a “classical” guanylyl cyclase system is also hard to establish (Astier *et al*., 2019), with still many facets of NO signalling in plants not fully understood (Leon and Costa-Broseta, 2020).

There is no doubt, however, that NO has many physiological effects in plants. NO has been implicated in seed germination (Arc *et al*., 2013), root development (Sanz *et al*., 2015), stomatal closure (Gayatri *et al*., 2013), plant reproduction (Hiscock *et al*., 2007), fruit ripening (Palma *et al*., 2019), and stress responses (Hu *et al.*, 2017) including pathogen challenge (Mur *et al*., 2006).

What is also clear is that NO does not act as a lone signal in biological systems, but rather is a suite of small reactive compound which orchestrate a range of activities in cells. Many of these reactive compounds have had to be tolerated during evolution, and it appears that cells have not only adapted to their presence, but have adopted them for positive activities, such as signalling (Hancock, 2017; Gutteridge and Halliwell, 2018). Enzymes has also evolved which appear to have dedicated roles in generating reactive compounds, such as the NADPH oxidase systems. Compounds of relevance here include the reactive oxygen species, such as superoxide anions ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) (Sies, 2017). Also amongst these compounds is hydrogen sulfide (H_2S) (Olas, 2015), and more recently molecular hydrogen (H_2) has been mooted as a signal (Li *et al*., 2018).

This review will discuss how NO interacts with other reactive compounds used in signalling, but will also look at how the redox status of the cell interacts with NO metabolism. Furthermore, the idea of reductive stress will be discussed, and how the alterations of redox may impinge on NO signalling.

NITRIC OXIDE AS PART OF A SUIT OF REACTIVE SIGNALS

NO can be produced endogenously by plants cells (reviewed by Kolbert *et al*., 2019), from NOS-like enzymes (Gupta *et al*., 2019), and with the enzyme nitrate reductase (NR) being a major protein undertaking this activity (Chamizo-Ampudia *et al*., 2017). It can also arrive at a plant cell from exogenous sources (Kanta Gaihre *et al*., 2019). However, other reactive compounds, also involved in signalling, can arrive from the outside of the cell too, as well as being endogenously produced. These include ROS, H_2S and H_2. Therefore, the interactions of such compounds with NO needs to be considered (Hancock and Whiteman, 2014) (Figure 1).

Of particular importance here, is that many reactive compounds are used in signalling and are generated in cells at the same time, and often in the same subcellular location, especially in times of cellular stress. Such stress may include the presence of heavy metals (Shivaraj *et al*., 2020), salt (Ahmad *et al*., 2016), temperature (Suzuki, 2019) light (Choudhury *et al*., 2017) or pathogens (Arasimowicz-Jelonek and Floryszak-Wieczorek, 2016). Reports can be found for the accumulation of many reactive signalling molecules in such conditions, including ROS, H_2S and NO. This has been the topic of other recent reviews (Hancock and Neill, 2019), so an overview will be given here.

ROS are produced in plants cells by a variety of enzymatic and non-enzymtic systems, including from the activity of the mitochondrial electron transport chain and from photosynthesis, but of significance is the action of the family of NADPH oxidases (Qu *et al*., 2017), the respiratory burst oxidase homologues (RBOHs). Products of ROS metabolism include free radicals such as superoxide anion, $O_2^{\cdot-}$, and hydroxyl radical ($^{\cdot}OH$), the latter which is probably the most reactive compound found in biological systems, and will react at a diffusion limited rate. Its reactions often proceeds by hydrogen abstraction, as investigated for DNA damage by this radical (Balasubramanian *et al*., 1998). ROS also includes non-radical forms such as hydrogen peroxide (H_2O_2). An over production, or accumulation, of ROS has traditionally been seen as detrimental, leading to oxidative stress. This notion will be discussed much further below, but there are a range of antioxidants which attempt to keep ROS in check (He *et al.*, 2017), including enzymes such as superoxide dismutase (SOD) and catalase (CAT). There are also a range of small compounds which are involved, not least of which is glutathione and its metabolism (Bachhawat and Yadav, 2018). Of importance here is also the cycling of compounds such as

ascorbate, as has been reviewed elsewhere (Gill and Tuteja, 2010). Many of the effects of ROS are mediated by redox proteins such as thioredoxins, which are immensely important for the control of a range of physiological and cellular events, including growth and development, metabolic control and gene expression (Gelhaye *et al*., 2005; Geigenberger *et al*., 2017).

A significant molecule in the group of reactive signals is H_2S. This too has been the subject of reviews (Hancock and Whiteman, 2016; Corpas, 2019; Corpas *et al*., 2019). H_2S can be made by plant cells and has been measured in response to a range of stresses, in a similar manner to ROS (Lisjak *et al*., 2013).

Perhaps surprisingly, H_2 has now been suggested as a plant signalling molecule, and like other mechanisms has been reviewed relatively recently (Wilson *et al*., 2017). This odourless, relatively insoluble and relatively inert gas has been mooted as a potential therapy in humans (Iida *et al*., 2016), but also useful for agriculture (Zeng *et al.*, 2014), as it confers stress resistance, for example (Ciu *et al*., 2014).

What is pertinent here is how the suite of reactive signals outlined above interact, especially with NO. It has long been known that NO and ROS can interact together to produce peroxynitrite ($ONOO^-$), which has many roles in cells including in signalling (Speckmann *et al*., 2016). However, this is not the only direct reaction of NO with reactive signalling compounds. NO and H_2S can react together to form nitrosothiol (Whiteman *et al*., 2006), another downstream product which can be used as signalling molecule. However, as well as producing new molecules, the direct reactions of NO with other compounds removes the NO from being bioavailable, and so may reduce NO signalling. Lastly, it has been suggested that NO and H_2 may interact, although the exact outcome has not been determined (Hancock and

Hancock, 2018). Certainly, NO has been mooted as being important in mediating some of the effects of H_2. For example, NO appears to mediate H_2 effects in the induction of adventitious root growth in cucumber (Zhu *et al.*, 2016).

One of the major antioxidants in cells is the tripeptide glutathione (GSH). NO can react to form *S*-nitrosoglutathione (GSNO), which not only removes NO but is potentially a method for NO transportation and storage (Hogg *et al*., 1996; Lindermayr, 2018).

As well as direct interactions with other reactive signals, NO may have several indirect effects. NO, through an action mediated by peroxynitrite, can alter superoxide dismutase (SOD) activity, and so alter the amount of both $O_2^{\cdot-}$ and H_2O_2 in a cell (Holzmeister *et al*., 2015). NO can also modulate the activity of catalase, so altering H_2O_2 mediated signalling (Bauer, 2015; Rodríguez-Ruiz *et al*., 2019). As already mentioned, the antioxidant GSH may be removed by NO (to make GSNO), so there is a significant capacity for NO to alter ROS metabolism and hence signalling. H_2S can increase glutathione levels in cells in animals (Parsanathan and Jain, 2018) and plants (de Kok *et al*., 1985) which can impinge on its availability for reactions with NO, as well modulating its role in ROS metabolism and controlling cellular redox (Schafer and Buettner, 2001).

NO may also have the ability to modulate the rates of the generation of reactive signalling molecules. For example, in human endothelial cells NO reduces NADPH oxidase activity (Selemidis *et al*., 2007). A similar effect is seen in plants, where NO donors and peroxynitrite both reduced RBOH activity (Chu-Puga *et al*., 2019).

The alteration of protein activity is often through the modification of thiol groups on cysteine residues (Figure 2). Oxidation can lead to the formation of

disulfide bonds, if two thiols are in the correct three dimensional orientation. Single thiols can be oxidised to the sulfenic acid, sulfinic acid and sulfonic acid groups, depending on the concentration of hydrogen peroxide in the vicinity of the protein. Sulfenic acid can be converted back to the thiol group, so this modification is akin to phosphorylation, in that the two states can be toggled between (Couturier *et al*., 2013). Significant signalling proteins, such as tyrosine phosphatase, can have their activity altered in this way; in this case, inhibition (Denu and Tanner,1998). Alternatively, the thiol group may be covalently modified by NO, to create to the –SNO group. This process is *S*-nitrosation, commonly referred to as *S*-nitrosylation (Lindermayr and Saalbach**,** 2005). Again, this can be converted back to the –SH group, allowing the activity of proteins to be toggled between two states. It should be noted that *S*-nitrosylation and *S*-nitrosation are chemically different as explained by Heinrich *et al*. (2013).

Other thiol modifications are seen too. H_2S can modify thiols in a process known as persulfidation (often referred to as *S*-sulfhydration: for a paper on terminology in plant NO research see Gupta *et al.,* 2019) (Aroca *et al.,* 2018). Alternatively, thiols are also able to react with glutathione, a process called *S*-glutathionlyation (Mailloux and Treberg, 2016). It can be seen therefore that the accessible thiol groups of proteins are a point of possible competition between a range of reactive molecules used in signalling, including NO. Which of the final protein products is created must be determined by the relative concentrations of the reactive compounds in the vicinity of that protein. This will be determined by a range of factors. This includes the rate of production, the rate of removal, or the other reactions in which that molecule may partake. All these factors can be influenced by the local concentration of NO, which itself will be affected by the presence of the

other signalling molecules. Therefore, it must be a complex interplay between all these compounds, and the final result will be determined by the local conditions prevailing for a particular polypeptide. This will, of course, be influenced by the likely compartmentalisation of the signals, a topic covered by a recent review on NO (Hancock, 2019).

NO may also alter a protein's activity through tyrosine nitration (Kolbert *et al*., 2017). As with *S*-nitrosation, nitration of an amino acid, in this case the addition of a NO moiety to a tyrosine side group to form a nitrotyrosine group (Corpas *et al*., 2009), can alter the conformation of a polypeptide and hence control its activity. Although originally thought to be an irreversible change to a protein, to be useful for signalling, such modifications should be a reversible reaction (Kolbert *et al*., 2017), as seen with *S*-nitrosation (Gould *et al*., 2013).

Lipids can also be modified by NO, and this may impact on whether NO can freely transverse membranes. The product may be a signalling molecule too, so this broadens the scope for NO-based signal transduction (Mata-Pérez *et al*., 2016).

It is clear, therefore, that NO cannot act alone. It will be produced and accumulate in cellular compartments in which other similarly reactive compounds will be generated, setting up possible competitions between this suite of signalling molecules. New products and signals are produced, whilst the end result may be the alteration of protein activities. A good example is that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which can be modified in numerous ways, including by NO, ROS and H_2S. This is a classic example of a moonlighting protein (Jeffery, 2018), i.e., that is one that has more than one defined activity. GAPDH would normally have activity in the cytoplasm, where it partakes in the sixth step of glycolysis. However, despite this being a crucial activity for cells, GAPDH can be

post-translationally modified, including by oxidation by ROS and *S*-nitrosation by NO. Once modified GAPDH can partake in a range of supplementary events in the cell, but one of the most important is that it can translocate to the nucleus where it is involved in the control of gene expression. Such GAPDH modifications, and the range of moonlighting activities of this enzyme, has been the subject of a recent review (Russell *et al*., 2020), and the influence of such mechanisms has been reported in plants (Wang *et al*., 2017). Here, the authors report that GAPDH is a direct target of NO and is involved in a mechanism which alters root growth.

REDOX AND ITS INFLUENCE ON NO METABOLISM

The redox environment within a cell is important to consider (Jones and Sies, 2015), especially when working with redox active compounds such as NO. This is also important to consider when a pharmacological approach is used to manipulate cell function. With nitric oxide, in most cases, it is assumed that the NO exists as the free radical ($NO^{\cdot}$), but it can gain and lose electrons so has in fact three redox states: the radical form $NO^{\cdot}$, nitroxyl (NO^{-}) and nitrosonium (NO^{+}) ions (Lancaster Jr, 2015). Different NO donor molecules may deliver different forms of NO and their chemistry is not the same. Therefore, even with NO itself, presence of absence of electrons is important, and emphasises why looking at the cellular redox state is crucial to an understanding of NO metabolism.

The intracellular redox state is relatively reducing. This is partly to maintain cofactors such as NAD(P)H in their reduced state so that they can partake in metabolism (Bücher *et al*., 1972). Glutathione plays a major role in the maintenance of the cellular redox. However, its influence is complex. Often the redox of the cell is measured by assaying the amount of glutathione present in both of its redox states.

Glutathione undergoes the reaction: 2GSH (reduced) → GSSG(oxidised). The concentration of these, and therefore total glutathione, can be measured (Rahman *et al*., 2006), and then the ratio of the reduced and oxidised forms can be used to measure the cellular redox (E_h) using the Nernst equation (Equation 1) and the reported E_m for glutathione (Rost and Rapoport, 1964):

Equation 1: The Nernst Equation (redox equation) assuming an intracellular pH of 7.4. E_h: redox potential; $E_{m(pH7.4)}$: midpoint potential of redox couple at pH7.4; R: Gas Constant; T: temperature in Kelvin; F: Faraday Constant; n: number of electrons used in oxidation/reduction.

$$E_h = E_{m(pH7.4)} + \frac{RT}{nF} \times 2.303 \text{Log} \frac{[\text{oxidised}]}{[\text{reduced}]}$$

However, as explored by Schafer and Buettner (2001), as there are two GSH molecules needed to make one GSSG the Nernst equation becomes a squared equation. This means that the overall redox state is also related to the total concentration of glutathione, and not just the ratio of oxidised to reduced molecules. Although the glutathione concentration in cells is often relatively high, often mM, it is also not static. Glutathione can be made by cells (Forman, 2016), which may be influenced by the presence of H_2S (de Kok *et al*., 1985; Parsanathan and Jain, 2018), and can be lost from cells (Ghibelli *et al*., 1995), so potentially altering cellular redox.

However, glutathione is not the only molecule which helps to control the cell's redox state. Other abundant low-molecular weight (LMW) thiols include cysteine (Cys), cysteinyl-glycine (Cys-Gly) and γ-glutamyl-cysteine (γ-Glu-Cys). Up to 25% of the total thiol concentration may be composed of such compounds, and therefore

their influence should not be underestimated. Their levels also change with time as physiology changes, such as in seed aging (Birtic *et al*., 2011).

On a background of the redox status of cells being controlled by thiols, the metabolism of ROS, RNS and H_2S has to be considered. There are two things to think about here: how does RNS metabolism influence cellular redox; how does cellular redox influence RNS metabolism (Hancock and Whiteman, 2018). If ROS is looked at as a parallel, it is usually the influence of the presence of ROS which is thought to be important, and an imbalance of ROS can be said to lead to oxidative stress. More recently literature has started to see the phrase nitrosative stress (Corpas *et al.,* 2007), although the term nitro-oxidative stress has also been suggested as being more all-encompassing (Gupta *et al*., 2020). The over accumulation of ROS and/or RNS can lead to alterations of the redox state of the cell. It was suggested by Schafer and Buettner (2001) that to get a true picture all the redox-influencing molecules should be considered, and they mooted Equation 2.

Equation 2: (E_i is the half-cell reduction potential of the redox couple of interest (Schafer and Buettner, 2001)).

$$\text{Redox environment} = \sum_{i-1}^{n\ (couple)} E_i \times [\text{reduced species}]_i$$

To get a full picture for this, not only will all the redox active compounds need to be known, but their interactions, and how they influence each other's accumulation will also need to be considered. Of course this is a very dynamic system too, as well as being compartmentalised.

The intracellular redox of cells has been estimated (Hwang *et al*., 1992; Jones *et al*., 1995; Hutter *et al*., 1997; Jones *et al*., 2000; Kirlin *et al*., 1999; Cai *et al*.,

2000), and in their review Schafer and Buettner (2001) suggested that the average status of the cell is approximately -242mV. It was suggested that as the redox potential rises cells are likely to differentiate, rather than be in a proliferate state (as the redox becomes approximately -200mV), but it if the redox is raised too far, to around -170mV, apoptosis is initiated (Schafer and Buettner, 2001). Generation by, or arrival of RNS at, a cell will therefore feed into this overall redox. It will not just be ROS which contributes to these fluctuating redox states, but all the other reactive compounds too, including NO and H_2S.

It has been suggested that the cellular redox, to sustain cellular health, is maintained in what has been dubbed the “Goldilocks Zone” (Figure 3: Alleman *et al*., 2014). Here, the redox status of the cell is suggested to be able to alter, perhaps fluctuate, but the intracellular E_h needs to stay within defined limits. Outside of those limits the activity of the cell may be altered. For example, if the E_h becomes too positive then oxidative stress will occur, leading to cell damage and even the initation of apoptosis (Schafer and Buettner, 2001). Alteration of the E_h in the opposite direction may lead to reductive stress. Both endogenous enzymes and small molecular antioxidants will help remove ROS and other reactive compounds to ensure cells are held within these redox limits.

Over accumulation of ROS and RNS may also increase downstream signalling, through the additional modifications of thiol groups, and perhaps elevated levels of tyrosine nitration of proteins (Corpas *et al*., 2009; Kolbert *et al*., 2017), or the formation of nitro-fatty acids (Mata-Pérez *et al*., 2016). Many cellular stress responses lead to enzymatic generation of ROS, RNS and H_2S, which can lead to altered gene expression and adaptation to manage future stress (Huang *et al*., 2019).

Effects of redox on NO have been considered previously (Hancock and Whiteman, 2018; Hancock, 2019). Using the published values for E_m for relevant couples (as also discussed and used below), the ratios of those redox couples was calculated at E_h values of -390mV, -242mV, -200mV and -170mV, which were values highlighted previously for the intracellular redox poise (Schafer and Buettner, 2001). The salient points which relate to NO metabolism will be revisited here. Some NO-based redox couples would not be affected if the intracellular redox became more oxidising. With an E_m of +1210 mV (Koppenol, 1997), an alteration of a redox from -240mV to -200mV (Schafer and Buettner, 2001) would have negligible effect on the $NO^+/NO^{\cdot}$ couple. The $NO^{\cdot}/NO^-$ (singlet) couple has an E_m of approximately -350 mV (Koppenol, 1997). This is relatively near the -242 to -170 mV redox of the cell (Schafer and Buettner, 2001) and potentially the ratio of forms in this couple will be influenced by this change. The $NO^{\cdot}$ form is favoured (Hancock and Whiteman, 2018; Hancock, 2019) under more oxidising conditions. As this is the form usually associated with NO signalling, it could be mooted that as the redox changes to become oxidising there would be an increase in NO signalling, perhaps allowing it to persist for longer. This could drive cells towards a programmed cell death state, as seen for example, in the hypersensitive response (HR). Plants can have mechanisms of microbe-associated molecular patterns (MAMP)-triggered immunity (MTI) and effector-triggered immunity (ETI), with the death of cells being an effective way to limit pathogen spread. NO is involved in such processes (Coll *et al*., 2011), so increased NO signalling may be significant here.

NO is involved in posttranslational modifications of proteins, as discussed above, and these can be influenced by redox states too. The RSNO/RSH couple has an E_m of -400 mV (Koppenol, 1997). This would favour the RSNO form as the

intracellular redox becomes more oxidising. Therefore, as the NO levels rises -SNO signalling increases, and if this is accompanied by rises in oxidising ROS such as H_2O_2, the NO signalling would be reinforced, and perhaps prolonged. Both ROS and NO signalling would be working in the same direction.

H_2S accumulation has been mooted to be a brake-point for NO and ROS signalling (Hancock and Whiteman, 2014), perhaps through the removal of NO by creating the nitrosothiol (Whiteman *et al*., 2006). With an E_m of -200 mV (Li and Lancaster Jr, 2013), the S/H_2S couple would not favour the H_2S form as the redox becomes oxidising, so favouring NO signalling, which again reinforces the NO signalling which the other effects of redox change are driving.

REDUCTIVE STRESS

Historically the literature on the detrimental effects of ROS and redox has focused on oxidative stress, that is, the intracellular environment becoming more oxidizing. However, the opposite can also take place, that is, reductive stress. A search in *PubMed* in January 2020 for oxidative stress listed 222379 articles, with the phrase in the title of 48660. In a similar search for reductive stress only 872 were listed, with the phrase in the title in only 66: 9 in 2019 and 4 in 2018. Interestingly, that is 20% in the last 2 years. It is a subject which is getting more traction, and therefore needs to be considered when discussing NO metabolism.

Reductive stress is when the redox in the cell becomes too reducing, rather than oxidising. This means that the redox drops outside of the Goldilocks Zone mooted by Alleman *et al*. (2014), as shown in Figure 3. This, like oxidative stress, can be detrimental to cells. It is mediated by changes in the ratios of the oxidized forms and reduced forms of the major redox mediators in cells, namely NADH,

NADPH and glutathione (Xiao and Loscalzo, 2019). Physiological responses may include increased autophagy (Pan *et al*. 2019a). The downstream signalling in reductive stress responses may involve MAP kinase and Akt pathways. (Carne *et al*., 2019), as well as events in the nucleus through Nrf2 and NF-κB mediated events (Quiles *et al.*, 2017; Bellazza *et al*., 2018). Hence, reductive stress has been implicated in the onset and progression of several human diseases (Pérez-Torres *et al*., 2017).

Much of the work on reductive stress has been carried out on animal systems. Several papers report on how reductive stress impinges on cancer progression. The increased production of GSH and reduced ROS accumulation in a breast cancer line led to reductive stress and increased cell growth (Kim *et al*., 2020). By measuring NAD(P)H levels as an assay for redox, Pan *et al*. (2019b) suggested that under hypoxia cancer cell death is induced by natural antioxidants, which is mediated by reductive stress. Others have also suggested methods for assaying reductive stress (Ao *et al*., 2017), and report that such techniques can distinguish between normal and cancer cells (Niu *et al*., 2019).

Induction of reductive stress has also be mooted as a treatment against cancer, where NADPH levels are altered by ascorbate, with cancer cells being particularly susceptible (Gao *et al*., 2019).

Reductive stress can be induced, either chemically (Tirla and Rivera-Fuentes, 2018), or physiologically in animals, as seen with exercise (Wadley *et al*., 2018), and has been reported to help against oxidative stress in mononuclear cells (Spanidis *et al*., 2018).

It is not only animals cells which have been studied. The effects of reductive stress in *Mycobacterium tuberculosis* has been reviewed recently (Singh *et al*.,

2019), with the suggestion that it may be a target for new therapies. Other species studied include *Candida albicans*, where, not surprisingly, glutathione was found to be major factor mediating the effects (Jia *et al*., 2019). In the yeast, *Saccharomyces cerevisiae*, thioredoxins were found to be used to mediate against reductive stress (Trotter and Grant, 2002).

To date, very little literature seems to be dedicated to reductive stress in plants. The effects on metallothioneins in *Quercus suber* (cork oak) were investigated by Raman spectroscopy and reductive stress was found to alter the proteins' structures (Torreggiani *et al*., 2009). More recently, in Arabidopsis the enzymes thimet metalloendopeptidase 1 (TOP1) and thimet metalloendopeptidase 2 (TOP2) were studied under redox stress conditions, including reductive stress. TOP1 was sensitive, whereas TOP2 was not (Almohanna, 2019). Clearly, there is still much to learn about how reductive stress contributes to redox metabolism in plants.

It will be important to determine if reductive stress is likely under physiological conditions in plants. As discussed, the cellular redox status will depend on the ratio of redox couples such as $NADH/NAD^+$, $NADPH/NADP^+$ and GSH/GSSG. It has been suggested that the redox state of $NADPH/NADP^+$ will vary under fluctuating light and that it would be most reducing when light levels are very high (Hashida and Kawai-Yanada, 2019). Although the study used mutants of *Arabidopsis thaliana*, high light increased antioxidant levels, particularly α-tocopherol and ascorbate (Golan *et al*., 2006), so potentially pushing the intracellular redox to be more negative. Plants will be exposed to H_2S from the atmosphere (Ausma and De Kok, 2019), or H_2S may be used as a treatment to mitigate against plant stress (e.g. Du *et al*., 2017). Increased H_2S in cells may increase glutathione, and so alter the redox status (de Kok *et al*. 1985). It would be important to know how such alterations effect the redox couples in

418 cells, and hence potentially alter NO and/or ROS signalling. Of course, not all plant
419 cells or tissues are the same, so not all are photosynthetic, and many may have
420 different conditions, such as low oxygen if roots are flooded, for example. It has
421 already been suggested that NO may be important as part of the response to
422 hypoxia in plant cells (Borisjuk and Rolletschek, 2008; Gupta *et al*., 2020), conditions
423 which may cause reductive stress in cells (Gores *et al*., 1989). Furthermore, as
424 discussed elsewhere in this paper, cells are compartmentalised (Hancock, 2019). Of
425 relevance here, it has been reported that the highest levels of glutathione are found
426 in the mitochondria, which have no glutathione biosynthesis (Zechmann *et al*., 2008).
427 Therefore reductive stress and NO metabolism may impinge on each other in certain
428 intracellular regions. It is therefore not unlikely that reductive stress can have
429 physiological relevance to plant cells, and it may therefore have an impact on redox
430 signalling.

431 NO metabolism was affected by oxidative stress, as discussed above.
432 Reductive stress would intuitively do the opposite, but it would depend on the E_m of
433 the redox couple being considered. If the cellular redox is around -240mV (Schafer
434 and Buettner, 2001), but the E_m of the redox couple of interest is very much higher,
435 perhaps +200mV, then the effects of moving the cellular redox 20-30mV more
436 reducing would be negligible. However, if the E_m is around the cellular redox, then
437 effects could be seen.

438 In the above discussion the effects of the intracellular redox becoming more
439 oxidising were discussed, so what happens if the redox becomes more reducing?
440 With an E_m of +1210 mV (Koppenol, 1997), an alteration of a redox to be more
441 negative will have no effect on the $NO^+/NO^{\cdot}$ couple. On the other hand, the $NO^{\cdot}/NO^-$
442 (singlet) couple has an E_m of approximately -350 mV (Koppenol, 1997), which is

relatively near the -242/-170 mV redox of the cell (Schafer and Buettner, 2001). A change to a more reduced state will potentially alter the ratio of forms in this couple. The $NO^{\cdot}$ form is favoured (Hancock and Whiteman, 2018; Hancock, 2019) on oxidation, which suggests that NO signalling may be enhanced. Going the other way, a reductive redox would lower the amount of NO signalling, perhaps curtailing it. It would potentially reduce the capacity for further interactions of NO with other reactive compounds too.

An over-reducing environment would also affect the way NO is involved in posttranslational modifications of proteins as well. As discussed above, the RSNO/RSH couple has an E_m of -400 mV (Koppenol, 1997). This would not favour the RSNO form as the intracellular redox becomes more reducing. Therefore, as the NO levels rises and -SNO signalling increases, a reductive environment would counter this and reduce the NO signalling which is mediated by this protein modification. A rise in GSH, perhaps because an accumulation of H_2S, would counter the NO signalling which is being initiated.

With H_2S accumulation being mooted to be a brake-point for NO and ROS signalling (Hancock and Whiteman, 2014), a reducing environment would favour the H_2S form of the S/H_2S couple (E_m of -200 mV: Li and Lancaster Jr, 2013), and so potentially reduced the capacity for NO signalling. Therefore, an accumulation of H_2S would increase GSH, drive the redox environment to be more negative, and therefore prolong the potential for H_2S to interfere with NO signalling.

Overall, an oxidising redox inside the cell will be helping to drive NO signalling to be prolonged, whereas a reductive environment will be curtailing NO signalling. Both these scenarios will be greatly influenced by the local generation, or arrival, of ROS and H_2S, which are likely to the produced spatially and temporally with NO,

especially under stress conditions. Further details on the effect of redox on NO couples, along many others involved in reactive molecule signalling, has been previously published (Hancock and Whiteman, 2018; Hancock, 2019).

CONCLUSION AND FUTURE PERSPECTIVES

It is clear from the literature that NO is a key molecule which aids in the orchestration of a wide range of plant physiological responses. However, the effects of NO should not be considered in isolation from other reactive signalling molecules. NO can be endogenously generated in a plant cell by non-enzymatic mechanisms as well as NOS-like enzymes or NR, but the increase of NO is often accompanied by the accumulation of other reactive molecules, such as ROS and H_2S. These may be being used as signals in their own right, but they may also react with NO (Figure 1). The corollary of this is that the bioactivity of NO may be reduced, whilst new signalling molecules may be being generated, such as peroxynitrite (Speckmann *et al*., 2016) and nitrosothiol (Whiteman *et al*., 2006). NO may also indirectly affect the metabolism of these other signals, for example by altering levels of antioxidants which can modulate ROS levels in cells, or modulating the activity of enzymes generating other reactive signals such as ROS. Therefore, there is a complex interplay between NO metabolism and the metabolism of ROS and sulfur-based compounds such as H_2S.

Many of the downstream effects of NO and other reactive signalling molecules are mediated by post-translational modifications of proteins. In particular are the alterations of thiol groups, which can be modified in a variety of ways, including oxidation, *S*-nitrosation and persulfidation (see Figure 2). This suggests that there is a potential competition for reacting with thiol groups and the ultimate result will

depend on the relative local concentrations of the reactants, which may be influenced how these reactive compounds react together but also by compartmentalisation of reactive molecules (Hancock, 2019). Bioavailability of molecules will be key to how they influence downstream signalling.

The influence of ROS and NO is often considered, with studies on oxidative stress, and more recently nitrosative stress and nitro-oxidative stress (Corpas *et al.,* 2007; Gupta *et al*., 2019) being regularly published. However, rarely are the effects of the intracellular redox status of the cell on NO considered (Hancock and Whiteman, 2018). It should be remembered that these redox compounds, such as NO, are being generated, or arriving, into a redox environment to which they will not be immune. If the redox mid-point potential of a redox couple is near the redox status of the medium in which it finds itself, then that redox medium might have an influence. This can impact on the severity and longevity of the signalling which that redox active molecule can enact.

Historically, studies have looked at what happens as the intracellular redox poise of a cell becomes more oxidising, in the process known as oxidative stress. However, many organisms, including yeast and humans, the idea of the intracellular redox becoming too reducing is being more fully understood. The process of reductive stress has not been widely discussed in plants. The over accumulation of reduced nicotinamide compounds such as NADH and NADPH, as well as the changing redox caused by glutathione can lead to the intracellular redox becoming more negative than it should, causing a stress which can be harmful. Alleman *et al*. (2014) have mooted the idea of a redox “Goldilocks Zone”. Cells would work to keep the redox within defined limits. Too oxidising and cells may change their behaviour, as suggested by Schafer and Buettner (2001), perhaps differentiating or entering a

programmed cell death mechanism. However, moving the redox from this defined zone to being over-reducing can also have negative effects. It may also potentially impinge on the signalling which can be transmitted by a range of reactive molecules, including ROS and NO. Clearly, the physiological conditions under which reductive stress in plant cells may be induced need to be understood, as well as the downstream effects of intracellular reduction. This may be of particular relevance if treatments mooted for plants impinge on redox metabolism, such as those based on H_2S, which may alter glutathione metabolism (de Kok *et al*., 1985).

Although it is clear that NO is a hugely important signalling molecule, there are still many aspects which are not fully understood. The reactions of NO, and other RNS, with a range of cellular components needs to be fully elucidated. The manner in which ROS, RNS and H_2S potentially alter the intracellular redox, and how that reciprocally alters signalling needs to be unravelled. Finally, the potential for cells to undergo reductive stress is clearly under studied.

The treatment of plants with NO or H_2S releasing compounds has been suggested to be advantageous to plant growth, survival and crop yield. Such treatments allow plants to survive stress (for example: da-Silva *et al*., 2018). However, to use such manipulations to full advantage, a better understanding of how these compounds behave in plant cells is needed.

COMPETING INTERESTS STATEMENT

The authors confirm that they have no competing interests.

DATA AVAILABILITY STATEMENT

No primary data was presented in this paper.

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AUTHORS' CONTRIBUTIONS

JTH initiated this paper and did most of the writing of the manuscript. DV was involved in discussions about the science and was instrumental in editing the manuscript for clarity.

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Figure Legends:

Figure 1: NO interacts with other reactive molecules.

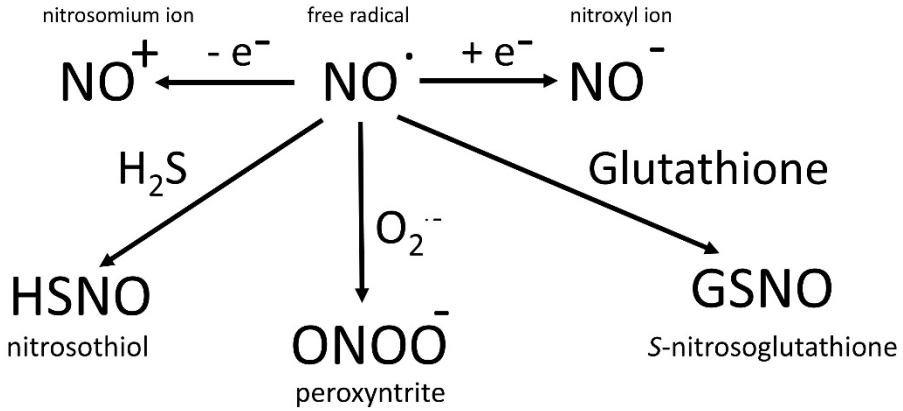

Figure 2: Thiol modifications: a simplified scheme to highlight the competition between NO and other reactive signalling molecules. The thiolate ($-S^-$), and interconversions of one form to another are not shown. Reaction with NO highlighted in black.

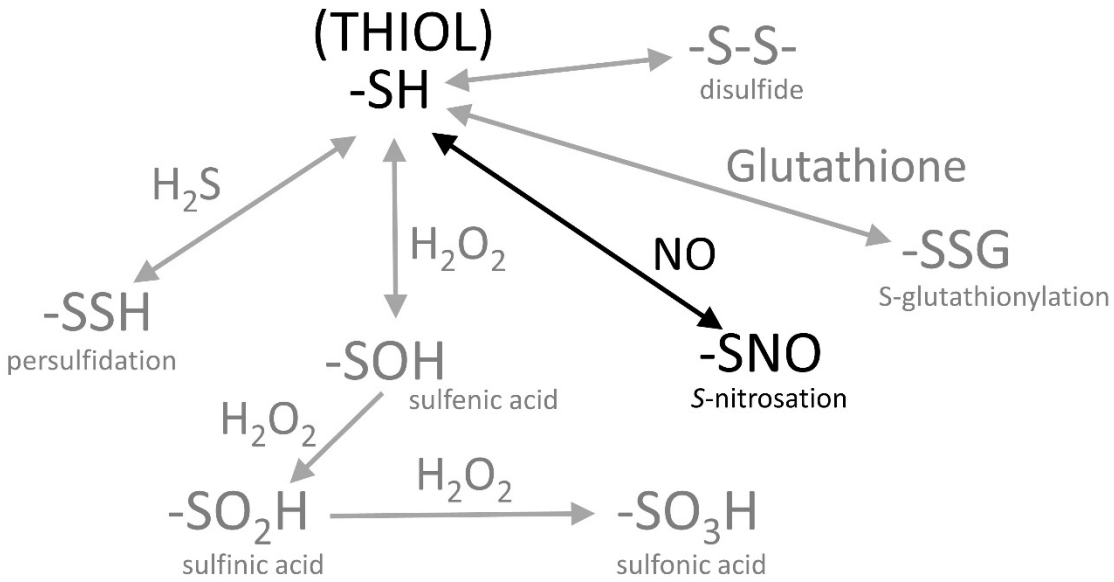

Figure 3: The redox "Goldilocks Zone". A schematic to show how the intracellular redox may fluctuate but stays within safe limits. If it becomes too reducing, reductive stress may result. Figure adapted from Alleman *et al*. (2014).