Considerations of the importance of redox state on reactive nitrogen species action

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Abstract

Nitric oxide (NO) and other reactive nitrogen species (RNS) are immensely important signalling molecules in plants, being involved in a range of physiological responses. However, the exact way in which NO fits into signal transduction pathways is not always easy to understand. Here, some of the issues that should be considered are discussed. This includes how NO may interact directly with other reactive signals, such as reactive oxygen and sulfur species, how NO metabolism is almost certainly compartmentalised, that threshold levels of RNS may need to be reached to have effects and how the intracellular redox environment may impact on NO signalling. Until better tools are available to understand how NO is generated in cells, where it accumulates and to what levels it reaches, it will be hard to get a full understanding

of NO signalling. The interaction of RNS metabolism with the intracellular redox environment needs further investigation. A changing redox poise will impact on whether RNS species can thrive in or around cells. Such mechanisms will determine whether specific RNS can indeed control the responses needed by a cell.

Introduction

Reactive oxygen species (ROS), such as the superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2), along with reactive nitrogen species (RNS) such as nitric oxide (NO) are instrumental in the way cells control their activities. With other reactive compounds such as hydrogen sulfide (H_2S), these compounds impact on the intracellular redox poise of cells and lead to post-translational modifications of proteins (Hancock, 2009; Mur *et al*., 2013; Forman *et al*., 2014; Schieber and Chandel, 2014). Therefore, how NO fits in to this intricate control system of cells is immensely important to fully understand.

Since the realisation that NO is important in cell signalling which came to light in 1987 (Palmer *et al*., 1987), and the further papers that NO was involved in plant physiology (Laxalt *et al*., 1997; Delledonne *et al*., 1998; Durner *et al*., 1998) it has been realised that NO has an instrumental role in controlling plant cell functions. This includes the control of development, seed germination and stomatal closure, as well as numerous stress responses including pathogen challenge, as previously reviewed (Romero-Puertas *et al*., 2004; Del Rio, 2015; Fancy *et al*., 2017).

The production of NO in plants is still controversial, with papers reporting NOS-like enzymes and NOS-like activity. However, no NOS gene or protein has been found to-date in higher plants (Jeandroz *et al*., 2016; Fröhlich and Durner, 2011), although NOS-like enzymes have been reported to exist in algae (Foresi *et al*., 2010). Other sources of NO in plants include nitrate reductase (Rockel *et al*., 2002) as discussed previously (Mur *et al*., 2013). It is likely that there are range of enzymatic and non-enzymatic sources of NO that contribute to the overall NO status in the cell, as shown in Table 1 and discussed by Astier *et al*. (2018).

The downstream effects of NO in plants include the modulation of levels of cyclic nucleotides, such as cGMP, but also the post-translational modification of proteins. The most widely reported modification is that seen with thiols, as can be detected by the biotin switch assay (Jaffrey and Snyder, 2001). This is a *S*-nitrosation (often referred to as *S*-nitrosylation) (Lindermayr and Saalbach**,** 2005). Here a thiol group is modified to the –*S*-NO state. This can be restored back to the –SH state and so this modification is akin to phosphorylation, being able to toggle between two states, with the concomitant potential alteration of activity or function. It is worth noting here that the terms nitrosation and nitrosylation are often used erroneously and sometimes interchangeably. Nitrosation refers to the addition of NO^+ (nitrosonium ion) to a group with nucleophilic properties, an example of relevance here being a thiolate group on amino acids. Nitrosylation on the other hand, refers to the direct addition of NO. For a much more in depth explanation of these terms, and in fact also of the term nitrosative stress, see Heinrich *et al*. (2013).

Other similar protein modifications involve nitration of tyrosine (Kolbert *et al*., 2017). Of particular relevance to this discussion is the thiol groups of proteins are also susceptible to modification by other reactive signalling molecules such as H_2O_2 and hydrogen sulfide (H_2S), as discussed further below and shown in Figure 1. Therefore, it is likely that NO is in competition with other intracellular reactive signals, as discussed previously (Hancock and Whiteman, 2014; 2016).

NO is difficult to measure in biological systems even though many methods have been employed over the years, as shown in Table 2 and discussed by Mur *et al*. (2011). These assays range from the Greiss reaction (Tsikas, 2007), NOS activity (Tischner *et al*., 2007), to fluorescent probes (Planchet and Kaiser, 2006). Even though it is recommended that at least two independent methods for measuring NO

are used (Gupta and Igamberdiev, 2013) is it still difficult to know specifically how NO is having its action and where in the cell NO accumulation is significant.

Here, the interaction of NO with other reactive species and in particular its effect on redox is discussed. It can be seen therefore that the generation and measurement of NO in plants is not without controversy. On top of this there it needs to be considered that NO is very reactive, will have sub-cellular locations and will impact on, and be impacted on by, the redox poise of the cell. Such considerations are further discussed below.

Interactions of NO with other reactive species

Biological systems have evolved in the presence of a range of reactive and potentially toxic compounds (Hancock, 2017). Therefore it should be considered that the presence of NO in cells will not be in isolation but with other reactive signalling compounds. Consequently, it is not surprising that there are several direct chemical interactions reported, as shown in Figure 2. NO and $O_2^{\cdot-}$ can react to form peroxynitrite ($ONOO^-$). This has several effects. Firstly $ONOO^-$ is created and this can act as a signal in cells in its own right (Speckmann *et al*., 2016), although its bioavailability is going to be reduced by the presence of carbon dioxide (Squadrito and Pryor, 1998). Secondly, as $ONOO^-$ is produced, the bioavailability of both NO and $O_2^{\cdot-}$ is reduced, lowering their significance in future cell signalling pathways. In a similar manner, NO and H_2S can react together, lowering each other's bioavailability and creating a new potential signalling molecule, nitrosothiol (Whiteman *et al*., 2006). Another extremely important molecule capable of being generated is GSNO, which may be seen as a method of storage and/or transport of NO in biological systems (Hogg *et al*., 1996). Furthermore, reactions such as those which can generate N_2O_3

may interfere with the measure of $NO^{\cdot}$ in biological systems, especially those using diaminofluorescein (DAF) (Planchet and Kaiser, 2006). Therefore interactions of NO with other compounds, many of which may be produced at the same time as NO, will have a major impact on whether NO signalling can proceed.

Compartmentalisation and thresholds

Assays of NO often give a measurement of overall NO levels, especially if the Griess assay or assay of NOS-like activity is used. However, both of these assays have issues. The Griess assay will suffer from interference from background nitrite/nitrate levels while the NOS assay may be measuring argininosuccinate lyase activity (Tischner *et al*., 2007). If a fluorescence-based method is used, such as DAF, then information of the subcellular location of NO may be gleamed, but this is at the sacrifice of quantification. Therefore it is hard to know exactly how much NO is present in any part of the cell in real time. A similar issue is seen with measuring redox status of a cell. Often glutathione levels are measured (Schafer and Buettner, 2001) as further discussed below, but again the actual quantification in subcellular locations is not easy and generally not done.

The location of signalling molecules such as NO in cells is important. As well as having individual organelles, even the cytoplasm cannot be considered uniform, and so therefore there are numerous specific locales in cells. It is perhaps not surprising, therefore, that hotspots have been reported for ROS for example (de Rezende *et al*., 2012; Noctor and Foyer, 2016). In a similar way hotspots of Ca^{2+} ions have been reported (Nunes *et al*., 2012). Compartmentalisation of calcium ions was discussed by Bononi *et al*. (2012), while cAMP-mediated pathways are often said to be compartmentalised (Zaccolo *et al*., 2002; Baillie *et al*., 2005). If second

messenger pathways are compartmentalised (McCormick and Baillie, 2014) it would be naive to think that NO-mediated pathways are not structured in a similar manner. It is often assumed that NO can freely move around the cell, but it is relatively reactive. Not only will *S*-nitrosation of proteins take place, but lipids too can be modified by NO (Mata-Pérez *et al*., 2016), suggesting that free movement through membranes may not be possible. Membranes have been suggested to enhance NO oxidation to create other reactive nitrogen-based compounds such as nitrogen dioxide and dinitrogen trioxide, so NO *per se* may be removed (Möller *et al*. 2007). Furthermore if NO reacts with lipids its availability to react with other biomolecules will be reduced while the modified lipids themselves may be able to act as signalling molecules (Mata-Pérez *et al*., 2016; 2018). On the other hand, aquaporins have been implicated as a way to facilitate NO movement in cells (Wang and Tajkhorshid, 2010).

The second issue which needs to be discussed here is the actual levels of signalling molecule that needs to be present to trigger a response. It would be hard to find in the literature data where NO or *S*-nitrosation is measured and the levels show that there is no NO or protein modification in the cell, and then these levels are often reported to increase to a higher and measureable amount. Data tends to show low levels of NO or *S*-nitrosation which rise. How much rise is needed? If *S*-nitrosation increases by 10% is that enough? Perhaps it is to push signalling over a threshold to create a response. Certainly thresholds have been discussed in NO research (Li *et al*., 2006). It has also been suggested that other reactive signals such as H_2S dampen down the signalling mediated by NO and ROS (Hancock and Whiteman, 2014), suggesting that signalling is kept below a threshold level until such time as that response is truly needed. Therefore a consideration of the significance

158 of NO metabolism changes in cells needs to be had, with small changes perhaps not
159 having the effects that might be assumed.
160
161 **Considerations of redox chemistry**
162 Probably the first consideration of how nitric oxide impacts on redox is to determine
163 which species is present. Nitric oxide chemistry discussions need to include the
164 radical form $NO^{\cdot}$, but also species such as nitroxyl (NO^{-}) and nitrosonium (NO^{+}) ions
165 (Lancaster Jr, 2015). The chemistry of these three nitrogen species is quite different
166 and methods to supply NO to experimental systems may deliver one of these
167 (Hughes, 1999), not necessarily $NO^{\cdot}$ as might be wanted or expected.
168 Despite the interaction of NO with redox, the redox status of the cell is
169 important to determine. Often the overall redox of the cell is estimated by the
170 measurement of glutathione (reviewed by Schafer and Buettner, 2001). It is not
171 sufficient to simply measure the concentrations of the reduced form GSH, but the
172 concentration of the oxidised form, GSSG, also needs to be determined. These two
173 pieces of data can then be used to calculate the total amount of glutathione present,
174 as well as the oxidised:reduced ratio (ox:red ratio).
175 To determine the redox status of the experimental solution being studied, E_h,
176 there are two numbers which are needed. It is important to know the ox:red ratio of
177 the chemical species under study, but the mid-point potential of the species is also
178 needed, that is the E_m. Once these numbers are known they can be used to
179 calculate E_h using the Nernst Equation, as shown in Equation 1.
180
181 Equation 1: The Nernst Equation (redox equation) assuming an intracellular pH of
182 7.4.

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

where: E_h is the redox potential; $E_{m(pH7.4)}$ is the midpoint potential of redox couple at pH7.4; R is the Gas Constant; T is the temperature in Kelvin; F is the Faraday Constant; n is the number of electrons used in oxidation/reduction.

Methods are available to measure concentrations of glutathione (Rahman *et al*., 2006), and the E_m for the glutathione ox/red couple has been determined (Rost and Rapoport, 1964) and therefore in theory the cellular redox can be estimated. But at best this is an estimation. The estimation of the GSH/GSSG E_m has not been measured in a more robust manner, whilst the redox reaction of glutathione can be summed up by Equation 2.

Equation 2: the simplified chemical reaction of the glutathione couple.

$$2GSH \longrightarrow GSSG$$

Therefore when the data is put into the Nernst Equation it becomes a squared relationship. The corollary of this is that the calculated E_h becomes dependent on the total glutathione concentration, as well as the GSH/GSSG ratio. The levels of glutathione in cells is not static. Glutathione can be generated in cells (Forman, 2016) but conversely cells can lose glutathione too (Ghibelli *et al*., 1995). Both scenarios will alter the cellular redox status. Furthermore, some reactive signalling molecules, such as H_2S, will induce an increase in intracellular glutathione (Parsanathan and Jain, 2018), and so modulate the redox status. The reaction of NO

with GSH, so forming GSNO, will also reduce the total GSH/GSSG concentration in the cell and potentially impact on the cellular redox, especially if this happens in a compartmentalised manner.

Furthermore, in a treatise by Schafer and Buettner (2001), it was argued that it should be all the redox active compounds in the cell that should be considered. Equation 3 has been mooted to encompass this.

Equation 3:

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

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

It is not just glutathione which will contribute to the intracellular redox status but the presence of many other abundant low-molecular weight (LMW) thiols (Birtic *et al*., 2011), including 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 so they could have a significant effect, and levels can change with time as physiology changes, such as in seed aging (Birtic *et al*., 2011). Therefore, a simple measurement of GSH/GSSG levels may be only an inaccurate estimate of the intracellular redox status.

A further compound which needs consideration here is ascorbate (AsA). The redox status of the ascorbate couple (AsA/dehydroascorbate (DHA): it should be noted that there is also an intermediate state; the semi-dehydroascorbic acid radical (SDA)) and its influence on biological systems has been known for some time

(Sapper *et al*., 1982). More recently the redox of AsA has been implicated in flowering for example (Chin et al., 2016) and plant stress responses (Jozefczak.*et al*., 2015). The distribution of AsA in plant tissues has been estimated to be in the mM range (Zechmann, 2011) and therefore the status of the AsS redox ratio needs to be taken into account too.

Having said this the intracellular redox poise has been estimated in several systems (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 it has been suggested that the average status of the cell is approximately -242mV (Schafer and Buettner, 2001). However, this is not static, and would not be expected to be. With influences of H_2S and glutathione generation and export, as discussed above, the redox state of the cell will flux. Whether it is a cause or effect, the redox state has been estimated to become more oxidising as cells differentiate, becoming approximately -200 mV, while as apoptosis is initiated the cellular redox may well be as oxidising as -170 mV (Schafer and Buettner 2001). It is into such environments that nitric oxide metabolism has to be interwoven.

The perceived dogma is that oxidative stress is the shift of redox in cells towards more oxidising states, and that this is caused by an imbalance in reactive oxygen species such as superoxide anions and hydrogen peroxide (H_2O_2). For example see Uttara *et al.* (2009). However, the intracellular glutathione levels are reported to be mM (Schafer and Buettner, 2001; Bhatla and Horvitz, 2015), and along with other low molecular weight thiols maintaining the intracellular redox too it is hard to envisage the generation of ROS being a huge influence, as argued previously (Hancock and Whiteman, 2018). It is more likely that the intracellular redox will have an influence on the maintenance and longevity of certain partners in

a redox couple, perhaps to the health of the cell, or in some cases the detriment of the cell. Using the Nernst Equation (Equation 1) this can be estimated for predicted intracellular redox states, such as at -242 mV, -200 mV or -170 mV. This has previously been reported (Hancock and Whiteman, 2018) and it can indeed be seen for some redox couples that there is a significant effect.

If intracellular signalling pathways (McCormick and Baillie, 2014), cAMP pathways (Zaccolo *et al*., 2002; Baillie *et al*., 2005), calcium ion metabolism (Bononi *et al*., 2012; Nunes *et al*., 2012) and ROS metabolism (de Rezende *et al*., 2012; Noctor and Foyer, 2016) can all be compartmentalised, then there is no reason to suspect that the intracellular redox status is not also compartmentalised, and this is indeed what has been reported (Go and Jones, 2008). Therefore the actual redox poise that many cellular components, including proteins and their thiol groups, are exposed to will not be known. A small amount – not concentration – increase in ROS in an organelle or cytoplasmic hotspot may be paramount to a large concentration increase which on a local scale may have a significant effect on redox status, and the corollary effects.

Which partner in a couple is able to be sustained in a cell may be important, as exemplified by cytochrome *c*. This can exist in an oxidised or reduced state, the toggling between such states allows it to carry out its function as an electron shuttle between Complex III and Complex IV in mitochondria (Hüttemann *et al*., 2011). When released into the cytoplasm of the cell during apoptosis (Liu *et al.,* 1996) cytochrome *c* will be exposed to the redox environment which is there, and it has been suggested that the subsequent redox state of the cytochrome protein will determine its ability to partake in apoptosis (Hancock *et al*., 2001). The same argument can be made for any redox couple which is in a changing redox

environment, either because it has moved in the cell or the redox environment has been impacted on by external factors.

As NO and chemical species involved in NO metabolism often exist in redox couples then the same arguments can be made here. To influence the intracellular redox environment the amount needed to overcome redox buffering by mM small molecular weight thiols such as GSH would be impossible, unless compartmentalisation allowed the small amount to translate into a significant concentration change. This could be possible in the locale of NO generating enzymes such as nitrate reductase. It has been suggested that many proteins have moonlighting functions and one of the reasons given was that the cell must have a very well controlled internal environment where proteins are packed very closely together (Irving *et al*., 2018). Therefore, if NO generating enzymes are in close proximity to NO perception proteins then significant changes in concentrations may be occurring, with concomitant changes in redox or reaction rates with thiols, but by measuring more global concentrations of NO and GSH/GSSG such local interactions would not be seen.

On the other side of the argument, NO would be produced into a highly buffered redox environment and therefore it is important to understand how this may impact on the sustaining of RNS species. Many RNS exist as redox couples and therefore there will be an E_m associated with their interconversion. If this E_m is in the region of that of the cell redox poise then changes in that cellular environment may have an impact. Alternately, if the E_m is significantly more positive than the cellular environment then one species of the redox couple will be favoured, the other perhaps never being able to exist in a stable state. Therefore, knowing the cellular redox at any location and time is really important to understand how other low

abundant redox species may be impacted upon. Such estimations have been reported recently for a range of ROS, RNS and other redox signalling components (Hancock and Whiteman, 2018). Some examples will be picked out here.

As an example of how redox impacts on RNS redox couples, $NO^+/NO^\cdot$ has an E_m of +1210 mV (Koppenol, 1997) and therefore the ratio of the couple will be very one sided, favouring one species over the other. As the redox changes towards an apoptotic state by approximately 70mV (Schafer and Buettner, 2001) such changes should be considered too. For the $NO^\cdot/NO^-$ (singlet) couple the E_m is approximately -350 mV (Koppenol, 1997) and this is near the -242/-170 mV redox of the cell and therefore will be influenced by this change, and in fact the $NO^\cdot$ form is favoured, which is usually thought of as the signalling form of NO. So it could be suggested here that as the redox changes to be more oxidising the increase in NO signalling here is helping to drive the apoptotic state once the cell initiates this action. On the other hand the reduced state of cytochrome *c* is favoured by the intracellular redox, and as it was the oxidation of cytochrome *c* which was mooted to drive apoptosis (Hancock *et al*., 2001) this seems to be counter to what is needed. Perhaps the answer once again comes down to compartmentalisation of all these redox active compounds.

Posttranslational modifications of proteins is not immune to the influence of redox either. The RSNO/RSH couple has been reported to have an E_m of -400 mV (Koppenol, 1997) which would favour the RSNO as the redox becomes more oxidising, so perhaps driving –SNO signalling as the cell moves towards differentiation and apoptosis. Therefore, as the NO levels rises, -SNO signalling becomes more prevalent, and if this is accompanied by rises in ROS and oxidation of the environment then reinforcement of the signalling would be seen, perhaps

above a vital threshold level, as the signalling all drives in the same direction. On the other hand, with a E_m of -200 mV (Li and Lancaster Jr, 2013), the S/H_2S couple would not favour the H_2S form, so removing its possible brake on RNS/ROS signalling which has previously been mooted (Hancock and Whiteman, 2014). This would therefore favour NO signalling, which would be the preferred outcome under these circumstances. It would be assumed that a threshold level of NO signalling has been reached and therefore NO-mediated signalling needs to proceed.

For a more full exploration of redox on a range of ROS, RNS and reactive signals see Hancock and Whiteman (2018).

Conclusions and future perspectives

Looking at the literature with regards to RNS metabolism and NO signalling it appears that often studies have a level of naivety. There are many considerations which are needed before data can be fully understood. NO is difficult to measure and knowing exactly how much NO has accumulated and where it exactly is in the cell is not possible to determine at the moment, as fluorescence probes which give location fail to be quantifiable. To further complicate this, although there are known intracellular sources of NO, such as NR (Rockel *et al.*, 2002), other sources such as NOS are still very controversial (Jeandroz *et al*., 2016). To complicate the situation further, NO is known to react with numerous other reactive signalling molecules such as $O_2^{\cdot-}$ and H_2S, producing $ONOO^-$ and nitrosothiols respectively, and hence not only affecting each other's bioavailability but also producing new signalling molecules in the cell (Whiteman *et al*., 2006; Speckmann *et al*., 2016).

It has been realised for some time that cAMP signalling is compartmentalised (Baillie *et al*., 2005), while this has similarly been reported for Ca^{2+} signalling (Nunes

et al., 2012) and ROS metabolism (de Rezende *et al*., 2012; Noctor and Foyer, 2016), so it would be expected that NO accumulation and signalling would show a similar pattern. NO levels would be expected to rise to significant levels, or thresholds, and it has been suggested that damping NO signalling down below should thresholds may be under the control of other reactive signals such as H_2S (Hancock and Whiteman, 2014), or the redox poise of the cell.

Lastly, but certainly not least, there is the fact that cells maintain their intracellular environment in a very reduced state (Schafer and Buettner, 2001). This is partly to enable the important co-factors such as NADH to be maintained appropriately in the cell (Bücher *et al*., 1972) but also to enable redox to partake in signalling (Forman *et al*. 2014). This redox state is not static and has been shown to change as the cells move between proliferative states and differentiation, and to become even more oxidising as apoptosis proceeds (Schafer and Buettner, 2001). Driving the cellular intracellular redox environment towards oxidation has always been thought to be partly an imbalance in ROS metabolism leading to oxidative stress (Sies *et al*., 2017), but with small molecular weight thiols being thought to amass to mM concentrations this is not going to be easy at a cellular level, suggesting that compartmentalisation is indeed key here. Furthermore, NO and other RNS will be produced into this reducing environment which will impinge on their longevity and ability to have an effect (Hancock and Whiteman, 2018).

In summary, the field of NO research needs better tools to understand exactly how NO species are produced, where they are able to accumulate and how they interact with numerous other reactive signalling components such as ROS and H_2S. By taking a holistic approach to NO signalling, encompassing accurate measures of

ROS and redox at a compartmentalised level a better understanding of how NO fits into the complex signalling pathways of cells may be gained.

Conflicts of interest

The author has no conflicts of interest.

References

Alber NA, Sivanesan H, Vanlerberghe GC. 2017. The occurrence and control of nitric oxide generation by the plant mitochondrial electron transport chain. Plant Cell and Environment **40**, 1074-1085.

Arasimowicz M, Floryszak-Wieczorek J, Milczarek G, *et al.* 2009. Nitric oxide, induced by wounding, mediates redox regulation in pelargonium leaves. Plant Biology **11**, 650-663.

Astier J, Gross I, Durner J. 2018. Nitric oxide production in plants: an update. Journal of Experimental Botany **69**, 3401-3411.

Astier J, Jeandroz S, Wendehenne D. 2018. Nitric oxide synthase in plants: The surprise from algae. Plant Science **268**, 64-66.

Baillie GS, Scott JD, Houslay MD. 2005. Compartmentalisation of phosphodiesterases and protein kinase A: opposites attract. FEBS Letters **579**, 3264-3270.

Bethke PC, Badger MR, Jones RL. 2004. Apoplastic synthesis of nitric oxide by plant tissues. Plant Cell **16**, 332-341.

Bhatla N, Horvitz HR. 2015. Light and hydrogen peroxide inhibit *C. elegans* feeding through gustatory receptor orthologs and pharyngeal neurons. Neuron **85**, 804-818.

Birtic S, Colville L, Pritchard HW, *et al*. 2011. Mathematically combined half-cell reduction potentials of low-molecular-weight thiols as markers of seed ageing. Free Radical Research **45**, 1093-1102.

Bononi A, Missiroli S, Poletti F, *et al*. 2012. Mitochondria-associated membranes (MAMs) as hotspot Ca^{2+} signaling units. Advances in Experimental Medicine and Biology. **740**, 411-437.

Bright J, Hiscock SJ, James PE, *et al*. 2009. Pollen generates nitric oxide and nitrite: a possible link to pollen-induced allergic responses. Plant Physiology and Biochemistry **47**, 49-55.

Bücher T, Brauser B, Conze A, *et al.*, 1972. State of oxidation-reduction and state of binding in the cytosolic NADH-system as disclosed by equilibration with extracellular lactate/pyruvate in hemoglobin-free perfused rat liver. European Journal of Biochemistry **27**, 301-317.

Byrnes CA, Bush A, Shinebourne EA. 1996. Measuring expiratory nitric oxide in humans. Nitric Oxide, Pt B **269**, 459-473.

Cai JY, Wallace DC, Zhivotovsky B, *et al*. 2000. Separation of cytochrome c-dependent caspase activation from thiol-disulfide redox change in cells lacking mitochondrial DNA. Free Radical Biology and Medicine **29**, 334-342.

Chamizo-Ampudia A, Sanz-Luque E, Llamas A, *et al*. 2017. Nitrate reductase regulates plant nitric oxide homeostasis. Trends in Plant Science **22**, 163–174.

Chamizo-Ampudia A, Sanz-Luque E, Llamas Á, *et al*., 2016. A dual system formed by the ARC and NR molybdoenzymes mediates nitrite-dependent NO production in Chlamydomonas. Plant, Cell and Environment **39**, 2097–2107.

Chin DC, Hsieh CC, Lin HY, *et al*. 2016. A low glutathione redox state couples with a decreased ascorbate redox ratio to accelerate flowering in Oncidium orchid. Plant and Cell Physiology **57**, 423-436.

Conrath U, Amoroso G, Kohle H, *et al.* 2004. Non-invasive online detection of nitric oxide from plants and some other organisms by mass spectrometry. Plant Journal **38**, 1015-1022.

Corpas FJ, Palma JM, del Rio LA, *et al*. 2009. Evidence supporting the existence of L-arginine-dependent nitric oxide synthase activity in plants. New Phytologist **184**, 9-14.

Cristescu SM, Persijn ST, te Lintel Hekkert S, *et al*. 2008. Laser-based systems for trace gas detection in life sciences. Applied Physics B **92**, 343–349.

Delledonne M, Xia Y, Dixon RA, *et al.* 1998. Nitric oxide functions as a signal in plant disease resistance. Nature **394**, 585-588.

Del Río LA. 2015. ROS and RNS in plant physiology: an overview. Journal of Experimental Botany **66**, 2827-2837.

Durner J, Wendehenne D, Klessig DF. 1998. Defense gene induction in tobacco by nitric oxide, cyclic GMP, and cyclic ADP-ribose. Proceeding of the National Academy of Science USA **95**, 10328-10333.

Fancy NN, Bahlmann AK, Loake GJ. 2017. Nitric oxide function in plant abiotic stress. Plant Cell and Environment **40**, 462-472.

Foresi N, Correa-Aragunde N, Parisi G, *et al*. 2010. Characterization of a nitric oxide synthase from the plant kingdom: NO generation from the green alga *Ostreococcus tauri* is light irradiance and growth phase dependent. Plant Cell **22**, 3816-3830.

Forman HJ. Ursini F, Maiorino M. 2014. An overview of mechanisms of redox signalling. Journal of Molecular and Cellular Cardiology **73**, 2-9.

Forman HJ. 2016. Glutathione – From antioxidant to post-translational modifier. Archives of Biochemistry and Biophysics **595**, 64-67.

Fröhlich A, Durner J. 2011. The hunt for plant nitric oxide synthase (NOS): Is one really needed? Plant Science **181**, 401-404.

Galatro A, Puntarulo S. 2016. Measurement of nitric oxide (NO) generation rate by chloroplasts employing Electron Spin Resonance (ESR). Methods in Molecular Biology **1424**, 103-112.

Ghibelli L, Coppola S, Rotlilio G, *et al.* 1995. Non-oxidative loss of glutathione in apoptosis via GSH extrusion. Biochemical and Biophysical Research Communications **216**, 313-320.

Go Y-M, Jones DP. 2008. Redox compartmentalization in eukaryotic cells. Biochimica et Biophysica Acta **1780**, 1273–1290.

Groß F, Rudolf EE, Thiele B, 2017. Copper amine oxidase 8 regulates arginine-dependent nitric oxide production in *Arabidopsis thaliana*. Journal of Experimental Botany **68**, 2149-2162.

Godber BL, Doel, JJ, Sapkota GP, *et al*. (2000) Reduction of nitrite to nitric oxide catalyzed by xanthine oxidoreductase. Journal of Biological Chemistry **275**, 7757-7763.

Gupta KJ, Igamberdiev AU. 2013. Recommendations of using at least two different methods for measuring NO. Frontiers in Plant Science **4**, 58.

Hancock JT. 2009. The role of redox mechanisms in cell signalling. Molecular Biotechnology **43**, 162-166.

Hancock JT. 2017. Harnessing evolutionary toxins for signaling: Reactive oxygen species, nitric oxide and hydrogen sulfide in plant cell regulation. Frontiers in Plant Science, **8**, 189.

Hancock JT, Desikan R, Neill SJ. 2001. Does the redox status of cytochrome *c* act as a fail-safe mechanism in the regulation of programmed cell death? Free Radical Biology and Medicine **31**, 697-703.

Hancock JT, Whiteman M. 2014. Hydrogen sulfide and cell signaling: team player or referee? Plant Physiology and Biochemistry **78**, 37-42.

Hancock JT, Whiteman M. 2016. Hydrogen sulfide signaling: Interactions with nitric oxide and reactive oxygen species. Annals of the New York Academy of Sciences **1365**, 5-14.

Hancock JT, Whiteman M. 2018. Cellular redox environment and its influence on redox signalling molecules. Reactive Oxygen Species **5** (14).

Heinrich TA, Da Silva RS, Mirandam KM *et al.* 2013. Biological nitric oxide signalling: chemistry and terminology. British Journal of Pharmacology **169**, 1417–1429.

Hogg N, Singh RJ, Kalyanaraman B. 1996. The role of glutathione in the transport and catabolism of nitric oxide. FEBS Letters **382**, 223-228.

Hughes MN. 1999. Relationships between nitric oxide, nitroxyl ion, nitrosonium cation and peroxynitrite. Biochimica et Biophysica Acta (BBA) – Bioenergetics **1411**, 263-272.

Hutter DE, Till BG, Greene JJ. 1997. Redox state changes in density-dependent regulation of proliferation. Experimental Cell Research **232**, 435-438.

Hüttemann N, Pecina P, Rainbolt M, *et al.* 2011. The multiple functions of cytochrome *c* and their regulation in life and death decisions of the mammalian cell: from respiration to apoptosis. Mitochondrion **11**, 369-381.

Hwang C, Sinskey AJ, Lodish HF. 1992. Oxidised redox state of glutathione in the endoplasmic reticulum. Science **257**, 1496-1502.

Igamberdiev AU, Ratcliffe RG, Gupta KJ. 2014. Plant mitochondria: source and target for nitric oxide. Mitochondrion **19 Pt B**, 329-333.

Irving HR, Cahill DM, Gehring C. 2018. Moonlighting proteins and their role in the control of signaling microenvironments, as exemplified by cGMP and phytosulfokine receptor 1 (PSKR1). Frontiers in Plant Science **9**, 415.

Jaffrey SR, Snyder SH. 2001. The biotin switch method for the detection of S-nitrosylated proteins. Science's STKE **2001**(86), pl1.

Jeandroz S, Wipf D, Stuehr DJ, *et al.*, 2016. Occurrence, structure, and evolution of nitric oxide synthase-like proteins in the plant kingdom. Science Signaling **9**, re2.

Jones DP, Carlson JL, Mody VC, *et al*. 2000. Redox state of glutathione in human plasma. Free Radical Biology and Medicine **28**, 625-635.

Jones DP, Maellaro E, Slater AFG, *et al*. 1995. Effects of N-acetyl-L-cysteine in T-cell apoptosis are not mediated by increased cellular glutathione. Immunology Letters **45**, 205-209.

Jozefczak M, Bohler S, Schat H, *et al*. 2015. Both the concentration and redox state of glutathione and ascorbate influence the sensitivity of Arabidopsis to cadmium. Annals of Botany **116**, 601-12.

Kirlin WG, Cai J, Thompson SA, *et al.* 1999. Glutathione redox potential in response to differentiation and enzyme inducers. Free Radical Biology and Medicine **27**, 1208-1218.

Kojima H, Sakurai K, Kikuchi K, *et al*. 1998. Development of a fluorescent indicator for nitric oxide based on the fluorescein chromophore. Chemical & Pharmaceutical Bulletin **46**, 373-375.

Kolbert Z, Feigl G, Bordé Á, *et al*. 2017. Protein tyrosine nitration in plants: Present knowledge, computational prediction and future perspectives. Plant Physiology and Biochemistry **113**, 56-63.

Koppenol WH. 1997. The chemical reactivity of radicals. K.B. Wallace (Ed.), *Free Radical Toxicology*, Taylor and Francis, London, 3–14.

Lancaster, Jr. 2015. Nitric oxide: a brief overview of chemical and physical properties relevant to therapeutic applications. Future Science OA. **1**, FSO59.

Laxalt AM, Beligni MV, Lamattina L. 1997. Nitric oxide preserves the level of chlorophyll in potato leaves infected by *Phytophthora infestans*, European Journal of Plant Pathology **103***,* 643–651.

Li Q, Lancaster JR Jr. 2013. Chemical foundations of hydrogen sulfide biology. Nitric Oxide **35**, 21-34.

Li CQ, Pang B, Kiziltepe T, *et al*., 2006. Threshold effects of nitric oxide-induced toxicity and cellular responses in wild-type and p53-null human lymphoblastoid cells. Chemical Research in Toxicology **19**, 399-406.

Lindermayr C, Saalbach G, Durner J, 2005. Proteomic identification of S-nitrosylated proteins in Arabidopsis. Plant Physiology **137**, 921–930.

Liu X, Kim CN, Yang J, *et al*. 1996. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome *c*. Cell **86**, 147–157.

Maia LB, Moura JJ. 2015. Nitrite reduction by molybdoenzymes: a new class of nitric oxide-forming nitrite reductases. Journal Biological Inorganic Chemistry **20**, 403-433.

Mata-Pérez C, Sánchez-Calvo B, Padilla MN, *et al*. 2016. Nitro-fatty acids in plant signaling: nitro-linolenic acid induces the molecular chaperone network in Arabidopsis. Plant Physiology **170**, 686-701.

Mata-Pérez C, Padilla MN, Sánchez-Calvo B, *et al*. 2018. Biological properties of nitro-fatty acids in plants. Nitric Oxide. **pii**: S1089-8603(17)30286-0.

McCormick K, Baillie GS. 2014. Compartmentalisation of second messenger signalling pathways. Current Opinions in Genetic Development **27**, 20-25.

Modolo LV, Augusto O, Almeida IM, *et al.* 2005. Nitrite as the major source of nitric oxide production by *Arabidopsis thaliana* in response to *Pseudomonas syringae*. FEBS Letters **579**, 3814-3820.

Mur LA, Mandon J, Cristascu SM, *et al*. 2011. Methods of nitric oxide detection in plants: a commentary. Plant Science **181**, 509-519.

Mur LA, Mandon J, Persijn S, *et al*., 2013. Nitric oxide in plants: an assessment of the current state of knowledge. AoB Plants **5**, pls052.

Mur LAJ, Santosa IE, Laarhoven LJJ, *et al*., 2005. Laser photoacoustic detection allows in planta detection of nitric oxide in tobacco following challenge with avirulent and virulent *Pseudomonas syringae* pathovars. Plant Physiology **138**, 1247-1258.

Mur LAJ, Sivakumaran A, Mandon J, *et al*. (2012) Haemoglobin modulates salicylate and jasmonate/ethylene-mediated resistance mechanisms against pathogens. Journal of Experimental Botany **63**, 4375-4387.

577 **Murphy ME, Noack E.** 1994. Nitric oxide assay using hemoglobin method. Methods
578 in Enzymology **233**, 240-250.
579 **Noctor G, Foyer CH**. 2016. Intracellular redox compartmentation and ROS-related
580 communication in regulation and signaling. Plant Physiology **171**, 1581-1592.
581 **Nunes P, Cornut D, Bochet V, *et al.*** 2012. STIM1 juxtaposes ER to phagosomes,
582 generating Ca^{2+} hotspots that boost phagocytosis. Current Biology **22**, 1990-
583 1997.
584 **Palmer RMJ, Ferrige AG, Moncada S,** 1987. Nitric oxide release accounts for the
585 biological activity of endothelium-derived relaxing factor. Nature **327**, 524-526.
586 **Parsanathan R, Jain SK.** 2018 Hydrogen sulfide increases glutathione biosynthesis,
587 and glucose uptake and utilisation in C_2C_{12} mouse myotubes. Free Radical
588 Research **52**, 288-303.
589 **Planchet E, Kaiser WM**. 2006. Nitric oxide (NO) detection by DAF fluorescence and
590 chemiluminescence: a comparison using abiotic and biotic NO sources.
591 Journal of Experimental Botany **57**, 3043–3055.
592 **Rahman I, Kode A, Biswas SK**. 2006. Assay for quantitative determination of
593 glutathione and glutathione disulfide levels using enzymatic recycling method.
594 Nature Protocols **1**, 3159-3165.
595 **de Rezende FF, Martins Lima A, Niland S**, ***et al*.,** 2012. Integrin α7β1 is a redox-
596 regulated target of hydrogen peroxide in vascular smooth muscle cell
597 adhesion. Free Radical Biology and Medicine **53**, 521-531.
598 **Rockel P, Strube F, Rockel A, *et al.*** 2002. Regulation of nitric oxide (NO)
599 production by plant nitrate reductase *in vivo* and *in vitro*. Journal Experimental
600 Botany **53**, 103-110.

Romero-Puertas MC, Perazzolli M, Zago ED, ***et al*.** 2004. Nitric oxide signalling functions in plant-pathogen interactions. Cell Microbiology **6**, 795-803.

Rost J, Rapoport S. 1964. Reduction-potential of glutathione. Nature **201**, 185.

Rumer S, Gupta KJ, Kaiser WM. 2009**.** Plant cells oxidize hydroxylamines to NO. Journal of Experimental Botany **60**, 2065-2072.

Sapper H, Kang SO, Paul HH*, et al.* 1982. The reversibility of the vitamin C redox system: electrochemical reasons and biological aspects. Zeitschrift für Naturforschung C **37**, 942-946.

Schafer FQ, Buettner GR. 2001. Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. Free Radical Biology and Medicine **30**, 1191-1212.

Schieber M, Chandel NS. 2014. ROS function in redox signaling and oxidative stress. Current Biology **24**, R453-R462.

Sies H, Berndt C, Jones DP. 2017. Oxidative stress. Annual Review of Biochemistry **86**, 715-748.

Squadrito GL, Pryor WA. 1998. Oxidative chemistry of nitric oxide: the roles of superoxide, peroxynitrite, and carbon dioxide. Free Radical Biology and Medicine **25**, 392-403.

Speckmann B, Steinbrenner H, Grune T, *et al.* 2016. Peroxynitrite: From interception to signaling. Archives Biochemistry Biophysics **595**, 153-160.

Stöhr C, Strube F, Marx G, *et al*. 2001. A plasma membrane-bound enzyme of tobacco roots catalyses the formation of nitric oxide from nitrite. Planta **212**, 835–841.

Tewari RK, Prommer J, Watanabe M. 2013. Endogenous nitric oxide generation in protoplast chloroplasts. Plant Cell Reports **32**, 31-44.

626 **Tischner R, Galli M, Heimer YM, *et al.*** 2007. Interference with the citrulline-based
627 nitric oxide synthase assay by argininosuccinate lyase activity in Arabidopsis
628 extracts. FEBS Journal **274**, 4238-4245.
629 **Tsikas D**. 2007. Analysis of nitrite and nitrate in biological fluids by assays based on
630 the Griess reaction: appraisal of the Griess reaction in the L-arginine/nitric
631 oxide area of research. Journal of Chromatography B Analytical Technologies
632 in the Biomedical and Life Sciences **851**, 51-70.
633 **Tun NN, Santa-Catarina C, Begum T, *et al*.** 2006. Polyamines induce rapid
634 biosynthesis of nitric oxide (NO) in *Arabidopsis thaliana* seedlings. Plant and
635 Cell Physiology **47**, 346–354.
636 **Uttara B, Singh AV, Zamboni P, *et al*.** 2009. Oxidative Stress and
637 Neurodegenerative diseases: a review of upstream and downstream
638 antioxidant therapeutic options. Current Neuropharmacology **7**, 65–74.
639 **Vitecek J, Reinohl V, Jones RL.** 2008. Measuring NO production by plant tissues
640 and suspension cultured cells. Molecular Plant **1**, 270-284.
641 **Wang Y, Tajkhorshid E.** 2010. Nitric oxide conduction by the brain aquaporin
642 AQP4. Proteins **78**, 661-670.
643 **Weaver J, Porasuphatana S, Tsai P, *et al*.** 2005. Spin trapping nitric oxide from
644 neuronal nitric oxide synthase: A look at several irondithiocarbamate
645 complexes. Free Radical Research **39**, 1027-1033.
646 **Whiteman M, Li L, Kostetski I, *et al*.,** 2006. Evidence for the formation of a novel
647 nitrosothiol from the gaseous mediators nitric oxide and hydrogen sulphide.
648 Biochemical and Biophysical Research Communications **343**, 303-310.
649 **Wilson ID, Hiscock SJ, James PE, *et al.*** 2009. Nitric oxide and nitrite are likely
650 mediators of pollen interactions. Plant Signaling and Behavior **4**. 416-418.

651 **Yamasaki H, Cohen MF**. 2006. NO signal at the crossroads: polyamine-induced
652 nitric oxide synthesis in plants? Trends in Plant Science **11**, 522-524.
653 **Yang B, Wu J, Gao F, *et al*.** 2014, Polyamine-induced nitric oxide generation and its
654 potential requirement for peroxide in suspension cells of soybean cotyledon
655 node callus. Plant Physiology and Biochemistry **79**, 41-47.
656 **Yang J, Giles LJ, Ruppelt C, *et al*.** 2015 Oxyl and hydroxyl radical transfer in
657 mitochondrial amidoxime reducing component-catalyzed nitrite reduction.
658 Journal of the American Chemical Society **137**, 5276–5279.
659 **Zaccolo M, Magalhães P, Pozzan T.** 2002. Compartmentalisation of cAMP and
660 Ca^{2+} signals. Current Opinion in Cell Biology. **14**, 160-166.
661 **Zechmann B.** 2011. Subcellular distribution of ascorbate in plants. Plant Signaling
662 and Behavior **6**, 360–363.
663 **Zweier JL, Samouilov A, Kuppusamy P.** 1999, Non-enzymatic nitric oxide
664 synthesis in biological systems. Biochimica et Biophysica Acta. **1411**, 250-262
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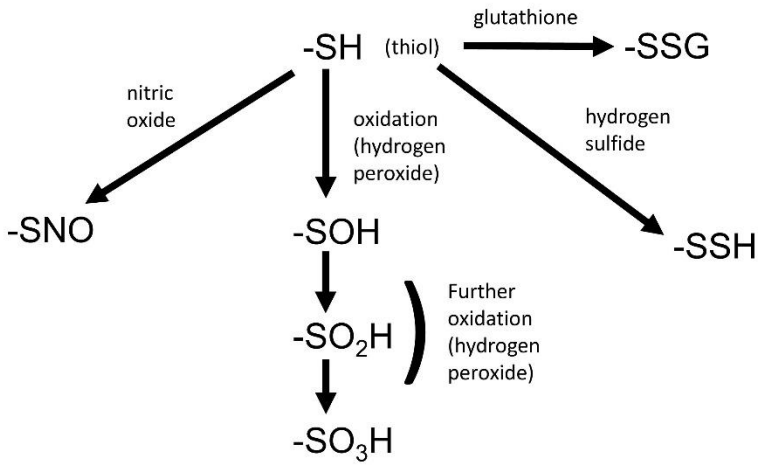
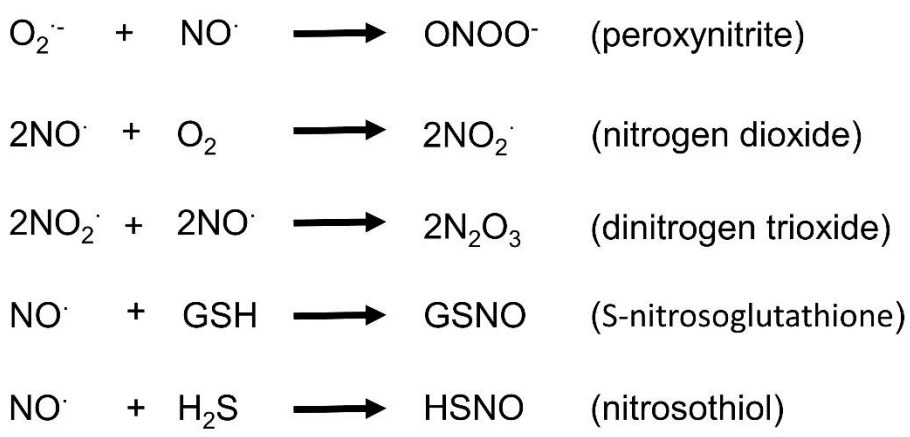
Figure legends

Figure 1: Some of the modifications which may take place on protein thiol groups in the presence of reactive signalling molecules. Thiols will be potentially open to attack by a range of intracellular reactive species, including NO, H_2O_2 and H_2S.



673 Figure 2: Reactions in which NO may partake in biological systems.



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676 **Tables**

677 Table 1: Non-enzymatic and enzymatic sources of NO reported in plants (also see
678 Astier *et al*., 2018).

Source of NO	Comment	Reference(s)
Nitric oxide synthase (NOS) or (NOS-like)	No gene or specific protein has been found in higher plants. NOS-like enzyme reported in algae.	Corpas *et al*. (2009) Foresi *et al*. (2010) Jeandroz *et al*. (2016)
Nitrate reductase (NR)	Probably one of the major sources of NO.	Rockel *et al*. (2002) Modolo *et al*. (2005 Chamizo-Ampudia *et al*. (2017)
Nitric oxide-forming nitrite reductase (NOFNiR)	Interacts with NR to generate NO from nitrite. Belongs to protein family called amidoxime reducing component (ARC).	Chamizo-Ampudia *et al*. (2016) Yang *et al*. (2015)
Membrane-bound nitrite reductase (Ni:NOR)	Associated with the plasma membrane. Produces NO from nitrite	Stöhr *et al.* (2001)
Xanthine oxidoreductase (XOR) and other molybdenum-based enzymes	XOR can generate NO under anaerobic conditions. Such enzymes have nitrite utilising capacity. Aldehyde oxidases (AOs), and sulfite oxidases (SOs) are involved here.	Godber *et al*. (2000) Maia and Moura (2015)
Polyamine reducing enzymes	Putrescine, spermidine and spermine can increase NO generation.	Tun *et al*. (2006) Yamasaki and Cohen (2006) Yang *et al*. (2014)
Non-enzymatic reduction of nitrate	NO can be generated from nitrite under acidic conditions.	Zweier *et al*. (1999) Bethke *et al*. (2004)
Hydroxylamine reducing enzyme	Production was lowered by anoxia or catalase but increased by hydrogen peroxide.	Rumer *et al*. (2009)
Mitochondrial electron transport chain	From nitrite through action of Complex III, Complex IV (cytochrome oxidase) and ETC components. Alternative oxidase activity may have controlling effects.	Igamberdiev *et al*. (2014) Alber *et al*. (2017)
Copper amine oxidase	Copper amine oxidase 8 (CuAO8) appears to regulate NO accumulation via an arginine-dependent mechanism.	Groß *et al*. (2017)
Chloroplast NO generation	Has been suggested to be NOS-like and not due to NR.	Tewari *et al*. (2013) Galatro and Puntarulo (2016)

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681 Table 2: Methods for measuring NO in plants.

682 For a more thorough review see Mur *et al*. (2011). It has been recommended that at
683 least two independent methods are used to ensure data obtained is robust (Gupta
684 and Igamberdiev, 2013).

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Method reported	Comments	Reference for use of method
Electron Paramagnetic Resonance (EPR) or Electron Spin Resonance (ESR)	Needs a probe to form an adduct but often seen as the gold-standard of techniques. Needs NO to be available, for example extracellular/ *in vitro*. Detection down to pmol.	Weaver *et al*. (2005) Bright *et al.* (2009) Galatro and Puntarulo (2016)
Oxyhaemoglobin assay	Can suffer interference from ROS. Detection limit of 1.3-2.8 nM.	Murphy and Noack (1994)
Diaminofluorescein-based fluorescence	Not quantifiable but variants can be used for intracellular or extracellular measurements. However, other RNS can cause interference. Detection limits at 5nM have been reported.	Kojima *et al*. (1998) Planchet and Kaiser (2006) Yang *et al*. (2014)
Rhodamine-based fluorescence	Not quantifiable. Good alternative to DAF-based methods.	Wilson *et al*. (2009)
Griess assay	Background nitrite/nitrate levels, which may be high in plants, can cause quantification problems. Sensitivity originally reported to be 0.5 µM.	Vitecek *et al*. (2008)
Ozone-chemiluminescence	Detection limits may be as low as 20-50 pmol.	Byrnes *et al*. (1996) Rumer *et al*. (2009)
NO electrodes/meter	Useful for extracellular or *in vitro* work. Often difficult for *in vivo* work.	Arasimowicz *et al*. (2009)
Mass spectrometry	Can be used to detect N isotopes.	Conrath *et al.* (2004)
NOS-like activity	Often based on citrulline production these assays may be measuring argininosuccinate lyase activity.	Tischner *et al*. (2007)
Laser photoacoustic detection (LAPD)	Detection limit approximately 20 pmol.	Mur *et al*. (2005) Cristescu *et al*. (2008)
Quantum cascade laser (QCL)-based sensor	NO measured by attenuation of laser intensity.	Mur *et al*. (2012) Cristescu *et al*. (2008)

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